Effects of pH and Cholesterol on DMPA Membranes: A Solid State ^{2}H- and ^{31}P-NMR Study

Tanja Pott, Jean-Christophe Maillet, and Erick J. Dufourc*
Centre de Recherche Paul Pascal, Centre National de la Recherche Scientifique, 33600 Pessac, France

ABSTRACT The effect of pH and cholesterol on the dimyristoylphosphatidic acid (DMPA) model membrane system has been investigated by solid state ^{2}H- and ^{31}P-NMR. It has been shown that each of the three protonation states of the DMPA molecule corresponds to a ^{31}P-NMR powder pattern with characteristic $\Delta\sigma$ values; this implies additionally that the proton exchange on the membrane surface is slow on the NMR time scale (millisecond range). Under these conditions, the ^{2}H-labeled lipid chains sense only one magnetic environment, indicating that the three spectra detected by ^{31}P-NMR are related to charge-dependent local dynamics or orientations of the phosphate headgroup or both. Chain ordering in the fluid phase is also found to depend weakly on the charge at the interface. In addition, it has also been found that the first pK of the DMPA membrane is modified by changes in the lipid lateral packing (gel or fluid phases or in the presence of cholesterol) in contrast to the second pK. The incorporation of 30 mol % cholesterol affects the phosphatidic acid bilayer in a way similar to what has been reported for phosphatidylcholine/cholesterol membranes, but to an extent comparable to 10–20 mol % sterol in phosphatidylcholines. However, the orientation and molecular order parameter of cholesterol in DMPA are similar to those found in dimyristoylphosphatidylcholine.

INTRODUCTION

Biological membranes may contain up to 20% ionizable lipids; thus one might expect electrostatic surface charges to be of great importance for various membrane-occurring processes. Depending on the electrolyte environment, their charge can be modified, which results in a change in the lateral packing of the lipids; i.e., it has been shown that the gel-to-fluid phase transition of model membranes can be triggered electrostatically, e.g., by varying the external pH (Eibl and Blume, 1979). On the other hand, a change in lipid packing can lead to a change in the electrolyte environment; i.e., the spontaneous lateral expansion at the phase transition gives rise to a proton pulse into the electrolyte medium (Vaz et al., 1978). The packing of the membrane hydrophobic interior can also be considerably altered by the presence of cholesterol, which regulates lipid chain dynamics over large temperature ranges. Thus the study of both the electrostatic interactions at the interface (variation of the surface net charge) and the hydrophobic effect in the membrane core is expected to elucidate some physical and chemical properties of biological membranes. Recently, it was demonstrated that charged polypeptides, or calcium ions, strongly modulate both the thermotropism and the ordering properties of dimyristoylphosphatidic acid (DMPA) model membranes (Laroche et al., 1990, 1991). In the same study, it was shown that peptide conformation (i.e., β-sheet or random coil) could be triggered by the negatively charged membrane.

As a model membrane, DMPA bilayers are also interesting because the surface charge can be easily modified by varying the pH; i.e., the phosphate group can lose one or two protons. The corresponding pK_a values are dependent on the ionic strength. For example, the pK_a values of DMPA were determined to be 3.5 and 8.0 (Träuble and Eibl, 1974), but an increase in the NaCl concentration (from 0 to 100 mM) leads to values of 4.0 and 8.5 (van Dijck et al., 1978). Its gel-to-fluid phase transition temperature, T_C, shows a strong dependence on its protonation state (Träuble and Eibl, 1974; Jacobson and Papahadjopoupos, 1975; van Dijck et al., 1978; Blume and Eibl, 1979; Eibl and Blume, 1979). To summarize the results in the absence of salt, for pH values lower than 4, DMPA transits between 45° and 48°C, which is followed by a sudden increase in T_C to 55°C when pH 4 is reached (Eibl and Blume, 1979). In the pH range 4–9, the variation of T_C versus pH is relatively flat, with a small decrease from 55° to 50°C with increasing pH. For pH >9, a sudden and strong decrease in T_C has been observed, leading to values of $T_C \approx 30$°C at pH 10 (Eibl and Blume, 1979). The profile of T_C versus pH seems to be generally valid for phosphatidic acid (PA)-lipids and is associated with two pK_a values of the phosphate group, i.e., with the dissociation state of the lipid. Moreover, in monolayer studies Sacré and Tocanne (1977) found that (in agreement with the Gouy-Chapman theory of the diffuse electric double layer) an increase in the concentration of monovalent cations will decrease the apparent pK values, and this effect is maximum at 100 mM NaCl, the salt concentration used in this work. As a consequence of such a screening effect, the transition temperature for a given pH was affected, which has indeed been reported at 100 mM NaCl by van Dijck et al. (1978), and values of T_C were found to be at ~52° and 42°C at pH 4.2 and 8.2, respectively.

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Address reprint requests to Dr. Erick J. Dufourc, Centre de Recherche Paul Pascal, Centre National de la Recherche Scientifique, 33600 Pessac, France. Tel.: 33-56-84-5638; Fax: 33-56-84-5600; E-mail: dufourc@crpp.u-bordeaux.fr.

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The effects of elevated cholesterol concentrations (≥22%) on phosphatidylcholine (PC) membranes in general may be summarized as follows. 1) The gel-to-fluid phase transition is reduced or eliminated; 2) there is a decrease and an increase in orientational order of the lipid chains in gel and fluid phases, respectively; and 3) below T_C, the lipid headgroup is markedly disordered but slightly affected above (Mabrey et al., 1978; Sankaram and Thompson, 1990; Vist and Davis, 1990; Léonard and Dufourc, 1991). However, few experiments have been performed to investigate the structure and dynamics of negatively charged lipids. Structural and dynamic changes at the headgroup level have been monitored on phosphatidylserine (PS) (Roux et al., 1989; De Kroon et al., 1991) and phosphatidylglycerol (PG) (Eklund et al., 1987; Marassi and Macdonald, 1991), but few correlations have been made between the motional behavior at the membrane surface and in the bilayer core. In addition, whether the charges at the interface influence the lipid chain ordering has never been investigated.

The goal of the present study is to follow the influence of surface charge density (pH) on the structure and dynamics of both the polar headgroup and the hydrophobic core of DMPA model membranes. The effect will be monitored in the bilayer core by ^{2}H-NMR of DMPA perdeuterated on the *sn*-2 acyl chain and at the bilayer surface by ^{31}P-NMR. As will be reported herein, under extreme pH conditions (pH 2.9 and 8.9) one detects small vesicles whose isotropic reorientation prevents the measurement of order parameters via ^{2}H-NMR. As a consequence, ^{31}P measurements will be performed on the entire pH range of our study, whereas ^{2}H-NMR will be performed on samples that still exhibit an extended lamellar structure that is as close as possible to the singly and doubly charged state, i.e., pH 4.2 and 8.2. The effect of 30 mol % cholesterol on the ordering properties of DMPA membranes will also be investigated for the two pH values mentioned above.

MATERIALS AND METHODS

Synthetic DMPA was purchased from Fluka (St. Quentin-Fallavier, France); [*sn*-2-$^2H_{27}$]-DMPA was purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Cholesterol was obtained from Sigma (St. Quentin-Fallavier, France); [2,2′,3,4,4′,6-$^2H_6$]-cholesterol was synthesized according to published procedures (Dufourc et al., 1984). Thin layer chromatography was performed before and after experiments. No lysophosphatidic acid was detected in any sample after completion of NMR experiments.

