Solvent Effect on Phosphatidylcholine Headgroup Dynamics as Revealed by the Energetics and Dynamics of Two Gel-State Bilayer Headgroup Structures at Subzero Temperatures

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ABSTRACT The packing and dynamics of lipid bilayers at the phosphocholine headgroup region within the temperature range of −40 to −110°C have been investigated by solid-state nuclear magnetic resonance (NMR) measurements of selectively deuterium-labeled H_2O/dimyristoylphosphatidylcholine (DMPC) bilayers. Two coexisting signals with ^2H NMR quadrupolar splittings of 36.1 and 9.3 (or smaller) kHz were detected from the $-CD_3$ of choline methyl group. These two signals have been assigned to two coexisting gel-state headgroup structures with fast rotational motion of $-CD_3$ and $-N(CD_3)_3$ group, respectively, with a threefold symmetry. The largest quadrupolar splitting of the NMR signal detected from the $-CD_2$ of Cα and Cβ methylene segment was found to be 115.2 kHz, which is 10% lower than its static value of 128.2 kHz. Thus, there are extensive motions of the entire choline group of gel-state phosphatidylcholine bilayers even at a subzero temperature of −110°C. These results strongly support the previous suggestion (E. J. Dufourc, C. Mayer, J. Stohrer, G. Althoff, and G. Kothe, 1992, *Biophys. J.* 61:42–57) that ^{31}P chemical shift tensor elements of DMPC determined under similar conditions are not the rigid static values. The free energy difference between the two gel-state headgroup structures was determined to be 26.3 ± 0.9 kJ/mol for fully hydrated bilayers. Furthermore, two structures with similar free energy difference were also detected for "frozen" phosphorylcholine chloride solution in a control experiment, leading to the conclusion that the two structures may be governed solely by the energetics of fully hydrated phosphocholine headgroup. The intermolecular interactions among lipids, however, stabilize the static headgroup structure as evidenced by the apparently lower free energy difference between the two structures for partially hydrated lipid bilayers. Evidence is also presented to suggest that one of the headgroup structures with trimethylammonium group rotation, which is not compatible with the static headgroup structure in crystals, is due to the dielectric relaxation of the slowly reorienting interbilayer water molecules near the physical edge of membrane surface. Finally, a molecular model of the hydration-induced conformational changes at the torsion angle α_5 of the O–C–C–N^+ bond is proposed to explain the two detected coexisting headgroup structures. These results emphasize the important role of the trimethylammonium group in monitoring the structure and dynamics of the lipid headgroup.

INTRODUCTION

We have previously reported that the ^2H nuclear magnetic resonance (NMR) spectra obtained from fully hydrated D_2O/sphingomyelin lipid bilayers exhibit a large isotropic signal at subzero temperatures (Wu et al., 1991). Although the molecular origin of the isotropic interbilayer D_2O signal at temperatures far below the homogeneous nucleation temperature of ice formation, i.e., −40°C (Bronshteyn and Steponkus, 1994; Echlin, 1992), is not clear, it has been suggested that water molecules are relatively free to rotate in polycrystalline ice if small orientation defects naturally occur (Auty and Cole, 1952). Tetrahedral reorientation of water molecules in ice has indeed been observed by both ^1H and ^2H NMR (Wittebort et al., 1988). Therefore, it is reasonable to assume that the isotropic water signals of hydrated lipid bilayer arise from the reorientation of the interbilayer water molecules with a high concentration of orientational defects created by the surface residues. The isotropic NMR signal of these "unfrozen" water molecules near the physical edge of bilayer surface may therefore serve as an important probe to study the solvent effect on the phosphatidylcholine (PC) headgroup dynamics in the gel phase.

The solvent effect on the structure and dynamics of PC headgroups in the liquid crystalline phase has been studied by ^2H NMR using lipids specifically ^2H-labeled at the choline headgroup (Bechinger and Seelig, 1991; Ulrich and Watts, 1994). The N^+ end of the phosphocholine headgroup dipole moves closer to the hydrocarbon layer with a decrease in hydration state; progressive hydration, however, induces a concerted change in headgroup conformation together with an increase in its state of motion. Information regarding the molecular response of such an effect previously inaccessible by the combined x-ray diffraction and neutron scattering studies (Buldt et al., 1979), can now be easily obtained because of the sensitivity of the headgroup structures to solid-state ^2H NMR spectroscopy (Seelig and Seelig, 1980; Seelig et al., 1987). A detailed description of the solvent effect at the molecular level still remains to be established, although an influence of the hydrates on the orientation of the phosphate group has been suggested (Scherer and Seelig, 1989; Roux et al., 1989).

Recent Fourier transform infrared (FTIR) spectroscopic studies of PC bilayer in the subgel phases suggest that the

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structural profile of the hydrophobic fatty acyl chain region of lipids "crystallized" from water is, in many respects, incompatible with that deduced from x-ray studies of dimyristoylphosphatidylcholine (DMPC) crystallized from organic solvent (Lewis and McElhaney, 1992). This incompatibility raises the possibility that the gel-state headgroup structure of PC lipid bilayers during the freezing process may not be the same as the regular static structure of the headgroup in crystals. A substantial amount of NMR data obtained from lipid dispersions in the liquid crystalline phase indicates that the preferred features of the phospholipid headgroup are, to a certain degree, in agreement with those determined by x-ray diffraction (Hauser et al., 1981, Pascher et al., 1992); however, the extensive mobility of the phosphocholine moiety in DMPC lipid bilayers is incompatible with any known static structure or packing arrangement of PC in crystals. In fact, analysis of the solid-state NMR data does not support the notion that there is only a single conformational solution to the NMR measurements of the headgroup in PC lipid bilayers (Skarjune and Oldfield, 1979). Evidence has also been presented to indicate the existence of at least two headgroup structures, based on the chemical shift of PC headgroup in the cross-polarization magic angle ^{13}C NMR spectrum of dipalmitoylphosphatidylcholine (DPPC) polycrystals (Wu and Chi, 1990). A study of the lipid bilayers in the gel phase would be a useful reference for the structure and dynamics of PC headgroup in the liquid crystalline phase.

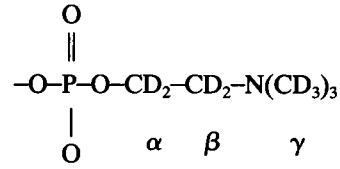
Furthermore, there are other reasons that interested us in the properties of the lipid headgroup in the gel phase, despite the fact that lipids in biological membranes are in the liquid crystalline phase. A comprehensive analysis of ^{31}P NMR lineshape and relaxation time measurements suggests that the previously determined ^{31}P chemical shift tensor elements for anhydrous DMPC and DPPC are not the rigid static values, since they are already averaged by internal motions (Dufourc et al., 1992). The magnitude of the internal motion, which was estimated to be ~20° with respect to a certain molecular axis, is nevertheless significant. It is, therefore, important to investigate whether a similar change could be detected by ^2H NMR in the choline moiety of the polar headgroup.

