Solid state ^{13}C NMR of unlabeled phosphatidylcholine bilayers: Spectral assignments and measurement of carbon–phosphorus dipolar couplings and ^{13}C chemical shift anisotropies

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ABSTRACT The direct measurement of ^{13}C chemical shift anisotropies (CSA) and ^{31}P–^{13}C dipolar splitting in random dispersions of unlabeled L_α-phase phosphatidylcholine (PC) has traditionally been difficult because of extreme spectral broadening due to anisotropy. In this study, mixtures of dimyristoyl phosphatidylcholine (DMPC) with three different detergents known to promote the magnetic orientation of DMPC were employed to eliminate the powder-pattern nature of signals without totally averaging out spectral anisotropy. The detergents utilized were CHAPSO, Triton X-100, and dihexanoylphosphatidylcholine (DHPC). Using such mixtures, many of the individual ^{13}C resonances from DMPC were resolved and a number of ^{13}C–^{31}P dipolar couplings were evident. In addition, differing line widths were observed for the components of some dipolar doublets, suggestive of dipolar/chemical shift anisotropy (CSA) relaxation interference effects. Oriented sample resonance assignments were made by varying the CHAPSO or DHPC to DMPC ratio to systematically scale overall bilayer order towards the isotropic limit. In this manner, peaks could be identified based upon extrapolation to their isotropic positions, for which assignments have previously been made (Lee, C. W. B., and R. G. Griffin. 1989. *Biophys. J.* 55:355–358; Forbes, J., J. Bowers, X. Shan, L. Moran, E. Oldfield, and M. A. Moscarello. 1988. *J. Chem. Soc., Faraday, Trans.* 1 84:3821–3849). It was observed that the plots of CSA or dipolar coupling versus overall bilayer order obtained from DHPC and CHAPSO titrations were linear. Estimates of the intrinsic dipolar couplings and chemical shift anisotropies for pure DMPC bilayers were made by extrapolating shifts and couplings from the detergent titrations to zero detergent. Both detergent titrations led to similar "intrinsic" CSAs and dipolar couplings. Results extracted from an oriented Triton-DMPC mixture also led to similar estimates for the detergent-free DMPC shifts and couplings. The results from these experiments were found to compare favorably with limited measurements made from pure L_α PC. This detergent-based method for assigning spectra and for determining dipolar couplings and CSA in detergent-free systems should be extendable to other lipid systems. The resulting data set from this study may prove useful in future modeling of the structure and dynamics of DMPC bilayers. In addition, the fact that experiments utilizing each of the three detergents led to similar estimates for the spectral parameters of pure DMPC, and the fact that spectral parameter versus bilayer order plots were linear, indicate that the averaged conformation and dynamics of DMPC in the presence of the three detergents are very similar to those of pure L_α DMPC.

INTRODUCTION

It has been demonstrated that the use of oriented membrane samples can greatly facilitate spectral assignments and the measurement of spectral parameters in solid state NMR studies of isotopically labeled biomolecules (1–6). In this contribution, possible sources of structurally useful anisotropic NMR data from *unlabeled* lipids are explored, using magnetically oriented model bilayers.

At least three detergent-like molecules are known, which will promote the magnetic orientation of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) bilayers above the gel to liquid crystalline phase transition of DMPC (24°): dihexanoyl phosphatidylcholine (DHPC) (7), 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPSO) (8), and α-[4-(1,1,3,3,-tetramethylbutyl)phenyl]-ω-hydroxypoly(oxy-1,2-ethanediyl) (Triton X-100) (Sanders, C. R. et al., manuscript submitted for publication). It is believed that the essential bilayer morphology and L_α-like properties of DMPC are preserved in the oriented mixtures at the appropriate temperatures (7, 8).

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Previous ^{13}C NMR studies employing magnetically orientable systems have focused on the spectra of ^{13}C-labeled additives into the oriented DMPC matrix (5, 6). In those studies the natural abundance ^{13}C spectrum arising from DMPC and the orientation-promoting detergents were not carefully analyzed and were generally regarded as a nuisance because of overlap with signals from the labeled molecule of interest. In this contribution, the neglected ^{13}C spectra from the DMPC matrices are considered in detail.

MATERIALS AND METHODS

Materials. CHAPSO and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO). L-α-DMPC was purchased from Sigma, while DHPC and d_{54}-DMPC were purchased from Avanti Polar Lipids, Inc. (Birmingham, ALA). Deuterium oxide was purchased from Cambridge Isotopes (Woburn, MA).

Experimental. Methods used for preparation of NMR samples and for the acquisition of ^1H-decoupled ^{13}C NMR spectra have been described previously (5–8). An MR-Resources refurbished Bruker AC-270 NMR spectrometer (Bruker Analytische Messtechnik GmbH, Karlsruhe, Germany) was employed for most measurements (^{13}C frequency of 67.9 MHz), except for a few spectra which were acquired at 125.8 MHz on a Bruker AM-500. WALTZ ^1H decoupling (90° decoupler pulse length = 13.6 μs) was used in the acquisition of all spectra.

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The referencing of ^{13}C spectra proved to be problematic because standard internal reference molecules are probably non-polar enough to interact with the amphiphilic aggregates (leading to non-isotropic averaging of their chemical shifts). External referencing was ruled out by the probability that the highly concentrated lipid samples possess different bulk magnetic susceptibilities than aqueous isotropic solutions. Thus, we generally used ethylene glycol (64.0 ppm) as an internal reference. Because its resonance overlaps with headgroup/glyceride peaks, spectra were first acquired in the absence of the reference (and are the ones presented in this paper) followed by addition of the reference and reacquisition of the spectrum.

Theory. In samples (such as those of this study) where bilayers are oriented with their normal perpendicular to the magnetic field of the spectrometer and the lipid molecules execute axially symmetric long-axis rotations about the normal, ^{13}C–^{31}P dipolar coupling ($\Delta\nu$, Hz) can be expressed:

$$\Delta\nu = -24473 \cdot -\tfrac{1}{2} \cdot S_{\text{bilayer}} \cdot \langle (3 \cdot \cos^2\theta - 1) \cdot r^{-3}/2 \rangle \quad (1)$$

where r is the instantaneous internuclear distance (Å) between the carbon and the phosphorus and θ is the instantaneous orientation of the internuclear vector with respect to the motional director (i.e., the bilayer normal). The $\langle\ \rangle$ brackets denote a time averaged function. The factor of $-\frac{1}{2}$ arises from the orthogonal orientation of the motional director (the bilayer normal) to the magnetic field. S_{bilayer} describes the *relative* degree of order of the normal axes with respect to the ideal 90° orientation (see reference 8). Because S_{bilayer} is here treated as a *relative* parameter, it is defined to be 1.0 in pure multilamellar L_α DMPC dispersions at a given temperature. At this temperature, when the bilayers acquire additional degrees of overall motional freedom due to the presence of a detergent, S_{bilayer} will be reduced until the overall motions become effectively isotropic and S_{bilayer} becomes 0 at high detergent contents.

