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Nature of the gel to liquid crystal transition of synthetic phosphatidylcholines

(lipid bilayers/phase transitions/purification/scanning calorimetry/synthesis)

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ABSTRACT **High sensitivity differential scanning calorimetry is employed in the study of the gel to liquid crystal phase transition of a highly purified sample of dipalmitoylphosphatidylcholine in multilamellar bilayer suspension. It is concluded from the calorimetric data that the purity of the sample is better than 99.94 mol % and that the transition closely approximates an isothermal first-order transition.**

The gel to liquid crystal transitions shown by synthetic phosphatidylcholines in multilamellar aqueous suspension have been found to possess a finite width, even when examined by a nonperturbing technique such as differential scanning calorimetry. Because such behavior has been observed with materials that by the usual criteria (such as thin-layer chromatography) appear to be quite pure, the question arises (1) as to whether these transitions deviate for some fundamental reason from the very nearly isothermal first-order melting transitions observed with pure crystalline substances. To investigate this question further we have studied by means of high sensitivity differential scanning calorimetry the transition shown by a highly purified sample of dipalmitoylphosphatidylcholine (DPPC).

MATERIALS AND METHODS

Preparation of DPPC. Very pure DPPC was prepared by synthesis from carefully purified components and subsequent recrystallization of the product (2).

Fatty Acids. Many commercial samples of palmitic acid are adequately pure and sufficiently crystalline to use as the starting material for zone refining. A critical part of the process is selecting the most efficient rate of zone movement, which is determined by the rate at which a planar crystalline interface of the material will advance. This was estimated from crystal growth measurements (3) to be 5 cm per day. The use of multiple zones with heaters and coolers and a rotating horizontal tube (4) enabled a purification cycle of 20 zone passes to be completed in 10 days. Analysis of the acid by gas–liquid chromatography during refining showed that the major impurities are other fatty acids, and that these can be reduced to less than 1:1000.

The purified acid was converted to the acid anhydride by reaction with dicyclohexyl carbodiimide (5). An acyl urea is formed as a byproduct; it was removed by zone refining, using a procedure similar to that employed with the acid.

Glycerophosphocholine. The glycerophosphocholine part of the lecithin molecule having the naturally occurring configuration was prepared by modification of a published procedure (6). Changes in the procedure included dehydration of the crude lecithin solution by passage through a molecular sieve column. The cadmium chloride complex was crystallized slowly in a controlled manner from aqueous ethanol solution after seeding the slightly undersaturated solution. This procedure significantly affected the purity of the final product. After isolation the glycerophosphocholine readily crystallized from an aqueous solution on evaporation; reports in the literature emphasize the reluctance of this compound to crystallize when it is prepared by the usual method.

Synthesis of DPPC. The acid anhydride and glycerophosphocholine were condensed in the presence of the potassium salt of the acid (7). Free fatty acid and unreacted anhydride and glycerophosphocholine were removed by diethyl ether extraction. The residue was dissolved in chloroform and filtered to remove the potassium salt, and, on the addition of ether, the lecithin was precipitated as anhydrous crystals. These were again dissolved in chloroform and precipitated with ether.

Recrystallization of DPPC. The product was dissolved in chloroform to make a 2.5% solution, and sufficient water was added to form the monohydrate. The crystals were later found to be the dihydrate. Pure hexane was then added to form a slightly supersaturated solution, which was seeded and allowed to crystallize at constant temperature (17°). More hexane was added slowly over a period of 10 days so that most of the lecithin was recovered as a crystalline powder. The material used in the calorimetric experiments was recrystallized twice more using conditions controlled so as to ensure slow crystallization. The crystals were isolated by filtration and the excess solvent was removed under reduced pressure. It should be noted that lecithin that is not in a highly crystalline state retains solvent tenaciously.

The degree of purification obtained by recrystallization is highly dependent on the rate of crystallization, which is itself critically influenced by several factors. These included the crystallization temperature and the ethanol content of the chloroform. Ethanol is added to the chloroform as a stabilizer in amounts that are variable. The water content of the solution appears to be significant but it is difficult to control. The optimal conditions for each crystallization were established by preliminary experiments. In the recrystallization, it is, of course, important to use solvents of the highest purity obtainable.

Calorimetric Procedure. A Privalov instrument (8) was employed. Before each experiment the cells were filled with dichromate/sulfuric acid cleaning solution, heated to 95°, and then thoroughly rinsed with water. Deionized, glass-distilled

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Abbreviation: DPPC, dipalmitoylphosphatidylcholine.

