The Membrane Teichoic Acid of *Staphylococcus lactis* I3

By A. R. ARCHIBALD, J. BADDILEY AND D. BUTTON

Microbiological Chemistry Research Laboratory, Department of Organic Chemistry,
University of Newcastle upon Tyne, NE1 7RU

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1. Teichoic acid was isolated by extraction with trichloroacetic acid of the membrane fraction of disrupted cells of *Staphylococcus lactis* I3. 2. The purified material contains glycerol, phosphate and alanine, but little or no sugar or amino sugar. 3. A study of the products of hydrolysis with acid and alkali established that the membrane teichoic acid is a (1→3)-linked poly(glycerol phosphate) that differs in structure from the glycerol teichoic acid in the wall of this organism. 4. The alanine ester residues show the characteristic high lability to alkali and are thus distinguishable from the more stable alanine ester residues of the wall teichoic acid. 5. The significance of these structural features and the possible function of teichoic acids are discussed.

The first glycerol teichoic acids to be described were (1→3)-linked polymers of glycerol phosphate containing D-alanyl ester residues and, in some cases, sugar or acetamido sugar substituents. They occur as components of the walls of several Gram-positive bacteria and are of widespread occurrence as 'intracellular' constituents of such organisms (for a review see Archibald & Baddiley, 1966), where they appear to be components of the cytoplasmic membrane (Hay, Wicken & Baddiley, 1963; Shockman & Slade, 1964; Smith & Shattock, 1964). Subsequently, glycerol teichoic acids from walls have been described in which the inter-unit phosphodiester linkages are believed to occupy positions 1 and 2 on glycerol (Naumova & Zaretskaya, 1964; Wicken, 1966), and in the poly(galactosylglycerol phosphate) and poly(glucosylglycerol phosphate) from the wall of *Bacillus licheniformis* A.T.C.C. 9945 the sugar residues form an integral part of the polymer chain (Burger & Glaser, 1966).

A further variation in structure was found in the wall teichoic acid of *Staphylococcus lactis* I3 in which both glycerol and *N*-acetylglucosamine form an integral part of the polymer chain, each being double-linked through phosphate (Archibald, Baddiley & Button, 1968). No such variations in structure have been observed for membrane teichoic acids examined in this Laboratory, all of which are (1→3)-linked poly(glycerol phosphate) polymers differing only in the nature and extent of glycosidic substitution (cf. Archibald & Baddiley, 1966). The present investigation of the membrane teichoic acid of *Staph. lactis* I3 was undertaken to establish whether this material was chemically similar to

the novel teichoic acid present in the wall of this organism.

EXPERIMENTAL

Materials

Alkaline phosphomonoesterase (type 1, from calf intestinal mucosa) and ribonuclease (type 1-A from bovine pancreas) were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A.

Analytical methods

Alanine, glycerol, phosphorus, formaldehyde and reducing sugars were determined as previously described (Archibald *et al.* 1968).

Chromatography

Paper chromatography was carried out on Whatman no. 1 and no. 4 papers. The following systems were used: *A*, propan-1-ol-aq. NH₃ (sp.gr. 0.88)-water (6:3:1, by vol.); *B*, butan-1-ol-pyridine-water (6:4:3, by vol.); *C*, propan-2-ol-aq. NH₃ (sp.gr. 0.88)-water (7:1:2, by vol.). Products were detected as previously described (Archibald *et al.* 1968).

Isolation of the particulate fraction

Staph. lactis I3 was grown, harvested and disrupted as previously described (Archibald *et al.* 1968; Davison, 1968). Intact cells and walls were removed by centrifugation at 60000g for 30 min. The supernatant was then centrifuged at 100000g for 1 hr., whereupon the particulate fraction of the cellular contents was deposited as a gelatinous mass.

Extraction of teichoic acid

The gel obtained by disruption of 140g. wet wt. of cells was suspended in 10% (w/v) trichloroacetic acid (15 ml.) and

stirred at 2° for 36 hr. Centrifugation (20000g for 30 min.) of the suspension gave a clear supernatant, which was mixed with cold ethanol (60 ml.). The resulting precipitate (60 mg.) was removed by centrifugation and dried with ethanol and ether. Addition of acetone (200 ml.) to the supernatant obtained after removal of the teichoic acid gave no further precipitation. The crude teichoic acid (60 mg.) was dissolved in water (10 ml.) and centrifuged (20000g for 30 min.), and the clear solution was freeze-dried. A sample (4 mg.) of the residue was hydrolysed in 2N-HCl (0.5 ml.) at 100° for 3 hr. and examined chromatographically with solvents *A* and *B*. The major products were glycerol, glucose, alanine and glycerol monophosphates, together with smaller amounts of ribose, inorganic phosphate, glycerol diphosphates and nucleotides.

