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Fatty Acids of Extractable and Bound Lipids of *Rhodomicrobium vannielii*[1]

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ABSTRACT

Cells of *Rhodomicrobium vannielii* grown at 29 C in a lactate-containing medium were extracted at room temperature with organic solvents. The extractable fraction contained the bulk of the simple lipid (1.87% of cell dry weight) and complex lipids (phospholipids, 4.2%; sulfolipid, 0.01%), coenzyme Q (0.09%), and pigments (carotenoids 1.2%; bacteriochlorophyll, 1.9%). The cell residue contained the bound lipids (nonpolar fatty acid fraction, 1.86%; polar hydroxy fatty acids, 0.49%). The residue also contained poly-β-hydroxybutyric acid (0.2%), which was extracted in boiling chloroform. In both the simple and complex lipids, vaccenic acid (11-octadecenoic acid) was the largest single component (approximately 90% in each fraction). The fatty acids of the bound lipid contained 35% vaccenic acid, even- and odd-numbered saturated and unsaturated straight-chain fatty acids, cyclopropane-, branched-, and α- and β-hydroxy fatty acids. The extractable lipids contained only straight-chain saturated and unsaturated even-numbered fatty acids. Nearly 60% of hydroxy fatty acid fraction was α-hydroxydodecanoic acid (24%) and β-hydroxydodecanoic acid (34.5%). Coenzyme Q was crystallized and identified as Q_9 on the basis of melting point and chromatographic properties. Q_{10} had been previously reported.

Until a few years ago, little was known of the fatty acids in photosynthetic bacteria. James and Nichols (11) reported the fatty acid components in four species of *Rhodopseudomonas*, in *Rhodospirillum rubrum*, in various algae, and in the leaves of higher plants. Wood et al. (36) compared the fatty acid composition of cells grown anaerobically in the light with those grown aerobically in the dark. Scheuerbrandt and Block (29) examined the position of double bonds in monounsaturated fatty acids of *Rhodopseudomonas spheroides* grown aerobically and anaerobically.

Carr and Exell (1) surveyed the ubiquinones of various photosynthetic bacteria, including *Rhodomicrobium*. The formation and utilization of poly-β-hydroxybutyric acid in photosynthetic bacteria was studied by Stanier et al. (33) and Doudoroff and Stanier (4). The amount of polymer which a cell produces was found to depend on the nature of the organic hydrogen donor and the CO_2 concentration. Duchow and Douglas (5) reported the presence in *Rhodomicrobium* of fat globules, but did not determine their nature.

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

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MATERIALS AND METHODS

The cultivation, harvest, and lyophilization of *Rhodomicrobium vannielii* have been previously described (28).

Extraction. A 30-g amount of lyophilized cells was extracted by the method of Huston and Albro (10). Extractions were done in 1.5-liter flasks by shaking at room temperature with 600-ml portions of acetone (extract 1) for 1.5 hr; this was followed by extraction three times (2 hr each time) with $CHCl_3$-methanol, 2:1, v/v (extract 2), and twice (2 hr each time) with $CHCl_3$-methanol, 1:1, v/v (extract 3). Each extract was separated from the cell residue by centrifugation for approximately 10 min at 2,300 × *g*. Extracts 1 through 3 were combined and evaporated to dryness in vacuo by use of a rotary evaporator; the residue was then taken up in $CHCl_3$-methanol (2:1, v/v). This solution was washed according to the method of Folch et al. (6) to remove nonlipid contaminants. The lower phase was evaporated to dryness in vacuo and weighed.

The cell residue was hydrolyzed in 3 N NaOH in methanol at reflux temperature for 2 hr. 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 for nonextractable or bound lipids. Poly-β-hydroxybutyric acid was extracted with three portions of boiling chloroform from the cell residue after the solvent extraction; the $CHCl_3$ solution was filtered, and the filtrate was concentrated in vacuo.

Silicic acid column chromatography. Lipids and solvent-extractable compounds were separated by column chromatography on silicic acid (28). Silicic acid (100 mesh; Mallinckrodt Chemical Works, St. Louis, Mo.) was dried for 48 hr at 110 C, made into a slurry with $CHCl_3$, and poured into three columns (A, B, and C). Column A was 5.5 by 120 cm and columns B and C were 2.5 by 45 cm. Packed columns were washed with acetone, diethyl ether, and chloroform, in that order.

