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Solubilization and Localization of Weakly Polar Lipids in Unsonicated Egg Phosphatidylcholine: A ^{13}C MAS NMR Study†

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ABSTRACT: The weakly polar lipids cholesteryl ester, triacylglycerol, and diacylglycerol incorporate to a limited extent into the lamellar structure of small unilamellar vesicles. The localization of the carbonyl group(s) at the aqueous interface was detected by [^{13}C]carbonyl chemical shift changes relative to the neat unhydrated lipid [Hamilton, J. A., & Small, D. M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6878-6882; Hamilton, J. A., & Small, D. M. (1982) *J. Biol. Chem. 257*, 7318-7321; Hamilton, J. A., Bhamidipati, S. B., Kodali, D. R., & Small, D. M. (1991) *J. Biol. Chem. 266*, 1177-1186]. This study uses ^{13}C NMR to investigate the interactions of these lipids with unsonicated (multilamellar) phosphatidylcholine, a model system for cellular membranes and surfaces of emulsion particles with low curvature. Magic angle spinning reduced the broad lines of the unsonicated dispersions to narrow lines comparable to those from sonicated dispersions. [^{13}C]Carbonyl chemical shifts revealed incorporation of the three lipids into the lamellar structure of the unsonicated phospholipids and a partial hydration of the carbonyl groups similar to that observed in small vesicles. Other properties of interfacial weakly polar lipids in multilayers were similar to those in small unilamellar bilayers. There is thus a general tendency of weakly polar lipids to incorporate at least to a small extent into the lamellar structure of phospholipids and take on interfacial properties that are distinct from their bulk-phase properties. This pool of surface-located lipid is likely to be directly involved in enzymatic transformations and protein-mediated transport. The ^{13}C magic angle spinning NMR method may be generally useful for determining the orientation of molecules in model membranes.

Cholesteryl esters (CE),[1] diacylglycerols (DAG), and triacylglycerols (TAG) are weakly polar water-insoluble lipids that form stable monolayers at an air–water interface but do not swell in water to form lamellar structures (Small, 1986). The esterified form of cholesterol is a key chemical component involved in the transport and metabolism of cholesterol. A structural derivative that decreases the polarity of unesterified cholesterol, CE can be sequestered into the core of plasma

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[1] Abbreviations: CE, cholesteryl ester; DAG, diacylglycerol; TAG, triacylglycerol; MAS, magic angle spinning; PC, phosphatidylcholine; TO, triolein; CO, cholesteryl oleate; DPG, dipalmitoyl-*sn*-glycerol; NOE, nuclear Overhauser enhancement; T_1, spin-lattice relaxation time.

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lipoproteins or into cellular lipid droplets to enhance the plasma or cellular solubility of cholesterol. The two forms of cholesterol are readily interconverted by enzymes acting in the plasma or within the cell, e.g., cholesterol acyltransferases and cholesterol esterases (Brown & Goldstein, 1986; Jones & Glomset, 1985). In human plasma CE and TAG are transferred between lipoprotein particles by the "cholesteryl ester transfer protein" (CETP) in a process that may facilitate transport of CE to the liver (Tollefson & Albers, 1986). Long-chain TAG serve as the main storage form of fatty acids in most animals and plants. Because of their low polarity, TAG (like CE) partition into oil phases consisting primarily of TAG and/or CE. During their metabolism and transport, TAG undergo enzymatic hydrolysis to release the constituent fatty acids, and the lipolytic enzymes must gain access to this water-insoluble substrate. DAG are produced at low levels by phospholipase C action on phospholipids in various types of cells in response to extracellular stimuli. Although they are normally minor lipid constituents in cells, DAG play key roles in several biological processes. Perhaps the most important role is their involvement as a second messenger in cellular signal transduction via activation of protein kinase C (Nishizuka, 1984; Berridge & Irvine, 1984). DAG are also important intermediates in TAG metabolism and will be present, at least as transient species, in cells containing TAG and in TAG-rich plasma lipoproteins. Hydrolytic enzymes such as pancreatic lipase, lipoprotein lipase, and hepatic lipase hydrolyze TAG at the primary carbonyl, producing 1,2- or 2,3-diacylglycerol (Quinn, 1982). These species are in turn metabolized to 2-monoacylglycerol, presumably before acyl-chain migration of the DAG occurs.

Thus, key events (enzymatic transformations, protein-mediated transport, and enzymatic activation) in the metabolic cycles of these weakly polar lipids involve protein interactions and most likely occur at interfaces rather than in oil phases. There is mounting evidence from polarizing light microscopy studies (Janiak et al., 1974), surface monolayer studies (Smaby & Brockman, 1987), and ^{13}C (Hamilton & Small, 1981, 1982) and ^{2}H NMR studies (Gorrissen et al., 1980) that even the most apolar of these lipids, CE, is not completely insoluble in phospholipids but incorporates to a small extent into the phospholipid lamellar structure because of the ability of the carbonyl group to interact with water at the aqueous interface. ^{13}C NMR is a direct method of assessing the localization of carbonyl-containing weakly polar lipids in bilayers because the hydration of carbonyl group(s) produces measurable chemical shift changes (Maciel & Netterstad, 1965; Schmidt et al., 1977). To date, ^{13}C NMR studies have been limited primarily to small unilamellar vesicles, which yield high-resolution spectra with conventional FT NMR methods (Hamilton & Small, 1981, 1982; Hamilton et al., 1983; Hamilton, 1989; Deckelbaum et al., 1989). While vesicles provide appropriate models for the surface monolayer of small lipoproteins and for highly curved regions of biomembranes, they may be inappropriate models for systems with low curvature. Unsonicated phospholipids, which consist of multibilayers of low curvature, may be better models for membranes and surfaces of large emulsion particles. However, these dispersions produce very poorly resolved ^{13}C spectra with high-resolution methods (Oldfield et al., 1987). It is important to compare the interfacial properties of weakly polar lipids in these two different model lamellar systems, and it would be highly desirable to employ the same, or very similar, methodology to make this comparison. In this study we use ^{13}C magic angle spinning (MAS) NMR to obtain high-resolution spectra of multilamellar systems (Haberkorn et al., 1978; Sefcik et al., 1983; Oldfield et al., 1987), making it possible to determine properties of lipids incorporated into the phospholipid lamellar phase.

