

This dissertation has been  
microfilmed exactly as received 67-13,709

PARK, Chong Eel, 1927-  
THE LIPIDS OF RHODOMICROBIUM VANNIELII.

University of Hawaii, Ph.D., 1966  
Microbiology

University Microfilms, Inc., Ann Arbor, Michigan

THE LIPIDS OF RHODOMICROBIUM VANNIELII

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE  
UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT  
OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN MICROBIOLOGY

SEPTEMBER, 1966

By

Chong Eel Park

Dissertation Committee:

Leslie R. Berger, Chairman  
O. A. Bushnell  
Albert A. Benedict  
Howard F. Mower  
Harry Y. Yamamoto

#### ACKNOWLEDGMENTS

The author wishes to thank Dr. H. Goldfine, Harvard University, for the suggestion that he look for ornithine-containing phosphatides in certain phospholipid fractions of Rhodomicrobium.

This investigation was partially supported by a grant AI-05907 from the National Institutes of Health of the United States.

## ABSTRACT

Cells of Rhodomicrobium vannielii grown at 29° in a lactate-containing medium were extracted at room temperature with organic solvents. This extractable fraction contains the bulk of simple lipids (1.8% of cell dry weight) and complex lipids (phospholipids, 4.2%, and sulfolipid, 0.01%) coenzyme Q (0.09%) and pigments (carotenoids, 1.2%, and bacteriochlorophyll, 1.9%). The cell-residue contains the bound lipids (2.7%) which are liberated by treatment with alkali. The residue also contains poly- $\beta$ -hydroxybutyric acid (0.2%), which was extracted with boiling  $\text{CHCl}_3$ . CoQ was determined to be  $\text{Q}_9$ . Bacteriochlorophyll was found to be type a. Vaccenic acid ( $\text{C}_{18:1}$ ) is the major fatty acid component in both simple lipid and phospholipid fraction (ca. 90% in each fraction). The bound lipids contain many fatty acid components, including cyclopropane-, branched-, and alpha( $\alpha$ ) and beta( $\beta$ )-hydroxy fatty acids.

Seven carotenoids were found. Rhodopin (61.3% of total carotenoids by weight) and lycopene (20.5%) are the major constituents; the presence of  $\beta$ -carotene (3.4%) makes R. vannielii unique among the photosynthetic bacteria. The rest of the minor carotenoids are spirilloxanthin (11.2%), anhydro-rhodovibrin (1.8%), rhodovibrin (0.5%), and monodemethylated spirilloxanthin (0.5%). R. vannielii synthesizes mainly the normal spirilloxanthin series and is characterized by a high content of hydroxylated carotenoids.

Crude phospholipids were separated from the bacteriochlorophyll by acetone treatment after fractionation on silicic acid columns. They

were refractionated by TLC on silica gel-H and 8 components were separated; 7 of these contained phosphorus. The nature and amounts of these components were determined. They are phosphatidic acid (1.83% of total phospholipids by weight), bis-phosphatidic acid (6.75%), phosphatidyl ethanolamine (4.5%), an ornithine ester of phosphatidyl glycerol (46.5%), phosphatidyl glycerol (9.7%), an ornithine amide of an undetermined fatty acid (0.95%), phosphatidyl choline (26.5%), and the ornithine ester of lysophosphatidyl glycerol (3.2%).

## LIST OF ABBREVIATIONS

APL . . . . .	Apiezon L
Aq . . . . .	Aqueous Solution
BCHL . . . . .	Bacteriochlorophyll
CoQ . . . . .	Coenzyme Q
C - M . . . . .	$\text{CHCl}_3\text{-CH}_3\text{OH}$
DEGS . . . . .	Diethylene Glycol Succinate Poly-ester
ft. . . . .	Foot or Feet
GLC . . . . .	Gas-liquid Chromatography
in. . . . .	Inch or Inches
mA . . . . .	Milliampere
PA . . . . .	Phosphatidic Acid
PC . . . . .	Phosphatidyl Choline
PE . . . . .	Phosphatidyl Ethanolamine
PG . . . . .	Phosphatidyl Glycerol
<u>R.</u> . . . . .	<u>Rhodomicrobium</u>
<u>Rps.</u> . . . . .	<u>Rhodopseudomonas</u>
<u>Rsp.</u> . . . . .	<u>Rhodospirillum</u>
Wt . . . . .	Weight
SF . . . . .	Separation Factor
TLC . . . . .	Thin-layer Chromatography
v/v . . . . .	Volume/Volume
w/v . . . . .	Weight/Volume

## TABLE OF CONTENTS

	Page
ABSTRACT . . . . .	v
LIST OF ABBREVIATIONS . . . . .	vii
LIST OF TABLES . . . . .	ix
LIST OF FIGURES . . . . .	x
INTRODUCTION . . . . .	1
HISTORICAL . . . . .	2
EXPERIMENTAL METHODS . . . . .	5
Growth of Bacteria	
Extraction and Purification	
Quantitative Chemical Analyses	
Infrared Analyses	
Isotopic Analysis	
RESULTS . . . . .	38
Extractable Lipids	
Non-extractable Lipids	
DISCUSSION AND CONCLUSIONS . . . . .	58
SUMMARY . . . . .	66
BIBLIOGRAPHY . . . . .	85

LIST OF TABLES

TABLE		Page
I	FATTY ACID COMPOSITION OF EXTRACTABLE LIPIDS . . . . .	68
II	CHARACTERIZATION OF PHOSPHOLIPIDS . . . . .	69
III	CHARACTERIZATION OF CAROTENOIDS . . . . .	70
IV	FATTY ACIDS OF BOUND LIPIDS . . . . .	71
V	SEPARATION FACTORS OF NON-POLAR FATTY ACIDS OF BOUND LIPIDS . . . . .	72



## LIST OF FIGURES

Fig.		Page
1	Extraction of Lipids . . . . .	73
2	Column Fractionation of Extractable Lipids . . . . .	74
3-1	Gas-liquid Chromatographs of Fatty Acids of Simple Lipids . . . . .	75
3-2	Gas-liquid Chromatographs of Fatty Acids of Complex Lipids . . . . .	76
4	Gas-liquid Chromatographs of Aldehydes . . . . .	77
5	Gradient Fractionation of Phospholipids . . . . .	78
6	Thin-layer Chromatogram of Phospholipids (one dimension) . . . . .	79
7	Thin-layer Chromatogram of Phospholipids (two dimensions) . . . . .	80
8	Gas-liquid Chromatographs of Non-Polar Fatty Acids from Bound Lipids . . . . .	81
9	Gas-liquid Chromatographs of Hydroxy Acids from Bound Lipids . . . . .	82
10	Thin-layer Chromatogram of Carotenoids . . . . .	83
11	Standard Curves of Retention Times of Fatty Acids by GLC . . . . .	84

## INTRODUCTION

Rhodomicrobium vannielii is an obligately anaerobic photosynthetic purple bacterium which requires an organic hydrogen-donor for growth. It differs, however, from other non-sulfur purple bacteria in several ways, for example, in its method of reproduction by budding and in its production of small amounts of B-carotene, which is usually associated with oxygen-evolving organisms. The pigment is confined to lamellar structures rather than to the more commonly-observed chromatophores or vesicles.

Until a few years ago, the lipids in photosynthetic bacteria were all but neglected. This investigation constitutes a complete analysis of solvent-extractable lipids and non-extractable lipids in R. vannielii through modern analytical techniques. The solvent-extractable fraction contains simple and complex lipids (phospholipids and sulfolipids), pigments (carotenoids and bacteriochlorophyll), and coenzyme Q. The latter fraction contains bound lipids (non-polar fatty acids and hydroxy-fatty acids) and poly-beta-hydroxybutyric acid.

The purpose of this study was to establish the analytical methods for the isolation and purification of each component from the mixture of lipids and pigments, and to determine the specific lipid composition of Rhodomicrobium vannielii.

## HISTORICAL

Rhodomicrobium vannielii was first described by Duchow and Douglas (1949). Their report includes a description of the isolation procedure, morphology, mode of cell division, general biochemical activity and physiology, a definition of the new genus and species, and its possible taxonomic position and relationship to Hyphomicrobium. Murray and Douglas (1950) reported on the reproductive mechanism of budding and nuclear division by this organism. Douglas and Wolfe (1959) demonstrated that single cells sometimes exist in the culture which are motile by means of peritrichous flagella in the early growth phase. Rhodomicrobium is usually found as a clumped mass of cells, attached to each other by branching extensions of the cell wall, the "stalks."

Volk and Pennington (1950) examined the pigments of Rhodomicrobium and identified B-carotene and 3 other carotenoids, rhodopin, rhodovibrin, and spirilloxanthin, and a typical bacteriochlorophyll. Conti and Benedict (1962) also found B-carotene and 7 other carotenoids, of which 4 were previously undescribed: lycopene, anhydro-rhodovibrin, monodemethylated spirilloxanthin, and lycophyll. Recently, Ryvarden and Jensen (1964) found B-carotene and 8 carotenoids, among which dihydroxy 432 was previously undescribed. Lyoxanthin and lycophyll reported by Conti and Benedict (1962) were established to be rhodopin and 1, 2, 1', 2'-tetra-hydro 1, 1'-dihydro-lycopene, respectively. Since beta-carotene is characteristic of oxygen-evolving organisms, the presence of beta-carotene makes R. vannielii unique. Bacteriochlorophyll a has been found in R. vannielii (Jensen, Aasmundrud, and Eimhjellen, 1964).

Like most photosynthetic bacteria and blue green algae, Rhodospirillum rubrum fixes nitrogen (Mortenson, 1962). Its hydrogenase is highly restricted in the array of inorganic complex ions which will be reduced (Woolfolk, 1960). Vatter, Douglas, and Wolfe (1959), Boatman and Douglas (1961), and Conti, Fuller, and Bergeron (1961) have shown that the organism does not possess the typical chromatophores which are characteristic of the other photosynthetic bacteria. The photosynthetic pigments are housed in lamellar structures similar to those found in the blue-green algae. Kinetic studies of pigment synthesis in non-sulfur purple bacteria were done by Cohen-Bazire, Sistrom and Stanier (1957). Their report includes useful methods for the analysis of pigments.

Since the start of this study, a few papers on the lipids of photosynthetic bacteria have been published. The total literature on this subject is scant. The following summarizes major contributions:

- (1) A study on phospholipid synthesis in relation to the formation of photosynthetic pigments by Rhodospseudomonas spheroides (Lascelles and Szilazyi, 1965). These authors found phosphatidyl choline, phosphatidic acid, phosphatidyl glycerol, and phosphatidyl ethanolamine.
- (2) The lipid and fatty acid metabolism in Rhodospirillum rubrum and 4 species of Rhodospseudomonas (Wood, Nichols, and James, 1965). In these organisms, phosphatidyl choline, cardiopin, phosphatidyl glycerol, phosphatidyl ethanolamine, and an unknown compound predicted as ornithine phosphatidyl glycerol were found. The major fatty acid was C<sub>18:1</sub>.
- (3) Ornithine-containing lipoamino acid in Rps. spheroides was

reported by Gorchein (1964). (4) Quantitative analysis data of ubiquinone in photosynthetic bacteria including Rhodomicrobium were reported by Carr and Exell (1965). (5) Poly-beta-hydroxybutyric acid metabolism in photosynthetic bacteria was studied by Stanier, Doudoroff, Kunizawa, and Contopoulou (1959). This substance is synthesized from the carbon of acetic acid. It is accumulated in the cell in the absence of CO<sub>2</sub> and converted to polysaccharides in the presence of CO<sub>2</sub>. (6) Sulfolipid of photosynthetic bacteria was reported by Benson and Wiser (1959) in Rsp. rubrum, and by Wood, Nichols, and James (1965) in Rsp. rubrum and four other species of Rhodopseudomonas. Isolation and purification procedures of plant sulfolipid were reported by O'Brien and Benson (1964).

## EXPERIMENTAL METHODS

Growth of Bacteria: Rhodomicrobium vannielii was isolated from Manoa Stream near the University of Hawaii, and was cultivated in a medium which was prepared in the following way. The final concentration of each ingredient is indicated in parentheses:

Solution A. 10 g (0.1%)  $(\text{NH}_4)_2\text{SO}_4$ ; 5 g (0.05%)  $\text{K}_2\text{HPO}_4$ ; 1 g (0.01%)  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; 50 ml (0.5% v/v) lactic acid; 10 g (0.1%) Bacto-yeast extract (Difco Laboratories, Detroit 1, Michigan); 1 ml of 0.01% (v/v) phenol red indicator solution (saturated in ethanol), dissolved in about 8 liters of distilled water and placed in 10 liter narrow-mouthed Pyrex bottles.

Solution B (stock). 20%  $\text{Na}_2\text{S}$  aqueous solution.

Solution C. 10%  $\text{NaHCO}_3$  aqueous solution.

All solutions were sterilized by steam at  $121^\circ\text{C}$ .

0.5 ml of sterile Solution B was added to the sterilized Solution A and the pH was adjusted to pH 7.2 with sterile Solution C. The medium was then inoculated with a young culture of Rhodomicrobium vannielii. 400-ml of inoculation was used per 10 liter bottle. Each culture bottle was completely filled with Solution A (adjusted to pH 7.2 with Solution C) and then was tightly sealed with a sterile rubber stopper.

The cultures were grown anaerobically at  $29^\circ\text{C}$  in a temperature-controlled cabinet made from a glass-fronted metal bookcase. Glass aquarium tanks (26x36x22 cm) filled with water were placed between incandescent flood lamps (150 w) and the cultures in order to trap the

heat from the lamps.

Stock cultures of the organisms were maintained in a screw-capped Pyrex tubes (1.5x15 cm) in a semi-solid medium prepared by adding 0.5% agar to the medium described above. These cultures were kept under light at room temperature for 7 to 10 days. Cotton-plugged 1 ml pipettes or hand-made pipettes of glass tubing (0.5 x 32 cm) were used for inoculation from the semi-solid culture to the 400 ml subculture bottles.

Cells were harvested from the 10-liter bottles near the end of logarithmic growth (usually on the sixth day) by use of a Sharples Super Centrifuge (The Sharples Corporation, Philadelphia, Pennsylvania) and were washed three times, once with 10 liters of 0.01 N HCl and twice with distilled water. Harvested cells were stored immediately in a freezer at  $-20^{\circ}\text{C}$ . When 2 kg of harvested cells were obtained, they were lyophilized in a commercial food freeze-dryer (Repp Industries, Inc., Gardiner, N. Y.). The lyophilized cells were then quickly powdered in a mortar and stored at  $-20^{\circ}\text{C}$  under nitrogen. The yield of cells was about 20 grams in wet weight or 4 grams in lyophilized dry weight per 10 liters of culture.

Sulfur-limited medium containing  $\text{S}^{35}$  for sulfolipid analysis. For the analysis of sulfolipid, 15 mc of  $\text{S}^{35}_{34}$ -form (A.E.C., Oakridge, Tennessee) was added to 10 l of a sulfur-limited medium which contained 0.1%  $\text{NH}_4\text{Cl}$ , 0.05%  $\text{K}_2\text{HPO}_4$ , 0.01%  $\text{MgCl}_2$ , 0.5% (v/v) lactic acid, 0.1% yeast extract, and 0.01% (v/v) phenol red solution (saturated in ethanol). The solution was neutralized with sterile  $\text{NaHCO}_3$  as described

for the mass culture of cells, and was inoculated with cells which were separated from the medium of a 400-ml bottle culture by decantation. Since the organisms grew poorly on this medium, 500 mg and 2 g of  $(\text{NH}_4)_2\text{SO}_4$ , respectively, were added later.

#### Extraction and Purification

##### a) Extraction

50 g of lyophilized cells were extracted by the method of Huston and Albro (1964). During the extraction, the contents of the glassware were protected from light by wrapping in aluminum foil. All procedures were carried out in dim light. Extractions were done in 1.5-liter flasks by shaking at room temperature with 600-ml portions of acetone (Extract 1) for 1.5 hours, followed by three times with  $\text{CHCl}_3$ -MeOH, 2:1 v/v (Extract 2), each time for 2 hours, and twice with  $\text{CHCl}_3$ -MeOH, 1:1 v/v (Extract 3), each time for 2 hours.

Each extract was separated from the cell residue by centrifugation for approximately 10 minutes at 2,300 g. Centrifuge tubes (250 ml bottles) were covered and sealed with aluminum foil.

Extracts 1 through 3 were combined and evaporated to dryness in vacuo using a rotary flash evaporator (Buchler Instruments Co., Fort Lee, N. J.); the residue was then taken up in  $\text{CHCl}_3$ -MeOH (2:1 v/v). This solution was washed according to the method of Folch, Lees, and Sloan-Stanley (1957) to remove non-lipid contaminants. The bottom layer of the chloroform portion was evaporated to dryness in vacuo and weighed.

The cell residue was hydrolyzed in 3 N NaOH in methanol at reflux



temperature for 2 hours. The mixture was cooled, acidified, and filtered through Celite; the residue was washed 4 times with 50-ml portions of diethyl ether. The combined filtrates were concentrated in vacuo at 50°C and weighed. This portion was examined separately for non-extractable lipids or bound lipids.

The acetone extract was reddish due to carotenoid pigments; the chloroform-methanol (2:1) extract was dark green due to bacteriochlorophyll and polar carotenoid pigments; chloroform-methanol (1:1) extracts were pale green due to residual bacteriochlorophyll. Because of these colored materials, the usual method of direct spot testing each extract with Rhodamine 6G could not be used to determine when all lipid had been extracted.

Thin-layer chromatography was very useful for this purpose. The plates were developed with a solvent system of  $\text{CHCl}_3$ -MeOH-AcOH- $\text{H}_2\text{O}$  (250:74:19:3 v/v/v/v), dried for a few minutes, and exposed to iodine vapor for 30 seconds by placing the plates in a jar saturated with iodine vapor. The size and intensity of the yellow spots were evaluated qualitatively to establish the amount of lipids in each extract. Such tests indicated that no further lipid was extracted with  $\text{CHCl}_3$ -MeOH (2:1 v/v) after three shakings. However, a small additional quantity of lipid and an appreciable amount of non-lipid material were extracted by the final extraction with  $\text{CHCl}_3$ -MeOH (1:1, v/v) (Fig. 1).

Poly-beta-hydroxy-butyric acid was extracted with three portions of boiling chloroform from the cell residue after lipid extraction; the  $\text{CHCl}_3$  solution was then filtered and the filtrate was concentrated in vacuo.

b) Silicic acid column chromatography

Lipids and solvent-extractable compounds were separated by column chromatography on silicic acid according to the scheme in Figure 2. Mallinckrodt silicic acid (100 mesh) was dried for 48 hours at 110°C, made into a slurry with  $\text{CHCl}_3$ , and poured into columns (a, b, c, and d). Column a was 5.5 x 120 cm, and columns b, c, and d were 2.5 x 45 cm. Each column contained a plug of glass wool at the bottom and a filter-paper disk. After addition of silicic acid another filter paper disk was placed on top of the absorbent. Columns a, c, and d were washed with acetone, ethylether, and chloroform in that order; column b was washed in the same way except that the final solvent was petroleum ether (30-60° b.p.). The volumes of the washing solvents were 600 ml of each solvent for column a, and 300 ml of each for columns b, c, and d. Care was exercised in pouring the solvents into the columns so as not to disturb the upper layer of absorbent.

The lipid extract, containing not more than 500 ug of lipid phosphorus per gram of silicic acid, was dissolved in 20 ml of  $\text{CHCl}_3$  and first applied to column a. At first, the neutral lipids were eluted with 1,800 ml of chloroform at a flow rate of about 6 ml per minute. Complex lipids were eluted with 2,400 ml of  $\text{CHCl}_3$ -MeOH (2:1), 1,500 ml of  $\text{CHCl}_3$ -MeOH (1:1), 1,000 ml of  $\text{CHCl}_3$ -MeOH (1:4), and finally with 600 ml of methanol for their complete elution from the column a.

The simple lipids were evaporated in vacuo at 45°C and refractionated into two components, Fraction I and Fraction II, from column b. Column b was developed with 600 ml of 4% diethylether in petroleum ether. This procedure yielded Fraction I which contained mainly beta-carotene,

lycopene, and coenzyme Q. Each component of this fraction was separated by Thin-Layer Chromatography (TLC) as will be described later (p. 11).

Column b was developed with 1,200 ml of chloroform, yielding Fraction II which contained all the simple lipids, polar carotenoids, and a small amount of bacteriochlorophyll. These individual compounds were purified by TLC (p. 11).

Complex lipids from column a were concentrated to 100 ml and divided into 3 portions, Fraction III-1 (30% of total complex lipids), Fraction III-2 (30%), and Fraction III-3 (40%). Fraction III-1 was refractionated through column c with an approximately linear gradient of methanol in chloroform (0 to 100% MeOH). Twenty fractions of 50 ml were collected in two 25-ml portions.

The flow-rate from the column was approximately 3 to 4 ml per minute. After the gradient, 150 ml of methanol was passed through column c and collected. A small portion of each fraction was then analyzed for phosphorus.

Fraction III-2 was refractionated through column d, first with  $\text{CHCl}_3$ -MeOH (9:1 v/v) until the chlorophyll band was completely eluted. This eluent contained all of the chlorophyll and a small portion of the less polar phospholipids. The solution was evaporated in vacuo at  $40^\circ\text{C}$  and 30 ml of acetone were added. After standing for 2 hours in a refrigerator ( $6^\circ$ ), a precipitate was separated from the acetone solution by centrifugation. This procedure was repeated three times. Part of the phospholipids which was retained on column d was then completely eluted with 400-ml portions of  $\text{CHCl}_3$ -MeOH (2:1, 1:1, 1:4 v/v) and finally with methanol. The acetone-treated phospholipids and chlorophyll-free

phospholipid fractions were combined and the solvents were evaporated in vacuo on a rotary evaporator at 40°C. Fatty acid esters of complex lipids were prepared from this portion by subsequent procedures of saponification, esterification, and purification which will be described in the section for fatty acid ester preparations (p. 24).

Fraction III-3 was used directly for the isolation of crude phospholipids after removing bacteriochlorophyll and polar carotenoids by precipitation with acetone. The crude phospholipids were dissolved in  $\text{CHCl}_3$  and used for further analysis of individual phospholipids.

Column chromatography of sulfolipid was made by combinations of columns of DEAE-cellulose, Florisil (60-100 mesh), and silicic acid (100 mesh) according to the method of O'Brien and Benson (1964).

Separation of coenzyme Q was done by developing a silicic acid column with 2 - 4% diethylether in petroleum ether (30 - 60°).

c) Thin Layer Chromatography (TLC)

Silica gel G and silica gel H (plain, without binder) plates (20 x 20 cm and 5 x 20 cm) were prepared by the method of Stahl (1965) and of Mangold (1961). Silica gel G (Research Specialties Co., Richmond, California) was made into a slurry with 1.5 parts w/w distilled water, while silica gel H (Research Specialties Co., Richmond, California) was mixed with 3 parts (w/w) distilled water. 20 x 20 cm plates were spread with a Desaga-Brinkman adjustable applicator to a thickness of 0.6 to 0.8 mm. A glass rod (0.5 x 40 cm) or tube was used for spreading the silica gel slurry on small plates (5 x 20 cm). Usually three 5 x 20 cm plates were made at the time. Thickness of the film was determined by layers of electrical insulating tape placed on plates at each side of the

experimental ones.

