Molecular Order and Hydration Property of Amine Group in Phosphatidylethanolamine and Its N-Methyl Derivatives at Subzero Temperatures

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ABSTRACT The molecular order and hydration properties of the amine group in phosphatidylethanolamine and its N-methyl derivatives were studied by ^{2}H-NMR at subzero temperatures. Three coexisting signals with ^{2}H-NMR quadrupolar splittings of 146, 106, and 28.8 KHz were detected from the fully hydrated phosphatidylethanolamine/D_2O at the lowest studied temperature of −120°C by using short recycle time in the applied NMR pulse sequence. These signals have been assigned to originate from frozen D_2O in the interbilayer space and the deuterated amine group, i.e., -ND, with and without threefold symmetric motions. Comparative ^{2}H-NMR studies of phosphatidylethanolamine/D_2O with different degrees of methylation over a temperature range between −40 and −120°C lead to the following conclusions. First, the bond angle of -D attached to the nitrogen atom of the amine group may be determined by the ^{2}H-NMR quadrupolar splittings, i.e., 106 and 28.8 KHz, of the two coexisting signals of the deuterated amine group and found to be 112.9 for the gel-state phosphatidylethanolamine. Second, assuming the applicability of the empirical equation for the hydrogen bond distance of N^+D—O with deuteron quadrupole coupling constants and using the intermolecular hydrogen bond distance of the amine group determined in single crystals of phosphatidylethanolamine bilayers, the largest measured quadrupolar splitting ($\Delta\nu_Q$) of N-D in this study, i.e., 106 KHz, is close to the static value. This interpretation is also consistent with the fact that the $\Delta\nu_Q$ value determined remains constant in the temperature range between −70 and −120°C. Third, the molecular order parameter of the amine group, as calculated from the ratio of the libration-averaged and static $\Delta\nu_Q$ value for the lipid with different degrees of methylation, suggests that the perturbation of the headgroup interaction is most significant for the final methylation step. Finally, measurement of the spectral intensity of isotropic unfrozen D_2O signals in D_2O/phospholipid dispersions at temperatures below the homogeneous nucleation temperature of ice formation for D_2O, i.e., below −34°C, suggests that the first methylation step perturbs the neighboring water most significantly. Assuming that the molecular order of the amine group and the amount of unfrozen water detected under the present experimental condition can be taken as a measure of the hydrogen-bonding ability and the extent of perturbation caused by the methyl group, respectively, the gradual methylation of the amine group perturbs the interactions of the N-methylated headgroups in a nonlinear fashion. The results provide a molecular explanation for the phase behavior of phospholipids with different degrees of methylation.

INTRODUCTION

There has been considerable interest recently in understanding the effect of N-methylated residues on the hydrogen-bonding ability of the amine headgroup and on the bulk of the headgroup in membrane bilayers (Domingo et al., 1994; Mason and O'Leary, 1990; Castelli et al., 1988; Gagne et al., 1985). Research in this direction can shed light on the phenomena underlying the marked differences in phase behavior and permeability of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Mulukutla and Shipley, 1984; Blume et al., 1982; Singer, 1981), the two major zwitterionic glycerolipids of mammalian cell membrane (Hirata and Axelrod, 1980). Furthermore, research in this direction would also reflect how membrane fluidity and chain-melting properties are regulated by the polar surface of the lipid bilayers (Sisk and Huang, 1992; Cevc, 1987, 1989; Mio et al., 1984; Sklar et al., 1977). For instance, a progressive increase in the number of the methyl groups in the PE headgroup gradually changes the bilayer melting transition from "PE-like" to "PC-like" (Casal and Mantsch, 1983; Vaughan and Keough, 1974). Based on the x-ray diffraction investigations, it seems that progressive increases in the number of methyl groups decreases gel phase bilayer thickness as a result of the increase in acyl chain tilting (McIntosh, 1980; Simon et al., 1991). Although the changes in the size and hydrogen-bonding ability of the headgroup regions are expected to play an important role in the aforementioned phenomena (Mason and O'Leary, 1990; Silvius et al., 1986), the exact nature of the effect of differences in headgroup structure on packing of the fatty acyl chain is unclear. We thus felt the need to carry out a detailed spectroscopic study of the effect of headgroup methylation.

^{2}H-NMR spectroscopy is a well established technique for the studies of conformation and dynamics of membrane lipids (Gally et al., 1975; Seelig and Gally, 1976; Ghosh, 1988). The ^{2}H-NMR spin-lattice relaxation time (T_1) of deuterium-labeled α (P-O-CD_2-CH_2) and β (P-O-CH_2-CD_2) headgroup segments of membrane lipids in the liquid-crystalline state suggests that the headgroup of PE is more rigid than that of PC (Browning, 1981a). In addition, increasing

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flexibility toward the free end of the headgroup has been observed for PC but not for PE, which exhibits essentially identical T_1 for both α and β methylene positions. This implies that the amine and ammonium groups of PE and PC exist in distinct dynamic states. The physical properties of the amine group may then serve as a sensitive probe for the study of the effect of headgroup methylation. Although abundant information has been deduced from x-ray studies of single crystals at the atomic level (for a recent review see Pascher et al., 1992) regarding the interactions of the amine group, it is not clear how the hydration and methylation exert their effects on the structure and dynamics of the amine headgroup. Because the complex ^{2}H-NMR spectra resulting from the slow dynamics of the headgroup in the gel-state at ambient temperatures (Blume et al., 1982) can be simplified by studying specifically deuterium-labeled molecular groups at subzero temperatures below the apparent freezing event of phosphate group (Hsieh and Wu, 1995a,b), an NMR study of the hydrated PE bilayer and its N-methylated derivatives at subzero temperatures may add a new piece of information about the molecular interactions of the phospholipids with different degrees of methylation. To the best of our knowledge, there has been no systematic investigation of the dynamics of the amine group by directly monitoring the spectroscopic properties of the -NH group in PE bilayers and their N-methylated derivatives.

In the present paper, we quantitate the hydrogen-bonding ability of the N-methylated headgroup by measuring the molecular order parameter of the amine groups in phospholipid bilayers. This information can be obtained by studying the magnitude and temperature-dependent behavior of ^{2}H-NMR quadrupolar splitting ($\Delta\nu_Q$) of the deuterated molecular group attached to the amine group at subzero temperatures between -40 and -120°C. Because of the fast proton exchange between the -NH and H_2O in PE dispersion at ambient temperatures (Ralph et al., 1985), deuterium exchange-labeled PE and its N-methyl derivatives can be easily obtained by simply dispersing lipid in D_2O.