Chemical shift anisotropy ($\Delta\delta$) can be defined:

$$\begin{aligned}\Delta\delta &= \delta_{\text{oriented}} - \delta_{\text{isotropic}} \\ &= -\tfrac{1}{3} \cdot S_{\text{bilayer}} \cdot \left[\left\langle \frac{3\cos^2\theta_1 - 1}{2} \right\rangle \cdot (\sigma_{11} - \sigma_{22}) \right. \\ &\quad \left. + \left\langle \frac{3\cos^2\theta_3 - 1}{2} \right\rangle \cdot (\sigma_{33} - \sigma_{22}) \right] \end{aligned} \quad (2)$$

where σ_{nn} are the principal components of the relevant static CSA tensor. θ_n are the direction cosines between the nth CSA principal axis and the motional director axis (i.e., the bilayer normal) (9). S_{bilayer} and the averaging brackets maintain the same definitions as given for Eq. 1.

If it is assumed that the presence of detergent in magnetically oriented samples does not significantly perturb the local averaged structures of *individual* DMPC molecules, differences between the observed splittings and CSAs from magnetically oriented samples and those from pure 90° oriented DMPC suspensions will arise solely from the reduction of S_{bilayer} caused by the presence of the detergent in the magnetically oriented samples. The validity of this assumption will be tested in the Results section.

Estimates of S_{bilayer} for magnetically oriented samples are extracted from the position of the ^{31}P resonance (δ) from DMPC:

$$S_{\text{bilayer}} = (\delta_{\text{oriented}} - \delta_{\text{isotropic}})/(\delta_{90,\ \text{powder pattern}} - \delta_{\text{isotropic}}) \quad (3)$$

where $\delta_{90,\ \text{powder pattern}}$ is the position of the 90° component of the ^{31}P powder pattern from pure DMPC dispersions at the same temperature used for the oriented sample (40°).

Nomenclature. The labeling of the carbons of DMPC is as follows and roughly conforms to previous usage (10): G1,2,3: the *sn*-1, 2, and 3 glycerol carbons, respectively; CO1 and CO2: the *sn*-1 and *sn*-2 carbonyls; α, β, and γ: the choline headgroup carbons in order from the phosphodiester linkage to the ammonium methyls; C2/C2′ and C14: the alpha carbons and the terminal carbons on the acyl chains, respectively.

Results

Key spectral parameters

The primary experiments described in this paper involve titrations of DMPC with DHPC, CHAPSO, and Triton X-100 followed by ^{13}C NMR (Figs. 1–3). The spectra at the bottom of Figs. 1 and 2 represent predominately isotropic mixtures of DMPC with CHAPSO and DHPC, respectively. All of the other spectra represent samples which are at least partially *oriented.* In the case of Triton X-100, the "titration" consisted of a single oriented sample because, unlike DHPC and CHAPSO, Triton promotes bilayer orientation without significantly perturbing overall bilayer order (Sanders C. R., et al., manuscript submitted for publication). A perusal of the data from these titrations immediately reveals three general spectral phenomena which must be addressed. It can be observed that sample orientation often results in significant shifts in resonance frequency and in the transformation of isotropic singlets into apparent doublets. Thirdly, the doublets are sometimes observed to be asymmetrically broadened (e.g., see the CO2 resonances in Figs. 1–3).

The changes in resonance frequency result from the anisotropic contribution to the chemical shift in oriented samples and require no additional explanation at this point. However, the splittings and the differential line broadenings of the doublet components were somewhat unanticipated and must be considered in greater detail. It should be noted that while Gaussian resolution-enhancement has been used in the production of Figs. 1–3, both differential line broadenings and splittings are reproducible and clearly discernible in unenhanced spectra. In the following sections, the nature of these spectral phenomena are addressed, presupposing ^{13}C spectral assignments which are described at a later point in this paper.

^{31}P–^{13}C dipolar coupling. *A priori,* the splittings observed for many resonances in Figs. 1–3 might be explained by the existence of two equally populated, spectroscopically distinct, slowly exchanging forms of DMPC in these samples (as in the formation of inside/outside lipid populations due to vesicularization, see reference 11). This possibility is ruled out by data (not shown) which demonstrates that the magnitudes of the splittings (in Hz) remain the same when spectra are acquired at magnetic field strengths varying by nearly a factor of 2 (11.7 and 6.3 T). Thus, the splittings must arise from a nuclear spin coupling phenomenon. This conclusion and the identification of this coupling as *dipolar* in nature is confirmed by the fact that the splittings

