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Polymorphism in Myristoylpalmitoylphosphatidylcholine

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Abstract

This study focuses on the mixed-chain lipid myristoylpalmitoylphosphatidylcholine (MPPC) near full hydration. The lipid, synthesized according to the procedure of (Mason et al., 1981a, has a low degree of acyl chain migration. When MPPC is temperature-jumped (T-jumped) from the L_α phase ($T = 38°C$) to $T = 20°C$ or below, a subgel phase forms; this formation takes less than 1 h at a temperature below $T = 12°C$. The subgel remains stable up to $T = 29°C$. When MPPC is T-jumped from the L_α phase to $T = 24°C$ or above, a ripple phase forms with coexisting ripple wavelengths of 240 Å and 130 Å. In contrast, when MPPC is melted from the subgel phase, the ripple phase is characterized by bilayers having a single ripple wavelength of 130 Å. In agreement with earlier studies (Stumpel et al., 1983; Serrallach et al., 1984. Structure and thermotropic properties of mixed-chain phosphatidylcholine bilayer membranes. Biochemistry 23:713–720.), no stable gel phase was observed. Instead, an ill-defined low-angle X-ray pattern is initially observed, which gradually transforms into the subgel phase below 20°C, or into the ripple phase above 24°C. In the wide-angle X-ray diffraction, a single peak is observed, similar to the ripple phase wide-angle pattern, that either persists above 24°C or transforms into a multi-peaked subgel wide-angle pattern below 20°C. The absence of a gel phase can be understood phenomenologically as the relative dominance of the subgel phase in mixed-chain PCs compared to same-chain PCs. The subgel structure and molecular interactions responsible for this comparative behavior are interesting open issues.

Keywords: Differential scanning calorimetry; Lipid bilayers; Mixed-chain lipids; X-Ray diffraction

1. Introduction

Pure phospholipids in the form of multilamellar vesicles (MLVs) have long been the system of choice to study the structure of biologically relevant fully hydrated bilayers. Saturated same-chain

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PCs, such as DPPC or DMPC, have well-characterized and robust L'_β gel phases. Wide-angle diffraction reveals a very sharp (20) reflection consistent with coherent domains in the plane of the bilayer extending about 2900Å (Sun et al., 1994). As has been known for many years, the chains are tilted (Tardieu et al., 1973; McIntosh, 1980), and the tilt angle has been shown to be $\theta_t = 32°$ for DPPC at $T = 19°C$ (Katsaras et al., 1992; Tristram-Nagle et al., 1993) and varies gradually with temperature (Sun et al., 1996). It has also been known, essentially from the width of the (11) wide-angle peak, that the chains in the two monolayers of the bilayer are tilted in the same direction (Keough and Davis, 1979); detailed modeling indicates that the chains are essentially colinear with a lateral offset that is only about 15% of the distance between chains (Sun et al., 1994). The colinearity suggests a trans-bilayer coupling between monolayers that transcends the obvious van der Waals attraction between two hydrocarbon regions and implicates specific tongue-in-groove packing of the terminal methyl ends of the chains. This would be expected if the two chains on the lipid molecule are sufficiently different in length that the ends of the longer all-trans interdigitating chains are forced to be parallel to those from the opposite monolayer. The energetics of this must be rather subtle, however. One extensive molecular dynamics (MD) simulation for DPPC bilayers (Tu et al., 1996) obtained pleated rather than colinear chain packing, with the azimuthal angle of chain tilt different in the two monolayers although the chain tilt angle θ_t agreed with experiment. Only recently has an MD simulation obtained the observed colinearity (Venable et al., in preparation). Another indication that the energetics of chain packing must be subtle is that the direction of tilt, towards nearest neighbors versus between nearest neighbors, changes with hydration (Smith et al., 1988) implicating the headgroup region.

As is well-known, in the fluid L_α phase the glycerol backbone is aligned perpendicular to the bilayer surface, so that the sn-1 chain penetrates one and a half carbons further into the interior of the bilayer than the sn-2 chain (Seelig and Seelig, 1975; Buldt et al., 1979). A similar glycerol orientation is seen in the crystal structure of DMPC (Pearson and Pascher, 1979), which also exhibits an offset of essentially colinear all-trans chains with the sn-1 chain penetrating three carbons further than the sn-2 chain. Such features can be systematically probed by studying mixed-chain lipids, which occur widely in nature and have been the subject of numerous investigations (Keough and Davis, 1979; Chen and Sturtevant, 1981; Mason et al., 1981b; Stumpel et al., 1983; Hui et al., 1984; McIntosh et al., 1984; Serrallach et al., 1984; Tummler et al., 1984; Mattai et al., 1987; Zhu and Caffrey, 1993; Lewis et al., 1994).

The dependence of the main transition temperature, T_M, and transition enthalpy, ΔH_M, reflect the hydrocarbon chain inequivalence. Mason et al. (1981a) quantitated this phenomenologically using the parameter $\Delta C/C_L$, where $\Delta C = (n_1 - n_2 + 1.5)$, and n_1 and n_2 are the number of carbons in the chains at the sn-1 and sn-2 positions of the glycerol backbone, respectively. The constant 1.5 is introduced to account for the chain length shortening of the sn-2 chain as a result of a bend at the second carbon. The quantity C_L is the effective length of the longer of the two chains. For small ΔC, T_M decreases proportional to $(\Delta C/C_L)^2$ (Eq. 1 in Huang et al. (1994)).

Assuming that the colinearity of the hydrocarbon chains in the gel phase of same-chain PCs is a result of partial interdigitation of the terminal methyl ends, then the molecular variation that is a priori most likely to obtain a different type of gel phase, is the one where the ends of the two chains penetrate equally into the bilayer, because then there would be minimal interaction between monolayers to induce colinearity. From the formula of Mason et al. (1981b), this suggests that mixed-chain lipids in which the sn-1 chain has one to two fewer carbons than the sn-2 chain might have a special gel phase. From the crystal structure of Pearson and Pascher (1979) three carbon difference might yield this special gel phase. A compromise, MPPC, with a two carbon difference, appears to be the most likely choice. However, earlier structural studies of MPPC, as well as PMPC, indicated no gel phase (Stumpel et al., 1983) or only a vestigial unstable gel phase (Serrallach et al., 1984). This result, that it is the

same-chain PCs that yield special gel phases, does not conform to expectations based on the $\Delta C/C_L$ concept and the inequivalence of the chains. An experimental re-examination of the previous remarkable result seemed to be justified by two considerations: First, the synthesis of mixed-chain lipids is subject to the problem of chain migration, so that MPPC is contaminated with PMPC, or even with the DMPC precursor. In this work we synthesize MPPC and show that the characteristics of the bilayers indicate high purity. Second, there are subtleties in thermal protocols that affect the formation of subgel phases (Tristram-Nagle et al., 1994) that might, for example, have resulted in the gel phase being minimized. Although our results basically confirm the absence of the gel phase in MPPC, we have obtained some new results for subgel kinetics and ripple phase structure and have quantitatively refined some old results. There remains, however, a major unresolved issue regarding the low temperature phases.

