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THE RIPPLED STRUCTURE IN BILAYER MEMBRANES OF PHOSPHATIDYLCHOLINE AND BINARY MIXTURES OF PHOSPHATIDYLCHOLINE AND CHOLESTEROL

BRUCE R. COPELAND * and HARDEN M. McCONNEL **

The John Stauffer Laboratory for Physical Chemistry, Stanford University, Stanford, CA 94305 (U.S.A.)

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Summary

Freeze-fracture electron microscopy is used to study the rippled texture in pure dimyristoyl and dipalmitoyl phosphatidylcholine membranes and in mixtures of dimyristoyl phosphatidylcholine and cholesterol. Evidence is presented that the apparent phase transition properties of multilamellar liposomes may be dependent on the manner in which liposomes are prepared. Under certain conditions the ripple structures as visualized by freeze-fracture electron microscopy for the pure phosphatidylcholines are observed to be temperature dependent in the vicinity of the pretransition. Thus the transition can sometimes appear to be a gradual transition rather than a sharp, first-order phase transition. In mixtures of dimyristoyl phosphatidylcholine and cholesterol, the ripple repeat distance is found to increase as the cholesterol concentration is increased between 0 and 20 mol%. Above 20 mol%, no rippling is observed. A simple theory is presented for the dependence of ripple repeat spacing on cholesterol concentration in the range 0—20 mol%. This theory accounts for the otherwise inexplicable abrupt increase in the lateral diffusion coefficients of fluorescent lipids in binary mixtures of phosphatidylcholine and cholesterol when the cholesterol concentration is increased above 20 mol%.

* Present address: Division of Medical Genetics, Department of Medicine, School of Medicine, University of Washington, Seattle, WA 98195, U.S.A.

** To whom correspondence should be addressed.

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

Introduction

In aqueous dispersions of saturated phosphatidylcholines a main (solid to fluid) phase transition and a pretransition (in the solid) have been observed by a variety of techniques (see, for example, Luzzati and Tardieu [1]. Mabrey and Sturtevant [2], Janiak et al. [3], Luna and McConnell [4], and Lentz et al. [5]). X-ray diffraction [3] and freeze-fracture electron microscopy [4] have shown that there is a regular rippled (or banded) texture in the solid phase between the pretransition and the main transition. The ripple repeat distance is between 100 and 200 Å. Previous workers [3,4,6,7] have thus interpreted the pretransition as a first-order transition from a smooth (lamellar) solid phase at low temperatures to a rippled (monoclinic) solid phase at higher temperatures; two theories have also been proposed for the rippling above the pretransition [6,8]. In mixtures of saturated phosphatidylcholines with other lipids, freeze-fracture electron microscopy has, on this basis, been used as a means of corroborating phase diagrams that have been constructed from other types of experimental data [9,10].

Mixtures of cholesterol and phosphatidylcholines have been extensively investigated by a number of techniques [6,11—29] and have also been used as model membranes in immune studies [30—34]. It is interesting that in mixtures containing dimyristoyl or dipalmitoyl phosphatidylcholine and less than 20 mol% cholesterol, a phase transition is observed that is very similar to the phase transition for the pure phosphatidylcholine [25,26]. A number of experiments have also suggested that there is a significant break in membrane properties at 20 mol% cholesterol for temperatures below the main phase transition temperature [13,15,17,25,26,28,30]. Of particular interest is a recent investigation of lateral diffusion in binary mixtures of cholesterol and dimyristoyl or dipalmitoyl phosphatidylcholine. In that study [28], a large increase in diffusion constant was observed near 20 mol% cholesterol at temperatures below the main phase transition temperature. Two freeze-fracture electron microscopy studies of rippling in mixtures of cholesterol and saturated phosphatidylcholines have been described. In one of these studies [15], rippling was found to disappear at cholesterol mol fractions greater than 20 mol%. In the other study, rippling was observed up to 35 mol% cholesterol [6].

In the work presented here, freeze-fracture electron microscopy is used to extend previous investigations of pure dipalmitoyl and dimyristoyl phosphatidylcholines. Certain complications in the interpretation of the pretransition are described. A simple theory is also advanced for the variation in ripple repeat spacing with cholesterol composition. The theory is particularly helpful for understanding lipid lateral diffusion coefficients, as well as immune responses to model membranes composed of binary mixtures of phosphatidylcholine and cholesterol.

Materials and Methods

Materials. Dimyristoyl and dipalmitoyl phosphatidylcholines were purchased from Sigma Chemical. Silica gel thin-layer chromatography showed a single spot for both lipids. The fatty acids of each phospholipid were more than 98% pure,

as evidenced by gas chromatography of the fatty acid methyl esters. Cholesterol, which had been recrystallized twice from ethanol, was kindly provided by Dr. James R. Sheats. It was at least 99% pure according to silica gel thin-layer chromatography and elemental analysis. The phospholipids and cholesterol were stored in dilute ethanol solutions at 4°C under argon atmospheres. The concentrations of the phospholipid solutions were determined by assaying for phosphate according to the method of McClare [35]. The concentrations of the cholesterol solutions were determined from gravimetric and volumetric measurements.

Lipid dispersions. Thin lipid films were prepared by the following procedure. Appropriate volumes of stock solutions of phospholipid and cholesterol were pipetted into 5-ml round-bottom flasks to give a total of 10 mg of lipid per flask. The ethanol was removed under vacuum, and $CHCl_3/CH_3OH$ (approx. 10 : 1, v/v) was added to each flask. This solvent was then carefully removed under vacuum to leave a thin film of lipid inside each flask. Mol% fractions of cholesterol could be determined for each flask with a precision of ±1%.

