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Comparative Studies on the Isolation of Membrane Lipoteichoic Acid from *Lactobacillus fermenti*

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Preparations of membrane lipoteichoic acid containing different amounts of protein were isolated from intact organisms of *Lactobacillus fermenti* NCTC 6991 by various procedures chosen for their ability to disrupt the hydrophobic interaction of lipoteichoic acid with other membrane components. The highest yield of lipoteichoic acid was obtained with hot aqueous phenol, and this preparation contained very little protein. Partial removal of cell lipids with chloroform-methanol followed by extraction with water at 100 C gave a lipoteichoic acid-protein complex that was a very effective immunogen; immunogenicity was shown to relate to protein content, though the specificity of the antibodies was directed against the teichoic acid component.

Membrane glycerol teichoic acids have been isolated from several species of lactobacilli by extraction of disrupted organisms with cold aqueous phenol; such preparations, referred to as lipoteichoic acid, differ from those obtained by trichloroacetic acid extraction in that they contain glycolipid and protein, and are immunogenic when injected into rabbits with adjuvant (10, 19, 20). The nature of these complexes is consistent with the lipoteichoic acid being an integral component of the common leaflet structure of the cell membrane through hydrophobic interaction of the glycolipid portion of the molecule with other membrane components. Electron microscopy evidence for this proposed membrane location has been obtained by the use of ferritin-labeled antibody (D. van Driel et al., J. Ultrastruct. Res., *submitted for publication*).

This paper describes the isolation of lipoteichoic acid from intact organisms of *Lactobacillus fermenti*. The procedures used were chosen for their ability to disrupt hydrophobic bonds, and, in particular, methods that had previously proved useful in extracting lipopolysaccharides from gram-negative bacteria were surveyed. In addition, the relation between protein content of these preparations and their immunogenicity has been examined.

MATERIALS AND METHODS

Organism. *L. fermenti* NCTC 6991 was originally obtained from the National Collection of Type Cultures, Colindale, London, and belongs to serological Group F (15). Organisms were grown in batch culture to the stationary phase (9), washed, and freeze-dried.

Chloroform-methanol-water extraction procedure. Organisms were extracted with chloroform-methanol (2:1, v/v), in the proportion 1:20 (w/v), by stirring at 18 C for at least 2 hr. Organisms were recovered by filtration through a No. 5 sintered-glass filter, and the extraction procedure was repeated twice. Residual solvent was removed in vacuo and the dry residue of organisms suspended in distilled water (1:10, w/v) at 100 C for 30 min. The aqueous extraction was repeated twice, and the combined aqueous extracts were concentrated by rotary evaporation at 30 C and dialyzed against three changes of distilled water. The indiffusible fraction, which contained crude lipoteichoic acid, was clarified by centrifugation and freeze-dried.

Comparison of other extraction procedures. Several methods that have been used in the extraction of lipopolysaccharides from gram-negative bacteria were compared for their efficacy in extracting lipoteichoic acid. In each case extraction was performed on 2 g of freeze-dried organisms from the same batch culture as follows. (i) Hot aqueous phenol—Organisms reconstituted in 30 ml of water were extracted with an equal volume of 90% (w/v) phenol at 65 to 68 C (17) and the aqueous phase was dialyzed. (ii) Hot water—Organisms were reconstituted in water (100 ml) and stirred vigorously at 80 C for 30 min (14). Extraction was repeated twice, and the combined extracts were dialyzed and concentrated by rotary evaporation. A further batch of organisms was extracted with chloroform-methanol (as described above) before extraction with water at

80 C. (iii) Pyridine formate—Organisms were extracted three times for 10 min at 100 C with 20 ml of pyridine-formic acid (76:49, v/v) (11). (iv) Water-ether—Organisms were extracted with water saturated with diethyl ether (11, 13). (v) Trichloroacetic acid—Organisms were extracted with 5% trichloroacetic acid by homogenization in the cold for short periods and also by gentle stirring at 4 C for 3 hr (11). In addition, the cells, after extraction with trichloroacetic acid, were treated with hot aqueous phenol (12). (vi) Cetyltrimethyl ammonium bromide—Organisms were extracted under the conditions described by Nowotny et al. (11) and Westphal and Jann (16).

