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Lack of Transbilayer Coupling in Phase Transitions of Phosphatidylcholine Vesicles[†]

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ABSTRACT: Praseodymium and ytterbium chlorides were used as nuclear magnetic resonance shift reagents to resolve the inner and outer choline methyl resonances of single-walled dimyristoylphosphatidylcholine bilayer vesicles. The gel to liquid-crystalline phase transition of these vesicles was monitored by observing the proton and carbon-13 nuclear magnetic resonance line widths of the choline methyl group nuclei. In the absence of ions the transition occurred at 21.5 °C in both halves of the bilayer. With Pr^{3+} or Yb^{3+} added to the outside of the vesicles, the phase transition temperature of the outer half of the bilayer was raised several degrees, while the transition temperature of the inner half was unchanged. In vesicles containing 20 mol % cholesterol the phase transition of the outer monolayer was considerably broadened, while the inner half still melted sharply at 21.6 °C. By use of dipalmitoylphosphatidylcholine vesicles with UO_2^{2+} added to the outside, phase transitions at 41.5 and 44 °C were detected by electron spin resonance with the spin-label 2,2,6,6-tetramethylpiperidinyl-1-oxy. These results imply that the two halves of the bilayer in phospholipid vesicles are so weakly coupled that they can undergo the gel to liquid-crystalline phase transition independently.

A natural consequence of the bilayer model of biological membranes is the question of whether or not there is distributional symmetry of the lipid components across the midplane. It is widely believed that the glycolipids are located primarily in the outer monolayer (Hakomori, 1981), but the situation for both cholesterol and the phospholipids is still a matter of some study. Erythrocytes have been clearly shown to possess an asymmetric phospholipid distribution, but other membranes have not presented such an easily unraveled pattern (Op den Kamp, 1979). It appears that cholesterol exchanges between the bilayers with a time constant of several hours at 37 °C (Backer & Davidowicz, 1979; Bloj & Zilversmit, 1977); the measured exchange rates were found to depend strongly on the amount of acyl chain unsaturation, with increasing unsaturation favoring faster exchange.

Proteins and ions are not symmetrically disposed across the bilayer. Given this and the probable lipid asymmetries, it becomes relevant to attempt to ascertain the functional consequences of these structural features of the bilayer. Since the fluidity of the bilayer depends, in part, on the phospholipid head group and acyl chain composition, transbilayer phospholipid asymmetry may result in fluidity asymmetry as well. For asymmetric fluidity to exist, there must be weak coupling between the monolayers. This paper reports that phase transitions, and hence fluidity changes, are poorly transmitted across the bilayer for saturated synthetic phosphatidylcholines.

Materials and Methods

DMPC[1] was obtained from Sigma, while cholesterol, DPPC, and DMPE were purchased from Calbiochem. Deuterium oxide (99.7%) was supplied by Aldrich (Milwaukee, WI). Anhydrous praseodymium and ytterbium chlorides were obtained from PCR Inc. (Gainesville, FL) and handled under a dry argon atmosphere. Merck (St. Louis, MO) supplied $^{13}CH_3I$ enriched to 90% in ^{13}C. 2,2,6,6-Tetramethyl-

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[1] Abbreviations: NMR, nuclear magnetic resonance; ESR, electron spin resonance; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy; DMPE, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine.

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piperidinyl-1-oxy (Tempo) was synthesized according to the method of Briere et al. (1965). The purity of the lipids used was checked by thin-layer chromatography as described below. There was no detectable lysolipid or free fatty acid (<1%). All other reagents were of the highest purity available.

