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Phospholipid aliphatic chain composition modulates lipid class composition, but not lipid asymmetry in *Clostridium butyricum*

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The phospholipid composition of the butyric acid-producing clostridia is responsive to the degree of enrichment of the lipids with *cis*-unsaturated fatty acids. When *Clostridium butyricum* and *Clostridium beijerinckii* are grown on oleic acid in media devoid of biotin, the acyl and alk-1-enyl chains of the phospholipids become highly enriched with 18:1 and C_{19}-cyclopropane. Under these conditions there is a marked increase in the glycerol acetals of the major plasmalogens of these organisms. We have grown both species on mixtures of palmitate and oleate in the absence of biotin. The alk-1-enyl chains were highly enriched with C_{18}-unsaturated and C_{19}-cyclopropane residues at all but the highest ratios of palmitate to oleate (80:20, w/w) added to the medium. At ratios of palmitate to oleate $\geqslant$ 40:60, the saturated acid was incorporated predominantly into the phospholipid acyl chains in both organisms. The effects of increasing unsaturation of the acyl chains as the ratio of oleate to palmitate was increased was examined in *C. butyricum*. In cells grown on mixtures of palmitate and oleate equal to or exceeding 40% palmitate, the ratio of glycerol acetal lipid to total phosphatidylethanolamine (PE) was relatively constant. As the proportion of oleic acid added to the medium was increased, the ratio of glycerol acetal lipid to PE increased from 0.7 to 2.0. Thus the ratio of the polar lipids appears to respond to the content of phospholipids that contain two unsaturated chains. The fraction of PE present as plasmalogen remained relatively stable (0.82 ± 0.05) at varying ratios of medium oleic and palmitic acids. Both the glycerol acetal of ethanolamine plasmalogen, and ethanolamine plasmalogen, are shown to be 80% or more in the outer monolayer of the cell membrane. These two polar lipids represent approx. 50% of the phospholipids in cells grown on exogenous fatty acid. The bulk of the remainder is polyglycerol phosphatides. We suggest that the ability of both species to grow with highly unsaturated membranes is related to their ability to modulate their polar lipid composition.

Introduction

In the study of the dynamics of biological membranes, considerable emphasis has been placed on the regulation of membrane fluidity [1,2]. The importance of phospholipid polar headgroup structures and of their aliphatic chains as determinants of membrane fluidity at the temperatures encountered by living organisms, has been intensively evaluated. Recently, theoretical and experi-

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Abbreviations: MGDG, monoglucosyldiacylglycerol; DGDG, diglucosyldiacylglycerol, TNBS, trinitrobenzenesulfonic acid.

Nomenclature: Use of the unmodified name of a phospholipid, for example, phosphatidylethanolamine or phosphatidylglycerol, implies the sum of the diacyl and alkenyl acyl (plasmalogen) species. The terminology plasmenylethanolamine, plasmenylglycerol is used to refer specifically to the plasmalogen forms of these phopholipids.

mental studies have begun to emphasize the importance of lipid shape in the maintenance of bilayer stability [3,4]. According to these concepts, the size of the polar headgroup, the hydrophobic volume occupied by the hydrocarbon chains, and their length, determine the shapes of membrane lipid molecules. The hydrophobic volume and length of the hydrocarbon chains is influenced by the presence or absence of double bonds and by temperature. When these features combine to produce a cylindrically shaped molecule, stable bilayers are formed, for example, with model membranes of phosphatidylcholine or dihexosyldiacylglycerols. When these parameters combine to produce a conical shape, lipids will aggregate to form reverse hexagonal and other non-lamellar configurations, as is the case for naturally occurring phosphatidylethanolamines and monohexosyldiacylglycerols [4–7]. Thus the need for a variety of lipids in biological membranes may result in part from the dual requirements of maintaining membrane fluidity and bilayer stability.

