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STAINING AND DRYING-INDUCED ARTIFACTS IN ELECTRON MICROSCOPY OF  
SURFACTANT DISPERSIONS;— III: EVIDENCE FROM OPTICAL MICROSCOPY AND  
A NEGATIVE STAINING CASE

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Previously, we have shown that the structures imaged in the electron microscope of air-dried aqueous mixtures of sodium 4-(1'-heptylnonyl) benzene sulfonate (SHBS) and sodium octanoate (NaOc) with uranyl acetate (UA) and barium chloride ( $\text{BaCl}_2$ ) were not present before drying; both SHBS and NaOc are positively stained by UA and  $\text{BaCl}_2$ . Here, we present results on aqueous dispersions of didodecyldimethylammonium bromide (DDDAB), which is stained negatively by UA. Additionally, we present further results on NaOc with UA which show that although there is a strong correlation among visual, optical microscopic, and electron microscopic appearance in the dried state, these structures bear no apparent relationship to the original, hydrated surfactant fluid microstructure. Evidence is drawn from electron and polarizing optical microscopy, nmr spectroscopy, and differential scanning calorimetry. We conclude that heavy metal stains can only be used as structure-preserving contrast agents in electron microscopy of surfactant dispersions if the sample is either fast-frozen to preserve the hydrated state or if the sample is frozen and then in-situ freeze-dried.

## INTRODUCTION

Electron microscopy has been widely used for direct determination of surfactant microstructures. Because of the high vacuum required in the electron microscope column ( $< 10^{-6}$  torr), the sample must either be chemically or thermally fixed before imaging. Talmon, Falls, and others<sup>1,2</sup> have shown that fast-freezing can preserve the structure of

liquid crystalline and vesicular dispersions and that these structures can be readily imaged in the electron microscope. With either air-drying or *in-situ* freeze-drying, however, there is not sufficient electron-density contrast to observe the surfactant. Consequently, it must be stained with a strong scatterer of electrons in some way to enhance contrast.

A common technique for staining surfactant microstructures is to mix the aqueous surfactant dispersion with an aqueous stain solution, such as uranyl acetate or phosphotungstic acid, place a drop of the mixed sample on a film-covered microscope grid, and allow the sample to dry in air before imaging. Depending on the drop size, the substrate, and the humidity of the air, the sample can take from a few minutes to an hour or so to completely dry to either crystals or crystal hydrates. During drying (usually several minutes) precipitation of surfactant and stain from molecular solution and gross structural rearrangements are constantly occurring. We have shown<sup>3</sup> that the "fringes" observed by others<sup>4,5</sup> in the electron micrographs of dried surfactant-stain samples and believed by them to be bilayers characteristic of the original, hydrated sample, are, in fact, produced by the precipitation of surfactant and stain from molecular solution during drying. They bear no relationship to the original structures and were not present in the original sample.

We present here evidence for the existence of drying- and staining-induced artifacts in aqueous dispersions of a surfactant, didodecyl-dimethylammonium bromide (DDDAB), which is stained negatively by UA. We also present further evidence of a strong correlation among visual, optical, and electron microscopic appearance of dried NaOc-UA samples which have no apparent connection to the original, pure, hydrated surfactant fluid microstructure. Our evidence for microstructure and micro-environment comes from electron microscopy, nmr spectroscopy, and differential scanning calorimetry. Our evidence for macrostructure comes from polarizing optical microscopy and visual appearance. DDDAB was obtained from Eastman and recrystallized twice from ethyl acetate.<sup>3</sup> The other experimental materials and methods are described elsewhere.<sup>3</sup>

## RESULTS

### DDDAB - Water

A 2 wt% DDDAB dispersion in water, pH 6.6, was prepared by adding the surfactant to water followed by gentle shaking of the dispersion. A drop of this dispersion was placed on a glass slide and observed in the optical microscope while drying. Initially, spherulites of positive optic sign which characterize the dispersed lamellar phase were observed (Figure 1a). As the sample dried, "oily streaks" (Figure 1b) characteristic of the concentrated lamellar phase appeared at the drop's edge. Oily streaks and concentrated spherulites persisted until the sample was nearly dry. These results were not pH-dependent upon the addition of either acetic acid (HAc) or sodium hydroxide (NaOH).