In this report, we present ^2H NMR studies of choline-perdeuterated DMPC (d_{13}-DMPC), methylene-deuterated DMPC (d_4-DMPC), perdeuterated phosphorylcholine salt, and D_2O/DMPC at the subzero temperature range from −40 to −110°C. At temperatures far below the apparent freezing event of phosphate group occurring at −34°C (Wu et al., 1991; Bronshteyn and Steponkus, 1994), complexity of solid-state ^2H NMR spectra due to phospholipid dynamics (Auger et al., 1990) can be largely minimized. This allows an investigation of the molecular order and dynamics of the entire choline segment without having to consider the motions of phosphate group. The results obtained suggest a coexistence of two gel-state PC headgroup structures distinguishable by the dynamics of trimethylammonium group. The relative populations of these two structures appear to correlate well with the number of interbilayer water molecules having slow tetrahedral reorientation. A molecular model of the hydration-induced conformational change in the PC headgroup is proposed to provide an explanation of the two coexisting headgroup structures detected by ^2H NMR.

MATERIALS AND METHODS

Spectra were obtained on a 7.05 T Bruker MSL-300 spectrometer using a broadband probe horizontally mounted with a 5 mm insert. ^2H NMR spectra were recorded with a quadrupole echo pulse sequence ($90°_{\pm x} - t - 90°_{y} - t$ - FID) using 90° pulses of 2.2~2.5 μs delay. The interpulse delay, t, was 20 μs unless mentioned otherwise. Recycle delays were varied from 100 ms to 300 s depending on the samples and the nature of experiments. Temperatures of the studied samples were controlled by liquid N_2 and monitored by a Bruker VT-1000 thermal system.

Dimyristoylphosphatidylcholine (DMPC), perdeuterated-choline DMPC (d_{13}-DPMC) and DMPC selectively deuterated at both Cα and Cβ methylene segments (d_4-DMPC) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL). D_2O and phosphorylcholine chloride selectively deuterated at the Cγ methyl group was obtained from Cambridge Isotope, Inc. The conventional notation used to specify the deuterated molecular group is as follows:



Hydration of the lipid samples with 2 ± 1 water per lipid was performed by the following two methods. In method 1, lipid was dissolved in chloroform, and solvent removed under a stream of nitrogen. The lipid thus obtained was then lyophilized in NMR tube under high vaccuum overnight. Under this condition one to two water molecules still remained bound to the lipid (Cevc, 1993). The amount of lipid was determined by phosphate assay. In method 2, a known amount of lipid in dry state was prepared gravimetrically and subjected to further drying under high vaccuum overnight, since it absorbs water quickly at room temperature. Because samples prepared by both the methods exhibited the x-ray diffraction pattern of PC bilayers (W. Huang, personal communication) and also because the ^2H NMR spectra obtained for d_{13}-DMPC were similar, we designated these samples as PC bilayers with 2 ± 1 H_2O/lipid molecule.

Lipids with a higher water content (4 ± 1, 8 ± 2, and 22 ± 2 H_2O per lipid) were prepared by directly adding a known amount of deuterium-depleted water (Aldrich Chemical Co., Milwaukee, WI) to samples obtained by the second method. These samples were septa sealed, vortexed and incubated at 50 and 4°C, respectively, for 30 min. This procedure was repeated three to four times to ensure homogeneity of the sample. The samples thus prepared appeared to be stable as judged from the reproducibility of the spectra and sample weight.

RESULTS AND DISCUSSION

Fig. 1 shows representative ^2H NMR spectra of d_{13}-DMPC at the indicated temperatures for samples with hydration state of 2 ± 1 (Fig. 1 *A*) and 8 ± 2 (Fig. 1 *B*) water molecules per lipid. The spectra reveal three overlapping ^2H NMR signals with distinct quadrupolar splittings ($\Delta\nu_Q$) of 115.2, 36.1 and 9.3 (or smaller) kHz as determined by the peak-to-peak separations of the three respective central doublets. It should be emphasized that the spectra shown were obtained during the heating mode of samples that followed

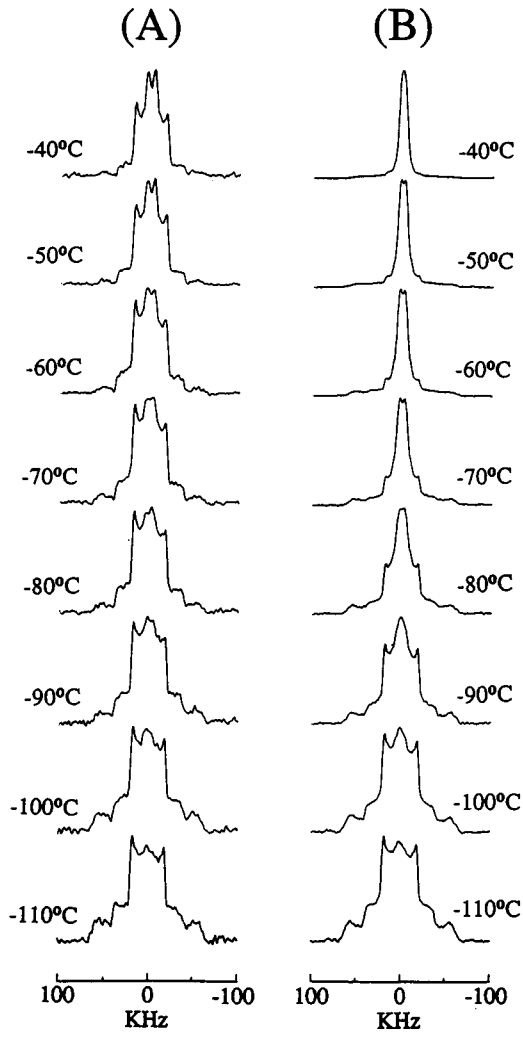


FIGURE 1 Representative ^2H NMR spectra for DMPC at two hydration conditions: H_2O/d_{13}-DMPC at molar ratio of 2 ± 1 (*A*) and 8 ± 2 (*B*). Variable temperature spectra were obtained with a two-pulse quadrupolar echo sequence with 90° pulse width of 2.5 μs duration and 20 μs spacing. The line broadening was 200 Hz. The repetition time for all the spectra is 500 ms. The designated repetition time is much longer than the T_1 values of the two narrow components with Pake splitting of 36.1 and 9.3 kHz, but is only comparable to the T_1 of the broad component (see Fig. 2). Therefore, the intensity ratio between the two narrow components can be reasonably estimated from their respective signal intensity shown in the figure without correcting the intensity loss during the interpulse delay.

cooling of the samples to the lowest studied temperature. These spectra were quantitatively the same as those obtained upon cooling samples from room temperature to the studied temperature indicated in the figure, using liquid nitrogen as a cooling agent. Thus, the spectra apparently represent the thermodynamic equilibrium states of the systems.

