

The Teichoic Acid from the Walls of *Lactobacillus buchneri* N.C.I.B. 8007

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Teichoid acids are widely distributed in the walls of lactobacilli (Baddiley & Davison, 1961; Ikawa & Snell, 1960) and the intracellular variety has been found in all members of the genus so far examined. The serological importance of these compounds as group precipitinogens is now well established (Baddiley & Davison, 1961; Sharpe, Davison & Baddiley, 1964), and the detailed chemical structure of individuals is significant in efforts to relate structure with immunological specificity. The teichoic acid from the walls of *Lactobacillus buchneri* N.C.I.B. 8007 is particularly interesting. This organism is in serological group E, and it has been shown that the group precipitinogen in this case is the wall teichoic acid (Sharpe *et al.* 1964); consequently, it would be valuable to compare structural features, especially the nature and configuration of the sugar residues, with those of group E precipitinogens from other members of the group.

Detailed chemical knowledge of the teichoic acid from the walls of this organism could also be valuable in efforts to establish the mechanism of biosynthesis of these polymers. *L. buchneri* would appear to be particularly suitable for such studies as it grows well in the laboratory, its walls are easily prepared in clean condition and they contain substantial amounts of teichoic acid. Moreover, as the polymer contains glycerol and glucose, isotopically labelled nucleotide precursors are reasonably available. The simple chemical degradations described below would ensure the unambiguous characterization of the teichoic acid formed in enzyme experiments.

MATERIALS AND METHODS

Materials. *L. buchneri* N.C.I.B. 8007 was grown and harvested and walls were prepared by Dr A. L. Davison according to the methods described by Sharpe *et al.* (1964). Alkaline phosphatase was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A., β -glucosidase from L. Light and Co. Ltd., Colnbrook, Bucks., and the Glucostat reagent from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Paper chromatography. Whatman no. 1 and no. 4 papers were used in the following solvent systems: *A*, propan-1-ol-aq. ammonia (sp.gr. 0.88)-water (6:3:1, by vol.) (Hanes & Isherwood, 1949); *B*, butan-1-ol-ethanol-water-aq. ammonia (sp.gr. 0.88) (40:10:49:1, by vol., organic phase) (Foster, Horton & Stacey, 1957); *C*, butan-1-ol-pyridine-

water (6:4:3, by vol.) (Jeanes, Wise & Dimler, 1951). Compounds were detected by the following reagents where-ever appropriate: periodate-Schiff reagent for α -glycols (Baddiley, Buchanan, Handschumacher & Prescott, 1956); molybdate reagents for phosphoric esters (Hanes & Isherwood, 1949); alkaline silver nitrate for reducing sugars (Trevelyan, Procter & Harrison, 1950); ninhydrin for amino compounds (Conden & Gordon, 1948).

Analysis. The following analytical procedures were used: for phosphorus, that of Chen, Toribara & Warner (1956); for alanine, that of Rosen (1957); for reducing sugars, that of Park & Johnson (1949); for D-glucose, the Glucostat reagent; for periodate oxidation, that of Dixon & Lipkin (1954).

Enzymic dephosphorylation. Phosphomonoesters (10 μ g.) were dephosphorylated by incubation with a solution of alkaline phosphomonoesterase (0.2 ml. of a 0.1% solution) in an ammonium carbonate buffer, pH 9, at 37° overnight.

Hydrolysis with β -glucosidase. Incubations of glucosides with β -glucosidase (0.2 ml. of an aqueous 1% solution) were carried out at 37° overnight.

EXPERIMENTAL AND RESULTS

Isolation of the teichoic acid

The walls (500 mg.) were suspended in 10% (w/v) trichloroacetic acid solution (20 ml.) and stirred for 70 hr. at 4°. The insoluble residue was removed by centrifugation and washed with 10% trichloroacetic acid solution (10 ml.). The combined supernatant and washings were extracted three times with an equal volume of ether and the aqueous layer was freeze-dried. The residue was dissolved in 10% trichloroacetic acid solution (5 ml.), 2 vol. of ethanol was added and the mixture was kept at 4° for 16 hr. The precipitate was removed by centrifugation and a further 5 vol. of ethanol was added to the supernatant solution. The combined precipitates were dissolved in the minimum volume of 10% trichloroacetic acid solution and reprecipitated by the addition of 3 vol. of ethanol. The teichoic acid was collected, washed with acetone and ether, and finally dried over phosphoric oxide, yielding a white powder (142 mg.). Before extraction the walls contained 4.9% of phosphorus; after extraction this value fell to 0.17%. Acid hydrolysis of the wall residues after extraction showed the absence of degradation products characteristic of teichoic acids.