Deacylation of lipoteichoic acid. Lipoteichoic acid was dissolved in water (1 volume), mixed with 10 volumes 0.2 M methanolic KOH at 37 C, and incubated at 37 C for 15 min (21). The solution was chilled in ice, diluted with water, and deionized by passage through a column of Dowex 50 (H^+ form). The acidic eluant was extracted three times with chloroform to remove fatty acids, and the aqueous phase was neutralized with ammonia. Solutions of deacylated teichoic acid were concentrated by rotary evaporation and centrifuged to remove precipitated protein before chromatography.

An alternative mild deacylation procedure used was incubation at 18 C for 16 hr of a mixture of teichoic acid in water (1 volume) and concentrated ammonia (d = 0.880, 2 volumes). Ammonia was removed by rotary evaporation, and fatty acids were extracted with chloroform following deionization as described above.

Partial removal of protein from lipoteichoic acid complex. Lipoteichoic acid obtained by chloroform-methanol-water extraction was subjected to various chemical and enzymatic procedures in an attempt to lower the protein content as determined by the subsequent analysis of products isolated by chromatography on 6% agarose. The two most satisfactory methods proved to be (i) extraction with hot aqueous phenol (17) and (ii) incubation of 100 mg of lipoteichoic acid with 10 mg of papain (Sigma Chemical Co., St. Louis) in 100 ml of 0.1 M sodium phosphate buffer, 0.01 M cysteine-hydrochloride, and 0.002 M ethylenediaminetetraacetic acid, pH 7.0, at 37 C for 20 hr, followed by extraction with cold phenol (19). Less-effective methods were digestion with pepsin, trypsin, and streptococcal Pronase (Sigma Chemical Co.) or treatment with 1% sodium dodecyl sulfate, 3 M LiCl, or 8 M urea (3).

Column chromatography. Gel chromatography was carried out on columns (40 by 2.6 cm) of 6% agarose (Litex, Denmark) and Sepharose 2b (Pharmacia) at 20 C with 0.2 M ammonium acetate, pH 6.9, as elutant (19).

Deacylated teichoic acids were fractionated on columns (initially 34 by 2 cm) of diethylaminoethyl (DEAE)-Sephadex A25 (Pharmacia) equilibrated in 0.01 M imidazole-hydrochloride buffer, 0.1 M NaCl, pH 6.8. Teichoic acid was applied in starting buffer and eluted with a linear gradient of 0.1 M to 1.0 M NaCl in the same buffer (total volume, 500 ml).

Comparison of molecular sizes of fractions eluted from gel columns were made, as previously (19), by estimating their distribution coefficients (Kd). Values ranging from 0 to 1 indicate, respectively, complete exclusion from and complete accessibility to the stationary gel phase.

Analytical methods. Conditions for the acidic and alkaline hydrolyses of teichoic acids, enzyme dephosphorylation, and identification of products by paper chromatography were essentially as previously described (5, 18). Protein was estimated with the Folin-Ciocalteau reagent (6) with bovine serum albumin as a standard. Polysaccharide in column eluants was estimated by phenol-concentrated H_2SO_4 (2). Other quantitative analytical procedures, solvent systems used for paper chromatography, and chromatographic spray reagents were as detailed previously (19).

Serological methods. Antisera to teichoic acid preparations were prepared by subcutaneous injection into rabbits of emulsions containing equal volumes of teichoic acid solutions and Freund complete adjuvant, and the antibody content was determined by the quantitative precipitin reaction (8). Gel immunoelectrophoresis was carried out with standard LKB equipment (Uppsala, Sweden) on microscope slides coated with 0.85% Oxoid Ionagar no. 2 in 0.01 M potassium phosphate buffer, pH 7.0, containing 0.02% sodium azide as a preservative (1). Electrophoresis of teichoic acid antigens (1 mg/ml) was routinely carried out in the same buffer at 200 v for 75 min. After addition of antiserum, maximum development of precipitin lines was obtained in 16 to 24 hr at 4 C in a water-saturated atmosphere. Where necessary, antisera were concentrated to approximately 2 mg of antibody protein per ml by "dialysis" in Aquacide II (Calbiochem) at 4 C.

