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Lipids of *Bacteroides melaninogenicus*

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The lipids of *Bacteroides melaninogenicus* were readily extractable with chloroform-methanol. Three per cent of the fatty acids were not extractable. The neutral lipids contained 4% of the extractable fatty acids, the stench characteristic of these organisms, and 0.5 μmole of vitamin K_2 isoprenologues K_2-35, K_2-40, and K_2-45 per g (dry weight). This is one-fifth to one-tenth of the vitamin K_2 level found in other bacteria. Ninety-six per cent of the extractable fatty acids were associated with the phospholipids (60 μmoles of lipid phosphate/g, dry weight), which consisted of the diacyl lipids phosphatidic acid, phosphatidyl serine, and phosphatidyl ethanolamine (with phosphatidyl glycerol and cardiolipin in one strain). The unusual phosphosphingolipids ceramide phosphorylethanolamine, ceramide phosphorylglycerol, and ceramide phosphorylglycerol phosphate accounted for 50 to 70% of the lipid phosphate. In protoheme-requiring strains, the protoheme concentration in the growth medium regulated the growth rate and the amount of enzymatically reducible cytochrome *c*. There were no gross changes in the lipid composition in cells containing different levels of enzymatically reducible cytochrome *c*.

Bacteroides melaninogenicus (*Fusiformis nigrescens*) is a gram-negative, nonsporulating anaerobe which forms a membrane-bound electron transport system (17). Many strains require protoheme (6, 18), and the growth rate and the level of enzymatically reducible cytochrome *c* depend on the protoheme supplied in the media (17). Many strains of this anaerobe also require vitamin K (6, 10, 11), for growth and have been shown to contain vitamin K_2 (5). *B. melaninogenicus* is one of the few true bacteria that are able to utilize lipids that are added to the growth medium. Vitamin K_1, menaquinone, and 1,4-naphthoquinone are transformed to vitamin K_2-45 by this organism (14). Vitamin K-requiring strains elongate and accumulate ribonucleic acid when deprived of vitamin K during growth (12).

In view of the remarkable lipid permeability and the possible involvement of lipids in the electron transport system, the lipid composition of this organism was examined. The phosphosphingolipids ceramide phosphorylethanolamine (CPE), ceramide phosphorylglycerol [CPG (9, 26)] and ceramide phosphorylglycerol phosphate [CPGP (D. C. White and A. N. Tucker, Lipids, *in press*)] have been characterized from the lipids of *B. melaninogenicus*. The latter two compounds are previously unknown natural products. Sphingolipids are exceedingly rare in bacteria (1), although another anaerobe, *B. ruminicola*, has been reported to contain an ethanolamine-containing sphingolipid (J. F. Kunsman, Jr., I. Katz, and M. Keeney, Abstr. Amer. Chem. Soc. 152nd Meeting, New York, 1966). In this study, the lipids of *B. melaninogenicus* have been identified and shown not to change significantly in bacteria grown with different amounts of protoheme.

MATERIALS AND METHODS

Growth of organisms. *B. melaninogenicus* strain CR2A1 was the gift of R. J. Gibbons. Strains CR2A2 and Lev were the gifts of M. P. Bryant. Strains CR2A1 and CR2A2 were isolated from the same infection, but have been carried in different laboratories. The Lev strain was isolated from the bovine rumen by M. Lev. Strain Dog was isolated from a dog with peridontal disease at the University of Kentucky Medical Center. This strain was isolated from a black colony on blood-agar. The media and growth conditions have been described (17). Cultural purity was checked by colonial morphology, pigmentation on blood-agar, and by Gram stain (17). Cultures were grown with 500 μc of $H_3{}^{32}PO_4$ per 450 ml of medium, and cells were harvested by centrifugation after the culture reached the stationary phase of growth. In the experiments in which growth was limited by the protoheme concentration added to the medium, the cells were depleted of protoheme by incubation in media in the absence of protoheme as described (17). Cultures were preserved with 15% glycerol added to the medium at −60 C or as prereduced (3) stabs made with the medium containing 2% agar.

