Magic-angle spinning NMR studies of molecular organization in multibilayers formed by 1-octadecanoyl-2-decanoyl-*sn*-glycero-3-phosphocholine

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ABSTRACT Magic-angle spinning ^1H and ^{13}C nuclear magnetic resonance (NMR) have been employed to study 50%-by-weight aqueous dispersions of 1-octadecanoyl-2-decanoyl-*sn*-glycero-3-phosphocholine (C[18]:C[10]PC) and 1-octadecanoyl-2-d_{19}-decanoyl-PC (C[18]:C[10]PC-d_{19}), mixed-chain phospholipids which can form interdigitated multibilayers. The ^1H NMR linewidth for methyl protons of the choline headgroup has been used to monitor the liquid crystalline-to-gel (LC-to-G) phase transition and confirm variations between freezing and melting temperatures. Both ^1H and ^{13}C spin-lattice relaxation times indicate unusual restrictions on segmental reorientation at megahertz frequencies for C(18):C(10)PC as compared with symmetric-chain species in the LC state; nevertheless each chemical moiety of the mixed-chain phospholipid exhibits motional behavior that may be classified as liquidlike. Two-dimensional nuclear Overhauser spectroscopy (NOESY) on C(18):C(10)PC and C(18):C(10)PC-d_{19} reveals cross-peaks between the ω-methyl protons of the C_{18} chain and the *N*-methyl protons of the phosphocholine headgroup, and several experimental and theoretical considerations argue against an interpretation based on spin diffusion. Using NMR relaxation times and NOESY connectivities along with a computational formalism for four-spin systems (Keepers, J. W., and T. L. James. 1984. *J. Magn. Reson.* 57:404–426), an estimate of 3.5 Å is obtained for the average distance between the ω-methyl protons of the C_{18} chain and the *N*-methyl protons of the phosphocholine headgroup. This finding is consistent with a degree of interdigitation similar to that proposed for organized assemblies of gel-state phosphatidylcholine molecules with widely disparate acyl-chain lengths (Hui, S. W., and C.-H. Huang. 1986. *Biochemistry.* 25:1330–1335); however, acyl-chain bendback or other intermolecular interactions may also contribute to the NOESY results. For multibilayers of C(18):C(10)PC in the gel phase, ^{13}C chemical-shift measurements indicate that *trans* conformers predominate along both acyl chains. ^{13}C Spin-lattice relaxation times confirm the unusual motional restrictions noted in the LC state; nevertheless, ^{13}C and ^1H rotating-frame relaxation times indicate that the interdigitated arrangement enhances chain or bilayer motions which occur at mid-kilohertz frequencies.

INTRODUCTION

The ordered structures adopted by phospholipid molecules in water have long been of interest because of their possible significance to the function of biological membranes. Although most physical and spectroscopic studies have focused on the bilayers formed by lipids containing two identical acyl chains (Silvius, 1982; Browning, 1981), natural lipids frequently have differing chain lengths and degrees of unsaturation (Holub and Kuksis, 1978). Recent work on phospholipids having acyl chains with unequal lengths, employing differential scanning calorimetry (DSC),[1] Raman spectroscopy, and x-ray diffraction has provided evidence for a variety of interdigitated packing arrangements in the gel phase. It is thought that assemblies with these irregular geometries might be important in membrane fusion and transport, lipid-protein interactions, cellular recognition, and information transmission across the bilayer (Huang and Mason, 1986; Slater and Huang, 1988).

For the diacyl phospholipid 1-octadecanoyl-2-decanoyl-*sn*-glycero-3-phosphocholine (C[18]:C[10]PC), which has one chain roughly twice as long as the other, a great deal of structural evidence for interdigitation (Fig. 1) has been obtained from several types of gel-state physical studies.

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[1] *Abbreviations used in this paper:* C(18):C(10)PC, 1-octadecanoyl-2-decanoyl-*sn*-glycero-3-phosphocholine; C(18):C(10)PC-d_{19}, 1-octadecanoyl-2-(d_{19}-decanoyl)-*sn*-glycero-3-phosphocholine; CP, cross polarization; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DP, direct polarization; DPPC, 1,2,-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSC, differential scanning calorimetry; ESR, electron spin resonance; LC, liquid crystalline; MAS, magic-angle spinning; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser spectroscopy; $T_1(C)$ and $T_1(H)$, ^{13}C and ^1H spin-lattice relaxation times; $T_1\rho(C)$ and $T_1\rho(H)$, ^{13}C and ^1H rotating-frame relaxation times; TLC, thin-layer chromatography; T_m, gel-to-liquid-crystalline melting temperature; T_r, reduced temperature.

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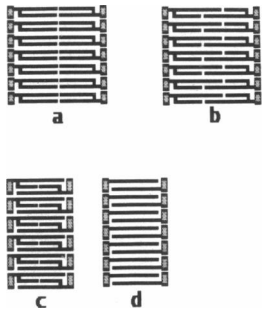

FIGURE 1 Phospholipid bilayer models for packing in the gel state. Dashed rectangular boxes represent the phospholipid headgroup, and the filled bars represent the hydrocarbon chains. (*a*) Noninterdigitated; (*b*) partially interdigitated; (*c*) mixed interdigitated; (*d*) fully interdigitated. Reprinted with permission of the American Chemical Society, from Hui and Huang (1986).

First, x-ray measurements of hydrated crystals have revealed unit cell dimensions equivalent to a single C_{18} chain (McIntosh et al., 1984; Hui et al., 1984). Moreover, Raman peak-intensity ratios have indicated a proportion of *gauche/* isomers and degree of chain–chain interaction that matches dioctadecanoyl-PC in the gel state, a situation that may again be achieved with a mixed interdigitated arrangement for C(18):C(10)PC (Huang et al., 1983). Finally, the large transition entropy obtained from DSC studies suggests that a new type of packing arrangement is adopted to overcome the distortions caused by the large inequivalence in chain length (Mason and Huang, 1981). Such a proposal is reasonable because the mixed interdigitated arrangement would maximize chain–chain interactions and minimize *gauche* conformations (Mattai et al., 1987).

Among these investigative approaches for the study of packing geometries in mixed-chain phospholipids, only x-ray diffraction provides *direct* evidence for interdigitated bilayers. Thus one motivation for the nuclear magnetic resonance (NMR) experiments described herein was the possibility of identifying those chemically distinct hydrogens within ~5 Å of each other in space, using two-dimensional ^1H-^1H nuclear Overhauser spectroscopy (NOESY) (Wuthrich et al., 1982; Keepers and James, 1984). Investigations of this type have been used, for instance, to evaluate the proximity of headgroup and terminal methyl protons within vesicles (Ellena et al., 1985; Xu and Cafiso, 1986; Gabriel and Roberts, 1987), micelles (Stark et al., 1986) and multilayers (Forbes et al., 1988) formed by egg PC and synthetic PC's in the liquid-crystalline (LC) state. Moreover, the utility of NMR properties (lineshapes, spin-relaxation times) for elucidation of model-membrane packing and dynamics of both gel and LC phases has been demonstrated (Browning, 1981; Chan et al., 1981; Blume et al., 1982; Yeagle, 1987; Rommel et al., 1988). Finally, recent advances in solid-state NMR techniques, especially magic-angle spinning (MAS), have opened the way for high-resolution spectral studies of phospholipid multilayers (Oldfield et al., 1987; Forbes et al., 1988).

