P-31 Nuclear magnetic resonance studies of the appearance of an isotropic component in dielaidoylphosphatidylethanolamine

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ABSTRACT We have utilized phosphorus nuclear magnetic resonance, which provides an excellent means of characterizing the physical state of lipids, to investigate the polymorphic phase behavior of pure dielaidoylphosphatidylethanolamine (DEPE). We have observed a sharp isotropic component in the typical bilayer and inverted hexagonal P-31 NMR spectra. This component appears in the spectra of both the bilayer and inverted hexagonal lipid phases after several cycles through the bilayer-to-hexagonal phase transition. The magnitude of the isotropic component increased as a function of the number of cycles through the transition. The appearance of this component was not a function of time at constant temperature, but only a function of the number of cycles through the transition. The isotropic component is stable at all temperatures above the gel-to-liquid crystal transition, but it abruptly disappears when the lipid is cooled below the gel-to-liquid crystal phase transition. It is suggested that this isotropic phase is similar to the isotropic phase observed in dioleoylphosphatidylethanolamine (DOPE) by x-ray diffraction and identified as a cubic phase (Shyamsunder, E., S. M. Gruner, M. W. Tate, D. C. Turner, P. T. C. So, and C. P. S. Tilcock. 1988. *Biochemistry.* 27:2332–2336).

INTRODUCTION

Recent studies have shown that fully hydrated, unsaturated chain phosphatidylethanolamines can adopt either the bilayer L_α or inverted hexagonal H_{II} phase organization depending upon the physical conditions (temperature, pH, ionic strength, etc.) (1–3). Several biophysical methods have been shown to be sensitive to the conversion of phospholipid bilayers to the inverted hexagonal phase. These methods include P-31 NMR (2), high-sensitivity differential scanning calorimetry (4), freeze fracture techniques (5, 6), x-ray diffraction (6) and infrared spectroscopy (7). In addition to the nonbilayer inverted hexagonal phase, mixtures of hexagonal preferring and bilayer preferring lipids have been shown to exhibit complex behavior including other nonbilayer phases such as lipidic particles (5, 8, 9) and inverted cubic phases (5, 6). Until recently these other nonbilayer phases have been observed only in mixtures of lipids, but a cubic phase has now been observed in pure dioleoylphosphatidylethanolamine (10). In the present report we describe the appearance of an isotropic phase in the P-31 NMR spectrum of DEPE as a function of the cycling of the lipid through the bilayer to inverted hexagonal phase transition.

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MATERIALS AND METHODS

Materials

Dielaidoylphosphatidylethanolamine (18:1, 18:1) was purchased from Avanti Polar Lipids, Inc., Birmingham, AL, and was found to produce a single spot by thin layer chromatography analysis. Deuterium oxide (99.8%), was obtained from Aldrich Chemical Co., Milwaukee, WI. Aqueous hand-shaken multilamellar liposomes were prepared according to the method of Bangham et al. (11).

Nuclear magnetic resonance

P-31 NMR measurements were conducted on a model WP-200 multinuclear NMR spectrometer (Bruker Instruments, Inc., Billerica, MA) at 81.03 MHz as described previously (12). The spectrometer is equipped with a Bruker BVT-1000 variable temperature unit with a resolution of one degree. The DEPE liposomal suspension (100 mg/ml) contained 10% deuterium oxide which prevented settling and also served as a deuterium signal for locking the NMR spectrometer. Samples in 10-mm NMR tubes were allowed to equilibrate at given temperatures for at least 10 min before data acquisition. P-31 NMR free induction decays were accumulated for up to 5,000 transients by employing a 13-μs 90° radio-frequency pulse, 50-kHz sweep width, and 4-K data points. The delay between transients was 0.5 s. A two-level (0.5W, 12W) gated broad-band proton decoupling scheme was used to minimize decoupler heating of the samples. Automated temperature sequence experiments and temperature cycling experiments were car-

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ried out under software control, with preequilibration for 10 min and data acquisition for ~45 min at each temperature. An exponential multiplication corresponding to 25-Hz line broadening was applied to the free induction decay before Fourier transformation.