In addition to diacylphosphoglycerides and the classical plasmalogens, 1-*O*-alkenyl 2-acyl glycerophospholipids, the butyric acid-producing clostridia contain a unique glycerol acetal of their major plasmalogens [8–10]. In *Clostridium butyricum* the polar headgroup base of these lipids is ethanolamine, and in *Clostridium beijerinckii* it is *N*-methylethanolamine, with smaller amounts of ethanolamine [10]. We have observed that the content of the glycerol acetals of the plasmalogens varies considerably in both of these species. When cells are grown in semi-synthetic media containing biotin, which permits endogenous synthesis of fatty acids and alk-1-enyl groups, the ratio of the glycerol acetal lipid to the analogous plasmalogen-rich phosphatidylethanolamine or phosphatidyl-*N*-methylethanolamine, is less than one. In a rich medium containing biotin, this ratio is less than 0.2 [9–11]. However, when cells are grown in a semi-synthetic medium devoid of biotin, which is supplemented with oleic acid, the ratio of the glycerol acetal lipid to the corresponding plasmalogen-rich phospholipid, is approx. 2.0 [11,12]. In these cells endogenous fatty acid synthesis is essentially abolished and the acyl and alkenyl chains are almost entirely 18:1 and the C_{19} cyclopropane chains derived from the unsaturated precursors [11–13]. Studies on the phase behavior of the glycerol acetal lipid showed that the liquid-crystalline to gel transition temperature of the elaidate-enriched glycerol acetal of plasmenylethanolamine was lower than that of the corresponding diacyl or alk-1-enyl acyl phosphoglyceride, and the gel to liquid-crystalline transition of the oleate-enriched glycerol acetal lipid was much higher than that of dioleoyl phosphatidylethanolamine [14]. There was a hysteresis of 13 to 18°C in these phase transitions. Based on these observations, we proposed that the oleate-enriched glycerol acetal lipid may in some way stabilize these highly unsaturated membranes, permitting the cells to attain a degree of membrane lipid unsaturation not tolerated by *Escherichia coli* fatty acid auxotrophs [13,15]. We have also shown that both plasmenylethanolamine and its glycerol acetal are predominantly located in the outer monolayer of the cell membrane of *C. butyricum* [16].

We now present studies on the effects of supplementing biotin-depleted media with mixtures of oleic and palmitic acids as a means of controlling the degree of unsaturation of the membrane phospholipids of *C. butyricum.* These experiments show that the ratio of the glycerol acetal lipid to plasmalogen responds to the degree of unsaturation of the aliphatic chains, and in particular the acyl chains of the phospholipids. Although the ratio of the two lipids can vary considerably, we show that both lipids continue to reside predominantly in the outer monolayer of the cell membrane. We propose that the glycerol acetal lipid of these highly unsaturated membranes may serve in part to prevent destabilization caused by the formation of hexagonal or other non-bilayer structures.

Experimental Procedures

Cells and culture conditions. Clostridium butyricum and *Clostridium beijerinckii* are separate species distinguishable by their cell wall compositions, DNA homology [17], and lipid compositions [10]. *C. butyricum* IFO 3852, obtained from the Institute for Fermentation, Osaka, has a lipid composition typical of *C. butyricum* [10]. Its reported origin from *C. beijerinckii* ATCC 6014 is questionable [10]. It has, therefore, been dropped from the Institute for Fermentation collection. *C.*

beijerinckii ATCC 6015 was formerly designated *C. butyricum,* but its listing is being revised [10,17]. Cultures were maintained on the Casamino acid-glucose medium of Broquist and Snell [18], and subcultured at least twice on oleate, 25 μg/ml, in a vitamin-free medium [12], prior to the experimental culture. Cultures were grown for analysis of acyl and alkenyl chain compositions in 100 ml volumetric flasks, which were made anaerobic by an alkaline pyrogallol double plug technique [19]. These cells were grown at 37°C for 16 to 19 h prior to harvest, as described [13].

For phospholipid asymmetry and lipid class composition studies, cell were grown overnight on 10 ml of the same oleic acid-supplemented vitamin-free Casamino acid medium. In the morning, 100 ml of the same medium containing the desired fatty acid(s) was freshly autoclaved in a volumetric flask, and inoculated with the overnight culture. It was made anaerobic as described above. After 2 to 3 h, when growth became evident through the appearance of gas bubbles, $^{32}P_i$, 2.5 μCi/ml, was added. The cultures were then harvested in logarithmic growth ($Klett_{66}$ = 70 to 100), washed and resuspended in 2.0 ml of protoplasting buffer: 0.4 M sucrose, 10 mM potassium phosphate, 15 mM $MgCl_2$, (pH 7.5) [16,20].

