X-Ray Studies on Phospholipid Bilayers. III. Structure and Morphology of L-α-Dilaurylphosphatidylethanolamine (DLPE)

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Powder samples and oriented films of the synthetic phospholipid L-α-dilaurylphosphatidylethanolamine (DLPE) were studied by X-ray diffraction over a wide range of hydration. Only small differences were found between the unit cell dimensions of DLPE powder and oriented-film specimens. These dimensions did not vary appreciably with hydration. In the multilayer DLPE structure, the molecules pack with polar groups parallel to the bilayer plane and hydrocarbon chains perpendicular to it. Examination of oriented films by scanning electron microscopy gave information on the surface morphology. Complementary data on the lamellar packing of the molecules and the average thickness of the bilayers was provided by thin-section and shadow-cast preparations studied by transmission electron microscopy.

Introduction

Phospholipids, in the form of asymmetric bilayers, are the main constituents of biological membranes forming the matrix where proteins and other components are inserted. In the human red-cell membranes, for instance, lecithins and sphingomyelin are located primarily at the outer surface of the cell whereas nearly all of the phosphatidylethanolamines and phosphatidylserine are found at the cytoplasmic surface.

Artificial membranes, built up by either pure phospholipids or accompanied by other membrane components, have been extensively studied as models for natural membranes. In previous papers we have described X-ray fiber structural studies on the phospholipids dimyristoyllecithin (DML) [1] and dipalmitoylphosphatidylethanolamine (DPPE) [2]. These phospholipids present two common features: bilayer structures and polymorphism. In fact, they exhibit different polymorphic forms under the same conditions of temperature and relative humidity, the only variable being the method of preparing the specimens or, as in the case of DPPE, its state of aggregation.

We have extended these studies to L-α-dilauryl phosphatidylethanolamine (DLPE). Powder and oriented specimens were prepared and studied at different hydrations by X-ray fiber and powder methods, at room temperature.

This paper also describes the appearance of DLPE oriented films as examined by scanning and transmission electron microscopy. From the former, information about the specimens surface morphology was obtained. Thin-sections and shadow-cast preparations provided complementary information to that obtained by X-ray diffraction concerning the lamellar packing of the molecules and the average thickness of the bilayers.

Materials and Methods

Synthetic L-α-dilaurylphosphatidylethanolamine from Calbiochem (Lots 901313 and 001023) was used. Oriented films were prepared by slow evaporation from chloroform solutions at 0 °C, which were collected on fine metallic rings. Small rectangular sections of about 1 by 2 mm^2 of oriented films were cut and kept at 0, 47 and 92% of relative humidy (r.h.) and room temperature (about 20 °C); Sikkon (0% r.h.) and saturated salt solutions of KSCN (47% r.h.) and sodium tartrate. $2H_2O$ (92% r.h.) were used. Equilibrium, controlled by gravimetry, was usually reached after about a month of exposure at each r.h. These specimens were X-ray diffracted in humidity controlled cameras as described elsewhere [3]. Dry specimens were obtained by heating over P_2O_5 in vacuum for four hours at 100 °C. Samples were also exposed to the highest possible hydration by immersing them in

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1.5 mm diameter glass capillaries filled with distilled water. X-ray diagrams of powder samples, equilibrated at the same humidities and hydrations, were obtained in Debye-Scherrer and flat-plate cameras. The degrees of hydration and densities of DLPE specimens at each r.h. studied were determined by gravimetry and flotation respectively. Molecular models were built from CPK space-filling components (1.25 cm = 1 Å).

The oriented films were also studied by scanning (SEM) and transmission electron microscopy (TEM). For SEM analysis the specimens were first placed in sample holders covered with Neoluble and then coated with a 240 Å thick gold film in a LADD 40000 vacuum evaporator operated at 10^{-2} torr, 20 mA and 0.6 KV for 4 min. The specimens were observed in an Autoscan U-1 scanning electron microscope operated at 20 KV.

Two different techniques of specimen preparation were used for TEM, shadow-casting and thin-sectioning. In the first, the oriented films were shadowed with carbon-platinum at an angle of 26°, with the vacuum evaporator operating at 10^{-5} torr, 50 mA for 20 s. The angle of shadow was checked by means of polystyrene spheres suspended in methanol. The specimens were then dipped in chloroform; after two hours the DLPE membranes dissolved and the floating shadow-casts were picked-up on copper grids and air-dried.

