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Deuteron Nuclear Magnetic Resonance Studies of Phase Equilibria in a Lecithin–Water System†

Jan Ulmius,* Håkan Wennerström, Göran Lindblom, and Gösta Arvidson‡

ABSTRACT: Water deuteron NMR spectra have been studied for the system dipalmitoyllecithin (DPL)–heavy water (D_2O) at different compositions and temperatures. From an analysis of the spectra in terms of quadrupole splittings, a phase diagram has been constructed for the temperature range 25–60 °C and the composition range 4–15 mol of D_2O/mol of DPL. Evidence is given that the "pretransition" observed by differential scanning calorimetry is caused by a crossing of a three-phase line. Strong support for a specific hydration of about 11 water molecules per lecithin molecule in the phase between the pretransition and main transition is also found.

In the investigation of the physical properties of membrane-lipid systems, studies of the phase equilibria for the different model systems are of considerable significance. Such studies have been concentrated on phospholipids and have shown that the polymorphism is generally large, and few, if any, complete phase diagrams are hitherto available.

A common feature of all membrane phospholipids is the existence of a phase transition with a large ΔH associated with the melting of the lipid chains. This order–disorder transition has been examined in detail with x-ray and calorimetric techniques (Chapman, 1968; Shipley, 1973; Luzzati and Tardieu, 1974; Chapman et al., 1967). Following the nomenclature of Tardieu et al. (1973), the ordered state is denoted β where the hydrocarbon chains are fully extended and relatively rigidly aligned, but the chains can undergo hindered rotation about their axes. In the Lβ phase, the chains are oriented perpendicularly; in the Lβ' phase, they are tilted with respect to the planar bilayer. The disordered state Lα has hydrocarbon chains with a liquid-like flexibility, but with an average orientation perpendicular to the bilayer plane.

Differential scanning calorimetry (DSC)[1] shows that at a high water–lecithin ratio the transition takes place over a very narrow temperature range, but at lower water contents the transition is smeared out (Ladbrooke and Chapman, 1969). Gottlieb and Eanes (1974) have reported an x-ray diffraction study of the thermal transition from the Lβ' phase to the Lα phase of dipalmitoyllecithin (DPL). They concluded that separate Lβ' and Lα phases coexist in varying proportions over the transition-temperature range. This observation is in agreement with the phase rule, but at higher water content Gottlieb and Eanes (1974) note that pure water phase coexists with the two hydrated lecithin phases. According to the phase rule, a two-component, three-phase system should have no degrees of freedom at constant pressure; i.e., the transition between the Lβ' phase and the Lα phase should be isothermal, contrary to the observed transition range.

Tardieu et al. (1973) have described several structures formed by phospholipid systems at temperatures below the order–disorder transition. Janiak et al. (1976) and Rand et al. (1975) have correlated the pretransition observed in synthetic lecithins with transitions between these structures. However, the two investigations do not agree as to the nature of the transition. Janiak et al. investigated the systems dimyristoyllecithin (DML)–water and DPL–water as a function of temperature and water content by x-ray and DSC techniques. They reached the conclusion that the pretransition is associated with a structural transformation from a one-dimensional lamellar to a two-dimensional monoclinic lattice consisting of lipid lamellae distorted by a periodic ripple while the hydrocarbon chains remain tilted with respect to the bilayer plane (Lβ' to Pβ'). Rand et al. studied the system DPL–water as a function of temperature by monolayer and x-ray techniques and concluded that the pretransition is associated with a conformational change in the hydrocarbon chains from a tilted to a perpendicular structure with respect to the lamellar plane (Lβ' to Lβ).

Hitherto x-ray and calorimetric techniques have been the prevailing methods for investigations of structures and transitions of phases in lecithin–water systems. X-ray diffraction provides information about the phase symmetry and characteristic distances in the system, while phase transitions are detected indirectly as abrupt changes in these parameters. On the other hand, calorimetric measurements reveal transitions that are accompanied by sizeable enthalpy changes.

