SOME ELECTRON MICROSCOPICAL OBSERVATIONS ON LIQUID-CRYSTALLINE PHASES IN LIPID-WATER SYSTEMS

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ABSTRACT

OsO_4 fixation preserves some liquid-crystalline phases of soaps and phospholipids to an extent that it is possible to observe their structure in electron micrographs of thin sections. Good agreement exists between the structure observed directly and that deduced from x-ray diffraction studies of the same systems.

In the preceding paper, Luzzati has summarized some of the extensive work done in his laboratory on the structure of lipid-water systems (2). By using, mainly, improved x-ray diffraction techniques he succeeded in demonstrating several new liquid-crystalline structures in soap-water systems in which only a lamellar structure had been assumed in the past. In our own work on the electron microscopy of phospholipids and soaps we sometimes encountered non-lamellar structures, but being unaware of the factors which control their existence, we were unable to get consistent results. Recently, however, Miss Husson, in Dr. Luzzati's laboratory, has obtained a complete phase diagram of our phospholipid extract (2), and her data have enabled us to fix the material under conditions where only the hexagonal phase existed. Since most of the work on the phase diagrams of soaps so far had been done on saturated compounds, which cannot be fixed with OsO_4, it was also necessary to extend the inquiry to highly unsaturated compounds which can be fixed with OsO_4 (3).

MATERIALS AND TECHNIQUES

The phospholipid extract used in this work has been described earlier (5). Na-linolenate was prepared by adding a solution of NaOH in ethyl alcohol to an alcoholic solution of linolenic acid (The Hormel Institute, Austin, Minnesota) with phenolphthalein as an indicator. The alcohol was subsequently evaporated *in vacuo*. To get the correct concentration and a homogeneous distribution of water in lipid, the latter was dried *in vacuo* over P_2O_5 for several days until the weight remained constant. Then a surplus of water was added and after a macroscopically homogeneous mixture had been obtained, the water was slowly evaporated in a desiccator under reduced pressure of N_2 until weighing indicated that the desired concentration had been reached. The material was then exposed to OsO_4 vapor for 8 to 17 hours. In the case of the phospholipid extract, the preparation of the lipid-water mixture and the fixation were carried out at 37°C in some experiments and at room temperature in others, dehydration and embedding being carried out always at room temperature. The electron microscopical techniques were the same as those described previously (5).

A Hilger microfocus unit was used for the x-ray diffraction work. A simple low-angle camera with slit collimator was built in the workshop of The Rockefeller Institute.

RESULTS AND INTERPRETATIONS

Phospholipid, Lamellar Phase

As can be seen in Fig. 5 of the preceding paper (2), the lamellar phase of the phospholipid-water system exists over a wide range of concentrations at room temperature. All the work on this phase was

therefore done at ~22°C. In a typical experiment the x-ray diffraction diagram of a sample containing 30 per cent water showed four orders of the fundamental spacing of 58 A, the ratios of the orders being $1:\frac{1}{2}:\frac{1}{3}:\frac{1}{4}$. It was thus ascertained that we were dealing with a lamellar structure. The absolute value of the spacing changed during the fixation, dehydration, and embedding process in the same way as has been described for myelin figures in an earlier paper (6), but the ratio of the orders and, therefore, the lamellar structure were preserved. The electron micrographs showed the expected pattern of alternating light and dense bands with a repeat period of 38 A (Fig. 1). This is the same pattern as that found in electron micrographs of myelin figures, and the molecular arrangement is known to be the same in both cases (2, 6) although the two systems differ in their gross structure. Myelin figures are liquid crystals, usually in the shape of thick walled tubes which are filled with and surrounded by another phase of nearly pure water. Phospholipid containing 30 per cent water forms one homogeneous liquid-crystalline phase consisting entirely of alternating and evenly spaced layers of lipid and water. The orientation of the layers is different in different regions of the specimen and they can be observed in the microscope only where they are oriented in such a way that the plane of the lipid leaflets is approximately parallel to the optical axis of the microscope. This limitation could account for the large areas in which no regular structure is found. But these may be due not only to unfavorable orientation. Imperfect fixation of the specimen may also give rise to areas in which no band pattern is visible. Indeed, after fixation the lines in the diffraction diagram sometimes become less sharp, fewer orders of the fundamental spacing can be picked up, and the diffuse scattering is increased. From a previous discussion (4, 6) on the interpretation of electron micrographs of OsO_4-fixed lipid structures, it follows that the dark bands represent the layers of hydrophilic groups and the light bands the central parts of the bimolecular leaflets originally containing the hydrocarbon chains of the fatty acids.

