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Minimal Size Phosphatidylcholine Vesicles: Effects of Radius of Curvature on Head Group Packing and Conformation†

Christie G. Brouillette,* Jere P. Segrest, Thian C. Ng, and James L. Jones

ABSTRACT: Egg phosphatidylcholine small unilamellar vesicles ranging from 150 to 270 Å in diameter have been studied by proton nuclear magnetic resonance (400 MHz) to investigate the relationship between phosphatidylcholine head group conformation and small changes in the vesicle radius of curvature. We find that as the vesicle size decreases, the split between the choline *N*-methyl resonances, corresponding to lipids residing in the outer and inner monolayer, becomes more pronounced. The increasing split is due to the dramatic upfield shift of the inner monolayer choline resonance with decreasing vesicle size. We also investigated the formation of deoxycholate–phosphatidylcholine mixed micelles by following the changes in the choline *N*-methyl resonances of small unilamellar vesicles with the progressive addition of deoxycholate. Our data provide additional support for the proposal by Mazer et al. [Mazer, N. A., Benedek, G. B., & Carey, M. C. (1980) *Biochemistry 19*, 601] of the existence of distorted bilayer structures at low deoxycholate:lipid molar ratios (<0.5 mM), which decay to mixed micelles at higher deoxycholate concentrations. Taken together, our results demonstrate that the choline *N*-methyl chemical shift is a sensitive indicator of head group surface area. Furthermore, we infer from our results that (1) the inner monolayer head group packing significantly influences the size limitations of a small unilamellar vesicle, (2) the inner phosphatidylcholine *N*-methyl chemical shift is indicative of the vesicle radius, and (3) the chemical shift of a phosphatidylcholine *N*-methyl residing in a planar bilayer will be very similar to that in an outer monolayer of a small unilamellar vesicle.

Small unilamellar vesicles (SUV)[1] are of interest from a biological as well as a physical chemical point of view due to the special properties a small radius of curvature imparts to them. In contrast to larger, multilamellar vesicles (MLV), SUV are unstable below the phase transition temperature (T_m), which results in spontaneous enlargement of the vesicles, probably via fusion (Suurkuusk et al., 1976; Kantor et al., 1977; Lichtenberg et al., 1981). The enthalpy associated with the SUV phase transition is 30–50% of that observed for MLV, and the T_m is depressed (Mabrey & Sturtevant, 1978; Gruenewald et al., 1979). The individual leaflets of SUV have been shown by NMR (Schmidt et al., 1978; Eigenberg & Chan, 1980) to respond independently to thermal perturbation.

The flexibility in the structure of a phosphatidylcholine (PC) molecule allows it to adapt somewhat to the geometric constraints imposed by a highly curved surface. On the basis of hydrodynamic and NMR data, the calculated lipid packing densities of the inner and outer monolayers of PC SUV are quite different, and both differ from values for phospholipids in a planar bilayer obtained by X-ray diffraction studies (Reiss-Husson, 1967; Small, 1967a). For an egg PC vesicle of Stokes radius 105 Å, the hydrated outer and inner surface area per lipid molecule has been calculated to be 84 and 56 Å^2, respectively (Cornell et al., 1980). The corresponding surface area of a maximally hydrated planar lipid molecule is about 72 Å^2 (Small, 1967a). The difference in packing requirements between the outer and inner monolayers is manifested in transbilayer compositional asymmetries of mixed lipid vesicles (Berden et al., 1975; Lentz & Litman, 1978) and a greater average freedom of motion for the hydrocarbon chains compared to MLV, deduced from Raman (Gaber & Peticolas, 1977) and NMR (Petersen & Chan, 1977) spectroscopies.

NMR spectroscopy has been successfully employed to gain information about phospholipid head group conformation in aqueous dispersions (Seelig, 1977; Hauser & Phillip, 1979).

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[1] Abbreviations: SUV, small unilamellar vesicle; MLV, multilamellar vesicle; PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; ^{1}H NMR, proton nuclear magnetic resonance; DOC, deoxycholate; O:I, outer monolayer:inner monolayer small unilamellar vesicle ratio; Tris, tris(hydroxymethyl)aminomethane; TLC, thin-layer chromatography.

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The main conformational features, derived from studies on phosphatidylcholines, are quite similar to those found in X-ray crystal structures (Hauser et al., 1981). The dipole between the choline quaternary amine and phosphate group is oriented parallel to the plane of the bilayer, while the C(1)–C(2) glycerol bond, which orients the head group relative to the hydrocarbon chains, is flexible and appears to have no preferred conformation. The difference in head group packing density between the outer and inner monolayers results in a different chemical environment, which is detected by ^{1}H NMR (Sheetz & Chan, 1972; Kostelnik & Castellano, 1973). The inner monolayer choline N–CH_3 resonance is seen upfield from the outer, consistent with the expectation that the smaller available surface area causes a closer juxtaposition of the positively charged choline quaternary amine and neighboring negatively charged phosphate (Hutton et al., 1977). The chemical shift split between the inner and outer carbonyls observed by ^{13}C NMR of ^{13}C-enriched PC acyl carbonyls is interpreted as resulting mainly from differences in carbonyl hydration in a manner that is consistent with a greater degree of hydration occurring with the outer (downfield) head group (Schmidt et al., 1977). Correlating the degree of carbonyl hydration with surface area led to the correct prediction of an intermediate chemical shift for MLV carbonyls. The magnetic nonequivalence of the inner and outer head groups allows the determination of the outer to inner phospholipid molar ratio from the area found for each respective peak by NMR (Hutton et al., 1977; Bergelson, 1979).

