Biochimica et Biophysica Acta 857 (1986) 217–228
Elsevier

BBA 73080

Solid- and liquid-phase equilibria in phosphatidylcholine / phosphatidylethanolamine mixtures. A calorimetric study

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(Received December 16th, 1985)

Key words: Phosphatidylcholine; Phosphatidylethanolamine; Lipid phase diagram; Phase behavior; Differential scanning calorimetry

Phase diagrams have been determined for mixing of binary mixtures of phosphatidylethanolamines (PE) with phosphatidylcholines (PC), using high-sensitivity differential scanning calorimetry and allowing extensive incubation times to equilibrate samples in the solid phase. All of the PE-PC systems examined, which contained saturated or *trans*-unsaturated PC components, showed limited solid-phase miscibility, chiefly because the PC component can adopt more solid phases than the PE component. For the dielaidoyl PE-PC system, the lamellar-to-hexagonal II transition endotherm seen at 63.5°C for the pure PE is shifted to considerably higher temperatures upon incorporation of even low mole fractions of PC. All of the PE-PC systems examined here reveal a complete miscibility in the liquid phase, including the dipalmitoyl PE-dielaidoyl PC system for which limited liquid-phase miscibility had previously been suggested (Wu, S-H. and McConnell, H.M. (1975) Biochemistry 14, 847–854). However, PE-PC mixing appears to be less nearly ideal than the mixing of either PE or PC with anionic phospholipids. Our results demonstrate that calorimetry can be useful in determining accurate phase diagrams for lipid mixtures of this type, but only if proper attention is given to the existence and the proper equilibration of multiple solid phases in these systems.

Introduction

Phosphatidylcholines (PC) and phosphatidylethanolamines (PE) constitute the major glycerophospholipid species found in the membranes of most animal cells. Aqueous dispersions of these two lipids differ markedly in such properties as their surface hydration energies, mean molecular areas and propensities to form nonlamellar phases [1–3]. The PE/PC balance in natural membranes may therefore be an important (and carefully regulated) determinant of its function. Accordingly, a number of recent studies have examined various aspects of the behavior of PE/PC mixtures, including bilayer permeability [4], formation of nonlamellar phases [5–8], and the ability to reconstitute protein-containing membranes with proper barrier and transport properties [9, 10]. To interpret the results obtained in studies of this type, it is important to characterize the thermodynamics of mixing of PE and PC species in lamellar phases.

While the mixing of many binary combinations of synthetic phosphatidylcholines has been studied by calorimetric and other methods [11,12], fewer binary PE-PC systems have been examined in

Abbreviations: DE-, dielaidoyl; DM-, dimyristoyl; DP-, dipalmitoyl; PA, 1,2-diacyl-*sn*-glycero-3-phosphate; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; PE, 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine; PS, 1,2-diacyl-*sn*-glycero-3-phosphoserine; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl] amino}ethanesulfonic acid.

similar detail. Studies of the thermotropic behavior of PE/PC mixtures have often failed to account adequately for the solid-phase polymorphism exhibited by one or both components, and complete phase diagrams have been reported to date only for the DMPC-DPPE and DPPC-DPPE systems [13,14]. Few studies have examined the phase diagrams for PE-PC pairs in which one or both of the components is an unsaturated species, but the results of Wu and McConnell [15] have raised the intriguing possibility that the DEPC-DPPE system may show fluid-fluid immiscibility. In view of the obvious importance of PE–PC interactions in natural membranes, and the relative paucity of thermodynamic data currently available to describe the miscibilities of PE and PC species, we have examined the phase diagrams for four binary PE-PC systems, using high-sensitivity differential scanning calorimetry. We have paid particular attention to details of sample equilibration, and to the multiple solid phases that can form in these mixtures. Our results demonstrate that limited solid-phase miscibility is a common feature in PE-PC systems, largely as a consequence of the solid-phase polymorphism of the PC component. However, we find no evidence for liquid-liquid immiscibility in these systems, in contrast to the previous report of Wu and McConnell [15].

Materials and Methods

Dimyristoyl- and dielaidoylphosphatidylcholine were synthesized and purified as described previously [16]. Dipalmitoylphosphatidylcholine was obtained from Sigma (grade I) and was twice precipitated from chloroform with an excess of cold acetone. Phosphatidylethanolamines were prepared from the corresponding phosphatidylcholines by phospholipase D-catalyzed transphosphatidylation as described by Comfurius and Zwaal [17], except that the reaction was carried out at pH 6.8, and the products were purified as described previously [16]. All lipid preparations were judged to be free of detectable phosphate-containing impurities (limit of detection approx. 2 μg phospholipid) after thin-layer chromatography in $CHCl_3$/methanol/H_2O/conc. NH_4OH (65 : 30 : 2.5 : 2.5) or $CHCl_3$/acetone/methanol/acetic acid/H_2O (50 : 15 : 10 : 10 : 5), loading at least 400 μg of lipid per sample. All preparations were further checked for purity by comparing their calorimetric properties (transition temperatures, enthalpies and widths) to those of samples of the same lipids that had been repurified by preparative thin-layer chromatography in the basic solvent system described above. All of the lipids used in this study showed essentially identical calorimetric behavior before and after such repurification. All common chemicals used were of reagent grade or better, and all solvents were redistilled before use.

