Eur Biophys J (1996) 24: 423–431

ARTICLE

Radoslav Marinov · Erick J. Dufourc

Thermotropism and hydration properties of POPE and POPE-cholesterol systems as revealed by solid state ^2H and ^{31}P-NMR

Received: 6 February 1996 / Accepted: 25 March 1996

Abstract The partial phase diagram and the hydration properties of the 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE)-water system, in the absence and presence of 30 mol% cholesterol, have been investigated by solid state phosphorus NMR of the lipid and deuterium NMR of heavy water. The POPE-D_2O phase diagram resembles other phosphatidylethanolamine (PE)-water systems: below water-to-lipid molar ratios (R_i) of 3 the lamellar gel (L_β or L_c)-to-hexagonal type II (H_{II}) phase sequence is observed on increasing the temperature. For $R_i \geq 3$ the thermotropic sequence (L_β or L_c)-L_α-H_{II} is detected. On increasing hydration from $R_i = 3$, the H_{II} phase is detected from 40 °C to 85 °C whereas the gel phase is observed from 40 °C to 30 °C. The limiting hydrations of the gel, L_α and H_{II} phases are $R_i \approx 3$, 17 and 20, respectively. The number of bound water molecules per lipid is ca. 8 in both the L_α and H_{II} phases. The presence of cholesterol stabilizes the hexagonal phase 20 °C below temperatures at which it is observed in its absence and reduces the limiting hydration of the fluid and hexagonal phases to $R_i \approx 9$ and 14, respectively. The structure and/or dynamics of the water bound to the interface are markedly modified on going from the L_α to the H_{II} phase.

Key words Phosphatidylethanolamine · Cholesterol · Phase diagram · Hydration · ^2H and ^{31}P NMR

Abbreviations

NMR, Nuclear magnetic resonance.
DDPE, 1,2-Didodecyl-*rac*-glycerol-3-phosphoethanolamine.
DHPE, 1,2-Dihexadecyl-*sn*-glycerol-3-phosphoethanolamine.
DOPE, 1,2-Dioleoyl-*sn*-glycerol-3-phosphoethanolamine.
POPE, 1-Palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphoethanolamine.
DAPE, 1,2-Diarachinoyl-*sn*-glycerol-3-phosphoethanolamine.
DMPC, 1,2-Dimyristol-*sn*-glycerol-3-phosphocholine.
DPPC, 1,2-Dipalmitoyl-*sn*-glycerol-3-phosphocholine.
T_c, lamellar gel-to-lamellar fluid transition temperature.
T_h, lamellar fluid-to-hexagonal transition temperature.

R. Marinov
Institute of Solid State Physics, Bulgarian Academy of Sciences, Sofia, Bulgaria

E. J. Dufourc (✉)
Centre de Recherche Paul Pascal, CNRS, F-33600 Pessac, France
(fax: (33) 56 84 56 38, email: dufourc@crpp.u-bordeaux.fr)

Introduction

The interactions between water and amphipathic molecules lead to their self-assembling into different structures. The most important structure from a biological point of view is the lamellar phase because of its similarity to cell membranes. Temperature- or concentration-driven phase changes in water-phospholipid systems are of great importance because the different non-lamellar structures (hexagonal, isotropic phases) which then appear can serve as a basis for a mechanistic explanation of biological processes such as fusion, fragmentation or transmembrane transport (Cullis and De Kruijff 1979; Verkleij 1984). The structure and dynamics of water near membrane surfaces is important for transport processes across the membrane, for dynamics of membrane components, for membrane-membrane interactions as well as for interactions of ions and other molecules with membranes.

It is possible to investigate the phase behavior of water-phospholipid dispersions in simple model systems, including only one lipid and water. Such systems have been examined for a long time by means of different experimental methods such as differential scanning calorimetry (Ulmius et al. 1977; Seddon et al. 1984); X-ray diffraction (Blaurock 1982; Seddon et al. 1984; König et al. 1994) and nuclear magnetic resonance (Finer 1973; Laroche et al.

1991; König et al. 1994). Two major phase transitions are observed in water-lipid systems: "gel-to-fluid" and lamellar-non-lamellar transitions. The "gel" state of lipids is a lamellar phase with fully extended and rigidly packed hydrocarbon chains. Molecules in this state can be oriented perpendicularly (L_β phase) or tilted ($L_{\beta'}$ phase) with respect to the bilayer plane. The lamellar "fluid" phase L_α is characterized by hydrocarbon chains with liquid-like flexibility and average orientation of molecules perpendicular to the bilayer surface. The gel-to-fluid phase transition is characterized by a considerable enthalpy, ΔH, change. Much weaker enthalpy changes are detected for lamellar-to-hexagonal (H_I or inverted H_{II}) transitions or lamellar-to-isotropic phases (cubic or rhombic) (Cullis and Kruijff 1978; Gullis and De Kruijff 1979). The transition temperatures and their dependence upon hydration vary for different lipids (Marsh 1990). Small changes (1–3 °C) in L_α-H_{II} phase transition temperatures are observed when exchanging H_2O with D_2O (Gawrisch et al. 1992). Phase behavior may be significantly altered by addition of different proteins, ions or other molecules to pure lipid-water systems (Cullis et al. 1976; Harlos and Eibl 1981; Blaurock 1982; Ellens et al. 1986; Batenburg et al. 1988; Laroche et al. 1991; Gaillard et al. 1992; Killian 1992).

Experimental phase diagrams are obtained in different model or biological membrane systems by changing the temperature and/or composition. Most investigations are concentrated with the "gel-to-fluid" transition. For instance, in the phase diagram of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC)-water a $L_{\beta'}$ phase is observed at temperatures lower than 12 °C and a L_α phase above 23 °C. An intermediate "rippled" phase $P_{\beta'}$ is observed in the temperature range 12–23 °C (Janiak et al. 1976). The same phase sequence is observed in the system 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine (DPPC)-water (Grabielle-Mandelmont and Perron 1983a, b). The existence of isotropic and lamellar phases was demonstrated for egg PC-water (Luzzati 1968) and for DPPC-water (Ulmius et al. 1977). An inverted hexagonal phase was reported for egg PC-water (Luzzati 1968).

