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Lipid Composition of the Electron Transport Membrane of *Haemophilus parainfluenzae*

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The principal lipids associated with the electron transport membrane of *Haemophilus parainfluenzae* are phosphatidylethanolamine (78%), phosphatidylmonomethylethanolamine (0.4%), phosphatidylglycerol (18%), phosphatidylcholine (0.4%), phosphatidylserine (0.4%), phosphatidic acid (0.2%), and cardiolipin (3.0%). Phospholipids account for 98.4% of the extractible fatty acids. There are no glycolipids, plasmalogens, alkyl ethers, or lipo amino acid esters in the membrane lipids. Glycerol phosphate esters derived from the phospholipids by mild alkaline methanolysis were identified by their staining reactions, mobility on paper and ion-exchange column chromatography, and by the molar glycerol to phosphate ratios. Eleven diacyl phospholipids can be separated by two-dimensional thin-layer chromatography. Each lipid served as a substrate for phospholipase D, and had a fatty acid to phosphate ratio of 2:1. Each separated diacyl phospholipid was deacylated and the glycerol phosphate ester was identified by paper chromatography in four solvent systems. Of the 11 separated phospholipids, 3 were phosphatidylethanolamines, 2 were phosphatidylserines, and 2 were phosphatidylglycerols. Phosphatidylcholine, cardiolipin, and phosphatidic acid were found at a single location. Phosphatidylmonomethylethanolamine was found with the major phosphatidylethanolamine. Three distinct classes of phospholipids are separable according to their relative fatty acid compositions. (i) The trace lipids consist of two phosphatidylethanolamines, two phosphatidylserines, phosphatidylcholine, phosphatidic acid, and a phosphatidylglycerol. Each lipid represents less than 0.3% of the total lipid phosphate. These lipids are characterized by high proportions of the short (C_{10} to C_{14}) and long (C_{19} to C_{22}) fatty acids with practically no palmitoleic acid. (ii) The major phospholipids (93% of the lipid phosphate) are phosphatidylethanolamine, phosphatidylmonomethylethanolamine, and phosphatidylglycerol. These lipids contain a low proportion of the short ($<C_{14}$) and long ($>C_{19}$) fatty acids. Palmitic and palmitoleic acids represent over 80% of the total fatty acids. (iii) The fatty acid composition of the cardiolipin is intermediate between the other two classes. Both palmitoleic and the longer fatty acids represent a significant proportion of the total fatty acid.

Haemophilus parainfluenzae has been shown to require a membrane-bound electron transport system for metabolic activity (24). The membrane-bound electron transport system consists of dehydrogenases, cytochromes, cytochrome oxidases, a respiratory quinone, and phospholipids (20, 29, 32). Of the total fatty acids that can be extracted with organic solvents, 98% are associated with phospholipids (26). However, treatment of the membrane with organic solvents that extract lipids produces a simultaneous disruption of electron transport (23). This property of the phospholipids in electron transport prompted an examination of the lipids in the membrane of *H. parainfluenzae*. The composition of the membrane-bound electron transport system can be extensively modified during growth in various environments (20, 21, 23, 25). The concentration of 2-demethyl vitamin K_2 has been shown to change during these environmental modifications (22). If the phospholipid composition is known, the changes in the phospholipid composition can be correlated to the modified composition of the respiratory pigments. This could lead, hopefully, to deeper understanding of the formation of the membrane complex.

This study will show that, in addition to the phospholipids expected in a typical gram-negative aerobe, *H. parainfluenzae* synthesizes small quantities of phospholipids which differ markedly in

fatty acid composition from the major lipids. *H. parainfluenzae* forms traces of a lipid with properties like phosphatidylcholine.

MATERIALS AND METHODS

Cultures. The medium, cultural conditions, bacterial strain, and harvesting conditions have been described (20, 24). The dry weight was measured as described previously (24). The lipids were labeled by growing the *H. parainfluenzae* in the presence of 100 μc of $H_3{}^{32}PO_4$ per 1.5 liters of media for at least 21 generations, unless otherwise stated.

Materials. Phosphatidylglycerol and phosphatidylethanolamine isolated from *Escherichia coli* were gifts from J. H. Law. Cardiolipin was obtained from Sylvana Chemical Corp., Englewood Cliffs, N.J., or was isolated from *Staphylococcus aureus* (27). Phosphatidylserine and phosphatidylcholine were obtained from the Applied Science Laboratories, Inc., State College, Pa. Labeled glycerolphosphorylmonomethylethanolamine and glycerolphosphorylcholine were gifts from R. L. Lester. Carrier-free $H_3{}^{32}PO_4$ was supplied in plastic bottles by Tracerlabs, Richmond, Calif. Other materials were of the best grade available commercially, or as described previously (23, 24).

Extraction of the lipid. The two methods used to extract the lipids have been described (26). For large-scale preparations, *H. parainfluenzae* was harvested, washed, and suspended in a mixture of chloroform and methanol (2:1, v/v) and extracted at room temperature for 2 hr with stirring. The extract was then filtered through glass wool, and the residue was reextracted with chloroform-methanol twice. The combined extracts were partitioned against salt water (3), and nonlipid components were removed by chromatography with Sephadex (18) from which the phospholipids were recovered quantitatively. The second method of extraction was the Bligh and Dyer procedure (2). Various extraction procedures were used in an attempt to maximize the total lipid phosphate that could be extracted. Exposure of the cells to ultrasonic vibration, boiling the cells in isopropanol before extraction, acidifying the growth medium before harvesting the bacteria, or boiling in isopropanol after acidification resulted in the recovery, respectively, of 39.1, 55.4, 57.9, and 70.2 μmoles of phospholipid phosphate per g (dry weight) of cells. The Bligh and Dyer and the chloroform-methanol methods (2, 26) resulted in the extraction of 76.6 $\pm$ 1.1 μmoles of phospholipid phosphate per g (dry weight) of cells. Boiling in isopropanol and acidification of the medium before harvest is necessary for the maximal recovery of the lipids of some bacteria (27). The proportions of the lipids, determined by chromatography of the deacylated derivatives on Dowex-1, were essentially the same for all modifications of the extraction procedure. Acidifying the chloroform-methanol does not extract additional phospholipid (26). These extractions leave a residue with distinctively different fatty acid composition from the fatty acid composition of the total cells (26).

