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ANALYSIS OF THE CRYSTALLIZATION PROCESS IN LECITHIN LIPOSOMES: A FREEZE–ETCH STUDY

P. H. J. TH. VERVERGAERT[a], A. J. VERKLEIJ[b], P. F. ELBERS[a] and L. L. M. VAN DEENEN[b]

[a] *Biological Ultrastructure Research Unit, State University of Utrecht and* [b] *Laboratory of Biochemistry, State University of Utrecht, Utrecht (The Netherlands)*

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SUMMARY

1. Regular band patterns are visualized by freeze–etch electron microscopy on fracture faces of lecithin liposomes and bulk preparations containing 25% water when quenched from below transition temperatures.

2. It has been demonstrated that this crystalline structure is a general phenomenon of saturated phosphatidylcholines and occurs also in some unsaturated lecithins.

3. Freeze–etch results with liposomes consisting of two lecithins are consistent with differential scanning calorimetric experiments. No segregation can be demonstrated by freeze–etching when the mixture shows only one thermotropic peak. On the other hand, in mixtures which display two peaks, band patterns are found along with smooth areas when the material is quenched from a temperature between the two peaks.

INTRODUCTION

Liposomes are widely used as models for biological membranes. Especially in permeability studies the liposome system shows remarkable similarities with some natural membranes[1–4]. In both types of membranes a temperature-dependent phase transition from the liquid crystalline to the gel state can be demonstrated by X-ray analysis and differential scanning calorimetry[5–7]. Freeze–etch electron microscopy has recently demonstrated that liposomes prepared from synthetic phosphatidylcholines have smooth fracture faces when quenched from above the transition temperature, whereas below that temperature band patterns were observed[8]. It was shown that the bilayers in these liposomes are corrugated[9]. From X-ray studies[10], it was concluded that below the transition temperature at least two phosphatidylcholines, (dilauroyl)-lecithin and (dimyristoyl)-lecithin, are organized in "undulated" bilayers, in which the molecules are rigid and tilted with respect to the normal of the bilayer plane. However, such detailed X-ray analysis can only be performed on systems containing 25% water or less. We were interested to establish whether the X-ray data could be directly related to the freeze–fracture results on liposomes. This would be the case if by freeze–fracturing of lipid preparations containing

25% water, comparable band patterns were found as in liposomes. Such experiments would confirm the reliability of the freeze–fracturing technique that was used in our investigation of membrane model systems.

To understand in more detail the phase transition that occurs in a biological membrane of mixed lipid composition like that of *Acholeplasma laidlaiwii*[11], we studied the crystallization in lecithin liposomes with both intra- en intermolecular mixed acyl chains. By differential scanning calorimetry single transition peaks have been found[12] with liposomes prepared from a phospholipid containing the same or two different acyl chains within one molecule. In intermolecularly mixed liposomes of two phospholipids one peak was found during the transition only when the components had a difference of no more than two CH_2 groups. It was concluded that the lipid molecules in that case crystallize simultaneously. This process is called cocrystallization[12]. When the difference in chain length is larger than two CH_2 groups or one species is completely saturated and the other contains an unsaturated acyl moiety, two thermotropic peaks in the differential scanning calorimetry curve are observed upon cooling. It was suggested that upon cooling the system, migration of lecithin molecules within the bilayers must occur to give crystalline regions corresponding to the two components. This process is called monotectic crystallization[12]. In the present paper, freeze–fracturing results are described from liposomes consisting of one species of phosphatidylcholine containing acyl groups with both equal and unequal length. In addition the crystalline structure of intermolecularly mixed liposomes consisting of two species of phosphatidylcholine is analysed.

MATERIALS AND METHODS

Materials

The phosphatidylcholines were synthesized as described before[13]. The phospholipids were dissolved in chloroform and aliquots were evaporated to dryness. 1 ml of water was added to about 10 mg of phospholipid and liposomes were formed by shaking the preparation above the transition temperature. The lipid dispersion was incubated for at least 1 h at the desired temperature. When this temperature was lower than 0 °C ethyleneglycol was added to the dispersion to prevent ice formation. Equimolarly mixed liposomes were obtained by first dissolving the two components in chloroform and evaporation of the mixture to dryness. 1 ml of water was added to 10 mg of the phospholipids. Liposomes were prepared by dispersing the material above the transition temperature of the highest melting compound.

