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Phase properties of the aqueous dispersions of *n*-octadecylphosphocholine

Mahendra Kumar Jain [a], Roger W. Crecely [a], Jan D.R. Hille [b], Gerard H. de Haas [b] and Sol M. Gruner [c]

[a] *Department of Chemistry, University of Delaware, Newark, DE 19711 (U.S.A.),* [b] *Department of Biochemistry, The State University, The Uithof, Transitorium 3, Utrecht (The Netherlands) and* [c] *Department of Physics, Princeton University, Princeton, NJ 08544 (U.S.A.)*

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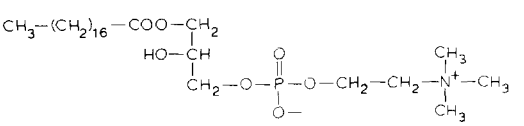
Properties of the aqueous dispersions of *n*-octadecylphosphocholine are examined by differential scanning calorimetry, fluorescence depolarization, light scattering, ^{31}P-NMR, pig pancreatic phospholipase A_2 binding, and X-ray diffraction. On heating, these dispersions exhibit a sharp lamellar to micelle transition at 20.5°C. The lamellar phase consists of frozen (gel-state) alkyl chains which do not bind phospholipase A_2. The kinetics of the transition are asymmetric: the micelle to lamellar transition is very slow and the lamellar to micelle transition is fast. It is suggested that the lamellar phase is a frozen chain bilayer in which the chains interdigitate.

Introduction

Lysophospholipids have been implicated in a variety of biological functions including lysis [1] and fusion [2]. More recently 2-acetyl analogs have been shown to influence the physiological role of platelets, leucocytes, and several other cell types [3], presumably by modulating arachidonate metabolism. The physical properties of their aqueous dispersions are also interesting. For example, the micelles of lysophospholipids are also known to bind phospholipase A_2 [4]. The bilayers formed from an equimolar mixture of lysophospholipids and fatty acids do not bind the enzyme [5].

Lamellar to micelle transitions require a reorganization of the time-averaged shape of the constituent molecules because molecules which pack into planar structures, such as bilayers, do not pack well in highly curved surfaces, such as micelles. However, it has recently been demonstrated that aqueous micellar dispersions of lysophospholipids can form a lamellar phase at low temperatures [6,7]. It was suggested that this phase consists of bilayers in which the acyl chains are highly interdigitated. Since little work has been done on micelle-lamellar transitions in phospholipids, we decided to examine if it occurs with the lysolipid analog *n*-octadecylphosphocholine (Fig. 1). This compound differs from 1-octadecanoyl-

a



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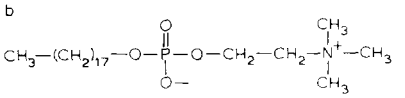

Fig. 1. A comparison of the structures of 1-octadecanoylphosphatidylcholine (a), and the analog *n*-octadecylphosphocholine (b).

phosphatidylcholine in that it has a simpler headgroup-to-alkyl chain linkage which cannot hydrogen bind in aggregated phases. Further, the analog does not have a hydrolyzable site; thus, it is well suited for studies of the binding of phospholipase A_2 to micelles and lamellae [5]. In the results shown below, X-ray diffraction is used to conclusively demonstrate that the micelle-lamellar transition occurs with this analog. It is also shown that transition kinetics of the micelle to lamellar transition are very different from the lamellar to micelle transition. The kinetics are similar to that of 1-octadecanoylphosphatidylcholine [7].

Materials and Methods

n-Octadecylphosphocholine [8] and pig pancreatic phospholipase A_2 [9] were prepared as described elsewhere. Differential scanning calorimetry [10], fluorescence depolarization [11], ^{31}P-NMR [11], phospholipase A_2 binding [5], light scattering measurements [8] and X-ray diffraction [12,13] were carried out by established procedures as described elsewhere. Unless stated otherwise, measurements were done in buffer solution containing 100 mM KCl, and 20 mM Tris at pH 8.0. The buffer for the phospholipase A_2 experiments additionally contained 10 mM $CaCl_2$.

