A ^{2}H NMR Study of Macroscopically Aligned Bilayer Membranes Containing Interfacial Hydroxyl Residues

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ABSTRACT The polar interface of membranes containing phosphatidylglycerol or cholesterol was studied by ^{2}H nuclear magnetic resonance (NMR) as a function of membrane hydration. The membranes were macroscopically aligned and hydrated with deuterium oxide. Water uptake and membrane annealing was achieved under NMR control, using a novel hydration technique. Well-resolved ^{2}H quadrupolar doublets were obtained from individual hydroxyl residues and from the interlamellar water. The response of the phosphatidylglycerol headgroup and of the cholesterol molecule to the spontaneous evaporation of interlamellar water could be thus monitored continuously. It is shown that the phosphatidylglycerol headgroup undergoes changes of conformation and average orientation with respect to the membrane surface and that the off-axis motion of the cholesterol molecule decreases. The deuteron exchange between hydroxyl residues and surface-associated D_2O was determined by an inversion transfer technique. The exchange rates of the hydroxyl residues in the phosphatidylglycerol headgroup were different and depended strongly on the total hydration of the membrane. Significantly lower and almost hydration-independent rates were obtained for cholesterol. These results will be discussed with reference to earlier reports on the headgroup dynamics of phosphatidylglycerol and on the interaction of cholesterol with the membrane–water interface.

INTRODUCTION

Much research has been invested into an understanding of the spontaneous water uptake by phospholipid membranes (Cevc, 1993), with particular emphasis on the molecular response of the phospholipid headgroup (Bechinger and Seelig, 1991; Lindblom et al., 1991; Ulrich and Watts, 1994b; Volke et al., 1994b; Zhou et al., 1999) and on the development of interbilayer forces (Cevc et al., 1995; Simon et al., 1995; Israelachvili and Wennerström, 1996). Biomembrane hydration has been frequently studied by deuterium (^{2}H) nuclear magnetic resonance (NMR) with ordinary water being replaced by deuterium oxide (Finer and Darke, 1974; Cornell et al., 1974; Ulmius et al., 1977; Finer, 1979; Borle and Seelig, 1983a; Bechinger and Seelig, 1991; Klose et al., 1992; Volke et al., 1994a,b; Ulrich and Watts, 1994b; Hsieh and Wu, 1996; Faure et al., 1997). This technique exploits the sensitivity of the ^{2}H quadrupolar interaction with respect to interfacial order and dynamics. Planar membrane alignment on a solid support further enhances the intrinsic sensitivity of the ^{2}H NMR experiment and circumvents problems associated with the inhomogeneity of unoriented multibilayers (Koenig et al., 1997). The majority of these studies have dealt with single-component model membranes using synthetic lipids with zwitterionic headgroups. Less data are available, however, on the hydration of complex lipid mixtures, including cholesterol and charged lipids.

The present study is focused on the interaction of surface-associated water with lipid hydroxyl groups in the polar membrane interface. Two different examples of such lipids were considered, i.e., phosphatidylglycerol and cholesterol. Exchange of hydroxyl hydrogens for deuterium in the presence of surface-associated D_2O and macroscopic membrane alignment yielded well resolved ^{2}H quadrupolar doublets for the deuterated hydroxyl residues and for the interlamellar D_2O. Lipid mixtures containing phosphatidylglycerol were chosen as a paradigm for energy-conserving biomembranes. Proton diffusion within the polar membrane interface, which is believed to be essential for energy coupling in mitochondria and prokaryotes (Williams, 1988; Teissié et al., 1993; Lechner and Dencher, 1994; Teissié, 1996), may involve the hydroxyl groups of phoshoplipids such as cardiolipin or phosphatidyglycerol. These lipids typically represent major components of energy-conserving membranes (Neidleman, 1993). A sort of "proton wiring" (Pomès and Roux, 1998) was envisaged, e.g., it has been argued that the negative net charge of the phosphodiester moiety and the unesterified glycerol hydroxyl residue in the cardiolipin headgroup represent a charge-relay system capable of propagating protons at the membranes surface (Hübner et al., 1991). Lipids with the potential for interfacial hydrogen bond formation may be also important for the defense against environmental stress, e.g., dehydration of procaryotic organisms (Dowhan, 1993). Combining the alignment technique with ^{2}H NMR, we obtained separate quadrupolar splittings from the nonequivalent deuterated hydroxyl residues in the phosphatidylglycerol headgroup, which made it

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Abbreviations used: POPC, 1-palmitoyl-2-oleyl phosphatidylcholine; POPG, 1-palmitoyl-2-oleyl phosphatidylglycerol; EYPG, phosphatidylglycerol obtained by headgroup exchange from egg yolk lecithin; PC, phosphatidylcholine; PG, phosphatidylglycerol.

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possible to determine the individual OD/D_2O deuteron exchange rates.

The current knowledge of the interaction of cholesterol with phospholipids and interfacial water is still incomplete, despite the fact that the molecule is ubiquitous in eukaryotic membranes (for recent review articles see McMullen and McElhaney, 1996; Finegold, 1993). It is customary to assume that the 3-β-OH group of the molecule resides in the interfacial region of the bilayer where it may form transient hydrogen bonds (McMullen and McElhaney, 1996). Interactions with the available OH-bond acceptors, i.e., fatty acids carbonyls, unesterified oxygens of the headgroup phosphodiester moiety and water, have been demonstrated by molecular modeling (Robinson et al., 1995; Tu et al., 1998) and NMR spectroscopy (Le Guerneve and Auger, 1995). The functional significance of these interactions has not been fully established, however. The deuterated OH group can be used as a probe for H-bond formation and hydrogen exchange. It is shown here that the OD/D_2O exchange is approximately five times slower for cholesterol than for phosphatidylglycerol at full membrane hydration. This difference indicates that cholesterol preferentially forms hydrogen bonds with acceptors other than water. At the same time, however, our data argue against a long-lived hydrogen bond, e.g., with the fatty acid carbonyls of the surrounding phospholipids (Wong et al., 1989).

The orientation of cholesterol in liquid crystalline phospholipid membranes has been previously studied in great detail using ^2H NMR of multiple deuterated cholesterol analogs (Dufourc et al., 1984). The rigidity of the sterol backbone, as opposed to the flexible PG headgroup, facilitates a unique interpretation of the residual quadrupolar splittings in terms of molecular orientation and rotational symmetry. Therefore, a second ^2H label was introduced here into C-3 of the sterol skeleton to explore the molecular dynamics of the membrane–water interface as a function of interfacial hydration.

