Phospholipids of *Clostridium butyricum*. V. Effects of growth temperature on fatty acid, alk-1-enyl ether group, and phospholipid composition

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Abstract Many anaerobic bacteria have a high proportion of 1-alk-1′-enyl ethers (plasmalogens) among their phospholipids. We have examined the effects of growth temperature on the phospholipid, fatty acid, and alk-1-enyl group compositions of *Clostridium butyricum*. When the growth temperature was decreased from 37°C to 25°C, the proportion of glycerol phosphoglycerides (the sum of phosphatidylglycerol and the corresponding plasmalogen) increased at the expense of the ethanolamine and *N*-methylethanolamine phosphoglycerides. Analysis of the proportion of these lipids present in the diacyl and 1-alk-1′-enyl-2-acyl forms has shown a substantial increase in the plasmalogen form of the glycerol phosphoglycerides and a decrease in the plasmalogen forms of the ethanolamine and *N*-methylethanolamine phosphoglycerides. An analysis of the fatty acids and alk-1-enyl groups isolated from the total phospholipids of cells grown at 25°C, 30°C, and 37°C has shown a general increase in the proportions of unsaturated and cyclopropane hydrocarbon chains at lower growth temperatures. When the temperature was lowered from 37°C to 25°C, the fatty acids had progressively more unsaturated and cyclopropane chains and fewer saturated chains. The alk-1-enyl groups, in particular those from the ethanolamine and *N*-methylethanolamine plasmalogens, were more saturated at 30°C than at 37°C. When the growth temperature was lowered to 25°C, there was little further change in the degree of unsaturation of the alk-1-enyl groups.

Supplementary key words plasmalogens · phosphatidylglycerol phosphatidylethanolamine · phosphatidyl-*N*-methylethanolamine · unsaturation · cyclopropane

Plasmalogens, derivatives of 1-alk-1′-enyl *sn*-glycerol 3-phosphate, constitute a large fraction of the phosphoglycerolipids of certain mammalian tissues, for example, central nervous system, peripheral nerves, heart, and skeletal muscle (1). They are also found in avian tissues, in other vertebrates, and in many invertebrates (1–3). Among microorganisms, however, alk-1-enyl ether lipids have only been found in obligately anaerobic bacteria, in which they often constitute more than half of the total phospholipids (4). Lipids in biological membranes are thought to be arranged predominantly as bilayers, with their polar head groups facing outward and their hydrocarbon chains toward the center (5). A number of workers have presented evidence for the importance of unsaturated, cyclopropane, branched-chain, and shorter-chain fatty acids for the maintenance of the hydrocarbon chains, at least in part in a liquid-expanded, highly mobile state (6–8). A number of organisms have been shown to adjust the fatty acid composition of their membrane lipids to lower growth temperatures in a manner that decreases the average melting point of the fatty acid chains. It is thought that this adjustment makes it less likely that all the lipids will become frozen and condensed, a condition that would interfere with such vital functions as membrane transport and growth.

To our knowledge there has been only one reported study on the effects of the surrounding temperature on the alk-1-enyl group composition of plasmalogens and the plasmalogen content of the tissue. Roots and Johnston explored this problem in a study of the phospholipid and alk-1-enyl group compositions of goldfish acclimated to different temperatures (9). Their measurements demonstrated a marked effect of temperature on the degree of unsaturation of the alk-1-enyl groups in goldfish neural tissue. We have carried out a study of the effects of growth

Abbreviations: PE, phosphatidylethanolamine; PME, phosphatidyl-*N*-methylethanolamine; PG, phosphatidylglycerol. The terms ethanolamine phosphoglycerides, *N*-methylethanolamine phosphoglycerides, and glycerol phosphoglycerides denote the mixtures of PE, PME, and PG and their corresponding 1-alk-1′-enyl-2-acyl derivatives (plasmalogens), respectively. Fatty acids and alk-1-enyl groups are designated by number of carbon atoms:number of double bonds. Cyc indicates a cyclopropane ring. GLC, gas–liquid chromatography.

temperature on the fatty acid and 1-alk-1′-enyl group compositions of *Clostridium butyricum* phospholipids. Our experiments showed changes in the phospholipid composition, in the proportions of alk-1-enyl ethers in the different phospholipid species, and in the fatty acid and alk-1-enyl group compositions, with changes in the growth temperature.

EXPERIMENTAL PROCEDURES

Materials

Fatty acids. Most of the fatty acid methyl ester standards used were purchased from Applied Sciences Laboratories, State College, Pa. *cis*-9,10-Methyleneoctadecanoic acid was purchased from Analabs, Inc., North Haven, Conn.

Fatty aldehydes. Palmitaldehyde was purchased from Aldrich Chemicals as the bisulfite addition product and was converted to the aldehyde by alkali treatment. *cis*-9-16:1, *cis*-9,10-methylenehexadecanal, *cis*-9,10-methyleneoctadecanal, and *cis*-9-18:1 were obtained by hydrolysis of the dimethylacetals, which were prepared in this laboratory (10).

Methods

Cells. Clostridium butyricum ATCC 6015 was grown in the Casamino acid medium of Broquist and Snell (11). Cells initially grown at 37°C were used as inocula for 30°C cultures. Cells grown at 30°C were used as inocula for 25°C cultures. The cells were grown for at least five generations at each temperature. We were not able to obtain growth below 22°C. Cells were harvested in the log phase of growth between Klett (66 filter) values of 90 to 110 and were stored at −20°C.

