

BBA 71014

THE POLYMORPHIC PHASE BEHAVIOUR AND MISCIBILITY PROPERTIES OF SYNTHETIC PHOSPHATIDYLETHANOLAMINES

C.P.S. TILCOCK and P.R. CULLIS

Department of Biochemistry, University of British Columbia, Vancouver, B.C. V6T 1W5 (Canada)

(Received August 27th, 1981)

Key words: Phosphatidylethanolamine; ³¹P-NMR; Lamellar phase; Hexagonal (H_{II}) phase; Phase behavior; Miscibility

(1) The polymorphic phase behaviour of aqueous dispersions of various synthetic phosphatidylethanolamines, both singly and in mixtures, has been investigated by ³¹P-NMR. (2) 14:0/14:0 PE remains in the lamellar phase up to 90°C. 18:1_t/18:1_t PE exhibits a lamellar to hexagonal (H_{II}) transition between 60°C and 63°C. For 18:1_c/18:1_c PE, the lamellar to hexagonal (H_{II}) transition occurs between 7 and 12°C, whereas for 18:2_c/18:2_c PE, the hexagonal (H_{II}) phase is the preferred structure above -15°C. (3) Mixtures of 18:1_c/18:1_c PE and 18:1_t/18:1_t PE exhibit near-ideal miscibility behaviour. For mixtures of 18:1_c/18:1_c PE and 14:0/14:0 PE there is evidence of fluid-solid immiscibility at temperatures below the gel-liquid crystalline transition temperature of the 14:0/14:0 PE component. Mixtures of 18:2_c/18:2_c PE and 18:1_t/18:1_t PE exhibit complex phase behaviour involving limited fluid-solid immiscibility at low temperatures and formation of a phase allowing isotropic motional averaging at higher temperatures. (4) ³¹P-NMR provides a graphic method for investigating the miscibility properties of mixed PE systems.

Introduction

Unsaturated phosphatidylethanolamines (PE's) of both synthetic [1,2] and natural [2-4] origin preferentially adopt the hexagonal (H_{II}) phase above a characteristic bilayer to hexagonal (H_{II}) transition temperature (T_{BH}). This temperature is dependent upon the degree of acyl chain unsaturation; the greater the acyl chain unsaturation, the lower the T_{BH} . This ability to adopt the hexagonal (H_{II}) phase appears restricted to unsaturated species although there is evidence that certain saturated PE's may also adopt hexagonal phases in the presence of very high concentrations of mono-

valent cations at elevated temperatures [5].

There have been numerous studies of the phase behaviour and miscibility properties of phosphatidylcholines [6-8] and also mixtures of PC's and PE's [9]. However, the miscibility properties of PE's have received little attention [9]. In this work we use the technique of ³¹P-NMR [10] to investigate the polymorphic phase behaviour of aqueous dispersions of 14:0/14:0, 18:1_t/18:1_t, 18:1_c/18:1_c and 18:2_c/18:2_c PE's in isolation. Subsequently, the behaviour of various binary mixtures of these compounds is examined. We show that in mixtures where both species are above their gel-liquid crystal transition temperatures, behaviour consistent with near ideal mixing is observed. Alternatively, in mixed systems at temperatures where one component prefers the crystalline gel phase, phase behaviour consistent with limited fluid-solid immiscibility occurs.

Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PG, phosphatidylglycerol; DMSO, dimethylsulphoxide.

Materials and Methods

Materials

Chromatographically pure oleic, elaidic and linoleic acids, dimyristoyl PC, DMSO (Grade 1) and 1,1'-carbonyldiimidazole were obtained through Sigma (St. Louis, MO). Tetrabutylammonium hydroxide (25% in methanol) was obtained from Eastman-Kodak (Rochester, NY), Tetrahydrofuran (99.5+) was obtained from Aldrich Chemical Company. Molecular sieve type 4A (8–12 mesh) and AR-grade ethanolamine were obtained through B.D.H. (Vancouver). Miscellaneous reagents were of reagent or analytical grade. Chloroform and methanol were distilled immediately before use. DMSO and tetrahydrofuran were stored over molecular sieve type 4A.

