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Effect of *n*-alkanes and peptides on the phase equilibria in phosphatidylcholine–water systems

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The phase equilibria in phosphatidylcholine (PC)-*n*-alkane-^2H_2O systems have been studied to elucidate the driving forces for the transition between a lamellar liquid-crystalline (L_α) phase and a reversed hexagonal (H_{II}) phase. A tentative phase diagram for the system dioleoyl-PC (DOPC)-*n*-dodecane-^2H_2O was determined. DOPC forms an L_α phase up to at least 90°C in excess water. However, an H_{II} phase was formed at room temperature at both low and high water concentrations in DOPC-*n*-dodecane-^2H_2O mixtures. The phase equilibria were also studied in PC-*n*-dodecane-^2H_2O systems containing PC with different degrees of acyl chain unsaturation. The water and dodecane concentrations required to induce the formation of an H_{II} (or isotropic) phase increase in the order dilinoleoyl-PC ~ DOPC < 1-palmitoyl-2-oleoyl-PC < dipalmitoyl-PC. The effect of *n*-alkanes with different chain lengths (C_8–C_{20}) on the phase equilibria in DOPC-*n*-alkane-^2H_2O mixtures was studied. Although the number of alkane carbon atoms added per DOPC molecule was kept constant, the ability of the alkanes to promote the formation of an H_{II} phase was strongly chain length dependent; the ability decreased when going from octane to eicosane. Finally, some PC-peptide-^2H_2O systems were investigated. Gramicidin (hydrophobic) had a similar influence on the phase equilibria as the alkanes. Melittin (amphiphilic) induced the formation of an isotropic phase, while insulin and duramycin (water soluble) had no, or a very limited, ability to induce a non-lamellar phase, respectively. Our results are discussed in the light of simple physical models dealing with the self-assembly of amphiphiles.

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1. Introduction

Numerous studies have been performed on the transitions between lamellar and non-lamellar phases in biological membrane lipid–water systems (see, e.g., [1–5] and references therein). Such studies are of biological relevance: (1) nearly all biological membranes contain significant amounts of at least one lipid species which, together with water, do not form a lamellar phase under physiological conditions [6, 7]; and (2) it has been speculated by several authors that non-bilayer structures play an important role for different membrane-associated processes such as exo- and endocytosis, fusion, and transport of proteins and nucleic acids through membranes [2, 4, 8–10].

In order to elucidate the principles underlying the lamellar–non-lamellar phase transitions some investigations of phosphatidylethanolamine (PE)-*n*-alkane-H_2O and phosphatidylcholine (PC)-PE-*n*-alkane-H_2O systems have been performed [11–14]. It was generally found that *n*-alkanes induce or facilitate the formation of a reversed hexagonal (H_{II}) phase. It has also been shown that the presence of alkanes in a biological membrane has a profound influence on its lipid composition [15]. Physical models dealing with the self-assembly of amphiphiles were used to interpret these results [10, 16].

In this paper we have studied the phase equilibria in the system PC-*n*-alkane-^2H_2O, in which we have systematically varied: (1) the degree of unsaturation of the acyl chains of PC; (2) the chain length of the alkane; and (3) the water concentration. We also compare the effect of the *n*-alkanes with the effect of hydrophobic, amphiphilic and water soluble peptides on the phase equilibria in the PC–^2H_2O system.

2. Results

2.1. *DOPC-n-dodecane-^2H_2O*

A tentative phase diagram for the system DOPC-*n*-dodecane-^2H_2O has been determined by ^2H- and ^{31}P N.M.R. ([17] figure 1). At 25°C DOPC–^2H_2O mixtures with water contents between 4 och 44 wt % ^2H_2O formed a lamellar liquid-crystalline (L_α) phase; above 44 wt % ^2H_2O (31 mol ^2H_2O per mol DOPC) the L_α phase stands in equilibrium with free water.

