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Membrane Lipoteichoic Acid of *Streptococcus pyogenes* and Its Stabilized L-Form and the Effect of Two Antibiotics upon Its Cellular Content

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Membrane lipoteichoic acid continues to be synthesized by an osmotically fragile, stabilized L-form of *Streptococcus pyogenes*. Chromatographic and electrophoretic comparisons indicate that the lipid component of lipoteichoic acid in this L-form and its parental streptococcus is glycerophosphoryldiglucosyl diglyceride and not phosphatidylkojibiosyl diglyceride. Based upon dry weight determinations, the yield of lipoteichoic acid from the L-form is 0.19%, as compared with 0.97% from the streptococcus. When grown with bacitracin, the L-form contains the same amount of teichoic acid as when grown without this antibiotic; however, its lipoteichoic acid content is reduced by 85%. Similarly, the L-form grown with novobiocin for 10 h contains only 17% of the teichoic acid found in control cells.

In an earlier publication, we detailed the initial finding of a glycerol teichoic acid (TA) in the membrane of a stabilized L-form of *Streptococcus pyogenes* (28). Also, we showed that this TA is smaller in size and is lacking D-alanine when compared with that isolated from the parental coccus (28). In a subsequent enzymic study, it was also shown that although this L-form possesses the required soluble components for D-alanine incorporation, its membrane cannot function as the acceptor even though it contains D-alanine membrane-deficient TA. It was concluded that a defect exists in the membrane of this stabilized L-form for D-alanine incorporation into membrane TA. (3).

In the membranes of most gram-positive bacteria, glycerol TA is associated with lipid as a lipid complex, lipoteichoic acid (Lp-TA) (15). Most recently, a phosphoglycolipid has been established as the lipid component of Lp-TA in *Streptococcus faecalis* (8). Such a lipid complex is capable of sensitizing erythrocytes (13, 19), and that from the group A streptococci has been suggested as a possible factor in the pathogenesis of rheumatic fever and glomerulonephritis (15, 19). The role of bacterial L-forms in pathogenesis is speculative; however, this speculation has been strengthened by recent findings with an osmotically fragile L-form of *S. pyogenes*. It has been shown that this L-form can be rapidly adapted to grow in physiologically isotonic media (physiological L-form) and is capable of destroying human heart cells in tissue culture (16), and survives in immunosuppressed mice (P. Bhat-Fernandes and C. Panos, Abstr. Annu. Meet. Am. Soc. Microbiol., 1976, D38, p. 57). In this regard, we have now shown that the lipid chain rigidity of the membrane of this streptococcus and these L-forms is in the order: physiological L-form > osmotically fragile L-form > streptococcus (4). This report establishes the continued presence of membrane Lp-TA in these stabilized L-forms of *S. pyogenes* and indicates the nature of its lipid component. Also, it documents the inhibition of Lp-TA by bacitracin in a bacterial L-form.

MATERIALS AND METHODS

Bacteria and media. *S. pyogenes*, type 12, its stabilized but osmotically fragile L-form (28), and this L-form recently adapted to grow in physiological isotonic media (physiological L-form) were used (16). Usually, from 20 to 60 liters of medium (in 5-liter batches) was incubated at 37°C for 16 h (late stationary growth phase) and harvested by centrifugation (17,000 × *g*, 4°C). The streptococcus was cultured in a medium of lipid-preextracted brucella broth (Pfizer Diagnostics, Brooklyn, N.Y.) and preextracted bovine serum albumin (fraction V, 8 g/liter, Armour Pharmaceutical Co., Chicago, Ill.) (11, 16), supplemented with (in grams per liter): sodium acetate, 10; glucose, 19; KH_2PO_4, 4.5; with pH adjusted to 7.3 with NaOH. Since no significant increase in the dry weight yield of the osmotically fragile L-form was demonstrable in this supplemented medium (28), this same but unsupplemented

