Biochem. J. (1966) **99**, 108

The Glycerol Teichoic Acid from the Cell Wall of *Bacillus stearothermophilus* B65

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(*Received* 28 *September* 1965)

1. A glycerol teichoic acid has been extracted from cell walls of *Bacillus stearothermophilus* B65 and its structure examined. 2. Trichloroacetic acid-extractable teichoic acid accounted for 68% of the total cell-wall phosphorus and residual material could be hydrolysed to a mixture of products including those characteristic of glycerol teichoic acids. 3. The extracted polymer is composed of glycerol, phosphoric acid, D-glucose and D-alanine. 4. Hydrolysis of the polymer with alkali gave glycerol, 1-*O*-α-D-glucopyranosylglycerol and its monophosphates, glycerol mono- and di-phosphate, as well as traces of a glucosyldiglycerol triphosphate and a glucosylglycerol diphosphate. 5. The teichoic acid is a polymer of 18 or 19 glycerol phosphate units having α-D-glucopyranosyl residues attached to position 1 of 14 or 15 of the glycerol residues. 6. The glycerol residues are joined by phosphodiester linkages involving positions 2 and 3 in each glycerol. 7. D-Alanine is in ester linkage to the hydroxyl group at position 6 of approximately half of the glucose residues. 8. One in every 13 or 12 polymer molecules bears a phosphomonoester group on position 3 of a glucose residue, the possible significance of which in linkage of the polymer to other wall constituents is discussed.

Teichoic acids occur widely in Gram-positive bacteria. Whereas glycerol teichoic acids appear to occur frequently, in small quantities, in a position considered to be intracellular to the cell wall, they are less commonly found than the ribitol teichoic acids in the cell wall itself (for review see Baddiley, 1962). The structure of only two cell-wall glycerol teichoic acids, those from *Staphylococcus lactis* and *Lactobacillus buchneri*, has been reported in detail (Ellwood, Kelemen & Baddiley, 1963; Shaw & Baddiley, 1964). In a survey of the cell-wall constituents of some thermophilic bacilli (Forrester & Wicken, 1966), the presence of a glycerol teichoic acid in the cell wall of *Bacillus stearothermophilus* B65 was reported. Alkali degradation of this teichoic acid gave a mixture of products including a glucosylglycerol that appeared to have glucose attached to a primary hydroxyl group of glycerol. It was suggested that this polymer might be linked by phosphodiester bonds involving positions 2 and 3 of glycerol rather than the more usual 1- and 3-positions. The present paper describes investigations into the detailed structure of this teichoic acid.

MATERIALS AND METHODS

Materials. Alkaline phosphomonesterase (EC 3.1.3.1), β-glucosidase (EC 3.2.1.21), D-amino acid oxidase (EC 1.4.3.3), D-glucose oxidase (EC 1.1.3.4), glycerol dehydrogenase (EC 1.1.1.6) and NAD were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.

Paper chromatography. The following solvent systems were used: A, propan-1-ol–aq. ammonia (sp.gr. 0·88)–water (6:3:1, by vol.) (Hanes & Isherwood, 1949), Whatman no. 4 paper, ascending; B, pyridine–ethyl acetate–water–acetic acid (5:5:3:1, by vol.) (Fischer & Nebel, 1956), Whatman no. 1 paper, descending; C, butan-2-ol–formic acid–water (7:1:2, by vol.) (Roberts, Cowie, Abelson, Bolton & Britten, 1955), Whatman no. 1 paper, descending; D, phenol–water–aq. ammonia (sp.gr. 0·88) (80:20:0·3, w/v/v) (Roberts *et al.* 1955), Whatman no. 1 paper, descending; E, butan-1-ol–ethanol–water–aq. ammonia (sp.gr. 0·88) (40:10:49:1, by vol., upper layer) (Foster, Horton & Stacey, 1957), Whatman no. 4 paper, descending; F, butan-1-ol–pyridine–water (6:4:3, by vol.) (Jeanes, Wise & Dimler, 1951), Whatman no. 4 paper, descending; G, ethyl acetate–pyridine–water (7:2:1, by vol.) (Viscontini, Hoch & Karrer, 1955), Whatman no. 4 paper, ascending. Whatman no. 4 paper was washed before use with 2N-acetic acid containing EDTA (0·1%, w/v) and then with water.

The following spray reagents were used where appropriate: periodate–Schiff reagent for polyols (Baddiley, Buchanan, Handschumacher & Prescott, 1956); perchloric acid–molybdate reagent for phosphate (Hanes & Isherwood, 1949); alkaline silver nitrate for sugars (Trevelyan, Procter & Harrison, 1950); ninhydrin for amino acids (Consden & Gordon, 1948).

