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Dependence of the Conformation of the Polar Head Groups of Phosphatidylcholine on Its Packing in Bilayers. Nuclear Magnetic Resonance Studies on the Effect of the Binding of Lanthanide Ions†

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ABSTRACT: Proton magnetic resonance spectra of vesicles of various sizes composed of egg phosphatidylcholine (PC) with varying concentrations of cholesterol differed in the apparent line width of the signal of the methylene protons of PC ($\Delta\nu_{1/2}$). They also varied in the extent of lanthanide-induced shifts of the ^{31}P and ^{1}H NMR signals of the corresponding nuclei of the polar head groups located on the outer surface of the vesicles ($\Delta\delta$). The differences in the lanthanide-induced shifts of the ^{31}P signals are fully accounted for by the ratio between the externally added lanthanide and the number of PC head groups available for interaction with the lanthanide ions. This was not the case for the changes in the ^{1}H NMR spectra. Here $\Delta\delta$ decreased with increasing $\Delta\nu_{1/2}$, suggesting that the packing of the PC paraffinic chains in the bilayer affects the conformation of the polar head groups; tightening of the packing probably results in a more extended conformation of the head groups. This conclusion is also supported by the larger effect lanthanides have on the ^{1}H chemical shift of the choline head groups on the outer surface of small unilamellar vesicles as compared to groups on the inner, tighter packed layer.

Small unilamellar phospholipid vesicles have been extensively used as models for biological membranes. Various techniques have been employed to study the mobility, viscosity, and local motions within the hydrophobic core of the bilayers and the dependence of these parameters on the composition of the liposomes (Bangham et al., 1974; Levine, 1972). Efforts have also been devoted to the investigation of surface properties of the model membranes, mainly the binding of cations to the membrane components. Thus, the binding of lanthanides, Ca^{2+}, and Mg^{2+} to phosphatidylcholine (PC)[1] vesicles has been investigated in terms of stoichiometry, apparent binding constants, and environmental conditions (Hauser et al., 1975, 1977). The affinity of cations for other phospholipids also has been measured, and a pronounced effect of negatively charged phospholipids has been demonstrated (Hauser et al., 1976a). Moreover, the effects of cation binding on both the conformation of the PC polar group (Hauser et al., 1976b) and the packing of the PC in the bilayer have been thoroughly studied (Hauser et al., 1975).

One question of importance, which has received relatively little attention, is how the surface properties of the membrane depend on the packing of the phospholipids in the bilayer. In a previous paper it has been shown that the polar head group exhibits a restricted flexibility, characterized by rapid transitions between two enantiomeric conformations (Seelig et al., 1977). Increasing the temperature resulted in a change in the average orientation of the polar head groups to a less extended conformation, with the *N*-methyl groups closer to the phosphorus atom (Gally et al., 1975). It is conceivable that any other process which leads to the loosening of the packing within bilayers might also result in a similar reduction of the distance between the *N*-methyl and the phosphate groups. The goal of the present work is to test this possibility.

Information on the conformation of the polar head groups of PC can be gained from the lanthanide-induced shifts of the NMR signals of various nuclei of these groups (Bystrov et al., 1971; Kostelnik & Castellano, 1972; Huang et al., 1974; Michaelson et al., 1974; Sears et al., 1976; Hauser, 1976; Hauser et al., 1976b). These measurements indeed reflect the conformation of those PC head groups to which the lanthanide is bound, which might differ from the conformation in the absence of bound ions (Yeagle et al., 1977; Brown & Seelig, 1977; Hauser et al., 1978).

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[1] Abbreviations used: PC, egg phosphatidylcholine; $[PC]_{tot}$, the total PC concentration in the dispersion; I_{in} and I_{out}, the intensities of the peaks of nuclei located on the inner and outer surfaces of the vesicles, respectively; $[PC]_{eff}$, the concentration of PC on the outer surface of the vesicles (equal to the multiplication of $[PC]_{tot}$ times the ratio of intensities $I_{out}/(I_{out} + I_{in})$); $\Delta\delta_H$ and $\Delta\delta_P$, the lanthanide-induced shifts of the signals of the polar groups of the phospholipid in the ^{1}H and ^{31}P NMR spectra, respectively; $\nu_{1/2}$, the full width at half-height of the ^{1}H NMR signal of the bulk methylene of PC.