DOPC-*n*-dodecane–^2H_2O samples with 1·6 mol ^2H_2O and 1·5–3·0 mol dodecane per mol DOPC formed a reversed hexagonal (H_{II}) phase at 25°C. When the temperature was raised to 40°C, samples containing 0·75 and 1·0 mol dodecane per mol DOPC also formed an H_{II} phase. Samples with 6, 13 and 20 mol ^2H_2O per mol DOPC formed an L_α phase with dodecane concentrations up to at least 2·5 mol per mol lipid in the temperature interval 25–55° C. Additional increases in the water concentration gave rise to the most remarkable feature of the phase diagram: an H_{II} phase was formed again at dodecane/DOPC molar ratios between 1·0 och 2·5 (figure 1). An L_α phase in equilibrium with an H_{II} phase was observed already at 25°C in a sample with 25 mol ^2H_2O and 1·0 mol dodecane per mol DOPC. This phase equilibrium is displaced towards the H_{II} phase by three different manipulations: (*a*) by increasing the temperature; (*b*) by increasing the water concentration; and (*c*) by increasing the

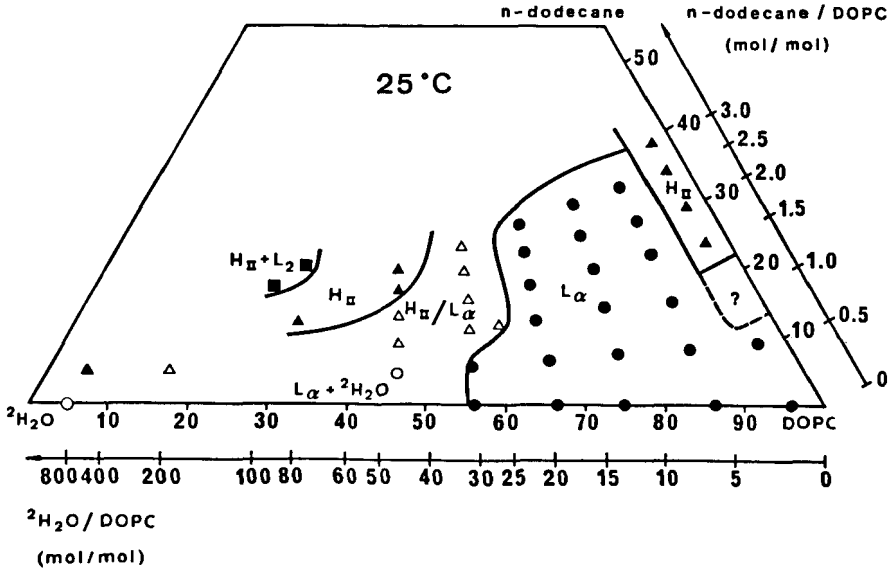

Figure 1. Part of a ternary phase diagram for the system DOPC-*n*-dodecane-^2H_2O at 25°C. ●, L_α (lamellar liquid-crystalline phase); ▲, H_{II} (reversed hexagonal phase); △, $H_{II} + L_\alpha$; ○, $L_\alpha + {}^2H_2O$; ■, L_2 (a phase giving rise to an isotropic ^{31}P N.M.R. signal) + H_{II}. The phase border lines drawn are tentative and are indicated only as an aid for the eye. Compositions are given as wt % and molar ratios.

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dodecane concentration. Three arguments favour our proposition that the hexagonal phase formed at high water contents is of the reversed type (H_{II}): (*a*) the relative amount of this phase increases when the temperature and the dodecane concentration is increased [2, 10]; (*b*) an H_{II} phase is obtained at high alkane concentrations even in some soap–alkane–water systems, i.e. with amphiphiles that, together with water, form micellar solutions and normal hexagonal phases [18]; and (*c*) normal hexagonal phases cannot be in equilibrium with a diluted micellar solution or free water [19, 20].

Investigations of DOPC-*n*-dodecane-d_{26}-H_2O mixtures with ^2H N.M.R. indicated that dodecane is partially oriented in the H_{II} phase formed at low water contents, probably with a preference to be oriented along the acyl chains of DOPC. However, in the H_{II} phase formed at high water contents the order parameter for dodecane is close to zero and the alkane is probably located between the lipid–water cylinders building up the phase [17].

2.2. *PC with varying degrees of acyl chain unsaturation*

According to the model presented by Gruner [16] concerning the aggregation of membrane lipids in the presence of hydrophobic molecules, an H_{II} phase should form at different water and dodecane concentrations in PC-*n*-dodecane-^2H_2O systems containing PC with different degrees of acyl chain unsaturation. This hypothesis was tested by exchanging DOPC for dipalmitoylphosphatidylcholine (DPPC), 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), or dilinoleoylphosphatidylcholine (DLiPC). The ability of PC-*n*-dodecane-^2H_2O mixtures, with a dodecane/PC molar ratio of 2 : 1, to form an H_{II} phase or an isotropic phase at different water concentrations is shown in figure 2. The system containing DPPC requires the highest water concentrations in order to form a non-lamellar phase. An H_{II} phase is formed at lower water