Protoplast preparation and phospholipase treatment. Protoplasts were obtained by lysozyme treatment as previously described [16]. After 30 to 45 min incubation at 37°C, protoplast formation was monitored by phase contrast microscopy, and was considered sufficient when $\geq$ 75% of cells were either rounded or no longer had the normal cylindrical cell shape and had lost the strongly demarcated cell outline. Phospholipase C from *Bacillus cereus,* 20 U, was then added and the protoplasts were incubated at 23°C for 90 to 120 min.

Determination of lipid composition before and after phospholipase treatment. At time zero, before addition of phospholipase C, two 0.2 ml aliquots of the protoplast suspension were removed to 0.6 ml of chloroform/methanol (1 : 2, v/v). At intervals, 0.2 ml samples were similarly removed to chloroform/methanol (1 : 2, v/v), and after all samples were collected on ice, the lipids were extracted by the method of Bligh and Dyer [21]. Usually the extracts were left overnight at 2°C. The phases were separated by 10 min centrifugation at 2000 rpm in a desk-top centrifuge. The lower phase was removed quantitatively, evaporated to dryness and taken up in 20 to 30 μl of chloroform for spotting on TLC plates. One- or two-dimensional TLC was carried out on plastic backed silica plates as described [16], except that exposure to HCl fumes was reduced to 20 to 30 s in order to minimize hydrolysis of diacylphosphatides and lysophosphatides formed by acid hydrolysis of the plasmalogen [22]. Lipids were located by exposure to X-Omat AR film (Kodak), and the spots were cut out and assayed for radioactivity by liquid scintillation in a minimal volume of OCS scintillation fluid (Amersham).

Trinitrophenylation of amino lipids. Cells, which were cultured and harvested in parallel with those subjected to protoplasting and phospholipase C treatment, were harvested, washed and treated with TNBS at 5°C as described [16]. The kinetics of trinitrophenylation were studied by removing aliquots of the incubation mixtures at intervals up to 18 h, and stopping the reaction as described [16]. TNBS penetration of intact cells at 5°C was measured as described [16,23].

Analyses and enzyme assays. Acyl and alkenyl chain compositions were determined by gas-liquid chromatography as previously described [10]. Alkenyl chains include those released from the glycerol acetals by acid hydrolysis. NADH oxidase was assayed spectrophotometrically at 340 nm in 1.0 ml of 0.1 M potassium phosphate (pH 7.5) containing 0.8 mM $MgCl_2$ and 204 μM NADH.

Materials. Vitamin-free Casamino acids were obtained as a sterile solution from ICN-Nutritional Biochemicals (Cleveland, OH). $^{32}P_i$, carrier-free, was obtained from Amersham Corp. (Arlington Heights, IL). TNBS was purchased from Aldrich Chemical Co. (Milwaukee, WI), and was recrystallized from 3 M HCl. Egg white lysozyme and phospholipase C from *B. cereus* (Type V) were from Sigma.

Results

Incorporation of mixtures of exogenous palmitic and oleic acids into phospholipids

When *C. butyricum* and *C. beijerinckii* are grown on an exogenous fatty acid in media devoid of biotin, the cellular phospholipids become highly

enriched with the fed fatty acid almost to the exclusion of endogenously synthesized aliphatic chains [12,13]. The composition of the acyl and alkenyl chains of the phospholipids of both organisms grown on mixtures of palmitic and oleic acids under these conditions is shown in Fig. 1. As we have reported previously, when grown on oleic acid alone, the acyl chains contained from 88 to 94% 18:1 plus 19:cyc chains and the alkenyl chains were somewhat more highly enriched. When mixtures of fatty acids were added to the medium, it can be seen that palmitic acid was predominantly incorporated into the acyl chains in both organisms. Only when the ratio of palmitate to oleate was 6:4 or greater was significant incorporation of palmitate into the alk-1-enyl chains seen in *C. butyricum*. There were differences in the two species with respect to conversion of unsaturated to cyclopropane chains. In *C. butyricum* approximately one-third of the 18:1 acyl chains and one-half of the alkenyl chains were converted, but in *C. beijerinckii*, methylenation of the acyl chains ranged from 43 to 57%; and that of the alkenyl chains was 87% or greater. Similar differences have been noted in biotin-grown cells [10].

