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The polymorphic phase behavior of dielaidoylphosphatidylethanolamine. Effect of *n*-alkanols

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The polymorphic phase behavior of dielaidoylphosphatidylethanolamine (DEPE) has been investigated using spectrophotometry and ^{31}P nuclear magnetic resonance (NMR). It has been demonstrated that the bilayer to inverted hexagonal phase transition can be observed by spectrophotometry. The effects of the methanol, ethanol, and propanol on both the gel to liquid crystal transition and the bilayer to inverted hexagonal transition were investigated by spectrophotometry. It was shown that these alcohols shift the gel to liquid-crystalline phase transition to lower temperature, whereas the bilayer to inverted hexagonal phase transition is shifted to higher temperatures by these alcohols. The structural transition between the bilayer and inverted hexagonal phase of pure DEPE was also investigated by ^{31}P-NMR.

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 ^{31}P-NMR [2], high-sensitivity differential scanning calorimetry [4], freeze-fracture techniques [5,6], X-ray diffraction [6] and infrared spectroscopy [7]. Mixtures of hexagonal preferring and bilayer preferring lipids have been shown to exhibit complex behavior including a new structure known as the lipidic particle [5,8,9], as well as an inverted cubic phase [5,6].

Membrane active substances such as calcium and anesthetics have been shown to influence the polymorphic phase behavior of several PE's [5,10,11]. Some of these molecules were found to stabilize while others destabilize the inverted hexagonal phase. However, the effect of these substances on the main gel to liquid-crystalline phase transition has not been examined to the same extent in these lipids.

In this report we have demonstrated the applicability of spectrophotometry to the study of both the gel to liquid-crystalline phase transition and the bilayer to inverted hexagonal phase transition of dielaidoylphosphatidylcholine (DEPE). DEPE was the model system chosen because both its gel to liquid-crystalline phase transition (37°C) and its bilayer to inverted hexagonal phase transition (55–65°C) occur at temperatures accessible for the techniques used. The effects of a series of short chain *n*-alkanols on both the transitions of DEPE have been investigated using spectrophotometry and ^{31}P-NMR.

Materials and Methods

Materials. Dielaidoylphosphatidylethanolamine (18:1,18:1) was purchased from Avanti Polar-Lipids and was found to produce a single spot by thin-layer chromatography analysis. Deuterium oxide (99.8%), methanol and propanol were obtained from Aldrich, and ethanol (200 Proof) from Publicker Industries Co., Linfield, PA. Aqueous multilamellar liposomes were

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Abbreviations: DEPE, dielaidoylphosphatidylethanolamine; PE, phosphatidylethanolamine; NMR, nuclear magnetic resonance.

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prepared according to the method of Bangham et al. [12].

Spectrophotometry. The change in optical density at 400 nm of dilute (0.67 mg/ml) DEPE multilamellar liposomal suspensions was used to monitor its phase transitions on a Cary 219 spectrophotometer as described previously [13,14]. A uniform heating and cooling scan rate of 0.75 C°/min was used unless stated otherwise.

Nuclear magnetic resonance. ^{31}P-NMR measurements were conducted on a Bruker WP-200 multinuclear NMR spectrometer at 81.03 MHz. 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 prior to data acquisition. ^{31}P-NMR free induction decays were accumulated for up to 5000 transients by employing a 13 ms 90° radio-frequency pulse, 50 kHz sweep width, and 4K data points. The delay between transients was 0.5 s. A two-level (0.5 W, 12 W) gated broad-band proton decoupling scheme was used to minimize decoupler heating of the samples. Automated temperature sequence experiments were carried out under software control, with pre-equilibration for 10 min and data acquisition for approx. 45 min at each temperature. An exponential multiplication corresponding to 25 Hz line broadening was applied to the free induction decay prior to Fourier transformation.

Results

Spectrophotometric studies

Fig. 1 shows the heating transition curve for an aqueous dispersion of dielaidoylphosphatidylethanolamine (DEPE) followed by the optical density at 400 nm and the corresponding first-derivative plot. Two distinct changes in optical density are observed centered at 37 and 65.6°C. The low-temperature change reflects the main gel to liquid-crystalline phase transition, and it appears to be sharp and relatively symmetrical. The temperature of the transition is in excellent agreement with values reported in the literature using differential scanning calorimetry [15,16]. The optical density change associated with this main transition is now well established and is primarily due to a change in the refractive index increment of the lipid as the lipid density changes during melting [17,18].

