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MULTIPLE PHASE EQUILIBRIA IN BINARY MIXTURES OF PHOSPHOLIPIDS

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Summary

Approximate phase diagrams describing lateral phase separations are given for binary mixtures of dimyristoyl phosphatidylcholine with dipalmitoyl phosphatidylcholine, distearoyl phosphatidylcholine, and dipalmitoyl phosphatidylethanolamine. These diagrams are based in part on freeze-fracture electron microscopic data presented here, as well as other earlier spin-label, calorimetric, and X-ray data. These phase diagrams represent an improvement over previous studies in that both solid phases ($P_{\beta'}$ and $L_{\beta'}$) of the phosphatidylcholines are included. Further consideration is given to the problem of binary mixtures in which there are two $P_{\beta'}$ phases that do not form a continuous range of solid solutions.

Introduction

In previous work we have described the phase diagrams of binary mixtures of lipids, where coexisting phases are present in the planes of the bilayer membranes [1—5]. Recently we have described the phase diagrams of lipid mixtures (phosphatidylcholines and dipalmitoyl phosphatidylserine) where one lipid component (the phosphatidylcholine) has two crystalline phases ($P_{\beta'}$ and $L_{\beta'}$) † and the second lipid component has only a single lipid phase ($L_{\beta'}$) [6]. Such phase diagrams are of considerable interest, since at some temperature-composition point, or line, a phase of one symmetry ($P_{\beta'}$) must disappear. In the phosphatidylcholine/phosphatidylserine binary mixtures this unique region

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† Nomenclature is according to Luzzati and Tadieu [7].

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl.

is an invariant point. The purpose of the present paper is to indicate the likelihood of a similar invariant point in binary mixtures of phosphatidylcholine and phosphatidylethanolamine, and further, to draw approximate phase diagrams for mixtures of phosphatidylcholines themselves, where both the $P_{\beta'}$ and $L_{\beta'}$ phases exist throughout the composition range.

Materials and Methods

Dimyristoyl phosphatidylcholine, dipalmitoyl phosphatidylcholine, distearoyl phosphatidylcholine, dipalmitoyl phosphatidylethanolamine, and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (ULTROL^{tm} grade) were purchased from Calbiochem and were used without further purification. Thin-layer chromatography showed no detectable impurities in the phospholipids. Dodecyltrimethylammonium bromide was prepared by the reaction of dodecyl bromide and trimethylamine, and was purified by recrystallization [8].

The phosphatidylcholines were stored as 0.5 or 1% ethanolic solutions; dipalmitoyl phosphatidylethanolamine was stored in a chloroform/ethanol. Lipid concentrations were verified by assaying aliquots of the stock solutions for phosphate by the method of McClare [9].

Large vesicles consisting of only a few lamellae were prepared by the detergent-dialysis method of Hong and Hubbell [10]. Ethanol or chloroform/ethanol solutions containing about 5 μmol of the desired lipids were evaporated under vacuum to dryness. The lipids were then dissolved at 50—60°C in 2 ml of 0.1 M dodecyl trimethylammonium bromide, 0.01 M sodium phosphate buffer, pH 7.0. The resulting mixtures were cooled to 4°C and dialyzed vs. 100 vols. of 5 mM HEPES buffer, pH 6.6. Dialysis was continued for 4—5 days with buffer changes approximately every 12 h. Large vesicles were harvested by centrifugation (10 000 × *g*, 20 min, 0°C). The supernatants were discarded; the pellets were stored at 0°C.

Samples were kept on ice until 1—2 μl droplets were pipetted onto copper planchets resting on a metal block at the desired quenching temperature in a room thermostated to within 1—2 degrees of this temperature. After equilibration for 2—3 min, the samples were quenched and replicated in a Balzers BAF301 Freeze-Etching Device as described previously [11]. Micrographs were taken on $3\frac{1}{4}$ inches × 4 inches Kodak electron microscope film with an initial magnification of 33 000× in an Hitachi HU-11E electron microscope.

Results

Dimyristoyl phosphatidylcholine-dipalmitoyl phosphatidylcholine

Fig. 1 shows freeze-fracture electron micrographs of dimyristoyl phosphatidylcholine/dipalmitoyl phosphatidylcholine vesicles of varying compositions quenched from different temperatures. At the lowest temperatures examined, fracture faces are unmarked except for an occasional random line (Fig. 1e). Samples heated further reveal fracture faces characterized by areas of unmarked lipid surface areas with periodic bands and areas marked by 'whorls', i.e. a texture apparently composed of a series of circular or spiral 'bands'