The neutral lipids were eluted with 1,800 ml of chloroform at a flow rate of about 6 ml/min. Complex lipids were subsequently eluted with chloroform-methanol and methanol (28). The simple lipid fraction was evaporated in vacuo at 45 C and refractionated on column B into two components, fraction I and fraction II. Column B was first developed with 600 ml of 4% diethyl ether in petroleum ether, which yielded fraction I; it contained mainly β-carotene, lycopene, and coenzyme Q. Column B was further developed with 1,200 ml of chloroform, yielding fraction II which contained all the simple lipids, polar carotenoids, and a small amount of bacteriochlorophyll.

Saponification. Portions of the refractionated simple lipids were transferred to 50-ml round-bottom flasks. The solvents were removed under a stream of nitrogen, 20 ml of 0.5 N KOH in methanol was added, and the material was refluxed in a water bath for 3 hr. The contents were transferred to a 60-ml separatory funnel, 10 ml each of distilled water and petroleum ether were added, and the mixture was shaken for 3 min. The petroleum ether portion was separated. This procedure was repeated several times until no further pigments were extracted into the petroleum ether layer. The petroleum ether fractions were combined and used for analyses of nonsaponifiable components, such as carotenoids, coenzyme Q (CoQ), and steroids. The aqueous portion was acidified with 6 N HCl to a *p*H between 2 and 3, and was extracted three times with 5-ml portions of petroleum ether. The petroleum ether portions were combined, washed twice with 5 ml of distilled water, and dried by passing through a column (0.5-cm diameter) of anhydrous Na_2SO_4 (3 g). The column was finally washed with chloroform.

Esterification. To solvent-free fatty acid materials was added 3 ml of BF_3—CH_3OH reagent (Applied Science Laboratories, Inc., State College, Pa.). The solution was boiled for 2 min on a steam bath, and was transferred to a separatory funnel which contained 5 ml of petroleum ether and 5 ml of water (25). After the solution was shaken for 2 min, the petroleum layer was removed; the procedure was repeated twice. The petroleum ether extracts were combined and dried with anhydrous Na_2SO_4 as described above. The fatty acids of bound lipids were prepared and esterified by the method of Kaneshiro and Marr (13).

Standard fatty acids. Various aldehydes from C_1 to C_{16} and straight-chain methyl esters of odd- and even-numbered saturated and unsaturated fatty acids C_8 to C_{24} were obtained from commercial sources. α-Hydroxy fatty acids (C_{10}, C_{12}, C_{14}, and C_{16}) were synthesized (2, 13). β-Hydroxy fatty acids (C_{10}, C_{12}, C_{14}, and C_{16}) were synthesized by the Reformatsky reaction (30) with octanal, decanal, dodecanal, and tetradecanal. Each of these substances was reacted with ethylbromoacetate with Zn as a catalyst. Cyclopropane fatty acids were synthesized by a modification (18) of Simmons and Smith's reaction (31).

Gas-liquid chromatography. Fatty acid methyl esters and aldehydes were analyzed by gas-liquid chromatography. Helium was the carrier gas at an outlet flow of 90 to 100 ml/min and a column inlet pressure of 50 psi. Columns (0.25 inch by 8 ft or 0.25 inch by 5 ft) of 30% diethylene glycol-succinic acid (DEGS) polyester on a support of 60/80 mesh firebrick and columns (0.25 inch by 5 ft) of 10% Apiezon L (ApL) on chromosorb P, 42/62, were used for the separation of both esters and aldehydes. The effluent was monitored by thermoconductivity in a gas chromatograph, model A-90-P2 (Varian Aerograph, Walnut Creek, Calif.). The methyl esters of the fatty acids were analyzed at 168 to 215 C, and fatty aldehydes were run at 130 C. 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 (8). The isolation of individual esters by gas-liquid chromatography was done as previously described (28).

Positions of the double bond in monounsaturated fatty acids were established by dihydroxylation (7, 35) and periodate oxidation of dihydroxy acids (9, 14). The fatty aldehyde product was extracted and analyzed by gas-liquid chromatography. Identification of branched-chain, hydroxy, and cyclopropane acids was done by hydrogenation, gas-liquid chromatography at various temperatures, and determination of the separation factors (15), and by gas-liquid chromatography through columns containing polar and nonpolar liquid stationary-phase absorbents (10).

