A Calorimetric and Fluorescent Probe Study of the Gel–Liquid Crystalline Phase Transition in Small, Single-Lamellar Dipalmitoylphosphatidylcholine Vesicles†

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ABSTRACT: The results of a calorimetric and fluorescent probe study of the thermotropic behavior of various types of dispersions of dipalmitoylphosphatidylcholine bilayer vesicles are reported. Bangham-type, multilamellar vesicles exhibit two distinct phase transitions at 34.6 and 41.2 °C. On the other hand, single-lamellar spherical vesicles appear to exhibit a single transition at 37 °C. The single-lamellar vesicles are thermodynamically unstable below 27 °C and slowly transform into a multilamellar structure with a single phase transition of 41.2 °C. These transformed structures resemble, but are not identical with, Bangham-type vesicles. An experimentally testable thermodynamic and kinetic model based upon these results is developed.

Considerable information about the physical properties and molecular organization of phospholipids in bilayers has been obtained from the study of various types of liposome dispersions. This information has provided a basis for understanding the contributions made by lipid components to the physiological properties of biological membranes. Two types of liposome dispersions have been utilized in such studies. The first, introduced by Bangham and co-workers (1965, 1967), consists of liposomes in which the lipid is organized into concentric bimolecular lamellae, each separated from its neighbor by an interspersed water lamella. Since each lipid lamella is a topologically closed surface, a single liposome is composed of many concentric vesicles. A typical aqueous dispersion of this type contains multilamellar liposomes which vary in size from several hundred angstroms in diameter to objects observable in the light microscope. The second type of phospholipid dispersion is that introduced by Huang (1969). This system is comprised of spherical vesicles, which are homogeneous in size and consist of a single continuous lipid bilayer enclosing a volume of aqueous solution.

Each type of dispersion has its own particular experimental advantages and frequently the two preparations are used interchangeably. There is, however, evidence that the physical properties of the bilayers in these two systems are markedly different (Sheetz and Chan, 1972; Thompson et al., 1974). An early report by Steim (1968) also suggested that the gel–liquid crystalline phase transition characters of sonicated and nonsonicated dipalmitoylphosphatidylcholine liposomes were different. This paper reports a calorimetric and fluorescent probe study of the gel–liquid crystalline phase transition of dipalmitoylphosphatidylcholine in aqueous dispersions of both types. The results of this study not only show that the characteristics of this transition are different in the two systems, but also suggest that the small vesicles are a metastable system at temperatures below approximately 27 °C.

Experimental Procedure

Materials. 1,2-Dipalmitoyl-3-*sn*-phosphatidylcholine was synthesized by the method of Cubero Robles and Van den Berg (1969) from L-α-glycerolphosphorylcholine prepared from egg phosphatidylcholine (Chadha, 1970) and from palmitic acid (purity 99%) obtained from Sigma. The crude dipalmitoylphosphatidylcholine was twice purified by silicic acid column chromatography in chloroform–methanol mixtures (Mallinckrodt Silicar CC-4). The fractions containing dipalmitoylphosphatidylcholine without traces of free fatty acid or lysolecithin were pooled. The solvent was removed under reduced pressure. The dipalmitoylphosphatidylcholine was dissolved in spectral grade chloroform. In order to remove traces of multivalent ions including heavy metals ions, 4 vol of methanol and 3 vol of EDTA solution (pH 7.2) were added to 8 vol of chloroform containing dipalmitoylphosphatidylcholine. The mole ratio of EDTA to phospholipid was 1 in the final mixture. After repeated mixing, the two phases were separated. The chloroformic lower phase was extracted three times with ideal upper phase to remove traces of EDTA. The organic solvent was then removed under reduced pressure. The resulting nearly pure dipalmitoylphosphatidylcholine was twice reprecipitated from chloroform by the addition of acetone. The final product was dissolved in spectral grade chloroform and stored at −20 °C until used.

Thin-layer chromatography with 2 μmol of loading gave one heavy spot and a faint second spot having a slightly larger R_f value than dipalmitoylphosphatidylcholine (developing solvent, $CHCl_3$–CH_3OH–H_2O, 65:25:4; I_2 staining). The ratio between the contaminant and the dipalmitoyllecithin was less than 0.01 as calculated from the relative amount of [^{3}H]palmitic anhydride incorporation during synthesis. All commercial samples examined gave several minor spots under similar conditions corresponding to this same contaminant, to lysolecithin, and to other faster moving components (B. Lentz, Y. Barenholz, and T. E. Thompson, manuscript in preparation).

Preparation of Multilamellar Liposomes. Dispersions of large, multilamellar liposomes were prepared by the method

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of Bangham (Bangham et al., 1967). When the dispersion was prepared for fluorescence studies, 50 mM KCl in 15% (w/v) sucrose was used as the aqueous phase. This was done to prevent settling of the liposomes in the fluorescent cuvette during the course of an experiment. Dispersions of this type were prepared as follows. A measured amount of dipalmitoylphosphatidylcholine solution was added to a 50-ml pear-shaped flask and the chloroform removed on a rotary evaporator at 30 °C. The phospholipid coating the flask wall was heated to 50–55 °C and a measured amount of preheated 15% sucrose (Ultra Pure, Schwarz/Mann) and 50 mM KCl (Extra pure, Heico, Inc.) solution was added. The water used to prepare this solution was first deionized, then distilled from alkaline $KMnO_4$, and finally redistilled from glass. The lipid dispersion was vortexed for 1 min at 50–55 °C. One microliter of a 2 mM solution of the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH)[1] in tetrahydrofuran was added with rapid agitation to one portion of this solution. A second portion was retained as a light scattering blank. The solutions were then gently swirled for at least 2 h at 48–50 °C before use to permit equilibration of the dye with the vesicle bilayers (the time for dye penetration at these temperatures is less than 60 min) (B. Lentz, Y. Barenholz, and T. E. Thompson, manuscript in preparation). Samples for the calorimetric studies were prepared in a similar fashion except that a 50 mM KCl solution, usually without sucrose or added DPH, was used.

Preparation of Small, Single-Lamellar Vesicles. Small, single-lamellar vesicles, homogeneous in size, were prepared following a modification of Huang's procedure (Huang and Thompson, 1974) which eliminates the molecular sieve fractionation step. Dispersions of these vesicles were prepared by sonication of the lipid in 50 mM KCl at a temperature of 50–55 °C using a Heat Systems W-350 Sonifier. Small vesicles were fractionated from larger liposomes by high-speed centrifugation. Extensive autocorrelation light scattering studies carried out on small vesicle dispersions of a variety of phospholipids prepared by this procedure indicate that these dispersions are equivalent to Huang dispersions in all respects (Y. Barenholz, D. Gibbes, B. J. Litman, J. Goll, T. E. Thompson, and F. Carlson, manuscript in preparation). For fluorescence measurements, 1 μl of 2 mM DPH in tetrahydrofuran was added to a portion of the supernatant with rapid stirring; the remaining portion was used as a light scattering blank. Comparative calorimetric and fluorescence studies were usually carried out on the same dispersion of small vesicles or Bangham-type liposomes.

