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Soluble Macromolecular Complexes Involving Bacterial Teichoic Acids

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Cell wall and membrane teichoic acids from several bacteria formed soluble complexes with polysaccharides and bovine plasma albumin in alkyl alcohol solutions. Polysaccharides which contain different monomeric units and anomeric configurations complexed with the teichoic acids, suggesting that the interaction is relatively nonspecific. Teichoic acids complexed glycogen or bovine plasma albumin in 50 to 97% ethanol solutions. The macromolecular association between teichoic acids and polysaccharides or proteins was independent of teichoic acid size over a threefold molecular weight range. Glycerol phosphates or an acid hydrolysate of teichoic acid would not complex to either glycogen or bovine plasma albumin in ethanol. The optimal interaction between glycogen and the *Bacillus subtilis* lipoteichoic acid occurred between pH 4.5 and 8.2. The ability of teichoic acids to bind polysaccharides and proteins in moderate dielectric constant solvents suggests that these polymers may serve as complexing agents for hydrophilic molecules found in membranes.

Bacterial teichoic acids are long-chain polymers containing repeating units of glycerol or ribitol phosphate with other components attached to the polyols. They behave as typical polyelectrolytes in aqueous solution (10) and occur in both the cell walls and cytoplasmic membranes of gram-positive bacteria (2). Membrane teichoic acids are usually referred to as lipoteichoic acids since they contain covalently bound lipid molecules. It is not known whether membrane and wall teichoic acids have similar functions. However, Fiedler and Glaser (12) found that lipoteichoic acid serves as a carrier for cell wall teichoic acid precursors. Because of their high charge densities, it is easily rationalized that teichoic acids could interact readily with polysaccharides or oppositely charged proteins.

Several reports have appeared which suggest that teichoic acids can form associations with other macromolecules. Young and Jackson (31) showed that deoxyribonucleic acid preparations from *Bacillus subtilis* contained contaminating teichoic acid which could not be readily removed by precipitation with alcohol or by phenol extraction. Cell wall and membrane teichoic acids which contain alpha-D-glucose residues precipitate with concanavalin A (8, 10, 24).

Teichoic acids adsorb to erythrocyte membranes and such sensitized cells form excellent indicators for the presence of anti-teichoic acid antibodies (3, 14). In addition, Kohoutová (21) has suggested that the competence factor of *Diplococcus pneumoniae* binds to cell wall teichoic acids. Brown et al. (4) found that the *N*-acetyl-muramyl-L-alanine amidase of *B. subtilis* associates strongly with teichoic acids. Recently, Höltje and Tomasz (16) showed that lipoteichoic acid inhibits the amidase of *D. pneumoniae*. It is well known that the adsorption of bacteriophage by many cell types involves an interaction between the virus and cell wall teichoic acids (5, 32). In this report, we show that both cell wall and membrane teichoic acids form stable soluble complexes with polysaccharides and proteins in alkyl alcohol solutions. The results suggest a new functional role for teichoic acids, namely the binding of hydrophilic molecules in hydrophobic areas of the cell surface.

MATERIALS AND METHODS

Polysaccharides, teichoic acids, and proteins. Bacterial dextrans with known modes of linkages (18), as well as a bacterial levan, were gifts from A. Jeanes, Northern Regional Research Lab., Peoria, Ill. Cell wall teichoic acids were prepared from *B. subtilis* 168 by affinity chromatography (10). Teichoic acids from the cell walls of *B. subtilis gtaC10*, *B. subtilis gtaB290*, *B. subtilis* W-23, *Staphylococcus aureus* H,

and *S. aureus* 052-2 were isolated by previously described methods (5, 20). Cell wall teichoic acids from *S. epidermidis* T_1 and *S. aureus* 406 were kindly provided by R. Ekstedt, Northwestern University Medical School, Chicago, Ill. A lipoteichoic acid from *B. subtilis gtaB290* was isolated by hot phenol extraction of whole cells as described by Wicken et al. (29). A membrane teichoic acid (6) from *Streptococcus sanguis* was kindly provided by T. H. Chiu, University of Pittsburgh, Pittsburgh, Pa. A. J. Wicken, University of New South Wales, Australia, provided a membrane teichoic acid preparation from *Lactobacillus fermenti* NCTC 6991 (30). I. Ofek, University of Tennessee Medical Units, Memphis, Tenn., generously supplied a lipoteichoic acid from *Streptococcus pyogenes* (23). Amylose, galactan and lichenan, and rabbit liver glycogen were purchased from Calbiochem., La Jolla, Calif., Pierce Chemical Company, Rockford, Ill., and Sigma Chemical Co., St. Louis, Mo., respectively. All polysaccharide samples were dissolved in water and precipitated by the addition of 5 volumes of absolute ethanol before use. Bovine plasma albumin (BPA), lot Y-4013, assay 100%, was obtained from Schwarz/Mann, Orangeburg, N.Y.

