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Chemical exchange between lamellar and non-lamellar lipid phases. A one- and two-dimensional ^{31}P-NMR study

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One- and two-dimensional ^{31}P-exchange NMR has been used to investigate chemical exchange between coexisting lamellar (L_α) and non-lamellar (hexagonal H_{II} and cubic I_2) lipid phases. Samples of DOPE, DOPE/DOPC (9:1 and 7:3), DOPE/cholesterol sulfate (9:1), DOPC/monoolein (MO) (3:7 and 1:1), and DOPC/DOPE/cholesterol (1:1:2) were macroscopically oriented on glass plates and studied at the 0° orientation (angle between the bilayer normal and the external magnetic field), where the L_α, H_{II}, and I_2 resonances are resolved. A reversible L_α to H_{II} transition was observed for all of the samples except for the DOPC/MO mixtures, which displayed a reversible L_α to I_2 transition. Near-equilibrium mixtures of L_α and either H_{II} or I_2 were obtained after prolonged incubation at a given temperature. Two-dimensional exchange experiments were performed on DOPE at 9–14°C for mixing times ranging from 500 ms to 2 s. For all samples, one-dimensional exchange experiments were performed for mixing times ranging from 100 ms to 4 s, at temperatures ranging from 3°C to 73°C. No evidence of lipid exchange between lamellar and non-lamellar phases was observed, indicating that if such a process occurs it is either very slow on the seconds' timescale, or involves an undetectable quantity of lipid. The results place constraints on the stability or kinetic behaviour of proposed transition intermediates (Siegel, D.P. (1986) Biophys. J. 49, 1155–1170).

Introduction

The ability of many membrane lipids to form nonbilayer structures, such as the hexagonal H_{II} and cubic I_2 phases, has prompted considerable research into the potential roles played by nonbilayer structures in biomembrane function [1–4]. Nonbilayer structures have been implicated in such phenomena as membrane fusion, transbilayer transport, and intermembrane communication [1]. Transient nonbilayer structures, such as those involved in fusion, are likely intermediates in the lamellar (L_α) to hexagonal (H_α) phase transition [5], even though hexagonal phase lipid has not been observed in a biological system except under pathological conditions [6]. Understanding the mechanism of this and other bilayer to nonbilayer transitions, therefore, has implications for a range of biological phenomena. Much progress has been made in this regard by Siegel [7–10].

One dynamic aspect of L_α to $H_{II}(I_2)$ phase transitions, which has received little experimental attention, is the rate of exchange of lipids between bilayer and nonbilayer phases at temperatures where they coexist. Because the L_α to H_{II} transition is rapid, reversible, and energetically favourable [11], it would be reasonable to expect a significant exchange of lipid at equilibrium on timescales similar to the phase transition times (0.3–3 s [11,12]). Similar considerations hold for the lamellar to cubic phase transition. This information could be important in verifying or refining the existing models for these transitions.

In the present paper, we investigate the chemical exchange between coexisting bilayer and nonbilayer phases, employing one- and two-dimensional solid-state ^{31}P-NMR techniques, which are included in the general class of magnetization transfer experiments (for a review, see Alger and Shulman, Ref. 13). Two-dimensional NMR is a powerful technique for studying exchange processes in both liquids and solids [14,15]. In

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Abbreviations: CS, cholesterol sulfate; DMPC, L-α-dimyristoylphosphatidylcholine; DOPC, L-α-dioleoylphosphatidylcholine; DOPE, L-α-dioleoylphosphatidylethanolamine; DPPC, L-α-dipalmitoylphosphatidylcholine; IMI, inverted micellar intermediate; MO, 1-monoolein; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine.

membrane systems, 2D ^{2}H-NMR has been used to probe slow molecular reorientations [16,17], and 2D ^{31}P-NMR to study phospholipid lateral diffusion [18]. Two-dimensional ^{31}P-NMR should be ideally suited to investigate the timescale of chemical exchange between coexisting L_α and $H_{II}(I_2)$ phases, as mixing times ranging from milliseconds to seconds can be probed. However, complications may arise if the membranes are studied as multilamellar dispersions, the 'standard' for most membrane NMR studies. Lateral diffusion of phospholipid over curved liposomal surfaces results in changes in the angle between the bilayer normal and the external magnetic field. In the 2D spectrum, this results in off-diagonal intensity between the regions of the powder pattern, which correspond to the orientations through which the lipids have diffused. Since the L_α and H_{II} powder patterns and the I_2 isotropic resonance overlap, chemical exchange between the phases could be obscured by exchange due to diffusion, as both processes may occur on similar timescales. While these problems may be surmountable, they can be avoided altogether by the use of macroscopically oriented samples [16,18–22]. When the angle between the bilayer normal and the external magnetic field is 0°, the L_α, H_{II}, and I_2 resonances will be maximally resolved. Thus, in the 2D experiment, any exchange would be revealed by off-diagonal cross-peak connectivities between the diagonal resonances. Furthermore, because the bilayers are flat and make only a single angle with respect to the magnetic field, the effects of lateral diffusion are removed.

