Effect of Surface Curvature on Stability, Thermodynamic Behavior, and Osmotic Activity of Dipalmitoylphosphatidylcholine Single Lamellar Vesicles†

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ABSTRACT: The size and surface curvature dependence of the properties and stability of single lamellar vesicles have been investigated by using a variety of physicochemical techniques. Dipalmitoylphosphatidylcholine single lamellar vesicles of sizes ranging between 200 and 900 Å in diameter have been prepared by the French press method and characterized with respect to their size distribution, stability, and thermotropic behavior by negative stain electron microscopy, molecular sieve chromatography, nuclear magnetic resonance spectroscopy, and differential scanning calorimetry. Vesicles with a diameter smaller than 400 Å are unstable below their transition temperature and fuse spontaneously to form larger single lamellar vesicles. Correlation analysis of experimentally obtained size distributions and calorimetric phase transitions profiles allowed estimation of the size dependence of the transition temperature. The phase transition temperature depends on the vesicle size in a sigmoidal fashion. Throughout the entire 200–700 Å diameter range, the phase transition parameters are sensitive to size; however, the size dependence is especially pronounced around 400 Å in diameter. The anomalous size dependence of the transition temperature for vesicles smaller than 400 Å in diameter has been attributed to a decrease in the effective bilayer curvature due to packing rearrangements of the lipid molecules. Changes in the fractional degree of self-quenching of trapped 6-carboxyfluorescein induced by osmotic stress indicate that large single lamellar vesicles are not spherical under isoosmotic conditions. These vesicles are relatively flexible and can sustain almost a 2-fold increase in their internal aqueous volume without any leakage of the internal content.

The effect of surface curvature on the properties of lipid bilayers is a subject of much interest. Highly curved, small unilamellar vesicles (SUV),[1] made by ultrasonic irradiation of phospholipids (Huang, 1969), have been extensively studied by various techniques and their properties compared with those of the essentially planar, large multilamellar liposomes (Bangham, 1967). The gel to liquid-crystalline phase transition temperature (T_m) as well as the enthalpy, entropy, and cooperativity associated with this transition is lower for the small unilamellar vesicles than for the multilamellar liposomes (Sturtevant, 1974; Melchior & Steim, 1976; Suurkuusk et al., 1976; Lentz et al., 1976; Freire & Biltonen, 1978; Gruenwald et al., 1979). NMR data (Sheetz & Chan, 1972; Lichtenberg et al., 1975; Petersen & Chan, 1977) and the results of Raman studies (Gaber & Peticolas, 1977; Spiker & Levin, 1976; Mendelsohn et al., 1976) suggest that the difference in surface curvature induces differences in the packing of the phospholipid hydrocarbon chains, which may in fact be responsible for the different thermotropic behavior of the two types of bilayers.

Small vesicles also differ from large multilamellar liposomes in their trapped volume and hydrodynamic properties. In addition, multilamellar phosphatidylcholine liposomes containing small amounts of charged lipid are osmotically active, while those without charged lipids are not (Bangham et al., 1967). Small unilamellar vesicles, either with or without charged lipid, are not osmotically sensitive (Johnson & Buttress, 1973). However, very large, probably oligolamellar ("thin-walled") vesicles are osmotically active with and without charged lipids (Reeves & Dowben, 1969; Deamer & Bangham, 1976). This indicates that the multilamellarity plays a role when large liposomes are not charged. More importantly, it would appear that it is the high curvature of the small vesicles that prevents them from being osmotically sensitive.

Recently, it has been shown that below T_m the small unilamellar vesicles are unstable and fuse spontaneously into larger vesicles (Suurkuusk et al., 1976; Kantor et al., 1977; Larrabee, 1979; Schullery et al., 1980; Schmidt et al., 1981). For dipalmitoylphosphatidylcholine (DPPC), this fusion leads to the formation of unilamellar vesicles of about 700 Å in diameter, which are stable below T_m (Schullery et al., 1980) and have osmotic activity and calorimetric characteristics that are very similar to those of the multilamellar liposomes. Thus, any size dependence of the thermodynamic properties of single lamellar vesicles is expected to be confined to a size interval between 200 and 700 Å.

