The Phospholipids in Membrane Ghosts from *Streptococcus faecalis* Protoplasts*

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The total lipid of the membrane ghost fraction prepared from *Streptococcus faecalis* (ATCC 9790) in the stationary phase of growth was found to be 21% of the membrane dry weight. Silicic acid chromatography indicated that two main types of lipid were present, polar lipid (77%) and a lipid resembling glyceride (22%). The polar lipid was further fractionated on a silicic acid column into two components. The first component (faster moving on thin-layer chromatography) constituted 54% of the total lipid and the second component (slower moving on thin-layer chromatography) constituted 23% of the total lipid. Reagents sprayed onto thin-layer plates failed to detect the presence of choline or amino nitrogen in either polar lipid, but did indicate the presence of carbohydrate in the slow polar lipid. Chemical analysis of the fast polar lipid showed that it was 3.75% phosphorus and had an ester-phosphorus ratio of 4.9:2. The slow polar lipid had an ester-phosphorus ratio of 6.1:1. Paper chromatography of the deacylated fast polar lipid before and after HCl hydrolysis indicated that the water-soluble moiety was diglycerolphosphorylglycerol. The fast polar lipid thus appears to be a diphosphatidylglyceride. The fatty acids of the fast polar lipid were found by gas-liquid chromatography to have the following structure codes: 14:0, 16:0, 16:1, 17:0 cyclopropane, *cis*- 18:1, and 19:0 cyclopropane. The fatty acids of the slow polar lipid were qualitatively identical to those of the fast polar lipid.

It is generally recognized that the plasma membrane (limiting membrane) of cells is composed principally of lipid and protein. However, information concerning the chemical structure of the lipid components of this complex organelle is on the whole quite limited. Such information is likely to be particularly important for the eventual understanding of the mechanisms underlying the functions of the plasma membrane such as selective permeation and specific mediated transport of solutes.

One of the principal obstacles to a study of the chemistry of the plasma membrane has been the difficulty in isolating it in a form reasonably free from other constituents of the cell. One of the few convenient sources of isolated plasma membranes is the membrane ghosts obtained by lysis of bacterial protoplasts. In recent years such membrane ghost preparations have been used to investigate the lipids in the plasma membrane of certain species of Gram-positive bacteria, namely, *Micrococcus lysodeikticus* (Gilby *et al.*, 1958; MacFarlane, 1961a,b), *Bacillus megaterium* M (Weibull, 1957), and *Bacillus megaterium* KM (Yudkin, 1962); see review by Kodicek (1962). Particularly noteworthy was the finding that a major component of the membrane ghosts from *Micrococcus lysodeikticus* is phosphatidylglycerol (GPGPG-lipid) (MacFarlane, 1961a), similar to the cardiolipins found in the tissues of plants (Benson and Strickland, 1960) and animals (Pangborn, 1947; Rose, 1964).

Membrane ghosts derived from protoplasts of *Streptococcus faecalis* have been under investigation for several years and have been found to contain a number of enzymes (Abrams *et al.*, 1960; Abrams and McNamara, 1962), and some membrane-bound ribonucleic acid (Abrams *et al.*, 1964). Electron microscopy of thin sections of these membrane ghosts shows that they are made up principally of an outer plasma membrane about 80 Å thick. Some internal structures are also present and these consist of some very thin filaments, some membrane-bound vacuoles, and a relatively thick tubular structure studded with ribosome-like particles running down its center (Abrams *et al.*, 1964).

The present report describes the isolation and characterization of the phospholipids in the membrane ghosts of *S. faecalis*. A preliminary account of this work has been presented (Ibbott and Abrams, 1964).

EXPERIMENTAL PROCEDURES

The cells (*S. faecalis* ATCC 9790) were grown in a medium containing 1% tryptone, 0.5% yeast extract, and 1% K_2HPO_4 (Abrams, 1958). They were harvested by centrifugation in the stationary phase of growth after 18 hours' incubation at 38°, then washed with cold distilled water and stored overnight at 4° as a thick suspension in water. For the preparation of the membrane ghosts (Abrams *et al.*, 1960; Abrams, 1959), the intact cells were first converted to protoplasts by incubation with lysozyme (0.18 mg per ml) in 0.4 M glycylglycine (*p*H 7.2) and 0.001 M $MgSO_4$. The protoplasts were then lysed by metabolic lysis and the resulting membrane ghosts were washed by centrifugation in cold 0.001 M $MgSO_4$. Finally the ghosts were lyophilized and stored under nitrogen at −20° until processed further.

