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Lipid Growth Requirement and Influence of Lipid Supplement on Fatty Acid and Aldehyde Composition of *Syntrophococcus sucromutans*

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Results concerning the ruminal fluid growth requirement of the ruminal acetogen, *Syntrophococcus sucromutans*, indicate that octadecenoic acid isomers satisfy this essential requirement. Complex lipids, such as triglycerides and phospholipids, can also support growth. The cellular fatty acid and aldehyde composition closely reflects that of the lipid supplement provided to the cells. Up to 98% of the fatty acids and 80% of the fatty aldehydes are identical in chain length and degree of unsaturation to the octadecenoic acid supplement provided in the medium. *S. sucromutans* shows a tendency to have a greater proportion of the aldehyde form among its 18 carbon chains than it does with the shorter-chain simple lipids, which may be interpreted as a strategy to maintain membrane fluidity. ^{14}C labeling showed that most of the oleic acid taken up from the medium was incorporated into the membrane fraction of the cells.

Syntrophococcus sucromutans, isolated as the most numerous ruminal bacterium demethylating methoxy groups of monoaromatic compounds (10^7 cells per ml), has a unique energy metabolism. It produces acetate and carbon dioxide, using caffeate reduction to hydrocaffeate, O demethylation of phenyl-methyl ethers, formate or hydrogenotrophic bacteria as electron acceptor systems with various sugars, and glycosides as possible electron donors. This dual requirement is absolute, since *S. sucromutans* will not grow effectively using sugars or pyruvate, unless the hydrogen partial pressure is kept very low (17). Further investigation of its physiology was nevertheless impaired by an unknown nutritional requirement satisfied by ruminal fluid, such that a medium containing more than 30% (by volume) of ruminal fluid was necessary for near-optimal growth.

Growth factor studies have been performed in parallel with the isolation of new species and genera of bacteria on nonselective media. It is quite understandable, knowing the complexity of the ruminal environment, that well-adapted microbes often have specific requirements for nutrients present in steady supply from the breakdown of dietary components or from the metabolic activities of other ruminal microbes.

Since none of the components of the most complex nonselective but still well-defined media previously developed, such as medium 10 of Caldwell and Bryant (5), could satisfy the growth requirement of *S. sucromutans*, we have investigated this requirement in order to develop more-defined culture conditions for physiological, biochemical, and genetic studies. We describe here a lipid requirement and further report on the incorporation of exogenous fatty acids into membrane fatty acids and aldehydes.

MATERIALS AND METHODS

Organisms and maintenance. *S. sucromutans* S195 and *Selenomonas ruminantium* HD4 were from our laboratory collection. Cellobiose (10 mM) and syringic acid (5 mM) replaced fructose and formate, respectively, in the solid medium described by Krumholz and Bryant (17), used for transfers of *S. sucromutans* every 3 weeks. *S. ruminantium* was maintained on RGCA slants (3) as modified by Bryant and Robinson (4).

Media and culture conditions. Anaerobic techniques were used throughout (18). The basal medium for *S. sucromutans* growth assays was that of Krumholz and Bryant (17), modified by the inclusion of only 30 mM bicarbonate and the replacement of Casitone by 0.2% (wt/vol) each of Trypticase and yeast extract. The sterile buffered basal medium was prepared in tubes (100 by 13 mm) in 80% of the final volume (4 ml) to allow the addition of sterile stocks of the desired supplement and reducing agent with a syringe prior to inoculation.

S. ruminantium HD4 was grown by using a similar basal medium, with the following modifications; 0.4% (wt/vol) Trypticase, 0.01% (vol/vol) *n*-valerate, 40 mM bicarbonate buffer, and 15 mM glucose as the main energy source.

For growth assays, two transfers were made in liquid medium before use in the test medium (2% inocula). Ruminal fluid (30%) in the transfer medium was later replaced by 200 μg of crude egg yolk phosphatidylcholine (Sigma Chemical Co., St. Louis, Mo.) per ml. Growth assays were performed with medium containing 0, 5, or 10% ruminal fluid as indicated. Ruminal fluid (5 and 10%) basal medium was used when possible inhibition or synergistic effects were to be tested, and 30% ruminal fluid controls were commonly used as near-optimal growth references. For large batch cultures, the medium was steamed, sparged with N_2-CO_2 gas, buffered and reduced while cooling, and autoclaved.

Cultures were incubated at 38 to 39°C.

Bacterial growth and cell extract preparation. Bacterial growth was measured spectrophotometrically (A_{600}; Spectronic 70, Bausch & Lomb, Inc., Rochester, N.Y.). Crude cell extracts were prepared in 10-ml samples (from 750 ml of a batch culture harvested in early stationary phase) by single passage through a French pressure cell (52,400 kPa) and centrifugation (12,000 × *g*, 15 min, twice). Ultracentrifugation conditions were 80,000 × *g* for 2 h at 4°C.

