MAGNETIC ORIENTATION OF SPHINGOMYELIN–LECITHIN BILAYERS

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ABSTRACT Phospholipid bilayers consisting of a 60:40 mixture of *N*-palmitoylsphingomyelin and dimyristoylphosphatidylcholine orient in a strong magnetic field. The orientation is easily observed in ^{31}P- and ^{2}H-nuclear magnetic resonance spectra where the intensity of the perpendicular edges of the powder lineshapes are enhanced. The lineshapes indicate that the long axis of the molecule is perpendicular to the magnetic field.

INTRODUCTION

In a magnetic field molecules may orient due to the presence of an anisotropy in their diamagnetic susceptibility. For small molecules the degree of orientation, β, is directly proportional to the diamagnetic anisotropy, $\Delta\chi$, and to the square of the magnetic field, H (Maret and Dransfeld, 1977)

$$\beta = \Delta\chi\, H^2/k_B T, \quad (1)$$

where k_B is Boltzmann's constant and T is the absolute temperature. The degree of orientation is usually quite small even for molecules with large values of $\Delta\chi$. However, in a system where diamagnetically anisotropic molecules are held parallel to each other, as in a liquid crystal, the anisotropy is additive and the degree of orientation is also proportional to the number, N, of molecules in the cluster (Maret and Dransfeld, 1977).

$$\beta = N\Delta\chi\, H^2/k_B T. \quad (2)$$

Thus substantial orientation is often observed in liquid crystalline phases.

By this reasoning, phospholipids, which form bilayers with their acyl chains held parallel to each other, should also exhibit ordering in a magnetic field. Yet, even though there have been a great number of studies on the nuclear magnetic resonance (NMR) and electron spin resonance spectra (ESR) of lipid bilayers, which, of course, require the lipid sample to be in a magnetic field, nearly all of the experiments show negligible orientation effects (Gaffney and McConnell, 1974). Generally, the degree of orientation is so small that it is observed only by the very sensitive technique of magnetic birefringence or Cotton-Mouton effect (Gaffney and McConnell, 1974; Maret and Dransfeld, 1977). Although relatively large effects have been observed in some nonaqueous lipid samples (Sakurai et al., 1980; Larsen et al., 1984), there has been only one report of substantial magnetic ordering of hydrated lipid bilayers (Seelig et al., 1985). Seelig and co-workers detected by ^{2}H- and ^{31}P-NMR the magnetic orientation of hydrated bilayers of the lipid extract from *Escherichia coli* strain T 131 GP and also of a synthetic lipid mixture of 17 wt% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) and 83 wt% 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE). The NMR indicated that the lipid molecules were orienting with their long axis perpendicular to the magnetic field.

We report here another hydrated phospholipid mixture that exhibits a high degree of orientation in a magnetic field. The mixture is composed of 60 mol% *N*-palmitoylsphingomyelin (NPSM) and 40 mol% 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine(DMPC) and hydrated with 50 wt% water. When taken above the gel to L_α-phase transition, the lipids orient in the magnetic field, yielding NMR powder spectra with enhanced intensity in the perpendicular edges of the lineshapes. The results indicate that magnetic alignment of phospholipid membranes may be more common than previously supposed and raise questions on the mechanisms of orientation.

EXPERIMENTAL

Materials

N-[4,4-$^{2}H_2$] and *N*-[7,7-$^{2}H_2$]palmitoylsphingomyelin (4,4-d_2-NPSM and 7,7-d_2-NPSM) were synthesized using the procedure described by Calhoun and Shipley (1979) using palmitic acid labeled with ^{2}H at the 4 and

7 positions, respectively, prepared according to previously published methods (Das Gupta et al., 1982). 1-Myristoyl-2[6,6-$^2H_2$]myristoylphosphatidylcholine (6,6-d_2-DMPC) was synthesized by Avanti Polar Lipids (Birmingham, AL) using 2-[6,6-d_2] palmitic acid provided by us. Unlabeled DMPC was purchased from Sigma Chemical Co. (St. Louis, MO). The purity of all the lipids was checked by differential scanning calorimetry (DSC) prior to sample preparation.