Growth of organisms and preparation of cell walls. B. stearothermophilus B65 was grown in a trypticase–yeast-extract broth of composition essentially as described by Forrester & Wicken (1966) with the exception that yeast

extract (Baltimore Biological Laboratories) was replaced by Basamin (Anheuser–Busch Inc., St Louis, Mo., U.S.A.) at the same concentration. Cells were grown in 10 l. batches of broth medium at 55° in a New Brunswick Fermentor (stirring rate 400 rev./min.; aeration rate 1 l. of air/l. of culture/min.) until near the end of the exponential phase of growth and rapidly harvested (within one generation time) through cooling coils, immersed in salt–ice, on to cracked ice. The cells were collected by centrifugation in the cold on a Sharples continuous-flow centrifuge and cell walls prepared from them as described by Forrester & Wicken (1966).

Analysis. The following analytical procedures were used: for phosphorus, that of Chen, Toribara & Warner (1956) and Fiske & Subbarow (1925); for alanine, that of Rosen (1957); for sugars, that of Dubois, Gilles, Hamilton, Rebers & Smith (1956); for D-glucose, the glucose oxidase procedure of Huggett & Nixon (1957); for periodate, that of Dixon & Lipkin (1954); for formic acid, that of Whelan (1962); for formaldehyde and glycerol, that of Hanahan & Olley (1958). Phosphomonoesters were dephosphorylated with alkaline phosphomonoesterase as described by Wicken & Baddiley (1963).

EXPERIMENTAL AND RESULTS

Isolation of the teichoic acid

Freeze-dried walls (860 mg.) were suspended in 10% (w/v) trichloroacetic acid solution (100 ml.) and stirred for 24 hr. at 4°. The insoluble residue was removed by centrifugation and extracted with fresh portions (100 ml.) of trichloroacetic acid solution at 4° for periods of 48 hr. and 72 hr. Teichoic acid was precipitated from the clarified extracts as a gum by the addition of 5 vol. of ice-cold ethanol. Dehydration of the gum by trituration with ice-cold acetone yielded a white powder which was washed with acetone and then ether and dried *in vacuo* over phosphoric oxide. The 24, 48 and 72 hr. periods of extraction gave 123, 95 and 54 mg. of teichoic acid respectively. The combined preparations were dissolved in 10% (w/v) trichloroacetic acid solution (5 ml.), centrifuged and teichoic acid was reprecipitated from the clear supernatant by the addition of 5 vol. of ice-cold acetone. The precipitate (238 mg.) was washed with acetone and ether and dried as before. Before extraction the cell walls contained 1·28 μmoles of phosphorus/mg. dry wt. of wall; after extraction this value fell to 0·4 μmole of phosphorus. Chromatograms of acid hydrolysates of the wall residues after extraction showed the presence of degradation products characteristic of glycerol teichoic acids.

Analysis of the teichoic acid

A sample of the teichoic acid (3 mg.) was hydrolysed with 2N-hydrochloric acid (0·2 ml.) for 3 hr. at 100°. The hydrolysate was neutralized with 0·2N-sodium hydroxide and the volume adjusted to 5 ml. The molar proportions of phosphorus, alanine, sugar and glycerol in the hydrolysate were 1:0·37:0·79:0·93. Estimation of sugar by the glucose oxidase method showed that at least 96% of the sugar was D-glucose. The ammonium salt of the polymer, after removal of alanine residues (see below), had $[\alpha]_D^{30}+70{\cdot}2°$ (*c* 0·98 in water).

Structural investigations

Acid hydrolysis. Hydrolysis of the teichoic acid (3 mg.) was carried out with 2N-hydrochloric acid (0·2 ml.) at 100° for 3 hr. After evaporation *in vacuo* over sodium hydroxide, the products were examined chromatographically in solvents A and B and identified by reference to known compounds. (The mobilities of teichoic acid-degradation products in these solvents have been well documented; cf. Critchley, Archibald & Baddiley, 1962; Ellwood *et al.* 1963.) Glycerol, glycerol mono- and di-phosphates, inorganic phosphate, alanine and glucose were detected. Enzymic dephosphorylation of material eluted from the glycerol mono- and diphosphate regions of parallel chromatograms yielded glycerol and inorganic phosphate as the sole products. Glycerol, obtained in a similar manner from a larger quantity of teichoic acid, was further characterized as the tribenzoate (m.p. 73°, undepressed by mixing with authentic glycerol tribenzoate; cf. Critchley *et al.* 1962) and also by its ready reduction of NAD in the presence of glycerol dehydrogenase.