There are several reasons for adopting the indirect ^{2}H-NMR instead of the direct ^{14}N-NMR measurement to study the molecular order of the amine group. First, the ^{14}N quadrupolar coupling constant is known to be sensitive to a chemical environment such as different degrees of methylation (Ragle and Minott, 1978), rendering it unsuitable as a probe for physical studies. In contrast, the principal component of the deuteron quadrupolar coupling tensor generally lies along the N-D bond. In addition, the $\Delta\nu_Q$ values obtained in the fast motionally symmetric regime are independent of the electric field gradient asymmetry parameter (η), in spite of the fact that η can be as high as 0.2 in an N-D bond (Prosser et al., 1994).

Second, the bond angle of the molecular group, i.e., -D and -CD_3, attached to the amine moiety can be unambiguously determined at subzero temperature if the ^{2}H-NMR signal representing the static and dynamic amine group with threefold symmetry can be measured under similar experimental conditions. As pointed out in the pioneering study of the PC headgroup structure using ^{2}H-NMR (Gally et al., 1975), and more recently in a study of the solvent effect on PC headgroup dynamics at subzero temperatures (Hsieh and Wu, 1995a), the threefold C_3 symmetry of the ammonium group provides a unique constraint to allow the determination of the bond angle for the -CD_3 attached to the amine group. Once the geometric contribution can be delineated, the average orientation order due to the presumed libration (or wobbling) of the amine group can be determined in principle, provided the N-D static $\Delta\nu_Q$ value, which is known to depend on the inverse cube of the hydrogen bond distance (Hunt and Mackay, 1976), can be measured or reasonably estimated. Such an approach has been successfully applied to study the dynamics of peptides in solid proteins and in monoclinic *N*-acetylglycine single crystals (Usha et al., 1991, 1992). Even if the absolute value of the calculated molecular order parameter may depend on the static $\Delta\nu_Q$ value used for calculation, comparative studies of these parameters for lipids at different degrees of methylation can still serve as a useful property, representing the intermolecular strength of the studied molecules.

It should be emphasized that both conformation and dynamics can contribute to order parameter changes; therefore, the interpretation of the order parameters obtained from the powder samples is not unique (see Browning, 1981b, and references therein). Nevertheless, if the order parameter obtained at subzero temperatures reaches its rigid limit, conformational differences will not contribute to the detected quadrupolar splitting differences measured in samples with randomly distributed lipids.

Finally, the water molecules near the methyl group can also be studied by ^{2}H-NMR if the experiments are performed at temperatures below the homogeneous nucleation temperature of ice formation, i.e., below -34°C. The isotropic heavy water signals of hydrated lipid bilayers arise from the reorientation of the interbilayer water molecules because of the orientation defects of the crystalline ice created by the methyl group in the headgroup region (Wittebort et al., 1988; Hsieh and Wu, 1995a). A combined ^{2}H- and ^{31}P-NMR study of several D_2O/phospholipid systems with different headgroups has also established that molecular groups attached to the phosphate segment, instead of the phosphate itself, are mainly responsible for the aforementioned unfrozen water (Hsieh and Wu, 1995b). The amount of perturbed unfrozen water in the neighborhood of the N-methylated headgroup could then be taken as a measure of the extent of perturbation caused by the introduced methyl group. It is not clear whether the introduction of the methyl group would enhance or decrease the interaction between the headgroup and water. An increase in the headgroup size due to methylation may lower the electrostatic field around the headgroup; furthermore, it also decreases the hydrogen bonding ability of PE. The results of the present study would help delineate the respective contribution of the two factors to the thermodynamic properties of PE and its N-methyl derivatives.

MATERIALS AND METHODS

Spectra were obtained on a 7.05 T Bruker MSL-300 spectrometer (Germany) using a broadband probe horizontally mounted with a 5 mm insert. ^{2}H-NMR spectra were recorded with a quadrupole echo pulse sequence ($90°_x$-τ-$90°_y$-τ-FID) using 90° pulses of 2.2–2.5 μs (depending on the temperatures) delay. The interpulse delay τ was 20 μs, unless mentioned otherwise. Recycle delays were varied from 100 ms to 300 s depending on the sample and the type of experiment. For instance, when only the water remaining in the interbilayer space was studied, a short recycle delay of 200 ms was used to eliminate signals from bulk water. The dwell time used to obtain the spectra was typically 0.5 μs. The temperature of the samples studied was controlled by evaporation of N_2 gas from a liquid nitrogen dewer and monitored by a Bruker VT-1000 thermal system. All of the temperature-dependent NMR spectra were obtained during the heating mode after the samples cooled to −120°C at a cooling rate of ~1°C/min. The obtained spectra were reproducible for each freshly prepared sample as long as the dispersions were frozen and thawed through the main lipid phase transition temperature within 24 hrs.

Samples used in this work, including dimyristoyl phospholipids containing PE, *N*-methylethanolamine (PMME), *N,N*-dimethyl ethanolamine (PDME), PC, or perdeuterated choline (d_{13}-DMPC) at the headgroup region, were obtained commercially (Avanti Polar Lipids, Alabaster, AL). Spectroscopic grade D_2O was from Cambridge Isotope Laboratory (Woburn, MA). Fully hydrated lipid samples were prepared in preweighed NMR tubes, using a known amount of lyophilized lipid. A known amount of heavy water was then added with a Hamilton microsyringe into the sample maintained at room temperature. After homogenization, the samples were ready for experiments and were sealed with septa.

RESULTS AND DISCUSSION

Fig. 1 shows a representative ^{2}H-NMR spectra of amine-containing phospholipids, at different degrees of methylation, at the indicated subzero temperatures. All of the samples were prepared at room temperature to a hydration state of ~19 ± 2 D_2O per lipid. The spectra were obtained during the heating mode, which followed the cooling of samples to the lowest temperature under study. The NMR signals of frozen bulk water were eliminated by a short recycle delay because of their relatively long T_1 (20 s) in comparison with that (20 ms) of the interbilayer water. Therefore, all of the signals shown in Fig. 1 arise from D_2O "trapped" in the interbilayer space. This is evident by the residual quadrupolar splitting of the expanded ^{2}H-NMR spectra obtained at −20°C. The bulk water remains frozen, in principle, at −20°C during the heating mode. Therefore, the detection of sharp NMR signals with a residual quadrupolar splitting clearly indicate that these water molecules are in the interbilayer space because they undergo fast, quasi-isotropic motions (Finer and Darke, 1974; Volke et al., 1994).

