Phase Diagram of Soybean Phosphatidylcholine-Diacylglycerol-Water Studied by X-Ray Diffraction and ^{31}P- and Pulsed Field Gradient ^{1}H-NMR: Evidence for Reversed Micelles in the Cubic Phase

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ABSTRACT The phase equilibria of the system soybean phosphatidylcholine, diacylglycerol, and water has been determined using a combination of classical methods together with x-ray diffraction and NMR techniques. In particular, the extent of the phase regions of the lamellar, the reversed hexagonal, and the cubic phases have been determined. By pulsed field gradient ^{1}H-NMR, the diffusion coefficients of all three components in a cubic phase composed of soybean phosphatidylcholine, diacylglycerol, and heavy water have been determined at 25 and 59°C and also for the corresponding cubic phase composed of the chemically more well defined synthetic components 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoylglycerol (DOG), and heavy water. The extension of the phase region of the cubic phase did not seem to change appreciably for the two ternary systems studied. The translational diffusion coefficient of DOPC in this cubic phase is more than an order of magnitude smaller (3×10^{-13} m^2 s^{-1}, 59°C) than the lateral diffusion coefficient of DOPC in an oriented lipid bilayer (5×10^{-12} m^2 s^{-1}, 35°C), whereas the diffusion coefficients of water and DOG were found to be about two orders of magnitude larger than of DOPC at 59°C. It is concluded that the cubic phase is built up of closed reversed micelles in accordance with the suggestion from previous x-ray diffraction studies.

INTRODUCTION

In signal transductions over biological membranes, 1,2-*sn*-diacylglycerol (DAG) serves as a second messenger by activating the membrane-interacting, Ca^{2+}-dependent, regulatory enzyme protein kinase C (Berridge, 1987; Newton, 1993). DAG is formed by enzymatic degradation of phosphatidylinositol-bisphosphate. There are at least 10 protein kinase C isozymes, all of which are activated by DAG and phosphatidylserine; however, only four isozymes are regulated by Ca^{2+} (Newton, 1993). It is assumed that because the relatively hydrophobic DAG molecule is produced in biological membranes, this molecule will remain solubilized in the lipid bilayer. The role of DAG as a membrane-bound second messenger, besides specifically binding to protein kinase C, may be to affect the physicochemical properties of the lipid bilayer. Several investigations have shown that DAG may cause structural changes in phospholipid bilayers (Das and Rand, 1986; Goldberg et al., 1994; Siegel et al., 1989a, 1989b), even where the proportion of DAG is relatively low (<2 mol%, similar to physiological concentrations).

A narrow symmetric signal has been observed in ^{31}P-NMR spectra upon addition of DAG up to about 15 mol% to a phospholipid bilayer, which was associated with an enhanced hydrolysis by phospholipase C (Dawson et al., 1984). Nonbilayer lipid phases induced by DAG have been demonstrated previously in various DAG/phospholipid systems (Das and Rand, 1986; De Boeck and Zidovetzki, 1989; Epand, 1985; Siegel et al., 1989a, 1989b). At high DAG concentrations in phospholipids, a reversed hexagonal (H_{II}) phase forms (De Boeck and Zidovetzki, 1989; Goldberg et al., 1994). Furthermore, dipalmitoyl-DAG increases the curvature of the cylindrical aggregates in the H_{II} phases formed by lipids with oleoyl acyl chains (Siegel et al., 1989b).

The structures of the normal hexagonal (H_I), the lamellar (L_α), and the H_{II} phases are well established (Fontell, 1990; Lindblom and Rilfors, 1989; Seddon, 1990b), but the structures of the many intermediate phases, which may be found between every other phase in the phase diagram, are much less well known. A majority of these intermediate phases are cubic phases. Several cubic lipid phases belonging to different space groups have been identified in a wide variety of amphiphile/water systems (Lindblom and Rilfors, 1989; Seddon, 1990b). There are mainly four possible locations in the phase diagram where cubic phases may occur: i) the cubic (I_1) phase located between the micellar solution (L_1) phase and the H_I phase; ii) the cubic phase located between the H_I and the L_α phases; iii) the phase located between the L_α and the H_{II} phases; and iv) the cubic phase located between the H_{II} and the reversed micellar solution (L_2) phases. The I_I cubic phases are usually composed of closed anisometric lipid aggregates like micelles (Eriksson et al., 1985a, 1987; Lindblom and Rilfors, 1989), and the cubic phases under ii and iii are normal and reversed bicontinuous cubic phases, respectively (Lindblom and Rilfors, 1989). There are rather few observations of cubic phases in the location iv. One may expect that a cubic structure in this location would be a complementary mirror structure to that occurring between the L_1 and the H_I phases.

