Fusion of Dipalmitoylphosphatidylcholine Vesicles†

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ABSTRACT: Small unilamellar dipalmitoylphosphatidylcholine vesicles formed by sonication are shown to fuse spontaneously below the phase transition temperature. The ultimate fusion products are unilamellar vesicles about 700 Å in diameter, which are stable and provide an intact ionic permeation barrier either above or below the phase transition. The fused vesicles have been characterized by gel chromatography, trapped volume, ^{31}P nuclear magnetic resonance, and negative stain and freeze-fracture electron microscopy.

Phospholipid multilamellar liposomes and single lamellar small vesicles have been intensively studied to provide a basic understanding of biological membrane structure and function (Bangham et al., 1974). More recently these systems have been examined as potential drug delivery vehicles (Papahadjopoulos, 1978).

The small vesicles produced by sonication (Huang, 1969) have often been the system of choice for physical studies because of their size homogeneity and their unilamellar structure. The bilayers of these vesicles have, however, been shown to differ in certain respects from the more nearly planar bilayers of multilamellar liposomes and biological membranes (Sheetz & Chan, 1972; Thompson et al., 1974; Mason & Huang, 1978; Suurkuusk et al., 1976). In addition, light scattering measurements have indicated that under some circumstances small vesicles prepared from saturated diacylphosphatidylcholines appear to form larger structures spontaneously (Tsong, 1974; Petersen & Chan, 1978). It is not possible, however, in the terms of light scattering data to decide if the larger structures result from simple vesicle aggregation or from fusion processes which result in an increased vesicle size. In a previous study of the thermal properties of small vesicles formed from dipalmitoylphosphatidylcholine, we observed time-dependent changes in the heat capacity vs. temperature function that were also reflected in fluorescence polarization studies and turbidity measurements on this system (Suurkuusk et al., 1976). These changes were interpreted as due to fusion of the vesicles below the gel–liquid-crystalline phase transition temperature. This process produced structures that were similar but not identical in their thermal properties with multilamellar liposomes. In this paper we present additional evidence that fusion of dipalmitoylphosphatidylcholine vesicles does in fact occur below but not above the phase transition temperature. Size and structure studies carried out by several methods show the fusion products to be a unilamellar vesicles about 700 Å in diameter. These vesicles appear to afford a surprisingly homogeneous and well-defined system with potential applications as model membranes and drug delivery vehicles.

Materials and Methods

Preparation of Vesicles. Dispersions of small unilamellar vesicles were prepared as follows. Sixty micromoles of 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine was dried in vacuo overnight (Suurkuusk et al., 1976). This material was then suspended in 3 mL of buffer or salt solution by vortexing at 45 °C, followed by sonicating for six 2-min bursts at 1-min intervals at 50–55 °C (Heat Systems W-350 sonifier). The sonicated material was then centrifuged for 15 to 30 min at 100000*g* (45–55 °C) to remove probe particles and the remaining multilamellar liposomes. All transfers were made by using pipets warmed to 45–55 °C.

Lipid concentrations were determined as inorganic phosphorus by the method of Bartlett (1959).

Gel Chromatography. Sepharose CL-2B (Pharmacia) columns (29 × 1.6 cm) were packed at a pressure of 30 cm of water and run at 25–28 cm of water with upward flow. Sephadex G-25-Coarse (Pharmacia) columns (13 × 1.6 cm) were run under similar conditions. For parallel studies of particle size and trapped volume, a Sepharose column and a Sephadex column were connected by a common valve (LV-3, Pharmacia) to the eluant and sample reservoirs. Aliquots of the same sample preparation were then analyzed on both columns at time intervals. For the high-temperature experiments, in addition to using water-jacketed columns, the sample and eluant reservoirs, the valves, the connecting tubing, and the lower ends of the columns were all immersed in a 47 °C water bath so that the phosphatidylcholine was never below its phase transition temperature until through the column (Suurkuusk et al., 1976). Column effluent was monitored continuously at 260 nm in a 1-cm flow cell with a Hitachi 100-10 spectrophotometer connected to a Sargent-Welch SRLG recorder (sensitivity 0.2 Å full scale). In some cases fractions were collected and analyzed for phosphorus.