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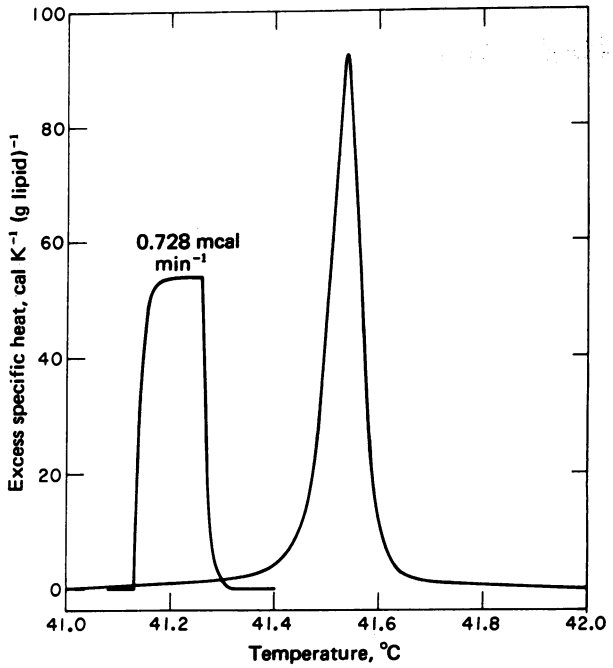

FIG. 1. Calorimetric phase transition curve for DPPC. DPPC at 0.508 mg per g of suspension was scanned at 0.0232 K min^{-1}. The excess apparent specific heat is plotted against the temperature, with the maximum occurring at 41.55°. Also shown is a calibration mark produced by supplying excess heat at a rate of 0.728 mcal min^{-1} to the reference cell containing water.

water was used throughout. The lipid was suspended in water by vortex-mixing for 2–3 min at about 50°.

The heating circuits of the calorimeter were modified to permit scanning at rates much lower than 0.1 K min^{-1}, the usual minimum for this instrument.

RESULTS AND DISCUSSION

Fig. 1 shows a calorimetric scan obtained with a suspension containing 0.508 mg of DPPC per g of suspension. The scan rate was 0.0232 K min^{-1}. The maximal excess specific heat was 91.9 cal K^{-1} g^{-1} (1 cal = 4.184 J), and the peak width at half-maximal excess specific heat was 0.067 K. Also shown in the figure is a calibration mark produced by suppling extra heat to the reference cell. This mark illustrates the response of the instrument to a step function, and it shows that only a minor fraction of the broadening evident in the transition peak can be attributed to thermal lags in the calorimeter. Curve A in Fig. 2 shows the variation with temperature of the fraction melted; it was obtained by planimeter integration of the transition peak in Fig. 1.

The excess specific heat curve is slightly asymmetric, with the onset of melting being somewhat less abrupt than the completion of melting. This asymmetry is of the form expected when the sample is slightly impure. It can be shown (9) that when a single impurity is present that forms ideal solutions in both the solid (gel) and liquid (liquid crystal) states of the main component, the fraction melted, α, can be expressed as a function of temperature, T, in the form

$$(T_0 - T)_{\text{impurity}} = \frac{RT_0^2}{\Delta H_f} X_2 \frac{1}{\frac{k}{1-k} + \alpha} \qquad [1]$$

in which T_0 is the melting point of the pure main component, ΔH_f is the enthalpy of fusion of the main component, X_2 is the

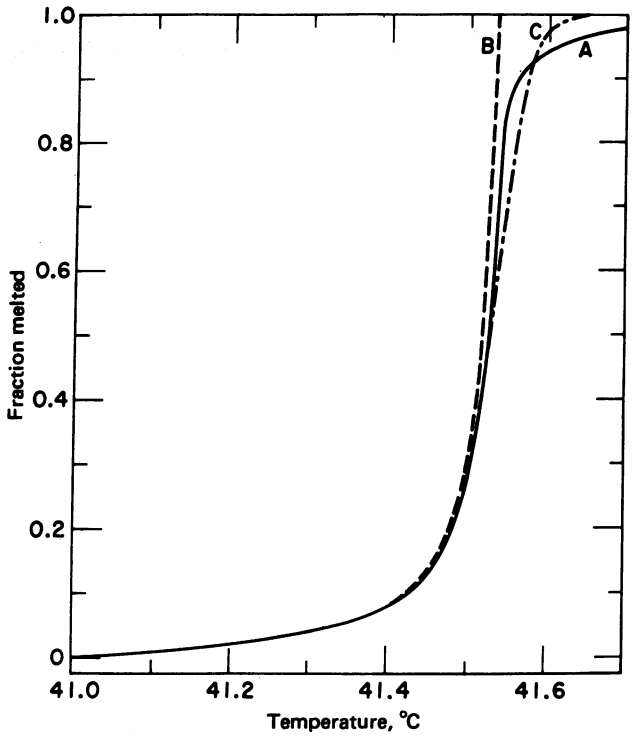

FIG. 2. The fraction of DPPC that was melted as a function of temperature. Curve A: the fraction melted, α, as a function of temperature obtained from the transition curve in Fig. 1 by planimeter integration. Curves B and C: theoretical variation of α with temperature assuming that the broadening terms given by Eqs. 1 and 2 are additive; Curve B: T_0 = 314.70 K, $\Delta H_{vH} = \infty$, X_2 = 0.00073, k = 0.029; Curve C: T_0 = 314.70 K, ΔH_{vH} = 1.2 × 10^7 cal mol^{-1}, X_2 = 0.00038, k = 0.0042.