Thin-layer chromatography. The lipids chromatographed over thin-layer plates of silica gel G were detected with reagents and recovered from the silica gel by methods described previously (27). The solvents used for separating the intact lipids were chloroform, methanol, and 6.7 M ammonium hydroxide (33:18.2:2.5, v/v) in the first dimension, and chloroform, methanol, acetic acid, and water (45:7:3.15:0.5, v/v) in the second dimension. The lipid was dried by successive evaporations with benzene and absolute ethyl alcohol (4:1, v/v; 33), and not more than 0.5 μmole of lipid phosphate was applied to each plate for maximal reproducibility. The silica gel was 0.5 mm thick. For separation of larger amounts of the phospholipids, the lipids were fractionated as follows: (i) phosphatidylcholine and neutral lipids (R_F value, 1.0 to 0.8); (ii) the major and two minor phosphatidylethanolamines and part of the cardiolipin (R_F value, 0.8 to 0.62); (iii) the major and minor phosphatidylglycerols, the phosphatidylserine, and the rest of the cardiolipin (R_F value, 0.62 to 0.38); and (iv) the phosphatidic acid and two trace phosphatidylethanolamines (R_F value, 0.38 to 0.01). This was accomplished by one-dimensional chromatography of a strip that contained 5 μmoles of lipid phosphate per plate in a solvent of chloroform, methanol, and 6.7 M ammonium hydroxide (33:18.2:2.5, v/v). The fraction that contained phosphatidylcholine was chromatographed on thin-layer plates in chloroform and isooctane (1:1, v/v) to remove the neutral lipid. Each of the fractions from the initial chromatography was then chromatographed in the two-dimensional system described above. Several plates were used for each fraction from the preliminary purification. The cardiolipin and the trace phosphatidylethanolamines from the preliminary fractionation were combined into three fractions, since they occupied similar positions on the two-dimensional thin layer plates. The lipids were then recovered. Those occupying the same position were pooled, and about 10% of the recovered lipid was subjected to mild alkaline methanolysis. The glycerol phosphate derivatives were identified by paper chromatography as described below. The pooled lipids contained only one glycerol phosphate ester in each spot.

Mild alkaline methanolysis. A modified procedure of alkaline methanolysis developed by R. L. Lester (*unpublished data*) was used to deacylate the extracted lipids. The lipid was dissolved in 1 ml of methanol and toluene (1:1, v/v), and an equal volume of freshly prepared methanolic 0.2 M KOH was added (20 μmoles per μmole of phosphate). The solution was incubated at 0 C until the reaction was completed. The solution was then neutralized by adding a suspension in water of Biorex-70 (Bio-Rad Laboratories, Richmond, Calif.) in the acid form (1 meq/10 μmoles of phosphate). The mixture was added to a column in a Pasteur pipette that contained an additional 3.3 meq of Biorex. The test tube that contained the mixture was rinsed twice with 1 ml of methanol and then twice with 2 ml of water. Each rinse was added to the top of the Biorex column. The solvents were forced out of the Pasteur pipette with compressed air. The eluate was *p*H 7.0 and it contained less than 1 μmole

of K^+, as measured with a Coleman flame photometer. This method gave the lowest proportion of α glycerol phosphate in the lipids.

The methanolysis was performed at 0 C to prevent the side reactions which result in cyclic glycerol phosphate and other secondary products. The time course of the methanolysis is illustrated in Fig. 1. The recovery of phosphate in the aqueous phase is 100 ± 0.5%. As will be established, the products of the methanolysis are glycerol phosphate esters in the aqueous phase, and methyl esters of the fatty acids in the organic phase. In early experiments involving column chromatography of the glycerol phosphate esters, the methanolysis was stopped by neutralization with acetic acid. Under these conditions, between 1.5 and 2.0% of the lipid phosphate was recovered as α-glycerol phosphate. The use of Dowex-50 to neutralize the base has been suggested by Benson (1). With these anhydrous conditions, neutralization with Dowex-50 resulted in large proportions of α-glycerol phosphate.

The following abbreviations are used to represent the glycerol phosphate esters derived from the phospholipids by deacylation: GPC, glycerolphosphorylcholine derived from phosphatidylcholine; GPE, glycerolphosphorylethanolamine derived from phosphatidylethanolamine; GPMME, glycerolphosphorylmonomethylethanolamine derived from phosphatidylmonomethylethanolamine; GPDME, glycerolphosphoryldimethylethanolamine derived from phosphatidyldimethylethanolamine; GPG, glycerolphosphorylglycerol derived from phosphatidylglycerol; GPS, glycerolphosphorylserine derived from phosphatidylserine; αGP, L-α-glycerolphosphate derived from phosphatidic acid; GPGPG, diglycerolphosphorylglycerol derived from cardiolipin.

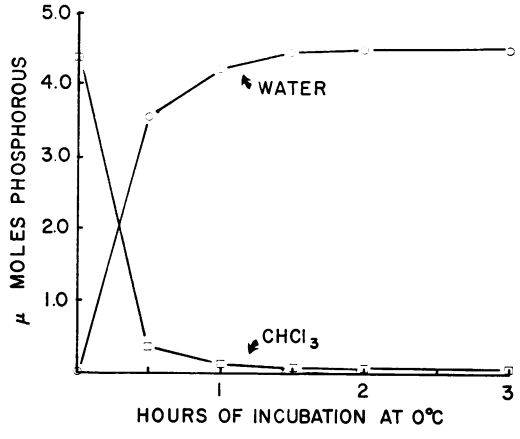

FIG. 1. *Time course of the mild alkaline methanolysis of the lipids of H. parainfluenzae. The lipid was dissolved in 1 ml of methanol and toluene (1:1). Freshly prepared 0.2 M methanolic KOH (1 ml) was added and the mixture was incubated at 0 C. At the times indicated, portions were removed, sufficient 1 M acetic acid was added to bring the mixture to pH 7.0, and equal volumes of both chloroform and water were added. The solution was mixed with a vortex mixer for 5 min. The aqueous phase was removed and combined with a second aqueous phase that also had been partitioned against the chloroform. The volumes were reduced to dryness in a stream of nitrogen; the lipids were digested and analyzed for phosphate.*

Column chromatography. The gradient fractionation of deacylated phospholipids on Dowex-1 columns with ammonium formate-sodium borate has been described (27). Components eluting in the early fractions of the column can be better resolved by the use of 0.02 M ammonium formate at *p*H 9.0 (R. L. Lester, *unpublished data*). This procedure was used to detect GPC. The individual glycerol phosphate esters separated by preparative columns were pooled and desalted, as will be described in the Results.

