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A LIPOPROTEIN FROM THE MEMBRANES OF *STREPTOCOCCUS PYOGENES* AND ITS STABILIZED L-FORM

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SUMMARY

The lack of cell wall formation by a stabilized L-form from *Streptococcus pyogenes* may be related to, or reflected in, changes of a particular type of membrane lipid. Therefore, this study details the first comparative investigation of isolated membranes from this *Streptococcus* and its stabilized L-form for isoprenoid-containing components. A lipoprotein present in minute amounts in the membranes from both this *Streptococcus* and its derived L-form was detected, isolated, purified and partially characterized. Lipoprotein from both membrane sources appeared to be identical, contained phosphorus and was electrophoretically homogeneous. A ratio of streptococcal to L-form membrane lipoprotein of at least 10 was observed. Chemical, physical and chromatographic studies of isolated and nonsaponifiable lipid of lipoprotein indicated the absence of quinones but the presence of isoprenoid units and hydroxyl group(s). Also, the spectral characteristics of lipid of lipoprotein and its chromatographic behavior, before and after acetylation, were similar to those of an isoprenoid alcohol isolated from lactobacilli and *Staphylococcus aureus* by others and known to be involved in bacterial cell wall peptidoglycan biosynthesis. Protein of lipoprotein, seemingly covalently linked to lipid, was unique because of its high ornithine content: with all of the ornithine of the coccal and L-form membrane apparently concentrated within this membrane component. Approximately one-half of this lipoprotein was composed of protein. The possibility of lipoprotein being related to an inability by this L-form to synthesize a rigid cell wall is indicated.

INTRODUCTION

A stabilized (*i.e.* non-reverting) L-form derived from *Streptococcus pyogenes* is unable to synthesize the rigid streptococcal cell wall but continues to form the sugar-nucleotide precursors necessary for cell wall peptidoglycan biosynthesis[1,2]. It is known that the stage of cell wall synthesis involving the transfer of sugar fragments from such nucleotide precursors to an isoprenoid lipid phosphate to form the disaccharide–pentapeptide–lipid intermediate occurs in the cell membrane[3]. Also

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membrane morphology (Fig. 12 in ref. 4) and fatty acid and glyco- and phospholipid changes have been documented upon conversion of this *Streptococcus* to its L-form[5,6]. To our knowledge, however, no examination of any stabilized bacterial L-form for possible lipid intermediates for the synthesis of cell wall polymers has been reported. This report details the first investigation of isolated membranes from *S. pyogenes* and its stabilized L-form for isoprenoid-containing components. Some of these results have been presented in preliminary form[7].

EXPERIMENTAL SECTION

Microorganisms, growth medium and cultivation

The *S. pyogenes* and its stabilized L-form are the same as those used recently[8]. These organisms were harvested at their respective mid to late logarithmic phases of growth from lipid preextracted media (Brucella broth, Pfizer, New York, N.Y., U.S.A., and lipid-free bovine albumin, Armour Co., Kankekee, Ill., U.S.A., 70-l batches) and their membranes obtained and treated essentially as detailed elsewhere[5]. The only change being that L-form whole cells were ruptured by sonic oscillation (10 kcycles) for 3–2 min intervals, instead of by freeze–thawing, followed by stirring in the cold for 1 h in phosphate buffer (0.05 M, pH 7.4), with Mg^{2+} and DNAase[5], prior to the membrane washing procedure. Membrane preparations were also subjected to disc gel electrophoresis for verification of existing quantitative differences between this coccus and its L-form[9].

Extraction, chromatographic and electrophoretic methods

A detailed scheme for the extraction of lipoprotein from *S. pyogenes* and its L-form appears in Fig. 1. The free lipids of lyophilized whole cells or membranes were removed according to Ames[10]. The remaining residue was then extracted with phenol[11] and the phenol removed by dialysis against glycerol[12]. Most of the sodium dodecyl sulfate was removed by dialysis against water. Fraction B contained the membrane lipoprotein whereas Fraction C was devoid of this component.

All descending paper chromatography was performed with Whatman No. 1 paper. The solvent systems for amino acids and amino sugars included (a) *n*-butanol–formic acid–water (75:15:10, by vol.) and (b) *n*-butanol–acetic acid–water (4:1:1, by vol.) while (c) *n*-butanol–pyridine–water (6:4:3, by vol.) served for glucose and glycerol. Amino acids were visualized by ninhydrin (0.2% in acetone), all sugars by aniline phthalate[13] and alkaline $AgNO_3$ (ref. 14), glycerol by the periodate benzidine method of Cifonelli and Smith[15] and by alkaline $AgNO_3$ and amino sugars by a modification of the Elson–Morgan technique[16].