Experimental Procedures

Materials. Egg phosphatidylcholine (PC) was obtained from Avanti Polar Lipids (Birmingham, AL) and used without further purification. Triolein (TO) with 99% ^{13}C enrichment in the three carbonyl carbons was purchased from Cambridge Isotopes Laboratory (Woburn, MA). Cholesteryl oleate (CO) with 90% ^{13}C enrichment in the carbonyl carbon was synthesized from the [^{13}C]carboxyl oleic acid by condensation with cholesterol (Sripada, 1988). 1,2-Dipalmitoyl-*sn*-glycerol (DPG) with 90% [^{13}C]carbonyl enrichment was prepared from the corresponding carbonyl-enriched phospholipid (PC) by treating with phospholipase C (*Bacillus cereus*; Sigma Chemical Co., St. Louis, MO) in moist ether medium (Mavis, 1972). Anhydrous ether (10 mL) was added to 10 mg of ^{13}C-labeled (*sn*-2) 1,2-dipalmitoyl-*sn*-glycerophosphocholine in 0.5 mL of methanol. To this lipid solution was added 50 units of phospholipase C in phosphate buffer (0.05 M NaH_2PO_4, pH 7.1), and the reaction mixture was constantly agitated for 4 h. After the organic layer containing the DAG was separated, the aqueous layer was washed with 3 mL of ether. The combined layers were evaporated on a rotary evaporator at 20 °C. Trace amounts of water remaining in the sample were removed by adding dry benzene and evaporating to dryness. DAG thus prepared was analyzed by TLC to assure that there was no contaminating 1,3-isomer (see below) and stored in chloroform solution at −20 °C until use. 1,2-DPG with ^{13}C enrichment in both carbonyls was obtained from the corresponding "double-labeled" 1,2-dipalmitoyl-*sn*-glycerophosphocholine. Isotopic labeling purity of all lipids was verified by ^{13}C NMR of C^2HCl_3 solutions.

Sample Preparation. Samples were prepared by mixing the appropriate amounts of lipids in organic solvent, followed by drying under N_2 and then under vacuum. A total of 200–250 mL of D_2O or buffered D_2O (10–100 mM K_2HPO_4, pH 7.4) was added to 200 mg of total lipid with vortexing at room temperature until the lipids were dispersed from the glass walls (0.5–1.0 h). Samples were stored at 4 °C prior to NMR analysis. Selected samples were analyzed chemically to determine composition and to check for breakdown products. Freshly prepared samples were milky with no visible yellow color, and there was very little or no change in color following NMR analysis. Phospholipid concentration was determined by the method of Bartlett (1959); cholesteryl ester was determined by measurement of cholesterol (Rudel & Morris, 1973); triacylglycerol was measured by the Sigma method (kit 355-UV) of enzymatic conversion to glycerol. The isomeric purity of diacylglycerol was determined by TLC analysis on silica gel G plates (without boric acid soaking) with 96% $CHCl_3$/4% acetone (Thomas et al., 1965). Thin-layer chromatography showed no free fatty acid or lysophospholipid in sample preparations. Sample compositions are given as weight percent of the total lipid, unless noted otherwise.

Electron Microscopy. Selected samples were examined by electron microscopy with a Hitachi 11-C electron microscope. EM samples were prepared by dilution of NMR samples with buffer to ~1 mg/mL lipid. This lipid solution was applied to 400-mesh copper grids covered with carbon-coated Formvar stained with 1% sodium phosphotungstate solution at pH 7.5 and dried in air.

NMR Spectroscopy. ^{13}C NMR spectra were obtained under standard "high-resolution" conditions, without MAS,

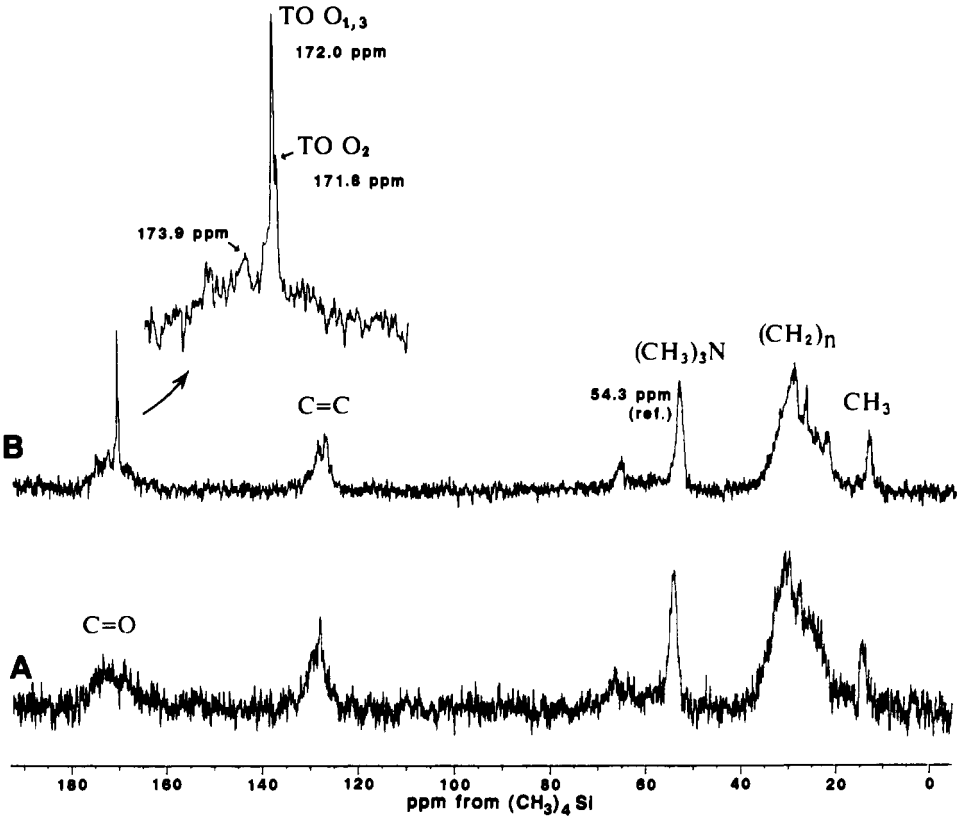

FIGURE 1: ^{13}C NMR spectra at 50.3 MHz of (A) 2.0 wt % and (B) 4.0 wt % [^{13}C]carbonyl TO and 98.0–96.0 wt % egg PC (unsonicated) in 50 wt % D_2O at 30 °C after 1000 and 2000 scans (spectra A and B, respectively) with a 2.0-s pulse interval. The same samples were used for MAS spectral analysis (Figure 2). The inset in spectrum B shows the carbonyl region with narrow peaks at ~172.0 and 171.8 ppm corresponding to oil-phase [^{13}C]carbonyl TO (TO $O_{1,3}$ and TO O_2; *sn*-1,3 and *sn*-2 carbonyls, respectively). Chemical shifts are referenced to the choline methyl peak [$(CH_3)_3N$] and are only approximately (±0.2 ppm). The carbonyl region of spectrum B contained an identifiable component at 173.9 ppm that is probably not a real peak. Other peaks designated correspond to phospholipid carbons at natural abundance: CH_3, terminal fatty acyl methyl; $(CH_2)_n$, various methylene carbons of the acyl chains; and C=C, olefinic carbons.

