Seelig, J. (1977) *Q. Rev. Biophys. 10*, 353–418.
Seelig, J. (1978) *Biochim. Biophys. Acta 515*, 105–140.
Seelig, J., & Gally, H. U. (1976) *Biochemistry 15*, 5199–5204.
Seelig, J., & Browning, J. L. (1978) *FEBS Lett. 92*, 41–44.
Seelig, J., & Seelig, A. (1980) *Q. Rev. Biophys. 13*, 19–61.
Seelig, J., Gally, H. U., & Wohlgemuth, R. (1977) *Biochim. Biophys. Acta 467*, 109–119.
Seelig, J., Tamm, L., Hymel, L., & Fleischer, S. (1981) *Biochemistry 20*, 3922–3932.
Shepherd, J. C. W., & Büldt, G. (1978) *Biochim. Biophys. Acta 514*, 83–94.
Skarjune, R., & Oldfield, E. (1979) *Biochemistry 18*, 5903–5909.
Smith, I. C. P. (1978) *Can. J. Biochem. 57*, 1–14.
Sundaralingam, M. (1972) *Ann. N.Y. Acad. Sci. 195*, 324–355.
Thomas, A. F. (1971) in *Deuterium Labeling in Organic Chemistry*, pp 232–233, Appleton–Century–Crofts, New York.
Träuble, H., Teubner, M., Woolley, P., & Eibl, H. (1976) *Biophys. Chem. 4*, 319–342.
Volino, F., & Dianoux, A. J. (1979) *Mol. Cryst. Liq. Cryst. 49*, 153–158.
Wieslander, A., Ulmius, J., Lindblom, G., & Fontell, K. (1978) *Biochim. Biophys. Acta 512*, 241–253.
Wohlgemuth, R., Waespe-Šarčevič, N., & Seelig, J. (1980) *Biochemistry 19*, 3315–3321.
Worcester, P. L., & Franks, N. P. (1976) *J. Mol. Biol. 100*, 359–378.
Yeagle, P. (1978) *Acc. Chem. Res. 11*, 321–327.
Yeagle, P., Hutton, W., Huang, C.-L., & Martin, R. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3477–3481.

Motions and Interactions of Phospholipid Head Groups at the Membrane Surface. 2. Head Groups with Hydroxyl Groups†

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ABSTRACT: A set of phospholipids with zero, one, or two hydroxyl groups at various positions in the head group were synthesized to approach the question of hydrogen bonding at the surface of phospholipid membranes. These lipids had as head groups the alcohols propanol, ethanediol, 1,3-propanediol, and glycerol esterified to the phosphate. The four different properties of these lipids that were studied were the following: phase transition temperatures, packing properties in monolayers, the relative rates of head-group motions, and the "ordering" of the head group. The gel-to-liquid-crystalline phase transition temperatures of these derivatives were measured by differential scanning calorimetry, and the effect of hydroxyl group addition was found to be small. The phase properties were examined with phosphorus-31 NMR, and all lipids formed normal bilayer phases in aqueous mixtures. Measurement of the surface pressure–area diagrams for monolayers of these lipids showed that incorporation of a hydroxyl group into the head group had a "condensing" effect. This effect was dramatic and was attributed to the formation of hydrogen bonds within the plane of the lipid surface. These lipids were synthesized with deuterium labels on essentially every carbon segment in the head group. Measuring the ^2H NMR spin–lattice (T_1) relaxation time allowed a determination of the relative rates of head-group segmental motions. The addition of hydroxyl groups to the propyl head-group "skeleton" substantially reduced the rates of motion in both the liquid-crystalline and gel states. The head groups could be ordered in terms of increasing rigidity as propanol (0 -OH) < 1,3-propanediol (1 -OH) < ethanediol (1 -OH) < glycerol (2 -OH). This trend was observed for labels attached to all of the head-group carbon segments of the lipids in this set. The rates of motion of the different segments within the ethanediol or glycerol head groups were almost identical, whereas those of the propyl head group increased as one progressed toward the free end of the head group. The average activation energy for the motions involved in T_1 relaxation increased upon introduction of a hydroxyl group. Measurement of the deuterium residual quadrupole splittings showed that the initial PO_2–O–CD_2– segments of the hydroxyl-containing head groups have very similar ordering properties which differed distinctly from those of the propyl head group. Thus, the introduction of one hydroxyl group appears to alter the head-group conformation to a specific conformation which is shared by all of these hydroxyl group containing head groups.

The properties of the water–lipid interface region of phospholipid membranes are important from the viewpoints of both the physical chemistry of lipid surfaces and the structure and function of biological membranes. In this paper, the question of noncovalent bonds or interactions between phospholipid head groups at this interface will be addressed. The properties of a set of phospholipids with simple, short alkyl chains as head groups were explored in the preceeding paper in this issue (Browning, 1981a). The data obtained from this "reference set" of head groups will be applied to a more complicated set, those head groups containing hydroxyl groups. In this set, hydroxyl groups were placed at various positions on either an ethyl or a propyl "skeleton". By making these slight alterations in these head groups and then determining the effects of the alterations on the properties of the head groups in membranes, one can hope to infer the existence or nonexistence of hydrogen

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bonds in the plane of the membrane surface. Only one member of this set of hydroxyl group containing lipids occurs in nature, namely, phosphatidylglycerol; nevertheless, it may be possible to extend the principles gained here to other naturally occurring members of this group such as phosphatidylinositols or glycolipids.

Hydrogen bonding at membrane surfaces has remained an elusive topic. Generally, the best approach has been to infer characteristics of the membrane surface from the study of a macroscopic property such as the thermodynamics of the phase transition. Several workers have studied head-group interactions using this method (Jacobson & Papahadjopoulos, 1975; Träuble et al., 1976; Eibl & Blume, 1979; Eibl & Woolley, 1979; Jähnig et al., 1979; Cevc et al., 1980). The phase transition enthalpy change has been calculated as the sum of a term due to the hydrophobic chain contribution and a term accounting for the repulsive force of the negatively charged phosphates of phosphatidic acid and phosphatidylmethanol (Träuble et al., 1976). With this simple treatment, poor agreement with experimental data was obtained. The deficiency of the model was presumed to be due to enthalpy contributions from nonelectrostatic interactions, i.e., hydrogen bonding (Eibl & Wooley, 1979; Cevc et al., 1980). Bond shortening characteristic of hydrogen bonds is observed in the head-group regions of several crystal structures such as phosphatidylethanolamine (Hitchcock et al., 1974; Elder et al., 1977), phosphoserine (Putkey & Sundaralingam, 1970), sphingolipids (Pascher, 1976), and even in a hydroxyl group containing head-group analogue, glycerol 3-phosphate (Fenn & Marshall, 1972). Hydrogen bonding in a crystalline form containing only a few molecules is readily conceivable; however, the important question as pointed out by Eibl & Woolley (1979) is whether or not these bonds are retained in the aqueous environment of the membrane surface. In one experimental system consisting of a hydrogen bond donor, diglyceride, and an acceptor molecule, phosphatidylmethanol, the existence of hydrogen bonding at the surface could be inferred (Eibl & Woolley, 1979).

In this paper, macroscopic properties such as the packing of lipids in monolayers and phase transition temperatures and some microscopic properties such as the head-group ordering and segmental motions in the head groups have been examined. Since all of the compounds studied here under these conditions bear formally one negative charge, only the capacity to hydrogen bond (or possibly to become more highly solvated) has been altered. These measurements were carried out on membranes composed of the pure phospholipid and in mixtures with cholesterol and phosphatidylcholine. These mixtures were studied since they occur in biological membranes, and these additives can theoretically disrupt interactions at the membrane surface. Phosphatidylcholine addition does not change the spacing of the phosphate groups and should not alter bonding to this group. On the other hand, cholesterol increases the separation between all of the interacting groups and should weaken the noncovalent interactions. While the actual effects of these lipid additions may be much more complex, they represent an attempt to distinguish between inter- and intramolecular interactions.

Experimental Procedures

Figure 1 gives the names of the phosphate-esterified portions of the head groups, structures, and abbreviations of the compounds discussed in this paper.

Syntheses. Perdeuterated ethylene glycol was purchased from Merck. Deuterated 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerols (DPPG's)[1] were the generous gift of Dr. J. Seelig. Synthesis of the deuterated DPPG derivatives has been described (Wohlgemuth et al., 1980). Thin-layer chromatography (TLC) was performed in a chloroform/methanol/H_2O (65/25/4) system.