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medium, with the addition of 0.35 or 3.0% (wt/vol) NaCl, was used to grow the physiological and osmotically fragile L-forms, respectively. Streptococcal medium readily caramelized when autoclaved (28). Thus, to minimize this effect, the medium was quickly chilled in cold water (10°C) after being autoclaved and before the addition of sterile (by filtration) albumin. The effect of antibiotics on Lp-TA formation was studied by using the L-form harvested from 40 to 60 liters of medium containing bacitracin (300 μg/ml of medium) or novobiocin (5 μg/ml of medium). Only L-form inocula for the bacitracin studies contained this antibiotic. Different lots of bacitracin behaved differently towards these L-forms (see Results). The lot used in these studies, while permitting continued growth, resulted in a reduction in the dry weight yield of L-form cells in large cultures by 38% at the concentration used. L-form cultures growing with novobiocin had to be harvested after 10 h to prevent lysis. For the isolation and identification of lipid of Lp-TA, the coccus and L-form were grown in the lipid-preextracted medium without acetate, glucose, and KH_2PO_4. Because of the extremely small yields of Lp-TA from the L-form (see Table 2), this complex was labeled by adding 250 μCi of [U-^{14}C]glycerol (specific activity, 46 mCi/mmol) to the undefined but lipid preextracted growth medium. The total amount of radioactivity incorporated was approximately 0.1%. Pieringer and Ambron (24) have discussed the specific labeling of glycerol in glyceride-containing lipids of a streptococcus with ^{14}C-labeled glycerol.

Extraction and purification of TA and Lp-TA. Washed cells of *S. pyogenes* and its L-form (28) were extracted with phenol at 4°C, and the crude Lp-TA was purified according to Wicken and Knox (35). Trichloroacetic acid extraction and purification of TA using a split column Sephadex gel and Dowex 2(Cl-) column chromatographic procedures has been described by us (28). In our earlier study (28), cellular yields of TA were calculated from column elution profiles, by determining the amount of phosphorus present excluding that ascribed to ribonucleic acid, and the results were expressed as milligrams of TA per gram (dry weight) of cells. Because of differences in molecular weight, 1 mg of TA phosphorus was found to be equivalent to 6.9 and 5.7 mg of TA from the coccus and L-form, respectively. However, since different methods were used for obtaining TA and Lp-TA in the present study, all chromatographic results are given as milligrams of phosphorus per gram (dry weight) of cells.

Analytical procedures and identification of hydrolytic products. Most of the methods indicated by us earlier for the characterization of TA from this streptococcus and its L-form were again used (28). For the isolation of lipid, Lp-TA was dissociated with 60% hydrofluoric acid (HF) at 4°C, neutralized after 6 h with LiOH and $LiCO_3$ (2, 31), and the resulting supernatant and precipitate were extracted for lipid with chloroform. Lipid was also obtained by mild hydrochloric acid hydrolysis and deacylated as described by Ganfield and Pieringer (8). Lipid was examined by thin-layer and paper chromatography and high-voltage electrophoresis (50 V/cm). For thin-layer chromatography, Silica Gel G plates (400 and 250 μm thick) were used with solvent systems: (A) chloroform-methanol (9:1, vol/vol) or (B) 8:2, vol/vol), (C) chloroform-methanol-water (65:35:8, vol/vol/vol), or (D) diisobutyl ketone-acetic acid-water (40:25:5, vol/vol/vol). Also, paper chromatography of intact lipid utilized silicic acid-impregnated paper, SG-81 (Whatman), ascending, with solvent system (D). For deacylated lipid, high-voltage electrophoresis was performed, as detailed elsewhere (4), and by paper chromatography with Whatman no. 1 paper, ascending, overnight in solvent system (E); butanol-pyridine-water (4:3:4, vol/vol/vol). Lipid and deacylated lipid were visualized by Rhodamine G (0.001% aqueous solution, wt/vol) spray and ultraviolet light, alkaline silver nitrate (32) by dipping or with the molybdate reagent (6, 10). After chromatography, radioactivity was detected by placing scrappings from segmented thin-layer plates or paper chromatograms (1- by 2-cm blocks) into 0.3% PPO (2,5-diphenyloxyzole) in toluene (10 ml) and counting in a Packard Tri-Carb liquid scintillation spectrometer model 3320 for 5, 10, and 50 min. Finally, after deacylation fatty acids were extracted, methylated, and analyzed by capillary column (150-foot length, Carbowax K-20M) gas chromatography as previously detailed (22).