The plates were activated at 110°C for 2 hours and stored in a desiccator over Drierite until needed. When the plates were stored in the air, they were reactivated at 105°C for 1 hour just prior to use. Silica gel chromatogram sheets (Eastman Co.) (20 x 20 cm) were conveniently used for TLC by cutting the desired pieces with scissors; it was also possible to cut out the spots after development.

The plates were developed in an ascending direction in unlined tanks (23 x 22 x 12 cm) for 20 x 20 cm plates and in cylinders (6 x 23 cm, Research Specialties Co., Richmond, California) for the 5 x 20 cm plates.

All column cuts of simple lipids were examined for members of the simple lipid class by developing the plates in n-hexane-ethylether-acetic acid (90:10:1, v/v/v; Malins and Mangold, 1960) and in a one dimensional, two-step TLC system with silica gel plain plates. Isopropyl ether-acetic acid (96:4) was the first step and light petroleum-ethylether-acetic acid (90:10:1 v/v) the second step (Skipski, Smolowe, Sullivan, and Barclay, 1965). Acetone-petroleum ether (1:7) was used for the separation of carotenoids from neutral lipids, CoQ, and steroids on a plate of silica gel, plain. The eluted material from various spots was checked for absorption at 275 mu and at 290 mu after reduction with  $\text{KBH}_4$  in ethanol. The maximum absorption of CoQ shifts from 275 to 290 mu, but that of carotenoid does not change. Lipids show no absorption at 260 mu.

Complex lipids were separated on plain silica gel with  $\text{CHCl}_3$ -MeOH- $\text{H}_2\text{O}$  (65:25:4 v/v/v; Horrocks, 1963),  $\text{CHCl}_3$ -MeOH-AcOH- $\text{H}_2\text{O}$  (250:74:19:3, v/v/v/v; Abramson and Blecher, 1964) and  $\text{CHCl}_3$ -MeOH-14%  $\text{NH}_4\text{OH}_{\text{aq}}$  (17:7:1 v/v/v). Complex lipids were also separated by two dimensional development

in  $\text{CHCl}_3$ -MeOH-AcOH- $\text{H}_2\text{O}$  (250:74:19:3, v/v/v/v) for the first dimension and in  $\text{CHCl}_3$ -MeOH-7 M  $\text{NH}_4\text{OH}_{\text{aq}}$  (230:90:15, v/v/v) for the second dimension on silica gel G plates (20 x 20 cm). It was found necessary to let the solvents equilibrate in the tanks for at least 4 hours for the larger containers (23 x 22 x 12 cm) and for 1 hour for the smaller one (6 cm diameter x 23 cm) before attempting development of the plates.

Reagents for visualization of spots on the developed plates were:

(i) iodine vapors: show yellow spots on with unsaturated compounds.

Plates are exposed to the iodine vapors for 30 seconds.

(ii) 0.001% aqueous Rhodamine 6G: fluoresces in various colors under UV light, depending upon the component present.

(iii) saturated solution of  $\text{K}_2\text{Cr}_2\text{O}_7$  in conc. sulfuric acid: by charring, yields dark brown or black permanent spots with organic compounds.

(iv) 0.25% ninhydrin in water-saturated n-butanol: turns violet or reddish color with amino or imino compounds after heating at  $105^\circ$  for 5 min. It does not react with quaternary amine compounds but it does react with inorganic ammonium salts.

(v) a molybdate reagent (3 g of ammonium molybdate in 50 ml  $\text{H}_2\text{O}$  plus 5 ml of 6 N HCl aq. and 13 ml of 70%  $\text{HClO}_4$ ): turns phospholipids blue after heating at  $80^\circ$  for 10 min. (Skipski, Peterson, and Barday, 1962).

(vi) Dragendorff's reagent for choline and quaternary amine compounds: as the plates are dried at room temperature, free choline produces a purple spot and choline-containing compounds produce orange spots (Wagner, Horhammer, and Wolff, 1961; and Skidmore and Entenman, 1962).

(vii) ammoniacal silver nitrate reagent, 0.1 N  $\text{AgNO}_3$  aq - 7 N  $\text{NH}_4\text{OH}$  aq

(1:1), to detect glycerol and/or inositol in phospholipids: the plates are heated at 110° until dark brown spots appear on a white background (Skidmore and Entenman, 1962).

(viii) 35% o-phosphoric acid aqueous solution for steroids: the plates are heated at 110-120°C for 7-15 min. after spraying; steroids fluoresce strongly under UV light (365 mu) (Stahl, 1965).

(ix) Liebermann-Burchard's reagent for steroids, mixed 5 ml of acetic acid anhydride with 5 ml conc. H<sub>2</sub>SO<sub>4</sub> in cold: the mixture is added slowly to 50 ml absolute alcohol. After spraying, the plates are heated at 110°C for 10 min. and then are observed under UV light.

d) Silicic acid-impregnated paper and paper chromatography

1) Chromatography with silicic acid-impregnated paper

Silicic acid-impregnated paper was prepared by the method of Marinetti (1962) and Rouser, O'Brien, and Heller (1961). The paper was used for the identification of S<sup>35</sup>-sulfolipid and for phospholipids. Chromatograms were developed with diisobutyl ketone-acetic acid-water (8:5:1 v/v/v; Lepage, 1964) and/or chloroform-methanol (9:1, O'Brien and Benson, 1964). Visualization procedures were the same as with TLC.

Activity of S<sup>35</sup>-sulfolipid was measured by radiochromatogram scanner Model 7201 (Packard Co., La Grange, Illinois).

2) Paper chromatography

Paper chromatography was used for the identification of products from hydrolyzed complex lipids.

i) Hydrolysis. Fraction III-2 (complex lipids from column a) was hydrolyzed to water-soluble products by strong or mild hydrolysis with acid or alkali. However, it was found that alkaline hydrolysis was better for separation of the fatty acid portion from the non-saponifiable polar pigments, such as polar carotenoids. A water mixture of lipid hydrolysates was acidified and extracted with petroleum ether to isolate fatty acids (total fatty acids of complexed lipids) which will be described in the section on GLC. For the study of completely hydrolyzed components in individual phospholipids, crude phospholipids from Fraction III-3 were resolved into individual components by TLC. 10 mg of lipid sample was hydrolyzed with 2 ml of 3 N HCl aqueous solution at 105° in sealed tubes for 24 hours for testing for amino acids and at 120° for 48 hours for the analysis of glycerol. The hydrolysate was extracted with ether; amino acid, glycerol, and phosphate separated to the aqueous phase, while fatty acids went into the ether layer. The aqueous phase was then evaporated in vacuo to remove excess H<sub>2</sub>O and HCl and the residue was redissolved in 0.2 ml of H<sub>2</sub>O.

Mild acid hydrolysis of phospholipids was done by the method of Kaneshiro and Marr (1962) using 0.2 N HCl in methanol at 60° for 2 hours.

#### Deacylation of phospholipids

For the study of deacylated phospholipids, deacylation was done by mild alkaline or acid hydrolysis.



10 mg of phospholipids was deacylated by addition of 0.5 ml absolute ethanol and 0.1 ml of 1 N NaOH aqueous solution for 0.5 hours at room temperature (Ikawa, 1963). More frequently, however, the following methods were used: 0.6 ml methanolic NaOH (0.1 N) and 0.4 ml  $\text{CHCl}_3$  were added to the sample and incubated for 20 minutes at  $37^\circ\text{C}$  (Lascelles and Szilagy, 1964), or heated in sealed tubes for 4 hours with 1.25 ml of methanolic 2 N HCl at  $100^\circ\text{C}$  (Dawson, Hemington, and Davenport, 1962). Acid hydrolysates were neutralized with formamide and alkaline ones with acetic or formic acid. 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 removed and freeze-dried, using Thunberg tubes.

#### Paper chromatography of deacylated phosphatides

The water-soluble deacylated phosphatides were separated by ascending chromatography on Whatman No. 1 paper with phenol-water (100:38 w/v) and with n-butanol-acetic acid-water (5:3:1 v/v, Matches, Walker and Ayres, 1964). Two-dimensional separation was also used for the deacylated phosphatides, descending partition chromatography in the first dimension and ionophoresis in the second dimension. The first dimension was developed with water-saturated phenol-acetic acid-ethanol (100:10:2 v/v/v, Dawson, 1960). After the chromatogram was dried, the paper was sprayed with a volatile buffer solvent of pyridine-acetic acid-water (1:10:89, v/v/v, at pH 3.6) and it was immediately introduced into the paper electrophoresis apparatus which was saturated with the vapor of the volatile buffer solvent. Ionophoresis was done at 100v/cm at  $6^\circ\text{C}$ .

The chromatograms were dried and sprayed first with the ninhydrin reagent for the detection of amino or imino compounds and heated for 5 min. at 85°C. Then they were sprayed with Hanes-Ischerwood's reagent (Hanes and Ischerwood, 1949) and heated for 7 min. at 90°C. The blue spots indicating presence of phosphorus were intensified with H<sub>2</sub>S and detected under UV light.

Paper chromatography of amino acids from lipids

Amino acids were detected by ascending paper chromatography on Whatman No. 1 paper and developed with various solvent systems: phenol solvent (phenol-H<sub>2</sub>O, 160:40, w/v), phenol solvent-ammonia (0.88) (200:1 v/v), phenol solvent-ethanol-water (150:40:10, v/v/v), t-butanol-H<sub>2</sub>O-methylethylketone-diethylamine (80:80:40:8, v/v/v/v), n-butanol-pyridine-water (60:60:60 v/v/v), water-saturated phenol, water-saturated solvent of collidine-2,6 lutidine (1:3, v/v), n-butanol-ethanol-water (4:1:1, v/v/v), and n-butanol-acetic acid-water (9:1:1, v/v/v).

After air drying the paper, various spray reagents were used to visualize the spots. Following reagents were prepared according to the methods described by Smith (1961):

- (i) 0.2% ninhydrin in water-saturated n-butanol for amino and imino compounds, peptides, proteins, and amino acid-breakdown materials,
- (ii) Sakaguchi reagent for guanido compounds,
- (iii) Pauly reagent, and
- (iv) p-anisidine reagent for histidine and imidazole compounds,
- (v) nitrosopruesside reagent for cysteine, cystine, cystamine, meso-cystine, homo-cystine and homo-cysteine,

- (vi) Elson-Morgan reagent for hexosamines;
- (vii) benzidine reagent,
- (viii) dinitrososalicylic acid reagent,
- (ix) silver nitrate reagent, and
- (x) periodate reagent for sugar test.

Before the chemical reagent tests, UV light was used to detect fluorescence by imidazoles, tyrosine (which fluoresced), and purine-pyrimidine derivatives (absorbed, but guanine and xanthine and their compounds fluoresced quite strongly in the acidic state).

#### Paper chromatography of CoQ

The chain length of the isoprenoid moiety of ubiquinone in Rhodomicrobium vannielii was examined by reverse phase paper chromatography with known ubiquinones as standards. Standard Q<sub>10</sub> was prepared from calves' liver by the method of Crane, Lester, Widmer, and Hatefi (1959). Whatman No. 1 paper was soaked in 5% (v/v) liquid paraffin in petroleum ether (30-60° b.p.) and allowed to dry at room temperature. The chromatogram was developed with water-N, N-dimethylformamide (1:39 v/v) in an ascending direction. The position of ubiquinone was revealed as dark areas when the chromatogram was examined under UV light, and the spots were visualized by the following procedure (Lester and Ramasarma, 1959): the chromatogram was first reduced by dipping the paper into a freshly-prepared 0.1% (w/v) KBH<sub>4</sub> aqueous solution for approximately 30 seconds or until the yellow spots had faded. The paper was then drained briefly, and the excess KBH<sub>4</sub> was hydrolyzed by dipping the paper into a 0.1 N HCl aqueous solution for a few seconds, or until gas evolution

ceased. The paper was then dipped for several seconds into 0.5% (w/v) neotetrazolium chloride aqueous solution and into 0.25 M potassium phosphate (pH 7.0) aqueous solution. The paper was then drained and heated at 80-100° on a clean glass plate for 60 seconds. Deep purple spots appeared on a slightly pink background.

e) Spot tests on filter paper and its disk chromatograms

1. Filter paper spot test. The lipid samples purified by TLC were located on the strip of Whatman No. 1 paper (0.8 mm spot size). After evaporation of the solvent, the spots were tested by reagents described below.
2. Paper disk chromatogram. Lipid sample solutions, containing 5 mg/0.5 ml, were added slowly to the center of the Whatman No. 1 paper disks (5.5 cm) under a stream of nitrogen, using Drummond disposable 5 ul pipettes microcaps (Kensington Scientific Co., Oakland, California), confining the diameter of sample spot to about 8 mm. Then 25 ul of  $\text{CHCl}_3$ -MeOH (2:1, v/v) was added twice (lesser polar solvents were desirable first) until the lipid solution was spread to a ring 20 mm in diameter, and later 25 ul of methanol was added three times to yield rings about 40 mm in diameter with a wet spreading-rate of about 2 mm/sec. Total time required was about 5 minutes. When the paper was dried under a stream of nitrogen, it was cut into several pieces of adequate size and tested with appropriate chemical reagents.

(a) Dipycrylamine test for choline lipid

The chromatogram was immersed for about 10 minutes in a saturated dipycrylamine solution which contained 1 liter of water and 50 ml of 10%  $\text{Na}_2\text{CO}_3$ , and was then transferred to flowing tap water until an intense yellow color appeared. This test is very sensitive and is a specific reaction for choline-containing phospholipids (Beiss, 1961). If ninhydrin reagent had been sprayed previously the pink color was completely removed by this test.

(b) Reinecke salt test for choline lipid

When the chromatogram was immersed, without previous hydrolysis, in aqueous 0.05 M Reinecke salt for 2 or more hours at room temperature, a pink ring appeared at the site of the choline-containing lipid. By rinsing in water the excess reagent was removed, and the chromatogram could be retained as a permanent record when dry.

(c) Phosphomolybdic acid -  $\text{SnCl}_2$  test for choline lipid.

Fresh or ninhydrin-treated papers were dipped for 30 seconds in 2% phosphomolybdic aqueous solution and washed thoroughly in tap water for at least 45 minutes. The washed papers were dipped in a fresh solution of 2%  $\text{SnCl}_2$  in 2.5 N HCl aqueous solution. A permanent deep blue spot appeared at the site of choline-containing substances.

(d) Periodate-Schiff's test for vicinal-OH groups

The chromatogram was sprayed with 2%  $\text{NaIO}_4$  aqueous solution and heated for 7 minutes at  $60^\circ\text{C}$  in a  $\text{N}_2$  atmosphere. The paper

was then transferred to a SO<sub>2</sub> chamber and sprayed with Schiff's reagent. Phosphatidyl glycerol stains pink in 3 to 24 hours. The Schiff's reagent was prepared by decolorization of 50 ml of 2% rosanilin (basic fuchsin) aqueous solution with SO<sub>2</sub> gas which was produced by addition of 10 N H<sub>2</sub>SO<sub>4</sub> aqueous solution to solid Na<sub>2</sub>SO<sub>3</sub> or Na HSO<sub>3</sub>, and the decolorized reagent was diluted with distilled water to 1 liter.

(e) Rhodamine 6G test for phospholipids

Chromatograms were immersed in a solution of 0.001% Rhodamine 6G in 0.25 M K<sub>2</sub>HPO<sub>4</sub> aqueous solution and excess reagent was washed out by flowing distilled water; papers were observed under UV light while wet. Spots or rings were identified by their blue or yellow colors.

f) Preparation of fatty acid methylesters from lipid extracts and synthesis of standards

Since Rhodomicrobium vanielii contains an appreciable amount of non-saponifiable pigments, such as carotenoids and CoQ, alkaline hydrolysis was preferable to acid hydrolysis for their elimination. Fatty acids of total-, neutral-, complex-, and bound-lipids were prepared for GLC by three procedures: (i) saponification, (ii) esterification, and (iii) elimination of chlorophyll compounds by absorption chromatography.

1) Preparation of total fatty acid esters from the extractable lipids.

(a) Saponification

10% (0.57 g) of the total washed extract was transferred to a 50 ml round bottom flask and the solvent was evaporated to

dryness under a  $N_2$  stream. 15 ml of 0.5 N KOH in MeOH was added to the flask and it was refluxed in a  $60^\circ\text{C}$  water bath for 3 hours. The contents were transferred to a 60 ml separatory funnel after the mixture had cooled to room temperature. Then, 10 ml of distilled water and 10 ml of petroleum ether were added and shaken for 3 minutes. The petroleum layer was dark red with non-saponified carotenoids, and the water layer was dark green with saponified bacteriochlorophyll. This water layer (the lower phase) was separated from the petroleum ether layer and this treatment was repeated until no colored material was extracted by the petroleum ether from the aqueous layer. The aqueous portion was acidified with 6 N HCl aqueous solution to pH 2 to 3, and extracted three times with 5-ml portions of petroleum ether. The petroleum ether portion was then washed twice with 5 ml of distilled water and it was dried by passing through a column (0.5 x 20 cm) of anhydrous  $Na_2SO_4$ . The column was finally washed with petroleum ether.

(b) Esterification

The solvent of the fatty acids portion was evaporated in vacuo at room temperature in a rotary evaporator (Labline Inc., Chicago, Illinois) or under a  $N_2$  stream on a water bath at  $40^\circ\text{C}$ . 3 ml of  $BF_3$ -MeOH reagent (Applied Science Labs., Inc., State College, Pa.) was added to the tube, boiled for two minutes on a steam bath, and the boiled mixture was transferred to a separatory funnel which contained 5 ml of petroleum ether and 5 ml of  $H_2O$  (Morrison and Smith, 1964). After shaking for 2

minutes, the petroleum layer was removed, and the procedure was repeated twice. The petroleum ether extracts were combined and dried by passing through an anhydrous  $\text{Na}_2\text{SO}_4$  column (0.5 x 20 cm) which was washed finally with petroleum ether.

(c) Decolorization

Since the fatty ester contained hydrolyzed bacteriopheophytin and bacteriochlorophyllide, the esterified solution was treated further for the elimination of the bacteriochlorophyll breakdown components by passing the sample through a silicic acid column and eluting it with 10% diethyl ether in petroleum ether. Alternatively, carbon columns (5 g) (0.5 x 20 cm) were used and the sample was eluted with 5% diethylether in n-hexane. The decolorized fatty acid ester solution was concentrated in vacuo to 1 ml at 35°C for GLC analysis.

2) Preparation of fatty acid esters from neutral lipids

20% of Fraction II from column b was used for preparation of fatty acid esters of neutral lipids. This fraction contained neutral lipids, most of the non-polar carotenoids, and a small amount of bacteriochlorophyll. Since non-saponifiable carotenoids were totally removed by saponification, decolorization of the acid esters was done simply by adding a small amount of active charcoal powder (20 mg) to the acid ester solution. The ester solution was filtered through Whatman No. 1 paper and the paper was washed with petroleum ether. Saponification and esterification procedures were done by the same methods which are described in the previous section for total fatty acid esters.



### 3) Preparation of fatty acid esters from complex lipids

Since the bulk of the bacteriochlorophyll is contained in the complex lipid fraction, the purification of fatty acids from the saponified mixture was an extremely tedious task. Therefore, chlorophyll was eliminated from the lipids as much as possible before saponification. Thus, Fraction III-2 was treated with acetone to remove chlorophyll; chlorophyll dissolves in it but the phospholipids are only sparingly soluble. By this procedure the phospholipids of Fraction III-2 were nearly freed of chlorophyll. They were then saponified, esterified, and purified by the same method described in the section for total fatty acid esters.

### 4) Preparation of fatty acid esters of bound lipids

Fatty acids of bound lipids were prepared by the method of Kaneshiro and Marr (1963), using 10 g of dried cell residue from the solvent extraction. The methylesters of fatty acids were prepared with  $\text{BF}_3\text{-MeOH}$  reagent by the same method described in the section for total fatty acid esters. Fatty acids of the bound lipids contained various kinds of less polar straight-chain fatty acids, cyclopropane fatty acids, branched fatty acids, and polar hydroxy fatty acids. Methylesters of hydroxyfatty acids were separated from other lipids by chromatography on columns (2.5 x 45 cm) containing 40 g activated silicic acid (Mallinkrodt Chemical Works, 100 mesh). Less polar fatty acid esters were eluted with 10 column volumes of diethyl ether-petroleum ether (4:96 v/v) and the polar hydroxy fatty acid esters were eluted with 12 column volumes of diethyl ether-petroleum ether (20:80, v/v).

## 5) Standard compounds for gas liquid chromatography (GLC)

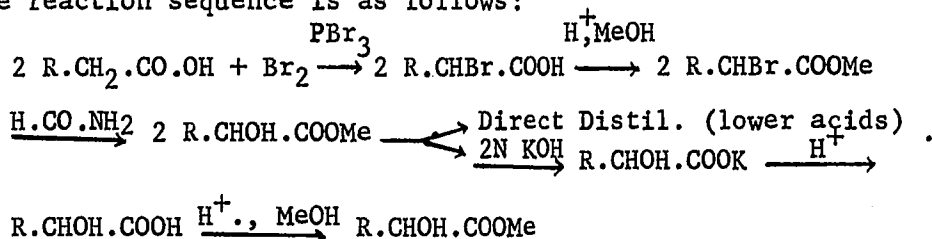
(a) Straight-chain methyl esters of odd- and even-numbered saturated and unsaturated fatty acids  $C_8$  to  $C_{24}$  were obtained from Applied Science Laboratories, Inc. (State College, Pa.); vaccenic acid, methyl-12-hydroxystearate, methyl-12 hydroxy-oleate, and various aldehydes from  $C_1$  to  $C_{16}$  were obtained from Calbiochem (Los Angeles, California).

(b) Alpha- and B-hydroxy fatty acid-esters, and cyclopropane fatty acid esters were synthesized.

## (i) Synthesis of alpha-hydroxy fatty acid esters

Alpha-OH fatty acids ( $C_{10:0}$ ,  $C_{12:0}$ ,  $C_{14:0}$ ,  $C_{16:0}$ ) were synthesized in 3 steps: (1) alpha-bromination of fatty acid, (2) esterification of alpha-bromofatty acid, and (3) alpha-hydroxylation with formamide. Purification was done by direct vacuum distillation of the lower alpha-OH fatty acid esters. However, higher alpha-OH fatty acid esters were purified by crystallization as the free acids after saponification and then chromatographed on silicic acid columns.