Phosphatidylethanolamines (PE) have been found to undergo not only chain melting phase transitions but also transitions to non-lamellar phases (Boggs et al. 1981; Harlos and Eibl 1981; Seddon et al. 1983). The inverted hexagonal phase was also observed in water mixtures of: 1,2-diarachinoyl-*sn*-glycero-3-phosphoethanolamine (DAPE), 1,2-didodecyl-*rac*-glycero-3-phosphoethanolamine (DDPE) (Seddon et al. 1984),1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine and lysophosphatidylcholine (Arvidson et al. 1985; Caffrey 1985); for completeness see Marsh (1990). Interestingly, Gawrisch et al. (1992) obtained a partial phase diagram of the system dioleoylphosphatidylethanolamine (DOPE)-water and measured the trapped water volumes in L_α and H_{II} phases as a function of osmotic pressure.

In the present work we have investigated the phase changes and hydration properties of the 1-palmitoyl-2-oleoyl-*sn*-glycero-3-PE (POPE)-D_2O and POPE-(30 mol%) cholesterol-D_2O systems. Phosphorus-31 NMR (^{31}P-NMR) is a convenient tool for the study phospholipid polymorphism (Cullis and De Kruijff 1978). The phosphate head group exhibits a large chemical shift anisotropy, $\Delta\sigma$, which leads, in the presence of proton decoupling, to characteristic axially symmetric powder patterns which may be used to identify $L_{\beta'}$, L_α and H_{II} phases. For instance, the lipids in the inverted hexagonal H_{II} phase do experience additional motional averaging as compared to those in the lamellar structures because of rapid lateral diffusion of molecules perpendicular to the cylinder axes of the hexagonal phase. This results in characteristic ^{31}P-NMR spectra which have a reversed symmetry as compared to the bilayer and are two times narrower (Seelig 1978). Lipids in isotropic phases (cubic or rhombic) undergo lateral diffusion on the highly curved structures which averages to zero the residual magnetic interaction and thus leads to narrow symmetric ^{31}P-NMR spectra. The structure and dynamics of water associated with the lipid headgroups have also been investigated by deuterium magnetic resonance (^{2}H-NMR) of D_2O. It has indeed been demonstrated that changes in the lipid lateral diffusion influence the structure and motions of the water molecules and hence the NMR parameters. Appearance of bound, swelling and free water was shown to affect equally ^{2}H-NMR spectra and nuclear relaxation times (Ulmius et al. 1977; König et al. 1994). Gel-to-fluid, bilayer-non-bilayer transitions as well as systems hydration were thus followed by ^{2}H-NMR on our systems.

The combination of ^{31}P-NMR of lipid molecules and of ^{2}H-NMR of heavy water offers the unique advantage of allowing one to simultaneously examine the lipid phase behavior and the corresponding changes in water structure. In what follows, the partial phase diagram of POPE-water and POPE-(30 mol%)cholesterol-water were thus determined together with the hydration properties of each of the phases.

Materials and methods

POPE was purchased from Avanti Polar Lipids (USA) and used without further purification. Possible lipid degradation was checked by thin layer chromatography after completion of NMR experiments. When lysophosphatidylethanolamine was detected in some samples, especially when they were kept for long periods of time at high temperatures, they were prepared again from pure POPE. Cholesterol was obtained from Sigma (Paris) and heavy water (99.8% isotopic purity) purchased from EURISO TOP (Paris).

Samples were prepared by mixing anhydrous POPE powder and appropriate quantities of heavy water in 10-mm diameter Pyrex tubes to obtain the desired molar concentrations. The systems were vigorously shaken on a vortex mixer, heated to 50 °C and cooled down to liquid nitrogen temperature. This cycle was repeated at least 10 times to ensure sample homogeneity, i.e. until reproducible ^{2}H- and ^{31}P-NMR spectra were obtained. In a glove

bag containing a dry nitrogen atmosphere, systems were transferred into 5 mm NMR sample tubes and then sealed. Samples containing cholesterol, POPE and 30 mol% sterol were dissolved in chloroform and the mixture dried under a nitrogen stream. Residual traces of chloroform were removed by overnight pumping under vacuum. The lipid dispersions were obtained by adding the required quantities of D_2O to the dry film in a glove bag containing dry nitrogen atmosphere as described above.

NMR measurements were performed on a Bruker ARX 300 spectrometer operating at 121.5 MHz and 46.07 MHz for ^{31}P and ^{2}H measurements, respectively. ^{31}P spectra were obtained with a phase-cycled Hahn echo pulse sequence (Rance and Byrd 1983; Dufourc et al. 1992) with proton broad band decoupling whereas ^{2}H measurements were performed by means of a phase-cycled quadrupolar echo sequence (Davis et al. 1976). Typical acquisition parameters were: 90° pulse width of 8.4 μs and 10 μs (^{31}P and ^{2}H NMR, respectively); interpulse delay of 40 μs; recycle delay of 6 s and 2 s for ^{31}P and ^{2}H, respectively; spectral window of 32 kHz (^{2}H NMR) and 50 kHz (^{31}P NMR); 1024 scans. Samples were allowed to equilibrate at a given temperature (±1 °C, Euroterm control unit) for at least 20 minutes before starting the acquisition. Because hysteresis was observed for the L_α-H_{II} transition, spectra were recorded on increasing the temperature. Data treatment was accomplished on a Bruker Station 1 and a VAX 4000 computer. A Lorentzian line broadening of 20 and 200 Hz was applied for ^{2}H- and ^{31}P-NMR to increase the signal-to-noise ratio. Spectral moments calculation and "de-Pakeing" were performed as described by Davis (1983).

