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THE INTERMEDIATE MONOCLINIC PHASE OF PHOSPHATIDYLCHOLINES

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Summary

Two pure phospholipids, dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine, have been studied using freeze-fracture electron microscopy and the partitioning of the spin label, TEMPO. It is found that the characteristic band pattern, corresponding to monoclinic symmetry in multilamellar liposomes, is observed only in freeze-fracture electron microphotographs when samples are quenched from temperatures intermediate between the chain melting transition temperature and the pretransition temperature of the membrane. Markings are also observed on fracture faces of samples quenched from below the pretransition, but these "bands" are few in number and are widely and irregularly spaced. The lipid membranes used for freeze-fracture were prepared using detergent dialysis and are thought to consist of one, two, or some small number of concentric bilayer shells. These observations are in excellent accord with the recent, prior studies of Janiak, M.J., Small, D.M. and Shipley, G.G., ((1976) Biochemistry 15, 4575—4580), who found monoclinic symmetry (P_{β}' structure) in multilamellar liposomes of these phospholipids only when the sample temperature was intermediate between the main, chain melting transition temperature, and the pretransition temperature. The significance of these results for relating freeze-fracture electron microphotographis to phase diagrams derived from spin label or calorimetric data is discussed briefly.

2,2,6,6-Tetramethylpiperidine-1-oxyl (TEMPO) partitioning data show distinct differences between liposomal preparations of these lipids, and other preparations having fewer bilayers per vesicular structure, with respect to the position, width, and hysteresis of the pretransition.

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

Introduction

A striking feature observed in early freeze-fracture electron microscopic and X-ray diffraction studies of phosphatidylcholine liposomal (multibilayer) membranes is "banding" [1—5]. These are periodic, lateral, wavelike features (with e.g., 100—300 Å periodicities) that confer monoclinic symmetry on multilamellar arrays of these bilayers. These bands are not only intrinsically interesting, but they have proven extremely useful in correlating freeze-fracture electron microphotographs with spin-label [6,7], fluorimetric [8,9], and calorimetric [10] derived phase diagrams for binary mixtures of lipids [11—14]. These correlations are particularly significant since only the freeze-fracture microphotographs offer the possibility of determining the domain sizes of the phases coexisting in equilibrium. In a recent X-ray study of liposomes of dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine, Janiak et al. [15] have reported that the banded (P_{β}') phase of these phospholipids is present only at temperatures intermediate between the main, chain melting transition temperature of these lipids, and the lower, "pretransition" transition temperature. The present work was undertaken to confirm and extend these results using freeze-fracture electron microscopy, and spin-label paramagnetic resonance.

Materials and Methods

Materials

Dimyristoyl phosphatidylcholine, dipalmitoyl phosphatidylcholine, and *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (HEPES) (ULTROL^{tm} grade) were purchased from Calbiochem and were used without further purification. TEMPO was the gift of Dr. Wolfgang Kleemann. Dodecyltrimethylammonium bromide was prepared by the reaction of dodecyl bromide and trimethylamine, and was purified by recrystallization [16].

Phospholipid dispersions

Dimyristoyl and dipalmitoyl phosphatidylcholine were stored as 1% ethanolic solutions. The interior of a 5 ml round-bottom flask was coated with a thin film of phospholipid by the evaporation to dryness under vacuum of a solution containing approximately 10 μmol of the desired lecithin. Dispersions containing lipid in the form of large, multilayered liposomes were prepared by adding a glass bead, 5 μl of a 0.01 M TEMPO solution, and 100 μl of 5 mM HEPES buffer, pH 6.6, and by vortexing this mixture at about 50° C for several minutes. The liposomes were allowed to settle briefly before transfer to a 50 μl capillary pipet which was used as a sample cell for electron paramagnetic resonance measurement.

Large vesicles consisting of only a few lamellae were prepared by the detergent-dialysis method of Hong and Hubbell [17]. The interior of a 5 ml round-bottom flask was coated with lipid as described above. About 5 ml of 0.1 M dodecyltrimethylammonium bromide, 0.01 M sodium phosphate buffer, pH 7.0, were added to the flask. After a brief incubation at room temperature, the mixture was cooled to 4° C and dialyzed vs. 40 vols. of 5 mM HEPES buffer,

pH 6.6. Dialysis was continued for 4–5 days with buffer changes approximately every 12 h. The resulting large vesicles were harvested by centrifugation (10 000 × *g*, 20 min, 0°C). The supernatant was discarded; the pellet was stored at 0°C. For electron paramagnetic resonance experiments, 100 μl of this pellet were mixed by vortexing with 5 μl of a 0.01 M TEMPO solution. After transfer to the 50 μl capillary pipet used as a sample cell, air bubbles were removed from the viscous mixture by a brief centrifugation at 800 × *g*. Samples for freeze-fracture electron microscopy were prepared as described for the magnetic resonance experiments except TEMPO was omitted.

