Electron diffraction studies of molecular ordering and orientation in phospholipid monolayer domains

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ABSTRACT The molecular order and orientation of phase separated domains in monolayers of DP(Me)PE and DP(Me)$_2$PE were determined by electron diffraction. Dark and bright fluorescent domains at the air-water interface were observed by fluorescence microscopy. The monolayers were transferred to Formvar coated electron microscope grids for electron diffraction studies. The positions of domains on the marker grids were recorded in fluorescence micrographs, which were used as guide maps to locate these domains in the electron microscope. Selected area electron diffraction patterns were obtained from predetermined areas within and outside the dark domains. Sharp hexagonal diffraction patterns were recorded from dark domains, and diffuse diffraction rings from bright areas in between dark domains. The diffraction results indicated that the dark domains and bright areas were comprised of lipid molecules in solid and fluid states, respectively. The orientation of diffraction patterns from adjacent locations within a dark domains changed gradually, indicating a continuous bending of the molecular packing lattice vector within these domains. Orientation directors in U-shaped DP(Me)$_2$PE domains followed the turn of the arm; no vortex nor branching was indicated by electron diffraction. Directors branching from the "stem" of highly invaginated DP(Me)PE domains usually occurred at twinning angles of $n\pi/3$ from the stem director, which would minimize packing defects in the development of thinner branches. Electron diffraction from local areas of individual domains proved that dark fluorescent domains were solid ones, and that pseudo-long range order existed in these solid domains.

INTRODUCTION

Recent advancement of low light level fluorescence microscopy enables phase separated domains in phospholipid monolayers to be observed and measured (Weis, 1991; Mohwald, 1990; McConnell, 1991). The nature and the factors controlling the formation and shaping of these domains are gradually being understood. A proper understanding of the physical chemistry of domain shaping and formation is essential in the development of monolayer coating technology for various applications. Knowledge of the factors controlling the formation and shaping of these domains may also help us to understand the complex behavior of lipids in bilayers and in biological membranes.

Under certain conditions, phase separated domains in monolayers may grow to tens or even hundreds of micrometers across. The shapes of these domains vary from lipid to lipid, and dependent on the temperature, surface pressure, impurity, speed of compression, and the subphase chemistry of the monolayers. The forces that shape the solid domains have been attributed to the interplay between intradomain molecular alignment and inter-phase line tension (McConnell and Moy, 1988; Keller et al., 1986; Florsheimer and Mohwald, 1989). In general, molecular alignment and dipole-dipole repulsion within a solid domain sets the patterns of domain growth, while the line tension at the domain boundaries tends to minimize the boundary-to-area ratio to favor a discoid geometry, countering the growth of domains in any particular direction.

The molecular arrangements in monolayer domains are subjects of speculation and debate. While x-ray and neutron diffractions offer some average molecular packing information on a large sampling area of monolayers (Mohwald, 1988; Helm et al., 1987; Lin et al., 1990), electron diffraction may, in addition, provide local structural information (Hui et al., 1975; Fischer et al., 1984) within and even along an individual domain. In this report, we present a point by point mapping study of individual solid domains in phospholipid monolayers, by applying the selected area electron diffraction technique. Solid and fluid natures of these domains, as well as the variation of molecular alignment along the domain centers and boundaries, are revealed by this approach.

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MATERIALS AND METHODS

Both L-α-dipalmitoyl-monomethyl-phosphatidylethanolamine [DP(Me)PE] and L-α-dipalmitoyl-dimethyl-phosphatidylethanolamine [DP(Me)$_2$PE] were purchased from Avanti Biochemicals (Birmingham, AL). All lipid samples were dissolved in chloroform at a concentration of 0.5 mg/ml before spreading on a Langmuir trough. A custom-built, environmentally controlled Langmuir trough was maintained within an enclosed chamber with thermostat wall, floor, and ceiling. The chamber was filled and continuously flushed with nitrogen gas. The size of the trough was 22 × 15 cm^2. Surface pressure versus area per molecule of monomolecular films (π-A isotherms) were measured by a π-A recording system. Surface pressure was measured with a Wilhelmy plate and a CAHN RG electric balance. The area was adjusted by a motor-driven Teflon bar moving along the surface of the trough; the movement of the bar is either manual or servo controlled (in the isobaric mode) by the output of the electrobalance. The barrier was driven at a rate of 1–6 mm^2/s during compression or decompression.