Extraction of the lipid. Lipids were extracted from the bacteria by a modified Bligh and Dyer procedure (2). A 30-ml suspension of bacteria in 50 mM phosphate buffer, *p*H 7.6, containing about 14 mg (dry weight) of cells was mixed with 75 ml of methanol

and 37.5 ml of chloroform and shaken vigorously. The one-phase system was allowed to stand overnight. Then 37.5 ml of chloroform and 37.5 ml of 1.0 M KCl solution containing 0.4% (v/v) glacial acetic acid was added and the mixture was shaken. After several hours, the mixture separated into two phases. The lower layer containing the lipid was filtered through a 4-cm piece of Whatman no. 12 filter paper.

Mild alkaline methanolysis. The mild alkaline methanolysis quantitatively cleaved the diacyl phospholipids into water-soluble glycerol phosphate esters and fatty acid methyl esters (23). The ceramides were not effected by this procedure. The methanolysis is complete in 2.5 hr at 0 C (9). The alkali was neutralized with the weakly acidic cation-exchange resin Biorex 70 (Bio-Rad Laboratories, Richmond, Calif.) as described (21, 25). The fatty acid methyl esters and ceramides were recovered from the methanolysis mixture by three extractions with diethyl ether and a final extraction with chloroform (9). The abbreviations used for the glycerol phosphate esters derived from the phospholipids are as follows: glycerolphosphorylethanolamine (GPE) from phosphatidyl ethanolamine, glycerolphosphorylglycerol (GPG) from phosphatidyl glycerol, diglycerolphosphorylglycerol (GPGPG) from cardiolipin, glycerolphosphorylserine (GPS) from phosphatidyl serine, and L-α-glycerol phosphate (αGP) from phosphatidic acid.

Fatty acid methyl esters were separated from ceramides on 1-g silicic acid columns (11 × 50 mm, Unisil, 100 to 200 mesh). The fatty acid methyl esters were eluted in 5 ml of chloroform. The ceramides were eluted with 5 ml of chloroform-methanol (1:1) followed by 5 ml of methanol.

Paper chromatography. Ceramides and diacyl phospholipids were separated on silica gel-impregnated paper (Whatman SG-81) with solvents of chloroform-methanol-diisobutyl ketone-acetic acid-water (23:10:45:25:4, v/v) in the first dimension and chloroform-methanol-diisobutyl ketone-pyridine-0.5 M ammonium acetate buffer, *p*H 10.4 (30:17.5:25:35:6, v/v) in the second dimension as described by Wuthier (27). Lipids were eluted from the silica gel-loaded paper with a solvent of chloroform-methanol-19 mM ammonium hydroxide (20:20:1) by soaking the paper in 3 ml of solvent for 1 hr. The paper was then rinsed in three 1-ml portions of solvent. The recovery was quantitative.

Glycerol phosphate esters were separated (21) on acid-washed amino-cellulose paper (Whatman AE-81). Solvents were 0.4% pyridine in 3 M formic acid and modified Wawszkiewicz solvent (21). This solvent contains 1.15 M ammonium acetate with 11.8 mM ethylenediaminetetraacetic acid (EDTA) made to *p*H 5.0 with acetic acid and diluted 3:7 with 95% ethanolic 0.25 M ammonium hydroxide.

Thin-layer chromatography. Glycerol phosphate esters were separated on cellulose thin-layer plates (Eastman Chromagrams 6064; Eastman Kodak Co., Rochester, N.Y.) with solvents of 3.8 mM EDTA and 0.7 M ammonium bicarbonate in 90 mM ammonium hydroxide containing 67% (v/v) ethyl alcohol in the first dimension and with isobutyric acid-water-concentrated ammonium hydroxide (66:33:1, v/v) in the second dimension.

Vitamin K_2 was separated from the neutral lipids and phospholipids by thin-layer chromatography on silica gel G with a solvent of chloroform-isooctane [2:1, v/v (6*a*)]. A portion of the plate was covered with Saran wrap, and the remainder was sprayed with $NaBH_4$ followed by neotetrazolium. The vitamin K_2 was recovered and the isoprenologues were separated by reversed-phase thin-layer chromatography on hexadecane-impregnated Kieselguhr G with a solvent of acetone-water (95:5, v/v) saturated with hexadecane (6*a*).

Gas chromatography. Preparations were saponified; the fatty acids were recovered and were methylated as described (22). In the lipid extract, neutral and phospholipids were separated on silicic acid columns (22). Fatty acid methyl esters were separated on ethylene glycol succinate and SE-30 columns under the conditions described previously (22). The proportions of each ester agreed whether determined on the polar or nonpolar gas chromatographic column.

