Time-Dependent Changes in the Size Distribution of Distearoylphosphatidylcholine Vesicles†

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ABSTRACT: The results of transmission electron microscopic and ultracentrifugal studies of the size distributions of sonicated distearoylphosphatidylcholine vesicles are reported. Small vesicles ($d \approx 300$ Å) were prepared by sonication of pure 1,2-distearoyl-3-*sn*-phosphatidylcholine in water and incubated at 4, 21, 40, 53, and 65 °C. The vesicle size distributions changed as a function of time at all temperatures below the phase-transition temperature but remained constant at the transition temperature and above. The sizes of structures to which the small vesicles are converted are the same at all temperatures, although the rates of conversion differ. The primary structures formed are identified as larger vesicles. The rate of loss of small vesicles is found to increase with decreasing temperature. At 4 and 21 °C small vesicles are converted to amorphous material, possibly irregular fragments of neat phase, in addition to being converted to larger vesicles. Trace amounts of an impurity commonly produced in the synthesis of 1,2-distearoyl-3-*sn*-phosphatidylcholine, 1,3-distearoyl-2-*sn*-phosphatidylcholine, are found to dramatically reduce the rate of loss of small vesicles at 21 °C.

Vesicles formed by sonication of saturated chain phosphatidylcholines in aqueous media have been used extensively as a model for the lipid component in the plasma membrane. Since these vesicles are created by raising the energy of a system from a thermodynamic equilibrium state (lamellar phospholipid plus water), reports of instabilities of the vesicle preparations are consistent with the expectation that a colloidal dispersion of small (~300-Å diameter) vesicles could revert to the equilibrium state on a time scale rapid enough to influence the outcome of model membrane studies. This suggests that an understanding of the kinetic behavior of the vesicle system itself may aid in interpretation of model experiments and define the conditions under which the sonicated vesicle is appropriate as a membrane model.

Lawaczeck et al. (1976) have observed that vesicles sonicated below the gel–liquid crystalline phase-transition temperature are permeable to trivalent cations and interact rapidly to form larger structures unless heated above the transition temperature, i.e., "annealed". Martin & MacDonald (1976) have described a sharp increase in the rate of change of size-related properties of a dimyristoylphosphatidylcholine vesicle suspension just above the transition temperature. Several calorimetric studies of homogeneous phospholipid vesicles (Suurkuusk et al., 1976; Papahadjopoulos et al., 1974; Van Dijck et al., 1978) have observed behavior which is consistent with the conversion of small vesicles to larger structures during the course of repeated calorimetric scans. Kantor et al. (1977) and Suurkuusk et al. (1976) have also observed that small vesicles of highly purified phospholipids retain the calorimetric behavior of freshly sonicated suspensions if maintained above the phase-transition temperature. Whether small vesicles of pure phosphatidylcholine are stable above, below, or at the phase-transition temperature has not been unambiguously established, nor has the nature of the particles to which the vesicles are converted or the mechanism for the conversion. Proposed mechanisms include vesicle–vesicle fusion (Lawaczeck et al., 1976; Van Dijck et al., 1978) and phospholipid exchange (Papahadjopoulos et al., 1976; Martin & MacDonald, 1976).

The present study was undertaken to examine the temperature dependence of the kinetic behavior of small distearoylphosphatidylcholine vesicles. Both the rate of loss of small vesicles and information about the structures to which the small vesicles are converted can be obtained from sedimentation velocity experiments. The sedimentation coefficients of different components in the vesicle suspension and the relative concentrations of the components as represented by the area under the Schlieren curves can be observed as a function of time. It is not possible, however, to distinguish on the basis of sedimentation coefficients alone between a sedimenting particle which is a single bilayer vesicle, an aggregate of small single bilayer vesicles, or a multi-bilayer vesicle because structures of each kind can give rise to the same sedimentation behavior. Consequently, vesicle samples which were incubated under conditions comparable to those used in the sedimentation experiments were examined by transmission electron microscopy and size distributions obtained. Combination of these two techniques has indicated that small single bilayer vesicles of pure distearoylphosphatidylcholine are unstable at all temperatures below the phase transition and are converted to larger single bilayer vesicles. This change in vesicle size below the phase transition is found to be nonreversible upon reheating the vesicles above the transition temperature.