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In previous papers it has been concluded that in the presence of lanthanide ions the polar head groups are oriented perpendicular to the PC bilayer (Hauser et al., 1976b), whereas in the absence of the lanthanide, the head group is oriented parallel to the surface of the bilayer due to intermolecular electrostatic interaction (Yeagle et al., 1977). Still, differences in the lanthanide-induced shifts between vesicles of various sizes and compositions may be indicative of conformational differences.

In the present communication, Pr^{3+}-induced shifts of ^1H and ^{31}P NMR signals (denoted $\Delta\delta_H$ and $\Delta\delta_P$, respectively) are used as a criterion for the head group conformation, and the correlation between the latter factor and the packing of the PC molecules within model membranes is investigated.

Materials and Methods

Egg yolk lecithin (PC; Makor Chemicals, Jerusalem) was chromatographically pure and was used without further purification. Cholesterol (Merck) was recrystallized from ethanol. Praseodymium chloride was the product of the Research Chemical Division of the Nuclear Corporation of America, and $EuCl_3$ was the product of Alpha Products. Both were dissolved in D_2O (99.8%; Merck) to give a stock solution of 0.3 M. A solution of PC and mixed solutions of PC and cholesterol in $CHCl_3$ were evaporated to dryness. Multilamellar liposomes were prepared by dispersing the residue in D_2O and mixing the formed dispersions with a vortex mixer for 2 min. Small unilameller vesicles (SUV) were prepared by sonication of the dispersions until clearness, using a Heat Systems W-350 sonicator. Larger vesicles of an average diameter of 400 Å (FUV) were obtained with an Aminco French pressure cell of the American Instrument Co., Inc., at 4 °C as described by Barenholz et al. (1979). ^1H NMR spectra were measured on a JEOL MH-100 instrument, the probe temperature being 31 ± 1 °C. Line widths ($\nu_{1/2}$) and the lanthanide-induced shifts ($\Delta\delta_H$) were measured by using a sweep width of 7.5 Hz/cm and a sweep rate of 1 Hz/s. Intensities were measured by weighing paper cutouts of the signals. The experimental errors in $\nu_{1/2}$ (which were mainly due to base line determination) depended on the widths and were estimated as 10% for samples in which narrow lines ($\nu_{1/2}$ < 25 Hz) of the paraffinic protons were observed and were up to 50% of the measured $\nu_{1/2}$ for much broader lines. The estimated error in $\Delta\delta_H$ and $\Delta\delta_P$ is 0.01, and in the areas of the inner and outer choline head group signals it is up to 10%. ^{31}P NMR spectra were recorded on a Brucker WP-60 instrument equipped with a Fourier transform system operating at 24.3 MHz and a probe temperature of 30 ± 1 °C. They were measured by using a sweep width of about 16.0 Hz/cm without proton broad band decoupling to avoid a possible interference of differences in the nuclear Overhauser enhancement in the determination of the ratio of intensities (out/in). The spectra were the result of accumulations of several thousand scans.

Results and Discussion

The addition of $PrCl_3$ to the dispersions of vesicles of various composition and sizes resulted in a shift of the ^1H and ^{31}P NMR signals of the head groups on the outer surface of the bilayer. In vesicles of pure PC, the concentration of PC available for interaction with lanthanide ions (denoted $[PC]_{eff}$) indeed depends on the size of the vesicles since, for any given total concentration of PC, the number of PC molecules on the surface of the vesicles must decrease with the increase in the vesicle size (Bystrov et al., 1971).

