Studies of Phospholipid Hydration by High-Resolution Magic-Angle Spinning Nuclear Magnetic Resonance

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ABSTRACT A sample preparation method using spherical glass ampoules has been used to achieve 1.5-Hz resolution in ^{1}H magic-angle spinning (MAS) nuclear magnetic resonance (NMR) spectra of aqueous multilamellar dispersions of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), serving to differentiate between slowly exchanging interlamellar and bulk water and to reveal new molecular-level information about hydration phenomena in these model biological membranes. The average numbers of interlamellar water molecules in multilamellar vesicles (MLVs) of DOPC and POPC were found to be 37.5 $\pm$ 1 and 37.2 $\pm$ 1, respectively, at a spinning speed of 3 kHz. Even at speeds as high as 9 kHz, the number of interlamellar waters remained as high as 31, arguing against dehydration effects for DOPC and POPC. Both homonuclear and heteronuclear nuclear Overhauser enhancement spectroscopy (NOESY and HOESY) were used to establish the location of water near the headgroup of a PC bilayer. ^{1}H NMR comparisons of DOPC with a lipid that can hydrogen bond (monomethyldioleoylphosphatidylethanolamine, MeDOPE) showed the following trends: 1) the interlamellar water resonance was shifted to lower frequency for DOPC but to higher frequency for MeDOPE, 2) the chemical shift variation with temperature for interlamellar water was less than that of bulk water for MeDOPE MLVs, 3) water exchange between the two lipids was rapid on the NMR time scale if they were mixed in the same bilayer, 4) water exchange was slow if they were present in separate MLVs, and 5) exchange between bulk and interlamellar water was found by two-dimensional exchange experiments to be slow, and the exchange rate should be less than 157 Hz. These results illustrate the utility of ultra-high-resolution ^{1}H MAS NMR for determining the nature and extent of lipid hydration as well as the arrangement of nuclei at the membrane/water interface.

INTRODUCTION

Water is essential to the structure and dynamics of biological and model membranes (Crowe and Crowe, 1984). Recent years have witnessed an increasing research effort to delineate properties of the membrane surface that determine the extent and nature of lipid hydration (Gawrisch et al., 1992; Israelachvili and Wennerstrom, 1992; McIntosh and Simon, 1993; Volke et al., 1995; Chen et al., 1996). The phase equilibria and the construction of phase diagrams of water/amphiphile systems were also studied by ^{2}H nuclear magnetic resonance (NMR) (Ulmius et al., 1977; Gutman et al., 1984; Sjolund et al., 1987; Lindblom and Oradd, 1994; Lindblom, 1996). The phase diagrams can be used to obtain the amount of water interacting with lipid. Undoubtedly, surfaces, including lipid bilayer surfaces, will influence the physical properties of nearby water molecules (Woessner, 1980). Conversely, progressive hydration of bilayer-forming phospholipids has been found by NMR to allow greater motional freedom of the polar lipid headgroups (Bechinger and Seelig, 1991; Ulrich and Watts, 1994) as well as the hydrophobic fatty-acid chains (Volke et al., 1982). Thus, changes can occur in both the structural and dynamic properties of lipids as a result of interactions with water. Hydration of phospholipid bilayers has also been investigated by several other biophysical techniques: adsorption isotherms (Jendrasiak et al., 1996), differential thermal analysis (Lundberg et al., 1978), infrared (IR) (Fookson and Wallach, 1978), and differential scanning calorimetry (DSC) (Ulrich et al., 1994; Bach and Miller, 1998).

The challenges of performing high-resolution proton NMR experiments on model biological membranes are well known, as aqueous lipid dispersions form extended bilayers for which residual (inhomogeneous) dipolar interactions between different proton pairs result in spectral patterns several kHz in breadth (Bloom et al., 1977). In the liquid-crystalline phase of phosphatidylcholines, the magic-angle spinning (MAS) technique can yield much improved ^{1}H NMR spectra that resolve the isotropic chemical shifts of all protons in the polar headgroup as well as the glycerol backbone (Forbes et al., 1988; Halladay et al., 1990). Although spinning speeds of just a few kHz are sufficient to remove spinning sidebands from these NMR spectra, sample preparation strategies that use cylindrical Kel-F inserts (Holte and Gawrisch, 1997) or spherical glass ampoules (Zhou et al., 1997) are required to achieve routine spinning rates of 10 kHz and linewidths as small as 1.5 Hz. Under these circumstances, it is possible to distinguish spectroscopically between slowly exchanging populations of bulk and interlamellar water in a variety of aqueous lipid suspensions.

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Following our initial MAS ^{1}H NMR study of hydration in monomethyldioleoyl-phosphatidylcholine (MeDOPE) dispersions (Chen et al., 1996), the current work exploits the enhanced experimental capabilities described above to address several questions about lipid hydration. First, we have assessed whether the centrifugal force created during fast magic-angle spinning measurements causes inadvertent alterations in the absolute and relative numbers of interlamellar and bulk water molecules. These numbers have been compared with previous studies of interlamellar water and water saturation numbers derived from differential scanning calorimetry (DSC) (Kodama et al., 1997), x-ray diffraction (Rand and Parsegian, 1989), and ^{2}H NMR (Ulrich and Watts, 1994). Second, we have used MAS-assisted two-dimensional nuclear Overhauser effects (NOESY and HOESY) (Halladay et al., 1990; Yu and Levy, 1983, 1984; Cistola and Hall, 1995) to investigate the location of water molecules within the PC lipid bilayers. Our findings have been evaluated in light of two surprising reports that water resides at the interior of dimyristoylphosphatidylcholine (DMPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) multibilayers (Forbes et al., 1988; Volke and Pampel, 1995). Possible correlations of hydration behavior with the phase and hydrogen-bonding properties of particular lipids are also discussed. Finally, we have estimated the exchange rates between bulk and interlamellar populations of water in multilayers of MeDOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and their mixtures.

EXPERIMENTAL

The phospholipids used in this study (DOPC, POPC, MeDOPE and dimethyldioleoylphosphatidylethanolamine (MeMeDOPE)) were purchased from Avanti Polar Lipids (Alabaster, AL). Deuterium oxide (99.9%) and $CDCl_3$ were purchased from Isotec (Miamisburg, OH). For qualitative ^{1}H NMR measurements, aqueous phospholipid dispersions were made by hydrating the lipid powder or film with water or buffer. For quantitative ^{1}H NMR measurements, DOPC and POPC were dissolved in $CHCl_3/CH_3OH$ (2:1, v:v), dried with N_2, and then lyophilized at high vacuum for 20 h. After addition of D_2O to well defined amounts of the lipids, the MLVs were taken through five cycles of vortex mixing, centrifuging, and freeze-thawing to obtain a uniform paste. Each sample was loaded into a Pyrex tube with a 7–12-μl bulb at the end; the bulb was then cut off at the stem, sealed with epoxy glue, and positioned near the center of a 5-mm cylindrical Zirconia MAS rotor filled with finely powdered dry KBr (Zhou et al., 1997).