Paper chromatography. Glycerol phosphate esters derived from the phospholipids by deacylation were separated by paper chromatography by the use of solvents and detection methods described previously (27). GPE, GPMME, and GPDME were separated by paper chromatography in a solvent of 3.8 mM ethylenediaminetetraacetic acid and 0.7 M ammonium bicarbonate in 90 mM ammonium hydroxide containing 67% ethyl alcohol. The components were detected by radioautography or an amine reagent (9). Amino cellulose paper (Whatman AE-81) was first treated with 3 M formic acid and dried. The samples were then chromatographed over a solvent system of 0.4% pyridine in 3 M formic acid in the first dimension, and in Wawszkiewicz solvent I (17) diluted 3 to 7 with 95% ethyl alcohol containing 0.26 M ammonia in the second dimension. Up to 2.5 μmoles of lipid phosphate can be chromatographed without streaking. Lipids can be localized by radioautography or by treatment of the papers with periodate followed by *o*-tolidine (27). These methods were developed by R. L. Lester (*unpublished data*).

Gas-liquid chromatography. Gas-liquid chromatography for the methyl esters of the fatty acids was performed as previously described (26). Fatty acids were designated with a subscript for the length of the carbon chain, and with "Br" for iso and anti-iso branched acid esters, "C" for cyclopropane, and 1 following a colon (:1) for monounsaturated (26). The fatty acid methyl esters were prepared and identified as described (26). Gas-liquid chromatography of the lipid acid hydrolysate was used to identify the ethanolamine-containing lipids (10).

Analysis of the lipids. Analysis of the lipids for glycerol, fatty acids, phosphate, carbohydrate, and amino nitrogen were performed as described previously (27).

Treatment with phospholipase D. Between 0.1 and 0.01 μmole of lipid phosphate was dissolved in 1 ml of ether in a test tube, and 0.42 ml of water that contained 20 μmoles of acetate buffer (*p*H 5.6), 40 μmoles of $CaCl_2$, and 1.6 mg of cabbage phospholipase D (Calbiochem, Los Angeles, Calif.) was added. The reaction mixture was agitated vigorously and incubated at 25 C for 4 hr. The ether was removed with a stream of nitrogen, and 1.85 ml of water, 2.5 ml of

chloroform, and 2.5 ml of methanol were added. After thorough mixing, the lower phase was recovered, and the lipid was deacylated. The water-soluble glycerol phosphate esters were identified by paper chromatography with a water-saturated phenol solvent.

Measurement of radioactivity. Samples were assayed for radioactivity in a model 2311 scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Deacylated phospholipids were counted on paper discs (1.5 to 2 cm in diameter) in a scintillation fluid of 9.28 mM 2,5[2 (5-tertbutyl benzoxazol)]-thiophene (BBOT) in toluene. Since all counts were made on similar paper discs, the influence of quenching was minimized. The efficiency of counting for ^{32}P was 90.6%. No radioactivity could be detected in the vial after removal of the paper disc. Water-soluble materials in solution were counted in a scintillator consisting of 1.236 M naphthalene and 6.99 mM BBOT in dioxane. Up to 1 ml of aqueous solution can be put with 10 ml of counting fluid. The efficiency of this scintillator for ^{32}P was 100%.

Radioautography. Dried papers or Saran-wrapped thin-layer plates were placed on Kodak no-screen X-ray film for 12 to 72 hr in film holders. The film was then developed, and the dark spots corresponding to radioactive components were used to find the components on the chromatogram.

RESULTS

Over 98% of the fatty acid extractable with organic solvents from *H. parainfluenzae* are bound to phospholipids (26). Consequently, phospholipids of this organism were analyzed. Mild alkaline methanolysis of diacyl phospholipids converts the lipids into water-soluble glycerol phosphate esters and methyl esters of the fatty acids that are soluble in organic solvents. The initial analysis of the lipids of *H. parainfluenzae* involved the separation and identification of the glycerol phosphate esters. Lipid phosphate can be quantitatively recovered in the aqueous phase after mild alkaline methanolysis, as illustrated in Fig. 1. This fact precludes the presence of alkyl ethers and plasmalogens in the lipids of *H. parainfluenzae*. These ether derivatives are not hydrolyzed under the conditions of mild alkaline methanolysis (19).

Analysis of a lipid sample containing 27 μmoles of lipid phosphate for carbohydrate with the anthrone reagent (27) indicates that less than 0.1 μmole of carbohydrate was present in the lipid. After thin-layer chromatography of 5 μmoles of lipid phosphate, no reactivity with diphenylamine reagent (16) was seen. The diphenylamine reagent used in this manner is sensitive to 0.05 μmole of monoglycosyldiglyceride per cm^2. Within experimental error, all of the fatty acids from the complex lipids recovered from a silicic acid column with methanol are accounted for by the phospholipids (26). Lipids isolated from *H. parainfluenzae* grown with ^{14}C-glucose or ^{14}C-glycerol and ^{32}P, and separated by chromatography, give no evidence for complex lipids other than phospholipids.

Separation of the glycerol phosphate esters. Water-soluble glycerol phosphate esters of the phospholipids were separated by chromatography on Dowex-1 with the gradient described previously (27). Deacylated phospholipids derived from bacteria grown in the presence of ^{32}P are illustrated in Fig. 2. Repeated analysis indicates that the proportions of deacylated phospholipids are reproducible to ±1% for GPE and GPG and to ±0.2% for the other lipids. A tentative identification of the lipids can be made by comparison of the volume necessary to elute a volume of specific glycerol phosphate ester. The elution volumes are reproducible.

