Structure of *Escherichia coli* Membranes. Deuterium Magnetic Resonance Studies of the Phosphoglycerol Head Group in Intact Cells and Model Membranes†

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ABSTRACT: Deuterium nuclear magnetic resonance was used to investigate the structure and dynamics of phosphatidylglycerol in intact cell membranes of *Escherichia coli*. Selectively deuterated glycerol was supplied to the growth medium of *E. coli* strain T 131 GP which was defective in endogenous glycerol synthesis and degradation enabling the stereospecific labeling of the head-group segments of phosphatidylglycerol (accounting for ~20 wt % of the membrane lipids) as well as the backbone segments of phosphatidylglycerol and phosphatidylethanolamine (~80 wt %). Well-resolved deuterium NMR spectra of cell membranes were observed for the β- and γ-head-group segments (*sn*-2′ and *sn*-3′ segments) and characterized by their quadrupole splittings and T_1 relaxation times. The α-segment (*sn*-1′ segment) could not be resolved in intact cell membranes due to overlapping backbone resonances. However, all three head-group signals were easily identified in pure lipid dispersions. In order to assess the influence of the fatty acid composition and lipid heterogeneity on the glycerol head group, the following bilayer model systems were also studied: (1) synthetic 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol, (2) purified *E. coli* phosphatidylglycerol, and (3) total lipid extract, i.e., *E. coli* phosphatidylglycerol in a mixture with phosphatidylethanolamine. The comparison of the various pure lipid membranes with *E. coli* cell membranes led to the following conclusions: (1) A large fraction, if not all, of the glycerol head groups in the *E. coli* cell membrane was NMR visible. The structural and dynamical parameters were typical of a liquid-crystalline bilayer environment and were taken as evidence that the glycerol head groups were not involved in strong interactions with membrane proteins. (2) The head-group conformation was found to be remarkably similar in pure lipid bilayer aggregates and intact *E. coli* cell membranes. By analogy with bilayers of phosphatidylethanolamine which exhibit similar quadrupole splittings it is suggested that the glycerol head group is extended parallel to the membrane surface. (3) The T_1 relaxation rates of the glycerol head group in *E. coli* membranes at 25 °C were identical with those of pure liquid glycerol at 40 °C and by about a factor of 20 shorter than those of glycerol (10 wt %) in aqueous solution. The reorientation rate of the glycerol head groups at the membrane surface thus corresponds to a viscosity of about 2.9 P which is the bulk viscosity of liquid glycerol at 40 °C. (4) The conformation and the dynamics of the phospholipid glycerol backbone also showed a close similarity in intact cell membranes and pure lipid bilayers. (5) The *E. coli* cell membrane spectra were characterized by a rather large intrinsic line width which appears to be typical for protein-containing bilayers. The line-width effect suggests the occurrence of protein-induced low-frequency motions in the range 10^5–10^7 Hz.

The membrane lipids of *Escherichia coli* consist of predominantly phosphatidylethanolamine (~80%), phosphatidylglycerol (~15%), and cardiolipin (~5%) (in the exponential growth phase). The head-group complexity can be reduced to only two phospholipid classes, i.e., phosphatidylethanolamine (PE)[1] and phosphatidylglycerol (PG), by using mutants which are defective in the synthesis of cardiolipin (Pluschke et al., 1978; Pluschke & Overath, 1981). In a previous study we have employed strain T 131 GP which is defective in both the synthesis of cardiolipin and the biosynthesis and degradation of glycerol. Strain T 131 GP incorporates externally added glycerol specifically into the membrane lipids. Addition of deuterated glycerol to the growth medium therefore allows the selective deuteration of the glycerol head group of PG and of the glycerol backbone of both PG and PE (Gally et al., 1981). In model membranes composed of the extracted lipids the two types of segments can easily be distinguished with deuterium magnetic resonance. The residual quadrupole splittings of the PG head-group segments are generally smaller than those of the backbone. Moreover, their intensity compared to the backbone signals is much reduced since only PG (20%) contributes to the head-group signal while the backbone signal arises from both PE and PG.

Our previous studies were limited to lipid bilayers composed of the total lipid extract of strain T 131 GP and provided the assignments of all the glycerol head-group resonances (Gally et al., 1981). However, in this earlier investigation it was not possible to observe the PG head-group signals in intact *E. coli* membranes. It remained open if this was simply a matter of spectrometer sensitivity or due to tight binding of phosphatidylglycerol to proteins or metal ions. Because of technical limitations, all earlier spectra had to be recorded under high-resolution conditions without using the quadrupole echo technique. These spectra therefore showed a distorted line shape even though the quadrupole splittings could be determined quite accurately. More seriously, the earlier method failed in the case of broad lines, and an immobilization of the phospholipids via binding would have made the glycerol head group NMR invisible under these conditions.

By use of an improved NMR spectrometer, the objectives of the present study were (1) to identify the phosphoglycerol head group with deuterium NMR in *intact E. coli* cell membranes, (2) to compare the ^2H NMR spectra of the cell membrane with those obtained from aqueous dispersions of the total lipid extract and purified phosphatidylglycerol, (3)

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[1] Abbreviations: NMR, nuclear magnetic resonance; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol; EDTA, ethylenediaminetetraacetic acid; Pipes, 1,4-piperazinediethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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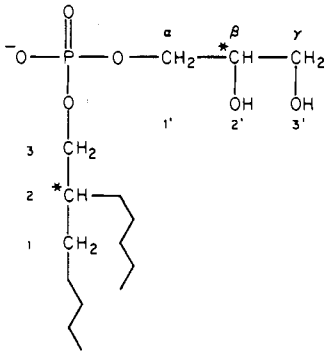
to contrast the behavior of *E. coli* phosphatidylglycerol which has a wild-type fatty acid composition with that of a synthetic, cis-unsaturated phosphatidylglycerol (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol, POPG), and (4) to obtain information about the dynamics of the phosphoglycerol head group by measuring ^2H spin-lattice (T_1) relaxation times.