Experimental Procedure

Materials. 1,2-Distearoyl-3-*sn*-phosphatidylcholine (DSPC) was synthesized by the method of Cubero Robles & Van den Berg (1969). Purity of the product was checked by thin-layer chromatography (developing solvent $CHCl_3$–CH_3OH–H_2O–HCO_2H, 80:25:3:1, H_2SO_4 spray and char staining). A single spot was observed with a 100-μg loading. Samples from which 1,3-distearoyl-2-*sn*-phosphatidylcholine (J. E. Thompson, personal communication) was not removed gave a second TLC spot with a slightly larger R_f value than that of the 1,2 compound. Low concentrations (<1%) of the 1,3-DSPC were not detected by TLC when I_2 was used for staining rather than H_2SO_4. The crystalline DSPC was stored in capped bottles over desiccant at 4 °C.

Cholesterol (99+% pure) was purchased from Sigma and used without further purification. All solvents were reagent grade and used as obtained. Water was triply distilled from a quartz apparatus.

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Vesicle Preparation. DSPC (200 mg) was dissolved in $CHCl_3$–CH_3OH (2:1, w/v) in a truncated conical glass sonication vessel of ~12-cm^3 volume. Solvent was removed under vacuum at 55 °C, and a film of the phosphatidylcholine was formed on the vessel wall. Triply distilled H_2O (10 cm^3) was added and the mixture sonicated in the closed vessel at 100-W output power with a Branson Model 350 sonifier equipped with a microtip. The microtip was filed flat before each sonication, and the tip depth in the liquid was set to maximize measured output power for a given nominal power setting. Sonication took place for 10 or 20 min at 65 °C under dry N_2. Samples appeared clear at the end of sonication. Schlieren curves obtained as quickly as possible after sonication for samples sonicated 10 and 20 min were indistinguishable. Consequently, most samples were prepared with 10-min sonication to minimize the possibility of hydrolysis of the phospholipid. Thin-layer chromatography of 100 μg of the sonicated DSPC showed no evidence of stearic acid or lysophosphatidylcholine, for which the detection limits were determined to be 0.5 μg in this system. Lysophosphatidylcholine and stearic acid were detected, however, at the trace levels in vesicle preparations which had been incubated at 65 °C for 24 h.

Some of the vesicle samples were held at 65 °C for 0.5 h after sonication for the purpose of annealing (Lawaczeck et al., 1976), but this was found to have no effect on the vesicle behavior as determined by both turbidity and sedimentation velocity measurements when the sample was subsequently cooled below the phase-transition temperature. In addition, vesicles used for turbidity studies were centrifuged (20 °C) for 0.5 h at 20000*g* to remove titanium particles and any multilamellar particles which may have remained at the end of sonication. Concentration of the suspended DSPC after sonication was determined from phosphorus content by a modification of the method of Fiske & SubbaRow (1925). For 200 mg of DSPC starting material, the sonicated suspension concentration was found to be 20.3 ± 1.5 mg/cm^3 for a 10-min sonication and 19.7 ± 0.7 mg/cm^3 for a 20-min sonication.

ζ potentials measured for both pure DSPC vesicles and those containing the 1,3-DSPC were found to be zero within experimental error (Laser Zee, Model 400, Pen Kem Co., Croton-on-Hudson, NY). This is within the region of ζ potential values (0 to +20 mV) found to most favor colloid flocculation.

Physical Methods. Turbidity was measured with a 0.2-cm path length at 280 nm and 21 ± 1 °C for all samples. A 0.25-cm^3 aliquot was taken for measurement at timed intervals from vesicle suspensions which were incubated at known temperatures. Thermal equilibration of the aliquot was complete within 5 min. Due to the time required to centrifuge the vesicles, it was not possible to obtain turbidity data in the time period immediately following sonication. No attempt was made to relate turbidity to particle size since the samples were expected to be heterogeneous. Turbidity experiments were used only to estimate the time frame in which major changes had taken place and the relative magnitude of the changes at each temperature.

Transmission electron microscopy of the vesicle samples was carried out by taking an aliquot from an incubating vesicle suspenson, diluting to ~0.1% DSPC, and placing a small drop of the dilution at room temperature on a freshly prepared formvar–silica coated grid. Phosphotungstic acid (2%, pH 7.4) was used as negative stain. Grids were air-dried and viewed with a Hitachi 12A electron microscope operating at 75 kV. Vesicle size measurements were made from micrographs with a Zeiss particle size analyzer TGZ3 (Karl Zeiss, NY). Approximately 1000 vesicles were sized for each distribution. Details of grid support film preparation and vesicle-sizing procedure are given by Larrabee et al. (1978). The larger vesicles appeared in the micrographs to have textured surfaces. This texture was also observed in freeze-fracture micrographs, but was absent from the negatively stained preparations when vesicles at 65 °C were stained at that temperature. The negative staining technique applied to DSPC vesicles is incapable of distinguishing between single and multi-bilayer vesicles due to the impermeability of the vesicle bilayer to the staining solution. Consequently, the only information obtained from the transmission electron microscopic experiments is the external diameter of the particle.