Sample Preparation

Labeled NPSM and unlabeled DMPC were used to prepare several NMR samples varying from 40 to 80 mol% NPSM. ~50 mg of the 4,4-d_2-NPSM or 7,7-d_2-NPSM was used for each sample with an appropriate amount of DMPC to achieve the desired molar ratio. The NPSM and DMPC were dissolved together in a 2:1 methanol/chloroform solution. The solvent was evaporated under N_2 and lyophilized. The dry lipid mixture was dispersed in an equal weight of deuterium-depleted water (Sigma Chemical Co.) inside a 7-mm glass sample tube with a constriction and sealed under high vacuum. Pure lipid samples were prepared similarly and consisted of ~50 mg of lipid and an equal weight of deuterium-depleted water. All the sealed samples were heated above their phase transitions before use to assure uniform hydration.

Samples for use in microscopy were prepared generally in the same manner as those for use in NMR experiments except that enough water was added to form a 2 wt% lipid solution. The lipid/water sample was heated above the phase transition and then vigorously stirred or shaken to disperse the lipid. A drop of the sample solution was placed on a glass slide and covered with a coverslip.

NMR Spectroscopy

The NMR spectra were acquired on a home-built solid state spectrometer with a 9.35 T magnet (397.7 MHz for ^1H, 160.99 MHz for ^{31}P, and 61.05 MHz for ^2H). All ^2H spectra were recorded using the quadrupole echo (Davis et al., 1976) with a pulse spacing of 36 μs and a 90° pulse length of 2.1 μs or less. The ^{31}P spectra were recorded using a Hahn echo with decoupling and a 90° pulse of 4.7 μs. The decoupling power was kept at the minimum level necessary to obtain adequate decoupling and was used with a recycle delay of 5–7 s to avoid sample heating.

Temperature control was achieved by a gas flow system where heated air was blown directly into an insulated chamber housing the rf sample coil. The temperature was kept constant within 0.5°C during the experiments. The sample was allowed to equilibrate in the heated chamber for at least 20 min after each temperature change before the spectra were collected.

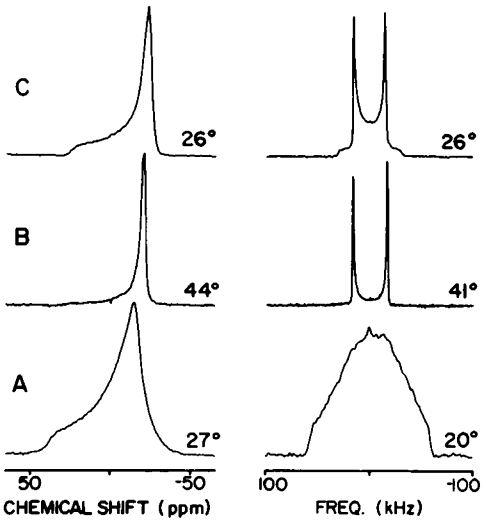

FIGURE 1 *A* and *B* show ^{31}P- (*left*) and ^2H- (*right*) NMR spectra of 60:40 NPSM/DMPC mixtures with 50 wt% water in the gel and L_α phases, respectively. The ^2H spectra were taken on mixtures containing 7,7-d_2-NPSM. The spectra in *A* are powder patterns typical of phospholipid membranes in the gel phase. The spectra in *B*, however, show the membranes to be oriented with the lipid long axis perpendicular to the magnetic field since nearly all the spectral intensity occurs at the perpendicular edges. The ^{31}P and ^2H spectra in *C* are of 6,6-$^2H_2$-DMPC in 50 wt% water in the L_α phase. They are typical motionally-averaged powder patterns and are shown for comparison to the spectra of oriented lipids in *B*. All ^{31}P spectra were taken at 160.99 MHz with proton decoupling and acquired using a Hahn echo. All ^2H spectra were taken at 61.05 MHz using a quadrupole echo.