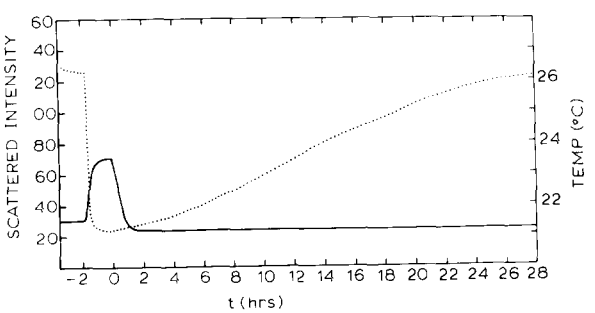

Fig. 2. Effect of temperature (solid line; right ordinate) on 90° light scattering (dotted line; left ordinate) of *n*-octadecylphosphocholine dispersions (6 mM). On heating (from −4 to 0 h) a sharp decrease in intensity is observed at 24°C. On cooling an increase in turbidity is observed, which reaches its maximum value in about 30 h at 20°C. These measurements were done in 100 mM NaCl, 10 mM $CaCl_2$, 20 mM Pipes at pH 6.0. Based on laserbeat spectroscopy, the particle size has been determined to have a hydrodynamic radius > 300 nm at 21°C and about 5 nm at 27°C.

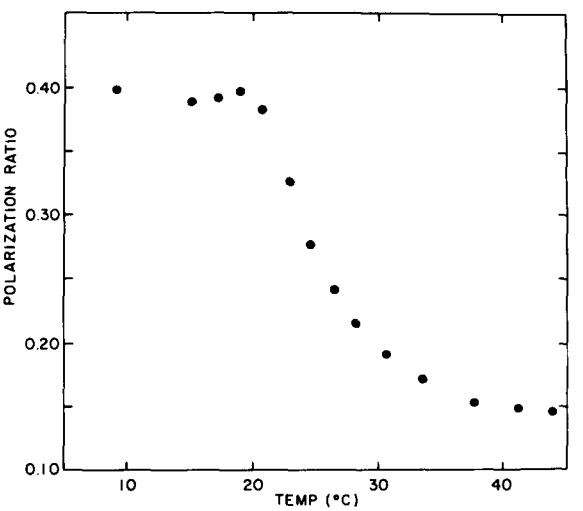

Fig. 3. The polarization ratio for diphenylhexatriene (1 μM) in the dispersions of *n*-octadecylphosphocholine (70 μM).

Results

Dry *n*-octadecylphosphocholine can be readily dispersed in aqueous solutions above 25°C. The solutions have been shown to be micellar and the critical micelle concentration is 1 μM [8]. The solutions remain optically clear for months if stored above 25°C; however, they become turbid if stored for a few hours below room temperature. Static light scattering measurements showed that, upon

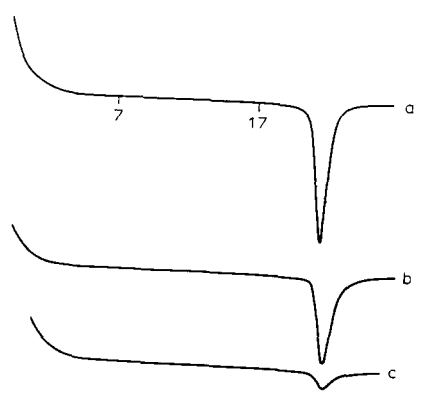

Fig. 4. Differential scanning calorimetric heating profiles for *n*-octadecylphosphocholine (2–3 mg/30 μl) dispersions in water (a); with phospholipase A_2 in 1:100 mole ratio after incubation at 4°C for 8 weeks (b); and rerun after incubation at 4°C for 10 min (c). The scan rate was 1 deg. C/min. The temperatures at 7 and 17°C are indicated.

TABLE I

PHASE TRANSITION CHARACTERISTICS OF THE AQUEOUS DISPERSIONS OF *n*-OCTADECYLPHOSPHOCHOLINE

Conditions	T_m (°C)	ΔH (kcal/mol)
Solid	199.4	19.2
+ 33% water	24 *	
+ 66% water	23.7	
+ 80% water	21	8.7
+ > 90% water or buffer (pH 8.0)	20.8	8.1
+ $CaCl_2$/buffer (pH 8.0)	22.7	6.4
+ $CaCl_2$/phospholipase A_2	22.7	–
100 mM in water (cooling scan)	below −5	6.7
100 mM, after 15 min at 4°C	20.8	1.9
100 mM, after 30 min at 4°C	20.8	3.4
100 mM, after 2 h at 4°C	20.8	7.4
100 mM, after 30 h at 4°C	20.8	8.3
100 mM, after > 20 days at 4°C	23.7	>11

* The half-height width in this case is about 4 deg. C compared to 1.2 deg. C for all other aqueous dispersions.

