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Phase equilibria of the ternary system 1-palmitoyl-*sn*-glycero-3-phosphocholine / oleic acid / water studied by NMR

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Part of a phase diagram for the system 1-palmitoyl-*sn*-glycero-3-phosphocholine (PamGroPCho) / oleic acid / water has been constructed from mainly ^{31}P-NMR data and a previous determination of the phase equilibria of the binary PamGroPCHo / water system. It was found that the appearance of the phase diagram is very similar to those found for several simple soap / fatty acid / water or soap / long-chain alcohol / water systems. The most striking features observed are: (1) the lamellar phase can swell towards very high water contents (2) vesicles are formed after sonication and (3) the cubic liquid crystalline phase disappears upon addition of very small amounts of oleic acid. The self-association of the amphiphiles and the shape of the aggregates are discussed in terms of existing first-order approximative theories.

1-Acyl lysophosphatidylcholines are zwitterionic lipids that are found in many biological membranes. The concentration of these lipids in the membranes is usually very low and their cytolytic properties are well known [1]. Recently, the phase behaviour of several lysophosphatidylcholine/water systems has been published [2,3]. In the present communication we report a study of the phase equilibria in the ternary system 1-palmitoyl-*sn*-glycero-3-phosphocholine/oleic acid-/heavy water at 30 ° C. A tentative phase diagram has been constructed, mainly be means of ^{31}P-NMR measurements. It is found that the lysolipid has physicochemical properties similar to ordinary detergents. Solubilization of a fatty acid or an alcohol leads to changes in the phase structures that can be understood by a consideration of the packing properties of the lipids in the aggregate

Palmitic and oleic acid of 99% + purity were obtained from Nu Chek Prep., Inc. (Elysian, MN, U.S.A.). 1-Palmitoyl-*sn*-glycero-3-phosphocholine, PamGroPCHo, was prepared and purified by standard procedures as previously described [2]. Thin-layer chromatography showed that the contamination of the lysoPC by other lipids was < 1%. Potassium oleate was prepared by neutralizing oleic acid with potassium hydroxide. The samples were prepared by mixing appropriate amounts of vacuum-dried lipids and heavy water (isotopic purity > 99.8 atom%, Ciba-Geigy) in glass tubes. Mixing was accomplished by stirring the contents of the tubes with a glass rod The tubes were flame-sealed and their contents packed at the bottom of the tubes by centrifugation. Before NMR measurements the samples were allowed to equilibrate for several days. ^{31}P- and ^{2}H-NMR spectra were recorded at the resonance frequencies 101.27 and 38.40 MHz, respectively, on a Bruker WM-250-NMR spectrometer operating in the pulsed Fourier transform mode. Inverse gated broad band

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proton decoupling with an effect of about 5 W was used during the measurements of ^{31}P. The sensitivity of the instrument was enchanced by using quadrature detection. The temperature of the NMR probe was regulated with a variable temperature control unit. Before the spectra were recorded the samples were thermally equilibrated at 30 ± 1°C for more than one hour. The number of scans required for obtaining a spectrum which was reasonably easy to interpret varied between 1000 and 18000 depending on the PamGroPCho content of the sample.

The phase equilibria were determined by recording ^{31}P- and ^{2}H-NMR spectra for a large number of samples with appropriate compositions as described previously [4]. In particular ^{31}P-NMR is convenient for such determinations since the spectral appearence depends on the symmetry of the liquid crystalline phase structure. Thus, in a lamellar phase the ^{31}P-NMR spectrum shows a high-field peak and a low-field shoulder, while for a hexagonal phase it has a low-field peak and a high-field shoulder (Fig. 1). Furthermore, for gemometrical reasons the magnitude of the chemical shift anisotropy for the lamellar phase is about twice as large as that of the hexagonal phase. For isotropic solutions (e.g. micellar phases) and isotropic liquid crystals (e.g. cubic phases) the ^{31}P-NMR spectrum usually exhibits a single narrow peak, the line width of which is considerably larger for cubic phases than for micellar solutions. When two or more phases are present in the sample the resultant NMR spectrum is a superposition of spectra originating from each of all the different phases [4].

Part of the phase diagram of the three component system PamGroPCho/oleic acid/heavy water at 30°C is drawn in Fig. 2. ^{31}P-NMR spectra of more than 150 samples together with the approximate phase boundaries from the binary system PamGroPCho/$^{2}H_2O$ constitute the main basis for the construction of the phase diagram. It is a very time-consuming and tedious work to find the exact location of the phase boundaries, and the lines in the diagram are drawn with a relative error of approx. ±5%. The composition of the samples ranged from 1.0 to 87.7% (w/w) PamGroPCho, from 0.8 to 63.0% (w/w) oleic acid and from 9.8 to 95.0% (w/w) heavy water.