Despite the usefulness of 2D exchange NMR, the technique suffers the drawback of the amount of time required to obtain an acceptable 2D spectrum. The same information can be achieved in much reduced time using a one-dimensional analogue of the 2D exchange experiment [23], thereby allowing longer mixing times to be probed (with acceptable S/N) in time periods where little or no change in the proportion of the phases occurs. We have applied both techniques to a sample of DOPE, and the latter technique to a wide variety of lipid mixtures, with lipids both neutral and charged. The results for both methods indicate that the exchange between L_α and H_{II} phases, and between L_α and I_2 phases, is either very slow (or nonexistent), or that only a small fraction of the lipid, less than the NMR detection limit, is undergoing exchange on these timescales. In either case, the two phases are seen to occupy essentially separate spatial and temporal domains at equilibrium or near-equilibrium conditions.

Materials and Methods

L-α-Dioleoylphosphatidylethanolamine (DOPE), L-α-dioleoylphosphatidylcholine (DOPC), and L-α-1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE) were obtained from Avanti Polar Lipids, Birmingham, AL. Cholesterol sulfate (CS) and 1-monooleoyl-*rac*-glycerol (MO) were obtained from Sigma.

Multilamellar dispersions were prepared for NMR by hydrating the lipid with at least a 3-fold excess of distilled water, and cyclically heating above the gel to liquid-crystalline phase transition temperature with vortex mixing and freeze-thawing to homogeneity (typically five cycles). Oriented samples were prepared essentially as described by Jarrell et al. [21] using method B. Approx. 40 glass slides, with dimensions varying from 3 × 13 mm to 8 × 13 mm, were cut from microscope cover slides. The plates were stacked in a 10 mm (o.d.) open-ended NMR tube, and rinsed several times with methanol, which was removed under high vacuum. The plates were then removed from the NMR tube. Between 10–50 mg of a given lipid or lipid mixture, dissolved in $CHCl_3$, was evenly applied to the plates, allowing the solvent to dry between applications. The plates were restacked in the NMR tube, and traces of $CHCl_3$ were removed under high vacuum overnight. Hydration of the lipid was accomplished by placing the tube in a sealed 25 ml liquid scintillation vial containing 1 ml water at room temperature for 3–4 days. Samples containing MO were hydrated under an atmosphere of N_2. Prior to sealing the NMR tube, 1–3 tiny drops of water were gently placed on the edges of the glass plates.

^{31}P-NMR spectra were acquired at 81.0 MHz on a Bruker MSL-200 spectrometer. One-dimensional spectra were recorded using a Hahn echo pulse sequence [24] with WALTZ decoupling (gated on during acquisition). The ^{31}P $\pi/2$ pulse length was 4.0 μs (10 mm solenoid coil), the pulse spacing was 60 μs, and the recycle time was 5.0 s. Two-dimensional spectra were recorded as described by Fenske and Jarrell [18], using the NOESY pulse sequence with TPPI (used on Bruker spectrometers) to give quadrature detection in both dimensions [25]:

$$[(\text{preparation}) - 90° - t_1(\text{evolution}) - 90° - t_{mix} - 90° - t_2(\text{detection}) - (\text{delay})]$$

WALTZ ^{1}H-decoupling was gated on during the evolution and detection periods. Preparation of the system was achieved by including 16 dummy scans at the beginning of each serial file, t_{mix} varied from 500 ms to 2 s, and the recycle delay was either 2 or 5 s. The evolution time t_1 was 3 μs for the first serial file, and was incremented by the dwell time (50 μs) for each of the 64 serial files. The datasets were 256 points zero-filled to 512 points in the F_2 dimension, and 64 points zero-filled to 512 points in the F_1 dimension. 96 transients were recorded for each serial file in a given 2D experiment. The spectral width in both dimensions was

10 kHz. Other parameters were as described for the one-dimensional experiments.

One-dimensional (1D) exchange experiments were performed essentially as described by Connor et al. [23]. The pulse sequence used was the NOESY sequence with WALTZ decoupling described above for the 2D experiments. The differences lie in the phase cycling, the placement of the transmitter frequency, and the use of a fixed evolution time t_1. For a system with two (potentially) exchanging sites L (lamellar) and H (hexagonal), with off-resonance frequencies ν_L and ν_H, the transmitter frequency is set such that $\nu_L = 2(\nu_H)$, and t_1 is set to satisfy $t_1 = (1/4)\ \nu_H^{-1} = (1/2)\ \nu_L^{-1}$. At the end of the evolution time, the magnetization vectors for the spins, M_L and M_H, are separated by 90°, and M_L has the same phase as the applied rf field of the second 90° pulse. Thus only M_H is rotated into z during the mixing time. If exchange processes occur during t_{mix}, then a magnetization vector M_L will form in z, and both a lamellar and hexagonal resonance will be recorded during t_2, with the intensity of the lamellar resonance being a function of t_{mix}. If no exchange occurs, then only a hexagonal resonance will be present in the final spectrum. For most samples, the value of t_1 calculated from the above relation required slight adjustment to null the L_α resonance for short mixing times. For example, the value of t_1 calculated for one of the DOPE experiments was 142 μs. For this value of t_1 and $t_{mix} = 100$ ms (for which no exchange would be observed in the 2D experiment), some negative intensity was observed for the lamellar resonance. A level baseline in the lamellar region of the spectrum was achieved using $t_1 = 135$ μs, which was, therefore, used for all longer mixing times. The phase cycling scheme used for the 1D exchange experiment is given below:

