Characterization of Magnetically Orientable Bilayers in Mixtures of Dihexanoylphosphatidylcholine and Dimyristoylphosphatidylcholine by Solid-State NMR†

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ABSTRACT: Mixtures of long-chain and short-chain phosphatidylcholine (PC) were characterized by multinuclear (^{13}C, ^{31}P, ^{2}H) solid-state nuclear magnetic resonance. This work complements and extends previous characterization of such mixtures by focusing on *concentrated* mixtures at temperatures *above* the gel to liquid crystalline phase transition temperature (T_m) of the long-chain PC component. Above T_m it was observed that highly oriented, bilayer-like assemblies could be formed of mixtures of dimyristoylphosphatidylcholine (DMPC) and dihexanoylphosphatidylcholine (DHPC) in molar ratios ranging from approximately 1:3.5 to 1:2 (DHPC:DMPC) over a considerable range of lipid concentrations (at least 3–40% w/v total lipid, for a 1:2.5 sample). Orientation was observed to occur only in an L_α-like phase. The NMR data can be accounted for by a general model of the DHPC–DMPC aggregates in which DHPC can be found in two distinct populations (one highly ordered, one not). The averaged conformations of the glycerol backbone/headgroup regions of the long- and short-chain PC composing the assemblies were judged by solid-state ^{13}C NMR to be similar to each other. The information gleaned about these mixtures and the quality of the oriented NMR spectra obtained suggest that DHPC–DMPC mixtures may prove to be useful as model membrane media in solid-state NMR studies of biomembranes.

Among the binary detergent–phosphatidylcholine (PC) mixtures hitherto characterized, mixtures of long- and short-chain PC are of peculiar interest because the "detergent" component is nearly isostructural to the lipid component. Previous studies of the interactions of short-chain phosphatidylcholine (dihexanoyl or diheptaonyl) with long-chain (dimyristoyl, dipalmitoyl, or distearoyl) phosphatidylcholine (PC) have focused primarily upon rather dilute mixtures of the two components (<50 mM) and have emphasized characterization of mixtures below the gel to liquid crystalline phase transition temperature (T_m) of the long-chained component (Bian & Roberts, 1990; Eum et al., 1989; Gabriel & Roberts, 1987, 1986, 1984). In this paper, we focus upon the characterization of mixtures of dimyristoylphosphatidylcholine (DMPC) and dihexanoylphosphatidylcholine (DHPC) using solid-state NMR. This study complements and extends earlier work by dealing primarily with *concentrated* mixtures at temperatures *above* the gel to liquid crystalline (L_α) phase transition of pure aqueous DMPC.

MATERIALS AND METHODS

Materials. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (99%) and ^{2}H-depleted water were purchased from Sigma (St. Louis, MO). 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine-d_{54} (99%) and 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (99%) were obtained from Avanti Polar Lipids (Birmingham, AL). Deuterium oxide was acquired from Cambridge Isotopes Lab (Woburn, MA). Lipids were used as purchased without further purification.

Sample Preparation. NMR samples were prepared by weighing the appropriate weights of lipids and 0.1 M KCl directly into 5-mm NMR tubes (12 cm long). The tubes were sealed with teflon tape, capped, and mixed by a combination of heating, cooling, vortexing, and centrifugation. Samples were stored at −20 °C when not in use. We found it difficult to add the waxy and hygroscopic DHPC directly to the NMR tubes. Therefore, titration of DMPC by DHPC was usually executed by adding a 1:1 (mol:mol) DMPC:DHPC stock solution (25% w/v total lipid in 0.1 M KCl) to the DMPC-rich mixtures in the NMR tubes (1:1 DHPC:DMPC solutions were found to be more fluid than pure DHPC solutions). Samples were allowed to equilibrate in the spectrometer probe for at least 20 min before acquiring spectra.

NMR Methods. All spectra were acquired on an MR Resources refurbished Bruker AC-270 spectrometer. Samples were not spun. ^{31}P and ^{2}H spectra (but not ^{13}C) were acquired with the deuterium field frequency lock off. A 10-mm broad-banded probe was used to acquire ^{2}H (41.5 MHz) and ^{31}P (109 MHz) spectra, while a 5-mm probe was employed for ^{13}C (67.8 MHz) spectra. The ^{31}P, ^{2}H, and ^{13}C 90° pulses were 13, 24, and 6 μs, respectively. ^{2}H NMR spectra were acquired using the solids echo pulse sequence (Davis, 1983) with a 45-μs pulse separation. ^{31}P spectra were acquired using 4-μs pulses and continuous wave ^{1}H decoupling during the acquisition time (7–10 W of decoupling power) and were externally referenced to 1 M H_3PO_4. ^{13}C spectra were acquired using 4-μs pulses and WALTZ ^{1}H decoupling (20 W of power, ^{1}H 90° decoupling pulse of 13.5 μs) and were referenced externally to the isotropic resonance frequency of the ammonium methyls of DMPC (55.4 ppm; Lee & Griffin, 1989). A Bruker variable temperature control unit was used to heat samples to temperatures having uncertainties of ±1.5 °C, as judged by direct thermometric calibration. In cases where the signal in the free induction decay (FID) was truncated prior to its full decay into noise (spectra containing very sharp peaks), FIDs were subjected to apodization by a cosine function to remove spectral distortion.

Theory. In L_α-phase bilayers oriented with normals oriented at 90° with respect to the magnetic field, lipid molecules will

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undergo rapid axial rotation about the bilayer normal, leading to averaging about this axis of the tensors associated with dipolar/quadrupolar interactions and chemical shift anisotropy. Under these conditions ^{31}P–^{13}C dipolar coupling (D_{CP}) can be expressed

$$D_{CP,90} = 12250\left\langle \frac{3\cos^2\theta - 1}{2r^{-3}} \right\rangle \quad (1)$$

where the brackets $\langle\ \rangle$ denote an average, θ is the instantaneous angle between the carbon phosphorus internuclear vector and the bilayer normal, and r is the instantaneous internuclear distance (Å). Similarly, for sp^3 deuterocarbons, ^2H quadrupolar coupling can be described

$$\Delta\nu_{90} = -129000\left\langle \frac{3\cos^2\theta - 1}{2} \right\rangle \quad (2)$$

where θ is the instantaneous angle between the normal and the C–D bond vector. The ^{31}P or ^{13}C chemical shift of such molecules is (Seelig, 1978)

$$\delta_{90} = 1/2[(\sigma_{11} + \sigma_{22} + \sigma_{33}) - \sigma_{11}\langle\cos^2\theta_1\rangle - \sigma_{22}\langle\cos^2\theta_2\rangle - \sigma_{33}\langle\cos^2\theta_3\rangle] \quad (3)$$

where σ_{nn} are the principal values of the chemical shift tensor and θ_n are the angles between the nth chemical shift anisotropy (CSA) tensor principal axis and the bilayer normal.

