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COMPLEX PHASE MIXING OF PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLSERINE IN MULTILAMELLAR MEMBRANE VESICLES

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Summary

The phase mixing in dipalmitoyl phosphatidylcholine and bovine brain phosphatidylserine mixtures suspended in aqueous salt solutions was studied by differential scanning calorimetry, freeze-fracture electron microscopy and X-ray and electron diffraction. The pure dipalmitoyl phosphatidylcholine has two well-defined solidus phases $P_{\beta'}$ and $L_{\beta'}$ and a liquidus phase L_{α} while the pure phosphatidylserine has a broad transition from L_{β} to L_{α}. The mixture exhibits some dipalmitoyl phosphatidylcholine characteristics up to 30% phosphatidylserine when pure $P_{\beta'}$ phase no longer exists. Phase mixing is observed at all compositions. An addition of 3% phosphatidylserine is found to be sufficient to destroy the sharp $L_{\beta'}$—$P_{\beta'}$ transition observed in pure dipalmitoyl phosphatidylcholine. The transition between $L_{\beta'}$ and $P_{\beta'}$ in these mixtures is displaced to lower temperatures and becomes gradual as depicted by all three techniques, but freeze-fracture gives more definite information of the continuous transition. The most striking observation on the morphology of the mixed membranes (3—30% phosphatidylserine in dipalmitoyl phosphatidylcholine) is the presence of banded patterns ($P_{\beta'}$) at temperatures well below the main transition peak as detected by differential scanning calorimetry.

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Abbreviations: DPPC, dipalmitoyl phosphatidylcholine; Tes, *N*-Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid.

Introduction

The structural phases of membrane lipids are believed to affect many functions of biomembranes. This relation has been well-demonstrated in the case of microorganisms [1,2]. In the membranes of higher animals, this relationship is not so well-defined, due perhaps to the complexity of the lipid components. Since membrane lipids of higher animals are well regulated to consist of many lipid components including cholesterol and several kinds of phospholipids with varying acyl chain length and degree of unsaturation, there is usually no clear-cut phase transition, but rather a broad range of transition temperature [3]. Within this temperature range, several phases of lipids may coexist. The heterogeneous state of the membrane lipid may provide different microenvironments for various other membrane components and therefore could affect membrane function. The fusion between membranes is believed to initiate in certain local regions of membrane where the lipid phases and other conditions are favorable [4]. Thus, the miscibility and the possible phase separation among various lipid components are of interest in the study of membrane structure and function.

Lipid phase separation was first suggested by Shimshick and McConnel [5] based on ESR evidence. Since then, many techniques have been applied to observe this phenomenon, namely, calorimetry [6,7], freeze-fracture electron microscopy [7—10], fluorescence spectroscopy [19] and X-ray and electron diffraction [11,12]. Recently, Luna and McConnell [13,14] have reported a detailed study of a mixed phosphatidylcholine/phosphatidylserine system using spin label ESR and freeze-fracture electron microscopy. By correlation of their results with other studies based on calorimetry [15] and X-ray diffraction [11], they were able to chart a phase diagram of this mixture system.

In order to further simulate a heterogeneous mixture of lipids in biomembranes, we have carried out a study of mixed membranes containing a synthetic homogeneous phospholipid-dipalmitoyl phosphatidylcholine (DPPC) and a heterogeneous natural phospholipid-phosphatidylserine isolated from bovine brain. The former has a sharp main phase transition and a definite minor transition at premelt temperatures while the latter has only a broad transition range. This mixture represents one step further from mixtures of two homogeneous lipids (with well-known transition temperatures), towards a more complex system involving many components. Differential scanning calorimetry, freeze-fracture electron microscopy and X-ray and electron diffraction studies were made on specimens of same compositions, and the results were integrated to produce a phase diagram for this particular system.

Materials and Methods

Lipid vesicles. Phosphatidylserine was purified from bovine brain [16] and dipalmitoyl phosphatidylcholine was synthesized [17] as described before. Both lipids were finally purified on silicic acid columns. In addition, phosphatidylserine was washed with EDTA and converted to the sodium salt [17]. Both lipids were shown to be pure by thin-layer chromatography [16] even on

overloaded plates (500 μg/spot), and stored in chloroform in sealed ampules under nitrogen at −50°C until use.

Vesicles were prepared as follows: the appropriate lipid or mixture was evaporated from a chloroform solution into a glass tube under vacuum, at 25°C for 10—20 min. The dry lipid was then hydrated by the addition of aqueous buffer solution (100 mM NaCl, 2 mM L-histidine, 2 mM Tes, 0.1 mM EDTA, pH 7.4), and dispersed by Vortex shaking at 45°C for 10 min. The concentration of phospholipid was 20 μmol in 5 ml based on phosphorus analysis [18]. The suspension was allowed to further equilibrate at 45°C for 2 h and then centrifuged for 10 min at 25°C in an Eppendorf minicentrifuge in three aliquots of 1.6 ml each. The supernatant was removed by a pasteur pipet and the wet pellets were used immediately for differential scanning calorimetry, freeze-fracture or diffraction studies.

Differential scanning calorimetry. An aliquot of the centrifuged (pellet) vesicle preparation (5—8 μmol in 20—50 μl volume) was placed in hermetically sealed calorimeter pans at 25°C, and transfered into a differential scanning calorimeter (DSC-2, Perkin-Elmer). The sample was initially cooled to an appropriate temperature (usually 1°C) and then heated at a rate of 5°C/min. The overall conditions were as described before [19].

