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CHARACTERIZATION OF THE SUB-TRANSITION OF HYDRATED DIPALMITOYLPHOSPHATIDYLCHOLINE BILAYERS

X-RAY DIFFRACTION STUDY

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The structural changes accompanying the recently described sub-transition of hydrated dipalmitoylphosphatidylcholine (Chen, S.C., Sturtevant, J.M. and Gaffney, B.J. (1980) Proc. Natl. Acad. Sci. USA 77, 5060–5063) have been defined using X-ray diffraction methods. Following prolonged storage at $-4°C$ the usual $L_{\beta'}$ gel form of hydrated dipalmitoylphosphatidylcholine (DPPC) is converted into a more ordered stable 'crystal' form. The bilayer periodicity is 59.1 Å and the most striking feature is the presence of a number of X-ray reflections in the wide angle region. The most prominent of these are a sharp reflection at $1/4.4\ Å^{-1}$ and a broader reflection at $1/3.9\ Å^{-1}$. This diffraction pattern is indicative of more ordered molecular and hydrocarbon chain packing modes in this low temperature 'crystal' bilayer form. At the sub-transition ($T_{sub} = 15–20°C$) an increase in the bilayer periodicity occurs ($d = 63.6$ Å) and a strong reflection at approx. $1/4.2\ Å^{-1}$ with a shoulder at approx. $1/4.1\ Å^{-1}$ is observed. This diffraction pattern is identical to that of the bilayer gel ($L_{\beta'}$) form of hydrated DPPC. Thus, the sub-transition corresponds to a bilayer 'crystal' → bilayer $L_{\beta'}$ gel structural rearrangement accompanied by a decrease in the lateral hydrocarbon chain interactions. Differential scanning calorimetry and X-ray diffraction show that on further heating the usual structural changes $L_{\beta'} \rightarrow P_{\beta'}$ and $P_{\beta'} \rightarrow L_\alpha$ occur at the pre- and main transitions, at approx. 35°C and 41°C, respectively.

Introduction

The structure, phase behavior and thermotropic properties of hydrated 1,2-dipalmitoyl-L-phosphatidylcholine (DPPC) bilayers have been the subject of numerous studies. The original structural and calorimetric studies of Chapman et al. [1] showing gel → liquid crystalline transitions between hydrated DPPC bilayer structures have been confirmed, with the more detailed X-ray diffraction [2–6] and scanning calorimetry [7,8] studies providing clarification of the structures present in different regions of the phase diagram and the thermodynamic changes characterizing their temperature-induced inter-conversions, respectively. These studies have resulted in a detailed description of the temperature-composition phase diagram of the DPPC-water system [4], the main features of which are two transitions at approx. 35°C and 41°C, corresponding to lamellar gel → gel ($L_{\beta'} \rightarrow P_{\beta'}$) and gel → liquid crystal ($P_{\beta'} \rightarrow L_\alpha$) bilayer transformations (for nomenclature, see Ref. 2).

A recent observation by Chen et al. [9], utilizing

Abbreviation: DSC, diferential scanning calorimetry.

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high precision scanning calorimetry demonstrates that when the DPPC-water 'gel' form is maintained at 0°C for prolonged periods (up to 3.5 days), a slow conversion to a different low temperature form occurs. This form exhibits a third transition at approx. 18°C. The enthalpy change accompanying this so-called sub-transition is approx. 3 kcal/mol. In this paper we investigate the structural changes which accompany this sub-transition utilizing low angle X-ray diffraction. Our data demonstrate clearly that the new low temperature form is a highly ordered structure in which the lateral hydrocarbon chain packing is more ordered than the quasi-orthorhombic chain packing which characterizes the usual $L_{\beta'}$ gel phase [2,3].