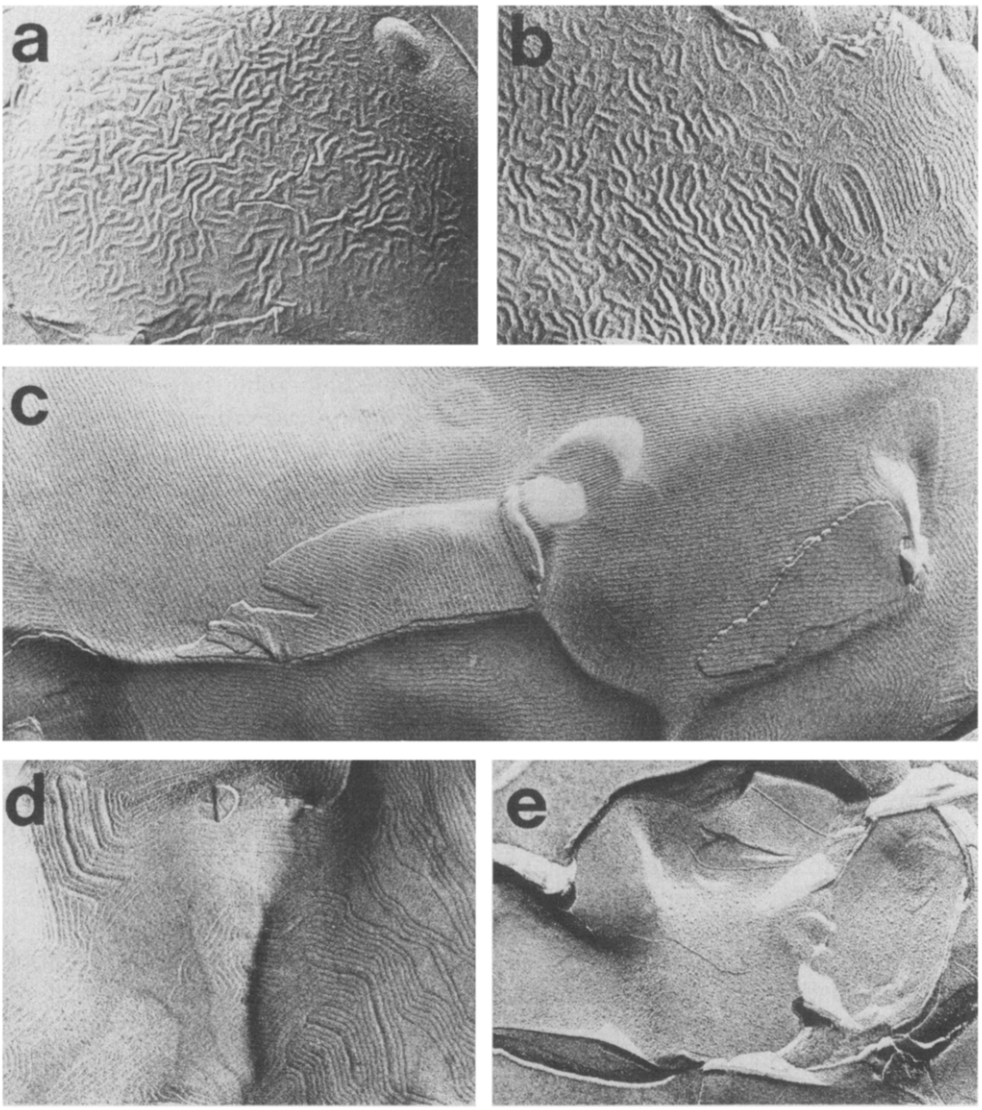

Fig. 1. Freeze-fracture electron micrographs of dimyristoyl phosphatidylcholine/dipalmitoyl phosphatidylcholine vesicles prepared by detergent dialysis. Mol% dipalmitoyl phosphatidylcholine and quench temperature are as follows: (a) 20%, 35°C; (b) 49%, 34°C; (c) 80%, 35°C; (d) 80%, 27°C; (e) 80%, 19°C. All samples were heated to the quench temperatures which are accurate to ±1—3°C. Magnifications are about 65 000×.

(Fig. 1d). Fig. 1c presents the banded texture characteristic of lipid surfaces quenched from still higher temperatures. Smooth areas and whorls are absent; the bands are periodic and form nearly regular arrays. Further heating results in fracture faces exhibiting the jumbled texture characteristic of fluid phosphatidylcholines as well as areas of banded lipid (Fig. 1b). Samples quenched from the highest temperatures examined reveal fracture faces exhibiting only the jumbled texture (Fig. 1a). These successive changes in texture with