Freeze-fracture electron microscopy. An aliquot of the pellet comprising of 5—8 μmol of phospholipid was resuspended in 30% glycerol and small volume was pipetted into Balzer's specimen holders (center recessed gold discs). The Balzer specimen holders containing lipid were placed on a metal block in a hydrated enclosure. The entire enclosure was then incubated at the desired temperatures and after equilibration for 15 min, the final temperature was read by three thermometers and an average temperature was recorded. The samples were quenched in Freon 22 from the desired temperature and fractured and replicated in a Polaron E 7500 freeze-fracture module pumped by a Perkin-Elmer Ultek TNB-X ion pump. All samples were fractured at a temperature of −115°C under a vacuum of $5 \cdot 10^{-7}$ Torr or better. Replicas were cleaned in full strength Clorox for 1 h, transferred to 10—20 ml of distilled H_2O containing three drops of 0.01% bacitracin and picked up on uncoated 200 mesh copper grids. The dried grids were then placed in a Ladd replica extractor (Ladd Res. Ind., Burlington, VT) and cleaned with chloroform vapors according to the method of Vail and Stollery [20].

Representative micrographs were taken at an initial magnification of 20 000 times on a Siemens 101 electron microscope.

Diffraction. Small and wide angle X-ray diffraction were carried out at set temperatures. Multilamellar vesicle samples identical to those used in above sections were used. The multilamellar vesicle suspensions were concentrated by centrifugation at $300 \times g$ for 10 min. The concentrated suspension was placed in an aluminum cell with Mylar windows. The temperature of the cell was controlled by a thermoelectric module (Melco) to an accuracy of 0.5°C. Both wide and small-angle diffraction lines were recorded on the same film using a Frank camera with slit focusing. The X-ray source was a Jerrel-Ash microfocusing unit with copper target and nickel filter. Each exposure requires about 10 h, therefore, diffraction measurements were carried out only at selected temperatures and compositions.

In order to accurately locate onsets of phase transition by wide-angle diffraction lines, an alternative technique, i.e. electron diffraction, was employed. The results of electron and X-ray diffraction are shown to be equivalent in most cases [21]. The strong wave-specimen interaction in electron diffraction experiments enables a large amount of data to be collected within a short time. The specimens used in electron diffraction experiments were unsupported bilayers formed by the dipping method [22], using the same buffer solution as in the multilamellar vesicle suspension. The bilayers were kept fully hydrated at all temperatures in an environmental stage [23] in a Siemens 1a electron microscope operating at 80 kV. Wide-angle diffraction patterns were recorded as a function of temperature. From a series of low dose (10^{-4} C/cm^2) patterns taken at 2°C intervals (2 min/degree, heating rate), the upper phase transition end was located as the disappearance of the sharp diffraction line at 4.2 Å. The onsets measured by X-ray and electron diffraction invariably agreed.

Results

The differential scanning calorimetry scans of pure phosphatidylcholine, pure (100%) phosphatidylserine and various mixtures of the two are shown in Fig. 1. The curves shown were obtained by heating the samples at a rate of 5°C/min.

Pure dipalmitoyl phosphatidylcholine shows two transitions in differential scanning calorimetry thermograms. The smaller endotherm is the premelt or pretransition [8]. The onset of the premelt is 33°C and the end point is 37°C. The peak area indicates a ΔH of 1.2 ± 0.2 kcal/mol [19]. The larger of the two peaks is the endotherm associated with the main (solid-fluid) phase transition [24]. The onset of this transition is 41°C and end point is 43°C. This narrow peak indicates a sharp transition and has a ΔH of 7.7 ± 0.5 kcal/mol [19].

The differential scanning calorimetry scans of pure bovine brain phosphatidylserine show a single broad peak centered at approx. 8°C and extends over the range of 0—15°C. This endotherm is interpreted as the main transition and measured 4.5 ± 0.5 kcal/mol [19].

The differential scanning calorimetry of mixtures containing a small percentage of phosphatidylserine resemble the scans of 100% phosphatidylcholine with the following differences. As the composition of the mixture is varied from 3% to 30% phosphatidylserine, the pretransition and main transition peaks are lowered and broadened with a noticeable downscale shift of both peaks. At a composition of greater than or equal to 30% phosphatidylserine, there is no longer a detectable pretransition by differential scanning calorimetry. The main transition of these mixtures shows a further broadening and lowering of the main peak temperature with a shift downscale, finally merging with the broad transition of 100% phosphatidylserine.

The upper end of the main transition was also determined by the temperature at which the 4.2 Å wide-angle diffraction spacing vanishes. The disappearance occurred abruptly within 2°C and had a hysteresis (upon cooling) of 2—3 degrees. The results from X-ray and electron diffraction agreed in all cases. Since electron diffraction data were taken at closer temperature intervals, they were useful in accurately locating the onset temperatures, which were plotted

