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Characterization of the Lipids of *Butyrivibrio fibrisolvens*

JOSEPH E. KUNSMAN

Division of Animal Science, University of Wyoming, Laramie, Wyoming 82070

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Butyrivibrio fibrisolvens strain D-1 was grown on a lipid-free chemically defined medium. The lipids were extracted with chloroform-methanol and separated into nonpolar and polar fractions by silicic acid column chromatography. Further separations were made by preparative thin-layer chromatography. The lipid fractions were identified by specific staining reactions and R_F values, by phosphorus and nitrogen determinations, by chromatography of hydrolysis products, and by the use of infrared spectroscopy. The major nonpolar lipid was free fatty acid. Four major polar lipids were identified: phosphatidylethanolamine, phosphatidyl glycerol, lipoaminoacid, and glycolipid. The lipoaminoacid contained alanine, leucine, and isoleucine. The glycolipid contained galactose. The major fatty acids identified were C16:0 and C18:1. The significance of the presence of lipoaminoacid is discussed.

Keeney et al. (17) interpreted the quantity and character of the microbial lipid isolated from rumen digesta as indicating that significant quantities of milk fatty acids originate from rumen microbial synthesis of long-chain acids from volatile fatty acids. Other studies of the bacterial fraction from rumen digesta showed that mixed ruminal bacteria are a rich source of aldehydogenic lipid (16). D. Abraham (Ph.D. Thesis, Univ. of Maryland, College Park, 1965) found nonpolar lipid, rich in carbonyls, as well as polar lipid consisting of mono- and trigalactosylglycerides, phosphatidylethanolamine, an ethanolamine-containing sphingolipid, and phosphatidyl glycerol in the lipids isolated from a mixed population of rumen bacteria. Using pure cultures of ruminal bacteria grown on a chemically defined, lipid-free medium, J. E. Kunsman (Ph.D. Thesis, Univ. of Maryland, College Park, 1966) demonstrated the presence of an ethanolamine-containing sphingolipid in *Bacteroides ruminicola*.

Other studies of rumen bacteria have shown a relationship between volatile fatty acid metabolism and the lipid portion of the cell (2, 23). These results suggest that detailed study of lipids in predominant ruminal bacteria may contribute to knowledge both of anaerobic bacterial metabolism and of the symbiotic relationship between the ruminal bacteria and their host. A detailed lipid analysis of several rumen bacteria has been undertaken in this laboratory. This paper reports on the lipid composition of *Butyrivibrio fibrisolvens*.

MATERIALS AND METHODS

Growth of bacteria. *B. fibrisolvens* strain D-1 was previously described (5). A pure culture was supplied by D. R. Caldwell (Microbiology and Veterinary Medicine, University of Wyoming, Laramie). Anaerobic culture maintenance, inoculum preparation, and growth estimation methods described by Bryant and Robinson (4) were used. The synthetic medium used for growth of *B. fibrisolvens* was essentially the same as the medium described by Caldwell et al. (6) with the exception that glucose rather than maltose was the energy source. The culture was grown to the stationary phase in a 12-liter flask at 37 C. The cells were harvested by centrifugation at 9,500 × *g* for 35 min, washed with 0.85% saline, resuspended, and recentrifuged.

Extraction of lipids. The packed cells were combined with 600 ml of chloroform-methanol (1:1, v/v) and stirred at room temperature for 30 min. A 300-ml quantity of chloroform was then added, and the suspension was stirred for 4 hr. The mixture was filtered over a medium-grade sintered-glass filter, and the residue was re-extracted for 18 hr in 750 ml of chloroform-methanol (2:1, v/v). The second extraction was filtered as before and combined with the previous extraction. The combined extracts were evaporated to dryness on a flash evaporator (Buchler Instruments, Fort Lee, N.J.) at 60 C, resuspended in chloroform-methanol (2:1, v/v), and washed as described by Folch et al. (11). The washed lipid extract was evaporated to dryness, suspended in a small volume of chloroform-methanol (2:1, v/v), and stored at −4 C under nitrogen (8).