MATERIALS AND METHODS

Chemicals

Phospholipids (POPC, POPG, sodium salt) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL). The sodium salt of EYPG was from Sigma-Aldrich (Deisenhofen, Germany). Cholesterol, cholest-5-en-3-on, $LiAlD_4$ and deuterated *tert*-butanol ($(CH_3)_3COD$) were also purchased from Sigma-Aldrich. Deuterated cholesterol (β-(3-^2H)-cholesterol) was prepared from cholest-5-en-3-on essentially according to a published procedure (Wheeler and Mateos, 1958). Briefly, 100 mg $LiAlD_4$ was dissolved in 8 ml carefully dried tetrahydrofuran, and 0.5 ml $(CH_3)_3COD$ was slowly added while stirring at 0°C. One hundred milligrams of cholest-5-en-3-on in 8 ml tetrahydrofuran was added, and the solution was incubated for 1 h at room temperature. The mixture was then poured into 8 ml 10% HCl while stirring and cooling on ice. The aqueous solution was extracted three times with chloroform (50 ml, 25 ml, and 25 ml) followed by washing of the combined chloroform phase with 25-ml portions of 10% HCl, saturated $NaHCO_3$, and water, respectively. After drying over $CaCl_2$, the solvent was evaporated and the residue was recrystallized from hot ethanol. The resulting β-(3-^2H)-cholesterol was checked for purity by TLC and ^1H-NMR.

Sample preparation

A total of 30 mg of the lipids was dissolved in 5 ml of deuterated methanol (CH_3OD). The solution was spread evenly on 50 ultrathin glass plates (8 × 18 × 0.08 mm; Marienfeld Lab. Glassware, Bad Mergentheim, Germany) and dried for 20 min under a flow of warm air and then at room temperature for at least 18 h in vacuo (20–30 Pa). The glass plates with the dried lipid deposits were stacked on top of each other with gentle pressure and inserted, along with a pair of glass cylinder segments, into an open glass tube of 9.8 mm i.d. as shown in Fig. 1. Two small paper strips were soaked in D_2O and then dried carefully. Three μl D_2O were spotted onto each of the dry strips and the strips were attached at the short sides of the stack

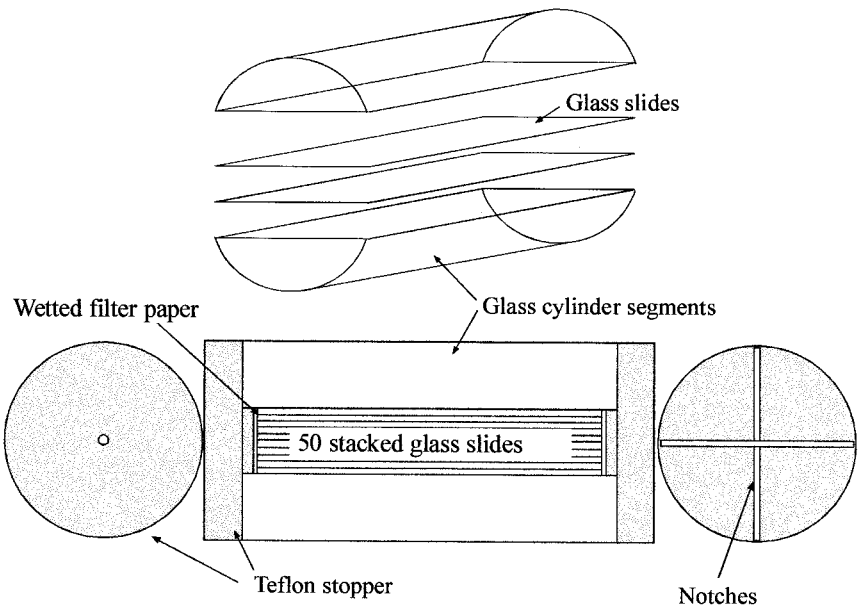


FIGURE 1 Arrangement of planar-oriented phospholipid membranes for ^2H NMR. Fifty lipid-coated glass plates were sandwiched between two glass cylinder segments and the nearly cylindrical assembly was inserted into a 10 mm o.d. glass tube. Two wetted paper strips were pressed against either side of the stacked plates by teflon stoppers. Solenoid coil and goniometer not shown.

(Fig. 1). The cylinder was rapidly stoppered by appropriately machined teflon plugs with silicon o-rings, and the membranes were annealed for 3 h at 40°C. Further hydration was achieved by repeating the process until the desired water/lipid ratio was achieved. The first three hydration steps yielded D_2O/lipid ratios of 10–15 mol/mol. The total time required for sample annealing was 12–15 h, depending on the number of hydration steps. Slow evaporation of the interlamellar water (from maximum hydration to a D_2O/lipid ratio of ~6 mol/mol; see Results) typically proceeded within two weeks. Stepwise hydration resulted in planar alignment of the membranes, whereas sudden addition of larger quantities of D_2O sometimes led to the formation of vesicular structures as shown by ^{31}P-NMR spectroscopy.

^{2}H-NMR

^{2}H-NMR spectra were recorded with a VARIAN VXR-400S spectrometer (^{2}H-frequency 61.4 MHz). ^{2}H-spectra of the macroscopically aligned lipid samples were acquired using a 10-mm i.d. flat wire solenoid coil. A home-built goniometer, attached to a stepper motor under software control, was used for accurate sample orientation with respect to the magnetic field. The annealed specimen was mounted into the goniometer and the orientation of the stack was varied systematically. The quadrupolar splittings $\Delta\nu_Q$ of the ^{2}H signal from surface associated D_2O were fitted according to $\Delta\nu_Q = \frac{1}{2}(3\cos^2\Theta - 1)\Delta\nu_Q^0$ where Θ denotes the angle between the normal to the membrane stack with respect to the magnetic field and $\Delta\nu_Q^0$ the maximum splitting. This yielded the required parallel orientation ($\Theta = 0°$) within $\pm 1°$. The progress of sample annealing could be also directly followed in the NMR spectrometer by the sharpening of the D_2O doublet components and by the minimization of a central D_2O signal (most probably arising from unoriented water). The quadrupolar echo sequence (Davis et al., 1976) was applied for signal excitation using composite pulses with a 90° pulse width of 7 μs, a pulse spacing of 20 μs and recycling time of 0.7 s.

The rate constants, k_{OD}, of the ^{2}H transfer from deuterated hydroxyl, OD, residues to D_2O were measured using the inversion transfer technique (Led and Gesmar, 1982). Selective inversion of the D_2O doublet was achieved by an appropriate low-power radio frequency pulse (90° pulse width, 140 μs) positioned at the center frequency in the spectrum. Excitation of lipid bound deuterons was excluded due to the large frequency dispersion in the macroscopically aligned membranes. The entire inversion transfer sequence was $(\pi)_{sel}$–Δ–$(\pi/2)_x$–τ–$(\pi/2)_y$–τ-acquisition, where the frequency selective 180° pulse, $(\pi)_{sel}$, inverts the D_2O signal and the nonselective 90° pulses, $\pi/2$, correspond to the usual quadrupolar echo sequence. The echo delay τ was typically 20 μs. Increasing the delay time Δ resulted in the expected time variation of the OD signals (cf. Fig. 6 *B*). Reference spectra were recorded after each acquisition in the inversion transfer series, and signal amplitudes were normalized with respect to the average amplitudes in the reference spectra. The D_2O signal was suppressed after signal acquisition by digital filtering (low-frequency signal suppression) to minimize baseline distortions and integration artifacts at the OD resonance positions. Thirty-five filter coefficients were used for a sharp filter cutoff (VNMR software, version 5.1). Inversion recovery experiments, i.e., $(\pi)_{nonsel}$–δ–$(\pi/2)_x$–τ–$(\pi/2)_y$–τ-acquisition, were performed immediately after the inversion transfer series to obtain the spin lattice relaxation time T_{1z} for the interbilayer D_2O, which was assumed to be independent of the deuteron exchange rate. Nine δ-increments and 64 transients per increment were sufficient for a reliable T_{1z} determination.