Two other observations deserve further consideration. First, the spectral lineshape and intensity of the central narrow component change significantly at different experimental conditions. For instance, the narrow component with smallest $\Delta\nu_Q$ at −40°C changed to a central band at −100°C for samples with 2 ± 1 waters per lipid (Fig. 1, *left*). A similar behavior was also detected for samples with higher hydration states of 8 ± 2 waters per lipid (Fig. 1, *right*). Although a similar change in the spectral lineshape has also been observed for *N*-acetyl-DL-(γ-d_6)-valine and other polycrystalline amino acids selectively deuterated at the methyl group (Batchelder et al., 1983; Beshah et al., 1987; Beshah and Griffin, 1989), it does not necessarily follow that the motional rates of the studied molecular groups at low temperatures have already entered the intermediate regime, where the lineshapes are unique and very sensitive to the dynamic processes and pulse spacing in the quadrupolar echo experiment. To test this possibility we obtained NMR spectra as a function of interpulse delay of quadrupolar echo sequence. As shown in Fig. 2 *A*, the central feature of the spectrum does not seem to be sensitive to the pulse spacing for spectra obtained at the indicated temperatures. In addition, the intensity ratio (A_s/A_b) of the spectral intensity between the sharp (9.3 kHz) and broad (36.1 kHz) signal remains relatively constant at least for interpulse delay shorter than 40 μs (Fig. 2 *B*). Therefore, variation of the signal intensity and lineshape of the narrower central signal probably reflects other properties of the DMPC bilayers under study. Further lineshape simulation analysis are necessary to determine whether the central band detected at the lowest studied temperature of −110°C is indeed related to the dynamic process in the intermediate motional regime.

Secondly, powder pattern ^2H NMR lineshapes with clear Pake doublets are detected for two other broad components with $\Delta\nu_Q$ of 36.1 kHz and 115.2 kHz between −40 and −110°C. Although the exact lineshape, i.e., the signal intensity distributed as different solid angles of the powder spectra, changes slightly as a function of temperature, the edges representing their respective Pake doublets are still detectable in the spectra shown in Fig. 1. Before commenting on the possible implications of these spectra, an assignment of these signals to the specifically deuterated molecular groups would be necessary.

We thus assigned the respective ^2H NMR signals to the corresponding $-CD_2$ and $-CD_3$ group of perdeuterated choline moiety based on both physical and chemical evidence. First, T_1 spin-lattice relaxation time measurements were performed to separate each component on the basis of their relaxation properties (Fig. 3 *A*). The two narrow components of the detected NMR spectra show similar T_1 relaxation times of 14 and 16 ms, respectively, at −110°C, while the broad component with $\Delta\nu_Q$ of 115.2 kHz exhibits a much longer relaxation time of 300 ms. Second, ^2H NMR spectra were obtained for d_4-DMPC specifically labeled at the methylene segment of the choline head group to facilitate the chemical assignment (Fig. 3 *B*). It is evident that only one Pake doublet powder pattern with $\Delta\nu_Q$ of 115.2 kHz is detected for d_4-DMPC. It can thus be concluded that the broad component with $\Delta\nu_Q$ of 115.2 kHz is due to the methylene $-CD_2$ segment and the two narrow components due to the methyl groups on the $N-(CD_3)_3$ moiety.

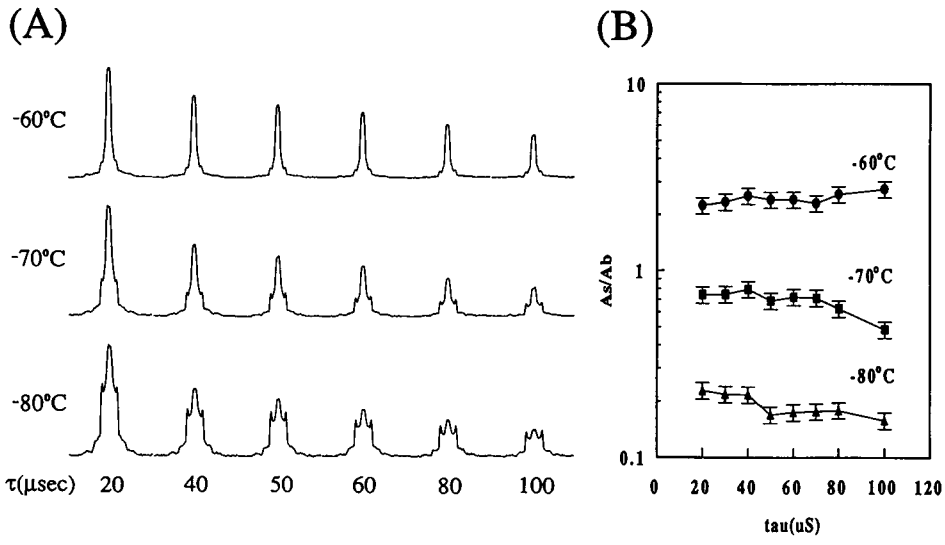


FIGURE 2 Variation of the ^2H NMR spectra (A) and the intensity ratio (A_s/A_b) between the sharp central and broad 36.1 kHz components (B) obtained from perdeuterated choline d_{13}-DMPC bilayers at indicated interpulse delays and temperatures. The spectral intensity of the broad 36.1 kHz component (A_b) was the integrated intensity obtained by computer simulation using the theoretical powder spectrum of Pake doublets, while that of the sharp central component (A_s) was the integrated intensity of the central signal after substrating the broad 36.1 kHz component from the experimentally obtained spectra.

The spectral signals with 36.1 and 9.3 kHz splitting obtained for d_{13}-DMPC at hydration state at 2 ± 1 water per lipid can also be explained readily on the basis of the C_3-symmetry axis of the terminal methyl and trimethylammonium group (Veksli et al., 1969; Skarjune and Oldfield, 1979). For an axially symmetric averaged powder pattern, the averaged $\overline{\Delta\nu_Q}$ is given by

$$\overline{\Delta\nu_Q} = \Delta\nu_Q S$$

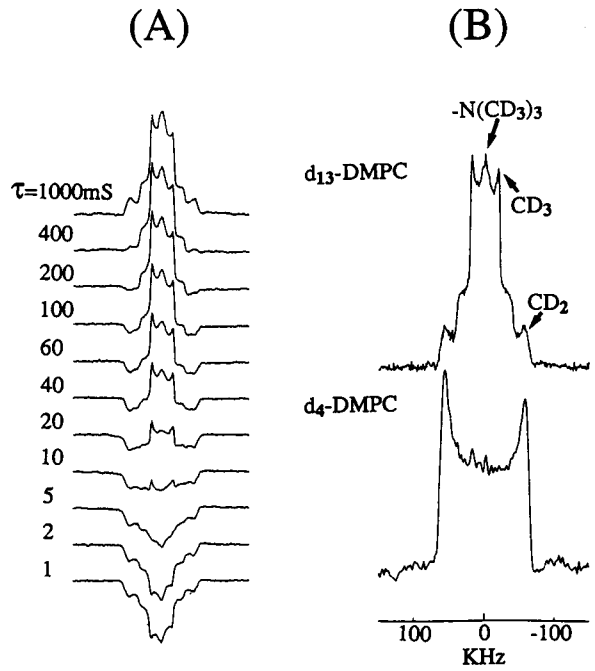


FIGURE 3 Assignment of the ^2H NMR spectra obtained from perdeuterated choline d_{13}-DMPC bilayers: physical assignment according to the differences in the T_1 relaxation behavior (A) and chemical assignment obtained from d_4-DMPC selectively deuterated at two methylene segment of the choline headgroup (B). The spectra shown in the figure were obtained at -110°C. Similar conclusions regarding the signal assignment can also be drawn from experiments performed at different temperatures. Please see text for the details of the assignment.

