# Aggregate Structure in Dilute Aqueous Dispersions of Oleic Acid/Sodium Oleate and Oleic Acid/Sodium Oleate/ Egg Phosphatidylcholine

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*Received December 22, 1994. In Final Form: April 5, 1995*[⊗]

Cryo-transmission electron microscopy was used to investigate the aggregate structure in dilute aqueous samples of oleic acid, as a function of pH. At pH 10.7, where the fatty acid is almost completely deprotonated, the micrographs show spherical or cylindrical micelles depending on the concentration. Upon a decrease of the pH to values just above 9, formation of unilamellar vesicles is induced. With decreasing pH the vesicles show an increasing tendency to aggregate. At pH between 8 and 7, large clusters of aggregated vesicles coexist with dispersed nonlamellar, presumably inverted hexagonal structures. Further decrease in pH results in a complete transition into nonlamellar liquid-crystalline structures and finally to the formation of oil droplets. Addition of high concentrations of oleic acid to small unilamellar lecithin vesicles induces, at pH 7.4 and lower, clustering and formation of particles with nonlamellar structure. At high pH, on the other hand, oleic acid acts like a conventional cationic surfactant. With increasing fatty acid: lipid molar ratio both significant vesicle growth and finally lipid solubilization into mixed micelles are observed.



## Introduction

Apart from their various industrial uses, fatty acids have important biological functions and are present in a variety of different forms in body tissue and fluids. Esterified to glycerol and stored in the cytoplasm of many cells, fatty acids serve as important sources for energy. Possibly even more important is their presence in phospholipids, the major building blocks of most biological membranes.

The level of free, unesterified, fatty acids is generally low in both body fluids and cell membranes, and the majority of the unesterefied fatty acid is found associated to albumin or lipoproteins, or bound to specific fatty acid binding proteins. However, in certain membranes, such as rat liver plasma membranes, the level of free fatty acids may be as high as 8%.[1] Furthermore, under conditions where fatty acids are produced at high rates, serum levels, as well as the concentration in underlying tissue, can rise to considerable values. Fatty acids are believed to help regulate important membrane-mediated cellular functions, such as membrane permeability[2] and fusion (refs 3 and 4 and references therein).

Another interesting function ascribed to free fatty acids concerns their role in the regulation of enzymatic activity. Under certain conditions free fatty acids are believed to accumulate in the phospholipid monolayer surrounding lipoprotein particles. This accumulation has been suggested to cause structural modifications of the lipoprotein surface which, in turn, decreases the binding affinity of certain hydrolytic enzymes.[5,6]

In order to understand this mechanism, as well as the role of fatty acids in membrane-mediated processes, knowledge about the phase behavior and aggregate structure in phospholipid/fatty acid systems is essential. However, the behavior in these systems has been found to be strongly influenced by the protonation state of the fatty acid component.[3,7] In systems containing low proportions of fatty acid, the observed pH-dependence is often satisfactorily explained by simple electrostatic stabilization. On the other hand, in mixtures containing high proportions of fatty acid, the aggregate structure at a specific pH is closely linked to the phase propensity of the fatty acid component. Thus, knowledge of the phase behavior and aggregate structure in pure fatty acid systems, as a function of pH, is a prerequisite to understand the behavior in the mixed systems. Although a number of studies concerning the phase behavior of fatty acid/soap systems have been presented[8–12] the aggregate structure in dilute dispersions has not yet been thoroughly investigated. Part of the explanation for this lies in the fact that many of the conventional methods used to determine phase structure in lipid/surfactant systems are ill-suited for investigations of very dilute samples.

Cryo-transmission electron microscopy (cTEM) offers unique possibilities for investigation of the labile microstructures formed by lipids and surfactants.[13] The method is ideally suited for dilute aqueous solutions, and the gentle sample preparation and quick cooling rates ensure minimum perturbation of the original sample structure.

In this work we have used cTEM to investigate how the aggregate structure in dilute solutions of oleic acid changes as a function of pH. In order to help understand, and possibly predict, the structural effects brought about by accumulation of free fatty acids in biological membranes and monolayers, we complemented the cTEM study of pure fatty acid/soap systems with a study of the effect of

[⊗] Abstract published in *Advance ACS Abstracts,* June 15, 1995.

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oleic acid on size and aggregate structure of small unilamellar vesicles composed of phosphatidylcholine.

## Materials and Methods

**Materials.** Fatty acids were bought from Nu-Chek-Prep, Inc. (Elysian, MN), and used without further purification. Concentrated stock solutions were prepared by dissolving the fatty acid in ethanol. Egg phosphatidylcholine (egg-PC) of grade 1 was purchased from Lipid Products (Nutfield, U.K.).