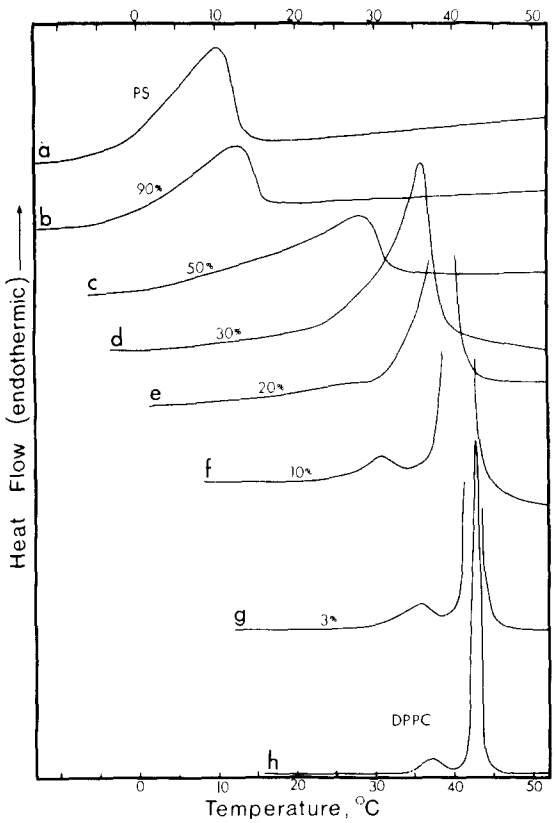


Fig. 1. Differential scanning calorimetry of multilamellar vesicles composed of bovine brain phosphatidylserine (PS) and dipalmitoyl phosphatidylcholine (DPPC) and various mixtures suspended in 0.1 M NaCl buffer. The details of preparation as described in Materials and Methods. (a) Pure phosphatidylserine; (b) 90% phosphatidylserine/10% phosphatidylcholine; (c) 50%, respectively; (d) 30%; (e) 20%; (f) 10%; (g) 3%; (h) pure phosphatidylcholine.

in Fig. 2. The lower onset should be observable as the disappearance of the diffuse 4.6 Å ring which is characteristic of liquid-crystalline diffraction. The disappearance of this diffuse diffraction ring, usually very weak at temperatures approaching the lower onset, cannot be pinpointed with accuracy. Long diffraction spacings in the order of 100 Å in addition to lamellar repeats, are sometimes observable by X-ray diffraction of samples containing high percentage of phosphatidylcholine. This spacing is believed to be related to the banded structure B, as interpreted by Janiak et al. [11]. Again, the disappearance of this spacing cannot be accurately located.

The differential scanning calorimetry data for limits of pretransition and main transition was used as a guide for selecting the points of interest for the freeze-fracture studies. In general, the points of interest were considered to be those regions of the phase diagram which fall below, between or above the pretransition and main transition. No attempt was made to study the exact onset or end-points of these transitions by freeze-fracture because the temperature regulation was not accurate enough to pinpoint the onsets.

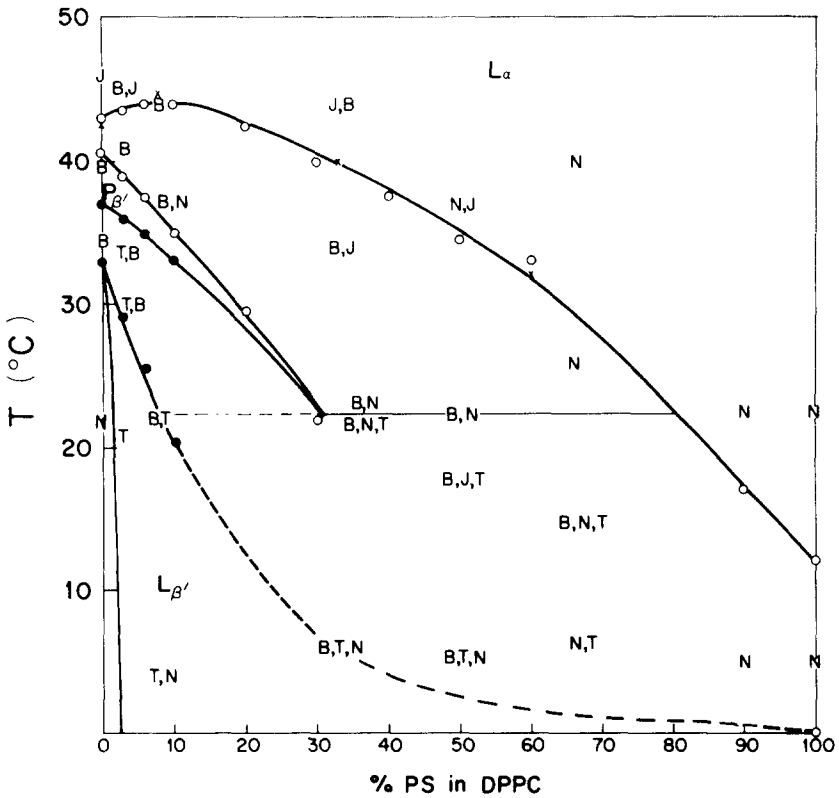


Fig. 2. Phase diagram for mixtures of dipalmitoyl phosphatidylcholine (DPPC) and phosphatidylserine (PS). ○, onsets and end-points of main transition by calorimetry; ●, onsets and end-points of pretransition by calorimetry; X, end-points of transition by X-ray and electron diffractions. Symbols for features seen in freeze-fracture micrographs (except T) follows that in Ref. 13. They are: B, banded; J, jumbled; N, smooth, and T, terrace. When more than one feature appears, the order of symbols indicates the order of predominance of each feature. Experimentally determined and extrapolated phase boundaries are indicated by continuous and broken curves, respectively.

The surface morphology of pure dipalmitoyl phosphatidylcholine as shown by freeze-fracture, is smooth (N) throughout the temperature range of 0—33°C. At approx. 33°C, there is the appearance of the banded texture (B) which persists until a quenching temperature of approx. 42°C (Fig. 3). At higher quenching temperatures, the surface texture exhibits a jumbled (J) appearance. In the case of pure phosphatidylcholine, the surface textures seen by freeze-fracture appear to follow the same trends as the differential scanning calorimetry scans *. Thus, the N textures appear in a temperature range below the premelt. From the onset of the pretransition endotherm till the end of the

* The presence of glycerol, which is added to the freeze-fracture samples as a cryoprotectant could conceivably complicate any correlations between differential scanning calorimetry and freeze-fracture data. In order to investigate this possibility, we have studied the effect of glycerol on the differential scanning calorimetry thermograms of both pure phospholipids used in this study. These experiments indicated that the addition of glycerol (30%, v/v) to dipalmitoyl phosphatidylcholine dispersions produced an upward shift of the transition temperature, which was 2 degrees for the premelt, and 0.5 degrees for the main transition. A similar small upward shift was noted for the phosphatidylserine dispersions.