Paramagnetic resonance spectroscopy

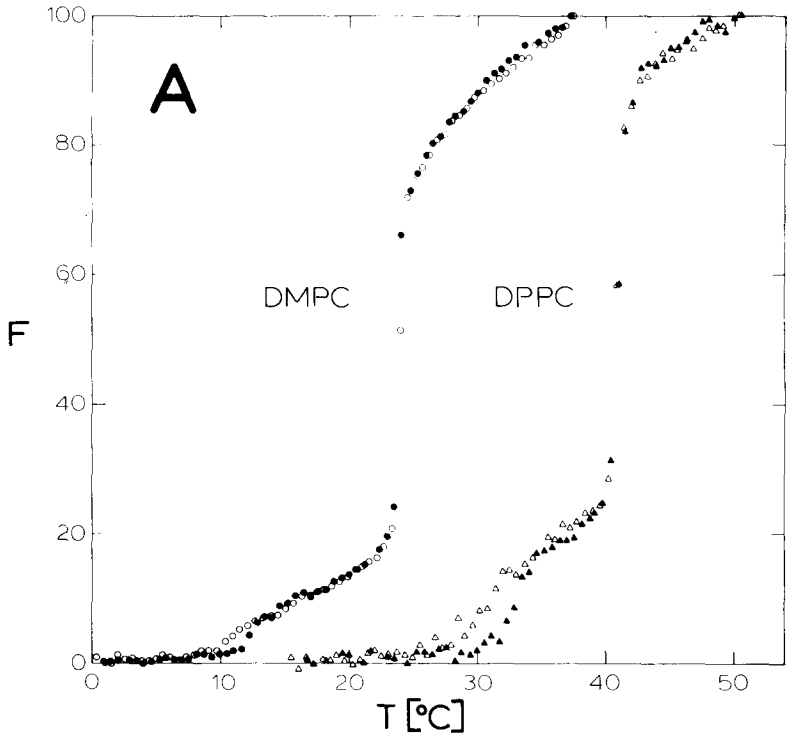
All measurements were made on a Varian E-12 spectrometer at X-band with the sample cell oriented horizontally to minimize the settling of the phospholipid liposomes. The sample cell holder and temperature control accessories described by Gaffney [18] were used to insure reproducible sample insertion and accurate temperature control. The temperature was measured with a copper-constantan thermocouple connected to a Smith-Florence potentiometric microvoltmeter. Spectral parameters were measured for both cooling and heating of the sample at rates of 5—10°C per h.

Freeze-fracture electron microscopy

Samples cooled to the quench temperature were obtained by heating a beaker of water above the transition temperature of the lipid and then allowing it to cool naturally. To minimize evaporation, samples to be heated to the quench temperature were kept on ice until 1—2 μl droplets were pipetted onto copper planchets resting on a metal block at the desired temperature in a room thermostatted to within a few degrees of this temperature. After equilibration for 2–3 min, the planchets were rapidly plunged into partially solidified Freon 22 (du Pont), then transferred to liquid nitrogen where they were stored until fracturing in a Balzers BAF301 Freeze-Etching Device. Fracturing was carried out at −114.5°C with no etching. Replicas were floated off on water, cleaned by a 1-h flotation on sodium hypochlorite solution (Chlorox), rinsed on water and transferred to 90% ethanol. After a few minutes, the replicas were picked up on bare 400 mesh copper grids. Micrographs were taken on $3\frac{1}{4} \times 4$ inch Kodak electron microscope film with an initial magnification of 33 000 × in an Hitachi HU-11E electron microscope.

Results

The spin-label molecule TEMPO is readily soluble in water and in phospholipid bilayers in the fluid, liquid-crystalline state, and is less soluble in bilayers in the solid or gel phase. The TEMPO spectral parameter, *f*, is defined as H/(H + P) where H is the amplitude of the high-field nitroxide hyperfine signal resulting from spin label dissolved in the membrane bilayer and P is the amplitude of the signal resulting from TEMPO in the aqueous phase. Thus, *f* is roughly proportional to the fraction of the lipid which is in the fluid state [6].