Materials and Methods

Naturally occurring phosphatidylglycerol has the L (backbone)–D (head group) conformation of the two optically active glycerols. According to the stereospecific numbering (sn) nomenclature the molecule is denoted as 1,2-diacyl-*sn*-glycero-3-phospho-1′-glycerol (cf. Bonsen et al., 1966). For simplification of the discussion and in order to facilitate the comparison with other polar groups, we also use the nomenclature α, β, and γ for the three glycerol head-group segments (asterisks denote asymmetric carbon atoms):



Synthesis of Deuterated Glycerols. Optically active *sn*-[1,1-$^2H_2$]glycerol and *sn*-[3,3-$^2H_2$]glycerol were synthesized as described by Lok et al. (1976) starting from L- and D-serine, respectively. As intermediates 2,3-*O*-isopropylidene-*sn*-[1,1-$^2H_2$]glycerol and 1,2-*O*-isopropylidene-*sn*-[3,3-$^2H_2$]glycerol were obtained and used for the synthesis of 3,1′- and 3,3′-phosphatidylglycerol, respectively. [2-^{2}H]Glycerol was prepared by reducing dihydroxyacetone with $NaBD_4$, and the racemic isopropylidene[2-^{2}H]glycerol was formed by condensation with acetone (Renoll & Newman, 1955). Employing this protected glycerol in the chemical synthesis of phosphatidylglycerol leads to an equimolar mixture of β-deuterated 3,1′- and 3,3′-phosphatidylglycerol. However, if the same racemic glycerol is added to the growth medium of *E. coli* only the optically pure diastereomer 3,1′-phosphatidylglycerol is produced by biochemical incorporation.

Chemical Synthesis of 1-Palmitoyl-2-oleoyl-sn-glycero-3-phospho-sn-1′-glycerol (POPG). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine was hydrolyzed with phospholipase A_2 (*Crotalus atrox*) to yield 1-palmitoyllysophosphatidylcholine (Chakrabarti & Khorana, 1975). The lysolecithin (cadmium salt) was esterified with oleic acid anhydride according to Gupta et al. (1977). After purification the unsaturated phosphatidylcholine was hydrolyzed by phospholipase D treatment (Eibl & Kovatchev, 1981) to yield 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoric acid. The latter was coupled with selectively deuterated isopropylidene-*sn*-glycerol (Aneja et al., 1970; Browning & Seelig, 1979; Wohlgemuth et al., 1980), and the protecting group was removed with boric acid in trimethyl borate (Mattson & Volpenheim, 1962; Bonsen et al., 1966).

Preparation of Selectively Deuterated Membranes and Membrane Phospholipids. Biochemical incorporation of selectively deuterated glycerol was achieved with an *E. coli* glycerol auxotroph (strain T 131 GP) which has been described in more detail previously (Gally et al., 1981). Briefly, cells were grown on mineral salt medium (Vogel & Bonner, 1956), 0.4% glucose, 0.002% deuterated glycerol, and 0.3% casamino acid in a 35-L fermentor with aeration (40 L of air/min). The cells were harvested by centrifugation at the end of the exponential growth phase (OD $\sim$ 5). A total phospholipid fraction was prepared from cells by chloroform/methanol extraction (Ames, 1968), and the phospholipids were further purified by precipitation from anhydrous acetone at 4 °C (Baer, 1951). The phospholipid extract was washed with a 0.1 M EDTA solution saturated with NaCl (pH 8.5) to yield the sodium salts.

PG ($\sim$20 wt % of total lipid) was separated from PE ($\sim$80 wt %) by gel filtration on Sephadex LH-20 (cf. Eichberg & Burnham, 1970). The fatty acid composition of purified PG was determined by gas chromatography on a 15% Silar 10 C column (cf. Gilkes & Weeks, 1977), yielding 42.0 mol % vaccenic acid, 33.9 mol % palmitic acid, and 12.2 mol % stearic acid as the major components. The fatty acid composition of pure PG appears to be similar to that of wild-type *E. coli* with palmitic acid predominantly in the *sn*-1 position and vaccenic acid in the *sn*-2 position (cf. Overath & Thilo, 1978). A total membrane fraction (inner and outer membrane) was prepared by suspending frozen cells in 20 mM Pipes buffer at pH 7.3 containing 0.2 M KCl. Some grains of DNase I were added, and the cells were passed twice through a French press. The suspension was centrifuged for 10 min at 5000 rpm in a Sorvall SS-34 rotor to remove unbroken cells. The supernatant was centrifuged for 1 h at 75000*g* and the pellet resuspended in the same buffer as above, containing in addition 5 mM EDTA. After centrifugation at 75000*g* (1 h) the pellet was again dispersed in the same type of buffer, this time prepared with deuterium-depleted water. The suspension was centrifuged for 1 h at 200000*g* and the wet pellet (containing $\sim$70 wt % H_2O) used for the NMR measurements.

NMR samples of pure phospholipids were prepared as follows. The phospholipids were dispersed at 40–50 °C in excess 10 mM Pipes buffer solution of pH 7.0 containing 2 mM EDTA. Deuterium-depleted water was used to minimize the isotropic ^2H NMR signal due to natural abundance of deuterium in water. After high-speed centrifugation the pellet was transferred in an NMR sample tube. Phospholipid spectra were also recorded for samples containing 0.1 M NaCl. Within experimental error the same type of spectra and quadrupole splittings were observed.

^2H and ^{31}P NMR Measurements. All measurements were made with a Bruker-Spectrospin CXP-300 spectrometer operating at 46.1 MHz for ^2H and 121.48 MHz for ^{31}P. The experimental conditions are the same as detailed before (Seelig et al., 1981; Tamm & Seelig, 1983).

Results

Phosphatidyl-β-CD-glycerol. β-Deuterated glycerol contains only a monodeuterated carbon segment. Compared to the CD_2 groups of α- and γ-deuterated glycerol the potential problem of the motional inequivalence of the two deuterons giving rise to two different quadrupole splittings is avoided. Incorporation of β-CD-glycerol ([2-^{2}H]glycerol) into the membrane lipids of *E. coli* should thus produce a rather simple ^2H NMR spectrum with just one quadrupole splitting for the β-segment of the glycerol head group (PG only) and another one for the *sn*-2 segment of the glycerol backbone (both PG and PE). Figure 1A shows the ^2H NMR spectrum of *E. coli* cell membranes (inner plus outer membrane) obtained from cells grown on β-deuterated glycerol. In order to minimize the sharp central peak caused by the natural abundance of deuterium in water,[2] the spectrum was recorded under partially

Table 1: Deuterium Quadrupole Splittings (kHz) of Phosphatidylglycerol Labeled at the Glycerol Head Group

temp (°C)	POPG[a,b]	*E. coli* PG[a,c] (purified)	*E. coli* PG[a,d] (in total lipid extract)	membrane (inner and outer *E. coli* membrane)	DPPG[f]
			α-Segment [1',1'-$^2H_2$]		
40	9.8		10.2		10–11 at temp above
25	10.0	11.1	10.5	not resolved	the phase transition
15	10.3	11.3	10.9		(T_c = 41 °C)
5	10.6	11.4	11.0		
−5	10.7	11.4			
			β-Segment [2'-^2H]		
40	4.1 (4.6)[e]		4.5	4.1[e]	4–3 at temp above
25	4.0 (4.6)[e]	4.4	4.2	4.1[e]	phase transition
15	3.8 (4.4)[e]	4.2	4.1	4.6[e]	
5	3.3 (4.0)[e]	3.7	4.2 (5.6)[e]		
−5	2.8 (6.2)[e]	2.8			
−15	(8.4)[e]				
			γ-Segment [3',3'-$^2H_2$]		
40			0.8[e]	0.4[e]	0.6; 0.8, two splittings
25	ND[g]	ND[g]	0.7	1.0[e]	
15			0.9	1.4[e]	
5			0.9 (1.95)[e]		

