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# INDUCTION OF AN INTERDIGITATED GEL PHASE IN FULLY HYDRATED PHOSPHATIDYLCHOLINE BILAYERS

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**Several surface active small molecules induce an unusual phase in dipalmitoylphosphatidylcholine (DPPC) suspensions. In this phase, the lipid hydrocarbon chains from apposing monolayers interpenetrate or interdigitate. A structural analysis by X-ray diffraction shows that with incorporation of the drug chlorpromazine, the bilayer thickness, or lipid headgroup separation, in DPPC liposomes is only 30 Å, which is about 20 Å smaller than two fully-extended DPPC molecules. This interdigitated phase may be a more general phenomenon than previously believed, as several other molecules, both charged and uncharged, such as tetracaine and benzyl alcohol, can cause the lipid hydrocarbon chains to interpenetrate.**

## Introduction

The phase behavior of saturated phosphatidylcholines has been studied by a variety of techniques [1–6]. Four distinct phases, all lamellar, have been reported for fully hydrated dipalmitoylphosphatidylcholine (DPPC). The three gel phases (the bilayer 'sub-transition' phase [7], the gel $L_{\beta'}$ phase [1], and the 'rippled' $P_{\beta'}$ phase [1,2,6]) have stiff, parallel hydrocarbon chains, while in the higher temperature liquid-crystalline phase ($L_\alpha$) the hydrocarbon chains are more fluid. In the three gel phases the width of the hydrocarbon region of the bilayer corresponds to the length of two fully extended, tilted lipid hydrocarbon chains [1,2,7]. The terminal methyl groups of the chains are localized near the geometric center of the bilayer, and the hydrocarbon chains from one monolayer do not appreciably interpenetrate, or interdigitate, with the hydrocarbon chains of the apposing monolayer [1,2,7]. In the melted $L_\alpha$ phase, the bilayer width is smaller due to the formation of rotational isomers or kinks [8,9], but, on the average, the chains are only slightly interdigitated [10].

Recently, Ranck and Tocanne [11,12] have shown that the addition of choline, acetylcholine, or polymyxin B induces a complete interdigitation of apposing hydrocarbon chains in hydrated bilayers of dipalmitoylphosphatidylglycerol (DPPG). This interdigitated gel phase was first described in 1966 for the potassium stearate-water system by Vincent and Skoulios [13] and in 1977 for hydrated DPPG by Ranck et al. [14]. A similar interdigitated phase has been found in DPPC at high concentrations of glycerol or ethylene glycol [15].

In this paper we show that several surface-active molecules, in the presence of excess water, cause the hydrocarbon chains of DPPC to interdigitate. Thus, this interdigitated phase may be more common than previously thought. In particu-

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Abbreviation: ANS, 1-anilino-8-naphthalene sulfonate.

lar, we analyze the structure of this phase when caused by the incorporation of the drug chlorpromazine-HCl. Chlorpromazine has a variety of pharmacological effects and several groups have analyzed its effects on biological and model membranes [16–18]. Our structural analysis shows that some of the previous model membrane studies with chlorpromazine and other amphiphilic molecules have been performed with this interdigitated DPPC phase.

## Materials and Methods

DPPC was used as obtained from Calbiochem. Chlorpromazine · HCl, tetracaine · HCl, and 2-phenylethanol (Sigma), 4-phenyl-1-butanol (Aldrich), and benzyl alcohol (Fisher Scientific) were used as obtained. Appropriate amounts of chlorpromazine or tetracaine and DPPC were codissolved in chloroform. The solvent was removed by rotary evaporation and a carefully measured amount of triply distilled water was added. The suspensions were vortexed and allowed to incubate for 2–3 h above the lipid phase transition temperature. In the case of benzyl alcohol, phenylethanol and phenylbutanol, 75 mM solutions were mixed with DPPC above the lipid phase transition temperature. The specimens were concentrated by a brief centrifugation with an Airfuge (Beckman). All samples were then sealed in X-ray capillary tubes and mounted in a point collimated X-ray camera. Diffraction patterns were recorded with a stack of three sheets of Kodak No Screen X-ray film in a flat plate film holder. Exposure times were between 2 and 7 h at room temperature. The X-ray films were densitometered with a Joyce-Loebl microdensitometer Model MK III C and integrated intensities were measured as described previously [19,20].

