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LATERAL PHASE SEPARATIONS IN BINARY LIPID MIXTURES: CORRELATION BETWEEN SPIN LABEL AND FREEZE-FRACTURE ELECTRON MICROSCOPIC STUDIES

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SUMMARY

1. Spin label measurement have been performed on the hydrated binary lipid system, dielaidoyl/dipalmitoyl phosphatidylcholine in order to determine the state of lipid fluidity as a function of temperature. The results are interpreted in terms of a binary phase diagram describing lateral phase separations within the plane of the lipid bilayer membrane.

2. For a given temperature and composition the phase diagram rationale imposes certain restrictions on local lipid fluidity and composition. At intermediate temperatures the lipid bilayers are expected to contain domains of different composition. Critical predictions of the spin label-derived phase diagram have been tested using freeze-fracture electron microscopy. The results show that the phase diagram approach can be profitably applied to the understanding of lipid distribution in bilayer binary lipid systems.

INTRODUCTION

Thermally induced fluid-gel (disorder/order) gel phase changes have been observed in a variety of lipid bilayer-containing membranes. These range from broad, often indistinct, changes in cell plasma membranes [1–6] to the sharp, cooperative transitions characteristic of pure phospholipids in the presence of water [7–11]. Recently Shimshick and McConnell [7, 12] have suggested that for systems containing two or more lipids the term "phase separation" is more appropriate than "phase transition". These spin label experiments gave little direct evidence that lateral phase separations could occur in lipid bilayers; however, the authors showed that equilibrium was reached very rapidly [7] and hence that bilayer fusion/fission processes were unlikely to be involved. More recent freeze-fracture electron microscopic work [13] has definitely linked phase separations to a lateral diffusion process. A better knowledge of such phenomena in simple mixed lipid systems, both with and

Abbreviation: TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl.

without membrane-bound macromolecules, should contribute to the understanding of lateral distribution of components in cell membranes.

A considerable number of physical and spectroscopic techniques have been used in the past to monitor the properties of hydrated lipids. Here we are concerned with lipids in the presence of excess water under conditions in which they maintain a bilayer array [14]. The phase-change of interest is the transition from gel phase (lipid acryl chains rigidly extended) to fluid phase (highly mobile acyl chains with a high proportion of gauche conformations). The spin label TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) has been used to monitor this phase change since it has been shown to be sensitive to both onset and completion of phase separations [1, 7, 15] and involves a relatively easy measurement. This technique is based on the partition of TEMPO between fluid hydrophobic regions of the lipid bilayers and aqueous regions: TEMPO is effectively excluded from regions of rigid acyl chains. The exclusion appears in a TEMPO spectral parameter which is approximately equal to the fraction of TEMPO dissolved in lipid at any one temperature [7].

Freeze-fracture electron microscopy with its extensive face views of lipid bilayer hydrophobic and hydrophilic regions offers a powerful tool for the investigation of component distribution in membrane systems [2, 12, 15–18]. There have been attempts using this technique to correlate protein distribution in cell plasma membranes with lipid behaviour. Pioneering work in this area has been done with *Acholeplasma laidlawii* [2, 19, 20] enriched in various single fatty acids so that their lipids exhibit detectable phase change phenomena. It has been shown [2, 19, 20] that the normally randomly distributed (protein-related) particles can become highly aggregated in such systems. Furthermore, this temperature-dependent particle aggregation seems to be related in some way to the formations of regions of gel-phase lipid in equilibrium with fluid regions. More recently Kleemann and McConnell [15] have studied particle distribution in plasma membranes of a β-oxidation-deficient unsaturated fatty acid auxotroph of *Escherichia coli* grown on elaidic acid. They have used the spin label TEMPO to detect the onset and completion of the lateral phase separation of a major membrane lipid component. The onset of this freezing out of lipid corresponds to the onset of particle aggregation as protein-related particles are squeezed out of rigid lipid regions.

