Membrane Lipid Composition of *Streptococcus pyogenes* and Derived L Form*

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ABSTRACT: The major lipids of *Streptococcus pyogenes* protoplast and L-form membranes have been isolated, characterized, and identified. Membranes were prepared from cultures harvested at their midlogarithmic phases of growth from a lipid preextracted growth medium. Protoplast membranes contained 15.3% lipid as compared with 35.6% found within membranes from the L form. The glycolipid content of the protoplast membrane lipid was one-half that of the L form whereas the per cent phospholipid of the former greatly exceeded that of the latter. The major carbohydrate-containing lipid from both membranes was identified as diglucosyl diglyceride. Similarly, the major phospholipid component was found to be of the cardiolipin type: polyglycerol phosphatide. Ester/phosphorus ratio determinations revealed that the diphosphatidylglycerol from protoplast membrane lipids is completely esterified whereas that from L-form membranes indicated the possibility of one esterification site still available. The long-chain fatty acid composition of these major glycolipid and phospholipid fractions, from both membrane sources, illustrated that *cis*-vaccenic acid is the predominating monoethenoid fatty acid of protoplast lipids while its isomer, oleic acid, prevails in the lipids from L-form membranes. The possibility of the elevated glycolipid but decreased phospholipid content of the L-form lipid being associated with its inability to synthesize a rigid cell wall is discussed.

The comparative biochemical study of *Streptococcus pyogenes* and its derived stable L form has been the interest of this laboratory for some years. Although cell wall biosynthesis is permanently blocked in this L form, the mucopeptide cell-wall precursor, uridine diphosphate–muramic acid–peptide, continues to accumulate (Edwards and Panos, 1962). Also, while membrane preparations from the parent streptococcus readily transfer rhamnose, a coccal cell-wall component, from thymidine diphosphate[1] rhamnose to polymeric rhamnose, similar L-form preparations can not (Panos and Cohen, 1966). The streptococcal membrane, therefore, apparently possesses both the transferring enzymes and receptor sites for this cell-wall carbohydrate and may be of primary importance in the biosynthesis of the rigid cell wall.

Recent advances in microbial lipid biochemistry have already provided an insight into the lipid composition and structural varieties present in bacterial membranes. Vorbeck and Marinetti (1965a) reported that 94% of the total lipids of *Streptococcus faecalis* are localized in the cell membrane. It had been established earlier that protoplast membranes from gram-positive bacteria possess a lipid content of from 15 to 30%. Likewise, membrane lipid phosphorus and membrane carbohydrate content have also been shown to range from 2 to 3.5 and from 2 to 10%, respectively (Macfarlane, 1964). Glycolipids such as galactose and/or glucose diglycerides have been found in numerous gram-positive bacteria. This has been confirmed by Brundish *et al.* (1965) who suggested that these type compounds might be more widely distributed in bacteria than hitherto suspected and may be instrumental in microbial polysaccharide biosynthesis.

Phospholipids such as phosphatidylglycerol and phosphatidylaminoacylglycerol (Vorbeck and Marinetti, 1965a) and diphosphatidylglycerol have also been identified in *S. faecalis* (Ibbott and Abrams, 1964) and *Micrococcus lysodeikticus* (Macfarlane, 1961) membrane preparations. Of considerable interest in this respect is the recent finding of a glycerol diphosphate disaccharide pentapeptide as a functional group of a lipid intermediate in bacterial cell-wall biosynthesis. This cardiolipin-type compound was isolated by Dietrich *et al.* (1965). Thus, although long abeyant, interest in microbial lipids as possible key intermediates in cell wall biosynthesis is being realized.

This report details the first comparative study of the lipid content and composition of membranes derived from a group A streptococcus and its stable L form. The lipoidal differences found within the L form are discussed in terms of its inability to synthesize a rigid cell wall.

Experimental Section

Preparation of Cell Membranes. S. pyogenes and its

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[1] Abbreviation used: TDP, thymidine diphosphate.

stable L form are the same as those used previously (Panos, 1965b). The dried ethereal extracted growth medium possessed a fatty acid content of 0.05–0.07% and was prepared as described elsewhere (Panos *et al.*, 1966). Both the streptococcus and its L form were harvested during their exponential phases of growth and washed, and membranes were prepared as detailed earlier (Panos *et al.*, 1966) except for two modifications: (1) protoplasts were formed utilizing a purified phage induced lysin (kindly supplied by Dr. S. S. Barkulis, Ciba Pharmaceuticals, Inc.) and (2) lysis was affected by vigorous stirring (4°) in a large volume (350 ml) of 0.05 M potassium phosphate buffer, pH 7.4. Protoplast formation was followed by optical density changes after dilution with distilled water and by phase microscopy. Membranes were lyophilized and stored *in vacuo* over phosphorus pentoxide and sodium hydroxide in the cold (4°) for periods not exceeding 2–3 weeks.