All NMR spectra were recorded on a Bruker Avance DRX-500 spectrometer. High-resolution liquid-state data were recorded with a 5-mm broadband inverse probe equipped with triple-axis gradient capability. Two-dimensional proton correlation spectroscopy (COSY) was carried out in the absolute value mode using the pulse sequence (90° - t_1 - 45° - ACQ) and pulsed field gradients for coherence selection (Hurd, 1990; von Kienlin et al., 1991). A single transient with a 2.841-kHz spectral width (F_2) defined by 2K data points was acquired for each of 256 free induction decays (FIDs) (F_1) in the two-dimensional (2D) data matrix. The ^{1}H 90° pulse width was 6.6 μs, and a 1.0-s relaxation delay was inserted between successive acquisitions. Zero-filling in the F_1 dimension produced a 1K $\times$ 1K data matrix with a digital resolution of 2.774 Hz/point in both dimensions. The transformed data were then symmetrized. High-resolution ^{13}C NMR spectra were recorded at 125.758 MHz, using a 28.985-kHz spectral width defined by 32K data points. The ^{13}C 90° pulse width was 12.7 μs, and a 1.0-s relaxation delay was also used. The FIDs were processed using exponential line broadening of 1.0 Hz and zero-filled to 64K points before Fourier transformation. The inverse-detected ^{1}H-^{13}C 2D chemical shift correlation spectra were acquired in the phase-sensitive mode using a pulsed-field-gradient version of the heteronuclear single quantum coherence (HSQC) experiment (Kontaxis et al., 1994). In the F_2 (^{1}H) dimension, 1K data points were recorded over a 3.788-kHz spectral width. In the F_1 (^{13}C) dimension, 128 FIDs of two scans each were obtained covering a 21.367-kHz spectral width. The fixed delays in the pulse sequence included a 1.0-s relaxation delay and a 1.786-ms polarization transfer delay. The resulting data were processed using a sine-bell squared window function shifted by $\pi/2$ in both dimensions and linear prediction to 256 data points in F_1 followed by zero-filling to 1K.

MAS NMR spectra were acquired using a 5-mm high-speed Doty solid-state probe. MAS speeds were varied between 2 and 10 kHz in separate experiments, and the sample temperature was equilibrated for at least 10 min before data acquisition. One-dimensional proton spectra were obtained with eight scans and 32K data points covering a 3.005-kHz spectral width. The FIDs were processed using exponential line broadening of 0.2 Hz and were zero-filled to 64K before Fourier transformation. MAS-assisted proton NOESY NMR spectra were acquired in the phase-sensitive mode using the pulse sequence (90° - t_1 - 90° - τ - 90°- ACQ) (Jeener et al., 1979) and a 90° pulse width of 5.8 μs. Phase-sensitive data were obtained using time proportional phase incrementation (TPPI) (Ernst et al., 1987). In the F_2 dimension, 2K data points were used with a 3.005-kHz spectral width, 32–128 scans, and a 4-s relaxation delay. In the F_1 dimension, 128 FIDs were extended by linear prediction to 256 data points and zero-filled to produce a 1K $\times$ 1K data matrix. This procedure gave a digital resolution of 2.934 Hz/point in both dimensions. During the 2D Fourier transformation a sine-bell window function shifted by $\pi/2$ was applied in both dimensions. The transformed data were not symmetrized. MAS ^{31}P-^{1}H HOESY 2D NMR spectra were also acquired in the phase-sensitive mode using TPPI. In the ^{31}P (F_2) dimension, a 4.045-kHz spectral width was defined by 2K data points,

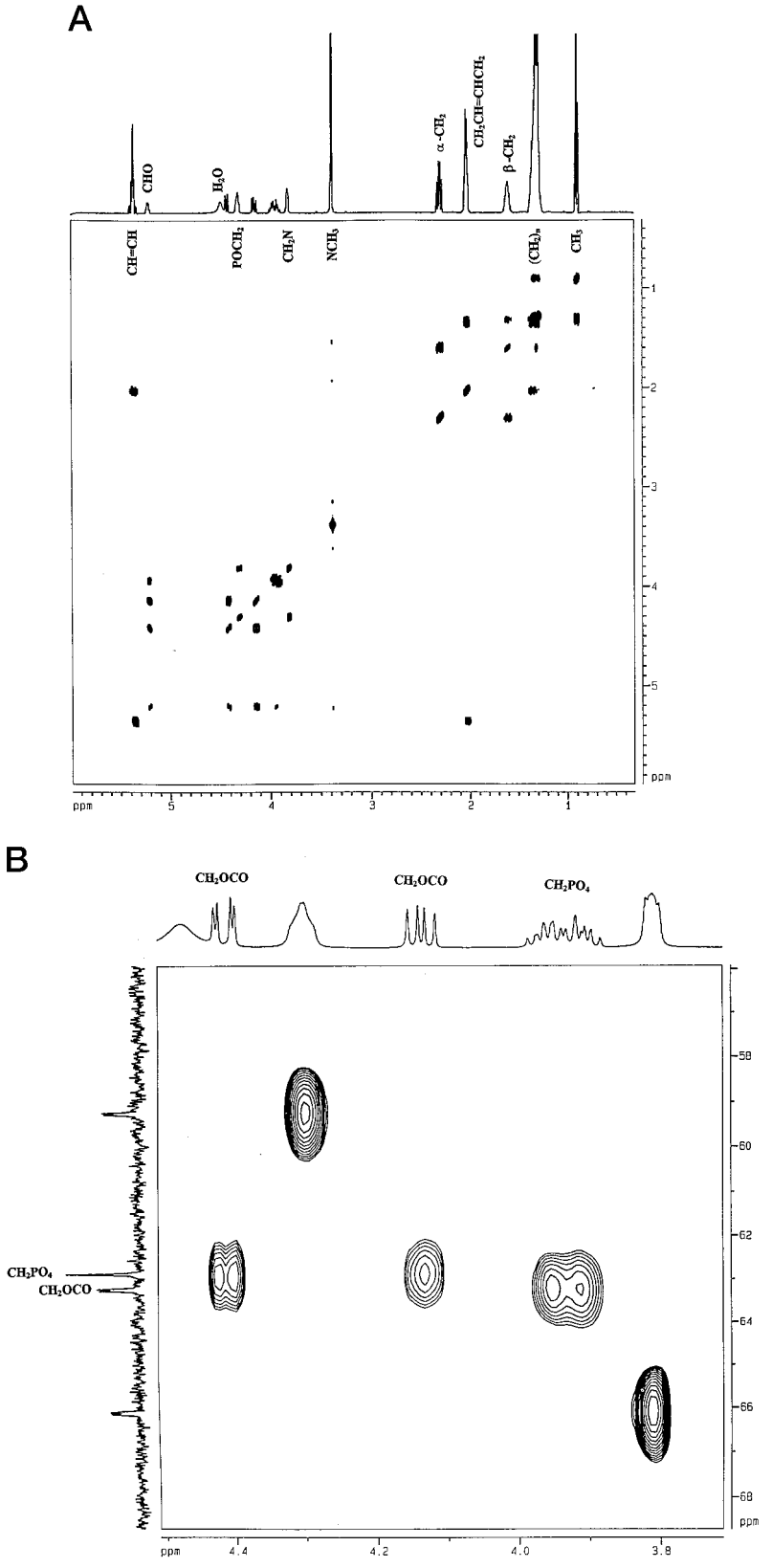

FIGURE 1 (*A*) Contour plot of ^{1}H gradient-assisted COSY-45 results for DOPC in $CDCl_3$ at 30°C. (*B*) Gradient ^{1}H-^{13}C HSQC data for DOPC in $CDCl_3$ at 30°C, providing assignments for CH_2OCO and CH_2PO_4 protons of DOPC as described in the text.

and each FID required 80–128 scans. In the F_1 dimension, 128 FIDs were linear predicted to 256 data points and zero-filled to 1K points covering a ^{1}H spectral width of 3.004 kHz. The ^{31}P 90° pulse width was 2.6 μs. The data were processed using exponential line broadening of 3.0 Hz in both dimensions.

