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Fusion of Dipalmitoylphosphatidylcholine Vesicles at 4 °C†

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ABSTRACT: Small sonicated dipalmitoylphosphatidylcholine vesicles when incubated at 4 °C and high concentrations are shown to fuse completely to vesicles about 700-Å diameter in 7 days, and these further fuse to about 950 Å diameter vesicles after 3–4 weeks. The 950 Å diameter vesicles are spherical, homogeneous, mostly unilamellar, have an internal aqueous space about 10 times that of small vesicles, and are stable for at least 6 months. The 950-Å vesicles are characterized by agarose gel chromatography, freeze–fracture electron microscopy, trapped volume measurements, differential scanning calorimetry, and diphenylhexatriene fluorescence polarization.

Phospholipid liposomes have been used extensively in physical studies of membrane properties (Lentz et al., 1976; Chen & Sturtevant, 1981; Martin & MacDonald, 1976), in delivery of materials to cells (Schneider et al., 1980; Pagano & Weinstein, 1978; Weinstein et al., 1978; Heath et al., 1980), in protein–lipid interaction studies (Leto & Holloway, 1979; Roseman et al., 1977), in reconstitution studies (Racker, 1979), and in membrane–membrane interactions (Parsegian et al., 1979; Nir & Bentz, 1978). Unilamellar small vesicles, homogeneous in size (Huang, 1969; Barenholz et al., 1977), have proven to be a very useful model membrane system but are less suitable for some applications because of their small internal aqueous compartment and large curvature (Cornell et al., 1980). In previous studies (Suurkuusk et al., 1976; Schullery et al., 1980) we incubated small sonicated vesicles of dipalmitoylphosphatidylcholine (DPPC)[1] below the phase

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transition at 21 °C and found that fusion to 700 Å diameter vesicles occurred with a rapid initial phase followed by a much slower phase over the next 8 days. The fusion process was monitored by using a combination of trapped volume measurements, gel chromatography, and electron microscopy. The fused 700-Å vesicles were found to be mostly unilamellar, based upon the outside/inside mass ratio determined by NMR. From gel chromatography profiles and electron micrographs, approximately one-third of the small vesicles remained after 12 days of incubation.

In this paper we present studies of fusion of DPPC small vesicles at 4 °C over the concentration range 20–120 mM (P_i). Small vesicles were found to fuse completely to 700-Å vesicles and these further fused to 950 Å diameter vesicles in 3–4 weeks. By comparison with the previous DPPC fusion studies at 21 °C and the distearoylphosphatidylcholine (DSPC)[1] studies by Larrabee (1979), it was found that increased concentration, decreased temperature, and increased hydrocarbon chain length increased the rate of fusion but did not appreciably change the size of the structures to which the small vesicles fused.

Characterization of the 950 Å diameter fused DPPC vesicles showed them to be mostly unilamellar, homogeneous, spherical, with a trapped volume approximately 10 times that of small sonicated vesicles, and stable for at least 6 months. Both the 700 Å and 950 Å diameter vesicles are well-defined systems with potential applications in further physical studies of bilayer structure and in drug delivery systems.

In the following paper (Wong & Thompson, 1982) we present studies of aggregation of DPPC vesicles.

Materials and Methods

Preparation of Vesicles. Small unilamellar vesicles were prepared at 55 °C as follows. 1,2-Dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC)[1] was dried in vacuo overnight (Suurkuusk et al., 1976) and then suspended in buffer (20 mM Pipes, 10 mM NaCl, and 0.02% NaN_3, pH 7.4) at a concentration of 100–120 mM phosphate by vortexing. The suspension was sonicated under N_2 (Schullery et al., 1980) and centrifuged for 30 min at 100000*g* to remove probe particles and multilamellar vesicles. Small unilamellar vesicles were obtained by a second centrifugation for 60 min at 100000*g* (Barenholz et al., 1977). The top one-third of the centrifuged material was carefully removed and used directly, or after dilution with warm buffer. Fusion was initiated by cooling to below the gel–liquid-crystalline phase transition temperature to 4 °C. A control sample was incubated at 50 °C for 5 days in a sealed glass and Teflon syringe, which contained no air space. All lipid concentrations are given as inorganic phosphate, determined by the method of Bartlett (1959). DPPC gave a single spot on thin-layer chromatographs and was >99.9% pure by gas chromatography. The chloroform and methanol extract (Folch et al., 1956) of sonicated vesicles which were incubated for 6 months at 4 °C gave a single spot on thin-layer chromatographs, with both iodine and primuline (Skipski, 1975) detection. Thin-layer plates were loaded with 0.5 μmol of lipid. Primuline sensitivity is less than 0.1 nmol of phosphatidylcholine.