A concentrated sample of DDDAB (50%) was also prepared and examined microscopically. Upon original placement, the specimen's appearance was featureless. After several hours under a coverslip, very large oily streaks began to form near the edge of the sample (Figure 1c). After several days, the entire sample exhibited large oily streaks as well as a mosaic pattern of lamellar positive units.<sup>6</sup>

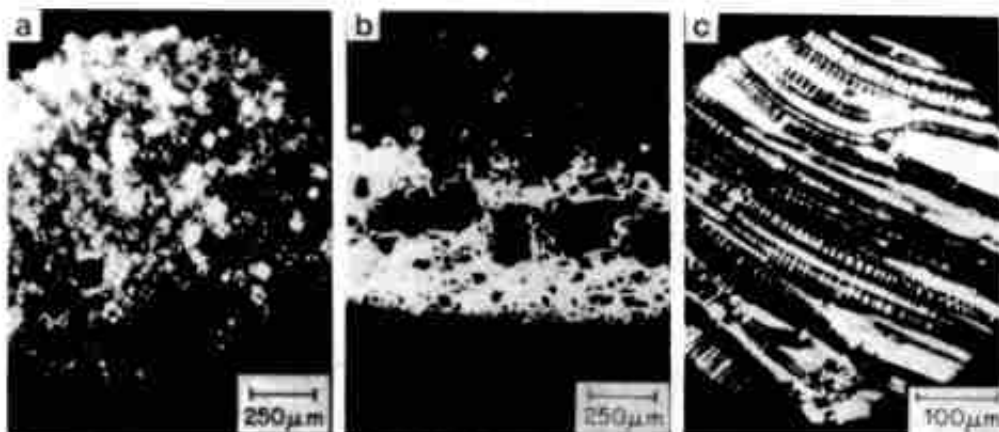


Figure 1. Optical photomicrographs between crossed polarizers of drying droplet of 2 wt% DDDAB in water, pH=6.6: (a) spherulites indicative of dispersed lamellar phase in drop initially upon placement; (b) oily streaks at drying edge of drop coexisting with spherulites; (c) 50 wt% DDDAB in water under coverslip, pH=7, exhibiting large oily streaks.

#### DDDAB-UA-Water

A 1 wt% DDDAB-1 wt% UA sample in water was prepared by combining equal volumes of stock solutions, and cooling in an ice-water bath for 30 minutes; the pH was 4.5. As with the 2% DDDAB solution, spherulites were immediately observed. After a short period of time, oily streaks became visible at the edge of the drying drop (Figure 2a). In contrast to the UA-free sample very distinctive needle-like textures, having the appearances of crystals, were seen throughout the sample (Figure 2b). Their appearance was similar in form to "batonnets" of a hexagonal phase observed by Rosevear<sup>6</sup> in concentrated alkali carboxylate samples. However, as the sample continued to dry, these textures disappeared. We must conclude they are either textures of a liquid crystalline phase not seen in the pure surfactant solution or that a region on the phase diagram was traversed in which a crystalline phase was present. Upon further drying of the sample, a very distinctive crystalline pattern not resembling the dried pure DDDAB dispersion was observed (Figure 2c).

The 1 wt% DDDAB-1 wt% UA sample was adjusted to pH 6.5 with NaOH. Dispersed liquid crystallites were evident as spherulites observed in the optical microscopic. This dispersion upon drying exhibited quite different textures than the parent dispersion of lower pH, or the 2 wt% DDDAB dispersion at pH 6.6. As the sample dried, small droplets (Figure 3a) of a separating phase were swept to the drying edge of the sample. Inside these droplets were concentrated the needle-like textures observed in the pH 4.5 sample. As these droplets dried, their edges became brightly birefringent (Figure 3b). In addition dendritic crystals similar in appearance to uranyl hydroxide hydrate began to form throughout the viewing area (Figure 3c). Characteristic crystalline textures, different from those observed in the sample of lower pH, appeared upon complete drying.

We also examined a concentrated DDDAB-UA sample (40% UA-40% DDDAB) in the optical microscope. After smearing the sample on a microscope slide and covering it with a coverslip, the sample was examined over several days. The large oily streaks which had been seen throughout the