Specimens containing 25% water were prepared by adding the required amount of water to the dry material and equilibration in a closed vessel for 24 h at a temperature below the transition temperature.

Electron microscopy–freeze–fracturing

Samples were transferred to specimen holders and rapidly quenched from the desired temperature. Specimens were fractured in a Denton machine at −196 °C while the shroud surrounding the specimen was also kept at this temperature. Replicas were floated off on water and cleaned with a hypochlorite solution (2% active chlorine). Replicas of specimens containing 25% water were stripped off at a toluene–water interface. The toluene was removed by evaporation, the replicas transferred to

water and cleaned with hypochlorite. Electron micrographs were made on a Siemens Elmiskop I A and a Philips EM 200.

For comparison with X-ray data the periodicity of the band-patterns was measured in specimen regions that, according to focus conditions together with the uniform appearance of the shadow distribution, are considered to be oriented approximately normal to the optical axis.

Small variations in orientation of the specimen with respect to the optical axis may still be present, which, depending on the orientation of the local tilting axis with respect to the direction of the band pattern, may result in small variations in the measured periodicities. It has been argued previously[9] that the maximum value found will be close to the real periodicity.

Differential scanning calorimetry

The differential scanning calorimetric measurements were carried out as described by de Kruyff *et al.*[16].

RESULTS

Samples of (dimyristoyl)-lecithin (transition temperature approx. 30 °C) containing 25% water were quenched from +5 °C. The fracture faces of this specimen display a band pattern (crystallization structure) with a periodicity lying in a range from 120–170 Å (Fig. 1A). This is a value close to that found by X-ray diffraction[10] for the undulation periodicity in specimens containing 20 and 25% water *viz.* 144 and 175 Å. Such a crystallization pattern, although with a different periodicity (233 and 117 Å) is also found with liposomes prepared from the same lipid[8,9].

Because we wanted to know whether the occurrence of this structure is characteristic for phosphatidylcholine below the transition temperature, we studied phosphatidylcholines different to those previously investigated, *i.e.* (dimyristoyl)-, dielaidoyl)- and (1-oleoyl-2-stearoyl)-lecithin[8,9]. We found that the saturated homologues (dilauroyl)-, (dipalmitoyl)-, and (distearoyl)-lecithin all exhibit fracture faces with band patterns when quenched from below their transition temperature. (Dilauroyl)-lecithin (transition temperature 0 °C) has a band periodicity of about 180 Å and one of 90 Å (Fig. 1B). In (dipalmitoyl)-lecithin (transition temperature +41 °C) we found many areas with a band periodicity of about 150 Å (Fig. 1C). In other regions we often observed patterns in which the band periodicity varies from 250–400 Å. Another typical feature of (dipalmitoyl)-lecithin preparations was the presence of regions in which the bands do not run parallel. The band patterns of (distearoyl)-lecithin (transition temperature +58 °C) are arranged in a rather angular way (Fig. 1D). Many parts of the replica exhibit a band pattern of about 150 Å and others show arrays of bands with varying spacings (300–500 Å). In liposomes of (dioleoyl)-lecithin (transition temperature −20 °C) we could not detect crystallization structures even when the material was quenched from −40 °C.

As an example of an intramolecularly mixed lipid next to (1-oleoyl-2-stearoyl)-lecithin we studied (1-palmitoyl-2-oleoyl)-lecithin (transition temperature about 0 °C[14]). Liposomes of this lipid also show a crystallization structure below the transition temperature with a single periodicity of about 150 Å (Fig. 1E).