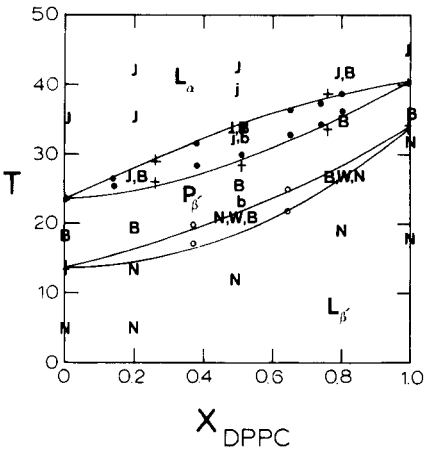

Fig. 2. An approximate phase diagram for aqueous dispersions of the dimyristoyl phosphatidylcholine/dipalmitoyl phosphatidylcholine binary system: temperature vs. mol fraction of dipalmitoyl phosphatidylcholine (DPPC). +, Data taken from Figs. 2 and 3A in Shimshick and McConnell [1]; •, data taken from Fig. 2B in Mabrey and Sturtevant [12]; ○, points estimated from the calorimetric curves in Fig. 2A in Mabrey and Sturtevant [12]. J, N, B and W denote the presence of jumbled, smooth, banded, and whorled (or wavy) textures on fracture faces of samples quenched from the designated locations on the phase diagram. Capital letters denote data presented in Fig. 1 and in Figs. 2, 3, and 4 in Luna and McConnell [11]; lower-case letters designate fracture faces observed by Kleemann [18]. The region of the phase diagram containing L_α phase only, $P_{\beta'}$, phase only, and $L_{\beta'}$, phase only are indicated.

increasing temperature are observed for each of the three dimyristoyl phosphatidylcholine/dipalmitoyl phosphatidylcholine mixtures examined. However, the temperatures at which the textural changes occur increase with increasing mol fractions of dipalmitoyl phosphatidylcholine.

The dimyristoyl phosphatidylcholine/dipalmitoyl phosphatidylcholine phase diagram has been investigated using the techniques of differential scanning calorimetry [12], fluorescence spectroscopy [13,14], and electron spin resonance spectroscopy [1]. With only one exception [14], no attempt has been made to incorporate the 'pretransition' characteristic of phosphatidylcholines [11,15—17] into phase diagrams for binary mixtures of phosphatidylcholines.

In Fig. 2 we present an approximate phase diagram for the dimyristoyl phosphatidylcholine-dipalmitoyl phosphatidylcholine system. This phase diagram is based on previously published data as well as the location and relative amounts of the different textures observed by freeze-fracture electron microscopy (Fig. 1). Circles denote points derived from the calorimetric data of Mabrey and Sturtevant [12]; crosses indicate data points obtained by Shimshick and McConnell [1] from 2,2,6,6,-tetramethylpiperidine-1-oxyl (TEMPO) curve break points. The letters "J", "B", and "N" are used to designate points on the phase diagram at which jumbled, banded, and smooth textures are observed. The letter "W" indicates the presence on fracture faces of whorls and/or 'wavy bands'. When two or more textures are observed on the fracture surfaces of a sample, this fact is recorded at the appropriate point on the temperature-composition diagram by listing the designating letters in order of decreasing predominance. Capital letters denote data obtained by us previously [11] or in

the present study; lower-case letters designate fracture surfaces observed by Kleemann [18]. The combined data suggest a phase diagram in which the $P_{\beta'}$-L_α two-phase region is either symmetric, or nearly so, about a line connecting the two pure-component transitions. The $L_{\beta'}$-$P_{\beta'}$ two-phase region, however, is probably asymmetric with a concave upper solvus line (Fig. 2). This phase diagram is also consistent with the general form, if not the transition temperatures, of the dimyristoyl phosphatidylcholine/dipalmitoyl phosphatidylcholine phase diagram constructed from fluorescence depolarization data [14].

Dimyristoyl phosphatidylcholine-distearoyl phosphatidylcholine

Freeze-fracture electron micrographs of dimyristoyl phosphatidylcholine/distearoyl phosphatidylcholine bilayers (Fig. 3) reveal the same changes in texture with increasing temperature as those observed for mixtures of dimyristoyl phosphatidylcholine/dipalmitoyl phosphatidylcholine (Fig. 1). At the lowest temperatures examined, fracture faces are smooth or crossed by only an occasional line (Fig. 3i). As temperatures are increased, the lines increase in number and whorls appear (Figs. 3g and 3h). At higher temperatures, the number of whorls increases and areas composed of more or less periodic bands become noticeable (Fig. 3f). At still higher temperatures, the whorls are replaced by 'wavy bands' and a regular band pattern becomes a prominent feature of fracture faces (Fig. 3e). Eventually, the regular band pattern becomes the only detectable feature (Fig. 3d). Further temperature increases lead to the formation of small patches of the distinctive jumbled texture indicative of fluid phosphatidylcholines (Fig. 3c). These jumbled patches grow larger at the expense of the banded areas (Fig. 3b) until the temperature approaches that of the fluidus curve at which point all bands disappear (Fig. 3a).

