Interactions of Lyso 1-Palmitoylphosphatidylcholine with Phospholipids: A ^{13}C and ^{31}P NMR Study†

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ABSTRACT: ^{13}C and ^{31}P NMR spectroscopy were used to monitor interactions of lyso 1-palmitoylphosphatidylcholine (LPPC) in the interfacial region of egg phosphatidylcholine (PC) bilayers and determine the effect of LPPC on the phospholipid bilayer structure. ^{13}C NMR spectroscopy of small amounts (0.5−10 mol %) of ^{13}C carbonyl-enriched LPPC cosonicated with egg PC to form small unilamellar vesicles (SUVs) revealed separate carbonyl signals for LPPC in the inner and outer leaflets of the vesicles. The ratio of LPPC in the outer leaflet to that in the inner leaflet was ≥3/1. Exchange of LPPC between bilayer leaflets ("flip-flop") was too slow to be measured ($t_{1/2}$ >12 h). Albumin added to the external buffer of LPPC/PC vesicles was shown by ^{13}C NMR to extract LPPC only from the outer leaflet. LPPC was a poor detergent in egg PC multilayers and SUVs. Stable SUVs were prepared by cosonicating egg PC with up to 30 mol % LPPC, and preformed SUVs incorporated up to 40 mol % of LPPC (added as an aqueous solution) without undergoing any morphological changes as evidenced by ^{31}P NMR spectroscopy. The presence of oleic or palmitic acid did not have observable effects on properties of LPPC in SUVs, such as the localization of the LPPC carbonyl in the interface, and the transbilayer distribution and movement of LPPC. The apparent pK_a of the fatty acid (FA) carboxyl at the membrane interface (7.7) measured by ^{13}C NMR was not affected by LPPC, but the FA carboxyl carbon resonance showed linewidth changes near the apparent pK_a that were dependent on the FA/LPPC ratio. These data suggest weak interactions in the interfacial region between FA and LPPC when both lipids are present at low levels in PC vesicles.

Lysophospholipid is a key intermediate in phospholipid metabolism (Van den Bosch, 1974) and occurs as a minor constituent in various cell membranes (Ansell et al., 1973). *In vivo*, phospholipase A_2 (PLA_2)[1] catalyzes the hydrolysis of the ester bond at the *sn*-2 position of 1,2-diacyl phospholipids, generating lyso 1-acyl phospholipid and free fatty acid (FA). Lysophospholipid is also produced by lecithin:cholesterol acyltransferase (LCAT) action on plasma lipoproteins. Lysophospholipid produced by either PLA_2 or LCAT under normal conditions is rapidly reacylated (Eibl et al., 1969; Van Golde et al., 1971) or further metabolized (Pelech & Vance, 1989). Abnormal accumulations of lysophospholipid in cell membranes induce morphological changes in cells (Klibansky & De Vrier, 1963), mediate cell fusion (Poole et al., 1970), and cause hemolysis (Reman et al., 1969; Weltzein et al., 1976). Elevation of lysophospholipid levels in ischemic myocardium has been correlated with cardiac arrhythmias in a number of clinical studies (Kinnaird et al., 1988; Katz & Messineo, 1981; Vadas & Pruzanski, 1986). However, in recent years, low levels of lysophosphatidylcholine (lysoPC) have been shown to exert quite different effects, for example, inhibition of model (Yeagle et al., 1994) and biological membrane fusion (Chernomordik et al., 1993). LysoPC generated from PLA_2 hydrolysis of PC in response to stimulation by growth factors and agonists has been suggested to potentiate the activation of protein kinase C by the second messenger diacylglycerol and thus may play a crucial role in cell proliferation and differentiation (Asaoka et al., 1992; Nishizuka, 1992; Sasaki et al., 1993). Lysophospholipid has also been shown to affect the permeability properties of phospholipid model membranes (Lee & Chan, 1977).

FA are formed in equimolar amounts along with lysophospholipid by PLA_2 action on membranes, and the properties and effects of lysophospholipid on membranes may depend on the amount of FA present. For example, in erythrocyte membranes lysophospholipid and FA generated by PLA_2 action did not destabilize the membrane even after extensive hydrolysis (Haest et al., 1981). LysoPC forms lamellar structures with long-chain FA as well as cholesterol when mixed in equimolar ratio (Jain et al., 1980; Klopfenstein et al., 1974; Ramsammy & Brockerhoff, 1982; Kumar et al., 1986; Ramsammy et al., 1984; Bhamidipati & Hamilton, 1993a). Jain and co-workers (1981) proposed that, in equimolar mixtures of lysoPC and FA, the acyl chains of the two lipid components pack parallel to one another as in diacyl phospholipid, and the free hydroxyl group of lysoPC and the carboxyl group of FA are in close proximity. ^2H

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[1] Abbreviations: BSA, bovine serum albumin; CSA, chemical shift anisotropy; FA, fatty acid; LCAT, lecithin:cholesterol acyltransferase; LPPC, lyso 1-palmitoyl-*sn*-3-phosphatidylcholine; MAS, magic angle spinning; NOE, nuclear Overhauser enhancement; OA, oleic acid; PA, palmitic acid; PC, phosphatidylcholine; PLA_2, phospholipase A_2; POPC, 1-palmitoyl-2-oleoyl-PC; SUVs, small unlamellar vesicles; TLC, thin layer chromatography; T_1, spin lattice relaxation time.

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and ^{31}P NMR studies of equimolar mixtures of LPPC and palmitic acid confirmed the parallel packing of the acyl chains of lysoPC and FA, but interactions between the carboxyl and the hydroxyl groups of these lipid components were not investigated (Allegrini et al., 1983; Brentel et al., 1987).

Despite accumulating evidence that lysophospholipids can exert a wide variety of physiological and morphological effects on cell membranes, the interactions of lysoPC in membranes are incompletely characterized. It has been shown that up to 30 mol % of lysoPC can be incorporated into multilamellar phospholipid dispersions before the bilayer structure is disrupted (Van Echteld et al., 1981) and that incorporation of 30 mol % of lysoPC into SUVs actually stabilizes the phospholipid bilayer structure (Kumar et al., 1989). ^{13}C NMR spectroscopy of lysoPC with ^{13}C enrichment in the choline methyl groups has been employed to measure lysoPC distribution and transbilayer movement in model membranes, although the use of paramagnetic shift reagents in these experiments may perturb the native behavior of lysoPC (De Kruijff et al., 1977; Van Den Besselaar et al., 1979). A less direct measurement of the distribution of lysoPC in the leaflets of SUVs was made by an enzymatic assay (Van Den Besselaar et al., 1977). The transbilayer distribution and movement of lysoPC in erythrocyte membranes have also been studied by extraction of radiolabeled lysoPC with bovine serum albumin (BSA) and enzymatic assays (Van Den Besselaar et al., 1977; Mohandas et al., 1982; Bergmann et al., 1984a,b). The protocols used in these studies required separation of the protein and the membrane components to quantitate the amount of lysoPC in these fractions, and their validity has not been tested in simple model systems.

In the present study, we have used ^{13}C NMR spectroscopy of LPPC with ^{13}C enrichment of the ester carbonyl group to monitor interactions in the interfacial region of model membranes. Egg PC was used as a model membrane because PC is the predominant phospholipid in most membranes and the mixed acyl chains of egg PC are representative of those in typical biological membranes. LPPC (lyso 1-palmitoyl-PC) represents the major species of lysoPC that would be generated from PC in membranes. The carbonyl carbon was chosen for ^{13}C enrichment because of the high sensitivity of the carbonyl ^{13}C chemical shift to changes in the local magnetic environment (e.g., H-bonding and proximity of polar groups) and the usefulness of ^{13}C NMR for monitoring small amounts of ^{13}C-enriched lipids in model membranes (Bhamidipati & Hamilton, 1989; Boylan & Hamilton, 1992; Cabral et al., 1986). ^{13}C NMR studies presented here examined low proportions of LPPC in phospholipid bilayers, in the presence and absence of FA. Egg PC vesicles containing large (up to 50 mol %) amounts of LPPC were examined by ^{31}P NMR and ^{13}C spectroscopy to monitor the disruption of lamellar structure, as postulated to occur in pathophysiological circumstances. Additionally, the partitioning of LPPC between vesicles and BSA was determined directly by ^{13}C NMR without separation of the components.

MATERIALS AND METHODS

Chemicals. Egg PC was purchased from Lipid Products (Nutley, England) or Avanti Polar Lipids (Pelham, AL) and unenriched LPPC from Avanti Polar Lipids (Pelham, AL). These lipids were used without further purification after analysis by thin layer chromatography (TLC) showed no detectable amounts of any impurities. Essentially FA-free BSA and phospholipase A_2 (*Crotalus adamanteus*) were obtained from Sigma Chemical Co. (St. Louis, MO). [1-^{13}C]-Palmitic acid and [1-^{13}C]oleic acid with 99% ^{13}C enrichment were obtained from Stohler Isotopes Inc. (Cambridge, MA).

Synthesis of lyso 1-[1-^{13}C]Palmitoyl-sn-3-phosphatidylcholine (LPPC). ^{13}C-enriched LPPC was prepared by PLA_2 (*C. adamanteus*) hydrolysis of 1,2-[1-^{13}C]dipalmitoyl-*sn*-3-phosphatidylcholine (DPPC) in moist ether medium (Mason et al., 1981). ^{13}C-enriched DPPC (20 mg) was dissolved in 1.0 mL of methanol and 19 mL of diethyl ether. PLA_2 enzyme (250 μL; equivalent to 20 units of activity) in 50 mM Tris buffer containing 20 mM $CaCl_2$ (pH 7.4) was added and the reaction mixture agitated by occasional manual shaking. LPPC precipitated as the hydrolysis proceeded, and the progress of the reaction was monitored by TLC in a polar solvent system (65:25:4:1 chloroform/methanol/water/acetic acid). At the end of the reaction, the LPPC precipitate was centrifuged to remove the ether layer containing FA. The residue was redissolved in a minimum amount of methanol (~2 mL) and distributed into six 20 mL conical test tubes. Cold diethyl ether (15 mL) was added to each of the tubes and stored at −20 °C for 4 h to precipitate LPPC. This process was repeated four times to remove trace amounts of FA. LPPC[2] thus prepared was isomerically pure as determined by TLC and analytical ^{31}P and ^{13}C NMR spectra.