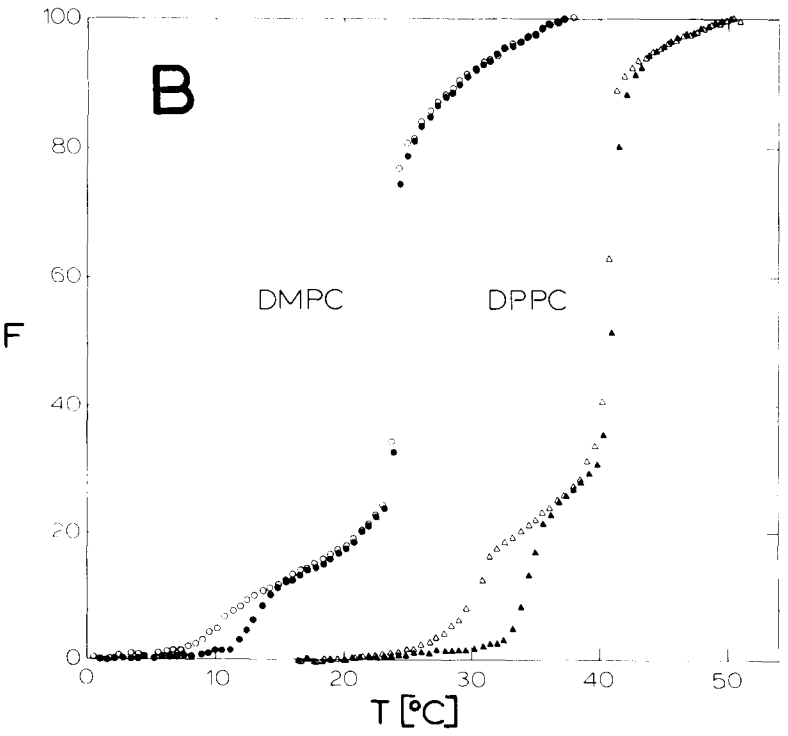


Fig. 1. For legend see opposite page.

The spectral parameter, f, is also a function of the relative concentrations of lipid and water. To facilitate comparisons between samples containing different amounts of lipid, we introduce a normalized TEMPO spectral parameter, F.

$$F = \frac{f - f_{min}}{f_{max} - f_{min}} \times (100)$$

where f is the TEMPO spectral parameter at a given temperature and f_{max} and f_{min} are the TEMPO spectral parameters measured at temperatures arbitrarily chosen above and below the lipid transition region. Fig. 1 gives plots of F vs. temperature for aqueous dispersions of the lecithins, dimyristoyl and dipalmitoyl phosphatidylcholine. Fig. 1A shows F vs. temperature plots for detergent-dialyzed vesicles made from each of these lipids. F vs. temperature plots for liposomal dispersions of the two lecithins are presented in Fig. 1B. In agreement with previously published results [6], all four plots exhibit abrupt decreases in the magnitude of the normalized TEMPO parameter at temperatures corresponding to the transition between the gel and liquid crystalline phases of the lipid. All four plots also exhibit a secondary transition, the "pretransition" observed with differential scanning calorimetry [19], approximately 10°C below the main transition temperature [6].

In Fig. 1, the F vs. temperature plots for dimyristoyl phosphatidylcholine are normalized between 37.3 and 1.3°C. Both dipalmitoyl phosphatidylcholine F curves are normalized between 50.4 and 17.2°C. This procedure allows us to compare the dimyristoyl phosphatidylcholine plots with each other and to comment on differences between the two dipalmitoyl phosphatidylcholine curves. However, our procedure does not necessarily normalize the dimyristoyl and dipalmitoyl phosphatidylcholine plots to the same scale. Thus, comparisons between F plots of different lipids are avoided in the following discussion of Fig. 1.

Although there are many similarities between the F plots of detergent-dialyzed vesicles (Fig. 1A) and the F plots of the corresponding liposomal suspensions (Fig. 1B), there are significant differences between the two sets of curves. One difference is found in the fluidity change associated with the pretransition relative to the total observed fluidity change. For each of the lecithins studied, the F parameter at temperatures intermediate between the two transitions is larger for the liposomal dispersion than for the detergent-dialyzed vesicles. This indicates that the relative fluidity change which occurs when multilamellar liposomes are heated through the pretransition is greater than the fluidity change observed when large vesicles containing only a few