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Previously, it has been reported that a cubic liquid crystalline phase forms in a fully hydrated mixture of egg phosphatidylcholine and egg DAG (Das and Rand, 1984; 1986). Stenius and co-workers (Stenius et al., 1984) found a cubic phase at the location iv in the system sodium oleate/oleic acid/water (with 0.25 M NaCl). It has been suggested from x-ray diffraction that this cubic phase belongs to the space group *Fd3m* (Q^{227}) and that the structure is composed of reversed micellar aggregates (Seddon et al., 1990). However, x-ray diffraction, the traditional technique for structural investigations of lyotropic liquid crystalline phases, does not *alone* provide data of sufficient distinction to determine conclusively the structure of a cubic phase or what type of lipid aggregates it is composed of (Fontell, 1990; Mariani et al., 1988). An excellent complementary method for such investigations is provided by the pulsed field gradient (PFG) NMR diffusion method (Lindblom and Orädd, 1994; Lindblom and Rilfors, 1989). With this technique, it is possible to discriminate conclusively between cubic phases built up of closed micelles and bicontinuous structures (Lindblom and Orädd, 1994; Lindblom and Wennerström, 1977).

The method is based on a comparison of lateral diffusion coefficients obtained from the lipids in different liquid crystalline phases. It is possible to determine whether the lipid lateral diffusion is subjected to restricted diffusional motion in the lipid aggregate structure (Lindblom and Orädd, 1994). This is feasible, because in a typical NMR diffusion experiment the time during which diffusion is observed is varied between 100 and 500 ms. This means that the average displacement of a freely diffusing molecule is in the order of 1 μm, which is much larger than the dimensions of the cubic unit cell. Thus, the *measured* diffusion coefficient of lipid molecules in a cubic phase built up of closed micelles will be that of an aggregate rather than of the lipid molecule itself, because the lipids can only move within the small volume of the closed micelle. On the other hand, the lipid translational diffusion in a bicontinuous cubic phase will be unrestricted over macroscopical distances. It is found that the lipid diffusion coefficient in a bicontinuous cubic phase is of about the same magnitude as the lateral diffusion coefficient in the corresponding lamellar phase, with which it is in equilibrium (Lindblom et al., 1981; Lindblom and Orädd, 1994; Lindblom and Wennerström, 1977).

Note that the comparison between diffusion coefficients has to be performed between *lipid diffusion* coefficients. It is not advisable to compare, for example, water diffusion coefficients, because these might be affected by a number of different interactions like hydrogen bonding and chemical exchange. Furthermore, it is well known that water diffusion usually is not restricted appreciably in lipid aggregates as measured by pulsed field gradient NMR. In a recent study of the transmembrane transport of water and various alcohol molecules incorporated in lipid bilayer vesicles, no restriction in the water diffusion was observed using the NMR diffusion method (G. Orädd and G. Lindblom, unpublished data).

The purpose of this investigation is twofold. First, a ternary phase diagram of a phospholipid system containing DAG and water is determined. Detailed knowledge of the phase equilibria of a lipid system provides an important clue to the macroscopic appearance of the system and offers a link with the molecular interactions occurring in the membrane bilayer (Guldbrand et al., 1982; Ulmius et al., 1977). A proper understanding of this connection signifies that the phase behavior can be controlled by changing interactions at the molecular level, or that interaggregate interactions can be studied. Therefore, it is crucial that one knows the detailed phase behavior of the system. Second, the structure of the cubic phase in the phospholipid/DAG/water system is determined. The present study also provides us with some missing information about cubic phase structures and should be welcomed by theoreticians working with amphiphile self-assembly. It should be noted that this cubic phase structure was thought not to form in membrane lipid systems (Kirk et al., 1984; Lindblom and Rilfors, 1989).

MATERIALS AND METHODS

Materials

In this study, we have used technical substances of both the phospholipid and the DAG components, namely, the soya phosphatidylcholine (SPC) and DAG from sunflower oil. One of the reasons for doing so is that for the determination of a phase diagram, ~150–200 samples have to be prepared that, if synthetic lipids had been used, would result in unwarranted high costs, because these substances are very expensive. Another reason is that we would like to use natural lipids, because the system studied might be useful in pharmaceutical applications. However, a number of samples were also prepared with synthetic dioleoylphosphatidylcholine (DOPC) and dioleoylglycerol (DOG), in particular, in the cubic phase region of the phase diagram, and these samples were found to be in remarkably good agreement with the phase behavior, structure, and lipid diffusion of the natural lipids.

For the determination of the phase diagram, we used the following technical phosphatidylcholine (PC) and diacylglycerol substances. The PC is a lipid extract of high quality from soya beans, called Epikuron 200, and was purchased from Lucas Meyer (Hamburg, Germany). It is a purified SPC with a linoleic acid content of more than 60% of the total fatty acid content, and the rest of the acyl chains are mainly palmitoyl and oleoyl chains. SPC is semicrystalline and contains 1–2 moles of crystal water.