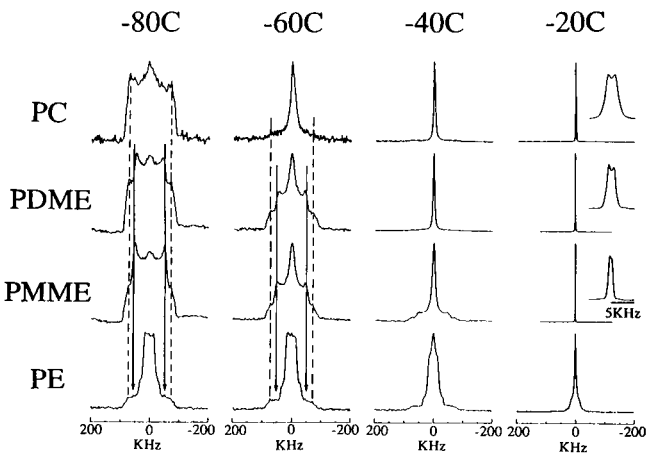

FIGURE 1 ^{2}H-NMR spectra of PC, PDME, PMME, and PE fully hydrated with D_2O at the indicated temperatures. The bulk ice signals were eliminated by using a short recycle delay of 200 ms because they exhibit relatively long T_1. (Please refer to the text for a detailed description of the figure.)

As seen in Fig. 1, the spectra obtained at −60 and −80°C exhibit at least two distinct anisotropic ^{2}H-NMR signals. The first, indicated by broken lines, corresponds to the quadrupolar splitting, $\Delta\nu_Q$, of 146 KHz, which is essentially the same as that of frozen D_2O (Wittebort et al., 1988; Hsieh and Wu, 1995b). The second, indicated by arrows, shows a $\Delta\nu_Q$ value of ~100 KHz. It should be noted, however, that the second quadrupolar splitting is barely detectable in PE (106 KHz), but it is clearly discernible in PDME (95 KHz) and PMME (100 KHz). In the case of PE, there is an additional strong signal with a $\Delta\nu_Q$ value of 28.8 KHz. Judging from the signal intensity and the $\Delta\nu_Q$ value, we tentatively attribute the broad anisotropic signal (146 KHz) to the frozen D_2O in the interbilayer space and the intermediate (~100 KHz) and narrow (28.8 KHz) anisotropic signals to the N-D attached to the amine group. We exclude the possibility that the intermediate quadrupolar splittings of 95, 100, and 106 KHz detected for PDME, PMME, and PE, respectively, may be due to bound water molecules hydrogen-bonded to the glycerol moiety or the phosphate group because signals with similar $\Delta\nu_Q$ values are not present in other phospholipid dispersion systems such as PC and phosphatidic acid (Hsieh and Wu, 1995a,b).

The quadrupolar splitting of 106 KHz, which is barely detectable for PE at the reported noise level (as indicated by the arrow shown in Fig. 1), deserves further attention. Its intensity is much lower than that of PDME and PMME, as one would expect from their respective exchange-labeled deuterium number. Fortunately, the signal can be enhanced further by accumulating data at a longer recycle delay (D_0) of 5 s (compare the PE spectra obtained at −80°C in Figs. 1 and 2 *A*). The spectral intensity obtained at longer recyle delay suggests that there may be some intensity loss for the signal with $\Delta\nu_Q$ value of 106 KHz due to the saturation effect caused by the short recycle delay. We should point out, however, that signals from the bulk water ice were also enhanced under the experimental condition of longer recycle delay.

The signal intensity with the $\Delta\nu_Q$ value of 106 KHz seems to decrease at higher temperature. This is evident from Fig. 2 *A*, which shows a series of spectra obtained under similar conditions at different temperatures. A similar phenomenon has also been observed for the ^{2}H-NMR spectra obtained from the deuterated trimethylammonium group of PC bilayers at subzero temperatures (Hsieh and Wu,

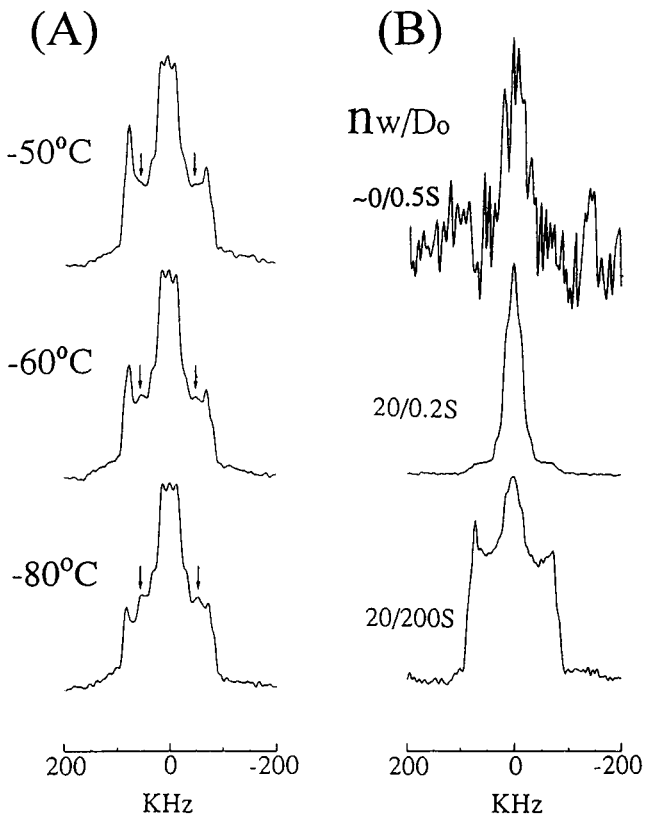

FIGURE 2 Comparison of ^{2}H-NMR spectra of fully hydrated D_2O/PC obtained under different conditions: (*A*) effect of temperature and (*B*) control experiment to assign the signal from the interbilayer D_2O and the effect of the hydration state (n_w: molar ratio of water to lipid). The spectra shown in B were obtained at −40°C. All of the spectra were obtained by Fourier transformation of the FID using the time domain of 256 words (W) except as indicated otherwise. Line broadening of 1 KHz was also used to enhance S/N of the spectra. The arrows shown in A indicate the quadrupolar Pake doublet signals from the deuterated molecular group of -ND in the absence of threefold symmetric motion of the amine group. The spectra shown in A were obtained using a recycle delay (D_o) of 5 s.

