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THE EXISTENCE OF A HIGHLY ORDERED PHASE IN FULLY HYDRATED DILAUROYLPHOSPHATIDYLETHANOLAMINE

HUA CHANG and RICHARD M. EPAND

Department of Biochemistry, McMaster University, Health Sciences Centre, Hamilton, Ontario L8N 3Z5 (Canada)

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Dilauroylphosphatidylethanolamine dispersion forms a crystalline phase at physiological pH and temperature and in the presence of excess water. This phenomenon was observed and studied by differential scanning calorimetry, scanning densitometry and X-ray diffraction. The crystalline phase is stable at pH 5.5–9.5 and below 40°C. The crystalline phase formed at pH 5.5 and pH 9.5 index according to orthorhombic cells with $a = 9.41$, $b = 8.15$, $c = 46.0$ and $a = 9.33$, $b = 8.05$, $c = 45.8$ (Å), respectively. Around 43°C, the crystalline phase is transformed into a multilayer liquid crystal phase. Cooling from 44°C results in the disappearance of the original transition at 43°C and the appearance of a second transition at around 30°C. Below 30°C the lipid forms a gel phase. This gel phase is metastable at pH 5.5 and a crystalline phase may be recovered from it by dispersing or aging methods. Suspensions of dilauroylphosphatidylethanolamine show similar phase transition behaviour at pH 5.5 and pH 9.5, although the transitions are somewhat broader at the higher pH. The thermotropic phase behaviour of dilauroylphosphatidylethanolamine dispersions may be governed by changes in hydration.

Introduction

It is well known that phospholipid dispersions and biological membrane exist in ordered structures, with one-dimensional or two-dimensional order [1]. Phospholipid dispersions have long been known to form crystalline phases in the presence of limited amounts of water [2,3]. Recently, it has been shown that several phospholipids can form more than one type of gel phase. Phosphatidylethanolamine at high pH and in the presence of calcium has been shown to exhibit this property [4]. Other phospholipids that form stable gel states that may involve the formation of crystalline phases are phosphatidycholines [5–7], sphingomyelin [8] and cerebrosides [9,10]. The demonstration of such a crystalline phase under physiological conditions would suggest that they may occur in biological systems.

Recently, Wilkinson and Nagle [11] have demonstrated unusual thermotropic behaviour of aqueous suspensions of dilauroylphosphatidylethanolamine (DLPE). They have obtained evidence from calorimetry and dilatometry that there is more than one phase transition observable with DLPE and that the transition behaviour depends on the thermal history of the sample. We have further investigated this phenomenon and with the use of X-ray diffraction have demonstrated the presence of a crystalline phase of DLPE in the presence of excess water. Our results also demonstrate the slow rate at which this crystalline phase

Abbreviations: DLPE, dilauroylphosphatidylethanolamine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DLPC, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine; DCPC, 1,2-dicaproyl-*sn*-glycero-3-phosphocholine; DSC, differential scanning calorimetry; Pipes, 1,4-piperazinediethanesulphonic acid.

is formed from aqueous dispersions of DLPE in other phases. The ability of a phospholipid to form a crystalline phase at physiological temperature and pH 5.5–9.5 and in the presence of excess water is unusual and is the first demonstration of such a phenomenon for phosphatidylethanolamine.

Materials and Methods

DLPE was obtained from Fluka Ag, Buchs SG, and used without further purification. The purity of the preparation was verified by thin-layer chromatography on silica gel H run with chloroform/methanol/water, 65 : 25 : 4. The lipid showed a single spot when developed with iodine vapour.

The buffers used in this work were 0.1 M acetic acid adjusted to pH 5.5 with ammonium hydroxide or 0.2 M potassium phosphate, pH 9.5. Similar results were also obtained using a Pipes buffer at pH 7.4 but the results are not described here for brevity.

Preparation of samples. The DLPE was suspended in buffer at about 35°C by vortexing and by breaking up larger particles by passing the suspensions through a 24-gauge hypodermic needle. Lipid concentrations of 10 mg/ml were generally used and pellets from these suspensions were used for the X-ray diffraction. For differential scanning calorimetry, suspensions of 2 mg/ml were used. In the case of the calorimetry experiments, lipid suspensions which had not previously been heated above 40°C were used within a few hours of preparation, while in the case of the X-ray diffraction experiments, the suspensions were stored for 48 h at 4°C before centrifugation to ensure maximal hydration.