In this communication we report the results of a systematic study of the stability and thermotropic behavior of dipalmitoylphosphatidylcholine single lamellar vesicles ranging between 200 and 900 Å in diameter. These vesicles were prepared by using the French press method which has previously been shown to yield egg phosphatidylcholine vesicles in this size range (Barenholz et al., 1979). Below T_m, DPPC vesicles prepared by the French press method undergo a fusion process similar to that exhibited by sonicated vesicles (Schullery et al., 1980) but limited to those vesicles that have a diameter smaller than 400 Å. The size distribution of the resulting vesicles is different from that of fused sonicated vesicles. They are single lamellar and average about 600 Å in diameter, which is intermediate between the sonicated vesicles and their fusion products. Analysis of the molecular sieve profile, electron micrographs, and scanning calorimetry

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[1] Abbreviations used: DPPC, dipalmitoylphosphatidylcholine; SUV, small unilamellar vesicles; C_p, heat capacity; T_m, gel to liquid-crystalline phase transition temperature.

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of these vesicles provides valuable information regarding the size and curvature dependence of the properties and stability of lipid bilayers.

Materials and Methods

Preparation of Vesicles. 1,2-Dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC) was synthesized and purified as described previously (Suurkuusk et al., 1976). Dispersions of vesicles were prepared in 100 mM KCl following the procedure described by Barenholz et al. (1979). The lipid suspended in the dilute salt solution was passed through the exit valve of a French pressure cell at a flow rate of 1 mL/min under a pressure of 20 000 psi at 45 °C. This procedure was repeated 4 times, and the resulting dispersion was centrifuged for 30 min at 20 000*g*. The resultant supernatant of these preparations contained only single lamellar vesicles as judged from negative stain electron micrographs and out/in ratios obtained from NMR measurements (see below).

Molecular Sieve 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 pressure with reverse flow. Column effluent was collected at 2-mL fractions. Lipid concentrations were determined as inorganic phosphorus by the method of Bartlett (1959).

Trapped Volume Determination. The trapped volume of the vesicles was determined by preparing the vesicle in the presence of [^{14}C]glucose or 6-carboxyfluorescein. External radiolabeled glucose or 6-carboxyfluorescence was removed by passage over a Sephadex G-25 column. The total trapped marker was then determined. The volume of the total internal aqueous compartment of the vesicle was calculated from the amount of trapped solute, the concentration of the trapped solute in the initial medium, and the molar concentration of phospholipid (Roseman et al., 1978).

Electron Microscopy. Negatively stained vesicle preparations were examined on carbon-coated Formvar films on copper grids in a Siemens Elmiscope IA electron microscope operating at 60 kV and usually at 40 000 magnification. The sample was mixed isotonically with ammonium molybdate solution (0.75–1.5%) at pH 8. After 1 min of incubation, it was applied to the grid, the excess solution was drained, and the preparation was examined immediately.

NMR Measurements. The NMR measurements were performed at 59.75 MHz for ^{1}H and 24.15 MHz for ^{31}P by using a JEOL FX60Q Fourier transform spectrometer. All spectra contained 4K data points after Fourier transformation. ^{1}H NMR spectra at 23 °C were obtained by using a 10-kHz sweep width and 0.41-s acquisition time. A 180°–τ–90° pulse sequence was used to minimize the residual HOD signal (Patt & Sykes, 1972). ^{31}P NMR spectra at 24 °C were obtained by using continuous ^{1}H noise decoupling, a 2-kHz sweep width, 0.26-s acquisition time, 1.5-s delay between acquisitions, and a 90° pulse of 16 μs. A total of 1000 scans was accumulated, so the time per spectrum was 0.5 h. The out/in ratio, between the number of molecules on the outside of the vesicles and that on the inside, was determined at 45 °C by ^{31}P NMR spectroscopy, using isoosmotic solutions of $PrCl_3$ as a shift reagent (Bergelson, 1978). Typically, a Pr^{3+}/lipid ratio of 0.05 was employed. Narrow sweep widths (600 Hz) were used in these measurements.

Scanning Calorimetry. A highly accurate scanning calorimeter of the heat conduction type was used for these studies (Suurkuusk et al., 1976). All the experiments in this communication were made at a scanning rate of 15 °C/h. Digitized heat capacity (C_p) data were recorded and stored at constant temperature intervals of 0.1 °C. All calculations were performed in a CDC Cyber 172 computer. The lipid concentration in the calorimetric cell ranged between 20 and 30 mM.

