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A CALORIMETRIC EXAMINATION OF STABLE AND FUSING LIPID BILAYER VESICLES

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Summary

Mixed lipid samples containing dimyristoylglycerophosphocholine and small amounts of myristic acid were examined calorimetrically. Examination of multilamellar and small vesicle samples indicated that upon heating small vesicles combine to form more extended structures. An exothermic peak (at 19.5°C) can be associated with the structural transformation. The enthalpy for this process, which may be interpreted as vesicle-vesicle fusion, is found to be approx. −2 kcal/mol.

Introduction

In efforts to elucidate the properties of biological membranes, model systems of phospholipid bilayers have been analyzed by several investigators [1—5]. Both the multilamellar and small vesicle forms of phospholipid bilayers have been studied in depth [6—9]. Instability in vesicle preparations has, however, hampered some investigations. This is particularly true for differential scanning calorimetric studies of vesicles prepared from homogeneous phospholipids since, as several studies have shown, these vesicles are prone to undergo a transformation to larger bilayer structures [10—14] during a temperature scan. We have found that through extensive purification procedures, a stable preparation of homogeneous phosphatidylcholine vesicles can be prepared, and that the addition of small concentrations of fatty acids to this preparation causes a reproducible alteration in vesicular properties. In the presence of free fatty acid a structural transformation to a more extended bilayer occurs when a sample is incubated at a temperature near the gel to liquid crystalline phase transition [15].

A study of this transformation is of particular interest because of its implications for vesicle-membrane fusion [16]. A true fusion process should lead in a

single event to merging of the two bilayer structures and retention in the product of the contents of both original structures. Most models under study including the present one cannot satisfy this rigorous definition. The term fusion is used here to mean only the merging of two bilayer structures.

In addition to merging as a result of fusion [13], other mechanisms leading to redistribution of lipids among bilayer structures have been proposed. These include multistep contact transfer of lipids and diffusive transfer of individual molecules [14]. Multistep contact transfer is unlikely in the present study because only structures larger than those in the original preparation are generated. The diffusion mechanism can in principle lead to a slow redistribution of lipids and such slow redistribution has been recently observed when phosphatidylcholine systems are incubated well above phase transition temperatures [17]. However, calculations based on a simple diffusion process show that dimyristoylglycerophosphocholine systems, such as those to be studied here, which have estimated critical micelle concentrations of 10^{-8} M [18], are not capable of substantial transfer of lipid by molecular diffusion in time periods less than several hours. Diffusion cannot therefore account for the rapid initial rates of transformation observed for the system to be studied here. Regardless of the fusion mechanism, conversion from one bilayer structure to another must be characterized by basic thermodynamic parameters. Calorimetric data relevant to those parameters are the focus of the present study.

Materials and Methods

1,2-Dimyristoyl-*sn*-glycero-3-phosphorylcholine was purchased from Calbiochem, Lot No. 400488, and was purified by alumina chromatography followed by silicic acid chromatography as previously described [15]. Myristic acid was purchased from Matheson, Coleman and Bell, and ^2H_2O from Bio Rad Laboratories. All mixed lipid preparations were first dissolved in chloroform, then dried in a vacuum oven for at least 6 h at 40°C. Multilayer suspensions were prepared by vortexing above the phase transition temperature for several minutes in a 0.01 M sodium phosphate/^2H_2O buffer at p^{2}H 7.3. Vesicles were made by sonication under nitrogen in capped vessels, using a Branson Model E bath sonicator for 2—4 h at 35—40°C. Sample concentrations were typically 0.1% (w/v). Sonication was considered to be complete when the sample became visually clear. Vesicle samples prepared under similar conditions and analyzed by thin-layer chromatography do not show detectable amounts of lysophosphatidylcholine or other hydrolysis products.

Sepharose 2B chromatography using a refractive index detector was employed to determine the size distribution of a representative sample. Refractive index detection is chosen because of its linear relationship to weight fraction and independence of particle size [14]. A size versus elution volume curve had been determined previously for the column using a series of proteins of defined size and an egg yolk phosphatidylcholine vesicle standard. The column was run at 30°C to maintain the system above the gel to liquid crystalline phase transition. Samples both with and without 4% myristic acid showed a peak corresponding to 5—10% of the sonicate being particles larger than 1000 Å.