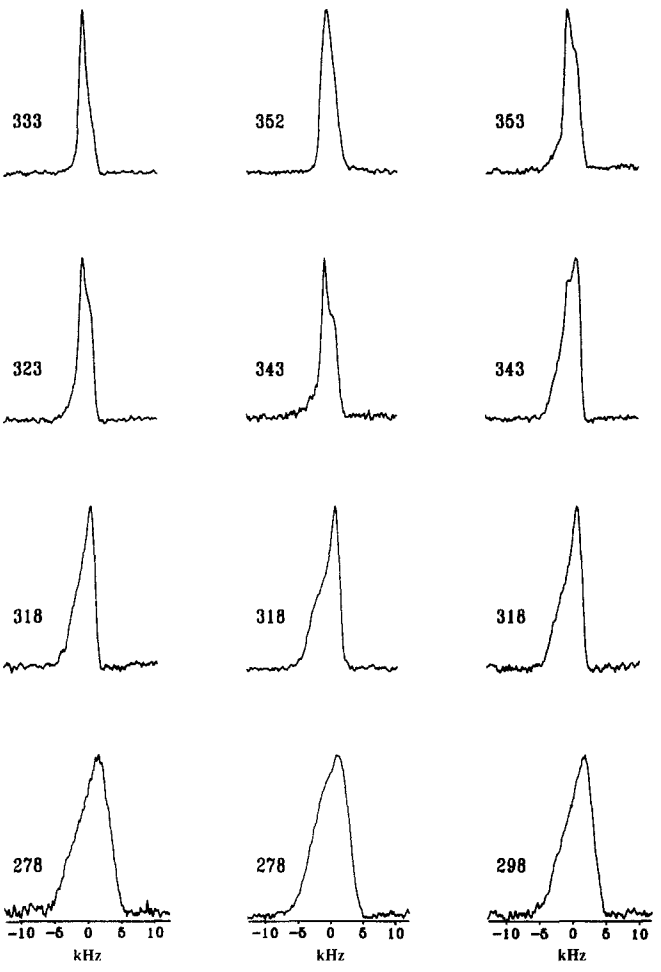

Fig. 1 Selected solid state ^{31}P-NMR powder spectra of the phospholipid in the system POPE-D_2O. Water content is $R_i = 6$ (*left column*), 13 (*middle column*) and 300 (*right column*). Numbers beside spectra indicate the temperature in degrees Kelvin

Results

The POPE-water system

^{31}P- and ^{2}H-NMR spectra of the system POPE-D_2O were recorded in the temperature range 278–357 K for D_2O-to-POPE molar ratios, R_i, of 3, 6, 8, 13, 15, 17, 20, 30 and 300. POPE ^{31}P-NMR spectra at $R_i = 6$, 13 and 300 and for selected temperatures are shown in Fig. 1. Spectra at lower temperatures (278 or 298 K) exhibit broad lineshapes which can be assigned to the so-called gel phase, $L_{\beta'}$ or $P_{\beta'}$ (Dufourc et al. 1992). At higher temperatures (318 K), narrower and axially symmetric phosphorus powder patterns of phospholipids in a L_α phase are clearly detected. Increasing the temperature leads to the onset of a new powder pattern of reversed chemical shielding anisotropy and of width half that observed in the L_α phase. This is the signature of the inverted hexagonal phase, H_{II} (Cullis and De Kruijff 1978). It may be noted in Fig. 1 that the spectrum of the H_{II} phase is superimposed on that of the L_α phase at 323, 343 and 353 K for $R_i = 6$, 13 and 300, respectively. This clearly indicates that the more the system is hydrated, the higher the temperature at which the L_α-H_{II} phase transition begins.

Determination of transition temperatures may also be performed from second moment, M_2, calculation of spectra in the entire temperature range. As an example, Fig. 2 shows the resulting thermal variation of M_2 for $R_i = 13$. M_2 smoothly reduces from low temperature to 298 K and then abruptly decreases to 303 K. One then observes a "plateau" of M_2, i.e. an almost temperature invariance, up to 333 K followed by another decrease to 353 K where a second plateau is reached. Interestingly, M_2 values for the two plateau regions scale with a factor ≈4 which is what one expects when there is a reduction by a factor two in the chemical shielding anisotropy ($\Delta\sigma$). This is again a good indication of the onset of the H_{II} phase, as already detected from spectra in Fig. 1. The two marked steps in this plot can thus be assigned to the onset of the lamellar gel-to-lamellar fluid and L_α-H_{II} phase transitions, respectively. The vertical lines in Fig. 2 tentatively indicate the domains where gel (G), lamellar fluid (L) and inverted hexagonal (H) phases are detected. The biphasic domain, L+H, is clearly identified because of the superimposition of spectra characteristic of each of the phases (see also deuterium

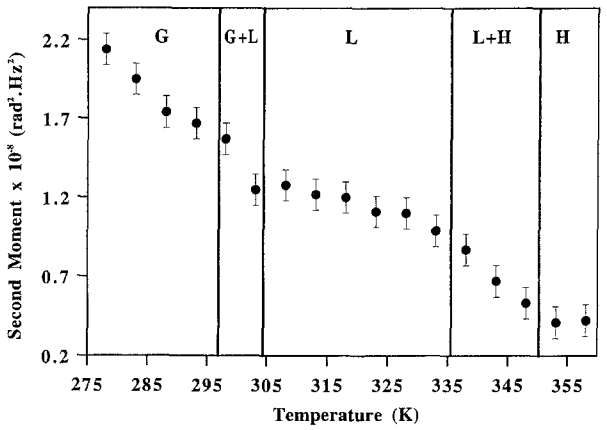

Fig. 2 Thermal variation of the second spectral moment, M_2, of ^{31}P-NMR powder spectra in the system POPE-D_2O for water content, $R_i = 13$. *Solid vertical lines* tentatively delimit the gel (*G*), lamellar fluid (*L*) and inverted hexagonal (*H*) phases (see text). Accuracy in second moment calculation is ±0.1 rad^{2}Hz^2

