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Transverse Nuclear Spin Relaxation in Phospholipid Bilayer Membranes†

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ABSTRACT: Experimental proof is presented that some of the motions responsible for transverse relaxation (T_2) in deuterium magnetic resonance (^{2}H NMR) experiments on acyl chains of a model membrane in the liquid-crystalline phase are extremely slow on the ^{2}H NMR time scale being characterized by a correlation time $\tau_2 \gg 10^{-5}$ s. The experiments used to investigate these slow motions involve a form of the Carr–Purcell–Meiboom–Gill pulse sequence modified so as to be suitable for ^{2}H NMR. The most plausible mechanism responsible for T_2 relaxation is the gradual change in the average molecular orientation due to lateral diffusion of the phospholipid molecules along curved membrane surfaces. A procedure for separating contributions to T_2 relaxation due to slow and fast motions is described.

The technique of deuterium nuclear magnetic resonance (^{2}H NMR) has provided important information on structure and molecular motion in model and biological membranes (Seelig & Seelig, 1980; Jacobs & Oldfield, 1981; Davis, 1983; Devaux, 1983; Bloom & Smith, 1985). Most ^{2}H NMR studies have concentrated on the determination of local orientational order from the spectrum of quadrupolar splittings. In this paper we are particularly concerned with the dynamic properties of these systems as determined from measurements of ^{2}H NMR longitudinal (T_1) and transverse (T_2) spin relaxation times. Interpretation of T_1 and T_2 measurements requires a knowledge of the correlation times for those molecular motions that modulate the quadrupolar interactions. The T_1 relaxation processes in ^{2}H NMR are only sensitive to "spectral densities", $J(\omega)$, of the fluctuating quadrupolar interactions at $\omega = \omega_0$ and $\omega = 2\omega_0$, where $f_0 = \omega_0/2\pi$ is the nuclear Larmor frequency, while the T_2 relaxation processes are also affected by $J(0)$. This dependence of T_2 on the low-frequency components of the spectral density means that T_2 is very sensitive to slow motions. As emphasized by Davis (1979), the observation that $T_1 \gg T_2$ for ^{2}H nuclei in the acyl chains of phospholipid molecules implies that there must exist motions with correlation times $\tau_1 \lesssim \omega_0^{-1}$ that are responsible for T_1 relaxation and other motions with correlation times $\tau_2 \gg \omega_0^{-1}$ that are dominant in T_2 relaxation.

It has been implicitly assumed up to now that the motions responsible for ^{2}H NMR relaxation in the liquid-crystalline phase of lipid bilayers are associated with local chain reorientation, either collective or noncollective (Brown, 1982, 1983; Kimmich et al., 1983; Bloom & Smith, 1985, p 70), and are characterized by correlation times that are short "on the NMR time scale for motional averaging", τ_M (Seelig & Seelig, 1980). If the relaxation is produced by a fluctuating interaction, which accounts for ΔM_2 of the ^{2}H NMR second moment (Davis, 1979, 1983), this would require that its correlation time $\tau_c \ll \tau_M = (\Delta M_2)^{-1/2}$. The hierarchy of correlation times implied by this interpretation is

$$\tau_1 \lesssim \omega_0^{-1} \ll \tau_2 \ll \tau_M$$

Such an interpretation of T_1 and T_2 is at the present time consistent with all published experimental data. Measured values of T_1 lead to $\tau_1 \leq 10^{-10}$ s while a typical value of the Larmor frequency in ^{2}H NMR, $f_0 \approx 35$ MHz, gives $\omega_0^{-1} \simeq 5 \times 10^{-9}$ s. Since the total second moment of ^{2}H spins on acyl chains is $M_2 \simeq 1.1 \times 10^{11}$ s^{-2}, a lower limit for τ_M is given by $\tau_M \gtrsim 3 \times 10^{-6}$ s and a reasonable estimate of τ_M corresponds to $\tau_M \simeq 10^{-5}$ s (Seelig & Seelig, 1980). The relatively shorter T_2 values in the liquid-crystalline phase are compatible with values of τ_2 in the range 10^{-7}–10^{-8} s. Indeed, with this interpretation of T_2, the reduction of T_2 always observed upon addition of proteins to phospholipid bilayer membranes may be interpreted as indicating a significant slowing down of the acyl chains at the lipid–protein interface (Paddy et al., 1981; Bienvenue et al., 1982).

