Comparative Effects of Cholesterol and Cholesterol Sulfate on Hydration and Ordering of Dimyristoylphosphatidylcholine Membranes

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ABSTRACT The comparative effect of cholesterol (CH) versus cholesterol sulfate (CS) on dimyristoylphosphatidylcholine (DMPC) membranes has been investigated by optical microscopy, freeze-fracture electron microscopy, x-ray diffraction, and solid state ^2H and ^{31}P nuclear magnetic resonance (NMR). The sulfate analogue extends the lamellar phase domain toward high water contents, and substitution of 30 mol % CH by CS in DMPC lamellae induces the trapping of 30 wt % additional water. The greater swelling of the CS-containing systems is evidenced by determination of lamellar repeat distances at maximal hydration: 147 ± 4 Å and 64 ± 2 Å in the presence of CS and CH, respectively. ^2H-NMR of heavy water demonstrates that CS binds ~12 more water molecules at the interface than CH whereas NMR of deuterium-labeled DMPC chains reveals that 30 mol % CS orders the membrane as 15 mol % CH at high temperature and disorders much more than CH at low temperatures. The various effects of CS versus CH are discussed by taking into account attractive Van der Waals forces and repulsive steric/electrostatic interactions of the negatively charged sulfate group.

INTRODUCTION

Cholesterol (CH) is found in many biological membranes and is the main sterol of animal organisms. It is equimolar with phospholipids in membranes of liver cells, erythrocytes (Van Deenen and De Gier, 1964), and myelin (Ansell and Hawthorne, 1964), whereas in human stratum corneum, i.e., the outermost layer of epidermis, it represents 20 wt % of the lipidic fraction (Elias, 1983; Lampe et al., 1983b). Other steroids such as cholesterol sulfate (CS) have also been detected in substantial amounts in various tissues. CS was found in the spermatozoa head where it can account for up to 20% of the human sperm head surface area (Langlais et al., 1981) and in the stratum corneum but in lesser amounts (<5 wt %) (Lampe et al., 1983a) than CH. CH has been extensively studied over the last two decades and is known as a regulator of membrane ordering (Oldfield et al., 1978; Taylor et al., 1981; Dufourc et al., 1984; Sankaram and Thompson, 1990; Léonard and Dufourc, 1991; for a review, see Yeagle, 1985). CS was also shown to be a membrane stabilizer: it protects erythrocytes against hypotonic hemolysis (Bleau et al., 1974), inhibits Sendaï virus fusion to both human erythrocytes and model membranes (Cheetham et al., 1990b) and reduces the fertilization efficiency of rabbit spermatozoa (Fayrer-Hosken et al., 1987).

In most organisms in which both CH and CS are present, e.g., the spermatozoon plasma membrane or the stratum corneum, a good regulation of their relative concentrations is essential. This regulation is ensured by hydrolysis of CS to CH via sulfatase activity. A dysfunction during this step can lead to dramatic changes in membrane properties and serious troubles. Indeed, an accumulation of CS in the spermatozoa heads results in the incapacity of spermatozoa to penetrate ovum (Langlais and Roberts, 1985; Roberts, 1987). This contraceptive effect has been tentatively explained by the CS-driven lamellar stabilization of the sperm membrane, which would thus be less fusogenic (Roberts, 1987; Cheetham et al., 1990a). At the skin level, an increase in the relative concentration of CS versus CH leads to a thickening of the stratum corneum. This clinical pattern of scaling results from disorders in desquamation, i.e., the loss of intercellular cohesion in the stratum corneum (Shapiro et al., 1978; Epstein et al., 1981). Both CH and CS effects in biological systems have thus been extensively studied from the biochemical viewpoint. Although the physicochemical properties of CH in biomembranes are well described (Yeagle, 1985), little is known about the structure and dynamics of its sulfate analogue in membranes. One must nevertheless mention the work of Le Grimellec and coworkers (1984) who showed by fluorescence polarization measurements using 1,6-diphenyl-1,3,5-hexatriene as a fluorescent probe that CS would abolish the gel-to-fluid transition and would be a membrane fluidity regulator. However, care must be taken when using 1,6-diphenyl-1,3,5-hexatriene as a probe for membrane ordering due to the presence of two membrane locations that may contribute in different ways to the total fluorescence (Pebay-Peyroula et al., 1994). Similar conclusions were drawn by Kitson and co-workers (1992) who studied by ^2H nuclear magnetic resonance (NMR) the effect of both steroids on 1-[$^2H_{31}$]palmitoyl-2-oleoyl-phosphatidyl-ethanolamine dispersions. The above authors partly explained their results by postulating a strong hydration of the CS headgroup with, however, no experimental proof to support this assertion.

To our knowledge, there is no information about the hydration properties of CS in membranes. A comparative investigation of the influence of CH and CS on the hydro-

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carbon ordering and hydration of model membranes was therefore undertaken on membranes made of dimyristoylphosphatidylcholine (DMPC). Partial phase diagrams on DMPC-CH-D_2O and DMPC-CS-D_2O were determined by x-ray diffraction and optical microscopy. Hydration of the interface was monitored by ^{2}H-NMR of heavy water. NMR experiments were also carried out on samples composed of *sn*-2-chain-perdeuterated DMPC associated with CH or CS in excess water to investigate membrane ordering. As CH roughly represents 20 wt % of the lipidic fraction of the stratum corneum and as a comparison between both systems is to be drawn, the membrane composition for the latter experiments was fixed by the DMPC-to-steroid molar ratio $N_D/N_S = 2.3$, which corresponds to a CH concentration of 30 mol %.

MATERIALS AND METHODS

Chemicals

DMPC was purchased from Interchim (Montluçon, France) whereas 1-myristoyl-2-[$^2H_{27}$]myristoyl-*sn*-glycero-3-phosphocholine was obtained from Avanti Polar Lipids (Birmingham, AL). CH was purchased from Sigma Chemical Co. (St. Louis, MO) and CS was from Genzyme (Suffolk, England). Deuterium-depleted water (H_2O) and heavy water (D_2O) were obtained from Aldrich Chemical Co. (Milwaukee, WI) and CEA (Saclay, France), respectively. The purity of DMPC and both CH and CS was checked by thin layer chromatography before and after completion of experiments; no degradation was detected.

Sample preparation

Mixtures of DMPC with CH and DMPC with CS were prepared by cosolubilization in chloroform/methanol (2/1, v/v) and methanol solutions, respectively. It was necessary to heat the CS methanolic solution to 60°C for complete dissolution. Once the mixture was achieved, the solvent was removed, first by blowing a stream of nitrogen over the sample and then by pumping the residue under high vacuum for 1 h. Afterwards, excess water was added to mixtures and samples were heated to 45°C for 30 minutes, vortexed, and cooled down in liquid nitrogen. This cycle was repeated several times to ensure a good homogeneity of the dispersion and the samples were lyophilized overnight. A white dry fluffy powder was thus obtained and transferred into 10-mm-diameter glass tubes. Eventually, the appropriate amount of water was added and the tubes were sealed. Homogenization of the samples was performed by successive heating, shaking, and cooling cycles as described before and then transferred either into a 1.5- or 2-mm-diameter x-ray capillary or into a 5-mm-diameter NMR glass tube according to the experiment to be carried out. To prevent the samples from being hydrated during transfers, preparations were made in a glove bag under a dry nitrogen atmosphere.

Light-Polarized Microscopy

Partial phase diagrams of DMPC-CH-D_2O and DMPC-CS-D_2O were realized by observations under a microscope. Samples were placed between thin plates and observed through crossed polarizers at room temperature. Because the lamellar phase is birefringent, its textural defects are easily recognizable as Maltese crosses. For biphasic samples such as lamellae in the presence of excess water, the latter appears as black domains of isotropic phase. When crystals are present, they appear as white parallelepipeds through polarized light.

Freeze-fracture electron microscopy

A drop of sample was placed onto a copper dish and frozen in liquid propane (−196°C) cooled in liquid nitrogen. After mounting the frozen specimen onto the specimen holder, it was inserted into the freeze-fracture apparatus (BAF 400) and fixed on the precooled holder. A vacuum of 10^{-6}-10^{-7} torr was then achieved. Fracturing was realized with a single edge scalpel blade precooled at −150°C. The fractured sample was then unilaterally shadowed by evaporation of platinum produced by an electron beam gun (1900 V, 90 mA). A platinum layer (2 nm thick) was thus deposited at an angle of 30°. An additional carbon layer (20 nm thick) was deposited vertically onto the replica (2400 V, 140 mA). The specimen holder, with object and replica, was then transferred into a tank containing a solution of water/ethanol (50/50, v/v) to remove the replica from the surface of the object. A rinse with tetrahydrofuran was done before cleaning the replica in a mixture of water and saturated solution of Cr(VI) oxide in sulfuric acid. Three additional cleanings were done in water.

