Vesiculation of Unsonicated Phospholipid Dispersions Containing Phosphatidic Acid by pH Adjustment: Physicochemical Properties of the Resulting Unilamellar Vesicles†

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ABSTRACT: Aqueous dispersions of phosphatidic acid and mixtures of phosphatidic acid with other phospholipids vesiculate when the pH is transiently increased to a pH near or above the second p*K* of the phosphatidic acid. Both small unilamellar vesicles (SUV) of a narrow size distribution (average diameter 25–30 nm) and large unilamellar vesicles (LUV) of a wide size distribution are formed. The fraction of SUV increases linearly with increasing pH from 6 to 12 and was also increased by increasing the rate of the pH change from 2 min to 1 s. For mixed phospholipid dispersions, the fraction of SUV appears to be linearly related to the phosphatidic acid content. Unilamellar vesicles formed by the transient pH increase are subsequently relatively stable to changes in pH and ionic strength. If, after vesiculation is induced, the dispersion (pH 7–8) is acidified to pH 3, the fraction of SUV decreases. However, the multilamellar structures present in the original dispersion do not re-form, and most of the reduction in the SUV fraction is reversible when the pH is returned to 7–8. The addition of NaCl to the dispersion after vesiculation has no effect on the fraction of SUV up to physiological NaCl concentrations. Subsequent addition of NaCl to a concentration in excess of 0.2 M reduces the SUV fraction; this disappears above 1 M NaCl due to aggregation or fusion. SUV of phosphatidic acid or mixed phospholipids containing phosphatidic acid can be stored at 4 °C for 14 days without detectable aggregation and/or fusion.

Liposomes, in the form of both multilamellar structures and unilamellar vesicles, have proved to be important for two reasons: (i) since the proposal by Gorter & Grendel (1925) that lipid bilayers are integral structures of biological membranes, liposomes have been used extensively as model membranes; (ii) more recently, they have become potentially important as drug carriers. The latter application has stimulated research into the development of new methods for the preparation of liposomes. Recently, a simple and quick method for producing unilamellar vesicles of phosphatidic acid (PA)[1] and of mixtures of PC and PA has been described (Hauser & Gains, 1982). The method is based on a transient increase in pH so that the phosphate group of the PA molecules becomes ionized. The resulting phospholipid dispersion is a mixture consisting of small unilamellar vesicles (SUV) of a homogeneous particle size distribution and large unilamellar vesicles (LUV) of a wide particle size distribution. The paper presented here is an extension of this work in which some of the physicochemical properties of these PA/PC and other vesicles, formed from different lipid mixtures, have been investigated.

Materials and Methods

Egg phosphatidylcholine (EPC), egg phosphatidic acid (EPA), and ox brain phosphatidylserine (PS) were purchased from Lipid Products (Surrey, U.K.). DMPC, DPPC, and DSPC were obtained from R. Berchtold (Biochemisches Labor, Bern, Switzerland). 1,2-Dilauroyl-*sn*-glycero-3-phosphoric acid (DLPA) and 1-lauroyl-*rac*-glycero-3-phosphoric acid (LLPA) were synthesized according to Baer (1963). The disodium salts of LLPA and DLPA were made from the dipyridinium salt dissolved in CH_3OH by adding an excess of sodium acetate. The analysis of the disodium salt that precipitated gave a mole ratio for Na/P of 2/1. When necessary, the disodium salt of DLPA was converted into the free acid by using the procedure of Folch et al. (1957). The purity of the lipids was monitored by TLC on silica gel 60 F/25 plates (Merck) using $CHCl_3/CH_3OH/H_2O$/acetic acid (65:50:4:1 v/v). The lipids were pure by TLC standards (0.3–1 mg applied as a 1-cm-wide band; under the conditions used, a contamination of 3–6 μg can be detected).

Preparation of Vesicles. Vesicles were made either from EPA or from mixtures of EPA and other phospholipids. Usually, 10–20 mg of phospholipid dissolved in 1–2 mL of $CHCl_3/CH_3OH$ (2:1 v/v) was rotary evaporated in a round-bottomed flask under reduced pressure at room temperature. The conditions were such that the solvent was removed quantitatively as determined by ^1H NMR. Phospholipid mixtures were readily dispersed in 1 mL of D_2O or salt solution in D_2O by vortex mixing at room temperature for 5 min or by hand shaking with one or two glass beads. For the production of dispersions of pure EPA, vortex mixing and freeze–thawing (about six cycles between −15 and 5 °C) were necessary. The pH of pure EPA dispersions in D_2O or salt solution was between 2.5 and 3; that of mixtures of EPA with other phospholipids depended on the amount of EPA present. The pH of the phospholipid dispersions was increased, unless otherwise stated, to 11–12 with 0.2 or 1 M NaOD delivered relatively slowly from an Agla syringe (Wellcome, Beckenham, U.K.) or rapidly from a micropipet, both into stirred dispersions. In the first case, titration from pH 3 to pH 11–12 took about 2 min and in the second case probably less than 1 s. The rate of NaOD addition from the Agla syringe was not controlled. Dispersions with properties similar to those produced by the rapid NaOD addition were obtained when the appro-

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[1] Abbreviations: PC, phosphatidylcholine; EPC, egg phosphatidylcholine; PA, phosphatidic acid; EPA, egg phosphatidic acid; PS, ox brain phosphatidylserine; DLPA, 1,2-dilauroyl-*sn*-glycero-3-phosphoric acid; LLPA, 1-lauroyl-*rac*-glycero-3-phosphoric acid; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; TSS, sodium 3-(trimethylsilyl)propanesulfonate; TLC, thin-layer chromatography; SUV, small unilamellar vesicle(s); LUV, large unilamellar vesicle(s).

