

# Phospholipids of *Clostridium butyricum*.

## IV. Analysis of the positional isomers of monounsaturated and cyclopropane fatty acids and alk-1'-enyl ethers by capillary column chromatography

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**ABSTRACT** The positional isomers of the cyclopropane fatty acids of *Clostridium butyricum* phospholipids have been analyzed by capillary column gas-liquid chromatography. Greater than 95% of the methylenehexadecanoic acids was the 9,10 isomer. On the other hand, 60–70% of the hexadecenoic acid precursors was the  $\Delta^7$  isomer, and the remainder was the  $\Delta^9$  isomer. Of the methylenooctadecanoic acids 75–80% was the 11,12 isomer, with the remainder being the 9,10 isomer. There were approximately equal amounts of the  $\Delta^9$ - and  $\Delta^{11}$ -octadecenoic acids in the phospholipids. This study reveals a surprisingly strong specificity of the cyclopropane synthetase for the (n-7) series of monoenoic fatty acids.

An analysis by capillary column chromatography of the monoenoic and cyclopropane aldehyde dimethylacetals derived from the plasmalogens (1-alk-1'-enyl-2-acyl-glycero-phosphatides) of *C. butyricum* revealed the presence of the same positional isomeric mixtures of the 16- and 18-carbon monoenoic residues in approximately the same ratios as were found in the fatty acids. In the formation of the cyclopropane alk-1'-enyl ethers there was also specificity for the (n-7) series, but it was not as strong as that seen in the fatty acids. The ratio of the 7,8 isomer to the 9,10 isomer was higher in the methylenehexadecanals than in the corresponding fatty acids. This paper extends the use of Golay capillary columns to the analysis of the positional isomers of plasmalogen aldehydes as their dimethylacetal derivatives.

**SUPPLEMENTARY KEY WORDS** plasmalogens  
aldehydes · cyclopropane synthetase · specificity

**T**HE SEPARATION of positional isomers of long-chain monounsaturated and cyclopropane fatty acids by the use of open capillary (Golay) column gas-liquid chromatography has recently been demonstrated (1–3). Since the lipids of *Clostridium butyricum* are known to contain positional isomeric mixtures of monounsaturated fatty acids (4, 5), one aim of this investigation was to determine the ring positions of the cyclopropane fatty acids, which are known to be formed by transfer of methyl groups from S-adenosylmethionine to phospholipid-bound monoenoic fatty acids (6, 7). In addition, *C. butyricum* contains plasmalogens (8), and the alk-1'-enyl chains are known to be derived from long-chain fatty acids (9, 10). Since the composition of the alk-1'-enyl chains is similar to that of the fatty acids, including substantial amounts of monounsaturated and cyclopropane aldehydes (8), we were also interested in determining the positions of the double bonds and the cyclopropane rings in these residues. The results of this investigation are presented here. The same positional isomers are shown to be present in the

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monounsaturated fatty acids and alk-1'-enyl ethers, and in the cyclopropane fatty acids and alk-1'-enyl ethers. In general, the relative amounts of the isomers of the monounsaturated compounds are similar in the fatty acids and alk-1'-enyl chains. Examination of the cyclopropane chains revealed that greater than 95% of the methylenehexadecanoic acids was the 9,10 isomer. Of the hexadecenoic acid precursors only 30–40% was the  $\Delta^9$  isomer, the remainder was the  $\Delta^7$  isomer. 75–80% of the methyloctadecanoic acids was the 11,12 isomer. Examination of the cyclopropane aldehydes revealed greater proportions of the  $C_{17}$  7,8 and the  $C_{19}$  9,10 isomers than were seen in the fatty acids, but the  $C_{17}$  9,10 and  $C_{19}$  11,12 isomers were the predominant species. The results are discussed in terms of an apparent specificity of the cyclopropane synthetase(s) for the (n-7) series of monoenoic fatty acids and aldehydes.

## EXPERIMENTAL PROCEDURES

### Materials

**Fatty Acids.** Most of the fatty acid methyl ester standards used were purchased from the Hormel Institute, Austin, Minn. *Cis*-11,12-methyleneoctadecanoic (lactobacillic) acid was synthesized from *cis*-vaccenic acid according to the procedures of Simmons and Smith (11). *Cis*-9,10-methyleneoctadecanoic (dihydrosterculic) acid and *cis*-9,10-methylenehexadecanoic acid were purchased from Analabs, Inc., North Haven, Conn.