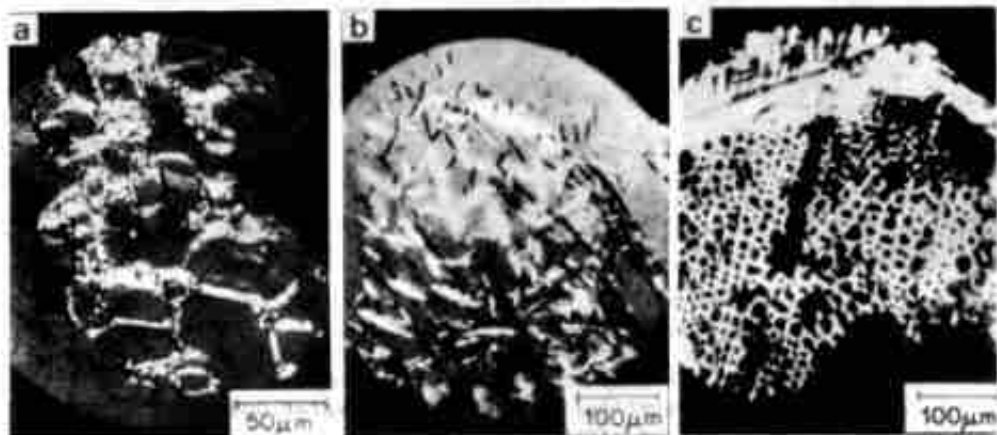


Figure 2. Optical photomicrographs of drying droplet of 1 wt% DDDAB-1 wt% UA in water, pH=4.1: (a) oily streaks near edge of drying drop; (b) needle-like textures; (c) distinctive crystalline pattern exhibited by dried specimen.

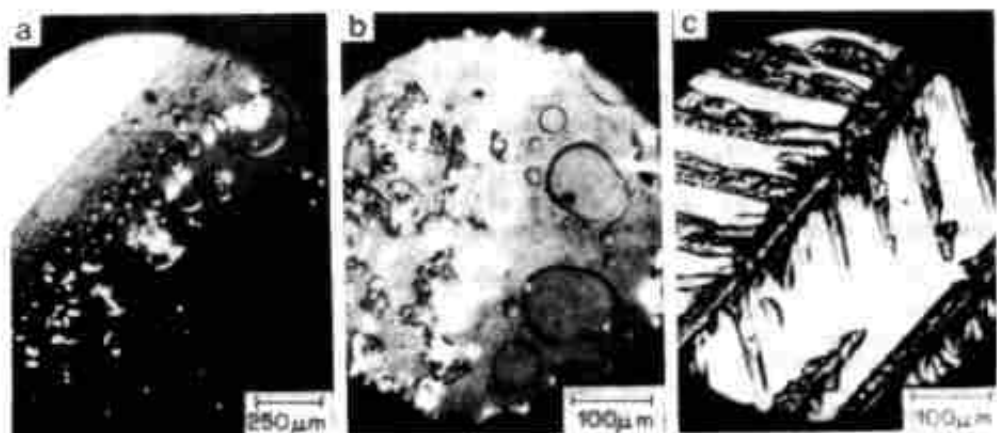


Figure 3. Optical photomicrographs in drying droplet of 1 wt% DDDAB-1 wt% UA in water, pH=6.5: (a) droplets of separating phase enclosing needle-like textures; (b) birefringent borders of drying droplets (note presence of spherulites); (c) large dendritic crystals formed upon drying.

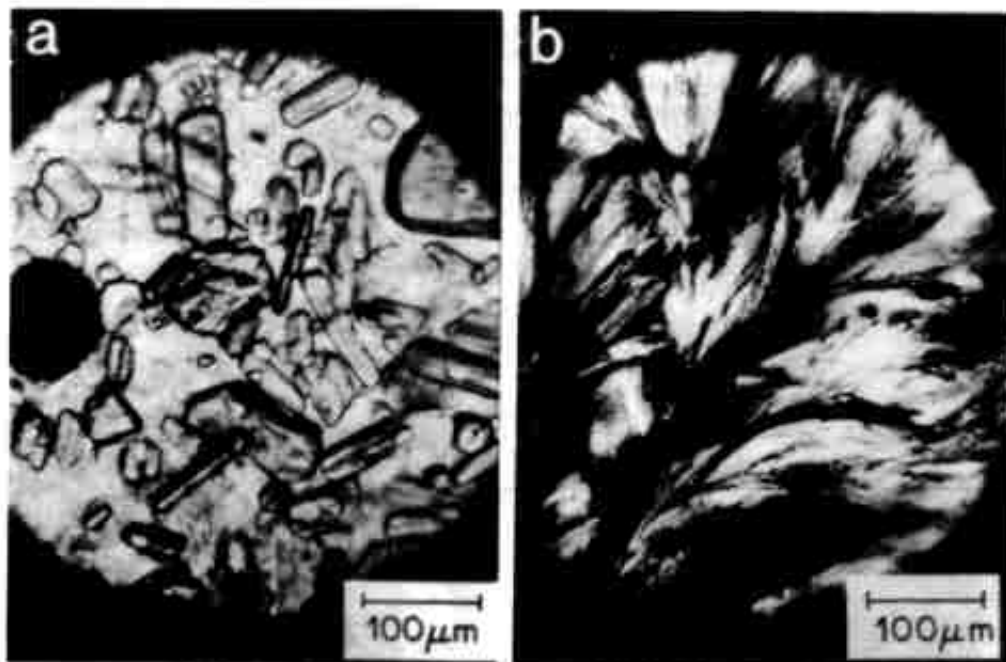


Figure 4. Optical photomicrographs of 40 wt% DDDAB-40 wt% UA in water under coverslip, pH=7: (a) polygonal crystals formed throughout sample; (b) distinctive feathery textures in isolated portions of sample.

