The Occurrence of Lipoteichoic Acids in the Membranes of Gram-positive Bacteria

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Glycerol teichoic acids have been found associated with the cytoplasmic membrane of all of the Gram-positive bacteria examined and it is believed that, unlike wall teichoic acids which are absent from some organisms, these membrane teichoic acids are characteristically present in all species (for reviews see Archibald, Baddiley & Blumsom, 1968; Baddiley, 1970, 1972). Wall teichoic acid is attached to the peptidoglycan of the wall through its terminal phosphate residue, but although it has been known for some time that membrane teichoic acid is associated with the outer surface of the membrane (Hay, Wicken & Baddiley, 1963; Shockman & Slade, 1964) the nature of this association has only recently been examined.

Under the acidic conditions usually employed to isolate membrane teichoic acids the linkage with the membrane is hydrolysed and the purified products comprise chains of glycerol phosphate residues to which D-alanine and sometimes glycosyl residues are attached. When disrupted preparations of *Bacillus licheniformis* or *B. subtilis* are treated with 80% (w/v) aqueous phenol the membrane teichoic acid can be recovered from the aqueous phase (Burger & Glaser, 1964), and it has been suggested that this gentle non-hydrolytic procedure would not disrupt the presumed covalent linkage between teichoic acid and membrane (Archibald *et al.* 1968). Material extracted in this way was free of much of the protein and nucleic acid of the organism and did not contain wall teichoic acid. A phenol-extracted membrane teichoic acid from *Lactobacillus fermenti* NCTC 6991 has been prepared (Wicken & Knox, 1970), and it was concluded from the nature of the products of alkaline hydrolysis that the teichoic acid was combined with glycolipid and phospholipid. This complex is antigenic when injected together with Freund's adjuvant into rabbits, whereas the lipid-free teichoic acid (extracted with trichloroacetic acid) is not antigenic (Knox, Hewett & Wicken, 1970). Similar results have been obtained from other strains of *Lactobacillus* (Wicken & Knox, 1971). A lipoteichoic acid has also been isolated from *Streptococcus faecalis* NCIB 8191 in which the teichoic acid, a polymer of 28 to 35 units of glucosylglucosyl (kojibiosyl)-glycerol phosphate, is covalently attached through its terminal phosphate to a diglucosyldiglyceride or more probably a phosphatidyl diglucosyldiglyceride (Toon, Brown & Baddiley, 1972). Thus the combination between teichoic acid and membrane in this organism takes the form of a lipoteichoic acid in which the lipid is a normal membrane component which intercalates with other phospholipids in the bilayer region of the cytoplasmic membrane. As this teichoic acid is the serological group-specific substance of *S. faecalis* (group D) and is accessible to antiserum in the intact organism, it is concluded that polymer chains in which serological specificity resides must project from the outer surface of the membrane where they participate in the control of bivalent cation concentration (Heptinstall, Archibald & Baddiley, 1970; Hughes, Stow, Hancock & Baddiley, 1971). We have now examined a range of bacteria and demonstrated in all cases that the membrane teichoic acid is lipoteichoic acid.

METHODS

RNase and DNase were obtained from Sigma Chemical Co., St Louis, Missouri, U.S.A. Sepharose 6B and Sephadex G-200 were obtained from Pharmacia Ltd, London.

The following organisms were used: *Lactobacillus buchneri* NCIB 8007, *L. plantarum* NIRD C106, *Staphylococcus aureus* H (Baddiley, Buchanan, RajBhandary & Sanderson, 1962), *Micrococcus* sp. 24 (from Dr A. L. Davison), *S. aureus* A1 (Davison & Baddiley, 1963) and *Bacillus subtilis* Marburg strain 168 (Hughes, 1971). They were grown in batch cultures under conditions similar to those described previously (Davison & Baddiley, 1963; Shaw & Baddiley, 1964; Archibald & Coapes, 1971; Hughes 1971). Washed bacteria were disrupted in a Braun disintegrator with cooling; walls, unbroken organisms and debris were removed by centrifugation (17000 g for 20 min) and the supernatant solution was freeze-dried. The residue was treated twice with chloroform-methanol (2:1, v/v) to remove free lipids, dialysed overnight at room temperature and again freeze-dried. The lipoteichoic acids were extracted from the freeze-dried powder by two different methods.