Lipid dispersions were prepared according to established procedures (Dufourc et al., 1984). The dry lipid powder (50 mg) was dispersed in 1 ml of the appropriate buffer and then was heated to 50–60°C, vortexed, and cooled down until freezing of the sample. This cycle was repeated at least five times until a homogeneous dispersion was obtained. In the case of cholesterol-containing systems, lipids and cholesterol were first dissolved in chloroform/methanol (2:1 v/v), mixed, and heated if necessary; the solvent was then removed by blowing a stream of nitrogen over the sample. To ensure that all the organic solvent was removed, the sample was pumped under vacuum overnight. A Tris buffer solution (100 mM Tris, 100 mM NaCl, 2 mM EDTA) was used for studies at neutral and basic pHs, whereas an acetate buffer (100 mM acetate, 100 mM NaCl, 2 mM EDTA) was used for acidic conditions. NaCl and EDTA were added to provide control conditions for other studies involving the above systems and the bee venom toxin melittin (T. Pott, J. C. Maillet, C. Abad, A. Campos, J. Dufourcq, and E. J. Dufourc, unpublished data).

Because relaxation times for immobilized systems are long (Dufourc et al., 1992) and lead to long experimental times, the principle tensor values of the ^{31}P shielding tensor were measured on dry powders. In order to reduce the risk of lipid hydrolysis during the experiment, samples were prepared at pH 3 and 8 by dropwise addition of HCl or NaOH to a dispersion of 50 mg DMPA. After pH control and adjustment, the samples were freeze-dried and used without additional treatment.

^{31}P-NMR was carried out on a Bruker WH270 (Wissembourg, France) implemented for high power solid-state spectroscopy and operated at 109.35 MHz; a phase-cycled Hahn-echo pulse sequence (Rance and Byrd, 1983) with gated proton decoupling was utilized. ^{2}H-NMR experiments were performed on a Bruker MSL200 operating at 30.7 MHz by means of a quadrupolar echo composite pulse sequence (Levitt and Freeman, 1981; Levitt, 1982). In the case of ^{2}H-labeled cholesterol, a quadrupolar echo pulse sequence with proton decoupling via spin lock was applied. Quadrature detection was used in all cases. Samples were allowed to equilibrate for at least 30 min at a given temperature before the NMR signal was acquired; the temperature was regulated to ±1°C. Typical acquisition parameters were spectral window of 50 kHz (^{31}P-NMR) or 500 kHz (^{2}H-NMR); $\pi/2$ pulse width of 8 μs and 4 μs (^{31}P-NMR and ^{2}H-NMR, respectively) and delay between the two pulses to form the echo of 40 μs; recycle delay for ^{31}P-NMR of 6–8 s and for ^{2}H-NMR of 2 s (lipid chains) and 0.1 s (cholesterol). The number of scans was 1000–1500 and 8000–100,000 (^{31}P-NMR and ^{2}H-NMR, respectively). Spectral "dePaking" was performed as described by Bloom et al. (1981) and Sternin et al. (1983) and calculated for bilayer normals oriented at 90° with respect to the magnetic field direction. C-^{2}H bond order parameters, $S_{C\text{-}^2H}$, were determined from the quadrupolar splittings according to Davis (1983) and traced as a function of labeled carbon positions. We used the "smoothed order profiles" method (Lafleur et al., 1990); i.e., first, a classic assignment assuming an increase of $S_{C\text{-}^2H}$ from the terminal methyl group to the region in which splittings are no longer individually resolved, the so-called "plateau" region; and second, by integrating this unresolved plateau doublet and fitting by the remaining quadrupolar splittings. In the fitting procedure, a monotonic variation of $S_{C\text{-}^2H}$ in the plateau was assumed, and each of the methylene groups was given the same integrated area. Labeled positions next to the carbonyl were barely detected and as a consequence were not accounted for in the procedure.

RESULTS

Analysis of pure DMPA versus pH and temperature

The influence of the surface charge was monitored by ^{31}P-NMR of aqueous dispersions of DMPA for various pHs (2.9, 3.5, 4.2, 5.0, 7.1, 8.2, and 8.9) and as a function of temperature. Fig. 1 shows selected ^{31}P-NMR spectra at various pHs and temperatures. Fig. 1, *A* and *B*, characterizes samples of extreme pH values in the fluid phase. Spectra are composed of powder patterns and isotropic lines, both spectral features being poorly resolved. The appearance of this isotropic line for these extreme pHs is already noticeable at 37°C, although rather weak (≈1%). Low amounts of isotropic lines are also observed in the high temperature region for pH 8.2 and 7.1. For the other pH values and temperatures, superposition of two axially symmetric powder patterns are observed (Fig. 1, *C–F*). This seems to be a general feature of the DMPA system and is in contrast to what is observed with PC bilayers.

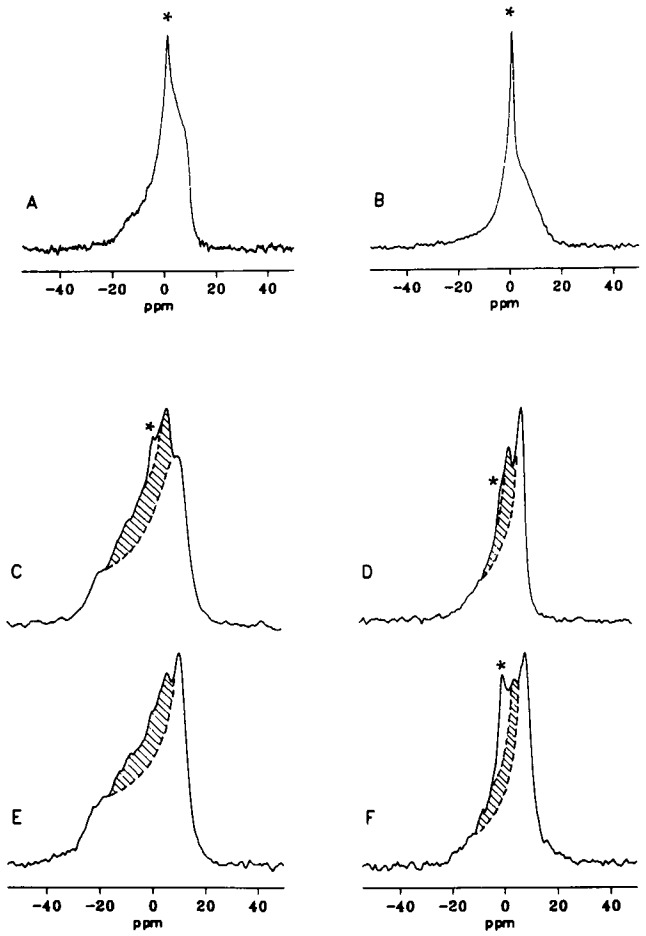

FIGURE 1 Selected ^{31}P-NMR spectra of DMPA membranes at different pH and temperatures. (*A*) and (*B*) show representative spectra with an isotropic compound: A, pH = 2.9, 57°C; B, pH = 8.9, 57°C. (*C–F*) Representative spectra are shown where two well-defined superimposed powder patterns are shown: C, pH = 2.9, 32°C; D, pH = 4.2, 57°C; E, pH = 5.0, 37°C; F, pH = 7.1, 57°C. Asterisks indicate the isotropic subspectra. The smaller powder patterns were shadowed for easier reading of the figure.

To ensure that the observed pH-dependent effects are not caused by a modification of the anisotropic chemical shift tensor, its eigenvalues (σ_{ii}) were determined from powder spectra at pH 3 and 8 obtained at −93°C . In Table 1, σ_{ii} values for dimyristoylphosphatidylcholine (DMPC) and those available for PAs are also reported. The dissimilarity between DMPA and DMPC demonstrates the different environment of the phosphorus atom in these two types of lipids. Although the temperature used in our study (−93°C) is low in comparison with that of Kohler and Klein (1977) and Herzfeld et al. (1978), we cannot exclude the presence of remaining motions around the O-P bond. However, solid state magnetic parameters for DMPA are about the same, within the experimental error, for both acidic and basic pHs. This indicates that the several features detected on spectra are not related to different electronic shielding of the phosphorus nucleus.

