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Electrostatically induced growth of spiral lipid domains in the presence of cholesterol

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Abstract. The formation of crystalline domains of the phospholipid L-α-dimyristoyl-phosphatidic acid containing 1 mol% cholesterol, was studied as a function of head group charge by fluorescence microscopy with monolayers at the air/water interface. It is shown that the usual dendritic growth occurs at low pH (8), whereas spiral domains are formed at high pH (11), where the head group contains two negative charges. The findings are ascribed to an electrostatically induced chain tilt that, in conjunction with an in-plane dipole moment, causes a ferroelectric state. This allows for domain aggregation and orientation originating in elongated domains that, additionally, are bent because of the chirality of the molecules. The structure is stabilized and further elongated due to the anisotropic edge activity of cholesterol.

Key words: Lipid monolayers, domaine structure, fluorescence microscopy, electrostatics, cholesterol

I. Introduction

Studies of cholesterol/lipid interactions are of the utmost importance for obvious physiological reasons. Such studies often concentrate on the phase behavior of cholesterol/lipid mixtures, and in this context monolayer studies are of interest, as this particular model system provides the advantage that many variables such as surface pressure, film physical state and ionic milieu can be independently varied. Fluorescence microscopy with monomolecular films at the air/water interface has proven to be a valuable tool in the characterization of phase separations and in the study of the formation of crystalline lipid domains (Lösche et al. 1983; Peters and Beck 1983).

Recent studies, performed with monolayers of dipalmitoylphosphatidyl-choline (DPPC) containing about 2 mol% cholesterol, showed very peculiar spiral crystalline domains (Weis and McConnell 1985; McConnell et al. 1986). This phenomenon indicates a possible role for cholesterol as being edge active and thus elongating the boundary of the solid domains. These studies elegantly considered the situation at chemical equilibrium, but did not answer questions about the molecular origin of its crystal asymmetry or the sequence of aggregation steps required to form the spiral patterns. In order to shed light on this peculiar behavior of cholesterol, we carried out further investigations. We found experiments with other lipids were less helpful and the formation of spiral domains with DPPC appeared to be unique. Our own prior fluorescence microscopy studies with dimyristoylphosphatidic acid (DMPA) at pH 6 showed roughly identical solid domains in the presence and absence of cholesterol (Lösche 1986), although the percentage of dendritic particles did appear to increase with increasing cholesterol content. Also electron microscopic measurements with cholesterol in DMPA or dimyristoylphosphatidylethanolamine (DMPE) (Fischer and Sackmann 1986) showed no snakelike domains but rather the familiar clover-leaf or coffee-bean type dendrites. Hence, the findings with DPPC may be due to the large choline head group. However, the effective area of the polar head group can be changed for example with DMPA, by changing the pH or the ionic strength and thus changing the head group charge between zero and two (Helm et al. 1986). Taking this approach, the present study shows that it is the head group size and, as a consequence, the symmetry of crystalline domains, that is responsible for a given domain shape.

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II. Experimental

The fluorescence microscope and film balance have been described elsewhere (Lösche and Möhwald 1984). Fluorescence excitation is achieved via a water immersion objective in the bottom of a Langmuir trough, emission is imaged on a SIT TV camera and textures are photographed from a TV screen. The lateral resolution is better than 2 μ, and the sensitivity of the optical set up is sufficient to measure textures with dye concentrations as low as 0.1 mol%. These differing textures are observed, because the dye used, DP-NBD-PE (dipalmitoyl-nitrobenzoxadiazolphosphatidylethanolamine) (Avanti Polar Lipids Inc.) is soluble in the fluid phase, but not in the solid domains. The phospholipids L-α-dimyristoylphosphatidic acid (DMPA) (Sigma, Taufkirchen, FRG) and L-α-dimyristoylphosphatidylethanolamine (DMPE) were checked for purity chromatographically. Dehydroergosterol (DHE) was prepared by D. M. Balthaser in Buffalo, New York. The purity of the DHE was approximately 96 mol% but the major impurity was ergosterol. Cholesterol was purchased from Fluka AG Buchs, Switzerland, and cholesterol antracene-carboxylate (cholanthracene) from Molecular Probes Inc., Junction City, Oregon.