Action of ammonia. Alanine residues were removed from the teichoic acid (2 mg.) by treatment with ethanol–aq. ammonia (sp.gr. 0·88) (1:1, v/v) for 5 hr. at room temperature (Wicken & Baddiley, 1963). Chromatography in solvent A showed alanine and alanine amide. Two-dimensional chromatography in solvents C and D confirmed the absence of any other amino acid or amide. Milder conditions of ammonolysis that have been utilized to remove alanine residues from other teichoic acids (Shaw & Baddiley, 1964) were not completely effective with *B. stearothermophilus* teichoic acid.

Alanine, isolated as above from a larger sample of teichoic acid (50 mg.), was converted into its hydrochloride by the method of Shaw & Baddiley (1964). It had m.p. 195–197°, undepressed by mixing with authentic D-alanine hydrochloride. The D configuration for this amino acid was established with D-amino acid oxidase by the method of Wicken & Baddiley (1963).

Potentiometric titration. Teichoic acid (100 mg.), from which alanine had been removed, was titrated with sodium hydroxide solution from an Agla micrometer syringe (cf. Wicken & Baddiley, 1963; Critchley *et al.* 1962). Two peaks were observed

when $\Delta pH/\Delta v$ was plotted against v, corresponding to the proportions of primary and secondary acidic groups in the molecule. The ratio of the two maxima was 17:1.

Alkaline hydrolysis. Teichoic acid (2mg.) was heated in N-sodium hydroxide (0·2ml.) at 100° for 3hr. Na^+ ions were removed by passage of the hydrolysate through a short column (2cm. × 1cm.) of Dowex 50 (NH_4^+ form) resin. Chromatography in solvent A revealed minor products with the mobilities of glycerol and a glucosylglycerol (R_F 0·67) and at least three organic phosphates, two of which corresponded in mobility to glycerol mono- and di-phosphate. Enzymic dephosphorylation of a similar alkali hydrolysate of the teichoic acid gave glycerol and the glucosylglycerol already referred to as the only readily detectable products. The glucosylglycerol gave a rapid purple colour with the periodate–Schiff reagent.

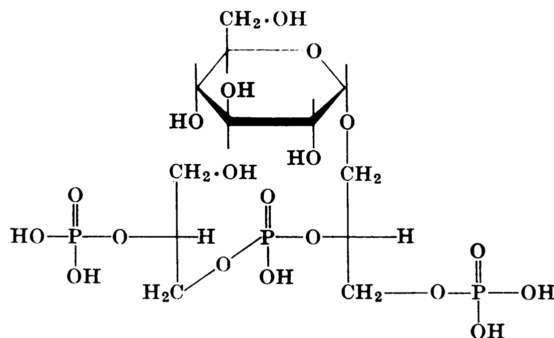
1-O-α-D-*Glucopyranosylglycerol.* Glucosylglycerol isolated from an enzymically dephosphorylated alkali hydrolysate of teichoic acid (40mg.) by chromatography in solvents A and E was further purified on charcoal–Celite (1:1, w/w) (Critchley *et al.* 1962). Yield, 13·1mg. In a sample of the glucoside that had been hydrolysed in 2N-hydrochloric acid for 3hr. at 100° the ratio glucose: glycerol was 1:1·05. Incubation of the glucoside with β-glucosidase at 37° for 24hr. (Shaw & Baddiley, 1964) followed by chromatography in solvents B and E showed no detectable reducing sugar or glycerol.

The glucoside (8mg.) in water (5ml.) was mixed with 0·375M-sodium metaperiodate (1ml.), diluted to 25ml. with water and kept at room temperature in the dark. Portions were removed at intervals for the measurement of the consumption of periodate and the production of formaldehyde and formic acid. After 48hr. oxidation was complete, 3·04mol.-prop. of periodate having been consumed with the production of 0·99 mol.prop. of formaldehyde and 0·98 mol.prop. of formic acid. The remainder of the oxidation mixture was treated with sulphur dioxide to destroy excess of periodate, aerated, and reduced with a small excess of sodium borohydride (10mg.) during 24hr. at room temperature. The solution was acidified with acetic acid, and Na^+ ions were removed by passage through a short column (1cm. × 5cm.) of Dowex 50 (H^+ form) resin. Borate was removed from the freeze-dried eluate by repeated evaporation with methanol. The residue was hydrolysed in 2N-hydrochloric acid for 3hr. at 100°. Chromatographic examination of the products in solvent G showed only glycerol and ethylene glycol. No glucose was detected.