Electron microscopy was carried out using a Phillips 301 microscope. For

negative staining a 1% solution of uranyl acetate was mixed with equal volumes of 0.1% dimyristoylglycerophosphocholine dispersions on a carbon-coated grid and excess liquid was removed with filter paper. Proton nuclear magnetic resonance spectra were obtained on a Bruker HX-270 spectrometer in the Fourier transform mode for the purpose of quantitating the extent of vesicle fusion. The probe temperature was maintained at 35°C and, because of the low vesicle concentration, 1000 scans were accumulated to provide an adequate signal-to-noise ratio. 98% deuterated chloroform and chromium acetylacetonate in a coaxial tube was used as an intensity standard. Percent fusion was determined by measuring the intensity of the narrow portion of the choline resonance assuming 100% contribution from vesicles. Integration was done using a computer routine when possible, and cutting and weighing when not.

Samples for calorimetry were prepared as described above. Sonicated samples were stored at 37°C for a maximum of 1 h before use, and were rapidly cooled by immersion in an ice bath immediately before being placed in the calorimeter. The differential scanning calorimeter used for these experiments was designed by P.L. Privalov. The calorimeter cell volume is 1 ml and the instrument has a sensitivity of approx. 50 μcal/K at a scan rate of 1 K/min [20]. Calorimeter cells cooled to 4°C were filled with sample (also at 4°C) and buffer solutions. Scans were started between 5 and 10 min after filling. A scan rate of 0.5 K/min was used for all experiments, and the baseline, which was determined prior to each experiment, was in all cases straight. Deviations in the experimental calorimetric scans are sample related and cannot be attributed to instrument instability. When reheats were required, solutions were cooled in the calorimeter. Enthalpies were determined by integrating the areas under the transition curves using a planimeter. As has been pointed out [21,22] there appears to be no unique way to assign enthalpies to two transitions when the excess apparent specific heat does not return to zero between the transitions. We have thus assumed that each transition is symmetric and have calculated the enthalpies accordingly. After calorimetric runs, care was taken to maintain the samples at temperatures where fusion is negligible (35—40°C) until further analysis could be undertaken.

Results and Discussion

The calorimetric scans of pure dimyristoylglycerophosphocholine multilayers and vesicles appear in Fig. 1. For multilayers, a small peak at 13.7°C, and a very sharp peak at 24.4°C, with an enthalpy of 6.2 kcal/mol, are observed. In H_2O buffer the sharp transition is at 23.9°C with an enthalpy of 5.5 kcal/mol (unpublished results).

The calorimetric scan of dimyristoylglycerophosphocholine vesicles shows a broad low intensity peak at 19.5°C and sharper transition at a slightly higher temperature than seen in the multilayer scan. This observation is in general agreement with observations of Suurkuusk et al. [10] but differs from those of de Kruijff et al. [23]. If we associate the sharp peak with multilayer contamination, the enthalpy of this peak corresponds to 14% of that expected if all the lipid were in multilamellar form. Sepharose chromatography in fact showed at least 3% multilayers present in the vesicle suspension before the calorimetry.

