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Characterization of a Third Phase Transition in Multilamellar Dipalmitoyllecithin Liposomes†

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ABSTRACT: The thermotropism of dipalmitoyllecithin in fully hydrated multilamellar dispersions has been reexamined by differential scanning calorimetry, X-ray diffraction, and ^{31}P nuclear magnetic resonance. Apart from the well-known pretransition and main transition, there exists a third transition at about 11 °C with a transition enthalpy of approximately 3.7 kcal/mol. Both adjoining phases are lamellar, but they differ in the lateral acyl chain packing of the lecithin molecules and in the dynamics of the polar head groups. The kinetics of this third phase transition are extremely slow in comparison with those of the other two transitions.

Different lamellar phases are spontaneously formed by pure synthetic phospholipids in aqueous dispersions. Their structural and dynamic properties have been investigated in detail (Tardieu et al., 1973; Janiak et al., 1976; Inoko & Mitsui, 1978; Seelig, 1977, 1978; Davis, 1979; Cameron et al., 1980; Marsh, 1980; Kawato et al., 1977), since phospholipid bilayers are believed to be a basic structure of many biomembranes. Within the context of liquid-crystal physics, purely physicochemical reasons have also promoted the study of lipid polymorphism.

For dipalmitoyllecithin (DPL)[1] and other structurally related lipids in excess water, two thermotropic phase transitions are well characterized by their thermodynamic (Ladbrooke & Chapman, 1969; Jacobson & Papahadjopoulos, 1975; Mabrey & Sturtevant, 1976; Albon & Sturtevant, 1978) and kinetic parameters (Tsong & Kanehisa, 1977; Lentz et al., 1978; Teissie, 1979). These are the so-called "main transition" between a "liquid-crystalline" phase and a "gel" phase at T_m = 41 °C (DPL) and the "pretransition" between two gel phases at T_p = 35 °C (DPL). On a molecular level, the main transition is better understood (Marcelja, 1974; Jähnig, 1979) than the pretransition, the nature of which is still under discussion.

This paper describes another thermotropic phase transition between two gel phases at T_s = 11 °C (DPL). It will be referred to as the "subtransition" in line with the nomenclature of a recent DSC study (Chen et al., 1980).

Materials and Methods

Commercially available DPL (obtained from Fluka, Buchs, Switzerland) was chromatographed on CM-cellulose (Comfurius & Zwaal, 1977) and recrystallized twice from acetone. Its purity was checked by TLC and ^1H and ^{31}P NMR. The homogeneity of the fatty acids was assured by cleaving them and by analyzing their methyl ester derivatives with gas chromatography (Eibl & Lands, 1970).

Sample Preparation. Multilamellar liposomes were prepared by incubating the dry lipid in doubly distilled water (ca. 10 mg/mL) at 45 °C for 1–2 h. During the incubation time, the dispersion was vortexed several times for about 1 min at the elevated temperature. The liposomes were then centrifuged at low *g* forces (1000–15000*g*, 10 min, 4 °C) to give lipid concentrations in the pellet between 30% and 60% (w/w).

Calorimetry. A differential scanning calorimeter (Perkin-Elmer DSC 2 with Intracooler I) was used for the calorimetric measurements. The sample pans (stainless steel, hermetically sealed) usually contained 8–12 mg of the liposomal pellet; an appropriate amount of water was taken as reference. Each sample was scanned several times at a heating/cooling rate of 0.313 °C/min and at a sensitivity of 1 mcal/s (full scale).

The lipid content of the samples was determined gravimetrically after completion of the measurements. Also, comparison of the initial and final total weights of the sample pans ensured that no water loss had occurred.

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[1] Abbreviations used: DPL, dipalmitoyllecithin; DSC, differential scanning calorimetry; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; FID, free induction decay.

