

Complex Lipids of *Rhodomicrobium vannielii*¹

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Received for publication 29 August 1966

ABSTRACT

Eight components, seven of which contained phosphorus, were found in the phospholipid fraction of *Rhodomicrobium vannielii*. The major components were lipo-amino acid (*o*-ornithine ester of phosphatidyl glycerol, 46.5%) and phosphatidyl choline (26.5%). The other six components were phosphatidyl glycerol (9.7%), bis-phosphatidic acid (6.7%), phosphatidyl ethanolamine (4.5%), phosphatidic acid (1.8%), lysophosphatidyl glycerol-*o*-ornithine ester (3.2%), and *N,N*-ornithine amide of unidentified fatty acid (0.95%). Total phospholipid accounted for 4.2% of cell dry weight. The major fatty acid was vaccenic acid, C_{18:1}, which accounted for approximately 90% of the total fatty acids of the complex lipid fraction. The other four fatty acids were C_{16:0} (6.25%), C_{18:0} (3.8%), C_{14:0} (0.7%), and C_{18:1} (0.35%). The sulfolipid content was 0.01% of the cell dry weight or 0.14 μmoles per g of dried cells, assuming that its fatty acid component is vaccenic acid. No steroids were detected.

Until a few years ago, the lipids in photosynthetic bacteria were all but neglected. James and Nichols (25) compared the fatty acids and phospholipid components of photosynthetic bacteria, marine algae, yellow and blue-green algae, and green algae. Lascelles and Szilagy (31) studied phospholipid synthesis in relation to the formation of photosynthetic pigments in *Rhodospseudomonas spheroides*. Among the lipid components, these authors found phosphatidyl choline, phosphatidic acid, phosphatidyl glycerol, and phosphatidyl ethanolamine. The lipid and fatty acid metabolism in *Rhodospirillum rubrum* and four species of *Rhodospseudomonas* were studied by Wood, Nichols, and James (48). They found phosphatidyl choline, cardiolipin, phosphatidyl glycerol, phosphatidyl ethanolamine, and an unknown compound predicted to be an *o*-ornithine ester of phosphatidyl glycerol. Ornithine-containing lipoamino acid was reported by Gorchein (15) in *R. spheroides*.

This paper reports studies on the nature of the phospholipids and amount of sulfolipid in *Rhodomicrobium vannielii*. A later report will deal with other lipid materials in this organism.

¹ This work constitutes part of a thesis submitted by Chong-Eel Park in fulfillment of the requirements for the Ph.D. degree.

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MATERIALS AND METHODS

Growth of bacteria. *Rhodomicrobium vannielii* was isolated from mud near the University of Hawaii and was cultivated in the following medium (all percentages represent grams per 100 ml of solution): 0.1% (NH₄)₂SO₄, 0.05% K₂HPO₄, 0.01% MgSO₄·7H₂O, 0.5% lactic acid, 0.1% yeast extract (Difco), and 0.001% phenol-red indicator. After steam sterilization at 121 C, 0.01% Na₂S was added, and the medium was neutralized with a sterile solution of NaHCO₃.

The cultures were grown anaerobically at 29 C. Cells were harvested from 10-liter bottles near the end of logarithmic growth (usually on the 6th day) with a Sharples Super centrifuge, washed three times, and immediately frozen. When a sufficient quantity of frozen cells was obtained, it was lyophilized.

Sulfur-limited medium contained 0.1% NH₄Cl, 0.05% K₂HPO₄, 0.01% MgCl₂, 0.5% (v/v) lactic acid, 0.01% yeast extract, 0.001% phenol red, and 5 mc of carrier-free Na₂S³⁵O₄ per 10-liter culture. The solution was neutralized with sterile NaHCO₃ and inoculated. A few days after inoculation, 2.5 g of (NH₄)₂SO₄ was added to increase the total mass of cells.

Solvent-soluble materials. A 50-g amount of lyophilized cells was extracted by the method of Huston and Albro (22). All procedures were carried out in dim light; tubes and flasks were wrapped in aluminum foil. Extractions were done in 1.5-liter flasks by shaking at room temperature with 600-ml portions of acetone (extract 1) for 1.5 hr; this was followed by extraction three times (2 hr each time) with CHCl₃-CH₂OH, 2:1, v/v (extract 2), and twice (2 hr each time) with CHCl₃-CH₂OH, 1:1, v/v (extract 3; see Fig. 1). Extracts 1 through 3 were combined and