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Table I: Chemical Shifts[a] of Phosphatidic Acid Dispersions[b] in D_2O

signal	sonicated EPA	unsonicated EPA	sonicated or unsonicated DLPA	micellar solution of LLPA in D_2O
terminal CH_3	0.88	0.88	0.88	0.865
$(CH_2)_n$	1.27	1.28	1.30	1.28
CH_2CCO	1.60	1.59	1.61	1.61
$CH_2C{=}C$	2.03	2.03		
CH_2CO	2.33	2.35	2.34	
	2.40	2.42	2.40	2.40
$(CH_2)(C{=}C)_2$	2.76	2.80		
	2.85			
$CH{=}CH$	5.33	5.31		
CH_2OCO	4.25	4.25	4.25	4.08
	4.44	4.47	4.43	4.15
CHOCO	5.33	5.31	5.28	4.03
CH_2OP	3.99	4.00	3.97	3.89
				3.82

[a] Expressed in ppm downfield from the internal standard TSS. [b] Unsonicated dispersions of EPA or DLPA in D_2O, apparent pH 7, were prepared by the slow addition of NaOD. DLPA and LLPA were used as the disodium salts. The error of the chemical shift measurement is basically ±0.01 ppm; the error between different preparations is ±0.02 ppm. The assignment is based on intensity measurements, pH titration, homonuclear double-resonance experiments, and changes in chemical shift and line width induced by shift and broadening probes of the lanthanide series.

priate amount of NaOD solution was added directly to the dry lipid film on the glass wall. When this procedure was used, EPA was readily dispersed without freeze–thawing. Unless otherwise indicated, after the addition of NaOD, the apparent pH was reduced within 2 min to 7–8 by the addition of 1 M DCl. When the aqueous dispersions were freeze–dried and the residue was dissolved in $CHCl_3/CH_3OH$ (2:1 v/v), no degradation of the phospholipids was detectable by TLC.

For the production of sonicated PA dispersions, the apparent pH of unsonicated PA dispersions was adjusted to 7–8 by adding NaOD, and ultrasonication was carried out under standard conditions (Brunner et al., 1978), using a Branson B 12 sonicator with a microtip.

NMR Measurements. ^1H NMR spectra were recorded at 90 and 360 MHz by using Bruker HXE 90 and HXS 360 Fourier-transform spectrometers. Spectral intensities were measured by integration using a known quantity of CH_3CO-ONa as an internal intensity standard. The spectral lines in the ^1H NMR spectra were such that the intensity of the $N(CH_3)_3$ signal of EPC and of the hydrocarbon chain signals of phospholipids could be determined reproducibly. The hydrocarbon chain signal intensity is here defined as the sum of all signals upfield of the CH_3COONa standard. The disodium salt of LLPA forms micelles in H_2O, and using CH_3COONa as an intensity standard, it can be shown that all LLPA molecules contribute to the ^1H NMR spectrum. Micellar solutions of LLPA in D_2O (10.5 mg/mL) were used to optimize the NMR parameters. For the quantitative determination of signal intensities, to avoid saturation of the acetate intensity standard, a relaxation delay of ~20 s before each 90° pulse was used. In the presence of $K_3Fe(CN)_6$ (~10 mM), delay times of a few seconds were sufficient. The error of the intensity measurement depends on the absolute value of the signal intensity; it is usually within ±20% but increases if the signal intensity drops below 25%. The apparent pH was measured in the 5-mm NMR tube by using a combined glass electrode with a diameter of about 3 mm. The apparent pH was adjusted by adding NaOD or DCl to the NMR tube.

Electron Microscopy. Cryofixation of the samples was carried out as follows: a gold grid was dipped into the aqueous lipid dispersion and placed between two low-mass copper platelets. The assembly was frozen in a propane jet (Müller et al., 1980a), and the cryofixed sample was freeze–fractured in a Balzers BAF 300 at a pressure of 10^{-5} Pa. Contamination of the fracture surfaces was prevented by starting the evaporation of platinum/carbon prior to fracturing (2.5 nM Pt/C and 20 nM C). The replica was rinsed with $CHCl_3/CH_3OH$ (1:1 v/v). Samples for thin sectioning (Müller et al., 1980b) were first cryofixed and freeze–fractured as described above. Freeze substitution was carried out by immersing the fractured sample in the substitution medium at −95 °C. The substitution medium consisted of methanol containing 1% OsO_4, 0.5% uranyl acetate, and 3% glutaraldehyde. After the sample was left for 8 h each at −95, −60, and −30 °C, the substitution medium was replaced by methanol. Low-temperature embedding and UV polymerization in Lowicryl HM 20 were performed at −30 °C according to Carlemalm et al. (1982). Thin sections were stained with uranyl acetate and lead citrate.

Results

Effect of pH and Rate of NaOD Addition on the Fraction (Percentage) of Small Unilamellar Vesicles (SUV). In Figure 1, ^1H NMR spectra of various unsonicated EPA dispersions (B–E) are compared with that of a sonicated dispersion (A). The assignments together with the chemical shifts of the ^1H NMR signals of PA dispersions are summarized in Table I. The assignment is in good agreement with previous work (Hauser et al., 1975, 1980). Similar chemical shifts were observed for synthetic monoacyl- and diacylphosphatidic acids. Integration of the PA spectrum between 3 ppm and the HDO signal indicated the presence of four protons. The two protons at 3.99 ppm were assigned to the CH_2OP group and the remaining two peaks, consisting of one proton each, to the CH_2OCO glycerol. The splitting of this signal is due to the nonequivalence of the two CH_2OCO glycerol protons (Figure 1A); this has been reported for both short- and long-chain phosphatidylcholines (Hauser et al., 1980) as well as for lysophosphatidylcholines (Hauser et al., 1978).