RESULTS

Fig. 1 *A* shows typical ^2H- and ^{31}P-NMR powder patterns of 60% 7,7-d_2-NPSM/40% DMPC in 50 wt% water in the gel phase. Fig. 1 *B* shows spectra of the same sample after heating above the phase transition to the L_α-phase. Above T_c = 32°C the sample no longer yields a powder pattern but gives instead a "single crystal-like" lineshape where nearly all the intensity is at the perpendicular edge. The spectra obtained from the melted 60:40 NPSM/DMPC mixture in Fig. 1 *B* can be compared with the classic ^2H and ^{31}P fast limit powder patterns shown in Fig. 1 *C* obtained from melted 6,6-d_2-DMPC dispersed in 50 wt% H_2O. The ^2H- and ^{31}P-NMR spectra of 100% 7,7-d_2-NPSM as well as those of 40:60 and 80:20 NPSM/DMPC mixtures indicate little or no orientation.

Fig. 2 illustrates that once orientation is achieved in the L_α phase it persists after cooling to the gel phase. Fig. 2 (*left*) shows ^{31}P spectra obtained as the sample is heated from 23.5° to 44°C. Initially unoriented, the sample does not show orientation until heated above the phase transition. Fig. 2 (*right*) shows spectra obtained as the now

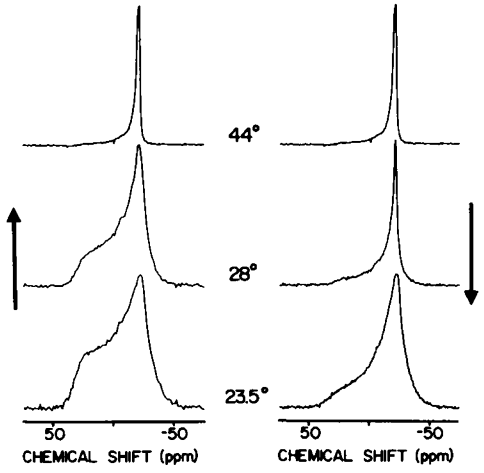

FIGURE 2 Proton-decoupled ^{31}P spectra of 60:40 NPSM/DMPC in 50 wt% water as a function of temperature, heating, and cooling. (*Left*) Spectra obtained as the sample was heated from 23.5° to 44°C. (*Right*) Side shows spectra as the sample was cooled from 44° to 23.5°C (*arrows*, direction of the temperature scan). In the gel phase the lipids are not oriented, but above T_c = 32°C in the L_α phase, orientation occurs and persists even after cooling back down into the gel phase. ^{31}P spectra were acquired at 160.99 MHz using a Hahn echo.

oriented sample was cooled from 44° to 23.5°C. With decreasing temperature, the lineshapes become broader due to decreased motional averaging, but they quite clearly retained a degree of orientation. The spectra of the oriented lipid in the gel phase were collected over ~20 h, indicating that in a strong magnetic field the orientation will persist in the gel phase for an extended period.

Spectra similar to those in Fig. 1 *B* can be erroneously obtained by beginning the Fourier transform of a free induction decay (FID) after the top of the echo; however, we were careful to eliminate that possibility by recording both sides of the echo and left-shifting to its peak (Griffin, 1981). In addition, the spectra exhibit other behavior characteristic of oriented samples. For example, in the L_α phase, the signal-to-noise ratio increases many times over that of unoriented samples, and the FID from an oriented sample persists for a longer time than an FID from, for example, melted DMPC.