X-ray diffraction

Experiments were carried out on an 18-kW Rigaku Rotaflex RU 300 rotating anode generator. A two-dimensional Marresearch imaging plate (18 cm diameter and 150 × 150 μm pixel size) was used for detection. Monochromation of the Cu radiation ($\lambda = 1.54$ Å) was achieved using a flat graphite crystal. The spot size, defined by three sets of vertical and horizontal slits, was approximately 0.25 mm^2. The sample holder was designed such that the temperature is controlled by a Haake water bath (Berlin, Germany). X-ray data were obtained at 25 ± 1°C. Samples were placed in glass cylindrical capillaries purchased from Glas Müller (Berlin, Germany). The latter were sealed once filled. The sample detector distance was fixed to $L = 303 \pm 1$ mm.

Nuclear magnetic resonance

Solid state ^{2}H-NMR experiments were performed at 46.1 MHz using a Bruker MSL 200 spectrometer. ^{2}H-NMR spectra were obtained on resonance using the quadrupolar echo pulse sequence with an eight-pulse cyclops sequence (Davis et al., 1976). Acquisition parameters for D_2O and [$^2H_{27}$]DMPC were, respectively, 50 kHz and 250 kHz spectral windows, 90° pulse durations of 10 and 8 μs, interpulse delays of 60 and 30 μs, and recycle times of 2 s in both cases. Spectral de-Paking was performed as described previously (Bloom et al., 1981; Sternin et al., 1983), and oriented-like spectra were calculated for bilayer normals at 90° with respect to the magnetic field direction. ^{31}P-NMR experiments were performed at 121.49 MHz on a Bruker ARX 300 spectrometer using the phase-cycled Hahn echo sequence (Rance and Byrd, 1983). Acquisition was performed on a 62-kHz spectral window with a 90° pulse duration of 9 μs and an interpulse delay of 40 μs. A recycle delay of 6 s was used together with proton high power decoupling during acquisition. Quadrature detection was used in all cases. Samples were allowed to equilibrate at least 30 min at a given temperature before signal acquisition. Temperature was regulated to ± 1°C.

Data treatment was performed on a Bruker Aspect 3000 and a VAX/VMS 4000 computer. A Lorentzian broadening of 100 Hz was applied to the free induction decays obtained in ^{31}P-NMR and ^{2}H-NMR of [$^2H_{27}$]DMPC before Fourier transformation.

THEORETICAL BACKGROUND

As it will be seen in the following and as already reported in the literature (Salsbury et al., 1972; Finer and Darke, 1973; Ulmius et al., 1977), two types of water molecules can be distinguished in monophasic lamellar systems (i.e., without water in excess): trapped and bound water. Trapped water, as defined by Finer and Darke (1973), is composed of

molecules incorporated in between bilayers with motional characteristics similar to those of free water. Bound water is partially oriented, in that the OH bond direction is maintained to a limited degree by hydrogen bonding. The latter, motionally hindered by the lipidic interface, constitutes hydration shells. Bound water molecules are exchanging between the different binding sites and with trapped water molecules. Our approach, however, does not allow us to differentiate between the different binding sites; only a global membrane-bound state can be defined. As a consequence, only two sites will be considered, one in which water is trapped and the other in which it is bound. The two-site exchange theory can thus be applied to the ^{2}H-NMR spectra of heavy water to determine the amount of water molecules in each site. This theory comes from the Chapman-Kolmogorov equation for a Markov process (Abragam, 1961; Rance, 1981). These two sites may be labeled B and T and the water fraction in each of the sites f_B and f_T, respectively. The probability per unit time for a molecule to jump from a state B to a state T is k_B whereas the reverse transition rate is k_T. The rates of change of f_B and f_T are given by the following equation (Rance, 1981; Ernst et al., 1990):

$$\frac{df_i}{dt} = -k_i f_i + k_j f_j, (i,j = B, T) \quad (1)$$

If $\Delta\nu_B$ and $\Delta\nu_T$ are the quadrupolar splittings of heavy water in sites B and T, respectively, then in the case of slow exchange, i.e. when $k_B \ll |\Delta\nu_B - \Delta\nu_T|$, two superimposed powder patterns are observed corresponding to both B and T sites. For fast exchange between bound water molecules at the interface (phospholipid and steroid sites) and trapped water, i.e., when $k_B \gg |\Delta\nu_B - \Delta\nu_T|$, only one quadrupolar splitting, $\Delta\nu_{obs}$, is detected in the resulting ^{2}H-NMR spectrum:

$$\Delta\nu_{obs} = f_B\Delta\nu_B + f_T\Delta\nu_T \quad (2)$$

As already mentioned, trapped water exhibits the motional characteristics of free water, in particular, $\Delta\nu_T$ is averaged to zero because of isotropic tumbling. Equation 2 then reduces to

$$\Delta\nu_{obs} = f_B\Delta\nu_B \quad (3)$$

in which the fraction of bound water f_B may be expressed as

$$f_B = \frac{N_D n_D + N_S n_S}{n} \quad (4)$$

where the indices D and S stand for DMPC and steroid, respectively; n_D and n_S are the number of water molecules bound to DMPC and steroid; N_D and N_S are the number of DMPC and steroid molecules; and n is the total number of water molecules in the sample. Introducing the water-to-phospholipid molar ratio, $R_i = n/N_D$, Eq. 3 can also be written as

$$\Delta\nu_{obs} = \frac{1}{R_i}\left(n_D + n_S\frac{N_S}{N_D}\right)\Delta\nu_B \quad (5)$$

From Eq. 5 it is seen that hydration properties of the membrane may be followed while recording $\Delta\nu_{obs}$ as a function of total water content. Two regimes can be distinguished, swelling and hydration. When $n > n_D N_D + n_S N_S$, bound and trapped water are present, this is the swelling regime. In such situations, n_D and n_S are constant, as hydration of the surface is completed, N_S/N_D is experimentally fixed and $\Delta\nu_B$ is likely to be constant, as Ulrich and coworkers (1994) showed that the quadrupolar splittings given by deuterated methylene in the phosphocholine headgroup are indeed constant in that regime. Consequently, the dependence of $\Delta\nu_{obs}$ on $1/R_i$ (Eq. 5) should be linear and cross the point of origin (Lindbloom et al., 1991). It is noteworthy that the above is valid only if the system remains in the same phase upon dilution. Caution must therefore be taken not to cross a phase transition when varying R_i. In situations in which swelling is not observed, i.e., when $n < n_D N_D + n_S N_S$, the system is in the hydration regime. Equation 5 is still valid but n no longer includes trapped water. Here, hydration shells are forming so that n_D and n_S as well as $\Delta\nu_B$ may be modified upon water addition (Ulrich et al., 1994). Henceforth, the dependence of $\Delta\nu_{obs}$ on $1/R_i$ will no longer be linear (Eq. 5). As a consequence of the above, one may predict that the variation of $\Delta\nu_{obs}$ as a function of $1/R_i$ should allow the determination of the water-to-DMPC molar ratio observed when the system passes from the hydration regime to the swelling regime:

$$R_i^* = \frac{n^*}{N_D} = n_D^* + n_S^*\frac{N_S}{N_D} \quad (6)$$

where n_D^* and n_S^* are the number of water molecules bound to a molecule of DMPC and steroid, respectively, when hydration of the interface is achieved. In our systems, R_i^* represents the number of bound water molecules per 1 molecule of DMPC and N_S/N_D molecules of steroid. Because N_S/N_D is experimentally fixed to 1/2.3, it is convenient to define the membrane unit cell as being composed of 1 molecule of steroid plus 2.3 molecules of DMPC. Therefore, 2.3 R_i^* will represent the number of water molecules bound to the membrane unit cell.

