Multinuclear and Magic-Angle Spinning NMR Investigations of Molecular Organization in Phospholipid–Triglyceride Aqueous Dispersions†

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Received March 4, 1993; Revised Manuscript Received July 1, 1993®

ABSTRACT: The supramolecular organization of multi-bilayers formed by aqueous egg phosphatidylcholine–triolein (PC–TO) mixtures has been investigated using ^{31}P, ^{13}C, and ^{1}H nuclear magnetic resonance (NMR). For these emulsions, which mimic substrates in the early hydrolytic stages of fat digestion, the NMR spectra obtained with magic-angle spinning (MAS) exhibit resolution comparable to that of sonicated vesicles and integrated peak intensities consistent with their chemical composition. Both ^{31}P line shapes and MAS sideband patterns from the phosphocholine group indicate that mixing with triolein produces a PC bilayer which remains predominantly liquid crystalline in its organization; nevertheless, anomalous spectral features in MAS spectra may be attributed to additional phases in which the headgroups adopt a different orientation with respect to the bilayer normal, and tight packing enhances phosphorus–phosphorus interactions. ^{13}C and ^{1}H line widths monitored as a function of PC mole fraction, spinning speed, and decoupling strength show that the choline headgroups and glycerol backbones are anchored preferentially in the phospholipid–triglyceride assemblies, whereas all acyl chains become very fluid. The average degree of chain order also decreases for the mixed dispersions, as judged from spinning-sideband intensities in ^{1}H MAS NMR spectra. The absence of proton spin-diffusion effects in the PC–TO multilayers is demonstrated by examination of their spinning sidebands and relaxation times, making it possible to use MAS-assisted two-dimensional NMR to assign overlapped ^{1}H resonances and to identify proximal interactions between the two constituents. The usefulness of these NMR strategies in mechanistic studies of gastric fat digestion is also discussed.

Mixtures of triglycerides and phospholipids have attracted considerable attention during the last decade because of their significance in the hydrolysis of both constituents in animals and plants. It is still an open question whether the small surface population of triglyceride that is typical of proposed aggregate structures (Hamilton & Small, 1981; Burns et al., 1983; Smaby & Brockman, 1987) renders these water-insoluble substrates sufficiently accessible to account for their high turnover rate (Carey et al., 1983; Brockman, 1984; Hamosh, 1990). For mixtures prepared in their dietary proportions, the molecular organization of surface triglycerides, core triglycerides, and phospholipids should be an essential element in the development of hypotheses for enzymatic hydrolysis.

NMR has proven to be a versatile tool for structural studies of triglyceride–phosphatidylcholine (TG–PC)[1] mixtures in various aggregation states. For tributyrin or trihexanoin solubilized by short-chain PC's, Roberts and co-workers observed no changes in ^{13}C chemical shift upon mixed-micelle formation and found spin-relaxation times that revealed substantial chain mobility for both components as well as altered motional anisotropy for the TG glycerol backbone (Burns & Roberts, 1981). They examined ^{13}C NMR relaxation trends, Mn^{2+}-induced line broadening, ^{31}P NMR line widths, quasieleastic light scattering data, and measurements of interfacial tension in order to develop a model in which 5–10% of tributyrin at its saturating concentration is present at the micellar aggregate surface (Burns et al., 1983).

For long-chain glycerides, such as triolein mixed with egg phosphatidylcholine, both sonicated vesicles and multilamellar dispersions were examined by NMR methods (Hamilton & Small, 1981; Hamilton, 1989; Hamilton et al., 1991). This work focused primarily on ^{13}C spectroscopy of the triglyceride carbonyl groups, which differ in chemical shift according to their position in the glycerol backbone and their location near the particle surface or within an oily core. A solubility limit of about 3% was established for TG molecules within PC matrices. Beyond this limit, the triglyceride phase separates but the PC bilayer structure is preserved, as indicated by NMR line shapes, chemical shifts, and intensities. Magic-angle spinning (MAS) NMR, a potent and practical method for obtaining high-resolution spectra of lipid multilayers (Haberkorn et al., 1978; Oldfield et al., 1987; Halladay et al., 1990), was essential in order to extend earlier work with triolein and tripalmitin in sonicated unilamellar vesicles to the multilayers more typical of biological membranes and emulsion particles.

We have examined the aggregate structure of stable emulsions formed by egg phosphatidylcholine–triolein (PC–TO) mixtures at a range of compositions, using both static and MAS multinuclear NMR and naturally abundant nuclear isotopes. The spinning-sideband intensities of ^{31}P MAS NMR spectra allow us to assess critically the impact of TO on PC bilayer structure. MAS ^{13}C NMR conducted with various decoupling conditions and spinning speeds identifies the mobile chemical groupings in these multilamellar dispersions. Supporting evidence regarding mobility and order, particularly for the PC bilayer, also comes from ^{1}H MAS

† This work was supported by a grant to R.E.S. from the National Institutes of Health (R01-DK36888). R.E.S. is also the recipient of an NIH Research Career Development Award (K04-DK01793).

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® Abstract published in *Advance ACS Abstracts,* September 1, 1993.

[1] Abbreviations: NMR, nuclear magnetic resonance; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine or phosphatidylcholine; TG, triglyceride; TO, triolein; MAS, magic-angle spinning; DP, direct polarization; CP, cross polarization; DD, dipolar decoupling; SD, scalar decoupling; T_1, spin-lattice relaxation time; COSY, two-dimensional correlated spectroscopy; X_{PC}, mole fraction of phosphatidylcholine.

0006-2960/93/0432-9926$04.00/0

NMR. Both spin-lattice relaxation times and spinning-sideband behavior are used to assess intermolecular interactions and to lay the groundwork for studies of emulsion structure by two-dimensional nuclear Overhauser spectroscopy. The possible relationship between PC–TO structures and glyceride digestion is also discussed.

EXPERIMENTAL PROCEDURES

Materials. Egg phosphatidylcholine (PC) and triolein (TO) were purchased from Sigma Chemical Company (St. Louis, MO) or Avanti Polar Lipids (Birmingham, AL). Their purities were confirmed to exceed 99% by thin-layer chromatography on 250-μm silica gel G plates (elution with 12:6:0.8 chloroform–methanol–water and toluene, respectively). Both materials were used as received. Deuterium oxide (99.96%) was obtained from Aldrich Chemical Co. (Milwaukee, WI).

Preparation of Aqueous Dispersions. Egg PC and TO were cosolubilized by combining a chloroform solution of PC, neat TO, and excess 2:1 chloroform–methanol; TO was omitted from some samples. After evaporation of the solvents under a stream of nitrogen and pumping at reduced pressure overnight, the lipid films were rehydrated with D_2O that was 150 mM in NaCl. The resulting aqueous dispersions [50:50 (w/w) lipid–water, typically 314 mg of each] were vortex-mixed for 1-2 min and taken through five freeze–thaw cycles to obtain a uniform paste. The hydrated mixtures were loaded into 7-mm Sapphire or Zirconia rotors equipped with Macor end caps and O-ring seals (Doty Scientific, Columbia, SC). All samples remained dispersed over the course of several months.

Nuclear Magnetic Resonance Studies. NMR experiments were carried out on an IBM Instruments WP-200 spectrometer augmented with high-power amplifiers and a Doty Scientific probe for magic-angle spinning. The resonance frequencies were 200.13, 81.02, and 50.33 MHz for ^1H, ^{31}P, and ^{13}C, respectively. Spinning speeds (ν_{rot}) were adjusted to 2100 ± 100 Hz except as noted otherwise. Sample temperatures were controlled to within 1 °C with a Bruker B-VT 1000 unit (Bruker Instruments, Inc., Billerica, MA) as described previously (Halladay et al., 1990).

Direct polarization ^{31}P NMR measurements, conducted under both static and magic-angle spinning conditions, were performed using 4–5-μs 90° pulses for phosphorus nuclei and a 50-kHz ^1H decoupling field during signal acquisition. Recycle delays of 4 s and auxillary air flow through the center of the probe were used to avoid sample heating during these experiments. Typically, 32–800 acquisitions of 4K time domain points spanning a spectral width of 24 kHz were zero-filled to 8K points, multiplied by a decaying exponential (line broadening of 20–100 Hz), and processed by Fourier transformation. Additional ^{31}P experiments were conducted at 121.5 MHz, using 0–40-kHz decoupling fields and MAS at 500–3500 Hz.