Extraction of the Membrane Total Lipid.—Dry membranes were extracted exhaustively with nitrogen-purged ethanol-ether, 3:1, at 25°. After centrifugation of the extract, the supernatant was transferred to a weighed beaker and evaporated to dryness with a stream of nitrogen. The beaker was then desiccated to constant weight and the quantity of lipid determined by difference.

Fractionation of the Total Lipid.—Silicic acid, Mallinckrodt, 100 mesh, suitable for chromatographic analysis, was prepared as described by Barron and Hanahan (1958). The resulting material was slurried in *n*-hexane and used to prepare chromatographic columns 1 cm in diameter and 4 cm high.

Owing to the low solubility of the membrane lipid in *n*-hexane, it was not possible to apply the lipid sample to the column directly. Therefore, about 10 mg of the lipid was dissolved in 1 ml of chloroform-methanol 2:1, and approximately 50 mg of silicic acid was added. The mixture was then swirled vigorously while a stream

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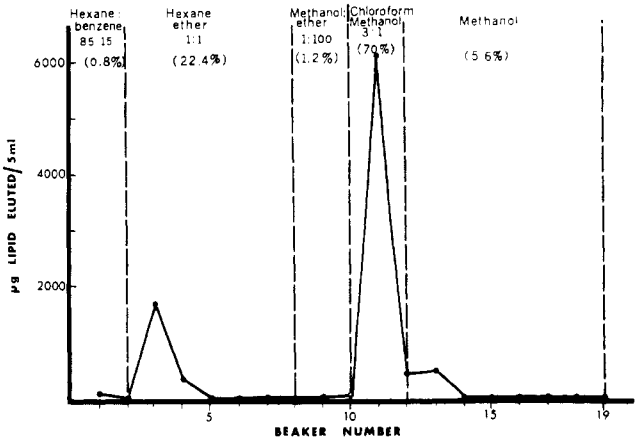

FIG. 1.—Silicic acid column chromatography of the total lipid. The fractionation was monitored on a weight basis and expressed in terms of μg lipid eluted per 5 ml solvent. The figures in parenthesis refer to percentages of the total lipid.

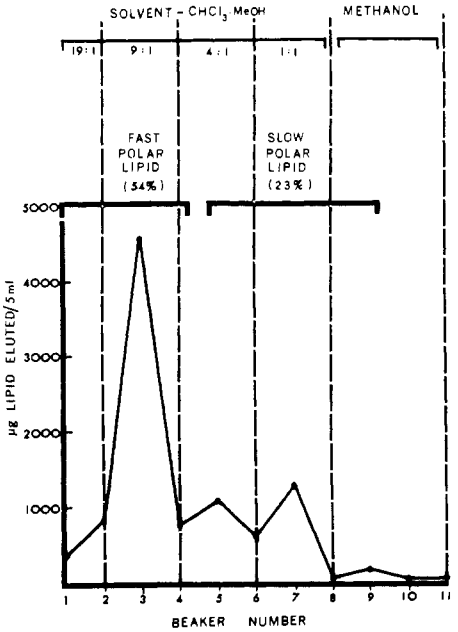

FIG. 2.—Silicic acid column chromatogram of the polar lipid fraction. The result is expressed in μg lipid eluted per 5 ml solvent. The figures in parentheses refer to percentage of the total lipid. (The analysis of each fraction on thin-layer chromatography is shown in Figure 3.)

of nitrogen accelerated the evaporation of the solvent. The lipid-coated adsorbent was then transferred to the top of the column.

The solvent series used for elution was hexane-benzene 85:15, hexane-ether 1:1, methanol-ether 1:100, chloroform-methanol 3:1, and methanol. Five-ml eluate fractions were collected which were immediately evaporated to dryness under nitrogen and desiccated to constant weight.

In order to fractionate further the polar lipids obtained from the first column, they were applied to a second column identical to the first but eluted with chloroform-methanol mixtures in the following proportions: 19:1, 9:1, 4:1, 1:1, and finally methanol alone.

The homogeneity of each fraction eluted from the columns was examined by thin-layer chromatography on silica gel G according to Stahl (1956), using as solvent chloroform-methanol-water 65:25:4. Lipid spots were demonstrated after thin-layer chromatography by spraying the plate with one of the reagents recommended by Wagner *et al.* (1961).

