

# COEXISTENCE OF RIGID CRYSTALLINE AND LIQUID CRYSTALLINE PHASES IN LECITHIN-WATER MIXTURES

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**ABSTRACT** X-ray diffraction measurements show that the  $\beta'$ -crystalline to liquid crystalline transitions of dipalmitoyl lecithin-water mixtures take place over temperature ranges of several degrees, and by the gradual disappearance of one crystalline state accompanied by the gradual appearance of the other. The narrowness of the low angle X-ray lines indicates that the two crystalline states exist in separate phases. At a given temperature, the liquid crystalline phase has the greater water content. The bilayers of the liquid crystalline phase are thinner than those of the  $\beta'$ -crystalline phase while the distances between bilayers are essentially equal in the two phases.

## INTRODUCTION

Lecithin-water mixtures form lamellar structures in which bilayers of the phospholipid are separated by water (1). Such mixtures have been extensively studied as models for biological membranes since at least the major part of the phospholipid in the latter is also in a bilayer structure (2).

The bilayers of the synthetic phospholipids exist primarily (1) in two states. In the *rigid crystalline*, or, more precisely (following Tardieu et al. [1]), the  $\beta'$ -crystalline state,<sup>1</sup> the hydrocarbon chains of the phospholipid undergo hindered rotation about their axes, but are relatively rigidly aligned. In the state stable at higher temperatures, to be referred to as the *liquid crystalline* state, the hydrocarbon chains have a liquid-like flexibility (1). Evidence has been accumulating for the existence of, and transition between, the two states in cell membranes (3-5).

<sup>1</sup> We use the term  $\beta'$ -crystalline state for dipalmitoyl lecithin-water mixtures below the transition temperature solely to follow Tardieu et al. (1), and without having worked out the detailed structure. Unlike these authors we did not obtain an X-ray diffraction band outside of the characteristic 4.2 Å line. This discrepancy may possibly be due to temperature differences; the X-ray patterns appearing in ref. 1 are for measurements made at 4°C and 33°C, while our studies were conducted above 40°C. Plate I of ref. 1 indicates that the band in question is less intense and closer to the main line at 33°C than at 4°C.

The transition between the two crystalline states of the synthetic phospholipids has not been considered in detail. While differential scanning calorimetry shows that with lecithin-water mixtures of water contents greater than 50%, the transition takes place over a temperature range of no more than a few tenths of a degree (6, 7), mixtures containing lesser amounts of water given transition ranges of several degrees (6). However, these latter results are not unequivocal, since broad transitions in scanning calorimetry may be merely the results of heat transfer limitations in the calorimeter cell (8).

We report here an X-ray diffraction study aimed at determining (a) whether the  $\beta'$ -crystalline to liquid crystalline transition is truly isothermal, and (b) if such is not the case, the nature of the crystalline states present in the transition temperature range. The lecithin used was dipalmitoyl lecithin (DPL), which unlike other lecithins exists only in the crystalline states mentioned. The presence of each crystalline state was ascertained from the appearance of wide angle X-ray diffraction lines, the  $\beta'$ -crystalline state giving a sharp line at a Bragg spacing of about 4.2 Å, and the liquid crystalline state giving a diffuse line at about 4.6 Å (1). Low angle X-ray diffraction measurements, which indicate repeat spacings between adjacent bilayers (1), were also made to determine some parameters of the crystalline states.

## EXPERIMENTAL

The DPL was used as obtained from Applied Sciences Laboratories, Inc., College Park, Pa. Thin-layer chromatography showed that the purity varied from sample to sample, some samples seeming completely pure and others having small amounts of an impurity, probably a lysolecithin. No significant differences were found between the behavior of pure and impure samples. No fatty acid residues other than palmitic were detectable by vapor phase chromatography (sensitivity 0.5%).

To form the mixtures with water, first a thin layer (about 20 mg/cm<sup>2</sup>) of lecithin was deposited at the bottom of a glass tube by evaporation of a CHCl<sub>3</sub> solution of the material. After overnight drying *in vacuo* at room temperature, water was added and the tube sealed and kept at about 5°C above the transition temperature for at least 16 h before being transferred into thin walled capillary tubes for the X-ray measurement proper. Standard X-ray diffraction techniques were used (9). Wide angle X-ray measurements required 1 h exposure time; low angle X-ray measurements required 16 h.