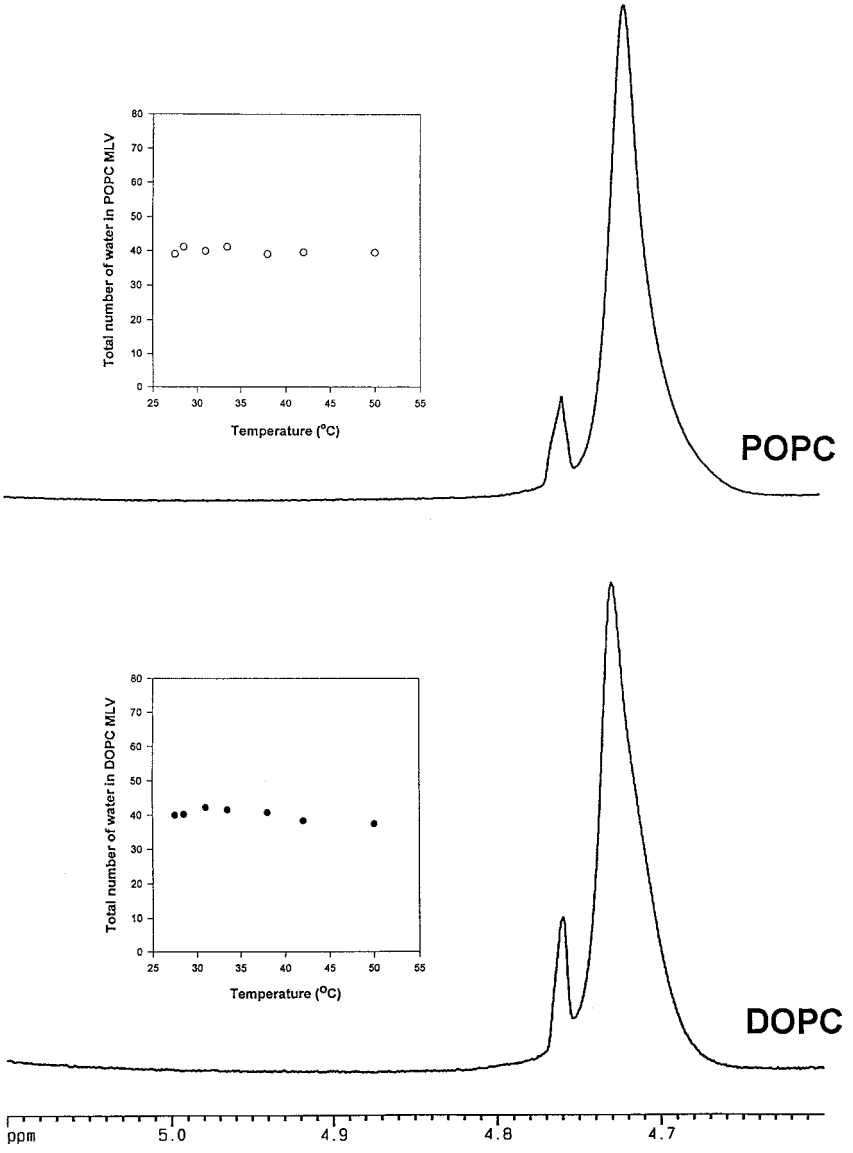

FIGURE 2 Proton MAS NMR spectra showing water resonances in DOPC/water (1:40 mol:mol (corresponding to 49.6 wt % of lipid)) and POPC/water (1:39 mol:mol (corresponding to 49.4 wt % of lipid)) liposomes, measured with a 3-kHz spin rate, 45° pulse width, and a 60-s delay between acquisitions. The insets show the dependence on temperature of the total number of detectable waters in DOPC and POPC MLVs.

Chemical shifts for the high-resolution NMR spectra are reported in ppm relative to tetramethylsilane (TMS) using the residual $CDCl_3$ signals at 7.24 and 77.0 ppm as internal references for the ^1H and ^{13}C spectra, respectively. The MAS ^1H NMR spectra were referenced by assigning the terminal methyl group of the phospholipid to 0.9 ppm (Halladay et al., 1990). The ^{31}P MAS spectra were referenced relative to external 85% phosphoric acid in D_2O.

RESULTS AND DISCUSSION

^1H NMR assignments for DOPC

The gradient-enhanced COSY results summarized in Fig. 1 *A* were used to make most of the phospholipid resonance assignments (Li et al., 1993). For CH_2OCO and CH_2PO_4 protons of the glycerol backbone that were not previously assigned (Volke and Pampel, 1995; Chen et al., 1996), unambiguous assignments were achieved using gradient ^1H-^{13}C HSQC, as shown in Fig. 1 *B*. Along the F_1 axis is displayed the methylene portion of the J-modulated spin-echo ^{13}C NMR spectrum. The singlet at 62.88 ppm is assigned to the CH_2OCO group, as it has no coupling with ^{31}P. Conversely, the doublet at 63.24 ppm may be assigned to the CH_2PO_4. The corresponding proton assignments for CH_2OCO and CH_2PO_4 groups follow from the HSQC spectrum as shown.

A spectral comparison was also made between MAS ^1H NMR of DOPC MLVs and conventional liquid-state ^1H NMR of DOPC small unilamellar vesicles (SUVs) (data not shown). As SUVs contain no population of interlamellar water, these results confirm the assignment of bulk water to the sharp downfield resonance in DOPC/D_2O MLV samples. Conventional spinning and MAS yielded comparable linewidths in ^1H spectra of the SUV sample, suggesting that SUV rotation had averaged the dipole-dipole interactions and chemical shift anisotropy effects.

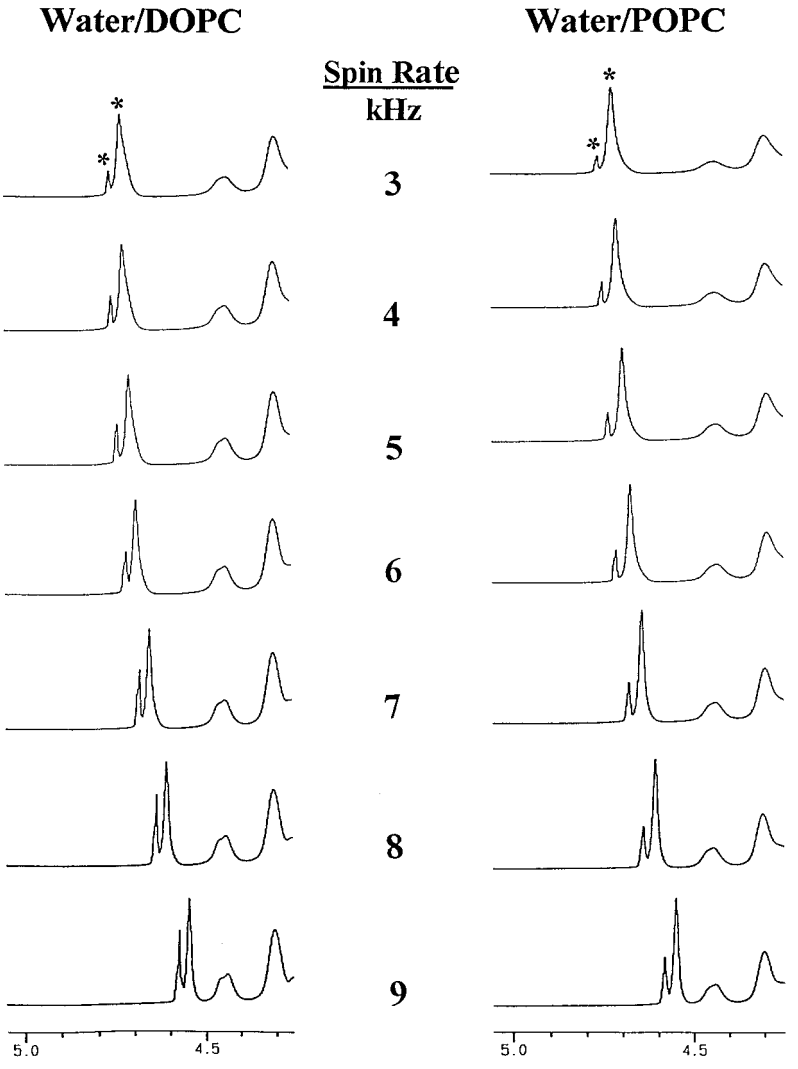

FIGURE 3 Quantitative ^{1}H NMR spectra of DOPC/water (1:40 mol:mol (corresponding to 49.6 wt % of lipid)) and POPC/water (1:39 mol:mol (corresponding to 49.4 wt % of lipid)) liposomes at spinning speeds of 3–9 kHz. Water peaks are marked with asterisks. No higher speeds were used because the water and lipid signals overlapped at 10 kHz (Zhou et al., 1997).