Lipid samples for calorimetry were lyophilized from cyclohexane, then dispersed in 200 mM NaCl/5 mM histidine/5 mM Tes/0.1 mM EDTA (pH 7.4) by vortexing at a temperature above the transition temperature of the higher-melting component of the sample. Samples containing DPPE were cooled to 50°C at 1 Cdeg/min, then cooled from this temperature to 2°C at 0.3 Cdeg/min. Samples containing DMPE were cooled from 52°C to 2°C at 0.3 Cdeg/min, and samples containing DEPE were cooled from 42°C to 2°C at the same rate. All samples were then incubated at 2°C for extended periods (7 days for DMPE/DMPC mixtures, 10 days for DEPC/DEPE and DEPC/DPPE mixtures or 40 days for DPPE/DPPC mixtures) prior to calorimetry. Samples were analyzed in a Microcal MC-1 high-sensitivity differential scanning calorimeter at scan rates of 12 Cdeg/h (for DPPE-DPPC samples) or 25 Cdeg/h (for other samples). Sample phospholipid concentrations and heat of transition were determined as described previously [16].

Results

In previous studies of PC/PS mixtures [18], we have shown that phase diagrams constructed from calorimetric data compare well with those obtained using more structurally based measurements, such as freeze-fracture electron microscopy [19], so long as the calorimetric samples are thoroughly equilibrated prior to analysis. In practice, such equilibration is accomplished by cooling hydrated lipid samples at slow rates (0.3 Cdeg/min) from the liquid-crystalline state to a low temperature, and by subsequently holding the samples at low temperatures for periods ranging from several

hours to many days [12,16,18,20,21]. The conditions of sample equilibration described in Materials and Methods for each PE-PC system examined here were chosen to allow full equilibration of samples in the gel state(s) prior to calorimetry. Further low-temperature incubations of the samples usually produced minimal further changes in their calorimetric behavior, except in a few exceptional cases that are explicitly noted below.

DMPE / DMPC mixtures

In Fig. 1 are shown a series of thermograms obtained with DMPE-DMPC dispersions of varying PE content. Pure DMPC exhibits a gel-gel 'pretransition' at 14.7°C and a gel-to-liquid-crystalline transition at 24.0°C, while pure DMPE exhibits a single transition at 50.1°C. Hydrated DMPE samples that were incubated at 4°C for

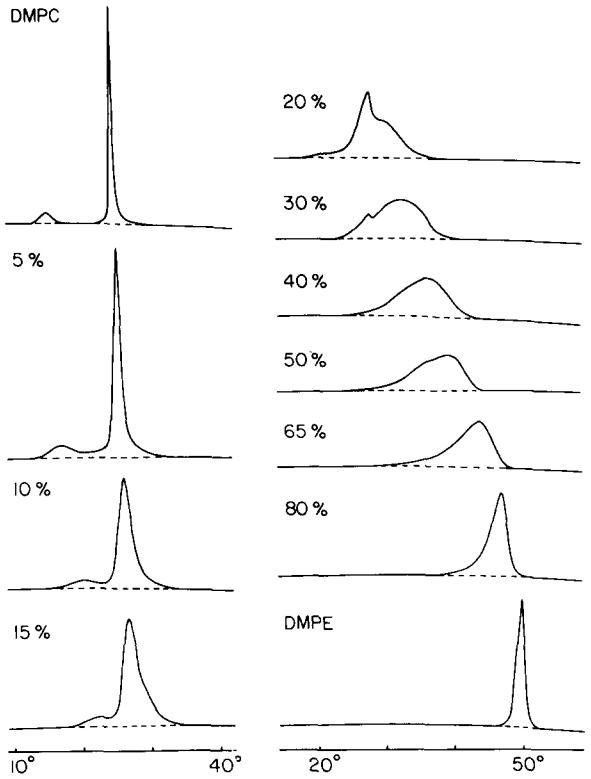


Fig. 1. Calorimetric thermograms for samples of DMPC containing the indicated mole fractions of DMPE. Details of sample preparation and calorimetric analysis (scan rate: 25 Cdeg/h) are described in the text. All traces for DMPC/DMPE mixtures were obtained with comparable amounts of lipid, while the traces for pure DMPC and DMPE were recorded with roughly one-half as much lipid.

times longer than approx. 12 h gradually relaxed to a second gel state which exhibits a highly endothermic melting transition at 57.3°C, as other workers have also observed previously [22–24]. By contrast, samples containing 20% or more DMPC showed no calorimetrically detectable changes in the gel phase when incubated at 4°C for periods up to at least 10 days. Therefore, all DMPE/DMPC mixtures were incubated for 7 days prior to calorimetric examination to allow possible (slow) lateral redistributions of lipids in the solid phase to take place.

As small amounts of DMPE are incorporated into DMPC bilayers, the DMPC pretransition appears to shift toward the main transition, and the latter transition gradually broadens and shifts upward in temperature. For samples containing about 15–40% DMPE, the shape of the main transition endotherm becomes more complex. A sharp endothermic feature at 27.5°C can be seen in samples containing 20% or 30% DMPE, and a shoulder is seen at this same temperature in the thermograms of samples containing 15% DMPE. This feature can be assigned to a line of three-phase coexistence in the DMPE-DMPC phase diagram at 27.5°C, representing the equilibrium of the liquid-crystalline L_α phase with the gel-state $P_{\beta'}$ and $L_{\beta'}$ phases. The liquidus curve bordering the L_α single-phase region, and the solidus representing the limit of the $L_{\beta'}$ one-phase region, can be determined from the limits of the endothermic features in the thermograms, correcting for the finite transition widths of the pure components as described by Mabrey and Sturtevant [25]. These results are summarized in the phase diagram shown in Fig. 3A.