Total Lipid Extracts. Lyophilized membrane preparations were extracted with redistilled chloroform–methanol (2:1, v/v) by hand grinding in an all-glass tissue homogenizer at 40° for 20 min. Extraction was repeated four times with a ratio of solvent/cell material of at least 10:1. Reextraction of membrane residues at an elevated temperature (65°) with methanol followed by chloroform failed to increase the initial lipid yield. Each lipid extract was concentrated *in vacuo* and dried with benzene–absolute alcohol (4:1, v/v) as described by Vorbeck and Marinetti (1965b) and brought to a known volume with chloroform. Lipid extracts were immediately placed on silicic acid columns after aliquots had been removed for chemical analyses.

Silicic Acid Column Chromatography. Unisil silicic acid (10 g), 100–200 mesh (Clarkson Chemical Co., Williamsport, Pa.), was placed into a large, scintered glass funnel and washed consecutively three times with distilled *n*-heptane, diethyl ether, and, lastly, chloroform. Chromatographic columns were prepared by pouring slurries of silicic acid in chloroform into columns (1.8 × 15 cm) previously fitted with glass wool plugs at the bottom. Quickly following an additional wash with 100 ml of chloroform, 2–5 ml of a lipid extract was placed onto a column and allowed to percolate in with the addition of small volumes (3 ml) of chloroform. All nonpolar lipids were collected in one 50-ml fraction with chloroform. Increasing amounts of acetone in chloroform followed by methanol in chloroform served to separate the remaining lipid components in each microbial membrane extract (Figure 1). Fractions (5 ml) were collected with an automatic fraction collector.

Analytical Procedures. Silicic acid impregnated paper was prepared with Whatman No. 1 filter paper, cut into sheets (5.5 × 18 cm) as described by Marinetti (1962). Paper chromatograms were developed for 20 hr with diisobutyl ketone–acetic acid–water (40:20:3, v/v/v). All chromatograms were spotted with a mixture (10 μg each) of phosphatidylcholine (synthetic dipalmitoyl) plus phosphatidylserine (both from Mann Research Labs, Inc., N. Y.) as standards. The remaining area of each chromatogram was utilized for multiple applications of the lipid fractions obtained by column chromatography. Upon air drying, each chromatogram was cut lengthwise into four equal strips for the various staining procedures. Strips stained with Rhodamine 6G were viewed while wet with an ultraviolet light. Acid phosphatides stained blue; neutral lipids, yellow. A solution of ninhydrin (0.25%) in acetone–lutidine (9:1, v/v) served for the detection of free amino groups. After dipping and air drying (5 min), strips were heated (80°) for 10 min prior to observation. Phosphatidylserine (10 μg) were easily perceptible by this method. Finally, vicinal hydroxyl groups were detected by the periodate–Schiff test as modified by Vorbeck and Marinetti (1965b). A commercial Schiff reagent (Fisher Scientific Co.) was used which colored the entire paper in approximately 15 min. A less sensitive reagent (Barka and Anderson, 1963) was also employed that produced better contrast between lipid and paper background.

The water-soluble glycoside–glycerol moiety was isolated by the deacylation procedure as utilized for the phosphatides and separated by ascending paper chromatography with butanol–pyridine–water (3:2:1.5, v/v/v) (Carter *et al.*, 1965). All paper chromatography was performed with Whatman No. 1 paper. Detection was by the alkaline silver nitrate method of Trevelyan *et al.* (1950). This was correlated with its reducing ability as determined with an aniline hydrogen phthalate reagent (Partridge, 1949). Glucose served as the standard and each R_F value was compared with that reported for comparable glycolipids (Brundish *et al.*, 1965). The nonreducing glycoside as well as glycosyl diglycerides were hydrolyzed with 3 N HCl for 90 min at 100° and the products were chromatographed with butanol–pyridine–water (45:25:40, v/v/v). Spots were viewed with the alkaline silver nitrate and aniline hydrogen phthalate reagents.