2. Materials and methods

2.1. Lipids

1-Myristoyl-2-palmitoyl-*sn*-3-phosphatidylcholine (MPPC) was synthesized using the procedure of Mason et al. (1981a), in which freshly prepared 1-myristoyl-lysolecithin is reacylated with palmitoylanhydride in the presence of 4-pyrrolidinopyridine. The standard method was used to analyze chain migration chemically. MPPC was enzymatically cleaved using phospholipase A2 which specifically cleaves the acyl chain from the sn-2 position; this fatty acid is then esterified and subjected to gas chromatography and mass spectrometry. The result indicated 5% acyl chain migration, which is an upper bound because of the possibility of lysolecithinase contamination. For comparison, a commercial sample of MPPC (Lot No. 140-160PC5 from Avanti Polar Lipids, Alabaster, AL, USA) was used as received. The commercial MPPC contains about 20–30% acyl chain migration (personal communication from Walt Shaw, Avanti Polar Lipids, Alabaster, AL, USA). Both lipids were analyzed using TLC using chloroform: methanol: 7N NH_4OH (46:18:3, v/v) as the solvent system and stained with molybdic acid stain. TLC revealed $<$ 0.1% lysolecithin contamination in both lipids when compared to a standard curve. Unless specifically indicated, all results are from our synthesized MPPC.

2.2. Differential scanning calorimetry (DSC)

MLVs were prepared by adding lyophilized lipids (4–5 mg) to Barnstead nanopure water (3 g) in 3 ml nalgene vials. These dispersions were cycled between 50 and 5°C with 5 min of vortexing at each temperature. After the third heating, the samples were held at room temperature, awaiting low temperature incubation. Short low temperature incubations were carried out in the calorimeter. Long low temperature incubations were carried out in a refrigerator and the samples were then transferred to the calorimeter with a cold syringe.

High sensitivity DSC (MC-2 from Microcal, Northampton, MA, USA) was used with typical heating scan rates of 13°C/h. The MC-2 is interfaced to a PC which uses Microcal software to collect data. Peaks were analyzed using Origin 4.1 and the Peak Fitting Module from Microcal Software (Northampton, MA, USA). Enthalpies of all peaks were obtained by comparing to the MC-2 calibration pulse.

2.3. X-Ray Diffraction of unoriented MLVs

Concentrated MLV suspensions, prepared as for DSC except with solvent to lipid weight ratio 3:1, were loaded into thin walled 1.0 mm glass capillaries. The capillaries were centrifuged for 10 min at $1100 \times g$ at room temperature to remove air bubbles and were then flame-sealed above the water layer.

X-ray diffraction spectra were obtained using a Rigaku fixed tube source operated at 2.3 kW with a graphite monochromator selecting Cu $K\alpha\lambda =$ 1.5418Å. The capillaries were mounted vertically and data were collected using a Bicron scintillation counter. Three sets of Huber slits (monochro-

mator exit, before sample, and before detector), all opened from 0.25 to 1 mm horizontally and 5 mm vertically, produced an in-plane resolution from 0.05 to 0.2° (FWHM) in 2θ.

2.4. X-Ray diffraction of oriented stacks of bilayers

Oriented samples of MPPC were prepared by dissolving 5 mg MPPC in 500 μl 3:1 chloroform: methanol and loading onto the outside of a cylindrical 100 ml glass beaker with a diameter of 5 cm. The solvent was allowed to evaporate for 1 day at room temperature. The temperature was controlled with two circulating water baths connected to the inner and outer chambers of a new humidity chamber for X-ray studies (Chalk River Labs, Ontario, Ont, Canada). The chamber was humidified from a sponge soaked in distilled water.

X-ray diffraction was carried out on the D1 line at the Cornell High Energy Synchrotron Source (CHESS) where the beam was collimated using three sets of slits set to 1, 0.25, and 1 mm in the horizontal direction and 1 mm vertically. X-rays with $\lambda = 1.3808$Å were selected and D spacings were calibrated using powdered crystals of silver behenate, $D = 58.4$ Å. (Blanton et al., 1995). A CCD camera (Tate et al., 1995) collected 16-bit greyscale TIF images on an array of 1024×1024 pixels each of 50 μ linear extent and with sample to camera distance of 58.1 cm. Typical exposure times were 5 sec and no indication of radiation damage was observed. The software Image Quant, from Molecular Dynamics, Sunnyvale, CA, USA was used to measure and quantitate the TIF images.

3. Results

3.1. Differential scanning calorimetry

Fig. 1 compares calorimetric scans of our synthesized sample of MPPC with the commercially available MPPC. In addition to chemical analysis, three aspects of calorimetry indicate sample purity, namely: (a) narrower transition widths ΔT; (b) higher transition enthalpies ΔH ;and (c) higher transition temperatures. Table 1 lists these three properties for the two samples we studied and also for three earlier studies (from which it is not possible to obtain transition widths). The data for the main transition (subscript M) show that this transition is relatively insensitive to acyl chain migration impurity when compared to the subtransition (subscript S). This is not surprising since disordered phases can accommodate impurities more easily than more ordered phases. Direct comparison of the subtransition results in Table 1 for the relatively impure commercial sample and our sample supports the purity criteria listed above. Comparisons with the results of Serrallach et al. (1984) are consistent with our sample being purer. Stumpel et al. (1983) obtained the same T_S, but their higher T_M suggests that their temperature may have been calibrated too high since T_M is not very sensitive to small migration impurity in the other studies. This is consistent with their lower ΔH_S and suggests that we may have higher overall purity (including lack of lysolecithin) despite the chemical assay test for acyl chain migration. Except for our larger ΔH_S our calorimetric results agree well with the results of Lin et al. (1991).

