Deuterium Nuclear Magnetic Resonance Studies on the Plasmalogens and the Glycerol Acetals of Plasmalogens of *Clostridium butyricum* and *Clostridium beijerinckii*†

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Received December 1, 1986; Revised Manuscript Received April 16, 1987

ABSTRACT: Deuterium nuclear magnetic resonance was used to investigate the structure of different lipid fractions isolated from the anaerobic bacteria *Clostridium butyricum* and *Clostridium beijerinckii*. The fractions isolated from *C. butyricum* were (1) phosphatidylethanolamine/plasmenylethanolamine and (2) the glycerol acetal of plasmenylethanolamine, and from *C. beijerinckii* similar fractions containing principally (1) phosphatidyl-*N*-monomethylethanolamine, along with its plasmalogen, and (2) the glycerol acetal of this plasmalogen were isolated. The third fraction from both species consisted largely of the acidic lipids phosphatidylglycerol and cardiolipin along with plasmalogen forms of these lipids. Palmitic acid with deuterium labels at C-2, C-3, or C-4 or oleic acid with deuterium labels at C-2 and C-9,10 was added to the growth medium and incorporated to various extents in the lipid fractions. Biochemical analysis showed that palmitic acid and oleic acid were preferentially bound to the *sn*-2 and *sn*-1 positions, respectively, of the glycerol backbone when both fatty acids were added to the medium. From the ^2H NMR spectra, the hydrocarbon chain ordering near the lipid–water interface could be determined and appeared to be similar for all three lipid fractions. The deuterium quadrupole splitting and order parameter were low at the C-2 segment and increased by almost a factor of 2 at positions C-3 and C-4 for cells fed with deuteriated palmitic acid along with unlabeled oleic acid. These results agree with previous findings on pure diacyl lipids in which the *sn*-2 chain was found to adopt a bent conformation at the carbon segment C-2. However, two unusual quadrupole splittings could be detected for the plasmalogens. First, a quadrupole splitting of 25 kHz was observed at the C-2 position of the *sn*-2 chain of plasmenylethanolamine, which suggests that the *sn*-2 chain of this lipid is perpendicular to the bilayer surface at all chain segments. Second, a quadrupole splitting of 8 kHz was detected for the C-2 segment of deuteriated oleic acid when bonded at the *sn*-1 chain in a vinyl ether linkage. By comparison with other model systems it could be concluded that the double bond is aligned essentially parallel with the long axis of the hydrocarbon chains. The glycerol acetal *sn*-1 alkyl chain C-2 segment produced an unusually wide quadrupolar splitting of 30.2 kHz.

Plasmalogens, 1-(alk-1-enyl)-2-acylphosphoglycerides, are frequently present in biological membranes together with diacylphosphoglycerides. The proportion of plasmalogens varies widely, from a few percent of the total phospholipid in mammalian liver, erythrocytes, and fibroblasts to 20–40% in such tissues as brain and heart. Many invertebrates are also rich in plasmalogens (Horrocks & Sharma, 1982). Among prokaryotes, plasmalogens have been found almost exclusively in obligately anaerobic bacteria, in which they represent from 12 to 80% of the total polar lipid (Johnston & Goldfine, 1983; Goldfine, 1984). Although there have been numerous studies on the chemical and physical behavior of these lipids, their specific functions in biological membranes are not well understood (Paltauf, 1983). *Clostridium butyricum* membranes contain substantial amounts of the plasmalogen forms of phosphatidylethanolamine (plasmenylethanolamine), phosphatidylglycerol (plasmenylglycerol), and cardiolipin. In addition to these lipids, *C. butyricum* contains the glycerol acetal of plasmenylethanolamine, 1-(1-glyceroalkyl)-2-acyl-*sn*-glycero-3-phosphoethanolamine (see Figure 6 for structures). In *Clostridium beijerinckii* the major base of the plasmalogen and the glycerol acetal is *N*-monomethylethanolamine. The specific functions of these molecules have been the subject of several studies (Goldfine et al., 1981, 1987; Johnston & Goldfine, 1985). Although the thermotropic phase behaviors of plasmalogens and their glycerol acetals have been studied (Goldfine et al., 1981, 1987; Boggs et al., 1981; Lohner et al., 1984), little is known about their conformation and dynamics in membranes.

Two of the butyric acid producing clostridia, *C. beijerinckii* and *C. butyricum*, require fatty acids when grown in the absence of biotin (Broquist & Snell, 1951; Goldfine et al., 1981), and the cellular phospholipids are highly enriched with the fed fatty acids under these conditions (Khuller & Goldfine, 1975; Goldfine et al., 1981; Johnston & Goldfine, 1985). We have taken advantage of these properties in order to incorporate deuterium-labeled fatty acids into the ether and diacyl lipids of clostridial membranes. Deuterium magnetic resonance spectroscopy was used to investigate the conformation and chain dynamics of plasmalogens and their glycerol acetal derivatives in artificial and biological membranes. The deuterium probe is nonperturbing and has revealed important information on diacyl polar lipids in membranes (Seelig, 1977; Seelig & Seelig, 1980). The results of our studies with ar-

† This investigation was supported by U.S. Public Health Service Grant AI-08903 (H.G.) and by Swiss National Science Foundation Grant 3.014.84 (J.S.). During part of this work, H.G. was a Senior International Fellow of the Fogarty International Center of the National Institutes of Health (F06 TW00884).

0006-2960/87/0426-5826$01.50/0

tificial membranes are presented here. Studies on whole cells and isolated membranes will be presented elsewhere.

Materials and Methods

Deuteriated fatty acids were prepared as described previously (Seelig & Seelig, 1974; Seelig & Waespe-Šarčevič, 1978).

C. beijerinckii ATCC 6015 and *C. butyricum* ATCC 19398 were maintained on the casamino acid–biotin–glucose medium of Broquist and Snell (1951). They were then subcultured a minimum of 3 times on the vitamin-free medium described elsewhere (Khuller & Goldfine, 1975; Goldfine et al., 1981). The source of the vitamin-free casein hydrolysate (ICN-Nutritional Biochemicals, Cleveland, OH) is important for the suppression of endogenous fatty acid synthesis. The media were supplemented with fatty acids dissolved as 5% solutions in ethanol to give a final total concentration of 20 mg/L. Fatty acids were usually added after sterilization of the media by autoclaving.