Materials. [^{14}C]glycerol-labeled intact and deacylated phosphatidylkojibiosyl diglyceride were gifts from R. Pieringer, Temple University. [U-^{14}C]glycerol was obtained from the Amersham/Searle Corp., Arlington Heights, Ill. Albamycin (sodium novobiocin, lot no. 334 BY) and the bacitracin (lot no. 416 BG K2) used during large-scale cultivations are products of the Upjohn Co., Kalamazoo, Mich. HF was a gift from the Pennwalt Corp. (Three Parkway, Philadelphia, Pa).

RESULTS

Detection of Lp-TA. In our earlier publication (28), the TA from this streptococcus and its L-form was characterized in great detail. The cellular yields and chemical composition of the TA component of the Lp-TA to be described below are in agreement with these past findings.

Table 1 compares the TA and Lp-TA content of *S. pyogenes* and its L-form. It indicates that neither adaptation of the osmotically fragile L-form to grow in physiological isotonic medium (physiological L-form) nor growth of the coccus in hypertonic medium changes the cellular TA content. Also, it suggests that all of the TA present in these two organisms is in the form of Lp-TA. Typical elution patterns of Lp-TA from *S. pyogenes* (S-Lp-TA) and its L-form (L-Lp-TA) are shown in Fig. 1 and 2, respectively. The coccal-to-L-form Lp-TA ratio, as calculated from such elution patterns, was 5.7:1 (Table 1). After column chromatography, the contaminating ribonucleic acid content of Lp-TA from the coccus was 0.4% and from the L-form 3.0%.

TABLE 1. *Teichoic acid and lipoteichoic acid content of Streptococcus pyogenes and its L-form with additions to the growth medium*[a]

Source and material obtained	Medium additions[b]		Antibiotics	
	Sodium chloride		Novobiocin (5 μg/ml of medium)	Bacitracin (300 μg/ml of medium)
	3.0% (wt/vol)	0.85% (wt/vol)		
Streptococcal TA	1.04 (1)[c]	1.24 ± 0.30 (5)[c]		
Streptococcal Lp-TA	ND	0.91 ± 0.06 (2)		
L-form TA	0.18 ± 0.09 (13)[c]	0.26 (1)	0.03 (1)	0.20 ± 0.05 (2)
L-form Lp-TA	0.16 ± 0.06 (2)	ND	ND	0.03 ± 0.02 (2)

[a] Expressed as milligrams of P per gram (dry weight) of cells.

[b] Additions to the lipid preextracted growth medium. Numbers in parentheses indicate number of different large batch cultures used for determining standard deviations. Abbreviations: ND, Not done; TA, teichoic acid; Lp-TA, lipoteichoic acid.

[c] From our earlier results (reference 28).

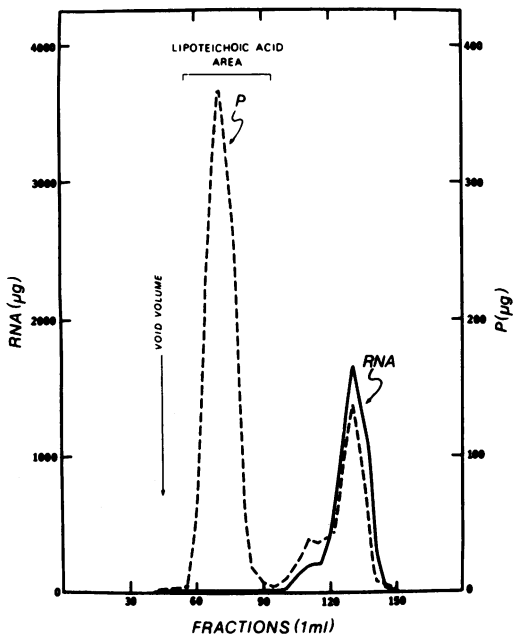

FIG. 1. *Sepharose 6B column (1.5 by 88 cm) chromatogram of lipoteichoic acid from Streptococcus pyogenes. Column loaded with 84 mg of crude material and eluted with 0.2 M ammonium acetate, pH 6.9, at a flow rate of 14 ml/h.*

