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TURBIDITY CHANGES OF LIPID VESICLES NEAR THE PHASE TRANSITION TEMPERATURE AS AN INDICATION OF FUSION

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Summary

Sonicated liposomes of dipalmitoyl phosphatidylcholine show sharp turbidity changes on heating at two distinct temperatures. A decrease in turbidity at the lower temperature (approx. 37°C) is thought to be associated with the phase transition of small vesicles and a decrease at about 44°C with larger vesicles or multilayer. An increase of turbidity between 38 and 43°C is attributed to the fusion of small vesicles. The turbidity changes were studied under various modes of vesicle preparation to confirm the interpretation of the turbidity data. Alternate interpretations are discussed.

Introduction

Thermotropic phase transition in both natural and model membranes dispersed in water have been observed by light scattering and turbidity methods [1—7]. Abramson [1] observed a decrease in turbidity of an aqueous dispersion of dipalmitoyl phosphatidylcholine in the range from 24 to 33°C and a sharp decrease at the transition temperature of 41°C.

Recently, Chong and Colbow [4] distinguished between the "normal" reversible changes in the scattered light intensity and turbidity with temperature, and the so-called anomalous irreversible changes. The normal change was mainly attributed to the change in the refractive index of the lipids as suggested earlier by Yi and MacDonald [5]. The anomalous change was attributed to fusion of smaller vesicles and is often only seen on the first temperature scan through the phase transition temperature following the lipid dispersion.

In the present paper we present further data of the anomalous turbidity changes, and suggest that these are caused by the fusion of small vesicles. The anomalous changes are large for well dispersed samples at higher lipid con-

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centrations than used previously in light scattering experiments [4]. At higher vesicle concentrations fusion may be expected to play a more important role. Light scattering and turbidity measurements are very sensitive to change in vesicle size [4], and provide a valuable and simple technique to study membrane fusion, a field of considerable current interest [6—12].

One may either observe the scattered light at right angles to the incident beam or measure the turbidity, which is the decrease by scattering of the transmitted light, and thus is equal to the scattering overal angles. Turbidity measurements are advantageous at high lipid concentrations (less than 30% light transmission) while 90° light scattering is more sensitive for relatively clear solutions. It is expected that the smallest vesicles of dipalmitoyl phosphatidylcholine have their phase transition temperature lowered by 2—5°C relative to unsonicated dispersions. A decreased cooperativity of the transition for small vesicles could arise from the reduced number of lipid molecules involved, or from a disruption of close packing of lipid molecules due to the small radius of the vesicle [13]. Recent calorimetic investigations by Sternberg et al. [15] produced two well separated peaks with transition temperatures at 39 and 43°C. Since the 39°C peak increased with length of sonication, it was attributed to small vesicles, while the 43°C transition was attributed to multilayers or, presumably, also larger vesicles.

We shall assume the validity of these interpretations in the following discussion of our data.

Experimental

β,α-Dipalmitoyl-DL-α-phosphatidylcholine, dipalmitoyl phosphatidic acid and phosphatidic acid from egg lecithin were purchased from Sigma Chemical. All lipids were stored dessicated below 0°C.

Lipid vesicles were prepared by sonication of the lipid dispersion or chloroform preparation in buffer or doubly-distilled water. The buffer used in this study contained 0.050 M *N*(trishydroxy methyl)-aminomethane and 100 mM NaCl and the pH was adjusted to 7.2. The desired amount of lipid was weighed in a Mettler H20 balance, dissolved in chloroform and placed in a dark chamber in which nitrogen gas was blown over the solution to evaporate the chloroform overnight and leave the lipid on the sides of the tube. Following this procedure, no residual chloroform was detectable by infrared absorption. After adding the buffer, 4 ml of the solution was mixed twice for 15 s in a Bronwill mixer, and was then placed in a water jacket (21°C) and sonicated by a Biosonic IV sonicator equipped with a low power probe. Alternatively, the lipid was added directly to the buffer before mixing and sonicating, and no chloroform was used. The samples were placed into 1-cm^2 cuvets, and the transmitted light was measured at 436 nm in the Jarell-Ash spectrophotometer similar to the one described previously [4], but rearranged for light transmission instead of 90° light scattering. The samples were usually heated from 22 to 45°C at a rate of about 1°C per min while the transmitted light was measured. The temperature was measured in the circulating bath, and a separate measurement in the sample cell using a thermistor indicated a temperature lag of less than 0.5°C at this heating rate.

For all diagrams the transmitted light is in the same arbitrary relative units and was divided electronically by the approximately constant incident light intensity. Other experimental conditions are specified under each figure.