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Extraction of lipids from ruminal fluid. In all experiments involving the manipulation of lipid extracts, all glassware was rinsed with chloroform-methanol. Teflon-lined screw caps were used.

Chloroform-methanol extracts of ruminal fluid were prepared by a modification of the technique of Bligh and Dyer (2). The results were termed crude lipid extract.

Before fractionation of lipid components, further removal of non-lipidic contaminants was made, where indicated, by passing the extracts through a Sephadex G25-50 (superfines, Sigma) column by the procedure of Wells and Ditmer (26). The resulting preparations were termed purified lipid extracts.

Extraction of lipids from bacterial cells. Total cellular lipids were extracted by using a modification of the above procedure described by Vorbeck and Marinetti (24). For storage, the extracts were concentrated extensively and stored under a N_2 gas phase at −20°C.

Fractionation of lipid extracts. Microbial cell lipid extracts (25 mg) were fractionated by liquid chromatography on silicic acid (100 mesh, Mallinckrodt Chemical Works, St. Louis, Mo.) with a column (22 by 1 cm). Three fractions were collected with a gravity flow setting of 1 ml/min as described previously by Kates (15). Ruminal fluid extracts were chromatographed in a similar manner by using silica gel flash chromatography (Baker Research Products, Phillipsburg, N.J.) with 20 mg of dry silica gel for 5 mg of lipid extract.

For preparative thin-layer chromatographic (TLC) fractionation of a mixture of phospholipids, the crude complex lipids (25 mg), dissolved in 0.5 ml chloroform, were spotted on a silica gel GF plate (20 by 20 by 0.1 cm) (Analtech, Inc., Newark, Del.) run once with chloroform-methanol-water (65:25:1) as the mobile phase. Bands, detected by exposure to UV before and after spraying with rhodamine 6G on one edge of the plate and with Zinzade reagent for phosphorus on the other edge, were scraped off the plate, and the lipids were recovered from the support with three 15-ml washes, using chloroform-methanol-water (1:2:0.8). Further reextraction proceeded as described above. The chloroform extracts were concentrated in a rotary evaporator (Rinco Instrument Company, Inc., Greenville, Ill.) and finally under a N_2 stream in glass tubes. Recovery was 80% or greater (by weight). Tentative identification was made with pure phospholipids as controls.

Mild alkaline deacylation was performed as described by Kates (15), and the products were separated by extraction as described above. Qualitative TLC analyses of treated phospholipids indicated that the deacylation was never complete, leaving a fatty acid-containing fraction enriched in lysophospholipids and a polar fraction free of fatty acids.

Preparation of lipid fractions for assay as growth supplement. Since traces of chloroform were inhibitory to the growth of *S. sucromutans*, a procedure to suspend chloroform extracts into aqueous suspensions for addition to the culture medium was developed.

Ruminal fluid lipid extracts were concentrated 200-fold (per volume) under N_2 for storage and suspended in ethanol to be added either directly to the medium as 40-fold concentrated stock or diluted back to the original ruminal fluid volume with water free of oxygen. In other cases, such as with cell extracts, the chloroform was removed by sequentially concentrating the sample under an N_2 stream and suspending the sample, first with ethanol, then with ethanol-water (1:1), and finally with water. The final concentration of ethanol (below 0.4%) did not affect growth.

Pure polar lipids were suspended in ethanol and added to the medium prior to autoclaving, whereas the fatty acids were suspended by sonic dispersion (Branson Sonic Power, setting 3, maximum output; Branson Instruments, Inc., Danbury, Conn.) in a sodium taurocholate solution (final concentration of 75 μg of bile salt) as described previously (11).

Analysis of simple lipids (fatty acids and aldehydes). Simple lipids were obtained from crude or purified extracts by direct *trans*-esterification with boron trifluoride-methanol, as described by Morisson and Smith (22). The fatty acid methyl esters (FAME) and dimethylacetals (DMA) obtained were separated by using analytical silica gel G TLC plates (Analtech) chromatographed once with benzene and recovered quantitatively by washing the gel in glass wool-plugged funnels with diethylether for FAME or chloroform for DMA. A 5985B Hewlett-Packard gas chromatograph-mass spectrometer equipped with a Supelcowax 10 capillary column (30 m long, 0.25-μm internal diameter), at a hydrogen pressure of 103.4 kPa with a split ratio of 100:1, was used. The oven temperature was programmed at 175°C for 1 min, rinsing to 200°C at a rate of 1.5°C/min, and 200°C for a further 15 min. Mass spectral characteristics were used in the identification of all simple lipids (21), in addition to Nu Check Prep (Nu Check Prep, Inc., Elysan, Minn.) and Supelco Bacterial standards (Supelco, Inc., Bellefonte, Pa.) for FAME and DMA prepared from cells of *S. ruminantium* grown with valerate (14).