Water-soluble phosphate esters of the polar lipids were obtained after deacylation (0.1 N methanolic KOH, 37° for 15 min) by the method of Maruo and Benson (1959). The reaction was neutralized by addition of washed AG 50W resin (hydrogen form), and water-soluble components were extracted after addition of water and chloroform. Phosphate esters were separated by two-dimensional paper chromatography (phenol–water, 100:38, v/v, and butanol–propionic acid–water, 142:71:100, v/v/v) according to Benson and Strickland (1960). These esters were visualized as white spots on a mauve background as described elsewhere (Vorbeck and Marinetti, 1965a). All R_F values were compared with literature values (Benson and Strickland, 1960), known standards, and those prepared from commercially available cardiolipin containing phosphatidylethanolamine.

Ester groups were determined by hydroxylaminolysis as described by Rapport and Alonzo (1955). It was found more reliable to prepare small amounts of alkaline hydroxylamine solution immediately before use. Lecithin (dipalmitoyl synthetic, Mann Research Labs, Inc., N. Y.) was used as a standard and a similar color yield to that reported in the literature was obtained (Rapport and Alonzo, 1955).

Glycerol was determined by the enzymatic method of Wieland (1963). Excellent recoveries were obtained in the range of 0.2 μmole. Column chromatographically eluted lipid fractions were prepared for these determinations by extensive acid hydrolysis as described by Smith and Henrikson (1965). Total phosphorus was determined by the Fiske and Subbarow method (1925). Total carbohydrate was assayed by the anthrone method of Radin *et al.* (1955) with galactose as the standard. Glucose and galactose were also assayed enzymatically (Glucostat and Galactostat reagents, Worthington Biochemical Corp., N. J.). Dry weight of the total lipids was determined by heating chloroform aliquots at 80° overnight followed by desiccation to constant weight.

The long-chain fatty acids were extracted with chloroform after deacylation and acidification (Maruo and Benson, 1959). Methylation of the free fatty acids was achieved with boron trifluoride in methanol. Methyl esters were identified by capillary (Golay) column chromatography (Panos, 1965a).

Results

Composition of Total Lipids from Protoplast and L-Form Membranes. The results of various chemical analyses of purified protoplast and L-form membranes are tabulated in Table I. The lipid content of L-form membranes (35.6%) was significantly greater than that of protoplast membranes (15.3%). While the phosphorus content in protoplast membrane lipids (1.49%) was almost twofold that of L-form membrane lipids (0.65%), the carbohydrate content was found to be reversed: 11.3 and 22.2% for protoplast and L-form membranes, respectively. The nonpolar lipid content of the total lipid extract was 23.4% for the protoplast and 34.7% for L-form membranes. Vorbeck and Marinetti (1965a) had reported a nonpolar lipid content at 5% for *S. faecalis*. These nonpolar lipid variations are probably the result of the growth media employed.

TABLE I: Lipid Composition of *S. pyogenes* Protoplast and Derived L-Form Membranes.

Procedure	Protoplast	L Form
Membrane (mg)	262.8	456.8
Total lipid	39.8	162.5
% lipid in membrane	15.3	35.6
Total lipid phosphorus (mg)	0.59	1.06
% lipid phosphorus in membrane	0.23	0.23
% phosphorus in lipid	1.49	0.65
Total carbohydrate (mg)[a]	4.5	36.0
% carbohydrate in membrane	1.7	7.9
% carbohydrate in lipid	11.3	22.2

[a] Anthrone method, galatose standard.

Silicic Acid Column Distribution of Total Lipids. A comparative elution diagram of the total lipid extracts from protoplast and L-form membranes is shown in Figure 1. The major carbohydrate-containing lipid from both sources (peak B) was eluted with acetone whereas the phosphorus-containing lipid was obtained with chloroform–methanol (peak D). A minor phosphorus-containing fraction (peak C) was found in lipids from both membrane preparations and was separable from peak D by increasing the concentration of chloroform. The final solvent, methanol, strips the column of all remaining lipid materials. It was observed that the position of elution of each peak was remarkably consistent with that reported by others (Vorbeck and Marinetti, 1965a). The R_F values indicated (Figure 1) were obtained from silicic acid impregnated paper stained with Rhodamine 6G. Peak D of the L form contained an additional minor spot (R_F 0.23) which was also present only as a trace streak in this protoplast lipid fraction. It is apparent from Figure 1 that, based on equivalent weights being taken for column chromatographic separation, the amount of glucose in both isolated peaks B is proportional to the magnitude determined by analyses of the total extracted lipids (Table I). This difference was also confirmed by assaying each peak enzymatically with glucose oxidase. Likewise, the phosphorus content of peaks C–E substantiated the difference obtained by direct analyses of the respective total membrane lipids (Table I).