Purification of teichoic acid

(a) A solution of the teichoic acid (40 mg.) in 0.1 M-tris-HCl buffer, pH 7.8 (30 ml.), was incubated with ribonuclease (5 mg.) at 37° for 14 hr. The solution was then dialysed against water and the non-diffusible fraction was applied to a column (20 cm. × 2 cm.) of DEAE-cellulose (HCO_3^- form). Material was eluted with a linear concentration gradient of $(\text{NH}_4)_2\text{CO}_3$; the mixing vessel contained water (300 ml.) and the reservoir 0.6 M- $(\text{NH}_4)_2\text{CO}_3$, pH 8.6 (300 ml.) Fractions (5 ml.) were collected automatically at a flow rate of 2 ml./min. The elution pattern was followed by analysis for phosphate and by measurement of extinction at 260 and 280 nm. (Fig. 1). Fractions 70–90 were combined and evaporated to dryness by rotary evaporation.

(b) A solution of teichoic acid (12 mg.) in water (1 ml.) was applied to a column (6 cm. × 1 cm.) of Dowex 2 (Cl^- form) resin. The column was washed with water (100 ml.) and material was eluted with a linear concentration gradient (0.1 M) of LiCl, pH 6. Fractions (50 × 2 ml.) were collected and samples (0.2 ml.) were analysed for phosphate. A single peak representing 85% of the applied phosphate was produced at a salt concentration of 0.8–0.9 M. The appropriate fractions were combined, dialysed against water and freeze-dried.

Examination of the teichoic acid

Hydrolysis in acid. A sample (3 mg.) of teichoic acid, purified by chromatography on DEAE-cellulose, was hydrolysed in 2N-HCl (0.5 ml.) at 100° for 3 hr. The acid was removed by evaporation *in vacuo* over KOH and the residue dissolved in water (2 ml.). A portion of this solution (1 ml.) was evaporated to dryness *in vacuo* and the residue was examined chromatographically with solvent *A*. Glycerol and its mono- and di-phosphates were the major products. The remainder of the hydrolysate (1.0 ml.) was mixed with a solution of phosphomonoesterase (0.1 mg.) in 0.04 M- $(\text{NH}_4)_2\text{CO}_3$, pH 9.3 (1 ml.). Samples were withdrawn for analysis of phosphate, reducing sugar and glycerol. The molecular proportions found were: phosphate:reducing sugar:glycerol, 1:0.07:1.01.

Hydrolysis in alkali. Purified teichoic acid (16 mg.) was hydrolysed in N-NaOH solution (4 ml.) in a sealed tube at 100° for 3 hr. The hydrolysate was passed through a column (10 cm. × 1 cm.) of Dowex 50 (H^+ form) resin and the eluate was adjusted to pH 7 by addition of dilute aq. NH_3 . The solution was applied to a column (20 cm. × 2 cm.) of DEAE-cellulose (HCO_3^- form) that was then washed with water

Fig. 1. Chromatography of teichoic acid preparation on DEAE-cellulose. Experimental details are given in the text. —, Phosphate ($\mu\text{moles/ml.}$); ----, E_{260} ; · · · ·, E_{280} .

Fig. 2. Chromatography on DEAE-cellulose of products of hydrolysis of teichoic acid with alkali. Details are given in the text.

(200 ml.); material was eluted with a linear concentration gradient of $(\text{NH}_4)_2\text{CO}_3$ [200 ml. of water and 200 ml. of 0.2 M- $(\text{NH}_4)_2\text{CO}_3$, pH 8.7]. Fractions (5 ml.) were collected and analysed for phosphorus (Fig. 2). The water eluate was evaporated to dryness and the residue was examined chromatographically with solvents *A*, *B* and *C*. Glycerol was the only product detected. The fractions corresponding to each of the three phosphate peaks were combined and evaporated to dryness, the $(\text{NH}_4)_2\text{CO}_3$ being removed by repeated evaporation with water.

Peak I. Material corresponding to this peak represented 68% of the total phosphate. Chromatography in solvents *A* and *C* showed the presence of a small amount of inorganic phosphate, the two major components having the mobilities and colour reactions of glycerol 1-phosphate and glycerol 2-phosphate, the latter having R_{GF} ($R_{\text{glycerol 1-phosphate}}$) 1.2 in solvent *C*.