Chemical analyses. Phosphorus was determined by the ashing procedure described by Ames (1). Protein was quantitated by the standard Lowry (22) technique using bovine plasma albumin as the standard. Polysaccharides were determined with the anthrone technique using the individual polysaccharides as standards.

Molecular weight determinations. Molecular weights of teichoic acid preparations were determined by sedimentation equilibrium or by the Archibald approach to equilibrium method using standard techniques. A partial specific volume of 0.57 ml/g was assumed in all calculations (9).

General procedures for complex formation. The interaction between teichoic acids and polysaccharides or protein was studied in the following manner. Polysaccharide or protein, dissolved in water (0.5 ml), was added to 0.5 ml of aqueous teichoic acid solution, followed by 5 ml of cold absolute alcohol. After an overnight incubation of 4 C, the solutions were centrifuged for 5 min at 15,000 × *g* and the precipitates were washed once with 10 ml of 83% (vol/vol) alcohol solution. The insoluble material was analyzed for polysaccharide (or portein) and phosphorus. Controls (samples lacking teichoic acid, protein, or polysaccharide) were run in parallel. Concentrations of the reactants and the kinds of alcohols used are given in the text.

The effect of hydrogen ion concentration on the interactions was studied using the following reagents: HCl, pH 2.8; acetic acid, pH 4.5; *N*-[tris(hydroxymethyl)-methyl]-glycine (tricine), pH 5.2; 4-morpholine-propanesulfonic acid, pH 5.6; tris(hydroxymethyl)aminomethane, pH 7.8; triethanolamine, pH 8.2; NaOH, pH 8.9. Glycogen and teichoic acid (0.5 ml) were mixed with 0.5 ml of 0.02 M acid, buffer, or base, and 5 ml of 95% ethanol was added. Apparent pH values were determined for the resulting aqueous ethanol solutions by use of a Corning model 7 pH meter equipped with a combination electrode.

RESULTS

Complexes soluble in 83 % ethanol. Normally, most polysaccharides precipitate in relatively concentrated alcohol solutions. The *B. subtilis* 168 cell wall teichoic acid prevented the precipitation of glycogen in 83% ethanol (Fig. 1). In the strictest sense, the teichoic acid "solubilized" the glycogen. Because solubilization resulted from the interaction of two macromolecules, we prefer to use the term "complex formation" to describe the association between glycogen and teichoic acid. The ability of the teichoic acid to form complexes with glycogen that were soluble in 83% ethanol depended on the relative weight ratio of the two reactants. For example, 20 μg of teichoic acid complexed 1,000 μg of glycogen. Smaller quantities of the teichoic acid complexed correspondingly smaller quantities of the polysaccharide. When 2.5 μg of teichoic acid was mixed with 1,000 μg of glycogen, none of the polysaccharide was complexed. However, 2.5 μg of teichoic acid complexed 60 μg of glycogen when the original mixture contained 100 μg of the glucan. Repeated efforts to centrifuge down the soluble complex were ineffective. Centrifugations for 30 min at 25,000 × *g* did not result in removing either glycogen or teichoic acid from the solution. Furthermore, glycogen precipitates were found not to contain phosphorus when small quantities of teichoic acid were present. Thus, insoluble glycogen-teichoic acid complexes were not formed in the presence of 83% ethanol.

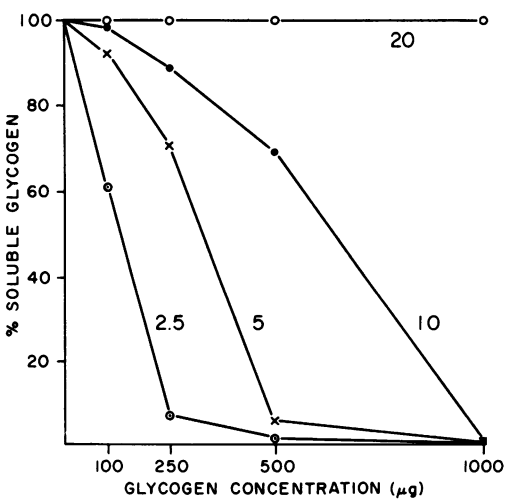

FIG. 1. *Glycogen-B. subtilis 168 cell wall teichoic acid interactions in 83% ethanol. The amounts of teichoic acid (in micrograms) are shown by the numbers on the curves. In the absence of teichoic acid, greater than 96% of the glycogen was insoluble.*