Multilamellar liposomes were prepared from the thin lipid films in the following manner. To each flask was added 140 μl of 0.1 M phosphate buffer, pH 7.0. Each flask was then stoppered under an argon armosphere and briefly heated above 80°C in order to hydrate the lipid. Next the flask was swirled in an oil bath at 90°C for a few minutes until no lipid remained on the glass surface. A glass bead was added, and the flask was again stoppered under argon. After brief heating to 90°C, the flask was vortexed for 1—2 s in order to disperse the lipid in the buffer. All manipulations were as gentle as possible. The resulting liposomes were large and contained many lamellae (according to freeze-fracture electron microscopy).

Small vesicles (0.1—1.0 μm), containing only a few lamellae, were prepared by two different procedures. Both procedures were modifications of the ether injection method of Deamer and Bangham [36]. The first procedure, which we shall call the standard method, has been described by Parce et al. [37]. For this study, 0.1 M phosphate buffer, pH 7.0, was used in place of sucrose solution and barbitol-buffered saline. In the second procedure, 2 mg of lipid was dissolved in 20 ml of diethyl ether/CH_3OH (4 : 1, v/v). This solution was injected at a rate of 0.25 ml/min into 4 ml of phosphate buffer, pH 7.0, at 62°C. The hot buffer suspension was then filtered through a Triton-free millipore filter and permitted to cool for 30 min. The vesicles were pelleted twice at 12 000 × *g* for 20 min at 20°C, and resuspended each time in 1 ml of buffer. The resulting suspension was then transferred to a dialysis bag and dialyzed at 4°C against 200 ml of buffer. The dialysis buffer was changed three times at approx. 12-h intervals. Finally the vesicles were harvested by centrifugation of the dialysis bag contents (12 000 × *g*, 4°C, 20 min).

Freeze-fracture electron microscopy. All samples to be quenched for freeze-fracture were kept under argon atmosphere in stoppered flasks, in order to prevent decomposition and growth of microorganisms. Prior to quenching, each flask was normally kept at 4°C overnight, then incubated for at least 4 h in a room that was thermostatted to within a few degrees of the quench temperature. A few samples, designated 'slow cooling', were prepared for quenching in a different manner. In these cases, the flask was suspended in a Thermos

bottle filled with water at a temperature several degrees above the main transition temperature for the lipid sample. The thermos was then partially covered, and the contents were allowed to cool slowly to the quench temperature. In no case did the rate of cooling exceed 0.5°C/h. For quenching, 1 μl drops of samples were pipetted onto 3-mm diameter copper planchets that were resting on a metal block at the quench temperature. The room temperature was always maintained within a few degrees of the quench temperature. After the planchets had equilibrated on the block for several minutes, they were rapidly plunged into partially solidified Freon 22 (Virginia Chemical) and transferred to liquid nitrogen.

Quenched samples were fractured in a Balzers BAF301 Freeze-Etching Device that was equipped with electron beam apparatus for platinum and carbon shadowing and a quartz-crystal monitor for regulating shadow thickness. Fracturing was performed at −114.5°C without etching. Platinum films were approx. 20 Å thick. Replicas were floated from the planchets onto water and cleaned by flotation on $NaHClO_3$ solution for at least 2 h. They were then rinsed on water and transferred to 85% ethanol. After a short time, replicas were picked up on 400-mesh copper grids.

Electron micrographs were taken on 3.25 by 4 inch Kodak electron microscope film in an Hitachi HU-11E electron microscope. The nominal initial magnification was 33 000×. For quantitative measurements, magnification was calibrated using a grating with 1000 lines per inch.

TEMPO partitioning experiments. The temperature dependence of the partitioning of 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) between liposomes and aqueous phase was measured as described by Luna and McConnell [4].

Results and Discussion

Pure phosphatidylcholines

In many studies by earlier workers [2—5,38—40], both a main transition (24°C) and a pretransition (10—14°C) have been observed in aqueous dispersions of dimyristoyl phosphatidylcholine. In Fig. 1a and b, we show freeze-fracture electron micrographs that are representative of dimyristoyl phosphatidylcholine liposomes and ether-injection vesicles that have been quenched from between 14 and 24°C. The results are in good agreement with earlier freeze-fracture electron microscopy studies [4,6]. In this temperature region, there is a primary ripple pattern with a ripple repeat distance of 120—140 Å for liposomes and 140—160 Å for ether-injection vesicles. A secondary ripple repeat pattern is also frequently observed. This pattern has a repeat distance that is 1.90—1.95 times the primary ripple repeat distance and a ripple amplitude that is much greater than the primary ripple amplitude. In some cases, features are observed that look like even higher repeat distances (on the order of 3—10 primary repeat distances). These higher order repeat distances appear to be associated with macroscopic variations in membrane curvature that occur near defects in primary and secondary ripple repeat patterns.

