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EXTRACTION AND PURIFICATION OF LIPOTEICHOIC ACIDS FROM GRAM-POSITIVE BACTERIA*

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ABSTRACT

Hot and cold, 80% aqueous phenol extraction procedures together with an aqueous extraction technique have been evaluated for the isolation of lipoteichoic acids from the cytoplasmic membrane of Gram-positive bacteria. Lipoteichoic acids of *Staphylococcus aureus* H, *Micrococcus* 2102, *Bacillus subtilis* 168, and *Bacillus subtilis* W-23 were examined as each of them emphasises a different problem of contamination. The purity of the lipoteichoic acids with respect to cell-wall material, nucleic acid, and protein is discussed together with the criteria of purity which enables critical structural analysis of lipoteichoic acids to be carried out.

INTRODUCTION

This paper describes optimum procedures for the preparation of lipoteichoic acids sufficiently pure for structural analysis. Emphasis is given to the difficulties encountered and the problems which occur if short cuts are taken. Lipoteichoic acids, which are widespread components of Gram-positive bacteria[1], are structurally uniform; all are polymers of glyceryl phosphate in which linkage is through phosphoric diester groups involving positions one and three of adjacent glycerol residues, and the chain is attached through a phosphoric diester linkage to a diglycosyldiglyceride or a phosphatidylglycolipid which intercalates with lipid chains in the cytoplasmic membrane[2,3]. The structural similarity and functional relationship between the wall and membrane teichoic acids could account for the frequent contamination of one type with the other in isolated preparations. The diagrammatic representation of the cell wall and membrane of Gram-positive bacteria, by van Driel *et al.*[4], suggests a close spatial relationship between wall and membrane teichoic acid. It is possible that, in this close relationship, Mg^{2+} ions form associations between chains of both wall and membrane polymers of the kind suggested previously for different types of binding sites within the same chain[5].

*Dedicated to Professor Michael Heidelberger in honor of his 87th birthday.

Membrane teichoic acids were first prepared by trichloroacetic acid extraction of the membrane-ribosome fraction of disrupted organisms[6]. After it was recognised[2] that the membrane teichoic acids contain lipid residues and that the acidic conditions employed for their isolation deacylate these polymers, milder extraction methods, for example with 80% (w/v) aqueous phenol at 0°, have been used. These methods lead to contamination with protein, nucleic acids, and in some cases wall material. The nucleic acid can usually be removed by nuclease digestion, and it has been reported[8] that preparations having a lower protein content can be obtained by a hot, aqueous phenol extraction and also by papain digestion followed by a cold, 80% (w/v) phenol extraction. Preparations having even greater proportions of protein have resulted from an aqueous extraction procedure[8]. Such preparations are so contaminated with protein, nucleic acid, and wall material that they are not suitable for analytical studies as the purification steps necessary to remove the contaminants results in considerable losses of material[9].

A comparison of various extraction methods and the purity of extracted lipoteichoic acids are given here for material from several different organisms.

RESULTS

Staphylococcus aureus *H.* — (*a*) *Cold, 80% aqueous phenol extraction.* This procedure gave excellent results with *Staphylococcus aureus* H and, hence, there was no advantage of carrying out an aqueous phenol extraction at 60°. The profile of an elution from a Sepharose 6B column is shown in Fig. 1. The lipoteichoic acid (Peak 1) contained 2.9% of protein, and 2.5% of the phosphorus in Peak-1 fractions was due to nucleic acid. Acid hydrolysis, followed by paper chromatography failed to reveal components of the cell wall in this material. Analysis showed that different preparations had consistent molecular proportions of glycerol to phosphorus to D-glucose to