Although the interpretation of T_2 described above is internally consistent with published experimental T_2 data, none of the experiments performed thus far rules out the possibility that $\tau_2 \gg \tau_M$. For this reason, we undertook an investigation

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of this possibility. The results of our study, described in this paper, indicate that, contrary to previous interpretations, a large fraction of the ^2H NMR transverse relaxation rate in model membranes is due to molecular motions having $\tau_2 \gg \tau_M$.

THEORY

Modified CPMG[1] Method of Measuring T_2. The transverse relaxation rate in ^2H NMR is normally denoted by T_{2e} and measured with a two-pulse quadrupolar echo (qe) sequence (Davis et al., 1976; Davis, 1979, 1983) corresponding to 90_x–τ–90_y–echo. We shall use the notation T_2^{qe} to distinguish such relaxation measurements from those obtained by other methods. Then, for exponential relaxation, the resulting echo that is peaked at a time $\approx 2\tau$ after the first pulse has an amplitude given by

$$A(2\tau) = A(0) \exp\left(-\frac{2\tau}{T_2^{qe}}\right) \quad (1)$$

where (Pauls et al., 1985)

$$1/T_2^{qe} = \Delta M_2 \tau_2 \quad \text{for } \tau_2 \ll \tau_M \quad (2a)$$

$$T_2^{qe} \propto \tau_2 \quad \text{for } \tau_2 \gg \tau_M \quad (2b)$$

Equations 2a and 2b are written on the assumption that a single molecular motion dominates the T_2 relaxation and that this motion modulates a portion, ΔM_2, of the second moment. The generalization of these equations to include more than one type of motion is obvious [see, for example, Paddy et al. (1981), eq 13].

A method of distinguishing between the two limiting regions of τ_2 in eq 2a and 2b is to use a form of the Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence (Meiboom & Gill, 1958; Abragam, 1961, pp 58–62) appropriate to ^2H NMR. We shall refer to this sequence, given by 90_x–τ–$(90_y$–2τ–$)_N$, as the "quadrupolar CPMG sequence" (q-cpmg), though Blicharski (1986), who has analyzed the transverse relaxation associated with this sequence theoretically, calls it the "MW-4 sequence", as do others (Mehring, 1983). If the echoes appearing at times $2n\tau$, $n = 1, 2, \ldots, N$, following the first pulse decay exponentially, then we denote the apparent transverse relaxation time, referred to by Blicharski as T_{2e}, by $T_2^{q\text{-cpmg}}$. With this notation the amplitude of the nth echo is given by

$$A(2n\tau) = A(0) \exp\left(-\frac{2n\tau}{T_2^{q\text{-cpmg}}}\right) \quad (3)$$

where for fluctuating quadrupolar interactions governed by a single correlation time

$$\frac{1}{T_2^{q\text{-cpmg}}} = \Delta M_2 \tau_2 \left[1 - \frac{\tau_2}{\tau} \tanh\left(\frac{\tau}{\tau_2}\right)\right] \quad (4)$$

(Blicharski, 1986). This result is identical with that obtained for the CPMG relaxation rate of spin $^1/_2$ nuclei undergoing chemical exchange between sites having different chemical shifts (Luz & Meiboom, 1963). In the limit $\Delta M_2 \tau_2^2 \ll 1$ (or, equivalently, $\tau_2 \ll \tau_M$) the experimental limit $\tau \gg \tau_2$ is invariably satisfied, in which case eq 4 yields the result $T_2^{q\text{-cpmg}} \simeq T_2^{qe}$; i.e., the quadrupolar CPMG sequence indicates the same transverse relaxation rate as the two-pulse sequence. Our interest in this pulse sequence is that the opposite limit, $\Delta M_2 \tau_2^2 \gg 1$ (or $\tau_2 \gg \tau_M$), permits the experimenter the possibility of using values of $\tau \ll \tau_2$. In this case, eq 4 gives

$$\frac{1}{T_2^{q\text{-cpmg}}} \simeq \frac{\Delta M_2 \tau^2}{3\tau_2} + \frac{1}{T_2'} \quad \tau \ll \tau_2 \quad (5)$$

where $1/T_2'$ is the transverse relaxation rate due to processes having correlation times $\tau_2' \ll \tau_M$. An exact expression for T_2' is implicit in the paper by Blicharski (1986) and also corresponds to eq 139 of Abragam (1961, p 315).