Fig. 1. The normalized TEMPO spectral parameter, F, vs. temperature for aqueous suspensions of dimyristoyl phosphatidylcholine (DMPC) and of dipalmitoyl phosphatidylcholine (DPPC). Dimyristoyl phosphatidylcholine heating (●) and cooling (○) curves. Heating (▲) and cooling (△) curves for dipalmitoyl phosphatidylcholine samples. (A) F plots of large vesicles prepared by detergent dialysis. f_{max} and f_{min} for the dimyristoyl phosphatidylcholine vesicle suspension (27 mg/ml) are 0.400 and 0.035, respectively. f_{max} and f_{min} for the dipalmitoyl phosphatidylcholine vesicle suspension (19 mg/ml) are 0.246 and 0.030. (B) F plots of multilamellar liposomes. f_{max} and f_{min} for the dimyristoyl phosphatidylcholine dispersion (100 mg/ml) are 0.535 and 0.034, respectively. f_{max} and f_{min} for the dipalmitoyl phosphatidylcholine sample (100 mg/ml) are 0.584 and 0.038.

lamellae are heated to similar temperatures.

Another important difference between TEMPO curves for liposomal dispersions and for large vesicles is the magnitude of the hysteresis associated with the pretransition. For both dimyristoyl and dipalmitoyl phosphatidylcholine, the pretransition as measured by the liposomal heating curve occurs about 3.5—4°C higher than the pretransition observed for the corresponding cooling curve. Detergent-dialyzed vesicles, which contain fewer lamellae than do liposomes, exhibit smaller pretransition hystereses; vesicular heating curves differ by about 2.5°C from the cooling curves in the pretransition region. Our observations agree with previous observations and the suggestion that the pretransitional hysteresis is a characteristic of multilamellar liposomes [20]. When the number of adjacent bilayers in the system is decreased either by the addition of dicetyl phosphate [20] or by the choice of preparative method, the hysteresis associated with the pretransition diminishes.

In addition to the pretransitional hysteresis, we have observed a small (0.1—0.3°C), but possibly significant, hysteresis in the main order-disorder transition of lecithin liposomes. This finding is in agreement with the previously reported observation that lecithin vesicles have a melting temperature about 0.3°C above their freezing temperature [21]. No such hysteresis was observed in the main transition of detergent-dialyzed vesicles.

Other workers demonstrated that freeze-fracture electron microscopy can be used to visualize regular band patterns on fracture faces of liposomes prepared from synthetic lecithins [2,3,22—24]. These researchers [2,22] found smooth fracture faces above and band patterns below the main order-disorder transition of each lecithin studied. However, these workers did not systematically investigate changes in the fracture faces of phosphatidylcholines associated with the pretransition. In the present study, we confirm their work and correlate the appearance of the fracture faces of dimyristoyl and dipalmitoyl phosphatidylcholines visualized by the freeze-fracture electron microscopy with the fluidity changes measured by TEMPO throughout the temperature range of interest.

Fig. 2 exhibits freeze-fracture electron micrographs of detergent-dialyzed vesicles of synthetic dimyristoyl phosphatidylcholine. When these vesicles are quenched from temperatures above the main transition (Fig. 2a), fracture faces exhibit smooth patches and jumbled lines with the amount and degree of jumbling dependent on the quenching rate of the sample. Samples quenched from between the main transition and pretransition temperatures have regular band patterns with a periodicity of about 130 Å (Figs. 2b and 2c). Below the pretransition temperature, fracture faces reveal the disappearance of the regular banding pattern (Figs. 2d and 2e). Large areas of the lipid fracture surfaces are completely devoid of bands. In those few areas in which some bands remain, the periodicity is irregular and several times greater than that observed for the same lipid at higher temperatures.

Freeze-fracture electron micrographs of large dipalmitoyl phosphatidylcholine vesicles are presented in Fig. 3. Samples quenched from above the main transition temperature have fracture faces composed of jumbled bands (Fig. 3a). When quenched from about 41.5°C, approximately the center of the main transition, fracture surfaces (Fig. 3b) reveal patches of jumbled lines inter-

