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Phase Behavior of Ether Lipids from *Clostridium butyricum*†

Howard Goldfine,* Norah C. Johnston, and Michael C. Phillips

ABSTRACT: Ether lipids have been isolated from the phospholipid fraction of *Clostridium butyricum* IFO 3852 cells which had been grown in media devoid of biotin with added elaidic acid or oleic acid. The plasmalogen form of phosphatidylethanolamine (plasmenylethanolamine) from elaidate-grown cells was highly enriched with 18:1 in both the alk-1-enyl and acyl chains. A transition from the gel to liquid-crystalline state, with a peak maximum (T_m) at 33 °C and enthaply ΔH = 5.7 kcal/mol, was observed by differential scanning calorimetry. With the fluorescent probes *cis*- and *trans*-parinaric acids, transitions were observed at 33 °C on heating and at 29 °C on cooling. These transition temperatures are 5-6 °C lower than those reported for the corresponding diacyl lipid, dielaidoylphosphatidylethanolamine. A similar study of the phase behavior of both the elaidate-enriched and oleate-enriched glycerol acetal derivative of plasmenylethanolamine from *C. butyricum* revealed a large hysteresis of 12.5-16 °C. Hysteresis in the polar head group motion was also observed by ^{31}P nuclear magnetic resonance. The elaidate-enriched lipid, which melted between 28 and 33 °C, appears to undergo supercooling prior to the transition to the gel state at about 18-13 °C, depending on the scanning rate. The formation of a more ordered gel state relative to plasmenylethanolamine was indicated by a 2-fold increase in ΔH. Electron microscopy revealed a marked reorganization from typical multilamellar liposomes above T_m to large needle-like structures below T_m. The oleate-enriched glycerol acetal lipid formed the gel phase at −4 °C, which is 10 °C above the transition temperature reported for dioleoylphosphatidylethanolamine. Stabilization of oleate-enriched glycerol acetal lipid bilayers may result from hydrogen bonding between polar head groups. The relationship of the phase behavior of the ether lipids to the lipid composition and phase behavior of *C. butyricum* membranes is discussed.

Plasmalogens, 1-(*O*-alk-1′-enyl)-2-acylglycerophospholipids, are major lipids of the membranes of many anaerobic bacteria including Gram-positive and Gram-negative organisms belonging to a variety of genera (Kamio et al., 1969; Goldfine & Hagen, 1972; Hagen, 1974; Clarke et al., 1976; vanGolde et al., 1973, 1975). The presence of these unsaturated ether lipids in animal tissues has been recognized for many years (Debuch & Seng, 1972), and in certain tissues, such as brain white matter and heart, they represent >30% of the total phosphoglycerides (Horrocks, 1972).

Although some work has been reported on the physical behavior of plasmalogen monolayers (Shah & Schulman, 1965), there have been no detailed studies on the phase behavior of these lipids. Presumably the difficulties encountered in the chemical synthesis of plasmalogens with the natural

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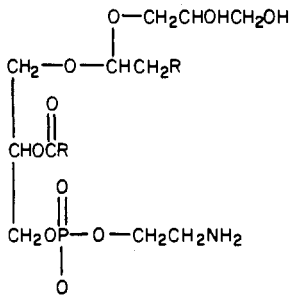
configuration have slowed progress in this area (Gigg, 1972).

We have previously reported that growth of *Clostridium butyricum* on exogenous fatty acids in the absence of biotin leads to a marked enrichment of the fed fatty acids in both the alk-1-enyl and acyl chains of the plasmalogens (Khuller & Goldfine, 1975). Physical studies on the total phospholipids of cells grown on elaidate or oleate with the spin probe 2,2,6,6-tetramethylpiperidinyl-1-oxy (Tempo) and with the fluorescent probe diphenylhexatriene (DPH) showed relatively narrow melting transitions in phospholipids from elaidate-grown cells and broad transitions in those from oleate-grown cells (Goldfine et al., 1977).

C. butyricum also has an unusual glycerol acetal derivative of the major plasmalogen (Matsumoto et al., 1971) which constitutes ~25% of the phospholipids in biotin- and elaidate-grown cells and nearly 50% in oleate-grown cells (Khuller & Goldfine, 1974; 1975; Goldfine et al., 1977). The structure of this lipid is



In this paper we describe the phase behavior of the plasmalogen form of phosphatidylethanolamine (plasmenylethanolamine) and of the glycerol acetal of this plasmalogen.

Materials and Methods

Materials. *cis*-9-Octadecenoic and *trans*-9-octadecenoic acids were obtained from Nu-chek Prep (Elysian, MN). *cis*- and *trans*-Parinaric acids (PnA)[1] were from Molecular Probes, Inc. (Plano, TX). They were purified as described by Fraley et al. (1980) and stored as described by Sklar et al. (1977). Glass-distilled solvents used for all final purification steps were obtained from Burdick & Jackson Laboratories, Inc. (Muskegon, MI). Bio-Sil A was obtained from Bio-Rad Laboratories (Richmond, CA). *Crotalus adamanteus* venom was obtained from Sigma Chemical Co. (St. Louis, MO).

Methods. *C. butyricum* IFO 3852 was obtained from Dr. M. Matsumoto, Schizuoka College of Pharmacy, Japan. Cells were grown anaerobically with casamino acids and a fatty acid in the absence of biotin as described (Goldfine et al., 1977). Cells were harvested by centrifugation at 8000*g* for 10 min and washed twice with potassium phosphate buffer, 0.05 M, pH 7.2. All cultures were examined by phase contrast microscopy for spores. Methods for lipid extraction have been described (Goldfine & Bloch, 1961).

The ethanolamine-containing diacylphosphatides, plasmalogens, and the glycerol acetal derivative of the plasmalogen from a 20-L culture were separated from the other phospholipids, mainly phosphatidylglycerol, by elution from DEAE-cellulose with $CHCl_3$–MeOH, 7:3 (v/v) (Law & Essen, 1969). The ethanolamine diacylphosphatides and plasmalogen were separated from the glycerol acetal derivative on a 1.8 × 8 cm column of Bio-Sil A. A total of 82 mg was added to the column in $CHCl_3$. The column was eluted with 55 mL of 10:1 (v/v), 108 mL of 7:2, and 60 mL of 1:1 $CHCl_3$–MeOH and 50 mL of MeOH. The fraction eluting with $CHCl_3$–MeOH, 7:2, gave a single spot corresponding to phosphatidylethanolamine, and the glycerol acetal derivative was found in the $CHCl_3$–MeOH, 1:1, fraction. Since the fraction was slightly contaminated with phosphatidylethanolamine, it was recromatographed on Bio-Sil A. Fractions were checked for contamination by thin-layer chromatography on silica gel G with the solvent system $CHCl_3$–MeOH–7 N NH_4OH (60:35:5 v/v/v).