50% DDDAB in water system were never observed in this sample. Large hexagonally-shaped crystals which had not been present in the UA-free sample were now seen (Figure 4a). In addition, distinctive feathery textures not seen in either the UA sample or concentrated DDDAB sample were observed (Figure 4b). The colorful uranyl acetate dihydrate crystals we had observed previously in drying uranyl acetate at lower pH were also evident in the thinner sections of the sample.

Changes in pH also affected strongly the structures as revealed by high resolution electron microscopy of dry specimens prepared from dispersions of 1% DDDAB + 1% UA. Dried specimens prepared from dispersions of pH 3.1 or pH 4.9 and higher produced no fringes such as reported by Kajiyama *et al.*<sup>7</sup> and taken as evidence of liposomes or lamellar liquid crystals. A solution of pH 3.9, however, produced small, round structures of concentric fringes (Figure 5a) similar to Kajiyama *et al.*'s so-called liposomes. At pH 6.0, only broad expanses of amorphous regions were observed, while at pH 7.0, needle-like crystals of ca. 0.1  $\mu\text{m}$  in size were seen (Figure 5b). Clearly, the appearance of fringes in electron microscopy of dried DDDAB-UA samples has little, if anything, to do with the original surfactant fluid microstructure. The sample pH, or pH modifier<sup>3</sup>, greatly affects the observed structure.

Although the macroscopic textures observed in the optical microscope appeared to be quite different in drying samples of DDDAB and DDDAB-UA, <sup>13</sup>C nmr spectra and differential thermograms indicated the surfactant remained predominantly in a lamellar phase in the hydrated state. Figure 6 shows <sup>13</sup>C nmr spectra of a 20 wt% DDDAB in CDCl<sub>3</sub> solution, a 20 wt% DDDAB in water dispersion, and 10% DDDAB - 10% UA dispersion at pH 5.3 and

11.3. The surfactant solution in chloroform was isotropic as indicated by the narrow and well-resolved carbon resonances. In water, the surfactant appears to be moving more slowly and anisotropically, consistent with the lamellar morphology indicated by optical microscopy. The carbon resonances do not change significantly in either chemical shift or line width upon addition of an equal weight of UA. The methyl resonance of the acetate group now appears as a shoulder on the unresolved DDDAB resonances while the carbonyl resonance of the acetate group is well-resolved. For all spectra of the DDDAB lamellar phase,  $^{13}\text{C}$  chemical shifts agreed with those reported by McNeil and Thomas.<sup>8</sup>

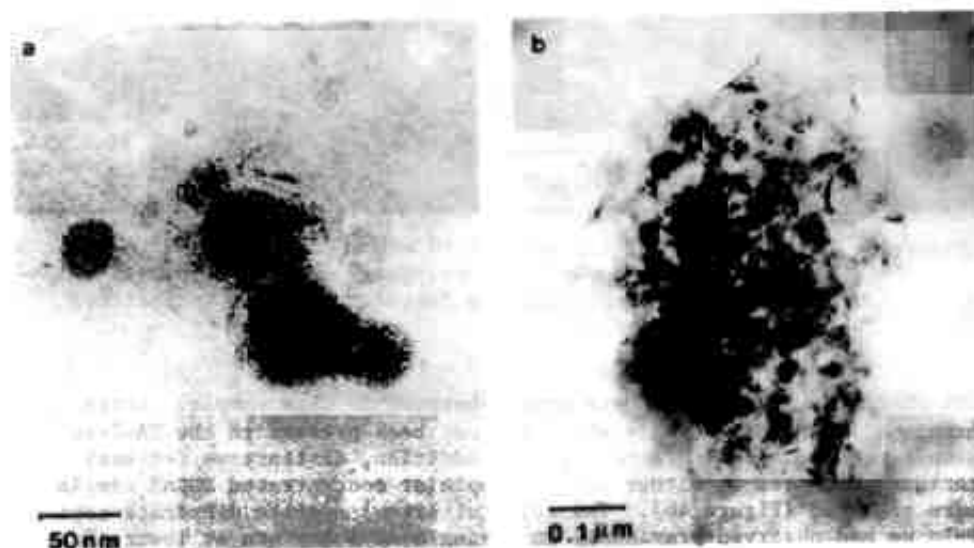


Figure 5. Transmission electron micrographs of air-dried specimens of 1 wt% DDDAB-1 wt% UA: (a) rounded fringes observed in sample of pH 3.9; (b) needle-like crystals in sample of pH 7.0.