Gel Chromatography. Sepharose CL-2B (Pharmacia) columns (30 × 1.6 cm) and Bio-Gel A-150 (Bio-Rad) columns (22 × 1.6 cm) were run at 30–33 cm of water pressure with upward flow at room temperature. Vesicles (8–10 μmol) were loaded in 0.5 mL of buffer. Columns were calibrated with uniform polystyrene latex beads [380 (±75) (carboxylated), 850 (±55) (Dow Chemical), and 1000 (±80) Å diameter (Polysciences)] and with small sonicated vesicles made from 1-palmitoyl-2-oleoylphosphatidylcholine. The latex beads were run in 1% Triton X-100 in buffer to prevent coagulation (Rhoden & Goldin, 1979). Latex beads were assayed by using absorbance at the following wavelengths: 320 (380 Å), 400 (850 Å), and 530 nm (1000 Å). Correction for the effects of detergent was made by running the 380 Å diameter carboxylated latex beads with and without detergent. With detergent, the beads eluted at a position closer to the void volume, which was interpreted as the elution volume of beads coated with a monolayer of detergent. In constructing the column calibrations curves, the latex beads were assumed to have diameters corrected by an additional 96 Å, the diameter of the Triton X-100 micelle (Helenius & Simons, 1975). The calibration curve used for the Sepharose CL-2B column was elution volume = $-21.71 \ln R + 157.6$, where R = radius in angstroms. From this curve, an elution volume of 32 mL equals a diameter of 651 Å, and an elution volume of 30 mL equals a diameter of 713 Å. The calibration curve used for the A-150 column was elution volume = $-6.83 \ln R + 71.69$. From this curve, an elution volume of 30 mL equals a diameter of 897 Å, and an elution volume of 28 mL equals a diameter of 1202 Å. Lipid distribution profiles were from column fractions assayed by inorganic phosphate or by [^{14}C]DPPC. Column fractions were 2 mL/tube.

Electron Microscopy. Freeze–fracture electron micrographs were prepared as described in a previously published procedure (Schullery et al., 1980). Etching by sublimation was to a depth approximately equal to the vesicle radius. Only fractured surfaces were counted in the determination of the vesicle diameter distribution profile. Diameter was measured in the direction perpendicular to the direction of shadowing. The number distribution was weighted by the radius to correct for the mass of lipid in a vesicle and the probability of a vesicle of a given radius being in the fracture plane (Schullery et al., 1980).

Trapped Volume Determination. The trapped volume of the vesicles was determined by equlibrating in either 6-carboxyfluorescein (5 mM) or [^{3}H]glucose for 18–24 h at 50 °C, pH 7.4, in a filled and covered test tube to prevent evaporation. Previous studies showed that marker solute equilibrium was reached in less than 10 h (Lichtenberg et al., 1981). The pH was raised to 8.5 by addition of an aliquot of concentrated buffer (5×) or NaOH in order to decrease membrane permeability to marker (Szoka et al., 1979). The sample was equilibrated for 1.5 h. Quasi-elastic light-scattering measurements of vesicle radius at this point showed no change from the original vesicles. External marker was removed with a Sephadex G-25 column (18.5 × 1.6 cm) at pH 8.5, 21 °C. Only the initial fractions off the column were used in order to assure maximum separation from external marker. Triton X-100 was added to release internal marker, which was assayed by using absorbance at 490 nm or by counting the radiolabel. Three separate measurements were made with each trapped marker. The internal aqueous volume was calculated from the amount of trapped marker, the initial marker concentration and equilibration, and the molar concentration of phospholipid.

Differential Scanning Calorimetry. A highly accurate scanning calorimeter of the heat conduction type was used in these measurements (Suurkuusk et al., 1976). The samples were scanned at a rate of 15 °C/h, and the excess heat ca-

[1] Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); DPH, diphenylhexatriene; (P_i), concentration of inorganic phosphate.

pacity was recorded at 0.1 °C intervals. The phospholipid concentration was 56 mM (P_i). All scans were made after equilibrating the sample to room temperature from 4 °C and then scanning to above the gel–liquid-crystalline transition temperature. The base line was corrected with a buffer blank. All calculations were performed on a CDC Cyber 172 computer.