**Fatty Aldehydes.** Samples of 16:0 and 18:0 aldehyde dimethylacetals were generous gifts of Dr. Manfred Karnovsky. Dihydrosterculic acid, *cis*-9,10-methylenehexadecanoic acid and palmitoleic acid were converted to aldehyde dimethylacetal derivatives by the following procedures. The fatty acids (50 mg) were reduced to the alcohols with  $LiAlH_4$  (12) and completeness of reduction was checked by thin-layer chromatography on Silica Gel G in hexane–chloroform–methanol 68:30:2 (v/v/v). The mesylates of the alcohols were synthesized as described by Baumann and Mangold and the reaction was monitored by thin-layer chromatography as suggested (13). The aldehydes were then obtained by oxidation of the mesylates in dimethylsulfoxide and sodium bicarbonate as described by Mahadevan, Phillips, and Lundberg (14). The aldehydes were purified on 0.5–1.0-g columns of Unisil by elution with 5% ethyl ether in petroleum ether (bp 30°–60°C) and approximately 1.0-ml fractions were collected. Their purity was checked by thin-layer chromatography (14). The dimethylacetals were obtained by heating the aldehydes in anhydrous methanolic HCl (5%), as described by Farquhar (12). Thin-layer chromatography on Silica Gel G in benzene was used to verify the formation of the dimethylacetals. The mesylate of oleyl alcohol was kindly provided by

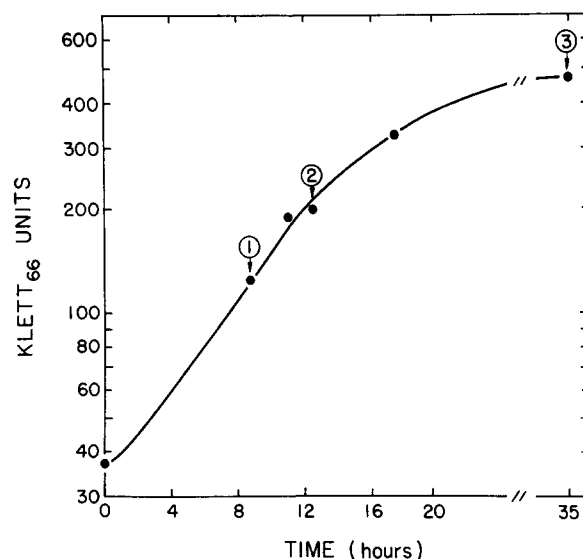


FIG. 1. Growth curve of *Clostridium butyricum*. The cells were harvested at the times indicated by the numbered arrows.

Dr. C. V. Viswanathan and oxidized by us to the aldehyde, and the dimethylacetal was made as described above. Unisil, a purified activated silicic acid, 200–325 mesh, was obtained from the Clarkson Chemical Co., Williamsport, Pa.

### Methods

**Cells.** *Clostridium butyricum* ATCC 6015 was grown at 37°C in a 20-l carboy in the Casamino acid (Difco) medium of Broquist and Snell (15). The growth curve is shown in Fig. 1. 4–8 l of culture were harvested at the times indicated by the arrows.

**Isolation of the Phospholipids.** Methods for washing cells and for extracting and washing the lipids were described in an earlier publication (16). The phospholipids were separated from the nonpolar fraction by chromatography on Unisil as previously described (9).

**Isolation of Methyl Esters and Aldehyde Dimethylacetals.** The phospholipid fraction was subjected to acid methanolysis, and the resulting methyl esters and dimethylacetals were resolved by saponification of the methyl esters followed by extraction of the dimethylacetals into petroleum ether (12). The fatty acids were remethylated in  $BF_3$ -methanol. Impurities in the resulting methyl esters were removed by chromatography on 1-g columns of Unisil (17). It has since been observed that esterification in  $BF_3$ -methanol may degrade cyclopropane rings.<sup>1</sup> Therefore, duplicate fatty acid samples 2 and 3 (see Fig. 1) were methylated with diazomethane, and the results were in excellent agreement with those obtained by  $BF_3$ -methanol treatment. Apparently no degradation of

<sup>1</sup> Panos, C. Unpublished experiments.

cyclopropane rings had taken place during esterification by either reagent.

