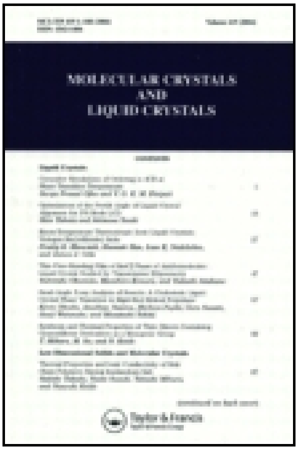

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Electron Microscopy of Lyotropic Mesomorphic Phases

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Electron Microscopy of Lyotropic Mesomorphic Phases

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Abstract—Electron microscopy can provide direct visual confirmation of mesomorphic phase structure in surface-active agent + water systems. Several different specimen preparative techniques have been used to examine the structures of the two most commonly occurring mesomorphic phases in such systems, neat phase and middle phase. Surface replicas of both anhydrous and quench-frozen specimens have clearly illustrated the domain structures which give rise to the characteristic optical textures of these two mesomorphic phases. In several systems osmium tetroxide fixation followed by thin sectioning has permitted the examination of neat and middle phase structures in cross-section. Polymerisation of a suitable compound in the mesomorphic state has provided non-labile structures for examination in the electron microscope as thin sections, using either pre- or post-staining with heavy metal reagents.

Introduction

Low-angle X-ray diffraction has been used to identify and classify the different lyotropic mesomorphic phases which occur over well defined temperature and concentration ranges in surface-active agent + water systems.[1] From the symmetry and spacings of the diffraction patterns it has also been possible to propose plausible structures[2,3] for the different lyotropic mesophases, and in favourable cases, electron microscopy has provided direct visual confirmation of mesophase structures.[4,5] The limitations of the electron microscopy approach are imposed by the preparative techniques which are required to produce a specimen for examination that is both thin and anhydrous, that possesses sufficient contrast and which preserves the original mesomorphic phase structure. It is important therefore to use several distinct specimen preparative techniques in order to reduce the chance of being misled by artefacts peculiar to any one preparative method. Variants of the two main specimen preparative techniques, (i) surface replication and (ii) chemical fixation and staining, have been used to illustrate the structures of neat phase and middle phase as they occur in different synthetic surface-active agent + water systems.

Experimental

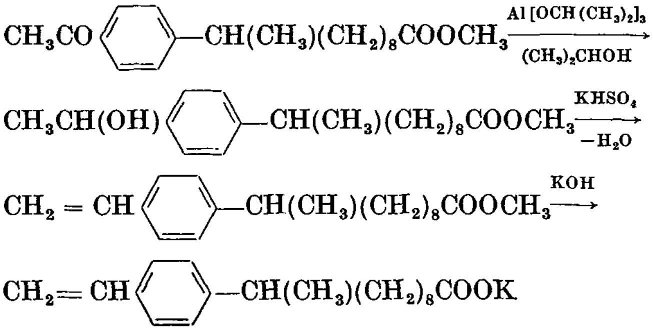
1. Preparation and Purification of the Surface-Active Compounds

(a) *Potassium* 10-*p-styrylundecanoate*

Potassium 10-*p*-styrylundecanoate was prepared by the following synthetic route[6] from methyl undecenoate, shown to be pure by gas-liquid chromatography (G.L.C.).

$$C_6H_6 + CH_2{=}CH(CH_2)_8COOCH_3 \xrightarrow{AlCl_3}$$

$$C_6H_5CH(CH_3)(CH_2)_8COOCH_3 \xrightarrow[AlCl_3]{CH_3COCl}$$



At the dehydration stage 1% picric acid was added to inhibit polymerisation of the methyl ester. The inhibitor was removed from the final product by adsorption on to charcoal. The potassium 10-*p*-styrylundecanoate was a pale yellow non-crystalline solid which was stored under vacuum in a refrigerator. Infra-red spectroscopy showed no detectable amount of the straight chain isomer, potassium 11-*p*-styrylundecanoate, which conceivably could have been formed during the Friedel-Crafts reaction.

(b) *Sodium di-2-ethylhexyl sulphosuccinate*

The synthesis of this compound has been described previously.[7]