**Preparation of Samples.** Fatty acid solutions were prepared by slow injection of a small amount of concentrated stock solution into a continuously stirred alkaline solution containing 150 mM NaCl. (The final concentration of ethanol never exceeded 1.0% by volume.) The salt solution was made alkaline by addition of NaOH and the amount of NaOH adjusted so that the pH, after addition of fatty acid, was close to 11. The soap solution was titrated with HCl and the pH measured with a Metrohm 632 pH-meter from Metrohm Ltd. (Herisau, Switzerland).

Small unilamellar vesicles were prepared by ultrasonic irradiation of samples containing about 30 mg of egg-PC in 4 mL buffer. A Soniprep 150 from MSE Scientific Instruments (Crawley, U.K.) was used for the irradiation. After sonication the samples were diluted to the desired concentration and filtered through a 0.2-$\mu$m Sartorius Minsart filter.

For measurements at pH 7.4 a buffer containing 10 mM Tris–HCl, 1 mM EDTA, 150 mM NaCl, and 0.01% $NaN_3$ was used, whereas at pH 6 and 10 the solutions were buffered with 90 mM $NaH_2PO_4$ and 25 mM Borax, respectively.

All samples were left to equilibrate for at least 24 h before cTEM investigations were performed.

**Cryo-Transmission Electron Microscopy (cTEM).** Specimens for cTEM examination were prepared in a controlled environment vitrification system (CEVS). The system, which comprises an improved version of the CEVS described by Bellare et al.,[14] ensures good temperature control and minimizes evaporation during sample preparation.

The preparation procedure has been described in detail elsewhere[14] but consisted in short of the following. The sample was equilibrated within the CEVS at the desired temperature (25 or 38 °C) and humidity (98–99% relative humidity). Thereafter a small (~2 $\mu$L) drop of sample solution was withdrawn and deposited on a holey polymer-film-covered TEM grid.[15] After careful spreading of the drop, excess liquid was blotted away with filter paper. By this technique thin (10–500 nm) sample films, spanning the ~5 $\mu$m large holes in the polymer film, were formed. After blotting the sample was immediately plunged into liquid ethane held at its freezing point. The vitrified sample was then transferred under liquid nitrogen to a Zeiss EM 902 electron microscope. The specimen temperature was kept below 108 K and all observations were made in zero loss bright field mode and at an accelerating voltage of 80 kV.

## Results

**Effect of Oleic Acid on Small Unilamellar Phospholipid Vesicles.** In an attempt to investigate the aggregation behavior in dilute mixtures of phospholipids and fatty acids, we used cTEM to study the morphological changes induced by addition of oleic acid to small unilamellar vesicles composed of egg phosphatidylcholine.

It is well-known that the phase behavior in mixtures containing fatty acids is sensitive to the protonation state of the fatty acid (see Discussion for details about the apparent p$K_a$). The aggregate structure may thus be expected to vary considerably with the pH of the solution and experiments were therefore conducted at neutral, slightly acidic, as well as alkaline pH. At 150 mM NaCl the aqueous monomeric concentration of fatty acid is extremely low. By means of static light scattering measurements we estimated a critical micelle concentration for oleic acid in the order of 10–20 $\mu$M at pH 7.4. Compared to the total concentration, the concentration of free fatty acid may thus be neglected and the molar ratios given in the figure captions represent the actual composition of fatty acid and phospholipid in the aggregates.

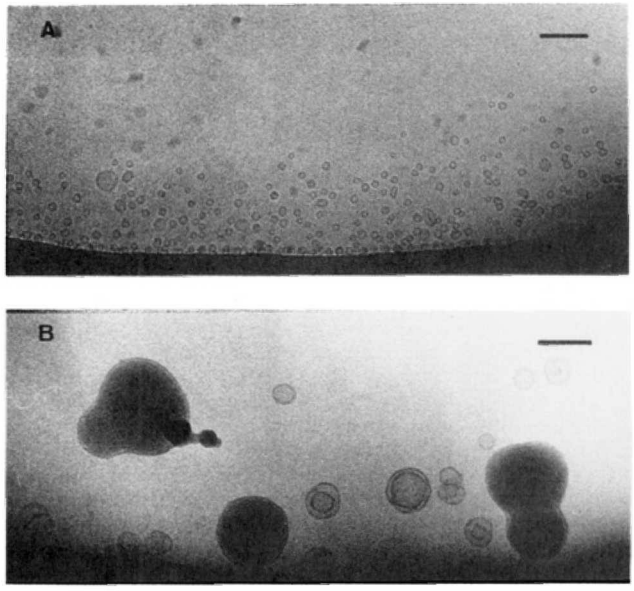


**Figure 1.** Sonicated phosphatidylcholine vesicles in buffer at pH 7.4: (A) no fatty acid added; (B) after addition of oleic acid to give an oleic acid:lipid molar ratio of 3:1. [Lipid] = 1 mM, $T$ = 25 °C, bar = 100 nm.