Also, electron paramagnetic resonance has given valuable information on phase equilibria in lecithin–water systems, mainly on hydrated binary lipid systems (Grant et al., 1974).

Deuteron magnetic resonance studies of lecithin–water systems have given information about the water associated with phospholipids (Salsbury et al., 1972; Finer and Darke, 1974), and Salsbury et al. relate their spectroscopic measurements to a crude phase diagram of the DPL–water system.

The purpose of this paper is to show how deuteron magnetic resonance can be conveniently used to obtain a more detailed picture of the phase diagram of the system DPL–D_2O in the concentration range 4–15 mol of D_2O/mol of DPL.

Materials and Methods

DPL was either synthesized according to Cubero Robles and van der Berg (1965) or purchased from Serdary Research Laboratories, London, Ontario, Canada. The synthesized lecithin was purified by silicic acid column chromatography

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[1] Abbreviations used are: DSC, differential scanning calorimetry; DPL, dipalmitoyllecithin; DML, dimyristoyllecithin; TLC, thin-layer chromatography.

until satisfactory thin-layer chromatographic (TLC) results were obtained, as described previously (Lindblom et al., 1976). Since the purchased lecithin gave a single spot on TLC, it was used without further purification. Deuteron NMR spectra showed no differences between the two preparations of lecithin. D_2O (99.7% isotopic purity) was used without further purification.

DPL was dried to constant weight at about 60 °C in high vacuum and then transferred to ampules (8-mm o.d. with a constriction of about 1-mm i.d. just above the sample volume). D_2O was added with a syringe and the ampules were then sealed. The samples were homogenized by centrifugation back and forth through the constriction while the temperature was kept above the gel to liquid crystalline transition temperature. The samples were then left for 1–2 days to equilibrate. To avoid evaporation of D_2O from the lipid–water mixture, the sample filled as much as possible of the space of the sealed ampule volume. The water content was determined both before and after the NMR measurements. The error of water concentrations given is estimated to ±1%.

The ^2H NMR spectra were recorded at 15.4 MHz on a Varian XL-100-15 NMR spectrometer. The sample temperature was determined with an estimated accuracy of ±0.2 °C with a thermocouple placed just above the ampule. For most samples, thermal as well as thermodynamic equilibrium was reached within 15 min after a change in the temperature. To ensure that thermodynamic equilibrium had been reached, spectra were recorded at intervals for several hours for some samples without any differences in appearence. No time dependence of spectra was observed, as has been reported for egg lecithin–water systems (Finer and Darke, 1974). Typical spectrometer parameters were: pulse width 40 μs (50 μs gives a 90° pulse), sweep width 5 kHz, acquisition time 0.2 s giving a resolution of 1.6 Hz, number of transients 1000–2000. Computer simulation of the spectra were performed with a Gaussian broadening on the powder pattern band shapes.

Results and Discussion

The deuteron NMR spectra of the system DPL–D_2O were investigated in the concentration and temperature range 4–15 mol of D_2O/mol of DPL and 25–60 °C, respectively. Representative water-deuteron spectra are shown in Figure 1a–h. The spectra a and c exhibit the typical doublet powder patterns, and spectrum d shows a superposition of two such powder patterns. The features of the spectra b, e and f–h are, however, not that distinct. Apparently, in e and f two doublets are present, while in b only one doublet appears having a very narrow splitting. The spectra g and h have no resolvable splitting. These examples show that there is a considerable variation in the ^2H NMR spectra with composition and temperature, and there is thus a wealth of information to be obtained from a thorough ^2H NMR investigation of the system. The interpretation of the NMR spectra was performed in two steps. First, an analysis of the spectrum was made in terms of quadrupole splittings Δ, effective asymmetry parameter η, number of peaks, and their relative intensities. The second step of the analysis of the experimental data was to make a chemical interpretation of the results from the spectral analysis.

The behavior of the quadrupole coupling constant in these types of systems has been discussed by several authors (Lindblom et al., 1976, Salsbury et al., 1972, Finer and Darke, 1974, Persson et al., 1974; Lindblom et al., 1974), and the present data are in full accordance with previous work.