(c) *Sodium linolenate*

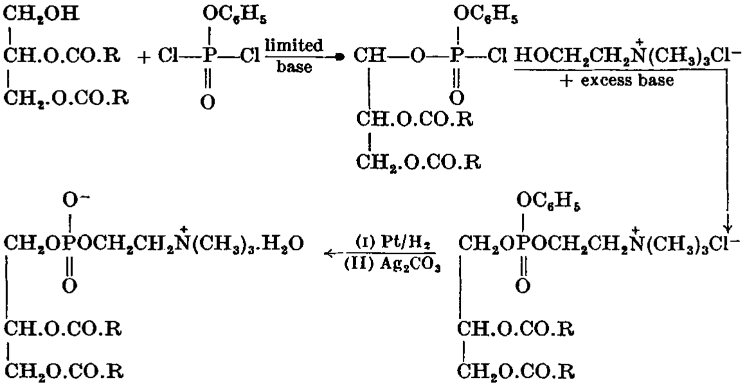
Linolenic acid (Fluka AG) was shown to contain at least 98% 9,12,15-octadecatrienoic acid by G.L.C. and thin layer chromatography (T.L.C.) of the methyl ester. Sodium linolenate was prepared by dissolving linolenic acid in ethyl alcohol and neutralising with the stoichiometric amount of sodium hydroxide; a slight excess (0.3%) of alkali was then added to ensure that an acid soap was not formed; 200 p.p.m. of 2,6-di-ter.butyl *p*-cresol were also added at this stage as anti-oxidant. The solvent was removed in vacuo while maintaining the temperature below 40 °C. The expected iodine value was obtained for the product.

(d) *Potassium oleate*

Oleic acid (Fluka AG) was shown to contain at least 99% 9-octadecenoic acid by G.L.C. and T.L.C. of the methyl ester. Potassium oleate was prepared by dissolving oleic acid in ethyl alcohol and titrating with potassium hydroxide to pH 9. The resulting clear solution was evaporated using a freeze-drying method and finally dried over phosphorus pentoxide to yield the product as a white powder.

(e) *DL-Dialkanoyl phosphatidyl cholines* (*Lecithins*)

Three racemic dialkanoyl lecithins were synthesized by Dr. F. E. Hardy in these laboratories using the following route, where $R = C_9H_{19}$, $C_{13}H_{27}$ or $C_{17}H_{35}$:



This method is a modification of that described by Baer and Mahadevan.[8] The lecithins were extremely hygroscopic and were stored as the monohydrate[9] in a vacuum desiccator over phosphorus pentoxide.

All compounds gave the correct elemental analyses. Table 1 gives, in summarised form, the concentration ranges for the occurrence at 25 °C of neat phase and middle phase in binary aqueous systems of these surface-active agents.

TABLE 1 Concentration Ranges for Middle Phase and Neat Phase Existence in Surface-Active Agent + Water Systems at 25 °C.

Surface-active agent	Middle Phase weight % surface-active agent	Neat Phase weight % surface-active agent
potassium 10-*p*-styryl-undecanoate	30 – 47	52.5–100
sodium di-2-ethyl hexyl-sulphosuccinate	83 –100	17 – 77
sodium linolenate	22.5– 48.5	60 – 80
potassium oleate	18 – 53.5	70 – 80
didecanoyl lecithin	—	41 – 92‡ (∼0 – 41)§

‡ Homogeneous neat phase.

§ Co-existing neat phase + isotropic solution.

2. MESOMORPHIC PHASE STRUCTURE

(a) *X-ray diffraction*

Low-angle X-ray diffraction patterns were recorded using the vacuum camera described previously.[3]

(b) *Electron microscopy*

All specimens were examined in a Siemens Elmiskop I, using a double condenser at 80k V and magnifications ranging from 10,000 to 80,000 × .

(i) Surface replication

Several branched chain surface-active agent + water systems are known which persist as neat phase or middle phase in the anhydrous state. This has permitted the preparation of platinum/carbon surface replicas of anhydrous neat phase and middle phase illustrating the respective domain characteristics and stratified morphology of these mesomorphic phases.[7] To remove objections associated with the examination of completely anhydrous systems a quench-freeze replication technique[4] has been used to examine the surface topography of mesomorphic specimens containing up to 50% water. This method is restricted to systems which, instead of crystallising at low temperatures, form a vitrified mesophase when quench-cooled to liquid nitrogen temperature. The low temperature phase behaviour has been confirmed by differential thermal analysis.

(ii) Chemical fixation and staining

Osmium tetroxide fixation. As described previously,[4] a Porter Blum MT-1 ultramicrotome was used to prepare thin sections from osmium tetroxide fixed mesomorphic phases given by surface-active agent + water systems. The sodium linolenate and potassium oleate mesomorphic specimens were prepared by isopiestic equilibration followed by fixation with osmium tetroxide vapour for 24 hours at 25 °C. Fixation of samples of the fully saturated lecithins was more difficult. Samples from the two phase region (neat + isotropic solution) were reacted with osmium tetroxide vapour at an elevated temperature of 65 °C for 48 hours in a closed vessel. The high temperature was found necessary since very little fixation occurred at 25 °C. The resulting sections still lacked contrast but this was enhanced using a supplementary staining method.[10] In this technique (OTO staining) the osmium in the sections complexes with a bidentate ligand, thiocarbohydrazide, which can then bind more osmium tetroxide.