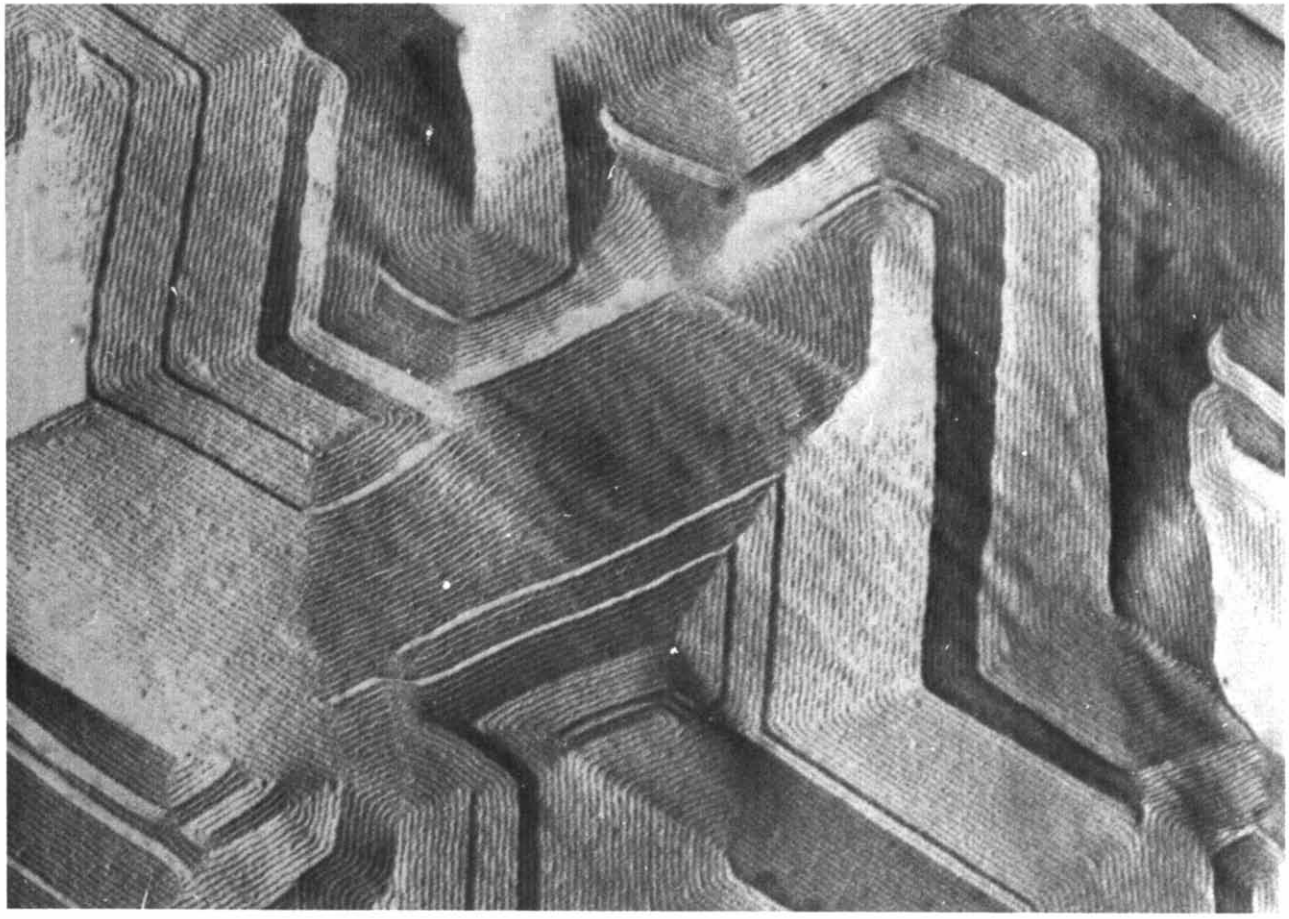


Fig. 3. Freeze-freacture micrographs of a multilamellar vesicle of 100% dipalmitoyl phosphatidylcholine quenched from 37° C. Magnification is 80 000×.

main transition endotherm the freeze-fracture texture is banded. At temperatures above the end of the main transition, the jumbled texture predominates.

In samples were phosphatidylserine is present as 3%, the freeze-fracture textures still appear to follow the differential scanning calorimetry results. Below the premelt temperature range, the texture is a mixture of smooth (N) and terrace-like (T) structures which were previously described by Luna and McConnell [13,14] as whorls. The banded (B) pattern appears at a quenching temperature corresponding to the premelt and gives way to a jumbled (J) texture at quenching temperatures higher than the end of the main transition. At the onset of premelt there is a gradual transition from T to B textures (Fig. 4).