Several column separations similar to that shown in Fig. 2 yielded a total of 394 μmoles of lipid phosphate distributed among the six tentatively identified glycerol phosphate esters. Each glycerol phosphate derivative was pooled and desalted on a second Dowex-1 column. Borate was removed by washing the column with several volumes of 0.05 M formic acid. The glycerol phosphate esters were then eluted with 0.03 M ammonium formate. The ammonium ion was removed by passing the eluate through a third

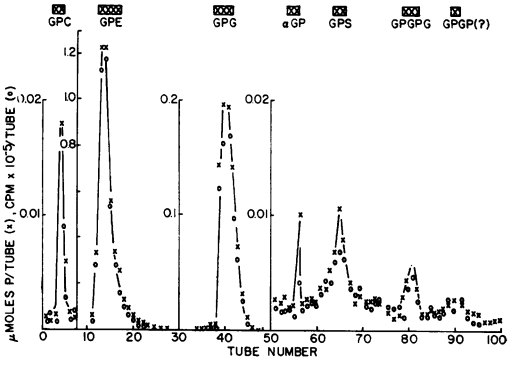

FIG. 2. *Chromatographic analysis of the deacylated phospholipids from H. parainfluenzae grown in the presence of ^{32}P. Lipids were eluted from a Bio-Rad AG-1 (Dowex-1) column in the formate form with the gradient described previously (27). The gradient was pumped through the column at a rate of 0.4 ml/min, and 2.64-ml fractions were collected. Each fraction was assayed for ^{32}P by removal of 0.5 ml, which was added to the dioxane scintillation mixture and the radioactivity determined. The remainder of the fraction was taken to dryness at 150 C, digested, and the phosphate determined. Recovery of ^{32}P added to the column was 97%. The R_F values of known deacylated lipids are illustrated at the top of the figure.*

Dowex-50 (acid form) column. The eluate was collected in ice and lyophilized as rapidly as possible. Recovery of the lipid phosphate after desalting for each deacylated lipid was GPC, 71%; GPE, 99%; GPG, 78%; GPS, 49%; αGP, 62%; and GPGPG, 74%.

After desalting, each component was subjected to paper chromatography in four solvent systems. The results of this chromatography are illustrated in Table 1. Each of the deacylated phospholipids gave the expected reactions for phosphate, amino nitrogen, choline, and vicinyl glycols.

Analysis of the deacylated phospholipids. The molar glycerol to phosphate ratios of the deacylated phospholipids used in the paper chromatography described above were GPE, 0.92:1.00; GPG, 1.98:1.00; αGP, 0.98:1.00; GPS, 1.05:1.00; and GPGPG, 1.56:1.00. Insufficient material was available for the analysis of GPC.

Enzymatic assays for αGP. The enzymatic assay of Wichert (30) for L-α-glycerol phosphate was used with the fraction that had the chromatographic and analytical characteristics of αGP. In the sample of αGP, 96.5% of the αGP determined by phosphate analysis can be accounted for by the enzymatic assay.

TABLE 1. *Paper chromatography of the deacylated phospholipids isolated by column chromatography from Haemophilus parainfluenzae*

Lipid[a]	R_F values			
	Solvent 1	Solvent 2	Solvent 3	Solvent 4
GPC	0.91	0.35	0.97	0.55
GPE	0.59	0.17	0.97	0.48
GPG	0.47	0.24	0.38	0.48
αGP	0.23	0.33	0.32	0.05
GPS	0.33	0.15	0.62	0.23
GPGPG	0.10	0.08	0.08	0.12

[a] Deacylated phospholipids were separated by column chromatography similar to that illustrated in Fig. 2. Each component was pooled and desalted as described in the text. Solvent 1 was water-saturated phenol adjusted to *p*H 5.5 (27); solvent 2 was butan-1-ol, acetic acid, and water (5:3:1, v/v) (27). These solvents were used with Schleicher and Schuell (no. 589) acid-washed paper. Solvent 3 was 3 M formic acid containing 0.4% pyridine (v/v); solvent 4 was Wawszkiewicz solvent (17) diluted 3 to 7 with 95% ethyl alcohol containing 0.26 M ammonia. The paper was Whatman AE-81 amino cellulose paper treated with 3 M formic acid. Each deacylated lipid was both compared and co-chromatographed with the corresponding deacylated authentic lipid by one-dimensional ascending chromatography. The deacylated lipids gave the expected reactions for amino-nitrogen with ninhydrin, phosphate with acid molybdate, and vicinyl glycol with periodate and *o*-tolidine using techniques described previously (27). The GPC reacted with Dragendorf's reagent (16).

Analysis of the nitrogen-containing phospholipids. The GPE fraction from the preparative Dowex-1 columns was hydrolyzed in 3 M HCl for 3 hr at 100 C. The hydrolysate was neutralized with solid $NaCO_3$, extracted with tertiary butanol, and subjected to gas-liquid chromatography as described (10). The molar ratio of ethanolamine to glycerol with the ethanolamine determined from gas-liquid chromatography was 1.02:1.00. The ethanolamine had a relative retention volume of 1.8 compared to an internal standard of cyclohexylamine.

In a total lipid mixture that was deacylated, acid-hydrolyzed, and analyzed by gas-liquid chromatography, monomethylethanolamine was determined to be 0.35% of the total lipid phosphate (10). The monomethylethanolamine isolated from the lipid had retention volume identical to that of an authentic sample of monomethylethanolamine.

A sample of *Haemophilus* lipid was partitioned twice against 4 M KCl at *p*H 2.0, chromatographed on a preparative thin-layer plate, eluted from the silica gel, and treated with Sephadex (19). This procedure was necessary to remove a peptide contaminant from the lipid. No amino acid methyl esters could be detected in the aqueous phase after mild alkaline methanolysis. Therefore, *o*-lipoyl esters of amino acids found in gram-positive bacteria by Macfarlane (11) were not present in *Haemophilus*. After removal of the peptide contaminant, the lipid was deacylated and hydrolyzed in 2 M HCl under nitrogen for 3 hr at 115 C. The amino nitrogen-containing components in the hydrolysate were separated and assayed with a Technicon amino acid analyzer. Two major components were detected. One had an R_F value identical to that of ethanolamine, and the second had an R_F value identical to that of serine. The ratio of GPE to GPS, calculated from the phosphate after separation of the glycerol phosphate esters by chromatography on Dowex-1, equaled the ratio of ethanolamine to serine measured after acid hydrolysis of the lipid and separation with the amino acid analyzer. About 99.2% of the ethanolamine of GPE was recovered, and about 94% of the GPS was recovered.. The remaining 0.03% of the total amino nitrogen-reacting material was glycine, which may have come from the serine during hydrolysis.

Presence of GPC. Phosphatidylcholine has been reported to be restricted to bacteria with extensive internal membrane systems (4). *Haemophilus* does not show extensive internal membranes, yet it contains traces of a lipid which

yields GPC upon deacylation. Glycerol phosphate esters containing ^{32}P were eluted from a Dowex-1 column with 20 mM ammonium formate (*p*H 9.0) (Fig. 3). The fractions containing the glycerol phosphate ester with the elution volume of GPC were pooled and the ammonium formate was removed by evaporation in vacuo. The sample was co-chromatographed with authentic GPC and separated from GPE (Fig. 4). The sample gave reactions characteristic of choline-containing lipids with phosphomolybdate after ninhydrin (15), Dragendorf's reagent (16), and hydrated cobalt-amine complex (9). *H. parainfluenzae* incorporated ^{32}P from the growth medium into a lipid characterized as containing a quaternary amine that has the chromatographic properties of GPC after deacylation. As yet, it has not been possible to isolate and identify choline as the sole nitrogen-containing component of acid-hydrolized GPC. Consequently, the designation of this deacylated lipid as GPC must be considered tentative.