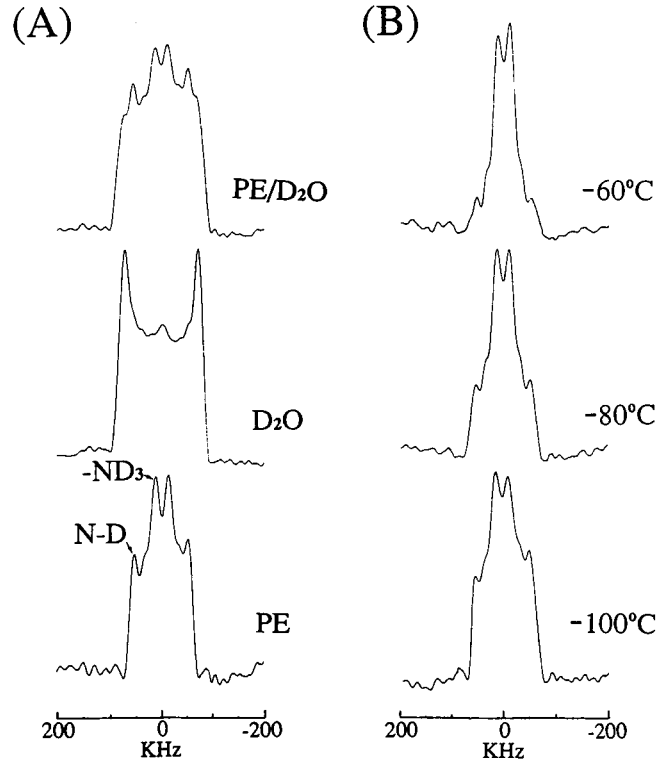

FIGURE 3 Assignment of the ^{2}H-NMR spectra obtained from PE/D_2O bilayers (*A*) and variation of the signal intensity for the two assigned lipid signals as a function of temperature (*B*). The spectrum obtained from PE/D_2O was carried out at −120°C, whereas that obtained from D_2O was performed at −30°C. All of the spectra shown in B were obtained according to the data processing procedure shown in A.

1995a), in which it was shown that the two distinct $\Delta\nu_Q$ values in the PC spectra are due to the coexistence of two gel-state bilayer structures in the presence and absence of a threefold symmetric motion of -$N(CD_3)_3$. Thus, by analogy, the two quadrupolar splittings of PE with $\Delta\nu_Q$ values of 28.8 and 106 KHz can be rationalized as two coexisting gel-state bilayer structures in the presence and absence of the threefold symmetric motion of -ND_3. The ratio of the two measured $\Delta\nu_Q$ values are also consistent with the estimated bond angle of the amine group based on Eqs. 1 and 2. Additional evidence to support this interpretation is presented later (Fig. 3 and 4).

The exact reason for the presence of two coexisting gel-state PE headgroup structures is not clear. We have attributed the presence of two coexisting gel-state PC headgroup bilayer structures to the interaction of the choline moiety with water molecules of distinct property near the membrane surface (Hsieh and Wu, 1995a). However, unlike PC, the signal to noise ratio (S/N) of the spectra of PE obtained at low hydration state and at temperatures below −40°C become too low to extract useful information even after 8 h of spectral accumulation time, as shown in Fig. 2 *B* (upper). Considerable intensity loss may occur if the correlation time of the dynamic process is in the vicinity of $(e^2qQ/h)^{-1}$ and/or insufficient recycle delay is applied to obtain the spectra (Beshah et al., 1987). Alternatively, in the absence of a hydrogen-bond acceptor from the nearby water molecules, some of the signals from N-D become too broad to be detected. The deuteron quadrupole coupling constant (QCC) of ND is sensitive to the N^+D—O hydrogen bond distance (Hunt and Mackay, 1976). Because we are not capable of describing the two putative coexisting gel-state structures for PE, it is important to provide sufficient evidence to indicate that there are indeed two coexisting signals for -ND_3, in view of the insuffient S/N of the signal with intermediate quadrupolar splitting of 106 KHz indicated by the arrows shown in Figs. 1 and 2.

We have tentatively determined that the signal with $\Delta\nu_Q$ values of 146 KHz shown in Fig. 1 arises from D_2O in the interbilayer space. Control experiments were carried out, using fully hydrated samples by changing the recycle delay, to show that the quadrupolar splitting of broad signal indeed resembles the quadrupolar splitting of the bulk water ice (Fig. 2 *B*, middle and bottom). A similar result has also been

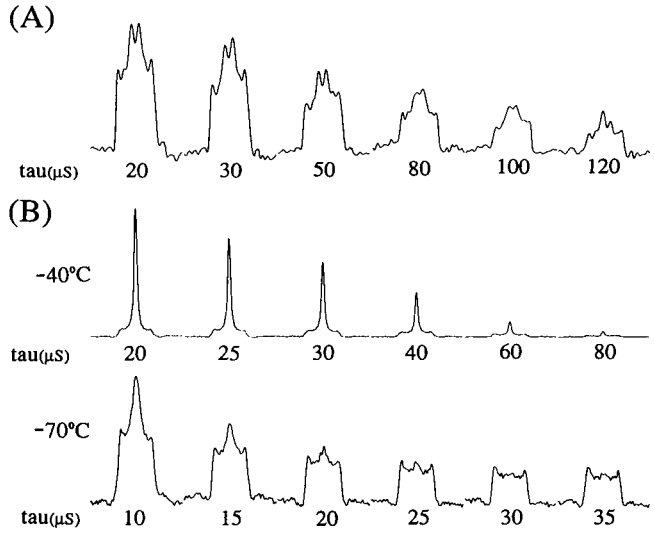

FIGURE 4 The effect of the interpulse delay (τ) on the ^{2}H-NMR spectra of the (*A*) PE/D_2O and (*B*) PMME/D_2O bilayers. The spectra presented are obtained after deleting frozen D_2O signal according to the procedure described for Fig. 3.

obtained with PC bilayers in which interbilayer D_2O molecules are the only source of the signal of ^{2}H-NMR spectra (Hsieh and Wu, 1995a). On the basis of this observation, we proceeded to perform spectral analysis to obtain a clear picture of the intermediate 106 KHz component by directly subtracting frozen D_2O signal from the presumed three-component ^{2}H-NMR spectra detected in the PE/D_2O system.

As shown in Fig. 3 *A*, a clear picture of our interpretation emerges when the ^{2}H-NMR signal of frozen D_2O ice is subtracted from that of PE/D_2O. In this particular case, in order to maximize the signal intensity of 106 KHz component, the spectrum shown in Fig. 3 *A* (upper) was obtained at the lowest studied temperature of −120°C, using a repetition time of 2 s. A short time domain of 128 words was also applied to emphasize the Pake doublet feature of the spectra. It is clear that only two ^{2}H-NMR signals assigned to -ND and -ND_3 of the amine group remained detectable (Fig. 3 *A*, bottom) after deleting the contribution of the D_2O component (Fig. 3 *A*, middle).