DISCUSSION

Powder patterns like those in Fig. 1, *A* and *C* arise from the orientation dependence of the chemical shift or quadrupole splitting combined with a distribution of lipids in the sample such that all orientations are equally populated. The spectra in Fig. 1 *B* are not powder patterns and, in fact, resemble spectra arising from single crystals because, as in a single crystal, only one orientation is appreciably populated. Unlike a single crystal, the lipids are not oriented with respect to a crystal fixed axis system but with respect to the magnetic field. The intensity at the perpendicular edge of a ^2H powder pattern arises from lipids oriented in such a way that the symmetry axis of the electric field gradient (EFG) tensor is perpendicular to the field. In a rigid molecule the symmetry axis of the EFG tensor lies along the C-D bond, but in the melted lipid there is fast axial diffusion about its long axis, and the C-D vector rapidly samples every orientation perpendicular to this axis. The long axis then becomes the symmetry axis of the motionally averaged EFG tensor. Since the spectra of the 60:40 NPSM/DMPC mixture contains only perpendicular edges, the lipids must be orienting with their long axis perpendicular to the field, which would put the magnetic field in the plane of the bilayer. Similarly, the ^{31}P lineshape of Fig. 1 *B* has nearly all the intensity at the perpendicular edge because the lipids are oriented such that the symmetry axis of the motionally averaged chemical shift tensor lies perpendicular to the magnetic field.

Lipids in water form closed multilamellar liposomes that are assumed to be spherical, and thus give rise to powder lineshapes observed in NMR. To achieve the oriented lineshape, the spherical liposome must be distorted by the magnetic field so that most of the lipids would lie with their long axis perpendicular to the field. Two shapes are possible: a prolate ellipsoid with the symmetry axis parallel to the field or an oblate ellipsoid with the symmetry axis perpendicular to the magnetic field. The other possibility is that the liposomes do not start out spherical but ellipsoidal. Outside the magnet these ellipsoids would be randomly dispersed so that overall no particular lipid orientation is favored, but in the magnetic field they would align to produce the same result as a deformation process. Such magnetic alignment has been observed by phase contrast microscopy of large cylindrical vesicles of egg lecithin (Boroske and Helfrich, 1978). Since only some of the vesicles in the sample formed cylinders, the orientation was not a bulk effect. Preliminary examination of the 60:40 NPSM/DMPC membranes under a cross-polarizing microscope reveals that these liposomes are also quite large, ranging from 12 to 45 μm. The size lends support to the orientation by deformation hypothesis since the larger the liposomes, the more easily they are deformed. Since some of the liposomes appeared round and some appeared greatly distorted outside the magnetic field, the actual orientation process may be a combination of both deformation and alignment.

Any magnetic orientation is due to the diamagnetic anisotropy of the orienting molecule. The negative value of their diamagnetic anisotropy dictates that lipids will orient with their long axis perpendicular to the field. A positive value would cause a molecule to align parallel to the field. For example, α-helices in proteins exhibit $\Delta\chi > 0$ and therefore purple membrane fragments align with the helices parallel to H_0 (Neugebauer et al., 1977). Most of the diamagnetic anisotropy of lipid molecules is due to the acyl chains. Crystalline 1,2-dipalmitoyl-*sn*-phosphatidylcholine (DPPC) has a molar anisotropy of about -68×10^{-6} EMU/mol (Sakurai et al., 1980). Since crystalline stearic acid has a molar anisotropy of $\Delta\chi = 26 \times 10^{-6}$ EMU/mol (Lonsdale, 1939), and since there are two chains in DPPC, only about -16×10^{-6} EMU/mol of the total molar anisotropy of DPPC is due to the headgroup (Sakurai et al., 1980). The values for hydrated lipid bilayers will almost certainly be different due mainly to differences in the ordering of the headgroups and chains. For example, egg yolk lecithin in the L_α phase has a $\Delta\chi$ of only about -2×10^{-6} EMU/mol (Boroske and Helfrich, 1978).

It is interesting and significant that magnetic orientation of liposomes does not occur in the gel phase. Our results are similar to the observations of Seelig et al. (1985) but are contrary to the findings of Maret and Dransfeld (1977) who, by using magnetic birefringence, found small DPPC vesicles to magnetically orient most while in the gel phase. Since there is a much higher degree of order in the gel phase bilayer, especially with regard to the hydrocarbon chains, the lateral correlation of the chains should be greater than in the L_α phase, resulting in enhanced additivity of the diamagnetic anisotropy of each molecule. Thus, a sample in the gel phase is expected to have a larger molar anisotropy than a sample in the L_α phase and, therefore, is also expected to have a greater orientability. The possible reasons why magnetic orientation is not initiated in the gel

