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On the Identification of Lamellar and Hexagonal Phases in Negatively Stained Phospholipid–Water Systems*

David O. Tinker† and L. Pinteric

ABSTRACT: The principles of projective geometry are used to predict the features of electron micrographs of negatively stained liquid crystals composed of lamellar and two hexagonal phases. A well-characterized phosphatidylethanolamine from pig erythrocytes was hydrated and the resulting liquid crystals negatively stained with phosphotungstic acid and examined at high magnification in the electron microscope. Three types of liquid crystal were identified in these preparations: lamellar (bimolecular lipid leaflets spaced at 46 Å units), H_I (lipid cylinders, diameter 40 Å units, hexagonally packed in an aqueous matrix with a spacing of 61 Å units), and H_{II} (aqueous cylinders, diameter 26 Å units, hexagonally packed in a lipid matrix with a spacing of 56 Å units). All the theoretically predicted features were evident in the micrographs.

In the early stages of hydration, tubules of irregular diameter were seen to grow out of the unstructured lipid particles, later attaining a more uniform diameter and aligning regularly. These tubules evidently give rise to the H_I phase. Tubular structures and H_I phase were evident in the micrographs of Korman *et al.* (Korman, E. F., de Pury, G., Asai, J., Allman, D. W., Kopaczky, K., and Green, D. E. (1970), *Biochemistry 9*, 1318), but the interpretations of their results given by these authors are refuted.

Naturally occurring phospholipids have been shown by X-ray diffraction techniques to form a variety of liquid crystalline phases in aqueous systems (Luzzatti *et al.*, 1960; Luzzatti and Husson, 1962; Luzzatti and Spegt, 1967; Luzzatti *et al.*, 1968a–d; Rand and Luzzatti, 1968; Reiss-Husson, 1967; Small and Bourges, 1966; Small 1967). The most commonly observed phases are the lamellar phase (L) and the two hexagonal phases H_I and H_{II}. Diagrammatic representations of these structures are shown in Figures 1–3.

The structural conclusions drawn as a result of the X-ray studies have been confirmed (at least in part) by electron microscopic investigations. Thus Bangham and his colleagues (Bangham and Horne, 1964; Papahadjopoulos and Miller, 1967) presented convincing evidence for the lamellar structure in negatively stained preparations of hydrated liquid crystals ("liposomes") of a variety of phospholipids. Recently Junger and Reinauer (1969) published high resolution micrographs of negatively stained phosphatidylethanolamine–water preparations in which a hexagonal phase (probably H_{II}) was correctly identified.

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† To whom correspondence should be addressed.

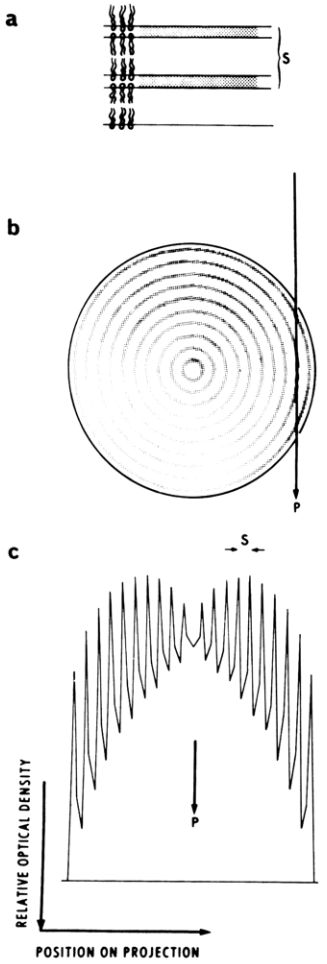


FIGURE 1: Structure of lamellar (L) phase in phospholipid–water systems. (a) Molecular arrangement in lipid lamellae; heads of molecules represent the polar phosphate groups and the two tails represent the hydrocarbon chains. (b) Cross section of spherical particle showing concentric layers of lamellae; shaded areas in a and b represent water- or stain-filled spaces. (c) Calculated variation of optical density along a diameter of a projected negative image of b; the abscissa corresponds in position and scale to b. P is the direction of the electron beam. S is the repeat spacing.