Extraction of lipoteichoic acid with aqueous phenol. The residue was suspended in water and extracted with an equal volume of 80% (w/v) aq. phenol at 4 °C for 40 min. The upper aqueous phase was collected, dialysed as before and incubated at 37 °C for 12 h with DNase (from bovine spleen) and RNase (from bovine pancreas) (10 µg/ml) in 5 mM-tris buffer, pH 8, containing 1 mM-MgCl₂. The aqueous-phenol extraction was then repeated to remove the enzymes and the aqueous layer was freeze-dried. At least 95% of the phosphorus in the chloroform-methanol-washed material was extracted into the aqueous phase in the initial phenol treatment and no lipids containing glycerol residues were detected in the phenol phase.

Extraction of lipoteichoic acid by an aqueous procedure. The freeze-dried powder was suspended in distilled water (10 mg/ml) and boiled gently for 30 min. The solution was then filtered and the filtrate collected. The residue was extracted twice with boiling water under the same conditions. The filtrates were combined, dialysed and freeze-dried. At least 95% of the total phosphorus was solubilized using this procedure.

Methods for paper and thin-layer chromatography, hydrolysis of fractions and characterization of hydrolysis products were similar to those used in previous studies (Toon *et al.* 1972).

RESULTS AND DISCUSSION

Lipoteichoic acids extracted by aqueous phenol were purified by chromatography on columns (25 mm × 450 mm) of Sepharose 6B using upward flow (15 ml/h) with 0.02 M-ammonium acetate containing 0.1% of sodium azide as eluant. The elution diagrams were of three types (Fig. 1); type A for *Lactobacillus buchneri*, *L. plantarum*, *Staphylococcus aureus* H and *S. aureus* A1; type B for *Bacillus subtilis*; type C for *Micrococcus* sp 24. In all cases peak 1 occurred in the void volume and represented the lipoteichoic acid; the molecular ratio glycerol/phosphorus was 1:1, but differences were noted in the amount and nature of the glycosyl substitution. Peak 2 represented an unidentified polymer containing glycerol, phosphorus and lipid, and peak 3 represented nucleic acid (from extinction at 260 nm). Occasionally a small peak showing extinction at 260 nm was seen before the lipoteichoic acid; this was due to light scattering from colloidal material originally present in the Sepharose. This small peak is similar to that eluted before the lipoteichoic acid from *Streptococcus faecalis* on Sepharose 6B (Toon *et al.* 1972) and is presumably an artefact.

The lipoteichoic acids extracted by aqueous phenol resembled in their physical properties

Fig. 1. Chromatography of extracts of bacteria on Sepharose 6B using 0.02M-ammonium acetate containing 0.1% sodium azide as eluant. Phenol extracts were used for A, B and C while an aqueous extract was applied in D. A represents extracts of *Staphylococcus aureus* H although *S. aureus* A1, *Lactobacillus buchneri* and *L. plantarum* gave the same type of separation; B, *Bacillus subtilis* and C, *Micrococcus* sp. 24; D is the elution diagram for *S. aureus* H, although all of the aqueous extracts used in this study gave similar elution curves.

that isolated from *Streptococcus faecalis* (Toon *et al.* 1972). They gave very sharp peaks in the ultracentrifuge (6 to 10 s) due to the formation of micelles. Hydrolysis in alkali (10 mg samples in 5 ml of 0.1 M-NaOH at 100 °C for 1 h) and preparation of methyl esters of the resulting fatty acids followed by gas chromatography showed that fatty acid ester residues (mainly C₁₆, with smaller amounts of C₁₂, C₁₄ and C₁₈) were present; the lipoteichoic acid from *Micrococcus* sp. 24 contained fatty acid:phosphate in the molecular ratio 1:14.3. The ester residues could not be removed by repeated chromatography on columns (16 mm × 250 mm) of Sephadex G-200 using upward flow (15 ml/h) with sodium dodecyl sulphate solution (1%, w/v) or 6 M-urea as eluant. In both these systems the lipoteichoic acids were eluted from the column just after the void volume. This and their stability to chloroform-methanol (2:1, v/v) indicates that the lipid and teichoic acid components are not held together by hydrogen bonds, electrovalent attraction or hydrophobic interactions. Fatty acid esters in similar proportions were also detected in material corresponding to peak 2.