Epifluorescence microscopic images were observed by a fluorescent microscope AO-2071 with a 40× (NA = 0.85) objective lens. A Teflon retaining ring with a small opening was attached to the objective lens, and immersed through the air-water interface. It was used to minimize monolayer drift. A Dage-MTI silicon intensified target (SIT) video camera MCP-SIT66 was attached to the microscope. The images were recorded on video tape cassette, and processed by a Datacube Maxvision system.

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Phospholipid monolayers were deposited on Formvar-covered marker grids for fluorescence microscopy and electron diffraction. The Formvar-covered grids were coated with gold on one side, and the coated side was pressed against a small piece of parafilm during monolayer deposition, to ensure only one side (the side with uncoated Formvar film) is exposed to the monolayer. The method for monolayer deposition has been described previously (Hui et al., 1975). It is necessary to deposit the monolayer on the uncoated side since the metal coating quenches the fluorescence label of the monolayer. Successful transfer of a monolayer from the air-water interface to the Formvar film was verified by comparing the fluorescent domain patterns of the air-water interface and that of the deposit on a Formvar film. The domain patterns on selected locations on the marker grids were recorded in photomicrographs, which were used as guide maps for selected-area electron diffraction.

The grids supporting the deposited monolayer were used in low dose electron diffraction experiments as previously described (Hui et al., 1975). Briefly, a 5 μm condenser aperture was used to limit the already low beam current to below 50 $\mu A/cm^2$ at the specimen level to minimize radiation damage. Areas of the marker grid adjacent to selected locations were moved to the beam, and diffraction conditions were set. The selected area was then quickly moved to the beam and the diffraction pattern was immediately recorded on Kodak DEF-5 x-ray film. The sampling area of ~ 10 μm in diameter was defined by the selected area diffraction aperture. The diffraction camera length was set at 1.6 m. The spacings of the lipid reflections were calibrated by the gold diffraction ring as the internal standard.

Over 50 diffraction patterns from dark and bright fluorescent areas were recorded. The vast majority of diffraction patterns recorded from dark areas have hexagonal symmetry. The orientation of the 100 diffraction spots (diffraction directors) are given by the lattice vector of the corresponding reflecting (100) planes. The diffraction director is defined between 0 and $\pi/3$. However, since the rotations of the image and the diffraction pattern in the electron optical system are different, the relative orientation of the diffraction pattern to that of the specimen varies with focusing conditions. Therefore, only the relative diffraction orientation $\Delta\phi$ against an arbitrary reference director, instead of the absolute orientation ϕ, was measured for a same set of data. The relative orientations of different sets of patterns are expressed as $\Delta\phi \pm n\pi/3$, where n is an assigned integer (see Results).

RESULTS

(a) Solid and fluid domains

DP(Me)PE and $DP(Me)_2PE$ monolayers were transferred from the air-water interface to Formvar supports, at 28° and 24°C, respectively, at a surface pressure of 12 dyn/cm. The domain geometry suffered a slight distortion during the transfer. In some areas, dark domains were found to be not as evenly distributed as they were on the air-water interface. However, the $DP(Me)_2PE$ and DP(Me)PE dark domains retained their respective round and branching shapes, as reported previously (Yu and Hui, 1992). The U-shaped domains of $DP(Me)_2PE$ sometimes appeared to close the loop to an O shape, with perhaps an opening too small to be resolved by fluorescence microscopy. The gaps between neighboring branches of DP(Me)PE domains were also found to be narrower than those at the air-water interface.

Two types of diffraction patterns were observed. When the selected diffraction area was greater than 100 μm in diameter, the patterns were either a sharp ring or a diffuse ring. The spacing of the sharp ring was 0.42 nm, while that of the diffuse one centered at $\sim$0.46 nm. If the selected diffraction area was reduced to $\sim$10 nm, the sharp ring became a set of arcs arranged in a hexagonal pattern. The intensities of the six arcs were equal in general, except in a few recordings (<5%) showing a two-fold instead of a six-fold symmetry. The rare occasions suggested that the deviation from the hexagonal symmetry was more of an experimental artefact than a property of the sample. The hexagonal pattern was typically that of a phospholipid monolayer deposit as previously reported (Hui et al., 1975; Hui, 1976). These types of patterns have been classified as hexatic B in liquid crystal terminology (Hui, 1989). If the patterns showed a two-fold instead of a six-fold symmetry, the samples would belong to a family of smectic liquid crystals with tilted molecular chains, or that the sample planes were tilted with respect to the electron beam (Hui, 1976; Hui, 1989).