Thin-layer chromatography. Silica gel G and silica gel H (plain, without binder) plates (20 by 20 cm and 5 by 20 cm) were prepared by the method of Stahl (34) and of Mangold (24). The plates were activated at 110 C for 2 hr and were stored in a desiccator over Drierite until needed. Thin-layer chromatography was used both analytically and for monitoring column cuts of simple and complex lipids. Plates were developed in the following solvent systems: for one-dimensional two-step thin-layer chromatography (32), isopropyl ether-acetic acid (94:4, v/v) followed by *n*-hexane-diethyl ether-acetic acid, 90:10:1, v/v, (23). The latter system was also used in simple thin-layer chromatography. Spots were made visible as previously described (28).

CoQ. CoQ was estimated by its decrease in absorbancy at 275 mμ after reduction with a small crystal of KBH_4 by the method of Lester et al. (19, 20). The following values for extinction coefficients were used: $E^{1\%}_{1cm}$ of 142 for CoQ_{10} and 158 for CoQ_9 (27). The melting points of crystallized CoQ samples were determined with a Fisher-Johns apparatus. 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*-dimethylformamide-water (39:1, v/v) in an ascending manner. The ubiquinone was located by ultraviolet light and by staining with neotetrazolium chloride (21).

Bacteriochlorophyll. The content of bacteriochlorophyll was estimated by the method of Cohen-Bazire et al. (3).

Poly-β-hydroxybutyric acid. Poly-β-hydroxybutyric acid was extracted from the solvent-extracted cell residue in boiling chloroform. It was determined spectrophotometrically as crotonic acid at 235 mμ (16).

RESULTS AND DISCUSSION

Extractable lipids of *R. vannielii* accounted for 6.08% of cell dry weight; simple lipid constituted 1.87%, and complex lipid (phospholipid, 4.2%, and sulfolipid, 0.01%), 4.21% (28). The extractable lipid fraction also contained CoQ (0.09%), carotenoids (1.2%), and bacteriochlorophyll (1.9%). From the cell residue, the bound lipids (nonpolar fatty acids, 1.86%, and polar hydroxy fatty acids, 0.49%) were liberated by treatment with alkali. Poly-β-hydroxybutyric acid (0.2%) was also extracted in boiling chloroform from the solvent-extracted cell residue.

Fatty acid components of extractable lipids. The fatty acid composition of simple and complex lipids is summarized in Table 1 and Fig. 1. Thin-layer chromatography also showed very strong yellow spots with iodine vapor, indicating considerable amounts of unsaturated compounds. The position of the double bond of the $C_{18:1}$ component was determined. The retention time of the resulting aldehyde was equal to that of standard heptanal. The aldehyde also reacted with 2,4-dinitrophenylhydrazine to form a yellow crystalline 2,4-dinitrophenylhydrazone (melting point, 108 C; reported 108 C). 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-octadecenoic, or vaccenic, acid. The major component of both the simple and complex lipids was vaccenic acid (approximately 90% in each fraction). This value is very close to that reported by Wood et al. (36) for *Rhodopseudomonas spheroides* and *R. capsulata*, although cells were grown in different media.

TABLE 1. *Fatty acid composition of extractable lipids of Rhodomicrobium*[a]

Compound	Neutral lipids (%, w/w)	Complex lipids (%, w/w)
$C_{10:0}$	0.10	—
$C_{12:0}$	0.30	—
$C_{14:0}$	1.75	0.70
$C_{16:0}$	5.40	6.25
$C_{18:0}$	0.11	3.80
$C_{14:1}$	0.50	—
$C_{16:1}$	1.08	0.35
$C_{18:1}$	88.20	89.0
$C_{18:2}$	2.70	—

[a] Fatty acid composition was determined by gas-liquid chromatography with polar and nonpolar columns. The amount of each component was measured by planimetry in comparison with standard compounds (8). The conditions for gas-liquid chromatography are described in the text.