The *B. subtilis* 168 cell wall teichoic acid also complexed with other polysaccharides containing different monomeric residues as well as different types of linkages (Table 1). Lichenan, a beta-linked polysaccharide containing D-glucose monomers, was poorly complexed by the teichoic acid. However, when 50 μg of teichoic acid was mixed with 500 μg of lichenan, complete solubilization of the D-glucan occurred. The results (Fig. 1 and Table 1) suggest that the teichoic acid displays little specificity in terms of the binding reaction. This was confirmed by interacting the teichoic acid with dextrans possessing well-characterized linkages (17). These results are shown in Table 2. It is apparent that the extent of complex formation cannot be correlated with a given type of linkage. One dextran, B-1298, interacted poorly with the teichoic acid. This dextran, however, was insoluble in water.

Effect of solvent. To investigate the specificity of solvent, complex formation was explored with other alcohols. The data in Table 3 show that complexes can form in aqueous solutions of other alcohols. Ethanol appears to be the most effective of the solvents. Tertiary butanol was effective, but the glycogen control was partially soluble in this solvent.

Teichoic acid specificity. It was important to assess whether teichoic acids derived from different bacterial walls or membranes would complex other macromolecules. Preliminary experiments showed that all teichoic acid preparations were completely soluble in 83% ethanol. When the teichoic acids were mixed with glycogen followed by the addition of alcohol, it was found that complex formation occurred with all the preparations (Table 4). Thus, both lipoteichoic acids and cell wall teichoic acids were effective in complexing glycogen. In addition, the type of sugar substituent on the teichoic acid was not important in the interaction. However, the sugar substituent could be significant in determining the relative affinities of the polymer interactions. Alpha- or beta-glycerol phosphate could not be substituted for teichoic

TABLE 1. *Complex formation between polysaccharides and the teichoic acid of B. subtilis 168*[a]

Polysaccharide	Principal monomer	Principal linkage	Complex with teichoic acid
Dextran	D-glucose	α-(1→6)	96.0
Amylose	D-glucose	α-(1→4)	47.5
Galactan	D-galactose	α-(1→4)	94.6
Lichenan	D-glucose	β-(1→3), β-(1→4)	16.7
Levan	D-fructose	β-(2→6)	99.1

[a] The amounts of teichoic acid and polysaccharides present were 10 and 500 μg, respectively. The amount of complexed polysaccharide refers to percentage of polysaccharide solubilized by the teichoic acid. In the absence of teichoic acid the polysaccharides were 97 to 100% insoluble. The reactions were run in 83% ethanol in a total volume of 6.0 ml.

TABLE 2. *Teichoic acid-induced solubilization of bacterial dextrans in ethanol*[a]

Dextran	Mode of linkage			Soluble dextran
	% α-(1→6)	% α-(1→4)	α-(1→3)	
B-1208	95	5	0	72.2
B-1254	93	7	0	97.2
B-1225	90	10	0	78.2
B-1255	89	7	4	92.3
B-1351	85	4	11	95.6
B-1298	64	36	0	1.1
B-1355(S)	57	8	35	99.1

[a] The reactions were carried out in a 6.0-ml total volume containing 83% ethanol (vol/vol), 500 μg of dextran, and 10 μg of *B. subtilis* 168 teichoic acid. The soluble dextran values were computed as the percentage of total dextran solubilized by the teichoic acid. In the absence of teichoic acid, the dextrans were 98 to 100% insoluble in the alcohol.

TABLE 3. *Teichoic acid-glycogen complex formation in alkyl alcohol solutions*

Reactants[a]	Solvent[b]	Soluble glycogen (μg)
Glycogen (100 μg)	Methanol	6.1
Glycogen (100 μg) + teichoic acid	Methanol	96.1
Glycogen (250 μg)	Methanol	8.7
Glycogen (250 μg) + teichoic acid	Methanol	190.4
Glycogen (100 μg)	Ethanol	4.7
Glycogen (100 μg) + teichoic acid	Ethanol	98.4
Glycogen (250 μg)	Ethanol	6.9
Glycogen (250 μg) + teichoic acid	Ethanol	219.2
Glycogen (100 μg)	Propanol	5.9
Glycogen (100 μg) + teichoic acid	Propanol	76.8
Glycogen (100 μg)	tert-Butanol	49.1
Glycogen (100 μg) + teichoic acid	tert-Butanol	85.5

[a] Teichoic acid (10 μg) from *B. subtilis* 168 cell wall was used.