A weakly positive reaction was obtained with peaks B (Figure 1) by the periodate–Schiff test as performed by Vorbeck and Marinetti (1965b). Although negative results were obtained with the remaining peaks from both the L-form and protoplast lipids, peaks D were pink when a more sensitive reagent (see Experimental Section) was employed. Chromatograms visualized with ninhydrin revealed only a trace of amino nitrogen content at the origin of each set of peaks D and E as well as a minor spot just above the origin (R_F 0.05) of peaks E.

Characterization of the Major Glycolipid (Peaks B). The results of the various determinations are listed in Table II. This carbohydrate-containing lipid, from both L-form and protoplast membranes, behaved identically in these analyses. They yielded comparable R_F values, a yellow color with Rhodamine 6G, and a positive periodate–Schiff test. Upon acid hydrolysis, the primary components identified were glycerol, glucose, and long-chain fatty acids by capillary gas chromatography.

After mild alkaline deacylation of peaks B, the aqueous portion was chromatographed as shown in a typical chromatogram tracing (Figure 2, part I). The hydrolysis product obtained had an R_F of 0.29 which was nonreducing and compared favorably with literature values of 0.25 (Vorbeck and Marinetti, 1965a) for glucosylgalactosylglycerol and 0.23 for digalactosylglycerol. In this solvent system (Figure 2, part I) the R_F of glucosyldiglycerol has been reported as being 0.49. Thus, the hydrolysate R_F values of 0.29 and 0.22 for peak B