Column chromatography. The washed total extractable lipid (405 mg) was fractionated on a silicic acid column of the type described by Vorbeck and Marinetti (28, 29). The silicic acid (200 to 325 mesh,

Unisil, Clarkson Chemical Co. Inc., Williamsport, Pa.) was activated at 100 C overnight. The activated silicic acid (12 g) was suspended in 75 ml of chloroform, poured into a 1.9-cm (outer diameter) column, and allowed to settle by gravity. The column was washed with 200 ml of chloroform, and a 2-cm layer of glass beads (0.45 to 0.50 mm in diameter) was placed on top of the column to prevent disruption of the surface. The lipid sample was dissolved in 10 ml of chloroform and placed on the column. Neutral lipids were eluted with 70 ml of chloroform. The phospholipids were eluted with the following solvents: 50 ml of chloroform-acetone (1:1, v/v), 50 ml of acetone, 30 ml of chloroform-methanol (8:2, v/v), 40 ml of chloroform-methanol (1:1, v/v), and 50 ml of chloroform-methanol (1:50, v/v). The neutral lipids eluted with chloroform were collected as a single fraction. The rest of the eluate was collected in 10-ml fractions, and the residue from each fraction was weighed. The lipid fractions were then pooled as shown in Fig. 2.

For the separation of the phosphorus-containing lipids, column chromatography was carried out according to the procedure of Kanemasa et al. (14). Unisil silicic acid (12 g) and 6 g of Celite (545 Fisher Scientific Co., Fair Lawn, N.J.) were mixed and slurried in 120 ml of chloroform-methanol (10:1, v/v). The material was placed in a glass column (3-cm outer diameter) and allowed to settle by gravity. The sample was placed on the column in 10 ml of chloroform-methanol (10:1, v/v). Four fractions were eluted from the column with chloroform-methanol (900:72, 900:90, 900:108, and 100:400, v/v).

Thin-layer chromatography. Thin-layer plates were prepared with Silica Gel HR, extra pure (E. Merck AG, Darmstadt, Germany). Plates used for identification were spread to a thickness of 0.25 mm. For preparative thin-layer chromatography, the slurry was spread to a thickness of 0.60 mm. The plates were allowed to air-dry for 1 to 2 hr, and were then activated by heating overnight at 100 C. The solvent used for nonpolar lipid separations was hexane-ether-acetic acid (70:30:1, v/v). The solvent used for polar separations was chloroform-methanol-water (70:30:4, v/v). Sugar analysis was carried out with *n*-butyl alcohol-isopropanol-water (5:3:1, v/v). For preparative thin-layer chromatography, the sample was streaked across the bottom of the plate, and, after development, the resulting bands were revealed by spraying the edges of the plates with an appropriate spray. The bands were marked and the sprayed area was discarded. The remaining portions of the bands were scraped into glass columns and eluted with chloroform-methanol (2:1, v/v).

In addition, thin-layer chromatography was carried out on microcrystalline cellulose (Avicel S., FMC Corp., American Viscose Division, Marcus Hook, Pa.) according to the method of Wolform et al. (33). Two-dimensional thin-layer chromatography was employed with the cellulose plates. The solvents were *n*-butyl alcohol-propionic acid-water (77:24:33, v/v) and phenol-water (100:38, w/v).

Sprays employed were ninhydrin for amino groups (20), molybdate for esterified phosphorus (9), Schiff reagent for vicinal hydroxyls (28), sulfosalicylic acid for water-soluble phosphate esters (29), orcinol for glycolipids (25), 50% sulfuric acid-water for charring, and Rhodamine 6 G and 2,7-dichlorofluorescein for neutral lipids. Migrations of unknown lipid fractions were compared to the migration of known lipid standards (Supelco Inc., Bellefonte, Pa.).