Negative staining. Negatively stained[11,12] neat phase specimens of the lecithins were prepared by dispersing the materials as dilute suspensions of neat phase in water using an ultrasonic device (Disontegrator system 40). The dilute suspensions (0.5%) were sprayed on to carbon coated grids which were then inverted on to a 2% solution of phosphotungstic acid adjusted to pH 6.5 with potassium hydroxide. The suspensions of tetradecyl and octadecyl lecithins were heated to ~80 °C (i.e. above their respective Krafft boundaries) before spraying. After a staining period of 10–15 minutes the excess stain was removed with a filter paper before examination.

Polymerisation and staining. Neat phase samples from the potassium 10-*p*-styrylundecanoate + water system were polymerised[13] in a sealed glass tube in the presence of 1% α-azobis-isobutyronitrile as catalyst by heating at 70 °C for 72 hours. Samples of neat phase polymer were stained by exposure to osmium tetroxide vapour for two days at 25 °C. The solid fixed material was extracted with water to remove the water soluble components and this resulted in considerable swelling of the polymer. However gradual replacement of the water with acetone restored the polymer to its original proportions and it was embedded in Araldite and sectioned. Samples of neat phase polymer were also directly embedded in Araldite, sectioned and then stained by floating for a period of twenty minutes on a freshly prepared 1% uranyl acetate solution in 50% ethanol. Both polymerised and unpolymerised middle phase samples were stained with osmium tetroxide vapour and examined as thin sections.

Results and Discussion

(i) Surface replication

Figure 1 shows a typical surface replica prepared at − 145 °C from a quench-frozen neat phase specimen containing 30% water given by the potassium 10-*p*-styrylundecanoate + water system. Electron micrographs of surface replicas from quench-frozen neat phase samples are very similar to those obtained from anhydrous neat phase samples,[7] showing the typical terraced drop structure of a smectic mesophase. Steps may be seen on the plateau region in the centre of the drop while the edges are fringed by focal conic features. At higher magnifications the height of individual steps can be obtained from the measured shadow lengths and the known shadowing angle. The smallest measured step heights correspond to a bimolecular layer thickness (34 ± 6 Å) in agreement with the measured X-ray periodicity (34 Å).

Figure 2 shows a surface replica prepared from a quench-frozen middle phase sample containing 20% water given by the sodium di-2-ethylhexyl sulphosuccinate + water system. This electron micrograph is almost identical to the corresponding anhydrous replica.[7] The angular texture[14] of middle phase is clearly revealed. A stepped growth pattern can be observed running across the major axes of the tactoidal features. The minimum step heights (25 ± 6 Å), measured at higher magnifications, correspond to the low-angle X-ray diffraction spacing (23 Å), being approximately equal to twice the length of the surface-active molecules.

(ii) Chemical fixation and staining

Osmium tetroxide fixation. Osmium tetroxide fixation of unsaturated surface-active agents in the lyotropic mesomorphic state yields insoluble cross-linked polymers, which when examined in the electron microscope as thin sections, show retention of the mesomorphic phase configuration.[4,5] For example, Fig. 3 clearly illustrates the lamellar structure of neat phase from the sodium linolenate + water system initially containing 28% water.

