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Temperature dependence of calcium-induced fusion of sonicated phosphatidylserine vesicles

(dynamic light scattering/bilayer phase transition)

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ABSTRACT **We have measured the temperature dependence calcium-induced fusion of sonicated phosphatidylserine vesicles. The vesicles were incubated in the presence of calcium at a specified temperature until the resulting aggregation or fusion process had gone to completion. EDTA was then added and the resulting final size of the vesicle population was measured by using dynamic light scattering. This final size was plotted against incubation temperature to show the temperature dependence of calcium-induced fusion. This curve has a peak near 11°C which may be associated with the phase transition of the sonicated phosphatidylserine vesicles in the presence of calcium prior to the aggregation or fusion process.**

There is great interest in understanding membrane fusion, both for its fundamental importance in biology and for its potential application in medicine. Fusion of phospholipid vesicle systems is important as a model of the fusion of more complicated biomembranes and as a prototype encapsulation system for the introduction of drugs and genetic material into living tissues.

Studies conducted over the past 6 years have clarified the conditions under which the model system of neutral phosphatidylcholine vesicles will fuse. There has been controversy over the relative importance of vesicle growth due to monomer diffusion between vesicles (1, 2) and that due to bilayer fusion after vesicle–vesicle collisions. The role of impurities was also unclear in the earliest studies (3, 4). It has now been shown, by Kantor *et al.* (5) among others, that pure phosphatidylcholine vesicles are stable at all temperatures, although Suurkuusk *et al.* (6) reported slow changes toward larger vesicles for pure sonicated dipalmitoylphosphatidylcholine vesicles held below 27° for hours. However, these neutral vesicles will fuse and at a dramatically enhanced rate at their phase transition temperature, provided that an appropriate "impurity" is present in the bilayer. Prestegard and coworkers (4, 5, 7) showed that myristic acid at 2–4 mol% will bring about fusion of phosphatidylcholine vesicles. Van der Bosch and McConnell (8) found that concanavalin A can cause dipalmitoylphosphatidylcholine vesicles to increase in size through fusion even when present at a concentration of only 1–20 concanavalin A molecules per vesicle. Again, the rate of size increase was maximal at the bilayer phase transition temperature. Finally, Martin and MacDonald (1) reported that dimyristoylphosphatidylcholine vesicles with 3% ganglioside increase in size due to membrane fusion, although no evidence of any temperature dependence for this phenomenon was given. In each of these studies, the size increase was not measured directly but was inferred from the effect of a size increase on a nuclear magnetic resonance, electron spin resonance, optical, or calorimetric signal. In the temperature studies it was the rate rather than the extent of the size increase that was shown to reach a maximum at the phase transition.

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We have been studying the model system of negatively charged phosphatidylserine vesicles which are known to fuse in the presence of Ca^{2+} above a threshold concentration. This system is likely to be of direct relevance to biological fusion which depends upon the presence of calcium (9). Sonicated phosphatidylserine vesicles have the added advantage of being stable in the absence of calcium, because of their negative charge. This model fusion system was first studied at 37° by Papahadjopoulos *et al.* (10) who found, using freeze-fracture electron microscopy, that large scroll-like cylinders, named cochleates, precipitated from a solution of sonicated phosphatidylserine vesicles (diameter *ca* 30–50 nm) upon the addition of 1 mM calcium. Subsequent addition of the chelating agent EDTA in solution above this precipitate resulted in the formation of large unilamellar vesicles (diameter *ca* 300–1000 nm). These events are summarized schematically in Fig. 1.

We have been using dynamic light scattering to study this system at slightly lower calcium concentrations to avoid precipitation of the cochleates (11). Dynamic light scattering measures average vesicle size by responding to the Brownian motion of a population of vesicles in solution (12).

MATERIALS AND METHODS

Highly purified phosphatidylserine from beef brain was supplied to us by D. Papahadjopoulos (Department of Experimental Pathology, Roswell Park Memorial Institute, Buffalo, NY) (13). Vesicles were prepared in 0.1 M NaCl/2 mM 2{[tris(hydroxymethyl)methyl]amino}ethanesulfonic acid/2 mM L-histidine/0.1 mM EDTA adjusted to pH 7.4 with NaOH. In a typical experiment, 20 μmol of phospholipid in chloroform was evaporated to dryness, 2 ml of buffer was added, and the mixture was shaken mechanically on a vortex mixer for 10 min and then sonicated for 1 hr in a bath-type sonicator maintained at 24°C. The sample was kept under nitrogen atmosphere at all times. The sample was next centrifuged at 50,000 × *g* for 15 min to eliminate any remaining large or multilamellar vesicles.