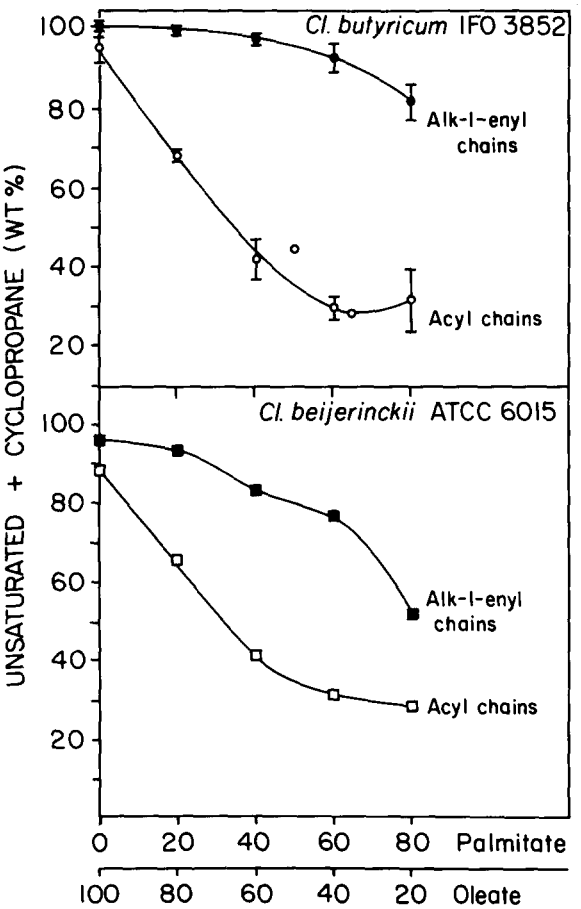

Fig. 1. Phospholipid acyl and alk-1-enyl chain composition of *C. butyricum* and *C. beijerinckii* grown on mixtures of palmitate plus oleate. The wt% of 16:0 is equal to 100% − (unsaturated + cyclopropane). Error bars represent mean deviations of analyses of two separate cultures each analyzed in duplicate by gas chromatography. The results for *C. beijerinckii* represent single cultures analyzed in duplicate. Alk-1-enyl chains include the aldehydogenic, C-1-linked chains, of the glycerol acetal lipid.

Modulation of lipid class composition

The effects of alteration of the alphatic chain composition on the phospholipid composition of *C. butyricum* was studied and the results of these experiments are shown in Fig. 2. It can be seen that protoplasts from cells grown on mixtures of palmitate and oleate containing 40% or more of the saturated fatty acid, had a relatively constant ratio of the glycerol acetal lipid to phosphatidylethanolamine (0.70 ± 0.01). As the proportion of oleic acid added to the medium was increased, the ratio of glycerol acetal lipid to phosphatidylethanolamine increased from 0.7 to 2.0. The proportion of phosphatidylglycerol declined continuously with increasing ratios of oleic to palmitic acid added to the medium, with only minor changes in the proportion of cardiolipin. By comparing Figs. 1 and 2, it can be seen that the reversal in ratio of the glycerol acetal lipid to phosphatidylethanolamine occurred as the proportion of unsaturated plus cyclopropane acyl chains in the phospholipids increased markedly. The proportion of phosphatidylethanolamine present as plasmalogen remained relatively stable at varying ratios of medium oleic and palmitic acids: $89 \pm 1\%$ at 100:0, $77 \pm 2\%$ at 80:20, and $80 \pm 4\%$ at 60:40.

Phospholipid asymmetry in cells grown on exogenous fatty acids

We have shown that phosphatidylethanolamine and the glycerol acetal lipid are predominantly localized in the outer monolayer of the cell membrane of *C. butyricum* IFO 3852 grown in biotin-containing media by treatment of intact cells with trinitrobenzenesulfonate and by phospholipase C