In this article, we report the effect of SUV size and lipid head group surface area on the chemical shift of the phosphatidylcholine N–CH_3. Analysis of our results from ^{1}H NMR studies at 400 MHz has led to conclusions regarding the relationship between surface curvature and head group conformation.

Experimental Procedures

Preparation of Small Unilamellar Vesicles. Egg phosphatidylcholine in ethanol was obtained from Avanti Biochemicals, Inc. (Birmingham, AL). After the removal of solvent by evaporation under reduced pressure, followed by a 50-μm vacuum treatment for 8 h, the dried lipid was suspended in a D_2O buffer (0.01 M Tris–0.15 M NaCl, pH 8.0) at a concentration between 30 and 50 mg/mL and sonicated at ice-bath temperatures under N_2. After a low-speed centrifugation step to remove sonicator probe tip particles, a 1-mL suspension was applied to a 1.2 × 95 cm Sephacryl 400 column (which has molecular sieve properties very similar to those of Sepharose 4B) and eluted at room temperature with a H_2O buffer (0.01 M Tris, 0.15 M NaCl, and 0.02% NaN_3, pH 8.0) through which helium was continuously bubbled during elution to inhibit lipid oxidation. Fractions of 1 mL were collected at a flow rate of 3 mL/h and stored under N_2 at 4 °C prior to use. Fractions corresponding to the peak for small unilamellar vesicles (Huang & Thompson, 1974) were used in the ^{1}H NMR studies after dialysis against the D_2O buffer. Eluted lipid was shown to be chromatographically pure by TLC on silica gel with chloroform–methanol–acetic acid–water (50:25:8:4 v/v) as eluent. Phospholipid concentrations were determined by the method of Stewart (1980). The column calibration method of Ackers (1967) was used to determine average Stokes radii; thyroglobulin and catalase served as column standards.

Electron microscopy was performed on a Zeiss EM-10. Samples were prepared by a variation of the method of Hamilton et al. (1971) with 2% potassium phosphotungstate for staining.

Deoxycholate Titration of Egg Phosphatidylcholine Vesicles. Egg phosphatidylcholine small unilamellar vesicles were prepared as above but without chromatography. Aliquots of 20 mM sodium deoxycholate were sequentially added to a 5-mm NMR tube containing a 10.7 mM suspension of vesicles. NMR spectra were recorded immediately after each addition of deoxycholate.

^{1}H NMR Spectroscopy. All ^{1}H NMR spectra were recorded on a Bruker WH400 operating at a radio frequency of 400 MHz. Free-induction decays (FID) were accumulated in a 16K data memory and stored on a disk with on-line minicomputer Aspect 2000. A 45° flip angle (corresponding to an 8-μs radio-frequency pulse) with a repetition rate of 2 s was used. Under these conditions no saturation of magnetization was observed. A 0.4-Hz line broadening was applied to each FID prior to the Fourier transform. Convolution difference spectra were obtained on the deoxycholate–phosphatidylcholine samples by applying 0.2-Hz and 5-Hz line broadening (Campbell et al., 1973). In the studies on vesicle size, 128 scans were collected for each spectra, while 16 scans were collected for each deoxycholate–phosphatidylcholine spectra. All experiments were carried out at 24 ± 0.5 °C. Sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$ was used as an internal standard.

The outer to inner phospholipid ratio of the vesicles was determined from the integrated areas of the respective choline N–CH_3 resonances in the presence of the shift reagent $K_3Fe(CN)_6$. Peak height ratios were also determined in the absence of shift reagent. It was observed that the outer to inner ratio was consistently overestimated by measuring peak height, probably due to the broader line width of the inner monolayer resonance compared to that of the outer (see Figure 4). A plot of peak height ratio in the absence of $K_3Fe(CN)_6$ vs. peak area ratio in the presence of $K_3Fe(CN)_6$ yielded a straight line with a slope = 1.45 and a correlation coefficient (r^2) = 0.99.

Calculation of Phosphatidylcholine Head Group Surface Areas. Hydrated lipid surface areas were calculated in a manner similar to that of Cornell et al. (1980). In order to calculate the change in head group surface area with vesicle size at the outer and inner surfaces, respectively, we have assumed the hydrated bilayer thickness and lipid volume are constant for all vesicle sizes. On the basis of the analysis of Small (1967a), we have distributed most of the water bound to the vesicle between the polar head groups. Chrzeszczyk et al. (1977) have shown for DPPC that the vesicular bilayer thickness is the same as that of a planar bilayer, although the outer and inner monolayers are thicker and thinner, respectively, than a planar monolayer. This is also likely true for egg PC (Cornell et al., 1980), and we have chosen the X-ray diffraction value of a hydrated bilayer (45.6 Å) for our calculations (Small, 1967a). Small's value of the hydrated volume of an egg phosphatidylcholine molecule was also used (1636.8 Å^3).