In this report, we present magic-angle spinning ^1H and ^{13}C NMR results for aqueous dispersions of C(18):C(10)PC in the liquid-crystalline and gel states. Measurements of signal intensity, linewidth, NOESY cross-peaks, and various spin-relaxation times have been employed together for the first time to assess the order, mobility, and interdigitated arrangements of C(18):C(10)PC multilayers. Parallel measurements on C(18):C(10)PC-d_{19}, in which the C_{10} acyl chain is perdeuterated, have also been made to clarify the structural interpretations of the NMR data. Results for the mixed-chain phospholipid have been compared with dipalmitoyl-PC (DPPC), a symmetric-chain-length lipid for which a wealth of NMR data is readily available (Browning, 1981).

EXPERIMENTAL PROCEDURES

Materials

DPPC was purchased from Calbiochem-Behring Corp. (San Diego, CA). Its purity (>99%) was confirmed by thin-layer chromatography (TLC) on 250-μm silica gel G plates (elution with chloroform-methanol-water [12:6:1]), and the material was used as received. Deuterium oxide (99.8%) was obtained from Aldrich Chemical Co. (Milwaukee, WI). 1-Octadecanoyl-2-decanoyl-PC (C[18]:C[10]PC) was synthesized from 1-octadecanoyllyso-PC (Avanti Polar Lipids, Birmingham, AL) as described previously (Ali and Bittman, 1989). For the preparation of 1-octadecanoyl-2-d_{19}-decanoyl-PC (C[18]:C[10]PC-d_{19}), acylation of 1-C_{18}-lyso-PC was carried out using perdeuterodecanoic anhydride, which was prepared from d_{19}-decanoic acid (Cambridge Isotope Laboratories, Woburn, MA) and dicyclohexylcarbodiimide in CCl_4 (Selinger and Lapidot, 1966). The purities of C(18):C(10)PC and C(18):C(10)PC-d_{19} were established by TLC (elution with chloroform-methanol-water [65:25:4]) and by DSC on a Hart Scientific (Provo, UT) calorimeter using a scan rate of 15°/h. The transition temperature (T_m) and transition width were 293.6 and 1.21 K, respectively, for vesicles from C(18):C(10)PC; 291.5 and 1.02 K, respectively, for C(18):C(10)PC-d_{19}. The mixed-chain PC's were lyophilized in a minimum volume of benzene and then stored desiccated at 263 K. TLC conducted after completion of the NMR experiments indicated that degradation to lyso-PC did not occur in any of these materials.

Preparation of aqueous dispersions

Samples for NMR were prepared by weighing 100–200 mg of dry lipid into a small glass vial and adding an equal mass of D_2O. Each dispersion was vortex-mixed for ~2 min at 5–10° above the phospholipid phase-transition temperature and then taken through a freeze–pump–thaw cycle. This mixing treatment was repeated until a uniform paste was obtained. The hydrated lipid samples were loaded into 7-mm cylindrical Macor rotors equipped with O-ring seals and openings to release pressure (Doty Scientific, Columbia, SC).

NMR studies

NMR measurements were carried out on an IBM Instruments WP-200 spectrometer equipped with high-power amplifiers and a Doty Scientific probe for magic-angle spinning. ^1H and ^{13}C Resonance frequencies were 200.13 and 50.33 MHz, respectively. Spinning speeds were adjusted to 2.2 ± 0.1 kHz except as noted otherwise. A Bruker Instruments, Inc. (Billerica, MA) B-VT 1000 unit was used to control sample temperatures to within 1.0°. Compressed air was dried and passed through a dry-ice bath for low-temperature spinning experiments. Exit-gas temperatures in the NMR probe were expected to provide a reasonable measure of the sample temperature for our range of 274–335 K (English, 1984), and the nominal phase-transition temperatures deduced from our ^1H NMR spectra varied by no more than 1.0° from the published values for each lipid sample (Huang and Mason, 1986). Results referring to the gel state, for instance, refer to NMR experiments conducted at temperatures between 8° and 40° below the phase transition for a given phospholipid.

Cross-polarization magic-angle spinning (CPMAS) ^{13}C NMR experiments were performed using 5-μs 90° pulses for both ^1H and ^{13}C spins and a 50-kHz ^1H decoupling field during data acquisition (dipolar decoupling). Recycle delays of 3–5 s were employed during signal averaging to permit repolarization of the ^1H spin reservoir and also to minimize sample heating. Proton spin-temperature alternation and quadrature phase cycling were used to avoid baseline distortions and other spectral artifacts (Stejskal and Schaefer, 1974, 1975). For cross-polarization (CP) times > 2 ms, distortions from preamplifier overload were avoided by storing the ^{13}C magnetization along the field direction for 20 ms before signal acquisition (Murphy, 1985).

Direct-polarization magic-angle spinning (DPMAS) ^{13}C NMR experiments were carried out using 5-μs 90° pulses for ^{13}C nuclei and a 50-kHz decoupling field during signal acquisition. Recycle delays of 3–20 s and quadrature phase cycling were used during data collection. For both DPMAS and CPMAS experiments, 80–800 transients of 2 K points each were accumulated, zero-filled to 4 K points, multiplied by a decaying exponential (line broadening of 20 Hz unless specified otherwise), and subjected to Fourier transformation. Typical spectral widths were 20 kHz (resolution of 0.1 ppm or 5 Hz).

^{13}C Spin-lattice relaxation times $T_1(C)$ were measured by DPMAS methods using an inversion-recovery sequence (Vold et al., 1968). ^{13}C Signal intensities in the CPMAS experiments were also monitored as a function of ^1H-^{13}C contact time (0.4–80 ms); the relaxation time $T_1\rho(H)$ was found by varying the spin-lock time *before* a 2-ms CP period which was followed by data collection (Dickinson et al., 1988). ^{13}C Rotating-frame relaxation times $T_1\rho(C)$ were found from signal intensities that remained following a spin-lock time of 0.05–40 ms after CP. The spin-locking field was varied between 29 and 43 kHz in separate relaxation experiments.

MAS ^1H NMR spectra were obtained with the radio-frequency amplifier normally used for solution-state experiments. A 5-μs pulse (~30°) and 10 kHz spectral width were employed to observe spinning sidebands as well as the central spectral region. Typically, 8–32 transients of 8 K points were accumulated, zero-filled to 16 K points, multiplied by an exponential (line broadening 2 Hz), and subjected to Fourier transformation. ^1H Spin-lattice relaxation times $T_1(H)$ were determined with the usual inversion-recovery sequence (Vold et al., 1968).

