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New phases of DPPC/water mixtures

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Hydration of DPPC at low temperatures yielded two new phases, a non-lamellar C1 phase and a lamellar C2 phase, as well as the normal gel phase, depending upon the initial physical state of the dry lipid. From the results of wide-angle diffraction and calorimetry the C2 phase appears very similar to the normal C phase, but the *D* spacing is considerably larger, suggesting that the C2 phase is a C phase with untilted chains.

The normal method of preparing hydrated multilamellar vesicles (MLVs) involves heating the lipid/water mixture above the main phase transition temperature. The normal procedure is ideal for many model membrane studies since it can be carried out rapidly with minimal resources. Our original motivation for using a non-standard hydration procedure was to obtain the fully hydrated subgel C phase of DPPC more rapidly. The normal procedure to form the C phase is to start with a fully hydrated gel phase, lower the temperature to below 7°C and wait for a long period of time [1,2], during which the sample partially dehydrates [3]. Instead we hoped to form the C phase by approaching from the other end, namely, to start from a dehydrated crystal form and introduce water at low temperature.

The following will be called the cold hydration procedure: Dry lipid was placed in a test tube on ice for one hour so that water condensed onto the lipid. The sample was then transferred to a 5°C cold room and a small amount of cold water (about the same mass as the lipid) was added to the lipid. The mixture was crushed with a glass rod to produce a homogeneous paste. Then excess cold water was added. The glass rod was used again to crush and stir the suspension.

Lipids that are not fully hydrated are characterized by a broadened main transition at higher temperature and absence of the pretransition [4]. Lyophilized lipid taken directly from the supplier (Avanti Polar Lipids) and cold hydrated exhibited basically the same main transition and pretransition in DSC scans (curve B in Fig. 1) as normally hydrated DPPC (curve A in Fig. 1), suggesting that cold hydration produces normal, fully hydrated bilayers of DPPC. However, the DSC traces for the cold hydrated samples exhibited complex behavior below 30°C. Three low temperature peaks are identified in Fig. 1 as C1, C2 and C3. For many variations of the cold hydration method DSC peaks occurred at similar temperatures as in Fig. 1B although the relative sizes of the peaks were different. (For more details on this and many other aspects of this report, see the Ph.D thesis of C.P. Yang.)

To investigate if cold hydration were the primary cause for the additional low temperature peaks, the experiment was repeated with a small

Abbreviations: DSC, differential scanning calorimetry; DPPC, L-α-dipalmitoyl-phosphatidylcholine.

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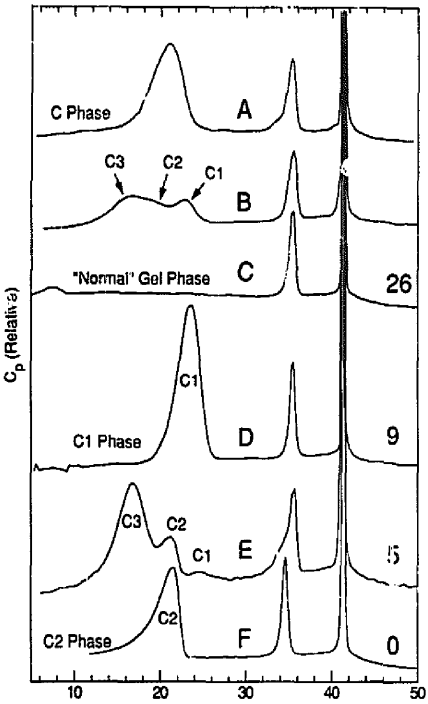

Fig. 1. Specific heat (displaced scale) versus temperature (°C) for DSC scans on DPPC/water at 13 C°/h. A. Normally hydrated sample after incubation at 0.2°C for 110 days. B. Cold hydrated sample taken directly from supplier (Avanti). C. Sample was normally hydrated, dried, then cold hydrated. D–F. Samples were cold hydrated after drying from chloroform/water mixtures where the numbers above the right hand side of the traces represent the number of waters per lipid molecule deliberately added before chloroform addition and drying.

variation. The dried lipid was first hydrated normally, then dried under various conditions, and then cold hydrated. The result was that none of the C1, C2 and C3 peaks was found. Clearly, the origin of the C1, C2 and C3 peaks must be in the physical state of the dried lipid before cold hydration. Since the stock lipid powder was lyophilized from chloroform, chloroform was an additional factor which could affect the states of the dried lipid. Indeed this was found to be the case. When the same lipid sample that gave the results in the previous paragraph was dried again, then mixed with excess chloroform, then dried again at room temperature and finally cold hydrated, essentially the same DSC trace as in curve B in Fig. 1 reappeared.

In order to address the original objective, whether the subgel C phase can be prepared by cold hydration, it is necessary to characterize the three peaks C1, C2 and C3. The C3 peak is not stable and grows into the C2 peak in the same fashion as the C* peak grows into the C peak, which has been described in Ref. 5. The C1 and C2 transitions are the fundamental ones and the phases that give rise to these transitions will be called the C1 and C2 phases.

To explore whether a sample could be prepared with only one of the low temperature phases instead of a mixture, variations were made on the chloroform evaporation procedure. The first variation was to carry out the chloroform evaporation at low (1–5°C) temperature. An isolated DSC peak, essentially identical to the one shown in curve D in Fig. 1, was found at 24°C. After this initial observation of an isolated C1 peak, reproducing it consistently proved difficult. We found that this was due to variations in the amount of water present during the chloroform evaporation.