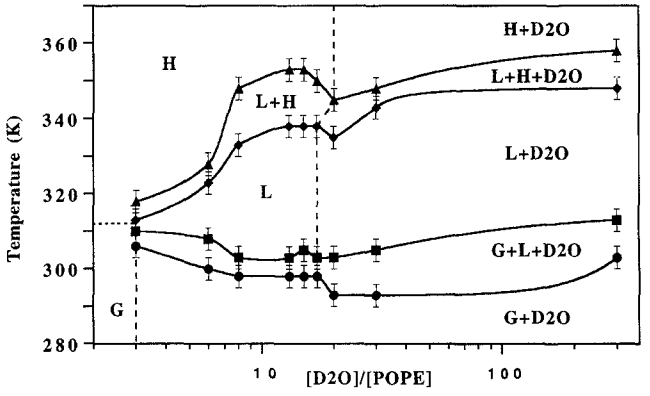

Fig. 3 Partial phase diagram of the POPE-D_2O system, as determined from ^{31}P and ^{2}H-NMR (see text for details). *Solid curves* are drawn to help reading the figure and come from interpolation between data values. *Dashed lines* indicate the swelling limit as obtained from ^{2}H-NMR of D_2O. The *dotted horizontal line* is also drawn to help reading the figure. Note that the water content, R_i, is plotted in a Log scale. Accuracy in phase boundaries is ±3 K

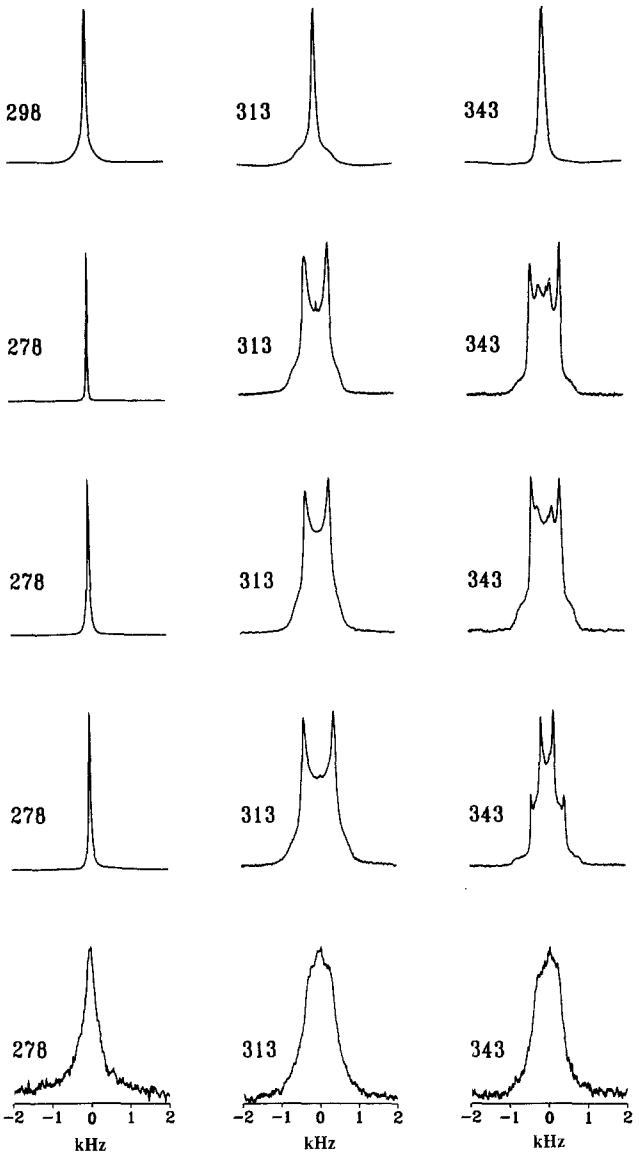

Fig. 4 Selected solid state ^{2}H-NMR powder spectra of D_2O in the system POPE-D_2O. Water content is, from top to bottom respectively, $R_i = 20, 17, 13, 8, 6$. Numbers beside spectra indicate the temperature in degrees Kelvin

results, *vide infra*). The G+L domain is harder to determine and phase boundaries in this case should be considered as rough estimates. Such M_2 calculations were performed for all ^{31}P-NMR spectra for all temperatures and R_i investigated. The determined phase boundaries allowed representation of the POPE-water phase diagram, Fig. 3. As general trends one notes that the L-to-H transition temperature increases with water content to reach some kind of plateau at $R_i \approx 8$–10 whereas the G-to-L transition always occurs at about 298 K. For very low water content, $R_i = 3$, it seems that the system transits almost directly from the gel phase to the hexagonal phase.

Selected ^{2}H-NMR spectra for POPE-D_2O systems are presented in Fig. 4. Low temperature (278 K) spectra are mostly characterized by a single sharp isotropic line ($\Delta\nu_{1/2} \approx 50$ Hz) except for $R_i = 6$ where the line is very broad ($\Delta\nu_{1/2} \approx 450$ Hz). At intermediate temperatures (313 K) a single axially symmetric powder pattern is observed for $8 \leq R_i \leq 17$. For $R_i \leq 6$ a broad, unresolved powder pattern is observed whereas for $R_i \geq 20$ an isotropic line ($\Delta\nu_{1/2} \approx 80$ Hz) is superimposed on a powder pattern. For high temperatures (343 K), a broad powder pattern is observed for $R_i = 6$ and two superimposed axially symmetric powder spectra are clearly detected when $6 < R_i < 20$. Interestingly, under these conditions, i.e. when the lamellar and hexagonal phases coexist, their respective quadrupolar splittings are in a ratio of 2–3 which indicates that the water molecules bound to lipids in each of the phases are in slow exchange and follow in part the dynamic behavior of the phospholipid molecules. A pattern suggesting chemi-

Table 1 Quadrupolar splittings measured on the system POPE-D_2O as a function of temperature and water content, R_i

R_i T(K)	6[a]	8	13	15	17	20[b]
278	–	0	0	–	0	–
288	–	0	0	–	0	–
293	–	0	0	150	0	–
295	–	150	150	–	–	–
298	300	230	270	230	420	–
303	460	380	310	380	530	620
308	530	690	490	490	570	680
313	610	760	610	570	570	680
318	–	800	610	610	610	680
323	–	840	660	610	650	680
328	460	880/380*	690	650	690	680
333	530	880/380*	690	650	690	680
338	530	880/340*	690/300*	690/380*	760/270*	680/200*
343	610	840/320*	690/340*	690/340*	760/300*	200*
348	–	300*	730/300*	690/270*	230*	–
353	–	–	730/270*	690/240*	230*	–
358	–	–	230*	–	–	–

Accuracy in measurements is ±20 Hz. [a] Directly estimated on powder patterns, accuracy of ±50 Hz. [b] Accuracy is estimated to be ±50 Hz due to exchange (see text). * Quadrupolar splitting measured for D_2O in the hexagonal phase. –, not determined

cal exchange in the intermediate time regime between the different types of water is observed for $R_i \geq 20$, T = 343 K.