Materials and Methods

Commercial grade DPPC (Calbiochem-Behring Corp., La Jolla, CA) was purified by silicic acid chromatography and shown to be >99% pure by thin-layer chromatography. Hydrated DPPC multilamellar dispersions were prepared by centrifuging weighed amounts of DPPC and water through a narrow constriction in a sealed tube at 50°C. DPPC dispersions were then transferred to calorimetry pans and/or X-ray diffraction capillary tubes and stored at −4°C for different periods of time. Following storage, the DPPC dispersions were transferred at low temperatures to the scanning calorimeter and/or X-ray diffraction camera. Both the calorimeter and the X-ray diffraction sample holder were pre-equilibrated at −3°C and +5°C, respectively, prior to transfer of samples. In this way the samples were maintained at temperatures less than 10°C at all times during the transfer process.

Differential scanning calorimetry (DSC) over the heating range −10 to 50°C was performed using a Perkin Elmer (Norwalk, Conn.) DSC-2 instrument. Each sample was heated and cooled at a rate of either 2.5°C/min or 5.0°C/min. Transition temperatures were determined as the onset of the endothermic or exothermic transition extrapolated to the baseline. Enthalpy measurements were determined from the area under the transition peak by comparison with those for a known standard, gallium.

X-ray diffraction data were recorded using both film and position-sensitive detector counter methods. For film recording, nickel-filtered CuK_α radiation ($\lambda = 1.5418$ Å) from an Elliot GX-6 rotating anode X-ray generator (Elliot Automation, Borehamwood, U.K.) was collimated by either double mirror [10] or toroidal [11] optics. Microdensitometry of X-ray diffraction photographs was carried out using a Joyce Loebl model III-CS microdensitometer. For counter recording, CuK_α radiation from a microfocus X-ray generator (Jarrel-Ash, Waltham, MA) was line focussed by a single mirror and collimated using the slit optical system of a Luzzati-Baro camera. X-ray diffraction data were recorded using a linear position sensitive detector (Tennelec, Oak Ridge, TN) and associated electronics (Tracor Northern, Middleton, WI). In all cases samples were contained in thin-walled capillary tubes (internal diameter 1.0 mm) and mounted in variable temperature sample holders. Temperature stability was ±1°C.

Results

DSC heating and cooling curves of hydrated (93.9 wt.% H_2O) DPPC multilamellar dispersions are shown in Fig. 1. Following storage at −4°C for 13 days, the initial heating run at 5°C/min (Fig. 1A) shows endothermic transitions corresponding to the sub-, pre-, and main transitions with onset temperatures at 19.8, 35.9, and 41.2°C, respectively. The low temperature, 'sub-transition' at 19.8°C has an enthalpy $\Delta H = 3.5$ kcal/mol DPPC, in good agreement with the values determined by Chen et al. [9]. As shown in Table I, the corresponding temperature and enthalpy values for the pre- and main transition are similar to those observed previously [1,2,9]. On cooling at 5°C/min (Fig. 1B) the pre- and main transitions are clearly reversible but, as predicted by the study of Chen et al. [9] there is no evidence of the sub-transition on cooling. Immediate reheating at 5°C/min (Fig. 1C) confirms that the sub-transition is absent but that the pre- and main transitions are identical to those observed on the initial heating run (see thermodynamic parameters listed in Table I). Finally, storage of this sample at −4°C for 4 days results in a heating curve (Fig. 1D) similar to that observed initially (Fig. 1A) and exhibiting the sub-transition, albeit with a lower transition temperature (18.0°C) and enthalpy ($\Delta H = 3.0$ kcal/mol).