Data and Discussion

The data presented in this section is a small selection from a series of experiments undertaken to study fusion and aggregation of dipalmitoyl phosphatidylcholine vesicles. Our preliminary experiments showed that after sonication the turbidity of the lipid dispersion changed little with time at 22°C and changed rapidly at 45°C. Fig. 1 shows the vesicle turbidity as the temperature is increased from 22 to 45°C at a rate of about 1°C per min. The reproducibility is often poor for the absolute intensity of the 39°C peak in the transmitted light on the first heating cycle. This is probably due to high sensitivity of vesicle fusion and aggregation on trace amounts of lysophosphatidylcholine, fatty acids [12], divalent ions [13], as well as the exact conditions of sonication. Different lipid samples gave different peak heights by as much as a factor of two, despite the fact that thin layer chromatography did not reveal any contamination. However, the peak positions were reproducible to within about 0.5°C. The general features of the curve may be interpreted as follows: negligible fusion or aggregation takes place between 22 and 35°C and the transmitted light is constant. Between 35 and 37°C small vesicles undergo the lipid phase transition, resulting in a lower turbidity mainly due to a smaller index of refraction in the liquid crystalline state [4,5]. Between 37 and 44°C small vesicles undergo fusion, which is triggered by their phase transition [7]. This is expected to produce a higher turbidity since scattering is proportional to the number of vesicles and the square of their volume [4]. Further changes in turbidity are observed at 44°C which are possibly associated with the phase transition of larger vesicles and multilayers. Keeping the sample at 45°C for the next 12 min one observes an increase of transmitted light due to sedimentation followed by flocculation, the appearance of visible lipid fragments, which results in a fluctuating signal and more rapid sedimentation. Depending on conditions,

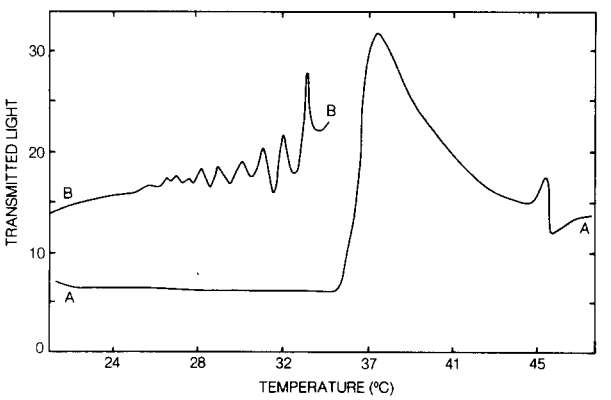

Fig. 1. Turbidity of lipid vesicles during heating from 22 to 45°C (A), and (B) sample was kept at 45°C for the next 12 min. (1.19 mg/ml lipid sonicated for 35 min in buffer.)

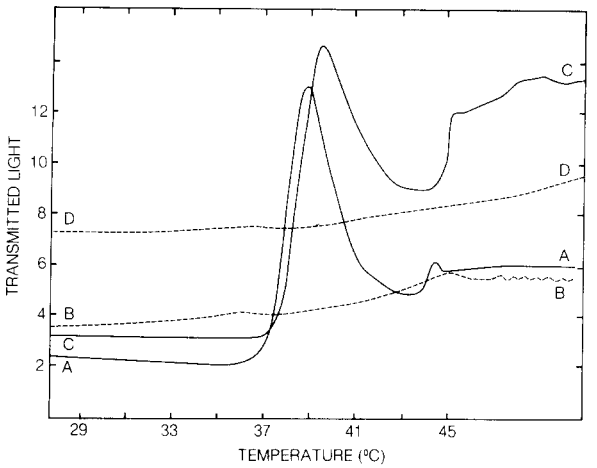

Fig. 2. The effect of chloroform preparation on the turbidity of lipid vesicles during heating from 22 to 45°C (solid curves) and after cooling to 22°C, mixing and heating a second time to 45°C (dotted curves). A and B, 10.42 mg lipid dissolved in chloroform, dried under nitrogen, and sonicated for 20 min in buffer; C and D, no chloroform was used but otherwise identical conditions.

the onset of flocculation may take hours or minutes, and considerably longer at lower lipid concentrations [4].