on a Bruker WP200 spectrometer (4.7 T) at 50.3 MHz. Low-power (~1 W) broad-band ^1H decoupling centered at 3.4 ppm downfield from tetramethylsilane was continuously applied while data were acquired. Other conditions were as before (Hamilton & Small, 1981) and as described in figure captions. MAS NMR spectra were obtained on a Bruker AM-300 instrument (7.05 T) equipped with a Bruker Solids Accessory Unit and a multinuclear magic angle probe, with spinning rates of ~2.5 kHz unless noted otherwise. ^{13}C spectra were independent of spinning rate (±500 Hz). To prevent seepage of the sample during spinning, the cap was glued to the sample rotor. High-power (~80 W) ^1H decoupling was applied during data acquisition (typically 0.27 s) only, followed by a waiting period of at least 20 times the acquisition time to dissipate decoupler heating. This gated decoupling cycle removes the NOE (Opella et al., 1976). ^{31}P NMR spectra were obtained at 7.05 T in the magic angle probe, with or without sample spinning. ^{13}C spin–lattice relaxation times (T_1) were measured by the fast inversion–recovery method (Canet et al., 1975) with magic angle spinning and high-power ^1H decoupling as above. The estimated uncertainty in carbonyl T_1 values is ±10% – 20%, on the basis of the signal-to-noise ratio for the fully relaxed carbonyl peak. Chemical shifts were measured in ^{13}C MAS spectra with the terminal fatty acyl methyl at 14.10 ppm as an internal reference (Hamilton & Small, 1981) and were reproducible to better than ±0.1 ppm.

Results

Without magic angle spinning, samples of unsonicated phospholipids with small amounts of weakly polar lipids produced broad-line spectra characteristic of unsonicated phospholipids without an additional component (Oldfield et al., 1987). Figure 1 shows ^{13}C spectra for dispersions containing 2% and 4% [^{13}C]carbonyl triolein in egg PC. The spectrum of a sample with 2% TO (Figure 1A) contains no narrow signals, while the spectrum of the 4% TO sample (Figure 1B) shows narrow carbonyl signals at ~171.8 and 172.0 ppm. These isotropic signals arise from triolein in an oil phase (Hamilton & Small, 1982). Since there is no isotropic signal from the 2% sample, these spectra suggest that there is also a less mobile and/or more anisotropic pool of TO for which the carbonyl signals are severely broadened. However, no carbonyl chemical shift information can be extracted from the broad carbonyl envelope in spectrum A.

A dramatically different spectrum (Figure 2) was obtained with magic angle spinning, with overall detail and resolution comparable to that for small unilamellar vesicles (Hamilton & Small, 1981; Oldfield et al., 1987). The improved resolution is the result of line narrowing from MAS and high-power decoupling; for example, line widths of 6–7 Hz are observed for the terminal fatty acyl methyl peak and the PC carbonyl peak (Figure 2). The phospholipid carbonyl carbons yield a single narrow resonance corresponding to the *sn*-1 and *sn*-2 carbonyls (expanded region). In contrast to highly curved small vesicles (Schmidt et al., 1977), there is no chemical shift difference between inner and outer leaflet phospholipids, a consequence of the low curvature of multilamellar PC.

The carbonyl region of the multilamellar dispersion with 4% TO (Figure 2, main spectrum) is quite similar to that for small vesicles with 4% TO (Hamilton & Small, 1981). There are two peaks for oil-phase TO [*sn*-1,3 ($O_{1,3}$) and *sn*-2 (O_2) carbonyl carbons] at chemical shifts nearly identical with those

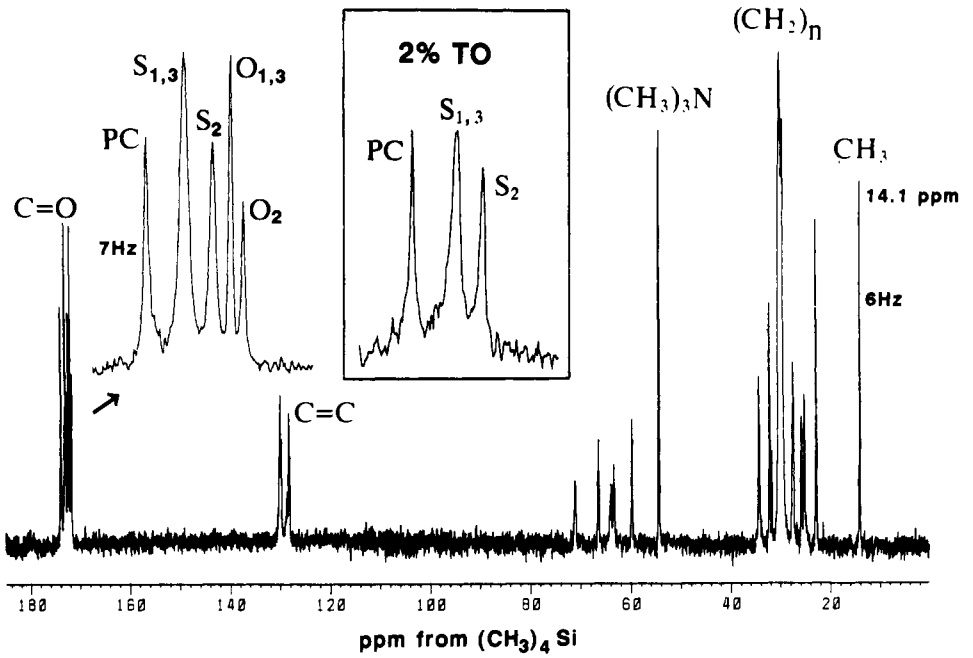

FIGURE 2: ^{13}C MAS NMR spectrum at 74.5 MHz of 4.0 wt % [^{13}C]carbonyl TO and 96 wt % egg PC (unsonicated) in 50 wt % D_2O at 30 °C (same sample as in Figure 1B). The spectrum was obtained after 320 accumulations over 8K time domain points with a 6.3-s pulse interval and a 200 ppm spectral width. Processing included exponential multiplication with 1.5-Hz line broadening. The boxed inset shows the ^{13}C MAS NMR carbonyl region of the sample with 2 wt % TO corresponding to the sample in Figure 1A. Peaks are labeled $S_{1,3}$ and S_2 for surface-phase TO and $O_{1,3}$ and O_2 for oil-phase TO (*sn*-1,3 and *sn*-2 carbonyls, respectively). There is a single carbonyl (designated PC) for the phospholipid *sn*-1 and *sn*-2 carbonyl carbons.