Direct polarization magic-angle spinning (DPMAS) ^{13}C NMR was conducted with 5-μs 90° pulses for ^{13}C and either 50-kHz decoupling (dipolar decoupling, DD) or 8-kHz decoupling (scalar decoupling) gated on during signal acquisition. In a typical experiment, 800 transients with 5-s recycle delays were collected. Spectra used in line-width studies had 4K time domain points zero-filled to 32K points, a line broadening of 10 Hz, and a spectral width of 20 kHz. Measured line widths have an estimated precision of 1–2 Hz.

MAS ^1H NMR spectra were obtained with 14-μs 90° pulses, a 10-kHz spectral width, and 1-s recycle delays. For a typical line-width determination, 32 acquisitions of 8K time domain points were zero-filled to 16K points, multiplied by a decaying exponential (line broadening 0.3 Hz), and subjected to Fourier transformation. Using the above conditions, spinning-sideband intensities were measured with respect to the $(CH_2)_n$ center band, and spin-lattice relaxation times $T_1(H)$ were determined with an inversion-recovery sequence (Vold et al., 1968).

MAS-assisted, two-dimensional NMR experiments (correlated spectroscopy, COSY) were performed with time-proportional phase incrementation (Marion & Wuthrich, 1983). COSY spectra were obtained with the pulse sequence $(90°–t_1–90°–\text{acquire}[t_2])_n$, $n = 32$, and 256 t_1 values. Double-quantum filtering (Piantini et al., 1982) was used in some COSY experiments. The resulting NMR data sets were processed with NMR2 software (New Methods, Inc., E. Syracuse, NY) using phase-shifted sine-bell window functions and double Fourier transformation. These experiments were carried out with 2.1-kHz MAS and required 3–4 h of spectrometer time.

RESULTS

^{31}P NMR Spectra. Figure 1 displays the motionally averaged powder patterns and variable-speed MAS data for the choline headgroup of egg phosphatidylcholine, which was present as pure PC multi-bilayers and in a mixed assembly with triolein. The line shapes observed in the static spectra are as expected for multilamellar dispersions in the liquid-crystalline state (Yeagle, 1987; Knowles & Marsh, 1991); Table I shows that the shift tensors obtained with standard spectral analysis have a trace and a residual anisotropy comparable to values reported for the liquid-crystalline phase of saturated PC's and egg PC dispersions containing 3% TO (Hamilton et al., 1991). The powder patterns for mixtures with $X_{PC} = 0.25$ and 0.75 have an overall shape similar to that of PC alone, but some unexpected additional signal intensity appears near 0 ppm for the mixture with $X_{PC} = 0.25$.

Although the static ^{31}P NMR spectra suggest that the average electronic environment, conformation, and motional properties of the choline headgroup are similar in all three PC-containing mixtures, this conclusion is not supported by their respective MAS spectra (Figure 1). At each of three rotation speeds, both PC–TO emulsions exhibit fewer and smaller spinning sidebands than the pure PC multilayers. The detailed comparisons presented in Table I also show the following trends: (a) all PC-containing multilayers have the expected dependence of sideband intensities on spinning speed; (b) relative sideband intensities are fairly similar for dispersions with $X_{PC} = 0.75$ and 1.00; (c) relative sideband intensities for $X_{PC} = 0.25$, $\nu_{rot} = 1000$ Hz roughly match those observed for $X_{PC} = 1.00$, $\nu_{rot} = 1200$. Under fast-spinning conditions (1000–2300 Hz),[2] the ^{31}P spectra of the mixture with $X_{PC} = 0.25$ have sharp lines even with no ^1H decoupling (spectra not shown). However, low-speed (335-1000 Hz) MAS data for this mixture display a broad powder pattern underlying the sharp set of MAS lines. Thus, although the multilayer organization remains predominantly liquid crystalline upon TO addition, dipole–dipole and/or chemical shift anisotropy

[2] ^{31}P spectra at 81 MHz were acquired with MAS at 800–1500 Hz; ^{31}P experiments at 121 MHz used 500–3500-Hz spinning speeds. For the sake of clarity, the data have been presented as if all experiments were carried out at 81 MHz.

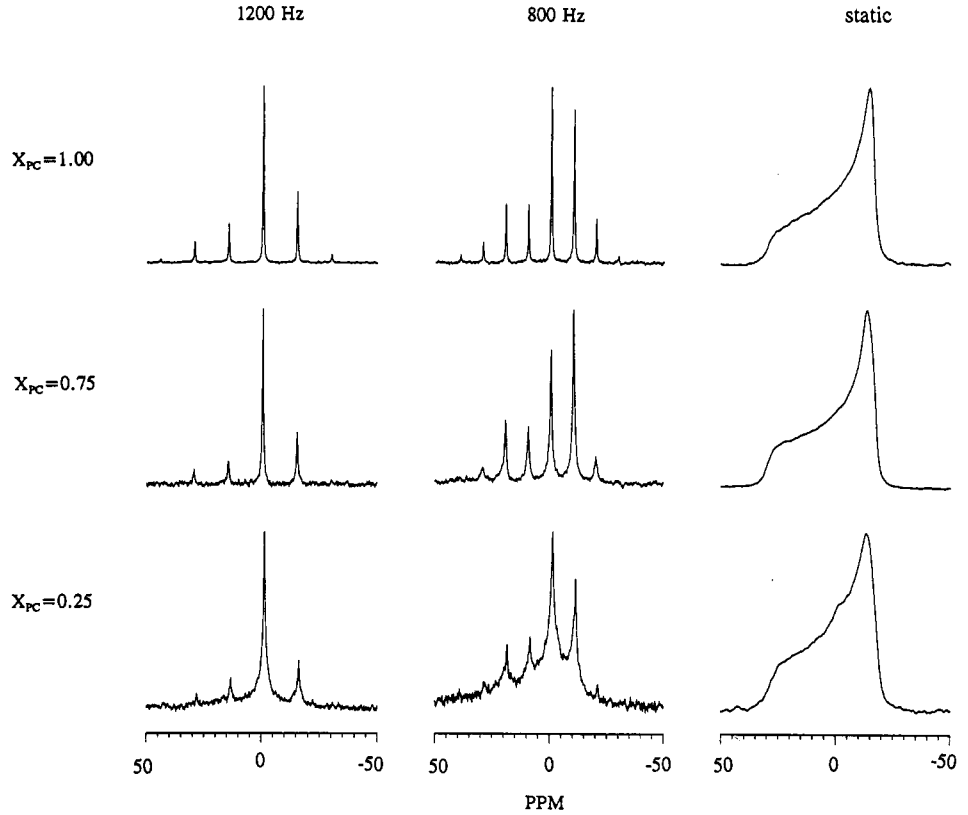

FIGURE 1: DPMASDD ^{31}P NMR spectra (81.02 MHz) of egg phosphatidylcholine–triolein mixtures at various spinning speeds. The chemical shifts are referenced to external 85% phosphoric acid at 0.00 ppm; negative shifts correspond to a more shielded environment. Exponential line broadening of 20 and 100 Hz was applied for spinning and static spectra, respectively. The speeds have an estimated precision of 5 Hz and accuracy of 6 Hz. The shoulder in the static spectrum for X_{PC} = 0.25 does not correspond to the central observation frequency.