Prior to flocculation one is also able to observe directly the formation of larger vesicles (over 100 nm radius) using incident-light dark ground illumination in an ultramicroscope. Our previous light scattering analysis [4] indicates that after 20 min sonications most vesicles have a single bilayer and a radius of about 200 Å. Few particles are observable in the ultramicroscope for freshly sonicated samples. However, after heating to 41°C larger particles appear, at which point some aggregation is also observable in the form of coupled diffraction patterns. So far this method has not produced quantitative results on vesicle fusion and aggregation. The dotted curves show that once heated to 45°C the 38—39°C peaks disappear, and one obtained the broader turbidity change at 41°C, as reported earlier [1—5]. In Fig. 2D the phase transition is somewhat obliterated due to the high lipid concentration causing some multi-

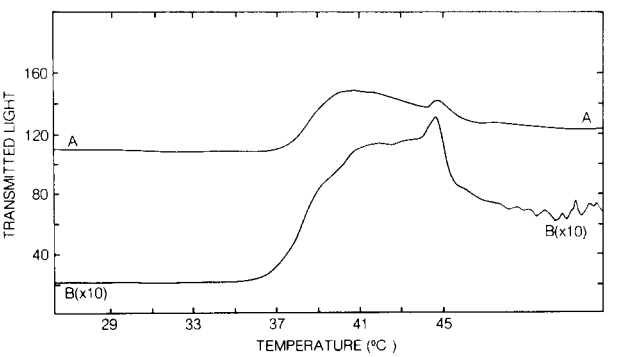

Fig. 3. The effect of centrifugation on the turbidity of lipid vesicles during heating. 11.37 mg lipid dissolved in chloroform sonicated for 20 min in buffer, after which the solution was centrifuged for 20 min at 10000 rev./min at 4°C. A, turbidity of the supernatant and B, of the pellet, which was resuspended in 3 ml of buffer by a mixer.

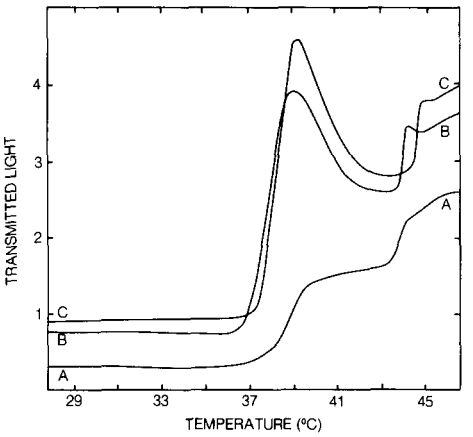

Fig. 4. The effect of lipid concentration on the turbidity of lipid vesicles during heating. The samples were sonicated for 20 min in buffer. A, 3.34 mg/ml; B, 2.83 mg/ml; C, 2.64 mg/ml.

ple scattering and simultaneous sedimentation due to aggregation and fusion. A larger percentage change of the transmitted light at 41°C is observed at lower lipid concentration [4,5]. To eliminate the possibility that air bubbles may be responsible for these peaks we repeated the experiment with vesicles made from egg lecithin and obtained a flat curve for the first and second heating cycle. To further test our interpretation we centrifuged one sample and found, as expected, a higher relative proportion of the 39°C peak in the supernatant than in the redispersed pellet (Fig. 3). Fig. 4 shows that a approx. 3 mg/ml the height of the 39°C peak becomes a sensitive function of concentration, with the lower concentration producing a larger height, probably due to less fusion during sonication and, thus, production of a larger fraction of small vesicles. A steep dependence of colloidal stability on cencentration is well known for metal colloids [16]. Fig. 5 shows that the 39°C peak is also observed when

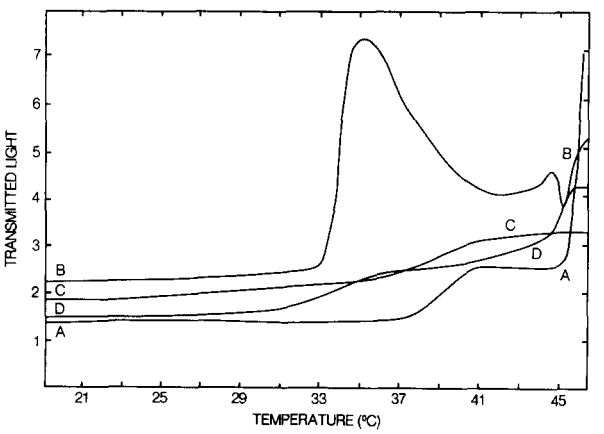

Fig. 5. The effect of the buffer, length of sonication and temperature of sonication on the turbidity of lipid vesicles during heating. A, 10.50 mg lipid sonicated 5 min in 4 ml distilled water; B, 5.98 mg lipid sonicated 20 min in 3 ml distilled water; C, 6.7 mg lipid dispersed (not sonicated) in 3 ml of buffer; D, 10.3 mg lipid dissolved in chloroform and sonicated 5 min in 4 ml buffer at approx. 50°C.