Because biomembranes contain a complex lipid mixture composed of many

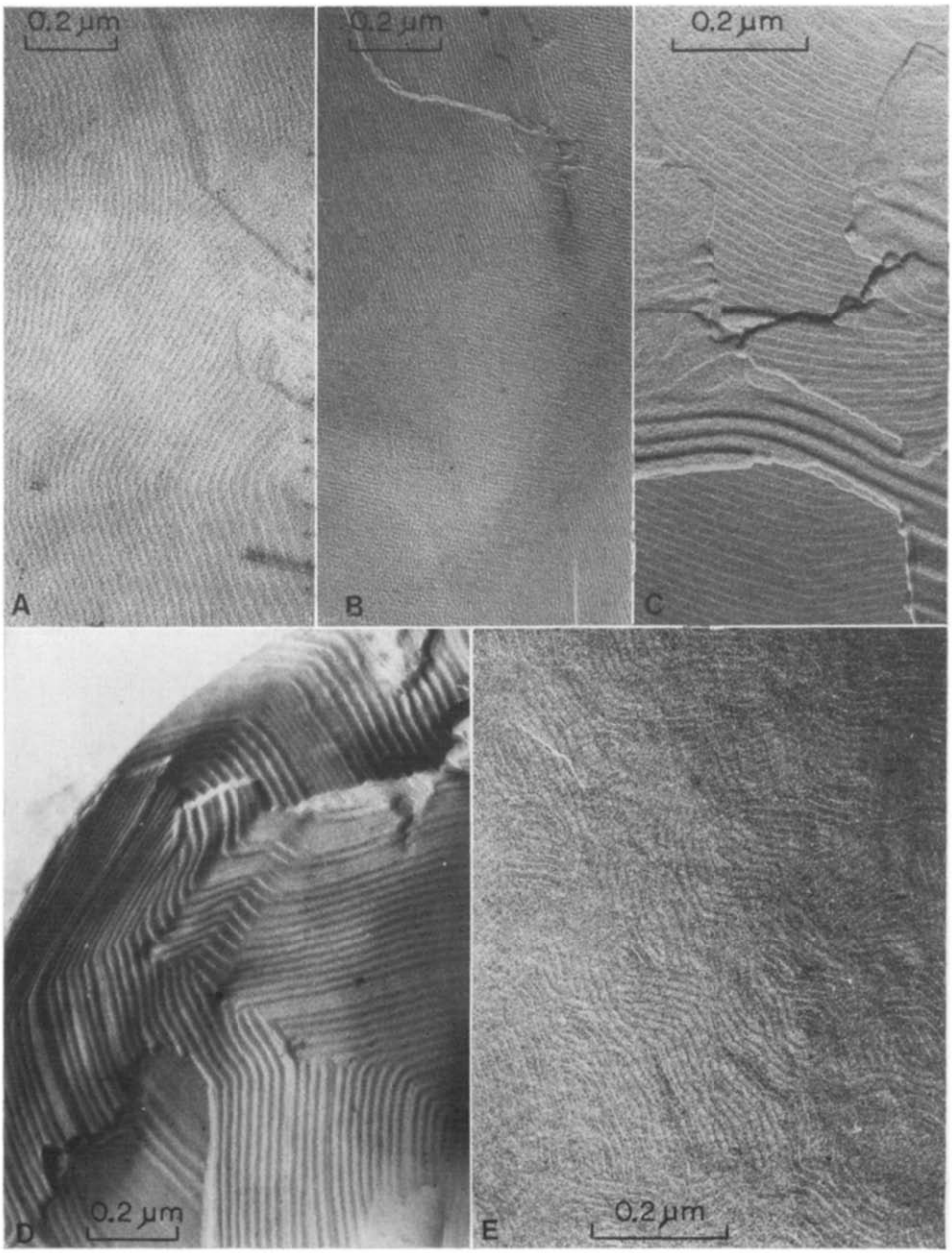

Fig. 1. (A) Fracture face of (dimyristoyl)-phosphatidylcholine containing 25% (w/w) water. Quenched from +5 °C. (B–E) 1% (w/v) lecithin dispersions quenched from below their transition temperatures. B, (Dilauroyl)-phosphatidylcholine at −10 °C; C, (dipalmitoyl)-phosphatidylcholine at +5 °C; D, (distearoyl)-phosphatidylcholine at +5 °C; E, (1-palmitoyl-2-oleoyl)-phosphatidylcholine at −10 °C.