RESULTS

Magnetic Orientation of DMPC: Titration of DMPC by DHPC. A 25% w/v dispersion of DMPC was titrated with DHPC such that the total lipid concentration in 0.1 M KCl (D_2O or H_2O) was maintained at 25%. At each point, ^{31}P and/or ^2H spectra were acquired, usually at multiple temperatures. It was observed that the ^{31}P spectra from otherwise identical samples were essentially the same regardless of whether the solvent was D_2O or H_2O. The titrations were accompanied by clarification of the samples. Samples cleared below room temperature at DHPC:DMPC ratios higher than 1:10. Beyond 1:4, optical clarity was observed over a wider range of temperatures (at least 5–50 °C). The ^{31}P spectra from these titrations at 35 °C are illustrated in Figure 1. The corresponding spectra taken at 39 °C were very similar.

The pure DMPC spectrum at the bottom left of Figure 1 illustrates the powder pattern which arises from DMPC in the L_α phase and exhibits the characteristic line shape resulting from axially symmetric motional averaging of the ^{31}P chemical shift tensor in nonoriented samples (Seelig, 1978). Addition of DHPC beyond 1:10 DHPC:DMPC is accompanied by the appearance of a second resonance with an intensity relative to DMPC which correlates with the DHPC:DMPC ratio, indicating that this peak almost certainly arises from DHPC. Besides the appearance of this second resonance, two other major changes are observed. First, there is a decrease in the powder pattern-like nature of the spectra relative to the intensity of a component which appears to "grow out of" the 90° component of the powder pattern. Near DHPC:DMPC ratios of 1:2.5, it can be observed that asymmetry of the line shapes is completely absent. Secondly, the DMPC peak gradually shifts downfield as DHPC is added and eventually converges upon the isotropic chemical shift exhibited by a pure DHPC (micellar) solution (data not shown). The DHPC resonance in these samples appears to mirror that of DMPC at each point in terms of resonance asymmetry and shift toward the isotropic position, until it finally merges with the DMPC peak. The scaling toward isotropicity of the DHPC and DMPC resonances during these titrations is illustrated further in Figure 2.

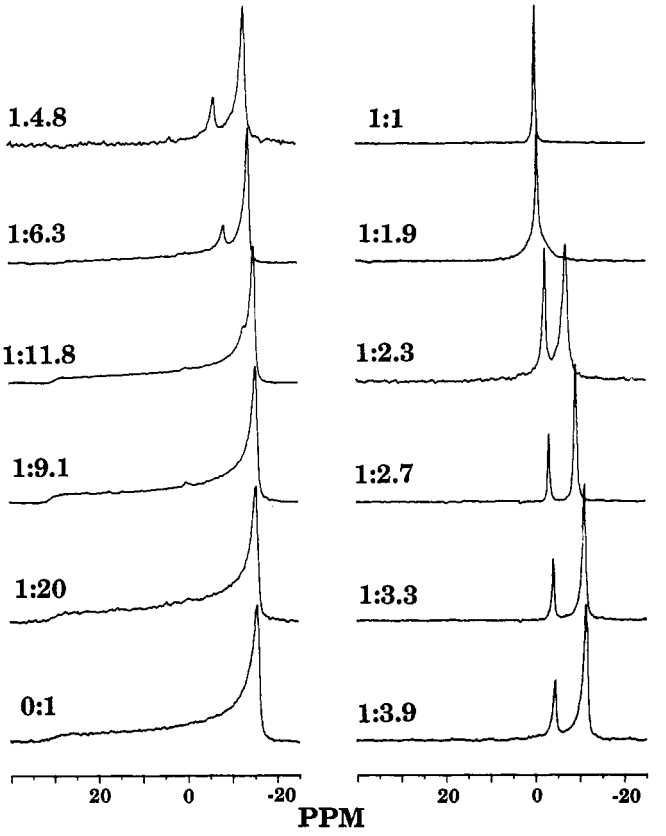

FIGURE 1: ^{31}P (^1H-decoupled) spectra taken during titration of DMPC by DHPC at 35 °C. The samples contained 25% (w/v) total lipid (DHPC + DMPC) in 0.1 M KCl–D_2O. Spectra are labeled with the DHPC:DMPC mol:mol ratio and are referenced to external 1 M H_3PO_4. Spectra were produced following exponential multiplication of the free induction decays (FID) with line broadening of 10–50 Hz and represent 40–6000 scans. The small glitch apparent in some of these spectra around 3 ppm is a quadrature image artifact. During the course of this titration, the concentration of DMPC decreased 0.37–0.22 M.

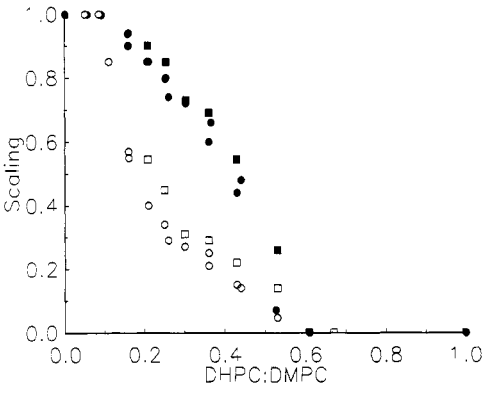

FIGURE 2: Scaling of the ^{31}P resonances from DMPC (closed circles and squares) and DHPC (open circles and squares) toward isotropicity as a function of the DHPC:DMPC molar ratio at 35 (circles) and 39 °C (squares). The scaling is defined as the observed δ divided by the difference in δ between isotropic PC and the position of the 90° component of the powder pattern in randomly dispersed DMPC. Points for which scaling = 0 represent both DHPC and DMPC resonances. The uncertainty in scalings is about ±0.05.