## Results

As reported previously [1,21], X-ray diffraction patterns from DPPC at 20°C in excess water contain several lamellar orders of a repeat period of 64 Å and a double wide-angle reflection, which consists of a sharp reflection at 4.2 Å surrounded by a diffuse band centered at 4.1 Å. In excess water, the addition of 0.10 mole fraction chlorpromazine to DPPC causes a small (3 Å) increase in lamellar repeat period, and a change in the wide-angle pattern to a single broad reflection at 4.2 Å. Higher concentrations of chlorpromazine (0.20 or 0.33 mole fraction chlorpromazine) cause a large increase in lamellar repeat period, up to 114 Å. Moreover, at these concentrations, the wide-angle pattern of DPPC/chlorpromazine suspensions consists of a single sharp reflection at 4.2 Å. To obtain further information on the structure of the lamellae at high chlorpromazine concentrations, a series of 'swelling' experiments were performed. The amount of water added to the dried DPPC/chlorpromazine (0.33 mole fraction chlorpromazine) was varied from 20% to 75%, by weight. Under these conditions, the lamellar repeat period changed from 42 to 114 Å. Structure amplitudes from these experiments have been normalized to the same relative scale [22] and are plotted versus reciprocal space coordinate $R$ in Fig. 1. All of the structure amplitudes fall on the same smooth curve which has been drawn in by eye in Fig. 1. This means that, at this resolution, the structure of the bilayer is the same for all of these experiments and the difference in repeat period in the experiments corresponds to a difference in the width of the fluid space between bilayers [23]. The structure amplitude curve of Fig. 1 is similar to that of DPPC in glycerol [15] in that both curves have a minimum at $R \simeq 0.05$ Å$^{-1}$. The difference in shape of the two curves for $R \simeq 0.03$ Å$^{-1}$ is due to the difference in electron density of the two swelling fluids, water and glycerol.

To calculate electron density profiles, the phase angle must be determined for each reflection. Since this is a centrosymmetric system, each phase must

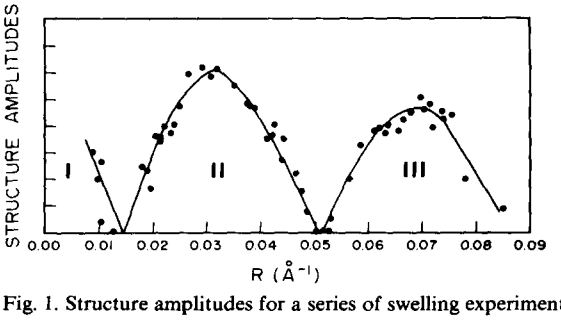


Fig. 1. Structure amplitudes for a series of swelling experiments with DPPC/chlorpromazine (2 : 1 mole ratio). The three nodes or regions of the curve are labeled I, II, and III.

be either 0 or $\pi$. The swelling curve of Fig. 1 simplifies the phase problem, since for $0 < R \leqslant 0.085$ Å$^{-1}$, there are only two possible points on the curve where the transform could go through zero and a phase change could occur, at $R \cong 0.013$ Å$^{-1}$ and $R \cong 0.05$ Å$^{-1}$. That is, there are three regions in the curve, region I ($0 \leqslant R \leqslant 0.013$ Å$^{-1}$), region II ($0.013 \leqslant R \leqslant 0.05$ Å$^{-1}$), and region III ($0.05 \leqslant R \leqslant 0.085$ Å$^{-1}$).