Cells were harvested by centrifugation at 3500*g* for 7 min and were washed twice with potassium phosphate buffer, 0.05 M, pH 7.2. Larger cultures, 10–40 L, which were needed for the isolation of individual lipids labeled with deuterium, were first concentrated on a Millipore (Bedford, MA) Pellicon cassette system. All cultures were examined by phase-contrast microscopy for contamination and the presence of spores. Methods for lipid extraction with chloroform–methanol, 2:1, have been described (Goldfine & Bloch, 1961). Phospholipids were separated from neutral and glycolipids by acetone precipitation (Kates, 1972), and the phospholipids were purified by column chromatography essentially as described (Goldfine et al., 1981). The combined phosphatidylglycerol and cardiolipin fraction was eluted from DEAE-cellulose columns with chloroform–methanol–concentrated NH_4OH, 4:1:0.1, containing 0.38% ammonium acetate.

The acyl and alkenyl chain compositions of the total phospholipids and of the individual fractions were determined as described (Johnston & Goldfine, 1983). The distribution of fatty acids in the *sn*-1 and *sn*-2 chains of diacylphosphatidylethanolamine was determined by phospholipase A_2 digestion. Eight milligrams of sample was first hydrolyzed in 90% acetic acid at 37 °C overnight (Johnston & Goldfine, 1983). The acetic acid was removed by lyophilization, and the products of hydrolysis were separated by chromatography on silicic acid. Aldehydes were eluted with 4 column volumes of chloroform followed by 1 volume of 2% methanol in chloroform. The diacylphospholipids were then eluted by 5 column volumes of 20% methanol in chloroform, and the lysolipids arising from the plasmalogens were eluted by 4 column volumes of chloroform–methanol, 1:2 v/v. The diacyl-PE[1] was dissolved in 3 mL of freshly redistilled ether, 0.1 mL of a solution of bee venom, 1 mg/mL (Sigma, St. Louis, MO), was added (Thompson, 1969), and the mixture was shaken at 30 °C for 50 min. After addition of 3 mL of methanol and evaporation to dryness, the products were separated on silicic acid columns, 0.2 g. The free fatty acids were eluted with chloroform–methanol, 3:1 v/v, and the lysolipids were eluted as described above. Alkaline methanolysis of the two samples of lyso-PE was by the method described by Kates (1972). After methylation of the free fatty acids with diazomethane, the methyl esters were purified by preparative thin-layer chromatography on Absorbosil +1 Prekotes (Applied Science, State College, PA) in petroleum ether–ether–acetic acid, 90:10:1 v/v/v. Phosphorus was measured by the method of Bartlett (1959). Vinyl ether was measured by a modification of the procedure of Gottfried and Rapport (1962) in which all volumes were reduced by 2.5-fold.

For deuterium NMR measurements of lipids, 5–50 mg of the desired lipid was dissolved in methylene dichloride–methanol, 2:1 v/v, and transferred to a NMR sample tube. After the solvent had been removed under a stream of nitrogen, the sample was placed under high vacuum for at least 48 h until no further change in weight was observed. The dry lipid was resuspended in either pure deuterium-depleted water or 10 mM potassium phosphate buffer made with deuterium-depleted water, pH 7.0, containing 5 mM EDTA. The final water content was adjusted to approximately 50% (w/w). To obtain hydrated dispersions, the sample was repeatedly subjected to vortex mixing under nitrogen with several freeze–thaw cycles. All NMR measurements were made with a Bruker Spectrospin CXP-300 spectrometer operating at 46.1 MHz and using a quadrupole echo sequence. The pulse width for a 90° pulse was 3.6 μs, the spectral width was 100 kHz, and the recycle time was 0.25 s. Typical measuring times were of the order of 1 h/spectrum for the purified lipids.

NMR spectra were simulated by a program provided by Dr. K. R. Jeffrey. In this program an angular independent intrinsic line width defined by T_2 is assumed, which is used to simulate the corresponding free induction decay signal (FID). The FID is then Fourier transformed and the corresponding line-shape function calculated (Siminovitch et al., 1984).

Results

Acyl and Alk-1-enyl Chain Compositions of Deuterium-Labeled Lipids. In order to properly assign the NMR spectra of deuteriated lipids, it was necessary to study the extent of incorporation of fed fatty acids into the acyl chains, the alk-1-enyl chains of plasmalogens, and the alkyl chains of their glycerol acetals and to measure the distribution of fatty acids in the *sn*-1 and *sn*-2 chains of the diacyl lipids and plasmalogens. We had previously shown that incorporation of unlabeled palmitic and oleic acids into the acyl and alkenyl chains of *C. butyricum* and *C. beijerinckii* differs somewhat in that a larger percentage of the saturated chains were found in the alkenyl moieties in the latter organism. Similar results have been obtained in the present experiments when deuterium-labeled palmitate and unlabeled oleate were fed to the two organisms in the absence of biotin (Table I). In general it can be seen that both organisms incorporate unsaturated moieties preferentially into the *sn*-1 acyl, alkenyl, and alkyl chains and saturated fatty acids into the *sn*-2 chains as previously shown for *C. beijerinckii* and endogenously synthesized acyl chains (Hildebrand & Law, 1964; Khuller & Goldfine, 1974). The main difference in the lipid compositions of the two species is the presence of *N*-monomethylethanolamine in *C. beijerinckii* and ethanolamine in *C. butyricum* as the major amino-containing bases (Johnston & Goldfine, 1983).

^2H NMR of Lipids from Clostridia Grown on [2,2-$^2H_2$]-Palmitic Acid. The ^2H NMR spectra of the purified lipid fractions of *C. butyricum* and *C. beijerinckii* grown on [2,2-$^2H_2$]palmitic acid–oleic acid (60:40 w/w) are shown in Figure 1. The glycerol acetal of plasmenylethanolamine from *C. butyricum* is a single chemical species, and most of the deuteriated palmitate is in the acyl chains at the *sn*-2 position (Table I). Two closely spaced resonances of 18.4 and 20.9 kHz could be resolved for this lipid, which are assigned to the

[1] Abbreviations: DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; FID, free induction decay; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PME, phosphatidyl-*N*-methylethanolamine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine.