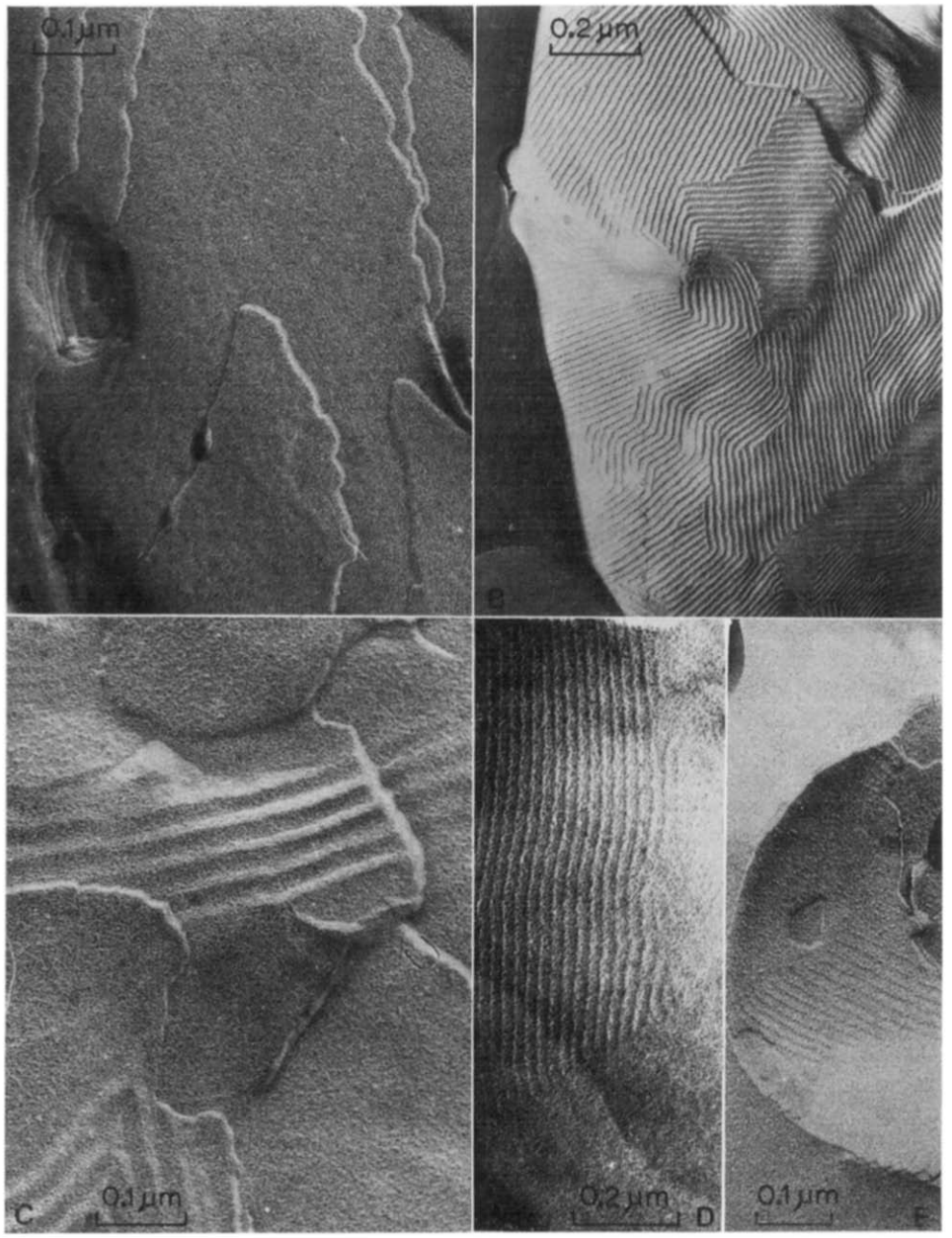

Fig. 2. (A and B) Mixed preparation consisting of equimolar amounts of (dilauroyl)-, and (dimyristoyl)-phosphatidylcholine. Quenched from +5 °C (A) and −10 °C (B). (C) Mixed preparation consisting of equimolar amounts of (distearoyl)- and (dioleoyl)-phosphatidylcholine. Quenched from +5 °C. (D and E) Liposomes from an equimolar mixture of (dipalmitoyl)- and (1-palmitoyl-2-oleoyl)-phosphatidylcholine. Quenched from +25 °C.