Similar results can also be obtained for the spectra obtained at higher temperature under identical experimental conditions and data treatment (Fig. 3 *B*). The signal intensities of the two components clearly vary as a function of temperature, as suggested before. Because of the possible intensity loss of the 106 KHz component under the present experimental conditions, no effort was made to estimate the free energy difference of the two components. Nevertheless, as in the case of PC headgroup, the intensity of the sharp signal component increases as the temperature of the lipid sample increases.

We should emphasize again that the two coexisting signals shown in Fig. 3 *B* originate from two coexisting gel-state PE headgroup structures. The detection of the two signals is not because the amine group undergoes motions in the intermediate regime. Both the spectral lineshape and the intensity of the central narrow component will change significantly if the deuterium-labeled molecular group undergoes motions in the intermediate regimen (Beshah et al., 1987). Although the signal intensity of the narrow component does vary as a function of temperatures, its lineshape remains similar (Fig. 3 *B*). In addition, the lineshapes shown in Fig. 4 *A* are not sensitive to interpulse delay in the quadrupolar echo experiment. It should be pointed out that all the spectra shown in Fig. 4 are the spectra obtained after deleting frozen D_2O signals, using the procedure described for Fig. 3 *A*, and therefore the broad components are from the respective ND group of the studied lipid molecules. The ratio of the spectral intensity of the sharp (28.8 KHz) and broad (106 KHz) signal remains relatively constant, at least for an interpulse delay shorter than 50 μs. Therefore, as in the case of PC bilayers, variation of the signal intensity reflects the property of the PE bilayer structures under study.

The quantitative aspect of isotropic signals in the PC, PDME, and PMME spectra and their assignments deserve more attention if these signals are going to be used to shed light on the lipid-water interactions. First, control experiments carried out by obtaining ^{31}P-NMR spectra of the samples under similar conditions showed an axially asymmetric powder pattern with a chemical shift anisotropy close to the rigid limit (data not shown). Therefore, it is unlikely that isotropic signals originate from D_2O tightly bound to the phosphate group. Because there is no deuterium label in the PC headgroup, the isotropic signal, at least for the one detected in PC bilayers at subzero temperature, can be assigned only to the unfrozen water, i.e., the water molecules undergoing tetrahedral reorientation, in the interbilayer space (Wu et al., 1991; Hsieh and Wu, 1995a,b).

Second, we have already designated signals with intermediate quadrupolar splittings of ~100 KHz as those from "static" ND groups of PDME, PMME, and PE. Therefore, the isotropic signals detected in PDME and PMME may be attributed partially to dynamic ND groups and/or to unfrozen interbilayer D_2O. In other words, it is possible that the isotropic sharp signal may actually consist of both water and lipid components. In order to exclude the latter possibility, we obtained a series of spectra as a function of interpulse delay (Fig. 4 *B*). Quantitative analysis of the intensity decay of isotropic signal did not show any indication of the presence of two superimposed components. The apparent spin-spin relaxation times as measured by two-pulse echo, T_{2e}, obtained at −40°C and −70°C are 38 μs and 15 μs, respectively. In addition, similalr T_{2e} values were also found for the isotropic signals of PDME, PMME, and PC under similar experimental condition. Therefore, the possible contribution of the isotropic signals of PDME and PMME from the dynamic -ND group can be considered small, if not negligible.

Third, it is not likely that fast exchange between ND of lipid and D_2O of interbilayer water can occur, in view of the

distinct T_{2e} measured for the sharp and broad component. For instance, the isotropic signals in PMME decay much faster than the broad signal of the static -ND at −70°C (Fig. 4 *B*). In addition, both the ND_3 with threefold symmetric motion and the static ND of the PE headgroup are seen to exhibit relatively long T_{2e}, with apparent values of ~100 μs (Fig. 4 *A*). The lack of exchange between -ND and D_2O is also consistent with the well defined lineshape of the characteristic ^{2}H-NMR powder spectra for the anisotropic signal with quadrupolar splitting of 100 KHz (for instance, see the spectra obtained at −70C and at 35 μs interpulse delay shown in Fig. 4 *B*). The NMR lineshapes detected at indicated interpulse delay also argue against the -ND dynamics being in the intermediate time regime. In conclusion, the sharp isotropic signals detected from PC, PDME, and PMME are mainly from the interbilayer D_2O exhibiting isotropic or quasi-isotropic motions. The detection of a negligible isotropic signal from PE suggests that the first methylation step may play an important role in inducing the unfrozen isotropic D_2O signal at temperatures below the homogeneous nucleation temperature of ice formation.

The knowledge of the $\Delta\nu_Q$ values for the ND_3 with and without C_3 symmetric rotation/jump allows us to obtain unique information on the bond angle of the -D attached to the nitrogen atom. By assuming that in addition to three-site rotation/jump the entire amine group librates rapidly with an angle of θ, the averaged $\Delta\nu_Q$ for an axially symmetric averaged powder pattern is given by

$$\overline{\Delta\nu_Q} = \Delta\nu_Q S(3\cos^2\phi - 1)/2 \quad (1)$$

where

$$S = \overline{(3\cos^2\theta - 1)/2} \quad (2)$$

and ϕ is the angle made by the N-D bond with the threefold axis. For a static molecular group without libration motion, the angle θ is equal to 0 and the corresponding $\overline{(3\cos^2\theta - 1)/2}$ value in Eq. 2 becomes 1. Because the static and three-site rotation-averaged $\Delta\nu_Q$ are determined experimentally as 106 KHz and 28.8 KHz, respectively, a value of 112.9 is obtained for ϕ between C_β-N and N-D on the basis of Eqs. 1 and 2. The angle ϕ between C_β-N and N-C_γ in PC has been determined previously to be 113.7, on the basis of the same principle (Hsieh and Wu, 1995a). Interestingly, the corresponding values of the two angles, as determined by x ray of PDME crystal (Pascher and Sundell, 1986), are 112 and 112.7, respectively. It is important to note that the two ϕ values determined for PE and PC are highly sensitive to the values of $\Delta\nu_Q$ used for calculation; therefore, the possible error in the value of ϕ determined by NMR is much smaller than that determined by x ray, provided that the determined values of $\Delta\nu_Q$ can be as accurate as ± 0.25 KHz.

