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Effect of Sonication on the Structure of Lecithin Bilayers†

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ABSTRACT: The properties of dipalmitoyllecithin vesicles of varying sizes have been compared employing proton and ^{31}P nuclear magnetic resonance spectroscopy and dilatometry. These studies indicate that small vesicles of about 250 Å in diameter are inherently disordered and that the well-known spectral differences generally observed for vesicles of different curvatures such as lecithin multilayers and sonicated bilayers arise from variations in the molecular packing of phospholipid molecules in the bilayer phase.

The increased interest in membrane structure and biological transport has stimulated the development of easily prepared model membrane systems, which can be readily manipulated to elucidate some of the physical and chemical properties of biological membranes. In particular there has been much interest in phospholipid bilayers. A simple suspension of bilayer-forming lipids in water produces a multilamellar structure containing a number of concentric vesicles of various sizes (Bangham and Horne, 1964). Although many structural questions concerning the bilayer phase of these model membranes can be deduced from studies of these multilamellar structures (Papahadjopoulos and Miller, 1967), they are unsuitable for transport studies (Bangham, 1972). Moreover, each multilamellar unit contains an unknown number of phospholipid bilayers separated by an unknown amount of water, whose state of order is unclear. It is, however, possible to obtain homogeneous phosphatidylcholine vesicles by prolonged ultrasonic irradiation. These vesicles are closed spheres about 250 Å in diameter, each composed of a single continuous lipid bilayer membrane enclosing a volume of aqueous solution (Huang, 1969). Such vesicles have been widely used in recent years as model systems for structural studies of the bimolecular lipid lamellar phase and, in contrast to the unsonicated preparation of Bangham (1968), have been used in numerous physiochemical and transport studies (Kornberg and McConnell, 1971a,b; Lee *et al.*, 1972; Papahadjopoulos and Watkins, 1967; Bangham, 1972).

During the last several years a number of attempts have been made to characterize phospholipid bilayers by nuclear magnetic resonance spectroscopy (Penkett *et al.*, 1968; Finer *et al.*, 1972). As a result of these studies it is now known that the nuclear magnetic resonance (nmr) spectrum of phospholipid bilayers is very different depending upon whether the bilayers are in a multilamellar state or whether they are in vesicles of 250 Å in diameter. Unsonicated lecithin suspensions (multilayers) exhibit only broad resonances (Chan *et al.*, 1971) in contrast to the proton magnetic resonance (pmr) spectra of sonicated vesicles which contain sharp resonances. The sharper resonances are presumably a manifestation of a faster and more complete averaging of the nuclear dipole–dipole interactions of the proton spins, but the way in which the sonication of lecithin leads to this motional averaging is not understood despite numerous attempts.

This paper describes an attempt to elucidate those factors responsible for the spectral differences between lecithin multilayers and vesicles of 250 Å in diameter. In this work dipalmitoyllecithin vesicles were prepared with a wide range of diameters and their nmr spectral as well as molal volume characteristics were studied under a variety of experimental conditions. These studies indicate that the small vesicles of about 250 Å in diameter are inherently disordered and that the observed differences associated with vesicles of different curvatures arise from differences in local mobility through variations in molecular packing.

Experimental Section

Preparation of Dipalmitoyllecithin Samples. L-α-Dipalmitoylphosphatidylcholine from Nutritional Biochemicals was checked for purity by thin-layer chromatography (tlc) and found to contain less than 1% impurity. It was also shown to have no effect on the conductivity of deionized and distilled water. This lecithin was used without further purification in the preparation of the following types of bilayers.

UNSONICATED BILAYERS. Dipalmitoyllecithin samples in the range of 100–300 mg/ml of lecithin in H_2O were suspended by repeated passage at 60° through a 6-in. long 20-gauge needle.

VESICLES CONTAINING HIGH SALT. A suspension of about 50 mg/ml of lecithin in D_2O (0.1 M NaCl–2 mM PO_4 at pD 7.8) was sonicated for five minutes at power level 6 with a Bronson sonifier Model S-75. Initially the temperature was 20° but during sonication the temperature of the sample rose above 50°. The sample was then centrifuged at 20,000*g* in a Sorvall RC-2 centrifuge for 30 min and the lower layer was drawn off since the larger particles centrifuged to the surface. The

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lower layer, containing the smaller particles, was then applied to a Sepharose 4B column (2.5 × 80 cm), which had been calibrated with bacteriophage ϕX/174, a nearly spherical virus of about 250 Å in diameter (donated by Anthony Zuccarelli, Division of Biology, Caltech). The phospholipid concentration in each fraction was monitored by measuring the amount of light scattered at 300 mμ with a Beckman D.U. spectrophotometer. From each major peak in the elution curve several of the 6-ml fractions were pooled and were then concentrated on an Amicon pressure dialysis apparatus with a UM-10 membrane. The concentrated small vesicle fraction was centrifuged again at 20,000*g* for 30 min and the supernatant, which contained the smaller vesicles, was withdrawn. All concentrated samples were then dialyzed in D_2O containing 2 mM PO_4 at pD 7.2. The purity of the lecithin in the vesicles was rechecked by tlc and the lipid was found to give a single spot. Divalent ions were added to the vesicles in the form of microliter amounts of a 0.1 M solution of the sulfate salts in D_2O. In the viscosity experiments the sample viscosity was increased by the addition 1.5% (w/v) of native calf thymus DNA (Worthington Biochemicals), and the solution was made homogeneous by passage through a 22-gauge syringe needle.

VESICLES WITH LITTLE OR NO SALT. Suspensions of 50–100 mg/ml of lecithin in water or in D_2O (2 mM PO_4 at pD 7.8) were sonicated for 10 min at 60° with a Bronson ultrasonicator using a microtip probe at power level 5. Samples were then centrifuged at 20,000*g* for 30 min. Samples of small vesicles were obtained directly from the supernatant in this procedure and used for nmr and dilatometry experiments. Larger vesicles were prepared by passage of a sample sonicated in 2 mM PO_4 at pD 7.8 through a Sepharose 4B column equilibrated in the same buffer. Considerable aggregation occurred on the column under these conditions and no small vesicle fraction could be obtained. These larger vesicles from the column were then concentrated by pressure dialysis and centrifuged at 20,000*g* for 10 min. For pmr studies the supernatant was dialyzed into D_2O (2 mM PO_4 at pD 7.8).