Analysis of the teichoic acid

A sample of the teichoic acid (2 mg.) was hydrolysed with 2N-hydrochloric acid (0.5 ml.) for 4 hr. at 100°. The hydrolysate was neutralized with sodium hydroxide solution and the volume adjusted to 10 ml. The proportions of phosphorus, alanine and reducing sugar were 1:0.45:0.26. These proportions were unchanged in a sample that had been passed through a column of Sephadex G-75. The reducing sugar was determined enzymically with the Glucostat reagent and shown to consist entirely of D-glucose. The ammonium salt of the polymer had $[\alpha]_D^{20} + 35.5^\circ$ (c 1.8 in water) measured after removal of alanine residues (see below). No reducing sugar was detected chromatographically after incubation of the teichoic acid with the β -glucosidase.

Identification of degradation products

Acid hydrolysis. The teichoic acid (5 mg.) was dissolved in 2N-hydrochloric acid (0.2 ml.) and heated at 100° for 3 hr. The solution was evaporated to dryness *in vacuo* over potassium hydroxide and the residue examined by paper chromatography in solvents A, B and C; glycerol, glycerol mono- and di-phosphates, inorganic phosphate, alanine and glucose were detected. The glycerol mono- and di-phosphates were eluted with water from an unsprayed paper run in solvent A and eluted material was incubated with phosphatase. Chromatography of the products in solvent A showed complete dephosphorylation to glycerol and inorganic phosphate.

Action of ammonia. The teichoic acid (2 mg.) was dissolved in 2N-ammonia (0.2 ml.) and kept at room temperature overnight. Chromatography in solvent A and spraying with ninhydrin revealed only alanine and alanine amide. Alanine was isolated and characterized in the following manner.

Teichoic acid (120 mg.) was treated with ammonia as described above. Excess of ammonia was removed by evaporation *in vacuo*. The residue was dissolved in the minimum volume of water and introduced on to a column of Dowex 50 (H⁺ form) resin (6 ml.). The polymer was eluted from the column with water (25 ml.) and recovered by freeze-drying (92 mg.). Other products were eluted with N-hydrochloric acid (40 ml.) in eight fractions. These were each evaporated to dryness *in vacuo* over potassium hydroxide and the residues examined by paper chromatography in solvent A. Fraction 4 contained most of the alanine hydrochloride (30 mg.), m.p. 194–196°, which was recrystallized from aqueous ethanol and was identical in all respects (m.p., mixed m.p. and infrared-absorption spectrum) with an authentic sample. The D-configuration was established with D-amino

acid oxidase according to the method described by Wicken & Baddiley (1963).

Hydrolysis in alkali. The polymer (75 mg.) from which alanine had been removed (see above) was hydrolysed with N-sodium hydroxide (0.5 ml.) at 100° for 3 hr., then Na⁺ ions were removed by passing the solution through a column (3 cm. × 0.5 cm.) of Dowex 50 (NH₄⁺ form) resin. Examination in solvents A, B and C revealed a complex mixture of organic phosphates as well as two neutral components having the mobilities of glycerol and a glucosylglycerol.

Separation and identification of glycerol and 2-O- α -D-glucopyranosylglycerol. The above hydrolysate was introduced on to a column (11 cm. × 1.75 cm.) of DEAE-cellulose (CO₃²⁻ form) and the neutral components were eluted with water (250 ml.). The solution was evaporated to a small volume and applied to a paper chromatogram. After running in solvent A, the two components were eluted from the appropriate areas of the paper with water which was then evaporated. Chromatography in solvents A, B and C showed the two components to be glycerol and a glucosylglycerol. Hydrolysis of the latter with 2N-hydrochloric acid at 100° for 3 hr. yielded glucose and glycerol. No reducing sugar was produced by incubating the glucosylglycerol with β -glucosidase. It was chromatographically indistinguishable from 2-O- α -D-glucopyranosylglycerol, obtained from intracellular teichoic acid from *L. arabinosus* and fully characterized by Critchley, Archibald & Baddiley (1962).

