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Metastability in the Phase Behavior of Dimyristoylphosphatidylethanolamine Bilayers†

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ABSTRACT: A new subgel phase is demonstrated to occur in hydrated dimyristoylphosphatidylethanolamine (DMPE) by using dilatometric and calorimetric techniques. The formation of the subgel phase takes place very slowly at temperatures near 0 °C, but it can still be observed at 25 °C. Once formed, the subgel phase melts (ΔH_h = 16.0 ± 0.6 kcal/mol and ΔV = 0.085 ± 0.014 mL/g) directly into the liquid-crystalline phase at a temperature, T_h = 56.3 °C, that is higher than the gel to liquid-crystalline transition temperature, T_m = 49.6 °C. Thus, the gel phase appears to be metastable over its entire temperature range. In this regard, DMPE behaves differently from dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine but similarly to dilaurylphosphatidylethanolamine. This unusual long-lived metastability provides cells an additional option in determining the properties of membranes.

Low-temperature phases in phospholipid bilayers and the phase transitions into such phases are important references with which to compare the biologically relevant high-temperature phase, L_α, and from which to obtain quantitative information concerning the molecular interactions that determine bilayer structure (Nagle & Wilkinson, 1978). The discovery by Chen and co-workers (Chen et al., 1980) of the subtransition in saturated phosphatidylcholines having 16, 17, or 18 carbons per chain has been followed by some detailed studies of the kinetics of formation and the density of the new subgel phase (Nagle & Wilkinson, 1982) and by structural studies of the subgel phase using diffraction and spectroscopy (Fuldner, 1981; Ruocco & Shipley, 1982; Cameron & Mantsch, 1982). Characterization of the subgel phase promises to provide an even simpler reference state than either the L_β or the P_β phase.

It is of interest, therefore, to determine if subgel phases occur in other lipids as well as in the phosphatidylcholines. In an

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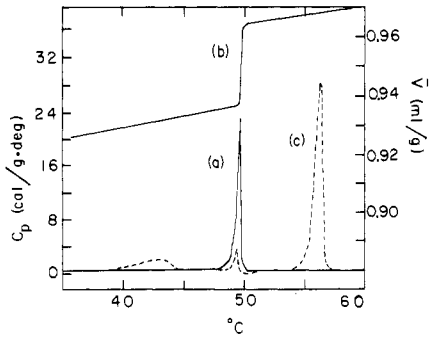

FIGURE 1: (a) Specific heat vs. temperature for gel and liquid-crystalline phases of DMPE. (b) Specific volume vs. temperature for gel and liquid-crystalline phases of DMPE. (c) DSC heating scan of DMPE incubated at 2 °C for 6 days (dashed line).

Table I: Thermal Transition Properties of DMPE at Neutral pH

transition	T_m (°C)	$\Delta T_{1/2}$	ΔH (kcal/mol)	ΔV [(mL/g) × 10^5]
gel, lc	49.6	0.3	5.7 ± 0.1	2633 ± 280
subgel, lc	56.3	0.6	16.0 ± 0.6	8484 ± 1400

earlier paper (Wilkinson & Nagle, 1981), we reported on additional polymorphism in fully hydrated dilaurylphosphatidylethanolamine (DLPE)[1] beyond the usual L_β to L_α transition. It was subsequently shown (Chang & Epand, 1983) that the new phase in DLPE was a highly ordered one with similarities to the subgel phase of the PC's. In this paper, we present evidence for a subgel phase in DMPE and report on some of its thermal properties.

Materials and Methods

The phosphatidylethanolamines used in this study were obtained from Calbiochem-Behring Corp. and Avanti Biochemicals. The half-widths of the main transition observed in scanning experiments were sufficiently small (<0.5 K) to assure high purity of samples.

Proper hydration of the samples is critical (Mantsch et al., 1983) and was performed as follows. To the weighed dry lipid powder was added either water or 20 mM buffer (phosphate, pH 7.0) to produce suspensions whose concentrations were 2–3% for dilatometry and 0.1% for calorimetry. The lipid and buffer were heated to 70 °C for several minutes, vortexed vigorously for approximately 30 s, and then cooled to 30 °C. This heating–cooling procedure was repeated an additional 3 times.

The specific volume as a function of temperature or time was determined by using a differential dilatometer (Wilkinson & Nagle, 1978). Calorimetry was performed with a Microcal MC-1 (Amherst, MA) differential scanning calorimeter. Heating rates of 3–4 °C/h for dilatometry and 10 °C/h for calorimetry were employed.