Electron Microscopy. Portions of the vesicle solutions were diluted to 1–5 mg/ml of lecithin and a drop of the solution was applied to a 400 mesh copper grid coated with parlodion sprayed with a thin film of carbon. After 30 sec the majority of the liquid was drawn off with a blotter and a drop of 2% phosphotungstic acid solution (pH 7.4) was applied. After another 30 sec the grid was blotted to remove excess liquid and allowed to dry. The grids were observed on a Phillips 300 electron microscope operating at 60 kV and pictures were recorded on a 35 mm camera at 10,000–50,000 × magnification. Polystyrene beads with a diameter of 1000 ± 30 Å were used to calibrate the electron micrographs and size distributions were obtained by sizing several hundred vesicles from the electron micrographs. Samples for electron microscopy were taken both prior to and after proton magnetic resonance measurements.

Dilatometry and Molal Volume Experiments. A simple volumetric dilatometer was used to measure the temperature dependence of the volume of the lipid–water system. The dilatometer had a capacity of 5.0 ml and was calibrated with distilled, deionized water over the temperature range of 25–45°. The temperature dependence of the sonicated and unsonicated systems were studied over the range of 26–45° by placing the dilatometer in a temperature-controlled water bath and by following the meniscus in the capillary tube (1.1-mm diameter) with a cathetometer. The full range of temperature was covered in 3–4 hr.

In these experiments only suspensions of sonicated and unsonicated dipalmitoyllecithin containing no salt were studied and the phospholipid concentration was varied from 4 to 16%. Two independent methods were used to determine the apparent molal volume of the lecithin at 27°. First, the volume of the sample in the dilatometer was measured at several lecithin concentrations and the apparent molal volume of the lecithin was obtained using the following formula: $\bar{V} = x_{H_2O}\bar{V}_{H_2O} + x_L\bar{V}_L$, where $\bar{V}$ is the apparent molal volume of the lecithin suspension as measured; and $\bar{V}_{H_2O}$ and $\bar{V}_L$ denote, respectively, the apparent molal volumes of the H_2O and lecithin in the two-component system. x_{H_2O} and x_L denote the mole fractions of the components. The phospholipid concentration was determined by two methods: by drying and weighing, and by colorimetric analysis for total phosphorus using the method of Bartlett (1959). Secondly, the apparent molal volume of the lecithin was obtained from the buoyant density of the lecithin in mixtures of D_2O and H_2O as measured by centrifugation to within ±0.0005 mg/ml. Ideal mixing of D_2O and H_2O was assumed in these experiments and the following formula was used to calculate the density of the lecithin at neutral buoyancy from which the apparent molal volume was determined.

$$\rho_{sample} = \frac{\text{vol \% } D_2O}{100}(\rho_{D_2O} - \rho_{H_2O}) + \rho_{H_2O}$$

Proton Magnetic Resonance Measurements. All pmr spectra were taken on a modified Varian HR-220 superconducting nmr spectrometer equipped with frequency sweep and multinuclear capabilities, and a C-1024 time-averaging computer was used to enhance the signal-to-noise ratio. Probe temperature was measured with an ethylene glycol sample using the calibration curve supplied by Varian. Chemical shifts were measured relative to an external acetone standard in D_2O doped with $MnSO_4$ which was in turn calibrated *vs.* temperature with DSS (3-(trimethylsilyl)propanesulfonic acid sodium salt).[1] The area of the acetone resonance was used as an area standard and was calibrated using a 0.003 M solution of tetrabutylammonium bromide.

^{31}P Nmr Measurements. ^{31}P nmr spectra of sonicated and unsonicated dipalmitoyllecithin bilayers were recorded at a magnetic field of 22 kg and the resonance frequency of 36.4 MHz on a Bruker H-90 spectrometer equipped with a Nicolet 1074-PDP-8e computer system operating in the Fourier transform mode, and at 53 kg and 89 MHz on a modified Varian HR-220 superconducting nmr spectrometer equipped with frequency sweep and multinuclear capabilities. Proton noise decoupling was used to remove any effects arising from phosphorus–proton spin–spin couplings, and either a C-1024 time-averaging computer or a Varian 620i computer operating in the accumulation mode was used to enhance the signal-to-noise ratio in the 89-MHz experiments.

Results

Size Distribution of Vesicles. The particle size distributions of vesicle samples were found to vary with the length of sonication and whether the vesicles were passed through a Sepharose 4B column in the presence or absence of salt. For this reason all of the vesicle samples were visualized by negative

[1] Abbreviation used is: DSS, 3-(trimethylsilyl)propanesulfonic acid sodium salt.