Infrared Spectrophotometry.—Potassium bromide pellets, 1 × 5 mm, containing lipid were scanned in a Beckman IR5 infrared spectrophotometer equipped with the micro condenser assembly.

Total Phosphorus.—About 50 μg of the lipid sample was digested at 180° with 100 μl of 60% perchloric acid, after which the determination was completed by the method described by McDonald and Hall (1957).

Ester Groups.—The hydroxamate procedure of Antonis (1960) was scaled down to one-tenth volumes and the final color read on the Beckman DU at 515 mμ.

Examination of the Water-Soluble Fractions of the Polar Lipids.—To characterize the water-soluble moieties of the polar lipids, they were first deacylated by the method of Wintermans (1960). The reaction was stopped by the addition of Dowex 50 X-8, and the water-soluble components were separated from the fat-soluble components by the addition of water and chloroform. Each phase was taken to dryness under nitrogen. The water-soluble fractions were then examined before and after HCl hydrolysis by paper chromatography using the procedure of Benson and Strickland (1960). The resultant phosphorus-containing spots were visualized by spraying with the molybdate reagent of Hanes and Isherwood (1949), followed by irradiation with a strong source of ultraviolet light (Dawson, 1960).

Gas-Liquid Chromatography of the Fatty Acid Methyl Esters.—The chloroform extract obtained at the deacylation step contained fatty acids as the methyl esters, and these were examined by standard methods in a gas chromatograph.

In order to characterize further two of the fatty acids, an octadecenoic acid and one believed to be lactobacillic acid, they were collected in 3.5 in. no. 10 stainless steel Luer hypodermic needles as they emerged from the outflow gasket of the chromatograph until sufficient material had been obtained for further analysis. The fatty acid methyl esters collected in this way were contaminated with thermal decomposition products from the diethyleneglycol polyester column, which would cause interference with infrared spectrophotometry. Further purification was effected by application of the material in *n*-hexane to a silicic acid column which was then eluted with hexane-benzene 85:15.

RESULTS

Analysis of Total Lipid by Silicic Acid Column Chromatography and Thin-Layer Chromatography.—The value found for the total lipid of the lyophilized membrane material was 21%. Figure 1 shows the elution pattern obtained by silicic acid column chromatography of this membrane lipid material. The majority of the lipid, comprising about 77% of the total, appeared in the methanol-containing solvents and will be referred to as polar lipid.

The next largest fraction, 22% of the total, was eluted by hexane-ether and appears to be glyceride, although no actual proof of its identity was obtained. These two major fractions thus account for 99% of the membrane total lipid.

Thin-layer chromatography showed that the polar lipid could be resolved into two components, both of which reacted strongly to the molybdate spray reagent. The second silicic acid column separated these two polar lipids (Fig. 2), and thin-layer chromatography indicated that each of these fractions migrated as a single spot (Fig. 3). The fast polar lipid comprised

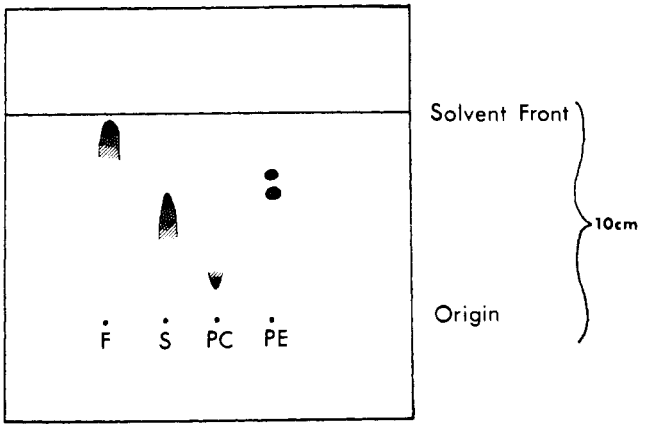

FIG. 3.—Thin-layer chromatogram of the pooled polar lipid fractions obtained from silicic acid column chromatography (Fig. 2). Solvent: chloroform-methanol-water, 65:25:4. Spray reagent: molybdate-perchloric acid. F = lipid from beakers numbers 1–4 (fast polar lipid). S = lipid from beakers numbers 5–9 (slow polar lipid). PC = phosphatidylcholine. PE = phosphatidylethanolamine.