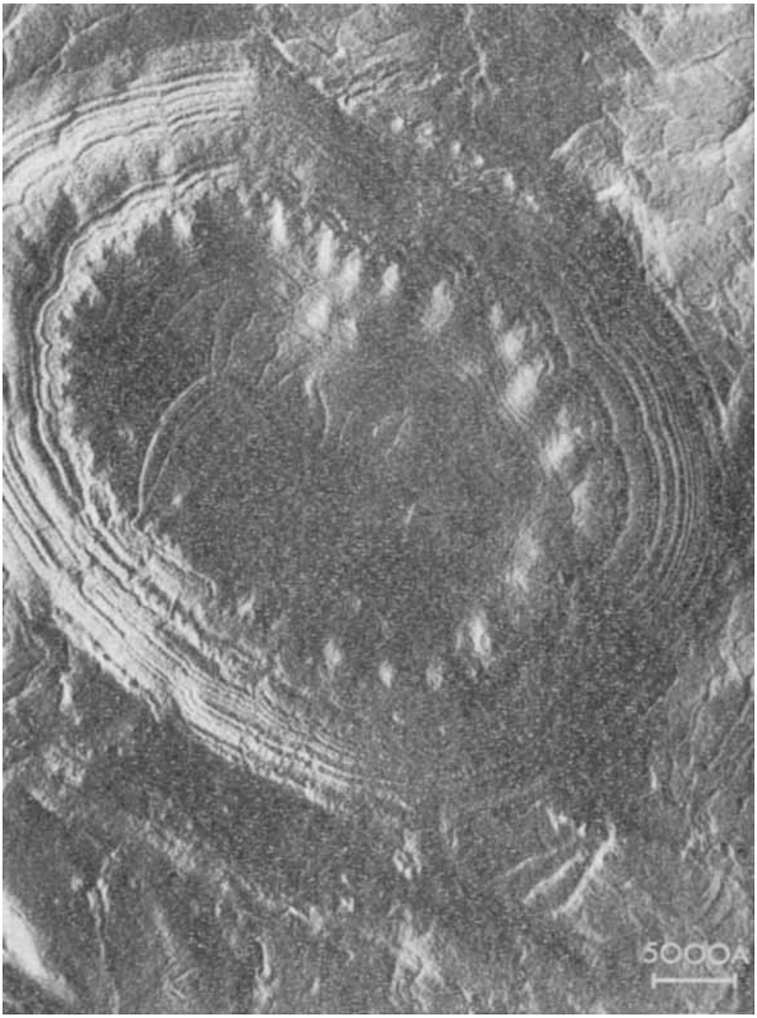


Figure 1. Replica of quench-frozen neat phase containing 30% water. Potassium 10-*p*-styrylundecanoate + water system.

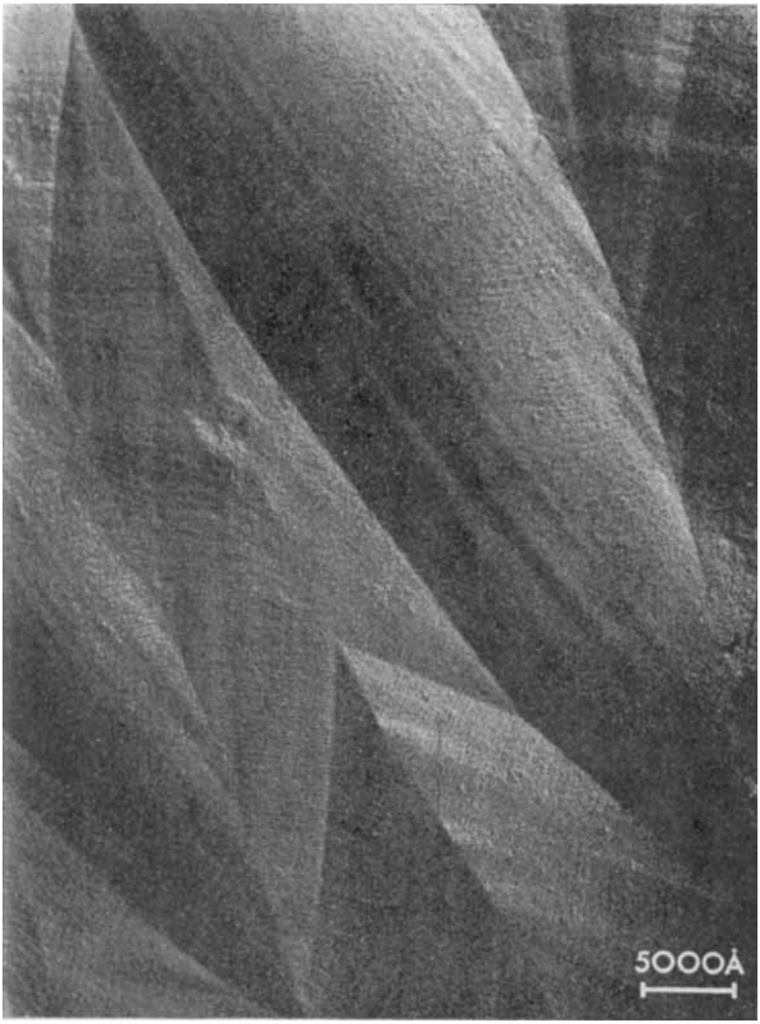


Figure 2. Replica of quench-frozen middle phase containing 20% water. Sodium di-2-ethylhexyl sulphosuccinate + water system.