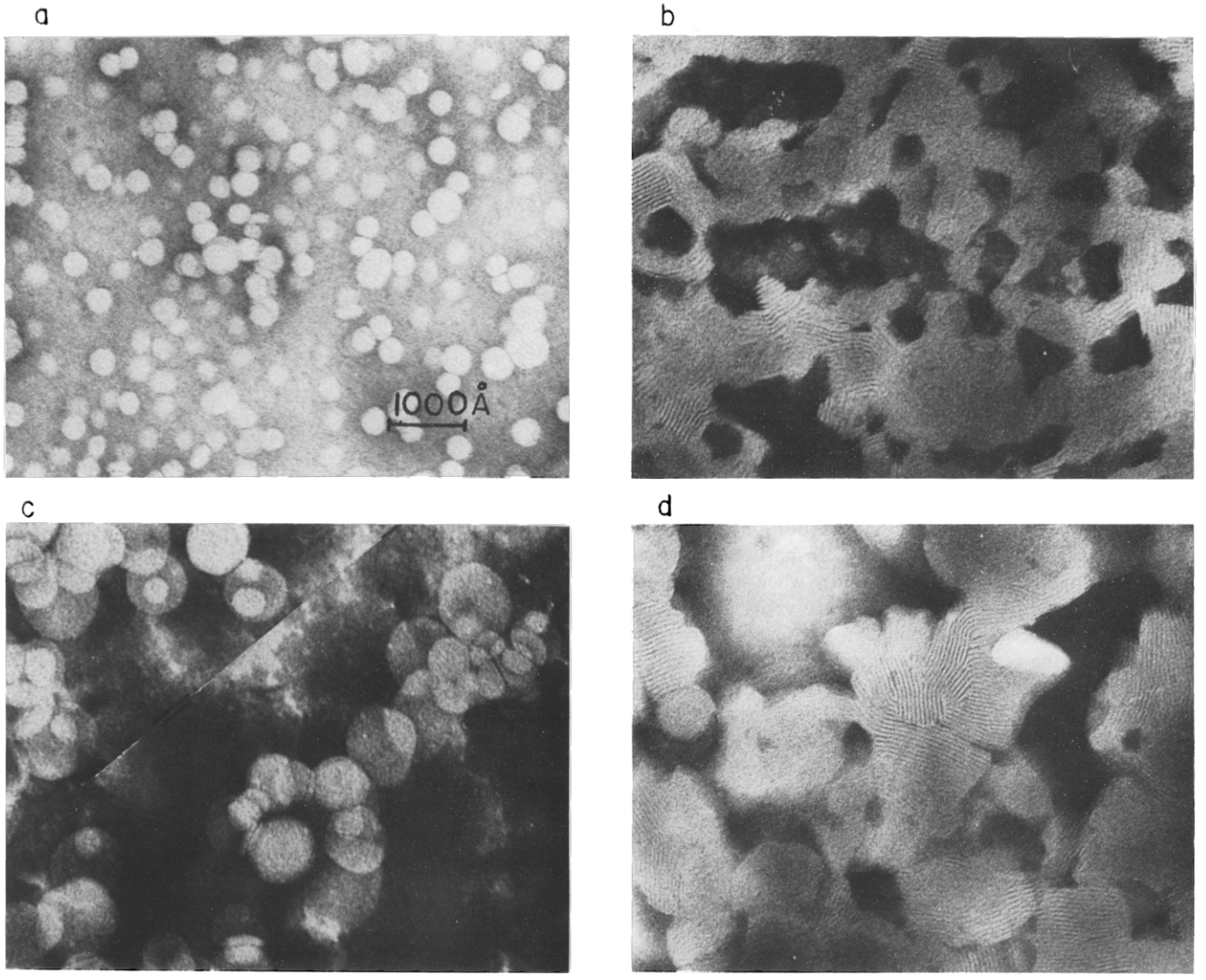

FIGURE 1: (a) Small vesicles containing 0.1 M NaCl before heating; (b) after heating to 45° for 0.5 hr; (c) larger vesicles containing 0.1 M NaCl before heating; (d) after heating to 45° for 0.5 hr.

TABLE I: Per Cent by Weight of Lecithin in Different Size Vesicles and the Average Vesicle Size.

Sample	150–350 Å	350–450 Å	450–600 Å	600–800 Å	800–1000 Å	1000–1400 Å	Av (Å)
Small vesicles containing high salt	69	22	9				300
Larger vesicles containing high salt		4	12	14	41	29	900
Small vesicles containing low salt	33	44	9	10	4		350
Larger vesicles containing low salt				16	43	41	1000
Sonicated dilatometry sample after 16 hr	7	21	22	17	16	17	600

staining in the electron microscope. Representative micrographs are shown in Figure 1. From such micrographs, size distributions and the average vesicle diameter were obtained for the various vesicle samples. These data are summarized Table I. The estimate of 300 Å for the average size of small vesicles containing high salt correlates well with the estimate of about 250 Å from the elution curve of the Sepharose 4B column as shown in Figure 2. The major fractions marked I and II, which will henceforth be labeled "larger vesicles" and "small vesicles," had average diameters of 900 and 300 Å, respectively. A similar elution curve was not obtained for vesicles in low salt since aggregation occurred in the column and thus only larger vesicles were obtained from the column under these conditions. These larger vesicles had an average diameter of 1000 Å. Small vesicles in low salt were therefore taken directly from the freshly sonicated samples after centrifugation. Electron microscopy pictures indicate an average diameter of 350 Å for these vesicles.

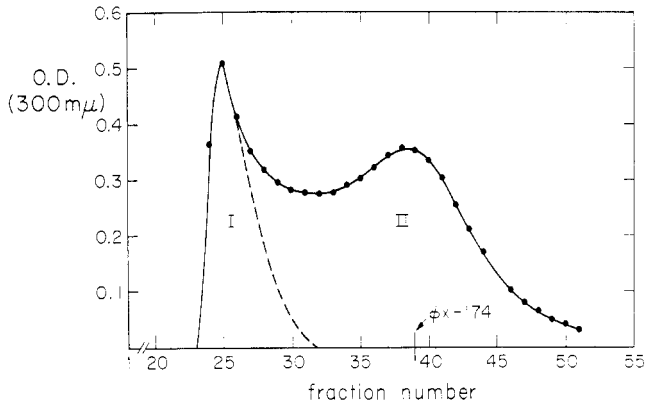

FIGURE 2: Optical density at 300 mμ of 6-ml eluent fractions from a Sepharose 4B column (2.5 × 80 cm) in 0.1 M NaCl and 2 mM PO_4 at pH 7.8 for vesicles containing 0.1 M NaCl.

The size distribution of all vesicle fractions changed gradually over the period of several days. In particular, vesicles prepared in high salt aggregated rapidly when heated above 40° as shown in Figure 1b,d. Since these vesicles have been dialyzed into D_2O containing only 2 mM PO_4, this aggregation appears to be the result of an osmotic pressure gradient of about 4 atm across the bilayer (Moore, 1962). Whereas vesicles containing high salt inside (0.1 M NaCl and 2 mM PO_4) and low salt outside (2mM PO_4) were found to burst within about 20 min above 40°, the vesicles in isotonic medium were stable for at least several hours at high temperatures. However, the dilatometry measurements indicate a decrease in the molal volume of lecithin when these vesicles are allowed to stand for a period of 16 hr just after sonication.