To complete the phase diagram, it is necessary to assign the boundaries of the $P_{\beta'}$ single-phase region. This can be done as follows. In samples containing from 0 to 10 mol% DMPE, the pretransition is sufficiently well resolved to allow the upper boundary of the pretransition, and the lower boundary of the main transition, to be assigned with reasonable accuracy. The boundaries of the $P_{\beta'}$ single-phase region are more difficult to determine reliably for samples containing higher proportions of DMPE. However, by observing the total heat absorbed below 27.5°C in samples of varying PE content, we can assign the point at

which these boundary curves intersect the three-phase line at 27.5°C. The conversion of lipids from the $P_{\beta'}$ to the L_α phase absorbs much more heat than does the conversion from the $L_{\beta'}$ to the $P_{\beta'}$ phase. Consequently, a large heat absorption will be seen below 27.5°C only for samples in which at least part of the lipids convert from the $P_{\beta'}$ to the L_α phase below this temperature. Examining the sample thermograms in the light of this consideration, we conclude that samples containing no more than 25 mol% DMPE are at least partly converted to the L_α phase below 27.5°C. Therefore, the boundaries of the $P_{\beta'}$ one-phase region must intersect the 27.5°C three-phase line at about 25 mol% DMPE.

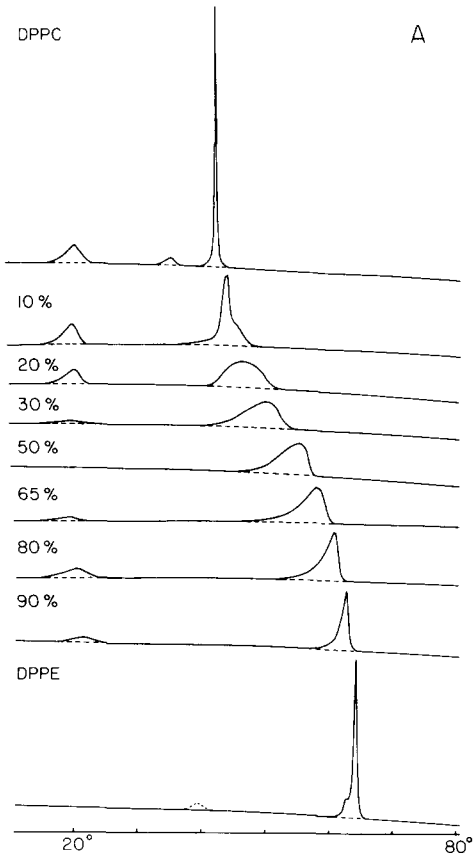
The total enthalpies of the thermograms shown in Fig. 1 were found to vary essentially linearly with the molar proportions of the two lipids, in accord with the predictions of ideal solution theory (not shown). Similar results were obtained for the DPPE-DPPC and DEPE-DEPC systems discussed below (see, for example, Fig. 2B), in agreement with findings reported previously for the DMPE-DPPC and DPPE-DPPC systems [32]. Therefore, we can conclude that the excess enthalpy of mixing of a diacyl-PE with the corresponding PC is similar in the gel and liquid-crystalline phases. This result does not necessarily imply, of course, that the excess enthalpies of mixing in both phases may not be substantial, but only that they are comparable in magnitude.

DPPE/DPPC

In Fig. 2A are shown thermograms obtained for mixtures of these lipids that were incubated for 40 days at 2°C before calorimetric analysis. DPPC dispersions prepared in this way exhibit a subtransition centered at 19.6°C, which develops over a period of a few days at 2°C [21,26,27,42] as well as the pre- and main transitions at higher temperatures. The integrated enthalpies of these transitions are plotted vs. the mole fraction of DPPE in Fig. 2B. The pre- and main transition endotherms change with increasing DPPE content in a manner qualitatively similar to that described above for DMPC-DMPE mixtures, and the equilibria of the L_α, $P_{\beta'}$ and $L_{\beta'}$ phases can be mapped as for the DMPC-DMPE system, giving the results summarized in Fig. 3B.

The behavior of the low-temperature (subtransition) endotherm in various DPPE/DPPC mixtures follows a more complex pattern. As the proportion of DPPE in these mixtures increases from 0 to 50 mol%, the shape and the peak temperature of the subtransition endotherm do not change, but the amplitude of this transition gradually falls off to zero at 40–50 mol% DPPE (Fig. 2B)). When the preincubation of a 50:50 DPPC/DPPE mixture was prolonged to 80 days at 2°C, there was still no evidence of any low-temperature transition in the sample thermogram. Curiously, however, samples containing 65 mol% DPPE again show an endotherm centered near 20°C, and this endotherm is still more prominent in samples containing 80 or 90 mol% DPPE (Fig. 2A). Unfortunately, this transition could not be examined in samples of pure DPPE that were incubated for many days at 2°C, as these samples gradually reverted to a 'dehydrated' gel phase during the preincubation [23]. However, samples of DPPE that were briefly hydrated at 70°C, then incubated for 4–7 days at 2°C did not revert to this higher-melting gel phase but did show a small endotherm at 39.7°C, which was not observed with freshly hydrated samples of this lipid.