TABLE 1 Chemical shielding tensor eigenvalues of the phosphorus nucleus in phosphatidic acids and in phosphatidylcholine systems

	DMPA (pH = 3)	DMPA (pH = 8)	UPA*	DSPA‡	DMPC§
T (°C)	−93	−93	20	0	−163
σ_{xx} (ppm)	−53	−53	−24	−40	−123
σ_{yy} (ppm)	−2	1	−5	−4	−24
σ_{zz} (ppm)	62	62	45	48	147
$\Delta\sigma$ (ppm)	89.5	88	59.5	70	220
η	0.85	0.92	0.48	0.77	0.67

Accuracy in σ_{ii} values is ~±5 ppm.
*From Herzfeld et al., 1978. UPA, urea phosphatidic acid.
‡From Kohler and Klein, 1977.
§From Dufourc et al., 1992.

Experiments with deuterated lipid (^{2}H-NMR) were performed at only two selected pHs (pH 4.2 and 8.2). These pH values were chosen as representative for the acidic and basic milieu and because they are characteristic of regions in which the system is under the form of large bilayer assemblies, i.e., low amounts of isotropic line, whatever the temperature. Fig. 2 *A* shows the powder pattern of *sn*-2-$^2H_{27}$-DMPA for selected temperatures (pH 4.2 and 8.2, *left and right*, respectively). For $T < T_C$, broad unresolved powder patterns are obtained, reflecting a typical gel phase bilayer. Contrariwise, for $T > T_C$ the fluid phase spectra are substantially smaller, and different quadrupolar splittings corresponding to different chain positions of the deuterium labels are nicely resolved. At pH 8.2 and high temperature, a weak amount of isotropic line is observed. Near T_C (42°C) and at pH 4.2, the superposition of a gel and a fluid phase bilayer spectrum is noticeable, whereas at pH 8.2 the membrane is already in the fluid state. In opposition to ^{31}P-spectra, the ^{2}H-spectra are always "homogeneous"; i.e., superposition of two or more anisotropic spectra in the gel or fluid phase was never detected.

Effect of cholesterol on DMPA

As it is known that cholesterol highly modifies the dynamics of the membrane hydrophobic interior, ordering properties were followed in systems in which no isotropic line is observed (vide supra). The influence of 30 mol % cholesterol on the DMPA system was thus investigated at pH 4.2 and 8.2 through ^{31}P- and ^{2}H-NMR.

In Fig. 3, ^{31}P-spectra of the system in the acidic and basic media in the presence (*solid lines*) and absence (*dashed lines*) of the 30 mol % sterol, as well as the DMPC system (data from Léonard, 1993), are presented for comparison. In acidic media, the spectral feature of the PA/cholesterol system is quasi-invariant with temperature and reflects fast axial rotation. The line width for $T < T_C$ is drastically reduced in comparison with the cholesterol-free system, and the second powder pattern almost completely disappears at high temperatures for pure DMPA. This is not the case at pH 8.2 where the presence of the two powder patterns is not

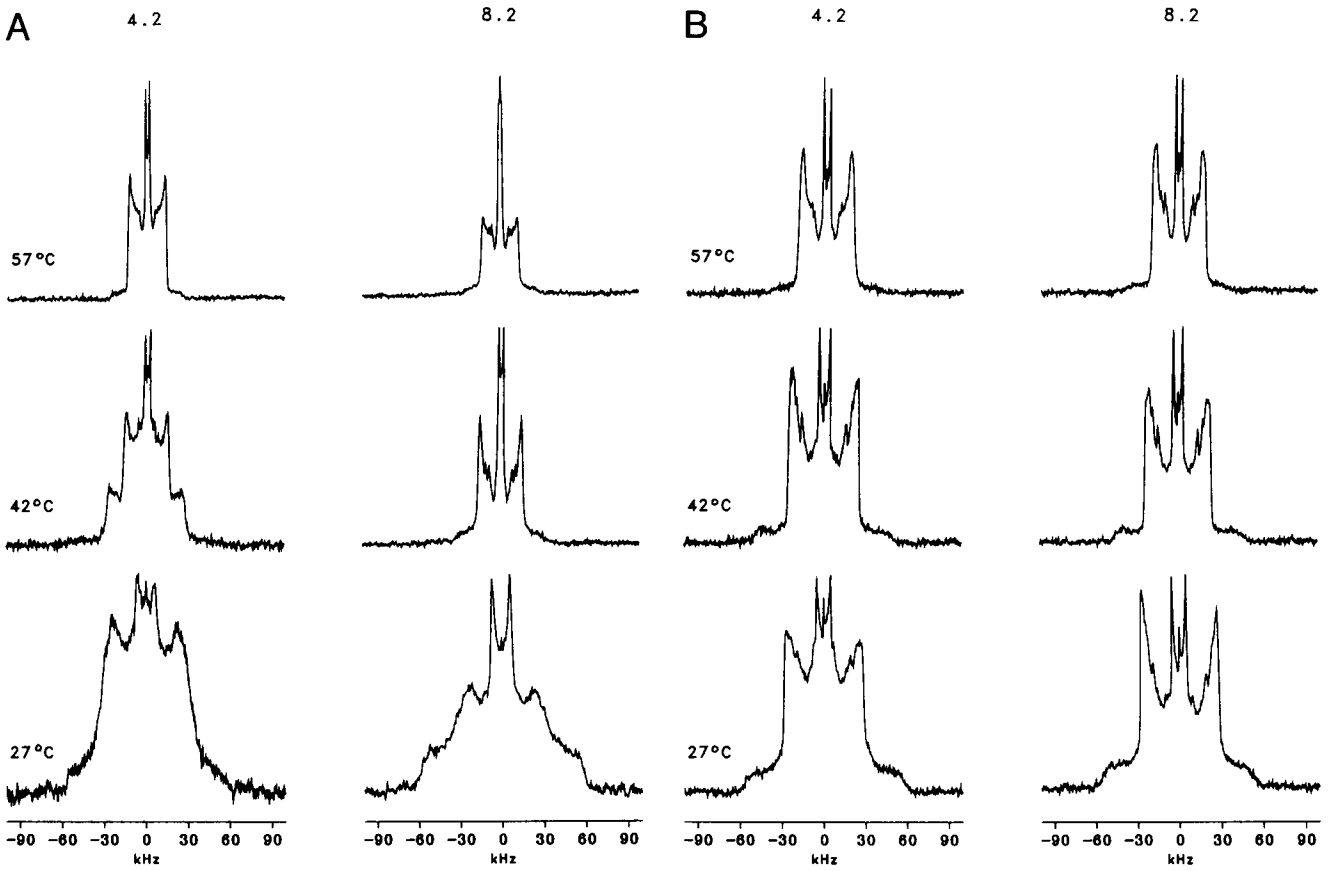

FIGURE 2 Representative ^{2}H-NMR spectra of [*sn*-2-$^2H_{27}$]-DMPA bilayers at pH = 4.2 (*left*) and pH = 8.2 (*right*) in the gel phase (*bottom*), near the gel-to-fluid transition of the pure system (*middle*), and in the fluid phase (*top*). (*A*) Cholesterol-free systems and (*B*) 30 mol % cholesterol-containing bilayers are shown.

affected by the sterol. At this pH there are only weak spectral differences between sterol-containing and free systems, and the presence of cholesterol does not inhibit the appearance of a small isotropic line at high temperatures. In general, the chemical shift anisotropy seems to be only slightly modified (if at all) by the sterol, which is in contrast to PC model membranes. In the case of ^{2}H-NMR spectra, the presence of 30 mol % cholesterol (Fig. 2 *B*) results in smaller spectral variations in temperature compared with the pure system. Typical gel phase spectra are no longer obtained, and at high temperature, spectra are wider than those observed with pure DMPA membranes. Differences between both media are rather small if spectra at pH 4.2 are compared with those at pH 8.2 at a 5°C higher temperature.