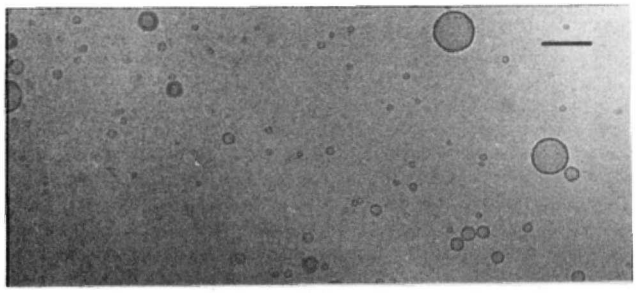

**Figure 2.** Phosphatidylcholine vesicles at pH 6. Oleic acid:lipid molar ratio 2:1. [Lipid] = 1 mM, $T$ = 25 °C, bar = 100 nm.

Figure 1A shows a cTEM micrograph of sonicated EPC vesicles, prepared in aqueous solution buffered at pH 7.4. At this pH, addition of oleic acid up to a fatty acid:phospholipid molar ratio of 1:1 does not change the appearance of the sample appreciably; the vesicles remain small, unilamellar, and unaggregated. Higher concentrations of fatty acid do, however, induce a dramatic change in the aggregate structure. As seen in Figure 1B, which depicts a sample having a fatty acid:phospholipid ratio of 3:1, small clusters of aggregated vesicles now coexist with large particles having a nonlamellar, material dense structure.

The behavior at pH 6 resembles that observed at pH 7.4. Vesicles containing a fatty acid:phospholipid ratio of 1:2 remain small and unaggregated (Figure 2). However, when the ratio is increased to 2:1, vesicles are no longer observed and the sample quickly sediments in the form of macroscopic droplets.

In contrast to the observations at neutral and slightly acidic pH, addition of oleic acid gives at pH 10 rise to a marked increase in vesicle size. The growth of the vesicles becomes significant above fatty acid:phospholipid ratios of about 2:1, and the maximum vesicle size is observed at a ratio of 9:1 (Figure 3A). Higher concentrations of fatty acid induce the formation of mixed micelles (Figure 3B).

From the micrographs shown in Figures 1–3 it is obvious that the protonation state of the fatty acid, as well as the sample composition, is a factor which determines the overall aggregate structure in phospholipid/fatty acid mixtures. In an attempt to elucidate as to what

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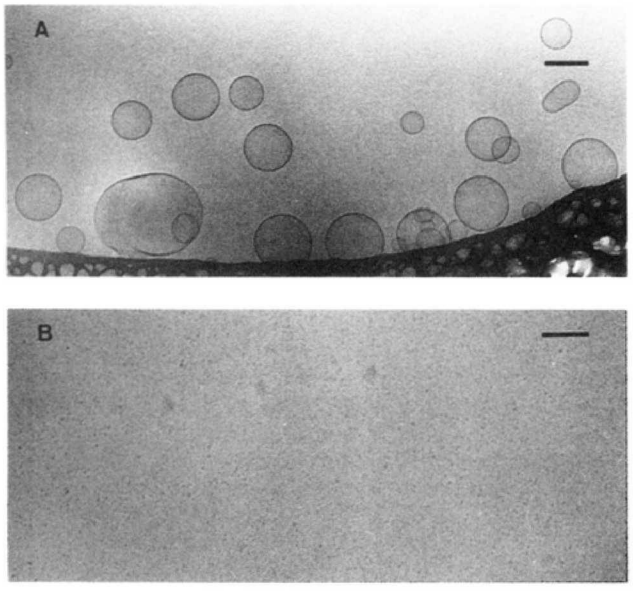


**Figure 3.** Phosphatidylcholine vesicles at pH 10: (A) oleic acid:lipid molar ratio 9:1, [lipid] = 1 mM; (B) oleic acid:lipid molar ratio 19:2, [lipid] = 0.25 mM. $T$ = 25 °C, bar = 100 nm.

extent the phase propensity of the fatty acid component governs the structures formed in the mixtures, we conducted a systematic cTEM study of aggregate structure, as a function of pH, in dilute solutions of oleic acid.

**Aggregate Structure in the Dilute Part of the Oleic Acid/Na-Oleate System.** Figures 4 and 5 show the aggregate structure in a sample containing 3.6 mM oleic acid at a number of different pH values. Under conditions were the fatty acid is close to fully ionized, the sample contains long cylindrical micelles, as exemplified by Figure 4A. The size and shape of the micelles were found to be very sensitive to the sample concentration. At pH 10.7 samples containing 0.5 mM oleic acid showed, for instance, only globular micelles with an appearance very similar to that seen in Figure 3B.