RESULTS

Partial phase diagrams

Fig. 1 displays the partial phase diagrams of the systems DMPC-CH-D_2O and DMPC-CS-D_2O. For both systems, all samples were hydrated with D_2O instead of H_2O because several samples were analyzed by ^{2}H-NMR of heavy water. Localization of the lamellar phase (hatched area in Fig. 1) in both systems was performed at 25°C by light-polarized microscopy. Fig. 1 indicates that lipidic lamellae can accept up to ~43 mol % CH and 37 mol % CS (30 wt % steroid). Interestingly, the presence of the sulfate group results in an extension of the lamellar phase domain toward elevated water contents. The CS-containing membrane can indeed admit 30 wt % of extra water

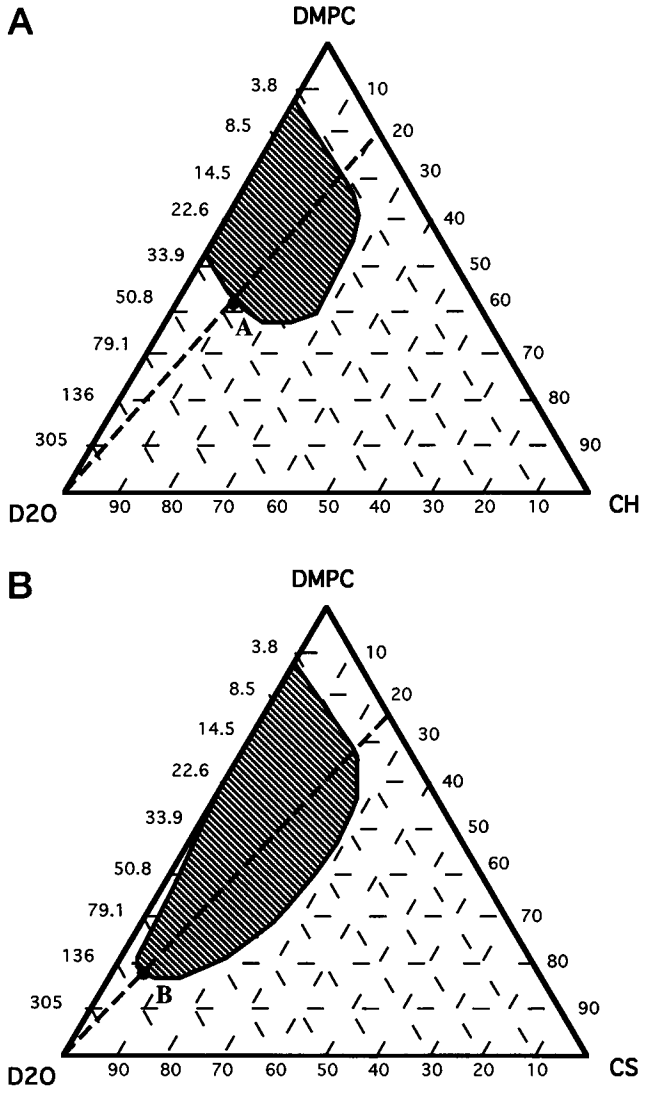


FIGURE 1 Partial phase diagrams of DMPC-CH-D_2O (*A*) and DMPC-CS-D_2O (*B*) at 25°C. Hatched areas represent the lamellar phase domain. Compositions are given in weight percentages or corresponding D_2O-to-DMPC molar ratio, R_i. Labels A and B indicate the composition for which freeze-fracture electron microscopy was performed. Dashed lines represent the dilution lines, i.e., DMPC plus 30 mol % steroid systems where the amount of water is progressively increased on going toward the D_2O corner. ^{2}H-NMR and x-rays were performed along theses lines.

compared with that with CH. This clearly indicates that the CS increases the hydration of the lamellar phase. To confirm the borders of the lamellar phase domain, some freeze-fracture micrographs were also made. Fig. 2 shows two micrographs of samples before separation from the bulk water phase. Their compositions, labeled A and B, are indicated in Fig. 1. Both were obtained at 25°C with the same magnification. They do not present any relevant differences; both are characteristic of a lamellar phase, but the interlamellar distance seems to be greater in the CS-containing membrane.

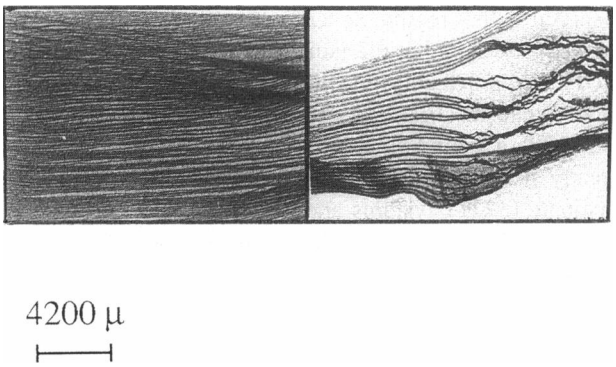


FIGURE 2 Freeze-fracture electron micrographs of the DMPC-CH (*left*) and DMPC-CS (*right*) systems hydrated in D_2O plus glycerol (30 wt %). The composition of each sample is given in Fig. 1 (A and B labels). Magnification, ×24,000.

Membrane swelling from x-ray diffraction

The maximal swelling of the lamellar phase composed of DMPC associated with 30 mol % steroid was determined by x-ray diffraction at 25°C. For each system, a set of samples in which the water content was progressively increased was analyzed (dotted lines in Fig. 1). Fig. 3 represents the intensity profile $I(q)$ as a function of the wave vector ($q = (4\pi\sin\phi)/\lambda$) for both systems. Several Bragg peaks are

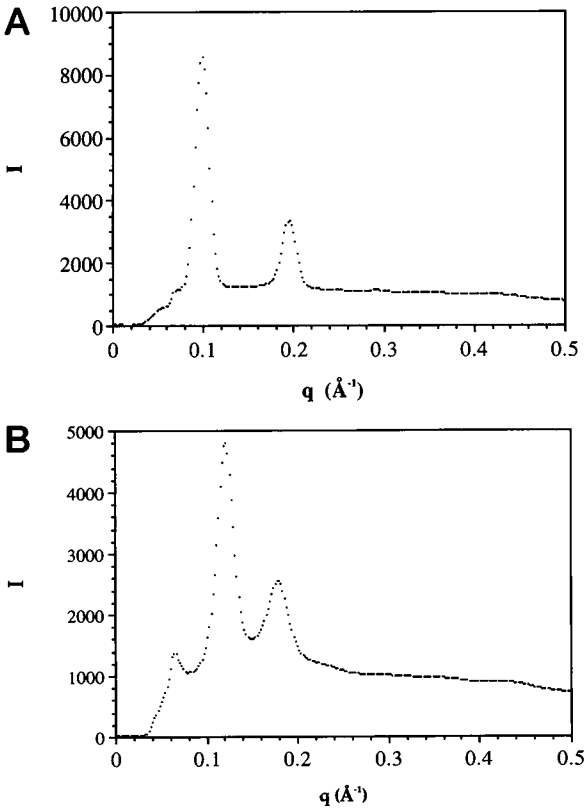


FIGURE 3 Intensity profile obtained at 25°C by x-ray diffraction on a DMPC-CH-D_2O sample (*A*) and DMPC-CS-D_2O sample (*B*) in excess water.

observed with a regular spacing characteristic of the lamellar phase from which the lamellar spacing, d, can be calculated. It is noticeable that the second order peak is more intense than the first order peak in the CS-containing system. Such a modulation of the Bragg peak intensities has already been observed in bis(2-ethylhexyl) sodium sulfosuccinate (AOT) systems and was attributed to the bilayer form factor as well as to different magnitudes of the bilayer elastic constants (Nallet et al., 1993). The variation of d with the water-to-DMPC ratio, R_i, is plotted in Fig. 4. The value of d first increases linearly with increasing water content and then reaches a plateau. The corresponding value is d_{max} = 64 ± 2 Å for DMPC-CH-D_2O and 147 ± 4 Å for DMPC-CS-D_2O. These data confirm unambiguously that lamellae are more swollen in the presence of CS. Experiments have also been performed on the DMPC-CS(30 mol %)-brine system. NaCl concentration in brine is 0.34 M (20 g/L). In excess water, the lamellar repeat distance is reduced to 86 ± 2 Å, reflecting the screening of electrostatic charges by sodium ions.

Determination of bound water at the interface by ^{2}H-NMR

Hydration of DMPC-CH-D_2O and DMPC-CS-D_2O was studied at three different temperatures, 10, 23, and 45°C for R_i = 2, 3.8, 5.8, 8.7, 11.3, 15.6, and 25.0. Selected ^{2}H-NMR spectra are represented on Fig. 5 (CH-containing systems) and on Fig. 6 (CS-containing systems). For both systems and for small R_i values, one observes a unique quadrupolar splitting that strengthens the hypothesis of fast exchange between water molecules bound to DMPC and steroid. In Fig. 5 and for R_i < 8.7, axially symmetric powder patterns are recorded on the temperature range of the study; their quadrupolar splitting clearly increases with temperature.

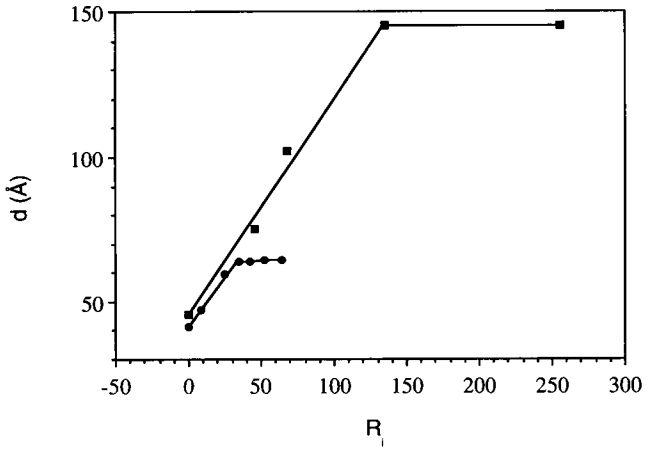


FIGURE 4 Evolution of the lamellar phase repeat spacing (d) with the D_2O-to-DMPC molar ratio, R_i. (●) DMPC-CH(30 mol %)-D_2O. (■) DMPC-CS(30 mol %)-D_2O. A linear fit (*solid line*) was performed for R_i < 30 and R_i < 130, in the CH- and CS-containing systems, respectively. Horizontal solid lines are drawn to help in reading the figure.