Separation of the lipids by thin-layer chromatography. Phospholipids isolated from *H. parainfluenzae* can be separated by two-dimensional chromatography with silica gel G thin-layer plates. A radioautogram of the chromatographed lipids is illustrated in Fig. 5. Each of the separated lipids

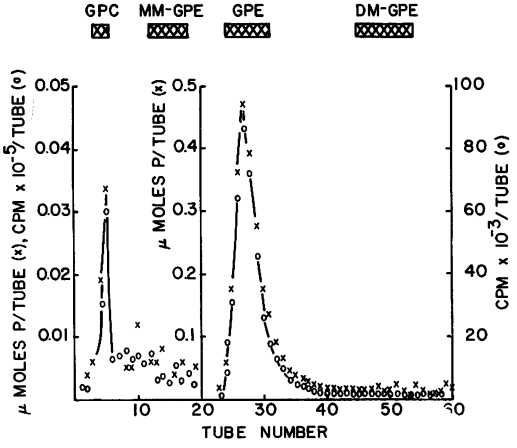

FIG. 3. *Chromatographic analysis of the ethanolamine deacylated phospholipids of H. parainfluenzae. A column like that described in Fig. 2 was loaded with deacylated phospholipids. The deacylated phospholipids were then eluted with 0.02 M ammonium formate, (pH 9.0) and 2.40-ml fractions were collected. The fractions were assayed as in Fig. 2, and 83.2% of the added phosphate was recovered. The GPG contaminated with αGP can be eluted after about 170 ml of ammonium formate has been used. The GPS and GPGPG can be eluted only if the pH of the eluting buffer is raised. The elution patterns of authentic deacylated lipids are illustrated at the top of the figure.*

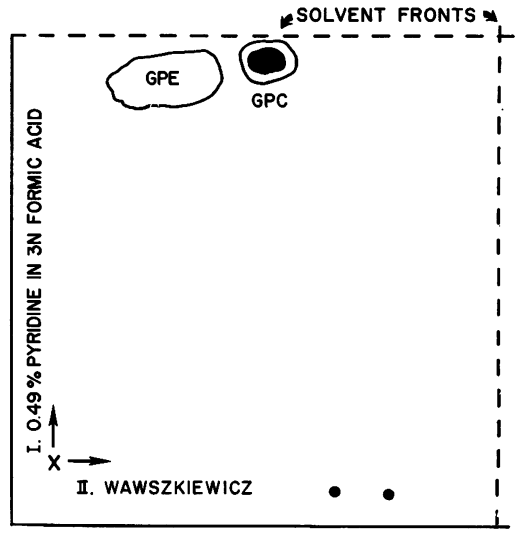

FIG. 4. *Two-dimensional chromatography of GPC-^{32}P on amino cellulose paper. The presumed GPC fraction of H. parainfluenzae grown in the presence of ^{32}P was collected from columns like those illustrated in Fig. 3. The ammonium formate was removed by successive evaporations in a vacuum oven; the material was combined with unlabeled GPE and GPC and subjected to chromatography on amino cellulose paper as described in Table 1. After chromatography, the paper was placed on Kodak no-screen X-ray film for 2 days. The paper was then treated with periodate followed by o-tolidine (27) to detect vicinyl glycols, and the positions of the unlabeled GPE and GPC were marked. The radioautogram was then placed over a tracing of the chromatogram and a picture was taken.*

was eluted from the silica gel as previously described (27), and was subjected to mild alkaline methanolysis. The resulting water-soluble glycerol phosphate esters were identified by paper chromatography with water-saturated phenol solvent and by two-dimensional chromatography on amino cellulose paper. In addition, paper chromatography in the Wawszkiewicz solvent, modified to contain ammonium carbonate, was used to separate GPE, GPMME, and GPDME. Since the specific radioactivity of the three major lipids was equal, the radioactivity was used to calculate the proportions of phosphate in each fraction (Table 2). The analyzed proportions of the major classes of phospholipids, after separation by thin-layer chromatography, were the same with glycerol phosphate esters determined after deacylation and paper chromatography (Tables 2 and 3).

Three classes of phosphatidylethanolamines, as well as two classes each of phosphatidylglycerol and phosphatidylserine, are separable by thin-layer chromatography. The phosphatidylmono-

methylethanolamine is found with the major fraction of phosphatidylethanolamine. The lipid used for the analysis shown in Table 2 and in Fig. 5 contained a somewhat larger proportion of phosphate and fatty acids at the origin. Usually the origin accounts for less than 0.01% of the lipid phosphate.

One obvious reason for the separable types of phosphatidylethanolamines, phosphatidylserines, and phosphatidylglycerols would be that the minor components were lyso-phospholipids. Determination of the fatty acid to phosphate ratio by three methods rules out lyso-phospholipids.

The mild alkaline methanolysis results in the quantitative liberation of fatty acid methyl esters. With $C_{20:1}$ methyl ester as an internal standard, a summation of the peak areas of all responses of the fatty esters detected after gas-liquid chromatography indicates a fatty acid to phosphate ratio of 2:1 (Table 2).