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pulse 1:  Y -Y
pulse 2:  8(X) 8(-X)
pulse 3:  X X -X -X Y Y -Y -Y
receiver: X -X -X X Y -Y -Y Y -X X X -X -Y Y Y -Y
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In choosing values of t_{mix} in the 1D exchange experiment, it is important that $t_{mix} \gg T_2$, otherwise the 'cross-peak' will be 90° out-of-phase with the 'diagonal' peak [23]. In phospholipid bilayers, an angular-dependent transverse relaxation rate of the form:

$$1/T_2(\beta) = A + B|3\cos^2\beta - 1|/2$$

is obeyed, with $A = 50$ Hz and $B = 200$ Hz [26] giving a reasonable fit to experimental spectra. Thus $T_2 < 10$ ms for all angles, which agrees with T_2 values estimated from the linewidths of the oriented sample. No t_{mix} values less than 100 ms were examined.

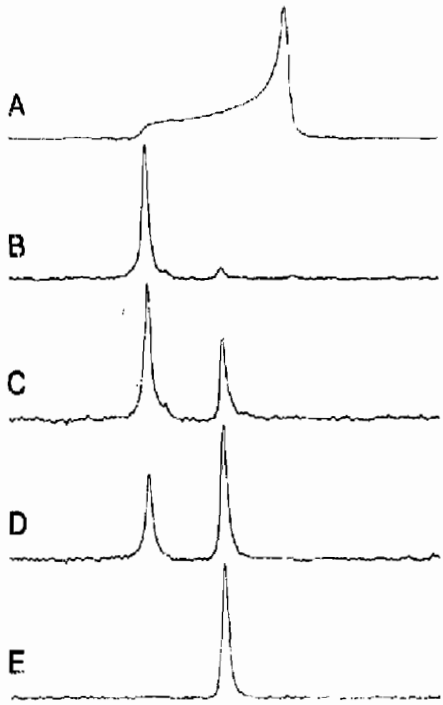

Fig. 1. ^{31}P-NMR spectrum of POPE multilamellar dispersions acquired at 30°C (A). Representative ^{31}P-NMR spectra of DOPE macroscopically oriented at an angle of 0° (angle between bilayer normal relative to external magnetic field) at 0°C (B), 8°C (C), 13°C (D) and 23°C (E). Number of acquisitions = 1024 (A), 64 (B–E). The plot width is 10 kHz.

Results and Discussion

Chemical exchange between lamellar and hexagonal phase lipid

DOPE is a well-characterized lipid which undergoes a lamellar to hexagonal phase transition over the range of 5–15°C [2,27]. Temperature-dependent ^{31}P-NMR spectra of DOPE macroscopically oriented on glass plates are shown in Figs. 1B–E for an orientation of 0° (angle between bilayer normal and magnetic field). At 0°C, the lipid exists in the lamellar phase (Fig. 1B), with a chemical shift identical to that of the 0° shoulder of the ^{31}P-NMR powder pattern of POPE shown in Fig. 1A. As the temperature is raised the proportion of the hexagonal phase increases, with the phase transition complete by 23°C (Figs. 1C–E).

The temperature range over which the DOPE transition occurs is somewhat greater than that observed in multilamellar dispersions. This is expected, as it is easier to detect small quantities of the L_α and H_{II} phases in an oriented spectrum, where the two peaks

are resolved, with the magnetization localized in a small frequency range, than in a powder, where the resonances overlap and are spread out over a much larger frequency range. However, results obtained from oriented samples and multilamellar dispersions may differ as a result of reduced hydration in the oriented systems. Variations in water content may affect the location of a mixture on the lipid-water phase diagram, thereby altering the relative proportions of bilayer and nonbilayer phases over a given temperature range. However, since the mechanism of the phase transition will remain unchanged, these variations should not alter lipid exchange rates. The fact that the transition occurs rapidly over a similar range with the same midpoint (about 10°C [27]) in both DOPE systems demonstrates that any differences are minor.

Previous investigations of the lamellar to hexagonal phase transition have yielded a great deal of mechanistic information, with which our results are in general agreement. Early ^{31}P-NMR studies on oriented egg PE bilayers provided evidence for the bilayer-hexagonal transition occuring as an interbilayer event [20], a conclusion supported by our similar results with DOPE and the other lipid systems. Furthermore, the linewidths of the L_α resonance remain constant throughout the transition (Fig. 1), indicating that the bilayers retain their orientation as they undergo the conversion to H_{II} phase at a rate rapid on the NMR time scale. If the bilayer orientation was altered during the transition, a broadening of the L_α resonance could be observed. Similar conclusions have come from real-time X-ray diffraction studies of the L_α to H_{II} transition [11]. Finally, as assessed by NMR, only the L_α and H_{II} phases are detectable during the transition; no intermediates are observed. This is also in agreement with real-time X-ray diffraction studies [11]. However, we cannot rule out the presence of intermediates too short-lived to be observed by NMR, or with the same symmetry as either the L_α or H_{II} phases.