The DAG used is an extract from sunflower oil and was obtained as a gift from Grindstedt Products (Brabrand, Denmark). It contains 81% of diacylglycerols (a racemic mixture), 15% monoacylglycerols, 3% triacylglycerols, and 1% free fatty acids. The sunflower oil has a high content of unsaturated fatty acids, 80% oleic acid, about 10% linoleic acid, and the remainder being mainly saturated acids. The supplied product is opaque at room temperature and becomes clear at a temperature above 40°C. By first melting the sample to an isotropic fluid and then centrifuging for about 40 min (4000 rpm) in an ordinary desk centrifuge at room temperature, a clear supernatant reduced in saturated chains was obtained. This lipid mixture was separated by thin layer chromatography (TLC) on 0.25 mm precoated plates of silica-gel 60 H (Merck, Darmstadt, Germany) developed with petroleum ether (40–60°C)/diethylether/acetic acid, 80:20:1. As judged from TLC, the purified lipid mixture now contained about 88% DAG, 10% monoacylglycerol, and 2% triacylglycerol (no free fatty acids were detected). The purified lipid mixture was used in the preparation of the samples for the determination of the phase diagram in Fig. 2. This purification procedure was necessary to avoid precipitation of saturated acyl chains otherwise present. Saturated fatty acids gave rise to small solid crystals clearly observed in the cubic phase samples examined by polarizing microscope.

Heavy water, from Dr. Glaser, AG (Basel, Switzerland), was used for two reasons. First, it was used to increase the density differences between the

phases to physically separate them by centrifugation. This was done to locate the phase borders. Second, the ^{1}H-NMR diffusion studies are performed preferably on such samples (Lindblom and Wennerström, 1977).

The components of the samples were weighed in glass ampules that were flame-sealed. Typically, a sample contained between 100 and 200 mg of the phospholipid. Equilibrium was obtained by thermal treatment, shaking, and centrifuging back and forth at 40–60°C. The samples were stored at 25°C for at least 2 weeks before any measurements were made. The phase behavior and the phase diagrams were determined as described, e.g., in Gutman et al. (1984). All samples were studied within 4 months.

The NMR diffusion studies were performed on cubic phases containing either compounds extracted from the natural products described above (SPC and DAG) or the synthetic substances 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-*sn*-dioleoylglycerol (DOG). These synthetic lipids were obtained from Larodan Fine Chemicals (Malmö, Sweden).

X-ray diffraction

The low angle x-ray diffractograms were obtained using a pin-hole camera of the Kiessig-design. The specimen-to-film distance was 200 or 500 mm, and copper K_α nickel-filtered radiation (λ = 1.542 Å) was used. The samples were held in thin walled, flame-sealed glass capillaries with a diameter of 0.7 mm. All samples were investigated at 25°C, except for some of the cubic phases, which were studied at both 25 and at 59°C.

NMR diffusion

The ^{1}H diffusion studies were performed on a Bruker ACP-250 spectrometer equipped with an HR-50 high resolution VT diffusion probe for 5 mm samples (Cryomagnet Systems, Inc., Indianapolis, IN). The gradient pulses, controlled by the spectrometer, were generated by a home-built gradient unit driven by a Kenwood PD35–20D power supply. Two different pulse sequences were used in the diffusion experiments, namely, the Hahn-echo sequence (SE) (Carr and Purcell, 1954) and a modification of the stimulated spin-echo sequence (LED) (Gibbs and Johnson, 1991). The two pulse sequences are illustrated in Fig. 1. In both sequences, two gradient pulses of strength g and duration δ were applied during the defocusing and refocusing periods, respectively. Δ is the time between the gradient pulses. The attenuation of the signal, $S(t)$, is described by Eq. 1, where γ is the magnetogyric ratio and R is the attenuation due to relaxation.

$$S(t) = S(0)e^{-(g\gamma\delta)^2 D(\Delta - (\delta/3))}e^{-R} \quad (1)$$

For the SE experiment $R = 2\tau/T_2$ and for the LED experiment $R = (2\tau_1/T_2) + (\tau_2 - \tau_1 + T)/T_1$, where τ, τ_1, τ_2, and T are defined in Fig. 1. In the diffusion experiments, all parameters except the gradient pulse width is kept constant so that the second exponent in Eq. 1 is constant during the experiment. Note that because of different relaxation times, T_1 and T_2, the attenuation caused by spin relaxation will be different for each NMR peak and the intensity of the peaks, therefore, cannot be compared directly. It is also important to note that the weight factors for DOG and DOPC obtained in the biexponential fits of the overlapping peaks at 0.7 and 1.1 ppm will be influenced by the spin relaxation. Therefore, these factors are not necessarily the same as the mole fractions of the components in the sample.