The present freeze-fracture data are combined with calorimetric, electron spin resonance, and earlier freeze-fracture data to produce the approximate dimyristoyl phosphatidylcholine/distearoyl phosphatidylcholine phase diagram shown in Fig. 4. Although there are many uncertainties associated with the solidus and solvus curves of this phase diagram, it is obvious that each of the two-phase regions is asymmetrically shaped. Freeze-fracture data suggest that the region in which $P_{\beta'}$ and L_α phases coexist is probably shaped essentially the same as in the dimyristoyl phosphatidylcholine/distearoyl phosphatidylcholine phase diagrams originally drawn by Shimshick and McConnell [1] and Mabrey and Sturtevant [12]. Both calorimetric [12] and freeze-fracture electron microscopic data indicate that the pretransitional region is characterized by extensive solid-solid (i.e. $L_{\beta'}$-$L_{\beta'}$) immiscibility.

The location of the upper solvus curves is the most ambiguous feature of the dimyristoyl phosphatidylcholine/distearoyl phosphatidylcholine phase diagram. However, it has been observed that the pretransition comes very close to and probably interferes with the $P_{\beta'}$-L_α transition for 36 mol% and higher distearoyl phosphatidylcholine concentrations [12]. Given this fact and the putative location of the solidus line in this system, it appears that the upper solvus curve undergoes a change of curvature at about 50 mol% distearoyl phosphatidylcholine. This deduction is incorporated into the phase diagram presented in Fig. 4.