The water used was distilled and filtered using a Milli-Q system. pH and ionic strength were established using NaOH and NaCl (Fluka, Buchs, CH. pA grade), respectively. Contamination by divalent ions was prevented by using 10^{-5} M EDTA (ethylenediamine tetraacetic acid, sodium salt, Sigma) in the subphase.

The phospholipid/cholesterol/dye mixtures were spread from a chloroform/methanol (3 : 1) mixed solvent. Varying the dye content between 0.3 and 1.8 mol% showed no influence on the observed textures.

Data analysis was performed using a graphic table and an Apple P.C.

III. Experimental results and analysis

Figure 1 shows typical fluorescence micrographs for a DMPA monolayer at pH 11.4 containing 1 mol% of cholesterol at various surface pressures just above that corresponding to the onset of the fluid/solid phase transition. Increasing the pressure, one observes the onset of nucleation, then crystal growth (Fig. 1 a and b) and after that an aggregation of spherical domains to form elongated crescent-moon shaped solids (Fig. 1 b and c). These solids increase in length and decrease in thickness to yield circular or S-shaped domains and finally become spirals. The solid domain shapes do not depend on the film compressional speed as was indicated by varying the latter between 10 and 0.3 Å^2/molecule/min. The surface texture is also reversible, i.e. on re-expanding the domain width increases again. The number of nuclei and the thickness of the spirals are, within a factor of two, dependent on both aging and material purity. This can be observed from Fig. 2 where the crystallization process was performed under virtually identical conditions. Here we found fewer but larger domains, however qualitatively very similar structures were observed. Spiral formation was observed for DHE but was not observed for cholanthracene. We have not been able to study cholesterol partitioning between solid and fluid phase using fluorescence microscopy since DHE, which we initially used in an attempt to study cholesterol's distribution via lateral fluorescence profiles, exhibited too weak a quantum yield, while cholanthracene is obviously a poor substitute for cholesterol.

The cholesterol concentration dependence will be reported in detail in a separate publication. Here we will only state that spirals were observed with DMPA at pH 11.4 for a cholesterol content between 0.5 mol% and 15 mol% with the number of spirals increasing and their thickness decreasing with increasing cholesterol content.

In order to understand spiral formation it is essential to take into account the variation of DMPA head group charge. We had previously shown that when working with Millipore water of low ionic strength, the degree of dissociation of the head group is below 0.2, whereas at high ionic strength and pH 5.5 it is unity. Varying the pH between 5.5 and 8 and the ionic strength between 10^{-4} M and 10^{-1} M we did not observe any spiral formation. The only difference from the situation that exists in the absence of cholesterol was an increase in the length of the boundary lines between fluid and condensed phases. Increasing the pH above 8 leads to dissociation of the second proton (Jähnig et al. 1979). It is in this case, where the head group exhibits a doubly negative charge, that spirals are observed. We found spirals were formed when either DHE or cholesterol concentrations were varied between 0.5 mol% and 15 mol%: for pH 8 we did not find any concentration window where spiral formation could be detected. The only noticable influence of cholesterol, especially at high concentration, was a significant increase in the dendritic character of the condensed phase i.e. an increase in the solid/fluid boundary, accompanied by an increase in the density of critical nuclei. Nevertheless at this point we would like to stress that we do not believe electrostatic interactions are *directly* responsible for spiral formation. The discus-