The X-ray cell consisted of a brass block through which temperature controlled water circulated. The only part of the (sealed) sample-containing capillary tube not in contact with the circulating water was the region of a 2 mm diameter hole in the cell through which the X-ray beam passed. To minimize temperature gradients across the sample here, each end of the hole was covered with a thin sheet of Mylar, thus immobilizing about 3-mm thick layers of air at the exposed surfaces of the capillary. As indicated by the sharpness of the melting of pure crystalline com-

pounds (thymol, m.p. 50°C and 1,3,5-trichlorobenzene, mp 61°C) temperature gradients along the path of the X-ray beam were less than 0.5°C. Sample temperature calibration was accomplished both by the above mentioned melting of pure compounds, and by substituting a thermocouple wire in the sample tube. Temperatures in the cell were constant to  $\pm 0.1^\circ\text{C}$  during the X-ray exposures. Measurements were made at 0.5°C intervals in the neighborhood of the transition, except for the mixture with the transition range of 0.75°C where measurements were made at about 0.2°C intervals.

## RESULTS AND DISCUSSION

The transitions were found to take place over finite temperature ranges for all mixtures studied, the temperature ranges varying from 0.75°C to about 4°C. The transitions took place as gradual changes in the relative amounts of the  $\beta'$ -crystalline and liquid crystalline states, with no indications of other crystalline states.

The occurrence of the transition over a temperature range was manifested in the wide angle X-ray measurement by the simultaneous presence of the diffraction lines characteristic of each crystalline state. In the low angle measurements two diffraction patterns, with repeat spacings about 5 Å apart, appeared in the transition range. The temperatures at which the lines characteristic of a given state appeared or disappeared were the same for the wide angle and low angle measurements, and no differences were observed between measurements made on heating or cooling. Also, as determined from the relative intensities of the low angle X-ray lines from the two crystalline forms, their relative amounts varied approximately linearly with temperature in the transition regions.

Fig. 1 shows for each mixture ( $c$  = weight percent water) the lowest temperatures at which traces of the liquid crystalline phase and the highest temperatures at which traces of the  $\beta'$ -crystalline phase can be discerned in the X-ray pattern.<sup>2</sup> These transition temperatures agree with those found by differential scanning calorimetry (11).

There are several strong arguments against the observed coexistence of the two

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<sup>2</sup> In the sample of the highest water content for which data are shown,  $c = 24.2\%$ , some of the water is probably present in a purely aqueous phase, which can coexist in thermodynamic equilibrium with a lecithin-water mixture (1). This sample was originally prepared with a water content of 30%, but water condensate appeared on the walls of the sample tube during storage at 45°C; the water content of 24.2% was arrived at by blotting off the condensate and reweighing. There are some indications that the mixture  $c = 24.2\%$ , and also the mixture  $c = 19.3\%$ , are metastable with regard to the loss of water to a purely aqueous phase. Thus, although these particular mixtures were stable and gave low angle X-ray spacings consistent with their water contents (*vide infra*, Table I), water condensed out from other mixtures with  $c > 19\%$ , until their water contents, as indicated both by weighing and by low angle X-ray measurements, were as low as 18.5%. A probably related metastability is implicit in the observation (10) that the maximal water content of egg lecithin (at 25°C) in contact with water vapor at 100% relative humidity is significantly smaller than for contact with liquid water (1).

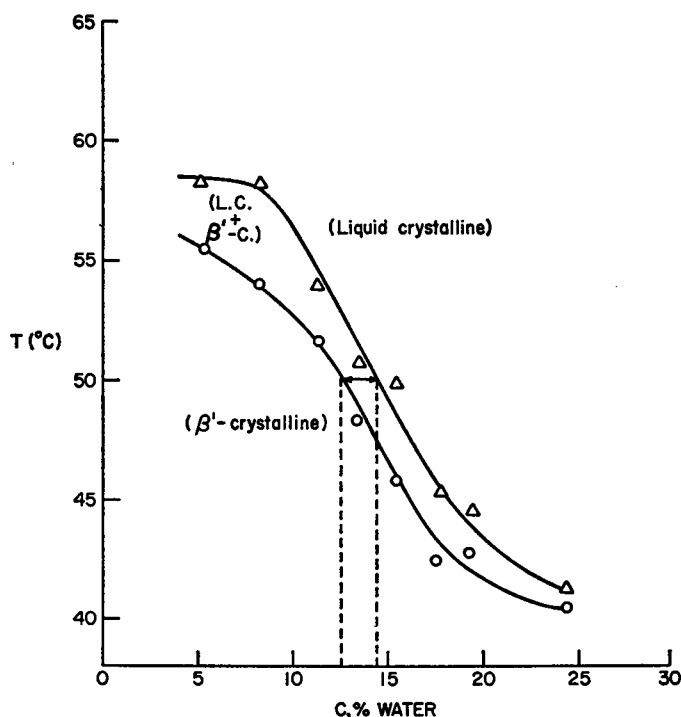


FIGURE 1 Temperatures at which X-ray measurements show traces of liquid crystalline (O) and  $\beta'$ -crystalline ( $\Delta$ ) states for DPL-water mixtures of various compositions.