Thermograms of a 50% DDDAB in water dispersion and a 40% DDDAB-40% UA in water dispersion are shown in Figure 7. The large thermal transition for bulk water at ca. 273 K indicates both dispersions are biphasic. The smaller thermal transition above this temperature are presumably associated with the gel-liquid crystal transition, in agreement with the transition temperature of 289 K reported by Kajiyama *et al.*,<sup>9</sup> for DDDAB-water. The addition of UA has no effect on this thermal transition.

**NaOAc-Water.** A 2 wt% micellar solution of NaOAc in water was prepared. Upon drying, distinctive birefringent textures were observed. These include large spherulites of positive optic sign (Figure 8a) and expanses of parallel striations (Figure 8b) indicative of the hexagonal

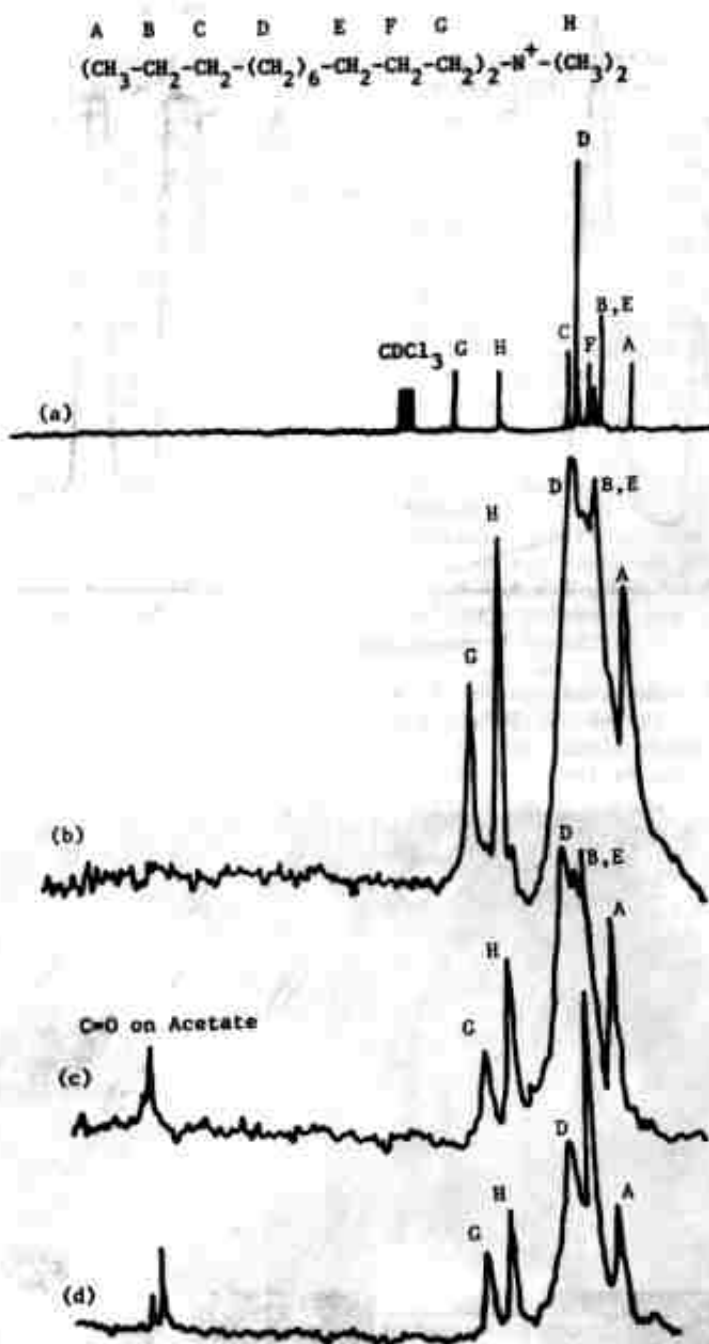


Figure 6. Proton-decoupled  $^{13}\text{C}$  nmr spectra of (a) 20 wt% DDAB in  $\text{CDCl}_3$ ; (b) 20 wt% DDAB in water; (c) 10 wt% DDAB-10 wt% UA in water, pH=5.3; (d) 10 wt% DDAB-10 wt% UA in water, pH=11.3. Structural formula shows resonance assignments.

