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LAMELLAR AND HEXAGONAL LIPID PHASES VISUALIZED BY FREEZE-ETCHING

DAVID W. DEAMER, ROBERT LEONARD, ANNETTE TARDIEU* AND DANIEL BRANTON**

*Department of Zoology, University of California, Davis, Calif. 95616 (U.S.A.), *Centre Génétique Moléculaire, Centre National de la Recherche Scientifique, Gif-sur-Yvette, 91 (France) and **Department of Botany, University of California, Berkeley, Calif. 94720 (U. S.A.)*

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SUMMARY

1. Rapid freezing of lipid-water preparations preserves the structure of the high temperature phases. Both lamellar and hexagonal phases can be readily demonstrated by electron microscopy and X-ray observations.

2. Without careful controls, contaminants are readily deposited on fresh fracture faces. The contamination can take the form of particles resembling those found on natural membranes.

3. Neither degree of saturation, degree of hydration, nor cholesterol admixture significantly affects the appearance of lamellar fracture faces which appear uniformly smooth. On uncontaminated specimens, no structures were found which resembled the particulate material of natural membranes.

INTRODUCTION

Many hypotheses of membrane structure are based, to some extent, on electron microscopic evidence. The electron microscope allows direct visualization at the near molecular level and makes it possible to examine specific areas within membranous structures. The freeze-fracture and freeze-etch methods of electron microscopy are particularly useful because they expose structure within the hydrophobic plane of biological membranes¹⁻⁷. In the freeze-fracture technique, a specimen is rapidly frozen to liquid nitrogen temperature, fractured *in vacuo*, shadowed and replicated with platinum and carbon. The specimen is dissolved away, and the replica may be examined in an electron microscope. In freeze-etching, ice is sublimed from the fractured specimen before it is shadowed. Etching sometimes reveals greater detail and may provide more information than fracture alone.

Since freeze-fracture planes and the resulting electron microscopic images depend on the physical state of membrane components, the significance of freeze-fracture and freeze-etch results would be greatly enhanced if it could be shown that phase transitions are avoided during rapid freezing. One way to test the technique in this regard is to examine fracture faces in bulk-phase lipid systems. X-Ray diffraction studies of model systems involving lipids, proteins and water^{8,9} reveal

a remarkable polymorphism which is highly sensitive to temperature and water concentration. It is known that the low-temperature equilibrium conformation of lipid hydrocarbon chains is stiff, frequently with rotational disorder; the only structure compatible with stiff chains is lamellar. At higher temperatures the chains become more liquid and less ordered; structures compatible with these more liquid chains include hexagonal, cubic and other phases in which the chains fill volumes of odd shape⁸. If rapid freezing could be demonstrated to preserve hexagonal phases, it would suggest that complex liquid-crystalline phases might also be preserved in frozen biological membranes.

In the present study, we addressed ourselves to the following questions. (1) Can the various lipid phases defined by X-ray methods be preserved and visualized by the freeze-etch technique? (2) What is the effect of hydration on the fracture faces? (3) What is the effect of unsaturation on the fracture faces? (4) Are there any structures in bulk-phase lipid systems that can account for the particulate appearance of biological membrane systems?

METHODS

Lipids

Fatty acids and cholesterol were purchased from Applied Science Laboratories, Inc. (State College, Pa.). Lysolecithin was purchased from Calbiochem, Inc. (Los Angeles, Calif.). L- α -Dipalmitoyl phosphatidyl choline was purchased from Mann Research Laboratories (New York, N.Y.). Strontium laurate and strontium palmitate were prepared according to the method of SPEGT AND SKOULIOS¹⁰. Egg lecithin was prepared by the method of LEA AND RHODES¹¹. Calcium cardiolipin (a gift of M. Faure) was precipitated from an aqueous solution of sodium cardiolipin prepared from beef heart¹².

Freeze-fracturing and freeze-etching

Specimens were affixed to 3-mm flat copper discs or dish-shaped nickel or copper discs and frozen in liquid Freon. Anhydrous lipids and fatty acids which tended to crumble during fracturing were either melted onto the discs, pressed into pellets or, in the case of calcium stearate and cholesterol, embedded in ethylene glycol prior to freezing. All materials with unsaturated bonds were handled in a dry nitrogen atmosphere. Specimens were fractured and replicated in a Balzers freeze-etch apparatus as described elsewhere^{1,13}. Etching conditions were varied and are specified in the figure legends. Shadows are white, and all figures are mounted with the shadow direction from bottom to top.

X-Ray diffraction

The X-ray experiments were carried out *in vacuo* in a Guinier-type camera, with bent quartz monochromator. The sample, 1 cm in diameter and 1 mm thick, was mounted between thin mica sheets in a 40-g vacuum-tight brass holder. The sample holder was supported by a stand in which the temperature could be controlled from -30 to 300°. In order to correlate freeze-etch and X-ray diffraction results, samples for X-ray diffraction were cooled as rapidly as possible by plunging the mounted specimens into liquid nitrogen and then remounting them in the diffraction

stand which had been precooled to -30° . Diffraction patterns were obtained before and after freezing.

Repeat distances

Light diffraction patterns of selected regions in high contrast transparencies of electron micrographs were produced with a 0.5-mW gas laser beam (Electro-Optics Inc., Palo Alto, Calif.) or a modified light microscope system¹⁴. The diffraction patterns were recorded on film, and spacings were measured from densitometer tracings. The electron microscopic magnifications were individually measured on photographs of gratings (2160 lines/mm) whose replicas were placed on the same electron microscope grid with the freeze-etch replica.