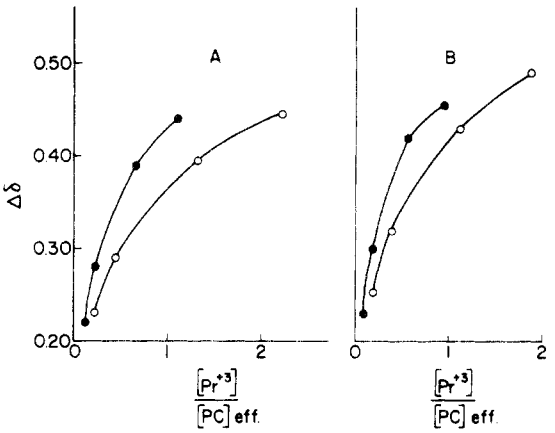

FIGURE 1: Pr^{3+}-induced downfield shift of the signal of the choline head group on the outer surface of PC vesicles as a function of the molar ratio of Pr^{3+} to PC available for interaction with it ($[PC]_{eff}$). The total PC concentrations were 46.6 (closed circles) and 23.3 mM (open circles). $[PC]_{eff}$ was calculated from the ratio of I_{in} to I_{out} (see abbreviations for definitions). The latter ratio had a value of 1.5 for FUV (A), prepared by the French pressure cell, and 2.2 in SUV (B), obtained after further sonication of the FUV.

Figure 1 describes the dependence of $\Delta\delta_H$ on the ratio between the total concentration of Pr^{3+} and $[PC]_{eff}$ in SUV and FUV, composed of pure PC, at two different total PC concentrations (denoted $[PC]_{tot}$). As expected from previously published data, $\Delta\delta_H$ in the vesicles increased with the increasing molar ratio of $[Pr^{3+}]$ to $[PC]_{eff}$ (Figure 1B). Also, for any given ratio of $[Pr^{3+}]$ to $[PC]_{eff}$, an increase in $[PC]_{tot}$ resulted in larger shifts of the choline head group signal (Hauser, 1976; Hauser et al., 1977). Most interestingly, $\Delta\delta_H$ also depended on the size of the vesicles, being always larger for SUV than for the corresponding FUV (compare Figure 1A with Figure 1B at equal $[PC]_{tot}$ and $[Pr^{3+}]/[PC]_{eff}$).

The capacity of the single bilayered PC to bind Pr^{3+} was analyzed for the various vesicle preparations according to the method of Hauser et al. (1977). For these calculations we adopted the conclusion of the above authors that lanthanides form a 1:2 complex with PC. The calculated binding constants (K_b) for SUV and FUV were very similar. For example, K_b for $\Delta\delta_H = 0.32$ had a value of 4.14×10^3 L^2 M^{-2} for SUV and 4.04×10^3 L^2 M^{-2} for FUV.

It can therefore be concluded that the effect of size on $\Delta\delta_H$ most likely does not reflect changes in the binding capacity but rather in the conformation of the choline head group. This conclusion is considerably strengthened by ^{31}P NMR measurements. The addition of $PrCl_3$ (30 mM) to SUV of PC (100 mM) shifted the signal of the ^{31}P of the phosphate group on the outer surface by 12.9 ppm, whereas in FUV of the same total concentration, a larger shift was observed (15.0 ppm). Only when the concentration of $PrCl_3$, added to SUV, was raised to 35 mM, did the molar ratio of $[PrCl_3]$ to $[PC]_{eff}$ equal that of the dispersion of the FUV with 30 mM $PrCl_3$ and so did $\Delta\delta_P$. On the basis of these results, we suggest that $\Delta\delta_P$ reflects mainly the binding of Pr^{3+} to the most probable site for its interaction, namely, the phosphate group of PC (Hauser et al., 1975), and is much less sensitive than $\Delta\delta_H$ to conformational changes.

The ^1H shifts are pseudocontact in origin and the site of binding has effective axial symmetry (Barry et al., 1971, 1974). The conformation of the head group should therefore largely affect the $\Delta\delta_H$ since the extent of this shift should depend on the third power of the distance between the choline head group and the attached praseodymium ion. The more extended the conformation of the head group is, the further apart should