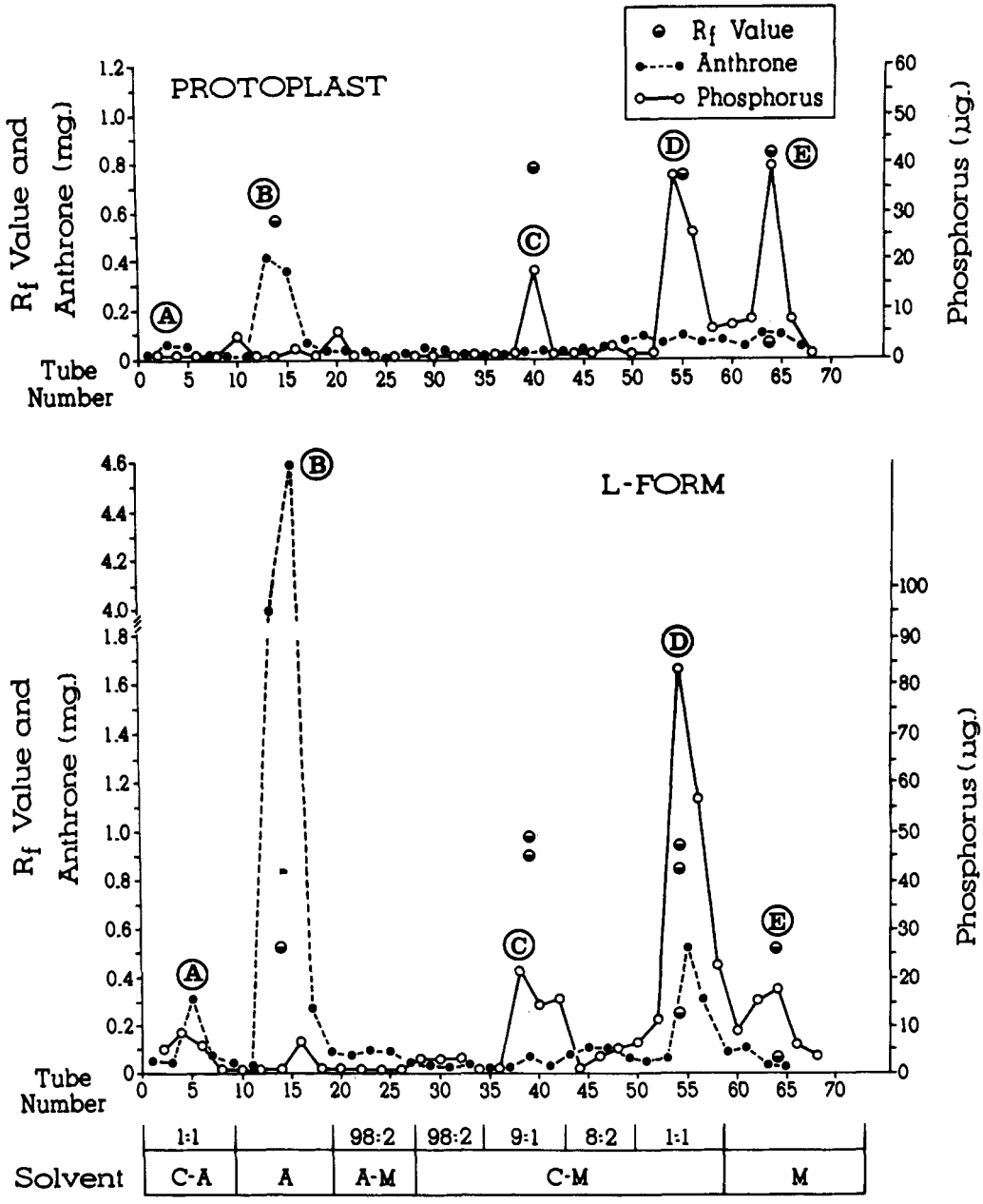

FIGURE 1: Silicic acid column chromatography of total lipid extracts from *S. pyogenes* protoplast and L-form membranes. Ordinate indicates anthrone content per tube and R_F values of fractions on silicic acid impregnated paper (diisobutyl ketone–acetic acid–water (40:20:3, v/v/v)); phosphorus per tube on right. Total lipid on column, 34.4 and 142.9 mg from protoplast and L-form membranes, respectively. Peak B identified as glucosylglucosyl diglyceride; peak D, major spots of higher R_F contain polyglycerol phosphatide with a possible trace of phosphatidylglycerol. Smaller peaks not characterized further. C = chloroform, A = acetone, M = methanol.

from the protoplast and L form, respectively, indicated a disaccharide glycerol compound. Acid hydrolysis of these disaccharide glycerol fragments yielded only glucose (Figure 2, part II). A slight streak was observed below the glucose spot (Figure 2, part II) which was not identified further. An ester/glucose molar ratio of close to 1:1 indicated a glucosylglucosyl diglyceride with two fatty acids per molecule attached (Table II).

Characterization of the Major Phosphatide (Peaks D). Peak D from the L-form lipids was rechromatographed on another silicic acid column with chloroform, chloroform–methanol (5:2), and methanol as eluting solvents. The rechromatographed lipid yielded one spot (R_F 0.79). This phosphatide, from protoplast and L-form membranes, had comparable R_F values (Table III). Both stained blue with Rhodamine 6G, indicating

TABLE II: Characterization of Major Glycosyl Diglyceride of *S. pyogenes* Protoplast and L-Form Membranes.[a]

Procedure	Protoplast	L Form
Chromatography, R_F[b]	0.55	0.51
Rhodamine 6G	Yellow	Yellow
Ninhydrin	Negative	Negative
Periodate–Schiff	Positive (weak)	Positive (weak)
Acid hydrolysis (3 N HCl, 100°, 90 min)	G,[c] FA,[c] Glu[d]	G,[c] FA,[c] Glu[d]
Deacylation (0.1 N KOH in methanol, 37°, 15 min)	Glu–Glu–G,[c] FA (nonreducing)	Glu–Glu–G,[c] FA (nonreducing)
Carbohydrate (after acid hydrolysis)	Glu[c]	Glu[c]
Ester/glucose molar ratio	0.84:1[d]	1.1:1[d]

[a] Abbreviations: G = glycerol, Glu = glucose, FA = fatty acids. [b] Silicic acid impregnated paper; diisobutyl ketone–acetic acid–water (40:20:3, v/v). [c] Identified by chromatographic procedures. [d] Identified by enzymatic assay.

anionic phospholipids. Only a faint pink was observed with the more sensitive commercial Schiff reagent, indicating the possibility of a small amount of phosphatidylglycerol. No ninhydrin-positive material was detected except for a trace spot at each of the chromatogram origins. Upon mild alkaline hydrolysis and extraction, the aqueous portion of the L-forms' peak D (before rechromatographing) yielded a spot, by two-dimensional paper chromatography, in the glycerol–phosphorus–glycerol–phosphorus–glycerol (G–P–G–P–G) area (Benson and Strickland, 1960) as well as the G–P–G area. The protoplast phospholipid yielded a major spot similar to that obtained with a commercial sample of cardiolipin. These chromatograms, by virtue of the characteristic elongated spots close to the origin and the negligible migration with the butanol–pyridine–water solvent system, indicated polyglycerolphosphoryl compounds. No spots were found in the glycerolphosphorylcholine or glycerolphosphorylethanolamine areas as seen with suitable standards. To identify the rechromatographed peak D more thoroughly, the glycerol/phosphorus molar ratio was determined and found to be 3.1:2 from both sources. This ratio plus the location of deacylated products by paper chromatography suggests that the polyglycerol phosphatide is of the G–P–G–P–G type. The ester/phosphorus molar ratio of 5:2 for the protoplast lipid indicates that all of the hydroxyl groups are esterified by fatty acids, whereas in the L form the ratio of 1.9:1 indicates that one hydroxyl group is not esterified. Ibbott and Abrams (1964) had reported a polyglycerol phosphatide from *S. faecalis* protoplast lipids as having an ester/phosphorus molar ratio of 5:2. They also indicated, however, that a variety of ratios had been reported by others for this cardiolipin-type phosphatide. It is felt that the difference in the ester/phosphorus molar ratio (5.0:2 and 1.9:1 for protoplast and L-form lipids, respectively) of this phosphatide may suggest a structural phospholipid modification between these two microbial membranes. However, absolute verification of this aspect must await the accumulation of sufficient quantities for further detailed analyses. It may be noted that the position of elution of peaks D by silicic acid column chromatography suggests a salt form (Figure 1). This type component is usually removed with chloroform–methanol (9:1 or 8:2) when present as the free acid.