Two-dimensional nuclear Overhauser spectroscopy (NOESY) was carried out in the phase-sensitive mode (Marion and Wuthrich, 1983). The pulse sequence (90°-t_1-90°-τ_m-90°-acquire $[t_2]$)$_n$ was employed with 128–156 t_1 values, mixing times (τ_m) of 200–500 ms, and $n = 16$. Random variation of the mixing time by 4–8% was used to remove scalar coupling effects. The resulting NMR signals were processed with Gaussian apodization and two-dimensional Fourier transformation (New Methods Research, Inc., E. Syracuse, NY). A typical experiment required 4 h of spectrometer time.

RESULTS

Liquid-crystalline state: proton spectra and relaxation times

Fig. 2 shows typical MAS ^1H NMR data for DPPC and C(18):C(10)PC multilayers in the smectic liquid-crystalline state. As expected from prior reports (Oldfield et al., 1987; Forbes et al., 1988), high-resolution spectra are obtained without sonication of the samples or lengthy signal averaging. Key structural features of the phospholipid headgroup, backbone, and acyl chains are easily resolved, although no spin–spin couplings are observed by us at 200 MHz or by other investigators working at higher

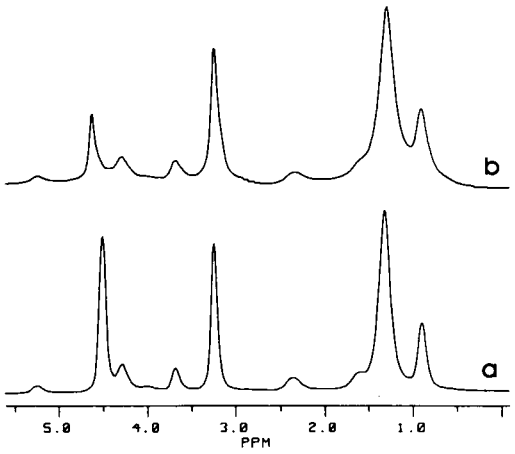

FIGURE 2 200 MHz MAS ^{1}H NMR spectra (centerband region) of 50 wt% phospholipid dispersions in the LC state. Samples were spun at 2.2 kHz. Exponential line broadening of 5 Hz was applied to each spectrum, and the chemical shifts were referenced with respect to ω-CH_3 groups set at 0.90 ppm. Peak assignments are made in Table 1. (a) DPPC at 325 K ($T_r = [T - T_m]/T_m = 0.032$); (*b*) C(18):C(10)PC at 300 K ($T_r = 0.048$).

resonance frequencies. The C_{18} and C_{10} chains are not distinguishable in these spectra.

At a spinning speed of 2.2 kHz and temperatures 5–6°C above T_m, most ^{1}H signal intensity appears in the centerband region for both DPPC and C(18):C(10)PC, as expected from previous 360-MHz results for 1,2-dimyristoyl-PC (DMPC) spinning at 2.5 kHz. Functional groupings from the headgroup, backbone, and acyl-chain regions *all* have large, narrow NMR centerbands, confirming prior suggestions that molecular motion reduces the magnitude of dipole–dipole interactions in liquid-crystalline multilayers (Wennerstrom, 1973). Additional sharp sidebands appear as the spinning speed is lowered to ~500 Hz, demonstrating that only *inhomogeneous* dipolar broadening of the NMR spectral lines remains after motional averaging in the phospholipid assembly (Forbes et al., 1988; Wennerstrom, 1973; Bloom et al., 1977). The latter observation indicates that the proton spins are isolated from one another in the LC state of C(18):C(10)PC.

No more than three sets of spinning sidebands may be measured reliably at 2.2 kHz for any chemical grouping of either phospholipid (data not shown). Bulk-methylene protons of the acyl chains do exhibit larger sidebands than *N*-methyl or ω-methyl groups of the acyl chains, 37% and 40% of the total signal intensity for DPPC and C(18):C(10)PC, respectively. Nevertheless, no variations in order or mobility of the LC phases of symmetric and mixed-chain phospholipids are revealed by these spectral measurements.

Table 1 summarizes the spin-lattice relaxation times T_1(H) measured for identical- and mixed-chain PC's in their LC phases. In both bilayer systems distinct T_1(H) values are found at various proton sites of the molecule, in agreement with previous studies of DMPC (Forbes et al., 1988). These T_1 values converge only near T_m (Fig. 3), achieving a common value within 2° and 10° of the

TABLE 1 Proton spin-lattice relaxation times for PC assemblies in the liquid-crystalline state

Chemical shift	Proton type‡	T_1(H), *s* DMPC multilayers§	DPPC sonicated vesicles‖	DPPC multilayers¶	C(18):C(10)PC multilayers¶
*ppm**					
0.90	ω-CH_3	0.67		0.97	0.50
1.30	$(CH_2)_n$	0.62	0.74	0.63	0.40
1.58	β-CH_2	—	—	0.49	—
2.34	α-CH_2	0.45	—	0.41	0.31
3.24	$N(CH_3)_3$	0.48	—	0.57	0.35
3.68	CH_2N	0.35	—	0.46	0.31
4.29	$POCH_2$	0.46	—	0.44	0.33
5.25	CHOCOR	—	—	0.40	0.35
			$\bar{x}$**	0.55	0.36
			$\sigma/\bar{x}$**	0.33	0.17

*Referenced to ω-CH_3 set at 0.90 ppm.
‡From Hauser et al. (1976).
§^{1}H resonance frequency = 360 MHz; $T_r = 0.04$. From Forbes et al. (1988).
‖^{1}H resonance frequency = 220 MHz; $T_r = 0.06$. From Chan et al. (1981).
¶Resonance frequency = 200 MHz; $T_r = 0.06$. This work. CH_2's at positions 1 and 3 of the glycerol backbone are not resolved in these spectra. Error limits, based on repeated measurements, are estimated at 10%.
**$\bar{x}$ = average, $\sigma/\bar{x}$ = normalized standard deviation.