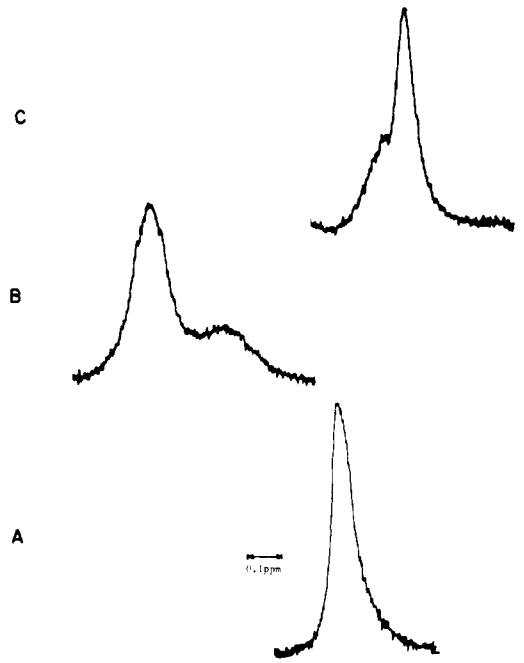

FIGURE 2: The ^1H NMR signals of the choline head groups of PC (60 mM) in SUV prepared by sonication in (A) D_2O, (B) a 60 mM solution of $PrCl_3$, and (C) a 80 mM solution of $EuCl_3$.

the Pr^{3+} ion be from the choline methyls. In FUV the hydrocarbon chains are packed more tightly than in SUV, probably due to the smaller curvature (Sheetz & Chan, 1973; Lichtenberg et al., 1975). The tighter packing might result in a more extended conformation of the polar head groups and consequently in lower $\Delta\delta_H$ values. Recently it has been shown that, in SUV, the polar head groups of PC on the outer surface are less tightly packed than those of the inner layer (Huang & Mason, 1978; Chrzeszczyk et al., 1977). It is therefore expected, in light of the above interpretation of our results, that $\Delta\delta_H$ (but not $\Delta\delta_P$) will be larger for the outer than for the inner monolayer.

Experimentally, when PC was sonicated in the presence of Pr^{3+}, the ^{31}P signal of the phosphate group was evenly shifted from its original field. On the other hand, in the ^1H NMR spectra, the choline head group signal of the inner layer was shifted less than that of the outer surface. This is evident from the apparent "splitting" of the signal, obtained upon sonication of PC in a solution of $PrCl_3$, which shifted the whole signal to a lower field (Figure 2B). The peak of the inner surface head groups appeared at a higher field than that of the outer surface. On the other hand, in the spectrum of a dispersion of PC that was sonicated in a solution of $EuCl_3$, which shifted the whole signal to a higher field, the signal of the inner surface choline head groups appeared as a shoulder on the low-field side of the choline signal (Figure 2C).

The elevation of the temperature also has a fluidizing effect on the packing of the hydrocarbon chains. In fact, raising it from 30 to 80 °C (for samples of $[PC]_{tot}$ = 50 mM and $[PrCl_3]$ = 20 mM) caused some increase in $\Delta\delta_H$ (0.03–0.04 in both SUV and FUV), the temperature effect being only slightly larger in FUV than in SUV. This small increase might still be considered significant since it is observed in spite of the plausible temperature-induced reduction of the cation binding.

Cholesterol is known to have a "stiffening" effect on the packing within bilayers (Darke et al., 1972). Consequently, increasing the concentration of cholesterol caused broadening of the bulk methylene signal (Gent & Prestegard, 1974). Sonication of mixed dispersions of PC and increasing amounts