Quadrupolar splittings, $\Delta\nu_Q$, were measured on depaked ^{2}H-NMR spectra for all temperatures and hydration conditions and are reported in Table 1. In cases were two quadrupolar splittings could be measured, the smaller was assigned to the H_{II} phase and identified with an asterisk. It must be mentioned that for $R_i = 3$ a broad unresolved pattern of triangular shape was detected on which measurement of quadrupolar splitting was not possible and that for $R_i = 6$ no depaking was performed owing to the non-axially symmetric lineshape. Quadrupolar splitting were then roughly estimated on the broad pattern. Except for low hydration conditions ($R_i \leq 6$) general behaviors can be observed. On increasing the temperature the quadrupolar splitting increases from 0 to 750 ± 100 Hz where a plateau is observed, i.e. there is no longer an increase in quadrupolar splittings with temperature. With a further increase in temperature, two quadrupolar splittings that can be assigned to the coexistence of the lamellar and hexagonal phase are detected over large temperature ranges. The range appears to get narrower as the R_i increases above 15. At very high temperatures, only one quadrupolar splitting of 250 ± 50 Hz is detected.

The POPE-cholesterol-water system

Similar experiments have been carried out on the same system in the presence of 30 mol% cholesterol, for water contents such that the D_2O-POPE molar ratio is $R_i = 10$, 15 and 20. Figures 5 and 6 display selected ^{31}P and ^{2}H-NMR spectra, respectively. It is clearly seen in Fig. 5 that broad spectra characterizing the gel phase at low temperature in

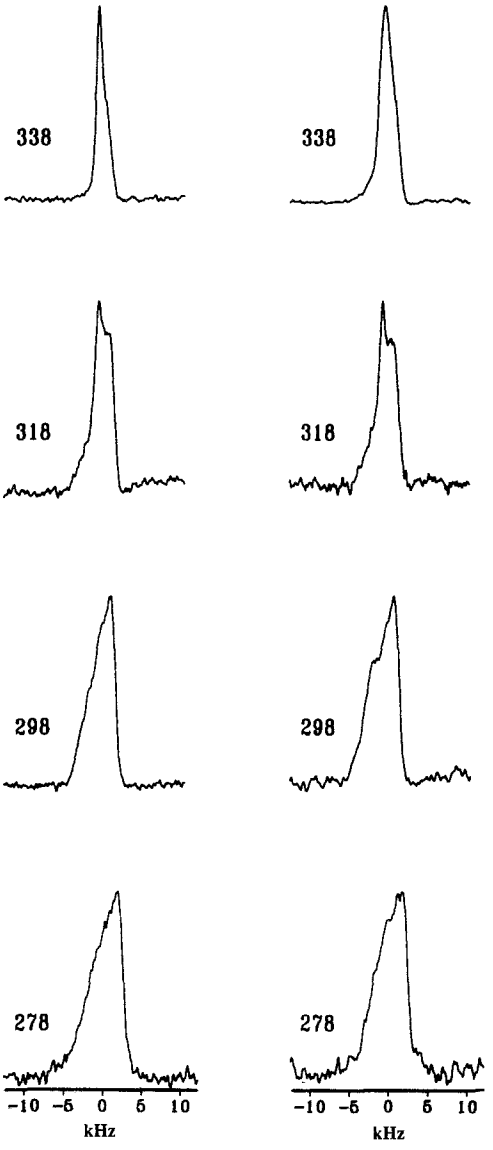

Fig. 5 Selected solid state ^{31}P-NMR powder spectra in the system POPE-(30 mol%)cholesterol-D_2O. *Left column*, $R_i = 10$; *right column*, $R_i = 15$. Numbers beside spectra indicate the temperature in degrees Kelvin

the absence of cholesterol (Fig. 1) have disappeared. Axially symmetric spectra reflecting intra and intermolecular motions of the liquid-ordered lamellar phase (Vist and Davis 1990) are observed from 278 to 308–313 K, in the presence of sterol. At 318 K a second powder pattern of reversed anisotropy appears to be superimposed on that associated with the lamellar phase. The onset of the L-H transition therefore appears for lower temperatures, in the presence of cholesterol. As described for pure systems, phase boundaries were determined by calculation of the second spectral moment. It appears that cholesterol stabilizes the L+H biphasic domain some 20 K below temperatures where it was detected in its absence. In contrast to what was observed for D_2O-POPE systems the width of the L+H domain is greater in the presence of cholesterol, i.e.

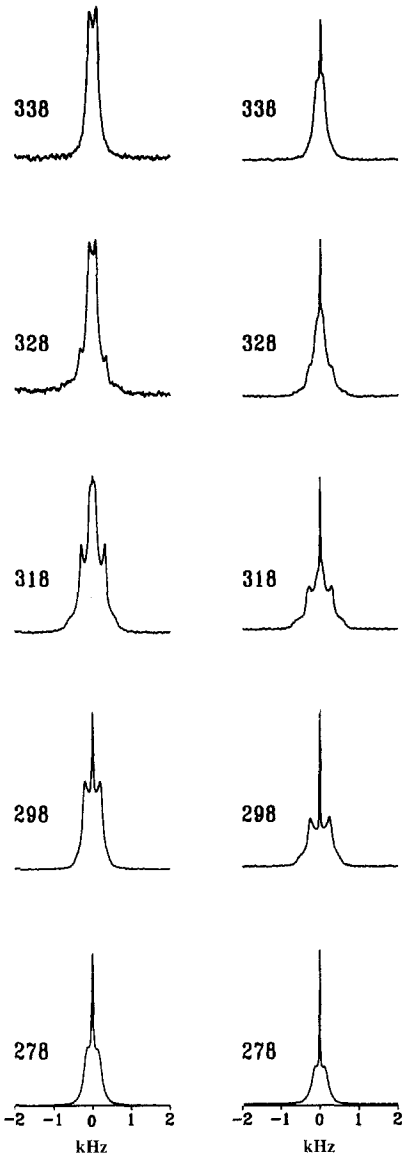

Fig. 6 Selected solid state ^{2}H-NMR powder spectra in the system POPE-(30 mol%)cholesterol-D_2O. *Left column*, $R_i = 10$; *right column*, $R_i = 15$. Numbers beside spectra indicate the temperature in degrees Kelvin

20–25 K vs. 5–15 K, and stays approximately constant for $10 \leq R_i \leq 20$.