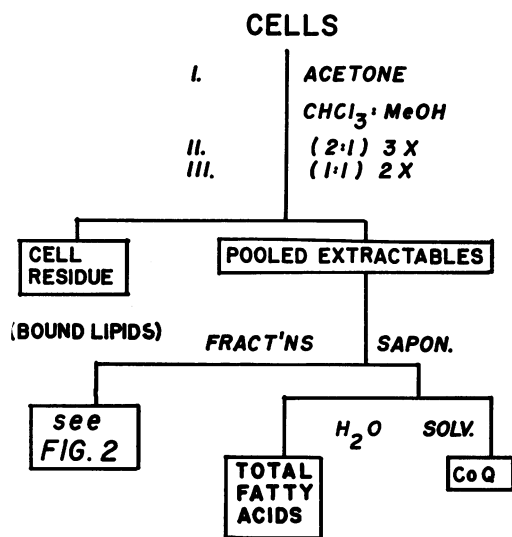


FIG. 1. Flow diagram for the extraction of lipids from lyophilized cells. Bound lipids and coenzyme Q (CoQ) will be characterized in a later report.

evaporated to dryness in vacuo; the residue was then taken up in CHCl_3 — CH_3OH (2:1, v/v). This solution was washed according to the method of Folch et al. (12) to remove nonlipid contaminants. The bottom layer of the CHCl_3 portion was evaporated to dryness in vacuo and weighed.

Silicic acid column chromatography. The extractable materials were separated on columns of 100 mesh, heat-activated silicic acid according to the scheme in Fig. 2.

The lipid extract, containing not more than 500 μg of lipid phosphorus per g of silicic acid, was dissolved in 20 ml of chloroform and applied to column *a*. The simple lipids were eluted first with chloroform; complex lipids were eluted with the addition of chloroform-methanol (2:1, 1:1, and 1:4, v/v).

Complex lipids from column *a* were concentrated and divided into three portions. Fraction III-1 was prepared by fractionation through column *c* with an approximately linear gradient of methanol in chloroform (0 to 100% methanol). Forty 25-ml fractions were collected. Each of the 40 samples was analyzed for phosphorus. Fraction III-2 was refractionated through column *d*, developed first with chloroform-methanol (9:1, v/v) until the bacteriochlorophyll was completely eluted. In addition to the bacteriochlorophyll, the effluent contained some of the less polar phospholipids. The solution was evaporated in vacuo at 40 C, and 30 ml of acetone was added. After standing in the cold for 2 hr, a precipitate of phospholipids was separated from the acetone solution by centrifugation. This procedure was repeated three times, effecting removal of most of the bacteriochlorophyll. The phospholipids remaining on column *d* were then eluted with portions of chloroform-methanol (2:1, 1:1, 1:4, 0:1, v/v). These solutions and the acetone-treated phospholipids were combined and taken to

dryness in vacuo at 40 C. Fraction III-3 was prepared by removing bacteriochlorophyll and polar carotenoids by precipitation with acetone. The crude phospholipids were dissolved in chloroform and used for the analysis of individual phospholipids.

Sulfolipid was purified by combination of columns of Florisil (60 to 100 mesh), diethylaminoethyl (DEAE) cellulose, and silicic acid (100 mesh) according to the method of O'Brien and Benson (38). It was further purified by thin-layer chromatography on silica gel G according to the method of Davies et al. (8).

Thin-layer chromatography. Silica gel G and H plates (20 by 20 cm and 5 by 20 cm) were prepared by the method of Stahl (45) and of Mangold (34). The plates were activated at 110 C. Phospholipids were separated on single-dimension ascending plates on plain silica gel with chloroform-methanol-water, 65:25:4, v/v (18), and chloroform-methanol-acetic acid-water, 250:74:19:3, v/v (1). Complex lipids were also separated by two-dimensional development on silica gel G plates, 20 by 20 cm (1), in chloroform-methanol-acetic acid-water (250:74:19:3, v/v) for the first dimension and in chloroform-methanol-7 M NH_4OH (230:90:15, v/v), for the second dimension. After development of the plates, components were visualized with various specific reagents.

Chromatography with silicic acid-impregnated paper. Silicic acid-impregnated paper was prepared by the method of Marinetti (35) and Rouser et al. (41). Chromatograms were developed with diisobutyl ketone-acetic acid-water, 8:5:1, v/v (32). Visualization