Using samples of DPPC at hydrations ranging from 37.0 to 93.9 wt.% water which show similar calorimetric behavior to that shown in Fig. 1, X-ray diffraction patterns, were recorded as a function of temperature starting at approx. 5°C. Fig. 2 shows X-ray diffraction patterns recorded using the double mirror focussing camera, of hydrated DPPC (62.9 wt.% water) at temperatures below (8°C) and above (21°C) the sub-transition. At 8°C, the low angle reflections index according to a lamellar lattice of periodicity $d = 59.1$ Å. In addition, there are reflections in the wide angle regions at 1/10.0, 1/9.30, 1/6.81, 1/4.90, 1/4.52, 1/4.43, 1/4.2, 1/3.83 $Å^{-1}$ (arrowed, Fig. 2A) which provide clear evidence that this low temperature form of DPPC has an ordered structure in which the hydrocarbon chain packing is more regular than that of the usual gel form. At 21°C, above the sub-transition, the low angle reflections index according to a lamellar lattice with an increased periodicity ($d = 63.6$ Å) and a single major reflection at 1/4.22 $Å^{-1}$ with a shoulder at approx. 1/4.1 $Å^{-1}$ is observed in the wide angle region (arrowed, Fig. 2B). This diffraction pattern is identical to that observed previously for the $L_{\beta'}$ phase [2–4]. Thus, the sub-transition represents a transition from a bilayer 'crystal' form with an ordered hydrocarbon chain packing mode to the usual 'gel' form characterized by a quasi-orthorhombic chain packing.

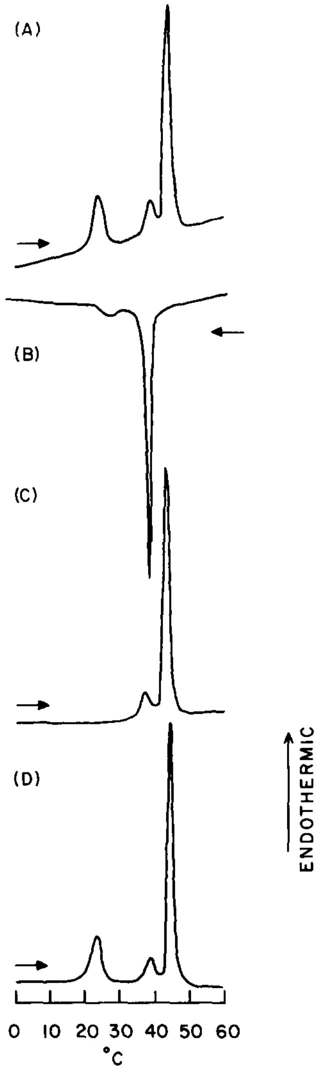

Fig. 1. DSC heating and cooling curves of a 93.9 wt.% water dispersion of DPPC. (A) initial heating run (5°C/min) following prolonged storage at −4°C, (B) cooling run (5°C/min) performed immediately after A; (C) heating run (5°C/min) performed immediately after B; (D) heating run (5°C/min) of same sample following storage at −4°C for 4 days.

TABLE I

TRANSITION TEMPERATURES AND ENTHALPIES OF HYDRATED DPPC

DPPC : H_2O 93.9 wt.% H_2O	1st heating run		1st cooling run		2nd heating run		1st heating run following storage	
	T_c (°C)	ΔH (kcal/mol)	T_c (°C)	ΔH (kcal/mol)	T_c (°C)	ΔH (kcal/mol)	T_c (°C)	ΔH (kcal/mol)
Sub-transition (T_{sub})	19.8	3.5	–	–	–	–	18.0	3.0
Pre-transition (T_{pre})	35.9	1.6	30.4	1.4	34.4	1.6	34.3	1.7
Main transition (T_{main})	41.2	8.7	38.9	9.0	40.9	8.7	40.8	9.1