Sample Preparation. For NMR spectra of LPPC[3] in organic solvents, about 1−2 mg of the sample was dried in the NMR tube and redissolved in 2.0 mL of the deuterated organic solvent. Aqueous micellar solutions of LPPC were obtained by diluting a stock solution (18 mM) of the lysoPC in 75 mM KCl (pH ~ 7.0). Aqueous lysoPC samples (~5 mM) were used to determine the ^{31}P NMR chemical shift as a function of temperature.

For phospholipid vesicle preparation, measured amounts of egg PC and other lipid components (i.e., ^{13}C-enriched LPPC, oleic acid, and palmitic acid) were co-dried from chloroform/methanol solutions under nitrogen to form a thin film and further dried under vacuum for 4−6 h to remove traces of organic solvents. The lipid film was hydrated by vortexing with 2.0 mL of 75 mM KCl or 10 mM Tris buffer (pH 7.4) containing 10−15% D_2O, and the lipid dispersion was sonicated in a pulsed mode on a Branson 350 type sonicator for 50−60 min (until the sample became almost clear with a blue tinge) under nitrogen atmosphere. The sonicated vesicle samples were centrifuged at 35 000 rpm

[2] The LPPC used in all the studies reported here is the 1-isomer (lyso 1-palmitoyl-*sn*-3-phosphatidylcholine) and contained no detectable amounts of the 2-isomer (lyso 2-palmitoyl-*sn*-3-phosphatidylcholine). Since acyl chain migration can occur under certain conditions (Dennis & Pluckthun, 1984), experiments were carried out to ascertain that the LPPC used in the present studies did not undergo significant acyl chain migration. Two samples of LPPC in aqueous solution were incubated in a water bath at 50 °C and monitored by ^{31}P NMR as a function of time. NMR spectra obtained after 0, 1, 2, and 7 days showed a strong signal at −0.24 ppm corresponding to the 1-isomer. A relatively small signal at −0.42 ppm corresponding to the 2-isomer was seen after 1 day of incubation and increased with time, but was <10% of the total signal after 1 week of incubation at 50 °C.

[3] ^{13}C-enriched LPPC was used in ^{13}C NMR experiments and unenriched LPPC in ^{31}P NMR experiments unless otherwise explicitly mentioned.

in a fixed angle 50.3Ti rotor for at least 1 h on a Beckman ultracentrifuge (Model L8-70) at 20 °C to remove small amounts of undispersed lipid and metallic particles. Vesicle samples containing ^{13}C LPPC were analyzed by ^{31}P NMR at 81.0 MHz on a Bruker WP-200 instrument to ensure that additional lysoPC was not produced during sonication. Lipid vesicle samples were subjected to qualitative analysis by TLC following a Folch extraction in a polar solvent, viz., chloroform/methanol/water/acetic acid (65:30:4:1 v/v), to ensure lipid integrity. Samples were also subjected to quantitative chemical analysis (Bartlett, 1959) for PC and lysoPC and gas chromatography for FA quantitation following separation by TLC in polar solvent. The concentration of egg PC in vesicle samples was 50−75 mg/mL. Sample concentrations are expressed as mol % LPPC with respect to total lipids.

Protein Sample Preparation. Aqueous BSA was prepared by dissolving the protein in 75 mM KCl solution and dialyzing against 75 mM KCl for 24 h. Protein samples were filtered at least once through 0.2 μm acrodisc filters to remove insoluble, light scattering fibers. Final protein concentration was determined after the pH was adjusted to 7.2 with 0.1 N KOH, using 100-fold diluted samples by measuring the absorbance at 279 nm (Janatova et al., 1968); typical protein concentrations used were 90−100 mg/mL (~1.5 mM).

NMR Measurements. ^{13}C NMR spectra were recorded at 50.3 MHz on a Bruker WP-200 NMR instrument or at 75.0 MHz on a Bruker AMX-300 NMR instrument. ^{13}C NMR spectra at 75.0 MHz were acquired with a 90° pulse (8.5 μs), 16K data points, a 2.0 s pulse interval, and composite pulse ^{1}H decoupling. ^{13}C NMR spectra at 50.3 MHz were acquired with a 90° pulse (14.0 μs), 16K data points, a 2.0 s pulse interval, and broad-band ^{1}H decoupling. Spin−lattice relaxation times (T_1) were measured using a fast inversion−recovery method (Canet et al., 1975) and calculated with a three-parameter exponential fitting (Sass & Ziessow, 1977). Nuclear Overhauser enhancement (NOE) was measured as the ratio of integrated intensities (±10% accuracy) with broad-band and inverse-gated decoupling (Opella et al., 1976). ^{31}P NMR spectra at 121.0 MHz were acquired with a 90° pulse (14.0 μs), 8K data points, a 1.5 s pulse interval, and composite pulse ^{1}H decoupling. ^{31}P NMR spectra of vesicle samples at 81.0 MHz were recorded on a Bruker WP-200 NMR instrument as described elsewhere (Bhamidipati & Hamilton, 1993b). All spectra were acquired at 30−32 °C unless mentioned otherwise. Data were typically processed using an exponential multiplication function with a line broadening factor of 1−3 Hz and in some cases using a Gaussian multiplication function (line narrowing). Chemical shifts and linewidths were measured digitally and referenced with respect to the following: (i) the FA methyl signal at 14.10 ppm, for ^{13}C spectra of phospholipid vesicle samples; (ii) internal TMS, for ^{13}C spectra in organic solvents; and (iii) external phosphoric acid, for ^{31}P spectra.

For magic angle spinning (MAS) ^{13}C NMR experiments, the sample was prepared as follows. A lipid film containing [^{13}C]LPPC (1.5 mg) and egg PC (75 mg) was hydrated in 0.2 mL of 75 mM KCl by gentle swirling of the sample (avoiding vigorous vortex mixing). The sample was stored overnight at 4 °C under argon atmosphere and then loaded into a CRAMPS (combined rotation and multiple pulse spectroscopy) insert. Spectra were obtained at room temperature on a Bruker AMX-300 instrument at 75.0 MHz equipped with a solid state MAS accessory. After MAS NMR experiments, the lipid sample was recovered as completely as possible from the insert by washing several times and pooling the washes. D_2O (0.2 mL) was added to this lipid dispersion, which was brought to a final volume of 2.0 mL with 75 mM KCl and sonicated under nitrogen atmosphere as described above to obtain SUVs for a solution ^{13}C NMR spectrum of the same sample.

Table 1: ^{13}C and ^{31}P NMR Chemical Shift Data for LPPC in Different Solvent Systems at 30 °C

solvent system	chemical shift (ppm)	
	^{13}C	^{31}P
C^2HCl_3	174.00[a]	−4.10[b]
$C^2HCl_3/C^2H_3O^2H$ (2:1)	174.73[a]	−3.27[b]
$C^2H_3O^2H$	175.60[a]	−2.27[b]
SDS micelles	175.25[c]	−0.15[e]
75 mM KCl (pH = 7.0)	175.00[c]	−0.35[e]
PC bilayers	174.73[c] (outer)	−0.53[e]
	174.55 (inner)	−0.66
BSA bound	175.40[d]	−0.45[e]

[a] Chemical shifts were measured with respect to TMS as internal standard, and the lipid concentration is about 2 mg/mL. [b] Chemical shifts were measured with respect to TMP as external standard. [c] Chemical shifts were measured with respect to the terminal methyl signal at 14.10 ppm. [d] Chemical shifts were measured with respect to external TMS. [e] Chemical shifts were measured with respect to external H_3PO_4.

Electron Microscopy. Cosonicated samples of egg PC and LPPC were examined by electron microscopy (EM). Samples were prepared by dilution of vesicle samples with buffer to 0.5−1.0 mg/mL lipid and applied to 400-mesh copper grids covered with carbon-coated Formvar and stained with 1% sodium phosphotungstate solution at pH 7.5. After drying in air, samples were examined with an Hitachi 11-C electron microscope. Egg PC vesicle samples containing small amounts of LPPC showed a uniform population with a size distribution in the range of 20−30 nm diameter (data not shown). Samples containing large amounts of LPPC could not be visualized because of uneven distribution of the stain.

RESULTS

LPPC in Organic Solvents. ^{13}C and ^{31}P NMR spectra of LPPC at a low concentration (5 mM) in three organic solvents were obtained to assess the sensitivities of the ester carbonyl ^{13}C chemical shift and the phosphate headgroup ^{31}P chemical shift to changes in solvent polarity. The chosen solvents were chloroform, chloroform/methanol (2:1), and methanol (all perdeuterated). The observed dependence of the carbonyl chemical shift (in ^{13}C NMR) with solvent polarity (Table 1) indicates solvent interactions with the expected trend of downfield shifts with increasing polarity/H-bonding (Hamilton & Small, 1981). The ^{31}P chemical shift showed a downfield shift with increasing solvent polarity that was larger than that for the carbonyl group (Table 1).