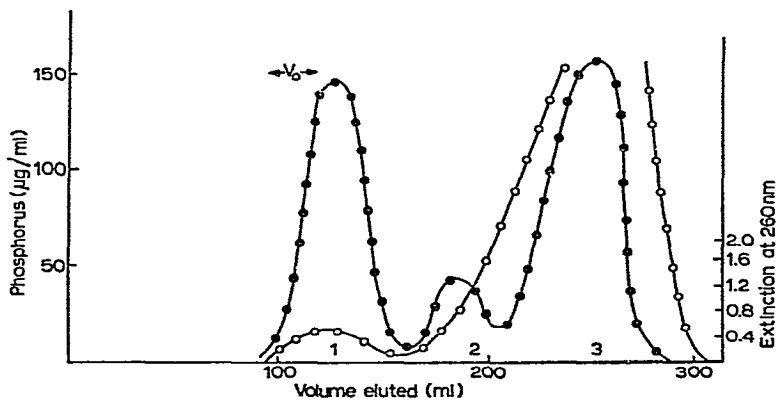

Fig. 1. Purification of the lipoteichoic acid of *Staphylococcus aureus* H (extracted by cold, 80% aqueous phenol) by chromatography on a Sepharose 6B column (for details, see the Experimental section); phosphorus (–●–●–●–) and extinction at 260 nm (–O–O–O–).

fatty acid (expressed as methyl palmitate) of 1.0:1.0:0.06:0.076. However, all preparations also contained *N*-acetyl-D-glucosamine in amounts varying between phosphorus to D-glucosamine 1:0.1 and 1:0.028. This suggests that not all of the lipoteichoic acid chains are identical with respect to substitution with D-glucosamine.

Ribitol teichoic acid from the wall was eluted from a Sepharose 6B column in a position corresponding to Peak 2 (Fig. 1), but its acid hydrolysis products were not present in hydrolysates of the material that was eluted at this position. In fact, the nature of this fraction is not clear; it was nondialysable and, on acid hydrolysis, gave glycerol, its mono- and bis-phosphates, and D-glucosamine. The possibility that the lipoteichoic acid might be contaminated with material corresponding to Peak 2 seemed unlikely from agarose gel-diffusion, which gave a clear separation of material corresponding to Peaks 1 and 2.

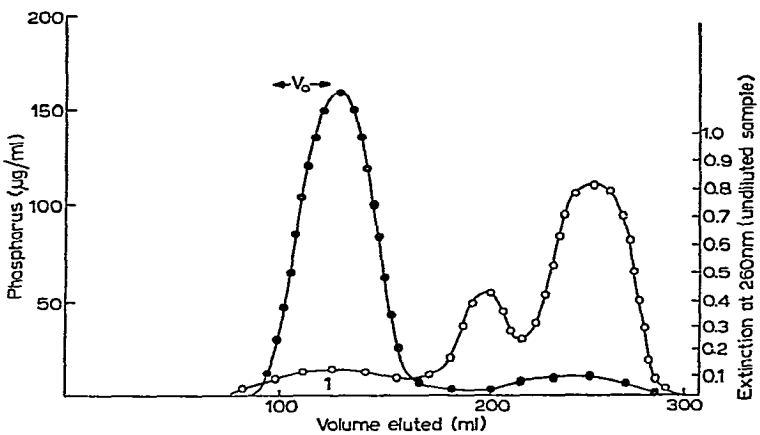

Fig. 2. Repurification of the lipoteichoic acid of *S. aureus* H (extracted by cold, 80% aqueous phenol) by chromatography on a Sepharose 6B column in the presence of 25% methanol (for details, see the Experimental section): phosphorus (–●–●–●–) and extinction at 260 nm (–O–O–O–).

Rechromatography of the lipoteichoic acid on Sepharose 6B in 25% methanol (Fig. 2) yielded material containing not more than 0.5% of protein and 0.29% of nucleic acid. Although glycerol analysis of defatted organisms indicates that the lipoteichoic acid content of *S. aureus* cells might be about 2% (by weight of whole dried cells), the weight of purified lipoteichoic acid actually obtained is ~0.3%.