The phosphatidylethanolamine (PE) fraction was treated with phospholipase A_2 in order to hydrolyze the diacyl form selectively (Waku & Nakazawa, 1972). Phosphatidylethanolamine (8 mg) was shaken at 30–32 °C in a two-phase system consisting of 20 mL of ethyl ether (freshly distilled), 2 mL of Tris, 0.1 M, pH 7.4, 0.5 mL of $CaCl_2$, 0.04 M, and 350 μg of *C. adamanteus* venom. Incubation was for 90 min. The reaction was stopped by the addition of 60 mL of MeOH. The mixture was evaporated on a rotary evaporator and fractionated on a 1-g Bio-Sil A column (0.8-cm i.d.) with the following solvents: $CHCl_3$–MeOH, 8:1 (v/v), 9 mL; $CHCl_3$–MeOH, 3:1 (v/v), 12 mL; MeOH. The plasmenylethanolamine was recovered in the second fraction.

The plasmalogen content was determined in two ways. Aliquots of the lipid were hydrolyzed in 90% acetic acid overnight and chromatographed to separate the lyso-PE arising from hydrolysis of plasmenylethanolamine from the unhydrolyzed PE (Khuller & Goldfine, 1974). This gave an average of 97 ± 3% hydrolysis. Vinyl ether assay by I_2 uptake combined with phosphate analysis indicated 1.00 ± 0.06 vinyl ether/phosphorus (mole ratio).

Fatty acids were determined as their methyl esters after saponification, extraction, and methylation with diazomethane as described (Khuller & Goldfine, 1974, 1975). Alk-1-enyl chains were analyzed as the aldehydes released by hydrolysis overnight at 37 °C in 90% acetic acid (Khuller & Goldfine, 1974). Fatty acid methyl esters and aldehydes were analyzed by gas chromatography on a 10% EGSS-X on Gas Chrom P column at 180 °C (Khuller & Goldfine, 1975). Proportions of each component were determined by multiplying the peak height by the retention time (Kates, 1972). This technique was verified with commerical fatty acid methyl ester mixtures. Lipid phosphorus was determined by the method of Bartlett (1959). Vinyl ether was determined by the method of Gottfried & Rapport (1962).

Fluorometric Measurements. Routinely, 137 nmol of phospholipid stored in CS_2 was placed in a 12-mL round-bottom tube. Free PnA (0.91 nmol) was added in ethanol and mixed, and the sample was dried under N_2. The lipid was suspended by vortexing in 3.5 mL of potassium phosphate buffer, 0.01 M, pH 7.0, which was purged with nitrogen and prewarmed to ~40 °C. The tube was covered with parafilm during vortexing. Lipid suspensions were transferred to a glass cuvette, which was capped with a rubber serum stopper. The suspension was sparged with nitrogen through a syringe needle with a second needle serving as an exit valve. Fluorescence data were obtained with a Hitachi Perkin-Elmer MPF-2A spectrofluorometer equipped with an X–Y recorder, and temperature was recorded on the *x* axis. Excitation was at 320 nm, and emission was measured at 410 nm. Heating and cooling at 0.5–2.0 °C/min were controlled by an external circulating water bath. Data were digitized and plotted on a Hewlett-Packard 9825-A calculator plus plotter. Phase

[1] Abbreviations used: PnA, parinaric acid; PE, phosphatidylethanolamine; DEPE, dielaidoylphosphatidylethanolamine; PC, phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DEAE, diethylaminoethyl; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

Table I: Phospholipid Acyl and Alk-1-enyl Chain Composition (wt %) of *C. butyricum* IFO 3852[a]

	elaidate grown			oleate grown	
	total phospholipids	phosphatidyl-ethanolamine plasmalogen	glycerol acetal of ethanolamine plasmalogen	total phospholipids	glycerol acetal of ethanolamine plasmalogen
Acyl Chains					
12:1	1.2				
14:0	4.5	6.5	3.8	5.2	
16:0	2.6	3.0	1.8	3.1	nd[b]
16:1	t[c]	t	1.7		
18:1	91.1	90.6	92.6	84.9	
19:cycl				6.3	
Alk-1-enyl Chains					
16:1			0.8		
17:cycl	1.0				
18:0					2.7
18:1	99	98.8	99	67.8	91.6
19:cycl		1.2		32.2	5.7

[a] The isolation of the lipids and analysis of the chains are described under Materials and Methods. The chains are described as follows: number of carbons:number of double bonds. 17:cycl and 19:cycl are the 17-carbon and 19-carbon cyclopropane fatty acids or aldehydes. [b] Not determined. [c] Trace.

transitions were analyzed as described by Tecoma et al. (1977). Briefly, a line was drawn through the linear region of the fluorescence intensity curve at higher temperatures. The slope of this line is θ_f. With decreasing temperature the slope of log I increased abruptly. A line with slope θ_t was drawn through the transition region. A curve was drawn parallel to θ_f through or tangent to the curve at low temperature (θ_s). The intersection of θ_f with θ_t is defined as T_f, and the intersection of θ_t with θ_s is defined as T_s. Both heating and cooling curves were used to determine transition parameters for each sample. Parameters are presented as mean values, with the hysteresis indicated in parenthesis. The mid-points of the transitions are defined as $(T_f + T_s)/2$.

Differential Scanning Calorimetry (DSC). Samples (0.3–2 mg) were dried in a vacuum oven at 40 °C for 2 h, weighed, and hydrated with 5 μL of water at 50 °C for 1 h in sealed aluminum pans. The samples were heated and cooled (usually at 10 °C/min) with a reference pan containing 5 μL of water in a Perkin-Elmer DSC-2 which had been calibrated with indium, dipalmitoyl-PC, cyclohexane, and water. T_c is defined as the temperature at which extrapolation of the leading edge of the transition intersected the base line. T_m is the temperature at which maximal excess heat capacity was seen. The phospholipid content of the pans was determined by phosphorus analysis using the method of Sokoloff & Rothblat (1974). The water content of the pans was monitored by weighing. The proportion of the water which was not bound to phospholipid and could freeze was determined by measuring the heat associated with melting the ice and calculating the weight of water involved, with the known enthalpy of fusion of ice.