Electron microscopy. Freeze-dried preparations were fixed in osmium tetroxide and uranyl acetate (7), dehydrated in acetone, and embedded in araldite. Sections were cut with a diamond knife on a LKB Ultratome III, stained sequentially in uranyl acetate and lead citrate, and examined in a Philips 300 electron microscope by M. Vesk (Electron Microscopy Unit, University of Sydney).

RESULTS

Chloroform-methanol-water extraction of lipoteichoic acid. The chloroform-methanol-water extraction procedure described above was found in preliminary experiments to be optimal for the extraction of lipoteichoic acid associated with protein. Precipitin ring tests indicated that most of the teichoic acid was removed in the first two aqueous extractions at 100 C. Aqueous extraction at 30 C also removed the complex, but six or more extractions of 30 min were required for complete extraction.

Aqueous extracts were separated in columns of 6% agarose into two phosphate-containing fractions. The first (Kd = 0.1) contained teichoic acid and protein, whereas the second (Kd = 0.5) was nucleic acid. Quantitative

TABLE 1. *Properties of lipoteichoic acid fractions isolated from Lactobacillus fermenti by various extraction procedures*

Extraction procedure	Fraction	Molecular size (Kd) of lipoteichoic acid fractions[a]		Yield (μmoles of teichoic acid phosphorus/g of cells)[b]	Analyses of lipoteichoic acid fractions after Sepharose chromatography			
		6% Agarose	Sepharose 2b		Mole ratio P:Glu:Gal[c]	Protein (%)	Nucleic acid (%)	Fatty acid ester (%)
Chloroform-methanol-water (100 C)		0.10	0.27	51.6	1:0.09:0.10	19.0	0.26	2.6
Chloroform-methanol-water (80 C)		0.10	0.27	51.6	1:0.12:0.08	36.0	11.1	11.7
Water (80 C)	I	ND[d]	0.0	3.1	1:0.51:0.18	28.8	6.96	13.7
	II	ND	0.27	40.6	1:0.15:0.09	35.0	2.20	6.1
Hot aqueous phenol		0.10	0.27	123.0	1:0.08:0.11	1.40	1.79	6.6
Pyridine formate	I	ND	0.0	2.95	1:2.65:1.47	3.75	5.80	22.7
	II	ND	0.27	16.75	1:0.23:0.17	8.26	3.28	12.8
	III	0.50	ND	ND	ND	4.75	74.1	ND
Water-ether		ND	0.0	7.25	1:0.24:0.07	57.5	5.04	10.7

[a] Values for fractions quoted from both 6% agarose and Sepharose 2b indicate sequential chromatography on columns of both gels.

[b] Yield calculated from total phosphorus in Sepharose fractions and corrected for nucleic acid phosphorus.

[c] Phosphorus-glucose-galactose, corrected for nucleic acid phosphorus. The lipoteichoic acid from *L. fermenti* has previously been characterized as a glycosyl-substituted polyglycerophosphate (19), and, consequently, analyses for glycerol are not included in these data.

[d] Not determined.

analyses of the lipoteichoic acid preparation extracted at 100 C (preparation 2a) are shown in Table 1. Similar analyses were obtained for preparations extracted at 30 C.

Figure 1 shows electron micrographs of thin sections of organisms at different stages during extraction. Intact wall, plasma membrane, and mesosomal elements are evident in sections of freeze-dried organisms (Fig. 1a); after solvent extraction the triple-layer membrane is still present (Fig. 1b), but this virtually disappeared after aqueous extraction at 30 C (Fig. 1c) although the wall still appeared intact.

Comparison of other extraction procedures. Extraction with hot aqueous phenol, hot water (80 C), pyridine formate, and water saturated with ether gave soluble fractions containing lipoteichoic acid (Table 1). Hot phenol extraction gave the highest yield of lipoteichoic acid-containing material, whereas the lowest yield was obtained with the water-ether method, by which dialysis of the fraction obtained by chromatography on Sepharose gave an insoluble phosphate-containing precipitate. Methods that used trichloroacetic acid or cetyltrimethyl ammonium bromide did not yield significant amounts of soluble lipoteichoic acid. Hot phenol extraction, following treatment of organisms with trichloroacetic acid, also failed to extract lipoteichoic acid in contrast to the high yields by hot phenol extraction alone.