Number of interlamellar waters in MLV samples

The water interacting with lipids has been classified variously as tightly bound, weakly bound, trapped, and free water (Walter and Hayes, 1971; Finer and Darke, 1974; Berendsen, 1975; Israelachvili and Wennerstrom, 1992) or simply as interlamellar water and bulk water (Chen et al., 1996). The interlamellar water itself has also been categorized as nonfreezeable and freezeable water (Kodama et al., 1997). By measuring the ^{2}H NMR of deuterated MLVs or of D_2O in MLVs, it has been reported that saturation of the lipids with water is essentially complete at 22 waters per PC (Gawrisch et al., 1985; Ulrich and Watts, 1994). Lipid structural changes have been observed for water concentrations up to 12–15 water molecules per lipid molecule, based upon analysis of the residual quadrupolar splittings in selectively deuterated lipids (Ulrich and Watts, 1994; Volke et al., 1994). The effects of low water content on bilayer structure were also studied by other researchers (Sackmann, 1983). The motional freedom (spin-lattice relaxation rate, R_1) of a $N^+(CD_3)_3$ deuterated DOPC was found to increase continuously with hydration up to a limit of 20–22 water molecules per lipid (Ulrich et al., 1990). It was hypothesized that as the water molecules partition into the polar region between the bilayers, opposing bilayers move apart to accommodate interlamellar water until they reach an equilibrium distance where their repulsive and attractive forces balance (Ulrich and Watts, 1994). Additional water added to the sample then remains in the lipid dispersion as bulk water. The water saturation number obtained with ^{2}H NMR should correspond to the number of tightly bound and weakly bound waters; thus, it may represent only a portion of the interlamellar water. In other words, the number of interlamellar waters present within the multilamellar assembly could be larger than the number interacting directly with lipids (Gawrisch et al., 1985; Ulrich and Watts, 1994).

Recently we reported that the bulk and interlamellar water of PC MLVs could be differentiated by using ^{1}H MAS NMR with samples in a spherical glass ampoule (Zhou et al., 1997). This protocol facilitates the counting of interlamellar waters, as shown in Fig. 2 for DOPC and POPC dispersions. Several conditions permit reliable interpretation of these data. First, all of the water added to the

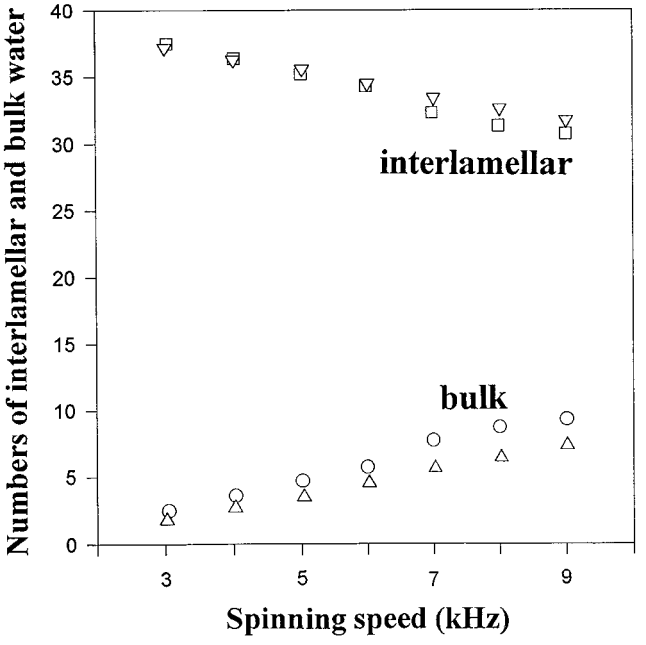

FIGURE 4 The dependence of the number of interlamellar and bulk waters in DOPC (□ and ○) and POPC (▽ and △) MLVs on spinning speed.

phospholipids should be detectable by NMR, as the experimental temperature of 27.5°C is well above the gel-to-liquid crystalline phase-transition temperatures for DOPC and POPC (−20°C and −5°C, respectively; Caffrey, 1993) and should therefore have sufficient motion. This assumption is confirmed by the constancy of total waters in each MLV preparation that we observe as a function of temperature (insets to Fig. 2). Second, the long waiting times between acquisitions in comparison with the T_1 spin-relaxation times for HDO protons (Zhou et al., 1997) should guarantee the validity of using integrated intensities for the two water resonances to derive the numbers of interlamellar waters. Finally, the calculations must account for the fact that one water molecule remains bound to the lipid headgroup under our sample preparation conditions (Cevc, 1992; Ulrich and Watts, 1994). At the 3-kHz spinning speed used in this set of experiments, we estimate an average of 37.5 ± 1 and 37.2 ± 1 moles of interlamellar water per mole of phospholipid for DOPC and POPC, respectively. These numbers are consistent with diffraction studies that indicate 30–44 water molecules per DOPC (Rand and Parsegian, 1989; McIntosh and Magid, 1993).

Variations in the number of interlamellar waters with spinning speed

If reliable structural information on multilamellar vesicles is to be obtained with MAS NMR techniques, it is crucial to understand to what extent the centrifugal forces induced by spinning the sample may remove water from the lipid preparation. This issue has not been addressed systematically during the 10 years that MAS NMR techniques have been used in studies of water/phospholipid systems. The decrease of interlamellar waters (increase of bulk waters) with spinning speed is obvious in Fig. 3 for DOPC and for POPC dispersions. For MLVs of both phospholipids, the number of interlamellar waters was found to decrease from ~37 to 31 as the MAS rate is varied between 3 and 9 kHz (Fig. 4). Three important conclusions follow from these results. First, we expect that static aqueous samples of DOPC and POPC will have more than 37 interlamellar waters per lipid molecule, in agreement with previous x-ray diffraction studies (Rand and Parsegian, 1989; McIntosh and Magid, 1993). Second, the modest drop in the degree of hydration with spinning speed may reflect the removal of interlamellar water that is not directly bound to lipid, but it is not expected to cause alterations in lipid dynamics (Ulrich et al., 1990) or structure (Ulrich and Watts, 1994; Volke et al., 1994). Thus, MAS NMR data collected on phospholipids at speeds below 9 kHz should not be compromised by artifacts from centrifugal forces. Third, the similar number of interlamellar waters in DOPC and POPC samples suggests that the amount of interlamellar water is not sensitive to acyl-chain structure.

Distribution of water within the PC bilayer

The well-known sensitivity of nuclear Overhauser effects (NOEs) to molecular geometry has been used in 2D NOESY experiments to delineate both intramolecular and intermolecular proximities in a variety of lipid systems (Forbes et al., 1988; Volke and Pampel, 1995; Zhou et al., 1996; Chen et al., 1996; Holte and Gawrisch, 1997). Based upon the observation of NOESY cross-peaks between HOD and protons of the lipid molecule, previous workers have made the surprising suggestion that substantial amounts of water reside at the interior of DMPC and POPC multibilayers (Forbes et al., 1988; Volke and Pampel, 1995). We chose to reinvestigate these phenomena in our DOPC and POPC systems, particularly in light of possible ambiguities arising from instrumental shortcomings, symmetrization applied during data processing, and spin diffusion effects (Chen and Stark, 1996).