The types of spectra in Figure 1a,c are powder patterns with an asymmetry parameter $\eta \simeq 0$, typical for the liquid-crystalline (Lα) and gel (Lβ') state, respectively. Below the transition temperature, the splitting decreases with increasing temperature, reflecting the decreasing order of the lipid polar groups. Above the transition temperature, the doublet splitting increases with temperature. This is probably due to a redistribution of bound water as a result of increasing area per polar group. New binding sites are exposed as the head groups move

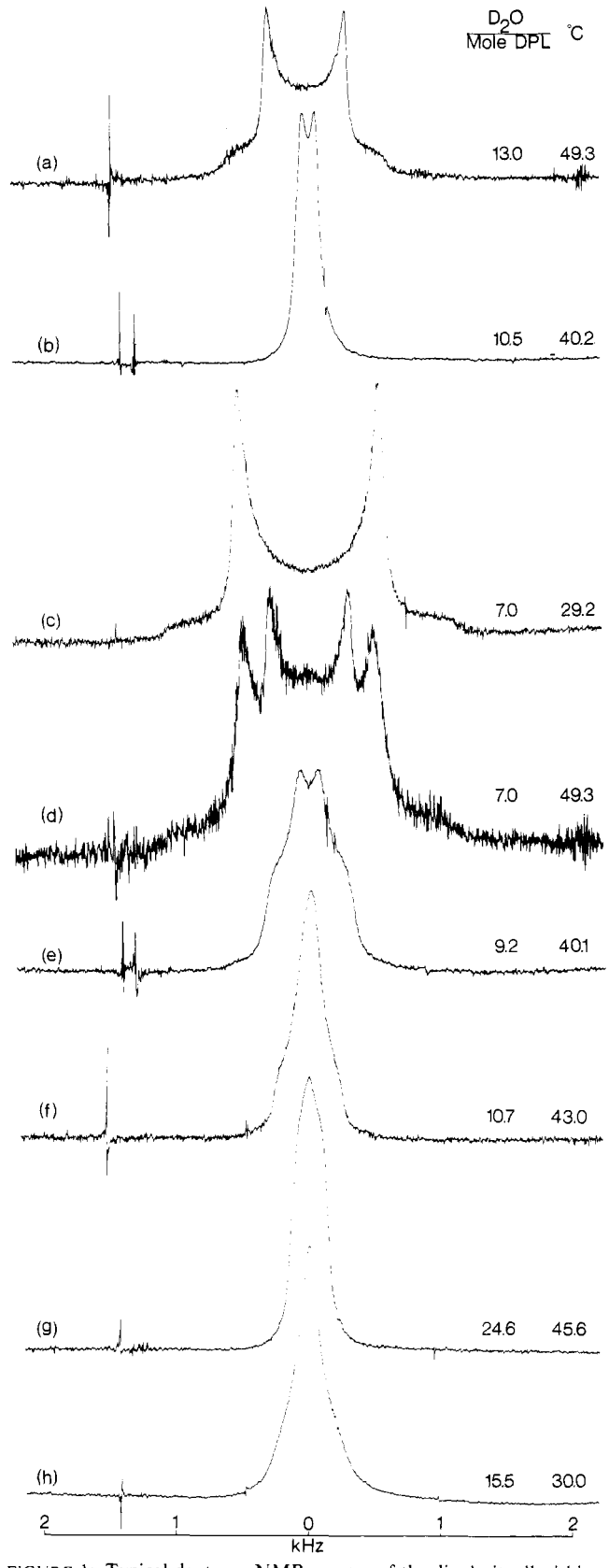

FIGURE 1: Typical deuteron NMR spectra of the dipalmitoyllecithin–D_2O system at different compositions and temperatures.