The peak height of the signals in the spectrum were fit to Eq. 1 using the curve-fitting feature in SigmaPlot (Jandel Scientific, Erkrath, Germany), from which the diffusion coefficients were obtained. Several experiments were made with different settings on τ (100–500 ms) and Δ (100–500 ms), whereas δ were varied typically over 20 different values (usually 1–20 ms). g was kept constant at 0.5734 Tm^{-1} according to calibrations performed on $H_2O/^2H_2O$, dodecane, and oleic acid. The experiments were preceded by three gradient pulses to create steady-state residual gradient conditions before acquisition (Gibbs and Johnson, 1991). The error in the measured diffusion coefficients was usually less than 3%.

From measurements of the phospholipid diffusion in the L_α and the cubic liquid crystalline phases, it is possible to discriminate between the two fundamentally different cubic phase structures, namely, those built up of closed aggregates and those having continuous hydrocarbon regions (Lindblom and Orädd (1994), and Lindblom and Rilfors (1989) and references therein). The simple idea behind the method is based on the fact that for a cubic phase consisting of reversed micelles, the phospholipid molecules will be restricted to diffuse within the micelle and, therefore, the phospholipid can only move a very short distance, whereas a lipid molecule in a bicontinuous phase can perform translational diffusion over macroscopical distances.

The temperature, which was monitored by a thermocouple placed close to the sample, was kept constant to within 1°C by a heated airstream passing over the sample.

^{31}P-NMR measurements

^{31}P-NMR measurements were made at 101.26 MHz on a Bruker ACP-250 NMR spectrometer, using the phase-cycled Hahn echo pulse sequence (Rance and Byrd, 1983). From the chemical shift anisotropies obtained for

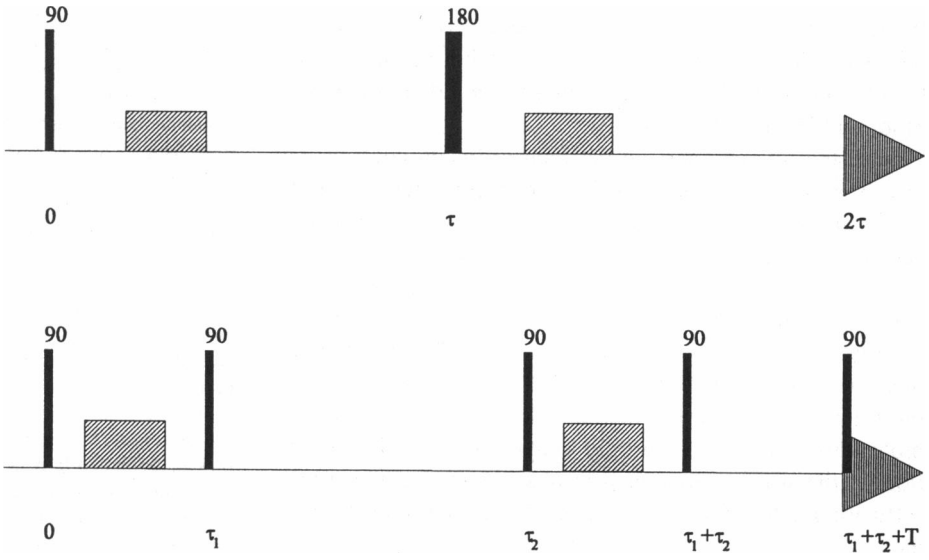

FIGURE 1 The pulse sequences used in the NMR diffusion experiments. The radio frequency pulses are shown as filled vertical rectangles with the flip angles denoted above each pulse. The magnetic field gradient pulses are shown as hatched rectangles, and the data acquisition is indicated with a vertically hatched triangle. (*top*) The SE experiment. (*bottom*) The LED sequence. For the meanings of the other symbols, see text.

the different samples, the phase equilibria could be determined as described, e.g., in Eriksson et al. (1985b).

RESULTS

The phase behavior at 25°C of the system SPC/DAG/^2H_2O is shown in Fig. 2. SPC incorporates 25 wt% DAG, but the phase structure in this "nonaqueous" region has not been studied in detail here. In the binary system SPC/^2H_2O, an L_α phase is obtained at water contents between 8 and 35 wt% (Gutman et al., 1984). This phase has a rather small capability (~5 wt%) to incorporate DAG without losing the lamellar structure. This low incorporation of DAG in lipid bilayers has also been observed previously in connection with investigations of membrane fusion (Siegel et al., 1989a). At higher DAG contents, there is, after a two-phase region, an H_{II} phase. At still higher amounts of DAG, a cubic I_{II} phase forms, which occupies a narrow region around the composition of 30:60:10 in wt% of SPC/DAG/^2H_2O. The cubic phase is in turn in equilibrium, with an L_2 solution phase of DAG containing some water and SPC. This sequence of the occurrence of the different phases in the diagram is the same as that reported for other DAG-PC-water systems (Das and Rand, 1984, 1986). Note that all of the liquid crystalline phases may be in equilibrium with excess water (Fig. 2).