Fluorescence Depolarization of DPH. Diphenylhexatriene (DPH)[1] was used as a hydrophobic probe in scanning fluorescence depolarization as in previous studies (Suurkuusk et al., 1976; Lentz et al., 1976, 1978). Fused 950 Å diameter vesicles were diluted to 0.5 mM in 5 mL of buffer, and 1 μL of 2 mL DPH in tetrahydrofuran was added at 50 °C while the sample was vortexed. The sample was incubated in a covered container for 1 h at 50 °C in a rotary shaker. Fluorescence measurements were made with an SLM 4800S in the A/B mode, with the A and B channels measuring light polarized parallel and perpendicular, respectively, to the incident beam. Both channels measured light at 90° to the incident beam through 389 nm sharp cutoff filters (Schottke). The A and B channels were balanced initially to a nonpolarizing sample of *N*-methylacridinium perchlorate in methanol. Light-scattering blanks identical with the samples but without DPH gave less than 0.3% of the sample signal over a 4–50 °C range. Samples required no sucrose to remain in suspension and were stirred continuously to minimize temperature gradients. Temperature was controlled with a NESLAB-Endocal refrigerated circulating bath (RTE-8), temperature programmer (ETP-3), and digital controller readout (DCR-1). The temperature monitor/set point was a platinum probe in a cuvette located in the same holder as the sample. Temperature was scanned at 20 °C/h by using a linear programmer. Slit widths were kept narrow to minimize bleaching effects. Data were stored and processed on a Hewlett-Packard 9825S computer and a 7225A digital plotter.

Fusion of 700-Å Vesicles Formed at 21 °C. Homogeneous 700-Å vesicles were prepared by incubating small vesicles at 21 °C for 8 days (Schullery et al., 1980) followed by separation from residual small vesicles with a Sepharose CL-2B column. The fractions from the 700-Å peak were pooled and diluted to 10 mM (P_i). A 2-mL sample was heated to 60 °C for 10 min and then cooled to 4 °C. After 2 days some of the vesicles had aggregated and settled to the bottom of the test tube, effectively increasing the local concentration to 80–100 mM (P_i). After 20 days, 1.3 mL of the upper solution was removed. The remaining 0.7 mL was shaken to suspend the fused vesicles, and 0.5 mL (6.8 μmol) was loaded on a Sepharose CL-2B column. Aliquots of the original 700 Å diameter fractionated fused vesicles, the upper solution, and the resuspended lower solution were assayed in a Model HN5-90 quasi-elastic light-scattering spectrometer and Model 6864 computing autocorrelator (Nicomp) (detailed in the following paper) at 41 °C to assay particle diameters. From four to six measurements were made on each sample.

Results

Sepharose CL-2B Chromatography. When small sonicated vesicles were cooled below the gel–liquid-crystalline phase transition temperature to 4 °C, fusion occurred to approximately 700 Å diameter vesicles. This is illustrated in Figure 1. Small vesicles at concentrations of 120, 50, and 20 mM inorganic phosphate were incubated at 4 °C for 0–20 days, and aliquots were applied to Sepharose CL-2B columns. For the first 7 days all three concentrations gave a bimodal distribution consisting of small and 700 Å diameter fused vesicles,