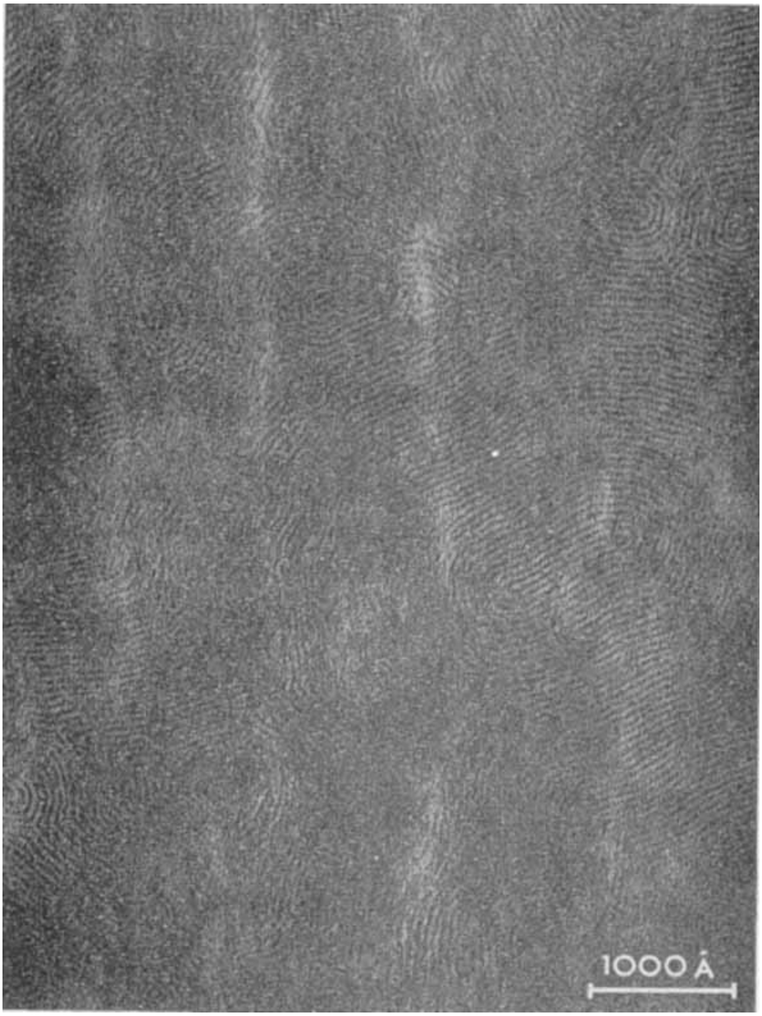


Figure 3. Thin section of osmium tetroxide fixed neat phase from sodium linolenate + water system.

The lamellar neat phase structure is shown to be convoluted in a characteristic pattern having a periodicity (49 ± 2 Å) comparable to the low-angle X-ray diffraction value of (35 Å) for the original composition. Within the sections it is possible to observe dislocations similar to those seen in a conventional crystal.

An unsaturated alkyl chain is desirable but not essential for the fixation of neat phase. Figure 4 shows an osmium tetroxide fixed, OTO stained, neat phase from the fully saturated didecanoyl lecithin + water system giving an interlayer spacing of 31 ± 2 Å. This result may be compared with the lamellar periodicity of 33 Å observed in an electron microscope by Elbers and Ververgaert[15] for the didecanoyl lecithin + water neat phase after " tricomplex fixation " with cobalt nitrate and ammonium molybdate. Interlayer spacings for the other homologues are given in Table 2.

TABLE 2 Comparison of X-ray Diffraction and Electron Microscopy Spacings for Neat Phase given by an Homologous Series of di-Alkanoyl Lecithins.

	X-ray Spacing (Å)	Electron Microscopy Spacing (Å) from neat phase	
Di-alkanoyl Lecithin	32 : 1 mole ratio water : lecithin (homogeneous neat phase)	Negative Staining	OTO Staining Technique
R = C_9H_{19}	54 ± 0.5	39 ± 2 Å	31 ± 2 Å
R = $C_{13}H_{27}$	63 ± 0.5	50 ± 3 Å	37 ± 7 Å
R = $C_{17}H_{35}$	74 ± 0.5	61 ± 3 Å	53 ± 4 Å

The technique of osmium tetroxide fixation has also been successful in illustrating aspects of middle phase structure in unsaturated surface-active agent + water systems. Thin sections from OsO_4 fixed middle phase given by sodium linolenate + water indicate a two-dimensional hexagonal array of discrete units (Fig. 5) with an inter-particle spacing (52 Å) of the order anticipated from low-angle X-ray diffraction (47–59 Å across the phase