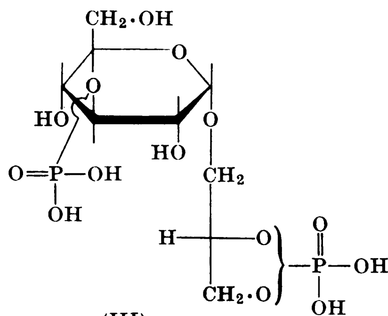
Separation and identification of the organic phosphates. Teichoic acid (50mg.), from which alanine had been removed, was hydrolysed with N-sodium hydroxide as described above. After removal of Na^+ ions, the hydrolysate was introduced on to a column (30cm. × 1·3cm.) of DEAE-cellulose (HCO_3^- form) and the neutral components were eluted with water (300ml.). The eluate was concentrated by rotary evaporation and examined by chromatography in solvents A and E. Glycerol and 1-*O*-α-D-glucopyranosylglycerol were the only components detected. Linear-gradient elution of phosphate esters from the column was carried out with water (500ml.) in the mixing vessel and 0·15M-ammonium carbonate solution (500ml.) in the reservoir according to the method of Shaw & Baddiley (1964). Fractions (5ml.) were collected automatically at a rate of 51ml./hr. Three phosphate fractions were eluted: fraction 1 eluted with 0·03M-ammonium carbonate represented 93·3% of the total phosphorus applied to the column; fractions 2 and 3 were eluted with 0·055M- and 0·08M-ammonium carbonate and represented 1·2% and 5·5% of the total phosphorus respectively.

Fraction 1. 1-*O*-α-D-Glucopyranosylglycerol monophosphates and glycerol monophosphates. Material from this fraction was chromatographed in solvent A and showed two components of R_F 0·31 and 0·23. The material of higher R_F gave a rapid purple colour with the periodate–Schiff reagent, was chromatographically indistinguishable from glycerol monophosphates and on incubation with alkaline phosphomonoesterase gave glycerol and inorganic phosphate. Material of R_F 0·23 gave a slow blue colour with the periodate–Schiff reagent and on enzymic dephosphorylation was converted into 1-*O*-α-D-glucopyranosylglycerol and inorganic phosphate. It had glucose:glycerol:phosphate proportions 1·06:0·98:1·00. In addition, fraction 1 also contained a trace amount of inorganic phosphate produced during hydrolysis of the polymer.

Fraction 2. Glucosyldiglycerol triphosphate (I). This component had R_F 0·12 in solvent A and gave a slow reaction with the periodate–Schiff reagent. Incubation with phosphomonoesterase completely converted this component into a compound of higher mobility (R_F 0·54 in solvent A) which gave rapidly a purple colour with the periodate–Schiff reagent. Hydrolysis of this new compound (II) in 2N-hydrochloric acid for 3hr. at 100° gave a mixture of glucose, glycerol and glycerol monophosphates. The glucose:glycerol:phosphate proportions for material eluted from paper chromatograms was found to be 1·2:2·2:1·0. Hydrolysis with N-sodium hydroxide gave 1-*O*-α-D-glucopyranosylglycerol and glycerol monophosphates. Further structural studies on this compound were not possible owing to the small amounts of fraction 2 present in alkali hydrolysates of the teichoic acid. Shaw & Baddiley (1964) reported R_F 0·53 in solvent A for the isomeric glucosyldiglycerol monophosphate.



(I)



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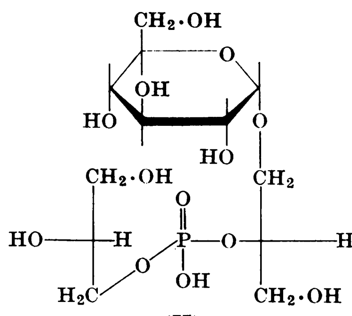


(II)

where glucose is attached to the 2 position of one of the glycerol residues.

Fraction 3. Glycerol diphosphates and 1-*O*-α-D-glucopyranosylglycerol diphosphates (III). Chromatography of this fraction in solvent A showed a double spot in the region of glycerol diphosphates (R_F 0·10) which did not react with the periodate–Schiff reagent. Incubation with phosphomonoesterase and rechromatography in solvent A showed glycerol and 1-*O*-α-D-glucopyranosylglycerol. Fraction 3 was dissolved in 0·1M-sodium metaperiodate solution (1ml.) and left at room temperature in the dark for 72hr. Solid sodium borohydride (5mg.) was added and the solution left at room temperature for a further 24hr. Sodium borate was removed as described above and the residue was hydrolysed with 2N-hydrochloric acid for 3hr. at 100°. Chromatography in solvents A, B and G showed glucose, glycerol, glycerol mono- and di- phosphate.