The reaction sequence is as follows:



(1) Bromination was done by the method of Gason, Allinger, and Sumrell (1953): to a two-necked 100-ml round-bottom flask were added 0.01 mole-equivalent of the sample fatty acid, 0.01 M-eq of  $PBr_3$ , 0.02 M-eq of Br, and a magnetic stirrer bar. The flask was attached to a condenser containing circulating ice water, the contents were refluxed for 3 hours, and then cooled to room temperature.

(2) Alpha-bromo fatty acid methyl esters were prepared as already described (p. 23).

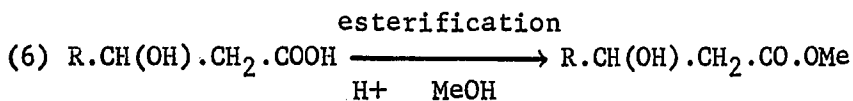
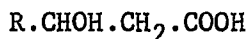
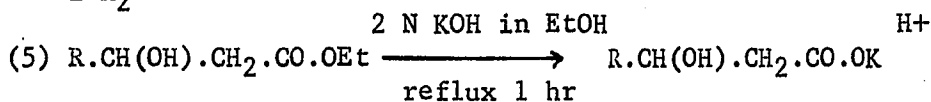
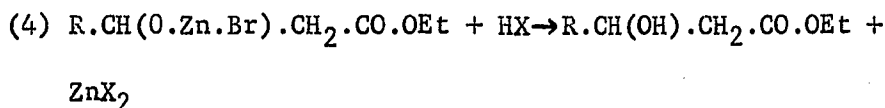
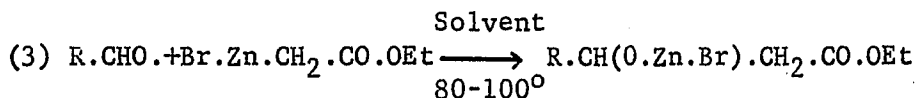
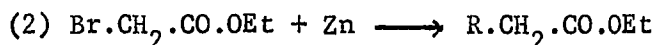
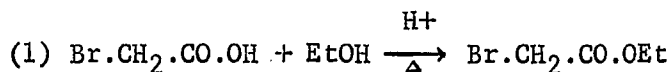
(3) Hydroxylation of the methyl ester of alpha-bromo fatty acid was done by the addition of formamide (0.02 M-eq) with heating at 180 - 200° for 20 hours.

(4) Purification. Lower alpha-OH fatty acid methyl esters ( $C_8$ ,  $C_{10}$ ) were separated by direct fractional distillation on a sand bath. Higher alpha-OH acid esters, however, were converted to potassium soaps by saponification with 15 ml of 2 N KOH in 95% ethanol heated under reflux for 1 hour. The mixture was then diluted with water, acidified, and the alpha-OH acids were extracted twice with 5-ml portions of diethyl ether which were then evaporated. Alpha-OH acids were crystallized from petroleum ether and purified by recrystallization from petroleum ether. Methyl esters were formed as already described (p. 22). The recrystallized esters were chromatographed on silicic

acid columns and eluted with diethyl ether-petroleum ether (20:80, v/v).

(ii) Synthesis of Beta-OH fatty acid esters

Beta-OH acid methyl esters ( $C_{10:0}$ ,  $C_{12:0}$ ,  $C_{14:0}$ ,  $C_{16:0}$ ) were synthesized separately by the Reformatsky reaction (Shriner, 1942) with octanal, decanal, dodecanal, and tetradecanal, and to each of these substances ethylbromoacetate was condensed with Zn as a catalyst, and the beta-OH acids were synthesized in 6 steps as follows:



Since bromoacetic acid boils at  $208^\circ$  and its methyl ester boils at  $52^\circ$  at 15 mm. ethylbromoacetate ( $168^\circ$  b.p.) had to be used for the Zn-coupling reaction with aldehydes at  $80 - 100^\circ$ .

This condensation reaction was done in a two-necked 100-ml round-bottom flask which contained a magnetic stirrer and 0.02

M-eq of ethylbromoacetate, aldehyde, and Zn in 30 ml of ethanol. A condenser was attached which was cooled with ice water. The other neck was used for supplying 0.02 M-eq of Zn-dust and aldehyde samples and finally 10 ml of 6 N HCl<sub>Aq</sub>. The ethyl ester of each tetra-OH fatty acid was concentrated and saponified with 2 N KOH in 95% ethanol at refluxing temperature for 1 hour. The solution was chilled in an ice bath, and the crystalline potassium salt of the hydroxy acid was recovered by filtration. The salt was washed once with cold ethanol and dissolved in a small volume of water. The cold, aqueous solution was acidified with 6 N HCl<sub>Aq</sub>, and the insoluble hydroxy acid was recovered by filtration. The acid was recrystallized from petroleum ether and methylated as described above (p. 23).

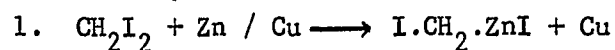
(iii) Synthesis of cyclopropane fatty acid

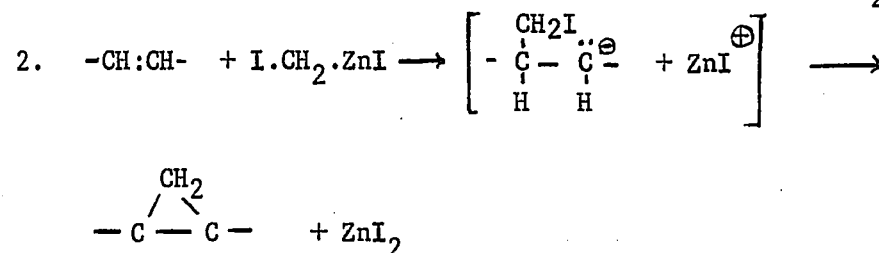
11, 12 methylene octadecanoic acid was prepared by a stereo-specific reaction using vaccenic acid (11-octadecenoic acid) with methylene zinc iodide by the method of Simmons and Smith (1959), and with the highly active zinc-copper couple.

(a) Cu-Zn couple. This was prepared according to the method of LeGoff (1964).

(b) Synthesis of the cyclopropane fatty acid.

Synthesis was done in 2 steps:





Cyclopropane ring formation (Simmond's and Smith's reaction) was done by the modified method of LeGoff (1964).

Cyclopropane fatty acid was esterified and purified by GLC. The appropriate fraction of effluent gas was collected in glass wool moistened with methanol.

#### 6) Gas-Liquid Chromatography (GLC)

Fatty acid methyl esters and aldehydes were analyzed by GLC.

Helium was the carrier gas at an outlet flow rate of 80 to 100 ml per minute and column inlet pressure of 50 psi. Columns ( $\frac{1}{8}$  in. x 8 ft. and  $\frac{1}{4}$  in. x 5 ft.) of 30% diethylene glycol-succinic acid polyester on a support of 60/80 mesh fire-brick and  $\frac{1}{4}$  in. x 5 ft. columns of 10% APL on chromosorb-P, 42 x 62 (Varian-Aerograph, Walnut Creek, California) were used for the separation of both esters and aldehydes.

The effluent was monitored with the 4-filament katharometer thermo-conductivity detector in an Aerograph Manual Temperature Programmer Gas Chromatograph, Model A-90-P2 (Varian Aerograph, Walnut Creek, California), the output of which was recorded on a millivolt recorder. Fatty acids were analyzed as their methyl esters at 170 - 180° and fatty aldehydes were run at 130°.

Unknown fatty acids were estimated from a standard curve plotting the logarithm of retention time versus carbon number, which was made with various standard 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 a suitable standard. For the isolation of individual esters, the desired methyl esters were trapped from appropriate fractions of the effluent gas in glass wool moistened with methanol. This operation was repeated until a sufficient quantity of the desired methyl ester had been collected. The individual esters were washed from the glass wool with methanol, and the solvent was evaporated.

(a) Position of the double bond in mono-unsaturated fatty acids was established by dihydroxylation (Swern, Billen, Findley, and Scanlan, 1945; and Hoffman, Lucas, and Sax, 1952) and by periodate oxidation of the dihydroxy fatty acids to a fatty aldehyde and an aldehydoacid (Huber, 1951; King, 1938). The fatty aldehyde extracted in a suitable solvent was dried with anhydrous  $\text{Na}_2\text{SO}_4$ , and concentrated to a small volume for analysis by GLC.

(b) Identification of branched chain-, saturated-, unsaturated-, OH-, and cyclopropane acids was done by (1) hydrogenation, (2) GLC at various temperatures (Landowne and Lipsky, 1961), and by (3) GLC through columns containing polar- and non-polar liquid-stationary phase absorbents.

(1) Hydrogenation: The olefinic acids or their methyl esters were hydrogenated at room temperature for 2 hours in methanol with a catalyst of 5% Pt on charcoal (Matheson, Coleman & Bell Co., Norwood, Ohio) under  $\text{H}_2$  at one atmosphere. This hydrogenation was selective in that cyclopropane rings were not opened under these conditions.

(2) GLC at different column temperatures: The branched fatty acids were characterized from other fatty acids by the

separation factors\* (SF) which were established through GLC at two different column temperatures (Landowne and Lipsky, 1961). The required temperature difference was more than 10°.

Saturated lipids showed highest separation factors with lower values at the higher temperature, branched acids gave lower values which decreased at the higher temperature, and unsaturated fatty acids gave lowest values which increased at the higher temperature. (See Table V)

(3) GLC with both columns of polar and non-polar liquid-

stationary phase: GLC with a polar column, such as diethylene glycol succinate polyester, gave increasing retention times for branched-, saturated-, mono-unsaturated or cyclo-, di-unsaturated-, and hydroxy fatty acids of a given carbon length, in that order. In the non-polar column, however, relative retention times increased in the reverse order.

Mono-unsaturated acids and cyclopropane fatty acids with the same carbon number had almost identical retention times; likewise, the branched saturated acids and the straight-chained monounsaturated compounds with one less carbon atom had very similar retention values in the 30% DEGS columns. The hydroxy acids had very long retention times on polar columns and very

---


$$*SF = \frac{\text{Rt. of sample F.A.}}{\text{Rt. of saturated F.A. preceded}}$$

e.g., Rt (12:0) /Rt (11:0); Rt (16:1) /Rt (16:0); Rt (18:iso) /Rt (17:0) etc.

Rt. - Retention time  
F.A. - Fatty acid



short values on the non-polar columns.

#### Quantitative Chemical Analyses

(1) Phosphorus determinations were made on samples of the total extracts (Fig. 1). The range of the test is 0.4 ug P (0.13 uM). Samples of individual phospholipids were eluted from thin-layer chromatograms. These and samples of each fraction of Fraction III-1 were analyzed.

After removal of the solvents by evaporation at 80°C, 0.1 ml of concentrated H<sub>2</sub>SO<sub>4</sub> was added to each sample. These samples were digested at 150 to 160°C for 4 hours and cooled to room temperature; two drops of 30% H<sub>2</sub>O<sub>2</sub> were then added to each sample tube and the digestion was continued for 2 hours. If needed, further additions of peroxide were made to achieve clear solutions (Bartlett, 1959).

The digests were diluted with 5 ml of water, 0.2 ml of 5% ammonium molybdate aqueous solution, and 0.2 ml of the Fiske-SubbaRow reagent (Fiske and SubbaRow, 1925). The mixture were mixed well and heated for 7 minutes in a boiling water with glass marbles covering the tubes. The resulting color had an absorption maximum at 800 mu.

(2) Nitrogen was determined after digestion of the sample by the procedure of Johnson (1941). After removal of the solvent from the lipid samples by evaporation at 80°C, 0.1 ml of concentrated H<sub>2</sub>SO<sub>4</sub> was added to the sample in a pyrex tube which contained from 10 to 100 ug of N. The tubes were heated with a microflame for 2 hours until fuming ceased, were cooled to room temperature, and then 2 drops of 30% H<sub>2</sub>O<sub>2</sub> were added to each tube and the digestion was resumed until a clear solution was obtained. Further additions of H<sub>2</sub>O<sub>2</sub> were made when needed. For the

lipid extracts, total digestion time required about 3 hours. The digests were then diluted with 5 ml of ammonia-free re-distilled water mixed with 18 ml of 0.2 N NaOH aqueous solution, and then neutralized to approximately pH 7. 0.2 ml of Nessler's reagent was rapidly added. After 10 min., the O D was determined photocolometrically at 490 mu. Spectral curves for the colored solutions showed an absorption increasing mainly linearly from 900 mu to 400 mu without any specific maxima. Digests should be diluted and cooled because Nessler's reagent tends to precipitate when warm or at high ionic strength.

(3) Amino nitrogen: The procedure of Cocking and Yenn (1954) was modified for use with unhydrolyzed phospholipids by Wheeldon and Collins (1957). The solvent from unhydrolyzed lipid samples was removed by evaporation, after which 1 ml of 2-ethoxyethanol, 0.5 ml of 0.2 M citrate buffer (pH 5.0), 0.2 ml of 5% (v/v) ninhydrin in 2-ethoxyethanol and 1 ml of KCN solution, made by dilution of 1.5 ml of 0.01 M KCN aqueous solution to 50 ml with 2-ethoxyethanol, were added to the samples. The tubes were covered with glass marbles and heated in a boiling water bath for 30 min. Standards were made with 0.25, 0.5, 0.75, and 1.0 umole of L-ornithine, because it was found that this was the major amino acid in the lipids of Rhodomicrobium vannieli. Maximum O D was found to be 570 mu.

(4) Ester bonds of lipids: For the determination of ester bonds, samples (1.5 mg) were reacted with alkaline hydroxylamine, and the resulting hydroxamic acids were determined colorimetrically as the ferric hydroxamates by the procedure of Rapport and Alonzo (1955). Synthetic

dipalmitoyllecithin (General Biochemicals, Chagrin Falls, Ohio) was used as the standard. Color intensity was determined at 520 mu.

(5) Lyso compounds were estimated by their hemolytic activity on erythrocytes (Bard and McClung, 1948). Fresh human erythrocytes were washed at least 5 times with saline (0.85% NaCl in H<sub>2</sub>O, pH 7.0) and made into a 3% suspension in saline. The sample lipids in solvent (0.2, 0.4, 0.6, 0.8, and 1 ml) were transferred to test tubes and the solvent evaporated by a stream of N<sub>2</sub>. They were then diluted with 0.5 ml of sterile calcium saline (0.005 M calcium acetate in 0.85% NaCl aqueous solution, pH 6.8). To each mixture, 0.5 ml of the saline erythrocyte suspensions was added, and agitated. The tubes were placed in a 37°C water bath for 3 hours and then refrigerated overnight. Control tubes were made similarly but without the lipid sample.

(6) Steroids were estimated by the Liebermann-Burchardt reaction (Cavanaugh and Glick, 1952) after alkaline hydrolysis and precipitation with digitonin according to the Sperry and Webb (1950) modification of the method of the Schoenheimer and Sperry (1934).

(7) Coenzyme Q was purified by TLC using the material which was contained in the non-saponified portion of Fraction-I eluted with 4% diethyl ether in petroleum ether from silicic acid column b. The solvent was evaporated, and the residue was dissolved in ethanol. The content of CoQ was estimated by the decrease in absorbancy at 275 mu after reduction with a small crystal of KBH<sub>4</sub> by the method of Lester and Crane (1959) and Lester, Hatefi, Widmer, and Crane (1959). The measurements of absorbance were carried out in a Beckman Model DK-2 recording

spectrophotometer. This spectrophotometric method was used as a basis of estimating the concentration of CoQ with  $\frac{\text{ethanol}}{0 \quad R}$  275 mu assuming the following values for extinction coefficients:

$E_{1\%}^{1 \text{ cm}}$  of 142 for CoQ<sub>10</sub> and 158 for CoQ<sub>9</sub> (Olson, Dialameh, Bentley, Springer and Ramsey, 1965). Melting points of crystallized CoQ were measured with a melting point apparatus (Fischer-Johns, Inc., N. Y.).

(8) Sulfate was determined colorimetrically with barium chloranilate (Bertolacini and Barney, 1957). This method was based on the reaction of solid barium chloranilate (2,5-dichloro-3,6-dihydroxy-p-benzoquinone barium salt, Eastman Organic Chemicals, Rochester 3, N. Y.) with sulfate ion at pH 4 in 50% ethanol solution to liberate highly colored acid-chloranilate ion. Color was measured at 520 mu.

(9) Bacteriochlorophyll: A sample from the total extract or the methanolic extract was estimated spectrophotometrically by the method of Cohen-Bazire, Sistrom, and Stanier (1957). The solvent evaporated from the total extract was determined without conversion to pheophytin. The optical density of an acetone solution was measured at 769 mu (Jensen, Aasmundrud, and Eimhjellen, 1964).

(10) Protein in the lipids extract was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951) using the Folin-Ciocalteu reagent (Folin and Ciocalteu, 1927). Bovine albumin (Pentex, Inc., Kankakee, Illinois) was used as the standard (diluted to 700-70 gamma/ml). O D readings were determined at 660 mu, and at 750 mu for dilute solutions.

(11) Hexose was assayed with anthrone (Radin, Lavin, and Brown, 1955).

(12) Glycerol in lipids was determined by the estimation of formaldehyde which resulted from periodate oxidation. To prepare the lipid sample for this analysis lipid containing 0.2 to 1.0 umole of glycerol was hydrolyzed in a sealed tube with 5 ml of 2 N HCl aqueous solution at 125° for 48 hours. 0.1 ml of 10 N H<sub>2</sub>SO<sub>4</sub> aqueous solution and 0.5 ml of 0.1 M NaIO<sub>4</sub> aqueous solution were added to the hydrolyzed sample. After 5 minutes the reaction was stopped by addition of 10% NaHSO<sub>3</sub> aqueous solution. Color was developed with 5 ml of 0.18% chromotropic acid (1, 8-dihydroxynaphthalene- 3,6-disulfonic acid, Distillation Products, Inc., Rochester, N. Y.) in 20N H<sub>2</sub>SO<sub>4</sub> aqueous solution in an oven at 100° for 135 minutes (Renkonen, 1962). After cooling the color was read at 570 mu against a reagent blank.

(13) Poly -B-hydroxybutyric acid was estimated by quantitative conversion to crotonic acid by heating in concentrated sulfuric acid with measurement of the ultraviolet absorption of the resulting compound (Law and Slepecky, 1961). For the spectrophotometric assay of the polymer, a sample containing 5 to 5 ug of the polymer in chloroform was transferred to a clean tube. The CHCl<sub>3</sub> was evaporated and 10 ml of conc. H<sub>2</sub>SO<sub>4</sub> was added, the tube was capped with a glass marble and heated for 10 minutes at 100°C in a water bath. The solution was cooled, and the absorbancy at 235 mu was measured against a sulfuric acid blank. The value of  $1.55 \times 10^4$  was used for the molar extinction coefficient (Slepecky and Law, 1960).

### Infrared Analysis

One-half inch transparent KBr pellets were made with ca. 200 mg of specially dried and spectroscopically pure KBr (K&K Laboratories, Inc., 177-10, 93rd Avenue, Jamaica 33, N. Y.) at 20,000 psi after the evacuation of gases for 5 minutes with a Carver Press (Fred S. Carver Co., N. Y.) and a Beckman pellet maker.

The sample, dissolved in dried, pure, redistilled reagent grade  $\text{CHCl}_3$  (Merck & Co., Inc., Rahway, N. J.), was layered as a thin film on the KBr pellet. The  $\text{CHCl}_3$  was allowed to evaporate. Infrared spectra of various fractions, standard compounds, and constituent lipids were obtained with the recording infrared spectrophotometer (Beckman Instruments, Model IR-5A).

### Isotopic Analysis

The total amount of  $\text{S}^{35}$ -sulfolipid was determined by measuring its activity with a Windowless Flow Counter (Tracerlab, Inc., Waltham 54, Mass.). The spot of  $\text{S}^{35}$ -sulfolipid on a silicic acid-impregnated paper chromatogram and on a thin-layer chromatogram was detected by the Radiochromatogram Scanner, Model 7201 (Packard Instrument Co., La Grange, Ill.).

## RESULTS

For purposes of analysis, the lipids of Rhodomicrobium vannielii were grouped into two categories: the solvent-extractable lipids and the non-extractable lipids.

The composition of the cells in per cent dry weight, is given below for each of the components which were analyzed:

I. Extractable materials	11.40%
a. Total glyceride	
1. Neutral lipids	1.87
2. Complex lipids ... phospholipids	4.20
... sulfolipid	0.01
b. Carotenoids	1.20
c. Coenzyme Q	0.09
d. Bacteriochlorophyll	1.94
e. <u>Steroids</u>	0
f. Proteins	0.73
g. Sugar	0
II. Non-extractable lipids (acids)	2.90
a. Bound lipid fatty acids	
1. Non-polar fatty acids	1.86
2. Hydroxy fatty acids	0.49
b. Poly-beta-hydroxybutyric acid	0.20

### I. Extractable lipids

The material obtained by extracting the lyophilized cells with acetone, chloroform, and chloroform-methanol according to the scheme shown in Fig. 1 accounted for 11.4% of the cell dry weight. These total

extracts were chromatographed on silicic acid columns according to the scheme in Figure 2, and three fractions were collected as follows:

Fraction	Eluting Solvent	% of Total Extract	Components
I	4% diethyl ether in petroleum ether	2.9	1. Nonpolar carotenoids ( $\beta$ -Carotene, Lycopene) 2. CoQ
II	$\text{CHCl}_3$	25.4	1. Simple lipids 2. Polar carotenoids 3. Bacteriochlorophyll
III	$\text{CHCl}_3$ -MeOH (2:1 1:1 1:4 0:1)	66.5	1. Complex lipids (phospholipids, sulfo- lipid) 2. Polar carotenoids 3. Bacteriochlorophyll

94.8% of the total solvent extract was recovered from silicic acid columns a and b.

- a. Total glycerides: Total glycerides are defined as the sum of the neutral and complex lipids. The amounts of neutral triglycerides were calculated from the amount and composition of the fatty acids in the neutral lipids fraction. This indirect calculation was necessary because the free neutral lipids could not be completely separated from carotenoids and bacteriochlorophyll. Saponification was necessary to eliminate these substances.



	% cell dry weight
neutral glycerides	1.87
complex lipids	4.20
total glycerides	6.07

Total glycerides accounted for 53% of the total solvent extract.

This value is based on the total fatty acid content of 4.8%, cell dry weight.

- b. Composition of lipid: The fatty acids in the neutral lipids and complex lipids were analyzed by GLC (Table I). Vaccenic acid (11-octadecenoic acid) was the major component of the neutral and complex lipids. Fatty acids in the neutral lipids show more variety than those in the complex lipids. Unsaturated compounds were determined by GLC, comparing retention times before and after hydrogenation (Fig. 3). The double-bond position in the C<sub>18:1</sub> component was established by performic dihydroxylation and periodate oxidation. The resulting aldehyde was characterized with 2,4-dinitrophenylhydrazine to form a yellow crystalline of 2,4-dinitrophenylhydrazone (m.p. 108°). The retention time of the free aldehyde was equal to that of standard heptanal (Fig. 4). From these data, it was concluded that the position of the double bond lay between the 11th and the 12th carbon atoms. This established the compound as 11-C<sub>18:1</sub>, vaccenic acid. The position of the double bonds of other unsaturated compounds was not determined because their amounts were too small. No branched acids and cyclopropane fatty acids were observed in the neutral and complex lipids of the solvent

extractable material.