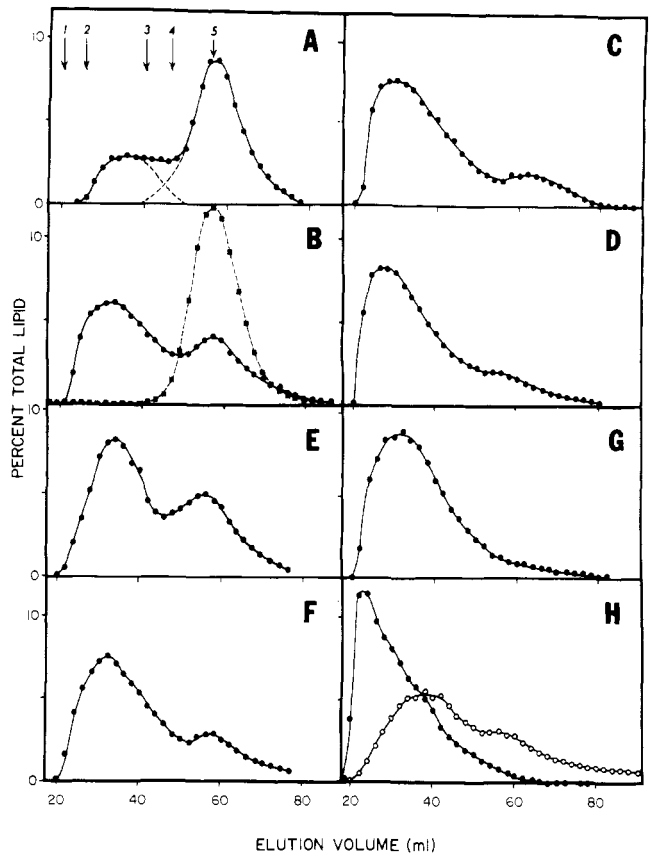

FIGURE 1: Sepharose CL-2B elution profiles for dipalmitoylphosphatidylcholine vesicles incubated at 4 °C: (A) 20 mM (P_i), 1 day; (B) 20 mM (P_i), 7 days; (C) 50 mM (P_i), 1 day; (D) 50 mM (P_i), 7 days; (E) 120 mM (P_i), 4 h; (F) 120 mM (P_i), 1 day; (G) 120 mM (P_i), 7 days; (H) 120 mM (P_i), 20 days [(●) 4 and (○) 21 °C]. 8–10 μmol of P_i were loaded per profile. Columns were calibrated with uniform polystyrene latex beads (see text). The arrows are located at the elution peaks of the standards: (1) 1000 ± 80 Å; (2) 850 ± 55 Å; (3) 380 ± 75 Å; (4) 380 ± 75 Å; (5) small sonicated vesicles. (1–3) were run with and (4) and (5) without 1% Triton X-100. Panel 1B shows the profile of 20 mM (P_i) small sonicated vesicles incubated for 5 days at 50 °C (■).

with little intermediate sized material. This same bimodal pattern was observed by Schullery et al. (1980) for fusion at 21 °C and 20 mM (P_i). Schmidt et al. (1981) analyzed the ^{31}P NMR line width and found a conversion from an original narrow line width (14 Hz) to a single broader line width (100 Hz), also suggesting a conversion from small to 700 Å fused vesicles with little intermediate sized vesicles. Calorimetric scans of vesicles during the fusion process are characterized by the growth of the 41.6 °C peak at the expense of the 37 °C small vesicle peak (Suurkuusk et al., 1976; Schmidt et al., 1981) with little material of intermediate transition temperatures produced, consistent with the bimodal column size distribution. The arrows in Figure 1A are located at the positions of the peaks in the elution profiles for uniform polystyrene latex bead standards. When a calibration curve of elution volume vs. ln (radius) is used, the peak of the elution profile for fused vesicles corresponds to 710 ± 60 Å diameter. Sepharose CL-2B column recoveries were >95% for samples incubated 1–5 days and decreased gradually to 70–75% for samples incubated 31 days. The decreased recovery may indicate more tightly bound large aggregates formed at longer times which do not pass through the agarose columns.

In contrast, vesicles stored in a sealed glass and Teflon container without any air space at 50 °C did not appreciably fuse over 5 days, as shown in Figure 1B (■). Recovery from this column was 94% of the applied lipid. The profile was the

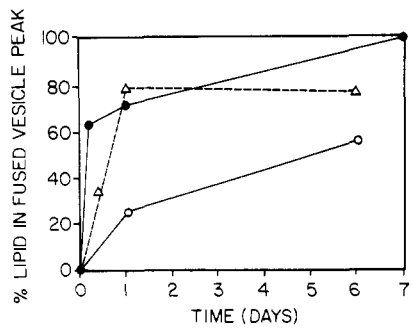

FIGURE 2: Fraction in the fused vesicle peak vs. time, for (●) 120, (△) 50, and (○) 20 mM (P_i). The percent lipid in the fused vesicle peak was estimated by measuring the area under the curve, as illustrated by the dashed lines in Figure 1A. The 50 mM (P_i) sample fused to entirely 700-Å vesicles and gave a profile identical with Figure 1H at day 31. The initial rates (4–24 h) are greater than first order in concentration (see text).

same at that obtained from freshly prepared small sonicated vesicles.

An estimate of the percent total lipid in the fused vesicle peak was obtained by cutting and weighing the area under the column profiles as illustrated in Figure 1A. The procedure used was to assume that the descending half of the fused vesicle peak was symmetric to the ascending half. It was found, as previously reported by Schullery et al. (1980), that reloading the fused vesicle peak on the column gave a symmetrical peak centered at the same position. The reproducibility for this procedure of estimating the percent total lipid in the fused vesicle peak was approximately ±5%. Figure 2 is a summary of the column chromatography for 0–7 days fusion at 4 °C. At all three concentrations, fusion occurred with an initial rapid conversion followed by a slower conversion over 1–7 days. A similar time course for trapped volume as a function of time of fusion at 21 °C was found by Schullery et al. (1980). The rate of the initial rapid conversion is a monotonic function greater than first order in concentration. The greater than first-order concentration dependence argues against monomer exchange through the aqueous phase as the mechanism of initial conversion from small to large vesicles at 4 °C (Lawaczeck, 1978). Schmidt et al. (1981) monitored the NMR ^{31}P peak height parameter P and found that for about the first 2 days of incubation at 21 °C, the reciprocal of P was linear with time. This argues for a second-order collisional mechanism for the conversion from small to large vesicles and is consistent with the concentration dependence seen in the initial points in Figure 2.