In order to elucidate whether the changes in the ^{31}P spectrum of DMPC induced by DHPC were caused by local perturbation of the headgroup structure (θ_n in eq 3) or of the ^{31}P static tensor (σ_{nn} in eq 3), we also followed the titrations by ^2H NMR (using acyl-chain perdeuterated DMPC, Figure 3) and

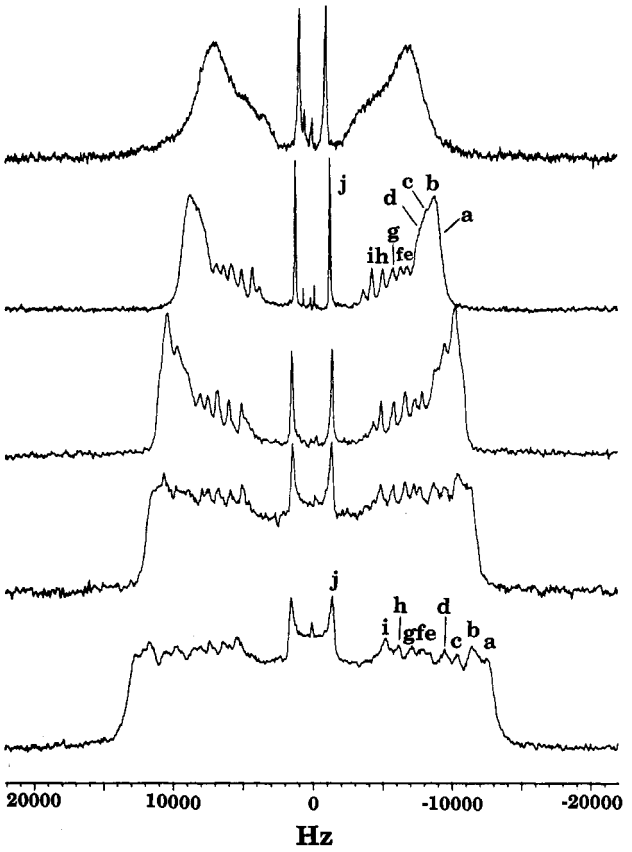

FIGURE 3: ^2H NMR spectra of DMPC-d_{54} in excess DMPC during titration by DHPC at 35 °C. The DHPC:DMPC ratios were (from bottom to top): 0:1, 1:6.3, 1:3.8, 1:2.8, and 1:2.3. The labeling of the peaks is described under Results. Samples contained 25% total lipid in 0.1 M KCl/^2H-depleted H_2O. Spectra represent 9000–36 000 scans and were produced following exponential multiplication of the FID using a line broadening of 30–50 Hz.

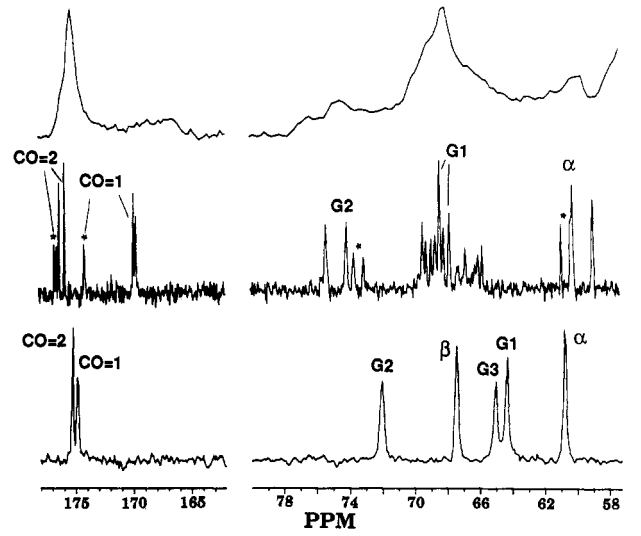

FIGURE 4: Carbonyl (left) and glycerol/headgroup regions (right) of ^{13}C (^1H-decoupled) spectra of randomly dispersed DMPC (top), an oriented 1:2.5 DHPC:DMPC mixture (middle), and an isotropic 1.33:1 DHPC:DMPC mixture (bottom), all at 25% total lipid, 40 °C, and in 0.1 M KCl/D_2O. The spectra were produced following 10 and 5 Hz of exponential line broadening of the FID for the top and bottom spectra, respectively. The middle spectrum was produced following Gaussian multiplication (resolution enhancement) of the FID. The top spectrum represents 40 000 scans, while the oriented and isotropic spectra represent 20 000 and 800 scans, respectively. The assignments of some of the peaks in the isotropic spectrum are based upon previous work (Oldfield et al., 1987; Lee & Griffin, 1989). Assignments of some resonances from the oriented sample were made as described elsewhere (Sanders, submitted). DHPC resonances in the oriented spectrum are labeled with an asterisk. Other labeling nomenclature is explained in Table I.

by ^{13}C NMR (Figure 4 and Sanders, in preparation). Addition of DHPC is accompanied by a scaling down of most of the ^2H quadrupolar splitting and by a reduction in the powder pattern character of the Pake doublets (see the central doublet from the terminal methyl groups). The broadening seen in the DMPC component in the ^{31}P spectrum of the 1:2.3 sample (Figure 1) is also observed in the corresponding ^2H spectrum. The ^{13}C spectra from these titrations (Figure 4 and Sanders, submitted) also exhibit titration-induced elimination of powder pattern character and shifts toward isotropic limits of both ^{13}C CSA and ^{13}C–^{31}P dipolar coupling. The fact that quadrupolar splitting from the acyl chains, ^{13}C CSAs from all parts of the molecule, ^{13}C–^{31}P dipolar couplings from headgroup/glycerol backbone regions, and the headgroup ^{31}P CSA are scaled down in a generally uniform fashion and are all accompanied by reductions in the axial powder pattern nature of the spectra suggests that the primary effect of DHPC upon DMPC bilayers is a change in the bulk properties of the DMPC bilayers, rather than a gross headgroup or acyl chain-specific perturbation of local structure. A more quantitative treatment is given in later sections.