[b] Final solvent concentrations were 83% (vol/vol) in a total volume of 6.0 ml.

TABLE 4. *Specificity of complex formation between bacterial teichoic acids and glycogen in ethanol*[a]

Teichoic acid origin	Polyol	Sugar substituent	Complexed glycogen
Staphylococcus epidermidis T_1	Glycerol	α-D-Glucose	496
S. aureus 406	Glycerol	*N*-Acetylgalactosamine	488
S. aureus H	Ribitol	*N*-acetylglucosamine	495
S. aureus 052-2	Ribitol	None	491
S. pyogenes	Glycerol (lipoteichoic acid)	None	485
Streptococcus sanguis ATCC 10556	Glycerol (lipoteichoic acid)	α-D-Glucose	489
Bacillus subtilis gtaB290	Glycerol	None	471
B. subtilis gtaB290	Glycerol (lipoteichoic acid)	Unknown	496
B. subtilis gtaC10	Glycerol	None	468
B. subtilis W23	Ribitol	β-D-Glucose	483
B. subtilis 168	Glycerol	α-D-Glucose	487
Lactobacillus fermenti NCTC 6991	Glycerol (lipoteichoic acid)	α-D-Galactose	498

[a] The quantities of teichoic acid and glycogen present were 20 and 500 μg, respectively. Total volume was 6.0 ml with a final ethanol concentration of 83%. "Complexed glycogen" refers to the amount of glycogen (micrograms) forming an ethanol-soluble complex with the teichoic acid.

acid and yield a soluble complex with glycogen. Thus, in terms of structure, any polymer containing glycerol or ribitol phosphate-repeating units would probably serve to complex polysaccharides in alcohol solutions.

Complex formation with proteins. Conceivably, proteins as well as polysaccharides should interact with teichoic acids to form soluble complexes in alcohol solutions. The cell wall teichoic acid from *B. subtilis* 168 was mixed with BPA and alcohol was added. The results shown on Fig. 2 show that BPA is also complexed by the teichoic acid. The binding of BPA by the teichoic acid is less efficient than that of glycogen (Fig. 1). For BPA, 250 μg of teichoic acid easily complexed 1,000 μg of the protein, whereas 20 μg of teichoic acid easily complexed 1,000 μg of glycogen. Although an exhaustive survey was not performed, it was found that the teichoic acid (amounts ranging from 5 to 500 μg) would not complex 250- or 500-μg quantities of human immunoglobulin G, concanavalin A, subtilisin, myoglobin, or trypsin. In addition, deoxyribonucleic acid and ribonucleic acid remained insoluble in the presence of either cell wall or membrane teichoic acids. However, all teichoic acids shown in Table 4 were capable of forming soluble complexes with BPA in 83% ethanol. With either BPA or polysaccharides the order of addition of the reactants was not critical. The end result was always the same, that is, in the presence of teichoic acid, the polymers were soluble in 83% ethanol, whereas in the absence of teichoic acid, they were insoluble. This can be considered as evidence for true complex formation.

Effect of molecular weight. The cell wall

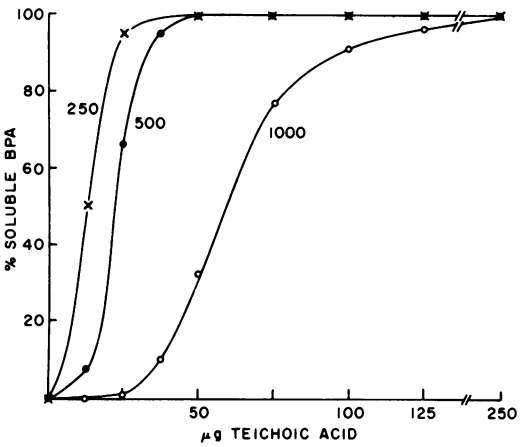

FIG. 2. *Complex formation between B. subtilis 168 cell wall teichoic acid and BPA. Numbers on the curves indicate the amount of BPA present (micrograms). The total volume was 6.0 ml with an ethanol concentration of 83%. In the absence of teichoic acid the albumin was 92 to 97% insoluble.*

teichoic acid of *B. subtilis* 168 was prepared by different procedures and the molecular weights of the products were determined by sedimentation equilibrium. The fringe displacement-concentration profiles showed that the teichoic acids prepared by chemical extractions were polydisperse. The teichoic acid preparations, along with an HCl hydrolysate, were interacted with glycogen or BPA in 83% ethanol. The data, shown in Table 5, show that there is no apparent molecular weight effect in either enhancing or destroying complex formation. The acid-hydrolyzed teichoic acid gave no significant binding to either glycogen or BPA. These results