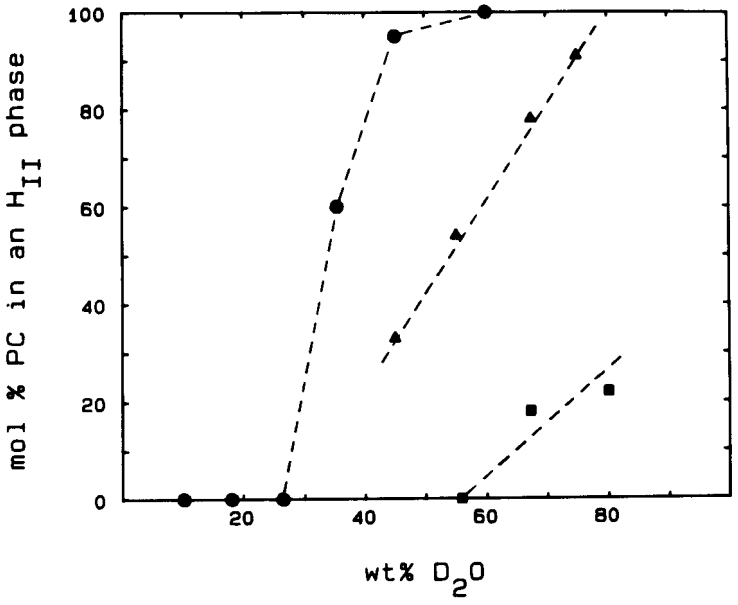

Figure 2. Phase equilibria at 45°C in different PC-dodecane-^2H_2O systems as a function of the water concent. The PC/dodecane ratio was 1 : 2. ●, DOPC; ▲, POPC; ■, DPPC. DPPC formed an isotropic phase at this temperature and composition but at higher temperatures an H_{II} phase was formed.

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concentrations with POPC, but the concentrations required for mixtures containing this lipid are still well above those needed for mixtures containing DOPC. On the other hand the phase equilibria in the PC-*n*-dodecane-^2H_2O system is only slightly affected by replacing the monounsaturated oleoyl chains of PC with the diunsaturated linoleoyl chains. It should be mentioned that the dodecane concentration required to induce the formation of a non-lamellar phase in PC-*n*-dodecane-^2H_2O mixtures with a fixed water content is increased in the order DLiPC ~ DOPC < POPC < DPPC.

2.3. *Variation of the chain length of the n-alkane*

The effect of alkanes with different chain lengths on the phase equilibria in DOPC-*n*-alkane-^2H_2O mixtures was also studied. The alkanes from octane to eicosane with an even number of carbon atoms were used, and the samples were prepared in such a way that the number of alkane carbon atoms added per DOPC molecule was kept constant. Figure 3 shows the results from mixtures containing 35 wt % ^2H_2O and 24 mol of alkane carbon atoms per mol DOPC. Although the added mass of alkane is kept constant, the ability of the alkanes to promote the formation of an H_{II} phase is strongly chain length dependent; the ability decreases monotonically when going from octane to eicosane.

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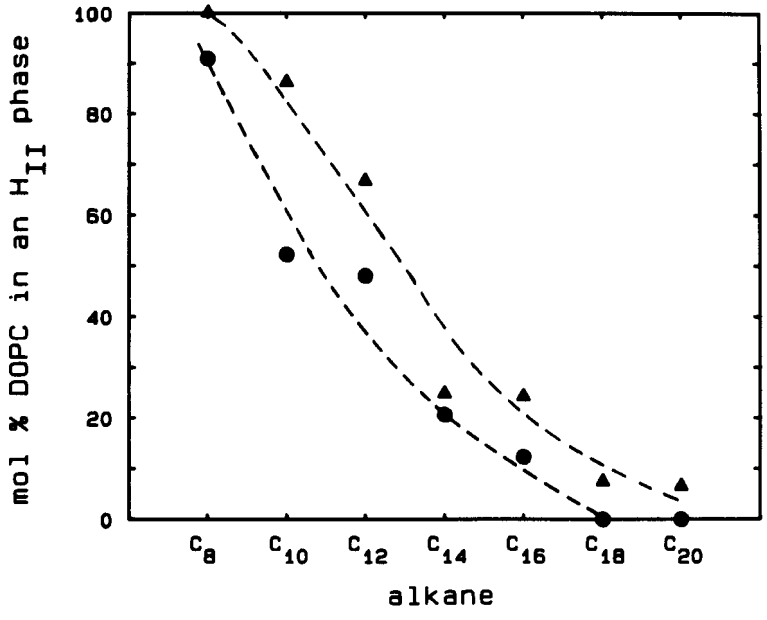