The thin-sectioning technique included the following operations: a) membrane fixation with 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for 1 h, at 4 °C, followed by three rinsings of 5 min each in phosphate buffer; b) post-fixation with 1% OsO_4 in 0.1 M phosphate buffer, pH 7.2, for 2 h at 0 °C, and three rinsings in bidistilled water for 5 min each; c) prestaining with 2% uranyl acetate solution for 15 min at room temperature; d) dehydration by immersion for 5 min each in 20, 40, 60, 80, 95, and 100% methanol; e) the membranes were embedded in Epon-Araldite, which was polymerized by heating at 60 °C for 24 h; f) the samples thus prepared were placed directly into an ultramicrotome for thin sectioning. They were oriented in such a way that their surfaces were perpendicular to the glass knife. Transverse sections of about 800 Å thick were cut. These, as well as the samples prepared by shadow-casting, were examined with a Philips EM 200 electron microscope operated at 80 KV with a 50 micron objective aperture.

Results

X-ray diffraction

The X-ray diagrams of oriented films of DLPE obtained at 0, 47, and 92% r.h., all at room temperature (20 °C) presented high degrees of crystallinity and orientation. In fact, more than one hundred reflections distributed in nine layer lines were measured in each of them. Figure 1 shows the pattern obtained at 47% r.h., which was more crystalline than the others. All the observed reflections in the three patterns were consistent with orthogonal unit cells. Their axial dimensions, water contents (expressed as the number of molecules of water per molecule of DLPE), both theoretical and experimental densities, the DLPE molecular area at the bilayer surface (S) and the angle of tilt of the molecules ($\varnothing$) with respect to the normal to the bilayer plane – calculated as explained elsewhere[3] – are presented in Table I for each relative humidity.

Table I. Unit cell parameters for oriented films of DLPE at 20 °C.

	Relative Humidity [%]		
	0	47	92
a [Å]	8.00 ± 0.03	7.99 ± 0.02	8.00 ± 0.06
b [Å]	9.65 ± 0.05	9.80 ± 0.04	9.85 ± 0.04
c [Å]	92.35 ± 0.5	92.10 ± 0.2	92.64 ± 0.2
H_2O/DLPE	0	1.0	1.7
d_t [g/cm^3]	1.08	1.10	1.11
d_x [g/cm^3]	1.06	1.07	1.08
S	39.5	40.4	39.9
$\varnothing$	0°	0°	0°

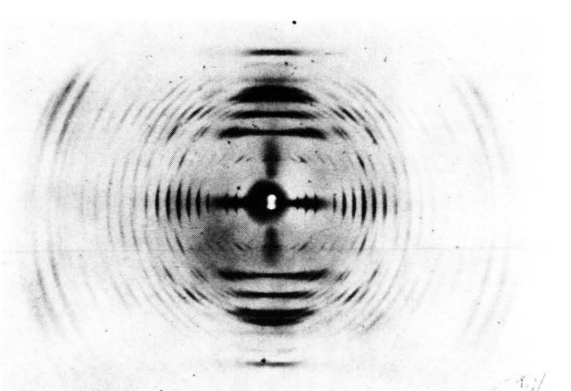


Fig. 1. X-ray diagram of an oriented film of DLPE at 47% r.h., 20 °C. D = 50.78 mm.

The oriented films were also exposed to the highest hydration by immersing them in distilled water. After four days of exposure, about fifteen poorly oriented reflections could be observed; at the end of thirty days only four unoriented reflections were visible. The *c* axis (which was the only one that could be determined) remained practically unchanged at about 95 Å.

Powder samples were also equilibrated and X-ray diffracted at the same humidities as the oriented films. Although fewer reflections were observed (about three to four dozen), their spacings did not differ too much of those of the oriented specimens. Table II presents their unit cell dimensions, degrees of hydration, densities, molecular areas and angles of tilt.

A comparison of Tables I and II indicates that there are very little differences between DLPE in powder with respect to its oriented films. About the same results were obtained when powder samples where exposed to excess liquid water. This is quite different from what was observed in the homologous phospholipid DPPE where remarkable differences were found between their powder and oriented-film specimens under the same conditions [2].