[a] Lipids were dispersed as sodium salts in 10 mM Pipes and 2 mM EDTA, pH 7.3. [b] POPG has a phase transition temperature of $T_c \simeq -5$ °C, and the spectra become very broad below T_c. [c] Well-resolved quadrupole splittings were measured at temperatures as low as −15 °C. At −20 °C the ^2H NMR signal became very broad. [d] Mixture of PG (~20 wt %) and PE (~80 wt %). The phase transition of the purified PE is centered around 25 °C with a width of about $\Delta T \simeq 30$ °C (Ghosh & Seelig, 1982). The head-group signal of PG in mixture with PE is broadened out at higher temperatures than in pure PG dispersions. [e] Width at half-height of resonance. [f] Taken from Wohlgemuth et al. (1980). [g] ND = not determined.

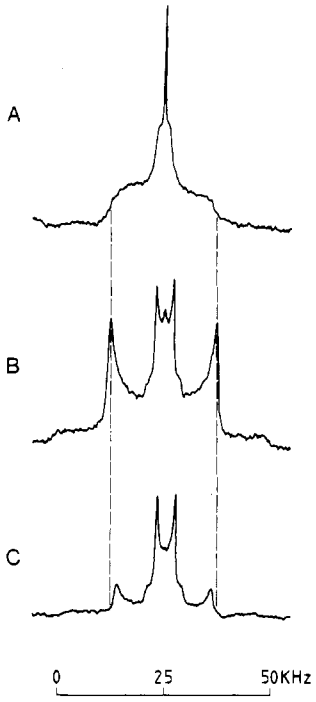


FIGURE 1: ^2H NMR spectra (at 46.1 MHz) of *E. coli* strain T 131 GP grown on [2-^2H]glycerol. Labeling of the β-head group and the *sn*-2 backbone segments. Measuring temperature 25 °C. (A) *E. coli* inner and outer membrane; 130K scans corresponding to about 9 h measuring time (~500 mg of wet membrane pellet containing 11–14 mg of PG). (B) Total lipid extract dispersed in buffer; 20K scans (~90 mg of lipid containing ~18 mg of PG). (C) Purified *E. coli* PG dispersed in buffer; 10K scans (~40 mg of lipid).

relaxed conditions by using a 180°–τ–90° sequence followed by the quadrupole echo. The spectrum clearly consists of two components with quadrupole splittings of about 4 and 24 kHz. Evaluation of the areas underneath the broad and small component by weighing cutouts of the respective traces yields a ratio of about 5:1 (broad component:small component). On the basis of this intensity ratio as well as on previous assignments obtained with deuterated 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (Wohlgemuth et al., 1980), the 4-kHz splitting must be ascribed to the β-CD segment (*sn*-2' segment) of the PG head group, while the 24-kHz component originates from the *sn*-2 segment of the PE and PG glycerol backbones. This is further supported by comparison with the ^2H NMR spectra of the total lipid extract (Figure 1B) and the purified PG alone (Figure 1C), both dispersed in deuterium-depleted

[2] Even though the membranes were dispersed in deuterium-depleted buffer, it was not possible to completely eliminate the DHO signal. The T_1 relaxation time of this signal was about 120 ms. The relaxation delay after the initial 180° pulse was chosen empirically between 60 and 80 ms in order to zero the water signal. The T_1 relaxation times of the deuterated lipid segments were in the range 5–30 ms.

Table II: Deuterium Quadrupole Splittings ν_Q (kHz) and Spin-Lattice (T_1) Relaxation Times (ms) of the Glycerol Backbone Segments of *E. coli* PG and PE

temp (°C)	*E. coli* PG (purified) $\Delta\nu_Q$	*E. coli* PG (purified) T_1	*E. coli* PG (total extract) $\Delta\nu_Q$	*E. coli* PG (total extract) T_2	membrane (inner and outer membrane) $\Delta\nu_Q$	membrane (inner and outer membrane) T_1	*E. coli* PE[c] (purified) $\Delta\nu_Q$	*E. coli* PE[c] (purified) T_1
			[3,3-$^2H_2$] Segment					
40			25.7[a]	8.6	25.6	7.1	24.5	7.4
25	ND	ND	26.3	5.1	24.1	5.3		
15			26.3	5.4	23.0	5.5		
5			broad					
			[2-^2H] Segment					
40	21.8	5.5	23.8	5.4	23.4	5.2	24.1	5.3
25	22.7	3.8	25.6	5.0	24.4	<7.0		
15	23.1	4.2	26.7	4.6	broad			
5	25.2	4.7	broad					
			[1,1-$^2H_2$] Segment					
40			16.2[b]	6.7			17.5	5.2
25	15.5		17.8	5.2		6.0		
15	16.6		18.2	5.3				
5	17.9		broad					

[a] Average of two splittings with about 2-kHz difference. [b] 1*R* deuteron; the splitting of the 1*S* deuteron is ~0 kHz. [c] Taken from Ghosh & Seelig (1982).

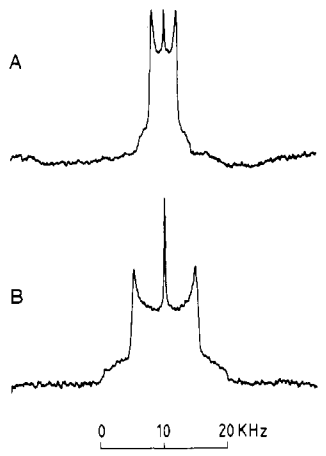


FIGURE 2: ^2H NMR spectra of multilamellar dispersions of synthetic 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol selectively deuterated at various head-group segments at 25 °C. (A) β-Deuterated POPG [$CH_2(OH)CD(OH)CH_2$–]. (B) α-Deuterated POPG [$CH_2(OH)CH(OH)CD_2$–].

buffer. The pure lipid dispersions gave rise to sharp, well-defined "powder patterns" characteristic of a random orientation of liquid-crystalline bilayer microdomains (cf. Seelig, 1977). The separation of the most intense peaks in the spectrum defines the quadrupole splittings, $\Delta\nu_Q$, of the head-group and backbone segments. Representative data are summarized in Table I for the head group and in Table II for the backbone segments, respectively. In contrast, the line shapes of *E. coli* cell membranes are distinctly broader and the sharp 90° orientations which are normally used to evaluate the quadrupole splittings are not resolved. As a first-order approximation, we have therefore measured the widths of the backbone and head-group signals at half-height ($\Delta\nu_{1/2}$) and have included these numbers in Tables I and II. A comparison of $\Delta\nu_{1/2}$ and $\Delta\nu_Q$ for pure lipid model systems demonstrates that $\Delta\nu_{1/2}$ is only 10–20% larger than $\Delta\nu_Q$ provided the membrane is in the liquid-crystalline state (cf. β-segment in Table I). Below the phase transition this comparison is no longer valid.