Measurement of radioactivity. ^{32}P was counted on paper discs in a scintillation spectrometer (21). Radioautograms were prepared with Kodak no-screen X-ray film (21).

Assays. Protoheme was measured as the reduced pyridine hemochrome (17). Cytochromes were measured by difference spectroscopy (17). Protein was measured by the procedure of Lowry et al. (13). Malate dehydrogenase activity was measured by determining the rate of disappearance of reduced nicotinamide adenine dinucleotide (16).

RESULTS

Extraction of the lipids. The Bligh and Dyer extraction procedure involves a one-phase extraction mixture of chloroform-methanol-water, and is rapid, convenient, and reproducible. If modified by the addition of acetic acid (9, 26), formation of emulsions with the lipids from *B. melaninogenicus* is minimized. When cells from stationary-phase cultures of *B. melaninogenicus* were extracted once by this procedure, 97% of the total fatty acids in the cells were recovered. About 3% of the fatty acids remained in the residue. The fatty acids in the residue were present in different proportions than in the lipid extract. No β-hydroxymyristic acid was detected in the residue. This fatty acid is found in the lipid A of the cell wall in many gram-negative bacteria (15). Nearly 96% of the extracted fatty acids were in the phospholipid portion of the extract. The fatty acids of the neutral lipid were present in different proportions from those in the phospholipids. These data are illustrated in Table 1.

Localization of the lipids. *B. melaninogenicus* cells grown to the early stationary phase were harvested and ruptured by passage through a Ribi-Sorvall refrigerated French pressure cell. The cells were forced through the Ribi valve at a

pressure of 40,000 psi, and the temperature at the value was maintained between 0 and 8 C. After treatment, no intact cells could be detected by phase-contrast microscopy. A portion of the cell-free suspension was then centrifuged at 48,200 × *g* (102,000 × *g* for phospholipid) for 60 min, and the supernatant portion was separated from the pellet. The pellet was resuspended to the same volume as before centrifugation in 50 mM phosphate buffer, *p*H 7.6. The suspension, the resuspended pellet, and the supernatant fraction were examined for total protein, protoheme, vitamin K_2, malate dehydrogenase, and lipid phosphate (Table 2). All of the vitamin K_2, 90% of the protoheme, 20% of the protein, and none of the malate dehydrogenase were recovered in the membrane fraction; 80% of the lipid phosphate in the broken cell suspension was recovered in the membrane fraction. Previous work has shown that the electron transport system is localized in the membrane fraction of the cells (17). *B. melaninogenicus* has an extraordinarily high malate dehydrogenase activity, and the complete recovery of this enzyme activity in the supernatant fraction indicates that cell rupture was complete.

Neutral lipids. The neutral lipids contained about 4% of the extractable fatty acids. The crude neutral lipid contained a small amount of yellow pigment, suggesting the presence of carotenoids, and a fraction that could be eluted from a silicic acid column. The latter fraction contained the characteristic stench associated with these organisms. The most prominent neutral lipids were the vitamin K_2 isoprenologues. Some strains of *B. melaninogenicus* are unable to synthesize vitamin K_2 and require menadione or vitamin K_2 in the growth medium (6, 10, 11). All strains used in this study can form vitamin K_2. The vitamin K_2 isoprenologues were purified by first spotting along the edge of a silica gel G thin-layer plate. Ascending chromatography in a solvent of chloroform-isooctane (2:1) separated the neutral lipids (solvent front), the vitamin K_2 detected by spraying with KBH_4 and neotetrazolium (6*a*; at R_F 0.6), and the phospholipids (at the origin). A portion of the vitamin K_2 band that had not been sprayed was recovered and had the spectral properties of vitamin K_2. Absorbancy maxima at 327, 271, 262, 250, and 246 nm, and minima at 266, 256, 248, and 224 nm were detected in a solvent of isooctane in the eluted quinone. When dissolved in ethyl alcohol containing 1% of 1 M ammonium acetate, *p*H 5.0, and reduced with KBH_4, the reduced minus oxidized difference spectrum showed a minimum at 270 nm and a maximum at 245 nm. These are the spectral properties of authentic vitamin K_2 (8). No other quinones were detected. The vitamin K_2 fraction from strain CR2A1 was chromatographed on hexadecane-impregnated Kieselguhr G in a solvent of acetone-water (95:5) saturated with hexadecane, which separated the isoprenologues of vitamin K_2 (6*a*). The chromatographic mobilities,