Thus, the ^2H NMR response to the quadrupolar CPMG pulse sequence provides an unambiguous indication of the relative magnitudes of τ_2 and τ_M.

MATERIALS AND METHODS

The phospholipid *sn*-2-($^2H_{31}$)dipalmitoylphosphatidylcholine (DPPC-d_{31}) was kindly provided by Dr. R. Cushley.

A standard method of preparing multilamellar phospholipid dispersions was followed. The required amount of dry lipid was dissolved in an organic solvent. By rotation of the flask in a warm bath while the solvent was pumped off, a thin homogeneous film was deposited on the walls. To remove the remaining solvent, the samples were then dried under high vacuum for a minimum of 8 h. Aqueous dispersions were formed by adding excess buffer (pH 7.0) to the films, vigorously mixing, and freeze–thawing a number of times. The resulting samples were centrifuged down to a pellet size, excess buffer was removed, and the pellets were transferred into sample tubes of $\simeq 0.5$ cm^3. Deuterium-depleted buffer was added in excess, samples were sealed, and additional freeze–thaw cycles were performed. Samples were vortexed every time they were thawed, including immediately before the experiment. Degradation of the samples was monitored by TLC; none was observed. Additional details may be found in Wallace (1986).

^2H NMR Methods. All NMR measurements were performed at 35 MHz on a home-built ^2H NMR spectrometer with its own automated data acquisition system described in detail elsewhere (Davis, 1979; Sternin, 1985). All measurements were performed above the gel-to-liquid-crystalline phase transition, at 44 °C. The modified Bruker temperature controller was used to maintain the stability of the sample temperature to within ±0.1 °C; the absolute error in temperature was ±1 °C.

Data acquisition of the time domain signal in Fourier transform NMR (FT NMR) is traditionally performed by converting the analog output of the radio frequency amplifier into a series of digital values produced by an analog-to-digital (A/D) converter at a constant rate, once every so-called "dwell time". Once triggered, the A/D converter usually uses its own free-running time base to determine when the next data point is taken relative to the previous one. The shorter the dwell time, the greater the spread of spectral frequencies that can be detected. However, for a fixed size of the computer memory, decreasing the dwell time also shortens the total time over which the data is collected. Due to the nature of FT NMR the signal must decay to zero due to destructive interference of the various signal components before the data collection is terminated; otherwise, a truncated data set is obtained in which components of similar frequency may be unresolved. Thus for a data set of a fixed size, the requirements of decreasing the dwell time to improve the spectral bandwidth and increasing the total time of the data acquisition to improve spectral resolution are contradictory.

In the case of a CPMG pulse sequence, the signal is refocused many times and the difficulty of acquiring a complete

[1] Abbreviations: CPMG, Carr–Purcell–Meiboom–Gill; TLC, thin-layer chromatography; FT NMR, Fourier transform nuclear magnetic resonance.