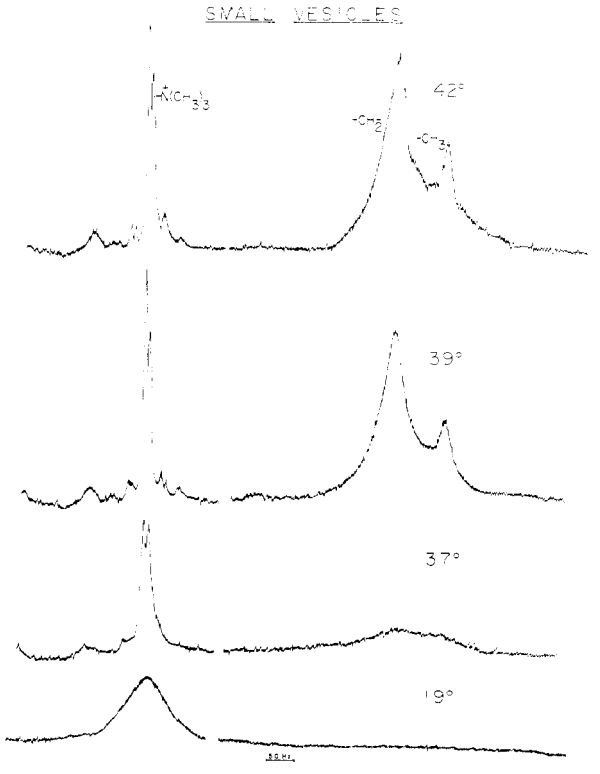

FIGURE 3: Pmr spectra (220 MHz) of small vesicles (~300 A) containing 0.1 M NaCl in the region from 0 to −3.5 ppm from DSS.

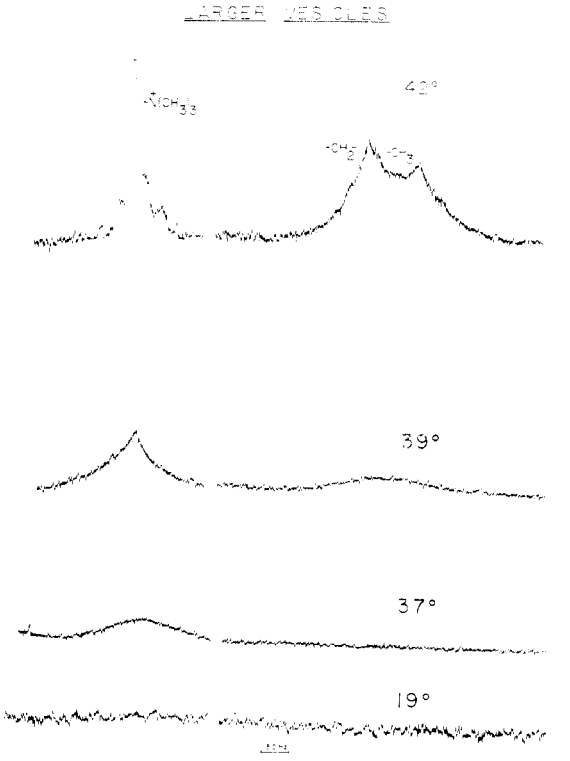

FIGURE 4: Pmr spectra (220 MHz) of larger vesicles (~900 A) containing 0.1 M NaCl in the region from 0 to −3.5 ppm from DSS.

Effect of Temperature on the Pmr Spectrum. We have studied the crystalline ⇌ liquid-crystalline-phase transition of dipalmitoyllecithin (Chapman *et al.*, 1967) by pmr for both the larger and small vesicles containing high and low salt. The pmr spectrum of dipalmitoyllecithin bilayers includes resonances from the choline methyl groups at −3.25 ppm, from the fatty acid methylene protons at −1.25 ppm and from the fatty acid methyl protons at −0.90 ppm from DSS (Penkett *et al.*, 1968). Glycerol and certain methylene group proton resonances are too broad to be observed under these conditions.

The effect of temperature on the spectrum of small and larger vesicles containing high salt in the spectral region 0 to −3.5 ppm is depicted in Figures 3 and 4. As expected the pmr spectrum of both vesicle samples showed marked changes in intensity and line width near 40° as a result of the first-order-phase change in the lecithin bilayer. However, striking differences in the spectral behavior of the two vesicle samples were noted.

Below the phase (Chapman) transition the larger vesicles exhibited only a very broad (~150 Hz) choline resonance of less than half of the expected intensity. As shown in Figure 5, only at temperatures near the phase transition were most of the choline protons detected. In addition, the transition from few to many cholines observed and from a broad to a narrow choline resonance occurred abruptly in the temperature range 37–42°. By contrast all of the choline protons were detected in the case of the small vesicles even at 19°. In both cases the choline methyl resonance exhibited appreciable narrowing at the Chapman transition.

There was no evidence of the fatty acid proton resonances in the spectra of the small and larger vesicles at low temperatures. In the case of the small vesicles these fatty acid resonances became apparent at 31° and in the case of the larger

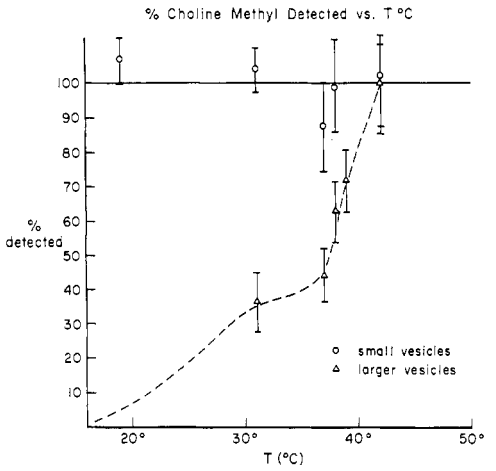

FIGURE 5: Percentage of the choline methyl proton resonance detected *vs.* temperature for (○) small vesicles containing 0.1 M NaCl and (△) larger vesicles containing 0.1 M NaCl.