LPPC in Aqueous Solution. In aqueous solution (75 mM KCl, pH 7.0) at 32 °C, LPPC showed a single ^{13}C carbonyl signal at 175.00 ppm and a single phosphate signal at −0.35 ppm. ^{13}C and ^{31}P chemical shifts and linewidths were independent of concentration (1−18 mM) and pH (4−10). The observed chemical shifts correspond to LPPC in micellar form, since the critical micelle concentration for LPPC is in

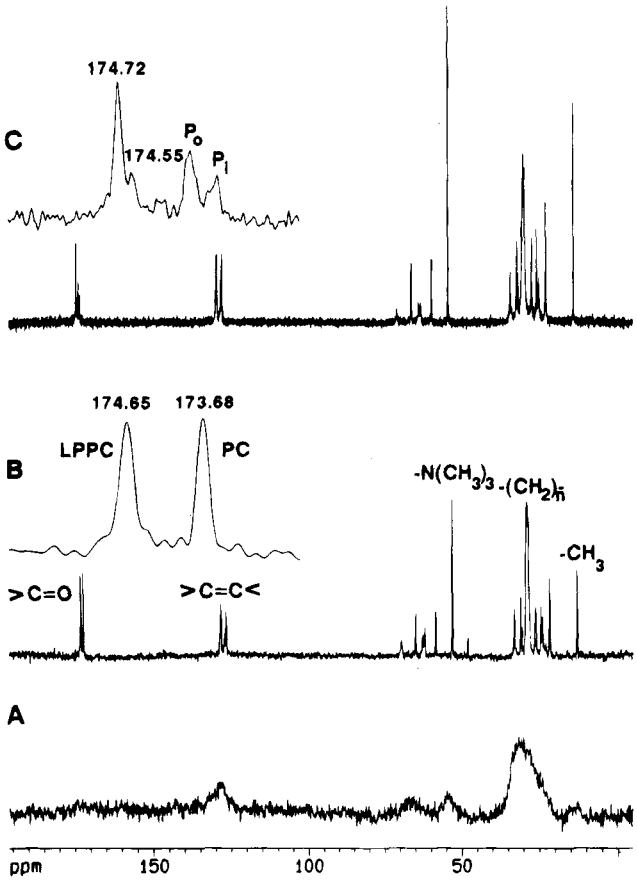

FIGURE 1: 75.0 MHz ^{13}C NMR spectra of 3 mol % [^{13}C]LPPC/97 mol % EPC mixture in 75 mM KCl (pH = 7.4) at 32 °C: (A) unsonicated dispersion of the sample oriented at magic angle but without spinning; (B) same as (A) but the sample was subjected to 2 kHz spinning; and (C) high-resolution ^{13}C NMR spectrum of the same sample following sonication as described under Materials and Methods. Insets show the expansions for the carbonyl region. These spectra correspond to 12 288, 2048, and 4096 accumulations, respectively. Spectra in (A) and (B) were processed using exponential multiplication function with 3.0 Hz line broadening, and the spectrum in (C) was processed using Gaussian multiplication function. In spectrum B, PC peaks are designated as CH_3 for the terminal methyl, $(CH_2)_n$ for the methylene, C=C for the olefin groups of the acyl chains, $N(CH_3)_3$ for the choline methyl groups, and C=O for the ester carbonyl groups. In spectrum C, P_o and P_i represent the PC carbonyl groups in the outer and inner leaflets of the bilayer. Although the chemical shifts were measured with reference to terminal methyl group of PC fatty acyl chains at 14.10 ppm in both panels B and C, the chemical shifts observed for PC and LPPC carbonyl groups were slightly different.

the micromolar range (Stafford et al., 1989). The phosphate ^{31}P chemical shift of aqueous LPPC shifted from −0.30 ppm at 38 °C to −0.50 ppm at 15 °C without any significant change in linewidth, whereas the ^{13}C carbonyl signal at 175.00 ppm was unaffected in this temperature range. The observed temperature-dependent ^{31}P NMR changes may be due to changes in the phosphate group orientation (Dennis & Pluckthun, 1984). The ^{13}C carbonyl and ^{31}P phosphate peaks shifted downfield to 175.25 and −0.15 ppm, respectively, when LPPC was incorporated in SDS micelles.

LPPC Interactions with Egg PC Bilayers. The interactions of LPPC with egg PC model membranes were studied at various LPPC/PC mole ratios in multilamellar dispersions or sonicated unilamellar vesicles by ^{13}C and ^{31}P NMR spectroscopy. Figure 1 compares ^{13}C NMR spectra of an unsonicated and sonicated dispersions of 3 mol % [^{13}C]-LPPC/97 mol % egg PC at pH 7.2 and 30 °C. The spectrum of the unsonicated sample (Figure 1A) without spinning showed broad lines characteristic of multilamellar dispersions of phospholipids (Hamilton et al., 1991b). When the sample was subjected to fast spinning (2 kHz) at the magic angle, the broad spectrum collapsed into a high-resolution spectrum with narrow signals corresponding to each functional moiety of the phospholipid (Figure 1B). The resolution in MAS spectrum is comparable to that for SUVs seen in Figure 1C, and the two spectra are very similar except for the carbonyl region. The carbonyl region of the MAS spectrum (Figure 1B) consisted of two signals at 173.67 and 174.57 ppm, which correspond to PC and LPPC carbonyl groups, respectively, since unsonicated dispersions of egg PC alone give rise to a signal at 173.7 ppm in MASNMR spectra (Hamilton et al., 1991b). The signal for LPPC was shifted ~0.40 ppm upfield from the signal for micellar LPPC (see Table 1). An upfield shift of a carbonyl signal signifies a less polar environment surrounding this group and is an expected consequence of LPPC binding to phospholipid bilayers, which causes partial dehydration of the carbonyl group (Hamilton & Small, 1981). Note that the MASNMR spectrum does not show separate signals corresponding to lipids in the two halves of the bilayer (see Discussion).

The high-resolution ^{13}C NMR spectrum of small unilamellar vesicles (SUVs) obtained after sonication of the same dispersion (see Materials and Methods) showed two carbonyl signals with an intensity ratio of ~2:1 (labeled P_o and P_i in Figure 1C), corresponding to phospholipids in the outer and inner leaflets of the vesicles. This chemical shift inhomogeneity is a consequence of the high curvature of sonicated vesicles and the differential hydration of carbonyl groups in the two leaflets (Schmidt et al., 1977). The carbonyl group of LPPC also showed two signals, but with an intensity ratio of ~3:1. These peaks at 174.73 and 174.55 ppm were assigned to LPPC in the outer and inner monolayers, respectively (see below). The difference in the observed chemical shifts for outer and inner monolayer LPPC (~0.20 ppm) is similar to that for PC carbonyl groups and is likely also a result of differential hydration. The intensity ratio of the LPPC carbonyl signals shows that LPPC does not have the same distribution in the bilayer leaflets as the phospholipid but prefers the outer monolayer. At this proportion of LPPC in PC vesicles, no detectable effects on the phospholipid bilayer structure, as reflected in the chemical shifts and linewidths of PC resonances, were seen in either the MASNMR spectrum of multilamellar dispersions or the high-resolution spectrum of sonicated vesicles.

Binding of low amounts of [^{13}C]LPPC to egg PC vesicles was studied in detail by ^{13}C NMR spectroscopy. These experiments also allowed evaluation of the distribution and transbilayer movement of LPPC in vesicle bilayers. SUVs containing 0.5−10 mol % of [^{13}C]LPPC in egg PC were prepared by cosonicating the two lipid components or by adding [^{13}C]LPPC in aqueous solution to preformed PC vesicles. Figure 2 shows the carbonyl region of the ^{13}C NMR spectra of egg PC vesicles at 32 °C with 0.6, 1.5, 3.0, and 5.0 mol % [^{13}C]LPPC prepared by cosonication (Figure 2A−D) and of PC vesicles to which aqueous [^{13}C]LPPC was added at the indicated molar compositions (Figure 2E−H). The two signals designated P_o and P_i at 173.85 and 173.55 ppm correspond to the carbonyl carbons of egg PC in the

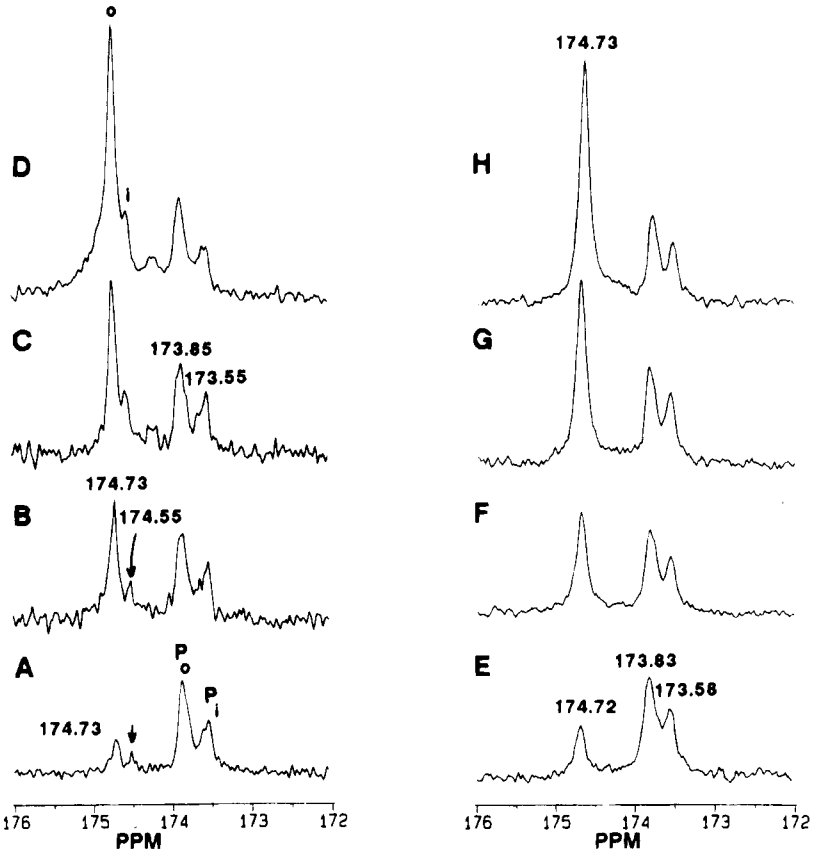

FIGURE 2: 75 MHz ^{13}C NMR spectra of LPPC incorporation in egg PC bilayers. Spectra in the left panel correspond to cosonicated samples of (A) 0.6, (B) 1.5, (C) 3.0, and (D) 5.0 mol % [^{13}C]LPPC and egg PC SUVs in 75 mM KCl (pH = 7.4) at 32 °C. Spectra in the right-hand panel correspond to egg PC vesicles to which [^{13}C]LPPC was added exogenously as an aqueous solution corresponding to 0.6, 1.9, 2.8, and 4.0 mol % LPPC (E−H). Spectra in A−D represent 10 240, 5120, 10 240, and 4096 accumulations, respectively, and were processed using a Gaussian multiplication function. Spectra in E−H correspond to 2000 scans each and were processed using an exponential multiplication function with 2 Hz line broadening. LPPC in the outer and inner monolayer is designated o and i in D.