Table I: Principal Aliphatic Chains (wt %) of Lipids Isolated from *C. butyricum* and *C. beijerinckii* Grown on Deuteriated Fatty Acids

		C. butyricum											
		[2,2-$^2H_2$]16:0/18:1 (60:40)				[2,2-$^2H_2$]18:1/16:0 (40:60)				[9,10-$^2H_2$]18:1			
fraction	chain	plas[a] (%)	16:0[b]	18:1	19:cyc	plas (%)	16:0	18:1	19:cyc	plas (%)	16:0	18:1	19:cyc
total PE	alkenyl	50.4	14.5	29.9	55.6	55.7	22.5	33.5	39.4	70	t[c]	36.8	63.2
	acyl		73.2	18.4	8.4		82.0	9.0	6.5		t	89.3	8.1
glycerol acetal	*sn*-1		5.7	84.4	10.0		4.4	89.3	6.3		t	93.8	6.2
	acyl		81.6	18.4			86.8	11.5	t		t	97.6	t
PG + Cl[e]	alkenyl	ND[d]	6.9	33.8	59.2	27.3	4.0	39.4	54.9	ND	ND	ND	ND
	acyl		60.6	23.4	16.0		54.4	18.8	24.1		t	78.0	18.5

		C. butyricum							
		[3,3-$^2H_2$]16:0/18:1 (50:50)				[4,4-$^2H_2$]16:0/18:1 (50:50)			
fraction	chain	plas (%)	16:0	18:1	19:cyc	plas (%)	16:0	18:1	19:cyc
diacyl-PE	*sn*-1		29.2	36.0	34.5		21.3	51.5	18.3
	sn-2		74.0	20.1	2.9		70.1	25.0	1.7
plas PE	alkenyl	62	23.4	7.5	68.3	59	35.7	47.4	16.4
	acyl		72.5	16.9	8.0		72.9	22.2	2.4
glycerol acetal	*sn*-1		1.6	93.6	4.8		1.9	92.9	5.3
	acyl		69.8	30.2			68.6	31.4	t
PG + CL	alkenyl	27	3.4	31.4	65.1	29	1.65	43.7	54.7
	acyl		53.9	26.3	19.8		48.8	35.2	15.9

		C. beijerinckii											
		[2,2-$^2H_2$]16:0/18:1 (60:40)				[3,3-$^2H_2$]16:0/18:1 (60:40)				[4,4-$^2H_2$]16:0/18:1 (50:50)			
fraction	chain	plas (%)	16:0	18:1	19:cyc	plas (%)	16:0	18:1	19:cyc	plas (%)	16:0	18:1	19:cyc
diacyl-PE	*sn*-1		17.5	9.2	72.2		29.2	30.0	34.5		35.6	47.4	16.4
	sn-2		76.6	13.7	8.1		74.0	20.1	2.9		72.9	22.2	2.4
plas PE	alkenyl	62.5	14.5	5.2	80.2	63	23.4	7.5	68.3	67	21.3	51.5	18.3
	acyl		77.4	9.9	10.2		72.5	16.9	8.0		70.1	25.0	1.7
glycerol acetal	*sn*-1		15.6	8.7	75.7		19.0	15.8	65.0		30.1	51.5	18.4
	acyl		71.4	18.4	6.5		70.3	23.0	5.0		70.1	25.4	1.8
PG + CL	alkenyl	ND	21.6	7.2	71.2	43	21.6	7.2	71.2	47	27.6	48.5	23.7
	acyl		59.3	21.0	17.1		59.3	21.0	17.1		60.0	30.1	7.4

[a] Numbers represent the percent plasmalogen in the total PE or PG + CL fractions. [b] The chains are described as follows: number of carbons: number of double bonds. 19:cyc is the C_{19}-cyclopropane fatty acid or aldehyde. Minor fatty acids included 14:0, 16:1, and 17:cyc. [c] Trace. [d] Not determined. [e] The PG + CL fraction contained variable amounts of an acidic phospholipid of unknown structure. In one sample from cells grown on palmitic plus oleic acids, the unknown lipid represented 18% of the weight, and in one sample from cells grown on oleic acid alone, it represented 29% of the weight of this fraction. The structure of this lipid is being studied (N. Johnston and H. Goldfine, unpublished results).

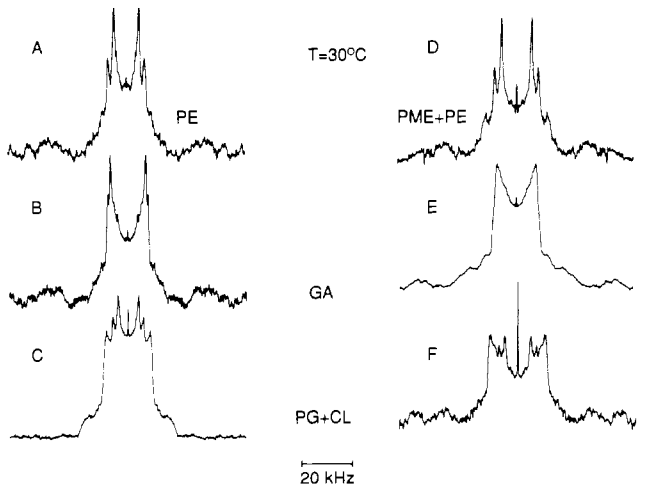

FIGURE 1: Deuterium NMR spectra (46.1 MHz) of liposomes composed of phospholipids isolated from *C. butyricum* (left) and *C. beijerinckii* (right) grown on [2,2-$^2H_2$]palmitic acid–oleic acid, 60:40 w/w. Measuring temperature, 30 °C. Lipids (20–30 mg) were dispersed in water (50:50 w/w), 20000 scans, 100-kHz spectral width. The spectra were symmetrized. PE, phosphatidylethanolamine; PME, phosphatidyl-*N*-monomethylethanolamine; GA, glycerol acetal of plasmalogens; PG, phosphatidylglycerol; CL, cardiolipin. See Table I for percent plasmalogen in the various fractions.