The results just presented indicate that DPPE-DPPC mixtures that are rich in DPPE can slowly form what appears to be a subgel phase so long as relaxation to the dehydrated solid phase can be avoided. This result agrees with the findings of Mulukutla and Shipley [43], who have previously reported similar behavior for hydrated dispersions of dimyristoyl-PE. However, some of the present calorimetric results suggest strongly that the subgel phase that forms in DPPE-rich mixtures is distinct from that formed by DPPC. First, we note that as DPPE is gradually introduced into DPPC bilayers, the subtransition endotherm does not change in shape or position from 0 to 40 mol% DPPE. This result alone could suggest either that the mixing of the two lipid species in the subgel phase is virtually ideal, or that DPPC phase-separates in these mixtures at low temperatures, forming a subgel phase composed of essentially pure DPPC which coexists with a second solid phase of higher DPPE content. The observation that the subtransition enthalpy falls off sharply as DPPE is introduced into DPPC bilayers (Fig. 2B) argues



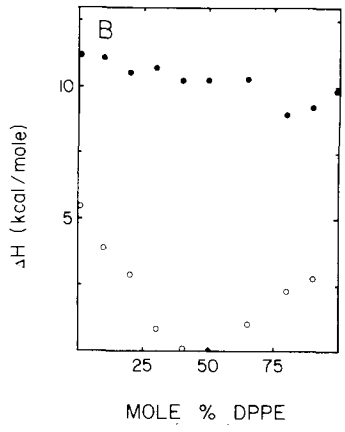


Fig. 2. (A) Calorimetric thermograms for samples of DPPC containing the indicated mole fractions of DPPE. Details of sample preparation and calorimetric analysis (scan rate: 12 Cdeg/h) are given in the text. Samples containing 90 mol% and 100 mol% DPPE were normally incubated at 2°C for only 14 days or 16 h, respectively, in order to avoid possible relaxation to a dehydrated solid phase [23]. The dashed curve in the thermogram for pure DPPE represents an endotherm that was observed in samples incubated for 4–7 days, but not overnight, at 2°C. (B) Integrated molar enthalpies for the endothermic transitions of DPPE/DPPC mixtures. Open circles, subtransition enthalpy; closed circles, sum of pre- plus main transition enthalpy (net $L_{\beta'}$ to L_{α} conversion). Enthalpies were determined as described in Materials and Methods.

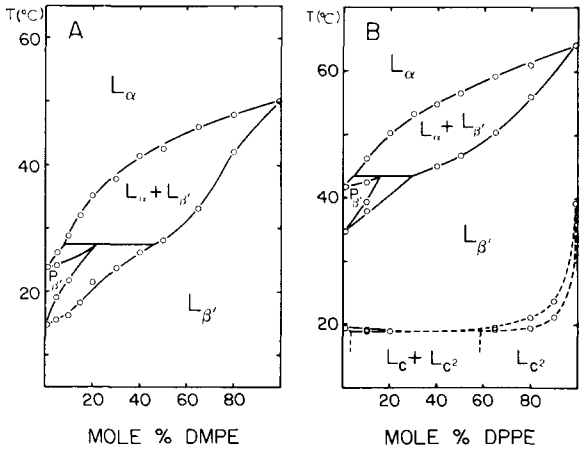


Fig. 3. Phase diagrams for (A) the DMPC-DMPE and (B) the DPPC-DPPE system, derived from calorimetric results as described in the text. Phases are designated by standard symbols: L_α, liquid-crystalline lamellar phase; $L_{\beta'}$, hydrated gel phase; $P_{\beta'}$, 'ripple' phase; L_c, 'subgel' phase of DPPC; L_{c^2}, low-temperature metastable solid phase of DPPE. In (B), the vertical boundaries of the $(L_c + L_{c^2})$ and L_{c^2} regions are indicated as dashed lines, as these boundaries can be mapped only approximately given the metastability of the L_{c^2} phase.

strongly against the former alternative. DPPC bilayers incorporating DPPE in proportions up to at least 50 mol% thus appear to be phase-separated at low temperatures. By contrast, mixtures containing $\geqslant 65$ mol% DPPE in DPPC exhibit a subtransition endotherm that changes in shape and position with increasing PE content, suggesting that a single solid phase exists both above and below the subtransition in this range of compositions. These features of the low-temperature phase behavior of DPPE/DPPC mixtures are summarized in the phase diagram shown in Fig. 3B.

It remains to explain why roughly equimolar mixtures of DPPE and DPPC fail to show any indication of a subtransition, even after incubation for times very much longer than those re-

quired to form the subgel phase in mixtures of either higher or lower DPPE content. It may be that a gel ($L_{\beta'}$) phase containing 50 mol% DPPE cannot form a 'subgel' phase, even on a time scale of several weeks, although it would do so at true equilibrium. Alternatively, it is possible that a subgel phase can form in a DPPE/DPPC mixture of this composition but is so close in enthalpy to the $L_{\beta'}$ phase that the transition between these phases is undetectable by calorimetry. It is not possible to decide between these alternatives from the present results. It is of interest in this regard that an equimolar mixture of D- and L-DPPC also fails to form a subgel phase on a time-scale considerably longer than is required to form a subgel phase from pure L- (or presumably, pure D-) DPPC [44]. Similar behavior has also been reported for an equimolar mixture of the even-chain species DPPC and the odd-chain dihexadecanoyl-PC [45], while an equimolar mixture of two even-chain species, DMPC and DPPC, forms a subgel phase on a time-scale intermediate between those required to form subgel phases from the pure components [46].