There are $2^3 = 8$ possible phase combinations, since each of these regions can have a 0 or $\pi$ phase. We have used the sampling theorem [24,25] to determine the correct phase combination. First, the structure factor at $R = 0$ was calculated for each phase combination by use of the method of King and Worthington [26]. Next, the continuous transforms were calculated for each possible phase combination for two data sets, with repeat periods of 47 Å and 102 Å. The two curves plotted in each graph in Fig. 2 represent the absolute values of the continuous transform for these two data sets. In Fig. 2A, alternate phases for the regions I, II, and III of the transform are shown; in Fig. 2B, regions II and III have the opposite phases of region I; in Fig. 2C, regions I and II have the opposite phase of region III; and in Fig. 2D, all regions have the same phase angle. Clearly, the two curves in Fig. 2A are much closer than the pairs of curves in Figs. 2B, 2C or 2D. Thus, the correct phase choice is either ($\pi$, 0, $\pi$) or (0, $\pi$, 0) for regions I, II, and III of the transform. These two remaining possibilities can be distinguished by using the fact that lipid hydrocarbon chains have a lower electron density than water. To make the electron-density profile reflect this, the phase choice (0, $\pi$, 0) must be chosen.

Using the correct phase combination, we have calculated electron-density profiles for the data sets of repeat periods $d = 47$ Å and $d = 102$ Å plus two additional specimens with repeat periods of $d = 80$ Å and $d = 95$ Å (Fig. 3). For all of the profiles, the region between $-20$ Å and $+20$ Å is the same. In each profile there are high-density peaks centered at approximately $-15$ and $+15$ Å. These peaks represent the high-density lipid

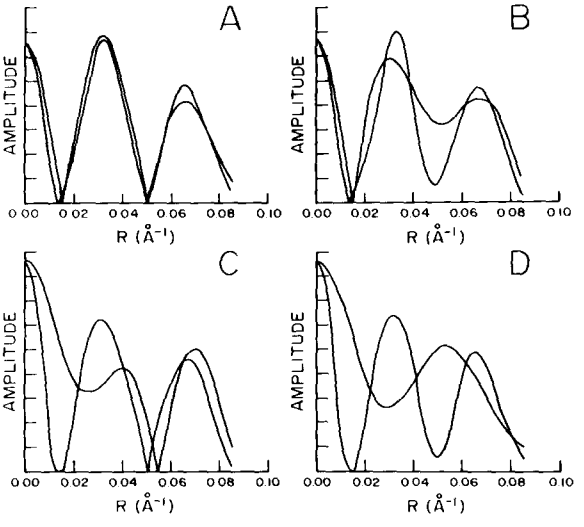


Fig. 2. Absolute values of continuous transforms calculated using Shannon's sampling theorem and two data sets of DPPC/chlorpromazine (2 : 1 mole ratio), $d = 47$ Å and $d = 102$ Å. The transform pairs were calculated for (A) alternating phases for regions I, II and III; (B) regions II and III with the opposite phase of region I; (C) regions I and II with the opposite phase of region III; and (D) regions I, II, and III all with the same phase.

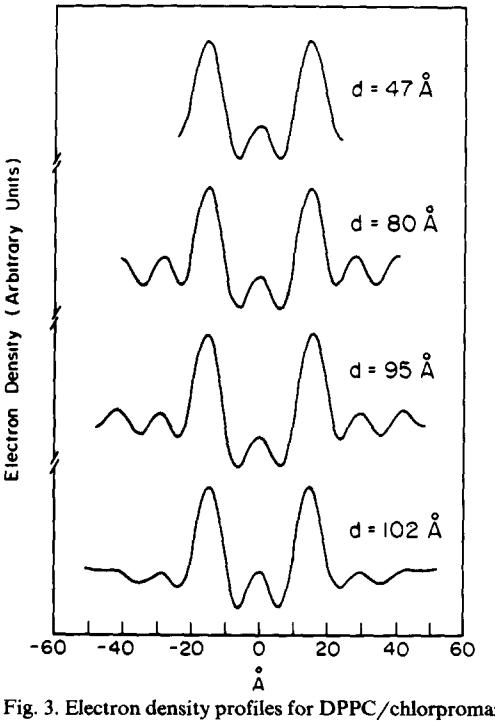


Fig. 3. Electron density profiles for DPPC/chlorpromazine (2 : 1 mole ratio) bilayers with different water contents. The high-density peaks centered at $\pm 15$ Å in each profile correspond to the lipid headgroup region. The fluid layers between bilayers increase in width as the repeat period increases.