where

$$S = (3\cos^2\theta - 1)/2$$

and θ is the angle made by the C–D bond with the threefold axis (Seelig and Seelig, 1980). Assuming the "static" $\Delta\nu_Q$ of the C–D bond before averaging to be the same as the value detected for $-CD_2$, i.e., 115.2 kHz, one can calculate the bond angle between N–Cγ and Cγ–D to be 110.6°. Proceeding along similar lines, the bond angle between Cβ–N and N–Cγ is found to be 113.7°. These two values are in excellent agreement with the experimental values of 111° and 112.7° determined by x-ray diffraction (Pascher and Sundell, 1986). This agreement in the values strongly suggests that the orientation geometry is indeed the major factor in determining the observed $\Delta\nu_Q$ value.

The $\Delta\nu_Q$ value (115.2 kHz) of the methylene segment in the phosphocholine headgroup is significantly lower than the typical static $\Delta\nu_Q$ values of the $-CD_2$ (Seelig, 1977). Several lines of evidence suggest that the appreciably smaller $\Delta\nu_Q$ value obtained by us is not a result of systematic errors. First, the $\Delta\nu_Q$ value of polycrystalline L-[3,3,3$^2H_3$]alanine at -150°C was found to be 125 kHz as determined by measuring the peak-to-peak separation of the Pake doublet adopted in this study (Batchelder et al., 1983). Therefore the smaller $\Delta\nu_Q$ value is not due to the adoption of a simple measuring procedure without lineshape simulation. Secondly, other technical problems such as insufficient excitation power can also be excluded. The ^2H NMR spectra of hexagonal ice with a $\Delta\nu_Q$ value of 146 kHz as obtained by Wittebort et al. (1988) is reproducible by us (data not shown). Finally, the $\Delta\nu_Q$ values determined by us are in excellent agreement with the conformational consideration on the basis of bond angle. We thus conclude that additional motions of the entire choline group are present even at the lowest studied temperature of -110°C. Two other lines of evidence suggest that a fast small angle librational motion is present, which causes further averaging in the static $\Delta\nu_Q$

value. First, the ^2H NMR T_1 value of the methylene segment was found to be 300 and 244 ms at −110 and −90°C, respectively, which are almost two orders of magnitude lower than those measured for the deuterated ice at similar temperature range. Second, the observed $\Delta\nu_Q$ values of the $-CD_2$ on the choline segment of PC bilayers are lower at higher temperature (Table 1). For instance, the $\Delta\nu_Q$ values of samples with hydration state of 2 ± 1 water per lipid decrease from 115.2 to 112.3 kHz as the temperature for that sample is increased from −110 to −50°C. The reduction factor is even higher for samples at higher hydration states. This is consistent with the expectation that the libration angle would vary with temperature and packing environment (Batchelder et al., 1983). Assuming that the C–D bond axis librates rapidly in a cone of semi-angle θc (Usha and Wittebort, 1992), the calculated θc values would change approximately from 21° to 24°, if the temperature is increased from −110°C to −50°C. It should be pointed out, though, that the values thus derived are only approximate because resolving the possible coupling effect among the libration motions of different molecular groups on the phosphocholine moiety has proved difficult for us. Nevertheless, our result is consistent with the suggestion (Dufourc et al., 1992) that previously determined "static" ^{31}P chemical shift tensor elements of anhydrous DMPC and DPPC (Kohler and Klein, 1977, Herzfeld et al., 1978) are indeed not the rigid limit values.

As pointed out earlier, ^2H NMR signal of the $-N(CD_3)_3$ group is sensitive to the experimental conditions. Not only the lineshape but also the $\Delta\nu_Q$ values of the observed ^2H NMR signal depends upon the temperature and hydration states of the samples. The simplest model, which can account for the observed narrow linewidth of the designated signal and its temperature-dependent profiles, is to assume that the trimethylammonium group undergoes extensive reorientation along the $C–N(CD_3)_3$ bond axis with rates approaching, but not yet reaching, those at the onset of the intermediate exchange regime within the reported temperature range. In contrast to the rotational motion of the bond along $N–CD_3$, hydration appears to have a significant effect on the motional mode of $C–N(CD_3)_3$. The $\Delta\nu_Q$ values detected for samples at low and high water contents are found to be 9.3 and 5.4 kHz respectively, at −70°C (Table 1). A further reduction in the detected $\Delta\nu_Q$ values with high water content suggests that in this case a wobble within the cone model is more applicable to the motional mode of $C–N(CD_3)_3$. We suspect this to be the reason for the obscure quadrupole splitting of the narrow signal arising from the dynamics of $C–N(CD_3)_3$. It should be noted that the fast rotation of methyl group would still dominate the T_1 relaxation process deduced for the trimethylammonium group. The T_1 relaxation values obtained for the two narrow components are quite similar within the temperature range studied (Fig. 3). The rate of reorientation of the $-N(CD_3)_3$ group is therefore expected to be slower than that of the $-CD_3$ group (10^{-8} s) but faster than the characteristic ^2H NMR time scale of the intermediate time regime (10^{-5} s) (Beshah and Griffin, 1989).

TABLE 1 Observed averaged quadrupolar splitting ($\Delta\nu_Q$) as a function of temperature for DMPC deuterated at the choline group

	$\Delta\nu_Q$ (kHz, 2 ± 1 waters/PC)			$\Delta\nu_Q$ (kHz, 8 ± 2 waters/PC)		
T (°C)	$-CD_2$‡	$-CD_3$	$-N(CD_3)_3$	$-CD_2$‡	$-CD_3$	$-N(CD_3)_3$
−110	115.2	36.1		115.2	36.1	
−100		36.1			36.1	
−90	113.8	36.1		113.3	36.1	
−80		36.1			36.1	
−70	112.8	36.1	9.3	110.4	36.1	5.4
−60		36.1	9.3		36.1	4.9
−50	112.3	36.1	9.3	110.4	36.1	4.4
−40		36.1	9.3		36.1	

*Uncertainty ±0.25 kHz; ‡measured from d_4-DMPC.