Separation and identification of the organic phosphates. After removal of the neutral components from the column, gradient elution of phosphates was carried out with water (300 ml.) in the mixing vessel and 0.15M-ammonium carbonate solution (300 ml.) in the reservoir. Fractions (4 ml.) were collected automatically at approx. 2 ml./min. The phosphorus in each fraction is shown in Fig. 1; the five peaks in the Figure represent, in order of ascending fraction numbers, 3, 52, 8, 30 and 7 %

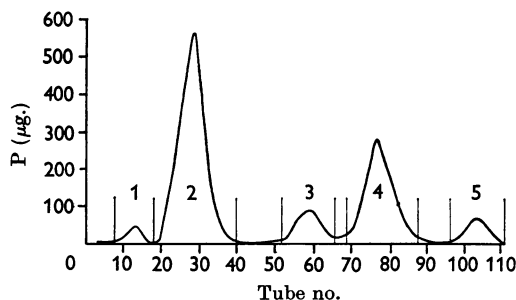


Fig. 1. Separation of the phosphate mixture produced by alkaline hydrolysis of the teichoic acid on a column of DEAE-cellulose.

respectively of the total phosphorus. The fractions were combined as shown (vertical lines) and ammonium carbonate was removed by repeated evaporation to dryness at 37°.

Peak 1: diglycosyldiglycerol phosphate (I). Material corresponding to this peak slowly gave a blue-grey colour with the periodate-Schiff reagents, similar to that given by 2-O- α -D-glucosylglycerol. Incubation with phosphatase did not produce inorganic phosphate and rechromatography indicated that the compound (R_f 0.43 in solvent A) was unchanged by the enzyme. Acid hydrolysis with 2N-hydrochloric acid at 100° for 3 hr. gave glucose, glycerol and glycerol monophosphates. Quantitative analysis gave the glucose:phosphorus ratio as 1.8:1.

Peak 2: glycerol monophosphates. Material from this fraction rapidly gave a purple colour with the periodate-Schiff reagents and was chromatographically indistinguishable from glycerol monophosphates. Incubation with phosphatase gave glycerol and inorganic phosphate. In addition, this fraction also contained a small amount of inorganic phosphate produced during hydrolysis of the polymer.

Peak 3: glucosyldiglycerol diphosphate (II). This component slowly gave a blue-grey colour with the periodate-Schiff reagents (R_f 0.25 in solvent A). After incubation with phosphatase, 50% of its phosphorus was converted into inorganic phosphate. Chromatography of the solution after treatment with the phosphatase showed that conversion into a compound of higher mobility (R_f 0.53 in solvent A) had been complete. This product rapidly gave a purple colour with the periodate-Schiff reagents, a reaction characteristic of compounds that give

formaldehyde on oxidation with periodate. Hydrolysis with 2N-hydrochloric acid at 100° for 3 hr. gave a mixture of glucose, glycerol and glycerol monophosphates, the glucose:phosphorus ratio being 0.92:1. Hydrolysis with alkali gave glucosylglycerol and glycerol monophosphates.

Peak 4: glycerol diphosphates. This fraction was chromatographically identical with authentic glycerol diphosphates and did not react with the periodate-Schiff reagents. Incubation with phosphatase gave glycerol and inorganic phosphate.

Peak 5: diglycerol triphosphate. The phosphate in this fraction had R_f 0.1 in solvent A and did not react with the periodate-Schiff reagents. Incubation with phosphatase converted 65% of its phosphorus into inorganic phosphate and yielded a product that rapidly gave a purple colour with the periodate-Schiff reagents and was chromatographically identical with authentic diglycerol phosphate (R_f 0.6 in solvent A). The amount of diglycerol triphosphate produced in the hydrolysis with alkali (7% of the total phosphorus) is in close agreement with the amount found in the hydrolysate of the intracellular glycerol teichoic acid from *L. arabinosus* (Critchley *et al.* 1962).

Length of the polymer. The three methods previously used in this Laboratory to determine average chain length were carried out on material from which the alanine had been removed (for full experimental details see Ellwood, Kelemen & Baddiley, 1963).