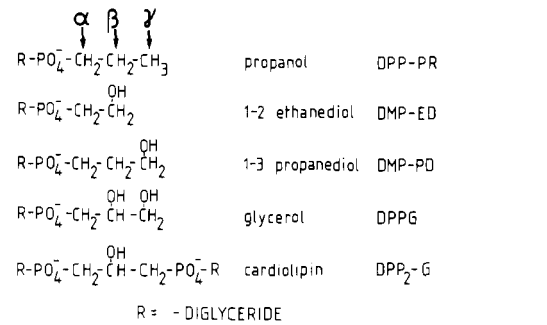

FIGURE 1: Structures and names of the esterified portions of the head groups used in this study as well as the abbreviation for the entire lipid. DMP and DPP refer to the 1,2-dimyristoyl-*sn*-glycero-3-phospho and 1,2-dipalmitoyl-*sn*-glycero-3-phospho derivatives, respectively.

1,3-(1,1-$^2H_2$)Propanediol. 3-Hydroxylpropionic acid lactone (Fluka, 5.6 mL, 0.08 M) was added dropwise to a stirred cold suspension of lithium aluminum deuteride (2 g) in 30 mL of diethyl ether. After addition, the solution was allowed to come to room temperature and stirred for 1 h. Water (2 mL) was added, and the suspension was filtered. The preciptate was stirred in 100 mL of acetone and filtered again. This extraction was repeated 3 times. The ether and acetone extracts were pooled and rotavaped, and the resultant oil was distilled (Büchi microdistillation apparatus). The 210–220 °C boiling fraction was taken (boiling point of pure 1,3-propanediol, 214 °C) and redistilled. Gas chromatography showed the product to be ~90% pure, and the ^1H NMR spectrum indicated the correct deuterated product, yield 2.4 g (45%).

(1,2-Dimyristoyl-sn-glycero-3-phospho)(1,1,2,2-$^2H_4$)(perdeuterated)ethanediol (DMP-ED). This compound was synthesized from dimyristin and perdeuterated ethanediol via a dioxaphospholane intermediate as described by Eibl (1978). Yield from 1 g of dimyristin was 0.4 g (32%). The product was pure as judged by TLC on silica gel, R_f = 0.40–0.45, in chloroform/methanol/H_2O (65/25/4). The sodium salt was prepared by titrating a stirred mixture of the lipid dissolved in dichloromethane and aqueous saturated sodium chloride with a Na_2EDTA solution (pH 10.0) to pH 7.0. The dichloromethane phase was collected and concentrated. The resultant solids were dissolved in dichloromethane, and any nonsoluble solids (salts) were discarded. The dichloromethane solution was concentrated to a small volume, acetone was added, and the precipitated product was collected. The product comigrated on TLC with authentic DMP-ED prepared by an enzymatic procedure (Comfurius & Zwaal, 1977) essentially as described for DMP-PD.

(1,2-Dimyristoyl-sn-glycero-3-phospho)-1,3-(1,3-$^2H_2$)-propanediol (DMP-PD). This compound was prepared by transesterification of phosphatidycholine with phospholipase D after the procedure of Comfurius & Zwaal (1977). 1,3-

[1] Abbreviations used: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPP-PR, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-1-propanol; DMP-PR, 1,2-dimyristoyl-*sn*-glycero-2-phospho-1-propanol; DMP-ED, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanediol; DPP-ED, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanediol; DMP-PD, 1,2-dipalmitoyl-*sn*-glycero-3-phospho-1,3-propanediol; DPPG, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol; DPP_2-G, 1,3-bis(1,2-dipalmitoylglycero-3-phospho)glycerol (cardiolipin); DMP_2-G, 1,3-bis(1,2-dimyristoylglycero-3-phospho)glycerol (cardiolipin); TPS-Cl, 2,4,6-triisopropylsulfonyl chloride; Pipes, 1,4-piperazinediethanesulfonic acid; CSA, chemical shift (or shielding) anisotropy.

Table I: Gel-to-Liquid-Crystalline Phase Transition Temperatures for Various Hydroxyl Group Containing Phospholipids[a]

head group (R = $-PO_4^-$-diglyceride)	lipid	transition temp (°C)
Diacyl Phospholipids		
palmitoyl chains (16:0)		
R-ethyl	DPP-ET	41
R-propyl	DPP-PR	42
R-ethanediol	DPP-ED	41
R-glycerol	DPPG	41[b]
myristoyl chains (14:0)		
R-propyl	DMP-PR	22
R-ethanediol	DMP-ED	26
R-1,3-propanediol	DMP-PD	23
R-glycerol	DMPG	23[b]
Tetraacyl Phospholipids		
palmitoyl chains (16:0)		
R-glycerol-R[c]	DPP_2-G	40[d]
myristoyl chains (14:0)		
R-glycerol-R[c]	DMP_2-G	24[e]

[a] From differential scanning calorimetry in excess 50 mM Pipes–Tris, 0.1 M NaCl, and 1 mM EDTA, pH 7.0. Data are accurate to within ±0.5–1.0 °C. [b] Jacobson & Papahadjopoulos (1975). [c] Cardiolipin. [d] Rainier et al. (1979). [e] Temperature was extrapolated from the chain length dependence of the transition temperatures of the NH_4^+ and Na^+ salts of cardiolipin; data of Rainier et al. (1979).

(1,1-$^2H_2$)propanediol (0.5 g) was mixed with 4.5 mL of 0.05 M sodium acetate buffer, pH 5.5, with 0.1 M $CaCl_2$ and 20 mL of alcohol-free diethyl ether. DMPC (1.5 g) was added followed by 100 mg of cabbage phospholipase D (Worthington). The lyophilized enzyme could be added directly to the ether solution. The mixture was stirred at 40 °C overnight. After 1 h, 1 mL of 1 M $CaCl_2$ was added to replace the calcium chelated by the product, which falls out of solution. The entire suspension was filtered, the precipitate was dissolved in 150 mL of warm chloroform/methanol/H_2O (65/25/4), and charcoal and Hyflo were added. The warm solution was filtered, and the clear solution was allowed to cool to room temperature whereupon the product precipitated. The precipitate was filtered and converted to the pure acid by treatment with Dowex 50 (Browning & Seelig, 1979). The sodium salt was formed as described for DMP-ED. At this point, the product was pure except for trace amounts of phosphatidic acid. Chromatography over silica gel as described for the alkyl derivatives (Browning, 1981a) yielded the pure product, 0.9 g (58%), R_f 0.42–0.47, in chloroform/methanol/water (65/25/4). Anal. Calcd for $C_{34}H_{64}D_2O_9PNa \cdot H_2O$ (M_r 692): C, 58.98; H, 10.11. Found: C, 58.49; H, 9.98. The product contains the CD_2 label equally distributed between the 1 and 3 positions of the head group. The phospholipase enzyme cannot incorporate 1,3-propanediol into a phospholipid in a stereospecific manner (Joutti & Renkonen, 1976; Wohlgemuth et al., 1980). This molecule will be described hereafter as α,γ-CD_2-DMP-PD but is actually 50% α-CD_2, 50% γ-CD_2 DMP-PD.

Sample Preparation. Multilamellar dispersions of these lipids were formed in 50 mM Pipes–Tris buffer, pH 7.0, with 1 mM EDTA and 0.1 M NaCl as described before (Browning, 1981a).

Measurements. Scanning calorimetry, ^{31}P and ^2H NMR, and monolayer measurements were carried out as described previously (Browning, 1981a).

Results

Phase Properties. Differential scanning calorimetry was carried out on these compounds to determine their phase transition temperatures. Additionally, phosphorus-31 NMR spectroscopy was employed to characterize the types of phases formed by these lipid–water mixtures [see review by Cullis & de Kruijff (1979)]. The gel-to-liquid-crystalline phase transition temperatures of this hydroxyl group containing series of phospholipds are summarized in Table I. A phase transition temperature of 35 °C was previously reported for DMP-ED which is in poor agreement with these data, although the exact conditions of the previous determination were not given (Eibl, 1978). Since each of these lipids bear formally one negative charge, the effect on the phase transition temperature of the additional hydroxyl groups is obtained directly. Table I shows that the effect is very small. The phase transition temperatures of two synthetic cardiolipins have been included for comparison (Rainier et al., 1979). It was seen (cf. Table I) that the cardiolipin compounds have phase transition temperatures similar to those for the corresponding phosphatidylglycerols.

^{31}P NMR spectra of some of these analogues are shown in Figure 2. These spectra are typical for dispersed phospholipids in a bilayer phase (Cullis & de Kruijff, 1979; Seelig, 1978). Under these conditions, no detectable amounts of an isotropic phase were observed. The ^2H NMR spectra also showed no indication of a large amount of isotropic (small vesicle) phase. Both phosphatidylglycerol (Wohlgemuth et al., 1980) and natural cardiolipins are known to form a bilayer phase in the absence of calcium (Cullis et al., 1978).