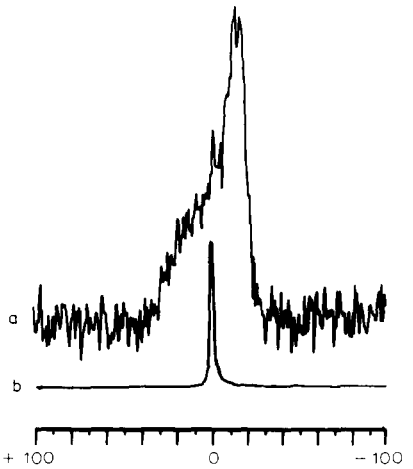

Fig. 5. ^{31}P-NMR spectrum (101.27 MHz) of aqueous dispersions of *n*-octadecylphosphocholine at 15°C (a) and 25°C (b). The abscissa is the frequency shift (ppm) relative to freely-tumbling phosphate.

heating the turbid dispersions, there was a rapid decrease in the scattered intensity at about 24°C (Fig. 2), indicative of a phase transition from a state of large scale lipid aggregates to dispersed micelles. The kinetics of the transition were very dependent on whether one heated or cooled through the transition temperature. The heating transition was observed to occur within the instrumental resolution time (seconds) at all concentrations. By contrast, the cooling transition was very slow, with a rate that increased with the concentration of the lipid. For example as shown in Fig. 2, at 20°C and at a concentration of 6 mM, the scattered light intensity continued to rise for more than 24 h. Even at 1 M concentration, the cooling transition took hours to go to completion.

The phase change in the dispersions of *n*-octadecylphosphocholine was accompanied by a large change in the fluorescence polarization of diphenylhexatriene (Fig. 3). The steady state fluorescence anisotropy in the aggregated phase is 0.4, compared to the value of 0.35 observed for dipalmitoylphosphatidylcholine bilayers in the gel phase. The fluorescence anisotropy of diphenylhexatriene in the aggregated phase of *n*-octadecylphosphocholine was the theoretically maximum value for a probe in a glassy solid [14], which strongly suggests that in this phase the acyl chains are packed tightly in the hydrophobic region where the probe resides. In the micellar phase, a lower polarization ratio of 0.120 was observed, which was consistent with the values observed for other micellar systems; as expected it was lower than the values observed for bilayers in the liquid-crystalline phase [14]. These results suggest that the anisotropy of the hydrophobic environment sampled by diphenylhexatriene in the aggregated *n*-octadecylphosphocholine phase is greater than it is for bilayers of diacylphosphatidylcholines.

Differential scanning calorimetry (DSC) also revealed the *n*-octadecylphosphocholine phase transition (Fig. 4a). The phase transition characteristics depend upon the thermal history of the sample. For example the transition characteristics which resulted upon heating after incubating 100 mM dispersions at 4°C for 0.25, 0.5, 2, and 30 h are shown in Table I. A thermotropic transition is seen to have occured at 20.8°C with an endothermic enthalpy which grew with the length of time the specimen was incubated at 4°C. After 24 h the enthalpy had reached its maximum value of 8.3 ± 0.3 kcal/mol, a value which is comparable to that observed for 1-octadecanoylphosphatidylcholine [7]. The incubation time required to achieve this maximum value decreased as the incubation temperature was lowered. For example only 20 min of incubation at −20°C were required to achieve the

maximum enthalpy. The time required to achieve half the maximum value was roughtly 5 min at −20°C, 10 min at −5°C, 30 min at 4°C and 4 h at 18°C (data not shown). Upon extended incubation (many days) at −10°C, a second transition was seen to arise in the DSC heating scans with T_m = 23.7°C and an enthalpy of 12 kcal/mol. This higher temperature transition appeared at the expense of the one at 20.8°C. Thus, the lower temperature transition may represent a metastable state, analogous to the gel phase of diacylphosphatidylcholine dispersions [15,16], and this state ultimately goes to a more stable L_c type of phase.

The low-temperature phase obtained after incubation at 4°C for 24 h yielded a ^{31}P-NMR spectrum (Fig. 5a) similar to that seen with lamellar phospholipids [17]. At 25°C, the sharp, isotropic ^{31}P-NMR peak expected for micellar dispersions was observed (Fig. 5b).