In ether-injection vesicles prepared by the standard procedure, surface area measurements have shown that 30% of the lipid is on the outside of the vesicles (determined by chemical reactivity of externally exposed spin-labeled lipid

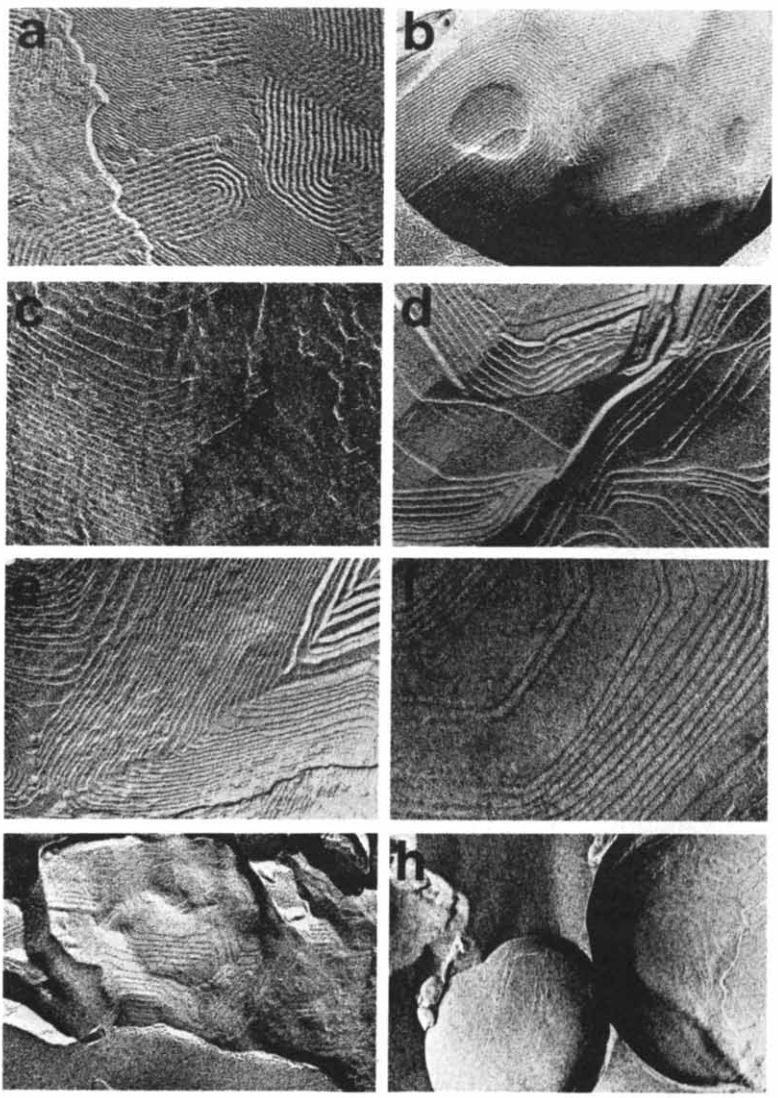

Fig. 1. Freeze-fracture electron micrographs of liposomes and vesicles containing either dimyristoyl or dipalmitoylphosphatidylcholine. Composition, structure, and quenching details are as follows: (a) dimyristoyl phosphatidylcholine liposome quenched from 23°C after heating; (b) dimyristoylphosphatidylcholine vesicle prepared by the standard ether-injection method (see Materials and Methods) and quenched from 17°C after heating; (c) dipalmitoyl phosphatidylcholine liposome quenched from 19°C after heating; (d) same as in (c) but quenched from 23°C; (e) same as in (c) but quenched from 25°C; (f) dipalmitoyl phosphatidylcholine liposomes quenched from 17°C after 'slow cooling' (see Materials and Methods); (g) dipalmitoyl phosphatidylcholine vesicle prepared by the standard ether-injection method and quenched from 20°C after heating, and (h) dipalmitoyl phosphatidylcholine vesicles prepared by the ether-injection/dialysis method and quenched from 19°C after heating. Magnification is approx. 43 000.

haptens; Wallace Parce, J., private communication). This implies that a substantial fraction of these vesicles must be unilamellar. In our study, hundreds of vesicles similar to Fig. 1b were viewed in the electron microscope. In all cases, the vesicles were rippled. This argues strongly that rippling is not simply

a result of interaction between more than one bilayer in a vesicle. This does not, however, mean that there is no interaction between different lamellae in vesicles or liposomes. In fact, interaction between lamellae is demonstrated quite clearly in liposome preparations (see, for example Fig. 1a), where in certain areas ripple patterns in one layer are identical with ripple patterns in an adjacent layer. This type of interaction is particularly common for second repeat patterns, which have a large amplitude. Occasionally 'interference patterns' are observed when rippling propogates along one direction in one layer and along another direction in an adjacent layer.

In aqueous dispersions of dipalmitoyl phosphatidylcholine, previous workers [2—5,38—40] have found a pretransition between 28 and 35°C and a main transition at 41°C. Fig. 1c—e show freeze-fracture electron micrographs of dipalmitoyl phosphatidylcholine liposomes that were heated from 4°C and then quenched at several temperatures below the pretransition measured by earlier workers. Significant amounts of rippling are observed in these liposomes. Moreover, dimyristoyl phosphatidylcholine liposomes quenched from 6°C (not shown here) have also demonstrated comparable rippling features. In general this rippling at lower temperatures differs from the rippling observed in samples quenched from temperatures above the pretransition. These differences can be summarized as follows. At lower temperatures, the ripple repeat spacing is larger; there is a decrease in correlation between ripple lines; and there appears to be a decrease in apparent ripple amplitude. These results differ from the results and interpretations in some previous freeze-fracture and X-ray diffraction studies [3,4,6]. However, Krbecek et al. [7] have also recently observed a type of rippling at temperatures below the pretransition.