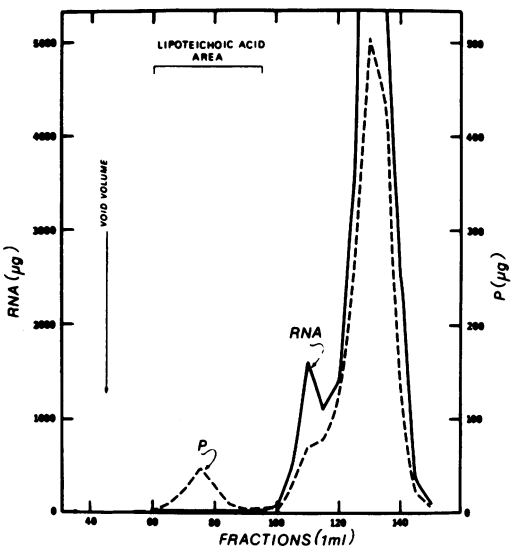

FIG. 2. *Sepharose 6B column (1.5 by 88 cm) chromatogram of lipoteichoic acid from stabilized L-form. Column loaded with 119 mg of crude material and eluted with 0.2 M ammonium acetate, pH 6.9, at a flow rate of 16 ml/h.*

Column chromatographic recoveries, in terms of phosphorus, were always better than 95%. Acid hydrolysates of Lp-TA from both organisms revealed glycerol monophosphate, glycerol diphosphate, inorganic phosphorus, glucose, glycerol, and traces of ninhydrin-positive material by paper chromatography. Also, traces of ester-linked alanine were present in only streptococcal preparations, as expected from the incubation at pH 8 during the phenol extraction procedure. As reported earlier (28), TA from only the L-form is devoid of D-alanine.

Figure 3 illustrates the extent of dissociation of S-Lp-TA into free TA and residual Lp-TA after treatment with 10% trichloroacetic acid for 48 h at 4°C. Identical results were obtained with L-Lp-TA. A similar dissociation of Lp-TA from *Lactobacillus fermenti* had been reported by Wicken and Knox (35).

Effect of antibiotics on Lp-TA formation. Table 1 tabulates the effect of bacitracin on Lp-TA formation in the osmotically fragile L-form. In such experiments, an overnight culture of the L-form grown with bacitracin (cell yield: 1.3 g [dry weight] per 10 liters of medium) was harvested, and divided in half, and TA was extracted by the trichloroacetic acid method,

and Lp-TA was extracted by the phenol procedure. Although the cell yield was reduced (by 38%) from that of cultures growing in the absence of bacitracin (control cells, 2.1 g [dry weight] per 10 liters of medium), there was no change in the content of TA when the L-form was grown with and without this antibiotic (Table 1, Fig. 4). However, of considerable interest was the finding that synthesis of Lp-TA was reduced by 85% (Table 1).

The minimal inhibitory concentration of bacitracin for L-forms of *Staphylococcus aureus* varies (36). Earlier, bacitracin had no effect upon this osmotically fragile L-form (20). During these studies, three different lots of this antibiotic inhibited growth of the parental streptococcus at the same level (3 μg/ml of medium). However, these same lots from the same manufacturer gave variable results when tested against the L-form. As already indicated, the particular lot number used for the isolation of TA and Lp-TA from the L-form also permitted continued growth of this organism and, also, of various osmotically fragile and physiological isotonic L-forms from our stock collection at a concentration of 300 μg/ml of medium. Likewise, these respective L-forms could be transferred in medium with this concentration of bacitracin for at least three sub-

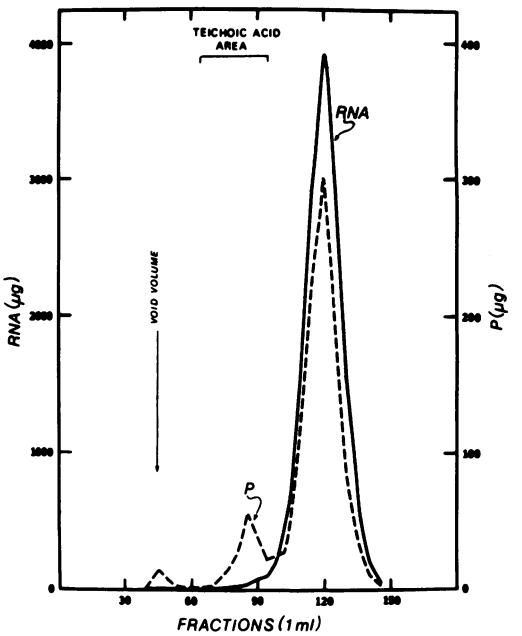

FIG. 4. *Stacked Sephadex column (1.5 by 18 cm G-25 M; 1.5 by 70 cm G-75) chromatogram of teichoic acid from stabilized L-form grown in presence of bacitracin (300 μg/ml). Column loaded with 75 mg of crude material and eluted with 0.2 M LiCl, pH 6.5, at a flow rate of 39 ml/h.*