Periodate oxidation of the teichoic acid. Teichoic acid (2mg.), from which alanine had been removed, was dissolved in 0·2M-sodium acetate buffer (1ml.), pH 5·8. 0·1M-Sodium metaperiodate (0·5ml.) was added and the solution allowed to stand at room temperature in the dark for 72hr. Teichoic acid (2mg.), with alanine residues still intact, was similarly treated. Further treatment with sodium borohydride, acid hydrolysis and chromatography in solvents A, B and G was carried out as described above. Glycerol and trace quantities of unoxidized glucose were the only products observed. Visual comparison of the intensity of the spots on these chromatograms indicated approximately equal concentrations of glycerol and glucose from both oxidized samples of teichoic acid. No erythritol was detected in either case.

Quantitative estimation of periodate consumed by teichoic acid in sodium acetate buffer (0·2M, pH 5·8) showed that oxidation was complete after 72hr. in the teichoic acid from which alanine had been removed, 2·1 moles of periodate being consumed/mole of polymer glucose. Under similar conditions teichoic acid with alanine residues still intact consumed 2·7 moles of periodate/mole of polymer glucose and the consumption of periodate still slowly increased after this time.

A parallel oxidation of a larger quantity of teichoic acid (10mg.) was carried out to determine the proportion of glucose in the polymer resistant to periodate oxidation. Suitable portions of the reaction mixture, taken before and after periodate oxidation and subsequent reduction with sodium borohydride, were hydrolysed with 2N-hydrochloric acid for 3hr. at 100°. Hydrolysates were neutralized by passage through short columns (1cm. × 2cm.)

of Dowex 3 (OH^- form) resin, and chromatographed in solvent F. Glucose regions were eluted with water and the eluates freeze-dried. Glucose was determined by the glucose oxidase method. Of the total polymer glucose 0·55% was found to be resistant to periodate oxidation.

DISCUSSION

The glycerol teichoic acid in the walls of *B. stearothermophilus* B65 was extracted with dilute trichloroacetic acid and purified by precipitation with ethanol. This procedure is now a standard one for the extraction of teichoic acids from bacterial cell walls. Other workers (cf. Shaw & Baddiley, 1964; Ellwood *et al.* 1963) have reported that such extraction procedures remove almost all of the phosphorus from cell walls as teichoic acid, the small amount of residual phosphorus in the extracted walls not being associated with teichoic acid. The teichoic acid from *B. stearothermophilus* cell walls proved much more resistant to extraction with trichloroacetic acid. A total of 144hr. of extraction removed 69% of the wall phosphorus as teichoic acid; the residual 31% of wall phosphorus was still associated, at least in part, with teichoic acid since chromatographic analysis of acid hydrolysates of extracted walls showed the degradation products typical of a glycerol teichoic acid.

Acid hydrolysis of the extractable teichoic acid yielded glycerol and its mono- and di- phosphates, which are the products expected from a polymer of glycerol units joined together through phosphodiester linkages. Glycerol derived from the polymer was characterized by its chromatographic mobility, the m.p. of its tribenzoate derivative and its ready oxidation by glycerol dehydrogenase. Glycerol mono- and di- phosphates, identified chromatographically, were completely converted into glycerol and inorganic phosphate by the action of phosphomonoesterase. The only other products of acid hydrolysis of the polymer were glucose and alanine. Both compounds were shown to have the D configuration by the action of D-glucose oxidase and D-amino acid oxidase respectively. Alanine was further characterized by the m.p. of its hydrochloride.

As is characteristic of teichoic acids, alanine was in ester linkage with a hydroxyl group of the polymer. Treatment of the teichoic acid with ammonia gave a mixture of alanine and its amide but the conditions of ammonolysis or hydrolysis required for complete removal of alanine were more rigorous than those reported for the hydrolysis of alanyl-(polyol)hydroxyl group bonds (cf. Shaw & Baddiley, 1964; Critchley *et al.* 1962). Analysis of the polymer showed that approximately 80% of the glycerol moieties were substituted with glucose. It is clear that there would be insufficient glycerol hydroxyl groups available for attachment of the amount of alanine found, the latter, by the conditions of extraction employed, being regarded as a minimal value. Attachment of some, if not all, of the alanine to the polymer must therefore be to a glucose hydroxyl group. Although unusual, a similar finding has been reported for the glycerol teichoic acids from group D streptococci (Wicken & Baddiley, 1963) where similar relatively vigorous conditions of ammonolysis were required for the complete removal of alanine.