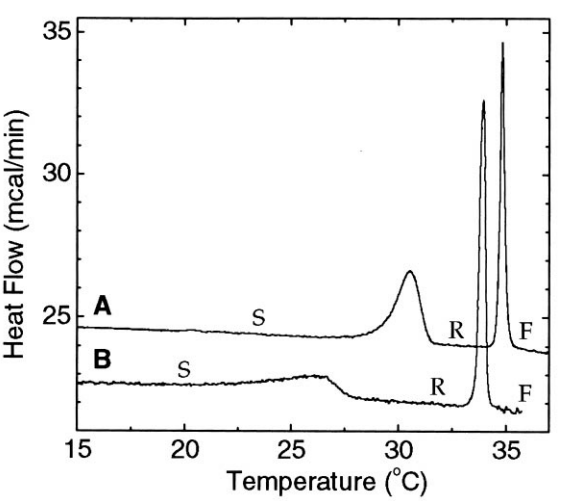

Fig. 1. Differential scanning calorimetry of our MPPC (scan A) and commercial MPPC (scan B). Lipid concentrations were 1.5 mg/ml; scan rate was 13°C/h. S, R, and F designate the subgel, ripple and fluid (L_α) phases, respectively. Curves have been shifted vertically.

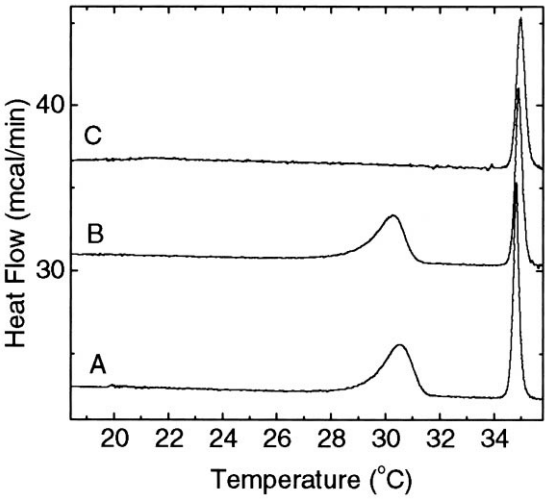

Fig. 2. Differential scanning calorimetry (DSC) study of kinetics of subgel formation in MPPC. Incubation for (A) 12 days at 0.5°C, (B) 1 h at 12°C, and (C) 1 h at 14°C. Scans have been shifted vertically.

All of the rest of the data is for MPPC synthesized by us. Fig. 2 shows the kinetics of subgel formation in our MPPC studied by DSC, scanned in the heating direction. Incubations were carried out for the times and at the temperatures indicated, and then the samples were scanned in the heating direction. It is remarkable that the subtransition enthalpy ΔH_S of the sample incubated for only 1 h at 12°C (curve B) is as large as for the sample incubated 12 days at 0.5°C (curve A). However, subgel transformation slowed considerably when incubated at 14°C (curve C), as indicated by the small, broad peak at 21.5°C in curve C in Fig. 2.

3.2. X-Ray diffraction

Low-angle scattering from the L_α phase of an unoriented sample is shown in Fig. 3. $D = 64$Å was obtained as determined from the first and second order Bragg peaks. No higher order peaks were observed and wide-angle data are devoid of sharp peaks (see Fig. 6).

A T-jump from 38°C to an incubation $T =$ 33°C gave the low-angle results shown in Fig. 4. There was no significant shift in position of these peaks up to 19 h, which indicated that the ripple

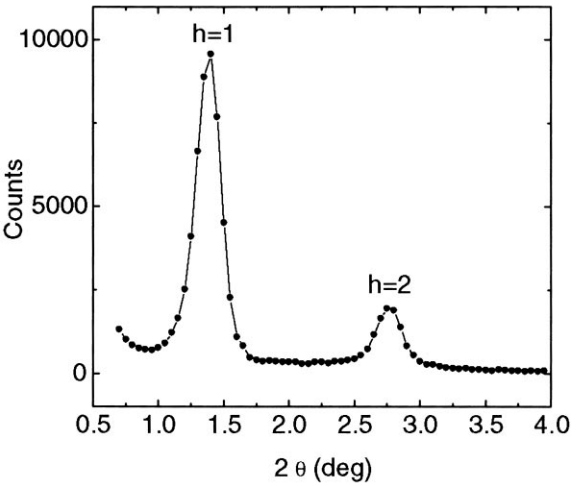

Fig. 3. Fluid phase low-angle X-ray diffraction at $T = 38$°C.

phase formed rapidly. Peaks are broader than those in the fluid phase, as a result of many close (hk) reflections which cannot be resolved at these typical resolutions, so the repeat spacing associated with the (h0) peaks, $D = 68$Å, is only apparent. The scan at 19 h was obtained at higher instrumental resolution in an unsuccessful effort to differentiate the ripple peaks. The duration

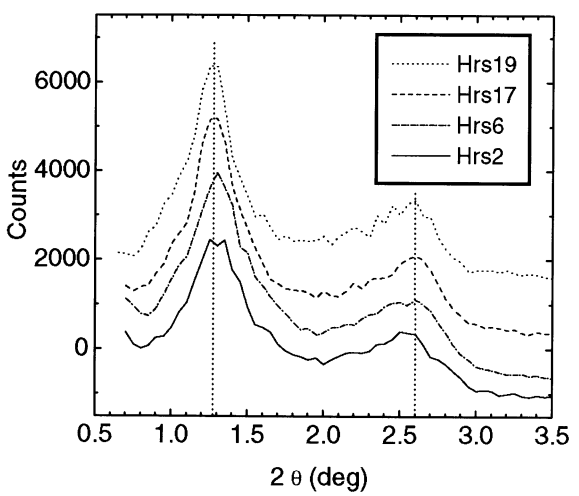

Fig. 4. Rippled phase low-angle X-ray spectra of MPPC quenched from the fluid phase to 33°C. The 19 h spectrum had higher instrumental resolution (FWHM = 0.05°). Scans have been displaced vertically.

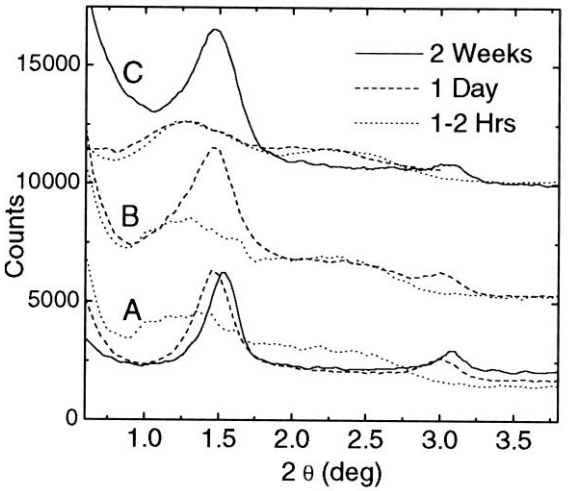

Fig. 5. Low-angle X-ray diffraction data of MPPC quenched from 38°C in the L_α phase to: (A) 15°C, (B) 18°C (data for 16.5°C were nearly identical) (C) 20°C. Dotted lines, 1–2 h incubation; dashed lines, 1 day incubation; solid lines, 2 week incubation. Curves have been displaced vertically.

times for data collection varied from 15 min for the 2 h incubation, to 1.5 h for the 19 h incubation.