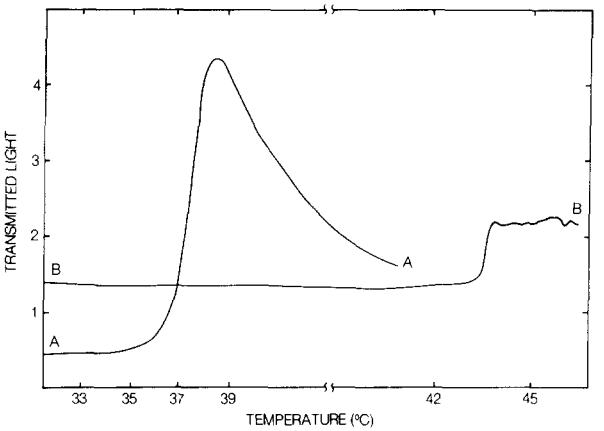

Fig. 6. The effect of incubation at 39.5°C on the turbidity of lipid vesicles. 10.66 mg lipid dissolved in chloroform and sonicated for 20 min in buffer. A, turbidity during the temperature rise from 22 to 39.5°C. The decrease in transmitted light occurred during constant temperature at 39.5°C for 10 min. B, incubation at 39.5°C for 30 min was followed by the temperature increase from 39.5 to 45°C.

dipalmitoyl phosphatidylcholine is sonicated in distilled water for 20 min (curve B) rather than in Tris buffer containing 0.1 M NaCl. The transition temperature is lowered by about 3°C to 34°C compared to sonication in buffer (compare Fig. 4). For only 5 min sonication in distilled water (curve A) at a somewhat higher lipid concentration, no small vesicle transition is observable; the turbidity change at about 39°C is believed to be due to larger vesicles and the 45°C change due to sedimentation. Dispersion without sonication in buffer does not produce a 39°C peak (curve C). Fig. 6 illustrates that once the temperature has been increased to 39.5°C, vesicle fusion produces an increase in turbidity for about 10 min at constant temperature (curve A), after which the turbidity stays constant until the temperature is raised again to about 44°C.

Fig. 7 shows that dipalmitoyl phosphatidylcholine kept at 4°C for about 2 years produces a relatively large peak at 37°C (curve A), compared with those in the previous figures. Furthermore, this peak persists to a larger degree through the second and third heating cycle (curves B and C). The simplest

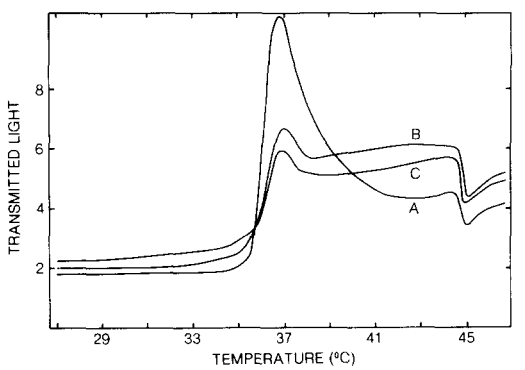

Fig. 7. Turbidity during heating for vesicles prepared from 2 year old lipid. A, 2.13 mg/ml sonicated for 35 min in buffer; B, after cooling the above sample to 22°C, mixing, and heating a second time to 45°C; C, cooling the sample to 22°C, mixing, and heating for the third time to 45°C.

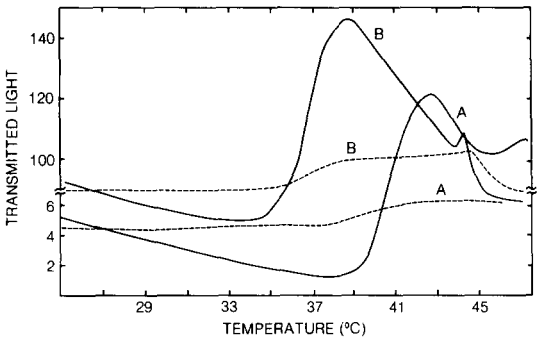

Fig. 8. The effect of dipalmitoyl phosphatidic acid on the turbidity of dipalmitoyl phosphatidylcholine vesicles during heating. A, 12.72 mg lipid and 0.84 mg of the phosphatidic acid were dissolved in chloroform, and sonicated for 15 min in buffer (without NaCl); B, 10.30 mg lipid prepared as above, except the phosphatidic acid was omitted. The dotted curves represent the second heating cycle after cooling to 22°C.