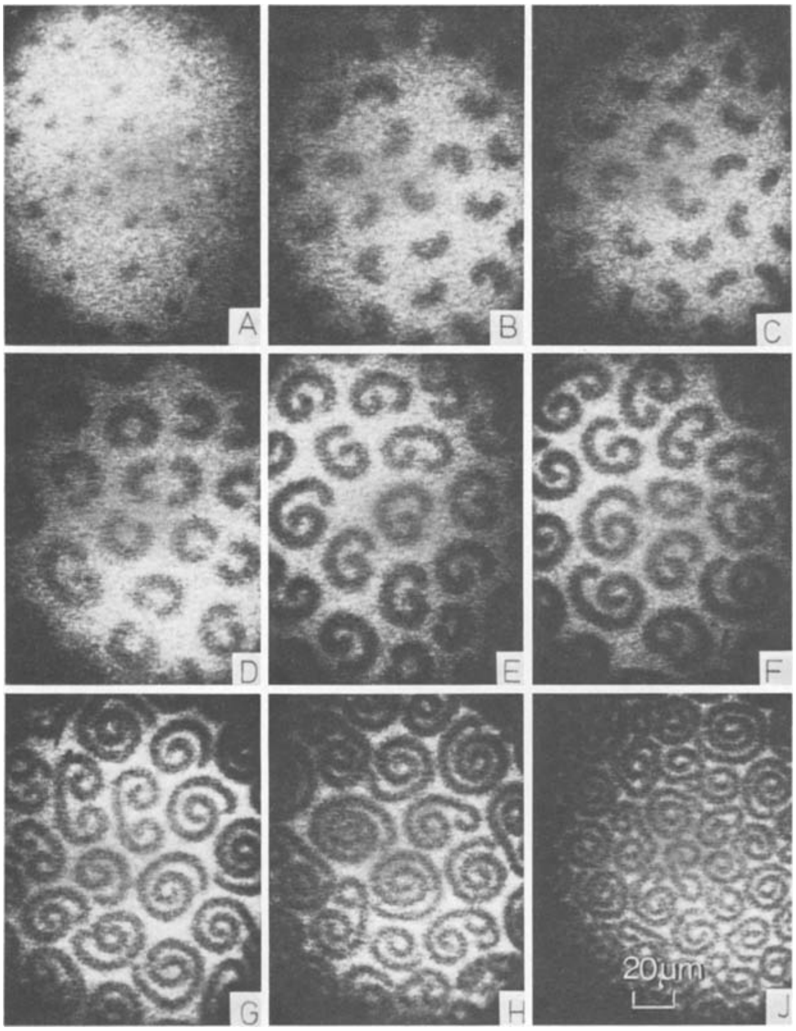

Fig. 1. Fluorescence micrographs of a DMPA monolayer containing 1 mol% of cholesterol for surface pressures increasing from A to J and indicated by arrows in Fig. 3. pH 11.4, $T = 10\,°C$, ionic strength 10^{-3} M

sion that follows will show that action occurs *indirectly* via a tilt of the hydrocarbon chains in the solid phase.

The pressure/area diagram in Fig. 3 shows that the pressure corresponding to the phase transition can clearly be distinguished. From the fluorescence micrographs of Figs. 1 and 2 the fraction of solid lipid Φ can be determined (with the solid regions being dark). Φ as a function of molecular area for $\Phi < 0.4$ can be described by a straight line corresponding to the equation:

$$\Phi = \frac{A_L - A}{A_L - A_S}, \tag{1}$$

where A = mean molecular area and $A_S = 42\,Å^2$, $A_L = 64\,Å^2$ are taken as the molecular areas corresponding to the solid and the fluid phase, respectively. For molecular areas below 52 Å/molecule the fraction of solid to fluid lipid no longer increases with mean density. This shows that the solid, as well as the fluid lipid, is compressible at high pressures.