outer and the inner monolayers of the vesicles, as in Figure 1C. The relative intensity ratio ($P_o/P_i \sim 2.0$) reflects the small size (~250 Å) of the vesicles (Schmidt et al., 1977). Cosonicated vesicles of all compositions studied here (Figure 2A−D) also showed signals at 174.73 and 174.55 ppm, corresponding to the *sn*-1 ester carbonyl of the [^{13}C]LPPC in the outer and inner leaflets of the vesicles. The chemical shifts of both LPPC peaks did not show any changes over the concentration range examined. On the other hand, the intensity of the larger signal at 174.73 ppm increased continuously with increasing mol % of [^{13}C]LPPC (0.5−10%) incorporated. The peak at 174.55 ppm was insufficiently resolved from the larger peak at 174.73 ppm to be quantitated accurately. However, the data in Figure 2 show that the signal at 174.55 ppm appears to increase in intensity for 0.6−3.0 mol % LPPC but not at higher compositions of LPPC (Figure 2D, 5 mol %; 10 mol % spectrum not shown). The peak height ratio of the two peaks was >3/1 in all spectra. Since the size of the egg PC vesicles is unaffected by low amounts of LPPC used in these experiments, as evidenced from the constant PC carbonyl intensity ratio, the observed change in intensity ratio as a function of LPPC concentration suggests a limit on the amount of LPPC that could be accommodated in the inner monolayer of SUVs. The observed chemical shift separation of 0.17 ppm (13 Hz at 75.0 MHz) for the two signals corresponding to LPPC location in the outer and inner leaflets of the bilayer constrains its flip-flop rate to less than 13 s^{-1}.

When aqueous [^{13}C]LPPC was added to sonicated PC vesicles, LPPC showed a signal only at 174.73 ppm (Figure 2E−H). The chemical shift remained constant, and the intensity increased with increasing concentration of added [^{13}C]LPPC, as illustrated by the spectra corresponding to 0.6, 1.9, 2.8, and 4.0 mol %. The chemical shift is close to that for LPPC generated by PLA_2 hydrolysis of the outer leaflet of palmitoyl-PC vesicles (Bhamidipati & Hamilton, 1989). The observation of this single signal confirms its assignment to LPPC in the outer monolayer of egg PC vesicles. The absence of a signal for LPPC in the inner leaflet also shows that LPPC incorporated into the outer leaflet does not flip to the inner leaflet during the time period of the NMR experiment. In fact, a ^{13}C NMR spectrum obtained after 24 h of adding LPPC to preformed PC vesicles did not show any signal corresponding to LPPC in the inner monolayer, indicating that the transbilayer movement of LPPC in egg PC SUVs is extremely slow.

Carbonyl carbon T_1 and NOE values were measured at 50.3 MHz for SUVs with 5 mol % [^{13}C]LPPC. The observed T_1 value (2.0 s) for LPPC in vesicles at 32 °C was similar to that of for PC carbonyls (Hamilton & Small, 1981). The measured NOE value (1.8) for LPPC was also similar to that for PC carbonyls.

^{31}P NMR spectra were also obtained for samples prepared by cosonication of LPPC and egg PC. For samples with <5 mol % LPPC, the spectrum consisted of signals predominantly from egg PC phosphate groups at −0.96 and

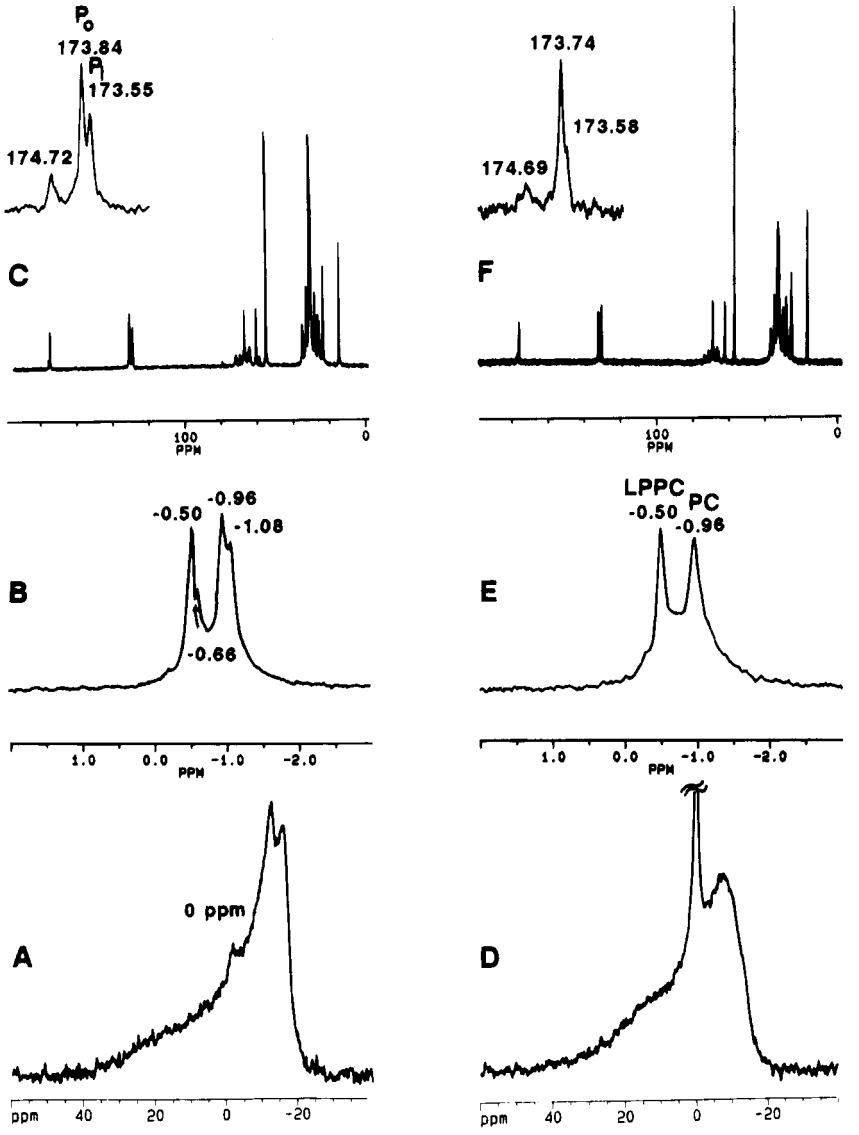

FIGURE 3: NMR spectra of egg PC dispersions containing 30 (left panel) and 40 mol % (right panel) LPPC in 75 mM KCl at 32 °C. (A and D) 121.0 MHz ^{31}P spectra of unsonicated dispersions; (B and E) 81.0 MHz ^{31}P spectra of sonicated dispersions; and (C and F) 75.0 MHz ^{13}C spectra of sonicated dispersions. Spectra correspond to 512 (A, D), 300 (B, E), and 10 000 accumulations (C, F). Spectra were processed using exponential multiplication function with 30 Hz (A and D) and 2 Hz (C, E, and F) line broadening factor except for (B), which was processed using a Gaussian multiplication function.

−1.07 ppm, corresponding to PC molecules in the outer and inner monolayers; signal(s) from LPPC could not be seen because of the high intensity of the signals from egg PC. At 5 mol % LPPC a signal with low intensity was seen at −0.52 ppm, which is significantly different from that of micellar LPPC (−0.35 ppm). This signal was assigned to LPPC located in the outer monolayer of the vesicles based on the observed chemical shift for LPPC when hydrolysis of egg PC vesicles by PLA_2 was monitored by ^{31}P NMR (Bhamidipati & Hamilton, 1988). In a sample with 10 mol % LPPC, however, two signals were seen for LPPC, corresponding to its location in the outer (−0.52 ppm) and the inner (−0.66 ppm) monolayers of the vesicles. The relative intensities of the LPPC signals in this spectrum show that LPPC was located preferentially in the outer monolayer of the SUVs.