The incubation time appropriate to a given preparation was determined by monitoring the kinetics after calcium addition at room temperature. We have performed separate kinetics experiments that indicated that the aggregation or fusion rate at temperatures down to 5°C is never less than one-half the rate of the room temperature rate. Typically, the supernatant from the centrifugation step was diluted 1:10 and mixed with an equal volume of 1.8 mM calcium in buffer to form a 1-ml sample having a phospholipid concentration of less than 0.5 mM and a nominal calcium concentration of less than 0.9 mM. Light from a Spectra-Physics 144 He/Ne laser was focused on this sample. The transmitted intensity was detected with a Spectra-Physics 385 photodiode whose output was recorded on a

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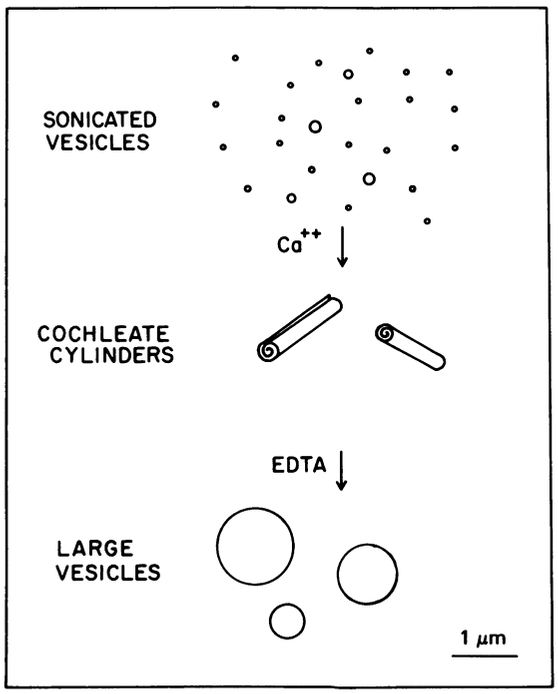

FIG. 1. Schematic representation of the morphological changes induced in sonicated phosphatidylserine vesicles by calcium and EDTA.

stripchart recorder. As the aggregation or fusion events after calcium addition proceeded, the transmitted intensity decreased. The incubation time, typically 3 hr, was chosen to exceed by more than a factor of 2 the time when this transmitted intensity of a room temperature sample leveled off. Therefore the aggregation or fusion process had gone to completion within the chosen incubation time over the entire temperature range of this study.

Inverted-Y shaped glass tubes were used to equilibrate the vesicles and calcium prior to mixing to allow mixing at constant temperature under nitrogen at the start of incubation. The two arms of each mixing tube were loaded with 0.5 ml of the 1:10 diluted supernatant and 0.5 ml of 1.8 mM calcium in buffer, respectively, and sealed under nitrogen. Each mixing tube was then equilibrated at the incubation temperature in a regulated water bath controlled to better than ±0.1°C. Temperatures were measured with a platinum resistance thermometer. The solutions in the separate arms were then mixed and held at the incubation temperature in the water bath, typically for 3 hr. The Ca^{2+} was next removed by injecting 0.1 ml of 0.1 M EDTA in buffer into the incubated sample. Each sample was removed from the water bath 0.5 hr after EDTA addition and measured at room temperature by using dynamic light scattering. The samples were turbid after the 3-hr incubation with calcium. Because this turbidity cleared in less than 5 min after EDTA addition at all temperatures, the 0.5-hr EDTA incubation time was more than adequate to ensure completion of chelation over the entire temperature range of this study.

Dynamic light scattering measurements were made by detecting the laser light scattered at 60° through collimating pinholes to a photomultiplier. The photocurrent $I(t)$ at time t was amplified and its time correlation function $R_I(\tau) = \langle I(t)I(t + \tau)\rangle$ was computed with a Honeywell SAI-42A correlation and probability analyzer for 100 values of delay time τ separated by a selectable constant value. The data were recorded on magnetic cassette tape with a data logger. Data analysis was performed by using a time-share terminal with dual cassettes connected to the University CDC Cyber 173 computer.