molecular species it is important to understand the mode of mixing of such molecules. Therefore we investigated the crystallization of intermolecularly mixed liposomes. We decided to study systems that are defined to a certain extent by differential scanning calorimetry[12,14]. For an example of cocrystallization in our freeze-fracture study we have chosen liposomes prepared from an equimolar mixture of (dilauroyl)- and (dimyristoyl)-lecithin. We found upon quenching from a temperature above the transition of this mixture that no band patterns are visible on fracture faces (Fig. 2A). When, however, the material is quenched just below the transition (0–20 °C, Fig. 4A) the fracture faces are covered with a regular band pattern with a spacing of about 180 Å (Fig. 2B). The same phenomenon was found with an equimolar mixture of (dimyristoyl) and (dipalmitoyl)-lecithin. A completely different crystallization behaviour was observed with mixtures of (dimyristoyl)-lecithin or (distearoyl)-lecithin and (dioleoyl)-lecithin. By differential scanning calorimetry two peaks are found in this case[12,14]. When we quenched our material from a temperature in between those peaks the fracture faces were partly covered with a band pattern, but in other areas smooth fracture faces were present (Fig. 2C). The band pattern in the mixture containing (dimyristoyl)-lecithin displayed a periodicity of 233 Å and a subperiod of 117 Å, which was also found in (dimyristoyl)-lecithin liposomes[8,9]. Fracture faces of mixed liposomes containing (distearoyl)-lecithin exhibited a band periodicity of about 400 Å. When we quenched the material from a temperature below the transition temperature of (dioleoyl)-lecithin the same fracturing pattern was still obtained.

It is well known that within the family of molecular species of lecithin from animal membranes, molecules with a combination of one saturated and one unsatu-

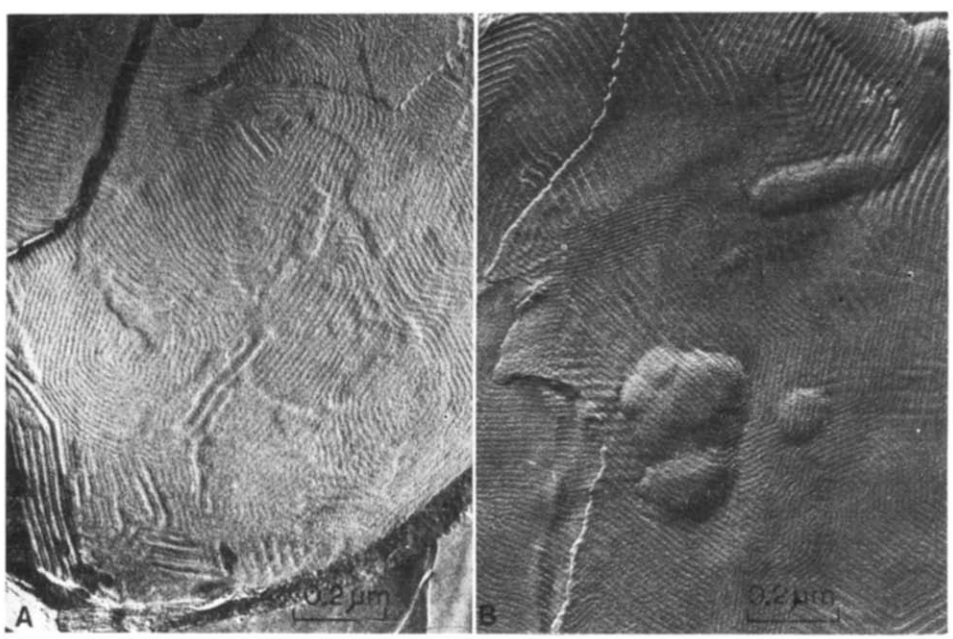

Fig. 3. (A and B) Liposomes from an equimolar mixture of (dipalmitoyl)- and (1-palmitoyl-2-oleoyl)-phosphatidylcholine. Quenched from −10 °C.

rated fatty acyl chain are dominant[4]. An example of such a species is (1-palmitoyl-2-oleoyl)-lecithin. Liposomes of this lipid alone show a crystallization structure below the transition temperature. An equimolar mixture of this compound with (dipalmitoyl)-lecithin displays two thermotropic peaks by differential scanning calorimetry (Fig. 4B), namely between 0 and 40 °C. When we quenched liposomes of this mixture from +25 °C, we found in the replicas band patterns along with smooth fracture faces (Figs 2D, 2E). Quenching from −10 °C resulted in fracture faces that are all covered with band patterns (Figs 3A, 3B). We detected periodicities of 150 Å and also larger ones from 250–400 Å. When, however, a more complicated mixture containing (1-palmitoyl-2-oleoyl)-lecithin is quenched from below its transition, band patterns are no longer observed. This is demonstrated with egg lecithin. According to Ladbrooke and Chapman[12], the transition of this mixture (between −5 and −15 °C) is principally that of (1-palmitoyl-2-oleoyl)-lecithin, which constitutes 60% of the lipid. In freeze–etch replicas we could not find any difference between liposomes quenched from above and those quenched from below the transition.