The products obtained by extraction with hot water (80 C), hot aqueous phenol, and pyridine formate were fractionated by column chromatography (results are compared in Table 1). Chromatography of the hot water extract on Sepharose 2b gave a minor fraction (I) that was excluded from the gel and apparently consisted of membrane fragments containing lipoteichoic acid (Wicken and van Driel, *unpublished observations*), and a major fraction (II) containing lipoteichoic acid and protein. The relative proportion of the two fractions varied from one batch of extracted organisms to another. The recovery of material obtained by aqueous extraction at 80 C could be increased by prior extraction of the cells with chloroform-methanol, though the resultant product contained significantly more nucleic acid (Table 1).

Chromatography of the aqueous phenol extract on 6% agarose yielded a crude lipoteichoic acid fraction low in protein but containing

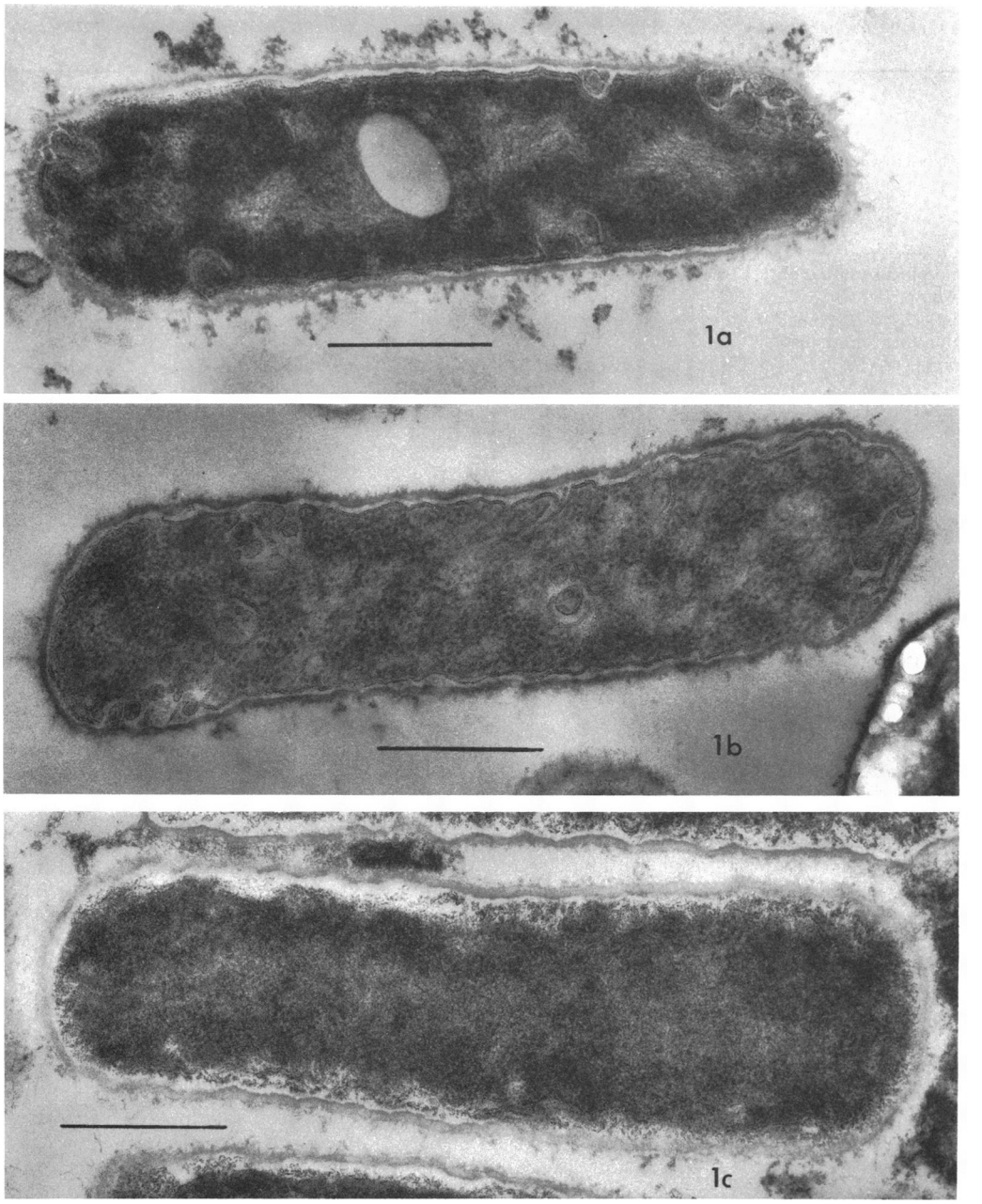

FIG. 1. *Electron micrographs of thin sections of Lactobacillus fermenti after various stages of lipoteichoic acid extraction. (a) Freeze-dried whole organisms before extraction, showing plasma and mesosomal membranes; (b) after extraction of lipid with chloroform-methanol, showing clear profiles of membranous elements; (c) after exhaustive aqueous extraction at 30 C of the lipoteichoic acid-protein complex from defatted organisms, showing an intact cell wall but the virtual disappearance of typical membrane profiles. Markers represent 400 nm.*

approximately 40% nucleic acid. Much of the nucleic acid was removed from the lipoteichoic acid by further chromatography on Sepharose 2b (Table 1), or by treatment of the extract with deoxyribonuclease and ribonuclease at *p*H 7.0 prior to chromatography.

Column chromatography of the pyridine-formate extract gave two lipoteichoic acid frac-

tions (I and II, Table 1) similar to those obtained by hot water extraction but in lower yields; a third fraction (III) contained nucleic acid as the major component.

The mole ratios of phosphorus, glucose, and galactose in the various preparations are compared in Table 1 with those for the previously isolated fraction (preparation 2a) obtained by extraction with chloroform-methanol-water (100 C). Variations are apparent in the amounts of carbohydrate present; subsequent studies, described below, on deacylated lipoteichoic acid indicate that the variations observed arise from contamination by a polysaccharide fraction containing glucose and galactose. Such a polysaccharide is a component of the cell wall (9).