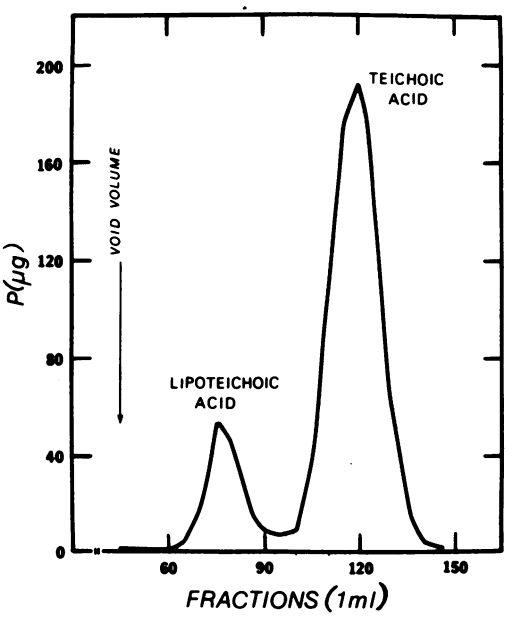

FIG. 3. *Sepharose 6B column (1.5 by 88 cm) chromatogram of lipoteichoic acid from Streptococcus pyogenes treated with trichloroacetic acid. Column loaded with 44 mg of material treated with cold 10% trichloroacetic acid for 48 h and eluted with 0.2 M ammonium acetate, pH 6.9, at a flow rate of 15 ml/h.*

transfers, the maximum number tested, without a continuing decrease in their growth being observed. A second lot (no. 839DC) behaved in an identical fashion. However, the activity of another lot (no. 268DB) of bacitracin ranged from at least 50% inhibition of growth at 80 μg/ml of medium, by the criteria established previously (20), to rapid cell lysis within 0.5 h, when greater than 80 μg/ml of medium was added to mid-logarithmically growing cultures of these same L-forms. Also, it was not possible to demonstrate continued viability of such cultures when this inhibition of growth occurred or when lysis was observed. Repeated consultations with the scientific personnel of the manufacturer failed to uncover reasons for this variability.

The L-form was grown in medium with novobiocin (5 μg/ml of medium) for one transfer (cell yield: 1.1 g [dry weight] per 10 liters of medium; 52% of control cells), and its TA content was determined with stacked Sephadex column chromatography (28). During this growth period, the L-form lacked detectable Lp-TA. Also, the trace amount of TA (17% of control cells) detected by chemical analyses (Table 1), was in addition to that from the growing inoculum (10% vol/vol; TA content, 0.02 mg of P). Growth of the coccus, in terms of optical

TABLE 2. *Typical cell and lipoteichoic acid yields from 60-liter cultures*[a]

Material	*Streptococcus pyogenes*	L-forms
Dry cells (g)	32.0	12.8
Crude extract after phenol extraction (mg)	774.0	359.0
weight in mg phosphorus	70.0	22.0
Pure lipoteichoic acid (mg)	310.0	24.5
weight in mg of phosphorus	37.0	2.9

[a] From overnight cultures of cells in lipid preextracted medium.

density, decreased by approximately 50% at a lower concentration of novobiocin (2 μg/ml of medium; see also reference 20).