The extensive mobility of choline moiety in DMPC lipid bilayers detected by us is incompatible to any known static structure and packing arrangement of PC in crystals (Pascher et al., 1992). This strongly suggests that the gel-state headgroup structure of PC lipid bilayers during the freezing process may not be the same as the regular static structure of the headgroup in crystals. In fact, since variation of the pulse spacing in the quadrupolar echo experiment (Fig. 2) does not modify the lineshape, the simplest explanation to account for the coexistence of two overlapping signals from the methyl group is that two phosphocholine bilayer headgroup structures, one exhibiting only $-CD_3$ rotation and the other exhibiting both $-CD_3$ and $-N(CD_3)_3$ rotation, are present at temperatures below −40°C. In view of the fact that the intensity ratio of the two populations, i.e., A_s/A_b, remains relatively constant at short interpulse delays, the free energy differences between the two populations can be obtained from the respective integrated areas of the lipid signals. Incidentally, with the possible exception at the lowest studied temperature, the intensity loss due to the dynamic process is negligible; therefore, the free energy difference can be reasonably estimated. As shown in Fig. 4, the free energy difference estimated from the ratio of two populations is found to decrease during the dehydration process. It should be stressed that the detection of two populations at different temperatures is not due to the freeze-induced dehydration process, but to the changing concentration of the freely rotating interbilayer water molecules at different temperatures, as we shall show later. The relative intensities of these two populations are completely reversible during heating and cooling cycles at least within the experimental time span of several hours. Any other freeze-induced dehydration within the interbilayer space would exhibit an irreversible behavior during the course of experimentation.

Above all, low hydration increases the population of the bilayer headgroup structure without involving trimethylammonium group motion. Therefore, the apparent free energy difference changes from 26.3 ± 0.9 kJ/mol for lipid with 8 ± 2 water molecules per lipid to 9.2 ± 0.2 kJ/mol for lipid

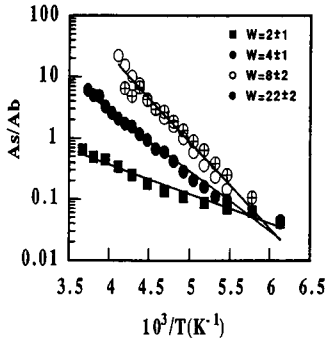


FIGURE 4 Estimation of the free energy difference for the two PC lipid bilayer headgroup structures as detected by two trimethylammonium group populations at subzero temperatures. As and Ab are same as that defined in the legend of Fig. 2. No correction for the intensity loss due to the T_{2e} relaxation process was included in the intensity ratio shown in the figure as justified by the relatively constant intensity ratio of the two populations obtained at short interpulse delays (Fig. 2). Free energy differences between the two gel-state PC headgroup structures as estimated from the fit shown in the figure are 9.2 ± 0.2, 18.9 ± 0.1, and 27 ± 3 kJ/mol. Please note that only one fitting line was used to simulate two sets of data obtained from samples with hydration state of 8 ± 2 and 22 ± 2 water molecules per lipid. A slightly different value (26.3 ± 0.5 and 23 ± 0.5 kJ/mol) would be obtained if two sets of data were fitted independently. It is noted that the intensity ratio obtained at low temperature exhibits significant deviation from the fitted line.

with 2 ± 1 water molecule per lipid. It should be noted, however, that there appears to be no significant effect of a water content higher than eight water molecules per lipid since the detected free energy difference for 8 ± 2 water molecules per lipid is almost the same as that for 22 ± 2 waters per lipid (23 ± 0.5 kJ/mol). Free energy of bilayer hydration as a function of the number of bound water molecules has been estimated from a nonlocal electrostatic model of hydration (Cevc, 1993), and the results agree very well with the values calculated from the sorption data (Jendrasiak and Mendible, 1976). Presumably, low hydration stabilizes the static trimethylammonium group of the phosphocholine head group by enhancing the intermolecular electrostatic interactions between the negatively charged phosphate and positively charged trimethylammonium group. These interactions could be between two neighboring phospholipid molecules within the same bilayer or between two molecules across the bilayers.

To search for the structural basis of the detected free energy difference, we obtained ^{2}H NMR spectra for the phosphorylcholine chloride solution selectively deuterated at the methyl group to eliminate the possible involvement of fatty acyl chain region. As shown in Fig. 5*A*, the spectra obtained at temperatures below −40°C are similar to those obtained for PC. Furthermore, the free energy difference for the two populations of the phosphorylcholine chloride solution is found to be ~23 kJ/mol, which is within the experimental errors of the values deduced for PC at hydration states higher than eight waters per lipid (Figs. 4 and 5 *B*). Unless the packing of the "frozen" phosphorylcholine chloride solution is the same as that of PC bilayers, the detected free energy difference of 23 kJ/mol can only be attributed to the intramolecular interaction of phosphorylcholine and/or to the intermolecular interaction between water and phosphorylcholine molecules. The saturation effect of the detected free energy difference suggests the presence of fewer than eight water molecules per lipid in the interbilayer space within the temperature range studied. Lower hydration states may allow stronger intermolecular interaction between lipids to stabilize the low energy state without involving the rotation of trimethylammonium group.

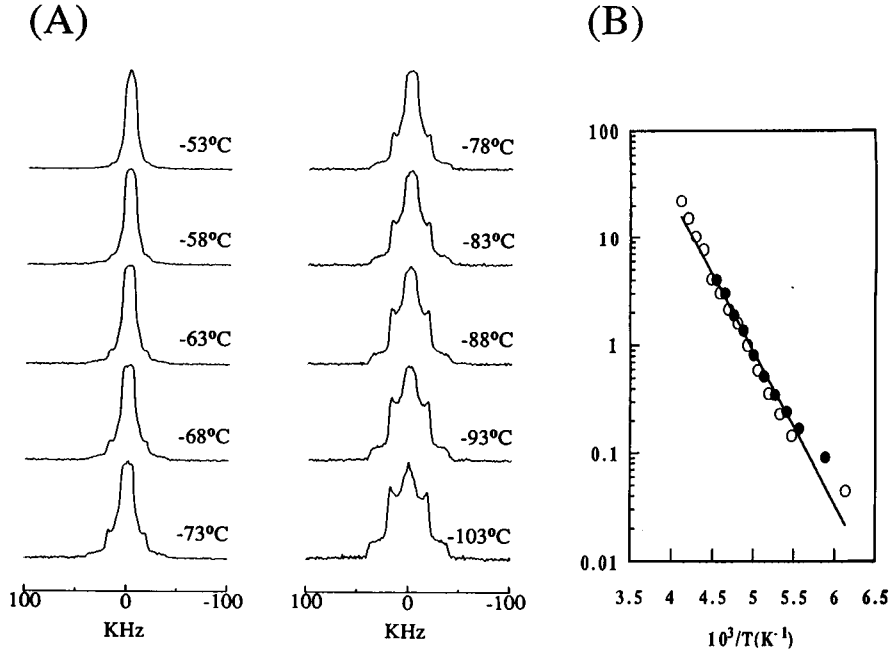


FIGURE 5 ^{2}H NMR spectra obtained at the indicated temperature from phosphorylcholine chloride solutions selectively deuterated at the methyl groups (*A*) and comparison of the free energy differences between phosphorylcholine solution and fully hydrated phosphatidylcholine bilayer (*B*). Closed and open circles shown in *B* represent relative intensity of the two coexisting NMR signals from phosphorylcholine and PC, respectively.