Compositions of 10, 20, and 30% of phosphatidylserine in DPPC show textures in freeze-fractured samples that also correspond reasonably well to the differential scanning calorimetry results. At quench temperatures immediately above the main transition, the texture is jumbled (J) with occasional banding (B). Furthermore the freeze-fracture data consistently shows the appearance of bands (B) at quench temperatures near and slightly lower than the onset of the pretransition as determined by differential scanning calorimetry. Banded texture is observed even in mixtures where the premelt is not clearly defined (30%). The location of these points are indicated on the phase diagram (Fig. 2). The B texture is gradually being replaced by a terrace-like texture (T) as the temperature is lowered from the region in between the premelt and the main

Fig. 4. Freeze-fracture micrograph of a multilamellar vesicle of 3% phosphatidylserine in DPPC, and quenched at 30°C. Magnification is 80 000X.

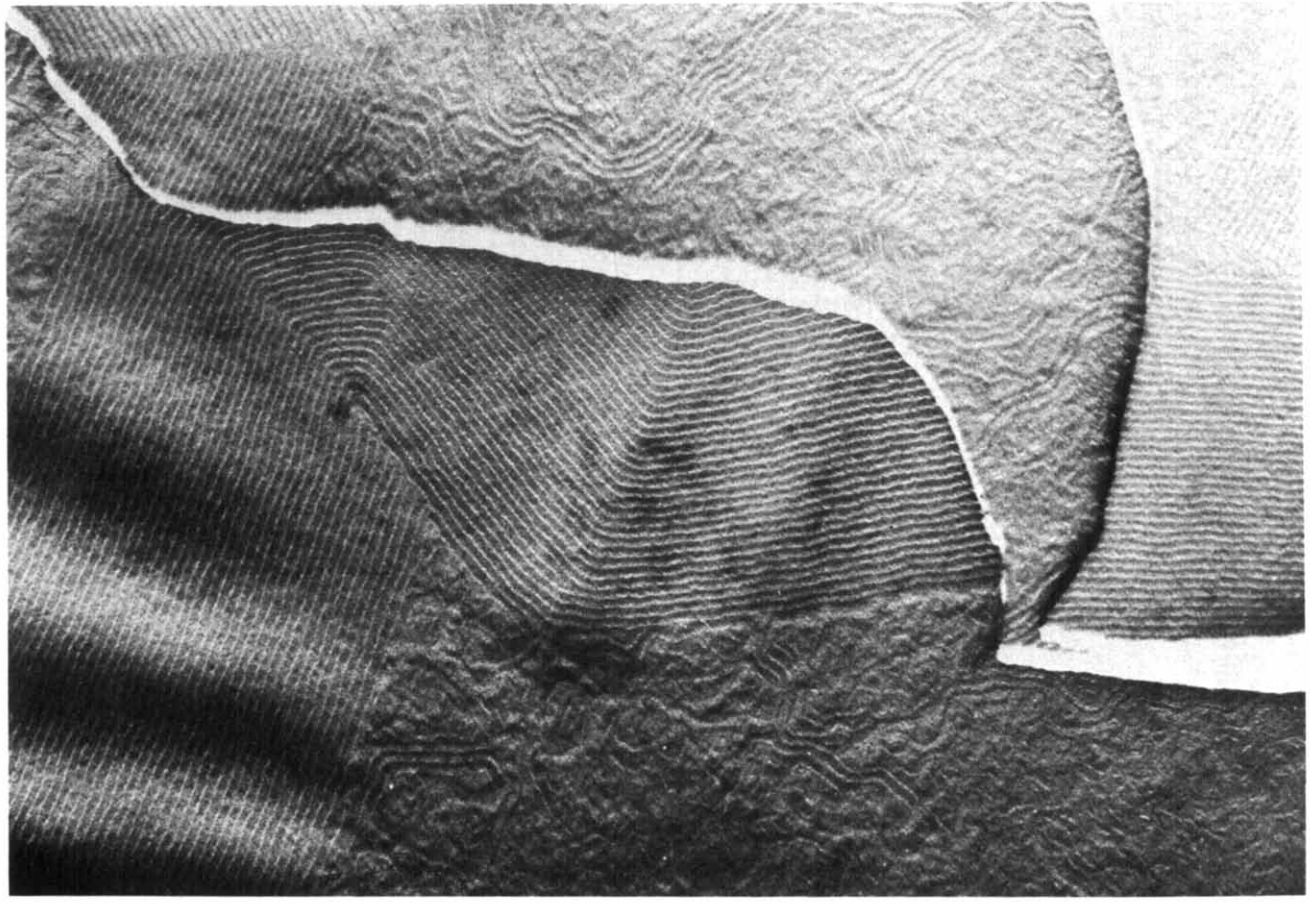
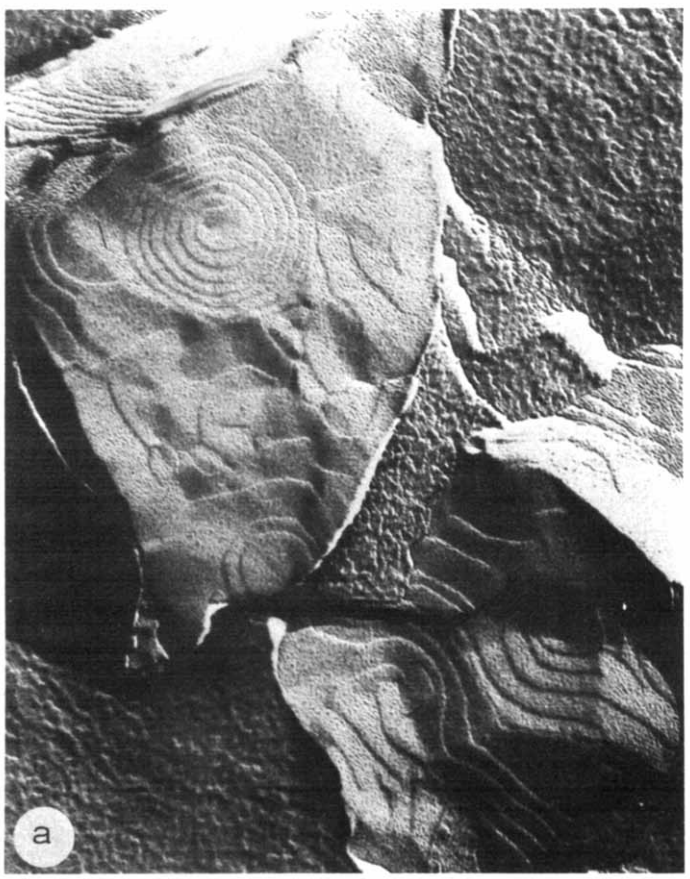