RESULTS

Freeze etching

During preliminary experiments we noted occasional variability in the appearance of identical lipids. For example, samples of phospholipid appeared either smooth (Fig. 1) or particulate (Fig. 2) depending upon which of two freeze-etch machines was used. A thorough analysis of the possible variables led us to ascribe the particles seen in Fig. 2 to contamination of the fracture face in the vacuum of the freeze-etch device. Specifically, we found that if the cooling system of the knife was shut off for more than 3 min during final cutting, the specimen assumed an orange-peel appearance (Figs. 3 and 4) similar to that observed by FLUCK *et al.*¹⁵. Condensable vapors trapped on the knife lead-in coils may have contaminated the specimen face when the knife cooling system was turned off. Specimens were also contaminated if the cutter was not positioned directly above the sample after the final fracture (Figs. 5 and 6). The pattern of contamination was specific and reproducible. Turning off the knife cooling resulted in contamination of the lipids but not the surrounding ice and always produced an orange-peel effect (Fig. 4). Improper positioning of the knife caused contamination of both lipid and ice, but the contaminant was always more particulate and sharply outlined on the lipid faces than on the ice surfaces (Fig. 6). We found that many of the lipids were more sensitive to contamination than our previous experience with biological membranes had led us to expect. Fatty acids and their salts were particularly troublesome and sometimes showed particles (Fig. 9) or plaques (Fig. 11) in spite of efforts to prevent contamination.

As a result of these and other preliminary experiments we learned to reduce contamination to a minimum. In most cases proper knife cooling and positioning prevented detectable contamination. Nevertheless, subtle differences in the quality of the fracture faces were evident from day to day and these variations rather than resolution problems defined the limits within which it was possible to say whether a given lipid was "the same as" or "different than" another lipid in appearance.

Lamellar phases

The simplest lipids were an obvious starting point for this study. Therefore, stearic acid, oleic acid, calcium stearate, calcium oleate and strontium palmitate were fractured and replicated (Figs. 7-11). Each formed lamellar fracture faces,

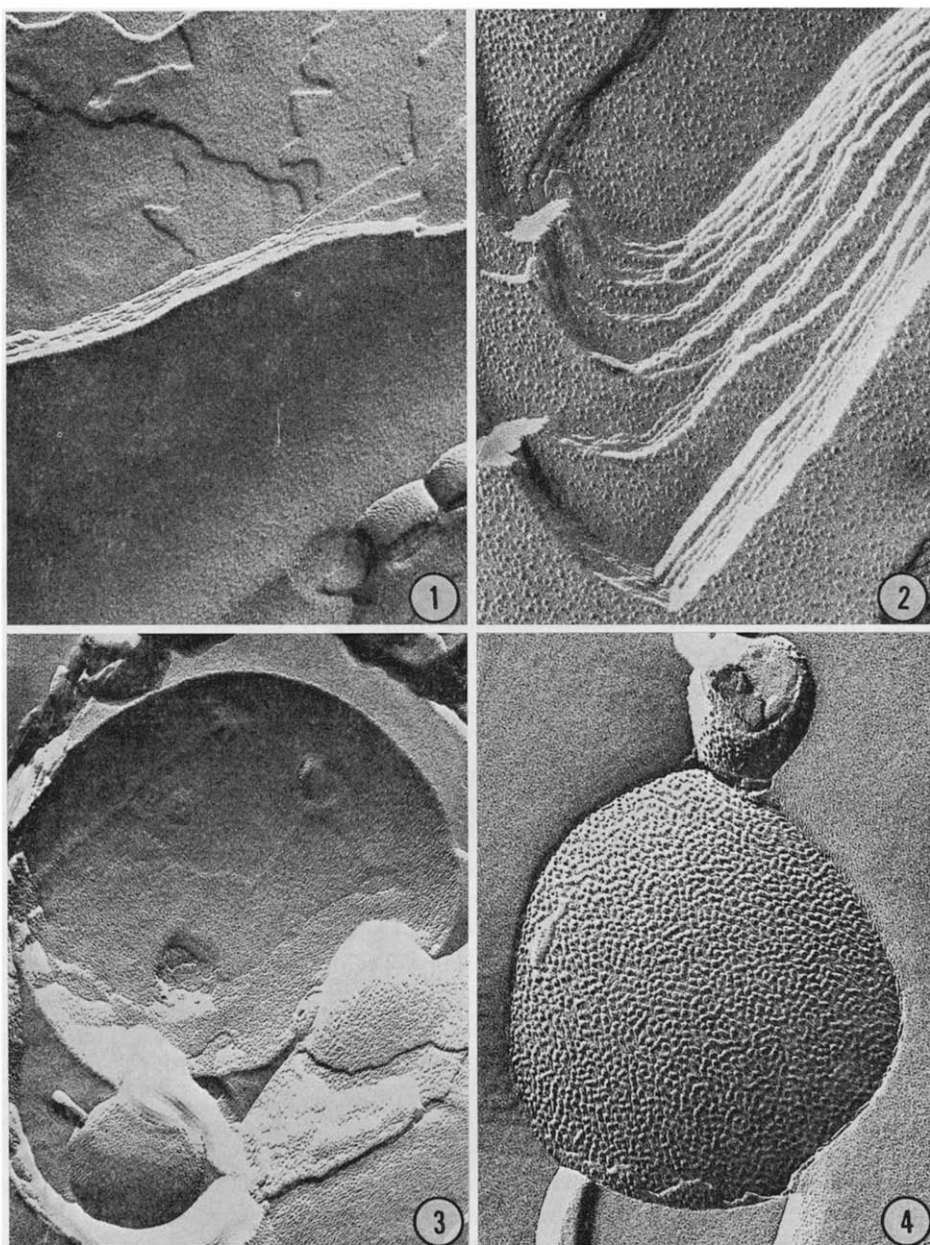
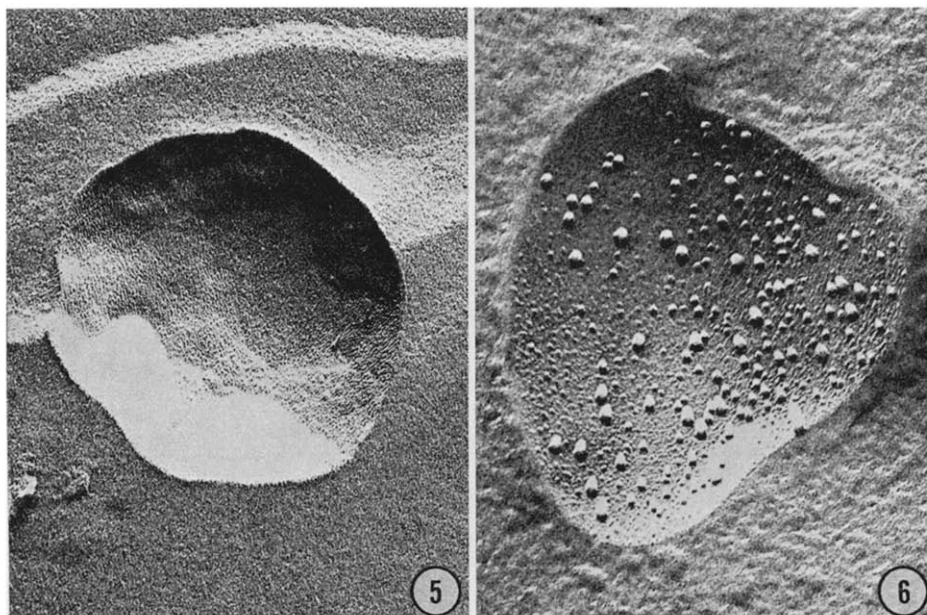