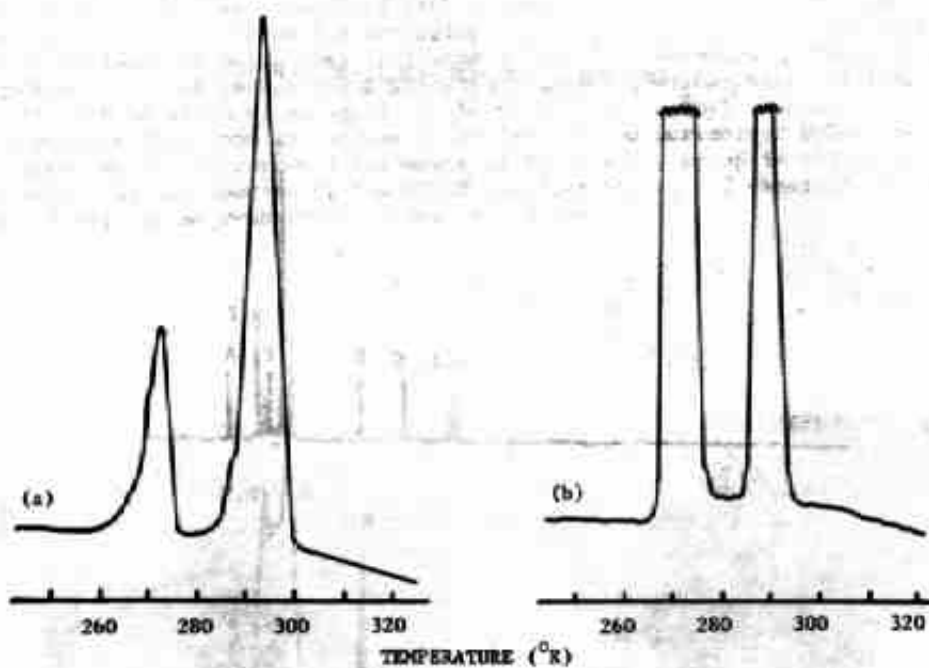


Figure 7. DSC thermograms of (a) 50 wt% DDDAB in water, pH=7 and (b) 40 wt% DDDAB-40 wt% UA in water, pH=7.0.

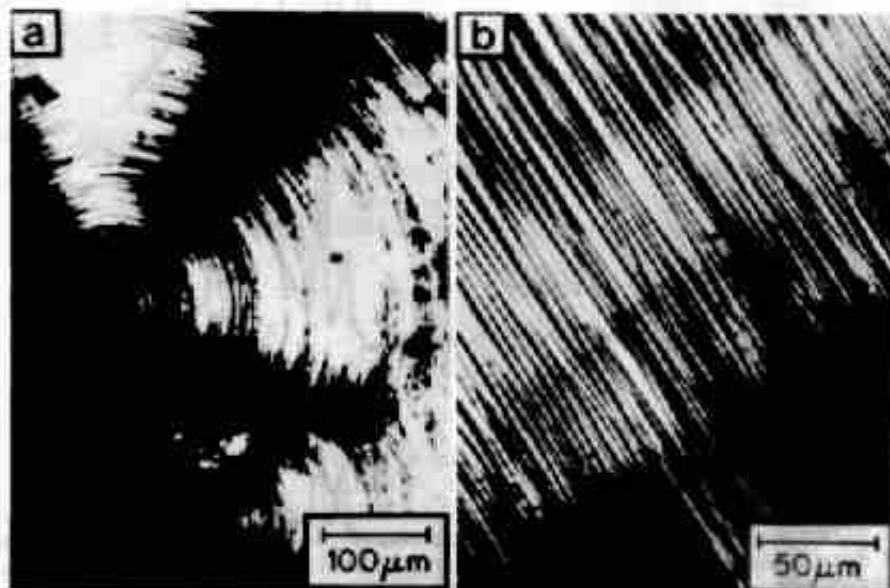


Figure 8. Optical photomicrographs of drying droplet of 2 wt% NaOe in water, pH=7: (a) partial spherulitic textures; (b) extremely uniform parallel striations of hexagonal phase.

phase.<sup>9</sup>  $^{13}\text{C}$  nmr spectra of 10 wt% NaOc solutions in water show well-resolved resonance lines of narrow linewidth indicating rapid and isotropic motion characteristic of the micellar phase.