For thin-layer chromatography, all plates (400 nm thick) were prepared with the silica gel G of Stahl (Merck Co., Darmstadt, Germany). Chloroform–methanol–water (65:25:4, by vol.) served as the solvent system for isolation of lipoprotein from Fraction B. *n*-Heptane–ethyl acetate (9:1 and 85:15, by vol.) or benzene–methanol (99:1, by vol.) also served for the isolation of the lipid of lipoprotein after hydrolysis. Solvent systems for the isolation of organic acids were *n*-propanol–conc. NH_4OH (70:30, by vol.) and *n*-butanol–formic acid–water (75:15:10, by vol.) following their detection by 2,6-dichlorophenolindophenol (0.1% in ethanol)[17] after heating (10 min at 105 °C) or by dichlorofluoroscein (0.2% in ethanol) under ultraviolet light.

Proteins in Fraction B and of lipoprotein were detected by ninhydrin (0.2% in acetone) and complex lipids by rhodamine 6G (0.01% in 0.25 M $NaHPO_4$), phosphomolybdate (5% in ethanol), the phosphorous reagent of Dittmer and Lester[18] and by iodine vapor.

Disc gel electrophoretic studies of lipoprotein and its component parts were performed with acidic and alkaline gels (Canalco apparatus, Rockville, Md., U.S.A.). For acetic acid gels the method of Takayama *et al.*[19] as described by Rottem and Razin[20] was used. For lipoprotein, each gel contained from 200–300 μg of protein which had been solubilized by phenol–acetic acid–water (4:2:1, by vol. (and subjected to a current of 5 mA for 3–4 h. Both large (internal diameter 0.7 cm × 6 cm) and small (internal diameter 0.5 cm × 5.5 cm) gels composed of 35% acetic acid, 5 M urea and 7.5% acrylamide were utilized. For large and small alkaline gels, the method essentially as described by Okuda and Weinbaum[12] was used. Material added to gels was solubilized in Tris–glycine buffer (0.05 M, pH 8.3) and run for 30–45 min, with bromophenol blue serving as marker. Each gel contained 7.5% acrylamide and 5 M urea in Tris–HCl buffer (0.05 M, pH 8.3). Proteins were stained by amido black (0.4% in 7% acetic acid) and coomassie blue[21] while sudan black was used to detect lipoprotein[22]. Destaining was done non-electrically with 7% acetic acid for protein and with acetic acid (15%) *plus* acetone (20%) in water for the lipoprotein. For elution from gels, from one to several gels were segmented with a razor blade, the appropriate bands collected and placed in a 3-ml syringe equipped with a stainless steel Millipore Swinny adapter (XX3001200, Millipore, Bedford, Mass., U.S.A.) without filter and macerated through the wire screen[23]. Intact lipoprotein was then extracted by the appropriate buffer to be described followed by dialysis against water (4 °C, 3–5 days) for removal of salts and gel constituents.

Chemical methods

All acid hydrolyses were performed in sealed tubes (2 mg/ml) as follows: for glucose and glycerol, 2 M HCl, 2 h; for amino sugars, 4 M HCl, 4 h; and for amino acids, 6 M HCl, 18 h. Either acid (1 M HCl, 1 h, 105 °C, sealed tube) or alkaline (5% KOH in methanol, reflux, 1 h) hydrolysis was used for release of lipid from lipoprotein. For larger amounts of this lipid from whole cells, the alkaline hydrolysis (20% KOH in ethanol) procedure of Thorne and Kodicek[24] was employed but with the addition of 0.25% pyrogallol[25]. Reduction of quinones was accomplished with excess borohydride[26]. The procedure of Wellburn and Hemming[27] with dry pyridine, served for the acetylation of lipid of lipoprotein. For oxidation of the isoprenoid chain, 1 mg of lipid in 2.5 ml *tert*-butanol *plus* 0.3 ml of 0.02 M Na_2CO_3 and 1 ml of oxidant (2.08 g sodium metaperiodate *plus* 0.158 g $KMnO_4$ in 100 ml water) was placed in a sealed tube at room temperature with vigorous shaking for 5 days. The oxidation mixture was decolorized dropwise with a saturated solution of sodium bisulfite then alkalinized with NaOH to pH 9.0 with indicator paper. *tert*-Butanol was removed, with mild heat under a stream of N_2, and the mixture acidified with HCl to pH 3.0 with indicator paper for conversion of aldehydes to acids. All salts were removed by preparative thin-layer chromatography (propanol–conc. NH_4OH (70:30, by vol.)), the band (R_F 0.35) staining with dichlorophenolindophenol removed, and the organic acids extracted with diethyl ether. Levulinic (R_F 0.60) and succinic (R_F 0.85) acids were resolved by thin-layer chromatography with *n*-butanol–formic acid–water (75:15:10, by vol.), with the former staining off-white and the latter pink

with dichlorophenolindophenol. Levulinic acid was also identified by gas chromatography (Porapak Q, 5 ft. × 1/8 inch, at 225 °C).

Ultraviolet spectra were obtained with a Zeiss PMQ II spectrophotometer using only spectroanalytical solvents. Infrared analyses were performed with a Perkin–Elmer 337 grating spectrometer as KBr pellets. Attempted detection of methylated (diazomethane) fatty acids was by capillary column gas chromatography on Carbowax K-20M columns as previously described[5,6]. Amino acid analyses of protein of lipoprotein were performed with an automatic Technicon apparatus. Protein determinations were according to Lowry *et al.*[28] with bovine serum albumin (Armour, Kankekee, Ill., U.S.A.) as the standard while phosphorus was quantitated by the method of Chen *et al.*[29]. Coenzymes Q_6 and Q_{10} (ubiquinone) and vitamin K_1 were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.