The liposomes used to obtain Fig. 1c—e were heated from 4°C and equilibrated at the quench temperature for at least 4 h prior to quenching. It therefore seems unlikely that hysteresis can explain the discrepancies between our results and the results of earlier workers. In order to test the possibility that rippling might depend on the rate of cooling through the pretransition, we investigated liposome samples that were prepared by very slow cooling prior to quenching. (See Materials and Methods.) Fig. 1f shows a representative freeze-fracture electron micrograph for a typical sample. Significant rippling is still observed, although the amount of rippling may be somewhat reduced in comparison with liposomes that were prepared for quenching by the normal method.

The spin label TEMPO was used to measure the pretransition and main transition in dipalmitoyl phosphatidylcholine liposomes. (See Shimshick and McConnell [38] or Luna and McConnell [4] for a discussion of the method.) The curve of TEMPO partitioning as a function of temperature was similar in shape to that obtained previously by Luna and McConnell [4]. However, in this case the main transition occurred at a slightly lower temperature (40.5°C), and the pretransition occurred at a significantly lower temperature (20—27°C) than in the study by Luna and McConnell. Since the lipids appeared to be quite pure, both before and after TEMPO partitioning experiments, we conclude that the phase transition properties of liposomes are dependent upon the manner in which liposomes are made. (The procedure used for preparing liposomes in this study differs from the procedure used by other workers. See for example Shim-

shick and McConnell [38] or Mabrey and Sturtevant [2].) This lower pretransition temperature may account for the fact that we observe rippling in liposomes at temperatures well below the pretransition temperatures reported by other workers.

In Fig. 1g we show a typical freeze-fracture electron micrograph of a dipalmitoyl phosphatidylcholine vesicle prepared by the standard ether-injection procedure and quenched from 20°C. Very regular and extensive rippling is observed, although the ripple repeat distance is larger than would normally be seen above the pretransition. (Similar results were obtained with dimyristoyl phosphatidylcholine vesicles prepared by the standard ether-injection procedure and quenched from 20°C.) Very regular and extensive rippling is observed, although the ripple repeat distance is larger than would normally be seen above the pretransition. (Similar results were obtained with dimyristoyl phosphatidylcholine vesicles prepared by the standard ether-injection procedure.) There was some reason to be concerned that the regular rippling in these vesicles at this temperature might be the result of a depression in the pretransition temperature caused by ether contamination of the membrane. Therefore, dipalmitoyl phosphatidylcholine vesicles were prepared by the ether-injection/dialysis procedure (see Materials and Methods) in order to eliminate ether contamination. In Fig. 1h we show a freeze-fracture electron micrograph of vesicles prepared by this latter method and quenched from 19°C. Only a relatively small amount of very low-amplitude rippling is observed in these vesicles. The difference between Fig. 1g and h may therefore indicate that there was ether contamination in the vesicles of Fig. 1g. Alternatively, the long incubation of vesicles at 4°C may have simply diminished the amount of rippling in the vesicles of Fig. 1h. At this point it is not possible to distinguish between these two explanations.

The region of greatest temperature dependence of rippling in liposomes (see Fig. 1c—e) is in the vicinity of the pretransition. If the pretransition were a simple, discontinuous, first-order phase transition from a rippled phase to a smooth phase, we should expect to see domains of smooth membrane coexisting with domains of rippled membrane in the pretransition region. Moreover the size of the rippled domains should decrease to zero as the temperature is reduced, and we should not expect to see any significant change in ripple properties (correlation, repeat distance, and amplitude) with temperature. However, significant variations in ripple properties with temperature are experimentally observed, and there is a decrease in ripple amplitude rather than a decrease in extent of rippling as temperature is decreased. These observations show that under certain conditions of sample preparation the pretransition may manifest itself as a gradual transition from a rippled texture to a smooth texture rather than a discontinuous transition. Such a gradual transition could still have a differential heat absorption over some temperature range. Moreover, a gradual transition is not necessarily inconsistent with the X-ray data of Janiak et al. [3]. At intermediate points in the transition it may be difficult to detect rippling by X-ray diffraction if the ripple lines are poorly correlated and/or have a low amplitude. The fact that under some conditions of sample preparation the pretransition appears to be gradual shows that particular caution is necessary in using banding or rippling to construct phase diagrams. In the

experiments of Luna and McConnell [4,9,10] sample preparation procedures were employed where no periodic ripple was observed below the pretransition temperatures. Thus, we have no reason to doubt the validity of the phase diagrams based on these studies [4,9,10]. However, the work presented above in the present paper, as well as that given below, shows that the relationship between ripple structure and thermodynamic phase equilibria may not be straightforward, either experimentally or theoretically.

Mixtures of dimyristoyl phosphatidylcholine and cholesterol

We have used freeze-fracture electron microscopy to investigate liposomes prepared from mixtures of dimyristoyl phosphatidylcholine and cholesterol. The cholesterol composition has been varied from 0 to 24 mol%, and the samples have been quenched from several temperatures between 14 and 23°C. All electron micrographs shown here were obtained from samples that were prepared for quenching by the normal method (see Materials and Methods). However, samples prepared by 'slow cooling' gave comparable results. There is reason to believe, therefore, that the 'pretransition' is below 14°C for the entire composition range studied here.