Using POPC samples similar to those studied previously (Volke and Pampel, 1995), we confirmed the observation of negative cross-peaks between interlamellar water and protons of the polar headgroup and backbone regions (Fig. 5, *A* and *B*), which indicate short water residence times at these lipid sites. In contrast with previous work, however, our spectra displayed only some small positive cross-peaks between water and the acyl-chain protons. These latter observations were confirmed for mixing times of 200–600 ms with a 500-MHz NMR spectrometer and for MAS-NOESY data collected at a ^{1}H frequency of 300 MHz (data not shown). As polar water molecules are not expected to have long residence times in the hydrophobic environment of the lipid, it is more likely that the small positive cross-peaks between water and the acyl chains arise from T_1 noise or

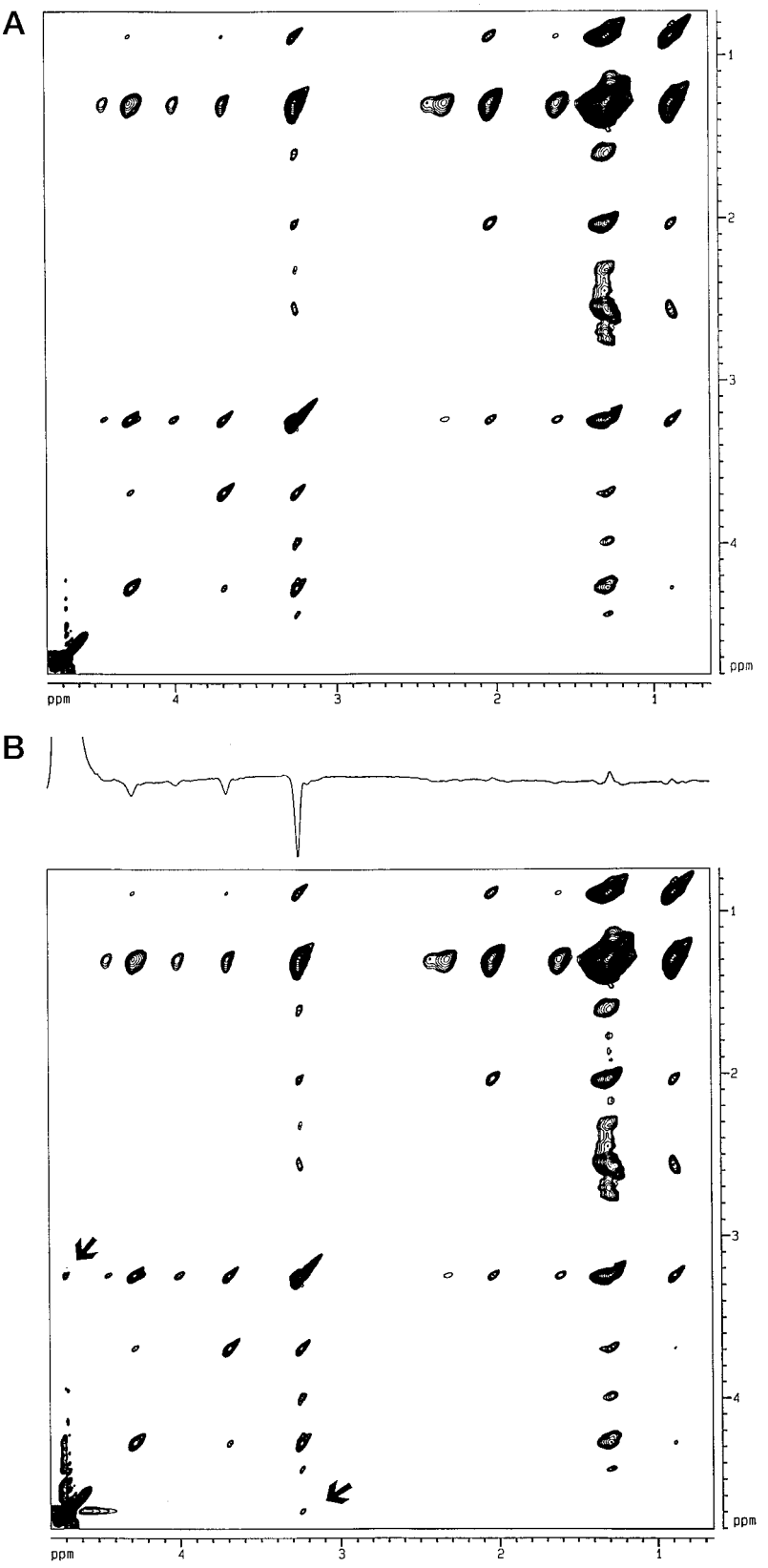

FIGURE 5 Phase-sensitive NOESY contour plot of POPC dispersions/water (1:9 mol:mol (corresponding to 81.2 wt % of lipid), 80% D_2O and 20% H_2O), measured with 4-kHz MAS, a 600-ms mixing time, and 4 s between successive acquisitions. (*A*) Positive cross-peaks; (*B*) Both positive and negative cross-peaks (negative cross-peaks are marked with *arrows*). The spectrum above *B* is a slice across the water peak. Negative cross-peaks are observed for water with the polar headgroup (*arrows*) and the glycerol backbone of the lipid. Similar results are obtained with mixing times of 200 and 400 ms.

spin diffusion that occurs after direct through-space interactions with the phospholipid headgroup and glycerol backbone. A similar phenomenon has been demonstrated previously by us for DMPC multilayers (Chen and Stark, 1996).

Independent evidence for the localization of water in the headgroup and backbone regions of the phospholipid in multilamellar dispersions comes from 2D heteronuclear Overhauser effect (HOESY) experiments (Rinaldi, 1983;

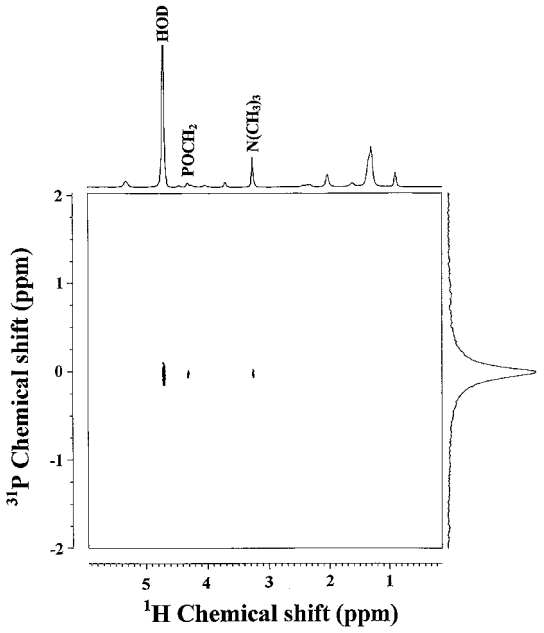

FIGURE 6 Phase-sensitive HOESY contour plot for DOPC/water (1:1, w:w, in 20 mM phosphate buffer, pH 5.2, 85% H_2O/15% D_2O), measured with 6-kHz MAS, a 300-ms mixing time, and 3 s between successive transients.