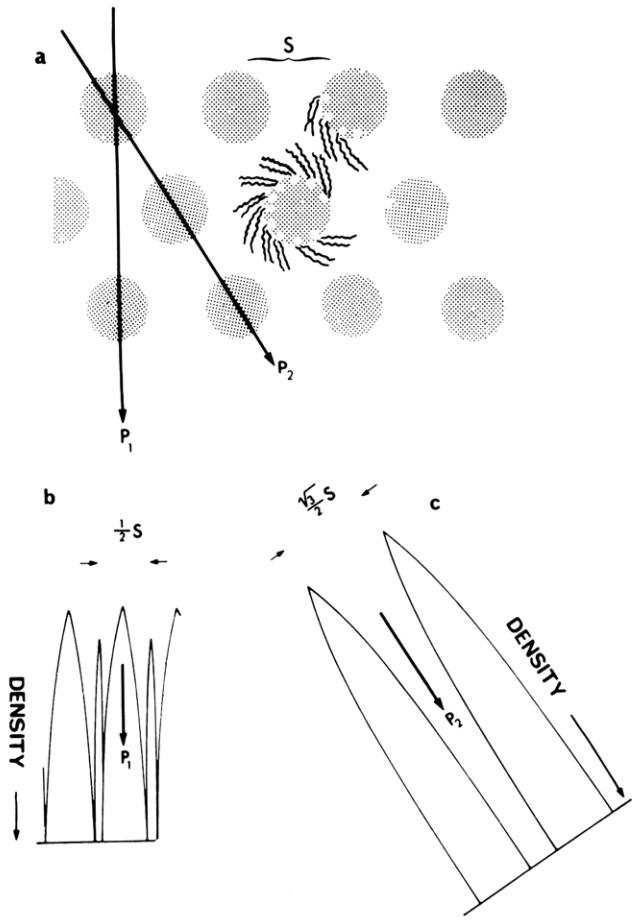


FIGURE 2: Structure of hexagonal (H_{II}) phase in phospholipid–water mixtures. (a) Cross section of hexagonal array of water- or stain-filled cylinders (shaded areas) with possible arrangement of phospholipid molecules shown in part. P_1 and P_2 are directions of electron beam producing two distinct projected images. The angle between P_1 and P_2 is 30°. S is the repeat spacing. (b) Calculated variation of optical density along a line perpendicular to the cylinder axes in a two-dimensional negative image of a, projected in the direction P_1. The center of each broad peak corresponds to the centres of cylinders in a column parallel to P_1. (c) Calculated variation of optical density along a line perpendicular to the cylinder axes in a two-dimensional image of a, projected in a direction P_2. Each peak corresponds to a column of cylinders parallel to P_2.

As was first pointed out by Bangham and Horne (1964), correct interpretation of micrographs of unsectioned, negatively stained material must be based on the principles of projective geometry, since such micrographs are two-dimensional plane projections of three-dimensional stain distribution functions. Failure to realize this fact has probably led to a number of misconceptions in the literature, to one of which (Korman *et al.*, 1970) we wish to draw attention at this time. In this paper we shall discuss the principles of identification of the three common liquid crystalline phases in negatively stained material. We have recently undertaken structural studies on the system phosphatidylethanolamine–water which have included electron microscopy of negatively stained samples and other studies, and the results which have a bearing on the present subject are also presented here.

Theoretical Section

Figures 1 and 2 present cross sections of idealized liquid crystalline particles. The lamellar-type particle consists of a regularly spaced array of bimolecular lipid leaflets. Because of the tendency for these lamellae to close on themselves (this has been discussed by Fergason and Brown, 1968), such particles have a structure analogous to an onion or, in the case of a cylindrical particle, to a tree trunk. A cross section of an idealized lamellar particle is shown in Figure 1b. The

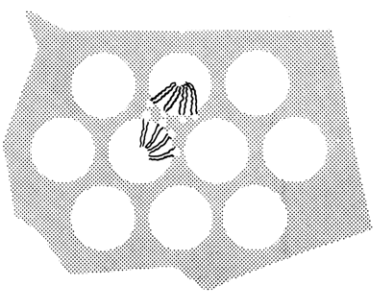


FIGURE 3: Structure of hexagonal (H_I) phase in phospholipid–water mixtures.