Methods

Lipids. Fatty acids were shown to be over 99% pure on the basis of gas chromatography and used without further purification. DMPC was purified by preparative liquid chromatography employing a Waters Prep-500 LC unit with silica as support and $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$ (60:30:4, v/v) as the mobile phase.

Egg phosphatidylcholine was prepared from hen egg yolks by preparative liquid chromatography of a $\text{CHCl}_3/\text{MeOH}$ (1:1, v/v) extract. Glycerophosphocholine was then derived from the egg phosphatidylcholine by the procedure of Brockerhoff and Yurkowski [11]. Dioleoyl, dielaidoyl, and dilinoleoyl PC's were synthesized by the procedures of either Warner and Benson [12] or Lammers et al. [13]. For procedures involving sodium methylsulfonylethylmethide catalyzed reacylation, excess DMSO and unreacted fatty-acyl imidazole were first removed by preparative liquid chromatography on silica using $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$ (60:30:3, v/v) as the mobile phase and the lipids were then purified by conventional low-pressure chromatography on Silica gel 60 (70–230 mesh) or carboxymethylcellulose. Dioleoyl, dielaidoyl, dilinoleoyl and dimyristoyl PE's were derived from their respective PC's employing the base exchange capacity of phospholipase D [14] and purified by preparative liquid chromatography on silica using $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}/\text{NH}_3$ (60:30:1:1, v/v) as the mobile phase.

All phosphatidylethanolamines were shown to be 1,2-diacyl-*sn*-glycero-3-phosphoryl conformers on the basis of $^1\text{H-NMR}$ [13], were over 99% pure with respect to lipid phosphorus as determined by phosphorus analysis following two-dimensional TLC and were over 99% pure with respect to their designated fatty acid as determined by gas chromatography and argentation thin-layer chromatography.

Lipids were stored as powders in liquid N_2 for up to 4 weeks without evidence of degradation as determined by TLC, GC or ultraviolet spectroscopy. Lipids were prepared as stock solutions in CHCl_3 before use and stored at -20°C under N_2 .

NMR. $^1\text{H-NMR}$ and $^{31}\text{P-NMR}$ spectra were obtained using a Bruker WP200 FT-NMR spectrometer operating at 200 MHz for ^1H and 81 MHz for ^{31}P . For $^1\text{H-NMR}$ lipids were dispersed (10% w/v) in C^2HCl_3 (containing 1% trimethylsilane) and spectra collected for up to 200 transients employing a $2\ \mu\text{s}$ 90° pulse, 4 kHz sweep width and 0.8 s interpulse delay.

For $^{31}\text{P-NMR}$ lipids were dispersed in 10 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, 10% v/v $^2\text{H}_2\text{O}$, pH = 7 by extensive vortexing at room temperature then stored overnight at -20°C . The next day, samples were transferred to the NMR, previously equilibrated at the lowest temperature of interest, without permitting the lipid sample to warm to room temperature. Spectra were accumulated for up to 2000 transients employing a $16\ \mu\text{s}$ 90° pulse, 20 kHz sweepwidth and 0.8 s interpulse delay, in the presence of broadband proton decoupling. Spectra were recorded at sequentially higher temperatures for a particular sample, 10 min being allowed for equilibration at each temperature prior to accumulation.

Following NMR, TLC and phosphorus analysis showed that in no instance did lyso or free-fatty acid contaminants comprise greater than 3% of the total lipid, for any lipid or lipid mixture.

