The Glycerol Teichoic Acid of Walls of *Staphylococcus lactis* I3

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1. The teichoic acid from walls of *Staphylococcus lactis* I3 was isolated by extraction with trichloroacetic acid and shown to contain glycerol, *N*-acetylglucosamine, phosphate and β -alanine in the molecular proportions 1:1:2:1. The alanine is attached to the polymer through ester linkages. 2. Hydrolysis with acid gave alanine, glucosamine and glycerol diphosphates. Under mild acid conditions a repeating unit was produced; this consists of glycerol diphosphate joined through a phosphodiester group to *N*-acetylglucosamine. 3. Hydrolysis with alkali gave glycerol diphosphates, saccharinic acid and two phosphodiesters containing glucosamine whose structures were elucidated; these both contain glucosamine 1-phosphate, and *N*-acetylglucosamine 1-phosphate was isolated by a degradative procedure. 4. The unusual properties of the teichoic acid are explained by a polymeric structure in which *N*-acetylglucosamine 1-phosphate is attached through its phosphate to glycerol phosphate. 5. The biosynthetic implications of this structure are discussed.

Since their discovery in cells of *Lactobacillus arabinosus*, *Bacillus subtilis* and *Staphylococcus aureus* (Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958), teichoic acids have been shown to occur as components of the walls of many Gram-positive bacteria and actinomycetes and additionally as membrane-bound constituents of possibly all Gram-positive bacteria. The first glycerol teichoic acids examined were (1 $\rightarrow$ 3)-linked poly(glycerol phosphate) compounds containing glycosyl and β -alaninyl substituents (for a review see Archibald & Baddiley, 1966). Such teichoic acids have been found in the walls of many bacteria, and all of the membrane teichoic acids so far examined in this Laboratory are of this structural type. More recently it has been claimed that the wall teichoic acids of *Actinomyces antibioticus* (Naumova & Zaretskaya, 1964) and of *Bacillus stearothermophilus* (Wicken, 1966) are poly(glycerol phosphate) compounds in which the phosphodiester linkages joining adjacent units occupy positions 1 and 2 on glycerol, glycosyl substituents being attached to position 3. Acid hydrolysis of both these structural types should give glycerol and its mono- and di-phosphates. The wall teichoic acids of *Bacillus licheniformis* A.T.C.C. 9945 differ from the above as they do not have a poly(glycerol phosphate) 'backbone'; in these teichoic acids, glycosylglycerol units are joined by phosphodiester linkages connecting the primary hydroxyl groups

of glycerol and the sugar of an adjacent repeating unit; the sugar residues thus form an integral part of the polymer chain (Burger & Glaser, 1966). Acid hydrolysis of such teichoic acids gives mainly glycerol monophosphates and sugar, together with small amounts of sugar phosphate and glycerol. Hydrolysis with alkali gives mainly glycosylglycerol phosphates; a glycosylated poly(glycerol phosphate) in which phosphodiester groups join positions 1 and 2 on adjacent glycerol residues would give similar products in alkali, whereas such compounds cannot arise as major products of the action of alkali on glycosylated (1 $\rightarrow$ 3)-linked poly(glycerol phosphates). Valuable structural information on teichoic acids can thus be obtained by preliminary chromatographic study of the products of hydrolysis with alkali and acid. Such studies on the teichoic acid from walls of *Staphylococcus lactis* I3 (Davison & Baddiley, 1963; Davison, 1968) suggested that this polymer differs fundamentally from previously examined teichoic acids. Neither acid nor alkali gave significant amounts of glycerol or its monophosphates, the major phosphates in each case being glycerol diphosphates; acid hydrolysis also gave alanine and glucosamine, whereas alkali gave alanine and a saccharinic acid. Although on these findings it was possible that the glucosamine and glycerol were in separate polymers, it was clear that the glycerol teichoic acid must possess a novel structure to account for the almost

exclusive formation of glycerol diphosphates. A detailed study of the polymer was therefore undertaken and it was found that the teichoic acid also differs from those previously described in having a glycerol:phosphate molecular ratio 1:2, indicating that incorporation of phosphate into the polymer by transfer of glycerol phosphate units from CDP-glycerol could account for only half of the phosphate groups present. Although this polymer differs significantly in structure from the first teichoic acids studied, its components, glycerol, phosphate, *N*-acetylglucosamine and *D*-alanine, and its location in the wall render appropriate its description as a teichoic acid. A preliminary account of some of the present observations has been published (Archibald, Baddiley & Button, 1965).

EXPERIMENTAL

Materials

The bacterium *Staph. lactis* 13 was grown from a culture isolated by Dr A. L. Davison (Davison & Baddiley, 1963). Sephadex was purchased from Pharmacia, Uppsala, Sweden. Whatman DEAE-cellulose was obtained from L. Light and Co., Colnbrook, Bucks. Calf intestinal phosphomonoesterase and *D*-amino acid oxidase (hog kidney) were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A., and *L*- α -glycerol phosphate dehydrogenase from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

Paper chromatography

This was carried out on paper that had been washed with 2*N*-acetic acid and water. Unless otherwise indicated Whatman no. 4 paper was used. The following solvent systems were used: *A*, propan-1-ol-aq. NH_3 (sp.gr. 0.88)-water (6:3:1, by vol.); *B*, butan-1-ol-pyridine-water (6:4:3, by vol.); *C*, butan-1-ol-acetic acid-water (4:1:1, by vol.); *D*, propan-1-ol-aq. NH_3 (sp.gr. 0.88)-water (7:1:2, by vol.); *E*, butan-1-ol-acetic acid-water (12:3:5, by vol.); *F*, phenol-water-aq. NH_3 (sp.gr. 0.88) (200:50:1, w/v/v).

Compounds were detected with the periodate-Schiff reagent for α -glycols (Baddiley, Buchanan, Handschumacher & Prescott, 1956), the molybdate reagent for phosphoric esters (Hanes & Isherwood, 1949), the AgNO_3 reagent for reducing substances (Trevelyan, Procter & Harrison, 1950), the ninhydrin reagent for amino acids and aminodeoxyhexoses (Consden & Gordon, 1948) and the FeCl_3 reagent for hydroxamates (Fink & Fink, 1949).

Analytical methods

Phosphorus was determined by the method of Chen, Toribara & Warner (1956), reducing sugars were determined by the method of Park & Johnson (1949) and glycerol and formaldehyde were determined by the method of Hanahan & Olley (1958). Periodate was measured spectrophotometrically (Dixon & Lipkin, 1954; Aspinall & Ferrier, 1957). Amino compounds were analysed by the method of Rosen (1957), optical rotations were measured with an ETP-NPL

type 143A automatic polarimeter with cells of light-path 1 cm. and gas-liquid chromatography was carried out on a Pye 104b chromatograph.

Preparation of cell walls

Cells were disrupted by shaking an 18% (wet wt./vol.) suspension in water with Ballotini no. 11 beads in a shaking centrifuge head (Shockman, Kolb & Toennies, 1957) on an International centrifuge at 1250 rev./min. for 30 min. The beads were removed by filtration through a no. 1 sintered-glass funnel; microscopic examination showed that more than 80% of the cells were ruptured. Alternatively, cells were dried with acetone and the resulting defatted material, as an aqueous 25% (w/v) suspension, was passed through a Ribl cell disintegrator (Ribl, Larson, List, Brown & Goodie, 1959) at 5° (cell pressure 250 kg./cm.²); more than 80% disruption was effected. Walls from either procedure were isolated by differential centrifugation in the usual way, washed with 0.1*M*-sodium phosphate buffer, pH 7.0, followed by water and then freeze-dried. In each case approx. 1.5 g. of walls was obtained from 200 g. wet wt. of cells.