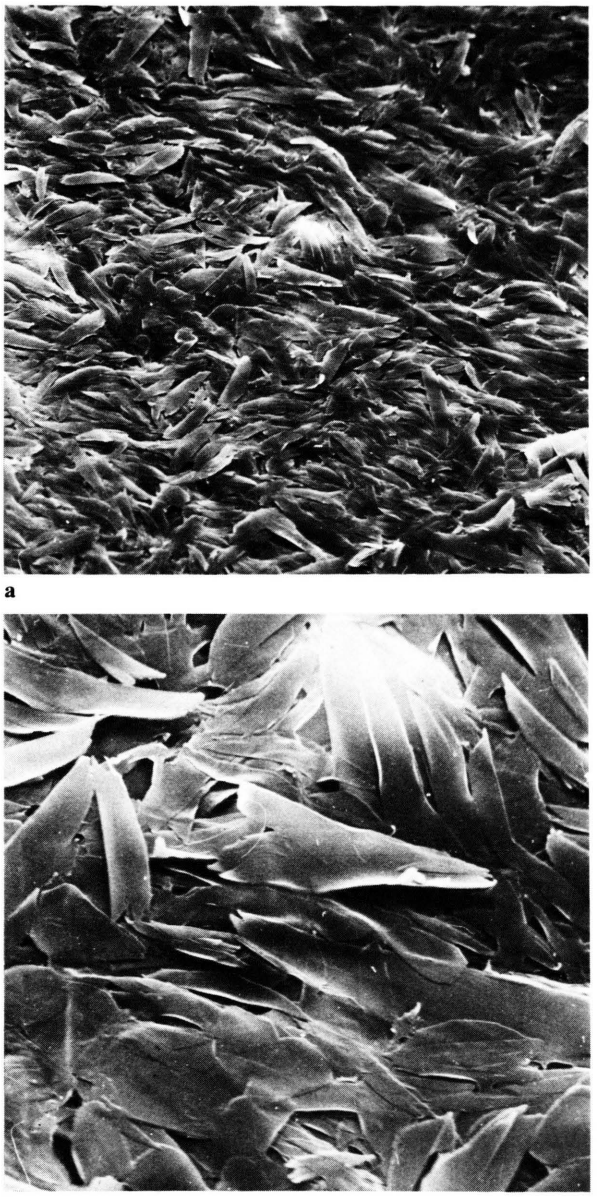
Electron microscopy

In Fig. 2 two SEM micrographs are presented. They show different magnifications of the faces of DLPE oriented films, prepared as for X-ray diffraction. As can be seen, their surfaces are not continuous but rather consist of a random arrangement of small sheets unidimensionally ordered in the plane of the film. Figure 3 shows a TEM micrograph of a small area of one of these sheets, obtained by shadow casting. Very noticeable are the lateral growths of the bilayers which form a multilayered system. Measurements indicated that the average bilayer thickness, about 42 Å, is close to the value found by X-ray diffraction (46 Å at 0% r. h.).

The transverse section micrograph of an oriented-film obtained by thin-sectioning is seen in Figure 4. Technically, the OsO_4 staining is imperfect for

Table II. Unit cell parameters for powder samples of DLPE at 20 °C.

	Relative Humidity [%]		
	0	47	92
a [Å]	8.49 ± 0.04	8.47 ± 0.04	8.50 ± 0.06
b [Å]	9.16 ± 0.06	9.21 ± 0.01	9.23 ± 0.04
c [Å]	91.7 ± 0.3	91.8 ± 0.2	91.7 ± 0.1
H_2O/DLPE	0	0.3	0.7
d*t* [g/cm^3]	1.08	1.09	1.10
d*x* [g/cm^3]	1.05	1.07	1.10
S	40.1	39.7	39.1
∅	0°	0°	0°



a

b

Fig. 2. SEM micrographs of the surface of a DLPE oriented-film, covered with a 240 Å gold coat. Magnification: a) 100 ×, b) 9600 ×.