Table I: [^{13}C]Carbonyl NMR Measurements

	chemical shift (ppm)		line width (Hz)		T_1 (s)	
lipid	sonicated vesicles	unsonicated dispersions	vesicles	unsonicated dispersions	vesicles	unsonicated dispersions
PC[a]	173.8[b-d]	173.8	10[e]	6	2.3[c]	2.5[g]
	173.6[b-d]				2.0[c]	
CO_s	172.0[b]	171.8	8–12[b,f]	8[g]	1.3[b]	2.4[g]
$TO_{s1,3}$	173.1[c]	172.8	6[c]	13[h]	2.2[c]	2.7[h]
TO_{s2}	172.4[c]	172.2	7[c]	9[h]	1.9[c]	2.1[h]
1,2-DPG	173.3[d]	173.45	7[d]	10[i]	2.4[d]	2.1[i]
1,3-DPG	173.5[d]	173.65	6[d]			

[a] Note that PC shows separate peaks for outer and inner monolayers of small unilamellar vesicles and a single peak for multibilayers (see text). [b] Hamilton and Small (1982); the T_1 of 1.3 s for the CO_s peak in vesicles is the only carbonyl T_1 in any system that is significantly less than 2.0 s and may represent an underestimation of the true value. [c] Hamilton and Small (1981) [d] Hamilton et al. (1991). [e] Estimate for outer leaflet resonance only. [f] Dependent on temperature and composition (Hamilton & Small, 1982). [g] 1% CO/99% PC at 35 °C. [h] 3% TO/97% PC at 35 °C. [i] 2% DPG/98% at 35 °C.

for neat TO (Hamilton & Small, 1981). Two additional broader peaks from the ^{13}C-labeled TO ($S_{1,3}$ and S_2; 172.8 and 172.2 ppm, respectively) downfield from the peaks for oil-phase TO closely resemble the peaks from TO in the surface phase of vesicles (Hamilton & Small, 1981; Hamilton, 1989). The peak area of the downfield peak ($S_{1,3}$) is twice that of the upfield peak (S_2), as expected; the peak height ratio is <2.0 because the line width of the $S_{1,3}$ peak (13 Hz) is greater than that of the S_2 peak (9 Hz). The boxed inset in Figure 2 shows that the carbonyl region of PC with 2% TO contains only two peaks, both of which represent surface-located TO. There are no oil-phase peaks, as predicted from examining the spectrum of the same sample without MAS (Figure 1A). The TO invisible to "high-resolution" NMR (Figure 1A) is now seen as a phospholipid-incorporated, surface-oriented pool.

The integrated intensities of carbonyl peaks can be used to calculate the amount of surface-phase (lamellar-incorporated) TO relative to PC (Hamilton & Small, 1981). Since NOEs are suppressed (Experimental Procedures) and since T_1 values are very similar for the carbonyl peaks PC, $TO_{s1,3}$, and TO_{s2} (Table I), peak intensities, with corrections for ^{13}C enrichment and the number of carbonyls, reflect chemical compositions (Hamilton & Small, 1981). For the 2% TO sample, all the TO signal is represented by surface-phase peaks, and the measured surface TO/PC peak area ratio (2.6) was close to the expected chemical ratio of 2.3. The 4% TO sample showed a surface TO/PC ratio of 3.3, corresponding to 3.0% TO, the maximum incorporation of TO in PC vesicles (Hamilton & Small, 1981). The total TO (oil + surface phase)/PC determined from the carbonyl integrals for the 4% TO sample also agreed closely with the expected composition. Additional samples containing <2% TO and 6% TO were examined by ^{13}C MAS NMR. Samples with <2% TO showed only surface-phase peaks for TO, while the sample with 6% TO showed intense oil-phase peaks in addition to the surface-phase peaks. In the latter spectrum the intense oil-phase peaks were not completely resolved from the weaker surface-phase peaks, and an accurate measurement of surface TO/PC was not possible; however, the integral showed ~3% TO incorporation in the lamellar phase. Taken together, the data from different compositions show that there was a fairly well-defined and reproducible solubility limit of 3% TO in multilayers, the same

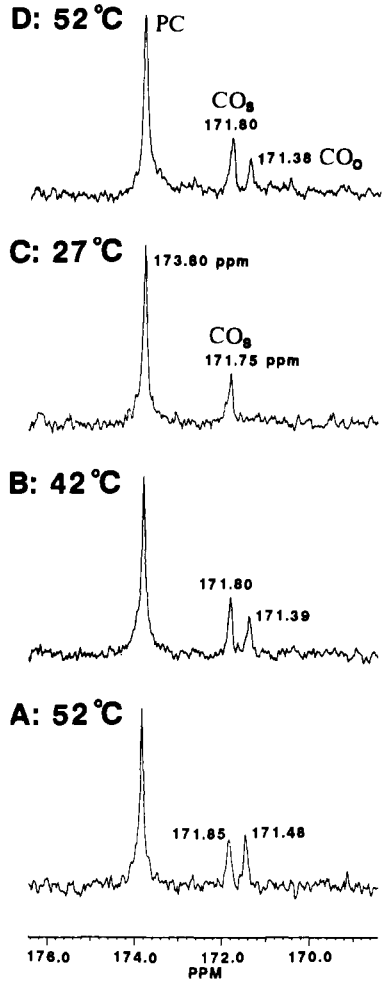

FIGURE 3: ^{13}C MAS NMR spectra (carbonyl region) at 74.5 MHz of unsonicated 1.0 wt % [^{13}C]carbonyl CO and 99 wt % egg PC in 50 wt % D_2O. Spectra were obtained with a spectral width of 200 ppm, a pulse interval of 6.3 s, 1024 scans, and 8K time domain points and were processed with 2-Hz line broadening. Spectra were acquired in the order A–D, at the temperatures indicated. Samples were equilibrated at each temperature for 45 min prior to collection of data. The carbonyl region shows a single peak for PC carbonyls (designated PC) and two peaks for [^{13}C]CO (CO_s and CO_o) corresponding to surface-phase and oil-phase CO, respectively.