The preferred conformation of the zwitterionic phospholipid headgroup as detected in crystals is highly restricted by its intramolecular interactions and is not appreciably affected by either *N*-methylation or by differences in interactions and packing pattern of the P-N dipoles (Pascher et al., 1992). Therefore, the parallel layer orientation of the headgroup is believed to be mainly due to a perpendicular bend, caused by strongly favored conformation of the O–P–O phosphate diester bond. The orientation of the choline moiety is further stabilized by electrostatic attraction between the positively charged nitrogen and negatively charged phosphate group such that a torsion angle of ~60° is always detected for the O–C–C–N^+ bond (α_5) in PC crystals (Fig. 6, Pascher et al., 1992).

The aforementioned intramolecular interactions and packing requirement of the headgroup observed in crystals serve as a good starting point to understand the properties of lipid in aqueous dispersion; however, evidence is also present to suggest that the intermolecular interactions may further modify the packing arrangement of PC headgroup in lipid bilayers. For instance, subtle changes in the dynamic and conformational properties of Cα–Cβ segment of chiral thiophosphocholine head group have been detected (Loffredo et al., 1990). In fact, two slowly exchanging conformational states are suggested to coexist based on the ^{2}H NMR spectra obtained for the Cβ segment of the Rp isomer. Such changes are believed to be due to the noncovalent intermolecular interaction between the quaternary ammonium group of choline and the phosphate group of a neighboring molecule in the PC bilayers. Since in natural PC bilayers both types of interaction are likely to be present, it is possible that the dynamics and packing arrangement of the PC headgroup "crystallized" from water could be significantly different from those obtained by x-ray determination of single crystals. Interestingly, as shown in Fig. 6 *A*, the torsion angles of O–C–C–N^+ bond (α_5) for PC bilayers in the liquid crystalline state, known to exist in a gauche state as evidenced by vibration spectroscopy (Akutsu, 1981; Akutsu and Nagamori, 1991), were determined by NMR to be comparatively larger than those determined by x-ray diffraction. Therefore, it is tempting for us to propose that the detected two gel-state PC structures may be related to the changes in the torsion angle of O–C–C–N^+ bond. The molecular model shown in Fig. 6 *B* illustrates the effects of a change in torsion angle of α_5 from 60° to 90° on the packing and orientation of the bulky trimethylammonium group as related to the phosphate segment. It is important to note first that the trimethylammonium group appears to rotate freely at α_5 = 90° but not at α_5 = 60° because of the steric hindrance as emphasized by the arrows shown in the Fig. 6, and secondly that the P-N dipole would change its orientation by ~10°, in conjunction with a small change in the magnitude of its dipole moment. Although future experiments on oriented sample may help to prove or disprove the proposed model, our preliminary T_{2e} relaxation measurements on d_4-DMPC do suggest the important role of the O–C–C–N^+ bond (data not shown; manuscript in preparation).

We next investigated the reason for extensive reorientation of the gel-state PC headgroup of the trimethylammonium group as a result of the putative conformational change at the O–C–C–N^+ bond, a fact that is clearly not compatible with any known static headgroup structure determined by x-ray studies of single crystals. As we have pointed out before, the other possibility is the interaction of neighboring water molecules with the phosphorylcholine group. In the studied temperature range from −40°C to −110°C, all ^{31}P NMR spectra of lipid bilayers exhibit similar anisotropic spectra with previously determined ^{31}P chemical shift tensor elements (Kohler and Klein, 1977).

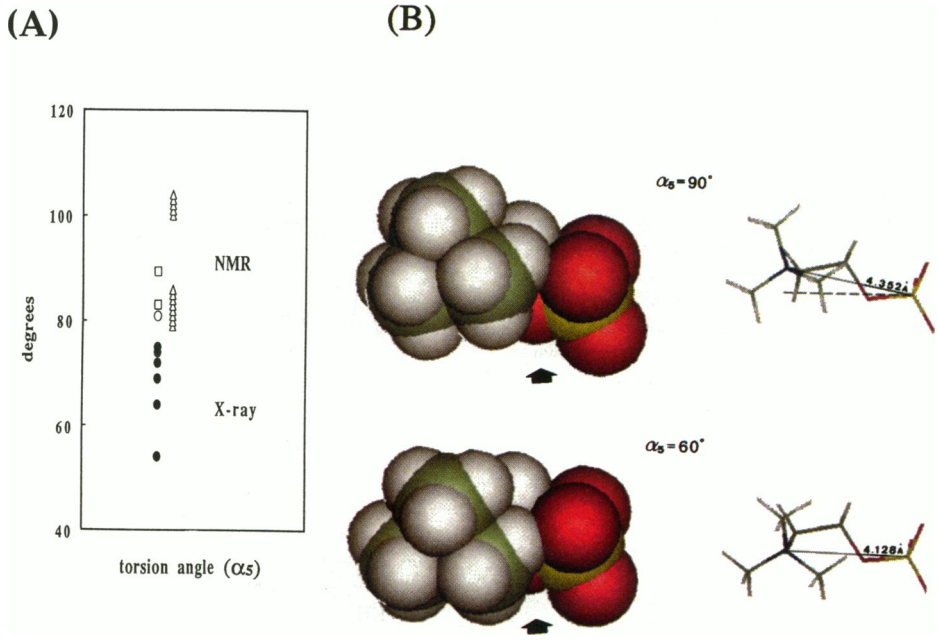


FIGURE 6 Summary of the torsion angle of O–C–C–N^+ bond (α_5) in PC lipid headgroup as determined by x-ray diffraction (●) and NMR (○, □, △) methods (*A*) and molecular model (*B*) to explain the different mobility of two coexisting trimethylammonium groups detected in this study. The data shown in *A* were obtained from Pascher et al., 1992; Seelig et al., 1977; Akutsu et al., 1991; and Skarjune et al., 1979.

