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PHASE TRANSITIONS OF PHOSPHOLIPID BILAYERS AND MEMBRANES OF *ACHOLEPLASMA LAIDLAWII* B VISUALIZED BY FREEZE FRACTURING ELECTRON MICROSCOPY

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1. By freeze fracturing electron microscopy regular band patterns were visualized on fracture faces of liposomes prepared from dimyristoylphosphatidylcholine, dielaidoylphosphatidylcholine, and 1-oleoyl-2-stearoylphosphatidylcholine below their respective transition temperatures. Above these temperatures only smooth fracture faces were observed.

2. Liposomes of these phospholipids prepared after addition of more than 20 mole % cholesterol exhibited no band patterns below their transition temperatures.

3. Fracture faces of the membrane of *Acholeplasma laidlawii* B (previously denoted as *Mycoplasma laidlawii*) below the transition temperature showed structural details that can be attributed to a redistribution of membrane molecules as a consequence of the solidification of the membrane.

INTRODUCTION

Phase transitions in both artificial phospholipid bilayers and native membranes can be detected by various physical methods. The transition of these membranes from the liquid-crystalline to the gel phase has been studied especially by means of differential scanning calorimetry[1,2,12] and X-ray diffraction[4,5].

For dimyristoylphosphatidylcholine it has been demonstrated that the fracture face of this phospholipid, quenched from +5 °C, exhibits a surface pattern of bands with a periodicity of 233 Å and a subperiod of 117 Å (ref. 10). It is known that at this temperature dimyristoylphosphatidylcholine occurs in the gel state[1]. The crystalline structure has already been characterized by X-ray diffraction[7].

In this paper we show that this morphological phenomenon of dimyristoylphosphatidylcholine is related to a transition from the liquid-crystalline to the gel phase, and that this is also the case with other phosphatidylcholines. Using the freeze-etching technique we have also investigated the membranes of *Acholeplasma laidlawii* B above and below the transition temperature, because it is known that these membranes are liable to similar phase transitions.

MATERIALS AND METHODS

Materials

Dimyristoylphosphatidylcholine, dielaidoylphosphatidylcholine and 1-oleoyl-2-stearoylphosphatidylcholine were synthesized as described before[8]. 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 lamellar structures were formed by shaking the mixture above the transition temperatures. The lipid–water mixture was incubated for 30 min at the desired temperature.

Organism and growth conditions

Acholeplasma laidlawii strain B was grown in 100-ml quantities of lipid-poor medium[9] containing 1 ml penicillin (100000 units/ml) per l of culture. Fatty acids were added to the growth medium as ethanolic solutions, giving a final concentration in the medium of 0.06 mM. After incubation at the experimental temperature the cells were concentrated by centrifugation for 15 min at 12000 × *g* at the same temperature

Electron microscopy — Freeze etching

Samples of liposomes and cells were transferred to specimen holders and rapidly quenched, starting from the incubation temperature, in a mixture of solid and liquid nitrogen as described by Sjöstrand[11]. In our experiments we used the Denton freeze-etch apparatus. The temperature control was calibrated by means of a small thermocouple frozen in ice on the specimen stage. The specimen was kept at − 150 °C without etching and at − 100 °C with etching, while the shroud surrounding the specimen in both cases was maintained at − 196 °C. 1 min after the fracturing operation the specimen was shadowed with Pt–C. The replicas were floated off on a cleansing hypochlorite solution and washed with distilled water. Electron micrographs of the replicas were made with a Philips EM 200 and a Siemens Elmiskop IA electron microscope.