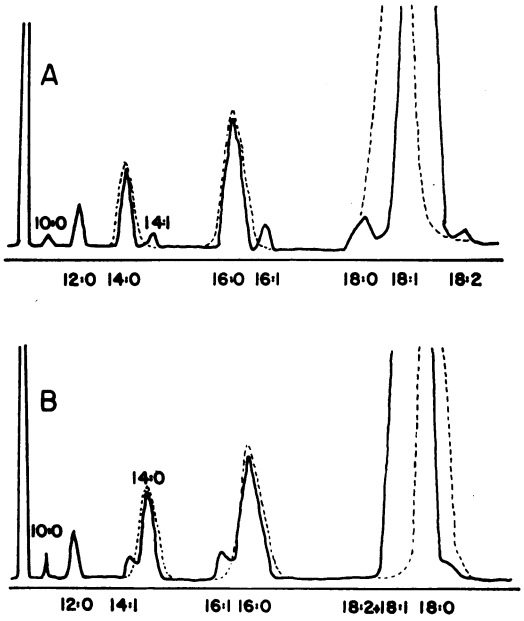

FIG. 1. *Gas-liquid chromatographs of the fatty acids from the simple lipids of Rhodomicrobium. Columns (0.25 inch by 5 ft) were packed with 30% DEGS on firebrick (A) and 10% Apiezon L on Chromosorb P (B) and were maintained at 182 C. Carrier helium with an inlet pressure of 50 psi flowed through the columns at 100 ml/min. Detection of components was by thermal conductivity. Solid lines show the separation of the fatty acid methyl esters before hydrogenation. Dotted lines show the esters after hydrogenation.*

The position of the double bonds in the tetradecenoic acid, $C_{14:1}$, and hexadecenoic acid, $C_{16:1}$, in the extractable lipids was not determined because of their trace amount. However, they could be predicted to be 7-tetradecenoic acid and 9-hexadecenoic acid from the study of Scheuerbrandt and Block (29) for *Rhodopseudomonas spheroides*, since *R. spheroides* and *R. vannielii* contain no 9-octadecenoic acid.

Fatty acids in the simple lipids show more

variety than those in the complex lipids. However, branched and cyclopropane fatty acids were not observed in either fraction. Only even-numbered fatty acids were found in the extractable fraction.

Fatty acids of bound lipids. By use of a silicic acid column, a nonpolar fatty acid fraction was eluted with diethyl ether-petroleum ether (4:96, v/v), and a polar hydroxy fraction was eluted with diethyl ether-petroleum ether (20:80, v/v). The composition of the bound fatty acids is summarized in Table 2 and Fig. 2 and 3. Of the bound lipids, 72.5% were in the nonpolar fraction. Vaccenic acid comprised more than 35% of the nonpolar fatty acid fraction. Except for branched fatty acids, fatty acids could be identified by conventional gas-liquid chromatography, before and after hydrogenation with polar and nonpolar columns. Branched fatty acids were established by their separation factor (15) by operation of the columns at different temperatures (Table 3).

Saturated fatty acids 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 the lowest values which increased at the higher temperature. Two branched fatty acids (C_{15} and C_{17}) were found in this fraction; these comprise a minor part of bound lipids. Huston and Albro (10) found that 40% of the total fatty acids in the extractable lipid fraction of *Sarcina lutea* were branched.

C_{19} and C_{21} cyclopropane fatty acids were found in *Rhodomicrobium*, but the positions of the methylene groups were not established because of the small amounts of these groups. It may be assumed, however, that the cyclopropane rings are derived from the methyl group of methionine, and from 11-octadecenoic and 13-eicosenoic acid (17, 22, 26).

Hydroxy acids were analyzed by gas-liquid chromatography and compared with authentic