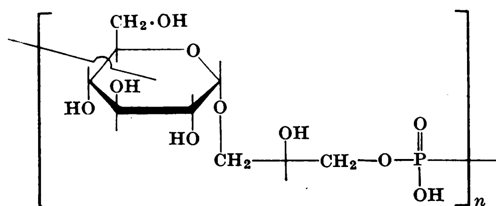
An attempt to determine the position of alanyl substitution of glucose was made by a comparison of the products of periodate oxidation and subsequent borohydride reduction of intact teichoic acid and teichoic acid from which alanine had been removed. (The use of acetate buffer at pH 5·8 has been shown, by Armstrong, Baddiley & Buchanan, 1961, to preserve alanyl ester bonds in ribitol teichoic acids during periodate oxidation.) Both experiments gave, after acid hydrolysis, approximately equivalent amounts of glycerol and traces of glucose; the significance of the latter is considered below. Assuming monosubstitution of glucose moieties by alanine, substitution of the latter on position 3 of glucose would have prevented oxidation of approximately half of the glucose residues. The quantitatively observed reduction of periodate by intact polymer and polymer from which alanine had been removed (2·7 and 2·1 moles of periodate/mole of polymer glucose respectively) suggests that substitution of position 2 or 4 of glucose is unlikely. Alanine on either of these positions would have caused half of the glucose residues to reduce only 1 mole of periodate instead of 2. The absence of erythritol from the hydrolysate of oxidized and borohydride-reduced intact polmer also precludes position 4 for substitution by an alanyl ester. Substitution of position 6 of glucose, however, would not be expected to affect the course of periodate oxidation of the polymer. Although not conclusive, the experimental evidence suggests that it is likely that the alanyl ester residues are attached to the primary hydroxyl groups of glucose moieties in the polymer.

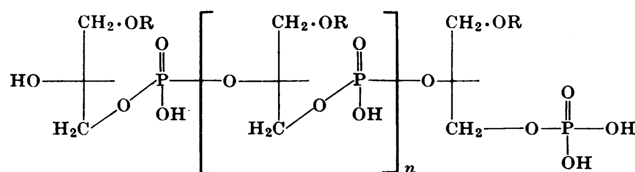
Studies on the products of hydrolysis of the teichoic acid with alkali established both the type of phosphodiester linkage present and also the location of the glucose residues. The mechanism of alkaline hydrolysis of glycerol teichoic acids has been fully discussed elsewhere (Kelemen & Baddiley, 1961; Critchley *et al.* 1962). It has been shown that increasing glycosidic substitution of a 1,3-phosphodiester-linked glycerophosphate polymer increases the resistance of the polymer to alkali degradation, substitution of the 2-hydroxyl groups of glycerol with a sugar preventing the necessary formation

of an intermediate cyclic phosphate (cf. Wicken & Baddiley, 1963). A 2,3-phosphodiester-linked glycerol teichoic acid on the other hand would be expected to be completely degraded in alkali, even if highly substituted with sugar, to a mixture of glycerol monophosphates and glycosylglycerol monophosphates together with small amounts of glycerol, glycosylglycerol and glycerol diphosphates. The teichoic acid from *B. stearothermophilus* was completely degraded in alkali to glycerol, glucosylglycerol and a mixture of five phosphate esters that were separated by a combination of paper and ion-exchange chromatography. Glycerol mono- and di-phosphates were recognized by their chromatographic mobility and ready degradation to glycerol and inorganic phosphate by phosphomonoesterase. The major phosphate ester was characterized by analysis as a glucosylglycerol monophosphate, which was converted into glucosylglycerol by the action of phosphomonoesterase.

The glucosylglycerol gave rapidly a purple colour with the periodate–Schiff reagent, which is indicative of the presence of an α-glycol end group within the molecule (Roberts, Buchanan & Baddiley, 1963). Analysis showed a glucose:glycerol ratio 1:1 and incubation with β-glucosidase failed to release reducing sugar from the glucoside. The positive specific rotation obtained for the polymer, after removal of alanine, supported an α-configuration for the glucoside. Final characterization of the glucoside as 1-*O*-α-D-glucopyranosylglycerol was obtained by periodate oxidation, the consumption of periodate and production of formaldehyde and formic acid being close to that expected. The alternative structures, 1- or 2-*O*-α-D-glucofuranosylglycerol, were precluded by these periodate-oxidation studies. Substitution with glucose of a large proportion of the glycerol residues in the polymer is strong evidence for the 2,3-phosphodiester linkage between the glycerol residues suggested by the ready degradation of the polymer in alkali (V).