Our first task was to establish the correspondence of the dark and bright areas in the fluorescence micrographs to the sharp hexagonal and diffuse ring patterns derived from these respective areas. We have examined more than 50 dark domains and neighboring areas in five experiments with DP(Me)PE and $DP(Me)_2PE$ monolayers, and found exact correspondence. A pair of diffraction patterns each from a DP(Me)PE and $DP(Me)_2PE$ monolayer, and their originating areas in the fluorescence micrographs are shown in Fig. 1 as examples. The diffraction patterns shown in Fig. 1 are similar to that reported by Fischer et al. (1984) for phosphatidic-acid monolayer domains which were visualized directly by an electrostatic contrast mechanism in the electron microscope. When the monolayers had been previously exposed to an electron beam radiation of more than 10^{-3} coulomb/cm^2 ($\sim$60 electrons/nm^2 at the specimen level), no hexagonal diffraction pattern could be detected from the dark domains. In such cases, a diffuse diffraction ring was seen in all areas, regardless of fluorescence. We presumed that the loss of pattern was due to the loss of molecular packing order after receiving a damaging radiation dose. The damaging dosage is similar to that previously reported for phospholipid monolayers (Hui, 1980).

(b) Local orientation in $DP(Me)_2PE$ domains

In order to avoid the arbitrary assignment of the $n\pi/3$ value in isolated orientational measurements, initial diffraction studies were from areas positioned closely together along a gradually curving periphery of a solid domains (Fig. 2, spots *a–c*), with the assumption that the change in orientation was gradual within this short distance, so that a common n value applied to all patterns derived from within this region. Indeed the change of orientation was found to be gradual, with a total change of $23 \pm 2°$ from end to end (Fig. 3, *a–c*). If the director at one end is chosen to be one that parallel to the domain boundary, the directors of neighboring areas, as derived