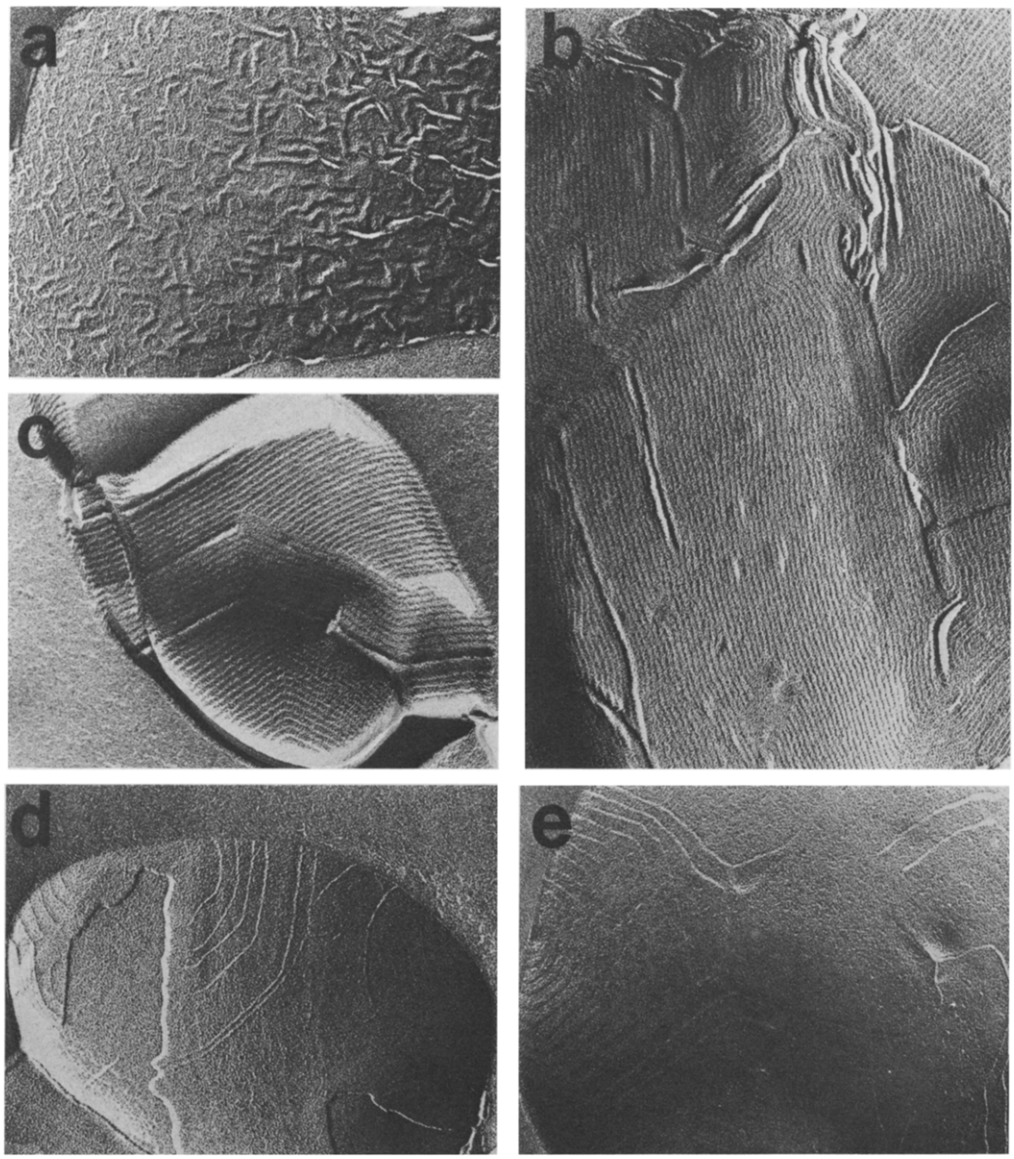


Fig. 2. Freeze-fracture electron micrographs of dimyristoyl phosphatidylcholine vesicles prepared by detergent dialysis. Vesicles were quenched from (a) 35 ± 2°C, (b) 17.5 ± 0.5°C after heating, (c) 14.5 ± 0.5°C after cooling, (d) 5.5 ± 0.5°C after heating, (e) 5 ± 1°C after cooling. Magnification is approx. 55 000×.

spersed with regions containing the regularly spaced bands which are characteristic of the lipid when quenched from between the two transition temperatures (Figs. 3c and 3d). Fracture faces of dipalmitoyl phosphatidylcholine vesicles quenched from below the pretransition temperature reveal few bands irregularly spaced (Figs. 3e and 3f).