TABLE I: Fatty Acid Composition of Pig Erythrocyte Phosphatidylethanolamine.[a]

Fatty Acid[b]	Mole %
16:0	26.8
18:0	6.7
18:1	38.9
18:2	19.3
20:4	4.9
22:6	1.9
Others	1.5

[a] Analytical methods were as described by Morgan *et al.* (1963). The material had nitrogen:acyl ester:glycerol:phosphorus molar ratios of 0.98:2.09:0.94:1.00. Vinyl ether analogs were not detectable and ethanolamine was the only nitrogenous component detected after hydrolysis and chromatography. [b] Fatty acid notation: first figure, number of carbons; second figure, number of double bonds.

hexagonal phases H_{II} and H_I consist, respectively, of indefinitely long, water-filled cylinders in a lipid matrix, and of indefinitely long, lipid cylinders in an aqueous matrix, as in Figures 2a and 3. The most probable dimension for the hydrocarbon regions in the L and H_I phase (the thickness of the lamellae and the diameter of the cylinders, respectively) is twice the mean length of the hydrocarbon chains, or about 40 Å. As was pointed out by Asunmaa *et al.* (1969), the thickness of the hydrocarbon regions in H_{II} phase may be less than 40 Å, since close-packing in the hydrocarbon region cannot be achieved if the long axes of the molecules are perpendicular to the hydrocarbon interface. A possible mode of packing of lipid molecules in the H_{II} phase is shown in Figure 2b.

We assume that in negatively stained particles the aqueous spaces are filled with a stain of a uniform electron-scattering power per unit length. If a uniform electron beam is projected through such a particle in a direction P onto a photographic plate normal to P, the reduction in electron density at the end of a ray P will be directly proportional to the total length of the stain space traversed by P. The variation of optical density on the photographic plate after exposure for a specified time, and development, depends on the variation of electron density in the rays incident on the plate.

Thus, the variation of optical density along a linear path through the two-dimensional projected image may be calculated by computing the length of stain space traversed by each ray P terminating on the path. This is equivalent to a densitometric scan across the image. Examples of such "scans" are shown for the L and H_{II} phases in Figures 1 and 2, in which the correspondence between stain distribution in the particles and density distribution in the projected images is apparent.