Some aspects of the materials and protocols employed should be emphasized. First, all lipid mixtures investigated in this work exhibited hysteresis with regard to their lamellar to hexagonal (H_{II}) phase transitions. While a mixture of $18:1_c/18:1_c$ PE and $18:1_l/18:1_l$ PE showed approximately ideal mixing behaviour (Fig. 2), using the above protocol quite dissimilar phase be-

haviour was observed if spectra were collected from sequentially higher to lower temperatures using the same equilibration times. Under these conditions, the bilayer to hexagonal (H_{II}) transition temperature was apparently depressed some 5–15°C depending upon the composition of the sample. Such hysteresis effects were even more marked with mixtures of 18:2_c/18:2_c PE and 18:1_t/18:1_t PE, where the isotropic resonance was observed to persist down to –25°C for certain samples.

There are also variations between the values for the temperatures of the lamellar to hexagonal (H_{II}) phase transitions for 18:1_c/18:1_c PE and 18:1_t/18:1_t PE reported here and other sources [1,2,15], to which the following observations pertain. As mentioned in the preceding paragraph, temperature hysteresis effects are potentially rich source of artifactual variation in T_{BH} . Beyond this, reproducibility between preparations is dependent upon fatty acid purity, lipid oxidation and the presence of degradation products such as lysophospholipid and free fatty acid. We have found that as little as 0.5% stearic acid contaminant in a batch of elaidic acid results in an elevation of T_{BH} for the final 18:1_t/18:1_t PE by some 5°C. Thus fatty acid purity is obviously a critical factor. We generally observe that oxidation of a given PE, whether synthetic or derived from natural sources such as soya bean or egg yolk, results in an increase in its T_{BH} and a decrease in transition cooperativity. Alternatively, the presence of fatty acid contaminants generally results in a decrease in both the T_{BH} and the cooperativity of the bilayer → H_{II} crystalline transition.

Results and Discussion

The polymorphic phase preference of aqueous dispersions of pure and mixed species of PE were investigated employing ³¹P-NMR techniques [16]. Briefly, broad (approx. 40 ppm wide) asymmetric ³¹P-NMR spectra with a high-field peak and low-field shoulder are observed for large (diameter > 2000Å) bilayer phospholipid systems, whereas ³¹P-NMR spectra a factor of two narrower with reversed asymmetry are observed for hexagonal (H_{II}) phase structures. On the other hand, structures allowing isotropic motional averaging (sonicated

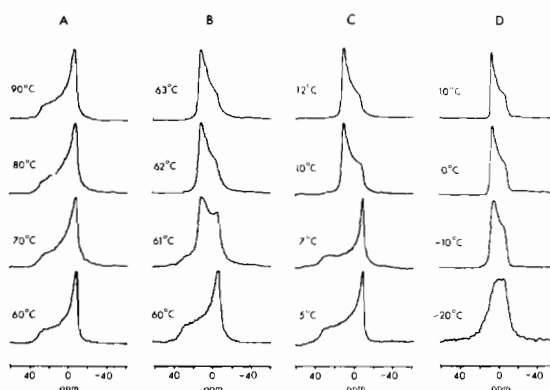


Fig. 1. 81.0 MHz ³¹P-NMR spectra for aqueous dispersions of (A) 14:0/14:0 PE; (B) 18:1_t/18:1_t PE; (C) 18:1_c/18:1_c PE; (D) 18:2_c/18:2_c PE. Temperatures are indicated adjacent to respective spectra. Spectra were collected under conditions as described in Methods.

vesicles, inverted micelles [17], or the cubic phase [18]) give rise to narrow symmetric spectra. On this basis the phase behaviour of the pure phosphatidyl-ethanolamine systems may be readily identified from the spectra presented in Fig. 1. The saturated 14:0/14:0 PE (which undergoes a gel to liquid crystalline phase transition at $T_c \approx 50^\circ\text{C}$ [19]) remains in the lamellar phase at temperatures up to 90°C, whereas 18:1_t/18:1_t PE ($T_c \approx 35^\circ\text{C}$ [20]) undergoes a bilayer to H_{II} transition between 60 and 63°C, close to previously reported values [2]. The *cis*-unsaturated 18:1_c/18:1_c PE ($T_c \approx -5^\circ\text{C}$ [15]) has a bilayer to hexagonal (H_{II}) transition