Certain pure phosphatidylcholines which form lipid bilayers in the presence of excess water offer interesting possibilities for model membrane lateral distribution studies because they can display characteristic, highly regular surface patterns in freeze-fracture electron micrographs [19, 21–23]. These regular patterns are presumably related in some way to the crystal packing of the phospholipid molecules upon passing from fluid to gel phase [14]. Regular patterns are not present in freeze-fracture electron micrographs when lipid samples are quenched (cryogenically fixed) from above their phase transition temperatures [22, 23]; instead, the lipid surface takes on a jumbled or even smooth appearance depending upon the quenching rate. An infinitely fast quenching rate would be expected to 'trap' the individual phospholipid molecules in their original positions, even when quenched from the fluid phase. In fact though, the process takes a finite time and the molecules have a finite period in which to begin to form their gel-lattice ordered array. By working with lipids displaying these surface patterns, one can readily distinguish between lipid samples which were fluid prior to quenching (smooth or jumbled) and those which

were in the gel phase (highly ordered pattern).

Freeze-fracture preparations of binary lipid mixtures may also show these striking patterns on their fracture and etch faces. In these cases, however, regions of different composition and fluidity may occur together in the same bilayer [13, 18]. Shimshick et al. [13] state that such behaviour is consistent with their spin label-derived phase diagrams. Ververgaert et al. [18] have related their observations to differential scanning calorimetry spectra.

We report here an attempt to describe the behaviour of mixtures of one saturated and one unsaturated phospholipid in the presence of excess water using the phase diagram approach of Shimshick and McConnell [7]. A phase diagram has been constructed for fully hydrated binary mixtures of dielaidoyl and dipalmitoyl phosphatidylcholines. This was done by studying the fluid/gel behaviour of various molar combinations of these lipids as a function of temperature, using the spin label, TEMPO. The predictions of this spin label-derived phase diagram have been carefully tested by freeze-fracture electron microscopy. Samples for microscopy were quenched from critical points in the diagram.

MATERIALS AND METHODS

Sample preparation

Dipalmitoyl phosphatidylcholine was obtained from Calbiochem and used without further purification. Impurity content was about 1 % as judged by thin-layer chromatography.

Dielaidoyl phosphatidylcholine was prepared by the method of Cubero Robles and Van den Berg [24] from *O*-(*sn*-glycero-3-phosphoryl)choline (available as the $CdCl_2$ adduct from Sigma), and the anhydride and sodium salt of elaidic acid (Analabs). The product was purified by column chromatography on silica gel (Bio-Sil A 200–325 mesh) eluted with a chloroform/methanol gradient. It emerged at 35–40 % methanol and gave a single spot on silica gel plates. The product was stored as a stock solution (in methanol under argon at −20 °C) whose concentration was determined by the method of McClare [25].

Lipids were mixed in chloroform/ethanol solution and coated on the inside of 10 ml flasks by removal of the solvent under vacuum. Each 30–35 μmole sample was resuspended in 150–200 μl of 0.01 M sodium phosphate buffer (pH 7.0) by vortexing at 45 °C. 75 μl of a $2 \cdot 10^{-3}$ M TEMPO solution was then added and a portion of the lipid suspension was sealed in a 50 μl pipette for EPR measurements.

EPR spectroscopy

The TEMPO spectral parameter, f [7], was measured as a function of temperature. All measurements were made on a Varian E-12 spectrometer operating at X-band with the sample in a horizontal position. The temperature was controlled with a Varian variable temperature accessory and measured with a copper-constantan thermocouple connected to a Smith-Florence potentiometric microvoltmeter. Measurements were made both heating and cooling the sample at rates of 8–10 °C per h.

Electron microscopy, freeze-fracturing

Samples were prepared for freeze-fracturing by equilibrating small droplets of the EPR liposome preparations at the temperature of interest on 3 mm copper planchets in a water saturated atmosphere. Specimens were quenched in Freon 22 cooled in liquid nitrogen. Specimens were fractured and platinum–carbon coated at −119 °C in a Balzers BAF 301 freeze-etch unit. Replicas were viewed with a Phillips EM 200 electron microscope.