TABLE I
ESTER-PHOSPHORUS RATIOS OF THE FAST AND SLOW POLAR LIPIDS[a]

	Ester (μM/mg)	Phosphorus (μM/mg)	Ester-Phosphorus
Fast	2.97 ± 0.11	1.21 ± 0.03	4.9:2
Slow	3.91 ± 0.07	0.64 ± 0.01	6.1:1

[a] Each result is the mean of two pairs of duplicate determinations.

54% by weight of the total membrane lipid and the slow polar lipid 23%.

The fast polar lipid gave no color reaction on thin-layer plates with the spray reagents for amino nitrogen, choline, and carbohydrate. Similar amounts of phosphatidylcholine, phosphatidylethanolamine, and cerebroside were easily demonstrable.

The slow polar lipid also failed to react with the ninhydrin reagent for amino nitrogen and with Dragendorff's reagent for choline. However, a faint blue-gray color was produced with the diphenylamine reagent, implying the presence of carbohydrate.

Infrared Spectra of the Polar Lipids.—The infrared absorption spectrum of the fast polar lipid shows a prominent band at 1740 cm^{-1} indicating the presence of ester groups, at 1250 cm^{-1} indicating P-O, and at 720 cm^{-1} signifying a long aliphatic chain.

The spectrum of the slow polar lipid resembles that of the fast compound, except for the presence of a very strong band at 1380 cm^{-1}, the significance of which is not known.

Ester-Phosphorus Ratios of the Polar Lipids.—The determinations of ester and phosphorus were carried out in duplicate on each of two independently prepared samples of the fast and the slow polar lipids. The results are given in Table I, from which it can be seen that the fast polar lipid had a phosphorus content of 1.2. μM per mg and an ester concentration of 2.97 μM per mg and thus an ester-phosphorus ratio of 4.9:2. These findings, together with others to be described below, indicate that the structure of the fast polar lipid is that of a diphosphatidylglyceride and that all potential sites for esterification are occupied.

The slow polar lipid showed a rather different constitution, with a phosphorus level of 0.64 μM per mg, while the concentration of ester was 3.91 μM per mg.

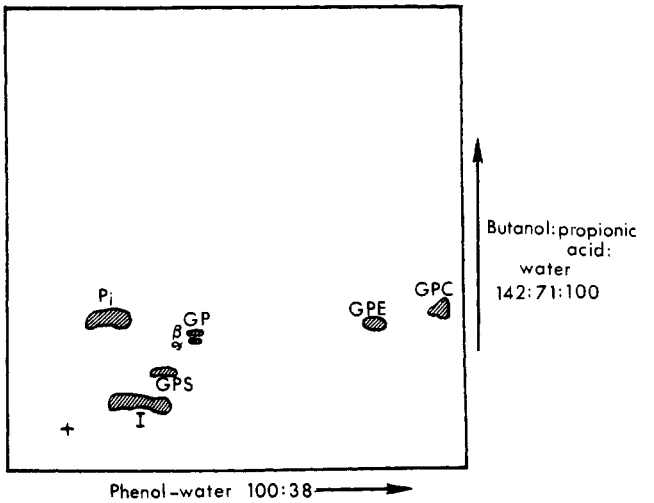

FIG. 4.—Paper chromatography of the deacylated fast polar lipid (I) and known glycerophosphate esters. I = deacylated fast polar lipid. GP = glycerophosphate. GPC = glycerophosphocholine. GPE = glycerophosphoethanolamine. GPS = glycerophosphoserine. P_i = inorganic phosphate.

The ester-phosphorus ratio was, therefore, 6.1:1 (see Discussion).

The Water-Soluble Products of Deacylation of the Polar Lipids.—The water-soluble phosphorus-containing compound obtained after removal of the fatty acids comprised approximately 32% of the fast polar lipid, assuming that the deacylation process went to completion. The result of two-dimensional paper chromatography of this water-soluble substance together with known glycerophosphate esters is shown in Figure 4. By comparison with the work of Benson and Strickland (1960), the deacylated fast polar lipid appears to behave in a manner apparently identical to that of diglycerolphosphorylglycerol with regard to R_F and the characteristically spread out spot close to the origin.