Determination of Phospholipid Concentration. The lipid concentration of each dispersion was measured as inorganic phosphate by the Bartlett method (1959). At least ten phosphate determinations were made on each sample with a resulting standard deviation of ±1% for small vesicle and ±2.5% for large vesicle dispersions. Phosphatidylcholine concentrations in dispersions examined calorimetrically were 7–10 mM and in dispersions studied fluorimetrically, 0.4–0.5 mM.

Calorimetry. A newly developed differential scanning calorimeter of the heat conduction type (Ross and Goldberg, 1974) was used for these studies. The calorimeter was designed for measuring heat capacities and heat effects accompanying thermally induced transitions of solute molecules in dilute solution. The temperature range for the instrument is 0–75 °C, and scanning rates from 3 to 50 deg/h can be selected. The total volume of the sample compartment is approximately 0.7 ml. The precision in terms of baseline noise is better than ±25 μcal/deg. Absolute temperature determination is better than ±0.05 °C.

The calorimeter consists of two rectangular aluminum cells each with a hole to contain a sample or reference ampule. Each cell is sandwiched between a pair of thermoelectric modules (Cambion, Inc.) in thermal contact with a copper heat sink by means of a wedge system. The thermopiles surrounding the two cells are connected electrically in series with opposing polarity so that when the cell temperatures are equal, the differential voltage signal is zero. The heat sink is positioned inside a cylindrical adiabatic shield. The assembly is insulated from the surroundings by 5 cm of polyurethane foam. The differential voltage signal from the thermopiles is amplified (Kiethly 150 B Voltmeter), digitized (Hewlett-Packard Digital Voltmeter, 3450-A; Coupler/controller, 2570-A), and recorded on a punch tape together with time and heat sink temperature (as measured by a Hewlett-Packard Quartz-Thermometer, 2801-A) at a rate of one reading every 13.1 s.

When the heat sink temperature is increased at constant rate, temperature differences are created because of thermal barriers between the heat sink and cells. The measured differential voltage signal is proportional to the temperature difference between the cells and thus to the heat capacity difference. For an ideal calorimeter the relation between heat capacity difference between cells, ΔC, and the voltage, V, is given by:

$$\Delta C = (\epsilon/\alpha)[V + (dV/dT)\tau\alpha] \quad (1)$$

where α is the scanning rate, ϵ is a calibration constant (calories volts^{-1} sec^{-1}), and τ is the time constant (seconds). By comparing ΔC values from buffer and sample experiments apparent partial heat capacities of the solute can be obtained. Due to imperfectly matched thermoelectrical module pairs, a temperature dependence of the calorimetric constants, and a correction of the measured heat sink temperature to the actual sample cell temperature, an extensive computer calculation is necessary to obtain ΔC as a function of sample cell temperature (J. Suurkuusk, D. M. Mountcastle, and R. L. Biltonen, manuscript in preparation).

All measurements were carried out by placing the sample in the calorimeter at 20–40 °C. After cooling to about 0 °C, the heat capacity was measured during heating at a rate of 15 °C/h. The time required to cool and equilibrate the sample in the calorimeter was about 3–6 h.

Fluorescence Measurements. Fluorescence measurements were made on a modified Perkin-Elmer MPF 3 spectrofluorometer operating in the ratio mode. The modifications included addition of two polarizers, one in the excitation beam and the other in the emission beam, and installation of a multiwave plate wedge depolarizer in the emission beam. This latter modification eliminated the correction for preferential photo tube sensitivity to horizontally or vertically polarized light. Instrument output was displayed on a digital voltmeter and strip chart recorder. The temperature of the sample chamber was controlled by means of a Lauda MK-2 thermoregulated bath. A Yellow Springs Instrument thermistor probe connected to a digital ohmmeter was used to measure the temperature of the fluorescence sample in the cuvette to within ±0.1 °C. When fluorescence depolar-

[1] Abbreviation used is: DPH, 1,6-diphenyl-1,3,5-hexatriene.

ization was determined as a function of temperature, the sample in the cuvette was heated to 55 °C and then cooled at a rate of 20 °C. Fluorescence intensity and temperature measurements were made continuously during cooling. DPH was excited at 360 nm and fluorescence recorded at 430 nm using instrument filter 39 as a cutoff for wavelengths below 390 nm.

The general theory of the fluorescence probe depolarization technique as applied to membrane structure has been outlined in detail by Cogan et al. (1973). The use of DPH as a fluorescent probe specific for the hydrophobic region of lipid bilayer has been described by Shinitzky and Barenholz (1974). The technique is based on determination of the rotational motion of the probe molecule. This motion is characterized by the anisotropy parameter, r, as defined by:

$$r = \frac{(I_\parallel/I_\perp) - 1}{(I_\parallel/I_\perp) + 2} = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp} \quad (2)$$

where $I_\parallel$ is the fluorescence intensity parallel to and $I_\perp$ is the intensity perpendicular to the plane of polarization of the excitation beam. Total fluorescence is given by $I_\parallel + 2I_\perp$. The theoretical upper limit for r when the probe is in a medium of infinitely high viscosity is 0.40. The lower limit in a medium of very low viscosity is zero. With DPH the experimentally determined upper limit for r was 0.362.

In all experiments the ratio was corrected for light scattering using the following equation (Shinitzky et al., 1971):

$$\frac{I_\parallel}{I_\perp} = \frac{I_\parallel^t - I_\parallel^s}{I_\perp^t - I_\perp^s} \quad (3)$$

Here $I_\parallel^t$ and $I_\perp^t$ are the measured intensities for liposome dispersions containing DPH and $I_\parallel^s$ and $I_\perp^s$ are the values of light scattering obtained for control dispersions without the DPH. The scattered light from small vesicle dispersions without DPH measured as $I_\parallel^s$ was always less than 1% of $I_\perp^t$ determined for vesicles containing DPH. For large, multilamellar liposomes $I_\parallel^s$ was less than 3% of $I_\perp^t$. These limits were obtained for both types of dispersions over the entire temperature range examined. Light scattering itself did not cause depolarization of fluorescence since dilution of each type of dispersion did not alter the value of $I_\parallel/I_\perp$.

Turbidity Measurements. The turbidities of the dipalmitoylphosphatidylcholine dispersions were measured as absorbances in a Gilford spectrophotometer.