RESULTS

Fractionation and yields

The manner in which the various Fractions (A–D) were obtained is shown in Fig. 1. It is apparent from Table I that the L-form membrane is devoid of Fraction A even though greater quantities of such membranes (up to 6 times) were repeatedly taken for extraction. Similarly, coccal membranes contained approx. 10 times more of Fraction B than L-form membranes on a comparable dry weight basis. Finally, although L-form membranes possessed considerably less of Fraction C than coccal membranes, the former contained almost twice as much insoluble material (Fig. 1, Fraction D) as the latter. This difference in weight distribution within fractions from coccal membranes was not apparent when whole cells of *S. pyogenes* were examined. The protein content of streptococcal and L-form membranes was 72 and 63%, respectively. Also, the nitrogen (Kjeldahl) content of whole cells of *S. pyogenes* was 81%; that of the intact L-form, 78%.

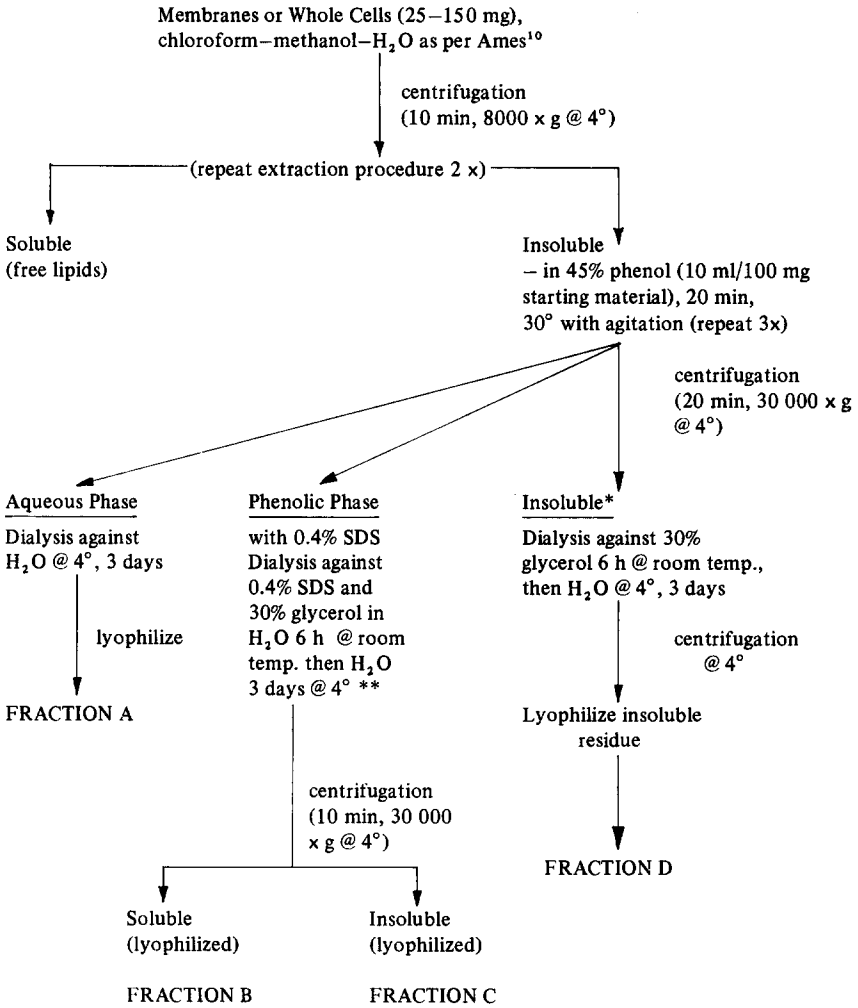
Table II indicates the chemical composition of these fractions. It should be pointed out that Fraction C contained only protein while Fraction B was devoid of nucleic acids but contained lipids and proteins. Fraction B was found to be soluble

TABLE I

DRY WEIGHT YIELDS OF THE VARIOUS FRACTIONS OBTAINED AFTER PHENOL EXTRACTION

The dry weight yields (%) are calculated from the weights of whole cells or membranes before lipid extraction.

Fraction	*Streptococcus*		*L-form membrane*
	Whole cells	*Membranes*	
A	4.2	<1	0.0
B	3.0	27.0	2.6
C	3.0	15.0	1.0
D	85.0	47.0	85.0



*With coccal membranes, solid white material present between water and phenol layers. With L-form membranes, brown and tacky product at bottom of centrifuge tube.
**Time of dialysis important for amount of insoluble material to be constant.