Upon addition of an equal amount of UA stock solution to the 2 wt% NaOc in water solution, a yellowish-green precipitate resulted. The visual appearance of these NaOc-UA-water mixtures was a strong function of pH. At pH 5.1, the yellowish-green precipitate sedimented to the bottom of the containing flask, leaving a clear colorless supernatant. At pH 6.0, there were both sediment and floating yellowish-green precipitate and the solution had considerable suspended precipitate throughout. At pH 7.0, there was much more precipitate than in the dispersions of lower pH and most of this solid floated on the solution. At pH 8.0, the color of the precipitate darkened to a deep yellow and all of the solid now sedimented. At pH 12.4, the sediment was quite dense and deep orange in color.  $^{13}\text{C}$  nmr spectra of these samples indicated the surfactant remains in isotropic solution. In anticipation of a corresponding variation in texture and microstructure, this sequence was examined by both polarizing and electron microscopy.

The NaOc-UA sample of pH 5.1 showed large angular crystals, probably uranyl acetate dihydrate (Figure 9a), immediately upon placement. As the solution dried, birefringent textures appeared but these were not of the same regular structure as in the UA-free sample. Rosettes seemed to nucleate about UA crystals, and a less bright birefringence was observed throughout the sample (Figures 9b and 9c). In the sample of pH 6.0, very similar yellowish-green crystals were observed immediately upon placement of the drop. As in the sample of lower pH, as the solution dried a birefringent texture appeared with the brightest regions seeming to nucleate about the UA crystals. The sample of pH 7.1 also had large yellowish-green crystals throughout before drying. As the sample dried, however, fibrous textures not seen in the two samples of lower pH appeared (Figure 9d). Many of these fibers appeared to grow from crystals, like the rosettes of the lower pH samples. It is not known, however, whether these structures are comprised of surfactant, UA, or a combination of the two. The pH 8.0 sample exhibited regular striations along its drying edge (Figure 9e). These textures were seen throughout the UA-free NaOc sample and are identified as hexagonal liquid crystalline. In some isolated regions of the sample, fibrous textures as seen in the pH 7.1 sample were observed. The pH 12.5 sample exhibited textures very similar to those in the pH 8.0 sample. Orange crystals were seen rather than yellowish ones, but the liquid crystalline textures observed upon drying were much like those in UA-free samples.

The pH dependence of electron microscopic observations of dried NaOc-UA samples corresponds well to visual and optical microscopic results; we have described these elsewhere.<sup>3</sup>

## DISCUSSION AND CONCLUSION

Talmon<sup>2</sup> has shown that air-drying of aqueous surfactant-stain samples can produce "fringes" even though the original hydrated surfactant sample is known to be a molecular or micellar solution. *In-situ* freeze-drying, on the other hand, has been shown to preserve the original surfactant fluid microstructure when crystals, liquid crystals, or vesicles are present.<sup>1, 2</sup> Here, we have shown the effects of air-drying and original sample pH on the macrostructures observed by optical microscopy during air-drying. The system chosen--DDDAB stained by UA in water--is an example of negative staining. These structures were then compared with results from electron microscopy of the dried structures and with results