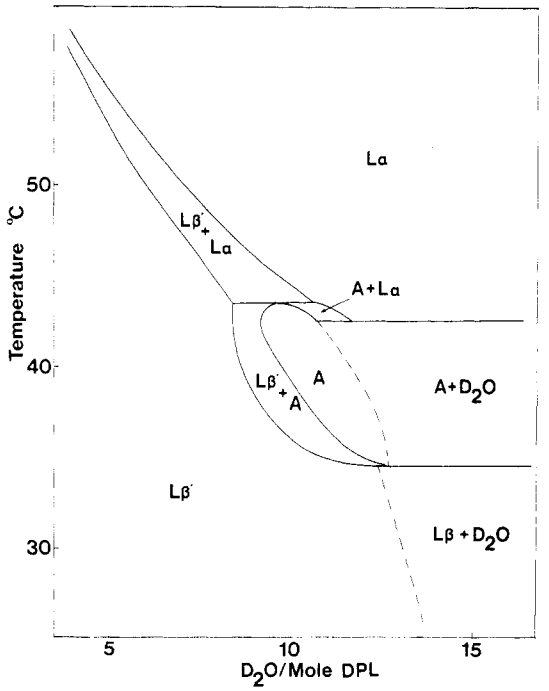

FIGURE 2: Part of the phase diagram for the DPL–D_2O system. The area labeled A is either a Pβ' phase or a Lβ phase. L denotes a one-dimensional lamellar lattice; P denotes a two-dimensional monoclinic lattice. In the α and β phases, the hydrocarbon chains are liquid-like and solid-like, respectively. The prime on β signifies that the carbon chains are tilted with respect to the lamellar plane. The broken lines indicate that the phase boundary is uncertain. Above the right-hand part of the three-phase line at 42 °C there should be a two-phase area, but we have been unable to detect it.

apart. In both these phases, the splitting decreases with increasing water content until the splitting cannot be resolved, which means that there is rapid exchange between water with isotropic motion ($\Delta = 0$) and bound water. This "isotropic" water must be trapped between the bilayers in order to give rapid exchange. The bound and trapped water is thus contained within the Lα phase and it is the increase in trapped water that is responsible for the increase in repeat distance observed in x-ray determinations at increasing water content. [For a review and discussion of the hydration of phospholipids, see Hauser (1975).]

At still higher water content, a sharp singlet line can be seen superimposed on a broader line. This is characteristic for free water in a separate phase exchanging slowly with trapped or bound water. The spectra in Figure 1g,h are examples of this behavior. These spectra are not as distinct as spectra from egg lecithin–water systems with free water present, which show a dependence of the splitting with time (Finer and Darke, 1974), and a well-resolved splitting can be seen a month after homogenization. In DPL–water samples with high water content, no such behavior is observed even after several months and the splitting decreases with increasing water content until it cannot be resolved. It is therefore hard to judge the maximum hydration (bound + trapped water) from the water deuteron spectra. However, by observing at what water content the spectra change from the type in Figure 1c to the type in Figure 1h, maximum hydration is estimated to 13–14 mol of water per mol of DPL below the transition temperature. Janiak et al. (1976) give the figure 14 mol of water per mol of DPL in the Lβ' phase.

The observation of two independent ^2H NMR signals (i.e., two doublets) for some DPL–D_2O compositions over a well-defined temperature range shows that there must exist physically distinguishable water sites with lifetimes of at least 10^{-4} s. Two explanations can be given for these water sites: either there are different binding sites within a homogenous system (i.e., on the same lamellae) or they can result from water binding in different phases. However, since the diffusion of water in lamellar mesophases containing lecithin has been shown (Rigaud et al., 1972) to be sufficiently rapid ($D \simeq 10^{-10}$ m^2/s) to give a fast exchange between different water-binding sites in a homogeneous phase, a situation with two independent binding sites in one and the same phase is unlikely to occur. Instead, the experimental result, that the populations in the different sites change from 0 to 100% within very narrow temperature and concentration ranges, strongly supports the idea that the two doublet peaks in the NMR spectra are due to different phases. For these types of spectra, computer simulations were used in order to obtain accurate values for the relative intensities.