From the micrographs one can count the number (N) of solid domains and hence $N' = N^*A$, being proportional to the total number of domains, as a function of area A varies as shown in Fig. 4, but dependent on the precise preparation conditions in that changes are more or less abrupt and the absolute numbers can differ by a factor of two. Nevertheless all observations qualitatively agree. N' increases slightly during the initial growth period (I) and then decreases in region II where crescent-moon shaped particles are created (corresponding to Fig. 1 b). On further decreasing the molecular area domains grow in size but the numbers remain almost constant (region III). N' increases again at very high pressures (region IV), apparently due to the breaking up of solid domains.

The shape and anisotropy of the crystalline areas are fairly similar to those reported for DPPC. The

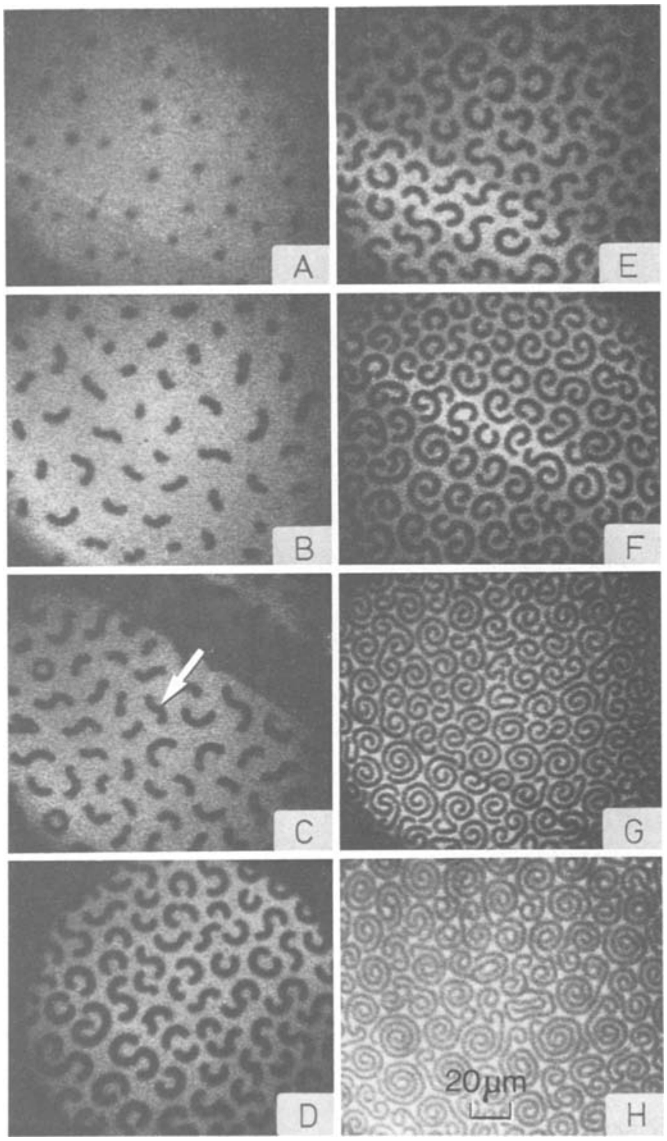

Fig. 2. Fluorescence micrographs of a DMPA monolayer containing 1 mol% of DHE for surface pressures increasing from *A* to *H*, pH 11.4, $T = 10\ ^\circ\mathrm{C}$, ionic strength 10^{-3} M

width W is uniform and decreases with decreasing molecular area (Fig. 4). The length L of a single spiral is less uniform. Its mean value obviously increases so that the anisotropy L/W, also given in Fig. 4, increases by more than a factor of forty during compression.

IV. Discussion

1. Mode of action of cholesterol

The work of Weis and McConnell (1985) and McConnell et al. (1986) has shown that cholesterol is edge active and thus tends to enlarge the fluid/solid boundary lines. These authors also developed an equilibrium model to account for the observed dependence of the spiral width on the degree of crystallization or fraction of solid, Φ. This model also seems to hold for our case of cholesterol in DMPA at pH 11, as a similar dependence of domain shape on Φ is observed. Their assumption of chirality being responsible for the formation of rounded edges also seems reasonable. Yet there is still a lack of understanding of the origin of the various forces on a molecular level and in this respect our work contributes considerably. Being performed with an acidic phospholipid, where intermolecular interactions can be varied to a large extent, we can address the question: Why does cholesterol stabilize different crystal faces selectively as is assumed in the above cited model?