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Table I: Calorimetric Data of the Three Transitions in Multilamellar DPL Liposomes

	subtransition			pretransition			main transition			
	T_s^o (°C)	T_s (°C)	ΔH (kcal/mol)	T_p^o (°C)	T_p (°C)	ΔH (kcal/mol)	T_m^o (°C)	T_m (°C)	ΔH (kcal/mol)	ref
annealed liposomes	18.0	19.8	3.70 ± 0.4	34.3	35.0	1.35 ± 0.1	40.7	41.3	7.9 ± 0.4	this work
		18.4	3.23		35.1	1.09		41.1	6.9	Chen et al. (1980)
		17.5 ± 0.3	2.65 ± 0.18		35.0 ± 0.1	0.93 ± 0.02		41.1 ± 0.03	6.4 ± 0.5	Chen et al. (1980)
unannealed liposomes				33.9	34.9	1.20 ± 0.1	40.7	41.4	8.1 ± 0.4	this work

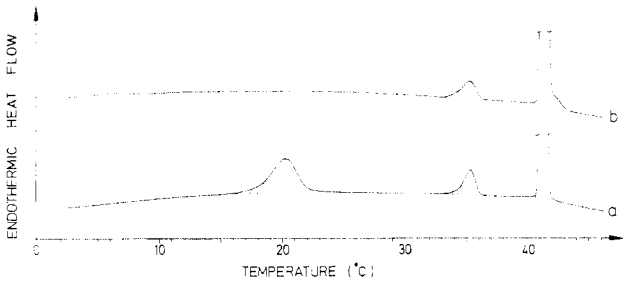

FIGURE 1: DSC endotherms of multilamellar DPL liposomes (a) first and (b) second heating scans after 5 days incubation at 4 °C (main transition not fully shown).

X-ray Diffraction. The X-ray diffraction experiments were performed by using a Guinier camera whose resolution and technical details are reported elsewhere (Harlos, 1978). The samples were contained in sealed glass capillaries (1-mm o.d., 0.01-mm wall thickness; Fa. Hilgenberg, 3509 Malsfeld, Federal Republic of Germany). X-ray diffraction patterns were recorded between 5 and 45 °C with a thermostatable specimen holder (temperature accuracy = ±0.5 °C). At any temperature, an equilibration time of at least 2 h was allowed before a cumulative exposure time of 10 h. Densitometry of the photographed reflections was done with a Joyce-Loebl 3 CS microdensitometer.

^{31}P NMR. ^{31}P NMR spectra were recorded on a Bruker WH 270 FT spectrometer operating in the quadrature detection mode. The intervals between the excitation pulses of 5 μs were set to 2 s, and the proton broad-band decoupling power (about 10 W) was switched on only for the first 1–3 ms of the acquisition time, during which the entire ^{31}P FID was collected. With this duty cycle of ~1 ppt, no heating of the sample could be detected, as judged from the ^{2}H NMR spectrum of external CD_3OD contained in a coaxial capillary (Van Geet, 1968). The accuracy of the temperatures (regulated by a Bruker B-VT 1000 gas thermostat) is estimated to be ±0.5 °C. The time course of the ^{31}P NMR measurements was similar to that of the X-ray diffraction experiments: After 4-h equilibration time at every new temperature, data were averaged for 6 h.

Results

Calorimetry. The samples were allowed to anneal for at least 5 days at 4 °C. After having loaded them at 0 °C into the precooled calorimeter, a first heating scan gave the result shown in Figure 1a. A first endothermic transition, the subtransition, occurs at T_s^o = 18 °C (onset temperature), followed by the well-known pretransition at 34 °C and the main transition at 41 °C. Subsequent cooling/heating scans showed the pre- and the main transitions with almost unchanged parameters[2] (Figure 1b). The subtransition only reappeared reproducibly (at 18 °C with ΔH = 3.7 kcal/mol) after another minimum annealing period of 5 days at 4 °C. Table I gives the calorimetric data of all three transitions.