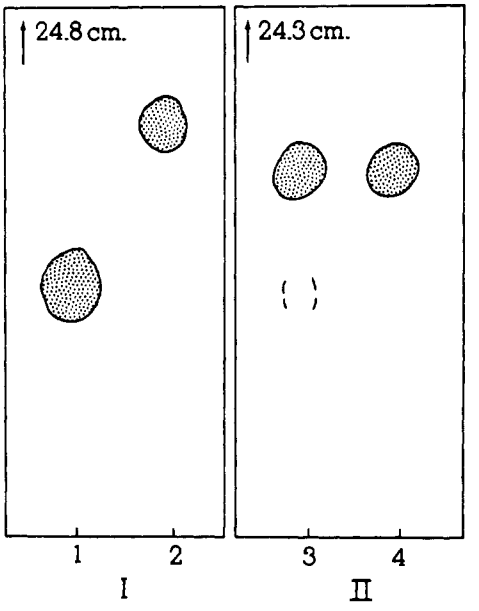

FIGURE 2: Chromatogram of glucosyl lipid from protoplast membrane after mild alkaline and acid hydrolysis. (I) Mild alkaline hydrolysate (0.1 M KOH in methanol, 37°, 15 min) of peak B lipid spotted at no. 1, R_F 0.29. Glucose standard at no. 2; solvent, butanol–pyridine–water (3:2:1.5, v/v/v). (II) Acid hydrolysate (3 N HCl, 100°, 90 min) of compound no. 1 spotted at no. 3, glucose standard at no. 4; solvent, butanol–pyridine–water (45:25:40, v/v/v). Both papers stained with alkaline silver nitrate.

Major Fatty Acid Components. The distribution of the major long-chain fatty acids of protoplast and

TABLE III: Characterization of Major Phosphatide of *S. pyogenes* Protoplast and L-Form Membranes.

Procedure	Protoplast	L Form
Chromatography, R_F[a]	0.73	0.83, 0.92 (minor 0.24)
Rechromatographed, R_F[a]	...	0.79
Rhodamine 6G	Blue	Blue
Periodate–Schiff[b]	Negative	Negative
Ninhydrin	Negative	Negative
Deacylation[c,d] (0.1 N KOH in methanol, 37°, 15 min)	FA, G–P–G–P–G, G–P–G	FA, G–P–G–P–G, G–P–G
Ester/phosphorus molar ratio	5.0:2	1.9:1
Glycerol/phosphorus molar ratio	3.1:2	3.1:2

[a] Silicic acid impregnated paper; solvent system, diisobutyl ketone–acetic acid–water (40:20:3, v/v). [b] Only trace positive spot revealed with more sensitive reagent. [c] Performed on total fraction before rechromatographed on silicic acid column. Spots detected in G-P-G-P-G and G-P-G areas by two-dimensional paper chromatography. Solvent system 1, phenol–water (100:38, v/v); 2, butanol–propionic acid–water (142:71:100, v/v). [d] Abbreviations: G = glycerol, P = phosphorus, FA = fatty acids.

L-form lipid fractions B and D are tabulated in Table IV. The fatty acid distribution was consistent in both fractions from the same source. The average oleic acid content of the L-form membrane lipid fractions (26%) is more than twofold that of the protoplast fractions (9.7%). The *cis*-vaccenic acid content, on the other hand, prevailed in these protoplast fractions. It is apparent that the C-16 monoolefinic fatty acids of the L-form fractions are approximately one-fourth that of similar fractions from the protoplast. These major acids account for at least 90% of the total fatty acids detected. Of interest was the finding of linoleic acid in the isolated membrane lipids from both microbial sources. Suitable controls had revealed the presence of this polyunsaturate in the extracted growth medium.