Pig pancreatic phospholipase A_2 is known to bind *n*-octadecylphosphocholine micelles [8] but not to pure bilayers of unhydrolyzable dual chain phospholipids [5]. Moreover, binding of phospholipase is accompanied by an increase in the protein trypotophan fluorescence [4]. Consequently, we investigated the binding of the phospholipase to *n*-octadecylphosphocholine above and below the 22.7°C phase transition temperature. If the lipid dispersion was cooled to below the phase transition temperature for many hours it becomes turbid due to the formation of lipid aggregates. If the phospholipase was now added and the fluorescence monitored as the sample was heated, a large change in the fluorescence intensity was found near the phase transition temperature (Fig. 6). Prolonged incubation of the phospholipase with the aggregated cold lipid affected the heating curve only marginally (Fig. 6). If, however, lipid was cooled through the phase transition and phospholipase was then added before the lipid aggregate was allowed to form (i.e., before the sample turned turbid), then the fluorescence intensity versus heating was relatively constant (Fig. 6). Thus, the phospholipase binding, as assayed by the fluorescence increase, was high for the micelle phase but low for the aggregated phase. It should be noted that whenever there was a large increase in the fluorescence, the sample was observed to go from turbid to clear. Also the phase transition temperature is shifted to 23°C in the presence of the 10 mM $CaCl_2$ required for the phospholipase A_2 binding studies. A similar shift is seen with dipalmitoylphosphatidylcholine dispersions with 100 mM $CaCl_2$ [10]. We note, incidentally that the effects on the transition of *n*-octadecylphosphocholine have not been fully explored for buffers containing divalent cations but without phospholipase A_2.

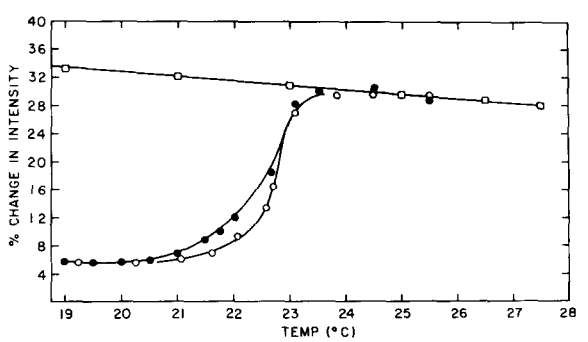

Fig. 6. The percent change in the tryptophan fluorescence intensity (excitation = 280 nm, emission = 328 nm) at various temperatures, as observed upon mixing pig pancreatic phospholipase (4 μM) with *n*-octadecylphosphocholine dispersions (250 μM) in the aggregated form and incubated for 2 min (○———○) and 2 h (●———●), and in the micellar form (□———□) incubated for 2 min. See text.

Phospholipase A_2 was not a completely benign probe in that it affected the phase behavior: in high protein to lipid molar ratios (1 : 100), the phospholipase slowed the micelle to aggregated phase transition. For example, it was necessary to incubate the phospholipid micelle mixture for weeks at 4°C before the sample became completely turbid. We postulate that the lipid is acting as a surfactant in solubilizing the protein and that the energy barrier toward the aggregated phase was raised by this interaction. Moreover, the protein slightly affected the behavior near the transition temperature if the protein and aggregated-phase lipid was heated (Fig. 4C).

Because lipid which was bound to the enzyme participated in the micelle to aggregated phase transition on a time scale of days, instead of hours as for the free lipid, a plot of the transition behavior vs. mole fraction of enzyme can be used to determine the number of lipid molecules bound per protein in the micelle. In this experiment, protein containing micelles were formed by mixing

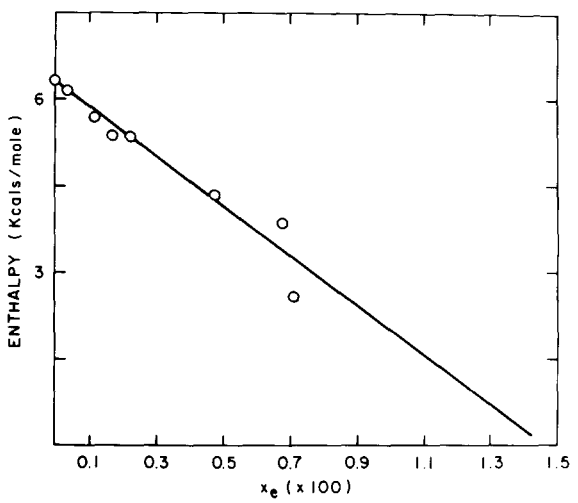

Fig. 7. Effect of varying mole fraction of phospholipase A_2 (X_e) in the aqueous codispersions of *n*-octadecylphosphocholine on the enthalpy of the lamellar to micelle transition.