From spectra B–E (Figure 1), it is clear that as the apparent pH of the unsonicated PA dispersions is raised by adding NaOD to ~8, a high-resolution spectrum is obtained with line widths similar to those of the sonicated dispersions (A). The spectral intensity increases as the apparent pH of the sample increases. Unsonicated mixed phospholipid dispersions containing PA and PC gave ^1H high-resolution spectra similar to those shown in spectra C–E of Figure 1 when their apparent pH was raised to 8–9 with NaOD (Figure 1F,G). It has been reported before (Finer et al., 1972; Hauser & Gains, 1982) that the signal intensity in the high-resolution spectrum of sonicated phospholipid dispersions correlates well with the

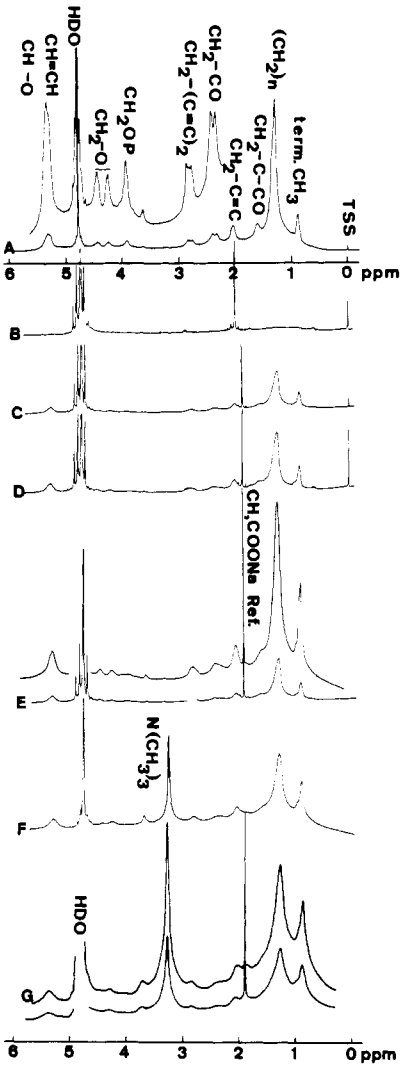

FIGURE 1: 360-MHz ^1H NMR spectra of various unsonicated EPA dispersions compared with that of a sonicated dispersion. (A) Sonicated EPA dispersion in D_2O, apparent pH 7.5 (14 mg/mL = 0.019 M). (B–D) Unsonicated EPA dispersions in D_2O (10 mg/mL = 0.013 M): (B) apparent pH ~3; (C) apparent pH ~8; (D) apparent pH ~11. (E) Same as (D) but the apparent pH was returned to ~8 with DCl; a vertically expanded version of (E) is also included. (F) Unsonicated EPC/EPA dispersion, mole ratio 2:1 (10 mg/mL = 0.013 M) in D_2O; the initial apparent pH of the dispersion was increased slowly to a final value of ~8.5. (G) Unsonicated phospholipid dispersion in D_2O (13 mg/mL = 0.02 M) containing EPC/PS/EPA, mole ratio 7:3:3; the apparent pH of the dispersion was adjusted by the slow addition of NaOD to a final value of ~8. Signal intensities in (B–G) are determined and normalized against 14.9 mM CH_3C-OONa (signal height = 20 cm) as an internal intensity standard.

fraction of the total phospholipid present as SUV (diameter ≲80 nm). Large multilamellar liposomes and LUV with diameters in excess of about 100 nm have been shown to give lines too broad to contribute to this high-resolution spectrum. Therefore, signal intensities in the ^1H high-resolution NMR spectra may be used as an approximate measure of the population of SUV present in phospholipid dispersions. This method has been used here to monitor the population of SUV present in phospholipid dispersions.

The main features of the unsonicated PA spectra (Figure 1) such as the number of signals and their line widths were independent of the rate of NaOD addition. However, when NaOD was added rapidly, the signal intensities were larger, and hence, a greater proportion of the total phospholipid was present as SUV. This is shown in Figure 2. Figure 2A shows the effect of pH on the fraction of PA contributing to the ^1H high-resolution spectrum. The signal intensity increases approximately linearly with pH above a threshold pH of ~5 and up to about pH ~12 (○). A further increase in pH led to a reduction in signal intensity which is probably a salt effect (see below). The fast addition of NaOD (●) has the effect of increasing the signal intensity (Figure 2A), indicating that in this case the population of SUV is larger than when NaOD was added slowly (○). Raising the apparent pH to 11–12 in less than 1 s produces 90–100% SUV. In contrast, the fraction of SUV produced by the slow addition of NaOD to the same pH is only 50–70%. The effect of the PA content of PC/PA mixed dispersions on the ^1H signals is shown in Figure 2B (○). The signal intensity and hence the population of SUV increase approximately linearly with PA content. For comparison, the signal intensity of the $N(CH_3)_3$ choline signal of PC is included (▲); above about 40% EPA, the $N(CH_3)_3$ signal intensity is 90–100% of that expected from the phosphorus determination. Figure 2B also shows that synthetic saturated LLPA (△) and DLPA (□) are approximately equally as effective in dispersing PC as the naturally occurring unsaturated EPA. As with EPA dispersions (Figure 2A), the fast addition of NaOD to PC/PA mixed dispersions produced higher signal intensities, i.e., larger proportions of SUV (Figure 2B, ●). For instance, the hydrocarbon chain signal intensity in the ^1H NMR spectrum of an unsonicated EPC/EPA dispersion (mole ratio 2:1, Figure 1F) was 20% when NaOD addition was slow. In contrast, the fast addition of NaOD produced a signal intensity that was increased by a factor of almost 2. The PC/PA dispersions shown in Figure 2B (○) were stored at 4 °C, and the signal intensity was examined as a function of time. Within the error of the measurement, no change in signal intensity was detected over a period of 7 days (data not shown).