Results

Calorimetry. Figure 1 shows a typical heat capacity vs. temperature scan of a fresh suspension of large, multilamellar Bangham-type liposomes prepared from dipalmitoylphosphatidylcholine. The scan is characterized by two transitions with T_m values of 35.4 and 41.2 °C and corresponding enthalpy changes of 1.6 and 8.2 kcal/mol, respectively. T_m is defined as the position of a maximum in the heat capacity function and the corresponding ΔH is the integral of the C_p function over the appropriate temperature range calculated as described below. These thermodynamic quantities are in reasonable agreement with T_m values of 34.0 ± 0.2 and 41.75 ± 0.06 °C and the corresponding values of ΔH = 2.3 ± 0.2 and 9.7 ± 0.2 kcal/mol obtained by Hinz and Sturtevant (1972) on a very similar preparation of dipalmitoylphosphatidylcholine liposomes. Our estimate of 8.2 kcal/mol for ΔH of the high-temperature transition is in better agreement with values reported by other workers (Ladbrooke and Chapman, 1969; de Kruijff et al., 1973; Klopfenstein et al., 1974; Vaughn and Keough, 1974; de Kruijff et al., 1975).

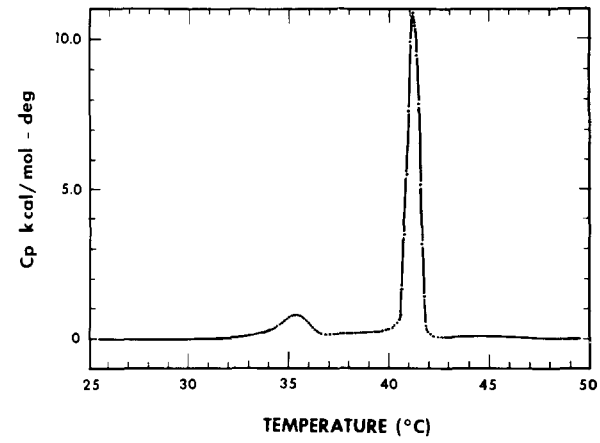


FIGURE 1: Calorimetric scan of a fresh dispersion of multilamellar Bangham-type liposomes prepared from dipalmitoylphosphatidylcholine; aqueous phase composition 50 mM KCl.

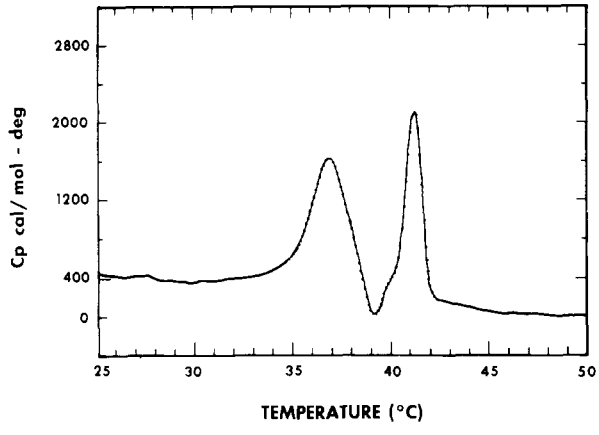


FIGURE 2: Calorimetric scan of small single-lamellar vesicles prepared from dipalmitoylphosphatidylcholine. The vesicle dispersion was introduced into the calorimeter immediately after preparation and allowed to reach temperature equilibrium at about 0 °C for 3.0 h before commencing the scan; aqueous phase composition, 50 mM KCl.

Although the temperature-dependent behavior of small vesicle preparations of varying degrees of homogeneity has been described using nuclear magnetic resonance (NMR) spectroscopy (Sheetz and Chan, 1972; Lee et al., 1972; Uhing, 1975), electron spin resonance (Hubbell and McConnell, 1971), dilatometry (Sheetz and Chan, 1972; Melchior and Morowitz, 1972), light scattering (Yi and MacDonald, 1973), and fluorescence (Lussan and Faucon, 1971; Vanderkooi and Chance, 1972; Cogan et al., 1973; Papahadjopoulos et al., 1973), the transition characteristics of a homogeneous population of small, single-lamellar phospholipid vesicles, prepared as described in the preceding section, have not previously been determined by direct calorimetric measurements. A calorimetric scan of such vesicles freshly prepared from dipalmitoylphosphatidylcholine is shown in Figure 2. Two distinct heat capacity maxima at 36.9 and 41.2 °C are observed. The enthalpy changes associated with each are 3.9 and 2.9 kcal/mol, respectively. It is clear that the thermal characteristics of the small vesicles are quite different from those displayed by multilamellar Bangham-type liposomes.

The thermal characteristics of Bangham-type liposomes are stable with respect to time; the basic features of the transitions, T_m and ΔH, are reproducible after several tem-

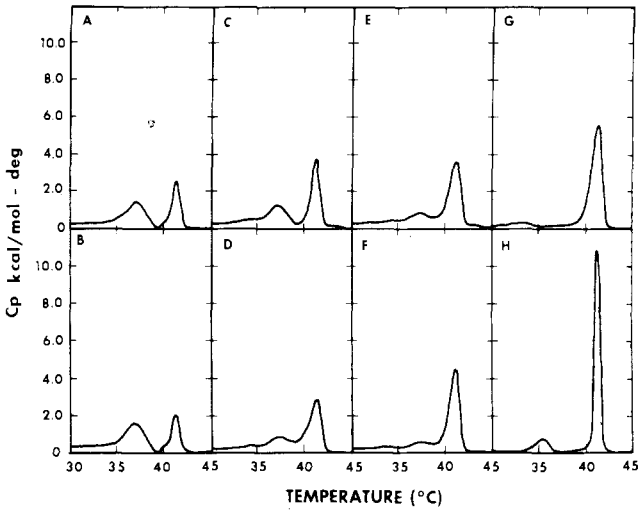


FIGURE 3: Calorimetric scans of small vesicle dispersions prepared from dipalmitoylphosphatidylcholine as a function of time under different conditions; aqueous phase composition 50 mM KCl: (A) sample held at 48–50 °C for 1 week before scanning; (B) identical with Figure 2, sample was introduced into the calorimeter immediately after preparation and allowed to equilibrate at about 0 °C for 3 h before scanning; (C, D, E, and F) samples after 2, 7, 13, and 19 cycles from 4 to 50 °C; (G) a small vesicle preparation stored in the frozen state at −10 °C for 90 h; (H) identical with Figure 1, scan of a fresh dispersion of Bangham-type liposomes.