The inversion transfer results were evaluated on the basis of the coupled differential equations for a two-side exchange (Led and Gesmar, 1982)

$$dM^{OD}/dt = k_w M^w - k_{OD} M^{OD} - (M^{OD} - M_\infty^{OD}) R_{1OD}, \quad (1)$$

$$dM^w/dt = -k_w M^w + k_{OD} M^{OD} - (M^w - M_\infty^w) R_{1w}, \quad (2)$$

where M^{OD}, M^w, M_∞^{OD} and M_∞^W denote the magnetizations and the equilibrium magnetizations, respectively, of the OD deuterons and of the partially ordered D_2O. The normalized experimental data were fitted to Eqs. 1 and 2 by manual parameter variation (cf. Fig. 6) using Mathematica (Wolfram Research, Inc., Campaign, IL). The parameters R_{1OD} and R_{1w} are the longitudinal relaxation rates of OD (obtained by the fitting procedure) and of D_2O (determined experimentally) and k_w and k_{OD} refer to the forward and backward deuterium exchange according to ROD + D*OD $\rightleftharpoons$ ROD* + DOD, where the asterisk denotes magnetic labeling by selective inversion. The corresponding second-order rate constants, $k_{\rightarrow}$ and $k_{\leftarrow}$, can be formally expressed in terms of the deuteron exchange rates, i.e., $k_{OD} = k_{\rightarrow}$ [D_2O] and $k_w = k_{\leftarrow}$ [ROD]. A further condition is provided by the chemical equilibrium, i.e.,

$$k_w M_\infty^w = k_{OD} M_\infty^{OD} \quad (3)$$

The fitting was used separately for both quadrupolar components of the respective OD resonance, and the results were averaged taking account of the error propagation involved.

RESULTS

Macroscopically aligned bilayers containing phosphatidylglycerol

An equimolar mixture of POPC and EYPG was macroscopically aligned and hydrated with deuterium oxide as described in Methods. The orientation of the glass plates was chosen to give the maximum D_2O quadrupolar splitting, indicating that the normal to the glass plates was parallel to the direction of the magnetic field. The sealing of the glass tube (Fig. 1) was deliberately not completely tight, which led to a continuously decreasing water/lipid molar ratio in the sample. The evaporation rate was sufficiently slow (even at 37°C) so that the ratio was always nearly constant within the acquisition period of approximately 1 h. Thus, the spontaneous water evaporation permitted an investigation of interfacial membrane properties from full hydration to very low hydration under NMR control.

A series of ^{2}H-NMR spectra recorded over a range of hydration values is shown in Fig. 2. The strong doublets in the center of the spectra are due to interlamellar D_2O (quadrupolar splitting $\approx$1 kHz), whereas the small signals with quadrupolar splittings of $\approx$28 kHz can be attributed to the headgroup hydroxyl moieties of the PG component (with OD replacing OH). The deuterium oxide/phospholipid molar ratio, denoted by n_w, was calculated from the signal integrals, using sufficiently long relaxation delays to warrant full recovery of the OD and D_2O signals.

The observation of separate signals indicates that the chemical exchange among hydroxyl and water deuterons is slow on the NMR time scale. With decreasing hydration ($n_w < 20$) the hydroxyl doublet splits into two subspectra, which are tentatively assigned in Fig. 2 to the individual hydroxyl residues (β and γ, corresponding to the central and terminal segments of the glycerol headgroup, respectively; cf. Scheme 1). It must be noted that this assignment is based on the assumption that the γ-OD moiety has more motional

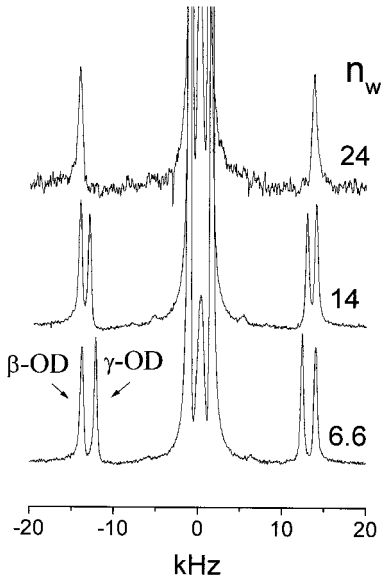
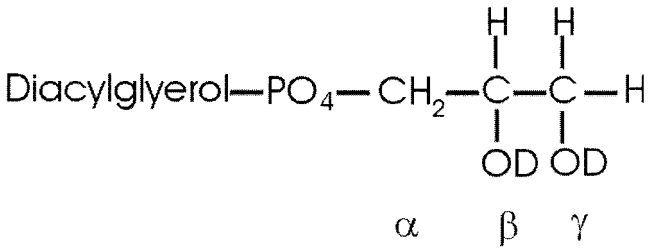


FIGURE 2 ^{2}H NMR spectra of planar-oriented membranes ($\Theta = 0°$) consisting of an equimolar mixture of POPC and EYPG (total amount of of phospholipid, 30 mg). The number of scans accumulated was 1024 for the top spectrum and 16,384 for the lower spectra. The hydration values n_w (molar ratio of D_2O/total phospholipid) were obtained by integration of the respective doublet signals. The singlett in the center of the spectrum arising from unoriented D_2O was removed by subtraction of an appropriate Lorentzian line before integration. Temperature, 30°C.



SCHEME 1

freedom at low hydration values (cf. Fig. 3 *A*) than the β-OD, resulting in a smaller quadrupolar splitting. Selective labeling of the PG headgroup would be necessary to confirm this assignment. The signal amplitudes of the β and γ-OD resonances are different as a result of varying line broadening.

The quadrupolar splittings $\Delta\nu_Q^\beta$ and $\Delta\nu_Q^{\gamma\beta}$ obtained over the entire experimentally accessible hydration range are summarized in Fig. 3 *A*. Three regions (designated as roman numerals in Fig. 3 *A*) can be identified where the PG headgroup signals respond differently to increasing hydration. Starting with $n_w \approx 5$, $\Delta\nu_Q^\beta$ increases with increasing hydration and reaches a plateau value of 28 kHz at $n_w \approx 9$, whereas $\Delta\nu_Q^\gamma$ increases monotonously until both resonances merge at $n_w \approx 20$. There is only one OD doublet when the interlamellar water level increases further. The increasing OD quadrupolar splittings in region I suggest that the development of a coherent layer of interfacial water results in a continuous change of the average headgroup orientation with respect to the membrane surface. Small changes of the dihedral angles within the PG headgroup probably lead to the coalescence of the OD-resonances in region II, whereas there may be hardly any change of the average headgroup conformation in region III, although the membranes take up another 20–30 mol of interlamellar water per lipid headgroup. Hydration of the mixed POPG/EYPC membranes was feasible up to $n_w \approx 60$ without loss of lamellar order, as was confirmed independently by ^{31}P NMR (data not shown).