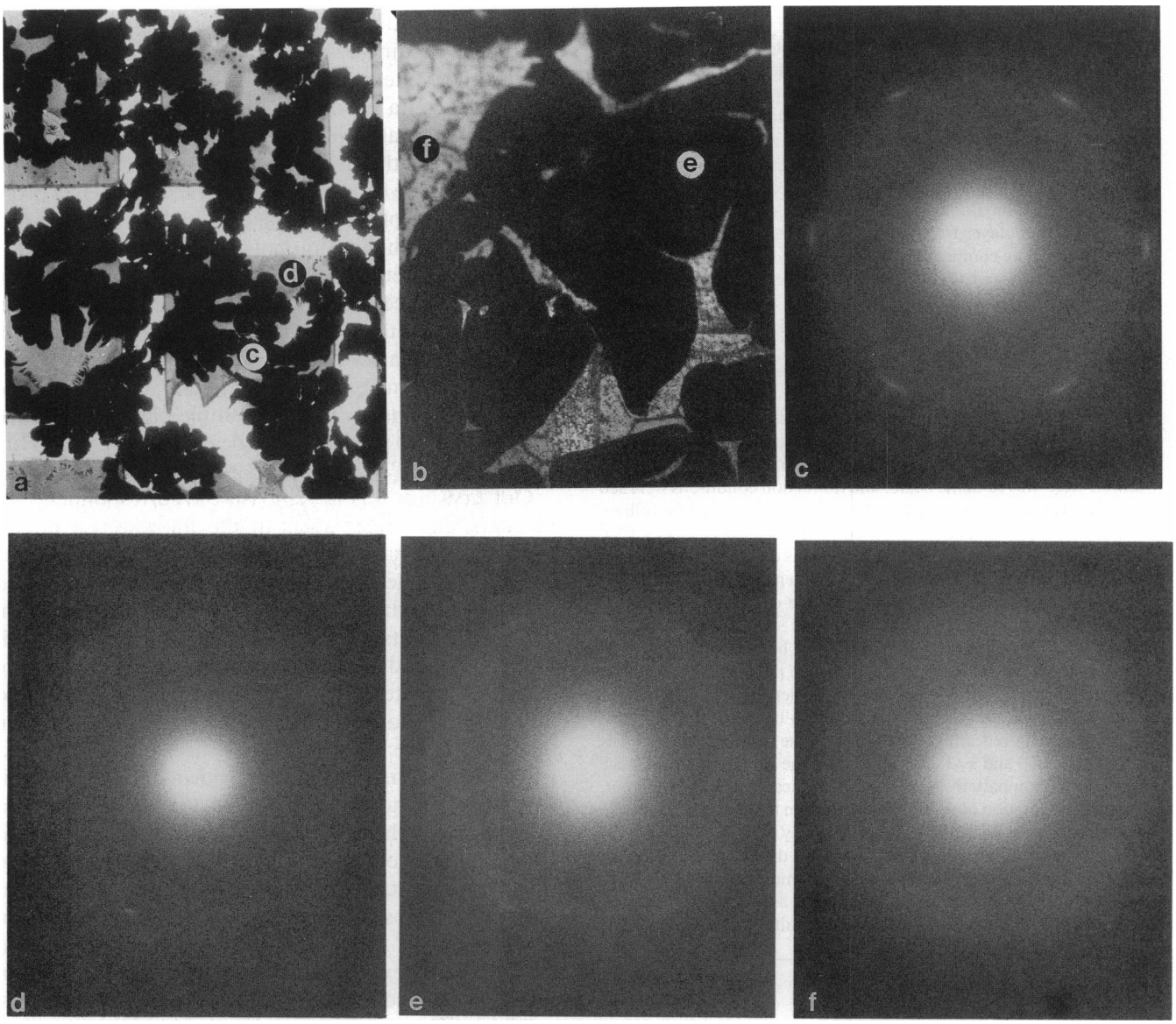

FIGURE 1 Electron diffraction patterns from corresponding areas marked on fluorescence micrographs of monolayers of (*a*) DP(Me)PE and (*b*) $DP(Me)_2PE$ deposited on Formvar-coated marker grids. Patterns *c–f* are derived from correspondingly areas marked in *a* and *b*.

from corresponding diffraction patterns, followed approximately the curve of the domain boundary.

The analysis was then applied to cover an area of higher curvature on another domain (Fig. 2, spots *d–f*). The directors of the patterns derived from these spots were found to rotate a total of $60 \pm 2°$, staying within the same *n* value. We then extended this analysis to trace the director around a U-shaped domain (Fig. 4). The total rotation of the directors was found to be $198 \pm 4°$. Several selected diffraction patterns from this domain are given in Fig. 5, their origins are marked correspondingly in the fluorescence micrograph in Fig. 4. As an additional check, the directors from a neighboring domain were also measured for comparison. These directors were also found to change along the curvature of the domain boundary.

All eight U-shaped domains measured by this method in two separate experiments showed that the directors followed the U turn. There was no significant change of director from one side of the U arm to the other side. When the selected diffraction area was widened to encompass the entire U-shaped domain, the six-fold symmetry of the diffraction pattern was lost, and was replaced by a homogeneous ring, indicating the director had taken at least a smooth rotation exceeding $\pi/3$, and perhaps exceeding π.

(c) Local orientation in DP(Me)PE domains

The dark domains in DP(Me)PE monolayers were branch shaped rather than U shaped. The shape was thought to be determined by the hydration property of

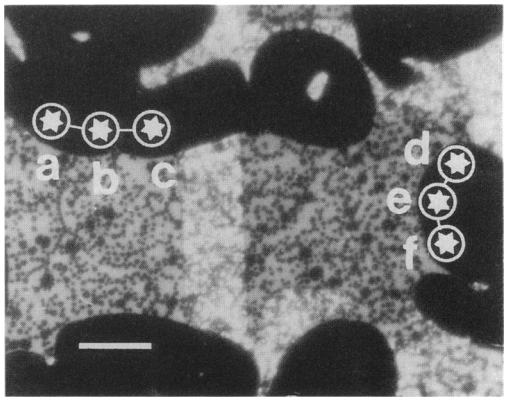

FIGURE 2 Fluorescence micrograph of $DP(Me)_2PE$ monolayers deposited on a Formvar-coated marker grid. Electron diffraction was obtained from the marked areas (*a–f*). The relative orientations of the diffraction patterns obtained from these areas are indicated by sixfold symmetric symbols on the micrograph. Bar = 20 μm.