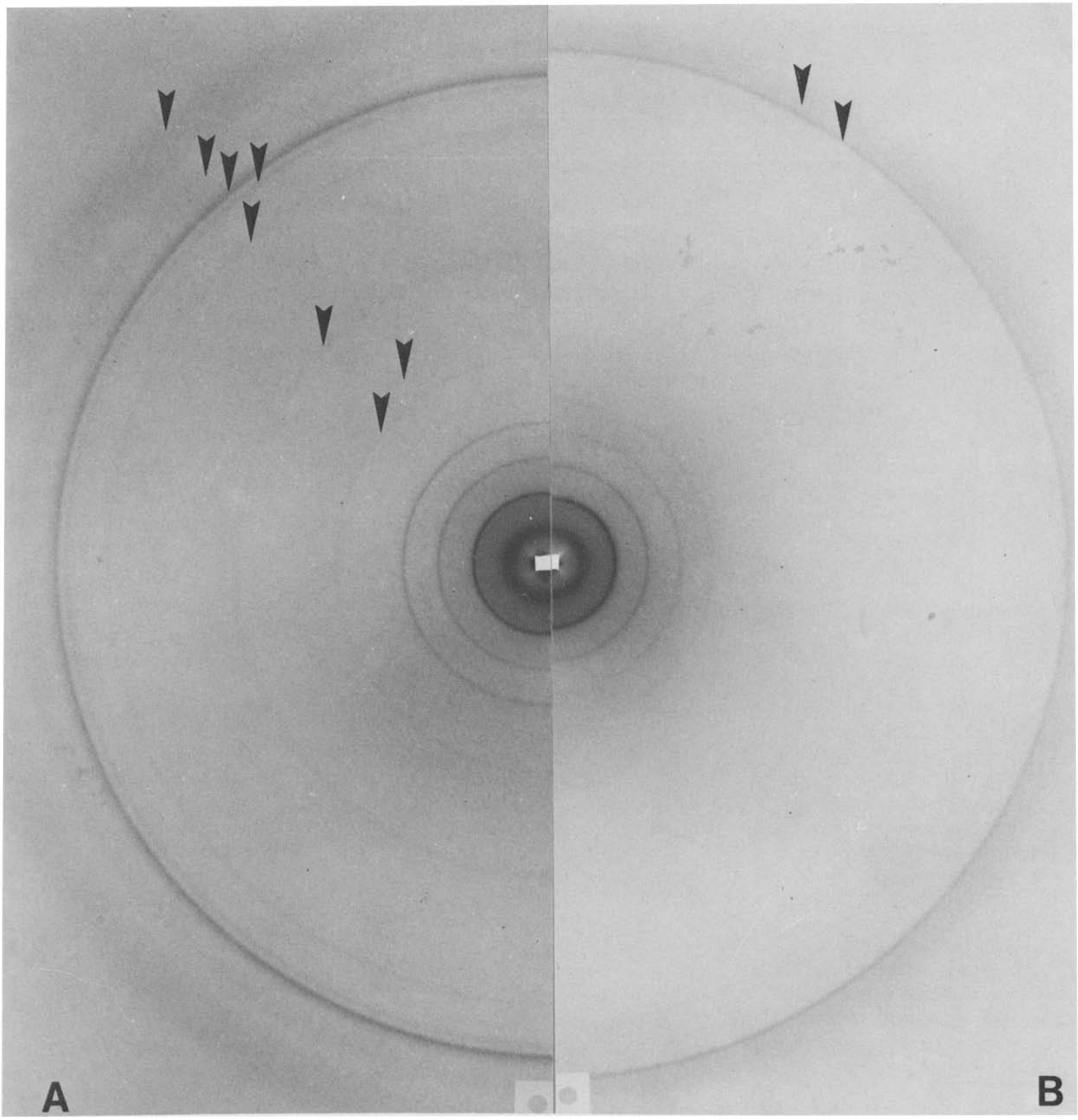

Fig. 2. X-ray diffraction patterns of a 62.9 wt.% water dispersion of DPPC at (A) 8°C; and (B) 21°C. The diffraction patterns were recorded using the Franks double mirror focussing camera with a sample to film distance of 198.3 mm.

