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Vesicle–Vesicle Interactions in Sonicated Dispersions of Dipalmitoylphosphatidylcholine†

C. F. Schmidt, D. Lichtenberg, and T. E. Thompson*

ABSTRACT: The time course of the size transformation of sonicated small unilamellar dipalmitoylphosphatidylcholine vesicles at 23 °C has been followed with ^{31}P and ^{1}H nuclear magnetic resonance (NMR) spectroscopy. Comparison of these results with turbidity measurements indicates that vesicle aggregation, monitored by turbidity, and size transformation, followed by NMR, occur on distinctly different time scales. For lipid concentrations in the 5–50 mM range, aggregation takes place on a time scale of minutes, whereas size transformation takes many hours. Aggregation, unlike size transformation, can be reversed by increasing the temperature above the phospholipid phase transition temperature. Analysis of the ^{31}P NMR line shapes provides evidence for a model in which the small vesicles transform into the product vesicles [characterized by Schullery, S. E., Schmidt, C. F., Felgner, P., Tillack, T. W., & Thompson, T. E. (1980) *Biochemistry 19*, 3919] without producing significant amounts of intermediate-size vesicles. Kinetic analysis indicates that the size transformation is apparently second order. ^{1}H NMR data indicate that the rate of transformation is decreased if trivalent ions are added to the dispersions and also if the temperature is periodically increased above the transition temperature. Analysis of the latter experiment provides some evidence that vesicle aggregation is a necessary precursor to size transformation. It was also found that increasing the average vesicle size decreases the extent of transformation.

Ultrasonic irradiation of phospholipid dispersions produces small unilamellar vesicles, 200–300 Å in diameter (Huang, 1969), which have been used in a variety of physical and biological studies (Papahadjopoulos, 1978). When the vesicles have been prepared from saturated fatty acid phosphatidylcholines, which undergo thermotropic phase transitions above 0 °C, there have been conflicting reports about their stability. Recently, however, it has become clear that if the phosphatidylcholines have been shown by heavy thin-layer chromatography spotting to be free of contaminants, then the small vesicles transform into larger species at an appreciable rate only below the gel to liquid-crystalline transition temperature, but not at or above it (Suurkuusk et al., 1976; Kantor et al., 1977; Larrabee, 1979; Schullery et al., 1980). This study, using ^{31}P and ^{1}H nuclear magnetic resonance (NMR) to follow changes in the size distribution of dipalmitoylphosphatidylcholine vesicles, was undertaken in an attempt to learn more about the mechanism of transformation, in the hope that this information will be applicable to more complicated systems.

We feel that much of the confusion associated with vesicle stability, aside from the purity problem mentioned above, stems from the use of light scattering or turbidity measurements as a primary tool. Light scattering is severely limited by the inability to distinguish between aggregation and size transformation. This has often been pointed out (Martin & MacDonald, 1976; Chong & Colbow, 1976; Avramovic & Colbow, 1978; Petersen & Chan, 1978), but the relative contributions have not been evaluated in an unambiguous manner. An equally important problem is caused by the presence of small amounts of multilamellar "contaminants" if the vesicles are not size fractionated before use (Huang, 1969; Barenholz et al., 1977). Marsh et al. (1977) have shown, by light scattering measurements on unfractionated preparations, that the transition due to the small dipalmitoylphosphatidylcholine vesicles, centered around 37 °C (Suurkuusk et al., 1976), cannot be easily distinguished from that due to the larger species at 41 °C.

The great advantage of using light scattering to follow vesicle transformation is, of course, that the sample can be monitored continuously without perturbation. Other methods (electron microscopy, trapped volume, ultracentrifugation, and gel chromatography) rely on the analysis of aliquots, which introduces the possibility of changes in the sample during analysis, and also has a tendency to limit time resolution. ^{1}H NMR has been used to monitor the fatty acid induced transformation of sonicated dimyristoylphosphatidylcholine vesicles (Prestegard & Fellmeth, 1974; Kantor & Prestegard, 1975; 1978) by taking aliquots and measuring the spectra above the transition temperature. More recently, ^{1}H NMR has been used isothermally to monitor Ca^{2+}-induced fusion of phosphatidylcholine–phosphatidic acid vesicles above the transition temperature (Liao & Prestegard, 1979). ^{31}P NMR has also been used to characterize the end product of transformation in both the latter system (Liao & Prestegard, 1979; Koter et al., 1978) and dipalmitoylphosphatidylcholine vesicles (Schullery et al., 1980). In the latter study, the ratio of the number of molecules on the outside of transformed vesicles to that on the inside, measured above the phase transition temperature using the shift reagent Pr^{3+} (Bergelson, 1978),