crystalline states being the trivial result of inhomogeneous distribution of water in the sample: (a) Where duplicate samples were examined, they gave identical results for the relative amounts of each state at each temperature. (b) The low angle X-ray lines, whose widths would be expected to be sensitive to inhomogeneities in water content since the line positions are determined by interbilayer distances (1), were very narrow. The line widths corresponded to broadening of  $0.22^\circ 2\theta$  (9) which is about equal to the estimated instrumental broadening (and in fact was somewhat less than the broadening obtained with dry DPL). (c) Since the diffusion coefficient of water in lecithin-water mixtures is about  $4 \times 10^{-6} \text{ cm}^2\text{s}^{-1}$  (12), and the X-ray beam was about 0.05 cm wide, inhomogeneities across the *entire* width of the beam would be evened out by diffusion in about  $(0.05)^2/(4 \times 10^{-6}) = 625 \text{ s}$ , i.e. in about 10 min. In some cases the measurements were done over a period of a week, with no changes in the X-ray patterns.

One conceivable interpretation of our findings is that  $\beta'$ -crystalline and liquid crystalline regions coexist within individual bilayers, the relative proportions of each being temperature dependent. We do not believe that this is the case, because of the above mentioned sharpness of the low angle X-ray diffraction lines. The line broadening of only  $0.22^\circ 2\theta$  indicates that the  $\beta'$ -crystalline and liquid crystal-

line regions producing the diffraction patterns are at least 1,000 Å thick (9). For this to occur with bilayers containing both types of regions,  $\beta'$ -crystalline regions would have to be stacked above  $\beta'$ -crystalline regions, and liquid crystalline regions above liquid crystalline regions, for about 20 bilayers. This type of long range ordering seems a priori improbable.

It seems most probable that separate, hydrated  $\beta'$ -crystalline and liquid crystalline phases coexist over the transition temperature range. On this basis, and assuming that the X-ray diffraction method is sufficiently sensitive to detect the very start of the second phase, the water contents of the coexisting phases at each temperature can be obtained from horizontal tie lines between the curves of Fig. 1. For example, at  $T = 50^\circ\text{C}$ , DPL-water mixtures with water contents of 12.5–14.3 % consist of a crystalline phase with 12.5 % water and a liquid crystalline phase with 14.3 % water.

The low-angle X-ray repeat spacings,  $\delta$ , measured for each phase in the two phase region, together with the compositions of the coexisting phases derived as indicated above from Fig. 1, permit computation of the bilayer thicknesses,  $\delta_L$ , and the thicknesses of the intervening water layers,  $\delta_w$ , in each phase. The bilayer thicknesses can be computed using the expression:

$$\delta_L = 0.86 \phi_v \delta, \quad (1)$$

where  $\phi_v$  is the *volume* fraction of DPL in the phase,<sup>3</sup> and the numerical factor, 0.86, arises from the convention of assuming that the phosphocholine groups of the lecithin are immersed in the aqueous interbilayer regions (1, 14–16). The thicknesses of these aqueous interbilayer regions,  $\delta_w$ , can be computed by subtracting the computed bilayer thickness from the total repeat spacing, i.e.:

$$\delta_w = \delta - \delta_L. \quad (2)$$

Table I lists, for DPL-water mixtures of total water content,  $c$ : the measured low-angle X-ray repeat spacings at temperatures at which two phases coexist; the water contents,  $w$ , of each phase as derived from Fig. 1; and the computed values of  $\delta_L$  and  $\delta_w$  for each coexisting phase. Assuming that the measured low-angle repeat spacings,  $\delta$ , are accurate to  $\pm 0.2$  Å, and the water contents of the coexisting phases as derived from Fig. 1 are accurate to  $\pm 1$  percentage units, the computed values of  $\delta_L$  and  $\delta_w$  would be accurate to  $\pm 1$  Å.

Although at all temperatures the water contents of the liquid crystalline phases are greater than those of the coexisting  $\beta'$ -crystalline phases, Table I indicates that the water layer thicknesses,  $\delta_w$ , of the coexisting phases are essentially equal. Since the equilibrium between the coexisting phases requires that the thermodynamic ac-

<sup>3</sup> The same density, 1.04, was used for DPL in the two crystalline states in computing  $\phi_v$  from the weight percent water since there is only a small density change with the transition (13).