Fig. 1. Egg lecithin-cholesterol, 1:1 mole ratio, 50% (w/v) dispersed in water. No etching. 100 000 \times .

Fig. 2. Egg lecithin-cholesterol, 1:1 mole ratio, 50% (w/v) dispersed in water. Compare with Fig. 1. No etching. 100 000 \times .

Fig. 3. Egg lecithin, 10% (w/v) dispersed in water. Shadowing was carried out immediately following fracture at -150° (no etching). Cutting arm and knife were cooled continuously for 30 min prior to fracture and replication. Fractured liposomes show smooth surfaces free of particles. 100 000 \times .

Fig. 4. Egg lecithin 10% (w/v) dispersed in water. Liquid nitrogen to cutting arm was turned off for 8 min prior to fracturing at -150° . Shadowing was carried out immediately following fracture as in Fig. 3. Particulate material has accumulated on all fractured liposome faces, but not on surrounding ice. 100 000 \times .



Figs. 5 and 6. Egg lecithin, 5% (w/v) dispersed in water. The specimens in Figs. 5 and 6 were fractured simultaneously at -150° on a multiple stage. Following fracture, the knife was placed over the specimen in Fig. 5 but not over the specimen in Fig. 6. Both specimens were shadowed 1 min later. Heavy contamination accumulated on the liposomes and the surrounding ice of the uncovered specimen (Fig. 6), but not on the covered specimen (Fig. 5). Both $100\,000\times$.

as would be expected from X-ray diffraction data. In general, the faces were smooth and there were no obvious differences between fracture faces of saturated and unsaturated fatty acids or soaps. Oleic acid presented an interesting case, since it is liquid at room temperature and could be conveniently frozen slowly or rapidly. When frozen slowly by cooling in air at 0° the specimen had typical lamellar fracture faces (Fig. 8). When frozen rapidly by immersion in liquid Freon, no fracture faces were apparent (Fig. 12). This suggests that slow cooling permits extensive crystalline planes to form, while rapid cooling fixes a relatively amorphous structure.

Dry dipalmitoyl phosphatidyl choline, a relatively simple phospholipid containing only saturated palmitic acid chains, was chosen as a lamellar phase phospholipid for fracturing and replicating. Replicas of this material (Fig. 13) showed extended, smooth lamellae similar to those of fatty acids and their soaps.

Unsaturated phosphatidyl choline (egg lecithin) was prepared at room temperature with varying amounts of water for comparison with the saturated dipalmitoyl phosphatidyl choline. At low water content, egg lecithin showed extensive lamellar planes and areas where lamellae had been cross fractured (Fig. 14). The fracture faces were smooth. When the lipids were dispersed in water as liposomes (Fig. 3), the smooth appearance of the lamellae was not affected. Vesicles of egg lecithin formed by the method of REEVES AND DOWBEN¹⁶ also showed smooth lamellar faces (P. PINTO DA SILVA, unpublished observations.) Therefore neither the degree of hydration nor the degree of saturation altered the appearance of the fracture plane in lamellar phase phospholipids.