Labeling experiment. *S. sucromutans* was grown in a medium containing [1-^{14}C]oleic acid (150 μM, 10 μCi/liter) suspended in crude phospholipids (50 μg/ml of medium). The label was monitored in the gas phase, the culture supernatant, two washes with 100 mM phosphate buffer (pH 6.8, the first of which contained 0.1% Triton X-100), and finally in the cells.

Half of the cells were subsequently subjected to lipid extraction, and the distribution of the label was determined in the simple lipid fraction by using the analytical procedures described above for fractionation. The other half was subjected to a differential fractionation of the cell components. The cells were treated with 50 μg of lysozyme per ml and 400 μg of EDTA per ml, added between two sonications (total time of 6 min) performed in a sonicator bath with water at 0°C. The reaction mixture was then incubated for 30 min at room temperature and diluted fivefold with water. Unbroken cells were removed (3,000 × *g*, for 3 min) before further fractionation into total soluble components and total membranes by ultracentrifugation (300,000 × *g*, 20 min, 4°C). Scintillation cocktails were from Dupont, NEN Research Products, Boston, Mass. CO_2 was trapped in redistilled phenylethylamine (Sigma) and counted in Oxifluor, aqueous samples were counted in Aquasol-2, and organic samples, as well as TLC strips, were counted in toluene-Omnifluor. Labeled samples were counted in a Beckman LS 5801 scintillation counter, and all counts were corrected for quench and background.

Chemicals. Organic solvents were reagent grade from various sources. All phospholipid and fatty acid preparations were from Sigma. [1-^{14}C]oleic acid was from Dupont NEN.

RESULTS

Ruminal fluid as growth-promoting supplement. When increasing concentrations of ruminal fluid were used as the growth supplement in a medium containing 0.2% Casitone and 10 mM fructose and 10 mM formate, *S. sucromutans*

exhibited a linear response up to the 30% (vol/vol) ruminal fluid in the medium (17; this study). Thus, the optimum level is somewhat higher. Little or no growth occurred in the absence of ruminal fluid, although the medium contained a complex mixture of vitamins, trace metals, and minerals. Similar results were observed when 0.2% yeast extract and 0.2% Trypticase were substituted for the Casitone. Yeast extract alone (0.2 and 0.4% [wt/vol]) was stimulatory for growth in 10% ruminal fluid basal medium but had no effect at all up to 0.4% (wt/vol) in the basal medium without ruminal fluid. A mixture containing 50 μM hemin and 1,4-naphthoquinone, 10 mM acetate, 0.5 mM isobutyric acid, 0.5 mM isovaleric acid, 0.5 mM DL-2-methylbutyric acid, 0.5 mM *n*-valeric acid, 0.5 mM phenylacetic acid, 0.5 mM phenylpropionic acid, 0.05% (vol/vol) Tween 40, and 0.05% (vol/vol) Tween 60 also was unable to replace ruminal fluid for growth, although it stimulated growth. The stimulatory effect in terms of final growth yield was almost twice as high in the medium containing 5% (vol/vol) ruminal fluid than with the complex mixture as the sole supplement, with an increase in absorbance of 0.35 and 0.20 U, respectively, above the values for the corresponding controls.

Ruminal fluid lipid extract as growth-promoting supplement. We observed that the growth-promoting effect of ruminal fluid could be consistently and almost quantitatively recovered in the total lipid extract (2). No growth stimulation for *S. sucromutans* was observed with the residual methanolic phase of ruminal fluid lipid extractions. Concentrating the chloroform extracts under a stream of nitrogen gas was impaired by the observation that complete dryness usually led to losses of material on the glassware and incomplete recovery of growth-promoting activity.

***S. ruminantium* cellular lipids as growth-promoting supplement.** When cells were subjected to total lipid extractions, the total lipid fractions replaced ruminal fluid. The best results were obtained with lipid extracts of *S. ruminantium*; and, since the cell extracts were less complex than the ruminal fluid lipid extracts, we fractionated the cellular lipid components by liquid chromatography with silicic acid. With *S. ruminantium* total lipids, both the least- and most-polar fractions, which eluted, respectively, first with chloroform and third with methanol, shared the growth-promoting activity. The chloroform and acetone second fraction exhibited little activity. Analytical TLC indicated that the prevailing phospholipid had an R_f similar to that for pure phosphatidylethanolamine (as expected for *S. ruminantium*) and was partially eluted in the first fraction.

Phospholipids as growth supplements. Crude phosphatidylcholine from egg yolk was highly stimulatory to growth, and pure phosphatidylglycerol and phosphatidylcholine were also stimulatory. Bovine brain crude lipid extracts were also highly stimulatory. The 60% pure egg yolk phosphatidylcholine totally replaced ruminal fluid (Fig. 1), with optimum levels of 200 μg/ml of medium, or 250 μM (final concentration), assuming an average M_w of 800.