Fig. 5 shows the time course of the low-angle diffraction peaks when MPPC was quenched to lower temperatures from the fluid phase. Spectra taken within 1–2 h indicate non-equilibrium. For MPPC held overnight well-defined low-angle peaks were present except for the sample at 20°C. After subsequent incubation for 2 weeks, all samples had well-defined low-angle peaks, with $D =$ 57.5Å at 15°C (based on the second order). Some corresponding wide-angle spectra are shown in Fig. 6. Overnight incubation at 15°C resulted in a prominent wide-angle peak corresponding to $D =$ 4.46Å (19.9°-dotted line in Fig. 6), which is a larger spacing than characteristic gel phase wide-angle peaks (Sun et al., 1996). Longer incubation at various temperatures of 20°C or less resulted in this peak splitting into two close peaks (19.8 and 20.2°). The broad wide-angle peak centered at 22.8° (3.91Å) also split into three sharper features as can be seen by the solid line in Fig. 6. One might consider interpreting the latter behavior as a gel to subgel transition. If so, the breadth of the wide-angle peak at 3.91Å in the putative gel phase after 1 day incubation at 15°C (dashed line, Fig. 5A) is consistent with a reflection indicating a chain tilt angle of about 30° and with no colinear coupling between the two monolayers (Keough and Davis, 1979). However, the persistence of the sharp peak corresponding to 4.46Å suggests a different interpretation, that the subgel phase is gradually being annealed into larger well-correlated domains (Tristram-Nagle et al., 1987, 1994). The lamellar $D = 59.2$Å after only 1 day incubation also supports this interpretation because a gel phase would presumably have D between that of DPPC (63.4Å) and DMPC (61.7Å) (Marsh, 1990). This latter interpretation concurs with that of Serrallach et al. (1984) in that they observed a similar pattern upon slow cooling. They also reported a metastable gel phase in the same cooling run; the wide-angle spectrum did not have a sharp peak at 4.47Å and the lamellar D was 63Å, but we did not see this pattern. The subgel low-angle lamellar D spacing decreased about 2Å over a 2-week incubation, also observed by Serrallach et al. (1984).

To investigate further the temperature region of a putative gel phase, a fresh sample, initially at $T = 38$°C was incubated for 3 weeks at 24°C. The

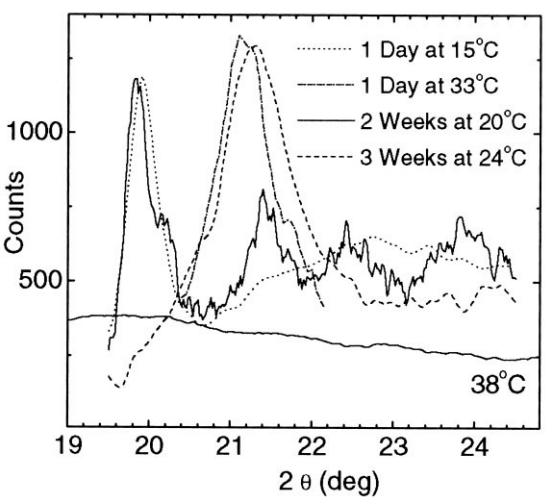

Fig. 6. Wide-angle diffraction data of MPPC quenched from the fluid phase for incubation times and temperatures indicated.

low-angle spectrum was similar to the ripple phase low-angle spectra in Fig. 4. The wide-angle spectrum (Fig. 6 dashed line) shows only a single, relatively sharp peak corresponding to $D = 4.17$Å. This pattern is identical, except for expected small thermal shifts, to the pattern formed at $T = 33$°C (Fig. 6, dash-dotted line). The prominent peak near 21° indicates hexagonal packing of the chains, characteristic of the ripple phase in PCs with little chain tilt, instead of the pseudo hexagonal (orthorhombic) packing of typical gel phases.

The next goal was to obtain the true subtransition temperature. Although the DSC heating scans give $T_s = 30$°C, such apparent values for T_s can be too high as a result of non zero scanning rate, as is well documented for DPPC where even the lowest extrapolated calorimetric T_s is 17°C (and much higher values have been reported), whereas the true equilibrium T_s is 14°C (Nagle and Wilkinson, 1982; Kodama et al., 1985; Tristram-Nagle et al., 1987). Fig. 7 shows diffraction results for the slow melting of the subgel. The subgel in this sample was formed by quenching the sample from the fluid phase to 2°C for 2 h and then equilibrating for 16 h at 15°C before the first scan. The wide-angle pattern (inset in Fig. 7) shows that this is a well-formed subgel. In agreement with the result of Serrallach et al. (1984), who used a similar procedure, the subgel melted less than 1° below the calorimetric T_s, although some ripple phase (near $2\theta = 2.5°$ in Fig. 7) began to be present at 27°C. Not only does the subgel form quickly at low enough incubation temperature, but it melts quickly in DSC scans. Nevertheless, there is a gap of 10°C, from $T = 30$°C (subgel melting) to $T = 20$°C within which the subgel does not form even with prolonged incubation. This is comparable to the analogous gap of 7°C in DPPC, which has been ascribed to very slow nucleation above $T = 7$°C (Nagle and Wilkinson, 1982; Tristram-Nagle et al., 1994).

Finally, we wished to verify unequivocally that the two phases separated by the subtransition are indeed subgel and ripple phases. Fig. 8 shows a scan obtained over a wide angular range for

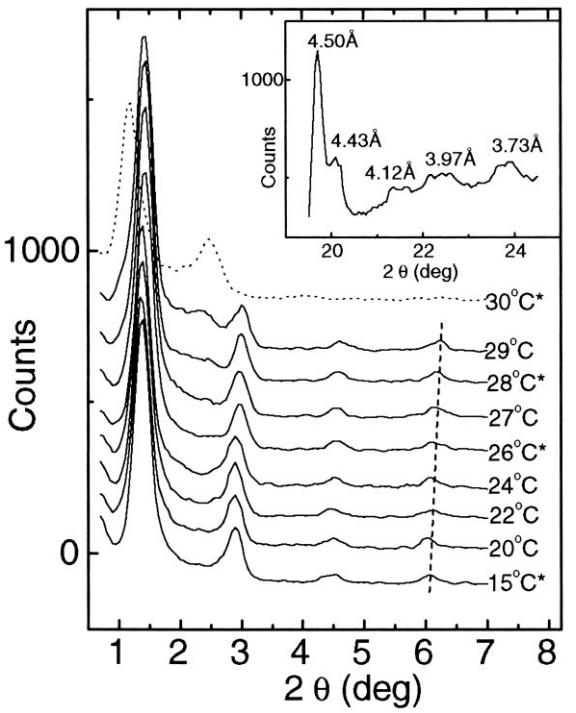

Fig. 7. Melting of the subgel of MPPC in near equilibrium steps, starting at 15°C. Subgel was formed by incubating for 2 h near 2°C. The sample was then held at the indicated temperatures for 2 h, or overnight (indicated with an asterisk) before each scan of duration 30 min. The inset shows the wide-angle data obtained at 15°C. Curves have been displaced vertically.