(*b*) *Aqueous extraction at 100°*. The profile of elution from the Sepharose 6B column is shown in Fig. 3. The lipoteichoic acid (Peak 1) contained 21% of protein and 10% of nucleic acid. Although the nucleic acid and protein could be removed almost completely by digestion with Pronase, DNase, and RNase, followed by an extraction with 80% aqueous phenol to remove the enzymes, the losses due to the additional steps made this technique unsatisfactory.

Micrococcus *sp. 2102*. — Both hot (60°) and cold (4°), 80% aqueous phenol extractions were carried out, followed by chromatography on Sepharose 6B. The

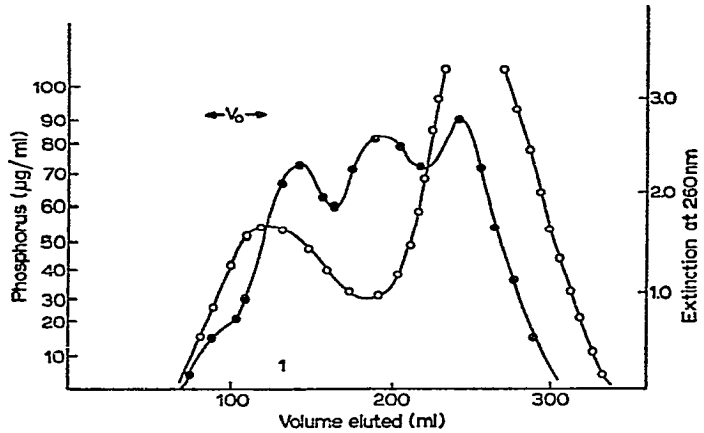

Fig. 3. Purification of the lipoteichoic acid of *S. aureus* (extracted by the aqueous extraction procedure) by chromatography on a Sepharose 6B column (for details, see the Experimental section): phosphorus (–●–●–●–) and extinction at 260 nm (–O–O–O–).

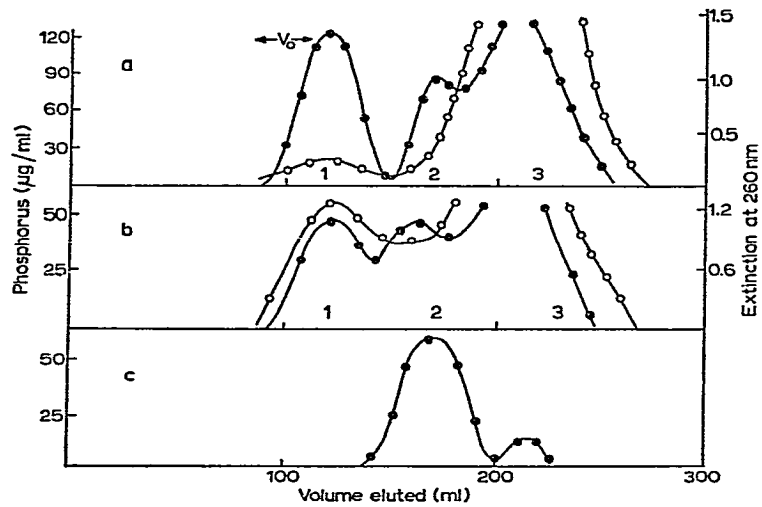

Fig. 4. Comparison of the lipoteichoic acids of *Micrococcus* 2102. (a) Hot, aqueous phenol extract purified by chromatography on a Sepharose 6B column. The lipoteichoic acid (Peak 1) was contaminated with 5.6% of nucleic acid and 1% of protein. (b) Cold, aqueous phenol extract purified by chromatography on a Sepharose 6B column. The lipoteichoic acid (Peak 1) was contaminated with 18.4% of nucleic acid and 13.7% of protein. (c) Poly(2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate) extracted from the cell wall of *Micrococcus* 2102 with alkali and chromatographed on a Sepharose 6B column under identical conditions as described in (a) and (b): phosphorus (–●–●–●–) and extinction at 260 nm (–O–O–O–).

elution curves are shown in Fig. 4. The hot phenol extraction gave a much sharper peak corresponding to the lipoteichoic acid peak than did the cold phenol procedure. Moreover, analysis showed that material from the 60° extraction contained less nucleic acid and protein than did that from the 4° extraction. As even the cold, 80% phenol extract was heavily contaminated with nucleic acid and protein, the aqueous extraction procedure was not performed. Rechromatography of material from the hot extraction on Sepharose 6B in 25% methanol gave the elution curve shown in Fig. 5. The extent of contamination of the lipoteichoic acid with protein remained unchanged, whereas there was a six-fold reduction in the content of nucleic acid.