Sedimentation velocity measurements were made with a Beckman Model E ultracentrifuge equipped with Schlieren optics using a 12-mm double sector cell. High-speed runs were made at 65000*g* (measured at the miniscus) and low speed at 7500*g*. All measurements were made at 23 °C regardless of the incubation temperature of the vesicle sample. Aliquots were taken from the incubating vesicle samples and remained at room temperature for about 15 min during the process of cell loading and bringing the rotor up to speed prior to the beginning of a sedimentation run. Areas under the Schlieren curves were determined by integration of tracings prepared from photographic plates of the patterns as they appeared after 16 min at maximum rotor speed. The integration was carried out with an Elographics Digitizer interfaced with a Monroe 1860 calculator. Each curve was integrated three times and the average of the three measurements used in the data analysis. Since all of the vesicle samples examined consisted of more than a single component (peak) and the relative concentrations of the components varied with time, it was not possible to correct for the Johnston–Ogston effect (Johnston & Ogston, 1946) or determine the $s°$ value for the components. A linear extrapolation of the peak areas observed over the course of the experiment with vesicles incubated at 40 °C gave an extrapolated value of s = 20 S for the peak of lowest s value and s = 48 S for the peak of intermediate s value. These extrapolated s values were used in lieu of $s°$ values for the calculation of $r_{external}$ for the vesicles (Johnson, 1973). For the calculation, a bilayer thickness of 45 Å, $r_{Stokes} = r_{external} + 10$ Å, ρ_{DSPC} = 1.075 g cm^{-3}, ρ_{H_2O} = 0.9976 g cm^{-3}, and η_{H_2O} = 0.9325 cp were assumed. The model on which the calculation is based assumes a single lipid bilayer. Variation of the vesicle radius calculated at each s value for a choice of bilayer thicknesses between 40 and 60 Å is ±15% of the mean. Variation of r from r_{Stokes} to $r_{external}$ + 20 with a 45-Å bilayer thickness results in a maximum variation of ±13% in the calculated radius. The density of DSPC in vesicles was determined from the partial molal volume of suspension of large (1000 Å) vesicles at 25 ± 0.5 °C. The assumption that the density of the DSPC does not vary as a function of vesicle radius is perhaps questionable since it is unlikely that the bilayer packing in 300-Å diameter vesicles is comparable to that in 1000-Å vesicles (Sheetz & Chan, 1972). This error would have the effect of underestimating $r_{external}$ for the small vesicles.

Quasielastic light scattering measurements were made at 22 °C with an instrument constructed in our laboratories utilizing a commercially available Malvern correlator which had been interfaced to a Hewlett-Packard 9825 calculator.

Results

Figure 1 shows the effect of incubation below the gel–liquid crystal transition temperature, T_c, on the turbidity of vesicle

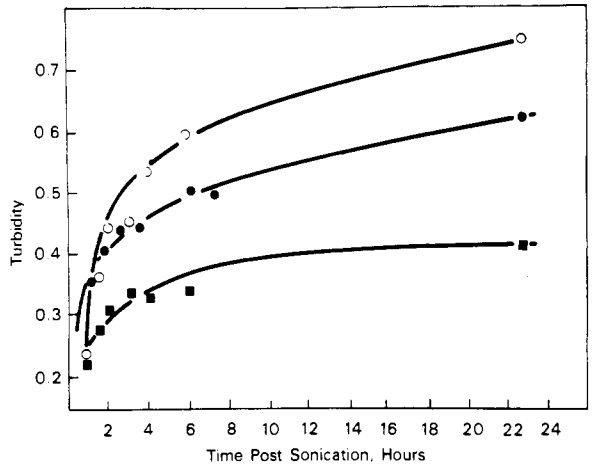

FIGURE 1: Turbidity of vesicle samples measured at 280 nm, 0.2-cm path length, and 21 ± 1 °C. Samples were prepared by a 10-min sonication of 2% DSPC in H_2O. (○) Incubated at 4 °C; (●) incubated at 21 °C; (■) incubated at 40 °C.