Purification of lipids. Lipid fractions isolated from columns or thin-layer plates were purified by dissolving them in 300 μliters of chloroform in 15-ml conical centrifuge tubes and centrifuging for 10 min at 1,500 $\times$ *g*. The chloroform solution was drawn off, and the procedure was repeated.

Gas-liquid chromatography. For separation of fatty acid methyl esters, gas chromatography with a Perkin Elmer model 881 gas chromatograph equipped with a dual flame ionization detector was used. Glass columns (1.8 meters) containing 20% diethylene glycol succinate polyester on Anakrom ABS (90 to 100 mesh) were operated at 180 C. Detector and injector temperatures were 210 C, and nitrogen flow was 30 ml/min. Methyl esters were tentatively identified by comparison of their retention times with standards (Supelco, Inc., Bellefonte, Pa.), and by use of semilog plots of chain length versus retention time. Fatty acid methyl esters were hydrogenated by the methods of Farquhar et al. (10). Gas chromatography of amino acids was done by use of the same instrument and the method of Coulter and Hahn (7). The amino acids were identified by comparison with standards (Mann Research Laboratories, Inc., New York, N.Y.).

Hydrolysis. Acid hydrolysis was carried out according to the method of Wenger et al. (30). The samples were transferred to 3-ml ampoules with chloroform. The chloroform was evaporated, and 2 ml of 3 N HCl was added. The ampoules were sealed and heated for 3 hr at 100 C. After cooling, the ampoules were opened and the contents were transferred to test tubes. The ampoules were washed with 1 ml of water and 3 ml of chloroform. The water and organic phases were mixed, centrifuged, and separated.

Mild basic hydrolysis was carried out by the method of Hübscher et al. (13). The samples were transferred to 15-ml screw-cap vials in 2 ml of chloroform; 4 ml of chloroform-methanol (2:1, v/v) and 4 ml of 0.5 N NaOH in methanol were added. After 15 min at room temperature, the contents of the vials were transferred to a separatory funnel, and 6 ml of 5 N HCl, 6 ml of water, and 20 ml of ether were added. After standing overnight, the water and organic phases were separated.

Analytical procedures. Phosphorus was determined by the method described by Bartlett (3) and modified by Marinetti (19). Amino nitrogen was determined by the method of Wheeldon and Collins (32). Plasmalogen was determined by the method of Katz and Keeney (16). Lipid content was determined gravimetrically in 10-ml beakers after drying to constant weight at room temperature under a stream of nitrogen.

RESULTS

Separation of the lipid extract. The procedure for the isolation of the lipid components of *B.*

fibrisolvens by use of column and preparative thin-layer chromatography is shown in Fig. 1. The distribution of the lipid fractions after silicic acid column chromatography is shown in Fig. 2. The R_F values determined by thin-layer chromatography and the staining characteristics of each fraction are shown in Table 1. Fraction D (Fig. 2) consisted of five subfractions. Further attempts to separate these subfractions by routine silicic acid column chromatography with various elution systems were unsuccessful. Fraction D (215 mg) was then chromatographed according to the method of Kanemasa et al. (14). Data in Table 2 show the results of column chromatography of this fraction.

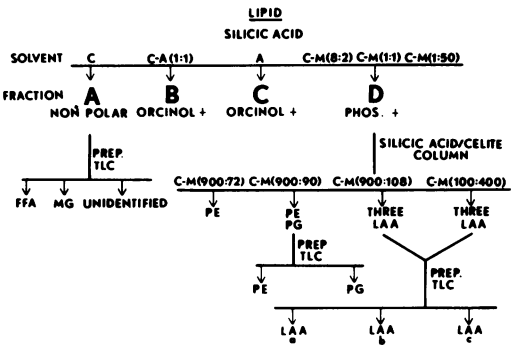

FIG. 1. *Procedure used for separation of lipid fractions. C = chloroform; A = acetone; M = methanol; FFA = free fatty acids; MG = monoglyceride; PE = phosphatidylethanolamine; PG = phosphatidyl glycerol; LAA = lipoaminoacid; TLC = thin-layer chromatography.*