The thermal reversibility of the main transition was also investigated, as shown in Fig. 2. There is a difference in the transition temperature for heating and cooling scans of approx. 2.5 C°. The transition temperature exhibited no detectable dependence on the rate of heating or cooling over the range 1.5 to 0.15 C°/min,

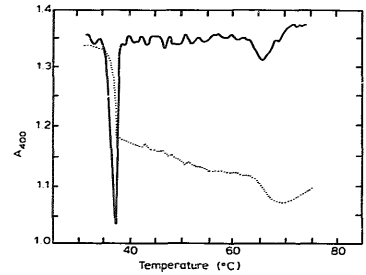

Fig. 1. Change in optical density at 400 nm (broken line) and the corresponding first-derivative curve (solid line) for the heating transition of DEPE multilamellar liposomes.

and reproducible results were obtained upon reheating. The reason for this observed hysteresis is not yet understood; however, it may be an indication of an unusual gel structure.

The heating scan exhibits an additional small change in optical density centered at approx. 30.2°C. The corresponding cooling curve shows only the optical density change associated with the main gel to liquid-crystalline phase transition. On reheating the additional transition reappears. The absence of the 30.2°C optical density change in the cooling curve and subsequent reappearance on reheating indicates slow reversible kinetics for this transition. Slow kinetics are characteristic of the pretransition in phosphatidylcholines [19,20]; however, pretransitions have only rarely been observed in phosphatidylethanolamines [21]. At present the nature of this transition in DEPE is not known.

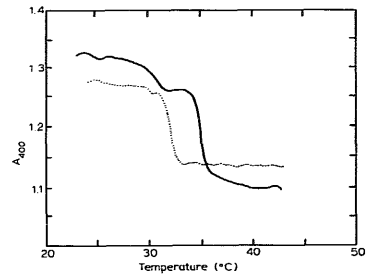

Fig. 2. Change in optical density at 400 nm of DEPE multilamellar liposomes as a function of temperature showing the gel to liquid-crystalline phase transition. Solid line, heating scan; broken line, cooling scan.

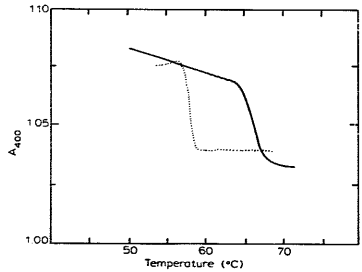

Fig. 3. Change in optical density at 400 nm as a function of temperature showing the bilayer to inverted hexagonal transition. Solid line, heating scan; broken line, cooling scan.

The high-temperature (65.6°C) optical density change shown in Fig. 1 is associated with the conversion of the bilayer phase of DEPE to the inverted hexagonal phase, as observed by ^{31}P-NMR (see below). The temperature of this transition is in excellent agreement with values reported in the literature using ^{31}P-NMR spectroscopy [1,3] and differential scanning calorimetry [4]. The bilayer to inverted hexagonal transition is fairly broad but symmetrical. These results demonstrate that the bilayer to hexagonal phase transition may be accurately detected by changes in optical density, although other methods such as ^{31}P-NMR and freeze-fracture electron microscopy are necessary to define the nature of the transition.

The reversibility of this transition was also examined by optical density as shown in Fig. 3. It exhibits significant thermal hysteresis (approx. 7.5 C°). As the scan rate was decreased from 1.5 to 0.15 C°/min, the transition temperatures were observed to decrease slightly. Hysteresis in the bilayer to inverted hexagonal transition has previously been observed in other PE's using ^{31}P-NMR and differential scanning calorimetry [1,15].

The effect of n-alkanols on the phase transitions of DEPE

Using the spectrophotometric optical density method, the effect of a series of short chain *n*-alkanols on both transitions of DEPE was investigated. Fig. 4 shows the alcohol concentration dependence of the main phase transition midpoint temperature of DEPE for heating scans in the presence of methanol, ethanol and propanol. In each case the apparent transition temperature is reduced as a linear function of alcohol concentration. This indicates a preferential interaction of alcohol with the liquid-crystalline lipid. It is also observed in Fig. 4 that the slope of each plot is dependent upon the alcohol chain length, such that the longer the chain length of the alcohol the greater the negative slope. The effect of each alcohol on the reversibility of the transition was also investigated; the presence of alcohol had no effect on the hysteresis of this transition.

The membrane/buffer partition coefficients for each alcohol were calculated from the slopes of Fig. 4 using the thermodynamics of freezing point depression as described previously [14]. The enthalpy of the transition used in the calculation was 6.3 kcal/mol [16]. The partition coefficient values obtained were: methanol, 2.9; ethanol, 3.5 and propanol, 24.9. As expected [22] the partition coefficients for the three alcohols into DEPE vary with the degree of hydrophobicity of the alcohol molecule. The partition coefficient values obtained for the three alcohols into DEPE are similar to those obtained for dipalmitoylphosphatidylcholine [22,23] and for some saturated PE's [22].