In contrast to fusion at 21 °C where the last one-third of small vesicles converts very slowly to larger vesicles (Schullery et al., 1980), at 4 °C and 120 mM (P_i) small vesicles fuse entirely to 700 Å diameter vesicles after 7 days, and these continue to enlarge, reaching a stable population of 950 Å diameter vesicles after 3 weeks. Figure 1G shows the profile of small vesicles fused at 4 °C and 120 mM (P_i) for 7 days. No evidence of a small vesicle shoulder is seen in the profile. Figure 1H contains two profiles, both prepared from a common small vesicle preparation at 120 mM (P_i) incubated for 20 days, half at 4 °C and half at 21 °C. The sample incubated at 21 °C contained approximately 60% fused and 40% small vesicles, the result expected based upon the work of Schullery et al. (1980). The sample incubated at 4 °C is shifted closer to the column void volume, to a distribution containing some 700 Å diameter material which appears as a shoulder on the approximately 1000 Å diameter material peak located near the column void volume.

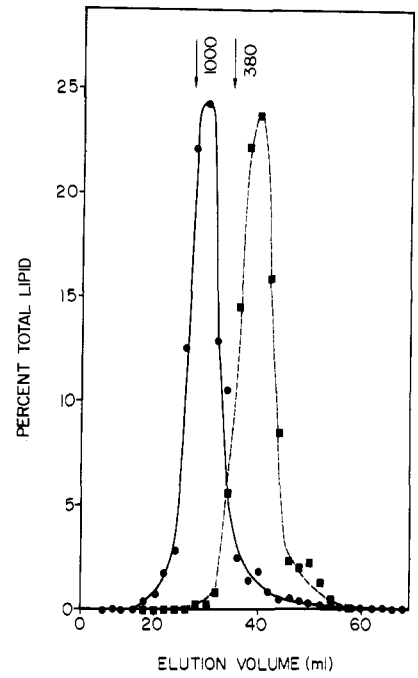

FIGURE 3: Bio-Gel A-150 column profile of fused vesicles (●) at 100 mM (P_i), 4 °C, after 55 days. The column was calibrated with uniform polystyrene latex beads run in 1% Triton X-100 and eluted at the peaks indicated by arrows: 1000 ± 80 Å and 380 ± 75 Å. Also shown is the profile of small sonicated vesicles (■).

Although Figure 2 shows the 50 mM (P_i) sample has apparently reached a plateau at approximately 80% 700 Å fused vesicles, it is clear that this sample also completely fused to 700-Å vesicles and then to larger vesicles because the 50 mM (P_i) sample after 31 days gave essentially the same column profile as in Figure 1H for the 4 °C, 120 mM (P_i) sample at 20 days. In this concentration range of 20–120 mM (P_i), the effect of increasing concentration is to decrease the time required to reach a given size distribution.

The longer time course elution profiles on Sepharose CL-2B were asymmetric because the larger fused vesicles were close to the void volume and were at least partially excluded from the gel matrix. Therefore, the larger vesicles were characterized on a calibrated A-150 column with a larger exclusion limit, as shown in Figure 3. The profile of small sonicated vesicles is also shown. This sample had been incubated 55 days at 4 °C and 100 mM (P_i) and gave a symmetric column profile with a peak corresponding to a diameter of 990 ± 150 Å.

Freeze–Fracture Electron Microscopy. Figure 4 shows the freeze–fracture electron micrographs of vesicles allowed to fuse at 4 °C and 100 mM (P_i) for (A) 17 and (B) 35 days. At 17 days both 700 and 950 Å diameter vesicles were found. At 35 days mostly 950 Å diameter vesicles were found. The vesicles appeared spherical with small amounts of aggregation. The size distribution profile measured from the 35-day incubated sample micrographs (sample size 270 vesicles) is shown in Figure 4C. The distribution was centered at 900–950-Å diameter and the half-width at half-maximum was approximately 115 Å. An estimate of the standard deviation of the distribution showed that about 68% of the area under the curve is within 175 Å of the peak. The freeze–fracture results correspond well with the gel chromatography measurements.

Trapped Volume Measurements of 950 Å Diameter Vesicles. The trapped volume measured using 6-carboxyfluorescein marker was 2.5 ± 0.3 (n = 3) L/mol. By use of a [^{3}H]glucose marker, the trapped volume was 2.8 ± 0.7 (n = 3) L/mol. These measurements agree well with the predicted trapped volume of 2.67 L/mol calculated for a spherical

Table I: Phase Transition Parameters for DPPC Multilamellar Liposomes and Fused Vesicles

	pretransition	T_m (°C)	ΔH (kcal/mol)	C_p^{max} [kcal/(mol·°C)]	$\Delta t_{1/2}$ (°C)	cooperative unit (n)[b]
DPPC multilamellar liposomes[a]	+	41.6	8.7	25	0.3	255
fused vesicles, 15 days[c]	–	41.6	6.2	6.2	0.3	128
fused vesicles, 35 days[c]	–	41.6	6.9	7.6	0.3	126

[a] Measurements for the same DPPC preparation (E. Freire, personal communication). [b] $n = \Delta C_p^{max}(4RT_m^2)/\Delta H^2$ (Mountcastle et al., 1978). [c] 4 °C, 100 mM (P_i).