Yu and Levy, 1983, 1984; Canet et al., 1992; Ganapathy et al., 1995; Cistola and Hall, 1995; Warschawski et al., 1996). First, phase-sensitive $^{31}P\{^1H\}$ HOESY was tested with an ATP solution (0.5 M ATP in 20 mM phosphate buffer, pH 5.2, 85% H_2O/15% D_2O), yielding the three anticipated interactions of water protons with phosphorus nuclei of the nucleic acid (Yu and Levy, 1983, 1984). An additional cross-peak to the phosphate buffer (presumably folded and attenuated under the earlier experimental conditions) indicated strong dipolar interactions between water and the buffer. Then, HOESY experiments with a range of mixing times were carried out on MLVs of DOPC/D_2O (1:1, w:w.) and DOPC/water (1:1, w:w; 20 mM phosphate buffer, pH 5.2, 85% H_2O/15% D_2O). Although our small sample size and large chemical shift anisotropy effects (Brauer and Sykes, 1981) preclude the observation of cross-peaks that originate from ^{31}P of the phospholipid, it was possible to use the phosphate buffer as a reporter of the water location. Fig. 6 shows the HOESY results, revealing cross-peaks between the ^{31}P of the buffer and protons of the CH_2OP, $POCH_2$, and $N(CH_3)_3$ groups, respectively. As no HOESY connectivities to the acyl-chain protons are found, this result strengthens our argument against the presence of water in the interior of the lipid bilayer.

The NOESY and HOESY results presented above indicate that few waters reside inside the membrane, but they should not be construed to mean that water cannot traverse the membranes. In fact, water diffusion through model and biological membrane structures constitutes the basis of osmotic lysis of membrane-entrapped structures. Our conclusion that water is distributed primarily at headgroup and backbone sites conforms to an extensive body of previous data, including a neutron diffraction study showing that water binds to the ester carbonyls, phosphate, and *N*-methyls of a phosphatidylcholine bilayer (Zaccai et al., 1975), a DSC finding of three kinds of binding sites (Kodama et al., 1997), and recent computational modeling results (Tieleman et al., 1997; Feller et al., 1997).

Lipid structure and hydration

Fig. 7 compares the MAS ^1H NMR spectra of several phospholipid-water systems. In the DOPC/D_2O system (Fig. 7 *a*), the interlamellar water is shifted to lower frequency (Zhou et al., 1997). By contrast, Fig. 7 *b* shows the broad interlamellar water peak at higher frequency in the ^1H NMR spectrum of MeDOPE/D_2O, consistent with a previous report (Chen et al., 1996). The chemical shift difference between the two water peaks in MeDOPE MLVs is 0.08 ppm. This value is smaller than the 0.13 ppm estimated previously without the enhancement of resolution afforded by the spherical inserts (Chen et al., 1996). Our findings may be rationalized by examining the three reported binding sites for water molecules: the ester carbonyls, the phosphate, and the $NH_n(CH_3)_m$ headgroup (Zaccai et al., 1975). For DOPC the waters should exchange rapidly because no positive NOESY cross-peaks were observed between the water proton and protons near the three binding sites. The waters interacting with ester carbonyls and phosphate should experience a deshielding effect because of hydrogen bonding, but these two kinds of water might also be affected by double bonds and neighbor anisotropy effects of carbonyls and phosphate. The interaction between water and the $N(CH_3)_3$ group should be relatively weak. The resulting effect on the water proton with DOPC is that it is shielded; i.e., its chemical shift is at lower frequency compared with bulk water opposite to the case of MeDOPE. For MeDOPE, waters that interact with carbonyls and phosphate might also form strong hydrogen bonding with NH_2CH_3. Water protons might also exchange rapidly with the NH protons of NH_2CH_3 resulting in a change in the chemical shift of interlamellar water. However, at a water-to-lipid molar ratio of 38 (corresponding to 49.5 wt % of lipid), the average chemical shift of this water should be very close to that of bulk water.

The nature of lipid hydration was also assessed for several types of mixed MLV samples. First, an aqueous dispersion prepared from a dry MeDOPE/DOPC mixture gave the MAS ^1H NMR spectrum shown in Fig. 7 *c*. As the two phospholipids reside in the same bilayer, water molecules interacting with MeDOPE and DOPC are expected to exchange rapidly on the NMR time scale. Moreover, as the bulk water has nearly the same chemical shift as interlamellar waters, only one water peak is observed.

In a second experiment, MeDOPE liposomes were packed on top of DOPC liposomes in a single glass ampoule. MAS ^1H NMR results for the resulting sample are

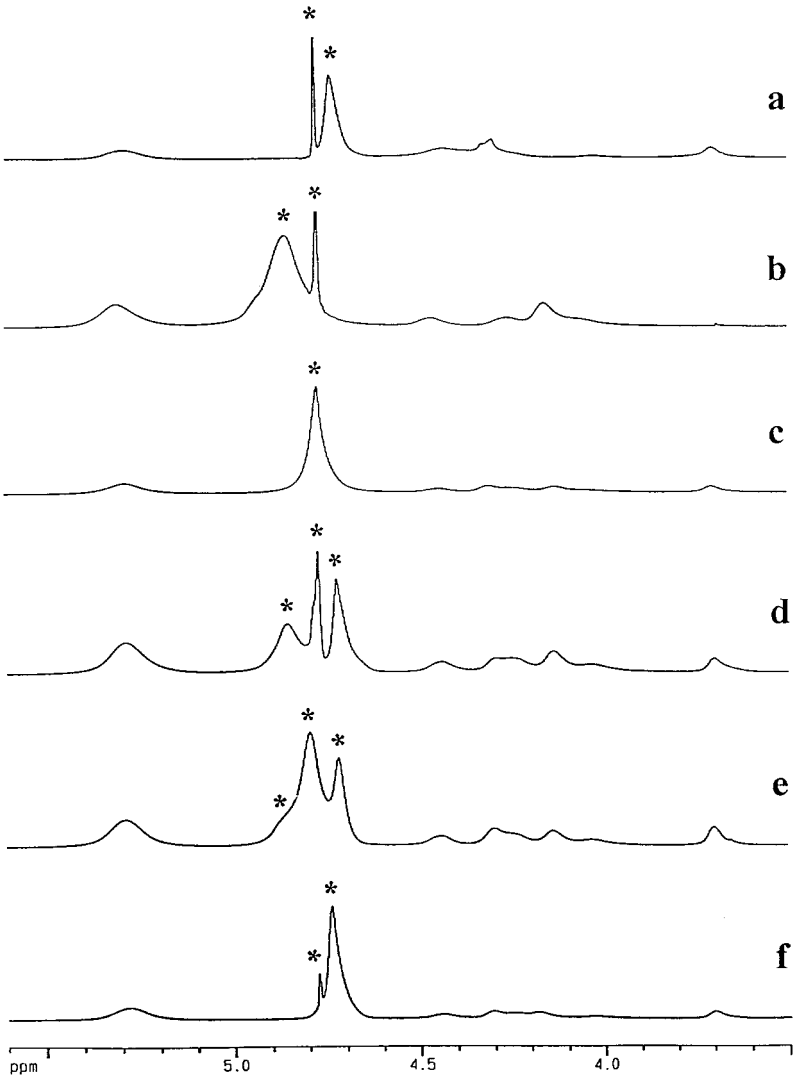

FIGURE 7 ^{1}H NMR spectra of several phospholipid/D_2O systems measured with a spinning speed of 3 kHz (water peaks are marked with asterisks). (*a*) DOPC/D_2O (1:1, w:w); (*b*) MeDOPE/D_2O (1:1, w:w); (*c*) MeDOPE/DOPC/D_2O (1:1:2, w:w:w); (*d*) MeDOPE/D_2O (1:1, w:w) liposomes packed on top of DOPC/D_2O (1:1, w:w) liposomes in a spherical glass ampoule; (*e*) MeDOPE/D_2O (1:1, w:w) liposomes mixed with DOPC/D_2O (1:1, w:w) liposomes; (*f*) MeMeDOPE/DOPC/D_2O (1:1:2, w:w:w) liposomes.

displayed in Fig. 7 *d*, which reveals three water peaks. Their chemical shifts correspond to the interlamellar water in MeDOPE liposomes, the bulk water, and the interlamellar water in DOPC liposomes, respectively. These three kinds of water are evidently in slow exchange even though they are present in the same ampoule. This is in agreement with previous ^{2}H NMR studies (Lindblom, 1996). As the spinning speed was increased from 3 to 10 kHz, the sample temperature increased and the peaks shifted to lower frequency (Zhou et al., 1997). It can be noted that the signals of the bulk water and of the interlamellar water of DOPC shifted to similar extents, but the signal from the interlamellar water of MeDOPE shifted relatively less (data not shown).