Monolayer Properties. Surface pressure–area diagrams for these phospholipids are shown in Figure 3. With pure water

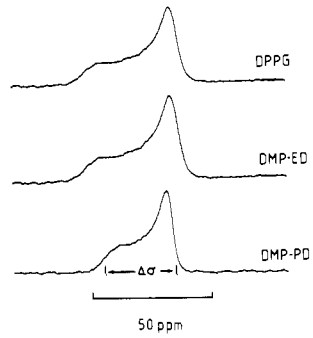

FIGURE 2: Phosphorus-31 NMR (121 MHz) spectra of several phospholipids with head groups containing hydroxyl groups. Spectra were measured at 40 ± 3 °C with about 1 kW of inverse gated proton decoupling power with a pulse repetition rate of 1 s.

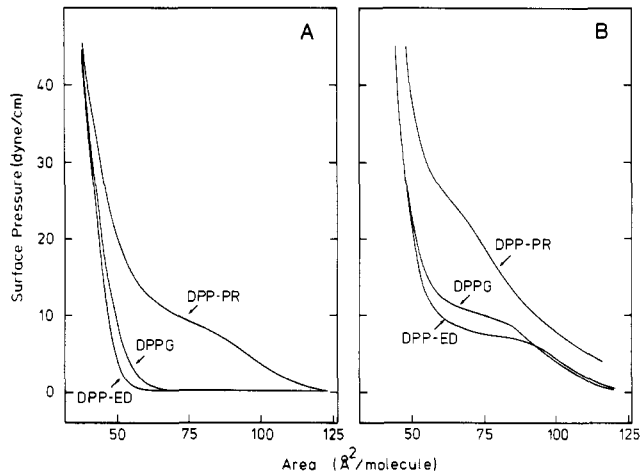

FIGURE 3: Comparison of the surface pressure–area diagrams for three dipalmitoyl phospholipids with varying numbers of hydroxyl groups in the head group. The subphase was pure water, pH 5.4–5.8 (A), or 2 mM Pipes–Tris buffer, pH 7.0, 0.1 M NaCl, and 1 mM EDTA (B). Curves are shown for monolayer compression at 22–24 °C.

Table II: Surface Areas of Various Phospholipids in Monolayers

		area (Å²/molecule)			
		pure water[a]		buffer, 0.1 M NaCl[b]	
lipid	head group	15 dyn/cm	30 dyn/cm	15 dyn/cm	30 dyn/cm
DPP-PR	propyl	53	44	81	54
DPP-ED	ethanediol	43	40	52	47
DPPG	glycerol	44	40	54	47

[a] The pH was consistently 5.4–5.8, and the temperature was 22–24 °C. [b] Pipes-Tris buffer (2 mM), pH 7.0, with 1 mM EDTA. Accuracy was about ±1 Å²/molecule.

Table III: Phosphorus-31 Chemical Shift Anisotropy (ppm) of a Hydroxyl Group Containing Series of Phospholipids (45 °C)[a]

		mol % cholesterol	
lipid	head group	0	50
DPP-PR	propyl	−22	−27
DMP-PD	1,3-propanediol	−31	−36
DMP-ED	ethanediol	−36	−39
DPPG	glycerol	−42	−40
natural[b]	cardiolipin	−(30–35)	

[a] When the data are obtained from a plot of CSA vs. temperature, the accuracy is about ±1–2 ppm. A consistent deviation may result when compared with values obtained with a computer fit to the spectra. [b] Cullis et al. (1978), bovine heart cardiolipin measured at 30 °C.

(pH 5.4–5.8) as the subphase, those lipids with hydroxyl groups in their head groups, DPP-ED and DPPG, were easily compressed to a small area whereupon the pressure increased sharply. In contrast, DPP-PR, lacking a hydroxyl group, offers resistance to compression even at relatively large surface areas. Essentially the same results were obtained with a subphase of buffer, pH 7.0, and 0.1 M NaCl (same buffer as used for the NMR measurements). However, at this pH and in the presence of the high salt concentration, the monolayers expanded considerably. Experiments performed with a pH 7.0 buffer and a salt concentration less than 1.0 mM Na^+ resembled the results shown in Figure 3B; thus, the bulk of the difference between the curves in Figure 3A,B can be attributed to the difference in pH. With the compression rates used in these experiments, some hysteresis was observed between the compression and expansion curves. Equilibrium values are presented in Table II. These values agree well with data for DPPG and dilauroylphosphatidylpropanol (Sacrē & Tocanne, 1977), and the shapes of the DPPG curves resemble those of previous studies (Sacrē & Tocanne, 1977; Sacrē et al., 1979; El Mashak & Tocanne, 1979).

Phosphorus-31 NMR. The motional and orientational properties of the phosphate segment of these lipids were examined by comparison of their chemical shielding anisotropies (CSA) [cf. Seelig (1978)]. The separation of the edges of the powder spectra shown in Figure 2 [measured as described by Browning & Seelig (1980)] yields the CSA value, and the data have been summarized in Table III. The CSA values of DMP-ED and DMP-PD were intermediate between those of DPP-PR and DPPG. In this sense, they are more akin to the CSA values of phosphatidic acid and a phospholipid with phosphomethanol as its head group (Browning, 1981a). The cardiolipin CSA was reported to be about −30 to −35 ppm (Cullis et al., 1978), which is similar to those of the 1,3-propanediol and ethanediol head groups. Among the diacyl phospholipids, cholesterol shifted the CSA values of all phospholipids of this series toward a common value of about

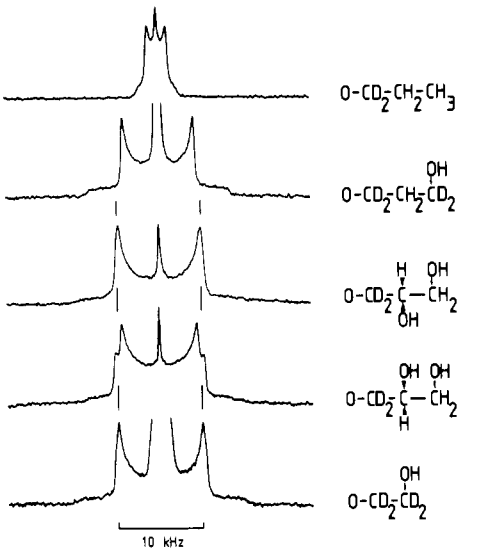

FIGURE 4: Comparison of the ^2H NMR spectra of phospholipids with hydroxyl groups in varying positions in the head group. The spectra are for the α-CD_2 label of each head group. In the DMP-ED and DMP-PD cases, the β and γ segments, respectively, also contained deuterium, accounting for the strong center signal. Samples were 1/1 molar mixtures with DMPC and were measured at a common reduced temperature of $\theta = 0.01$. In the calculation of the reduced temperature, the phase transition temperature was assumed to vary linearly between the transition temperatures of the pure components. Lines mark the quadrupole splitting of the 3,1′-DPPG compound.

−36 to −40 ppm. The CSA values of the pure compounds which were less than this value, i.e., DPP-PR, DMP-ED, and DMP-PD, became smaller with cholesterol addition while the opposite effect was observed with DPPG.

^2H Quadrupole Splittings. ^2H spectra of selectively deuterated DPP-PR (Browning, 1981a) and DPPG (Wohlgemuth et al., 1980) have been well characterized. From a structural viewpoint, the compounds DMP-ED and DMP-PD with ethanediol and 1,3-propanediol head groups, respectively, can be considered as intermediates between the propyl (DPP-PR) and glycerol (DPPG) head groups. In Figure 4, the deuterium spectra of multilamellar dispersions of α-deuterated preparations of these compounds are compared. These spectra were obtained from DMPC/lipid mixtures (1/1 molar ratio) at a common reduced temperature, θ, where $\theta = (T - T_c)/T_c$, T_c being the phase transition temperature of the lipid (Seelig & Browning, 1978). The phase transition temperature of a lipid mixture was approximated as $T_{c\,\text{mixture}} = X_1 T_{c1} + X_2 T_{c2}$ where X_i is the mole fraction of the ith component (Findlay & Barton, 1978). Similar deuterium spectra were obtained with the pure lipids. The most striking feature of Figure 4 is the similar size of the quadrupole splittings (the separation of the two peaks) for those head groups containing hydroxyl groups. A quadrupole splitting of about 10 kHz was found for these lipids which is distinctly different from the α-CD_2 quadrupole splitting of the ethyl, propyl, or butyl head groups. Second, in some spectra, two signals were observed at this position. This apparent splitting is commonly observed and results from the inequivalence of the two CD_2 deuterons (Wohlgemuth et al., 1980; Browning & Seelig, 1980; Gally et al., 1981). In pure bilayers of 3,3′-DPPG and DMP-ED, both α-CD_2 deuterons were observed, whereas only one signal was found for this segment in DPP-PR, DMP-ED, and 3,1′-DPPG.