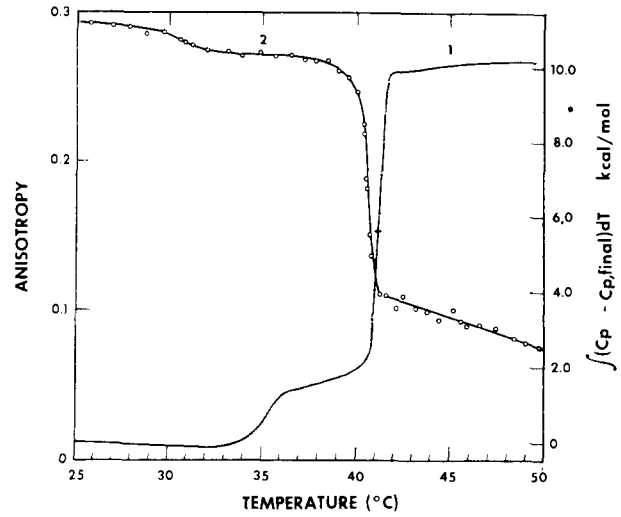


FIGURE 4: Comparison of calorimetric and fluorescence results for Bangham-type liposomes: (curve 2) fluorescence anisotropy of DPH as a function of temperature; (curve 1) calorimetrically determined excess enthalpy as a function of temperature. These data are the integral under the curve shown in Figure 2 after a baseline correction.

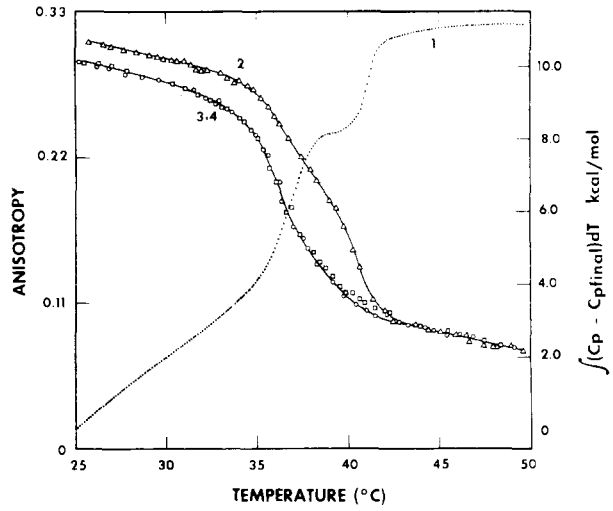


FIGURE 5: Comparison of calorimetric and fluorescence results for small vesicle preparations: (curve 1) calorimetrically determined excess enthalpy as a function of temperature; (curve 2) fluorescence anisotropy of DPH vs. temperature after the small vesicle preparation has been held below the transition temperature for 6 h; (curve 3,4) fluorescence anisotropy of DPH vs. temperature for freshly prepared small vesicles (○) and for small vesicles held at 48–51 °C for 5 days (□).

perature cycles. This, however, is not the case for the small vesicles whose thermotropic behavior is highly dependent upon the history of the sample. With successive calorimetric scans of the same vesicle preparation, it was observed that the heat associated with the transition at 41.2 °C increased while that associated with the 36.9 °C transition decreased.

The changing thermotropic behavior of the small vesicles as a function of time under different conditions is illustrated in Figure 3. Scan A was performed with a sample stored above the highest transition temperature for 1 week. Scan B, which is identical with Figure 2, was initiated within 3 h after preparation. The thermal characteristics of the two samples are very similar indicating that the small vesicle preparation is essentially stable by this criterion if maintained above the highest transition temperature. However, as described below, a distinct increase in turbidity is observed after prolonged storage above the transition temperature.

Scans C, D, E, and F were performed after increasing numbers of temperature cycles from 0 to 70 °C with the same sample used in scan B. It is quite evident that the enthalpy change of the transition at 41 °C grows at the expense of that associated with the transition at 37 °C. Scan G was performed on a sample of the small vesicle preparation stored frozen at −10 °C for approximately 80 h. The thermotropic behavior of this sample is similar to that shown in scan F, and to that of a fresh multilamellar Bangham-type preparation shown in scan H and Figure 1. These results demonstrate that small vesicle preparations stored at temperatures below T_m are unstable. While it is clear that the thermotropic behavior of small vesicles is substantially different from that of multilamellar Bangham-type liposomes, it appears that upon exposure to temperatures below the T_m, species slowly form spontaneously which resemble in some respects multilamellar Bangham-type liposomes in their calorimetric characteristics. The absence of a well-defined low-temperature transition in scan G clearly indicates that this species is not, however, identical with Bangham-type liposomes.

Fluorescence Depolarization Measurements. Fluorescence depolarization measurements were carried out on preparations similar to those examined calorimetrically. The resulting DPH fluorescence anisotropies and the calorimetrically determined excess enthalpies (the integral of the C_p curve in Figure 2 corrected for baseline heat) are plotted as a function of temperature in Figures 4 and 5.

The correspondence between calorimetric and fluorescence data for multilamellar Bangham-type liposomes is excellent in the region of the main phase transition as shown in Figure 4. The transition temperature determined from fluorescence depolarization measurements, 41.1 ± 0.5 °C, is in good agreement with the value of 41.2 °C determined calorimetrically. However, a significant difference between the calorimetrically and fluorometrically measured range of

the lower temperature transition in multilamellar Bangham-type liposomes is apparent in Figure 4. While the temperature range for the lower temperature transition measured by the fluorescence polarization technique was 25.2–33.9 °C, a range of 33.2–36.7 °C was obtained by the calorimetric method. Instrumental uncertainties cannot account for this difference, nor can the fact that in the fluorometric studies sucrose was present in the aqueous phase and the vesicles contained DPH but both were absent in the calorimetric experiments. Identical calorimetric scans were obtained in systems with and without 15% sucrose or DPH. Since the calorimetric data were taken during a heating scan, and the fluorometry data during a cooling scan, this difference could be due to a real histeresis associated with the low-temperature transition. It should be mentioned that the values of this transition temperature range obtained by studies of tempo partitioning into dipalmitoyllecithin dispersions (Shimshick and McConnell, 1973) (25–31 °C) are also lower than those obtained calorimetrically. Work is now in progress in order to resolve this point.

A comparison of the calorimetric and fluorometric results for small vesicles is shown in Figure 5. Curve 1 is the integrated calorimetric scan for a small vesicle preparation held below the transition temperature for 6 h. Curve 2 is a plot of the fluorescence anisotropy as a function of temperature for a similar preparation maintained at 4 °C for 6 h. The similarity between curves 1 and 2 is apparent. Both show clear transitions at 37 and 41–42 °C. Curves 3 and 4, which are completely coincident, are fluorescence anisotropy data obtained for freshly prepared small vesicles and for small vesicles held at 48–51 °C for 5 days. Curve 3,4 shows a single transition centered at 37 °C with no indication of the high-temperature transition at 41–42 °C seen in curves 1 and 2.