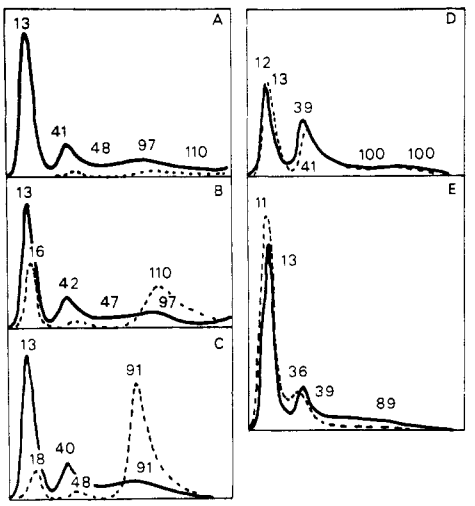

FIGURE 2: Tracings of Schlieren patterns of vesicle samples prepared by a 10-min sonication of 2% DSPC in H_2O. Left margin of each frame represents the meniscus. Numbers above peaks are sedimentation coefficients. (—) Pattern within 45 min of end of sonication and (---) after 24-h incubation at (A) 4, (B) 21, (C) 40, (D) 53, and (E) 65 °C.

samples. No data are shown for samples incubated above T_c since the effect of cooling the samples below T_c for centrifugation and then reheating through the transition temperature is not comparable to maintaining the vesicles above T_c. The effect of cooling and reheating will be discussed in relation to the sedimentation experiments. Each sample for which turbidities were measured appeared clear to visual inspection at the end of sonication and became opaque and milky within hours. The largest total change and the most rapid initial change were observed for the sample incubated at the lowest temperature, 4 °C.

The tracings in Figure 2 were obtained from Schlieren patterns of vesicle samples observed at times after sonication which correspond approximately to the first and last data points on the turbidity curves, 0.75 (solid curve) and 24 (dashed curve) h postsonication, respectively. Three well-defined peaks appeared in the samples incubated below T_c. These peaks are identified with approximately the same s values at the three different temperatures. A significant shift in area from one peak to another occurred over the 24 h for the samples below T_c while areas for the samples at T_c (53 °C) and above (65 °C) remained virtually unchanged.

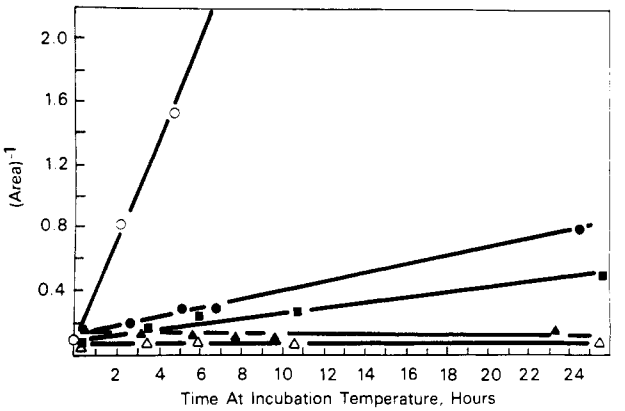

FIGURE 3: Reciprocal of area (arbitrary units) under Schlieren curve due to vesicles with sedimentation coefficients of 11–20 S. Samples were prepared by a 10-min sonication of 2% DSPC in H_2O and incubated at (○) 4, (●) 21, (■) 40, (▲) 53, and (△) 65 °C. Solid lines represent a linear least-squares fit of data.

The behavior of the total area under the Schlieren curves as a function of time shows no change for the samples incubated at 65, 53, and 40 °C. Changes after 24 h of incubation in the areas under the Schlieren curves for samples at 21 and 4 °C indicated that 36 and 94%, respectively, of the material in the samples sedimented too rapidly under the experimental conditions for measurement of sedimentation coefficients. Low-speed sedimentation experiments were run with samples incubated at 21 and 4 °C in an attempt to measure sedimentation coefficients for the material which sedimented rapidly at high speed. These experiments were intended to distinguish, if possible, between the conversion of the phospholipid vesicle population to large closed multilamellar structures (liposomes) or aggregates of small vesicles for which sedimentation coefficients could theoretically be measured and bulk lamellar liquid crystalline material. The Schlieren patterns for the low-speed experiment showed only peaks due to the particles observed in the high-speed experiments and no evidence for large liposomes or large aggregates of small vesicles. The behavior of the peak with the lowest sedimentation coefficient is shown in Figure 3. The peak areas changed only for those samples incubated below the pretransition temperature. For the samples incubated at 40, 21, and 4 °C, the time dependence of the area (concentration) is consistent with second-order kinetics but inconsistent with zero- or first-order kinetics. No simple zero-, first-, or second-order dependence was observed for the changes in area of the peaks of intermediate or largest sedimentation coefficient.