That the method of vesiculation described here is of wider applicability is shown in Figure 2C. When this is applied to unsonicated mixed phospholipid dispersions containing EPC, PS, and EPA (mole ratio 7:3:x, ▲), the resulting intensity of the hydrocarbon chain signal increases approximately linearly with PA content. The signal intensities for unsonicated mixed dispersions of asolectin and EPA as a function of EPA content are included in Figure 2C (●).

As shown in Figure 2A, the extent of SUV formation depends, among other factors, on the pH to which the phospholipid dispersion is exposed: a maximum is reached at about pH 11–12 (Figure 2A). Figure 3 confirms this for a EPC/EPA dispersion (mole ratio 1.7:1). Similar results were obtained with other PC/PA dispersions provided the PA content exceeded ~30% (PC/PA mole ratio ≤2). However, at PA contents <25%, the maximum signal intensity is reached at pH 8–9 with little further increase upon raising the pH to 11–12. Figure 3 shows that reducing the apparent pH from 11.5 to 3 produced a reduction in signal intensity from about 45 to 25%. In pure EPA dispersions in which vesiculation was induced by the slow addition of NaOD to pH 11–12, the SUV fraction decreased upon acidification to pH ~3 from 60 to 50% (data not shown). Figure 3 shows that when NaOD was added to the acidified dispersion and the apparent pH was brought back to neutrality, the original signal intensity and hence the original population of SUV were restored. This reversibility suggests that the reduction in signal intensity is probably due to vesicle aggregation, but fusion cannot be ruled out.

Effect of NaCl on the Population of SUV Contributing to the High-Resolution Spectrum. It can be shown that sonicated EPA dispersions consist of SUV [Figure 1A and Hauser & Gains (1982)]. All of the PA molecules contribute to the

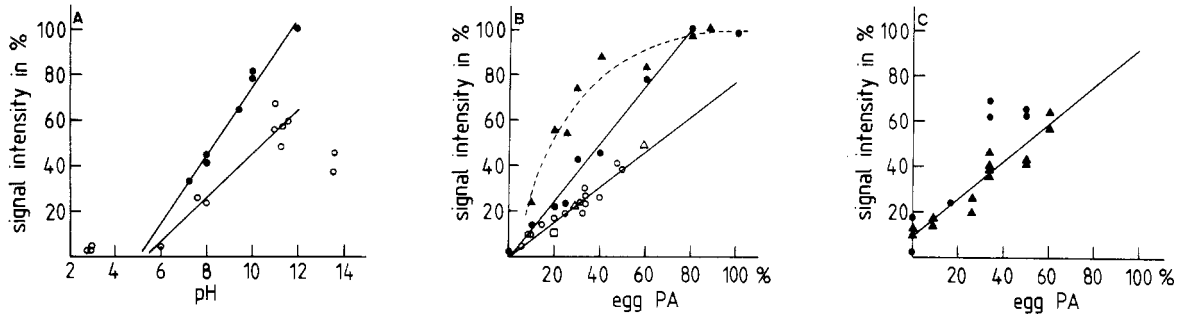

FIGURE 2: (A) Hydrocarbon chain signal intensity in ^1H NMR spectra of EPA as a function of the maximum apparent pH to which the EPA dispersion was adjusted. (A) Hydrocarbon chain signal intensity in ^1H NMR spectra of EPA as a function of the maximum apparent pH to which the EPA dispersion was adjusted. The hydrocarbon chain signal intensity as defined under Materials and Methods is used as an approximate measure of the proportion of total lipid present as SUV. The hydrocarbon chain signal intensity is expressed as the percent of the total hydrocarbon chain signal intensity expected from the phospholipid composition. Unsonicated EPA dispersions (~10–14 mM) in D_2O were prepared as described under Materials and Methods. Spontaneous vesiculation was induced by raising the apparent pH either by adding the NaOD slowly (~2 min) from an Agla syringe (○) or by adding NaOD rapidly (~1 s, ●) from a micropipet. The solid lines through the data points between pH 6 and 12 are linear regression lines with slopes of 9.6 and 15 for the slow and fast NaOD addition, respectively. (B) Hydrocarbon chain signal intensity or fraction of total phospholipid (in percent) present as SUV as a function of EPA content. Unsonicated dispersions of EPA and EPC (total phospholipid concentration ~13 mM) were prepared as described under Materials and Methods. Vesiculation was induced by transiently raising the apparent pH to 11–12 either by the addition of NaOD slowly from an Agla syringe (○) [included in the plot are mixed dispersions containing instead of EPA LLPA (△) and DLPA (□), both as the disodium salt] or by the fast addition of NaOD from a micropipet in less than 1 s (●). Subsequently, the apparent pH was returned to 7–8 by adding DCl. For the fast addition of NaOD, the fraction of the $N(CH_3)_3$ choline signal of EPC contributing to the ^1H high-resolution spectrum is also shown (▲). The solid lines are least-squares fits with slopes of 0.77 (○) and 1.26 (●). The dashed curve fitted to the triangles is arbitrary in the sense that it could equally well be replaced by two straight lines. (C) ^1H hydrocarbon chain signal intensity expressed as the percent of total phospholipid (10–14 mM) in unsonicated phospholipid dispersions prepared as described under Materials and Methods. Vesiculation was induced by the fast addition of NaOD, raising the apparent pH transiently to 11–12; then, the apparent pH was brought back to 7–8 by adding DCl. EPC/PS/EPA mixtures containing EPC/PS at a constant mole ratio of 7:3 (▲); asolectin/EPA mixtures (●). The line is a least-squares fit to the data for the EPC/PS/EPA mixtures.