^{31}P Nuclear Magnetic Resonance. Spectra were recorded on a Brucker WH360/180 spectrometer operating at 145.7 MHz., which was used without proton decoupling. Accumulated free-induction decays were obtained from 6000 to 22000 transients employing an interpulse time of 0.819 s. A sample of 10 mg of lipid was suspended in 0.5 mL of ^2H_2O buffer as described by Cullis & DeKruyff (1978), except that vortexing with a glass bead for ~5 min was sufficient to produce the desired suspension.

Electron Microscopy. Lipid dispersions were negatively stained with 2% w/v sodium phosphotungstate (pH 7) on Formvar-coated 200-mesh copper grids. The grids were electrostatically charged in a Denton DV-502 vacuum evaporator prior to applying the sample (0.6 μL of a dispersion containing ~0.25 mg of lipid/mL) and the stain in order to facilitate spreading over the grid. Micrographs were then obtained by standard procedures with a Zeiss 10 transmission electron microscope operating at 80 kV.

Results

Acyl and Alk-1-enyl Chain Composition. In these studies we have used *C. butyricum* IFO 3852, a strain reported to have ethanolamine but have no *N*-methylethanolamine among its polar head groups (Matsumoto et al., 1971), which was confirmed in this laboratory (H. Goldfine and N. C. Johnston, unpublished experiments). This is in contrast to strain ATCC 6015, which has a mixture of *N*-methylethanolamine and ethanolamine phosphatides, with the former predominating (Baumann et al., 1965). Since lipids with these two polar head groups are difficult to separate on a preparative scale, use of strain IFO 3852 simplified the isolation of the pure lipid classes needed for physical measurements. We have analyzed the acyl and alk-1-enyl composition of the ethanolamine phosphatides purified from cells grown on elaidic acid or oleic acid (Table I).

The acyl chains are enriched over 90% and the alk-1-enyl chains 99% in 18:1 in elaidate-grown cells, and both chains are over 90% 18:1 plus the C_{19} cyclopropane chains derived from 18:1 in oleate-grown cells. The enrichment is slightly higher than previously seen in strain ATCC 6015, and the formation of C_{19} cyclopropane from oleate is somewhat less. Therefore, these lipids have more homogenous chains than were previously obtained. The virtual absence of C_{19} cyclopropane chains in elaidate-grown cell lipids argues that the 18:1 chains are virtually all *trans*-9-18:1, as was demonstrated earlier for strain ATCC 6015 grown on elaidate (Goldfine et al., 1977).

Phase Transitions in Total Phospholipids Detected by Free Parinaric Acids. Free *cis*- and *trans*-PnA were incorporated into liposomes as described by Tecoma et al. (1977). The results of measurements of fluorescence intensity as a function of temperature during heating and cooling scans of the total phospholipids from elaidate-grown cells are shown in Figure 1A. As previously observed with the total phospholipids from elaidate-grown ATCC 6015 cells probed with Tempo and DPH, relatively narrow transitions are seen, with midpoints at 24 °C with *cis*-PnA and 25.5 °C with *trans*-PnA. These

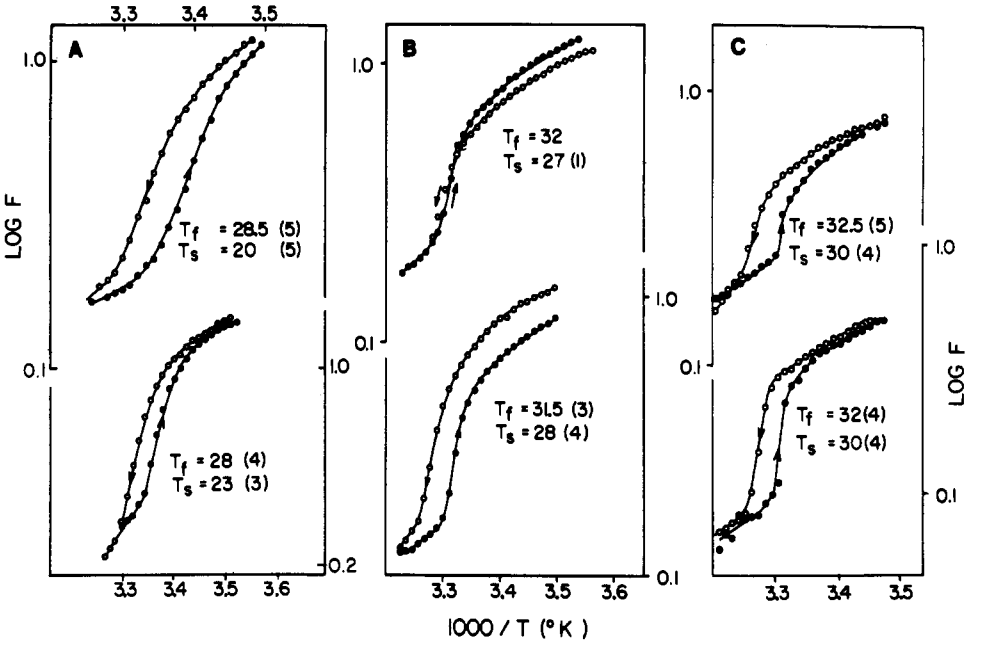


FIGURE 1: Transitions detected in elaidate-enriched *C. butyricum* phospholipids by free *cis*-PnA (top of each panel) and *trans*-PnA (bottom of each panel). The acyl and alk-1-enyl chain compositions are given in Table I. (A) Total phospholipids. The samples were prepared as described under Materials and Methods, except that 133 nmol of lipid was used with 2.5 nmol of *cis*-PnA and 266 nmol of lipid was used with 2.5 nmol of *trans*-PnA. The slit widths were 3 nm for excitation and 20 nm for emission. (B) Total phosphatidylethanolamine. The samples were prepared as described under Materials and Methods. Slit widths were as described for panel A. (C) Plasmalogen fraction of phosphatidylethanolamine (plasmenylethanolamine). The samples were prepared as described under Materials and Methods except that *trans*-PnA and lipid were twice the standard concentrations. The slit widths were 5 nm for excitation and 20 nm for emission.