The coalescence of the OD resonances reflects the changing headgroup conformation rather than an increasing interresidue deuteron exchange rate. This has been verified by running ^{2}H spectra of oriented PG/PC samples over a temperature range from 0°C to 50°C (experiments not shown). In samples with $n_w < 20$, there was no signal coalescence, even at 50°C, whereas the signal splitting did not reappear in the low temperature range in samples with $n_w > 20$. Moreover, the different line widths of the two signal pairs, as noted above (Fig. 2), suggest that exchange with the interlamellar D_2O occurs at different rates.

A plot of the quadrupolar splitting of the interlamellar D_2O versus n_w may be also tentatively divided into different regions (Fig. 3 *B*), i.e., there is a sudden increase of the splitting at low hydration (up to $n_w \approx 6$) followed by a region where the splitting decreases monotonously (from $n_w \approx 6$ to $n_w \approx 40$). It can be recognized, however, that this division differs from that in Fig. 3 *A*.

Bilayers containing cholesterol

A mixture of 30 mol% of double deuterated cholesterol (after exchange labeling of the hydroxyl group of β-(3-^{2}H)-cholesterol, the molecule will be denoted by cholesterol-d_2) with POPC was macroscopically oriented and hydrated with D_2O as described above. The covalent deuteration at C-3 and the exchange deuterated hydroxyl residue at the same carbon position yielded quadrupolar splittings of 87 kHz and 51 kHz, respectively, at 37°C and $n_w = 48$ (see top trace in Fig. 6 for a representative spectrum). These assignments were confirmed by hydration with H_2O instead of D_2O or by hydration of unlabeled cholesterol with D_2O, where only the respective resonance pairs remained. Likewise, addition of small quantities of deuterochloric acid (DCl) led to drastic line broadening of the OD component only (quadrupolar splitting ~51 kHz), indicating that acid catalysis results in rapid deuteron exchange between the cholesterol OD and the surrounding D_2O.

The D_2O quadrupolar splitting and the splittings of the cholesterol CD and OD deuterons ($\Delta\nu_Q^{D20}$, $\Delta\nu_Q^{CD}$ and $\Delta\nu_Q^{OD}$, respectively) decreased monotonously with increasing n_w (Fig. 4). The ratio $\Delta\nu_Q^{CD}/\Delta\nu_Q^{OD}$ was nearly constant over the entire hydration range ($10 \leq n_w < 60$; see insert in Fig.

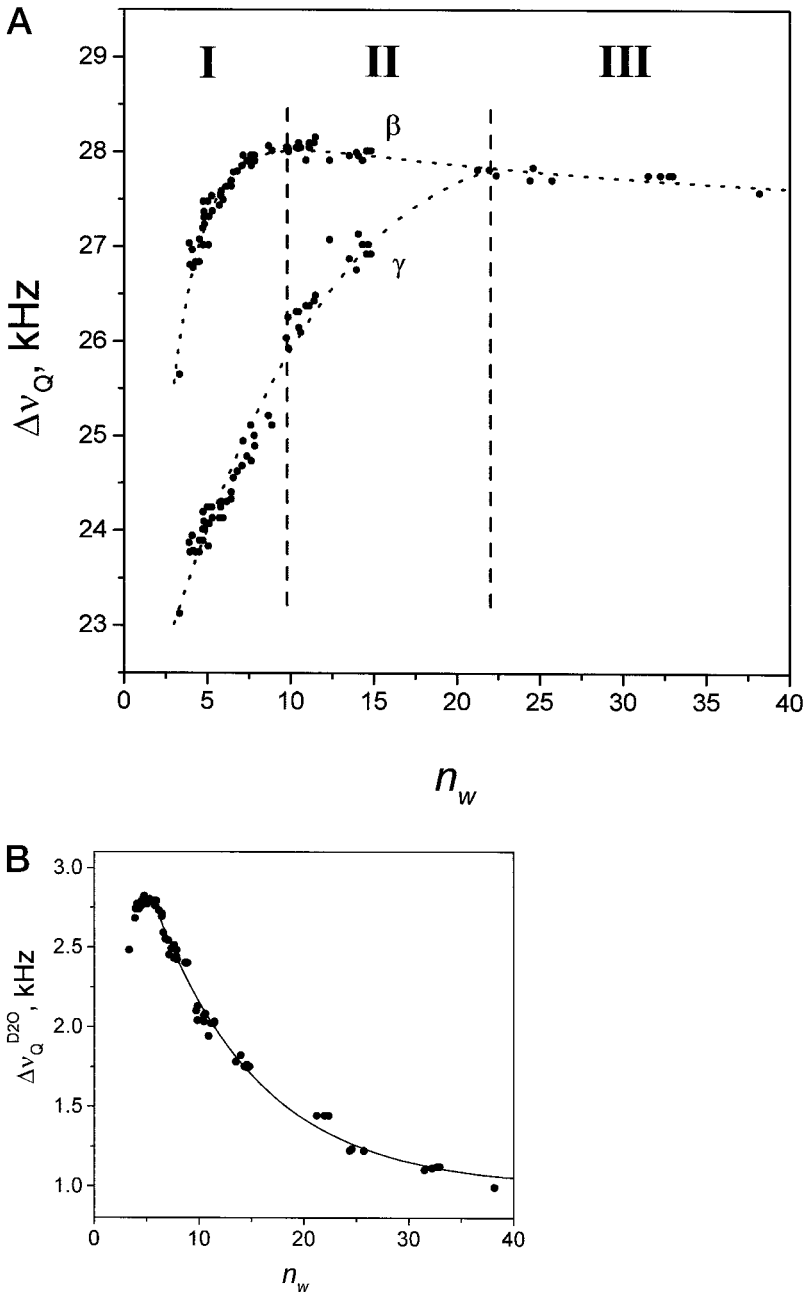


FIGURE 3 (*A*) Hydration dependence of the ^{2}H quadrupolar splittings of exchange deuterated PG. A mixture of 50 mol% of EYPG and 50 mol% of POPC was macroscopically oriented and slowly dehydrated within the receiver coil (cf. Fig. 1). Dotted lines connecting the data points are used to guide the eye and dashed bars to delineate the tentative hydration regions. For the signal assignment, see text. (*B*) Quadrupolar splittings of interlamellar D_2O in the same sample. The solid line represents a single exponential fit to the data (excluding data for $n_w < 10$), according to $\Delta\nu_Q^{D20} = A + B \exp(-n_w/C)$ where *A*, *B*, and *C* are adjustable parameters (see text). Temperature, 37°C.