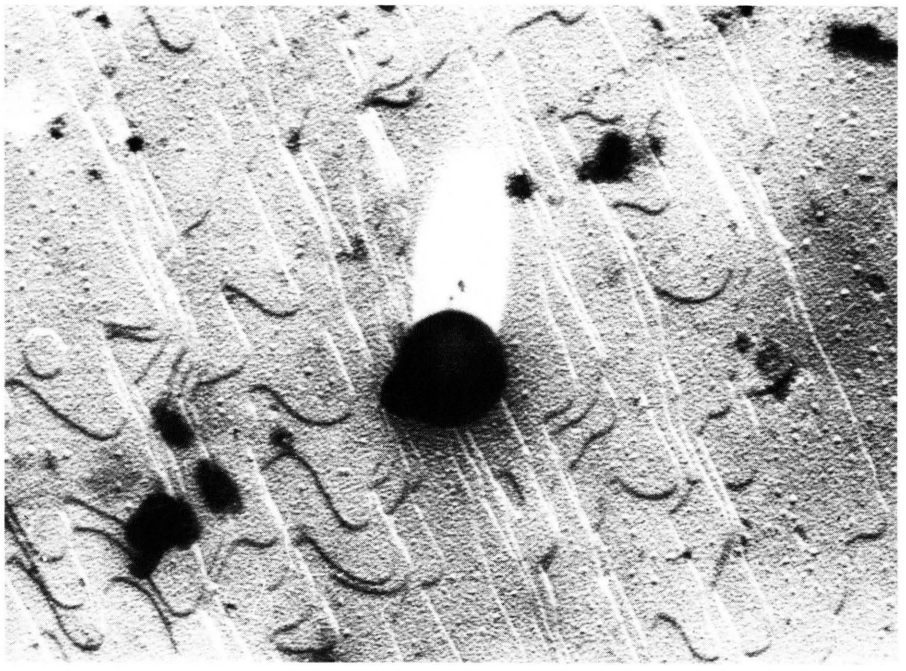


Fig. 3. TEM micrograph of a DLPE oriented-film shadowed with C-Pt at an angle of 26°. Specimen unstained, unfixed and unembedded. Magnification: 48 800×.

reasons that will be explained later, and consequently the picture is somewhat diffuse. Nevertheless, it is still possible to see the dark and light zones characteristic of a multilayer structure of phospholipids. In fact, those zones correspond to the hydrophilic and hydrophobic groups respectively. Measurements indicate a 48 ± 5 Å bilayer periodicity, close to the value found by X-ray diffraction.

Discussion

From X-ray diffraction and electron microscopy studies of oriented films and powder samples it is possible to propose a bilayer structure for DLPE. Such a molecular arrangement is not surprising at all as it has been found in other acylphosphatidylethanolamines [3–8] and in single crystals of DLPE [9]. Nevertheless, the dimensions of the long crystallographic *c* axes are about twice of those reported for analogous phospholipids. This implies two bilayers instead of one in the *ac* plane (Fig. 5). In fact, unit cell dimensions and experimental densities indicate eight DLPE molecules in the unit cells in the range of humidity between 0 and 92%. Although such a unit cell has previously been reported for a lecithin [10] we do not have an explanation for it. On the other hand, powder and films of DLPE consistently present some differences in their unit cell dimensions at each r.h., although not as marked as in dipalmitoylphosphatidylethanolamine [3]. Perhaps these differences can be explained by their different water contents. In fact, oriented films exhibit higher hydrations than powder samples of DLPE, situation that might affect its molecular packing.

Molecular models for oriented films of DLPE at 47% r.h. were built on the basis of the previous considerations and in agreement with an electron density profile of the homologous phospholipid dimyristoylphosphatidylethanolamine (DMPE) [8]. The most satisfactory model is the one shown in Fig. 5, where the molecules of DLPE form a typical bilayer structure 46.05 Å thick. The phosphatidylethanolamine groups are oriented parallel to the bilayer plane; this arrangement allows electrostatic interactions between the negatively charged phosphate and positive amino terminal groups. On the other hand the hydrocarbon chains are extended perpendicular to the bilayer plane, closely packed along the *a* axis. Because one of the hydrocarbon

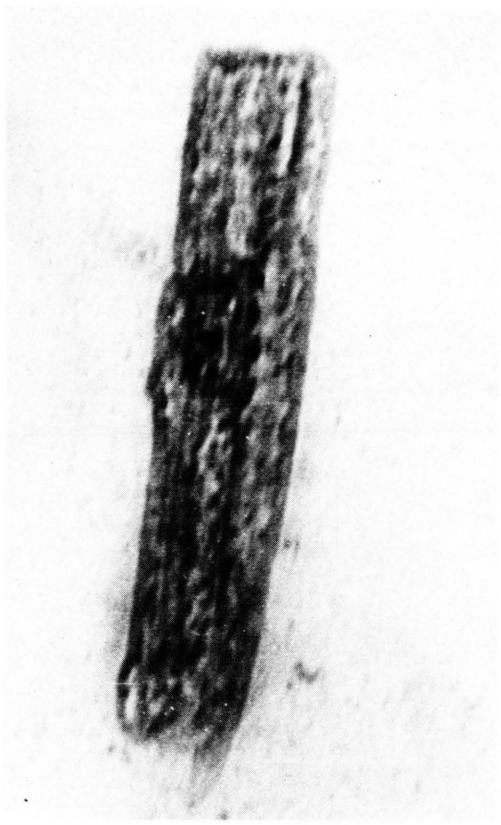


Fig. 4. TEM micrograph of a thin section of DLPE multilayer cut perpendicular to the oriented-film surface. Fixed with glutaraldehyde and OsO_4, stained with uranyl acetate. Magnification: 208 000 ×.