The deuteron quadrupole coupling constant (QCC, e^2Qq/h) for N-D can also be estimated from its $\Delta\nu_Q$ value (QCC = 4/3 $\Delta\nu_Q$) to be 141 KHz, which is similar to that determined from the amide bond (Ragle and Minott, 1978). Two lines of evidence indicate that this experimental value is indeed close to the static one. First, the hydrogen bond distance for P-O—H-N has been determined in the single crystal of PE bilayers to be ~1.74 Å (Hitchcock et al., 1974; Pascher et al., 1992). Assuming the applicability of the empirical equation derived from nuclear quadrupolar resonance work on deuterated amino acids at 77 K (Hunt and Mackay, 1976)

$$e^2Qq/h = 253 - 572/R^3 \text{ KHz} \quad (3)$$

where R represents the D-O hydrogen bond length in angstroms, the expected QCC can be roughly calculated to be ~144 KHz, which is practically the same as that determined for -ND from −60 to −120°C. Therefore, it is reasonable to assume that the measured value is indeed close to the static value of QCC. A similar argument has also been applied to assign the deuterium quadrupolar couplings in *N*-acetylglycine single crystals (Usha et al., 1991). Alternatively, accepting this argument at face value, our results suggest that the hydrogen bond distance determined from the crystal with minimum hydration is similar to PE under a fully hydrated condition. Another line of evidence is obtained from the temperature- dependent studies of $\Delta\nu_Q$, a topic dealt with in the following section.

Fig. 5 shows the temperature-dependent profile of the $\Delta\nu_Q$ value of -ND determined from the exchange-labeled amine group with progressive methylation (*closed symbols*). The values determined from the selectively deuterated trimethylammonium group in PC bilayers are also included for comparison (*open circle*). The estimated molecular order parameters (S) shown in the figure were obtained from the ratio of the presumed libration-averaged and static $\Delta\nu_Q$ values. We believe that the $\Delta\nu_Q$ value of 106 KHz is indeed close to a static one because it remains constant for PE at temperatures below −60°C. As shown in Fig. 5, a stepwise methylation reduces the molecular order parameter of the amine group in a nonlinear fashion, and the most significant change occurs at the final methylation step.

It should be emphasized that there is no evidence to suggest that only a single type of libration motion and/or conformational change is responsible for the reduced quadrupolar coupling constant at higher temperature and higher methylation of the PE headgroup. Both conformation and dynamics can contribute to order parameter changes. Additionally, there may be several different anisotropic motions of the headgroup, which can all contribute to the reduction of the detected order parameter. Therefore, the interpretation of the order parameter determined from membrane lipid at ambient temperature is not unique because of the contribution of several factors (Browning, 1981b). It would be simpler, however, to determine the order parameter of the lipid headgroup at subzero temperatures because of the apparent freezing event of the phosphate dynamics. But complications would still remain if the conformational contributions from the different methylated headgroups are not constant. Keeping these in mind, we first discuss the order

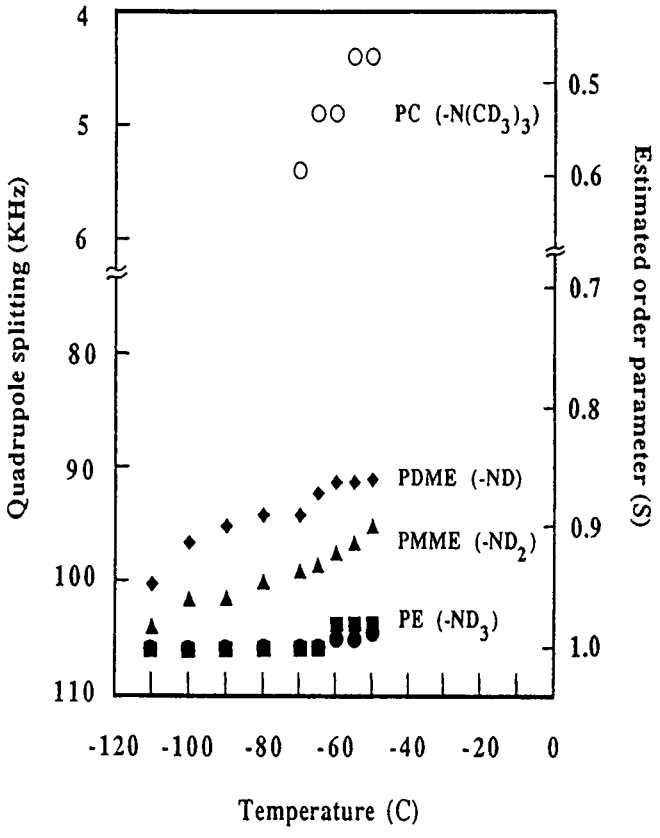

FIGURE 5 Temperature-dependent profile of the measured quadrupolar splittings and estimated molecular order parameter of the amine group and its N-methyl derivatives. The order parameters were obtained by assuming the static quadrupolar splitting of the -ND group (106 KHz) to be the same for PDME, PMME, and PE. The solid triangle and rhombus represent the parameters determined from the deuterated amine group of the indicated lipids. The open circle represents the parameters for $-CD_3$ in the choline headgroup moiety.

parameters obtained from the $-N(CD_3)_3$ and $-ND_3$ group of PC and PE because their C_3 symmetry allow us to delineate the respective contributions from the conformation and dynamics of the studied molecular groups.

The much lower values (in the range of 0.5) of the order parameter obtained from the trimethylammonium group of PC can be attributed to two dynamic processes (Hsieh and Wu, 1995a): libration of the entire choline group, as evidenced by the reduced order parameter of the methylene segments, and wobbling motion of the trimethylammonium group, as evidenced by a much higher slope of its temperature-dependent profile (*open circle* in Fig. 5). It should be mentioned that the quadrupolar splitting of the $-CD_2-CD_2-$ in the choline group was found to be ~115 KHz, which was ~10% lower than its static value of 125 KHz in the studied temperature range (Hsieh and Wu, 1995a). To illustrate the aforementioned dynamic processes, we presume that the obtained molecular order parameter can be translated into a root-mean-square angle of the average orientational order according to Eq. 2, i.e., $S = \overline{(1/2)(3\cos^2\theta - 1)}$ where θ defines the libration/wobble angle of the C-D bond (Usha and Wittebort, 1992). A simple calculation reveals that the the methylene segment may undergo a libration motion with an angle of $\theta \sim 15°$, and the ammonium group of PC may undergo an additional wobbling motion with an angle of $\theta \sim 30°$.