The following conclusions can be drawn from the data in Figure 5. (i) Comparison of curve 1 with curve 2 indicates that the fluorescence probe accurately monitors the phase transition characteristics of small vesicles as defined by the calorimetric scan. (ii) Comparison of either curve 1 or 2 with curve 3,4 clearly shows that storage at 4 °C alters the transition characteristics of small vesicles, while storage at temperatures above the transition region (48–51 °C) produces no alteration in these characteristics. (iii) The fact that curve 3 shows only a single transition at 37 °C strongly suggests that *pure* small vesicles exhibit a single distinctive transition. A calorimetric scan of small vesicles immediately after preparation and thus completely equivalent to the fluorescence data of curve 3 was impossible to obtain. For technical reasons the scan in the calorimeter employed in the study must be made from low to high temperature. Since the equilibration time in the calorimeter below T_m is about 3 to 6 h, it is clear that at the initiation of the scan, the phase transition characteristics of the small vesicle preparations have already been altered. In contrast to this situation, the fluorescence spectrometer is best suited for a high- to low-temperature scan. Thus, in this instrument the sample is above the transition range during the initial temperature equilibration, a condition under which alteration in the thermal characteristics does not occur as is shown by the identity of curves 3 and 4, Figure 5.

The effect of repeated cycling of a sample of small vesicles from 4 to 50 °C is illustrated in Figure 6. Curve 1 in this figure is a plot of the fluorescence anisotropy of the DPH probe vs. temperature obtained for freshly prepared vesicles. Curve 2 is a similar plot of data obtained after the same preparation has been cycled 13 times between 4 and 50 °C. The appearance of a transition at about 41 °C is in agreement with the calorimetric data for the same sample shown in Figure 3E. Curve 3, Figure 6, is a plot of data obtained after the cycled sample was frozen at −10 °C and then gently stirred at 48–50 °C for 12 h before fluorescence depolarization was measured.

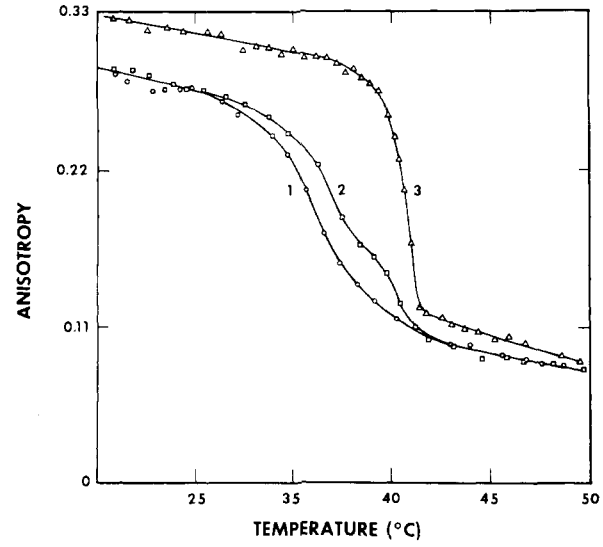


FIGURE 6: Plot of fluorescence anisotropy of DPH vs. temperature: (curve 1) freshly prepared small vesicle dispersion; (curve 2) the same small vesicle preparation shown in curve 1 but after 4.3 h in the temperature range 4–50 °C (cycled 13 times); (curve 3) the same sample shown in curve 2 after freezing at −10 °C followed by 12 h at 48–50 °C.

It is apparent that this treatment of small vesicles produces a species whose temperature-dependent behavior is similar to that displayed by multilamellar Bangham-type liposomes as shown in Figure 4, curve 2. Curve 3, Figure 6, however, shows no evidence of the low-temperature transitions displayed by Bangham-type liposomes. These results are in agreement with the calorimetric data discussed above.

Turbidity Measurements. An independent assessment of spontaneous changes in small vesicle structure was obtained from turbidity measurements at 350 and 400 nm. In general, for objects which are less than ¼ the wavelength of light, an increase in either particle size or relative refractive index will produce an increase in turbidity (Mysels, 1959). Thus, the simple aggregation of small vesicles would be expected to lead to an increase in turbidity.

Figure 7A is a plot of turbidity measured at 400 nm vs. time for dispersions of small vesicles stored below the transition at 4 °C and above the transition at 48–50 °C. It is apparent that the turbidity increases with time under both sets of conditions. However, the time dependence of the turbidity increase in the two samples is markedly different. Small vesicles stored above the phase transition at 48–50 °C exhibit a tenfold increase in turbidity over a 25-h period and then show very little if any additional change for as long as 192 h. Storage below the transition temperature at 4 °C on the other hand leads to a nearly linear rise in turbidity over a 60-h interval.

Additional information can be obtained from the ratio of turbidities measured at two different wavelengths. For Rayleigh scatters the following theoretical relation obtains (Mysels, 1959):

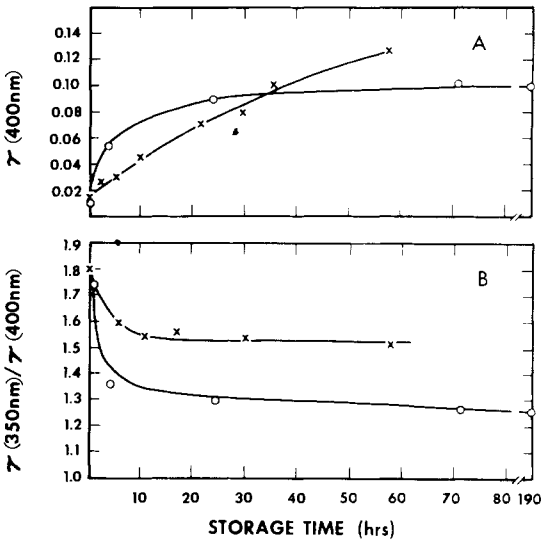


FIGURE 7: (A) Plot of turbidity of a small vesicle preparation as a function of time at 4 °C (×) and at 48–50 °C (○). (B) Plot of the ratio of turbidities at 350 and 400 nm for a small vesicle preparation as a function of time at 4 °C (×) and 48–50 °C (○). Turbidities are the optical density per micromole of lipid phosphorus in 1 ml of dispersion.

$$\tau_1/\tau_2 = [\lambda_2/\lambda_1]^4 \quad (4)$$

Here, τ_1 and τ_2 are the turbidities measured at wavelengths λ_1 and λ_2, respectively. This ratio is relatively insensitive to changes in refractive index of the scattering particle (Yi and MacDonald, 1973).

The small vesicles have been shown to have a radius of approximately 110 Å and to behave as Rayleigh scatters (Y. Barenholz, D. Gibbes, B. J. Litman, J. Goll, T. E. Thompson, and F. D. Carlson, manuscript in preparation). Thus, for measurements carried out at 400 and 350 nm, the expected turbidity ratio is 1.7. For large, multilamellar liposomes prepared according to the Bangham procedure, it has been found empirically that the ratio of the turbidities at these two wavelengths is 1.1.