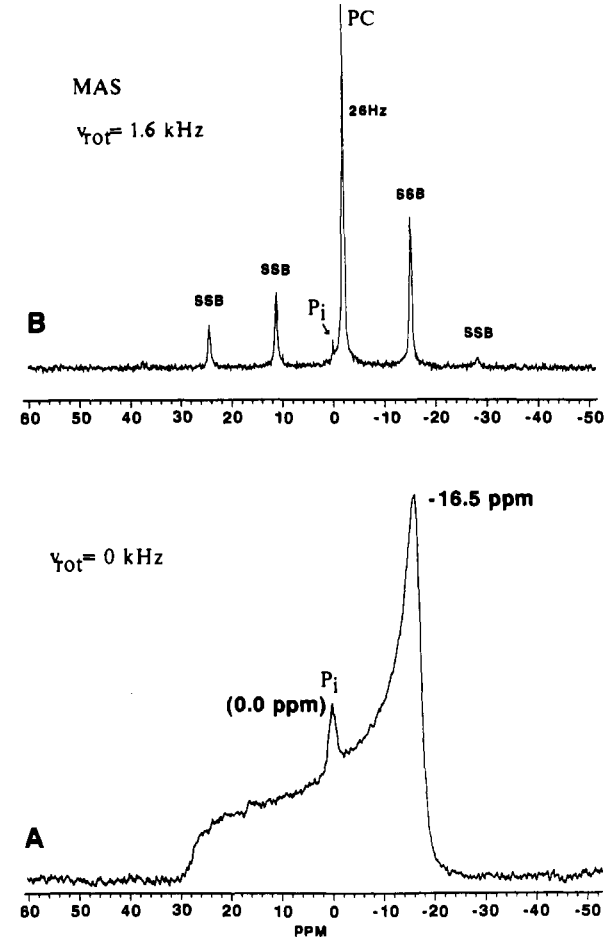

FIGURE 4: ^{31}P MAS NMR spectra at 121.5 MHz of 1.0 wt % [^{13}C]carbonyl CO and 99 wt % egg PC (unsonicated) in 50 wt % D_2O (10 mM phosphate buffer, pH 7.4) at 25 °C. Spectra were obtained with a spectral width of 25 000 Hz, a pulse interval of 4.1 s, and 4K time domain points. The narrow peak from inorganic phosphate was used as an internal reference (0.0 ppm). (A) The spectrum was acquired with sample rotation of 1.6 kHz at the magic angle after 128 scans and processed with 2-Hz line broadening. (B) The spectrum was acquired in the magic angle probe without sample spinning after 1024 scans and processed with 25-Hz line broadening. There are four spinning side bands, three of which are labeled SSB and the fourth is unlabeled at −28 ppm, which have unequal intensities as expected (Haberkorn et al., 1978; Herzfeld, 1980). The same sample was used for ^{13}C studies in Figure 3.

solubility measured for TO in PC vesicles (Hamilton & Small, 1981).

NMR characterizations of an unsonicated mixture of egg PC with 1 wt % [^{13}C]carbonyl cholesteryl oleate are illustrated in Figures 3 and 4. The carbonyl region of the ^{13}C MAS NMR spectrum at several temperatures is shown in Figure 3. The first ^{13}C spectrum (Figure 3A), obtained at 52 °C, above the crystal-to-isotropic transition of CO (50 °C; Small, 1970), shows two peaks from the ^{13}C-labeled CO. With cooling, the upfield peak decreased in intensity (Figure 3A–C). Most of the intensity was recovered when the sample was reheated to 52 °C (Figure 3D); other samples with CO showed a more complete recovery of intensity in this peak, indicating this sample may not have been fully equilibrated. The downfield peak, designated CO_s, was unaffected by temperature and has a chemical shift close to that for CO solubilized in PC vesicles (171.8–172.0 ppm; Hamilton & Small, 1982). The downfield shift indicates partial hydration of the carbonyl as a consequence of the proximity of the carbonyl to the aqueous interface (Hamilton & Small, 1982). The pool of CO represented by the upfield peak (CO_o) is apparently from an oil-phase pool that undergoes a phase transition(s) in the temperature range of 27–52 °C. Because the phase properties of CE are altered upon incorporation into the bilayer structure, the CO_s peak is not affected in this temperature range.

Additional ^{13}C MAS NMR data were obtained for a second sample of 1% CO and for two samples with 2% CO/98% PC (data not shown). All samples showed a peak at ~171.8 ppm corresponding to bilayer-incorporated CO. Results for the maximum solubility of CO in PC multibilayers (based on carbonyl integrals; see above) were not consistent, as they were for TO. Three CO samples showed a surface incorporation of 0.5% CO (as in Figure 3), while one of the 2% CO samples showed incorporation of ~1.5 wt % CO into the surface phase. The latter result is close to the solubility (2 mol %; 1.6 wt %) determined for CO in PC vesicles (Hamilton & Small, 1982). The lower incorporation may reflect incomplete equilibration of CO in those samples.

Representative samples of unsonicated PC/weakly polar lipids were examined by electron microscopy. Electron micrographs showed a heterogeneous population of multilamellar lipids and some large (apparently) unilamellar vesicles. ^{31}P spectra were obtained to determine whether the unsonicated mixed lipid systems showed the broad-line CSA pattern

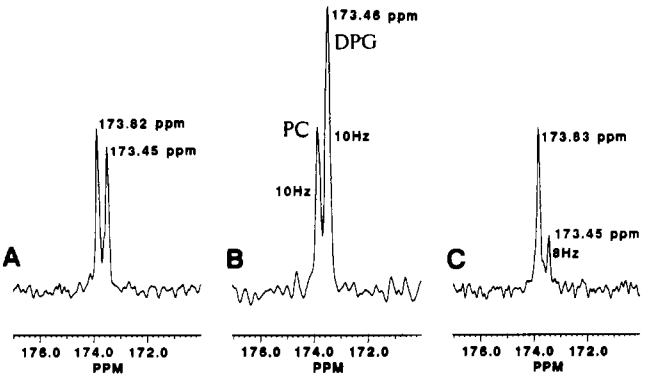

FIGURE 5: ^{13}C MAS NMR spectra (carbonyl region) at 74.5 MHz of unsonicated egg PC with 1,2-dipalmitoyl-*sn*-glycerol: (A) 1.0 wt % [1-^{13}C,2-^{13}C]carbonyl DPG, (B) 2.0 wt % [1-^{13}C,2-^{13}C]carbonyl DPG, and (C) 0.5 wt % [1,2-^{13}C]carbonyl DPG, in 50 wt % D_2O at 25 °C. Conditions are the same as in Figure 4, except for the number of scans: (A) 933, (B) 1000, and (C) 730. Spectra were processed with Gaussian multiplication (GB = 0.1 and LB = −5 Hz).

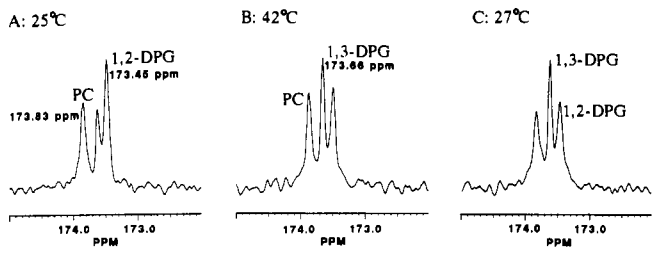

FIGURE 6: ^{13}C MAS NMR spectra (carbonyl region) at 74.5 MHz of 1.5 wt % [^{13}C]carbonyl DPG and 98.5 wt % egg PC in 50 wt % D_2O. Spectra were acquired at the temperatures indicated. The sample was equilibrated at each temperature for ~1 h prior to collection of data. Conditions were the same as in Figure 5, except for number of scans (1024). A total of 8K time domain point data were zero-filled to 16K and processed with Gaussian multiplication (GB = 0.15 and LB = −2 Hz) to enhance resolution. The acyl migration of 1,2-dipalmitoyl-*sn*-glycerol to its 1,3-isomer is seen as an irreversible intensity increase in the peak for the 1,3-isomer.

characteristic of multilamellar PC (Haberkorn et al., 1978) and whether there was a detectable population of isotropic lipid (e.g., small or large unilamellar vesicles). Figure 4 shows ^{31}P NMR spectra of 1% CO in PC at ~27 °C obtained in the magic angle probe with and without MAS. Without MAS (Figure 4A), the spectrum shows the chemical shift anisotropy pattern characteristic of multilamellar liquid-crystalline PC, with a width of ~50 ppm and an intense upfield shoulder at −16.5 ppm. There is a small isotropic signal in the spectrum, but it comes from inorganic phosphate in the buffer (P_i) and was not present in ^{31}P spectra of lipid mixtures in D_2O without phosphate buffer. The broad-line ^{31}P spectrum was the same before and after ^{13}C MAS NMR studies, indicating that spinning did not alter the lamellar structure in the sample. ^{31}P spectra obtained without MAS for mixtures of PC with ≤2 wt % TO or DAG showed similar results: (1) the spectrum was the same as in Figure 4, (2) there was no isotropic component (except for buffer, when present), and (3) there was no difference in spectra obtained before and after MAS NMR studies.