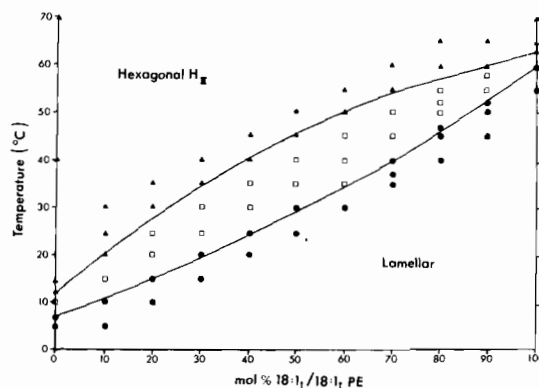


Fig. 2. Partial phase diagram for mixtures of 18:1_c/18:1_c PE and 18:1_t/18:1_t PE. ●, lamellar phase only; □ mixed lamellar and hexagonal (H_{II}) phases; ▲ hexagonal (H_{II}) phase only.

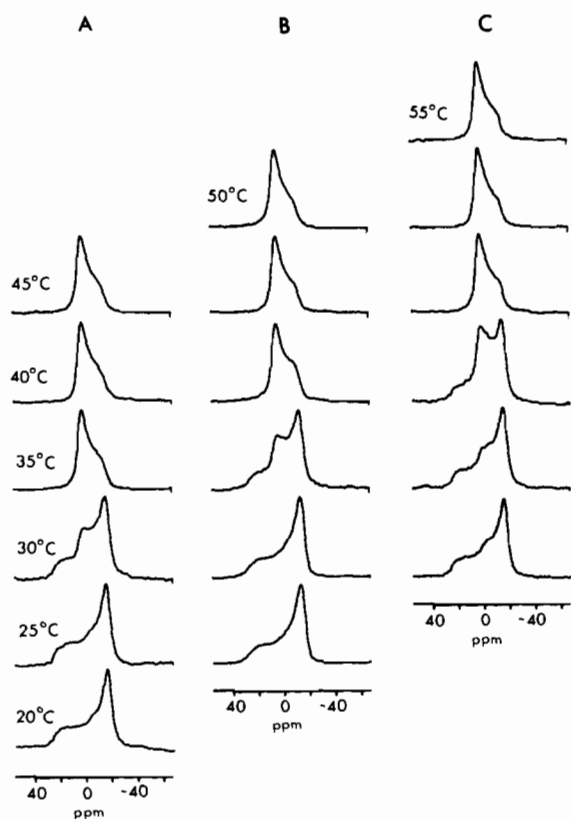


Fig. 3. Selected 81.0 MHz ^{31}P -NMR spectra for aqueous dispersions of mixtures of 18:1_c/18:1_c PE with (A) 40 mol%; (B) 50 mol%; (C) 60 mol% of 18:1_t/18:1_t PE.

temperature $T_{\text{BH}} \approx 10^\circ\text{C}$ whereas for 18:2_c/18:2_c PE T_{BH} is approx. -15°C .

The polymorphic phase behaviour of mixtures of 18:1_t/18:1_t PE and 18:1_c/18:1_c PE is summarized in the phase diagram of Fig. 2. Data for the construction of Fig. 2 were taken from ^{31}P -NMR spectra of the various mixtures obtained at sequentially higher temperatures as illustrated in Fig. 3 for mixtures containing 40, 50 and 60 mol% 18:1_t/18:1_t PE. It appears from the results presented in Fig. 2 that 18:1_c/18:1_c PE and 18:1_t/18:1_t PE exhibit nearly ideal miscibility.