Effect of Large Amounts of LPPC on Bilayer Structure. The interactions of large amounts of lysoPC with egg PC bilayers were examined by ^{13}C and ^{31}P NMR spectroscopy. Mixtures of 20−40 mol % LPPC and 80−60 mol % egg PC were hydrated together in 75 mM KCl to form multilayered dispersions. LPPC/PC vesicles of similar compositions were prepared by cosonication or by addition of LPPC to preformed PC vesicles. Figure 3 shows ^{31}P and ^{13}C NMR spectra of egg PC samples containing 30 and 40 mol % LPPC. The bottom spectra (Figure 3A,D) correspond to unsonicated phospholipid dispersions. The spectrum of 30 mol % LPPC/70 mol % egg PC (Figure 3A) showed a chemical shift anisotropy (CSA) pattern and value (52.0 ppm) typical of multilamellar phospholipids (Haberkorn et al., 1978; Hamilton et al., 1991b). The spectrum also contained a low intensity isotropic signal at 0 ppm, indicating the presence of a small amount of a second phase. The spectrum of 40 mol % LPPC/60 mol % egg PC (Figure 3D) showed a higher intensity isotropic signal and a powder pattern with a significantly reduced CSA (42.0 ppm). The sample with 30 mol % LPPC was sonicated to clarity, and the ^{31}P NMR spectrum (Figure 3B) showed signals for LPPC and egg PC on the outer and inner monolayers of the SUVs. The sample with 40 mol % LPPC was slightly turbid after sonication, and the ^{31}P spectrum (Figure 3E) showed signals corre-

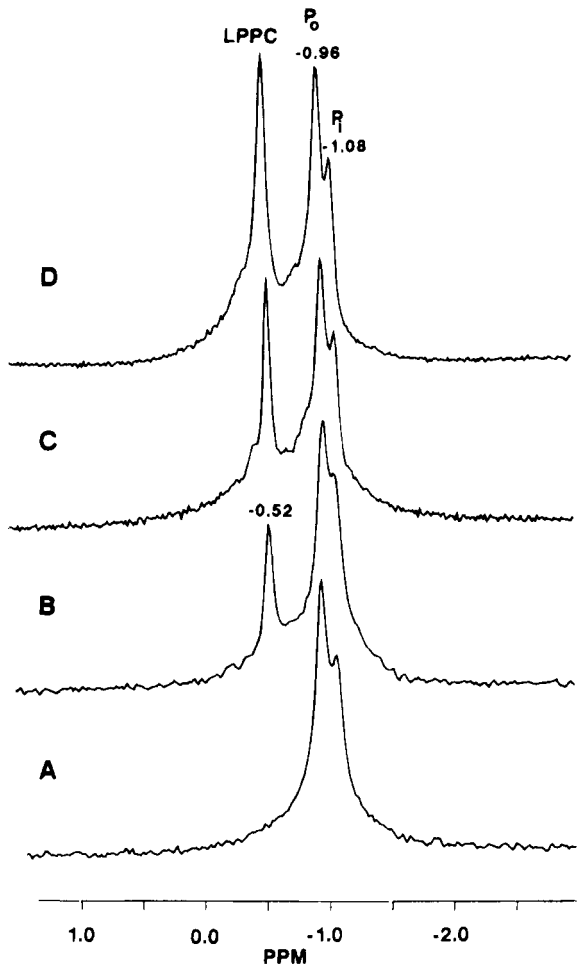

FIGURE 4: 81.0 MHz ^{31}P NMR spectra of sonicated egg PC vesicles in 75 mM KCl containing (A) 0, (B) 20, (C) 30, and (D) 40 mol % externally added aqueous LPPC in solution at 32 °C. Spectra correspond to 500 scans each in (A) and (B) and 1000 scans each in (C) and (D) and were processed using a Gaussian multiplication function.

sponding to LPPC and PC without any resolution of outer and inner monolayer lipid components. These samples were also analyzed by ^{13}C NMR (Figure 3C,F). The spectrum of 30 mol % LPPC in egg PC showed PC carbonyl signals typical of SUVs (Figure 3C), whereas the spectrum of 40% LPPC in egg PC showed somewhat broader lines with reduced separation of the PC carbonyl signals.

The effects of LPPC on phospholipid structural organization in vesicles were further investigated by addition of large amounts of LPPC to preformed egg PC SUVs (Figure 4). The ^{31}P NMR spectrum of egg PC SUVs (Figure 4A) showed the characteristic signals for PC in the outer and inner monolayers at −0.96 and −1.08 ppm. Addition of 20 mol % LPPC to the PC vesicles yielded a new signal at −0.52 ppm (Figure 4B) corresponding to LPPC incorporated into the outer monolayer of the vesicles (see above). Further addition of aqueous LPPC to the same sample to obtain 30 mol % (Figure 4C) and 40 mol % (Figure 4D) LPPC resulted in an increase in intensity of the signal at −0.52 ppm. These spectra showed no signal for LPPC in the inner monolayer of the vesicles (see Figure 3). The lack of any detectable changes in egg PC signals indicates that the structural organization and the average size of the PC vesicles did not change significantly.

LPPC Interactions with Fatty Acids. Because lysoPC is often formed together with unesterified FA *in vivo* (e.g., by PLA_2 action) and specific molecular interactions between these two lipid components have been postulated to occur (Jain et al., 1980; Jain & DeHaas, 1981), we investigated the interactions of LPPC and FA in egg PC bilayers. These studies were carried out by incorporating relatively small amounts of [^{13}C]LPPC and [^{13}C]carboxyl enriched FA (2−10 mol %) into egg PC bilayers by cosonication of the three lipids. When samples of egg PC vesicles containing 5 mol % [^{13}C]LPPC and an equimolar amount of ^{13}C-enriched oleic acid (OA) or palmitic acid (PA) were examined by ^{13}C NMR at pH 7.4, the LPPC carbonyl peaks exhibited the same chemical shifts and intensities (spectra not shown) as in vesicles without added FA (Figure 2D). Thus, the microenvironment and the asymmetric distribution of LPPC were not affected by either of these fatty acids. The corresponding FA gave a single carboxyl signal with a linewidth similar, though not identical, to that observed for FA alone in PC vesicles at the same pH. ^{13}C NMR spectra were obtained as a function of pH to determine the ionization behavior of FA (Kantor & Prestegard, 1978; Hamilton & Cistola, 1986) in the presence of equimolar amounts of LPPC. The chemical shift titration curve and the apparent pK (pK_a) determined from these data were identical to that of FA alone in egg PC vesicles, as illustrated in Figure 5A. However, the linewidth of the fatty acid carboxyl signal showed significant decreases near the pK_a (i.e., pK_a ± 1.0) of the FA (Figure 5B).

Since membranes may also contain nonequimolar ratios of FA/LPPC, we studied PC vesicles with various molar ratios of these constituents. Figure 6 shows the chemical shift and linewidth changes as a function of pH for egg PC vesicles containing 1:2, 1:5, and 2:1 molar ratios of PA/LPPC. The NMR titration curve of PA was identical for all the molar ratios studied (Figure 6A). On the other hand, the observed linewidth changes near the pK_a were dependent on the FA/LPPC mole ratio (Figure 6B). At a mole ratio of 1:2 (PA/LPPC), the carboxyl linewidth was larger than that observed for PA in the absence of LPPC (~50 Hz at the apparent pK_a), whereas at a mole ratio of 2:1 (PA/LPPC), the linewidth was smaller (11 Hz at the pK_a). At the lowest ratio of PA to LPPC (1:5), the linewidth changes near the pK_a were too large to be measured reliably. The measured linewidths of 12 and 18 Hz at pH values of 5.0 and 8.4, respectively, are also significantly larger at this mole ratio than with other compositions examined. These experiments were repeated at least three times with PA and with oleic acid, with results similar to those in Figure 6. However, in some instances, additional lysoPC and FA were generated during sonication, as revealed from quantitative chemical analyses, and the intended molar ratio of FA/LPPC was altered. In these cases, the linewidth changes observed for FA were similar to those for FA with equimolar LPPC in egg PC vesicles.

Partitioning of LysoPC between PC Vesicles and BSA. Albumin binds lysoPC and may serve to transport lysoPC in plasma (Switzer & Eder, 1965). Bovine serum albumin (BSA) has been used to characterize lysoPC distribution and transbilayer movement in erythrocyte membranes (Bergmann et al., 1984a,b) and to extract lysoPC from erythrocyte membranes hydrolyzed by PLA_2 to understand bilayer stability and cell shape changes following lipid removal

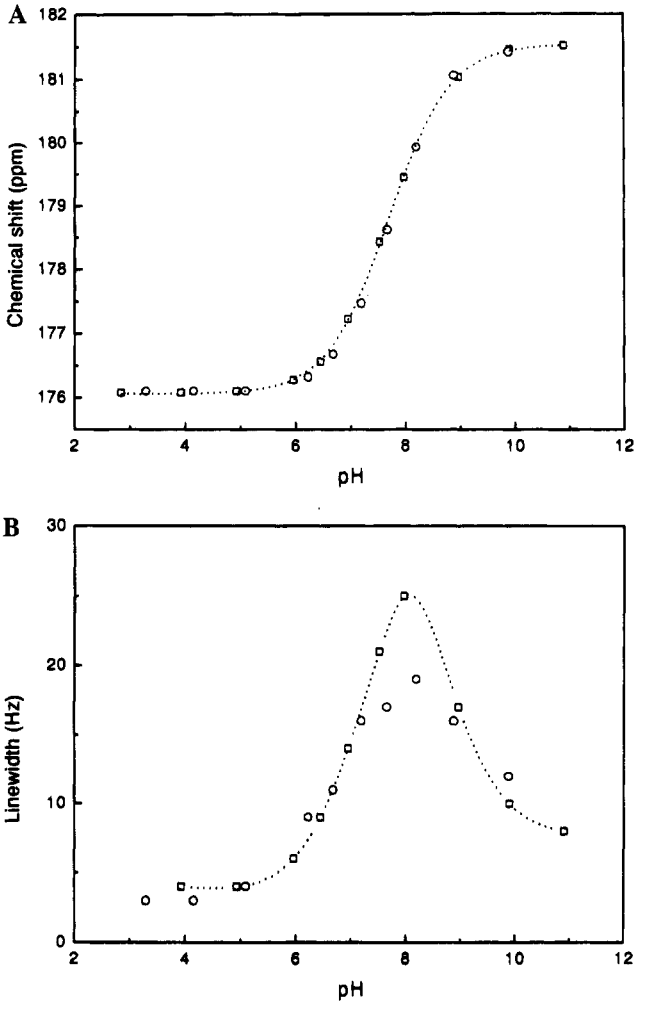

FIGURE 5: ^{13}C NMR chemical shift (A) and linewidth (B) changes for the carboxyl group of [^{13}C]carboxyl-enriched palmitic acid in egg PC vesicles as a function of pH and at 32 °C in the absence (□) and presence (○) of equimolar amounts of LPPC.