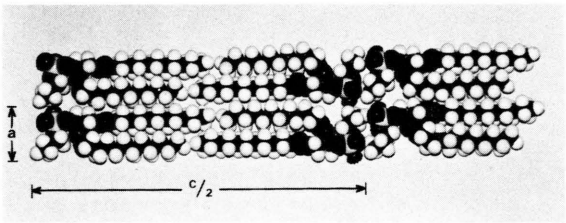


Fig. 5. Two-dimensional molecular model of a DLPE bilayer.

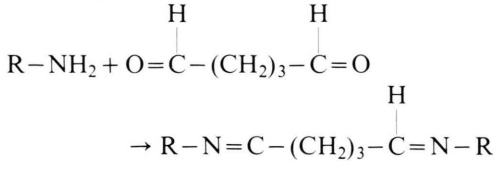
chains is slightly bent it is about three methylene groups shorter than the other chain. Contacts between monolayers occur via their terminal methyl groups. This type of packing of the hydrocarbon chains favors intra- and intermolecular hydrophobic interactions.

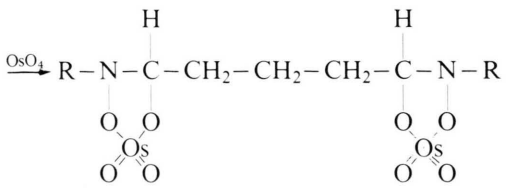
The dimension of the perpendicular *b* axis (9.80 Å) is about twice the expected repeat distance. This value results from the presence of an additional bilayer at about *b*/2, shifted by *a*/2 with respect to the neighboring bilayers. The molecule of water present in DLPE at 47% r.h. is located in the highly polar pocket formed by the phosphatidylethanolamine chain and the glycerol. The only element of symmetry present in this molecular arrangement is a two-fold rotation axis parallel to *b*. Therefore, although the unit cell has orthogonal axes, it is monoclinic P2, pseudo C-centered.

It is interesting to compare these results with those derived from the single crystal studies of 1,2-dilauryl-DL-phosphatidylethanolamine recrystallized from acetic acid and containing one molecule of acetic acid per molecule of phospholipid [9]. Its unit cell is also monoclinic, with $a = 47.7$ Å, $b = 7.77$ Å, $c = 9.95$ Å, $\beta = 92°$ and $Z = 4$. However, the space group is $P2_1/c$. Because the crystals are racemic they can present glide planes, which are not possible in the enantiomer L of DLPE. Otherwise, their molecular conformations and packing arrangements are very similar. These results are somewhat different to those reported for DML [1] and DPPE [3]. Both phospholipids present polymorphism (see Introduction) whereas crystals, powder and oriented films of DLPE all have practically the same structural features.

The close packing of DLPE, which favors strong molecular interactions, might explain the small effect of water on its unit cell dimensions, even when present in excess. About similar results have been reported for DLPE dispersions at the same conditions [11] as well as at physiological pH and temperature [12]. On the other hand, they differ of those observed in DML [13], which is not as closely packed as DLPE [1].

To enhance the contrast of the image obtained by transmission electron microscopy, DLPE oriented films were stained with OsO_4. This compound is known to react with double-bonds of the aliphatic chains; OsO_3 is formed which, being more polar than OsO_4, migrates to the polar regions of the lipid bilayers [14]. As DLPE lacks double-bonds, OsO_4 might interact with its polar groups according to the following reaction:





In the proposed reaction, above, glutaraldehyde, a reagent used in the fixation, reacts with the amino-terminal groups of DLPE forming Schiff bases; OsO_4 is then bound to the C=N double-bonds producing, in this way, the dark regions of the multilayers observed in the micrographs.

To test the putative reaction above several assays were performed. In one of them, no glutaraldehyde was used in the fixation, and indeed, OsO_4 failed to stain the oriented film of DLPE. In another experiment, DLPE was replaced by dimyristoyllecithin (DML), a phospholipid which lacks the terminal NH_2-group. In the absence of glutaraldehyde, OsO_4 did not stain the film. When glutaraldehyde was added, the sample dissolved, indicating that no stabilizing Schiff bases were formed.

Acknowledgements

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