the lipid headgroup; DP(Me)PE is similar to phosphatidylethanolamine, whereas $DP(Me)_2PE$ is similar to phosphatidylcholine (Yu and Hui, 1992; Sen and Hui, 1988). It was more difficult to trace the directors along the highly invaginated DP(Me)PE domain peripheries than the smoother $DP(Me)_2PE$ ones. Our strategy was then to trace initially the directors along the "stem" or the central portion of an elongated, branch-shaped domain, in order to establish the continuity of orientation within a neighboring region. Fig. 6 shows the director map of such a domain. Points *b* and *d* were picked along the "stem" of the domain, the directors of the corresponding diffraction patterns (Fig. 7, *b* and *d*) were found to differ by only 4°. If the points were picked moving out on a "limb," the directors were found to grow at an angle of $\sim n\pi/3$ from that of the trunk, as indicated by points *a* and *c* in Fig. 6. Again, some orientational flexibilities were observed along neighboring areas. Similar orientation changes were observed in other domains (Fig. 8).

DISCUSSION

Phase-separated solid and fluid domains in monolayers have been predicted and observed in recent years. The pressure-area (π-A) isotherms of many phospholipid monolayers contain a segment within which the monolayers are infinitely compressible. The invariance of pressure in this region of the pressure-area (π/A) curve implies the existence of two phases by the Gibbs phase rule. The hypotheses that dark areas in fluorescence micrographs represent solid domains, and bright areas fluid domains, are based on the expected partitioning properties of fluorescent dyes. The increasing area of dark domains at high surface pressure also correlates with increasing molecular order in monolayer as found by x-ray diffraction, using synchrotron radiation (Mohwald, 1988; Helm et al., 1987). However, present x-ray technology does not allow the beam to be focused in the dimension of a monolayer domain, therefore it is not possible to examine the molecular organization within single domains. Electron diffraction, using a focusable electron beam, is more suitable for this purpose. Indeed, the direct observation of bilayer domains (Hui et al., 1975; Hui, 1981), and the observation of hexatic local melting in single liquid crystal domains (Cheng et al.,

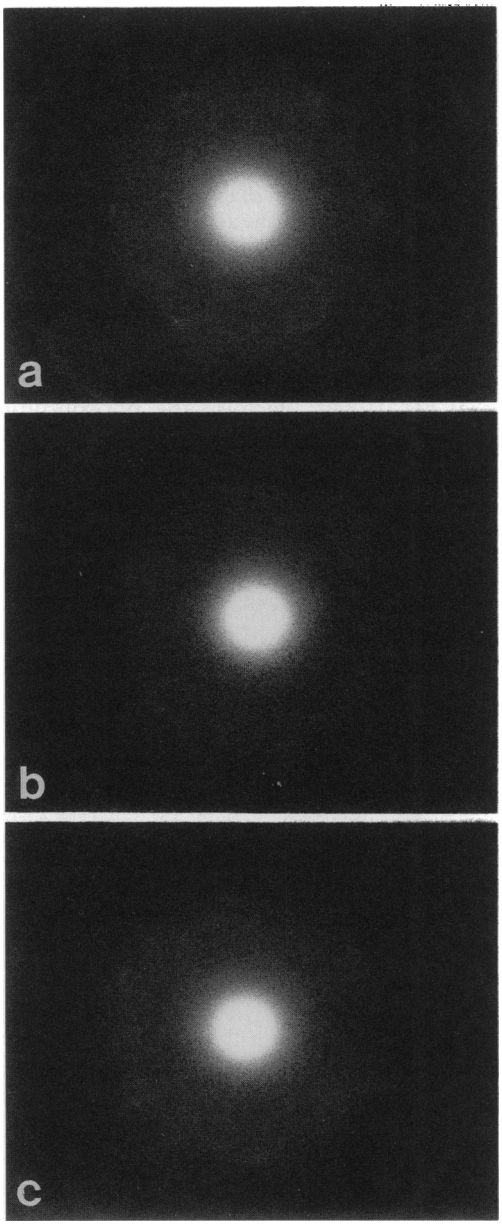

FIGURE 3 Electron diffraction patterns (*a–c*) derived from respective areas marked on Fig. 2. The relative angles *a–b*, *b–c*, *d–e*, and *e–f* are 15.5°, 7.5°, 34°, and 26°, respectively, with an uncertainty of ±2°. The relative orientations of the diffraction patterns obtained from these areas are retained in the display. The outer circle is the (111) powder diffraction ring from evaporated polycrystalline gold on the reverse side of the supporting film, serving as an internal calibration standard of 2.35 Å.