Phospholipids, Hexagonal Phase

The hexagonal phase of the phospholipid-water system is only stable at elevated temperatures (2). Most of the work on this phase was, therefore, done at 37°C. In choosing the concentration and temperature we relied on Dr. Luzzati's results obtained with the same phospholipid preparation (2), for our x-ray camera was not equipped with a device for heating the specimen, hence samples prepared for electron microscopy could not be checked by x-ray diffraction. The water concentration in all experiments was kept between 2.5 and 3.5 per cent.

The electron micrographs show a hexagonal array of dark dots on a lighter background (Figs. 2 and 3). The center-to-center distance between the dots is found to be 42 to 45 A. The size and shape of the dots vary considerably and no definite value for their diameter can be given. In general, it seems to be somewhat less than half of the center-to-center distance. A comparison of this pattern with the structure deduced from x-ray diffraction (Fig. 4) indicates that the dark dots correspond to the areas containing the hydrophilic groups of the lipid molecules and, therefore, should represent cross-sections through cylinders.

Besides the hexagonal pattern, a line pattern of alternating light and dark bands with a spacing of approximately 36 A is frequently found in these specimens. The numerical value of this spacing, as compared to the center-to-center distance of the dots, suggests that this pattern arises where short pieces of dense cylinders in the section are slightly inclined with respect to the optical axis of the microscope, either in the 11 or the 20 plane of the hexagonal lattice (see Fig. 4). This assumption is further supported by the observation that the rows of dots which mark these planes in the lattice are often continuous with dark bands which form repetitive patterns and which in different regions of the same microscopic field often run at an angle of approximately 60° to each other (Fig. 2). Smaller spacings of the lattice cannot be expected to give rise to a line pattern since the next smaller spacing, that of the 02 or 31 planes, is already of the same size as the diameter of the dense dots, so that in case of an inclination in this plane no light interspace could appear between the dense lines caused by an "overlapping" of the short pieces of cylinders contained in the section (see Fig. 5).

Pictures of the hexagonal phase identical with those described above were also obtained from samples with a water content below 5 per cent which were fixed at room temperature. This is not surprising since the x-ray diffraction diagram indicates that under these conditions the system consists of a mixture of the hexagonal and the lamellar phases (2), and in the electron microscope small areas

FIGURE 1

(1114/59) Phospholipid in the lamellar phase. The sample contained 30 per cent water when fixed with OsO_4. Most of the area in this micrograph shows the band pattern which is seen when the material is cut approximately normal to the plane of the lipid lamellae. × 450,000.