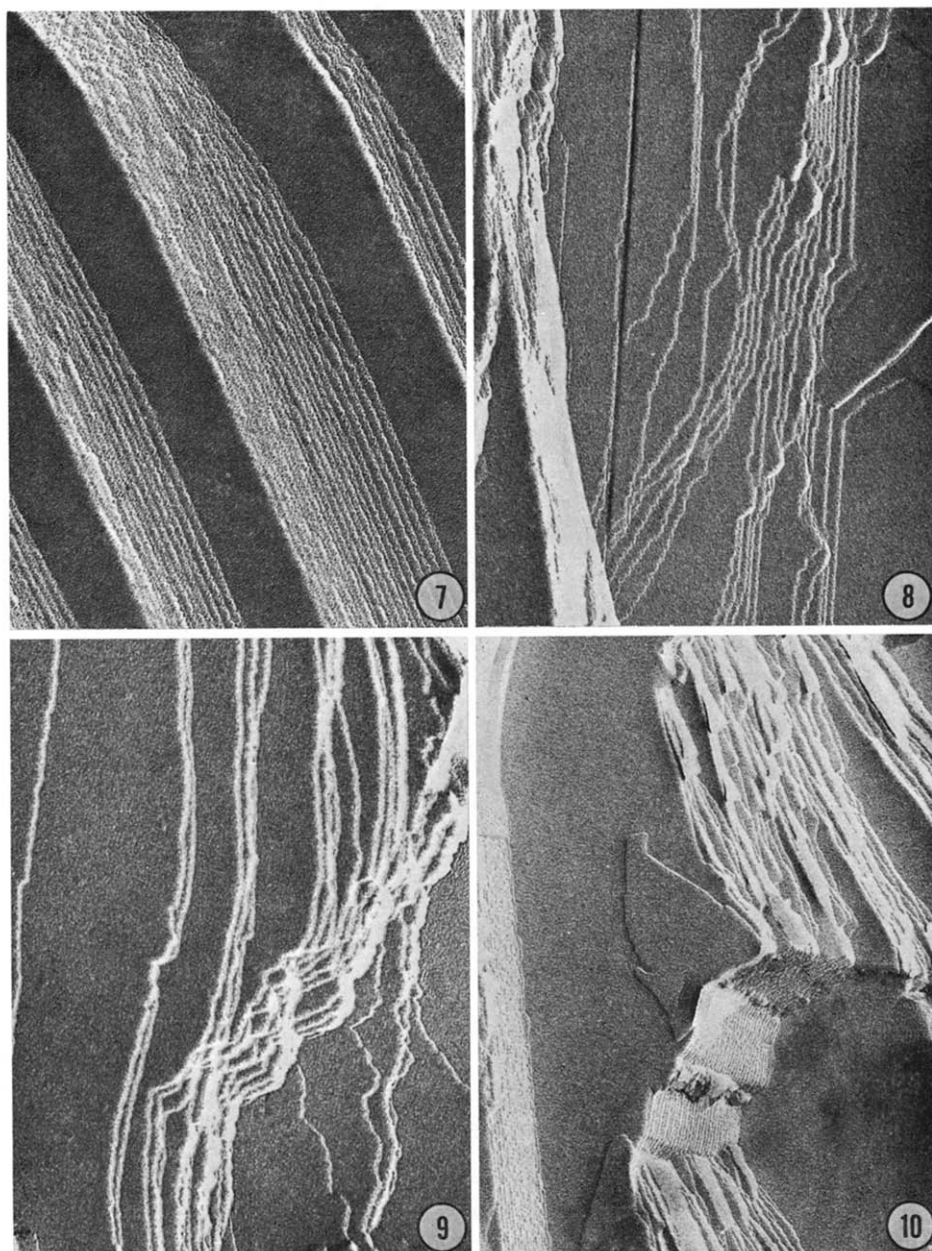


Fig. 7. Crystalline stearic acid. Stearic acid was melted and allowed to recrystallize on the specimen disk. No etching. $120\,000\times$.

Fig. 8. Oleic acid, slowly frozen. A drop of liquid oleic acid was crystallized on the specimen disk at 4° . The disk was then plunged into liquid Freon. No etching. $120\,000\times$.

Fig. 9. Calcium stearate. Crystals of calcium stearate were embedded in ethylene glycol prior to freezing in liquid Freon. No etching. $120\,000\times$.

Fig. 10. Calcium oleate. Crystals of calcium oleate were pressed onto a specimen disk and frozen in liquid Freon. No etching. $120\,000\times$.