The total lipid extract and the purified PG dispersions behave rather similar as far as the quadrupole splittings are concerned. However, there are also two obvious differences. First, the total lipid extract shows a broad gel-to-liquid crystal phase transition centered around $T_c \sim 25$ °C with a width of about $\Delta T \simeq 30$ °C, whereas purified PG remains in the liquid-crystalline state down to $T_c \sim -5$ °C. Second, the integrated intensity ratio head group:backbone signal changes from about 1:5 for the total lipid extract to about 1:1 for purified PG, in accordance with the change in the chemical composition of the bilayer.

According to the fatty acid composition as determined by gas chromatography, the major single lipid species of *E. coli* PG is 1-palmitoyl-2-vaccenoyl-*sn*-glycero-3-phospho-1′-glycerol which accounts for as much as 50% of the total PG. In order to assess the influence of the fatty acid heterogeneity on the head-group behavior of PG, we have also investigated a well-defined synthetic phosphatidylglycerol, i.e., 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG). In contrast to the biochemical incorporation the chemical synthesis allows a specific labeling of the head-group segments only. Hence, as shown in Figure 2A, the ^2H NMR spectrum of β-CD-POPG consists of just one quadrupole splitting of about 4 kHz while the backbone signal is missing. Synthetic POPG exhibits a relatively sharp phase transition around −5 °C as indicated by a broadening of the ^2H NMR signal below this temperature. Numerical values of the quadrupole splitting of β-CD-POPG are listed in Table I. For comparison with the *E. coli* cell membrane spectra we have also included the width at half-height.

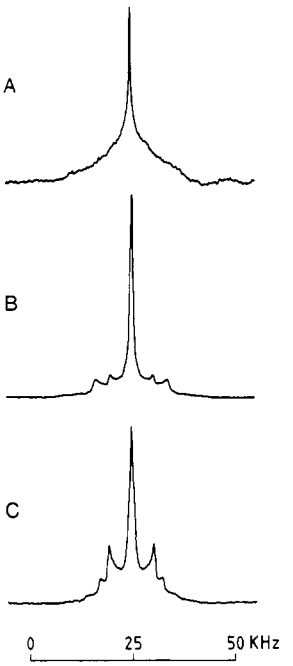


FIGURE 3: ^2H NMR spectra of *E. coli* strain T 131 GP grown on [1,1-$^2H_2$]glycerol. Labeling of the α-head group and the *sn*-1 backbone segments. Measuring temperature 25 °C. (A) *E. coli* cell membranes. (B) Total lipid extract dispersed in buffer. (C) Purified *E. coli* PG.

Phosphatidyl-α-CD_2-glycerol. Addition of [1,1-$^2H_2$]glycerol to the growth medium of strain T 131 GP leads to the selective labeling of the *sn*-1 segment of the glycerol backbone as well as the α-segment (*sn*-1′ segment) of the PG head group. Figure 3 compares the ^2H NMR spectra of intact cells (Figure 3A) with those of aqueous dispersions of the total lipid extract (Figure 3B) and purified PG (Figure 3C). These spectra are more complicated due to the motional inequivalence of the two backbone deuterons. One of the C–D bonds containing the 1*S* deuteron is close to the magic angle giving rise to the sharp central component, while the other deuteron (1*R*) produces a splitting of about 17 kHz (cf. Gally et al., 1981). In contrast, the ^2H NMR of α-CD_2-POPG is quite simple and consists of just one quadrupole splitting of about 10.5 kHz (Figure 2B). This head-group signal is also well-resolved in dispersions of the *E. coli* total lipid extract (Figure 3B) and in purified *E. coli* PG (Figure 3C) but is broadened in intact membranes. The shape of the cell membrane spectrum indicates that the head group contributes to the total spectrum, but an evaluation of the quadrupole splittings is not possible. For the other bilayer systems the splittings of the α-head-group segment and the *sn*-1 backbone segment are listed in Tables I and II, respectively.

Phosphatidyl-γ-CD_2-glycerol. A specific labeling of the γ-segment is achieved by addition of [3,3-$^2H_2$]glycerol to the growth medium. The ^2H NMR spectra of dispersions of the total lipid extract consist of a superposition of two large quadrupole splittings (25 and 27 kHz) and a narrow component (~0.8 kHz) (Figure 4B). The outer splittings arise from the two backbone deuterons of the *sn*-3 segment which are motionally inequivalent (Gally et al., 1981). The intense narrow splitting must be assigned to the γ-segment (*sn*-3′ segment) of the glycerol head group since its quadrupole splitting agrees with previous results obtained for the γ-segment of 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol

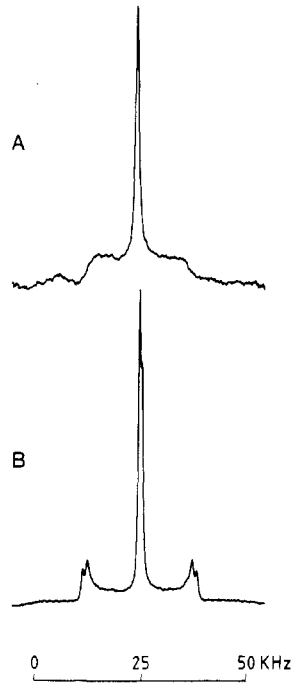


FIGURE 4: ^2H NMR spectra of *E. coli* strain T 131 GP grown on [3,3-$^2H_2$]glycerol. Labeling of the γ-head group and *sn*-3 backbone segments. Measuring temperature 25 °C. (A) *E. coli* cell membranes. (B) Total lipid extract dispersed in buffer.

(Wohlgemuth et al., 1980). The ^{31}P NMR spectrum of the same sample shows the characteristics of a fluid lipid bilayer and rules out the possibility that the intense inner component originates from micellar lipids or other isotropic structures (Seelig, 1978; Cullis & de Kruijff, 1979).