The following is a sample calculation of the outer and inner hydrated surface areas per lipid molecule for a vesicle with our experimentally derived values of the Stokes radius, 114.2 Å, and outer to inner ratio, 1.72: (1) volume of the bilayer $(V_b) = (4/3)\pi[r_s^3 - (r_s - 45.6)^3]$, where r_s, the Stokes radius, is the radius at the outer surface and $r_s - 45.6$, the radius at the inner surface, is r_i; (2) total number of lipid molecules $(n_t) = V_b/V_{pc}$, where V_{pc} is the volume of a phosphatidylcholine molecule; (3) number of lipid molecules in the inner monolayer $(n_i) = n_t/2.72 = 1097$; (4) number of lipid molecules in the outer monolayer $(n_o) = n_t - n_i = 1886$; (5) hydrated lipid area at the inner surface $= 4\pi r_i^2/n_i = 53.9$ Å^2; (6) hydrated lipid

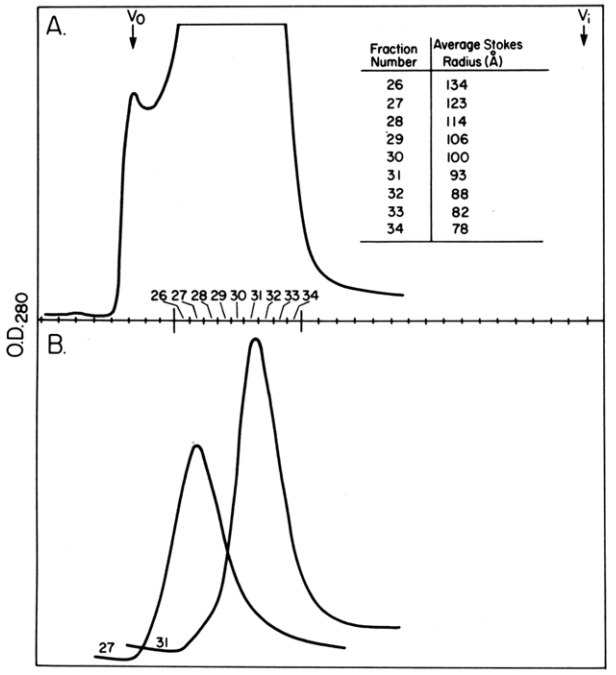


FIGURE 1: Elution profiles from Sephacryl 400 gel filtration of small unilamellar vesicles monitored at OD_{280}. (A) Gel filtration of a sonicated dispersion of egg phosphatidylcholine. The average Stokes radius calculated for vesicles collected in fractions 26–34 is indicated. (B) Gel filtration of fractions 27 and 31 shown in (A).

area at the outer surface = $4\pi r_s^2/n_o$ = 86.9 Å^2.

Results

Vesicle Size. Sonicated egg phosphatidylcholine was chromatographed on Sephacryl 400, and nine fractions were collected across the peak representing small unilamellar vesicles (Huang & Thompson, 1974). The elution profile is shown in Figure 1A. The vesicles ranged in radius from 75 to 135 Å, based on a calculated average Stokes radius of the vesicles collected in each fraction (Ackers, 1967). Several vesicle fractions were rechromatographed after NMR spectra were obtained, as a means of determining whether degeneration in vesicle size had occurred, and two representative elution profiles are shown in Figure 1B. All rechromatographed vesicle fractions ran very close to their original positions. Negative-stain electron microscopy of the vesicle fractions also indicated that there is a distribution in vesicle size about the gel filtration peak center. Three typical electron micrographs are shown in Figure 2. Fraction 31 is located at approximately the center of the vesicle peak.

Effect of Vesicle Size on Chemical Shift, O:I Molar Ratio, and Peak Line Width. As the vesicle size decreases, the split between the choline N–CH_3 resonances, characteristic of small unilamellar vesicles, becomes more pronounced (Figure 3). The results of two different experiments are illustrated in Figure 4 and show that the increasing split between the N–CH_3 resonances is due to the dramatic upfield shift of the inner monolayer choline resonance with decreasing vesicle radius; the outer monolayer cholines also shift upfield but to a much lesser degree. In response to the decrease in vesicle size the inner and outer choline N–CH_3 peak line widths are also observed to narrow (Figure 5). There is a corresponding, although less dramatic, splitting of the hydrocarbon methylene and methyl resonances with decreasing size as well (Figure 6).