DMPA at high pH has a doubly charged head group which occupies a large area. Thus to increase the attractive forces of the chains the latter are tilted

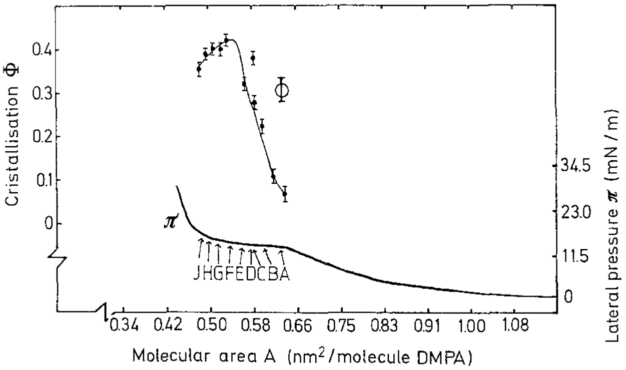

Fig. 3. Surface pressure, π, and fraction of solid area, Φ, as a function of molecular area for the DMPA monolayer of Fig. 1. The *arrows* correspond to the fluorescence micrographs of Fig. 1

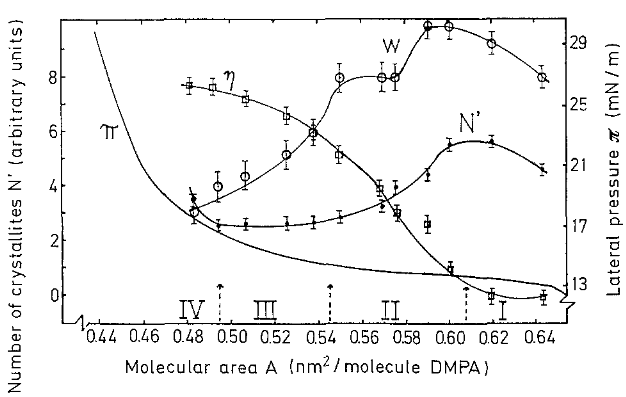

Fig. 4. Number, N', of crystallites (in arbitrary units), domain width W (μm) and anisotropy η $\left(\eta = \frac{2 \cdot \ln \textit{medium length } L}{W}\right)$ as a function of molecular area for the monolayer of Figs. 1 and 3 in the solid/fluid phase coexistence range. For comparison the corresponding part of the pressure/area diagram is included. Regions I to IV are defined in the text according to changes of N'

(Jähnig et al. 1979). In this respect the system resembles DPPC.

Therefore, for both systems, the hexagonal symmetry observed for lipids with small head groups is reduced essentially to zero symmetry and a solid domain preferentially grows in one, and only one, direction. The faces normal to that corresponding to the growth direction are stabilized and thus elongated domains are formed if the chains are tilted. On the other hand, one expects no domain elongation for a zero or very small chain tilt. This is indeed observed by us for cholesterol in DMPA at pH 8 and also for cholesterol in DMPE. Hence it is the chain tilt that is responsible for the formation of elongated domains, and in the present case this formation can be controlled by the ionic milieu.