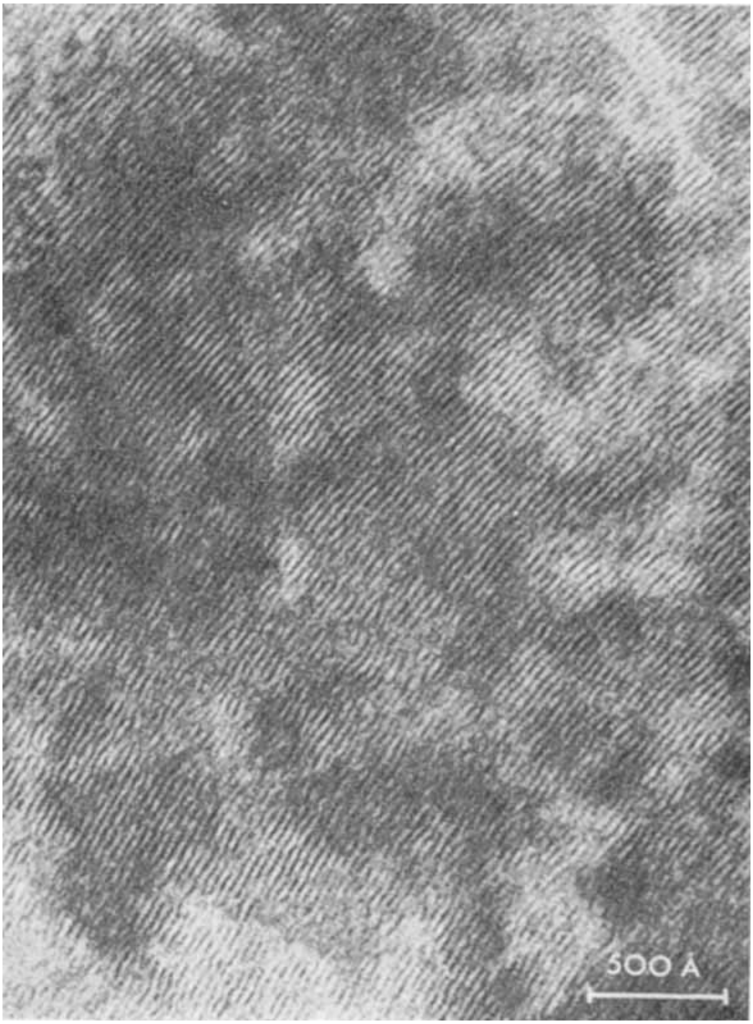


Figure 4. OTO stained thin section of osmium tetroxide fixed neat phase from didecanoyl lecithin + water system.

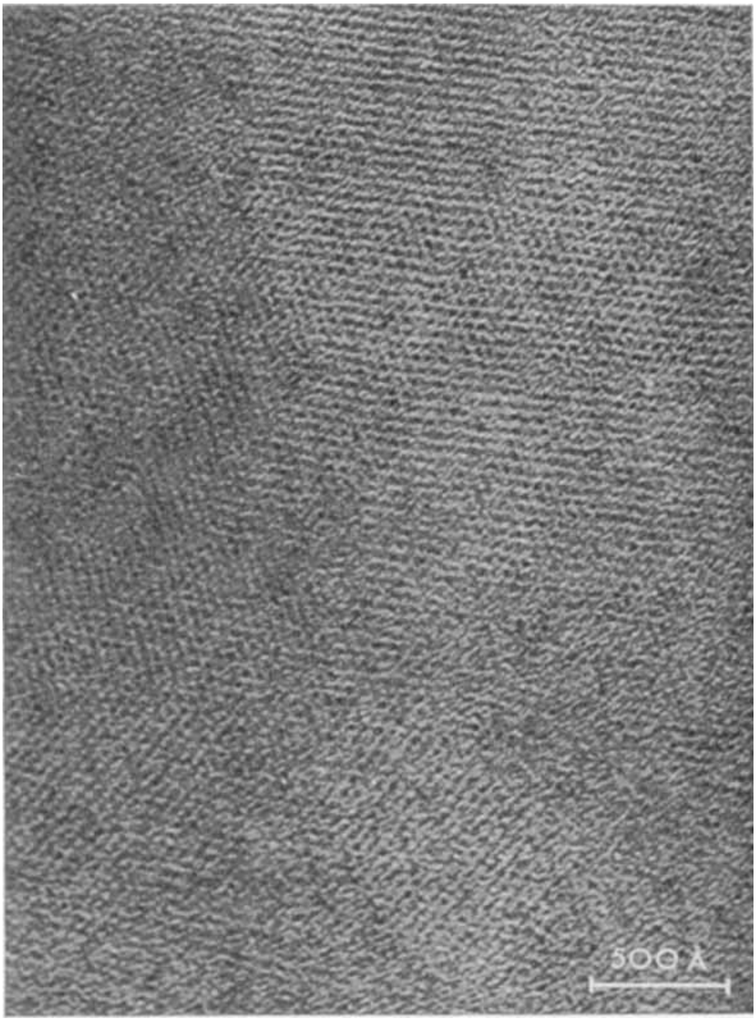


Figure 5. Thin section of osmium tetroxide fixed middle phase from sodium linolenate + water system.

region). The contrast in this middle phase image, as with all periodic images, can be varied with the focus[16] and a complete reversal of contrast is obtained on examining a through-focal series. This micrograph presents the in-focus image contrast which shows a two-dimensional hexagonal array of black units on a lighter background. The contrast in this micrograph is the reverse of that found by Stoeckenius[5] for this system. The reason for the discrepancy may be due to differences in specimen preparation or focusing. The micrograph obtained here may be compared with that obtained from the osmium tetroxide fixed middle phase given by the potassium oleate + water system (Fig. 6) where there is only one ethylenic double bond per octadecyl chain instead of three. This micrograph again represents the in-focus image contrast from a through focal series, and shows an hexagonal arrangement of white units on a dark background. As the preparation of both our middle phase specimens was carried out under identical conditions we may conclude that the resulting contrast of the in-focus image depends on the relative degree of unsaturation of the surface-active species.

Negative staining. Negative staining[11,12] has been applied to the homologous series of saturated lecithin + water systems. Figure 7 shows a mixture of crystals + neat phase trapped during the drying stage. The black areas are composed of lecithin crystals + stain, while the stained neat phase layers are seen to follow the crystal contours fairly closely. The periodicities obtained by this technique are compared to those obtained by low-angle X-ray diffraction and OTO staining technique in Table 2. Differences in the absolute values are observed, but the incremental values by any single technique are of the expected order.