Examination of cell walls

The composition of the walls was not detectably affected by the method used for disintegration of the cells; they contained 3.9% of phosphorus. A sample (4 mg.) was hydrolysed in 2*N*-HCl (0.5 ml.) at 100° for 3 hr. The acid was removed by evaporation *in vacuo*, and chromatographic examination in solvent *A* showed the presence of large amounts of glycerol diphosphates, glucosamine, alanine and glycine. Two-dimensional chromatography in solvents *E* and *F* showed that the major amino acids were alanine, glycine, glutamic acid and lysine; no degradation products of nucleic acids were detected. A sample (2 mg.) was shaken at 22° for 1 hr. with 0.4 ml. of methanol-aq. NH_3 (sp.gr. 0.88) (1:1, v/v). Chromatographic examination of the extract in solvent *A* showed the presence of alanine and alanine amide.

Configuration of the alanine ester residues

A suspension (17 mg.) of walls in *N*-NaOH (4 ml.) was shaken at 37° for 18 hr. A portion (1 ml.) of the extract was passed through Dowex 50 (NH_4^+ form) resin and examined chromatographically in solvent *A*; alanine was the only amino acid present. The remainder of the extract was neutralized with formic acid, evaporated to dryness and dissolved in water (2 ml.). The concentration of alanine was determined colorimetrically (Rosen, 1957) and the concentration of *D*-amino acid was determined manometrically with *D*-amino acid oxidase (Burton, 1955); 93% of the alanine had the *D*-configuration.

Action of acid on the cell walls

(a) 2*N*-HCl (0.5 ml.) was added to samples (0.5 ml.) of a suspension of walls in water (2 mg./ml.) and the mixtures were kept in sealed tubes at 37°. Tubes were removed at intervals and the phosphorus content of samples (0.2 ml.) of suspension and of supernatant after centrifugation (2000*g* for 30 min.) was determined. The rate of production of soluble phosphates is shown in Fig. 1.

Fig. 1. Solubilization of teichoic acid from walls by treatment with acid. Walls were suspended in *N*-HCl and centrifuged and the amount of organic phosphate in the supernatant was measured at intervals. Details are given in the text.

Fig. 2. Formation of inorganic phosphate by heating walls at 100° at pH4. Details are given in the text.

(b) 0.2M-Sodium acetate buffer, pH4 (12ml.), was added to a suspension of walls (9mg.) in water (4ml.). The mixture was homogenized and phosphorus was determined. Samples (0.5ml.) were heated at 100° in sealed Pyrex tubes, at intervals samples were removed, cooled and centrifuged (20000*g* for 30 min.), and inorganic phosphate was determined in the supernatant solutions. Results are expressed as the percentage of wall phosphate that appears in the supernatant as inorganic phosphate (Fig. 2).

Isolation of teichoic acid

Walls (437mg.) were stirred with 10% (w/v) trichloroacetic acid (20ml.) for 24hr. at 4°. After centrifugation (20000*g* for 10 min.) the supernatant was decanted and the walls were again stirred with trichloroacetic acid for 24hr. The combined extracts were mixed with ethanol (2 vol.) and acetone (2 vol.). Very little precipitate was formed by the addition of ethanol, but with acetone the solution became cloudy and a white precipitate (67mg.) was recovered after the mixture had been kept for 24hr. at 6°. The residual walls contained 1.3% of phosphorus, and 27% of the extracted phosphorus remained soluble after the addition of acetone

to the extract. In later preparations a milder procedure (5% trichloroacetic acid for 16hr.) was used to minimize degradation of the teichoic acid.

Teichoic acid

Electrophoresis. Samples of teichoic acid were subjected to electrophoresis on Whatman no. 1 paper at pH5.7 and at pH9 for 3–4hr. at 20 v/cm. In each the teichoic acid (detected by the molybdate spray reagent) migrated towards the anode, but streaks rather than discrete spots were obtained.

Gel filtration. An aqueous solution of teichoic acid (20mg. in 0.5ml.) was applied to a column (diam. 2cm.) of Sephadex G-25 (40ml.) and material was eluted with water. A single broad peak was observed from phosphate analysis of fractions and this accounted for 90% of the applied phosphorus. The polymer was recovered by freeze-drying as a white powder (containing 11.6% of phosphorus) that gave the same products on acid hydrolysis as did the original sample.

Analysis. (a) Teichoic acid (2mg.) was hydrolysed in 2*N*-HCl (0.5ml.) for 3hr. at 100°. The hydrolysate was neutralized with *N*-NaOH and adjusted to 5ml. with water. The solution was analysed for phosphorus, reducing sugar (as glucosamine) and amino compounds. The molecular proportions found were: phosphate:reducing sugar (as glucosamine):amino compounds (as alanine), 1.0:0.5:1.1.

(b) Teichoic acid (0.8mg.) was hydrolysed in 2*N*-HCl (0.2ml.) for 3hr. at 100°. Water (2ml.) was added and the solution was adjusted to pH9 by the addition of 2*N*-NH₃. The solution was incubated with phosphomonoesterase (0.1mg.) for 12hr. at 37° and samples were removed for analysis. The molecular proportions found were: phosphate:reducing sugar:glycerol, 1:0.56:0.54.

Ammonolysis. Teichoic acid (1mg.) was shaken for 4hr. at 22° with 0.2ml. of methanol-aq. NH₃ (sp.gr. 0.88) (1:1, v/v). Paper chromatography in solvent *A* showed the presence of alanine and alanine amide (*R*_{A1a} 1.2) as the only products that reacted with the ninhydrin reagent.

Action of hydroxylamine. (a) An aqueous solution (0.05ml.) of teichoic acid (1mg.) was shaken with 0.5M-hydroxylamine solution (1ml.) at pH7 for 4hr. at 37° (Kelemen & Baddiley, 1961). The products were examined by paper chromatography in solvent *C*. The FeCl₃ reagent for hydroxamates showed only one product, with the mobility of alanine hydroxamate.

(b) A series of tubes containing salt-free 0.1M-hydroxylamine (0.5ml.) at pH7.4 and an equal volume of an aqueous solution of teichoic acid (2mg./ml.) were heated on a water bath at 45°. Samples were removed at intervals and mixed with *N*-HCl (1ml.) followed by *m*-FeCl₃ (1ml.). The concentration of hydroxamate was determined colorimetrically at 540nm. with alanine hydroxamate as the standard. A similar experiment was carried out with alanine ethyl ester (2.8 μmoles/ml.) in the place of teichoic acid. Results are given in Fig. 3.