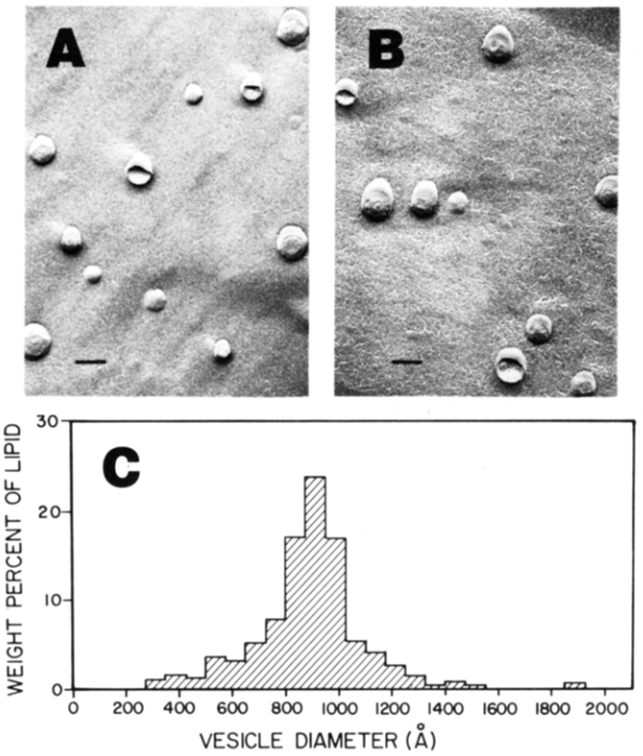

FIGURE 4: Freeze–fracture electron micrograph of fused vesicles incubated at 100 mM (P_i) at 4 °C (A) for 17 and (B) 35 days. The fractured surfaces were etched to a depth approximately equal to the vesicle radius. (C) Size distribution profile of 35-day fused vesicles weighted by radius to correct for the mass of a vesicle and the probability of a vesicle appearing in the fracture plane. The profile was from a sample of 270 vesicles. The markers correspond to 1000 Å.

950 Å diameter vesicle, assuming a membrane thickness of 37 Å, a specific volume per phospholipid molecule of 1223 Å^3 (based on egg phosphatidylcholine measurements), and free access of the marker molecules to the internal hydration layer (Mason & Huang, 1978). Trapped volume is a sensitive indicator of the presence of oligo- or multilamellar vesicles. Together with the absence of void volume material in the A-150 elution profile and the absence of multiple fracture planes in the freeze–fracture electron micrographs, the trapped volume measurements indicate that the 950 Å diameter fused vesicles are mostly unilamellar and close to spherical in shape.

Calorimetry. The calorimetric scan of vesicles fused for 35 days, at 4 °C and 100 mM (P_i), is shown in Figure 5. The sample was diluted to 56 mM (P_i) prior to measurement and this was the first upward scan following fusion at 4 °C. One symmetric main transition was seen at 41.6 °C with a half-width at half-maximum of 0.3 °C. The enthalpy of the transition is 6.9 kcal/mol, and the calculated cooperative unit is 126 (Mountcastle et al., 1978). Table I contains the comparable data for DPPC multilamellar liposomes and for the same sample fused for 15 days at 4 °C. The fused vesicles had a smaller change in enthalpy, C_p^{max}, and calculated co-

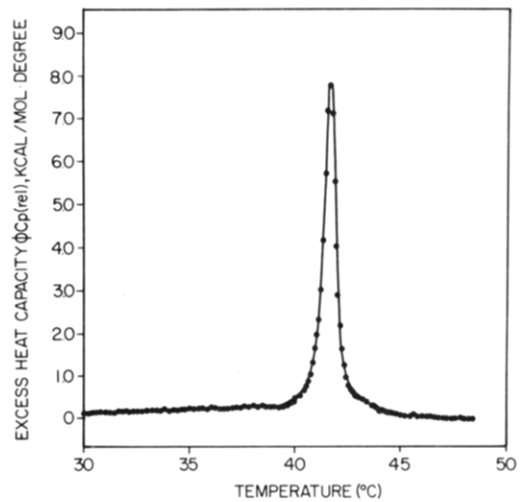

FIGURE 5: Excess heat capacity vs. temperature function of fused vesicles incubated 35 days at 100 mM (P_i) at 4 °C. This is the first upward scan following fusion at 4 °C. The sample was diluted to 56 mM (P_i) in buffer for the scan. The scan base line was corrected by using a buffer blank.