Figure 3. Phase equilibria in DOPC-*n*-alkane-^2H_2O systems as a function of the alkane chain length at 25°C ●, and 55°C ▲. The water concentration was 35 wt %, and the alkane concentration corresponds to 24 carbon atoms per lipid. Eicosane (C_{20}) induced the formation of a phase giving rise to an isotropic ^{31}P N.M.R. signal.

2.4. *PC-peptide-^2H_2O systems*

Some investigations were also performed on PC-^2H_2O mixtures to which hydrophobic, amphiphilic or hydrophilic peptides had been added. The influence of the hydrophobic peptide gramicidin (15 amino acids) on the phase equilibria of samples prepared with PC containing acyl chains with varying degrees of unsaturation were

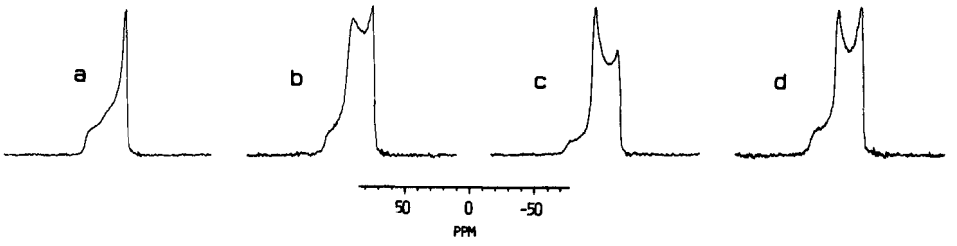

Figure 4. ^{31}P N.M.R. spectra recorded at 25°C from different PC–gramicidin–^2H_2O systems with 35 wt % ^2H_2O and a gramicidin/PC molar ratio of 1 : 10. (*a*) DPPC (45°C); (*b*) POPC; (*c*) DOPC; (*d*) DLiPC.

studied. The results from mixtures with 35 wt % ^2H_2O and with a gramicidin/PC molar ratio of 1 : 10 is shown in figure 4. The DPPC-gramicidin-^2H_2O sample formed only an L_α phase. When one (POPC) and two (DOPC) oleoyl chains were introduced into the PC molecule gradually larger fractions of the samples transformed to an H_{II} phase. The phase equilibria in the sample containing DLiPC was similar to those obtained with POPC.

The effect of gramicidin on the phase equilibria in the system DOPC-^2H_2O was compared with the effects of the amphiphilic peptide melittin (26 amino acids), the water soluble peptide duramycin (19 amino acids) and the small water soluble protein insulin (51 amino acids). Samples with 80 wt % ^2H_2O and a peptide/lipid molar ratio of 1 : 10 were investigated. Melittin reduced markedly the chemical shift anisotropy of DOPC and a considerable fraction of the sample formed an isotropic phase. Duramycin reduced slightly the chemical shift anisotropy of DOPC, and induced the formation of two lamellar phases and a small fraction of an isotropic phase. Only an L_α phase was observed with insulin.

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3. Discussion

Here we will confine our discussion to the conditions under which the H_{II} phase is formed. The base for our investigations is the phase diagram shown in figure 1, which has been dealt with in detail in a previous paper [17]. The most interesting observation is the formation of the H_{II} phase at *high* water contents when dodecane is added to the system. The formation of different phase structures in amphiphile– or lipid–water systems has been treated theoretically by many previous authors [10, 16, 19, 21, 22].