Isolation of the phospholipids. Extraction and washing of the lipids was as described elsewhere (12). The phospholipids were separated from the nonpolar fraction by column chromatography on silicic acid as described previously (13).

Separation of phospholipids. The polar lipids were fractionated by preparative thin-layer chromatography on silica gel G (E. Merck, obtained from Brinkmann Instruments, Inc.) in the solvent mixture chloroform–methanol–7 N ammonia 60:35:5 (v/v/v) as described previously (13). They were located by spraying with water and eluted as described (13).

Isolation of alk-1-enyl groups and diacyl phosphatides. In order to hydrolyze plasmalogens, phospholipids were treated with 90% acetic acid at 37°C for 18 hr, followed by lyophilization (14). The lyophilized products were dissolved in a small volume of chloroform and subjected to thin-layer chromatography as described above. The aldehydes, derived from the alk-1-enyl groups, diacyl phosphatides, and lysophospholipids, were extracted from the plates with chloroform–methanol 1:2 (v/v). The fatty aldehydes were stored in carbon disulfide at −20°C until analyzed (15).

TABLE 1. Acyl chain composition of phospholipids of *C. butyricum* grown at different temperatures

Chain Length	37°C (3)[a]	30°C (3)	25°C (4)
	wt %	*wt %*	*wt %*
12:0	0.4 ± 0.2	0.3 ± 0.1	0.3 ± 0.1
14:0	4.7 ± 0.5	3.8 ± 1.2	3.3 ± 0.9
14:1	0.5 ± 0.1	0.9 ± 0.2	1.1 ± 0.3
16:0	49.3 ± 2.1	46.8 ± 3.3	43.8 ± 1.0
16:1 + 17:cyc	30.6 ± 1.1	38.0 ± 5.2	41.0 ± 2.2
18:0	1.1 ± 0.4	0.8 ± 0.2	0.7 ± 0.1
18:1 + 19:cyc	13.3 ± 3.2	9.4 ± 0.9	9.3 ± 0.7
Total saturated	55.5 ± 2.2	51.7 ± 4.7	48.2 ± 1.7
Total unsaturated plus cyclopropane	44.5 ± 2.2[b]	48.3 ± 4.7	51.9 ± 1.7[b]

[a] The numbers in parentheses represent the number of separate samples, each analyzed twice by GLC. Values are means ± SD.
[b] Using Student's *t* test, the difference in the unsaturated plus cyclopropane fatty acid content at 37°C and 25°C is significant, $P = 0.02$.

Determination of positional distribution of fatty acids. The PE plus PME diacyl lipids, isolated as described above, were treated with *Crotalus adamanteus* venom (Sigma Chemical Co.) as a source of phospholipase A_2 according to the method of van Golde and van Deenen (16), except that tris(hydroxymethyl)aminomethane–HCl (0.1 M, pH 7.2) replaced borate (0.1 M, pH 7.0) as a buffer in the hydrolysis reaction. The lyso-PE plus lyso-PME and fatty acid products were separated by thin-layer chromatography as described above. The hydrolysis was continued until no further diacyl phosphatides could be detected by chromatography on thin-layer plates as described above.

Preparation of fatty acid methyl esters and gas–liquid chromatography. Fatty acids were obtained by saponification of lipids as described by Nesbitt and Lennarz (17) and were methylated with diazomethane. Fatty acid methyl esters and aldehydes were resolved on a column (⅛ inch × 6 ft) of 10% EGSS-X on Gas-Chrom P (100–200 mesh, Applied Science Laboratories) at 180°C. Amounts of fatty acids were determined by triangulation or by an Infotronics (model CR3-208) electronic integrator. Fatty acids and aldehydes were identified by comparison of retention times with those of standards (10).

Analytical procedures. Phosphorus was determined by the method of Bartlett (18). Alk-1-enyl ethers were determined by colorimetric estimation of iodine uptake (19) and by alkaline hydrolysis (20). Because alkyl ether lipids constitute 0.2% of the phospholipids (4), they do not interfere with the measurement of alk-1-enyl ethers as alkali-stable lipids. The two methods agreed within ±10%.

TABLE 2. Alk-1-enyl chain composition of phospholipids of *C. butyricum* grown at different temperatures

Chain Length	37°C (4)[a]	30°C (3)	25°C (6)
	wt %	*wt %*	*wt %*
16:0	46.5 ± 1.9	53.7 ± 4.3	50.6 ± 3.6
16:1 + 17:cyc	34.0 ± 2.0	32.7 ± 1.8	35.8 ± 4.0
18:0	3.5 ± 0.5	2.7 ± 0.8	3.3 ± 1.1
18:1 + 19:cyc	16.0 ± 0.8	10.8 ± 2.8	10.5 ± 1.5
Total saturated	50.0 ± 2.2	56.3 ± 3.6	53.7 ± 4.2
Total unsaturated plus cyclopropane	50.0 ± 2.1[b]	43.4 + 3.8[b]	46.3 ± 4.3[b]

[a]The numbers in parentheses represent the number of separate samples, each analyzed twice by GLC. Values are means ± SD.
[b]Using Student's *t* test, the difference in the unsaturated plus cyclopropane alk-1-enyl content at 37°C and 25°C is not significant, $P > 0.1$; the difference in the unsaturated plus cyclopropane alk-1-enyl content at 37°C and 30°C is significant, $P < 0.05$.