Determination of O- and N-acetyl groups. Samples (0.2ml.) of 22mM-*N*-acetylglucosamine, 37mM-methyl 6-*O*-acetyl-α-D-galactoside and teichoic acid (7.5mg./ml.) solutions were heated at 100° in sealed tubes with 0.2*N*- and 2*N*-HCl (0.2ml.). At intervals tubes were removed and cooled in water and then in acetone–solid CO₂. The contents were mixed with 14.5mM-propionic acid (0.2ml.) as an

Fig. 3. Reaction between teichoic acid and alanine ethyl ester with hydroxylamine. Curve A, formation of alanine hydroxamate from teichoic acid; curve B, formation of alanine hydroxamate from alanine methyl ester. Details are given in the text.

internal standard. Samples (0.5–1 μl.) of the mixture were examined by gas-liquid chromatography on a column (1.52 m.) of 15% Tween 80 on Celite (80–100 mesh) at 105° and N₂ pressure of 1.1 kg./cm.²; the retention times of acetic acid, propionic acid and butyric acid were 6.25, 10.25 and 32 min. respectively. The ratio of peak areas of equimolar amounts of acetic acid and propionic acid was 0.58:1. After 30 min. in 0.1 N-HCl no acetic acid was detected from the *N*-acetylglucosamine standard, but about 70% of the theoretical amount was obtained from methyl 6-*O*-acetyl- α -D-galactopyranoside. The production of acetic acid from *N*-acetylglucosamine was almost complete in N-HCl after 1 hr. at 100°. Acetic acid was not detected when the teichoic acid was hydrolysed in 0.1 N-HCl for 30 min. but 0.8 molecular equivalent was formed for each glucosamine by hydrolysis in N-HCl for 1 hr.

Acid hydrolysis of the teichoic acid

(a) Teichoic acid (10 mg.) was heated with 2 N-HCl (1 ml.) in a sealed tube at 100° for 3 hr. The hydrolysate was dried *in vacuo* over KOH and examined chromatographically in solvent A. The major products had the mobility and colour reactions of alanine, glucosamine and glycerol diphosphates; small amounts of glycerol monophosphates and of a reducing phosphate giving a reaction with ninhydrin were also present, but no glycerol was detected. These products were further characterized by paper chromatography of the hydrolysate as a band and elution from the appropriate areas after development for 14 hr. Three products were obtained at (1) 12–15 cm., (2) 5.5–6.5 cm. and (3) 2–3 cm.

Compound (1) had the chromatographic mobility and colour reactions of glucosamine. A sample (about 1 mg.) was heated at 100° for 2 hr. with aq. 2% (w/v) ninhydrin solution (0.2 ml.) containing 4% (v/v) of pyridine; chromatographic examination in solvent C showed the presence of arabinose. A further sample (about 1 mg.) was dissolved in 2 N-HCl (0.2 ml.) and NaNO₂ (2 mg.) was added. The mixture was left at room temperature for 4 hr. and then evaporated *in vacuo* over KOH. The resulting solid was dissolved in water (0.5 ml.) containing NaBH₄ (5 mg.) and the solution was kept at room temperature overnight. It was acidified with acetic acid and then passed through a

Fig. 4. Formation of reducing groups by the action of 0.1 N-HCl at 100° on the teichoic acid. Details are given in the text.

column (2 ml.) of Dowex 50 (H⁺ form) resin. The eluate was evaporated to dryness and borate was removed by repeated dissolution in methanol and evaporation to dryness. The product had chromatographic properties in solvent A identical with those of 2,5-anhydromannitol prepared from an authentic sample of glucosamine by the same method.

Compound (2) was a reducing phosphate that reacted with the ninhydrin reagent. It was dissolved in 0.01 M-(NH₄)₂CO₃, pH 9.3 (1 ml.), containing phosphomonoesterase (0.1 mg.) and incubated at 37° for 10 hr. Chromatography in solvents A and B showed glucosamine and inorganic phosphate as the only products.

Compound (3) was an organic phosphate that gave no colour with the AgNO₃, ninhydrin or periodate-Schiff spray reagents. A sample (containing 6.2 μg. atoms of P) was dissolved in 0.01 M-NH₄Cl (2 ml.) containing phosphomonoesterase (0.2 mg.) and incubated at 37° for 16 hr. Chromatographic examination showed glycerol and inorganic phosphate as the only products; inorganic phosphate and glycerol were present in the molecular ratio 2:0.96.

(b) Samples (0.2 ml.) of an aqueous solution of teichoic acid (1.5 mg./ml.) were mixed with 0.2 N-HCl (0.2 ml.) and heated at 100° in sealed Pyrex tubes. Samples were examined at intervals for reducing power (against glucosamine as standard) and total phosphate. The rate of formation of reducing groups is given in Fig. 4.

Examination of the product (1) of partial acid hydrolysis. Teichoic acid (1 mg.) was heated in a sealed tube with 0.1 N-HCl (0.4 ml.) for 10 min. at 100° and the solution evaporated to dryness *in vacuo* over KOH. Chromatographic examination in solvent A showed the almost quantitative formation of a reducing phosphate (1) with *R*_{AGP} (*R*_{glycerol 1-phosphate}) 0.9 together with traces of glycerol diphosphates and *N*-acetylglucosamine.

A further sample of teichoic acid (18 mg.) was hydrolysed under similar conditions in 4 ml. of 0.1 N-HCl. The hydrolysate was neutralized by the careful addition of N-NH₃ and then evaporated to 0.4 ml. *in vacuo*. The resulting solution was applied as a band to Whatman 3MM paper and chromatographed in solvent A for 16 hr. The reducing phosphate, which had run 4–6 cm. from the base line, was eluted with water. A sample of the eluate was rechromatographed in solvent D; the material appeared to be homogeneous (*R*_{AGP} 0.87). A further sample was hydrolysed in N-HCl for 3 hr. at 100°; chromatography showed the presence of glucosamine, glycerol diphosphates and traces of glycerol monophosphates and a glucosamine phosphate. A third sample was hydrolysed in N-NaOH for 3 hr. at 100°. The

hydrolysate was passed through a small column of Dowex 50 (NH_4^+ form) resin and examined chromatographically; glycerol diphosphates and glucometasaccharinic acid were detected.

Analysis of the phosphate (I). Samples (0.1 and 0.2 ml.) of a solution of the phosphate (containing 0.6 $\mu\text{g. atom}$ of P/ml.) were adjusted to 1 ml. with water and the reducing power was determined; it was equivalent to 0.19 mole of *N*-acetylglucosamine/mole of phosphate.

A sample (containing 0.75 $\mu\text{g. atom}$ of P) of the phosphate was hydrolysed in 2*N*-HCl (1 ml.) for 3 hr. at 100°; the solution was evaporated *in vacuo* over KOH and the residue was dissolved in 0.01*M*-(NH_4)₂CO₃, pH 9.4 (2 ml.), containing 0.2 mg. of phosphomonoesterase and then incubated overnight at 37°. Analyses were carried out for phosphorus, reducing sugars (as glucosamine) and glycerol; the molecular proportions phosphate:glucosamine:glycerol were 1:0.52:0.47.

Action of phosphomonoesterase on the phosphate (I). A solution of the phosphate (I) (containing 1.26 $\mu\text{g. atoms}$ of P) in 0.01*M*-(NH_4)₂CO₃, pH 9.4 (1 ml.), containing phosphomonoesterase (0.2 mg.) was incubated at 37° for 16 hr.; 53% of the phosphorus was converted into inorganic phosphate. After this dephosphorylation, treatment of the product with 0.1*M*-NaIO₄ solution gave 0.48 mol. prop. of formaldehyde/mol. prop. of total phosphate, whereas before dephosphorylation 0.07 mol. prop. of formaldehyde/mol. prop. of phosphate was produced.