In Figs. 2 and 3, we show representative series of micrographs for different cholesterol compositions that have been quenched from along the isotherms 17 and 20°C, respectively. As can be seen, the variation in rippling with cholesterol concentration is quite similar for the two temperatures. Comparable results are also obtained at 14°C (not shown). Thus, for the entire composition range studied here, there is no significant temperature dependence of rippling between 14 and 20°C. The effect of cholesterol concentration on rippling can be characterized as follows. From 0 to 15 mol% cholesterol, there is a gradual increase in primary ripple repeat distance, which is approximately linear in cholesterol mol fraction. Secondary ripple repeats are also observed, but only for cholesterol mol fractions less than approx. 5 mol%. In all cases, the secondary ripple repeat distance is approx. 1.9 times the primary repeat distance, and the apparent amplitude is much larger than the apparent amplitude for the primary repeat. Above 15 mol%, the primary ripple repeat distance begins to increase more rapidly with cholesterol mol fraction; there is more scatter in the observed ripple repeat distance; and the ripple lines begin to show local correlated deviations from parallel straight lines (see, e.g., Figs. 2 and 3). At and above 20 mol% cholesterol liposomes show no rippling and appear to have extremely smooth surfaces. These results are in qualitative agreement with the work of Verkleij et al. [15]. The absence of rippling above 20 mol% does not, however, agree with the work of Gebhardt et al. [6], in which rippling was observed up to 35 mol% cholesterol. Since we are normally able to see even very low-amplitude rippling, through the use of thin platinum films, and since in this study we are completely unable to observe any rippling above 20 mol% cholesterol, we believe that the discrepancies between our results and the results of Gebhardt et al. are probably caused by differences in sample preparation.

The apparent primary ripple repeat amplitude does not appear to change significantly with cholesterol mol fraction between 0 and 20 mol%. However, because this apparent amplitude is relatively low, it is sometimes difficult to discern rippling in certain membrane regions where platinum shadowing is

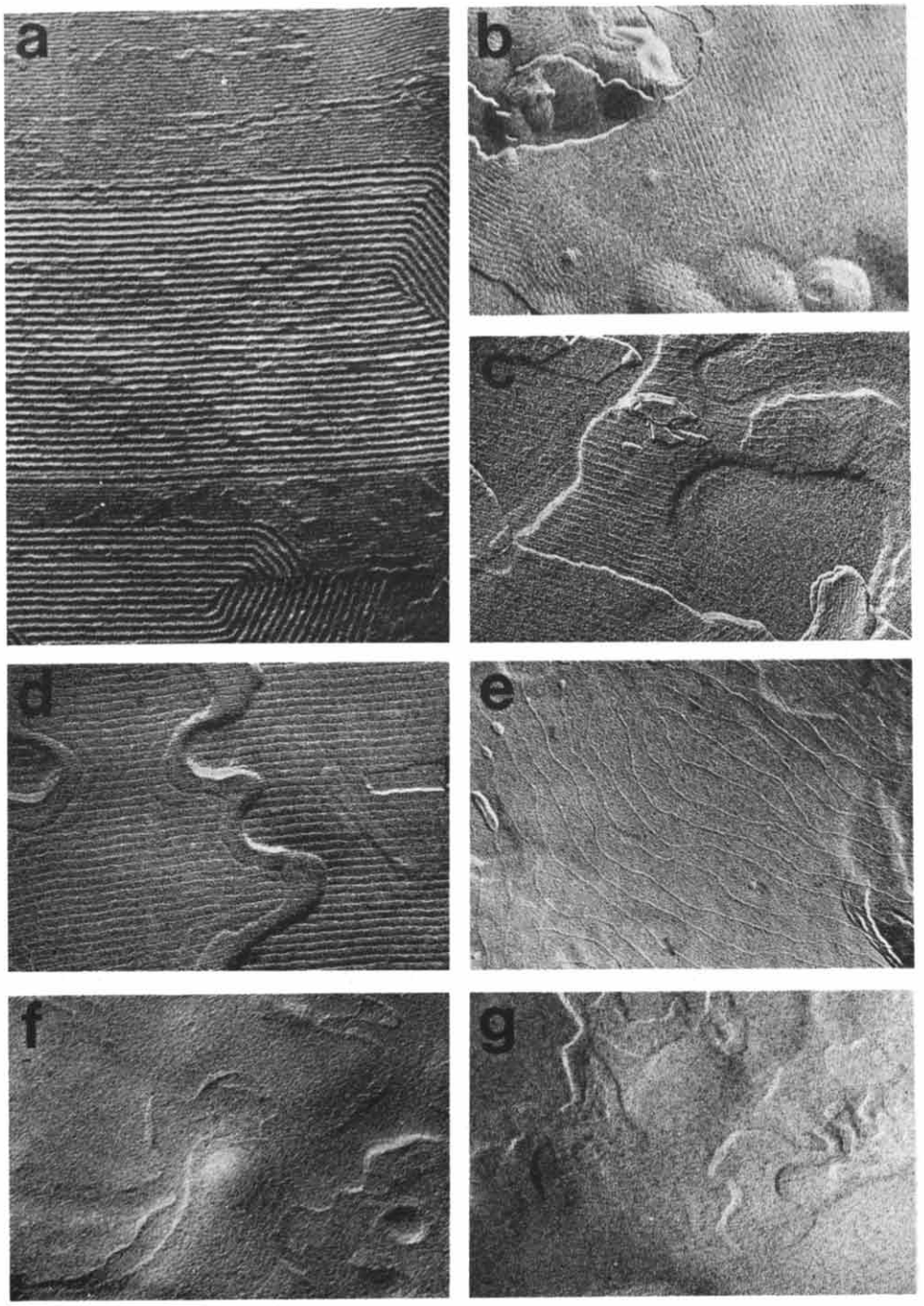

Fig. 2. Freeze-fracture electron micrographs of liposomes containing mixtures of dimyristoyl phosphatidylcholine and cholesterol. All liposomes were quenched from 17°C after heating. Compositions of cholesterol are (a) 0 mol%; (b) 4 mol%; (c) 8 mol%; (d) 12 mol%; (e) 16 mol%; (f) 20 mol%, and (g) 24 mol%. Magnification is approx. 54 000.