Fig. 5. Freeze-fracture micrographs of a multilamellar vesicle of 33% phosphatidylserine in DPPC, and quenched at 34°C. Magnification is 80 000X.

transitions. This terrace-like pattern seems to be a transition between the B and N textures, but is definitely not a mixture of two distinct textures like B and J (Fig. 5) at the temperatures within the main transition. At first, the regular bands transform gradually to a monoclinic saw tooth pattern. It seems that one side of the saw tooth expands irregularly to form a series of terrace-like features (see for instance Fig. 4). The change from B to T spreads over a large temperature range within which regular saw tooth bands intermix with irregular terraces. The irregular patterns become very difficult to observe by X-ray diffraction. Before the T pattern is completely extended to a smooth surface (Fig. 6a), the topography of some vesicles becomes angulated (Fig. 6b). Straight ridges and valley lines start to form, which distort an otherwise smooth texture N.

At a composition of 50% phosphatidylserine, even though there is no premelt shown by differentical scanning colorimetry, bands (B) are seen at quench temperatures just below the main transition as indicated on the phase diagram. At quench temperatures within the main transition range, a mixture of banded (B) and jumbled (J) textures is seen. T texture appears below a temperature of approx. 22°C. Quench temperatures above the main transition show a jumbled



(J) and/or smooth (N) freeze-fracture textures.

At a composition of 70% phosphatidylserine, the differential scanning calorimetry shows a single broad curve centered at 13°C and covering the range of 3—29°C. A quench temperature of 5°C shows a terraced (T) texture. Quench temperatures of 18°C show the banded as well as the smooth (N) and terraced (T) textures. At a quenching temperature of 25°C and above, the textures are smooth (N).

At compositions of 90% and 100% phosphatidylserine, the freeze-fracture textures are smooth regardless of quenching temperature (Fig. 7).

Discussion

Our results have substantiated in general those reported by Luna and McConnell [13], but differ in two respects. The phosphatidylserine we used has a lower T_c than the other lipid component, dipalmitoyl phosphatidylcholine, and has a broad range of T_c rather than sharp points as for dipalmitoyl phos-

Fig. 6. (a, b). Freeze-fracture micrograph of multilamellar vesicles of 10% phosphatidylserine in DPPC, and quenched at 5°C. Magnification is 80 000X.