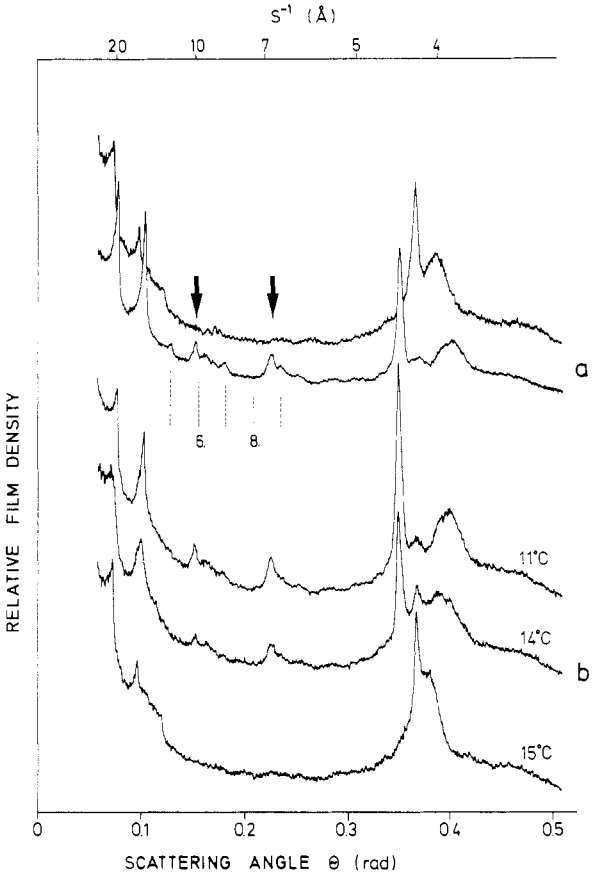

FIGURE 2: (a) X-ray diffraction pattern of DPL liposomes, taken at 5 °C, before (upper trace) and after (lower trace) annealing at 4 °C. (b) Temperature dependence of the diffraction pattern of the annealed sample, through the subtransition temperature region.

X-Ray Diffraction. The X-ray diffraction patterns of DPL liposomes before and after annealing at 4 °C are shown in Figure 2a. The pattern obtained before annealing agrees with earlier published ones (Tardieu et al., 1973). After being annealed, a new pattern appears with the following characteristics: (i) The reflections in the small angle region, $\theta < 0.13$ rad, are still typical of a lamellar periodicity; they only change their angular positions (first and second order not shown). (ii) In the range 0.13 rad $< \theta <$ 0.3 rad, there are several new reflections. The two most prominent ones are indicated by arrows; the positions expected for the fifth to ninth orders of the lamellar periodicity are marked by dashed lines. (iii) The superimposed narrow and broad reflections in the wide angle region ($\theta > 0.3$ rad) become well separated.

In the first heating cycle, the new pattern of the annealed liposomes remains unchanged up to 11 °C. Then its intensity decreases continuously and vanishes completely between 14 and 15 °C (Figure 2b). Simultaneously, there is a steady increase of the pattern already known from unannealed liposomes which has fully built up at 15 °C. The overlap of both

[2] In second heating scans like that in Figure 1b, the main transition endotherms occasionally showed a high-temperature shoulder, which was observed neither in later scans nor in freshly prepared dispersions.

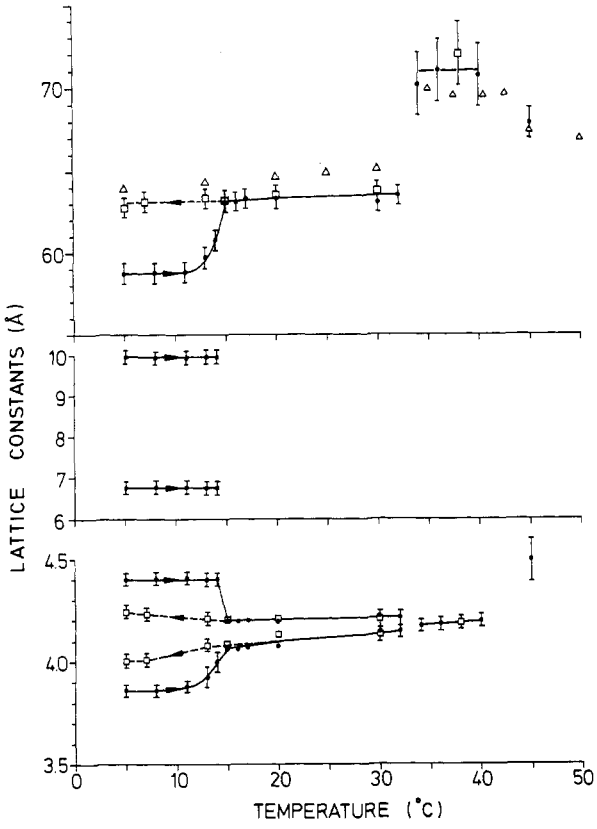

FIGURE 3: Lattice constants of multilamellar DPL liposomes after 5 days annealing at 4 °C (●) and without annealing (□). For comparison, the data from Inoko & Mitsui (1978) are included (△).

patterns is clearly visible at 14 °C.