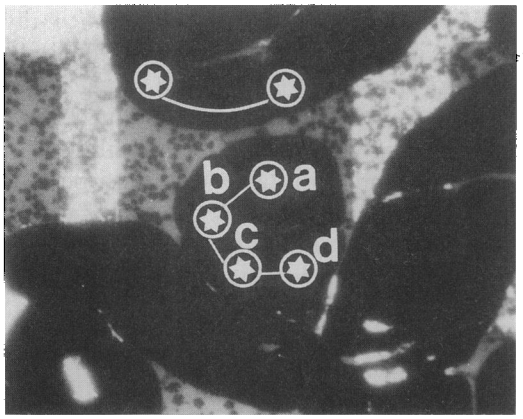

FIGURE 4 Fluorescence micrograph of DP(Me)$_2$PE monolayers deposited on a Formvar-coated marker grid. Electron diffraction was obtained from the marked areas (*a–d*) in a large, curved domain. Symbols and magnification are the same as in Fig. 2.

1987) were made by electron diffraction. Diffraction from a single phospholipid monolayer domain deposited on solid film was also obtained by using electron beam (Fischer et al., 1984). Because the phospholipids we used carried no net electric charge, the method of electrostatic contrast for visualizing domains (Fischer et al., 1984) cannot be applied. We therefore resorted to use fluorescence labelled monolayers on marker grids to help locating the domains. The method is reliable as shown by the exact correspondence of dark domains to sharp, hexagonal patterns (Fig. 1).

The shape of monolayer domains depends to a large extend on the molecular dipole alignment. The theory (McConnell, 1988; Keller et al., 1986; Florsheimer and Mohwald, 1989) predicts that continuous dipole alignment runs through most solid domains, while the line tension between solid and fluid regions minimizes the domain boundary, forcing the domain shape to be more round and the boundary to be smooth. When the repulsion between dipoles exceeds line tension, domains become thin and branching. DP(Me)PE and PE monolayers express thinner and branching domains, perhaps due to the dominance of the dipole-dipole repulsion of their stronger headgroup alignment, which is thought to be related to intermolecular hydrogen bonding. On the other hand, DP(Me)$_2$PE and PC monolayers express coffee bean shape domains, which is a compromised curvilinear dipole organization. The basis of this reasoning, i.e., the long range order and continuity of molecular alignment within a domain, has been shown by fluorescence polarization measurement (Moy et al., 1986).

The ordering of in-plane dipole component of phospholipid monolayers is closely related to the headgroup