TABLE IV: Major Fatty Acid Components of Polar Lipids of Protoplast and L-Form Membranes.[a]

	Fraction B[b]		Fraction D[b]	
Fatty Acid	Protoplast	L Form	Protoplast	L Form
Palmitic	42.2	38.4	35.8	31.8
C_{16} Monoolefinic[c]	23.5	6.2	23.7	7.4
Stearic	7.5	8.5	6.9	12.6
Oleic	9.1	27.2	10.4	25.1
cis-Vaccenic	14.5	9.1	13.6	12.6

[a] Per cent of total fatty acids. Determined by capillary gas chromatography (Golay), Carbowax 1540, 150 ft, at 185°. Fatty acids obtained by mild alkaline hydrolysis. [b] From silicic acid column chromatography, B = glucosyl diglyceride; D = phosphatide. [c] Mixture of three positional isomers.

Discussion

As is apparent from the data, the major membrane lipid differences between *S. pyogenes* and its derived L form were quantitative rather than qualitative. Under the mild extraction conditions employed for the preservation of lipid structure, it was observed that the L-form membrane contained more total lipid and glycolipid than protoplast membranes. However, L-form membrane lipids had significantly less phospholipid than those from the protoplast. Previously, it had been shown that their protein content was similar (59 and 68% for L-form and protoplast membranes, respectively) but that the L-form membrane fatty acid content was 57% greater than protoplast membranes (Panos *et al.*, 1966). These quantitative lipid differences probably account for the increased fragility and susceptibility to osmotic or vibrational stress of newly formed protoplasts, as compared with the L form, so apparent during the preparation of membranes for these studies. To minimize the effect of cultural conditions, the streptococcus and its L form were harvested during their midlogarithmic phases of growth from a lipid-extracted growth medium.

The membrane lipid results from this streptococcus differed from those reported by others for the taxonomically related *S. faecalis*. *S. pyogenes* membrane lipids were found to contain significantly less total lipid and phosphorus but more carbohydrate than *S. faecalis* (Vorbeck and Marinetti, 1965a). As was found by others (Ibbott and Abrams, 1964), these studies failed to indicate the presence of the usual phospholipids present in plant and animal tissues, *i.e.*, those containing choline or ethanolamine, in the major lipid fractions examined.

The diglucosyldiglyceride structure for the carbohydrate containing fraction (peak B) from both protoplast and L-form membranes was determined from chromatographic and staining characteristics and by examination of hydrolytic products. Glucosylgalactosyl

diglyceride had been obtained from a similarly eluted fraction from *S. faecalis*. By comparison, however, diglucosyl diglycerides are purported to be more widespread in the bacterial and plant kingdoms.

The phospholipid water-soluble moiety of *S. faecalis* has been found to be either diglycerolphosphorylglycerol and/or glycerolphosphorylglycerol, depending upon the age of the culture and/or growth medium employed. The data concerning the major phospholipid present in these streptococcal protoplast and L-form membranes indicate that a mixture of these is present. While the major water-soluble moiety of both peaks D suggests its probable structure to be diglycerolphosphorylglycerol, a trace spot obtained with a more sensitive Schiff reagent also suggested the presence of minute quantities of the diglycerol phosphate as well. Similar but opposite results have been reported for exponential phase cells of *S. faecalis* (Vorbeck and Marinetti, 1965a). A second major phospholipid fraction (peak C) obtained by Vorbeck and Marinetti (1965a) contained phosphatidylaminoacylglycerol. A comparable fraction, peaks D in these studies, however, proved to be ninhydrin negative, indicating the absence of aminoacyl groups in the major phospholipids from these streptococcal protoplast and L-form membranes. Earlier, Freimer (1963) had reported the presence of phosphatidylcholine as a major component in the membrane lipids of two strains of a group A streptococcus obtained after 18 hr of incubation from a crude medium. Choline, however, was not demonstrable in the major membrane phospholipid (peaks D) characterized in this study.

The long-chain fatty acid distribution of the two major fractions (peaks B and D) from protoplast and L-form membranes proved of interest. The presence of five major fatty acids in each fraction (Table IV) denotes an excess of the maximal sites available for lipid substitution, indicating a mixture in which each polar lipid carries a multiplicity of fatty acids. Similar results have been obtained by others (Smith and Henrikson, 1965). It has been found that, as opposed to mammals, bacteria distribute their fatty acids in an almost irrelevant fashion among their various lipid classes (Macfarlane, 1964). Although this has been confirmed, of importance was the finding of the reversed but consistent prevalence of oleic acid or *cis*-vaccenic acid in fractions B and D from each membrane. This substantiates our earlier report that conversion to the L form results in a concomitant shift in its octadecenoic acid content. A correlation of these fatty acid alterations with the L forms, inability to synthesize a cell wall, is discussed elsewhere (Panos *et al.*, 1966). Further, it had been shown that a growth medium constituent, linoleic acid, was also present in these isolated protoplast and L-form membranes (Panos *et al.*, 1966). The finding of this polyunsaturate as a component of these two membrane lipid fractions from both membrane sources proves that its incorporation was enzymatic rather than by adsorption.