The trapped volume of the vesicles (volume of the total internal aqueous compartment) was determined by preparing and incubating the vesicles in the presence of 50 mM Na_2CrO_4. After a portion of the vesicle sample was passed over the Sephadex column and solubilized by Triton X-100, the volume of the total vesicle interior per mole of phospholipid was determined by spectrophotometric measurement of CrO_4^{2-}. To eliminate possible contamination of the vesicle fraction by the trailing CrO_4^{2-}, only the leading half of the vesicle peak was collected for analysis.

Isoosmotic and constant ionic strength conditions were maintained on all of the gel columns. When the sample contained Na_2CrO_4, the columns were prewashed and then eluted with an equivalent Na_2SO_4 concentration in the same buffer.

A Sepharose CL-2B column was calibrated for solute radii determination using the method of Ackers (1967). Sonicated egg phosphatidylcholine vesicles purified by the method of Barenholz et al. (1977) and turnip yellow mosaic virus kindly donated by Dr. D. Kupke were used as calibration standards.

Electron Microscopy. Samples at 21 °C were placed on gold planchets and rapidly frozen by plunging into liquid

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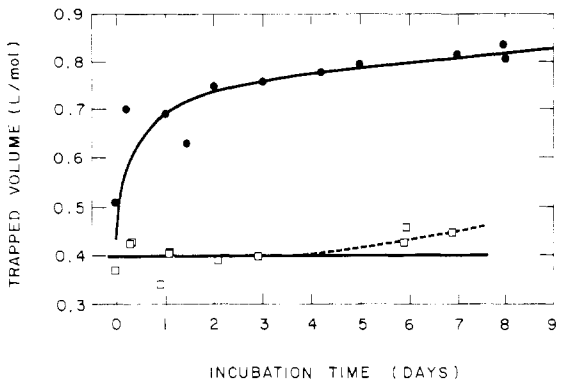

FIGURE 1: Trapped volume of dipalmitoylphosphatidylcholine vesicles incubated at 21 °C (○) and 47 °C (□) for various times. The vesicles were prepared and incubated in a solution of 0.050 M Na_2CrO_4, 0.01 M Tris (pH 8.2), 1 mM EDTA, and 0.02% NaN_3. The initial time is taken as the time when the vesicles are removed from the warm centifuge head. The apparent slight rise for the trapped volume of the 47 °C preparation (dashed line) is not thought to be statistically significant.

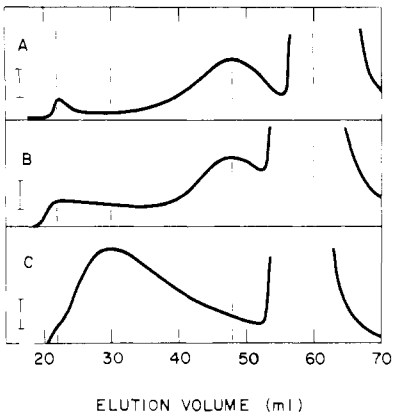

FIGURE 2: Turbidity-monitored Sepharose CL-2B elution profiles for dipalmitoylphosphatidylcholine vesicles incubated at 47 °C for 0 to 7 days (A), at 21 °C for 5 h (B), or at 21 °C for 1 day (C). The vertical markers correspond to a 0.02 optical density change at 260 nm. Vertical dashed lines indicate the void volume (22 mL), fused vesicle peak (30 mL), small vesicle peak (48 mL), and the solvent volume, in this case marked by the CrO_4^{2-} (60 mL). The sample loaded onto the column was 0.2 mL of 20 mM dipalmitoylphosphatidylcholine in 0.050 M Na_2CrO_4, 0.010 M Tris (8.2), 1 mM EDTA, and 0.02% NaN_3. Other experimental conditions are given in the text.

Freon. Fracturing, etching, and shadowing were performed in a Balzers BAF 300 Freeze-etch apparatus under a vacuum of 2×10^{-6} mbar. Fracturing was done at −100 °C followed by etching by sublimation for 30 s. Platinum was shadowed at a 45° angle to a depth of 20 Å. A 250-Å carbon film was then applied to the replica. The original sample was cleaned from the replicas by floating on Clorox bleach for 24 h.

The replicas were examined in a Philips 300 electron microscopy operating at 60 kV and at 41 000 magnification. Vesicles were sized by measuring the diameter at the widest part of the shadow. This procedure slightly overestimates the vesicle size because of the thickness of the platinum shadow.