headgroups, while the low-density region between the peaks represents the lipid hydrocarbon chains. The medium-density regions outside the headgroup peaks correspond to the water layers between bilayers. The layers increase in width as the repeat period increases.

Diffraction patterns from DPPC with a 33% mole fraction of tetracaine are very similar to those of DPPC/chlorpromazine, as the structure factors fall on the same transform shown in Fig. 1. Thus, the electron density profiles for DPPC/tetracaine are the same as those shown in Fig. 3.

Several other surface-active molecules, when added to DPPC liposomes, give diffraction patterns with a lamellar period of about 50 Å and a single sharp wide-angle reflection at 4.2 Å. These include glycerol, ethylene glycerol, and methyl alcohol at low water content [15] and 75 mM solutions of benzyl alcohol, phenylethanol, and phenylbutanol. Since these molecules are not charged, they do not increase the fluid layer as chlorpromazine or tetracaine do in excess water. In all cases the electron density profiles, at comparable resolution, resemble the profiles of DPPC/chlorpromazine in that the high-density headgroup peaks have a separation of about 30 Å.

## Discussion

The diffraction patterns and electron-density profiles (Fig. 3) from DPPC/chlorpromazine at 33% mole fraction chlorpromazine strongly indicate that the lipid hydrocarbon chains are interdigitated. The single sharp wide-angle reflection at 4.2 Å indicates that the chains are extended and perpendicular to the plane of the bilayer [1]. The electron-density profiles show that the distance between headgroup peaks is about 30 Å, in contrast to the 49 Å expected for two fully extended ultilted DPPC chains which are not interdigitated [21]. Moreover, the electron density profiles of Fig. 3 do not have the deep terminal methyl trough in the center of the profile which is observed when the hydrocarbon chains are not interdigitated and the methyl groups are localized in the bilayer center. The profiles of Fig. 3 are very similar to those derived by Ranck et al. [14] for the interdigitated phase of gel state DPPG. The difference between the DPPC bilayer width for fully extended, untilted, non-interpenetrating chains (49 Å) and the bilayer width for DPPC/chlorpromazine (30 Å) is 19 Å. Assuming 1.25 Å per $CH_2$ group, this corresponds to a difference of about 15 $CH_2$ groups. Thus, the hydrocarbon chains have penetrated up to the first or second $CH_2$ group of the lipid of the apposing monolayer when chlorpromazine is present.

Previously, DPPC/chlorpromazine suspensions have been analyzed by differential scanning calorimetry and NMR techniques [17]. It was shown that chlorpromazine causes a mobility reduction in the headgroups of the DPPC molecule and a model has been proposed in which the dialkylaminoalkyl chains are located near the polar headgroup and the ring system penetrates only a few angstroms into the hydrocarbon region of the bilayer [17]. This model must now be modified to take into account the X-ray results. Chlorpromazine, in its location in the headgroup region, must, at sufficient concentrations, increase the area per lipid molecule enough that hydrocarbon chains from apposing monolayers can interpenetrate (see below). These X-ray results may also explain the concentration dependence of the phase transition width for DPPC with chlorpromazine observed by Frenzell et al. [17]. In the concentration range of 5–15 mol% chlorpromazine, they observed a broadened transition which became narrow again above 20 mol% chlorpromazine. Our data show that the sharpening of the transition at the higher chlorpromazine concentrations may be correlated with the induction of the interdigitated phase.