Phosphate esters are converted to alcohols by anionic displacement through the action of 60% (w/v) HF at 0 °C for 6 h. Under these conditions neither glycosidic linkages nor fatty

acid ester linkages are hydrolysed (Glaser & Burger, 1964), and with the lipoteichoic acid from *Streptococcus faecalis* the products from such treatment were glycosides of glycerol (from the teichoic chain) together with diglycosyldiglyceride and diglyceride (from phosphatidyl glycolipid) (Toon *et al.* 1972). The products obtained by treatment of material corresponding to peak 1 with HF were extracted with chloroform and examined by thin-layer chromatography on silica gel (chloroform-methanol-water, 65:25:4, by vol.). Using the periodate-Schiff spray reagents for polyols and glycosides spots corresponding to diglycosyldiglycerides were observed from the lipoteichoic acid from *Lactobacillus buchneri*, *L. plantarum*, *Staphylococcus aureus* A1 and *S. aureus* H. These products were indistinguishable from the corresponding material prepared from lipids extracted from isolated membranes by chloroform-methanol (2:1), but the presence of diglyceride in the lipid extracts after HF treatment has not yet been established unequivocally.

The lipids obtained by the action of HF on the lipoteichoic acids from *Bacillus subtilis* and *Micrococcus* sp. 24 differed in their behaviour on thin-layer chromatography from the products of action of HF on the lipids that were extracted from the membranes of these organisms with chloroform-methanol. The reasons for these differences are unknown; nevertheless as these lipoteichoic acids also give fatty acids on treatment with alkali it seems probable that they have a general structural similarity to those obtained from other bacteria.

All the preparations of lipoteichoic acids extracted by aqueous phenol contained small amounts of protein (0.5 to 3.0%) after the first chromatographic purification, but after repeated purification on columns of Sepharose 6B this protein was separated from the lipoteichoic acid.

The lipoteichoic acid preparations extracted by the aqueous procedure were also purified by chromatography on Sepharose 6B, using the conditions already described. For each species studied the elution diagram was the same (Fig. 1 D). Three peaks corresponding to phosphate were obtained similar to those observed with the phenol extraction procedure. However, the separation of material showing extinction at 260 nm was different from that in the phenol extracts. The lipoteichoic acid fraction (Fig. 1 D, peak 1) contained 30 to 40% (w/w) of protein together with RNA and DNA. The nucleic acids and protein could be removed almost completely by digestion with pronase, DNase and RNase followed by chromatography. Although losses on extraction and purification were considerable the purified lipoteichoic acid represented about 2.0% of the dry weight of *Micrococcus* sp. 24 after removal of walls and cell debris. Ultracentrifugation of the lipoteichoic acid fraction obtained by aqueous extraction and chromatography did not show a sharp peak corresponding to a micelle. On the other hand, phenol-extracted lipoteichoic acids (Fig. 1 ABC, Peak 1) were prepared virtually free of protein and these preparations gave very sharp peaks in the ultracentrifuge. We conclude that, although it is unlikely that lipoteichoic acid is covalently attached to either protein or nucleic acids, these molecules nevertheless profoundly influence their physical properties, especially with respect to micelle formation.

The present and previous work shows that lipoteichoic acids are widespread components of the membrane of Gram-positive bacteria and, taken together with the more extensive surveys of the occurrence of membrane teichoic acids, it is likely that they are present in all cases. In the organisms examined here no evidence was obtained for the occurrence of free membrane teichoic acid and so, as was shown for *Streptococcus faecalis*, it is likely that all membrane teichoic acid is in the form of lipoteichoic acid.

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