MPPC that incubated for two weeks at 15°C. Taking 'slit smear' into account, the four low-angle peaks index to $D = 56.4$Å, while the wide-angle peaks are similar to those shown in Fig. 7. In addition, Fig. 8 shows the intermediate-angle peaks (8–17°); these peaks are exclusively characteristic of subgel phases and are associated with ordering of the entire molecule including the headgroups, as well as of the hydrocarbon chains (Katsaras et al., 1995).

Although there is no doubt that the low temperature phase in unoriented samples is indeed a subgel phase, our unoriented data for the ripple phase could be considered to be ambiguous in that a single wide-angle peak could also be obtained from a gel phase with untilted chains packed hexagonally. Unambiguous confirmation

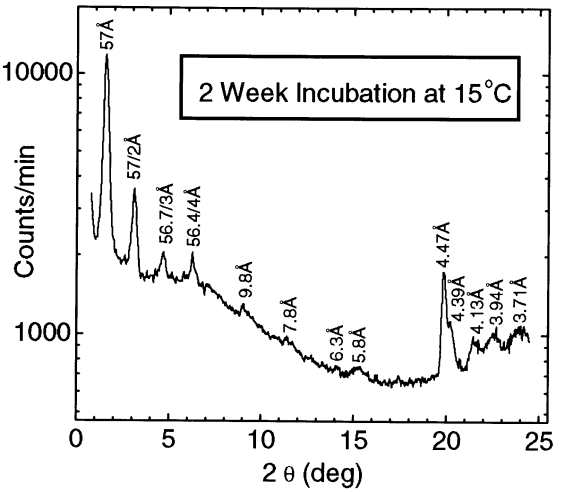

Fig. 8. X-ray diffraction of MPPC that incubated at 15°C for 2 weeks.

of the ripple phase is shown in Fig. 9 for an oriented sample of MPPC. Fig. 9A shows a rippled pattern at 33°C after cooling from 38°C, and Fig. 9B shows another rippled pattern at 33°C after heating from 5°C. The data in Fig. 9A are complicated by the coexistence of two lamellar D spacings, 82Å and 69Å. The $D = 82$Å spacing has a strong ripple repeat with $\lambda_r = 240$Å (strong reflections easily seen). The $D = 69$Å spacing also has ripple repeats with $\lambda_r = 130$Å that are difficult to see at the contrast chosen but can be easily seen when the 82Å ripple peaks are overexposed. When the sample temperature was reduced to 5°C and then raised back to 33°C within 1.5 h, only a single lamellar $D = 69$Å was observed (Fig. 9B),

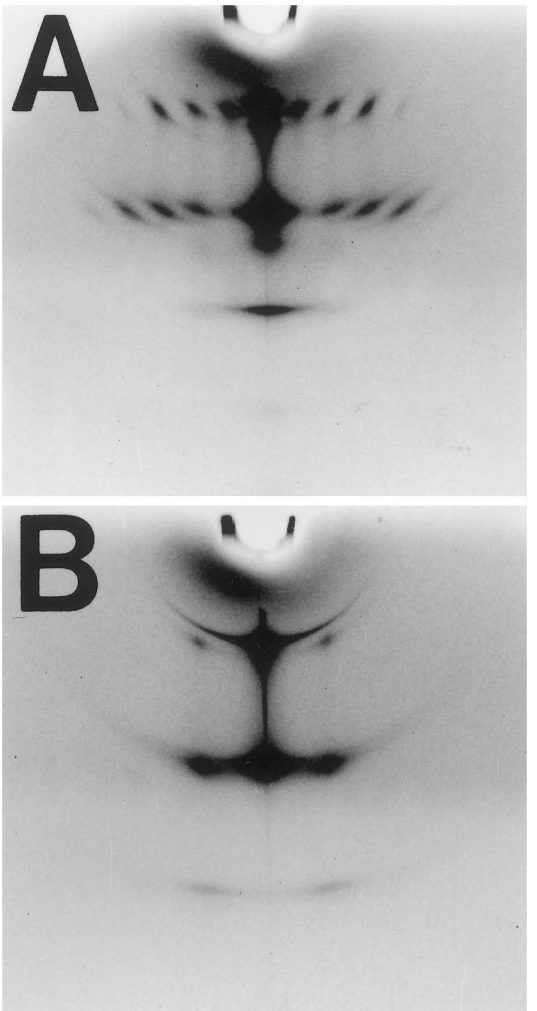

Fig. 9. X-ray diffraction at CHESS from oriented MPPC. The sample was maintained near 100% relative humidity. (A) $T =$ 33°C after cooling from 38°C. (B) $T = 33$°C after heating from 5°C.

Table 1
Differential scanning calorimetry (DSC) comparison of MPPC Samples

MPPC	Migration[a]	Incubation	ΔH_M	T_M[b]	ΔT_M	ΔH_S	T_S	ΔT_S
This study	≤5%	1 h—12 days@0.5°C	8.3	34.8	0.22	10.9	30.5	1.3
Commercial	20–30%	6 days@0.5°C	7.7	33.9	0.32	6.4	26.2	4.2
Serrallach et al.	6%	3 months@−3°C	8	33.9	–	8	26	–
Stumpel et al.	1%	2 weeks@4°C	7.3	37	–	6.4	30	–
Lin et al.	1%	9 months@0°C	8.1	34.9	–	7.7	30.1	–

[a] % acyl chain migration by chemical assay.
[b] Temperatures are in °C and enthalpies in kcal/mole.

having a single ripple wavelength of $\lambda_r = 130$Å. It may first be noted that the coexistence of the patterns shown in Fig. 9A vitiate obtaining reliable D spacings from powder samples, such as those shown in Figs. 4 and 7, which is why we emphasize that those D spacings are only apparent. Furthermore, the proportions of the coexisting patterns are highly history dependent, so the apparent D spacings are, not surprisingly, different in Figs. 4 and 7. It should also be noted that it is often quite difficult to fully hydrate oriented samples from the vapor. However, using a newly developed hydration X-ray oven similar to that recently reported for neutron diffraction (Katsaras, 1997), this sample gave rise to fully hydrated D spacings, even in the L_α phase. A summary of X-ray results for all phases is given in Table 2.