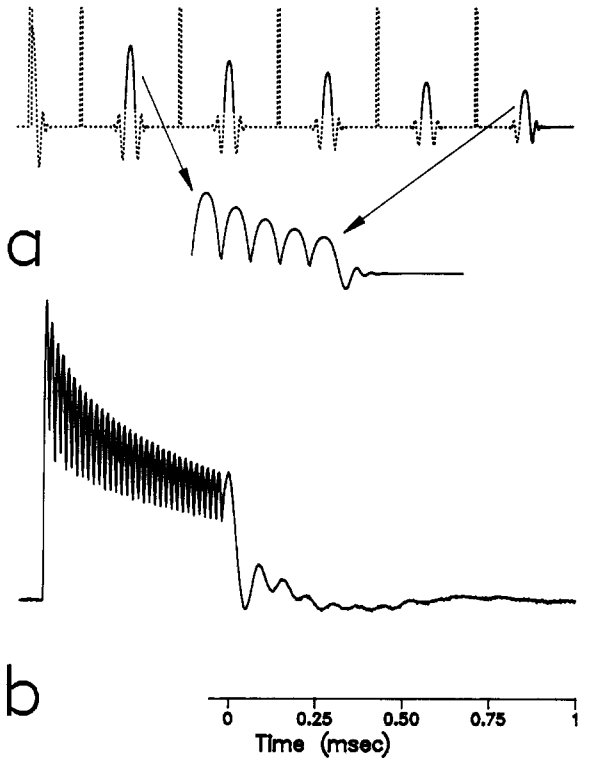

FIGURE 1: (a) Schematic representation of the selective data acquisition scheme used in the quadrupolar CPMG experiments, $90_x-\tau-(90_y-2\tau-)_N$, shown by the dotted line. Only the data points in the regions of interest—near the peaks of the echoes—were recorded, as indicated by the solid line. At the last echo the digitizer is switched into a free-running mode, and the entire echo is recorded. For illustrative purposes we chose $N = 5$. (b) An example of an actual experimental data set acquired as in (a). Here, $N = 32$ and $\tau = 100$ μs. The time scale is only shown from the last echo where it becomes continuous.

data set rises accordingly. With the help of a new pulse programmer (Sternin, 1985) we were able to control the time base of the A/D converter externally and to selectively acquire the data points only in the regions of interest. For a CPMG pulse sequence these regions are in the vicinity of the peaks of the echoes occurring at times $2n\tau$, $n = 1, 2, ..., N$. This is schematically indicated in Figure 1a. In fact, this efficient use of the computer memory enabled us to obtain in the same experiment the partially relaxed spectra, in addition to measuring the $T_2^{q\text{-}cpmg}$, by switching to a free-running time base at the Nth echo.

A partial CYCLOPS phase alternation was used in all experiments to get rid of some of the imperfections in the phases and the lengths of the radio frequency pulses (Rance & Byrd, 1983). A typical 90° pulse length was ≈3 μs.

The echo amplitudes were plotted (on a logarithmic scale) vs. the actual time of the echoes, $\approx 2\tau$ for T_2^{qe} and $\approx 2n\tau$ for $T_2^{q\text{-}cpmg}$.

Experimental Results

A qe experiment gives a single value of $A(2\tau)$; several measurements for various values of τ are required to determine T_2^{qe}. In contrast, a single quadrupolar CPMG experiment (see, for example, Figure 1b) gives a number of points, $A(2n\tau)$, $n = 1, 2, ..., N$, and thus is sufficient to determine $T_2^{q\text{-}cpmg}$. A summary of experiments performed during this study of a model membrane of pure DPPC-d_{31} is presented in Figure 2. As can be seen, the data from the qe experiments fit well to a single exponent; a least-squares fit yields $T_2^{qe} = 214 \pm 5$ μs. The data from the quadrupolar CPMG experiments is strongly nonexponential. At short times (first few echoes), the relaxation rates exhibit a strong τ dependence, while at longer times the relaxation rates appear to become independent of τ. The results of least-squares fits to the initial few echoes and to the echoes falling into the interval between 2.5 and 4 ms are presented in Table I.