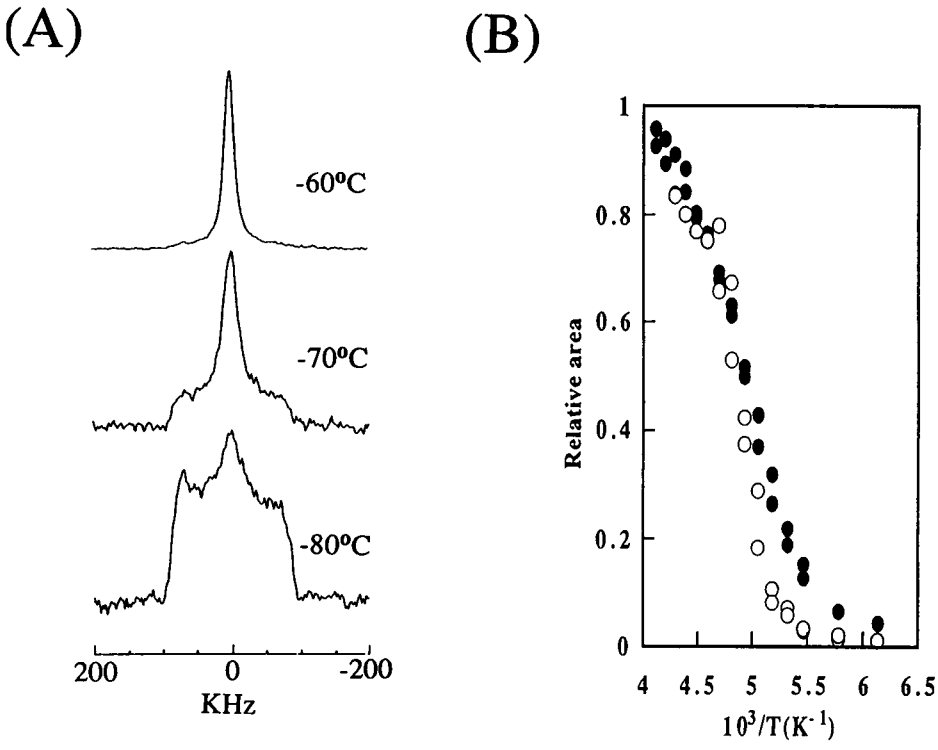


FIGURE 7 Representative ^2H NMR spectra obtained at the indicated temperatures for D_2O/DMPC bilayers at a hydration state of eight water molecules per lipid (*A*) and correlation of the intensity ratio between sharp and broad components of the ^2H NMR signals obtained from D_2O/DMPC (○) and H_2O/d_{13}-DMPC (●) lipid bilayers with hydration states of eight water molecules per lipid (*B*).

Therefore, the most likely reason for the presence of two coexisting headgroup bilayer structures is the interaction of choline moiety with water molecules of distinct property near the membrane surfaces.

Fig. 7 *A* shows the representative ^2H NMR spectra obtained from D_2O/DMPC with hydration states of eight water molecules per lipid. We emphasize again that all the spectra are reversible within the experimental time span of several hours. Fast repetition time of 200 ms was applied to eliminate signals from bulk water ice, and therefore the signal intensities represent those from the interbilayer water molecules. Since the T_{2e} values detected for the isotropic water signals are in the range of 50 μs (Wu et al., 1991), significant loss of the isotropic signal intensity may result from irreversible evolution due to the reorientation of interbilayer water molecules during the echo delay period of 20 μs used to obtain these spectra. Therefore, we correct the intensity loss during the echo delay by plotting the relative area between the isotropic and anisotropic water signals as a function of interpulse delay similar to the procedure adopted as shown in Fig. 2 *B*. The relative populations between the isotropic and anisotropic water signals are then plotted as a function of reverse temperatures (Fig. 7 *B*). A close correlation can be found for the relative area of two populations detected in both the trimethylammonium group (*closed circles*) and interbilayer waters (*open circles*). Since the characteristic Debye correlation times of dielectric relaxation in ice is related to dipole moment reorientation resulting from molecular rotation, it is not surprising that the positively charged trimethylammonium group would also respond to the reorientation of neighboring water molecules. As suggested earlier, the detected dynamic change may also be related to the change in the torsion angle of O–C–C–N^+ bond. Please note again that the phosphate group does not undergo significant dynamic change in the studied temperature range since ^{31}P NMR spectra obtained between −40°C and −110°C are similar.

The solvent effect on the gel-state PC headgroup dynamics can now be summarized as in Fig. 8, where the representative ^2H NMR signals of the trimethylammonium group at −40°C with three hydration states are depicted. The presence of two coexisting headgroup bilayer structures, exhibiting fast rotational motions along the $-CD_3$ and $-N(CD_3)_3$ bond axes with threefold symmetry, can be detected for DMPC bilayers with 2 ± 1 water molecules per

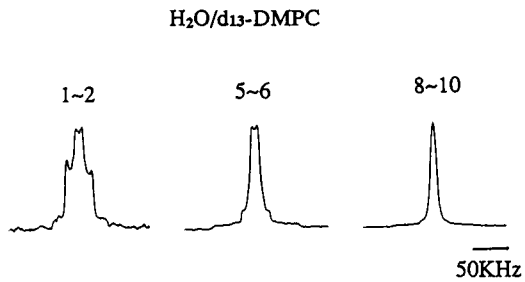


FIGURE 8 ^2H NMR spectra obtained from d_{13}-DMPC bilayers at −40°C at the indicated hydration states. The repetition time and interpulse delay of 100 ms and 20 μs, respectively, were used to obtain the spectra. The signal intensity of methylene $-CD_2-CD_2-$ was lower than theoretically expected value because of the intensity loss occurring under the experimental conditions. Therefore, the major portion of the spectra represents signals originating from $-N(CD_3)_3$.

lipid. This is mainly because the hydrates of gel-state bilayers are not in crystal form. At slightly higher water content of 4 ± 1 water molecules per lipid, the interaction of reorienting water molecules with their neighboring trimethylammonium group enhances the population of headgroup structure with trimethylammonium group rotation as indicated by the increasing intensity of the sharp component. At even higher water content of 8 ± 2 water molecules per lipid, the rotational motion along the axis of the trimethylammonium group become more wobble-like, causing the detected ^2H NMR spectra to appear quasi-isotropic. These solvent effects on the headgroup dynamics are suggested to be due to the reorientation of non-crystalline water molecules within the interbilayer space of two membrane surfaces.

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REFERENCES

Akutsu, H. 1981. Direct determination by Raman scattering of the conformation of the choline group in phospholipid bilayers. *Biochemistry.* 20:7359–7366.

Akutsu, H., and T. Nagamori. 1991. Conformational analysis of the polar head group in phosphatidylcholine bilayers: a structural change induced by cations. *Biochemistry.* 30:4510–4516.

Auger, M., M-R. Van Calsteren, I. C. P. Smith, and H-C. Jarell. 1990. Glycerolipids: common features of molecular motion in bilayers. *Biochemistry.* 29:5815–5821.

Auty, R. P., and R. H. Cole. 1952. Dielectric properties of ice and solid D_2O. *J. Chem. Phys.* 20:1309–1314.