A sample containing about 50 $\mu\text{g.}$ of phosphorus was incubated with phosphomonoesterase (0.1 mg.) in 0.01 M- $(\text{NH}_4)_2\text{CO}_3$, pH 9.3 (4 ml.), for 12 hr. at 37°. Chromatographic examination showed the presence of glycerol and inorganic phosphate. Samples were removed for the determination of total phosphorus and inorganic phosphate; a further sample was analysed for glycerol. Results are given in Table 1.

Table 1. Action of phosphomonoesterase and periodate on the products of alkali hydrolysis of the teichoic acid

Methods and conditions are described in the Experimental section.

Peak	Inorganic phosphate after phosphomonoesterase (%)	Formaldehyde formed by oxidation of phosphomonoesterase-treated sample (mole/mole of phosphate)	Products of dephosphorylation
I	98	1.91	Glycerol, inorganic phosphate
II	100	1.04	Glycerol, inorganic phosphate
III	69	0.60	Diglycerol phosphate, inorganic phosphate

Peak II. Chromatography of material corresponding to this peak gave a single spot with the mobility of glycerol diphosphate. Enzymic dephosphorylation (Table 1) gave glycerol and inorganic phosphate.

Peak III. Material corresponding to this peak represented 9% of the total phosphorus. Chromatography gave a single spot (R_{FGP} 0.13 in solvent *C*) having a lower mobility than glycerol diphosphate. On enzymic dephosphorylation (Table 1) it produced inorganic phosphate and a single organic phosphate (R_{glycerol} 0.68 in solvent *A*) that gave a colour immediately with the periodate-Schiff spray reagents.

Chain length. A solution of teichoic acid (containing about 5 μg . atoms of P) was incubated with phosphomonoesterase (2mg.) in 0.04M-(NH_4)₂CO₃, pH 9.3 (4ml.), for 12hr. Portions were analysed for total phosphate and inorganic phosphate. The molecular ratio inorganic phosphate:total phosphate was 0.105:1.

A further sample of the original teichoic acid (containing 2.6 μg . atoms of P) and a sample of the material that had been treated with phosphomonoesterase (containing 0.98 μg . atom of P) were oxidized with 0.1M-NaIO₄ and the formaldehyde formed was measured. The molecular ratio phosphate:formaldehyde for the original teichoic acid was 1:0.079, and for the material that had been treated with phosphatase the molecular ratio was 1:0.155.

Ammonolysis. A sample (1mg.) of teichoic acid that had been purified by chromatography on Dowex 2 (Cl⁻ form) resin was shaken with 0.5ml. of methanol-aq. 2N-NH₃ (1:1, v/v) at room temperature for 3hr. Chromatographic examination in solvent *A* showed the presence of alanine and alanine amide.

Action of hydroxylamine. A sample of the teichoic acid bearing alanine (about 3mg.) was dissolved in water (2ml.). Portions (0.2ml.) were mixed with a solution of 0.1M-hydroxylamine (0.2ml.) at pH 7.4 and heated on a water bath at 45°. Tubes were removed at intervals, N-HCl (0.4ml.) and M-FeCl₃ solution (0.4ml.) were added and the extinction was measured at 540nm. (Fig. 3).

DISCUSSION

The membrane teichoic acid of *Staph. lactis* 13 was isolated from the particulate fraction of the cytoplasmic contents by extraction with cold trichloroacetic acid. The crude teichoic acid obtained by precipitation with ethanol contained alanine, glycerol, phosphate and glucose together with some nucleic acid. Purification of the polymer was achieved by ion-exchange chromatography,

Fig. 3. Action of hydroxylamine on teichoic acid. The extinction at 540nm. is due to the colour given by alanine hydroxamate with the ferric chloride reagent. Details are given in the text.

when more than 80% of the total phosphate was recovered as a teichoic acid that was free from glucose. It is unlikely that the glucose derivative in the crude teichoic acid preparation is the diglucosyl diglyceride associated with the membrane of this organism (Brundish, Shaw & Baddiley, 1967) because of the relatively large amount present and because similar amounts of glucose were present in crude samples of teichoic acid prepared by extraction of the particulate fraction of defatted cells. It seems probable that a glucan or polysaccharide rich in glucose is associated with the particulate or membrane fraction of *Staph. lactis* 13.