TABLE I  
PARAMETERS OF COEXISTING PHASES

Water content of mixture, <i>c</i>	Temperature of low angle X-ray measurements	$\beta'$ -crystalline phase				Liquid crystalline phase			
		Low angle X-ray spacing, $\delta$	Water content from Fig. 1, <i>w</i>	Computed bilayer thickness, $\delta_L$	Computed water layer thickness, $\delta_w$	Low angle X-ray spacing, $\delta$	Water content from Fig. 1, <i>w</i>	Computed bilayer thickness, $\delta_L$	Computed water layer thickness, $\delta_w$
%	°C	Å	%	Å	Å	Å	%	Å	Å
5.5	55.4	58.6	5.5	47.2	11.4	52.2	10.6	40.0	12.2
8.1	55.4	57.0	5.5	46.2	10.8	52.4	10.6	40.1	12.3
11.3	51.6	57.1	11.2	43.4	13.7	53.0	13.2	39.3	13.7
13.3	49.8	57.5	12.8	42.9	14.6	53.1	14.4	38.9	14.2
15.4	48.9	57.9	13.5	42.8	15.1	53.0	15.5	38.2	14.8
15.4	45.8	58.1	15.4	42.0	16.1	53.3	17.6	37.5	15.8
17.5	44.8	58.6	16.4	41.9	16.7	54.2	18.5	37.7	16.5
17.5	42.3	58.9	18.0	41.3	17.6	55*	22	36	18
19.3	42.8	61.2	18.2	42.7	18.5	55.7	21.0	37.5	18.2
24.2	41.0	62.8	†	†	†	58.1	†	†	†

\* The uncertainty in the X-ray spacing for this sample is particularly large,  $\pm 0.5$  Å, because of the faintness of the line.

† Computation not feasible because tie line cannot be drawn.

tivities of water in them be equal, this implies that the thermodynamic activity of water in these lamellar bilayer systems is determined primarily by the water layer thickness.

Table I also shows that the thicknesses of the bilayers of the liquid crystalline phases are less than those of the  $\beta'$ -crystalline phases. This is consistent with data of other workers (1, 6) and is undoubtedly a reflection of the rigid, extended configuration of the hydrocarbon chains in the  $\beta'$ -crystalline state, as opposed to their flexibility and/or tilting in the liquid crystalline state.

However, our results are not completely consistent with the equilibrium coexistence of two hydrated crystalline phases. First as indicated in footnote 1, some of the water in the mixture  $c = 24.2\%$  is probably present in a purely aqueous phase coexisting with the hydrated lecithin phases. According to the phase rule this two-component (i.e. DPL and water), three-phase (i.e.,  $\beta'$ -crystalline, liquid crystalline, and pure water phases) system should have no degrees of freedom (at constant pressure), i.e. the transition between the two crystalline states for the mixture  $c = 24.2\%$  should be truly isothermal. Although the transition range of  $0.75^\circ\text{C}$  at  $c = 24.2\%$  is significantly smaller than for mixtures with lower water contents, where it ranges from  $2\text{--}4^\circ\text{C}$ , it is much greater than the experimental variation in temperature ( $\pm 0.1^\circ\text{C}$ ). The finite transition range for this mixture may have been a result of an impurity in the DPL, although no impurity could be detected (by thin-

layer chromatography) in the DPL used for this particular mixture. Conceivably, the anomalous behavior may be related to the metastable behavior exhibited at high water contents, described in footnote 2.

A second point of inconsistency with the equilibrium coexistence of the two hydrated phases arises from the data for the mixtures  $c = 5.5\%$  and  $c = 8.1\%$  (Table I). The low angle X-ray diffraction measurements for these mixtures were made at the same temperature,  $55.4^\circ\text{C}$ . Accordingly, only the relative amounts of each phase should be different in the two mixtures, while the compositions, and hence low-angle X-ray spacings should be the same. As seen from Table I, although the low-angle X-ray spacings for the liquid crystalline phases for the two mixtures are essentially equal, the X-ray spacings for the  $\beta'$ -crystalline phases differ by the large value of  $1.6 \text{ \AA}$ . We offer no explanation for this finding, but will point out that the low-angle X-ray rings from the  $\beta'$ -crystalline phase for the mixture  $c = 5.5\%$  were the only ones of the many examined during the course of this study which exhibited the "spottiness" (9) characteristic of large crystallite size; we estimate crystallite sizes of the order of  $10 \mu\text{m}$  from this spottiness. Also, Tardieu et al. have reported the presence of several phases in this region of low water contents (1).

Some incidental observations made in the course of this work were that the wide angle X-ray spacings for the  $\beta'$ -crystalline phase were essentially independent of water content at constant temperature; their temperature coefficient at constant water content was  $\simeq +0.01 \text{ \AA}/^\circ\text{C}$ . The wide angle X-ray lines for the liquid crystalline phase were too diffuse for accurate measurement of repeat spacing. The low angle X-ray spacings for both crystalline phases had temperature coefficients of about  $-0.1 \text{ \AA}/^\circ\text{C}$  at constant water contents.

Finally, our conclusion that the two crystalline states do not coexist within the same bilayers in the case of DPL-water mixtures supports the idea that the observed coexistence of these states in biological membranes is a consequence of the lipids being present in the membranes in at least two segregated regions (17).

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