The lattice constants of unannealed and of annealed liposomes are plotted against temperature in Figure 3. When the unannealed samples were cycled through the whole temperature range, a close agreement with previously reported results was obtained (Tardieu et al., 1973; Inoko & Mitsui, 1978; dashed lines in Figure 3).

As a result of annealing at 4 °C, the lamellar periodicity ("long spacing") changes from 63 to 58.8 Å; the constants usually related to interchain distances ("short spacings") change from 4.0 to 3.85 Å and from 4.23 to 4.4 Å, respectively. Of the newly appearing reflections, only the two most significant ones (labeled by arrows in Figure 2) have been evaluated, giving constants of 10.0 and 6.75 Å. The densitometric peaks of the other reflections are too weak for a precise determination of their constants. Furthermore, two of them could simply be the seventh and ninth orders of the lamellar repeat (see Figure 2).

All the new lattice parameters associated with the annealed samples stay constant up to 11 °C (solid lines in Figure 3). Between 11 and 14 °C, their values depend on how well the overlapping reflections are resolved: Three lattice constants (10, 6.75, and 4.4 Å) are unchanged up to 14 °C; the continuous increase of the two other constants (58.8 → 63 Å and 3.85 → 4.05 Å) mainly arises from a shift in the reflection maximum due to severe overlap.

^{31}P NMR. Figure 4a shows the proton-decoupled ^{31}P NMR powder spectra of annealed and unannealed liposomes at 5 °C (upper and lower traces, respectively). The spectra in Figure 4b demonstrate that ^{31}P NMR detects the subtransition between 11 and 12 °C. In order to clarify the significance of the ^{31}P spectra from below and above the subtransition, Figure 4c shows the non-axially symmetric powder pattern of dry DPL (upper trace) and the axially symmetric pattern of the lipid

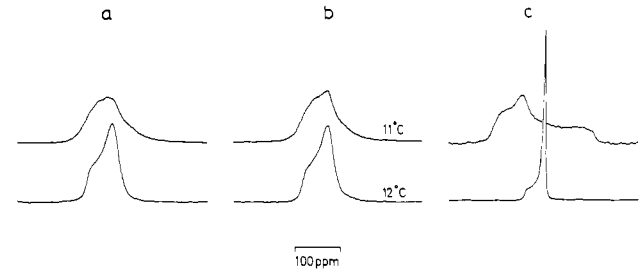

FIGURE 4: ^{31}P NMR powder spectra (a) of annealed (upper trace) and unannealed (lower trace) DPL liposomes at 5 °C, (b) of annealed liposomes on heating through the subtransition temperature region, and (c) of dry DPL powder (upper trace) and of liposomes at 45 °C (lower trace).

in the L_α phase at 45 °C (lower trace). On this basis, the spectra from below the subtransition are classified as "slow-motion" spectra, indicating an incomplete motional averaging of the non-axially symmetric ^{31}P shift tensor (Seelig, 1978). In contrast, the spectra from above the subtransition and those from unrelaxed liposomes approach an axially symmetric pattern.

Discussion

The kinetics of the subtransition are much slower than those of the other two transitions in both directions, heating and cooling. This is concluded from the following observations:

(i) Calorimetry, performed at a scan rate of 0.313 °C/min, detects the onset of the subtransition at T_s^o = 18 °C. On the other hand, X-ray diffraction and ^{31}P NMR, for which about 10 h are spent at every temperature (thus leading to an "average scan rate" on the order of 0.005 °C/min), reveal the onset at 11–12 °C. The same trend is observed by Chen et al. (1980), who found that the calorimetric transition temperature drops from 18 to 15 °C on reducing the scan rate from 0.5 to 0.1 °C/min.

(ii) Neither calorimetry, X-ray diffraction, nor ^{31}P NMR detects the subtransition in unannealed liposomes, under the experimental conditions given here.