TABLE 1. *Extraction of fatty acids from Bacteroides melaninogenicus CR2A2*

Fatty acids	Intact cells	Extract	Residue	Phospholipid	Neutral lipid
Total[a]	90.4	88.5	2.9	80.7	3.4
13:0, Br[b]	2.4	2.3	12.0	14.6	2.5
14:0	1.4	1.5	9.6	1.2	6.3
15:0, Br	84.4	90.8	9.6	79.0	69.9
16:0	2.5	2.3	36.6	2.5	17.0
17:0, Br	1.3	0.4	12.6	0.7	4.2
18:0	1.4	0.5	14.7	0.8	—
18:0, Br	6.5	1.1	4.8	1.2	—

[a] Total fatty acids were determined after saponification in whole cells, in Bligh and Dyer extract, in residue, and in the phospholipids and neutral lipids of the extract after separation with silicic acid chromatography. Total fatty acids were expressed as micromoles per gram (dry weight), and were measured colorimetrically with palmitic acid as standard.

[b] Proportions of the fatty acids were determined with gas chromatography of the methyl esters on ethylene glycol succinate columns and expressed as a percentage of total. Br, branched.

TABLE 2. *Cellular localization of components in Bacteroides melaninogenicus CR2A1*

Fraction	Protein[b] (mg/ml)	Heme[c] (nmoles/ml)	Vitamin K_2[d] (nmoles/ml)	Malate[e] dehydrogenase (μmoles per min per ml)	Lipid P_F (nmoles/ml)
Supernatant fluid[a]	2.59	<0.03	<0.1	1.64	91
Pellet	0.71	0.45	16.0	<0.01	25
Suspension	3.34	0.50	20.0	1.13	117

[a] A cell suspension of *B. melaninogenicus* was ruptured with a refrigerated French pressure cell and the membranes were collected after centrifugation at 48,200 × *g* (102,000 × *g* for the phospholipid) for 1 hr.

[b] Measured by the procedure of Lowry et al. (13).

[c] Measured as the pyridine hemochrome (17).

[d] Measured by its absorption spectrum after purification (6*a*).

[e] Measured by determining the rate of disappearance of reduced nicotinamide adenine dinucleotide (NADH) in the presence of 0.2 mM NADH, 0.2 mM oxalacetate, with 0.03 mg of protein in 200 mM phosphate buffer at *p*H 6.9.

[f] Measured after extraction of the lipids (23).

when compared with those of synthetic isoprenologues, indicated that strain CR2A1 contained K_2-45 (26%), K_2-40 (35%), K_2-35 (39%), and K_2-30 (0.1%). Each isoprenologue was recovered and the quantity in each band was determined by the absorbancy at 245 nm (Fig. 1).

Phospholipids. The phospholipids of *B. melaninogenicus* have been shown to be unusual, as most of the lipid phosphate is not hydrolyzed by mild alkali. The lipids which are stable in mild alkali can be recovered quantitatively in the organic phase after mild alkaline methanolysis. These lipids have been shown to be phosphosphingolipids. CPE, CPG, and CPGP have been characterized in these bacteria (9; D. C. White and A. N. Tucker, Lipids, *in press*). The long-chain bases from the ceramides of these organisms have been shown to be 15-methyl-hexadecasphinganine, normal octadecasphinganine, 17-methyl-octadecasphinganine and possibly 16-methyl-heptadecasphinganine by gas chromatography and mass spectrometry in strain CR2A1 (26). The phospholipids of *B. melaninogenicus* were separated by chromatography on silica gel-impregnated paper (Fig. 2). The lipids were extracted from the bacteria grown in medium containing ^{32}P. After chromatography, the lipids were located by radioautography and the spots corresponding to the dark areas were cut out. The lipid was eluted from the paper, and the proportion of each lipid rendered water-soluble by mild alkaline methanolysis was determined (Table 3). The ceramides were separated from the fatty acid methyl esters derived from the diacyl phospholipids by silicic acid chromatography, and the ceramides were identified by their mobility on silica gel-impregnated paper (26). The glycerol phosphate esters derived from the diacyl phospholipids by mild alkaline methanolysis were identified by their chromatographic mobility on aminocellulose paper (Fig. 3) and cellulose thin-layer plates (Fig. 4). Esters with the chromatographic mobility of GPGPG, GPG, GPE, GPS, and αGP were detected. They were present in the same proportions after separation by the two methods (Table 4). With these methods, the proportions of the phospholipids in four strains of *B. melaninogenicus* (in the stationary growth phase) isolated from widely differing sources were compared (Table 5). The proportions were remarkably similar. Only strain CR2A1 contained phosphatidyl glycerol and cardiolipin. The total fatty acid composition of the strains