A plot of the outer to inner phospholipid molar ratio vs. vesicle radius is shown in Figure 7. The solid line is the relationship between the calculated outer to inner monolayer

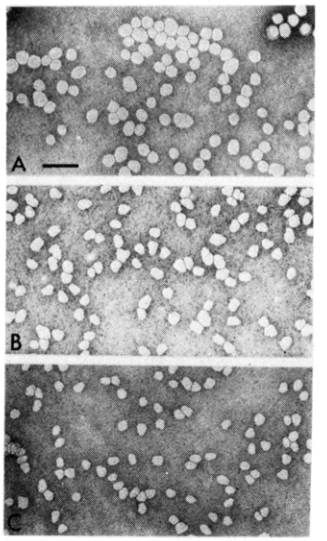
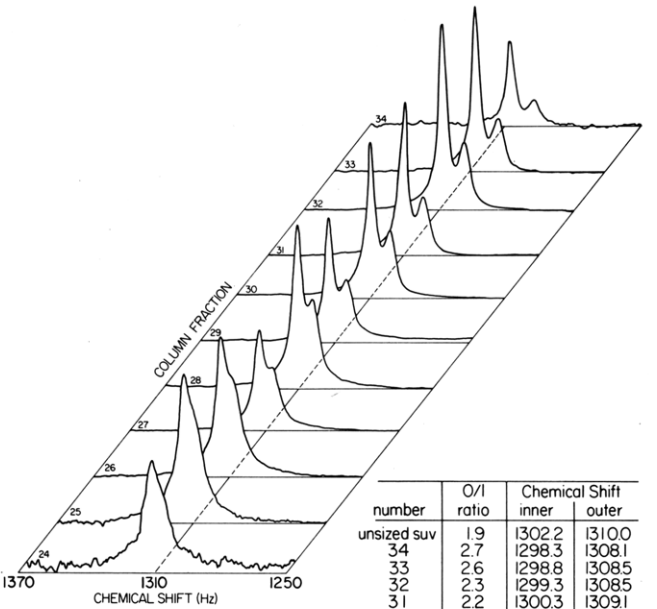


FIGURE 2: Negative-stain electron micrographs of chromatographed egg phosphatidylcholine small unilamellar vesicles: (A) fraction 29; (B) fraction 31; (C) fraction 33. Bar equals 1000 Å.



number	O/I ratio	Chemical Shift inner	Chemical Shift outer
unsized suv	1.9	1302.2	1310.0
34	2.7	1298.3	1308.1
33	2.6	1298.8	1308.5
32	2.3	1299.3	1308.5
31	2.2	1300.3	1309.1
30	2.0	1300.7	1309.5
29	1.9	1301.7	1309.5
28	1.7	1302.7	1309.5
*27	1.4	1304.7	1310.5
*26	1.4	1305.6	1310.5
*25	–	1306.6	1310.5
*24	–	—	1310.4

FIGURE 3: ^{1}H NMR spectra at 400 MHz, 24 °C, of choline N–CH_3 region of egg phosphatidylcholine small unilamellar vesicles subjected to gel filtration. Fractions 24–34 refer to the elution profile shown in Figure 1A. Tabulated chemical shifts are represented as the open and closed circles in subsequent figures. The asterisk denotes data that are not included in plots due to the potential contamination of these fractions with void volume vesicles. The outer:inner monolayer lipid molar ratio was obtained from NMR peak areas in the presence of $K_3Fe(CN)_6$.

surface area (see Experimental Procedures for the method used in these calculations) and the vesicle radius; this relationship has been shown by Lichtenberg et al. (1981) to be a hyperbolic function. The experimental points reflect the ratio of the amount of phospholipid in each half of the bilayer. This ratio also appears to be a nonlinear function of vesicle radius but, for a given radius, is significantly smaller than the respective surface area ratio. This discrepancy between lipid ratio and surface area ratio can be explained in one of three ways: by decreasing the inner head group surface area, by increasing

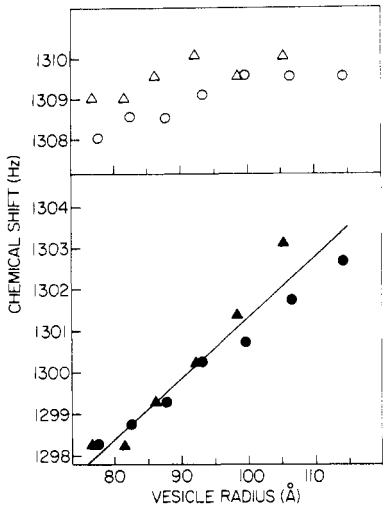


FIGURE 4: Change of choline N–CH_3 chemical shift with vesicle radius. Vesicle radii are derived from calculated Stokes radii of egg phosphatidylcholine small unilamellar vesicles sized by gel filtration. Two different experiments are represented by circles and triangles, respectively: (●, ▲) inner monolayer choline N–CH_3; (○, △) outer monolayer choline N–CH_3.

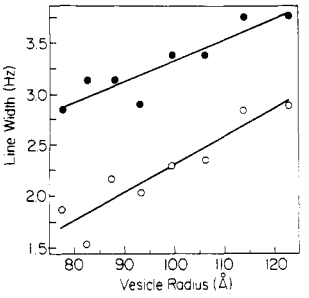


FIGURE 5: Change of half line width at half-height with vesicle radius. The half line widths were measured on plotted spectra from the downfield side of the outer monolayer choline N–CH_3 peak (○) and the upfield side of the inner monolayer choline N–CH_3 peak (●).