The entire ^2H NMR spectra of DMP-ED and DMP-PD in bilayers composed of only the labeled lipid and in mixtures with cholesterol or DMPC (1/1 molar ratio) are shown in Figure 5. The data are collected in Table IV. There are two signals with large (8–11 kHz) and small (0–2 kHz)

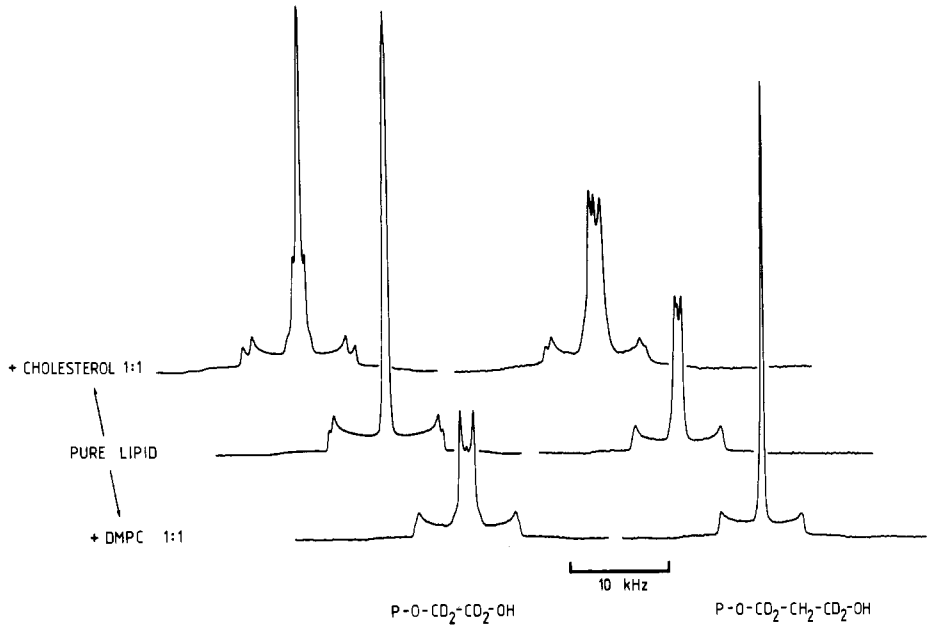

FIGURE 5: ^2H/NMR spectra of phospholipids containing either α,β-deuterated ethanediol (DMP-ED) or α,γ-deuterated propanediol (DMP-PD) as head groups. Spectra are shown for the pure lipid ($\theta = 0.01$) or in 1/1 molar mixtures with DMPC ($\theta = 0.01$) or cholesterol (same temperature as for the pure lipid).

Table IV: ^2H Quadrupole Splittings (kHz) of a Series of Phospholipids with Hydroxyl Group Containing Head Groups[a]

deuterated position in head group	lipid	head group	DMPC (mol %)		cholesterol (mol %)
			0	50	50
α	DPP-PR	propyl	0.3	1.8	4.4, 3.1
	DMP-ED	ethanediol	10.6, 11.4	9.6, 10.3	9.1, 11.1
	DMP-PD	1,3-propanediol	8.7	8.5	8.7, 9.9
	DPPG	glycerol (3,1')[b]	9.3, 11.3	10.5, 8.8	
	DPPG	glycerol (3,3')	10.3	9.3	9.9
β	DPP-PR	propyl	4.9	5.9	1.2, 1.7[c]
	DMP-ED	ethanediol	0.5	1.3	1.1, 0.4
	DPPG	glycerol (3-*rac*)	3.3	3.3	1.4
γ	DPP-PR	propyl	1.3	1.5	1.2, 1.7[c]
	DMP-PD	1,3-propanediol	0.7	0.3	1.4
	DPPG	glycerol (3-*rac*)	0.6		

[a] At a reduced temperature $\theta = 0.01$, where $\theta = (T - T_c)/T_c$; cholesterol mixtures were measured at the same temperature as the pure lipid. [b] Optical configuration, 3-(stereonomenclature)-*sn*-glycerol, 1'-*sn*-glycerol (head group). Data for 0% DMPC are from Wohlgemuth et al. (1980). [c] Two signals were observed (β and γ) with quadrupole splittings of 1.2 and 1.7. An exact assignment was not possible.

quadrupole splittings associated with α-CD_2, β-CD_2 DMP-ED and α-CD_2, γ-CD_2 DMP-PD. The larger signal (shown in detail in Figure 4) was assigned to the α-CD_2 position. The quadrupole splittings of the terminal segments of the propyl, ethyl, and glycerol head groups were always less than 2 kHz; thus, the smaller signal can be attributed with certainty to the terminal β (DMP-ED) and γ (DMP-PD) segments.

In Figure 6, the temperature dependence of the quadrupole splittings of these pure lipids is presented. As is generally the case for such data, the size of the quadrupole splittings decreased with increasing temperature. The exceptions were the β-CD_2 position of DMP-ED and the β-CD position of DPPG (Wohlgemuth et al., 1980). Both signal sizes increased with increasing temperature, an effect which must be attributed to a temperature-induced conformational change. The behavior of this segment suggests considerable structural similarity between these two head groups.

In mixtures of these lipids with cholesterol or DMPC, it is important to consider whether all the components are properly mixed. Although the thermodynamic properties of these mixtures were not examined, DPPG is known to mix well with

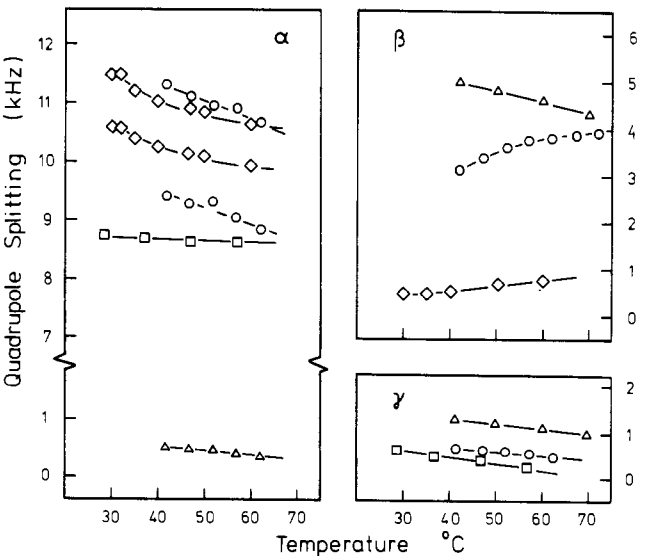

FIGURE 6: Temperature dependence of the quadrupole splittings of various deuterated segments of the hydroxyl group containing series of phospholipids. Data are from multilamellar dispersions of pure DPP-PR (△), DMP-ED (◇), DMP-PD (□), and DPPG (○).

DMPC without the occurrence of any phase separation (Findlay & Barton, 1978; Van Dijck et al., 1978). Generally, when the lengths of the hydrocarbon chains of the two components differ by less than four carbon segments, good miscibility is observed. The effects of cholesterol on the phase transition have not been reported, although by comparison with studies on other mixtures with cholesterol, one can expect that the phase transition is broadened and shifted to lower temperatures [cf. van Dijck (1979), van Dijck et al. (1978), and Demel & de Kruijff (1976)]. The behavior of DMP-ED and DMP-PD is assumed to be similar to that of DPPG in these mixtures.

The size of the α-position quadrupole splitting of all three compounds remained relatively constant in the presence of DMPC and cholesterol. In contrast, the β-CD_2 DMP-PD quadrupole splitting increased considerably in the order lipid/DMPC < lipid < lipid/cholesterol (Figure 5). If the β-CD_2 signal of DMP-ED changes in a continuous manner like the γ-CD_2 signal of DMP-PD in this sequence, then there appears to be a reversal in the sign of this quadrupole splitting. As discussed in the first paper of this series (Browning, 1981a), the absolute sign of the quadrupole splitting cannot be determined with this technique, and a sign reversal can only be inferred. Cholesterol and DMPC in mixtures with the alkyl head-group phospholipids altered the average orientation of the alkyl head group (Browning, 1981a). Cholesterol creates more space for the head groups and relieves steric crowding associated with the larger head groups. As a result, the head group could assume a more parallel conformation relative to the bilayer surface. The phosphatidylcholine head group is rather bulky and creates a more sterically hindered situation. In comparison with the propyl head group, these perturbants had relatively little effect on the ordering of the α segment; however, the effects encountered at the β segment are similar to those described for the alkyl head groups.