TABLE 5. *Effect of teichoic acid molecular weight on complex formation with glycogen and BPA*

Teichoic acid preparation[a]	Mol wt	Soluble glycogen (μg)[b]	Soluble BPA (μg)[c]
Affinity column	24,800	370 ± 30	470 ± 27
Affinity column, degraded	18,400	388 ± 33	473 ± 29
Trichloroacetic acid (60 C) soluble	8,500	380 ± 36	485 ± 18
Alkali soluble	7,800	374 ± 41	466 ± 37
Acid hydrolysate[d]	—	35 ± 10	51 ± 23

[a] Teichoic acids were from the cell wall of *B. subtilis* 168. Trichloroacetic acid extraction was for 3 h at 60 C. Alkali extraction (17) (0.1 N NaOH) was performed 10 h at room temperature under nitrogen. The teichoic acid prepared by affinity chromatography was degraded by sonic treatment as described in another study (9).

[b] The amounts of glycogen and teichoic acid present were 500 and 10 μg, respectively. Values refer to the amount of glycogen complexed with the teichoic acid in 83% ethanol. A minimum of four separate determinations were made and the standard deviations shown. Total volumes were 6.0 ml.

[c] The amount of BPA present was 500 μg, whereas 37.5 μg of teichoic acid was employed. Values (with standard deviations) refer to the amount of protein complexed with the teichoic acid in 83% ethanol.

[d] Teichoic acid was hydrolyzed with 4 N NCl at 100 C for 6 h. HCl was removed with a rotary evaporator.

show that only macromolecular teichoic acid can form ethanol-soluble macromolecular complexes.

Effect of hydrogen ion. Complex formation between glycogen and the lipoteichoic acid of *B. subtilis gtaB290* was studied in buffered-aqueous ethanol solutions. The results, shown in Fig. 3, show that the glycogen was solubilized readily by the teichoic acid between pH 4.5 and 8.0. However, at both the lower and higher pHs (pH 2.8 versus 8.9) the teichoic acid itself was insoluble. Thus, soluble complexes with glycogen could not be detected. In addition, high concentrations of buffer (0.1 M and above) induced precipitation of the teichoic acid control.

Effect of ethanol concentration. Figure 4 shows that lipoteichoic acid can complex glycogen and BPA over a wide range of ethanol concentrations. At ethanol concentrations above 90%, the extent of soluble complex formation appears to diminish. Because of the necessity of preparing the polymers in aqueous solution, the highest practical ethanol concentration used was 97%. Below 70% ethanol, the glycogen and BPA controls were partially soluble. Nevertheless, complex formation could still be detected since in the presence of lipoteichoic acid both glycogen and BPA were completely soluble. These results show that macromolecular associations between teichoic acids and

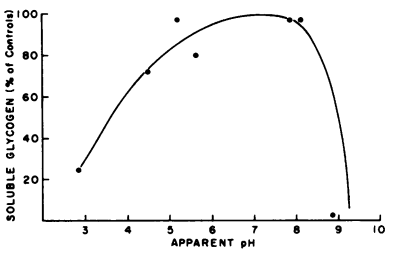

FIG. 3. *Interaction between glycogen and the lipoteichoic acid of B. subtilis gtaB290 as a function of pH. The amounts of teichoic acid and glycogen present were 50 and 500 μg, respectively. Final volumes were 6.0 ml. The ethanol concentration was 79.1%.*