Table I: ^{31}P Chemical Shifts and Relative Spinning-Sideband Intensities for Egg Phosphatidylcholine–Triolein Mixtures[a]

mole fraction of phospholipid	shift tensor trace (ppm)[b]	shift tensor anisotropy (ppm)[c]	rotation speed (Hz)	sideband number[d]								
				−4	−3	−2	−1	0	+1	+2	+3	+4
1.00	−1.5	−45.1	1200		1.8	11.6	21.9	100	41.5	4.4		
			1000		5.1	20.2	26.6	100	60.6	10.0		
			800	4.3	10.7	33.9	32.9	100	88.7	25.3	3.3	
0.75	−1.7	−46.3	1000			17.0	25.8	100	60.3	5.9		
0.25	−1.9	−45.7	1000			13.0	25.0	100	44.5			

[a] From DPMASDD spectra acquired at 298 K under conditions of 50-kHz inverse-gated decoupling and referenced to external 85% phosphoric acid at 0.0 ppm. [b] From one-third the sum of tensor elements $(\sigma_{\parallel} + 2\sigma_{\perp})/3$ measured from static spectra. Negative shifts correspond to a more shielded environment. The estimated precision of these measurements is 0.3–0.5 ppm. [c] From the difference, $\sigma_{\perp} - \sigma_{\parallel}$, measured from static spectra. [d] Intensities are referenced to the largest sideband, which is set to 100% and designated with the number 0. A positive sideband number corresponds to a more negative chemical shift.

interactions of the PC headgroup are incompletely removed at modest spinning speeds. A variety of structural explanations for this observation are considered in the Discussion section.

^{13}C NMR under DD Conditions: Chemical Shifts, Intensities, and Line Widths. More detailed structural information concerning the PC headgroup, as well as the acyl chains and glycerol backbones of both PC and TO constituents, is available from the MAS ^{13}C NMR spectra presented in Figure 2. Molecular motion reduces the efficiency of cross polarization in liquid-crystalline multi-bilayer systems (Halladay et al., 1990), but the combination of direct polarization (DP), magic-angle spinning (MAS), and high-power decoupling (DD) produces well-resolved spectra (Figure 2).

The ^{13}C chemical shifts summarized in Table II are in accord with those reported previously for phospholipids and glycerides as monomers, micelles, and multilayers (Haberkorn et al., 1978; Oldfield et al., 1987; Burns & Roberts, 1981; Rabiller & Maze, 1989). No changes in the average electronic environment or carbonyl hydration state are evidenced from chemical shifts monitored as a function of mixture composition. The spectral resolution is sufficient to distinguish many of the acyl chain carbons and also the corresponding carbonyl, glycerol, and α-CH_2 sites for the TO and PC components of the aqueous mixtures. The carbon signals attributable to TO carbonyls of the 1, 3 and 2 triglyceride chains, respectively, are well resolved from each other and from the PC carbonyl resonance; nevertheless, distinct spectral features from surface-oriented TO carbonyl groups are not observed because of their small relative population when X_{TO} = 0.25 (Hamilton, 1981). [Both oil and surface carbonyl signals are observed in analogous mixtures of trioctanoin and egg PC (M. Guo and R. E. Stark, unpublished results).]

Despite the well-known difficulties associated with quantitation of ^{13}C NMR spectra (Sotak et al., 1984), the use of inverse-gated dipolar decoupling to suppress ^{13}C{^{1}H} nuclear Overhauser effects and the use of 5-s recycle delays to permit spin relaxation allow us to obtain reliable peak intensities. For example, Table III compares observed and predicted ^{13}C

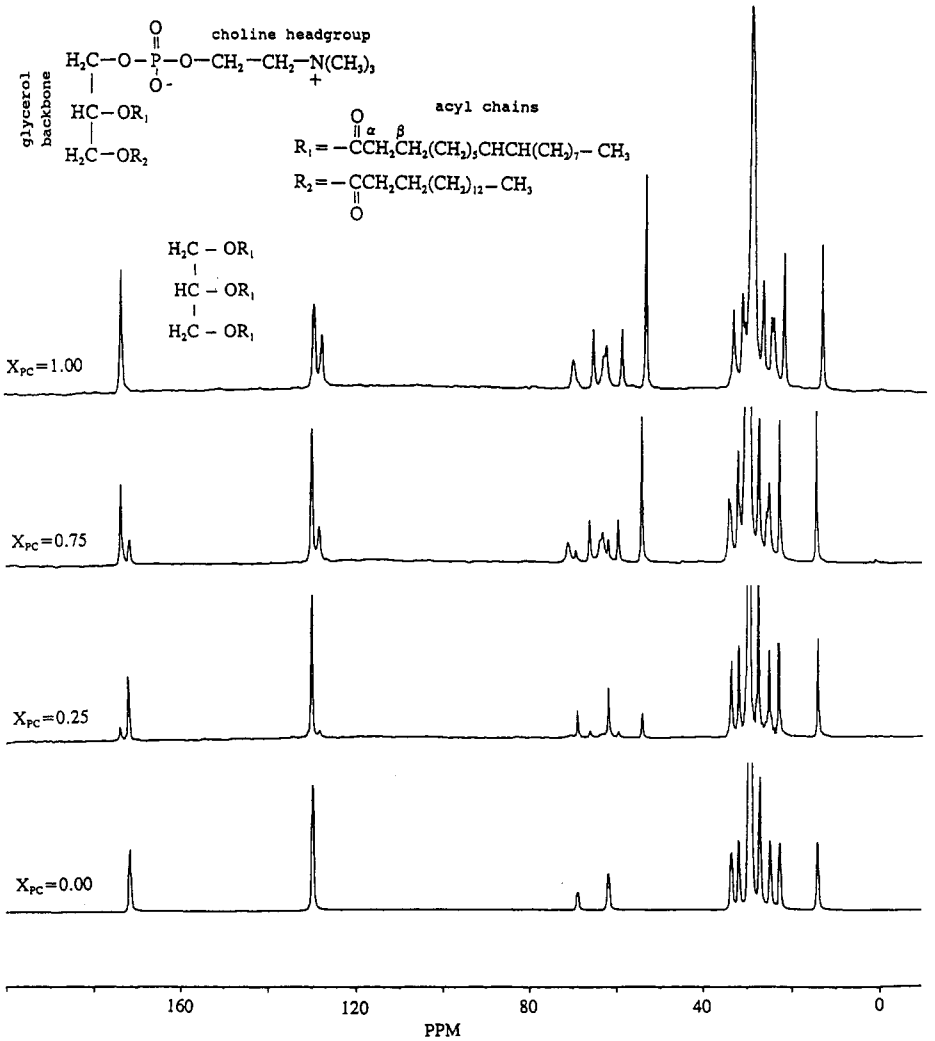

FIGURE 2: DPMASDD ^{13}C NMR spectra (50.33 MHz) of egg phosphatidylcholine–triolein aqueous dispersions with varying mole fractions (X_{PC}), obtained with inverse-gated decoupling and a recycle delay of 5 s to preserve quantitative relationships among the signal intensities. TO was examined as a neat oil. Magic-angle spinning at 2100 Hz was used in the acquisition of all spectra. The chemical shift scale is referenced to chain-terminal methyl groups at 14.1 ppm, following Hamilton and Small (1981); the spectral assignments are summarized in Table II.

integrated intensities for a mixture with $X_{PC} = 0.75$. The relative heights are as expected from compositional considerations, with a precision matching that reported for micellar mixtures of a bile salt, fatty acid, and monoglyceride (Wang et al., 1992). These quantitative results demonstrate the usefulness of DPMASDD ^{13}C NMR for the acquisition of spectral information on all carbon types represented in aqueous multilayer assemblies. They also indicate, as expected for lipid bilayers in the liquid-crystalline phase, that no carbon segments are rigid enough to have long spin-lattice relaxation times.

The question of motional narrowing, along with its possible implications for high-resolution ^1H NMR measurements in mixed-lipid systems, prompted a series of ^{13}C spectral linewidth measurements conducted with various choices of mixture composition and spinning speed. In agreement with results reported for the carbonyls and chain methyls of PC multilayers containing up to 3% TO (Hamilton et al., 1991), we find line widths of 6–12 Hz for these carbons if the spectra are obtained with the DPMASDD experiment and 2000-Hz spinning. By contrast, line widths 3–6 times larger are observed for the glycerol backbone and *nearby* chain methylenes in pure PC multilayers, suggesting that bilayer packing restricts their motions more than headgroups, carbonyls, and more *distant* acyl chain carbons. In the pure TO oil, all of the carbon lines are 3–7 Hz in width, including the glycerol backbone, α-CH_2, and β-CH_2 groups.