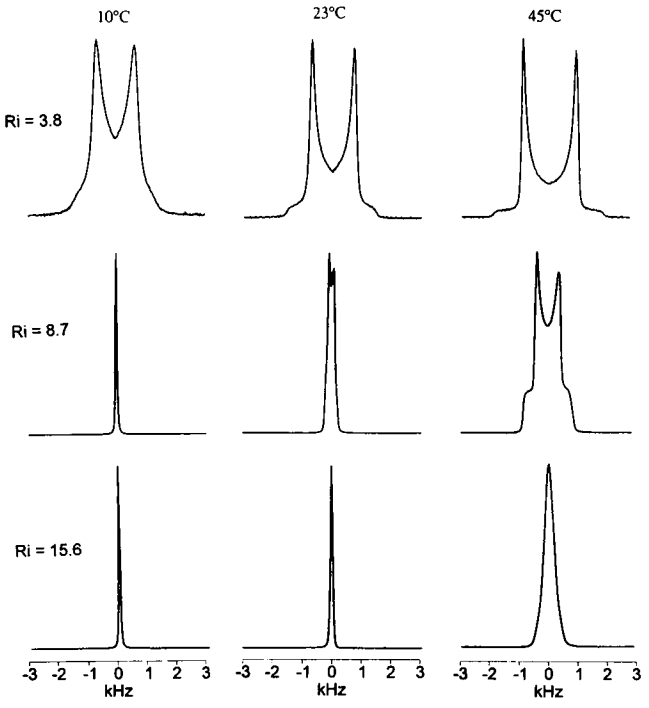


FIGURE 5 ^{2}H-NMR powder spectra of D_2O in DMPC-CH(30 mol %)-D_2O systems for selected temperatures (°C) and D_2O-to-DMPC molar ratio (R_i).

For 8.7 ≤ R_i < 15.6, a sharp isotropic line is observed at low temperature. This line is converted to an axially symmetric powder pattern on increasing the temperature. Interestingly, the spectrum observed at 45°C shows an enhancement in the intensity of the shoulders (0° orientations with respect to the external magnetic field). The same phenomenon is observed with solid state ^{31}P-NMR (not shown), indicating that this sample partially orients in the magnetic field. When R_i ≥ 15.6, the isotropic sharp line can be seen at 10 and 23°C and a broadened pattern is detected at 45°C. The latter might reflect an exchange in the intermediate time scale regime between an axially symmetric powder pattern and an isotropic line.

In the CS-containing system (Fig. 6), the water behavior differs. On increasing the temperature, independently of R_i, a minimum in quadrupolar splitting appears around 23°C. For 10 and 23°C, the variation of $\Delta\nu_{obs}$ versus R_i reaches a maximum for R_i = 8.7. At 45°C, $\Delta\nu_{obs}$ appears to decrease when increasing R_i. For R_i ≥ 15.6, an exchange in the intermediate time scale might also account for the spectral broadening, as already mentioned for the CH-containing system, in the same conditions. As already mentioned for the CH-containing system, marked intensity modifications are observed for the powder pattern shoulders at R_i = 8.7 and 15.6, independently of the temperature. Control was performed with ^{31}P-NMR, suggesting that, here also, there are partial macroscopic orientations of the sample in the magnetic field.

Fig. 7 displays the evolution of the D_2O quadrupolar splitting versus the reciprocal of R_i for both types of mem-

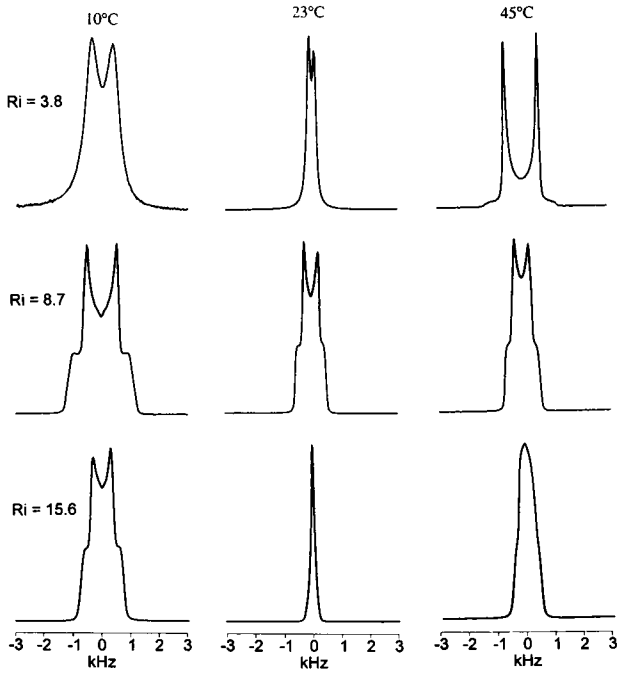
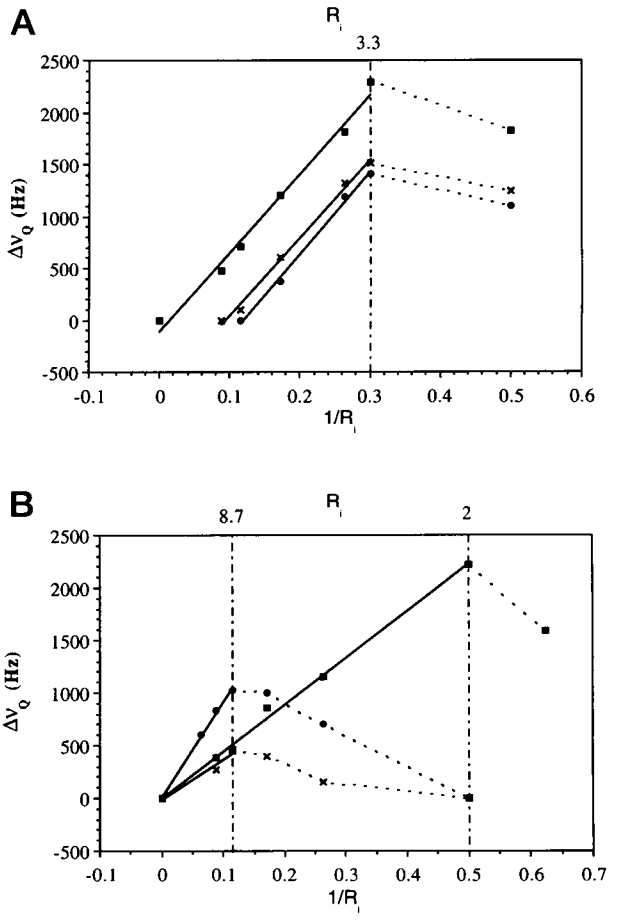


FIGURE 6 ^{2}H-NMR powder spectra of D_2O in DMPC-CS(30 mol %)-D_2O systems for selected temperatures (°C) and D_2O-to-DMPC molar ratio (R_i).



Faure and al., 1995, fig. 7

FIGURE 7 Evolution of the D_2O quadrupolar splitting with $1/R_i$ for the DMPC-steroid(30 mol %)-D_2O system at (●) 10, (×) 23, and (■) 45°C. (*A*) CH-containing systems. (*B*) CS-containing systems. Vertical dotted lines approximately indicate the water-to-DMPC molar ratio (R_i*) for which there is a passage between hydration and swelling regimes (see text). A linear fit (*solid lines*) was performed for $R_i \leq R_i$*. Dotted lines are drawn to help in reading the figure.

brane. It must be mentioned here that $\Delta\nu_{obs}$ values were considered for plotting only when spectra exhibited fast exchange regimes; in other words, quadrupolar splittings for $R_i \geq 15.6$ were discarded. For CH-containing systems, the data can be fitted with a linear regression (solid lines on Fig. 7 *A*) going through the origin at 45°C and for $R_i \geq 3.3$. At 10 and 23°C and for $R_i \geq 3.3$, a linear regression can still account for the data but does not pass through the origin (vide infra). For CS-containing membranes (Fig. 7 *B*) the variation of $\Delta\nu_{obs}$ versus $1/R_i$ is linear and goes through the origin for $R_i \geq 8.7$ at 10 and 23°C and for $R_i \geq 2.0$ at 45°C. For R_i less than these specific values, the quadrupolar splitting decreases when decreasing R_i. The linear behavior of $\Delta\nu_{obs}$ versus $1/R_i$ together with the crossing of the origin of the fitting line is in complete accordance with Eq. 5. This establishes that the number of bound water molecules as well as the corresponding quadrupolar splitting remain constant in the swelling regime for a given temperature. R_i^* values can thus be obtained from such graphs. It is found that $R_i^* = 3.3$ for CH-containing systems at 45°C and 2.0 and 8.7 for CS-containing systems at 45°C and 10 or 23°C, respectively.