operative unit through the main transition than the multilamellar liposomes. About a 10% increase in the enthalpy and C_p^{max} was found between the 15- and 35-day incubated fused vesicles, while the cooperative unit was essentially unchanged. One other difference found was that the 15-day fused vesicles scan was skewed with a small tail toward the lower temperature part of the heat capacity curve. This is consistent with previous calorimetric studies (Suurkuusk et al., 1976; Lichtenberg et al., 1981) monitoring the conversion from small to large vesicles.

Fluorescence Measurements. The DPH polarization was analyzed in terms of the anisotropy parameter r

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

where $I_\parallel$ is the intensity parallel to and $I_\perp$ is the intensity perpendicular to the incident beam, measured by channels A and B, respectively. Anisotropy as a function of temperature is plotted in Figure 6 for a preparation of 950 Å diameter fused vesicles. The sample was scanned in both heating and cooling modes, and no hysteresis was found. A single transition centered at 41.1 °C was obtained, in agreement with the transition temperature measured with DPH in DPPC multilamellar liposomes (Lentz et al., 1976). Compared with the previously published anisotropy scans for DPPC small sonicated vesicles and multilamellar liposomes (Suurkuusk et al., 1976), the scan for 950 Å fused vesicles is shifted upward from the small vesicle transition but is broader than the multilamellar liposome transition.

Fusion of 700-Å Vesicles Formed at 21 °C. Fractionated 700-Å vesicles, formed at 21 °C and then heated for 10 min at 60 °C, fused to 950-Å vesicles after 20-day incubation at

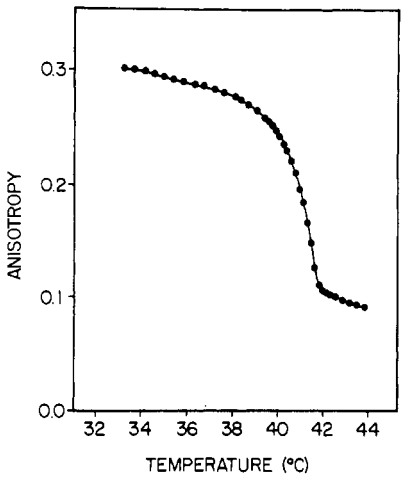

FIGURE 6: DPH fluorescence depolarization temperature scan of 950 Å diameter fused vesicles. Identical traces were obtained for heating and cooling scans.

4 °C. The Sepharose CL-2B column profile was similar to Figure 1H for small vesicles incubated for 20 days at 4 °C. The distribtion contained mostly 950 Å diameter vesicles with a small shoulder at the 700 Å diameter vesicle position. The quasi-elastic light-scattering diameter was 755 ± 7 Å for the fractionated 700-Å vesicles, 755 ± 30 Å for the upper solution following 20 days at 4 °C, and 887 ± 38 Å for the resuspended lower solution. The results show that the aggregated material which settled to the bottom of the test tube and was concentrated to 80–100 mM (P_i) fused to larger vesicles, while the dilute upper solution did not, consistent with the greater than first-order concentration dependence found in the column studies.

Discussion

The agarose gel chromatography, freeze–fracture electron microscopy, and trapped volume data show that small sonicated vesicles incubated at 4 °C fuse entirely to vesicles about 700 Å in diameter in approximately 7 days and that these vesicles further enlarge to vesicles about 950 Å in diameter after 3–4 weeks. After the vesicles are cooled to 4 °C, an initial rapid phase of fusion occurs within 24 h, followed by a much slower phase. The kinetics of the initial rapid phase is higher than first order in concentration, and this together with previously published NMR data (Schmidt et al., 1981) argues for a second-order collisional mechanism and against an aqueous transfer of monomer mechanism for the conversion from small to large vesicles (Lawaczeck, 1978). Conversion from small to about 700 Å fused vesicles occurs with little evidence of intermediate sized material, and the same bimodal size distribution pattern is seen at all concentrations [20–120 mM (P_i)]. Figure 1G,H demonstrate that the conversion from 700 Å to 950 Å diameter vesicles requires only an interaction between 700-Å vesicles and that an interaction between 700 Å and smaller diameter vesicles is not required.