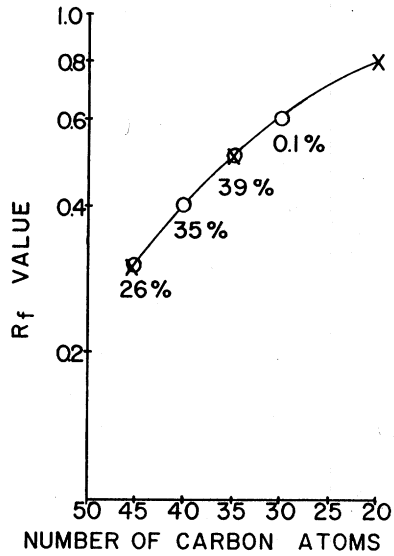

FIG. 1. *Relative chromatographic mobilities of vitamin K_2 isoprenologues of Bacteroides melaninogenicus CR2A1. Vitamin K_2 isoprenologues from the bacteria (○) and synthetic standards (×) were separated by ascending chromatography with an acetone-water (95:5) solvent on hexadecane-impregnated Kieselguhr G thin-layer plates. A portion of the plate was sprayed with KBH_4 and then neotetrazolium to locate the quinones. Quinones were recovered from the unsprayed portion of the plate, and the proportions were determined by the absorbancy at 245 nm in isooctane.*

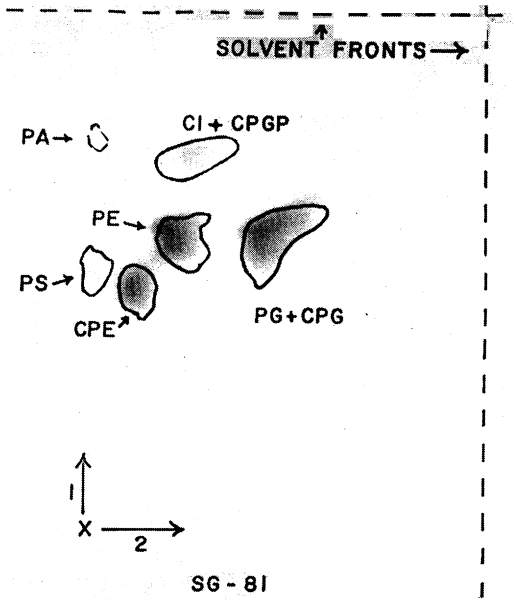

FIG. 2. *Radioautogram of the separation of the lipids of Bacteroides melaninogenicus CR2A1 on silicic acid-impregnated paper (Whatman SG-81). The solvents were (1) chloroform-methanol-diisobutyl-ketone-acetic acid-water (23:10:45:25:4, v/v) and (2) chloroform-methanol-diisobutyl-ketone-pyridine-0.5 M ammonium acetate buffer, pH 10.4 (30:17.5:25:35:6, v/v).*

was remarkably like that of CR2A2 illustrated in Table 1.

Lipids and the electron transport system. *B. melaninogenicus* requires the function of a membrane-bound electron transport system for growth (17). The organism has an absolute requirement for protoheme in the growth medium. The growth rate and the level of enzymatically reducible cytochrome *c* in the membrane are proportional to the protoheme concentration in the growth medium (17). The total amount of vitamin K_2 and phospholipid in the membrane in bacteria grown with different levels of protoheme supplementation in the medium did not change (Fig. 5). There was not much change in the proportions of the major phospholipids of *B. melaninogenicus* during growth with different levels of protoheme supplementation (Fig. 6). There are no glucolipids in these bacteria (9).

DISCUSSION

The lipid composition of *B. melaninogenicus* is similar to that of other gram-negative bacteria in that the major portion of the extractable fatty acids is linked with phospholipids (Table 1). *B. melaninogenicus* is unusual in the small amount of fatty acids in the residue after extraction and in the absence of β-hydroxy fatty acids. The non-extractable fatty acids are usually associated with the lipopolysaccharide. Hofstad (7) has noted the low level of esterified fatty acids in the lipopolysaccharide and the unusual nature of the lipopolysaccharide of this organism.