**Gas-Liquid Chromatography.** Fatty acid methyl esters and aldehyde dimethylacetals were resolved by capillary column gas-liquid chromatography using a polar column (150 ft  $\times$  0.01 in.) coated with Carbowax K20-M plus V-930 (99/1) as has been described (1, 18). The column temperature was 186°C, and the detector was at 230°C. The column was obtained from the Perkin-Elmer Corp., Norwalk, Conn. The relative amounts of the fatty acid and aldehyde derivatives were determined by copying the chromatograms by xerography and cutting out and weighing the individual peaks. Corrections for attenuation were applied when necessary. For several runs the proportions of fatty acids were also determined by computation of the area from the peak height times the width at half-height. The results by both methods were in good to excellent agreement. The average deviation of the weight % for all peaks in two randomly selected runs was  $\pm 0.41$ .

## RESULTS

As expected from the results obtained by Scheuerbrandt and Bloch (4), the monounsaturated fatty acids, 16:1 and 18:1, of *Clostridium butyricum* were resolved by capillary column into mixtures of 16:1  $\Delta^7$  plus 16:1  $\Delta^9$ , and 18:1  $\Delta^9$  plus 18:1  $\Delta^{11}$  (Fig. 2 and Table 1). Examination of the cyclopropane fatty acids, however, revealed that the 17:cyc was almost entirely the 9,10 isomer and that the 19:cyc was a mixture of 9,10 and 11,12 isomers in a

TABLE 1 *Clostridium butyricum* PHOSPHOLIPIDS.  
FATTY ACIDS

Chain Length: Number of Double Bonds and Position	1*	2*	3*
	wt %	wt %	wt %
12:0	0.51	1.1	1.29
14:0	4.14	4.9	6.47
14:1	1.08	0.88	0.89
16:0	50.6	47.4	52.1
16:1 $\Delta^7$	13.4	16.1	12.9
16:1 $\Delta^9$	9.35	6.96	6.03
17:cyc† 7,8	0.36	0.07	0.30
17:cyc 9,10	8.45	12.1	9.15
18:0	1.80	0.77	1.95
18:1 $\Delta^9$	1.6	1.27	1.29
18:1 $\Delta^{11}$	1.7	1.13	0.92
19:cyc 9,10	0.57	0.90	1.07
19:cyc 11,12	3.03	3.43	3.47
Unknown	3.42	3.09	2.28
Total saturated	57.1	54.1	61.8
Total unsaturated	27.1	26.3	22.0
Total cyclopropane	12.4	16.5	14.0

\* Sample number (see Fig. 1).

† Cyc, cyclopropane.

ratio of between 1:4 and 1:5 in the three samples examined.

With the exception of 12:0, 14:0, and 14:1, a similar assortment of chain lengths and positional isomers was also revealed on examination of the aldehydes derived from the plasmalogens (Fig. 3 and Table 2). The 16:1 and 18:1 aldehydes had 16:1  $\Delta^7$  to 16:1  $\Delta^9$  and 18:1  $\Delta^9$  to 18:1  $\Delta^{11}$  ratios similar to those in the fatty acids. However, examination of the cyclopropane aldehydes re-