Purification of the teichoic acid by treatment with ribonuclease, dialysis and ion-exchange chromatography on DEAE-cellulose gave a preparation containing 5–10% of nucleic acid (based on extinction measurements at 260 and 280nm.). Acid hydrolysis gave glycerol and its mono- and di-phosphates, the products expected from a polymer of glycerol units joined together through phosphodiester linkages. Analysis of the enzymically dephosphorylated hydrolysate showed the molecular proportions glycerol:phosphate:reducing sugar to be 1:1.01:0.07. The products from hydrolysis of the teichoic acid with alkali were

separated by ion-exchange chromatography into a neutral fraction (glycerol) and three mixtures of phosphates, identified as glycerol monophosphates, glycerol diphosphates and an alkali-stable diglycerol triphosphate, all of which were characterized by methods described previously (Kelemen & Baddiley, 1961; Critchley, Archibald & Baddiley, 1962). No glucosylglycerol or glucosylglycerol phosphates were detected, and it is likely that the small amount of reducing sugar present in acid hydrolysates of the teichoic acid (detected by the method of Park & Johnson, 1949) is derived from the contaminating nucleic acid. The products of hydrolysis in both acid and alkali are thus consistent with a (1→3)-linked poly(glycerol phosphate).

Crude teichoic acid, and the purified polymer obtained by chromatography on Dowex (Cl⁻ form) resin, contained alanyl ester groups that showed characteristic high lability to ammonia and hydroxyl amine; they differed in this respect from the alanyl ester groups present in the wall teichoic acid. Studies with periodate and phosphomonoesterase indicated a chain length of ten units; this is somewhat lower than the values observed for membrane teichoic acids examined previously, although it is likely that degradation had occurred during the extraction procedures and the native polymer may well contain appreciably more than ten units.

The membrane teichoic acid from *Staph. lactis* I 3 closely resembles all previously examined membrane teichoic acids and consists of a (1→3)-linked glycerol phosphate polymer to which alanyl ester residues are attached. Few or no sugar residues are present and the polymer is thus similar to that in *Lactobacillus casei* A.T.C.C. 7469. Membrane teichoic acids from other organisms contain various amounts of sugar (cf. Archibald & Baddiley, 1966), although, irrespective of the nature of the wall teichoic acid, they have in common the (1→3)-linked poly(glycerol phosphate) backbone. It is perhaps too soon to assert the universality of this type of structural arrangement in membrane teichoic acids, but the contrast with the considerable structural variation in wall teichoic acids is noteworthy. It has been suggested (Archibald & Baddiley, 1966) that the function of teichoic acids might be protective, in that these highly charged polymers might control the approach of ions towards or through the wall membrane. A comparable role could be assigned to other negatively charged compounds, e.g. phospholipids and lipopolysaccharides, in the outer regions of Gram-negative bacteria and in the cells of higher animals and plants. It is possible that the membrane teichoic acids are essential for cell survival, and so are of reasonably constant general structure, whereas the wall teichoic acids provide additional security and consequently their detailed

structure is less important. These suggestions are supported by the following observations: membrane teichoic acids have been found in all Gram-positive bacteria examined so far; bacteria containing large amounts of wall teichoic acid are particularly able to withstand high concentrations of salts in the surrounding medium (Archibald, Armstrong, Baddiley & Hay, 1961); bacilli grown in a medium deficient in phosphate fail to synthesize wall teichoic acid but produce in its place large amounts of acidic polysaccharide (Ellwood & Tempest, 1967; Tempest, Dicks & Ellwood, 1968), but membrane teichoic acid is still produced. Teichoic acids may also act by affording protection to the wall against lytic enzymes. Thus the composition of the wall teichoic acid (C-substance) (Brundish & Baddiley, 1967, 1968) of pneumococci greatly influences the susceptibility of the wall to autolytic enzymes (Tomasz, 1968); the wall teichoic acid of cells grown in a medium containing ethanolamine (instead of choline) contains ethanolamine, and these walls are completely resistant to the autolytic enzymes that hydrolyse choline-containing walls. During growth in ethanolamine, daughter cells remain associated after division and chains containing several hundred cells are produced. This effect is presumably due to the inability of autolytic enzymes to digest the ethanolamine-containing wall and suggests that a controlled autolysis of wall, necessary for separation of daughter cells during normal growth, may be influenced by the teichoic acid component of the wall.

A ribitol teichoic acid has also been shown to act as a specific inhibitor of the lytic agent produced by strains of *Streptococcus zymogenes* (Davie & Brock, 1966). Most Gram-positive bacteria are sensitive to the lytic agent, but the producing strains of *Strep. zymogenes* are protected by an inhibitor that has been shown to be a ribitol teichoic acid containing glucose and D-alanine. The integrity of the D-alanine ester residues was necessary for the activity of the inhibitor.

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