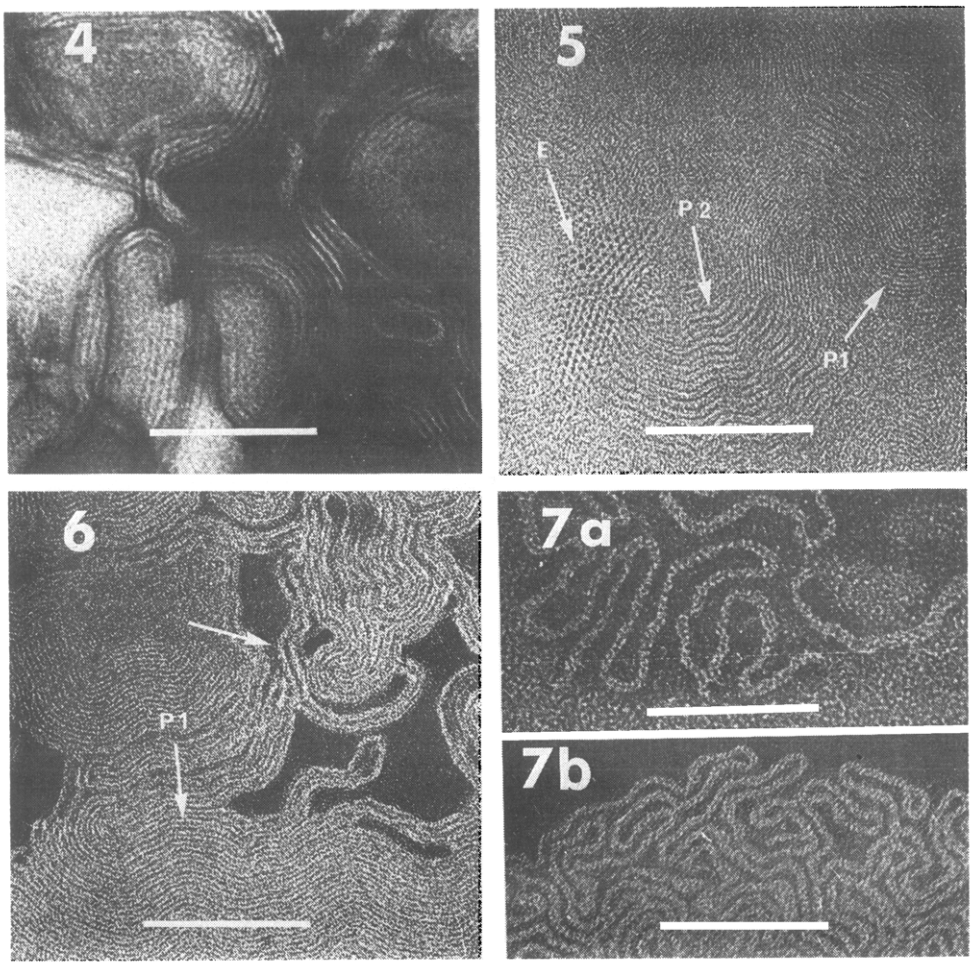
There is only one possible projection of a lamellar sphere as shown in Figure 1. The image corresponding to this scan consists of alternating light and dark bands of uniform thickness and spacing; however the contrast between light and dark bands disappears rapidly toward the center of the particle, and this feature together with the existence of only one repeat spacing is diagnostic of the L phase. In the case of H_{II} (and H_I) phase, however, there are three principle projection directions, an "end-on" projection and two "side views" as shown in Figure 2. The densitometric scan corresponding to projection P_2 (Figure 2c), shows alternating light and dark bands of constant thickness, spacing, and contrast. The repeat spacing in the image is $\sqrt{3}/2$ times the repeat spacing in the parent structure. The scan corresponding to the projection P_1 is shown in Figure 2b. The fine structure of the scan is highly sensitive to the ratio r = (cylinder diameter)/(nearest-neighbor spacing) in the parent structure; this ratio is 0.57 in Figure 2. (It is apparent that the image corresponding to Figure 2b could be interpreted as a micrograph of an array of lipid cylinders with stain-filled cores, an interpretation which is obviously false in this instance). The narrow bands of low optical density, which arise because of overlap of the edges of cylinders in adjacent layers, are absent if $r < 0.5$ and become more pronounced at the expense of the broad bands as r increases. Because of this feature, the only reliable measurement which can be obtained from a P_1 projection is the repeat spacing, which is always half the repeat spacing in the parent structure. The corresponding projections of the H_I phase can be obtained by simply inverting Figures 2b and 2c. In practice the projected images of H_I and H_{II} phases are very similar.

Theoretical scans for projections in directions other than P_1 and P_2 have also been constructed; for the sake of brevity, these are not shown here. In the case of hexagonal phases having $r \geq 0.5$, the contrast between areas of high and low optical density is relatively small for projections intermediate between P_1 and P_2. Thus it is to be expected that the latter projections will be the most prominent in electron micrographs of these phases.

Experimental Section

Phosphatidylethanolamine was prepared from pig erythrocytes which had been collected and extracted with chloroform–methanol exactly as described for human erythrocytes by Ways and Hanahan (1964). The lipid extract was chromatographed on a column of silicic acid (Mallinckrodt, 100 mesh)–Hiflo Super-Cel (Johns-Manville), 2:1 (w/w). Lipids were loaded in chloroform and eluted with the sequence of solvents: chloroform, acetone, acetone–methanol (9:1, v/v), and chloroform–methanol (1:1, v/v). The latter solvent eluted two phospholipid peaks, the first of which was chromatographed on a column of silicic acid treated with ammonium hydroxide as described by Rouser *et al.* (1961). Chromatographically homogeneous phosphatidylethanolamine was eluted from this column with chloroform–methanol (4:1, v/v), dissolved in a minimum of chloroform and precipitated with redistilled acetone. The resulting white solid, stored at −20° under acetone in a nitrogen atmosphere, was stable for over a year. The yield was about 3 g from 30 l. of pig blood. Analytical characteristics of this material are presented in Table I; pertinent methods have been cited (Morgan *et al.*, 1963). A chloroform solution was almost colorless and exhibited no pronounced light absorption at 230–280 mμ, indicating freedom from peroxidation products. The water used to prepare lipid dispersions was twice glass distilled, the second time over alkaline potassium permanganate, and stored in a carbon dioxide free atmosphere. It had a conductivity of 1.5 μmhos cm.