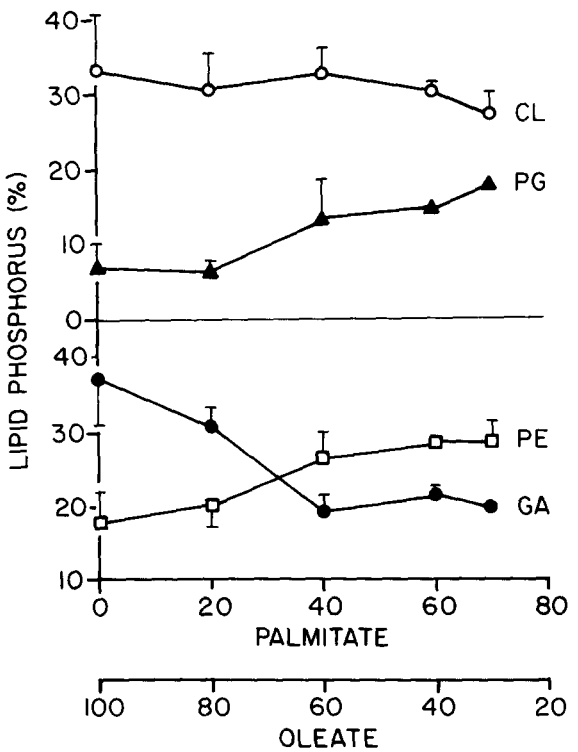

Fig. 2. Lipid class composition of *C. butyricum* protoplasts grown on mixtures of palmitate plus oleate. Abbreviations: CL, cardiolipin; PG, phosphatidylglycerol; PE, phosphatidylethanolamine (diacyl + plasmalogen); GA, glycerol acetal of ethanolamine plasmalogen. Each value represents the mean of two or three separate determinations and the error bars are either standard or mean deviations. In points that have no error bar, the deviation was within the symbol.

hydrolysis of protoplast phospholipids [16]. We have applied these same techniques to a study of phospholipid asymmetry in cells grown on exogenous fatty acids in the absence of biotin. The results are summarized in Table I. With phospholipase C, we observed rapid hydrolysis of 81 to 88% of the phosphatidylethanolamine and 85 to 94% of the glycerol acetal lipid followed by slower hydrolysis of the remaining 5 to 20% (Fig. 3). We and others have interpreted the rapidly hydrolyzed pools as representing the outer monolayer phospholipid, followed by slower hydrolysis of the inner monolayer phospholipid after either phospholipid flip-flop or increased permeability of the membrane to phospholipase C [16,24].

Treatment of intact cells with TNBS at 5°C showed trinitrophenylation of almost identical proportions of the two phospholipid classes (Table I). The kinetics of trinitrophenylation of both amino-lipids in cells grown on oleate/palmitate 60 : 40 or 80 : 20 were similar to those previously published for biotin-grown cells [16], with 60 to 70% of the two lipids modified at 7.5 h. However, in cells grown on 100% oleate, this level of modification was reached or exceeded by 2 h. By 7 h, trinitrophenylation of phosphatidylethanolamine was $86 \pm 4\%$ and that of the glycerol acetal was $91 \pm 2\%$. Whether this increased rate of reaction reflects changes in lipid spacing or mobility, remains to be determined.

As in biotin-grown cells [16], little or no

TABLE I

PHOSPHOLIPID ASYMMETRY IN *CLOSTRIDIUM BUTYRICUM* GROWN ON OLEIC AND PALMITIC ACIDS

Growth supplement	% of ^{32}P			
	Total phosphatidylethanolamine		Glycerol acetal lipid	
	Phospholipase C hydrolysis	TNBS modification	Phospholipase C hydrolysis	TNBS modification
Oleate	85 ± 5 [a]	92 ± 3 [b]	94 ± 3	98 ± 1
Oleate/palmitate (80 : 20)	81 ± 1	79	87 ± 4	88
Oleate/palmitate (60 : 40)	88 ± 3	86	85 ± 7	90

[a] Represents the fraction of each lipid class in protoplast membranes that was rapidly hydrolyzed by phospholipase C.

[b] Represents the fraction of each lipid class modified after 18 h treatment of whole cells incubated with TNBS. The kinetics of the reaction are described in the text.

cardiolipin was hydrolyzed by phospholipase C treatment of intact protoplasts (Fig. 3). In cells grown on palmitate/oleate 40 : 60 (Fig. 3C), about 30% of phosphatidylglycerol was hydrolyzed rapidly. In cells grown on mixtures containinig more of the unsaturated fatty acid, the smaller amounts of phosphatidylglycerol precluded accurate measurement of the rapidly hydrolyzable pool. We have shown that over 70% of the two acidic lipids combined was hydrolyzed in lysed protoplasts of biotin-grown cells [16], and a control experiment with sonicated ^{32}P-labeled lipids extracted from *C. butyricum* showed that phospholipase C from *B. cereus* hydrolyzed both acidic phospholipids, although cardiolipin was hydrolyzed slowly (data not shown). In the absence of independent confirming data, we tentatively conclude that phosphatidylglycerol and cardiolipin in cells grown on fatty acids without biotin are predominantly localized in the inner membrane.