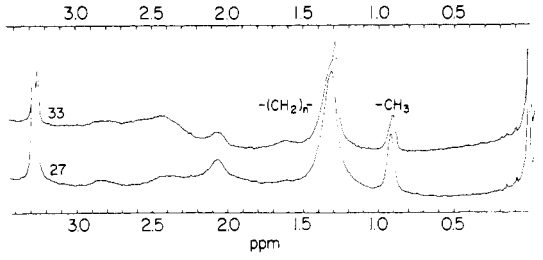


FIGURE 6: ^1H NMR spectra (400 MHz, 24 °C) of fractions 27 and 33 from gel filtration of egg phosphatidylcholine small unilamellar vesicles depicted in Figure 1.

the outer head group surface area, or by a combination of both. A plot of outer to inner phospholipid molar ratio as a function of the vesicle outer to inner surface ratio (not shown) is linear and intersects close to the (1,1) origin, as expected.

Effect of Vesicle Size on Head Group Surface Area. It is difficult to calculate the surface area occupied by each head group without making some assumptions. In our view, the most difficult decision involved the choice of model for distribution of bound water associated with the vesicles since our calculated vesicle radii include water of hydration. This problem has been discussed by Cornell et al. (1980), who, on the basis of the analysis of Small (1967a), chose to distribute the water largely between the head groups and, thereby, calculate hydrated surface areas. Huang & Mason (1978), on the other hand, assumed the presence of hydration shells

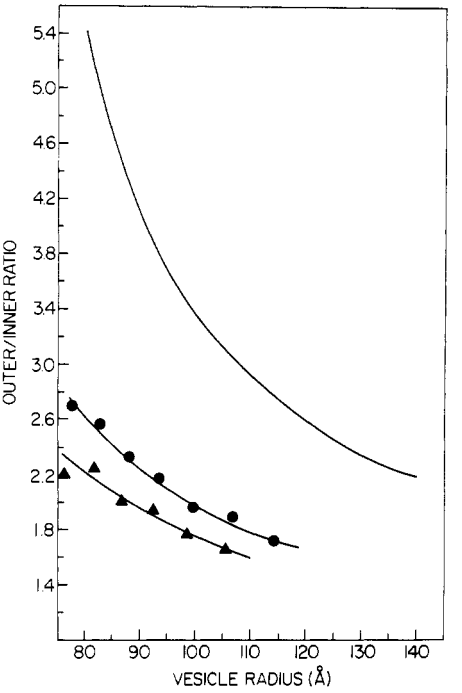


FIGURE 7: Vesicle radius dependence of calculated surface area outer:inner monolayer ratio and experimentally derived lipid molar outer:inner ratio of egg phosphatidylcholine small unilamellar vesicles. The solid curve is the surface area ratio calculated from the radii of the outer and inner surfaces, which are the Stokes radius (r_s) and r_s − 45.6 Å, respectively (see Experimental Procedures for details). The experimentally derived lipid molar outer:inner ratios for isolated vesicles of specific radii were determined for two different experiments (●, ▲) from NMR peak area ratios. The NMR peak area ratios were either measured directly (●) or interpolated from the linear plot of peak height ratio vs. peak area ratio (▲) (see Experimental Procedures for details).

surrounding the vesicle surface. Our data were more simply analyzed in terms of hydrated surface areas—the details of our calculations can be found under Experimental Procedures. As illustrated in Figure 8, with a decrease in vesicle radius, the inner head group area decreases and the outer increases. In Figure 9, the inner and outer head group areas are plotted vs. the chemical shift of the respective choline N–CH_3. An upfield shift accompanies a decreasing inner head group area and an increasing outer head group area.

Titration of Egg Phosphatidylcholine Vesicles with Deoxycholate. Deoxycholate is an amphipathic molecule that is able to solubilize aqueous dispersions of phosphatidylcholine by disrupting the characteristic bilayer molecular arrangement, leading to the formation of smaller, mixed micelles shaped like disks (Small, 1967b; Mazer et al., 1980). On the basis, of quasi-elastic light scattering studies, Mazer et al. (1980) have deduced a detailed molecular model for the formation, structure, and size of several bile salt–egg phosphatidylcholine mixed micelles, including deoxycholate. Their data were found to be most consistent with polydisperse bilayer disks that contain bile salt around the perimeter of the disk as well as intercalated as hydrogen-bonded dimers within the interior. According to the authors, an uncharacterized monodisperse bilayer structure exists as a distinct phase at low bile salt: phosphatidylcholine ratios, which decays to the mixed micelle phase at higher ratios.