A more detailed temperature study of a hydrated DPPC sample (37.0 wt.% H_2O) is shown in Fig. 3. Diffraction patterns recorded using the linear position sensitive detector are shown at 3°C intervals in the temperature range 5–20°C in Fig. 3. The diffraction patterns at 5, 8 and 11°C are almost identical; the left hand side shows four orders of a lamellar bilayer periodicity approx. 60 Å and the wide angle region shows reflections at approx. 1/9.8, 1/6.8, 1/4.4, and 1/3.9 Å^{-1}. For the two strong reflections, the one at approx. 1/4.4 Å^{-1} is sharp while that at approx. 1/3.9

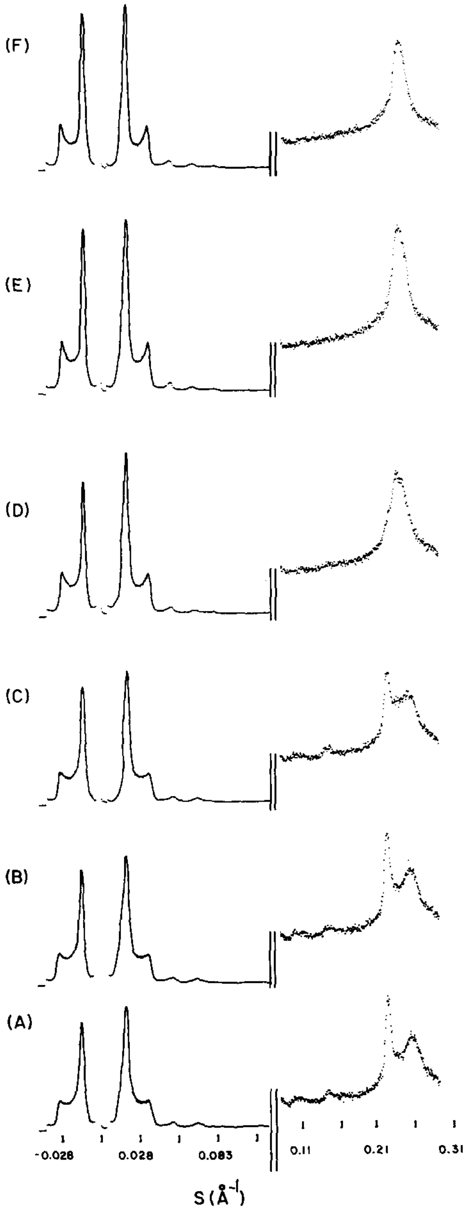

Fig. 3. X-ray diffraction patterns of a 37.0 wt.% water dispersion of DPPC as a function of temperature. (A) 5°C; (B) 8°C; (C) 11°C; (D) 14°C; (E) 17°C and (F) 20°C. The diffraction patterns were recorded using a line-focussed X-ray beam and linear position sensitive detector. The sample to detector distance was 116.2 mm.

Å^{-1} is significantly broader (see also Fig. 2A). At 14°C an obvious change in the diffraction pattern has occurred. The bilayer periodicity increases to approx. 63 Å and significant changes occur in the wide angle region. The wide angle reflections characteristic of the low temperature form have all disappeared and are replaced by a reflection at 1/4.17 Å^{-1} with a poorly defined shoulder at approx. 1/4.07 Å^{-1} (see also Fig. 2B). It is probable that the low temperature 'crystal' form of DPPC adopts a complex hybrid orthorhombic sub-cell with precisely defined hydrocarbon chain conformations and mutual orientations. This is in contrast to the $L_{\beta'}$ form where the rotationally disordered chains pack in a quasi-orthorhombic lattice. An essentially unchanged diffraction pat-