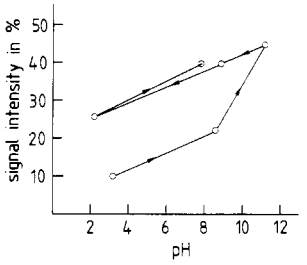

FIGURE 3: Signal intensity of the ^1H hydrocarbon chain signal (upfield of the CH_3COONa reference) of an EPC/EPA mixed dispersion (~10 mg/mL = 0.013 M, mole ratio 1.7:1) in D_2O as a function of the apparent pH. The apparent pH of the original dispersion, which was 3.1, was increased by the addition of NaOD and decreased by the addition of DCl from an Agla syringe. At each pH indicated, a ^1H NMR spectrum was recorded within 10 min. The titration of the sample was carried out in the direction indicated by the arrows. The dilution of the sample by the addition of NaOD or DCl was minimized by using 2 M solutions. Within experimental error, the same plot was obtained if separate lipid dispersions were made up for each point shown on the graph.

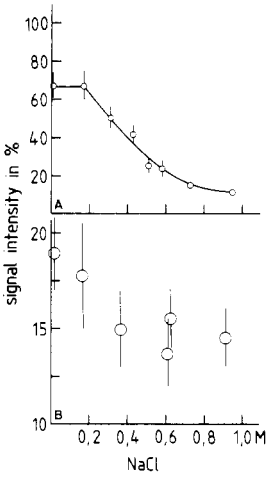

FIGURE 4: Effect of NaCl concentration on the ^1H signal intensity and hence on the fraction of SUV present in unsonicated EPA (A) and EPC/EPA, mole ratio 3:1 (B), dispersions. The intensity of the hydrocarbon chain signal is expressed as the percent of the total signal intensity expected if all the phospholipid was present as SUV. EPA and EPC/EPA vesicles were made by slowly raising the apparent pH of unsonicated dispersions in D_2O with NaOD to pH 11–12, followed by the addition of DCl to pH 7–8. The initial concentration (EPA, 14 mM; EPC/EPA, 12 mM) was diluted by the addition of 2 M NaCl. The initial Na concentration of the EPA dispersion, i.e., the Na^+ concentration of EPA before the addition of NaCl, was 0.03 M.

high-resolution spectrum. The addition of NaCl to such dispersions led to a reduction in signal intensity; for instance, physiological concentrations of NaCl produced a 10–30% reduction. This reduction was by and large reversible, suggesting that it is probably due to vesicle aggregation. Figure 4A shows the effect of increasing the NaCl concentration added to unsonicated SUV of EPA formed by pH-induced vesiculation. In contrast to sonicated EPA vesicles, there was no reduction, at least within experimental error, in the population of SUV up to [NaCl] $\simeq$ 0.2 M. Above this concentration, the percentage of SUV contributing to the high-resolution NMR spectrum decreased continuously, and at [NaCl] > 1 M, it was reduced to ~10%. Figure 4B shows the effect of NaCl concentration on SUV of EPC/EPA (mole ratio, 3:1) formed by pH-induced vesiculation. The percentage of SUV decreased, but, as expected with a less negatively charged surface, the NaCl concentration effect was smaller than with pure PA vesicles. It is important to note that the presence of NaCl during the process of pH-induced vesiculation reduces the population of SUV. In the presence of NaCl at 0.15 M, the fraction of SUV formed by the fast addition of NaOD was approximately half of that formed in the absence of NaCl (data not shown).

Ion Permeability and Stability. The ion permeability of SUV produced by spontaneous vesiculation of EPC/EPA bilayers was tested by using $K_3Fe(CN)_6$. The presence of the paramagnetic $Fe(CN)_6^{3-}$ anion produced splitting of the $-N-(CH_3)_3$ and $-CH_2N$ choline signals of PC. This kind of