Yields and lipid content of Lp-TA. Typical yields of materials from 60 liters of medium are tabulated in Table 2. The yield of Lp-TA from intact cells of the streptococcus and its L-form was 0.97 and 0.19%, respectively. Furthermore, whereas the amount of Lp-TA in phenol-extractable material from the coccus was 40%, similar extractable material from the L-form contained only 7% Lp-TA. HF treatment attacks phosphomono- and phosphodiester groups (31). The glycolipid content of purified Lp-TA from the coccus and its L-form, after HF treatment and gravimetric analyses, was 14.8 and 27.1%, respectively. Thin-layer chromatography of this lipid from both organisms gave identical results. Only one major spot was detected which was iodine and $AgNO_3$ positive and appeared yellow after detection with Rhodamine. No molybdate-positive material was noted, indicating absence of phospholipids. The R_f's of this lipid in solvent systems A and B were 0.08 and 0.35, respectively, and agreed with those for authentic diglucosyl diglyceride isolated and characterized by us earlier from this L-form and its parental streptococcus (5). Finally, molar ratios of this lipid from the parental streptococcus indicated a glucose-to-glycerol ratio of 2.22:1.00. Insufficient material prevented a similar determination with lipid from the L-form. Fatty acid analyses of the methyl esters of the glucolipid of Lp-TA from these two organisms were qualitatively identical to our earlier findings (5, 21). Again, palmitic acid predominated. Also, the oleic-to-*cis*-vaccenic acid ratios were reversed; 0.79 for the coccus, 6.99 for its L-form (21).

Evidence to be presented below indicates that glycerophosphoryldiglucosyl diglyceride is the lipid component of S-Lp-TA and L-Lp-TA. It is known that diglucosyl diglyceride is a degradative product of glycerophosphoryldiglucosyl diglyceride after HF treatment (see above and reference 31). Therefore, assuming that (i) the average fatty acid chain length for this phosphoglycolipid is 18 carbon atoms and (ii) considering that the TA from the coccus and its L-form is composed of 25 and 11 glycerophosphate units, respectively, with the former containing D-alanine and the latter devoid of this amino acid (28), the theoretical diglucosyl diglyceride content of Lp-TA from the coccus and its L-form is calculated to be 14.9 and 32.7%, respectively. Although excellent agreement between our experimental gravimetric value (14.8%) and the theoretical yield (14.9%) for glucolipid was obtained with coccal preparations, the observed value for this lipid from L-form Lp-TA was somewhat low (84% of theoretical). This is undoubtedly due to the extremely low content of Lp-TA from the L-form (Table 2) and the subsequent unavoidable loss of material during purification and isolation of its lipid component, making quantitative recoveries difficult.

Based upon (i) and (ii) above, the calculated content for glycerophosphoryldiglucosyl diglyceride in S-Lp-TA is 17.0% and for L-Lp-TA, 36.1%. Because of the close agreement between calculated and observed values for diglucosyl diglyceride after HF treatment, gravimetric analyses of lipid obtained after mild HCl hydrolysis was not performed with S-Lp-TA preparations. However, mild HCl treatment of L-Lp-TA indicated a lipid content of 31.4% (87% of theoretical). Lack of closer agreement is probably due, again, to the small amount of L-Lp-TA available (Table 2). From the calculated values for intact lipid of Lp-TA, the maximum content of glycerophosphoryldiglucosyl diglyceride in 1 g (dry weight) of the coccus and its L-form is 0.16 and 0.07%, respectively. The maximum yield of this lipid from a 60-liter culture of the L-form would be 8.9 mg; actual recovery was 7.7 mg. Finally, considering (ii) above, the calculated milligrams of phosphorus per milligrams of Lp-TA from the coccus and L-form is 8.02 and 8.14, respectively. From Table 2 it can be seen that the actual experimental value for the coccus is 8.38 and for the L-form, 8.45.

Nature of intact lipid of Lp-TA. Deacylated derivatives of glycerophosphoryldiglucosyl diglyceride and phosphatidylkojibiosyl diglyceride cannot be separated by the chromatography and electrophoresis systems used (4). However, these two intact lipids are separable by thin-layer chromatography in solvent system (C); with an R_f of 0.6 for phosphatidylkojibiosyl diglyceride and an R_f of 0.4 for glycerophosphoryl diglucosyl diglyceride. Earlier, Fischer et al. (7) had established the presence of glycerophosphoryldiglucosyl diglyceride in *S. haemolyticus (S. pyogenes)*. Therefore, the purpose of