As the pH is decreased to values just above 9, unilamellar vesicles begin to form. With decreasing pH the vesicles show an increasing tendency to aggregate (Figure 4B,C), and at values close to pH 8, large clusters of aggregated vesicles dominate the sample. Depsite the tight aggregation of the vesicles, no increase in vesicle size was observed with time.

Below pH 8 nonlamellar structures start to appear and are often observed in close contact with the vesicles. Some appear oil-like in their character (Figure 5A) whereas others show a more structured interior (Figure 5B,C) and closely resemble the nonlamellar aggregates observed in fatty acid/phospholipid mixtures (Figure 1B).

Below pH 7, finally, the sample quickly precipitates in large sticky droplets, and consequently the aggregate structure is difficult to investigated by means of cTEM.

To complement the morphological study performed above the melting temperature, we also wanted to investigate a crystalline sample by means of cTEM. Below the melting temperature, fatty acid/soap mixtures may form a number of different crystalline states depending on the pH, temperature, and composition.[8] Hargreaves and Deamer[16] have reported that, at pH values where the fatty acid is close to half protonated, needle-shaped crystals form in dilute aqueous samples of various fatty acids when the temperature is about 7−10 °C below the melting point for the corresponding anhydrous fatty acid. In the oleic acid/oleate system crystal formation is not expected until the temperature is brought down to values around 10 °C,

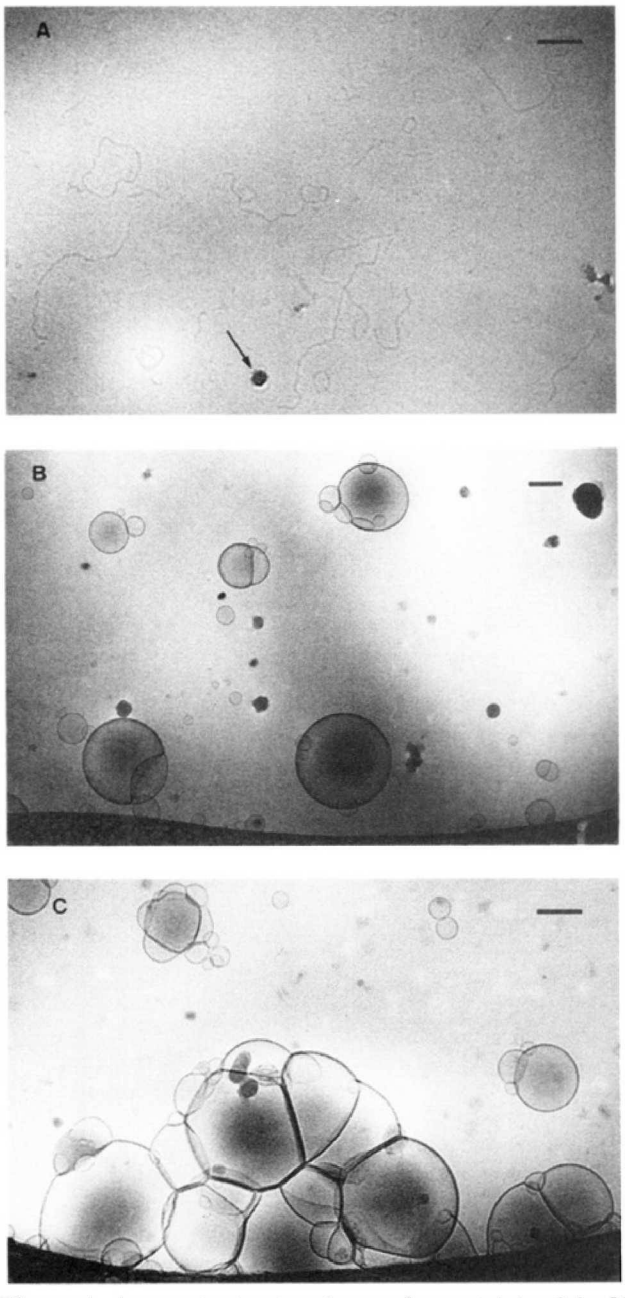


**Figure 4.** Aggregate structure in samples containing 3.6 mM oleic acid at pH 10.7 (A), 8.8 (B), and 8.1 (C). The arrow in (A) shows a water crystal deposited on the sample surface after vitrification. $T$ = 25 °C, bar = 100 nm.

and unfortunately this is below the minimum temperature accessible with the current CEVS. However, lauric acid has a melting temperature of about 44 °C (see compilation by Small[8]), and in aqueous dispersions containing no salt, crystal formation is expected at temperatures around 34 °C.[16] Figure 6 shows a sample containing 20 mM lauric acid at 38 °C and pH 9.2. Note that in addition to thin elongated crystals, a network of long, entangled cylindrical micelles can be seen in the micrograph.