Aqueous dispersions of phosphatidylethanolamine at a concentration of 10 mg/ml were prepared as follows: the material was removed from storage as a slurry in acetone and transferred to a tarred test tube with a Pasteur pipet. The acetone was removed under a stream of nitrogen and the tube was dried to constant weight *in vacuo* over phoshorus pent-



FIGURES 4–7: Electron micrographs of pig erythrocyte phosphatidylethanolamine–water mixtures negatively stained with phosphotungstic acid solution. Darker areas are stain and the markers are 1000 Å units in each case. See text for details. (4) Lamellar phase; note loss of contrast in centers of particles. The repeat spacing is 45.8 ± 2.7 Å units. (5) H_{II} phase; lettered arrows point to end-on view of stain-filled cylinders (E), and P_1 and P_2 projections of side view. (6) H_I phase; lettered arrow points to P_1 projection. Unlettered arrow points to lipid cylinders which are not in regular hexagonal array. (7) (a) Early stage of hydration of phosphatidylethanolamine. Tubular structures are "growing" out of unstructured lipid masses. Mean diameter of tubes ± standard deviation is 63 ± 16.8 Å units. Branching of the tubes is evident at two points. (b) Early stage of hydration of phosphatidylethanolamine. Tubes have now aligned in a roughly parallel fashion. Several branch points and closed loops can be seen. Diameter of tubes ± standard deviations is 43.2 ± 8.8 Å units.

oxide, care being taken to keep the dry material in a nitrogen atmosphere when not *in vacuo*. The lipid dried as a colorless wax at the bottom of the tube. An appropriate amount of nitrogen-saturated water was then pipetted over the lipid and frozen using an acetone–Dry Ice mixture. The tube was then sealed under a reduced pressure of nitrogen and the ice allowed to thaw. The tube was then subjected to a few minutes of ultrasonic irradiation in a "Sontegrator" (Ultrasonic Industries Inc., Plainview, N. Y.) cooled with an ice–water mixture, and set aside to equilibrate at room temperature, usually for 12 hr. The lipid then appeared as a flocculent white material which could be redispersed by shaking and settled again in several minutes. After opening the tubes, the material was kept under nitrogen until no longer required and examined within 30 min. By adding four volumes of methanol and five volumes of chloroform the mixture in the tube could be dissolved; thin-layer chromatography then revealed a single charring, ninhydrin-positive spot with the mobility of phosphatidylethanolamine.

Negatively stained samples for electron microscopy were prepared in one of three ways. In the first method, 0.5 ml of phosphotungstic acid solution (1% phosphotungstic acid, w/v, in distilled water, adjusted to pH 7.0 ml with sodium hydroxide) was placed as a drop on a Teflon sheet and a few microliters of the shaken lipid dispersion pipetted on top of the drop. The lipid together with some liquid was picked up off the surface of the drop with a carbon-coated copper electron microscope grid (rendered hydrophilic by ionization under an ultraviolet source), the grid was touched to a clean filter paper to remove excess liquid and inserted into the entry port of the electron microscope. In the second method a few microliters of lipid dispersion was pipetted onto the grid, an equal volume of phosphotungstic acid solution layered on top, and the grid was blotted and inserted into the entry port as before. In both these techniques a maximum of 10 sec elapsed between removing the lipid from the tube and insertion of the grid into the port. In the third technique, equal volumes of lipid dispersion and phosphotungstic acid solution were mixed in a test tube and after several minutes a few microliters of the mixture removed and treated as before. We found the first method easiest to carry out, but all methods gave comparable results and will not be compared here.

The electron microscope used was a Philips Model EM-200.

Results

Figures 4–7 are micrographs of PE-water samples that had been equilibrated 12 hr before staining. Figure 4 is lamellar phase; the progressive loss of contrast between dark and light layers in the centers of the several particles is clearly evident. Moreover, there is a single repeat spacing of 45.8 ± 2.7 Å units (mean ± standard deviation).