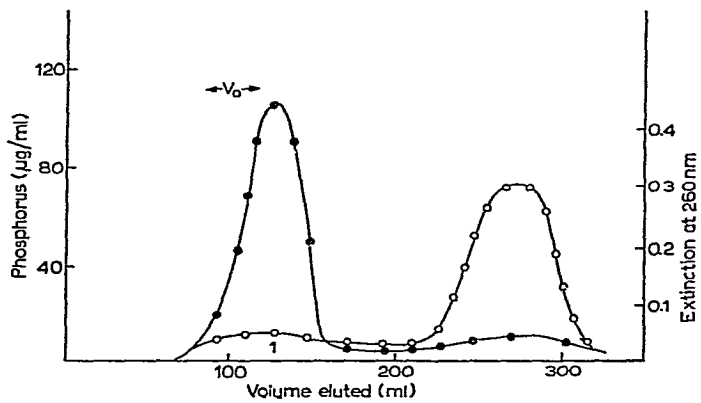

Fig. 5. Repurification of the lipoteichoic acid of *Micrococcus* 2102 (extracted by hot, 80% aqueous phenol) by chromatography on a Sepharose 6B column in the presence of 25% methanol: phosphorus (–●–●–●–) and extinction at 260 nm (–O–O–O–).

Wall teichoic acid in the lipoteichoic acid preparation from Micrococcus *sp. 2102.* — The lipoteichoic acid fraction (Peak 1, Fig. 4a) was heavily contaminated with poly(2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate)[10] from the cell wall. The amount of contaminant was measured by hydrolysis with 4M hydrochloric acid, followed by determination of 2-amino-2-deoxy-D-glucose 6-phosphate in the amino acid analyser, and correction for hydrolysis of the latter to 2-amino-2-deoxy-D-glucose and inorganic phosphate. This showed that 23% of the phosphorus in the preparation was due to the cell-wall polymer. Rechromatography in the presence of methanol was unsuccessful, and no method has yet been found for the removal of this impurity. It is noteworthy that although pure poly(2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate) behaves differently from the lipoteichoic acid on a Sepharose 6B column, the two could not be separated by this procedure. However, it is unlikely that the polymers are covalently linked together, because agarose gel-diffusion of the preparation against diethylaminoethyl(DEAE)dextran showed two precipitin lines, the faster-moving line forming a line of identity with wall teichoic acid. After correction for phosphorus due to wall polymer, the lipoteichoic acid had the following molecular

proportions of phosphorus to glycerol to D-glucose to fatty acid (as methyl palmitate), 1:1.1:0.29:0.07.

Bacillus subtilis *168*. — The cold (4°), 80% aqueous phenol extraction was followed by chromatography on a Sepharose 6B column. The elution curve is shown in Fig. 6a. Material corresponding to Peak 1 contained 2% of nucleic acid and 1% of protein, and hence in this case it was unnecessary to carry out an aqueous or hot