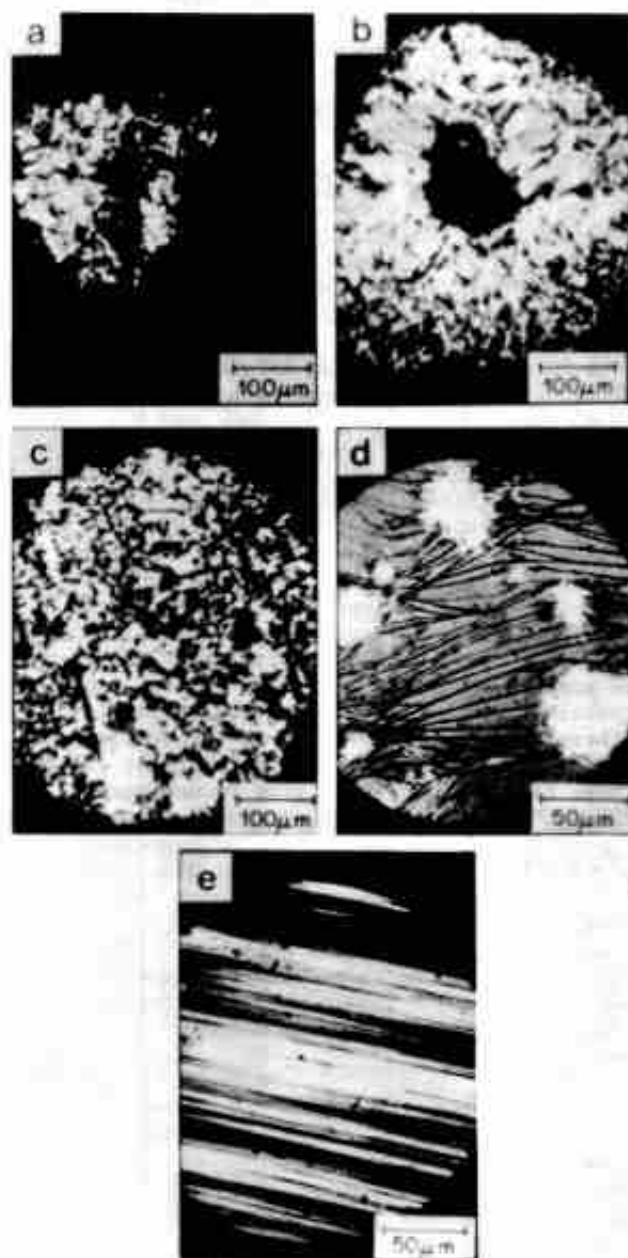


Figure 9. Optical photomicrographs of drying droplets of 1 wt% NaOCl-1 wt% UA in water: (a) crystal in sample of pH 4.2; (b) birefringent rosette of hexagonal phase centered about angular crystal in sample of pH 5.2; (c) birefringent pattern of non-geometric texture in sample of pH 5.2; (d) fibrous texture in dried sample of pH 7.1; (e) parallel striations of hexagonal phase in sample of pH 8.0.

from nmr and differential scanning calorimetry of the hydrated systems. There did not appear to be any correlation between the observed presence of lamellar liquid crystals in the hydrated state and the presence or absence of fringes in the electron microscope in the dried state. We also systematically observed phase transitions during drying and the effect of pH on those transitions.

Dilute dispersions of DDDAB and UA (1 wt%-1 wt%) exhibited optical textures consistent with a dispersion of lamellar liquid crystallites. Slightly more concentrated dispersions (10 wt% DDDAB-10 wt% UA) gave  $^{13}\text{C}$  nmr spectra which showed a motional gradient along the surfactant alkyl tail consistent with a lamellar morphology. Concentrated dispersions (40% DDDAB-40% UA) showed thermal absorption peaks consistent with a gel-liquid crystal transition in agreement with the pure surfactant in water. Thus it must be concluded that the presence of uranyl acetate does not significantly perturb the lamellar phase in the hydrated state. Nonetheless, optical micrographs did indicate that crystalline and liquid crystalline structures, not present in the original pure surfactant dispersion, precipitated from solution as the sample dried in air. Moreover, the form of these precipitating structures depended on the pH of the original dispersion. pH is a variable rarely controlled or monitored by others<sup>4,5,10</sup> who use staining-drying techniques.

The fringes observed in the electron microscope also appeared to depend on the pH of the original dispersion, but there was no obvious connection between the pH dependence of textures observed in the optical microscope and these fringes. Ideally, we would like to understand the connection between the appearance of fringes in air-dried samples and the associated phase behavior during drying. In the dried sample the fringes are produced from stain-surfactant structures no more than 50-100 Å thick. In the original liquid crystalline dispersions of DDDAB in water, the liposome size varies from about 0.05 to several hundreds of microns. Thus, the fringes observed in the dried sample bear no obvious relationship to the original hydrated sample. Rather these fringes are a result of precipitation of surfactant and stain as the sample dries.

That the existence and nature of these fringes are strong functions of pH indicates that the phase behavior during drying in these systems is not simple. This is clear from both the unpredictable occurrence of fringes in the electron microscope as well as the unusual dependence on pH of optical microscopic textures during drying for both DDDAB and NaOc in the presence of UA. Nonetheless, the original hydrated samples of DDDAB remain lamellar liquid crystalline in the presence of UA. Similarly, the original hydrated samples of NaOc remain micellar in the presence of UA. Thus, it is the drying in the presence of UA and not just the presence of the stain which renders this preparation technique for electron microscopy unusable. These observations are made from the combined evidence of electron and optical microscopy,  $^{13}\text{C}$  nmr, and differential scanning calorimetry.

It is clear from our work reported here and elsewhere<sup>2,3</sup> that staining with heavy-metal ions and air-drying is a totally unsatisfactory technique for preserving surfactant fluid microstructures or enhancing contrast in electron microscope specimens. Nonetheless, Kunitake and others<sup>5,10</sup> persist in using this method for establishing structure in hydrated surfactant specimens. Their electron micrographs are almost certainly artifact-ridden. Alternatives to this technique are fast-freeze, cold-stage electron microscopy of hydrated specimens (with or without *in situ* freeze-drying), freeze-fracture followed by replication, and polymerization of surfactant fluid microstructures followed by solvent exchange and drying. These techniques are reviewed in the proceedings of

this conference<sup>11,12</sup> and have been shown to successfully preserve liquid crystalline and vesicular dispersions for electron microscope imaging.

#### ACKNOWLEDGEMENTS

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