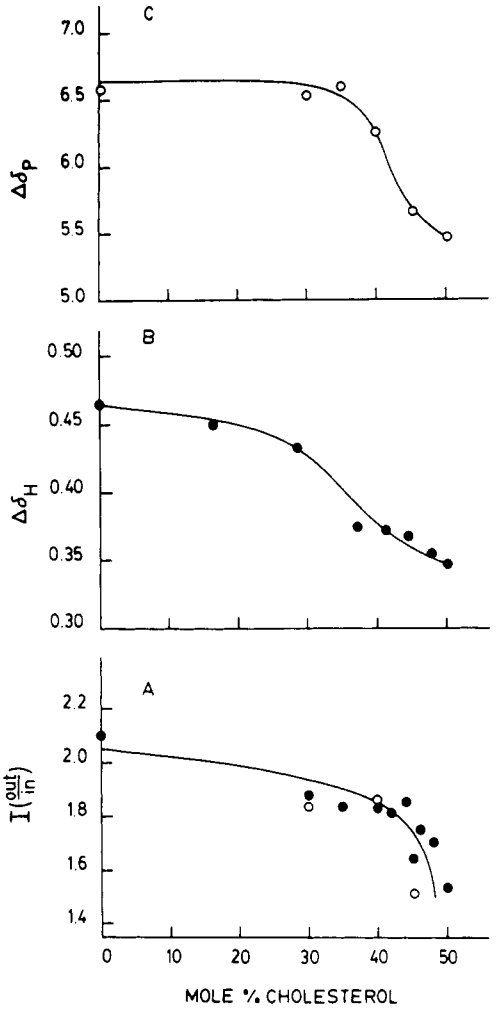

FIGURE 3: The dependence of spectral parameters on the mole percent cholesterol in PC–cholesterol vesicles. (A) The ratio of PC_{out}/PC_{in} (I) as determined from the ^1H (closed circles) and ^{31}P (open circles) NMR spectra. (B) The shift of the ^1H NMR signal of the choline head group on the outer surface of the bilayer ($\Delta\delta_H$), induced by 30 mM $PrCl_3$. (C) The shift of the ^{31}P NMR signal ($\Delta\delta_P$), induced by 7.5 mM $PrCl_3$. $[PC]_{tot}$ was 20 mM in all the dispersions, and the cholesterol concentration was varied from 0 to 20 mM.

of cholesterol, above 35 mol % of the latter lipid, resulted in the formation of increasingly larger vesicles (Gent & Prestegard, 1974; Newman & Huang, 1975; Forge et al., 1978). It also leads to a growing asymmetry of cholesterol distribution between the two layers, with a relatively higher content of cholesterol in the inner layer and a higher amount of PC in the outer layer (de Kruijff et al., 1976; Huang et al., 1974). The latter two phenomena should affect the ratio of intensities (I = out/in) in a contradictory fashion, which might explain the variability of the effect of cholesterol on this ratio for various phospholipids (de Kruijff et al., 1976).

In our experiments, inclusion of less than 40 mol % cholesterol caused only slight changes in I, whereas at higher cholesterol concentrations I decreased (Figure 3A). This decrease means that a lower fraction of PC becomes available for interaction with Pr^{3+}; namely, the ratio of $[Pr^{3+}]$ to $[PC]_{eff}$ and consequently the lanthanide-induced shifts should increase. The experimental reduction of $\Delta\delta_H$ (Figure 3B) and $\Delta\delta_P$ (Figure 3C), above 40 mol % cholesterol, can therefore not be explained by stoichiometric considerations. It might possibly reflect reduced binding of Pr^{3+} to the surface, which can be caused by an increase in the spacing of the choline head groups, since a 2:1 complex of PC and lanthanide ions is formed (Hauser et al., 1977). Alternatively, it may be caused

by an increase in the average distance between bound Pr^{3+} cations and ^{31}P and ^{1}H nuclei of adjacent PC molecules, due to cholesterol-induced spacing of the PC molecules on the surface of the membranes. This "spacing" can also account for the increase of motion of the choline head groups, observed upon inclusion of the elevated amounts of cholesterol in multilamellar PC (Oldfield et al., 1978), and for the slight alteration in the head group conformation due to reduction of intermolecular interactions (Brown & Seelig, 1978). The increased spacing of the polar head groups indeed depends on the concentrations of cholesterol. At low cholesterol concentration, the rigidizing effect of cholesterol might cancel any possible spacing. Thus, low concentrations of cholesterol may restrict the motion of the choline head groups, and in the Pr^{3+}-bound PC it may lead to a more extended conformation of these groups.

Experimentally, in small unilamellar vesicles with 30–40 mol % cholesterol, $\Delta\delta_H$ decreased while neither I (Figure 3A) nor $\Delta\delta_P$ (Figure 3C) was altered. It is unlikely that the decrease in $\Delta\delta_H$ is due to increased spacing since such an effect would have probably been accompanied by a reduction of $\Delta\delta_P$. Therefore, the lowering of $\Delta\delta_H$ may be due to a more extended conformation of the lanthanide-bound head groups. Thus, in this range of cholesterol concentrations, the cholesterol-induced tightening of packing causes conformational change to which $\Delta\delta_H$ (but not $\Delta\delta_P$) is sensitive.

This leads to the conclusion that tightening of packing, due to reduced curvature, decreasing temperature, or inclusion of cholesterol, may result in a more extended conformation of the polar head groups in the presence of lanthanides.

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