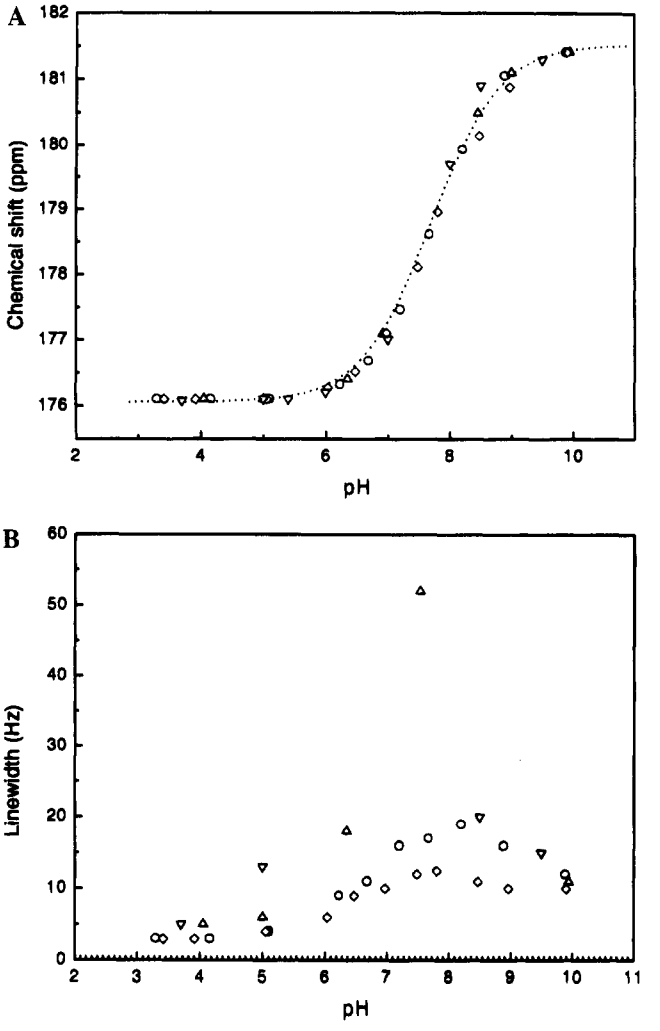

FIGURE 6: ^{13}C NMR chemical shift (A) and linewidth (B) changes for the carboxyl group of [^{13}C]carboxyl-enriched palmitic acid in egg PC vesicles as a function of pH and at 32 °C with different mole ratios of FA to LPPC: 1:1 (○), 1:2 (△), 1:5 (▽), and 2:1 (◇).

(Haest et al., 1981). However, there is no evidence from physical methods that do not involve separation procedures for the ability of BSA to extract lysoPC from membranes or deliver it to membranes. In studies to be reported elsewhere (Bhamidipati and Hamilton, unpublished), we investigated binding of lysoPC to BSA in detail and found that the [^{13}C]-carbonyl signal of LPPC bound to BSA is shifted downfield (175.40 ppm; Table 1) relative to that observed for LPPC bound to PC vesicles (174.73 and 174.55 ppm; Table 1). Here we exploited these chemical shift differences to determine the partitioning of ^{13}C LPPC between PC vesicles and BSA by a protocol similar to that developed to monitor the partitioning of FA between PC vesicles and BSA (Hamilton & Cistola, 1986).

Figure 7 shows the carbonyl region of the ^{13}C NMR spectrum of 5 mol % [^{13}C]LPPC/95 mol % egg PC vesicles (prepared by cosonication) in the absence and presence of BSA. Prior to addition of BSA, the spectrum (Figure 7A) showed the [^{13}C]LPPC signals at 174.73 and 174.57 ppm with the expected intensity ratio (Figures 1 and 2). Following addition of BSA to obtain a 3:1 LPPC/BSA ratio, the intensity of the signal for LPPC in the outer monolayer of the vesicle (174.73 ppm) was greatly reduced, whereas that of inner monolayer (174.57 ppm) remained unchanged (Figure 7B). Although this result suggested that BSA extracted LPPC from the outer monolayer, a signal for LPPC bound to BSA (expected to be at ~175.40) ppm was not seen. This is not surprising since signals from LPPC bound to BSA are significantly broader and more difficult to detect at high than at low temperature (Bhamidipati and Hamilton, unpublished). In addition, it is also possible that LPPC exchange between protein and vesicle environments causes some line broadening. Therefore, the sample temperature was lowered to 15 °C to enhance the signals for LPPC bound to BSA, and two signals were seen at chemical shifts (175.50 and 175.10 ppm) similar to those for LPPC bound to BSA in the absence of PC vesicles (Bhamidipati and Hamilton, unpublished results). Since the intensity of the signal for LPPC on the inner leaflet was unchanged and the spectrum at 15 °C was obtained after the 32 °C spectrum, it can be concluded that LPPC on the inner monolayer did not move to the outer monolayer over a period of hours.

DISCUSSION

Binding to Vesicles. Binding of LPPC to phospholipid (egg PC) bilayers was observed by monitoring the carbonyl chemical shift of LPPC. The upfield change in chemical shift (~0.3 ppm) compared to micellar LPPC signifies a less polar environment of the carbonyl in the vesicle interface.

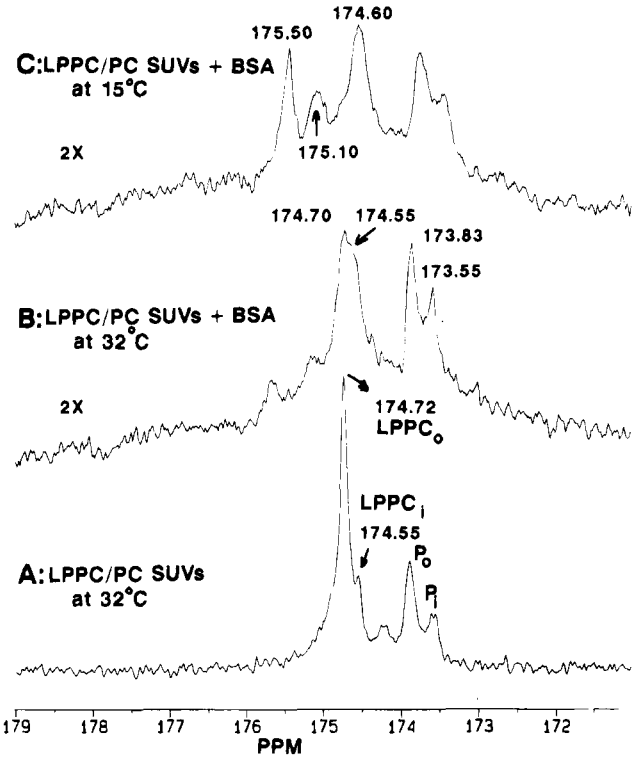

FIGURE 7: ^{13}C NMR spectra of [^{13}C]LPPC partitioning between PC bilayers and BSA at pH = 7.4. (A) 5 mol % [^{13}C]LPPC/egg PC cosonicated vesicles at 32 °C; (B) same sample as in (A) with BSA added in 3:1 mole ratio with respect to total [^{13}C]LPPC; and (C) same sample as in (B) at 15 °C. Spectrum A was obtained with 4096 accumulations and processed using a Gaussian multiplication function. Spectra in (B) and (C) were obtained with 6144 scans each and processed using exponential multiplication function with 2 Hz line broadening. Spectra in (B) and (C) were plotted with a 2-fold increased vertical scale relative to the spectrum in (A). There is a difference of 0.10 ppm relative to the signals observed for an equimolar complex of [^{13}C]LPPC/BSA at this temperature due to difference in the signal used for internal reference.

This change is expected, since localization of the carbonyl group of LPPC in the PC interface will result in partial dehydration of the carbonyl relative to the more loosely packed micellar phase, as in the case of phospholipid carbonyls (Schmidt et al., 1977). Other water-soluble lipids such as bile acids (Cabral et al., 1986) and acyl CoA (Boylan & Hamilton, 1992) also show an upfield shift on binding to PC vesicles compared to the aqueous monomeric or micellar form. In contrast, the carbonyl signals of water-insoluble weakly polar lipids such as diacylglycerols, cholesteryl esters, and triacylglycerols shift downfield upon going from the fully unhydrated form in their neat state to a partially hydrated state in a phospholipid interface (Hamilton & Small, 1981; Hamilton, 1989; Hamilton et al., 1991a,b).

In egg PC SUVs the [^{13}C]carbonyl chemical shift of LPPC was also sensitive to its localization within the two bilayer leaflets. The carbonyl signal for LPPC on the inner leaflet was shifted upfield by ~0.15 ppm from the signal for LPPC on the outer leaflet, without addition of exogenous shift reagents. A similar separation is observed for PC carbonyls (e.g., Figure 2) and for the carbonyl of oleoyl CoA bound to PC vesicles (Boylan & Hamilton, 1992). The presence of two signals is explained by curvature differences in the two leaflets of SUVs and the decreased hydration of molecules on the inner leaflet (Schmidt et al., 1977); flip-flop of the molecules must also be slow (see below). In multilayers, for which the average curvature is low and the two leaflets are not distinguished by curvature differences, a single signal was seen by MASNMR for both LPPC and PC carbonyls. Assignment of the two resonances for LPPC in vesicles was supported by experiments in which (i) LPPC was added to preformed PC vesicles and (ii) LPPC was extracted by adding albumin to LPPC/PC vesicles made by cosonication. In the former experiments (Figure 2) only the downfield (and more intense) resonance was seen. The sensitivity of the LPPC carbonyl chemical shift to environment also results in distinct chemical shifts upon binding to BSA, making it possible to identify binding of LPPC to BSA and phospholipid bilayers without the need to separate the different pools of LPPC. Addition of BSA to vesicles with LPPC incorporated into both leaflets resulted in a decrease only in the downfield LPPC resonance, showing selective extraction from the outer leaflet pool of LPPC. This experiment also provides the biophysical foundation for experiments with cell membranes, in which albumin is added to the external medium and presumed to extract only lysophospholipids in the external leaflet of the membrane (Bergmann et al., 1984a,b; Mohandas et al., 1982; Besterman & Domanico, 1991).