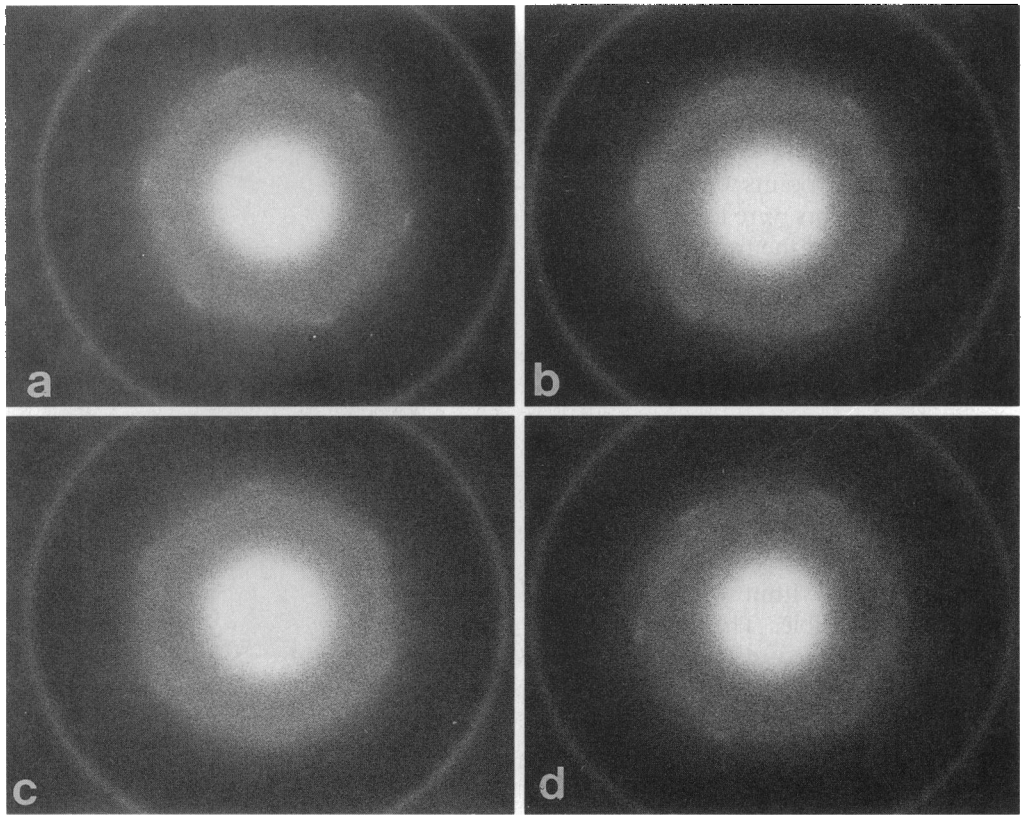

FIGURE 5 Electron diffraction patterns (*a–d*) derived from respective areas marked on Fig. 4. The relative angles *a–b*, *b–c*, and *c–d* are 119°, 47°, and 32°, respectively. The relative orientations of the diffraction patterns obtained from these areas are retained in the display.

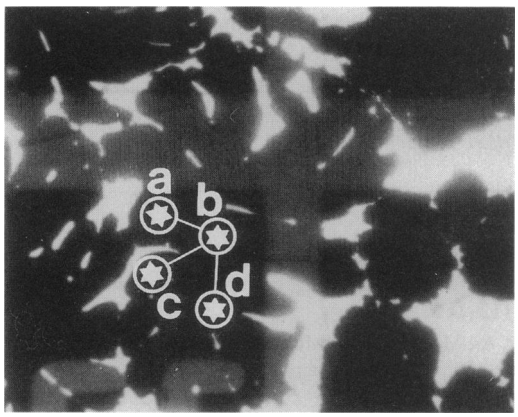

FIGURE 6 Fluorescence micrograph of DP(Me)PE monolayers deposited on a Formvar-coated marker grid. Electron diffraction was obtained from the marked areas (*a–d*). Symbols and magnification are the same as in Fig. 2.

alignment. A long range headgroup alignment is usually associated with some degree of acyl chain ordering in the solid domains. Therefore, the director of chain packing is a good indication, though not a determinant of the headgroup alignment. The electron diffraction patterns of monolayers are dominated by the acyl chains of the lipid molecules; measuring the orientation of the diffraction pattern thus gives us a possible continuous mapping of the dipole alignment of the molecules within that domain. The exact hexagonal symmetry of the diffraction patterns indicate that the chains are normal to the monolayer plane (Hui, 1976; Hui, 1989). This is in disagreement with some x-ray diffraction results from monolayers on the air-water interface (Mohwald, 1988; Helm et al., 1987; Lin et al., 1990). The difference may be due to the deposition and drying of monolayers on a solid substrate.

In $DP(Me)_2PE$ monolayers, we found that the directors of acyl chain alignment run continuously along the U-shaped domains, which is an exaggeration of the giant coffee bean shape domains observed from these monolayers on the air-water interface (Yu and Hui, 1992). Because we have never found any extensive azimuthal broadening of the reflection arcs greater than an orientation disorder of a few degrees, we may conclude that there is no orientational singularities such as a vortex within the arms. This conclusion concurs with our observation that no 2π turn of directors at the ends of arms of U-shaped domains was detected. The gradual turning of the director indicates that the orientational order in chain packing is only pseudo-long range. The picture is consistent with that depicted by McConnell and Moy (1988), showing dipoles pointing from one end of the