The reduction in the powder-like nature of the spectra in Figure 1, 3, and 4 can be accounted for by full or partial orientation of the lipid bilayers present such that the axis of axially symmetric motional averaging (the bilayer normal) becomes, on the average, perpendicular to the direction of the magnetic field of the spectrometer. Thus, the 90° components of the ^2H and ^{31}P powder patterns become increasingly dominant for partially oriented samples and is the only visible component for samples with a very high degree of orientation (see 1:2.8 DHPC:DMPC in Figure 1 and middle spectrum of Figure 4). The residual asymmetry observed in some of the partially oriented samples can be accounted for by the lipid in such samples existing in a statistical distribution of orientations about the ideal 90° (Moll & Cross, 1990) or may reflect a mixture of fully oriented and randomly dispersed components. The broadening in the spectra which occurs as the isotropic limit is approached (1:2.3 in Figures 1 and 3) has been observed in other oriented membrane systems (Sanders & Prestegard, 1990) and likely arises from motional or exchange phenomena related to the transition of the oriented assemblies to isotropic mixed micelles.

The quadrupolar splittings and ^{31}P chemical shifts of the 90° components in the partially or fully oriented samples do not correspond to those in the powder patterns from pure DMPC. Rather, they appear to be scaled down by additional motional averaging brought on by the presence of DHPC. This averaging probably rises from the global motions of the bilayers as a whole since ^{13}C, ^{31}P, and ^2H data from all parts of DMPC exhibit similar scalings (Figures 2–4 and Sanders, submitted).

The isotropic nature of the final point of the titration (1:1 in Figure 1) was confirmed by ^{13}C NMR (Figure 4) and ^1H NMR (data not shown). The optical clarity and high fluidity of this sample is suggestive of a isotropic solution rather than an ordered state (such as the cubic phase) which involves isotropic-like motional averaging.

Given these interpretations of the phenomena accounting for the spectral changes observed in Figures 1 and 3, generalized equations can be used to describe the observed spectral parameters in the oriented spectra. These treat the molecular orientation, conformation, and dynamics of pure DMPC at a given temperature in randomly dispersed L_α-

phase multilamellae as "standard" and describe the spectral parameters from oriented samples as arising from DHPC-induced perturbations of the structure and dynamics of "standard" DMPC. The difference ($\Delta\delta$) between the ^{31}P chemical shift from a sample oriented with the motional director 90° with respect to the field direction and its isotropic chemical shift can be described

$$\Delta\delta = S_{bilayer}S_{mol}S_{loc}(-1/3)(\delta_{0,PP} - \delta_{90,PP}) \quad (4)$$

while ^2H quadrupolar splitting can be defined

$$\Delta\nu = S_{bilayer}S_{mol}S_{loc}\Delta\nu_{90,PP} \quad (5)$$

An equation analogous to eq 5 applies to ^{31}P–^{13}C dipolar coupling as well. In these equations, $\delta_{90,PP}$ and $\delta_{0,PP}$ represent the chemical shifts associated with the orthogonal and parallel components of the axially symmetric ^{31}P powder pattern arising from a pure DMPC dispersion, while $\Delta\nu_{90,PP}$ is the splitting of the 90° components of the ^2H quadrupolar coupling powder pattern arising from a particular site. $S_{bilayer}$ is a scaling factor for averaging of CSA and quadrupolar splitting resulting from *overall bilayer* motions in excess of those in pure DMPC multilamellae. This factor is 1.0 for untitrated multilamellae and converges upon 0.0 when overall aggregate motions become isotropic. S_{mol} is very similar to $S_{bilayer}$ except that is describes whole-body motions of the molecules within the bilayer. This factor is assigned a value of 1.0 for disoriented multilamellar DMPC and could conceivably get larger or smaller, depending on whether the additive causes the individual lipid molecules to become more or less orientationally mobile within the bilayers. Finally, S_{loc} describes *local* orientational or dynamic perturbations of the CSA tensor or C–D vector from its "standard" state due to a perturbation DMPC's conformation or dynamics as a result of addition of DHPC. It should be noted that while these equations are generalized, they are not merely symbolic and can, in fact, be combined with eqs 1–3 and reexpressed in quantitative, analytical forms. These equations explain the nature of "scaling" (Figure 2) as being equivalent to the product of the three order parameters in eq 4.

As will be described, the data of Figures 1–4 are consistent with a gradual reduction in $S_{bilayer}$ being the primary source of the scaling down of spectral anisotropy for *DMPC* resonances rather than S_{mol} or S_{loc}. This matter will be examined in greater depth in a later section.

Variable Temperature Studies. The results of our titrations (Figures 1–4, Sanders, in preparation) are consistent with maintenance of an L_α-like phase at DHPC:DMPC > 2.0 and 35–40 °C. Indeed, the ^2H spectra illustrated in Figure 3 exhibit a distribution of quadrupolar splittings generally consistent with the well-known order profile exhibited by the acyl chains of L_α-phase PC (Davis, 1983; Seelig, 1977; Jacobs & Oldfield, 1981). Further confirmation was sought by examining the temperature dependence of spectra from several compositions of DHPC and DMPC.