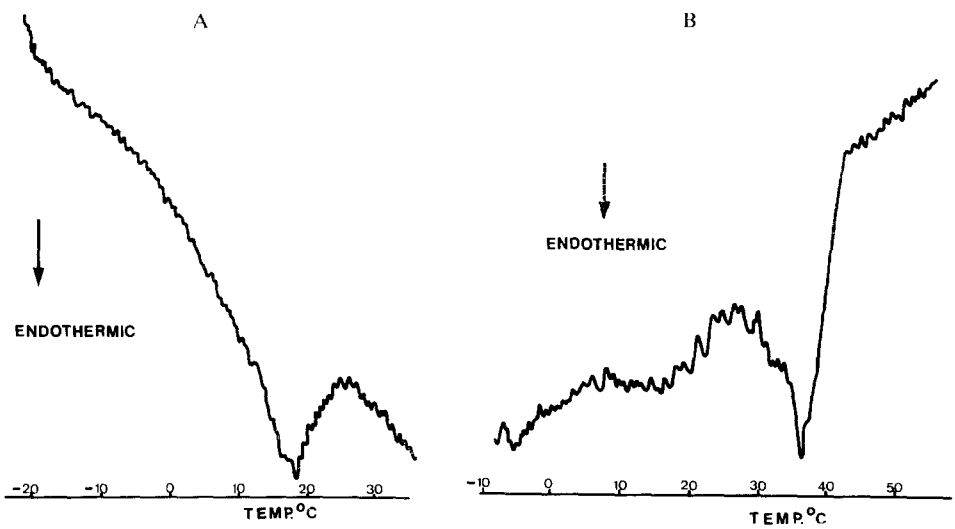

Fig. 4. (A) Differential scanning calorimetric curve for an equimolar mixture of (dilauroyl)- and (dimyristoyl)-phosphatidylcholine. (B) Differential scanning calorimetric curve for an equimolar mixture of (dipalmitoyl)- and (1-palmitoyl-2-oleoyl)-phosphatidylcholine.

DISCUSSION

In a previous paper it was concluded that some phosphatidylcholines show band patterns below the transition temperature[8]. It was suggested that the bilayers under these conditions are undulated[9]. The same structure was proposed by Tardieu[10] for two phosphatidylcholines containing 25% water and has been called the Pβ' phase. Our freeze–etch results demonstrate that in such a bulk preparation a band pattern is also visible; the periodicity appears to be close to that found by X-ray diffraction. The difference in periodicity between bulk and liposomal preparations could perhaps be related to the typical onion structure of the liposomes in comparison with the bulk preparation, which consist of infinitely extended stacks of lamellae.

It is known from X-ray studies[10,15] that three crystalline conformations of the lipid bilayers are possible. One is the $L\beta$ structure in which the fatty acid chains are rigid and perpendicular to the bilayer plane. This structure is proposed for the crystalline biological membranes.

Another is the $L\beta'$ structure in which the acyl chains are rigid but tilted with respect to the bilayer plane. This structure is suggested for (dipalmitoyl)-lecithin and (distearoyl)-lecithin. The third possible conformation is the $P\beta'$ structure that can be considered as an undulated $L\beta'$ structure.

We have shown that this $P\beta'$ structure is not confined to only a few species of phosphatidylcholine. Band patterns were found in the saturated species, (dilauroyl)-(dimyristoyl)[8,9]-, (dipalmitoyl)-, and (distearoyl)-lecithin and in the unsaturated species, (dielaidoyl)-lecithin[8]. We were unable to find this structure in liposomes of (dioleoyl)-lecithin even at a temperature as low as -40 °C (transition temperature -20 °C). Furthermore, two intramolecular mixed lecithins were found to display a $P\beta'$ structure: (1-oleoyl-2-stearoyl)-lecithin[8] and (1-palmitoyl-2-oleoyl)-lecithin.