Action of sodium borohydride on the phosphate (I). A 5 mg. portion of NaBH₄ was added to a solution of the phosphate (containing 4.5 $\mu\text{g. atoms}$ of P) in water (3 ml.) and the mixture was kept overnight at room temperature. It was acidified with *N*-acetic acid solution and passed through a column (3 cm. $\times$ 1 cm.) of Dowex 50 (H^+ form) resin. Borate was removed from the eluate by repeated evaporation with methanol. The product was chromatographed as a band for 16 hr. in solvent *A* on Whatman 3MM paper. The reduced product was eluted from the appropriate area, and samples of the original phosphate and the reduced product were chromatographed on Whatman no. 4 paper in solvent *D* for 36 hr. Both compounds appeared homogeneous; the original phosphate travelled 20.2 cm. and the reduced material travelled 19.4 cm.

Examination of the reduced phosphate (II). A sample was hydrolysed in *N*-HCl for 3 hr. at 100°. After evaporation *in vacuo* over KOH the hydrolysate was examined by chromatography in solvent *A*. Glycerol monophosphates and organic phosphate (R_{ZGP} 0.78) were the only products detected. A further sample was hydrolysed in *N*-NaOH for 3 hr. at 100°, and the hydrolysate was passed through a small column of Dowex 50 (NH_4^+ form) resin and examined chromatographically in solvent *A*. Glycerol monophosphates and a phosphate with R_{ZGP} 0.83, together with traces of glucosaminitol and glycerol diphosphates, were detected.

Periodate oxidation of the reduced phosphate (II). A solution (1 ml.) of the reduced phosphate (containing 0.75 $\mu\text{g. atom}$ of P) and NaIO₄ (1.5 μmoles) was kept at room temperature. Samples (0.15 ml.) were removed and adjusted to 2.5 ml. with water, and the amount of periodate was determined. Oxidation was complete after 26 hr., when 2.1 mol. prop. of periodate was reduced/2 mol. prop. of phosphate.

Hydrolysis of the teichoic acid with alkali

A sample of teichoic acid (1 mg.) was hydrolysed in *N*-NaOH (0.2 ml.) for 3 hr. at 100°; the solution became dark brown. The hydrolysate was passed through a column (2 ml.) of Dowex 50 (NH_4^+ form) resin and evaporated to dryness *in vacuo*. Chromatography in solvent *A* showed the presence of alanine, glucometasaccharinic acid, glycerol diphosphates and other phosphates (R_{ZGP} 0.55–0.6). A duplicate hydrolysate was dissolved in water (1 ml.) and a sample (0.2 ml.) was mixed with 0.1*M*-NH₄Cl, pH 9.4 (0.8 ml.), containing phosphomonoesterase (0.1 mg.) and incubated at 37° for 16 hr. Portions (0.1 ml.) were analysed for inorganic and total phosphate; 84% of the phosphate was inorganic phosphate. The remainder of the hydrolysate was heated with 4*N*-HCl (1 ml.) for 3 hr. at 100° in a sealed tube. The sample was dried *in vacuo* over KOH and examined chromatographically in solvent *A*; the AgNO₃ spray reagent showed the presence of glucosamine.

A further sample of teichoic acid (2 mg.) was dissolved in water (1 ml.) containing NaBH₄ (5 mg.) and kept for 4 hr. at room temperature. Excess of borohydride was destroyed by cautious addition of acetic acid, and the sample was evaporated to dryness *in vacuo* and then heated at 100° in *N*-NaOH (0.2 ml.); extensive browning occurred. In a companion experiment the reduced teichoic acid was heated in *N*-NaOH containing 20 mg. of NaBH₄ (sealed tube); under these conditions the solution remained colourless.

Isolation of the saccharinic acid. The teichoic acid (6 mg.) was hydrolysed in alkali and Na⁺ ions were removed by Dowex 50 resin as described above. The dried residue was subjected to preparative paper chromatography in solvent *A* and the saccharinic acid was isolated from the appropriate area. Samples of glucometasaccharinic acid were prepared by similar alkali treatment of glucose and laminaribiose. Alkali degradation of maltose gave a mixture of glucometasaccharinic acid and glucoisosaccharinic acid. These two compounds have slightly different chromatographic mobilities, but are clearly distinguished by the periodate-Schiff spray reagents, with which glucoisosaccharinic acid gives an intense yellow colour clearly distinct from the blue colour given by glucometasaccharinic acid.

The compound isolated from the teichoic acid had colour reactions and chromatographic mobilities in solvents *A*, *B*, *C* and *E* and mobility on paper electrophoresis in veronal buffer, pH 7.2 (15 cm. in 20 hr. at 2 v/cm.), identical with those of glucometasaccharinic acid.

Isolation of the products of hydrolysis in alkali followed by enzymic dephosphorylation. Teichoic acid (103 mg.) was heated in *N*-NaOH (5 ml.) in a sealed tube at 100° for 3 hr. The solution was passed through a column (10 cm. $\times$ 1 cm.) of Dowex 50 (NH_4^+ form) resin that was then washed with water (100 ml.). Then NH₄Cl (50 mg.) and phosphomonoesterase (10 mg.) were added to eluate and washings and the pH was adjusted to 9.4; the solution was kept at 37° for 18 hr. The mixture was applied to a column (60 cm. $\times$ 2 cm.) of DEAE-cellulose (HCO_3^- form) resin; the column was washed with water (200 ml.) and material was eluted with a linear concentration gradient of (NH_4)₂CO₃, pH 8.4. The mixing vessel contained water (2 l.) and the reservoir 0.15*M*-(NH_4)₂CO₃ (2 l.); fractions (25 ml.) were collected automatically at a flow rate of 2 ml./min. Samples (0.2 ml.) were analysed for phosphate (Fig. 5). A major component

Fig. 5. Chromatography on DEAE-cellulose of products obtained by treatment of teichoic acid with alkali and then phosphomonoesterase. Details are given in the text. —, Total phosphate ($\mu\text{mole/ml.}$); ----, concn. of $(\text{NH}_4)_2\text{CO}_3$ (M).

(fractions 60–75) was inorganic phosphate. The $(\text{NH}_4)_2\text{CO}_3$ was removed from fractions 10–30 by repeated evaporation at 37° ; chromatographic examination in solvent A showed the presence of two organic phosphates. Phosphate (V) (R_{AGP} 2.0) gave no reaction with the ninhydrin spray reagent, whereas phosphate (IX) (R_{AGP} 1.65) gave a blue colour with this spray. The two products were isolated by preparative paper chromatography in solvent A.

Hydrolysis of phosphate (V) in 0.1 N-HCl at 100° for 2 hr. gave products with the chromatographic mobilities and colour reactions of glucosamine, *N*-acetylglucosamine and glycerol monophosphates; the phosphate (IX) gave only glucosamine and glycerol monophosphates under these conditions. On hydrolysis with N-NaOH at 100° for 3 hr. both compounds gave glucometasaccharinic acid and glycerol monophosphates. A sample of phosphate (V) (0.2 mg.) was hydrolysed in N-HCl for 3 hr. at 100° and the solution was evaporated to dryness *in vacuo* over KOH. The products were dissolved in 0.01 M- $(\text{NH}_4)_2\text{CO}_3$, pH 9.3 (3 ml.), containing phosphomonoesterase (0.1 mg.) and incubated at 37° for 16 hr. Samples were analysed for phosphorus, reducing sugar (as glucosamine) and glycerol. The molecular proportions found were: phosphate:reducing sugar:glycerol, 1.0:0.93:0.96.