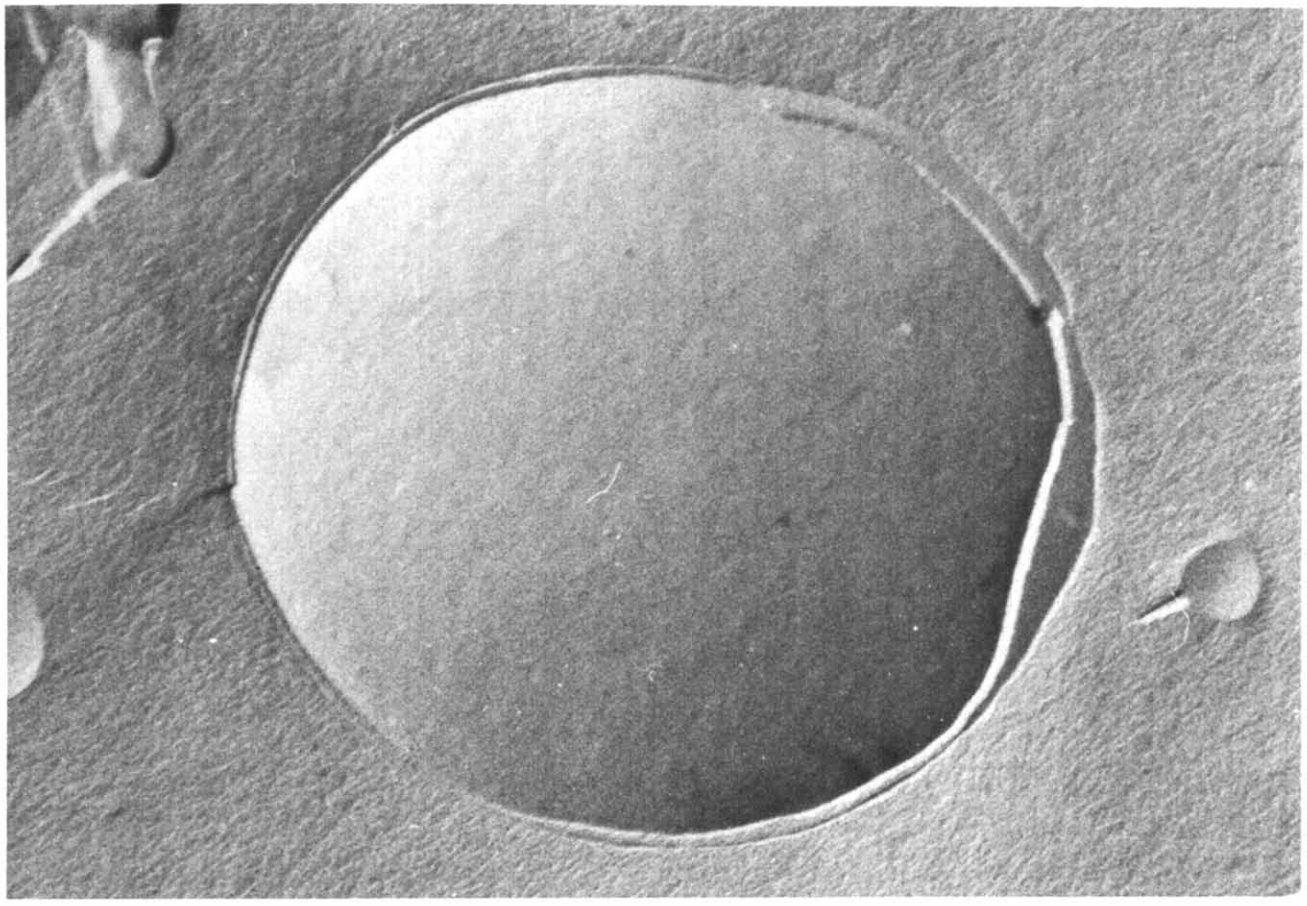


Fig. 7. Freeze-fracture micrograph of a multilamellar vesicle whose composition is 100% phosphatidylserine and quenching temperature is 20° C. Magnification is 80 000×.

phatidylserine and dimyristoyl phosphatidylserine used in Ref. 13. This provides a new dimension in analysis since the phosphatidylserine alone is already a miscible system of molecules differing in chain length and degree of unsaturation. As a result, these phase diagrams are open ended on one side.

The addition of phosphatidylserine has remarkable effects on the phase of dipalmitoyl phosphatidylcholine. On calorimetry measurements alone, the endothermic peaks become broadened as more phosphatidylserine is mixed in with phosphatidylcholine, until the premelt and the main transition peaks merge at 30% phosphatidylserine. The combined 'peak' then gradually shifts to that of pure phosphatidylserine as its percentage continues to increase. The downward shift of the onsets of phase transition, as depicted by both calorimetric and diffraction measurements, indicate that pure phosphatidylcholine domains do not exist in any compositions beyond 20% phosphatidylserine. The low temperature limit of the transition range is less clearly defined because of the complication of the premelt range. The onsets of this small ΔH peak cannot be accurately determined by differential scanning calorimetry. The disappearance of the 4.6 Å diffraction band at the lower onset of the main transition is not as easily observable as the disappearance of the 4.2 Å sharp ring at the upper end point. At this lower temperatures boundary, freeze-fracture parameters give better insight into the system.

As pointed out by Luna and McConnell [13,14], freeze-fracture electron microscopy provides an additional means to characterize the phase diagram, and to certain extent, to delinate new phase boundaries unobservable by other methods. The most important parameter characterized by freeze-fracture is the surface undulation. The precision of quenching temperature can be controlled to 2°C, and the hysteresis is about 3°C [13], therefore there is some uncertainty in the phase boundaries outlined by freeze-fracture. In pure phosphatidylcholine, the J, B, and N features are characteristic of the region of the temperature above the main transition, between main and premelt, and below the premelt, respectively. These features were also observed by X-ray diffraction [11] and corresponded to calorimetry measurements. When bovine brain phosphatidylserine was added to dipalmitoyl phosphatidylcholine, the phase boundary became less clearly defined. In our system, as in the case of dipalmitoyl phosphatidylcholine/dipalmitoyl phosphatidylserine mixtures [13,14], the B and N phase boundary cannot be defined precisely at the low temperature end. The change from T to N is even more ambiguous. The transition does not take place quantitatively where one distinct phase grows at the expense of the other, but is rather a continuous qualitative transformation. Apparently, there is a small energy barrier between the B, T and N phases. Therefore, little thermal transition is expected to be detectable. On the other hand, when B and J coexist in a two phase system, the two patterns are distinctly different. The growth of the B phase seems to be subject to the constraint of the J phase, perhaps through the 'frayed' band lines (Fig. 5). The mechanical constraints appear to cause the ripples to form parallel waves or waves around a 'hexagonal' domain. A detailed analysis of these particular features will be dealt with elsewhere. The N texture shown in Fig. 6b is more jagged than any undulations observed in the N pattern in pure phosphatidylcholine. It is possible that at low temperatures, the mechanical stress forces

deformation of the surface of the solid solution to bend along structurally weak lines, thus forming ridges and valleys. Structurally, the vesicles made of mixed lipids in the solid state are weaker and more heterogeneous than the pure phosphatidylcholine vesicles.

The presence of a heterogeneous phosphatidylserine (with a lower transition temperature than the phosphatidylcholine) has decreased the lower onset of the premelt transition to such an extent that the addition of small amount (3%) of phosphatidylserine is sufficient to change the N surface feature to BT. The steep fall of the N-BT boundary and the extensive BT region, due to the addition of small amounts of heterogeneous components, may have accounted for the earlier observations of B phase in phosphatidylcholine at temperatures below the premelt [8]. The existence of B pattern at temperatures below the premelt seems to depend on impurity rather than purity of the phosphatidylcholine sample.