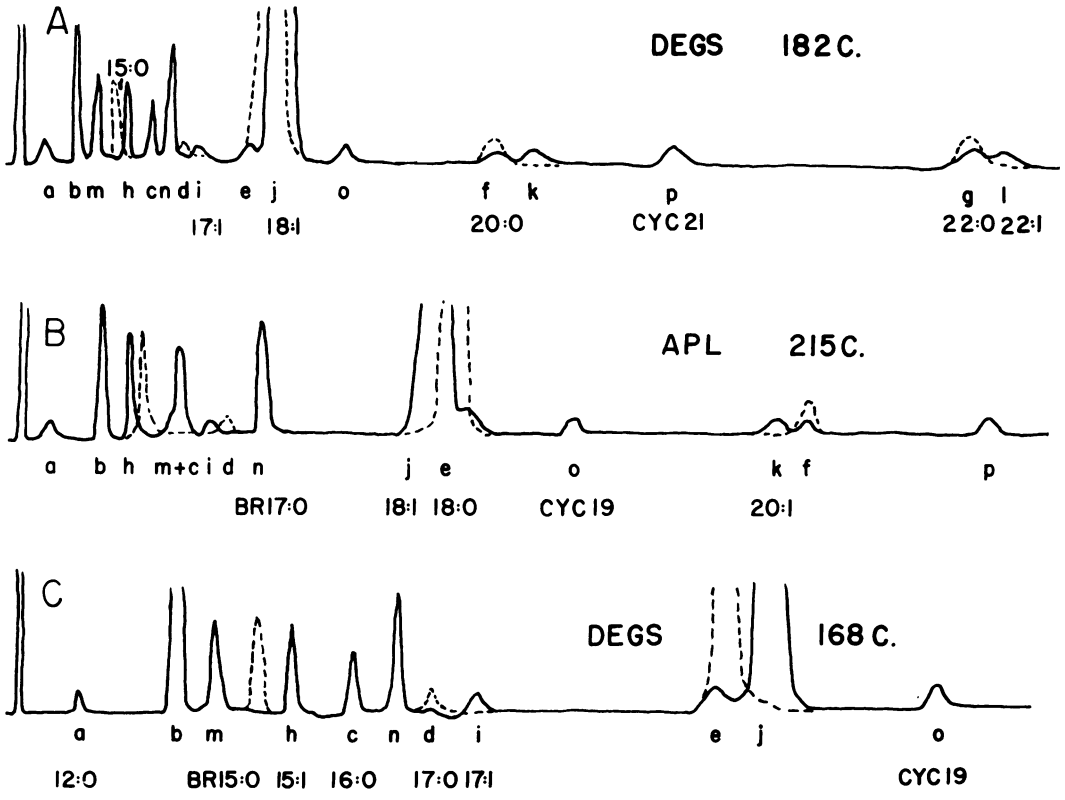

FIG. 2. *Gas-liquid chromatographs of nonpolar fatty acids of the bound lipids on polar (A, C) and nonpolar (B) columns at different temperatures. The operating conditions for the columns are described in Fig. 1. Solid lines show the resolution of the fatty acid methyl esters before hydrogenation. Dotted lines show the methyl esters after hydrogenation. Better resolution of the many components in this fraction was obtained by operating the column (C) at reduced temperature.*

standards. The results are summarized in Table 2 and Fig. 3. The retention time of β-hydroxy acids is greater than that for the corresponding α-hydroxy acid on DEGS columns. These compounds

TABLE 2. *Fatty acids of the bound lipids of Rhodomicrobium*[a]

Fraction I				Fraction II	
Compound	Per cent of fraction I	Compound	Per cent of fraction I	Compound	Per cent of fraction II
$C_{14:0}$	10.3	$C_{15\text{-}B}$	6.9	α-OH-$C_{12:0}$	24.0
$C_{16:0}$	3.7	$C_{17\text{-}B}$	9.8	α-OH-$C_{13:0}$	10.3
$C_{17:0}$	1.0	$C_{15:1}$	6.9	α-OH-$C_{14:0}$	6.9
$C_{18:0}$	2.5	$C_{16:1}$	1.1	β-OH-$C_{12:0}$	34.5
$C_{20:0}$	0.9	$C_{18:1}$	35.7	β-OH-$C_{14:0}$	6.9
$C_{22:0}$	8.0	$C_{20:1}$	1.6	α-OH-$C_{13\text{-}B}$	17.2
$C_{19\ CP}$	4.4	$C_{22:1}$	4.4		
$C_{21\text{-}CP}$	8.0				

[a] Fatty acid composition of bound lipids was determined by gas-liquid chromatography with polar and nonpolar columns at different temperatures. The amount of each component was measured by planimetry in comparison with standard compounds (8). The conditions of gas-liquid chromatography are described in the text.