Synthesis of $[(^{13}CH_3)_3N]$*DMPC.* Carbon-13-labeled DMPC was prepared by alkylation of DMPE with $^{13}CH_3I$ by the following procedure. In 15 mL of a 1:1 (v/v) mixture of chloroform–methanol, was dissolved 250 mg of DMPE. To this was added 1 g of $KHCO_3$. The resulting mixture was frozen in liquid nitrogen in a thick-walled glass tube and attached to a vacuum line. The $^{13}CH_3I$ (1.0 g) was similarly frozen and transferred to the reaction mixture on the vacuum line. The reaction tube was flame sealed, rotated in a water bath at 45 °C for 2 days, and applied to a Bio-Sil A (Bio-Rad Laboratories, Richmond, CA) 200–400 mesh silicic acid column (2 × 30 cm) packed in chloroform. The column was developed with a chloroform–methanol gradient beginning at 9:1 (v/v) and finishing at pure methanol. The column fractions were monitored by thin-layer chromatography on silica gel plates developed with chloroform–methanol–acetic acid–water (65:40:2.6:10, by volume). The spots were visualized with I_2 vapor. Unreacted DMPE eluted at 1:1 chloroform–methanol. The $[(^{13}CH_3)_3N]$DMPC was eluted with methanol. Its R_f (0.33) on thin-layer chromatography was identical with that of authentic DMPC and different from that of DMPE (R_f = 0.76). The yield was 162 mg or 61%. The product, dissolved in $CDCl_3$, gave a proton-coupled ^{13}C NMR spectrum consisting of a quartet centered at 55.1 ppm downfield from tetramethylsilane, with the intensity ratios 1:3:3:1, and coupling constant J_{CH} = 143.2 ± 0.8 Hz, consistent with authentic DMPC. A 1 mg/mL multilamellar dispersion, in water, of the reaction product, when heated in a differential scanning calorimeter, gave a phase transition temperature of 23.6 °C, identical with that of pure DMPC.

Vesicle Preparation. Dry phospholipid was weighed into a 5-mL pear-shaped flask to which was added 2 mL of degassed D_2O. The flask was flushed with argon and shaken at a temperature 4 °C higher than the gel to liquid-crystalline phase transition of lipid. The lipid concentration was 10–25 mg/mL for Fourier-transform NMR and 100–200 mg/mL for continuous wave and ESR studies. The lipid suspensions were sonicated for 5–10 min at a power level of 25 W by using a Branson W185 sonifier with a microtip. The sonication flask was thermostated at 4 °C above the phase transition temperature in a circulating water bath. The suspensions were then centrifuged at 10000*g* for 20 min to remove titanium particles and undispersed lipid. This procedure yielded reproducible vesicle suspensions that were almost transparent. The suspensions were placed on ice, and aliquots were taken for electron microscopy, thin-layer chromatography, NMR, and ESR immediately after preparation.

Vesicles containing cholesterol were prepared by adding dry, solid cholesterol to previously sonicated vesicle preparations of DMPC and sonicating the suspension in 1-min bursts, alternating with 1-min cooling periods, until the suspension cleared. The sonication flask was thermostated in a 27 °C circulating water bath. Ten pulses of sonicaton were usually sufficient.

After sonication the vesicles were checked for purity by thin-layer chromatography with a solvent system of chloroform–methanol–acetic acid–water (65:40:2.6:10 v/v). The spots were visualized with iodine vapor or by spraying with 6 N H_2SO_4 and heating at 180 °C. No lysolipid or fatty acid could be detected (<1%).

Electron Microscopy. Copper grids (300 mesh) were coated with formvar and carbon. The samples were diluted to 5–10 mg/mL lipid, and a drop was applied to the grid. After 30 s the excess liquid was withdrawn with a piece of filter paper. The sample was negatively stained with a drop of 2% uranyl acetate for 30 s, the excess liquid removed with filter paper, and the grid allowed to dry. The grids were examined with a Hitachi Hu-11C-1 transmission electron microscope operating at 75 kV.

Nuclear Magnetic Resonance. The ^1H NMR experiments were carried out on a Varian XL100 spectrometer operating at 100 MHz equipped with a Varian 620L computer and a Varian variable temperature controller. Protons were observed in both continuous wave and Fourier-transform modes with an internal deuterium lock. Because of the limited dynamic range of the computer when the instrument was used in the Fourier-transform mode, a π–τ–$(\pi/2)$ pulse sequence was used to eliminate the peak for residual HDO in the sample (Patt & Sykes, 1972). The interpulse pulse delay, τ, was set to $0.69T_1$ for the water protons, typically 5 s for the undoped and 2.5–3 s for the Pr^{3+}-doped samples. There was no effect upon the phospholipid resonances, which had spin–lattice relaxation times at least 10 times smaller than HDO. For example, the undoped choline methyl protons of DPPC have a T_1 of 0.5 s (Levine et al., 1973), while for DMPC at 31 °C in the presence of Pr^{3+} we found a value of T_1 = 0.28 ± 0.02 s for both resonances. The probe temperature was monitored at least 5 times during each run with a Fluke 2100A digital thermometer with a precision copper–constantan thermocouple. Although it is difficult to estimate the accuracy of temperature measurements because the sample tube must be spinning, the reproducibility (precision) of the phase transition temperature of dimyristoylphosphatidylcholine was 0.2 °C. Chemical shifts are reported relative to sodium 3-(trimethylsilyl)-2,2,3,3-tetradeuteriopropionate.