Scanning densimetry. The density difference between the lipid dispersion and the buffer was measured with the use of two DMA 602 external cells using a DMA 60 measuring unit in the phase lock loop mode (A. Paar, K.G., Graz, Austria). The temperature of the external cells was controlled by circulating fluid from a Neslab RTE-4 constant-temperature bath. The temperature of the bath could be changed at a constant rate with a Neslab ETP-3 temperature programmer, as previously described [12]. For most runs the temperature was varied at a rate of 4 K/h unless otherwise noted in the text, and the lipid concentration was 10 mg/ml.

Differential scanning calorimetry. Differential scanning calorimetry was performed with the high-sensitivity instrument described by Privalov et al. [13]. The scanning rate was generally 0.5 K/min, but no difference in the appearance of the transition was obtained when the scan rate was 0.2 K/min.

X-ray diffraction. The technique has been described in detail by Rand and Luzzati [14]. The samples for X-ray diffraction were lipid pellets obtained by centrifugation of aqueous dispersions. These were sealed between mica windows 1 mm apart and mounted in a temperature-controlled (± 0.2°C) Guinier camera. The X-ray diffraction pattern was recorded photographically using the CuKα line (1.54 Å) isolated by a bent quartz crystal monchromator. The intensity distribution was obtained using a Joyce-Loebl scanning microdensitometer.

Results

Differential scanning calorimetry

Fresh suspensions of DLPE in buffer which had never been heated above 40°C were analyzed by

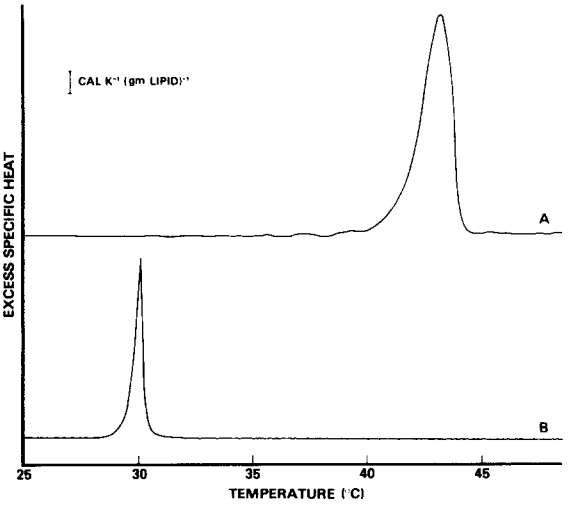

Fig. 1. DSC heating scan of the transition of DLPE at pH 5.5. (A) First heating. (B) Second heating done immediately after first heating and an elapsed time of about 30 min for recooling. Lipid concentration 2 mg/ml, scan rate 0.5 K/min.

DSC. Scans of a suspension at pH 5.5 are given in Fig. 1. The first scan shows only a single transition at 43.2°C while a second scan, run immediately after the first and at a scan rate of 0.5 K/min, showed only a single transition at 30.1°C. Previous DSC analysis of this lipid generally showed the presence of both the 43°C and the 30°C transition in a single sample which was heated at a rate of about 0.1 K/min, but showed only a single transition when the scan rate was 0.5 K/min [11]. The ability to prepare samples exhibiting only one of the two transitions has greatly aided in their characterization. Samples which show only the 30°C transition must be observed at a moderately rapid scan rate and immediately after the sample has been heated about 45°C. To obtain a sample showing only the 43°C transition, the lipid must be suspended in buffer while maintaining the temperature below 40°C. The phase which gives rise to the high temperature transition appears to be the more stable one but it reforms slowly after having been melted. Thus, a sample which shows only the presence of the 30°C transition must be maintained at 4°C for several days as an aqueous suspension for the 43°C transition to reappear. The kinetics of this conversion was not investigated in detail. In most of these aged samples some of the 30°C transition remains. Similar behaviour was observed for a sample at pH 9.5. The van't Hoff enthalpy was calculated from the width of the transition at half height [15]. The ratio of the van't Hoff enthalpy to the calorimetric enthalpy gave the size of the cooperative unit (Table I). Assuming that the 43°C transition is not kinetically limited, it appears to exhibit a degree of cooperativity lower than that of most other phospholipid phase transitions. The calorimetric ΔH is somewhat lower than that previously determined [11,15] for the 30°C transition. This discrepancy may arise because of the difficulty in obtaining a uniformally dispersed sample of DLPE without heating the sample over 40°C. In any event, the comparison of ΔH_{cal} for the two transitions is accurate, as it was done with the same sample.