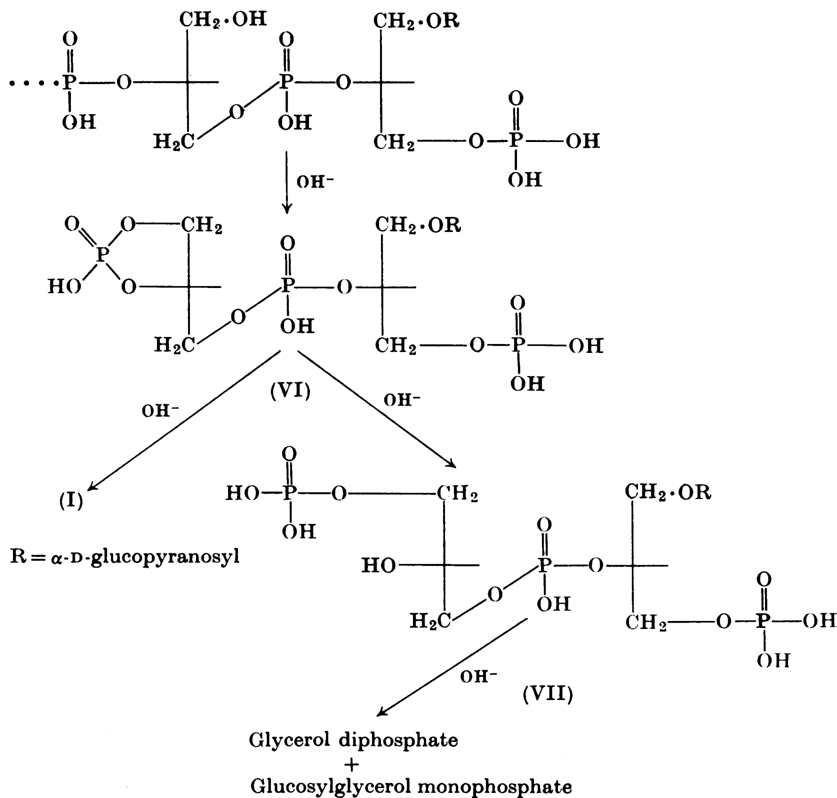
A possible alternative structure for the teichoic acid is one (IV) involving linkage of glycerol phosphate units through glucose. Such a polymer would be expected to be degraded in alkali to glucosylglycerol monophosphates almost exclusively. A structure of this general type is considered possible for a teichoic acid from the cell walls of a strain of *Lactobacillus plantarum* (J. Baddiley, personal communication). Structure (IV) can, however, be eliminated for the *B. stearothermophilus* teichoic acid by the following considerations. The lower proportion of glucose to that of glycerol in the teichoic acid and the production of both glycerol and glucosylglycerol as well as their phosphate esters on alkaline hydrolysis would demand either an irregular phosphate ester repeating unit for (IV) or a mixture of (IV) and non-glucosylated polyglycerophosphate. The former possibility is unlikely from what is known of the mechanism of biosynthesis of these polymers (cf. Burger & Glaser, 1964) and no evidence of a mixture of different polymers was obtained. Strong evidence that the extracted teichoic acid was a single polymer (V) with a backbone of glycerol phosphate residues joined by 2,3-phosphodiester bonds came from the isolation, from an alkali hydrolysate, of a small quantity of a phosphate ester having the properties of a glucosyldiglycerol triphosphate (I). Alkaline phosphomonoesterase converted (I) into glucosyldiglycerol monophosphate (II), which was characterized by its reaction with the periodate–Schiff reagent, quantitative analysis and degradation in alkali and acid. The triphosphate (I) could arise from the free phosphomonoester end of the polymer during alkaline hydrolysis (Scheme 1). Cyclization of the phosphate group in diester linkage to the secondary hydroxyl group of the penultimate glycerol residue with the free primary hydroxyl group of the same glycerol moiety would produce an intermediate cyclic phosphate (VI). (VI) could either break down to the alkali-stable triphosphate (I) or to an isomer bearing the phosphate group in a terminal position (VII), which would undergo further degradation in alkali to glycerol diphosphate and glucosylglycerol monophosphate. The finding that the glycerol residue at the phosphomonoester end of the polymer is substituted with glucose is supported by the slow and incomplete release of phosphate from the polymer by the action of





(IV)

(V)



Scheme 1. Formation of glucosyldiglycerol triphosphate (I) by alkaline hydrolysis of teichoic acid.

phosphomonoesterase. Inhibition of enzymic dephosphorylation by bulky sugar substituents has been observed in other teichoic acids (Armstrong, Baddiley & Buchanan, 1960; Baddiley, Buchanan, RajBhandary & Sanderson, 1962; Wicken & Baddiley, 1963).

The remaining phosphate ester produced by alkaline hydrolysis of the teichoic acid was not separated from glycerol diphosphates by ion-exchange chromatography and only partially separated by paper chromatography. Incubation of the mixture with phosphomonoesterase gave glycerol, from glycerol diphosphate, and 1-*O*-α-D-glucopyranosylglycerol. It was assumed, from its chromatographic behaviour, that the phosphate ester was a diphosphate of the glucoside. A glucosylglycerol diphosphate bearing phosphate groups on each of the two remaining glycerol hydroxyl groups could not conceivably arise during alkaline hydrolysis of the polymer, the necessary intermediate cyclic phosphates being unable to form. The supposition that one of the phosphate groups in this compound is present on the 3 position of the glucose moiety was supported by the lack of reactivity with the periodate–Schiff reagent (contaminating glycerol diphosphate would not be expected to be attacked by periodate) and more specifically by the observed resistance of the glucose residue to periodate oxidation. It is therefore proposed that this diphosphate has either of the structures illustrated (III) or is a mixture of the two isomers.