RESULTS

The effect of growth temperature on the degree of saturation of the total phospholipid acyl and alk-1-enyl groups is given in **Tables 1** and **2** and illustrated in **Fig. 1.** An increase in the proportion of 16:1 + 17:cyc fatty acids from 30.6% to 38.0% was seen when the growth temperature was decreased from 37°C to 30°C. At 25°C the trend towards increased unsaturation and decreased saturation continued, but these differences between 25°C and 30°C were not statistically significant. Among the alk-1-enyl groups there was a noticeable increase in 16:0, from 46.5% to 53.7%, when the growth temperature was decreased from 37°C to 30°C. The major compensating change was a decrease in 18:1 + 19:cyc from 16.0% to 10.8% of the total. When the growth temperature was decreased to 25°C, the alk-1-enyl groups appeared to be slightly more unsaturated than at 30°C, but these changes were not statistically significant. The net effect on the total phospholipid hydrocarbon chain composition is shown by the dashed line in Fig. 1. The computation for this composite curve takes into account the fact that there are more

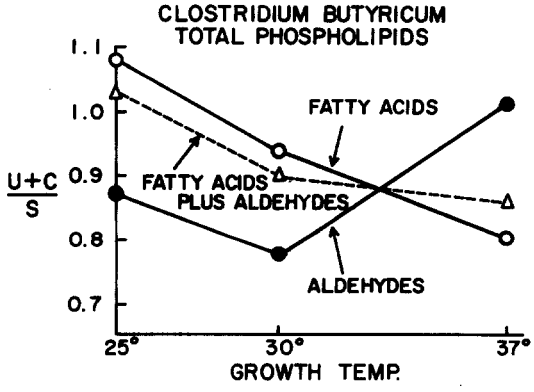


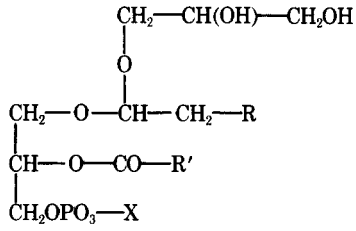
Fig. 1. Ratio of unsaturated (U) plus cyclopropane (C) to saturated (S) fatty acids and alk-1-enyl chains (aldehydes) isolated from *C. butyricum* total phospholipids in relation to growth temperature (°C).

TABLE 3. Phospholipids of *C. butyricum* at 37°C and 25°C

	Glycerol Phosphoglycerides		Ethanolamine and *N*-Methylethanolamine Phosphoglycerides	
	Diacyl	Plasmalogen	Diacyl	Plasmalogen
	% of total phospholipids			
37°C	16	9.7	12	30
25°C	15	22	13	22

fatty acyl chains than alk-1-enyl chains at all three temperatures (see below). It can be seen that the net effect is a gradual increase in the proportion of unsaturated and cyclopropane chains as the growth temperature is lowered.

In order to determine how these changes in total phospholipid hydrocarbon chains were accomplished, we examined the effect of growth temperature on the phospholipid class composition, the proportion of each phospholipid class containing alk-1-enyl ethers, and the alk-1-enyl and fatty acid chain compositions of the major phospholipid species. Some changes were noted in the phospholipid classes. At 37°C the ethanolamine and *N*-methylethanolamine phosphoglycerides represented 42% of total phospholipid phosphorus, whereas at 25°C their combined total represented 35% of phospholipid phosphorus. The glycerol phosphoglycerides, on the other hand, increased from 25.7% in cells grown at 37°C to 37% of the total phospholipids in cells grown at 25°C (**Table 3**). The sum of all the other phospholipid components did not change markedly at the two growth temperatures. A major constituent among them has recently been identified as a glycerol hemiacetal of the ethanolamine and *N*-methylethanolamine plasmalogens (21).



This structure was established for *C. butyricum* strain IFO 3852, which has ethanolamine but no *N*-methylethanolamine phosphoglycerides (21); however, the glycerol hemiacetal structure has been confirmed by Dr. P-O. Hagen[1] for a similar lipid fraction containing ethanolamine and *N*-methylethanolamine, which we observed in earlier studies on strain ATCC 6015 (13).

Marked changes in the proportions of alk-1-enyl ethers were also observed at lower growth temperatures (**Fig. 2**). It can be seen that the glycerol phosphoglycerides, which were 38% alk-1-enyl ether at 37°C, were 60% alk-1-enyl

[1] Personal communication.