Table II: Thermotropic Behavior of Phospholipids

lipid	heating		cooling		ΔH (kcal/mol)	hydration (mol of H_2O/mol of lipid)
	T_c	T_m	T_c	T_m		
dielaidoyl-PE[b]	34 ± 0.5[a,c]	38 ± 0.5[a]	37	34.5, 36.5	4–7	12[d]
dielaidoyl-PE plasmalogen	28 ± 0.2	33	28.3 ± 0.3	27	5.7	nd[e]
dielaidoyl-PE glycerol acetal						
after storate at −18 °C	28.8 ± 1.5	34.5 ± 0.8	15.8 ± 0.3	13	12	25
immediate reheating	30.0 ± 1.5[f]	33.6 ± 0.6				
oleate PE glycerol acetal[g]	6.7 ± 0.5	12	−4.1 ± 0.3	−6	9	nd

[a] T_c measured at linear extrapolation of the leading edge of the peak to the base line. T_m is the temperature at peak maximum. [b] From van Dijk et al. (1976), Jackson & Sturtevant (1977), and Yang et al. (1979). [c] Temperatures are ±SEM (N = 4–20). Other T_m values are reproducible to within ±1 °C. [d] Hydration of egg yolk phosphatidylethanolamine (Hauser & Phillips, 1979). [e] nd = not determined. [f] This value relates to a second peak appearing as a shoulder rather than an extrapolated temperature. [g] From oleate-grown cells (see Table I).

transitions are 6–7 °C higher than those seen with Tempo and diphenylhexatriene in the total phospholipids from strain ATCC 6015 (Goldfine et al., 1977). That the difference is not entirely due to to a change in probes was shown by measuring the fluorescence polarization of diphenylhexatriene as described (Goldfine et al., 1977) in liposomes prepared from phospholipids of strain IFO 3852 grown on elaidate. T_s was 16 °C and T_f was 24 °C, about 4–5 °C lower than observed with *cis*- and *trans*-PnA.

Phase Transitions in the Plasmalogen Form of Phosphatidylethanolamine (Plasmenylethanolamine). The phosphatidylethanolamine fraction from elaidate-grown cells was obtained by sequential column chromatography on DEAE-cellulose and silicic acid. The diacyl and plasmalogen forms were present in the ratio of 30:70. Figure 1B shows that narrow transitions were observed with both *cis*- and *trans*-PnA with midpoints at 29.5 and 30 °C, respectively. Some hysteresis was seen with *trans*-PnA, which partitions preferentially into the solid phase (Tecoma et al., 1977; Sklar et al., 1979).

The elaidate-enriched plasmenylethanolamine was obtained by selective deacylation of the diacyl lipid with *C. adamanteus* phospholipase A_2. Sharp transitions centered at 31 °C were

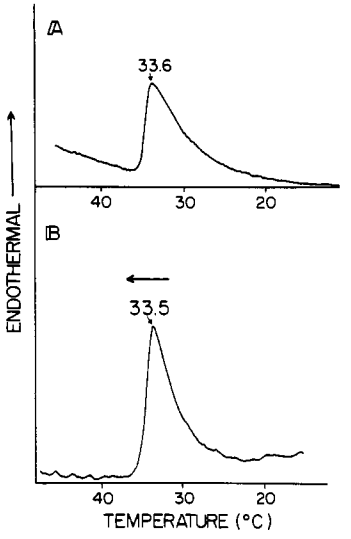


FIGURE 2: DSC heating curves for total phosphatidylethanolamine fraction (A) and plasmenylethanolamine (B) from *C. butyricum* enriched in elaidate. See Materials and Methods for sample preparation. Scanning rates were (A) 5 °C/min and (B) 10 °C/min.

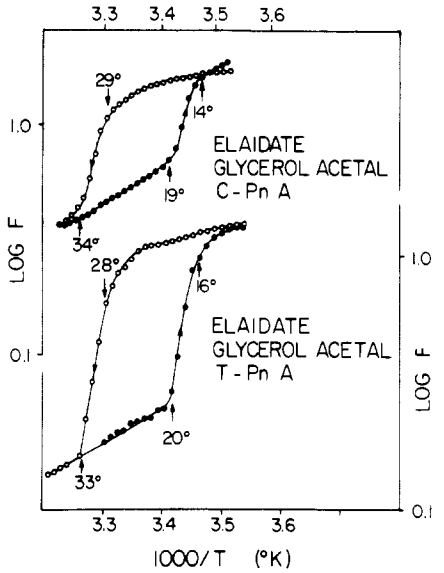


FIGURE 3: Transitions detected by free *cis*-PnA (top and *trans*-PnA (bottom) in the glycerol acetal of plasmenylethanolamine from *C. butyricum* enriched in elaidate. The composition is given in Table I. The samples were prepared as described under Materials and Methods. Slit widths were as described in the legend to Figure 1.

observed with both *cis*- and *trans*-PnA (Figure 1C).

Differential scanning calorimetry traces of the total phosphatidylethanolamine fraction and the plasmalogen form of phosphatidylethanolamine are shown in Figure 2. T_c for the former was 27.2 ± 0.1 °C and for the latter 28 ± 0.2 °C; no further transitions were observed on heating the total phosphatidylethanolamine to 70 °C. Table II summarizes the calorimetric data obtained in heating and cooling scans of these lipids. The enthalpy for chain melting of both the mixed and plasmalogen form of phosphatidylethanolamine is within the range previously reported for dielaidoyl-PE (DEPE) (van Dijk et al., 1976; Jackson & Sturtevant, 1977; Yang et al., 1979). The hydration of the mixed fraction is also close to that previously reported for phosphatidylethanolamine from egg yolk (Hauser & Phillips, 1979).