TABLE I

CHARACTERISTICS OF THE HIGH- AND LOW-TEMPERATURE THERMAL TRANSITION OF DLPE

pH	t_m (°C)	ΔH_{cal} (kcal/mol)	Size of cooperative unit (molecules)	$\Delta \bar{V}$ (ml/g)
5.5	43.2	10.0	50	0.050
	30.1	2.5	700	0.015
9.5	41.0	12.0	20	0.055
	28.0	3.0	250	0.015

Scanning densimetry

Similar results were obtained with scanning densimetry and the volume change accompanying each of the transitions could be characterized (Table I). The values of ΔV are precise to ± 0.002 ml/g and are reproducible on successive heating scans. In addition, cooling scans could also be performed with this instrument. No transition was observed in cooling a fresh sample of DLPE from 40°C, and the 43°C transition disappeared in the direction of cooling, but the low temperature transition at 30°C was observed. At pH 5.5, the gel phase is metastable and may be transformed to another phase by dispersing or aging methods. More than 50% of this other phase is recovered immediately by dispersing the pellet of DLPE with a small stainless-steel spatula against the sides of the test tube into a more homogeneously dispersed pellet. This other phase can also be almost completely recovered by aging at 4°C for several days. A partial recovery of this phase from the metastable gel phase was also observed by using a slow rate of scanning of 2 K/h. These scans showed two peaks, similar to that which has been already reported [11].

X-ray diffraction

A crystalline diffraction pattern was obtained with samples which were not heated above 40°C. The crystalline structure at pH 5.5 and 9.5 are very similar (Figs. 2 and 3 and Table II), but somewhat broader linewidths are obtained for all of the reflections of the sample at pH 9.5. This may be caused by the smaller crystal size or by a larger number of crystal defects at the higher pH and is consistent with the smaller size of the cooperative unit observed under these conditions (Table I). The crystalline phase has a bilayer structure with a repeat distance of about 46 Å (Table II). When the

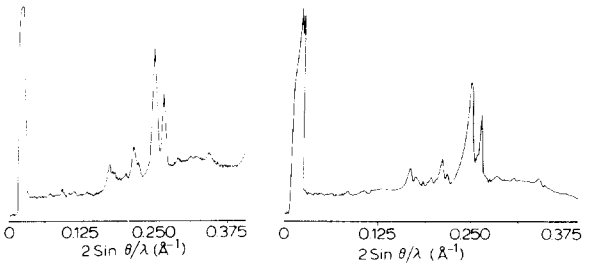

Fig. 2. (Left.) The crystalline diffraction pattern from DLPE at pH 5.5 and 20°C.

Fig. 3. (Right.) The crystalline diffraction pattern from DLPE at pH 9.5 and 20°C.

temperature was raised above 44°C, a multilayer liquid phase, L_α, was observed (Table III). Below 30°C the diffraction pattern corresponds to an L_β gel phase for a sample measured immediately after heating to 48°C (Table III). The repeat distance of this bilayer phase is about 51.3 Å and 53.0 Å at pH 5.5 and 9.5, respectively, and the wide angle spacing is at 4.2 Å. For the crystalline form we observe several lines with spacings around 4 Å, the most intense occurring at 4.05 Å. This is an important difference between the DLPE crystalline phase and that of the phosphatidylcholines (DMPC, DLPC and DCPC) whose strongest reflections occur around 4.2 Å (unpublished data). It is possible that the hydrocarbon chains are more closely packed in the DLPE crystalline phase, rendering it particularly stable, even in the presence of excess solvent.

TABLE II

X-RAY DIFFRACTION SPACING, S^{-1} (Å), FOR THE HYDRATED CRYSTALLINE PHASES OF DLPE AT 20°C

hkl	pH 5.5		pH 9.5	
	S^{-1} obs.	S^{-1} calc.	S^{-1} obs.	S^{-1} calc.
001	46.1	46.0	45.8	45.8
002	–	23.0	–	22.9
003	15.3	15.3	15.3	15.3
004	11.5	11.5	11.4	11.5
100	9.43	9.41	9.34	9.33
006	7.68	7.67	–	7.63
106	5.95	5.94	5.93	5.91
113	5.72	5.72	5.66	5.66
114	5.42	5.43	5.38	5.38
115	5.12	5.12	5.06	5.07
116	4.78	4.80	4.76	4.76
202	4.60	4.61	4.56	4.57
211	4.05	4.06	4.03	4.02
207	3.82	3.82	3.79	3.80
a		9.41		9.33
b		8.15		8.05
c		46.0		45.8
$a \cdot b/4$		19.2		18.8

TABLE III

X-RAY DIFFRACTION SPACING (Å) OF DLPE

t is the temperature of measurement; the 20°C measurement was taken directly after heating to 48°C. (d), diffuse.

t (°C)	pH 5.5		pH 9.5	
48	46.2	4.5 (d)	47.4	4.5 (d)
	23.1		23.7	
	15.4		15.8	
	11.6		11.9	
20	51.3	4.2	53.0	4.2
	25.7		26.5	
	17.1		17.7	
	12.8		13.3	