We have studied the formation of egg phosphatidylcholine–deoxycholate mixed micelles by ^1H NMR and have followed the change in chemical shift and line width of the choline N–CH_3 resonance with the incremental addition of deoxycholate to small unilamellar vesicles. Figure 10 shows

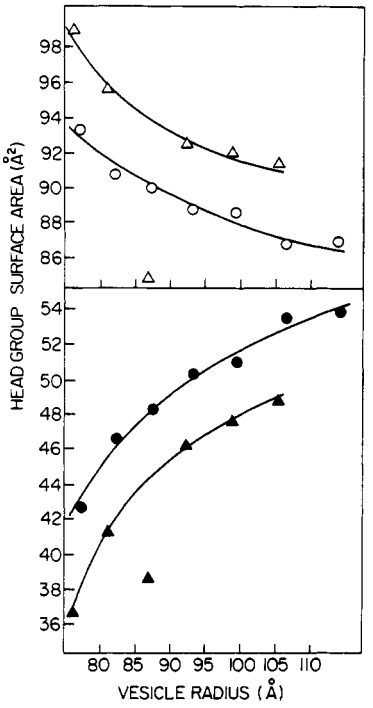


FIGURE 8: Dependency of outer monolayer (O, △) and inner monolayer (●, ▲) polar head group surface areas on vesicle radius. Details of the calculations can be found under Experimental Procedures. We have assumed that the hydrated bilayer thickness and hydrated lipid volume remain constant with changing vesicle size, but if the hydration state of the lipid changes, the differences between the inner and outer monolayer may not average in a manner that keeps the bilayer thickness and lipid volume constant. If one assumes water is added to the outer monolayer and subtracted from the inner as the vesicles become smaller, the absolute values obtained for the surface areas will change, but the trend depicted in Figure 8 should remain the same.

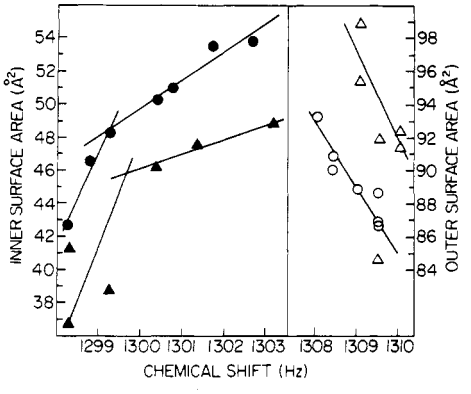


FIGURE 9: Change in outer monolayer (O, △) and inner monolayer (●, ▲) head group surface areas with chemical shift of respective choline N–CH_3.

deconvoluted spectra of the outer and inner monolayer choline N–CH_3 resonances with the progressive addition of deoxycholate. The resolution between the peaks is gradually lost due to a combination of line broadening and the downfield shift of the inner N–CH_3 resonance. The single resonance formed is then observed to narrow and shift upfield with increasing deoxycholate:lipid ratios, reaching an apparent chemical shift limit of approximately 1299 Hz at deoxycholate:lipid molar ratios greater than 2. After the conversion of the inner and outer choline N–CH_3 peaks to a single resonance, the chemical shift and line-width changes were analyzed and plotted as a function of the deoxycholate:lipid ratio (Figure 11). Nondeconvoluted spectra were used to measure the peak widths. Initially, addition of deoxycholate causes a large upfield chemical shift in the single resonance that is paralleled by a

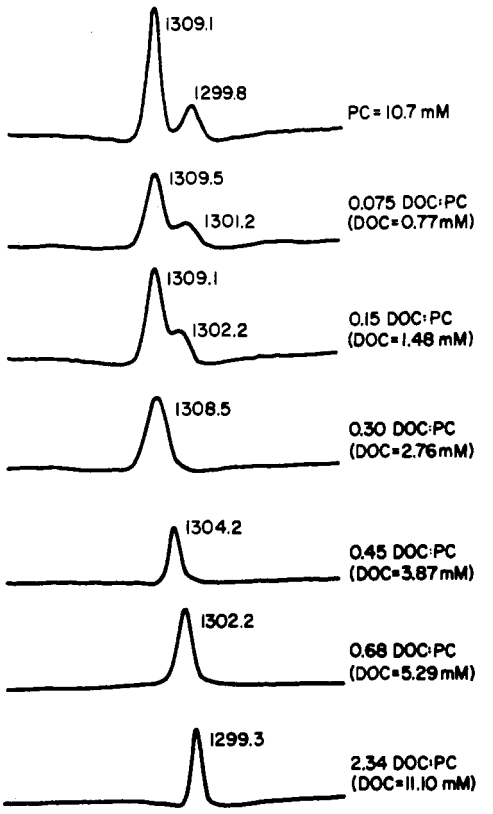


FIGURE 10: Deconvoluted ^1H NMR spectra (400 MHz, 24 °C) of choline N–CH_3 region of egg phosphatidylcholine small unilamellar vesicles as a function of deoxycholate:PC molar ratio. Aliquots of 20 mM deoxycholate were sequentially added to a 10.7 mM dispersion and the spectra recorded after each addition. Chemical shifts are in hertz.

rapid decline in line width. Further increases in the deoxycholate:lipid ratio lead to smaller changes in both chemical shift and line width.

Discussion

Although the average minimal size of a small unilamellar vesicle is approximately 200 Å in diameter, we have taken advantage of the fact that a significant fraction of the vesicles produced by prolonged sonication of aqueous lipid dispersions are both larger and smaller than the mean. Our calculations indicated the vesicle prepared ranged from 150 to 270 Å in diameter. The purpose of this study was to observe, through ^1H NMR, the effect of small changes in the vesicle radius of curvature on the phosphatidylcholine head group conformation. It has been generally accepted that the difference in lipid packing densities between the outer and inner monolayers of a small unilamellar vesicle is responsible for the different chemical shifts of the outer and inner choline N–CH_3 resonances observed by high field ^1H NMR (Sheetz & Chan, 1972). In fact, as shown in Figure 5, the different packing densities apparently influences the choline N–CH_3 peak line width as well. The inner choline N–CH_3 peak is much broader than the outer, suggesting a restricted inner head group motion.