RESULTS AND DISCUSSION

Fig. 1 shows the TEMPO spectral parameter [7] plotted as a function of $1/T$ for dipalmitoyl/dielaidoyl phosphatidylcholine dispersions of various compositions. Sample behaviour was reversible and the curves contain points obtained both while heating and cooling the samples. Abrupt changes in slope of the f versus $1/T$ curves were used to define points on the fluidus and solidus curves of the phase diagram. The phase diagram arrived at by this method is shown in Fig. 2. Because the break points in Fig. 1 are not precise, there is an experimental error involved of up to several degrees Centigrade.

It is instructive to follow the behaviour of a 50/50 mole % sample of dielaidoyl and dipalmitoyl phosphatidylcholines. Pure dipalmitoyl phosphatidylcholine is in the solid or gel phase at or below 41 °C. However, the phase diagram in Fig. 2 predicts that a 50/50 mixture will be fluid down to about 33 °C. A freeze-fracture electron micrograph of such a sample quenched from 36 °C is shown in Fig. 3A. The lipid appearance in this micrograph was typical for the sample: no liposomes could be found with fracture faces displaying the ordered lines characteristic of gel phase lipid.

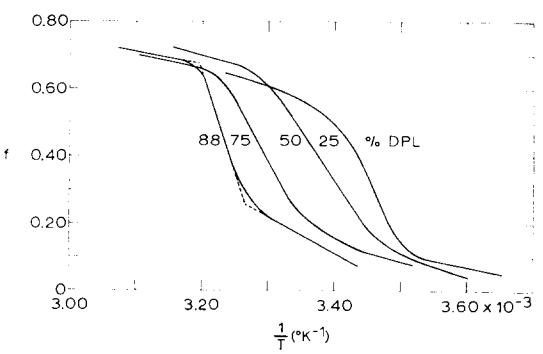

Fig. 1. Plots of the TEMPO spectral parameter, f, versus $1/T$ for mixtures of dielaidoyl and dipalmitoyl phosphatidylcholine (DPL) in the presence of excess water. Curves for mixtures of composition 25, 50, 75 and 88 mole % dipalmitoyl phosphatidylcholine are shown. Each curve exhibits two major changes in slope (break points) which have been taken as the onset and completion of the lateral phase separation. Dashed lines on the 88 % curve indicate how the actual break points were defined by extension of the experimental curves. A different such curve could be constructed for every possible combination of the two lipids. At temperatures above the upper break point all the lipid is in a fluid state. Below the lower break point all the lipid is in a solid state. At intermediate temperatures lateral separations of domains of fluid and solid lipid coexist. Data points were taken at less than 1 °C intervals. Point scatter was no greater than the line thickness of the curves in the figures.

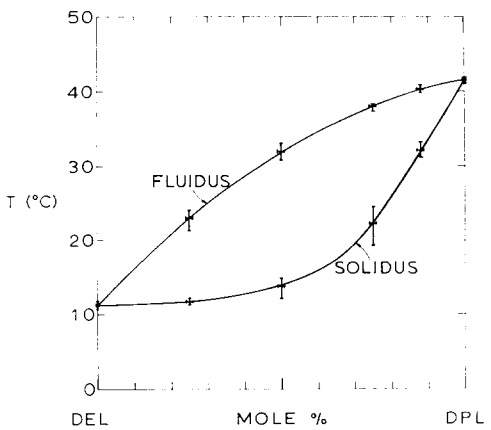

Fig. 2. Fluid/gel phase diagram for hydrated dielaidoyl/dipalmitoyl phosphatidylcholine mixtures as constructed from the EPR spin label data of Fig. 1. The x-axis shows the mole ratio of the phospholipid mixture, ranging from pure dielaidoyl (DEL) to pure dipalmitoyl phosphatidylcholine (DPL). The upper or fluidus curve was drawn through points dictated by the upper break points of Fig. 1. Similarly, the lower or solidus curve was drawn through points representing the lower break points of Fig. 1. Conditions corresponding to points above the fluidus represent totally fluid lipid mixtures while points below the solidus represent totally solid lipid. For points between these two curves, fluid and solid coexist, the domain compositions being predicted by the intersection points with the fluidus and solidus, respectively, of a horizontal through the point.