Samples prepared at 65 °C were cooled to 21 and 4 °C, respectively, incubated at the lower temperature for 1.5 h, and then reheated to 65 °C to determine if the time-dependent changes in the vesicle samples which occurred at low temperatures could be reversed by subsequent heating of the vesicles. Comparison of the Schlieren patterns obtained for the cooled and the reheated samples indicated that reheating the vesicles stabilized the size distribution which had been established at the lower temperature rather than reestablishing the distribution observed for the sample immediately following sonication and maintained when incubation took place only at 65 °C.

To aid in the identification of the vesicle structures observed in the sedimentation experiments, we obtained size distributions from transmission electron micrographs of negatively stained vesicle preparations. Size distributions were obtained for vesicle samples immediately following sonication and after

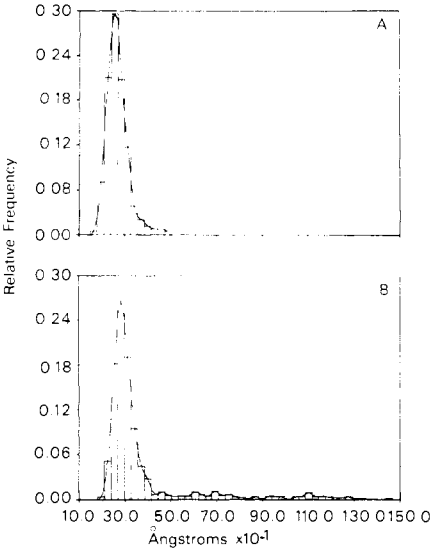

FIGURE 4: Size distributions measured from micrographs of negatively stained vesicles prepared by a 10-min sonication of 2% DSPC in H_2O: (A) 5-min postsonication, 1015 vesicles sized; (B) centrifuged sample at 21 °C for total of 45 min, 903 vesicles sized.

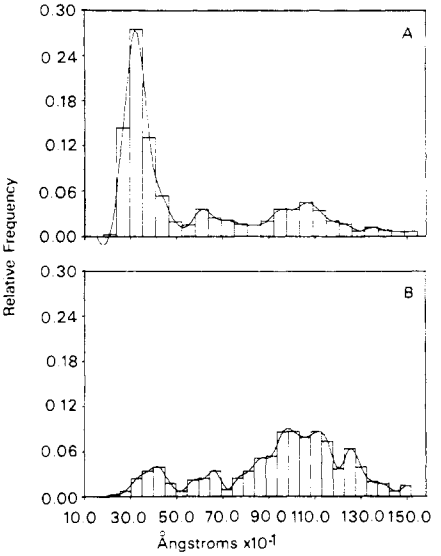

FIGURE 5: Size distribution measured from micrographs of negatively stained vesicles prepared by a 10-min sonication of 2% DSPC in H_2O. Samples were centrifuged prior to incubation at (A) 21 °C for 1 h, 938 vesicles sized, and at (B) 4 °C for 1 h, 406 vesicles sized.

incubation at the same temperatures at which the sedimentation experiments were carried out. Figure 4A represents the vesicle size distribution within 5 min after sonication. Most of the vesicles are between 200 and 300 Å in diameter. No data obtained as rapidly after the end of sonication are presented from other techniques. The earliest sedimentation observation is made at least 0.5 h after sonication. The vesicle sample in Figure 4B, where in addition to the 250-Å diameter vesicles small populations of ~600- and ~1000-Å diameter vesicles are apparent, represents a sample which has been at room temperature for 45 min. Figures 5A and 5B show portions of the same sample which were incubated at 21 and 4 °C, respectively, for 1 h following a 0.5-h centrifugation at 21 °C. In the 4 °C sample, the bulk of the vesicles is in the range of 900–1200 Å in diameter with few small vesicles evident, while the 21 °C sample consists primarily of small vesicles.

Figure 6 includes the vesicle size distributions which correspond to the dashed curves in Figure 2 for 21, 40, 53, and

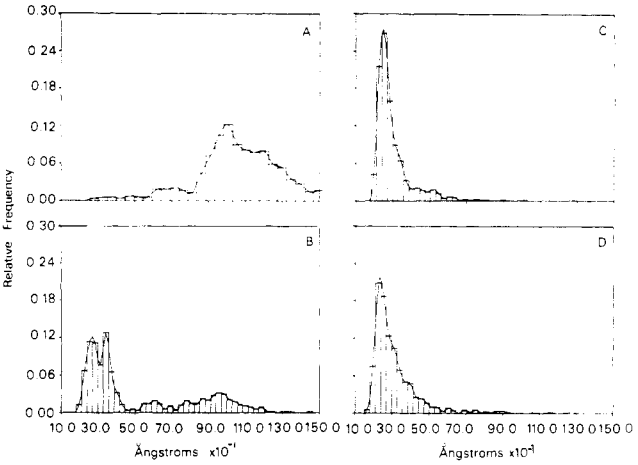

FIGURE 6: Size distributions measured from micrographs of negatively stained vesicles prepared by a 10-min sonication of 2% DSPC in H_2O. Samples were incubated for 24 h prior to examination: (A) 21 °C, 543 vesicles sized; (B) 40 °C, 564 vesicles sized; (C) 53 °C, 824 vesicles sized; (D) 65 °C, 733 vesicles sized.