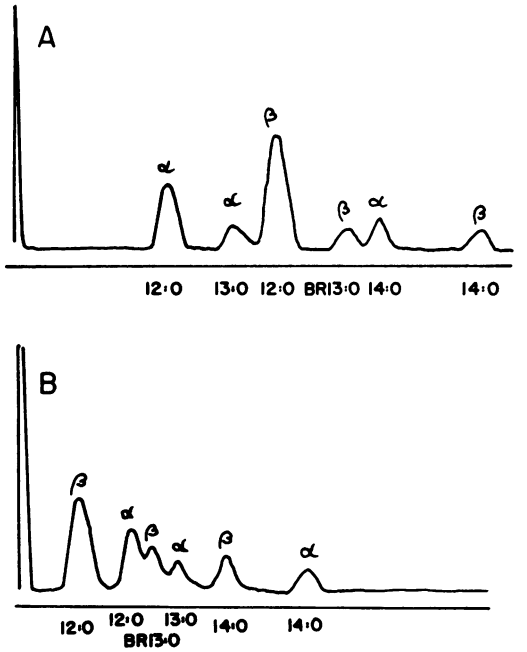

FIG. 3. *Gas-liquid chromatographs of the hydroxy acids of the bound lipids of Rhodomicrobium on polar (A) and nonpolar (B) columns. Operating conditions and composition of the columns are described in Fig. 1.*

TABLE 3. *Separation factors of the methyl esters of nonpolar fatty acids in the bound lipids of Rhodomicrobium*[a]

GLC peak	Sample-to-standard ratio	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

[a] Column temperatures are indicated; other conditions for GLC (gas-liquid chromatography) are described in the text. Separation factors were defined by Landowne and Lipsky (15).

are eluted in reverse order on the nonpolar ApL columns. In the hydroxy acid fraction, $C_{12:0}$ hydroxy acids form approximately 60% of the total hydroxy acid fraction; 24% is 2-hydroxydodecanoic acid and 34.5% is 3-hydroxydodecanoic acid. These compounds were also found in the bound lipids of *Azotobacter agilis* (13).

CoQ. CoQ of *R. vannielii* was previously reported to be CoQ_{10} by Carr and Exell (1). In this study, however, CoQ_9 was found. The material was crystallized. It was identified on the basis of its absorption maxima at 275 mμ in the oxidized form and at 290 mμ in the reduced form. It had an R_F of 0.22 on reverse-phase paper chromatograms; that of authentic CoQ_{10} from calf liver was 0.13. These data indicate that the CoQ from *Rhodomicrobium* has a shorter isoprenoid chain than does CoQ_{10}. The ratio, 0.590 (0.13:0.22), for the R_F of calf liver CoQ_{10} to that of *Rhodomicrobium* CoQ is comparable to that (0.585) previously reported for Q_{10}-Q_9 (1). The melting point of CoQ from *Rhodomicrobium* was 45.0 C. Reported values are CoQ_8, 37 C; CoQ_9, 45.2 C; and CoQ_{10}, 49.9 C (20). The finding of CoQ_9 here rather than the previously reported Q_{10} (1) may be due to differences in strains or cultural conditions. The CoQ content in *Rhodomicrobium* varied from 0.620 to 0.884 mg/g of dried cell material, which is approximately 1 μmole/g of dried cells, or 0.08% of cell dry weight.

Amounts of 16 to 20 μmoles of bacteriochlorophyll type a were found per g of dry *Rhodomicrobium* cells. Type a was previously reported in this organism (12). The ratio of bacteriochlorophyll to CoQ (18:1 to 21:1) is comparable to the results of Carr and Exell (1) for other photosynthetic organisms.

Poly-β-hydroxybutyric acid was determined as 0.2% of cell dry weight from the cell residue after solvent extraction. The amount of poly-β-hydroxybutyric acid in *Rhodomicrobium* no doubt depends on the conditions of cultivation. In *Rhodospirillum rubrum*, only trace amounts are synthesized when cells are grown on propionate and Krebs cycle intermediates, whereas large quantities are formed when acetate or butyrate is the organic substrate (33). It is probable that the fat globules originally reported in *Rhodomicrobium* (5) are actually poly-β-hydroxybutyric acid.

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