4. Discussion

A major concern recognized by investigators who have worked on mixed-chain lipids has been the purity of the synthesized lipids, in particular, the percentage of acyl chain migration, yielding PMPC as a contaminant in MPPC (Keough and Davis, 1979; Chen and Sturtevant, 1981). The main innovation of Mason et al. (1981a) was to use the catalyst 4-pyrrolidinopyridine, which significantly reduces acyl chain migration compared to other catalysts. Although our chemical analysis gave a maximum 5% contamination with the unwanted PMPC positional isomer, our larger values for subtransition enthalpy ΔH_M and subtransition temperature T_M summarized in Table 1 strongly suggest that our MPPC is overall as pure or purer than MPPC used in previous studies.

Table 2
Summary of X-Ray D-Spacings in MPPC

Temperature	Phase	Lamellar D(Å)	λ_r(Å)
38°C	Fluid	64	–
33 from 38°C	Ripple	82	240
		69	130
33 from 5°C	Ripple	69	130
15°C (2 weeks)	Subgel	56.4[a]	–

[a] Subgel D-spacing based on the fourth order in Fig. 8.

The lamellar repeat spacing for L_α phase MPPC, $D = 64$Å, falls between that of DPPC (65—67Å, Nagle et al., 1996) and that of DMPC (62.7Å, Petrache et al., 1998), as one would expect. The L_α phase generally appeared to be normal, including the usual anomalous dependence of D as T approached T_M (Nagle et al., 1998).

The phase at lowest T is clearly a subgel phase because it has intermediate-angle peaks, small lamellar D and a well-developed wide-angle pattern with a characteristic sharp peak corresponding to $D = 4.47$ Å (Fig. 8). Careful melting of this subgel phase, as was also carried out by Serrallach et al. (1984), gives an equilibrium subtransition temperature $T_S = 29$°C; this is surprisingly close to the DSC transition temperature (30.5°C), which indicates more rapid melting equilibration than for same-chain lipids such as DPPC (Tristram-Nagle et al., 1987). Nevertheless, formation of subgel phase in MPPC can not be accomplished in reasonable times unless incubated at temperatures T_I substantially below T_S; this is similar to same-chain lipids. We found that it took MPPC only 2 weeks to convert to the subgel phase for T_I even as high as 20°C. Quite possibly, longer incubations would allow subgel transformation a few degrees higher, but the maximum T_I would have to be less than 24°C at which our MPPC definitely transformed to a well-formed ripple phase. Therefore, there is a gap of at least 6°C, from 24 to 30°C, within which the subgel phase is thermodynamically stable but will not form spontaneously upon quenching from the L_α phase. Furthermore, when the subgel was formed by nucleating at 2°C for 1 h and then jumping to 15°C and holding overnight, a wide-angle pattern similar to that seen in the 2-week incubation at 15°C is observed (Fig. 7). This confirms that the rate limiting step for subgel formation is nucleation, as is the case for DPPC, and such nucleation becomes very slow for incubation temperatures in the gap (Tristram-Nagle et al., 1994). It is interesting to compare this behavior to DPPC for which the equilibrium $T_S = 14$°C (Nagle and Wilkinson, 1982; Kodama et al., 1985; Tristram-Nagle et al., 1987) and the maximum

incubation temperature for subgel formation is $T_I = 8°C$. For DPPC, growth occurs rapidly in the gap when nuclei have already been formed at lower temperature, while nucleation is so slow as to be immeasurable (Yang and Nagle, 1988). Another interesting comparison is for the kinetics of subgel formation at lower temperatures. MPPC subgel forms in less than 1 h for T_I less than 12°C (Fig. 2). There is no T_I at which DPPC subgel forms in less than days. Indeed, MPPC forms subgel more rapidly than any same-chain lipid we have studied (Wilkinson and Nagle, 1984; Tristram-Nagle et al., 1987, 1994), except for 2-[4,4-F_2]-DMPC, which formed a subgel phase in 1.5 h below 5°C (Dowd et al., 1993; Tristram-Nagle and Dowd, 1994).

In agreement with previous studies, the stable phase between the subgel and fluid phases is the ripple phase, as is most dramatically revealed by oriented samples (Fig. 9). Just as for DPPC (Tenchov et al., 1989; Yao et al., 1991; Mason et al., 1998), there are actually two different ripple phases, one obtained upon cooling the sample from the L_α phase and one obtained upon heating the sample from lower T. (See Table 2 for structural differences.) The reasons for the differences in these two phases, of which one is the stable phase, are still unknown. The present ripple data in MPPC are part of a more comprehensive study of the ripple phase of several oriented lipids (Katsaras et al., in preparation). Despite this uncertainty regarding ripple phases, it is likely that MPPC is not fundamentally different from same-chain PCs with regard to the ripple phase.

Our initial interest in MPPC was to compare the gel phase of a very pure mixed-chain lipid with that of DPPC. Although we were aware that there might not be a gel phase, Serrallach et al. (1984) reported a metastable gel phase and we hoped that we could stabilize it long enough for study, analogously to the metastable gel phase in DLPE (Chang and Epand, 1983), and this accounted for some of the temperature protocols we tried. However, we were unsuccessful in finding a gel phase (Figs. 5–7). This is unfortunate in that the gel phase of DPPC has been the most thoroughly characterized phase and would have been the best phase for detailed comparison with MPPC.

Why is there no gel phase in MPPC? The most obvious thermodynamic answer is that the subgel phase is so stable that T_s is higher than the highest temperature at which the gel phase is stable relative to the ripple phase. This refocusses the question from gel phases to subgel phases. Why is the subgel phase in MPPC more stable than in same-chain lipids such as DPPC, which have even more carbons in the chains, and so would be expected to have higher transition temperatures? The simplest, but incorrect, answer is that the equivalent penetration of the sn-1 and sn-2 chains in MPPC promotes subgel phase stability. The reason this answer is incorrect is that PMPC, which should have greater penetration inequivalence of the two chains, also has enhanced subgel stability as shown by Stumpel et al. (1983) and Serrallach et al. (1984). Although the transition temperatures are lower for PMPC ($T_s = 17°C$ and $T_M = 27°C$), the differences are similar and there is no gel phase, only a ripple phase, between the subgel and fluid phases. Therefore, in regard to subgel stability and the subgel transition, it appears that same-chain lipids are special in comparison to lipids where either the sn-1 or the sn-2 chain has two more carbons. In particular, we see no way to describe subgel versus gel phase stability phenomenologically using the $\Delta C/C_L$, parameter that works so well for ripple versus fluid phase stability at the main transition (Huang et al., 1994). The only special value of this parameter is zero, which occurs when the sn-2 chain has one and a half more carbons. This is also the special (minimal) value of $(\Delta C/C_L)^2$, which is the leading term in the T_M dependence (Huang et al., 1994).