A more detailed analysis of the effect of cholesterol might be given by examination of the first (M_1) and second (M_2) spectral moments in the case of ^{2}H- and ^{31}P-NMR, respectively. For comparison, M_2 values of DMPC and DMPC/(30 mol %)cholesterol membranes are also shown in Fig. 4 (from Léonard, 1993). The M_2 values for the PC system are markedly higher than those for PA. For all pure systems, M_2 values for $T < T_C$ are elevated and M_2 values at about T_C are decreased, reflecting the onset of the gel-to-fluid phase transition. For $T > T_C$, M_2 is low and weakly affected by changes in temperature. At pH 4.2 (Fig. 4 *B*) the decrease of M_2 at about T_C is well defined but covers a wide temperature range (from ≈37° to ≈52°C). Passing from acidic to basic medium (Fig. 4 *C*), the fall-off of M_2 is more abrupt and occurs at lower temperature. For the acidic model membrane, the presence of cholesterol affects the system in such a way that the evolution of M_2 with temperature is described by a straight line, exhibiting only a slight decrease with increasing temperature. At first approximation, a linear decrease of M_2 with temperature is also observed at basic pH, but with a steeper slope. Furthermore, for both cholesterol-containing media the variation of M_2 versus temperature crosses that of the sterol-free systems. This behavior is in contrast to what is found for PC systems in which M_2 in the presence of cholesterol and for $T > T_C$ is about the same (DMPC, Fig. 4 *A*) or remarkably lower (DPPC, data not shown) than when in its presence. In the case of ^{2}H-spectra, the presence of cholesterol results in a decrease of M_1 for $T < T_C$ and an increase for $T > T_C$ (Fig. 4, *E* and *F*). In contrast to DMPC membranes (Fig. 4 *D*), the incorporation of 30 mol % cholesterol does not result in an almost linear decrease of M_1 with increasing temperature, but is similar to that of the DPPC/cholesterol system containing 15–20 mol % cholesterol (Vist and Davis, 1990) or

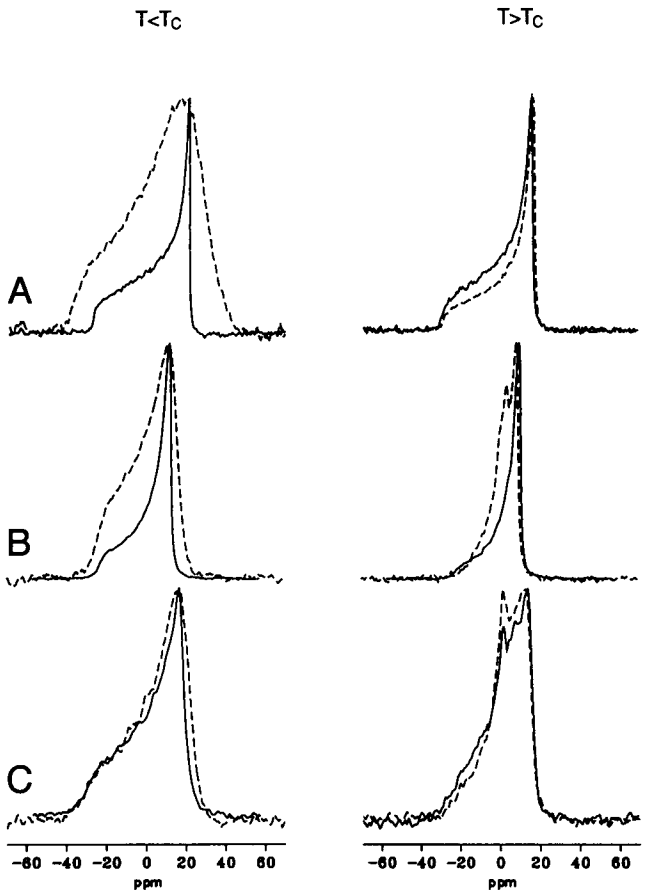

FIGURE 3 Superpositions of ^{31}P-NMR spectra of cholesterol-free (- - -) and 30 mol% cholesterol-containing (——) membrane systems at $T \approx T_C - 17°C$ (*left*) and $T \approx T_C + 17°C$ (*right*). (*A*) DMPC, (*B*) DMPA pH = 4.2, and (*C*) DMPA pH = 8.2. DMPC data are taken from Léonard, 1993.

that of DMPC/cholesterol model membranes containing 10–20 mol % sterol (Fig. 4 *D*) (Léonard and Dufourc, 1991).

To test the complete miscibility of the sterol in the systems studied herein, ^{2}H-NMR was performed on DMPA at pH 8.2 containing 30 mol % ^{2}H-labeled cholesterol. The labeled sterol leads to a typical axially symmetric bilayer powder pattern; no isotropic lines were detected at any temperature (data not shown). The molecular order parameter was calculated according to Taylor et al. (1981) and Dufourc et al. (1984) and led to S_{mol} values decreasing from 0.91 at 22°C to 0.81 at 62°C. By evaluating the changes in cholesterol orientation in the bilayer according to the above authors, the cholesterol orientation stays invariant and perpendicular to the bilayer surface within the measured temperature range.

Fig. 5 shows the smoothed order profiles of DMPA at both pHs in the presence and absence of cholesterol at 57°C. For pure systems the order parameters ($S_{C\text{-}^2H}$) of the plateau region (C-3 to C-9/10), as well as $S_{C\text{-}^2H}$ of the methyl group, are similar. Between these positions, $S_{C\text{-}^2H}$ is lowered when pH increases, and the plateau region seems to be less extended. Nonetheless the transition temperature for pure systems is not exactly the same. In the presence of cholesterol, differences in $S_{C\text{-}^2H}$ for positions near the bilayer core are no longer evident, whereas at the plateau level values of $S_{C\text{-}^2H}$ are slightly but significantly lower at pH 8.2 than at pH 4.2. For comparison, order parameters for the plateau positions are also reported for pure DMPC and DMPC/30 mol % cholesterol at 40°C (Léonard and Dufourc, 1991), i.e., $T \approx T_C + 17°C$. It is noteworthy that the increase in $S_{C\text{-}^2H}$ caused by 30 mol % cholesterol is not equivalent to that promoted by the same amount of cholesterol in PC model membranes. For both DMPA systems, 30 mol % sterol causes a ~40% increase in the order parameter of the plateau region at 57°C, i.e., at $T \approx T_C$ + (15–20°C), whereas the same cholesterol concentration leads to an increase in $S_{C\text{-}^2H}^{plateau}$ of ~80% for DMPC at about the same temperature relative to T_C (40°C, Fig. 5). T_C is taken as the transition temperature of pure lipid systems. Lower sterol concentrations of 20 and 10 mol % lead to an increase in $S_{C\text{-}^2H}^{plateau}$ of ≈50% and 20% in the DMPC cholesterol system, respectively (Léonard and Dufourc, 1991). As a result of the M_2 analysis as well as the cholesterol-induced effects on the order profile, the effect of 30 mol % cholesterol on DMPA is equivalent to that of 10–20 mol % on PC membranes, as far as the ordering properties of the membrane are concerned.