DEPE/DEPC

Unlike most saturated phosphatidylethanolamines, DEPE exhibits a lamellar-to-hexagonal II transition as well as a gel-to-liquid-crystalline phase transition within the temperature range examined in these experiments. DEPC exhibits a small transition at 9.3°C in addition to the main transition at 12°C. While this second transition of DEPC is small in amplitude ($\Delta H \approx 0.3$ kcal · mol^{-1}), its existence has important consequences for the thermotropic behavior of DEPE/DEPC mixtures, as the thermograms shown in Fig. 4 illustrate. Samples containing 5–30% DEPE in DEPC exhibit a sharp endothermic feature at 13.2°C, which can be attributed to a line of three-phase coexistence in the phase diagram for the DEPE-DEPC system. Using the same approach as was outlined above to determine the DMPE-DMPC phase diagram from calorimetric data, the lamellar-phase equilibria of the DEPE-DEPC system can be mapped to give the phase diagram shown in Fig. 5A. The DEPE-DEPC phase diagram is remarkably similar in form to the DMPE-DMPC phase diagram described above, suggesting that the energetics of PE–PC interactions in lamellar phases are fundamentally quite similar for the two systems.

The introduction of small amounts of DEPC into DEPE samples has much more dramatic effects on the lamellar-to-hexagonal II transition of DEPE than on the main (gel-to-liquid-crystalline) transition, as the thermograms shown in Fig. 4 illustrate. As the molar percentage of DEPC in DEPE increases from 0 to 15, the small 63.5°C endotherm observed for pure DEPE gradually broadens and shifts upward in temperature, with a barely visible peak at 82°C in samples containing 15 mol% DEPC. Because the limits of this small endotherm are difficult to assign precisely, we have plotted only the peak temperature of this transition on the phase diagram shown in Fig. 5A. Our demarcation of the high-temperature portion of the phase diagram into 'lamellar' and 'hexagonal-II' regions neglects two potential complications. First, ^{31}P-NMR and freeze-fracture electron microscopy has shown the presence of 'isotropic' structures in PE/PC mixtures at temperatures where the samples are neither purely lamellar nor purely hexagonal [6–8]. Secondly, the formation and reversion of isotropic structures in PE/PC mixtures containing higher mole fractions of PC have been shown to be relatively slow and to be prone to marked hysteresis [8]. These factors can in principle greatly complicate the interpretation of our calorimetric results. However, we have previously observed with *N*-alkylated PEs and similar compounds (Ref. 26, and unpublished results) that calorimetry appears to be insensitive to the formation of isotropic structures but faithfully reports the temperature at which the overall lipid organization changes from lamellar to hexagonal II. Moreover, Kirk and Gruner [29] have concluded from X-ray diffraction measurements that 25 mol% DOPC elevates the lamellar-to-hexagonal II phase transition temperature of DOPE by about 45 Cdeg. The magnitude of this shift is comparable to that which we predict for a sample containing 25 mol% DEPC in DEPE if we extrapolate the dashed curve in Fig. 5A to 25 mol% DEPC. Therefore, we believe that the calorimetric results describe adequately the effects of low molar fractions of DEPC on the tendency of DEPE to form the hexagonal II phase. However, the calorimetric results give no