## Discussion

For long-chain fatty acids at physiological salt concentrations, p$K_a$ for the carboxylate group has been determined to be about 5.[17] However, the apparent p$K_a$ is shifted to considerably higher values when the fatty acid is incorporated into a micelle, or situated at the surface of a bilayered aggregate. This effect, first explained by

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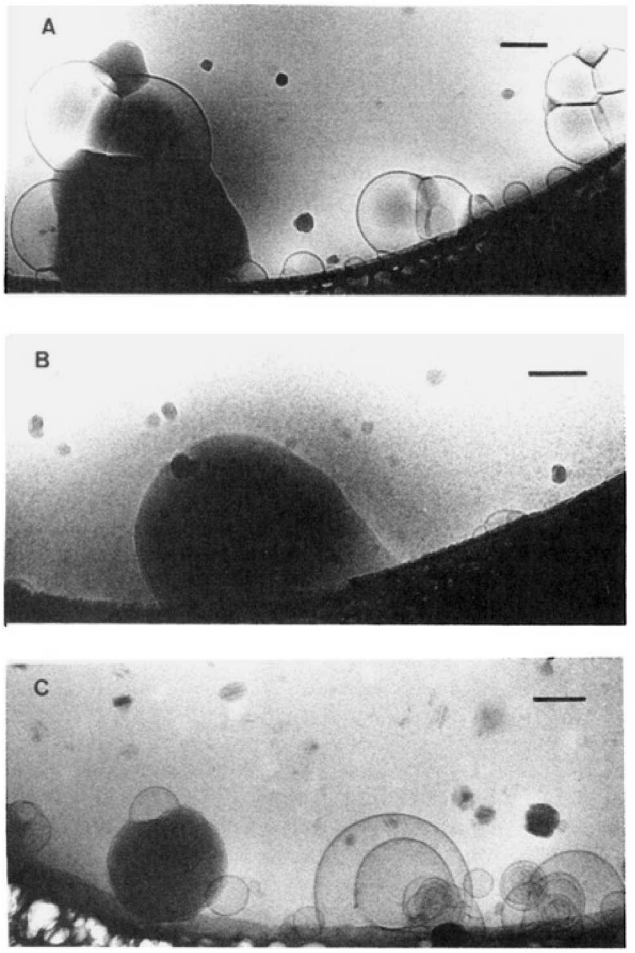


**Figure 5.** Micrographs showing coexistence between vesicles and inverted structures in samples containing 3.6 mM oleic acid at pH 7.9 (A and B) and pH 7.4 (C). $T = 25$ °C, bar = 100 nm.

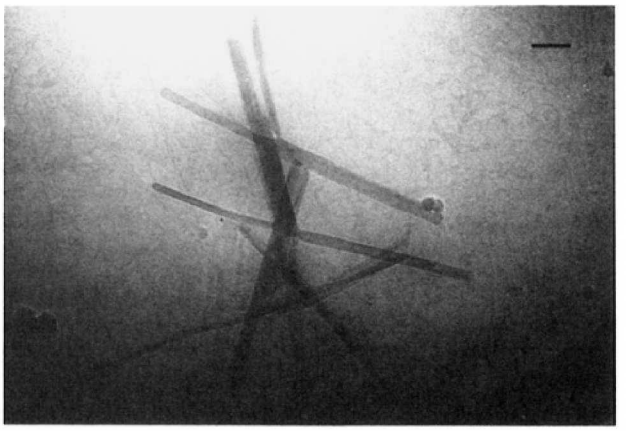

**Figure 6.** Needle-shaped crystals and threadlike micelles observed in sample containing 20 mM lauric acid at pH 9.2. $T$ = 38 °C, bar = 100 nm.

Gouy,[18] is due to the well-known capacity of a polyanionic surface to attract cations. At low ionic strength the accumulation of predominantly hydronium ions leads to a lower pH at the aggregate surface compared to that in the bulk. At high ionic strength, on the other hand, cations of the salt dominate and the pH at the aggregate surface remains closer to that in the bulk. Apart from the ionic strength, other factors, such as temperature and type of aggregate may influence the apparent p$K_a$. Differences in fatty acid chain length or unsaturation have as expected little influence on the p$K_a$.[19] A detailed analysis of the