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was established as an unambiguous indicator of size transformation. This out/in ratio should not be influenced by vesicle aggregation. In this study, we have used both ^{31}P and ^{1}H NMR to monitor the size transformation of dipalmitoylphosphatidylcholine vesicles *below* the transition temperature over the entire time course. Used in this manner, NMR has the advantages of light scattering, but it is much less sensitive to aggregation (see Results).

Using this nonperturbing method, we have studied the effects of variables such as temperature, concentration, radius of curvature, and added ions. These studies lead to new insights into the kinetics and mechanism of the size transformation. A preliminary report of this work has appeared elsewhere (Lichtenberg et al., 1980).

Kinetic analysis of transformation data should theoretically allow discrimination among the various mechanisms that have been proposed. Lipid transfer via monomers or micelles should be first order in phosphatidylcholine concentration (Martin & MacDonald, 1976; Lawaczeck, 1978), while vesicle–vesicle collision mechanisms (fusion), in their simplest form, would be expected to be second order (Lansman & Haynes, 1975; Lawaczeck, 1978). This picture could be complicated by a rate-limiting step or by the effect of vesicle aggregation. Petersen & Chan (1978) have speculated about the possible roles of aggregation in fusion. They distinguish between loose, reversible flocculation and tight, irreversible coagulation, the latter being a prerequisite for fusion. Unfortunately, all of the above studies employed light scattering or unfractionated vesicle populations and are thus difficult to interpret.

Experimental Procedures

1,2-Dipalmitoyl-3-*sn*-phosphatidylcholine was synthesized and purified, and small unilamellar vesicles were prepared and fractionated according to previously described procedures (Suurkuusk et al., 1976; Barenholz et al., 1977; Schullery et al., 1980), except that the samples for ^{1}H NMR were prepared in 50 mM KCl in D_2O and used without centrifugation. $N(CD_3)_3$–dipalmitoylphosphatidylcholine was prepared from dipalmitoylphosphatidylethanolamine (Sigma) and purified as previously described (Stockton et al., 1974; Shaw et al., 1977). Lipid concentrations were determined as inorganic phosphate by the Bartlett method (Bartlett, 1959).

Turbidity measurements were made by adding the vesicle dispersion at 45 °C to an equal volume of buffer at 4 °C, so the vesicles were rapidly cooled to room temperature. The absorbance increase due to the change in the refractive index at the lipid phase transition (Yi & MacDonald, 1973) therefore contributes only to the first time point.

The NMR measurements were performed at 59.75 MHz for ^{1}H and 24.15 MHz for ^{31}P 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). A total of 20 and 40 scans were accumulated for the 40 and 10 mM samples, respectively. ^{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 were accumulated, so the time per spectrum was 0.5 h.

The ratio of the number of molecules on the outside of sonicated vesicles to that on the inside was determined at 45 °C using Pr^{3+} as a shift reagent with either ^{1}H or ^{31}P NMR. Narrow sweep widths (600 Hz) were used for both nuclei. For the transformed vesicles, it was necessary to add isoosmotic solutions of $PrCl_3$ because of the osmotic sensitivity of these vesicles. ^{31}P NMR was used exclusively for these measurements because of the higher sensitivity of the ^{31}P chemical shift to Pr^{3+} (Bergelson, 1978). Typically, a Pr^{3+}/lipid ratio of 0.05 was employed.