the enzyme with lipid at room temperature. The specimen was then cooled to 4°C for several hours. During this time, most of the unbound lipid reverted to the aggregated phase. If the sample was now heated in a scanning calorimeter, it was found that the transition enthalpy decreased, relative to the same mass of lipid without enzyme, because only the unbound lipid was contributing to the transition. As shown in Fig. 7, the enthalpy of transition decreased linearly with increasing mole fraction of phospholipase A_2. Such a concentration dependence was not observed with the enzyme precursor prophophospholipase A_2, which does not bind to lipid-water interface in micelles or in bilayers [5]. The decrease in enthalpy was due to formation of an stoichiometric complex between phospholipase A_2 and the amphipath [4,8], and only the excess amphipath was available for the aggregated phase to micelle transition. Indeed, it has been demonstrated by independent techniques that over a large concentration range of *n*-octadecylphosphocholine and phospholipase only two distinct micellar species exist: those containing 200 monomers, and those containing two enzyme molecules with 140 monomers [8]. From the intercept of the line in Fig. 7 on the abscissa the stoichiometry of the complex is calculated at 70 : 1, which is quite consistent with the values obtained by hydrodynamic and spectroscopic methods [8].

Low angle X-ray diffraction was used to prove that the low-temperature phase of *n*-octadecylphosphocholine consisted of stacked lamellae and wide-angle diffraction was used to probe the lipid hydrocarbon chains. Fig. 8 shows the diffraction which resulted in both the low (Fig. 8a–j) and wide-angle (Fig. 8k–t) regimes when the temperature was rapidly vaired on a 33% lipid by weight dispersion. The specimen was held in a thermoelectrically servoed copper jacket such that a 10°C temperature step could be achieved in under a minute. The TV X-ray detector required only two minutes of X-ray exposure to accumulate the patterns. Thus, only several minutes elapsed between each of the successive low or wide-angle patterns of Fig. 8. At 30°C (Fig. 8a) the low-angle diffraction consisted of a stable, single relatively sharp ring at a spacing corresponding to 63 ± 2 Å. The absence of higher orders was consistent with a non-periodic specimen, as would be expected for a micelle solution. The wide-angle diffraction (Fig. 8k) consisted of a broad band with a peak at 4.4–4.6 Å, depending on how one chose to define the position of the peak. This band is commonly observed with lipids above the chain melt temperature and arises from the disordered hydrocarbon chains [18–20]. It is a reliable indicator that the lipid tails are melted. When the temperature was rapidly dropped step-wise to 0°C, the intensity of the low-angle (Fig. 8b) ring began to decrease and the wide-angle band (FIg. 8l) started to sharpen. Other experiments (not shown) indicated that these changes occured slowly whenever the specimen temperature was lowered to roughly 20°C. Further lowering of the temperature to −10 and −20°C (Fig. 8c–d) led to a progressive sharpening of sampled low-angle diffraction of equidistantly spaced rings. At −10°C and below, four orders (three are shown in Fig. 8f) were easily visible in the 2-dimensional detector display. Equidistantly spaced rings are a conclusive demonstration of the existence of stacked, periodic lamellae. Simultaneous with the rise of the low-angle rings, the broad wide-angle band decreased in intensity and was replaced by a relatively sharp band at 4.2 Å (Fig. 8m–s). (The two intense rightmost peaks in Fig. 8 n–q are from ice and are at 3.9 and 3.7 Å).