Isolation of *N*-acetylglucosamine 1-phosphate. The phosphate (V) (corresponding to $2\mu\text{g. atoms of P}$) was oxidized with 0.01 M-NaIO₄ solution (0.2 ml.) for 15 min. at room temperature. The reaction was terminated by the addition of 0.2 M-ethylene glycol solution (0.1 ml.). A 1% solution of *NN*-dimethylhydrazine (5 ml.) was added and the solution was adjusted to pH 6.5 with acetic acid. The mixture was shaken for 12 hr. at 37° and then exhaustively extracted with chloroform. The aqueous layer was freeze-dried, redissolved in water and applied as a band on Whatman no. 4 paper. The chromatogram was then developed for 60 hr. in solvent A, and *N*-acetylglucosamine 1-phosphate was run as a standard. The band had a mobility identical with that of standard *N*-acetylglucosamine 1-phosphate; both travelled 31.5 cm. and gave a yellow colour, that later turned grey, with the molybdate reagent. After elution with water and rechromatography in solvent D, the product was homogeneous and again had the mobility of *N*-acetylglucosamine 1-phosphate (R_{AGP}

Fig. 6. Chromatography on DEAE-cellulose of products of hydrolysis of teichoic acid with alkali. Details are given in the text. —, Total phosphate ($\mu\text{mole/ml.}$); ----, concn. of $(\text{NH}_4)_2\text{CO}_3$ (M).

1.38). After hydrolysis in 0.1 N-HCl at 100° for 10 min. it was converted entirely into *N*-acetylglucosamine and inorganic phosphate; these products were identified by chromatography in solvent A. On incubation with phosphomonoesterase the phosphate gave only *N*-acetylglucosamine and inorganic phosphate; these were formed in the ratio 1.08:1.

Examination of the phosphate (IX). The optical rotation of this phosphate ($4.0\mu\text{g. atoms of P/ml. of water}$) was measured in a 1 cm. cell in the automatic polarimeter and found to be $[\alpha]_D^{25} +90.5^\circ$. Its reducing power was equivalent to 0.06 mole of *N*-acetylglucosamine/mole of phosphate.

A solution of the phosphate (containing $1.73\mu\text{g. atoms of P}$) in water (0.4 ml.) was mixed with 18.2 mm-NaIO₄ (0.6 ml.) and kept in the dark at room temperature; reduction of periodate and production of formaldehyde were measured at intervals. The oxidation was complete after 72 hr. when 3.3 mol.prop. of periodate was reduced and 1.15 mol.prop. of formaldehyde was formed; formaldehyde production was complete after 45 min.

Isolation of the phosphates (IV and VIII) produced by hydrolysis in alkali. Teichoic acid (109 mg.) was dissolved in N-NaOH (5 ml.) and heated in a sealed tube at 100° for 3 hr. The cooled hydrolysate was passed through a column (12 cm. $\times$ 1 cm.) of Dowex 50 (NH_4^+ form) resin, evaporated to dryness, dissolved in water and applied to a column (60 cm. $\times$ 2 cm.) of DEAE-cellulose (CO_3^{2-} form). The column was washed with water and products were eluted with a linear concentration gradient (0–0.15 M) of $(\text{NH}_4)_2\text{CO}_3$, pH 9.0 (total volume 4 l.). Fractions (25 ml.) were collected and analysed for phosphorus (Fig. 6). Appropriate fractions were combined and evaporated to dryness by rotary evaporation, and the residues were dissolved in water and freeze-dried.

Peak 1. Chromatographic examination of this fraction showed it to consist of isomeric glycerol phosphates. On enzymic dephosphorylation, glycerol and inorganic phosphate were produced.

Peak 2. This consisted of one chromatographically homogeneous component, phosphate (IV), with R_{AGP} 0.77 in solvent E; it gave no colour with the ninhydrin spray reagent. Hydrolysis in 2 N-HCl for 3 hr. at 100° gave glycerol diphosphates and glucosamine. The molecular proportions found were: phosphate:glucosamine:glycerol, 1.0:50:0.53.

Fig. 7. Oxidation of compounds (IV) and (V) with periodate. Details are given in the text.

An aqueous solution (0.4 ml.) of phosphate (IV) (containing $2\mu\text{g. atoms of P}$) was heated with 0.1 N-HCl (0.4 ml.) for 1 hr. at 100° . Half of the resulting solution was evaporated to dryness over KOH and examined chromatographically in solvent A; N-acetylglucosamine and glycerol diphosphates were the only compounds detected. The remainder of the solution was examined chromatographically on Whatman no. 1 paper in solvent D (6 days development). The two isomeric phosphates separated in this system; glycerol 1,2-diphosphate, which was the major component, travelled 17.5 cm. and glycerol 1,3-diphosphate travelled 15 cm.

A solution (0.4 ml.) of phosphate (IV) (containing $1.8\mu\text{g. atoms of P}$) was adjusted to 2.0 ml. with 0.1 M- $(\text{NH}_4)_2\text{CO}_3$, pH 9.3, containing phosphomonoesterase (0.1 mg./ml.) and the mixture was incubated at 37° for 16 hr., when 53% of the phosphorus was converted into inorganic phosphate. Chromatographic examination in solvent A showed that the phosphate (IV) had been converted entirely into a product that was identified as phosphate (V) from its chromatographic mobility and colour reactions.

Phosphate (IV) ($4.4\mu\text{g. atoms of P}$) was mixed with NaIO_4 ($3.37\mu\text{moles}$) in water and adjusted to 25 ml. Phosphate (V) ($0.35\mu\text{g. atom of P}$) was mixed with NaIO_4 ($1.34\mu\text{moles}$) in water and adjusted to 10 ml. The solutions were kept in the dark at room temperature together with appropriate controls. The reduction of periodate is shown in Fig. 7. Phosphate (V) yielded 1.03 mol. prop. of formaldehyde/mol. prop. of phosphate, whereas phosphate (IV) gave no formaldehyde.

Peak 3. This consisted of a chromatographically homogeneous substance, phosphate (VIII), with R_{AGP} 0.69 in solvent D; it gave colours with both molybdate and ninhydrin spray reagents. A sample of compound (VIII) (containing $0.6\mu\text{g. atom of P}$) was heated in 0.05 N-HCl (0.4 ml.) at 100° for 1 hr. The hydrolysate was evaporated to dryness over KOH and examined chromatographically in solvent A; glucosamine and glycerol diphosphates were the only products.

A solution of phosphate (VIII) (1 ml., containing $3.2\mu\text{g. atoms of P}$) was mixed with 0.02 M- $(\text{NH}_4)_2\text{CO}_3$, pH 9.3 (1 ml.), containing phosphomonoesterase (0.2 mg.) and the mixture was incubated at 37° for 16 hr.; 47% of the original phosphate was converted into inorganic phosphate. The dephosphorylated material was identified

as the phosphate (IX) from its chromatographic mobility and colour reactions.

Action of nitrous acid on phosphates (VIII) and (IX). Samples (containing $2\mu\text{g. atoms of P}$) of phosphate (VIII) and of the compound obtained from it by the action of phosphatase were dissolved in 0.1 N-HCl (0.2 ml.) and the solutions were evaporated to dryness over KOH. The residues were dissolved in water (0.5 ml.), NaNO_2 (4 mg.) was added and the mixtures were heated at 40° in sealed tubes for 3 hr. The solutions were passed through small columns of Dowex 50 (NH_4^+ form) resin and evaporated to dryness. The resulting solids were dissolved in water (0.5 ml.) containing NaBH_4 (5 mg.) and kept for 16 hr. Excess of borohydride was destroyed by the addition of acetic acid and the solutions were passed through small columns of Dowex 50 (H^+ form) resin. Borate was removed by repeated evaporation with methanol and samples of the products were examined by chromatography in solvent A. Both phosphates gave 2,5-anhydromannitol (R_{AGP} 2.9) identified by comparison with an authentic sample prepared by deamination of glucosamine. Glycerol 1-phosphate was formed from phosphate (IX) and glycerol 1,2-diphosphate from phosphate (VIII).