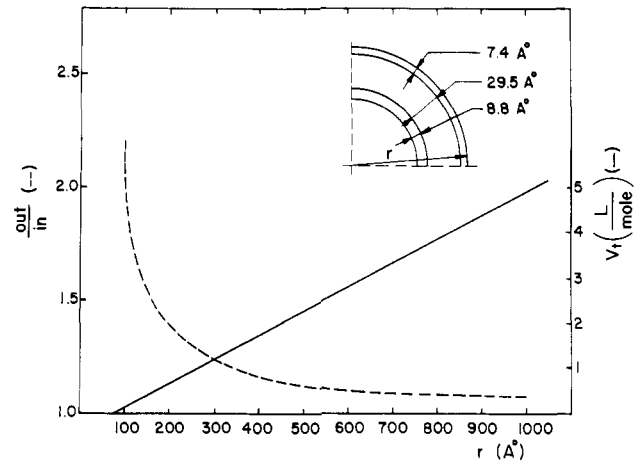

FIGURE 1: Calculated size dependence of the trapped volume and out/in ratio of spherical vesicles. The inset to the figure presents the results of calculations based on the model of Cornell et al. (1980) for sonicated egg phosphatidylcholine vesicles (Huang & Mason, 1978). In this model, the surface area per phospholipid molecule at the hydrophobic/hydrophilic interface is the same for the inner and outer monolayers and does not depend on the vesicle size. The out/in ratio is therefore approximately equal to the ratio of surface area: out/in = $[(r - 7.4)/(r - 36.3)]^2$. The calculated size dependence of this ratio is given by the broken line. The volume, in Å^3, trapped by 1 Å^3 of the hydrated bilayer is given by $(r - 45.7)^3/[r^3 - (r - 45.7)^3]$. If it is assumed that the size variation of the partial specific volume of the hydrated bilayer ($\bar{v}$ = 0.988 mL/g; Cornell et al., 1980) is small in comparison to changes in the trapped volume, the equation $V_t = (0.988\ M_r/1000)[(r - 45.7)^3/(r^3 - (r - 45.7)^3)]$, where M_r is the molecular weight of the phospholipid, describes the size dependence of the trapped volume (V_t; in units of liters per mole of lipid). The dependence of V_t on the radius r (full line) is approximately linear and can be described by the formula $V_t = 5.3 \times 10^{-3}(r - 75)$ L/mol.

Results and Discussion

Size Dependence of Vesicle Stability in the Gel Phase. Vesicles formed in the French pressure cell from synthetic dipalmitoylphosphatidylcholine above the phase transition temperature (T_m) undergo a spontaneous fusion process when held for several days below T_m. This can be followed by the decrease in the measured out/in ratio determined at 45 °C by using ^{31}P NMR and a shift reagent. In spherical vesicles, this ratio should depend on the vesicle size, as described in Figure 1. For vesicles made by the French press, it varied within the range 1.4–1.7 from preparation to preparation. However, it always approached a value of 1.30 ± 0.05 after 2 days of incubation, with no further change after 2 additional weeks of incubation below T_m. As shown in Figure 2, this size transformation is also reflected in the decreasing height of the choline methyl signal in the proton NMR spectra obtained below T_m (Schmidt et al., 1981). A time-dependent change is also observed in the calorimetric curves, as indicated in Figure 3. It is clear that the peak maximum in the heat capacity vs. temperature function shifts to higher temperatures with time. The extent of fusion of the French press vesicles, however, is much less than that observed for vesicles obtained by sonication, as indicated by the much smaller decrease in the proton NMR signals below T_m (Figure 2).

The end products of the fusion processes of small sonicated vesicles and French press vesicles differ in their size distribution. Molecular sieve chromatography and analysis of the ^{31}P line shape at 24 °C show that the distribution of fusion products of the sonicated vesicles is bimodal; i.e., small (~200 Å) and much larger vesicles coexist in the dispersion without

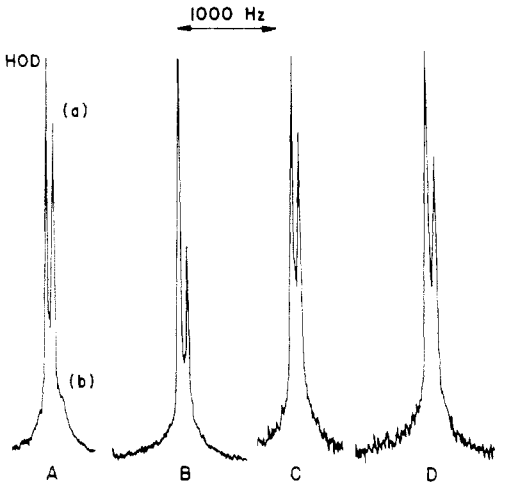

FIGURE 2: ^1H NMR spectra of dipalmitoylphosphatidylcholine (12 mM) vesicles prepared by sonication (A and B) or by the French press (C and D) after incubation at 24 °C for 1 h (A), 1.6 h (C), 79 h (B), or 92.5 h (D).