For those PC and TO peaks that coincide in mixed dispersions, most ^{13}C line widths have values intermediate between those of their constituents; the unique PC resonances from backbone, α-CH_2, and β-CH_2 sites remain the broadest in the spectrum. Most of the line widths also remain unchanged when the spinning speed is reduced to 500 Hz, suggesting that the shift anisotropy has been substantially removed by molecular motion (heteronuclear dipolar interactions are removed by high-power decoupling). Inexplicably, the signals from CHO backbone carbons of PC appear broader at 2000 Hz (the integrated intensities are comparable at the two speeds).

^{13}C NMR under SD Conditions: Line Widths and Intensities. High-resolution CPMAS ^{13}C spectra with low-power decoupling (SD) have been reported for semisolids such as intact fruit tissue and liquid-crystalline multilayers of dilauryl-PC (Ni & Eads, 1992; Sefcik et al., 1983), but line broadening under scalar-decoupling conditions has been observed in phospholipid dispersions by both us and others (Hamilton et al., 1991). This sensitivity of ^{13}C line widths to the NMR acquisition parameters, which provides a useful means of discriminating between the lipid constituents in PC–TO systems, is summarized in Figure 3.

Table II: ^{13}C Chemical Shifts for Egg Phosphatidylcholine–Triolein Mixtures[a]

carbon type[b]	mole fraction (X_{PC}) 1.00	0.75	0.25	0.00
C=O	173.72	173.74	173.77	
C=O (1,3)		171.83	171.86	171.75
C=O (2)		171.55	171.58	171.47
HC=CH	129.92	129.83	129.76	129.78
=CHCH$_2$CH=	128.18	128.15	128.24	
CHO	70.90	70.81		
CHO		69.15	69.30	69.09
CH$_2$N	66.35	66.22	66.40	
CH$_2$O	63.88	63.78		
CH$_2$O		61.99	62.07	61.95
CH$_2$OP	59.77	59.72	59.75	
N(CH$_3$)$_3$	54.39	54.27	54.46	
α-**CH$_2$**[d]	34.32	34.26	34.41	
α-CH$_2$ (2)[d]		33.99	34.07	33.94
α-CH$_2$ (1,3)[d]		33.82	33.90	33.76
(*ω*-2)CH$_2$	32.27	32.19	32.17	32.15
unassigned	31.81	31.80	31.83	
(CH$_2$)$_n$	30.19	29.98	29.98	29.93
		29.85	29.80	29.81
		29.60	29.56	29.53
		29.37	29.36	29.32
CH=CHCH$_2$CH$_2$	27.49	27.37	27.40	27.32
=CHCH$_2$CH=[c]	25.82	25.82	25.89	
β-**CH$_2$**[d]	25.27	25.25		
β-CH$_2$[d]		25.00	25.04	24.95
(*ω*-1)*CH$_2$*	22.90	22.90	22.87	22.85
ω-CH$_3$	14.10	14.10	14.10	14.10

[a] From DPMASDD ^{13}C NMR spectra acquired at 298 K for aqueous dispersions and a neat TO oil. Chemical shifts are referenced to the chain-terminal methyl groups at 14.10 ppm, following Hamilton and Small (1981). [b] Chemical structures for the major constituent of egg PC and for TO are shown in Figure 2: PC carbons are shown in boldface, and carbons assigned to both PC and TO are italicized. [c] Chromatographic analysis by the supplier indicates that 17.72% of the unsaturated acyl chains are derived from linoleic acid. The chemical shift assignment was made from standard empirical formulas (Kalinowski et al., 1988) and supported by the retention of this resonance in DPMASSD spectra. [d] These assignments are supported by DPMASSD spectra, in which the *α*-CH_2 and *β*-CH_2 of the PC constituent are severely broadened, whereas the corresponding resonances of TO are retained.

Table III: ^{13}C Signal Intensities for a Triolein–Egg Phosphatidylcholine Mixture[a]

carbon type[b]	relative number of carbons[c]	relative integrated intensity[d]
C=O	6	5.9
C=O	3	3.0
CHO	3	3.4
CHO	1	1.2
CH$_2$N	3	3.4
CH$_2$O	6	5.9
CH$_2$O	2	1.7
CH$_2$OP	3	3.0
N(CH$_3$)$_3$	9	9.0
α-*CH$_2$*	9	9.1
(*ω*-2)*CH$_2$*	9	9.2
(CH$_2$)$_n$	78	77.8
(*ω*-1)*CH$_2$*	9	9.4
ω-CH$_3$	9	8.9

[a] From a DPMASDD spectrum acquired at 295 K for an aqueous dispersion with X_{PC} = 0.75 (3 PC:1 TO) under conditions of inverse-gated decoupling. [b] PC carbons are shown in boldface; carbons assigned to both PC and TO are italicized. Egg PC contributions from linoleic acid chains, which enhance the signal intensity at 130 ppm and overlap partially with *β*-CH_2's from PC and TO, have been omitted from this analysis. Chemical structures for the two species are shown in Figure 2. [c] The CHO carbon of triolein has been set to 1. The bulk methylene carbons, which exclude sites near methyl groups, carbonyls, and double bonds, include 10 carbons for each palmitoyl chain and 8 carbons for each oleoyl chain. [d] The sum of integrated intensities corresponds to 171 carbons.

For the carbonyl groups, resonances from the TO oil remain very narrow in SD spectra, but signals from the pure PC multilayer broaden by a factor of 3, presumably reflecting the motional restrictions present in the organized PC structures. Upon mixing, the PC carbonyl signals become even more sensitive to decoupling strength, but the corresponding TO peaks remain almost unaffected. Thus, the carbonyl regions in the majority of TO molecules retain their basic oil character, whereas PC carbonyls of the lipid multilayer become more immobilized. This evident change in the organization of the phospholipid ester groups complements suggestions of headgroup alterations derived from the ^{31}P MAS spectra presented above. The line-width enhancements observed in DPMASSD experiments conducted with ν_{rot} = 2000 Hz are paralleled with 500 Hz MAS.

The spectral region between 50 and 75 ppm includes well-resolved peaks from both PC and TO glycerol backbone carbons. Substantial broadening of the PC backbone resonances, in both pure PC and the PC–TO emulsions, is both expected and observed with SD acquisition at both spinning speeds: the lines were broad due to motional restrictions even in DPMAS spectra acquired under DD conditions (see above). The anchoring effect for glycerol groups of the phospholipid results in lines up to 3 times broader under SD conditions, virtually eliminating their resonances from even the ^{13}C spectra of PC-rich mixtures. By contrast, the line widths of the TO backbone peaks exhibit no sensitivity to decoupling strength.

As for the carbonyls, the comparison of DPMAS spectra under SD and DD conditions shows that the PC backbone is immobilized in the presence of TO, but that the triglyceride retains its liquid (oily) character.

Also present in this spectral region are resonances from the PC choline headgroup. All three carbon peaks are retained in DPMASSD ^{13}C NMR spectra, but as the emulsions become rich in triolein, both CH_2OP and CH_2N peaks of the PC headgroup become broader than the TO backbone signals and very difficult to observe (data not shown). Thus, portions of the choline moiety, for which broad spectral features were evidenced in slow-speed ^{31}P MAS, exhibit similar characteristics in the DPMASSD ^{13}C data. Only carbons of the $N(CH_3)_3$ groups retain mobility in the mixed aggregates sufficient to fully average heteronuclear dipolar interactions.