The ^2H NMR spectra of *E. coli* membranes (Figure 4A) resemble closely those of the total lipid extract, consisting also of a broad component of about 25 kHz splitting (width at half-height) and an intense narrow signal with a width of about 1 kHz (cf. Tables I and II). The intensity ratio of the two signals is also very similar in the cell membrane and the pure lipid bilayer. As noticed before, the intrinsic line width is increased in the ^2H NMR spectra of the intact cell membrane, and the two backbone deuterons are no longer resolved.

The ^{31}P NMR spectra of *E. coli* cell membranes (at 121.4 MHz) were consistent with those obtained at lower field strength (van Alphen et al., 1980; Burnell et al., 1980). In addition to a bilayer component originating from the membrane lipids the spectrum also contained an isotropic component which was assigned to the phosphate groups of lipopolysaccharides of the outer membrane.

^2H Spin-Lattice Relaxation Times. Spin-lattice (T_1) relaxation times were measured by inverting the magnetization by a 180° pulse and observing the return to equilibrium by using the quadrupole echo sequence. Figure 5 shows a selected set of ^2H NMR spectra obtained with *E. coli* cell membranes grown with addition of [3,3-$^2H_2$]glycerol. Variation of the relaxation delay reveals three classes of deuterons. The slowest relaxing component ($T_1 \sim 120$ ms) arises from residual HDO which cannot be completely eliminated, even not by dispersing the membranes in deuterium-depleted buffer. The two faster relaxing components are most clearly seen in the two spectra of Figure 5 with relaxation delays of $\tau = 9$ and 16 ms. Under these conditions the magnetization of the HDO peak is still negative whereas the broad outer splitting of the *sn*-3 backbone segment and the narrow component of the γ-segment have already returned to a positive magnetization. The evaluation of T_1 relaxation times from these membrane spectra is difficult

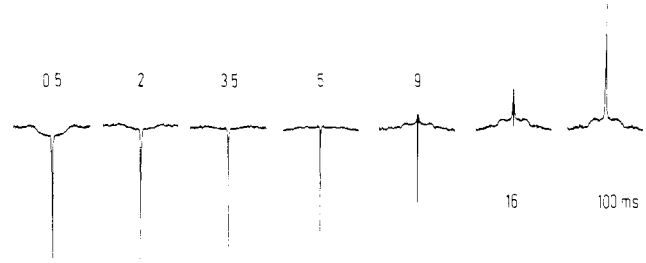


FIGURE 5: T_1 relaxation time measurement (at 46.1 MHz) of *E. coli* cell membranes. Growth medium supplied with [3,3-$^2H_2$]glycerol leading to a selective labeling of the γ-head group segment and the *sn*-3 backbone segment. The membrane suspension also contains traces of HDO. Measuring temperature 25 °C. The numbers refer to the relaxation delay in milliseconds.

Table III: Deuterium Spin-Lattice (T_1) Relaxation Times (ms) of Phosphatidylglycerol Labeled at the Glycerol Head Group

temp (°C)	POPG	*E. coli* PG (purified)	*E. coli* PG (in total lipid extract)	*E. coli* membrane (inner and outer *E. coli* membrane)
		α-Segment [1',1'-$^2H_2$]		
40	17.0	ND	18.7	resonances
25	10.2		10.7	not resolved
15	7.3		7.5	
5	5.8			
		β-Segment [2'-^2H]		
40	15.3	19.5	18.7	10.2
25	9.8	10.9	11.1	7.3
15	6.9	7.8	7.4	~5
5	5.6	6.0		
		γ-Segment [3',3'-$^2H_2$]		
40			22.4	21.5
25	ND	12.5 (20 °C)	11.5	8.4
15			7.9	5.5
5			6.0	

for overlapping resonances such as the γ-segment and the HDO resonances. This problem is not encountered for pure lipid bilayers where the T_1 times can hence be determined with higher accuracy. Representative T_1 relaxation time data for the three PG head-group segments in synthetic POPG, purified *E. coli* PG, total lipid extract, and cell membranes are summarized in Table III. Corresponding data for the backbone segments are found in Table II. Both the backbone and the head-group segments relax with very short T_1 times. All three backbone segments have T_1 relaxation times of the order 5–8 ms independent of the type of membrane studied and with little variation with temperature. The T_1 relaxation times of the head-group segments are also fairly similar to each other and fall in the range of 5–22 ms. However, in contrast to the backbone segments the head-group T_1's exhibit a much stronger dependence on temperature. Finally, it should be noted that the T_1 relaxation times are shorter in the cell membrane than in the pure lipid dispersion.

Discussion

Bilayers Composed of Synthetic POPG and DPPG. POPG was synthesized in order to study the influence of cis unsaturation on the behavior of the glycerol head group. The gel-to-liquid crystal phase transition of POPG is centered around −5 °C, and POPG bilayers are therefore in the fluid state at physiological temperatures. Since most naturally occurring phospholipids carry a cis-unsaturated fatty acid at the *sn*-2 position, POPG bilayers are more closely related to biological cell membranes than the fully saturated 1,2-di-

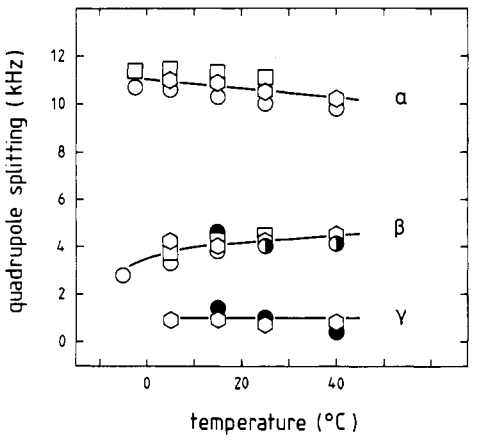


FIGURE 6: Variation of the quadrupole splittings of the three glycerol head-group segments with temperature. (○) POPG; (□) purified *E. coli* PG; (○) total lipid extract; (●) *E. coli* cell membranes.

palmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG) which has been studied before (Wohlgemuth et al., 1980; Browning, 1981). However, if compared at temperatures above the phase transition (which for DPPG is T_c = 41 °C), the quadrupole splittings of the two lipids are in quantitative agreement, with $\Delta\nu_\alpha$ = 10–11 kHz and $\Delta\nu_\beta$ = 3–4 kHz. Moreover, the α- and β-segments retain their characteristic temperature behavior in both bilayers: a rise in temperature decreases the α-splitting but increases the β-splitting. Since the quadrupole splittings are quite sensitive indicators of even small structural changes, it can be concluded that the cis double bond has no or little effect on the conformation of the phosphoglycerol head group.