Fig. 1. Scheme for the fractionation of streptococcal and L-form membranes and whole cells. SDS, sodium dodecyl sulfate.

in both water and organic solvents: indicating a direct association of lipid to protein since all soluble lipids had been removed previously during the initial extraction procedure. Fraction A probably contained teichoic acids as well as other components, no proteins, and only 2 or 3 amino acids of which alanine predominated. Fractions A, C, and D were not characterized further. Lipoprotein, of which this report is concerned, was found only in Fraction B from both membrane sources.

The ratio of streptococcal to L-form membrane Fraction B was at least 10 (Table I). However, the extremely small yields of lipoprotein within each Fraction B

TABLE II

COMPOSITION OF THE VARIOUS FRACTIONS OBTAINED AFTER AQUEOUS PHENOL EXTRACTION

Nucleic acids present in Fractions A and D from whole streptococcal cells only. Otherwise, results identical regardless of membrane source. +, present; −, absent; N.D., not done.

Fraction	*Lipid*	*Protein*	*Rhamnose*	*Glucose*	*Amino sugars*	*Glycerol*	*P*
A	+	−*	−	+	+	+	+
B	+	+	−	+	−	+	+
C	−	+	−	−	−	−	N.D.
D	−	+	+	+	+	−	N.D.

* Two or three amino acids only.

was difficult to estimate because of the isolation and purification procedures utilized. Nonetheless, a similar difference in magnitude between coccal and L-form lipoprotein content was also apparent, as judged by the initial amounts of each Fraction B necessary for lipoprotein detection and isolation.

Presence of lipoprotein in Fraction B

After lyophilization, Fraction B was soluble in water and in chloroform, methanol–chloroform (2:1 and 1:1, v/v) and phenol but insoluble in acetone and non-polar solvents such as diethyl ether. Thin-layer chromatography of this fraction with chloroform–methanol–water revealed the presence of 5 spots, 4 of which migrated and all of which were rhodamine positive. Since all of the free lipids had been removed previously, these migrating spots suggest the separation of weakly or non-covalently linked lipids from proteins present within this fraction. The fastest of these spots proved to be a glycolipid and two were phospholipids. All of these lipids behaved similarly to those characterized by us earlier[6]. Streptococcal and L-form lipoprotein remained at the origin when using this solvent system. It proved to be ninhydrin and phosphorus positive and could be selectively extracted with chloroform from such plates, leaving behind all other water soluble proteins. The fifth spot was sodium dodecyl sulfate, a contaminant from the extraction procedure, and migrated (R_F 0.28) in this solvent system. It appeared white after rhodamine spray and without ultraviolet.

Polyacrylamide gel electrophoresis and thin-layer chromatography of Fraction B

This fraction, when subjected to acetic acid disc gel electrophoresis, displayed 5 bands, 4 of which migrated and all of which were amido black and coomassie blue positive. The non-migrating band (top of gel) was found to be protein positive and to contain lipid; *i.e.* sudan black positive. This band proved to be the lipoprotein.

With alkaline gels, lipoprotein migrated with the marker (bromophenol blue); to within approx. 1 cm from the end of the gel. This component migrated as a single and distinct band of strong intensity regardless of whether gels were prepared with or without urea (5 M) and with or without sodium dodecyl sulfate (0.1%). A non-migrating band of considerably weaker protein intensity was always discernible

on top of such gels that was also lipid positive. Since mixtures of sodium dodecyl sulfate and lipoprotein gave two very close but distinct bands which stained with sudan black, the use of sodium dodecyl sulfate was omitted in these experiments. Alkaline gel electrophoretic results indicated that the molecular weight of lipoprotein was below 10000 (ref. 21).

Lipoprotein isolated from coccal and L-form membranes by selective extraction with chloroform from thin-layer chromatographic plates and subjected to alkaline gel electrophoresis, always displayed a single migrating band that was protein and lipid positive; indicating the absence of other proteins extracted with lipoprotein. Also, this lipoprotein, when extracted from such gels with phosphate buffer (0.1 M, pH 8.3) and reexamined by thin-layer chromatography as indicated above, continued to remain at the origin. Subsequent studies were to indicate that thin-layer chromatography and/or alkaline gel electrophoresis are satisfactory methods for obtaining this membrane lipoprotein sufficiently pure for characterization studies.

Characterization of lipoprotein

The linkage between protein and lipid in lipoprotein from these two organisms is thought to be stronger than in most lipoproteins[31]. The possibility of covalent bonding is suggested from the following; (a) no migration of lipid of lipoprotein by thin-layer chromatography without prior acid or alkaline hydrolysis, (b) classical methods for isolation of lipid from lipoproteins such as chloroform–methanol (2:1, v/v) or diethyl ether–ethanol (1:2 or 1:3, v/v), for example[21,30–33], did not yield free lipid, (c) after acetic acid gel electrophoresis and elution of most of a surface and non-migrating band containing lipoprotein (with 0.05 M Tris–glycine buffer (pH 8.3)), this component migrated during alkaline gel electrophoresis: indicating that the acetic acid concentration (4.5 M) used in its initial solubility for acetic acid gel electrophoresis was insufficient to dissociate lipid from protein. Subsequent studies showed that lipoprotein could also be extracted from coccal and L-form membranes by an acidic butanol technique used by others[34] for obtaining isoprenoid alcohol from *S. aureus*. Freed lipid of lipoprotein did not migrate upon alkaline gel electrophoresis and remained on the surface of such gels.