The effect of pure phospholipids as well as that of fractions of the egg yolk phosphatidylcholine obtained by preparative TLC (Table 1) ranged from no stimulation to high activity, although no fraction was as good as the crude phosphatidylcholine control for supporting growth of *S. sucromutans*. The results indicated that the various complex lipids present, together with phosphatidylcholine, in this preparation were additive in their effects.

A chemical deacylation of the crude phosphatidylcholine gave a maximum A_{600} of 0.92 for the fatty acid-containing fraction and 0.13 for the deacylated fraction compared with

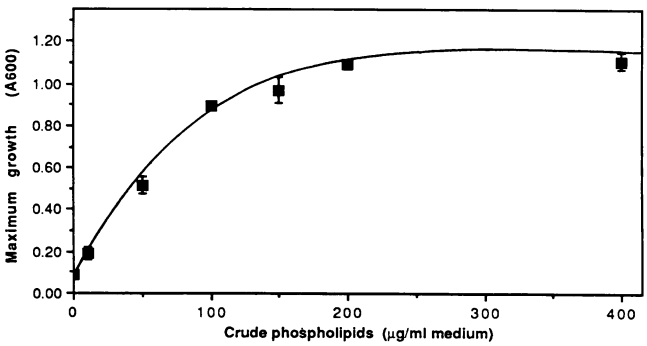

FIG. 1. Growth response of *S. sucromutans* to the addition of crude phospholipids to a basal medium containing no ruminal fluid. Crude phospholipids (phosphatidylcholine; Sigma type IX-E) were added in an ethanolic solution to give a maximum of 0.4% (vol/vol) ethanol in the medium. Results are means of three tubes. Vertical bar represents standard deviation.

0.10 for the control basal medium. The untreated control allowed growth (A_{600} of 1.05). When pure phosphatidylcholine was similarly treated, the fatty acid fraction supported even heavier growth than the untreated phospholipid (A_{600} of 0.76 versus 0.28, respectively, and 0.12 for the deacylated fraction, over the same control as above).

Fatty acids and tripalmitin as growth supplements. The results for single fatty acids are shown in Fig. 2. A preliminary assay of palmitic and vaccenic acid at various concentrations (0, 50, 100, 150, and 200 μM) had showed a bell-shaped response for vaccenic acid with optimum levels of 150 μM and no stimulation at all palmitic acid concentrations, but pure tripalmitin (250 mM) was moderately stimulatory (data not shown). Finally, linoleic ($C_{18:2}$), linolenic ($C_{18:3}$), as well as palmitoleic acid ($C_{16:1}$) were almost completely inhibitory.

Effect of the lipid supplement on *S. sucromutans* simple lipid composition. A comparison of the fatty acids and fatty

TABLE 1. Growth response of *S. sucromutans* to preparative TLC fractions of crude phosphatidylcholine[a]

Preparation assayed[b]	Maximum growth	
	A_{600}	SD
Basal medium	0.36	0.01
TLC fraction[c] (% dry weight recovery)		
Highly polar lipids (8)	0.43	0.01
Phosphatidylserine (14)	0.58	0.04
Sphingomyelin (7)	0.50	0.02
Phosphatidylinositol (11)	0.44	0.02
Lyso- and phosphatidylcholine (5)	0.45	0.01
Phosphatidylcholine and phosphatidylglycerol (31)	0.47	0.02
Phosphatidylethanolamine and phosphatidic acid (10)	0.40	0.02
Phosphatidyl *n*-methyl-ethanolamine (4)	0.40	0.02
Less-polar lipids (9)	0.40	0.02
Pooled fractions	0.96	0.01
Crude phosphatidylcholine	1.02	0.05

[a] All results are from triplicate tubes. Maximum growth was reached after 4.5 to 5.5 days.

[b] All fractions were assayed in a basal medium with cellobiose and formate (10 mM each) and 10% ruminal fluid.

[c] TLC fractions were separated on a Silicagel G plate by using chloroform-methanol-water (65:25:4) as the mobile phase, detected, and reextracted as detailed in the text. They were prepared for assay to give a final concentration equivalent to 100 μg of original material per ml of medium.

FIG. 2. Growth response of *S. sucromutans* to the addition of fatty acids in the medium. Fatty acids were suspended in a sodium taurocholate solution by sonic dispersion at melting temperature, and added to a basal medium (bm) at a final concentration of 150 μM. Abbreviations: N:0, N being the number of carbons in the straight-chain saturated fatty acid; N:1, monoenoic acid, with position and isomer cis [c] or trans [t] indicated for octadecenoic acid; br, branched fatty acid. No growth stimulation was found with straight-chain saturated acids of 6 to 11 carbons or with 5-undecenoic acid. Myristic ($C_{14:0}$) was contaminated with branched and unsaturated 14 and 15 carbon isomers. The isostearate contained only 50% isostearate contaminated with branched and unsaturated 17 and 18 carbon acids.

aldehydes of the cells and the various lipid supplements used for growth indicated that the major chain lengths were the same in a lipid supplement and in the cellular simple lipids after growth on that supplement (Table 2). In addition, *S. sucromutans* synthesized fatty aldehydes (Table 3), with a consistent tendency to make more aldehydes of the 18 carbon chain lengths (longest), whereas this tendency was reversed for the fatty acids, of which it made a greater proportion of the shorter chain lengths. Furthermore, this observation was independent of the lipid supplement, especially since it still held true with ruminal fluid as supplement, in which these proportions were reversed, i.e., more long-chain fatty acid and more short-chain aldehydes.