Fractionation and partial characterization of lipoteichoic acid. The preparation obtained by chloroform-methanol-water extraction (preparation 2a) bound irreversibly to ion-exchange celluloses and gels unless fatty acids were previously removed by mild deacylation. Such deacylated material could then be separated on DEAE-Sephadex A25 into a polysaccharide-containing fraction, eluted between 0.3 M and 0.5 M NaCl, and a teichoic acid-containing fraction (preparation 2b) eluted between 0.75 M and 0.8 M NaCl. The polysaccharide fraction contained glucose, small amounts of galactose and phosphorus, and amino acids and hexosamines typical of peptidoglycan.

The teichoic acid fraction (preparation 2b) contained phosphorus, glucose, and galactose in the proportions 1:0.06:0.12. Chromatography of hydrolysates (acid, and alkali followed by phosphomonoesterase) showed the same degradation products, typical of a glycosyl-substituted glycerol teichoic acid as described previously (19) for similar hydrolysates of cold phenol-extracted material. Preparation 2b contained 6.9% protein, and was eluted from 6% agarose as a single peak with Kd = 0.48. This value is consistent with the preparation having a higher molecular weight than the previously described protein- and lipid-free fraction (Kd = 0.56) obtained by trichloroacetic acid extraction (19).

Lipoteichoic acid extracted with cold phenol (19) or hot aqueous phenol also bound irreversibly to ion-exchange celluloses and gels. Deacylation and elution from DEAE-Sephadex A25 showed that these preparations were free from contamination by polysaccharide.

Protein content and immunogenicity of lipoteichoic acid. The lipoteichoic acid-protein complex, designated preparation 2a, contained 19% protein, whereas the fraction obtained on deacylation (preparation 2b) contained 6.9% protein. From the point of view of the immunogenicity of teichoic acid, it was of interest to attempt to remove protein from preparation 2a while still retaining the hydrophobic and therefore high-molecular-weight character of the lipoteichoic acid moiety. Of several procedures that were examined, the two most suitable were extraction with hot aqueous phenol (preparation 2c), and digestion with papain followed by cold phenol extraction (preparation 2d); preparations 2c and 2d contained 3.5% and 1.3% protein, respectively.