A first glance at the acyl chain resonances in PC–TO mixtures suggests that broadening under low-power decoupling conditions is quite modest, but the ^{13}C DPMAS spectra of pure PC multilayers belie this conclusion. Signals from the chain carbons in pure egg PC dispersions are substantially broadened under SD conditions, in contrast to the modest line-width enhancements observed for the acyl chain resonances of pure TO. For the mixtures, ^{13}C NMR spectra obtained with scalar decoupling show a broad "lump" under a "comb" of well-resolved peaks, though the integrated intensity of this region is in accord with compositional predictions. The appearance of the acyl chain spectral region may be attributed to chemical shift heterogeneity and/or incomplete motional averaging of dipolar interactions involving directly bonded C–H pairs (Chan et al., 1981). [Chemical shift anisotropies for the chain methylenes, which have been estimated at 10 ppm in phospholipid multilayers (Urbina & Waugh, 1974), will be removed under our experimental conditions.] As described above for carbonyl and glycerol functionalities, the spectral distinction between acyl chains in multilamellar and oil phases appears to be retained upon the formation of PC–TO mixed aggregates.

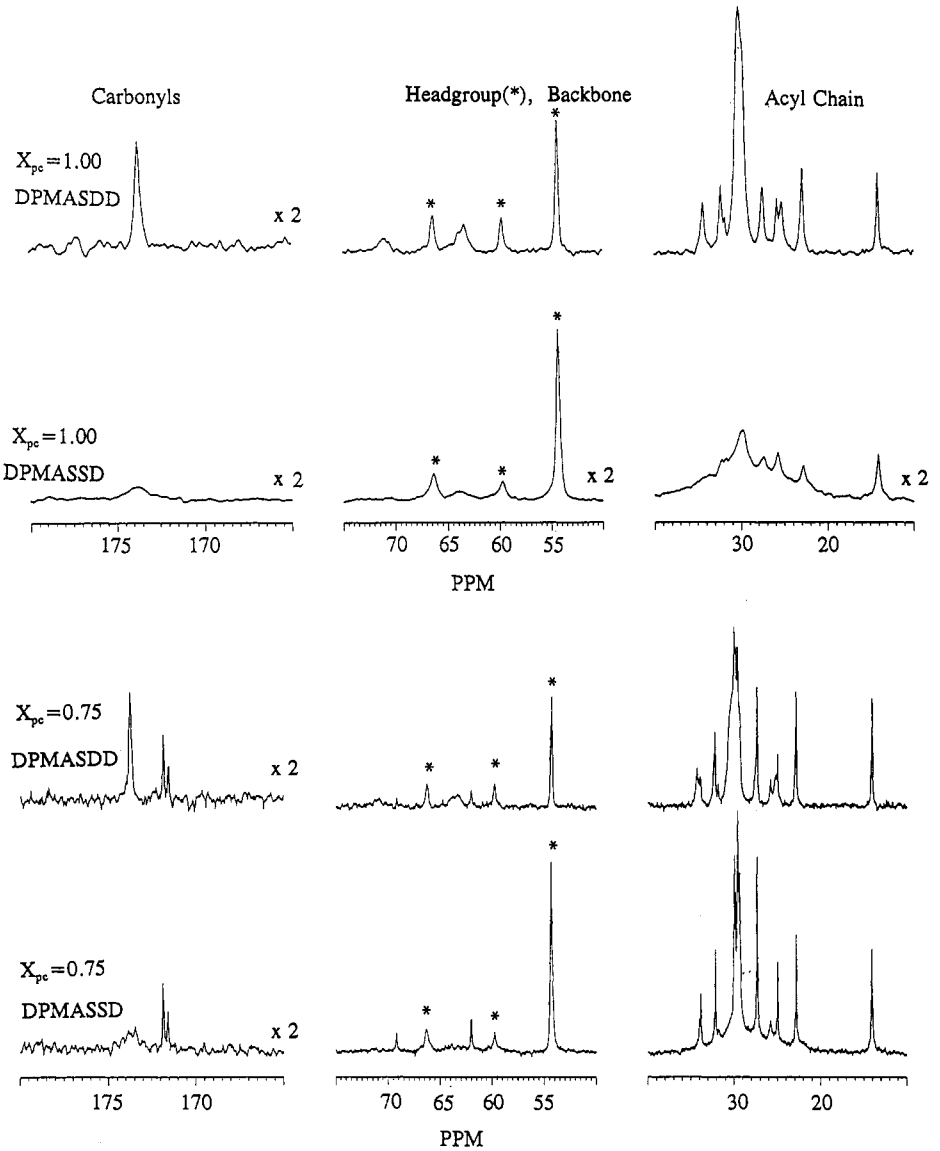

FIGURE 3: DPMAS ^{13}C NMR spectra (50.33 MHz) acquired with high-power decoupling (DD, 50 kHz) and low-power decoupling (SD, 8 kHz), respectively, for a pure PC multilamellar dispersion (X_{PC} = 1.00) and a PC–TO mixture (X_{PC} = 0.75). The spinning speed was 2100 Hz, and an exponential line broadening of 2.5 Hz was used to process the data. Spectral assignments are summarized in Table II.

^1H NMR: Chemical Shifts, Line Widths, Spin Relaxation, and Spinning Sidebands. A complementary view of molecular organization in PC–TO mixtures is available from MAS ^1H NMR spectra such as those displayed in Figure 4 and Table IV. These data illustrate the good spectral resolution and fairly narrow lines (3–30 Hz) reported previously for lipid multilayers by us and by others (Forbes et al., 1988; Halladay et al., 1990), although the modest chemical shift dispersion of proton nuclei results in serious overlap for chemically similar resonances of the phospholipid and triglyceride constituents. Nonetheless, resonance assignments may be made for many proton types in the PC–TO mixtures since most peaks are narrowed, and it is possible to carry out two-dimensional correlated NMR experiments (COSY) (Aue et al., 1976). Figure 5 illustrates how $POCH_2$–CH_2N scalar interactions distinguish $POCH_2$ groups from overlapping CH_2O's; CHO–CH_2O cross peaks distinguish CHO protons from overlapping HC=CH's. PC groups that have unique signals in the ^1H NMR spectra of PC–TO mixtures include $POCH_2$, CH_2N, and $N(CH_3)_3$. Additional distinctions between α-CH_2 and β-CH_2 groups of the PC and TO constituents are revealed in two-dimensional nuclear Overhauser experiments (C. A. Tihal, K.-L. Li, and R. E. Stark, unpublished results). With the possible exception of the backbone CH_2O groups, no significant changes in chemical shift accompany formation of the mixtures.

It should be emphasized that 2-kHz MAS suffices to produce well-resolved ^1H signals for both TO and PC constituents of each mixture. Prior work on phospholipid dispersions and other smectic liquid crystals showed that less than 10% of the signal intensity was missing when the spectra were acquired with 2–3-kHz MAS (Forbes et al., 1988). For spectra of PC–TO mixtures such as those displayed in Figure 4, the ^1H NMR integrated intensities are in full accord with compositional predictions. Thus, coincident ω-CH_3 signals from the two components exhibit the expected intensities when compared with phospholipid $N(CH_3)_3$ and CH_2N signals, respectively. CH_2CH= protons from the PC and TO together have the anticipated integrals as compared with $N(CH_3)_3$ and CH_2N, respectively; the $N(CH_3)_3$ signal has the predicted intensity with respect to CH_2N.