The measured correlation function $R_I(\tau)$ for a homogeneous population of vesicles can be fitted with the expression (12)

$$R_I(\tau) = R_I(\infty) + B \exp(-2\tau/\tau_c)$$

or equivalently,

$$\ln [R_I(\tau) - R_I(\infty)] = \ln B + (-2/\tau_c)\tau$$
$$= A_0 + A_1\tau.$$

To extract the hydrodynamic diameter of the vesicles from the correlation time τ_c, we calibrated the instrument over the size range of interest (30–2000 nm) by measuring the correlation times at the same scattering angle for various monodisperse polystyrene spheres of known radii (obtained from Dow Diagnostics). The calibration was performed with the polystyrene spheres suspended in the buffer solution at the same temperature as used for vesicle size measurements. For the heterogeneous population of vesicles resulting from the present fusion experiment, we fitted the measured intensity correlation function $R_I(\tau)$ to the expression (12)

$$\ln [R_I(\tau) - R_I(\infty)] = A_0 + A_1\tau + A_2\tau^2.$$

All the data reported here are based on an average hydrodynamic size found using the first-degree coefficient A_1 of this second-degree fit.

In the case of a monodisperse sample the measured size is that of the vesicles. In the case of heterogeneous population of vesicles, such as the fusion products of this study, the meaning of the measured average size can be ambiguous without independent information on the nature of the size distribution. For this reason we previously (11) checked our results for consistency with the freeze-fracture electron microscopic study of the same system by Papahadjopoulos *et al.* (10). Moreover, the central result of our present study depends not on the absolute size of the fusion products but on the dramatic changes of this measured average size with calcium incubation temperature. This relative measurement of average size against calcium incubation temperature is meaningful even if the absolute size at a given temperature is somewhat ambiguous.

RESULTS

Fig. 2 gives the final average vesicle size as a function of the calcium incubation temperature for two different preparations. The final size was measured after the initial sonicated vesicles had first been incubated in the presence of calcium for, typically, 3 hr at the specified incubation temperature and then EDTA added to remove the calcium. These curves were selected to represent the type of variability we have obtained upon repetition of this experiment. What is striking about the curves is the reproducibility of the temperature at which the peak occurs. Both preparations showed a peak at 11°C as did other preparations. The variation in the maximal measured size at the peak does not puzzle us because the concentration of phospholipid after sonication and centrifugation varied with the preparation. The sharpness of the peak may be exaggerated by our dynamic light scattering technique which weights the largest vesicles more strongly than the smallest ones.

DISCUSSION

A large final vesicle size is indicative of extensive fusion during calcium incubation. The measured initial size of these sonicated vesicles ranges from 30 to 80 nm, depending on the preparation. Were calcium addition to cause reversible aggregation without fusion, subsequent EDTA addition would yield a final size similar to this small initial size. Any increase over this initial size