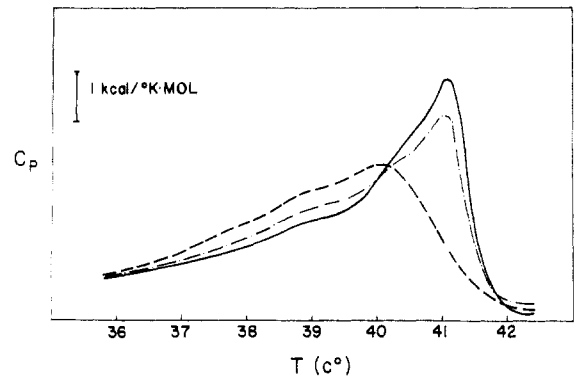

FIGURE 3: Successive calorimetric scans of a dispersion of French press vesicles immediately after preparation (---), after 6 h (-·-·-) and after 24 and 48 h (—) of incubation at 24 °C.

any significant population of intermediate size vesicles (Schullery et al., 1980; Schmidt et al., 1981). This pattern is very different from the distribution of sizes resulting from the fusion of French press vesicles. As shown in Figure 4, for the French press vesicles, only a minor fraction of the vesicles are of the size obtained by sonication, and about 75% of the lipid mass is found in vesicles whose diameters lie between the mean diameter of sonicated vesicles, 210 Å (Huang, 1969; Chrzeszcyck et al., 1977), and the mean diameter of the fusion product of these vesicles, 700 Å (Schullery et al., 1980). Electron micrographs of negatively stained fused French press vesicles show that the size distribution is Gaussian, with about 70% of the phospholipid in vesicles of diameters between 400 and 800 Å (Figure 5). The calculated mean weight-average vesicle diameter is 620 Å, with a standard deviation of 180 Å, in good agreement with the value of 640 Å obtained with a Coulter light scattering autocorrelation "nano sizer" system. The two fusion products also differ markedly in their heat capacity vs. temperature function (Figure 3), both in terms of the position of the maxima and the shape of the transition profile. The transition of fused sonicated vesicles is bimodal, with a narrow transition at about 41.5 °C, due to the 700-Å fused vesicles, and a somewhat broader transition at 37 °C, due to the remaining population of small vesicles (Suurkuusk et al., 1976; Schullery et al., 1980). On the other hand, a dispersion of fused French press vesicles, which is mostly an ensemble of vesicles with sizes between 400 and 800 Å, gives rise to a broad transition curve, with a maximum at 41 °C but asymmetric toward the low-temperature region (Figure

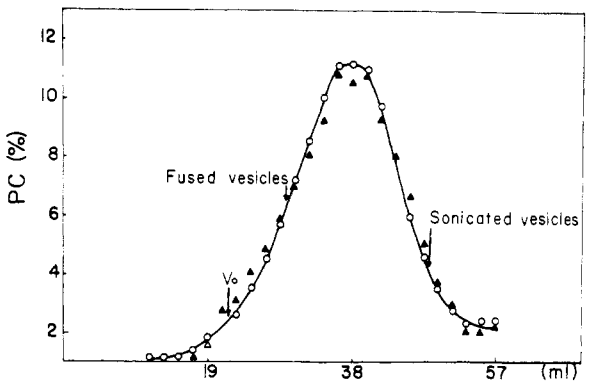

FIGURE 4: Eluton pattern of French press vesicles on Sepharose 2B column after 6 h (▲) and 48 h (O) of incubation at 24 °C. Small unilamellar vesicles (Huang, 1969) and their fractionated fusion products (Schullery et al., 1980) were used as markers.

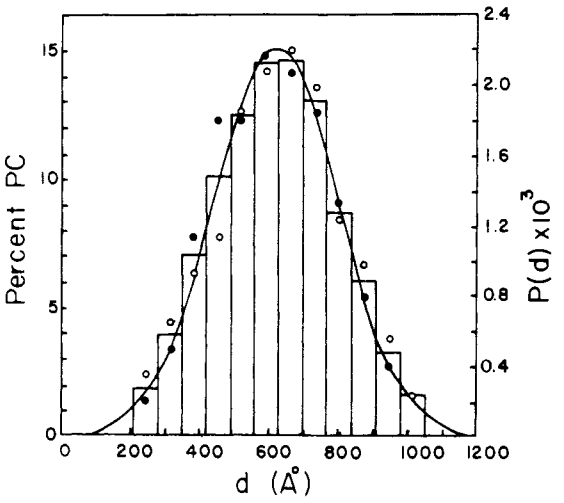

FIGURE 5: Size distribution of DPPC vesicles, obtained by counting ~600 vesicles in electron micrographs of each of two different preparations (●, O). The dispersions were incubated for 48 h at 24 °C prior to being negative stained by ammonium molybdate.