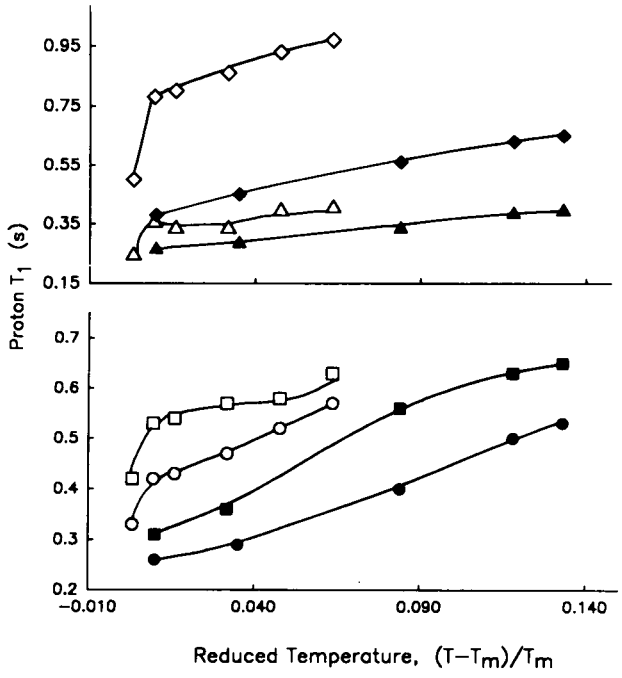

FIGURE 3 Proton spin-lattice relaxation times obtained at 200 MHz for phospholipid multilayers in the liquid-crystalline phase. (*Open symbols*) DPPC; (*solid symbols*) C(18):C(10)PC. Representative groupings are methyl protons of the choline headgroup (●), α-protons of the acyl chains (▲), bulk methylenes of the acyl chains (■), and terminal methyl protons of the acyl chains (♦).

transition temperature for DPPC and C(18):C(10)PC, respectively. Both the distinct values of T_1(H) and the inhomogeneous nature of the dipole–dipole interactions suggest that spin diffusion does not complicate the usual motional interpretations of NMR relaxation data for the LC phase of these phospholipid dispersions. Moreover, many ^{2}H and ^{1}H NMR studies on similar multilayers indicate that local rather than collective reorientations are the principal contributors to relaxation at NMR frequencies of 10–300 MHz (Rommel et al., 1988, and references therein).

The T_1 data presented in Table 1 are in reasonable agreement with results obtained previously for phospholipids assembled as multibilayers and sonicated vesicles (Forbes et al., 1988; Rommel et al., 1988; Chan et al., 1981). The temperature dependence of T_1(H) suggests that individual reorientations for protons of the chain, backbone, and headgroup regions occur on the fast-motion side of the T_1 minimum (tumbling rates in excess of 200 MHz), though even this inference must be made with caution if the motional processes are complex (Stark et al., 1986). Prior field-dependent studies confirm that the effective tumbling times for phospholipid aggregates are far from the extreme narrowing limit (Rommel et al., 1988; Stark et al., 1986; Brown et al., 1983), as does our observation of modest negative NOEs (see the NOESY results below).

In aggregates of both lipid molecules, short T_1's at the rigid glycerol backbone contrast with longer T_1's for the flexible chain-terminal methyl groups, suggesting it is appropriate to assume liquidlike motion when making qualitative interpretations of the spin-relaxation data. Shorter T_1's, and thus more sluggish reorientation, are indicated at *every* site of C(18):C(10)PC when compared with DPPC. As discussed below, the general motional trends derived from T_1(H) are also supported by ^{13}C relaxation data on the two phospholipids.

Liquid-crystalline state: nuclear Overhauser effects

Fig. 4 and Table 2 display the results of two-dimensional nuclear Overhauser effect experiments (NOESY) per-

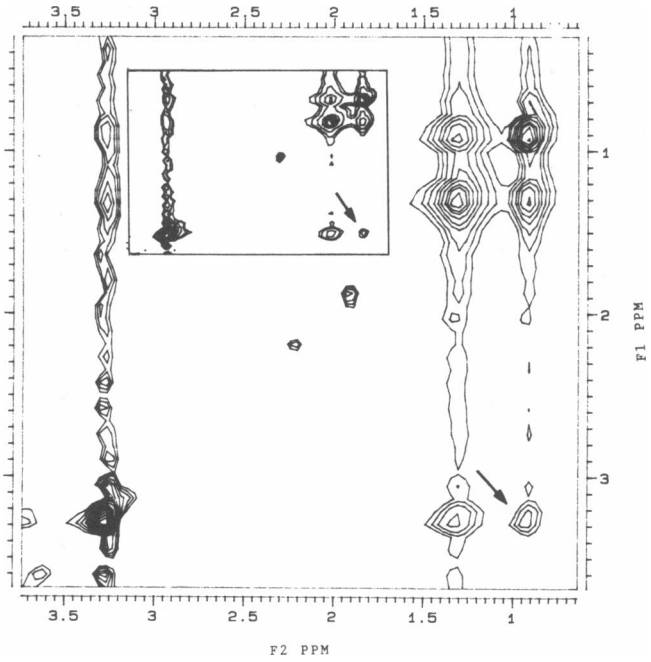

FIGURE 4 Two-dimensional nuclear Overhauser enhancement contour plot (negative-going signals) obtained for C(18):C(10)PC multilayers (T = 310 K, T_r = 0.07) with MAS at 1.6 kHz. Spectral widths were minimized to improve digital resolution, but care was taken to avoid folding of spinning sidebands into the centerband spectrum. A mixing time of 500 ms was employed; the data were processed with Gaussian apodization (3 Hz Lorentzian, 6 Hz Gaussian) but without symmetrization. The lowest contour represents a signal with an intensity 2% of the largest spectral peak. ^{1}H spectral assignments are summarized in Table 1. (*Main plot*) Both acyl chains fully protonated. (*Inset*) C_{10} chain fully deuterated, same spectral region as main plot. In both the main plot and inset, the arrow designates a cross-peak between ω-CH_3 and $N(CH_3)_3$ groups.

TABLE 2 Proton dipolar connectivities from NOESY of PC aggregates*

Chemical moieties	Lipid type: DMPC multilayers‡	DPPC sonicated vesicles§	DPPC-Di-C_7PC vesicles‖	DPPC multilayers**	C(18):C(10)PC multilayers**
$(CH_2)_n - \omega\text{-}CH_3$	x	x	x	x	x
$N(CH_3)_3 - (CH_2)_n$	x	x	x	x	x
$CH_2N(CH_3)_3$	x	x	nr	x	x
$POCH_2CH_2N$¶	—	—	nr	—	—
$POCH_2CH_2N(CH_3)_3$	x	x	nr	x	x
$N(CH_3)_3 - \omega\text{-}CH_3$	weak	weak	—	weak	weak

*Measurements were conducted with a 500-ms mixing time (except as noted otherwise) on lipid dispersions in the liquid-crystalline state. The data for DMPC multilayers and DPPC vesicles were symmetrized.

‡^{1}H resonance frequency = 500 MHz, from Forbes et al. (1988).

§^{1}H resonance frequency = 360 MHz, from Xu and Cafiso (1986).

‖^{1}H resonance frequency = 500 MHz, mixing time of 400 ms. The designation "nr" means the data were not reported. A weak cross-peak between the $N(CH_3)_3$ and ω-CH_3 protons is observed for this sample at a mixing time of 800 ms. From Gabriel and Roberts (1987).

¶The absence of this peak between chemically bonded residues has been attributed to mobility of the CH_2N group (Stark et al., 1986).