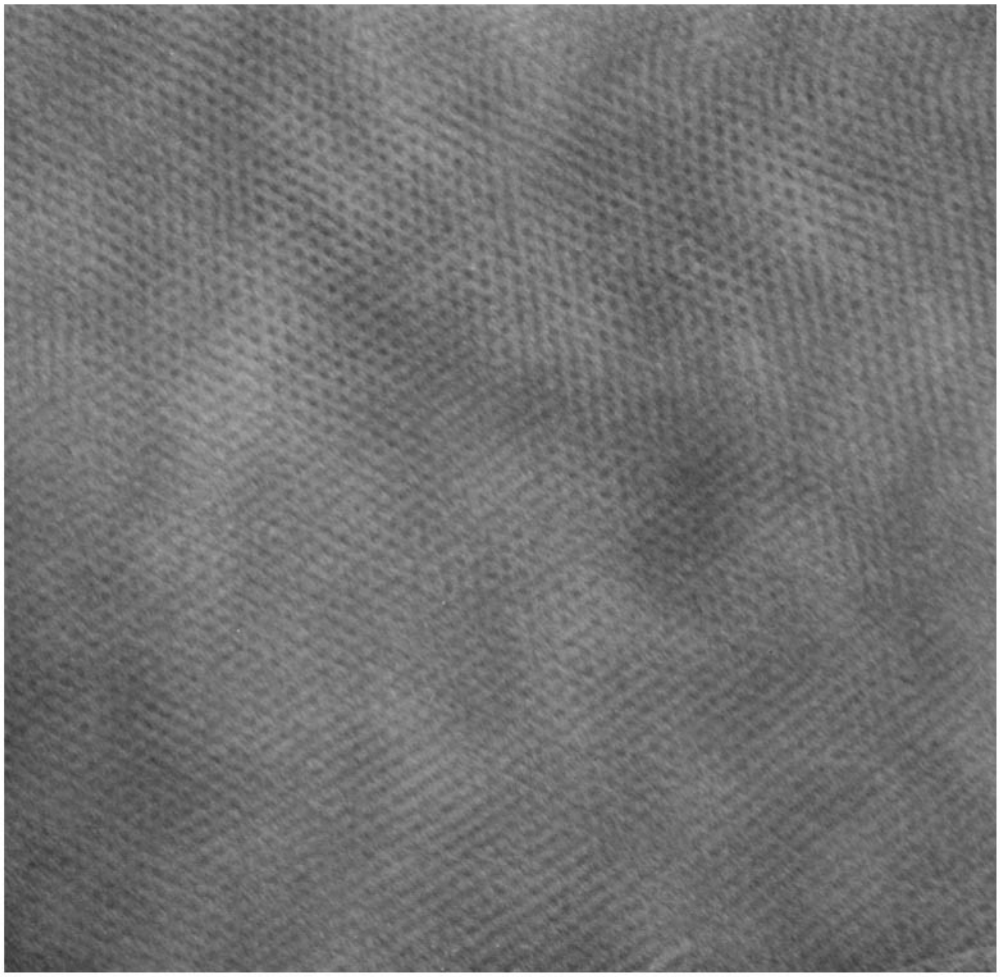

FIGURE 2

(968/60) Phospholipid in the hexagonal phase. The sample contained 3 per cent water when fixed with OsO_4. Most of the area is occupied by hexagonal array of dark dots, which represent cross-sections through cylinders. In other areas a band pattern is visible which probably arises from a slightly different orientation of the cylinders with respect to the direction of view (see text). × 560,000.

which contain only one phase, can be selected. In this case, it was often impossible to decide whether a band pattern in the micrographs was caused by an inclined hexagonal array or by a true lamellar phase. We can not be completely sure, therefore, that the band pattern found in the material fixed at 37°C is always caused by an inclination of the cylinders with respect to the optical axis, and may not sometimes be due to a transformation of the hexagonal into the lamellar phase, occurring during fixation. But in most instances the continuity of dense bands with rows of dots and the intersection of band patterns at 60° angles make the latter possibility appear rather unlikely.