DISCUSSION

It has been shown herein that the surface charge density, related to the ionization state of PA, modulates the transition temperature of the system, the ordering properties of the bilayer core, and most important, the structural and dynamical properties of the phosphate headgroup. One of our major findings is that the phosphate headgroup senses up to three different magnetic environments as a function of pH, whereas the chains do not. The incorporation of 30 mol % cholesterol does not cause large variations as far as the ^{31}P-NMR observables are concerned, but alters the dynamical properties at the chain level. All of these properties will be discussed in what follows.

pH-dependence of DMPA thermotropism and vesicle formation

The first moment of ^{2}H-NMR spectra of chain deuterated lipids is usually pictured as a good tool to determine T_C because it directly reflects lipid chain motions. Transition temperatures estimated from M_1 are at ~42° and 37°C at pH 4.2 and 8.2, respectively, which differs from what is reported by van Dijck et al. (1978) (52° and 42°C in 0.1 M NaCl, respectively). This may be attributable to an isotope effect on T_C because shifts in the transition temperature to lower temperatures for deuterated lipids have been reported to be of the order of a few degrees Celsius (Reinl et al., 1992). To account for the elevated transition temperatures

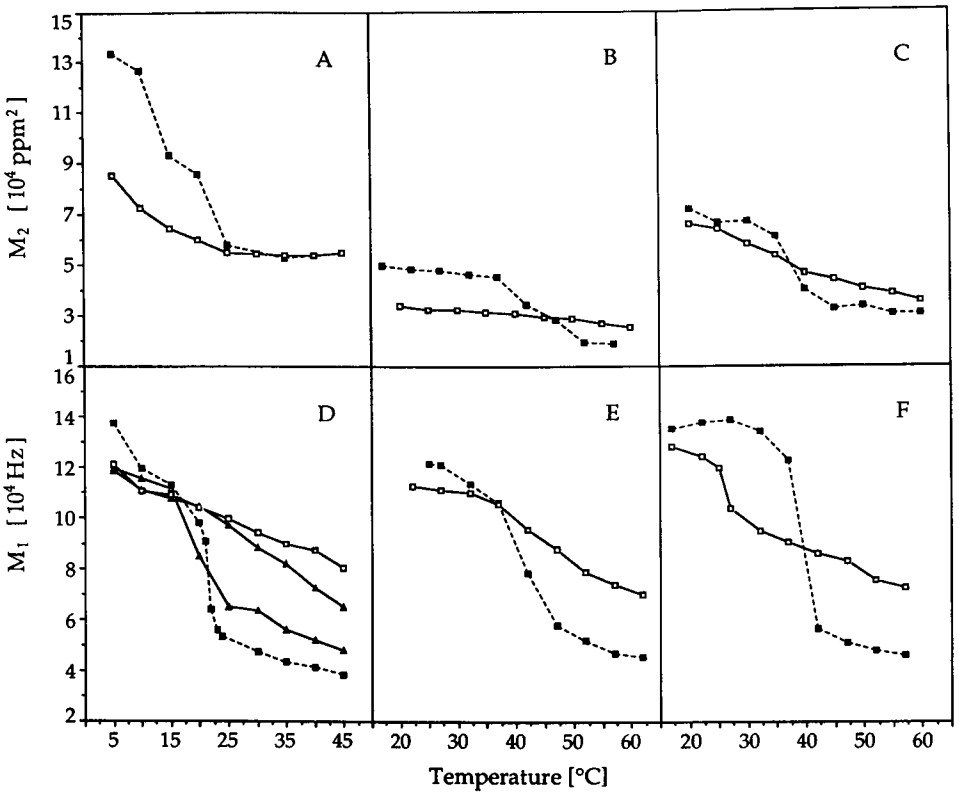

FIGURE 4 Second (*top*) and first moments (*bottom*) of ^{31}P- and ^{2}H-NMR spectra, respectively, as a function of temperature for lipid dispersions in the presence (*open symbols*) and absence of 30 mol % cholesterol (*filled symbols*). (*A*) and (*D*) DMPC values for 10 (△) and 20 mol % (▲) sterol are also presented in (*D*); (*B*) and (*E*) DMPA, pH = 4.2; (*C*) and (*F*) DMPA, pH = 8.2. DMPC data are taken from Léonard, 1993.

of DMPA when compared with DMPC, it is necessary to include intermolecular hydrogen bonding forces between lipid headgroups in order to account for the high transition temperature of PA-lipids in the pH range of 4–9 (Eibl and Woolley, 1979; Berde et al., 1980; for an overview see Boggs, 1987). However, remarkable differences between $\Delta\sigma$ values in the polycrystalline sample at very low temperatures ($\Delta\sigma$ = 88–90 ppm, η = 0.85–0.92) and in the gel phase ($\Delta\sigma$ = 20–60 ppm, η = 0) suggest that lipid headgroups exhibit fast rotation in that phase in a way similar to what has been reported for DMPC (Dufourc et al., 1992). Consequently, such intermolecular hydrogen bonds cannot be long-lived and should be pictured as "dynamic" rather than "rigid"; i.e., they are continuously broken and reformed (Boggs, 1987).

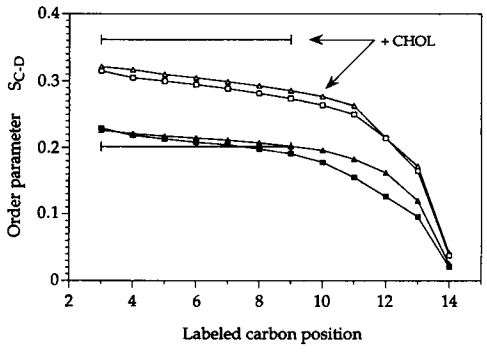

FIGURE 5 Representation of the smoothed order profiles of DMPA dispersions at pH = 4.2 (▲,) and pH = 8.2 (■,) at 57°C in the presence (*open symbols*) and absence (*filled symbols*) of 30 mol % cholesterol. For comparison, the plateau positions of DMPC at 40°C in the absence and presence of 30 mol % cholesterol are also shown (*lines, no symbols*).

The transition occurring at pH 4.2 is clearly broadened. Such a broadening of the transition (i.e., the loss in cooperativity) has already been reported for DMPA in the presence of 0.1 M NaCl solution (Blume and Eibl, 1979); it seems to be important between pH 3.3 and 5.8. In addition, these authors (in reference to the work of Träuble et al., 1976, and Jähnig et al., 1979) report that the first pK changes during the transition lead to a special broadening of the transition.

The appearance of an isotropic line in the basic or acidic media can be explained by the formation of cubic structures (Lindblom and Rilfors, 1989), discs (Dufourc et al., 1986), or vesicles of a size less than 2000 Å (Burnell et al., 1980). In the case of DMPA, spontaneous formation of small vesicles has been reported in the basic medium (Hauser, 1989) and can be explained by formation of pH gradients (Hauser et al., 1983, 1986; Hauser, 1989). Because the molecular area of DMPA increases with the increasing ionization state (Jähnig et al., 1979), the addition of buffer might generate a pH gradient across the membrane, i.e., an asymmetric distribution of lipids with different headgroup size on both sides of the model membrane. Such an asymmetric distribution may also be the result of different electrostatic repulsion forces between the charged DMPA species (Israelachvili, 1973). This obviously results in curvature of the membrane and thus in the formation of small vesicles. Because we also obtained an isotropic line of

comparable line shape in the acidic medium, we believe that for sample preparations in extreme pH conditions (pH <3 and pH >8), pH gradients may be generated that would induce membrane asymmetry and then highly curved surfaces, as in small vesicles. As seen in Fig. 1, *A* and *B*, the spectra cannot simply be decomposed into a single isotropic line of defined width plus a powder pattern, but rather can be decomposed into a distribution of isotropic lines of variable width superimposed on a powder pattern. This peculiar line shape can be accounted for by a size distribution of small vesicles (Douliez et al., 1994).