The results of turbidity ratio measurements at 400 and 350 nm made on small vesicles as a function of time at 4 and 48–50 °C are presented in Figure 7B. It is apparent that although the small vesicle preparation has a turbidity ratio essentially equal to the value expected for Rayleigh scatters, the value of the ratio decreases with time under both temperature conditions. The time course of the change in τ_1/τ_2 for the preparation at 48–50 °C closely parallels the turbidity change with time observed for that preparation. Such is not the case, however, for the dispersion at 4 °C. Although the turbidity rise shown in Figure 7A is linear, the decrease in τ_1/τ_2 is small and appears to plateau after 10 h.

The data of Figure 7 indicate that small dipalmitoylphosphatidylcholine vesicles slowly associate to form larger objects when stored either above or below the liquid crystalline phase transition. The calorimetric and fluorescence results, however, clearly show that, while association of small vesicles above the phase transition produces no alteration in the thermal behavior of the dispersion, association below the phase transition range ultimately results in thermal characteristics similar but not identical with those of Bangham-type liposomes. This similarity suggests that the association of small vesicles at low temperature results in the formation of multilamellar structures by a fusion process. The invariant characteristics of the small vesicle dispersions above the transition temperature suggest that association under these conditions results in an aggregate of small vesicles which retain their individual characteristics.

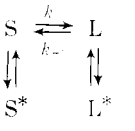
If this interpretation is correct, the relatively large increase in turbidity and the concomitant small change in the turbidity ratio obtained for small vesicles at 4 °C are most simply explained if it is assumed that an increase in refractive index of the bilayer accompanies the formation of multilamellar structures. Thus, the turbidity change at 4 °C reflects both a size and refractive index increase while the turbidity ratio change reflects mainly the increase in size. In contrast, the aggregation of the small vesicles at 48–50 °C produces objects of increased size but no concomitant refractive index change occurs. As a result, the time courses of the turbidity and turbidity ratio changes are similar in this temperature range.

Although complete refractive index data are not available for well-defined small vesicles and large multilamellar liposomes, there is evidence that the specific volume of the lipid in small vesicles is about 4% larger than it is in larger liposomes (Sheetz and Chan, 1972; B. Sears and T. E. Thompson, unpublished observation). Since this volume difference must be associated with a corresponding refractive index difference of opposite sign (Yi and MacDonald, 1973), it is reasonable to expect that an increase in refractive index occurs with the fusion of small vesicles to produce multilamellar structures.

Discussion

The observations presented in the preceding section strongly suggest that the time- and temperature-dependent behavior of small vesicle dispersions reflects a fusion process occurring at temperatures below the gel–liquid crystalline phase transition. The relation between vesicle fusion and the time-dependent thermal characteristics of the system can be formulated as a model with the following characteristics. (i) A pure dispersion of pure small vesicles, S, exhibits a single phase transition at 37 °C with an enthalpy change ΔH_S. (ii) A dispersion of multilamellar structures, L, containing no small vesicles has a phase transition at 41 °C with an enthalpy change ΔH_L. (iii) At temperatures below the phase transition, the L forms are thermodynamically more stable than small vesicles. In this temperature range the small vesicles slowly associate until an equilibrium mixture of S and L of minimum free energy is formed. (iv) At temperatures above the transition, where both L and S are in the liquid crystalline state, a kinetic barrier prevents interconversion between forms. (v) The calorimetric scans obtained for small vesicle preparations maintained below the transition are a composite of contributions from both S and L forms.

The following scheme summarizes this model:



Here S and L represent the gel forms of the small vesicles and aggregates and S* and L* the liquid-crystal states. k_1 and k_{-1} are the forward and reverse apparent first-order rate constants for the interconversion of S and L. It should be stressed that, although the thermal characteristics of the L form are similar to the main transition of Bangham-type liposomes, the two types of liposomes are not identical. Both the fluorescence anisotropy measurements and the calori-

Table I: Thermodynamic Characteristics of Various Liposome Dispersions.[a]

Preparation[b]	Time (h)	Transition 1 T_m = 33-36 °C ΔH_1 (kcal mol^{-1})[c]	Transition 2 T_m = 36.9-37 °C ΔH_2 (kcal mol^{-1})[d]	Transition 3 T_m = 41.2 °C ΔH_3 (kcal mol^{-1})[e]
A	5	0.8	3.7	3.2
B	4.7	0.8	3.9	2.9
C	17	0.8	3.1	4.9
D	25	0.6	2.2	5.1
E	43	0.9	1.9	5.5
F	80	0.8	1.7	6.7
G	90	0.9	(0.4)[f]	8.3
H		1.6	(0.2)[f]	8.2
I	8.8	1.1	3.6	4.3
J	26	0.7	2.0	5.2
K	80	0.4	1.9	5.5

[a] ΔH values were estimated as described in the text. [b] Samples A-G were all small vesicle preparations with different histories as described below. The time which each preparation spent in the 0-30 °C temperature range is given in the second column. Sample A was stored at about 50 °C for 80 h prior to cooling and initiation of the calorimetric scans. The calorimetric scan for sample B was initiated within 3 h after preparation. C was a repeat scan of sample B. D was a repeat scan of sample B after seven 0-50 °C temperature cycles, E after 13 cycles, F after 19 cycles. Sample G was a preparation of small vesicles stored in the frozen state for ~90 h. Sample H was a fresh preparation of Bangham-type liposomes. Samples I, J, and K were small vesicles stored at 5 °C for the approximate length of time indicated in column 2. [c] ΔH_1 refers to the enthalpy change obtained by integration of the 33-36 °C transition. The width at half-height of this transition was estimated to be between 1.4 and 2.6 °C for all experiments. [d] ΔH_2 is the estimated enthalpy change for the transition at 37 °C. The width at half-height for this transition was estimated to be between 2.3 and 3.0 °C for all small vesicle preparations. [e] ΔH_3 is the enthalpy change estimated for the transition at 41.2 °C. The width at half-height for the transition varied between 1.0 and 1.6 °C for all small vesicle preparations and was equal to 0.7 °C for the Bangham-type liposomes. The latter value provides an estimate of the cooperative melting unit of 126, in good agreement with the results of Hinz and Sturtevant (1972). [f] These values are subject to large error and could in fact be equal to zero; i.e., preparations G and H do not give clear evidence for the existence of the 33 °C transition. See text and figures for details.

metric studies clearly show that the low-temperature transition at $T < 36$ °C observed in Bangham-type liposomes is absent in the L form. In addition the width at half-height of the main transition at 41.2 °C is 1.3 ± 0.3 °C for the L type vesicles whereas freshly prepared Bangham-type liposomes exhibit a width at half-height of 0.7 °C.