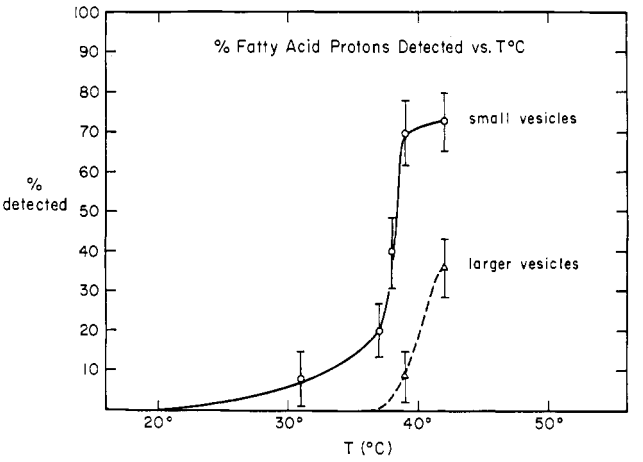

FIGURE 6: Percentage of the fatty acid proton resonances detected *vs.* temperature for (○) small vesicles containing 0.1 M NaCl and (△) larger vesicles containing 0.1 M NaCl.

vesicles at 39°. Although both samples underwent a transition from the crystalline to liquid-crystalline phase near 40°, the actual transition temperature for the fatty acids in the small vesicles was about 2° lower than for the larger vesicles and the transition was broader for the small vesicles covering 3–4°, as shown in Figure 6. In addition a higher percentage of the protons of these resonances were melted in the smaller vesicles. On the basis of these intensities at 42° it was estimated that along with the terminal methyl group, 9–11 of the 13 methylene groups became sufficiently mobile to give a high-resolution spectrum in the small vesicles whereas only 3–5 methylene groups were melted in the larger vesicles.

Near the phase transition the small vesicles also exhibited two choline resonances of unequal intensity separated by ~0.04 ppm. The high-field resonance was less intense and like the choline resonance in the larger vesicles, was chemically shifted −514 Hz from the aliphatic methyl resonance. The more intense resonance appeared at −522 Hz from the aliphatic methyl absorption. These choline resonances appeared to coalesce at temperatures above 70°.

Other than the greater tendency of the vesicles containing high salt to aggregate above the phase transition, there were only minor differences between the spectra of vesicles with the high-salt concentration inside and those which were isoosmotic with the 2 mM PO_4 outside the vesicles. Because of the aggregation tendencies of the high-salt vesicles at the phase transition they were not studied at temperatures above 42°.

Effect of Mn^{2+} on Pmr Spectrum. As an aid in the assignment of the lecithin resonances, Mn^{2+} ion was added to the solution of intact vesicles in order to cause extreme broadening of the proton resonances from groups contacting the extravesicular water space. The result was a radical decrease in the intensity of the choline resonance while the fatty acid resonances were unaffected. In the case of the small vesicles addition of Mn^{2+} caused a decrease of the choline resonance area to 30–40% of the total at temperatures below the phase transition while the line width of the residual signal remained constant. This implies that 60–70% of the lipid is on the outside of the vesicle, in rough agreement with calculations of the relative areas of the inside and outside surfaces assuming a thickness of 46 Å for the bilayer. The percentage of the cholines observed with Mn^{2+} present decreased at the transition temperature in the samples in high salt. Because the loss of choline intensity was not accompanied by any change in line width, the change probably reflects the breaking open of vesicles.

For both samples the observed choline resonances in the presence of Mn^{2+} were chemically shifted −514 Hz from the aliphatic methyl peak. This chemical shift may be compared to the chemical shift of choline methyl protons in the absence of Mn^{2+}, which was −514 Hz for the larger vesicles and the less intense resonance from the small vesicles, and −522 Hz for the more intense resonance from the small vesicles. Thus the chemical shift of the choline methyl resonance from the inside of the small vesicles was the same as that from the inside and outside choline groups of the larger vesicles, whereas the outside choline resonance in the small vesicles was chemically shifted to lower field with respect to the other choline resonances.

Effect of Viscosity on the Pmr Spectrum. In order to ascertain whether the pmr line widths were controlled by the vesicle tumbling rate, 1.5% of calf thymus DNA was added to a sample of small vesicles to create a gel of extremely high solution viscosity. In these experiments the water resonance was broadened to a width of ~3 Hz. This indicates a decrease in the rotational tumbling rate of the water molecules by 20- to 30-fold although from estimates of the viscosity there has been a much greater increase in the viscosity of the medium.

In Figure 7 it is seen that the addition of DNA to the small vesicle sample caused only a slight increase in line width and slight decrease in intensity (<10%) in the pmr spectrum. It is interesting to note that these small vesicles in DNA which are now rotating at least as slowly as the larger vesicles in aqueous solution exhibit quite a different pmr spectrum from the larger vesicles at the same temperature.

Dilatometry and Molal Volume Experiments. In order to confirm our pmr observations that the Chapman transition appeared at a lower temperature and was broader for lecithin in the small vesicles, we have also made dilatometry measurements. Träuble and Haynes (1972)[2] have shown that the transition of a lecithin bilayer from the crystalline to liquid-crystalline phase leads to an abrupt increase of ~1.4% in the apparent molal volume of lecithin. We have exploited this

[2] To be published.

TABLE II: Densities and Partial Molal Volume of Sonicated and Unsonicated Lecithin Bilayers at 27°.

Type of Bilayer	Method of Concn Detn	No. of Detn	$\rho_{27°}$	$\bar{V}_{27°}$
Sonicated	Bartlett	12	1.056 ± 0.003	695 ± 1
	Drying and weighing	3	1.0506 ± 0.0005	698.5 ± 0.3
	Drying and weighing after 16 hr	3	1.0536 ± 0.0005	696.6 ± 0.3
Unsonicated	Bartlett	12	1.063 ± 0.003	690 ± 1
	Drying and weighing	4	1.0600 ± 0.0005	691.6 ± 0.3
	Buoyant density	1	1.0585 ± 0.0005	693.4 ± 0.3

method with more precision and accuracy in the hope that these measurements might shed further light on the pmr observations. Figure 8 summarizes the change in the apparent molal volumes of both lecithins in the region of the Chapman transition. It is evident from these apparent molal volumes that the melting behavior of sonicated and unsonicated lecithins is different. As in the nmr observations, the dilatometry measurements indicate that the fatty acids in the small vesicles melt at a lower temperature and have a broader Chapman transition. Moreover, these experiments reveal that the apparent molal volume of sonicated lecithin is nearly 1% greater than that of unsonicated lecithin below the Chapman transition (27°), although above the phase-transition temperature the apparent molal volumes of both lecithins are equal within experimental error. Table II lists the densities and apparent molal volumes of sonicated and unsonicated lecithin at 27° in the absence of salt. Upon the melting of the fatty acid chains the apparent molal volume increased by 3.1% in the case of sonicated lecithin and about 4.0% for the unsonicated.