Figure 6 shows that deuterium powder patterns are detected over the entire temperature range of our study. For low temperatures (278–298 K), a narrow isotropic line appears on the powder spectrum. On increasing the temperature and only for $R_i = 10$ this line disappears, the system being then composed of two axially symmetric power spectra. These two spectra coexist for temperatures between 313 and 328 K, in accordance with the presence of L and H phases as detected by ^{31}P-NMR. Again, this indicates that the water molecules engaged in these two phases are in slow exchange on the NMR time scale. Increasing the temperature further ($T \geq 328$ K) leads to the powder spectrum associated with D_2O in a pure hexagonal phase. Increasing the water content does not significantly modify the above behavior except for the appearance of isotropic lines at elevated temperatures. For $R_i \geq 15$, the amount of isotropic line is greater in the L phase and appears as a third spectral component above 318 K. When the system is composed of a single H phase, this sharp line still appears on the powder pattern. This indicates that in these conditions there is free water in slow exchange with water molecules that have fully hydrated the hexagonal phase. Quadrupolar splittings were measured on dePaked spectra. $\Delta\nu_Q$ increases slowly from ca. 300 Hz at 278 K to ca. 600 Hz when the system enters the L+H biphasic domain. The quadrupolar splitting associated with the hexagonal phase does not appears to vary with temperature and stays approximately constant at ca. 200 Hz.

Discussion

Our investigations showed that ^{31}P and ^{2}H NMR spectra are sensitive to phase changes and to hydration properties of POPE-water and POPE-cholesterol-water systems. It what follows we will discuss these two aspects separately and consider the effect of cholesterol in each case.

Partial phase diagram

Information on the general phase properties of a given system is usually obtained from differential scanning calorimetry or X-ray data. Detailed phase diagrams of DDPE, DAPE and DHPE reported by Seddon et al. (1984) and Caffrey (1985) are very similar to what we report for POPE-D_2O. In particular we also found that for low water content ($R_i \leq 3$), the system undergoes a lamellar gel-to-hexagonal (G-H) transition on increasing the temperature, i.e. without the intermediate lamellar phase we denoted F. The thermotropic sequence G-F-H is only observed on increasing hydration above $R_i = 3$,i.e. above 8 weight % D_2O in POPE. The above authors report similar sequences for water contents greater than 5–12 weight % H_2O in DDPE, DAPE or DHPE which compares well with our data. Transition temperatures stay approximately constant above $R_i = 8$ (18 wt% D_2O) which is again in good agreement with what has been reported by Seddon and Caffrey. The L_β phase of the system DDPE – water was reported to be metastable, reverting spontaneously to the unhydrated crystalline phase, L_c. It is possible that the phase which we denoted G is actually such a crystalline phase. This would indeed explain the observed ^{2}H-NMR isotropic lines at lower temperatures in the POPE-D_2O system which reflect the presence of free water and therefore membrane dehydration (vide infra). In excess water, the gel-to-fluid and the fluid-to-hexagonal transition temperatures are respectively, $T_c = 35 \pm 5$ °C and $T_h = 80 \pm 5$ °C. This is to be compared with those reported for DPPE ($T_c = 64$ °C and $T_h = 123$ °C) (Seddon et al. 1983) and DOPE ($T_c = -10$ °C and $T_h = 10$ °C) (Cullis and Kruijff 1978). The presence of

one oleoyl chain in position sn-2 therefore drops the transition temperatures by ca. 30 and 40 °C. It appears however that the effects are not additive since the presence of a second oleoyl chain promotes a further decrease in transition temperatures by ca. 45 and 70 °C, respectively. The zone of coexistence of both G+F and F+H phases varies from 5 to 20 °C which compares well with what was reported for DHPE (Caffrey 1985).

Very interestingly, in conditions where two phases coexist, it appears that the water is in fast exchange between the gel and fluid phases and in slow exchange ($<10^3$ Hz) between the fluid and hexagonal phases. One indeed detects one average ^{2}H-NMR spectrum for D_2O in the G+F zone and a superimposition of two powder spectra in the F+H zone (Fig. 4). The difference in rates of exchange clearly derives from the different nature of the phases in coexistence. Fast exchange of water between lamellar gel and fluid phases is easily understood because water can diffuse freely from one bilayer to the other. In contrast, in hexagonal type II phases, water constitutes the core of the lipid cylinders. It is therefore trapped inside cylinders and can only exchange with other water molecules, from other cylinders or from coexisting lamellar phases, by passive diffusion across bilayers, which is energetically unfavorable. This accounts well for slow D_2O exchange between lamellar and hexagonal type II phases. Incidentally this situation is an indirect proof of a type II hexagonal phase, since one would expect a fast exchange situation for type I hexagonal phases coexisting with lamellar phases, the water molecules in the former being outside the lipid cylinders.

In the presence of cholesterol, the most drastic effect is a decrease of the F to H transition temperature by 20 °C. The steroid can therefore be considered as a hexagonal type II phase stabilizer. This is easily understood on the basis of the Israelashvili concept of self-assembly of lipid bilayers (Israelachvili et al. 1977). Cholesterol increases the volume of the lipidic chains much more than that of the headgroups, therefore promoting an earlier L-to-H_{II} transition as the temperature of the system is increased. The domain of existence of both phases appears to have the same width as in the absence of cholesterol. There is also a situation of slow water exchange between the lamellar and hexagonal type II phases in the presence of cholesterol and as for phosphatidylcholines, it appears that the G-to-F transitions has been abolished.