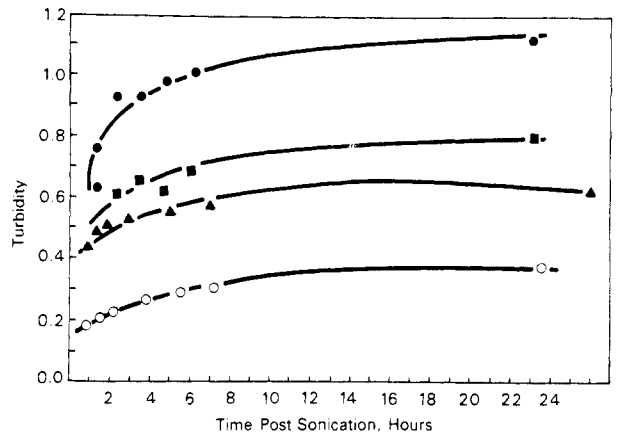

FIGURE 7: Turbidity of vesicle samples measured at 280 nm, 0.2-cm path length, and 21 ± 1 °C. Samples were prepared by a 10-min sonication of 5% solid in H_2O: (●) 1,2-DSPC only; (■) 1,2-DSPC, ~1 mol % 1,3-DSPC; (▲) 1,2-DSPC, 6 mol % cholesterol; (○) 1,2-DSPC, ~2 mol % 1,3-DSPC.

65 °C (24-h incubation). The proportion of small vesicles has been vastly reduced at 21 °C while the samples incubated at 53 and 65 °C consist almost entirely of small vesicles. The distribution for the 40 °C sample is not consistent with the sedimentation data in that a large population of small vesicles is still evident in the electron micrographs.

The sedimentation coefficients observed for the DSPC vesicles can be related to the external vesicle diameter if it is assumed that the vesicle is enclosed by a single bilayer (Johnson, 1973). Using the s values extrapolated to zero concentration for each Schlieren peak, 20 S, 48 S, and 110 S, we can predict vesicle diameters of 260, 525, and 1050 Å, respectively. Comparison of these predictions from the sedimentation coefficients to the size distributions determined from the transmission electron micrographs (Figures 4–6) suggests that the vesicles observed micrographically possess only a single bilayer, and the Schlieren peaks at $s \approx 48$ and 100 can be identified with large vesicles rather than with aggregates of small vesicles or multi-bilayer vesicles.

Behavior of vesicles prepared from 1,2-distearoyl-3-*sn*-phosphatidylcholine (1,2-DSPC) is altered significantly by the presence of small amounts of 1,3-distearoyl-*sn*-2-phosphatidylcholine (1,3-DSPC). Figure 7 illustrates the effect of the 1,3-DSPC on the turbidity of 1,2-DSPC at 21 °C.

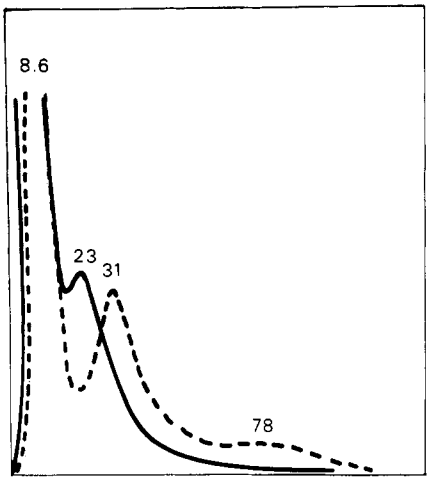

FIGURE 8: Tracings of Schlieren patterns of vesicle samples prepared by a 10-min sonication of 1,2-DSPC with ~2 mol % 1,3-DSPC, total phospholipid 2% in H_2O. Incubation temperature 21 °C. (—) Pattern 45-min postsonication; (---) 24-h postsonication.

The concentration of the 1,3-DSPC was estimated by a visual comparison of the spot intensity on the TLC plate due to the 1,3 isomer relative to that due to the 1,2 isomer. It was assumed that under the conditions of the H_2SO_4 spray and char that the response of both isomers would be equivalent. The concentration estimates may be uncertain as much as ±1 mol %.

