

EFFECTS OF DIVALENT CATIONS AND pH ON PHOSPHATIDYL SERINE
MODEL MEMBRANES: A ^{31}P NMR STUDY

M.J. Hope and P.R. Cullis

Department of Biochemistry, University of British Columbia
Vancouver, B.C., Canada, V6T 1W5

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SUMMARY

The influence of divalent cations, and pH on the behaviour of phosphatidylserine, derived from egg phosphatidylcholine, has been examined employing ^{31}P -NMR techniques. The addition of Ca^{2+} results in the observation of a "rigid lattice" ^{31}P -NMR spectra and more than an order of magnitude increase in the spin-lattice relaxation time T_1 . This corresponds to a strong and specific headgroup immobilization by Ca^{2+} , similar to that observed for anhydrous phosphatidylserine. At pH 7.4 the hydrated sodium salt of (egg) phosphatidylserine adopts the bilayer phase, whereas when the pH is decreased through 3.5 a bilayer to hexagonal (H_{II}) polymorphic phase transition is observed at 50°C , which is unaffected by equimolar cholesterol. The same transition is shown to occur at 37°C for phosphatidylserine isolated from human erythrocytes.

Phosphatidylserine, the most abundant acidic phospholipid found in mammalian membranes, is also one of the most intriguing and has been implicated to play a functional role in processes as diverse as membrane fusion (1) and activation of membrane bound enzymes (2). Characteristics of phosphatidylserine which are of basic interest concern the ability of pH, as well as monovalent and divalent cations to modulate such features as the hydrocarbon transition temperature (3) and, most importantly, the strong and specific interaction it experiences with Ca^{2+} (4-6). This latter interaction can have dramatic manifestations, including Ca^{2+} -induced precipitation of pure phosphatidylserine to form (crystalline) cochleate lipid structures (6) as well as Ca^{2+} induced lateral phase separation of phosphatidylserine in mixed lipid systems (4).

In this work we have investigated the behaviour of aqueous dispersions of phosphatidylserine with two objectives in mind. First, given the specificity of the Ca^{2+} phosphatidylserine interaction, it is of interest to ascertain the influence of Ca^{2+} on the headgroup motion and conformation. Secondly, in

the light of the observation that many other phospholipid species adopt the hexagonal (H_{II}) or other non-bilayer arrangements (8-11) on hydration, it is important to clearly establish the polymorphic preferences of phosphatidylserine under varying conditions such as pH, which may be directly related to functional roles in vivo.

Using ^{31}P NMR techniques we show that Ca^{2+} interacts strongly and specifically with phosphatidylserine to induce relatively complete immobilization of the head group. At physiological pH values, phosphatidylserine derived from egg yolk phosphatidylcholine and phosphatidylserine isolated from human erythrocytes, assume the bilayer phase on hydration. Lowering the pH below 4.0 results in a transition from the bilayer to the hexagonal (H_{II}) configuration.

MATERIALS AND METHODS

(Egg) phosphatidylserine was derived from hen egg yolk phosphatidylcholine, employing the headgroup exchange capacity of phospholipase D (12), whereas human erythrocyte phosphatidylserine was isolated from a total lipid extract of red blood cells (13). Both lipids were purified using carboxymethylcellulose low pressure column chromatography. The sodium salt was obtained by dissolving the dry lipid in a Bligh and Dyer monophasic (chloroform/methanol/ H_2O in the ratio 1:2.1:1 by vol.) where the aqueous component contained 0.4 M HCl. This was subsequently titrated to pH = 8.0 with a Bligh and Dyer monophasic where the aqueous phase contained 0.5 M NaCl and 0.5 M NaOH. The (egg) phosphatidylserine was shown to be >99% and the erythrocyte phosphatidylserine >97% pure, with respect to phosphorus, as determined by two dimensional thin layer chromatography (14). Fatty acid analyses were performed using a Hewlett Packard 7610A high efficiency gas chromatograph (fitted with a column of ethylene glycol succinate) operated at 170°C. Methyl esters were prepared by heating phosphatidylserine dissolved in 5% H_2SO_4 in methanol at 70°C under nitrogen for 2 hrs (15).