Bilayer Distribution and Transbilayer Movement. The relative intensities for LPPC carbonyl ^{13}C signals in PC vesicles indicated a preference of LPPC for the outer monolayer of SUVs. In our studies with high levels of LPPC in egg PC vesicles, ^{31}P spectra also showed two signals for LPPC and a higher proportion of LPPC on the outer monolayer (Figure 3B). Similar results were obtained by ^{31}P NMR spectroscopy of lysoPC in 1-palmitoyl-2-oleoyl-PC (POPC) vesicles and explained on the basis of the wedge shape of lysoPC which allows a better fit into the outer leaflet (Kumar et al., 1989). A preference of long-chain acyl CoA for the outer leaflet of SUVs has also been found (Boylan & Hamilton, 1992); in this case both size and charge of the CoA moiety may limit the incorporation of acyl CoA into the inner leaflet.

The ability to detect narrow ^{13}C or ^{31}P signals from LPPC located on the two leaflets of the bilayer of SUVs also indicates that transbilayer movement is slow. By the criterion of chemical shift, the two peaks with a separation of 0.17 ppm (13 Hz) in the ^{13}C spectrum represent an exchange rate of much less than 13 s^{-1}; in the ^{31}P spectrum the peak separation of 0.14 ppm (11 Hz) constrains the exchange to be <11 s^{-1}. Additional experiments provided more insight into the exchange rate limit. First, addition of LPPC to preformed vesicles did not give rise to ^{13}C or ^{31}P signals for LPPC on the inner leaflet, even in samples observed for long periods of time. Second, after removal of LPPC from the outer leaflet of SUVs, the signal for LPPC on the inner leaflet did not decrease with time, nor did the signal for LPPC on the outer leaflet increase with time (Figure 7). Thus, both movement of LPPC from the outer to inner monolayer and in the reverse direction is extremely slow ($t_{1/2}$ >hours). This result is consistent with previous reports showing very slow or immeasurable flip-flop; for example, a study monitoring hydrolysis of LPPC in egg PC SUVs estimated a $t_{1/2}$ for flip-flop of ~100 h (Van den Besselaar et al., 1977). However, faster flip-flop rates have been measured in vesicles with incorporated proteins and in membranes. For example, addition of glycophorin to phos-

pholipid vesicles has been shown to enhance flip-flop of lysoPC (Van Zoelen et al., 1978), and transbilayer movement of lysophospholipids in erythrocytes on the time scale of hours has been inferred from albumin extraction experiments (Bergmann et al., 1984a; Mohandas et al., 1982).

The exchange rate of LPPC between albumin and the outer monolayer of PC vesicles must be considerably faster than the flip-flop rate. ^{13}C NMR spectra of mixtures of LPPC, BSA, and vesicles were unchanged after minutes of signal averaging, indicating exchange was complete within this time. The observation of separate binding sites on BSA and vesicles with a signal separation of ~1 ppm (75 Hz) means that the rate constant for exchange is much less than 75 s^{-1} ($t_{1/2}$ >20 ms). McLean and Phillips (1984) found by a chromatographic separation approach that transfer of LPPC between vesicles occurs with $t_{1/2}$ <2 min. It is reasonable to predict that the $t_{1/2}$ for spontaneous desorption of LPPC from vesicles is in the range of 0.1−100 sec. The rate of desorption of lysoPC from vesicles or albumin is expected to vary significantly with the acyl chain length, based on data for unesterified fatty acids (Daniels et al., 1985).

Membrane Disruption. No changes in phospholipid structural organization or molecular mobility were detected with low levels (<10 mol %) of LPPC. Therefore, higher ratios of LPPC/PC were used to assess conditions for membrane disruption, and both unsonicated and sonicated lipid mixtures were examined by ^{13}C and ^{31}P NMR spectroscopy. Samples were prepared by two different protocols, one in which LPPC and PC were co-dried and hydrated from a mixed film to form multilayers, which were then sonicated to form vesicles. In a second protocol sonicated PC vesicles were first prepared, and LPPC was subsequently added in increasing concentrations. The wide-line ^{31}P NMR spectrum in Figure 3A shows that an unsonicated mixture comprised of 30 mol % LPPC and 70% PC is almost completely multilamellar. Upon sonication, this sample formed SUVs, as evidenced by the narrow ^{31}P and ^{13}C spectra and resolution of inner and outer leaflets PC and LPPC (Figure 3B,C). With 40 mol % LPPC a multilamellar phase predominated, but a second (isotropic) phase was seen by ^{31}P NMR spectroscopy (Figure 3D). For samples prepared by addition of LPPC to preformed vesicles, up to 40 mol % LPPC was incorporated into the vesicles without disruption of the vesicles and without flip-flop of LPPC to the inner leaflet. This represents a ratio of ~1/1 LPPC/PC in the outer leaflet. The linewidths of the ^{31}P resonances, which are very sensitive to vesicle size (Smith & Ekiel, 1984), did not change with increasing LPPC, indicating preservation of the SUV structure. Furthermore, the samples were stable and nonturbid; these results are consistent with previous NMR results of model mixtures with other phospholipids. Wide-line ^{31}P NMR studies have shown that LPPC can be incorporated up to 30 mol % into liquid crystalline multilayers of dipalmitoyl-PC and up to 35 mol % into dioleoyl-PC multilayers before the appearance of an isotropic signal signifying the onset of micellization (Van Echteld et al., 1981). Kumar et al. (1989) showed that stable SUVs could be formed with up to 30 mol % LPPC in POPC. Thus, rather than destabilize curved surfaces, lysoPC may stabilize highly curved bilayers because of its wedge shape (Kumar et al., 1989).

LysoPC and Fatty Acid. Whereas previous NMR studies (Allegrini et al., 1983) examined equimolar lysoPC and fatty acids in the absence of phospholipids, we studied small proportions (equimolar and nonequimolar) of lysoPC and fatty acid in a phospholipid bilayer matrix. The presence of long-chain fatty acids (oleic or palmitic acid) did not detectably affect properties (local magnetic environment, distribution, mobility, flip-flop) of lysoPC in SUVs. The apparent pK_a of the fatty acid was not affected by lysoPC in varying ratios to fatty acid; thus, the fraction of ionized and un-ionized species of fatty acid was not modulated by lysoPC.[4] Un-ionized fatty acid can flip-flop rapidly across phospholipids (Kamp & Hamilton, 1992), and the rapid flip-flop is not affected by lysoPC (Kamp & Hamilton, 1993). Therefore, when fatty acids and lysophospholipids are generated in membranes by PLA_2, the fatty acid will equilibrate across the membrane much more rapidly than the lysophospholipid. Interestingly, the presence of lysoPC in molar excess of fatty acid, a situation which will occur soon after PLA_2 action on one side of a membrane, resulted in larger linewidths of the fatty acid carboxyl signal at pH values near the pK_a. This effect could be the result of a decrease in mobility of the fatty acid or in the on/off rate of the proton. Taken together, our experiments suggest only weak interactions between lysoPC and fatty acids in the interfacial region of PC bilayers.

Biological Implications. Lysophospholipids are normally minor constituents of membranes because their rate of catabolism is much greater then their rate of production (Corr et al., 1984). Under conditions of ischemia, however, their levels are increased, and in myocardial tissue elevated levels of lysophospholipids are thought to produce electrical effects in membranes that lead to lethal arrhythmias (Corr et al., 1984; DaTorre et al., 1991; Katz & Messineo, 1981). Lysophospholipids can enter a membrane directly as a result of PLA_2 activity, which is known to be increased under conditions of ischemia (Katz & Messineo, 1981). Alternatively, lysophospholipids may be produced by one cell type (e.g., endothelial cells), from which they are secreted into the extracellular medium, and insert into the outer leaflet of other cells (e.g., myocytes; Man et al., 1990), where they cause pathophysiological alterations (McHowatt & Corr, 1993).

The molecular mechanism(s) for deleterious effects of lysophospholipids on membrane structure and function are not known, and several mechanisms have been hypothesized. A popular explanation is that lysophospholipids act as detergents to make mixed micelles with phospholipids in membranes, thus grossly disrupting membrane structure (Katz & Messineo, 1981).[5] However, lysoPC, which is the most abundant lysophospholipid, is a poor detergent for membrane phospholipids, as shown by previous studies (e.g., Small, 1969; van Echteld et al., 1981; Kumar et al., 1989) and the systematic ^{13}C and ^{31}P NMR studies presented here. We have also used ^{13}C NMR spectroscopy to monitor the disruption of egg PC bilayers by bile acids (Cabral et al., 1986) and acyl CoA's (Boylan & Hamilton, 1992); these lipids have much stronger detergent effects and begin to make

[4] Jain and DeHaas (1981) found two calorimetric transitions for aqueous dispersions of LPPC and palmitic acid, the relative enthalpic contribution of which was pH-dependent with an apparent pK_a of 7.5. This correlates well with our direct determination of an apparent pK_a of 7.6 for palmitic acid in SUVs with LPPC.

[5] This theory was advanced to explain the lysis of erythrocyte membranes, although lysis could occur without a detergent effect (Weltzein, 1979).

mixed micelles at levels of ~10 mol % (cholic acid) and ~15 mol % (oleoyl CoA) with respect to PC. Another explanation presented for the biological effects of lysophospholipids is that they accumulate in extracellular fluids as micelles which extract proteins as well as lipids from the membranes (see Weltzien, 1979). However, aqueous phase lysophospholipids desorb quickly from micelles and bind to phospholipid bilayers, as exemplified by our NMR binding studies above. Therefore, lysophospholipids would be expected to partition into membranes, and their aqueous concentration would then be very small.