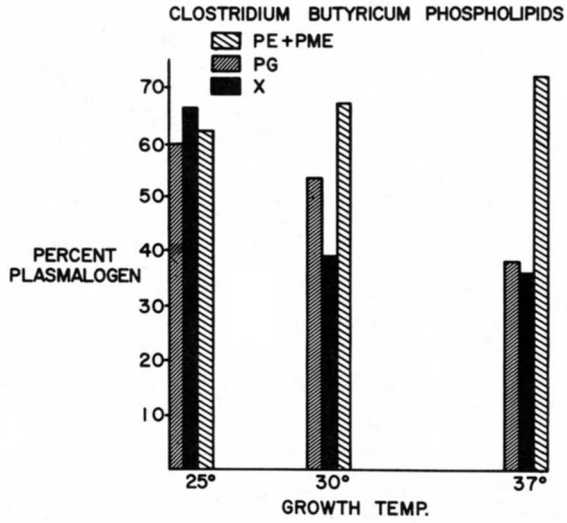


Fig. 2. Changes in the proportions of alk-1-enyl ether lipids (plasmalogens) isolated from *C. butyricum* at different growth temperatures. Results are given as means of colorimetric estimations of I_2 uptake and alkaline hydrolysis methods except for X lipids, as described in the Results section.

ether at 25°C. The other components, including the glycerol hemiacetal of the alk-1-enyl ethers, increased from 36% alkali-stable at 37°C to 66% alkali-stable when the cells were grown at 25°C. The hemiacetal component cannot be measured by iodine uptake (13, 21), but it does release an aldehyde on acid hydrolysis and is alkali stable. A slight decrease in the proportion of ethanolamine and *N*-methylethanolamine phosphoglycerides in the plasmalogen form from 75% at 37°C to 64% at 25°C was also seen.

The ethanolamine and *N*-methylethanolamine phosphoglycerides were isolated as a group by thin-layer chromatography, and the plasmalogens were hydrolyzed in 90% acetic acid. The fatty acid and alk-1-enyl group compositions of these plasmalogens at different growth tempera-

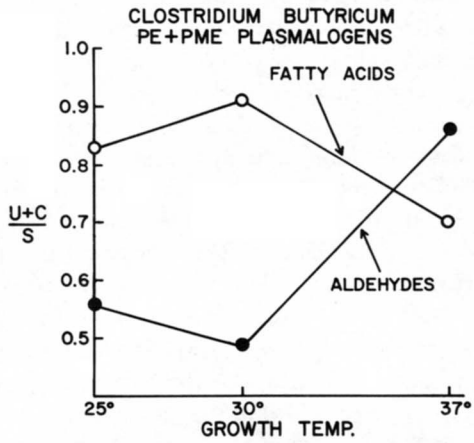


Fig. 3. Ratio of unsaturated (U) plus cyclopropane (C) to saturated (S) fatty acids and alk-1-enyl chains (aldehydes) isolated from *C. butyricum* ethanolamine and *N*-methylethanolamine plasmalogens in relation to growth temperature (°C).

TABLE 4. Acyl chain composition of the ethanolamine plus *N*-methylethanolamine plasmalogens of *C. butyricum* grown at different temperatures

Chain Length	37°C (3)[a]	30°C (3)	25°C (3)
	wt %	*wt %*	*wt %*
12:0		0.3	0.2 ± 0.1
14:0	2.8 ± 0.8	3.9 ± 0.1	3.6 ± 0.6
14:1	0.3	1.34 ± 0.3	1.1 ± 0.4
16:0	54.0 ± 2.2	47.9 ± 3.3	50.1 ± 1.4
16:1 + 17:cyc	31.9 ± 5.8	38.4 ± 2.7	37.2 ± 1.8
18:0	1.9 ± 0.7	0.5 ± 0.4	0.7 ± 0.5
18:1 + 19:cyc	12.6 ± 3.4	7.7 ± 1.1	7.0 ± 1.0
Total saturated	58.8 ± 2.8	52.6 ± 3.4	54.6 ± 1.3
Total unsaturated plus cyclopropane	41.2 ± 2.8[b]	47.4 ± 3.4[b]	45.4 ± 1.3

[a]The numbers in parentheses represent the number of separate samples, each analyzed twice by GLC. Values are means ± SD.
[b]Using Student's *t* test, the difference in the unsaturated plus cyclopropane fatty acids at 37°C and 30°C is not significant, $P > 0.1$.

tures are recorded in **Tables 4** and **5** and illustrated in **Fig. 3.** As in the total phospholipids, there appeared to be an increase in fatty acid unsaturation at 30°C as compared with 37°C. When the temperature was lowered further to 25°C, little further change was observed. These changes were not statistically significant. The aldehydes derived from the alk-1-enyl ethers (Table 5) were observed to become considerably more saturated at 30°C as compared with 37°C, again with little further change at 25°C. It should be noted, however, that the increased aldehyde saturation observed at 30°C was accomplished by an increase in 16:0 partly at the expense of 18:1 + 19:cyc; thus, there was an overall decrease in chain length.

It can be seen in **Fig. 4** and **Table 6** that the C-1-linked fatty acids in the diacyl PE + PME, like the alk-1-enyl groups, were more saturated at 30°C than at 37°C. Little further change was observed in the degree of unsat-

TABLE 5. Alk-1-enyl chain composition of the ethanolamine plus *N*-methylethanolamine plasmalogens of *C. butyricum* grown at different temperatures

Chain Length	37°C (4)[a]	30°C (2)	25°C (5)
	wt %	*wt %*	*wt %*
16:0	49.6 ± 3.6[b]	65.1 ± 3.0[c]	61.4 ± 4.0[b]
16:1 + 17:cyc	30.6 ± 2.6	25.9 ± 2.7	29.3 ± 4.8
18:0	4.4 ± 0.4	2.0 ± 0.1	3.0 ± 1.0
18:1 + 19:cyc	15.5 ± 1.4	6.2 ± 0.2	6.1 ± 2.4
Total saturated	53.9 ± 4.0	67.2 ± 3.1	64.4 ± 4.1
Total unsaturated plus cyclopropane	46.1 ± 4.0[d]	32.4 ± 2.6	35.7 ± 4.2

[a]The numbers in parentheses represent the number of separate samples, each analyzed twice by GLC.
[b]Values are means ± SD.
[c]Values are means ± mean deviations.
[d]Using Student's *t* test, the difference in the unsaturated plus cyclopropane alk-1-enyl content at 37°C and 25°C is significant, $P < 0.01$, as is the difference at 37°C and 30°C, $P < 0.02$.