- c. Phospholipids: The phospholipids content of R. vanniellii represented about 70% of the lipids extracted by solvents. This fraction contained seven phosphorus-containing phospholipids and one phosphorus-free compound, an amide of fatty acids with ornithine.
1. Gradient elution pattern. 30% of the complex lipids were fractionated on column c on silicic acid (100 mesh) which was eluted with an approximately linear gradient of methanol in chloroform. The phosphorus of each fraction was analyzed. Fig. 5 shows the gradient elution pattern of phospholipids. These data reveal that more than two thirds of the phospholipids are relatively weak polar material (Tube No. 1-24) and less than one third are polar phospholipids (Tube No. 29-40). The former portion gave 6 spots (A, B, C-1, C-2, D-1, and D-2) and the latter portion gave 2 spots (E-1 and E-2) on silica gel H thin-layer plates, in which spots C-1, C-2, D-2 and E-2 were ninhydrin-positive and all spots were ammonium molybdate-positive except D-2 (see Fig. 6). Identification of each spot will be discussed in the next section.

Most of the bacteriochlorophyll was confined to tubes No. 5 through 10, and most of the carotenoids were fractionated into tubes 2 to 13, with small amounts of polar carotenoids eluting farther through tubes to No. 24. The high phospholipid content in tubes No. 33 to 35 were freed from

pigments, and this portion was concentrated. The material was identified as phosphatidylcholine by infrared analysis (strong absorption at  $968\text{ cm}^{-1}$ ) and by a positive test with Dragendorff's reagent. Further preliminary confirmations for a choline-containing phosphatide were positive tests with dipicrylamine and phosphomolybdic- $\text{SnCl}_2$ .

2. Identification of individual phospholipids. From fraction III-3, crude phospholipids were prepared by acetone precipitation. Nearly all the carotenoids and most of the bacteriochlorophyll were removed by this procedure. However, this crude phospholipid solution had a pale greenish color due to slight contamination with bacteriochlorophyll. Individual phospholipids were freed from the residual bacteriochlorophyll by TLC on silica gel H eluting with chloroform-methanol-acetic acid-water (250:74:19:3, v/v/v/v). 5 spots (A, B, C, D, and E) were observed by the iodine vapor and phosphorus tests. Spot D and E each showed as two distinct spots with ninhydrin; ninhydrin-negative components (D-1 and E-1) and ninhydrin-positive ones (D-2 and E-2). Single-dimensional and two-dimensional thin-layer chromatograms of the phospholipids are shown in Figures 6 and 7, respectively. Spot C separated as a single component in the one dimensional thin-layer chromatogram, but it revealed two ninhydrin-positive spots on the plates developed in two dimensions; one was small (C-1), the other was large (C-2).

Rhodomicrobium vannielii, therefore, contains at least 8 different compounds. Various tests for identification of each compound were done and these are listed in Table II.

The compounds in each spot were identified as follows:

Solvent front component: Neutral lipid carryover. This spot showed a strong yellow color in iodine vapor and a strong positive reaction for the ester bond, but no reactions with ninhydrin, ammonium molybdate, Dragendorff's reagent, and the Lieberman-Burchardt reagent. Rhodamine 6G gave a yellow color to this spot under UV-light. Based on the above tests, the spot was identified as neutral lipid carryover.

Component-A: Phosphatidic acid. This component always appeared in the upper part of a greenish spot which resulted from some residual bacteriochlorophyll. It gave a weak blue color with rhodamine 6G, a positive test with ammonium molybdate, but a negative ninhydrin reaction. Its R<sub>f</sub> value of 0.77 on silicic acid-impregnated paper is very close to that of 0.78 reported by Marinetti (1962) for phosphatidic acid. Quantitative analysis of this spot gave an ester-bond/P molar ratio of 1.97. From these data this component was identified as phosphatidic acid.

Component B: Bisphosphatidic acid. On the basis of TLC data and column chromatographic properties, as well as its reaction with various of the detection reagents, this spot could have been either a bis-phosphatidic acid or a cardiolipin-type of polyglycerol phosphatide. However, R<sub>f</sub> values

on silicic acid-impregnated paper were obtained from 0.56 to 0.68; these values are virtually identical (0.57 to 0.67) with those reported by Marinetti (1962) for synthetic bis-phosphatidic acid for material from the spores of Bacillus polymixa (Matches, Walker, and Ayres, 1964). Ammoniacal silver nitrate reduced this compound but did not reduce authentic cardiolipin (Walter Reed Army Institute of Research, Washington 12, D. C.). The ester-bond/P molar ratio of 4:1 was obtained from a quantitative analysis of the compound. From these data, this compound (spot-B) was identified as bis-phosphatidic acid.

Component-C (C-1, C-2): Phosphatidyl ethanolamine and o-ornithine phosphatidyl glycerol ester. In one-dimensional TLC, 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 TLC by two-dimensional development.

Spot C (C-1 + C-2) showed a positive reaction with ninhydrin, ammonium molybdate, and strong absorption of iodine. Rhodamine 6G gave a strong yellow color under UV-light. The R<sub>f</sub> values of spot C were the same as those for authentic phosphatidyl ethanolamine. Therefore, initially this single spot was erroneously identified as a single component, phosphatidyl ethanolamine.

However, water-soluble products of the hydrolysis of this spot gave two ninhydrin-positive spots which were identified

as ethanolamine and L-ornithine on a paper chromatogram developed with water-saturated phenol. The relative amounts of ethanolamine and L-ornithine were estimated with a paper strip densitometer, Analytrol (Beckman Instruments, Inc., Spinco Division, Palo Alto, California) as 8% and 92%, respectively.

If spot C-1 is phosphatidyl ethanolamine, then the C-2 compound is the o-ornithine phosphatidyl glycerol ester. This may be deduced from the quantitative analysis of component-C, which had a N/P molar ratio of 2:1 and an ester-bond/P molar ratio of 2.7:1 for spot C-2. The low value for the ester-bond in spot C may be due to a slight overlapping of the upper portion of component D-1 and the tail portion of component C. Component D had a lower ester-bond/P molar ratio of 2:1. This contention is supported by the weak positive reaction of Component C with the Periodo-Schiff's reagent for vicinal hydroxy groups and the strong positive reaction in Component D-1.

Component D-1: Phosphatidyl glycerol. This component gave a negative reaction with ninhydrin but gave a positive test with ammonium molybdate. It gave a very strong blue color with Rhodamine 6G under UV light. Periodo-Schiff's reagent gave a strong positive reaction. The R<sub>f</sub> of 0.47 on silicic acid-impregnated paper was close to the value of 0.45 (Kates, Sehgal, and Gibbons, 1961; Matches, Walker, and

Ayres, 1964) and of 0.49 (Sehgal, Kates and Gibbons, 1962).

Quantitative analysis of this component indicated an ester-bond/P molar ratio of 2.2:1. The deacylated phosphoryl compound of this component showed an  $R_f$  of 0.45 on the first-dimension of a two-dimension paper chromatogram developed with water saturated phenol-acetic acid-ethanol (100:10:2, v/v/v) and an  $R_{pi}$  of 0.64 on the second-dimension paper chromatogram by ionophoresis with volatile buffer solvent of pyridine-acetic acid-water (1:10:89, v/v/v at pH 3.6, 100 v/cm). These  $R_f$ s were the same as the values reported by Dawson (1960). Based on these data, compound D was identified as phosphatidyl glycerol.

Compound D-2: Ornithine amide of fatty acid. This compound is not completely separable from Compound D-1 on the thin-layer chromatogram of silica gel H with the same solvent system. It is ninhydrin-positive but phosphorus-negative by ammonium molybdate reagent. Iodine vapor reacted with this compound and gave a yellow color. Water-soluble products of the hydrolysis of this compound gave only one ninhydrin-positive spot, which was identified as ornithine on a paper chromatogram developed with various kinds of solvent systems. This compound was not analyzed quantitatively, but it is most likely the fatty acid amide of ornithine (N, N-diacyl ornithine) as reported by Gorchein (1964) in Rhodopseudomonas spheroides and by Laneelle,

Laneelle, and Asselineau (1963) in Mycobacterium phlei.

Component E-1: Phosphatidyl choline. This component was identified as phosphatidyl choline on the basis of positive reactions with Dragendorff's reagent, dipicrylamine, phosphomolybdic-SnCl<sub>2</sub> reagents, and the Reinecke salt test. Tubes No. 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 of the tubes were combined and concentrated, and gave an infrared absorption spectrum typical of phosphatidyl choline with a prominent band at 968<sup>cm</sup><sup>-1</sup>, an ester absorption at 1740<sup>cm</sup><sup>-1</sup>, a P-O stretch at 1225<sup>cm</sup><sup>-1</sup>, and a P-O-C stretch at 1075 and 1030<sup>cm</sup><sup>-1</sup>.

Rhodamine 6G gave a yellow spot at an R<sub>f</sub> of 0.38 on silicic acid-impregnated paper. This is close to the R<sub>f</sub> of 0.37 reported by Marinetti (1962). It reacted strongly with ammonium molybdate but not with ninhydrin. Quantitative analysis of component E-1 gave an ester-bond/P molar ratio of 1.9:1 and an N/P molar ratio of 1.1:1.

Component E-2: O-ornithine ester of lysophosphatidyl glycerol.

The smallest spot, E-2, was attached to the tail of spot E-1, and was observed as a single spot, spot E, in the reactions of iodine vapor and ammonium molybdate on a thin-layer plate and on silicic acid-impregnated paper. However, the ninhydrin spray gave a positive reaction only at



spot E-2 and not at spot E-1.

The phospholipid of spot E-2 hemolyzed human erythrocytes. It gave no reaction with Periodo-Schiff's reagent and alkaline silver nitrate. Hydrolysates of component E-2 revealed two ninhydrin-positive spots having Rf values of 0.85 and 0.18 on paper chromatograms developed with water-saturated phenol solvent. However, in the system of n-butanol-acetic acid-water (120:30:50, v/v/v), only one spot (Rf=0.16) was found.

The compound (Rf=0.18) was identified as L-ornithine by comparing it with the authentic compound, using paper chromatograms developed with various kinds of solvent systems. The material with an Rf of 0.85 gave negative reactions with the Sakaguchi reagent for guanido compounds; Pauly reagent and P-anisidine reagent for histidine and imidazole compounds; nitroprusside reagent for cysteine, cysteine, and their derivatives; Elson-Morgan reagent for hexosamines; benzi-dine reagent and dinitrososalicylic acid reagent for sugars. Under UV light the Rf 0.85 spot on the paper chromatogram developed with the water-saturated phenol system fluoresced slightly, but this material did not possess quite the same Rf as tyrosine and tryptophane-breakdown components. But it is most likely a breakdown component of an amino acid which is unstable during acid hydrolysis.

On the basis of these various tests, component E-2 was tentatively identified as the o-ornithine ester of lysophosphatidyl glycerol.

Component at the base line: Proteins. The component (Rf=0) on a thin-layer chromatogram developed with the same solvent system reacted positively with ninhydrin and gave a weak blue color with Folin-Ciocalteu reagent. No reactions with ammonium molybdate, rhodamine 6G, alkaline silver nitrate, and Periodo-Schiff's reagent were given. On these bases, it was identified as protein.

d. 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 (1964), probably because too little material was present.

Cells were cultivated, therefore, in  $S^{35}O_4^{=}$  added to a sulfur-limited medium and extracted with chloroform-methanol (2:1, 1:1, 1:4, v/v) and purified through combined columns of florisil, DEAE-cellulose, and silicic acid.  $S^{35}$ -sulfolipid was assayed on planchets and the activity determined\* using a

---

* Cells (dry weight)	1.866
Total counts of original medium	$5.3 \times 10^9$ CPM/10 l.
$SO_4^{=}$ in original medium	4.35 g/10 l. = 0.0444 moles
Sulfolipid (total counts)	29.516 CPM = $3.0 \times 10^4$ CPM
M.W. of sulfolipid (Based on R = (C <sub>17</sub> H <sub>33</sub> )	= 792

windowless geiger tube detector after evaporation of the solvents. It was found that the sulfolipid content of Rhodomicrobium vanniellii was 0.01% of the cell dry weight, or 0.14  $\mu$  mole per gram of dried cells by assuming that the fatty acids are vaccenic acids.

e. Carotenoids

Total carotenoids of Rhodomicrobium vanniellii accounted for 1.2% of the cell dry weight. Fraction I from column b consisted of two non-polar carotenes, beta-carotene and lycopene, and coenzyme Q. However, the carotenoids of Fraction II were the same types as were present in Fraction III, although the amount of each component was different. This means that the silicic acid column was not adequate for the separation of polar carotenoids, because it absorbed the polar carotenoids strongly and increased the elution time with the lipid-extracting solvents. Therefore, carotenoids in the non-saponified portion of Fraction II were pooled in diethyl ether and combined with the non-saponifiable portion of Fraction III-2 and were refractionated on a deactivated aluminum oxide column with increasing amounts of acetone in petroleum ether (Table III). Each fraction was further refractionated by TLC on silica gel H with acetone-petroleum ether (1:7), and the 8 components were obtained and identified (see Fig. 10). It was found that three carotenoids, rhodopin, lycopene, and spirilloxanthin, comprised 93% of the total carotenoids of R. vanniellii. It is remarkable that the

simple compound, rhodopin, was the predominant component, comprising nearly two thirds of the total carotenoids.

#### Identification of individual components

Component 1: Beta-carotene. This component was identified as beta-carotene on the basis of a yellow spot with an Rf of 0.93, spectral maxima at 449 and 478 mu in hexane, and a partition coefficient of 100:0 (petroleum ether-95%-methanol); beta-carotene was a minor component of the carotenoids (3.4%, using  $E_{1\text{ cm}}^{1\%} = 2505$  at 451 mu in petroleum ether; Davies, 1965).

Component 2: Lycopene. This component gave a large orange pink spot on the chromatogram with an Rf of 0.78, maximum spectral absorption at 446, 472, and 503 mu in acetone, and a partition coefficient of 100:0 (petroleum ether-95%-methanol). Lycopene is the second largest component of the carotenoids (20.5%, using  $E_{1\text{ cm}}^{1\%} = 2940$  at 472 mu in acetone (Ryvarden and Jensen, 1964).

Component 3: Anhydro-rhodovibrin. This spot showed as a pale orange because it was present in trace amounts. Maximum spectral absorption at 460, 488, and 520 mu in acetone. The partition coefficient was 95:5 (petroleum ether-95%-methanol). Its Rf value was 0.65, and it was 1.8% of total carotenoids ( $E_{1\text{ cm}}^{1\%} = 2700$  at 482.5 mu in petroleum ether; Davies, 1965).

On this basis, this component was identified as anhydro-rhodovibrin (Ryvarden and Jensen, 1964).

Component 4: Spirilloxanthine. This spot was red, with an Rf of

0.46, spectral maxima at 468, 497, and 530 mu in acetone, and a partition coefficient of 90:10 (n-hexane-MeOH). This compound was 11.2% of the total carotenoids ( $E_{1\text{ cm}}^{1\%} = 2470$  at 510 mu in benzene, Davies, 1965).

Component 5: Rhodopin. This was the major component of the carotenoids of R. vanniellii. The spot was orange pink, with an Rf of 0.32, spectral maxima at 448, 472, and 502 mu in acetone, and a partition coefficient of 75:25 (petroleum ether-95%-MeOH). Rhodopin comprised 61.3% of the total carotenoids ( $E_{1\text{ cm}}^{1\%} = 2980$  at 472 mu in acetone; Ryvarda and Jensen, 1964).

Component 6: Rhodovibrin. This component was orange yellow, with an Rf of 0.23, and spectral maxima at 457, 487, and 520 mu in acetone. The partition coefficient was not determined, because of the small amount, estimated as 0.5% of the total carotenoids ( $E_{1\text{ cm}}^{1\%} = 2700$  at 483 mu in petroleum ether; Davies, 1965). This component was tentatively identified as rhodovibrin.

Component 7: Monodemethylated spirilloxanthin. This component was tentatively identified on the basis of its orange color, an Rf of 0.09, spectral maxima at 466, 497, and 530 mu in acetone, and a partition coefficient of 42:58 (petroleum ether/95% MeOH). This compound comprises 0.8% of total carotenoids ( $E_{1\text{ cm}}^{1\%} = 3360$  at 472.5 mu in petroleum ether; Davies, 1965).

Component 8: Unidentified. This component was yellow, with an Rf of 0.03. Because of its trace amount, spectral maxima and the partition coefficient could not be determined.

## f. Coenzyme Q

Coenzyme Q (CoQ), contained in Fraction-II from column b and eluted with 4% diethyl ether in petroleum ether, was purified by TLC on silica gel-H developed with 5% acetone in petroleum ether. CoQ was identified on the basis of its absorption maxima at 275 m $\mu$  in the oxidized form and at 290 m $\mu$  in the reduced form. It was reduced with  $\text{KBH}_4$  crystals in ethanol solution.

The length of the isoprenoid chain of CoQ was determined by reverse-phase paper chromatography, using Whatman No. 1 paper coated with paraffin oil and developed with N, N-dimethyl formamide- $\text{H}_2\text{O}$  (39:1). An Rf of 0.22 was found for the sample CoQ, and an Rf of 0.13 for that of authentic  $\text{CoQ}_{10}$  from calf liver. From these Rf values, one can assume that the sample CoQ has a shorter isoprenoid chain than is that of  $\text{CoQ}_{10}$ . The ratio\* of Rf of calf liver  $\text{CoQ}_{10}$  to Rf of sample CoQ was comparable to the ratio\*\* of Rf  $\text{CoQ}_{10}$  to Rf  $\text{CoQ}_9$  (Carr and Exell, 1965). The melting point of the purified crystalline material was also determined. The melting point of CoQ from R. vanielii was 45.0 $^\circ$ , which confirmed that  $\text{CoQ}_9$  was present (reported values are  $\text{CoQ}_8 = 37.0^\circ$ ,  $\text{CoQ}_9 = 45.2^\circ$ ,  $\text{CoQ}_{10} = 49.9^\circ$ , according to Lester, Hatefi, Widmer, and Crane, 1959).

---

\*  $0.13/0.22 = 0.590$

\*\*  $0.17/0.27 = 0.585$

The Coenzyme Q content was found to be 0.884 mg/g dried cell material, or 1.1  $\mu$ mole/l g dried cells, or 0.0884% = 0.09% of cell dry weight.

g. Steroids

No steroids were detected by the Lieberman-Burchardt reaction after alkaline hydrolysis and precipitation with digitonin of the sample from the non-saponified fraction of the lipid extract. No steroid compounds were detected on TLC by various tests.

h. Sugars

Quantitative tests failed to show any sugars using the anthrone reaction on neutral and complex lipid fractions and on their water-soluble hydrolysis products. No sugars were detected on TLC and paper chromatography by various tests.

i. Proteins

Proteins were present in the complex lipid fraction and they amounted to 0.73% of cell dry weight by the method of Lowry et al. (1951), using the Folin-Ciocalteu reagent.

j. Bacteriochlorophyll

Rhodospirillum rubrum contained only bacteriochlorophyll-a with absorption maxima at 358, 390, 577, and 769  $\mu$ m in acetone (Jensen, Aasmundrud, and Eimhjellen, 1964). The content of bacteriochlorophyll was estimated by using the mean value of  $O.D._{769} \times 2.58$  and  $O.D._{769} \times 2.19$ . This value expresses mg of bacteriochlorophyll per 100 ml of culture (Cohen-Bazire,

Sistrom, and Stanier, 1957). Because of the yield of cells in culture, 2 mg of dried cells were extracted with acetone-MeOH (7:2, v/v) instead of using a 5-ml aliquot of culture (4 g. dried cells is usually obtained from 10 liters of culture). It was found that the content of bacteriochlorophyll was 1.94% of the cell dry weight, or 21 umole per gram of dried cells.

## II. Non-extractable lipids

Non-extractable lipids are defined as the sum of the bound lipids which are liberated by alkaline hydrolysis and the poly-beta-hydroxybutyric acid which is extracted by boiling chloroform from the cell residue after extraction of free lipids. R. vannieli contained 2.9% of non-extractable lipids in dried cells.

### a. Bound lipids

The cell-residue after extraction of the free lipids was hydrolyzed in 3 N NaOH in methanol, acidified, pooled in diethyl ether, and esterified. The methyl esters of fatty acids in the bound lipid fraction were chromatographed on a silicic acid column to yield two fractions; fraction i contained nonpolar fatty acids and fraction ii contained polar hydroxy fatty acids which were eluted with 4% diethyl ether in petroleum ether and 20% diethyl ether in petroleum ether, respectively. The fatty acid components of each fraction were analyzed by GLC. Total fatty acids of bound lipids comprise 2.7% of the cell dry weight, or 36% of the total fatty acids of Rhodomicrobium vannieli. It was found that 1.8% of the total fatty acids of the



bound lipids were hydroxy acids. Fatty acids of the bound lipids were many in number and great in variety and they comprise 72.5% of the total bound lipids by weight (Table IV).

1. Fraction i: The non-polar fatty acids of the bound lipids

Fraction i contained odd-numbered, branched, and cyclopropane fatty acids along with straight-chain even-numbered saturated and unsaturated fatty acids. 72.5% of the bound lipids were in this fraction. Vaccenic acid comprised more than 35% of this fraction. Fatty acids were identified by conventional methods of GLC, by hydrogenation, by use of polar and non-polar columns, and by operation of the columns at different temperatures, comparing retention times with authentic compounds (Table V, Fig. 8).

The positions of the side chains of branched compounds were not established by  $\text{CrO}_3$ -oxidation because of limited sample material. Determination of the position of the cyclopropane ring by hydrogenation in glacial acetic acid and by establishing the presence of an infra-red absorption band at  $1026 \text{ cm}^{-1}$  characteristic of cyclopropane compounds could not be made for the same reason.

2. Fraction ii: Hydroxy fatty acids of the bound lipids

Fraction ii contained hydroxy fatty acids which represented 16.4 to 18% by weight of the bound lipid fatty acids. Three alpha-hydroxy and two beta-hydroxy fatty acids were identified by GLC, as described in the previous

section, using authentic hydroxy compounds synthesized for this purpose. It was found that beta-hydroxy fatty acids had a much longer retention time than did alpha-hydroxy acids on polar columns (DEGS), but had shorter retention times on the non-polar column (APL). Hydroxy compounds were easily distinguished from non-hydroxy compounds by this large difference in retention time on the different columns (see Fig. 9 and Table IV).