Results

When DMPE is hydrated as described under Materials and Methods, only one transition representing the melting of the gel phase is observed (see curves a and b of Figure 1). The thermodynamic parameters which characterize this transition

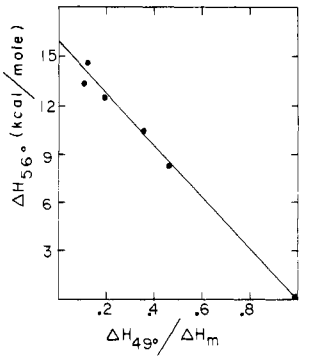

FIGURE 2: Enthalpy change at 56 °C [$\Delta H_{56}(t)$] as a function of the ratio of $\Delta H_{49}(t)$ to ΔH_m for six incubation times (t).

are listed in Table I. Compared to our previous study (Wilkinson & Nagle, 1981), the present data reflect certain improvements. Thus, the midpoint of the transition occurs at a slightly higher temperature, and the half-width is slightly smaller than that seen earlier, indicating a somewhat purer lipid. Our value for the enthalpy change is unaltered and is essentially the same as other literature values (Mabrey & Sturtevant, 1978) while that for the specific volume change (ΔV) is now larger. It should be noted that ΔV for DMPE is now nearly the same as it is for DMPC (Nagle & Wilkinson, 1978).

Dilute DMPE samples stored at 2 °C for long periods of time display additional endothermic peaks during the first heating scan in the calorimeter. Besides the usual one at T_m = 49.6 °C, there are endotherms with maxima in the heat capacity curves at T_l = 43.3 °C and T_h = 56.3 °C. An example is shown in Figure 1c where the DMPE suspension had been incubated for approximately 6 days at 2 °C. When a sample such as the one shown in Figure 1c is rescanned, only one transition at T_m is observed as shown in Figure 1a. With increasing incubation time at low temperature, the enthalpy change at T_m (ΔH_m) becomes smaller and the enthalpy change at T_h (ΔH_h) correspondingly larger. By plotting the enthalpy change at 56 °C as a function of the ratio of the observed $\Delta H_{49}(t)$ at time t to ΔH_m [$=\Delta H_{49}(0)$] (i.e., no incubation at low temperature) and extrapolating to the point where the enthalpy changte at 49 °C is zero, one can obtain ΔH_h. The result is shown in Figure 2. ΔH_h is then found to be 16.0 ± 0.6 kcal/mol. The linearity of Figure 2 supports the concept that the sample is divisible into two fractions, one melting at T_m and the other melting at T_h.

Similar results were obtained by using dilatometry. That is, the volume change associated with the chain melting at T_m decreased with time when samples were incubated at low temperatures, while the volume change at T_h increased. Once again, if one assumes that all the lipid melts at either T_m or T_h, one can calculate the maximum volume change associated with T_h. The value so calculated is, in fact, within experimental error of the volume change measured at T_h when there is no detectable transition at T_m. These values are summarized in Table I along with the calorimetric data.

More complicated polymorphism was evident in certain samples of DMPE. Occasionally, we observed a splitting of the DSC peak at T_h. However, after a longer time at low temperature (i.e., 2 °C), only one endothermic peak appeared at T_h. Mantsch and co-workers (Mantsch et al., 1983) described two polymorphs of anhydrous DLPE that gave a double peak at T_h. Our results suggest that such polymorphs may exist also in hydrated DMPE and furthermore that they can either interconvert, as do certain polymorphs of DLPE (Seddon et al., 1983), or perhaps convert into a third polymorphic type.

[1] Abbreviations: DLPE, dilaurylphosphatidylethanolamine; DMPE, dimyristoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; DSC, differential scanning calorimetry.

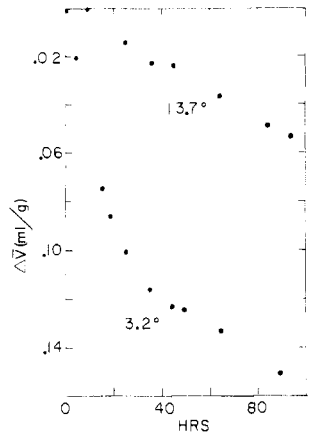

FIGURE 3: Relative specific volume as a function of incubation time at 13.7 and 3.2 °C.