**^{1}H resonance frequency = 200 MHz (this work). The indicated cross-peaks were observed at temperatures between 4° and 35° above the respective values of T_m.

formed on multilamellar dispersions of C(18):C(10)PC. As reported previously for related lipid assemblies (Forbes et al., 1988; Ellena et al., 1985; Stark et al., 1986; Gabriel and Roberts, 1987), magnetization exchange is selective, e.g., no dipolar connectivities are found between protons of the $POCH_2CH_2N$ headgroup moiety and weak dipolar interactions occur between protons of the phospholipid headgroup ($N[CH_3]_3$) and acyl chains (ω-CH_3, $[CH_2]_n$). Cross-peaks between the ω-CH_3 and $N(CH_3)_3$ groups, in both C(18):C(10)PC and DPPC multilayer samples well above T_m, are observed only with mixing times of 500 ms or more (see arrows in Fig. 4). These spectral features are retained, with similar intensity, if the NOESY experiment is repeated for C(18):C(10)PC-d_{19} (see Fig. 5). Thus it is the terminal methyl group of the C_{18} chain that appears to exhibit an intra- or intermolecular interaction with $N(CH_3)_3$ protons of the choline headgroup.

It is tempting to conclude that these NOESY cross-peaks constitute the first demonstration of interdigitated packing in the LC state, though x-ray scattering measurements of the C(18):C(10)PC bilayer thickness appear inconsistent with the particular packing scheme shown in Fig. 1 *c* (McIntosh et al., 1984). Molecular models corresponding to the mixed interdigitated arrangement do permit the protons in question to be located within 3–4 Å of each other. Alternative interpretations of the NOESY results and possible interferences from spin diffusion are considered in the Discussion.

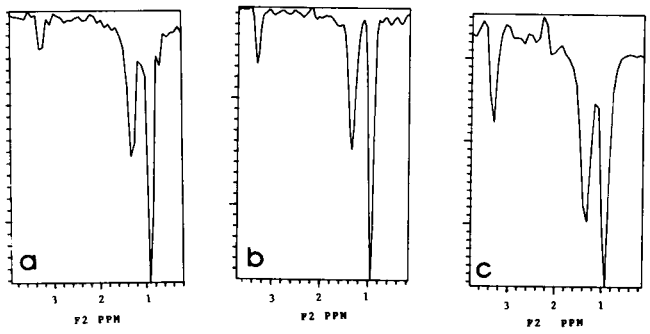

FIGURE 5 Slices of the NOESY plots for phospholipid multilayers, obtained under the experimental conditions described in Fig. 4. (*a*) C(18):C(10)PC, T_r = 0.074; (*b*) C(18):C(10)PC, T_r = 0.118; (*c*) DPPC, T_r = 0.067. In each plot the diagonal ω-CH_3 peak has been set to full scale.

Liquid-crystalline state: carbon spectra and relaxation times

The superior spectral resolution that may be achieved with MAS ^{13}C NMR, demonstrated previously for a variety of PC dispersions (Haberkorn et al., 1978; Sefcik et al., 1983; Oldfield et al., 1987), is illustrated in Fig. 6 *a* for C(18):C(10)PC in the LC state. Direct polarization (with high-power decoupling) is the method of choice for spectral acquisition in these samples; CP procedures are expected and found to be inefficient because of the motional averaging allowed by acyl-chain melting in these multibilayers.

The signal-to-noise ratio is also high enough to permit measurement of the spin-lattice relaxation times T_1(C) shown in Fig. 7 and Table 3. As with T_1(H), short values are taken to indicate considerable segmental motion for

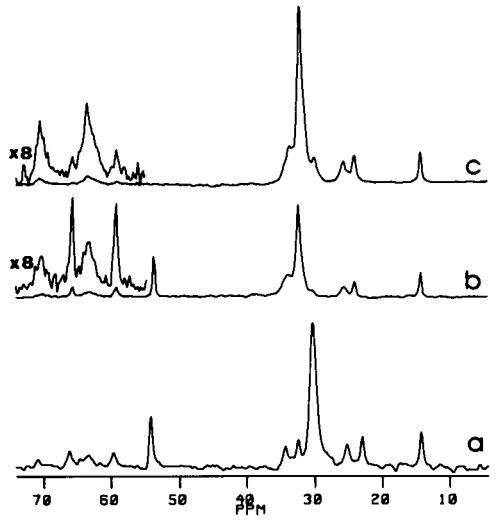

FIGURE 6 50-MHz MAS ^{13}C NMR spectra of a 50% wt% dispersion of C(18):C(10)PC under varying experimental conditions. All experiments were carried out with magic-angle spinning at 2.2 kHz with high-power decoupling. Exponential line broadening of 20 Hz was applied to each spectrum, and the chemical shifts were referenced to TMS, via *p*-di-*tert*-butylbenzene as a secondary substitution reference. Peak assignments are summarized in Table 3. (Carbonyl resonances at 173 ppm are not shown.) (*a*) Direct polarization in the LC state at 297 K, with a recycle delay of 10 s; (*b*) direct polarization in the gel state at 278 K ($T_r = -0.048$), with a recycle delay of 10 s; (*c*) CP in the gel state at 278 K, with a recycle delay of 3 s and a ^1H-^{13}C contact time of 1.5 ms. Spectra *b* and *c* were plotted to allow an absolute comparison of signal intensities.

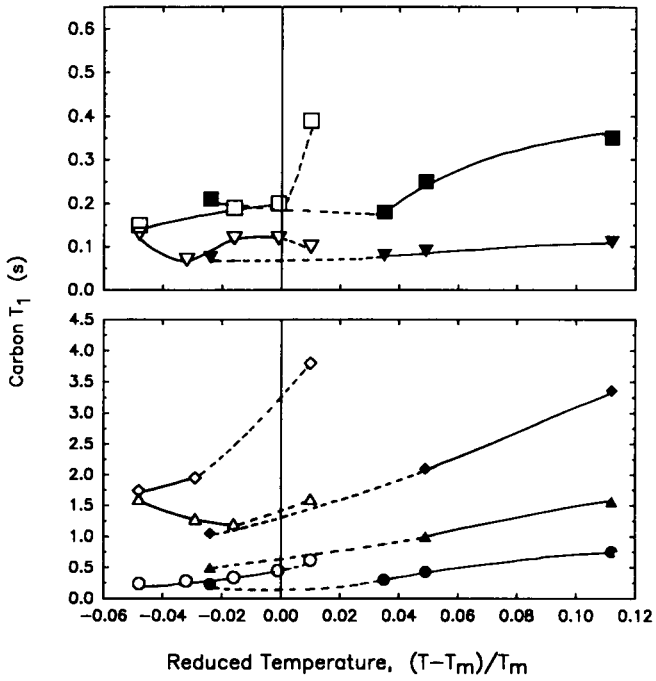

FIGURE 7 Carbon spin-lattice relaxation times obtained at 50 MHz for phospholipid multilayers in gel and liquid-crystalline phases. (*Open symbols*) DPPC; (*solid symbols*) C(18):C(10)PC. Representative groupings in the top figure are choline CH_2N (■), glycerol C-1 and C-3 (▼). The bottom figure includes choline methyls (●), (ω-1) methylenes (▲), and terminal methyls (♦).

both phospholipid assemblies. Above T_m, the values of T_1(C) rise as the temperature rises, suggesting liquidlike segmental reorientation for headgroup, backbone, and acyl-chain regions of both symmetric and mixed-chain phospholipids. Even more clearly than with measurements of T_1(H), there appears to be a motional gradient from the glycerol backbone anchor to the unrestricted terminal chain methyls. The rigidity of the backbone is indicated both by short T_1(C)'s and by a shallow dependence of these values on temperature.