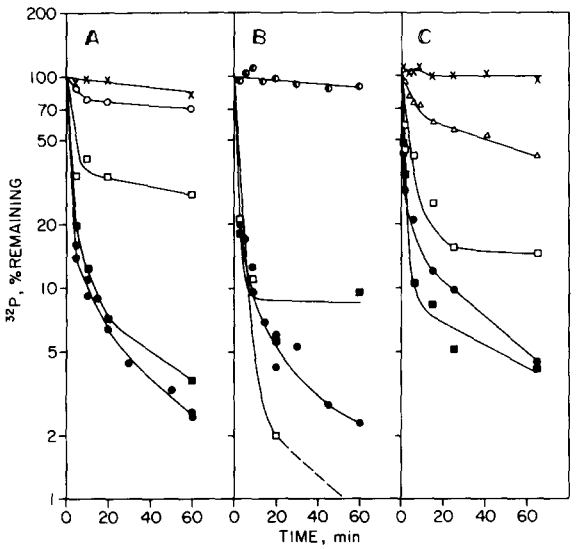

Fig. 3. Hydrolysis of ^{32}P-labeled phospholipids in *C. butyricum* protoplasts. Protoplasts, prepared as described in Experimental Procedures, were incubated with 20 U of phospholipase C from *Bacillus cereus* in 2.0 ml of protoplasting buffer at 23°C. At intervals, 200-μl aliquots were withdrawn and added to chloroform/methanol (1 : 2, v/v). The lipids were then extracted as described and spotted on silica gel 60 plates (10 × 10 cm). One- or two-dimensional TLC with HCl vapor treatment between the first and second dimension was performed as described under Experimental Procedures. The cells were grown on: Panel A, oleate alone; panel B, palmitate/oleate (20 : 80, w/w); panel C, palmitate/oleate (40 : 60, w/w). ●———●, Glycerol acetal lipid; ■———■, plasmenylethanolamine; □———□, diacyl phosphatidylethanolamine; ○———○, alkenyl acyl cardiolipin; ×———×, tetraacyl cardiolipin, ◐———◐, total phosphatidylglycerol plus total cardiolipin; △———△, total phosphatidylglycerol. The results for the glycerol acetal lipid in panels A and B were obtained from both one- and two-dimensional chromatograms of the same samples.

As noted above, the proportion of total phosphatidylethanolamine present as plasmalogen varies from 77 to 85% in cells grown on oleate alone or palmitate/oleate 20 : 80 and 60 : 40. Fig. 3 shows that at least 80% of plasmenylethanolamine is rapidly hydrolyzed by phospholipase C in protoplasts from these cells. These results for plasmalogens are consistent with the results for the total phosphatidylethanolamine fraction (Table I). The fraction of diacylphosphatidylethanolamine in the rapidly hydrolyzed pool varies from 60 to 90%. In protoplasts from oleate-grown cells, in which the diacyl form represented 3.3% of total lipid phosphorus, the remaining unhydrolyzed diacyl lipid after 5 min of phospholipase C treatment was significantly contaminated with a minor phospholipid component that runs near diacylphosphatidylethanolamine in the two-dimensional TLC system. In the two other experiments, this contaminant represented a smaller proportion of this fraction. Thus the values for the rapidly hydrolyzed fraction of diacylphosphatidylethanolamine in these experiments should be regarded as minimal figures.

Tests for leakiness of cells and protoplasts

As in the case of cultures grown in biotin-containing media [16] the cells grown on oleic acid alone or on mixtures of oleic and palmitic acids were only slightly permeable to TNBS. Comparison of protein trinitrophenylation showed only 2.3 and 1.9% as much protein modification in intact cells compared to lysed protoplasts of cells grown on oleic acid alone or on palmitic acid/oleic acid (60 : 40), respectively. Protoplast leakiness at the beginning of phospholipase C treatment was tested for by measuring the release of NADH oxidase during treatment with lysozyme, the accumulation of NADH oxidase in the supernatant of cells after the completion of protoplast formation was only 1.1 ± 0.3% of the amount released on osmotic lysis of the protoplasts.