Batchelder, L. S., C. H., Niu, and D. A. Torchia. 1983. Methyl reorientation in polycrystalline amino acids and peptides: a ^2H NMR spin-lattice relaxation study. *J. Am. Chem. Soc.* 105:2228–2231.

Bechinger, B., and J. Seelig. 1991. Conformational change of the phosphatidylcholine headgroup due to membrane dehydration: a ^2H NMR study. *Chem. Phys. Lipids.* 58:1–5.

Beshah, K., and R. G. Griffin. 1989. Deuterium quadrupole echo NMR study of methyl group dynamics in *N*-acetyl-DL-(γ-d6)-valine. *J. Mag. Reson.* 84:268–274.

Beshah, K., E. T. Olejniczak, and R. G. Griffin. 1987. Deuterium NMR study of methyl group dynamics in L-alanine. *J. Chem. Phys.* 86: 4730–4736.

Bronshteyn, V. L., and P. L. Steponkus. 1994. Calorimetric studies of freeze-induced dehydration of phospholipids. *Biophys. J.* 65:1853–1865.

Buldt, G., H.-U. Gally, J. Seelig, and G. Zaccai. 1979. Neutron diffraction studies on phosphatidylcholine model membranes. *J. Mol. Biol.* 134: 673–691.

Cevc, G. 1993. Lipid hydration. *In* Hydration of Macromolecules. E. Westhof, editor. MacMillan Press, New York. 338–390.

Dufourc, E. J., C. Mayer, J. Stohrer, G. Althoff, and G. Kothe. 1992. Dynamics of phosphate head group in biomembranes: comprehensive analysis using phosphorus-31 nuclear magnetic resonance lineshape and relaxation measurements. *Biophys. J.* 61:42–57.

Echlin, P. 1992. Low-Temperature Microscopy and Analysis. Plenum Press, New York. 12.

Hauser, H., I. Pascher, R. H. Pearson, and S. Sundell. 1981. Preferred conformation and molecular packing of phosphatidyl-ethanolamine and phosphatidylcholine. *Biochim. Biophys. Acta.* 650:21–51.

Herzfeld, J., R. G. Griffin, and R. A. Haberkorn. 1978. Phosphorus-31 chemical shift tensors in barium diethylphosphate and urea-phosphoric acid: model compounds for phospholipid headgroup studies. *Biochemistry.* 17:2711–2718.

Jendrasiak, G. L., and J. C. Mendible. 1976. The phospholipid headgroup orientation: effect on hydration and electrical conductivity. *Biochim. Biophys. Acta.* 424:149–158.

Kohler, S. J., and M. P. Klein. 1977. Orientation and dynamics of phospholipid head groups in bilayers of membranes determined from ^{31}P nuclear magnetic resonance chemical shielding tensors. *Biochemistry.* 16:519–526.

Lewis, R. N. A. H., and R. N. McElhaney. 1992. Structures of the subgel phases of *n*-saturated diacyl phosphatidylcholine bilayer: FTIR spectroscopic studies of C=O and ^2H labeled lipids. *Biophys. J.* 61:63–77.

Loffredo, W. M., R.-T. Jiang, and M.-D. Tsai. 1990. Phospholipid chiral at phosphorus: dynamic effect of phosphorus chirality on the deuterium NMR properties of the choline headgroup of phospholipids in the liquid crystalline phase. *Biochemistry.* 29:10912–10918.

Pascher, I., M. Lundmark, P. G. Nyholm, and S. Sundell. 1992. Crystal structures of membrane lipids. *Biochim. Biophys. Acta.* 1113: 339–373.

Pascher, I., and S. Sundell. 1986. Membrane lipids: preferred conformational states and their interplay. The crystal structure of dilauroylphosphatidyl-*N*, *N*-dimethylethanolamine. *Biochim. Biophys. Acta.* 855: 68–78.

Roux, M., J. M. Neumann, R. S. Hodges, P. Devaux, and M. Bloom. 1989. Conformational changes of phospholipid headgroup induced by a cationic integral membrane peptides as seen by deuterium magnetic resonance. *Biochemistry.* 28:2313–2321.

Scherer, P. G., and J. Seelig. 1989. Electric charge effects on phospholipid headgroup: phosphatidylcholine in mixtures with cationic and anionic amphiphiles. *Biochemistry.* 28:7720–7728.

Seelig, J. 1977. Deuterium magnetic resonance: theory and application to lipid membranes. *Q. Rev. Biophys.* 10:353–418.

Seelig, J., H.-U. Gally, and R. Wohlgemuth. 1977. Orientation and flexibility of the choline head group in phosphatidylcholine bilayers. *Biochim. Biophys. Acta.* 467:109–119.

Seelig, J., P. M. Macdonald, and P. G. Scherer 1987. Phospholipid headgroup as sensores of electric charge in membranes. *Biochemistry.* 26: 7535–7541.

Seelig, J., and A. Seelig. 1980. Lipid conformation in model membranes and biological membranes. *Q. Rev. Biophys.* 13:19–61.

Skarjune, R., and E. Oldfield. 1979. Physical studies of cell surface and cell membrane structure. Determination of phospholipid head group organization by deuterium and phosphorus nuclear magnetic resonance spectroscopy. *Biochemistry.* 18:5903–5909.

Ulrich, A. S., and A. Watts. 1994. Molecular response of the lipid headgroup to bilayer hydration monitored by ^2H NMR. *Biophys. J.* 66: 1441–1449.

Usha, M. G., and R. J. Wittebort. 1992. Structural and dynamical studies of the hydrate, exchangeable hydrogens and included molecule in β- and γ-cyclodextrin by powder and single crystal deuterium magnetic resonance. *J. Am. Chem. Soc.* 114:1541–1548.

Veksli, Z., N. J. Salsbury, and D. Chapman. 1969. Physical studies of phospholipids. XII. Nuclear magnetic resonance studies of molecular motion in some pure lecithin-water systems. *Biochim. Biophys. Acta.* 183:434–446.

Wittebort, R. J., M. G. Usha, D. J. Ruben, D. E. Wemmer, and A. Pines. 1988. Observation of molecular reorientation in ice by proton and deuterium NMR. *J. Am. Chem. Soc.* 110:5668–5671.

Wu, W., and L.-M. Chi. 1990. Comparison of lipid dynamics and packing in fully interdigitated monoarachidoyl-phosphatidylcholine and non-interdigitated dipalmitoyl-phosphatidylcholine bilayers: cross-polarization/magic angle spinning ^{13}C-NMR studies. *Biochim. Biophys. Acta.* 1026:225–235.

Wu, W., L.-M. Chi, T.-S. Yang, and S.-Y. Fang. 1991. Freezing of phosphocholine headgroup in fully hydrated sphingomyelin bilayers and its effect on the dynamics of nonfreezable water at subzero temperatures *J. Biol. Chem.* 266:13602–13606.