A similar conclusion is reached for the dynamics of the head-group motion as reflected in the deuterium spin-lattice (T_1) relaxation times. A comparison of POPG (cf. Table III) and DPPG bilayers (Browning, 1981) in the liquid-crystalline state reveals a close quantitative agreement of the T_1 relaxation times of the corresponding head-group segments. The incorporation of a cis double bond has therefore little influence on the reorientation rates of the glycerol head-group segments as long as the bilayers are kept at temperatures above the gel-to-liquid crystal phase transition.

Lipid Bilayers Containing E. coli PG. The temperature dependence of the quadrupole splittings of the various systems is summarized in Figure 6. One notes a close similarity between the segmental splittings of synthetic POPG, purified *E. coli* PG, *E. coli* PG in mixture with *E. coli* PE, and *E. coli* PG in intact cell membranes. For a given segment the differences in the quadrupole splittings amount to at most 10%. This result is in contrast to a recent study by Sixl & Watts (1982), who report distinct changes in the α- and β-head-group splittings of 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol upon addition of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine. In order to understand this discrepancy we have added 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine to bilayers of *E. coli* PG deuterated at both the β-head group and the glycerol *sn*-2 backbone segment. Variation of the PG/PC weight ratio from 0.25 to 4 induced at most a 10% increase in both quadrupole splittings. Hence, the differences between our results and those of Sixl & Watts (1982) remain unexplained at present.

As an additional control we also investigated pH effects on the conformation of the glycerol head group. Changing the pH from pH 5.7 (Pipes buffer) to pH 8.5 (Tris buffer) reduced the quadrupole splitting of the β-segment from 4.4 to 4.25 kHz (at 18 °C). This small change in $\Delta\nu_\beta$ may not even be a pH effect but could be brought about by the change in buffer composition.

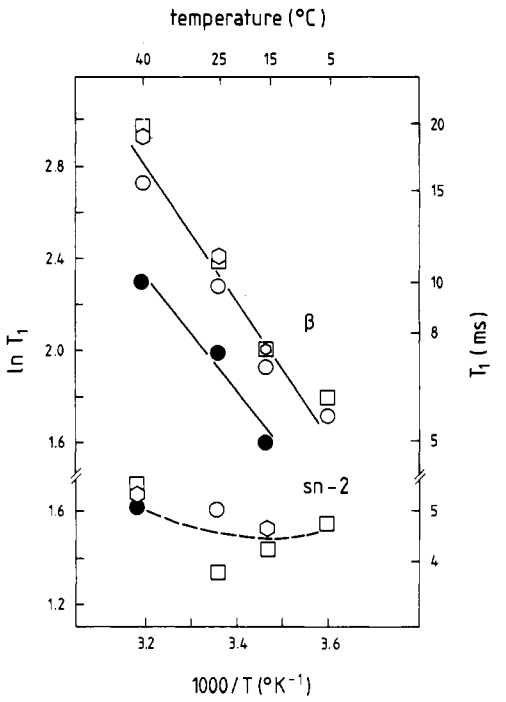


FIGURE 7: Variation of the deuterium T_1 relaxation time with temperature. Data are shown for the β-segment of the glycerol head group and the *sn*-2 segment of the glycerol backbone. (○) POPG; (□) purified *E. coli* PG; (○) total lipid extract; (●) *E. coli* cell membranes.

A comparison of the T_1 relaxation times of the various bilayer systems including *E. coli* membranes is displayed in Figure 7. For the sake of clarity only the data of *sn*-2′ (head group) and *sn*-2 (backbone) segment are shown. However, the essential features of Figure 7 are also representative for the other segments (cf. Tables II and III).

Inspection of Figure 7 reveals that the T_1 relaxation times of the head-group segments increase with a rise in temperature, yielding approximately linear Arrhenius plots with an activation energy of about 25 kJ/mol. The Arrhenius plots of the α- and γ-segment (not shown) are almost superimposable on those of the β-segment. Thus all three head-group segments are characterized by very similar motional properties. This is quite different from the behavior of CH_2 segments in the hydrophobic membrane interior where one observes a distinct increase in the T_1 relaxation times toward the methyl terminal of the fatty acyl chains (Brown et al., 1979).

The negative slope of the Arrhenius plots demonstrates that the motion of the head-group segments falls into the fast correlation time regime with $\omega_0\tau_c \ll 1$ (ω_0 = Larmor precessional frequency in rad s^{-1}; τ_c = correlation time of the segment reorientation). To a first approximation T_1 may be evaluated in terms of a single correlation time τ_c, neglecting the effects of anisotropic diffusion (Brown et al., 1979; Ghosh & Seelig, 1982). The segmental correlation times of the PG head group are found to be in the range of 0.1–0.5 ns (40–5 °C).

The T_1 relaxation times of the backbone segments are fairly short (5–8 ms) and exhibit a rather weak temperature dependence. In fact, the experimental T_1 values are close to the T_1 minimum which for isotropic motion is $(T_1)_{min}$ = 2.4 ms (at 46.1 MHz) and can be expected to be somewhat larger for anisotropic motions. Bearing this in mind the relaxation times of the *sn*-2 segment of pure *E. coli* PG (squares in Figure 7) have been interpreted tentatively in terms of a relaxation minimum around 20 °C although the scatter of the data is appreciable. The magnitude and the flat temperature de-

pendence of the backbone T_1 relaxation times are consistent with earlier studies on bilayers of *E. coli* PE (Ghosh & Seelig, 1982). The reorientational correlation times of the backbone segments are $\tau_c \sim 0.3$–0.5 ns. At the low-temperature end of our measurements the glycerol backbone and head-group segments appear to move equally fast in the bilayer membrane.

E. coli Cell Membranes. The pertinent aspects of the data which must be considered are as follows: (1) It was possible to identify specific glycerol head-group segments in intact cell membranes. (2) Only small differences in the quadrupole splittings and somewhat larger differences in the T_1 relaxation times were observed between cell membranes and pure lipid bilayer aggregates. (3) The apparent ^{2}H NMR line width was found to be distinctly larger in cell membranes than in lipid bilayers.