these studies was to determine which of these two complex lipids is the lipid component of Lp-TA in *S. pyogenes* and its osmotically fragile L-form. Extremely small yields of purified and labeled Lp-TA from the L-form (Table 2) limited our studies with the lipid component of this complex. Conversely, much greater yields of Lp-TA from the streptococcus obviated the need for labeling S-Lp-TA (Table 2). Radioactive L-Lp-TA was mixed with nonlabeled Lp-TA from the coccus and subjected to mild acid hydrolysis. Labeled lipid, after thin-layer chromatography in solvent system (C), had an R_f of 0.38 and did not co-chromatograph with authentic phosphatidylkojibiosyl diglyceride (R_f = 0.63). Also, lipid of S-Lp-TA, after thin-layer chromatography in solvent system (D), showed two spots (R_f's = 0.25 and 0.50) staining blue with Rhodamine, indicating the presence of phosphorus. But, five spots appeared when solvent system (C) was used; of these, four were barely visible. The only intensely staining, phosphorus-containing spot observed had the same R_f as for glycerolphosphoryldiglucosyl diglyceride (R_f = 0.40). Using silicic acid-impregnated paper chromatography in solvent system (D), intact lipid of L-Lp-TA appeared blue with Rhodamine and had an R_f of 0.39. The lipid of S-Lp-TA, after similar chromatographic treatment, gave two phosphorus-containing spots with R_f's of 0.16 and 0.37; with the latter having the greatest intensity. In our hands, lipid of Lp-TA from the coccus and its L-form and standard phosphatidylkojibiosyl diglyceride had an R_f of 0.34 ± 0.03 (n = 5) after silicic acid-impregnated paper chromatography in solvent system (D); the same R_f (0.37) as reported by others (18) for phosphatidylkojibiosyl diglyceride. Using high-voltage electrophoresis, deacylated derivatives of phosphatidylkojibiosyl diglyceride (standard) and lipid of L-Lp-TA gave migration values of 17 and 18.5 cm, respectively (at 90 mA, 80 min), whereas deacylated lipid from S-Lp-TA gave the same migration value as the labeled standard (10 cm at 79 mA, 85 min). Paper chromatography of deacylated streptococcal lipid in solvent system (E) and from phosphatidylkojibiosyl diglyceride gave identical results (R_f = 0.22).

DISCUSSION

Our earlier results illustrated that most, if not all, of the glycerol TA in this group A coccus is probably associated with the membrane and is situated between the membrane and the rigid cell wall (28). This study also suggests that all of this TA is probably associated with lipid. Formation of mucopeptide and TA within the rigid cell wall appears to be interdependent (18). By comparison, these studies have shown a continued formation of Lp-TA in this L-form which lacks a rigid cell wall, indicating that its synthesis like that for TA formation at the membrane level is independent of cell wall formation in this group A streptococcus. However, the absolute concentration of this membrane component drops appreciably (to about one-sixth of its normal concentration) when synthesis of the bacterial cell wall is inhibited (i.e., in the stabilized L-form).

Whereas the primary site of action of bacitracin appears to be inhibition of dephosphorylation of undecaprenol pyrophosphate during bacterial cell wall formation (27), other effects include cell lysis and suppression of induced enzyme synthesis (9, 29). To our knowledge, these findings represent the first report of a drastic inhibition of Lp-TA formation by this antibiotic. At present, it is not possible to state whether this inhibition of Lp-TA formation is due to inhibition of formation of the essential lipid or to an enzymic defect resulting in an inability to combine available essential lipid with TA. Regardless, these results suggest that TA synthesis is not dependent upon formation of the essential lipid of Lp-TA. By comparison, most recent enzymic studies have shown that this L-form is unable to synthesize significant quantities of the lipid substrates for peptidoglycan formation (26).

Lipid of Lp-TA is believed to be the anchor by which TA is attached to the bacterial membrane (25). However, the results obtained with the use of bacitracin suggest that attachment of this polyglycerophosphate polymer might be possible by other means (i.e., in lieu of lipid or noncovalently bound). In these particular studies, the Lp-TA content of the L-form is drastically reduced while its TA content remains essentially constant. Therefore, combination of lipid with TA does not seem to be absolutely necessary for attachment of TA or for viability of the L-form if one assumes that the minute amount of Lp-TA still present (during growth with bacitracin) is insignificant. It has been stated that a primary purpose of glycerol TA is the accumulation of divalent cations (34) for membrane stability and for enzymic reactions. If so, these results with bacitracin seem to imply that this function may also occur when TA is not associated with lipid.