The two crystalline phases at 20°C index according to orthorhombic cells with $a = 9.41$, $b = 8.15$, $c = 46.0$ and $a = 9.33$, $b = 8.05$ and $c = 45.8$ (Å) at pH 5.5 and 9.5, respectively (Table II). The crystalline diffraction patterns obtained at 37°C were practically identical with those obtained at 20°C. A small variation in dimensions of about 0.03 Å was probably due to thermal effects. There is a large decrease in the bilayer repeat distance between the crystalline phase and the gel phase from 45.8 Å to 53.0 Å (Tables II and III) at 20°C, and a pH of 9.5. Using the diffraction from the crystalline phases, a tentative correlation in both the indices (*hkl*) and the intensities, within a 3.7 Å resolution, was established (see Table II). Because of the limited number of diffraction lines and their resolution, this assignment may not be unique.

Discussion

Our results on the thermotropic transitions of DLPE are best explained by the scheme shown in Fig. 4. This scheme indicates that the crystalline phase melts at 43.2°C but can reform only slowly,

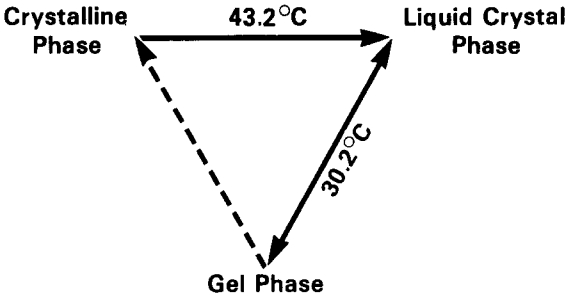

Fig. 4. Thermotropic phase transition scheme for DLPE. Dotted line indicates a transition with very slow rate.

leading to the observed hysteresis of this transition. DLPE is not unique in being able to form more than one kind of solid state. A very similar situation occurs with stearoylsphingomyelin [8]. In addition, gluco- and galactocerebrosides [9,10,16] and dipalmitoyl- and distearoylphosphatidylcholine [5] undergo transitions to different gel states. However, the higher homologues of DLPE may not exhibit this phenomenon as readily [11] because of a larger kinetic barrier to their formation.

The high-temperature transition is accompanied by a particularly large volume change. This is quite different from transitions to the hexagonal phase which do not exhibit any detectable volume change [12]. We have termed the phase which melts at 43°C as the crystalline phase. The structure of this phase may be related to that of single crystals of DLPE which were grown from glacial acetic acid [3]. The bilayer repeat distance of the crystalline phase obtained in our studies, 46 Å, is close to the value for the single crystal [3]. The cross-sectional area of the crystalline phase is 19 $Å^2$ ($a \cdot b/4$ in Table II) which is close to 19.3 Å found for the acetic acid-DLPE single crystal [3, 17]. We find, however, that the two structures are not identical. In particular, the strong wide-angle reflection occurs for a spacing of 4.18 Å for the single crystal structure, while it occurs at 4.0 and 3.8 Å in our structure (Table II). Thus, our crystalline structure may be stable even in the presence of excess water as a result of the close packing of the hydrocarbon chains. The single crystal structure, in contrast, has an interchain spacing similar to that in the L_β phase and remains in a crystalline state only in the presence of limited amounts of solvent.

Although our crystalline phase is not identical in structure to that of the single crystal of DLPE, it is nevertheless typical of lipid in a crystalline phase, since it exhibits a large number of diffraction lines, some of which are sharp, and it shows a small bilayer spacing (Table II). The decreased bilayer spacings could be caused by either a dehydration of the lipid or a change in the angle tilt of the hydrocarbon chain with respect to the plane of the bilayer. We believe the former explanation to be more likely. Crystallization processes in general are accompanied by desolvation. Partial dehydration caused by the presence of limited amounts of water (2–4% and 9–14%), is known to cause other phospholipids to exhibit crystalline diffraction patterns characterized by small bilayer spacings [18,19]. In addition, a change of tilt angle is unlikely because it could not be accommodated in an orthorhombic unit cell. Thus, although a change in tilt can be a contributing factor to the decreased bilayer thickness, it is most probable that the major factor leading to a decreased bilayer spacing is dehydration, i.e., less solvent between bilayers. This implies that the dehydration process which accompanies the formation of the crystalline phase can occur even in the presence of excess water. Since it can also occur under physiological conditions of temperature and pH, it is conceivable that analogous phenomena occur in biological systems.

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Note added in proof: (Received January 21st, 1983)

We have recently become aware of an infrared study on saturated phosphatidylethanolamines by Mantsch et al. [20]. Their conclusions reached in this study are similar to ours.

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