Hydration dependence

As recently demonstrated for DMPC (Faure et al. 1996), water can occupy three different situations while interacting with membranes: it can be i) tightly bound to the polar head groups at the interface, ii) trapped in the intermembrane space where it swells the system and iii) free from the membrane phase, when it is present in excess and macroscopically separated from the hydrated system. In such situations, ^{2}H-NMR of D_2O can be used to determine the number of bound and trapped water molecules per lipid, denoted n_B and n_T. Determination of the maximal number of trapped water molecules, i.e. just before the appearance of free water, is easily performed because of slow exchange between free water and membrane water (trapped + bound). In this situation an isotropic sharp line is observed superimposed on an axially symmetric powder pattern. Integration of both the isotropic signal and the powder pattern leads directly to the number of trapped water molecules at maximal hydration (Gawrisch et al. 1992). For POPE-D_2O we find $n_T = 17 \pm 1$ and 20 ± 2 in the F and H phases, respectively. For the G phase a broad unresolved powder pattern appears for $R_i = 3$ and an isotropic line shows up already at $R_i = 6$ suggesting that only few water molecules per lipid are retained by this phase, $n_T \leq 3$. This further suggests that this phase is a dehydrated phase, such as the L_c phase detected in DDPE-water system (Seddon et al. 1984) where n_T was reported to be 1–2. Although hydration data on PE system is scarce our findings generally agree with what was reported by Seddon on DDPE ($n_T \approx 14$ and 18 for the L_α and H_{II} phases, respectively) and on DAPE ($n_T \approx 10$ and 17). Gawrish et al. (1992) also reported n_T values of 11 ± 1 and 18 ± 1 for DOPE-water at 22 °C. It appears that POPE, which possesses both saturated and unsaturated acyl chains, is able retain a little bit more water than a PE system with both alkyl or acyl chains fully saturated (DDPE, DAPE) or both acyl chains unsaturated (DOPE). In any case, the POPE system is much less hydrated than PC systems where n_T is reported to be 27 ± 1 in the L_α phase (Marsh 1990).

In the presence of cholesterol, n_T can be estimated to be 9 ± 1 for the F phase and to 14 ± 1 in the H phase. Interestingly, the sterol molecules promote dehydration of the system, both in F and H phases, the effect being more pronounced for the F phase. This is to be compared with what has been reported in the presence and absence of 30 mol% cholesterol in DMPC where n_T was respectively found to be 28 and 30 in the fluid phase (Faure et al. 1996). Thus as a corollary of an H_{II} stabilizing effect, cholesterol can act as a strong dehydrating agent, presumably by reducing the POPE chain area and hence the headgroup surface.

The number of bound water molecules per lipid, n_B, may be determined in cases of fast exchange between bound and trapped water in a single phase by plotting the observed quadrupolar splitting for D_2O as a function of $1/R_i$ (Faure et al. 1996). Once adsorption of water molecules at the interface is completed the addition of water molecules in the interbilayer space leads to a linear decrease of $\Delta\nu_Q$ and the slope leads to n_B. Alternatively n_B may be determined from the change in hydration regime, i.e. on going from adsorption to swelling the quadrupolar splitting often passes through a maximum. The first method cannot be applied to our data because of the limited number of R_i values. However, a rough estimate of n_B can be obtained by considering that at $R_i = 8$, in the F phase (T = 313–328 K), $\Delta\nu_Q$ has a maximum value of ca. 800 Hz (Table 1). Hence there would be 8 water molecules bound to each POPE molecule. Interestingly this value is close to those reported for L_α phases on phosphatidylcholines (Ulmius et al. 1977; Faure et al. 1996). As a consequence, it appears that

PE and PC systems in the lamellar fluid state bind approximately the same number of water molecules but that the PE lamellar phase, on the other hand, swells much less than the PC phase. In the pure hexagonal phase, is also appers that the maximum in quadrupolar splitting (300 Hz) is reached near $R_i \approx 8$ suggesting that the number of bound molecules is about the same in both the fluid lamellar and hexagonal type II phases although the amount of trapped water is greater in the H phase than in the F phase (*vide supra*).

The D_2O quadrupolar splittings increase with the temperature to reach a plateau value when the system is in the F phase. Then a second quadrupolar splitting more than two times smaller appears on appearance of the H phase. The first increase is easily accounted for by a progressive hydration of the lamellar phase, starting with the poorly hydrated $G(L_c)$ phase to end up with the F phase. Interestingly, the quadrupolar splittings of D_2O in the L and H regions are in a ratio of 3.0 ± 0.4, in the presence or absence of cholesterol. This value is markedly greater than the value of 2 one finds when comparing the phosphorus-31 $\Delta\sigma$ of lamellar and hexagonal phases. This clearly indicates that the orientation and/or dynamics of the phospholipid headgroup is slightly modified, if at all, by the F to H transition, whereas the structure and/or dynamics of bound water at the surface is appreciably affected upon the phase transition.

Conclusion

We have demonstrated herein that structural information and hydration properties of lipid-water systems can be obtained with only one technique, solid state NMR. The phase diagram, the nature of the phases, the number of bound and trapped water molecules in each of the phases have thus been obtained. It noteworthy that the same level of information could have been obtained by using a combination of several other techniques such as X-rays diffraction, DSC or freeze-fracture electron microscopy. In the present study, instead of a multitechnique approach, we have taken advantage of a multinuclear analysis by using nuclei reporters both on the lipid and on the water.