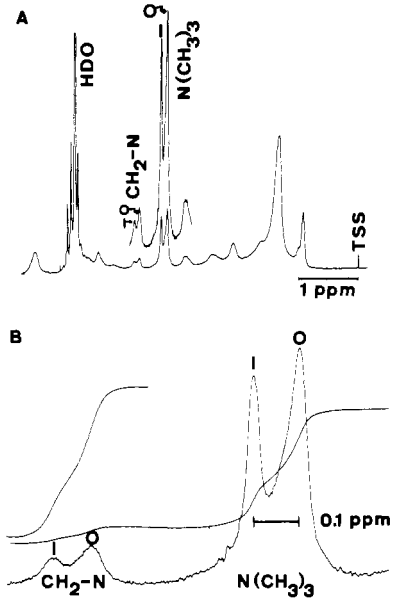

FIGURE 5: (A) ^1H NMR spectrum recorded at 360 MHz of a sonicated dispersion of EPA/EPC (~10 mg/mL = 0.013 M, mole ratio 3:1) in D_2O containing 0.1 M $K_3Fe(CN)_6$. In the presence of paramagnetic $Fe(CN)_6^{3-}$, the $-N(CH_3)_3$ and $-CH_2N$ signals from the choline group of EPC are split into two parts denoted O (outside) and I (inside). The resonance O is assigned to the molecules located on the outer layer of the bilayer as exposure to $Fe(CN)_3^{3-}$ shifts this resonance upfield whereas the chemical shift of resonance I from molecules on the inner layer is unaffected. (B) Expanded polar group region of spectrum A showing the splitting of the $-N(CH_3)_3$ and $-CH_2N$ choline signals of EPC. The upfield shift of the external $-N(CH_3)_3$ signal (O) is exactly 0.1 ppm. For both resonances, the intensity ratio of outside to inside (O/I) = 1.6 (cf. integrals superimposed on the spectrum).

splitting has been discussed in detail for phosphatidylcholine vesicles and is characteristic of SUV. The polar group resonances of the PC molecules on the external side of the bilayer, which is exposed to $Fe(CN)_6^{3-}$, are shifted to higher fields. In unsonicated EPC/EPA dispersions (mole ratio 3:1) in which vesiculation was induced by the fast addition of NaOD, the ratio of external (O) to internal (I) PC was O/I = 1.6:1. This ratio was identical with that of a sonicated EPC/EPA dispersion of the same mole ratio (Figure 5). The value for O/I $\simeq$ 1.6 is smaller than the range of values of 1.9–2.2 measured for sonicated SUV of EPC (Barsukov et al., 1974; Hauser et al., 1975; Hutton et al., 1977). The reduction of O/I as compared to pure EPC may be due to the asymmetric distribution of EPC and EPA over the two bilayer halves and/or a larger average vesicle diameter. The O/I ratio of the above EPC/EPA dispersion in the presence of $K_3Fe(CN)_6$ was measured as a function of time. Over a period of 48 h, neither the absolute signal intensities nor the O/I ratio changed significantly. Similar results were obtained for mixed EPC/EPA dispersions with an EPA content varying between 20 and 50%. In one instance, such a dispersion (EPA content ~30%), to which $[K_3Fe(CN)_6]$ = 0.12 M had been added, was stored at 4 °C for 14 days. ^1H NMR spectra were recorded as a function of time. The spectra were practically superimposable onto the original one obtained after sample preparation (data not shown).

Discussion

Here we have used ^1H high-resolution NMR spectroscopy to monitor the proportion of phospholipid present in aqueous phospholipid dispersions as SUV. It has been shown before that the signal intensity of the high-resolution spectrum correlates well with the percentage of phospholipid present as SUV. Evidence for this has been obtained by comparison of the NMR signal intensities with the percentage of SUV determined by gel filtration on Sepharose 4B (Hauser & Gains, 1982). Furthermore, a reasonable estimate of the fraction of phospholipid present as SUV can also be obtained from ultracentrifugation. LUV and multilamellar structures sediment when aqueous dispersions are centrifuged at 160000*g* for 3 h (Barenholz et al., 1977); the fraction of SUV recovered in the supernatant is similar to or slightly larger than that giving a high-resolution NMR spectrum. We have reported (Gains & Hauser, 1983) that aqueous EPA dispersions vesiculate when their pH is increased transiently to 10.5–11. The same is true for mixed dispersions of EPC and EPA when the pH is raised to 8–9. The procedure described previously, i.e., the slow addition of NaOD to an unsonicated EPA dispersion in D_2O, yields a vesicle population consisting of 50–70% of SUV of a narrow size distribution with the remainder consisting mainly of LUV and some multilamellar structures,[2] both of a wide size range. The modified procedure described here is a useful extension of the existing methodology. Either by the rapid addition of NaOD to the ready-made aqueous EPA dispersion or by the direct addition of NaOD solution (apparent pH ~12) to the EPA film dried down on the glass wall, the ratio of SUV to LUV can be increased. In this way, it is possible to convert practically all the EPA into SUV of a relatively narrow size range.

That the method of vesiculation based on the transient pH increase is not restricted to EPA and mixed EPC/EPA dispersions but is of wider applicability is demonstrated in Figure 2. With all phospholipid mixtures containing EPA, which were tested, the transient increase in pH to ~11–12 produced vesiculation yielding essentially a mixture of SUV and LUV. This is also true for mixtures of synthetic, saturated PC's and EPA. When unsonicated mixed dispersions of DMPC, DPPC, and DSPC containing EPA (weight ratio 1:1) were transiently exposed to high pH by the rapid addition of NaOD, vesiculation was induced as with natural phospholipids. However, the fraction of SUV as determined by ^1H NMR was less: 36 ± 3% for the DMPC dispersion and about 10 ± 4% for the DPPC and DSPC dispersions (data not shown). As shown in Figure 2B,C, the resulting fraction of SUV is directly proportional to the PA content. It could therefore be argued that during vesiculation the components of the phospholipid mixture separate from one another, the PA forming the SUV and the PC the larger vesicles. However, the concomitant increase in the $N(CH_3)_3$ signal with that of the hydrocarbon chain signal (Figure 2B) shows that EPC is a component of the SUV. The intensity of the $N(CH_3)_3$ signal in fact increases more rapidly with increasing EPA content than the hydrocarbon chain signal. However, this does not necessarily indicate an enrichment of EPC in the SUV; the enhanced signal arises from the relatively isotropic motion of the $N(CH_3)_3$ group [see Finer et al. (1972)].