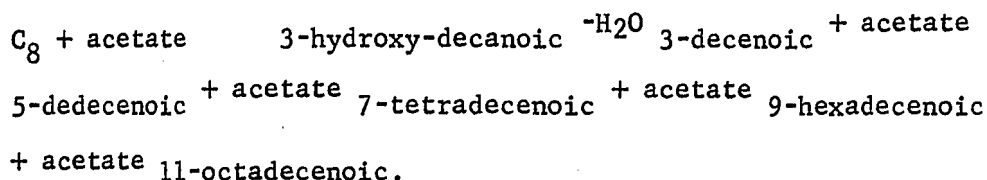
b. Poly-beta-hydroxybutyric acid

Poly-beta-hydroxybutyric acid was extracted from the solvent-extracted cell residue in boiling chloroform. By heating in concentrated sulfuric acid the material was converted to crotonic acid, which absorbs strongly at 235  $\mu$  and from which the content of poly-beta-hydroxybutyric acid was determined. It was found that the poly-beta-hydroxybutyric acid content of R. vanniellii was 0.2% of cell dry weight.

## DISCUSSION AND CONCLUSIONS

The principal fatty acid from the simple and complex lipids of R. vannielii is vaccenic acid, or 11-octadecenoic acid, and it forms approximately 90% of each fraction. This value is very close to that reported for Rps. spheroides and Rps. capsulata, although cells were grown in different media (Wood, Nichols, and James, 1965). Except for Micrococcus halodenitrificans, no other heterotrophs have been reported to have a vaccenic acid content greater than 50%. A high content of vaccenic acid appears to be characteristic of photosynthetic bacteria, although there are two exceptions, Rsp. rubrum with 37.3%, and Rps. gelatinosa with 6.2% (Wood, Nichols, and James, 1965). However, bound lipids of Rhodomicrobium contained only 35.8% vaccenic acid.

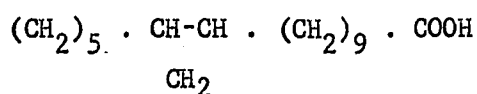
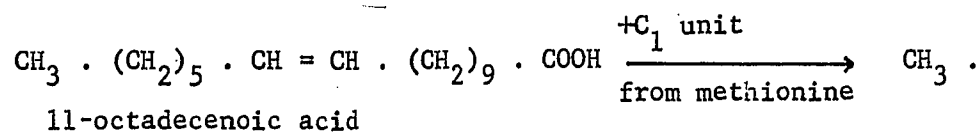
The position of the double bond tetradecenoic acid (C<sub>14:1</sub>) and hexadecenoic acid (C<sub>16:1</sub>) in the extractable lipids was not determined because of their trace amount (less than 1%). However, they could be predicted to be 7-tetradecenoic and 9-hexadecenoic acid from the summary of various reports (Kates, 1964). The pathway of monoene synthesis in R. vannielii probably may be fitted to the schemes of Block, Baronowsky, Goldfine, Lenarz, Light, Norris, and Scheuerbrandt (1961) and of Wood, Nicholes, and James (1965):



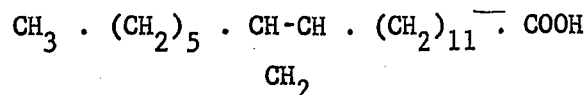
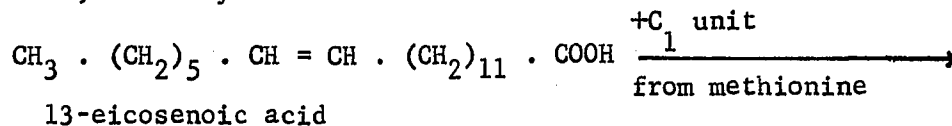
It was found that the fatty acids of the extractable lipids are

only even-numbered straight-chain forms of saturated and monounsaturated compounds, whereas the bound lipids contain many fatty acid components including cyclopropane-, branched-, odd- and even-numbered saturated and unsaturated fatty acids, and alpha- and beta-hydroxy acids. In the hydroxy acid fraction, C<sub>12:0</sub> hydroxy acids form approximately 60% of the total: 24% is 2-hydroxydodecanoic acid and 34.5% is 3-hydroxydodecanoic acid. These compounds were found in the bound lipids of Azotobacter agilis (Kaneshiro and Marr, 1963).

C<sub>19</sub> and C<sub>21</sub> cyclopropane fatty acids were found in Rhodomicrobium, but the positions of the methylene groups were not established because of their small amounts. However, it may be assumed that the cyclopropane rings are derived from the methyl groups of methionine, and from 11-octa-decenoic and 13-eicosenoic acid, respectively (O'Leary, 1959 and 1962; Liu and Hofman, 1962; Law, Zalkin, and Kaneshiro, 1963).



11, 12-methylene octadecanoic acid



13, 14-methylene eicosanoic acid

Two branched acids (C<sub>15</sub> and C<sub>17</sub>) were found in the cell residue of solvent extraction; these comprise a minor part of bound lipids, 16.7%

of total non-polar fatty acids. This contrasts with the report of Huston and Albro (1964), who found that the major group of fatty acids in the extractable lipid fraction of Sarcina lutea were branched acids which comprise over 40% of the total fatty acids.

The phospholipid content of R. vanniellii is 70% of the extractable total glycerides. The phospholipids fraction yields 8 components, 7 of which contain phosphorus. The major phospholipid of R. vanniellii is the lipoamino acid consisting of only one amino acid, L-ornithine, i.e. the o-ornithine ester of phosphatidyl glycerol (ca. 50% of the total phospholipids). The compound has the same R<sub>f</sub> as phosphatidyl ethanolamine on TLC by single dimension development. It was, therefore, initially identified erroneously as phosphatidyl ethanolamine, even when using authentic phosphatidyl ethanolamine. Macfarlane (1964) defined the term "lipoamino acid" as those compounds characteristically lipid in solubility, in which a single amino acid is linked either through the amino- or carboxyl group to a lipid moiety containing a long chain fatty acid (C<sub>10</sub> or more). Ornithine-containing lipoamino acids in bacteria have been recently reported in Bacillus megaterium, B. cereus, Serratia marcescens, and Pseudomonas stutzeri (Houtsmuller and Van Deenen, 1963), Clostridium perfringens (Macfarlane, 1962), Rps. spheroides (Gorchein, 1964), and Rps. rubrum, Rps. gelatinosa, Rps. capsulata and Rps. palustris (Wood, Nichols, and James, 1965). Other amino acids found in other bacterial lipoamino acids were reported to be basic diamino acids such as lysine, ornithine, and arginine. The phosphorus-free lipoamino acid, N, N-diacyl ornithine, in R. vanniellii is probably

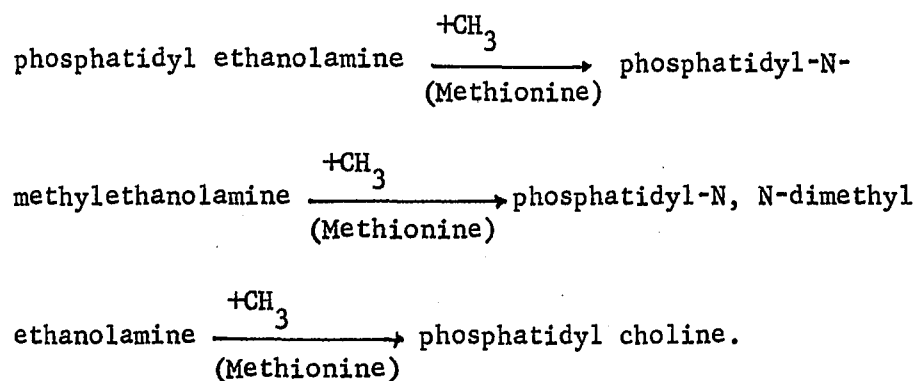
similar to that in Rps. spheroides reported by Gorchein (1964) and in Mycobacterium phlei reported by Laneelle, Laneelle, and Asselineau (1963). The role and biosynthetic pathway of lipoamino acid are of considerable interest.

Phosphatidyl ethanolamine of R. vanniellii is an unusually minor component, whereas it is a major phospholipid component in other organisms, e.g., Serratia marcescens (Kates, Adams, and Martin, 1964), B. megaterium (97% of phospholipids; Opden Kamp, Houtsmuller, and Van Deenen, 1965), E. coli (Law, 1961), Azotobacter agilis and Agrobacterium tumefaciens (Kaneshiro and Marr, 1962), and Rps. spheroides (40% of phospholipids; Lascelles and Szilagyi, 1965). It is, however, entirely absent in some organisms, such as Sarcina lutea (Huston, Albro, and Grindey, 1965) and in some lactic acid bacteria (Ikawa, 1963).

Cardiolipin in the photosynthetic bacteria was reported in Rps. spheroides, Rps. palustris, and Rsp. rubrum (Wood, Nichols, and James, 1965). It is absent in R. vanniellii. Bis-phosphatidic acid is present, however. The Rfs of these two components in TLC are quite close to each other. Bis-phosphatidic acid was reported as the phospholipid component in vegetative cells and spores of B. polymixa (Matches, Walker, and Ayres, 1964).

Among the heterotrophs, phosphatidyl choline had been reported in only one family of bacteria, the Agrobacteriaceae (A. tumefaciens, Kaneshiro and Marr, 1962; A. rhizogenes and A. radiobacter, Goldfine and Ellis, 1964). Phosphatidyl choline, however, was recently reported in Rps. spheroides (Lascelles and Szilagyi, 1965) and Rps. spheroides, Rps. capsulata, and Rps. palustris (Wood, Nichols, and James, 1965). In

R. vannielii, phosphatidyl choline is the second-largest component of the phospholipids (26.5% of the total). From these data, photosynthetic bacteria may be partially characterized as organisms which synthesize an appreciable amount of phosphatidyl choline. N-methyl ethanolamine and N, N-dimethylethanolamine were identified by transmethylation reaction in various bacteria grown in the presence of methyl-labeled methionine (Law, Zalkin, and Kaneshiro, 1963; Goldfine and Ellis, 1964; and Goldfine, 1962). These studies confirmed the proposed metabolic pathway for metabolism of phospholipids and methyl groups by Bremer, Higard, and Greenberg (1960), and for the stepwise conversion of phosphatidyl ethanolamine to phosphatidyl choline by transmethylation reaction involving S-adenosyl methionine (Bremer and Greenberg, 1961).



It is likely that R. vannielii also synthesizes the choline-containing phospholipid through the same pathway, although hydrolyzate of phospholipids was tested by paper chromatography with authentic N-methyl ethanolamine and N, N-dimethyl ethanolamine without detecting these intermediates. Confirmation might be made by using methyl-labeled methionine or S-adenosyl methionine- $\text{C}^{14}\text{H}_3$ .

CoQ of R. vanniellii was previously reported as CoQ<sub>10</sub> by Carr and Exell (1965). In this study, however, different results were obtained. CoQ was found. It was crystallized and its identity confirmed by M. P. and its properties on paraffin oil-coated reverse phase chromatograms. This discrepancy may be due either to strain differences or to differences in cultural conditions. CoQ is found characteristically in gram negative bacteria, whereas it is absent or present in trace amounts in gram positive bacteria, which contain vitamin K instead (Hatefi, 1963). It was reported that CoQ is especially concentrated in the electron transport particles of Azotobacter vinelandii (Lester and Crane, 1959). Vernon (1963) studied photooxidation and photoreduction reactions in purple photosynthetic bacteria, and suggested that CoQ is a cofactor of photophosphorylation in photosynthetic bacteria which transfers electrons between ferredoxin and/or flavoprotein and respiratory heme proteins, the cytochrome system.

The bacteriochlorophyll of R. vanniellii was found to be type a. It is present in the amount of 20  $\mu\text{M}$  per g. of cell dry weight. The ratio of BChl./CoQ is 20:1. This is comparable to the result of Carr and Exell (1965), who obtained 14  $\mu\text{M}/\text{g}$ . cell dry weight and a ratio of BChl./CoQ of 36:1. The difference may be due to the difference in strain or in cultural conditions. Caution should be paid to protecting the preparation from direct light as much as possible during isolation, because BChl. is very unstable in light.

Eight carotenoid components were separated from R. vanniellii and 7 of them were identified: lycopene (20.5%) and rhodopin (61.3%) are the major components, and the others were B-carotene, anhydroxanthin,



spirilloxanthin, rhodovibrin, and monodemethylated spirilloxanthin. B-carotene is characteristic for R. vannielii. This carotenoid has been found only in oxygen-evolving organisms. Further studies on structural and chemical properties of individual carotenoids were not made, because during the course of this study Ryvarden and Jensen (1964) reported the complete analysis of the carotenoids of this organism. They found that the components and their amounts were markedly influenced by the cultural conditions.

Carotenoid synthesis in R. vannielii is mainly in the normal spirilloxanthin series (Goodwin, 1965), comprising lycopene, rhodopin, 3,4-dihydroxyrhodopin, anhydro-rhodovibrin, rhodovibrin, monodemethylated spirilloxanthin, and spirilloxanthin, although a minor component of B-carotene is synthesized as the bicyclic carotenoid. Therefore, it can be concluded that R. vannielii is characterized by a high content of hydroxylated carotenoids and the major components contain the lycopene chromophore. Knowledge of the biosynthetic pathway of B-carotene would be of further interest, that is, whether it occurs by the same pathway as in the algae and higher plants or by some alternate pathway.

Carotenoids were found to function with chlorophyll in photochemical reactions (Lundegardh, 1966) in converting light energy to form polyphosphate high energy bonds, in photophosphorylation (Arnon, 1963). They function in phototaxis (Goodwin, 1959), and in the protection of cells from damage caused by the incidental absorption of visible light by other pigments in bacteria (Stanier and Cohen-Bazire, 1957).

Poly-B-hydroxy butyric acid was determined as 0.2% of cell dry weight from the cell residue after solvent-extraction. This value is

quite low in comparison with some organisms, e.g., 6% in Rsp. rubrum has been reported (Stanier, Doudoraff, Kunizawa, and Contopoulou, 1959). They found that practically all the acetate assimilated was converted to poly-B-hydroxy butyrate in the absence of CO<sub>2</sub>. However, acetate was not used as the major hydrogen donor in this study. Further studies will be needed to determine what effect CO<sub>2</sub> and various H-donors have on poly-B-hydroxy butyric acid metabolism.

No steroids were detected in R. vanniellii. No definitive report exists for the presence of steroids in bacteria. Levin and Block (1964) failed to detect steroids in blue green algae. It has been suggested that sterols are involved in the structure of intracellular membranes such as the nuclear membrane in eukaryotic cellular organisms (Stanier, 1961), and it may be significant that both sterols and such a membrane are absent from the bacteria and blue-green algae, the prokaryotic organisms.

Sulfolipid is found in R. vanniellii. 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.

## SUMMARY

1. Cells of Rhodomicrobium vannielii grown at 29° in a lactate-containing medium were first extracted at room temperature with organic solvents; this extractable fraction contains the bulk of the simple lipids (1.8%) and complex lipids (phospholipids, 4.2%, and sulfolipid, 0.01%), coenzyme Q (0.09%), and pigments (carotenoids, 1.2%, and bacteriochlorophyll, 1.9%). The cell residue contains the bound lipids (2.7%) which are liberated by treatment with alkali. The residue also contains poly-beta-hydroxybutyric acid (0.2%) which is extracted in boiling chloroform.

2. The fatty acids in the solvent-extractable material were determined by gas-liquid chromatography. Olefinic acids were identified by their susceptibility to hydrogenation. The position of the olefinic bond in monounsaturated acids was determined by performic and periodate oxidations and characterized by the resulting free-aldehyde cleavage products. In both the simple and complex lipids, vaccenic acid (11-octadecenoic acid) is the largest single component (approximately 90% in each fraction). The bound lipids contain many fatty acid components, including cyclopropane-, branched-, and alpha- and beta-hydroxy fatty acids. They were identified by comparison with authentic compounds which were synthesized for this purpose.

3. Coenzyme Q from R. vannielii was previously reported as CoQ<sub>10</sub>. Different results were obtained, however, from this strain. Purification of CoQ resulted in a crystalline product which melted at 45° (the reported value for Q<sub>9</sub> is 45.2°C) and which migrated on paraffin-oil

coated reverse-phase paper chromatograms with an  $R_f$  corresponding to that reported for  $Q_9$ .

4. Seven carotenoids were separated and characterized by their fractionation patterns (polarity), absorption spectra, and partition coefficients; as previously reported, rhodopin (61.3%) and lycopene (20.5% of total carotenoids by wt.) are the major constituents. The production of a small amount of beta-carotene is unique in *R. vannielii*; this substance is usually associated with oxygen-evolving organisms. The other four minor carotenoid components are anhydro-rhodovibrin, rhodovibrin, monodemethylated spirilloxanthin, and spirilloxanthin, which are found in the biosynthesis pathway of the normal spirilloxanthin series. Carotenoids of *R. vannielii* are characterized by a high content of hydroxylated carotenoids; the major components represent the lycopene chromophore.

5. Of the bacteriochlorophylls, only type a was found; its content was determined to be 20 umole/gram cell dry weight.

6. Steroids were not detected.

7. Eight components in the phospholipid fraction were isolated and identified; seven of these contained phosphorus with a lipoamino acid as the major component. This material, an o-ornithine ester of phosphatidyl glycerol, was about 50% by weight of the total phospholipids. Phosphatidyl choline was present in appreciable amounts (26.5%). The rest of the minor components are phosphatidic acid (1.83%), bis-phosphatidic acid (6.75%), phosphatidyl ethanolamine (4.5%), phosphatidyl glycerol (9.7%), an ornithine amide of an undetermined fatty acid (0.95%), and an ornithine ester of lysophosphatidyl glycerol (3.2%).

8. The sulfolipid of R. vanniellii represents 0.01% of cell dry weight.

TABLE I. FATTY ACID COMPOSITION OF EXTRACTABLE LIPIDS

Compound	% (by weight) of Neutral Lipids	% (by weight) of Complex Lipids
C <sub>10:0</sub>	0.10	-
C <sub>12:0</sub>	0.30	-
C <sub>14:0</sub>	1.75	0.70
C <sub>16:0</sub>	5.40	6.25
C <sub>18:0</sub>	0.11	3.80
C <sub>14:1</sub>	0.50	-
C <sub>16:1</sub>	1.08	0.35
C <sub>18:1</sub>	88.20	89.0
C <sub>18:2</sub>	2.70	-

Fatty acid composition was determined by GLC using polar- and non-polar columns (see Figs. 3-1 and 3-2). The amount of each component was measured by planimetry in comparison with standard compounds by the methods of Horning et al. (1964). The conditions for GLC are described in Figs. 3-1 and 3-2.

TABLE II. CHARACTERIZATION OF PHOSPHOLIPIDS

Spot	A	B	C-1	C-2	D-1	D-2	E-1	E-2	
TLC Rf*	0.87	0.72		0.53		0.38	0.31	0.22	0.09
Relative amount %**	1.83	6.75	4.50		46.5	9.70	0.91	26.5	3.2
Rhodamin 6G***	Blue	Blue	Yellow		Yellow	Blue	Yellow	Yellow	Blue
Reinecke Salt	-	-	-		-	-	-	+	-
Dragendorff's Reagent	-	-	-		-	-	-	+	-
Dipicrylamine	-	-	-		-	-	-	+	-
Ninhydrin	-	-	+		+	-	+	-	+
NH <sub>4</sub> -Molybdate	+	+	+		+	+	-	+	+
1 R 968 <sup>cm-1</sup>	-	-	-		-	-	-	+	-
Hemolysis	-	-	-		-	-	-	-	+
Iodine vapor	+	+	+		+	+	+	+	+
Esterbond/P Molar Ratio	1.97:1	4:1	1:1		2.7:1	2.2:1	-	1.9:1	?
P:Amino N: Total N, Molar Ratio	-	-	1:1:1		1:2:2	-	-	1.1:0:1	?

\*Rf values were measured on silica gel-H plate (5 x 20 cm) developed with CHCl<sub>3</sub>-MeOH-AcOH-H<sub>2</sub>O (250:74:19:3, v/v/v/v).

\*\*Based on vaccenic acid as the fatty acid component.

\*\*\*Rhodamine 6G tests were done on chromatograms of silicic acid impregnated paper developed with diisobutyl ketone-acetic acid-water (40:20:3, v/v/v). The spots were observed under UV light.

TABLE III. CHARACTERIZATION OF CAROTENOIDS

Component	(1) Rf	Absorbent in Column	Color of Column Zone	(2) Eluting Solvent	(3) Absorption Maxima mu	(4) % of Total Carotenoids	(5) Partition Coefficient	Identified as
1	0.93	Silicic acid	Yellow	1.5%	449, 478	3.4	100:0	B-carotene
2	0.78	Silicic acid	Orange-Pink	4%	446, 472, 503	20.5	100:0	Lycopene
3	0.65	Al <sub>2</sub> O <sub>3</sub>	Orange-Red	4%	460, 485, 520	1.8	95:5	Anhydro- rhodovi- brin
4	0.46	Al <sub>2</sub> O <sub>3</sub>	Red	5-7%	468, 497, 530	11.2	90:10	Spirillo- xanthin
5	0.32	Al <sub>2</sub> O <sub>3</sub>	Orange-Pink	8-12%	448, 472, 502	61.3	75:25	Rhodopin
6	0.23	Al <sub>2</sub> O <sub>3</sub>	Orange-Yellow	15%	457, 487, 520	0.5	--	Rhodo- vibrin
7	0.09	Al <sub>2</sub> O <sub>3</sub>	Orange	30%	466, 497, 530	0.8	42:58	Monode- methyl- ated spirillo- xanthin
8	0.03	Al <sub>2</sub> O <sub>3</sub>	Yellow	40%	---	trace	--	Unidenti- fied

TABLE IV. FATTY ACIDS OF BOUND LIPIDS

Compound	Fraction i		Fraction ii		
	% of Fraction i	Compound	% of Fraction i	Compound	
C <sub>14:0</sub>	10.3	C <sub>15:B</sub>	6.9	Alpha-OH-C <sub>12:0</sub>	24.0
C <sub>16:0</sub>	3.7	C <sub>17:B</sub>	9.8	Alpha-OH-C <sub>13:0</sub>	10.3
C <sub>17:0</sub>	1.0	C <sub>15:1</sub>	6.9	Alpha-OH-C <sub>14:0</sub>	6.9
C <sub>18:0</sub>	2.5	C <sub>16:1</sub>	1.1	Beta-OH-C <sub>12:0</sub>	34.5
C <sub>20:0</sub>	0.9	C <sub>18:1</sub>	35.7	Beta-OH-C <sub>14:0</sub>	6.9
C <sub>22:0</sub>	8.0	C <sub>20:1</sub>	1.6	Alpha-OH-C <sub>13:B</sub>	17.2
C <sub>19:CP</sub>	4.4	C <sub>22:1</sub>	4.4		
C <sub>21:CP</sub>	8.0				

Fatty acid composition of bound lipids was determined by GLC, using polar and non-polar columns at different temperatures (see Figs. 8 and 9). The amount of each component was measured by planimetry in comparison with standard compounds by the method of Horning *et al.* (1964). The conditions of GLC are also described in Figs. 8 and 9.