The samples containing a cubic phase are isotropic (dark) between crossed polarizers, and they are transparent and stiff. A cubic phase composed of either SPC/DAG/^2H_2O or DOPC/DOG/^2H_2O showed the same behavior as studied by either x ray or NMR. The x-ray pin-hole diffraction patterns are spotty (a very common feature observed in diffractograms of cubic phases from lipid/water systems), a fact that indicates the occurrence of rather large monodomains. Our pin-hole diffraction patterns are also very similar in appearance to that published by Seddon (Fig. 1 in Seddon, 1990a). For samples of the cubic phase with the composition in wt% of DOPC/DOG/^2H_2O equal to 26.33:60.87:12.80 and of SPC/DAG/^2H_2O equal to 29.9:59.7:10.4, we have obtained nine and eight separate reflections, respectively, which have been indexed according to the space group *Fd3m* (Table 1). The indexing of the diffraction data was assessed as shown in Fig. 3 by a plot of $1/d_{hkl}$ vs. $(h^2 + k^2 + l^2)^{1/2}$. For the correct choice of space group, this plot gives a straight line passing through the origin and having a slope of $1/a_0$, where a_0 is the unit cell lattice parameter. The reflections listed in Table 1 gives a_0 = 158.0 Å for the SPC/DAG/^2H_2O system and a_0 = 130 Å for the DOPC/DOG/^2H_2O system.

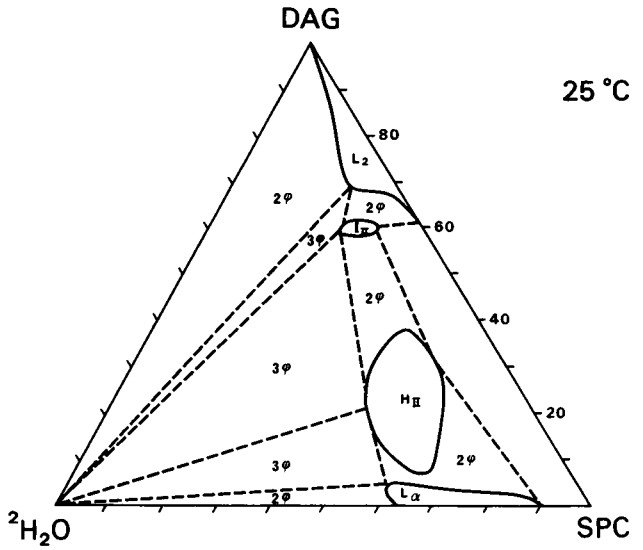

FIGURE 2 The phase diagram of the system soya phosphatidylcholine (SPC), diacylglycerol (DAG), and ^2H_2O at 25°C. The various phase regions are denoted by: L_2, reversed micellar solution phase; I_{II}, cubic liquid crystalline phase; H_{II}, reversed hexagonal phase; and L_α, lamellar liquid crystalline phase. The number of phases are indicated in the various two- and three-phase regions by 2φ and 3φ, respectively.

In the diffusion experiments, five different peaks in the ^{1}H-NMR spectrum were used, namely, those with chemical shifts of 0.7, 1.1, 3.1, 4.6, and 5.2 ppm. These five NMR peaks had T_2 relaxation times sufficiently long for a successful application of the NMR diffusion method. The assignments of the NMR signals arising from the lipid peaks are: 0.7 ppm, the terminal methyl groups of the hydrocarbon chains of PC and DAG; 1.1 ppm, the methylene groups of the hydrocarbon chains of PC and DAG; 3.1 ppm, the methyls of the choline headgroup of PC; and 5.2 ppm, the hydroxyl group of DAG. The ^{1}H-NMR peak at 4.6 ppm at 25°C was observed to move continuously toward lower chemical shift with increasing temperature, which is a typical behavior when chemical exchange occurs in the system. The diffusion coefficient measured from this peak was also found to be larger than that obtained from the other NMR signals. Therefore, the signal at 4.6 ppm was assigned to water.

The diffusion experiments were practiced on several samples embodying a cubic phase from either of the systems SPC/DAG/^2H_2O or DOPC/DOG/^2H_2O. A summary of typical results of both systems at similar compositions and at different temperatures is shown in Table 2.

TABLE 1 Data from x-ray diffraction studies of cubic phase samples

d_{hkl}, (Å)									
a)	46.3	39.9	37.9	32.9	29.8	26.3	25.2	23.0	19.8
b)	46.3	39.9	38.4	33.4	29.9	26.4	25.2	23.1	19.8
c)	55.1	46.7		39.0	35.7	32.1	30.2	27.5	23.7
hkl	220	311	222	400	331	422	511/333	440	622

Composition in wt% of a) DOPC/DOG/^2H_2O equal to 26.33/60.87/12.80 at 25°C, b) at 59°C, and c) of SPC/DAG/^2H_2O equal to 29.9/59.7/10.4 at 25°C.