The fact that the L_α and H_{II} resonances are resolved by NMR demonstrates that the two phases are in slow exchange on the NMR timescale. This provides an upper limit to the molecular exchange rate between the two phases. The spectral lines of a phospholipid in two phases, L and H, with frequencies ν_L and ν_H, respectively, will show well-resolved peaks if the molecular exchange rate $\tau_{ex}^{-1} \ll |\nu_L - \nu_H|$ [28]. The L_α and H_{II} resonances are separated by 1740 Hz; thus the exchange time $\tau_{ex} \gg 0.6$ ms. Any exchange between the two phases must occur on timescales significantly longer than 1 ms. This is in the regime where molecular motions are too slow to be studied by lineshape and spin-lattice NMR approaches. In contrast, 2D NMR (and 1D experiments based on similar pulse sequences) provide information on slow motion processes which occur in the frequency range of 10^3–10^0 Hz [15,17]. Aside from ^{2}H-NMR spin echo experiments [29,30], few other NMR techniques are suitable to the problem of exchange in the slow motion regime.

The principles of the 2D exchange experiment are described in the original work by Jeener et al. [14], and the application of ^{31}P 2D solid-state NMR to membrane systems is given by Fenske and Jarrell [18]. For the present discussion, it is sufficient to note that, in the 2D spectrum, components which do not exchange are located on the diagonal. The presence of off-diagonal cross-peaks connecting diagonal elements indicates chemical exchange between the connected resonances.

In order to measure chemical exchange between the lamellar and hexagonal phases, it is necessary that the system be at (or close to) equilibrium, where the rate of the forward and reverse transitions are equal. In practice, this is difficult to achieve. Although the L_α and H_{II} phases will coexist over a period of days in the mixed phase region, it takes several hours for the intensity ratio of the L_α and H_{II} resonances (L/H) to stabilize at a given temperature, and then slight variations in the intensities can be observed over a period of hours or days following. As the L/H ratio is temperature sensitive, this may be due to drift in the temperature control unit (which fluctuates over +/− 1 C°). Thus, prior to and immediately following each experiment, the 1D spectrum was recorded. For most of the experiments discussed below, the L/H ratio was essentially constant over the course of the experiment, which in some cases required as long as 14 h. However, in a few cases the L/H ratio changed significantly over a span of 5–12 h. Nevertheless, the results were the same in these cases as in the former, demonstrating that the L/H ratio was constant over t_{mix}, the time regime during which exchange was measured.

Two-dimensional exchange experiments were performed between 9 and 14°C for t_{mix} values ranging from 500 ms to 2 s; representative stacked plots are shown in Fig. 2. The L_α resonance is located in the upper left corner. No cross-peaks are observed, indicating no exchange between the L_α and H_{II} phases on a time scale up to 2 s. For $t_{mix} = 500$ ms (Fig. 2A), the ratio L/H changed from 0.9 prior to the experiment to 2.3 following the experiment, a period of 5 h. In this case, the rate of the transition L_α to H_{II} is greater than H_{II} to L_α, the latter of which would better approximate the equilibrium condition. The lack of any observable exchange indicates that none would be observed for the equilibrium condition. This is verified by the results for $t_{mix} = 1.5$ s (not shown) and 2 s (Fig. 2B), whose L/H ratios were essentially unchanged over 14 h. The decrease in S/N observed for the longer mixing time is due to intensity losses during t_{mix} resulting from longitudinal relaxation. The ^{31}P T_1 is of the order of 2 s at this field, and thus significant intensity loss will be observed for $t_{mix} > 2$ s. This illus-

trates a serious limitation in the 2D experiment, which is the time required to acquire a spectrum. Probing $t_{mix} > 2$ s would require a significant increase in the number of acquisitions to achieve an acceptable S/N, requiring spectrometer time in the order of days. Clearly this is unrealistic. In cases like this, where the timescale of interest is known from the 2D results, further investigation of the exchange processes is best achieved using the 1D analogue of the 2D exchange experiment [23]. In the present case, this allows mixing times up to 4 s to be probed, and spectra for $t_{mix} = 2$ s can be obtained in 2 h with 10-times the number of scans as were obtained in a 2D experiment which required 14 h.

In order to ensure the suitability of the 1D experiment for probing exchange processes in membrane systems, preliminary studies were performed on multilamellar dispersions of POPE at 30°C (not shown). The information obtained from this method should be equivalent to that obtained from 2D NMR; a given 1D exchange spectrum will simply be a slice of a 2D spectrum corresponding to that particular value of t_1. Approx. 3300 Hz separate the 0° shoulder and 90° edge of the POPE ^{31}P-NMR powder pattern (see Fig. 1). By setting the transmitter frequency 3300 Hz upfield from the 90° edge, and choosing $t_1 = 70$ μs, the magnetization near the 0° shoulder was observed to disappear for short mixing times (10–100 ms), and reappear for longer

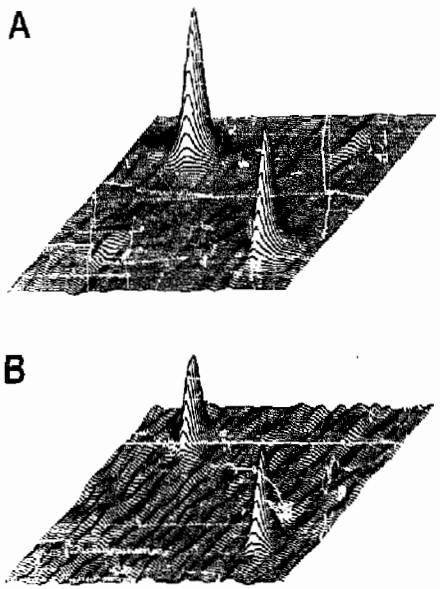

Fig. 2. 2D exchange spectra of oriented DOPE (0°-orientation) at 9°C and 14°C for $t_{mix} = 500$ ms and 2 s, respectively (A, B). The plot width is 3.2 kHz in both dimensions.