Figure 5 illustrates the temperature dependence of the ^{31}P and ^2H spectra from a 1:2.7 DHPC:DMPC sample. At 35 °C this sample was clear but highly viscous. It can be observed that as the temperature is lowered toward T_m for DMPC [24 °C (Mason & Huang, 1981)] the ^{31}P and ^2H signals from DMPC broaden and exhibit considerable asymmetry. Below T_m, the signals appear to collapse into isotropic spectra, and the samples become highly fluid. Similar results were obtained when the temperature was lowered through T_m for samples having DHPC:DMPC ratios of 1:4.8 and 1:3.3 (not shown). At 1:1.9 a similar transition to an isotropic phase was observed

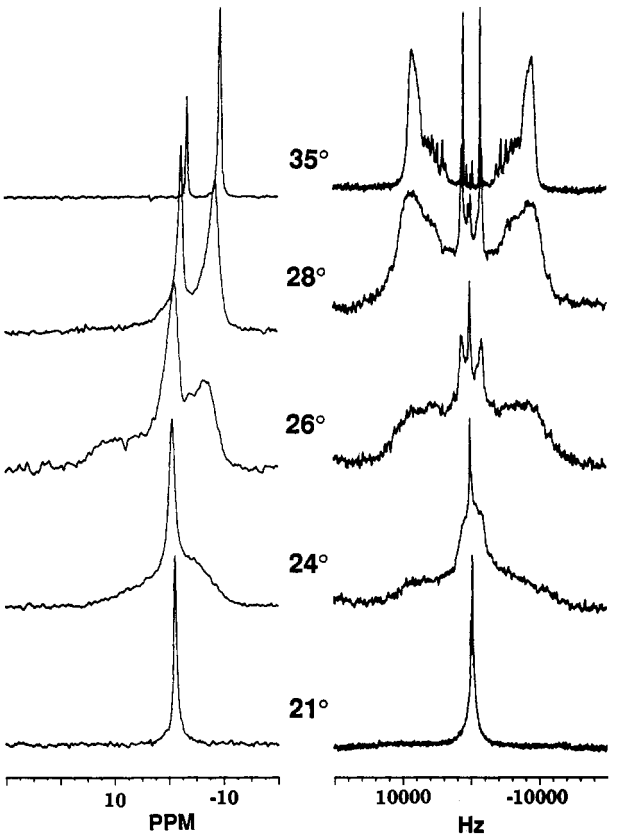

FIGURE 5: ^1H-decoupled ^{31}P (left) and ^2H NMR spectra from a 1:2.7 DHPC:DMPC (0.3 M DMPC, 0.11 M DHPC) sample at various temperatures (labeled, °C). Samples contained 25% total lipid in 0.1 M KCl/^2H-depleted H_2O. Spectra were produced following exponential multiplication of the FID using 10–100 Hz line broadening and represent 80–500 and 20 000–30 000 scans for ^{31}P and ^2H, respectively.

to occur at a somewhat elevated temperature (between 32 and 35 °C).

Both the spectra of Figure 5 and the observed change in viscosity indicate that the DHPC–DMPC samples undergo a phase transition near the T_m of pure DMPC. As confirmed by ^{13}C NMR (not shown), the fluid phase below room temperature is isotropic. On the basis of the work of Bian and Roberts (1990), this fluid phase is likely to be composed of phase-separated DHPC–DMPC aggregates in which DMPC exists in a bilayered gel-like state. The inhomogeneous broadening seen in the ^{31}P spectra in the 24–28 °C samples can arise from changes in DMPC dynamics and/or disorientation of the lipid aggregates. However, the data are insufficient to discern between the possibilities.

The temperature-dependent spectra are of significance for two reasons. First, the observation of a phase transition occurring at a temperature (or narrow range of temperatures) very close to the T_m of pure DMPC is consistent with the notion that the DMPC within the oriented 35–40 °C DHPC–DMPC aggregates is L_α bilayer-like. This suggestion is confirmed by detailed analysis of the conformation and dynamics of the individual DMPC molecules within the oriented assemblies (see later section). Secondly, these data indicate that the DHPC–DMPC assemblies are suitable as a media for oriented-sample solid-state NMR studies *only* above T_m for DMPC.

A Semiquantitative Evaluation of the Average Conformation and Dynamics of DMPC in the Oriented Assemblies. The structure and dynamics of PC within L_α bilayers has been the subject of extensive investigation [cf. Hauser et al.

(1988, 1981), Buldt and Wohlgemuth (1981), De Loof et al. (1991), and Weiner and White (1992)]. We employed both ^{13}C and ^{2}H NMR to compare the structures of DMPC within pure DMPC bilayers and DHPC-containing mixtures. ^{13}C CSA and dipolar coupling constants are capable of providing considerable structural information from membranous molecules because of their dependence upon internuclear distance, orientation, and dynamics (eqs 1 and 3). Figure 4 illustrates examples of ^{13}C spectra from unoriented DMPC (top) and from oriented (middle) and isotropic (bottom) DHPC–DMPC. DHPC resonances are often clearly distinguished from DMPC peaks in the oriented-sample spectrum by their relative integrals. The full ^{13}C spectral series from the DHPC titration of DMPC has been reported elsewhere (Sanders, submitted) along with details of assignment and measurement of CSA and ^{13}C–^{31}P dipolar couplings. In that study, some eight dipolar couplings were measured and assigned along with 10 CSAs. It was observed that, as DMPC was titrated with DHPC, all 18 parameters were *uniformly* scaled down as a *linear* function of $S_{bilayer}$. These data related primarily to the headgroup, glycerol backbone, carbonyl, and α-carbon regions of DMPC and indicate that the average conformation, dynamics, and orientation of these regions of the DMPC molecules within the oriented assemblies is, within experimental error, unperturbed by addition of DHPC.

Analysis of the quadrupolar splittings of Figure 3 is complementary to the ^{13}C data in that it provides some light upon the DHPC-induced changes in the dynamics of the acyl chains of DMPC. A number of the 90° components of the Pake doublets from pure randomly dispersed DMPC are labeled (a–j) in Figure 3. It is possible to follow these peaks to the 1:2.8 titration point, and the peaks are labeled accordingly. On the basis of previous work (Davis, 1983; Seelig, 1977; Jacobs & Oldfield, 1981), it is possible to ascertain that peaks a and b represent deuterons from methylenes in the high order parameter plateau region of the acyl chain near the ester linkages. Peaks c–i represent methylenes near the end of the methylene chain where order is falling off, while j represents the terminal methyl deuterons. In addition to the labeled peaks, at least one of the deuterons from the *sn*-2 2-methylene can be identified with reasonable certainty (by its integral) between i and j. This positioning is expected on the basis of the splitting observed for the *sn*-2 2-methylene in pure DMPC dispersions (Jacobs & Oldfield, 1981).