Polymerisation and staining. Greater structural control over mesophase composition is afforded by the technique of polymerisation of suitable amphipathic monomers in the mesomorphic state.[13] Thin sections of the polymerised neat phase stained with osmium tetroxide showed a regular lamellar structure with a periodicity (31 ± 2 Å) in good agreement with that obtained by

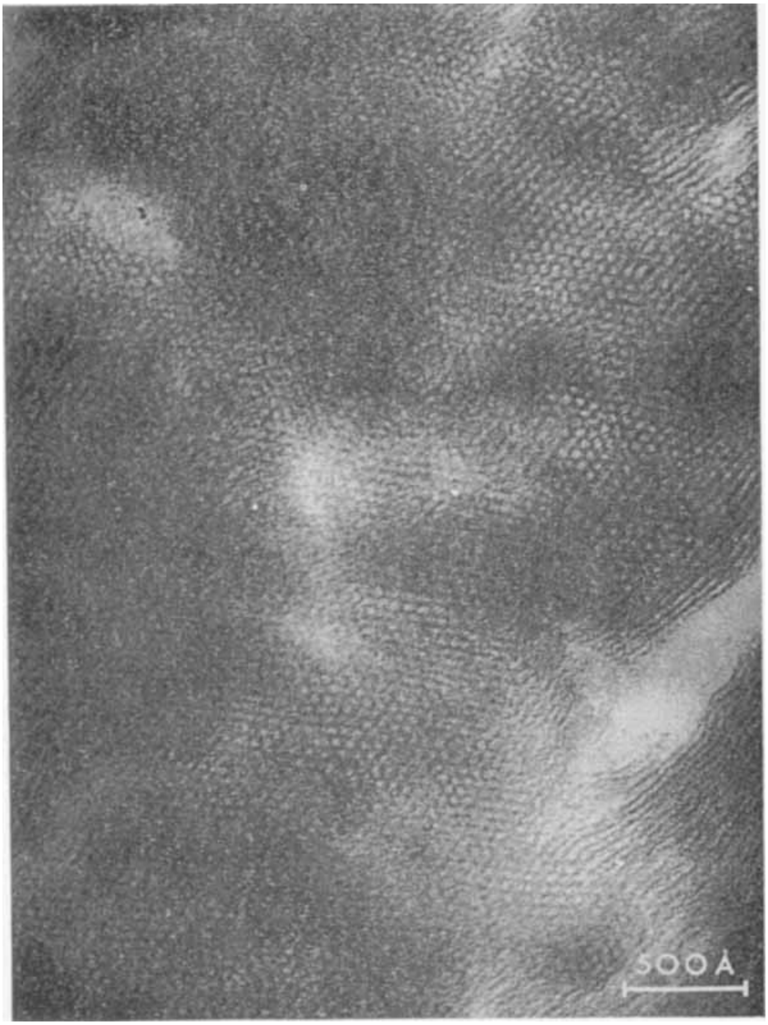


Figure 6. Thin section of osmium tetroxide fixed middle phase from potassium oleate + water system.