3). The shape of this curve strongly suggests that within this size range the phase transition is very sensitive to vesicle size. The size dependence of T_m can be estimated by correlating the calorimetric curves with the size distribution obtained from electron micrographs, as discussed in the following section.

For sonicated vesicles, the fusion of 210-Å vesicles into 700-Å vesicles is accompanied by a growth of the calorimetric peak at 41.5 °C, at the expense of the 37 °C peak, without producing significant amounts of vesicles that have transitions at intermediate temperatures. For the French press vesicles, the 41.5 °C peak also increases at the expense of the low-temperature part of the heat capacity curve, indicating again that the smaller, lower melting temperature vesicles in these dispersions undergo transformation to larger ones upon incubation below T_m. But in contrast to sonicated vesicles, vesicles remain which melt at temperatures between 38 and 41.5 °C (Figure 3). This observation indicates that only vesicles in which the phospholipid has a phase transition lower than ~39 °C fuse spontaneously, suggesting that the tendency of unilamellar vesicle to undergo vesicle–vesicle fusion depends on the vesicle size. This tendency is relatively small for vesicles of diameters larger than 400 Å, as can be deduced by correlating the calorimetric results with the observed size distribution.

Size Dependence of the Gel to Liquid-Crystalline Transition. The thermodynamic parameters associated with the phase transition of unilamellar vesicles are a function of the

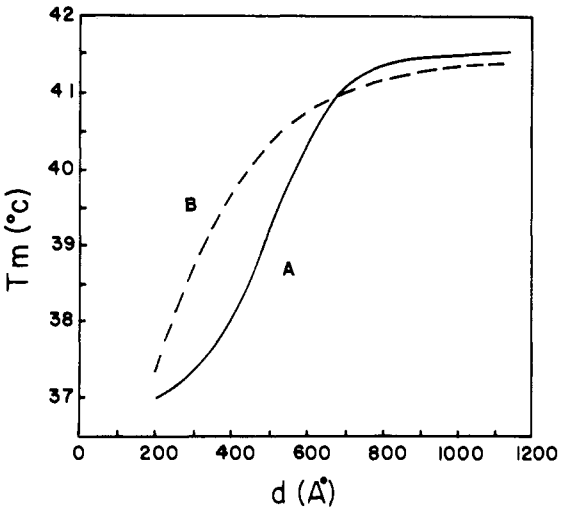

FIGURE 6: (Curve A) Calculated dependence of the transition temperature (T_m) on the vesicle diameter. This calculated dependence, in conjunction with the size distribution of Figure 5, yields a calculated calorimetric scan that is identical with that of Figure 3 (at equilibrium). (Curve B) Hypothetical exponential size dependence of T_m.

vesicle size and curvature. However, this dependence is restricted to vesicle sizes smaller than some critical value above which the phase transition parameters become independent of size. The transition temperature of small DPPC vesicles of 210-Å diameter is 37 °C whereas that of DPPC vesicles of 700-Å diameter obtained by fractionation of fused sonicated vesicles (Schullery et al., 1980) is 41.5 °C. This is identical with the T_m of large multilamellar liposomes (Suurkuusk et al., 1976). Beyond 700-Å diameter, the transition temperature is independent of size, which is also demonstrated by the fact that very heterogeneous populations of multilamellar liposomes give rise to a sharp phase transtion.

The fused French press vesicles cover an intermediate size range between small (200-Å) and large (700-Å) unilamellar vesicles. Their thermotropic behavior is therefore expected to be very sensitive to the vesicle size distribution. This is in fact the case, as can be observed in the calorimetric scans shown in Figure 3. As mentioned earlier, a prominent feature of these scans is the persistent skewness of the heat capacity function toward the low-temperature end of the transition. Even after several days of equilibration, the calculated fraction of DPPC molecules with a transition temperature lower than 38 °C is larger than 15%. On the basis of the size distribution of Figure 5, this calorimetric result indicates that vesicles of up to ~400 Å in diameter have a transition temperature at or below 38 °C. The immediate implication of this observation is that for vesicles smaller than 400 Å the mean rate of increase of the transition temperature with size is at most 0.005 °C/Å whereas for those between 400 and 700 Å the mean rate of increase is 0.01 °C/Å, i.e., at least twice of that observed for vesicles with diameters smaller than 400 Å. This is a surprising result since it assigns the maximum size dependence of T_m to vesicles of intermediate size, as indicated in Figure 6 (curve A), and not to the smallest vesicles, as would have been predicted by a simple power law (Figure 6, curve B).