Potentiometric titration of the teichoic acid indicated an average chain length of 17. This is about the same size as glycerol teichoic acids previously studied (Critchley *et al.* 1962; Shaw & Baddiley, 1964; Wicken & Baddiley, 1963). It has, however, been shown that some of the glucose residues in this teichoic acid bear a phosphomonoester group which would make the above estimate of chain length a minimal one. If the amount of periodate-resistant glucose in the polymer, approximately one glucose residue in 182, is taken as a measure of the extent of glucose phosphorylation, it would appear unlikely that each molecule of the teichoic acid is phosphorylated on a glucose residue. A teichoic acid with a chain length of 18 units and having one chain in every 13 bearing a phosphate

group on a single glucose residue would be expected to have a ratio of phosphomonoester to phosphodiester groups 1:16·7. Fourteen of the 18 glycerol residues would be glucosylated and the amount of expected periodate-resistant glucose in such a preparation would be in agreement with that found. Similar calculations for an average chain length of 19 units, one chain in every 12 having the two phosphomonoester groups, show a theoretical phosphomonoester:phosphodiester ratio 1:17·5. It is concluded that the probable average chain length of the polymer is 18–19 units.

Until recently it was considered that separation of teichoic acid from other cell-wall constituents in trichloroacetic acid was due to a simple dissociation of ionic bonds or hydrogen bonds or both (Archibald, Armstrong, Baddiley & Hay, 1961). The possibility that ribitol teichoic acids in the walls of some organisms may be covalently linked to glycosaminopeptide by an acid-labile phosphodiester bond involving the phosphomonoester group at the end of an extracted teichoic acid chain has been examined in several Laboratories. The ribitol teichoic acid from the walls of *B. subtilis*, when isolated by autolysis of the walls, appeared covalently linked to fragments of glycosaminopeptide and had less than one phosphomonoester group per 500 residues of organic phosphorus (Young, Tipper & Strominger, 1964). Similar findings have been reported from *Staphylococcus aureus* cell walls (Strominger & Ghuysen, 1963). Burger & Glaser (1964) have reported a synthesis of a glycerol teichoic acid by extracts of *B. licheniformis*, where the product contains small amounts of muramic acid. It appears unlikely that multiple linkages between ribitol teichoic acids and glycosaminopeptide exist (Rogers & Garrett, 1963, 1965) and phosphodiester linkage to an alcoholic hydroxyl group of glycosaminopeptide has also been discounted (Hay, Davey, Archibald & Baddiley, 1965). Archibald & Baddiley (1965) have presented evidence for a phosphoramidate linkage between the phosphomonoester group of the ribitol teichoic acid of *L. arabinosus* and a hexosamine amino group of the glycosaminopeptide of these walls. The slow release of the glycerol teichoic acid from the walls of *B. stearothermophilus* B65 during extraction with trichloroacetic acid would be consistent with a covalent linkage between the teichoic acid and some other constituent of the wall. Autolysis of these walls resulted in complete solubilization of the phosphorus and ion-exchange chromatography of autolysates on DEAE-cellulose gave a teichoic acid fraction that was still closely associated with glycosaminopeptide fragments (A. J. Wicken, unpublished work). That some of the extractable teichoic acid from these walls has a phosphomonoester group attached to a glucose residue is thought to be of some possible significance in the linkage of this polymer to other wall constituents.

It is concluded that the trichloroacetic acid-extractable teichoic acid from the walls of *B. stearothermophilus* B65 is a polymer comprising 18 or 19 glycerol phosphate residues joined through phosphodiester linkages at positions 2 and 3 on each glycerol, and that to position 1 of 14 or 15 of these residues are attached α-D-glucopyranosyl substituents. D-Alanine is in ester linkage to approximately one-half of the glucose residues and is probably attached to position 6 of the sugar. A phosphomonoester group is attached, probably at position 3, to a glucose residue in 1 in every 13 or 12 polymer chains. The position of the phosphodiester linkages in this polymer is unusual, all other glycerol teichoic acids that have been reported in detail having been shown to be linked between positions 1 and 3 of each glycerol. The possible existence of another 2,3-phosphodiester-linked glycerol teichoic acid in *B. licheniformis* has, however, been reported (Burger, 1963).

This work was supported by grants from the Nuffield Foundation and the N.Z. University Grants Research Committee, for which grateful acknowledgement is made.

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