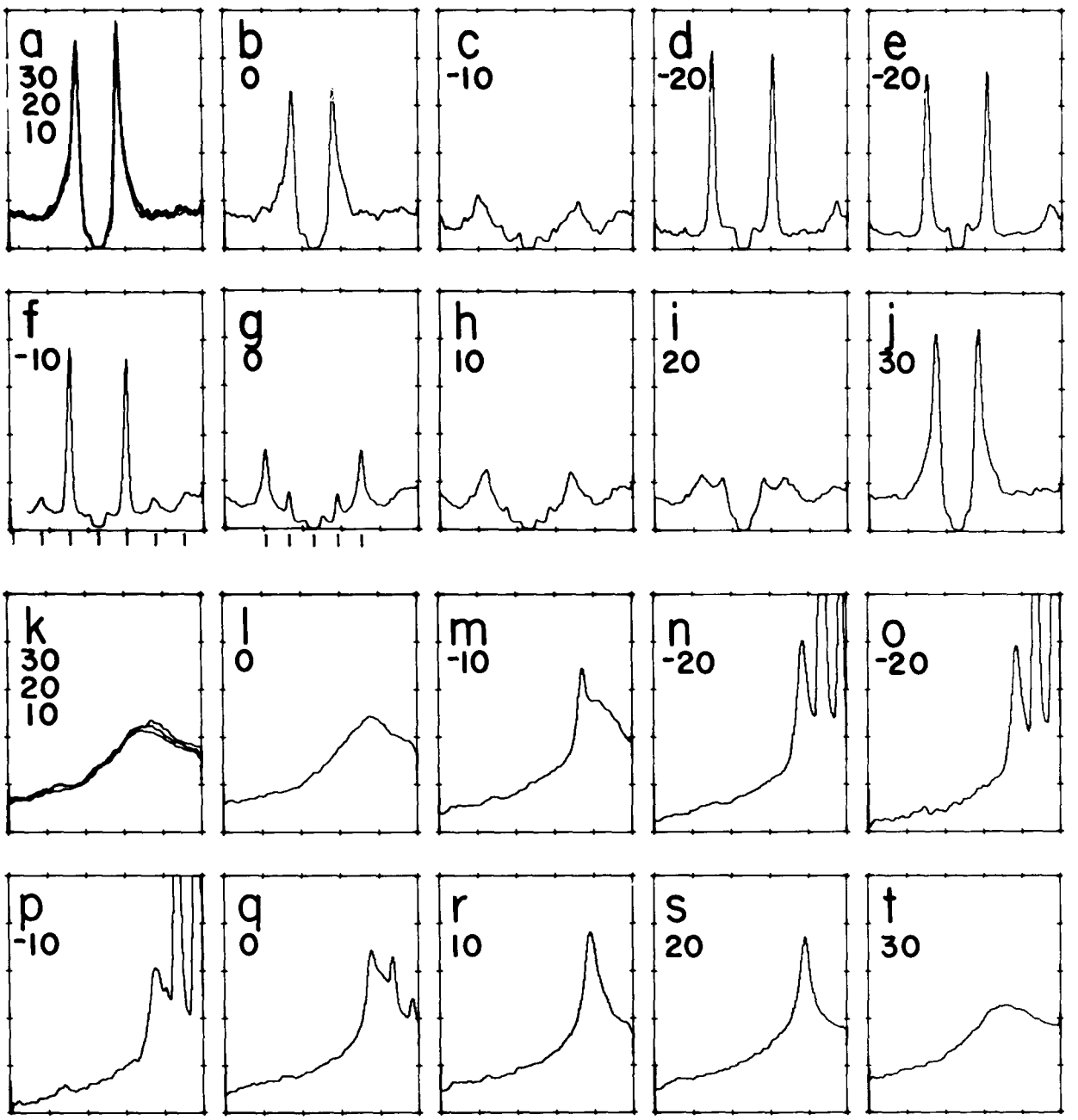

Fig. 8. The low (a–j) and wide-angle (k–t) X-ray diffraction intensity is graphed vs. the reciprocal space coordinate as the temperature is varied. The numbers in the upper left hand corners show the temperature of the specimen. In all cases, the ordinate is the diffracted intensity in arbitrary unit. For the low-angle patterns (a–j), the dip near the center is the beam stop shadow at 0° scattering angle and the abscissa tic marks (from left to right) are at -0.067, -0.038, -0.009, $+0.020$, $+0.050$, and $+0.079$ $Å^{-1}$. Tic marks representing the position of lamellar orders are seen below f and g. For the wide-angle patterns (k–t), the abscissa tic marks (from left to right) are at 0.095, 0.131, 0.167, 0.204, 0.240, and 0.276 $Å^{-1}$. The peak which is traceable in graphs m through s is the frozen lipid hydrocarbon peak at $(1/4.2)Å^{-1}$. The two rightmost peaks in n through q are from crystalline ice. This is from a 33% by weight dispersion of *n*-octadecylphosphocholine. The actual data taking sequence was a to d, then k to t, followed by e to j.

The 4.2 Å signal is a reliable signature of frozen ('gel') state lipids [18–20]. Thus, it appeared that the growth of the lamellar diffraction was associated with frozen lipid tails. Upon reheating to 20°C the lamellar diffraction (Fig. 8f–i) became increasingly ill-defined. Throughout this heating period the 4.2 Å signal remained sharp (Fig. 8p–s). Thus, heating the specimen had the effect of degrading the lamellar diffraction, perhaps by disordering the lattice, but without melting most of the lipid chains. Upon heating to 30°C the low and wide-angle diffraction (Figs. 8j and 8t) immediately reverted to the pervious form (Figs. 8a and 8k).