Phase Transitions in Glycerol Acetal of Plasmenylethanolamine. When the elaidate-enriched glycerol acetal lipid was probed with *cis*- and *trans*-PnA, an unusually large hysteresis was observed (Figure 3). The average separation of the transitions between heating and cooling scans was 12.5 °C. These scans were performed at 0.5–1.0 °C/min. That this phenomenon was not strongly dependent on the scanning rate was shown in two ways. In experiments with *cis*-PnA the sample was heated from 4 to 26 °C at 0.5 °C/min. The temperature was held at 26 °C for 32 min with only a 14% decrease in fluorescence intensity, whereupon heating above 28 °C resulted in a sharp decrease in fluorescence intensity. In a similar experiment a sample was cooled slowly and held at 20.5 °C for 0.5 h. No increase in fluorescence intensity was observed until the sample was cooled below 19 °C, after which time a rapid increase was observed. The results obtained upon differential scanning calorimetry showed small changes in the degree of hysteresis, depending on the scanning rate. As shown in Figure 4C and Table II, after storage at −18 °C overnight, on being scanned at 10 °C/min, the (elaidate) glycerol acetal lipid melted at 34.5 ± 0.8 °C (T_m) and became solid at 13 °C (Figure 4D). At slow scan rates, transition to the solid phase occurred at somewhat higher temperatures, for example, T_m = 18 °C at a scan rate of 0.62 °C/min, which agrees well with the fluorescence intensity data obtained at comparable scan rates. When the lipid was immediately re-

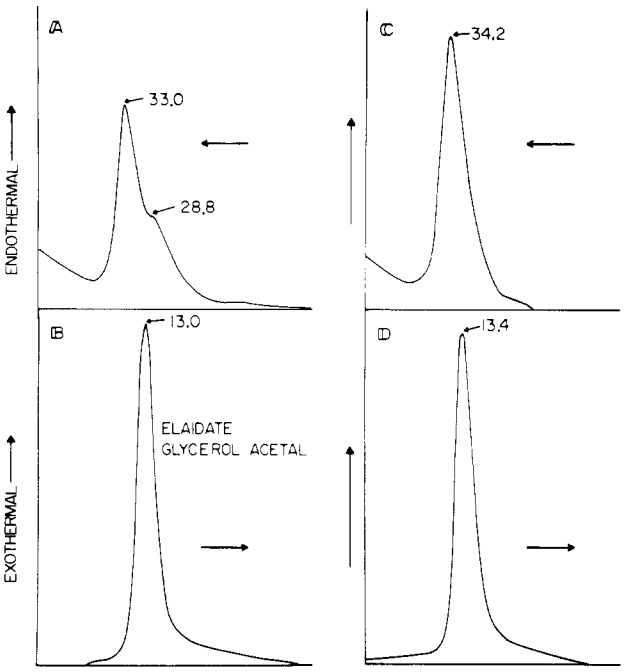


FIGURE 4: DSC curves for the glycerol acetal of plasmenylethanolamine from *C. butyricum* enriched in elaidate. (A) Heating, immediately after cooling, 10 °C/min. (B) Cooling curve obtained immediately after the heating scan shown in (A), 10 °C/min. (C) Heating after storage overnight at −18 °C, 10 °C/min. (D) Cooling curve obtained after the heating scan shown in (C), 10 °C/min. Arrows indicate the direction of the scan.

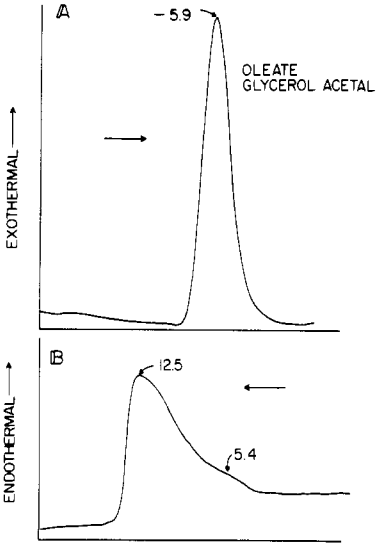


FIGURE 5: DSC curves for the glycerol acetal of plasmenylethanolamine from *C. butyricum* enriched in oleate. See Materials and Methods for sample preparation. (A) Cooling, 5 °C/min. (B) Heating, 5 °C/min. Arrows indicate the direction of the scan.

heated, a shoulder at 30 ± 1.5 °C was observed on the main endotherm at 33.6 ± 0.6 °C (Figure 4A). Differential scanning calorimetry of the glycerol acetal lipid from oleate-grown cells revealed a similarly broad hysteresis (Figure 5, Table II). Transitions were observed at 12 °C on heating and at −6 °C on cooling.

The elaidate-enriched glycerol acetal lipid gave an enthalpy change at the 33 °C endotherm about twice as large as that of the plasmalogen and that reported for DEPE (Table II). The ΔH for the glycerol acetal lipid from cells grown on oleic acid (9 kcal/mol) was also twice as large as that reported for dioleoyl-PE (4.5 kcal/mol) (van Dijk et al., 1976). The glycerol acetal lipid from elaidate-grown cells also had a large

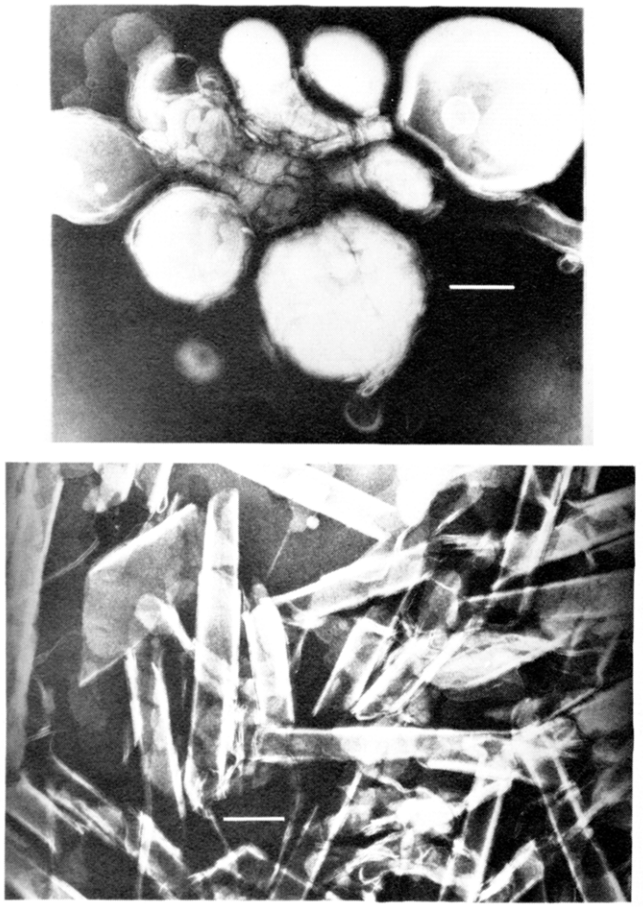


FIGURE 6: Electron micrographs of aqueous dispersions of the glycerol acetal of plasmenylethanolamine from *C. butyricum* enriched in elaidate, above and below the chain-melting transition. (A, upper) Phospholipid was dispersed in water by low-power sonication at 37 °C and negatively stained at the same temperature. (B, lower) The dispersion was prepared as described in (A) except that the sample was held at 4 °C for several hours before staining at that temperature.

hydration value compared to PE from egg yolk (Hauser & Phillips, 1979) and the PE fraction from *C. butyricum* (Table II).