The kinetics of formation of the subgel phase can be followed by using the dilatometer as shown in Figure 3. The rate $R(T)$ is defined as the initial slope of these graphs. At 3.2 °C, $R(T) = 48 \times 10^{-5}$ mL/(g·h); at 13.7 °C, $R(T) = 7 \times 10^{-5}$ mL (g·h). While these rates appear much larger than those measured for DPPC (Nagle & Wilkinson, 1982), it must be kept in mind that the overall volume change is approximately 8 times larger for DMPE. Thus, the relative rates (fractional change per unit time) are in fact quite comparable. A DSC sample incubated at 25 °C also showed evidence for subgel formation at a rate that was comparable to the one measured at 13.7 °C.

In order to provide further evidence that a nucleation phenomenon is present in subgel phase formation, "incubated T-jump" experiments (Nagle & Wilkinson, 1982) were performed. That is, the sample is first incubated at a low temperature (T_I) to initiate the formation of the subgel phase. The temperature is then quickly raised to a new value (T_J). For $T_I = 3.2$ °C and $T_J = 17.3$, 25.2, and 45.2 °C, the volume continues to decrease at T_J, corresponding to further conversion into the subgel phase. Hence, once it is nucleated, the subgel phase is more stable than the gel phase at any temperature.

To determine which phase is stable in the region between 49 and 56 °C, the following experiment was done. A sample was incubated for 50 h at 2 °C to nucleate the subgel phase and then scanned in the calorimeter up to 52 °C, where the temperature was held constant for 20 h. At that point, the sample was cooled and immediately rescanned through T_m and T_h. It was found that ΔH_{49} decreased by nearly 50% following the incubation of 52 °C. Thus, at 52 °C, conversion of the liquid-crystalline phase into the subgel phase was occurring. Therefore, the subgel phase is more stable than the liquid-crystalline phase between T_m and T_h.

Discussion

The polymorphism displayed by DMPE and described in this paper includes, besides the usual gel and liquid-crystalline phases, a slowly forming low-temperature phase which melts at a higher temperature than the gel phase. Because of the similarity to the phase behavior of hydrated DLPE, we assign the endothermic peak at T_h to the melting of a more ordered, less hydrated subgel phase into the liquid-crystalline phase. Rather surprisingly perhaps, the parameters describing this transition, T_h and ΔH_h, are quite similar to corresponding values ascribed to the melting and hydration of poorly hydrated DMPE (Mantsch et al., 1983). The subgel phase of DMPE (and DLPE) then is like the subgel phases of saturated PC's in being more crystalline and less solvated than the gel phase,

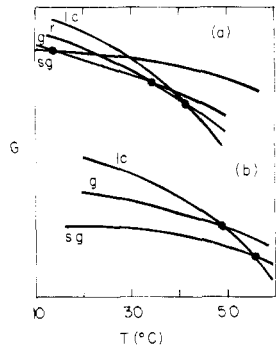

FIGURE 4: Schematic free-energy (G) vs. temperature (T) diagram for the phases lc (liquid crystalline), r (rippled), g (gel), and sg (subgel). Phase transitions that have been observed are marked with dots at the intersections of the free-energy curves: (a) DPPC; (b) DMPE.

but in many ways it is more complicated and interesting.

A schematic free-energy diagram (G vs. T) for the four phase [lc (liquid crystalline), r (rippled), g (gel), and sg (subgel)] is shown in Figure 4a for DPPC. The stable phase at a given temperature is the one with the lowest free energy. For the PC's, the succesion of stable phases as T is increased is sg, g, r, and lc, with each phase having a temperature range in which it is absolutely stable. The reason that the subgel phase was not discovered until very recently is that it forms only very slowly at low temperature. Because of its low-temperature character, the subgel phase in the PC's probably has no direct biological relevance, although it is relevant as the most ordered phase to which the other phases should be compared.