Intact lipoprotein from coccal and L-form membranes contained the same amount of phosphorus (Table III). However, mild alkaline hydrolysis (0.1 M KOH in 90% methanol, 37 °C) with time (0.5, 2.0 and 5.0 h) followed by thin-layer chromatography resulted in the complete release of lipid after 2 h which migrated but was phosphorus negative; indicating a probable ester linkage of lipid to protein. Phosphorus remained with protein at the origin of such plates. Ultraviolet spectra of intact lipoprotein (215–400 nm) in water or chloroform failed to show any absorbance while infrared patterns illustrated bands characteristic of both lipid and protein. After acid or alkaline hydrolysis, chromatographic methods failed to confirm the presence of long chain fatty acids, glycerol or glucose as components of coccal or L-form membrane lipoprotein. After hydrolysis, lipid of lipoprotein was always located in the non-saponifiable fraction. The absorption spectrum of this lipid appears as part of Fig. 2.

Lipid of lipoprotein, isolated after acid or alkaline hydrolysis, proved to be fluorescent (blue) and unstable. Fig. 3 is a composite tracing of the chromatographic results illustrating this instability with time. As control, ubiquinone (Q_{10}) also

TABLE III

COMPOSITION OF FRACTIONS B AND LIPOPROTEIN FROM STREPTOCOCCAL AND L-FORM MEMBRANES

Amino acid	*Number of residues per 1000 residues*			
	Fraction B		*Lipoprotein*	
	Coccus	*L-form*	*Coccus*	*L-form*
Aspartic	110	100	84	63
Threonine	60	66	42	43
Serine	55	60	117	151
Glutamic	85	88	134	117
Proline	35	45	42	48
Glycine	102	100	168	195
Alanine	102	100	75	78
Valine	75	72	42	39
Isoleucine	85	82	42	29
Leucine	95	90	54	39
Tyrosine	17	18	4	8
Phenylalanine	35	35	25	19
Ornithine	2	1	60	78
Lysine	85	100	64	48
Histidine	17	10	25	24
Arginine	42	28	25	14
Percent P	3.2	2.5	4.0	3.7
Percent lipid	N.D.*	N.D.*	45.0±2	47.0±2

* N.D. = not done.

behaved like lipid of lipoprotein after hydrolysis; *i.e.* was fluorescent and resulted in the formation of two spots upon chromatography, one of which had an identical R_F (0.36) to that of lipid of lipoprotein. Fig. 4 illustrates this instability by changes in the ratio of $A_{272\,nm}/A_{230\,nm}$ with time and coincides with the appearance of the multiple spots observed by thin-layer chromatography (Fig. 3). Chromatography of isolated lipid after more than 24 h resulted in the detection of no less than 5–6 spots. Three of these were yellow (Fig. 3, C and E) and non-fluorescent. One of these appeared earlier (R_F 0.25) and was insoluble in heptane. Its absorption spectrum appears in Fig. 5 and displays the appearance of a new band at 430 nm for the chromogen. Because of the instability of lipid of lipoprotein and the most minute amounts of lipoprotein obtained from these organisms, all attempts to obtain definitive mass spectra were unsuccessful.

Infrared spectra (Fig. 6) of acid-released lipid from purified (after gel electrophoresis) lipoprotein after 24 h was decidedly different from those characteristic of quinones[35,36]. Although there are no strong bands specific for isoprenoid units, certain absorption frequencies are indicative of their presence and appear here since

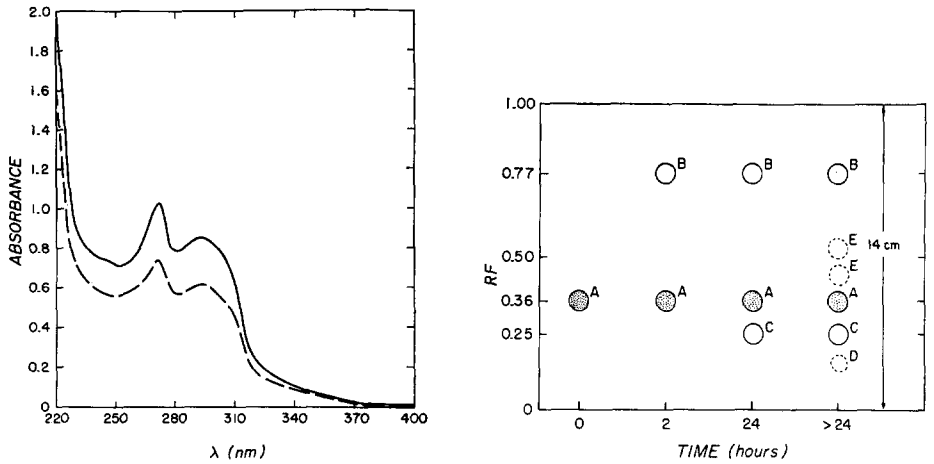


Fig. 2. Spectrum of lipid of lipoprotein (0.01% in heptane) after thin-layer chromatography (R_F 0.35; heptane–ethyl acetate (85:15, v/v)). Before (———) and after (– – – –) borohydride treatment.