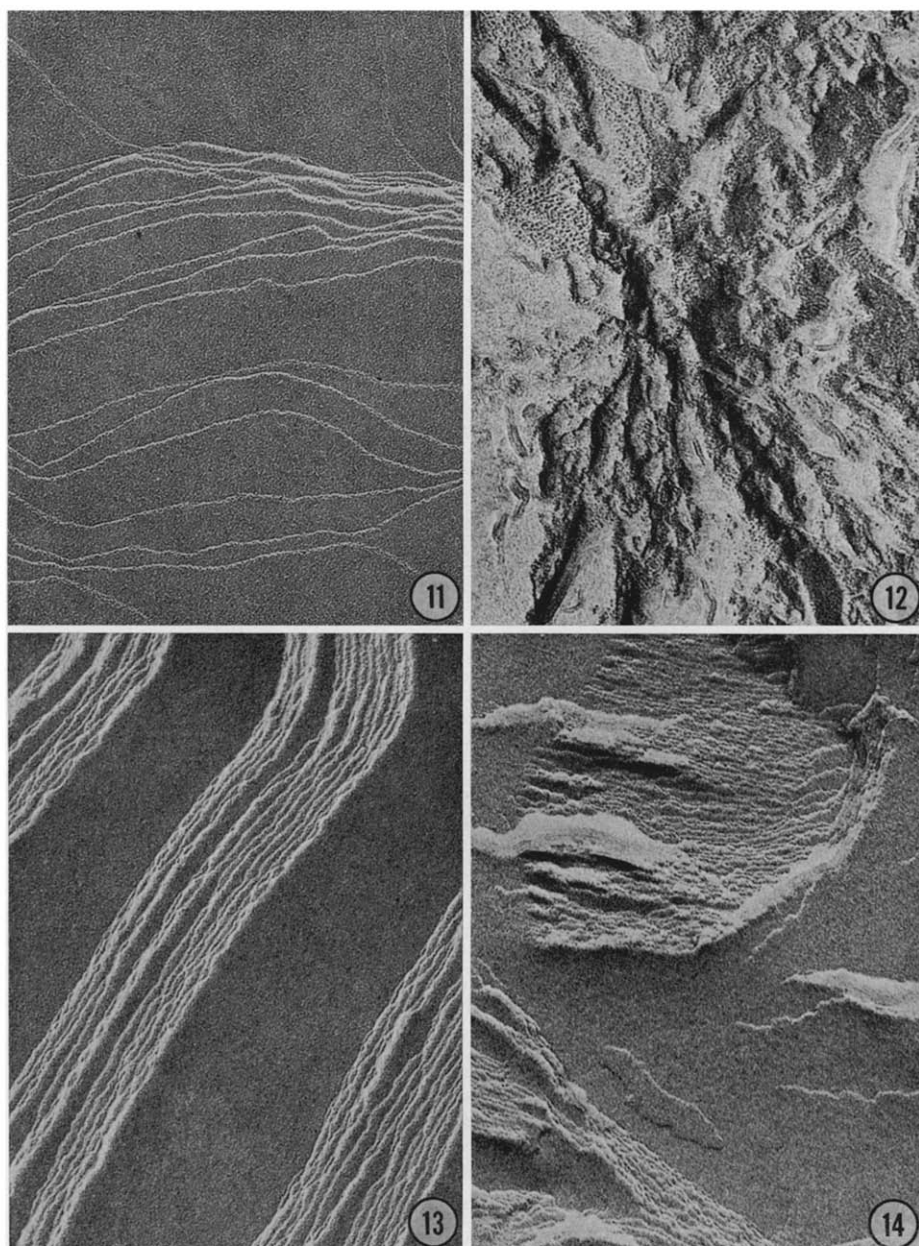


Fig. 11. Strontium palmitate. Crystals of strontium palmitate were embedded in ethylene glycol prior to freezing. No etching. 120 000 \times .

Fig. 12. Oleic acid, rapidly frozen. A drop of liquid oleic acid was frozen on the specimen disk by plunging it directly into liquid Freon. No etching. 120 000 \times .

Fig. 13. L- α -Dipalmitoyl lecithin. Dipalmitoyl lecithin was pressed onto a specimen disk and frozen in liquid Freon. 1 min etching at -100° . 120 000 \times .

Fig. 14. Egg lecithin. Water content was minimized by carrying out all manipulations under dry nitrogen. The dry lecithin was pressed onto a specimen disk and frozen in liquid Freon. No etching. 120 000 \times .

Hydrated egg lecithin-cholesterol containing more than 35 % water is known to be a two-phase system of lamellar phase vesicles of variable size at equilibrium with water^{17,18}. Mixtures of hydrated egg lecithin-cholesterol (mole ratio 1:1) were fractured. The fracture faces (Fig. 15) were indistinguishable from those of other lamellar phases. Although admixture of cholesterol prevents the phase transition that normally occurs in the hydrocarbon chains when hydrated egg lecithin alone is cooled below 3° (ref. 19), this admixture did not alter the nature of the fracture plane.

Crystals of recrystallized cholesterol were fractured and replicated in the usual manner (Fig. 16). Again, the fracture faces were smooth lamellae, although fracture edges were often straight, in contrast to the curved edges of fractured phospholipid.

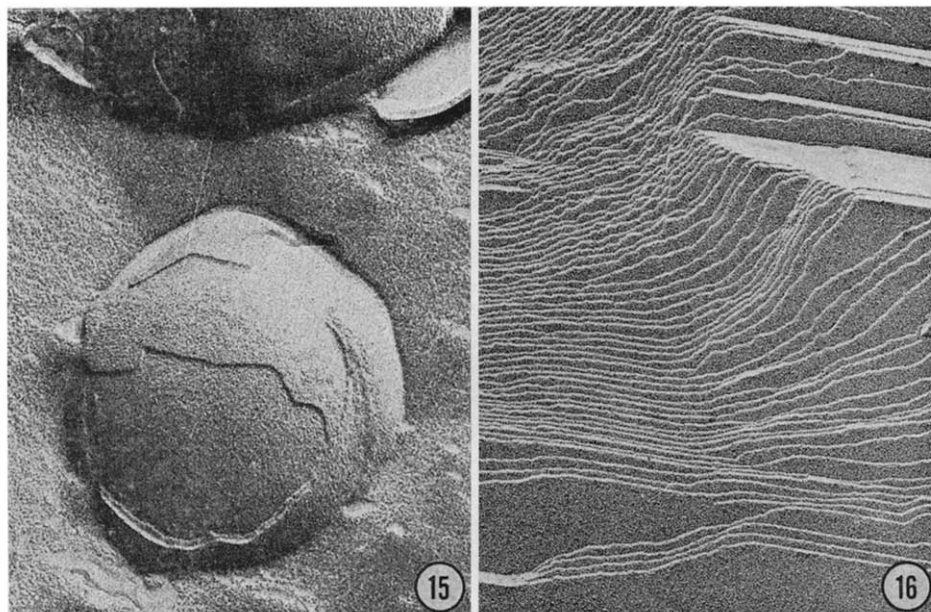


Fig. 15. Egg lecithin-cholesterol, 1:1 mole ratio, 10 % (w/v) dispersed in water. Compare with Fig. 3. 1 min etching at -100° . 90 000 \times .

Fig. 16. Cholesterol. Recrystallized cholesterol was embedded in ethylene glycol prior to freezing in liquid Freon. No etching. 90 000 \times .