Besides the rate of NaOD addition, another important parameter determining the ratio of SUV to LUV is the pH to which the dispersion is exposed. If the PA content exceeds 25–30%, the pH has to be raised rapidly to 11–12 in order to

[2] The term multilamellar is used here to indicate that many bilayers are packed in a concentric fashion, as is observed with unsonicated aqueous dispersions of phosphatidylcholine. The term LUV is used for vesicles with a diameter of ≥100 nm that are surrounded by a single closed bilayer. The larger these vesicles are, the more likely it is that they entrap smaller unilamellar vesicles. Since the entrapped vesicles are randomly distributed and are not concentrically packed, we consider them and the larger vesicle that entraps them as being unilamellar and not oligo- or multilamellar.

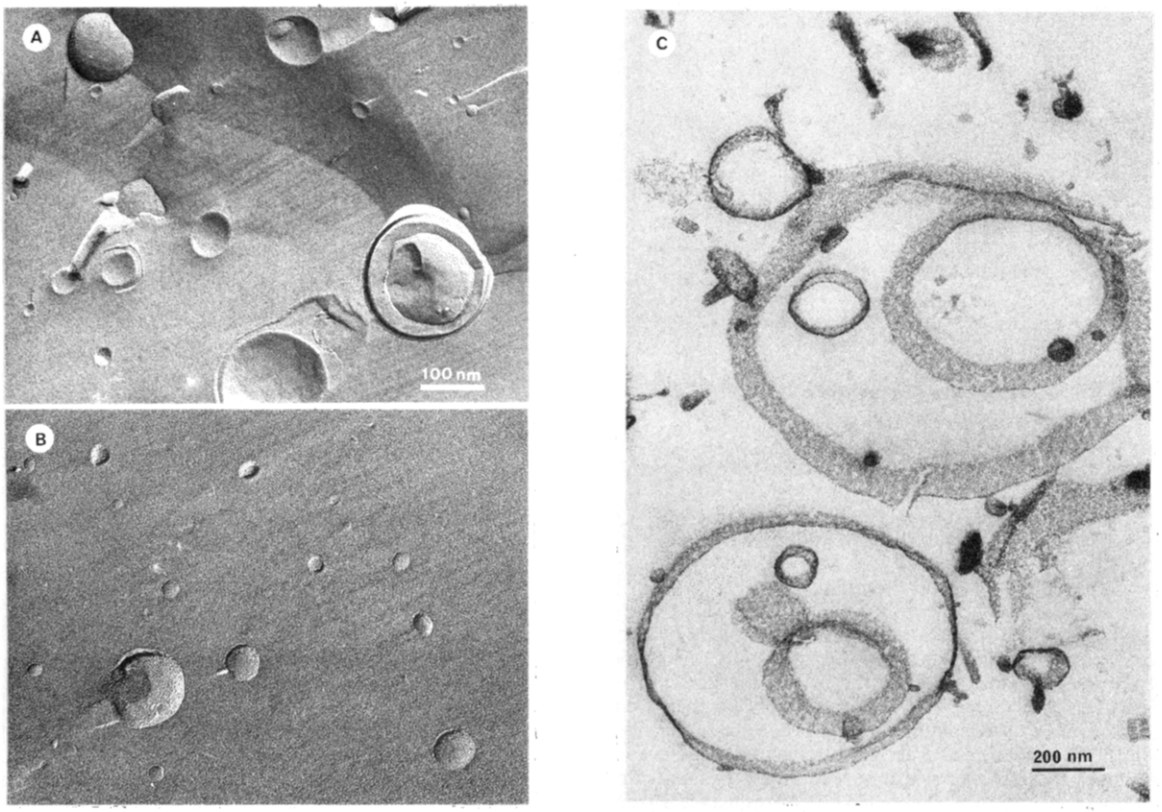

FIGURE 6: Electron microscopy of freeze–fractured samples (A, B) and of a thin-sectioned sample (C) of unsonicated EPC/EPA dispersions in H_2O. Freeze–fracturing (samples A and B) and freeze–substitution (sample C) were carried out as described under Materials and Methods. Samples A and B are unsonicated EPC/EPA dispersions (mole ratio 3.3:1, 10 mg/mL). Vesiculation was induced by the slow addition of NaOH to pH 8.6 and 12, respectively. Sample C is an unsonicated EPC/EPA dispersion (mole ratio 7:3, 10 mg/mL). Vesiculation was induced by the addition of NaOH (pH 12) to the dry lipid film followed by neutralization to pH 7.5 with HCl.

produce a maximum of SUV. With phospholipid dispersions containing less PA (≤25–30%), a pH of 8–9 suffices to maximize the SUV fraction. A possible explanation for the higher pH required for pure PA is that, in bilayers of pure PA, due to the larger surface potential, the second pK of PA is higher than in mixed bilayers of PC and PA with randomly distributed PA molecules.

That aqueous dispersions of mixed phospholipids containing EPA vesiculate when transiently exposed to high pH can be shown by electron microscopy of freeze–fractured samples. Figure 6 demonstrates that, besides SUV, mainly LUV are formed by this procedure. Onion-like, multilamellar structures, as are typical for the lamellar phase of PC, are absent. Frequently, smaller vesicles are entrapped in larger ones, but all vesicles appear to be bounded by a single bilayer.