Most of the spin-lattice relaxation times are shorter in both multilayer samples than for corresponding sites in micellar phospholipids (Burns and Roberts, 1980). The T_1's exhibited by DPPC in multilayer dispersions are quite similar to those reported previously for symmetric phospholipids in sonicated vesicles (Lee et al., 1972; Brown et al., 1983). Along its acyl-chain segments, the proportionate change in T_1(C) values for C(18):C(10)PC corresponds to that of DPPC; the shallow fluidity gradient deduced from electron spin resonance (ESR) data on spin-labeled C(18):C(10)PC (Boggs and Mason, 1986) is not apparent in our T_1(C) results. The shorter relaxation times for C(18):C(10)PC do indicate extra motional restrictions at each molecular site. Thus the carbon measurements fully confirm the dynamic information deduced above from ^1H relaxation data. Possible structural interpretations for these trends are discussed in the following section.

Liquid-crystalline-to-gel phase transition

MAS ^1H NMR spectra of phospholipid dispersions undergo dramatic changes as the sample is cooled to the gel state (Forbes et al., 1988). It is possible, moreover, to use the linewidth of the $N(CH_3)_3$ resonance to monitor the phase transition (Fig. 8). Upon either heating or cooling of the C(18):C(10)PC sample, for instance, the presence of the gel phase is indicated by loss of MAS NMR signals from other protons of the molecule. The fact that MAS at 2–3 kHz fails to produce high-resolution spectra below the phase transition is consistent with ^2H NMR data which demonstrate a slowdown of chain rotation, chain fluctuation, and *trans-gauche* isomerization (Meier et al., 1983; Meier et al., 1986).

TABLE 3 **Carbon spin-lattice relaxation times for liquid-crystalline PC assemblies**

Chemical shift	Carbon type‡	Di-C_8PC micelles§	DPPC vesicles‖	DPPC multilayers¶	C(18):C(10)PC multilayers¶
*ppm**					
14	ω-CH_3	1.9	3.1	3.8	1.7**
22	(ω-1)CH_2	1.6	1.0	1.6	0.93**
25	β-CH_2	0.43	0.49	0.51	0.24
29–32	$(CH_2)_n$	0.71	0.57	0.54	0.39**
34	α-CH_2	0.41	0.30	0.38	0.20
54	$N(CH_3)_3$	0.68		0.62	0.30
59	$POCH_2$	0.53		0.34	0.15
63	CH_2O's	0.11		0.10	0.08
66	CH_2N	0.49		0.39	0.18
71	CHOCOR	0.22		0.12	0.14
173	C = 0	2.4		2.3	2.0

*Referenced to external TMS set at 0.0 ppm.
‡From Birdsall et al. (1972); Burns and Roberts (1980).
§^{13}C resonance frequency = 68 MHz; T = 303 K. From Burns and Roberts (1980).
‖^{13}C resonance frequency = 45 MHz; T_r = 0.03. From Brown et al. (1983).
¶^{13}C resonance frequency = 50 MHz; T_r = 0.10 for DPPC, 0.035 for C(18):C(10)PC. This work. Direct polarization was used for spectral acquisition, with recycle delays exceeding five times T_1 for all carbon nuclei. Error limits, based on repeated determinations, are estimated at 15%.
**Interpolated from measurements at 284 and 300 K.

In agreement with prior DSC studies (Mattai et al., 1987), we find that C(18):C(10)PC exhibits (*a*) a gel-to-liquid crystal transition centered at 289.8 K; and (*b*) a liquid crystal-to-gel transition centered at 286.5 K. The melting and freezing behavior monitored by ^1H NMR occurs over a temperature range of 3–5° for C(18):C(10)PC (as compared with ~1° for DPPC); this effect is not fully attributable to temperature gradients in the NMR sample. A DSC exotherm has also been found at 283.2 K (Mattai et al., 1987), but no further changes in linewidth of the $N(CH_3)_3$ resonance are observed in the ^1H NMR spectrum.

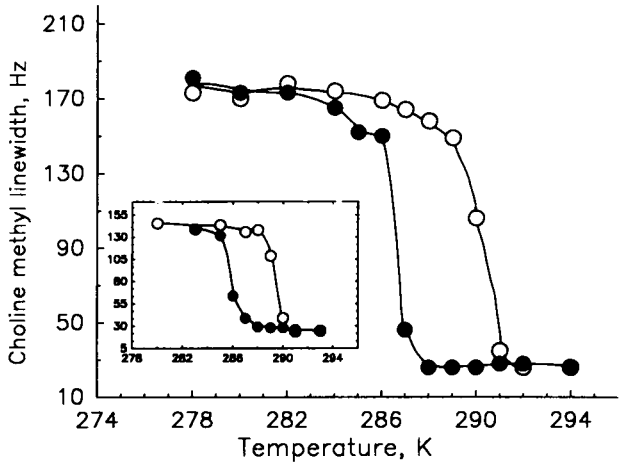

FIGURE 8 ^1H NMR linewidths as a function of temperature for methyl protons of the choline headgroup in aqueous dispersions of C(18):C(10)PC. All values have been corrected for contributions from digital line broadening. Each measurement was made after 10 min of equilibration at the indicated temperature, and repeated determinations revealed no spectral changes. (*Open circles*) Heating; (*solid circles*) cooling. (*Inset*) C(18):C(10)PC-d_{19}.

Additional information comes from MAS ^{13}C NMR, as illustrated in Fig. 6. Despite a slowing of molecular motion in the gel state, direct polarization methods are sufficient for acquisition of high-resolution spectra if high-power ^1H-^{13}C decoupling is employed and the recycle delay exceeds a few seconds (Fig. 6 *b*). Nevertheless, only signals from the choline headgroup of either lipid remain narrow and intense. Acyl-chain and backbone resonances are broadened within a few degrees below the phase transition, until finally they are detected more efficiently with CP methods (Fig. 6 *c*).

If the spectra of C(18):C(10)PC are compared at 318 and 284 K (or lower), it is possible to discern chemical-shift changes at several acyl-chain sites. The $(CH_2)_n$ signals, which appear at 29.7 ppm above T_m, shift to 32.1 ppm below T_m. Similar downfield shifts for the (ω-1) and β-CH_2 signals are also observed. These effects probably indicate an enhanced proportion of *trans* conformers in the gel state (Earl and VanderHart, 1979; Tonelli and Schilling, 1981). Alterations in chain packing may also contribute to the changes in chemical shift (VanderHart, 1981). Corresponding carbon signals from the two acyl chains remain coincident in the CPMAS ^{13}C NMR spectra. Concomitant upfield shifts of ~0.5 ppm may occur for the carboxyl carbons of both phospholipids, but

broadening of the gel-state peaks makes such effects difficult to confirm.