Carbon-13 Nuclear Magnetic Resonance. The ^{13}C NMR measurements were obtained with the aid of a Varian CFT20 Fourier-transform spectrometer, operating at 20 MHz, equipped with a Varian temperature controller. The ^{13}C NMR experiments were performed in as similar a fashion as possible to the ^1H NMR experiments above. No solvent resonance was present to interfere with the dynamic range of the computer, so data could be accumulated at a faster rate. Our measured T_1 for the choline methyl carbons was 0.31 ± 0.03 s, in agreement with Sears et al. (1976). Pulse repetition rates of 0.75 s^{-1} with a sweep width of 1000 Hz were utilized. At each temperature 100 90° pulses were accumulated in 8K data points, from 1.5-mL samples, in 8-mm sample tubes. Aliquots of a 100 mM solution of $YbCl_3$ were added to the vesicle solution to produce the desired cation to lipid ratio.

Electron Spin Resonance. The ESR spectra were recorded on a Varian EM500 spectrometer. The sample temperature was regulated with a custom-built regulator. The sample temperature was continuously monitored with a copper–constantan thermocouple attached to the sample with silicone thermal contact grease. The reproducibility of the temperature measurements was 0.1 °C. The spin-label Tempo was used to probe phospholipid vesicle fluidity. The high-field line of the ESR spectrum of this probe is partially resolved into a hydrophobic (lipid) and an aqueous component (Polnaszek et al., 1978). After correction for line-width differences and ^{13}C satellite peaks, the partition coefficient, K, of the probe between the phospholipid bilayer and the aqueous phase can be calculated as

$$K = [\mathrm{Tempo}]_{\mathrm{phospholipid}}/[\mathrm{Tempo}]_{\mathrm{water}}$$

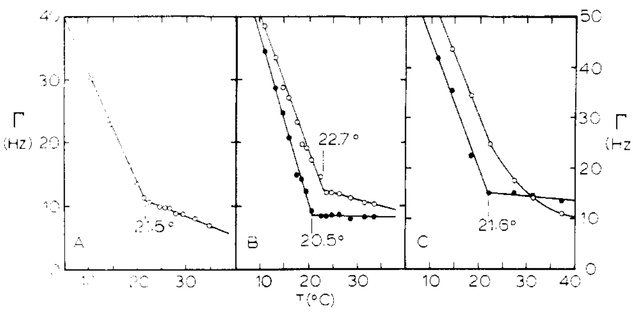

FIGURE 1: Variation with temperature of the line width of the choline methyl proton resonances of DMPC vesicles at 100 MHz. (A) Control sample, 28 mM phospholipid; Γ is the full width at half-maximum. (B) DMPC vesicles, 28 mM; $PrCl_3$, 9.1 mM; the open circles refer to the outer while the filled circles refer to the inner monolayer resonances. (C) DMPC vesicles, 28 mM; cholesterol, 5.6 mM; $PrCl_3$, 9.1 mM.

Results

The electron micrographs (not shown) revealed that the DMPC vesicles were single walled and homogeneous in size. The vesicle size was 28 ± 5 nm in the absence of cholesterol and 30 ± 5 nm in its presence. Neither praseodymium chloride nor ytterbium chloride had any effect on vesicle size.

Proton Nuclear Magnetic Resonance. Proton magnetic resonance spectra of the DMPC vesicles closely resembled those previously reported (Barsukov et al., 1975). We utilized the data of Finer et al. (1972) to assign the seven resolved resonances.

The ^1H NMR spectrum of 28 mM DMPC vesicles with the addition of 9.1 mM Pr^{3+} showed a shift of the outer choline methyl resonance 43 Hz downfield; the glycerol backbone protons shifted 21 Hz, and the choline ($-CH_2-$) protons shifted 7 Hz (Andrews et al., 1973; Yabusaki & Wells, 1975). The inner and outer choline methyl resonances were partially resolved even in the absence of added Pr^{3+} (Kostelnik & Castellano, 1973; Uhing, 1975). The resolution was temperature dependent and maximal near the phase transition temperature. The resolution was not good enough to make accurate line-width measurements for the separate resonances. The transition temperature was estimated from the intersection of least-squares lines as 21.5 ± 0.2 °C (Figure 1A).