The micrographs in Figs. 2 and 3 suggest the hypothesis that the pretransition is associated with the appearance/disappearance of periodic ripples in the

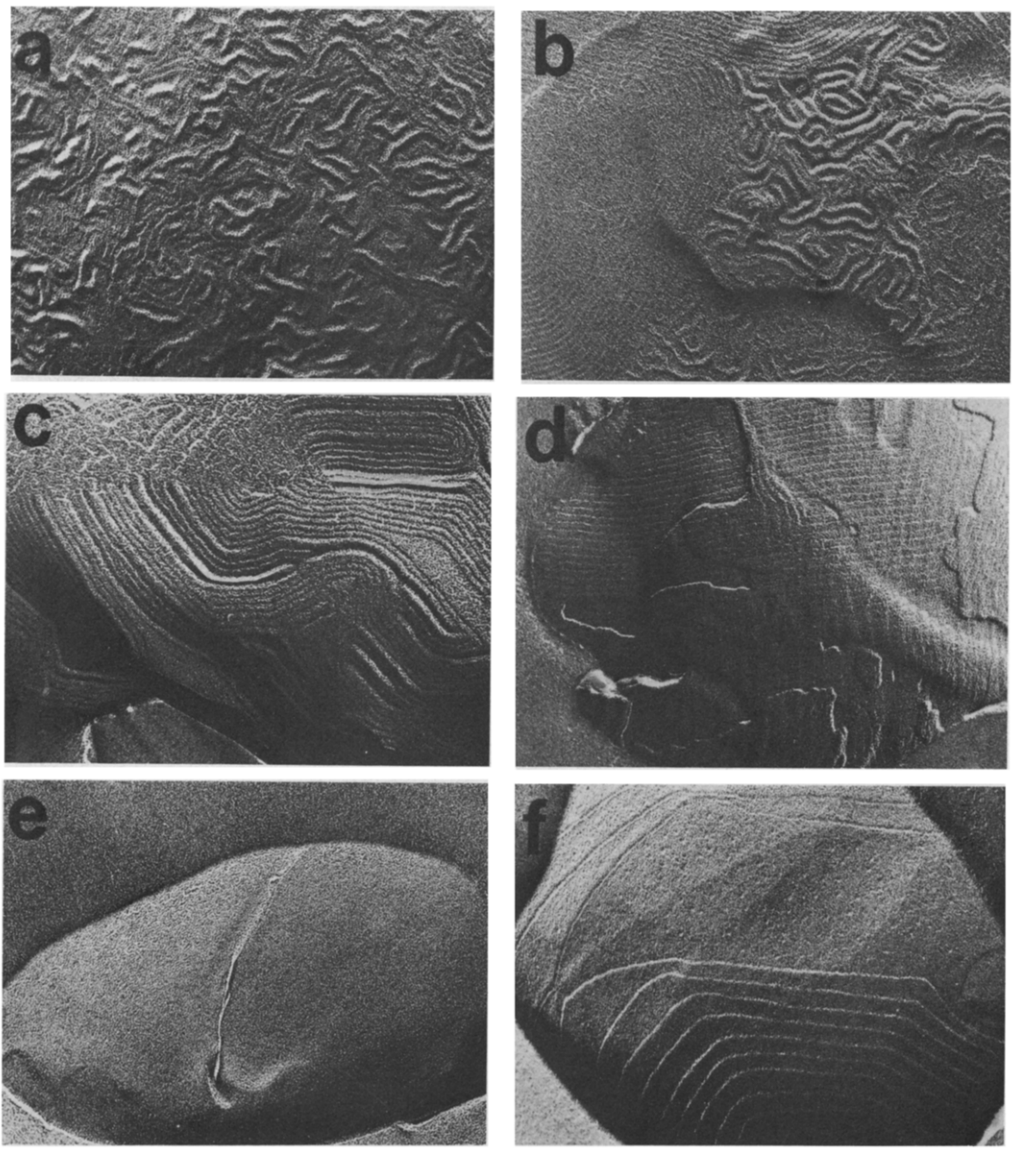


Fig. 3. Freeze-fracture electron micrographs of dipalmitoyl phosphatidylcholine vesicles prepared by detergent dialysis. Vesicles were quenched from (a) 45 ± 2°C, (b) 41.5 ± 0.5°C, (c) 35 ± 2°C after heating, (d) 35 ± 2°C after cooling, (e) 17.5 ± 0.5°C after heating, (f) 14.5 ± 0.5°C after cooling. Magnification is approx. 55 000×.

lecithin bilayer and that the large structural rearrangements involved with this transformation may be responsible for the hysteresis observed in the plots of the TEMPO *F* parameter vs. temperature. This hypothesis is supported by freeze-fracture electron micrographs of dipalmitoyl phosphatidylcholine vesicles quenched from within the pretransitional temperature range (Fig. 4). Samples heated from 0 to 32°C before quenching (Fig. 4a) have fracture faces containing only a few bands. Fracture faces of samples cooled to 32 from 56°C