Configuration of the glycerol 1-phosphate from phosphate (IX). A sample of glycerol 1-phosphate prepared as described above from phosphate (IX) was isolated by preparative chromatography in solvent A on Whatman no. 1 paper. A solution of the eluate (containing $0.4\mu\text{g. atom of P}$) was incubated with NAD^+ ($5\mu\text{moles}$) and D-glycerol 1-phosphate dehydrogenase (0.1 mg. of protein) at pH 9.5. The extinction of the solution at 340 nm. remained constant, whereas rapid reduction of NAD^+ took place in a control experiment containing DL-glycerol 1-phosphate. Addition of DL-glycerol 1-phosphate to the sample caused rapid reduction of NAD^+ .

DISCUSSION

Because of the unusual hydrolytic behaviour of the wall teichoic acid of *Staph. lactis* I3 a detailed study of this polymer was undertaken. It was readily extracted from walls by aqueous trichloroacetic acid, although with 10% trichloroacetic acid under the usual conditions rather poor yields were obtained on precipitation with ethanol and acetone. With 5% trichloroacetic acid, however, better yields were obtained, but about 20% of the extracted phosphate was not precipitated by the addition of organic solvents. These observations suggested that the polymer was unusually labile to acid, and this was supported by the rapid release of teichoic acid when walls were suspended in N-hydrochloric acid at 37° . When walls were heated at pH 4, they gave inorganic phosphate at a much higher rate than did walls that contain ribitol teichoic acid (Hay, Archibald & Baddiley, 1965); these conditions are especially favourable for the hydrolysis of phosphomonoesters and the observation confirms the conclusion that the teichoic acid is extensively degraded under mild conditions.

The precipitated teichoic acid contained 11.3%

of phosphorus and no further purification was achieved by electrophoresis or gel filtration. If all of the phosphorus in the wall (3.9%) is present as teichoic acid then this polymer constitutes about 35% of the wall. The precipitated material contained phosphate, glycerol, *N*-acetylglucosamine and alanine in the molecular proportions 2:1.08:1.1:1.1, and on acid hydrolysis it gave alanine, glucosamine, glycerol diphosphates and small quantities of glycerol monophosphates and a glucosamine phosphate. The phosphates were isolated by preparative paper chromatography and their identities confirmed by enzymic dephosphorylation; the glucosamine gave 2,5-anhydromannose on degradation with nitrous acid (Foster, Horton & Stacey, 1956) and arabinose with ninhydrin (Gardell, Heijkenskjöld & Rochnorlund, 1950). Gas-liquid chromatography was used to detect acetic acid in the products of acid hydrolysis of the polymer; no acetic acid was produced in 0.1 *N*-hydrochloric acid at 100° for 20 min., whereas under these conditions almost quantitative production of acetic acid was observed from methyl 6-*O*-acetylgalactoside but none from *N*-acetylglucosamine. With *N*-hydrochloric acid the production of acetic acid from *N*-acetylglucosamine was complete in 1 hr. at 100°, and the amount formed from the teichoic acid indicated that at least 80% of the glucosamine possesses *N*-acetyl groups, but *O*-acetyl groups are absent. Treatment of cell walls and isolated teichoic acid with methanolic ammonia gave alanine and alanine amide; alanine hydroxamate was formed similarly by treatment with hydroxylamine. The rate of formation of alanine hydroxamate under standard conditions (Kelemen & Baddiley, 1961) was similar to that observed with alanine ethyl ester but was appreciably lower than that for teichoic acids having activated alanyl ester residues (Shabarova, Hughes & Baddiley, 1962). The alanine was shown by incubation with D-amino acid oxidase to have the D-configuration.

The precipitated teichoic acid had little reducing power, but this increased rapidly on treatment with 0.1 *N*-hydrochloric acid until a value equivalent to 0.35 mole of glucosamine/mole of phosphate was reached after 10 min.; subsequently the reducing power increased only slowly (Fig. 4). These conditions would be expected to effect the hydrolysis of sugar 1-phosphates. Chromatographic examination of the products showed that after 10 min. the teichoic acid had been degraded to a reducing phosphate (see Scheme 1), together with only very small amounts of *N*-acetylglucosamine and glycerol diphosphates. The reducing phosphate (I) was isolated by preparative paper chromatography and shown to contain phosphate, glycerol and glucosamine in the molecular proportions 1:0.94:1.04. Acid hydrolysis gave glycerol di-

Fig. 8. Formation of colour in the Park & Johnson (1949) method with *N*-acetylglucosamine (0.02 μ mole) and compound (I) (0.045 μ mole). The procedure was carried out according to the published method and colour was measured at intervals.

phosphates and glucosamine, together with traces of glycerol monophosphates and a glucosamine phosphate; small amounts of alanine were present in some preparations. Hydrolysis of the phosphate (I) with alkali gave saccharinic acid and glycerol diphosphates. After reduction of the phosphate with sodium borohydride both acid and alkali converted it into glycerol monophosphates and an organic phosphate presumed to be a glucosaminitol phosphate. No saccharinic acid was formed on hydrolysis of the reduced phosphate with alkali but small amounts of glucosaminitol and glycerol diphosphate were detected. These hydrolysis products are consistent with a structure (II) for the reduced phosphate; the glucosaminitol phosphate is joined to glycerol phosphate through a phosphodiester linkage and hydrolysis would proceed through intermediate cyclic phosphates involving hydroxyl groups adjacent to the phosphodiester group. It is a common observation that cyclic phosphate formation occurs predominantly with the retention of phosphate on the larger polyol. The reducing power of compound (I) was equivalent to 0.38 mole of *N*-acetylglucosamine/2 moles of phosphate, when determined under standard conditions. This difference was due mainly to differing rates of reduction of potassium ferricyanide by the phosphate and by standard *N*-acetylglucosamine, the half-times of the two reactions being 30 and 5 min. respectively (Fig. 8). The reducing power of substituted acetamido sugars is influenced by the nature and position of substituents; thus the reducing power of *N*-acetylglucosamine 6-phosphate is 0.42 relative to that of glucose (Suzuki, 1962).

These observations establish that phosphate (I) consists of glycerol diphosphate linked to an alcoholic hydroxyl group of *N*-acetylglucosamine through a phosphodiester linkage. The location of the phosphodiester group on the primary hydroxyl

group of glycerol follows from studies on the compound produced from it by the action of the phosphomonoesterase. This treatment converted 50% of the phosphorus in phosphate (I) into inorganic phosphate, and the resulting phosphodiester yielded on oxidation with periodate 0.46 mol.prop. of formaldehyde/mol.prop. of phosphate in the original compound; oxidation of phosphate (I) itself gave only small amounts of formaldehyde (0.07 mol.prop./mol.prop. of phosphate). Oxidation of the reduced compound (II) also gave 1 mol.prop. of formaldehyde/2 mol.prop. of phosphate and resulted in the reduction of 2 mol.prop. of periodate. These results are consistent with the structure (II) for the reduced compound and indicate phosphate substitution at the 3-hydroxyl group of the *N*-acetylglucosamine. Formation of formaldehyde on oxidation of compound (II) rules out substitution at position 6 of *N*-acetylglucosamine and the 4-substituted compound would reduce only 1 mol.prop. of periodate.