phase all involve considerations of membrane curvature and elastic properties. A liposome will assume the shape that minimizes its curvature elastic energy and any change from that shape will raise the energy (Helfrich, 1973). If the liposome minimizes the energy with a spherical shape, then deformation is necessary to achieve orientation. The degree of orientation will depend not only on the effective diamagnetic anisotropy, the magnetic field strength, and k_BT, but also on the elasticity and initial curvature of the membrane, and also on the bilayer thickness (Helfrich, 1973). Thus, although the effective diamagnetic anisotropy may be greater in the gel phase, it may not be large enough to overcome the energy required to change shape due to the gel state membrane's curvature elastic properties. As the lipid melts, substantial changes in the membrane's physical properties take place. The membrane is more disordered but also more fluid and perhaps more easily deformed. The lowered effective anisotropy due to decreased lateral correlation among the chains may be offset by changes in the elasticity, permitting easier deformation so that orientation is seen in the L_α phase but not in the gel phase.

If the liposome is naturally ellipsoidal or cylindrical outside the magnetic field, then only alignment in the magnetic field is needed to achieve orientation. Alignment of gel phase liposomes may be hindered by the increased viscosity of the sample, but upon melting the resistance to alignment is decreased, allowing orientation to occur. Finally, again due to changes in the curvature elastic energy, it is possible that the natural shape of the liposome changes upon melting from spherical to nonspherical. Thus, orientation by alignment could only occur in the L_α phase.

The "supercooling" effect illustrated in Fig. 2 is easily explained within an alignment process. If the liposomes are naturally ellipsoidal or cylindrical in shape in both phases, then once aligned they should remain aligned in the magnetic field since the diamagnetic anisotropy still favors orientation and since increased viscosity now hinders motion away from alignment. On the other hand, the same energy considerations that would prevent deformation of a spherical liposome to an ellipsoidal shape in the gel phase should still apply to an oriented liposome after subsequent cooling to below the phase transition. This suggests that if deformation is occurring, then the deformed liposome is resistant to changing back to its original shape. This would be a very interesting situation that would imply a large activation energy barrier to any shape change while in the gel phase.

Differences is membrane shape, elasticity, curvature, and viscosity can explain why only some and not all lipid membranes orient in high magnetic fields. Membranes that are similar in composition may not have the same orientability. A 60:40 NPSM/DMPC mixture orients, but an 80:20 or 40:60 NPSM/DMPC mixture does not. The differences between these two membranes is almost certainly not in the values of their diamagnetic anisotropy but in the physical properties that determine their shape or ability to undergo deformation. Similarly, Seelig et al. (1985) found the percentage of water mixed with lipid to be a critical item in the ability to orient a particular lipid mixture. Again, small differences in composition seem to result in very significant changes in physical properties. In comparing DMPC liposomes and 60:40 NPSM/DMPC liposomes, we can offer a reason why the former do not orient and the latter do. Preliminary microscopy results show that the diameter of a 60:40 NPSM/DMPC liposome is on average approximately three times the diameter of a 100% DMPC liposome. The larger size results in a smaller curvature and, for this reason, the larger structures are more easily deformed than smaller ones. Thus, the size difference alone may explain why one orients and the other does not. Further microscopy studies are in progress to clarify both the mechanism of orientation and the determining factors for achieving magnetic orientation.

CONCLUSIONS

^{31}P- and ^{2}H-NMR of a 60:40 NPSM/DMPC mixture dispersed in 50 wt% water yielded single crystal-like spectra that showed that the liposomes will spontaneously orient in a strong magnetic field so that the long axis of the lipid is perpendicular to the magnetic field. This is the second report of a strongly orienting lipid membrane detected by NMR. As different lipids and different lipid mixtures are studied by NMR, and as higher magnetic fields become increasingly common, it may be that other cases of magnetic orientation will be found. The mechanism for orientation must involve either deformation of a spherical liposome into a prolate or oblate-shaped liposome, or alignment of nonspherical liposomes in the magnetic field. To date, it appears that orientation occurs most readily when the membrane is in the melted L_α phase but persists after cooling to the gel phase.

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