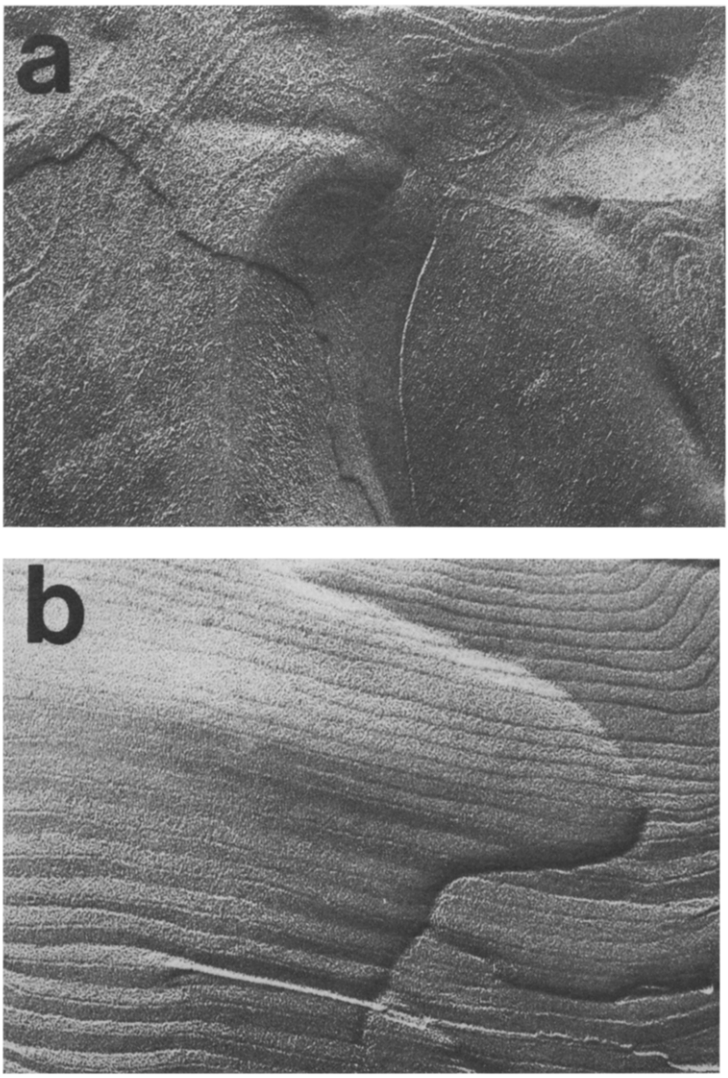


Fig. 4. Freeze-fracture electron micrographs of dipalmitoyl phosphatidylcholine vesicles prepared by the detergent dialysis method. Vesicles were quenched from 32 ± 1°C after (a) heating from 0°C and (b) cooling from 56°C. Magnification is approx. 82 000×.

(Fig. 4b) exhibit extensive banding. Although the band spacings tend to be somewhat irregular and greater than those observed in Fig. 3, it is obvious that there is a major structural difference between dipalmitoyl phosphatidylcholine bilayers heated to 32°C, the approximate center of the pretransition range, and bilayers cooled to this temperature.

The topographical features observed below the pretransition temperature may be associated with imperfections in the bilayer structure. These features often appear to radiate or spiral from points (Figs. 2d and 2e, and Fig. 3f), somewhat reminiscent of the spiral growths of screw disclocations observed in crystals [25].

In a recent paper, Janiak et al. [15] used the techniques of X-ray diffraction and differential scanning calorimetry to investigate the structural change associated with the pretransition of hydrated synthetic lecithins. These researchers found that both below and above the pretransition the lipid chains are stiff, fully extended, and tilted with respect to the plane of the bilayer. Below the pretransition, the lipid chains are packed in a one-dimensional lamellar lattice, the $L_{\beta'}$ conformation described by Tardieu et al. [4] and Ranck et al. [5]. At temperatures above the pretransition but below the main transition, the hydrocarbon chains are packed on a two-dimensional monoclinic lattice with a periodic ripple distorting the lipid lamellae ($P_{\beta'}$ conformation). In agreement with previous findings [5], Janiak et al. [15] found that the main transition of synthetic lecithins is associated with a conformational change from the $P_{\beta'}$ phase to a phase (L_α) characterized by melted hydrocarbon chains on a one-dimensional lamellar lattice.