^{31}P Nuclear Magnetic Resonance. The ^{31}P NMR spectra provided evidence for hysteresis in the polar head group motion. As shown by Cullis & deKruyff (1976), the width at half-height ($\Delta\nu_{1/2}$) of the 129-MHz ^{31}P NMR spectra for dimyristoylphosphatidylcholine (DMPC) increases from 12 ppm above its phase transition temperature to 49 ppm below its phase transition. With our spectrometer settings and above T_m, $\Delta\nu_{1/2}$ of the glycerol acetal was 9.1 ppm. On lowering the temperature to 23 °C, $\Delta\nu_{1/2}$ only increased slightly, to 11.7 ppm. Below T_m (9 °C) $\Delta\nu_{1/2}$ was 21.9 ppm, and raising the temperature to 23 °C resulted in little change (20.6 ppm). Thus, the motion of the phosphorus atom also exhibits the hysteresis shown in the chain-melting transition so that the width at half-height at 23 °C depends on the prior state of the lipid. It is also of interest that the width at half-height at 9 °C was less than one-half that seen under similar spectral conditions with solid-state DMPC or DMPE (Cullis & de-Kruyff, 1976), indicating that the motion of the phosphate group is relatively unrestricted below the transition temperature in the glycerol acetal system.

Electron Microscopy. Dispersions of the elaidate-enriched glycerol acetal stained at 20 °C showed typical multilamellar phospholipid liposomes (Figure 6A). When dispersions were cooled below the transition temperature to 4 °C, a marked reorganization was seen (Figure 6B) in which large needle-like or tubular structures were the predominant form. These appeared to be bounded by phospholipid bilayers.

Phase Behavior of Mixtures of Phosphatidylethanolamine and Glycerol Acetal Lipid. When the mixed (diacyl plus plasmalogen) phosphatidylethanolamine fraction from elaidate-grown *C. butyricum* was added to the elaidate-enriched glycerol acetal lipid there was a progressive decrease in the hysteresis (Figure 7). Even at 25% glycerol acetal and 75% PE there was greater hysteresis than in the PE alone, suggesting that the level of the glycerol acetal lipid in elaidate-grown cells could affect the phase behavior of the membranes.

Discussion

The thermotropic transition of the elaidate-enriched plasmalogen form of phosphatidylethanolamine observed by differential scanning calorimetry is compared in Table II with the data obtained by several laboratories with synthetic dielaidoylphosphatidylethanolamine (DEPE). The gel to liquid-crystalline transition temperatures seen with the plasmalogen were 6 ± 1 °C lower. Comparison of the phase transitions observed with *cis*- and *trans*-parinaric acid for the plasmalogen with those obtained for DEPE (Yang et al., 1979) also reveal a 5–6 °C difference. Since the plasmalogen from elaidate-grown *C. butyricum* had 9.5% of 14:0 plus 16:0 acyl chains, the effect of this heterogeneity needs to be considered.

Several laboratories have examined the phase behavior of *Escherichia coli* phospholipids obtained from unsaturated fatty acid auxotrophs grown on elaidate. Overath & Trauble (1973) examined PE from strain K1062 which had 65.1% *trans*-18:1, 16.6% 14:0, 12% 16:0, and smaller amounts of 12:0 and

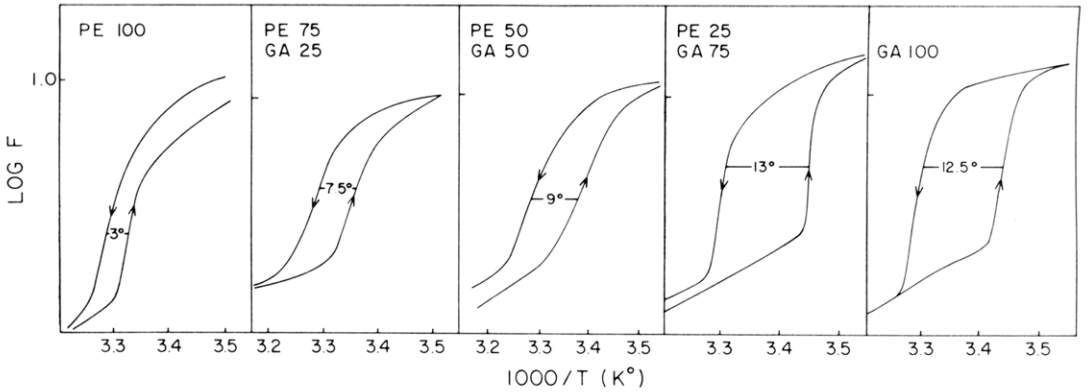


FIGURE 7: Transitions detected with *trans*-PnA in mixtures of the glycerol acetal of plasmenylethanolamine and the total phosphatidylethanolamine fraction, both derived from *C. butyricum* enriched in elaidate. The quantities of each (in micrograms) are indicated on the figure. The samples were prepared as described under Materials and Methods. Slit widths were as described in the legend to Figure 1A.

trans-16:1. Using 90° light scattering, they observed a thermal transition at 37–39 °C. Since the total phospholipids isolated from these cells also exhibited a transition at 38 °C, they concluded that the phosphatidylethanolamine fraction largely determined the transition temperature. In a similar study with *trans*-PnA, Tecoma et al. (1977) observed a thermal transition at 39 °C in *E. coli 30Eβox* phospholipids enriched 86% in *trans*-18:1. Jackson & Sturtevant (1977), using differential scanning calorimetry of elaidate-enriched *E. coli* lipids, observed an asymmetric transition with a gradual onset from 32 °C and an abrupt end at 40 °C (T_m = 39 °C). Finally, X-ray diffraction of elaidate-enriched *E. coli* lipids revealed a broader transition from 29 to 40 °C (T_t = 35 °C) (Shechter et al., 1974). Thus, all measurements obtained with lipids from *E. coli* enriched from 65% to 86% with *trans*-18:1 produced higher transition temperatures than those we have observed with the more homogeneous elaidate-enriched PE plasmalogen. We conclude that the lower melting temperature of the plasmalogen represents a real difference.