4 *A*). This is to be expected when the rate of deuteron chemical exchange remains slow and the reorientation rate of the OD about the CO bond is fast with respect to the quadrupolar splitting. The deuteron exchange is indeed negligible with respect to the residual quadrupolar splittings as shown below. Thus, both deuterons reflect the increasing motional freedom of the sterol molecule when the membrane interface becomes increasingly hydrated.

It has been shown previously by ^{2}H NMR, that the cholesterol molecule undergoes axially symmetric motion in membranes with 30 mol% of cholesterol and well above the main phase transition of the host phospholipid (Dufourc et al., 1984). Axial molecular motion implies that the quadrupolar splitting of the 3-α-CD is associated with an order parameter S_{CD}, i.e., $\Delta\nu_Q^{CD} = \frac{3}{4}\chi(3\cos^2\Theta - 1)S_{CD}$, where χ denotes the quadrupolar coupling constant and Θ the orientation of the membrane normal with respect to the magnetic field. A molecular order parameter can be obtained providing that the principal axis of motion of the cholesterol molecule as a whole is known, i.e. $S_{mol} = S_{CD}/S_\beta$, where $S_\beta = \frac{1}{2}(3\cos^2\beta - 1)$ and β represents the rigid angle between the CD bond vector and the molecule fixed principal axis (Seelig, 1977; Dufourc et al., 1984). Dufourc et al. obtained $\beta = 84 \pm 2°$ in a mixture of

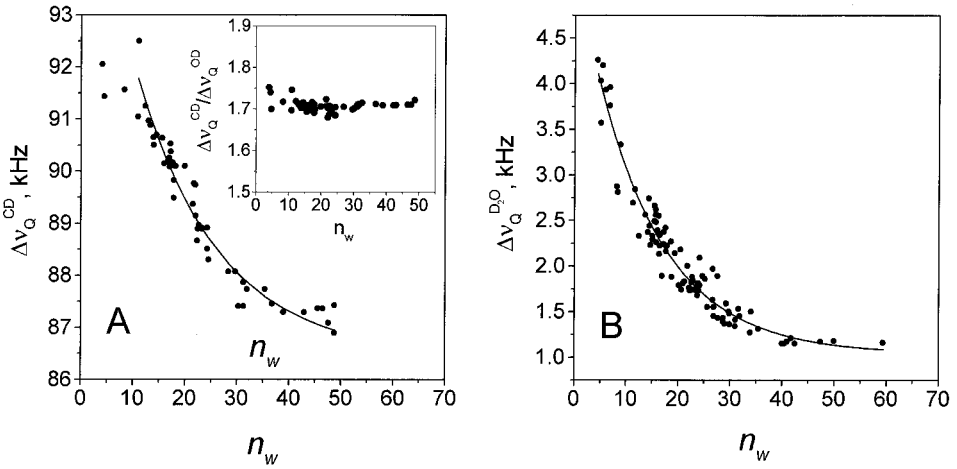


FIGURE 4 (*A*) Hydration dependence of the quadrupolar splittings $\Delta\nu_Q^{CD}$ of 30 mol% of cholesterol-d2 in POPC. The ratio of the splittings, viz. $\Delta\nu_Q^{CD}/\Delta\nu_Q^{OD}$, is shown in the insert. (*B*) Hydration dependence of the D_2O quadrupolar splittings. Temperature, 37°C. The solid lines are single exponential fits to the data as in Fig. 3 *B*.

1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) with 30 mol% of multiply deuterium-labeled cholesterol. Using this result and taking account of the membrane orientation $\Theta = 0$, the quadrupolar splittings obtained at $n_w = 48$ ($\Delta\nu_Q^{CD} = 87$ kHz) and $n_w = 10$ ($\Delta\nu_Q^{CD} = 91.5$ kHz) yielded $S_{mol} = 0.71$ and 0.74, respectively (37°C). This is less than the value of 0.79 observed in a mixture of 30 mol% cholesterol in DMPC at the same temperature (Dufourc et al., 1984) which may be a consequence of the disordering effect of the double bond in the sn-2 chain of the POPC molecule.

There was no reversal of $\Delta\nu_Q^{D20}$ on going to very low hydration ($n_w < 5$) as in the PG system (Fig. 4 *B*; cf. Fig. 3 *B*). The splittings were also slightly larger in the cholesterol membranes, e.g. $\Delta\nu_Q^{D20}$ was 3 kHz at $n_w = 10$ and 37°C, when n_w refers to the phospholipid only or 2.6 kHz when n_w includes the cholesterol component, as compared to 2.2 kHz in the POPC/EYPG mixture at the same hydration and temperature (both phospholipid components included in the calculation of n_w).

Further insight into the dynamics of the membrane interface was obtained from the spin lattice relaxation time T_{1z} of the interlamellar D_2O and of the sterol deuterons (Fig. 5). The T_{1z} values of the D_2O component increased from 18 ms at $n_w = 4$ to ~90 ms at $n_w = 20$. The spin lattice relaxation of the cholesterol OD resonance changed from 20 ms to an almost constant level of 50 ms, whereas the corresponding CD relaxation was less hydration dependent (~5 to 10 ms). The former observation indicates that the OD bond vector attains free rotation when the hydration ratio exceeds $n_w \approx 8$. The spin lattice relaxation times tended to a common value when $n_w \lesssim 5$, suggesting that partial breakdown of a continuous layer of interlamellar water severely restricts the free rotation of the interlamellar D_2O and of the entire cholesterol molecule.

Chemical exchange among hydroxyl and water deuterons

Inversion transfer experiments were performed at 30°C to determine the deuteron exchange rate according to Eqs. 1–3. The total time required for a set of inversion transfer and inversion recovery experiments was approximately 4.5 h. The water/lipid ratio decreased within this time range by a few percent, even when n_w was > 25. This error was accounted for by interpolation between the first and last spectrum of the series.

Representative ^{2}H-spectra of cholesterol-d_2 (30 mol% in POPC) and the corresponding fit to the experimental signal amplitudes according to Eqs. 1–3 are shown in Fig. 6, *A* and *B*, respectively. Only the signal pair with the smaller quadrupolar splitting (3-β-OD) is affected by the inversion transfer sequence, in agreement with the above signal assignment. The exchange rate constants were determined analogously for pure POPG membranes (spectra not shown). The measurements were made with pure POPG rather than with the PG/PC mixture to keep the total time for the experiment as short as possible. The hydration dependence was similar to that in the PC/PG mixture, except that the OD quadrupolar splittings were larger by approximately 5%. As expected, both ^{2}H doublets were affected by the inversion of the D_2O signal.