RESULTS

In order to confirm that the peculiar band pattern found with liposomes of dimyristoylphosphatidylcholine is related to the gel state of this phospholipid and that this phenomenon is not characteristic for this phospholipid alone, we investigated, in addition to dimyristoylphosphatidylcholine, both dielaidoylphosphatidylcholine and 1-oleoyl-2-stearoyl phosphatidylcholine above and below their transition temperatures. The transition temperatures of these three lecithins have been determined by differential scanning calorimetry[1,12,13,20]. All these phospholipids gave band patterns (crystallisation patterns) below their transition temperatures, only the periodicity of the bands being different. Dimyristoylphosphatidylcholine gives a periodicity of 233 Å and a subperiod of 177 Å (ref. 10), dielaidoylphosphatidylcholine a periodicity of about 160 Å, and 1-oleoyl-2-stearoylphosphatidylcholine a periodicity of about 500 Å below the transition temperatures (Fig. 1a, b and c). If the lipid–water mixture, prepared above the transition temperature, was rapidly quenched from above the transition temperature (+ 40 °C), the fracture faces appeared to be smooth (Fig. 1d).

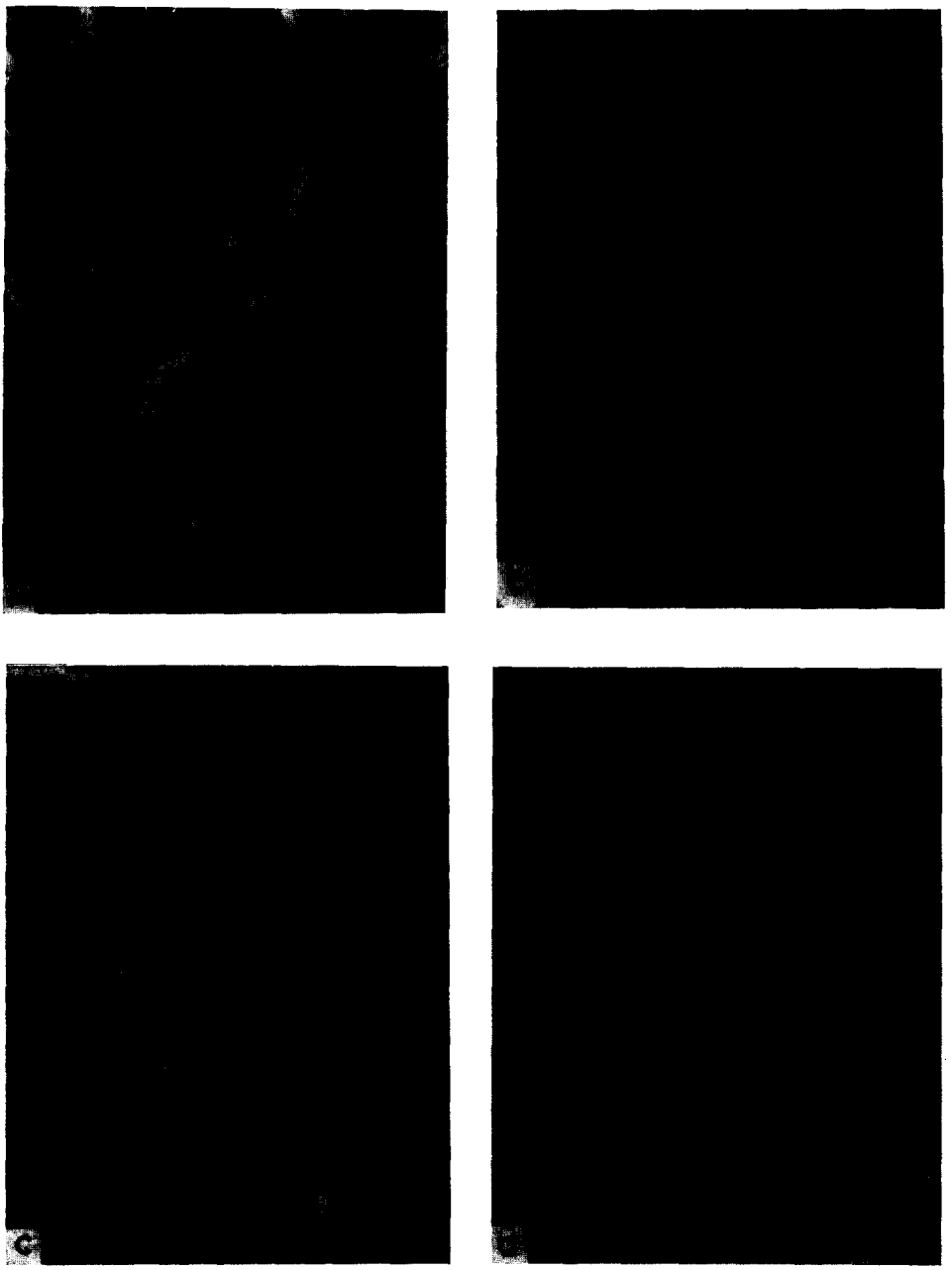

Fig. 1. a, b, c. Fracture faces of liposomes prepared from phospholipids dispersed in water. These liposomes have been quenched below their respective transition temperatures. a, Dimyristoylphosphatidylcholine at + 5 °C; b, dielaidoylphosphatidylcholine at + 5 °C; c, 1-oleoyl-2-stearoylphosphatidylcholine at − 5 °C. d. This micrograph is characteristic for fracturing faces of liposomes prepared from these three phosphatidylcholines above their respective transition temperatures. This micrograph is also characteristic for liposomes prepared from a mixture of each of these three phosphatidylcholines with 20 mole % cholesterol. Magnification, 60000 × .

Cholesterol can affect the fracture pattern obtained from specimens below the transition temperature. We investigated liposomes prepared from a mixture of dimyristoylphosphatidylcholine with 5, 10, 20 and 40 mole % cholesterol. Above the transition temperature the fracture faces consistently appeared to be smooth, but below the transition temperature the bands became less clear with increasing cholesterol concentration and disappeared when more than 20 mole % of cholesterol was present. The other two lecithins when mixed with 20 mole % cholesterol also displayed smooth fracturing faces below their transition temperatures.