On the basis of this model, the calorimetric scans of the various vesicle preparations were resolved into three separate transitions to yield estimates of ΔH_1, ΔH_2, and ΔH_3, the enthalpies associated with the transitions at 34.6, 36.9, and 41.2 °C. The procedure used is illustrated in Figure 8. First, the average high-temperature baseline was extrapolated to lower temperature. Next symmetrical peaks, centered at 34.6 and 36.9 °C, were constructed in a manner to account for essentially all the area under the C_p curve from approximately 34 to 38 °C. The curve associated with the highest temperature transition was then constructed by subtraction. Finally, ΔH_1, ΔH_2, and ΔH_3 were calculated from the measured areas under the three component curves. Although this procedure is somewhat arbitrary, the errors associated with it are relatively small. Thus, the error in ΔH_3 is ±10% while the error in ΔH_2 is less than ±0.5 kcal mol^{-1}. Errors of this magnitude do not seriously affect the principal conclusions discussed below.

It should be noted that the low-temperature baselines obtained with preparations of small vesicles were generally irregular and were not used in the analysis. This was permissible since the baselines at temperatures above 42 °C obtained for all systems and the baselines at low temperatures obtained for the Bangham-type liposomes did not exhibit such irregularity. In all cases heat capacity maxima were observed at the previously indicated transition temperatures as well as occasionally at temperatures below 34 °C. These latter "maxima" were not generally reproducible and were ignored in the analysis.

The results of the resolution of the heat capacity curves

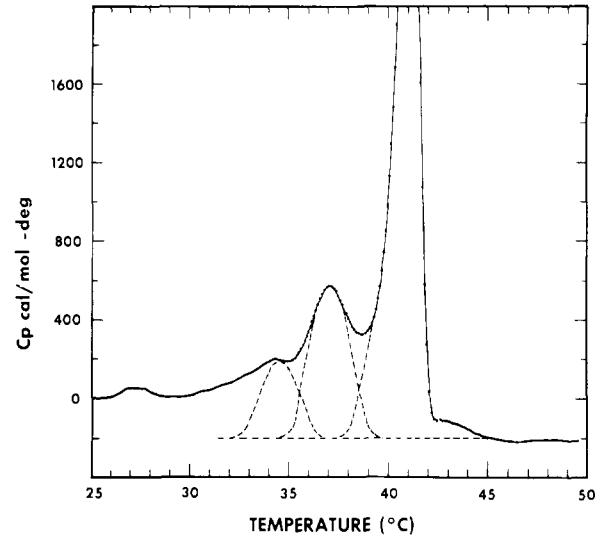


FIGURE 8: Illustration of the method used to resolve calorimetric scans of small vesicle preparations into contributions from S and L forms.

for several experiments are summarized in Table I. The enthalpy changes associated with the 34.6°C transition are highly susceptible to error and were not utilized in the subsequent analysis. The results in Table I clearly show the inverse correlation between ΔH_2 and ΔH_3, the enthalpy changes associated with the transitions at 36.9 and 41.2 °C. The quantitative nature of this correlation is demonstrated in Figure 9 in which ΔH_2 is plotted vs. ΔH_3. The solid curve is the linear least-squares regression line. The value of ΔH_3 extrapolated to $\Delta H_2 = 0$ is 8.4 kcal/mol. This is very similar to the value of 8.2 kcal/mol experimentally obtained for a fresh preparation of Bangham-type liposomes. These results suggest that ΔH_2 and ΔH_3 are in fact independent

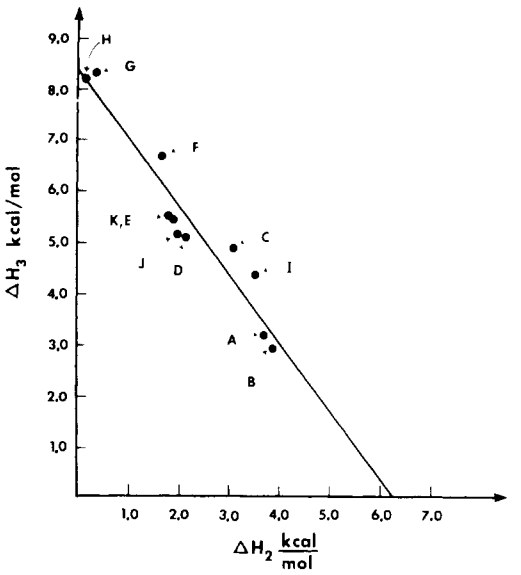


FIGURE 9: Plot of ΔH_2 vs. ΔH_3 calculated from calorimetric scans obtained for vesicles under different conditions. The points labeled A–K correspond to the correspondingly labeled data listed in Table I.

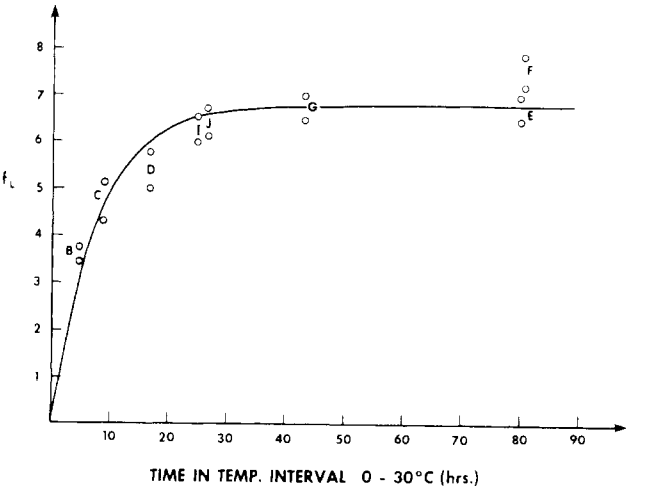


FIGURE 10: Plot of the fraction, f_L, of phosphatidylcholine molecules in multilamellar L structures vs. time. The sample was held in the temperature interval 0–30 °C. The pairs of points labeled B–K are calculated from the correspondingly labeled data listed in Table I.

measures of the relative amounts of small vesicles, S, and multilamellar structures, L, in each preparation. The results further suggest that the pure small vesicles are characterized by a single phase transition at 36.9 °C, whereas the L form is characterized by a phase transition at 41.2 °C. These results are consistent with the basic assumptions of the model.

Within the terms of this model the results in Figure 9 can be represented by:

$$\Delta H_3 = \Delta H_L - (\Delta H_L/\Delta H_S)\Delta H_2 \qquad (5)$$

Values of ΔH_L = 8.4 kcal/mol and ΔH_S = 6.3 kcal/mol were estimated from the parameters of the least-squares regression line.

Although these results are consistent with the hypothesis that small vesicles spontaneously transform into L structures at temperatures below the main transition, because these preparations experienced several 0–70 °C temperature cycles it was impossible to define time independently of temperature. However, since the transformation process appears to be slow we shall, for the purposes of kinetic analysis, define time as that time the small vesicle preparation was in the temperature range 0–30 °C.