Because quantitatively reliable high-resolution MAS ^1H NMR spectra are obtained for these dispersions, any line widths measured for overlapping acyl chain resonances will reflect the dynamic characteristics of both phospholipids and triglycerides in their mixed aggregates. The results of such

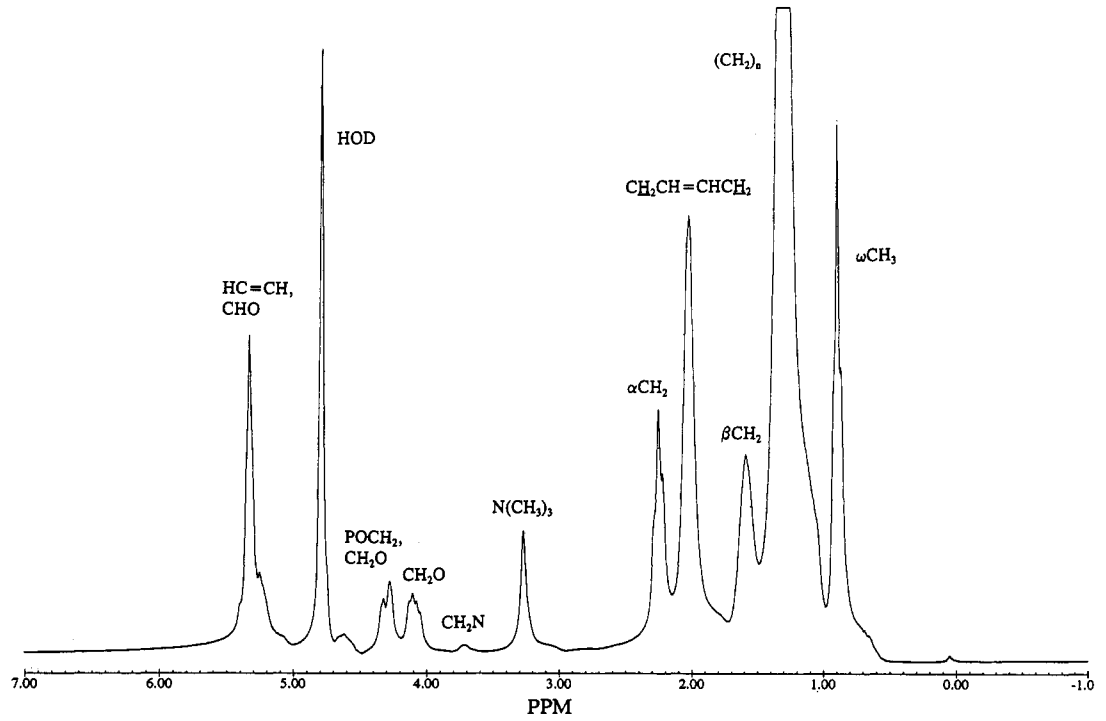

FIGURE 4: Center band region of the 200.13-MHz MAS ^{1}H NMR spectrum of an aqueous egg phosphatidylcholine–triolein dispersion with X_{PC} = 0.25 and ν_{rot} = 2030 Hz. The chemical shift scale is referenced to the ω-CH_3 resonance at 0.90 ppm, following Halladay et al. (1990). Complete spectral assignments are summarized in Table IV.

Table IV: ^{1}H Chemical Shifts (ppm) for Egg Phosphatidylcholine–Triolein Mixtures[a]

	X_{PC}			
proton type[b]	1.00	0.75	0.25	0.00
ω-CH_3	0.90	0.90	0.90	0.90
$(CH_2)_n$	1.30	1.30	1.33	1.32
β-CH_2	1.61[c]	1.59[c]	1.60[c]	1.60[c]
CH_2CH=$CHCH_2$	2.04[d]	2.02[d]	2.05[d]	2.04[d]
α-CH_2	2.36[c]	2.26[c]	2.27[c]	2.26[c]
C=CCH_2C=C	2.82	2.92	2.79[h]	
$N(CH_3)_3$	3.28	3.28	3.27[h]	
CH_2N	3.78[e]	3.70[e]	3.71[h]	
CH_2O	3.95[f,g]	4.08[f,g]	4.10[f,g]	4.08[f,g]
$POCH_2$	4.32[e]	4.30[e]		
CH_2O	4.22[f,g]	4.29[f,g]	4.32[f,g]	4.31[f,g]
CHO	5.26[g]	5.22[g]	5.25[g]	5.25[g]
HC=CH	5.35[d]	5.36[d]	5.36[d]	5.36[d]

[a] From MAS ^{1}H NMR experiments (phase-sensitive COSY and double-quantum-filtered phase-sensitive COSY) at 298 K. Chemical shifts are referenced to the ω-CH_3 resonance at 0.90 ppm, following Halladay et al. (1990). [b] Chemical structures for the major constituents of egg PC and for TO are shown in Figure 2. PC protons are shown in boldface; protons assigned to both PC and TO are italicized. [c] Verified by a β-CH_2–α-CH_2 cross peak. [d] Verified by a CH_2CH=$CHCH_2$–HC=CH cross peak. [e] Verified by a CH_2N–$POCH_2$ cross peak. [f] Verified by a CH_2O–CH_2O cross peak. [g] Verified by a CH_2O–CHO cross peak. [h] From 1D MAS spectrum.

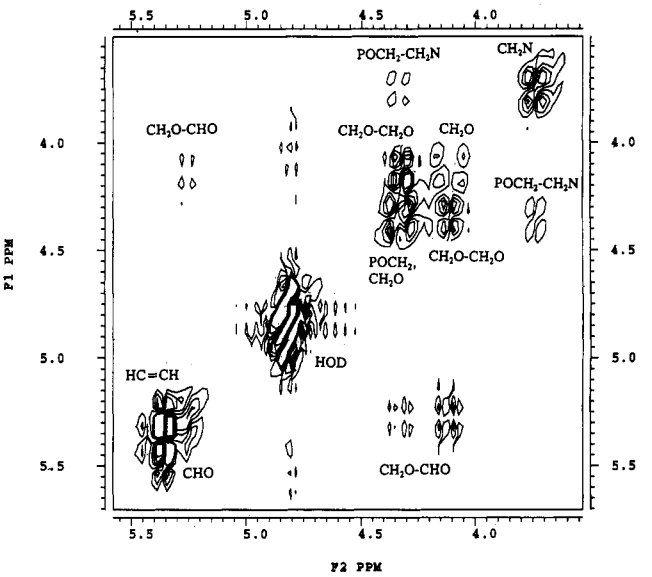

FIGURE 5: Downfield portion of the phase-sensitive COSY spectrum of a PC–TO mixture with X_{PC} = 0.75, acquired with 2100-Hz MAS. Complete spectral assignments are summarized in Table IV.

line-width determinations, made as a function of mixture composition and chemical grouping, are summarized in Figure 6. With rapid spinning (2000 Hz), we expect and find, in pure PC multilayers, that headgroup protons give the narrowest lines and that backbone proton signals are ~20 times broader (data not shown). This trend parallels the results described above in DPMASDD ^{13}C experiments. Whereas line widths for the headgroup protons remain constant as triolein is added to the PC multilayers, the acyl chain signals for the mixture with X_{PC} = 0.25 become even narrower than those in pure TO. In contrast to the ^{13}C data discussed above, the acyl chain regions of these MAS ^{1}H spectra exhibit no broad components from the PC, suggesting that neither chemical shift heterogeneity nor homonuclear dipolar interactions contribute significantly to the proton line widths.

That narrow methylene resonances for the X_{PC} = 0.25 mixture correspond to a highly mobile acyl chain organizational state is confirmed by monitoring the ^{1}H line width as a function of rotation speed (Figure 7, top). The narrow lines observed with 2000-Hz MAS are retained even with rotation as slow as 500 Hz, indicating a liquidlike state analogous to that reported for fruit tissue (Ni & Eads, 1992) and providing a distinct contrast with the chain behavior of pure PC liquid-crystalline dispersions (Figure 7, bottom). The concomitant loss of headgroup mobility upon PC–TO mixing is indicated by the sensitivity of $N(CH_3)_3$ line widths to rotation speed—again in contrast to the choline headgroup of pure PC multilayers. Thus, the 2000-Hz rotation speed is sufficient

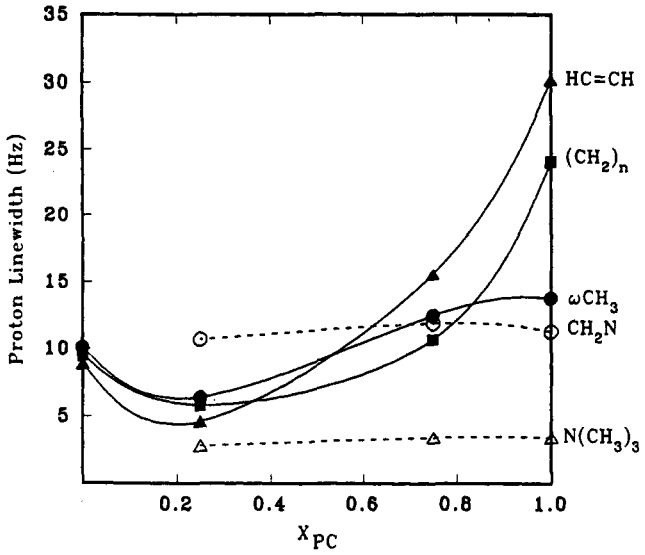

FIGURE 6: ^{1}H NMR line widths plotted as a function of PC–TO mixture composition for the center band region of spectra obtained with 2100-Hz MAS. Contributions from magnetic field inhomogeneities have been subtracted from the measured values.

to remove dipolar interactions among the choline methyl protons regardless of mixture composition, but spinning at 500 Hz produces fully narrowed resonances only in the mixtures.