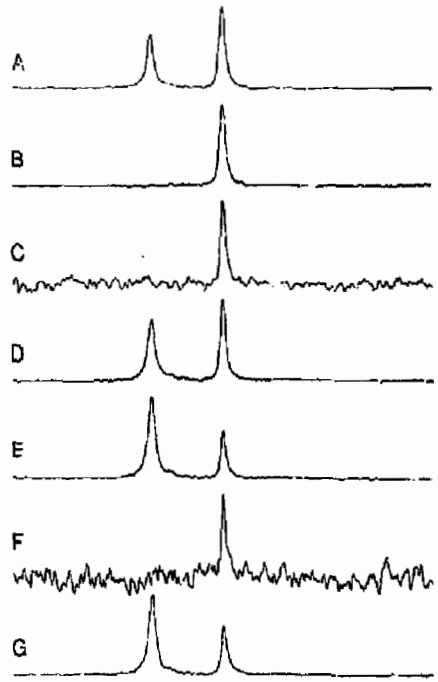

Fig. 3. Hahn echo (A, D, E, G) and 1D exchange spectra (B, C, F) of oriented DOPE (0°-orientation) at 15°C (A–D) and 18°C (E–G) for $t_{mix} = 500$ ms (B), 3 s (C) and 4 s (F). (A) and (D) were acquired immediately prior to and following the acquisition of (B) and (C), respectively and (E) and (G) were acquired immediately prior to and following the acquisition of (F). Number of acquisitions = 32 (A, D, E, G), 64 (B), 256 (C), 1024 (F). The plot width is 10 kHz.

mixing times (of the order of 1 s) as orientational exchange due to lateral diffusion caused the transfer of magnetization into that region of the spectrum. This is analagous to the recovery of the lineshape which occurs in 'hole-burning' experiments [26,31].

1D exchange spectra of the oriented DOPE were acquired for ratios of L/H which were as close to unity as possible. The 1D exchange experiment gives a spectrum which is the equivalent of a slice of the 2D spectrum, passing through one diagonal peak ($H_{||}$) and one cross-peak ($L_{\perp}$) [23]. Chemical exchange is revealed by the growth of the cross-peak with increasing t_{mix}. Hahn echo and 1D exchange spectra acquired at 15 and 18°C are shown in Fig. 3 for mixing times of 500 ms (Fig. 3b), 3 s (Fig. 3C), and 4 s (Fig. 3F). The spectra in Figs. 3A, D ($L/H = 0.7$) were acquired before and after the exchange experiments in Figs. 3B, C, respectively, and Figs. 3E, G ($L/H = 1.6$) were acquired before and after Fig. 3F. The L/H ratios were unchanged over the course of the experiments. The scale and peak positions are the same as in Fig. 2; it is

clear that no increase in the lamellar peak is observed for mixing times up to 4 s.

To investigate whether lipid exchange is observable in mixed lipid systems, 1D exchange studies were also performed on 9:1 and 7:3 (molar ratio) mixtures of DOPE/DOPC. The addition of DOPC and other PCs to DOPE results in an increase in the spontaneous radius of curvature of the bilayers [32], thereby raising the L_α to H_{II} phase transition temperature [32–34]. The rates of the forward and reverse phase transitions may be increased at higher temperatures. Furthermore, DOPE/DOPC mixtures exhibit metastable behaviour [33], which may have some effect on exchange rates. In the present study, the presence of 10 mol% DOPC in DOPE raises the L_α to H_{II} transition range approx. 25°C, from 0–23°C to 19–50°C (not shown). The chemical shifts of the L_α and H_{II} resonances were the same as in DOPE. The transition is reversible, but after several days a small quantity (< 7%) of an isotropic component was observed approx. 6 ppm upfield from the H_{II} resonance, the quantity of which remained relatively constant over a further period of several days, even after extended incubations at 30 and 40°C. This differs from multilamellar dispersions containing 20 mol% DOPC, where prolonged incubation at 40°C results in a transition from L_α to the isotropic phase [33]. There were some indications of metastable behaviour in the DOPE/DOPC, in that the L_α/H_{II} ratio showed a greater propensity to change at a given temperature, which ruled out the use of the longer 2D experiment. All exchange experiments were performed prior to the appearance of the isotropic component.

For DOPE/DOPC (9:1), Hahn echo and 1D exchange spectra were acquired in the range 27°C to 32°C for mixing times ranging from 100 ms to 3 s. Slight variations in the L/H ratios (10–15%) were observed in three separate experiments. At all temperatures and mixing times, no increase in the lamellar peak was observed.