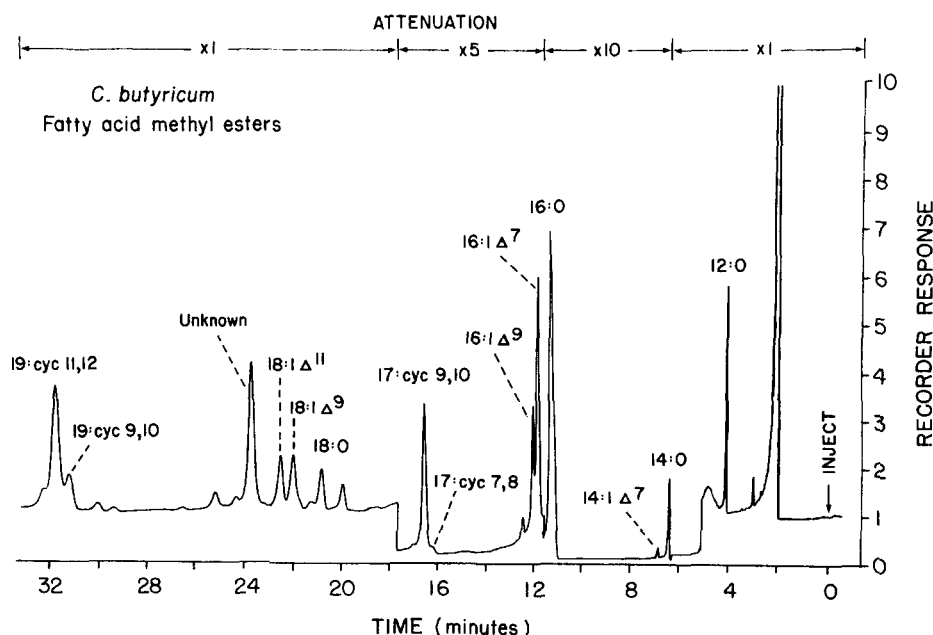


FIG. 2. Capillary column gas-liquid chromatography of the methyl esters of the fatty acids isolated from the phospholipids of *Clostridium butyricum*. Attenuations are given at the top of the figure.

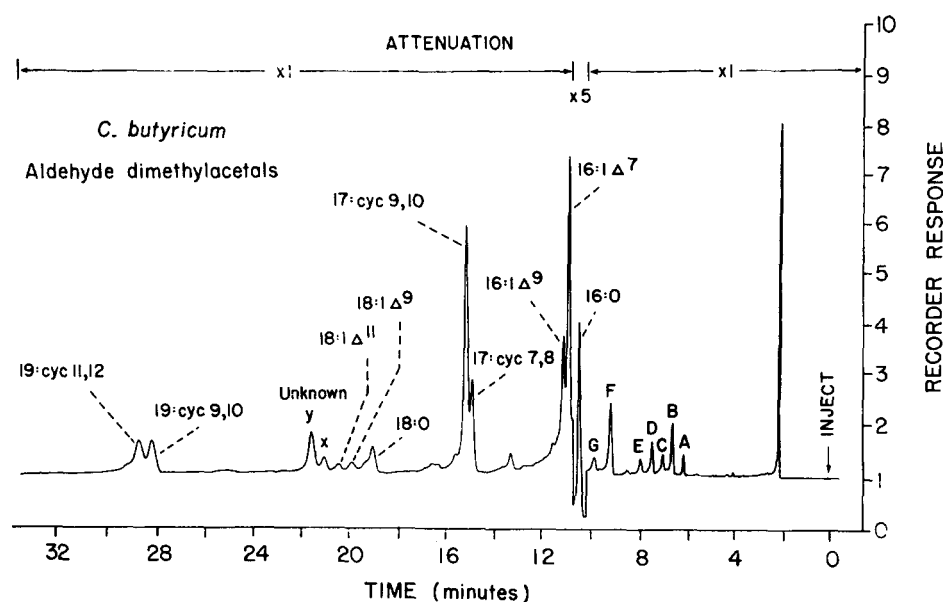


FIG. 3. Capillary column gas-liquid chromatography of the aldehyde dimethylacetals isolated from the phospholipids of *Clostridium butyricum*. All peaks with the exception of 16:0 were at the same attenuation. The peaks labeled A-G probably resulted from the breakdown of the dimethylacetals during gas-liquid chromatography.<sup>2</sup>

vealed a higher percentage of the 7,8 isomer of the 17:cyc aldehyde than was found in the 17:cyc fatty acids and a higher percentage of the 9,10 isomer of the 19:cyc aldehyde than was found in the 19:cyc fatty acids. The significant ratios of isomeric mixtures are given in Table 3.