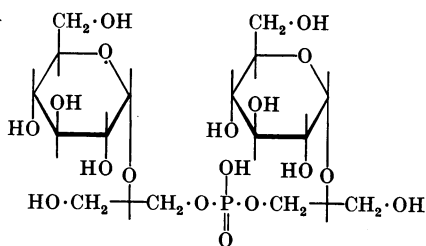
(i) Treatment with phosphatase. The teichoic acid was incubated with the phosphatase and the total phosphorus:inorganic phosphorus ratio was determined at intervals. A value of 13.5:1 was quickly reached; subsequently over several hours this slowly decreased, presumably because of a small amount of phosphodiesterase in the enzyme preparation.

(ii) Potentiometric titration. The teichoic acid was titrated against standard alkali and the curve $\Delta\text{pH}/\Delta v$ against v was plotted. The ratio of the two maxima, corresponding to primary and secondary acid groups, was 14:1.

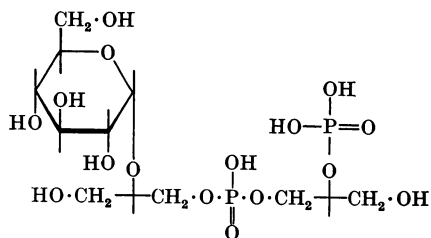
(iii) The teichoic acid was oxidized with 0.2 mM-sodium metaperiodate solution and the periodate consumed was measured at intervals. Under these conditions oxidation of the glucose residues is negligible (cf. Critchley *et al.* 1962). The ratio phosphorus:periodate consumed by the terminal glycol residue is 14.3:1.

DISCUSSION

The glycerol teichoic acid in the walls of *L. buchneri* N.C.I.B. 8007 was extracted with dilute trichloroacetic acid and purified by precipitation with ethanol. The polymer, which represents



(I)



(II)

approx. 30% of the dry weight of the walls, accounts for nearly all of the phosphorus present in the walls of this organism although, as with other organisms, a small amount of phosphorus not associated with teichoic acid could not be extracted. Acid hydrolysis yielded glycerol and its mono- and di-phosphates, the products expected from a polymer of glycerol units joined together through phosphodiester linkages; alanine and glucose were also produced. In common with other teichoic acids, alanine was attached to the polymer by labile ester linkages that could be hydrolysed with aqueous ammonia at room temperature to give alanine and its amide. Paper chromatography of samples taken at intervals during the reaction with ammonia indicated that the alanine residues are removed at a rate comparable with those for other teichoic acids, and so the alanine ester linkages are attached to glycerol and not to the sugar residues. The identity of the amino acid was confirmed by isolation as its hydrochloride and the D-configuration was established by enzymic methods. Quantitative analysis showed that in the isolated polymer alanine was attached to about half of the glycerol residues; the lability of these amino acid ester linkages may have caused losses during isolation and it is likely that the polymer in the wall would possess more alanine residues.

The three methods previously developed to determine the length of the teichoic acids suggested a molecule composed of 14 glycerophosphate units. This value is somewhat smaller than those previously observed for other glycerol teichoic acids (Critchley *et al.* 1962; Ellwood *et al.* 1963; Wicken & Baddiley, 1963).

Studies on the products of hydrolysis with alkali enabled the location and distribution of the glucose residues to be established. The mechanism of hydrolysis of glycerol teichoic acids with alkali, and the conclusions to be drawn from the structure of the products, have been fully discussed elsewhere (Kelemen & Baddiley, 1961; Critchley *et al.* 1962; Ellwood *et al.* 1963). Hydrolysis in alkali of the polymer lacking alanine residues yielded two neutral components, glycerol and 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, and a diglycerol triphosphate that was stable towards alkali, were readily identified and all have been previously isolated from glycerol teichoic acids by hydrolysis with alkali. The formation of the diglycerol triphosphate is diagnostic for a polymer in which glycerol residues are joined through phosphodiester linkages involving positions 1 and 3; it follows that the glucose and alanine substituents must occupy 2-positions on glycerol residues.

Analysis for glucose showed that of the 14 glycerol units in the polymer only four possess a D-glucose residue. As the glucosylglycerol produced by the action of alkali accounted for most of the glucose in the polymer, it is likely that most of the glucose residues are attached to glycerol units within the chain. The glucosylglycerol was chromatographically identical with 2-O- α -D-glucopyranosylglycerol and gave the blue-grey colour characteristic of α -glucosides with the periodate-Schiff reagents. No reducing sugar was formed from the glycoside by incubation with a β -glucosidase and the α -configuration was supported by the positive specific rotation of the polymer.