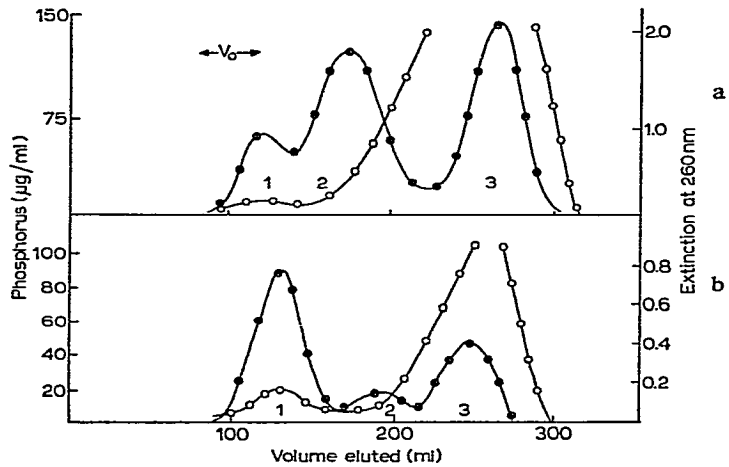

Fig. 6. Purification of the lipoteichoic acid of *Bacillus subtilis* 168 (extracted by cold, 80% aqueous phenol) by chromatography on a Sepharose 6B column: (a) Extracted from the membrane fraction of normal whole cells of *B. subtilis* 168 (top curves). (b) Extracted from the L-form of *B. subtilis* 168 (bottom curves). For complete experimental details, see the Experimental section: phosphorus (–●–●–●–) and extinction at 260 nm (–O–O–O–).

phenol extraction. However, the lipoteichoic acid (Peak 1) contained small amounts of muramic acid and diaminopimelic acid. Gel-diffusion experiments indicated that the material corresponding to Peak 2 (which contained substantial amounts of muramic acid and diaminopimelic acid) formed a 'line of identity' with the wall teichoic acids of this organism, and that the lipoteichoic acid itself was also heavily contaminated with wall teichoic acids. As both wall and membrane teichoic acids in this organism are polymers of glycerol, structural analysis of the lipoteichoic acid is difficult. Other fractionation techniques were no more successful. The difficulty was overcome by preparing the lipoteichoic acid from the L-form of this organism[11]. The lipoteichoic acid was extracted with 80% aqueous phenol at 4°, and the extracted material was fractionated on a Sepharose 6B column; the elution curve is shown in Fig. 6b. Neither nucleic acid nor protein could be detected in the fraction (Peak 1) that was free from cell-wall material and corresponded to lipoteichoic acid. It contained phosphorus, glycerol, D-glucose, and fatty acids (as methyl palmitate) in the molecular proportions 1.0:1.01:0.060:0.077, and small but variable amouts of glucosamine.

Bacillus subtilis *W-23*. — Extraction with either hot or cold, aqueous phenol gave a lipoteichoic acid preparation containing substantial amounts of DNA (Fig. 7) and wall teichoic acid. The DNA constituted about 93% of the material in the fraction containing lipoteichoic acid, and it was not removed by nucleases. Attempts to

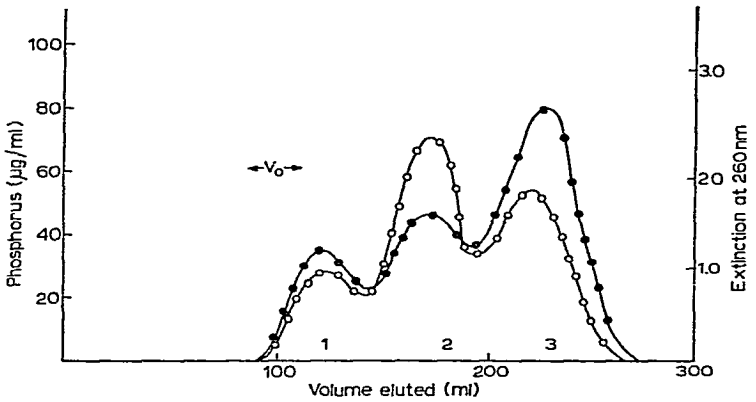

Fig. 7. Purification of the lipoteichoic acid of *Bacillus subtilis* W-23 (extracted by either hot or cold, 80% aqueous phenol) by chromatography on a Sepharose 6B column (for details, see the Experimental section): phosphorus (–●–●–●–) and extinction at 260 nm (–O–O–O–).