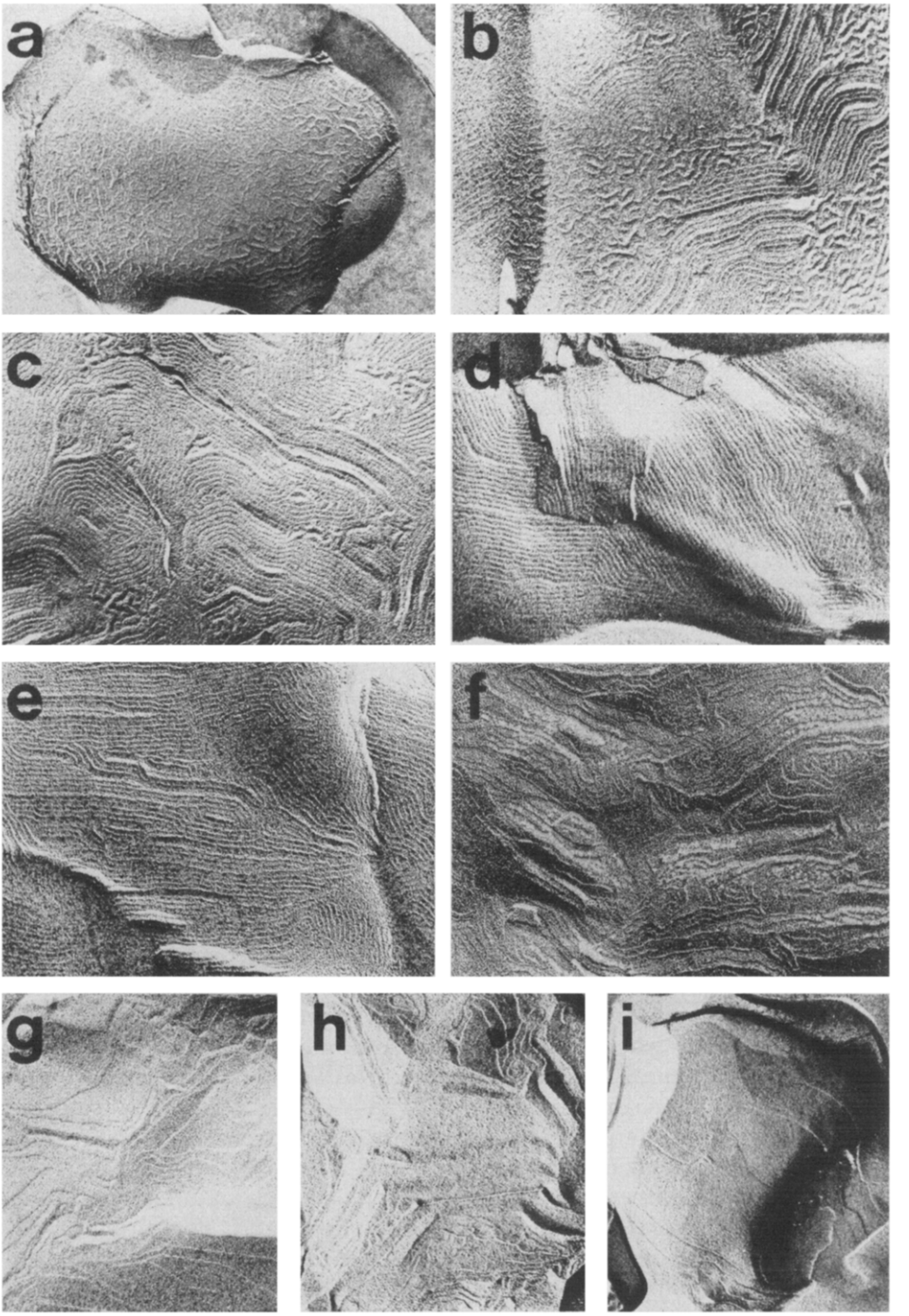

Fig. 3. Freeze-fracture electron micrographs of dimyristoyl phosphatidylcholine/distearoyl phosphatidylcholine vesicles prepared by detergent dialysis. Mol% distearoyl phosphatidylcholine and quench temperatures are as follows: (a) 20%, 42.5°C; (b) 40%, 33°C; (c) 60%, 33°C; (d) 40%, 26°C; (e) 60%, 26°C; (f) 60%, 21°C; (g) 80%, 26°C; (h) 20%, 13.5°C; (i) 20%, 5°C. All samples were heated to the quench temperatures which are accurate to ±1—3°C. Magnifications are about 65 000X.

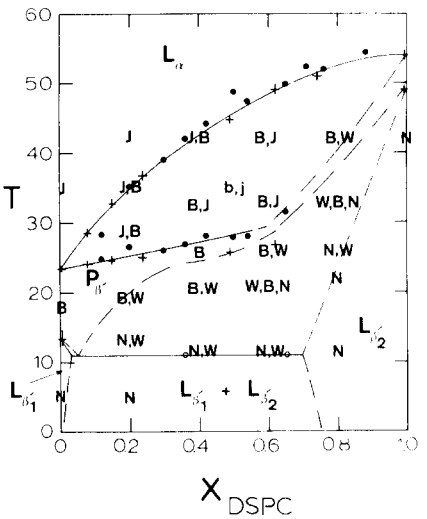

Fig. 4. An approximate phase diagram for aqueous dispersions of the dimyristoyl phosphatidylcholine-distearoyl phosphatidylcholine binary system: temperature vs. mol fraction of distearoyl phosphatidylcholine (DSPC). +, Data taken from Figs. 2 and 7 in Shimshick and McConnell [1]; •, data taken from Fig. 3B in Mabrey and Sturtevant [12]; ○, points estimated from the calorimetric curves in Fig. 3A in Mabrey and Sturtevant [12]. J, N, B and W denote the presence of jumbled, smooth, banded, and whorled (or wavy) textures on fracture faces of samples quenched from the designated locations on the phase diagram. Capital letters denote data presented in Fig. 3; lower-case letters designate fracture faces observed by Shimshick et al. [2]. The regions of the phase diagram containing L_α phase only, $P_{\beta'}$ phase only, each of the postulated $L_{\beta'}$ phases only and coexisting $L_{\beta'1}$ and $L_{\beta'2}$ phases are indicated.

Dimyristoyl phosphatidylcholine-dipalmitoyl phosphatidylethanolamine

Fig. 5 exhibits freeze-fracture electron micrographs of dimyristoyl phosphatidylcholine/dipalmitoyl phosphatidylethanolamine mixtures quenched from different temperatures. At low temperatures, only smooth fracture surfaces are observed (Fig. 5h). At higher temperatures and concentrations of 30 mol% or less dipalmitoyl phosphatidylethanolamine, domains of wavy, and sometimes periodic, bands appear in coexistence with areas of unmarked lipid surfaces (Figs. 5f and 5g). Regular band patterns uninterrupted by smooth areas are observed on fracture faces of samples containing 11 mol% phosphatidylethanolamine when these samples are quenched from about 24°C (Fig. 5e). Coexisting jumbled and banded textures appear on fracture surfaces when this sample is quenched from approx. 28.5°C (Fig. 5d). Further heating of dimyristoyl phosphatidylcholine/dipalmitoyl phosphatidylethanolamine samples results in the complete disappearance of bands, lines and whorls. Fracture faces of samples containing 11 mol% phosphatidylethanolamine reveal only the jumbled texture characteristic of fluid phosphatidylcholines (Fig. 5a). Fracture surfaces of samples containing higher concentrations of phosphatidylethanolamine exhibit this jumbled texture in apparent equilibrium with smooth lipid surfaces (Figs. 5b and 5c).