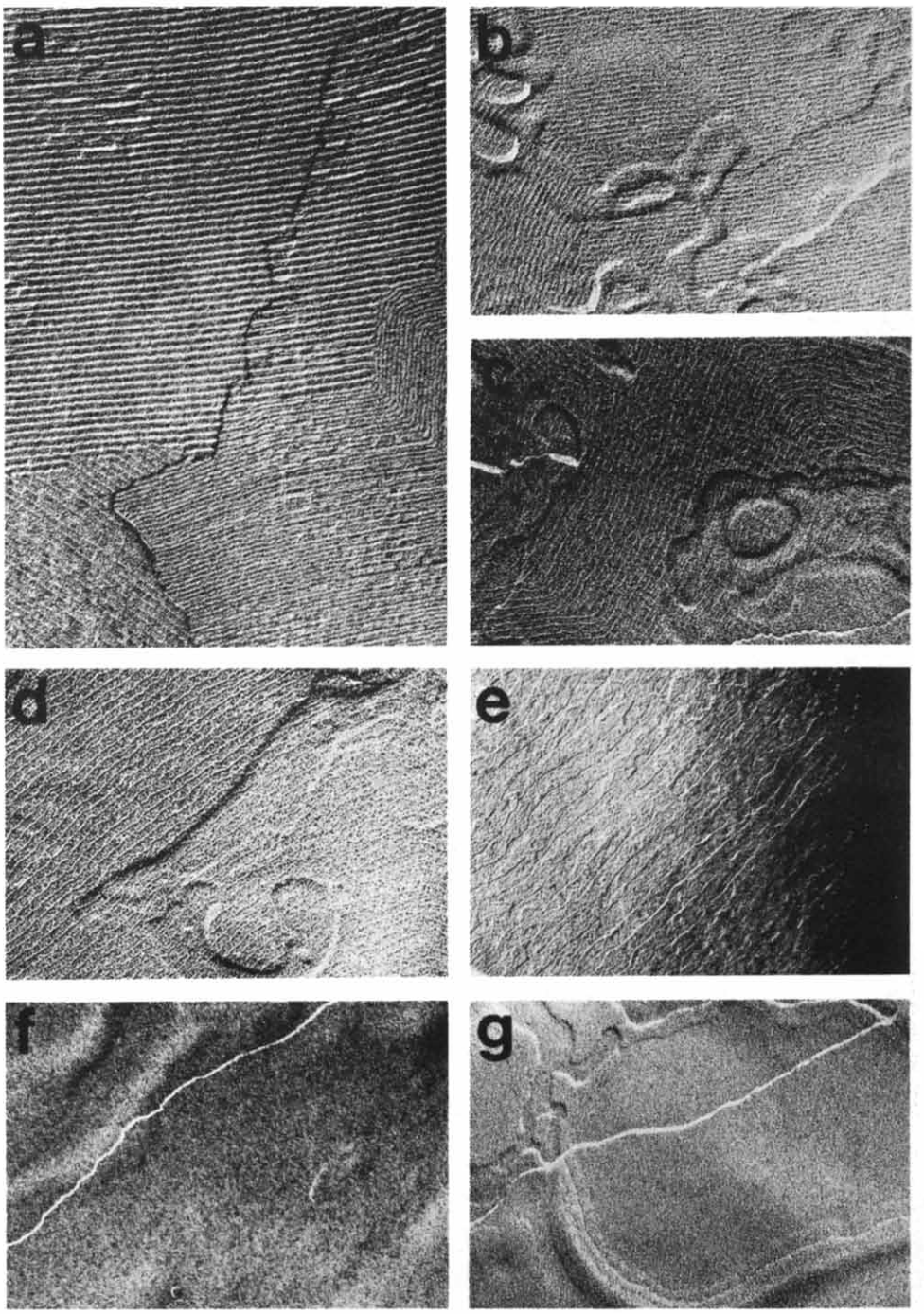

Fig. 3. Freeze-fracture electron micrographs of liposomes containing mixtures of dimyristoyl phosphatidylcholine and cholesterol. All liposomes were quenched from 20°C after heating. Compositions of cholesterol are: (a) 0 mol%; (b) 4 mol%; (c) 8 mol%; (d) 12 mol%; (e) 16 mol%; (f) 20 mol%, and (g) 24 mol%. Magnification is approx. 54 000.

heavy or where the shadowing direction is unfavorable. This problem is clear in the lower right corner of Fig. 3c. We emphasize that this is a shadowing artifact. The effect can be minimized by shadowing with thin platinum films.