Fig. 3. Composite of thin-layer chromatographic results illustrating instability of lipid of lipoprotein with time. Solvent system: heptane–ethyl acetate (85:15, v/v). 0 h, lipid immediately after release from protein (H^+ or OH^- hydrolysis); 2, 24, >24 h, spot at 0 h eluted with heptane and reapplied after times indicated. A, lipoprotein, fluorescent with ultraviolet light; rhodamine, iodine, *p*-anisaldehyde (green) positive. Decreased with time. B, rhodamine, iodine, *p*-anisaldehyde (blue) positive; no absorption with ultraviolet light. Constant with time. C, visible, yellow; rhodamine and iodine positive, nonfluorescent with ultraviolet light. D, rhodamine positive; weak or not always present. E, visible, yellow; rhodamine positive; weak or not always present.

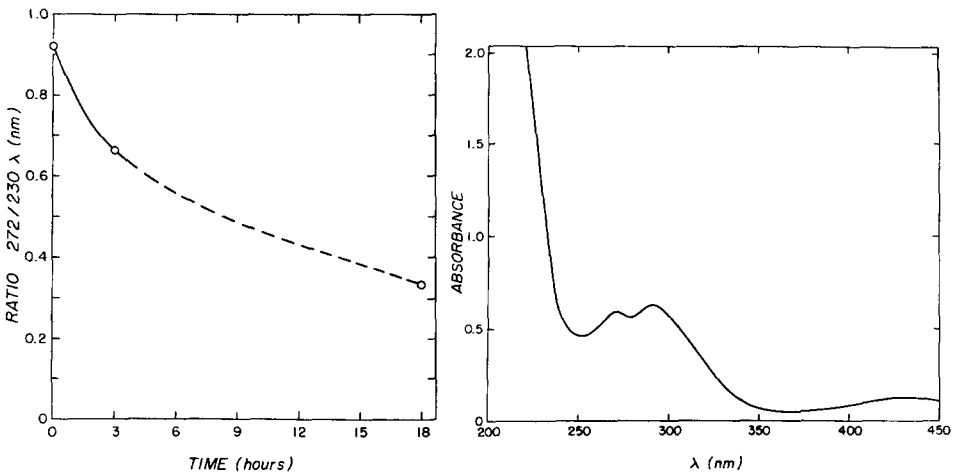


Fig. 4. Spectral change of lipid of lipoprotein with time coinciding with thin-layer chromatographic data.

Fig. 5. Absorption spectrum of new yellow compound (0.01% in isopropanol) from lipid of lipoprotein with time after isolation by thin-layer chromatography (R_F 0.25, heptane–ethyl acetate (85:15, v/v)).

this complex lipid was shown to be devoid of long chain fatty acids. They are: 745 cm^{-1} for skeletal vibrations, 990 cm^{-1} for unsaturation, 1375 cm^{-1} for C–CH_3, 1450 cm^{-1} for C–CH_2 as well as the presence of hydroxyl group(s) at 3425 cm^{-1}.

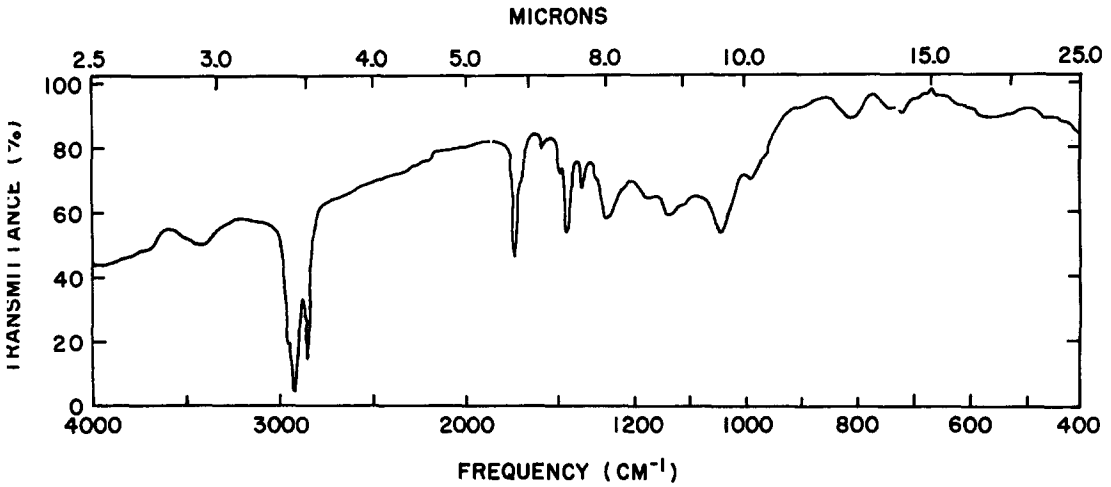


Fig. 6. Infrared spectrum of lipid of lipoprotein 24 h after isolation. Spectrum as KBr pellet with Comb attenuator.