Negatively stained vesicle preparations were examined on carbon-coated formvar films on copper grids in a Siemens Elmiscop IA electron microscope operating at 60 kV and usually at 40 000 magnification. The sample was applied to the grid, allowed to remain for 1 min, and then drained. Isoosmotic ammonium molybdate solution (0.75 to 1.5%) at pH 8 was added. After 1 min the excess molybdate was drained and the preparation was examined immediately.

^{31}P Nuclear Magnetic Resonance. ^{31}P nuclear magnetic resonance was performed at 24.15 MHz in a JEOL FX60Q Fourier transform NMR spectrometer. Usually 2000 scans were accumulated with an acquisition time of 0.512 s per scan. A delay of 6.0 s between pulses with the ^1H decoupler off was used to supress the Nuclear overhauser effect. The transformed spectra had 4K data points. The sample was 0.8–1.0 mL of 25–30 mM phospholipid prepared in 0.10 M KCl and 0.02% NaN_3 as bacteriocide and 10% D_2O to provide a lock signal. Spectra were obtained with the sample at 45 °C to obtain a conveniently narrow line width. Pr^{3+}, added as an isoosmotic solution of $PrCl_3$ to give a final ratio of Pr^{3+}/phospholipid of 0.054, was used as a shift reagent. Pr^{3+} shifts downfield and broadens the resonance of the exposed phosphorus nuclei on the external surface of the vesicles. The ratio of the area under the shifted peak to the unshifted peak corresponds to the molar ratio of exposed to unexposed phospholipid (Bergelson, 1978). This value reflects vesicle size, number of bilayers in the vesicle, and leakiness of the vesicle to Pr^{3+}.

Results

Vesicle Fusion Indicated by Increased Trapped Volume. When small vesicles were incubated in the presence of 0.050 M K_2CrO_4 at 21 °C, an initial rapid rise was observed in the trapped volume followed by a very slow increase which continued for at least 8 days. This is illustrated in Figure 1. Over this same period there was no significant change in the trapped volume of identical vesicles incubated at 47 °C. The increase in trapped volume at 21 °C can only be explained by fusion of vesicles to form new structures, whose volumes per mole of lipid were greater than the original small vesicles. It is apparent that the interiors of the fused vesicles were at least partially equilibrated with the chromate in the bulk solution. It is, however, not possible to distinguish between equilibration during fusion and fusion followed by equilibration as a result of permeation of chromate into the fused vesicles. A test was performed for CrO_4^{2-} permeability and vesicle leakiness while on the Sephadex column by halting the flow for various times during passage of samples down the column. There was no significant loss of trapped chromate at either 21 or 47 °C under the normal experimental conditions (3 and 10 min on the column for 47 and 21 °C, respectively). This result supports the validity of the chromate-trapping method for detecting fusion but does not rule out the possibility that the very slow long-term increase in trapped volume might be due to rapid fusion with limited equilibration followed by slow but continued permeation of more chromate into the fused vesicles.

Vesicle Size Increase Determined by Sepharose CL-2B Chromatography. Agarose gel chromatography performed on aliquots of the same preparations used for the trapped volume studies confirmed an increase in vesicle size for vesicles incubated at 21 °C. Representative turbidity-monitored elution profiles are shown in Figure 2. Also, consistent with the trapped volume results, no change was seen in the size of the same vesicles incubated at 47 °C. The time scale of the 21 °C fusion process seen on the Sepharose column roughly corresponded to that found for the trapped volume changes. The turbidity elution profile was essentially unchanged for times longer than 24 h at 21 °C. The phosphorus elution profiles for vesicles fused for 5 and 12 days at 21 °C are shown in Figure 3. Phosphorus analysis of column fractions revealed a very slow conversion of the last one third of the small vesicles to the larger forms. Complete conversion was never seen, since 12 days was the longest time studied. It is interesting that the difference in specific turbidity of the small and large