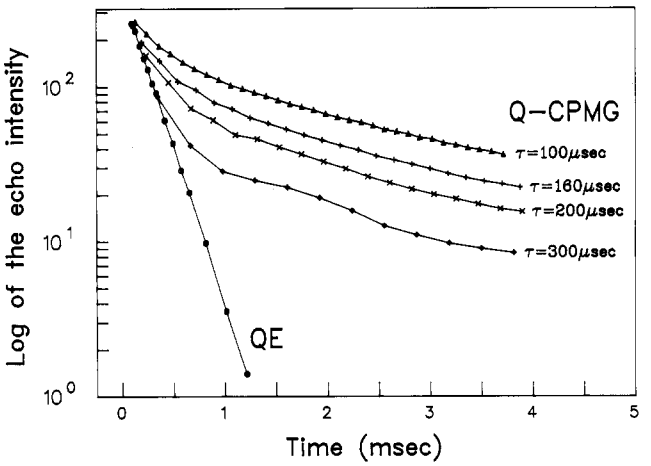

FIGURE 2: Comparison of transverse relaxation rates in DPPC-d_{31} membranes at 44 °C. The intensity of the peak of the signal is plotted vs. the actual time it occurs for the quadrupolar echo (QE) measurements (●) and for the quadrupolar CPMG (Q-CPMG) measurements for various values of τ: 100 (▲), 160 (+), 200 (×), and 300 μs (♦). The full data set for $\tau = 100$ μs is shown in Figure 1b.

Table I: Comparison of Relaxation Times $T_2^{q\text{-}cpmg}$ As Determined by Least-Squares Fits to Initial Slopes of the Quadrupolar CPMG Echo Envelopes (First Few Echoes) and to the Same Echo Envelopes at a Later Time (2.5 ms $\lesssim t \lesssim$ 4 ms)[a]

τ (μs)	$T_2^{q\text{-}cpmg}$ (μs) (initial slopes)	$T_2^{q\text{-}cpmg}$ (μs) (2.5 ms $\lesssim t \lesssim$ 4 ms)
100	643 ± 15	1322 ± 25
160	609	1277
200	551	1290
260	483	1250
300	439	1256

[a] Measurements for various values of τ are presented. By comparison, $T_2^{qe} = 214 \pm 5$ μs.

Note that the first echo of a quadrupolar CPMG experiment is exactly equivalent to the appropriate qe experiment; thus for all τ values the quadrupolar CPMG echo curves should start from the T_2^{qe} line, as they do to within experimental error. In all of the quadrupolar CPMG experiments 32 echoes were recorded but only the echoes occurring at times shorter than ≈4 ms are presented in Figure 2.

Discussion

Transverse Relaxation due to Diffusion along Curved Membrane Surfaces. As may be seen from Figure 2, the characteristic relaxation in a quadrupolar CPMG experiment is nonexponential. Thus the results cannot be interpreted in terms of a single relaxation time as implied by eq 3. Nevertheless, the experimental results indicate unambiguously that correlation times much longer than hundreds of microseconds play an important role in the transverse relaxation of the ^2H NMR signal of DPPC-d_{31} in the liquid-crystalline state.

The motion that is a prime candidate for such long correlation times is diffusion along curved membrane surfaces. For example, the correlation time of quadrupolar interactions for molecules diffusing on the surface of a sphere of radius R with a diffusion constant D is given by (Abragam, 1961, pp 298–300; Bloom et al., 1978)

$$\tau_2 = R^2/6D \qquad (6)$$

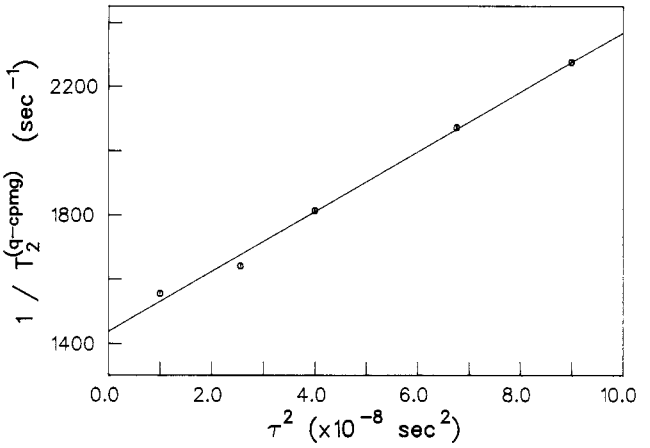

FIGURE 3: Initial slopes of the relaxation curves of Figure 2 vs. τ^2. The actual values of $T_2^{q\text{-cpmg}}$ are listed in Table I. The solid line is the result of a least-squares fit to eq 7, yielding $\langle 1/T_2' \rangle = 1/695$ μs and $\tau_2 \approx 100$ ms.