One might ask if this result is a result of small amounts of acyl chain migration in even the purest samples. However, this concern is easily dismissed. Our results (Fig. 1 and Table 1) indicate that T_s decreases with impurity and this is consistent with the general concept that impurities destabilize the more ordered phase more than the less ordered one. Another concern is that we are comparing DPPC, which has a total of two additional carbons, to MPPC and PMPC. DSC studies of $DC_{15}PC$ show a small pretransition

near 22°C upon cooling from ripple to gel phase and a larger subtransition near 19°C upon heating (Lewis et al., 1987). After taking into account thermal lags inherent in DSC transition temperatures (the pretransition temperature upon heating is near 24°C) there is still a temperature range in which the gel phase is stable.

We tentatively consider another hypothesis, one that involves the headgroup, which has previously been shown to undergo ordering in the subgel phase (Katsaras et al., 1995). This new hypothesis is that the glycerol backbone (which is part of the headgroup in the gross division of the lipid molecule into heads and tails) may be oriented parallel to the bilayer in the subgel phase, rather than perpendicular, as in the other phases, including the nearly dehydrated crystal phase. If so, then the chain penetration of the sn-1 and sn-2 chains would be nearly equal for same-chain PCs which would make them special compared to MPPC and PMPC and other mixed-chain lipids with small differences in number of carbons per chain. The new phenomenological parameter ΔC_S for comparing the sn-1 and sn-2 chains in the subgel phase would then naturally be $n_1 - n_2$, which differs from the usual $\Delta C = n_1 - n_2 + 1.5$ by not having a 1.5 carbon offset. This parameter (and its square) has its special value (zero) for same-chain lipids. The physical hypothesis to support greater subgel stability for mixed-chain PCs would be that partial interdigitation of the chains from the two monolayers would stabilize the subgel phase of MPPC and PMPC relative to the subgel phase of DPPC. However radical this hypothesis may seem, it appears that the phenomena that have led us to consider it are not easily understood otherwise. Testing this hypothesis requires obtaining more experimental information about the structure of subgel phases, which would be interesting in any case, and would be especially desirable if no other hypotheses emerge to explain the phenomena.

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References

Blanton, T.N., Huang, T.C., Toraya, H., Hubbard, C.R., Robie, S.B., Louer, D., Gobel, H.E., Will, G., Gilles, R., Raftery, T., 1995. JCPDS-International Centre for Diffraction Data round robin study of silver behenate: a possible low-angle X-ray diffraction calibration standard. Powder Diffr. 10, 91–95.

Buldt, G., Gally, H.U., Seelig, J., Zaccai, G., 1979. Neutron diffraction studies on phosphatidylcholine model membranes: I: headgroup conformation. J. Mol. Biol. 134, 673–691.

Chang, H., Epand, R.M., 1983. The existence of a highly ordered phase in fully hydrated Dilauroylphosphatidylethanolamine. Biochim. Biophys. Acta 728, 319–324.

Chen, S.C., Sturtevant, J.M., 1981. Thermotropic behavior of bilayers formed from mixed-chain phosphatidylcholines. Biochemistry 20, 713–718.

Dowd, S.R., Sturtevant, J.M., Simplaceanu, V., Ho, C., 1993. ^{31}P-NMR and calorimetric studies of the low-temperature behavior of three ^{19}F-labeled dimyristoylphosphatidylcholines. J. Phys. Chem. 97, 2946–2951.

Huang, C., Wang, Z.-q., Lin, H.-q., Brumbaugh, E.E., Li, S., 1994. Interconversion of bilayer phase transition temperatures between phosphatidylethanolamines and phosphatidylcholines. Biochim. Biophys. Acta 1189, 7–12.

Hui, S.W., Mason, J.T., Huang, C.-h., 1984. Acyl chain interdigitation in saturated mixed-chain phosphatidylcholine bilayer dispersions. Biochemistry 23, 5570–5577.

Katsaras, J., Yang, D. S.-C., Epand, R M., 1992. Fatty-acid chain tilt angles and directions in dipalmitoyl phosphatidylcholine bilayers. Biophys. J. 63, 1170–1175.

Katsaras, J., 1997. Highly aligned lipid membrane systems in the physiologically relevant 'excess water' condition. Biophys. J. 73, 2924–2929.

Katsaras, J., Raghunathan, V.A., Dufourc, E.J., Dufourcq, J., 1995. Evidence for a two dimensional molecular lattice in subgel phase DPPC bilayers. Biochemistry 34, 4686–4688.

Keough, K.M.W., Davis, P.J., 1979. Gel to liquid-crystalline phase transitions in water dispersions of saturated mixed-acid phosphatidylcholines. Biochemistry 18, 1453–1459.

Kodama, M., Hashigami, H., Seki, S., 1985. Static and dynamic calorimetric studies on the three kinds of phase transition in the systems of L- and DL-dipalmitoyl-phosphatidylcholine/water. Biochim. Biophys. Acta 814, 300–306.

Lewis, R.N.A.H., McElhaney, R.N., Monck, M.A., Cullis, P.R., 1994. Studies of highly asymmetric mixed-chain diacylphosphatidylcholines that form mixed-interdigitated gel phases: Fourier transform infrared and ^{2}H NMR spectroscopic studies of hydrocrabon chain conformation and orientational order in the liquid-crystalline state. Biophys. J. 67, 197–207.

Lewis, R.N.A.H., Mak, N., McElhaney, R.N., 1987. A differential scanning calorimetric study of the thermotropic phase behavior of model membranes composed of phosphatidylcholines containing linear saturated fatty acyl chains. Biochemistry 26, 6118–6126.

Lin, H.-n., Wang, Z.-q., Huang, C.-h., 1991. The influence of acyl chain-length asymmetry on the phase transition parameters of phosphatidylcholine dispersions. Biochim. Biophys. Acta 1067, 17–28.

Mason, J.T., Broccoli, A.V., Huang, C.-h., 1981a. A method for the synthesis of isomerically pure saturated mixed-chain phosphatidylcholines. Analytical Biochemistry 113, 96–101.