RESULTS

Fig. 1 shows the P-31 NMR spectra of DEPE in both the bilayer liquid crystalline phase and the inverted hexagonal phase. The P-31 spectrum of the bilayer phase has a characteristic line shape that is broad and asymmetrical with a high-field peak and a low-field shoulder. The bilayer to inverted hexagonal phase transition can be detected in the P-31 spectra as a reversal of the powder pattern and a reduction (narrowing by a factor of approximately two) of the chemical shift anisotropy (9).

In the course of investigating the reversibility of the phase transition from the bilayer to the inverted hexagonal phase, the appearance of an isotropic component in the P-31 NMR spectrum was detected. Fig. 1 *c* shows the P-31 NMR spectrum of DEPE in the bilayer phase after several cycles through the bilayer to inverted hexagonal phase transition. The presence of a sharp isotropic signal is apparent in this spectrum.

Because the isotropic signal appeared only after several passes through the bilayer-to-hexagonal phase transition, a systematic study of the effect of cycling of the sample through this transition was undertaken. The experiment was carried out in the NMR spectrometer by an automated sequence in which the temperature was alternated between 55 and 62°C. After each temperature change the sample was equilibrated for 10 min and then spectra were accumulated for 45 min. This was carried out for 48 cycles, with spectra accumulated both above and below the phase transition. Fig. 2, *a* and *b*, shows the spectra obtained at eight cycle intervals for the high and low temperature states, respectively. Fig. 2 clearly shows the increase in the proportion of the isotropic signal as a function of the number of cycles through the phase

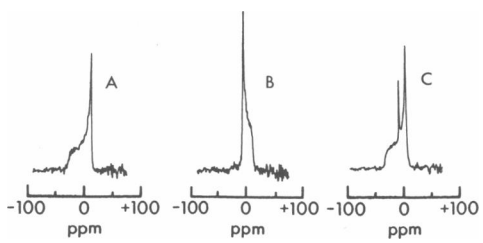

FIGURE 1 81.0 MHz P-31 NMR spectra of DEPE as a function of temperature illustrating the spectra obtained (*A*) in the bilayer phase, (*B*) in the inverted hexagonal phase, (*C*) in the bilayer phase after several cycles through the transition.

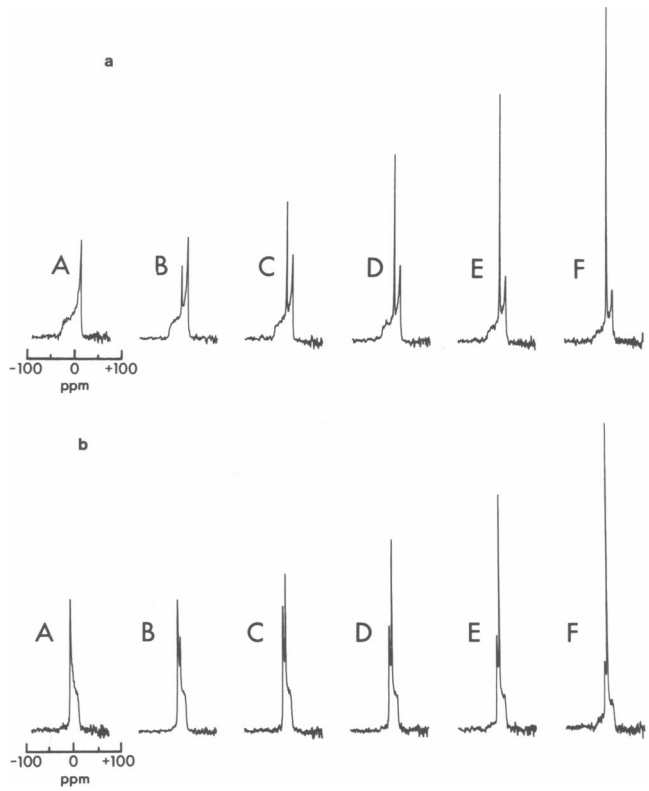

FIGURE 2 (*a*) 81.0 MHz P-31 NMR spectra of DEPE at 55°C in the bilayer phase after various numbers of cycles through the bilayer-to-inverted hexagonal transition. In this experiment an automatic program was employed which cycled the sample between 55 and 68°C, equilibrating for 10 min and then acquiring spectra for 5,000 scans, resulting in the sample remaining at each temperature for 55 min per cycle. The number of cycles are as follows: (*A*) 0; (*B*) 8; (*C*) 16; (*D*) 24; (*E*) 32; (*F*) 48. (*b*) 81.0 MHz P-31 NMR spectra of DEPE at 68°C, in the inverted hexagonal phase, after (*A*) 0; (*B*) 8; (*C*) 16; (*D*) 24; (*E*) 32; (*F*) 48 cycles through the the bilayer-to-inverted hexagonal phase transition. The methodology is as described for *a*.