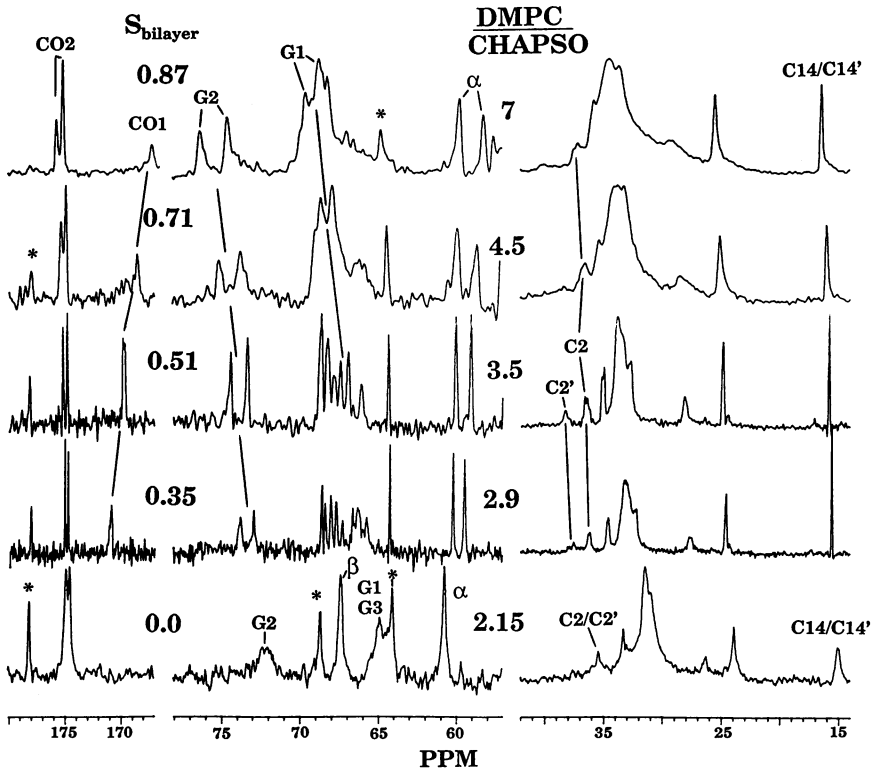

FIGURE 1 67.9 MHz ^{13}C (^{1}H-decoupled) spectra from titration of DMPC by CHAPSO at 40°C. The total amphiphile (CHAPSO + DMPC) wt/vol content was maintained at 25% in 0.1 M KCl/D_2O. Spectra are labeled with the DMPC:CHAPSO molar ratios and were processed following zero filling of the initial 2,048 or 4,096 data points, Gaussian multiplication (resolution enhancement) and Fourier transformation. Select CHAPSO resonances are marked with an asterisk. In the case of the isotropic sample (*bottom spectrum*) exponential multiplication (5 Hz line broadening) was used rather than Gaussian enhancement. The carbonyl region of the isotropic (*bottom*) spectrum was actually taken at 16° because the lines were more narrow at this temperature. Because assignments of the CO1 and CO2 resonances in the isotropic spectrum could not be made, the isotropic δ used in the calculation of $\Delta\delta$ was the average of the δ for the carbonyl resonances (174.7 ± 0.2 PPM). The spectra represent 500–25,000 scans. The nomenclature of carbon labeling is described in the Materials and Methods section. The choline methyl signal is not shown in these spectra because it was always a singlet unless very high resolution enhancement was used, in which case the peak took on a complex line shape similar to that shown in the inset of Fig. 3.

scale uniformly with changes in $S_{bilayer}$ (see Eq. 1) as demonstrated in following sections.

^{2}H (from solvent D_2O) or ^{14}N (choline) dipolar interactions with ^{13}C are ruled out as the source of the observed coupling because their interaction (if observed) would led to dipolar triplets (since I = 1 for these nuclei). Variable decoupling power experiments (data not shown) eliminated the possibility that the splittings could result from incompletely decoupled ^{1}H–^{13}C dipolar/scalar interactions. This leaves dipolar coupling of the isotopically dilute carbons to the headgroup ^{31}P as the only possible source of the splittings in Figs. 1–3. This is as expected, based on Eq. 1. The fact that such splittings have not previously been reported reflects the relatively poor resolution afforded by unoriented samples and, apparently, from mechanically oriented samples (see reference 12).

Differential line broadening (DLB). As noted, a number of the splittings in the spectra of Figs. 1–3 were observed to be asymmetric in terms of the linewidths of each component of the doublet in question. This effect is particularly pronounced in the carbonyl-2 signal from samples having relatively low detergent contents (high $S_{bilayer}$). This DLB is probably the consequence of interference effects between the ^{13}C CSA tensor (rank 2) and the phosphorus–carbon dipolar tensor (rank 1).

The interplay of dipolar and CSA tensors can result in two classes of spectral effects in NMR spectra. The first effect is simply related to the orientational dependencies of both dipolar coupling and chemical shift and can be observed as asymmetry in the Pake dipolar powder patterns from coupled spins having significant CSAs and associated with randomly dispersed molecules undergoing axially symmetric motions (13, 14). This effect is not observed in fully oriented or isotropic systems (but can play a role in spectra from *partially* oriented samples). The second type of interference phenomena is relaxation-based and is theoretically observable in the spectra of dipolar interacting spins due to cross-correlation interactions between dipolar and CSA tensors. It is theoretically observable in both solids and solution NMR spec-

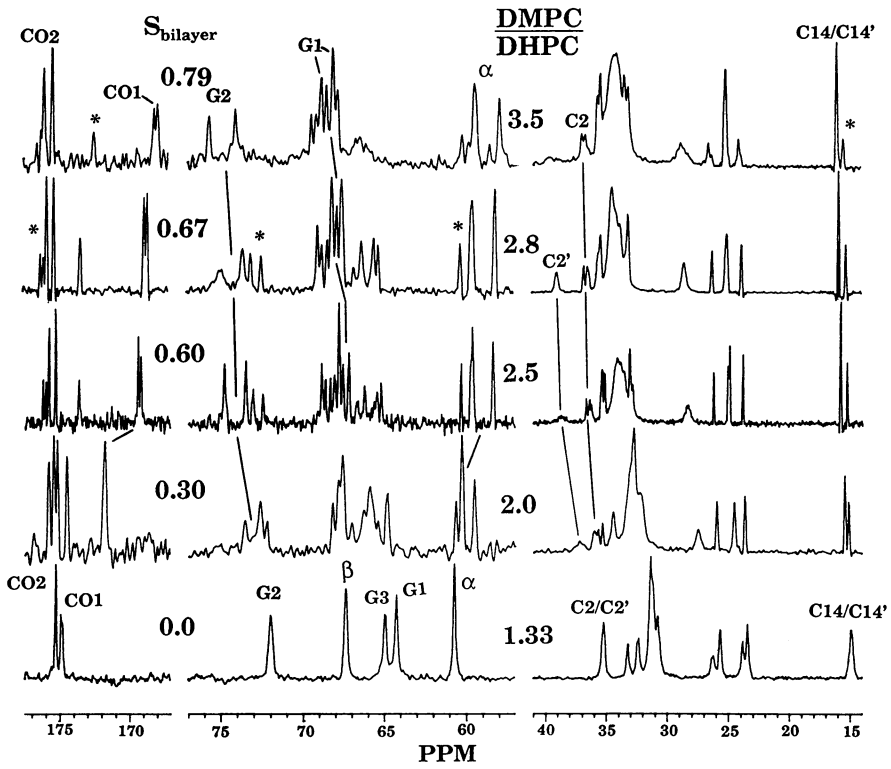

FIGURE 2 67.9 MHz ^{13}C (^1H-decoupled) spectra from titration of DMPC by DHPC at 40°. The total amphiphile (DHPC + DMPC) wt/vol content was maintained at 25% in 0.1 M KCl/D_2O. Spectra are labeled with the DMPC:DHPC molar ratio and were processed as described in the caption of Fig. 1. Select DHPC peaks are labeled with an asterisk. When the DMPC:DHPC ratio in isotropic samples was varied, it was observed that the relative integral of the 175 PPM peaks did not change, indicating that the carbonyl peaks represent CO1 and CO2, rather than DHPC (CO1 + CO2) and DMPC (CO1 + CO2). The assignment of the upfield isotropic resonance to CO1 (for both DHPC and DMPC) was based upon its relative breadth. The spectra represent 1,000–25,000 scans. The choline methyl signals from DHPC and DMPC are not shown, but were resolvable in the oriented samples. The DMPC choline methyl was always a singlet, unless very high resolution enhancement was used, in which case the peak took on a complex line shape similar to that shown in the inset of Fig. 3.

tra (15–17). The result of this second effect is that the individual components of both dipolar and scalar multiplets exhibit differing relaxation rates (and hence linewidths). Under isotropic conditions, and in the absence of scalar coupling, this effect can also be detected by non-exponential relaxation of singlets[1] (18).