The kinetics of the mesomorphic changes in the specimen, as seen in Fig. 8, were consistent with the DSC data discussed earlier. The specimen was stable at 30°C. Below 20°C, it slowly evolved into a different mesomorphic state. As the specimen became colder, the pace of this evolution quickened. Indeed, the −20°C data did not change substantially from Fig. 8e and 8o even if the specimen was allowed to remain at −20°C for many hours. Upon heating to 30°C, the diffraction immediately reverted to the stable high temperature form. The kinetics of the mesomorphic transition are more clearly seen in Fig. 9. Figs. 9a and f show the low and wide-angle diffraction, respectively, at 30°C. The patterns of Figs. 9b and 9g were taken within 3 min of a sudden drop to 6°C. No substnatial change had yet taken place. The succeeding patterns (FIgs. 9c–e and 9h–j) were then taken at 0.5-h intervals. The frozen chain lamellar patterns are seen to rise slowly. The reverse of this experiment was to suddenly increase the temperature from 6 to 30°C. The resultant patterns (data not shown) are indistinguishable from Figs. 9a and 9f within the instrumentally limited time scale of 60 s. Thus, freezing of the lipid chains is a slow event but melting and reversion to the high temperature form is fast.

The lamellar repeat distance is the sum of the lipid layer thickness and the thickness of the interlamellar water. The method usually used to determine these thicknesses via X-ray diffraction requires a precise knowledge of the amount of inter-

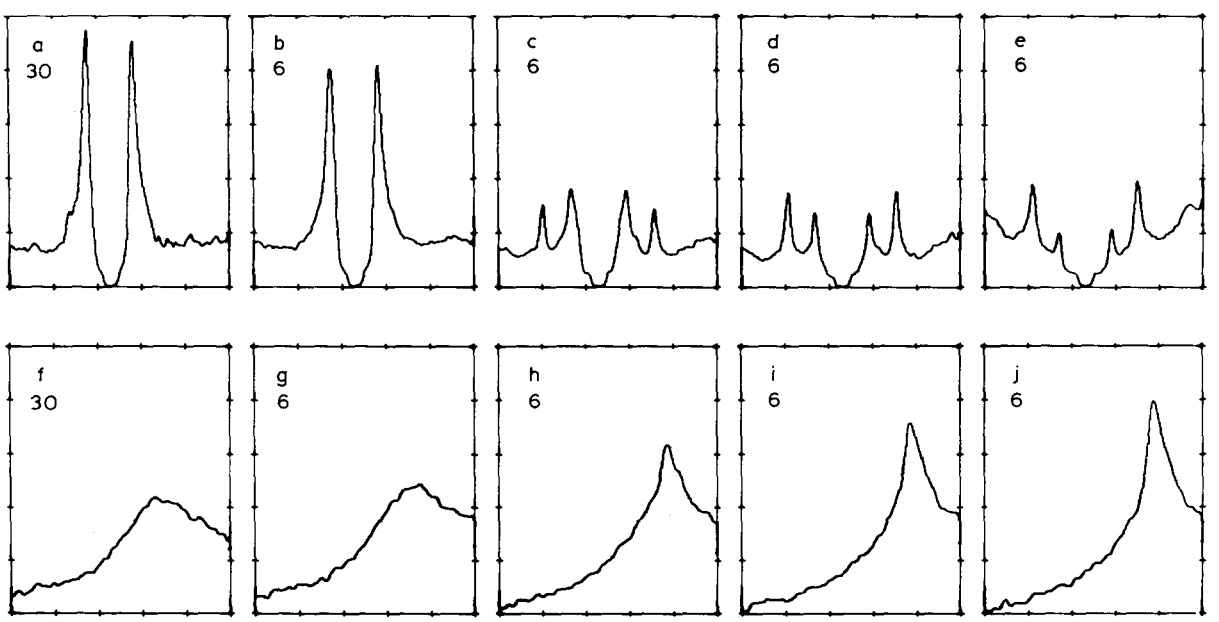

Fig. 9. The low (a–e) and wide-angle (f–j) X-ray diffraction intensity is shown as a function of time after a rapid decrease in the specimen temperature from 30°C (a and f) to 6°C (b and g). Patterns b and g were taken within 3 min of a and f, respectively. Thereafter, patterns c through e and h through j were taken at 0.5-h intervals. Note the gradual development of low-angle sampled diffraction (c–e) and the concommitant rise of the $1/4.2)Å^{-1}$ lipid hydrocarbon peak (h–j). See the caption for Fig. 8 for an explanation of the abscissas and ordinates. The data taking sequence was a through e, followed by f through j.