transition. It was also observed that after a large number of cycles the gross appearance of the samples changed from a milky fluid suspension to a highly viscous suspension, which seemed almost solid in nature.

To compare the effects of cycling through the phase transition with the effect of time alone, we held a sample in a heating sequence at 59°C for a period of 19 h, and spectra were accumulated each hour. At this temperature the lipid is in the phase transition region, with both the bilayer and inverted hexagonal phases coexisting. The results of this experiment are shown in Fig. 3. This figure demonstrates that there is little if any change in the proportion of the isotropic signal over this time period at constant temperature in the transition region. This is in contrast to the results shown in Fig. 2 over a similar time period during cycling. (This 19-h time period is compara-

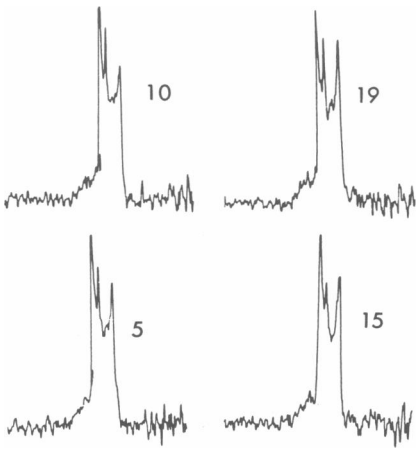

FIGURE 3 P-31 NMR spectra of DEPE as a function of time at 59°C, in the middle of the bilayer to hexagonal phase transition. The spectra were obtained after the number of hours indicated on the figure.

ble with the time required for eight cycles in the experiment of Fig. 2.)

Fig. 4 shows a plot of the fraction of isotropic phase as a function of the number of cycles, obtained from integrals of the data of Fig. 2. This plot demonstrates that the appearance of the isotropic signal is well fitted to an exponential plot against the number of cycles, and that it would take a large number of cycles to arrive at a maximal isotropic signal. The limiting value from such an exponential fit was found to be 64% isotropic, although the physical basis for this extrapolation is not clear.

The isotropic signal was very stable over time, as long as the temperature was maintained above the temperature of the gel-to-liquid crystal phase transition (32°C for DEPE). However, if the temperature was lowered to below this transition, the isotropic signal completely disappeared, and the sample then gave results similar to those of a fresh uncycled sample. This experiment rules out irreversible factors, such as degradation of the lipid,

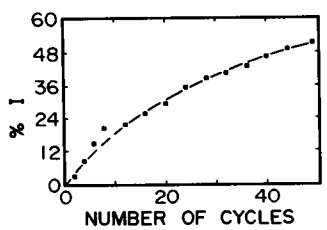

FIGURE 4 The integral of the isotropic resonance, given as apparent percentage *I* of the total P-31 NMR spectrum integral in the bilayer phase, as a function of cycles through the bilayer-to-inverted hexagonal transition.

as being factors in the appearance of the isotropic component.

DISCUSSION

In this report we have demonstrated the appearance of an isotropic signal in the P-31 NMR spectrum of DEPE which occurs as a function of the cycling of the lipid through the bilayer to inverted hexagonal phase transition. A number of possible structures are capable of isotropic motional averaging resulting in the sharp symmetrical signal observed in the P-31 NMR spectra. These include structures with high curvature such as small vesicles, micelles, conical and spherical lipidic particles, as well as a cubic phase (5, 6, 8, 9, 13). A recent x-ray diffraction study of the *cis* isomer of DEPE, DOPE (dioleoylphosphatidylethanolamine), showed that after repeated cycling through the phase transition, the sample converted to a cubic phase (10). A P-31 NMR spectrum of the end product showed that it was nearly totally isotropic. The similarity of DOPE and DEPE and the similar observations of the dependence upon cycling of the conversion to an isotropic P-31 NMR signal suggests that similar events are occurring in the two systems. It seems reasonable to assume that the appearance of the isotropic resonance in our spectra corresponds to the conversion of the lipid to a cubic phase.