In the absence of salt, heating the vesicle samples through the phase transition and then cooling to below the phase transition revealed no evidence of hysteresis over the time course of the experiments (~8 hr), as shown in Figure 8. Under these conditions the vesicle size distribution also remained relatively stable over a period of weeks. This was not true for vesicles in 0.03 M NaCl. In that case the lecithin vesicles tended to aggregate to form larger vesicles even below the Chapman transition. In fact, after 2 weeks, their melting behavior approached that of the unsonicated bilayers. If, in addition, there was a salt concentration gradient, as when the vesicles had been dialyzed into low-salt D_2O so that an osmotic pressure existed across the bilayer, the vesicles became unstable and above the Chapman transition aggregated in a matter of minutes.

^{31}P Nmr Studies. In order to confirm the pmr results and to obtain an additional handle on their interpretation, we have examined the ^{31}P nmr spectrum of lecithin bilayers in both the sonicated and unsonicated states. In these experiments we have exploited the fact that the magnetic shielding of the phosphorus nucleus in the glycerophosphocholine moiety is anisotropic (Figure 9). Consequently, to obtain a rotationally averaged spectrum the motion of this group must be sufficiently isotropic and quick so that the motional modulation of this chemical shift anisotropy is fast compared to the chemical shift anisotropy expressed in hertz. Since the chemical shift anistropy is proportional to the magnetic field strength, these experiments must be undertaken at sufficiently high fields so that the effect is manifested in the presence of dipolar effects.

The ^{31}P spectra of unsonicated multilayers of phosphatidyl-

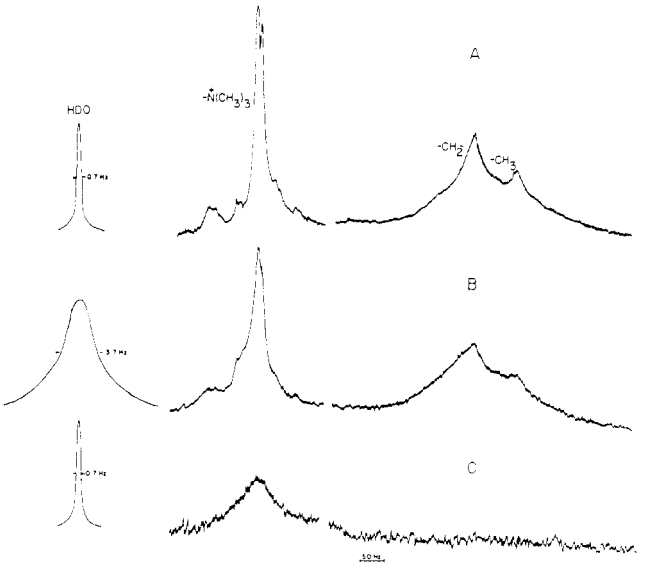

FIGURE 7: Pmr spectra (220 MHz) at 38° of (A) small vesicles containing 0.1 M NaCl, (B) small vesicles containing 0.1 M NaCl in a 1.5% DNA solution, and (C) larger vesicles containing 0.1 M NaCl.

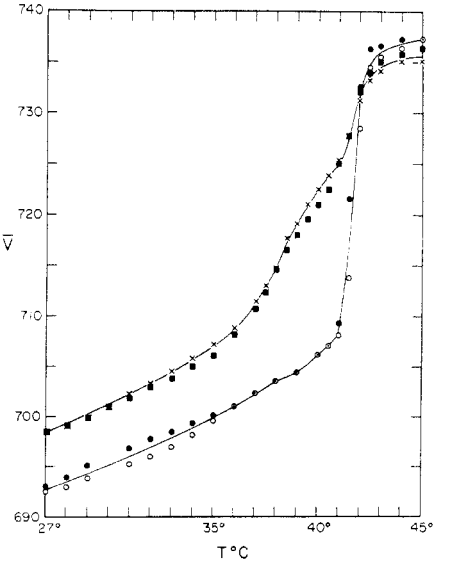

FIGURE 8: The apparent molal volume of dipalmitoyllecithin ($\bar{V}$) *vs.* temperature for (×) a sonicated sample being heated, (■) being cooled, and (○) an unsonicated sample being heated, (●) being cooled.

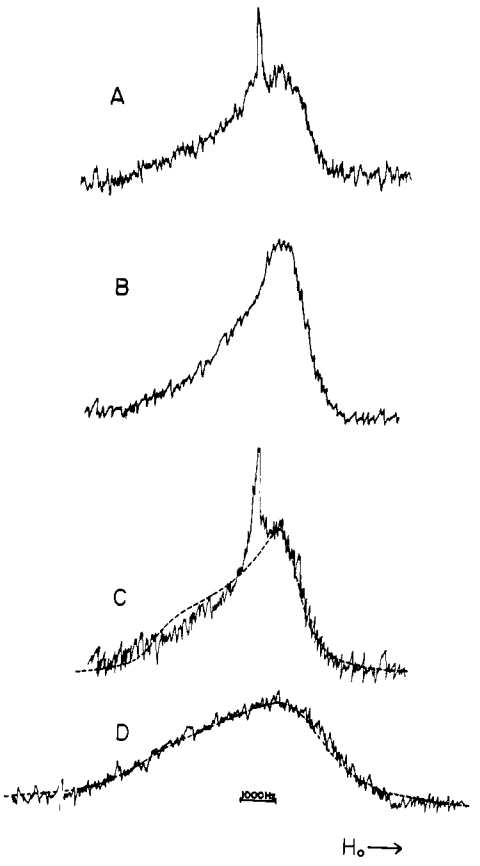

FIGURE 9: ^{31}P nmr spectra (89 MHz) of unsonicated (A) dimyristoyllecithin at 38°, (B) dipalmitoylphosphatidylethanolamine at 85°, (C) dipalmitoyllecithin at 55° with the computed spectrum (- - -), and (D) dipalmitoyllecithin at 18° with the computed spectrum (- - -). The symmetrical resonance in A and C is presumably from lecithin in a different configuration since the intensity of this resonance varies from sample to sample and the resonance was only found in saturated lecithins above the phase transition.