When the cells were grown with pure *trans*-vaccenic acid as the sole lipid supplement, 98% of the fatty acid and 80% of the aldehydes recovered were of the octadecenoic type ($C_{18:1}$).

Incorporation of [1-^{14}C]oleic acid. Cells grown with [1-^{14}C]oleic acid incorporated 19% of the label (402,871 dpm) of which 50.2% was in unbroken cells and 49.8% was in disrupted cells (the latter value obtained by the difference between total incorporation and undisrupted cell incorporation). Of the total incorporation into cells that were disrupted, 75.1% was recovered, 56.3% in the membrane fraction and 18.8% in the cell soluble fraction. This suggested

TABLE 2. Fatty acid composition of lipidic growth supplements and cells of *S. sucromutans*

FAME structure[a]	% Integrator signal in cells grown with:						
	Ruminal fluid		Phosphatidylcholine		Branched $C_{18:0}$ supplement	Phosphatidylcholine and branched $C_{18:0}$ supplement	*trans*-Vaccenic acid
	Supplement[b]	Alone	Supplement	Alone			
<13:0	0.62	0.77	–[c]	tr[d]	–	2.47	–
13:0	tr	1.68	tr	0.91	–	0.87	–
b14:0	–	1.94	–	–	–	–	–
14:0	1.99	8.42	tr	0.64	–	1.50	–
14:1	–	0.88	–	–	–	–	–
i15:0	1.94	3.08	tr	–	–	–	–
a15:0	4.01	4.19	tr	–	–	–	–
15:0	3.16	4.50	tr	0.59	tr	0.83	–
15:1	–	1.27	1.23	1.26	–	0.86	–
i16:0	1.22	1.98	–	tr	–	–	–
16:0	27.06	29.22	39.65	59.68	3.62	45.00	–
16:1	–	1.53	0.78	1.72	2.03[e]	1.57[e]	–
i17:0	0.63	0.97	–	tr	1.72	0.50	–
a17:0	0.98	1.25	–	tr	4.26	3.33	–
17:0	1.12	0.66	tr	0.71	4.03	2.74	–
17:1	–	–	tr	2.05	23.78[e]	7.01[e]	–
b18:0[f]	tr	1.38	–	2.47	6.24	1.57	–
b18:0[f]	–	–	–	–	4.69	1.25	–
i18:0	–	–	–	–	4.67	1.82	–
a18:0	–	–	–	–	3.28	1.84	–
18:0	40.72	14.43	27.27	23.60	4.29	13.31	–
18:1/9c	2.48	2.97	27.31	3.59	0.57	5.13	–
18:1/9t	2.84	4.20	1.21	0.52	0.85	0.74	–
18:1/11t	0.85[f]	–	–	–	–	–	98.20[g]
18:2	3.09	1.76	0.99	tr	4.50	2.32	–
18:3	tr	–	–	–	–	–	–
Others	7.30	12.92	1.56	2.26	31.47	5.34	1.80

[a] Fatty acid methyl esters. Abbreviations: b, branched; i, iso; a, anteiso; c, *cis*; t, *trans*.
[b] Analysis of lipidic growth supplement separately. These were used at concentrations of 30% (vol/vol) for ruminal fluid, 200 μg of phosphatidylcholine per ml, and 150 μM for the fatty acids.
[c] –, Not detected.
[d] Trace (below 0.5%).
[e] Monoenoic acid or branched isomer.
[f] These peaks were too close to others for adequate identification and quantification.
[g] Result of two complete procedures (standard deviation, 0.91). Substrate was 98% pure.