Our freeze-fracture results correlate very well with the data obtained by Janiak et al. [15]. At temperatures below that of the pretransition, X-ray diffraction data suggest that lecithin bilayers should appear smooth and unbanded when visualized by freeze-fracture electron microscopy. For the most part, this has been verified by our experiments. At temperatures intermediate between those of the pretransition and main transition, the techniques of X-ray diffraction and freeze-fracture electron microscopy both indicate the presence of periodic undulations on the bilayer surface. X-ray diffraction data indicate that the periodicities of these banding patterns are 120 Å and 140 Å for dimyristoyl and dipalmitoyl phosphatidylcholines, respectively. The corresponding periodicities obtained from Figs. 2 and 3 are 130 ± 10 Å for dimyristoyl phosphatidylcholine and 150 ± 20 Å for dipalmitoyl phosphatidylcholine vesicles, values in good agreement with those obtained by X-ray diffraction. Finally, the smooth bilayer surfaces observed by the X-ray diffraction technique at temperatures above the main transition are visualized by freeze-fracture electron microscopy as jumbled bands or smooth surfaces with the smoother texture predominant at very rapid quenching rates [26].

Our results do not agree with experiments reported by van Deenen et al. [22—24]. These authors find regular band patterns on the fracture surfaces of dimyristoyl [22—24], dipalmitoyl [23], and distearoyl [23] phosphatidylcholine bilayers when these lecithins are quenched from 5° C. Since this quench temperature is below the pretransition temperature of each of the lipids studied, our results predict that only irregularly distributed bands, if any, should have been observed. Hand-shaken suspensions prepared as described by these workers [23] (10 mg phospholipid per ml of aqueous suspension) exhibit regular band patterns which disappear upon cooling through the pretransition in a manner completely analogous to that observed for detergent-dialyzed vesicles. However, either sodium phosphate or HEPES buffers were used in all our experiments.

To investigate the possibility that different cooling rates were responsible for the discrepancy between our results and those of van Deenen et al. [22—24], we cooled dipalmitoyl phosphatidylcholine vesicles to 15° C at rates varying from 1—2° C/h to over 1000° C/h and after equilibration periods varying from a few minutes to over an hour. In no case did we observe a regular band pattern

on the bilayer fracture faces. Thus, the discrepancy between our results and those obtained by van Deenen et al. remains unexplained. One possibility is that their cooling rates are even greater than ours.

Discussion

The present work serves two immediate purposes. First, freeze-fracture results are in excellent agreement with the X-ray data of Janiak et al. [15] in that the freeze-fracture microphotographs show a banded pattern only between the temperatures of the pretransition and the main transition in dimyristoyl phosphatidylcholine and in dipalmitoyl phosphatidylcholine. Second, the present study clearly stresses that caution must be exercised in using such banding patterns to identify "solid" domains in lipid mixtures where there are lateral phase separations. By extrapolation from the present study it can be concluded that the excellent agreement found between spin-label derived phase diagrams for binary mixtures of phosphatidylcholines and the freeze-fracture results [11—14] is due to the fact that in these experiments the quench temperatures were high enough that the solid solution phase was always in the banded $P_{\beta'}$ phase.

This agreement between the spin-label derived phase diagrams and the freeze-fracture microphotographs of binary mixtures of phosphatidylcholines is also related to the fact that TEMPO partitioning is most sensitive to the $P_{\beta'} \rightarrow L_{\alpha}$ phase transition, and the microphotographs exhibit equilibria between the $P_{\beta'}$ and L_{α} phases. The phase diagrams, thus far, provide little information on equilibria involving the $L_{\beta'}$ phases, for example, $L_{\beta'}$ phases might show immiscibility in binary mixtures when $P_{\beta'}$ phases show miscibility.

The present work also shows a significant difference between liposomes and vesicles produced by dialysis from detergent, which evidently have one or a relatively small number of bilayer shells. In liposomes the pretransition is sharp and shows a strong hysteresis, whereas the opposite is true for the structures arising from detergent dialysis. The TEMPO binding data thus suggest that cooperative interactions between the bilayers affect the width of the pretransition. On the other hand, the net increase in TEMPO solubility throughout the transition region is about the same in all cases, so that reducing the number of bilayers may increase the width of the transition, without causing it to disappear entirely, or even affecting the net heat absorption.

The origin of the hysteresis in the pretransition is not understood at present. The hysteresis is not an artifact related to the properties of the TEMPO spin label since, in unpublished work in this laboratory, J. van der Bosch has observed similar hysteresis effects with a number of other spin labels including fatty acid and phospholipid spin labels (van der Bosch, J. and McConnell, H., unpublished).

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