Instead, the liposomes were smooth or displayed jumbled patterns characteristic of fluid lipid. It can be seen from Figs 1 and 2 that 36 °C corresponds to a point just above the onset of the lateral phase separation.

If the above 50/50 mixture is cooled slowly to about 33 °C a trace of solid lipid rich in dipalmitoyl phosphatidylcholine would be expected from Fig. 2 to appear in equilibrium with fluid of composition about 50/50 dielaidoyl/dipalmitoyl phosphatidylcholine. Micrographs of freeze-fracture replicas of this sample quenched from 30 °C show appreciable amounts of lipid patterned with highly ordered lines while the bulk of the lipid is still fluid. Fig. 3C shows such a liposome containing fluid and solid regions in the same bilayer.

At 25 °C a statistical survey of liposomes in freeze-fracture replicas shows roughly equal areas of fluid and solid lipid for the 50/50 mixture. Presumably the fluid regions are rich in dielaidoyl phosphatidylcholine while the solid regions are rich in dipalmitoyl phosphatidylcholine.

Similarly at 17 °C, a point just above the completion of the lateral phase separation for a 50/50 mixture (see Figs 1 and 2), the bulk of the lipid appears solid in freeze-fracture micrographs. As the temperature is lowered still more, the phase diagram predicts that at about 13–14 °C the lipid mixture should be totally solid. Samples quenched from 10 °C indicate that this is so (see Fig. 3B).

Other lipid mixtures were tested in a similar fashion. For instance, 15 °C is well above the transition temperature of pure dielaidoyl phosphatidylcholine. Yet when a sample of 75 % dipalmitoyl phosphatidylcholine – 25 % dielaidoyl phosphatidylcholine is quenched from 15 °C, there is no trace of fluid lipid in the freeze-fracture electron micrographs. This is predicted from Fig. 2.