Recently, Oldfield et al. (19) reported DLBs in scalar multiplets of ^{13}C (^1H-coupled) magic angle spinning (MAS) spectra of randomly dispersed membrane systems which appear to result from ^1H–^{13}C dipolar–^{13}C CSA interference. It is not totally clear which of the two phenomena described in the above paragraph is responsible because the authors imply that sample spinning may not have been sufficient to fully axially average ^{13}C CSA and/or ^{13}C–^1H dipolar interactions about 54.7° in their experiments.

[1] This may seem confusing, because dipolar *coupling* is averaged to zero under solution conditions. However, dipole–dipole interactions do not vanish and are manifested in such cross-correlation interference effects and in dipolar relaxation mechanism-related phenomena (including the Nuclear Overhauser Effect).

As noted, the DLBs observed in some of the spectra of Figs. 1–3 are reproducible and do not result from the apodization involved in producing the spectra depicted. ^1H dipolar coupling is unlikely to be involved, since decoupling was complete at the decoupling power used. However, unequivocal discernment of the exact nature of the DLB is not trivial and extends beyond the scope of this contribution. This matter is complicated by the fact there is no quantitative theoretical description of relaxation-based DLB under anisotropic conditions. Nevertheless, Oldfield's data and the observation of such effects in Figs. 1–3 are worth noting as a point of departure for future investigations because such DLBs can potentially yield important structural information through their dependence upon the relative orientations of the dipolar and CSA tensors.

Determination of $S_{bilayer}$

^{31}P spectra were acquired at most points of titrations of 25% DMPC by DHPC and CHAPSO at 40° (data not shown). From this data, $S_{bilayer}$s were calculated using Eq. 2. This approach assumes that the local orientation, dynamics, and static eigenvalues/vectors of the ^{31}P ten-

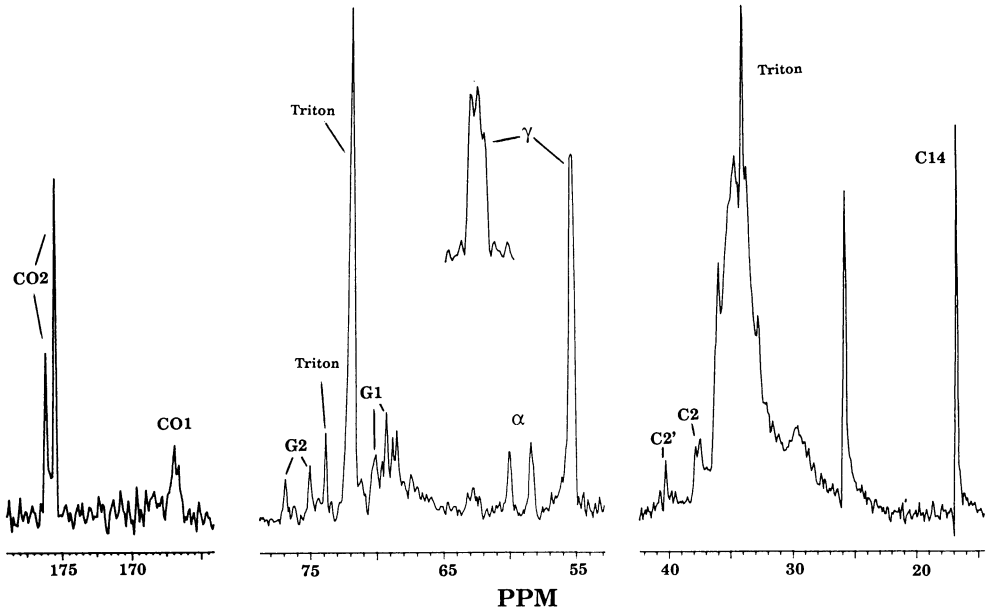

FIGURE 3 67.9 MHz ^{13}C (^{1}H-decoupled) spectrum of a 1:5 Triton X-100:DMPC sample at 25% total amphiphile in 0.1 M KCl/D_2O and 40°C. The free induction decay contained 2,048 points and was both zero-filled and Gaussian multiplied prior to Fourier transformation. This spectrum represents 27,000 scans.

sor of DMPC headgroups are not significantly perturbed by the added amphiphile. The validity of this approach has been generally confirmed by comparing scaling of the ^{31}P CSA and scaling of the quadrupolar splittings from acyl-perdeuterated DMPC for points in these titrations (7, 8). Nevertheless, this method for estimating $S_{bilayer}$ is not exact, and based on previous results it can be estimated that the uncertainty of each measured $S_{bilayer}$ is about 0.05. In the case of Triton X-100, ^{31}P and ^{2}H results led to an estimation of $S_{bilayer}$ of 0.95 ± 0.05 for the sample represented in Fig. 3.

Determination of isotropic ^{13}C chemical shifts

25% total amphiphile samples containing DHPC:DMPC at molar ratios greater than about 1:1.8 yield an isotropic spectra at 40° (bottom spectra of Fig. 2). This also appears to be the case for CHAPSO at ratios greater than 1:2.5, except that some of the resonances are very broad (see bottom spectrum of Fig. 1). The source of this spectral broadening cannot be identified with certainty, but is also observed in the ^{31}P spectrum (see reference 7). In any case, except for the presence of extra peaks from the detergents, these spectra are similar to those observed in isotropic PC vesicles or measured in unoriented dispersions using MAS methods (10, 20). Accordingly, the isotropic resonances in the spectra at the bottom of Figs. 1 and 2 can be assigned, based upon previous work. In all cases, the corresponding isotropic resonances in Figs. 1 and 2 are nearly equivalent in resonance frequency (largest observed difference of 0.3 ppm) and are very similar to those reported for pure DMPC samples (10, 20). We used the isotropic DHPC–DMPC shifts to determine $\Delta\delta$ for DHPC–DMPC mixtures and for Triton X-100–containing samples. For resonances which are narrow in the isotropic CHAPSO–DMPC mixtures, the shifts were used in determining $\Delta\delta$ from oriented CHAPSO–DMPC samples. However, in cases where the "isotropic" CHAPSO–DMPC resonances were broad, the corresponding shifts from the isotropic DHPC–DMPC mixture were employed.