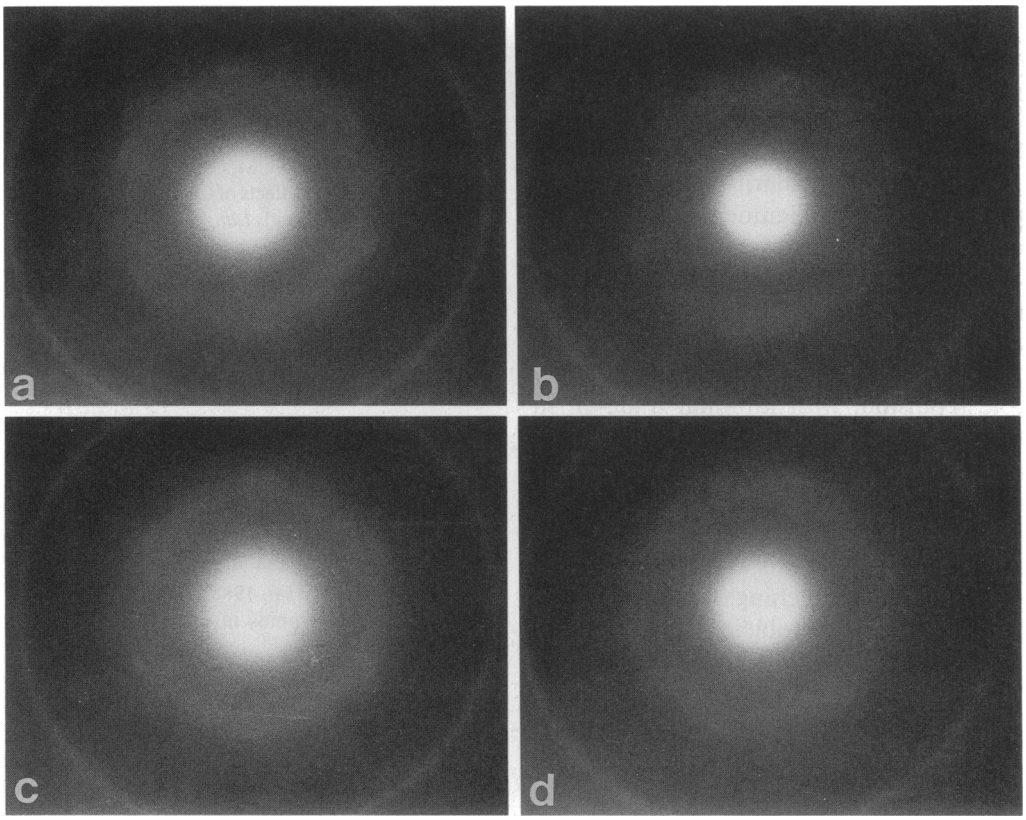

FIGURE 7 Electron diffraction patterns (*a–d*) derived from respective areas marked on Fig. 6. The relative angles *b–a*, *b–c*, and *b–d* are 21°, 8° and $-4° \pm n\pi/3$, respectively. The relative orientations of the diffraction patterns obtained from these areas are retained in the display.

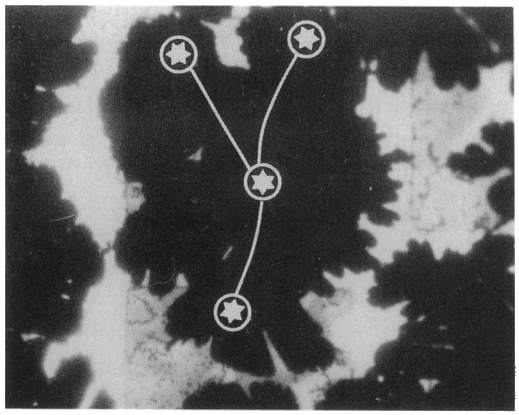

FIGURE 8 Fluorescence micrograph of DP(Me)PE monolayers deposited on a Formvar-coated marker grid. The symbols and magnification are the same as in Fig. 2.

coffee bean domain to the other end. If this type of dipole alignment is indeed true, it is energetically economical to transform an elongated coffee bean or U-shaped domain into a donut shape one, since the line tension is compensated by the attractive dipole alignment force at the point of union. Indeed many U-shaped domains were transformed into circular ones upon deposition from the air-water interface to solid films. Two such examples are shown in Figs. 2 and 4.

The same argument may also be applied to DP(Me)PE monolayers. We found that directors of acyl chain alignment run continuously from one end of the "stem" to another, and at branches, outward at a twinning angle of $\pi/3$. The branching at a twinning angle would avoid a chain packing singularity at the branching point, although the headgroup alignment at the branching point is not certain. Hexagonal symmetry favors this type of twin growth. This method of branching would explain the fact that, with no exception, only one orientation was detected at each selected area. Branching by twinning accomplishes the reduction of domain width to minimize dipole repulsion, with minimal trade-off in packing constraints.

In summary, this study verified the assumption that the dark areas in fluorescence micrographs indeed represent solid domains. Furthermore, the molecular packing lattice vectors in these solid domains were found to bend along their boundaries, and branching occurred at twinning angles. Similar structural problems may be solved by the application of selected area electron diffraction on monolayers.

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