The size dependence of the transition temperature was estimated by fitting the experimental heat capacity curve with theoretical curves (Biltonen & Freire, 1978). The curves were calculated on the basis of previous results for the enthalpy changes and cooperative units for small (200-Å) and large (700-Å) unilamellar vesicles (Suurkuusk et al., 1976; Biltonen & Freire, 1978). For these calculations, it was assumed that the enthalpy change and the cooperative unit for the transition

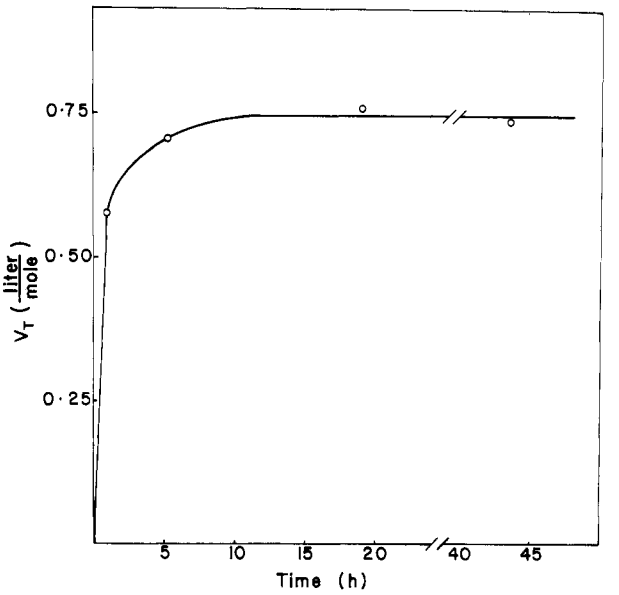

FIGURE 7: Kinetics of the entrapment of extravesicular 6-carboxyfluorescein (6-CF) in dipalmitoylphosphatidylcholine French press vesicles. The vesicles were incubated in the presence of 6-CF (10 mM) at 50 °C, and at various times, nontrapped 6-CF was removed by using Sephadex G-25 columns. Fluorescence intensity was measured after addition of Triton X-100. The trapped volume is given in units of liters of the original solution per mole of phospholipid.

have the same functional form as T_m.

The absence of a size dependence of T_m for vesicles larger than 700 Å is expected from the vanishing vesicle curvature in this size range, and so is the increased dependence as the vesicle size decreases. What is unexpected is the decreased slope of this dependence for vesicles smaller than 400 Å in diameter. This phenomenon could be the result of a different packing arrangement of the phospholipid molecules below certain vesicle size. In this respect, it has been suggested that below T_m small DPPC vesicles assume a polygonal shape (Blaurock & Gamble, 1979). If this is so, the near-planar packing of the molecules within the facets would have the net effect of decreasing the effective bilayer curvature per molecule over that of a perfect sphere. This observation, in conjunction with the assumption that T_m is primarily determined by the effective bilayer curvature, may explain the anomalous size dependence for vesicles smaller than 400-Å diameter.

Osmotic Activity of Vesicles: Dependence on Size. Trapped volume measurements using both [^{14}C]glucose and 6-carboxyfluorescein gave a value of 0.6 L/mol of lipid for the French press vesicles after 2 days of incubation below T_m. With the assumption that the vesicles are spherical, this value can be used to calculate an average vesicle diameter of about 380 Å (Figure 1). The trapped volume represents an average over all the vesicles in the dispersions, and it is weighted toward the vesicles of larger sizes. However, the trapped volume diameter of 380 Å is considerably smaller than the diameter estimated from molecular sieve chromatography, autocorrelation light scattering data, electron microscopy, and ^{31}P NMR determination of the out/in ratio, which are weighted averages based on surface areas. The data shown in Figure 7 demonstrate that this is not due to a difference of the marker concentration inside and outside the vesicles. Vesicles containing the trapped solute marker were incubated in the presence of external marker solute above T_m. At successive times, the external solute was removed from aliquots on a Sephadex G-25 column and the internal volume determined as described. The time dependence of the trapped volume determined under these conditions (Figure 7) clearly shows that marker solute equi-