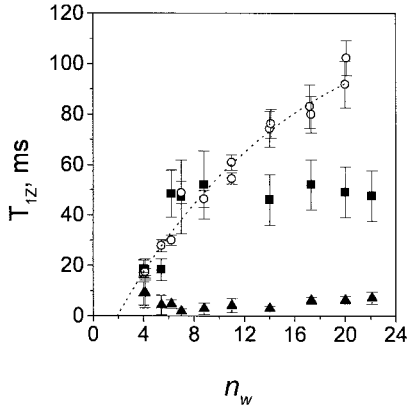


FIGURE 5 Spin lattice relaxation times (T_{1Z}) of interlamellar D_2O (*open circles*) and of the 3-β-OD (*squares*) and 3-α-CD deuterons (*triangles*) in a mixture of 30 mol% cholesterol-d_2 in POPC. *Dotted line*: second-order polynomial fit to the D_2O data to guide the eye. Temperature, 37°C.

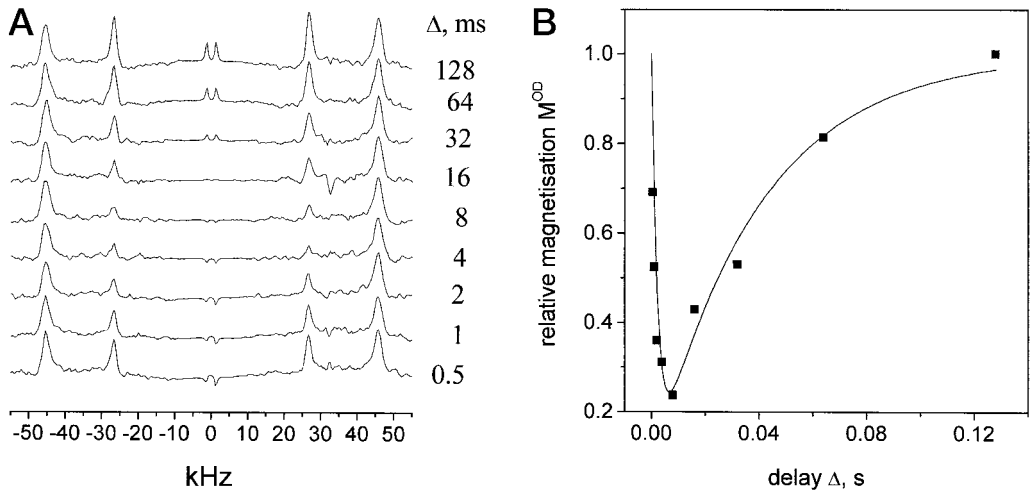


FIGURE 6 (*A*) Representative inversion transfer experiment for the determination of deuteron exchange in a 30 mol% cholesterol-d_2/POPC mixture. Δ, delay times in the inversion transfer experiment. Hydration ratio $n_w = 19$ mol of D_2O per mol of POPC. The D_2O signal was suppressed by digital filtering (see Methods). Temperature, 30°C. (*B*) Simulation according to Eqs. 1–3 to obtain a best fit to the normalized data from Fig. 6 *A*. An exchange rate $k_{OD} = 200\ s^{-1}$ was obtained from the data shown.

The results obtained for POPG and for cholesterol are summarized in Fig. 7. It can be recognized that the k_{OD} values for the POPG-headgroup strongly depend on the hydration ratio n_w, i.e., k_{OD} increases from 200 s^{-1} at $n_w \approx 6$ to $>1000\ s^{-1}$ at $n_w \approx 45$ for both OD signals in POPG. Specifically, k_{OD} is slightly smaller below $n_w \approx 8$ for the γ- than for the β-OD, whereas the opposite is true between $n_w \approx 10$ and 20, which is in qualitative agreement with the variable line broadenings of the individual resonances (cf. Fig. 2).

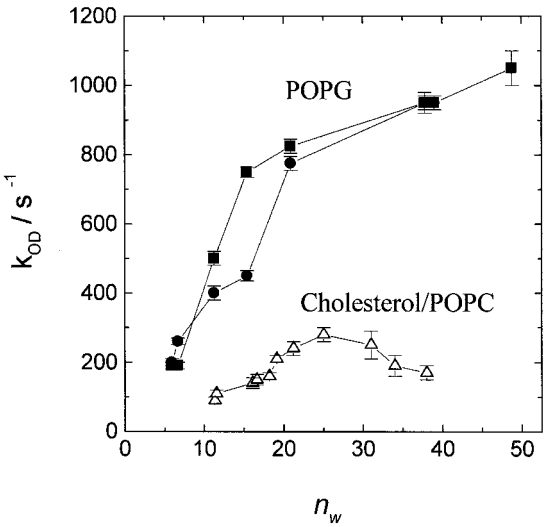


FIGURE 7 Summary of deuteron exchange rates k_{OD} versus hydration ratio n_w. *Solid circles*, β-OD of POPG; *solid squares*, γ-OD of POPG; *open triangles*, 30 mol cholesterol-d_2 in POPC. The n_w values represent the D_2O/POPC molar ratio. Temperature, 30°C.

The exchange rates for the cholesterol 3-β-OD deuteron are smaller, with a flat maximum at $n_w \approx 27$, and less dependent on the hydration ratio than those obtained for the PG headgroup residues. At the highest hydration level studied ($n_w \approx 38$) the OD–D_2O exchange rate was more than five times larger for the POPG hydroxyls than for the 3-β-hydroxyl of cholesterol-d_2 (Fig. 7).

DISCUSSION

The technique of lipid hydration and membrane alignment described in the present study differs from the conventional procedure where hydration is achieved by incubation of predried lipid deposits in an atmosphere of constant vapor pressure (Wieslander et al., 1978; Klose et al., 1992; Ulrich and Watts, 1994a; Volke and Pampel, 1995). The very slowly dehydrating lipid–D_2O multibilayers (cf. Fig. 1) are not strictly at thermodynamic equilibrium. This has been accounted for by keeping the change of the water/lipid molar ratio n_w during the total acquisition time of the NMR experiments <5%, whereas experiments with a higher evaporation rate were discarded. Advantages of this "close to equilibrium" hydration include the possibility to monitor water uptake and membrane annealing and to measure quadrupolar splittings, relaxation parameters, and deuteron exchange rates continuously over a broad range of water/lipid molar ratios in a single sample after the annealing has been established.

The short average lifetime of labile deuterons in amino- or hydroxyl residues makes it difficult to observe the corresponding ^2H NMR signals in randomly oriented lipid dispersions at physiological temperatures. It is shown here that homogeneous alignment of membranes between glass

slides yields well-resolved ^{2}H-signals from deuterium-exchanged hydroxyl residues, even if the water/lipid molar ratio is close to the maximum uptake capacity of the particular membrane. The exchangeability of headgroup and water deuterons affords an accurate determination of n_w by signal integration. The parallel orientation of the membrane normal with respect to the magnetic field results in the maximum effective quadrupolar splitting ($\Delta\nu_Q \propto 3\cos^2\Theta - 1$ with $\Theta = 0$), which meets the basic requirement $k_{OD} \ll \Delta\nu_Q$ for slow hydroxyl–water exchange on the NMR time scale. Water that is not associated with the lipid interface (isotropic water) can be easily excluded from signal integration, which represents a further benefit of using oriented versus nonoriented membranes.