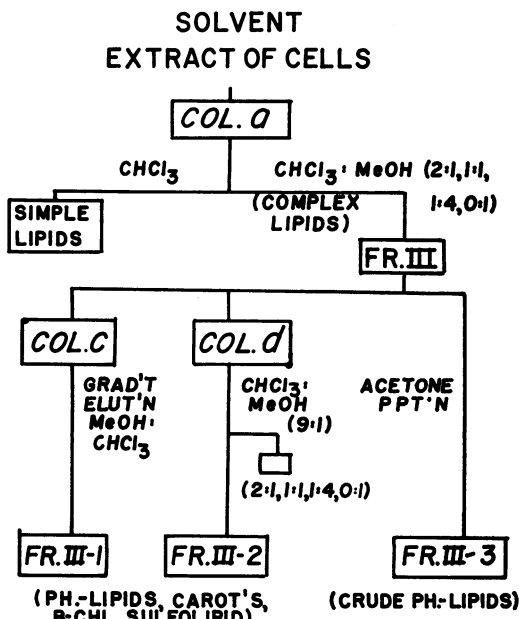


FIG. 2. Flow diagram for the fractionation of extractable lipids. The complex lipids were fractionated by three methods. Simple lipids will be characterized in a later report.

procedures were the same as with thin-layer chromatography.

Hydrolysis and paper chromatography. Paper chromatography was used for the identification of water-soluble products of hydrolysis.

Mild acid hydrolysis was done by the method of Kaneshiro and Marr (27) with the use of 0.2 N HCl in methanol at 60 C for 2 hr. Phospholipids were deacylated by several methods (10, 24, 31). The deacylated mixture was neutralized with formamide when acidic and with formic acid when alkaline. The fatty acids were separated from the water-soluble components by extraction with ether or chloroform. The aqueous phase, containing the deacylated phosphatides, was then freeze-dried in Thunberg tubes.

The water-soluble deacylated phosphatides were separated by ascending chromatography on Whatman no. 1 paper with phenol-water (100:38, v/v) and with *n*-butanol-acetic acid-water, 5:3:1, v/v (36). Two-dimensional separation, descending partition chromatography in the first dimension and ionophoresis in the second dimension, was also used for the deacylated phosphatides (9). The chromatograms were dried and sprayed, first with ninhydrin and later with a reagent for phosphorus (17).

Amino acids were characterized by ascending paper chromatography on Whatman no. 1 paper and were developed with as many as eight different solvent systems (43).

Paper disc chromatography. One per cent solutions of lipid samples were applied to Whatman no. 1 paper discs (5.5 cm) and developed by the method of Hack (16). When the paper was dry, it was cut into pieces and spot-tested as follows: for choline, the dipicrylamine test (4), Reinecke salt test (16), and phosphomolybdc-stannous chloride test (43); for vicinal hydroxy groups, a modified periodate-Schiff's test; for distinguishing various classes of lipids, Rhodamine 6G.

Gas-liquid chromatography. Fatty acid methyl esters and aldehydes were analyzed by gas-liquid chromatography. Columns (0.25 inch by 8 ft) of 30% diethylene glycol-succinic acid polyester (DEGS) on a support of 60/80 mesh fire-brick and columns (0.25 inch by 5 ft) of 10% Apiezon L (ApL) on Chromosorb P (42/62, respectively) were used for the separation of both esters and aldehydes; helium was the carrier gas. The effluent was monitored by thermoconductivity in an Aerograph chromatograph, model A-90-P2. Fatty acids were analyzed as their methyl esters at 170 to 180 C and fatty aldehydes at 130 C. Unknown fatty acids were tentatively identified from a curve plotting the logarithm of retention time versus carbon number, which was made with authentic compounds. For quantitative analysis, the area of the chromatograph under a peak for a given component was compared with that for a known amount of the same compound (18). For the isolation of individual esters, the desired methyl esters were trapped from the effluent gas in glass wool moistened with methanol.

Position of the double bond in mono-olefinic fatty acids was established by dihydroxylation, and the subsequent periodate oxidation of the dihydroxy acid

(21). The resulting fatty aldehyde, extracted in a suitable solvent, was dried with anhydrous Na₂SO₄, and concentrated for analysis by gas-liquid chromatography.

Methyl esterification of fatty acids was done by the method of Morrison and Smith (37) with the use of BF₃—CH₃OH reagent. The olefinic acids or their methyl esters were hydrogenated at room temperature for 2 hr in methanol with a catalyst of 5% Pt on charcoal under H₂ at 1 atm.

Quantitative analyses. Samples for phosphorus analysis were first digested by the method of Bartlett (3) and were then determined colorimetrically with the Fiske-SubbaRow reagent (11).

Total nitrogen was determined with Nessler's reagent after digestion of the sample (26). Amino nitrogen in unhydrolyzed phospholipid was determined by a modification of the procedure of Cocking and Yenn (47). L-Ornithine was used as standard after it was found to be the major amino acid in the lipids of *R. vannielii*.

Ester bonds were determined colorimetrically as ferric hydroxamates (40). Synthetic dipalmitoyllecithin was used as standard.