Such ideal mixing is not, however, reflected by mixed systems composed of 18:1_c/18:1_c PE and 14:0/14:0 PE, as illustrated in Fig. 4. In particular, equimolar mixtures of these lipid species (Fig. 4(a)) exhibit a strong H_{II} phase component superimposed on a broad bilayer component at temperatures below 50°C, whereas predominantly bilayer

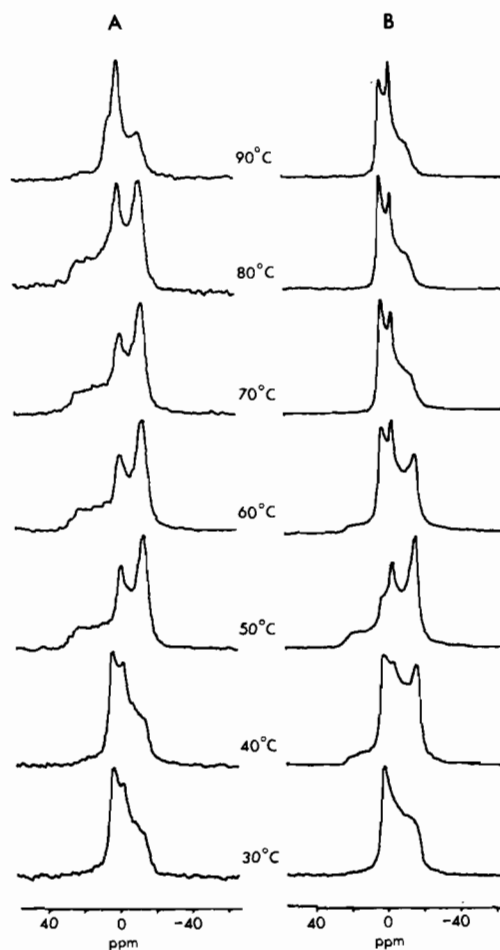


Fig. 4. 81.0 MHz ^{31}P -NMR spectra for aqueous dispersions of mixtures of 18:1_c/18:1_c PE and 14:0/14:0 PE, between 30 and 90°C. Molar ratios of 18:1_c/18:1_c PE to 14:0/14:0 PE were (A) 1:1; (B) 2:1.

^{31}P -NMR spectra are observed at higher temperatures. This anomalous behaviour is attributed to lateral segregation of 14:0/14:0 PE at temperatures below its gel-liquid crystalline transition temperature, which is a situation analogous to that observed via calorimetric techniques for mixtures of 18:0/18:0 PC and 18:1_c/18:1_c PC [21]. Thus below 50°C the 18:1_c/18:1_c PE would also be segregated and therefore free to adopt the H_{II} phase this component prefers at these temperatures. Stabilization of bilayer structure at 50°C and above may then be attributed to melting of the 14:0/14:0 PE domains, allowing better mix-

ing and a net stabilization of bilayer structure due to the distributed effect of the liquid crystalline 14:0/14:0 PE, which prefers the lamellar organization (see Fig. 1). Such net stabilization of bilayer structure for an H_{II} phase phospholipid by phospholipids preferring the bilayer phase is well documented, occurring for unsaturated PE's in the presence of PC [22], PS [23,24], sphingomyelin [25], phosphatidylglycerol [26] and cardiolipin [27]. The small narrow spectral component indicates the presence of structure allowing isotropic motional averaging for which, as indicated earlier, there are a number of possible sources. It is of interest to note that at the highest temperature (90°C) an H_{II} phase component is reappearing, indicating a diminished ability of the 14:0/14:0 component to stabilize bilayer structure and/or an increased proclivity of the 18:1_c/18:1_c PE for the H_{II} phase.