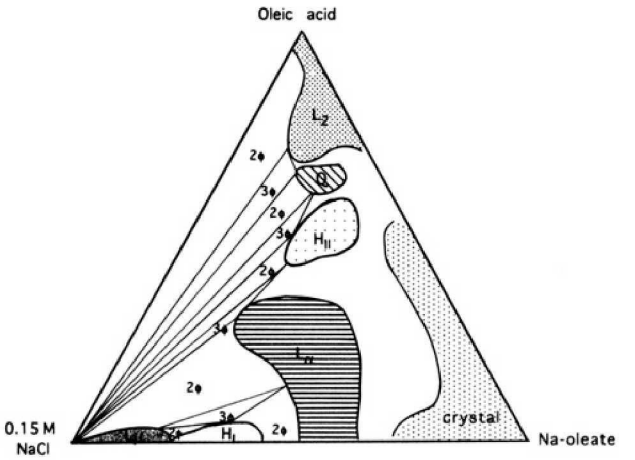


**Figure 7.** Schematic phase diagram of the ternary system oleic acid/oleate/brine, based on diagrams published by Stenius et al.,[9] Engblom et al.,[12] and Small.[20] Note that the location of the one-phase areas is not absolute and that the lines delimiting the two- and three-phase areas are arbitrary and mainly added to show the phase sequence.

protonation state in the present system would require systematic surface charge measurements at different pH and aggregate compositions. However, at 25 °C and physiological salt concentration p$K_a$ values between 7.2 and 8 have been reported for fatty acids in phosphatidylcholine vesicles (see compilation by Cevc et al.[3]). These values pertain to fatty acid:lipid molar ratios in the same range as those explored in the present study, and similar values have also been determined in negatively charged micelles.[17] Thus, although monomeric fatty acids are fully ionized at physiological pH, the solution will contain a mixture of fatty acid and soap if the concentration is above the monomer solubility limit or the fatty acid participates in aggregates formed by other lipids or surfactants present in the solution.

The phase behavior in pure fatty acid/soap systems has been studied quite extensively (see review by Small[8]). For the oleic acid/oleate/water system a number of phase diagrams have been presented in the absence[10-12,20] and presence[9] of added salt. Above the acid/soap melting temperature, three different liquid crystalline phases have been identified in between the two solution phases (see schematic phase map in Figure 7). With increasing concentration of oleate the inverse micellar ($L_2$) solution transforms first to an isotropic cubic (Q) phase, then to the inverse hexagonal ($H_{II}$) phase, and finally to the lamellar ($L_\alpha$) phase. At high water concentration all the above phases may be in equilibrium with oleate solution ($L_1$) below the cmc, and between the two-phase regions, three-phase areas involving two liquid crystalline phases in equilibrium with $L_1$ phase are expected, albeit not yet proven. At temperatures above the melting temperature for oleate, lamellar phase may be formed also in the absence of acid. Thus, in the oleate/water system a sequence of phases involving $L_1$, $H_I$, $L_\alpha$, and finally a number of gel or crystalline phases are observed with decreasing water concentration.[8]

Up to now, little has been reported about the aggregate structure in very dilute dispersions of fatty acid/soaps, and no systematic study of the change in aggregate structure as a function of pH has yet been published. Under conditions were the fatty acid is completely ionized, long chain fatty acids are known to form micelles above the melting point. Small-angle X-ray scattering data indicate

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that the micelles are spherical at low concentration and become rodlike at higher concentration.[21] The cTEM results presented in this study confirm the existence of spherical micelles at low concentrations and show that these indeed grow and become cylindrical when the concentration is increased. However, the micrographs show that the long cylindrical micelles formed at high concentration are not rodlike but very flexible and better described as threadlike.

As the pH is decreased, and the fatty acid becomes partly protonated, the micellar $L_1$ phase region is left, and the sample enters a two-phase area were dilute $L_1$ stands in equilibrium with lamellar $L_\alpha$. The aggregate structure in the dilute part of this phase area has been subject to some speculation. Ekwall showed early by light microscopy that acid soaps in water form myelin figures above the chain melting temperature.[22] Some 40 years later Gebicki and Hicks found that partly ionized unsaturated fatty acids in excess water were able to form closed, spherical, bilayer structures.[23,24] Shortly thereafter it was shown that closed bilayers could be formed also from saturated fatty acids.[16] A number of electron microscopy techniques were used in order to elucidate the morphology of the aggregates. For mixtures of oleic acid/oleate, samples prepared by freeze-etching[23] indicated a predominance of multilamellar vesicles, whereas negative staining and freeze fracture[16] showed mostly unilamellar vesicles. Cryo-transmission electron microscopy, which avoids the introduction of artifacts due to staining and drying procedures during sample preparation and offers a more straightforward interpretation of the micrographs, shows unambiguously that the vesicles formed in partly protonated samples of oleic acid are unilamellar. With decreasing pH, and ionization, the vesicles become more prone to aggregation and eventually large clusters of tightly aggregated vesicles dominate the sample. The extremely close packing of the vesicles observed in the clusters is somewhat surprising and demands some explanation. At the pH where tight packing is observed, the fatty acid is close to, but still above, the apparent $pK_a$,[3] and the vesicles are thus expected to carry considerable net negative charge. The repulsion between the vesicles could theoretically be reduced if a separation of protonated and unprotonated species, in such a way that the charged oleate molecules were expelled from the areas of membrane contact, took place. It is, however, difficult to see how such a separation mechanism could lead to the formation of the large, three-dimensional clusters observed. Furthermore, the fact that no significant growth of the vesicle size with time is observed seems to indicate that the clusters are stabilized by some type of intervesicle interaction. It is possible that the vesicles are held together by hydrogen bonds between acid–anions in opposing bilayers. Very strong hydrogen bonds between carboxyl and carboxylate groups of fatty acid and soap, respectively, are known to exist in the anhydrous crystalline state for 1:1 acid soaps.[25,26] A number of authors have suggested formation of stable acid–anion dimers by strong hydrogen bonds also in hydrated samples and that these help to stabilize the liquid crystalline states.[24,27,28] However, as discussed by Cistola,[11] hydrogen bonded dimers are neither needed nor particularly likely to occur in the liquid crystalline structures. A strong argument against intra-aggregate hydrogen bonds in hydrated samples is based on the fact that, at the surface of a bilayer, carboxyl/carboxylate hydrogen bonding with water would be more entropically favorable than hydrogen bonding between carboxyl and carboxylate groups. The same argument seems to rule out inter-aggregate hydrogen bonding.