Na-Linolenate, Hexagonal Phase

Na-linolenate at 22°C shows an extended hexagonal phase (middle soap) at water concentrations between 50 and 80 per cent, consisting of cylinders of soap with a constant diameter of 40.5 A (2, 3). A sample prepared in our laboratory, containing 54 per cent water, gave an x-ray diffraction diagram consistent with these findings. After exposure to OsO_4 vapors, however, only a diffuse scatter was found. In spite of this indication of poor preservation the material was embedded and sectioned. In the corresponding electron micrographs,

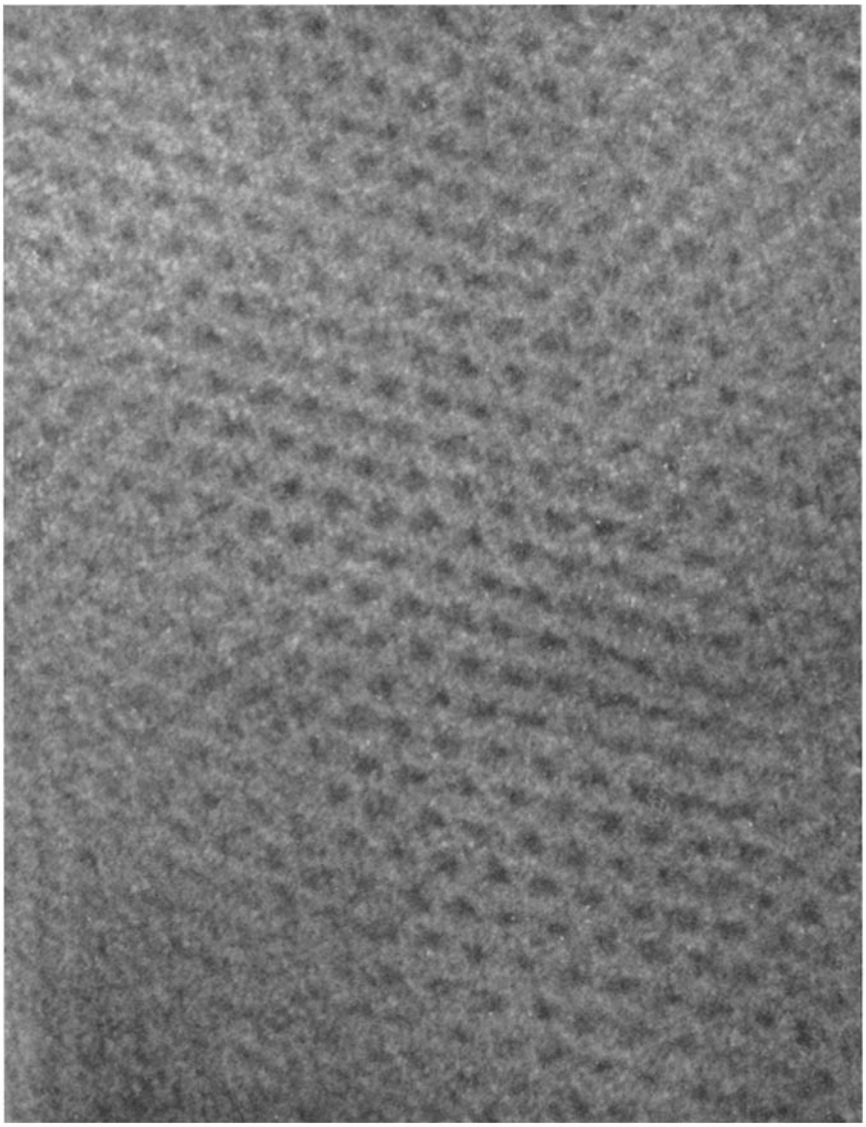

FIGURE 3

(956/60) Same as Fig. 2 at higher magnification to show the varied size and shape of the dark dots. × 1,300,000.

indications of an ordered structure could be found in some areas (Figs. 6, 7). It can best be described as a close packing of rather imperfect hexagons outlined in black. The light central area of the hexagons has a diameter of 25 to 30 A, the dense outline being somewhat less than 10 A wide. No other ordered structure was found.