Lyso compounds were estimated by their hemolytic activity (2). Steroids were estimated colorimetrically (44), and sulfate was determined colorimetrically with barium chloranilate (5).

Infrared analysis. Samples dissolved in dried, redistilled reagent-grade chloroform were layered as a thin film on 0.5-inch (1.27-cm) KBr pellets. The chloroform was allowed to evaporate. Infrared spectra of various fractions and authentic compounds were made with a Beckman IR-5A recording infrared spectrophotometer.

S³⁵ activity in sulfolipid was determined with a windowless gas-flow counter. The S³⁵-sulfolipid spots on silicic acid-impregnated paper chromatograms and on thin-layer chromatograms were detected with a radio-chromatogram scanner.

RESULTS

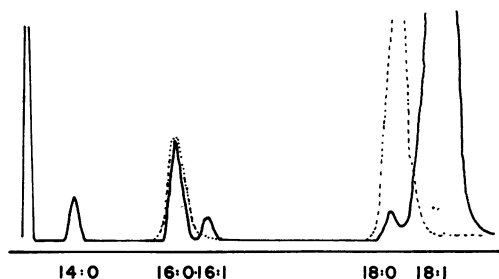
Fatty acid composition. A typical analysis of the fatty acids in the phospholipid fraction is shown in Table 1 and Fig. 3. The retention times of unsaturated compounds were compared before and after hydrogenation. The double bond position in the C_{18:1} component was established by performic dihydroxylation and periodate oxidation. The retention time of resulting free aldehyde was equal to that of standard *n*-heptanal by gas-liquid chromatography; the aldehyde was also characterized as a yellow crystalline 2,4-dinitrophenylhydrazone (melting point, 108 C). These data establish the position of the double bond between the 11th and the 12th carbon atoms and show the compound to be vaccenic acid. The position of the double bond of C_{16:1} was not determined because the amount was too small (0.35%). No branched acids and cyclopropane fatty acids were observed in the phospholipid fraction.

TABLE 1. Fatty acid composition of phospholipids^a

Compound	Per cent of complex lipids
C _{10:0}	—
C _{12:0}	—
C _{14:0}	0.70
C _{16:0}	6.25
C _{18:0}	3.80
C _{14:1}	—
C _{16:1}	0.35
C _{18:1}	89.0
C _{18:2}	—

^a Fatty acid composition was determined by gas-liquid chromatography using polar and non-polar columns (see Fig. 3). The amount of each component was estimated by planimetry in comparison with standard compounds (18). The conditions for gas-liquid chromatography are described in Fig. 3.

A. DEGS COLUMN



B. APL COLUMN

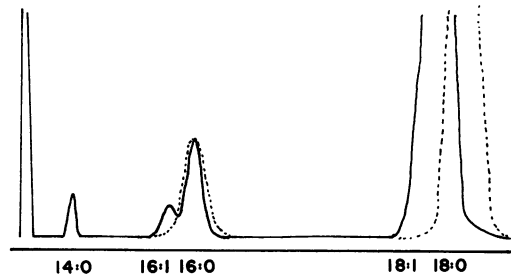


FIG. 3. Gas-liquid chromatography of the fatty acids from the complex lipids of *Rhodomicrobium*. Flow rate of carrier helium was 100 ml per min at an inlet pressure of 50 psi. A polar column and a nonpolar column, 0.25 inches by 5 ft, were charged with 30% diethylene glycol-succinic acid on firebrick or 10% Apiezon L on Chromosorb P and were maintained at 182 and 215 C, respectively. The solid lines show elution of the methyl esters of the fatty acids; the dashed lines show the elution of the esters after catalytic hydrogenation.

Gradient elution of the complex lipids on silicic acid columns with methanol-chloroform showed that more than two-thirds of the P-containing lipids was relatively weak polar material (Fig. 4).

The effluent fractions were concentrated and their contents were further resolved. Six spots were obtained from pooled material from tubes 1 to 24 (A, B, C-1, C-2, D-1, and D-2) on silica gel H plates; two spots (E-1 and E-2) were obtained from tubes 29 to 40 (Fig. 4). Except for component D-2, all contained phosphorus; C-1, C-2, D-2, and E-2 were ninhydrin-positive. Bacteriochlorophyll was confined to tubes 5 to 10. After freeing the material from carotenoids, the contents of tubes 33 to 35 were identified as phosphatidyl choline by strong absorption at 968 cm^{-1} and by positive tests with the Dragendorf and dipicrylamine reagents and by formation of a Reinecke salt complex.