The ^{31}P MAS NMR spectrum (Figure 4B) of the same sample (1% CO, 99% PC) as in Figure 4A shows a single PC peak just upfield from inorganic phosphate and four spinning side bands. This spectrum is similar to the ^{31}P MAS NMR spectrum of multilamellar dipalmitoyl-PC reported by Haberkorn et al. (1978). The characteristic spinning side bands contain information about the principal values of chemical shift tensors (Herzfeld & Berger, 1980). Thus, the ^{31}P spectra monitored the structural organization of the phospholipid matrix and revealed no effect of the minor lipid component on the PC lamellar structure in our mixed lipid systems.

^{13}C MAS NMR spectra were obtained for unsonicated mixtures of PC and 0.5%–2.0% 1,2-dipalmitoyl-*sn*-glycerol (DPG). The carbonyl region (Figure 5) shows two narrow (10 Hz) peaks, one readily identified as PC carbonyls (173.83 ppm) and an additional peak slightly upfield, at 173.46 ppm. The upfield peak increased in intensity with increasing DPG content. A spectrum of PC and DPG with ^{13}C enrichment only in the *sn*-2 position shows a DPG carbonyl peak at 173.45 ppm (Figure 5). Thus, the two DPG carbonyls must coresonate in a narrow peak at ~173.45 ppm. The same result was found for low levels of 1,2-DAG in small unilamellar vesicles (Hamilton et al., 1991). By contrast, unhydrated oil-phase DAG gives rise to two [^{13}C]carbonyl signals at 172.61 and 172.95 ppm (Hamilton et al., 1991). The downfield shift in unsonicated PC indicates greater hydrogen bonding of DAG carbonyls as a result of incorporation into the hydrated lamellar phase with PC and a preferential hydrogen bonding at the *sn*-2 carbonyl (see below).

Figure 6 shows the carbonyl region of the ^{13}C MAS NMR spectrum of a sample of multilamellar PC containing DPG that is isomerically impure. The initial spectrum at 25 °C showed a small peak between the PC carbonyls at 173.8 ppm and the 1,2-DPG carbonyls at 173.45 ppm. When the sample was heated to 42 °C, the relative intensity of this peak increased, while that for 1,2-DPG decreased. After the sample was cooled to 27 °C, the spectrum showed a slightly higher proportion of the new peak and did not revert to the original spectrum at 27 °C. Therefore, this new signal does not represent a reversible phase change of the lipids but likely represents an irreversible chemical change to the 1,3-isomer of DPG, as identified previously in vesicle systems (Hamilton et al., 1991). After MAS NMR studies, TLC analysis of this sample verified the presence of 1,3-DPG and showed a higher amount of 1,3-DPG than 1,2-DPG. Other NMR samples showing no peak at 173.66 ppm (e.g., Figure 5) showed no 1,3-isomer by TLC.

Discussion

The goal of this study was to examine properties of weakly polar lipids incorporated into unsonicated phospholipids. The hydration of carbonyl groups can be assessed directly and the conformation indirectly from [^{13}C]carbonyl shifts (Schmidt et al., 1977; Hamilton & Small, 1981). When weakly polar lipids are incorporated into an interface, the carbonyls are partially hydrated. ^{13}C enrichment of the carbonyl carbon(s) provides a nonperturbing probe to detect interactions of the minor lipid components with water by ^{13}C NMR spectroscopy. However, samples such as multilamellar phospholipids, which do not have rapid isotropic tumbling, give rise to broad NMR lines (Figure 1) because the angular dependence of the local magnetic field is not efficiently averaged (Haberkorn et al., 1978; Oldfield et al., 1987; Bovey, 1988). The spectrum in Figure 1B is a good illustration of this principle: this sample (4% TO/96% PC) contains, in addition to highly anisotropic components (PC and some triolein), an isotropic component (liquid triolein), which does give rise to narrow ^{13}C signals. Rapid sample rotation at the magic angle and high-power decoupling ("dipolar decoupling") can greatly reduce line broadening from proton dipolar coupling and chemical shift anisotropy to yield NMR spectra with narrow lines (Haberkorn et al., 1978; Sefcik et al., 1983; Oldfield et al., 1988; Yeagle et al., 1987).[2] The frequency of such a resonance

represents the isotropic chemical shift (Bovey, 1988). We were thus able to obtain carbonyl chemical shifts of weakly polar lipids in phospholipid multibilayers that are directly comparable to carbonyl chemical shifts obtained in single bilayers of vesicle systems.

Table I summarizes the [^{13}C]carbonyl NMR data from this study together with comparative data for sonicated vesicle systems (Hamilton & Small, 1981, 1982; Hamilton et al., 1990). The assignment of carbonyl resonances in multilayers is simplified by the fact that there is no distinction between lipids in inner and outer monolayers because of low bilayer curvature. In small unilamellar vesicles the high bilayer curvature (coupled with slow transbilayer movement of lipid) gives rise to phospholipid signals for outer and inner monolayers (Table I). The PC carbonyl chemical shift (173.8 ppm) in the unsonicated sample is similar to that for PC on the outer monolayer of a small unilamellar vesicle (Hamilton & Small, 1981; Schmidt et al., 1977). In previous studies of vesicle systems, separate signals for weakly polar lipids on outer vs inner monolayers were not observed, but carbonyl line widths and chemical shifts may have been affected by such a distribution (see below).

The [^{13}C]carbonyl shift(s) for all weakly polar lipids in bilayer systems occur(s) significantly downfield from the shifts for the neat unhydrated lipid (Hamilton & Small, 1981, 1982; Hamilton et al., 1991). This change in chemical shift proves that the weakly polar lipid is not simply trapped in the lipid dispersion as a separate oil phase. Deshielding of the carbonyl carbon nucleus is a result primarily of hydrogen-bonding interactions with water when the lipid is incorporated into the phospholipid lamellar structure with the carbonyls orientated toward the aqueous interface. The net hydrogen bonding at the carbonyls is determined by the interfacial conformation.[3] The rationale for this conclusion, previously discussed in detail for each lipid in vesicle systems (Hamilton & Small, 1981, 1982; Hamilton et al., 1989, 1991), is equally applicable to the multilamellar systems in this study. Results in Table I show that the chemical shift of a given carbonyl is generally similar in both multibilayers and single bilayers, demonstrating that the highly curved vesicles do not produce substantial alterations of hydration or conformation of weakly polar lipids at interfaces. Moreover, other NMR properties are similar in both systems (see below). However, there are small but measurable chemical shift differences in the two systems (Table I). The single carbonyl resonance from cholesteryl oleate and the two carbonyl resonances from triolein are slightly more deshielded (shifted downfield) in vesicles. A downfield shift in vesicle systems relative to multibilayers probably indicates that the carbonyl groups of weakly polar lipids experience a slightly greater degree of hydrogen bonding in the vesicle. This could occur if water penetration into the outer monolayer (where the majority of the lipid is presumed to be located) were increased by bilayer curvature or if the carbonyls of the lipid were positioned slightly higher in the bilayer. An effect in the opposite direction is seen for both 1,2-DPG and 1,3-DPG, suggestive of slightly increased hydration and hydrogen bonding of the carbonyls in multilamellar dispersions. A possible explanation in this case is that the chemical shift of DAG in vesicles reflects a preferred distribution on the inner leaflet of the bilayer, where the hydrogen bonding of DAG would be slightly reduced (Hamilton et al., 1991). In any case the small differences between vesicles and multilayers in the chemical shifts for the hydrated carbonyl carbons would be difficult to attribute to a specific structural effect. The important conclusion is that the chemical shifts in multilayers occur well downfield from those of the neat lipids and are similar to those for vesicles.