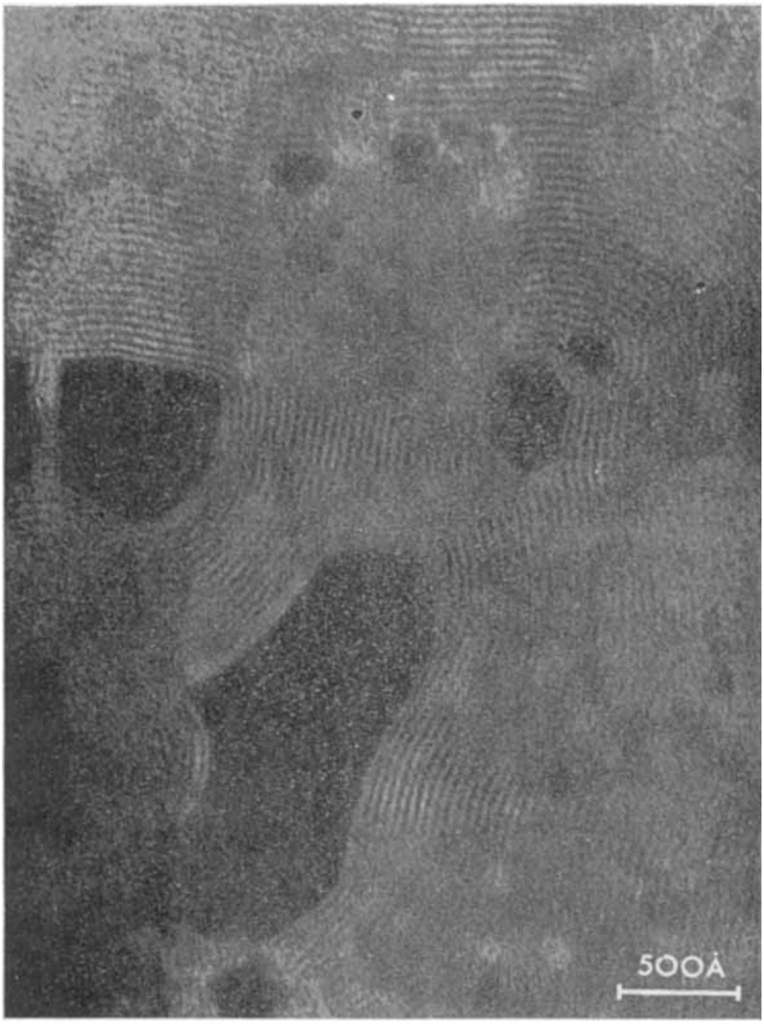


Figure 7. Negatively stained crystals + neat phase from didecanoyl lecithin + water system.

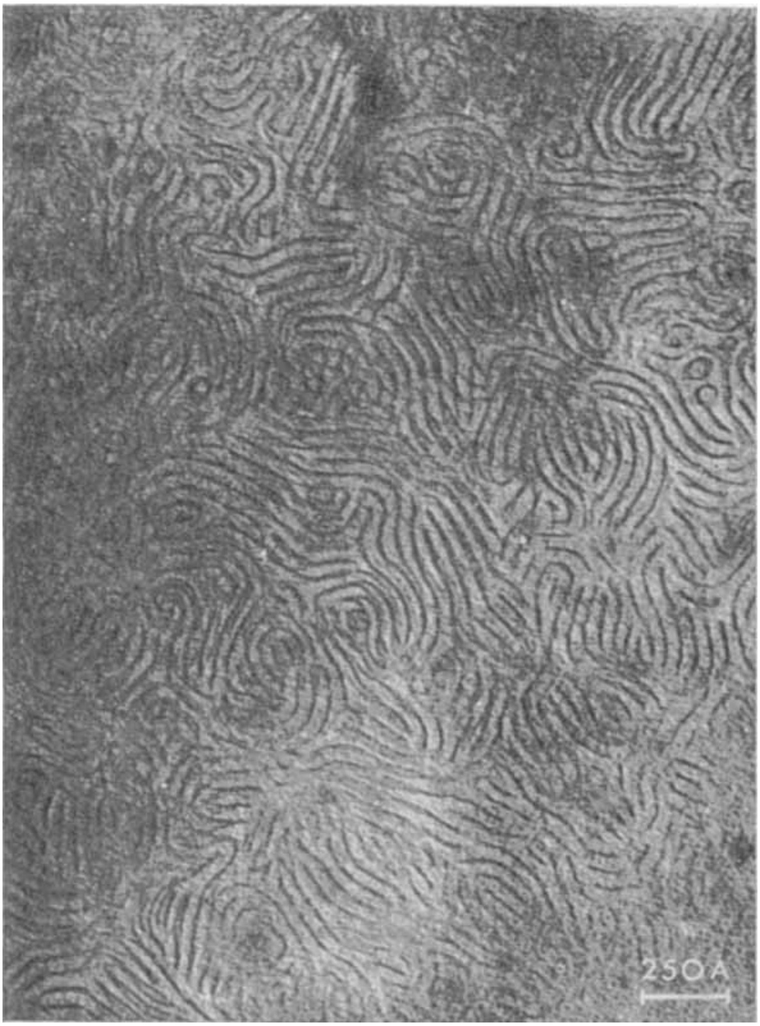


Figure 8. Uranyl acetate stained thin section of neat phase polymer from potassium 10-*p*-styrylundecanoate + water system.

X-ray diffraction (32–38 Å across the phase region). The polymerised neat phase sections post-stained with uranyl ions by ion-exchange showed a layered structure with greater overall dimensions (41 ± 2 Å) than those shown by the osmium tetroxide staining technique. This expansion may be attributed to swelling of the polymer when in contact with aqueous solutions. Figure 8 shows a uranyl acetate stained neat phase polymer section having many convolutions and bearing a formal resemblance to the osmium tetroxide fixed neat phase from sodium linolenate (Fig. 3).

We have confirmed the observations of Herz[13] that attempted polymerisation of middle phase in this particular system leads to structural disruption of the mesophase. However, osmium tetroxide fixation of the monomer middle phase was successful, the in-focus image contrast being the same as that found for the potassium oleate + water system.

Conclusion

All the specimen preparative techniques suffer some limitations. The conventional surface replication technique can only be applied to those systems which give anhydrous mesophases, while freeze replication must be restricted to systems which do not crystallise at low temperatures. Negative staining and polymerisation are not universally applicable and we conclude that the osmium tetroxide fixation and thin sectioning technique appears to be the most versatile currently available for examining thin sections.

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