Identification of individual phospholipids. With the use of fraction III-3, individual phospholipids were resolved by thin-layer chromatography. Five spots (A, B, C, D, and E) were stained with iodine vapors and the phosphorus test. Both spots D and E were shown to contain two distinct components with ninhydrin: ninhydrin-negative components D-1 and E-1 and ninhydrin-positive components D-2 and E-2 (Fig. 5). A two-dimensional thin-layer chromatogram of the phospholipid fraction is shown in Fig. 6. Spot C separated as a single component in the one-dimensional thin-layer chromatogram and showed two ninhydrin-positive spots when developed in two dimensions; one spot was small (C-1) and the other was large (C-2).

Rhodomicrobium vannielii, therefore, contains at least eight different compounds in the phospholipid fraction. The results of various tests for the identification of each compound are summa-

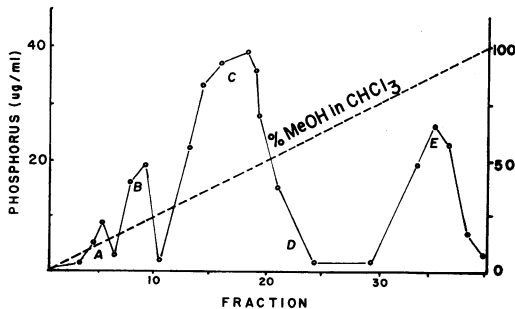


FIG. 4. Gradient fractionation of phospholipids was made through a silicic acid column with an approximately linear gradient of methanol in chloroform. Fractions (25-ml) were collected. The mixed components in the peaks were identified as: (A) phosphatidic acid, (B) bis-phosphatidic acid, (C) phosphatidyl ethanolamine and *o*-ornithine ester of phosphatidyl glycerol, (D) phosphatidyl glycerol and an unidentified fatty acid amide of ornithine, and (E) phosphatidyl choline and an *o*-ornithine ester of lysophosphatidyl glycerol.

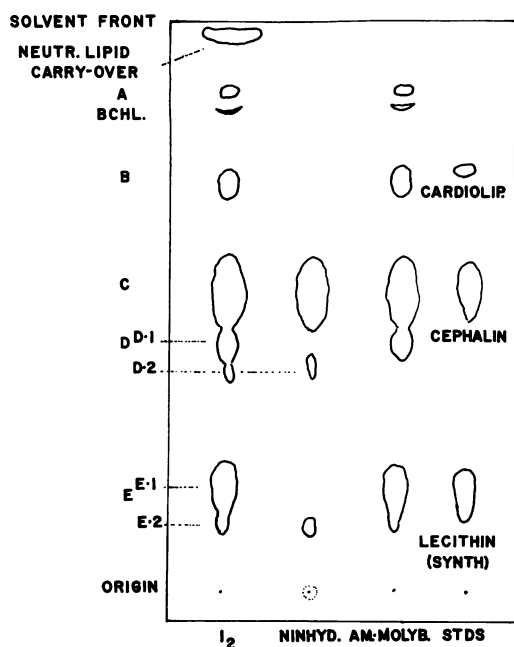


FIG. 5. Thin-layer chromatograms of phospholipids were made by one-dimensional development with CHCl_3 -methanol-acetic acid-water (250:74:19:3, v/v) on silica gel-H plates in an ascending manner at room temperature. After evaporation of the solvents, the spots were visualized with I_2 vapor for unsaturated lipids, ninhydrin for amino groups, and NH_4 -molybdate for phosphorus. The phospholipids were identified as: (A) phosphatidic acid, (B) bis-phosphatidic acid, (C) phosphatidyl ethanolamine and an *o*-ornithine ester of phosphatidyl glycerol, (D-1) phosphatidyl glycerol, (D-2) an unidentified fatty acid amide of ornithine, (E-1) phosphatidyl choline, and (E-2) an ornithine ester of lysophosphatidyl glycerol.

rized in Table 2. Based on these and other criteria the spots were identified as follows.

The component at the solvent front is *neutral lipid carryover*. This spot showed a strong yellow color in iodine vapor and a strong positive reaction for the ester bond, but no reaction with ninhydrin, ammonium molybdate, Dragendorff's reagent, or the Lieberman-Burchard reagent. Rhodamine 6G gave a yellow color to this spot under ultraviolet light.

Component A, phosphatidic acid. This component always appeared in the upper part of a greenish spot which resulted from some residual bacteriochlorophyll (Fig. 5). Its R_F value of 0.77 on silicic acid-impregnated paper is very close to that of 0.78 reported for phosphatidic acid (35). Quantitative analysis of this component gave an ester bond to phosphorus molar ratio of 1.97.