The scalings ($S_{bilayer}S_{mol}S_{loc}$, see eq 5) of the labeled splittings from their initial magnitudes to their values in the 1:2.8 sample were measured and given uncertainties of ±0.02 for a–d and ±0.01 for e–j. It was observed that a and b scale by factors of 0.73 and 0.75, respectively. These values are in reasonable agreement with the scaling of ^{31}P CSA observed for the samples of the same composition, as given in Figure 2. However, the scaling for peaks representing the order parameter transition region, c–i, all fall between 0.80 and 0.82, while of that the terminal methyl group is 0.85. These values are a little higher than expected on the basis of doublets a and b, the ^{31}P results (Figure 2), and ^{13}C results (Sanders, submitted), indicating that S_{loc} has probably increased for the deuterons giving rise to peaks c–j (eq 5). This suggests that DHPC causes the interior of the bilayers in the oriented aggregates to become slightly more ordered than in pure DMPC dispersions. While the mechanism of this limited perturbation is difficult to establish with certainty, a small amount of interdigitation by the acyl chains of the juxtaposed leaflets to fill voids created in the bilayer core by insertion of short-chained PC into the bilayer interfaces might account for this affect.

These ^{13}C and ^{2}H NMR results suggest that aggregation with DHPC results in only minor changes in the structure and dynamics of DMPC. This result supports our earlier assertion that the DHPC–DMPC aggregates appear to be dominated by a bilayered L_α-like morphology. A DHPC-induced change to another morphology and/or phase would be expected to be accompanied by much larger perturbations in the conformation and dynamics of the individual DMPC molecules.

General Characteristic of the DHPC Resonances. When DHPC is added to DMPC at low levels, it gives rise to a ^{31}P resonance which is virtually superimposable with that of DMPC (see 1:20 and 1:11.8 spectra in Figure 1). This observation suggests that most of the added DHPC in these two samples binds to the DMPC interface where it adopts an averaged conformation similar to that of DMPC. A certain amount also probably partitions into solution micellar or monomeric states, which probably accounts for the small isotropic component near 0 ppm in the 1:11.8 spectrum. This latter point was confirmed by reducing the bulk aqueous phase volume (38% total amphiphile instead of 25%) and observing that the isotropic component disappeared with no other changes being observed in the spectrum (data not shown). Initial interdigitation of DHPC into the DMPC bilayers is accompanied by little orientation of the sample.

Above 1:10 DHPC:DMPC, the 90° components of the ^{31}P resonances from both amphiphilic components begin to shift toward the isotropic limit. However, the scaling of the two components becomes unequal, and the relative scaling throughout the titrations is not constant. The degree of asymmetry of DHPC peaks generally reflects that of the corresponding DMPC components, indicating that both molecules are members of the same oriented or partly oriented assemblies. ^{13}C spectra from these titrations exhibit similar trends in both CSAs and dipolar couplings (see next section). Only a single set of DHPC peaks is observed in both ^{13}C and ^{31}P spectra, indicating that if DHPC exists in multiple states and/or conformations, they must rapidly exchange on the resonance frequency time scale. Both DMPC and DHPC will experience the same $S_{bilayer}$. The fairly uniform scaling observed for both ^{31}P and ^{13}C DHPC resonances from all parts of DHPC indicates that the *additional* scaling observed for the short-chained PC probably arises from reductions in S_{mol} (eqs 4 and 5). This decreased S_{mol} could arise from differences in the averaged whole-body orientation and/or dynamics within the oriented assemblies and and/or from rapid physical exchange of oriented DHPC with low order components.

As observed, addition of small amounts of DHPC leads to insertion of DHPC into the bilayers with no significant perturbation of the multilamellae above T_m. In this state, S_{mol} of DHPC appears to equal that of DMPC (ca. 1.0, see above). The onset of inequivalence of DHPC resonances (which generally correlates with sample orientation) suggests that addition of DHPC beyond some critical level leads to a second population of DHPC which is less highly ordered than the interfacial population but which undergoes rapid exchange between the two states. The possible aggregate morphologies which would be consistent this general model are described under Discussion. If we assume that the scaling of the DMPC resonance is approximately equal to $S_{bilayer}$, it can be

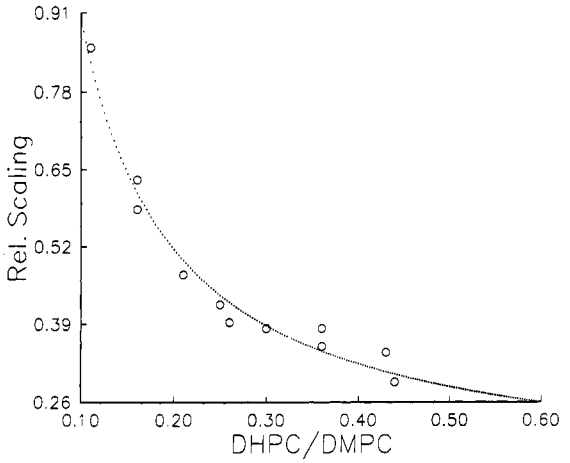

FIGURE 6: Relative scaling of DHPC and DMPC ^{31}P resonances toward isotropicity as a function of the DHPC:DMPC molar ratio at 35 °C. "Scaling" is defined in the caption of Figure 2. The curve represents the best fit of the data to eq 6.

demonstrated that this simple two-state model predicts the relative scaling for DHPC and DMPC resonances:

$$\text{scaling}_{DHPC}/\text{scaling}_{DMPC} = [DHPC]_{crit}/[DHPC]_{tot} + S_{mol}(1 - [DHPC]_{crit}/[DHPC]_{tot}) \quad (6)$$

where $[DHPC]_{crit}$ is the DHPC:DMPC ratio in DHPC-saturated DMPC bilayers, $[DHPC]_{tot}$ is the overall DHPC:DMPC ratio, and S_{mol} is the effective whole-body order parameter for the nonbilayer surface-associated population of DHPC. This equation applies only to spectra from samples in which the bilayered DMPC is fully DHPC-saturated (above ca. 1:10 DHPC:DMPC). This model presumes that the whole-body order parameter for the non-surface-associated population of DHPC remains constant throughout the titration.