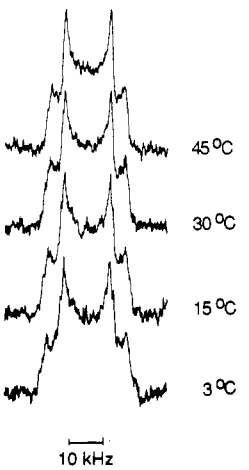

FIGURE 2: Deuterium NMR spectra (46.1 MHz) of liposomes composed of the glycerol acetal of plasmenylethanolamine isolated from *C. butyricum* grown on [2,2-$^2H_2$]oleic acid alone. Lipid (30 mg) was dispersed in water, 50:50 w/w, 20000 scans, 62.5-kHz spectral width. 150-Hz line broadening was applied. The measuring temperatures are shown.

sn-2 deuterons (Figure 1B). Computer simulation confirms that both observed signals are present with equal intensity. An experiment was also done with *C. butyricum* grown on [2,2-$^2H_2$]oleic acid alone, which labeled both of the chains of the glycerol acetal equally. An average quadrupolar splitting of 17.7 kHz for the *sn*-2 chain was seen at 30 °C, but at lower temperatures it could be resolved into two splittings of 17.7 and 20 kHz (Figure 2), in agreement with the results obtained for [2,2-$^2H_2$]palmitate grown cells (Figure 1B). In addition we observed a new splitting of about 29 kHz at 30 °C, which originates from the C-2 deuterons of the *sn*-1 chain (see be-

Table II: Assignments of Quadrupolar Splittings Obtained by Deuterium Nuclear Magnetic Resonance of Lipids from *C. butyricum* and *C. beijerinckii* Grown on [2,2-$^2H_2$]Palmitic Acid–Oleic Acid, 60:40 w/w

	quadrupolar splitting $\Delta\nu_Q$ (kHz)					
	C. butyricum			*C. beijerinckii*		
fraction	*sn*-2(1)	*sn*-2(2)	*sn*-1	*sn*-2(1)	*sn*-2(2)	*sn*-1
diacyl-PE[a]	13.0	19.0	25.0	12.7	18.6	25.5
plasmalogen PE	25.0			25.5		
glycerol acetal[b]	18.4	20.9		16.4		
glycerol acetal[c]	17.7					
PG + CL	10.9	16.0	23.8	11.1	15.8	24.0
POPC (T_m = 27 °C)	10.8	16.4	25.8			
DPPC (T_m = 57 °C)	12.1	17.0	26.2			

[a] The measuring temperature for all sample lipids was 30 °C. [b] Glycerol acetal of plasmenylethanolamine (*C. butyricum*); plasmenyl-*N*-monomethylethanolamine plus plasmenylethanolamine (*C. beijerinckii*). [c] Grown on [2,2-$^2H_2$]oleic acid.

Table III: Gel to Liquid Crystal Phase Transition Temperatures and Reduced Temperatures for Lipids from *C. butyricum*

lipid	gel to liquid crystal phase transition temp T_c (°C)	reduced temp Θ at T_m = 30 °C
PE	13[a]	0.059
glycerol acetal of plasmenylethanolamine	31 on heating[a] −0.6 on cooling[a]	0.12
PG + CL	0[b]	0.11
POPC	−5	0.12 (at T_m = 27 °C)

[a] Determined by DSC (J. Mattai and G. G. Shipley, unpublished results). [b] Estimated by comparison to the corresponding diacyl lipid (POPC).

low). The glycerol acetal lipids of *C. beijerinckii* have mostly *N*-methylethanolamine in the polar head group, with a small amount of ethanolamine (Baumann et al., 1965; Johnston & Goldfine, 1983). One broad resonance with a quadrupolar splitting of 16.4 kHz was seen (Figure 1E). The data are compared with those of diacyl lipids in Table II. In the latter, the C-2 deuterons of the *sn*-2 chain are characterized by two different quadrupole splittings of about 5-kHz separation. In the glycerol acetal of the plasmalogen, this inequivalence is reduced or completely absent.

The gel to liquid-crystalline transition of the PE from *C. butyricum* was about 13 °C by differential scanning calorimetry (J. Mattai, G. G. Shipley, N. C. Johnston, and H. Goldfine, unpublished results). T_c for the same fraction from *C. beijerinckii*, which is mainly phosphatidyl- and plasmenyl-*N*-monomethylethanolamine, should be several degrees lower (Vaughn & Keough, 1974). Two major splittings of 13.0 and 19.0 kHz and one weak splitting of 25.0 kHz for the total PE from *C. butyricum* (Figure 1A) and a similar set of splittings of 12.7, 18.6, and 25.5 kHz for the total PE plus PME fraction from *C. beijerinckii* (Figure 1D) were observed when measurements were made at 30 °C. Since these lipids were mixtures of plasmalogens (50–70%) and diacyl lipids (30–50%), the assignment of these resonances is complex. In principle, six separate signals could contribute to the observed line shape (one resonance from each of the *sn*-1 chain deuterons of ether and diacyl lipids and two resonances from each of the *sn*-2 chain deuterons). In the experimental spectra only three resonances can be observed. The quadrupolar splittings of the spectra can be assigned as follows: Since two of these resonances, 13 and 19 kHz, correspond to those already seen with synthetic and natural phosphatidylethanolamines labeled with [2,2-$^2H_2$]acyl chains (Seelig & Browning, 1978; Gally et al., 1979) and have been attributed to the *sn*-2 deuterons, we assume that these are the quadrupole splittings of the diacyl *sn*-2 chains. The 25-kHz splitting is more problematic. As is clear from the data in Table I at most only about 16% of the deuterons could be in the *sn*-1 chains of diacyl-PE. However, inspection of the spectrum (Figure 1A) clearly shows that the intensity of the splitting is at least 50% of the 13- and 19-kHz splittings, and computer simulation of the spectrum indicates that the 25-kHz splitting could be as much as 1.4 times as intense as the 13- and 19-kHz splittings. This leads to the conclusion that most of the 25-kHz splitting probably arises from the *sn*-2 chain of the plasmalogens (Table II).