It is possible that the contact areas between the tightly packed vesicles represents shared lamellar regions, rather than close bilayer adhesion. The formation of fused doublets, so-called hemifusion, has been suggested as an intermediate step in the process of vesicle fusion (ref 29 and references therein). However, fusion of vesicles normally occurs very rapidly, within less than 1 ms, and the fusion intermediates have so far not been visualized by electron microscopy. In this study, the observed long-term stability of the clusters, and lack of vesicle growth, seems to speak against the idea of shared lamellar regions. On the other hand, it is possible that the hemifused vesicles are particularly long-lived metastable structures in our system. Anyway, solely on the basis of the electron micrographs, it is difficult to discriminate between close bilayer adhesion and hemifusion, and the origin of the tight packing of vesicles composed of near half ionized fatty acids will have to be further investigated.

As the pH in the fatty acid/soap mixture is decreased to values below 8, nonlamellar structures appear. The material dense aggregates seen in Figure 5 very likely represent inverted hexagonal structures and/or transition structures formed during the change from lamellar to inverted hexagonal structures. The aggregates are similar to inverted hexagonal structures observed by cTEM in other systems.[30–33] Furthermore, the inverted structures are seen to coexist with aggregated vesicles over a fairly wide pH range. This agrees well with the phase diagram from which a three-phase region containing $L_1$, $L_\alpha$, and $H_{II}$ is expected. The inverted structures visualized by cTEM are probably covered by a monolayer, and/or bilayer, of lipid, so as to keep them dispersed in the aqueous solution. Large, uncovered assemblies of fatty acid/soap in the inverted hexagonal state precipitate quickly and are thus difficult to investigate by the cTEM method. Fast sedimentation was observed for all samples below pH 7, and no micrographs could consequently be obtained of the sample at pH values corresponding to those where cubic and/or $L_2$ phase is expected to coexist with monomeric $L_1$.

Below the melting temperature fatty acid, acid–soap, or soap crystals form depending on the protonation state of the fatty acid.[8] The micrograph (Figure 6) of lauric acid at pH 9.2 shows coexistence between threadlike micelles and thin, elongated crystals, indicating that the sample is close to the melting temperature at 38 °C. This seems reasonable; Hargreaves and Deamer[16] report crystal formation at temperatures below 34 °C and a slight increase in melting temperature is expected for systems containing physiological salt concentrations.

Investigations of phase behavior and aggregate structure in pure fatty acid systems are important not only from a fundamental point of view. The significant morphological changes observed upon comparably small alterations in pH may be important in many of the

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biological processes where fatty acids are involved. Knowledge of the preferred aggregate structure at a specific pH is, for example, valuable in order to understand, and possibly predict, the structural effects brought about by fatty acid accumulation in phospholipid monolayers and membranes.

Our results indicate that accumulation of fatty acids in the surface monolayer of lipoprotein particles may well lead to the formation of bilayer extensions, as has been suggested.[5,6] In the experiments carried out by Blanchette-Mackie and Scow the pH was kept just above 8, i.e., in the region where lamellar structures were found to be stable in the current study.