A similar approach can also be used to estimate the wobble angle of the ND_3 group of the PE bilayer. In addition to the threefold symmetric motion of the ND_3 group, the amine group seems to undergo a small angle of libration ($< 5°$) inasmuch as the quadrupolar splitting of the ND detected at temperatures above $-60°C$ is only slightly smaller than the presumed static value of 106 KHz. Therefore, the major difference between the PC and PE headgroups is that the threefold symmetric motion of ND_3 does not exhibit any significant additional wobbling because the smallest quadrupolar splitting of $-ND_3$ (~28.8 KHz) remains relatively constant within the experimental error in the studied temperature range. It also suggests that there is no detectable change of the bond angle of C-N-D for PE in the studied temperature range, in view of the sensitivity of the quadrupolar splitting to the bond angle, and that the quadrupolar splittings of both $-ND_3$ and -ND signals remain constant over a wide temperature range.

The interpretation of the order parameters obtained from PDME with the -ND group and PMME with the $-ND_2$ group is more complex because both the bond angle of C-N-D and the hydrogen bond distance of $N^+D—O$ may be significantly different in the respective -ND group. It should be pointed out that the possible conformational differences in the average bond angle of the C-N-D groups of PE, PMME, and PDME are not expected to cause the reduction of the quadrupolar splitting detected from the static -ND group for the ^{2}H-NMR spectra obtained from the randomly oriented powder sample. However, both the changes of the hydrogen bond distance and the dynamics of the -ND group can cause the reduction of the detected quadrupolar splitting. A shorter $N^+D—O$ hydrogen bond distance for the ND group of PDME may partially account for the smaller quadrupolar splitting value of PDME in comparison with that of PMME or PE. It is also possible that the bond distance is the same as in PE and that the order parameter differences are only due to the dynamic differences. Because the order parameter decreases significantly as the temperature increases, the most likely explanation for the temperature-dependent profiles would be the contribution from the dynamic process, such as libration of the ND group. Therefore, by assuming the static quadrupolar splitting of -ND, $-ND_2$, and $-ND_3$ in PDME, PMME, and PE, respectively, to be the same, i.e., 106 KHz, it is possible to estimate the effect of methylation on the libration angle as a result of the hydrogen-bonding ability of the amine group in N-methylated PE bilayers.

On the basis of the aforementioned argument, a stepwise methylation of PE was found to allow the N-methylated headgroup to move with 15°, 20°, and 35° amplitudes at temperatures near $-40°C$. It seems that the libration/wobble angles of the amine groups in PE, PMME, and PDME are even smaller than those (~20°) of the methylene segment in

PC. In fact, if the static quadrupolar splitting of ND in PDME is larger than 106 KHz, the estimated wobbling angle will be even smaller. Therefore, the final methylation step, in contrast to the first two methylations, seems to produce the most significant effect by allowing not only a much larger amplitude in the motion of the ammonium group, but also an extra degree of freedom for the entire choline moiety. Above all, there is no doubt that the stepwise methylation produces a nonlinear effect on the order parameter of the molecular group attached to the amine group.

It should be pointed out that an apparently contradictory picture may arise regarding the dynamic behavior of "frozen" interbilayer D_2O and "static" -ND bond if one considers that their spin-lattice relaxation times (T_1) are relatively short (~100 ms) but their quadrupolar coupling constants are close to static values. For instance, the $\Delta\nu_Q$ value of interbilayer frozen D_2O was detected to be 146 KHz, which is essentially the same as the one detected for hexagonal ice, despite the fact that their T_1 values were found to be in the range of 100 ms (Hsieh and Wu, manuscript in preparation). Similar behavior was also found for the deuterium NMR signal of the -ND bond. One possible explanation is that characteristic time scales sensitive to spin-lattice relaxation and quadrupolar splitting of deuteron are different by three orders of magnitude. Therefore, the fast small angle librational motion of the molecule in the glassy state may not be picked up by the measured ^{2}H- NMR quadrupolar splitting, despite the fact that it is clearly detected by the T_1 process (Dubinskii et al., 1994; Spiess, personal communication, 1995). We are currently investigating this possibility.

Quantitation of the isotropic unfrozen water signals at −40°C can provide, in principle, a measure of the effective perturbation of the introduced methyl group in the lipid samples under study. We therefore obtained a fully relaxed ^{2}H-NMR signal of D_2O (Fig. 6) in D_2O/phospholipid samples at −40°C to allow the determination of the relative intensity of isotropic unfrozen water signals. After correcting for the loss of signal intensity during the interpulse delay of the quadrupole echo pulse sequence, we estimated that there were approximately four unfrozen water molecules per lipid at −40°C for a lipid containing one methyl group, i.e., PMME (Fig. 7). In contrast, no significant isotropic unfrozen water signal can be detected for PE. It should be noted that the central band of the PE spectra shown in Fig. 6 originates mainly from -ND_3 with threefold symmetric motion (compare the PE spectra obtained at −40°C shown in Fig. 1 and Fig. 2 *B*), but the central isotropic signals of PC, PDME, and PMME are mainly from unfrozen interbilayer D_2O. There are no discernible signals from static ND because all of the samples are in an excess amount of D_2O. In fact, despite the quantitative feature of the unfrozen water molecules, it is clear that methylation of the PE headgroup induces the formation of unfrozen water molecules in a nonlinear fashion. The first methylation step seems to affect its nearby water molecules most significantly because unfrozen water molecules are negligible in the PE/D_2O system.