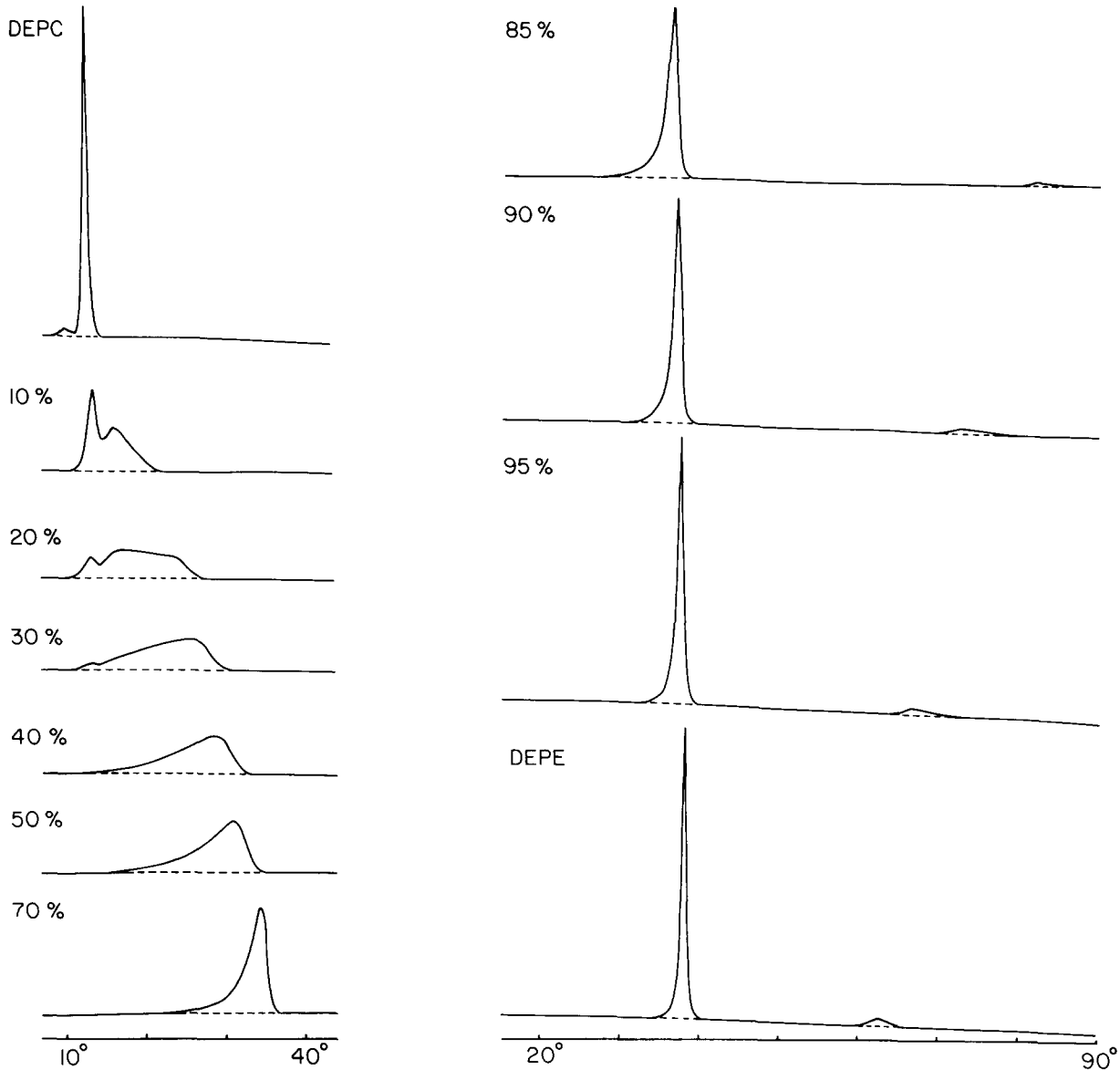


Fig. 4. Calorimetric thermograms for samples of DEPC containing the indicated mole fractions of DEPE. Details of sample preparations and calorimetry (scan rate: 25 Cdeg/h) are given in the text.

information on the local formation of 'isotropic' structures in DEPE/DEPC mixtures as a function of temperature.

DPPE/DEPC

The thermograms shown in Fig. 6 give evidence for poor miscibility of these two species. A distinct endothermic component is seen centered at 12.5°C in samples containing 10–50% DPPE, followed by a gradual absorption of excess heat over a broad temperature range. The 12.5°C feature can be assigned to a line of three-phase coexistence ($L_{\beta'}$, $P_{\beta'}$ and L_{α}) in the DPPE-DEPC phase diagram. Significantly, the upper temperature limit of the excess heat absorption in various thermograms increases continuously with increasing DPPE con-

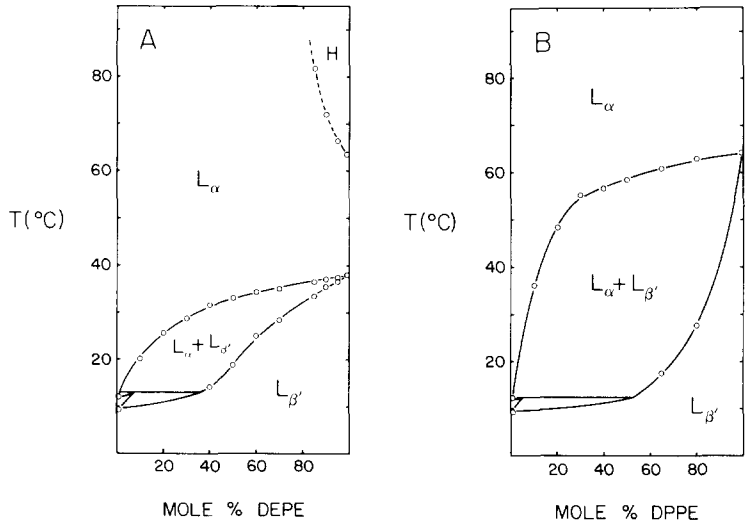


Fig. 5. Phase diagrams determined from calorimetric data for (A) the DEPC-DEPE system and (B) the DEPC-DPPE system. The dashed curve in (A) represents the peaks of the endotherms for the lamellar-to-hexagonal II phase transition in DEPC/DEPE mixtures of the indicated compositions. Because these endotherms are relatively small, we cannot map accurately the limits of the region of coexistence of lamellar and hexagonal II phases, which should replace this dashed curve in a rigorous phase diagram. Phases are designated as in Fig. 3, or as H for the hexagonal II phase.

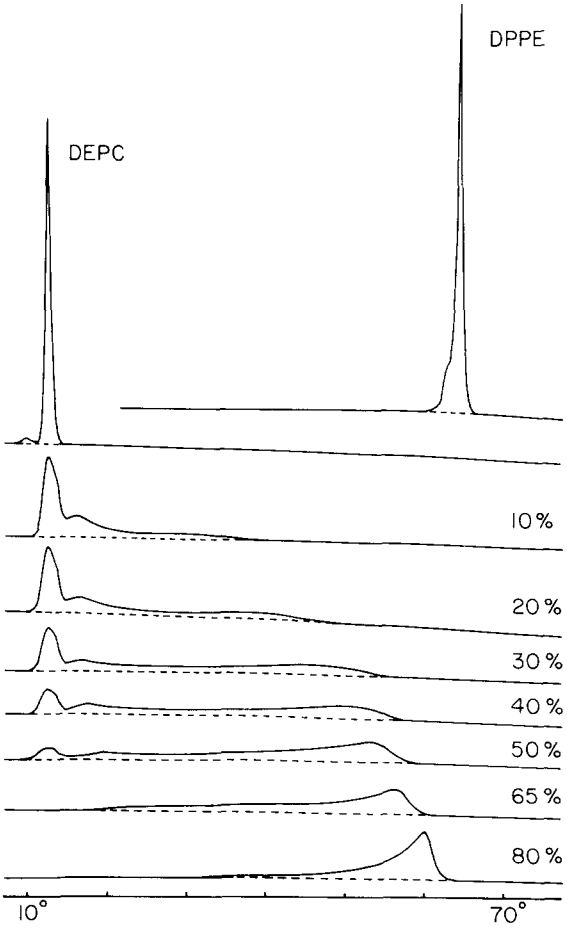


Fig. 6. Calorimetric thermograms for samples of DEPC containing the indicated mole fractions of DPPE. Details of sample preparation and calorimetry (scan rate: 25 Cdeg/h) are given in the text.