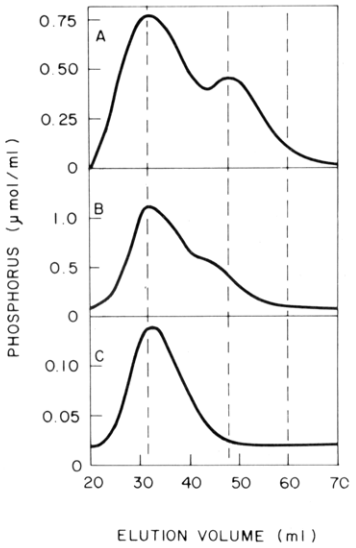

FIGURE 3: Phosphorus elution profiles from Sepharose CL-2B for dipalmitoylphosphatidylcholine vesicles incubated at 21 °C for 5 days (A) and at 21 °C for 12 days (B). Curve C is a rerun of the peak fraction from curve B after 2 more days at 21 °C. The vertical dashed lines indicate the void volume (22 mL), fused vesicle peak (31.5 mL), small vesicle peak (48 mL), and the solvent volume (60 mL). A profile identical with C was obtained when an aliquot of the B peak fraction was incubated overnight at 51 °C and then run on a 47 °C Sepharose CL-2B column. The sample loaded was 0.2 mL of 20 mM dipalmitoylphosphatidylcholine in 50 mM KCl and 0.02% NaN_3. Other experimental conditions are given in the text.

vesicles is such that the distinct small vesicle peak in the turbiditiy elution profile seen in Figure 2 disappeared after only 24 h.

The fused vesicle peak fractions from Figure 3B were collected for further study; these pooled fractions are designated purified fused vesicles. The purified fused vesicles were rechromatographed after 2 days of further incubation at 21 °C. As seen in Figure 3C, there was no change in the vesicle size. This indicates that the small and large forms are not undergoing rapid equilibration. Subsequent rechromatography of this sample showed that it was unchanged after storage at room temperature for 7 weeks. Furthermore, incubation of the purified fused vesicles for 18 h at 51 °C followed by rechromatography on a 47 °C Sepharose CL-2B column did not change the elution profile. Taken together these results rule out the possibility that the putative larger vesicles are in part simply aggregates of the initial small vesicles. It is clear that the large objects detected by these methods, which form spontaneously below the gel–liquid-crystalline phase transition temperature, are all fusion products.

It is important to note in Figures 2 and 3 that no material large enough to elute in the void volume was formed by the fusion process. Thus the vesicles appear to obtain a maximum size and then the fusion process either stops or assumes a much slower rate. This maximum size is thus either an equilibrium state or a highly stable intermediate state. Calibration of the Sepharose column indicated that the average diameter of the purified fused vesicles is 660 Å.

Vesicle Size and Shape Determined by Electron Microscopy. Freeze-fracture electron micrographs of dipalmitoylphosphatidylcholine vesicles allowed to fuse at 21 °C for varying times are shown in Figure 4. The distribution of fused vesicle size as a function of time is shown in Figure 5. These results are consistent with the agarose chromatography data. Thus, some large fused vesicles are formed within 1 day, but approximately one-third of the lipid is still in vesicles under 500-Å diameter after 9 days. Also, the maximum in the distribution occurs in the 700-Å diameter fraction in agreement with the Sepharose column results. The vesicles appeared to be spherical, and there was no evidence of aggregation. Multilamellar material was not seen in any of the samples, although the resolution was probably not adequate to distinguish between vesicles with one or two lamellae.

An electron micrograph of negatively stained purified fused vesicles is shown in Figure 6A. The sample appears to be remarkably homogeneous. Measurement of vesicle diameters gave an average diameter of 740 Å with a standard deviation of 105 Å. This is in reasonable agreement with the Sephadex column and freeze-fracture results.

Generally the interiors of the vesicles are not stained, so the number of lamellae could not be assessed with confidence. This is presumably because the bilayers are impermeable to the stain and because the vesicles did not collapse upon drying, which would have created a pocket of stain in the center that would contrast with the bilayers (Johnson et al., 1971). However, unequivocally unilamellar vesicles were sometimes observed, and neither oligo- nor multilamellar structures were ever seen. The fused vesicles used for NMR experiments clearly showed their single lamellar structure, as seen in Figure 6B.