In the presence of 9.1 mM Pr^{3+} the resonances for the inner and outer choline methyl groups were completely resolved, which allowed the accurate determination of the transition temperature for both halves of the bilayer. The outer monolayer, which had bound Pr^{3+}, melted at 22.7 while the inner monolayer melted at 20.5 °C (Figure 1B), a difference of 2.2 °C. The chemical shift difference induced by Pr^{3+} between the inner and outer choline methyl group resonances decreased about 20% above the phase transition temperature (Figure 2). The same percentage change was found for three different Pr^{3+} concentrations.

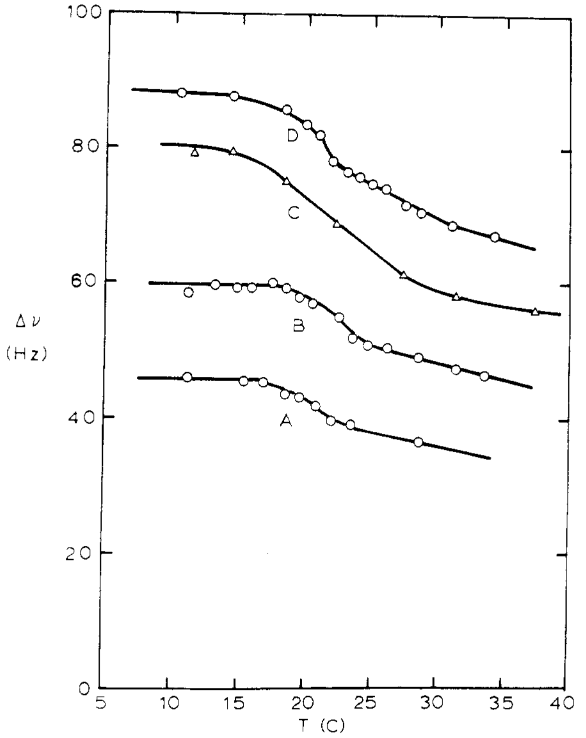

FIGURE 2: Effect of temperature variation on the chemical shift difference, Δν, between the outer and inner choline methyl proton resonances at 100 MHz of DMPC vesicles plus $PrCl_3$. (A) 4.8 mM $PrCl_3$; (B) 9.1 mM $PrCl_3$; (C) 19 mM $PrCl_3$ plus 20 mol % cholesterol; (D) 19 mM $PrCl_3$.

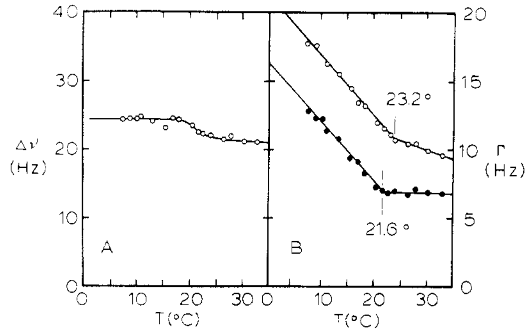

FIGURE 3: Variation with temperature of the NMR properties of 30 mM $[(^{13}CH_3)_3N]$DMPC vesicles in the presence of 10.5 mM $YbCl_3$. (A) The chemical shift difference, Δν, between the monolayer resonances. (B) The outer (open circles) and inner (closed circles) line widths of the choline methyl carbon resonances at 20 MHz.

The phase transition behavior of vesicles containing 20 mol % cholesterol is shown in Figure 1C. The inner monolayer melted sharply at 21.6 °C while the outer half showed a broad transition covering at least 20 °C. It is likely that the known bilayer expansion on melting (Trauble & Haynes, 1971; Marsh, 1974) of ~20% is responsible for the temperature-dependent decrease in Δν (Figure 2). The inclusion of 20 mol % cholesterol decreased the difference in chemical shift between the resonances from the inner and outer monolayers by about 8%, at a constant concentration of Pr^{3+} (Figure 2C,D). The data of Marsh (1974) showed that the inclusion of 20 mol % cholesterol in DPPC bilayer vesicles increased the area per phospholipid head group by about 6%, a value that is of the same order as our result. The effect of the cholesterol molecules is probably to increase the distance between polar heads (Marsh & Smith, 1973), leading to a decreased shift difference. We found that the average cholesterol-induced decrease in the chemical shift difference between the inner and outer monolayers was 9.5 ± 1.5 Hz. This agrees favorably with a previously reported measurement of Huang et al. (1974) of 8.0 ± 1.0 Hz.