Identification of the monounsaturated fatty acid positional isomers by comparison of their retention times on capillary columns with authentic standards agrees with the conclusions of Scheuerbrandt and Bloch, which were based on oxidation in permanganate-periodate followed by gas-liquid chromatography of the resulting degradation products (4). Scheuerbrandt and Bloch reported equal amounts (8% of total fatty acids) of  $\Delta^7$ - and  $\Delta^9$ -hexadecenoic acids in this organism (4). The difference between their analyses and ours may be related to differences in the samples analyzed. They examined the total fatty acids from cells grown in a minimal medium for 36 hr, whereas our study was done on the phosphatide fatty acids from cells grown on a medium supplemented with a complete amino acid mixture. The 14:1 fatty acid is

probably the  $\Delta^7$  isomer based on its position relative to and the separation factor from an authentic 14:1  $\Delta^9$  standard (1). Scheuerbrandt and Bloch reached the same conclusion on the position of the double bond in the 14:1 fatty acid (4). Identification of the 17- and 19-carbon cyclopropane fatty acids was also based on a comparison of their retention times with those of authentic standards (2). The identity of the small peak or shoulder on the leading edge of the 17:cyc 9,10 peak as 17:cyc 7,8, for which no standard was available, is assumed on the basis of its position relative to 17:cyc 9,10 and the separation factor between the two (2). Several unknowns were seen

TABLE 2 *Clostridium butyricum* PHOSPHOLIPIDS. FATTY ALDEHYDES

Chain Length: Number of Double Bonds and Position	1*	2*	3*
	wt %	wt %	wt %
16:0	43.2	44.1	57.9
16:1 $\Delta^7$	16.4	15.1	10.3
16:1 $\Delta^9$	9.62	7.71	4.97
17:cyc† 7,8	2.93	4.43	4.03
17:cyc 9,10	12.96	14.10	9.54
18:0	2.44	2.08	2.81
18:1 $\Delta^9$	1.74	1.22	1.12
18:1 $\Delta^{11}$	1.39	0.73	0.61
19:cyc 9,10	2.09	2.56	2.48
19:cyc 11,12	3.00	3.59	2.95
Unknown	4.25	4.34	3.31
Total saturated	45.7	46.2	60.7
Total unsaturated	29.1	24.8	16.96
Total cyclopropane	20.98	24.7	19.0

\* Sample number (see Fig. 1).

† Cyc, cyclopropane.

<sup>2</sup> Stein and Slawson (19) and Mahadevan, Viswanathan, and Phillips (20) have concluded that the breakdown of aldehyde dimethylacetals on gas-liquid chromatography results in the formation of varying amounts of the corresponding *cis*- and *trans*-alk-1-enyl methyl ethers as well as the free aldehydes. The amounts of conversion varied with the columns used. It appears that there was 10–20% breakdown with the columns used in the present study. Peaks B, D, and F were seen when standard 16:0 aldehyde dimethylacetal was chromatographed. Peaks C, E, and G were seen when standard 16:1  $\Delta^9$  aldehyde dimethylacetal was chromatographed. Other small unidentified peaks are assumed to be associated with the breakdown of the other aldehyde dimethylacetals.

TABLE 3 *Clostridium butyricum* PHOSPHOLIPIDS.  
RATIOS OF POSITIONAL ISOMERS

	1*	2*	3*
<b>Fatty acids</b>			
16:1 $\Delta^7$ to			
16:1 $\Delta^9$	1.43	2.31	2.1
17:cyc 7,8			
to 17:cyc 9,10	0.04	0.01	0.03
18:1 $\Delta^9$ to			
18:1 $\Delta^{11}$	0.96	1.12	1.4
19:cyc 9,10 to			
19:cyc 11,12	0.19	0.26	0.31
<b>Fatty aldehydes</b>			
16:1 $\Delta^7$ to			
16:1 $\Delta^9$	1.7	1.96	2.1
17:cyc 7,8 to			
17:cyc 9,10	0.23	0.31	0.42
18:1 $\Delta^9$ to			
18:1 $\Delta^{11}$	1.25	1.67	1.8
19:cyc 9,10			
19:cyc 11,12	0.70	0.71	0.84

\* Sample number (see Fig. 1).

in the fatty acid methyl ester mixture. The major one, with a retention (relative to 16:0) of 2.373, represents 2.8–3.4% of the total fatty acids. Mild hydrogenation of the fatty acids (21) did not result in a change in the amount or position of this fatty acid methyl ester. Its retention time did not correspond to any of the saturated straight-chain fatty acid methyl esters. We assume, therefore, that it is a branched-chain compound. It should also be noted that mild hydrogenation followed by gas-liquid chromatography of the fatty acid methyl esters provided confirmatory evidence for the identification of unsaturated and cyclopropane fatty acids. The increase in percentage composition of 14:0, 16:0, and 18:0 was as predicted from the amounts of unsaturated fatty acids of each chain length.