Hutton et al. (1977) have postulated that, due to the closer packing of the inner monolayer head groups of a small unilamellar vesicle, the quaternary amine of one molecule would be much closer to an adjacent phosphate than the quaternary amine in the outer monolayer, causing the observed upfield shift of the inner choline N–CH_3 resonance. This postulate is supported by the observation that dimethyl sulfoxide causes an upfield shift of the N–CH_3 proton resonances of substituted 1-methylpiperidine methiodides (Casey, 1971). In view of this

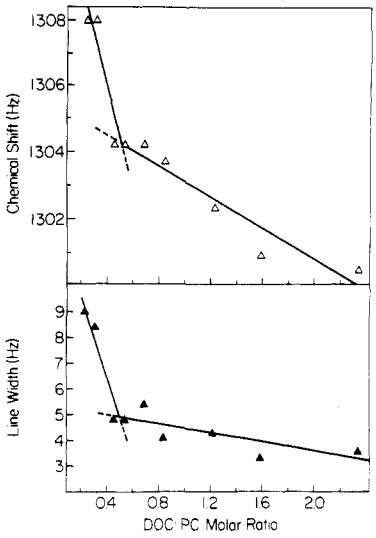


FIGURE 11: Change of choline N–CH_3 chemical shift (△) and full line width at half-height (▲) with deoxycholate:PC molar ratio. The analysis begins with the formation of a single choline N–CH_3 resonance at a deoxycholate:lipid molar ratio of 0.23. Line widths were measured on nondeconvoluted spectra.

hypothesis, it seems reasonable to predict that a vesicle smaller than 200 Å in diameter would show an even larger upfield shift of the inner monolayer N–CH_3 resonance, assuming that the inner head group surface area shrinks with decreasing vesicle size. This prediction has been born out by the plot of inner choline N–CH_3 chemical shift vs. vesicle radius (Figure 4). This plot is linear over the range of vesicle size studied and thereby allows the estimation of vesicle radius from the chemical shift of the inner choline N–CH_3 resonance. Furthermore, the progressive upfield shift of the inner choline N–CH_3 is associated with a decreasing head group surface area as seen in Figure 9. Our results are consistent with those of Eigenberg & Chan (1980), who observed a dramatic upfield shift of the inner choline N–CH_3 resonance of distearoylphosphatidylcholine vesicles over the narrow temperature range corresponding to the liquid-crystalline to gel phase transition. Since the surface area of a lipid decreases significantly when the temperature falls below its phase transition, their results lead to the same conclusion regarding the relationship between surface area and chemical shift.

In Figure 9 the plot of outer head group area vs. outer choline N–CH_3 chemical shift appears to be linear over the range of vesicle size studied. However, the plot of inner head group area vs. inner choline shift is clearly nonlinear. It seems likely that dehydration of the inner polar head group, resulting in a progressively closer positioning of positively charged cholines and adjacent negatively charged phosphates, is responsible for most of the upfield shift, with decreasing vesicle radius, of the inner choline N–CH_3 resonance. Additionally, we feel the nonlinearity in the Figure 9 plot is best interpreted as a conformational change in the head group as the surface area decreases. The phosphorylcholine head group is likely to maintain a bilayer-parallel orientation as long as possible since this is an energy-minimizing conformation. However, as the available surface area continues to decline, a more space-saving tilted orientation may be adopted (Hauser et al., 1981). It could be significant, therefore, that by fitting the data in Figure 9 to two intersecting lines, a break in the curve occurs at approximately an inner lipid surface area of 47 $Å^2$, which is in the lower range of the minimal area (47–54 $Å^2$) required for a phosphatidylcholine head group oriented parallel to the bilayer plane (Hauser et al., 1981). One could postulate

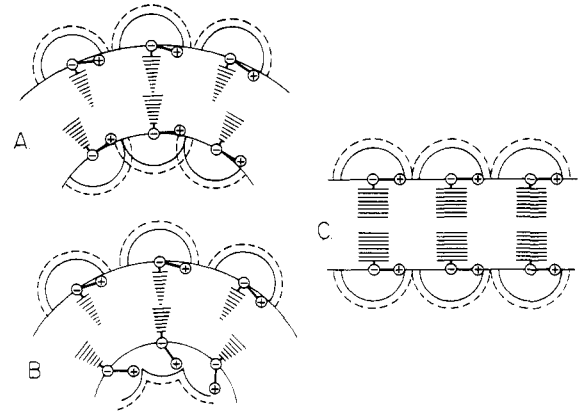


FIGURE 12: Schematic illustration showing postulated phosphatidylcholine head group packing in two different diameter small unilamellar vesicles [(A) and (B)] and a planar bilayer (C).

from this that the inner head group packing plays a major role in determining the limiting size of a small unilamellar vesicle.