Such graphic visualizations of the effects of lateral phase segregation are also apparent for 14:0/14:0 PE – 18:1_c/18:1_c PE (1:2) systems, as observed in Fig. 4(b). In comparison to the mixture of Fig. 4(a) two effects are clear. First, appreciable bilayer stabilization occurs at a somewhat lower temperature (40°C), presumably due to a reduced T_c for the 14:0/14:0 PE component due to a disordering effect of the majority 18:1_c/18:1_c PE. Second, at higher temperatures (60°C and above) the H_{II} phase is the preferred organization for an increasing majority of the phospholipids. Again, this presumably reflects a reduced ability of the minority 14:0/14:0 PE bilayer preferring species to stabilize a net bilayer organization as the acyl chain disorder increases.

The phase behaviour of mixtures of 18:1_t/18:1_t PE with 18:2_c/18:2_c PE is rather different, as illustrated in Fig. 5 for mixtures containing 30, 40, 50, 70, 80 and 90 mol% of 18:1_t/18:1_t PE. In particular, the dominant spectral feature for most mixtures is a narrow symmetric peak indicative of phospholipid in structures allowing isotropic motional averaging. Before discussing this in greater detail, several features of Fig. 5 are more amenable to interpretation. First, it may be observed that for temperatures below 60°C (the bilayer to H_{II} T_{BH} for 18:1_t/18:1_t PE, see Fig. 1) increasing amounts of 18:1_t/18:1_t PE increasingly result in bilayer stabilization, as may be expected. Second, there is

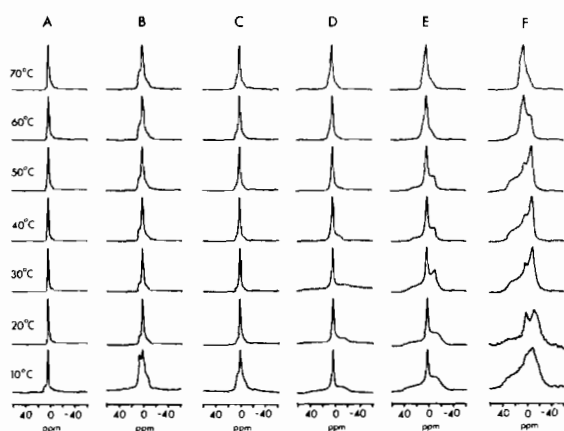


Fig. 5. 81.0 MHz ^{31}P -NMR spectra for mixtures of 18:2_c/18:2_c PE and 18:1_t/18:1_t PE, between 10 and 70°C. Mixtures contained the following mol% 18:1_t/18:1_t PE: (A) 30; (B) 40; (C) 50; (D) 70; (E) 80; (F) 90.

little evidence for lateral phase segregation at temperatures below the gel-liquid crystalline transition temperature of 18:1_t/18:1_t PE (35°C), in contrast to the situation for 14:0/14:0 PE. At 20°C for the equimolar 18:1_t/18:1_t PE – 18:2_c/18:2_c PE system for example (Fig. 5(c)), segregation of gel phase 18:1_t/18:1_t PE would be expected to result in an appreciable H_{II} phase component arising from the 18:2_c/18:2_c PE, contrary to experiment. It would therefore appear that co-crystallization of these two lipid species can occur to some extent, particularly in the samples containing equimolar or higher amounts of 18:1_t/18:1_t PE.

The prevalent narrow isotropic resonance of Fig. 5 cannot be unambiguously attributed to any one structure, a detailed understanding of which awaits X-ray studies. However, there are several points of interest. First, in all cases the dispersion consisted of large visible aggregates of lipid. It is therefore unlikely that this resonance arises from small vesicles or micelles, which give rise to more translucent dispersions. Second, the isotropic resonance persists in situations where both lipid components prefer the H_{II} phase in isolation (see the 70°C spectra of Fig. 5). It may be suspected that below 60°C where the lipid mixture is composed of a bilayer preferring species and an H_{II} preferring species that an alternative organization is

formed (such as the cubic phase [18], inverted micelles [17] or the honeycomb arrangement [28]) which gives rise to isotropic motional averaging. Such structures appear to be commonly available to mixtures of bilayer and H_{II} phase lipids [16] and often exhibit a strong temperature hysteresis, indicating that transitions between these arrangements and the bilayer or H_{II} phases may not be readily executed.