Detection of the three ionization states of DMPA by solid state ^{31}P-NMR

Regarding the superposition of different well defined powder patterns (Fig. 1, *C–F*), it must be emphasized that such axially symmetric sharp line shapes cannot be obtained by tumbling of vesicles that have different sizes. It has indeed been clearly demonstrated (Burnell et al., 1980) that NMR lines caused by vesicle tumbling become broader and more symmetric about the carrier frequency than those shown in Fig. 1, *C–F*.

In order to analyze the observed superposition of powder patterns, ^{31}P-spectra were dePaked, and the $\Delta\sigma$ values of the different powder patterns were determined. In the pH range 2.9–8.9 and at a given temperature, we found three values of $\Delta\sigma$. Whatever the pH, the superposition always consisted of up to two (and possibly three in the case of pH 5, $T > T_C$) of these powder patterns, with varying amounts. In Fig. 6, all different $\Delta\sigma$ values are plotted as a function of temperature without differentiation of the pH. The chemical shift anisotropy varies weakly with temperature and presents steps in accordance with the transition temperatures of the systems. In relation to the three dissociation states of PA-lipids, we were tempted to correlate the three observed $\Delta\sigma$ values with the charge borne by the lipid molecule, i.e., a

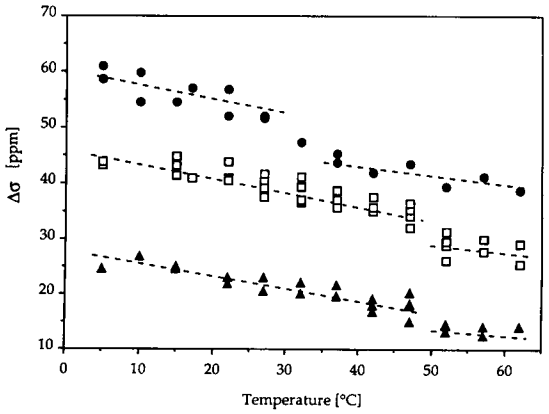

FIGURE 6 Values of the ^{31}P-NMR chemical shift anisotropys, $\Delta\sigma$, of the pure DMPA systems for all investigated pHs as a function of temperature. DMPA molecule bearing zero (▲), one (□), and two (●) negative charges (see text). Dashed lines are drawn for easier reading of the figure.

value of $\Delta\sigma_{gel}$, $\Delta\sigma_{fluid}$ of ~22.5 ± 2.5 ppm, 13.5 ± 1 ppm for the uncharged species; 39.5 ± 4.5 ppm, 27.7 ± 2.3 ppm for the lipid bearing one negative charge; and 56.5 ± 4.5 ppm, 41.8 ± 3.2 ppm for the completely deprotonated molecule. The decrease in $\Delta\sigma$ values with decreasing pH is in agreement with an earlier study (Cullis and De Kruijff, 1976), although these authors discussed neither the appearance of a second ^{31}P-environment in their spectra with smaller $\Delta\sigma$ values nor the presence of a phase undergoing fast isotropic tumbling in the basic medium.

The association of protonation state with $\Delta\sigma$ values implies that proton exchange between phosphate groups in the layer or between phosphate groups and the bulk has to be slow on the NMR-time scale, i.e., in the range of milliseconds. It is interesting here to relate our study to that of Nachliel and Gutman (1988) who measured proton transfer rates between the bulk and the phospholipid surface of PC, phosphatidylserine (PS), and PA lipids. In their analysis they were able to calculate the rate constants, k_j, for protonation or deprotonation of the various species. Hence it is possible to calculate the residence time, τ_D, of a proton on a given site ($\tau_D = \{k_j \cdot 10^{pK_a}\}^{-1}$). The authors report τ_D values for protons on a PC and PS lipid to be $\tau_D(PC) \approx 30$ ns and $\tau_D(PS) \approx 4$ μs. From their data $\tau_D(PA) \approx 30$ ms can be calculated by taking $pK_a = 8$ and $\tau_D(PA) \approx 10$ μs for $pK_a = 3.5$. If the slower residence time is the limiting factor in all proton exchanges occurring between the bulk and the surface, proton exchange in PA systems is clearly slow on the ^{31}P-NMR time scale. In other words, NMR is able to detect different charge states at the membrane surface through variations in the chemical shielding anisotropy of the phosphorus nucleus. In this context, it is noteworthy that there may be cases in which this exchange becomes fast on the NMR-time scale when triggered by temperature and a positively charged membrane effector like melittin (T. Pott, J. C. Maillet, C. Abad, A. Campos, J. Dufourcq, and E. J. Dufourc, unpublished data). This demonstrates further that the proton diffusion on PA-membranes, or on phospholipid bilayers in general, can be very sensitive to surface active matter.

All of the following will be discussed under the assumption that the correlation between the different ^{31}P-powder patterns and the lipid charge made above is valid. Several explanations are possible to account for the decrease in $\Delta\sigma$ values with the increasing protonation state of the phosphate group: 1) a change in the principal values of the chemical shift anisotropy tensor attributable to a change in the chemical shielding with modification of the phosphate group protonation; 2) changes in the orientation of the phosphate group with respect to the bilayer normal for different dissociation states; and 3) electrostatically induced changes in the motional freedom of the headgroup of the lipid. Additional effects, such as modification of the motion of the entire molecule caused by a change in the net surface charge density, can be excluded because this implies cluster formation with different charge densities and would cause different ^{2}H-NMR spectra (as in a phase separation), which

is not observed. The principal values of the DMPA chemical shielding tensor are close, within the experimental error, at pH 3 and 8 (Table 1); therefore, the first hypothesis can be excluded. In addition, P-OH bonds in urea phosphatidic acid are 1.55–1.56 Å (Herzfeld et al., 1978), whereas PO^- bonds measure 1.54 Å in the ion (Kohler and Klein, 1976). Because a linear correlation exists between the principal values of the ^{31}P chemical shift tensor and the P-O bond length (Un and Klein, 1989), we believe that the small variations in the P-O bond length on protonation or deprotonation will cause small modifications, if any, in the principle tensor values of the H_2PO_4, $H_2PO_4^-$, and PO_4^{2-} groups of DMPA in its three different ionization states.

Regarding a change in orientation, it has already been demonstrated that the membrane surface charge density induces conformational changes of the PC headgroup (Sixl and Watts, 1983; Seelig et al., 1987; Scherer and Seelig, 1989). As a result, PCs are already described as behaving like "molecular voltmeters." Charge-induced conformational changes have also been reported for PS (Roux et al., 1989; De Kroon et al., 1991) and PG (Marassi and Macdonald, 1991). For PC, the orientational change is believed to involve an alignment of the P^--N^+ dipole of the headgroup under the influence of the electrostatic field emanating from the membrane surface. It was suggested that this argument may also be valid for anionic lipids, when considering the P^--M^+ dipole of their metal-ion-associated form (Tocanne and Teissié, 1990). This is certainly true for PA in the anhydrous crystal, where different headgroup packings have been observed as a function of the lipid ionization state (Pascher et al., 1992). However, the existence of such a regular phosphate-Na^+ network in the aqueous phase remains questionable. Moreover, in the case of DMPA bearing different charges, such a (conformational) change cannot be induced by a net surface charge density but has to be intramolecularly induced. From the above it seems that explanation 2 should not apply to the DMPA headgroup. Nevertheless, one cannot totally exclude conformational changes of the phosphate with respect to the bilayer normal.