For the mixture with X_{PC} = 0.75 (data not shown), the proton line widths are generally intermediate between values observed with mole fractions of 0.25 and 1.00, but the dependence of line width on rotation speed follows the behavior of the X_{PC} = 0.25 mixture described above. An aggregate organized so as to enhance acyl chain mobility and restrict headgroup motion is clearly indicated.

The observation of well-resolved MAS ^{1}H NMR spectra in liquid crystalline lipid bilayers has been attributed to axial rotation and lateral diffusion that remove both chemical shift anisotropy and intermolecular dipolar interactions, leaving only inhomogeneous dipolar effects as a broadening mechanism (Forbes et al., 1988; Wennerstrom, 1973; Bloom et al., 1977). The notion that numerous proton spins in PC–TO mixtures are nevertheless isolated from one another is confirmed in two ways. First, the spin-lattice relaxation results summarized in Table V show a sizeable dispersion in the values of T_1(H). Rather than a convergence of relaxation rates due to spin diffusion, pronounced differences are observed even for some protons on chemically bonded carbons (e.g., *H*C=CH and H_2CCH=CH; N(CH_3)$_3$ and CH_2N). Similar values of T_1(H) are measured for chain methylene protons proximal to the carbonyl groups (α-CH_2, β-CH_2), a trend that follows what is observed for the pure TO oil. Although these (nonselective) T_1 values should not be taken as a direct indicator of local molecular mobility, they do provide convincing evidence regarding the absence of spin diffusion.

Secondly, the spinning-sideband intensities displayed for the mixtures in Figure 8 increase monotonically as the rotation speed is lowered, yet the center-band peaks remain narrow. This type of behavior, which has been reported previously in other phospholipid multilayers (Halladay et al., 1990), suggests that *intermolecular* dipolar interactions are unimportant in the PC–TO systems. The MAS ^{1}H NMR spectra exhibit spinning sidebands only for the $(CH_2)_n$ resonances as expected; their magnitudes in pure egg PC are comparable to those of liquid-crystalline dispersions of saturated phospholipids (Forbes et al., 1988). The progressive decrease in sideband intensities,

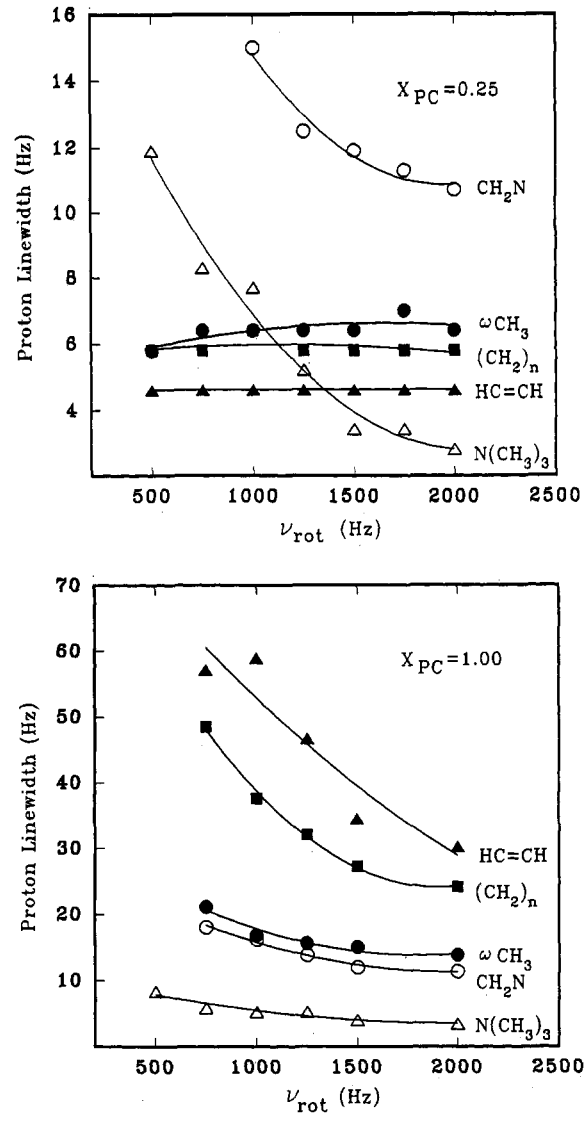

FIGURE 7: ^{1}H NMR line widths in the center band spectra of PC–TO aqueous dispersions, shown as a function of MAS conditions. The plots summarize measurements made for mixtures with X_{PC} = 0.25 and 1.00, respectively. Contributions from magnetic field inhomogeneities have been subtracted from the line widths.

which occurs as TO becomes a more important constituent, has two possible interpretations: (i) the PC acyl chain segments retain their bilayer order but are mixed with increasing proportions of disordered TO oil or (ii) acyl chains from both PC and TO are ordered to a smaller degree in the mixed aggregates.

DISCUSSION

The complementary use of ^{31}P, ^{13}C, and ^{1}H solid-state NMR methods represents a potent investigative strategy for lipid mixtures that form bilayer assemblies. With appropriate confirmation that all spins in the sample are observed and that the effects of spin diffusion are minimal, these methods provide a detailed and informative picture of molecular organization that may enhance our understanding of gastric and intestinal glyceride hydrolysis. To a surprising extent, this information is available without the introduction of isotopic labels and with the use of standard magic-angle spinning experiments. For egg PC–triolein mixtures, we conclude that most of the triglyceride remains in a mobile oil state, but its presence nevertheless produces significant alterations in the phospholipid bilayer.

Table V: ^{1}H Spin-Lattice Relaxation Times for Egg Phosphatidylcholine–Triolein Mixtures[a]

	T_1(H) (ms)				
proton type[b]	X_{PC} = 0.00	X_{PC} = 0.25	X_{PC} = 0.75	X_{PC} = 1.00[c]	$C_{18}C_{10}PC$[d]
ω-CH_3	697	792	798	1076	500
$(CH_2)_n$	314	322	367	517	400
β-CH_2	233	273	307		
$CH_2CH{=}CHCH_2$	260	304	307	370	
α-CH_2	239	278	284	258	310
$C{=}CCH_2C{=}C$				332	
$N(CH_3)_3$		269	295	353	350
CH_2N			262	212	310
CH_2O	186	219	257	187	330
$POCH_2$		221	258	187	
CH_2O	189	223		187	
HOD		821	996	696	
CHO	469	492	483	525	350
CH=CH	469	543	483	525	
$\bar{T}$[e]	323	396	508	488	364
$\sigma/\bar{T}$[e]	0.509	0.524	0.933	0.752	0.171

[a] Prepared as described under Experimental Procedures, with spin-relaxation times measured using an inversion-recovery experiment (Vold et al., 1968). The estimated precision, based on repeated measurements, is 10%. None of the recovery curves shows nonexponential behavior suggestive of multiple phases. [b] Chemical structures for the major constituents of egg PC and for TO are shown in Figure 2. [c] This work; reduced temperature (T_r) = 0.104. [d] From Halladay et al. (1990); T_r = 0.06. [e] $\bar{T}$ = average; $\sigma/\bar{T}$ = normalized standard deviation.