In contrast to the subgel phase in the PC's, the subgel phase in DMPE and DLPE might be biologically important. In Figure 4b, the schematic G vs. T diagram for DMPE shows that there are only two absolutely stable phases, sg and lc. The gel phase is metastable at all temperatures, but the formation of the subgel phase is so slow in pure lipid bilayers that the gel phase has previously been assumed to be the stable one. Furthermore, there is a temperature interval, from 49.5 to 56.3 °C, in which the liquid-crystalline phase has usually been assumed to be stable but where it too is metastable with respect to the subgel phase. However, in a cell living in this temperature range and containing DMPE in its membrane, heterogeneous nucleation might possibly catalyze the transition to the sg phase in much shorter times than reported in this paper. (This temperature range is somewhat high for most organisms. DLPE which appears to have similar phase behavior might be a better example.) Although it is generally thought that cell membrane lipids are mainly in a fluidlike state at physiological temperatures, patches of rigid solidlike lipids would not necessarily be harmful. In particular, since lateral diffusion in lipid bilayers (Fahey & Webb, 1978) and lipid membrane permeability (Tse & Singer, 1983) are strongly affected by lipid fluidity, it is feasible that phases such as the subgel could be important in the regulation of certain membrane functions. More interesting is the existence of a long-lived metastable state which stores a great deal of free energy which could be released and coupled to functional events by an appropriate mechanism which would catalyze the transition. Of course, this line of speculation presently has thermodynamic support only for two highly pure PE's. But it does suggest that further search for a subgel phase in a wide variety of lipids is warranted.

Another example of the more complicated phase behavior of DMPE is the small transition observed at 43 °C. Its origins are not at all clear. This transition occurs only in conjunction

with T_h (i.e., after low-temperature incubation), and when ΔH_h is zero, there appears to be no endothermic peak at 43 °C either. However, we were unable to measure the growth of it as a function of $\Delta H_{49}/\Delta H_m$, for example, because of its relatively small size and the large relative error. Further characterization of it by diffraction or spectroscopic techniques is necessary.

The slightly larger volume change reported here for the main transition of DMPE when compared to our previous data is most likely a result of using the more vigorous hydration procedure. It has been shown that the upper transition in DLPE can occur if the lipid is not fully hydrated (Mantsch et al., 1983). Assuming that DMPE behaves in a similar fashion leads one to conclude that our previous determination of the volume change for this lipid was too small because a fraction of it was incompletely hydrated and would not have melted until 56 °C had the scans gone that high in temperature.

As described in detail elsewhere (Nagle & Wilkinson, 1978), the volume change can be used in computing the energetics of the transition. The nearest-neighbor chain–chain separation just above the transition, r_a, is calculated to be 5.00 Å by using the new ΔV and the previous value of the chain separation below the transition, r_b. With these values of r_a and r_b, the van der Waals interaction energy contribution (U_{vdW}) is 3.5 kcal/mol. When ΔU_{vdW} and ΔU_{other} are subtracted from the measured enthalpy change, the remainder, ΔU_{rot}, is 1.6 kcal/mol. Thus, the change in the number of gauche rotamers, Δn_g, is 3.2, or about one fewer per molecule than we had previously obtained (Wilkinson & Nagle, 1981). This means that the energetics of the main transition in DMPE and DMPC are even more similar than we had once thought.

A similar calculation can be performed for the upper transition. To estimate r_b, we shall use X-ray diffraction data on DLPE for the subgel phase (Chang & Epand, 1983), although the volume per methylene group is probably slightly lower for DMPE than for DLPE (Wilkinson & Nagle, 1981). Their value for the cross-sectional area per chain at 20 °C (i.e., 23 °C below T_h) is 19 Å^2 which corresponds to a volume per methylene group of 24.1 Å^3. Allowing for thermal expansion over a temperature interval of 23 °C, we calculate the volume per methylene group to be 24.8 Å^3, just below T_h for DLPE and DMPE. For the measured ΔV, we obtain ΔU_{vdW} = 12.6 kcal/mol, which accounts for three-fourths of ΔH_h. When this and ΔU_{other} (10% of ΔH_h) are subtracted from ΔH_h, the remainder, ΔU_{rot}, equals 1.8 kcal/mol. Δn_g then is 3.6, which is slightly more than that for the melting of the gel phase. This suggests that the subgel phase, when compared to the gel phase, involves closer packing of chains and only a small additional amount of conformational order.

Since our earlier work on saturated PE's, the adiabatic compressibility of a series of saturated PE's and PC's has been measured (Russell & Collings, 1983) and compared to values calculated by using the Clausius–Clapeyron relationship, viz., $dP/dT_m = \Delta H/(T_m \Delta V)$. While there was good agreement between observed and calculated values for all of the PC's, the calculated values of dP/dT using our earlier volumetric data for DLPE and DMPE were considerably higher than what was measured. However, the larger ΔV reported here makes the discrepancy much less severe for DMPE as the value we calculate for dP/dT_m from our ΔV and ΔH values is 46 ± 3 bar/K compared to 39.0 ± 0.7 bar/K, the value measured by Russell & Collings (1983).

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