Gel state: spectra and spin-relaxation times

Within the gel state, DSC measurements have indicated an additional transition for C(18):C(10)PC at 283 K (Mattai et al., 1987). We observe no new spectral features or changes in chemical shift under these conditions, although alterations in dynamic behavior are inferred from the CP enhancements and ^{13}C spin-relaxation behavior (see below). For instance, if bilayer packing constraints required *trans-gauche* isomerizations along the C_{10} chain, a $(CH_2)_n$ peak might appear at 29.7 ppm.

Given the fact that high-resolution ^1H NMR spectra are not obtained in the gel state of either C(18):C(10)PC or DPPC, their ^{13}C relaxation behavior is expected to reveal diminished molecular mobility on the megahertz timescale. As both samples are cooled below T_m, the values of $T_1(C)$ go through a minimum (tumbling time $\sim 10^{-9}$ s) for the acyl-chain carboxyl groups and some glycerol carbons (Fig. 7). Such observations are not surprising in light of the short T_1's and restricted motion found at these sites even in the liquid-crystalline state. For DPPC, solidlike behavior generally appears well below T_m for the backbone and chain carbons, and it does not appear at all for molecular segments at headgroup or ω-CH_3 sites. For C(18):C(10)PC, in which anomalous motional restrictions were found in the high-temperature state, all carbon nuclei except the terminal chain methyl group go through a broad T_1 minimum just below the phase transition. The motional gradient typical of acyl chains in organized phospholipid assemblies is preserved in the gel state of both DPPC and C(18):C(10)PC dispersions.

The dynamic picture with regard to mid-kilohertz motions may be derived from determinations of $T_1\rho(C)$ if spin–spin contributions to the relaxation are assumed to be negligible (Sefcik et al., 1983). Table 4 summarizes the results for gel-state DPPC and C(18):C(10)PC multilayers along with reference data on related phospholipid systems (Sefcik et al., 1983; de Haan et al., 1985). The importance of mid-kilohertz motions in the relaxation process is evidenced, for instance, by the substantial dependence of $T_1\rho(C)$ for DPPC on the magnitude of the spin-locking field. Although it is difficult to compare values of $T_1\rho(C)$ for different physical states and spin-locking field strengths, one surprising result for DLPC and DPPC is that slow motions of the acyl chains appear to occur less efficiently in the LC phase. For gel-state DPPC vs. C(18):C(10)PC at the same reduced temperature (-0.05), the mid-kilohertz fluctuations occur to a greater extent in multilayers of the mixed-chain lipid. Given the restrictions on megahertz motions deduced from $T_1(C)$ data in both liquid-crystalline and gel phases, it is in fact reasonable that mid-kilohertz motions are enhanced in gel-state multilayers of the mixed-chain lipid species. Nevertheless, it does not necessarily follow that the dynamic processes which contribute to $T_1\rho$ are collective order fluctuations (Rommel et al., 1988).

Finally, a complementary view of gel-state motions and spin diffusion in these multibilayers is obtained from determinations of $T_1\rho(H)$. Fig. 9 demonstrates the difficulty of making such measurements through the traditional contact-time dependence of ^{13}C signal intensities (Schaefer and Stejskal, 1979). The oscillatory variation in signal intensities, which is most pronounced for the relatively mobile methyl groups, is reminiscent of that reported previously in J-CP experiments for liquids (Bertrand et al., 1978). It suggests that even though reorientation on the megahertz timescale is not efficient enough to

TABLE 4 ^{13}C **Rotating-frame relaxation times for aqueous PC dispersions**

	$T_1\rho(C)$, *ms**				
Carbon type	30 *kHz* DLPC liq. xtal‡	29 *kHz* DPPC§	43 *kHz* DPPC§	43 *kHz* C(18):C(10)PC§	85 *kHz* DPPC·$2H_2O$‖
ω-CH_3	40.0	—	—	—	30
$(CH_2)_n$	33.1	16	48	34	8.5
β-CH_2	28.6	16	59	50	—
glycerol	—	—	17	8	—
$N(CH_3)_3$	—	—	>100¶	>100¶	16

*From intensities of ^{13}C magnetization held in the indicated fields after spin locking and cross polarization from ^1H.
‡From Sefcik et al. (1983).
§$T_r = -0.048$ for DPPC; $T_r = -0.055$ for C(18):C(10)PC. This work. Error limits are estimated as 10% for $(CH_2)_n$'s, 20% for other carbon sites.
‖From de Haan et al. (1985). DPPC and H_2O were present in the proportions noted, in the gel phase.
¶Decreases in signal intensity were very modest, even at the longest spin-lock times (40 ms).

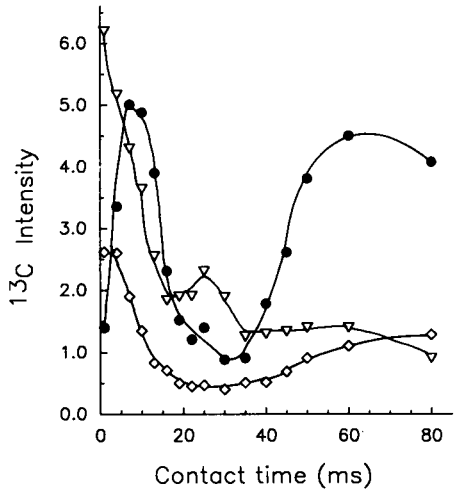

FIGURE 9 Variation in CPMAS ^{13}C NMR signal intensities for 50% DPPC in water at $T_r = -0.048$. Representative groupings are glycerol C-1 and C-3 (◇), terminal methyls (▽), and choline methyls (●). For contact times which exceeded 2 ms, recycle delays of 5 s were inserted between successive transients to avoid sample heating. The intensity measurements for a phospholipid were typically made within a single 8 h session, but oscillations remained observable if a new CP match was established on a different day. Similar variations in ^{13}C signal intensity were observed for C(18):C(10)PC at a comparable reduced temperature.

yield high-resolution ^1H MAS NMR spectra, there is enough motion in the mid-kHz regime to interfere with polarization transfer from ^1H to ^{13}C.

An alternative measurement of $T_1\rho(H)$ through the carbon signal intensities is made by varying the spin-lock time before a 2-ms fixed CP time (Dickinson et al., 1988). For lipid dispersions at a T_r of -0.05 as before, we find $T_1\rho(H)$ values of 14 and 15 ms for C(18):C(10)PC and DPPC, respectively. Within our experimental error of 10–20%, all carbon signal intensities yield a common $T_1\rho(H)$ for each gel-state phospholipid sample. Thus spin diffusion among the gel-state protons appears to occur throughout each molecule, despite the anomalous CP behavior discussed above. By contrast, relatively inefficient mid-kilohertz motions characterize DMPC in the LC state, where an average value of 85 ms has been reported in the absence of magic-angle spinning (Cornell et al., 1982).