Hexagonal phases

Several hexagonal lipid-water phases were examined. Previous work has shown that in hexagonal I phase the lipid molecules are radially arranged in rods of indefinite length, with the non-polar hydrocarbon tails directed inward and the polar heads outward. Water fills the interstices, and the rods are packed hexagonally⁸. In hexagonal II phase the hydrocarbon tails are directed radially outward from the central axis of the lipid rods, and the polar heads surround water channels in the center of the rods. Again the rods are hexagonally packed⁸.

Calcium cardiolipin was chosen as a stable hexagonal II phase for freeze-fracture because the only liquid-crystalline phase present in the calcium cardiolipin-

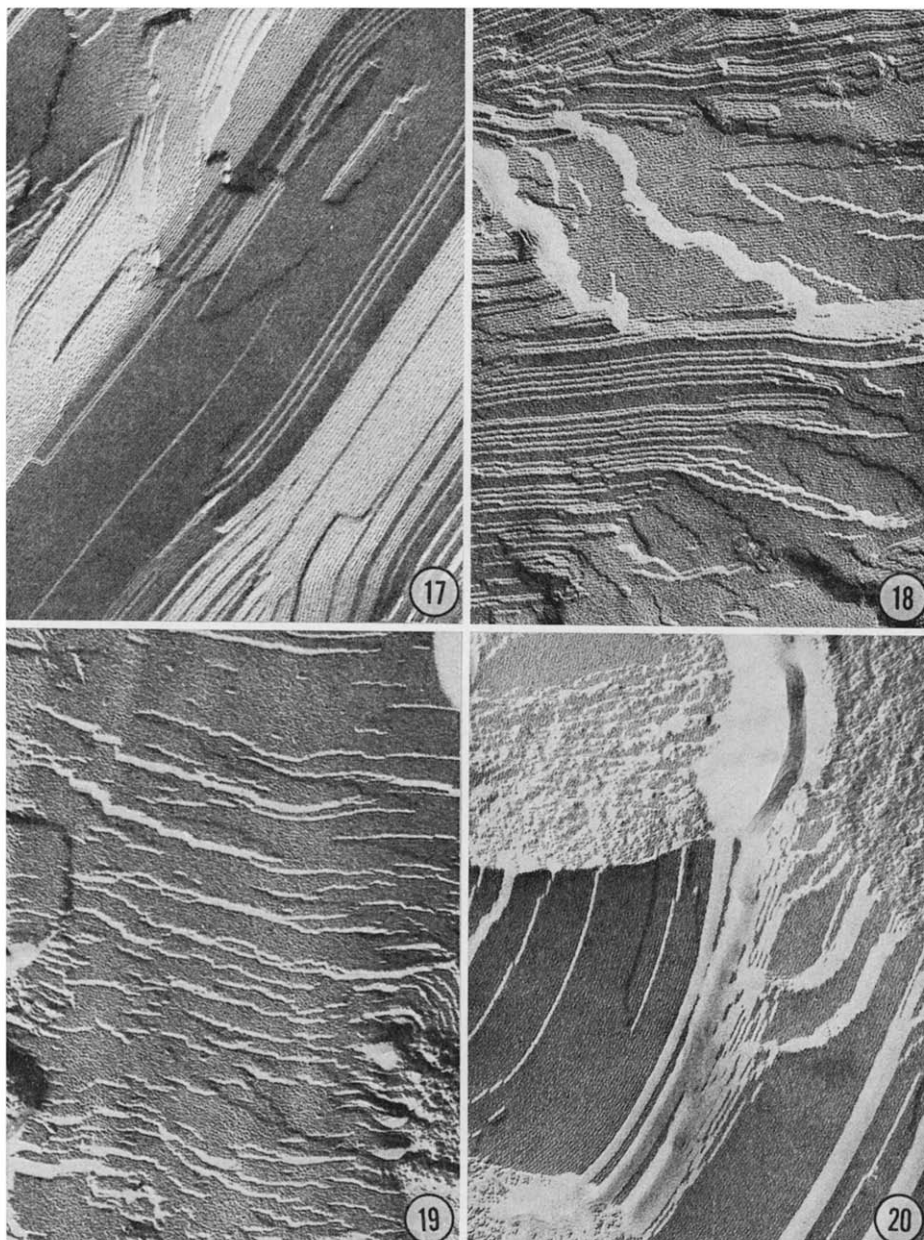


Fig. 17. Calcium cardiolipin. Calcium cardiolipin was precipitated from an aqueous suspension of sodium cardiolipin by addition of 0.1 M CaCl_2 . The precipitate was dried by filtration and pressed onto a specimen disk. 1 min etching at -100° . $90\,000\times$.

Fig. 18. Egg lecithin-cholesterol, 1:1 mole ratio. Water content was minimized as described in Fig. 14. No etching. $90\,000\times$.

Fig. 19. Strontium laurate. Melted strontium laurate at 300° was rapidly frozen in liquid Freon. No etching. $90\,000\times$.

Fig. 20. Total spinach chloroplast lipids. 50% (w/v) gel in water. 1 min etching at -100° . $90\,000\times$.

water system from room temperature to -30° is the hexagonal II phase (T. GULIK-KRZYWICKI, unpublished results). A replica fractured at -100° is shown in Fig. 17. Calcium cardiolipin freeze-fractured at -30° , the lowest temperature at which our equipment permitted X-ray diffraction analysis, appeared similar to the material fractured at -100° . In both cases, the fracture faces appear distinctly different from those of the lamellar phase. Although there were preferred fracture planes, the fracture faces were not smooth but were composed of long, parallel lines. The fracture faces thus took on a ribbed appearance. Furthermore, fractures occurred along at least two fracture planes at approx. 120° to each other.