Based on the differential scanning calorimetry, freeze-fracture and diffraction data, we may now attempt to construct a phase diagram. From the common onsets of the main exothermic peak and the 4.2 Å sharp diffraction ring at various compositions, one may define the upper end of the transition region. Above this line, the lipid mixture is in the L_α phase, and shows J-type surface feature in freeze-fracture micrographs. Below this line, there exists a region where both L_α and $P_{\beta'}$ coexist, as indicated by the BJ pattern and the coexistence of the 4.2 Å diffraction ring and the 4.6 Å diffraction band. From 30% phosphatidylserine on, the upper end of the transition range decreases monotonically as in the case of ideal mixtures. Between 0% and 30% phosphatidylserine, this main transition region is bordered on the lower edge by an interim phase that is characterized by the pure B feature. This phase, in pure dipalmitoyl phosphatidylcholine was shown to be the $P_{\beta'}$ phase by X-ray diffraction experiments [11]. This is the region between the main endothermic peak and the premelt peak. The extent of this region ends at 22°C and 30% phosphatidylserine. The region below the premelt peak is characterized by the coexistence of T and B features. This could mean that both $P_{\beta'}$ and $L_{\beta'}$ coexist. Since the lower edge of this region is not clearly marked by differential scanning calorimetry, electron diffraction or freeze-fracture, the extent of this region along the temperature and composition is uncertain. The transition between the $L_{\beta'}$ and $P_{\beta'}$ phase involved not the 'solidification' or 'melting' process, but a transformation of a 'solid' form to another. This transition may have a long relaxation time and an ill-defined boundary. The highly angulated pattern which occurs only at temperatures below 22°C is perhaps a low temperature deformation effect on the system of solid solution at the $L_{\beta'}$ phase, and is not directly related to any different molecular organization. At temperatures higher than 22°C, and at phosphatidylserine composition greater than 30%, the area under the main transition depicts two phases by tie lines joining the L_α and $P_{\beta'}$ phases. Since the transition in the heterogeneous phosphatidylserine is very broad, and at no temprature exhibits a banded pattern, it is reasonable to assume that within the transition temperature, the phosphatidylserine alone consists of a mixture of L_α and L_β (or $L_{\beta'}$) phases. Thus, any point at the region below 22°C in the main transition range of the mixture would be connected by a tie line joining L_α, L_β, and $L_{\beta'}$ phases. The appearance

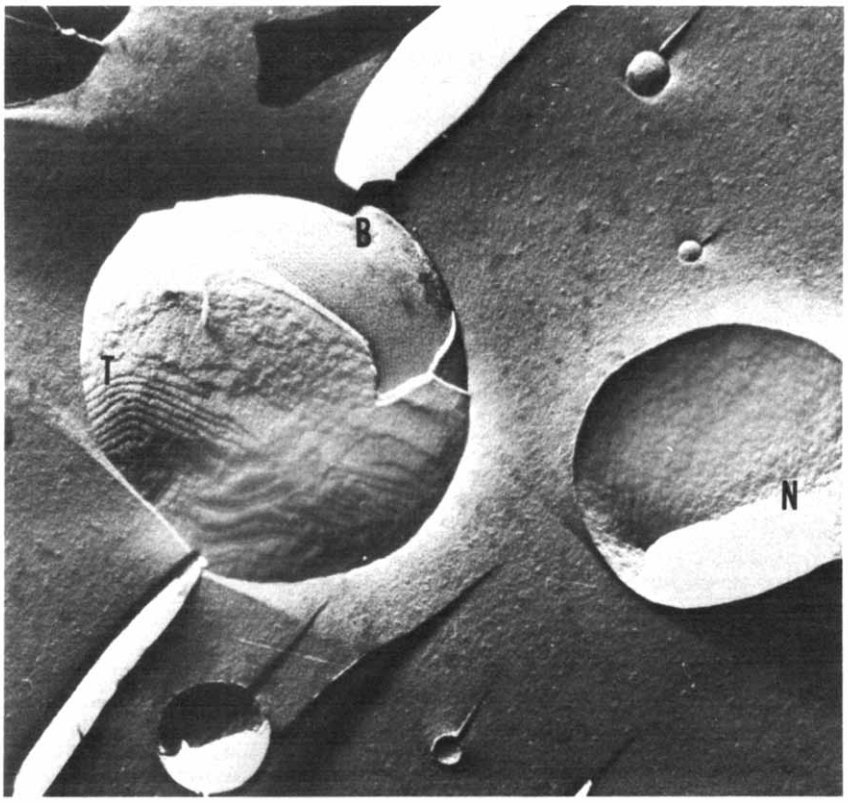


Fig. 8. Freeze-fracture micrograph of multilamellar vesicles whose composition is 50% phosphatidylserine and quenching temperature is 18°C. Magnification is 30 000X. Symbols are explained in Fig. 2.

of the B texture in this region suggests that some remnant of the $P_{\beta'}$ phase may also exist. The freeze-fracture micrographs do show all three features B, T, and N, sometimes in the same vesicle (Fig. 8).

In conclusion, it appears that the three different techniques we have used, agree reasonably well in the assignment of different phases for the mixtures of bovine brain phosphatidylserine and dipalmitoyl phosphatidylcholine. We have observed by freeze-fracture the coexistence of 2—3 phases at temperatures within the phase transition region, the presence of a 'terraced' configuration at very low temperatures with most mixtures, and its gradual transition into a 'banded' pattern at temperatures well below the main transition even with mixtures where the premelt transition is not apparent by differential scanning calorimetry. The presence of even small amounts of phosphatidylserine (3 mol% ratio) has considerable effects on the freeze-fracture morphology, inducing the appearance of 'terraced' and 'banded' patterns, by lowering the onset of the premelt transition.

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