Since the sample has a distribution of values of R, ΔM_2, and bilayer orientations, the nonexponential relaxations observed in Figure 2 are not unexpected. However, the initial slopes of the relaxation curves of Figure 2 are exactly equal to $\langle 1/T_2^{q\text{-cpmg}} \rangle$, where $\langle ... \rangle$ denotes the average over all of these parameters. A plot of these initial slopes of the relaxation curves of Figure 2 vs. τ^2 is shown in Figure 3 to be linear as predicted by eq 5.

Since the motions associated with the long correlation times are too slow to contribute to motional averaging, they are only capable of gradually modulating the quadrupolar splittings, which are measured in the ^{2}H NMR spectrum. For spherical bilayers we may, therefore, identify $\langle \Delta M_2 \rangle$ with the "residual second moment", M_{2r}, of the ^{2}H NMR spectrum (Bloom et al., 1978; Davis, 1979). Substituting eq 6 into eq 5 and defining an effective radius for diffusional relaxation, R_{eff}, by $R_{eff}^{-2} = \langle R^{-2} \rangle$, we obtain

$$\left\langle \frac{1}{T_2^{q\text{-cpmg}}} \right\rangle = \frac{2M_{2r}D}{R_{eff}^2}\tau^2 + \left\langle \frac{1}{T_2'} \right\rangle \qquad (7)$$

The linear plot in Figure 3 gives $2M_{2r}D/R_{eff}^2 = 93 \times 10^8$ s^{-3} and $\langle 1/T_2' \rangle = 1/695$ μs. For lipid bilayers having a diffusion constant $D \approx 4 \times 10^{-12}$ m^2 s^{-1} (Bloom et al., 1978) and a value of M_{2r} that we measured to be $M_{2r} = 3.0 \times 10^9$ s^{-2}, in agreement with Davis (1979), we obtain $R_{eff} = 1.6$ μm and, substituting into eq 6, $\tau_2 \approx 100$ ms. As noted by Hope et al. (1986), multilamellar phospholipid dispersions prepared in the manner we have described "exhibit broad size distributions centered around diameters of a micron or more". An independent measurement of the average size of the dispersions in our DPPC sample by means of light scattering (Sternin, unpublished results) confirms this estimate.

CONCLUSIONS

We have demonstrated in this paper that ^{2}H NMR transverse relaxation in DPPC bilayers is strongly influenced by molecular motions having correlation times much longer than hundreds of microseconds. The most plausible candidate for such slow motions is diffusion of the phospholipid molecules along the curved membrane surfaces. This motion modulates the precession frequency in a random manner because the ^{2}H NMR quadrupolar splitting is proportional to $3\cos^2\theta - 1$, where θ is the angle between the local bilayer normal and the external magnetic field. Indeed, the theoretical results of Blicharski (1986) fit the τ^2 dependence of the average CPMG relaxation rate for a plausible membrane radius of curvature of about 1.6 μm. In this section we mention briefly additional theoretical and experimental results which indicate that lateral diffusion is, indeed, responsible for the long correlation times established by our experiments. These points will be described more fully in a subsequent paper.

We have developed a theory of transverse relaxation in which the diffusion equation is explicitly solved for the lateral diffusion along a curved surface. Our calculation shows that the relaxation curve in the two-pulse qe experiment should be nonexponential in analogy with the case of diffusion in an inhomogeneous magnetic field (Abragam, 1961). It also confirms the use of eq 7 for the average relaxation rate, assuming a single correlation time model. This more general treatment predicts the dependence of the relaxation rate on bilayer orientation to be $\langle 1/T_2^{q\text{-cpmg}} \rangle \propto \sin^2\theta \cos^2\theta$ at short times. Our unpublished results on specifically labeled lipid chains are in agreement with this prediction. We find that the initial relaxation rate is slowest near the spectral edges of the ^{2}H NMR powder spectra corresponding to $\theta = 90°$ and near the shoulders at $\theta = 0°$. Such an angular dependence has been observed empirically in ^{2}H-labeled lipids by Perly et al. (1985, see Figure 4). A similar behavior was observed for ^{2}H NMR of heavy water, ^2H_2O, in contact with membrane surfaces (Volke, 1984). In addition, our sample consists of 31 ^{2}H nuclei per molecule, many of which have different quadrupolar splittings; this results in an additional distribution of relaxation rates. A complicated superposition of nonexponential relaxation contributions leads in this case to the exponential behavior observed in the qe experiment.