Membrane structure and dynamics by ^{2}H-NMR

The dynamics of DMPC chains in the presence of steroid was studied by ^{2}H-NMR of DMPC deuterated on the *sn*-2 chain. Spectra were realized from 0 to 60°C by 5°C steps. Fig. 8 displays selected ^{2}H-NMR spectra of the thermal variation for both systems at 5, 25, and 60°C. In the whole range of temperatures, spectra show similar axially symmetric powder line shapes indicative of a random bilayer distribution with respect to the magnetic field (Davis, 1983). The central isotropic peak (1–2% of total spectral area) observed in the CS-containing sample was not detected in the corresponding ^{31}P-NMR powder pattern so that it is attributed to residual D_2O in the samples. Both quadrupolar splittings and chemical shift anisotropy of ^{31}P-NMR spectra (data not shown) are larger in the CH-containing systems, revealing a more ordered system in the presence of CH than in the presence of CS. At 5°C, the CH-containing spectrum exhibits very wide features that depart from axial symmetry, whereas for the CS-containing analogue, axial symmetry is still observed. From powder spectra, the first spectral moments, M_1, were calculated (Davis, 1979) and reported on Fig. 9 *A*. In this figure are also reported M_1 values for pure DMPC (data from Léonard, 1993). The first moment of

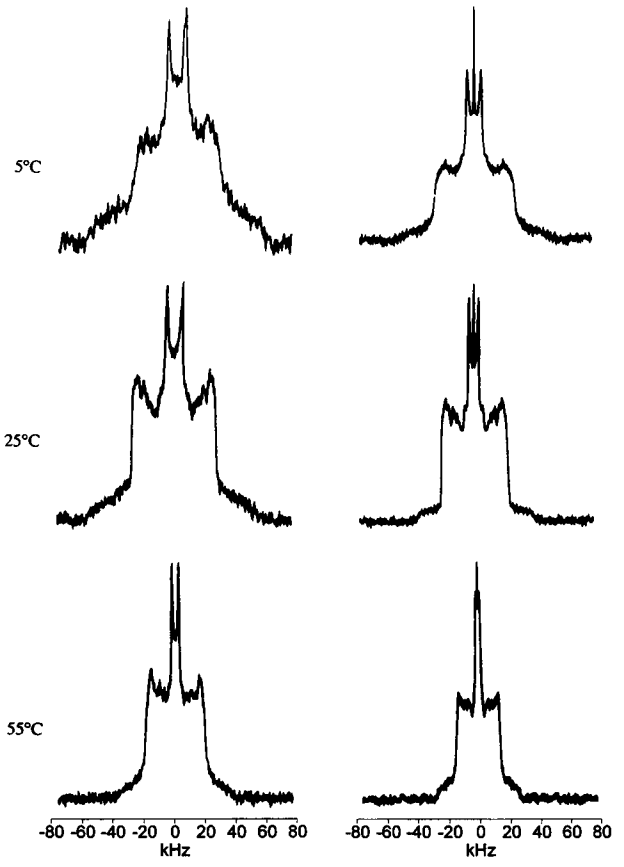


FIGURE 8 ^{2}H-NMR powder spectra of [*sn*-2-$^2H_{27}$]DMPC excess water dispersions in the presence of 30 mol % CH (*left*) or CS (*right*), at 5, 25, and 55°C. Data for the cholesterol-containing system are taken from Léonard (1993).

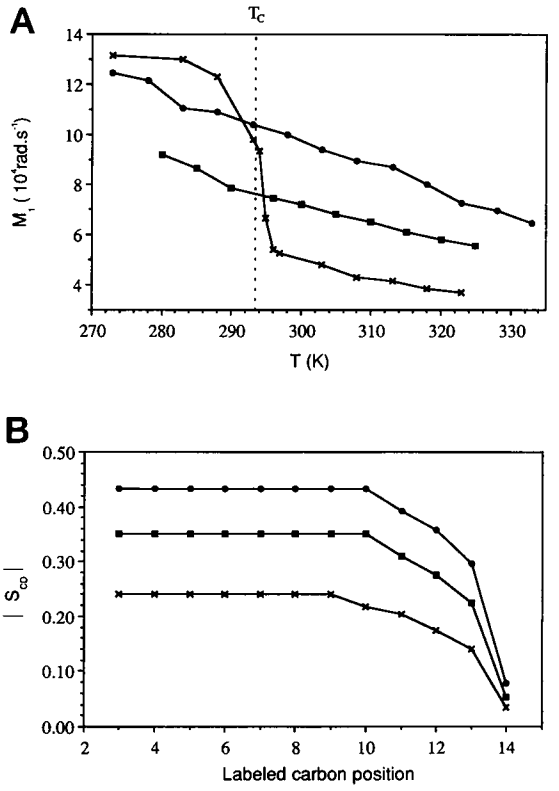


FIGURE 9 Comparative evolution of the first spectral moment, M_1, and the *sn*-2 chain segment order parameter, $|S_{CD}|$, for DMPC in the presence and absence of 30 mol % steroid. (*A*) Thermal evolution of M_1, T_C is the DMPC main phase transition temperature. (*B*) $|S_{CD}|$ as a function of the labeled carbon position at 25°C. (×) DMPC. (●) DMPC-CH (30 mol %). (■) DMPC-CS (30 mol %). DMPC-CH and pure DMPC data are taken from Léonard (1993). Data points are connected to help in reading the figure.

chain-perdeuterated lipid spectra is proportional to the mean chain order parameter (Davis, 1983; Ipsen et al., 1990), $\langle|S_{CD}|\rangle$, and thus gives information about the ordering properties of the hydrophobic core. The thermal variation of M_1 reveals that, whereas the DMPC chains are experiencing an order-disorder transition at $T_C \sim 21$°C, the presence of CH or CS seems to abolish that transition. Moreover, whatever the steroid incorporated in the membrane, chains are less ordered than in the native gel state ($L_{\beta'}$), for $T < T_C$, and more ordered than in the native fluid phase (L_α), for $T > T_C$. It is remarkable that this ordering effect is much more pronounced with CH whereas the disordering action at low temperature is greater with CS. Axially symmetric powder patterns can be de-Paked to afford the determination of order parameter profiles. ^{2}H-NMR spectra of perdeuterated lipid chains indeed consist of overlapping powder patterns (Fig. 8) arising from the inequivalent deuterated segments along the acyl chains (Davis, 1979). The de-Paking algorithm eliminates the powder part of the Pake doublet and thereby makes the determination of quadrupolar splittings, $\Delta\nu_Q$, easier. From each $\Delta\nu_Q$, the order parameter of each carbon-deuterium, $|S_{CD}|$, was calculated by taking a value of 168 kHz for the electric quadrupole coupling constant (Burnett and Müller, 1978). Assignment of order parameters to labeled positions was performed according to already published data (Oldfield et al., 1978). The resulting values are plotted in Fig. 9 *B*, as a function of labeled carbon position for the pure system (data from Léonard, 1993) and for the CH- and CS-containing systems, at 50°C. $|S_{CD}|$ values for labeled position 2 on the *sn*-2 chain are not reported because of the nonequivalence of deuterons at this position (Engel and Cowburn, 1981). As already mentioned with the main chain order parameter (Fig. 9 *A*), CH orders more the DMPC chains than CS at high temperature. Fig. 9 *B* shows that the effect is perceived in the same manner for all labeled positions.

DISCUSSION

From the above, results can be separated into two main parts: i) membrane hydration as evidenced by optical microscopy, x-ray diffraction, freeze-fracture electron microscopy, and ^{2}H-NMR of heavy water and ii) structure and dynamics of the membrane core as investigated by ^{2}H-NMR

of chain-perdeuterated phospholipids. In the following we will split the discussion accordingly.