The effect of the *n*-alkanols on the bilayer to inverted hexagonal transition midpoint temperature of DEPE was investigated using spectrophotometry. Fig. 5 shows that the alcohols increase the transition temperature, indicating that they stabilize the liquid-crystalline bilayer phase relative to the inverted hexagonal phase. It is also shown in Fig. 5 that the longer the chain length of the alcohol molecule, the greater is its effectiveness in increasing the temperature for this transition. These results indicate that the alcohols interact preferentially with the bilayer phase relative to the inverted hexagonal phase, and that these differential interactions increase with the increasing hydrophobicity of the alcohol. It was also found that the presence of alcohol did not affect the hysteresis of this transition (data not shown).

^{31}P-NMR studies

The polymorphic phase behavior of aqueous dispersions of pure DEPE was also investigated using ^{31}P-

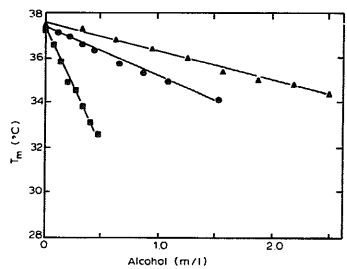

Fig. 4. Shift in the main gel to liquid-crystalline phase transition midpoint temperature (T_m) in heating scans of DEPE as a function of alcohol chain length and concentration for methanol (triangles), ethanol (circles) and propanol (squares) measured by spectrophotometry.

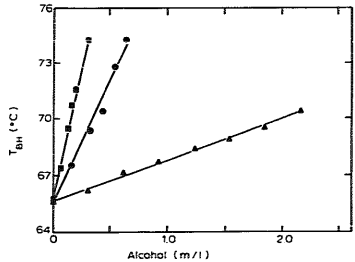

Fig. 5. Shift in the bilayer to inverted hexagonal phase transition midpoint temperature (T_{BH}) of DEPE for heating scans as a function of alcohol chain length and concentration for methanol (triangles), ethanol (circles) and propanol (squares) measured by spectrophotometry.

NMR spectroscopy. The ^{31}P spectrum of the bilayer phase has a characteristic line shape which 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 ^{31}P spectra as a reversal of the powder pattern and a reduction (narrowing by a factor of approx. 2) of the chemical shift anisotropy [9].

Fig. 6 shows the ^{31}P-NMR spectra of DEPE in the bilayer liquid-crystalline phase and the inverted hexagonal phase. The transition between these two phases is steep, as shown in Fig. 7 in the transition curves constructed from data such as shown in Fig. 6. The hysteresis of the transition is demonstrated in Fig. 7 where it is seen that there is a temperature difference of several degrees between the transition determined from an ascending temperature sequence of spectra as compared to that taken from a descending temperature sequence. This hysteresis is also illustrated by comparison of Figs. 6A and 6C, which shows that at the same temperature (60°C) the lipid in the ascending temperature sequence (6A) exhibits the bilayer phase whereas the spectrum from the descending sequence (6C) shows that the lipid is in the inverted hexagonal phase. In the course of these experiments an isotropic signal appeared in the ^{31}P-NMR spectra as a function of the cycling of the sample through the bilayer to hexagonal phase transition; a preliminary account of this has been presented elsewhere [29].

Comparison of the transition curves from spectrophotometry (Fig. 3) and ^{31}P-NMR (Fig. 7) shows a difference of a few degrees in the temperatures of both the heating and cooling transitions measured by the two methods. This discrepancy is probably due to the extremely slow kinetics of the transition, which gives rise to the hysteresis, and to the different 'scan' rates used

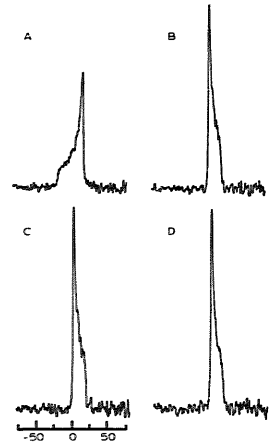

Fig. 6. 81.0 MHz ^{31}P-NMR spectra of DEPE as a function of temperature illustrating hysteresis exhibited by the bilayer to inverted hexagonal transition. Heating: A, 60°C; B, 62°C; cooling: C, 60°C; D 62°C.

in the two methods. In the NMR experiments, data are acquired for approximately one hour at each temperature, giving an effective 'scan' rate of 1 C°/h. In contrast, for the spectrophotometric experiments the practical limits for scan rates are from 9 C° to 90 C° per hour. In an experiment designed to simulate the NMR conditions in the spectrophotometer, a sample