TABLE V. SEPARATION FACTORS OF NON-POLAR FATTY ACIDS OF BOUND LIPIDS

GLC Peak	Methyl Esters	Separation Factors		Identified as
		168°C	182°C	
a	a/11:0	1.43	1.35	12:0
b	b/13:0	1.41	1.33	14:0
c	c/15:0	1.44	1.35	16:0
d	d/16:0	1.43	1.34	17:0
e	e/17:0	1.43	1.35	18:0
f	f/19:0	1.42	1.35	20:0
g	g/21:0	1.43	1.35	22:0
h	h/15:0	1.15	1.17	15:1
i	i/17:0	1.15	1.16	17:1
j	j/18:0	1.16	1.17	18:1
k	k/20:0	1.16	1.18	20:1
l	l/22:0	1.15	1.17	22:1
m	m/14:0	1.20	1.18	15:Branch
n	n/16:0	1.20	1.18	17:Branch
o	o/19:0	1.28	1.27	19:Cyclo
p	p/21:0	1.28	1.27	21:Cyclo

Column size:  $\frac{1}{4}$ " x 5'

Solid support: Fire Brick

Liquid phase: 30% DEGS

Carrier gas: He

Flow rate: 100 ml/min

Column temp.: 168°C

182°C

Detector temp.: 277°C

282°C

Injector temp.: 222°C

239°C

Detect current: 150 mA

150 mA

Gas-liquid chromatographs (see Figs. 8, 9, and 11).

FIG. 1 EXTRACTION OF LIPIDS

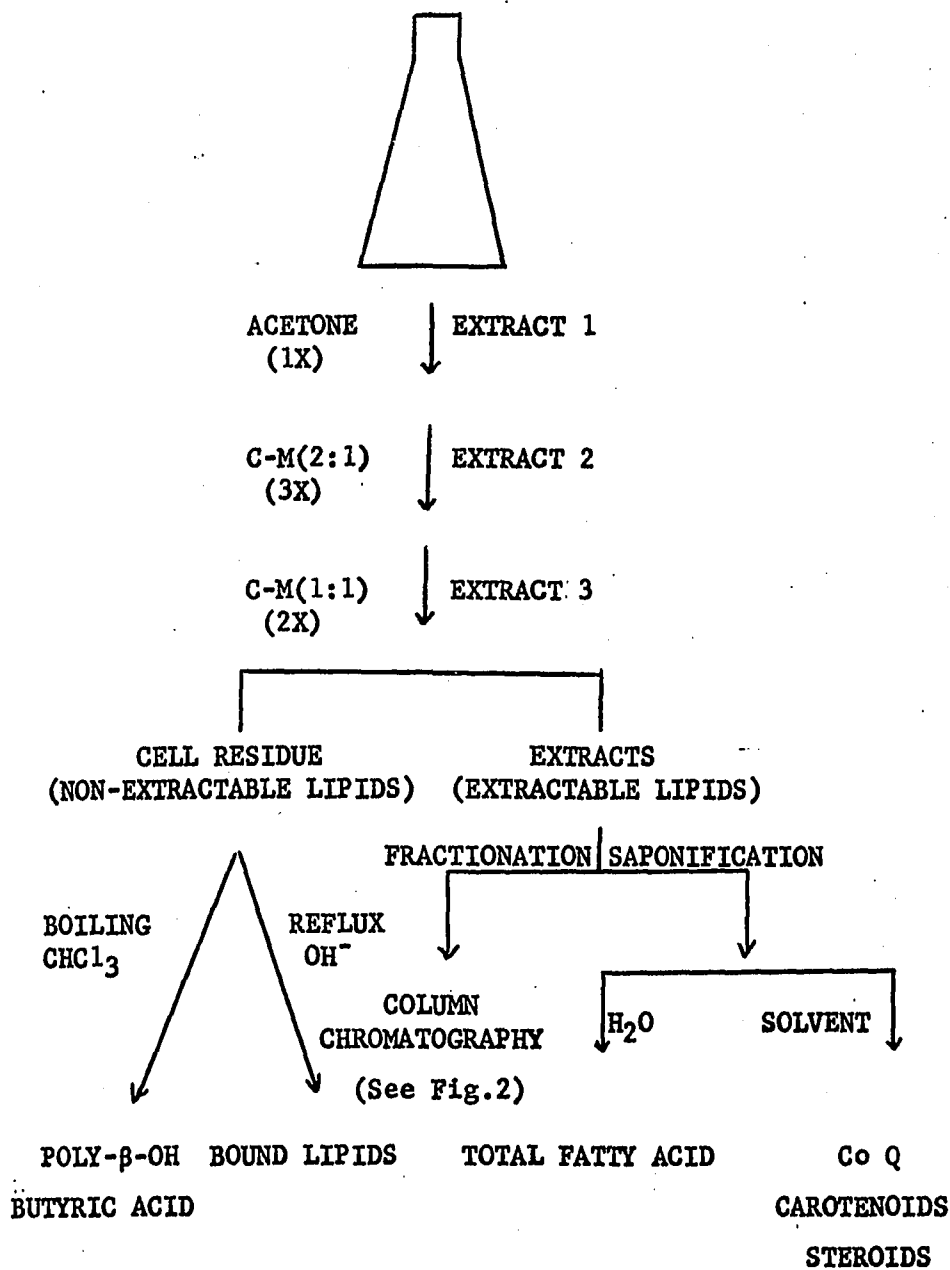


FIG. 2 COLUMN FRACTIONATION OF EXTRACTABLE LIPIDS

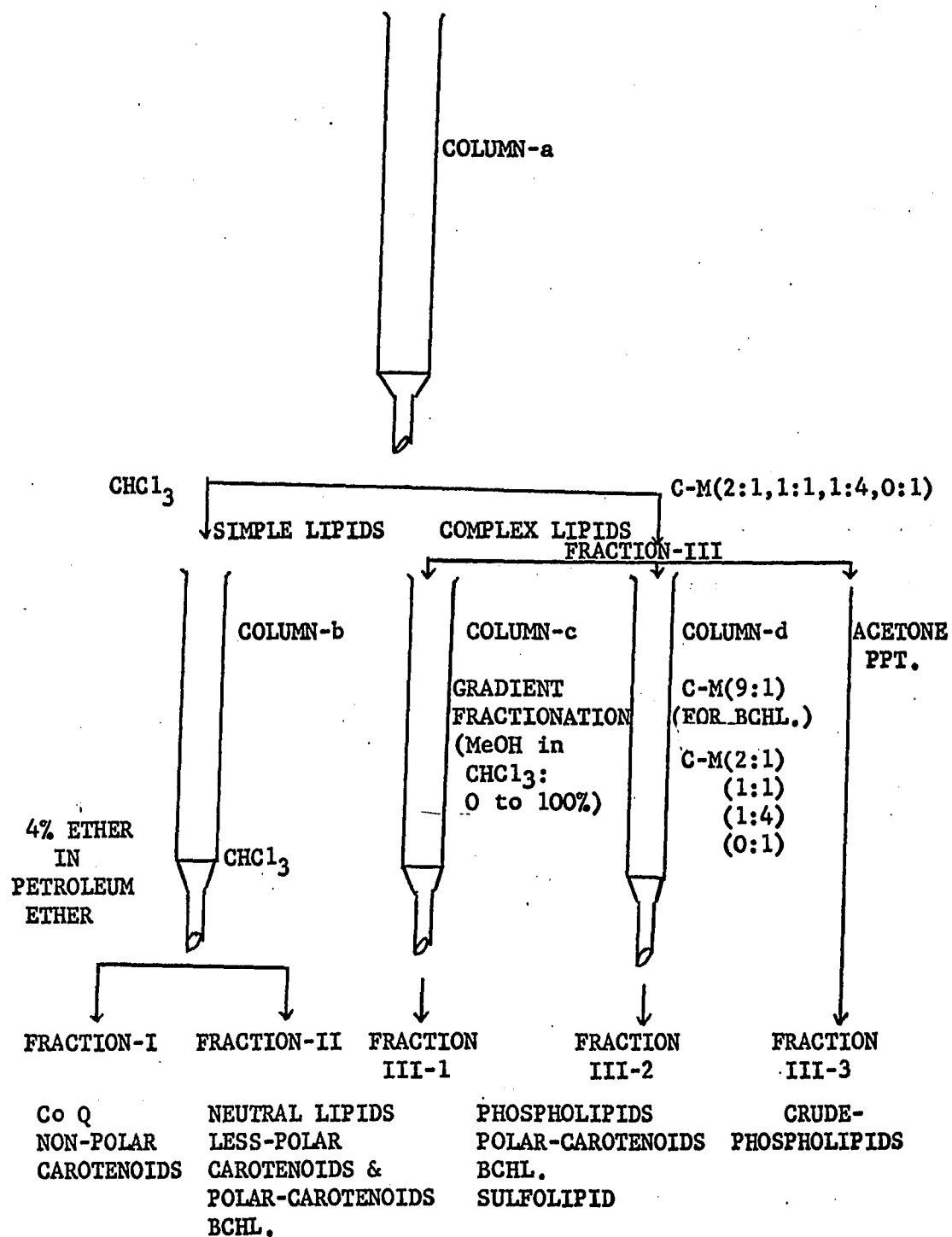
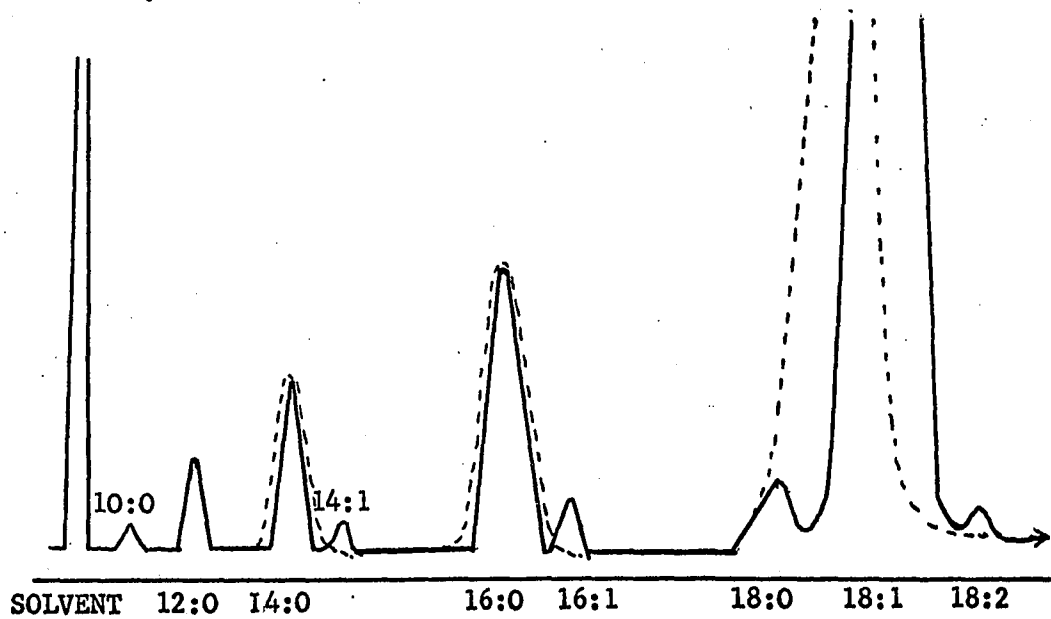


FIG. 3-1 GAS-LIQUID CHROMATOGRAPHS OF FATTY ACIDS OF  
SIMPLE LIPIDS

A. DEGS COLUMN



B. APL COLUMN

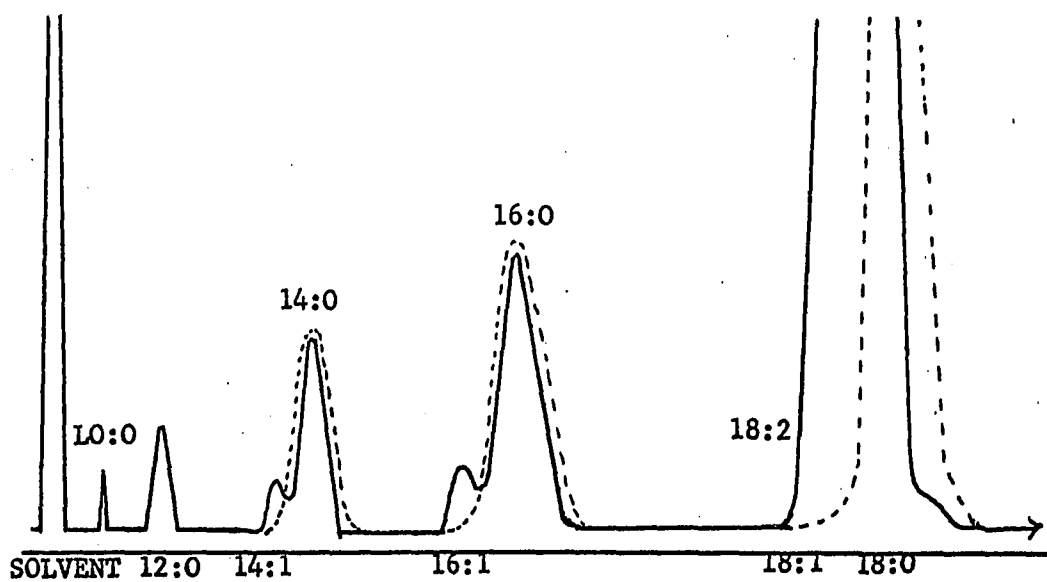
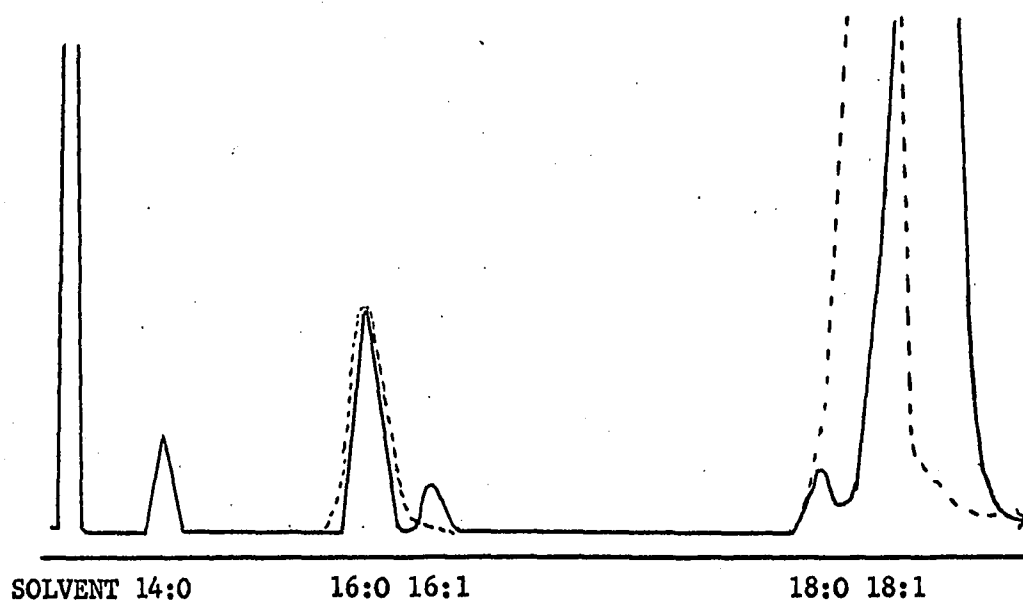


FIG. 3-2 GAS-LIQUID CHROMATOGRAPHS OF COMPLEX LIPIDS  
(METHYL ESTERS OF FATTY ACIDS)

A. DEGS COLUMN



B. APL COLUMN

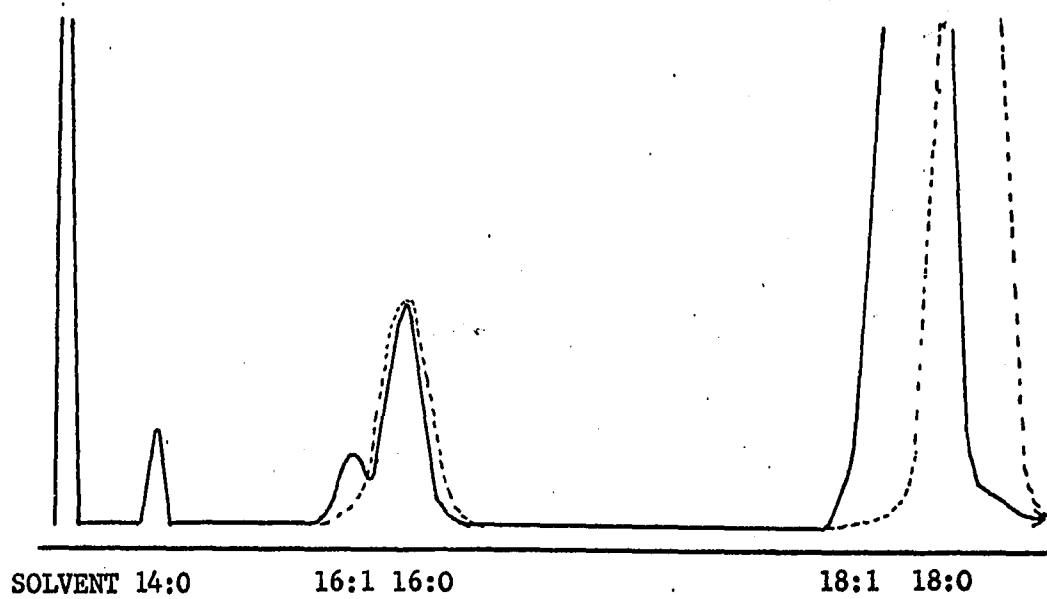
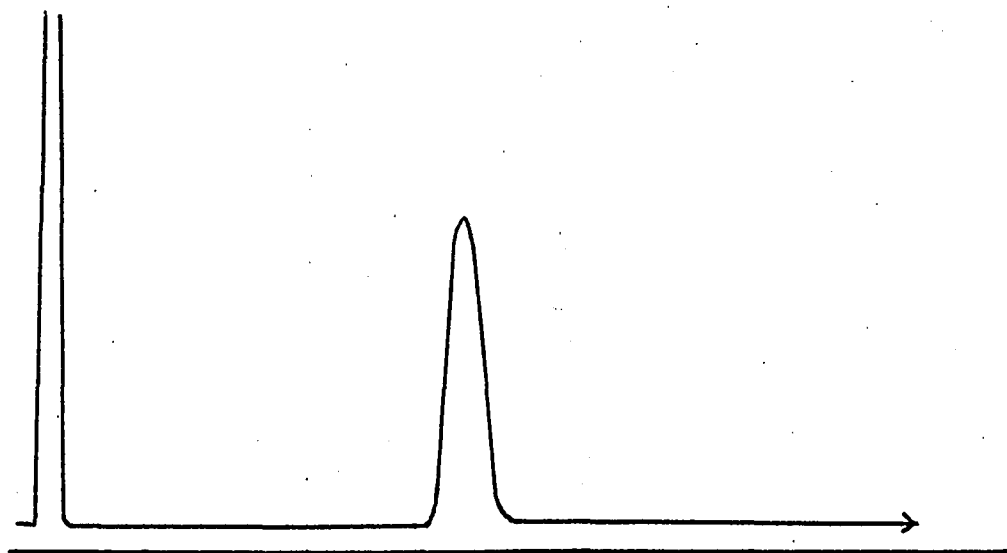


FIG. 4 GAS-LIQUID CHROMATOGRAPHS OF ALDEHYDES

## A. FROM 18:1 FATTY ACID



## B. STANDARDS

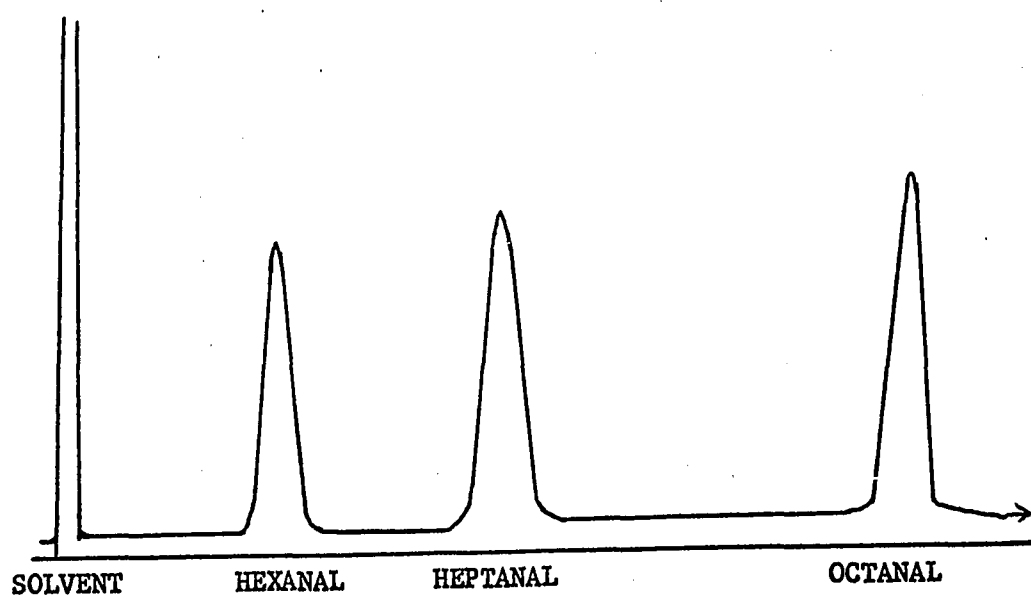


FIG. 5 GRADIENT FRACTIONATION OF PHOSPHOLIPIDS

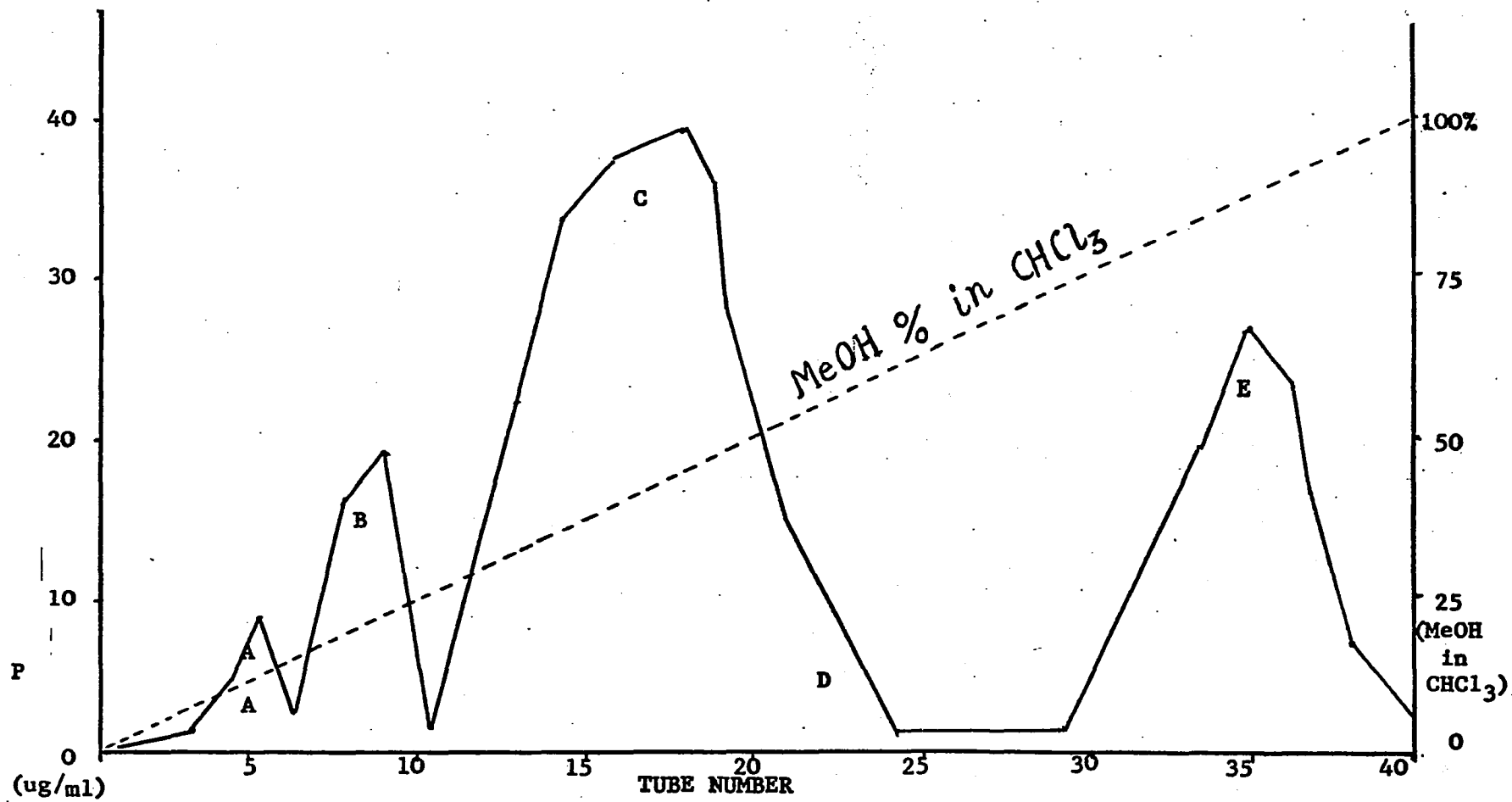


FIG. 6 THIN-LAYER CHROMATOGRAM OF PHOSPHOLIPIDS

(ONE DIMENSION)

