Calorimetric Studies of Dilute Aqueous Suspensions of Bilayers Formed from Synthetic 1-α-Lecithins*

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SUMMARY

The gel-liquid crystal transitions in lipid bilayers formed from synthetic dimyristoyl, dipalmitoyl and distearoyl 1-α-lecithins have been studied by high sensitivity differential scanning calorimetry in dilute aqueous suspensions ranging in concentration from 0.4 to 6.6 mg ml⁻¹. Each lipid shows two endothermic transitions, an extremely sharp main transition and a broader transition accompanied by a smaller heat absorption at a temperature 5-10° below the main transition. The enthalpy increases in the main transition are respectively 6.3, 0.7, and 10.8 kcal per mole of monomeric lipid. The main transition widths, which are not very reproducible, reach a minimum in the case of dimyristoyl lecithin of as little as 0.2° for 10% to 90% conversion, suggesting that these transitions would be truly isothermal with completely pure lipids. The apparent heat capacities of the lipids in the liquid crystal state are, with the possible exception of dipalmitoyl lecithin, no more than 5 cal deg⁻¹ (mole of lipid)⁻¹ larger than in the gel state, indicating that the hydrocarbon chains have much less mobility in the liquid crystalline state than in the corresponding liquid normal paraffins. This conclusion was also reached by Phillips et al. (PHILLIPS, M. C., WILLIAMS, R. M., AND CHAPMAN, D. (1969) Chem Phys. Lipids 3, 234) on the basis of entropy comparisons.

Many reports have been published of studies of phospholipid bilayers in the presence and absence of water, using a wide variety of techniques. Most of these techniques, when applied to lipid-water mixtures, require high concentrations of lipids. The technique which at present appears best adapted to working with low concentrations (below 1% by weight) is differential scanning calorimetry.

We have applied this method to dilute aqueous suspensions of three 1-α-lecithins, dimyristoyl, dipalmitoyl, and distearoyl. In a previous communication (5) we have given the results of a calorimetric study of aqueous suspensions of mixtures of cholesterol with dimyristoyl lecithin and dipalmitoyl lecithin.

MATERIALS AND METHODS

Lipids were purchased from Analabs, North Haven, Connecticut, Serdary Laboratories, London, Ontario, and Calbiochem, San Diego, California. Considerable differences, particularly with respect to the sharpness of transitions, were observed with different preparations, to the extent of having no observable transition in one or two cases. All the results reported here were obtained with dimyristoyl lecithin, Lot 100822, dipalmitoyl lecithin, Lot 100653, and distearoyl lecithin, Lot 100296, all obtained from Calbiochem. These preparations appeared homogeneous in silica gel thin layer chromatography (using chloroform methanol water in volume ratios 60:30:5). Suspensions were in most instances prepared by heating the solid lipid in water to a temperature well above its transition temperature and then shaking the mixture vigorously for 10 to 15 min in a Super-mixer (Lab-Line Instruments, Melrose Park, Illinois). The suspension was then reheated above its transition temperature and cooled rapidly in ice water. The pH of the suspensions was always within the range 6.5 to 7.2, and underwent no significant change as a result of two or more successive heatings and coolings in the calorimeter.

In some experiments lipid concentrations were estimated by phosphate determinations (6) on the suspensions. An equally valid measure of concentration appeared to be afforded by the weight makeup of the suspension, and this more convenient measure was used in many of the experiments. The lipid preparations were shown to be anhydrous by the fact that

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† Note Added in Proof.—It has been found that prolonged sonication of the lipid suspensions leads to disappearance of the transitions discussed in this paper. This matter will be more fully considered in a future publication.
Fig. 1. The variation with temperature of the excess specific heat (Curve A, right hand ordinates) and the excess enthalpy (Curve B, left hand ordinates) during the gel to liquid crystal transition of dipalmitoyl L-α-lecithin in aqueous suspension at a lipid concentration of 3.88 mg per ml. The points on Curve B are the experimental data recorded digitally at 1-min intervals. The evaluation of the heat changes $\Delta q_1$ and $\Delta q_2$ is described in the text. The slope of the line after the upper transition corresponds to an apparent heat capacity increase, relative to the situation below the lower transition, of 13 cal deg$^{-1}$ per mole of lipid.