Alternatively, one may account for the different $\Delta\sigma$ values for each charged species by invoking electrostatic modulations of the headgroup dynamics (explanation 3) and make use of the well known intramolecular dynamics of the DMPC headgroup, where three local motions are used to describe the reorientation of the phosphocholine moiety. Two hindered rotations of very high frequency ($\geq 10^8$–10^9Hz) are around the glycerol C-C and C-O bonds adjacent to the phosphate, and a free rotation (10^4–10^9Hz) is around the subsequent O-P bond. In the case of DMPA, it can be assumed that there are still the same intramolecular motions and that electrostatic constraints (zero, one, or two charges on the phosphate) affect the hindered rotations so that the average of the residual chemical shift anisotropy is different for each of the charged species. Furthermore, the different $\Delta\sigma$ values observed in the gel phase undergo a parallel decrease as the temperature increases. The proportional drop-down for each $\Delta\sigma$ value (11–12%) (Fig. 6) at the onset of the phase transition can be related to the appearance of molecular motions (rotation and wobbling) by analogy with DMPC (Dufourc et al., 1992).

To summarize, in the temperature range of our study, the intramolecular constrains would determine the differences in $\Delta\sigma$ values for each species, and the temperature-driven modifications of the ^{31}P-NMR observables would be attributable only to the onset of molecular motions.

pK estimation from solid state ^{31}P-NMR

Unfortunately it seems to be impossible to determine with satisfying accuracy the percentages of zero, one, and two charged lipids via solid state ^{31}P-NMR. Therefore only a qualitative analysis concerning the variation of each of the PA species as a function of temperature and pH will be given. As already mentioned, pK values for PA vary significantly according to different studies and different methods. This is because of the difference between the behavior of an ionizable group when in true solution or when located at an interfacial plane, like charged lipid bilayers. Interfacial pK_i values cannot be determined directly because the interfacial pH might be quite different from the bulk pH, i.e., the measurable pH. Yet the bulk pH only provides the determination of the apparent pK_a. Intrinsic and apparent pK values can be correlated for a given surface charge density via the electrostatic surface potential (Tocanne and Teissié, 1990). Although in our study evaluation of the surface charge density is not possible because of the reasons mentioned above, we report in Fig. 7 the schematic evolution of the ionization state versus pH in the gel and the fluid phase of the DMPA molecules. The criterion in building Fig. 7 was simply the detection of a charge-associated ^{31}P-envi-

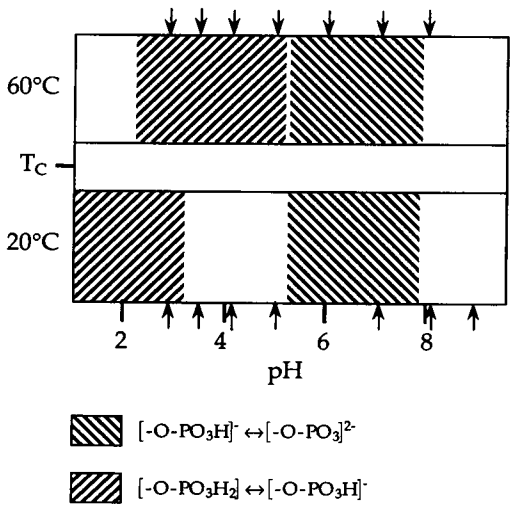

FIGURE 7 Schematic representation of the ionization state of DMPA model membranes in fluid and gel phase as estimated by solid state ^{31}P-NMR (see text). Arrows indicate the investigated pHs. For basic media, the temperature dependence of the Tris buffer was taken into account.

ronment. Although it was obtained by using rough estimates for the amount of each of the species, the fact that for the first pK one clearly finds $pK_a(gel) < pK_a(fluid)$ is beyond any experimental error, whereas for the second pK_a no variations are detected on phase transition. The result for the first pK_a is at variance with what is usually known on phase transitions; on going to fluid phases, there is a surface expansion and therefore a concomitant decrease of the amount of charges per unit area. This is the usual explanation of the well reported increase in pK_a (or pK_i) for negatively charged lipids on going from gel to fluid phases (for a review, see Table 1 in Tocanne and Teissié, 1990). However, in the same review, it is stated that for DMPA, $pK_i(gel) = 2$ whereas $pK_i(fluid) = 2.3$ (Copeland and Andersen, 1982).

For a full understanding of these behaviors, it is important to realize that pK_a and pK_i depend on temperature (T), electrostatic surface potential at the interface (ψ_0), water dielectric constant (ϵ), concentration of ions (C_b), and surface charge density (σ) (Tocanne and Teissié, 1990). When a phase transition occurs, the only two variable parameters are σ and ϵ; the possible contributions to a pK shift may be of an electrostatic and a polarity (interfacial dielectric constant) nature. In what follows we will show that hydrogen bonding may also play an important role.

In general, σ decreases and it is therefore expected that ψ_0 diminishes; i.e., it becomes less negative and hence $pK_a(gel) > pK_a(fluid)$ as observed for PS and PG. However, as computed by Tocanne and Teissié (1990), phase expansion ($|\sigma_{gel}| > |\sigma_{fluid}|$) accounts for only 20% of the observed change in pK between gel and fluid phases. The water dielectric constant apparently plays a major role in the ΔpK = pK(fluid)-pK(gel) associated with the transition. Indeed, ϵ depends on the precise part of the interface layer on the chemical structure and hydration of the lipid headgroup, on the lipid molecular packing, and on the nature and concentration of ions at the interface region. For usually negatively charged lipids (PS, PG), ΔpK_a and ΔpK_i are negative (Tocanne and Teissié, 1990) because $\sigma_{gel} < \sigma_{fluid}$ (taking into account the negative sign of σ) and $\epsilon_{fluid} > \epsilon_{gel}$ (Kimura and Ikegami, 1985) (DPPC values). As already mentioned, it has been reported for PA that $\Delta pK_i = 0.3$. Because it is likely that $\sigma_{gel} < \sigma_{fluid}$ even for PA lipids, the only way to obtain $pK_a(gel) < pK_a(fluid)$ is to have $\epsilon_{fluid} < \epsilon_{gel}$, i.e., the water dielectric constant of the gel phase greater than that of the fluid phase. Unfortunately there are no data on PA lipids concerning water dielectric constants. However, at pH 4 (in the region of the first pK) Boggs (1987) reports that most of the negatively charged molecules are involved in continuously broken and reformed hydrogen bonds. A maximum of hydrogen bonding at pH $\approx$4 leads to a stabilization of the lamellar gel phase (elevated transition temperatures). The network of water molecules stabilized in the gel phase by hydrogen bonding may thus be responsible for an elevated water dielectric constant in the gel phase and therefore may lead to the observed positive ΔpK_a. However, both ϵ and σ must be measured on PA lipids in order to settle the question.

Effect of cholesterol on the DMPA headgroup

As already described, the influence of cholesterol on the effective chemical shift anisotropy in the case of DMPA seems to be quite small. To quantify this, simulations have been carried out. For DMPA at pH 4.2 and $T < T_C$ (Fig. 3), the spectra exhibit at first glance the maximum of cholesterol influence. Simulation of such spectra in the presence and absence of the sterol shows that they can be fitted with approximately the same $\Delta\sigma$ value, assuming only line-width changes. In the basic medium it seems obvious from the spectra that for $T < T_C$, $\Delta\sigma$ values are about the same. This is also valid for $T \geq T_C$ and whatever the pH. The changes in line width observed in acidic conditions and low temperatures may be correlated with modification of intermolecular motions. In mixtures of DMPC and cholesterol, the sterol increases the frequency of lipid rotation and wobbling at low temperatures (Weisz et al., 1992). The sterol-induced decrease in line width for DMPA may thus be correlated with an increase in the rate of low frequency motions.