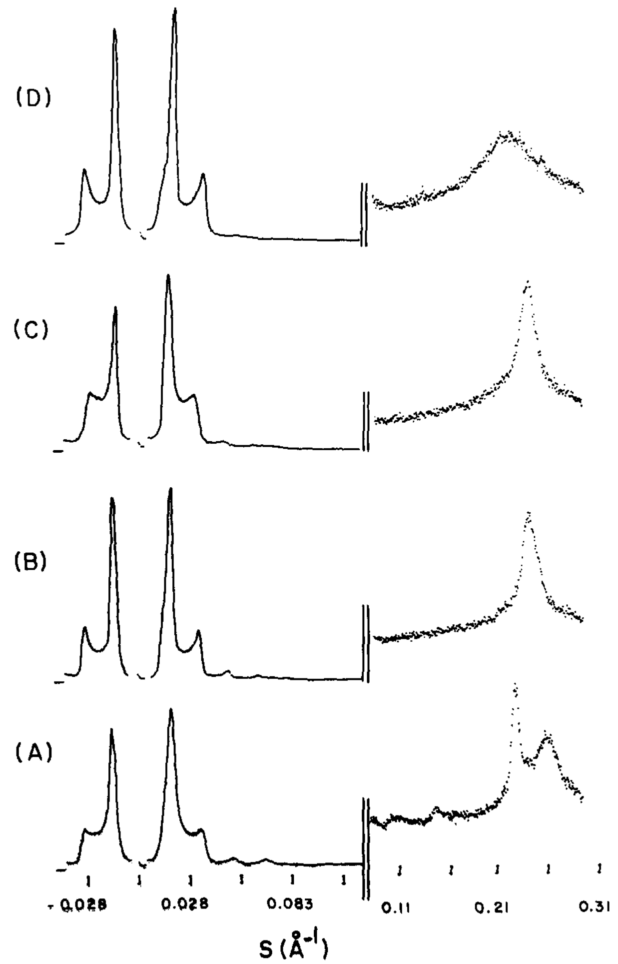

Fig. 4. X-ray diffraction patterns of a 37.0 wt.% water dispersion of DPPC as a function of temperature. (A) 5°C; (B) 23°C; (C) 38°C; and (D) 60°C. The diffraction patterns were recorded using a line-focussed X-ray beam and linear position sensitive detector. The sample to detector distance was 116.2 mm.

tern characteristic of the $L_{\beta'}$ form is observed at 17 and 20°C.

Diffraction patterns were recorded at 3°C intervals up to 44°C and finally at 60°C. Diffraction patterns representative of the four different phases are shown in Fig. 4; at 5°C, the low temperature 'crystal' form observed in the temperature range 5 to 11°C; at 23°C, the $L_{\beta'}$ form present in the temperature range 17 to 32°C; at 38°C the $P_{\beta'}$ form observed in the temperature range 35–41°C; and finally, at 60°C the liquid crystalline form L_α. The diffraction patterns of the 'crystal' and the $L_{\beta'}$ forms have been described above (see also Fig. 3). The $P_{\beta'}$ form shows the typical increase in bilayer periodicity to approx. 67 Å (note that the *hk* reflections characteristic of the two-dimensional oblique lattice are not clearly resolved with this recording camera geometry). As noted previously the wide angle reflection at 1/4.19 $Å^{-1}$ is now symmetrical, showing no evidence of a shoulder on the high angle side (cf. earlier studies, Refs. 2, 3). Transformation to the L_α phase is marked by a decrease in the bilayer periodicity ($d \simeq 60$ Å) and the presence of a single broad reflection at approx. 1/4.5 $Å^{-1}$ in the wide angle region. Cooling to 5°C shows the diffraction pattern characteristics of the $L_{\beta'}$ form, consistent with the extremely slow reversibility of the sub-transition [9].