Fig. 2. The variation with temperature of the excess specific heat in the lower transition shown in Fig. 1, plotted on a greatly enlarged scale. The points are experimental data recorded digitally at 1-min intervals. The dashed curve is drawn to give a symmetrical specific heat curve.

they suffered no significant loss in weight after several hours of heating at 80° in a vacuum (10 to 15 mm Hg).

The differential scanning calorimeter used in these experiments has been previously described (7, 8). Recent modifications of the equipment include provision for digital recording of all pertinent data on punched paper tape for convenient computer work-up. The calorimeter holds 1.94 ml of suspension, and can detect heat absorption, in excess of that required for the usual heating rate of 18° per hour, amounting to as little as $2 \times 10^{-3}$ cal per deg of temperature rise. In a few experiments a heating rate of 4.5° per hour was employed.

RESULTS

The results of a typical experiment with dipalmitoyl lecithin are illustrated in Fig. 1. Curve A is a plot of the excess specific heat of the lipid suspension as a function of temperature and Curve B is the corresponding integral curve. Both curves are plotted from data recorded digitally at 1-min intervals, the experimental points being shown only for Curve B. A small heat absorption centered at 33.5° precedes the main transition at 41.5°. The existence of these two transitions complicates the evaluation of the corresponding enthalpy changes since the excess specific heat does not go to zero between them. In low sensitivity scanning calorimetry, where the base line tends in any case to be rather irregular, the usual procedure adopted is to draw a line such as dashed line b in Fig. 1, and to evaluate the integral above this line. In the present case this gives $\Delta H_2 = 8.56$ kcal mole$^{-1}$ for the upper transition, in good agreement with the value 8.66 reported by Phillips et al. (9).

An alternative interpretation is suggested by the fact that the excess specific heat is very nearly zero below the first transition and above the second transition. If the excess specific heat is plotted on a larger scale as in Fig. 2, it appears that the over-all curve can be resolved into two transitions each with base line
TABLE I

| Lipid | Experiments | Concentration range | Lower transition | Upper transition |
|-------|-------------|---------------------|------------------|------------------|
|       |             | mg/ml               | $T_m^2$          | $\Delta H_1$     | Coop. unit | $T_m^2$ | $\Delta H_2$ | Coop. unit |
| DML   | 9           | 0.40-6.56           | 13.5 ± 0.2       | 1.1 ± 0.2        | 200 ± 50 | 23.70 ± 0.09 | 6.23 ± 0.18 | 200 ± 40 |
| DPL   | 6           | 0.78-4.33           | 34.0 ± 0.2       | 2.3 ± 0.2        | 70 ± 10  | 41.73 ± 0.06 | 9.09 ± 0.21 | 70 ± 10  |
| DSL   | 10          | 0.87-5.38           | 49.1 ± 0.2       | 1.4 ± 0.2        | 230 ± 40 | 38.24 ± 0.03 | 10.84 ± 0.17 | 80 ± 10  |

* Numbers in parentheses refer to Reference 9.

The baseline problem is less prominent than in that of dipalmitoyl lecithin, in the former case because the lower and upper transitions are more widely separated in temperature and there is a close approach to zero excess specific heat between them, and in the latter case because the transitions are close together so that little enthalpy is absorbed in the region between them. This accounts for the fact that our mean values of $\Delta H_1$ for dimyristoyl lecithin and distearoyl lecithin agree well with those of Phillips et al. (9) while our value for dipalmitoyl lecithin does not. Although in our earlier report (5) on the effect of added cholesterol on lipid transitions we used the first method considered above for evaluating enthalpies, and thus arrived at a considerably smaller value for the transition enthalpy of dipalmitoyl lecithin than given in the present paper, the relative effects produced by cholesterol are not significantly in error.