The identities of the aldehyde dimethylacetal derivatives are based on comparisons with the retention times of standards in the cases where standards were available (Table 4), and in those cases where a standard of only one of two positional isomers was available, the relative position on the chromatogram of the second isomer to the first was used as a guide to its identity. In all cases the relative chromatographic positions and the separation factors for the aldehyde positional isomers were in agreement with those observed in this and in previous studies for the fatty acid positional isomers (Fig. 4) (1, 2, 18).

## DISCUSSION

The results of our study of the positional isomers of the cyclopropane fatty acids of *Clostridium butyricum* revealed a surprisingly strong specificity of the cyclopropane synthetase of this organism for the (n-7) series of mono-unsaturated fatty acids. Despite the high ratio of 16:1

TABLE 4 RETENTION TIMES OF ALDEHYDE DIMETHYLACETALS OBTAINED FROM THE PLASMALOGENS OF *C. butyricum*

	Relative Retention Time*	
	<i>C. butyricum</i>	Standards
16:0	0.897	0.895
16:1 $\Delta^7$	0.948	
16:1 $\Delta^9$	0.969	0.970
17:cyc 7,8	1.380	
17:cyc 9,10	1.411	1.409
18:0	1.833	1.836
18:1 $\Delta^9$	1.928	1.929
18:1 $\Delta^{11}$	1.985	
19:cyc 9,10	2.820	2.821
19:cyc 11,12	2.880	

\* Methyl palmitate = 1.00. Retention times were corrected for column dead volume by observation of a solvent peak.

$\Delta^7$  to 16:1  $\Delta^9$ , almost all of the 17:cyc fatty acids have the ring at the 9,10 position. In the 19-carbon fatty acids, despite the availability of approximately equal amounts of 18:1  $\Delta^9$  and 18:1  $\Delta^{11}$ , 75–80% of the 19:cyc fatty acids have a ring at the 11,12 position. The studies of Law and his associates (6, 7, 22, 23) have clearly shown that the cyclopropane fatty acids are formed by transfer of a methyl group from *S*-adenosylmethionine to fatty acids, already bound to phospholipids. From our studies it appears that the activity of the enzyme *in vivo* is determined in part by the distance of the double bond from the methyl terminal of the acyl esters. Thomas and Law (22) have studied the substrate specificity of the enzyme from *C. butyricum* with regard to the structure of the polar head groups and the stereochemistry of the phospholipids. They also demonstrated the formation of cyclopropane rings on monounsaturated alkyl ether

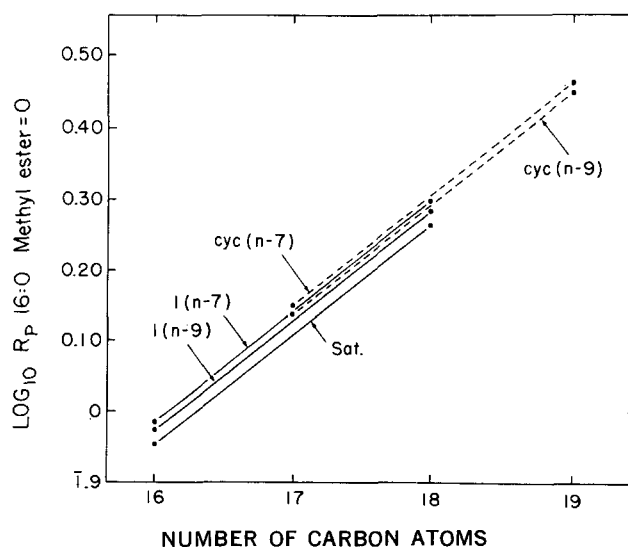


FIG. 4. Relationship between the log of the relative retention time and the position of the double bond or the cyclopropane ring in the aldehyde dimethylacetals.



chains. Thomas also examined the chain-length specificity of the enzyme using a synthetic phosphatidylethanolamine made from crude monoenoic fatty acids (24). No work on the specificity of the enzyme for various positional isomers of phospholipid-bound monoenoic fatty acids has been reported.