^{31}P NMR measurements were performed on aqueous dispersions of lipid (prepared by vortex mixing) employing a Bruker WP 200 NMR spectrometer. Two methods of adjusting the pH of the aqueous dispersions of phosphatidylserine were used, both gave similar results. Firstly, pH was altered by the direct addition of 0.1 M HCl or 0.1 M NaOH to an unbuffered dispersion of the lipid in a 10 mm NMR tube. pH was measured at the temperature of signal accumulation, following repeated freeze-thawing of the sample. The second procedure utilized buffered solutions of the required pH; the lipid was dispersed in 50 ml of buffer and incubated at room temperature for 10 min. The dispersion was concentrated by centrifugation, frozen and thawed, and the process repeated until the correct pH was achieved. Degradation of phosphatidylserine to the lyso derivative, during a typical experiment, was found to be <1%.

RESULTS AND DISCUSSION

The ^{31}P NMR spectrum obtained from anhydrous (egg) phosphatidylserine is shown in Fig. 1(a). The "rigid lattice" (no motion) lineshape observed is

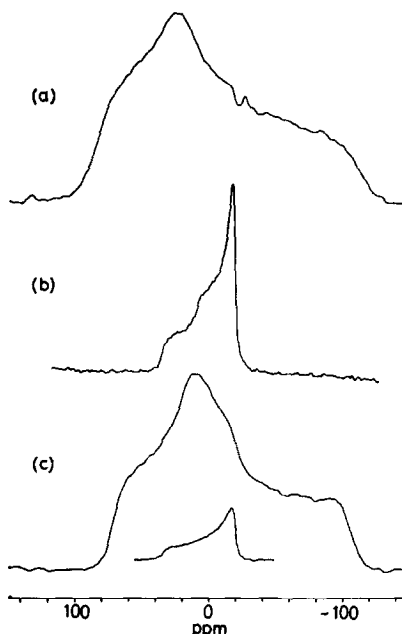


Fig. 1. 81.0 MHz ^{31}P NMR spectra at 30°C arising from (a) 200 μmol anhydrous (egg) phosphatidylserine (sodium salt), (b) 50 μmol hydrated (egg) phosphatidylserine ($\text{pH} = 8.0$) and (c) 200 μmol hydrated (egg) phosphatidylserine (sodium salt) to which 100 μmol Ca^{2+} was added subsequently. The insert in (c) shows the spectra obtained for the sample of (b) to which Mg^{2+} was added to a Mg^{2+} /phosphatidylserine ratio of 3.0. Fig. 1(b) and the insert of 1(c) were collected under the same conditions as indicated for Fig. 2. Fig. 1(a) and 1(c) were obtained from 200 transients employing a 20 sec interpulse time and a 50 KHz sweep width.

characteristic of an axially asymmetric ^{31}P chemical shift tensor. The principal values of this tensor are similar to those observed for anhydrous phosphatidylcholine (16) and sphingomyelin (17) and thus suggest that the head-group conformation in the phosphate region is similar for these different phospholipid species.

Hydration of (egg) phosphatidylserine resulted in the spectrum of Fig. 1 (b), which has a familiar asymmetric lineshape with a high field peak and low field shoulder characteristic of (liquid crystalline) lamellar phospholipids. This is consistent with X-ray studies of bovine brain phosphatidylserine (18) dispersed in excess standard buffer at $\text{pH} 7.4$, which also reveal a lamellar organization. A small hexagonal (H_{II}) phase spectral component is also visible.