^{31}P NMR Studies. The value of the outside/inside ratio, determined at 45 °C, decreased from 2.1 ± 0.1 to 1.3 ± 0.1 for vesicles incubated at 21 °C for 4 days. Vesicles kept at 21 °C for 19 days gave the same result. The out/in ratio did not increase appreciably over a period of 12–24 h, indicating that the fused vesicles are impermeant to Pr^{3+} on this time scale at 45 °C. An approximate calculation based on the assumption of equal packing densities for the two monolayers of a vesicle indicates that an out/in ratio of 1.3 corresponds

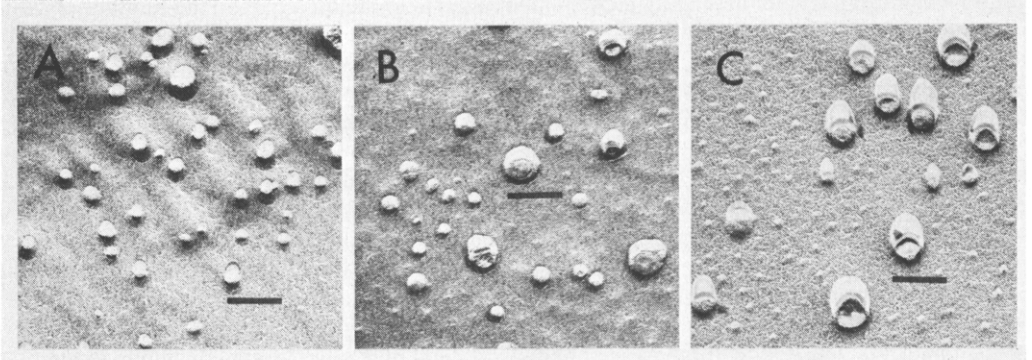

FIGURE 4: Freeze-fracture electron micrographs of dipalmitoylphosphatidylcholine vesicles that have been incubated at 21 °C for 40 min (A), 1 day (B), and 9 days (C). Fields representative of the particle size distributions are shown. A, B, and C are at the same magnification; the marker corresponds to 1000 Å. The direction of shadowing was from the bottom to the top of the page (the shadows are white). The sample in C was adjusted to contain 5% glycerol in order to minimize production of any freezing or sublimation artifacts. The glycerol made no significant difference.

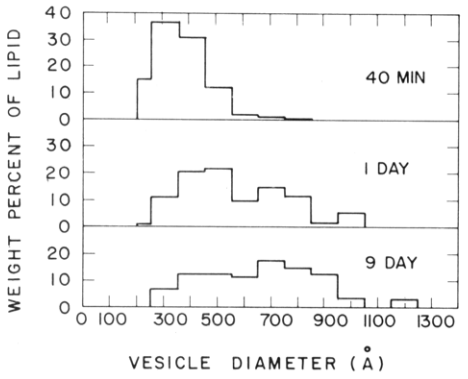

FIGURE 5: Estimated lipid distribution over the range of vesicle sizes as determined from freeze-fracture electron micrographs of the samples is shown. The weight percent of lipid in each size range was calculated by weighting the number percent of vesicles by the average radius, r, of the size range. The basis for this involved the assumptions that (1) the probability of a vesicle appearing in a fracture plane was proportional to r and (2) the mass of lipid in a vesicle (unilamellar) was proportional to r^2. Hence, the proper weighting factors to correct for undercounting of small vesicles and for the greater mass of large vesicles was $(1/r) \times r^2 = r$. The number of vesicles measured for each histogram was $N = 294$, 40 min; $N = 190$, 1 day; $N = 116$, 9 days.

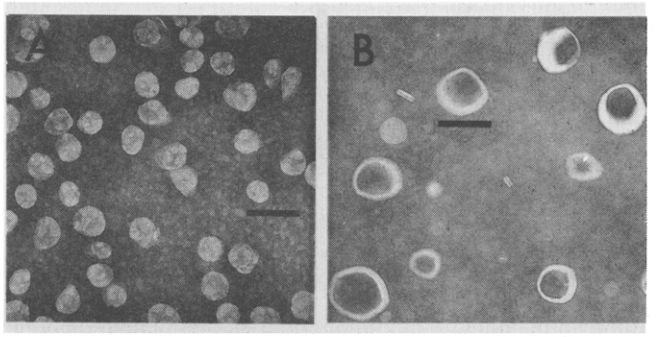

FIGURE 6: Negative stain electron micrographs of purified fused vesicles (A) and a vesicle sample used for NMR (B). The NMR sample had been allowed to fuse at 21 °C for 19 days, run in the NMR at 45 °C, mixed with isoosmotic $PrCl_3$ to a Pr^{3+}/phospholipid ratio of 0.054, rerun in the NMR, and then stored for 7 more days at 45 °C. Both micrographs are at the same magnification as those in Figure 4; the marker corresponds to 1000 Å.