explanation is to postulate the production of smaller vesicles with a net charge due to the presence of lipid decay products. Charged vesicles would more likely go through reversible phase transitions without fusion or aggregation due to their Coulomb repulsion. This hypothesis led us to the experiment of adding negative charge in the form of dipalmitoyl phosphatidic acid to vesicles prepared from fresh dipalmitoyl phosphatidylcholine. NaCl was omitted from the buffer to enhance the effect of Coulomb repulsion between vesicles. The results in Fig. 8 show that the omission of NaCl produced the turbidity changes below 35°C, which was not observed in the other experiments with NaCl nor with distilled water. In the absence of dipalmitoyl phosphatidic acid (curve B) the transmitted light decreases between 39 and 44°C at a slower rate than in the previous figures, which may be due to a slower rate of fusion. In the presence of dipalmitoyl phosphatidic acid (curve A) we are presently not sure whether the 41°C change represents large vesicles and multilayers, or smaller vesicles whose transition temperature is increased due to the phosphatidic acid. Further experiments with mixed lipids are in progress.

Conclusion

The results presented in this paper show that the sharp increase in the transmitted light on heating is related to the phase transition of lipid vesicles. The irreversible increase in transmitted light at about 39°C may be explained by the disappearance of small vesicles on repeated heating. Due to a lower phase transition temperature and a larger kinetic energy of Brownian motion small vesicles are more likely to undergo vesicle-vesicle fusion than large ones. Vesicle-vesicle fusion on heating has also been suggested on the basis of calorimetic studies by Kantor et al. [11] and on the basis of NMR and light-scattering data by Van Dijck et al. [7]. Martin and MacDonald [6] agree that large vesicles grow at the expense of small ones, but suggest a diffusive transfer of individual lipid molecules for dimyristoyl phosphatidylcholine vesicles. Their conclusions are based on the linear dependence of the change in absorbance with lipid concentrations and the somewhat weak assumption that turbitidy

changes are linearly proportional to the vesicle growth rate.

To account for the turbidity changes at 39°C one could further speculate that freshly sonicated vesicles may contain excess water and/or disorder in their membranes. This could produce the lowered transition temperature, and vanish after heating produced an annealing of the membranes and displacement of water from the membrane interior, both of which should lead to increased turbidity.

Acknowledgement

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References

1 Abramson, M.B. (1971) Biochim. Biophys. Acta 225, 167—170
2 Overath, P. and Träuble, H. (1973) Biochemistry 12, 2625
3 Sackman, E. and Träuble, H. (1972) J. Am. Chem. Soc. 94, 4482—4491
4 Chong, C.S. and Colbow, K. (1976) Biochim. Biophys. Acta 436, 260—282
5 Yi, P.N. and MacDonald, R.C. (1973) Chem. Phys. Lipids 11, 114—134
6 Martin, F.J. and MacDonald, R.C. (1976) Biochemistry 15, 321—327
7 Van Dijck, P.W.M., de Kruijff, B., Aarts, P.A.M.M., Verkleij, A.J. and de Gier, J. (1978) Biochim. Biophys. Acta 506, 183—191
8 Papahadjopoulos, D., Hui, S., Vail, W.J. and Poste, G. (1976) Biochim. Biophys. Acta 448, 245—264
9 Papahadjopoulos, D., Vail, W.J., Pangborn, W.A. and Poste, G. (1976) 448, 265—283
10 Papahadjopoulos, D., Vail, W.J., Newton, C., Nir, S., Jacobson, K., Poste, G. and Lazo, R. (1977) Biochim. Biophys. Acta 465, 579—598
11 Kantor, H.L., Mabrey, S., Prestegard, J.H. and Sturtevant, J.M. (1977) Biochim. Biophys. Acta 466, 402—410
12 Kantor, H.L. and Prestegard, H.J. (1975) Biochemistry 14, 1790—1794
13 Marsh, D., Watts, A. and Knowles, P.F. (1977) Biochim. Biophys. Acta 465, 500—514
14 Day, E.P., Ho, J.T., Kunze, Jr., R.K. and Sun, S.T. (1977) Biochim. Biophys. Acta 470, 503—408
15 Sternberg, B., von Löwis of Menar, M., Preusser, E., Grupe, R. and Göring, H. (1977) Stud. Biophys. 64, 53—64
16 Overbeek, J.Th.G. (1952) in Colloid Science I (Kruyt, H.R., ed.), p. 304, Elsevier Publishing Co., Amsterdam