The neutral lipids represent a small portion of the extractable fatty acids (Table 1). The neutral lipid fraction contains the characteristic stench of these organisms. Vitamin K_2 isoprenologues with side chains of 35, 40, and 45 carbon atoms make up the bulk of the neutral lipids. Martius and Leuzinger (14) found that the K_2-45 isoprenologue predominated, with traces of K_2-50 and K_2-40, in another strain of this organism. *B. melaninogenicus* contains one-fifth of the vitamin K_2 found in aerobically growing *Staphylococcus aureus* (4), and one-tenth of the 2-demethyl vitamin K_2 found in *Haemophilus parainfluenzae* (20).

The most unusual feature of the lipids of *B. melaninogenicus* is the large concentrations of phosphosphingolipids. Sphingolipids have been documented only in *Mycoplasma gallisepticum*

TABLE 3. *Separation of the phospholipids of Bacteroides melaninogenicus strain CR2A1 on silica gel-impregnated paper*

Lipid[a]	^{32}P[b]	Water-soluble after MAH[c]	Glycerol ester[d]	Ceramide[e]
	%	%		
Phosphatidyl serine	1.5	100	GPS	—
CPE	27.1	0	—	CPE
Phosphatidyl glycerol + CPG	50.8	30	GPG	CPG
Phosphatidyl ethanolamine	15.6	100	GPE	—
Phosphatidic acid	0.2	100	αGP	—
Cardiolipin + CPGP	4.7	43	GPGPG	CPGP

[a] Lipids were separated as in Fig. 2.

[b] Each lipid was eluted and the percentage of total ^{32}P was determined.

[c] Proportion of ^{32}P that was water-soluble after mild alkaline methanolysis (MAH).

[d] Identification based on the chromatographic mobility of the ester on aminocellulose paper as in Fig. 3.

[e] Identification of the ceramide from the organic phase of the MAH from the chromatographic mobility on silica gel-impregnated paper (D. C. White and A. N. Tucker, Lipids, *in press*.).

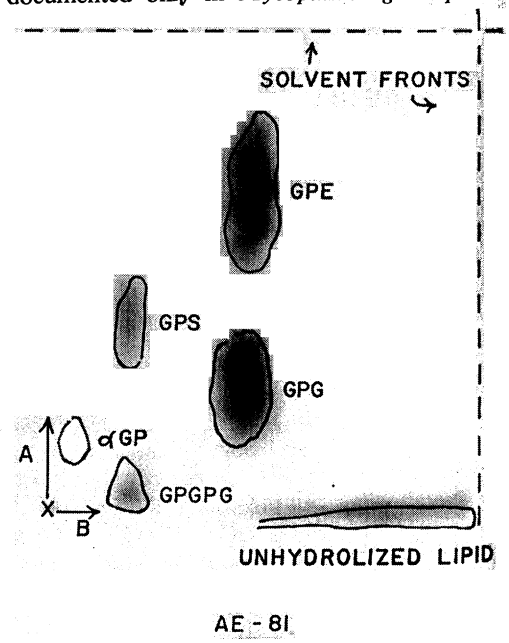

FIG. 3. *Radioautogram of the glycerolphosphate esters containing ^{32}P from the diacyl phospholipids of Bacteroides melaninogenicus CR2A1. Chromatography was on acid-washed aminocellulose paper (Whatman AE-81) with solvents: (A) 0.4% pyridine in 3 M formic acid, and (B) 1.15 M ammonium acetate containing 11.8 mM ethylenediaminetetraacetic acid made to pH 5.0 with acetic acid and diluted 3:7 (v/v) with 95% ethanolic 0.26 M ammonium hydroxide.*