Although the various phosphate environments detected on pure systems at pH 8.2 are still observed in the presence of cholesterol, the disappearance of the zero-charge powder pattern for the DMPA/cholesterol system at pH 4.2 and $T <$ 55°C (at 60°C the zero-charge pattern is just detectable) can be explained by a change in the pK_a, inasmuch as a change in the lateral packing leads to a change of both the surface charge density and the water dielectric constant. The presence of cholesterol can be expected to lead to a pK_a intermediate between that of the pure DMPA fluid and the gel phase, showing some temperature effects nevertheless. This is consistent with the appearance of the zero-charge pattern with increasing temperature for the pure system at pH 4.2 and its almost complete disappearance in the sterol-containing membrane. It emphasizes again that the first pK is sensitive to changes in the lateral packing, which has already been reported elsewhere (Copeland and Andersen, 1982). For the basic medium, such an effect seems not to exist, which is in accordance with the study mentioned above.

Influence of pH and cholesterol on the chain level

Values of the first moment (Fig. 4) barely differ between the pure DMPA bilayers in the basic and acidic media and are even comparable with those of DMPC for temperatures relative to T_C. However, the gel-to-fluid transition at pH 4.2 seems to be of lower cooperativity when compared with pH 8.2 or DMPC. Concerning the order profiles (Fig. 5), note that the order of the methylene segments near the end of the labeled chain is decreased at pH 8.2 in comparison with pH 4.2. The differences are quite small compared with those observed at the headgroup level, demonstrating that even

strong electrostatic modifications in the headgroup region do not extend very far into the hydrophobic region of the bilayer.

The action of cholesterol is easily explained by its "special ordering" effect; i.e., the lateral interactions of the lipid molecules are removed. As cholesterol perturbs the hexagonal packing of the lipids in the gel phase, there is fast long-axis rotation of the molecules, and the acyl chains are in some intermediate state with respect to gel and liquid-crystalline phases of pure lipids (Chapman and Wallach, 1968; Wittebort et al., 1982). These effects are less pronounced for DMPA in comparison with PC bilayers and are comparable with a cholesterol incorporation lower than 20 mol % for PC bilayers. The thermal evolution of the first moment for both PA systems in the presence of cholesterol still indicates the existence of a kind of phase transition, but the cooperativity of the transition between the gel- and the fluid-like state is strongly reduced. This is especially obvious with regard to the low temperature spectra ($T \leq 15°C$; data not shown), which are gel-like. This behavior has been described as a second order transition, occurring at cholesterol concentrations higher than 20 mol % (Blume and Hillmann, 1986). At this point, the 30 mol % cholesterol is completely incorporated into the DMPA-bilayer, inasmuch as no isotropic lines in the [2,2′,3,4,4′,6-$^2H_6$]-cholesterol spectra were detected. It is interesting to compare the cholesterol order parameter in DMPA with that obtained on DMPC (Dufourc et al., 1984; Léonard and Dufourc, 1991); similar values are found, indicating that the molecular ordering of this molecule seems not to depend on the membrane nature. The fact that the experiment with labeled sterol has been carried out only for the basic medium should not restrict the conclusion, inasmuch as it has been shown that cholesterol influences the chain level in a manner that is similar for basic and acidic media.

Thus we conclude that the presence of cholesterol causes no modification on the phosphate moiety but affects the acyl chain packing in a way similar to what has been observed for PC lipids. This is somewhat surprising; although the sterol does not extend into the phosphate region, one expects that it acts as a spacer, facilitating the headgroup rotation. Assuming that in the cholesterol-free PA system no sterical hindrances (but electrostatical ones) between the headgroup already exist, these may not be strong enough to be affected by the headgroup "dilution" by the sterol.

Concerning the biological relevance of the cholesterol incorporation, it is interesting to calculate the bilayer hydrophobic thickness (d_h) in the presence and absence of cholesterol and compare it with the DMPC system. d_h was calculated according to Seelig and Seelig (1974) and Schindler and Seelig (1975) by $d_h = d_0\ (\langle|S_{C\text{-}^2H}|\rangle + 0.5)$, where the average chain order parameter $\langle|S_{C\text{-}^2H}|\rangle$ was determined from the first moment by $\langle|S_{C\text{-}^2H}|\rangle = \sqrt{3}\ M_1/\pi A_Q$ (Bloom et al., 1978; Davis, 1979), and d_0 stands for the bilayer thickness for chains in all *trans*-conformation (d_0 = 34.4 Å for myristoyl chains). The result is presented in Fig. 8. For the pure systems, the d_h for the two DMPA membranes for same temperatures relative to T_C are quasi-identical and even the DMPC bilayers do not differ much for such relative temperatures, which is in accordance with the so-called chain-length scaling (Morrow and Lu, 1991). On the other hand, at the same absolute temperature the DMPC bilayer has a hydrophobic thickness 1.5 Å lower than that of the DMPAs. The incorporation of 30 mol % sterol diminishes this difference, especially in the high temperature region. As a consequence, the sterol-caused increase in d_h is somewhat less important for the DMPA system than for the DMPC. This effect is further pronounced when the systems are compared relative to their transition temperatures.

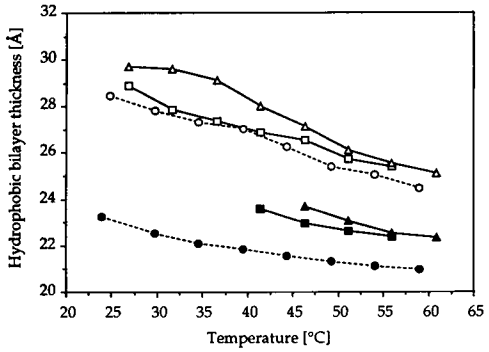

FIGURE 8 Thermal variation of the bilayer hydrophobic thickness as evaluated from ^{2}H-NMR spectra (see text) in the presence (*open symbols*) and absence (*filled symbols*) of 30 mol % cholesterol: ● - - - ●, ○ - - - ○, DMPC; ▲ and △, DMPA pH = 4,2; ■ and □, DMPA pH = 8.2. DMPC data are taken from Léonard, 1993.

In addition, it may be speculated that DMPA causes a sterically unfavorable environment for the cholesterol molecule. At this point one has to keep in mind the difference in the crystal structure of PA with respect to other phospholipids. In contrast to lipids, such as PC, PS, and phosphatidylethanolamine, the glycerol backbone of PA is found to be almost parallel to the bilayer normal (Harlos et al., 1984; Pascher et al., 1992). This results in an important conformational derivation: the bend in one of the hydrocarbon chains, which in molecules with layer perpendicular glycerol orientations occurs at C-2 of the *sn*-2-chain, is shifted to C-2 of the *sn*-1-chain. Assuming that this conformation is also valid for the fully hydrated bilayer and because for PC membranes it has been shown that the cholesterol molecule is located so that its hydroxyl group is in the immediate vicinity of the phospholipids *sn*-2-carbonyl group, it may be asked where the hydroxyl group of the sterols is located in the PA bilayer and whether a displacement of cholesterol with respect to the bilayers surface when compared to PCs is responsible for its lower effectiveness.

CONCLUSION

As a result of the slow proton hopping on the NMR time scale, solid state ^{31}P-NMR is able to discern the different

charged species of DMPA by taking advantage of the distinct chemical shielding interactions for the three protonation states of the PA headgroup. Although the strong changes in the surface charge density locally affect the DMPA headgroup and modify their influence on the gel-to-fluid transition temperature, the order of the hydrophobic interior of the bilayer is not markedly affected. On the other hand, strong variations of the chain packing, as they occur because of the phase transition, can result in an alteration of the pK_a. This is also reflected by the influence of cholesterol. Although it acts locally on the lipid acyl chains, this modulation of the lipid packing properties affects the charged surface. The finding that hydrophobic interactions are able to govern the electrostatic properties of the membrane may be of important biological relevance.

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