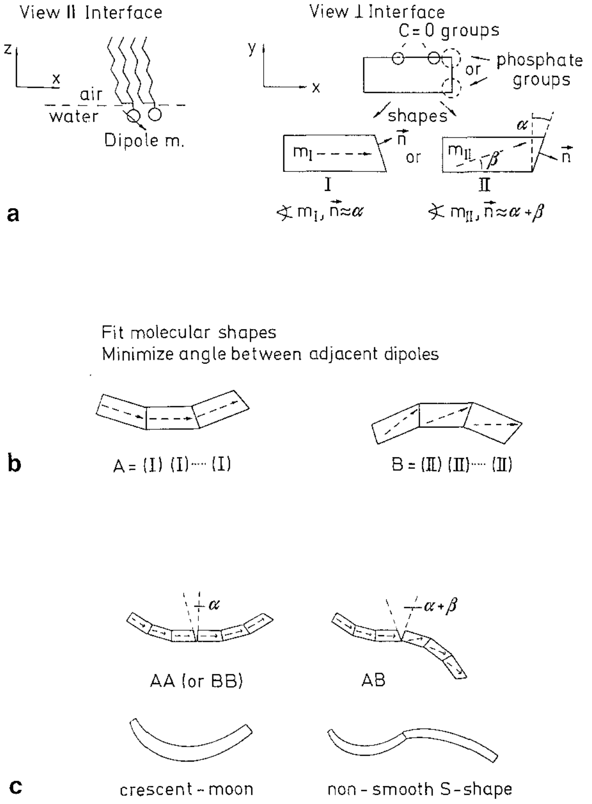

Fig. 5 a – c. Sketch of molecular arrangement and shape, domain formation and aggregation. **a** Molecule and molecular arrangement; **b** crystallization principles; **c** aggregation of crystallites (align dipoles)

2. *Domain aggregation and growth*

It is also reasonable to assume chirality to be responsible for the formation of bent, rather than rectangular, domains (McConnell et al. 1986). This could be due to chiral diffusion (Langer 1985) but may also be due to special molecular interactions based on essential molecular features, as we propose and sketch in Fig. 5. Due to the tilt of the chains and the presence of an asymmetric head group, the arrangement of the molecular cross section, viewed along the membrane normal, is elongated and the crystal grows preferentially in one direction, presumably along the x-axis (Fig. 5 a). The asymmetric electron distribution in the head group region creates a permanent dipole moment with components parallel to the membrane plane. The in-plane moment is oriented nearly along the x-axis but, due to the asymmetric head group arrangement, deviates slightly from this direction. The asymmetrical orientation of the head groups additionally causes a deviation of the molecular cross section from a rectangular to a trapezoidal shape. Due to packing constraints the molecules crystallize with the longest edge on one side of the growth direction yielding structures A and B in Fig. 5 b.

The molecular dipoles are nearly but not exactly parallel and form a non-zero angle with respect to the local growth direction (Fig. 5 b). We assume there are two possible dipole orientations with respect to the molecular axis. At this point one should state that the ferroelectric state is linked to a chain tilt: In the absence of a tilt an antiparallel arrangement of molecular dipoles and thus an antiferroelectric state would probably be favored. Condensation would then result in rounded faces.

Besides explaining this feature, the model accounts for two further observations: *(1)* Whereas usually crystalline domain patterns of phospholipids are governed by strong repulsive forces, due presumably to electrostatic interactions, we observe aggregation of domains in this system. This is most clearly demonstrated by the number N' decreasing with decreasing molecular area in region II of Fig. 4. The attractive interaction necessary for this to happen, could result from an alignment of the dipole moments of two aggregating crystallites (Fig. 5 c). A detailed comparison of electrostatic repulsion and attraction forces will be given elsewhere. The basic result is that, due to the lower dielectric constant near the carbonyl groups attached to the hydrocarbon chains, dipolar attraction may overcome repulsion for special orientations. *(2)* During condensation one observes in Fig. 2 b – g both cresent-moon and S-shaped domains. Their formation is sketched in Fig. 5 c. Assuming them to result from the A and B structures in Fig. 5 b, one realizes that AA structures are easy to merge: the angle between two faces for aligned dipole moments is as small as for any single molecule. This should yield a continually changing slope for the boundary lines. Again assuming aligned dipoles, a continuous slope is not expected for AB structures, as the angle between the faces is larger $(\alpha+\beta)$. One indeed observes discontinuities as sketched in Fig. 5 b for the S-shaped solids (see arrows in Fig. 2 c).