Carbonyl T_1 values of weakly polar lipids in unsonicated dispersions were greater than or equal to T_1 values in vesicle systems (Table I). The increased T_1's in several cases may reflect the higher field (7.05 T vs 4.7 T) used for the ^{13}C MAS NMR studies. T_1 values of protonated ^{13}C resonances from phospholipids measured at the same field in unsonicated systems with MAS and in vesicles (without MAS) were quite similar (Sefcik et al., 1983; Yeagle et al., 1987). It is likely that T_1 values reflect isotropic components of motion and that these motions are similar in vesicles and multibilayers (Yeagle et al., 1987).

Line widths for corresponding carbonyl carbons were generally similar in vesicles and unsonicated dispersions (Table I). The narrowest resonances in any of these systems were the oil-phase triolein carbonyl peaks (Figure 2). Line widths of the fatty acid methyl (6 Hz) and the choline methyl resonances (10–12 Hz) are close to those for sonicated PC [e.g., Hamilton and Small (1981)], showing effective dipolar decoupling in our MAS NMR experiments. In multilayers the carbonyl peak for bilayer-incorporated CO was narrower and the line shape more symmetric than in vesicles (Hamilton & Small, 1982). In vesicles there may be a distribution of CO between the inner and outer leaflets and a small (but unresolved) chemical shift difference for CO on the two leaflets, giving rise to a broadening from chemical shift inhomogeneity. In multibilayers the width of the TO carbonyl $S_{1,3}$ peak is slightly greater than that for the TO S_2 peak, while in vesicles the reverse is observed (Table I). Since T_1 was longer for the $S_{1,3}$ resonance, the relative broadening of the $S_{1,3}$ resonance may be a result of a small chemical shift inhomogeneity of the *sn*-1 and *sn*-3 carbonyls. However, this line broadening is a small effect and cannot be attributed at this point to a specific cause.

Figure 7 shows the orientation of the three classes of weakly polar lipids in phospholipid bilayers. The polar glycerol backbone region is a key part of the molecule for determining the overall molecular orientation. The carbonyl group(s) must be in the polar interfacial region in order to be appreciably hydrated. The long acyl chains are too hydrophobic to protrude into the water and must be embedded in the hydrophobic interior, most likely in the parallel manner schematized. The least polar lipids (the long-chain TAG and CE) have a low solubility in the surface phase and separate into oil-rich phases at >2–3 mol %.[4] Similar conclusions regarding the orientation and solubility of the same three lipids in PC have been reached in studies of mixed lipid monolayers at the air–water interface (Smaby & Brockman, 1987). A recent study of PC at a TO–saline interface also showed quantitative agreement with the above studies with respect to TO orientation and solubility in the phospholipid surface phase (Handa et al., 1990). Although the monolayer systems are qualitatively different from

[2] Cross polarization is not needed to reduce line widths because liquid-crystalline- and even gel-phase phospholipids have residual motions compared to true organic or inorganic solids (Oldfield et al., 1987). Another study reported that dipolar decoupling was not essential for liquid-crystalline-phase phospholipids (Sefcik et al., 1983), but we observed minimal line widths only after high-power dipolar decoupling.

[3] We adopt the thesis of Schmidt et al. (1977) that carbonyl conformation per se does not make a significant independent contribution to the isotropic chemical shift.

[4] The somewhat more polar DAG molecules have a much higher surface solubility in phospholipid bilayers (Epand, 1985; Das & Rand, 1986; Hamilton et al., 1991), but this study did not attempt to determine the solubility limit.

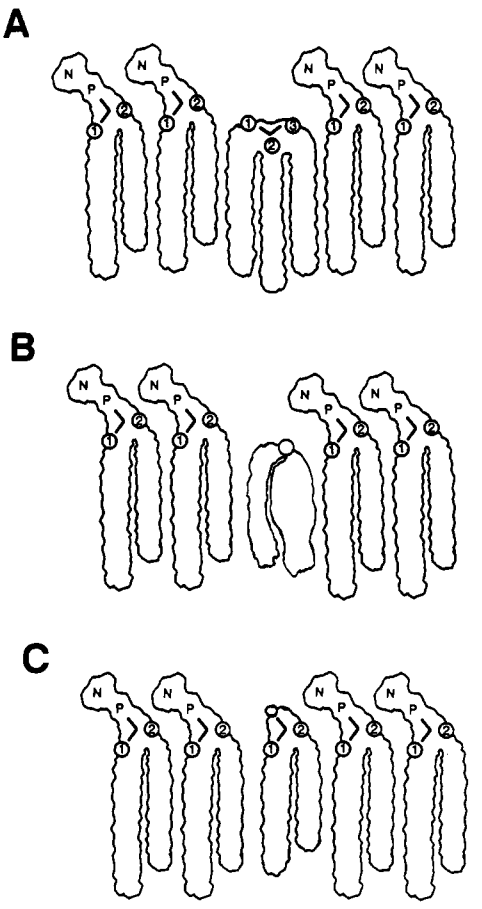

FIGURE 7: Schematized drawings of phospholipid bilayers containing weakly polar lipids. (A) Orientation of TAG in the monolayer of a PC bilayer. Since our results show partial hydration of the carbonyls (labeled according to the *sn*-1, *sn*-2, and *sn*-3 positions), these groups must be placed at the interface, and the long acyl chains must be aligned with those of the PC molecules, as shown. The solid line in the polar region of the structures shows the orientation of the glycerol backbone, and the N and P stand for the nitrogen and phosphorus of PC. (B) Orientation of CE in a PC monolayer. The carbonyl must be close to the aqueous interface and the water-insoluble acyl chain folded over the steroid ring. (C) Orientation of 1,2-DPG in a PC monolayer. Alternative conformations would include one in which the two carbonyls are equally exposed to the aqueous milieu and one in which the *sn*-1 carbonyl is closer than the *sn*-2 to the aqueous milieu. Our predicted conformation is based on analysis of chemical shift data (Hamilton et al., 1991) and places the *sn*-2 carbonyl closer to the aqueous interface than the *sn*-1 in a manner analogous to that of the PC carbonyls.