Some minor anomalies were observed in some of the experiments. For example it was found that a suspension of distearoyl lecithin freshly prepared and rapidly cooled as described above showed a small, sharp heat absorption between the first and second transitions, as shown in Fig. 3. This phenomenon was absent if the material was heated after having been slowly cooled from above its higher transition temperature.

The samples in the calorimeter were in nearly all cases cooled in situ during a period of 1 to 8 hours and reheated. The behavior on reheating was always similar to that observed in the first heating except that the transitions were usually somewhat broadened, though without any significant change in total heat absorption.

Table I summarizes the results obtained for dilute aqueous suspensions of dimyristoyl lecithin, dipalmitoyl lecithin, and distearoyl lecithin. Data are given for both the lower and upper transitions. The error estimates shown in the table are standard errors of the mean, indicating primarily the calorimetric reproducibility, and are thus minimum estimates of the uncertainties in the data, particularly those pertaining to the lower transitions. $T_m^1$ and $T_m^2$ are the temperatures at which the enthalpy changes are half completed. The evaluation of $\Delta H_1$ and $\Delta H_2$ from the experimental data was outlined above; they are expressed in kcal per mole of monomeric lipid. The sizes for the apparent cooperative units given in columns 6 and 9 are simply the ratios of the enthalpies derived from the van't Hoff equation to the corresponding calorimetric enthalpies. If heat absorption is assumed to be a linear measure of the extent of the cooperative phenomena, the term $\Delta H_1$ can be expressed as follows.

The significance of the term cooperative unit can be expressed as follows. The temperature course of the transition is approximately that to be expected for an assembly of independent units of the indicated size each one of which shows a strictly two state, or all-or-none, transition.
transition under observation, the van't Hoff expression for a
two state process in which no dissociation takes place can be
put in the form
\[
\left( \frac{d\alpha}{dT} \right)_{T=T_m} = \frac{\Delta H_{\text{fH}}}{4 RT_m^2}
\]
where \( \alpha \) is the fractional completion of the enthalpy absorption.
In point of fact some of the transition curves deviated signif-
ificantly from the symmetrical form expected for a two state transi-
tion with no change in heat capacity, so that application of Eq-
uation 1 is only approximately valid.

**DISCUSSION**

As mentioned above, the values for \( T_m \) and \( \Delta H_2 \) given in Table
I agree moderately well with those reported by Phillips et al. (9).
The largest discrepancy, in the value of \( \Delta H_2 \) for dipalmitoyl
lecithin, presumably results from the base line problem frequently
encountered in scanning calorimetry. The agreement between
these independent sets of data is particularly interesting in that
the suspensions used in the work quoted by Phillips et al. (9)
were as much as four orders of magnitude more concentrated
than our most dilute suspensions, and were thermally scanned at
rates an order of magnitude or more higher than we em-
ployed.

An outstanding feature of these transitions is their sharpness.
The transition width was to some extent dependent on the pre-
vious history of a particular sample, and varied considerably
with different preparations of the same lipid, so that the transi-
 tion widths indicated by the cooperative unit \( \Theta \) sizes given in Table
I cannot be considered as quantitatively determined charac-
teristic properties of the lipids. In some experiments dimyristoyl
lecithin was observed to melt (10% to 90%) within about 0.2\( ^\circ \),
though the usual transition width was larger than this. Our
observed main transition widths are much smaller than those
previously reported on the basis of scanning calorimetry (10) and
of observations of changes in the fluorescence of a probe incor-
porated in the bilayer (11). Even the lower transitions, which
are broader than the main transitions, turn out to be char-
acterized by the same high degree of cooperativity as the main
transitions, if it is assumed that the lower transition also involves
all of the molecules present. It was shown by experiments at
one-fourth the usual scanning rate that the transition of dimy-
ristoyl lecithin is not significantly broadened by calorimetric
lags. However, since any impurities would broaden the transi-
tions, it seems likely that the gel to liquid crystal transition in
pure lipid bilayers is truly isothermal.