Exchange labeling of the hydroxyl groups may help to elucidate the dynamics and the structural changes within the PG headgroup as a function of membrane hydration. The headgroup dynamics of phosphatidylglycerols in excess water have been studied previously by ^{2}H NMR after selective deuteration of the three glycerol carbons (Wohlgemuth et al., 1980; Borle and Seelig, 1983b; Marassi and Macdonald, 1991). Spin lattice relaxation-time measurements of labeled dipalmitoyl phosphatidylglycerol (Wohlgemuth et al., 1980) or of *E. coli* PG (Borle and Seelig, 1983b) led to the conclusion that the segmental fluctuations of the headgroup are similar to those of the zwitterionic lipids and even not much different from the mobility of neat gycerol (Borle and Seelig, 1983b). At 40°C, the quadrupolar splittings of the α, β, and γ segments of *E. coli* PG were 10.2, 4.5, and 0.8 kHz, respectively. Using order parameters rather than quadrupolar splittings makes a comparison with our aligned and exchange deuterated membranes practicable (cf. Figs. 2 and 3). Order parameters for the C_β-O and C_γ-O bond vectors in the headgroup glycerol moiety can be calculated from the observed OD quadrupolar splittings, taking account of the macroscopic alignment at $\Theta = 0$ (which yields twice the quadrupolar splitting of an unoriented sample). An order parameter $|S_{OD}|$ follows from $\Delta\nu_Q^{OD} = \frac{3}{2}\chi_{OD}|S_{OD}|$, where the bars denote absolute values. The quadrupolar coupling constant χ_{OD} has a value of 220 kHz for a deuteron in an OD bond (Soda and Chiba, 1969). Transformation according to $|S_{CO}| = (3\cos^2\beta - 1)^{-1}|S_{OD}|$ yields the desired order parameter of the C–O bond vector (Seelig, 1977). An average order parameter $|S_{CO}| = 0.12$ for the β and γ segments can be obtained from the OD splitting of 27.7 kHz, using a COD bond angle $\beta = 108.5°$ (see top spectrum in Fig. 2). The respective order parameters $|S_{CD}|$ in the selectively labeled *E. coli* PG at full hydration (Borle and Seelig, 1983b) were 0.08, 0.04, and 0.006 for the α, β, and γ segments. The larger order parameter value obtained from the OD quadrupolar splitting is likely to be due to the membrane alignment on a solid support and to the solidifying effect of intermolecular hydrogen bonding in the absence of excess water.

An evaluation of the OD quadrupolar splittings in structural terms is not straightforward. The orientation of the PG-headgroup with respect to the membrane surface has been examined earlier by small-angle x-ray (Watts et al., 1981) and neutron scattering techniques (Mischel et al., 1987). These experiments were performed in the ordered phase of DPPG or *E. coli* PG, respectively, at very low hydration ($n_w \approx 2.6$). An approximate tilt of 30° was derived from this data for the PG headgroup, although an alternative interdigitated structure could not be strictly excluded (Mischel et al., 1987). The scattering results and the NMR data may be not directly comparable, however, considering the different experimental conditions.

The counterdirectional change of the the β- and γ-quadrupolar splittings in region II of Fig. 3 *A* resembles the behavior of the α- and β-methylene headgroup signals in selectively ^{2}H labeled phosphatidylcholines where the α-splitting increases and the β-splitting decreases when the interlamellar water content is reduced below $n_w = 20$ (Ulrich et al., 1992; Bechinger and Seelig, 1991). The observation that a plot of $\Delta\nu_Q^\alpha$ versus $\Delta\nu_Q^\beta$ was almost linear with a negative slope was taken as evidence that the quadrupolar splittings reflect slight changes of the average orientation of the phosphocholine dipole rather than variations of the fluctuational amplitude of the headgroup (Bechinger and Seelig, 1991). The changes of $\Delta\nu_Q^\beta$ and $\Delta\nu_Q^\gamma$ shown in Fig. 3 *A* are more complicated, however. A plot of $\Delta\nu_Q^\beta$ vs. $\Delta\nu_Q^\gamma$ is not linear and its slope changes sign on going from region I to region II. A straightforward interpretation in terms of a unique conformational change in the PG headgroup is therefore not warranted here. The parallel decrease of the splittings with decreasing hydration (region I) is probably related to the breakdown of a continuous primary hydration shell, accompanied by the formation of an interheadgroup H-bonding network that replaces the headgroup–water interaction. The concomitant steep decrease of $\Delta\nu_Q^{D20}$ at low hydration values (Fig. 3 *B*) may be also a result of the reorganization of the membrane interface at $n_w < 8$. Borle and Seelig identified a primary hydration shell (bound water) of 10–16 water molecules per mol of PG by measuring relaxation times and D_2O line widths, whereas the D_2O quadrupolar splittings were not resolved in their nonoriented samples (Borle and Seelig, 1983b). Thus, it seems reasonable to assume that the counterdirectional change of $\Delta\nu_Q^\beta$ and $\Delta\nu_Q^\gamma$ in region II ($8 < n_w < 20$) is the result of slight changes of the headgroup torsion angles, whereas the decrease of $\Delta\nu_Q^\beta$ in region I ($n_w < 8$) is associated with a restructuring of the interface and probably with the formation of a more strongly hydrogen bonded state of the headgroup as noted above. Another possibility that must be considered is the replacement of D_2O molecules in the hydration shell of the sodium counterions with OD residues of the PG headgroup.

The majority of earlier studies on the modulation of the phospholipid bilayer by cholesterol were performed in the

presence of disaturated phosphatidylcholines. The prime focus was on the alteration of lipid-chain ordering and on the concomitant suppression of the phase transition in these systems (Finegold, 1993; McMullen and McElhaney, 1996). Comprehensive ternary phase diagrams, including unsaturated phospholipids and phospholipids other than phosphatidylcholine are still scarce, however (Vist and Davis, 1990; Thewalt and Bloom, 1992; McMullen and McElhaney, 1995). Thus, lateral segregation of the membrane into liquid-crystalline and liquid-ordered domains, which has been observed in mixtures of cholesterol with DMPC and DPPC, must be considered when dealing with hydration in mixed membranes. In a recent monolayer study, it has been shown that POPC with 30 mol% cholesterol remains in the fluid phase at applied lateral pressures of up to 40 mN/m, without phase separation (Worthman et al., 1997). This is also in line with an earlier study using small-angle x-ray scattering where lateral phase separation of the components was not detected at 21°C and 50 mol% cholesterol in egg PC (McIntosh et al., 1989). Therefore, it seems justified to assume that the POPC/30 mol% cholesterol membrane studied here at 30 and 37°C represents a homogeneous mixture even at low hydration values.