Positions of the double bond in the monounsaturated fatty aldehydes of the plasmalogens mirror those found in the fatty acids. The aldehyde mixtures show almost the same ratio of 16:1  $\Delta^7$  to 16:1  $\Delta^9$  that is seen in the fatty acids. A slightly higher ratio of 18:1  $\Delta^9$  to 18:1  $\Delta^{11}$  was seen among the aldehydes than was found in the fatty acids. These findings strengthen the impressions gained from earlier work with this organism that the alk-1'-enyl chains are derived from the corresponding fatty acids (9, 10). Recent work has shown that this organism contains soluble enzymes that interconvert long-chain acyl thioesters, aldehydes, and alcohols (25). The mechanism of formation of the alk-1'-enyl ether lipids of *C. butyricum*, however, is not known (9, 10).

Chung and Goldfine (26) showed that a crude cyclopropane synthetase from *C. butyricum* was capable of forming cyclopropane alk-1'-enyl ethers on the phosphatide plasmalogens from this organism in vitro. Our results show that the cyclopropane rings are preferentially formed from the 16:1  $\Delta^9$  alk-1'-enyl chains, but not with as strong a specificity as in the fatty acids. A slight preference for 18:1  $\Delta^{11}$  alk-1'-enyl chains over the 18:1  $\Delta^9$  chains was also seen. It appears that the (n-7) series of alk-1'-enyl chains are favored over the (n-9) series. The question posed before (26), whether this represents a single cyclopropane synthetase that acts on acyl esters, alk-1'-enyl ethers, and alkyl ethers, or a series of cyclopropane synthetases, has not been resolved.

Because the synthesis of monounsaturated fatty acids in bacteria by the "anaerobic" pathway described by Hofmann and Bloch and their coworkers (4, 5, 27) generally results in the synthesis of 16:1  $\Delta^9$  and 18:1  $\Delta^{11}$  rather than in the mixtures of isomers seen in *C. butyricum* and group A streptococci (21), no choice need be made by the enzyme in the formation of the cyclopropane rings. Indeed, the preferred monoenoic acid isomers of the cyclopropane synthetase(s) of *C. butyricum* are the ones usually provided by the "anaerobic" pathway, i.e., the (n-7) series. Although there are few complete analyses of the positions of the cyclopropane rings, in most cases, as expected, the 17:cyc 9,10 and 19:cyc 11,12 have been reported (27-29). An exception is Gray's finding that the 19:cyc of *Salmonella typhimurium* is 80% lactobacillic acid and 20% dihydrosterculic (19:cyc 9,10) acid (30).

There have been recent reports that the (n-7) monoenoic series occurs in the alk-1'-enyl ethers of higher organisms. Schmid, Bandi, Mangold, and Baumann

(31) reported that the major unsaturated aldehyde chain of the alk-1'-enyl diglycerides of ratfish (*Hydrolagus colliet*) liver is 20:1  $\Delta^{13}$ . Large amounts of 18:1  $\Delta^{11}$  and 22:1  $\Delta^{15}$  were also found. In the fatty acids, on the other hand, the (n-9) isomers were the major constituents. Dubuch and Winterfeld (32) recently reported that the 18:1 fatty aldehydes derived from human placenta plasmalogens contained almost equal mixtures of 18:1  $\Delta^9$  and 18:1  $\Delta^{11}$  as the major isomers. Small amounts of 20:1 aldehydes were also found and the ratio of 20:1  $\Delta^{11}$  to 20:1  $\Delta^{13}$  was approximately 3:1. Thus, in a human tissue the (n-7) series is also a major aldehyde type. From the results we have presented on *C. butyricum* lipids and the recent finding of high concentrations of plasmalogens in many anaerobic bacteria (33), we would like to suggest that bacterial and dietary sources may be providing some of the aldehyde chains for animal tissue alk-1'-enyl ether lipid biosynthesis.

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