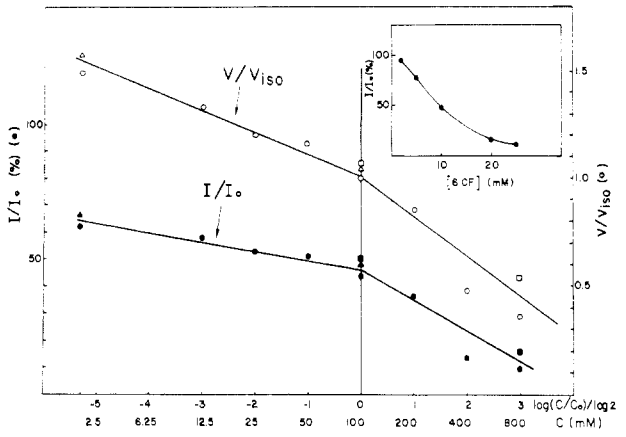

FIGURE 8: The dependence of the volume trapped in French press vesicles on the osmolarity of the external medium, as measured by quenching of trapped 6-CF. Trapping of 6-CF was done under isoosmotic conditions (total osmolarity equivalent to that of 100 mM KCl), as described for Figure 7. The osmotic pressure was changed by changing the extravesicular salt concentration. The fraction of unquenched fluorescence (I/I_0, in percent, full symbols) was calculated from the fluorescence intensity measurements before (I) and after (I_0) the addition of Triton X-100. The empty symbols represent V/V_{iso}, the ratio between the trapped volume (V) and that trapped under isoosmotic conditions (V_{iso}). V and V_{iso} were determined from the calibration curve of the inset, obtained by trapping different concentrations of 6-CF inside French press vesicles under isoosmotic conditions. The squares represent experiments in which the concentration of salt in the dispersions was first raised by dilution with concentrated salt solutions and then readjusted by dilution with water. The triangles represent experiments in which the dispersions were first diluted in water and then in concentrated salt solutions.

librium is reached in less than 10 h at a value of about 0.75 L/mol.

A plausible explanation for the apparent discrepancy between the vesicle size determined from trapped volume measurements and the size determined by other means is that the vesicles are not spherical. This possibility was tested by subjecting the vesicles to osmotic stress and simultaneously monitoring the fractional degree of self-quenching of 6-carboxyfluorescein trapped inside the vesicles. The results of these experiments are shown in Figure 8. In this figure, the ratio of intensity of the partially quenched fluorescence I to the full intensity I_0, observed after solubilizing the vesicles with Triton X-100, has been plotted as a function of extravesicular KCl concentration. As can be seen, dilution of the vesicle dispersion with hypotonic KCl solution at room temperature results in an increase in the nonquenched 6-carboxyfluorescein fluorescence. This increase in intensity can be reversed by the addition of concentrated KCl solution, which indicates that the observed increase is not the result of leakage of 6-carboxyfluorescein but is due to a dilution of the fluorophor trapped inside the vesicle. Figure 8 also shows that addition of hypertonic KCl solution to an isoosmotic vesicle dispersion causes a decrease in I/I_0. This decrease is also reversible by dilution of the external medium. The upper curve in Figure 8 shows the calculated change in vesicle internal volume based on the changes in the ratio of I/I_0 (inset to Figure 8). These results clearly indicate that the shape of these vesicles at osmotic equilibrium is nonspherical. The vesicles, as prepared, can sustain an increase in internal aqueous volume by a factor of 1.6 without induced leakage of 6-carboxyfluorescein. It should be noted that under these conditions the vesicle internal volume becomes similar to that expected from the NMR, molecular sieve chromatography, and electron microscopy results. We also note that the osmotic behavior of these vesicles, prepared without charged lipid, is qualitatively the same as that of the much larger "thin-walled" vesicles (Reeves & Dowben, 1969). This emphasizes the difference between multilamellar vesicles, small unilamellar vesicles, and larger unilamellar vesicles.