tent, indicating that the liquidus curve has no truly horizontal portion, although this curve flattens markedly above about 30% DPPE. As further evidence that the liquidus has no horizontal portion, we note that none of the calorimetric traces for DEPC/DPPE mixtures shows a sharp and distinct endothermic component in the

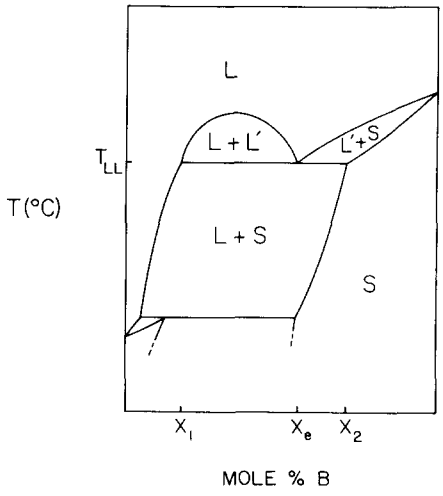


Fig. 7. Hypothetical phase diagram for a system exhibiting liquid-liquid as well as solid-solid phase separation. As samples with contents of component B ranging from mole fraction X_1 to mole fraction X_2 are heated through T_{LL}, an abrupt melting of a fraction of the lipids will be seen at T_{LL}, resulting in a sharp absorption of appreciable heat at this temperature. The amplitude of the resulting endotherm at T_{LL} will vary linearly with composition from zero at X_1 to a maximum at X_e, then fall linearly to zero at composition X_2.

upper-temperature portion of the thermogram. The importance of this observation can be appreciated by reference to the theoretical phase diagram shown in Fig. 7 for a hypothetical binary lipid mixture that exhibits liquid-liquid immiscibility. Applying the lever principle to this phase diagram, we predict that when a sample whose composition lies within the indicated range is gradually heated, a substantial fraction of the lipid will abruptly convert from the gel to the liquid state at the temperature denoted T_{LL}. This conversion will result in an abrupt absorption of substantial excess heat at T_{LL}; the amount of excess heat absorbed at this temperature will be maximal at the composition indicated as X_e in Fig. 7. In fact, no such behavior was observed at higher temperatures in our thermograms for the DEPC-DPPE system. By contrast, exactly this type of behavior was observed at lower temperatures, where solid/solid/liquid three-phase coexistence is observed for all four PE-PC systems examined in this study, as already discussed above. We conclude that DEPC and DPPE are in fact completely miscible in the liquid-crystalline phase, although the shape of the liquidus curve suggests that lipid mixing in this phase is strongly nonideal.

Discussion

The physical properties of PE/PC mixtures, and more specifically the mixing behavior of binary combinations of PCs with PEs, have long been of interest to membrane workers, and the first phase diagrams for such systems were reported over a decade ago [7,11,13,15,25,30–32]. However, two important sets of findings appearing in recent years have underscored the need for considerable care in the determination and analysis of such phase diagrams. First, the solid-phase behavior of saturated phosphatidylcholines has been found to be quite complex, with as many as three or even four solid phases demonstrated for some species, such as DPPC [21,26,27,33,34]. Solid-phase polymorphism has recently been demonstrated for branched-chain PCs [21,35,36] and, as discussed in this paper, for *trans*-unsaturated PCs as well. It is conceivable that still other types of PC, such as species with *cis*-unsaturated chains, may also exhibit solid-state polymorphism which has gone undetected in the studies carried out to date on these systems. The existence of multiple solid phases for many PCs has important consequences for the mixing of these species with other lipids, particularly when the second species exhibits a single solid phase or a different set of solid phases, as Luna and McConnell [13] have discussed previously.

A second complication that attends the determination of complete phase diagrams for lipid mixtures is that the equilibration of lipids between various solid phases can be quite slow. This problem arises both from the relatively slow rates of lateral diffusion of lipids in gel-state bilayers [37] and from the inherently slow kinetics of formation of certain types of solid phase, such as the 'subgel' phase(s) [26,27]. When we consider these points, as well as the precautions in sample preparation that must be observed for accurate determination of even simple gel-liquid-crystalline phase equilibria [11,12], it is clear that accurate phase diagrams can be obtained only by using lipid samples that are cooled very gradually from the liquid-crystalline to the gel state, incubated for long times in the solid state and finally heated at the lowest practical scan rates to examine their thermotropic behavior.