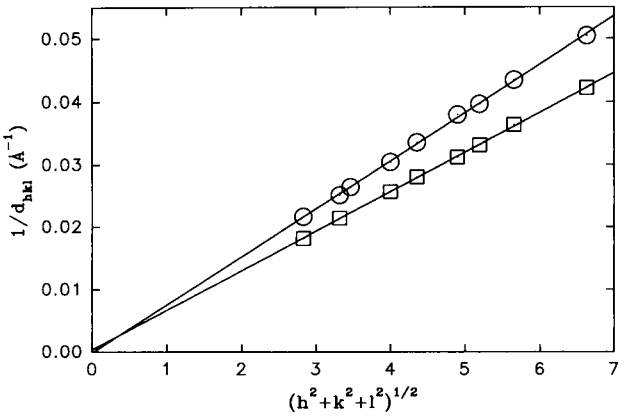

FIGURE 3 Plot of $1/d_{hkl}$ vs. $(h^2 + k^2 + l^2)^{1/2}$ of the reflections obtained (*a*) for the cubic sample of composition SPC/DAG/^2H_2O = 29.9/59.7/10.4 wt% at 25°C (□), and (*b*) for the cubic sample of composition DOPC/DOG/^2H_2O = 26.33/60.87/12.80 wt% at 59°C (○). The unit cell lattice parameters obtained are 158 and 130 Å, respectively.

DOPC/DOG/^2H_2O

The diffusion coefficients for all five NMR signals were measured between 25 and 59°C. The choline peak of DOPC was rather broad at low temperature but gradually narrowed with increasing temperature. Fig. 4 shows a stacked plot of the spectra recorded in a typical NMR diffusion experiment at 59°C, and Fig. 5 shows the fit to Eq. 1 of the experimental data obtained from the ^{1}H-NMR peaks of the lipids. The water diffusion coefficient was higher than that of the lipid at all temperatures, and at 59°C it was equal to 3×10^{-11} m^2 s^{-1}. In particular, it has been observed that the lipid lateral diffusion coefficient of a typical phospholipid at about 60°C is about 10^{-11} m^2 s^{-1} in bilayers and in bicontinuous cubic phases (Lindblom and Orädd, 1994), which should be compared with 10^{-12} to 10^{-13} m^2 s^{-1} determined for DOPC in the cubic phase studied here.

SPC/DAG/^2H_2O

For the SPC/DAG/^2H_2O system, the protons of the choline group at 25°C were not observable. However, at 30°C a wide peak appears at 3.1 ppm, and this peak narrows with increasing temperature. In the spin-echo signal, this component shows up at 35°C and then grows with increasing temperature, reflecting an increase in the T_2 relaxation time with increasing temperature. The diffusion coefficient measured of the choline peak could only be obtained with sufficient accuracy at temperatures above 40°C (Table 2). The water signal was not observed at any temperature for this system. This is probably due to the inhomogeneity in the lipids of this system, resulting in a possible broadening of the water peak. The high resolution NMR spectra exhibited rather broad signals at low temperatures, which made it impossible to apply the NMR diffusion method to all of the different molecular residues, as was possible for the DOPC/DOG/^2H_2O system.

The ^{31}P-NMR spectra of the cubic phases at 25°C exhibit a symmetrical and narrow signal, typically observed for isotropic solutions and cubic liquid crystalline phases, and the linewidth at half-height was found to be $\Delta\nu_{1/2} \approx 1.5$ ppm. Thus, the ^{31}P-NMR lineshape of this reversed cubic phase differs from the lineshape obtained for the cubic I_1 phase of lysopalmitoylphosphatidylcholine, where a strong asymmetrical ^{31}P-NMR peak with a chemical shift anisotropy of −3.6 ppm was observed (Eriksson et al., 1985a). In the latter cubic phase, such a lineshape is due to anisometric micelles, a geometrical shape that most probably does not pertain to the reversed micelles in the cubic phase studied in this work. Finally, the linewidth was found to decrease with increasing temperature to $\Delta\nu_{1/2} \approx 0.3$ ppm at 60°C.

DISCUSSION

Already for reasons of symmetry, one would expect (Fontell, 1990; Lindblom and Rilfors, 1989; Seddon, 1990b; Tiddy,

TABLE 2 Diffusion coefficients (10^{-12} m^{2}s^{-1}) at different temperatures for two samples of cubic phases built up of reversed micellar aggregates

DOPC/DOG/H_2O 26.5/61.3/12.3 wt%				
Temp (°C)	Acyl chains* (0.7 and 1.1 ppm)	DOPC choline group (3.1 ppm)	Water (4.6 ppm)	DOG hydroxyl (5.2 ppm)
25	2.8	0.5	4.3	2.8
30	4.2/0.4	0.4	6.1	3.9
42	8.7/0.9	0.5	8.8	6.1
59	15/0.3	0.3	30	15
SPC/DAG/H_2O 25.4/59.6/15.0 wt%				
Temp (°C)	Acyl chains* (0.7 and 1.1 ppm)	SPC choline group (3.1 ppm)	Water (4.6 ppm)	DAG hydroxyl (5.2 ppm)
25	2.2/0.7	‡	‡	1.7
30	3.1/0.7	‡	‡	3.4
42	7.2/0.8	0.5	‡	5.9
59	9.4/1.7	1.6	‡	8.8/1.6