The failure to detect a glucosylglycerol phosphate in the hydrolysate shows that few or no glucose residues are situated at the P-terminal end of the chain. On the other hand, a minor component was isolated having the properties expected of the diglucosyldiglycerol phosphate (I); it was unaffected by the phosphomonoesterase, gave the expected products on hydrolysis with acid and contained glucose and phosphorus in the ratio about 2:1. Such a product could arise only from a polymer containing some glucose residues on adjacent glycerol units. This feature has not previously been recognized in a glycerol teichoic acid.

The remaining product of hydrolysis with alkali was a glucosyldiglycerol diphosphate (II). This structure follows from analysis and from the behaviour of the compound towards the phosphomonoesterase; half of its phosphorus was converted into inorganic phosphate and the product contained an unsubstituted glycerol phosphate residue. This new product was labile towards alkali, giving glucosylglycerol and glycerol monophosphates. An analogous compound containing galactosamine instead of glucose has been observed in hydrolysates of the teichoic acid from the walls of *Staphylococcus lactis* (Ellwood *et al.* 1963).

The formation of the diphosphate (II) is analogous to the formation of the alkali-stable diglycerol triphosphate; both arise through intermediate cyclic phosphates according to well-established mechanisms for the hydrolysis of phosphodiester in alkali. Isomers bearing a phosphomonoester residue at the terminal position (1 or 3) would also be produced during further hydrolysis of the intermediate cyclic phosphates, but as these would now possess a hydroxyl group adjacent to the phosphodiester residue they would be unstable under the alkaline conditions.

It is concluded that the teichoic acid from the walls of *L. buchneri* N.C.I.B. 8007 is a polymer comprising about 14 glycerol phosphate residues, joined through phosphodiester linkages at positions 1 and 3 on each glycerol, and to the 2-position of four of these residues are attached α -D-glucosyl

pyranosyl substituents; to most of the remaining glycerol 2-hydroxyl groups are attached D-alanine ester residues. From the nature of the products of hydrolysis in alkali, it is probable that the sugar residues are more or less randomly distributed along the polymer chain, with the occasional occurrence of glucose attached to adjacent glycerol units.

Of the glycerol teichoic acids whose structures have been examined in detail, that from *L. buchneri* N.C.I.B. 8007 is intermediate in its structural features. The sugar content of these polymers varies from undetectable amounts in the intracellular one from *L. casei* A.T.C.C. 7469 (Kelemen & Baddiley, 1961) to high proportions in those from streptococci in group D, where in some strains tri-saccharide residues are attached to each glycerol unit in the polymer (Wicken & Baddiley, 1963). Its sugar content is higher than that of the intracellular teichoic acid from *Staphylococcus aureus* H, which contains traces of *N*-acetylglucosamine and a small number of gentiobiosyl residues attached to glycerol (RajBhandary & Baddiley, 1963). Similarly, it is higher than that of the intracellular one in *L. arabinosus* 17-5 where two α -glycosyl residues occur in a polymer of about 18 glycerol phosphate units (Critchley *et al.* 1962). In this respect it resembles more closely that from the walls of *S. lactis* (*S. albus*) N.T.C.C. 7944 in which an α -*N*-acetylgalactosaminyl residue is attached regularly to every third glycerol phosphate unit in the chain (Ellwood *et al.* 1963).

SUMMARY

1. The teichoic acid that represents approx. 30% of the dry weight of the walls of *Lactobacillus buchneri* N.C.I.B. 8007, and is the group E precipitinogen, has been isolated and its structure examined.

2. The polymer is composed of glycerol, phosphate, D-glucose and D-alanine.

3. Hydrolysis with alkali gave glycerol, 2-O- α -D-glucopyranosylglycerol and a mixture of organic phosphates. These were separated and identified as

glycerol mono- and di-phosphates, a diglycerol tri-phosphate, a diglucosyldiglycerol phosphate and a glucosyldiglycerol diphosphate. The detailed structures of the last three were established.

4. The teichoic acid is a polymer of about 14 glycerol phosphate units, four of which have α -D-glucosyl residues on position 2 of the glycerol. The glycerol residues are joined through phosphodiester linkages involving positions 1 and 3 in each glycerol. A few of the sugar residues are situated on adjacent glycerol units, and most of the remaining glycerol units bear D-alanine ester residues at position 2.

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