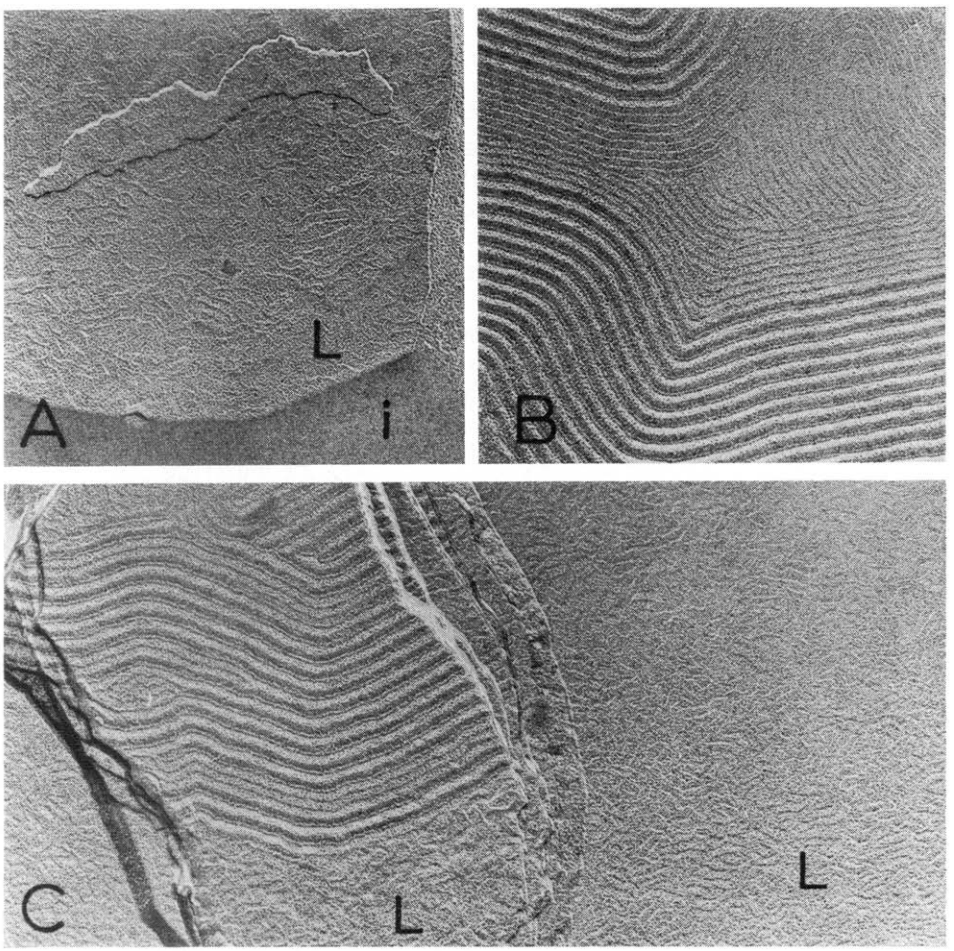

Fig. 3. Platinum–carbon replicas of liposome fracture faces for 50/50 mole % mixtures of dielaidoyl and dipalmitoyl phosphatidylcholine quenched from various temperatures. The direction of shadowing is from bottom to top of page. × 81 000. (A) Liposome quenched from 36 °C showing the typical fluid lipid appearance (disordered surface pattern). i, ice; L, lipid. (B) Typical liposome fracture face appearance when quenched from 10–11 °C. Note the highly ordered pattern characteristic of gel phase lipid and that no fluid lipid is present. (C) A multilamellar structure quenched from 30 °C showing appreciable areas of highly ordered (gel phase) lipid in equilibrium with fluid lipid. L, lipid.

It is convenient to consider lipid bilayer fluid/gel behaviour in a fashion analogous to crystal growth in solids. Crystallization in the case of lipid bilayers is two-dimensional and can actually be visualized in freeze-fracture electron micrographs of lipids such as those discussed here. The ripples seen in micrographs of gel phase lipid are a result of a more rigid 2-dimensional packing than is present in fluid lipid. For instance, vesicles of dimyristoyl phosphatidylcholine which have been cooled very slowly through the lipid transition temperature appear to be "annealed": whole bilayer vesicles with a surface area of several μm^2 may be one single lipid "crystal" of parallel ripples (our observation). Vesicles cooled more rapidly may show

several nucleation sites from which crystal growth has spiralled outward. Inefficient quenching of fluid lipid produces many nucleation sites from which crystal growth may have extended only several hundred Å or less before encountering another crystal. The fact that lipid quenched from a fluid state can be distinguished from solid lipid in freeze-fracture electron micrographs reflects the finite time required for lipid crystal growth. Quenching rates for freeze-fracture microscopy are typically of the order of 100 °C/s [26] and may be increased to better than 10 000 °C/s by special methods [23]. The surface appearance of hydrated phospholipids such as those considered here is determined by the rate of passage through the region where fluid and solid coexist.

Hydrated lipid mixtures comprising phospholipids with both saturated and unsaturated [27, 28] fatty acid side chains are of interest as models of biological membranes. In order to understand the behaviour of the dielaidoyl/dipalmitoyl phosphatidylcholine system we considered it in the light of a spin label-derived phase diagram (Fig. 2). This approach was highly successful as shown by freeze-fracture electron microscopy (Fig. 3). The electron micrographic data demonstrates very dramatically that lateral separation of lipid domains of different composition does occur between the onset and completion of a phase separation and that the amounts of each are in agreement with phase diagram predictions.

Some, if not all, cells appear to regulate their lipid composition so as to be close to a state in which solid lipid is just beginning to appear (i.e., a region analogous to a point on the fluidus curve of Fig. 2.) (See for example, Linden, et al. [1] and references contained therein.) One might therefore expect that phenomena such as those discussed above could play a part in component distribution and function in cell membranes.

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