The diffusion coefficient from five different peaks in the NMR spectrum have been measured. Note that the measurements for the peaks at 0.7 and 1.1 ppm are made on overlapping signals from both DAG and PC, and a biexponential decay of the signal is observed with diffusion coefficients corresponding to each lipid. For the biexponential decays obtained from the peaks of the acyl chain, two diffusion coefficients are calculated. These are separated by a slash.
*The diffusion coefficients of the acyl chains of the lipids were measured both of the terminal methyl groups at 0.7 ppm and of the methylene groups at 1.1 ppm.
‡Not observed.

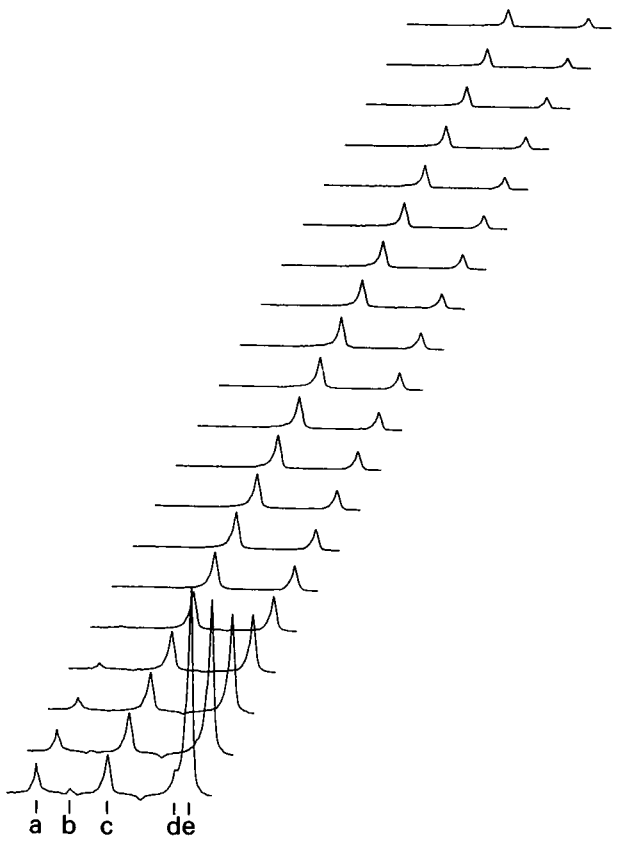

FIGURE 4 Stacked plot from a SE ^{1}H-NMR diffusion experiment of a cubic phase of the DOPC/DOG/^2H_2O system at 59°C. The ^{1}H-NMR peaks in the spectrum are from the left to the right: (*a*) DOG hydroxyl group (5.2 ppm); (*b*) water (4.6 ppm); (*c*) DOPC choline head group (3.1 ppm); (*d*) acyl chain methylenes of DOPC and DOG (1.1 ppm); and (*e*) the terminal methyl groups of the hydrocarbon chains of DOPC and DOG (0.7 ppm). The following settings were used: $\tau = \Delta = 300$ ms, $g = 0.5734\ Tm^{-1}$ and $\delta = 1$ ms for the spectrum at the bottom in the figure, and δ is then increased by 1 ms between successive NMR spectra from the bottom to the top.

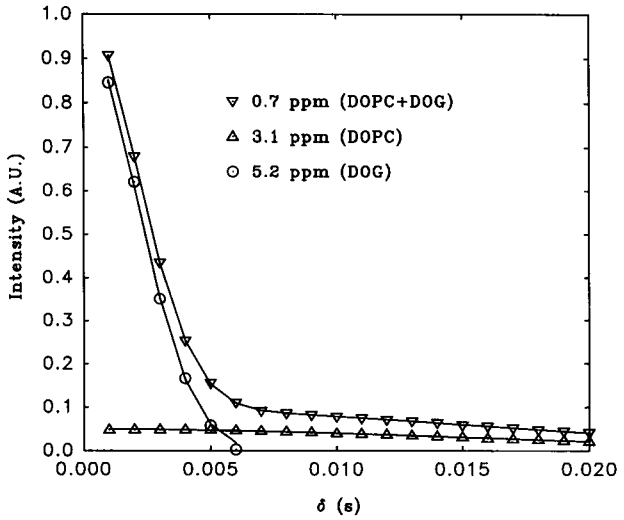

FIGURE 5 Fit of the intensities of the peaks in the NMR spectra shown in Fig. 4. The amplitudes of the different peaks have been arbitrarily chosen. The results obtained from the fits are: 5.2 ppm, $D = 1.5 \times 10^{-11}$ m^2 s^{-1}; 3.1 ppm, $D = 3 \times 10^{-13}$ m^2 s^{-1}; and 0.7 ppm, $D_1 = 1.5 \times 10^{-11}$ m^2 s^{-1} (90%); and $D_2 = 3 \times 10^{-13}$ m^2 s^{-1} (10%).