The physicochemical properties of SUV produced by the vesiculation method described here, particularly their stability and permeability, are important when assessing their potential application as drug delivery systems or as model membranes. Once formed at pH 11–12, they are relatively stable to subsequent pH changes and the addition of NaCl up to concentrations of 0.2 M (Figures 3 and 4). When the apparent pH of a mixed EPC/EPA dispersion containing 37% PA is reduced from ~11 to its original pH of ~2–3, the signal intensity is not reduced to its initial value (Figure 3). The reduction in signal intensity is much less, indicating that the vesiculation induced by the transient increase in pH is by and large irreversible. The irreversible nature of the vesiculation process can be demonstrated by ^{31}P NMR. The ^{31}P powder-type NMR spectrum obtained from an unsonicated PA dispersion (pH 7) is characteristic of large lamellar (bilayer) structures (data not shown). The ^{31}P spectrum of the same sample after the pH is raised to 12 and then reduced to 8 consists of a single narrow peak; it is indistinguishable from that of a sonicated PC or PA dispersion in which the ^{31}P chemical shielding anisotropy is effectively averaged out. Returning the pH of this dispersion to its original value of 3 does not produce a gross change in the ^{31}P NMR spectrum as compared to that of pH 8 (data not shown). Furthermore, the relatively small reduction in signal intensity on reducing the apparent pH from 11 to 2.5 (Figure 3) is reversible, indicating that it is probably mainly due to vesicle aggregation.

Figure 4 shows that, for pure EPA and for phospholipid mixtures, NaCl concentrations >0.3 M are required before a significant loss in signal intensity is observed. These results are in good agreement with the data of Abramson et al. (1964), who reported the coagulation of a phosphatidic acid dispersion, pH 5, at [NaCl] ≥0.3 M. The loss in intensity is at least partially reversible upon dilution of NaCl. The reversible loss in intensity is probably due to vesicle aggregation rather than fusion which would be expected to be irreversible.

The independence of the ^1H NMR spectra in the presence of $K_3Fe(CN)_6$ indicates that phospholipid dispersions containing EPA are stable and practically impermeable to ions. Recent efflux measurements carried out with these vesicles support this conclusion. It can be shown that the efflux of radiolabeled $^{22}Na^+$ and $^{35}SO_4^{2-}$ from EPA vesicles and EPC/EPA vesicles (EPA content 14%) follows first-order kinetics with a half-life time of the order of days (Gains & Hauser, 1983).

The preparative method described here produces unilamellar lipid vesicles. This method is easy and quick and has a number of significant advantages compared to other methods (Tyrell et al., 1976; Szoka & Papahadjopoulos, 1980; Hauser, 1982): it does not require elaborate laboratory equipment, it avoids time-consuming procedures such as ultrasonication and de-

tergent removal by dialysis or gel filtration, and it is therefore commercially economic. It can be applied to a variety of phospholipid mixtures containing PA and gives reproducible results. Preliminary experiments show that the method can be applied to large quantities of lipids. The ratio of SUV to LUV depends on a number of factors; foremost are the pH to which the phospholipid dispersion is exposed and the rate of the NaOD addition. Furthermore, the ionic strength and the nature of the ions present are important in determining this ratio and, for mixed phospholipid dispersions, the PA content. By varying these parameters, it is possible not only to control the ratio of SUV to LUV but also to vary the surface charge density of the bilayer and hence the surface potential within wide limits. By selecting suitable mixtures, it should, in principle, be possible to control another important bilayer property: lipid fluidity. In summary, the vesicles produced by pH adjustment as described here have characteristics which make them potentially useful as drug carriers. Questions of bilayer permeability and stability are important when considering such applications. Our experiments show that the unilamellar vesicles produced by this method are sufficiently stable and relatively insensitive to changes in pH and ionic strength. In their permeability to ions, these vesicles resemble sonicated PC vesicles (Gains & Hauser, 1983).

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Conformational States of *N*-Acylglycine Dithioesters in Solution: Resonance Raman Studies of Isotopically Substituted Models for Enzyme–Substrate Complexes†

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ABSTRACT: Resonance Raman (RR) and FTIR spectroscopic studies, taken with X-ray crystallographic data, are used to define the three major conformational states of *N*-acylglycine dithioesters in solution and to set up spectra–structure correlations. Importantly, each conformer has a characteristic RR signature, and thus the RR spectrum can be used to follow conformational events within dithioester enzyme–substrate intermediates. The signatures are further defined in the present work by the synthesis and spectroscopic characterization of ^{13}C and ^{15}N derivatives of *N*-benzoylglycine ethyl dithioester and *N*-(β-phenylpropionyl)glycine ethyl dithioester. The observed isotope shifts offer insight into the normal mode character of the RR bands and provide standards with which to compare the shifts in the corresponding enzyme–substrate intermediates.

The impetus for the present work originates in the finding that the resonance Raman (RR) spectra of dithioacyl enzymes, of the type $RC(=O)NHCH_2C(=S)S$–papain, provide a means of monitoring the vibrational spectrum of those bonds undergoing catalytic transformation in the enzyme's active site (Storer et al., 1979). The major features in the RR spectra of the enzyme intermediates occur in the 500–1200-cm^{-1} region and, in principle, give precise information on the conformational state or states of the enzyme-bound substrate. However, to elicit this information, it has been necessary to undertake extensive investigations into the spectroscopic and conformational properties of dithioesters. These studies include de-

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