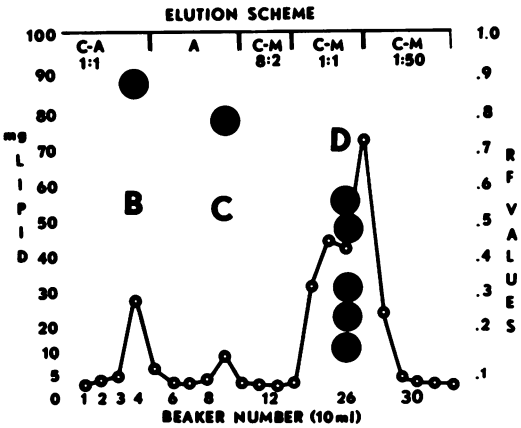

FIG. 2. *Silicic acid column chromatography of polar lipid from B. fibrisolvens strain D-1. Fraction A, not shown, was collected in bulk and contained the nonpolar lipid. Fractions B, C, and D denote the major polar fractions. Elution was with the solvents shown at the top of the figure (C = chloroform; A = acetone; M = methanol). Beakers 2 through 5 were pooled as fraction B; 8 through 10, as fraction C; and 14 through 20, as fraction D. These three fractions were chromatographed on thin-layer plates with chloroform-methanol-water (70:30:4, v/v).*

Characterization of phosphatidylethanolamine. Fraction D_1, containing 4.2% phosphorus, was ninhydrin-positive and migrated on thin-layer chromatography with the same R_F as phosphatidylethanolamine. Infrared analysis of this fraction revealed a spectrum similar to that of authentic phosphatidylethanolamine. Acid hydrolysis of both fraction D_1 and authentic phosphatidylethanolamine followed by thin-layer chromatography of the water-soluble hydrolysis products showed the presence of a ninhydrin-positive phosphate-negative spot which had the same R_F as the ninhydrin material from authentic phosphatidylethanolamine. Thus, fraction D_1 appeared to be phosphatidylethanolamine. The reaction of this fraction with 2,4-dinitrophenylhydrazine and the subsequent calculation of the phosphorus-to-aldehyde molar ratio revealed that the concentration of plasmalogen was less than 1%.

TABLE 1. *Staining reactions and R_F values of polar lipids on silicic acid thin-layer plates*[a]

Spot	R_F[b]	Staining reaction			
		Phosphate	Ninhydrin	Orcinol	Periodate Schiff
B	.86	−	−	+	+
C	.75	−	−	+	+
D_1	.52	+	+	−	−
D_2	.43	+	−	−	+
D_3	.27	+	+	−	−
D_4	.20	+	+	−	−
D_5	.14	+	+	−	−

[a] Thin-layer chromatography on silicic acid HR with chloroform-methanol-water (70:30:4).
[b] Average of three determinations.

TABLE 2. *Lipid distribution after column chromatography on silicic acid-Celite (2:1, w/w)*

Fraction	Solvent		Amt eluted
	Chloroform	Methanol	
	ml	*ml*	*mg*
D_1	900	72	110.5
D_2	900	90	26.1
D_3	900	108	32.5
D_4	100	400	48.9

Characterization of phosphatidyl glycerol. Fraction D_2 exhibited two spots upon thin-layer chromatography. One spot, ninhydrin- and phosphate-positive, migrated with the same R_F as authentic phosphatidylethanolamine. The second spot was ninhydrin-negative but phosphorus-positive, and had an R_F value similar to that of authentic phosphatidyl glycerol.

Preparative thin-layer chromatography was used to separate these two components. After elution from the plate and purification by centrifugation, the phosphorus-positive ninhydrin-negative material exhibited an infrared spectrum similar to that of authentic phosphatidyl glycerol.