fully hydrated bilayer systems, they do represent a model for surface monolayers with very low curvature. The present NMR study shows, by comparison to previous studies in vesicles (Hamilton & Small, 1981, 1982; Hamilton et al., 1983; Hamilton, 1989), that bilayer curvature has only minor effects on the properties of interfacial weakly polar lipids. Thus, all the above methodologies appear to be monitoring the same fundamental properties of lipids at an interface. By contrast, ^{2}H NMR studies of cholesteryl palmitate in egg PC have shown very different solubilities in vesicles (5%) and multibilayers (0.2%), and these results were taken to suggest that the solubility and packing of cholesteryl esters differed substantially between the two phospholipid systems (Valic et al., 1979; Gorrissen et al., 1980). While the quantitation of bilayer-incorporated lipid from [^{13}C]carbonyl intensities is probably more accurate than the ^{2}H-based method, the solubility difference observed with ^{2}H may reflect problems in equilibrating the higher melting cholesteryl palmitate with PC. We observed a variation in the incorporation of CO in PC multilayers, which might have been a result of incomplete equilibration (Results).

[^{13}C]Carbonyl chemical shifts also give indirect information about the interfacial conformation of the polar region. For TO and other TAG (Hamilton, 1981, 1989; Deckelbaum et al., 1989), the chemical shift separation between the *sn*-1,3 and *sn*-2 carbonyl carbons in the unhydrated oil phase increases on going to the interfacial orientation. Thus, the *sn*-1,3 carbonyls are shifted more than the *sn*-2 carbonyl as a result of hydrogen-bonding interactions. This differential hydration could be achieved if the primary carbonyl groups were slightly more proximal to the aqueous medium, as shown in Figure 7. The conformation of TAG in vesicles and multibilayers appears to be fundamentally the same.

1,2-DAG have two chemically inequivalent carbonyl carbons and exhibit two well-separated ^{13}C chemical shifts in organic solvents, with the secondary carbonyl upfield from the primary (Hamilton et al., 1991), as for TAG and phospholipids in organic solvents (Schmidt et al., 1977; Hamilton & Small, 1981). In contrast to the case for TAG, the chemical shift separation between carbonyls of DAG *decreases* after solubilization in a PC bilayer. For example, in dilute CCl_4, where minimal solvent–solute and solute–solute hydrogen bonding is present, DAG has chemical shifts of 171.62 ppm (*sn*-2) and 172.03 ppm (*sn*-1) (Hamilton et al., 1991). Upon incorporation into the bilayer, the *sn*-2 carbonyl undergoes a larger downfield shift and coincides fortuitously with the *sn*-1 carbonyl resonance; hence, only one (narrow) resonance is observed in multibilayer systems, as in vesicles (Hamilton et al., 1991). Analogous differential chemical shift changes are observed for PC carbonyls (Schmidt et al., 1977), and only a single carbonyl resonance is seen in hydrated PC bilayers (Figures 2, 4–6). The conformation of 1,2-DAG in the bilayer must allow a greater degree of hydration at the *sn*-2 carbonyl than at the *sn*-1. This differential hydration can be achieved by positioning the glycerol backbone perpendicular to the bilayer plane, pulling the *sn*-2 toward the interface, and submerging the *sn*-1 carbonyl. Thus, we propose that both 1,2-DPG and PC have similar conformations in the multibilayer system (Figure 6); furthermore, the conformations are the same as in small unilamellar bilayer systems (Hamilton et al., 1991). The 1,3-DPG isomer shows a single carbonyl resonance, meaning that the chemically equivalent primary carbonyls remain structurally equivalent (equally hydrated), in contrast to the 1,2-isomer. These predicted interfacial conformations for weakly polar lipids can be tested by independent techniques, and we have initiated ^{13}C solid-state NMR studies with [^{13}C]carbonyl-labeled lipids to make a more direct measurement of interfacial conformation.

The localization in bilayers of weakly polar lipids, irrespective of the precise conformation of the polar portion of the molecule, has implications for key physiological processes. Upon incorporation into a surface phase, the weakly polar lipids take on properties of an interfacial lipid rather than those of a bulk-phase lipid. For example, as shown for CO (Hamilton & Small, 1982, and above) and for the saturated TAG, tripalmitin, in vesicles (Hamilton et al., 1989), thermotropic transitions characteristic of the bulk phase are no longer observed for these lipids. Properties of the lamellar membrane, such as phospholipid physical state (Hamilton, 1989) and cholesterol content (Spooner et al., 1986; Spooner & Small, 1987), can substantially affect the interactions of weakly polar lipids. Localization of the carbonyl group at the aqueous interface of a membrane or emulsion particle, and its hydrogen-bonding with water, should greatly facilitate enzymatic

hydrolysis of the carbonyl group(s). In addition, the polar region of these molecules provides a recognition site for protein-mediated transport into the aqueous medium, a process known to occur for TAG and CE (Tollefson & Albers, 1986). In both of the above general cases, restriction of (nearly) isotropic rotation of CE and TAG in oil phases to highly anisotropic rotations in the phospholipid monolayer (with the correct molecular orientation) should also greatly facilitate lipid–protein interactions. In the case of 1,2-DAG, interaction with protein kinase C most likely occurs at a membrane interface in a complex with Ca^{2+} and phosphatidylserine (Ganong et al., 1986). The importance of the primary and secondary carbonyl groups and the single OH group for protein activation was inferred from structure–activity studies (Ganong et al., 1986). Thus, the localization of the carbonyls at the interface (and possibly their precise conformation) is critical for DAG involvement in signal transduction.

This study also provides direct evidence that acyl-chain migration can occur for dilute 1,2-DAG in multilamellar PC. The conversion of 1,2-DAG to 1,3-DAG is catalyzed by temperature. Previous NMR studies of DAG in vesicles showed similar results (Hamilton et al., 1991), and chemical assays of DAG in PC vesicles and multilayers have further elaborated conditions under which isomerization is favored (Kodali & Small, 1990). It is clearly critical to check for 1,3-DAG in physical studies of 1,2-DAG with phospholipids, particularly when protocols involve elevated temperatures and/or require long times for data accumulation.

The number of applications of ^{13}C MAS NMR to membranes and model membranes has been fairly limited (Sefcik et al., 1983; Oldfield et al., 1987; Yeagle & Frye, 1987; Forbes et al., 1988; Yeagle et al., 1990) since its first demonstration as a method to obtain high-resolution spectra of unsonicated phospholipids (Haberkorn et al., 1978). Moreover, studies to date have focused mainly on the major constituents of membranes. This study shows that the technique could be a powerful one for determining properties of minor constituents of model membranes and possibly biomembranes.

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Registry No. TO, 122-32-7; CO, 303-43-5; DPG, 30334-71-5.

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