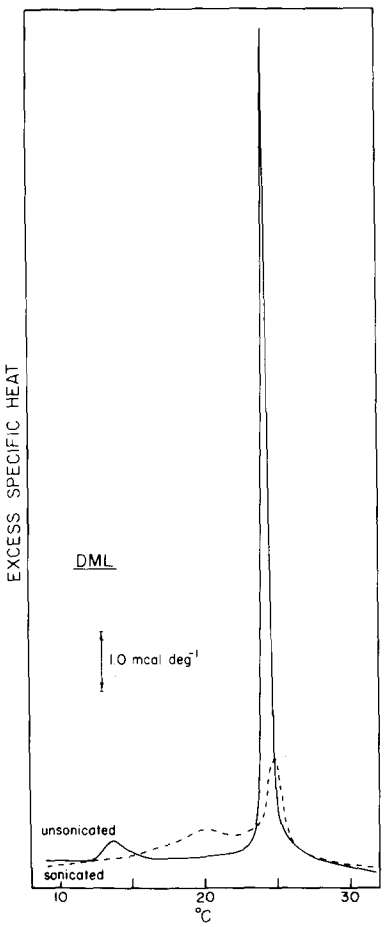

Fig. 1. Differential scanning calorimetry curves for unsonicated multilamellar and sonicated vesicle suspensions prepared from dimyristoylglycerophosphocholine (DML), 0.1% (w/v).

This is likely a lower limit since the largest structures may not pass through the column. If we assume only the broad peak to be characteristic of vesicles, we obtain an enthalpy of 0.9 kcal/mol for the vesicle transition.

The vesicle sample in Fig. 1 was cooled and reheated a second time in the calorimeter. There was essentially no change in the thermal behavior indicating that the vesicle suspension was stable over the time period of the experiment. This is consistent with our previous NMR data [15]. The samples may not have remained at 5°C long enough to show the changes recently reported by Suurkuusk et al. [10], in which case the stability would be kinetic rather than thermodynamic in origin.

In order to create a situation where structural transformations might be observed, a dimyristolylglycerophosphocholine sample containing 11 mol% myristic acid (4%, w/w) was prepared and examined calorimetrically, yielding the profiles illustrated in Figs. 2 and 3. It can be seen that the vortexed sample has a broad asymmetric transition with a peak temperature at 26.6°C and an enthalpy of 5.6 kcal/mol. The transition temperature is higher than for a simi-

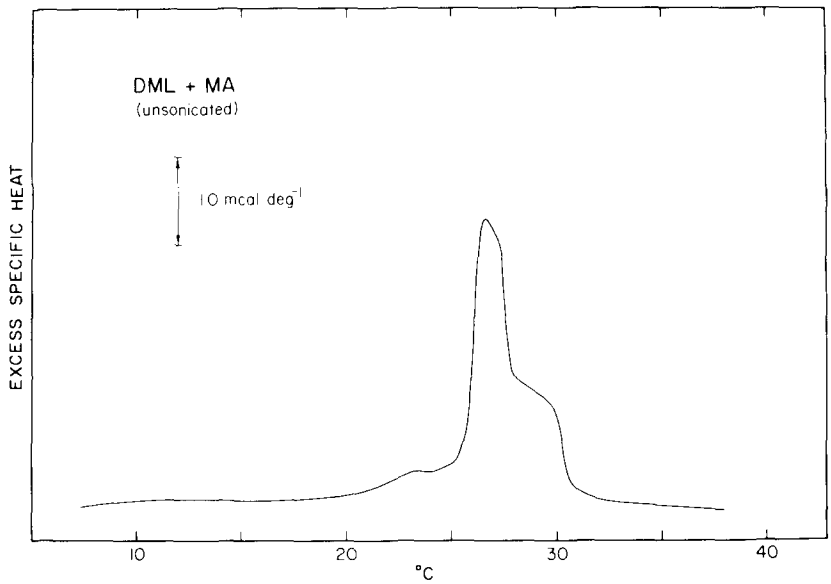

Fig. 2. Calorimetric transition curve for a 0.1% (w/v) suspension of multilamellar dimyristoylglycerophosphocholine (DML) containing 11 mol % myristic acid (MA).

larly prepared suspension of pure dimyristoylglycerophosphocholine which is consistent with data indicating a progressive increase in the transition temperature of dimyristoylglycerophosphocholine multilayer preparations as the myristic acid content is increased (manuscript in preparation).