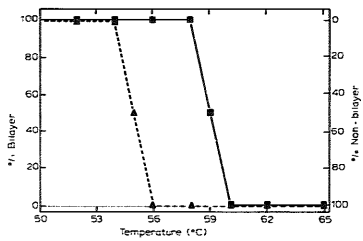

Fig. 7. The percentage of DEPE in bilayer or non-bilayer phases as a function of temperature determined from the integrals of the ^{31}P-NMR spectra. The squares represent measurements made in a heating sequence, and the triangles represent measurements from a cooling sequence.

was heated to 63°C and held in the spectrophotometer for 150 min. By subsequent sequential heating and cooling it was found to have been in the inverted hexagonal phase after 150 min at 63°C, more than two degrees below the transition temperature observed at the slowest scan rate. Thus the apparent discrepancy in transition temperatures between the two methods may be explained by the extremely slow kinetics of the transition at intermediate temperatures.

The effect of *n*-alkanols on the bilayer to inverted hexagonal transition of DEPE was investigated using ^{31}P-NMR (results not shown). The results were similar to those obtained spectrophotometrically; the *n*-alkanols increased the transition temperature as a function of alcohol concentration without significantly affecting the thermal hysteresis.

Discussion

The results presented in the current investigation demonstrate that spectrophotometry can be used to monitor the bilayer to inverted hexagonal phase transition of DEPE. Although spectrophotometry does not give structural information, it is very convenient method to measure the transition temperature of a large number of samples when supported by NMR or other physical verification. The precise origin of the optical density change arising from the bilayer to inverted hexagonal transition is not certain at present. However, it may be associated with a change in surface topography of the lipid leading to a change in the light scattering by the lipid.

We have shown by both NMR and spectrophotometry that the bilayer to inverted hexagonal phase transition of DEPE exhibits hysteresis, which has been noted by others [1,15]. This indicates that the transition is extremely slow in one or both directions. These slow kinetics are not readily evidenced by direct scan rate studies, because scan rates cannot be varied over a sufficiently wide range. However, comparison of our two methods, NMR and spectrophotometry, which have widely different effective scan rates, clearly show the rate effect. The reason for these slow kinetics at intermediate temperatures (the transition goes rapidly at temperatures outside the narrow transition region) is not understood.

The *n*-alkanols were found to increase the bilayer to inverted hexagonal phase transition temperature, indicating that they interact preferentially with the bilayer liquid-crystalline phase, and thus stabilize this phase relative to the inverted hexagonal phase. This stabilization of the bilayer may be explained in terms of the geometry of the alcohol moiety in the lipid matrix according to the shape hypothesis of Israelachvilli [24]. The short chain alcohols may bind in the PE headgroup region of the bilayer, complementing the cone-like shape of the PE molecules, and reducing their tendency to go into the inverted hexagonal phase. Our results are consistent with the observations of Hornby and Cullis [11], who showed that ethanol and butanol stabilized the bilayer phase in egg yolk phosphatidylethanolamine.

Our results of the study of the main gel to liquid crystal transition of DEPE were unusual in terms of the hysteresis observed. This is in contrast to the main transition of the saturated PE's, which we have previously shown to be thermodynamically reversible [22]. There are several reports in the literature of lipids whose gel to liquid-crystalline phase transition exhibits hysteresis. These include phosphatidylcholines which have become interdigitated due to interactions with ethanol [22], and unsaturated phosphatidylcholines which are in the crystalline subgel state [28]. Thus our finding of hysteresis in this transition raises the possibility that the structure of the gel state of DEPE is different from that of the saturated PE's.

The decrease in the main gel to liquid-crystalline phase transition temperature of DEPE in the presence of the short chain *n*-alkanols indicates preferential interactions of the alcohol molecules with the fluid phase relative to the gel phase. Similar simple freezing point depression effects have been observed with other phospholipid species including the saturated PE's and saturated PC's [22]. The similarity of the calculated partition coefficients for the alcohols in DEPE reported here with the values reported in the literature for the saturated PE's and DPPC multilayers [22,23] suggests that the depression of the main transition temperature is due to non specific partitioning of the alcohol into the hydrophobic region of the liquid-crystalline phase, which is not significantly affected by the characteristics of the headgroup region.

In conclusion, these studies have shown that the bilayer to hexagonal phase transition is strongly affected by short chain alcohols. These alcohols increase the temperature of the transition, indicating that they have preferential interactions with the lipid in the bilayer phase, thus stabilizing this phase relative to the inverted hexagonal phase. Comparison of Figs. 4 and 5 show that the bilayer to hexagonal phase transition is considerably more sensitive to the alcohols than is the gel to liquid crystal transition. Non-bilayer phases may have important biological roles [9,26,27]. If this is the case, then it is possible that the sensitivity of the phase distributions to the presence of alcohols could play a role in the mechanism of anesthesia or intoxication.

Acknowledgement

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