Authentic phosphatidic acid, phosphatidyl glycerol, cardiolipin, and the unknown material were subjected to mild alkaline hydrolysis. The phosphorus esters were isolated and subjected to cellulose thin-layer chromatography (Table 3). The results indicated that the water-soluble phosphorus ester resulting from alkaline hydrolysis of the unknown was glycerylphosphoryl glycerol. Thus, the phosphorus-positive ninhydrin-negative material in fraction D_2 was presumed to be phosphatidyl glycerol.

Characterization of the lipoaminoacids. Fractions D_3 and D_4 each showed three spots that were phosphate- and ninhydrin-positive. To separate these spots, D_3 and D_4 were pooled, streaked on silicic acid preparative plates, and developed in chloroform-methanol-water (70:30:4, v/v). Three individual bands were scraped into glass columns, and the lipids were eluted with several volumes of chloroform and chloroform-methanol (2:8, v/v). The individual fractions were evaporated to dryness and purified by centrifugation. The amount of phosphorus and amino nitrogen in each fraction was determined (Table 4). The infrared spectra in chloroform of these three fractions showed peaks in the region of 1,740 cm^{-1}. A typical spectrum of the lipoaminoacids is shown in Fig. 3.

TABLE 3. *R_F values of alkaline hydrolysis products from phospholipids on monocrystaline cellulose thin-layer plates*

Hydrolysis product of	R_F	
	Solvent 1[a]	Solvent 2[b]
Phosphatidic acid	.17	.15
Phosphatidyl glycerol	.19	.45
Cardiolipin	.22	.55
Fraction D_2	.20	.46

[a] *n*-Butyl alcohol-propionic acid-water (77:24:33).

[b] Phenol-water (100:38).

TABLE 4. *Results of preparative thin-layer chromatography of fractions D_3 and D_4*

Lipoaminoacid	Amt	R_F[a]	Phosphorus[a]	Phosphorus-amino nitrogen (molar ratio)[a]
	mg			
A	19.0	.27	3.5	1.0:0.9
B	18.8	.20	3.1	1.3:1.0
C	16.1	.14	3.5	1.0:1.1

[a] Average of three determinations.

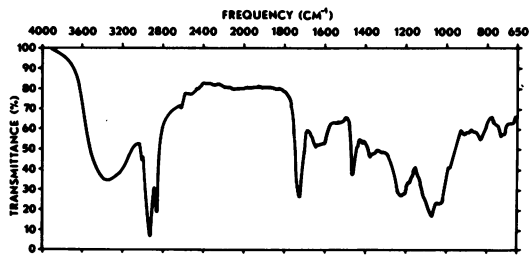

FIG. 3. *Infrared spectrum of lipoaminoacid A from Perkin-Elmer model 700 spectrophotometer. Sodium chloride cells; path length, 0.5 mm. Sample dissolved in chloroform.*

All three fractions were subjected to mild basic hydrolysis, and the water-soluble components were isolated. The water-soluble phosphate esters were subjected to thin-layer chromatography on cellulose plates; however, because of the small amounts of material, difficulty was encountered in identifying the spots. The phosphate esters from lipoaminoacids A, B, and C seemed to exhibit the same R_F as the hydrolysis product from authentic phosphatidyl glycerol. The water extracts containing the amino nitrogen were dried overnight in a desiccator, and the residues were subjected to the derivatization procedure of Coulter and Hahn (7). The esters were gas-chromatographed as described by these authors, with the results presented in Table 5. Table 6 summarizes and compares the experimental results and the theoretical values (calculated for the *o*-leucinyl ester of dipalmitylglycerylphosphorylglycerol).