It was noted that cholesterol addition tends to enhance the differences between the two deuterons at the α position (Browning, 1981a). The same trend was observed with these compounds (cf. Figure 5). With DMP-PD, the two deuterons were well resolved only in the presence of cholesterol. The α-CD_2 signal of the 3,1′ diastereomer of DPPG in a 1/1 mixture with cholesterol was also split into two signals. In the DMP-ED/cholesterol mixture, two signals were observed not only at the α segment but also for the β-CD_2 segment. This result can be attributed either to two long-lived conformations or from an inequivalence of the two CD_2 deuterons. By comparison with DPPG, one can be confident that the two signals associated with the α-CD_2 segment of DMP-ED must also arise from inequivalent deuterons. It is unlikely that the two β-CD_2 signals of DMP-ED result from two conformers because some evidence of two distinctly different conformers should have been observed at the neighboring α segment. At a particular segment, a lack of certain symmetrical motions relative to the bilayer normal introduces inequivalence, and two signals are observed [cf. Browning (1981a) and Gally et al. (1981)]. With the alkyl head groups, two signals for the α-CD_2 group were found in mixtures with cholesterol. This observation was rationalized on the basis of the more parallel orientation obtained in the presence of cholesterol. It was proposed that this parallel orientation results in increased steric interactions with the region lying under the head groups (i.e., the glycerol backbone region), leading to a reduced number of conformational possibilities. With the hydroxyl group containing head groups, the difference in the quadrupole splittings of the two deuterons decreased in the order lipid/

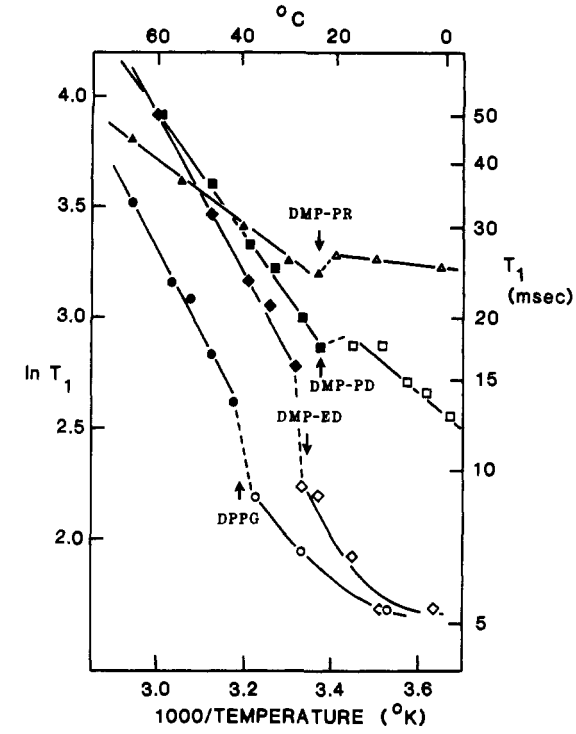

FIGURE 7: Temperature dependence of the α-CD_2 segment spin–lattice relaxation times of *dispersed* pure phospholipids in the liquid-crystalline (solid symbols) and gel states (open symbols): DPP-PR (△), DMP-ED (◇), DMP-PD (□), and DPPG (○). Data are expressed in the form of an Arrhenius plot of ln T_1 vs. 1/temperature. Arrows show phase transition temperatures.

cholesterol > lipid > lipid/DMPC, which was the order found with the alkyl head groups. A similar trend is suggested by the β-CD_2 data for DMP-ED. Thus, at the α segments of these hydroxyl-containing head groups, steric factors do not appear to appreciably affect the overall head-group orientation (size of the quadrupole splitting), yet steric crowding is still manifested in the motional freedom (inequivalence of the two CD_2 deuterons). At the β segments of these lipids, steric factors begin to influence both aspects.

The effects of cholesterol and DMPC on the quadrupole splittings of all three hydroxyl group containing lipids were quite small. At the α position of all three lipids, the quadrupole splittings became smaller. If there is a sign reversal at the β position of DMP-ED with cholesterol addition (as discussed above), then the changes at the β position of DPPG and DMP-ED are also in the same direction. These data suggest that the addition of cholesterol causes the DPPG and DMP-ED head groups to approach similar physical states. Similar behavior was found with the phosphatidylcholine and phosphatidylethanolamine head groups (Brown & Seelig, 1978). This tendency was also shown by the phosphorus chemical shielding anisotropy data.

^2H Spin–Lattice (T_1) Relaxation Times. ^2H spin–lattice relaxation times of multilamellar dispersions of this set of phospholipids were measured with a standard inversion–recovery experiment. The recovery of magnetization could be characterized by one exponential as indicated by linear ln ($M_0 - M_\tau$) vs. τ plots, where τ is the interpulse delay in a 180°–τ–90° experiment. Arrhenius plots of the data from the α position of several head groups are shown in Figure 7. As was discussed (Browning, 1981a), the data for the α position of DMP-PR and DPP-PR (and likewise DMPE and DPPE) overlap on an Arrhenius plot. Thus, on an empirical basis, an absolute temperature scale rather than a reduced scale is more appropriate for the comparison of head-group T_1 data

Table V: ^2H Spin-Lattice (T_1) Relaxation Times (ms) of a Series of Phospholipids with Hydroxyl Group Containing Head Groups

deuterated position in head group	lipid	head group	DMPC (mol %) 0 (20 °C)[a]	DMPC (mol %) 0 (47 °C)	DMPC (mol %) 50 (47 °C)	cholesterol (mol %) 50 (47 °C)
α	DPP-PR	propyl	22	32 ± 1	27 ± 1	33 ± 1
	DMP-PD	1,3-propanediol	16	37 ± 1	30 ± 2	38 ± 2
	DMP-ED	ethanediol	11	32 ± 1	32 ± 2	37 ± 2
	DPPG	glycerol (3,1′)	6.0	17 ± 1	22 ± 1	
	DPPG	glycerol (3,3′)			22 ± 1	21 ± 1
β	DPP-PR	propyl		56 ± 10	53 ± 3	
	DMP-ED	ethanediol	6.0	32 ± 1	33 ± 1	40 ± 1
	DPPG	glycerol (3-*rac*)		19 ± 2	22 ± 1	21 ± 1
γ	DPP-PR	propyl		195 ± 20	173 ± 20	
	DMP-PD	1,3-propanediol	21	54 ± 1	50 ± 1	66 ± 1
	DPPG	glycerol (3-*rac*)	14	28 ± 2		

[a] Extrapolated.

from various lipids. Such a comparison is presented in Table V. The T_1 relaxation times for all positions decreased with decreasing temperature, indicating that relaxation was in the short correlation time regime ($\omega_0\tau_c \ll 1$, where τ_c is the effective molecular correlation time and ω_0 is the NMR resonance frequency, $\omega_0 = 2.9 \times 10^8$ rad s^{-1}).

When compared at 47 °C (Table V), the α-segment T_1 value for the glycerol head group was the shortest followed by those of the ethanediol and the propanediol and propyl head groups. Thus, increasing numbers of hydroxyl groups decreased the T_1 relaxation times. Within this sequence, the T_1 values varied by a factor of 1.5–2.0 and are dependent on the presence of hydroxyl groups in the head group. At the β segment of these head groups, essentially the same trend was observed, only the effect of hydroxyl group addition was more pronounced and the T_1 relaxation times varied 2–3-fold at 47 °C. At the γ segment, hydroxyl group addition again dramatically reduced the relaxaton times. A direct comparison of relaxation times at the γ position of DMP-PD and DPPG with that of the propyl group is not strictly valid since the addition of a hydroxyl group disrupts the freer motion of the $-CD_3$ group independent of any interactions with the surrounding molecules. Nevertheless, a comparison between the 1,3-propanediol and glycerol head groups shows a reduced T_1 value for the glycerol γ segment. The data can be extrapolated to 20 °C (see Table V), where a very clear dependence on the number of hydroxyl groups and their proximity to the α segment was observed. This dependence was found in both the liquid-crystalline and gel states.

Also dependent on the same factors were the apparent activation energies for the motions involved in spin–lattice relaxation (obtained from the slopes of the lines in Figure 7). The activation energy of the α segment increased from 11–12 kJ/mol to over 30 kJ/mol upon going from the propyl to the glycerol head group (Table VI). In the absence of hydroxyl groups, the activation energy decreases with increasing head-group length; i.e., at the α position of the methyl, ethyl, and propyl head groups, values of 26, 21, and 12 kJ/mol were obtained. It is felt that a comparison with the propyl head group is valid since all the head groups are at least three bonds in length. On the other hand, steric factors are clearly important and cannot be easily separated. One trivial explanation for the increased activation energies is that the addition of a hydroxyl group to the α segment alters the energy barrier to rotation about the C_α–C_β bond, and simple steric restrictions result in the observed changes. However, in the 1,3-propanediol head group, C_α–C_β bond rotation should not be appreciably altered; nevertheless, the α-segment activation energy was still larger.