remove these contaminants had limited success, the most successful procedure being to prepare a wall-free membrane by lysis of cells with lysozyme. The membranes were then washed extensively in 0.03M magnesium chloride to remove wall materials and washed four times with 0.03M EDTA; the EDTA washings were combined and extracted with 80% aqueous phenol. After centrifugation, the upper aqueous phase was dialysed and kept at 4° under toluene for three days. A white precipitate was removed by centrifugation at 100,000*g* for 30 min. Impure lipoteichoic acid was recovered from the supernatant by chromatography on Sepharose 6B. This preparation was free of wall teichoic acid, but 20% of its phosphorus represented DNA. After correction for the DNA content, the preparation gave the proportions of phosphorus to glycerol to D-glucosamine to fatty acid as 1.0:1.1:0.26:0.12.

DISCUSSION

The examples emphasise particular aspects of the problems encountered in the preparation of lipoteichoic acids from the cytoplasmic membranes of Gram-positive bacteria. Purification of the lipoteichoic acid from *S. aureus* H is relatively simpler when the 80% aqueous phenol method is used. However, it is possible that the preparation could comprise more than one molecular species, especially in view of its small variable content of *N*-acetyl-D-glucosamine.

Preparations from *Micrococcus* 2102 contained minimal amounts of protein and nucleic acid when the hot, 80% aqueous phenol procedure was followed by

chromatography on Sepharose 6B in 25% methanol. However, such preparations contain up to 25% of wall teichoic acid. Although the wall and membrane teichoic acids are not covalently linked to each other in this preparation, nonbonded association might explain the chromatographic behaviour of this preparation. Similarly, preparations from *Bacillus subtilis* 168 could be obtained almost free from nucleic acid and protein, but wall teichoic acid was difficult to remove. The presence of wall teichoic acid in this case may arise from the ease of autolysis of this organism. As both wall and membrane polymers are derivatives of glyceryl phosphate, contamination is not easy to recognise. However it is possible to prepare the material uncontaminated by wall polymers by isolation from the L-form of this organism. This supports the observation by Slabyj *et al.*[12] that L-forms of Gram-positive bacteria do in fact still possess a membrane teichoic acid. Preparations from *Bacillus subtilis* W-23 were contaminated with large amounts of nucleic acid and, although various attempts were made to remove it, these were not completely successful.

For the preparation of both wall and membrane teichoic acids, it is always necessary to start from clean walls or membranes that have been isolated and separated from each other by effective physical and enzymic methods. Despite the losses usually encountered in centrifugation, failure to separate wall from membrane, or extraction of whole cells, invariably gives preparations that are contaminated. In a recent report by Chiu *et al.*[13] on the lipoteichoic acids from *Streptococcus sanguis*, sonication of cells was followed by centrifugation at 31,000*g*, and the pellet was then extracted with aqueous phenol. The lipoteichoic acid obtained after chromatography on Sepharose 6B was described as 'wall lipoteichoic acid'. From our experience, centrifugation at this relatively high speed would sediment both walls and fragmented membranes; consequently, it is more likely that the lipoteichoic acid described by Chiu *et al.*[13] is a mixture of wall and membrane teichoic acids and does not necessarily indicate the existence of a lipoteichoic acid in a bacterial wall.

We conclude that no general method is reliable for the extraction and purification of lipoteichoic acids from different organisms. The aqueous extraction procedure is of little value for the preparation of polymers suitable for analysis. When the preparation is likely to be contaminated with protein, hot aqueous phenol has advantages over cold aqueous phenol for the initial extraction step. In cases where contamination with wall polymers occurs, considerable difficulties may be encountered. Membrane teichoic acids prepared by the earlier method of extraction with cold trichloroacetic acid contain considerable proportions of D-alanine in ester linkage, *e.g.* that from *S. aureus*[6] gave a ratio of D-alanine to phosphorus as 0.87:1. This component is lost during the isolation steps described here for lipoteichoic acids, most of the loss occurring during the incubation with nucleases.