(iii) The subtransition occurs only in the first heating cycle after an annealing period below the transition temperature, which is extremely long compared to the longest relaxation times associated with the other two transitions (Tsong & Kanehisa, 1977; Lentz et al., 1978; Teissie, 1979; Clegg et al., 1975).

Therefore, the process taking place during the incubation at 4 °C is interpreted as a very slow relaxation into a new phase below the subtransition.

Following the interpretation of previous X-ray diffraction studies (Tardieu et al., 1973; Janiak et al., 1976; Rand et al., 1975), the powder patterns of unannealed liposomes below the pretransition (e.g., at 5 °C in Figure 2a) and those of both annealed and unannealed liposomes, obtained between the sub- and pretransition (e.g., at 15 °C in Figure 2b), correspond to the $L_{\beta'}$ phase [nomenclature following that of Tardieu et al. (1973)]. The phase structure below the subtransition differs from the $L_{\beta'}$ lattice in that it shows additional reflections in the range 0.13 rad $< \theta <$ 0.3 rad, corresponding to 10–5-Å spacings. Such spacings are most easily attributed to a lattice with at least one whole lipid molecule as motif, in contrast to the $L_{\beta'}$ structure where the motif consists of just one single acyl chain. However, on the basis of the limited information available from the powder patterns, the symmetry of the new lattice cannot be determined unambiguously.

The results of the ^{31}P NMR experiments are interpreted within a model where the PO_4 group of the DPL molecule rotates about a unique "effective" diffusion axis (Campbell

et al., 1979). Within the frame of this model, it is concluded that the PO_4 moieties (and therefore the whole polar head groups as well) reorient at a significantly slower rate in the phase below the subtransition as compared with the $L_{\beta'}$ phase. In addition, the principal axes of the ^{31}P shielding tensor may change their orientation with respect to the effective diffusion axis during the subtransition. This could contribute to the observed shape differences of the ^{31}P powder spectra as well.

In summary, the processes taking place during the subtransition may be visualized as follows: In parallel to changes in the lateral acyl chain packing, the reorientation of the head groups becomes faster. This is possibly accompanied by a conformational change in the head-group region. Finally, some preliminary ^2H NMR results (Füldner, 1980) hint toward a change in the hydration of the head groups at the subtransition.

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Messenger Ribonucleic Acid Transcripts of Pea Chloroplast Deoxyribonucleic Acid†

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ABSTRACT: The total ribonucleic acid (RNA) from pea chloroplasts has been found to hybridize with 50% of the base sequences of pea chloroplast deoxyribonucleic acid (ctDNA). The specificity of hybridization has been analyzed by competition experiments and thermal stability studies of ctDNA–RNA hybrids. The ctDNA-specific RNA does not hybridize with the nuclear DNA. There is about 2–3% reduction in the total hybridization of ctDNA with RNA from the dark grown leaves. About 0.15–0.2% of the RNA from chloroplasts has been found to contain poly(A) tracts. About 20–25% of the ctDNA has been found to hybridize with poly(A+) RNA. Poly(A−) RNA, however, has been found to hybridize with 50% of the ctDNA. The molecular sizes of the mRNA transcripts have been found to range from 0.3×10^6 to 3×10^6.

Chloroplast deoxyribonucleic acids (ctDNAs)[1] from higher plants have been characterized for their structural organization and conformation (Tewari et al., 1977). The molecular sizes of ctDNA from higher plants have been found to range from 85×10^6 for corn ctDNA to 95×10^6 for lettuce ctDNA (Kolodner & Tewari, 1975). The genetic information contained in ctDNA has been studied by carrying out molecular hybridizations between ctDNA and purified stable species of ribonucleic acids (RNAs). Such experiments between pea

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[1] Abbreviations used: ctDNA, chloroplast deoxyribonucleic acid; RNA, ribonucleic acid; rRNA, ribosomal RNA; mRNA, messenger RNA; tRNA, transfer RNA; Tris, tris(hydroxymethyl)aminomethane; poly(A), poly(adenylic acid); poly(U), poly(uridylic acid); $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; DNase, deoxyribonuclease; RNase, ribonuclease; BSA, bovine serum albumin.

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