Previous studies with the same *E. coli* glycerol auxotroph failed to detect specific PG head-group resonances (Gally et al., 1981). In the light of the present results this can be explained by the sensitivity limitations of the high-resolution spectrometer employed before. By use of the quadrupole echo technique (Davis et al., 1976), it was now possible to resolve unambiguously the β- and the γ-segment of the phosphoglycerol head group in intact cell membranes. Likewise, the *sn*-2 and *sn*-3 segments of the glycerol backbone were also observed. The backbone-to-head group intensity ratio was 1:0.2 for growth on [2-^{2}H]glycerol and 1:0.3 for growth on [3,3-$^2H_2$]glycerol. When the considerable error associated with such intensity measurements is taken into account, the results are in rather good agreement with the biochemically expected value of 1:0.2. This analysis can be extended further by evaluating the signal-to-noise ratio of the three PG head-group signals displayed in Figure 1. It should be noted that the amount of PG increases from the membrane pellet (containing $\sim$ 11–14 mg of PG) over the total lipid extract ($\sim$18 mg of PG) to the pure PG sample ($\sim$40 mg). However, after correction for this difference and also for the different number of scans, the relative signal-to-noise ratios are 1–0.85 (membrane):1 (total lipid extract):0.85 (purified PG). Within the experimental error of $\sim$20% (as judged from a comparison of the total lipid extract with purified *E. coli* PG) the membrane spectrum has the same signal-to-noise ratio as the pure lipid bilayer aggregates. Hence, it is safe to conclude that a large fraction (>80%), if not all, of the glycerol head groups in *E. coli* is NMR visible.

No well-defined quadrupole splittings were detected for the α-segment due to the overlap with the backbone *sn*-1 deuterons. However, the line shape of the membrane spectrum, in particular the intensity around $\pm$5 kHz from the center, suggests that the α-segment contributes appreciably to the overall line shape.

The fact that all or most PG head groups in the *E. coli* membrane can be detected by ^{2}H NMR is nontrivial in view of the various possible modes of lipid–protein interaction which may range from pure lipid solvation involving only weak hydrophobic forces to a specific and tight binding of lipids to membraneous proteins. Due to its net negative charge phosphatidylglycerol is a potential candidate for electrostatic interactions, and specific interactions with metal ions and proteins have indeed been demonstrated in pure PG model membranes (cf. Paphadjopoulos, 1977; van Dijck et al., 1975; Findlay & Barton, 1978; Dombrose et al., 1979). A particularly strong binding was observed for Ca^{2+} ions. Addition of Ca^{2+} at concentrations >10 mM to dispersions of *E. coli* PG or POPG leads to an immediate loss of the ^{2}H NMR signal (F. Borle and J. Seelig, unpublished results). This can be explained by a "crystallization" of the PG bilayers and a shift of the gel-to-liquid crystal phase transition from –5 to 40 °C (cf. also van Dijck et al., 1975; Findlay & Barton, 1978). In contrast, the PG head groups remain NMR visible in *E. coli* membranes down to 10 °C which is even below the phase transition temperature of *E. coli* PE. This is taken as evidence that the NMR-visible PG head groups are not involved in any strong interactions neither with proteins nor with multivalent ions.

A comparison of the quadrupole splittings of *E. coli* cell membranes with those of the extracted lipids (cf. Tables I and II) reveals a close similarity between the two systems. This holds true for the glycerol head group as well as for the backbone. Since the quadrupole splittings are structural parameters and as such are quite sensitive to even small changes in the head group or backbone conformations (Seelig & Seelig, 1980; Akutsu & Seelig, 1981), it follows that the glycerol head group must assume the same average conformation in intact cell membranes as in pure lipid model membranes. It should further be noted that the quadrupole splittings of the PG α- and β-segments are almost identical with those observed for the ethanolamine head group (Seelig & Gally, 1976), suggesting a similar head-group conformation of the two lipid classes. From ^{2}H NMR and neutron diffraction studies it is known that the phosphoethanolamine dipole is extended parallel to the surface of the membrane exhibiting only a limited flexibility (Seelig & Gally, 1976; Büldt & Seelig, 1980). The present ^{2}H NMR data suggest that the same model may also be applicable to the glycerol head group. Neutron diffraction studies are in preparation in order to further investigate this hypothesis.

A further point of interest is the magnitude and the observed changes in the T_1 relaxation times. The β- and γ-segments are characterized by T_1 relaxation times in the range of 20–5 ms (40–15 °C). The segmental motions fall in the fast correlation time regime since the Arrhenius plots of the T_1 relaxation times yield negative slopes with activation energies of about 20 (β-segment) to 40 kJ/mol (γ-segment). This allows a direct comparison with ^{2}H T_1 relaxation times of liquid glycerol ([1,1,3,3-$^2H_4$]glycerol) which were measured at a lower frequency (9.2 MHz) but also fall in the fast motional regime (Wolfe & Jonas, 1979). This comparison is of particular relevance because the T_1 relaxation times of liquid glycerol correlate with the bulk viscosity and may thus provide a measure of the effective viscosity at the membrane surface. Typically, T_1 relaxation times of 7–9 ms were observed for the β- and γ-segments at 25 °C, and this value was also measured for liquid glycerol at 40 °C (T_1 = 9 ms) corresponding to a glycerol viscosity of η = 2.9 P. In view of the identity of the molecular structures and segments investigated we suggest that equal T_1 relaxation times also reflect equal viscosities.[3] The effective viscosity at the membrane surface as probed by the glycerol head group is thus in the range of a few poise. To our knowledge, this is the first quantitative estimate of the motional restrictions experienced by a specific lipid polar group in an intact cell membrane.

The observed viscosity at the membrane surface is much larger than might be anticipated in a naive view from the location of the glycerol head group at the lipid–water interface and the bulk viscosity of water which is $\eta \sim 0.01$ P. Moreover, we have also measured the T_1 relaxation time of [3,3-$^2H_2$]-

[3] The motion of liquid glycerol is isotropic, while the head-group segments move anisotropically. However, the influence of the order parameter on the T_1 relaxation time is usually negligible except for extremely well-ordered systems (Brown et al., 1979).

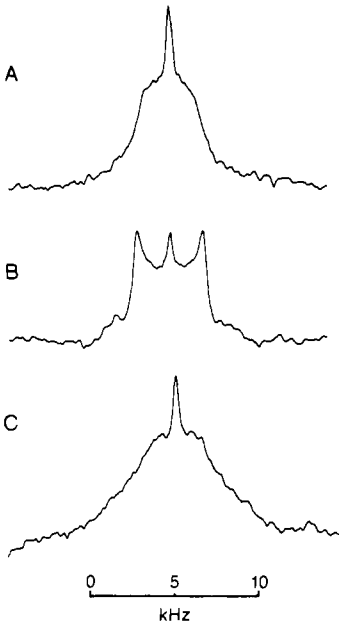


FIGURE 8: ^2H NMR spectra of the β-segment of the phosphatidylglycerol head group. (A) Intact *E. coli* cell membranes at 40 °C. (B) Coarse dispersions of synthetic POPG above the gel-to-liquid-crystalline phase transition (40 °C). (C) Same sample as (B) but measured immediately below the phase transition temperature (~−5 to −6 °C).