Fracture faces resembling those of calcium cardiolipin were also found in anhydrous mixtures of egg lecithin-cholesterol (mole ratio 1:1) (Fig. 18). X-Ray diffraction studies of egg lecithin-cholesterol confirmed that it was in a hexagonal II phase. When water was added (50 % by weight), the hexagonal phase completely disappeared and was replaced by a lamellar phase (Fig. 15). A similar pattern of fracture planes was also found in strontium laurate heated to 300° and rapidly cooled by plunging into liquid Freon (Fig. 19). However, the spacings were too small to resolve the individual rods. X-Ray diffraction studies have shown that strontium laurate is in the hexagonal II phase at 300° (ref. 20).

The same ribbed pattern was also found in hydrated total lipid extracts of spinach chloroplasts (Fig. 20), but it is unknown which lipids of this complex mixture are involved in the structures shown. The replicas of the spinach lipids also contained smooth lamellar areas and other unidentified configurations. X-Ray diffraction of such preparations suggests the existence of several different phases, none of which could be clearly resolved²¹.

Lysolecithin-water mixtures form hexagonal I phases²² and were prepared for freeze-etching by incubating a mixture of 60 % lipid-40 % water at 37° for several days. The incubation was carried out in a closed vessel with the lipid samples already on discs so that they could be rapidly frozen from the 37° incubation temperature. Replicas of this phase are shown in Figs. 21 and 22. As in the hexagonal II phase, the fracture faces are composed of indefinitely long, parallel lines (Fig. 21). However, it was not possible to determine whether the rods were fracturing through centers or along surfaces. If the samples were air-dried for 2 min or more before being frozen, extensive regions reverted to the lamellar phase.

Areas of replicas which apparently represented cross-fractures through the hexagonal arrays of lipid rods (Fig. 22) were also found. If the reader will view Fig. 22 by sighting along the plane of the page and simultaneously rotate the figure, three distinct linear arrays will be seen. These form angles of approx. 120° with each other. Evidence for the spacing and angles of the arrays seen in Fig. 22 was also provided by light diffraction patterns (Fig. 22, inset).

X-Ray diffraction

X-Ray diffraction patterns of dry or nearly dry phospholipid samples before and after freezing were quite similar, and the 4.5-Å diffuse band typical of liquid paraffins was found in both. This demonstrates that after freezing the liquid structure was preserved and crystallization of the paraffin chains was avoided.

On the other hand, the hexagonal I phase of lysolecithin with 40 % water became lamellar upon freezing, suggesting that the chain conformation had been

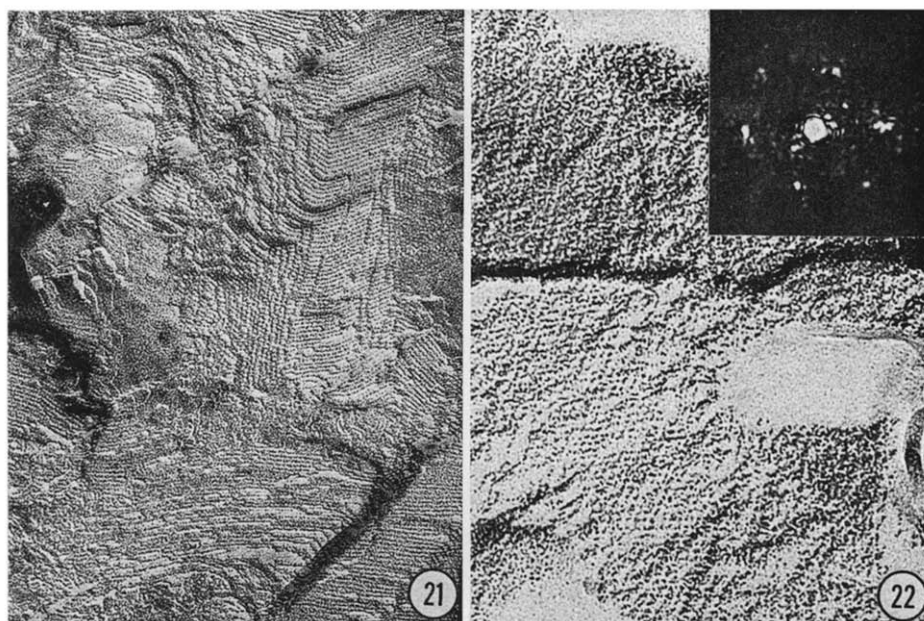


Fig. 21. Lysolecithin, 60 % (w/v) dispersed in water. Lipid rods in face view. No etching. 90 000 \times .

Fig. 22. Lysolecithin, 60 % (w/v) dispersed in water. Lipid rods cross fractured. 1 min etching at -100° . 180 000 \times . Inset: laser diffraction pattern of the same area.

transformed from a liquid to a stiff conformation. This finding was contrary to freeze-fracture observations which suggested that extensive hexagonal regions were preserved after freezing. The apparent discrepancy may be explained by the fact that time-dependent phase transitions occurred in the X-ray experiments but not the freeze-etch experiments because cooling was slower in the X-ray diffraction experiments where the mass of the sample and sample holder was much greater than in the freeze-etching experiments.

Repeat distances

Table I compares X-ray and freeze-etch measurements of repeat distances for lamellar and rod-like structures. The freeze-etch measurements are subject to substantial error because the angle of fracture varies from one part of the sample to another. In general, however, the repeat distances found in freeze-fractured material closely approximate those derived from X-ray data.