The osmotically fragile L-form grew reasonably well with a low level of novobiocin for one transfer. In contrast to the results obtained with bacitracin, cells grown with novobiocin contained very little TA. Thus, an explanation for the subsequent death of these cells, as found

by others using a cell-free system (34), may be the inhibition by novobiocin of the polyglycerophosphate backbone for glycerol TA formation. If one assumes this to be the only mode of action of novobiocin, then it becomes tempting to speculate that the results obtained with this antibiotic and bacitracin may be implying that whereas membrane glycerol TA is essential for survival of this L-form, Lp-TA may not be.

As is known, glycerol TA is nonimmunogenic, whereas Lp-TA is immunogenic (15). Since this L-form can be made almost devoid of antigenic Lp-TA, with the aid of bacitracin, one wonders how such changes in the surface structure of the membrane might affect the persistence of this organism in vivo or its cytopathic effects on human heart cells in tissue culture (16). It is known that repeated injection into rabbits of "membrane associated teichoic acid" from *S. pyogenes* results in tubular necrosis of the kidney (R. L. Waltersdorff and R. W. Jackson, Bacteriol. Proc., p. 117, 1970). Also, we have recently shown that this streptococcal L-form when adapted to grow in physiological isotonic medium can survive for long periods of time in immunosuppressed mice. (Bhat-Fernandes and Panos, Abstr. Annu. Meet. Am. Soc. Microbiol., 1976, D38, p. 57). Thus, the use of this L-form and bacitracin as a model system for investigating possible changes in the TA to Lp-TA content within the membrane of this streptococcal L-form as related to its persistence in vivo and destructive capabilities in vitro seems worthy of note.

Others have shown that some bacteria excrete Lp-TA into the growth medium (14, 17); however, no attempt was made to determine if excretion of this polymer was occurring during growth of the L-form. Earlier attempts to detect TA by chemical means in the growth medium of this L-form proved unsuccessful (28); i.e., chromatographic and chemical analysis of at least 100 mg of precipitates from spent medium.

Some serological evidence is at hand suggesting an association between M protein on the cell surface and membrane TA in the group A streptococci (K. W. Wittner and E. N. Fox, Abstr. Fed. Meet. Fed. Am. Soc. Exp. Biol. 1970 Abstr. 2199). Also, the work of Swanson et al. (30) with electron microscopy has revealed M protein seemingly excreted through the cell wall of group A streptococci. Finally, recent work with certain lactobacilli have shown that the glycerol phosphate polymer of Lp-TA, the primary immunogenic site of the molecule, extends from the membrane into the cell wall (33). Stabilized L-forms of the group A streptococci do not contain M protein. Our findings clearly indicate that the inability to detect M protein in this L-form (unpublished data) is not due to the absence of Lp-TA.

Glycerophosphoryldiglucosyl diglyceride, a relatively new phosphoglycolipid, is a component of *S. haemolyticus* (*S. pyrogenes*) (7). Most recently, phosphatidylkojibiosyl diglyceride was characterized as the lipid component of Lp-TA from the membrane of *S. faecalis* (8). The deacylated derivatives of these two phosphoglycolipids are structural isomers of one another (8). The results of chromatographic and electrophoretic comparisons with authentic phosphatidylkojibiosyl diglyceride and its deacylated product now suggest that the lipid component of Lp-TA in *S. pyogenes* and this stabilized L-form is glycerophosphoryldiglucosyl diglyceride.

The extremely small yield of glycerophosphoryldiglucosyl diglyceride from *S. pyogenes* and its L-form probably accounts for its lack of detection earlier when the complex membrane lipids of these two organisms was detailed (5). However, in that study it was shown that the glucolipid content (diglucosyl diglyceride) in the membrane of the L-form was two times greater than that of the parental coccus. Pieringer and co-workers (1, 23, 25) have now shown that this glyceride glycolipid is involved in the synthesis of phosphatidylkojibiosyl diglyceride of Lp-TA in *S. faecalis*. Therefore, accumulation of diglucosyl diglyceride in the L-form membrane may also be indicative of a precursor role for lipid of Lp-TA in *S. pyogenes*.

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