The quadrupolar splittings observed with the PG plus cardiolipin fraction are assigned in Table II. These lipids contain less plasmalogen than the PE fraction. The 24-kHz splitting is thus largely, if not entirely, attributable to the *sn*-1 chain, in agreement with diacyl lipids.

The values obtained thus far can be normalized by referring them to a reduced temperature [$\Theta = (T_m - T_c)/T_c$, where T_m denotes the measuring temperature and T_c the gel to liquid crystal phase transition temperature, both in kelvin]. It was found that plots of the quadrupolar splitting vs. the position of the labeled chain segment almost coincide for different diacyl lipids if they are measured at the same reduced temperature (Seelig & Browning, 1978). For the different samples, T_c and the reduced temperatures corresponding to a measuring temperature of 30 °C are given in Table III. Hence, it is reasonable to compare the quadrupolar splitting of the glycerol acetal of plasmenylethanolamine at 30 °C to those of POPC at 27 °C. The former has a large hysteresis of melting, as observed previously with the dioleyl and dielaidoyl forms (Goldfine et al., 1981). Since the lipid was melted at the time of the measurement, it had been heated above T_c, and the reference transition temperature can be taken as 0.6 °C. The corresponding temperature for the PE from *C. butyricum* would be 44 °C. Measurements made with PE liposomes at 40 °C yielded quadrupolar splittings of 12.8 and 17.9 kHz for the *sn*-2 acyl chains of the lipid from *C. butyricum*. These splittings are somewhat higher than those for POPC at the same reduced temperature, Θ = 0.12, and similar to those of DPPC at 57 °C, Θ = 0.051 (Table II).

Hydrocarbon Chain Ordering near the Lipid–Water Interface. The essential difference between diacyl lipids and plasmalogens rests in the linkage of the *sn*-1 hydrocarbon chain to the glycerol backbone. The transition from an ester linkage to a vinyl ether bond could be accompanied by a conformational change of the lipid molecule or by a change in the motional freedom of the hydrocarbon chains. Since these changes were expected to be most pronounced near the lipid–water interface, additional experiments were performed with cells grown on [3,3-$^2H_2$]- and [4,4-$^2H_2$]palmitic acid. The liposome samples prepared from the various head-group fractions exhibited well-resolved ^2H NMR spectra with the typical bilayer line shape. Spectra for each sample were measured at 20, 30, and 40 °C, and from each quadrupolar splitting the molecular order parameter S_{mol} was calculated. A plot of the order parameter S_{mol} vs. the segment position provides an order profile. These are depicted in Figure 3 for the various lipid fractions measured at 30 °C. All order profiles exhibit essentially the same shape, with S_{mol} being low at the C-2 position and increasing considerably toward the hydrophobic interior of the membrane. It should be recalled, however, that a quadrupole splitting of about 25 kHz was observed for the C-2 position of the *sn*-2 chains of the ethanolamine and *N*-monomethylethanolamine plasmalogens. The order profile of these lipids in pure form would exhibit rather

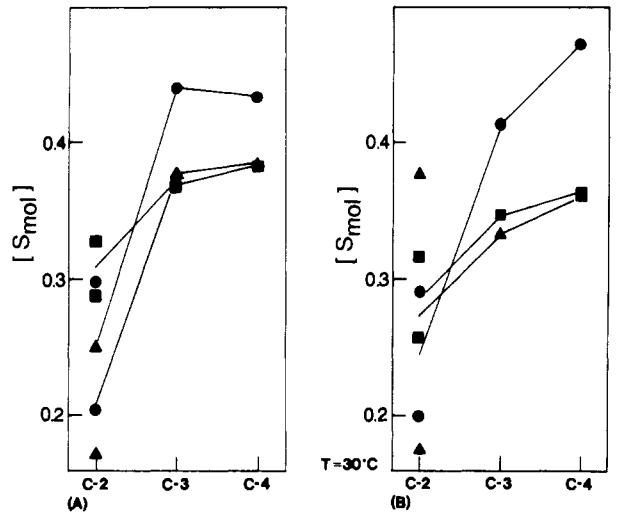

FIGURE 3: Variation of the deuterium order parameter S_{mol} with segment position. Lipids purified from (A) *C. butyricum* and (B) *C. beijerinckii*: (●) PE, *C. butyricum* or PME plus PE, *C. beijerinckii*; (■) glycerol acetal of plasmalogens; (▲) PG plus cardiolipin. Measuring temperature 30 °C. Samples were prepared as described under Materials and Methods. S_{mol} was calculated from the quadrupolar splittings according to the equations below (Seelig, 1977),

$$\Delta\nu = (3/4)(e^2qQ/h)S_{C-^2H}$$

$$|S_{mol}| = |2S_{C-^2H}|$$

where (e^2qQ/h) represents the static deuterium quadrupole coupling constant, which was determined to be 170 kHz for aliphatic deuterons (Burnett & Muller, 1971).

similar S_{mol} values for C-2, C-3, and C-4. The qualitative appearance of the profiles shown in Figure 3 is independent of temperature and parallels that of diacyl lipids. The S_{mol} values of PE or PME fractions are always larger than those of the glycerol acetal lipid and the phosphatidylglycerol plus cardiolipin fractions. The shape and position of the latter profiles almost coincide. This upward shift of the PE or PME profiles may be due to the fact that plots are not normalized with respect to the phase-transition temperature. As noted above, T_c for PE is higher than that for the other two fractions. When the order profiles of the two bacterial strains are compared quantitatively, it is found that in general the S_{mol} values of the lipids of *C. beijerinckii* are somewhat lower.

The two deuterons on C-3 and on C-4 of the PE (or PME plus PE) and the PG plus cardiolipin fractions were found to give a single splitting, showing their equivalence. Under certain conditions a barely resolvable inequivalence at the C-3 segment of the glycerol acetals of the plasmalogens was noted, which was ignored in the discussion of the order profiles below.