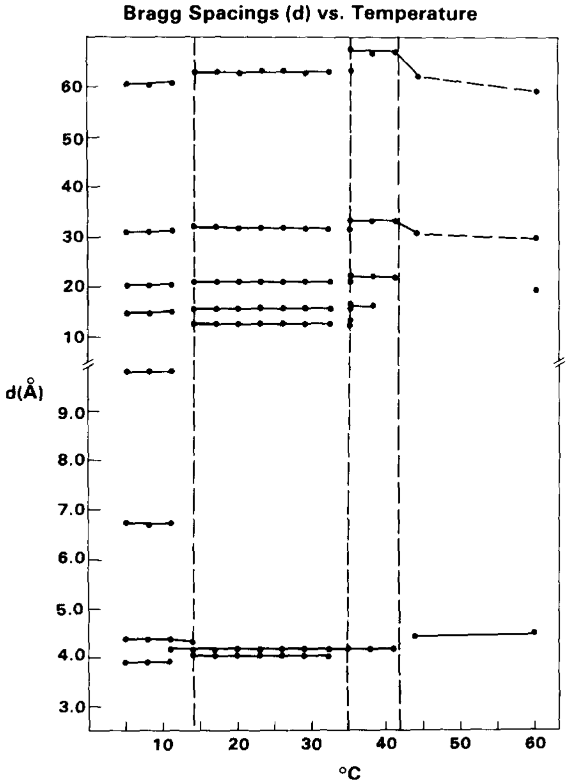

Fig. 5. Temperature dependence of the diffraction spacings *d* of a 37.0 wt.% water dispersion of DPPC. Transition temperatures of the sub-, pre-, and main transitions are indicated by the dashed lines.

A summary of the temperature dependent changes in the X-ray diffraction parameters for DPPC:H_2O (37.0 wt.%) is given in Fig. 5. Similar calorimetric behavior and changes in the X-ray diffraction pattern have been observed for DPPC containing 62 wt.% water as well as a very dilute aqueous dispersion of DPPC. In agreement with the studies of Chen et al. [9], the temperature at which the sub-transition occurs decreases with the DSC heating rate and increases with the time of storage at −4°C (data not shown).

Discussion

Until recently it was thought that the stable low temperature form of hydrated phospholipids and sphingolipids was the gel (L_β or $L_{\beta'}$) structure. Recent studies on stearoyl sphingomyelin showed that in this case the gel form was metastable and converted slowly to a more ordered, stable 'crystal' form [12]. Similar studies on hydrated cerebrosides [13,14] again indicate slow conversions of metastable to stable bilayer forms, in this case changes in hydration being important [14]. The interesting observation by Chen et al. [9] showing a low temperature sub-transition for DPPC (and other diacylphosphatidylcholines) after prolonged storage at low temperatures indicated very slow conversion of the $L_{\beta'}$ gel form to another, more stable form. In this study we have defined the structural changes that accompany the sub-transition. The presence of a number of sharp reflections in the wide angle region indicate a more highly ordered form below the sub-transition. For simple lipids, strong reflections in this region usually indicate the mode of hydrocarbon chain packing and the lattice geometry of the simple sub-cell describing this chain packing mode is obtained [15,16]. The interpretation with diacyl lipids is more difficult since they adopt more complex hybrid sub-cells