TABLE 3. Fatty aldehyde composition of lipidic growth supplements and cells of *S. sucromutans*[a]

DMA structure	% Integrator signal for:				
	Ruminal fluid	Cells grown with:			
		Ruminal fluid	Phosphatidylcholine	Phosphatidylcholine and branched $C_{18:0}$ supplement	*trans*-Vaccenic acid
<13:0	tr	–	–	–	–
13:0	1.34	2.21	–	tr	tr
13:1	tr	–	–	–	–
b14:0	11.04	1.18	–	–	–
14:0	9.52	2.24	tr	0.65	tr
14:1	6.40	–	–	–	–
i15:0	tr	1.40	–	–	–
a15:0	6.14	4.03	–	–	–
15:0	19.59	2.64	tr	0.67	–
15:1	6.35	–	–	–	–
b16:0	tr	–	–	0.54	–
i16:0	4.88	1.33	–	–	–
16:0	24.29	17.62	14.18	43.83	0.62
16:1	0.94	1.74	1.76	1.65[c]	tr
b17:0	–	–	–	tr	–
i17:0	0.94	0.99	–	6.56	3.02
a17:0	0.86	1.31	–	0.75	–
17:0	tr	1.03	tr	1.06	1.53
17:1	–	–	tr	2.19[c]	0.57[c]
b18:0	–	–	–	tr	1.50
b18:0	–	–	–	tr	–
i18:0	–	–	–	2.54	–
18:0	1.99	36.49	11.81	3.33	0.80
18:1/9c	1.95	8.80	32.67	25.14	–
18:1/8t	2.52	10.39	3.17	3.37	–
18:1/11t	–	–	–	–	80.26
18:2	1.18	5.70	2.22	1.28	–
Others	–	–	34.19	6.44	11.70

[a] For abbreviations amd concentrations, see footnotes *a* and *b*, respectively, in Table 2. Phosphatidylcholine, branched $C_{18:0}$, and *trans*vaccenic acid contained no aldehydes.

[c] Monoenoic acids or branched isomers.

that most of the label of [1-^{14}C]oleate was incorporated into membranes.

Of the [1-^{14}C]oleate label, 14% was recovered in the cellular fraction of *S. sucromutans*, with an overall recovery in this first fractionation of 86% in cells used to study incorporation of label into simple lipids. From the cells, 46% of the label was recovered in the total lipid extract versus 43% in the residual phase. The recovery for a [^{14}C]oleic acid control through *trans*-esterification, hexane extraction, and TLC separation of FAME and DMA was, on average, 72%. Using this procedure, it was observed that all the label from the lipid extract was recovered in the simple lipids, with respective proportions of FAME and DMA of 72 and 26% in this fraction. Of the label from the residual phase, 19% could be recovered in the same manner, presumably corresponding to material which had escaped extraction. FAME and DMA represented 7 and 12% of this fraction, respectively. Furthermore, all the label in the residual fraction (92%, with a recovery of 96% overall) was observed to be trichloroacetic acid precipitable.

DISCUSSION

Our investigation of the ruminal fluid growth factor required for growth of *S. sucromutans* led to the study of various classes of lipids. Phospholipids, particularly in crude preparations such as 60% pure phosphatidylcholine, could substitute for ruminal fluid. The recombined fractions of preparative TLC fractionation of this crude phosphatidylcholine showed that little loss occurred during the procedure and made it possible to identify the active fraction even if only one of these were to correspond with the growth requirement of the bacterium. In this case, we observed a partial stimulation of growth. No attempts were made to assay individual fractions at high concentrations, but assays of a variety of pure phospholipids and tripalmitin gave similar indications. In general, pure egg yolk complex lipids seemed to be more stimulatory than bovine brain components at the same concentration, and the most-polar molecules appeared more effective.

That the fatty acids might be the fraction of the phospholipid molecule actually required for growth of *S. sucromutans* was supported by the results of chemical deacylation. These results indicated that the deacylated glycerophosphate backbone or pure glycerophosphate had no stimulatory effect in itself, whereas the fatty acid-containing fraction retained all the activity for growth. The early attempts to replace ruminal fluid by phospholipid preparations were motivated by the results of fractionations of lipid extracts of *S. ruminantium* on silicic acid. The most-polar fractions, expected to contain the phospholipids, consistently stimulated growth, and furthermore, the least-polar fraction from extracts (neutral lipids, triglycerides, and free fatty acids), which was also stimulatory, was contaminated by the major phospholipid. The variable effect of a given phospholipid for growth of *S. sucromutans*, which seemed to be a function of its origin, also led us to further investigate the effect of pure fatty acids on growth. The results suggested that the fatty acid supplement might be used as precursors, incorporated intact into the cell, which was confirmed by the labeling experiment.

Growth factor requirements satisfied only by long-chain unsaturated fatty acids, such as those described here have rarely been documented for ruminal bacteria. *Butyrivibrio* sp. strain S2 is capable of degrading phospholipids or glycolipids and will grow with a whole range of long-chain fatty acids as individual lipid supplements (11). Long straight-chain acids and/or their iso or anteiso branched isomers support growth of many other ruminal bacteria, in the absence of short chains. If *S. sucromutans* has a growth requirement similar to *Butyrivibrio* sp. strain S2, it is unique in its ability to use only octadecenoic acids in place of ruminal fluid among all the free fatty acids tested. Interestingly, oleic acid, one of the octadecenoic acid isomers that supports growth of *S. sucromutans*, was found to inhibit the major ruminal cellulolytic components in pure culture, while it slightly stimulated growth of other bacteria (12, 19). The growth requirement of *S. sucromutans* when fatty acids are used as lipid supplement is thus far more restricted than that described for *Butyrivibrio* sp. strain S2.