Component B, bis-phosphatidic acid. On the basis of data from thin-layer chromatography and column chromatographic properties, as well as

its reaction to various reagents, this spot could contain either a bis-phosphatidic acid or a cardiolipin-type of polyglycerol phosphatide. However, on silicic acid-impregnated paper R_F values of 0.56 to 0.68 were obtained. These values are virtually identical (0.57 to 0.67) with those reported for synthetic bis-phosphatidic acid (35) and for material from the spores of *Bacillus polymyxa* (36). Ammoniacal silver nitrate reduced this compound but did not reduce authentic cardiolipin.

Component C (C-1 and C-2), phosphatidyl ethanolamine and *o*-ornithine phosphatidyl glycerol ester. In one-dimensional thin-layer chromatography, C-1 and C-2 were unresolved from each other, but the small C-1 spot was partially separated from the large C-2 spot on the thin-layer chromatogram by two-dimensional development (see Fig. 6).

Since the R_F value of spot C was the same as

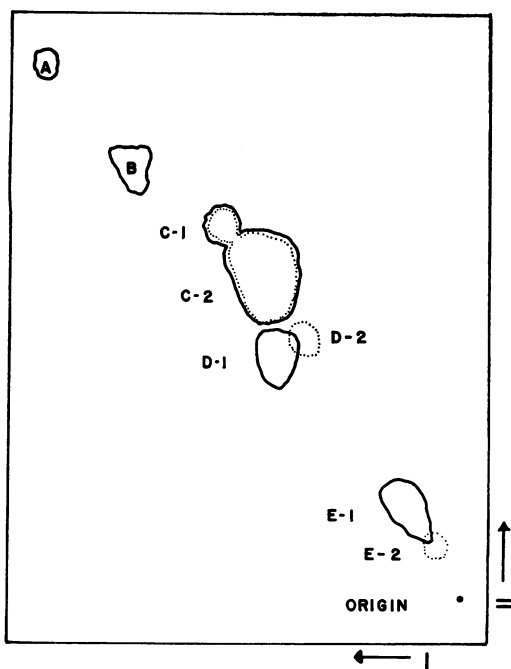


FIG. 6. Thin-layer chromatograms of phospholipids by two-dimensional development were done in CHCl_3 -methanol-acetic acid-water (250:74:19:3, v/v) for the first dimension and in CHCl_3 -methanol-7 M $\text{NH}_4\text{OH}_{\text{aq}}$ (230:90:15, v/v) for the second dimension on silica gel-G. The solid lines indicate phosphorus stained by NH_4 -molybdate reagent and the dotted lines show ninhydrin-positive compounds. The spots are identified as: (A) phosphatidic acid, (B) bis-phosphatidic acid, (C-1) phosphatidyl ethanolamine, (C-2) an *o*-ornithine ester of phosphatidyl glycerol, (D-1) phosphatidyl glycerol, (D-2) an unidentified fatty acid amide of ornithine, (E-1) phosphatidyl choline, and (E-2) an ornithine ester of lysophosphatidyl glycerol.

TABLE 2. *Characterization of phospholipids*

Spot	R_F^a	Per cent ^b	Rhodamine 6G ^c	Reinecke salt complex	Dragendorff	Dipicrylamine	Ninhydrin	NH ₄ molybdate	IR ^d	Hemolytic	Iodine vapor	Ester bond/P ^e	P/amino N/total N ^e
A	0.87	1.83	Blue	—	—	—	—	+	—	—	+	1.97:1	—
B	0.72	6.75	Blue	—	—	—	—	+	—	—	+	4:1	—
C-1	0.53	4.50	Yellow	—	—	—	+	+	—	—	+	1:1	1:1:1
C-2	0.53	46.5	Yellow	—	—	—	+	+	—	—	+	2.7:1	1:2:2
D-1	0.38	9.70	Blue	—	—	—	—	—	—	—	+	2.2:1	—
D-2	0.31	0.91	Yellow	—	—	—	+	—	—	—	+	—	—
E-1	0.22	26.5	Yellow	+	+	+	—	+	+	—	+	1.9:1	1.1:0:1
E-2	0.09	3.2	Blue	—	—	—	+	+	—	+	+	—	—

^a Thin-layer chromatography R_F values were measured on silica gel H plate (5 by 20 cm) developed with CHCl₃-methanol-acetic acid-water (250:74:19:3, v/v).

^b Based on vaccenic acid as the fatty acid component.

^c Rhodamine 6G tests were done on chromatograms of silicic acid-impregnated paper. The spots were observed under ultraviolet light.

^d Infrared spectrum at 968 cm⁻¹.

^e Molar ratio.

that for authentic phosphatidyl ethanolamine, it was at first erroneously identified as a single component, phosphatidyl ethanolamine (Park and Berger, *Bacteriol. Proc.*, p. 38, 1966).