The curve for a vesicle sample containing 6 mol % cholesterol is included for comparison of the effect on vesicle stability of a material known to moderate bilayer fluidity. At this cholesterol concentration the state of fluidity of the phospholipid bilayer has been significantly altered (Hinz & Sturtevant, 1972). The turbidity of the sample which contains ~2 mol % 1,3-DSPC changes very little. Tracings of the Schlieren patterns for a DSPC vesicle sample containing ~2 mol % 1,3-DSPC at 0.75 (solid curve) and 24 (dashed curve) h of incubation at 21 °C are shown in Figure 8. This sample has 0.4 times the total phospholipid concentration of the samples on which the turbidity measurements were made and shows at 24 h the beginning of a peak with a high sedimentation coefficient. Size distributions obtained from transmission electron micrographs of 1,2-DSPC vesicles containing 1 mol % 1,3-DSPC immediately following sonication, after 1 h at 4 °C, and after 48 h at 21 °C are shown in Figure 9. The size distributions of both incubated samples indicate the presence of large vesicles, but small vesicles remain the primary constituents of the samples. Particularly notable is the low concentration of large vesicles after 48 h at 21 °C.

Long-term storage data were obtained only for samples incubated at 4 and 21 °C. Examination 6 months after sonication of samples prepared from the pure 1,2-DSPC which had been stored in sealed vials indicates complete phase separation of the water and phospholipid. Analysis of the phospholipid content of the supernatant from these samples following mild centrifugation showed a maximum of 0.09 mg cm^{-3} of 1,2-DSPC compared to 15 mg cm^{-3} immediately following sonication. Because of the low phospholipid concentration, no other characterization of the supernatant was attempted. The vesicle samples prepared from 1,2-DSPC containing 2 mol % 1,3-DSPC still had a bluish slightly milky appearance. Diffusion coefficients were determined for the samples by quasielastic light scattering both before and after centrifugation for 30 min at 20000*g*. The data were fit to both a single and a double exponential. The better fit was obtained for the double exponential with particle diameters of 2336 and

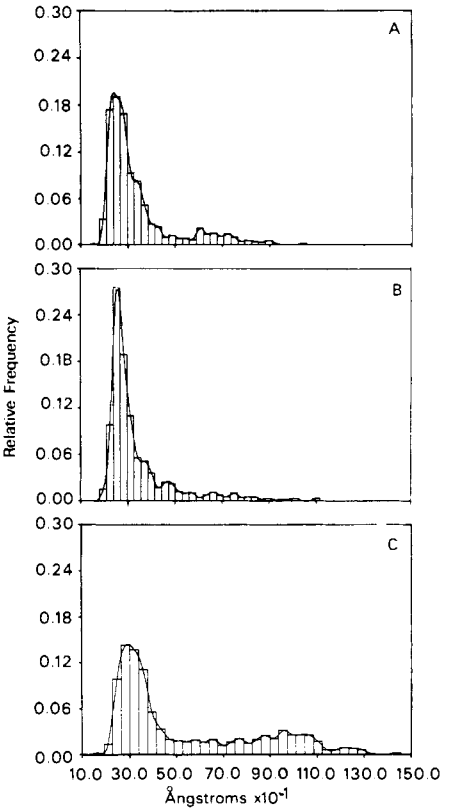

FIGURE 9: Size distributions measured from micrographs of negatively stained vesicles prepared by a 10-min sonication of 1,2-DSPC with ~2 mol % 1,3-DSPC, total phospholipid 2% in H_2O: (A) 5-min postsonication; (B) 1-h postsonication, incubation temperature 4 °C; (C) 48-h postsonication, incubation temperature 21 °C.

886 Å before and 1640 and 774 Å after centrifugation. Since no quasielastic light scattering data were obtained on these samples at the time of preparation, no direct comparison can be made. Assignment of an error limit to the calculated diameters is not straightforward; however, the fit of the data to the double exponential can be taken as a good indication of the polydispersity of the sample. It can be concluded, however, that the vesicle sample prepared with 1,3-DSPC still consisted primarily of suspended particles after 6 months of storage at 21 °C and the average particle size had increased from the initial 300-Å diameter during that time.

Discussion

The turbidity, sedimentation velocity, and electron microscopic data demonstrate the conversion of small (250 Å) vesicles of pure DSPC to larger particles at temperatures below the phase transition.