Yb^{3+}-Induced Asymmetric Melting of $[(^{13}CH_3)_3N]$DMPC Vesicles. ^{13}C NMR experiments aimed at acquiring data on the asymmetric melting of $[(^{13}CH_3)_3N]$DMPC in the presence of Yb^{3+} complemented the ^1H NMR results. We found that $YbCl_3$ provided adequate ^{13}C spectral resolution without the strong aggregation given by the use of large amounts of Pr^{3+} (Sears et al., 1976). Yb^{3+} also shifts the outer monolayer resonances upfield, opposite to that of Pr^{3+}. Asymmetric melting of the $[(^{13}CH_3)_3N]$DMPC vesicles was found from an examination of the temperature-dependent line width of the choline methyl carbon resonances (Figure 3B). The outer

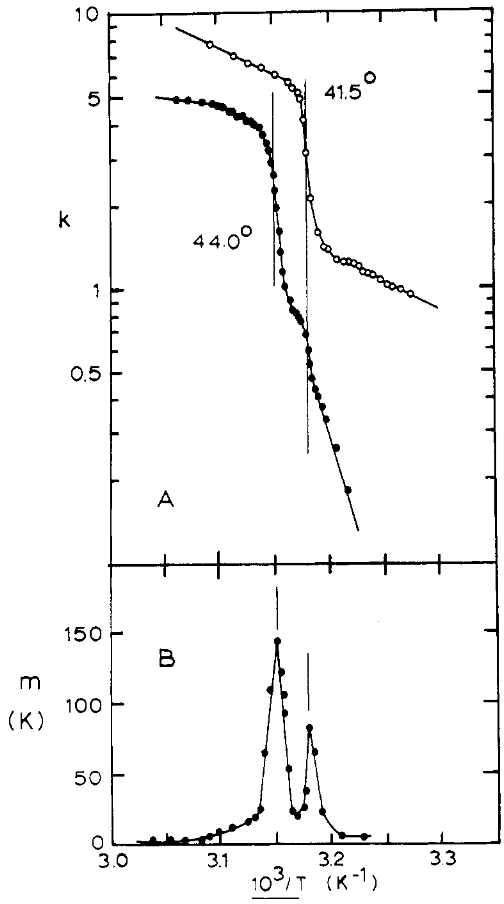

FIGURE 4: Effect of UO_2^{2+} on the temperature-dependent Tempo partition coefficient K in 263 mM DPPC vesicles. (A) The open circles are the control run without UO_2^{2+}, while the closed circles refer to the presence of 55 mM uranyl acetate. (B) The slope, m, of the Tempo partition coefficient data from (A) in the presence of UO_2^{2+}.

monolayer melted 1.6 °C higher than the inner, in the presence of a mole ratio of $r = 0.3$, Yb^{3+} to DMPC. The temperature-dependent ^{13}C chemical shift difference between the choline methyl carbon resonances from the outer and inner monolayers decreased with increasing temperature (Figure 3A), behavior that agrees with the ^1H NMR data (Figure 2). The decrease of 14% in the chemical shift difference compares favorably with that mentioned previously for the ^1H NMR results. The expansion of the bilayer on melting is the most probable cause of this decrease. An inflection point near the temperature of the gel to liquid-crystalline transition was also observed for these vesicles (Figure 3A).

Effect of UO_2^{2+} on Temperature-Dependent Tempo Partitioning in DPPC. The cation UO_2^{2+} is not permeable to membrane vesicles and can increase the phase transition temperature of phosphatidylcholine (Levine et al., 1973). Chapman (1973) and Chapman et al. (1974) used calorimetry to investigate the effect of uranyl cations (UO_2^{2+}) on the transition of DPPC. Their data clearly showed biphasic melting curves. In an attempt to extend these observations, we used sonicated DPPC vesicles and measured the Tempo partition coefficient as a function of temperature in the absence and presence of UO_2^{2+} (Figure 4A). The control DPPC vesicles melted at 41.5 °C. There are two visible transitions when UO_2^{2+} is present outside the vesicles, one at 41.5 °C and another transition at 44.0 °C.

The derivative, m, of the natural logarithm of the Tempo partition coefficient, K, is proportional to the excess heat capacity of partitioning. This slope is shown (Figure 4B) for the UO_2^{2+}-containing sample. The transition at 44.0 °C is due to the fatty acyl chains in the outer monolayer whose polar heads are binding UO_2^{2+}, and the transition at 41.5 °C is due to the inner unperturbed monolayer. The areas under the two peaks in Figure 4B are proportional to the enthalpy and are in the ratio of 2.21:1.0 (peak area at 44.0 °C to the peak area at 41.5 °C). This gives a value of 68.8% of the enthalpy of melting associated with the higher temperature transition and 31.2% associated with the lower transition, values that are consistent with the ratio of outer to inner DPPC molecules in our vesicles (de Kruijff et al., 1975).