In addition we studied cell membranes of *Acholeplasma laidlawii* B, an organism that cannot synthesize and does not require cholesterol[14,16]. The fatty acid composition of the membrane lipids of this organism can be varied by supplementing the growth medium with the desired fatty acids[15]. Thus membranes with different transition temperatures can be obtained. We have grown cells with elaidic acid, myristic acid or oleic acid. Differential scanning calorimetric analysis has shown that the membranes of oleate-grown cells can be considered to be completely in the liquid-crystalline state at the growth temperature of 37 °C. The membranes of cells grown on elaidic acid are in the transition between the liquid-crystalline and the gel state[12]. Steim[2] showed that membranes of cells grown on unsupplemented tryptose medium are also between the liquid-crystalline and the gel state at 37 °C. Since the fatty acid composition of membrane lipids of cells grown on myristic acid and cells grown on unsupplemented tryptose medium is similar (B. van Golden, unpublished), we can conclude that the transition of cells grown on myristic acid and unsupplemented tryptose medium is the same.

The membranes of cells grown on oleate, elaidate or myristate gave identical fracture faces when rapidly quenched from 37 °C (Fig. $2a_1$ and a_2). A smooth background with a lace-like distribution of particles was observed, in accordance with findings by other authors[17,18]. When the culture was chilled to 5 °C during 30 min, centrifuged at the same temperature and then rapidly quenched in the freezing medium, the membranes of the oleate-grown cells exhibited the same pattern as when quenched from 37 °C, but the fracture faces of the membranes of the elaidate- or myristate-grown cells were completely different (Fig. $2b_1$ and b_2). In the last two cases the particles appeared to be aggregated, as Tourtelotte *et al.*[17] found with cells grown on stearic acid, and, together with this phenomenon, ridges (R arrows) could be observed on the inner (convex) fracture face of the membrane only and fissures (F arrows) on the outer (concave) fracture face only.