On acid hydrolysis, however, the water-soluble products of this spot gave two ninhydrin-positive spots which were identified as ethanolamine and ornithine by paper chromatography. The relative amounts of ethanolamine and ornithine were estimated with a paper strip densitometer to be 8 and 92%, respectively. The molar ratios of amino N/P and ester bond/P were 2:1 and 2.7:1.

Component D-1, phosphatidyl glycerol. In addition to the tests shown in Table 2, the periodic acid-Schiff reagent gave a strong positive reaction. The R_F of 0.47 on silicic acid-impregnated paper is close to the reported R_F values of 0.45 (29, 36) and 0.49 (42). Quantitative analysis of this component showed an ester bond/P molar ratio of 2.2:1. On deacylation, the phosphoryl compound showed an R_F of 0.45 on the first dimension of a two-dimension paper chromatogram developed with water-saturated phenol-acetic acid-ethyl alcohol (100:10:2, v/v) and an R_{PI} of 0.64 on the second dimension by ionophoresis. These R_F values are comparable to the values reported by Dawson (9).

Compound D-2, an ornithine amide of an unidentified fatty acid. This compound was not completely separable from compound D-1 on the thin-layer chromatograms. Water-soluble products of the hydrolysis of this compound gave only one ninhydrin-positive spot, which was identified as ornithine on paper chromatograms developed in several solvent systems. This compound was not analyzed further, but it is believed to be similar to the fatty acid amide of ornithine (*N, N'*-diacyl

ornithine) reported by Gorchein (15) in *Rhodospseudomonas spheroides* and by Laneelle et al. (30) in *Mycobacterium phlei*.

Component E-1, phosphatidyl choline. Tubes 33 to 35 from the linear gradient fractionation of phospholipids on column C contained only one compound, which was found to be identical with the material in component E-1. The fractions from the tubes were combined and concentrated, and gave an infrared absorption spectrum typical of phosphatidyl choline, with a prominent band at 968 cm⁻¹, an ester absorption at 1,740 cm⁻¹, a p-o stretch at 1225 cm⁻¹, and a p-o-c stretch at 1,075 and 1,030 cm⁻¹. Rhodamine 6G gave a yellow spot at an R_F of 0.38 on silicic acid-impregnated paper [reported to be 0.37 by Marinetti (35)]. Other supporting data are summarized in Table 2.

Component E-2, o-ornithine ester of lysophosphatidyl glycerol. The spot (E-2) attached to the tail of spot E-1 was observed as a single spot in the reactions of iodine vapor and ammonium molybdate on thin-layer plates and on silicic acid-impregnated paper. However, only spot E-2 (not E-1) reacted with ninhydrin. E-2 hemolyzed human erythrocytes. It gave no reaction with periodic acid-Schiff reagent or alkaline silver nitrate. Acid hydrolysates of E-2 revealed two ninhydrin-positive spots having R_F values of 0.85 and 0.18 on paper chromatograms developed with water-saturated phenol solvent. However, in *n*-butanol-acetic acid-water (120:30:50, v/v), only one spot ($R_F = 0.16$) was found. The compound ($R_F = 0.18$) was identified as ornithine by comparing it with the authentic amino acid, with the use of paper chromatograms developed with several solvent systems. The material with an R_F

of 0.85 could not be identified positively with any of the eight solvent systems employed. It gave negative reactions with the Sakaguchi, Pauly, P-anisidine, nitroprusside, Elson-Morgan, benzidine, and dinitrososalicylic acid reagents. Under ultraviolet light it fluoresced slightly, but its R_F values differed slightly from tyrosine and tryptophan breakdown products. Most likely, it is a breakdown component of an amino acid which is unstable during acid hydrolysis and which is further modified in the presence of phenol.

Sulfolipid (6-sulfoquinovosyl diglyceride). Sulfolipids contained in the complex lipid fraction of cells grown in regular culture media were not detectable after using the purification procedure of O'Brien and Benson (38) and determination as sulfate with barium chloranilate because too little material was present. Cells were cultivated, therefore, with S^{35} -sulfate added to a sulfur-limited medium. After extraction and purification, the S^{35} -containing sulfolipid was separated by thin-layer chromatography (8) and found to have R_F values similar to those for unhydrolyzed sulfolipid. The sulfolipid content of *R. vannielii* was 0.01% of the cell dry weight or 0.14 μ mole per gram of dried cells, assuming that the glycerol is esterified with vaccenic acid. No steroids were detected in the extractable lipids.

DISCUSSION

The principal fatty acid in the complex lipids of *R. vannielii* is vaccenic acid, or 11-octadecenoic acid, and it forms approximately 90% of the fraction. This value is very close to that reported in *R. spheroides*, and *R. capsulata*, although cells were grown in different media (48). In bacteria, the $C_{18:1}$ fatty acid is generally vaccenic acid, rather than oleic acid.