Liposomes with different cholesterol mol fractions have also been quenched from 23°C and investigated by freeze-fracture electron microscopy. The electron micrographs are not shown here. In general the rippling in these samples is comparable to that seen in the temperature range 14—20°C, except that some amount of 'jumbling' (loss of long-range ripple correlation) is observed between 15 and 20 mol% cholesterol. Jumbling is normally characteristic of fluid membranes. Hence this observation is consistent with the recent differential scanning calorimetry work of Estep et al. [25] and Mabrey et al. [26], in which a slight depression in the main (solid to fluid) transition was observed as the cholesterol concentration was increased between 0 and 20 mol%.

The disappearance of rippling at about 20 mol% cholesterol implies a significant break in some membrane properties at this composition. This is in agreement with a number of different studies [13,15,17,25,26,28,30]. In particular, Rubenstein et al. [18] have found a dramatic increase in lipid lateral diffusion coefficient around 20 mol% cholesterol at temperatures below 20°C for dimyristoyl phosphatidylcholine mixtures and below 39°C for dipalmitoyl phosphatidylcholine mixtures. This is important because we observe a loss in short-range ripple correlation as the cholesterol concentration approaches 20 mol% at constant temperature. Under these conditions long-range ripple correlation is maintained. Such behavior is quite different from the loss in long-range correlation (jumbling) that occurs as the temperature is increased at constant cholesterol concentration in the vicinity of the main transition.

The variation in ripple repeat distance with cholesterol concentration appears to be continuous between 0 and 20 mol% cholesterol. This is true even though the change in repeat distance with cholesterol concentration becomes greater at concentrations above 15 mol%. Furthermore, we do not ever observe coexisting macroscopic domains of different ripple repeat distance (other than primary and secondary repeats) in liposomes. These facts argue strongly that

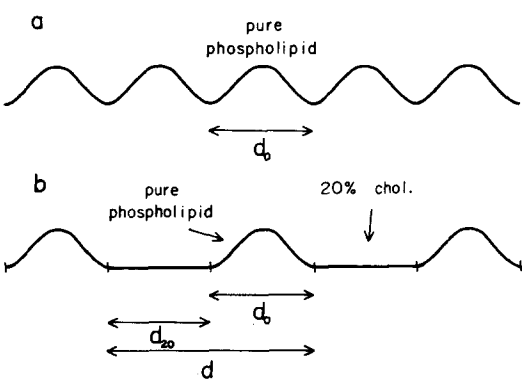

Fig. 4. Schematic cross-sectional view of the bilayer ripple pattern. The pattern for pure phosphatidylcholine is shown in (a); the ripple repeat distance is d_0. The hypothetical 'ordered, microscopic phase separation' is shown in (b); ripple strips of pure phosphatidylcholine of width d_0 are separated by strips of 20 mol% cholesterol phase of width d_{20}, and the total distance between ripple lines is d.

there is not any simple macroscopic phase separation into different phases of 0 and 20 mol% cholesterol concentration in the temperature composition range between 0 and 20 mol% cholesterol and 14 and 20°C. It has been suggested previously [18] that there might be a phase separation in which thin strips of smooth, 20 mol% cholesterol phase are separated by individual ripple lines of pure phosphatidylcholine, so as to form a regular, ripple-like pattern (see Fig. 4). In Appendix we derive an approximate equation relating the ripple repeat spacing d to the cholesterol mol fraction X_C for such a pattern. This equation is

$$\frac{d}{d_0} = \frac{0.2}{0.2 - X_C} \tag{1}$$

where d_0 is the ripple repeat spacing for pure phosphatidylcholine. In Fig. 5, a plot of experimental values of d_0/d vs. X_C is compared with the predictions of Eqn. 1. (For $X_C = 0.2$, we have taken the experimental value of d to be infinity.) The agreement between the experimental data and Eqn. 1 is fairly good. This suggests that the increasing smooth areas that are observed between ripple lines as the cholesterol concentration is increased are indeed regions containing approx. 20 mol% cholesterol, and the ripple lines themselves are largely phosphatidylcholine. For some purposes this could be viewed as an 'ordered, microscopic phase separation', but from a strictly thermodynamic point of view the system is doubtless a single phase. The possible analogy of this kind of system to infinitely adaptable structures [41,42] should be noted.

If the above description of an 'ordered, microscopic phase separation' is taken literally, there are obvious thermodynamic and kinetic implications. For example, the sharp, endothermic component that is observed in the scanning calorimetry studies in Mabrey et al. [26] and Estep et al. [25] could be interpreted as the 'melting' of the phosphatidylcholine ripples. The macroscopic, lateral diffusion coefficients of fluorescent lipid molecules in phosphatidylcholine/cholesterol mixtures are large for cholesterol concentrations above 20

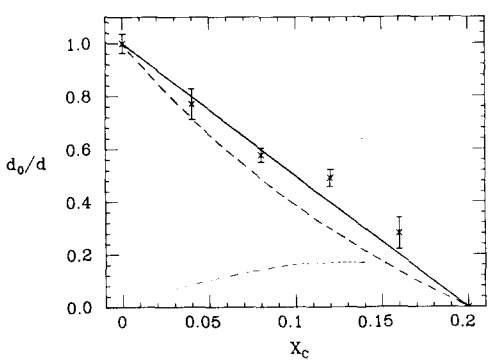

Fig. 5. Comparison of experimental (‡) and calculated (———) values of d_0/d as a function of cholesterol mol fraction. The experimental data points are averages from many measurements. The value of d_0 is 128.8 Å; the experimental value of d for 20 mol% cholesterol is assumed to be infinity. Eqn. 1 was used to calculate the theoretical line. - - - - - -, the results of a theoretical calculation where the ripples are assumed to have a semicircular profile and the surface area of the semicircle rather than the projected area of the ripples is used in the calculation. See Appendix, Eqn. A.8.

mol% ($D \approx 10^{-8}$ cm^2/s), and are at least one order of magnitude smaller at lower cholesterol concentrations, as noted earlier. These results are based on photobleaching patterns where the bleach period is approx. 5 μmin. At the microscopic level, with distances between ripples on the order of 500 Å, the diffusion coefficients would be non-uniform for an 'ordered, microscopic phase separation'. The ripples should then act as barriers for macroscopic, lateral diffusion, especially if the domain size for the ripples regions is small compared to 5 μmin, for cholesterol concentrations less than 20 mol%.