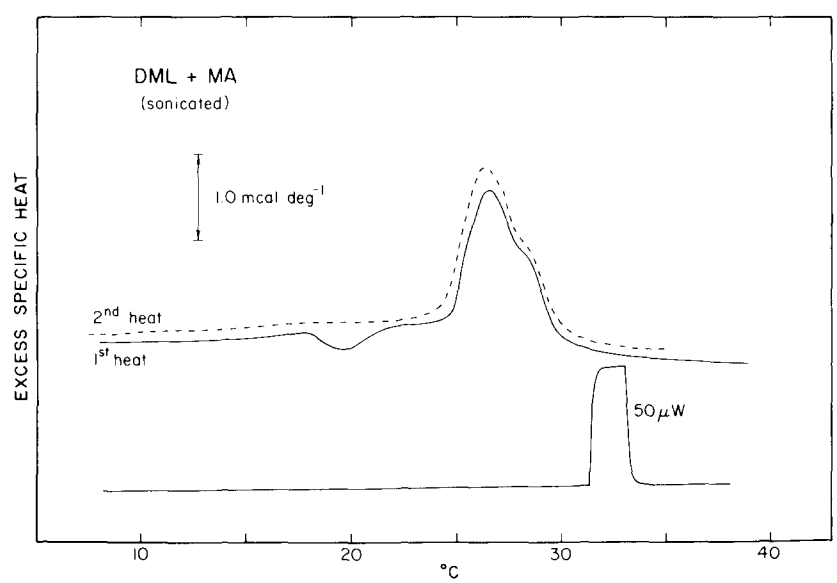

Fig. 3. Calorimetric scans of two successive heatings of a sonicated dispersion of dimyristoylglycerophosphocholine (DML) containing 11 mol % myristic acid (MA). The bottom scan is a baseline recording and calibration mark obtained on the solvent.

The corresponding dimyristoylglycerophosphocholine and myristic acid vesicle sample also differs from a pure dimyristoylglycerophosphocholine vesicle preparation upon calorimetric examination. The mixed lipid preparation has a broad high temperature transition at 26.6°C, closely resembling the dimyristoylglycerophosphocholine and myristic acid multilayer transition. The lower transition, if it exists at all, is very broad. The most interesting feature of the scan is a small exothermic peak at 19.5°C. This is consistent with isothermal flow calorimetric observations seen by other workers (Pabo, C. and Fellmeth, B., personal communication.) On a second scan, shown in Fig. 3, the exothermic peak disappears while the remainder of the profile is unchanged. Similar samples indicate that an additional scan displays no further alteration. Of the possible origins for this exothermic peak, one of the most intriguing is that it corresponds to an exothermic transformation from vesicles to a more extended bilayer structure. The 26.6°C peak would then characterize the extended structure. Comparison of electron micrographs (Fig. 4) of a myristic acid-containing sample with those of vesicle and multilayer samples supports this interpretation.

The fact that the exothermic peak occurs at 19.5°C also agrees quite well with previous information indicating that the most rapid destruction of vesicles occurs at 20°C [15]. The previous results, however, would lead one to believe that fusion which occurs at an appreciable rate in a 4°C temperature range around 20°C would not be complete in the approx. 8 min time span that the calorimeter spends in this range. The repeat calorimetric scans suggest that if fusion is associated with the exothermic peak, the sample fuses no further after the initial scan, or perhaps after the hour or so between the first and second heatings. Yet the enthalpy of the upper peak indicates that only 50% of the sample is non-vesicular if we assume molar enthalpies of the extended bilayer product to equal those of a multilamellar preparation. To clarify this situation NMR spectra were taken before and after each set of calorimetric scans, and after holding the samples at 20°C for 4 days.

^{1}H NMR spectra of vesicles show a narrow (5 Hz) choline methyl peak. Multilayers or other extended structures, on the other hand, show a broad complex choline peak of much greater width. Fusing samples produce few intermediate size structures [15], so all spectra can be approximated as a weighted average of the broad and narrow choline peaks. The narrow portion is easily integrated to give a measure of residual vesicles. A comparison of the NMR spectra shows that fusion indeed did occur, was only partially complete after the second calorimetric scan, and that further fusion did continue over the next several days. A 24% decrease in narrow choline signal had occurred after the second heating indicating that the calorimetric multilayer peak should have represented the 24% produced by fusion on heating plus that present in the original preparation. The estimated 38% is in reasonable agreement with the 50% taken from the measured calorimetric enthalpy.