The nature of the saccharinic acid formed on degradation of phosphate (I) and of the intact teichoic acid in alkali is also consistent with 3-substitution. The saccharinic acid had chromatographic and electrophoretic properties identical with those of glucometasaccharinic acid. It is known that 3-*O*-substituted glucose gives glucometasaccharinic acid on treatment with alkali (Kenner & Richards, 1954), whereas 4-*O*-substituted glucose gives glucoisosaccharinic acid (Kenner & Richards, 1955). Degradation of *N*-acetylglucosamine with alkali gives glucometasaccharinic acid and similar degradation of tri-*N*-acetylchitotriose gives largely glucoisosaccharinic acid (Whistler & BeMiller, 1963). These two saccharinic acids are readily distinguished by the periodate-Schiff spray reagents, since oxidation of glucoisosaccharinic acid yields a malondialdehyde intermediate and therefore gives a bright yellow colour (Hardy & Buchanan, 1963); no such intermediate arises during oxidation of glucometasaccharinic acid and so this gives a purple-blue colour with the reagent. Control experiments in the present work have shown that degradation of *N*-acetylglucosamine with alkali under the conditions used in the study of the teichoic acid yields a saccharinic acid that gives a purple-blue colour with the periodate-Schiff reagents, whereas degradation of chitobiose yields in addition a saccharinic acid giving an intense yellow colour. No yellow colour was given by the saccharinic acid from the teichoic acid or from phosphate (I), and it is concluded that glucoisosaccharinic acid is not produced. Caution, however, must be used in the interpretation of this finding, since during the treatment with alkali some or all of the phosphodiester groups could hydrolyse

through the usual cyclic phosphate route involving the secondary hydroxyl group of glycerol and thus produce free *N*-acetylglucosamine that would itself give metasaccharinic acid under the alkaline conditions.

In addition to saccharinic acid, hydrolysis of the teichoic acid with alkali produces substantial amounts of glycerol diphosphates. On incubation of such a hydrolysate with phosphomonoesterase 84% of the phosphorus was converted into inorganic phosphate. Acid hydrolysis showed that a small but significant amount of the *N*-acetylglucosamine had not been destroyed by the alkali, and ion-exchange chromatography gave two organic phosphates (V and IX; Scheme 2) each containing glucosamine and glycerol; these were isolated by preparative paper chromatography. Compound (V) contained equimolar amounts of phosphate, *N*-acetylglucosamine and glycerol, and was hydrolysed under controlled acidic conditions to *N*-acetylglucosamine and glycerol monophosphates; with alkali it gave glucometasaccharinic acid and glycerol monophosphates. It reduced 2 mol.prop. of periodate/mol.prop. of phosphate, giving 1 mol.prop. of formaldehyde. Under controlled conditions with 1 mol.prop. of periodate oxidation of the glycerol residue took place preferentially, and treatment of the resulting glycolaldehyde derivative (VI) with *NN*-dimethylhydrazine to effect α -elimination (LeCocq & Ballou, 1964) gave *N*-acetylglucosamine 1-phosphate (VII). This was isolated chromatographically and characterized by enzymic dephosphorylation and by treatment with acid under mild conditions; equimolar amounts of *N*-acetylglucosamine and inorganic phosphate were formed in each case.

Compound (IX) was also non-reducing and labile to acid and alkali. With acid glucosamine was formed but no *N*-acetylglucosamine was detected. Oxidation with sodium periodate gave 1 mol.prop. of formaldehyde/mol.prop. of phosphate and 3 mol.prop. of periodate was reduced. These results indicate that compound (IX) is similar to compound (V) but lacks the *N*-acetyl group, removal of the *N*-acetyl group having occurred during the initial hydrolysis in alkali. Compound (IX) gave a blue colour with the ninhydrin spray reagent, confirming the presence of a free amino group. On treatment with sodium nitrite (Foster, Martlew & Stacey, 1953) its hydrochloride was decomposed to glycerol 1-phosphate and 2,5-anhydromannose (X); the latter was identified by reduction with sodium borohydride to 2,5-anhydromannitol. The exclusive production of glycerol 1-phosphate indicates that the phosphodiester linkage is to the primary hydroxyl group of glycerol and confirms the glycosidic attachment of the glucosamine to the phosphate group. The glycerol phosphate was

Scheme 2. Degradation scheme for the products of hydrolysis of the teichoic acid with alkali. Ac, Acetyl.

isolated and found not to act as a substrate for D-glycerol 1-phosphate dehydrogenase; its stereochemistry is therefore that shown in structure (XI). The optical rotation of the phosphate (IX), $[M]_D^{18} + 30200^\circ$, is comparable with that of α -D-glucosamine 1-phosphate, $[M]_D^{18} + 29730^\circ$ (Maley, Maley & Lardy, 1956), and the anomeric linkage therefore has the α -configuration.

Phosphates containing glucosamine were also isolated by ion-exchange chromatography of an alkali hydrolysate that had not been treated with phosphatase. Gradient elution from DEAE-cellulose (CO_3^{2-} form) with ammonium carbonate gave glucometasaccharinic acid, glycerol mono- and di-phosphates, and two phosphodiester (IV and VIII) containing glucosamine. Treatment of the phosphate (IV) with phosphomonoesterase converted 50% of its phosphate into inorganic phosphate and produced compound (V). The phosphate (IX) was formed from compound (VIII) in a similar manner. The phosphate (IV) contained phosphate, *N*-acetylglucosamine and glycerol in the molecular proportions 2:1:1, was stable towards alkali but labile to dilute acid. A sample of the glycerol diphosphates formed from compound (IV) by hydrolysis in 0.05*N*-hydrochloric acid at 100° for 1 hr. was chromatographed together with the glycerol diphosphates formed by hydrolysis in 2*N*-hydrochloric acid at 100° for 3 hr. Both samples contained glycerol 1,2-diphosphate and glycerol 1,3-diphosphate, but there was considerably more of the 1,2-diphosphate in the more dilute acid hydrolysate of compound (IV), showing that complete equilibration of the phosphate ester groups does not take place under the mild hydrolytic conditions. Oxidation of both compound (IV) and compound (VIII) takes place without the production of formaldehyde; compound (IV) reduced 1 mol.prop. of periodate and compound (VIII) reduced 2 mol.prop. of periodate. On treatment of compound (VIII) with nitrous acid, glycerol diphosphate and 2,5-anhydromannose are formed.

These results confirm the structures given for compounds (IV) and (VIII). About 30% of the total phosphate and glucosamine in the teichoic acid is converted into these compounds on degradation with alkali. The results described above show that all of the *N*-acetylglucosamine residues in the teichoic acid are linked glycosidically to phosphate and indicate that the teichoic acid has structure (XII). Treatment of the polymer with acid under controlled conditions causes hydrolysis of the *N*-acetylglucosamine 1-phosphate bonds, but has little effect on the other, more stable, phosphodiester linkages. During the reaction 50% of the phosphodiester groups are converted into monoesters, and the polymer is degraded to compound (I); further hydrolysis gives glycerol diphosphates and glucosamine.