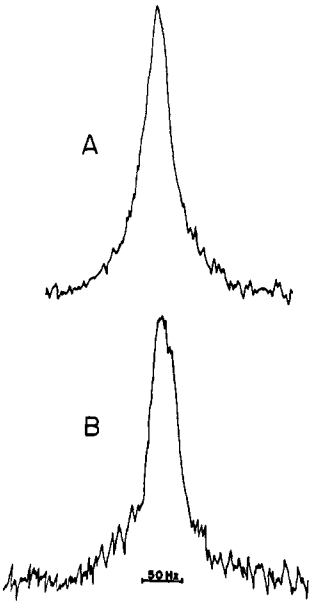

FIGURE 10: ^{31}P nmr spectra at 35° of sonicated dipalmitoyllecithin at (A) 36.4 MHz and (B) 89 MHz.

choline and phosphatidylethanolamine at 89 MHz clearly reveal an asymmetric resonance (Figure 9). This resonance, which is spread over 5000 Hz, is reminiscent of a powder spectrum of nuclei with a chemical shift anisotropy, and in fact it was possible to obtain a theoretical fit to these spectra assuming a chemical shift anisotropy $\delta_\perp - \delta_\parallel \approx 5000 \pm 1000$ Hz below and 4000 ± 1000 Hz above the phase transition plus the appropriate dipolar line width. For the spectrum below the Chapman transition, the dipolar line width giving the best fit is 2800 ± 400 Hz. This is reduced to 1100 ± 200 Hz above the transition.

Sonicated lecithin gave a single symmetrical resonance with a line width of ~70 Hz at 18° which sharpened gradually to ~35 Hz at 55°. This line width was field independent as determined by measurements at 22 and 53 kG (Figure 10). When 2% (w/v) DNA was added to a concentrated sonicated suspension (~10% lecithin w/v), the line width of the ^{31}P resonance remained ~70 Hz at 18° even though some aggregation occurred in addition to the viscosity increase.

Discussion

The pmr spectrum of lecithin in unsonicated bilayers is characterized by the following distinct features (Chan *et al.*, 1971, 1972; Seiter *et al.*, 1972). Below the Chapman transition no high-resolution features are apparent in the spectrum. Above the Chapman transition only the resonances of the choline methyl groups and the chain terminal methyl groups are narrow enough to appear in a delayed Fourier transform experiment. The observed intensities of these resonances are always less than 100% of their expected intensities. These resonances have line widths of about 150–200 Hz. The spin–lattice relaxation times of these protons have also been measured and have been found to be about 0.5 sec and to increase with increasing temperature. By contrast, the signal due to the bulk of the methyl protons is broad. These protons are characterized by a transverse relaxation time of 10^{-4} sec and they appear to undergo spin–lattice relaxation with a single T_1 of the order of 0.5 sec.

It is known that sonication has a profound effect on the pmr spectrum of lecithin bilayers (Penkett *et al.*, 1968; Finer *et al.*, 1972). The spectrum is now characterized by sharp resonances (line widths ~10–50 Hz), with intensities approaching 100% of those expected. More recently it has also been shown that different protons along the length of the fatty acid chains exhibit different spin–lattice relaxation times T_1's (Lee *et al.*, 1972). This distribution of T_1's indicates greater mobility of the methylene groups as one approaches the methyl end of the fatty acid chain.

There now seems to be little question that the principal source of the line broadening observed for the pmr spectrum of unsonicated bilayers is the nuclear dipole–dipole interactions among spins in the sample. The effective transverse relaxation time of the methylene protons has been shown to be independent of magnetic field, at least up to field strengths of 20 kG. In addition Finer *et al.* (1972) have observed that the CW (continuous wave) spectrum of oriented multilayers is narrowed considerably when the chain axis is oriented with respect to the field near the magic angle (54° 44′). Both of these observations strongly suggest that the breadth of the CW spectrum and the short transverse relaxation time in the free induction decay of lecithin bilayers is dipolar in origin. Moreover, recent work in this laboratory has indicated that it is possible to understand the pmr spectral behavior observed for the unsonicated lecithin bilayers above the phase transition in terms of a model in which the motion of the

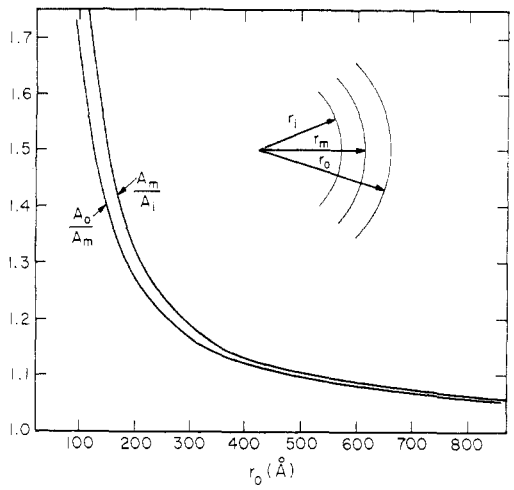

FIGURE 11: The ratios of the area available for the charge head to that for the fatty acid end of the lecithin molecule in a vesicular lamella *vs.* the outside radius of the vesicle for both halves of the bilayer assuming a bilayer thickness of 46 Å.

fatty acid chains is hindered and anisotropic (Chan *et al.*, 1972). Since the overall tumbling motion of the bilayer units is extremely slow (seconds or longer in the case of unsonicated lecithin) and there is a uniform distribution of orientation of the chain axis with respect to the magnetic field, the pmr spectrum of the lecithin bilayer should approach that of a powder in the absence of local motion (S. I. Chan and C. H. A. Seiter, 1972, personal communication). This is indeed the case below the Chapman transition. It is a reasonable approximation even above the Chapman transition, although it is necessary to invoke some local mobility of the fatty acid chains and the choline head groups to account for the results of the delayed Fourier transform experiments and the T_1 data. The observation of a powder ^{31}P nmr spectrum both above and below the Chapman transition for the multilayers corroborates this interpretation. These ^{31}P nmr data indicate that whatever local motion does exist is not sufficiently isotropic and rapid to average out the small chemical shift anisotropy of the phosphorus nucleus of the glycerophosphocholine moiety let alone the larger dipolar interactions in the multilayers.