If the sample from Fig. 7 *d* is mixed with a glass rod, the resonance from interlamellar water associated with MeDOPE becomes a broad shoulder on the high-frequency side of bulk water, as shown in Fig. 7 *e*. Although three water peaks are still visible in the spectrum, their broader appearance suggests that the rate of chemical exchange is now more comparable to their respective chemical shift differences. In physical terms, mixing may enhance the rate of exchange; alternatively or additionally, it may cause some MeDOPE headgroups to be moved away from the bilayer surface, thus diminishing hydrogen bonding to the NH_2CH_3 and shifting the water signal to lower frequency. Notably, interlamellar waters of the DOPC and MeDOPE liposomes still exchange slowly on the ^{1}H NMR time scale.

Finally, an aqueous dispersion prepared from a dry MeMeDOPE/DOPC mixture produced the MAS ^{1}H NMR spectrum shown in Fig. 7 *f*. As for the MeDOPE/DOPC sample prepared in an analogous fashion (see above), interlamellar water molecules that interact with the two lipids will exchange rapidly because MeMeDOPE and DOPC reside within the same bilayer. The resulting averaged peak appears at lower frequency, as the single NH group in MeMeDOPE diminishes hydrogen-bonding opportunities to the headgroup and their consequent deshielding effects. The interlamellar water is then observed as a distinct ^{1}H resonance from bulk water.

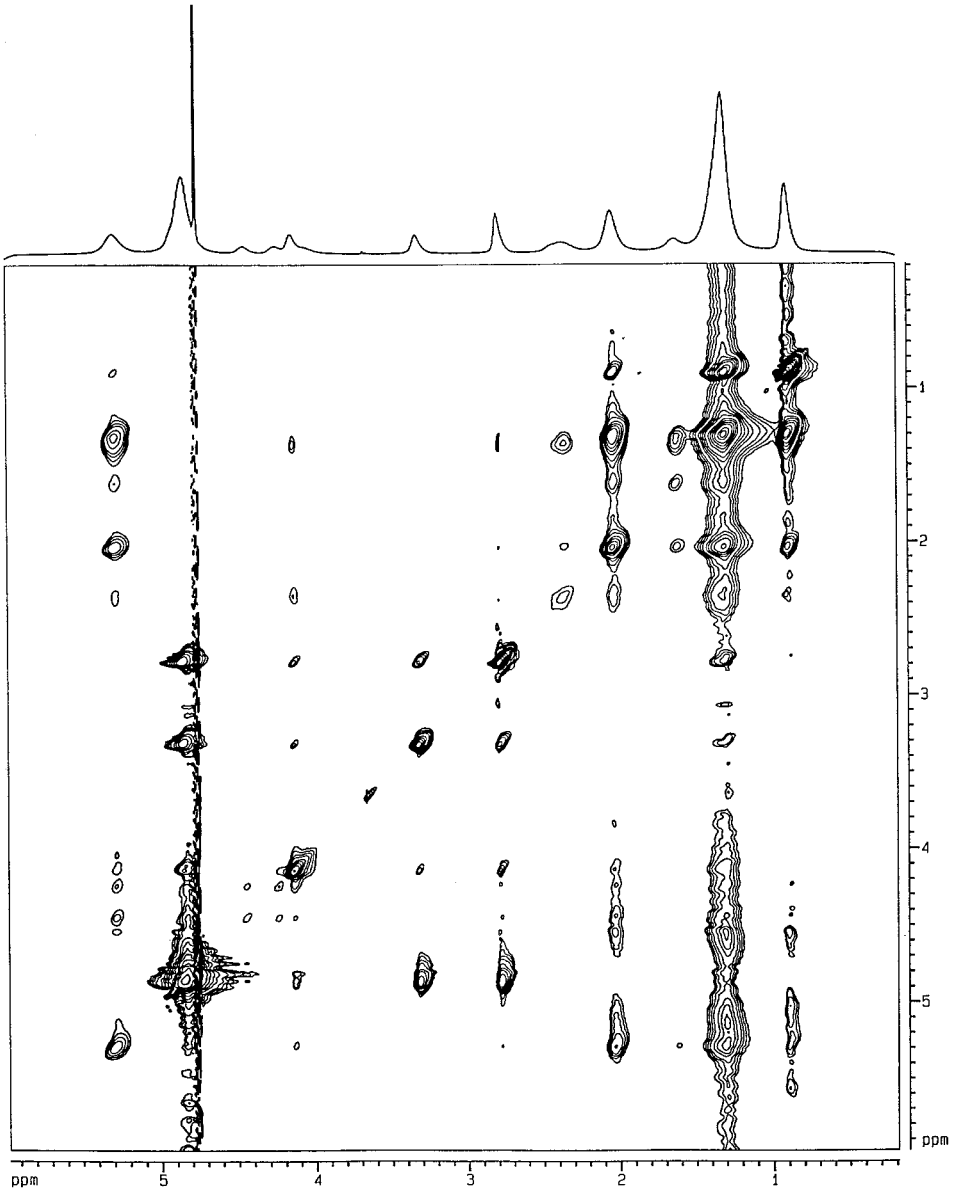

FIGURE 8 Phase-sensitive NOESY of MeDOPE/D_2O (1:1, w:w) liposomes measured with a 3-kHz MAS, a mixing time of 300 ms, and 4 s between transients.

Membrane permeation

The observation of distinct proton resonances from bulk and interlamellar water puts limits on their exchange rate, i.e., slower than $2 \times \pi \times 0.05 \times 500 = 157\ s^{-1}$ for a chemical shift difference of 0.05 ppm in aqueous DOPC dispersions. To refine this observation, MAS-assisted 2D exchange spectroscopy (2D EXSY) (Jeener et al., 1979; Riddell and Zhou, 1994) was conducted for the sample used in Fig. 7 *d*. With 3-kHz MAS and mixing times of 300, 800, and 1500 ms, no magnetization exchange was detected between the water sites (data not shown). Thus, the exchange occurs slowly, consistent with the limit quoted above.

Both the 1D spectra and the 2D EXSY indications of slow chemical exchange are surprising in light of previous electrical conductance measurements that yielded a water permeability coefficient of $\sim 5 \times 10^{-3}$ (cm/s) (5×10^5 Å/s) and indicated that water traverses the membrane rapidly in planar bilayers (Stryer, 1988). Nevertheless, they are in accord with a recent report of distinct water resonances in MAS ^1H NMR spectra of red blood cell suspensions, in which there exists a single bilayer membrane but where the exchange rate between intracellular and extracellular water must be less than $151\ s^{-1}$ (Humpfer et al., 1997).