DISCUSSION

Our results show that consistent and recognizable features distinguish different lipid-water phases when prepared by the freeze-etch technique. The extended smooth regions are characteristic of the lamellar phases. Alterations in the character of either the hydrophilic or hydrophobic portions of the lipid do not detectably alter the appearance of the smooth fracture faces of the lamellar phase. Similarly, the ribbed fracture faces are found only in hexagonal phases. Thus, certain characteristic

TABLE I
PHYSICAL PARAMETERS OF LIPID SYSTEMS

Phase	Repeat distances (\AA)	
	Freeze-fracture	X-Ray diffraction
<i>Lamellar</i>		
Strontium palmitate	44	43
Dipalmitoyl phosphatidyl choline	54	55
Egg lecithin (dry)	57	50–60
<i>Hexagonal</i>		
Calcium cardiolipin	52	52
Lecithin-cholesterol (dry)	48	48.5
Strontium laurate	< 30	23.2
Chloroplast lipids	49	46.7
Lysolecithin	60	66

patterns when seen in freeze-etch replicas, can be considered diagnostic for certain molecular arrangements within the specimen, and many aspects of the long-range molecular order detected by X-ray diffraction can be visualized by freeze-etching.

Initially we considered the possibility that unsaturated fatty acid chains might produce some of the particulate textures seen on fracture faces of biological specimens. However, our results with unsaturated lipids do not support this possibility. Although higher-resolution shadowing techniques might reveal subtle differences between the fracture faces of saturated and unsaturated lipids, under our conditions both were fairly smooth and did not contain structures that could account for the particles found in some biological membranes.

We also considered the possibility that some of the roughness in freeze-etched biological membranes could be produced by lipid hydration. However, hydration of phospholipid had little effect on the appearance of the fracture faces, and all of our evidence shows that bulk-phase lipids alone do not contain discontinuities which can account for the particulate appearance of fractured biological membranes. The particles in biological membranes may be due to proteins or localized and specific protein-lipid interactions.

We have examined only a few lamellar and hexagonal bulk-phase lipids. More work is required to determine whether other lipids and conformations can survive rapid freezing. However, it is encouraging to note that specimen hydration does not appear to be a critical factor in determining whether a specimen will fracture or what the fracture faces will look like. Even where hydration decreases the stability of a high-temperature liquid phase, our results with lysolecithin suggest that phase transitions can be inhibited by very rapid freezing.

The present observations are consistent with the notion that fractures occur along non-polar regions. Non-polar fractures for lamellar and hexagonal phases are diagrammed in Fig. 23. The lamellar phase fracture is easily understood in terms of non-polar fracture planes (Fig. 23a). Fractures would part lipid bilayers along the plane of hydrocarbon tails where only weak Van der Waal's interactions act to stabilize the bilayer⁴. Non-polar fracture planes also provide a ready explanation for the appearance of fracture faces in hexagonal II phase. The fracture mode illus-

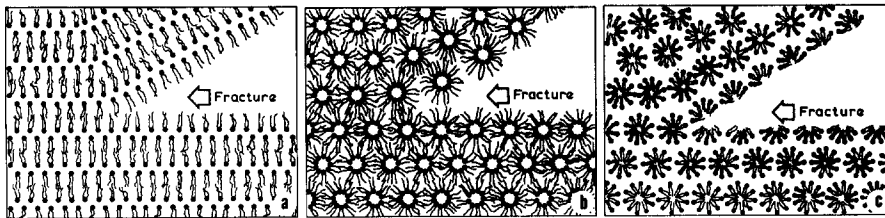


Fig. 23. Possible fracture modes in the various lipid phases. a. Lamellar. b. Hexagonal II. c. Hexagonal I.

trated in Fig. 23b would result in linear arrays of lipid rods in the plane of the fracture. A polar fracture mode does not provide a satisfactory basis for this image. However, it is more difficult to account for the appearance of the hexagonal I phase following fracture. It might be expected that lipid rods of this phase would not be visible without subliming away some of the surrounding ice, but the hexagonal phase is clearly visible in non-etched preparations (Fig. 21). Etching did not noticeably alter the appearance of the lipid. One possible fracture mode is shown in Figure 23c, where fractures are assumed to jump from rod to rod. This suggestion assumes that the exposed half-rods shadow differently than the surrounding ice.

End views of rods in hexagonal array are rare because there are no clearly defined fracture planes normal to the rod axes. However, it is possible to find such arrays in hydrated specimens where etching has lowered the ice table between the lipids, exposing hexagonally packed rods in sufficient relief to be shadowed and replicated (Fig. 22).

Previous workers have used positive staining^{23,24} and negative staining^{25,26} as well as freeze-fracturing^{27,28} to study lamellar phases and hexagonal phases of lipids. Our results extend their observations and demonstrate that the smooth lamellae seen by freeze-fracturing are characteristic of the lamellar phases of a variety of lipids regardless of hydration, saturation or cholesterol content. Positive staining²⁴ and negative staining^{25,29} have also been used to examine hexagonal phases. Our results, as well as preliminary observation of RUSKA³⁰ show that this phase can also be characterized by freeze-fracture techniques.

Unlike positive or negative staining techniques which show mostly cross sections of the lamellae or end views of the hexagonally packed rods, freeze-fracturing shows extensive face views of these structures. In this sense the techniques complement each other. Although staining techniques can provide an image of a hydrated specimen²⁶, fixation or drying artifacts as well as the possibility of interactions between the specimen and the aqueous staining solution are hazards that cannot be ignored³¹. Because a rapid temperature drop is the only treatment required for freeze-fracturing, and because freeze-fracturing appears to preserve the structure of lipid-water phases, it should be particularly useful in future studies of lipids and lipoproteins.

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