Since it has been shown that in electron micrographs of linolenic acid soaps fixed with OsO_4 the dense areas correspond to the layers of carboxyl groups (4, 6, 7), one would expect to see in suitably oriented sections of the middle soap a hexagonal array of circles, each encompassed by a dense band. The angular shape actually observed may be due to close packing of the cylinders after removal of the water and to a compression of the material probably during polymerization of the meth-

acrylate or during exposure to the electron beam in the microscope.

Na-Linolenate, Complex Hexagonal Phase

Several attempts to fix the complex hexagonal phase, which in this soap is found at 22°C and water concentrations between 41 and 35 per cent [(3), see also Fig. 3 *d* in the preceding paper (2)] were unsuccessful. After exposure to OsO_4, no indication of an ordered structure could be observed either by x-ray diffraction or in electron micrographs of the embedded material.

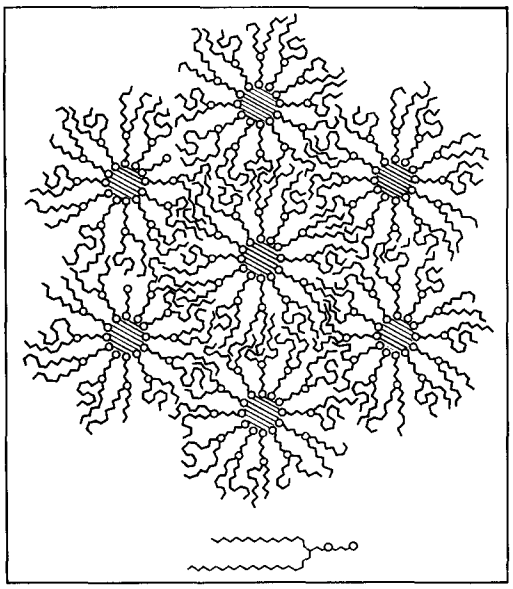

FIGURE 4

The arrangement of lipid and water in the hexagonal phase as seen in cross-section. The hatched areas represent the space occupied by water. The symbol at the bottom is used to represent a phospholipid molecule; the circles mark the site of the hydrophilic groups. Nothing is known about the actual configuration of the hydrophilic end of the molecule in this phase. The radial orientation given here is arbitrary. The irregular shape of the rest of the molecule is meant to indicate the essentially fluid state of the hydrocarbon chains in the liquid crystal.

DISCUSSION

The structures observed in electron micrographs of OsO_4-fixed lipid-water systems closely agree with the structures of the liquid-crystalline phases as deduced from x-ray diffraction data. This shows that OsO_4 acts as a good fixative for the lipids used. It actually preserves the general features of the molecular arrangement as it existed in the native material. Preservation is, however, not perfect. If the water content of the system to be fixed is high, as, for instance, in the middle soap, or if the liquid-crystalline phase is stable only over a very narrow range of concentrations, as in the complex hexagonal phase of Na-linolenate, the structure may be preserved only in small regions of the specimen or not at all. This, of course, is easily recognized and should not lead to wrong conclusions. For a study of such systems by electron microscopy, it would be much more dangerous if the fixation with OsO_4 by itself could transform one ordered structure into another. So far we have no conclusive evidence that this actually happens if OsO_4-vapor is used for fixation. According to Luzzati's evidence, however, it is to be expected