The 35 °C DHPC data were fit by the above equation using nonlinear least-squares analysis to produce the results depicted in Figure 6. It can be observed that the simple two-state model is fully consistent with the data. This analysis determined the critical DHPC:DMPC ratio to be 0.088 ± 0.002 (1:11 DHPC:DMPC) and the effective S_{mol} for the nonbilayer surface-associated population of DHPC to be 0.13 ± 0.02.

It should be noted that the concentration of free DHPC in solution as monomers or micelles is probably very low throughout the titrations because of the relatively low amount of water present in these samples. A test of this point was provided by ^{31}P spectra taken from a 1:2.5 DHPC:DMPC sample over a range of total lipid (w/v) from 3 to 40% (data not shown). By dramatically changing the bulk water volume of the samples, the population of free DHPC should vary substantially. We observed no significant differences in the spectra (which are similar to the 1:2.7 point of Figure 1), indicating that the free population must be very small.

Comparison of the Average Structures of DMPC and DHPC in the Oriented Bilayers. Because the ^{13}C CSAs and the ^{31}P–^{13}C dipolar couplings arising from both DHPC and DMPC are functions of the structure and dynamics of these molecules (eqs 1 and 3), comparison of the corresponding parameters from the two molecules can shed light on whether they exhibit similar averaged conformational and local dynamic structures within the oriented assemblies. As described earlier, it has been established that DMPC exhibits similarly averaged structures in both pure and DHPC-aggregated forms.

Table I: Observed and Predicted ^{13}C CSA ($\Delta\delta$,[a] ppm) and ^{13}C–^{31}P Dipolar Couplings (D_{C-P}, Hz) for Assigned DHPC Resonances from a 1:2.5 DHPC:DMPC Sample at 40 °C (see Figure 4)

carbon[b]	$\Delta\delta_{obs}$	$\Delta\delta_{predict}$[c]	$D_{C-P,obs}$	$D_{C-P,pred}$
C=O1	−2.5 ± 1.0	−6.8 ± 1.0	<17[d]	13
C=O2	+2.7 ± 1.0	+1.4 ± 1.0	31	30
G3	>+1.0	+2.9 ± 2.0	ND[e]	ND
G1	>+2.9	+6.3 ± 0.8	ND	ND
G2	+2.8 ± 0.5	+2.8 ± 0.5	96	89
α	−1.1 ± 0.4	−1.1 ± 0.4	86	86
Me	+1.2 ± 0.3	1.4 ± 0.3	ND	ND

[a] $\Delta\delta$ is the observed (oriented) chemical shift minus the corresponding isotropic chemical shift obtained from the spectrum at the bottom of Figure 4. [b] C=O1 and C=O2 represent the *sn*-1 and -2 carbonyls, G1–3 are the glycerol *sn*-1 to -3 backbone carbons, α and β are the first and second headgroup carbons beyond the phosphodiester linkage (respectively), and Me represents the signal arising from the degenerate acyl chain methyls. [c] Predicted on the basis of the parameters observed for DMPC (see text). [d] The uncertainty for all values of both observed and predicted couplings is 5 Hz. The dipolar coupling are absolute valued. [e] ND, not determined.

The measured CSAs and dipolar splittings observed in the middle of Figure 4 for a number of carbons of DHPC are listed in Table I.

From the ^{31}P results of Figure 2, $S_{bilayer}S_{mol}$ can be estimated to be 0.60 and 0.25 for DMPC and DHPC, respectively, in the 1:2.5 sample. If DHPC exhibits an averaged structure similar to that of DMPC, its $\Delta\delta$ and dipolar splitting should be reduced by a factor of 2.4 relative to its long-chain counterpart. The splittings predicted for DHPC based on this factor are listed in Table I. It is evident that while the observed and predicted splitting for DHPC are not always within experimental error, there is general agreement. Variation appears to be most pronounced for data related to the *sn*-1 and -2 ester linkages. However, it should be noted that eqs 1 and 3 dictate that even minor changes in averaged θ and/or r can lead to large variations in CSA or dipolar coupling. Thus, despite some differences, the overall agreement of predicted and observed data suggests that the variations between the averaged conformational structures of DHPC and DMPC in the oriented assemblies are fairly minor. Because it is known that L_αPC molecules are extremely flexible (De Loof et al., 1991; Weiner & White, 1992) and because we have reason to believe that DHPC exists in at least two distinct (but rapidly exchanging) morphological populations (see above section), a more quantitative description of specific structural differences presents a challenging problem which is beyond the scope of this paper.

Overall Concentration Dependence of Sample Orientation. Previous characterization of mixtures of short- and long-chained PC focused on mixtures considerably more dilute than those dealt with in this study. In this study we serially diluted a 1:2.5 mixture from an initial concentration of 40% total lipid to 1% (data not shown). This range spans DHPC/DMPC millimolarities of 180/480 to 4.6/12. At 39 °C and over a span of concentrations from 40% to 3%, the ^{31}P spectra were observed to be very similar to the 1:2.7 spectrum illustrated in Figure 1. These samples were optically clear above and below room temperature. A 2% sample was clear below room temperature and very turbid above. The 20 °C ^{31}P spectrum exhibited only a single isotropic component, while at 40 °C an isotropic peak was observed to be superimposed upon an axially symmetric powder pattern. Further dilution to 1% resulted in a sample which was visibly inhomogeneous at all temperatures. These results suggest that the oriented assemblies maintain a constant morphology over a wide range of concentration as long as a certain critical concentration is

exceeded (i.e., >2–3%). The loss of orientation and the appearance of both isotropic and powder pattern components above T_m and below 3% are probably the result of depletion of DHPC from the DHPC–DMPC aggregates due to partitioning of DHPC into isotropic solution phases (monomers and micelles). These observations help to account for why previous examinations of *dilute* short/long-chain PC mixtures failed to observe assembly orientation.