The complex lipids include phospholipids (4.20% of cell dry weight) and sulfolipid (0.01%). The major phospholipid of *R. vannielii* is the lipoamino acid consisting of only one amino acid, ornithine. It is the *o*-ornithine ester of phosphatidyl glycerol and represents about 50% of the total phospholipids. The compound has the same R_F as phosphatidyl ethanolamine by thin-layer chromatography with single-dimension development. Ornithine-containing lipoamino acids in bacteria have been recently reported in *B. megaterium*, *B. cereus*, *Serratia marcescens*, and *Pseudomonas stutzeri* (20), in *Clostridium perfringens* (33), in *Rhodopseudomonas spheroides* (15), and in *Rhodospirillum rubrum*, *Rhodopseudomonas gelatinosa*, *R. capsulata*, and *R. palustris* (48). The amino acids reported in other bacterial lipoamino acids are all basic diamino acids such as lysine, ornithine, arginine. The phosphorus-free lipo-

amino acid, *N,N*-diacyl ornithine, in *R. vannielii* is probably similar to that in *Rhodopseudomonas spheroides* (15) and in *Mycobacterium phlei* (30). The role and biosynthetic pathways of these lipoamino acids are of considerable interest.

Phosphatidyl ethanolamine in *R. vannielii* is an unusually minor component, whereas it is a major phospholipid component in other organisms, e.g. *Serratia marcescens* (28), *Bacillus megaterium* (97% of phospholipids; 39), *Escherichia coli* (J. H. Law, Bacteriol. Proc., p. 129, 1961), *Azotobacter agilis* and *Agrobacterium tumefaciens* (27), and *R. spheroides* (40% of phospholipids; 31). It is, however, entirely absent in some organisms, such as *Sarcina lutea* (23), and in some lactic acid bacteria (24).

Cardiolipin in the photosynthetic bacteria was reported in *Rhodopseudomonas spheroides*, *R. palustris*, and *Rhodospirillum rubrum* (48). It is absent in *R. vannielii*, but bis-phosphatidic acid is present. The R_F values of these two components by thin-layer chromatography are quite similar. Bis-phosphatidic acid was reported as a phospholipid component in vegetative cells and spores of *Bacillus polymyxa* (36).

Among the heterotrophic bacteria, phosphatidyl choline had been reported in only one family, the *Agrobacteriaceae* [*A. tumefaciens* (27), *A. rhizogenes* and *A. radiobacter* (14)]. Phosphatidyl choline, however, was recently reported in *Rhodopseudomonas spheroides*, 22% (31, 48), and in *R. capsulata* and *R. palustris* (48). In *R. vannielii*, phosphatidyl choline is the second largest component of the phospholipids (26.5% of the total). Thus, photosynthetic bacteria may be partially characterized as organisms which synthesize an appreciable amount of phosphatidyl choline.

N-methyl ethanolamine and *N,N*-dimethyl ethanolamine were identified by transmethylation reaction in various bacteria grown in the presence of methyl-labeled methionine (13, 14). Goldfine and Ellis (13, 14) confirmed the proposed metabolic pathway for metabolism of phospholipids and methyl groups proposed by Bremer et al. (7) and for the stepwise conversion of phosphatidyl ethanolamine to phosphatidyl choline by transmethylation reaction involving *S*-adenosyl methionine (6). *N*-methyl ethanolamine and *N,N*-dimethyl ethanolamine were not detected in hydrolysis products of the phospholipids of *R. vannielii*. Confirmation should be made by use of isotopically labeled methionine or *S*-adenosyl methionine- $C^{14}H_3$ as methyl donors.

No steroids were detected in *R. vannielii*. No definitive report exists for the presence of steroids in bacteria. Stanier (46) suggested that sterols are involved in the structure of intracellular mem-

branes such as the nuclear membrane in eukaryotic organisms, and it may be significant that both sterols and such membranes are absent from the bacteria and blue-green algae, the prokaryotic organisms.

Sulfolipid is found in *R. vannielii*. On the basis that sulfolipid is concentrated in the quantasomes of the chloroplasts in plants, it is predicted that sulfolipid is associated with the photosynthetic process, although direct evidence is lacking and its function is unknown. For these reasons the metabolism of sulfolipid and its role in photosynthesis are topics for continued investigation.

ACKNOWLEDGMENTS

We are indebted to Howard Goldfine for the suggestion that we look for lipoamino acids in *Rhodospirillum rubrum*.

This investigation was supported by Public Health Service grant AI-05907 from the National Institute of Allergy and Infectious Diseases.

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