The freeze-fracture electron microscopic data presented in Fig. 5 clearly indicate the existence of at least three two-phase regions in the dimyristoyl phosphatidylcholine/dipalmitoyl phosphatidylethanolamine phase diagram. Assuming first-order transition behavior throughout the temperature-composi-

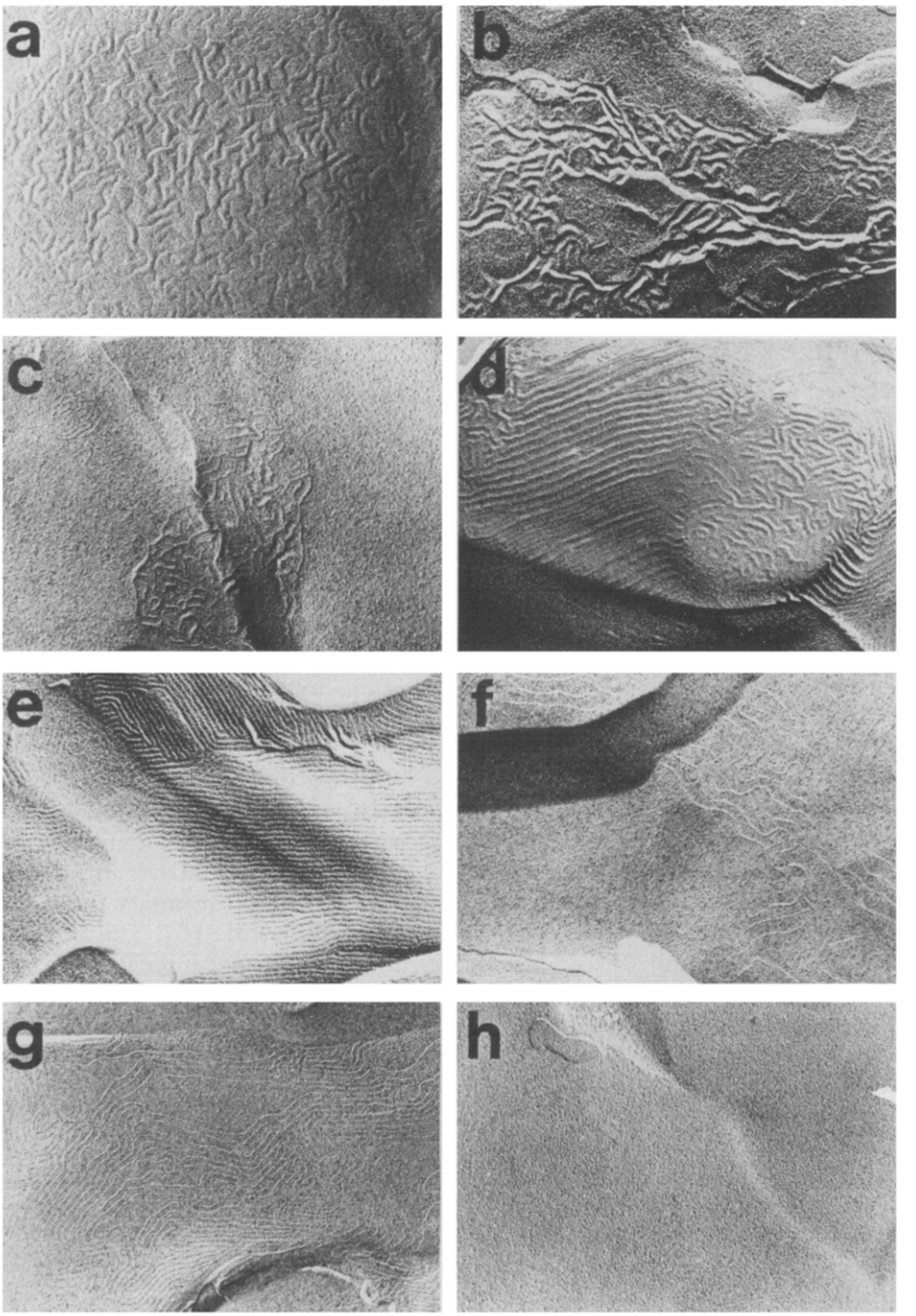

Fig. 5. Freeze-fracture electron micrographs of dimyristoyl phosphatidylcholine/dipalmitoyl phosphatidylethanolamine vesicles prepared by detergent dialysis. Mol% dipalmitoyl phosphatidylethanolamine and quench temperature are as follows: (a) 11%, 35°C; (b) 30%, 42°C; (c) 51%, 42°C; (d) 11%, 28.5°C; (e) 11%, 24°C; (f) 30%, 26°C; (g) 11%, 19°C; (h) 30%, 11.5°C. All samples were heated to the quench temperatures which are accurate to ±1—3°C. Magnifications are about 65 000×.