glycerol (10 wt %) in aqueous solution. The T_1 relaxation times were found to be 194 and 287 ms at 25 and 40 °C, respectively, which is by about a factor of 20 larger than the relaxation times of the same glycerol segment in the phosphatidylglycerol bilayer. This comparison again demonstrates the considerable difference between the membrane–water interface and the bulk aqueous phase. As to the molecular origin of this surface viscosity the most obvious explanation is to assume an extensive hydrogen-bonding network comprising lipid polar groups as well as proteins and water. The involvement of proteins is suggested by the observation that the T_1 relaxation times in intact cell membranes are shorter (corresponding to larger viscosities) by a factor of 2–1.5 than in the pure lipid extract dispersions (cf. Figure 7). It should further be realized that the T_1 relaxation times of the glycerol head-group segments are shorter by a factor of 2–3 than those measured for the methylene segments of the hydrophobic part of the lipid bilayer (cf. Brown et al., 1979). If the T_1 relaxation times are interpreted in terms of a microviscosity (cf. Seelig & Seelig, 1980), this comparison suggests that the microviscosity at the lipid–water interface is at least of equal magnitude, if not larger, than the microviscosity estimated for the hydrophobic interior of cell membranes.

An as yet unresolved problem is the question of the intrinsic line width of the cell membrane spectra. Regardless of the specific segment investigated the ^2H NMR spectra of the intact cell membranes were always found to be broadened compared to those of the corresponding pure lipid bilayers. This is illustrated in more detail in Figure 8 where the spectrum of the β-segment of the PG head group in *E. coli* membranes is displayed on an extended scale. Also included in Figure 8 are the corresponding spectra of synthetic β-CD-POPG measured above (Figure 8B) and immediately below (Figure 8C) the gel-to-liquid crystal phase transition. The *E. coli* cell membrane spectrum exhibits approximately the same width at half-height and the same relative signal-to-noise ratio as the model membrane in the liquid-crystalline state whereas the gel state spectrum is broader and has a lower signal-to-noise ratio. On the basis of these two criteria it can be concluded that the *E. coli* cell membrane spectrum bears a closer resemblance to the liquid-crystalline state of POPG than to the gel state. The same type of line broadening was also observed for reconstituted membranes containing a single type of protein. In fact, the protein-induced line broadening is the most obvious qualitative difference between pure lipid bilayers and protein-containing membranes (cf. Tamm & Seelig, 1983, and references therein). The increase in intrinsic line width is indicative of an additional type of lipid motion with considerably lower frequencies than the fast segmental movements which determine the spin-lattice relaxation time. Quantitative estimates place the rate of the protein-induced low-frequency motions in the range of 10^5–10^7 Hz (Paddy et al., 1981; Cornell et al., 1982). Several suggestions have been made as to the nature of the low-frequency motions, but the molecular origin of this effect and its significance for membrane function remain unclear at present.

Concluding Remarks. Nature has provided us with a vast diversity of lipid polar groups, the functional role of which remains largely unknown. There seems to be a great deal of interplay between many different forces which operate at the levels of thermodynamic stability, kinetic control, membrane potential, etc., and very few general principles have emerged to be helpful in understanding the complexities of lipid head-group structures. Physical–chemical studies on lipid polar groups have been restricted to pure lipid bilayer aggregates, for the most part, and there is a dearth of information on head groups in intact biological membranes (Oldfield et al., 1976; London et al., 1979). The present analysis of phosphatidylglycerol is the first systematic characterization, from segment to segment, of a specific lipid head group in an intact biological membrane. In spite of the negative electrostatic charge most PG head groups were found to move freely in a fluidlike lipid bilayer environment. The function of the glycerol head group may therefore be sought more in the ability of glycerol to stabilize networks of "structured" water molecules which, in turn, could be important for the stability of membrane-bound proteins. It may also be noted that the PG fraction of the total lipid pool is largest during the growth of the cell membrane (exponential phase) and decreases at the expense of cardiolipin in the stationary phase. It may be speculated that a highly structured membrane surface is particularly important for the process of protein insertion into the membrane.

Acknowledgments

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Registry No. POPG, 87246-80-8; DPPG, 74300-16-6; 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoric acid, 62600-81-1.

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Pressure Dependence of 1,6-Diphenyl-1,3,5-hexatriene Fluorescence in Single-Component Phosphatidylcholine Liposomes†

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ABSTRACT: Small unilamellar vesicles (SUV) and multilamellar vesicles (MLV) of dimyristoyl-L-α-phosphatidylcholine (DMPC) and dipalmitoyl-L-α-phosphatidylcholine (DPPC) and the MLV of dioleoyl-L-α-phosphatidylcholine (DOPC) were examined by steady-state polarization fluorometry under pressure in the range 10^{-3}–2 kbar. Isothermal pressure induced phase transitions were observed in DPPC and DMPC vesicles incorporated with 1,6-diphenyl-1,3,5-hexatriene. The temperature to pressure equivalences, dT/dP, estimated from the transition point, $P_{1/2}$, are 29.5 °C kbar^{-1} for DPPC(SUV), 22.7 °C kbar^{-1} for DPPC(MLV), 22.2 °C kbar^{-1} for DMPC(SUV), and 25.9 °C kbar^{-1} for DMPC(MLV) in an aqueous phase containing 0.1 M KCl and 0.01 M tris(hydroxymethyl)-aminomethane at pH 8.2. Even though there is no phase transition, we are still able to estimate a dT/dP of about 21 °C kbar^{-1} for DOPC(MLV). All the values of dT/dP obtained from this study are within the range typical for lipid-involving processes. The fluidity changes in the pretransition in DMPC(MLV) and in DPPC(MLV) observed in isobaric temperature studies are not detected in the isothermal pressure study, suggesting that the thermal pretransition may not involve an appreciable volume change in the hydrocarbon regions.

The temperature-induced gel to liquid-crystalline phase transition has been observed in many phospholipid vesicles by different physical techniques such as electron spin resonance (ESR)[1] (Hubble & McConnell, 1971), light scattering (Abramson, 1971), nuclear magnetic resonance (Sheetz &

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[1] Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; PRODAN, 6-propionyl-2-(dimethylamino)naphthalene; DPPC, dipalmitoyl-L-α-phosphatidylcholine; DMPC, dimyristoyl-L-α-phosphatidylcholine; DOPC, dioleoyl-L-α-phosphatidylcholine; SUV, small unilamellar vesicles; MLV, multilamellar vesicles; ESR, electron spin resonance; Tris, tris(hydroxymethyl)aminomethane.

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