Each of the preparations (2a-d) was injected into three rabbits, and the amount of precipitating antibody reacting with preparation 2a was determined. Individual rabbit variations were apparent but the results are indicative of a direct relation between antibody production and protein content (Fig. 2). Also included for comparison are previously reported results for lipoteichoic acid extracted with cold phenol, containing 8.0% protein (8); this preparation previously designated P-teichoic acid is here termed preparation 1.

Each of the preparations tested, except 2b, reacted equally with the antisera (Fig. 3). This preparation, which had been deacylated, precipitated only 40% of the antibody; it has been shown previously (8, 10) that the diminished serological reactivity of deacylated lipoteichoic acid antigens relates to their lower molecular weight rather than loss of antigenic determinants. The cell wall polysaccharide from *L. fermenti*, which contains glucose and galactose, did not react with any of the sera.

The specificity of the reactions between antibodies and preparations 2a to d was examined by immunoelectrophoresis and inhibition of the precipitin reaction. Immunoelectrophoresis of the three lipid-containing preparations (2a, 2c, 2d) showed a single electronegative component; the deacylated preparation (2b) also showed a single component but with a higher mobility.

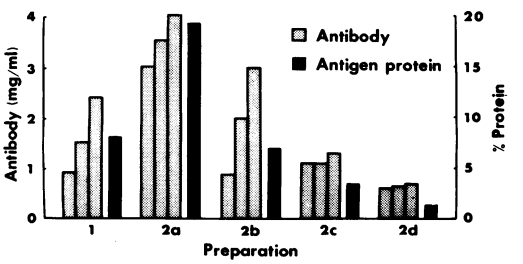

FIG. 2. *Comparison of protein content of lipoteichoic acid preparations and their immunogenicity on injection into groups of three rabbits.*

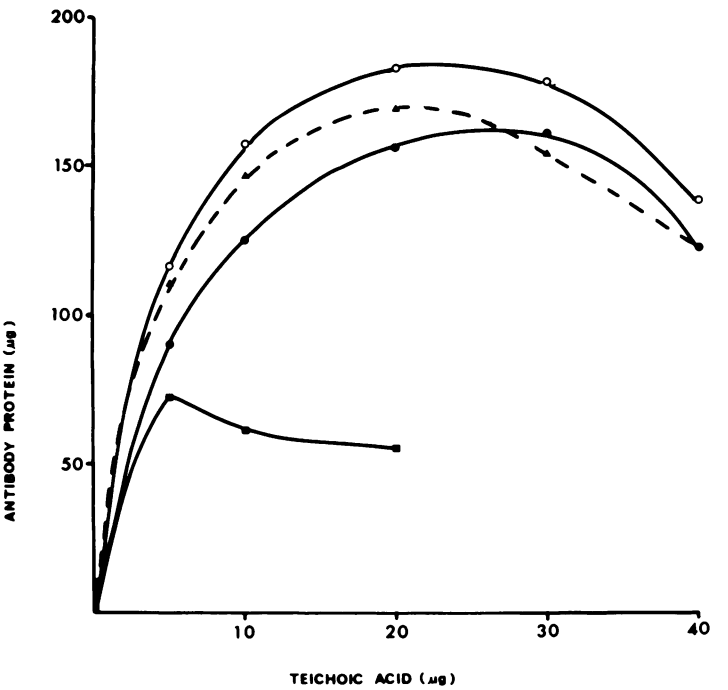

FIG. 3. *Precipitation of L. fermenti teichoic acid preparations by antiserum (40 μliters) against L. fermenti lipoteichoic acid (preparation 2a). Symbols:* ▲, *preparation 1;* ●, *preparation 2a;* ■, *preparation 2b;* ○, *preparation 2d.*

Prior absorption of antiserum with any one of the four preparations removed all detectable reaction with each of the other preparations. These results indicate that the preparations differing in electrophoretic mobility have the same serological specificity. Other preparations of lipoteichoic acid obtained by the action on preparation 2a of pepsin, trypsin, or Pronase showed a pair of fused lines, without spurring, that corresponded with the two components previously detected; this result is also consistent with the two components being serologically identical.

The components common to all the above preparations are teichoic acid and protein. To determine whether specificity depended on the teichoic acid component alone, sufficient protein- and lipid-free teichoic acid (2) was added to the sera to precipitate all the antibody that would react with this component. Such absorbed sera did not react with any of the other preparations (2a-d), indicating that the only antibodies detectable were those reacting with the teichoic acid component.