The addition of Ca^{2+} to hydrated (egg) phosphatidylserine to achieve a Ca^{2+} -phosphatidylserine molar ratio of 0.5 has remarkable effects, as indi-

cated in Fig. 1(c). Precipitation of the lipid occurred and the ^{31}P NMR spectrum obtained from the hydrated sodium salt had reverted to the rigid lattice spectrum obtained from the anhydrous sodium salt (Fig. 1(a)). The similarity of Figs. 1(a) and (c) would suggest that the headgroup conformation in the phosphate region of phosphatidylserine is not markedly affected by the presence of Ca^{2+} . A dramatic increase in the spin-lattice relaxation time T_1 (measured by a saturation recovery technique) was observed, in association with the Ca^{2+} induced spectral changes, from 0.5 ± 0.1 sec. for the sodium salt to 16 ± 3 sec. for the Ca^{2+} -phosphatidylserine precipitate. These results certainly suggest a strong Ca^{2+} -phosphatidylserine interaction. The specificity of this interaction is indicated by the fact that while Mg^{2+} also induced precipitation, normal "bilayer" ^{31}P NMR spectra were obtained (see insert on Fig. 1(c)) even at Mg^{2+} /phosphatidylserine molar ratios of 3.0. This latter observation strongly suggests that Ca^{2+} does not induce its effects by non-specific charge neutralisation, but rather binds specifically to the serine headgroup. This information, coupled with the two to one phosphatidylserine- Ca^{2+} stoichiometry in the condensed precipitate, provides support for models such as that of Portis et al. (18) who suggest that Ca^{2+} forms an anhydrous complex between two molecules of phosphatidylserine.

Variation of the pH of the phosphatidylserine dispersion also has strong effects, as summarised in Fig. 2. Between pH $\approx$ 4.0 and 8.0 the bulk of the phospholipid adopts the bilayer phase, as indicated previously. However, if the pH is reduced below the pK of the phosphatidylserine carboxyl group (approximately 4.0 (19)), a polymorphic phase transition is induced. In Fig. 2(a) (egg) phosphatidylserine, at 50°C , is shown to proceed from the bilayer phase, at pH $\approx$ 3.9, to the hexagonal (H_{II}) phase at pH $\approx$ 3.0. At pH $\approx$ 3.3 an intermediate state is obtained where isotropic motion occurs. Equimolar cholesterol appears to confine the transition from bilayer to hexagonal (H_{II}) to a narrower pH range as shown in Fig. 2(b). In Fig. 2(c) a similar polymorphic phase transition is shown for erythrocyte phosphatidyl-

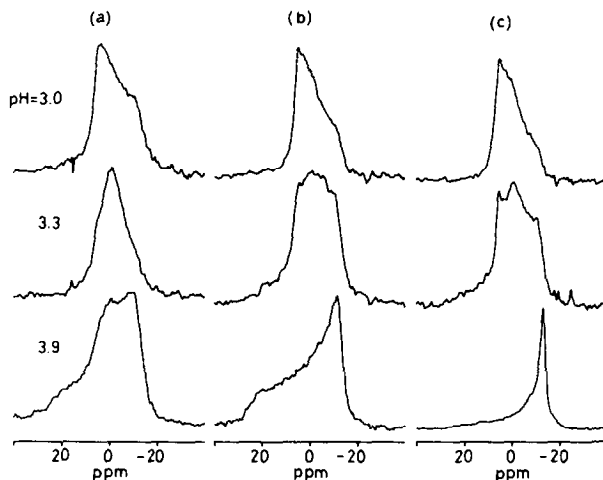


Fig. 2. 81.0 MHz ^{31}P NMR spectra arising from 50 μmol of lipid at pH = 3.0, 3.3 and 3.9. (a) (egg) phosphatidylserine at 50°C, (b) (egg) phosphatidylserine with equimolar cholesterol at 50°C and (c) phosphatidylserine isolated from human erythrocytes. The phospholipid was hydrated in 1 ml of water, containing 2 mM EDTA and 20% D_2O , the pH was adjusted using 0.1 M HCl. Spectra were collected from 2000 transients employing a 0.5 sec repetition rate, a 10 μsec 90° pulse and 20 KHz sweep width.

serine at 37°C. This observation that the protonated form of phosphatidylserine prefers the hexagonal (H_{II}) configuration may explain the small hexagonal (H_{II}) component seen at pH = 8.0 for the sodium salt, Fig. 1(b), which can be attributed to a fraction of the phosphatidylserine remaining in the acidic form. This would be consistent with the finding that in the presence of 0.5 M NaCl this component was not apparent, whereas the bilayer to hexagonal (H_{II}) phase transition in the region pH = 4.0 to 3.0 was unaffected by increasing the salt concentration (results not shown).