The decrease in T_c of 6 °C for the dielaidoylplasmalogen bilayer compared to phosphatidylethanolamine must arise from the combined perturbation in intermolecular interactions from both the cis double bond and the ether link of the 1-*O*-alk-1′-enyl group. The occurrence of a double bond near the end of both 18-carbon chains would be expected to decrease T_c of a phosphatidylcholine bilayer by 15 °C and less than this when the unsaturation occurs only in one chain (Barton & Gunstone, 1975); a qualitatively similar effect might be expected for a phosphatidylethanolamine bilayer. The ether bond probably has a relatively small effect in the alteration of T_c in the plasmalogen because replacement of acyl chains by alkyl chains in phospholipid monolayers does not affect the average molecular packing significantly and has only a minor effect on the phase transition (Paltauf et al., 1971). The perturbation in bilayers is also small because the T_c values for acyl and alkyl phospholipids are similar. Thus Vaughan & Keough (1974) found that dihexadecyl (di-*O*-alkyl ether) forms of phosphatidylethanolamine, phosphatidylcholine, and *N*-mono- and *N*,*N*′-dimethylethanolamine phospholipids displayed T_c values 2–5 °C higher than the corresponding diester phospholipids. With different hydrocarbon chains substituted in the 1 and 2 positions, a reduction of 1.5 °C in T_c was seen when the 1-*O*-acyl bond in 1-palmitoyl-2-oleoylphosphatidylcholine was replaced with a 1-*O*-alkyl bond (Lee & Fitzgerald, 1980). In summary, it seems that the observed decrease in T_c of a plasmalogen is probably due primarily to the presence of the double bond.

Examination of the phase behavior of the elaidate-enriched glycerol acetal derivative of plasmenylethanolamine with the parinaric acid probes and by differential scanning calorimetry revealed an unusually large hysteresis. The lipid melts at 31 ± 1 °C as determined with the fluorescent probes and at 34 °C by calorimetry. These temperatures are very close to those obtained with the plasmalogen, suggesting that the two elaidoyl chains are arranged in a similar manner in the glycerol acetal lipid and that van der Waals interactions between the hydrocarbon chains are dominant in determining the melting temperature. Therefore, the large decrease in the temperature of the transition to the gel can be attributed to supercooling, presumably due to the presence of a second polar group in the polar head group region. Steric hindrance between the acetal-linked glycerols or between the acetal-linked glycerols and the phosphorylethanolamine head groups of neighboring molecules may inhibit crystallization by preventing the necessary close approximation of the hydrocarbon chains while the lipid is supercooled. The acetal-linked glycerols are highly hydrated, since the number of water molecules bound to this lipid is twice that of PE or the plasmalogen. Consistent with this higher degree of hydration and spacing in the polar group region, the phosphate group motion is less restricted in the glycerol acetal than in bilayers of a simple PE below T_c.

Besides causing hysteresis of T_c, addition of the second glycerol group to the PE plasmalogen to form the acetal leads to a doubling in the enthalpy (ΔH) of the chain-melting transition which may arise from a change in either the gel or liquid-crystalline states, or both. Since a simple PE such as egg PE and the glycerol acetal lipid (Figure 6) form multilamellar liposomes above T_c, the liquid-crystalline states of both lipids are probably similar. It is likely that variations in the gel states give rise to the differences in ΔH. These may be associated with the altered morphology when the glycerol acetal lipid is cooled below T_c (Figure 6). The magnitude of ΔH is proportional to the number of carbon–carbon bonds about which trans–gauche isomerizations occur (Phillips, 1972), and the enhanced ΔH of the glycerol acetal compared to the plasmalogen implies that its gel phase is more ordered. If the hexagonally packed hydrocarbon chains in the gel phase become tilted with respect to the bilayer plane [i.e., a polymorphic transition from β to β' occurs; see Tardieu et al. (1973) for a definition of terms], then ΔH per methylene group should increase by about one-third (Phillips et al., 1969). This transition is likely to occur on changing from PE to the glycerol acetal because increasing the polar group size of a phospholipid (for a given chain length) favors the β' polymorph (Hauser & Phillips, 1979). The remaining two-thirds of the increase in ΔH of the glycerol acetal could be due to a reduction in the number of gauche conformers below T_c. Yellin & Levin (1977) have found an effective enthalpy difference of ~1.7 kcal/mol between the trans and gauche conformers about a carbon–carbon bond and that there are two gauche bonds per molecule in hydrated dipalmitoyl PC gels. The absence of these gauche conformers in the glycerol acetal below T_c would account for the remaining increment in ΔH in this system (Table II). Although these highly ordered gel phases have not been observed with PC and PE, modified hydrocarbon chain packing and more stable gel phases have been observed recently with sphingomyelin (Estep et al., 1980) and cerebrosides (Bunow, 1979). As we find with the glycerol acetals, the liquid crystal in these systems supercools and converts to a metastable gel phase ~16 °C below the T_c observed on heating; this gives rise to the hysteresis observed with the glycerol acetal but not the plasmalogen. On storage at low temperature the metastable gel transforms to a more stable form with a higher temperature and enthalpy of melting. For steric reasons, the second glycerol group in the glycerol acetals must hinder the close-packing of the hydrocarbon chains during cooling, but on storage at low temperature for several hours there is a slow change in packing during which the hydrocarbon chains adopt a more nearly perfect all-trans planar configuration. There is no change in the number of water molecules bound to the glycerol acetal molecules during this process. The purity of the lipid, as monitored by thin-layer chromatography, does not alter detectably during these steps, but the heterogeneity of the hydrocarbon chain distribution (Table I) may play some role in facilitating the transition to the more ordered gel phase [cf. Estep et al. (1980)].