Characterization of the glycolipid. As shown in Table 1, fractions B and C contained no phosphorus or amino nitrogen; however, the spots were orcinol-positive, suggesting a sugar lipid. Infrared spectroscopy of both fractions produced a spectrum similar to that of monogalactosyldiglyceride. However, thin-layer chromatography of fractions B and C with monogalactosyldiglyceride and digalactosyldiglyceride yielded R_F values for fractions B and C which did not agree

with those of either galactolipid. Fraction 2 (26 mg), fraction 3 (7 mg), and 15 mg of authentic monogalactosyldiglyceride were subjected to acid hydrolysis for 3 hr. The chloroform extracts were dried, resuspended in 50 μliters of chloroform, and spotted on a thin-layer plate with authentic samples of 1,2-diglyceride and 1,3-diglyceride. Trace quantities of diglyceride were detected in the chloroform extracts from fractions B and C and from monogalactosyldiglyceride. The water extract was dried and resuspended in 150 μliters of water. The water extracts were spotted on a thin-layer plate along with standard sugars. After development, the plate was sprayed with orcinol. Trace quantities of galactose were detected in the water extract of fractions B and C and of the monogalactosyldiglyceride (Table 7). Under the conditions used, diglyceride and galactose are the expected hydrolysis products from a galactolipid (30). These results suggest that fractions B and C contained mono- or digalactosyldiglyceride, or closely related compounds. Since the infrared spectra showed no band in the area of 1,640 to 1,680 cm^{-1}, an aliphatic amide was not present, which precluded a cerebroside-type structure.

Characterization of the nonpolar lipid. The nonpolar material (68 mg) was subjected to thin-layer chromatography with the use of the neutral solvent. The definite appearance of free fatty acids was observed as well as several other slower moving fractions. One spot, which moved just above the origin, corresponded in migration to monoglyceride. The nonpolar material was streaked onto a preparative plate and developed in the neutral solvent. Four areas of the plate were scraped into glass columns, and the material was eluted from the silica gel. The free fatty acid fraction had an infrared spectrum similar to that of stearic acid. The free fatty acids were methylated and gas-chromatographed.

The material for which the movement on thin-layer chromatography was similar to that of monoglyceride weighed only 2.3 mg. Its infrared spectrum was similar to that of authentic monoglyceride. No further work was done with this fraction.

The remaining material scraped from the plate (13 mg) was reacted with 2,4-dinitrophenylhydrazine. With the assumption of a molecular weight corresponding to that of keto palmitic acid, the carbonyl content of this fraction constituted less than 5%. Comparison of this fraction on

TABLE 5. *Gas chromatography of the amino acids isolated from the lipoaminoacids*

Lipoaminoacid	Amino acid	Per cent by (wt)
A	Alanine	41.9
	Isoleucine	6.1
	Leucine	52.0
B	Alanine	61.0
	Isoleucine	8.5
	Leucine	30.3
C	Alanine	37.4
	Isoleucine	15.1
	Leucine	47.7

TABLE 7. *Staining reaction and R_F values[a] of standard sugars and hydrolysis products from glycolipids*

Compound tested	R_F[b]	Periodate-Schiff reaction
Sugar		
Mannose	.55	Violet
Glucose	.49	Dull purple
Fructose	.46	Brown
Galactose	.38	Violet
Hydrolysis products		
Monogalactosyldiglyceride	.38	Violet
Fraction B	.38	Violet
Fraction C	.37	Violet

[a] Thin-layer chromatography on silicic acid HR with *n*-butyl alcohol-isopropanol-water (5:3:1).
[b] Average of three determinations.

TABLE 6. *Summary of data on lipoaminoacids A, B, and C, compared with the theoretical data for the o-leucinyl ester of dipalmitylglyceryl-phosphoryglycerol*

Determination[a]	Alkaline hydrolysis[b]	Phosphorus	Phosphorus-nitrogen (molar ratio)
Theoretical	GPG, amino acids	3.7	1:1
Lipoaminoacid A	GPG, alanine, leucine, isoleucine	3.5	1.0:0.9
Lipoaminoacid B	GPG, alanine, leucine, isoleucine	3.1	1.3:1.0
Lipoaminoacid C	GPG, alanine, leucine, isoleucine	3.5	1.0:1.1

[a] All compounds were ninhydrin-positive and gave a negative periodate-Schiff reaction.
[b] GPG = glycerylphosphoryglycerol.

thin-layer chromatography with that of hydroxy fatty acids was inconclusive.