CHAPSO titration of DMPC

When L_α-DMPC is titrated with CHAPSO at 25% total amphiphile, substantial magnetic orientation begins to occur near CHAPSO:DMPC ratios of 1:7 ($S_{bilayer}$ of ca. 0.9) and persists until the isotropic limit is reached below ratios of 1:2.7 as shown in Fig. 1. (Other points for which spectra are not shown are represented in Fig. 5). It can be observed that the peaks narrow considerably as the CHAPSO:DMPC ratio is lowered. This is primarily due to the elimination of residual sample disorientation.

In some cases, it is a trivial matter to follow changes in resonance position throughout the titrations, while in other cases it is difficult because of spectral overlap and large changes in chemical shifts. The variations in chemical shifts and in dipolar couplings for carbons CO1, CO2, G2, α, γ, and the degenerate C14/C14′ can be followed in a straightforward manner. No attempt is made herein to sort out the C3–C13 region. In the isotropic spectrum the acyl C2 and C2′ are degenerate. However, in oriented samples the two carbons become spectroscopically distinct. One of them (C2) undergoes only

small changes in chemical shift, but exhibits significant dipolar coupling. The other (C2′) undergoes a large upfield shift and is quite broad. Whether this broadening is due to unresolved coupling or to some other effect cannot be ascertained from the data. The C2/C2′ resonances can be tentatively assigned. The static tensors of these methylenes should have approximately the same orientation in their molecular frames. It is well known (21, 22) that the *sn*-2 C2 methylene of lamellar PC exhibits a very different average orientation with respect to the bilayer motional director than the bulk of the methylenes along the acyl chains (including the *sn*-1 C2). The *sn*-1 acyl C2 shares approximately the same averaged orientation as most of the other acyl carbons. The bulk acyl chain envelope clearly undergoes a large upfield shift from its isotropic range upon sample orientation (by about 2.2 ppm in the 1:3.5 sample). Because C2′ undergoes a similar upfield shift (3.0 ppm in the 1:3.5 sample) while C2 shifts by only 1.2 ppm, C2′ can be tentatively assigned to the *sn*-1 chain. This conclusion is supported by DHPC titration data (below) where the main acyl chain envelope and C2′ peak in the 1:2.5 spectrum are observed to shift +2.6 and +3.4 ppm, respectively, while C2 shifts only 1.1 ppm.

In order to assign the oriented β, G1, and G3 resonances, the 1:3.5 CHAPSO:DMPC spectrum taken at 67.9 MHz was compared with a spectrum of a sample having a similar $S_{bilayer}$ taken at 125.8 MHz (Fig. 4). By comparing the two spectra it was possible to tentatively ascertain which resonance pairs represent dipolar doublets. One doublet was found as having a large coupling constant (125 Hz). The size of this doublet and the constraint that the averaged term of Eq. 1 cannot exceed $1.0/\langle r^{-3}\rangle$ dictates that this doublet must be assigned as G3 (the *sn*-3 carbon). One of the other two doublets can be followed rather easily throughout the titration and can be observed to converge upon the G1/G3 isotropic shift range. This doublet can be assigned to G1. The remaining doublet must be β. The β and G3 carbons are not readily identified at other non-isotropic points in the titration (for which no 125.8 MHz data is available) such that their assignment is made only for the 1:3.5 sample and should be regarded as tentative.

Both chemical shifts and dipolar couplings can be observed to converge upon their isotropic values (0 for dipolar coupling) as CHAPSO content is increased. As described in the Theory section, if this scaling results only from a decrease in overall system order, both $\Delta\delta$ and $\Delta\nu$ should scale linearly toward 0 as a function of $S_{bilayer}$. Dipolar couplings and CSA are plotted in Fig. 5 as a function of $S_{bilayer}$. It should be noted that most spectra were processed a number of times with different degrees of resolution enhancement. Thus, couplings, which are not evident in the spectra explicitly shown in Fig. 1, were sometimes observable and measurable at other levels of

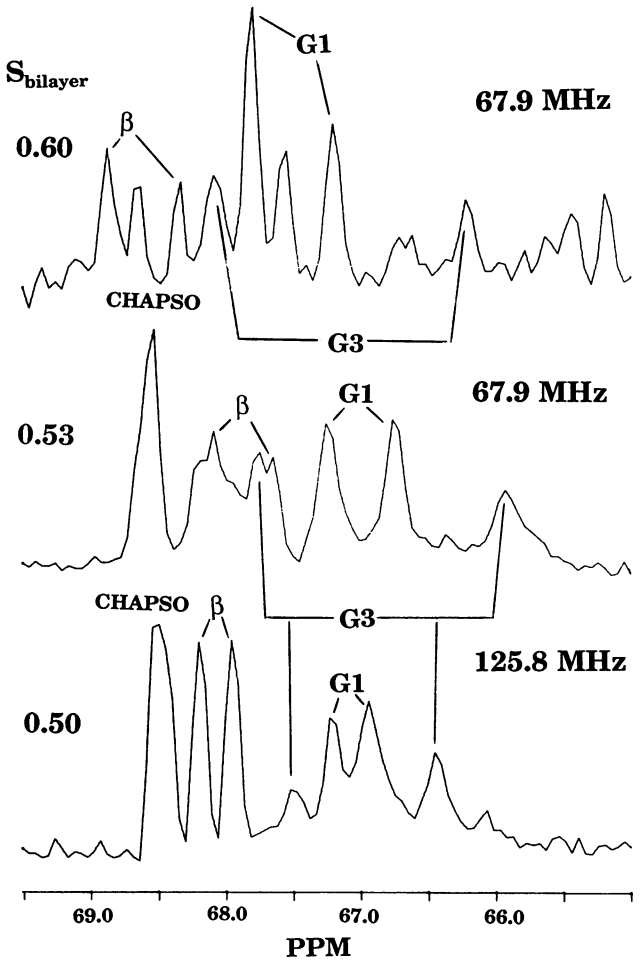

FIGURE 4 ^{13}C (^1H-decoupled) G1/G3/β spectral region from oriented DMPC samples at 40° having similar $S_{bilayer}$s at differing magnetic fields (see *labels*). *Top:* 1:2.5 DHPC:DMPC, 25% total amphiphile. *Middle:* 1:3.5 CHAPSO:DMPC, 25% total amphiphile. *Bottom:* 1:3.0 CHAPSO:DMPC, 30% total amphiphile. All three spectra were produced following zero-filling, Gaussian multiplication, and Fourier transformation of 4,096 point free induction decays. The sample represented by the bottom spectrum also had a small amount of β-dodecyl 6-deoxyglucopyranoside present (glycoside:DMPC molar ratio = 1:17) which did not appear to perturb the ^{13}C spectrum of the DMPC matrix. The assignments should be regarded as tentative.

enhancement. Error bars have not been drawn in Fig. 5, but are typically ±5 Hz for the coupling data and ±0.3 ppm for CSA. Additional data for peaks which underwent little change in $\Delta\delta$ during the titrations are summarized in Table 1. It can be observed in Fig. 5 that average lines can be drawn having an origin at (0,0) which fit virtually all points within experimental error bounds. This data suggests that perturbation of the averaged structure of the glycero/headgroup regions of DMPC within CHAPSO–DMPC aggregates is minimal. This result also indicates that linear extrapolation of the average line through each data set (and through 0,0) can provide a reasonable approximation of the splitting in CHAPSO-free DMPC bilayers. These values are given in Table 1.