Table VI: Apparent Activation Energies for ^2H T_1 Relaxation

lipid	head group	no. of hydroxyl groups	E_a (kJ/mol)[a] fluid	E_a (kJ/mol)[a] gel
α position				
DPP-PR	propyl	0	11.2	
DMP-PR	propyl	0	11.8	~5
DMP-PD	1,3-propanediol	1	22.9	~13.5[b]
DMP-ED	ethanediol	1	29.8	
DPPG	glycerol	2	30.6	
γ position				
DMP-PD	ethanediol	1	27.1	~13.5[b]
DPPG	glycerol	2	20.8	

[a] Data are accurate generally to ±2 kJ/mol. [b] Only approximate due to overlap of the α and γ signals. However, both signals appeared to relax at the same rate.

Distinct breaks in the Arrhenius plots occurred at the phase transition temperatures of all the lipids studied. Below the phase transition, the activation energy and the magnitude of the T_1 relaxation time changed. Activation energies were lower for the both the propyl and 1,3-propanediol head groups in the gel state. The linearity of the data for these two head groups and the decreasing T_1 values with decreasing temperature indicate that relaxation is still occurring in the short correlation time regime. With the glycerol and ethanediol head groups, relaxation may not be in this region. As the motions slow, the condition $\omega_0\tau_c = 1$ is reached, and further reductions in the rates of motion lead to increasing T_1 values with decreasing temperature. Clearly $\omega_0\tau_c$ is not greater than 1, but with the quality of these data, a proper distinction cannot be made.

At the α segment of phosphatidylglycerol, the ^2H spectra of the two diasteromers, 3,1′ and 3,3′, differ (Wohlgemuth et al., 1980). The relaxation time of this segment was the same in both diastereomers in a 1/1 mixture with DMPC, and in the pure state, no difference was noted with a perdeuterated head group which contained a racemic mixture of these diastereomers. A 1–3-ms difference could have been discerned in these experiments.

These data and trends are summarized in the form of an α-position vs. β-position T_1 relaxation time plot in Figure 8. One important characteristic of these data concerns the equivalence of the α-T_1 and β-T_1 values. In Figure 8, the dashed line indicates the condition where α-T_1 = β-T_1. The propyl head-group deviates substantially from this line, yet the introduction of hydroxyl groups, i.e., DMP-ED or DPPG, brings the "α,β" coordinates onto the α-T_1 = β-T_1 line. In the third paper in this series (Browning, 1981b), it will be seen that only the choline and propyl head groups deviate from this

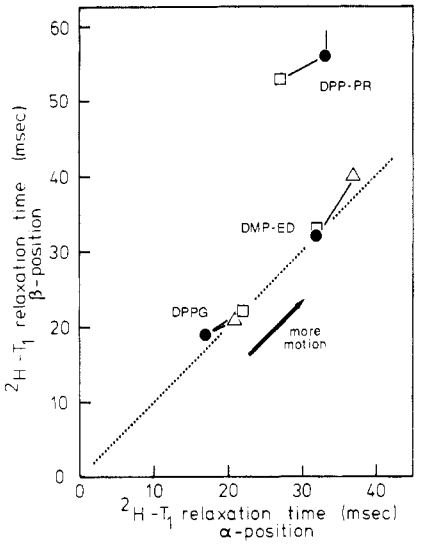

FIGURE 8: Plot displaying the α- and β-position ^{2}H spin–lattice (T_1) relaxation times of various hydroxyl group containing phospholipids. Data are presented for pure lipid (●) and in 1/1 molar mixtures with DMPC (□) and cholesterol (△) at 47 °C. Dotted line shows the point where the α and β splittings are identical.

condition. The question of inter- vs. intramolecular interactions can be approached by perturbing the system with either cholesterol or phosphatidylcholine. With DMP-ED, cholesterol addition resulted in longer relaxation times, while DMPC addition had little effect. The T_1 relaxation times of the glycerol head group were not appreciably perturbed by these agents (cf. Figure 8).

Discussion

This set of experiments was undertaken to determine whether hydrogen bonds exist at the membrane surface. Hydrogen bonds are postulated to form between an acceptor group, the nonesterified oxygens of the phosphate, and a donor group, i.e, the hydroxyl groups. None of the experiments described here can distinguish between a bond of this form, i.e., $-PO_4^-\cdots H-O-R$, and a similar bond with a water molecule bridging the donor and acceptor groups. Interhydroxyl bonds of the type R–O–H···OH–R′ are also possibilities. Toward this goal, four different independent aspects of the head-group properties have been examined. Upon formation of noncovalent bonds, the following effects could be expected: (i) interactions between head groups should result in a better packing of the lipids in monolayers; (ii) head groups should have less motional freedom, and the rates of segmental motions in the head group should decrease; (iii) inter-head-group interactions may result in a head group oriented parallel to the membrane surface; and (iv) the formation or breaking of noncovalent bonds at the phase transition may alter the phase transition temperature.

Packing Properties in Monolayers. Monolayer properties appear to be very sensitive to the formation of intermolecular noncovalent bonds at the aqueous interface. Upon introduction of hydroxyl groups into a head group, the molecular areas decreased dramatically. Since all three of the lipids compared, DPP-PR, DPP-ED, and DPPG, formally bear one negative charge and have similar phase transition temperatures (at least under the high salt concentration conditions), the experiments have been carried out effectively at a reduced temperature. Differences in the monolayer packing must reflect differences in the head-group region. The ethanediol head group is similar in bulk to the propyl head group, while that of phosphatidylglycerol is even larger.[2] There were no trends which could be explained on the basis of steric effects or simply the relative size of the head groups [steric effects can be quite large; cf. Browning (1981a)]. The lack of obvious steric effects emphasizes the importance of the hydroxyl groups on the packing properties. It is felt that hydrogen bonding in the plane of the membrane surface is a likely cohesive force leading to the observed condensation effect. This effect could be attributed to an intramolecular hydrogen bond which reduced the size of the head group and improved packing. However, it is difficult to imagine how such bonds could reduce the size of the relatively bulky glycerol head group to a size smaller than the propyl or butyl head groups. Furthermore, the deuterium data (see below) indicate that these hydroxyl group containing lipids have head-group conformations parallel to the membrane surface. To obtain condensation, it is expected that these bonds are to some extent intermolecular. Similar conclusions were reached from monolayer studies comparing mono- and digalactosyldiacylglycerols (Bishop et al., 1980).

Head-Group Motions. Rates of segmental motion can be conveniently measured with deuterium NMR spin–lattice (T_1) relaxation time measurements. Spin–lattice relaxation for deuterium occurs via a quadrupole mechanism which is sensitive primarily to the motions of the C–D bond. Neither intermolecular motions, the number of bound protons, nor the distances to neighboring nuclei contribute to this mechanism [cf. Brown et al. (1979) and Seelig (1977)]. Thus, a comparison of T_1 relaxation times from different head groups can tell us about the relative and to some extent the absolute rates of segmental motion in these head groups. The term "segmental motions" will be applied in a broad sense to describe those head-group motions whose frequency components contribute to spin–lattice relaxation. Only relatively high-frequency motions induce T_1 relaxation, and these motions are most likely to be fast rotations about the segmental bonds.

In the simplest treatment of deuterium T_1 relaxation in a membrane system, it is assumed that the motions involved in relaxation are isotropic. With this assumption and for data in the short correlation time regime ($\omega_0\tau_0 < 1$, which is the case for these data), the relaxation time is related to the correlation time for segmental reorientation (τ_c) by (Brown et al., 1979)

$$\frac{1}{T_1} = \frac{3}{8}\left(\frac{e^2qQ}{\hbar}\right)^2\left(1 + \frac{1}{2}S_{CD} - \frac{3}{2}S_{CD}^2\right)\tau_c \qquad (1)$$

where e^2qQ/h is the static quadrupole coupling constant (170 kHz for a C–D bond). S_{CD} is the C–D bond order parameter which is obtained directly from the quadrupole splitting ($\Delta\nu_q$) as

$$S_{CD} = \frac{4}{3}\left(\frac{e^2qQ}{h}\right)^{-1}\Delta\nu_q \qquad (2)$$

Equation 1 is valid only for those microdomains whose bilayer normal is perpendicular to the magnetic field, i.e., the peaks of the powder spectra. An angular dependence of the T_1 relaxation time is predicted but not observed. All parts of the head-group powder spectra were found to relax at the same rate, and a similar results have been reported for deuterium labels in the fatty acyl chains (Brown & Davis, 1981; Seelig et al., 1981). Brown & Davis (1981) have indicated that

[2] Surface areas of these head groups (including the phosphate) were estimated from the projection of space-filling models (CPK) onto the membrane surface to be approximately (Å^2) propyl 25, butyl 29, ethanediol 23, propanediol 26, and glycerol 28. A parallel head-group conformation was assumed.

lateral diffusion of the microdomains is sufficiently fast to account for a lack of an angular dependence of the T_1 value. The simplified treatment leading to eq 1 appears to be useful in describing the T_1 relaxation behavior in deuterated segments in the fatty acyl chains (Brown, 1979). In the head-group segments, the motions are undoubtedly more complex, and several correlation times may be required to properly describe the motions contributing to T_1 relaxation. However, lacking such detailed knowledge, one must employ an apparent or averaged correlation time. From eq 1, it is seen that the ordering of the C–D segment can affect the measured T_1 relaxation times. Experimentally it is found that the contribution of the C–D bond ordering to T_1 is small in the fatty acyl chains. The 9,10 deuterons in oleic acid containing phospholipids have very different geometries relative to the bilayer normal and hence different S_{CD} order parameters (0.12 and 0.02), yet both deuterons relax at the same rate (Brown, 1979; Seelig et al., 1981). Likewise in the head group of phosphatidylserine, both deuterons of the α-CD_2 segment have identical relaxation behavior despite quite different geometries (Browning, 1981b). The S_{CD} order parameters of the deuterons examined in this study are at the most 0.08. The anisotropic contribution to τ_c is very small, and eq 1 reduces to simply the expression for isotropic motion [cf. Abragam (1961)]. Thus, under these conditions, shorter T_1 values indicate slower rates of motion for those motions contributing to relaxation.