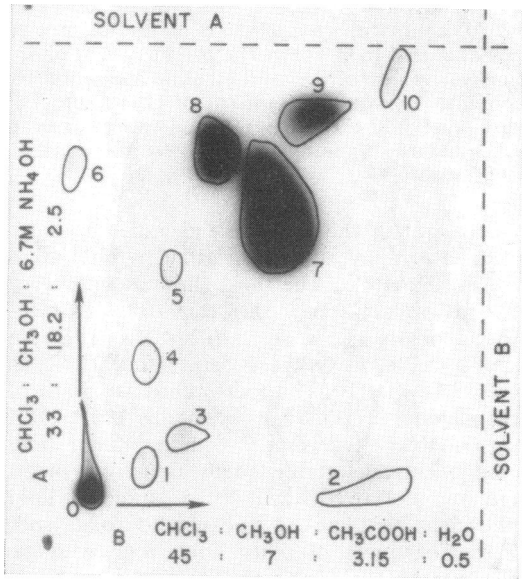

FIG. 5. *Thin-layer chromatography of intact phospholipids of H. parainfluenzae. Silica gel G thin-layer plates were prepared (27), and less than 0.5 μmole of lipid was spotted in the lower left hand corner. The plate was then chromatographed in two dimensions with the solvents indicated in the figure. The plate was dried in the vacuum oven, wrapped in Saran wrap, and placed in contact with X-ray film. After 24 hr, the film was developed and the lipids were recovered from the plate (27). Radioactivity was determined after deacylation by spotting the glycerol derivatives on paper discs and counting the paper discs in the toluene scintillation fluid. The proportion of the total lipid phosphate in each lipid is given in Table 2. The recovery of ^{32}P from the plate was 97%.*

Cabbage chloroplast phospholipase D, called phospholipase C by Kates (7), is specific for diacyl phospholipids. Incubation of the phospholipids with phospholipase D resulted in production of phosphatidic acid (measured as αGP in Table 4) from all the lipids but cardiolipin. Authentic cardiolipin from *S. aureus* was not attacked by phospholipase D.

Collection of larger amounts of the trace lipids allowed colorimetric analysis of the fatty acids and phosphate. The lipids were first fractionated by one-dimensional thin-layer chromatography as described in Materials and Methods. Then each fraction was chromatographed in the usual two-dimensional system. Spots corresponding to the same position after two-dimensional chromatography were pooled and 10% of the sample was used to confirm the identification by paper chromatography of the deacylated phospholipid. Only one glycerol phosphate ester was found in each spot. Spots 1 and 3 in Fig. 5 corresponded to a single phosphatidylserine, and the origin contained less fatty acid than was found in Fig. 5. The remainder of the lipid (90%) was saponified in 3 M KOH in 47% ethyl alcohol at 100 C for 4 hr. The saponification mixture was acidified to *p*H 2.0 with HCl, and the fatty acids were extracted with three portions of petroleum ether and analyzed by the Novák procedure (13). The aqueous portion of the saponification mixture was acidified further and the phosphate was determined colorimetrically (27). Both the phosphate and the fatty acid assays are accurate to 0.01 μmole per sample. At least 0.02 μmole of phosphate was present in each sample. The fatty acid to phosphate ratio of the phospholipids isolated by two-dimensional thin-layer chromatography was 2:1 (Table 5).

Evidence from the following experiments strongly suggests that hydrolytic breakdown is not the source of the minor lipids. The minor lipid components apparently are not generated by the thin-layer chromatography. A total phospholipid sample was applied to two thin-layer plates. One was subjected immediately to chromatography and the other was kept at room temperature for 72 hr before being placed in the chromatography solvents. Radioautograms from the two plates were essentially identical. If a slightly overloaded plate (0.7 μmole of lipid phosphate) is chromatographed and the major lipid (spot 7) is eluted and chromatographed repeatedly, there is progressive purification of the phosphatidyl ethanolamine. After the second chromatography, traces of GPGPG, GPG, αGP, and GPC in the appropriate positions can be

TABLE 2. *Separation of the phospholipids of Haemophilus parainfluenzae by thin-layer chromatography*

Spot[a]	Counts/min	Percentage of phosphate	Components							
			GPC	GPE	GPMME	GPG	αGP	GPS	GPGPG	Ratio of fatty acid to PO_4
0	457	0.04		50.5		39.3			10.2	5.6:1
1	711	0.06						100		2.1:1
2	2,073	0.19				3.4	94.2		2.4	2.0:1
3	3,170	0.29					2.0	98.0		1.6:1
4	664	0.06		100						1.9:1
5	495	0.04		100						2.3:1
6	1,145	0.10				100				1.6:1
7	886,500	77.60	0.7	98.4	0.7		0.1		0.1	1.9:1
8	169,300	15.52	0.1	0.4		98.7	0.5		0.1	1.8:1
9	56,000	5.77	0.5	1.3		2.8	3.0		92.4	1.7:1
10	3,142	0.29	67.6			17.4				2.2:1

[a] Spots were removed from four plates like those illustrated in Fig. 5 and deacylated by mild alkaline methanolysis (27). The water phase from the methanolysis was subjected to chromatography in four solvent systems as described in the text. The glycerol phosphate esters were detected by radioautography and assayed by counting the paper disc in the toluene scintillation fluid. Phosphate was then determined after digestion of the paper in perchloric acid. The specific activity of the deacylated lipid from spot 7 was 366,300 counts/min per μmole of P; of spot 8, 349,800 counts/min per μmole of P; and of spot 9, 311,500 counts/min per μmole of P. These were averaged and used to calculate the proportion of phosphate in the minor lipids from the radioactivity. The organic phase of the mild alkaline methanolysis was used to determine the fatty acid methyl ester content. An internal standard of $C_{20:1}$ (1 nmole/μliter) was added and the methyl esters were subjected to gas-liquid chromatography. The areas of the responses were calculated and compared to the internal standard. Remethylation (26) established that all the fatty acids released from the methanolysis were methyl esters.

TABLE 3. *Comparison of the proportions of the total lipids of Haemophilus parainfluenzae determined by thin-layer chromatography of the intact lipids and by chromatography of the deacylated lipids on amino cellulose paper*

Lipid[a]	Thin-layer chromatography (intact lipids)	Paper chromatography (deacylated lipids)
GPE	79.7 (0.2-GPC)	78.6
GPG	17.5	18.2
GPGPG	2.1	2.3
αGP	0.3	0.4
GPS	0.3	0.5

[a] A total of 1.899 μmoles of lipid phosphate was subjected to thin-layer chromatography (Fig. 5) on four plates. The lipids were detected by radioautography and recovered, deacylated, and identified by paper chromatography (Table 1). The total phosphate in each lipid was then calculated. The recovery of phosphate for the entire procedure was 89.2%. A second portion of the lipid was deacylated and separated with amino cellulose paper. The lipids were located by radioautography and cut out. The radioactivity was determined and the papers were digested for phosphate analysis. The recovery of phosphate was 101%.

demonstrated. The lipids were identified by paper chromatography after deacylation by mild alkaline methanolysis. The third chromatography of the material in spot 7 showed 99.35% as GPE with mobility like spot 7, 10.24% as GPE like spot 4, 0.20% as GPE like spot 5, and 0.17% as GPC like spot 10. The fourth chromatogram of the material in spot 7 was essentially 100% GPE with mobility like spot 7 with no minor lipid components detectable. With thin-layer plates that are not overloaded (<0.5 μmole of lipid phosphate per plate), it is possible to isolate spot 7 free of traces of other lipids after a single chromatography (Table 5). The minor lipids are not generated by the separation procedures