Preparatory to presenting our final results, a more detailed account of the protocol for preparing samples is given as follows: The lipid was first dried thoroughly in a vacuum oven at 50–60°C for 24 h for maximal removal of residual water. Small amounts of water (0 to 23 μl), measured by a calibrated Hamilton micro syringe, were added to 10 to 70 mg of DPPC in a 10 ml test tube. An excess amount (approx. 2 ml) of chloroform (Aldrich, HPLC grade) was added and the sample was vortexed on a Bronwill mixer briefly to disperse the lipid/water mixture as thoroughly as possible in the chloroform. This mixture was then immediately cooled to about 2°C and dried under low vacuum. After the sample turned into a white powder, which usually took 12 h, it was placed under high vacuum for 15 min, while keeping the sample cold. This material was then cold hydrated as described in the second paragraph of this report.

DSC results for samples prepared according to the above procedure are shown in curves C-F in Fig. 1. Curve D (with 9 water molecules added per lipid molecule) and curve F (with no added water) show that the C1 and C2 peaks can be produced

separately. When 26 waters per lipid were added curve C was reproduced, so the normal gel phase can also be produced. Curve E shows that mixed phases can be produced when the amount of water added is intermediate between those amounts that give the pure phases. The numbers above the curves in Fig. 1 are not absolute measures of the hydration level during the final removal of chloroform. In particular, when a bigger vial with flat bottom was used, an initial addition of 29 water molecules per lipid produced the same result as 9 waters per lipid when a test tube was used.

It is interesting to note that the C2 phase is also characterized by a 1 C° lower pretransition temperature, as can be seen by comparing curves F and E in Fig. 1 with the other curves. This suggests that the gel phase after the C2 transition is not 'normal'. Incubation of this abnormal gel phase at 23°C for 4 h raised the pretransition temperature about 0.3 C°, suggesting conversion into the normal gel phase on this time scale.

Once a procedure was established for preparing samples that were either in the C1 phase or in the C2 phase and not in some complicated mixture, diffraction studies were performed to determine whether either of these phases is the normal C phase. Structural information provided by X-ray diffraction performed at 5–10°C is given in Table I for the C1 and C2 phases as well as the normally observed C phase. For the C1 phase there is only one low angle peak that can be resolved at 64.9 Å. Even if one assumes the disappearance of the second and third reflections of a lamellar pattern, the reflections at 15.3 Å and 9.19 Å do not index with the 64.9 Å peak. From this we suggest that the C1 phase is a nonlamellar structure, although the structure of the C1 phase is otherwise unknown at this time.

The X-ray diffraction experiment on the C2 phase, on the other hand, did show a lamellar repeat pattern. The second, third and fourth orders were found at 32.9, 22.0 and 16.6 Å and an identifiable first order was found near 66 Å. Although C2 and C have similar chain packing, as evidenced by the wide angle spacings, the *D*-spacings are considerably different. We suggest that the C2 phase is a more dehydrated form of the C phase, thus reducing the head group area of the lipid, so that the chains can be less tilted [6,7]. For a tilt angle of 34° for the C phase [8] and an all-*trans* chain length of 20 Å, complete untilting of the chains adds an additional 8 Å to the bilayer thickness. This would increase the *D*-spacing of the C phase to 67 Å, close to what is observed for the C2 phase.

TABLE I

DIFFRACTION AND DSC DATA FOR THE C1, C2 AND C PHASES

Strong reflections are represented by italic type face numbers. The C phase X-ray data are from Ref. 4. T_S is the apparent transition temperature at a scan rate of 13°C/h, not the true equilibrium transition temperature.

Phase	C1	C2	C
D spacing (Å)	None [a]	*66.1 ± 0.5*	*59.1 ± 0.1*
Wide-angle spacings (Å)	*15.3 ± 0.1*	10.0	
	9.19 ± 0.04	9.08 ± 0.2	9.30
	8.11 ± 0.03	8.56 ± 0.2	
	6.31 ± 0.03	6.56 ± 0.2	6.81
	5.86 ± 0.02	6.04 ± 0.09	
	5.38 ± 0.03		
	5.03 ± 0.02		4.90
	4.73 ± 0.02		4.52
	4.59 ± 0.01	*4.44 ± 0.03*	*4.43*
	4.38 ± 0.01		
	4.23 ± 0.01	4.21 ± 0.09	4.2
	4.05 ± 0.01		
	3.96 ± 0.01	3.91 ± 0.08	
	3.82 ± 0.01		3.83
Calorimetric data			
T_S (°C)	24	21.4	21.5
ΔH (kcal/mol)	9	4.2	4.1

[a] No lamellar repeat was observed. All observed reflections are listed under wide-angle except for one at 64.9 ± 1.5 Å.

The similarity of the DSC results for the C2 transition and the C subtransition is quite striking as shown in Table I. Without the results of low angle X-ray diffraction, one could be misled to believe that the C2 phase is the same as the C phase. The similarities in the packing of the chains between the C2 phase and the C phase (see Table I) may account for the similarities in the thermodynamics of the two transitions since theories indicate that the cohesive forces holding the membrane together are relatively insensitive to chain tilt [9].

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