Implications. The results presented in this communication constitute an initial attempt to characterize the effects of vesicle size and bilayer curvature on the stability and physicochemical properties of single lamellar vesicles. These studies demonstrate that single lamellar vesicles smaller than 400 Å in diameter are unstable below their phase transition temperature and that they spontaneously fuse into larger single lamellar vesicles. Apparently, the final vesicle size in this fusion process is dictated by the fusion mechanism itself and not by the disappearance of the vesicle instability. This instability does not exist or is very small for vesicles larger than 400 Å, as demonstrated by the fact that vesicles of this size do not fuse. On the other hand, it has been previously demonstrated that small sonicated vesicles of 200-Å diameter fuse into 700 Å diameter vesicles (Schullery et al., 1979), i.e., a final size larger than that required to relieve the packing strains. Blaurock & Gamble (1979) have concluded that small single lamellar vesicles adopt a polygonal, faceted configuration below T_m; this molecular arrangement could be related to the instability of these vesicles and could also be associated with the anomalous size dependence of the thermotropic behavior of single lamellar vesicles smaller than 400-Å diameter. The results presented in this paper also indicate that vesicles with diameters larger than 400 Å are osmotically sensitive and, in general, have a nonspherical equilibrium configuration.

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Mechanism of Inactivation (Desensitization) of Acetylcholine Receptor. Investigations by Fast Reaction Techniques with Membrane Vesicles†

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ABSTRACT: Exposure of the acetylcholine receptor to acetylcholine, or its stable analogue carbamylcholine, inactivates (desensitizes) the receptor. Inactivation of receptor-controlled ion ($^{86}Rb^+$) flux in the presence of different concentrations of carbamylcholine (12.5 μM to 28 mM) was measured in the millisecond to minute time region, using a quench flow technique and membrane vesicles prepared from the electric organ of *Electrophorus electricus*. Three different kinetic measurements were made to establish the relationship between carbamylcholine concentration and the ion translocation process: (i) the rate of inactivation of the ion translocation process; (ii) the rate of recovery of the inactivated receptor upon removal of carbamylcholine; and (iii) the rate of the ion flux mediated by equilibrium mixtures of active and inactive receptor forms. The kinetics of these three processes follow single-exponential rate laws, and simple analytical expressions for their ligand concentration dependence could be used. Therefore, it was possible to determine the value of the rate constants in a scheme relating the ligand binding steps to ion translocation, and to predict the dependence of these rate constants on carbamylcholine concentration over the 200-fold range investigated.

Electrical signals play an important role in the function of nervous systems and in muscle contraction. Such signals are determined by the concentrations of inorganic ions on both sides of a cellular membrane and by the rates with which specific inorganic ions move through the membrane (Hodgkin & Huxley, 1952). The rates of the ion movements through the membranes are controlled by membrane-bound proteins, receptors, and are determined by the concentration of specific small molecules which bind to these receptors (Nachmansohn & Neumann, 1975). The important relationship between the ligand binding processes and the transmembrane ion fluxes, which determine the amplitude and duration of electrical signals and thereby the transfer of information between cells, is not fully understood. Determining this relationship for the acetylcholine receptor is the purpose of the experiments described.

Measurements of electrical signals controlled by the acetylcholine receptor indicated that the ligand binding process may be complex (Katz & Thesleff, 1957). Addition of acetylcholine or carbamylcholine to muscle cells produces an electrical signal which subsequently disappears even when the concentration of the ligand remains constant. Two different ligand-induced conformational changes of the receptor have been proposed to account for these observations: (i) Nachmansohn (1952, 1955) suggested that a conformational change which results in channel opening causes the signal, and (ii) Katz & Thesleff (1957) suggested a subsequent conformational change which leads to inactivation (desensitization) of the receptor and disappearance of the signal. More recently, evidence for the complexity of the ligand binding mechanism and of the ligand-induced isomerization of receptor forms has come from two types of studies: (i) the reaction of specific neurotoxins (Chang & Lee, 1963) with receptor-rich membrane preparations from *Electrophorus electricus* (Bulger & Hess, 1973; Bulger et al., 1977; Maelicke et al., 1971; Hess et al., 1975b); (ii) investigations of the binding of various ligands to both the isolated and the membrane-bound receptor (Weiland et al., 1977; Grünhagen & Changeux, 1978; Barrantes, 1978; Heidmann & Changeux, 1978; Rübsamen et al., 1978; Hess, 1979; Quast et al., 1978, 1979; Eldefrawi & Eldefrawi, 1979; Jürss et al., 1979; Dunn et al., 1980). Recently, it has become possible to investigate the correlation between ligand binding processes and the receptor-controlled ion translocation (Hess et al., 1979).

The measurements reported here were made with receptor-rich membrane vesicles, chosen because they allow one to investigate receptor-mediated ion translocation under controlled conditions which are not limited by the metabolic restrictions present in intact cells. The internal and external ion

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