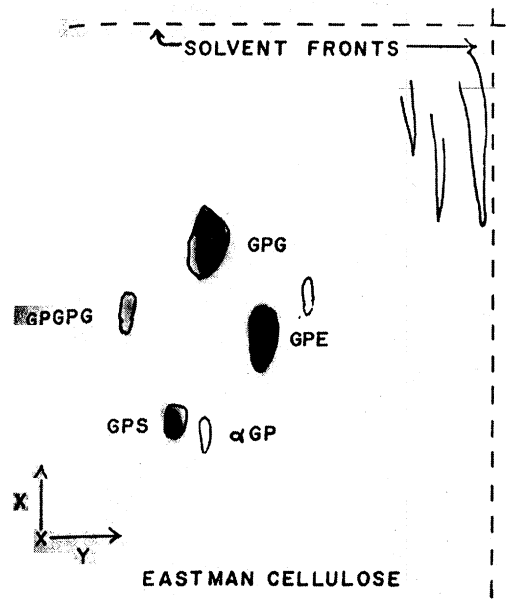

FIG. 4. *Radioautogram of glycerol phosphate esters containing* ^{32}P *from the diacyl phospholipids of Bacteroides melaninogenicus CR2A1. Chromatography was on Eastman cellulose chromagrams (6064) with solvents: (X) 3.8 mM ethylenediaminetetraacetic acid, 0.7 M ammonium bicarbonate in 90 mM ammonium hydroxide containing 67% ethyl alcohol in the first dimension and (y) isobutyric acid-water-13 M ammonium hydroxide (66:33:1, v/v).*

(19) and *B. ruminicola* (J. F. Kunsman, Jr., I. Katz, and M. Keeney, Abstr. Amer. Chem. Soc. 152nd Meeting, New York, 1966). Not only are sphingolipids rare in bacteria, but in *B. melaninogenicus* the phosphosphingolipids are unusual.

TABLE 4. *Diacyl phospholipids of Bacteroides melaninogenicus CR2A1*

Lipid[a]	Deacylated product	Per cent	
		AE-81	Cellulose thin-layer chromatography
Cardiolipin	GPGPG	2.16	2.60
Phosphatidyl glycerol	GPG	45.12	45.19
Phosphatidyl serine	GPS	6.09	7.26
Phosphatidyl ethanolamine	GPE	46.61	44.93
Phosphatidic acid	αGP	<0.05	<0.05

[a] The lipids were deacylated and the glycerol phosphate esters were separated by chromatography on aminocellulose paper (AE-81) as in Fig. 3 or on cellulose thin-layer plates as in Fig. 4. The lipids were recovered and the radioactivity was determined in a scintillation spectrometer.

TABLE 5. *Phospholipids of Bacteroides melaninogenicus strains CR2A1, CR2A2, Lev, and Dog*

Phospholipid	Per cent lipid phosphate			
	CR2A1	CR2A2	Lev	Dog
Total phosphosphingolipids[a]	*64.7*	*72.5*	*51.5*	*73.6*
Ceramide phosphorylethanolamine	27.1	37.0	25.3	43.0
Ceramide phosphorylglycerol	35.6	20.1	22.9	24.1
Ceramide phosphorylglycerol phosphate	2.0	2.4	—	2.6
Unidentified[b]	<0.1	12.6	1.4	3.9
Total diacyl phospholipids[c]	*34.5*	*27.5*	*48.5*	*25.8*
Phosphatidyl ethanolamine	15.6	26.5	46.2	24.7
Phosphatidyl glycerol	15.2	—	—	—
Cardiolipin	2.0	—	—	—
Phosphatidyl serine	1.5	0.7	1.9	0.9
Phosphatidic acid	0.2	0.1	0.3	0.2
Unidentified	<0.1	0.2	<0.1	<0.1

[a] Cells were grown in a medium containing ^{32}P, and the lipids were extracted. Phosphosphingolipids were identified by two-dimensional chromatography on silica gel-impregnated paper after mild alkaline methanolysis. The lipids were detected by radioautography and the proportion of lipid phosphate was determined.

[b] Unidentified ceramides in CR2A2 consisted of four lipids with chromatographic mobility different from that of the three that have been identified.

[c] Glycerol phosphate esters derived from the diacyl phospholipids were separated after mild alkaline methanolysis as in Fig. 3.