Specific Labeling of the Alk-1-enyl Chain of a Plasmalogen and the Alkyl Chain of a Glycerol Acetal of a Plasmalogen. In the experiments reported above, most of the deuteriated saturated fatty acid was incorporated into the *sn*-2 acyl chains of the plasmalogens and the glycerol acetals of the plasmalogens (Table I). In order to obtain labeling of the *sn*-1 chains, an experiment was performed in which [2,2-$^2H_2$]oleic acid was fed to *C. butyricum* along with unlabeled palmitic acid. The spectra of the isolated lipids are shown in Figure 4. Two principal splittings at 8.1 and 26.0 kHz were seen in the PE fraction (Figure 4A). This lipid was 57% plasmalogen and was largely labeled in the *sn*-1 chains (Table I). Since a 25-kHz splitting has already been seen with the acyl-labeled *sn*-1 chain of diacyl-PE and the *sn*-2 chain of the plasmalogen, we can assign most of this signal intensity to the *sn*-1 acyl chain of diacyl-PE. It follows that the 8.1-kHz splitting arises from the alk-1-enyl chain. Clearly, it is very different from the same

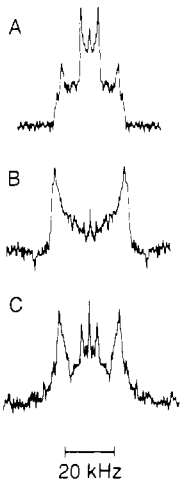

FIGURE 4: Deuterium NMR spectra (46.1 MHz) of aqueous dispersions of phospholipids isolated from *C. butyricum* grown on [2,2-$^2H_2$]oleic acid–palmitic acid, 40:60 w/w, 20 000 scans, spectral width 62.5 kHz for PE and 100 kHz for the other lipids. 150-Hz line broadening was applied. The measuring temperature was 40 °C for PE and 37 °C for the other lipids. The spectra were symmetrized: (A) phosphatidylethanolamine; (B) glycerol acetal of plasmenylethanolamine; (C) phosphatidylglycerol plus cardiolipin. See Table I for percent plasmalogen in the various fractions. Spectra were recorded at 20, 30, and 40 °C with no change in shape.

carbon of the *sn*-1 acyl chain, and the interpretation of this narrow quadrupolar splitting is discussed below.

The glycerol acetal of plasmenylethanolamine obtained from cells grown on [2,2-$^2H_2$]oleic acid plus unlabeled palmitic acid showed only one principal quadrupolar splitting at 30.2 kHz (Figure 4B). Since it is labeled at the carbon next to the point of attachment of the glycerol moiety, it is not surprising that its conformation and dynamics may differ from those of the C-2 segments of the *sn*-1 chains of both diacyl- and alkenylacyl-PE. In the experiment described above, in which the glycerol acetal of plasmenylethanolamine from *C. butyricum* was labeled by growth of cells in [2,2-$^2H_2$]oleic acid alone, we observed a quadrupolar splitting of 29.0 kHz at 30 °C, confirming the result obtained with the higher melting saturated/unsaturated species.

The phosphatidylglycerol plus cardiolipin fraction gave two principal splittings at 6.7 and 26.1 kHz (Figure 4C). Since these lipids are 60–70% diacyl and only 30–40% plasmalogen, the 26.1-kHz splitting of the *sn*-1 acyl chains is dominant, while the 6.7-kHz quadrupolar splitting of the C-2 of the alkenyl chain is weaker. This value can be compared to 8.1 kHz seen with alkenyl chain labeled PE.

^2H NMR of Lipids Containing [9,10-$^2H_2$]Oleic Acid. C. butyricum was also grown on media supplemented with [9,10-$^2H_2$]oleic acid in the absence of biotin. The lipids were separated, and acyl and alkenyl chain compositions were determined. The analyses showed, as expected, that both the *sn*-1 and *sn*-2 chains contained almost exclusively 18:1 and the 19-carbon cyclopropane chain derived from 18:1 (Law, 1971) (Table I). Therefore, both labeled hydrocarbon chains are expected to be visible in the ^2H NMR spectra. The appearance of the spectra of the glycerol acetal lipid and the phosphatidylglycerol plus cardiolipin fraction were very similar to those observed for 9,10-labeled POPC and DOPC (Seelig & Waespe-Šarčevič, 1978; Gally et al., 1979), allowing an immediate assignment. Table IV lists the quadrupolar splittings observed for the two lipid fractions. The oleate-enriched PE produces a reversed hexagonal phase close to the gel to liquid crystal boundary (Goldfine et al., 1987), and it was not possible to obtain useful spectra with the pure lipid.

Table IV: Assignments of Quadrupolar Splittings Obtained by Deuterium Nuclear Magnetic Resonance of Phospholipids from *C. butyricum* Grown on [9,10-$^2H_2$]Oleic Acid

fraction	quadrupolar splitting $\Delta\nu_Q$ (kHz)		
	C-9	C-10 (*sn*-1)	C-10 (*sn*-2)
glycerol acetal[a]	15.2, 12.7	7.3	2.2
PG + CL	13.3	6.5	3.0
DOPC (27 °C)	12.9	6.4	2.1

[a] Glycerol acetal of plasmenylethanolamine.

Table IV also lists the quadrupolar splittings obtained with DOPC labeled at C-9 and C-10. The main difference between the glycerol acetal lipid and the other two lipids is the appearance of two quadrupolar splittings attributable to the C-9 position. This indicates some inequivalence of the *sn*-1 and *sn*-2 chains at the level of the C-9 segment. Otherwise, the conformational difference between the chains of the glycerol acetal lipid, the phosphatidylglycerol plus cardiolipin fraction, and DOPC was found to be rather small at the level of the double bond.