Figure 5 is identified as H_{II} phase. An end-on projection of the stain-filled channels is a prominent feature in this field together with P_1 and P_2 projections. The diameter of the aqueous channels seen in end-on projection is 26.4 ± 3.5 Å units (mean ± standard deviation). In Table II we have tabulated the repeat spacings measured for the various projections together with their ratios. The mean repeat spacing in this phase calculated from the spacings of the P_1 and P_2 projections is 56.7 ± 2.7 Å units, which compares well with the value 56.0 ± 2.1 Å measured directly from the end-on projection. The minimum thickness of the hydrocarbon regions is calculated to be 30 ± 6 Å units and the ratio r is 0.47 ± 0.08.

TABLE II: Repeat Spacings in Various Projections of H_{II} Phase.[a]

Projection	Spacing (Å)[b]	Ratio[b,c]	Theoretical Ratio
End on	56.0 ± 2.1	1.00	1.00
P_1	27.2 ± 1.3	0.486 ± 0.041	0.50
P_2	51.1 ± 2.4	0.912 ± 0.077	0.866

[a] Measurements taken from Figure 6. [b] Results given as mean ± standard deviation of 50 measurements in random sections of the appropriate projection. [c] Ratio of repeat spacing to spacing of end-on projection.

Figure 6 is identified as H_I phase. Unfortunately, clear cut end-on and P_2 projections are not evident in this field, however the major structure can be identified as a P_1 projection by the constant contrast between dark and light bands and the close similarity to the P_1 projections in Figure 6. The repeat spacing measured in Figure 6 is 30.7 ± 1.9 Å units (mean ± standard deviation) corresponding to a spacing of 61 ± 3.8 Å units in the parent phase. At the water–liquid crystal interfaces there can be clearly seen the individual lipid cylinders from which the H_I phase is formed. These have a diameter of about 40 Å units. Figure 6 is qualitatively similar to Figure 1c of Korman *et al.* (1970).

It is difficult to quantitate the relative proportions of these phases in the micrographs, but a random selection of fields indicates that L phase predominates, especially in larger particles. We have, however, observed many small particles (100–300 mμ in diameter) which are exclusively composed of H_I phase. On the basis of the present evidence we must count all three phases as major structural components of the lipid particles. Several other, comparatively rare structures were observed which are not shown here.

In an effort to study the process of formation of these structures, micrographs were prepared from phosphatidylethanolamine–water mixtures that had been equilibrated for times of only 10–30 min. In such micrographs the predominant forms appeared to be unstructured lipid masses. In favorable fields, however, it was possible to observe tubular structures apparently growing out from the edges of the particles. In the earliest stages these tubules had a large and variable diameter and appeared to grow and branch in a random fashion. At an apparently later stage the tubules were aligned more regularly and the diameter attained a fairly uniform value of about 40 Å units. Figure 7 shows these features. It is probable that the structures in Figure 7 may subsequently pack into H_I phase and then rearrange into H_{II} and lamellar phases, but we have not yet observed intermediate stages in the latter processes.

In some cases, phosphatidylethanolamine–water mixtures were oxidized by slowly bubbling air through the dispersion overnight. No regular structures were observed in such dispersions after negative staining and electron microscopy, except for occasional small particles which appeared to be disordered aggregates of lamellae. The effects of partial oxidation were not examined; however, the procedures employed to prevent oxidation of the lipid seemed adequate to eliminate the presence of artefacts due to this cause.

Discussion

On the basis of the qualitative and quantitative arguments presented here, it is possible to conclusively identify the structures in Figures 4 and 5 as corresponding to L and H_{II} phases, respectively. The existence of these structures in negatively stained preparations of phosphatidylethanolamine was previously reported by Junger and Reinauer (1969), although the repeat spacings reported by these authors were 50 Å for the L phase and 80 Å for the H_{II} phase as compared to the values of 46 Å and 56 Å reported here. Junger and Reinauer studied phosphatidylethanolamine from rat liver however, while in the present study the pig erythrocyte lipid was employed. The quantitative differences between the two preparations may thus arise from differences in fatty acid distribution.