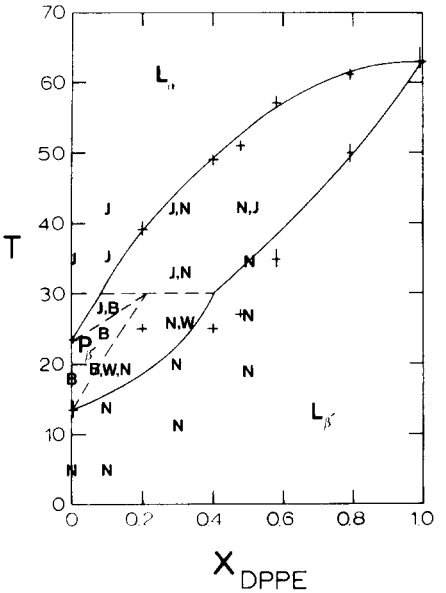

Fig. 6. An approximate phase diagram for aqueous dispersions of the dimyristoyl phosphatidylcholine-dipalmitoyl phosphatidylethanolamine binary system; temperature vs. mol fraction of dipalmitoyl phosphatidylethanolamine (DPPE). +, Data taken from Figs. 2 and 5A in Shimshick and McConnell [1]. J, N, B and W denote the presence of jumbled, smooth, banded, and wavy textures on fracture faces of samples quenched from the designated locations on the phase diagram. The regions of the phase diagram containing L_α phase only, $P_{\beta'}$ phase only and $L_{\beta'}$ phase only are indicated.

tion range studied here, the simplest possible phase diagram for this system is composed of three two-phase regions which meet at a three-phase line (Fig. 6). While the three-phase line almost certainly exists at a temperature between 28 and 33°C, we have obtained no direct experimental evidence for it in this system. However, three-phase lines are documented for binary mixtures of phosphatidylcholines with dipalmitoyl phosphatidylserine [6]. The relative extents of the L_α-$P_{\beta'}$ and $P_{\beta'}$-$L_{\beta'}$ regions are unknown, a fact indicated by the broken lines in Fig. 6.

The location of the fluidus curve as deduced from freeze-fracture electron microscopic data agrees well with the fluidus curves for this system previously presented by Shimshick and McConnell [1] and Lee [13]. However, our data are not compatible with the low-temperature break points determined by these researchers for low concentrations of phosphatidylethanolamine. A possible source for this discrepancy in results is the previously-held assumption of only one 'solid' phase (and thus, only one two-phase region) in mixtures containing a phosphatidylcholine. These workers ignored temperatures below 15—20°C and, therefore, may have overlooked additional break points.

Discussion

In this work, we extend previously studied phase diagrams to include the lower as well as upper phase boundaries for the $P_{\beta'}$ phases in binary mixtures of dimyristoyl phosphatidylcholine with dipalmitoyl phosphatidylcholine,

distearoyl phosphatidylcholine, and dipalmitoyl phosphatidylethanolamine. Since quench temperatures are only certain to within 1—3°C, freeze-fracture electron microscopy is not, by itself, a good technique for locating precise phase boundaries. However, freeze fracture provides information on the extent of the $P_{\beta'}$ phase and, by indicating the approximate locations of two-phase regions, can greatly facilitate the interpretation of data obtained with more accurate techniques.

There is a strong evidence that the chain-melting transition, $P_{\beta'}$-L_{α} in dimyristoyl phosphatidylcholine (at 23°C) and in dipalmitoyl phosphatidylcholine (at 41°C) is first order. The transitions are associated with substantial changes in enthalpy (ΔH) and volume (ΔV) [12,19,20]. There is evidence that the pretransition in phosphatidylcholines is also first order, there being a reported ΔH of transition [12], as well as extensive evidence for hysteresis in this transition [11,21] (not possible for second-order transitions [22]). It is, therefore, surprising that the pretransition in mixtures consisting of two phosphatidylcholines is visualized by freeze-fracture electron microscopy as a gradual change in texture of lipid surface (Figs. 1 and 3). Smooth fracture faces become marked with whorls which are replaced by wavy bands which give way to a regular band pattern in a seemingly continuous progression. This phenomenon is even more surprising since distinct banded and smooth domains are observed on fracture faces of dimyristoyl phosphatidylcholine/dipalmitoyl phosphatidylethanolamine mixtures quenched from the putative $P_{\beta'}$-$L_{\beta'}$ two-phase region (Figs. 5f and 5g). ($P_{\beta'}$-$L_{\beta'}$ regions in mixtures of dipalmitoyl phosphatidylserine with phosphatidylcholines are also frequently observed to have large (up to 5 μM wide) domains of each of the two phases [6]).