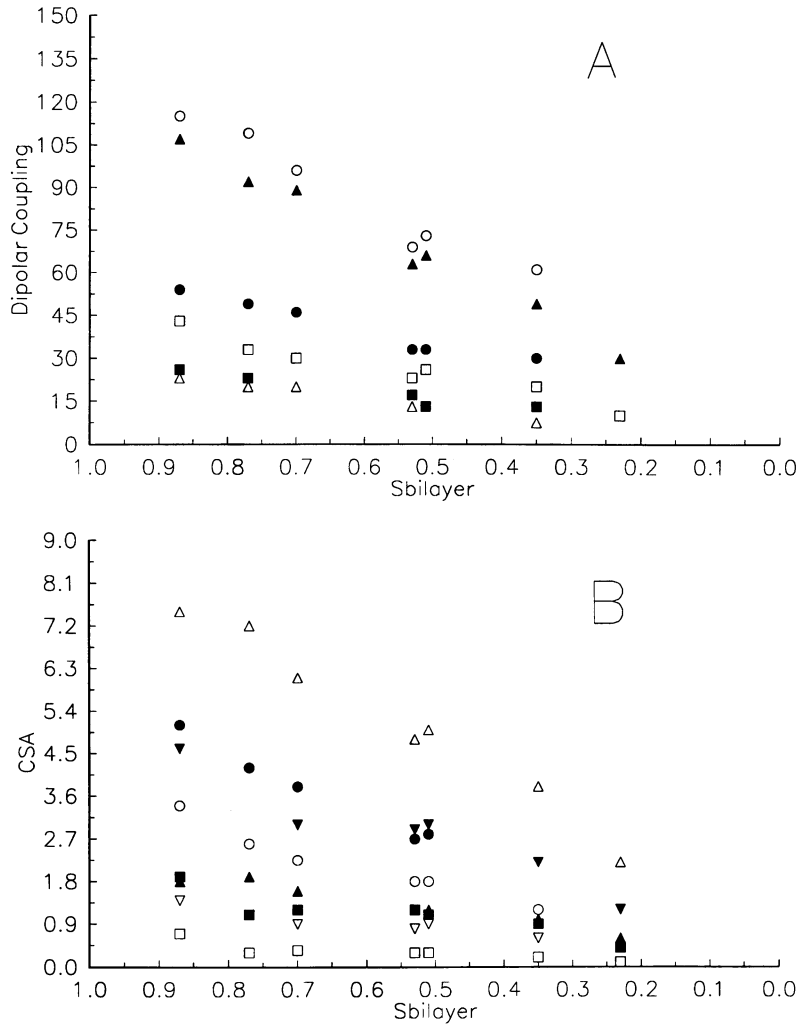

FIGURE 5 Dependence of dipolar coupling (*A*) and $\Delta\delta$ (*B*) upon overall bilayer order in the 40° titrations of DMPC by CHAPSO. Open circle: G2, closed circle: G1, open square: CO2, closed square: C2, open triangle: CO1, closed triangle: α, open inverted triangle: C14, closed inverted triangle: C2′. Absolute values of the data are plotted.

The signs of the dipolar couplings are not known. It should be noted that the fact that only two measurements were made for the β and C3 carbons, (at S_{bilayer} = 0.53 and 0.51) dictates that estimated parameters for these peaks have substantial uncertainty.

The validity of the above approach can be probed by repeating titrations using DHPC instead of CHAPSO and comparing results between titrations involving the two detergents as shown below.

DHPC titration of DMPC

Spectra from titrations of DMPC by DHPC are illustrated in Fig. 2; additional points not shown are represented in Fig. 6. It can be observed that the features of these spectra are similar to those of the CHAPSO series, except that all DMPC peaks are now "mirrored" by DHPC peaks. As described elsewhere (8), the DHPC peaks generally exhibit couplings and CSAs which are scaled somewhat towards their isotropic extremes relative to their DMPC counterparts. Despite some increased spectral cluttering, most DMPC peaks can still be identified and followed throughout the titrations. In cases of uncertainty, assignments could sometimes be made by comparing CHAPSO–DMPC spectra from samples having similar S_{bilayers}. The 125.8 MHz S_{bilayer} = 0.5 CHAPSO–DMPC spectrum shown in Fig. 4 was again of assistance in identifying DMPC β, G1, and G3 doublets (see previous section).

The splittings and CSA from this titration are illustrated in Fig. 6. Experimental uncertainty is the same as for the CHAPSO titrations. It is evident that each data set can be fit, within experimental error, by a straight line having an origin at 0,0. Thus, the perturbation of the averaged structures of the glycerol/headgroup region of DMPC molecules by DHPC in aggregates of the two am-

TABLE 1 **Direct measurement of ^{13}C chemical shift anisotropies and ^{31}P–^{13}C dipolar couplings in DMPC**