EXPERIMENTAL

Growth of organisms. — The organisms studied were *Staphylococcus aureus* H, *Micrococcus* sp. 2102, *Bacillus subtilis* 168, and *Bacillus subtilis* W-23. The organisms

were grown in each case as described previously[10,14–16], attempts being made to reduce autolysis by collecting cells in the late log phase and disrupting them immediately after collection. If autolysis occurred, severe contamination of the membrane teichoic acid with wall material resulted. It is important that extractions should not be carried out directly on whole cells; not only is the extraction more difficult owing to the viscosity of the cell contents, but greater contamination of the lipoteichoic acid occurs.

Preparation of membrane fractions. — Cell walls and membranes were prepared by disruption with glass beads. Cells (30 g, wet weight) were suspended in sodium chloride solution (0.9%, w/v, 100 ml) and shaken in an MSK Braun homogeniser with Ballotini No. 11 beads for 2.5 min. Beads were removed by filtration on a No. 1 sintered-glass funnel, and the filtrate was centrifuged at 16,000*g* for 30 min. The supernatant solution containing fragmented membranes was decanted and freeze-dried. Although the pellet contained cell walls heavily contaminated with cytoplasmic membranes, no attempt was made to isolate the lipoteichoic acid from such pellets, as initial investigations indicated that they would be heavily contaminated with wall material.

Earlier workers[6], instead of freeze-drying the supernatant solution containing the membranes, recentrifuged it at 100,000*g* for 1 h; the membrane teichoic acid was then extracted from the resulting clear gel of ribosome-membrane preparation. In our experience, not only is this step unnecessary but it also causes a reduction in yield, possibly due to the fact that the lipoteichoic acid only has a weak, magnesium ion-controlled association with the cytoplasmic membrane. It is known that the teichoic acid can be completely removed from protoplasts or from membranes by washing the preparations with buffers in the absence of magnesium ions[4]. It is, therefore, not surprising that, when ribosome-membrane pellets are prepared, lipoteichoic acid can always be detected in the supernatant.

Extraction of lipoteichoic acid. — The freeze-dried supernatant containing the membranes (10 g) was stirred overnight with chloroform–methanol (500 ml, 2:1, v/v), and then the residue was washed with chloroform–methanol. This procedure was repeated three times to ensure that all lipids had been removed. Traces of solvents were removed by drying the residue in air. The lipoteichoic acids were extracted from the defatted membranes by three different procedures.

(a) *Cold, aqueous phenol extraction.* The residue (about 8 g) was suspended in water (250 ml) and stirred with an equal volume of 80% (w/v) aqueous phenol for 40 min at 4°. The resulting emulsion was centrifuged at 16,000*g* for 30 min, and the upper aqueous phase collected and dialysed overnight against running water. The insoluble material at the interface and the lower phenol phase containing much of the protein were discarded. The volume of the dialysate was reduced to about 80 ml by rotary evaporation at 30–40°, and the solution was mixed with an equal volume of 0.1M-Tris buffer, pH 8.0, containing 0.02M magnesium chloride. Ribonuclease and deoxyribonuclease (10 μg/ml) were added, and the mixture was incubated at 37° under toluene in order to hydrolyse the nucleic acids. The incubation was continued for

48–72 h, thereby removing alanine ester residues from the teichoic acid. The volume of the incubation mixture was reduced to 80 ml by rotary evaporation, and the aqueous phenol extraction procedure was repeated in order to remove the enzymes and protein not removed in the first extraction. After centrifugation, the upper aqueous phase was dialysed as described earlier and freeze-dried. Analysis indicated that at least 95% of the phosphorus, in the material washed with chloroform–methanol, was extracted into the aqueous phase. The weight of the freeze-dried powder was 8–10% of the initial weight of the membrane material.

(b) *Hot, aqueous phenol extraction.* The procedure was identical to that just described, but the first aqueous phenol extraction was carried out at 60°. The second extraction to remove the nucleases was carried out at room temperature.