At pH = 2.5, well below the pK of the phosphatidylserine carboxyl group, phosphatidylserine undergoes a transition from the bilayer to hexagonal (H_{II}) phase as the temperature is increased through 40°C for (egg) phosphatidylserine and 20°C for erythrocyte phosphatidylserine, the spectra are shown in Fig. 3(a) and (b), respectively. This is similar behaviour to that observed for natural phosphatidylethanolamines (10). Phosphatidylethanolamine derived from egg yolk phosphatidylcholine also undergoes a polymorphic phase transition at about 40°C (authors unpublished results). The lower temperature at

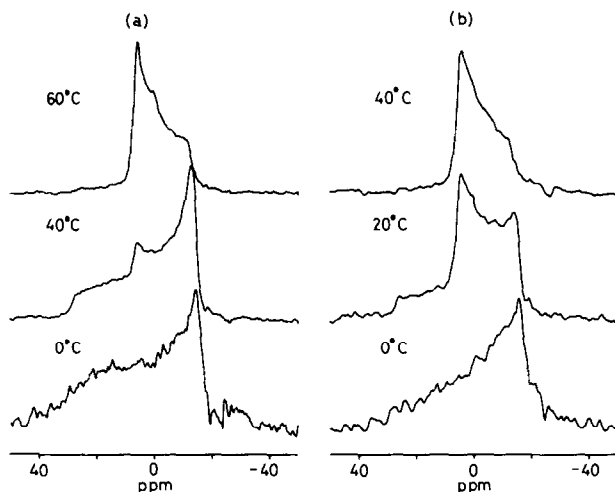


Fig. 3. 81.0 MHz ^{31}P NMR spectra arising from 50 μmol of (a) (egg) phosphatidylserine at 0°C, 40°C and 60°C and (b) erythrocyte phosphatidylserine at 0°C, 20°C and 40°C. The lipid was hydrated in 1 ml of water, containing 2 mM EDTA and 20% D_2O , the pH of both samples was adjusted to 2.5 with 0.1 M HCl. Spectra were collected as described for Fig. 2, following a 10 min interval to allow for equilibration of the temperature.

which erythrocyte phosphatidylserine undergoes a bilayer to hexagonal (H_{II}) phase transition is likely related to its higher degree of unsaturation (10). Fatty acid analysis showed approximately 2.0 unsaturated bonds per molecule of erythrocyte phosphatidylserine compared to approximately 1.0 per molecule of the (egg) phosphatidylserine used in this study.

In summary, the results presented here show that the hydrated sodium salt of phosphatidylserine prefers the bilayer phase at physiological pH values. This is consistent with the ability of phosphatidylserine to stabilize the bilayer configuration in mixed systems containing "non-bilayer" phospholipids (20,21). Secondly, reducing the pH to below the pK of the carboxyl group causes the phosphatidylserine to behave in much the same manner as phosphatidylethanolamine with a similar fatty acid composition, encouraging formation of the hexagonal (H_{II}) phase at higher temperatures. It is doubtful that the phase transition induced by decreasing the pH below 4.0 is significant in a biological system. However, it is not inconceivable that within the micro-environment of a membrane, conditions could arise which would result in an

increase in the pK of phosphatidylserine to a physiologically relevant range. Finally, Ca^{2+} interacts strongly with hydrated phosphatidylserine to immobilize the headgroup in the phosphate region inducing a motional state similar to that observed for the anhydrous sodium salt. The specificity of this interaction is implicit in the inability of Mg^{2+} to induce similar effects.

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