A lamellar liquid-crystalline phase in equilibrium with free water is formed at water contents above 31 mol ^2H_2O per mol DOPC in the absence of alkane (figure 1). Also the more unsaturated DLiPC [23] as well as the more saturated PC species like POPC [23] and DPPC [24] form only lamellar phases in excess water up to at least 90°C. The maximum hydration of the lamellar phases and the area per polar head group increases with the unsaturation, where the greatest step is taken between saturated and monounsaturated acyl chains [24–26]. Despite the fact that the molecular shape should change from a cylindrical-like to a more wedge-like one as the degree of unsaturation is increased, only lamellar phases are formed. Thus, non-lamellar phases are expected to form but this does not occur. This problem was recently solved by Gruner, who realized that although the lipid monolayers would prefer to curl they cannot do so because a bending of the bilayers, to form an H_{II} structure, would create

regions of vacuum between the water–hydrocarbon cylinders [16]. Thus, in spite of the fact that there is a natural tendency for the lipid monolayers to assume an intrinsic radius of curvature, this cannot be expressed by the lipid molecules themselves for geometrical reasons. The formation of lipid aggregates with large radii of curvature is consequently prevented by lipid packing constraints and the amount of water available in the system. However, if a hydrophobic substance, such as some alkanes or certain peptides, is added to the lipid–water system, the packing constraints can be removed since these substances can fill out the 'empty volumes' between the cylinders in the H_{II} phase structure. According to this model it is expected that the larger the constraint, i.e. the more saturated the acyl chain, the larger the water cylinder in the H_{II} phase must be and the higher the water content must be for the formation of this phase. This was experimentally observed (figure 2). The water content needed for the L_α–H_{II} phase transition increases from DLiPC to DPPC.

In this discussion we have implicitly assumed that the 'empty volumes' between the water–hydrocarbon cylinders may be filled by any hydrophobic molecule, regardless of its size. A certain number of CH_2 groups should be sufficient to induce the formation of the H_{II} phase independent of the chain length of the alkane. This simple assumption was, however, shown to be incorrect (figure 3). It was found that the chain length of the alkane plays a very important role. An increase in the number of methylene groups in the hydrocarbon chain eventually abolishes ($> C_{18}$) the ability of the alkane to promote the formation of the H_{II} phase. A probable explanation of this observation is that the location of the alkane in the hydrophobic region varies with the chain length, so that the shorter ones are located in the 'empty volumes', while the longer ones, perhaps for entropic reasons, extend into the lipid acyl chains. This assumption is supported by experiments, which showed that *n*-alkanes with less than 12 carbons are probably situated mainly in the centre of a bilayer at low concentrations, and the longer alkanes (C_{12}–C_{16}) seem to align parallel to the lipid acyl chains [27–29].

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Since small peptides frequently interact with biological membranes we have started a programme where studies of these interactions play a dominant role. Our studies of the effect of alkanes on lipid–water systems can be seen as a first step of investigations of the physico-chemical properties of the more complex lipid–peptide–water systems. It is therefore interesting to compare the lipid–alkane–water systems with lipid–peptide–water systems. Several studies of the system DOPC–gramidicin–water have been published by de Kruijff *et al.* ([30] and references therein). Our preliminary results show that the water soluble peptide and protein had no, or a very limited, capability to induce a non-lamellar phase in the DOPC–water system. However, the amphiphilic peptide melittin induced an isotropic phase, and the hydrophobic peptide gramicidin exhibited a behaviour very similar to that found for the alkanes. Figure 4 shows that the unsaturation of the lipid plays an important role in the L_α–H_{II} phase transition; gramicidin does not induce an H_{II} phase with DPPC while increasing amounts of the hexagonal phase are obtained with POPC and DOPC, respectively. However, further studies are needed before a definite explanation can be given to the mechanism behind the L_α–H_{II} phase transition induced by hydrophobic peptides.

4. Materials and methods

The different phosphatidylcholines were obtained from Avanti Polar Lipids, Inc., Birmingham, AL. The purity was given as greater than 99 per cent and no further

purification was done. Deuterium oxide was purchased from Ciba-Geigy, Basel, Switzerland. The *n*-alkane solvents, gramicidin, insulin and melittin, were obtained from Sigma Chemical Co., St Louis, Missouri.

The peptide containing samples were prepared as follows: the peptide and PC were dissolved in chloroform–methanol (2 : 1, *v*/*v*). After evaporation of the solvent, the samples were dried to constant weight in vacuum. Deuterium oxide was then added and the samples were mixed by centrifugation. The other samples were prepared as described by Sjölund *et al.* [17]. ^{31}P N.M.R. spectra were obtained with a Bruker WM-250 Fourier transform spectrometer at 101·3 MHz. Inverse gated high power proton decoupling was applied and an exponential multiplication corresponding to 20 Hz line broadening was applied before Fourier transformation. It has been shown in many previously published works that ^{31}P N.M.R. can be conveniently used to determine phase diagrams of lipid–water systems [5].

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