Lysophospholipids most likely exert their damaging effects within the membrane but without gross disruption of the membrane. Further evidence for this hypothesis is that pathological electrophysiological effects are produced in cells *in vitro* by low concentrations of lysophospholipid (2−3 mol %) relative to phospholipid (DaTorre et al., 1991). Lysophospholipid at these concentrations has been shown to change the order parameter of spin-labeled fatty acids in membranes, leading to the suggestion that altered physical properties of the membrane may lead to membrane dysfunction (Fink & Gross, 1984; DaTorre et al., 1991). However, the spin-labeled fatty acids used (Fink & Gross, 1984) contain a bulky polar group, and other biophysical data should be sought to support these interesting findings. As shown by our results and those of others (Jain et al., 1980; Fink & Gross, 1984), native fatty acids, which often accompany the production of lysophospholipids, could modulate the effects of lysophospholipids on membrane structure. Direct molecular effects of lysophospholipids on membrane proteins (e.g., on channels, receptors, and enzymes) are also a plausible explanation for their physiological effects; experimental approaches to evaluate direct effects should include fatty acids in combination with lysophospholipids.

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REFERENCES

Allegrini, P. R., Scharrenburg, G. V., DeHaas, G., & Seelig, J. (1983) *Biochim. Biophys. Acta 731*, 448−455.

Ansell, G. B., Hawthorne, J. M., & Dawson, R. M. C. (1973) in *Form and Function of Phospholipids*, 2nd ed., Elsevier, Amsterdam.

Asaoka, Y., Nakamura, S., Yoshida, K., & Nishizuka, Y. (1992) *Trends Biochem. Sci. 17*, 414−417.

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466−468.

Bergmann, W. L., Dressler, V., Haest, C. W. M., & Deuticke, B. (1984a) *Biochim. Biophys. Acta 769*, 390−398.

Bergmann, W. L., Dressler, V., Haest, C. W. M., & Deuticke, B. (1984b) *Biochim. Biophys. Acta 772*, 328−336.

Besterman, J. M., & Domanico, P. L. (1992) *Biochemistry 31*, 2046−2056.

Bhamidipati, S. P., & Hamilton, J. A. (1988) *Biophys. J. 53*, 320a.

Bhamidipati, S. P., & Hamilton, J. A. (1989) *Biochemistry 28*, 6667−6672.

Bhamidipati, S. P., & Hamilton, J. A. (1993a) *Biophys. J. 64*, 67a.

Bhamidipati, S. P., & Hamilton, J. A. (1993b) *J. Biol. Chem. 268*, 2431−2434.

Boylan, J. G., & Hamilton, J. A. (1992) *Biochemistry 31*, 557−567.

Brentel, I., Arvidson, G., & Lindblom, G. (1987) *Biochim. Biophys. Acta 31*, 401−404.

Cabral, D. J., Hamilton, J. A., & Small, D. M. (1986) *J. Lipid Res. 27*, 334−343.

Canet, D., Levy, G. C., & Peat, I. R. (1975) *J. Magn. Reson. 18*, 199−204.

Chernomordik, L. V., Vogel, S. S., Sokoloff, A., Onaran, H. O., Leikina, E. A., & Zimmerberg, J. (1993) *FEBS Lett. 318*, 71−76.

Corr, P. B., Gross, R. W., & Sokel, B. E. (1984) *Circ. Res. 55*, 135−154.

Daniels, C., Noy, N., & Zakim, D. (1985) *Biochemistry 25*, 2013−2021.

DaTorre, S. D., Creer, M. H., Pogwizd, S. M., & Corr, P. B. (1991) *J. Mol. Cell Cardiol. 23*, Suppl. I, 11−22.

De Kruijff, B., Van Den Besselaar, A. M. H. P., & Van Deenen, L. L. M. (1977) *Biochim. Biophys. Acta 465*, 443−453.

Dennis, E. A., & Pluckthun, A. (1984) in *Phosphorous NMR: Principles and Applications* (Gorenstein, D., Ed.) pp 423−446, Academic Press, Orlando, FL.

Eibl, H., Hill, E. E., & Lands, W. E. (1969) *Eur. J. Biochem. 9*, 250−258.

Fink, K. L., & Gross, R. W. (1984) *Circ. Res. 55*, 585−593.

Haberkorn, R. A., Herzfeld, J., & Griffin, R. G. (1978) *J. Am. Chem. Soc. 100*, 1296−1298.

Haest, C. W. M., Plasa, G., & Deuticke, B. (1981) *Biochim. Biophys. Acta 649*, 701−708.

Hamilton, J. A. (1989) *Biochemistry 28*, 2514−2520.

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*, 7318−7322.

Hamilton, J. A., & Cistola, D. P. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 82−86.

Hamilton, J. A., Bhamidipati, S. P., Kodali, D. R., & Small, D. M. (1991a) *J. Biol. Chem. 266*, 1177−1186.

Hamilton, J. A., Fujito, D. T., & Hammer, C. F. (1991b) *Biochemistry 30*, 2894−2902.

Jain, M. K., & DeHaas, G. H. (1981) *Biochim. Biophys. Acta 642*, 203−211.

Jain, M. K., Van Echteld, C. J. A., Ramirez, F., De Gier, J., De Haas, G. H., & Van Deenen, L. L. M. (1980) *Nature 284*, 486−487.

Janatova, J., Fuller, J. K., & Hunter, M. J. (1968) *J. Biol. Chem. 243*, 3612−3622.

Kamp, F., & Hamilton, J. A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 11367−11370.

Kamp, F., & Hamilton, J. A. (1993) *Biochemistry 32*, 11074−11085.

Kantor, H. L., & Prestegard, J. H. (1978) *Biochemistry 17*, 3592−3597.

Katz, A. M., & Messineo, F. C. (1981) *Circ. Res. 48*, 1−16.

Kinnaird, A. A. A., Coy, P. C., & Man, R. Y. K. (1988) *Lipids 23*, 32−35.

Klibansky, C., & De Vries, A. (1963) *Biochim. Biophys. Acta 70*, 176−187.

Klopfenstein, W. E., De Kruyff, B., Verkleij, A. J., Demel, R. A., & Van Deenen, L. L. M. (1974) *Chem. Phys. Lipids 13*, 215−222.

Kumar, V. V., & Baumann, W. J. (1986) *Biochem. Biophys. Res. Commun. 139*, 25−30.

Kumar, V. V., Malewicz, B., & Baumann, W. J. (1989) *Biophys. J. 55*, 789−792.

Lee, Y., & Chan, S. I. (1977) *Biochemistry 16*, 1303−1309.

Man, R. Y. K., Kinnaird, A. A. A., Bihler, I., & Choy, P. C. (1990) *Lipids 253*, 450−454.

Mason, J. T., Broccoli, A. V., & Huang, C.-H. (1981) *Anal. Biochem. 113*, 96−101.

McHowatt, J., & Corr, P. B. (1993) *J. Biol. Chem. 268*, 15605−15610.

McLean, L. R., & Phillips, M. C. (1984) *Biochemistry 23*, 4624−4630.

Mohandas, N., Wyatt, J., Mel, S. F., Rossi, M. E., & Shohet, S. B. (1982) *J. Biol. Chem. 257*, 6537−6543.

Nishizuka, Y. (1992) *Science 258*, 607−614.

Opella, S. J., Nelson, D. J., & Jardetsky, O. (1976) *J. Chem. Phys. 64*, 2533−2535.
Pelech, S. L., & Vance, D. E. (1989) *Trends Biochem. Sci. 14*, 28−30.
Poole, A. R., Howell, J. I., & Lucy, J. A. (1970) *Nature 227*, 810−813.
Ramsammy, L. S., & Brockerhoff, H. (1982) *J. Biol. Chem. 257*, 3570−3574.
Ramsammy, L. S., Mertz, P. A., & Brockerhoff, H. (1984) *Chem. Phys. Lipids 34*, 127−138.
Reman, F. C., Demel, R. A., De Gier, J., Van Deenen, L. L. M., Eibl, H., & Westphal, D. (1969) *Chem. Phys. Lipids 3*, 221−233.
Sasaki, Y., Asaoka, Y., & Nishizuka, Y. (1993) *FEBS Lett. 320*, 47−51.
Sass, M., & Ziessow, D. (1977) *J. Magn. Reson. 25*, 263−276.
Schmidt, C. F., Barenholz, Y., Huang, C., & Thompson, T. E. (1977) *Biochemistry 16*, 3948−3954.
Small, D. M. (1969) in *The Metabolism of Bile Acids* (Schiff, L., Ed.) pp 223−252, Charles C. Thomas.
Smith, I. C. P., & Ekiel, I. H. (1984) in *Phosphorous NMR: Principles and Applications* (Gorenstein, D., Ed.) pp 447−475, Academic Press, Orlando, FL.
Stafford, R. E., Fanni, T., & Dennis, E. A. (1989) *Biochemistry 28*, 5113−5120.
Switzer, S., & Eder, H. A. (1965) *J. Lipid Res. 6*, 506−511.
Vadas, P., & Pruzanski, W. (1986) *Lab. Invest. 55*, 391−404.
Van Den Besselaar, A. M. H. P., Van Den Bosch, H., & Van Deenen, L. L. M. (1977) *Biochim. Biophys. Acta 465*, 454−465.
Van Den Besselaar, A. M. H. P., De Kruijff, B., Van Den Bosch, H., & Van Deenen, L. L. M. (1979) *Biochim. Biophys. Acta 555*, 193−199.
Van Den Bosch, H. (1974) *Annu. Rev. Biochem. 43*, 243−277.
Van Echteld, C. J. A., De Kruijff, B., Mandersloot, J. G., & De Gier, J. (1981) *Biochim. Biophys. Acta 649*, 211−220.
Van Golde, L. M. G., Fleischer, B., & Fleischer, S. (1971) *Biochim. Biophys. Acta 249*, 318−330.
Van Zoelen, R. J. J., De Kruijff, B., & Van Deenen, L. L. M. (1978) *Biochim. Biophys. Acta 508*, 97−108.
Weltzein, H. U. (1979) *Biochim. Biophys. Acta 559*, 259−287.
Weltzein, H. U., Arnold, B., & Kalkoff, H. G. (1976) *Biochim. Biophys. Acta 455*, 56−65.
Yeagle, P. L., Smith, F. T., Young, J. E., & Flanagan, T. D. (1994) *Biochemistry 33*, 1820−1827.

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