mole fraction of the impurity, and k is the equilibrium constant for the distribution of the minor component between the solid and liquid phases. The data obtained from Fig. 1 up to α = 0.125 lie on a straight line (Fig. 3) when plotted according to Eq. 1 if k is assigned the value of 0.029. The slope of the line is 0.0164 K; when ΔH_f = 8740 cal mol^{-1} (10) and T_0 = 314.70 K as indicated by the intercept, X_2 = 0.00073. Curve B in Fig.

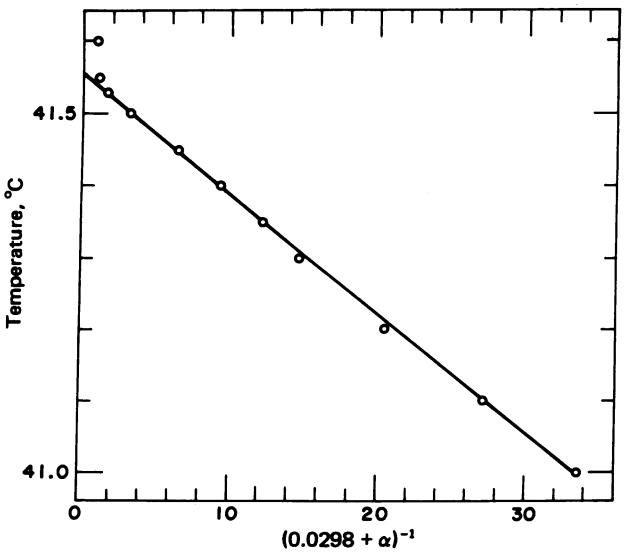

FIG. 3. The fraction melted, α, plotted as a function of temperature in the form suggested by Eq. 1, with k = 0.029.

2 is calculated by means of Eq. 1 using these values, and it agrees well with the observed curve A up to $\alpha = 0.3$.

There is undoubtedly some broadening of the experimental curve as a result of the gel phase's being composed of small domains, so that perfect cooperativity in the transition of the entire sample is not realized. In other words, the apparent or van't Hoff enthalpy for the transition is finite. If it is assumed that the transition is governed by a single van't Hoff enthalpy, integration of the van't Hoff equation gives a good approximation for a sharp transition

$$(T_0 - T)_{vH} = \frac{RT_0^2}{\Delta H_{vH}} \ln \frac{1-\alpha}{\alpha}. \qquad [2]$$

Curve C in Fig. 2 is calculated on the basis of the approximation that the impurity and van't Hoff broadenings are additive, with the following parameters: $T_0 = 314.70$ K; $X_2 = 0.00038$; $k = 0.0042$; $\Delta H_{vH} = 1.2 \times 10^7$ cal mol^{-1}. This value for ΔH_{vH} corresponds to an average cooperative unit of 1400 lipid molecules.

Consideration of the curves in Fig. 2 leads us to suggest that the sample of DPPC used in this work has a purity of at least 99.94 mol %. It also appears that the van't Hoff enthalpy for the transition is considerably larger than 1.2×10^7 cal mol^{-1}.

Above about 85% completion of the transition, the experimental transition curve deviates from any curve that can be constructed on the basis of Eqs. 1 and 2, with a single value of ΔH_{vH} used in the latter. Several reasons can be suggested for this deviation: a contribution to the deviation can be attributed to instrumental lag, but presumably not a major contribution; the impurity may not form an ideal solution in either or both of the main component phases; the distribution of domain sizes in the gel phase may be quite broad so that a distribution of values for ΔH_{vH} should be used in Eq. 2.

We believe that the transition behavior of this highly purified sample of DPPC gives convincing support to the view that the gel to liquid crystal transition in suspensions of pure phosphatidylcholines is a true isothermal first-order transition.

The enthalpy of the transition as measured in this work was 8.50 kcal mol^{-1}, 2.8% below the value, 8.74 kcal mol^{-1}, reported earlier (10). We believe the earlier value is the more reliable one because there are indications that the calorimeter does not function quantitatively correctly at extremely low scan rates, for reasons we do not understand.

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