As indicated earlier, there is little evidence for lateral segregation of the $18:1_t/18:1_t$ PE component in the temperature range $10\text{--}30^\circ\text{C}$ where this species adopts the gel (crystalline) phase in isolation. We therefore extended the temperature range investigated for the samples containing 30 and 40 mol% $18:1_t/18:1_t$ PE to include -10°C and 0°C , and the results obtained are indicated in Fig. 6. For both samples it appears that segregation occurs at these lower temperatures as a strong H_{II} phase component is observed which is reduced in favour of the isotropic resonance as the temperature is increased (see Fig. 6(a)). By analogy with the results obtained for the $14:0/14:0$ PE – $18:1_c/18:1_c$ PE mixture this may be suggested to reflect melting of the $18:1_t/18:1_t$ PE component resulting in mixing of the bilayer ($18:1_t/18:1_t$) PE and hexagonal ($18:2_c/18:2_c$) PE, leading to formation of some intermediate structure.

In summary, the results presented here lead to the following conclusions. First, the more

unsaturated the PE species, the more readily the H_{II} phase is adopted. This behaviour is fully consistent with previous suggestions [16] that the dynamic molecular shape of lipids dictates the preferred structure. In particular, at a given temperature increased unsaturation would be expected to lead to larger cross-sectional area subtended by the acyl chains, thereby imparting a more pronounced 'cone' shape to the molecule. Second, at temperatures above the gel-liquid crystalline temperatures of component lipids, binary mixtures of the PE species investigated here are readily miscible. This leads to stabilization of the bilayer organization or to formation of some intermediate structure in temperature regions where one component prefers the bilayer and the other the H_{II} phase. Below the T_c of one component lateral segregation can occur, which appears to proceed more readily when this component is a saturated species. Finally, it has been noted elsewhere [2] that naturally occurring PE's exhibit bilayer – H_{II} transitions which are relatively cooperative (i.e. occur within a 5°C temperature interval) when compared with the gel-liquid crystal transition. The results obtained with $18:1_c/18:1_c$ PE – $18:1_t/18:1_t$ PE systems allow some understanding of this behaviour, as a fairly discrete transition is observed in these mixtures where the net T_{BH} depends on the relative proportions of component species.

Acknowledgements

C.P.S.T. is a Postdoctoral Fellow of the Canadian Medical Research Council (MRC) and P.R.C. is a Research Scholar of the MRC. This research was supported by the MRC under grants MA 6475 and ME 6535.

References

- 1 Cullis, P.R. and De Kruijff, B. (1976) *Biochim. Biophys. Acta* 436, 523–540
- 2 Cullis, P.R. and De Kruijff, B. (1978) *Biochim. Biophys. Acta* 513, 31–42
- 3 Reiss-Husson, F. (1967) *J. Mol. Biol.* 25, 363–382
- 4 Rand, R.P., Tinker, D.O. and Fast, P.G. (1971) *Chem. Phys. Lipids* 6, 333–342
- 5 Harlos, K. and Eibl, H. (1981) *Biochemistry* 20, 2888–2892
- 6 Ladbroke, B.D. and Chapman, D. (1969) *Chem. Phys. Lipids* 3, 304–367

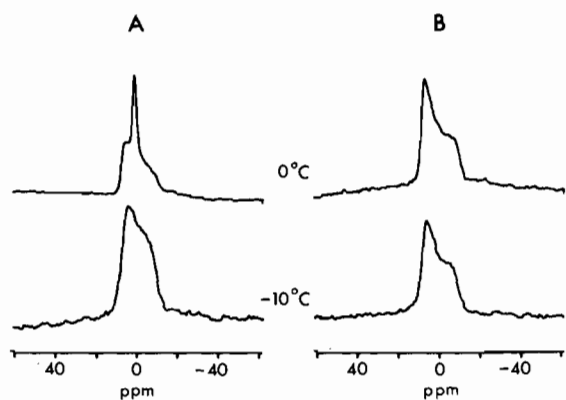


Fig. 6. 81.0 MHz ^{31}P -NMR spectra for mixtures of $18:2_c/18:2_c$ PE and $18:1_t/18:1_t$ PE, at -10 and 0°C . Mixtures contained the following mol% $18:1_t/18:1_t$ PE: (A) 30; (B) 40.