The presence of 30 mol% DOPC results in a further increase in the L_α to H_{II} transition range to 63 to > 78°C. A representative oriented L_α spectrum (33°C) is shown in Fig. 4A. The DOPC and DOPE resonances are resolved, as DOPC has a slightly greater chemical shift anisotropy than DOPE [35]. The ratio of the (downfield) DOPC resonance to that of DOPE is 3:7. As the L_α to H_{II} transition proceeds, this ratio remains constant, indicating that the PC/PE ratio is the same in both phases. The lipids are not resolved in the H_{II} phase. Exchange studies were performed at 73°C, with the experiment set up to null the DOPE resonance at short mixing times, resulting in slight inversion of the DOPC resonance (not shown). No exchange was observed for mixing times up to 2 s. It should also be noted that no isotropic resonance was observed in this sample.

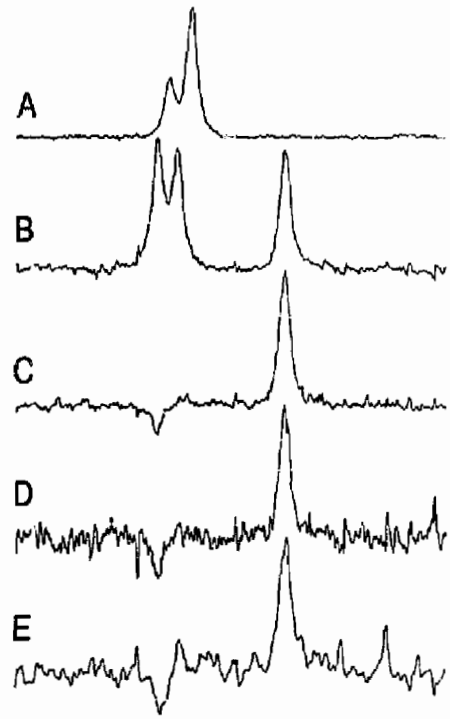

Fig. 4. (A) Representative ^{31}P-NMR spectrum of DOPE/DOPC (7:3) in the L_α phase at 33°C (0° orientation). (B) Representative ^{31}P-NMR spectrum of DOPC/DOPE/cholesterol (1:1:2) at 33°C (0° orientation). (C–E) 1D exchange spectra of DOPC/DOPE/cholesterol (1:1:2) at 33°C (0° orientation) for t_{mix} = 100 ms (C), 2 s (D), 3 s (E). Number of acquisitions = 96 (A), 128 (B, C), 800 (D), 1648 (E). The plot width is 6 kHz. The slight inversion of the DOPC resonance is a result of setting the experiment to nullify the DOPE resonance at short mixing times.

To examine a more complicated lipid mixture, we chose the system DOPC/DOPE/cholesterol (1:1:2, molar ratio), as cholesterol is an important constituent of biological membranes. This system is also known to display complex metastable behaviour [33], which has resulted in contradictory observations in the literature. Thus, when incubated at 30°C, dispersions of DOPC/DOPE/cholesterol (1:1:2) were found to display a mixture of H_{II} and isotropic phase [36]. However, at 40°C, the same mixture, in another study, gave a mixture of L_α and H_{II} phases [33]. These discrepancies were explained by an observed time-dependence in the phase preference of DOPC/DOPE/cholesterol mixtures [33]. In the present study, an L_α to H_{II} transition was observed over 23°C–48°C. A representative oriented sample spectrum, obtained at 33°C, is shown in Fig. 4B. The DOPC and DOPE resonances are resolved in the L_α phase but not in the H_{II}. The ratio of

DOPC/DOPE (1:1) is constant throughout the transition, indicating that the DOPC and DOPE partition equally between the two phases, in agreement with earlier studies (Tilcock et al., 1982). 1D exchange spectra were acquired at 33°C, with DOPE nulled at short mixing times. As mentioned above, this results in a slight inversion of the DOPC resonance, which is clearly seen in Figs. 4C–E, for which the mixing times were 100 ms (4C), 2 s (4D), and 3 s (4E). The ratio of DOPC/H_{II} remained constant at 1.1 over the period that the experiments were performed. As in the other systems, no increase in the L_α resonance was observed with increasing mixing times.

In contrast to results obtained by Cullis and coworkers [33,36], we did not observe an isotropic resonance in this sample at any temperature, even after cycling through the phase transition (alternating between 18°C and 38°C) 49 times. It is conceivable that the metastable behaviour exhibited by DOPC/DOPE, alone and in combination with cholesterol, is modulated in the oriented samples, where the hydration and lipid morphology (i.e., planar versus curved bilayers) differ.