Acid Hydrolysis of the Deacylated Fast Polar Lipid.—The HCl hydrolysis products of the deacylated fast polar lipid gave a chromatogram (Fig. 5) which was apparently identical to that described for the hydrolysis products of diglycerolphosphorylglycerol (Benson and Strickland, 1960). Two spots appeared to be unhydrolyzed diglycerolphosphorylglycerol, as they were identical in location with those obtained from the unhydrolyzed material applied to the paper in the presence of chloride.

Identification of the Fatty Acids Associated with the Polar Lipids.—Table II lists the retention times relative to palmitic acid ester for the fatty acids from the fast polar lipid, together with those of reference substances. The behavior of the components labeled A, B, C, and E were identical with reference fatty acid methyl esters of the following codes: 14:0, 16:0, 16:1, and 18:1, respectively. Similar results were obtained on the nonpolar column, SE-30, which provided additional evidence that the code assignments were correct.

The relative retention time of component F was essentially identical with that reported for the C-19 cyclopropane fatty acid lactobacillic acid when run under conditions essentially identical to those used in the present work (Kaneshiro and Marr, 1961). Accordingly, the infrared spectrum of component F was examined for the cyclopropane peak at 1020 cm^{-1}. The spectrum showed an absorption peak in the position of the cyclopropane band and hence component F is thought to be the C-19 cyclopropane fatty acid, lactobacillic acid, and component D is thought to be

TABLE II
RELATIVE RETENTION TIMES OF STANDARD AND UNKNOWN FATTY ACID METHYL ESTERS[a]

Standard Fatty Acid Esters				Fatty Acid Esters from Fast Polar Lipid		
Common Name	Code	Relative Retention Time		Component	Relative Retention Time	
		DEGS[b]	SE-30		DEGS[b]	SE-30
Methyl laurate	12:0	0.37				
Methyl myristate	14:0	0.59	0.47	A	0.59	0.47
Methyl palmitate	16:0	1.00	1.00	B	1.00	1.00
Methyl palmitoleate	16:1	1.16	0.90	C	1.16	0.91
				D	1.53	1.35
Methyl stearate	18:0	1.70				
Methyl oleate	18:1	1.94	1.87	E	1.97	1.91
Methyl linoleate	18:2	2.30				
Methyl linolenate	18:3	3.00				
				F	2.58	2.92
Methyl arachidate	20:0	2.90				
Methyl eicosaenoate	20:1	3.30	4.00			

[a] Retention time of palmitic acid methyl ester taken as unity. Temperature of the gas-liquid chromatography columns: DEGS, 198°; SE-30, 190°. [b] Diethyleneglycol polyester.

TABLE III
CONCENTRATION AND PROBABLE IDENTITY OF FATTY ACIDS FROM FAST POLAR LIPID

Component	Probable Code	Relative Molar Concentration[a]
A	14:0	1.0
B	16:0	1.5
C	16:1	0.7
D	17:0c[b]	0.05
E	18:1 (*cis*-)	1.0
F	19:0c	0.9

[a] Molar concentration of 18:1 taken as unity. [b] c following the figure indicates cyclopropane.

TABLE IV
CONCENTRATION AND PROBABLE IDENTITY OF FATTY ACIDS FROM THE SLOW POLAR LIPID

Component	Probable Code	Relative Molar Concentration[a]
A	14:0	1.1
B	16:0	2.1
C	16:1	0.7
D	17:1c[b]	0.06
E	18:1 (*cis*-)	1.0
F	19:0c	1.2

[a] Molar concentration of 18:1 taken as unity. [b] c following the figure indicates cyclopropane.

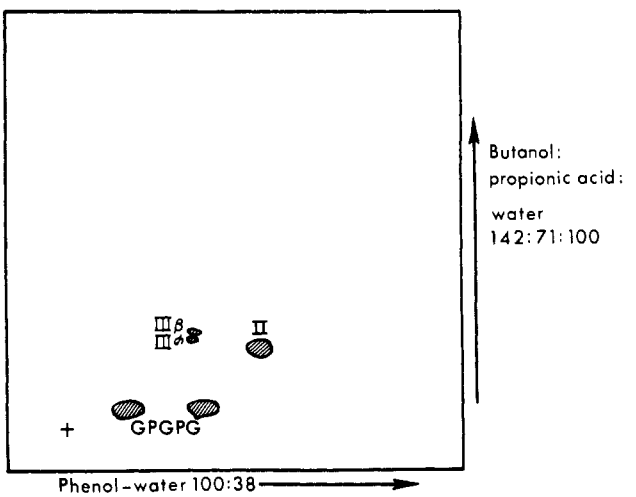

FIG. 5.—Two-dimensional paper chromatography of the compounds observed after HCl hydrolysis of the deacylated fast polar lipid. The spots due to unhydrolyzed material are labeled GPGPG (diglycerolphosphorylglycerol). II is at the position anticipated for GPG (glycerophosphorylglycerol). IIIα and IIIβ are α- and β-glycerophosphates.