1980) the occurrence of a cubic phase in the region between the H_{II} and L_2 phases in the phase diagram. The occurrence of cubic phases with that location in a number of lipid systems has been confirmed recently (Luzzati et al., 1992). One would expect the structure of this cubic phase to mirror the structure of that occurring between the L_1 and H_I phases. The structure of such a cubic phase has been determined conclusively (Eriksson et al., 1985a, 1987; Fontell et al., 1985; Lindblom et al., 1992) to consist of small, rod-like, micellar aggregates. The cubic phases studied in this work for the DOPC/DOG/water and SPC/DAG/water systems should be composed, therefore, of closed reversed aggregates with the polar headgroups of the phospholipids located at the interface of separate water globules, residing in a continuous matrix of hydrocarbon.

As mentioned in the introduction, the traditional method for structural studies of cubic liquid crystalline phases is x-ray diffraction, but this technique may sometimes give ambiguous results. One reason for this is that the x-ray diffraction patterns of cubic phases quite often are not so distinctive to determine the space group and the structure (Lindblom and Rilfors, 1989; Seddon et al., 1990). As an example of the difficulties, we mention the attempt made by one of the leading groups in the field to solve the structure for the cubic phase obtained from a lipid extract from *Pseudomonas fluorescens* (Mariani et al., 1988). It was suggested that the space group was either *Fd3m* or *Fd3*, and it was assumed for symmetry reasons that *Fd3m* was the correct one. The structure was proposed to be a composite one consisting of a three-dimensional network of rods, tetrahedrally joined 4 by 4 and organized in a diamond lattice, and a system of disjointed micelles. However, the view was changed later, and now a structure consisting of two types of disjointed reversed micelles embedded in a three-dimensional hydrocarbon matrix is the prevailing one (Luzzati et al., 1992).

Seddon and co-workers (Seddon et al., 1990) have adopted the view that the *Fd3m* structure is common in low water systems of fatty acids or DAG together with lipids such as phosphatidylcholine, phosphatidylethanolamine, monooleoylglycerol, or sodium oleate. Seddon et al. have also mentioned that they occasionally have observed another x-ray diffraction pattern for cubic samples with somewhat decreased water content from the fully hydrated condition that they have studied.

The x-ray diffraction patterns of the cubic phase with the location between the H_{II} and the L_2-phases in the systems DOPC/DOG/^2H_2O and SPC/DAG/^2H_2O investigated in the present work are in excellent agreement with a *Fd3m* or *Fd3* structure (Table 1; Fig. 3). As discussed above, it is necessary to complement this information with NMR diffusion studies to obtain an unambiguous structure of the cubic phase (Lindblom et al., 1992; Lindblom and Orädd, 1995; Lindblom and Rilfors, 1989).

For the DOPC/DOG/water system, five peaks, including the the methyl groups of the choline headgroup of DOPC, are

observed in the NMR spectrum (Table 2). All of these peaks can be used in the diffusion experiments. As can be inferred in Table 2 and Fig. 5, the translational diffusion coefficients obtained at 59°C for the choline headgroup of DOPC (and of SPC at higher temperatures) is more than an order of magnitude smaller than for the diffusion coefficient of DOG or water. The slow diffusion coefficient of the phospholipid could be determined accurately at 25°C for the system with synthetic lipids only. However, at higher temperatures (>40°C) the cubic phase in the SPC/DAG/water system could also be investigated by the NMR diffusion method. Because the x-ray diffraction pattern did not change in the temperature interval studied, it can safely be concluded that the structure of the cubic phase stayed intact within the actual temperature interval.

In the NMR measurement of the diffusion of the methylene groups of the acyl chains, two components of diffusional motion were observed, and these differed by about an order of magnitude (Table 2; Fig. 5). Note that the difference between the rapid and slow components of the measured diffusion coefficients increase with increasing temperature. The component with the rapid diffusion is comparable with the translational diffusion coefficients obtained in lipid bilayers (Lindblom et al., 1979; Lindblom and Orädd, 1995; Lindblom and Rilfors, 1989; Lindblom and Wennerström, 1977). The much slower diffusional motion is compatible with that measured for the cubic phase consisting of closed micellar aggregates of lysophospholipids (Eriksson et al., 1987; Lindblom and Orädd, 1994). Therefore, the small diffusion coefficients of the DOPC molecules in the cubic phase are attributed conclusively to restricted lipid translational motion caused by closed lipid aggregates.

It can be concluded, based on the NMR diffusion and x-ray investigations presented here, that the cubic phases located between the H_{II} and L_2 phases in the PC/DAG/water systems are built up of reversed micelles of mainly PC, surrounded by a continuous matrix of mainly DAG.

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