Membrane fusion is an essential event in a number of cellular processes, such as endocytosis, vesicle mediated intracellular transport, and secretion. For all fusion processes two basic requirements must be fulfilled: close approach of the two membranes that are to fuse, as well as destabilization of the bilayers at the point of fusion. However, within the cell a variety of different pathways are utilized for the control and regulation of the fusion process. Many of the intracellular events involved in receptor-mediated endocytosis are for instance regulated by proton-induced membrane fusion, whereas a number of other fusion processes appear to require the interaction between negatively charged lipids and positively charged ions, such as $Ca^{2+}$.[34] Phospholipid vesicles, or liposomes, are often used as models in studies of the mechanisms involved in various membrane processes, and vesicles composed of a mixture of phospholipids and fatty acids have been successfully used in studies of proton-induced fusion. It has been shown that liposomes composed of a 3:7 mixture of oleic acid and phosphatidylethanolamine (PE) aggregate and fuse upon a decrease in pH from 7.4 to below 6.5.[7] The same behavior has been observed also with mixtures of PE and other fatty acids.[19] The behavior observed for liposomes composed of phosphatidylcholine (PC) and fatty acids is, however, markedly different. Acidification of liposomes with fatty acid:lipid ratios of 3:7 does, in this case, not give rise to any significant aggregation or fusion.[17] However, liposomes containing high fatty acid concentrations, i.e., lipid:fatty acid ratios above 1:2, have been reported to aggregate quickly when subjected to a pH-jump from 10 to 4.[3] Futhermore, the aggregation was in this system followed by a transition from lamellar to nonlamellar structures.

The results presented in this study indicate that the very different results obtained in PC and PE systems are not caused by any fundamental difference in the phospholipid/fatty acid interaction in the two systems. Instead the difference may be explained by a combination of electrostatic effects and the inherent different aggregation behavior in the two systems.

In contrast to vesicles made from PC, vesicles composed of PE are well-known to aggregate and fuse under conditions were the membranes carry no net charge. The pH at which aggregation and subsequent fusion is induced depends primarily on the $pK_a$ of the amine group (approximately 9.5[35]), and for egg-PE vesicles increasing the pH to values in the region of 7.4−8 introduces enough net negative charge to prevent aggregation.[36] However, addition of cations, such as $Na^+$, screens the electrostatic repulsion and in buffers containing physiological salt concentrations aggregation is often induced at pH well above 7.4. Incorporation of fatty acids, with considerably lower $pK_a$ than that reported for PE, adds additional negative charge which helps prevent aggregation. Accordingly, the pH has to be lowered to values below 6.5 before appreciable fusion of PE vesicles, containing an oleic acid:lipid ratio of 3:7, is observed in buffer containing 150 mM NaCl.[7] The above described effects of fatty acids on vesicle fusion are valid at temperatures were the system exists in a true lamellar state. The behavior in the fatty acid/lipid mixtures may be further complicated by transitions into nonlamellar phases. Above a well-defined temperature pure PE undergoes a thermotropic transition to the inverted hexagonal ($H_{II}$) phase[36] and low concentrations of various fatty acids have been shown to decrease the transition temperature.[19] Lowering the pH to the point were membrane contact is allowed may thus, in these systems, induce not only fusion of the vesicles but also formation of $H_{II}$ phase.

In conclusion, vesicles composed of PE and low concentrations of fatty acid fuse because of the inherent tendency for fusion in the PE system. The fatty acids in themselves do not promote vesicle fusion. On the contrary, as shown in this study vesicles formed in pure fatty acid/soap mixtures seem very resistant against fusion at pH and temperatures were the lamellar phase is stable.

In line with the above, no fusion is observed upon acidification of solutions containing vesicles having a fatty acid:PC ratio of 3:7. However, when the molar ratio of fatty acid:lipid becomes high enough the phase propensity of the fatty acid will rule the overall behavior of the system. Thus, the aggregation and conversion to nonlamellar structures observed at high fatty acid ratios is merely a consequence of the fact that the fatty acid itself forms nonlamellar structures at pH 7.4 and lower.

Oleic acid is not appreciably ionized until the pH is raised to values above 10. At pH values this high the phase behavior in fatty acid/phospholipid mixtures does not have much physiological relevance. Nevertheless, it is interesting to compare the effects of oleate on vesicle size and structure with the effects of other surfactants. The experimental results show that the same general sequence of morphological changes, as previously observed by cTEM for both nonionic[37,38] and ionic[39] surfactants, takes place also in this system. Thus, oleate was found to induce significant vesicle growth at concentrations just below those needed for membrane solubilization and at higher concentrations a transition into mixed micelles. In the present study no systematic study of the structures formed during the vesicle to micelle transition was carried out. It thus remains to be investigated whether the transition takes place via the formation of "holey lamellar" structures, as found for CTAC[39] and SDS[40], or via a region of coexistence between lamellar, $L_\alpha$, and micellar phase, as observed for nonionic surfactants such as $C_{12}E_8$[38] and octyl glucoside.[41]

**Acknowledgment.** We are grateful to Professor Mats Almgren for helpful discussions. This work was financially supported by the Swedish Technical Research Council and by the Knut and Alice Wallenberg Foundation.

LA941034L

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