In addition to chlorpromazine and tetracaine, which are positively charged under these conditions, several neutral molecules induce the interdigitated phase in DPPC bilayers–benzyl alcohol, phenylethanol, and phenylbutanol with excess water present and methanol, ethylene glycol, and glycerol with small amounts of water present [15]. A common property of these molecules is that they are amphiphilic and tend to reside at the DPPC/water interface. Previously we found that alkanes [20] and benzene [27] which are located in the acyl chain region and are not anchored to the interface, do not induce an interdigitated phase for DPPC in excess water, even though they can increase the area per lipid molecule. To produce interdigitation it is also clear that there must be a size constraint

on the added amphiphile, since DPPC will not form an interdigitated phase in the presence of cholesterol [19,28] or long chain fatty acids [29]. The incorporation of these molecules can satisfy the van der Waals interaction so that the gel state lipid is not destabilized to the extent that the interdigitated phase is formed. Moreover, since phenylethanol and phenylbutanol cause interdigitation, while short chain fatty acids with the same number of carbon atoms, octanoic and decanoic acid, do not (McIntosh, McDaniel, and Simon, unpublished data), the shape or width of the molecule must be important. We do not know the precise requirements for a small molecule to induce the interdigitated phase in DPPC bilayers, but the following generalizations appear to be valid for all molecules tested. First, the molecule must displace water from a particular location(s) in the interfacial region [15], and second, its non-polar moiety cannot extend too deeply into the bilayer interior.

When small amphiphilic molecules are located in the interfacial region of gel state DPPC liposomes, they anchor to the interface by virtue of their polar moiety, with the non-polar part of the molecule intercalating between the rigid acyl chains. In the case of short amphiphilic molecules whose non-polar moieties are not as long as the DPPC hydrocarbon chains, this would potentially cause voids between chains in the bilayer interior. Since the energy of formation of holes in hydrocarbons is extremely large, the chains must eliminate the voids [30]. To do this, the chains could either bend cooperatively or else interdigitate. Apparently, at least for the several bulky amphililic molecules listed above, the lowest energy phase is the interdigitated phase.

The induction of the interdigitated phase may be more common than previously considered. From the above considerations, it might be predicted that other bulky amphiphilic molecules, several of which have previously been incorporated in DPPC bilayers for spectroscopic and calorimetric analysis, could induce interdigitation. With this type of molecule present, lipid phases which have been considered standard bilayer structures may be in fact an interdigitated phase. For example, we previously noted that benzyl alcohol reduces the thickness of hydrated DPPC bilayers by as much as 14 Å while eliminating chain tilt [31]. At that time we suggested that this decrease in thickness was caused by the lipid acyl chains bending around the benzyl alcohol [31]. In light of the new evidence presented in this paper, we now believe that benzyl alcohol [31,32], as well as other physiologically important molecules which have been studied in model membrane systems, can cause interpenetration of apposing lipid hydrocarbon chains. As another example, Lesslauer et al. [33,34] found that the anionic fluorescent probe 1-anilino-8-naphthalene sulfonate (ANS) decreases the thickness of DPPC bilayers to 30 Å. The electron density profiles of DPPC/ANS are very similar to those of DPPC/chlorpromazine (Fig. 3). Lesslauer et al. [33,34] noted that the sulfonate group in ANS is in the plane of the DPPC headgroups and the non-polar fluorescent moiety of the ANS penetrates for a short distance into the hydrocarbon interior. Although they did not state that ANS causes interdigitation, as now seems likely to us, they did point out that 'the structure of the hydrocarbon core is completely different from that of the lecithin bilayer' [34]. Since ANS is negatively charged, and chlorpromazine and tetracaine are positively charged, the ability to induce the interdigitated phase does not depend on the charge on the incorporated molecule.

Finally, we suggest that caution should be used in interpreting reconstitution experiments where transport proteins are found to be active in the liquid-crystalline phase, but inactive in the gel phase. Should a particular protein induce the interdigitated phase, its ability to transport might be significantly altered.

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