Three aspects of these relaxation experiments support a concept of noncovalent head-group binding. First, increasing the number of hydroxyl groups in the head group decreased the rates of head-group motions. At the α segment, the T_1 values are roughly proportional to the molecular weight of the esterified portion of the head group, a tendency which was previously noted in the study of the alkyl head groups (Browning, 1981a). A plot of T_1 vs. molecular weight of the head groups for all the lipids studied in these three papers showed a weak correlation with molecular weight at the α segment, but at the β position, there was no correlation. Hydroxyl group insertion lowered the T_1 values by factors of 2–3 in the liquid-crystalline phase and even more dramatically in the gel phase. The expected size of a decrease in T_1 values will be treated in the third paper (Browning, 1981b).

A second criterion for the evaluation of the relative dynamics of these head groups is provided by differences between T_1 values for various segments within one head group. The propyl head-group T_1 values increase in the order $\alpha < \beta < \gamma$ as would be expected when the flexibility increases along this alkyl chain. In this sense, the data resemble results obtained for the ends of the fatty acyl chains of the phospholipids (Brown et al., 1979; Lee et al., 1976) or simple alkanes (Levine et al., 1974). Adding one hydroxyl group leads to equivalent α- and β-segment T_1 values for this alkyl chain. A likely interpretation of this phenomenon would be that hydrogen bonds are limiting the motion of the normally free end of the head group. Consequently, the head group moves as a single entity, and those rates of motion which are measured with T_1 experiment become independent of the position in the head group. The glycerol head group is a good example of this effect where the ^2H T_1 values are almost identical at all three segmental positions.

A third aspect of the relative motional properties lies in changes in activation energies accompanying the addition of hydroxyl groups to these head groups. These "apparent" activation energies correspond to an average energy barrier for the various head-group motions which contribute to the relaxation process. These data indicate some increase in the activation energy upon introduction of a hydroxyl group. However, at this point, it is not clear to what extent steric factors enter into this parameter. Similar data presented in the third paper in this series for phosphatidylcholine suggest that steric factors are quite important.

If interactions at the membrane surface underlie these observations, the inter- or intramolecular nature of the bonding can be approached by increasing the distance between phosphates (cholesterol addition) or by diluting the hydroxyl groups (phosphatidylcholine addition). While the addition of phosphatidylcholine does not alter the spatial distribution of phosphate groups, the choline portion of the head group cannot hydrogen bond, and inter-head-group bonds of the type R–O–H···OH–R′ should be less prevalent. The observed lack of any large effect of DMPC on the T_1 values suggests that these interhydroxyl bonds may not be common. By separating the hydrogen-bonding head groups with equimolar cholesterol, one can expect to increase the area per head group from about 60 to 90 Å (equimolar mixture, Demel & de Kruijff, 1976). This corresponds to an average increase in the separation between phosphate groups of about 1.9 Å (assuming a hexagonal lattice). Although the magnitude of the effect cannot be predicted, this expansion should affect a network of intermolecular but not intramolecular bonds. The increased DMP-ED T_1 relaxation times upon cholesterol addition indicate a contribution from intermolecular interactions. However, in mixtures with phosphatidylglycerol, neither phosphatidylcholine nor cholesterol had an appreciable influence on the dynamic behavior. Probably some intramolecular elements are involved within this head group. Such an intramolecular bond is present between the β-hydroxyl group and the phosphate in the crystal structure of glycerol 3-phosphate (Fenn & Marshall, 1972). One problem with the use of cholesterol to differentiate between these two types of bonds is the possibility that cholesterol itself participates in a bonding network or alters the hydration layer at the surface. The existence of an interfacial phosphatidylcholine–cholesterol bond has been disputed (Demel & de Kruijff, 1976; Müller-Landau & Cadenhead, 1979), and in mixtures with phosphatidylglycerol, little relevant data are available.

Head-Group Ordering. From the ^2H NMR quadrupole splitting, an order parameter for the C–D bond can be determined (Seelig, 1977). This order parameter depends on the *orientation* of the C–D bond axis relative to the normal to the membrane surface and *fluctuations* of the bond about this average orientation. While one cannot evaluate a priori the relative contributions of these two aspects to the order parameter, empirically it is found that the head-group order parameters are extremely sensitive to the head-group conformation. The resolution of small differences between diastereomers of phosphatidylserine and phosphatidylglycerol as well as the often observed inequivalence of the two deuterons of a CD_2 segment testifies to this sensitivity (Wohlgemuth et al., 1980; Browning & Seelig, 1980). On the other hand, head-group quadrupole splittings are always in the range of 0–20 kHz, which is only a fraction of the possible static value of 127 kHz. Thus, considerable motional averaging is occurring, leading to the small residual quadrupole splittings. It was found here that adding one or two hydroxyl groups to an alkyl skeleton resulted in the same ordering at the segment irrespective of the number or location of the hydroxyl groups. It was shown that for a particular quadrupole splitting there is a family of segmental orientations compatible with that quadrupole splitting (Skarjune & Oldfield, 1979). While there

is not a unique correspondence between conformation and residual quadrupole splitting, the number of possible conformations is vastly reduced. In this case, it is felt that the similarity of these quadrupole splittings represents similar head-group conformations and not simply similar quadrupole splittings for unrelated geometries.

Parallel head-group conformations have been established for both phosphatidylcholine and phosphatidylethanolamine by using several techniques [for reviews, see Seelig & Seelig (1980), Büldt & Wohlgemuth (1981), Hauser & Phillips (1979), and Yeagle (1978)]. Preliminary data on selectively deuterated cardiolipin labeled in the α position of the glycerol moiety of the head group show a quadrupole splitting of about 13.5 kHz (H. U. Gally, G. Plutschke, P. Overath, and J. Seelig, unpublished results). This value is very close to the 9–11 kHz observed at the same position in these hydroxyl group containing lipids. Similar trends are seen in the phosphorus chemical shift anisotropies. Because of the chemical bond linking the two diglyceride sections of cardiolipin, this head group is restrained in a parallel conformation. These data taken together suggest that the cardiolipin, phosphatidylglycerol, phosphatidylethanediol, and phosphatidylpropanediol head groups share a conformation which is oriented parallel to the membrane surface. Since a perpendicular head-group conformation will not allow extensive intermolecular noncovalent bonding, it is possible that a hydrogen-bond network fixes the head group in a parallel orientation.

This bonding network appears to make the head group less perturbable to factors such as cholesterol and phosphatidylcholine, irrespective of whether one considers dynamic or ordering parameters. Thus, in the bulk lipid phase of biological membranes with up to 50% cholesterol and with other lipid species, the properties defined for the head groups in homogeneous systems may be retained. Studies on the *Escherichia coli* phosphatidylglycerol head group in membranes composed of total extracted *E. coli* lipids have supported this prediction (Gally et al., 1981).

Phase Transition Temperatures. Changes in the head-group region of a phospholipid bilayer can affect the packing of the lipids and hence their thermodynamic properties. The phase transition temperature is one such parameter which can be correlated with differences in the head-group properties between the gel and liquid-crystalline states. In favorable circumstances, some of the details of the head-group interactions can be deduced. Inherent to this method is the problem of the complexity of the head-group interactions and their effects on the thermodynamics of the systems [cf. Jacobson & Papahadjopoulos (1975)].