The quadrupolar splittings (Fig. 4) and the spin lattice relaxation data (Fig. 5) suggest that the cholesterol molecule acquires increasing motional freedom with increasing hydration of the membrane–water interface. At low hydration ($n_w \lesssim 10$), the deuteron signals from the doubly labeled sterol are rather broad, which results in ill-defined quadrupolar splittings. The spin lattice relaxation times, T_{1z}, in this region reflect the motional restriction of the cholesterol molecule and of the residual D_2O. Increasing hydration ($n_w > 10$) leads to a monotonous decrease and, eventually, to almost invariable $\Delta\nu_Q^{CD}$ and $\Delta\nu_Q^{OD}$ values, the ratio of which remains constant up to the maximum hydration attainable in the oriented system. An analysis of these relations in terms of the off-axis motion of the cholesterol molecule is beyond the scope of this article and will be given elsewhere. It may be noted that the maximum hydration value obtained in the present study ($n_w \approx 60$ with respect to the phospholipid component) is significantly higher than values reported previously for lecithin membranes without cholesterol (Rand and Parsegian, 1989; Morrison, 1993).

The dependence of $\Delta\nu_Q^{D20}$ on the degree of membrane hydration has been described by some authors in terms of a simple two-state model, assuming surface "bound" and interlamellar "trapped" water. This model predicts a linear relation between $\Delta\nu_Q^{D20}$ and $1/n_w$, providing that both bound and trapped water are present in the system (Finer and Darke, 1974; Lindblom et al., 1991; Faure et al., 1996; Wassall, 1996). The earlier assumption of discrete hydration shells with a unique $\Delta\nu_Q^{D20}$ in each individual hydration layer (Finer and Darke, 1974; Finer, 1979) certainly represents an oversimplification, however. Another model assumes exponentially decreasing individual D_2O splittings, leading to an average splitting $\langle\Delta\nu_Q^{D20}\rangle = A/n_w\ (1 - \exp(-n_w/C))$ with A and C being adjustable parameters (Volke et al., 1994b). This model is physically sound and gave acceptable results in nonoriented POPC dispersions (Volke et al., 1994b). A satisfactory fit to the present data was not obtained, however, probably as a result of the planar membrane alignment. Specifically, our values tend to a level of ~1 kHz at the highest hydration values (both for the PC/PG and for the PC/cholesterol membrane systems) rather than to zero quadrupolar splitting as suggested by the above equation. A different behavior was reported for a nonoriented dispersion of PG in D_2O where the quadrupolar splitting vanishes at $n_w \approx 40$ (Lindblom et al., 1991), probably as a result of diffusion along the curved multibilayer surfaces and exchange with water in packing defects.

Therefore, single exponentials were used here according to $\Delta\nu_Q^{D20} = A + B\exp(-n_w/C)$, without alluding to a particular hydration model (cf. Figs. 3 *B* and 4 *B*). The decay constant C for the POPC/cholesterol mixture (in mol D_2O per mol POPC, neglecting the cholesterol component of the mixture) from a fit to the data in Fig. 4 *B* (37°C) was 13.3 mol/mol and the limiting quadrupolar splitting $A + B$ was 5.35 kHz (a decay constant of 9 mol/mol is obtained when both components are considered in the calculation). A similar decay constant (15.7 mol/mol) was obtained when $\Delta\nu_Q^{CD}$ versus n_w was fitted analogously (Fig. 4 *A*), i.e. both quadrupolar splittings reflect the relaxation of the system into a fully hydrated equilibrium state. This may be compared with the decay of $\Delta\nu_Q^{D20}$ in the PC/PG mixture (Fig. 3 *B*) where the fit yielded $C = 10.0$ mol/mol and $A + B = 4.15$ kHz, respectively. An analogous experiment performed at 37°C with POPC alone (not shown) gave a decay constant of 6.9 mol/mol and a limiting splitting of 5.9 kHz. A similar difference between egg phosphatidylcholine and egg phosphatidylcholine/cholesterol mixtures has been previously observed by small-angle x-ray scattering with respect to the decay constants of the force-distance relation at hydration values $n_w \leq 10$ (Jendrasiak and Hasty, 1974; Marsh, 1989), whereas, to our knowledge, the PG/PC system has not been studied so far.

Proton exchange via chains of hydrogen-bonded surface residues is believed to be an essential step of the energy transduction in mitochondrial, bacterial, and photosynthetic membranes (Williams, 1988), although the extent to which this process involves the lipid headgroups is still a matter of debate (Teissié et al., 1993; Teissié, 1996). Hydrogen exchange between water and phospholipid ethanolamine headgroups has been studied previously in small sonicated vesicles, using the transverse relaxation of the water protons (Ralph et al., 1985). These authors presented a model where a rapid intrasurface H^+ exchange among the NH_2 groups is catalyzed by internal, hydrogen-bonded water molecules. This assumption accounts for the fact that the overall exchange rate was almost two orders of magnitude larger than expected when only catalysis by OH^- ions was considered

(Ralph et al., 1985). The overall exchange rates obtained here in an unbuffered PG membrane are similar to those reported for the vesicle system, e.g., at $n_w = 40$ the exchange rate $k_{OD} \approx 950\ s^{-1}$ (30°C), which compares favorably with $\approx 1500\ s^{-1}$ obtained by Ralph et al. at pH6 (25°C). Thus, it may be assumed that the exchange is accelerated by a hydrogen bond network that comprises both water molecules and headgroup residues, including the negatively charged phosphodiester moiety. A mechanism like this may be responsible for the steep decrease of k_{OD} in the aligned PG membrane when water evaporation ($n_w \leq 20$) results in the breakdown of a continuous hydrogen bond network (Fig. 7). It is noteworthy that the decrease of the exchange rates roughly coincides with the onset of the conformational change in the PG headgroup as indicated by the quadrupolar splittings (see regions I and II in Fig. 3 *A*). It may be also noted that, for an assessment of the effective hydration in the PG interface, it would be necessary to consider water binding by the sodium counterions, which reduces the number of water molecules available for headgroup hydration and intermolecular hydrogen bonding.

The exchange rate obtained in the POPC/cholesterol mixture is approximately five times lower at $n_w \approx 40$ than the rate obtained in the POPG membranes. This may be attributed to the embedment of the cholesterol molecule within the membrane, rendering the hydroxyl group much less accessible to water. It is also customary to assume strong hydrogen bonding with the phospholipid carbonyl groups. Neither of these assumptions is satisfactory, however. It has been demonstrated recently, using quasielastic neutron scattering, that diffusive motion in the direction of the membrane normal increases drastically when going from 20 to 36°C (Gliss et al., 1999). This is in line with a molecular dynamics study, where it was shown that the distribution profile of the hydroxyl oxygen along the bilayer normal has a width at half height of 7 Å and that the hydroxyl group interacts exclusively with water about half of the time (Tu et al., 1998). Although, in these studies, the cholesterol molecule was embedded in DPPC and the cholesterol concentration was significantly different (40 and 12.5 mol%, respectively), it cannot be maintained that cholesterol forms strong hydrogen bonds with the phospholipid carbonyl oxygens. It must be therefore assumed that the cholesterol exchange differs mechanistically from the deuteron exchange observed in the PG membrane surface, where the exchange may be catalyzed by the neighboring phosphodiester moiety.

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