	Triton X-100		CHAPSO		DHPC		Estimate for DMPC‡		Cornell	25% DMPC at 40° §	
Carbon	D_{C-P}	$\Delta\delta$	D_{C-P}	$\Delta\delta$	D_{C-P}	$\Delta\delta$	D_{C-P}	$\Delta\delta$	$\Delta\delta$	D_{C-P}	$\Delta\delta$
CO1	21 ± 6	−8.4 ± 1.0	26 ± 3	−9.2 ± 1.0	23 ± 4	−9.2 ± 1.0	23 ± 4	−9.0 ± 1.0	−9.0 ± 1	ND‖	−8.5 ± 1.0
CO2	47 ± 6	1.0 ± 0.6	50 ± 8	0.6 ± 0.3	53 ± 5	0.6 ± 0.5	50 ± 8	0.7 ± 0.5	0.5 ± 1	55 ± 20	0.9 ± 0.5
Glyc-2	132 ± 12	4.1 ± 0.6	142 ± 20	3.6 ± 0.4	148 ± 20	3.6 ± 0.4	141 ± 20	3.8 ± 0.5	3.8 ± 1	130 ± 25	3.2 ± 0.7
head-α	118 ± 12	−1.6 ± 0.6	130 ± 15	−2.4 ± 0.4	144 ± 20	−3.0 ± 0.5	131 ± 20	−2.3 ± 1	−2.9 ± 1	130 ± 25	−2.4 ± 0.7
Glyc-1	56 ± 15	5.6 ± 0.8	67 ± 12	5.4 ± 0.5	64 ± 8	5.4 ± 0.8	62 ± 15	5.5 ± 0.7	3.7 ± 1	ND	ND
Glyc-3	ND	ND	230 ± 30	4.0 ± 1.2	208 ± 30	3.9 ± 2.0	219 ± 30	4.0 ± 1.5	3.7 ± 1	ND	ND
head-β	ND	ND	57 ± 15	1.1 ± 1.0	60 ± 15	2.1 ± 2.0	58 ± 15	1.6 ± 1.5	1.4 ± 1	ND	ND
C2	28 ± 6	2.4 ± 0.8	31 ± 7	2.0 ± 0.6	28 ± 4	1.9 ± 0.5	29 ± 8	2.1 ± 0.6	ND	ND	ND
C2′	ND	5.3 ± 1.5	ND	5.3 ± 1.1	ND	5.5 ± 0.8	ND	5.4 ± 0.8	ND	ND	4.8 ± 1.0
head-γ	<15	0.0	<15	−0.8 ± 0.4	<15	−0.8 ± 0.5	<15	−0.5 ± 0.5	0.5 ± 1.0	ND	0.0 ± 0.5
C14	ND	1.8 ± 0.5	0.0	1.5 ± 0.4	0.0	1.3 ± 0.3	0.0	1.5 ± 0.5	1.0 ± 1.0	0.0	1.4 ± 0.5

Intrinsic 40° DMPC ^{31}P–^{13}C dipolar couplings* (Hz) and ^{13}CSAs (PPM) derived from titrations of DMPC with the three detergents, from Braach-Maksvytis and Cornell (12), and directly from DMPC (this study). *The signs of the dipolar couplings are known. ‡The final estimates for DMPC are the average of the results from the three detergents. §See text and Figure 7. ‖ND: not determined.

phiphiles appears to be fairly minor, validating extrapolation of data to pure DMPC ($S_{bilayer}$ = 1.0) as shown in Table 1.

Comparison of the coupling and CSA parameters for detergent-free DMPC bilayers estimated from both DHPC and CHAPSO titrations shows excellent agreement (Table 1).

Triton–DMPC mixture

In order to provide an additional test of the validity of our determination of "pure DMPC" spectral parameters, oriented Triton X-100–DMPC mixtures were also examined. The spectrum of an oriented 1:5 Triton:DMPC mixture is illustrated in Fig. 3. CSA and dipolar couplings which can be measured with certainty are listed in Table 1 and have been corrected for the $S_{bilayer}$ (0.95) determined as described in a previous section for this mixture. It can be observed that the splittings and dipolar couplings estimated for pure DMPC from the Triton experiments agree reasonably well with the estimates made from CHAPSO and DHPC titrations.

One unusual feature of the Triton X-100–DMPC spectrum shown in Fig. 3 is the γ-methyl resonance. At a high level of resolution-enhancement (see inset) it appears to be split more than once and is asymmetric. A similar pattern can be observed in the γ resonances from DHPC and DMPC titrations when spectra are processed with a higher degree of resolution enhancement than used to produce the spectra in Figs. 1 and 2 (not shown). The complexity of this resonance may arise from ^{13}C–^{14}N scalar and/or dipolar interactions superimposed upon a small ^{31}P–^{13}C dipolar coupling, though this is difficult to establish with certainty. In any case, it appears that the γ carbon–phosphorus dipolar interaction must be very small (<15 Hz).

The estimates for the intrinsic spectral parameters of pure DMPC at 40° from Triton, DHPC, and DMPC experiments can be averaged and assigned uncertainties as shown in Table 1.

Comparison of detergent-derived results with data obtained from detergent-free DMPC

The results of the detergent titration experiment were used as guides in the reinterpretation of the ^{13}C spectrum from unoriented DMPC (Fig. 7). While it is unorthodox to apply resolution enhancement techniques to powder pattern spectra, such apodization can be employed to help locate the most intense (90° director orientation) component of axially symmetric powder patterns. In this manner, the $\Delta\delta$ of C2′, C14, G2, α, CO2, and CO1 resonances could be roughly measured along with dipolar couplings for CO2, α, and G2 (Table 1). It can be observed that both couplings and CSA agree with the results from the detergent titrations, within the substantial experimental uncertainty. $\Delta\delta$ for CO1 and CO2 have been previously observed to be −10.7 and 1.0 for CO1 and CO2, respectively, in labeled DMPC dispersions at 30° (23). These values are in reasonable agreement with those determined in this study as shown in Table 1. The small difference between current and previous results for CO1 is probably due both to the 10° difference in temperatures used in the two studies and to the inclusion of 100 mM KCl in the samples of this work. $\Delta\delta$s for glass-plate oriented egg yolk PC in an L_α-like state at 20° in the absence of salt have been measured by Cornell and his co-workers (12). Their results are listed in Table 1 and display excellent agreement with the results of this study, despite differences in sample contents and conditions. It should be noted that while *oriented* samples were used in both the previous and the present studies, the resolution of the spectra from magnetically oriented samples ap-