TABLE 6. Positional distribution of the acyl chains of phosphatidylethanolamine plus phosphatidyl-*N*-methylethanolamine of *C. butyricum* grown at different temperatures

Chain Length	37°C			30°C			25°C		
	Total	C-1	C-2	Total	C-1	C-2	Total	C-1	C-2
	wt %			*wt %*			*wt %*		
12:0							0.1		
14:0	0.9	0.7	1.3	2.0	1.0	3.1	1.5	1.1	3.2
14:1	0.1			0.6			0.5		0.9
16:0	38.9	17.7	50.7	41.5	25.2	60.3	42.4	25.5	54.0
16:1	20.7	17.5	14.9	30.4	32.7	21.7	33.5	41.8	27.6
17:cyc	14.8	22.9	8.2	12.7	18.9	5.7	9.3	14.0	5.4
18:0	1.9	4.5	1.3	0.9	3.0	0.8	1.0	2.4	0.6
18:1	10.1	14.0	18.8	8.0	12.1	5.9	8.8	11.5	6.8
19:cyc	12.7	22.6	5.0	4.0	7.1	1.8	2.8	3.7	1.5
Total saturated	41.7	23.0	53.3	44.4	29.3	64.2	45.0	29.0	57.8
Total unsaturated	30.9	31.5	33.6	38.4	44.8	27.6	42.9	53.3	35.3
Total cyclopropane	27.5	45.6	13.2	16.7	26.0	7.6	12.1	17.7	6.9

Values are averages of two separate runs with the same sample. There are some discrepancies between the values for individual fatty acids at C-1 and C-2 when the values are compared with the values given for the same fatty acids in the total columns. By comparison with the standard deviations given in Tables 1 and 4, it is clear that most of these discrepancies fall within experimental error. Two exceptions to this are the 16:1 and 18:1 of PE plus PME from cells grown at 37°C. We have no explanation for these discrepancies. The total saturated, unsaturated, and cyclopropane fatty acids at each growth temperature, however, agree within experimental error for each set of data.

uration of the C-1-linked fatty acids in cells grown at 25°C, but again it should be noted that, at lower temperatures, the shorter chains, 16:0, 16:1, and 17:cyc, represented a greater proportion of the total. The C-2-linked fatty acids of diacyl PE + PME appeared to become more saturated at 30°C and more unsaturated at 25°C, but the statistical significance of these changes has not been tested. This unusual distribution, with more unsaturated fatty acids than saturated fatty acids at C-1 and more saturated fatty acids than unsaturated acids at C-2, has been noted for the diacyl PE of *C. butyricum* grown at 37°C by Hildebrand and Law (22). This distribution is seen to prevail at lower growth temperatures (Table 6 and Fig. 4).

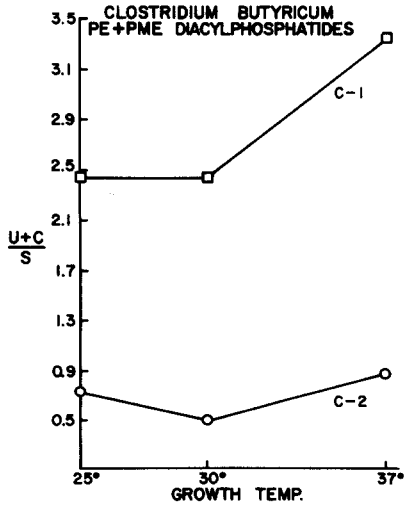


Fig. 4. Ratio of unsaturated (U) plus cyclopropane (C) to saturated (S) fatty acids of C-1 and C-2 positions of diacyl phosphatidylethanolamine plus phosphatidyl-*N*-methylethanolamine isolated from *C. butyricum* grown at 37°C, 30°C, and 25°C.

The fatty acids and alk-1-enyl chains in the glycerol phosphoglycerides were also analyzed. As can be seen in **Table 7**, there was little change in the degree of unsaturation in the total fatty acids of PG plus the C-2 fatty acid residue of the corresponding plasmalogen. The increase in unsaturation seen in alk-1-enyl chains at lower temperatures is not statistically significant (**Table 8**). At lower temperatures, the shorter chains 16:1 + 17:cyc increased from 32.6% to 41.5% of the total.