Taken together, the considerations just noted place rather stringent restrictions on the methods of sample preparation and data analysis that can be used to obtain accurate thermodynamic data for two- (or, more generally, multi-) component systems. At present, thermodynamic parameters describing lipid mixing in liquid-crystalline lamellar phases are usually evaluated from data that describe equilibria between liquid and solid phases (e.g., by simultaneously fitting the liquidus and solidus curves in an experimental phase diagram, using an appropriate thermodynamic model [11, 12,38]). This fact dictates that careful attention must be paid to the details of solid-phase mixing of lipids, including the possibility of multiple solid phases, even in cases where the liquid-phase mixing of the lipids may be of greater interest, as is the case for biological membranes. Unfortunately, quantitative analyses of experimental phase diagrams for PE-PC and other systems (see, for example, Ref. 11) have often failed to account for the complicating effects of multiple solid phases in

the phase diagrams, and thermodynamic values derived from these analyses (e.g., excess free energies of mixing) may therefore be unreliable.

One of the more surprising findings in our study is the fact that the solid- and liquid-phase mixing of DEPE and DEPC is very similar to the mixing of the disaturated PE-PC pairs examined here. While the pretransition of pure DEPC has a much lower heat content than that of DMPC or DPPC, the solid-phase miscibility of DEPE and DEPC is not significantly better than that observed for the DMPE–DMPC and DPPE-DPPC systems. This result conflicts with the intuitive notion that bilayers formed from *trans*-unsaturated lipids, which exhibit lower phase transition temperatures than their saturated counterparts, will be somewhat 'looser' in their packing requirements and therefore will exhibit better miscibility in solid phases than do fully saturated lipid mixtures. It is possible that other types of PC (e.g., *cis*-unsaturated species), for which no clear pretransition has been observed, may also be able to adopt gel phases whose structure is quite distinct from that of the $L_{\beta'}$ phase formed by saturated phospholipids at lower temperatures. If this is the case, the extensive solid-phase immiscibility that is frequently observed in mixtures of *cis*-unsaturated and disaturated phospholipids is readily understandable.

While limited solid-phase miscibility is clearly characteristic of binary PE/PC mixtures, we have obtained no evidence for liquid-liquid immiscibility of these species in any of the systems examined, in contrast to the findings of Wu and McConnell [15] for the DEPC-DPPE system. In fact, however, the TEMPO partitioning data reported by these authors for this system appear to be fundamentally compatible with the calorimetrically determined phase diagram shown in Fig. 5B of this paper. Most importantly, the previously reported ESR data provide no clear evidence for an abrupt melting of substantial amounts of lipid in samples of any composition around 50°C, the temperature assigned by Wu and McConnell to a putative line of three-phase coexistence (liquid/liquid/solid) in the DEPC-DPPE phase diagram. An abrupt melting of a significant fraction of the lipid should occur at about 50°C for at least some sample compositions if a region of liquid-liquid immiscibility, and hence a line of liquid/liquid/solid three-phase coexistence, were present in the DEPC-DPPE phase diagram at about 50°C. In fact, the TEMPO data provide no such evidence for a line of three-phase coexistence in the phase diagram at any temperature above about 10°C. We therefore believe that both the present calorimetric data and the previous TEMPO partitioning results can be interpreted most satisfactorily in terms of a DEPC-DPPE phase diagram whose fluidus line becomes relatively flat, but is nowhere truly horizontal, above about 30 mol% DPPE, as diagrammed in Fig. 5B. It thus appears that DEPC and DPPE are miscible (but not ideally so) in the liquid phase.

Since all of the PE/PC mixtures examined in this study exhibit limited solid-solid phase separations, the phase diagrams obtained from these systems cannot readily be analyzed quantitatively to evaluate the thermodynamic parameters governing lipid mixing in the solid and liquid-crystalline phases. However, the PE-PC phase diagrams obtained here can be compared to phase diagrams for other mixtures of lipids exhibiting similar transition temperatures and enthalpies for the pure components. If we compare the PE-PC phase diagrams determined here to the phase diagrams determined previously for PS-PC and PA-PC mixtures of like acyl chain composition [18–20], it appears that PC mixes more nearly ideally with PS or PA than it does with PE. It may be that lipids whose headgroups can serve as hydrogen-bond donors as well as acceptors, such as PE, PS and singly ionized PA, exhibit a net tendency to self-associate preferentially in mixtures with PC. However, this tendency will be more pronounced for PE, a neutral species, than for PS and PA, whose negative charges make such like-like interactions less energetically favorable.

If we consider the experimentally determined phase diagrams for a variety of phospholipid pairs with different headgroups but similar acyl chain composition [11,13–16,18–20,30–32], we can rank the miscibilities of various phospholipid pairs in the following order: (PE, PS) ≈ (PE, PA) > (PC, PS) ≈ (PC, PA) > (PE, PC). In light of this result, it is most interesting to note that anionic phospholipids and PE are often found to be concentrated on one side of a mammalian cell mem-

brane while choline phospholipids are typically distributed preferentially to the other face of the membrane [40]. This transbilayer distribution of lipids, while it is obviously not determined simply by the miscibilities of the lipids themselves, nonetheless reflects an optimal combination of the most compatible phospholipid species at each face of the bilayer. We would conclude in accord with the recent suggestions of Tenchov and Koynova [41], that the relative energies of mixing of the various phospholipid headgroup species in mammalian cell membranes may play a significant role in the stabilization (albeit not in the establishment) of the transverse asymmetry of the lipid distribution in these membranes.

Acknowledgements

This work was supported by grants from the Medical Research Council of Canada (Grants MA-7580 and ME-7776) and les Fonds pour la formation de chercheurs et l'aide à la recherche du Québec (Grant 86-EQ-2954). I thank Ms. Rania Leventis for her expert assistance in the preparation of some of the lipids used in this study.

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