Membrane hydration

The localization of the lamellar phase domain in the DMPC-CH-D_2O system is in agreement with the phase diagram built by Bourgès and co-workers (1967) on the egg lecithin-CH-H_2O system at room temperature. Differences between both diagram boundaries is less than 10 wt % in composition and may be partly accounted for by molecular weight differences between H_2O and D_2O. The strong similarities of both phase diagrams tend to prove that lamellar domain localization is little dependent on the phosphatidylcholine chain length, provided the system is above T_C of the pure phospholipid. From Fig. 1, it is clear that CS stabilizes the lamellar phase toward higher water contents. This indicates that the CS-containing membrane can accept much more water. Such a conclusion could be inferred from micrographs (Fig. 2), with, however, a lower accuracy, but is clearly confirmed by x-ray results. The lamellar repeat period of the CS-containing system at maximal hydration (label B, Fig. 1) is more than twice that of the CH-containing system: 147 ± 4 Å vs. 64 ± 2 Å, respectively. The latter value agrees well with the work of Mortensen and co-workers (1988) who found a repeat distance of 58 Å in DMPC-CH (24 mol %) systems at 30°C and that of Hui and He (1983) who measured a repeat distance of 66 Å in DMPC-CH (25 mol %) systems at 23°C. In our experimental conditions, CS is ionized, as the measured pD of D_2O is 5.2, whereas the sulfate moiety has a pK_a of approximately 3.3 (Bleau et al., 1974). Electrostatic repulsions between membrane surfaces due to the negative charge of the sulfate group may thus account for the greater swelling. This is clearly evidenced when heavy water is replaced by brine, as the lamellar repeat period at maximal hydration of a sample of the same composition (label B, Fig. 1) then drops from 147 to 86 Å. The screening of the sulfate moiety negative charge by sodium ions of the brine solution is therefore sufficient to markedly reduce electrostatic repulsions and hence membrane swelling as evidenced on other systems (Auguste et al., 1994). However, it remains to be determined whether the CS-driven increase in water incorporation results only from an increase in the amount of trapped water or whether there is also more water bound at the interface.

The number of bound water molecules per membrane unit cell can be easily determined from R_i^*(Eq. 5) but before discussing its values, we must comment upon the peculiar behavior of $\Delta\nu_{obs}$ versus $1/R_i$ observed for CH-containing lamellae at 10 and 23°C. Here indeed, Eq. 5 does not account for the observed behavior. The variation of the water quadrupolar splitting is linear but does not go through the origin; i.e., $\Delta\nu_{obs}$ follows a law of the type $\Delta\nu_{obs} = a/R_i + b$, where a and b are constants. Such an equation can indeed be derived (not shown) if one considers that there is a second lamellar phase, the amount of which linearly grows upon dilution. Such a situation could be encountered for DMPC-CH at 10 and 23°C. It has indeed been proposed in a recent publication (McMullen and McElhaney, 1995), a revised form of the DPPC-CH phase diagram. For 30 mol % CH, two liquid-ordered phases could be distinguished, $L_{o\beta}$ for low temperatures and $L_{o\alpha}$ for high temperatures, which would coexist over a large temperature range (25–58°C). Assuming that this phase diagram can be transposed to the DMPC-CH system providing an 18°C temperature shift (T_C(DPPC)-T_C(DMPC)) of phase boundaries, the $L_{o\beta}$ and $L_{o\alpha}$ would be present at 10 and 23°C. At 45°C, the system would be monophasic. From the above, it is clear that the number of bound water molecules per membrane unit cell, $2.3R_i^*$, can still be determined from the loss of linearity in the graph $\Delta\nu_{obs}$ versus $1/R_i$. However, this value has to be taken as the water globally bound to the membrane interface and does not provide hydration information about individual phases.

To summarize, the membrane unit cell made of 1 molecule of CH associated with 2.3 molecules of DMPC binds ~8 water molecules, whatever the temperature. When CH is substituted by CS, the membrane unit cell binds ~20 water molecules at 10 and 23°C and less than 5 water molecules at 45°C. These hydration differences could be attributed to i) an increase in DMPC hydration and/or ii) an increase in CS hydration versus CH. Case i could be explained by a conformational change of the choline headgroup. It has indeed been proven by ^{2}H-NMR of the deuterated choline group that incorporation of a charged amphiphilic molecule in a phosphatidylcholine membrane leads to a conformational change of the polar headgroup (Seelig et al., 1985; Marassi and Macdonald, 1991). Scherer and Seelig (1989) showed that the presence of the anionic molecule sodium didodecyl phosphate in a 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) membrane forces the N^+ end of the choline headgroup to reorient toward the membrane interior. The sulfate group may therefore influence the DMPC headgroup in a similar way, i.e., in moving the P^--N^+ dipole away from the water phase. If such a situation were to occur in the CS-containing system, the water-binding sites, i.e., the phosphate group and the *sn*-2 carbonyl group (Chiou et al., 1992) of the choline group would be less accessible so that DMPC would be less hydrated in the presence of CS than in the presence of CH. Although a change in the choline conformation cannot be totally excluded, it clearly cannot account for a greater hydration of the CS-containing membrane. It therefore appears that the hydration difference observed for both types of membranes can be attributed to a greater hydration of CS (case ii). By assuming that the number of water molecules bound to DMPC stays approximately the same in the presence of CS or CH at a given temperature, then CS would bind ~12 extra water molecules at 10 and 23°C. This is quite reasonable as the sulfate group contains more electron pairs, i.e., more potential hydrogen-binding sites, than the CH hydroxyl group.

A drastic drop in the amount of bound water to the CS-containing interface occurs at 45°C. The membrane unit cell would bind only 5 water molecules (see Results). Such a situation could be explained by a burying of CS in the membrane even though this is difficult to envisage because of the negative sulfate charge. We have to admit that up to now we have no satisfactory explanation of that peculiar hydration behavior. Further discussion is postponed until the determination of the accurate location of CS in the membrane. This can be implemented by neutron diffraction experiments of ^{2}H-labeled CS, as it has been performed for other membrane host molecules (Zaccai et al., 1979; Léonard, 1993; Pebay-Peyroula et al., 1994). Such experiments are in progress.

CS was shown to stabilize the bilayer organization in phosphatidylethanolamine or phosphatidylethanolamine/sphingomyelin mixtures whereas CH promotes the lamellar-hexagonal transition (Cheetham et al., 1990a,b; Kitson et al., 1992). The fact that CS can inhibit the hexagonal phase formation was attributed to its molecular shape according to the Israelachvili concept. Indeed, all factors that tend to increase the membrane area at the lipid/water interface versus that of the acyl chain region contribute to the lamellar phase stabilization (Israelachvili et al., 1977; Cullis and de Kruijff, 1979). Thereby, the stabilizing effect of CS, as compared with CH, was partly explained by postulating a greater hydration of the interface (Cheetham et al., 1990b; Kitson et al., 1992). We demonstrated herein that this is indeed the case and that, in addition, the negatively charged sulfate group promotes membrane swelling.

Dynamics of the membrane core

^{2}H-NMR of perdeuterated phospholipids allows us to follow the chain order in the bilayer by measurement of the first spectral moment, M_1, which affords calculation of the mean chain order parameter. Deuterium order parameters are influenced by intramolecular motions such as isomerizations between *trans* and *gauche* isomers as well as intermolecular motions such as the reorientation of the molecule as a whole (Meier et al., 1986; Douliez et al., 1995). When a lipid undergoes a transition from a well ordered phase (gel or crystalline) to a less ordered one (fluid), a decrease in the mean order parameter is observed in experiments (Fig. 9 *A*). This decrease has been interpreted as chains experiencing more conformational freedom (Davis, 1983). As can be seen in Fig. 9 *A*, this drastic decrease in M_1 values is no longer observed when 30 mol % CH or CS is incorporated into the membrane. Most of published work postulates that the incorporation of 30 mol % CH in dipalmitoylphosphatidylcholine (DPPC) membrane (Recktenwald and McConnel, 1981; Ipsen et al., 1987) or DMPC membrane (Almeida et al., 1992) leads to the formation of a liquid-ordered phase or β phase (Vist and Davis, 1990), whatever the temperature. As already mentioned, McMullen and McElhaney (1995) have postulated the presence of two liquid-ordered phases for that CH concentration in the DPPC-CH system. Two weakly energetic transitions have been detected by high-sensitivity differential scanning calorimetry (DSC) for DPPC membranes. One may then wonder why the evolution of the first moment with temperature displayed in Fig. 9 *A* does not present such transitions for CH-containing DMPC lamellae. A possible explanation is the absence of such transitions in CH-containing DMPC lamellae in the studied temperature range. However, this hypothesis seems to be denied by the anomaly observed at 10 and 23°C for the CH-containing system in the swelling regime. Therefore, it is likely that ^{2}H-NMR of chains is not sensible enough to detect transitions with very low cooperativity. Indeed, DSC analysis as well as ^{2}H-NMR of D_2O (Faure et al., 1995) show that the 30 mol % CS-containing system experiences a transition that is not observed in the temperature dependence of M_1 (Fig. 9). Its enthalpy (1.6 kcal/mol) is very comparable to that of a CH(15 mol %)-DMPC system (1.8 kcal/mol), the transition of which is still detected by ^{2}H-NMR of perdeuterated chains. Although both systems have approximately the same ΔH, they are characterized by different thermograms. The CS-containing system presents a broad asymmetric DSC peak sprayed over 35°C whereas the transition peak obtained in the presence of 15 mol % CH has a 15°C width, thus reflecting a greater cooperativity (data not shown). Therefore, the temperature dependence of M_1 does not seem to be sensitive enough to show broad transitions and would fail in the case of the DMPC-CH(30 mol %)-water system.