Assuming that at time zero the small vesicle preparation was *pure* small vesicle, S, and that transformation into an S and L mixture proceeded according to first-order kinetics, the fraction of phosphatidylcholine molecules in multilamellar L structures and the fraction of phosphatidylcholine molecules in small vesicles in the mixture are:

$$f_L = (k_1/k_{-1})[1 - \exp(k_1 + k_{-1})t] \qquad (6)$$
$$f_S = 1 - f_L$$

Here f_L and f_S are the fractions of phospholipid molecules in L structures and small vesicles at time t, and k_1 and k_{-1} are the apparent first-order rate constants previously defined.

According to the model, $f_L = \Delta H_3/\Delta H_L$ and $f_S = \Delta H_2/\Delta H_S$. These quantities, estimated from the results in Table I and the linear regression analysis shown in Figure 9, are plotted as a function of time in Figure 10. The solid line was computed using values of k_1 = 0.0878 h^{-1} and k_{-1} = 0.0412 h^{-1}. Thus, the conversion of small vesicles of dipalmitoylphosphatidylcholine into an equilibrium mixture of small vesicles and L structures follows apparent first-order kinetics with a half-time of about 5.4 h in the temperature range 0–30 °C.

The apparent standard Gibbs energy change for the S → L transformation at 15 °C can be estimated from the ratio of k_1/k_{-1}. If the sizes of the S and L vesicles are identical then $\Delta G^{\circ\prime} = -RT \ln (k_1/k_{-1}) = -0.4$ kcal/mol is the standard Gibbs energy change per mole of vesicle. If L is larger than S, which is most likely the case, it follows that $\Delta G^{\circ\prime} < -0.4$ kcal/mol.

Because of our lack of knowledge regarding the size of the L liposomes, this calculation cannot be exact, but within the terms of the model, an important conclusion can be derived from it: the L-type vesicle is thermodynamically more stable than the S type at 15 °C, but the Gibbs energy difference per mole of phospholipid is small.

Calculations of the difference in the standard Gibbs energy change for the gel–liquid crystalline transition of the S and L type vesicles also lead to some interesting consequences within the terms of the proposed model. For these transitions ΔH_S° = 6.3 and ΔH_L° = 8.4 kcal/mol of phospholipid. The corresponding standard entropy changes can be calculated from $\Delta S_S^\circ = \Delta H_S^\circ/T_{m_2}$ to be 20 cal/(mol deg) and $\Delta S_L^\circ = \Delta H_L^\circ/T_{m_3}$ to be 27 cal/(mol deg). The difference in the Gibbs energy changes is given by:

$$\delta(\Delta G^\circ) = (\Delta H_S^\circ - T\Delta S_S^\circ) - (\Delta H_L^\circ - T\Delta S_L^\circ)$$

At 15 °C $\delta(\Delta G^\circ) = -0.4$ kcal/mol of phospholipid. This result suggests that at 15 °C the free-energy difference per mole of phospholipid between the liquid crystalline forms S* and L* is also very close to zero. If this is indeed true and is maintained at all temperatures, then two important conclusions follow from the model. First, since at high temperatures the S* and L* forms do not appear to interconvert, then a substantial kinetic barrier between them must exist. Second, since the differences in the thermodynamic quantities associated with the gel–liquid crystal transitions must be accounted for in the gel states, it is necessary that the enthalpy and entropy of the S state must be greater than the L state. Therefore, the L-type liposomes will be more stable at low temperatures and the S-type liposomes will be more stable at high temperatures. The temperature

at which this inversion in stability will occur can be estimated from $(\Delta H_L^\circ - \Delta H_S^\circ)/(\Delta S_L^\circ - \Delta S_S^\circ)$ to be approximately 27 °C.

Although the above calculations are approximate, and in addition depend upon some restrictive assumptions, they lead to conclusions which can be experimentally tested.

Recently, de Kruijff and coworkers (1975) have reported that the calorimetric behaviors of sonicated and unsonicated liposomes are indistinguishable. These results are apparently not in agreement with our data nor are they consistent with data in the literature. For example, Sheetz and Chan (1972) clearly showed that the phase transition in small dipalmitoylphosphatidylcholine vesicles lies well below that recorded for larger liposomes. Calorimetric studies on large liposomes have established that the main phase transition for this lipid is centered at about 41 °C (Ladbrooke and Chapman, 1969; Hinz and Sturtevant, 1972; Vaughn and Keough, 1974). Many NMR studies necessarily carried out on small vesicle dispersions have shown the transition for dipalmitoylphosphatidylcholine to be centered at about 37 °C (e.g., Uhing, 1975).

The basis for this discrepancy between the results of de Kruijff and others is unknown. Several points are, however, worth noting in this connection. (1) Although de Kruijff and co-workers (1975) show similar calorimetric scans for sonicated and unsonicated liposomes which have a transition centered at about 45 °C, their NMR studies on sonicated liposomes clearly show a transition centered at about 37 °C. (2) Their thermal data show no pretransition for large vesicles, a characteristic feature of the thermal behavior of dipalmitoylphosphatidylcholine liposomes (Ladbrooke and Chapman, 1969). (3) Since the sonicated preparation of small vesicles utilized by de Kruijff and co-workers (1975) was not fully characterized, it may possibly contain an appreciable quantity of large, multilamellar liposomes. Because of the relative sharpness of their transition, these multilamellar structures may well dominate the calorimetric behavior of the system.

The results reported in this paper add to the evidence already in the literature that the physical properties of phospholipid bilayers in small, single-lamellar vesicles are different than they are in multilamellar liposomes. The variation in these physical and thermodynamic properties may be a manifestation of the difference of the radii of curvature or a reflection of single- vs. multilamellar character of the two types of liposome. The small single-lamellar vesicles described in this report have been shown by autocorrelation light scattering to be homogeneous in size with a radius of approximately 110 Å (Y. Barenholz, D. Gibbes, B. J. Litman, J. Goll, T. E. Thompson, and F. Carlson, manuscript in preparation). If it is assumed that the bilayer thickness is about 40 Å, then the radii of curvature of the two faces of the bilayer comprising the vesicle wall differ by a factor of about 2. In addition, the signs of the radii of curvature in opposite bilayer faces are different. This situation suggests that the molecular packing in the bilayer of a small vesicle will, on the average, be different than it is in bilayers of large Bangham-type liposomes.

It is not known whether the differences in bilayer properties that exist between small single-lamellar vesicles and large multilamellar Bangham-type liposomes have functional significance in biological membranes. All available evidence suggests that biological membranes are comprised of single bilayers. Multilamellar systems exist only in such specialized multimembrane structures as the myelin sheath of myelinated nerve fibers. Biological membranes frequently, however, exhibit minimum radii of curvature structures of the order of 100 Å. It is possible that the alteration in the properties resulting from the small radii of curvature of the component bilayers has functional significance in these biological membrane structures (Thompson et al., 1974).

Acknowledgment

We gratefully acknowledge the technical assistance of Dr. D. B. Mountcastle in performing some of the calorimetric experiments.

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