Because these cells grow more slowly at lower temperatures, we examined the effect of a nutritional shift-down on fatty acid and alk-1-enyl group compositions. Cells were grown at 37°C in the minimal medium described by Broquist and Snell (11) without casein hydrolysate and supplemental amino acids. This increased the doubling time by slightly more than a factor of two, which was similar to the doubling time seen at 25°C in the amino acid-enriched medium. The degree of unsaturation of the fatty acids was not affected by growth in minimal medium. The ratio of unsaturated plus cyclopropane to saturated fatty acids was 0.80. The alk-1-enyl groups were, however, more saturated in cells grown in minimal medium at 37°C than they were in cells grown in the amino acid-supplemented medium. The ratio of unsaturated plus cyclopropane to saturated aldehyde was 0.78 in minimal medium as opposed to 1.0 in the enriched medium. Thus, the increased saturation of alk-1-enyl chains seen in 25°C cells may have been the result of slower growth. The observation that the alk-1-enyl chains were even more saturated at 30°C in the enriched medium (Fig. 1), at which

TABLE 7. Acyl chain composition of the glycerol phosphoglycerides, diacyl plus plasmalogens, of *C. butyricum* grown at different temperatures

Chain Length	37°C (3)[a]	30°C (2)	25°C (4)
	wt %	*wt %*	*wt %*
12:0	0.1	0.3	0.2 ± 0.1[b]
14:0	2.5 ± 0.5[b]	2.1 ± 0.6[c]	2.6 ± 0.8
14:1	0.4 ± 0.1	0.7 ± 0.2	0.7 ± 0.3
16:0	44.0 ± 2.8	44.6 ± 0.7	45.3 ± 1.8
16:1 + 17:cyc	37.0 ± 5.3	40.4 ± 0.2	39.0 ± 2.3
18:0	1.2 ± 0.4	0.9	1.1 ± 0.3
18:1 + 19:cyc	15.0 ± 2.4	11.1 ± 0.4	11.2 ± 1.5
Total saturated	47.7 ± 3.1	47.9 ± 0.1	49.1 ± 1.3
Total unsaturated plus cyclopropane	52.4 ± 3.1	52.1 ± 0.1	50.9 ± 1.3

[a]The numbers in parentheses represent the number of separate samples, each analyzed twice by GLC.
[b]Values are means ± SD.
[c]Values are means ± mean deviations.

TABLE 8. Alk-1-enyl chain composition of the glycerol phosphoglycerides of *C. butyricum* grown at different temperatures

Chain Length	37°C (3)[a]	30°C (2)	25°C (6)
	wt %	*wt %*	*wt %*
16:0	45.5 ± 0.1[b]	46.4 ± 3.0[c]	43.2 ± 7.5[b]
16:1+ 17:cyc	32.6 ± 0.9	40.7 ± 3.7	41.5 ± 7.8
18:0	4.1 ± 0.9	1.5 ± 0.2	3.0 ± 0.7
18:1 + 19:cyc	17.6 ± 0.5	11.0 ± 0.1	12.4 ± 2.9
Total saturated	49.6 ± 1.0	48.0 ± 4.2	46.1 ± 7.5
Total unsaturated plus cyclopropane	50.2 ± 1.5	51.7 ± 3.8	53.9 ± 7.5

[a]The numbers in parentheses represent the number of separate samples, each analyzed twice by GLC.
[b]Values are means ± SD.
[c]Values are means ± mean deviations.

temperature the growth rate is almost as fast as at 37°C, argues against growth rate as the controlling factor.

DISCUSSION

In bacteria, as in higher organisms, phospholipids are found essentially only in membranes, and in many bacteria, especially gram-positives, the major membrane is the cytoplasmic membrane (23). It is now widely recognized that changes occur in the fatty acid composition of complex lipids in microorganisms, plants, and animals in response to the temperature of the environment (24, 25). The general rule is: as the growth temperature is lowered, the composition of the fatty acid chains is modified in the direction of lower average melting points. In gram-negative organisms, this adaptation is usually accomplished by an increased proportion of unsaturated and in some cases cyclopropane fatty acids at the expense of saturated fatty acids at lower growth temperatures (26–30). In certain gram-positive bacteria there are increased amounts of lower-melting branched-chain fatty acids at the expense of the higher-melting branched and straight-chain fatty acids at lower growth temperatures (31). In addition, a fatty acid desaturating system is induced in some bacilli at lower growth temperatures (32, 33). These changes have been shown by a number of workers to result in a lowering of the temperature at which the bulk of the membrane lipids change from a melted or fluid state to a frozen or condensed state (6, 7, 34).

The question we have asked is: What are the effects of growth temperature on the fatty acid and alk-1-enyl group compositions of an anaerobic bacterium in which more than 50% of the phospholipids contain alk-1-enyl chains? Our results show that the general rule is obeyed. At lower growth temperatures there is a higher proportion of unsaturated plus cyclopropane hydrocarbon chains and a slightly higher proportion of shorter chains ($<C_{18}$). Although the increased unsaturation is accomplished through a complex series of changes in the composition of the fatty acids and aldehydes, it should first be noted that the degree of change is almost identical with that seen in *Escherichia coli,* an organism that contains no plasmalogens, over the same temperature range. If we define the unsaturation index as the ratio of unsaturated plus cyclopropane chains to saturated chains $\left(\frac{U+C}{S}\right)$ we note that the unsaturation index of *C. butyricum* phospholipids goes from 0.86 at 37°C to 1.04 at 25°C (Fig. 1). In *E. coli* strain CR34 (28) or in an unsaturated fatty acid auxotroph derived from CR34, which was grown on oleate or *cis*-vaccenate (27), the unsaturation index changed from 1.2 at 37°C to 1.4 at 25°C or 27°C. In the same auxotroph grown on palmitoleate, the unsaturation index increased from 0.8 at 37°C to 1.0 at 27°C (27). In another strain of *E. coli,* ML30, which had a higher proportion of unsaturated fatty acids, the index went from 1.5 at 37°C to 2.2 at 25°C (26).