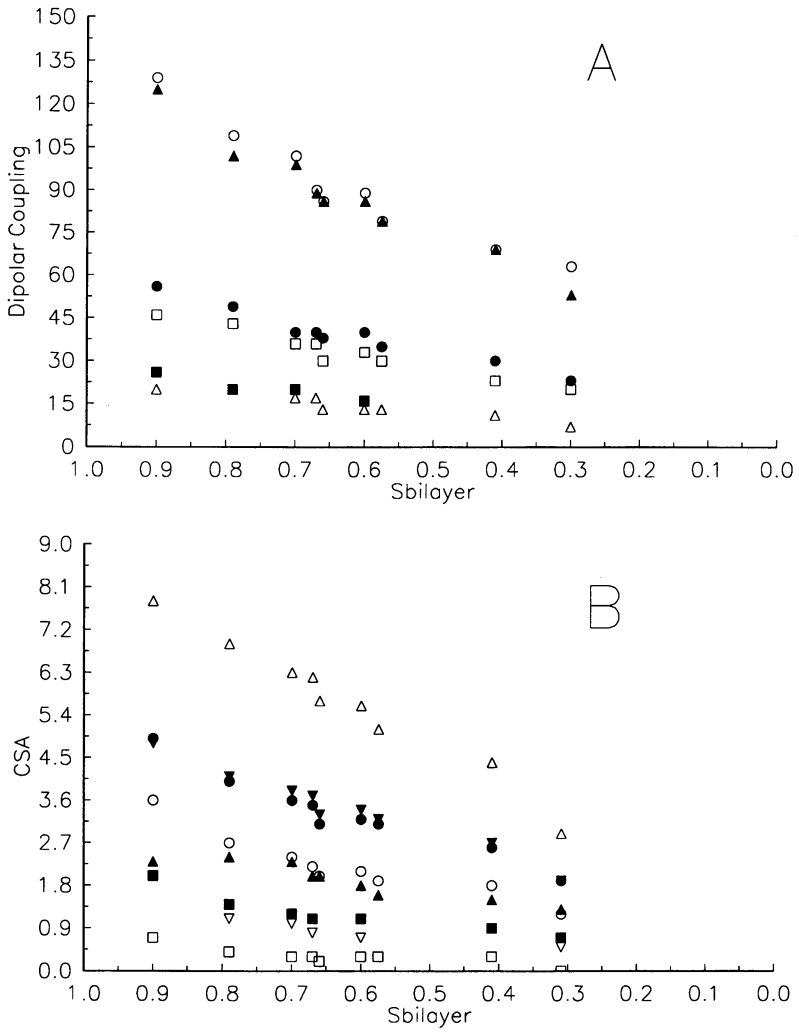

FIGURE 6 Dependence of dipolar coupling (*A*) and the anisotropic contribution to chemical shift (*B*) upon overall bilayer order in the 40° titrations of DMPC by DHPC. Open circle: G2, closed circle: G1, open square: CO2, closed square: C2, open triangle: CO1, closed triangle: α, open inverted triangle: C14, closed inverted triangle: C2′. Absolute values of the data are plotted.

pears to be much higher than that from glass-plate oriented samples.

DISCUSSION

The titration-based methodology presented in this paper led to a considerable body of CSA and dipolar coupling data for DMPC. While the uncertainties in the parameters thus determined are significant, it can be argued that a large volume of data of moderate accuracy is sometimes preferable to a few very precise measurements. This is particularly the case when experimental data is used in conjunction with computational techniques such as molecular dynamics, rather than being solely relied upon to provide an analytical solution of structure.

The success of this method is based upon the fact that despite the presence of substantial quantities of three different detergents, DMPC appears to maintain a remarkably constant averaged structure. A simple analysis of Eq. 1 demonstrates that even small perturbations of the average P–C internuclear distance or vector orientation with respect to the bilayer normal can produce dramatic changes in splitting. The agreement of parameters obtained from each of the three detergent titrations and from pure DMPC, as well as the linearity of the data from DHPC and CHAPSO titrations, indicate that any perturbations in local structure are minor. The molecular origins of this structural resiliency are difficult to assess, since the nature of the detergent-DMPC aggregates is not exactly known (7, 8).

Extension of this titration-based method to other problems of assignment and measurement can be envisioned along two primary pathways. First, extension can be made to the study of dilute additives to DMPC bilayers. Barring sensitivity problems, such studies may often be fairly straightforward, especially since most de-

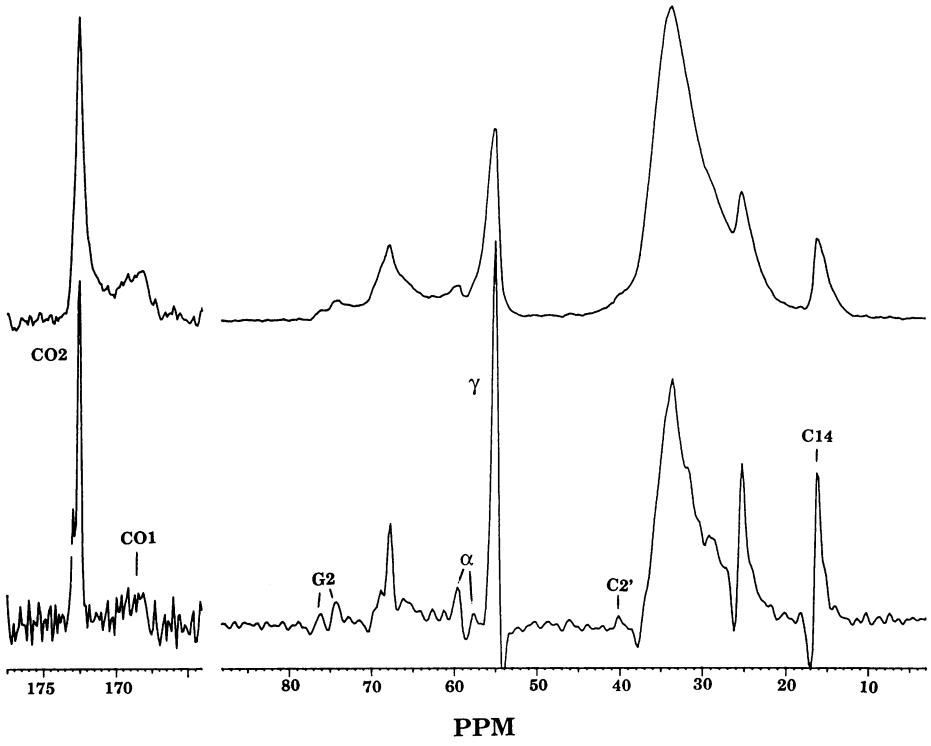

FIGURE 7 ^{13}C (^{1}H-decoupled) spectra of a dispersion of DMPC (25% by wt) at 40° in 0.1 M KCl. These spectra represent 40,000 scans and were produced following Gaussian multiplication (*bottom*) or exponential multiplication (20 Hz line broadening) of the 2,048 data points following zero-filling.

tergent and DMPC peaks can be identified in advance from the spectra in Figs. 1–3.

A second potential extension of the method would be to bilayer matrices composed predominately of lipids other than DMPC. However, this will first require the development of detergent-based, magnetically orientable bilayers composed of other lipids such as phosphatidylethanolamine. Whether this is possible remains to be seen.

Finally, it should be noted that the data set generated in this experiment may be of use in future modeling of the highly complex conformational dynamics of L_α-DMPC. Dipolar coupling data is especially useful because it is effectively a vectorial interaction and, unlike CSA, has a static magnitude which is defined by invariant nuclear properties rather than by a chemically-based (and perturbable) static tensor. However, even CSA data is becoming more usable in structural analyses due to progress in the determination of static CSA tensors in biomolecules and suitable model compounds (24, 25).

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