Structure (XII) also accounts for the observed products of hydrolysis in alkali. The two major paths (see Scheme 3) are those in which the secondary hydroxyl groups of the glycerol residues engage in cyclic phosphate formation with one or other of the phosphodiester groups attached to each primary hydroxyl group. Cyclic phosphate formation with the phosphate attached to the reducing group of the sugar gives rise to compound (XIII), and this is further degraded to glycerol diphosphates and saccharinic acid. Cyclic phosphate formation with the phosphate attached to the hydroxyl group at position 3 of the *N*-acetylglucosamine gives compound (XIV); this can open to give the alkali-stable compound (XV) or to give compound (XVI), which is further hydrolysed to saccharinic acid and glycerol diphosphates. This pathway accounts for the formation of appreciable proportions of compounds (IV) and (VIII). It is likely that both these routes would be followed during the degradation of the teichoic acid with alkali. Teichoic acid obtained by extraction of walls with trichloroacetic acid terminates at one end of the chain in an *N*-acetylglucosamine residue with a free reducing group; this is thus directly susceptible to alkali degradation. Similarly compound (XIII) may undergo saccharinic acid formation before or after hydrolysis of the phosphodiester.

The location of the *D*-alanyl ester on the primary hydroxyl group of the *N*-acetylglucosamine is based on its comparative stability towards hydroxylamine. Studies with model compounds have shown (Shabarova *et al.* 1962) that aminoacyl ester linkages adjacent to either hydroxyl or phosphate groups show greatly enhanced lability towards the nucleophile. This lability is shown by both glycerol and ribitol teichoic acids in which alanyl ester groups are attached directly to the polyol units. In the membrane teichoic acids of group D streptococci (Wicken & Baddiley, 1963), where each glycerol unit bears glycosidic sub-

(XII; Ac = acetyl)

Scheme 3. Mechanism of hydrolysis of the teichoic acid in alkali. Ac, Acetyl.

stituents and the alanyl esters are thus attached to sugar rather than to polyol, no enhanced lability is observed and the rate of hydrolysis of the ester linkages is comparable with that of alanine ethyl ester. Thus it is improbable that alanine could be attached to the secondary hydroxyl group of glycerol in the *Staph. lactis* teichoic acid and the observed stability is consistent with attachment of alanine to the primary hydroxyl group of the *N*-acetylglucosamine residues. More direct evidence could be obtained by further study of the products of carefully controlled acid hydrolysis of the teichoic acid, since hydrolysis of the sugar phosphate linkages can be achieved without complete hydrolysis of the alanyl linkages. Under neutral or slightly acid conditions for the isolation of the hydrolysis products it has been shown (experiments with Dr J. Anderson) that appreciable quantities of a repeating unit bearing alanine ester residues can be obtained.

The occurrence of sugar 1-phosphate linkages in microbial polymers has previously been reported in yeast phosphomannans and phosphogalactans (Wickerham & Burton, 1962; Slodki, 1966). Knox & Hall (1965) suggest that the polysaccharide in walls of *Lactobacillus casei* N.I.R.D. RO94 is attached to the peptidoglycan by a sugar 1-phosphate linkage. The wall teichoic acid of *Staphylococcus hyicus* A.T.C.C. 11249 gives chromatographically similar products to that of *Staph. lactis* I3 on hydrolysis with both acid and alkali (Oeding, Myklestad & Davison, 1967) and is probably similar in structure. Evidence for *N*-acetylglucosamine 1-phosphate linkages has also been obtained in studies on polymers in the walls of *Staph. lactis* N.C.T.C. 2102 and *Micrococcus* sp. 24 (Archibald, Baddiley, Button, Heptinstall & Stafford, 1968).

The distribution of polymers containing sugar 1-phosphate linkages may be more widespread than present reports suggest, since the conditions that have been used in the isolation and purification of bacterial cell-wall material are frequently sufficiently drastic to bring about hydrolysis of these labile linkages. An early indication of the nature of the *Staph. lactis* teichoic acid followed from the observation that, even after reduction with borohydride, alkali caused extensive browning and formation of saccharinic acid. This behaviour is readily observed and should be valuable in the rapid characterization of similar compounds in bacterial walls. Non-reducing oligosaccharides are stable towards alkali, so that the ability to form saccharinic acid after reduction with borohydride indicates that reducing groups of sugar residues are liberated by alkali. Glycosidic linkages are normally stable towards alkali, and consequently the formation of reducing groups in the present case is

due to hydrolysis through intermediate cyclic phosphates of sugar 1-phosphate linkages.

The biosynthetic implications of a teichoic acid with the structure (XII) are of interest. The stereochemistry of the substituted glycerol di-phosphate residues is such that the phosphate linking glycerol to the 3-hydroxyl group of the *N*-acetylglucosamine should originate in the glycerol phosphate unit from CDP-glycerol, i.e. D-glycerol 1-phosphate. The configuration of the glycerol phosphate moiety attached to the reducing group of the acetamido sugar is that of the 'unnatural' L-glycerol 1-phosphate, and this phosphate must therefore originate by a process other than incorporation of glycerol phosphate from CDP-glycerol. Biosynthetic studies (Baddiley, Blumsom & Douglas, 1968) have shown that this is indeed the case, the *N*-acetylglucosamine 1-phosphate residues arising by incorporation into the polymer of both *N*-acetylglucosamine and phosphate from UDP-*N*-acetylglucosamine.

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APPENDIX

The Characterization of a *Micrococcus (Staphylococcus lactis)* sp. I3 with an Atypical Teichoic Acid in its Wall

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From a study of the wall and membrane teichoic acids in staphylococci and micrococci (Davison & Baddiley, 1963; Davison, Baddiley, Hofstad, Losnegard & Oeding, 1964; Davison & Baddiley, 1964) it was concluded that all contain membrane teichoic acid in which the polyol is glycerol. On the other hand, the presence and structure of the wall teichoic acids are characteristics of the different species. Thus walls of strains of *Staphylococcus aureus* contain ribitol teichoic acids in which the configuration of the *N*-acetylglucosaminyl substituents determines serological specificity. Similarly, in strains of *Staphylococcus epidermidis* the sugar is glucose and the polyol is glycerol; the configuration of the glucosyl substituents determines the serological specificity (Oeding, Myklestad & Davison, 1967).

Acetoin-negative cocci described collectively in these studies as *Staphylococcus lactis* did not conform to a systematic pattern in the composition of their walls; some contained no teichoic acid, whereas others contained either glycerol or ribitol teichoic acid. One freshly isolated coccus (strain I3)

contained in its wall a glycerol teichoic acid the structure of which differed fundamentally from the structures of all examples hitherto encountered. This organism has been the subject of much chemical and biochemical investigation in these Laboratories (Archibald, Baddiley & Button, 1965, 1968*a,b*; Button, Archibald & Baddiley, 1966; Blumsom, Douglas & Baddiley, 1966; Brundish, Shaw & Baddiley, 1967; Baddiley, Blumsom & Douglas, 1968). Its isolation and characterization are described.

Strain I3 is a Gram-positive catalase-positive coccus isolated from the uropygeal gland of an eviscerated chicken. The cocci are encapsulated and form tetrads. They grow in 15% (w/v) sodium chloride and do not produce coagulase and phosphatase. Glucose is oxidized when examined by the recommended method (Subcommittee on Bacteriological Nomenclature, 1965), indicative of a micrococcus. Similarly, acid is produced aerobically from mannitol, but not from lactose; no acetoin is formed from glucose. Since, however, cocci with these physiological characteristics (sub-group 5)