Discussion

In previous studies on the effects of substituting exogenous oleic acid for endogenously synthesized mixtures of saturated and unsaturated phospholipid acyl and alkenyl chains in *C. beijerinckii* [11,12] and *C. butyricum* [13], we have observed a marked increase in the proportion of the glycerol acetal lipids with concomitant decreases in phosphatidyl-*N*-methylethanolamine or phosphatidylethanolamine, respectively. Growth on the *trans* isomer, elaidic acid resulted in smaller changes in the same direction in the ratios of these lipids (Refs. 11, 12; and unpublished observations of Johnston, N.C.)

The experiments presented here demonstrate that when fed mixtures of palmitate and oleate, the increase in the content of the glycerol acetal lipid in *C. butyricum* occurs when the acyl chains in addition to the alkenyl chains of the phospholipids become highly enriched with 18 : 1 and 19 : cyc (Figs. 1 and 2). In addition, we show that these marked changes in lipid class composition are largely localized in the outer monolayer of the cell membrane. In cells grown on oleate alone or on mixtures of oleate and palmitate, from 80 to 90% of the total phosphatidylethanolamine and 85 to 95% of the glycerol acetal lipid appear to be concentrated in the outer monolayer (Table I). The results obtained with phospholipase C hydrolysis and by TNBS modification are entirely consistent with one another. The plasmalogen component of the phosphatidylethanolamine fraction, which represented from 77 to 85% of the phosphatidylethanolamine, was similarly shown to be 80 to 90% localized in the outer monolayer, as determined by the kinetics of hydrolysis with phospholipase C (Fig. 3).

In order to understand the significance of the induced changes in lipid composition, it is necessary to consider the possible consequences of a high concentration of phosphatidylethanolamine with two *cis*-unsaturated chains in a single monolayer of the cell membrane. Dioleoyl phosphatidylethanolamine, which melts at −14°C, undergoes a lamellar to reversed hexagonal (L → H) phase transition at around 10°C [4]. Although the liquid crystalline to gel phase transition temperature of oleate-enriched ethanolamine plasmalogen is not known precisely, plasmenylethanolamine highly enriched with elaidate chains melts 6°C lower than dielaidoyl phosphatidylethanolamine [13]. Boggs et al. [25] have presented evidence for facilitation of the L → H transition, which occurred at 18°C in bovine brain ethanolamine phosphoglyceride. This lipid fraction is 77% plasmalogen and 7% 1-alkyl 2-acyl glycerolipid. A similar finding was made with a semi-synthetic plasmenylethanolamine containing mainly saturated alkenyl chains and oleate acyl chains, which assumed the hexagonal phase at 30°C compared to 69°C for a similar diacyl analog [26]. It is, therefore, reasonable to conclude that the presence of large amounts of di-*cis*-unsaturated ethanolamine plasmalogens in the outer monolayer of the cell membrane would lead to bilayer instability. The corresponding oleate-enriched glycerol acetal lipid melts at 7°C and reverts to the gel state at −4°C, on cooling, temperatures which are about 20°C higher than the corresponding transitions for dioleoylphosphatidylethanolamine [13,14]. Along with this increase in T_c there is an increase in ΔH to approximately twice that of the corresponding phosphatidylethanolamine. We have attributed the higher melting temperature and enthalpy to increased intermolecular interactions, presumably involving hydrogen bonding mediated by the free hydroxyls of the glycerol moiety in acetal linkage. Molecular model studies have shown that such hydrogen bonding is feasible [27].