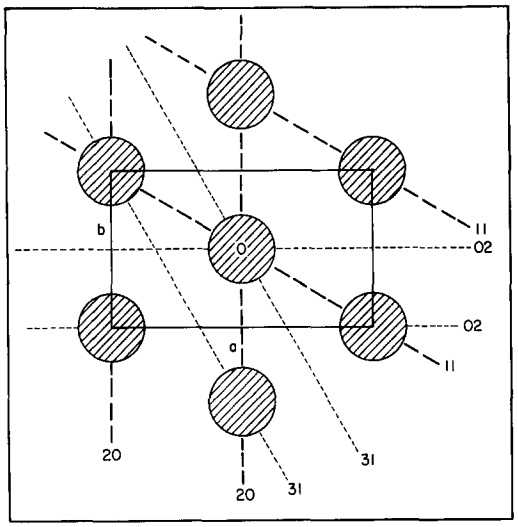

FIGURE 5

Hexagonal net with the unit cell. For convenience of description a non-primitive orthorhombic cell was chosen. The 11, 20, 02 and 31 planes indicated. If the hatched areas are assumed to be cylinders viewed end on, an inclination in the 11 or 20 direction would result in a pattern of alternating light and dark bands of approximately equal width. An inclination in the 02 and 31 direction would give to the same dark bands, but the light interspace would be extremely narrow. Given the actual dimensions of the hexagonal phase of the phospholipid in the section, one cannot expect to see it in an electron micrograph.

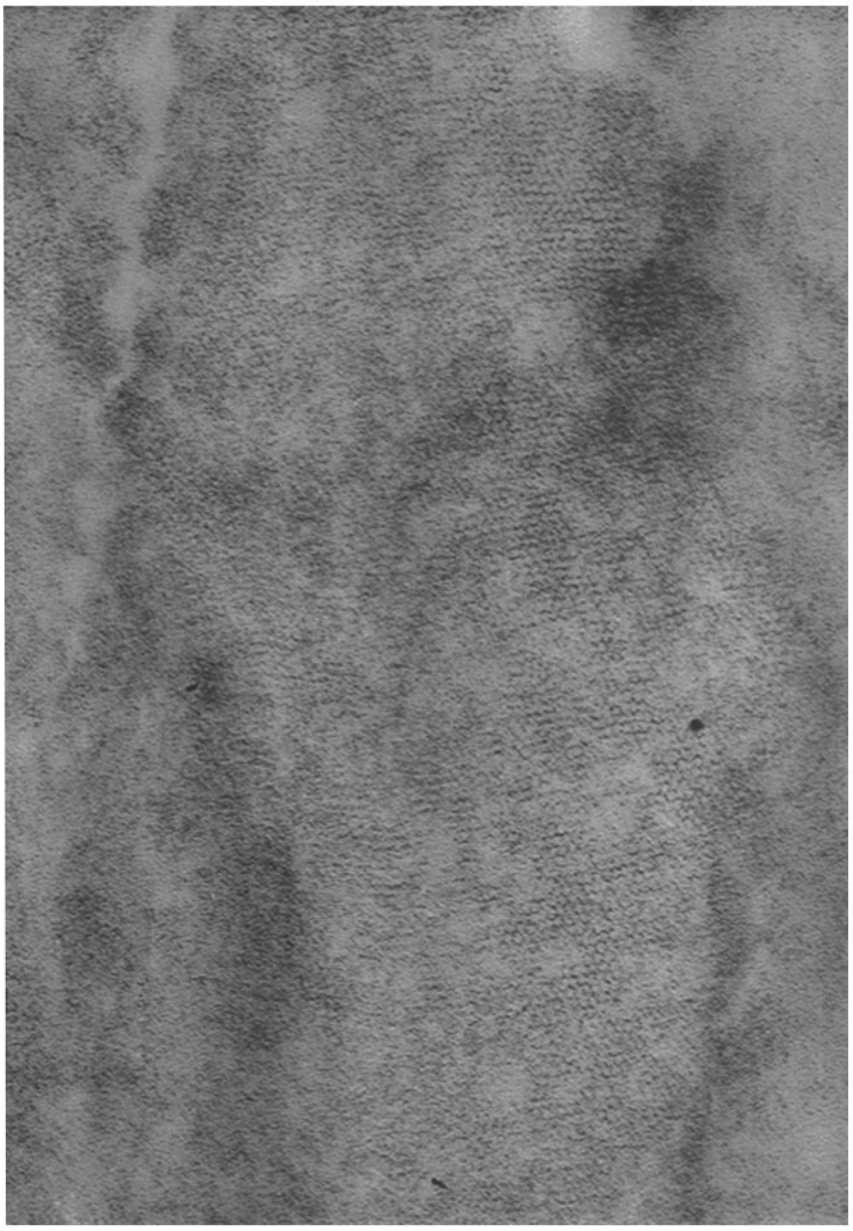

FIGURE 6

(1415/60) Middle soap phase of Na-linolenate. The sample contained 54 per cent water when fixed with OsO_4. Only few areas were found like the one shown here, where a regular structure could still be recognized. × 400,000.