Resolution of the problem involving the apparent absence of an exothermic peak on the second heating lies in a detailed examination of the kinetics of the fusion process. Data for a 0.1% sample in which 4 wt. % of the lipid is myristic acid are presented in Fig. 5. They indicate that in 8 min fusion is approx. 27% complete, but that fusion proceeds at a much slower rate after this level is

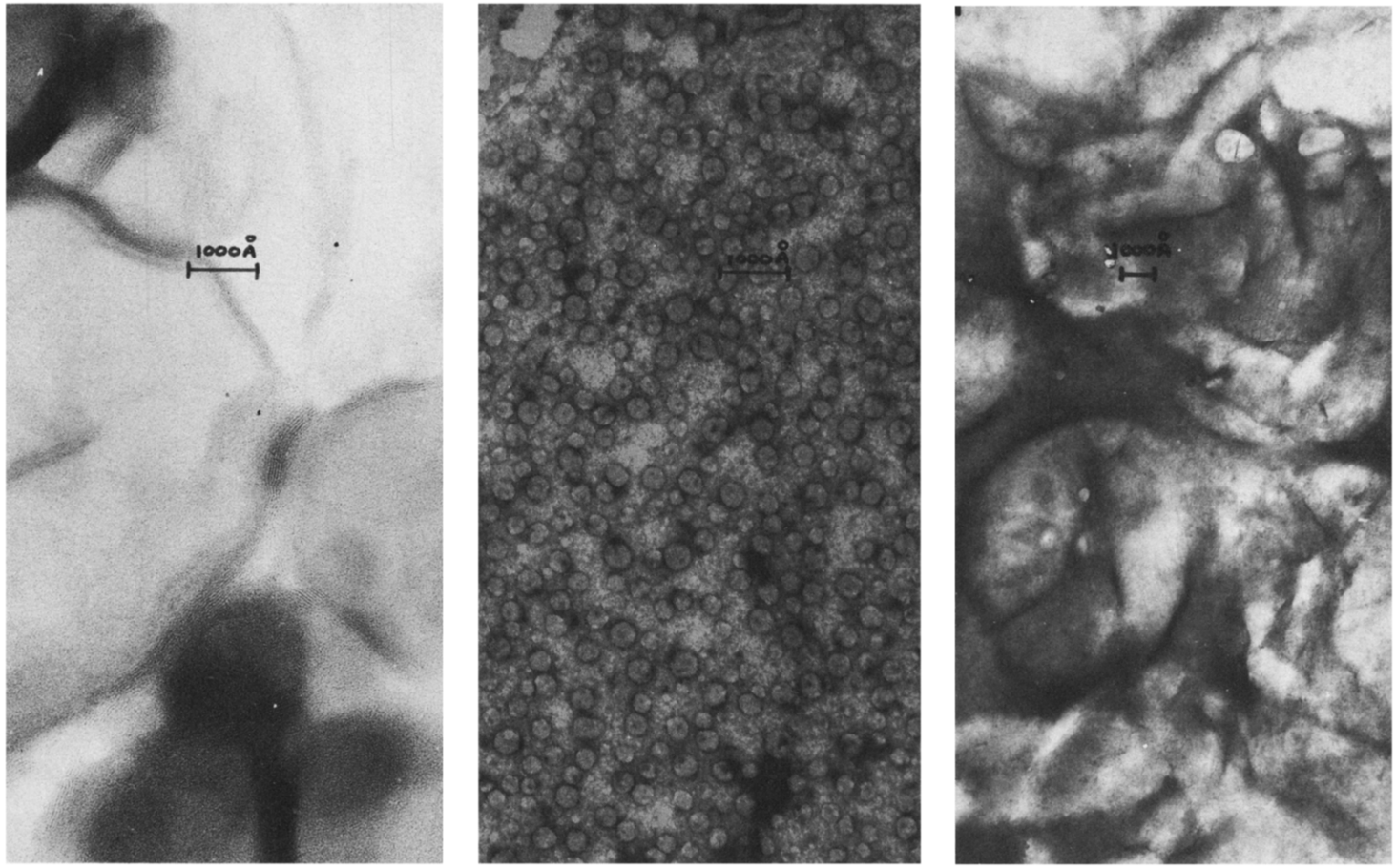

Fig. 4. Electron micrographs of dimyristoylglycerophosphocholine bilayer systems using negative staining with 1% uranyl acetate, 0.1% (w/v). a, multilamellar dispersion; b, sonicated dispersion of vesicles; c, fused product of myristic acid containing vesicles (10 mol %) after two heatings in the calorimeter.

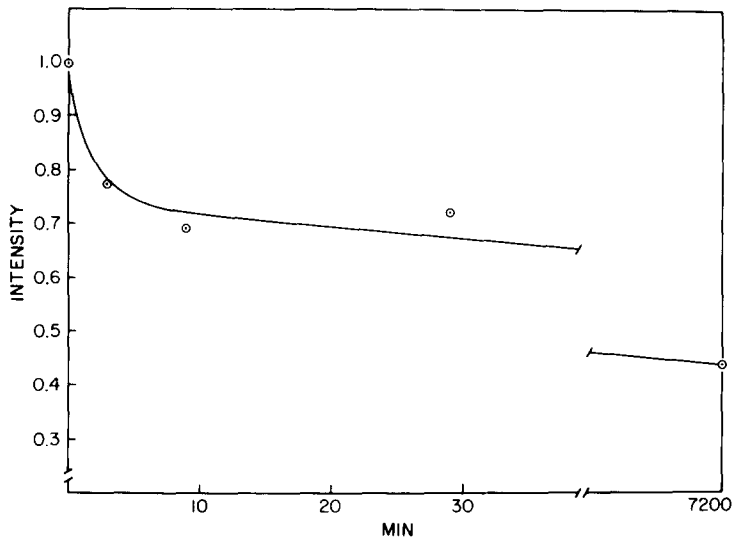

Fig. 5. Kinetics of fusion of 0.1% (w/v) lipid vesicles. Suspensions composed of 11 mol % myristic acid in dimyristoylglycerophosphocholine were sonicated at 37°C then incubated at 20°C for the times designated on the abscissa. The content of small vesicles was assayed by NMR choline peak intensity. The ordinate represents the relative intensity as compared to the initial sonicated sample.

reached. An additional 8 min would lead to less than another 5% conversion. The slow portion of the decay can therefore account for our inability to observe an exothermic peak after completion of the initial calorimeter heating. We would presumably see an exothermic peak on a second heating if we were able to heat and cool more rapidly. We have mimicked an initial rapid heating and cooling external to the calorimeter and do see an exothermic peak of reduced amplitude on a reheat in the calorimeter.