CPE is rare in nature (9) and CPG and CPGP have hitherto not been described. These lipids are synthesized by the cells, as both ^{32}P and ^{14}C are incorporated into the lipids during bacterial growth. ^{14}C is incorporated into the amide-linked fatty acid, the long-chain base, and the phosphate ester (Rizza and White, *unpublished data*). These phosphosphingolipids are not unique to strain CR2A1, as isolates from three different sources contained these lipids (Table 5). In strains Lev, Dog, and CR2A2, there are ceramide derivatives as yet uncharacterized. They have the chromatographic mobility suggestive of ceramide phosphate (CP) and ceramide phosphoryl serine (CPS). Should they prove to be present, synthetic pathways involving CP → CDP-ceramide → CPS → CPE and CP → CDP-ceramide → CPGP → CPG in homology with the usual CDP-diglyceride pathway in bacteria may be present. The amount of lipid phosphate per gram (dry

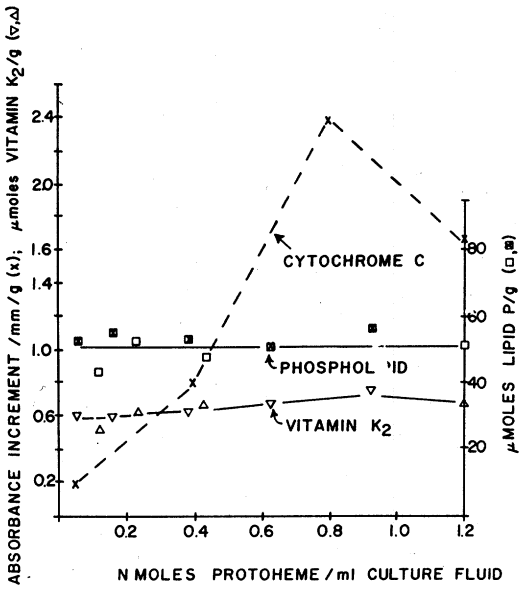

FIG. 5. *Vitamin K_2, phospholipid, and cytochrome c content of Bacteroides melaninogenicus CR2A1 grown with increasing amounts of heme. The cytochrome c data were taken from reference (17). The ⊠ and □ or ▽ and △ indicate different experiments.*

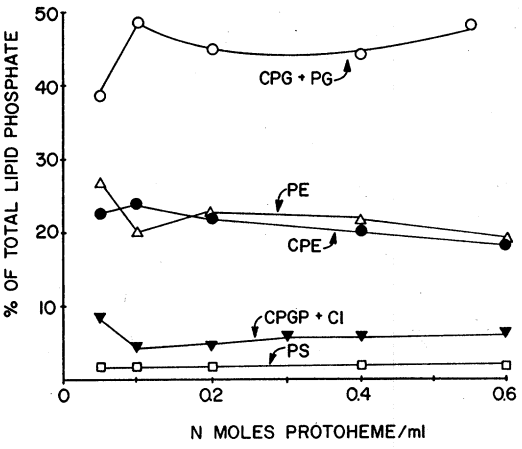

FIG. 6. *Proportion of the phospholipids in the membrane of Bacteroides melaninogenicus CR2A1 grown with increasing amounts of heme. Lipids were separated as in Fig. 2 and the proportions of lipid phosphate were measured.*

weight) in *B. melaninogenicus* and the fact that phosphatidyl ethanolamine is the predominant diacyl phospholipid are typical of a gram-negative bacterium. The absence of phosphatidyl glycerol and cardiolipin from three of the strains is unusual. The lipids seem to be localized in the membrane fraction with the cytochrome *c* (Table 2). The phospholipid found in the supernatant fraction may represent very small membrane fragments.

During the modifications of the electron transport system in *H. parainfluenzae*, there are changes in the proportions of the lipids, in the total content of phospholipid, and in the turnover rate and the incorporation rate of ^{32}P (24). Much more striking changes occur in the lipids of *S. aureus* during the induction of the membrane-bound electron transport system (4). In the staphylococcal system, there is a doubling of the phospholipids and the vitamin K_2 isoprenologues concomitant with the development of respiratory activity. In the protoheme-requiring strains of *B. melaninogenicus*, the amount of protoheme supplied regulates the growth rate and the amount of enzymatically reducible cytochrome *c* (17). The regulation of the respiratory activity by the protoheme concentration supplied to the cells does not appear to affect the gross lipid composition of the membrane (Fig. 5 and 6). Lev (12) found that the elongation of the cells and the accumulation of ribonucleic acid that occurred with vitamin K-depleted cells or in the presence of inhibitors of protein synthesis did not occur with protoheme-depleted cells. Perhaps there is little alteration in the membrane necessary for the insertion of cytochrome *c* into the preformed electron transport complex. Inhibition of lipid metabolism in the absence of vitamin K in auxotrophs apparently has a much greater effect on membrane function.

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