MLVs have many layers and may also be packed tightly together by the MAS so that there is no bulk water between them. Together, these factors may reduce the exchange rate between bulk water and interlamellar water, allowing for the observation of separate water signals. The appearance of distinct water signals might also be caused by the centrifugal force on water during MAS. It was shown in Fig. 4 that the interlamellar water could be pushed out of MLVs and be changed to bulk water by using a higher spinning speed. Because the density of D_2O is higher than that of the phospholipid, the free water could be localized near the

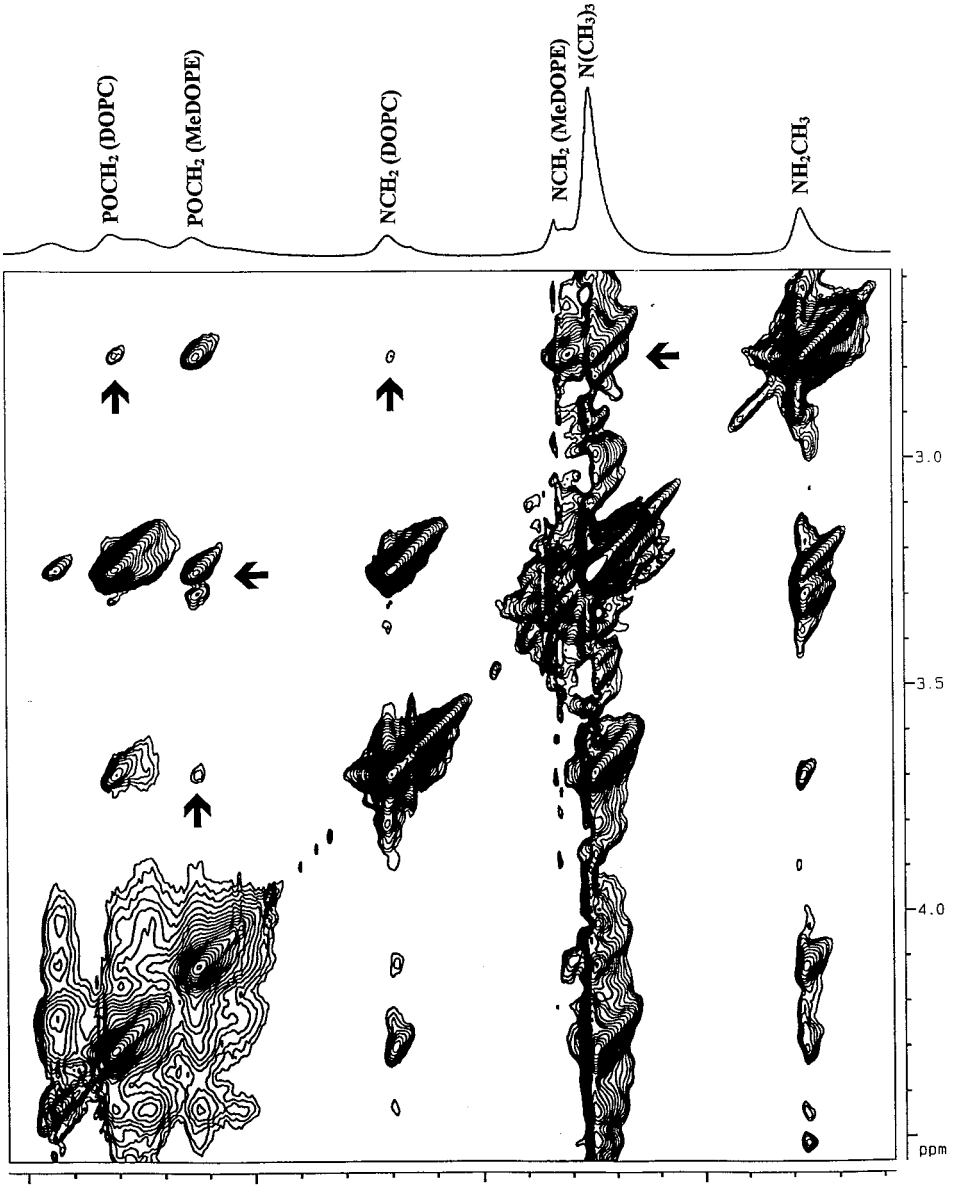

FIGURE 9 Phase-sensitive NOESY of MeDOPE/DOPC/D_2O (1:1:2, w:w:w) liposomes, measured as described in Fig. 8. The intermolecular cross-peaks are marked with arrows.

inner surface of the spherical glass ampoule by MAS. The exchange between interlamellar waters in different MLVs is also slow (see Fig. 7, *d* and *e*). The explanation for distinct water resonances in MAS ^{1}H NMR spectra of red blood cell suspensions may be that cells are packed tightly together upon removal of intracellular water, and as a consequence the water exchange rate between the intracellular and extracellular (bulk) water is reduced (Humpfer et al., 1997).

NOESY of MeDOPE liposomes

In addition to yielding information on the location and residence times of water within phospholipid MLVs, MAS-NOESY experiments can reveal organizational details in model membrane systems composed of one or more chemical components. For MeDOPE liposomes (Fig. 8), cross-peaks were observed between the following headgroup protons: POCH_2 and CH_2N, POCH_2 and NCH_3, and CH_2N and NCH_3. As reported previously (Chen et al., 1996), positive cross-peaks were found for interlamellar water with NCH_3 and CH_2N protons of the lipid headgroup; an additional small cross-peak to POCH_2 was observed in the current set of experiments. The cross-peak intensities with water decrease as NCH_3 > CH_2N > POCH_2. This suggests that water in MeDOPE/D_2O exchanges rapidly with NH_2CH_3 on the proton NMR time scale. Another explanation is that the water interacts strongly with NH_2CH_3 (Chen et al., 1996).

For mixed MLVs incorporating both MeDOPE and DOPC, NOESY experiments gave the results presented in Fig. 9. DOPC cross-peaks were found between POCH_2 and CH_2N, POCH_2 and NCH_3, CH_2N and NCH_3. Thus, despite previous reports that an expected cross-peak between POCH_2 and CH_2N was absent in many phospholipid systems (Forbes et al., 1988; Xu and Cafiso, 1986; Halladay et

al., 1990; Chen et al., 1996), this spectral feature was observed in MLVs containing both MeDOPE and DOPC. In contrast with MeDOPE, no NOE cross-peak was observed between water and the headgroup of DOPC, even though DOPC and MeDOPE are in same bilayer. This means that one of the reasons for observing the positive cross-peaks between water and the headgroup of MeDOPE in MeDOPE/DOPC might be the exchange of water with NH_2CH_3. No positive cross-peaks were found between water and $N^+(CH_3)_3$ of DOPC, although a strong cross-peak between NH_2CH_3 and $N^+(CH_3)_3$ was observed. This suggests the close physical proximity of these headgroups, likely resulting from their orientation in a direction close to the plane of the bilayer.

An intriguing feature of the MAS-NOESY spectra for MeDOPE/DOPC mixed MLVs concerns the intermolecular lipid cross-peaks designated by arrows in Fig. 9. In particular, cross-peaks are evident for $POCH_2$ of MeDOPE with NCH_3 and NCH_2 of DOPC and for NH_2CH_3 of MeDOPE with NCH_3, NCH_2, and $POCH_2$ of DOPC. These results show that the distances between these proton pairs are less than 5 Å. We are currently using such distance information in molecular dynamics simulations of membrane structures.

In summary, the current study demonstrates the ability of ultra-high-resolution MAS NMR to provide diverse types of molecular information regarding phospholipid structure and membrane organization. It is possible to determine the number of interlamellar waters in MLV preparations, assess the importance of dehydration effects that accompany spinning, deduce the location and exchange rate of water in lipid bilayers, and compare the magnetic environment of interlamellar water for phospholipids of related molecular structure.

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