Active biohydrogenation of polyunsaturated fatty acids by the ruminal microbiota has been well documented (6, 9, 16). Although linoleic and linolenic acids are two major components of plant fatty acids, they are found only in low concentrations in the rumen because of microbial hydrogenation (9). Indeed, high concentrations of vaccenic acid, a major intermediate, have been observed in the rumen shortly after the animal was fed. While we did not observe any hydrogenation of polyunsaturated fatty acids by *S. sucromutans*, vaccenic acid was the most effective of the three octadecenoic acid isomers tested. In fact, polyunsaturated acids ($C_{18:2}$ and $C_{18:3}$) proved to be inhibitory. In addition,

little β-oxidation of fatty acids usually occurs in the rumen because the syntrophic bacteria that carry out this activity have a growth rate which is too slow for these to be maintained in large enough numbers (20).

Catabolic activities towards complex lipids have been described for ruminal bacteria. Phospholipases, glycolipases, and in a more general sense, esterases, are known to be present and release fatty acids from dietary or bacterial complex lipids (9, 11, 13). Although in *S. sucromutans* these were not assayed directly, they are likely to be present. The major support for this statement comes from the observation that certain complex lipids can support growth of the bacterium while they do not contain free octadecenoic acid isomers, the only simple lipids that can replace ruminal fluid in the fatty acid form. Since the cells have a lipid composition that closely reflects that of the lipid supplement, they show the ability to use an array of fatty acids, provided these are supplied in their esterified form. When tripalmitin was prepared for assay as were the fatty acids and tested in parallel at levels of about 200 μg/ml of medium (250 μM), growth stimulation was as high as when using the most effective pure phospholipids tested. Similarly, egg yolk sphingomyelin, which stimulated growth in our assay contained almost exclusively palmitic and stearic acid. Furthermore, when specifically acylated phosphatidylcholines were used as the sole lipid supplement, dioleoylphosphatidylcholine was more efficient in supporting growth than were dipalmitoyl- and distearoylphosphatidylcholine. The two latter phospholipids were still slightly stimulatory for growth of *S. sucromutans* when tested in a basal medium without ruminal fluid (data not shown).

The synthesis of aldehydes is a common trait among anaerobes (7) where they are inserted in the plasmalogen fraction of the phospholipids. The first mention of bacterial plasmalogens came with the description of the fate of volatile fatty acids required for growth of ruminococci (1). Some other ruminal bacteria containing plasmalogens include *Fibrobacter* (*Bacteroides*) *succinogenes* (25), *S. ruminantium* (14), and *Megasphaera elsdenii* (23). The presence of plasmalogens may in fact be a very interesting criterion of evolutionary phylogeny (7). It has been observed that the phase transition temperatures are actually lower in bilayers of plasmalogens versus phospholipids after preparation with elaidate-enriched complex lipids (8). The tendency shown by *S. sucromutans* to make a greater proportion of fatty aldehydes of 18 carbons than of shorter chains and the contrary tendency for fatty acids may be a strategy to maintain given proportions of fatty aldehyde to fatty acid by chain length, ensuring optimal membrane fluidity.

Although when grown in the presence of both 150 μM oleic acid and 50 μg of phospholipids per ml, *S. sucromutans* assimilated the fatty acid, our study did not indicate whether the bacterium will preferentially use the octadecenoic acid or the complex lipids in its natural habitat. In either case, *S. sucromutans* may have to compete for its growth requirement with other bacteria or with the characteristic of lipids to adhere to particulate matter (10). This is clearly indicated by the total absence of growth-promoting activity of incubated ruminal fluid (27), in which incubation for several days at a controlled neutral pH potentially causes a depletion in lipid components by assimilation or adsorption and probably increases the degree of hydrogenation. In turn, the association with particulate matter (with a slower turnover rate in the rumen) may help the bacterium to maintain itself, especially if nutrients become limiting.

Although they do not reproduce the exact fatty acid composition of its original habitat, crude egg yolk phospholipids will be used further as lipid supplement for *S. sucromutans*, together with volatile fatty acids which were observed to slightly enhance the growth rate. This will provide a chemically defined medium suitable for further physiological, biochemical, and genetic studies of *S. sucromutans*.