In examining individual phospholipids isolated from *C. butyricum* grown at different temperatures, the changes in the degree of unsaturation of the fatty acid and alk-1-enyl chains of ethanolamine and *N*-methyethanolamine plasmalogens appear to cancel each other out. As the fatty acid chains at C-2 became more unsaturated, the alk-1-enyl chains at C-1 became more saturated (Fig. 3). The effects of such changes on intramolecular and intermolecular hydrocarbon chain mobilities are unknown. The fatty acids in the PE + PME diacylphosphatides became slightly more saturated at lower growth temperatures. This group of phospholipids represents 12% of the total at 37°C and 13% of the total at 25°C (Table 3). As can be seen in Table 7, the fatty acids of the glycerol phosphoglycerides, diacyl plus 1-alk-1′-enyl-2-acyl, are relatively constant with respect to the degree of unsaturation at different

temperatures. There was a trend towards greater unsaturation in the fatty acids of a phospholipid fraction containing the glycerol hemiacetals of the plasmalogens. The ratio of unsaturated plus cyclopropane to saturated fatty acids increased from 0.98 at 37°C to 1.16 at 25°C (data not shown), which accounts in part for the increased unsaturation of the total fatty acids seen in Fig. 1.

A study of the effects of environmental temperature on plasmalogens was carried out by Roots and Johnston (9) on the brain lipids of goldfish acclimated to different temperatures. These authors found an increase in the percentage of PE as plasmalogen (moles aldehyde per mole P × 100) at higher temperatures. At 5°C, PE was 35% plasmalogen, and this figure increased to 49% at 25°C and then declined slightly to 46% at 30°C. The change between 25°C and 30°C may not have been significant. These authors also observed a striking increase in the degree of unsaturation of the alk-1-enyl groups of this plasmalogen at lower temperatures. At 30°C the ratio of 18:0 to 18:1, the major alk-1-enyl components, was 1.69, whereas at 5°C the ratio was 0.68. These results were interpreted along with previous similar findings on goldfish brain fatty acids as an adaptation of neural membranes to achieve a greater degree of plasticity and permeability at lower temperatures in this poikilothermic organism.

Although the results with *C. butyricum* are less amenable to this interpretation, it is clear that the hydrocarbon chain composition of the plasmalogens is affected by growth temperature. The changes we have observed in the fatty acid and alk-1-enyl group compositions of *C. butyricum* phospholipids grown at different temperatures may eventually require a more complex interpretation. It will be necessary to distinguish between lipids that contribute mainly to the bulk lipid phase and those that are more tightly associated with the various proteins of the cell membrane (35). The effects of growth temperature on the hydrocarbon chain compositions of these two groups of lipids may be quite different. Physical studies on the membranes and on the isolated lipids and isolation of protein–lipid complexes will be needed to shed more light on these problems.

This work was supported by a research grant from the National Institute of Allergy and Infectious Diseases (AI 08903). We wish to acknowledge the contributions of Dr. Seamus Rooney in the initial parts of this work. For paper IV of this series see Ref. 10.

Manuscript received 4 February 1974; accepted 6 May 1974.