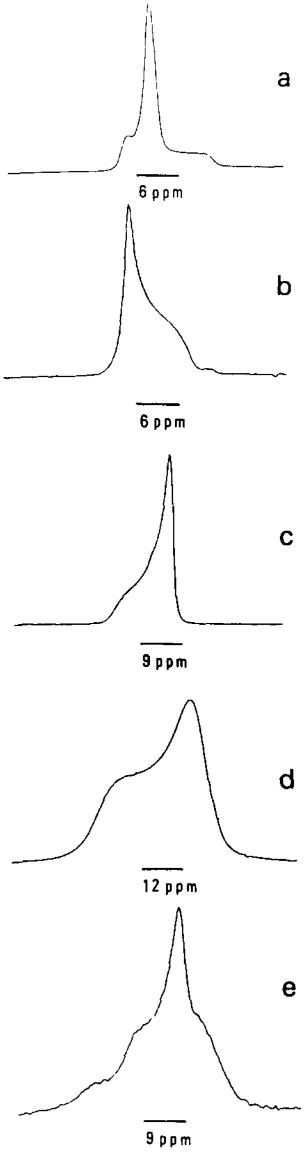

Fig. 1. ^{31}P-NMR spectra of the ternary system PamGroPCho/oleic acid/$^{2}H_2O$ (compositions in weight percentage) (a) 41.1 0.8 58.1, cubic + hexagonal phases, (b) 25.6 4.3 70.1, hexagonal phase, (c) 59.6 25.4 15.0, lamellar phase, (d) 87.7 1.4 10.9, 'gel' phase, (e) 81.1 8.7 10.2, lamellar + 'gel' phases

The clear isotropic micellar solution extending up to about 37% (w/w) PamGroPCho of the binary system PamGroPCho/$^{2}H_2O$ can solubilize at most about 2% (w/w) oleic acid (Fig. 2). The locations of the two- and three-phase areas have not been determined. The cubic liquid crystalline phase located between the micellar solution and the hexagonal liquid crystalline phase can hardly incorporate any oleic acid at all without breaking down. In the binary system PamGroPCho/^2H_2O the hexagonal phase, H_1 extends between roughly 48 and 80% (w/w) PamGroPCho. This phase can take up at least 5% (w/w) oleic acid and it extends with a narrow tail as far as up to about 70% (w/w) heavy water.

The ^{31}P-NMR spectrum from the phase area between 82 and 93% (w/w) PamGroPCho shows a band shape that is typical for a phase structure not having uniaxial symmetry (cf. Fig. 1d). However, when a sample in this area is heated to about 50 °C, the ^{31}P-NMR spectrum shows a typical line shape of a lamellar phase. This indicates that this phase at a temperature below 50 °C most probably is a 'gel' phase. In the region between about 8 and 13% (w/w) oleic acid and between about 10 and 20% (w/w) heavy water there is a two-phase region consisting of a lamellar phase and this 'gel' phase (cf. Fig. 1e). At 50 °C the two-phase area disappears and only a lamellar liquid crystalline phase is obtained.

Above the two-phase region there is an extensive lamellar liquid crystalline phase area that can take up at least as much as 90% (w/w) heavy water. It is likely that this lamellar phase extends even further towards the heavy water corner, but due to experimental difficulties at these very high water contents the exact limit cannot be firmly determined.

The region above the lamellar phase area towards the oleic acid corner has not been investigated. However, the ^{31}P-NMR spectrum from a liquid sample containing PamGroPCho, oleic acid, and heavy water in the ratio 27.0 : 63.0 : 10.0 (%, w/w) shows a large narrow peak superimposed on an exceedingly small lamellar line shape. This indicates that an isotropic micellar solution exists in a neighbouring region, and because of the low water content this is most probably a reversed micellar solution.

The aggregates of several different lysolecithins in the micellar solutions and cubic phases have been investigated in previous work [3] It was found that PamGroPCho formed small globular micelles in water and that the aggregates building up the cubic phase were slightly elongated or rodlike [3].