It appears therefore that the exothermic transition can be associated with fusion of fatty acid-containing dimyristoylglycerophosphocholine vesicles to more extended structures, and that the upper endothermic peak (26.6°C) is characteristic of the extended bilayer products. The percentage fusion giving rise to the exothermic peak can now be estimated and a molar enthalpy of fusion calculated. A sample cooled and heated rapidly indicated approx. 8% fusion would have occurred on cooling prior to the first calorimetric scan. We would therefore estimate that 16 of the 24% fusion gave rise to the exothermic peak. Using this value we obtain an enthalpy change for the structural transformation from vesicle to extended bilayer of −2 kcal/mol. This figure is probably a lower limit to the true value due to the uncertainty in fusion occurring during the initial cooling.

We believe this to be the first piece of thermodynamic data related to a possible vesicle-vesicle fusion. Because of the temperature at which it occurs it is difficult to say whether this enthalpy represents a transition from vesicles in a liquid crystalline state to extended structures in a gel state, or from vesicles in a gel state to extended structures in a gel state. If the former were true, a substantial part of the driving enthalpy could be viewed as coming from the gel to liquid crystalline transition. If the latter were true increasing the radius of cur-

vature of a bilayer would be an exothermic process which would be a determining factor in vesicle-membrane fusion.

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