Apparently the minor lipid classes exist in *H. parainfluenzae*, and their separability can be explained by the differences in proportions of the fatty acids. The relative proportion of each fatty acid in each type of phospholipid separated by thin-layer chromatography is given in Table 6. There appear to be three classes of lipids. The trace lipids contain less than 0.3% of the total lipid phosphate; a high proportion (10 to 50%) of C_{19}, C_{20}, and C_{22}; relatively less C_{16} and far less $C_{16:1}$ (0.6 to 3%); more $C_{14:1}$ than C_{14}; and higher proportions of either C_{12}, $C_{12:1}$, C_{13}, or $C_{14:1}$. The

TABLE 4. *Hydrolysis of Haemophilis phospholipids by phospholipase D*[a]

Spot	Component	Percentage of αGP after enzymatic hydrolysis
0	GPE, GPG	67
2	αGP	105
3	GPS	90
4	GPE	100
5	GPE	110
6	GPG	72
7	GPE	32
8	GPG	70
9	GPGPG	0
10	GPC	22

[a] Phospholipids labeled with ^{32}P were separated on eight thin-layer plates (Fig. 5). The lipids were located by radioautography and recovered. One-third of the sample was deacylated and the water-soluble glycerol derivatives were identified by paper chromatography with a water-saturated phenol solvent. The remainder of the lipid was treated with cabbage phospholipase D in acetate buffer in the presence of ether (7) for 4 hr at room temperature. The lipid was then deacylated and subjected to paper chromatography. The deacylated phospholipids were detected by radioautography, cut out, and the radioactivity was determined in the scintillation spectrometer. The material in spot 7 amounted to 0.16 μmole of lipid phosphate. When this material was treated with the enzyme at 100-fold dilution, the hydrolysis was complete in 4 hr.

TABLE 5. *Fatty acid to phosphate ratios of phospholipids of Haemophilus parainfluenzae separated by thin-layer chromatography*[a]

Spot	Lipid	Ratio of fatty acid to phosphate
0	Mixed	1.47:1.00
1 + 3	GPS	1.69:1.00
2	αGP	1.99:1.00
4	GPE	1.99:1.00
5	GPE	2.07:1.00
6	GPG	2.02:1.00
7	GPE	1.98:1.00
8	GPG	2.03:1.00
9	GPGPG	1.83:1.00
10	GPC	1.84:1.00

[a] Lipids containing ^{32}P were extracted and fractionated by thin-layer chromatography into four fractions in the chloroform–methanol–6.7 M ammonium hydroxide solvent, as described in the text. Each fraction was then divided into several portions and chromatographed on thin-layer plates in the two-dimensional system illustrated in Fig. 5. Lipids with similar positions in this chromatography were pooled and a portion of each was deacylated by mild alkaline methanolysis. The deacylated derivatives were then identified by paper chromatography with the phenol-water solvent (Table 1). Each pooled fraction gave rise to a single type of glycerylphosphoryl derivative. The remaining lipid from the thin-layer plates was saponified; the fatty acids were extracted; the residue was digested; and the fatty acid to phosphate ratio was determined by colorimetric analysis as described in the text. Spots were numbered as in Fig. 5 according to their position after the two-dimensional chromatography. The designation was confirmed by identification of the deacylated lipid.

major lipids are phosphatidyl ethanolamine (spot 7) and phosphatidyl glycerol (spot 8). These two lipids account for 93.12% of the total lipid phosphate. The C_{16} and $C_{16:1}$ comprise 80% of the total fatty acids, and the short and very long fatty acids comprise less than 5% of the total fatty acids. The cardiolipin (spot 9) seems from its fatty acid composition to be a mixture of the two lipid classes. Cardiolipin has high proportions of C_{16} and $C_{16:1}$ as well as high proportions of the long fatty acids.

DISCUSSION

The membrane of *H. parainfluenzae* contains diacyl phospholipids as the major components of the extractable lipids. These lipids are present at concentrations of 60 to 70 μmoles of phosphate per g of bacterial dry weight. No lipo amino acids, plasmalogens, alkyl ethers, glycolipids or lysophospholipids are present. A trace of phosphatidylmonomethylethanolamine and a lipid very much like phosphatidylcholine are the major differences between the lipids of *H. parainfluenzae* and *E. coli* (5).

The striking feature of the phospholipids of *H. parainfluenzae* is that 11 phospholipids can be separated into three classes of lipids that differ in fatty acid composition. The trace lipids, which together comprise less than 0.3% of the total lipid phosphate, have large proportions of the long and short fatty acids with very little $C_{16:1}$. In the major lipids, which comprise 93% of the lipid phosphate, 80% of the fatty acids are $C_{16:1}$ and C_{16}. The short and long fatty acids comprise a small proportion of the fatty acids of the major lipids. The cardiolipin appears to have features of both classes of lipids in having both $C_{16:1}$ and the short-chain fatty acids in significant proportions. The proportions of phosphate among the 11 phospholipids are quite reproducible and the

TABLE 6. *Fatty acid composition of the phospholipids of Haemophilus parainfluenzae separated by thin-layer chromatography*