[17,18] and a direct derivation of these complex sub-cells and, thus chain packing modes, from the 'powder' diffraction patterns is not yet reliable. However, the presence of a weak reflection at approx. 1/9.3 $\mathrm{\AA}^{-1}$ is similar to the a axis of the hybrid sub-cell of the crystalline form of dilauroyl-DL-phosphatidylethanolamine [17]. In addition a number of crystalline forms of hydrated diacylphosphatidylcholines have lattice parameters in the range 8–10 Å [18–20]. Of particular interest is the monoclinic form of dimyristoylphosphatidylcholine (DMPC) dihydrate described by Pearson and Pascher [18] with cell dimensions $a = 8.72$, $b = 8.92$, $c = 55.4$ Å, $\beta = 97.4°$. Increasing the c axis by 5 Å to convert from DMPC to DPPC gives $c \simeq 60$ Å in reasonable agreement with the bilayer periodicity ($d \simeq 59$ Å) of the low temperature 'crystal' form of hydrated DPPC (see Figs. 2A, 3, 4 and 5). The observed reflection at approx. 1/3.85 $\mathrm{\AA}^{-1}$ would result from chain stacking along the ($21l$) lattice planes. The space group $P2_1$, dictates that the $0k0$ reflections are absent for $k = 2n + 1$, therefore the 010 reflection (1/8.92 $\mathrm{\AA}^{-1}$) is absent and the 020 reflection (1/4.46 $\mathrm{\AA}^{-1}$) is permitted. The diffraction spacing corresponding to the 020 reflection of this crystal form is in good agreement with the strong sharp reflection at 1/4.43 $\mathrm{\AA}^{-1}$ observed for the low temperature 'crystal' form of hydrated DPPC (Figs. 2A, 3 and 4). However, although it is probable that the low temperature form present below the sub-transition has some of the packing characteristics of one of the polymorphic crystal forms of diacylphosphatidylcholines, a combination of the complexity of the hybrid sub-cells adopted and the paucity of detailed crystallographic analyses of phospholipids precludes a definitive assignment of the molecular or hydrocarbon chain packing at this stage. It is probable that the low temperature 'crystal' form of DPPC adopts a complex hybrid orthorhombic sub-cell with precisely defined hydrocarbon chain conformations and mutual orientations. This is in contrast to the $L_{\beta'}$ form where the rotationally disordered chains pack in a distorted orthorhombic lattice.

An additional point to be raised concerns the hydration state of DPPC. In an earlier study we have determined the hydration state of DPPC in different phases [4]. At maximum hydration DPPC incorporates approx. 27 water molecules per molecule DPPC in the liquid crystalline L_α phase; both the $P_{\beta'}$ and $L_{\beta'}$ phases incorporate 15 water molecules per molecule DPPC. Thus, although no major change in hydration occurs at the $L_{\beta'} \rightarrow P_{\beta'}$ transition, the $P_{\beta'} \rightarrow L_\alpha$ transition is accompanied by increased hydration. On the basis of a more ordered hydrocarbon chain lattice together with the observation of small reduction in the lamellar periodicity, some decrease in the hydration state of DPPC, perhaps to the dihydrate, would be predicted as the $L_{\beta'}$ form slowly converts to that of the low temperature 'crystal' form. Conversely, at the sub-transition an increase in DPPC hydration would be expected as the 'crystal' $\rightarrow L_{\beta'}$ transition occurs.

In summary, the X-ray diffraction data show convincingly that low temperature incubation converts the usual hydrated gel ($L_{\beta'}$) form to a more ordered bilayer structure. This results from an alteration in both the molecular packing and hydrocarbon chain packing and is probably accompanied by a decrease in hydration at the polar group interface. On heating this stable low temperature 'crystal' form it reverts to the $L_{\beta'}$ form at 14–20°C. At the sub-transition, energy is absorbed and the specific hydrocarbon chain packing mode characteristic of the low temperature form is converted to the approximate 'orthorhombic' chain packing mode of the $L_{\beta'}$ form. In this form the chains are rotationally disordered and precise chain-chain interactions are unlikely. Above the sub-transition hydrated DPPC exhibits the same thermotropic behavior as described previously with reversible transitions to the $P_{\beta'}$ (gel, hexagonally packed chains) and L_α (melted, hexagonally packed chains) bilayer structures.

Note added in proof: (Received November 16th, 1981)

Since the submission of this manuscript, a DSC, X-ray diffraction and ^{31}P-NMR study of the changes occurring at the sub-transition of hydrated DPPC has been reported [21]. This study concludes similarly that the ordered 'crystal' $\rightarrow L_{\beta'}$ gel transformation associated with the sub-transition involves a change in the hydrocarbon chain packing. A change in the polar headgroup hydration and dynamics is also indicated by the ^{31}P-NMR data.

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