By means of the quadrupolar CPMG pulse train described here, it is now possible to separate the contributions of extremely long correlation times to transverse relaxation. Systematic study of the remaining contribution, denoted by T_2' in eq 5 and 7, will give information on the slowest of the conformational motions. This will be useful in the study of lipid–protein interactions. The methods used in this paper could also be applied to detect a wide class of slow motions in membranes, such as conformational changes in integral membrane proteins or motions associated with lipids bound to such proteins.

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Articles

Destabilization of Phosphatidylethanolamine-Containing Liposomes: Hexagonal Phase and Asymmetric Membranes†

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ABSTRACT: We have measured the temperature of the L_α–H_{II} phase transition, T_H, for several types of phosphatidylethanolamine (PE), their binary mixtures, and several PE/cholesteryl hemisuccinate (CHEMS) mixtures. We have shown for liposomes composed of pure PE and in mixtures with CHEMS that there is an aggregation-mediated destabilization which is greatly enhanced at and above T_H. We now ask the question: How well can a dioleoylphosphatidylethanolamine/CHEMS liposome, for example, destabilize TPE (transesterified from egg phosphatidylcholine)/CHEMS liposome and vice versa? We use Ca^{2+} and H^+ to induce aggregation and to provide different values of T_H: the T_H of the PE/CHEMS mixture is much lower at low pH than with Ca^{2+}. We find that if the temperature is above the T_H of one lipid mixture, e.g., A, and below the T_H of the other lipid mixture, e.g., B, then the destabilization sequence [measured by the fluorescent 1-aminonaphthalene-3,6,8-trisulfonic acid/*p*-xylylenebis(pyridinium bromide) leakage assay] is AA > AB ≫ BB. That is, the bilayer of the lipid A (which on its own would end up in the H_{II} phase) destabilizes itself better than it destabilizes the bilayer of lipid B (which on its own would remain in the L_α phase). The BB contact is the least unstable. From these experiments, we conclude that the enhanced destabilization of membranes provided by the polymorphism accessible to these lipids above T_H is effective even if only one of the apposed outer monolayers is H_{II} phase competent. The surprising result is that if the temperature is above the T_H of both lipid mixtures, then the destabilization sequence is AB > AA, BB. That is, the mixed bilayers are destabilized more by contact than either of the pure pairs. We believe that this is due to specific differences in the kinetics of aggregation or close approach of the membranes. Similar results were obtained with pure PE liposomes induced to aggregate by Ca^{2+} at pH 9.5. We also found that the kinetics of low-pH-induced leakage from PE/CHEMS liposomes were initially faster when the CHEMS on both sides of the bilayer is fully protonated. However, in a citrate buffer, which cannot cross intact membranes, the leakage was eventually faster. Flip-flop of the protonated CHEMS to the inner monolayer can explain this observation.

The ability of many naturally occurring lipids to undergo a bilayer L_α to hexagonal H_{II} phase transition has led to much speculation about the putative roles of this polymorphism in cell function [for reviews, see Cullis & de Kruijff (1979), Verkleij (1984), Siegel (1984, 1987a), Rilfors et al. (1984), Cullis et al. (1985), Gruner et al. (1985), Lindblom et al. (1986), Weislander et al. (1986), and Quinn et al. (1986)]. For the L_α–H_{II} phase transition to be relevant for biological membrane fusion, it must satisfy three criteria: (i) it must occur after the contact of the two membranes; (ii) it must result in the mixing of aqueous contents between the membrane-bound compartments; and (iii) it must function between

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