Discussion

The use of deuterium NMR to study natural membranes has been limited to those organisms in which the lipid aliphatic chains can be readily labeled with deuteriated fatty acids. Since the deuterium signal is relatively weak, incorporation has to yield lipids that are highly enriched in the labeled chains. Previous studies have utilized the natural fatty acid auxotroph *Acholeplasma laidlawii* (Stockton et al., 1977) and fatty acid auxotrophic mutants of *Escherichia coli* (Gally et al., 1979, 1980; Davis et al., 1979; Kang et al., 1979). Both of these organisms contain polar lipids of the diacyl type, and studies with them have shown that the information previously obtained with chemically synthesized deuteriated phospholipids is applicable to natural membranes in which the principal lipids are phospholipids, as is the case in *E. coli*, or mixtures of phospholipids and glycosyldiacylglycerols, as is the case in *A. laidlawii*. To date, there have been no deuterium NMR studies on synthetic or natural membranes containing plasmalogens. In part, this may reflect difficulties encountered in their chemical synthesis (Paltauf, 1983a). Thus the organisms we have studied, *C. butyricum* and *C. beijerinckii*, which are natural fatty acid auxotrophs in the absence of biotin and are rich in ether lipids, provided an important opportunity to study the behavior of artificial and natural membranes containing plasmalogens. In addition, these organisms have a unique lipid, the glycerol acetal of a plasmalogen, which has been shown to play a role in stabilizing the bilayer arrangement of the membranes of these cells (Goldfine et al., 1987). Studies with deuterium-labeled fatty acids also provided an opportunity to study the conformation and dynamics of this unusual lipid in membranes.

In order to simplify the discussion, the chemical structures of a few selected lipids as well as characteristic quadrupole splittings are summarized in Figure 5. It should be noted that the *sn*-1 chain is usually connected to glycerol by an alk-1-enyl ether linkage in plasmalogens but that the double bond is substituted in the glycerol acetal of the plasmalogens. In both lipids only one deuteron is retained at the C-2 carbon segment of the *sn*-1 chain, assuming the glycerol acetal lipid is derived from the plasmalogen. As has already been mentioned, it is the C-2 segment that gives rise to two unusual quadrupole splittings in plasmenylethanolamine.

The fractions that are richest in plasmalogens are the PE from *C. butyricum* and the PME plus PE from *C. beijerinckii*.

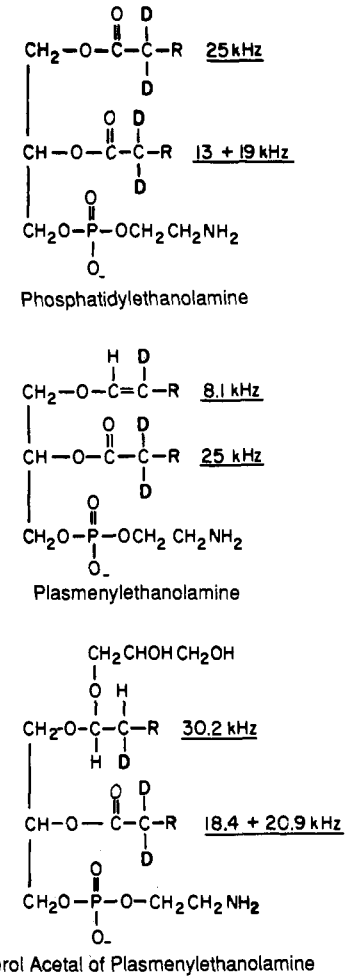

FIGURE 5: Structures of some of the lipids studied and the quadrupole splittings observed with deuterium at the C-2 segments of the *sn*-1 and *sn*-2 chains.

When the latter species is grown with biotin, the ratio of *N*-monomethylethanolamine to ethanolamine in this fraction is 4:1 (Baumann et al., 1965). In both organisms grown without biotin in media supplemented with fatty acids, the PE (or PME) fraction has from 50 to 70% plasmalogen (Table I). Our studies with palmitic acid labeled with deuterium at carbons 2, 3, and 4 have shown that the *sn*-2 acyl chains of both the plasmalogens and the diacyl lipids of the PE (or PME) fractions are the site of incorporation of most of the labeled fatty acid and that the quadrupolar splittings produced by the *sn*-2 acyl chains of the diacyl lipids and the plasmalogens are somewhat different (Table II). The C-2 segment of the plasmalogen *sn*-2 chain produced a single quadrupolar splitting of 25.0 kHz compared to two splittings of 13 and 19 kHz obtained for the *sn*-2 chain of diacyl-PE.[2] Only single quadrupole splittings were seen for the two deuterons at C-3 and C-4, and they appear to be identical for the diacyl lipids and plasmalogens.

The specific labeling of the *sn*-1 chains of the plasmalogens of *C. butyricum* with [2,2-$^2H_2$]oleate revealed quadrupolar splittings, which had not been previously observed. The C-2 segment of the alk-1-enyl chain, which can have only one deuteron, produced a narrow quadrupolar splitting of 8.1 kHz in the PE fraction and 6.7 kHz in the mixed phosphatidyl-

[2] Recent experiments with a semisynthetic plasmenylethanolamine labeled in the *sn*-2 acyl chain show that the C-2 segment produced a quadrupolar splitting of 23 kHz (Malthaner et al., 1987).

glycerol plus cardiolipin fraction. Since the C-2 deuteron is at a double bond and its splitting is similar to that of the C-10 deuteron of the oleic acid *sn*-1 chain (Table IV), we suggest that the small splitting reflects a similar average orientation of the double bond in both molecules with respect to the bilayer normal, i.e., the double bond lies essentially parallel to the bilayer normal (Seelig & Waespe-Šarčevič, 1978). These data represent the first direct information on the orientation of the alk-1-enyl ether double bond in membranes.

The additional *N*-monomethyl moiety of the polar head group of *C. beijerinckii* has a small disordering influence on the structure of the lipid bilayer in proximity to the polar head group as evidenced by the lower S_{mol} values for the relevant fractions (Figure 3). The PE fraction of *C. butyricum* grown on palmitate–oleate, 60:40, appeared to initiate a transition to a hexagonal phase between 30 and 40 °C, as evidenced by a decrease in the quadrupolar splitting to half the width of that found in the corresponding lamellar phase (Gally et al., 1980). This transition temperature corresponds closely to that of a pure plasmalogen containing mainly saturated alk-1-enyl chains and oleic acid acyl chains (Lohner et al., 1984). The corresponding *N*-monomethylethanolamine fraction from *C. beijerinckii* did not exhibit a lamellar to hexagonal transition at the temperatures examined (data not shown). This stabilizing effect of the addition of a single methyl group has been recently reported with the corresponding diacylphospholipids (Gagné et al., 1985).