Several important parameters are affected when small vesicles are formed by sonication. First the small vesicles can undergo faster rotational Brownian motion. For a 250- to 350-Å diameter vesicle this vesicle tumbling time becomes of the order of 10^{-6} sec (Finer *et al.*, 1972) compared to seconds or longer for the multilayers. Secondly, the effects of lateral diffusion can become important. Lateral diffusion of a phospholipid molecule on the surface of a sphere, like vesicle tumbling, tends to average out chemical shift anisotropies and dipolar interactions. Whereas in the case of a large multilayer it takes many seconds to undergo an angular displacement of $\pi/2$, this process could take place in 10^{-4} sec for a 250- to 350-Å vesicle, if we assume a lateral diffusion constant of 10^{-8} cm^2/sec. Both of these changes, however, are not expected to lead to the dramatic changes observed in the pmr spectrum of sonicated bilayers. A vesicle tumbling rate of 10^6 sec^{-1} predicts a dipolar line width of 3000 Hz instead of the 10 Hz observed. The fact that we observe no viscosity effect indicates that the line widths are not controlled by vesicle tumbling, although this isotropic vesicle tumbling is fast enough to average out the ^{31}P chemical shift anisotropy in the ^{31}P spectrum of these vesicles. Unless the lateral diffusion constant of phospholipid molecules in the bilayer phase of lecithin is grossly underestimated, the lateral diffusion process is obviously too slow to have any profound effect on the pmr spectrum. Two independent investigations have estimated the translational diffusion constant of lecithin in a bilayer to be about 10^{-8} cm^2/sec (Devaux and McConnell, 1972; Träuble and Sackmann, 1972). Moreover, if this lateral diffusion is important, it is difficult to understand why certain protons in a molecule should appear to be more mobile than others in the pmr spectrum. Both vesicle tumbling and lateral diffusion involve motion of the entire phospholipid molecule, and therefore any differential broadening of the resonances could only arise from differences in local or segmental motion.

Sonication can lead to subtle changes in the bilayer structure as a consequence of the increase in the bilayer curvature. This structural change can come about as the result of the pressure which must be maintained across a highly curved surface or from molecular packing considerations. For a 300-Å vesicle a pressure of about 2 atm exists across the bilayer surface. This pressure is probably not large enough to affect the bilayer structure, as is evident when we compare the pmr spectra of vesicles with the high-salt concentration inside and those which are isoosmotic to the 2 mM PO_4 outside the vesicles. There is no evidence for gross differences between the pmr spectra of these vesicles although an osmotic pressure of 4 atm exists across the bilayer for the vesicles containing high salt.

More important, perhaps, is the change in molecular packing which must presumably accompany the fitting of phospholipid molecules to form a small vesicle. In a flat bilayer the lateral area available per lecithin molecule is uniform from the surface to the center of the bilayer. For a small vesicle the area available for the polar head group and the fatty acid chains must necessarily be different. A larger or smaller area will be available to one end of the molecule rather than the other end, depending on which half of the bilayer the phospholipid molecule is located. Figure 11 depicts the ratio of the area available for the charge head to that for the fatty acid chain of the lecithin molecule in a vesicular lamella versus the outside radius of the vesicle for both halves of the bilayer. This simple geometric calculation illustrates that for a molecule on the inside of a 300-Å vesicle, the methyl ends of the fatty acids can occupy an area 1.5 times that covered by the charge group, whereas the opposite is true for a molecule on the outside of the bilayer. The most obvious conclusion which one can derive from these considerations is that the packing arrangement of the phospholipid molecules in the small vesicles is different or less regular than in the multilayers. This possibility is clearly indicated by the presence of two choline peaks from the small vesicles and by our dilatometry measurements, which showed the apparent molal volume of lecithin to be larger in the small vesicles than in the multilayers, at least below the Chapman transition. Thus, the small vesicles are probably somewhat disordered. The onset of the Chapman transition at lower temperatures and the greater breadth of this transition in the small vesicles, as monitored both by pmr and dilatometry, provide further evidence for this disorder. This disorder, or nonuniform molecular packing, undoubtedly facilitates the formation of kinks *via* gauche–trans isomerization along the the polymethylene chain (Träuble, 1971). When kinks are formed, thinning of the bilayer (45 to 41 Å, Chapman *et al.*, 1967) is accompanied by lateral expansion (51–59 Å^2, present data). Above the phase transition the number of kinks

formed per chain may or may not be greater in the small vesicles, but it is very likely that these kinks extend further up the chain than is possible in the case of unsonicated lecithin, where the packing is more uniform. Although the apparent molal volume of the lecithin is the same within experimental error between the larger vesicles and the small vesicles above the Chapman transition, one should not assume that consequently there are no structural differences between the two types of bilayers above the phase transition. This is because a large difference in the molal volume is not expected if the less regular structure in the smaller vesicles merely permits the kinks to extend further up the chain. However, this important difference most certainly would lead, on the average, to a greater segmental motion of the polymethylene chain.

The above differences in the bilayer properties between vesicles of different curvatures are also reflected in other measurements. We have performed experiments in this laboratory showing differences in the kinetics of water diffusion across the bilayer phase between small and larger vesicles. We have also found that small and larger vesicles have different affinities for local anesthetics. All these observations point to the possibility that the small vesicles have a looser structure than the larger ones. It is also likely that the small vesicles are metastable thermodynamically in view of their greater tendency to aggregate.

Our results suggest that many properties of the bilayer membrane are sensitive to the bilayer curvature. Such sensitivity may have important implications for biological membranes. It is possible that curvature provides a means for regional differentiation of membrane function. For example, it is possible that inner mitochondrial membranes and rod outer segment membranes have different transport properties in regions of high curvature as a result of the preferential adsorption of certain transport proteins to these bilayer regions.

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