The presence of more than a twofold increase in the diglucosyl diglyceride (peak B) content of membrane lipids from the L form, coupled with a significant decrease in the percentage of phospholipids, as compared with the reverse order noted in protoplast lipids, tempts speculation of the involvement of these lipids in cell-wall biosynthesis. The widespread occurrence of glycolipids in gram-positive bacteria has led to speculation of the role of these type lipids in polysaccharide biosynthesis. We had demonstrated earlier that (a) TDP rhamnose, a proposed intermediate in streptococcal cell-wall formation, is utilized to form polymeric rhamnose in protoplast but not L-form membranes, (b) rhamnose as a structural component is absent in the L form, and (c) cell-free extracts from the intact protoplast and derived L form retain the capacity to synthesize TDP rhamnose from glucose 1-phosphate. It is conceivable, therefore, that diglucosyl diglyceride utilization is inhibited and, in turn, accumulates as a result of the L-forms' inability to synthesize a rigid cell wall. This is consistent with the suggestion of Macfarlane (1964) that membrane glycolipids may serve to transport carbohydrates to sites of highest activity. The fact that group A streptococcal cell walls contain appreciable quantities of rhamnose and that this methylpentose is formed directly from glucose without scission of the carbon skeleton supports a hypothesis implicating this accumulated glycolipid in coccal cell-wall biosynthesis. Studies are currently in progress to determine whether there is interference in the enzymatic utilization of the diglucosyl diglyceride in these L-form membranes. On the other hand, it is conceivable that the increased total lipid content found in the L form may reflect a compensation for the lack of a rigid cell wall.

The decreased percentage of polyglycerol phospholipid in L-form membrane lipids, as compared with protoplast lipids, is of considerable interest due to the recent finding of a similar cardiolipin-type intermediate in microbial cell-wall formation (Anderson and Strominger, 1965). However, since an appreciable amount of this phospholipid still remains in L-form membranes, its decrease cannot be the primary cause of the L-form cell-wall inhibition defect. However, in other membrane–enzyme complexes, such as mitochondria, it has been found that lipids may orient enzymes into a favored active configuration (Edwards and Ball, 1954). Similarly, the decreased phospholipid content of L-form membrane lipids may have a profound effect upon this organism's ability to form its outer rigid layer. The importance of the ester/phosphorus molar ratio difference of this lipid (Table III) from each membrane source, in this respect, remains to be determined.

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Glutamate Biosynthesis in an Organism Lacking a Krebs Tricarboxylic Acid Cycle. V. Isolation of α-Hydroxy-γ-ketoglutarate (HKG) in *Acetobacter suboxydans**

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ABSTRACT: An enzymatic condensation of glyoxylate with oxaloacetate has been studied in *Acetobacter suboxydans*, and differentiated at pH 6.0 from a nonenzymatic condensation occurring at 40° and pH 7.4.

The nonenzymatic reaction was employed for synthesis of oxalomalate and through it, by decarboxylation, for α-hydroxy-γ-ketoglutarate. From the latter γ-hydroxyglutamate was obtained, by reductive amination with glutamic dehydrogenase. α-Hydroxy-γ-ketoglutarate has been isolated from incubation mixtures of equimolecular amounts of glyoxylate and oxaloacetate with cell-free extracts under conditions where nonenzymatic condensation is virtually zero. The identity of this compound has been established by comparison with the product of the nonenzymatic transamination of γ-hydroxyglutamate and of the synthetic α-hydroxy-γ-ketoglutarate from oxalomalate.

In *Acetobacter suboxydans*, the Krebs tricarboxylic acid cycle appears to be largely nonfunctional (Cheldelin, 1961; Rao, 1958) and the nonessential amino acids can be formed through alternate pathways (Sekizawa *et al.*, 1962). The metabolic interest in α-hydroxy-γ-ketoglutarate arose from the fact that it was envisioned as an intermediate of glutamic acid biosynthesis (Sekizawa *et al.*, 1962; Maragoudakis *et al.*, 1964).

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