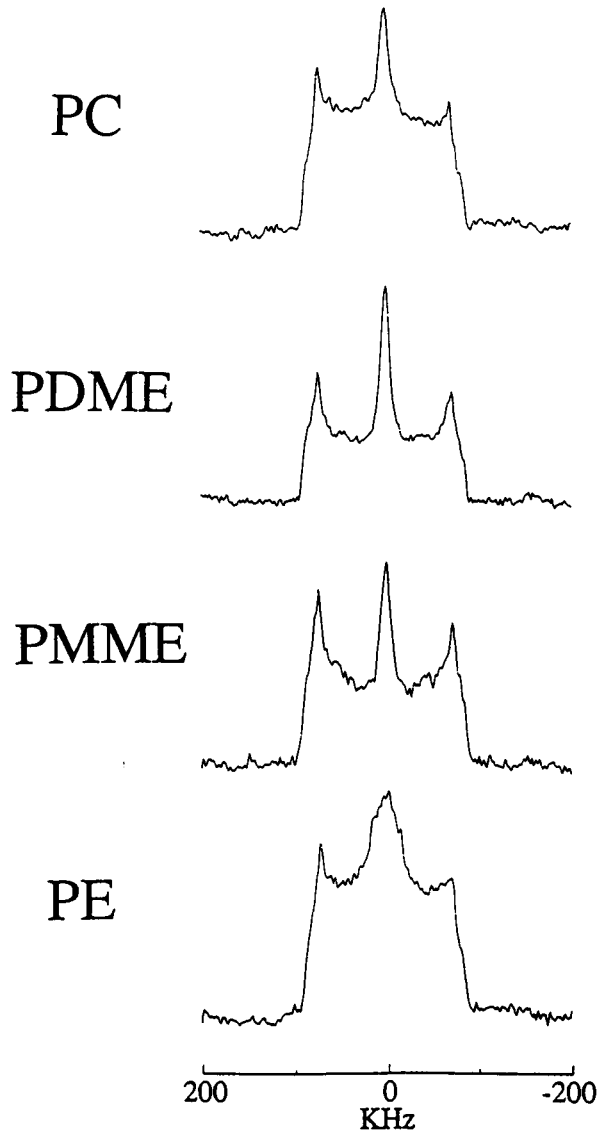

FIGURE 6 Fully relaxed ^{2}H-NMR spectra of PE and its N-methyl derivatives hydrated with 19 ± 2 D_2O per lipid. The lipids used in this study were the same as those in Fig. 1, but a long recycle delay of 200 s was used to obtain fully relaxed spectra at −40°C. The interpulse delay was 20 μs. The isotropic signals detected in PC, PDME, and PMME are from D_2O, but the corresponding signal with much broader line width detected in PE is mainly from exchange-labeled ND_3.

The effect of methylation on the phospholipid packing has been studied previously mainly by monitoring their phase behavior (Chowdhry and Dalziel, 1985; Brown et al., 1986; Dorfler et al., 1990; Sisk and Huang, 1992). Because the phase behavior properties of lipid dispersion are sensitive to the packing in the fatty acyl region, previous results represent the combined effects of both the headgroup and the hydrocarbon regions. In light of this, we herein investigated the effect of methylation on the molecular order of the amine group and on the amount of isotropic unfrozen

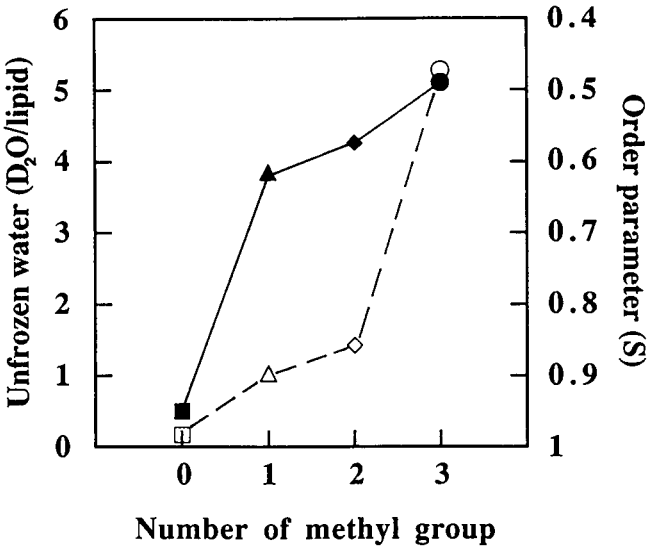

FIGURE 7 Effect of the progressive methylation on the amount of unfrozen water molecules and the order parameter of the amine group in fully hydrated PE and its N-methyl derivatives at −40°C. Solid symbols represent the amount of unfrozen water determined from fully hydrated PE, PMME, PDME, and PC with 0, 1, 2, and 3 methyl groups, respectively. The corresponding open symbols represent the estimated order parameter, S, of the amine groups in PE and its N-methyl derivatives. The absolute value of order parameter and the amount of unfrozen water amount may vary slightly depending on the static value of quadrupole splitting used for calculation and on whether one considers the water bound to the phosphate group in the lyophilized lipids. But the relative change of the studied parameters as a result of stepwise methylation is not expected to differ.

water signal from the interbilayer space, thus completely excluding the contribution or involvement of the fatty acyl region. The results, as summarized in Fig. 7, show that although the first methylation step perturbs the nearby water molecules most significantly, it changes the molecular order of the amine group only slightly. Therefore, the hydrogen-bonding ability, although clearly lowered by the reduced number of N-H hydrogen bonds in the N-methyl derivatives of PE, persists at the studied subzero temperature range as long as at least one N-H bond remains. In contrast, although the molecular order of the amine group is perturbed most significantly at the final methylation step, the magnitude of perturbation remains similar to prior methylation as measured by the amount of isotropic unfrozen water signals.

These results, although predictable, are useful for understanding the molecular mechanism of the effect of headgroup methylation on the phase behavior. First, the melting volume for a series of N-methylated dipalmitoyl PE was found to decrease monotonously and was followed by an abrupt increase in the final methylation step (Mason and O'Leary, 1990). This biphasic effect, which has been rightly interpreted in the original work, is a clear manifestation of the effectiveness of the hydrogen-bonding ability of the N-H-containing lipids.

Second, the effect of methylation on the lamellar repeat distance has been studied by x-ray diffraction measurement of hydrated multilamellar dispersions (Mulukutla and Shipley, 1984). The first methylation step of PE increases the lamellar repeat distance by ~10 Å, but the following methylation steps do not exhibit any significant effect. The result can be interpreted simply by assuming the perturbation effect of the introduced methyl group, as suggested in this study. The perturbation effect of the bulky methyl group, which is expected to give rise to a repulsive force between the two membrane surfaces, is most effective on the first methylation step. The fact that the magnitude of the repulsive hydration pressure for PC and PDME is comparable (Simon et al., 1991) is also consistent with the observation that the effective perturbation created by the introduced methyl groups for PC and PDME are similar (Fig. 7).

Finally, the effect of methylation of PE on the enthalpy and entropy of the lipid phase transition has been studied in several phospholipid species (Vaughan and Keough, 1974; Casal and Mantsch, 1983; Mulukutla and Shipley, 1984; Gagne et al., 1985; Chowdhry and Dalziel, 1985; Silvius et al., 1986; Sisk and Huang, 1992). In all the cases, both enthalpy and entropy increase significantly with the addition of the first methyl group, level off with the addition of the second methyl group, and finally decrease with the addition of the last methyl group. According to our model, the initial increase seems to be due mainly to the perturbation of the headgroup bulk and the final decrease to the perturbation of the hydrogen-bonding ability. Because abolishing the final hydrogen-bonding ability would decrease the enthalpy and entropy of the lipid phase transition, it would perturb the intermolecular interaction of the phospholipid more significantly in the gel-state than in the liquid-crystalline state of lipid bilayers. Thus, progressive methylation seems to perturb lipids packed in different physical states to different degrees.

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