The kinetics of the appearance of the isotropic phase in DEPE are remarkable in that they appear to depend upon the cycling process itself. If the spectra of Fig. 3 represented the dynamic equilibrium between two phases in the phase transition region, then one would expect the isotropic phase to build up even at constant temperature. However, as we (12) and others (1) have previously shown, the bilayer-to-hexagonal phase transition exhibits hysteresis, and the phase distribution at any temperature depends on the sample history. Thus the sample in Fig. 3 is not at equilibrium, but rather represents a mixture of the bilayer and inverted hexagonal phases, containing a small amount of a third phase represented by the isotropic signal.

The observation that the conversion to the presumed cubic phase depends upon the cycling of the temperature through the transition suggests that the conversion from the bilayer to the inverted hexagonal phase and the reverse are complex processes, involving one or more intermediates, and that a cubic phase may arise from one or more of these transient intermediates. Various features of nonlamellar phases have been observed in electron microscopy, including lipidic particles and interlamellar attachments and similar structures (5, 8, 9). However, most cases where such structures have been observed

involved mixtures of lipids. The similarity of the present work to that on DOPE (10) suggests that these two studies represent the first observations of the cubic phase arising from the bilayer-to–inverted hexagonal phase transition in a pure lipid. The further study of this phenomenon in pure lipids should make it possible to further elucidate the mechanism of the structural rearrangement which must occur in the transitions from bilayer-to-nonbilayer phases.

A theoretical treatment of the balance of forces which determine whether a lipid is in the L_α or H_{II} phase has been developed by Gruner and co-workers (14–16). In this treatment, the formation of the H_{II} vs. the L_α phase can be understood in terms of competition between the curling tendency or intrinsic radius of curvature of the bilayer, determined primarily by the headgroups, and the packing constraints of the hydrocarbon chains in the H_{II} phase, determined by the acyl chains. This group has also presented an analysis of a cubic phase showing that the cubic phase may represent another configuration where the balance between these two forces leads to a thermodynamically stable or a metastable state (17). Siegel has gone further to propose a mechanism for the transition for the bilayer-to-hexagonal phase transition which involves intermediates with cubic properties (18). Cubic phases have recently been reviewed (13). Based on the theoretical treatments of these various models, it would be expected that both the resulting phases and the pathway the phase changes take would be very sensitive to small differences in acyl chains or head groups and the presence of ligands which interact with either the head groups or the acyl chains.

In these theoretical models it is clear that the structures are complex and the processes involved in the transitions among them are complicated. The potential energy curves for these various phases as a function of temperature probably have several relative minima separated by large activation energies, so that the complexity of the processes involved in conversions among the phases as a function of temperature gives rise to the pathway-dependent, hysteretic behavior at temperatures in the region of the bilayer-to-hexagonal transition. Because of these considerations, it is not possible to determine whether the cubic phase we presumably have observed in DEPE is thermodynamically the most stable state or whether it is a trapped metastable state.

The possible biological roles for nonbilayer phase structures have been widely discussed (cf. references 9, 19, 20). A most interesting demonstration has recently been reported in which *A. laidlawii* strain A appears to regulate its membrane lipids in response to changes in growth conditions to keep the transition of its lipids from the bilayer-to-nonbilayer phases at a constant temperature ~20°C above the growth temperature (21). This constant temperature of the nonbilayer transition is maintained while the overall lipid composition varies widely and the gel-to-liquid crystal transition also varies. If the bilayer-to-nonbilayer transition is specifically being regulated, then this suggests that there is some property of the lipids 20°C away from the phase transition which is correlated with it. It could be that due to lateral phase separations there are small regions of nonbilayer lipids present 20°C below the transition for the bulk lipids. An alternative hypothesis, suggested by Gruner (14), is that the intrinsic bilayer curvature is itself the regulated property. Thus the presence of the bilayer-to-nonbilayer transition 20°C above the growth temperature is then a measure of this but is not itself biologically relevant.

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