(c) *Aqueous extraction.* The defatted membranes were suspended in water (25 mg/ml) and kept for 30 min at 100° with constant stirring. The solution was 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 phosphorus of the defatted membranes was solubilized.

Initial purification of the lipoteichoic acid. — The freeze-dried extract (100 mg) was dissolved in water (2 ml). The insoluble material was removed by centrifugation, the solution was applied to a column of Sepharose 6B (450 mm × 25 mm), and the material was eluted by use of an upward flow (15 ml/h) with 0.2M ammonium acetate, pH 6.9, containing 0.01% of sodium azide, the column having been previously prepared in this buffer. Fractions (3 ml) were collected automatically. The content of every third tube was analysed for phosphorus and nucleic acid (260 nm). Appropriate fractions were combined, dialysed against several changes of distilled water to remove the ammonium acetate, and then freeze-dried. The column void-volume (V_0) was determined in a separate experiment by measuring the elution volume of Blue Dextran. Occasionally a small peak showing both absorption at 260 nm and the presence of phosphorus was seen before the peak that occurred in the void volume. This component was initially thought to arise from an impurity in the Sepharose, but it is due to colloidal material in the freeze-dried extract. This component could be removed by centrifuging the sample at 12,000*g* before chromatography. A typical elution diagram is shown in Fig. 1.

Refractionation of the lipoteichoic acid (Peak 1) in the presence of methanol. — Freeze-dried material (20 ml) corresponding to Peak 1 was dissolved in water containing 25% of methanol (2 ml) and chromatographed on a column (25 mm × 450 mm) of Sepharose 6B by use of an upward flow (15 ml/h) with 0.02M ammonium acetate containing 0.1% of sodium azide and 25% of methanol, the column having been previously prepared in this buffer. Fractions were collected and analysed as described before.

General analytical methods. — Nucleic acid was determined by absorption at 260 nm, protein by the method of Lowry *et al.*[17], phosphate ions by the method of Chen *et al.*[18], glycerol by the method of Hanahan and Olley[19], D-glucose by glucostat, and fatty acids by the method of Snyder and Stephens[20]. The presence of wall

polymers in preparations from *Micrococcus* 2102 was examined by hydrolysis with 0.1M hydrochloric acid for 10 min at 100°, whereupon the poly(2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate) is hydrolysed to 2-acetamido-2-deoxy-D-glucose 6-phosphate[10]. The presence of wall teichoic acid in the preparations from *S. aureus* H and *B. subtilis* W-23 (both these organisms having ribitol wall teichoic acids) was examined by hydrolysis with 2M hydrochloric acid for 3 h at 100°, after dialysis to remove buffer. Acid was removed *in vacuo* over potassium hydroxide before paper chromatography. Muramic acid and diaminopimelic acid were determined after hydrolysis with 4M hydrochloric acid for 16 h at 100° with a JEOL amino acid analyser.

Paper chromatography. — Paper chromatography was performed on Whatman No. 1 paper (descending). System A, butan-1-ol–ethyl acetate–pyridine–water (5:5:1:3), followed by spraying with silver nitrate reagent[21], was used to detect 2-acetamido-2-deoxy-D-glucose phosphate. System B, propan-1-ol–aqueous ammonia (sp. gr. 0.88)–water (6:3:1), followed by spraying with periodate–Schiff reagent[22], was used to detect glycerol and glyceryl monophosphates. System C, butan-1-ol–ethanol–water–aqueous ammonia (sp. gr. 0.88) (40:10:49:1), followed by spraying with periodate–Schiff reagent, was used to distinguish between glycerol and 1,4-anhydro-D-ribitol.

Agarose-gel diffusion with concanavalin A or DEAE-dextran. — Concanavalin A (5 mg/ml) in Tris buffer (0.05M, pH 7.3) or DEAE-dextran (10 ml/ml) in the same buffer[23] were used in gel-diffusion studies in agarose (1%) in 0.05M Tris buffer. Precipitation zones were usually seen after 24 h with concanavalin A or 48 h with DEAE-dextran.

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