to an average vesicle diameter of 500–600 Å. This also assumes that the vesicles are unilamellar. A simple calculation shows that the out/in ratio should be rather sensitive to the presence of oligo- or multilamellar vesicles, i.e., if 5–10% of the lipid were inaccessible to Pr^{3+}, the out/in ratio would decrease by several tenths, and an overestimate of the vesicle size would result. These results are therefore consistent with, and provide further support for, the electron microscopy and molecular sieve chromatography results described above.

The ^{31}P line widths for the fused vesicles were 7 Hz for the total line width and 6 Hz for the inside (unshifted by Pr^{3+}) resonance, so that the out/in chemical-shift difference is ~1 Hz. For sonicated dipalmitoylphosphatidylcholine vesicles at 45 °C and 24 MHz, the inside line width is 3 Hz and the out/in chemical-shift difference is 3 Hz. The latter splitting has been attributed to packing differences between the two monolayers (Sheetz & Chan, 1972; Schmidt et al., 1977; Bergelson, 1978) and therefore would be expected to decrease as the radius increases, as observed. The assumption of equal packing densities of the two monolayers thus is more valid for the larger vesicles. In addition, the line-width increase produced by an increase in vesicle diameter from about 200 to about 600 Å is rather small. This is in agreement with preliminary calculations based on the analysis of McLaughlin et al. (1975) (C. F. Schmidt, unpublished results).

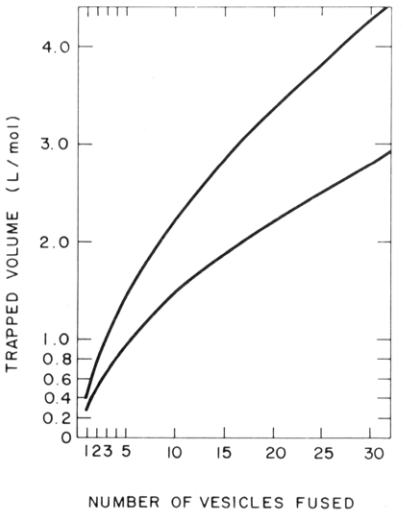

FIGURE 7: Theoretical dependence of vesicle trapped volume on the number of original small vesicles that have fused. The lower curve is based on egg phosphatidylcholine dimensions (Mason & Huang, 1978). The upper curve is based on unfused vesicle dimensions adjusted so as to predict our observed trapped volume of 0.40 L/mol. An unfused dipalmitoylphosphatidylcholine vesicle was assumed to have (1) an internal chamber accessible to solute of 82-Å radius, (2) a 6-Å thick internal layer of hydration water (Mason & Huang, 1978; J. Mason, personal communication) that excludes solutes, (3) an unhydrated bilayer thickness of 37 Å, and (4) 3500 dipalmitoylphosphatidylcholine molecules per vesicle. Both models assumed constant bilayer lipid density, so bilayer volumes were simply additive upon vesicle fusion. Temperature effects on bilayer thickness (Janiak et al., 1976) were ignored.

Discussion

The trapped volume measurements, molecular sieve chromatography, electron microscopy, and ^{31}P NMR results all strongly indicate that small vesicles of dipalmitoylphosphatidylcholine spontaneously form larger vesicles at temperatures below the gel–liquid-crystalline phase transition. No such process occurs above this temperature. Electron microscopy, molecular sieve chromatography, and ^{31}P NMR data lead to the conclusion that the small vesicles ultimately form a stable population of unilamellar vesicles about 700 Å in diameter. A simple calculation shows that these fused vesicles would contain the lipid of 15 to 20 small vesicles. These large vesicles present intact barriers to ion permeation both above and below the phase transition.