It is also important to consider the shapes of these molecules. Another effect readily attributable to the addition of the glycerol in acetal linkage is an increase in the effective size of the polar headgroup [27]. The acetal-linked glycerol is known to be highly hydrated [13]. According to lipid self-assembly theories [3,4], the increased size of the polar headgroup of dioleoyl phosphatidylethanolamine provided by the addition of the three *N*-methyl groups of phosphatidylcholine changes the shape of this molecule at physiological temperatures from one which may be best represented as a truncated cone to one with a more cylindrical shape. The theoretical prediction that the conical shape of dioleoyl phosphatidylethanolamine increases its propensity to undergo an L → H transition, whereas the cylindrical shape of the corresponding phosphatidylcholine prevents this transition, has been confirmed experimentally [4]. By

analogy, the formation of the glycerol acetal would predictably serve to enhance bilayer stability at 37°C. We have recently shown by ^{31}P-NMR that the oleate-enriched glycerol acetal from *C. butyricum* forms stable bilayers at temperatures up to 60°C and that addition of the glycerol acetal to egg yolk phosphatidylethanolamine (1 : 2, w/w), prevented formation of the H_{II} phase at temperatures up to 50°C. The egg phosphatidylethanolamine alone underwent an $L \rightarrow H$ transition at 15° to 20°C (Goldfine, H. and Johnston, N.C., unpublished data).

The important experiments of Wieslander and co-workers on the regulation of membrane lipid composition in *Acholeplasma laidlawii* have provided a biological test for lipid-packing theories [28,29]. These workers observed that growth of *A. laidlawii,* an obligate unsaturated fatty acid auxotroph, with varying mixtures of palmitate plus oleate, affects the ratio of the two principal membrane non-ionic polar lipids, monoglucosyldiacylglycerol (MGDG) and diglucosyldiacylglycerol (DGDG). When grown at constant temperature with increasing ratios of oleate to palmitate, the ratio of DGDG to MGDG increases from 0.42 at oleate/palmitate 30 : 120 to 1.75 at 100% oleate. They have more recently shown that the phase behavior of MGDG/DGDG mixtures follows the self-assembly rules discussed above. When highly enriched with oleate chains, 2.5/1.0 mixtures of MGDG/DGDG began to form a reversed cubic phase between 0 and 15°C, whereas a 1.2/1 mixture did not do so below 20°C. On the other hand, mixtures of MGDG/DGDG with approx. 50% saturation, formed a lamellar phase at temperatures up to 60°C [29]. Thus, these authors believe that the ratio of the two glycolipids is regulated in a manner which prevents the formation of bilayer-destabilizing cubic or hexagonal phases.

At this time it is not clear how the ratios of MGDG to DGDG in *A. laidlawii* and of the plasmalogen-rich phosphatidylethanolamine to the corresponding glycerol acetal lipids in *C. butyricym* and *C. beijerinckii* are regulated. MGDG is thought to be a precursor of DGDG in *A. laidlawii* [28], and plasmenylethanolamine is likely to be the precursor of the glycerol acetal lipid in *C. butyricum* [30]. Thus, a mechanism that promotes conversion of the precursors with smaller polar head groups to their products with larger polar headgroups in response to the formation of microdomains of nonbilayer structures, should be considered. If such domains occur in the vicinity of the enzymes involved in these conversions, they may affect protein conformations and, thereby, possibly the accessibility or the affinity of the enzymes with respect to one or both of their substrates. Alternatively, enhanced isotropic motion of the lipid substrates in the vicinity of the enzymes could promote these transformations by bringing the point of addition on the lipid substrate closer to the enzyme active site.

Our studies on the butyric acid clostridia and those of Wieslander and coworkers on *A. laidlawii* focus attention on properties of lipids distinct from their contributions to membrane fluidity. The need for lipids that stabilize the lamellar phase is especially important in *Acholeplasma,* which naturally depends on exogenous unsaturated fatty acids for growth. In the butyric acid group of clostridia, it is not clear that analogous conditions exist in nature. However, their ability to grow in the presence of either biotin or unsaturated fatty acids may be important where there is a supply of fatty acids and low biotin availability, perhaps because biotin is bound to molecules such as avidin. We have shown that *C. beijerinckii* can be grown in the presence of oleate or linoleate when suboptimal levels of biotin permit a limited amount of saturated fatty acid synthesis. Under these conditions, the proportion of glycerol acetal lipid was also increased [12]. The evolution of mechanisms that stabilize the lamellar phase when there are high levels of unsaturated fatty acids in the membrane lipids, may enable certain groups of bacteria to take advantage of naturally abundant fatty acid species. Perhaps closer examination of other species will reveal other adaptations.

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