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LITERATURE CITED

1. **Allison, M. J., M. P. Bryant, I. Katz, and M. Keeney.** 1962. Metabolic function of the branched chain fatty acids, growth factors for ruminococci. J. Bacteriol. **83:**1084–1093.
2. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37:** 911–917.
3. **Bryant, M. P., and L. A. Burkey.** 1953. Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. J. Dairy Sci. **36:**205–217.
4. **Bryant, M. P., and I. M. Robinson.** 1962. Some nutritional characteristics of predominant culturable ruminal bacteria. J. Bacteriol. **84:**605–614.
5. **Caldwell, D. R., and M. P. Bryant.** 1966. Medium without rumen fluid for nonselective enumeration and isolation of rumen bacteria. Appl. Microbiol. **14:**794–801.
6. **Fulco, A. J.** 1983. Fatty acid metabolism in bacteria. Prog. Lipid Res. **22:**133–160.
7. **Goldfine, H.** 1982. Lipids of prokaryotes—structure and distribution. Curr. Top. Membr. Transp. **17:**1–43.
8. **Goldfine, H., N. C. Johnston, and M. C. Phillips.** 1981. Phase behavior of ether lipids from *Clostridium butyricum*. Biochemistry **20:**2908–2916.
9. **Harfoot, C. G.** 1978. Lipid metabolism in the rumen. Prog. Lipid Res. **17:**21–54.
10. **Harfoot, C. G., M. L. Crouchman, R. C. Noble, and J. H. Moore.** 1974. Competition between food particles and rumen bacteria in the uptake of long chain fatty acids and triglycerides. J. Appl. Bacteriol. **37:**633–641.
11. **Hazlewood, G., and R. M. C. Dawson.** 1979. Characteristics of a lipolytic and fatty acid requiring *Butyrivibrio* sp. isolated from the ovine rumen. J. Gen. Microbiol. **112:**15–27.
12. **Henderson, C.** 1973. The effect of fatty acids on pure cultures of rumen bacteria. J. Agric. Sci. Camb. **81:**107–112.
13. **Hespell, R. B., and P. J. O'Brian-Shah.** 1988. Esterase activities in *Butyrivibrio fibrisolvens* strains. Appl. Environ. Microbiol. **54:**1917–1922.
14. **Kamio, Y., S. Kanegasaki, and H. Takahashi.** 1970. Fatty acid and aldehyde compositions in phospholipids of *Selenomonas ruminantium* with reference to growth conditions. J. Gen. Appl. Microbiol. **16:**29–37.
15. **Kates, M.** 1972. Techniques in lipidology: isolation, analysis and identification of lipids, p. 269–610. *In* T. S. Work and E. Work (ed.), Laboratory techniques in biochemistry and molecular biology, vol. 3. Elsevier Publishing Co., Inc., New York.
16. **Katz, I., and M. Keeney.** 1966. Characterization of the octadecenoic acids in rumen digesta and rumen bacteria. J. Dairy Sci. **49:**967–970.
17. **Krumholz, L. R., and M. P. Bryant.** 1986. *Syntrophococcus sucromutans* sp. nov. gen. nov. uses carbohydrates as electron donors and formate, methoxybenzenoids or *Methanobrevibac-*

ter as electron acceptor systems. Arch. Microbiol. **143**:313–318.
18. **Krumholz, L. R., and M. P. Bryant.** 1986. *Eubacterium oxidoreducens* sp. nov. requiring H_2 or formate to degrade gallate, pyrogallol, phloroglucinol and quercetin. Arch. Microbiol. **144**: 8–14.
19. **Maczulak, A. E., B. A. Dehority, and D. L. Palmquist.** 1981. Effect of long chain fatty acids on growth of rumen bacteria. Appl. Environ. Microbiol. **42**:856–862.
20. **McInerney, M. J., M. P. Bryant, and N. Pfennig.** 1979. Anaerobic bacterium that degrades fatty acids in syntrophic association with methanogens. Arch. Microbiol. **122**:129–135.
21. **McLafferty, F. W.** 1980. Interpretations of mass spectra, 3rd ed. University Science Books, Mill Valley, Calif.
22. **Morisson, W. R., and M. Smith.** 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol. J. Lipid Res. **5**:600–608.
23. **VanGolde, L. M. G., J. Akkermans-Kryswijk, W. Franklin-Klein, A. Lankhorst, and R. A. Prins.** 1975. Accumulation of phosphatidylserine in strictly anaerobic lactate fermenting bacteria. FEBS Lett. **53**:57–60.
24. **Vorbeck, M. L., and G. V. Marinetti.** 1965. Separation of glycosyl diglycerides from phosphatides using silicic acid column chromatography. J. Lipid Res. **6**:3–6.
25. **Wegner, G. H., and E. M. Foster.** 1962. Incorporation of isobutyrate and valerate into cellular plasmalogen by *Bacteroides succinogenes*. J. Bacteriol. **85**:53–61.
26. **Wells, M. A., and J. C. Ditmer.** 1963. The use of Sephadex for the removal of nonlipid contaminants from lipid extracts. Biochemistry **2**:1259–1263.
27. **Zeigler-Leedle, J. A., and R. B. Hespell.** 1980. Differential carbohydrate media and anaerobic replica plating techniques in delineating carbohydrate-utilizing subgroups in rumen bacterial populations. Appl. Environ. Microbiol. **39**:709–719.