Although the outer monolayer N–CH_3 resonance is found downfield of the inner one in small vesicles (Figure 4), it shifts slightly upfield with decreasing vesicle size, not downfield, as might be expected on the basis of an increasing outer head group surface area with decreasing vesicle size. It is not immediately obvious from viewing molecular models why the upfield shift in the outer N–CH_3 resonance should occur, although it may in some way be related to a head group reorientation by inward tilt; this reorientation would maximize the head group area as the curvature increases (see Figure 12).

As illustrated in Figure 6, we observed a splitting in the fatty acyl chain methylene and methyl resonances in very small vesicles. While the splitting is slight, it appears that a small fraction of the methylenes and a large fraction of the methyls are shifting upfield. Intermolecular van der Waal's interactions may be responsible for this splitting since the methyls and terminal methylenes of the outer monolayer lipid chains should experience closer packing as the vesicle size decreases.

Our studies on deoxycholate–phosphatidylcholine micelles were initiated with the idea of correlating macromolecular structure with choline N–CH_3 chemical shift. The response of phosphatidylcholine vesicles to increasing concentrations of deoxycholate takes place in three observable steps. The first step is the apparent loss of the small unilamellar vesicular structure, indicated by the conversion of the two choline N–CH_3 resonances to a singlet at a deoxycholate:lipid ratio of approximately 0.25 (deoxycholate 2.3 mM). This resonance is very close to the chemical shift of an outer monolayer N–CH_3 resonance. During the second step, the rapid linewidth narrowing of the single resonance with increasing deoxycholate concentration is most simply explained as a decrease in size of the newly formed structure. This interpretation is based on the data of Mazer et al. (1980), which showed that at very low bile salt:phosphatidylcholine ratios (the exact ratio depends on the experimental conditions), increasing the concentration of bile salt led to dramatic decreases in the size of the structure. Mazer et al. (1980) believe this structure to be bilayer in nature. The third step, which occurs at the break in the plot of line width vs. deoxycholate:lipid ratio can also be interpreted in terms of the Mazer et al. model as the formation of a new structure, the mixed micelle, which has a different dependency of size on deoxycholate:lipid ratio.

Combining the observations of Mazer et al. (1980) with our own, we believe the bilayer structure that they described at low deoxycholate:lipid ratios could be represented as a flattened vesicle with deoxycholate intercalated in the regions of high

curvature around the edges (Segrest, 1977). The rationale for this hypothesis comes mainly from our observation that this structure displays only one choline N–CH_3 resonance. We infer from this that the inner and outer head groups now have the same head group surface area, as would be found in a planar bilayer, since the respective choline N–CH_3 are magnetically equivalent. This structure would then be converted to a mixed micelle with increasing concentrations of deoxycholate. An alternative explanation for the single N–CH_3 resonance is a rapid flip-flop of lipid molecules between the two monolayers, which would cause an averaging of the two resonances. However, the flip-flop would have to be fast compared to the NMR time scale, which seems rather unlikely for a vesicular structure.

The continuing upfield shift of the mixed micelle choline N–CH_3 resonance can be explained by either one or a combination of two effects. At higher deoxycholate:lipid ratios, more lipid molecules will be in direct contact with deoxycholate. A close contact could lead to electrostatic interaction between the side-chain carboxylate of deoxycholate and the quaternary amine of the phospholipid or induce a conformational change in the choline head group, bringing the quaternary amine in closer contact with an adjacent phosphate.

An important conclusion that stems from our interpretation of the NMR results is that the phosphatidylcholine N–CH_3 of a planar bilayer will display a chemical shift very close to that of an outer monolayer choline N–CH_3. At the point where the choline N–CH_3 resonances collapse to a singlet with the addition of deoxycholate, the chemical shift of the singlet is approximately 1309 Hz. The resonance of the N–CH_3 of our largest vesicles (Figure 3) is unresolved and also occurs close to 1309 Hz.

Our model for the differences in head group packing and conformation between planar and curved bilayers is schematically illustrated in Figure 12. The solid circle represents the volume excluded by the head group alone, and the dashed circle represents the volume in which hydrational changes will affect the chemical shift of the choline N–CH_3 resonance. We postulate that the chemical shifts of the SUV outer monolayer head group (Figure 12A) and planar bilayer head group (Figure 12C) are similar mainly because each is surrounded by a critical amount of water that shields the head group from the effect of intercalating additional waters between the head groups. On the other hand, the closer packing of the inner monolayer head group is hypothesized to cause a reduction in the critical hydrational water volume (Figure 12A), leading to an upfield chemical shift of the inner choline N–CH_3 resonance. When the radius of curvature is further decreased (Figure 12B), the inner head group is required to tilt in order to avoid an overlapping contact with the neighboring head groups.

The data presented here reveal a close tie between choline N–CH_3 chemical shift and average head group surface area. Changes in the available phosphorylcholine surface area most likely lead to a combination of hydrational and conformational changes. The results of this study are consistent with the hypothesis that two key effects limiting the size of small vesicles are restricted cross-sectional areas for (a) the inner phospholipid head groups and (b) the outer phospholipid fatty acyl tail methylenes and methyl groups.

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