Mason, J.T., Huang, C.-h., Biltonen, R.L., 1981b. Calorimetric investigations of saturated mixed-chain phosphatidylcholine bilayer dispersions. Biochemistry 20, 6086–6092.

Mason, P.C., Gaulin, B.D., Epand, R.M., Wignall, G.D., Lin, J.S., 1998. Small-angle scattering studies of the fully hydrated phospholipid, DPPC. Phys. Rev. E. 59, 921–928.

Marsh, D., 1990. CRC Handbook of Lipid Bilayers. CRC Press, Boca Raton, p. 166.

Mattai, J., Sripada, P.K., Shipley, G.G., 1987. Mixed-chain phosphatidylcholine bilayers: structure and properties. Biochemistry 26, 3287–3297.

McIntosh, T.J., 1980. Differences in hydrocarbon chain tilt between hydrated phosphatidylethanolamine and phosphatidylcholine bilayers; a molecular packing model. Biophys. J. 29, 237–246.

McIntosh, T.J., Simon, S.A., Ellington, J.C., Porter, N.A., 1984. New structural model for mixed-chain phosphatidylcholine bilayers. Biochemistry 23, 4038–4044.

Nagle, J.F., Wilkinson, D.A., 1982. Dilatometric studies of the subtransition in dipalmitoylphosphatidylcholine. Biochemistry 16, 3817–3821.

Nagle, J.F., Zhang, R., Tristram-Nagle, S., Sun, W., Petrache, H.I., Suter, R.M., 1996. X-ray structure determination of fully hydrated L_α-dipalmitoylphosphatidylcholine. Biophys. J. 70, 1419–1431.

Nagle, J.F., Petrache, H.I., Gouliaev, N., Tristram-Nagle, S., Liu, Y., Suter, R.M., Gawrisch, K., 1998. Multiple mechanisms for critical behavior in the biologically relevant phase of lecithin bilayers. Phys. Rev. E. 58, 7769–7776.

Pearson, R.H., Pascher, I., 1979. The molecular structure of lecithin dihydrate. Nature 281, 499–501.

Petrache, H.I., Tristram-Nagle, S., Nagle, J.F., 1998. Fluid phase structure of EPC and DMPC bilayers. Chem. Phys. Lipids 95, 83–94.

Seelig, A., Seelig, J., 1975. Bilayers of Dipalmitoyl-3-*sn* – phosphatidylcholine, conformational differences between the fatty acyl chains. Biochim. Biophys. Acta 406, 1–5.

Serrallach, E.N., deHaas, G.A., Shipley, G.G., 1984. Structure and thermotropic properties of mixed-chain phosphatidylcholine bilayer membranes. Biochemistry 23, 713–720.

Smith, G.S., Sirota, E.B., Safinya, C.R., Clark, N.A., 1988. Structure of the L_β phases in a hydrated phosphatidylcholine multimembrane. Phys. Rev. Lett. 60, 813–816.

Stumpel, J.H., Eibl, H., Nicksch, A., 1983. X-ray analysis and calorimetry on phosphatidylcholine model membranes. Biochim. Biophys. Acta 727, 246–254.

Sun, W.-J., Suter, R.M., Knewtson, M.A., Worthington, C.R., Tristram-Nagle, S., Zhang, R., Nagle, J.F., 1994. Order and disorder in fully hydrated unoriented bilayers of gel phase dipalmitoylphosphatidylcholine. Phys. Rev. E 49, 4665–4676.

Sun, W.-J., Tristram-Nagle, S., Suter, R.M., Nagle, J.F., 1996. Structure of gel phase saturated lecithin bilayers: temperature and chain length dependence. Biophys. J. 71, 885–891.

Tardieu, A., Luzzati, V., Reman, F.C., 1973. Structure and polymorphism of the hydrocarbon chains of lipids; a study of lecithin-water phases. J. Mol. Biol. 75, 711–733.

Tate, M. W., Eikenberry, E.F., Barna, S.L., Wall, M.E., Lowrance, J.L., Gruner, S.M., 1995. A large-format high-resolution area X-ray detector based on a fiber-optically bonded charge-coupled device (CCD). J. Appl. Cryst. 28, 196–205.

Tenchov, B.G., Yao, H., Hatta, I., 1989. Time-resolved X-ray diffraction and calorimetric studies of low scan rates. Biophys. J. 56, 757–768.

Tristram-Nagle, S., Wiener, M., Yang, C.-P., Nagle, J.F., 1987. Kinetics of the subtransition in dipalmitoylphosphatidylcholine. Biochemistry 26, 4288–4294.

Tristram-Nagle, S., Zhang, R., Suter, R.M., Worthington, C.R., Sun, W.-J., Nagle, J.F., 1993. Measurement of chain tilt angle in fully hydrated bilayers of gel phase lecithins. Biophys. J. 64, 1097–1109.

Tristram-Nagle, S., Suter, R.M., Sun, W.-J., Nagle, J.F., 1994. Kinetics of subgel formation in DPPC: X-ray diffraction proves nucleation-growth hypothesis. Biochim. Biophys. Acta 1191, 14–20.

Tristram-Nagle, S., Dowd, S., 1994. X-ray diffraction study of three ^{19}F-labeled dimyristoylphosphatidylcholines. J. Phys. Chem. 98, 4469–4472.

Tu, K., Tobias, D.J., Blasie, J.K., Klein, M.L., 1996. Molecular dynamics investigation of the structure of a fully hydrated gel-phase dipalmitoylphosphatidylcholine bilayer. Biophys. J. 70, 595–608.

Tummler, B., Herrmann, U., Maass, G., Eibl, H., 1984. Kinetics of the subtransition of asymmetrically substituted phosphatidylcholines. Biochemistry 23, 4068–4074.

Wilkinson, D.A., Nagle, J.F., 1984. Metastability in the phase behavior of dimyristoylphosphatidylethanolamine. Biochemistry 23, 1538–1541.

Yang, C.P., Nagle, J.F., 1988. Phase transformations in lipids follow classical kinetics with small fractional dimensionalities. Phys. Rev. A 37, 3993–4000.

Yao, H., Matuoka, S., Tenchov, B., Hatta, I., 1991. Metastable ripple phase of fully hydrated dipalmitoylphosphatidylcholine studied by small angle X-ray scattering. Biophys. J. :255 59, 252.

Zhu, T., Caffrey, M., 1993. Thermodynamic, thermomechanical, and structural properties of a hydrated asymmetric phosphatidylcholine. Biophys. J. 65, 939–954.