However, if Fig. 9 cannot provide information on transitions, it gives an idea of the mean order of the DMPC chains. It indicates that the effect of CH and CS on chain order is similar in that both steroids induce motion of the lipid acyl chains below T_c and inhibit them above T_c as compared with the pure phospholipid. The particular effect of CH to chain order is mainly attributed to the rigidity of its fused ring system that causes *gauche* conformers in surrounding chains to be less probable (Stockton and Smith, 1976; Oldfield et al., 1978; Douliez et al., 1995). Fig. 9 shows that the ordering effect of CS parallels that of CH and confirms the fundamental role of the hydrophobic part of the steroid for the chain order regulation. However, M_1 values indicate that the action of CS on chain order is lesser in magnitude than that of CH above T_C. This is in agreement with previous fluorescence experiments (Le Grimellec et al., 1984). The ordering effect of both steroids can be better estimated by calculation of the average DMPC acyl chain length, $\langle L \rangle$, in situations in which the system undergoes axially symmetric motion, i.e., in the high temperature phases. It can be calculated from intramolecular and molecular order parameters according to Douliez and co-workers (1995). At 45°C, the hydrophobic length from the C3 to C14 carbon is equal to 13.0 and 11.8 Å for the DMPC membrane associated with 30 mol % CH and CS, respectively, suggesting that the membrane containing CH is thicker than that containing CS. The acyl chains then adopt a less extended conformation in presence of CS.

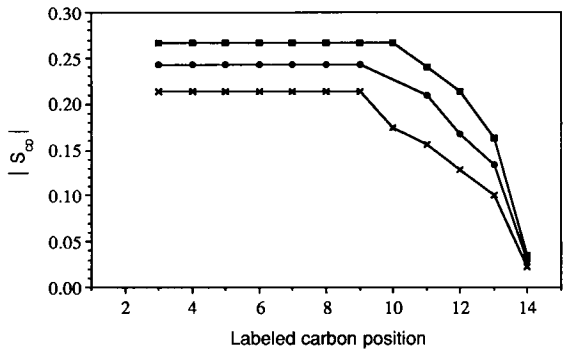


FIGURE 10 Comparative evolution of the *sn*-2 chain segment order parameter, $|S_{CD}|$ as a function of the labeled carbon position at 50°C. (×) DMPC-CH (10 mol %). (●) DMPC-CS (30 mol %). (■) DMPC-CH (20 mol %). DMPC-CH data are taken from Léonard (1993). Data points are connected to help in reading the figure.

To quantify the effect of CS with respect to that of CH, we plotted in Fig. 10 the evolution of the segment parameter order as a function of the labeled carbon position at 50°C in the presence of 30 mol % CS or in the presence of 10 and 20 mol % CH (data from Léonard and Dufourc, 1991). Quantitatively, Fig. 10 shows that, above T_C, the amount of CH necessary to produce an ordering effect comparable to that of 30 mol % CS is approximately 15 mol %. This may be attributed to different properties of the hydroxyl versus the sulfate group. Because of the greater hydrophilic character of the sulfate moiety, CS is more likely to be less embedded in the membrane than CH. This could lead to more disordered DMPC chains. However, a large difference in vertical location of both steroids would be reflected in order profiles; the plateau of order parameters would not include the same number of methylene groups. The other possible explanation to the order parameter difference is the headgroup hydration. We showed that the sulfate moiety binds more water than the hydroxyl moiety. The hydrated sulfate group is thus much more bulky than the hydroxyl group and CS would then act as a spacer; because of this steric hindrance, the DMPC acyl chains would be more away from the CS fused ring system than from that of CH. Therefore, the attractive van der Waals forces are likely to be less intense and hence the chains more disordered. Although our experiments seem to favor the latter explanation, the determination of CS location in the membrane by neutron diffraction experiments will definitely allow a conclusion to be made.

CONCLUSION

It has been shown herein that several physico-chemical techniques are needed to shed light on the hydration and ordering properties of membranes containing CS. A combination of x-rays and solid state NMR of heavy water was thus necessary to clearly attribute the greater hydration effect promoted by the sulfated molecule to an increase both in swelling and in water binding at the interface. Comparison of DSC and ^{2}H-NMR of chain-perdeuterated lipids regarding the detection of order-disorder transitions indicates that NMR may not be sensitive enough to detect transitions of weak energy and of poor cooperativity. It also appears that ordering properties of the bilayer core depend on a delicate balance between the attractive van der Waals forces and the repulsive steric and/or electrostatic interactions.

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REFERENCES

Abragam, A. 1961. Principles of Nuclear Magnetism. Oxford University Press, London.

Almeida, P. F. F. F., W. L. C. Vaz, and T. E. Thompson. 1992. Lateral diffusion in the liquid phases of dimyristoylphosphatidyl-choline/cholesterol lipid bilayers: a free volume analysis. *Biochemistry.* 31: 6739–6747.

Ansell, G. B., and J. N. Hawthorne. 1964. Phospholipids. Plenum Press, Amsterdam.

Auguste, F., P. Barois, L. Fredon, B. Clin, E. J. Dufourc, and A. M. Bellocq. 1994. Flexibility of molecular films as determined by deuterium solid state NMR. *J. Phys. II France.* 4:2197–2214.

Bleau, G., F. H. Bodley, J. Longpré, A. Chapdelaine, and K. D. Roberts. 1974. Cholesterol sulfate: occurrence and possible biological function as an amphipathic lipid in the membrane of the human erythrocyte. *Biochim. Biophys. Acta.* 352:1–9.

Bloom, M., J. H. Davis, and A. L. MacKay. 1981. Direct determination of the oriented sample NMR spectrum from the powder spectrum for systems with local axial symmetry. *Chem. Phys. Lett.* 80:198–202.

Bourgès, M., D. M. Small, and D. G. Dervichian. 1967. Biophysics of lipidic associations in the ternary systems cholesterol-lecithin-water. *Biochim. Biophys. Acta.* 137:157–167.

Burnett, L. J. F., and B. H. Müller. 1978. Deuteron quadrupolar coupling constants in three solid deuterated paraffin hydrocarbons: C_2D_6, C_4D_{10}, C_6D_{14}. *J. Chem. Phys.* 55:5829–5831.

Cheetham, J. J., R. J. B. Chen, and R. M. Epand. 1990a. Interaction of calcium and cholesterol sulfate induces membrane destabilization and fusion: implications for the acrosome reaction. *Biochim. Biophys. Acta.* 1024:367–372.

Cheetham, J. J., R. M. Epand, M. Andrews, and T. D. Flanagan. 1990b. Cholesterol sulfate inhibits the fusion of Sendai virus to biological and model membranes. *J. Biol. Chem.* 265:12404–12409.

Chiou, J. S., P. R. Krishna, H. Kamaya, and I. Ueda. 1992. Alcohols dehydrate lipid membranes: an infrared study on hydrogen bonding. *Biochim. Biophys. Acta.* 1110:225–233.

Cullis, P. R., and B. De Kruijff. 1979. Lipid polymorphism and the functional roles of lipids in biological membranes. *Biochim. Biophys. Acta.* 559:399–420.

Davis, J. H. 1979. Deuterium magnetic resonance study of the gel and liquid crystalline phases of dipalmitoylphosphatidylcholine. *Biophys. J.* 27:339–358.

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

Davis, J. H., K. R. Jeffrey, M. Bloom, M. I. Valic, and T. P. Higgs. 1976. Quadrupolar echo deuteron magnetic resonance spectroscopy in ordered hydrocarbon chains. *Chem. Phys. Lett.* 42:390–394.

Douliez, J. P., A. Léonard, and E. J. Dufourc. 1995. Restatement of order parameters in biomembranes: calculation of C-C bond order parameter from C-D quadrupolar splitting. *Biophys. J.* 68:1727–1739.

Dufourc, E. J., E. J. Parish, S. Chitrakorn, and I. C. P. Smith. 1984. Structural and dynamical details of cholesterol-lipid interaction as revealed by deuterium NMR. *Biochemistry.* 23:6062–6071.

Elias, P. M. 1983. Epidermal lipids, barrier function, desquamation. *J. Invest. Dermatol.* 80:44–49.

Engel, A. K., and D. Cowburn. 1981. The origin of multiple quadrupole couplings in the deuterium NMR spectra of the 2 chain of 1,2-

dipalmitoyl-*sn*-glycero-3-phosphorylcholine. *FEBS Letters.* 126: 169–171.

Epstein, E. H., M. L. Williams, and P. M. Elias. 1981. Steroid sulfatase, X-linked ichtyosis, and stratum corneum cell cohesion. *Arch. Dermatol.* 117:761–763.

Ernst, R. R., G. Bodenhausen, and A. Wokaun 1990. Principles of Nuclear Magnetic Resonance in One and Two Dimensions. Oxford University Press, London.