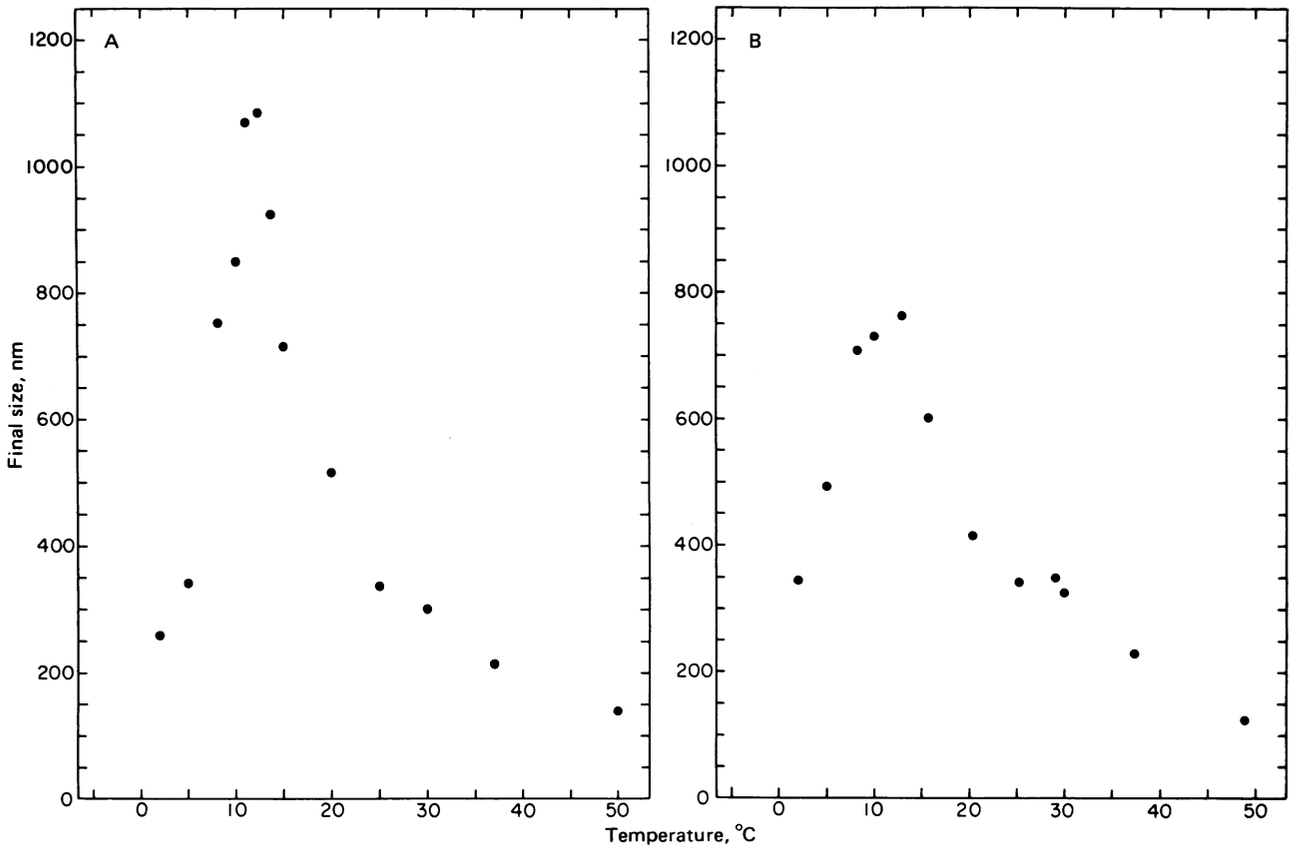

FIG. 2. Final average size as a function of the calcium incubation temperature for two separate preparations (*A* and *B*) of sonicated phosphatidylserine vesicles. The final size was measured by using dynamic light scattering after first incubating the sonicated vesicles (<0.5 mM) for, typically, 3 hr in the presence of calcium (<0.9 mM) and then adding EDTA (10 mM).

is indicative of an irreversible change in morphology brought about by fusion during incubation in calcium. The more extensive the fusion during incubation, the larger the final size will be. Therefore, we interpret our final size measurement as a measure of the extent of calcium-induced fusion.

The peak at 11°C in the temperature dependence of the extent of calcium-induced fusion of sonicated phosphatidylserine vesicles is suggestive of a role being played by the vesicle bilayer phase transition in the fusion process. This temperature, 11°C, is a reasonable value for the transition temperature of sonicated phosphatidylserine vesicles in the presence of a nominal calcium concentration of less than 0.9 mM.† Vesicles incubated at this temperature would be at their phase transition temperature as they collided and aggregated or fused. The fact that the final size is largest at this temperature indicates the importance of the phase transition in the fusion process. Further study is needed to identify which property of the phase transition is important to the fusion process.

The maximal measured average sizes are really quite large (>1 μm) and may be of interest to those designing vesicle encapsulation systems in which large vesicle size is of value.

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† Differential scanning calorimetry data for multilamellar phosphatidylserine vesicles indicate a broad peak in the vicinity of 6°C (14). Sonicated vesicles typically have a phase transition temperature shifted 5°–10°C lower than that of the corresponding multilamellar vesicles (2, 5, 6, 15, 16). This would place the sonicated phosphatidylserine vesical phase transition in the region of 0°C. The presence of divalent ions at a concentration giving 1:2 ion association with acidic phospholipids results in an upward shift of 6°–20°C in the bilayer phase transition temperature, unless there is substantial fusion which causes the peak to shift well above this temperature region (14, 17–21). The 11°C peak we are observing is therefore probably associated with the phase transition of the sonicated vesicles in the presence of calcium. This phase transition temperature in the presence of calcium cannot be readily measured by using standard techniques because of the rapid formation of cochleates under these conditions. The cochleate structure is an extremely anhydrous association of phospholipid and calcium (22) and therefore has a very high phase transition temperature (14, 17, 19, 20, 23).

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