REFERENCES

1. Horrocks, L. A. 1972. Content, composition, and metabolism of mammalian and avian lipids that contain ether groups. *In* Ether Lipids: Chemistry and Biology. F. Snyder, editor. Academic Press, New York. 177–272.
2. Mallins, D. C., and U. Varanasi. 1972. The ether bond in marine lipids. *In* Ether Lipids: Chemistry and Biology. F. Snyder, editor. Academic Press, New York. 297–312.
3. Thompson, G. A., Jr. 1972. Ether-linked lipids in protozoa. *In* Ether Lipids: Chemistry and Biology. F. Snyder, editor. Academic Press, New York. 321–327.
4. Goldfine, H., and P-O. Hagen. 1972. Bacterial plasmalogens. *In* Ether Lipids: Chemistry and Biology. F. Snyder, editor. Academic Press, New York. 329–350.
5. Singer, S. J., and G. L. Nicolson. 1972. The fluid mosaic model of the structure of cell membranes. *Science.* **175:** 720–731.
6. van Deenen, L. L. M. 1965. Phospholipids and biomembranes. *Progr. Chem. Fats Other Lipids.* **8(3):** 1–127.
7. Overath, P., H. U. Schairer, and W. Stoffel. 1970. Correlation of in vivo and in vitro phase transitions of membrane lipids in *Escherichia coli. Proc. Nat. Acad. Sci. USA.* **67:** 606–612.
8. Tourtellotte, M. E. 1972. Mycoplasma membranes: structure and function. *In* Membrane Molecular Biology. C. F. Fox, and A. D. Keith, editors. Sinauer Associates, Stamford, Conn. 439–470.
9. Roots, B. I., and P. V. Johnston. 1968. Plasmalogens of the nervous system and environmental temperature. *Comp. Biochem. Physiol.* **26:** 553–560.
10. Goldfine, H., and C. Panos. 1971. 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. *J. Lipid Res.* **12:** 214–220.
11. Broquist, H. P., and E. E. Snell. 1951. Biotin and bacterial growth. I. Relation to aspartate, oleate, and carbon dioxide. *J. Biol. Chem.* **188:** 431–444.
12. Goldfine, H., and K. Bloch. 1961. On the origin of unsaturated fatty acids in clostridia. *J. Biol. Chem.* **236:** 2596–2601.
13. Baumann, N. A., P-O. Hagen, and H. Goldfine. 1965. Phospholipids of *Clostridium butyricum.* Studies on plasmalogen composition and biosynthesis. *J. Biol. Chem.* **240:** 1559–1567.
14. Gray, G. M. 1958. The structure of the plasmalogens of ox heart. *Biochem. J.* **70:** 425–433.
15. Wood, R., and R. D. Harlow. 1969. Gas–liquid chromatographic analysis of free long-chain aldehydes. *J. Lipid Res.* **10:** 463–465.
16. van Golde, L. M. G., and L. L. M. van Deenen. 1967. Molecular species of extracellular phosphatidylethanolamine from *Escherichia coli. Chem. Phys. Lipids.* **1:** 157–164.
17. Nesbitt, J. A., III, and W. J. Lennarz. 1965. Comparison of lipids and lipopolysaccharide from the bacillary and L forms of *Proteus* P18. *J. Bacteriol.* **89:** 1020–1025.
18. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:** 466–468.
19. Williams, J. N., Jr., C. E. Anderson, and A. D. Jasik. 1962. A sensitive and specific method for plasmalogens and other enol ethers. *J. Lipid Res.* **3:** 378–381.
20. Tarlov, A. R., and E. P. Kennedy. 1965. The β-galactoside permease system and the metabolism of phospholipids in *Escherichia coli. J. Biol. Chem.* **240:** 49–53.
21. Matsumoto, M., K. Tamiya, and K. Koizumi. 1971. Studies on neutral lipids and a new type of aldehydogenic ethanolamine phospholipid in *Clostridium butyricum. J. Biochem.* (Tokyo). **69:** 617–620.
22. Hildebrand, J. C., and J. H. Law. 1964. Fatty acid distri-

bution in bacterial phospholipids. The specificity of the cyclopropane synthetase reaction. *Biochemistry.* **3:** 1304–1308.
23. Lennarz, W. J. 1972. Studies on the biosynthesis and functions of lipids in bacterial membranes. *Acc. Chem. Res.* **5:** 361–367.
24. Farrell, J., and A. H. Rose. 1967. Temperature effects on microorganisms. *Annu. Rev. Microbiol.* **21:** 101–120.
25. Hunter, K., and A. H. Rose. 1972. Influence of growth temperature on the composition and physiology of microorganisms. *J. Appl. Chem. Biotechnol.* **22:** 527–540.
26. Marr, A. G., and J. L. Ingraham. 1962. Effect of growth temperature on the composition of fatty acids in *Escherichia coli. J. Bacteriol.* **84:** 1260–1267.
27. Esfahani, M., E. M. Barnes, Jr., and S. J. Wakil. 1969. Control of fatty acid composition in phospholipids of *Escherichia coli:* response to fatty acid supplements in a fatty acid auxotroph. *Proc. Nat. Acad. Sci. USA.* **64:** 1057–1064.
28. Hechemy, K., and H. Goldfine. 1971. Isolation and characterization of a temperature-sensitive mutant of *Escherichia coli* with a lesion in the acylation of lysophosphatidic acid. *Biochem. Biophys. Res. Commun.* **42:** 245–251.
29. Sinensky, M. 1971. Temperature control of phospholipid biosynthesis in *Escherichia coli. J. Bacteriol.* **106:** 449–455.
30. Kates, M., and P-O. Hagen. 1964. Influence of temperature on fatty acid composition of psychrophilic and mesophilic *Serratia* species. *Can. J. Biochem.* **42:** 481–488.
31. Weerkamp, A., and W. Heinen. 1972. Effect of temperature on the fatty acid composition of the extreme thermophiles, *Bacillus caldolyticus* and *Bacillus caldotenax. J. Bacteriol.* **109:** 443–446.
32. Fulco, A. J., R. Levy, and K. Bloch. 1964. The biosynthesis of Δ^9- and Δ^5-monounsaturated fatty acids by bacteria. *J. Biol. Chem.* **239:** 998–1003.
33. Fulco, A. J. 1967. The effect of temperature on the formation of Δ^5-unsaturated fatty acids by bacilli. *Biochim. Biophys. Acta.* **144:** 701–703.
34. Podo, F., and J. K. Blasie. 1973. Mobility of membrane components. MTP International Reviews of Science. Biochemistry Series: Biochemistry of Cell Walls and Membranes. C. F. Fox, editor. MTP, Oxford. In press.
35. Träuble, H., and P. Overath. 1973. The structure of *Escherichia coli* membranes studied by fluorescence measurements of lipid phase transitions. *Biochim. Biophys. Acta.* **307:** 491–512.