The thermotropic behavior of the oleate glycerol acetal parallels that of the elaidate glycerol acetal in that the degree of hysteresis of T_c on heating and cooling is similar in both systems and ΔH is approximately twice that of the equivalent

PE. However, in contrast to bilayers of elaidate glycerol acetal, which require cooling 18 °C below the T_c of dielaidoyl-PE before the hydrocarbon chains crystallize, bilayers of the oleate glycerol acetal form the gel phase on cooling at −4 °C (Table II), which is 10 °C higher than the T_c = −14 °C of dioleoyl-PE (van Dijck et al., 1976). This interesting anomaly is due to the presence of the two cis-unsaturated chains which gives rise to different bilayer melting behavior compared to saturated or trans-unsaturated systems. Thus, bilayers of disaturated PE or dielaidoyl-PE have T_c values ~20 °C higher than the equivalent PC, whereas bilayers of dioleoyl- or dipalmitoleoyl-PE have T_c values within 3 °C of the equivalent PC. Van Dijck et al. (1976) have explained this difference, and a reduction in ΔH, in terms of a looser packing induced by the presence of two cis-unsaturated chains so that the greater intermolecular attraction between the PE polar groups relative to PC (Hauser & Phillips, 1979) is removed. Since addition of the second glycerol group in oleate glycerol acetal facilitates formation of the gel phase on cooling compared to dioleoyl-PE, the acetal group must provide some extra intermolecular attraction to offset the looser packing induced by the two cis-unsaturated chains. This effect probably involves some hydrogen bonding mediated by the second glycerol group. This observation is of particular interest since the glycerol acetal lipid increases from ~25% of the total phospholipid in biotin-grown and elaidate-grown *C. butyricum* to nearly 50% in oleate-grown cells (Khuller & Goldfine, 1975). Additional glycerol acetal lipid may serve to offset the marked decrease in T_c in the membranes of these cells which would otherwise occur if the PE was simply enriched in monounsaturated and cyclopropane fatty acids. In *E. coli* fatty acid auxotrophs an increase in unsaturated acyl chains above ~85% at 37 °C leads to slowing of growth and increased passive permeability of the membranes to small molecules (Davis & Silbert, 1974).

Apart from these harmful effects due to formation of a too loosely packed bilayer, excessive crystallization of the phospholipid molecules would also cause failure of membrane function, so it is apparent that membrane fluidity has to be maintained within some limits. The limits seem to be fairly wide for *C. butyricum* because elaidate- and oleate-enriched membranes have very different thermotropic behavior. Thus, heating a pellet of membranes from elaidate-enriched strain IFO 3852 reveals a single phospholipid melting transtion between 23 and 43 °C, with T_m at 33 °C. On cooling the transition occurs in the range 30–17 °C with T_m at 25 °C. Oleate-enriched membranes exhibit five endothermic transitions in the range from −7 to 36 °C on heating and between 15 and about −20 °C on cooling (unpublished results). Therefore, it seems that although the occurrence of glycerol acetals in the membranes may prevent excessive changes in fluidity, it is not involved in maintaining T_c within narrow limits.

Further work utilizing X-ray diffraction and Raman spectroscopy will be needed in order to understand the structure of the gel state of the glycerol acetal more fully. This unusual lipid has so far been reported in only two strains of *C. butyricum*. We have recently begun to examine other clostridia closely related to *C. butyricum* (Cummins & Johnson, 1971) and have observed an acid-labile phospholipid of similar mobility on thin-layer chromatography in several of these strains.

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Further Studies of the Riboflavin-Binding Immunoglobulin IgG^{Gar}. Resolution into Fractions of Different Riboflavin Content and Aspects of Reassembly†

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ABSTRACT: A previously described human immunoglobulin with unusual flavin-binding activity, IgG^{Gar} [Farhangi, M., & Osserman, E. F. (1976) *N. Engl. J. Med. 294*, 177], is further characterized. The protein can be fractionated into two subpopulations, one of which is nearly completely saturated with riboflavin and one in which the binding sites are largely vacant. Possible differences between these fractions and/or their binding sites are explored. While electrophoretically distinct, the IgG^{Gar}–riboflavin complexes possess a basic similarity in the binding sites of both fractions as evidenced by spectroscopic examination. However, an important difference exists in that added riboflavin equilibrates reversibly with the vacant sites of native IgG^{Gar}, while the riboflavin in the occupied sites is essentially irreversibly bound. The tight association may be due to an in vivo combination of riboflavin with protein of different conformation than occurs in vitro, such as an incompletely assembled or folded tetramer. Accordingly, in vitro renaturation was examined. Studies of renaturation revealed that the reduced interchain disulfides within a tetramer reoxidize smoothly, although inter-heavy-chain bonds form less readily than inter-heavy–light-chain disulfides. Renaturation of IgG^{Gar}, unlike previously studied IgG molecules, does not proceed under conditions in which the protein structure had previously been significantly disrupted. The assembly defect is localized in the inability of the denatured heavy chain to refold into a stable species capable of combining with the light chain.

Several years ago, Farhangi & Osserman (1976) discovered and characterized an unusual human immunoglobulin. The protein IgG^{Gar} was isolated from a patient with multiple myeloma and shown to be an IgG2, with λ light chains. This immunoglobulin was remarkable because it occurred in the serum with bound hapten, riboflavin, which remained tightly associated throughout standard isolation and purification procedures. The average occupancy of antigen binding sites in the native protein was about 1.5 equiv of riboflavin/mol of IgG^{Gar} in the preparations Farhangi and Osserman studied.

Among both murine and human monoclonal antibodies described until then, IgG^{Gar} was unique in being the only documented example of an antibody–hapten system in which the hapten was associated with the protein in vivo as well as in the isolated, purified antibody. However, as the authors noted, the discovery was strongly favored by the fact that the hapten is a chromophore, with a bright color and a characteristic visible spectrum. Accordingly, they suggested the possibility of instances of colorless haptens bound to circulating myeloma and normal immunoglobulins.

In addition to identifying the chromphore as riboflavin, Farhangi and Osserman localized the binding site to the Fab fragment and demonstrated that additional riboflavin was bound to unoccupied sites up to a saturating level of 2 equiv/mol. Moreover, although riboflavin was the exclusive chromophore on the native protein, the vacant sites bound

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