The fatty acid content of the isolated phosphatidyl glycerol, phosphatidylethanolamine, lipoaminoacid, and free fatty acid is shown in Table 8.

TABLE 8. *Component fatty acids of the major lipids of Butyrivibrio fibrisolvens*

Fatty acid	Per cent (by wt)			
	FFA[a]	PE	PG	LAA
C13:0[b]	—	Tr[c]	Tr	Tr
C14:0	12.8	17.7	14.1	13.5
C15:0	—	11.3	12.2	3.0
C16:0	80.0	31.7	38.4	57.2
C16:1	7.3	10.4	7.9	6.9
C17:0	—	Tr	3.4	20.6
C17:BR	—	1.7	1.4	
C18:1	—	27.2	22.6	5.6

[a] FFA = free fatty acids; PE = phosphatidylethanolamine; PG = phosphatidyl glycerol; LAA = lipoaminoacids.

[b] Number preceding colon indicates number of carbons and number following designates degree of unsaturation. BR means branched chain.

[c] Trace, peak less than 1%.

DISCUSSION

Although glycolipids and lipoaminoacids have been previously observed in bacteria (12, 18, 21, 22, 24–27, 31), it is of interest to note their presence together in the same bacterium. The anomalous migration of the glycolipids on thin-layer chromatography may have resulted from degradation of the sample in storage, or the sugar moiety may have been different from that observed in monogalactosyldiglyceride. In previous work, Abraham (Ph.D. Thesis, Univ. of Maryland, College Park, 1965) proposed that the glycolipids from a mixed population of rumen bacteria isolated from rumen digesta were endogenous to the rumen bacteria rather than exogenous from the diet. Evidence for this was suggested by the analysis of the fatty acids bound to the glycolipids, which revealed a fatty acid pattern typical of rumen bacteria rather than one typical of patterns obtained from plants. The present work seems to substantiate this proposition. *B. fibrisolvens* was found to synthesize glycolipid from a lipid-free chemically defined medium.

The amino acid content of the lipoaminoacid fraction is interesting for several reasons. Robinson and Allison reported that 67% of the radioactive carbon from 2-methylbutyrate incorporated into the cells of *B. fibrisolvens* was found in the lipid material (23). They also reported that the reductive carboxylation of 2-methylbutyrate is a pathway for isoleucine biosynthesis. Previously, it was reported that the carbon skeleton of isovalerate was used for the biosynthesis of leucine (1). It has been suggested that these branched-chain volatile fatty acids, when incorporated into the lipid portion of the cell, were used as precursors of branched-chain aldehydes and fatty acids (23). Allison et al. (2), using *Ruminococcus flavefaciens*, reported the incorporation of radioactive carbon from isovalerate-*1*-^{14}C in a branched-chain 15-carbon fatty acid, with some in a 17-carbon acid. About 7.5% of the ^{14}C was recovered in a branched-chain 15-carbon aldehyde. Since *B. fibrisolvens* incorporates the radioactivity from 2-methylbutyrate-*1*-^{14}C into lipid, and the organism is almost entirely free from branched-chain acids and aldehydes, another pathway must be involved. This pathway could be the reductive carboxylation of the branched-chain volatile fatty acids and subsequent formation of the branched-chain amino acids which, in turn, are incorporated into the lipoaminoacids. Isobutyric, isovaleric, and DL-2-methylbutyric acids were all present in the medium.

A second point of interest involving the lipoaminoacid fraction is the relatively small amounts of branched-chain or unsaturated fatty acids. If branched-chain and unsaturated fatty acids have an influence on orientation of lipid molecules and on membrane function, as has been discussed (15), then it is interesting to speculate on the role of these lipoaminoacids in membrane function.

Work is currently in progress to elucidate the structures of the glycolipids and to study the possibility that radioactive carbon from volatile fatty acids is incorporated into the lipoaminoacids of *B. fibrisolvens*.

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