These results provoke questions with respect to the explanation of this peculiar crystallization. Finean and Millington[17] and, recently, Philips *et al.*[18] concluded from X-ray and nuclear resonance studies that the polar headgroup of lecithin is perpendicular to the bilayer plane. Such a conformation with mutually repelling dipoles will be susceptible to small modifications in the apolar part. X-ray studies[10,12,15] on thermal polymorphism of phospholipids have shown that the acyl chains of the saturated homologues of lecithin and phosphatidylethanolamine become condensed and tilted when the lipids are cooled below the transition. This reorientation of the fatty acid chains will perturbate the balance between the repulsive forces within the polar layer and the van der Waals attractive forces in the apolar layer. As a consequence, the polar headgroups possibly have to be rearranged to obtain the most favorable position. We suggest that the undulation of the bilayers can be attributed to this rearrangement of the polar headgroups. The situation is different in phosphatidylethanolamines. The zwitterions of these lipids are arranged tangential to the bilayer plane[18]. Such a conformation contributes to a strong cohesion in the polar region. Therefore a reorientation of the polar groups is less likely to be expected when the acyl chains become tilted upon cooling*.

Contrary to our finding that bilayers of saturated lecithins including (dipalmitoyl)- and (distearoyl)-lecithin are undulated, it was inferred from X-ray data that (dipalmitoyl)- and (distearoyl)-lecithin are in a $L\beta'$ conformation below the transition[10,12]. We think that this inference is due to the absence of a strict periodicity in the undulation as can be seen in freeze–fracture replicas. These show a variety of crystallization features, even on one and the same fracture face.

Apparently, undulations are also present in intermolecularly mixed lecithins. Our results with freeze–etching are generally consistent with conclusions from differential scanning calorimetric experiments. In the case of (dilauroyl)- and (dimyristoyl)-lecithin we only found one regular band pattern at a temperature below the complete transition of the mixture. This is in agreement with the view that when the difference in acyl chain length is no more than two CH_2 groups the lipids tend

* This view is supported by the observation that liposomes of (dipalmitoyl)- and (dioleoyl)-phosphatidylethanolamine display smooth fracture faces below their transition temperatures.

to crystallize simultaneously in one crystalline lattice. Separate crystallization is suggested by electron micrographs showing smooth fracture faces along with band patterns. These were found only when mixtures characterized by two thermotropic peaks, *viz.* (dimyristoyl)- or (distearoyl)-lecithin + (dioleoyl)-lecithin and (dipalmitoyl) + (1-palmitoyl-2-oleoyl)-lecithin, were quenched from a temperature between those peaks.

The question can be raised whether the crystalline areas that give rise to the particular band patterns indeed contain only one phospholipid, as was suggested in a previous paper[9]. The similarity of the band pattern in the mixture and that of the saturated compounds alone indeed suggests that the lipids segregate. Besides, if one supposes that a large amount of the lowest melting lipid remains included in the crystalline region, one would expect a change in periodicity, as was found in the case of cocrystallization. However, this was not observed. Therefore we assume that in the cooled liposomes the two lipids are no longer randomly distributed. At the present state of our investigation we cannot prove conclusively whether a complete segregation has occurred. Quenching from below the complete transition reveals a difference between the mixtures. The mixture containing (dioleoyl)-lecithin still displays smooth fracture faces along with band patterns. This result once more supports the idea that the lipids crystallize separately. On the other hand the mixture with (1-palmitoyl-2-oleoyl)-lecithin now becomes characterized by band patterns on all fracture faces. The periodicity of this band pattern is 150 Å. In this case it is impossible to distinguish separate crystalline areas because each of the phospholipids by itself displays a periodicity of 150 Å. In this respect it would be interesting to find a mixture characterized by two thermotropic peaks and consisting of lecithins, that by themselves show sufficiently different band periodicities to be recognized in adjacent areas on the micrographs.

No band patterns could be demonstrated in egg lecithin. This complex mixture contains a high amount of (1-palmitoyl-2-oleoyl)-lecithin. Contrary to the result with the binary mixture, we found no indication that the bilayers were completely or partly undulated below the transition. This is in agreement with the idea that instead of a $P\beta'$ structure these bilayers have a $L\beta$ conformation[15]. It seems likely that the higher unsaturated compounds in egg lecithin prevent tilting of the acyl chains. The present results stimulate the systematic investigation of other lipids and lipid mixtures by freeze-etching. In this respect it may be recalled that membranes of *Acholeplasma laidlaiwii* and *Escherichia coli* display extended regions with band patterns along with smooth regions. It was suggested that these reflect crystalline lipid areas[8].

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