that a phospholipid-water mixture in the hexagonal phase, which is stable only at low water concentrations and elevated temperature, should change its structure if exposed to a cold OsO_4 solution. The drop in temperature alone should partly transform it into the lamellar phase, and water penetrating more rapidly than osmium into the specimen would have the same effect. One wonders, therefore, if the common practice of fixing organs of warm-blooded animals in cold

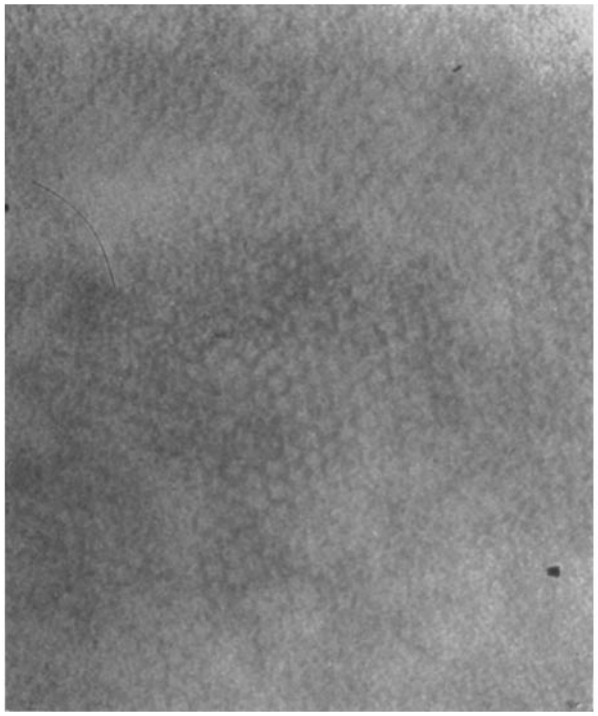

FIGURE 7

(145/60) Same as Fig. 6. Higher magnification of another area. × 800,000.

OsO_4 solutions insures the best preservation of all structures in the tissue.

Another important question is how well the spacings measured in electron micrographs agree with the spacings determined by x-ray diffraction. We shall not consider here the swelling and shrinkage that occur during fixation, dehydration, and embedding, which have been described and discussed before (6). We are here only concerned with the differences observed between the x-ray spacings of the lipid material included in the polymerized embedding medium and the spacings measured in the micrographs. In our experience, an x-ray diffraction diagram of a thick section from a block of lipid in the lamellar phase, embedded in methacrylate, regularly gives a slightly larger spacing than that found in an electron micrograph of a thin section from the same block. The same finding has been reported by Finean for Araldite-embedded material (1). Also, the calculated diameter of the cylinders in the middle soap of Na-linolenate is 40.5 A, compared to the 25 to 30 A we measure in the electron micrographs. These differences cannot be attributed to compression occurring during sectioning, since they are not related to the direction of cutting. We rather assume that they are due to a shrinking of the material under the electron beam. In the phospholipids it usually amounts to 15 to 20 per cent, whereas in the soaps the effect is stronger and the spacing in the micrographs may be only 60 to 70 per cent of that obtained from the x-ray diagram. A possible explanation would be that the shrinkage takes place mainly in the light regions originally filled by the hydrocarbon chains, which occupy a larger part of the total spacing in the soaps than in the phospholipids. But further investigations are needed before definite conclusions can be drawn.

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