Comparison of the observations on samples after incubation for 24 h at 4 and 40 °C suggests that the end point for the changes in the vesicle system does not consist of discrete vesicle or liposome-like structures. The high turbidity seen for the 4 °C sample relative to the 40 °C sample would be consistent with either large suspended structures or a higher concentration of small structures in the lower temperature sample. The comparable Schlieren patterns, however, indicate that the concentration of all identifiable suspended structures ($s \sim 13$ S, 40 S, and 100 S) is higher at 40 °C. Since the low-speed sedimentation velocity experiments on samples incubated at 4 °C identified no peaks due to large liposomes or larger aggregates of small vesicles, it is likely that the suspended material responsible for the high turbidity in the 4 °C sample consists of sheets or bilayer fragments. In addition, no distinct

structures in the range of 2000–5000 Å in diameter are observed in electron micrographs of vesicle samples incubated at 4 °C.

Since there is a significant time delay between the end of sonication and the first measurements made in the sedimentation experiments, the electron microscopy experiments provide the only available information about the composition of the vesicle sample immediately following sonication. The vesicle population observed at 5-min postsonication at 21 °C is represented by a distribution skewed slightly to large diameters with a mean of 252 Å and a standard deviation of 62 Å. The appearance of larger diameter (>500 Å) vesicles in the micrographs after 45 min at 21 °C (corresponding to the earliest obtainable Schlieren patterns) implies that the larger vesicles are formed in the sample (at 21 °C) after the end of sonication. The absence of 600-Å diameter vesicles in the micrographs of the vesicles incubated at 65 °C is also suggestive that the Schlieren peak observed at $s = 36$ in the 65 °C samples was formed when the sample was cooled to 23 °C for the sedimentation velocity experiment.

The existence of two distinct populations of large vesicles to which the 250-Å vesicles are converted at all temperatures below T_c strongly suggests that these larger size vesicles represent local potential energy minina. It is not possible to determine from the data available whether or not the ~600-Å vesicles represent an intermediate in the conversion of the 250-Å to the 1000-Å vesicles. It is also to be noted that the 600-Å vesicles could not be formed by the fusion of two 250-Å vesicles, but would require the combination of four or five small vesicles. Likewise, the 1000-Å vesicle would require the combination of four to five 600-Å vesicles for formation. No Schlieren peak that could be attributed to a vesicle formed by the combination of two small vesicles is observed in the preparations of pure 1,2-DSPC, but the second peak in the patterns for the vesicles containing the 1,3-DSPC impurity (Figure 8) might be interpreted as due to the combination of only two vesicles.

The experimental observations made for small DSPC vesicles are consistent with the model proposed for the interpretation of calorimetric and fluorescent probe studies on small dipalmitoylphosphatidylcholine vesicles by Suurkuusk et al. (1976) except that the process of small vesicle conversion is described by second-order kinetics rather than by first-order kinetics. The large structures to which the small DSPC vesicles are converted below T_c, which may be similar in the molecular packing of the bilayer (low curvature) to liposomes, are not identical by virtue of the presence of only a single bilayer. As proposed for the dipalmitoylphosphatidylcholine vesicles (Suurkuusk et al., 1976), the small and large DSPC vesicles do not appear to interconvert above T_c.

Although the turbidity and sedimentation data alone cannot rule out the possibility that aggregates of small vesicles are responsible for the experimental observations, the electron micrographic observation of three size populations of vesicles in approximately the same proportions as predicted from the sedimentation experiments argues against major contributions to the turbidity or the Schlieren peaks by aggregates. The existence of large vesicles rather than aggregates is also supported by the failure upon reheating to 65 °C of vesicles which had been cooled below T_c to 4 and 21 °C to restore the population to that which had been observed for samples incubated only at 65 °C.

The drastic change in the behavior of small DSPC vesicles below T_c when a small amount of 1,3-DSPC is present in the phospholipid is presumed to be the result of changes in bilayer packing. Although these vesicles do convert to larger vesicles, the rate of conversion is relatively slow and reduction of the sample temperature to 4 °C does not significantly alter the size distribution. The conversion of small vesicles of pure 1,2-DSPC to larger vesicles by whatever mode of interaction is operative is expected to be dependent upon exposure of hydrocarbon portions of the bilayer to the aqueous medium. It is not unreasonable that the changes occurring in the lipid packing in the bilayer confined to the surface of a sphere which accompany the phase transition may result in packing disorders that increase the contact of the hydrocarbon chains and water. If the presence of the 1,3-DSPC in the vesicle bilayer has an effect analogous to cholesterol, i.e., broadening of the phase transition and moderating of the bilayer fluidity, it may simply act to reduce the degree of molecular rearrangement necessary when the vesicle is cooled through the phase transition and consequently minimize the exposure of the hydrocarbon portion of the bilayer. It is apparent that a vesicle of well-defined bilayer properties is desirable to fully exploit the potential of this model system.

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