A lamellar phase usually forms when a fatty acid or a long-chain alcohol is added to a micellar solution or a hexagonal phase in an aqueous system of a soap or other detergents (see e.g. a review by Ekwall [5]) The factors governing the formation, shape and structure of different amphiphile aggregates have been discussed by several authors [6,7]. From a simple first-order approximation theory, provided by these authors, it appears that

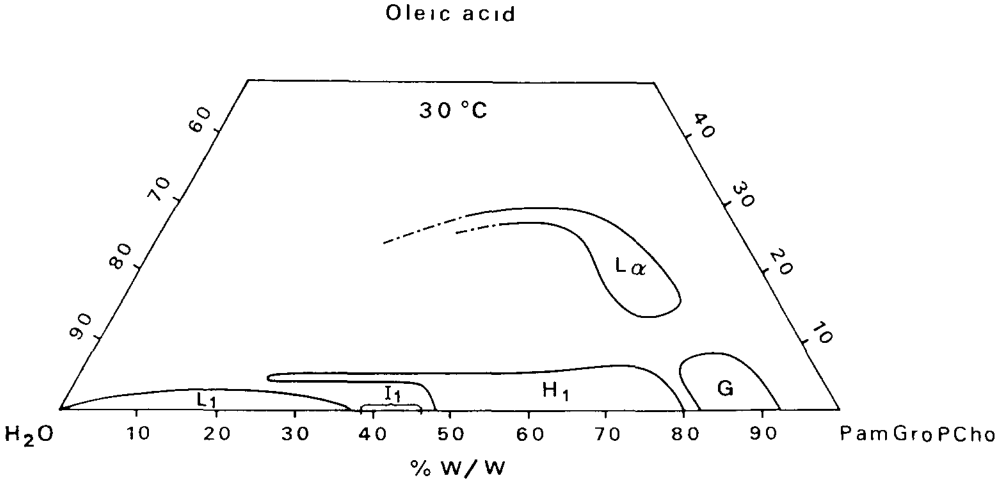

Fig 2 Part of the phase diagram of the PamGroPCho/oleic acid/^2H_2O system The composition is given in % (w/w) Notations L_1, isotropic aqueous solution, H_1, hexagonal liquid crystalline phase, I_1, cubic liquid crystalline phase, L_α, lamellar liquid crystalline phase; G, 'gel' phase

the effective geometry of the amphiphile often determines the shape of the aggregates. Thus, the packing of the amphiphiles into different aggregates depends on the hydrophobic volume, the hydrocarbon-water interfacial area and the hydrocarbon chain length of the participating molecules. An amphiphile forming a globular or spherical aggregate therefore has a conical shape with a large polar head group area A fatty acid or an alcohol, on the other hand, has a rather small polar head group, but occupies a relatively large hydrophobic volume The addition of an alcohol or a fatty acid to a sphercial aggregate thus results in a relative increase in the hydrophobic volume but only a minor change in the interfacial area. This eventually leads to the formation of a lamellar aggregate, since the effective molecular shape now becomes more cylindrical. According to these considerations it is not surprising to find that the phase diagram of the ternary system of PamGroPCho/oleic acid/water is rather similar to that of sodium octanoate/octanoic acid/water and analogous systems. It is also found that the addition of av long-chain alcohol to the hexagonal phase in the PamGroPCho/^2H_2O system promotes a transition to a lamellar phase For example the ^{31}P-NMR spectrum from a sample containing PamGroPCho, decanol, and heavy water in the ratio 76.0 : 3.0 : 21.0 (%, w/w) exhibits a line shape, typical for a lamellar phase. However, the cubic phase between the micellar solution and the hexagonal phase occurs only in the binary system PamGroPCho/^2H_2O This is probably due to the fact that the micellar aggregates building up this phase structure have a very critical size and shape as discussed in a previous publication [8]. A further strong indication of the crucial importance of the size of the aggregates in this phase is that it can take up only minute amounts of oleic acid, since addition of the fatty acid will induce a growing of the aggregates

The lamellar phase extends to very high water contents. This can be easily understood, if it is assumed that a small percentage of the fatty acid is ionized It has been shown previously [9,10] that a lamellar phase may swell to an extremely high degree even by a very small increase in the surface charge density. It has also been shown in a previous investigation by Jain and De Haas [11] that a lamellar phase could be obtained at high water content with lysoPC and fatty acid. It was speculated that vesicles might form after sonication This has been verified in this work by the addition of so called shift reagents to the vesicular solution. Addition of 0.01 M Eu^{3+} to a sonicated sample containing PamGroPCho, oleic acid and heavy water in the ratio 3.2 : 1.8 : 95.0 (%, w/w) shows a difference in the ^{31}P chemical shift between the inner and outer phosphorus of the vesicles of about 17 ppm. Since a lamellar phase was formed Jain and De Haas [11] draw the conclusion that lysoPC and fatty acid form a complex that looks similar to ordinary PC due to a hydrogen bond between the carboxylic acid and the hydroxylic group of the glycerol backbone. However, our data show that the lamellar phase forms for the same reasons as in ordinary soap systems as discussed above and it is thus not necessary to invoke the formation of specific complexes. It is important to realize that this conclusion also should apply to cell membranes containing lysoPC and fatty acids.

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