DISCUSSION

These studies demonstrate the usefulness of NMR methods in obtaining detailed structural and dynamic information for multibilayers of C(18):C(10)PC, which is the most extensively studied phospholipid known to pack in an interdigitated fashion. The combined methods of MAS ^1H and ^{13}C NMR provide high-resolution spectra for this type of organized assembly in both liquid-crystalline and gel phases, allowing measurements of site-specific and structurally informative parameters including chemical shifts, spin-relaxation times, and dipolar connectivities.

The variation in linewidth of the $N(CH_3)_3$ resonance in MAS ^1H NMR spectra serves as a straightforward diagnostic tool to monitor the phase transition in C(18):C(10)PC and also DPPC, a symmetric-chain phospholipid that serves as a reference material. The disparities between heating and cooling behavior, as well as the particular transition temperatures, are in good agreement with prior DSC studies. The number and intensity of spinning sidebands in our MAS ^1H NMR spectra suggest that neither type of multilayer has strong intermolecular interactions in the LC phase; the response of sidebands to changes in spinning speed indicates that motional averaging is sufficient to leave only inhomogeneous broadening of the spectral lines. The notion that spin diffusion is of minor importance is confirmed by the observation of distinct values of $T_1(H)$ for both lipids above their respective T_m values.

The spectral characteristics summarized above make possible two key types of experiments in the LC state: $T_1(H)$ assessments of local molecular mobility and NOESY determinations of near-neighbor proton interactions within the multilayer structure. Parallel measurements of $T_1(H)$ and $T_1(C)$ (Figs. 3 and 7) demonstrate that although segmental reorientation is liquidlike for all chemical moieties of C(18):C(10)PC and DPPC, the mixed-chain-length lipid is notable for its motional restrictions. The acyl chains appear to be packed more tightly in C(18):C(10)PC, but not so much so that the spin-relaxation behavior becomes solidlike or that spinning sidebands are numerous with 2–3 kHz MAS. The usual motional gradient along each chain is preserved in C(18):C(10)PC, but the slowed reorientation of both ω-CH_3 and $N(CH_3)_3$ end groups is suggestive of extra intra- or intermolecular interactions within the mixed-chain bilayer. In this latter case energetic considerations may favor a bending over of the headgroup to maximize hydrophobic interactions of $N(\underline{CH_3})_3$ groups with the ω-$\underline{CH_3}$ group of the C_{18} chain on an adjacent PC molecule.

Is the molecular organization of C(18):C(10)PC multibilayers one in which $N(CH_3)_3$ and ω-CH_3 groups are, on the average, close to one another in space? This question cannot be addressed easily by NMR methods for lipid dispersions in the *gel state*. Despite some *conformational* disorder associated with interdigitated bilayers, both *orientational* order and motional constraints below the T_m abolish high-resolution ^1H spectra and preclude geometrically informative MAS ^1H NOESY experiments.

In the LC state, however, small NOESY cross-peaks are observed between the ω-CH_3 and $N(CH_3)_3$ protons in both C(18):C(10)PC and DPPC samples. The possibility that spin diffusion along the length of the acyl chains accounts for this interaction has been ruled out in some vesicle systems (Xu and Cafiso, 1986) but implicated in other cases (Gabriel and Roberts, 1987). For multibilayers of C(18):C(10)PC and DPPC in the LC state, both the distinct values of T_1(H) and the response of spinning sidebands to rotation speed (described above) demonstrate that these systems are not true solids and also argue against the importance of a spin-diffusion mechanism of magnetization exchange between the ω-CH_3 and $N(CH_3)_3$ protons. The fact that NOESY cross-peaks for these $N(CH_3)_3$ and ω-CH_3 protons require 500 ms to exceed an observable threshold of ~10% is also consistent with current theoretical models.[2] A precise determination of the distances must await measurements of cross-peak volumes as a function of NOE mixing time.

At least two physical pictures are consistent with the proximity of ω-CH_3 and $N(CH_3)_3$ protons deduced from the NOESY experiments. First, chain ends are predicted theoretically to spend a significant fraction of their residence time near the aqueous interface in some vesicle systems (Dill and Flory, 1981), and it has been noted that bent-chain configurations should reduce packing constraints for lipids within bilayer assemblies with a small radius of curvature (Xu and Cafiso, 1986). Such a bendback phenomenon, though not obligatory in extended bilayer systems, would make possible dipolar interactions between the ω-CH_3 and $N(CH_3)_3$ protons in both C(18):C(10)PC and DPPC dispersions. Judging from ^{13}C chemical shifts measured at several acyl-chain sites, the relative abundance of *gauche* conformers in the LC state provides at least a necessary condition for chain bendback.

Alternatively, it is possible to interpret the NOESY cross-peaks as the first experimental evidence for an interdigitated packing mode in the LC state. The bilayer thickness determined by x-ray methods for C(18):C(10)PC just above T_m would seem to rule out the packing arrangement in Fig. 1 *c*, but significant interpenetration of chains from apposing monolayers has been proposed for aqueous dispersions of both C(18):C(10)PC and C(18):C(12)PC in the LC state (McIntosh et al., 1984; Hui et al., 1984). The NOESY results reported herein do indicate the proximity of ω-CH_3 protons of the C_{18} chain and $N(CH_3)_3$ protons of the choline headgroup, supporting a compact bilayer structure in which interdigitation of the C_{18} chains brings some of their termini close to the choline headgroups. Following this latter interpretation, the NMR results would also imply that the packing of DPPC multilayers incorporates a significant degree of interdigitation.

Information regarding the molecular organization of C(18):C(10)PC in the gel state is forthcoming from measurements of ^{13}C chemical shifts and a variety of spin relaxation times. The notion that interdigitation can occur without *trans-gauche* isomerization if one chain is twice as long as the other (Mattai et al., 1987) is supported by our NMR results: all-*trans* acyl chains are indicated for C(18):C(10)PC below T_m. The restriction of local reorientational motions characteristic of this mixed-chain phospholipid in the LC state is further accentuated below its T_m, as many ^{13}C segments go through their T_1 minima. By contrast, the interdigitated gel-state arrangement is distinguished by enhanced mid-kilohertz motions, as judged from shorter values of $T_1\rho$(C) (compared with DPPC at the same T_r) as well as oscillatory polarization transfer from ^1H to ^{13}C. Spin diffusion among the ^1H spins is nevertheless efficient enough to produce a common value of $T_1\rho$(H) for both DPPC and C(18):C(10)PC in the gel phase. Taken together, these rotating-frame relaxation results indicate that even though specific interactions of the lipid regions hinder motions on the megahertz timescale, the interdigitated packing arrangement facilitates chain or bilayer dynamics which occur at mid-kilohertz frequencies.

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[2]Calculations on a four-spin system make such a prediction, for instance, if the effective tumbling time is 2.5×10^{-8} s and the internuclear distance is ~3.5 Å (Keepers and James, 1984).

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