Discussion

The primary finding reported in this paper is that the two monolayers of a phospholipid bilayer, composed of lipids with saturated side chains, can undergo the gel to liquid-crystalline transition independently of one another. The presence of paramagnetic multivalent cations (Pr^{3+} and Yb^{3+}) on the outside of DMPC vesicles separated the inner and outer choline methyl nuclear magnetic resonances and raised the temperature of the outer monolayer chain transition. With Tempo, which dissolves primarily in the fatty acyl chain region of phospholipid bilayers (Sillerud & Barnett, 1977), as a probe of dipalmitoylphosphatidylcholine phase transitions, two phase transition temperatures were detected in the presence of the membrane-impermeable ion UO_2^{2+}.

These properties of praseodymium, ytterbium, and uranium ions enabled us to show that the outer monolayer of a symmetric bilayer vesicle can be induced, by an asymmetric metal ion distribution, to melt at a higher temperature than the inner, unperturbed monolayer. Incorporation of cholesterol into the phospholipid vesicles resulted in a broadening of the phase transition of the outer half of the bilayer alone, implying that cholesterol was enriched in the outer monolayer. The finding of asymmetric head group melting raises the question of how strongly the fatty acyl chains are coupled across the midplane of the bilayer. The data presented here imply a very weak coupling. This result might be expected since the coupling must occur primarily through the terminal methyl regions. The mobility of the fatty acyl chains has been shown by both ESR and NMR (Levine et al., 1972; Hubbell & McConnell, 1971) to be greatest in the terminal methyl region. The 20% lateral expansion that occurs on melting (Trauble & Haynes, 1971) apparently does not produce enough packing strain to give coupling nor does insertion of cholesterol into just one-half of the bilayer.

In this study, asymmetry across the plane of the bilayer was induced by the asymmetric binding of Pr^{3+}, Yb^{3+}, and UO_2^{2+} and by the asymmetric incorporation of cholesterol. These treatments gave rise to a bilayer vesicle with differing fluidities in the two monolayers. Such a phenomenon has been suggested to occur naturally in erythrocyte (Tanaka & Ohnishi, 1976) and viral membranes (Wisnieski et al., 1974) from the results of ESR spin-label studies. If the phospholipid spin-labels employed in the cited studies distribute like natural phospholipids across the bilayer, then the outer phosphatidylcholine-containing monolayer was found to be less fluid than the inner phosphatidylserine-containing monolayer. Wisnieski et al. (1974) also noted differences in the temperature dependence of the fluidities of the two monolayers.

Membrane lipid asymmetry may have implications for the regulation of membrane-bound enzymes through changes in fluidity of the separate monolayers. Fluidity changes in either of the monolayers separately could alter and, therefore, regulate the activities of only those proteins that penetrate that particular monolayer.

Arnold (1974) also has described the increase in the transition temperature of the outer monolayer of DPPC vesicles in the presence of Pr^{3+}. Since our work was first submitted for publication, several other laboratories have presented evidence confirming our discovery of the weak coupling of the monolayers of vesicles prepared from synthetic lecithins. Hunt & Tipping (1978) used Pr^{3+} and Gd^{3+} to examine the effects of various agents, including cholesterol, on the separate monolayers of DPPC vesicles. Direct comparison of our results with theirs is difficult due to the differences in vesicle preparation methods used. However, our data for 20 mol % cholesterol resemble theirs for 7.5–15 mol %. It seems likely that our method of incorporation of cholesterol was less than 100% efficient.

The flip-flop of cholesterol, or the exchange of cholesterol from one vesicle to another, has been measured recently. Time constants of 4 h have been reported between DMPC vesicles at 37 °C and erythrocytes (Bloj & Zilversmit, 1977). Our experiments were performed in 3 h by measuring heating curves on vesicle samples that had been stored on ice since their preparation. It is likely that these conditions would have favored maintenance of an initially asymmetric cholesterol distribution.

Weak coupling of the monolayers appears to be a consequence of the poor interdigitation of the equal lengths of the acyl chains of DMPC. Schmidt et al. (1978) have demonstrated that transbilayer coupling is present in vesicles formed from *N*-lignocerylsphinomyelin, a phospholipid whose acyl chains differ by 11 carbons. Natural biomembranes are heterogeneous; hence, it is interesting that differences in fluidity between bilayer halves of LM cell membranes have been reported (Schroeder, 1978).

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