Fatty acid[a] methyl esters	Percentage of the total area of the recorder response for each fatty acid ester in the 11 separated phospholipids										
	0	1	2	3	4	5	6	7	8	9	10
12:1	0.54		0.22	0.45	0.32	0.40	0.17	0.03	0.03	0.33	
12	0.36	0.07	0.31	3.25	0.67	0.63	0.16	0.38	0.26	0.19	0.27
13:1	9.80	0.75	14.84	0.04	1.09	2.77	11.18	0.05	0.09	0.96	0.10
13:Br	0.36		0.11	0.16			0.11	0.02			
13	0.36	0.13	0.39	0.12	0.13	0.23	0.03	0.01	0.02	0.04	0.10
13:C	1.81	0.20	3.93	0.02	0.18	0.03	0.12	0.01	0.01	0.03	0.08
14:1	4.36	3.52	1.18	8.44	22.73	5.04	2.95	0.56	0.55	2.13	0.88
14:Br	0.73		1.23	0.45		0.13	0.06				0.06
14	4.36	1.63	0.22	2.28	1.15	1.58	0.93	9.85	1.62	1.91	1.74
14:C	0.73	0.29	0.22	0.02	0.15	0.26	0.09	0.01	0.03	0.02	0.14
15:1	2.72	1.14	4.21	0.18	0.37	0.46	0.92	0.09	0.14	2.76	1.06
15:Br	0.36		1.18	0.89	0.04	1.29		0.46	0.01	0.57	0.66
15	1.45	1.82	0.34	0.09	0.53	0.07	0.56	0.01	0.35	0.06	1.06
15:C		0.26	0.09	0.09	0.07	0.15	0.12	0.01	0.01	0.06	1.06
16:1	8.17	2.93	2.67	2.90	1.32	2.90	1.06	41.32	38.12	22.93	0.67
16:Br	1.09	0.49	0.34		0.04		0.23				0.26
16	25.41	26.34	18.38	17.98	7.74	13.85	8.77	39.93	41.85	31.97	44.23
16:C		0.78	0.17	0.27	0.18	0.26	1.01	0.13	0.14	0.12	0.43
17:1	13.61	3.91	2.47	2.28	0.95	2.24	30.28	0.07	0.31	0.89	2.81
17:Br	0.91	4.69	1.26	2.61	0.70	3.43	1.63	0.04	0.23	1.08	2.03
17	0.36	2.28	0.84	0.86	0.44	1.19	2.17	0.09	0.32	0.40	1.60
17:C	1.45	1.95					2.42	0.42	0.30	0.62	0.03
18:1	0.73	0.52	2.36	0.43	1.06		0.62	1.56	2.29	3.17	2.20
18:Br			0.22	0.09	0.12		0.19	0.05	0.05		0.82
18		8.99	4.52	4.35	33.04	5.34	5.81	2.37	3.60	4.70	6.06
18:C											
19:1		13.25		0.61	3.64		9.91	0.01	1.52		0.67
19:Br				16.16				0.57		5.98	4.88
19	0.91	0.46					0.22	0.07	0.36	9.74	3.84
19:C									0.06		
20:Br				0.27	3.97		0.65			1.62	0.20
20	9.26	4.07	0.56	0.33			2.80	0.05	4.82	1.21	0.58
20:C											1.27
21:1				32.19							2.50
21											
22	9.26	19.54	20.74	10.10	19.38	49.46	15.50	1.12	2.96	6.56	18.86

[a] Fatty acids methyl esters were taken from the organic phase of the mild alkaline methanolysis of the lipids isolated after chromatography illustrated in Table 2. The volume was reduced in a stream of nitrogen, and the methyl esters were subjected to gas-liquid chromatography as described previously (26). The fatty acid methyl esters were identified by their retention times on polar and nonpolar columns before and after hydrogenation (26). The numbers represent the percentage of the total areas of the recorder response for each fatty acid ester taken after separation with a SE-30 column. The molar response of the hydrogen flame detector to the internal standard $C_{20:1}$ methyl ester did not differ significantly from that expected for the saturated and monounsaturated methyl esters of C_{12}, C_{14}, C_{16}, C_{18}, and C_{20} determined with known mixtures. Fatty acid methyl esters are designated as the number of carbon atoms, with ":1" for monounsaturated, ":Br" for branched, and ":C" for cyclopropane.

trace components are neither lyso-phospholipids nor are they generated during thin-layer chromatography itself. The possibility that the trace lipids could represent different salt forms still does not change the fact that the fatty acid composition of the three lipid classes is different.

The interesting feature of the fatty acid differences is that precursor lipids such as phosphatidylserine, which is decarboxylated to phosphatidylethanolamine; phosphatidylglycerol, which is the precursor of cardiolipin; and phosphatidic acid, which is the precursor of all the phospholipids, have different fatty acid compositions from the end product lipids. The synthesis of phospho-

lipids, as illustrated with *E. coli* (6), does not consider different relative fatty acid compositions between precursor and end product lipids. Preliminary experiments indicate that phosphatidylethanolamine is synthesized by decarboxylation of phosphatidylserine in *H. parainfluenzae*, yet the fatty acid composition of the major phosphatidylethanolamine is different from the phosphatidylserine. Either there is remarkable fatty acid specificity in the enzymes that form the major phospholipids and rapid turnover of the trace precursor lipid with the acid composition of the major lipid class, or there is modification of the fatty acid composition after synthesis of the lipids. Preliminary experiments with *H. parainfluenzae* have not demonstrated a very rapid turnover or incorporation of radioactivity into phosphatidic acid or phosphatidylserine. It seems clear that the fatty acid compositions are modified after the synthesis of the phospholipids. It could be that the trace fraction of phosphatidylethanolamine and phosphatidylglycerol represents lipid where the fatty acid composition has not been modified significantly from that of the precursor phosphatidic acid.

Recently, the activity of fatty acyl transferase has been suggested by the differences in the fatty acid composition of the phosphatidylglycerol, lysyl-phosphatidylglycerol, cardiolipin, mono- and diglucosyldiglyceride in *S. aureus* (28). This type of transferase activity appears to be especially active during the lipid modifications that are coordinate with the formation of the membrane-bound electron transport system (28). Although the proportions of eight fatty acids in phosphatidylglycerol, phosphatidylethanolamine, and cardiolipin isolated from *E. coli* are similar, the level of $C_{17:C}$ is significantly higher in the phosphatidylethanolamine (5). The higher level of $C_{17:C}$ appears significant as the level of $C_{16:1}$ is not depressed. If the high $C_{17:C}$ represented a conversion of the $C_{16:1}$, the increase in $C_{17:C}$ would have been accompanied by a decrease in $C_{16:1}$. Significant differences in the fatty acid composition between isolated phosphatidylethanolamine and cardiolipin have been detected in several species of mycobacteria (14). In the photosynthetic bacterium *Rhodopseudomonas capsulata*, the fatty acid composition of the phosphatidylethanolamine and phosphatidylcholine were similar, but they differed remarkably from the fatty acid composition of the phosphatidylglycerol (31). In *Chlorella vulgaris*, isolated mono- and digalactosyldiglyceride, sulfoquinovosyldiglyceride, phosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and cardiolipin differ significantly in the proportions of 10 fatty acids and in the changes in proportions in these fatty acids between light- and dark- grown cells (12). Although the fatty acid composition of isolated lipids of only a few microbial species has been examined, the possibility that an active modification of the fatty acid composition of the complex lipids after synthesis appears to be fairly common and suggests that transferase activity might play an important role in the matabolism of bacterial complex lipids.

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