lamellar water [19], a quantity which is not readily determined if the specimens, such as those discussed here, contain excess water. However, the overall repeat distance is already so small that it places strong constraints on how the lipid chains can be packed. Tardieu et al. [18] have measured the lipid thickness of the $L_{\beta'}$ phase of disteroylphosphatidylcholine (DSPC). From the values of the lipid thicknesses (d_1) and tilt angles (θ) of their Table I, one obtains a length along the chains of

$$d_c = d_1/\cos\theta = 61.5 \text{ Å}$$

At -20 to -10°C we commonly observe a lamellar repeat for *n*-octadecylphosphocholine of about 40 Å, with a several angstrom variation, depending on the concentration and temperature to which the sample has been cooled. Assume, for the moment, that the length of DSPC is a conservative approximation to the length of *n*-octadecylphosphocholine. This is a good assumption because both lipids have 18-carbon chains, are being considered below the chain melt temperature and because it is now generally believed that the DSPC headgroup lies mostly parallel to the plane of the membrane [22]. If we assume, for the sake of argument, that the thickness of the lipid layer with a 40 Å repeat is 40 Å, i.e., that the lipid is locally anhydrous, then we arrive at a lower limit of the tilt angle of

$$\theta = \cos^{-1}(40/61.5) = 49°$$

In fact, the lipid lamellae are probably separated by substantial water thicknesses because extensive vacuum pumping is required to dry out *n*-octadecylphosphocholine. Additional water thickness would make the tilt angle even greater than 49°C if it is to maintain a 40 Å repeat. The X-ray investigation of concentrated *n*-octadecylphosphocholine dispersions is outside the scope of this paper and will be reported in another publication. However, one result of that study was that the total lamellar repeat at -20°C was still about 40 Å up to at least 80% lipid concentration. At high concentrations there was no bulk water and the lipid thickness could be determined. It was about

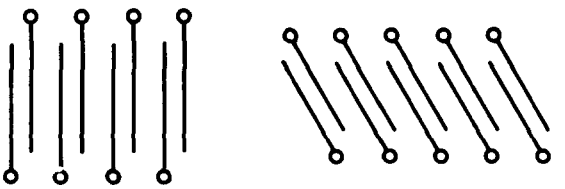

Fig. 10. Two highly schematic representations of *n*-octadecylphosphocholine in interdigitated, lamellar phases.

31 Å. This would imply a

$$\theta = \cos^{-1}(31/61.5) = 60°$$

Tilts of 49° or greater imply that the stiff lipid chains are more parallel to the membrane plane than perpendicular to it. This would be unprecedented. A greater objection to such large tilts is that Tardieu et al. [18] have calculated the effect of tilt on the 4.2 Å diffraction band. The band broadens and becomes asymmetric as the tilt increases, much more so than is seen in the wide-angle diffraction of Fig. 8n. These objections may be circumvented if the chains, are, in fact, not so steeply tilted but highly interdigitated (Fig. 10).

The most plausible interpretation of the X-ray data is that at low temperatures the lipid molecules are predominantly in a lamellar form of interdigitated frozen-chain bilayers. Note that the data does not rule out a minor component of melted or tilted chains, especially at temperatures just below the temperature at which the micelles form.

Discussion

The observations summarized, above, demonstrate that the dispersions of *n*-octadecylphosphocholine exhibit a thermotropic micelle-lamellar transition similar to that observed for dispersion of 1-hexadecanoylphosphatidylcholine [6] and for 1-octadecanoylphosphatidylcholine [7]. Based on a large anisotropy of polarization, and in analogy with the properties of the dispersions of 1-octadecanoylphosphatidylcholine, we believe that the lamellar phase we see are bilayers with at least partially interdigitated chains (Fig. 10). The fundamental objection to a micelle-bilayer transition is obviated if the chains interdigitate. Molecules pack

into micelles because the effective projected headgroup area is larger than that of the tails. Crudely speaking, the molecules are 'cone shaped' with the tails at the small end. The micelle is favored by this shape because it allows optimum packing while effectively shielding the hydrophobic chains from water. This shape cannot be effectively packed into a normal (non-interdigitated) bilayer. This is also why diacylphosphatidylcholines form bilayers and not micelles: the projected headgroup and tail (two chains) areas are roughtly equal, i.e., the molecule is 'cylinder shaped' [22]. However, if the chains of lyso-choline lipids interdigitate in a bilayer, then the effective projected areas are two chains per choline headgroup. Interdigitation is additionally supported by our X-ray observations that the lipid thickness in the lamellar phase of *n*-octadecylphosphocholine in excess water is certainly less than about 40 Å and probably closer to 30 Å. It is difficult to envision how two 18-carbon gel chains can be accomodated in this length without interdigitation or extraordinary chain tilt. In this regard, Hauser et al. [23] have solved the crystal structure of the related compound 3-dodecanoylpropandiol-1-phosphocholine/H_2O and find it to be a tilted and interdigitated-chain lamellar structure. Finally, we note the *n*-octadecylphosphocholine system has parallels to the structures of the micelle-gel transition of soaps as observed by X-ray diffraction [24].

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