We realize that to account for the S-shape we have to postulate two isomeric lipid forms with different orientations of dipole moments relative to molecular shape. This is at present somewhat speculative and will be studied further by us in future experiments by applying electric fields to lipids.

3. *Spiral formation*

Irrespective for the speculative explanation of the crescent-moon and S-shaped domains one would expect dipolar attraction to attempt to form circular domains. This is obviously not the case and the failure to do so can be ascribed to a repulsion of newly crystallized domain parts from the center of a domain. This repulsion would increase with domain size. Thus, although there is local attraction, the radius of curvature will increase. In favourable cases, where the distance from neighbouring dendrites is still small one can even observe an increasing distance between each spiral arm starting from the inner one, as expected for a logarithmic spiral (Kleine Enzyklopädie Mathematik 1974).

The origin of these repulsive forces has already been discussed (Helm et al. 1986; Fischer et al. 1984; Lösche et al. 1985). It results from the increased charge density of solid compared to fluid domains.

The thinning of domains was already discussed as being due to the reduction in the chemical potential of the fluid phase due to cholesterol enrichment during crystallization (McConnell et al. 1986). Hence, to continue to be in equilibrium, the solid phase has to lengthen the boundary line stabilized by cholesterol.

V. Concluding remarks

An essential feature of the above model is the existence of ferroelectric lipid domains requiring an in-plane dipole moment due to the phospholipid head group. Such a moment is obvious when considering the choline head group where the countercharge of the negative phosphate group protrudes along the plane in the water subphase. Yet, although Raman active ferroelastic modes have been discussed (Jürgens et al. 1986) one may argue that the in-plane component of the dipole moment will compensate due to high mobility in the head group region.

On the other hand for DMPA, having a negatively charged phosphate group but lacking a positive counter charge, the above origin for a dipole moment would not at first seem feasible. Yet large dipolar contributions to the surface potential have been observed and measured for charged lipids (Lakhadar-Ghazal et al. 1983). This demonstrates that an asymmetric charge distribution of the lipid or the surrounding water can exist, even if it is difficult to give a precise molecular interpretation. An interesting point in this regard, may be the idea of McLaughlin (1977) that replacement of ester by ether groups, i.e. removal of 2 carbonyl groups, will change the surface potential (Paltauf et al. 1971) by up to 200 mV. Hence the $C=0$ groups are a major contributor to the overall dipole moment. The in-plane dipole moment cannot yet be measured, but assuming that these groups are mainly responsible for the in-plane dipole moment would be plausible for the following reasons: the $C=0$ groups are asymmetrically arranged with respect to the molecular center of gravity; these groups are immobile in a crystalline domain thus avoiding motional averaging; they constitute the polar parts of the molecule farthest removed from the water surface and thus their electrostatic interactions will be less screened than those of dipoles protruding deeply into the water phase.

We have to point out, however, that we do not expect molecular dipole moments larger than a few Debye. This would be sufficient to allow for a ferromagnetic state but in most cases would not be sufficient to overcome dipolar repulsion due to the molecular dipole moments perpendicular to the surface. We are presently studying this concept of varying dipole moments, but also welcome completely different theoretical approaches (Andelman et al. 1985) minimizing the surface energy under various electrostatic conditions as has been presumed to account for domain formation in two-dimensional magnets (Garel and Doniach 1982).

Finally we also realize (cf. Fig. 3) that the solid area ratio remains constant (0.5) on increasing the pressure above a certain value. This points to the fact that solid lipid domains experience a decrease in size due to compressibility, similar to that observed for fluid domains. For the solid domains, this could be achieved by a decrease in tilt and thus in the lattice constant, but further X-ray and electron diffraction studies will have to be carried out to prove this point.

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