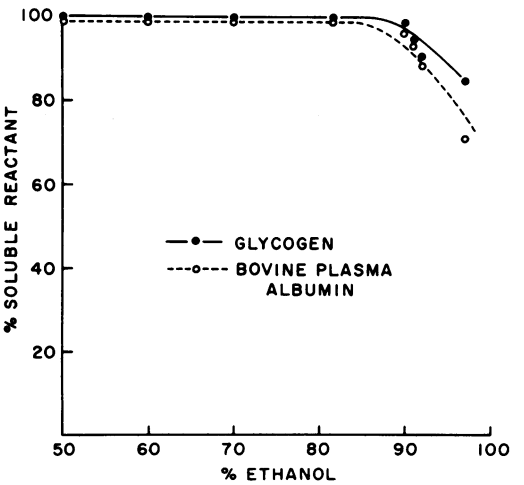

FIG. 4. *Effect of ethanol concentration on the interaction between lipoteichoic acid and glycogen or BPA. Lipoteichoic acid (20 μg), from L. fermenti NCTC 6991 was used. Glycogen (500 μg) and BPA (250 μg) were in a total volume of 6.0 ml.*

polysaccharides or proteins can occur in solvents with dielectric constants lower than that of water.

DISCUSSION

The results of these studies show that bacterial teichoic acids can complex or solubilize polysaccharides and BPA in alcohol solutions. Normally, both polysaccharide molecules and BPA are insoluble in relatively concentrated solutions of alkyl alcohols. The teichoic acid molecules interact with these polymers to form soluble complexes. The interactions described in this study are probably nonspecific in nature, because several polysaccharides, containing different monomeric units as well as different linkages, were rendered soluble by the teichoic

acids. Moreover, specificity is also lacking in terms of teichoic acid structure, since both glycerol and ribitol teichoic acid, possessing different sugar substituents, as well as lipoteichoic acids, formed soluble complexes with polysaccharides and BPA. Monomeric glycerol phosphates did not interact, indicating that polymer-polymer interactions were essential for complex formation. We suggest that these interactions are mediated through hydrogen bonds. Such bonds would probably be stable in 83% alcohol solutions, but not in aqueous solutions due to the competitive effects of water (25). In addition, polymer-polymer interactions may have favorable entropy effects, in which the stability of one hydrogen bond is enhanced by other similar bonds (25). What then is the biological significance of these types of interactions? We suggest that teichoic acids can form soluble complexes with a variety of substances in or on the membranes of gram-positive bacteria. This would be a means by which enzymes or cell wall precursors could remain soluble in membrane phases. Higashi and Strominger (15) isolated an isoprenoid alcohol phosphokinase which required phospholipid for activity and was stable in hydrophobic solvents. Crane and Lampen (7) suggested that certain enzymes, such as the penicillinase of *B. licheniformis*, were fixed into membranes by the binding of phospholipids. Our concept is similar, except we believe that teichoic acids would exhibit little specificity for proteins. A growing body of literature has shown that various anionic polyelectrolytes complex with enzymes in vitro to either stimulate or depress hydrolytic activity (see review by Elbein, reference 11). Holtje and Tomasz (16) have suggested that lipoteichoic acids may be involved in the in vivo control of autolysin activity.

The compositional symmetry of bacterial cytoplasmic membranes has not been studied in any significant detail. Lipoteichoic acids are probably located on the external face of the cytoplasmic membrane (28). Thus, lipoteichoic acid may most efficiently complex proteins in the periplasmic areas of the membrane. However, Theodore et al. (27) recently presented evidence which suggests that most of the lipoteichoic acid found in gram-positive bacteria is located in mesosomal vesicles. We are not in a position to offer a model showing spatial relationships between membrane-bound proteins and teichoic acids. We simply suggest that lipoteichoic acids may have the potential for complexing hydrophilic molecules under relatively hydrophobic conditions. Whether cell wall teichoic acids can play a similar role is doubtful. The fact that wall teichoic acids demonstrate the ability to complex polysaccharides and proteins in alcohols is probably a reflection of structural similarities, e.g., repeating phosphate groups separated by either glycerol or ribitol.

If one considers parallels to the kinds of interactions we propose for lipoteichoic acids of gram-positive bacteria, gram-negative cells must also possess similarly functioning polymers. However, in the gram-negative cell, many proteins are loosely held to the membrane, presumably in the periplasmic spaces (13). It is possible that lipopolysaccharide molecules or lipopolysaccharide precursors, as well as phospholipids (26), could complex proteins near the surface of cytoplasmic membranes. Jung et al. (19) have shown that phospholipids complex with glucose in chloroform. These kinds of complexes may serve as models for the transport of sugars and related molecules across cell membranes.

The suggestion that lipoteichoic acids complex with hydrophilic molecules in membranes is admittedly speculative. It is known, however, that proteins are difficult to remove from lipoteichoic acid preparations (29). We are presently attempting to isolate lipoteichoic acid-protein complexes from purified *B. subtilis* 168 membranes using hydrophobic solvents.

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