Fig. 2 illustrates the ultraviolet spectrum of lipid of lipoprotein before and after borohydride treatment. It is known that borohydride reduces quinones to phenol with a corresponding spectral shift from 272 to 293–295 nm (ref. 26). As is apparent, this treatment had no effect upon this lipid from coccal or L-form membranes. The maxima noted, although weak, were similar to those of others[24] for bactoprenol isolated from lactobacilli (230, 272 and 290 nm). As control, ubiquinone was subjected to alkaline hydrolysis and heptane extraction. In contrast to lipid of lipoprotein, a spectral shift was observed, from 272 to 290 nm, for this standard after borohydride reduction.

Table IV compares the thin-layer chromatographic data of lipid of lipoprotein from streptococcal and L-form membranes with those of known bacterial isoprenoid-containing lipids. As is apparent, the coccal and L-form lipid behaved in similar fashion, before and after acetylation, with such lipids from other microorganisms[24,34,37,38], confirming the earlier detection of hydroxyl groups by infrared analyses. Also, lipid of lipoprotein stained green by anisaldehyde, a semispecific reagent for the detection of polyprenols[39]. It should be mentioned that this reagent also stained one of the degradative products (R_F 0.77, blue) which began to appear 2 h after the initial isolation of lipid from lipoprotein (see Fig. 3). Oxidation of lipid from lipoprotein and of coenzyme Q_6 and Q_{10} (ubiquinone) by permanganate all yielded complex mixtures which contained levulinic and succinic acids. These organic acids had been isolated as a single band (R_F 0.35) by preparative thin-layer chromatography (propanol–NH_3 solvent system) before their resolution by butanol–formic acid–water. Because of its volatile nature, no attempt was made to detect acetic acid, another oxidative product of polyprenol destruction. Suitable controls

TABLE IV

THIN-LAYER CHROMATOGRAPHIC COMPARISON OF VARIOUS ISOPRENOID-CONTAINING LIPIDS

Solvent system	*Lipid treatment*	*R_F values*					
		*Strepto-coccal and L-form lipid**	*Staphylo-coccal C55 isoprenoid***	*Lacto-bacillus C55 bactoprenol****	Q_6	Q_{10}	*Vitamin* K_1
Heptane–ethyl acetate (9:1, v/v)	—	0.30	0.25	0.34	0.21	0.29	0.40
	After acetylation	0.70	—	0.73	—	—	—
Heptane–ethyl acetate (85:15, v/v)	—	0.36	0.40	—	0.25	0.35	0.48
	After acetylation	0.78	0.82	—	—	—	—
Benzene–methanol (99:1, v/v)	—	0.71	0.56	—	0.71	0.65	0.82
Chloroform–methanol–water (65:25:4, v/v/v)	—	0.66 and 0.98	0.72 and 0.90	—	0.97	0.98	0.95

* Lipid after initial isolation (0–2 h).

** Data from Higashi *et al.*[37] and Higashi and Strominger[42].

*** Data from Thorne and Kodicek[38].

indicated that under these oxidative conditions, levulinic acid was not degraded further to succinic acid. Finally, extraction of lipid of lipoprotein from whole streptococcal cells as detailed by Thorne and Kodicek[38] with pyrogallol[25,40] for the protective extraction of bacterial quinones (one band, 272 nm) yielded the same isoprenoid-containing lipid as from isolated membranes and negates this lipid being an artifact resulting from the destruction of a quinone. Therefore, these chromatographic and chemical results together with the physical data obtained suggest that the lipid of lipoprotein from streptococcal and L-form membranes is devoid of quinones and to contain isoprenoid units.

For protein studies, lipoprotein was obtained by alkaline disc gel electrophoresis and the amino acids released by acid hydrolysis (6 M HCl, 18 h). Lipid was removed by diethyl ether extraction before lyophilization of the aqueous phase. Table III compares the amino acid composition of each Fraction B and its lipoprotein component. The most significant differences noted were in the elevated content of serine, dicarboxylic acids, glycine and especially ornithine in lipoprotein as compared with their corresponding Fractions B. It is apparent from the amino acid composition of lipoprotein from either source that the method of Lowry *et al.*[28] is not suitable for a true estimation of the protein content of this component (*i.e.* few aromatic amino acids). Since 47% of lipoprotein is composed of lipid, an almost equal amount must be attributed to its protein component.

Protein of lipoprotein was subjected to acetic acid gel electrophoresis. It was observed that this protein, and in contrast to intact lipoprotein, now entered the gel *in toto* and without the appearance of any band remaining on the gel surface. However, any subunit compositional differences between protein from coccal and L-form membrane lipoprotein must await accumulation of sufficient quantities for definitive study.