The specificity of the serological reaction with the teichoic acid component of the lipoteichoic acid-protein complex (preparations 1 and 2a) was examined by inhibition of the quantitative precipitin reaction. For each of the antisera to preparation 2a, galactose was a more effective inhibitor than glucose, indicating that specificity was directed primarily against the galactose component. For instance, with antiserum 200 the reaction between 50 μliters of serum and 20 μg of preparation 2a was inhibited 8% by 100 μmoles of D-glucose and 30% by 100 μmoles of D-galactose; in the reaction with preparation 1, the corresponding values were 7% and 33%. These results are similar to those previously obtained for antisera to preparation 1 (8).

DISCUSSION

The previous isolation of a lipoteichoic acid-protein complex from the cytoplasmic fraction of disintegrated cells of *L. fermenti* was consistent with the proposition that teichoic acid is a component of the cytoplasmic membrane (19), and more direct evidence for such a location was subsequently obtained by electron microscopy using ferritin-labeled antibody to the teichoic acid component (D. van Driel et al., J. Ultrastruct. Res., *submitted for publication*).

In the present study it has been shown that lipoteichoic acid associated with varying amounts of protein can be isolated from intact

organisms by a number of procedures that had been used for extracting lipopolysaccharides from gram-negative bacteria. The most frequently used method for obtaining lipopolysaccharide free from protein involves extraction with hot aqueous phenol, and this procedure has also proved to be the most efficient for extracting lipoteichoic acid from *L. fermenti*. Cold emulsions of phenol-water were used previously to extract lipoteichoic acid from subcellular fractions (19), but these preparations contained more protein (8%) than those currently obtained with hot aqueous phenol. Similar findings have been reported for the phenol extraction of lipopolysaccharides (16), and it has been proposed that under the conditions of hot phenol extraction a protein-lipid-polysaccharide complex is degraded to a lipoprotein and a lipopolysaccharide (22). Whether the hot aqueous phenol method will be generally applicable to the extraction of lipoteichoic acid from intact gram-positive organisms is not yet known; very low yields have been obtained on extraction of some streptococcal strains.

For obtaining lipoteichoic acid associated with protein, a milder procedure involves partial removal of cell lipids with chloroform-methanol followed by aqueous extraction. Chloroform-methanol extraction would produce a lipid-depleted membrane though no change is detectable by electron microscopy; similar findings have been reported for lipid-depleted mitochondrial membranes (4). The subsequent treatment of cells with water, which extracts lipoteichoic acid, results in the cells no longer having a detectable membrane system. The cell wall still appears intact so that the results are consistent with the conclusion that membrane teichoic acid has been extracted from intact organisms. Sufficient damage to the membrane can also be imparted by freeze-drying to allow for a partial release of lipoteichoic acid by aqueous extraction.

A comparison of the lipoteichoic acid preparations obtained by extraction with phenol (hot or cold) and chloroform-methanol-water shows that preference for one particular method would relate to specific requirements. The lipoteichoic acid obtained by the aqueous extraction procedure is contaminated with a component that contains the cell wall polysaccharide. The isolated polysaccharide contains 36% glucose and 47% galactose (9), so that the amount of contaminant, although sufficient to influence the analyses, cannot be very great.

The product extracted with chloroform-methanol-water has the advantage that it is a very good immunogen. Immunogenicity correlates directly with protein content, the preparation obtained by cold phenol extraction (preparation 1) being less effective as an immunogen (8), whereas hot aqueous phenol extraction gives a product of very low protein content and poor immunogenicity (preparation 2c).

Previous results (8) showed that the lipid component of lipoteichoic acid influences the immunogenicity of teichoic acid, presumably by virtue of the lipoteichoic acid molecules forming a high-molecular-weight-micellar structure through hydrophobic interaction of the lipid components. The present results show the influence of the protein component on immunogenicity. In each situation, however, the specificity of the antibodies is towards the teichoic acid component, and no precipitating antibodies have been detected with specificity towards the lipid or protein components; all precipitating activity in these sera was removed by absorption with lipid- and protein-free teichoic acid.

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