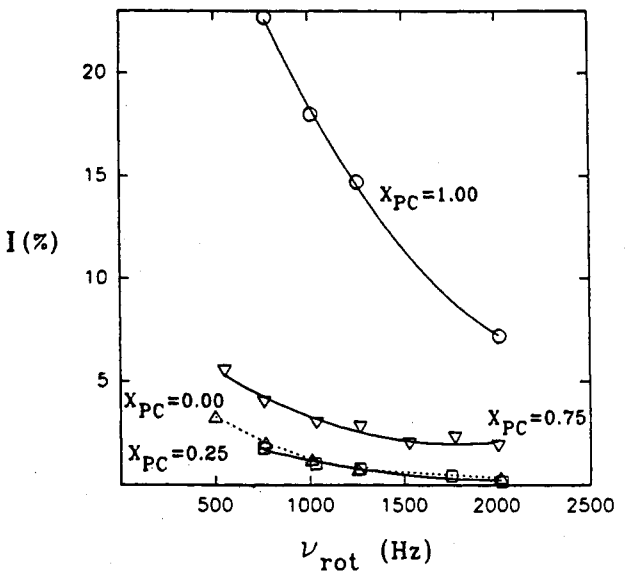

FIGURE 8: ^{1}H NMR spinning-sideband intensities (I) plotted as a function of rotation speed for the bulk methylene resonance in a series of PC–TO mixtures. The second set of sidebands, which are well resolved from the main spectrum at all spinning speeds, are measured as a percentage of the center band peak.

For the choline headgroup of egg PC, a hydrophilic moiety that is located at the aqueous interface of typical bilayer aggregates, ^{31}P MAS sideband intensities are clearly diminished in both mixed dispersions. These changes could be attributed to more rapid reorientation (motional averaging of the chemical shift tensor), but this possibility is ruled out by changes in the corresponding regions of both ^{13}C and ^{1}H NMR spectra. For instance, the sensitivity of ^{13}C line widths to decoupling strength implies that headgroup motions are not rapid enough to fully average heteronuclear dipolar interactions in the CH_2OP and CH_2N groups. The broadening of ^{1}H resonances for these groups at slow MAS rotation speeds, which probably reflects residual homonuclear dipolar interactions, shows that the various headgroup protons experience *greater* motional restrictions in PC–TO assemblies than in pure PC.

The perturbed static line shape and correspondingly altered ^{31}P sideband intensities in TO-rich mixtures could also stem from changes in the headgroup orientation with respect to the phospholipid bilayer normal. For instance, Thayer and Kohler (1981) have demonstrated the sensitivity of ^{31}P NMR line shapes to headgroup conformation in liquid-crystalline multilayers. Several groups of researchers have noted the sensitivity of ^{31}P shift anisotropies to headgroup conformation in both pure and mixed PC bilayer assemblies (Gally et al., 1975; Banerjee et al., 1985). Nonetheless, the ^{31}P spectral changes observed in the present study would indicate modest changes in headgroup conformation.

A number of structural arrangements could account for the anomalous ^{31}P NMR spectra observed for the mixed-lipid dispersion with X_{PC} = 0.25. If a small amount of non-bilayer PC phase (monolayer or hexagonal structure) were present, its static ^{31}P powder pattern could appear as a "bump" superimposed on the liquid-crystalline spectrum. Because such structures have diminished chemical shift anisotropy but identical average resonance position with respect to the liquid-crystalline phase, the resulting MAS spectra would have smaller sidebands at the same frequencies as pure PC.

The broad spectral feature observed with slow spinning is not attributable to residual ^{31}P chemical shift anisotropy, since this effect is sufficiently small that it fails to contribute to the spin-lattice relaxation of hydrated phospholipids at these field strengths (Yeagle, 1987). Neither is it likely to result from insufficient proton decoupling, since even static P–H dipolar interactions for phospholipids are no more than one-eighth the magnitude of our decoupling field (Chan et al., 1980). If the choline headgroups are tightly packed and motionally restricted in mixed PC–TO multilayers (*vide supra*), P–P dipolar interactions could exceed 1000 Hz and thus produce a broad background at spinning speeds below this threshold. The P–P broadening effects are expected to be insensitive to ^{1}H decoupling; in contrast to shift anisotropy, they are not removed with low-speed spinning (Stejskal et al., 1977).

Irrespective of whether additional phases are present, it appears from ^{31}P, ^{13}C, and ^{1}H measurements that bilayer-incorporated TO produces a more crowded headgroup region near the aqueous interface, and the TO oil at the interior of the aggregate has a similar impact on headgroups of the inner phospholipid monolayer. Thus, despite its hydrophobic character, the triolein constituent changes both the structure and dynamic properties (orientation, angular excursions, rates of motion) for the polar phospholipid headgroup.

The ability of triolein to influence the organizational state of the phospholipid bilayer, over a broad compositional range and without undergoing its own alterations, is also demonstrated for the glycerol backbone and nearby carbonyl groups. When the ^{13}C spectra obtained with dipolar and scalar decoupling are compared, motional restrictions are evident in both pure PC dispersions and their mixtures with TO. In particular, the formation of mixed assemblies renders the restricted PC carbonyl motions even more sluggish, yet leaves the backbone and carbonyls of the TO quite fluid. Both ^{1}H and ^{13}C NMR line-width measurements provide evidence for the rigidity of glycerol backbone carbons, confirming the conclusions of spin-relaxation experiments in related lipid systems (Halladay et al., 1990; Wang et al., 1992). It is notable that, whereas PC backbone resonances are often too broad to be observed in the mixed dispersions, the corresponding TO signals are strong and well resolved. Even in a mixture with

$X_{PC} = 0.75$, these observations confirm that most of the triolein is found within a pool of oil, whereas the phospholipid is exposed to both aqueous and oily environments by its bilayer organization.

Finally, the spectral properties of the acyl chain resonances indicate enhanced mobility but retention of much of the PC bilayer order. Regardless of the mixture composition, the chains of the triglyceride molecule remain highly mobile as judged from the sensitivity of ^{13}C line widths to decoupling strength and the magnitude of ^{1}H line widths at all rotation speeds. The acyl chains of the phospholipid contribute fully to the ^{1}H MAS spectrum and appear to be very mobile as judged from line widths and spin-lattice relaxation times. These results are expected for the liquid-crystalline state, since ^{1}H–^{1}H dipolar interactions will be reduced by lateral diffusion and axial rotation (Wennerstrom, 1973). Nevertheless, the retention of molecular order, i.e., restricted angular excursions for the chain segments (Chan et al., 1981; Davis, 1983), is confirmed by the observation of ^{1}H spinning sidebands and incompletely resolved ^{13}C resonances under DPMASSD conditions. The resulting physical picture for the acyl chains is that of a loosely packed but intact phospholipid bilayer, perhaps with small amounts of another ordered structure.

Although a recent ^{13}C NMR study found that small amounts of triolein exerted no effect on egg PC bilayer organization (Hamilton et al., 1991), even small amounts of unsaturated diglycerides promote the formation of hexagonal or isotropic phases (Dawson et al., 1984; Siegel et al., 1989; De Boeck & Zidovetzki, 1989). With the larger proportions of triolein used in our studies, the mixing tendency of the PC and TO constituents presumably overcomes the unfavorable TO–water interactions that accompany triglyceride insertion within the PC bilayer structure. Most TO molecules remain sequestered from their aqueous environment, but our results demonstrate the organizational impact of incorporating a small fraction of them within the PC bilayer. We find more rigid phosphodiester carbonyl groups, glycerol backbones, and choline headgroups in the mixed dispersion, arguing against a structural model in which the polar part of the triglyceride spreads the PC headgroups further apart at the aqueous interface (Hamilton et al., 1991). Moreover, our measurements of spin relaxation and order indicate loose packing of the PC acyl chains, which could in turn facilitate the shuttling of triglyceride substrate molecules to an interfacial hydrolysis site. Thus, despite the modest equilibrium population of triglyceride which is accessible to water-soluble lipases, loosening of the PC bilayer may permit rapid replenishment of those substrate molecules at the aggregate surface. Additional 2D NMR studies and kinetic assays are in progress to verify these hypotheses.

ACKNOWLEDGMENT

We thank Dr. A. Bielecki and Bruker Instruments, Inc., for performing ^{31}P experiments at 121 MHz. We also acknowledge useful discussions with Drs. A. Bielecki, G. S. Harbison, and J. E. Roberts during the course of this work.

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