The change in vesicle size distribution as a function of incubation time was monitored by gel chromatography, and then the 950 Å diameter fusion product was characterized by freeze–fracture electron microscopy and by trapped volume measurements. Consistent results were obtained by the three methods. The 950 Å diameter vesicles appeared to be essentially stable. Beyond 35 days, the agarose gel chromatography profile did not change appreciably, and freeze–fracture electron micrographs taken at 6 months were similar to those taken at 35 days, although an extensive size distribution profile was not measured at the longer time.

Schullery et al. (1980) followed fusion at 20 mM (P_i), 21 °C, for up to 12 days and found that 30–40% of the vesicles did not fuse, implying a very slow rate of conversion for this remaining fraction. We incubated vesicles at 100 mM (P_i), 21 °C, for 20 days (Figure 1H, open circles) and also found approximately the same fraction of small vesicles. After 6.5 months, however, a sample of 100 mM (P_i), 21 °C, incubated vesicles gave a gel chromatography profile similar to Figure 1G, that is, entirely fused to 700 Å vesicles with no shoulder at the small vesicle position. Therefore, the same progression of size changes appears to be occurring at 21 °C as was found at 4 °C, but at a much slower rate. Furthermore, the fusion properties of the vesicles do not depend on how they were made, only on their dimensions. This was shown in the equal ability of 700-Å vesicles formed either at 21 °C or at 4 °C to further fuse to 950-Å vesicles when cooled below the T_m to 4 °C. It can be calculated that about 18 small vesicles fuse to form a 700 Å diameter vesicle containing approximately 4.2×10^4 phospholipid molecules and that two 700 Å diameter vesicles fuse to form a 950 Å diameter vesicle containing approximately 7.9×10^4 phospholipid molecules (membrane thickness 37 Å, 1223 Å^3/phospholipid molecule, from egg phosphatidylcholine data; Mason & Huang, 1978). Changes in concentration and temperature apparently change the rate of conversion but do not appreciably alter the product of small vesicle fusion.

Using negative-stain electron microscopy, turbidity, and sedimentation velocity ultracentrifugation, Larrabee (1979) studied the time-dependent size changes of DSPC vesicles at 25 mM (P_i) over the temperature range of 4–65 °C. She concluded that changes in temperature affect the rate of size conversion, but not the dimensions of the products, a conclusion we have extended to the effects of temperature and concentration of DPPC size conversions. The DSPC fusion products were measured to be about 600 Å and about 1000 Å in diameter, very close to the approximate 700- and 950-Å diameters found for DPPC. In turbidity measurements, an initial fast phase followed by a slower phase was seen at all temperatures. The fast phase for DSPC lasts 4–6 h compared to 24 h for DPPC. Therefore, if the DSPC and DPPC fusion products are taken to be approximately the same size, then a comparison with Larrabee's data shows that the effect of increasing hydrocarbon chain length is to increase the rate of size conversion, without changing the dimensions of the products.

Incubation at 4 °C and high concentration provides a convenient method of preparing fused vesicles. After 7-day incubation, one obtains 700 Å diameter vesicles and, after 35 days, 950 Å diameter vesicles. Since conversion to fused vesicles is complete, a column purification is not required to remove small vesicles. The fused vesicles can be prepared at high concentrations, which is an advantage for subsequent study by many techniques, for example, calorimetry and NMR. If a preparation of fairly stable 700-Å vesicles is desired, after incubation for 7 days the sample can be diluted with buffer, centrifuged at low speed to remove any aggregates, and stored at 21 °C.

The agreement between the calculated and measured trapped volumes for 950 Å diameter vesicles indicates that they are mostly unilamellar and spherical. The vesicles appeared spherical in freeze–fracture electron micrographs, with a size profile which was fairly sharply distributed about 900–950-Å diameter. The enthalpy change, the width of the transition, and the cooperative unit correspond well with the expected characteristics of 950 Å diameter vesicles on the basis of

changes in thermodynamic characteristics with diameter measured in ethanol-injection vesicles, small and 700 Å fused vesicles, and French-press vesicles (Marsh et al., 1977; Van Dijck et al., 1978; Lichtenberg et al., 1981). The DPH fluorescence depolarization scan contained a transition centered at the mulitlamellar liposome transition temperature, but somewhat broader. The transition is sharper than and shifted upward from the small sonicated vesicle transition (Suurkuusk et al., 1976; Lentz et al., 1978). The trapped volume of 2.5–2.8 L/mol is about 10 times greater than that of small sonicated vesicles and may be an advantage in pharmaceutical drug delivery applications. The fused vesicle system is free of any of the residual small molecules used in preparing vesicles by other techniques, yet the 700 Å and 950 Å diameter vesicles are large enough to be relatively free of the molecular packing effects due to the high curvature present in small sonicated vesicles.

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