There have been a number of seemingly discordant results in studies of phosphatidylcholine/cholesterol mixtures in which dynamical, spectroscopic techniques that are sensitive to motions over different time and distance scales have been used [28,29,43]. The existence of a single thermodynamic phase having ordered solid- and fluid-like regions may account for these results. Owicki and McConnell [44] have recently developed a theory for long-range lateral diffusion in inhomogeneous membranes such as the one described here, and calculations based on this theory, and the observed diffusion data [28] are in substantial agreement.

We have used the term 'ordered, microscopic phase separation' to describe the ripple pattern in mixtures of phosphatidylcholine and cholesterol. However, it is probably a great oversimplification to think of the system in terms of a conventional thermodynamic phase separation. For one thing, there is significant long-range correlation between domains of the different phases. This long-range correlation may be electrostatic in origin. Even so, on a microscopic level the model that we have described may provide a useful way of viewing binary mixtures of cholesterol and phosphatidylcholines in this particular temperature composition region.

Appendix

Consider the regular ripple pattern that is observed in pure phosphatidylcholine bilayers at temperatures below the main phase transition. We represent the ripple repeat distance by d_0. (See Fig. 4a.) Next consider a binary mixture of cholesterol and phosphatidylcholine in which the cholesterol composition is less than 20 mol%. Let us imagine that there is an 'ordered, microscopic phase separation' in which strips of pure phosphatidylcholine of width d_0 (one ripple) are separated by uniform strips of 20 mol% cholesterol phase of width d_{20}, as indicated in Fig. 4b. Since the strips are presumed to be of equal length, the ratio d_{20}/d_0 is identical to the ratio of the total areas of the two different kinds of strips:

$$\frac{d_{20}}{d_0} = \frac{N_{20}a_{20}}{N_0 a_0} \tag{A.1}$$

where N_0 and N_{20} are respectively, the total number of molecules in pure phosphatidylcholine phase and in 20 mol% cholesterol phase, and a_0 and a_{20} are the average areas per molecule in these phases. The total distance from one ripple line to the next is then simply

$$d = d_0 \left(1 + \frac{N_{20}a_{20}}{N_0 a_0}\right) \tag{A.2}$$

The ratio N_{20}/N_0 can be related to the total cholesterol composition in the mixture by noting that 4/5 of the molecules in the 20 mol% cholesterol strips are phosphatidylcholine molecules, and the remaining molecules are cholesterol. Thus the total number of cholesterol molecules in the mixture is

$$N_C = N_{20}/5 \tag{A.3}$$

and the total number of phosphatidylcholine molecules is

$$N_L = N_0 + 4N_{20}/5 \tag{A.4}$$

The mol fraction of cholesterol X_C is related to N_C and N_L by

$$X_C = N_C/(N_C + N_L) \tag{A.5}$$

Hence Eqns. A.3—A.5 can be combined with Eqn. A.2 to give the result

$$\frac{d}{d_0} = \left[\frac{0.2 - X_C(a_0 - a_{20})/a_0}{0.2 - X_C}\right] \tag{A.6}$$

If the average area per molecule is the same in each phase, then Eqn. A.6 reduces to the particularly simple expression

$$\frac{d}{d_0} = \left[\frac{0.2}{0.2 - X_C}\right] \tag{A.7}$$

When the reciprocal of Eqn. A.7 is plotted vs. X_C a straight line is obtained, as shown in Fig. 5. If the areas a_0 and a_{20} differ by 10%, a plot of the reciprocal of Eqn. A.6 (not shown) gives a curve that is only slightly different from the straight line in Fig. 5.

A referee for the present paper has correctly pointed out that in deriving Eqn. A.7 we have employed the projected area of the ripple curve rather than the actual surface area. If one takes a 'worst possible case', and assumes the ripple profile is a semicircle, then the projected area is $2/\pi$ times the actual area. In this case Eqn. A.7 is modified to yield

$$\frac{d}{d_0} = \frac{0.2 + 0.57X_C}{0.2 - X_C} \tag{A.8}$$

A plot of d_0/d corresponding to Eqn. A.8 is given in Fig. 5 as a dashed curve. It is entirely possible that there are other sources of theoretical and experimental errors of this magnitude, such as the assumption concerning the equality of areas of lipid molecules in the two phases, variations in ripple amplitudes and uncertainties in measuring ripple repeat distances when these distances are large. We do not believe that any of these uncertainties are large enough to blemish our central conclusion: the dependence of ripple repeat spacing on lipid composition is such as to indicate strongly the ripples themselves are largely phosphatidylcholine, and the planar regions between the ripples contain approx. 20 mol% cholesterol.

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