The data in this paper suggest the existence of some form of hydrogen bonding in the plane of the membrane surface. It would be expected that the nature of these interactions would change at the phase transition and hence would have some consequence on the phase transition temperature. Experimentally, it was seen, at least in the presence of 0.1 M salt, that adding one or two hydroxyl groups to a propyl or ethyl head group had no substantial effect on the transition temperature. The phase transition temperature is given by

$$T_c = \Delta H/\Delta S$$

where ΔH and ΔS are phase transition enthalpy and entropy changes, respectively. Hydrogen bonding will shift T_c only if the liquid-crystalline phase is stabilized or destabilized relative to the gel phase. Hydrogen bonding should contribute to ΔH; however, the effects on ΔS are difficult to predict. An enthalpy contribution to T_c could be compensated by entropy changes at the transition. One possibility for a large entropy contribution is water restructuring at the transition. Such water restructuring has been suggested on the basis of extrapolation of ΔS to zero chain lengths (Seelig, 1980).

Cardiolipin can be considered to be a phosphatidylglycerol where the γ-hydroxyl group is chemically bonded instead of hydrogen bonded to the phosphate of a neighboring molecule. Although bond breaking or formation at the phase transition cannot occur, one would expect that the packing of such a tetraacyl phospholipid would be altered. When lipids with homologous fatty chains are compared, little difference in T_c is found between phosphatidylglycerol and cardiolipin (Ranier et al., 1979). This comparison suggests that the use of changes in T_c may not be a good indicator of noncovalent interactions in some cases.

Is it feasible that a hydrogen-bonding network exists within the plane of the membrane surface with these lipids? In this regard, it is of interest to examine the crystal structure of glycerol 3-phosphate, disodium salt (Fenn & Marshall, 1972). In this structure, almost every oxygen atom participates in two noncovalent interactions with neighboring atoms. The distance between phosphates (along the *a* or *c* crystal axes) is about 6.5 Å, which is close to the corresponding distance of 8.3–8.5 Å in the fluid-state bilayers [phosphatidylcholine, cf. Shepherd & Büldt (1978)] and even closer to the gel-state separation of 7.5 Å [L_β phase of phosphatidylcholine, see Tardieu et al., (1973)]. With this packing density and the assumption that the three-dimensional hydrogen-bonding network is similar to the quasi-two-dimensional lattice of the membrane–water interface, some noncovalent bonding should be present. At high temperatures in the liquid-crystalline state (for these data above 60 °C), the bonding is probably considerably weakened and does not contribute to the surface properties.

In the present analysis of the surface of these membranes, rigid noncovalent bonds are not expected. Rather within this short-lived network, there is fast exchange between various ligands, most likely involving both intra- and intermolecular arrangements. At any one moment, there are many ligands in a bound form representing a net contribution to the surface properties. The fast exchange allows for some motional flexibility in the head group and certainly lateral diffusion. Since no individual states are resolved in the ^2H NMR spectra, these exchange rates must be faster than 10^3–10^5 s^{-1}.

Acknowledgments

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References

Abragam, A. (1961) in *Principles of Nuclear Magnetic Resonance*, p 314, Oxford University Press, London.

Bishop, D. G., Kenrick, J. R., Bayston, J. H., MacPherson, A. S., & Johns, S. R. (1980) *Biochim. Biophys. Acta 602*, 248–259.

Brown, M. F. (1979) *J. Magn. Reson. 35*, 203–215.

Brown, M. F., & Seelig, J. (1978) *Biochemistry 17*, 1381–1384.

Brown, M. F., & Davis, J. H. (1981) *Chem. Phys. Lett.* (in press).

Brown, M. F., Seelig, J., & Häberlen, U. (1979) *J. Chem. Phys. 70*, 5045–5053.

Browning, J. L. (1981a) *Biochemistry* (first of three papers in this issue).

Browning, J. L. (1981b) *Biochemistry* (third of three papers in this issue).

Browning, J. L., & Seelig, J. (1979) *Chem. Phys. Lipids 24*, 103–118.
Browning, J. L., & Seelig, J. (1980) *Biochemistry 19*, 1262–1270.
Büldt, G., & Wohlgemuth, R. (1981) *J. Membr. Biol. 58*, 81–100.
Cevc, G., Watts, A., & Marsh, D. (1980) *FEBS Lett. 120*, 267–270.
Comfurius, P., & Zwaal, R. (1977) *Biochim. Biophys. Acta 488*, 36–42.
Cullis, P., & de Kruijff, B. (1979) *Biochim. Biophys. Acta 559*, 399–420.
Cullis, P., Verkleij, A. J., & Ververgaert, P. H. (1978) *Biochim. Biophys. Acta 513*, 11–20.
Demel, R. A., & de Kruijff, B. (1976) *Biochim. Biophys. Acta 457*, 109–132.
Eibl, H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4074–4077.
Eibl, H., &Blume, A. (1979) *Biochim. Biophys. Acta 553*, 476–488.
Eibl, H., & Woolley, P. (1979) *Biophys. Chem. 10*, 261–271.
Elder, M., Hitchcock, P., Mason, R., & Shipley, G. G. (1977) *Proc. R. Soc. London, Ser. A 354*, 157–170.
El Mashak, E. M., & Tocanne, J. F. (1979) *J. Colloid Interface Sci. 70*, 56–66.
Fenn, R. H., & Marshall, G. E. (1972) *Biochem. J. 130*, 1–10.
Findlay, E. J., & Barton, P. G. (1978) *Biochemistry 17*, 2400–2405.
Gally, H. U., Pluschke, G., Overath, P., & Seelig, J. (1981) *Biochemistry 20*, 1826–1831.
Hauser, H., & Phillips, M. C. (1979) *Prog. Surf. Membr. Sci. 13*, 297–413.
Hitchcock, P. B., Mason, R., Thomas, K. M., & Shipley, G. G. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3036–3040.
Jacobson, K., & Papahadjopoulos, D. (1975) *Biochemistry 14*, 152–161.
Jähnig, F., Harlos, K., Vogel, H., & Eibl, H. (1979) *Biochemistry 18*, 1459–1467.
Joutti, A., & Renkonen, O. (1976) *Chem. Phys. Lipids 17*, 264–266.
Lee, A. G., Birdsall, N. J., Metcalfe, J. C., Warren, G. B., & Roberts, G. C. K. (1976) *Proc. R. Soc. London, Ser. B 193*, 253–274.
Levine, Y. K., Birdsall, N. J. M., Lee, A. G., Metcalfe, J. C., Partington, P., & Roberts, G. C. K. (1974) *J. Chem. Phys. 60*, 2890–2899.
Müller-Landau, F., & Cadenhead, D. A. (1979) *Chem. Phys. Lipids 25*, 329–343.
Pascher, I. (1976) *Biochim. Biophys. Acta 455*, 433–451.
Putkey, E., & Sundaralingam, M. (1970) *Acta Crystallogr., Sect. B B26*, 782–789.
Rainier, S., Jain, M. K., Ramirez, F., Ioannou, P. V., Marecek, J. F., & Wagner, R. (1979) *Biochim. Biophys. Acta 558*, 187–198.
Sacrē, M.-M., & Tocanne, J. F. (1977) *Chem. Phys. Lipids 18*, 334–354.
Sacrē, M.-M., Hoffmann, W., Turner, M., Tocanne, J. F., & Chapman, D. (1979) *Chem. Phys. Lipids 25*, 69–83.
Seelig, J. (1977) *Q. Rev. Biophys. 10*, 353–418.
Seelig, J. (1978) *Biochim. Biophys. Acta 515*, 105–140.
Seelig, J. (1980) in *Biophysics of Membranes and Intercellular Communication* (Balian, R., et al., Eds.) Les Houches, Session XXXIII, North-Holland Publishing Co., Amsterdam.
Seelig, J., & Browning, J. (1978) *FEBS Lett. 92*, 41–44.
Seelig, J., & Seelig, A. (1980) *Q. Rev. Biophys. 13*, 19–61.
Seelig, J., Tamm, L., Fleischer, S., & Hymel, L. (1981) *Biochemistry 20*, 3922–3932.
Shepherd, J. C. W., & Büldt, G. (1978) *Biochim. Biophys. Acta 514*, 83–94.
Skarjune, R., & Oldfield, E. (1979) *Biochemistry 18*, 5903–5909.
Tardieu, A., Luzzati, V., & Reman, F. C. (1973) *J. Mol. Biol. 75*, 711–733.
Träuble, H., Teubner, M., Woolley, P., & Eibl, H. (1976) *Biophys. Chem. 4*, 319–342.
van Dijck, P. (1979) *Biochim. Biophys. Acta 555*, 89–101.
van Dijck, P., de Kruijff, B., Verkleij, A., van Deenan, L., & de Gier, J. (1978) *Biochim. Biophys. Acta 512*, 84–96.
Wohlgemuth, R., Waespe-Šarčevič, N., & Seelig, J. (1980) *Biochemistry 19*, 3315–3321.
Yeagle, P. (1978) *Acc. Chem. Res. 11*, 321–327.