The *sn*-2 acyl chains of the glycerol acetals of the plasmalogens exhibited a different behavior from that of the related plasmalogens and diacylphospholipids. The two deuterons at the C-2 segment appear to be almost equivalent. There are at least two possible explanations for this behavior. One assumes an increased motional averaging in the two resonances at the two C-2 methylene deuterons. The second postulates a conformational change at the carboxyl end of the *sn*-2 chain, leading to reduced inequivalence of the two deuterons. For example, a bending of the *sn*-2 hydrocarbon chain at C-2 by less than 90° with respect to the bilayer plane could produce this effect. Since the glycerol acetal linkage is at the C-1 segment of the *sn*-1 chain, its effect on the conformation of that chain (see below) would most likely be expressed near the carboxyl end of the *sn*-2 chain, resulting in changes in motion or conformation or both. Such effects may also affect the C-3 deuterons of the *sn*-2 chains, and we have observed a slight inequivalence of these deuterons in the glycerol acetals of plasmalogens isolated from both species.

Our studies on the quadrupolar splittings observed with the glycerol acetal lipid labeled in both the *sn*-1 and *sn*-2 chains with [9,10-$^2H_2$]oleate showed that, unlike the results obtained previously with diacylphosphoglycerides (Seelig & Waespe-Šarčevič, 1978; Gally et al., 1979), there was evidence for an inequivalence of the two chains at the level of the C-9 segment, as well as at the level of the C-10 segment. This suggests that the influence of the glycerol moiety attached to C-1 of the *sn*-1 chain extends beyond the C-3 segment.

The glycerol acetal alkyl chain C-2 segment labeled with [2,2-$^2H_2$]oleate produced an unusually wide quadrupolar splitting of 30.2 kHz compared to 25 kHz in diacyl-PE (Figure 4). At the *sn*-2 chain C-2 segment we observed a narrowing of the differences in the two quadrupolar splittings. In principle, these differences can be explained by a decrease in flexing motion at the *sn*-1 chain and an increase in flexing motion at the *sn*-2 chain. However, as noted above, a conformational change of the lipid molecule could explain the observed differences without invoking differential flexibility arguments. In view of the unusual head group of the glycerol acetal of plasmalogens, differences in conformation appear to be more likely.

The finding that one of the fatty acid C-2 deuterons is retained in the alk-1-enyl bond of the plasmalogen and in the glycerol acetal provides information of value in studies on the formation of this bond in anaerobes. While this subject is outside the scope of this paper, it should be noted that the biosynthesis of the alk-1-enyl bond of plasmalogens in obligately anaerobic bacteria differs significantly from the pathway existing in the tissues of higher organisms (Horrocks & Sharma, 1982; Paltauf, 1983b; Koga & Goldfine, 1984; Watanabe et al., 1984), and any mechanism proposed for the formation of the alk-1-enyl bond should take into account the retention of a hydrogen at C-2 of the precursor fatty acids.

Added in Proof

In a recent paper, Tate and Gruner (1987) discuss the effects of lipid chain length on formation of the inverted hexagonal (H_{II}) phase. The increased tendency of plasmenylethanolamine to undergo the L_α to H_{II} transition may be in part related to the greater effective chain length of the *sn*-2 chain resulting from its postulated extended conformation.

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Monoclonal Antibodies as Probes of High-Density Lipoprotein Structure: Identification and Localization of a Lipid-Dependent Epitope†

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Received August 27, 1986; Revised Manuscript Received April 8, 1987

ABSTRACT: Eight stable murine monoclonal antibodies (mabs) were raised against human high-density lipoproteins (HDL). Three different antibody reactivities were demonstrated by immunoblotting. A group of five antibodies were specific for apolipoprotein A-I (apoA-I) and bound to similar or overlapping epitopes. The second type of reactivity, shown by mab-32, was specific for apoA-II. In the third group, two antibodies showed high reactivity with apoA-II and slight cross-reactivity with apoA-I. The properties of two antibodies, mab M-30 specific for apoA-I and mab M-32 specific for apoA-II, were characterized in detail as probes of HDL structure. The association of ^{125}I-labeled HDL or synthetic complexes of apoA-I and phosphatidylcholine with mab M-30 was lipid dependent. Mab M-32 binding to apoA-II was independent of lipid. The lipid-dependent epitope bound by mab M-30 has been localized to an 18 amino acid synthetic apoA-I peptide. Moreover, studies with HDL_2, HDL_3, and immunoadsorbed HDL subfractions indicate that binding of mab M-30 to HDL is influenced by some component within the microenvironment of individual HDL particles. These lines of evidence suggest that the molar ratio of apoA-I to apoA-II is the critical determinant. Binding of mab M-32 to HDL increased the reactivity of HDL to mab M-30 in a dose-dependent manner, indicating an unusual form of cooperativity between two mabs that recognize different proteins in HDL. These monoclonal antibodies will be valuable in studies of the metabolic significance of protein–protein and lipid–protein interactions in HDL.

The physiological functions of HDL include regulation of cholesterol transport in the blood and cellular maintenance of cholesterol homeostasis. The two major proteins of human plasma high-density lipoproteins (HDL)[1] apolipoprotein A-I (apoA-I) and apolipoprotein A-II (apoA-II), have been investigated intensely since 1975, when HDL was rediscovered as a negative predictor of atherosclerosis. Some studies have suggested that the concentrations of these apolipoproteins are better discriminators of coronary disease than is HDL cholesterol (Maciejko et al., 1983; Brunzell et al., 1983; Sniderman et al., 1980). ApoA-I and apoA-II comprise more than 90%

†Supported by Contract 90649 from Triton Biosciences Inc. and Grant 12605 from the American Heart Association, Texas Affiliate.
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[1] Abbreviations: HDL, high-density lipoprotein(s); apoA-I, the major protein component of HDL; apoA-II, the disulfide-containing protein component of HDL; sHDL, synthetic HDL; mab, monoclonal antibody; VLDL, very low density lipoprotein(s); LDL, low-density lipoprotein(s); BSA, bovine serum albumin; PBS, phosphate-buffered saline; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; LCAT, lecithin:cholesterol acyltransferase; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; RIA, radioimmunoassay; Boc, *tert*-butoxycarbonyl; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase.

0006-2960/87/0426-5833$01.50/0