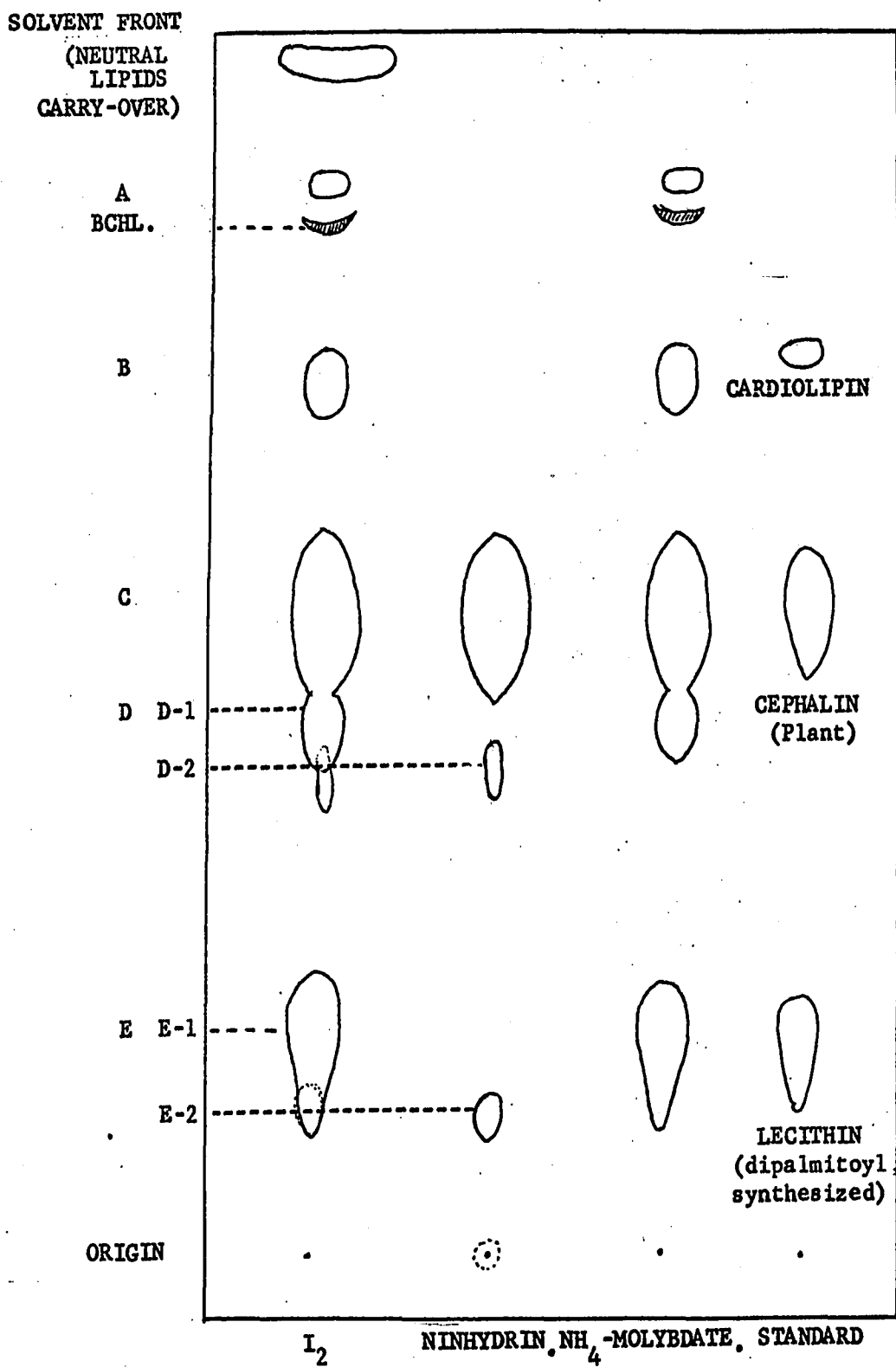




FIG. 7 THIN-LAYER CHROMATOGRAM OF PHOSPHOLIPIDS  
( TWO DIMENSIONS )

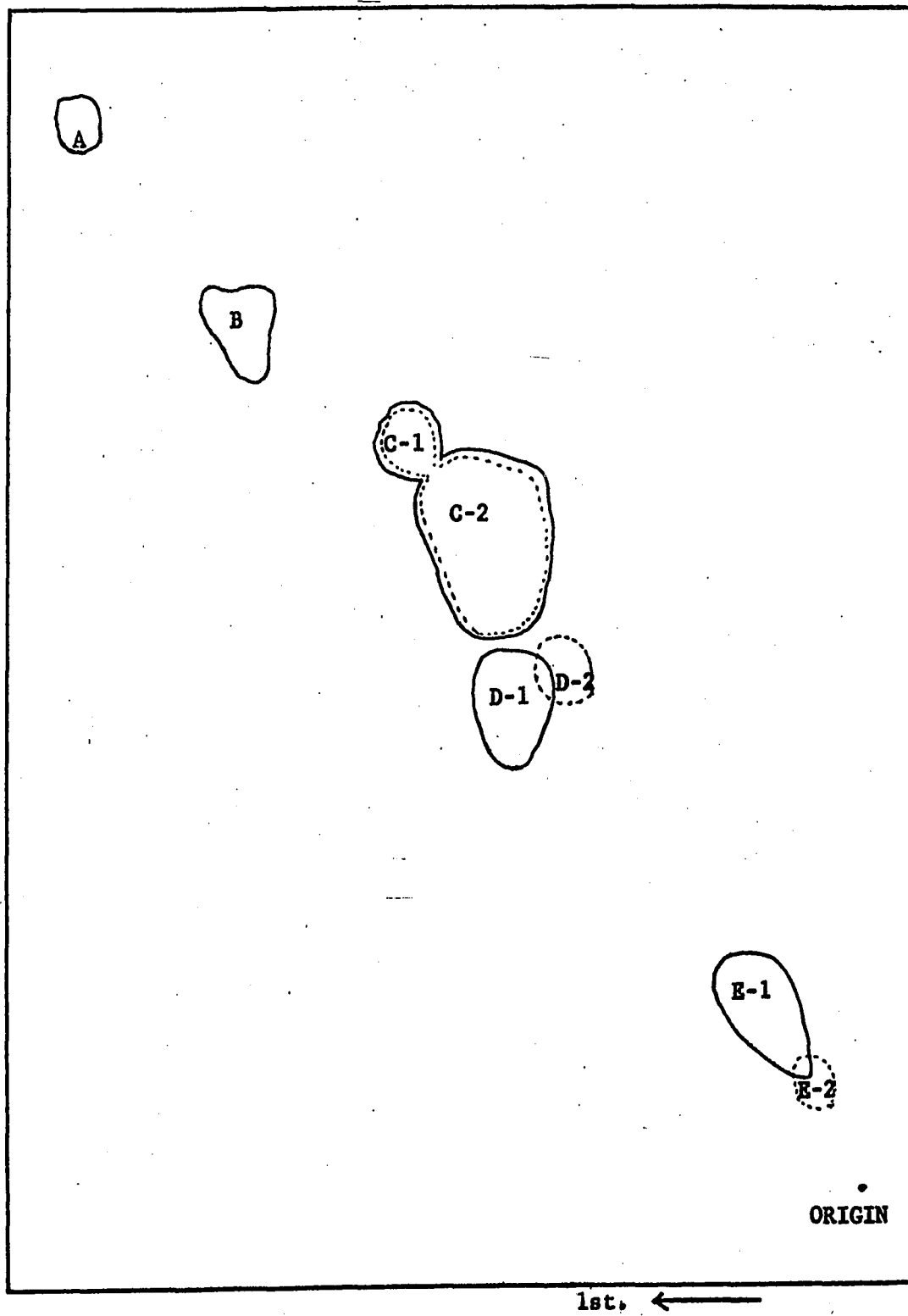
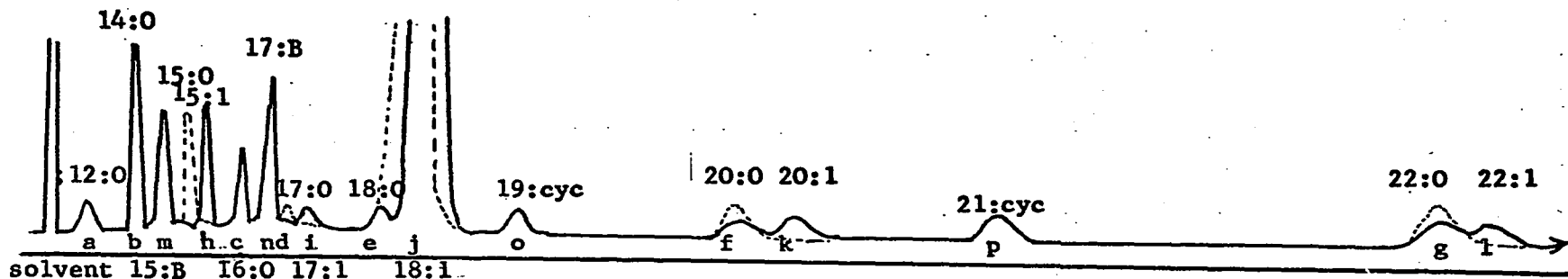
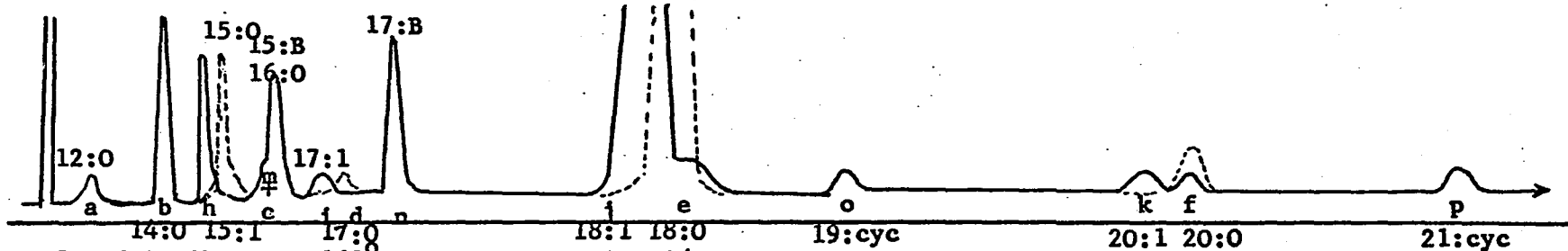


FIG. 8 GAS-LIQUID CHROMATOGRAPHS OF NON-POLAR FATTY ACIDS FROM BOUND LIPIDS

A. DEGS COLUMN AT 182°C IN FLOW RATE 100ml/min.



B. APL COLUMN AT 215°C IN FLOW RATE 100ml/min.



C. DEGS COLUMN AT 168°C IN FLOW RATE 100ml/min.

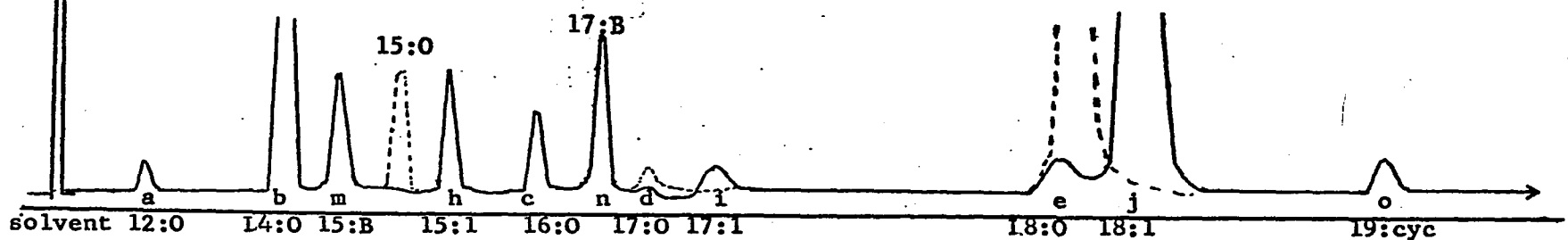
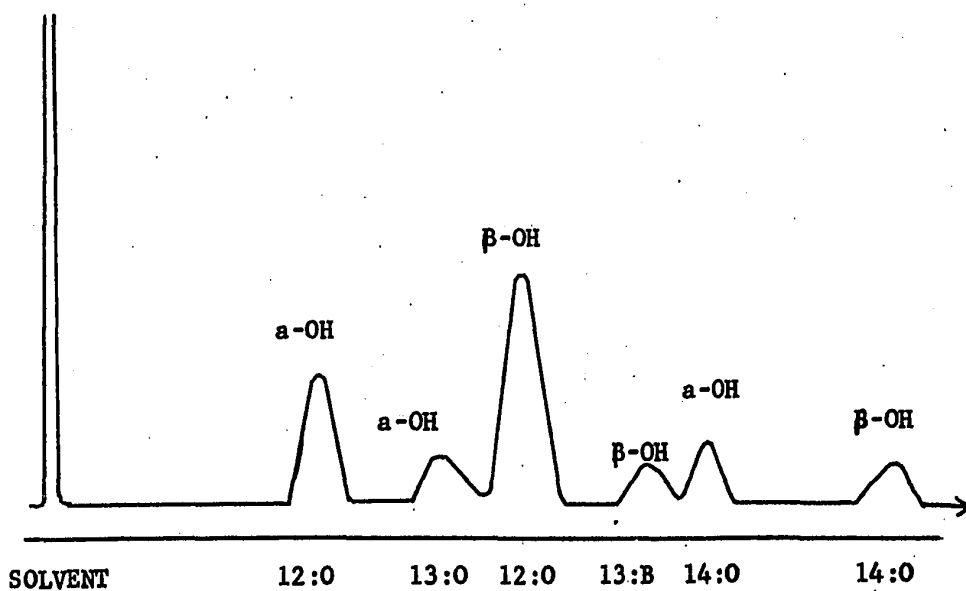


FIG. 9 GAS-LIQUID CHROMATOGRAPHS OF HYDRXY ACIDS  
FROM BOUND LIPIDS

A. DEGS COLUMN



B. AFL COLUMN

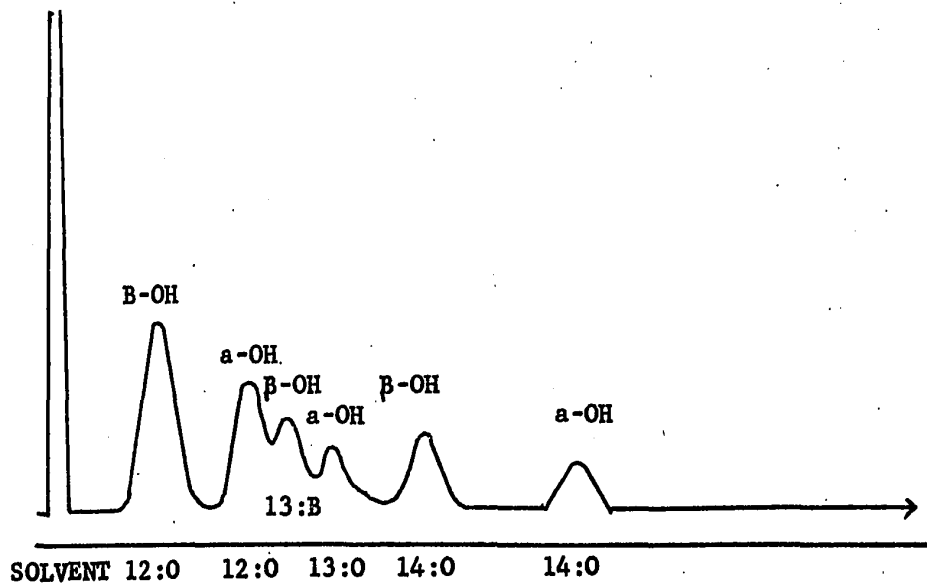


FIG. 10 THIN-LAYER CHROMATOGRAM OF CAROTENOIDS

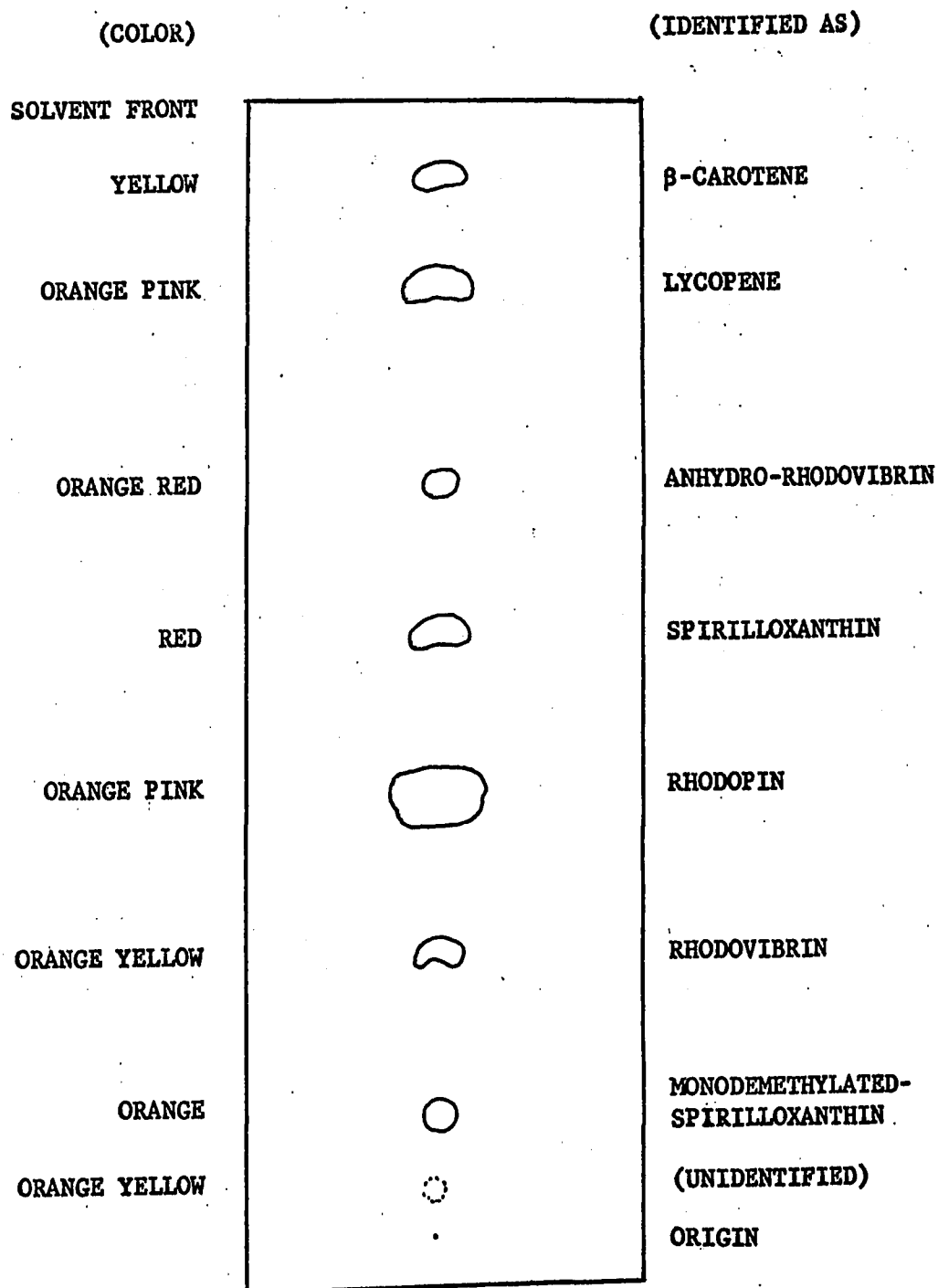
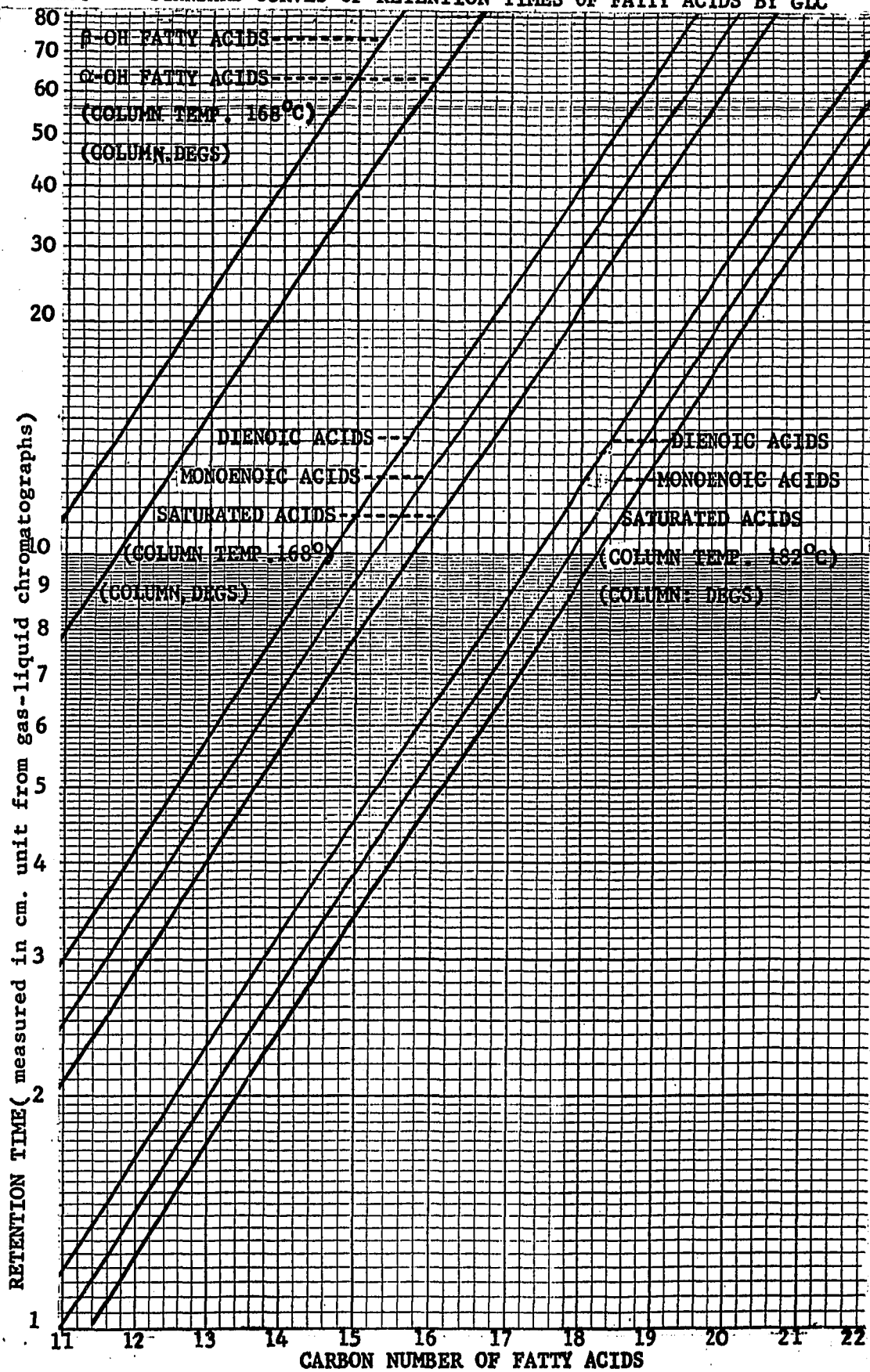