Faure, C., J. F. Tranchant, and E. J. Dufourc. 1995. An efficient method for detection of energetically low lipid phase transitions: deuterium NMR of D_2O. *J. Chim. Phys. France.* 92:1733–1737.

Fayrer-Hosken, R. A., B. G. Brackett, and J. Brown. 1987. Reversible inhibition of rabbit sperm-fertilizing ability by cholesterol sulfate. *Biol. Reprod.* 36:878–883.

Finer, E. G., and A. Darke. 1973. Phospholipid hydration studied by deuteron magnetic resonance spectroscopy. *Chem. Phys. Lipids.* 12: 1–16.

Hui, S. W., and N.-B. He. 1983. Molecular organization in cholesterol-lecithin bilayers by x-ray and electron diffraction measurements. *Biochemistry.* 22:1159–1164.

Ipsen, J. H., G. Karlström, O. G. Mouritsen, H. Wennerström, and M. J. Zuckermann. 1987. Phase equilibria in the phosphatidylcholine-cholesterol system. *Biochim. Biophys. Acta.* 87:162–172.

Ipsen, G. H., O. G. Mouritsen, and M. Bloom. 1990. Relationships between lipid membrane area, hydrophobic thickness and acyl-chain orientational order: the effects of cholesterol. *Biophys. J.* 57:405–412.

Israelachvili, J. N., D. J. Mitchel, and B. W. Ninham. 1977. Theory of self-assembly of lipid bilayers and vesicles. *Biochim. Biophys. Acta.* 470:185–201.

Kitson, N., M. Monck, K. Wong, J. Thewalt, and P. Cullis. 1992. The influence of cholesterol 3-sulfate on phase behaviour and hydrocarbon order in model membrane systems. *Biochim. Biophys. Acta.* 1111: 127–133.

Lampe, M. A., A. L. Burlingame, J. A. Whitney, M. L. Williams, B. E. Brown, E. Roitman, and P. M. Elias. 1983a. Human stratum corneum lipids: characterization and regional variations. *J. Lipid Res.* 24: 120–130.

Lampe, M. A., M. L. Williams, and P. M. Elias. 1983b. Human epidermal lipids: characterization and modulations during differentiation. *J. Lipid Res.* 24:131–140.

Langlais, J., and D. Roberts. 1985. A molecular membrane model of sperm capacitation and the acrosome reaction of mammalian spermatozoa. *Gamete Res.* 12:183–224.

Langlais, J., M. Zollinger, L. Plante, A. Chapdelaine, G. Bleau, and K. D. Roberts. 1981. Localization of cholesterol sulfate in human spermatozoa in support of hypothesis for the mechanism of capacitation. *Proc. Natl. Acad. Sci. USA.* 78:7266–7270.

Le Grimellec, C., A. Daigneault, G. Bleau, and K. D. Roberts. 1984. Cholesterol sulfate-phosphatidylcholine interactions. *Lipids.* 19: 474–477.

Léonard, A. 1993. Influence des stéroïdes sur les propriétés dynamiques et structurales de membranes biologiques modèles: une étude par RMN des solides et diffraction des neutrons. Ph.D. thesis. University of Bordeaux I, Bordeaux, France.

Léonard, A., and Dufourc, E. J.. 1991. Interactions of cholesterol with the membrane lipid matrix: a solid state NMR approach. *Biochimie.* 73: 1295–1302.

Lindbloom, G., L. Rilfors, J. B. Hauksson, I. Brentel, M. Sjölund, and B. Bergenstahl. 1991. Effect of head-group structure and counterion condensation on phase equilibria in anionic phospholipid-water systems studied by ^2H, ^{23}Na and ^{31}P NMR, and x-ray diffraction. *Biochemistry.* 30:10938–10948.

Marassi, F. M., and P. M. Macdonald. 1991. Response of the headgroup of phosphatidylglycerol to membrane surface charge as studied by deuterium and phosphorus-31 nuclear magnetic resonance. *Biochemistry.* 30:10558–10566.

McMullen, T. P. W., and R. N. McElhaney. 1995. New aspects of the interaction of cholesterol with dipalmitoylphosphatidylcholine bilayers as revealed by high-sensitivity differential scanning calorimetry. *Biochim. Biophys. Acta.* 1234:90–98.

Meier, P., E. Ohmes, and G. Kothe. 1986. Multipulse dynamic nuclear magnetic resonance of phospholipid membranes. *J. Chem. Phys.* 85: 3598–3614.

Mortensen, K., W. Pfeiffer, E. Sackmann, and W. K. Knoll. 1988. Structural properties of a phosphatidylcholine-cholesterol system as studied by small-angle neutron: ripple phase and phase diagram. *Biochim. Biophys. Acta.* 945:221–245.

Nallet, F., R. Laversanne, and D. Roux. 1993. Modelling x-ray or neutron scattering spectra of lyotropic lamellar phases: interplay between form and structure factors. *J. Phys. II France.* 3:487–502.

Oldfield, E., M. Meadows, D. Rice, and R. Jacobs. 1978. Spectroscopic studies of specifically deuterium labeled membrane systems: nuclear magnetic resonance investigation of the effects of cholesterol in model systems. *Biochemistry.* 17:2727–2740.

Pebay-Peyroula, E., E. J. Dufourc, and A. G. Szabo. 1994. Location of diphenyl-hexatriene and trimethylammonium-diphenyl-hexatriene in dipalmitoylphosphatidylcholine bilayers by neutron diffraction. *Biophys. Chem.* 53:45–56.

Rance, M. 1981. Deuterium nuclear magnetic resonance studies of unsaturated lipids in the membranes of *Acholesplasma laidlawii.* Ph.D. thesis. University of Guelph, Guelph, Canada.

Rance, M., and R. A. Byrd. 1983. Obtaining high-fidelity spin 1/2 powder spectra in anisotropic media: phase-cycled Hahn echo spectroscopy. *J. Magn. Res.* 52:221–240.

Recktenwald, D. J., and H. M. McConnel. 1981. Phase equilibria in binary mixtures of phosphatidylcholine and cholesterol. *Biochemistry.* 20: 4505–4510.

Roberts, K. D. 1987. Sterol sulfates in the epidermis: synthesis and possible function in the reproductive process. *J. Steroid Biochem.* 27: 337–341.

Salsbury, N. J., A. Darke, and D. Chapman. 1972. Deuteron magnetic resonance studies of water associated with phospholipids. *Chem. Phys. Lipids.* 8:142–151.

Sankaram, M. B., and T. E. Thompson. 1990. Modulation of phospholipid acyl chain order by cholesterol.: a solid state ^2H nuclear magnetic resonance study. *Biochemistry.* 29:10676–10684.

Scherer, P. G., and J. Seelig. 1989. Electric charge effects on phospholipid headgroups: phosphatidylcholine in mixtures with cationic and anionic amphiphiles. *Biochemistry.* 28:7720–7728.

Seelig, J., F. Borle, and T. A. Cross. 1985. Magnetic ordering of phospholipid membranes. *Biochim. Biophys. Acta.* 814:195–198.

Shapiro, L. J., R. Weiss, and D. Webster. 1978. X-linked ichtyosis due to steroid sulphatase deficiency. *Lancet.* 1:70–72.

Sternin, E., M. Bloom, and A. L. MacKay. 1983. De-Paking of nuclear magnetic resonance spectra. *J. Magn. Res.* 55:274–282.

Stockton, G. W., and I. C. P. Smith. 1976. A deuterium magnetic resonance study of the condensing effect of cholesterol on egg phosphatidylcholine bilayer membranes. *Chem. Phys. Lipids.* 17:251–263.

Taylor, M. G., T. Akiyama, and I. C. P. Smith. 1981. The molecular dynamics of cholesterol in bilayer membranes: a deuterium NMR study. *Chem. Phys. Lipids.* 29:327–339.

Ulmius, J., H. Wennerstrom, G. Lindblom, and G. Arvidson. 1977. Deuteron NMR studies of phase equilibria in a lecithin water system. *Biochemistry.* 16:5742–5745.

Ulrich, A. S., and A. Watts. 1994. Molecular response of the lipid headgroup to bilayer hydration monitored by ^2H-NMR. *Biophys. J.* 66: 1441–1449.

Van Deenen, L. L. M., and J. De Gier. 1964. The Red Blood Cell. Academic Press, New York.

Vist, M. R., and J. H. Davis. 1990. Phase equilibria of cholesterol/dipalmitoylphosphatidylcholine mixtures: ^2H-nuclear magnetic resonance and differential scanning calorimetry. *Biochemistry.* 29:451–464.

Yeagle, P. L. 1985. Cholesterol and the cell membrane. *Biochim. Biophys. Acta.* 822:267–287.

Zaccai, G., G. Büldt, A. Seelig, and J. Seelig. 1979. Neutron diffraction studies on phosphatidylcholine model membranes. II. Chain conformation and segmental disorder. *J. Mol. Biol.* 134:693–706.