DISCUSSION

As is known[34,41–48], isoprenoid-containing lipids are involved in the synthesis of assorted polymers that, together, may comprise the rigid bacterial cell wall or envelope. The purpose of this study was to examine and compare the membranes of *S. pyogenes* and a stabilized L-form derived from it for the presence of lipids with isoprenoid units. This report details the first finding of an unusual lipoprotein in the membrane of this group A *Streptococcus* and of its continued but barely detectable presence in the membranes and whole cells of the L-form, an organism unable to synthesize any cell wall. Isolation and partial characterization of this lipoprotein from these two organisms has shown it to be identical, it being electrophoretically homogeneous, to have a high ornithine content and to contain isoprenoid unit(s). Also, the linkage between protein and lipid of this lipoprotein appears to be stronger than that of most lipid–protein complexes[49]. Finally, the isolation of this lipoprotein from whole cells and membranes of *S. pyogenes* by two different methods negates it being a procedural artifact and indicates that it is an integral component of the membrane.

The use of phenol as an agent for the preferential solubilization of proteins and membranes and for the extraction of bacterial cell walls has been detailed[11,12,50]. In the present study, the successful extraction of a lipoprotein containing isoprenoid

units from *S. pyogenes* and L-form membranes and from whole cells by phenol illustrates the specificity of this procedure for the coccal membrane. These findings also negate the inability of new cell wall formation by this L-form as being related to a complete absence of isoprenoid-containing lipids.

Lipid of lipoprotein from *S. pyogenes* and its L-form possessed chromatographic and chemical characteristics which were identical and strikingly similar to those of an isoprenoid alcohol from *S. aureus*[37,42] and certain lactobacilli[38]. Some of the degradative products observed after isolation of this lipid undoubtedly represent rearrangement and/or dehydration products known to be formed from isoprenoid-containing lipids under certain hydrolytic conditions[34]. Results of the effect of ageing upon a polyisoprenoid, bactoprenol, has yielded similar results[38]. Thus, the continued appearance of new products with time from lipid of lipoproteins from the coccus and L-form was expected and only attests to an instability which along with insufficient material has, thus far, prevented its complete characterization. At present, the size as well as the portion of lipid of lipoprotein yielding succinic acid upon oxidation and the nature of the phosphorus (pyrophosphate?) within this streptococcal membrane component are unknown.

With regard to protein of lipoprotein, the amount of ornithine found was surprising. Proteins with a high concentration of ornithine are unusual. When found, this amino acid is usually attached to a lipid or present as a component of small peptides. This was reconfirmed recently by the finding of only ornithine attached to lipids from such diversified microorganisms as *Brucella melitensis* and *Mycobacterium bovis*[51]. Thus, the apparent concentration of all of the ornithine of the membrane within lipoprotein only amplifies the association of this amino acid with lipid and emphasizes the uniqueness of this streptococcal and L-form membrane component. As is usually the case with bacterial membranes and as found with those from this coccus and its L-form, protein of lipoprotein was also completely devoid of amino acids containing sulphydryl groups.

Results of amino acid analyses suggest that protein of lipoprotein from *S. pyogenes* and its L-form may be a polypeptide composed of rather small peptides with few amino acids; possibly representing repeating units. Since the molecular weight of lipoprotein is small and since lipid constitutes approximately one-half of its total weight, it is possible that several lipid molecules may be attached to each protein of this membrane component. However, confirmation of this must await the complete characterization of the lipid moiety, especially its size. Although small, lipoprotein subjected to acetic acid gel electrophoreses remained on the surface of such gels. Therefore, the subsequent ability of only its protein component to migrate after acid hydrolysis probably reflects a change in protein charge and not in solubility after its release from lipid.

Although the presence of an isoprenoid-containing lipoprotein within the membranes of a representative member of the group A *Streptococci* has been demonstrated, only indirect evidence suggests its possible involvement in streptococcal cell wall biochemistry. Chemically, this stems from the especially close chromatographic similarity of lipid of lipoprotein with one already implicated in staphylococcal cell wall formation[3], the detection of isoprenoid units in this lipid by chemical means, and the great decrease in Fraction B and of lipoprotein in the membrane of the L-form, *i.e.* after loss of cell wall synthesis, as compared with that of the parental

Streptococcus. Biologically, it has been established that the antibiotics ristocetin, vancomycin and bacitracin inhibit the phospholipid cycle (C55 carrier-lipid) leading to linear bacterial peptidoglycan synthetis[3]. Also that growth of another *Streptococcus*, *S. faecalis*, and protoplasts derived from it are equally as sensitive to minute concentrations of these antibiotics[52]. However, the complete lack of inhibition of growth of this L-form, as compared with the complete sensitivity of its parental *S. pyogenes* to all of these antibiotics[53,54] also points to the probable presence of a phospholipid cycle for cell wall formation in this coccus, and of its cessation in the L-form. Nevertheless, whether or not the decreased content of lipoprotein in the membrane of the L-form is directly related to an inability to synthesize a cell wall must await proof of the involvement of this lipoprotein in cell wall formation in the group A *Streptococci*.

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