In a final experiment dealing with L_α/H_{II} exchange, a system was chosen (DOPE/cholesterol sulfate, 9:1) in which one of the lipids was negatively charged. Cholesterol sulfate is thought to 'stabilize'

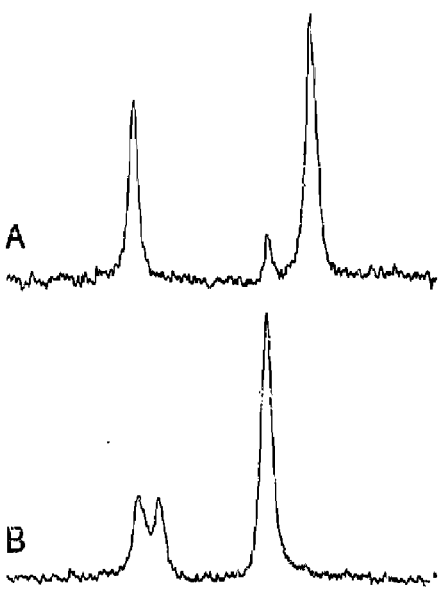

Fig. 5. Representative ^{31}P-NMR spectra of DOPC/MO (1:1) (A) and DOPC/DOPE/cholesterol (1:1:2) (B) at 43°C (0° orientation). The cubic I_2 phase of (A) is distinguished from the L_α and (transient) H_{II} phases, as seen by comparison with the L_α/H_{II} phases in (B). The plot width is 6 kHz.

biological and model membranes [37–39], and plays an important role in the differentiation of the outermost layer of human epidermis, the stratum corneum [40]. In the present study, the addition of 10 mol% CS to DOPE resulted in a slight increase in the L_α to H_{II} transition range, from 0°C–24°C to 3°C–33°C; thus CS is seen to stabilize the L_α phase (not shown). No lipid exchange was observed for experiments performed at 9°C (t_{mix} = 2 s) and 21°C (t_{mix} = 1 s).

Chemical exchange between lamellar and cubic phase lipid

A second class of non-lamellar phases, which may have important physiological significance, specifically with regard to membrane fusion, are the cubic phases, which give rise to 'isotropic' structures [3]. A number of lipid systems are known to or thought to form cubic phases under certain conditions; these include mixtures containing DOPC and/or DOPE [3]. As mentioned above, we did not observe significant quantities of isotropic phase lipid in any of the mixtures examined. Only in DOPE/DOPC (9:1) was a small quantity observed (< 7% of total lipid). In order to examine the question of lipid exchange between the L_α and cubic phase, oriented samples of DOPC/MO were prepared. DOPC/MO mixtures form a bicontinuous cubic phase I_2 [3], and MO is known to be an effective fusogenic lipid [41]. Because the I_2 phase has a bilayer structure, it is possible that exchange could occur at the contact points between the L_α and I_2 phases.

A reversible L_α to I_2 transition was observed over the range of 23°C to > 43°C for DOPC/MO (1:1). A representative spectrum acquired at 43°C is shown in Fig. 5A. Assignment of the upfield peak as cubic was obtained by comparison with DOPC/DOPE/cholesterol (1:1:2) at 43°C (Fig. 5B), where only L_α and H_{II} phases are present. In addition to the L_α and I_2 resonances, a small quantity of transient H_{II} phase is observed in the DOPC/MO spectrum. This H_{II} resonance was only observed at this temperature, and only for a short period of time; after 40 min only the L_α and I_2 phases remained. The cubic phase resonance is approximately 6 ppm upfield from the H_{II} resonance.

A reversible L_α to I_2 transition was observed over the range of 0°C to 22°C for DOPC/MO (3:7). The transition displayed considerable hysteresis. Following sample preparation, which involved incubation under a humid atmosphere of N_2 for 4 days, the sample was cubic at 18°C. After 1 h at 0°C, the ratio of L_α/I_2 was 4.3. The temperature was incrementally increased, and the sample was completely cubic by 23°C. The temperature was then decreased to 18°C for 15 min and then to 3°C for a further 30 min, following which the L_α/I_2 ratio was 1.1. After a further 5 h the ratio had only changed slightly (to 1.4), with most of this change

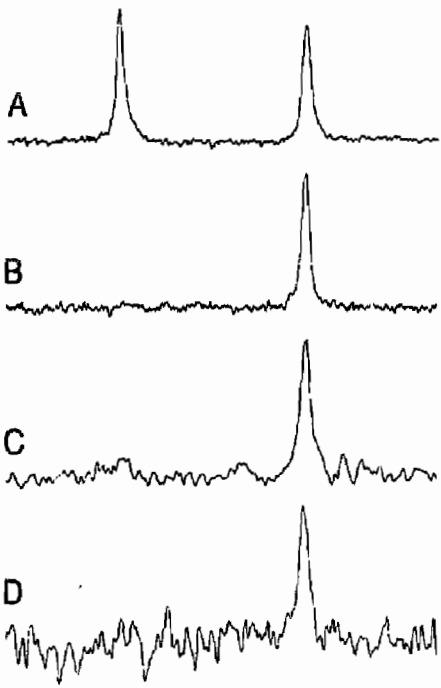

Fig. 6. Hahn echo (A) and 1D exchange spectra (B–D) of DOPC/MO (3:7) (0°-orientation) at 3°C for t_{mix} = 100 ms (B), 2 s (C) and 4 s (D). Number of acquisitions = 128 (A, B), 256 (C), 1200 (D). The plot width is 6 kHz.

occurring during the exchange experiment with $t_{mix} = 4$ s (see below).