The lower transition, or pretransition peak, was observed by
Chapmann et al. (10) and was suggested by Ladbrooke and
Chapman (12) to be due to a rotation of the polar head portion of
lipid molecules. As noted by Ladbrooke and Chapman, the tem-
perature separation between the pretransition and the main
transition decreases with increasing chain length, extrapolating
to zero at dibehenoyl \( \alpha \)-lecithin with chains 22 carbons

Precise heat capacity data (13) for the normal paraffins up to
\( C_{29} \) show that those having an odd number of carbon
atoms greater than seven undergo a phase transition between
orthorhombic and hexagonal (14) crystal forms a few degrees
below the melting point. The heat capacities of these com-
 pounds in the hexagonal form are higher than in either the or-
thorhombic or liquid states, and increase rapidly with tempera-
ture. Infrared data (15) and other evidence have led to the view
(14) that the paraffin chains, in fully extended form, undergo co-
operative rotation about their long axes in the hexagonal crystal.
In view of the fact that the enthalpies of these transitions are
similar in magnitude to those of the pretransitions observed with
phospholipids, the question arises as to whether the pretransitions
may not also lead to cooperative rotation of the hydrocarbon
chains. If this is indeed the case, and if the intermediate state
has a relatively high heat capacity as in the case of the normal
paraffins, then the procedure we have employed in evaluating
\( \Delta H_1 \) and \( \Delta H_2 \) is incorrect, and the values of \( \Delta H_2 \) given in Table I
are too high by approximately 2, 7, and 4% for dimyristoyl leci-
thin, dipalmitoyl lecithin and distearoyl lecithin, respectively.

Phillips et al. (9) found a linear relation between the length of
the hydrocarbon chains in the lecithins dimyristoyl lecithin
through dibehenoyl lecithin and the enthalpy of the main
transition, whereas our data suggest a nonlinear relation. Con-
ideration of the base-line problem, and of the fact that the transi-
ton enthalpy for dibehenoyl lecithin presumably includes an
unknown contribution from the pretransition, may alter
somewhat the enthalpy-chain-length relation given by Phillips
et al. (9).

In nearly all our experiments the excess specific heat returned
very closely to zero above the main transition. In a few experi-
ments with dipalmitoyl lecithin there was a slight continuing
apparent absorption of heat above the transition amounting to
10 to 20 cal deg\(^{-1}\) (mole of lipid)\(^{-1}\), or 5 to 10 cal deg\(^{-1}\) (mole of
hydrocarbon chain)\(^{-1}\). This is illustrated in Fig. 1. In other
experiments the excess heat capacity above the transition amounted to
no more than a fifth of this amount. The increase in heat capacity on melting a normal alkane, on the other hand, is of the order of 17 cal deg\(^{-1}\) mole\(^{-1}\) (13). It is thus evident
that many fewer degrees of freedom become excitatable as a re-
sult of the lipid transition than in the melting of an alkane, and
that the hydrocarbon chains in the lipid liquid crystal have con-
siderably less mobility than in a liquid alkane. This conclusion
is supported by the corresponding entropy data, as shown by
Phillips et al. (9).

Transfer of lipid molecules from the bilayer to the aqueous
phase would be expected to be accompanied by a large increase
in apparent specific heat because of the structuring of water
around the released hydrocarbon chains. An estimate of this
effect is difficult to make because of lack of data for appropriate
model systems, but it seems safe to conclude that the small values
for \( \Delta C_p \) actually observed in the transitions mean that no more than
1%, and probably considerably less than this, of the hydro-
carbon chains can leave the bilayer during the transition.

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