DISCUSSION

The liquid-crystalline to gel phase transition of phospholipid–water mixtures can be detected by X-ray diffraction[5] and differential scanning calorimetry[1,12,20]. This transition is affected by the length and the degree of unsaturation of the hydrocarbon chains of the phospholipid. Cholesterol disturbs the packing of the hydrocarbon chains and causes a reduction of the transition energy[1,12,19]. Intramolecular mixing of two different hydrocarbon chains in one phospholipid molecule exhibits one sharp transition[6]. Intermolecular mixing of two phospholipids the transition temperatures of which are far separated from each other give rise to a broad transition temperature region. The possible formation of clusters of the phospholipid species

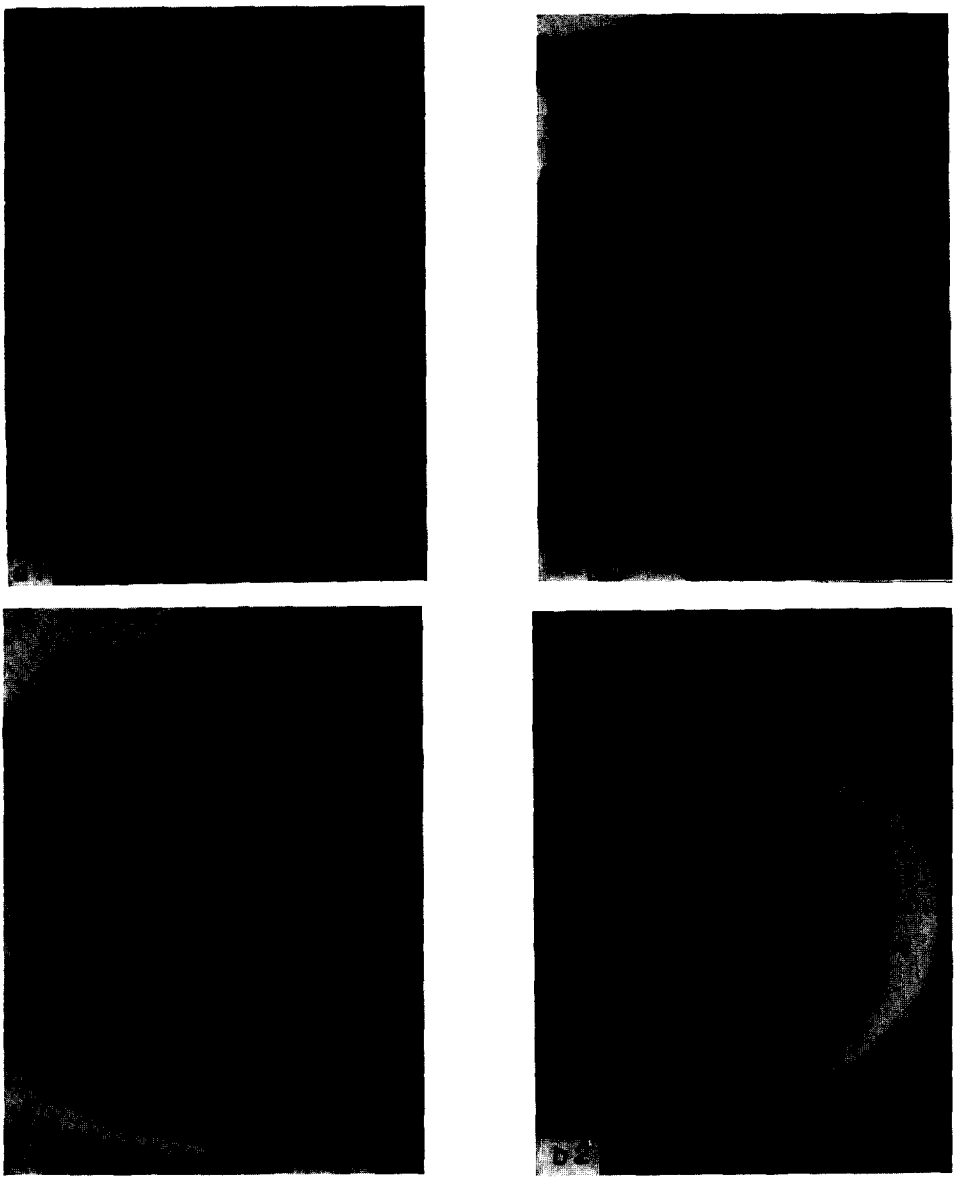

Fig. 2. Fracture faces of *Acholeplasma laidlawii* B membranes. a1, a2. These micrographs are characteristic for membranes at growth temperature. No differences are observed between the membranes of cells grown on oleate, myristate or elaidate. b1, b2, These micrographs are characteristic for membranes below the transition temperature. Membranes of cells grown on elaidate or myristate are quenched starting from + 5 °C. R, ridges; F, fissures; OFF, outer (concave) fracture face of the membrane; IFF, inner (convex) fracture face of the membrane. Magnification, 100000 × .

below the transition temperature was suggested as an explanation of this phenomenon[20].

In this article we showed that the liquid-crystalline to gel phase transition

of three phosphatidylcholines can be visualized by freeze etching. For dipalmitoyl-lecithin Pinta da Silva[22] found a regular band pattern also below the transition temperature, but when this phospholipid was quenched from above the transition temperature no smooth fracture faces were observed. We obtained similar results when the quenching speed was too low. We suggest that the quenching speed during the transition area was insufficient to preserve the phase above the transition temperature.

The liquefying effect of cholesterol could be confirmed, because no band patterns were found when the phospholipid–water mixture was quenched from any temperature below the transition of the phospholipid. Ververgaert *et al.