An estimate of the size of the larger vesicles can also be obtained from the trapped volume measurements as follows. Although the dipalmitoylphosphatidylcholine small vesicle has not been well characterized by size and molecular weight, an approximate estimate of the number of vesicle fusions that correspond to the observed trapped volume increase can be obtained using the information in the literature (Chrzeszczyk et al., 1977; Mason & Huang, 1978). The lower curve in Figure 7 is calculated assuming (1) the original vesicle dimensions and molecular weight are the same as egg phosphatidylcholine and (2) the fused vesicles are unilamellar spheres. From this curve the largest trapped volume obtained experimentally (Figure 1) corresponds to a vesicle resulting from the fusion of four or five of the original small vesicles. However, the trapped volume of 0.26 L/mol of P calculated for the original vesicles by this model is lower than the 0.4 L/mol of P initial trapped volume actually observed for the vesicles and is higher than that reported for small vesicles formed from other phospholipids (Roseman et al., 1978). A model based on an initial vesicle with 0.40 L/mol of P trapped volume is shown in the upper curve in Figure 7. Based on this

calculation, only two to three vesicles need fuse to give the observed increase in trapped volume. The vesicle size estimated from these trapped volume measurements is thus substantially smaller than the size estimated more directly by the other methods. This discrepancy is most easily explained if the early steps in the fusion process permitted equilibration of the internal vesicle compartment with the external medium, while subsequent fusion steps were essentially nonleaky. In this system it is clear that while trapped volume measurements give a qualitative indication of increased vesicle size, they do not permit a quantitative estimate of the size of the fusion product.

Several other phospholipid systems have been reported to give vesicle fusion under certain experimental conditions. Dimyristoylphosphatidylcholine vesicle fusion has been well studied by Prestegard and co-workers (Kantor & Prestegard, 1978, and references therein). Unlike our results, dimyristoylphosphatidylcholine vesicle fusion occurs only in the phase transition region and appears to require a fusogen such as free fatty acid. Recently, Larrabee (1979) reported increases in vesicle size for distearoylphosphatidylcholine vesicles maintained below the phase transition but not at or above the transition. Fusion of sonicated distearoylphosphatidylcholine vesicles produces a final state with two distinct large vesicle populations of ~600 and ~1000 Å diameters. No fusogenic component was known to be present in this system, although 1,3-distearoylphosphatidylcholine was found to inhibit the process. No known fusogenic component was present in our vesicle system.

Calcium ions have been shown to produce fusion of egg phosphatidylcholine vesicles containing phosphatidic acid (Koter et al., 1978; Liao & Prestegard, 1979), of phosphatidylserine vesicles either pure (Portis et al., 1979) or containing less than 50 mol % phosphatidylcholine (Papahadjopoulos et al., 1974), and in the mixed phospholipid vesicles from soybean asolectin (Ingolia & Koshland, 1978). Our initial work with dipalmitoylphosphatidylcholine vesicles was done in the presence of 1 mM ethylenediaminetetraacetic acid (EDTA) to eliminate effects due to trace amounts of divalent cations. Identical results were obtained when EDTA was omitted.

Sonicated phosphatidylcholine vesicles have previously been reported to be stable in size only below the transition temperature (Tsong, 1974), below or above but not at the transition (Prestegard & Fellmeth, 1974), at or above the transition (Larrabee, 1979), or under no conditions (Sheetz & Chan, 1972); now our results indicate size stability only above the transition. Part of this disagreement may represent real differences in the vesicle systems used including contamination by trace amounts of fusogens or antifusogens (Dunham et al., 1977; Kantor & Prestegard, 1978; Larrabee, 1979). However, the problem is traditionally complicated by the difficulty of distinguishing "true" fusion of topologically distinct bilayers to produce a single bilayer from aggregation of vesicles that otherwise remain intact. Most physical methods for particle size determination (most notably, turbidity changes) cannot distinguish fusion from aggregation. Certain gel filtration media have even been found to catalyze a fusion or aggregation process for vesicles passing through a column (Chen & Schullery, 1979). The role of salts in producing various states of aggregation has been analyzed in detail by Petersen & Chan (1978). In the work presented here, the increase in trapped volume for dipalmitoylphosphatidylcholine vesicles incubated at 21 °C together with the other results provide strong evidence that vesicle fusion occurred.

Dipalmitoylphosphatidylcholine-fused vesicles provide an attractive model system for studying bilayer membranes or a homogeneous vesicle population in which the strain induced by the small radius of curvature in sonicated vesicles has been largely relaxed. The system should be valuable for further fundamental physical studies of bilayer properties as well as possibly useful as a drug delivery system.

Acknowledgments

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