References

Arvidson G, Brentel I, Kahn A, Lindblom G, Fontell K (1985) Eur J Biochem 152:753

Batenburg AM, Van Esch JH, De Kruijff B (1988) Melittin-induced changes of the macroscopic structure of phosphatidylethanolamines. Biochemistry 27:2324–2331

Blaurock AE (1982) Evidence of bilayer structure and of membrane interactions from X-ray diffraction analysis. Biochim Biophys Acta 650:167–207

Boggs JM, Stamp D, Hughes DW, C.M. D (1981) Influence of ether linkage on the lamellar to hexagonal phase transition of ethanolamine phospholipids. Biochemistry 20:5728–5735

Caffrey M (1985) Kinetics of the lamellar gel/lamellar liquid-crystal and lamellar/inverted hexagonal phase transition in phosphatidylethanolamine: a real-time X-ray diffraction study using synchrotron radiation. Biochemistry 24:4826–4844

Cullis PR, De Kruijff B (1978) Polymorphic phase behaviour of lipid mixtures as detected by 31P NMR. Biochim Biophys Acta 507:207–218

Cullis PR, De Kruijff B (1979) Lipid polmorphism and the functional roles of lipids in biological membranes. Biochim Biophys Acta 559:399–420

Cullis PR, De Kruyff B, Richards RE (1976) Factors affecting the motion of the polar headgroup in pospholipid bilayers. A ^{31}P NMR study of unsonicated phosphatidylcholine liposomes. Biochim Biophys Acta 426:433–446

Cullis PR, Kruijff B (1978) The polymorphic phase behaviour of phosphatidylethanolamines of natural and synthetic origin. Biochim Biophys Acta 513:31–42

Davis JH (1983) The description of membrane lipid conformation, order and dynamics by ^{2}H-NMR. Biochim Biophys Acta 737:117–171

Davis JH, Jeffrey KR, Bloom M, Valic MI, Higgs TP (1976) Quadrupolar echo deuteron magnetic resonance spectroscopy in orderted hydrocarbon chains. Chem Phys Letters 42:390–394

Dufourc EJ, Mayer C, Stohrer J, Althoff G, Kothe G (1992) Dynamics of phosphate head groups in biomembranes. A comprehensive analysis using phosphorus-31 nuclear magnetic resonance lineshape and relaxation time measurements. Biophysical J 61:42–57

Dufourc EJ, Mayer C, Stohrer J, Kothe G (1992) ^{31}P and ^{1}H-NMR pulse sequences to measure lineshapes, T_{1Z} and T_{2E} relaxation times in biological membranes. J Chim Phys 89:243–252

Ellens H, Bentz J, Szoka FC (1986) Destabilization of phosphatidylethanolamine liposomes at the hexagonal phase transition temperature. Biochemistry 25:287–294

Faure C, Tranchant JF, Dufourc EJ (1996) Comparative effects of cholesterol and cholesterol sulfate on hydration and ordering of DMPC membranes. Biophysical J 70:1380–1390

Finer (1973) Phospholipid hydration studied by deuteron magnetic resonance spectroscopy. Chem Phys Lipids 12:1–16

Gaillard S, Dufourc EJ, Bonnet M, Renou JP (1992) Dantrolene prevents or repairs the halothane induced membrane restructuration. A ^{31}P-NMR and DSC study. Eur Biophys J 21:13–19

Gawrisch K, Parsegian VA, Hajduk DA, Tate MW, Gruner SM, Fuller NL, Rand RP (1992) Energetics of a hexagonal-lamellar-hexagonal-phase transition sequence in dioleoylphosphatidylethanolamine membranes. Biochemistry 31:2856–2864

Grabielle-Mandelmont C, Perron R (1983a) Calorimetric studies of phospholipid-water systems. I. DL-dipalmitoylphosphatidylcholine (DPPC)-water system. J Colloid Interface Sci 95:471–482

Grabielle-Mandelmont C, Perron R (1983b) Calorimetric studies of phospholipd-water systems. II. Study of water behavior. J Colloid Interface Sci 95:483–493

Harlos K, Eibl H (1981) Hexagonal phases in phospholipids with satured chains: Phosphatidylethanolamines and phosphatidic acids. Biochemistry 20:2888–2892

Israelachvili JN, Mitchell DJ, Ninham BW (1977) Theory of self-assembly of lipid bilayers and vescicles. Biochim Biophys Acta 470:185–201

Janiak MJ, Small DM, Shipley GG (1976) Nature of the thermal pretransition of synthetic phospholipids: dimyristoyl- and dipalmitoyllecithin. Biochemistry 15:4575–4580

Killian JA (1992) Gramicidin and gramicidin-lipid interactions. Biochim Biophys Acta 1113:391–425

König S, Sackmann E, Richter D, Zorn R, Carlile C, Bayerl TM (1994) Molecular dynamics of water in oriented DPPC multilayers studied by quasielastic neutron scattering and deuterium nuclear magnetic resonance relaxation. J Chem Phys 100: 3307–3315

Laroche G, Dufourc EJ, Pézolet M, Dufourcq J (1991) Structure and dynamics of dimyristoylphosphatidic acid/calcium complexes by ^{2}H-NMR, Infrared, and Raman spectroscopies and small-angle X-ray diffraction. Biochemistry 30:3105–3114

Luzzati V (1968) X-ray diffraction studies of lipid-water systems. Academic Press, London New York

Marsh D (1990) CRC Handbook of Lipid Bilayers. CRC Press, Boca Raton, Florida

Rance M, Byrd RA (1983) Obtaining high-fidelity spin-1/2 powder spectra in anisotropic media: Phase-Cycled Hahn echo spectroscopy. J Magn Res 52:221–240

Seddon JM, Cevc G, Kaye RD, Marsh D (1984) X-Ray diffraction study of the polymorphism of hydrated diacyl and dialkylphosphatidylethanolamines. Biochemistry 23:2634–2644

Seddon JM, Cevc G, Marsh D (1983) Calorimetric studies of the gel-fluid (L_β-L_α) and lamellar-inverted hexagonal (L_α-H_{II}) phase transitions in dialkyl and diacylphosphatidylethanolamines. Biochemistry 22:1280–1289

Seelig J (1978) Phosphorus-31 nuclear magnetic resonance and the head-group structure of phospholipids in membranes. Biochim Biophys Acta 515:105–140

Ulmius H, Wennerstrom G, Lindbloom G, Arvidson G (1977) Deuteron NMR studies of phases equilibria in a lecithin water system. Biochemistry 16:5742–5745

Verkleij AJ (1984) Lipidic intramembraneous particles. Biochim Biophys Acta 779:43–63

Vist MR, Davis JH (1990) Phase equilibria of cholesterol dipalmitoylphosphatidylcholine mixtures: ^{2}H-nuclear magnetic resonance and differential scanning calorimetry. Biochemistry 29:451–464