A spectrum of DOPC/MO (3:7) acquired at 3°C is shown in Fig. 6A. Below are exchange experiments with t_{mix} = 100 ms (Fig. 6B), 2 s (Fig. 6C), and 4 s (Fig. 6D), acquired at the same temperature. No exchange is observed on these timescales, as there is no increase in the intensity of the L_α resonance with increasing t_{mix}.

The key result of these experiments, for both the L_α to H_{II}, and L_α to I_2 transitions, is the complete lack of observable lipid exchange between lamellar and non-lamellar phases on timescales of several seconds. This implies that no exchange would occur over periods of tens of seconds, and perhaps longer. This is surprising, given the reversibility of the transitions and the rapid timescales on which they occur [8,11]. When PE's in the L_α phase were subjected to a rapid increase in temperature, the L_α to H_{II} transition times, measured by time-resolved X-ray diffraction, were found to be 0.3 to 3 s [8,11,12]. The theoretical L_α to H_{II} transition times calculated by Siegel fall in the range of 0.1 to 10 s, and the H_{II} to L_α transition rates are similar [8]. If the exchange at equilibrium involved sufficient lipid, and occurred at a rate comparable to the transition rates of the unidirectional processes, it should be easily detected by NMR. The failure to do so demonstrates that in the mixed phase domain, at equilibrium or conditions approaching equilibrium: (i) there is no exchange of lipid, or; (ii) the lipids exchange very slowly on the seconds' timescale, i.e., the rates of the forward and reverse transitions are greatly reduced, or; (iii) only a small proportion of lipids, less than the NMR detection limit, are undergoing exchange at an undetermined rate. Regardless of which option may be correct, there appears to be little communication between the lamellar and non-lamellar phases when they coexist.

Several models of the L_α to H_{II} transition have been proposed which are supported by experimental data [8,11]. Perhaps the most well-developed theory is that of Siegel [7–10], who provides a unified description of both L_α to H_{II} and L_α to cubic transitions. The theory is consistent with much experimental data, and is supported by the recent visualization via cryo-transmission electron microscopy of the proposed intermediates of the L_α to cubic transition [5]. Our results may be useful in placing some constraints on the stability and kinetic behaviour of certain proposed intermediates. The first intermediates in the process are thought to be IMI, which form between apposed bilayers at temperatures close to T_{II}. The IMI rapidly reach a steady state concentration, following which they either assemble into H_{II}-phase precursors, or (in some lipid mixtures) into interlamellar attachments (ILAs), which are cubic phase precursors, thought to be involved in membrane fusion. It is production of the H_{II}-phase precursors which determines the kinetics of the transition. Two intermediates are proposed to form, but the most important are line defects (LD), whose existence is supported by experimental data [8] and that is required to explain the observed rapid transition times. It is suggested that LDs provide a rapid, low energy pathway between L_α and H_{II} phases, and that their stability in the H_{II} phase allows for rapid reversion of H_{II} to L_α in the reverse transition [8]. The model suggests that the proportion of the two phases at equilibrium could be modulated by the elongation or shrinkage of LD, which terminate H_{II} tubes. This would appear to provide a pathway for chemical exchange between the L_α and H_{II} phases, which is clearly not supported by the present study. Thus, our results suggest either that LD are not stable in the bulk H_{II} phase, or that the lipid and water transport required to modify the length of a LD does not occur as easily and rapidly as proposed [8]. The present study thus places some limitations on the behaviour of a class of proposed intermediates, thereby demonstrating the potential usefulness of lipid exchange studies in the refinement of the theoretical description of bilayer to

nonbilayer transitions. Clearly, further modeling of both lamellar to non-lamellar transitions would be desirable, with the emphasis placed on what happens when the system is at or near equilibrium. Such modeling, in conjunction with the results of the present and related studies, may provide support for a given mechanism of lamellar to non-lamellar transitions [8,11].

The results from the present study are specific for bilayer to nonbilayer transitions. An obvious extension of this work would be to examine chemical exchange in lipid-peptide or lipid-protein systems where the protein induces the formation of nonbilayer structures. We hope to address this problem in the near future. In addition, it would be of interest to examine lipid exchange in other systems where lipid domains coexist; biologically important examples may include coexisting regions of gel and liquid crystalline membrane, or regions of bulk lipid and lipid which may be phase separated by ions. A knowledge of the exchange in these systems could have implications in a wide range of membrane-related phenomena.

Conclusions

In the present paper, a combination of one- and two-dimensional ^{31}P-NMR has been used to investigate chemical exchange between a wide variety of systems undergoing lamellar to non-lamellar transitions. Oriented samples were used to effect a separation of the resonances originating from each phase. The key result of these studies is the complete lack of observable chemical exchange on timescales up to 4 s (and therefore significantly longer), for temperatures ranging from 3°C to 73°C. This contradicts expectations based on the reversibility of the transitions and the rapid timescales on which they occur [8,11]. The results demonstrate that in mixed phase domains, at equilibrium or conditions approaching equilibrium: (i) there is no exchange of lipids, or; (ii) the lipids exchange very slowly on the seconds' timescale, or; (iii) only a small proportion of lipids, less than the NMR detection limit, are undergoing exchange. Lamellar and non-lamellar phases (L_α and H_{II} or L_α and I_2) are seen to occupy essentially separate spatial and temporal domains when they coexist.

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