The very narrow splittings observed for DPL–water mixtures with 9–13 mol of D_2O/mol of DPL and in the temperature range 35–42 °C are, however, more difficult to explain. Several interpretations can be given based on the structures given by Janiak et al. (1976) and Rand et al. (1975). Janiak et al. conclude that the pretransition at 34 °C is a structural transformation from the Lβ' to the Pβ' phase, while Rand et al. claim that it is a transition from the Lβ' to the Lβ phase. Both these phases are compatible with the observed narrow splittings. The amount of water that can be incorporated in the Lβ' phase is larger than in the Lβ phase (Tardieu et al., 1973). For a given water concentration, this results in an overall decrease in the degree of anisotropy of motion of the water molecules when going from the Lβ' to the Lβ phase and, therefore, a smaller splitting will be observed. The Lβ' and Pβ' phases have different symmetries which may lead to different apparent splittings in the two phases. If a water molecule moves in a surrounding that has a threefold or higher axis of symmetry, $\eta = 0$ otherwise $\eta \neq 0$ (Wennerström et al., 1975). For the liquid-crystalline phase Lα and the gel phase Lβ', the shapes of the NMR spectra strongly indicate $\eta = 0$, thus justifying the notation L for these phases. The Pβ' phase has a twofold symmetry around the director axis, which should result in an overall asymmetry parameter $\eta \neq 0$. However, including an $\eta \neq 0$ in the analysis does not significantly improve the fit between experimental and simulated spectra. Another mechanism that could cause smaller splittings in the Pβ' phase is the diffusion of water molecules along the lamellae. The critical time scale for the averaging process is about 10^{-6} s. The diffusion constant for water in lecithin systems is about 10^{-10} m^2 s^{-1} (Rigaud et al., 1972), giving a diffusion path of ~200 Å in 10^{-6} s. If the water molecule can have several different orientations with respect to the plane of the bilayer along this diffusion path, this will narrow the splitting further. The wavelength of the periodic ripples in the Pβ' phase is about 150 Å (Janiak et al., 1976), so the diffusion mechanism cannot be neglected.

From the dependence on temperature and water content of the splittings and the relative amounts of the different phases in the two-phase regions, it is very convenient and straightforward to construct a phase diagram for the DPL–D_2O system, and such a diagram is shown in Figure 2. There is good agreement between the data of Gottlieb and Eanes (1974) and our data regarding the two-phase region Lβ' + Lα. The most striking feature of the phase diagram in Figure 2 is the form of the one-phase region between 34 and 43 °C, which is similar to that found for intermediate compounds in a simple two-component system. This indicates that this phase (Pβ' or Lβ) has a very specific hydration requirement of about 11 mol of

D_2O per mol of lecithin. The existence of this phase thus seems to demand a well-defined water polar head group interaction. The $L\beta'$ phase does not have any specific hydration requirements for its existence, and it is also more stable than the $P\beta'$ or $L\beta$ phase at lower temperatures in agreement with the recent x-ray diffraction study by Janiak et al. (1976).

The two phase areas between $L\beta'$ and $L\beta$ or $P\beta'$ phase and between the $L\beta$ or $P\beta'$ and $L\alpha$ phase are well defined. However, the vanishing splitting at higher water content makes it difficult to construct the boundaries at such water contents for the $L\beta'$ and for the $L\beta$ or $P\beta'$ phase. When the two-phase areas have been determined, the phase diagram can be completed by drawing three-phase lines connecting one-phase areas. It follows that the transitions found at approximately 42 and 34 °C by DSC are due to crossings of three-phase lines. The transition at 34 °C is from the $L\beta'$ to the $L\beta$ or $P\beta'$ phase and this pretransition should disappear at 9–10 mol of D_2O/mol of DPL, as is also observed by Janiak et al. (1976).

Acknowledgments

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Supplementary Material Available

The temperature dependence of the deuterium splittings and the relative amounts of the different phases in the two-phase areas at different water contents (4 pages). Ordering information is given on any current masthead page.

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