its C-17 homolog. Further confirmation by comparison with authentic samples is desirable.

Component E, designated a monounsaturated C-18 fatty acid, was shown by infrared spectrophotometry to be of the *cis*- configuration but may be composed of one or more octadecenoic acids. This finding is in accordance with the synthetic pathway for lactobacillic acid reported by Liu and Hofman (1962) in which it was shown that *cis*-vaccenic acid together with methionine or formate were the precursers for this cyclopropane fatty acid in *Lactobacillus arabinosus*.

The configuration of component C, designated as a 16:1 fatty acid, was not investigated.

Tables III and IV indicate the probable code identities and the relative concentrations of the fatty acids in the fast polar lipid and the slow polar lipid as calculated from the areas under the peaks of the gas chromatograph charts.

DISCUSSION

Lipid constitutes 21% of the weight of the membrane ghost fraction of *Streptococcus faecalis* (ATCC 9790) prepared under the particular conditions we have described. About three quarters of the lipid is phospholipid, while the remainder, except for a trace quantity, appears to resemble glyceride. The phospholipid is separable by silicic acid–column chromatography into two components, the fast polar lipid and the slow polar lipid, by use of a solvent series similar to that used by Gray and MacFarlane (1958) for the separation of cardiolipin from cephalin. The fast polar lipid resembles cardiolipin in its behavior on silicic acid columns and on thin-layer chromatography (Wagner *et al.*, 1961); the infrared spectrum is similar to that obtained by Rose (1964) for rat liver cardiolipin, and the water-soluble products of acid and alkali degradation are these expected from diglycerolphosphorylglycerol.

In previous accounts concerned with phospholipids of the cardiolipin type, the number of hydroxyl groups in the molecule esterified with fatty acids was reported as three in cardiolipin from dog liver (McKibbin and Taylor, 1952), four in ox heart cardiolipin (Gray and MacFarlane, 1958), and four or five in material from plants (Benson and Strickland, 1960), while Rose (1964) reported an ester-phosphorus ratio of 2.03 in rat

liver cardiolipin. In the present work, the ester-phosphorus ratio of 5:2 makes it likely that all five potential sites for esterification are occupied and that the fast polar lipid has the structure of a diphosphatidylglyceride. Furthermore, the phosphorus content of the intact fast polar lipid was found to be 3.75%, which compares favorably with the calculated value of 3.82% for a diphosphatidylglyceride containing the molar proportions of the fatty acids found in this lipid fraction. As the six fatty acids found outnumber the maximum locations for substitution, the fast polar lipid cannot be a single substance but is more probably a mixture in which each member carries a varying complement of fatty acids.

In contrast to the fast polar lipid, the slow polar lipid apparently contains carbohydrate and has an ester-phosphorus ratio of 6:1. Compounds which resemble the slow polar lipid with respect to their ester-phosphorus ratio have been reported occasionally. Thus, a lipid with an ester-phosphorus-carbohydrate ratio of 5.7:1.0:3.2 has been reported by MacFarlane (1961b) to exist in *M. lysodeikticus* cells, and Marinetti *et al.* (1957) described a lipid derived from rat liver mitochondria with an ester-phosphorus ratio of 6.04. At the present time, the nature of the slow polar lipid remains obscure.

Vorbeck and Marinetti (1964) have reported an analysis of the membrane fraction of *S. faecalis* which is somewhat different from that which we have reported here. However, the two studies are not necessarily comparable for two reasons: first, the growth phase of the organism was stationary in our work and exponential in the work of Vorbeck and Marinetti, and, second, the compositions of the two culture media were different. It has been shown that such factors can influence profoundly the lipid composition of bacteria (Kanfer and Kennedy, 1963; Houtsmuller and van Deenan, 1964).

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