*[10] already showed by freeze etching that a mixture of dioleoyl- and dimyristoyl-phosphatidylcholine, when rapidly quenched from +5 °C (that is, below the transition of dimyristoyl- and above the transition of dioleoylphosphatidylcholine), displays smooth areas alternating with areas showing band patterns. We have to realize that these lipid–water mixtures are rather poor membrane model systems, in view of the fact that divalent cations and proteins may have important consequences with regard to the lipid phase transition. Still, there are remarkable similarities with biological membranes. Reversible phase transitions of the cell membrane of *Acholeplasma laidlawii* B can be detected by X-ray diffraction[4] and differential scanning calorimetry[2,3,12]. It is interesting that the transition in the membrane occurs at about the same temperature as in the isolated lipids[2]*.

When the freeze fracturing technique is used the difference between the membranes of *Acholeplasma laidlawii* above and below the transition temperature is very radical. The two striking phenomena observed below the transition temperature, *viz.* the aggregation of the particles and the presence of ridges on the inner fracture face which are accompanied by fissures on the outer fracture face, are not yet completely understood. One explanation may be that cooling a membrane to a point where solidification of the hydrocarbon chains starts results in squeezing out the protein groups that have penetrated into the hydrocarbon layer. The particle-free areas of the cell membrane micrographs are then to be considered as lipid bilayers. (During freeze etching the membranes split along the interface of the hydrocarbon chains[21].) The ridges and fissures are perhaps comparable with the bands of the artificial phospholipid bilayers. In the biological membrane there is a complex mixture of lipids, with many different polar headgroups, so that the pattern of bands (ridges) understandably is not as regular as in liposomes.

In order to establish whether the liquid-crystalline to gel phase transitions described in this article are of a universal character, other biological membranes will be subjected to further investigation**.

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* At present we have not been able to form liposomes from lipids extracted from *Acholeplasma* membranes.

** Preliminary results show that also the cell membrane of *Escherichia coli* exhibits aggregation of particles and band patterns below the transition temperature.

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