FIG. 11 STANDARD CURVES OF RETENTION TIMES OF FATTY ACIDS BY GLC



## BIBLIOGRAPHY

- Abramson, D. and M. Blecher. 1964. Quantitative two-dimensional thin-layer chromatography. *J. Lipid Res.* 5: 628-631.
- Arnon, D. J. 1963. Photosynthetic electron transport and phosphorylation in chloroplasts. National Academy of Science - National Res. Council publ. 1145: 195-212.
- Bard, R. C. and L. S. McClung. 1948. Biochemical properties of the toxins of Clostridium novyi and C. hemolyticum. *J. Bacteriol.* 56: 665-670.
- Bartlet, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* 234: 466-468.
- Beiss, U. 1963. Phosphatide und Glykolipide, 6 Band P. 52-82. In K. Paech und M. V. Tracey (ed.), *Moderne Methoden der Pflanzen Analyse*. Springer-Verlag, Berlin.
- Benson, A. A., H. Daniel, and R. Wiser. 1959. A sulfolipid in plants. *Proc. Natl. Acad. Sci.* 45: 1582-1587.
- Bertolacini, R. J. and J. E. Barney II. 1957. Colorimetric determination of sulfate with barium chloranilate. *Anal. Chem.* 29: 281-283.
- Block, K., P. Baranowsky, H. Goldfine, W. J. Lennarz, R. Light, A. T. Norris, and G. Scheuerbrandt. 1961. Biosynthesis and metabolism of unsaturated fatty acids. *Federation Proc.* 20: 921-927.
- Boatman, E. S. and H. C. Douglas. 1961. Fine structure of the photosynthetic bacterium Rhodomicrobium vannielii. *J. Biophys. Biochem. Cytol.* 11: 469-483.

- Bremer, J. and D. M. Greenberg. 1961. Methyl transferring enzyme system of microsomes in the biosynthesis of lecithin. *Biochem. Biophys. Acta* 46: 205-216.
- Bremer, J., D. H. Higard, and D. M. Greenberg. 1960. The biosynthesis of choline and its relation to phospholipid metabolism. *Biochim. Biophys. Acta* 43: 477-488.
- Carr, N. G. and G. Exell. 1965. Ubiquinone concentration in *Athiorhodaceae* grown under various environmental conditions. *Biochem. J.* 96: 688-692.
- Cason, J. N., L. Allinger, and G. Sumrell. 1953. Branched-chain fatty acids XXV. Methods of synthesis of 2-alkenoic acid. *J. Org. Chem.* 18: 850-856.
- Cavanaugh, D. J. and D. Glick. 1952. Determination of cholesterol in microgram quantities of tissue. *Anal. Chem.* 24: 1839-1841.
- Cocking, E. and E. W. Yenn. 1954. Estimation of amino acids by ninhydrin. *Biochem. J.* 58: xii.
- Cohen-Bazire, G., W. R. Sistrom, and R. Y. Stanier. 1957. Kinetic studies of pigment synthesis by non-sulfur purple bacteria. *J. Cell. Comp. Physiol.* 49: 25-68.
- Conti, S. F. and C. R. Benedict. 1962. Carotenoids of *Rhodomicrobium vanniellii*. *J. Bacteriol.* 83: 929-930.
- Conti, S. F., R. C. Fuller, and J. A. Bergeron. 1961. Observation on growth and structure of *Rhodomicrobium vanniellii*. *Bacteriol. Proc.* p. 79.

- Crane, F. L., R. L. Lester, C. Widmer, and Y. Hatefi. 1959. Studies on the electron transport system. XVIII. Isolation of CoQ from beef heart and beef heart mitochondria. *Biochim. Biophys. Acta* 32: 73-79.
- Davies, B. H. 1965. Analysis of carotenoid pigments. P. 529-531. In T. W. Goodwin (ed.), *Chemistry and biochemistry of plant pigments*. Academic Press, New York.
- Davies, W. H., E. I. Mercer, and T. W. Goodwin. 1965. The occurrence and intracellular distribution of the plant sulfolipid in maize, runner beans, plant tissue cultures and Euglena gracilis. *Phytochem.* 4: 741-749.
- Dawson, R. M. C. 1960. A hydrolytic procedure for the identification and estimation of individual phospholipids in biological samples. *Biochem. J.* 75: 45-53.
- Dawson, R. M. C., N. Hemington, and J. B. Davenport. 1962. Improvements in the method of determining individual phospholipids in a complex mixture by successive chemical hydrolysis. *Biochem. J.* 84: 497-501.
- Douglas, H. C. and R. S. Wolfe. 1959. Motility of Rhodospirillum rubrum. *J. Bacteriol.* 78: 594-598.
- Duchow, E. and H. C. Douglas. 1949. Rhodospirillum rubrum, a new photosynthetic bacterium. *J. Bacteriol.* 58: 409-416.
- Fiske, C. H. and Y. Subbarow. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* 66: 375-400.



- Folch, J., M. Lees and G. H. Sloan-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226: 497-509.
- Folin, O. and V. Ciocalteu. 1927. On tyrosine and tryptophane determination in proteins. *J. Biol. Chem.* 73: 627-650.
- Goldfine, H. 1962. The characterization and biosynthesis of an N-methylethanolamine phospholipid from Clostridium butyricum. *Biochim. Biophys. Acta.* 59: 504-506.
- Goldfine, H. and M. E. Ellis. 1964. N-methyl group in bacterial lipids. *J. Bacteriol.* 87: 8-15.
- Goodwin, T. W. 1959. The biosynthesis and function of the carotenoid pigments. 21: 295-368. In, F. F. Nord (ed.), *Advances in Enzymology*. Interscience Publishers, Inc., New York.
- Goodwin, T. W. 1965. *Chemistry and biochemistry of plant pigments*. P. 161. Academic Press, New York.
- Gorchein, A. 1964. Ornithine in Rhodopseudomonas spheroides. *Biochim. Biophys. Acta* 84: 356-358.
- Hack, M. H. 1953. Analysis of lipids by spot tests on filter-paper disk chromatograms. *Biochem. J.* 54: 602-605.
- Hanes, C. S. and F. A. Isherwood. 1949. Separation of the phosphoric esters on the filter paper chromatogram. *Nature* 164: 1107-1112.
- Hatefi, Y. 1963. Coenzyme Q (ubiquinone). 25: P289. In, F. F. Nord (ed.), *Advances in Enzymology*. Academic Press, New York.
- Hofmann, K., R. A. Lucas, and S. M. Sax. 1952. The chemical nature of the fatty acid of Lactobacillus arabinosus. *J. Biol. Chem.* 195: 473-485.

- Horning, E. C., E. H. Ahrens, S. R. Lipsky, F. H. Mattson, J. F. Mead, D. A. Turner, and W. H. Goldwater. 1964. Quantitative analysis of fatty acids by gas-liquid chromatography. *J. Lipid Res.* 5: 20-27.
- Horrocks, L. A. 1963. Thin-layer chromatography of brain phospholipids. *J. Amer. Oil Chemists' Soc.* 40: 235-236.
- Houtsmuller, U. M. T. and L. L. M. Van Deenen. 1963. Identification of a bacterial phospholipid as an o-ornithine ester of phosphatidyl glycerol. *Biochim. Biophys. Acta* 70: 211-213.
- Huber, W. F. 1951. A study of n-octadecenoic acids. I. Synthesis of cis- and trans-7-through 12- and of 17-octadecenoic acids. *J. Amer. Chem. Soc.* 73: 2730-2733.
- Huston, C. K. and P. W. Albro. 1964. Lipid of Sarcina lutea. I. Fatty acid composition of the extractable lipids. *J. Bacteriol.* 88: 425-432.
- Huston, C. K., P. W. Albro, and G. B. Grindey. 1965. Lipids of Sarcina lutea. III. Composition of the complex lipids. *J. Bacteriol.* 89: 768-775.
- Ikawa, M. 1963. Nature of the lipids of some lactic acid bacteria. *J. Bacteriol.* 85: 772-781.
- Jensen, A., O. Aasmundrud, and K. E. Eimhjellen. 1964. Chlorophylls of photosynthetic bacteria. *Biochim. Biophys. Acta* 88: 466-479.
- Jensen, S. L. 1962. The constitution of some bacterial carotenoids and their bearing on biosynthetic problems. P. 111-112. *Det KGL Norske Videnskabers Selskabs Skrifter.* 1962 NR.8. Trondheim.

- Johnson, M. J. 1941. Isolation and properties of a pure yeast polypeptidase. *J. Biol. Chem.* 137: 575-586.
- Kaneshiro, T. and A. G. Marr. 1961. Cis-10-methylene hexadecanoic acid from the phospholipids of Escherichia coli. *J. Biol. Chem.* 236: 2615-2619.
- Kaneshiro, T. and A. G. Marr. 1962. Phospholipids of Azotobacter agilis, Agrobacterium tumerfaciens and Escherichia coli. *J. Lipid Res.* 3: 184-189.
- Kaneshiro, T. and A. G. Marr. 1963. Hydroxy fatty acids of Azotobacter agilis. *Biochim. Biophys. Acta* 70: 271-277.
- Kates, M. 1964. Bacterial lipids. Vol. 2. P. 73. In, R. Paoletti and Dr. Kritchevsky (ed.), *Advances in Lipid Research*. Academic Press, New York.
- Kates, M., G. A. Adams, and S. M. Martin. 1964. Lipids of Serratia marcescens. *Can. J. Biochem.* 42: 461-479.
- Kates, M., N. Sehgal, and N. E. Gibbons. 1961. The lipid composition of Micrococcus halodenitrificans as influenced by salt concentration. *Can. J. Microbiol.* 7: 427-435.
- King, G. 1938. The oxidation of the 9,10-dihydroxy stearic acids with periodic acid. Eta-aldehydro-octic acid. *J. Chem. Soc.* 1826-1828.
- Laneelle, M. A., G. Laneelle, and J. Asselineau. 1963. Sur la presence d'ornithine dans de lipides bacteriens. *Biochim. Biophys. Acta* 70: 99-101.

- Landowne, R. A. and S. R. Lipsky. 1961. A simple method for distinguishing between unsaturated and branched fatty acid isomers by gas chromatography. *Biochim. Biophys. Acta* 47: 589-592.
- Lascelles, J. and J. F. Szilagyi. 1965. Phospholipid synthesis by Rhodopseudomonas spheroides in relation to the formation of photosynthetic pigments. *J. Gen. Microbiol.* 38: 58-64.
- Law, J. H. 1961. Lipids of Escherichia coli. *Bacteriol. Proc.* P. 129.
- Law, J. H. and R. A. Slepecky. 1961. Assay of poly-B-hydroxybutyric acid. *J. Bacteriol.* 82: 33-36.
- Law, J. H., H. Zalkin, and T. Kaneshiro. 1963. Transmethylation reactions in bacterial lipids. *Biochim. Biophys. Acta* 70: 143-151.
- LeGoff, E. 1964. Cyclopropanes from an easily prepared, highly active Zn-Cu couple, dibromoethane and olefins. *J. Org. Chem.* 29: 2048-2050.
- Lapage, M. 1964. The separation and identification of plant phospholipids and glycolipids by two-dimensional thin-layer chromatography. *J. Chromatog.* 13: 99-103.
- Lester, R. L. and F. L. Crane. 1959. The natural occurrence of Coenzyme Q and relative compounds. *J. Biol. Chem.* 234: 2169-2175.
- Lester, R. L., Y. Hatefi, C. Widmer, and F. L. Crane. 1959. Studies on the electron transport system. XX. Chemical and physical properties of the Coenzyme Q family of compounds. *Biochim. Biophys. Acta* 33: 169-185.

- Lester, R. L. and T. Ramasarma. 1959. Chromatography of the Coenzyme Q family of compounds on silicone-impregnated paper. *J. Biol. Chem.* 234: 672-676.
- Levin, E. Y. and K. Block. 1964. Absence of sterols in blue-green algae. *Nature* 202: 90-91.
- Liu, T. Y. and K. Hofmann. 1962. Cyclopropane ring biosynthesis. *Biochem. J.* 189-191.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.* 193: 265-275.
- Lundegardh, H. 1966. The role of carotenoids in the photosynthesis of green plants. *Proc. Natl. Acad. Sci.* 55: 1962-1965.
- Lynen, F., L. Wessely, O. Wieland and L. Rueff. 1952. Zur B-oxidation der Fettsauren. *Angew. Chem.* 64: 687.
- MacFarlane, M. G. 1962. Characterization of lipoamino acids as o-amino acid ester of phosphatidyl glycerol. *Nature* 196: 136-138.
- MacFarlane, M. G. 1964. Phosphatidylglycerols and lipoamino acids. Vol. 2. P. 116. In, R. Paoletti and D. Kritchevsky (ed.), *Advances in Lipid Research.* Academic Press, New York.
- Malins, D. C. and H. K. Mangold. 1960. Analysis complex lipid mixtures by thin-layer chromatography and complementary methods. *J. Amer. Oil Chemists' Soc.* 37: 576-578.
- Mangold, H. K. 1961. Thin-layer chromatography of lipids. *J. Amer. Oil Chemists' Soc.* 38: 708-727.
- Marinetti, G. V. 1962. Chromatographic separation, identification, and analysis of phosphatides. *J. Lipid Res.* 3: 1-20.

- Matches, J. R., H. W. Walker, and J. C. Ayres. 1964. Phospholipids in vegetative cells and spores of Bacillus polymixa. J. Bacteriol. 87: 16-23.
- Morrison, W. R. and L. M. Smith. 1964. Preparation of fatty acid methyl esters and dimethyl acetals from lipids with  $\text{BF}_3\text{-CH}_3\text{OH}$ . J. Lipid Res. 5: 600-608.
- Mortenson, L. F. 1962. Inorganic nitrogen assimilation and ammonia incorporation. Vol. 4, P. 123. In, I. C. Gunsalus and R. Y. Stanier (ed.) The Bacteria. Academic Press, New York.
- Murray, R. G. E. and H. C. Douglas. 1950. The reproductive mechanism of Rhodomicrobium vanniellii and the accompanying nuclear change. J. Bacteriol. 59: 157-167.
- O'Brien, J. S. and A. A. Benson. 1964. Isolation and fatty acid composition of the plant sulfolipid and galactolipids. J. Lipid Res. 5: 432-436.
- O'Leary, W. M. 1959. Studies on the utilization of  $\text{C}^{14}$ -labeled octadecenoic acids by Lactobacillus arabinosus. J. Bacteriol. 77: 367-373.
- O'Leary, W. M. 1962. S-Adenosylmethionine in the biosynthesis of bacterial fatty acids. J. Bacteriol. 84: 967-972.
- Olson, R. E., G. H. Dialameh, R. Bentley, C. M. Springer, and V. G. Ramsey. 1965. Studies on coenzyme Q. J. Biol. Chem. 240: 514-523.
- Op Den Kamp, J. A. F., U. M. T. Hautsmuller, and L. L. M. Van Deenen. 1965. On the phospholipids of B. megaterium. Biophys. Biochem. Acta 106: 438-441.

- Radin, N. S., F. B. Lavin, and J. R. Brown. 1955. Determination of cerebrosides. *J. Biol. Chem.* 217: 789-796.
- Rapport, M. M. and N. Alonzo. 1955. Photometric determination of fatty acid ester group in phospholipids. *J. Biol. Chem.* 217: 193-198.
- Renkonen, O. 1962. Determination of glycerol in phosphatides. *Biochim. Biophys. Acta* 56: 367-369.
- Rouser, G., I. O'Brien, and D. Heller. 1961. The separation of phosphatidylethanolamine and phosphatidyl serine by column chromatography. *J. Amer. Oil Chemists' Soc.* 38: 14-19.
- Ryvardin, L. and S. L. Jensen. 1964. Bacterial carotenoids. XIV. The carotenoids of Rhodomicrobium vanniellii. *Acta Chemica Scandinavica* 18: 643-654.
- Schoenheimer, R. and W. M. Sperry. 1939. A micromethod for the determination of free and combined cholesterol. *J. Biol. Chem.* 106: 745-760.
- Sehgal, S. N., M. Kates, and N. E. Gibbons. 1962. Lipids of Halo-bacterium cutirubrum. *Can. J. Biochem. Physiol.* 40: 69-81.
- Simmons, H. E. and R. D. Smith. 1959. A new synthesis of cyclopropanes. *J. Amer. Chem. Soc.* 81: 4256-4264.
- Skidmore, W. D. and C. Entenman. 1962. Two-dimensional thin-layer chromatography of rat liver phosphatides. *J. Lipid Res.* 3: 471-475.
- Skipski, V. P., R. F. Peterson, and M. Barclay. 1962. Separation of phosphatidyl ethanolamine, phosphatidyl serine, and phospholipids by thin-layer chromatography. *J. Lipid Res.* 3: 467-470.

- Skipski, V. P., A. F. Smolowe, R. C. Sullivan, and M. Barclay. 1965. Separation of lipid class by thin-layer chromatography. *Biochim. Biophys. Acta* 106: 386-396.
- Slepecky, R. A. and J. H. Law. 1960. A rapid spectrophotometric assay of alpha, beta-unsaturated acids and beta-hydroxy acids. *Anal. Chem.* 32: 1697-1699.
- Smith, I. 1960. P. 82-142, 212-230, and 363-378. Chromatographic and electrophoretic techniques. Vol. 1. Interscience Publishers, Inc., New York.
- Sperry, W. M., and W. Merrill. 1950. A revision of the Schoenheimer-Sperry method for cholesterol determination. *J. Biol. Chem.* 181: 97-106.
- Stahl, E. 1965. Thin-layer chromatography: a laboratory handbook. Springer-Verlag, Berlin and New York.
- Stanier, R. Y. 1961. La place des bacteries dans le monde vivant. *Ann. Inst. Pasteur.* 101: 297-312.
- Stanier, R. Y. and G. Cohen-Bazire. 1957. P. 56. In, R. E. O. Williams and C. C. Spicer (ed.), *Microbial Ecology*. Cambridge University Press, London.
- Stanier, R. Y., M. Doudoroff, R. Kunizawa, and R. Contoulou. 1959. The role of organic substrates in bacterial photosynthesis. *Proc. Natl. Acad. Sci.* 45: 1245-1260.
- Swern, D., G. N. Billen, T. W. Findley, and J. T. Scalan. 1945. Hydroxylation of monounsaturated fatty materials with hydrogen peroxide. *J. Amer. Chem. Soc.* 67: 1786-1789.



- Vatter, A. E., H. C. Douglas, and R. S. Wolfe. 1959. The structure of Rhodomicrobium vannielii. J. Bacteriol. 77: 812-813.
- Vernon, L. P. 1963. Photooxidation and photoreduction reactions catalyzed by chromatophores of purple photosynthetic bacteria. P. 235-268. In, H. Gest, A. San Pietro, and L. P. Vernon (ed.), Bacterial Photosynthesis. The Antioch Press, Yellow Springs, Ohio.
- Volk, W. H. and D. Pennington. 1950. The pigments of the photosynthetic bacterium Rhodomicrobium vannielii. J. Bacteriol. 59: 169-170.
- Wagner, H., L. Horhammer, and P. Wolff. 1961. Dunschichtchromatographic von Phosphatiden und Glykolipiden. Biochem. Z. 334: 175-184.
- Wheeldon, L. W. and F. D. Collins. 1957. Studies on phospholipids I. The determination of amino-nitrogen in unhydrolyzed phospholipids. Biochem. J. 66: 435-441.
- Wood, J. B., B. W. Nichols, and A. T. James. 1965. The lipids and fatty acid metabolism of photosynthetic bacteria. Biochim. Biophys. Acta 106: 261-273.
- Woolfolk, C. A., E. J. Ordal, and H. R. Whiteley. 1960. The reduction of inorganic compounds by Micrococcus lactilyticus. Bacterial Proc. P. 152.