- 7 Mabrey, S. and Sturtevant, J.M. (1978) in *Methods in Membrane Biology* (Korn, E.D., ed.), vol. 9, pp. 237–274, Plenum Press, New York
- 8 Van Dijck, P.W.M., Kaper, A.J., Oonk, H.A.J. and De Gier, J. (1977) *Biochim. Biophys. Acta* 470, 58–69
- 9 Van Dijck, P.W.M., De Kruijff, B., Van Deenen, L.L.M., De Gier, J. and Demel, R.A. (1976) *Biochim. Biophys. Acta* 455, 576–587
- 10 Cullis, P.R. and De Kruijff, B. (1978) *Biochim. Biophys. Acta* 507, 207–218
- 11 Brockerhoff, H. and Yurkowski, M. (1965) *Can. J. Biochem.* 43, 1777
- 12 Warner, T.G. and Benson, A.A. (1977) *J. Lipid Res.* 18, 548–552
- 13 Lammers, J.G., Liefkens, T.J., Bus, J. and Van der Meer, J. (1978) *Chem. Phys. Lipids* 22, 293–305
- 14 Comfurius, P. and Zwaal, R.F.A. (1977) *Biochim. Biophys. Acta* 488, 36–42
- 15 Gally, H.U., Pluschke, G., Overath, P. and Seelig, J. (1980) *Biochemistry* 19, 1638–1643
- 16 Cullis, P.R. and De Kruijff, B. (1979) *Biochim. Biophys. Acta* 559, 399–420
- 17 De Kruijff, B., Verkleij, A.J., Van Echteld, C.J.A., Gerritsen, W.J., Mombers, C., Noordam, P.C. and De Gier, J. (1979) *Biochim. Biophys. Acta* 555, 200–209
- 18 Wieslander, A., Rilfors, L., Johansson, L.B.A. and Lindblom, G. (1981) *Biochemistry* 20, 730–735
- 19 Gally, H.U., Niederberger, W. and Seelig, J. (1975) *Biochemistry* 14, 3647–3652
- 20 Jackson, M.B. and Sturtevant, J.M. (1977) *J. Biol. Chem.* 252, 4749–4751
- 21 De Kruijff, B., Demel, R.A., Slotboom, A.J., Van Deenen, L.L.M. and Rosenthal, A.F. (1973) *Biochim. Biophys. Acta* 307, 1–11
- 22 Cullis, P.R., Van Dijck, P.W.M., De Kruijff, B. and De Gier, J. (1978) *Biochim. Biophys. Acta* 513, 21–30
- 23 Cullis, P.R. and Verkleij, A.J. (1979) *Biochim. Biophys. Acta* 552, 546–551
- 24 Tilcock, C.P.S. and Cullis, P.R. (1981) *Biochim. Biophys. Acta* 641, 189–201
- 25 Cullis, P.R. and Hope, M.J. (1980) *Biochim. Biophys. Acta* 597, 533–542
- 26 Farren, S.B. and Cullis, P.R. (1980) *Biochem. Biophys. Res. Commun.* 97, 182–191
- 27 De Kruijff, B. and Cullis, P.R. (1980) *Biochim. Biophys. Acta* 602, 477–490
- 28 Cullis, P.R., De Kruijff, B., Hope, M.J., Nayar, R. and Schmid, S.L. (1980) *Can. J. Biochem.* 58, 1091–1100