DISCUSSION

Morphology of the Highly Oriented DMPC Assemblies. The results provide several important constraints upon possible morphologies for the oriented DHPC–DMPC aggregates. The DMPC in these aggregates appears to maintain an L_α-like bilayer state, while the DHPC appears to populate two fractions: one in which the DHPC interdigitates into the DMPC bilayer surface and a second in which the effective whole-molecule order is fairly low.

The magnetic orientation of lipid-like assemblies have been described in greater detail elsewhere (Forrest & Reeves, 1981; Sanders & Prestegard, 1990; Jannson et al., 1990; Mops et al., 1992; Seelig et al., 1985; Speyer et al., 1987; Rosenblatt et al., 1987; Boidart et al., 1988). There are several overall aggregate morphologies which can sometimes be magnetically oriented. While it is difficult to *prove* the existence of a particular morphology from NMR data, we can at least determine which possibilities are consistent with the data and which are not.

Multilamellar vesicles and tubules can sometimes be partially oriented by a magnetic field (Seelig et al., 1988; Mops et al., 1992; Rosenblatt et al., 1987; Speyer et al., 1987). While the observed bilayer-like state maintained by DMPC in DHPC–DMPC mixtures is consistent with these morphologies, such superstructures cannot account for the second (low-order) population of DHPC and are generally inconsistent with both the very high degree of orientational homogeneity and the optical clarity of some of the DHPC–DMPC samples.

A second class of magnetically orientable structures is based upon extended rod-like micelles in which the long-chained molecules are radially distributed (tails inward) about the long, oriented, symmetry axis of the rods (Forrest & Reeves, 1981; Boidart et al., 1988). These structures account for two populations of DHPC: one population "capping" the ends of the rod-like aggregates and the other population interdigitating with long-chained molecules along the main part of the rods. However, these structures are not bilayered. Furthermore, in addition to executing axial rotation about the long *molecular* axes, the lipids in such aggregates would also execute rotations about the long axis of the rods (via whole-rod rotation and molecular diffusion). This would lead to averaging of the ^{31}P headgroup tensor so that the oriented sample ^{31}P signal would appear *downfield* of the isotropic position [see Seelig (1978)]. This was not observed.

Finally, it is known that "discoidal micelles" in which the edges of bilayer discs are stabilized by edge interactions with the detergent component can sometimes be magnetically oriented (Forrest & Reeves, 1981; Boidart et al., 1988; Sanders & Prestegard, 1990). This morphology appears to be consistent with our data. The interior of such disks is bilayered, consistent with our observation that DMPC exists in a bilayer-like state with some DHPC also being interdispersed. The edges of these putative bilayers would be stabilized by association of a second population of DHPC (which appears to have the correct topological balance of polar and nonpolar moieties for such interactions). Rapid reorientation of DHPC about the edge of the discoids would account for the observed low order of the second DHPC population. A discoidal micelle morphology would also account for the decrease in $S_{bilayer}$ which accompanies DHPC titration: once discoidal structures are formed, continued addition of DHPC would be expected to break the bilayer discs into smaller (and less highly ordered) discs until they eventually are transformed into classical mixed micelles.

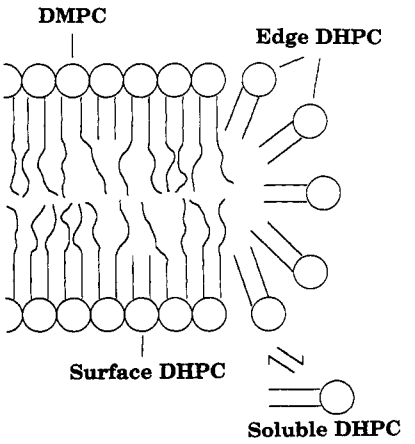

FIGURE 7: Cross section of the edge of a *hypothetical* discoidal micelle.

A *highly idealized* model for a 2-D cross section of the edge of the postulated discoids is illustrated in Figure 7. It should be reemphasized that the NMR data do not *prove* this model to be correct, but that it is consistent. It has previously been proposed that such discoidal micellar structures form in mixtures of short- and long-chain PC mixtures at compositions too *DHPC-rich* for maintenance of a multilamellar vesicular state and too *DMPC-rich* to form classical mixed micelles (Lasic, 1988; Bian & Roberts, 1990). Furthermore, most of our NMR results mirror observations previously obtained for mixtures of DMPC with a bile salt derivative, CHAPSO, a system which is also believed to be dominated by bilayered discoidal micelles (Sanders & Prestegard, 1990; Sanders, submitted).

Implications for Future Solid-State NMR Studies of Membrane Phenomena. The use of solid-state nuclear magnetic resonance (NMR) in studies of biomembranes is becoming increasingly widespread, partly as a result of methods for eliminating the broad powder patterns typically associated with the NMR spectra of large, unoriented amphiphilic assemblies such as multilamellar liposomes. Unlike magic-angle spinning (in its most basic form) or employment of isotropic vesicles, use of oriented bilayers allows spectral resolution to be enhanced without removing structurally relevant quadrupolar/dipolar couplings and chemical shift anisotropy. The primary magnetically orientable phospholipid system which has been used is based upon mixtures of DMPC with the bile salt derivative CHAPSO (Sanders & Prestegard, 1990, 1991, 1992).

The primary drawback to the use of the CHAPSO–DMPC system as a model system for biological membrane bilayers lies in the concern that the synthetic detergent constitutes a potential source of experimental artifact. While control experiments were developed to screen for CHAPSO-specific effects (Sanders & Prestegard, 1992), there is clearly room for improvement of orientable membrane media. The results of this paper constitute a significant step forward because they demonstrate that the detergent required to promote orientation need not be exotic in structure, but can be nearly isostructural with the long-chain lipid component. This should

significantly reduce the potential for experimental artifacts in studies employing DHPC–DMPC mixtures as model membrane media. Similar mixtures have already been employed in non-NMR studies of phospholipase A_2 (Gabriel & Roberts, 1987; Gabriel et al., 1987). This contribution should provide an impetus both for the further characterization and for the future employment of short-chain/long-chain PC mixtures.

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