It is possible that different rates of lateral diffusion are responsible for the appearances of different lipid surfaces observed by freeze-fracture electron microscopy. Diffusion may occur faster in, for example, dimyristoyl phosphatidylcholine/dipalmitoyl phosphatidylethanolamine bilayers than in bilayers composed of dimyristoyl phosphatidylcholine/dipalmitoyl phosphatidylcholine. Support for this hypothesis is obtained by studying the appearance of freeze-fracture lipid surfaces as a function of the equilibration time at the quench temperature prior to quenching. Preliminary experiments indicate that fracture surfaces of binary mixtures of phosphatidylcholines quenched after a 24 h incubation at a temperature within the pretransitional region exhibit large, well-defined areas of regularly spaced bands in apparent equilibrium with large, completely unbanded areas. (Samples quenched after only a 3 h incubation are almost indistinguishable from samples equilibrated for only a few minutes at the quench temperature.) However, a better test of this theory would be the direct measurement of rates of lateral diffusion in each of the lipid mixtures discussed here. It is also possible that rates of domain equilibration depend on nucleation and other factors in addition to the rates of lateral diffusion.

A significant feature of the dimyristoyl phosphatidylcholine/dipalmitoyl phosphatidylethanolamine phase diagram presented in Fig. 6 is the postulated presence of a three-phase line, a temperature at which fluid-, banded-, and smooth-textured solid lipid domains coexist. Three-phase lines have also been observed in binary mixtures of dipalmitoyl phosphatidylserine with dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine. Similarly, three-

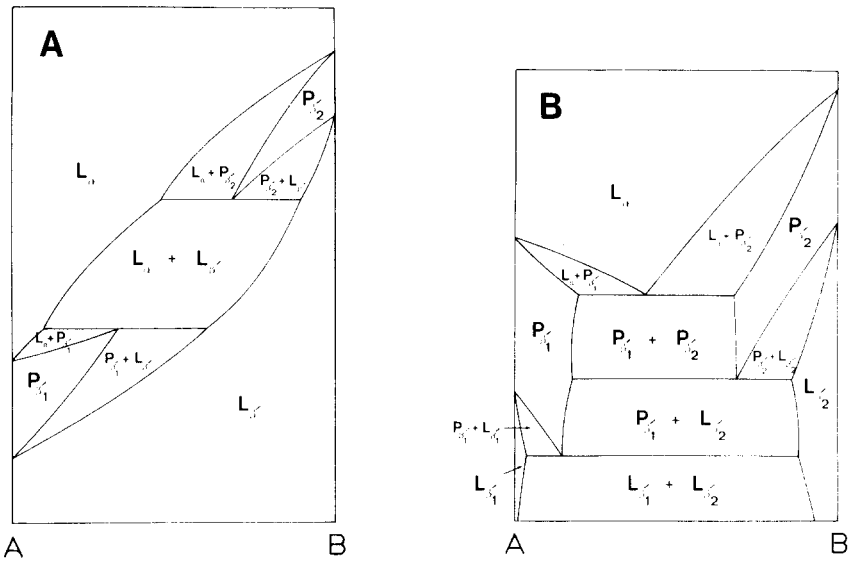

Fig. 7. Theoretical, speculative phase diagrams for binary mixtures of lipids, each of which has a $P_{\beta'}$ phase. (A) Phase diagram showing complete absence of $P_{\beta'}$ phase for some lipid compositions. (B) Phase diagram illustrating immiscibility of $P_{\beta'}$ phases.

phase lines may occur in other phase diagrams in which one component is a phosphatidylcholine, a molecule with two solid phases with different crystalline conformations, and the other component is a molecule with only one gel phase.

In conclusion it may be noted that the limited temperature stability range of the $P_{\beta'}$ phase for pure phosphatidylcholines raises the possibility that this phase may not be stable for some temperatures and compositions in binary mixtures of phosphatidylcholines. Indeed, in Fig. 4 the reported stability region of the $P_{\beta'}$ phase is quite narrow for some compositions. This suggests that as the chains of the two lipids become even more different from one another, immiscibility in the $P_{\beta'}$ phase may appear, or the $P_{\beta'}$ phase may disappear entirely for some composition range.

Fig. 7a gives a schematic phase diagram showing how the $P_{\beta'}$ phase may be entirely missing for some compositions of two pure lipids that individually have $P_{\beta'}$ phases. Fig. 7b gives a schematic phase diagram illustrating immiscibility in the $P_{\beta'}$ phases. Such schematic phase diagrams may prove to be helpful in designing efficient protocols for future studies of such lipid mixtures.

Acknowledgments

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