The structures observed in Figures 6 and 7 were not reported by Junger and Reinauer (1969). They are clearly based on a structural unit consisting of long cylinders of lipid with a diameter of about 40 Å. In Figure 6 these cylinders are aggregated in a regular hexagonal array, although only the P_1 projection of this array is evident. We have tentatively concluded that the arrays of cylinders seen "side-on" in Figure 6 are only two or three cylinders deep; this appears to be the case at the edges of the aggregates, and would account for the absence of P_2 and E projections in Figure 6, since most of the aggregates are lying flat on the surface of the grid. In Figure 7 we observe nonordered and semiordered groups of lipid cylinders which are common in the early stages of swelling of this lipid in water before extensive ordering of the cylinders into H_I phase has occurred.

It is somewhat beyond the scope of this report to enquire as to the relation between the structures observed in negatively stained preparations and the structures of unstained phospholipid aggregates in water. We have eliminated the possibility that the hexagonal phases arise because of oxidative degradation of the lipid. We cannot rule out the possibility, however, that interaction of the phospholipid with the negative stain and/or dehydration of the sample causes alterations in the "native" structure of the aggregates. An estimate of the amount of water remaining in the specimens when they are observed is not available, although it has been found that if the specimens in the electron microscope are heated by increasing the intensity of the electron beam, additional water

can be "boiled off" with disruption of the structures present. At the lower beam intensities used for normal observation and photography, therefore, an unknown amount of water is retained in the lipid aggregates, and the liquid crystalline phases observed may be characteristic of the composition that exists on the electron microscope grid. Further evidence bearing on this point will be presented later (R. P. Rand, D. O. Tinker, and P. Fast, in preparation). The coexistence of two or three distinct phases in a system which must be considered to have at least two components does not contradict the phase rule.

The present results have a distinct bearing on the conclusions reached in a recent report by Korman *et al.* (1970). These authors presented electron micrographs (their Figure 1) of negatively stained mitochondrial phospholipid which clearly contained cylindrical lipid structures having a diameter of about 40 Å (called "spaghetti" by these authors), as well P_1 projections of hexagonally packed (H_I) aggregates of these cylinders. The micrographs presented were in fact almost exactly similar to Figures 6 and 7b of the present report. From the dimensions quoted by these authors, we calculate the H_I phase of their Figure 1c consisted of lipid cylinders packed with a center-to-center spacing of between 53 and 61 Å. (The variability could easily arise because of the heterogeneous chemical composition of the phospholipid preparation.)

Unfortunately Korman *et al.* misidentified the H_I phase (their Figure 1c) as being a novel lamellar structure which they called a "doublet bilayer." This conclusion could only have been reached through the neglect of the principles of projection presented here and elsewhere. A close examination of the aforementioned Figure 1c reveals that if it were indeed a lamellar particle of the structure proposed, then all the lamellae must be standing on edge exactly parallel to the electron beam. Moreover the authors have neglected the fact that the so-called "singlet bilayers" on the edges of the supposed "doublet bilayer": aggregates are continuous with tubular structures in the "spaghetti" regions, a fact which is not surprising if the structures in Figure 1c are merely ordered arrays of these tubes.

One interesting feature of H_I phase observed by Korman *et al.* in their Figure 1c is that the dark lines corresponding to stain are alternately broad and narrow. This feature is predicted to occur in projections of the H_I phase in which the direction of projection differs only slightly from P_1. We assume that as in our Figure 6, the H_I phase consisted of only a few layers of cylinders lying flat on the grid, which may have inclined very slightly from a position normal to the electron beam. Korman *et al.* interpreted the narrow lines as "fissures" between the hydrocarbon regions of adjacent monolayers of lipid, into which stain penetrates during drying. This is an unlikely as well as unnecessary hypothesis.

In view of the misinterpretation of the structures of the phospholipid aggregates in Figure 1 of Korman *et al.*, the ensuing discussion of the significance of the "doublet bilayer" in mitochondrial structure seems to be invalidated. Perhaps it is possible that the structures of the complexes formed by phospholipid and mitochondrial headpiece–stalk sectors are as proposed by Korman *et al.*, but the present writers admit their inability to discern in the micrographs of these complexes, the structural features attributed to them. Again, the broader implications of the "doublet bilayer" structure of phospholipid aggregates, brought forth by Korman *et al.* in their summarizing discussion, cannot be accepted at this time.

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