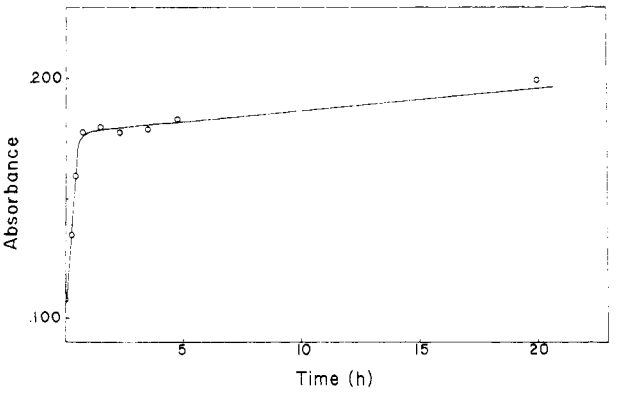

FIGURE 1: Plot of turbidity at 660 nm of 5 mM sonicated dipalmitoylphosphatidylcholine vesicles as a function of time of incubation at room temperature.

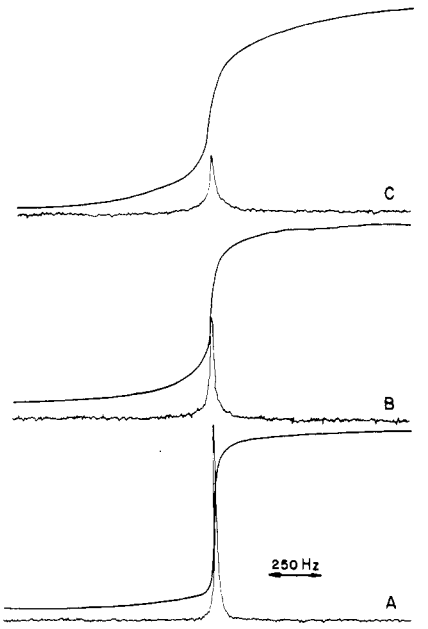

FIGURE 2: ^{31}P NMR spectra at 40 mM sonicated dipalmitoylphosphatidylcholine vesicles measured at 24 °C after (A) 0.5, (B) 35.5, and (C) 149 h at that temperature.

Results

The turbidity of a typical preparation of sonicated dipalmitoylphosphatidylcholine vesicles, cooled to 23 °C, showed an initial rapid increase for a period of about 0.5 h and a much slower increase at longer times (Figure 1). The out/in ratio of a sample kept at 23 °C for 0.5 h, and then reheated for measurement, decreased slightly but was still within the limits of error of the original measurement (2.0 ± 0.1), indicating that the rapid turbidity increase is not due to size transformation but predominantly to vesicle aggregation. NMR spectra, recorded at 24 °C, were taken at times, as defined by the accumulation starting time plus one-half of the total accumulation time, that were at least 0.5 h after cooling, so that changes in the spectra monitor only the changes reflected in the slow turbidity increase. Typical ^{31}P NMR spectra, taken at 24 °C after 0.5, 25.5, and 149 h, are shown in Figure 2. The values for the relative peak height $[100h(t)/h(0)]$ and the apparent line width (width at one-half of the total height)

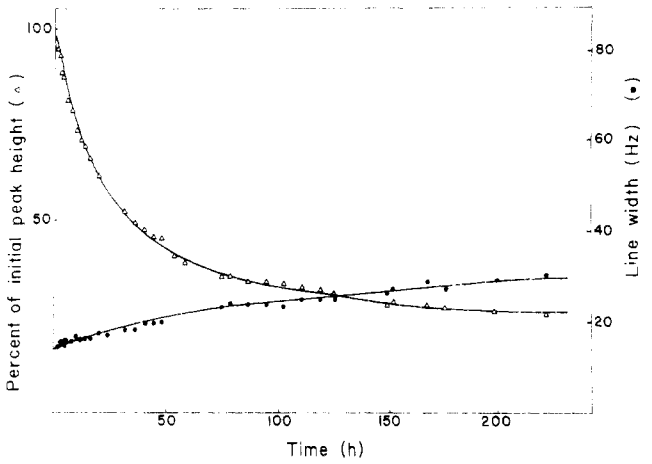

FIGURE 3: Plots of the time dependence of the relative peak height [$100h(t)/h(0)$; left ordinate] and the line width at one-half of the total height (right ordinate) of the ^{31}P NMR signal at 24 °C.

over the entire time course of transformation for a 40 mM sample are shown in Figure 3. Both the general appearance of the spectra at times greater than about 10 h and the fact that the decrease in the peak height is much more dramatic than the increase in the apparent line width are consistent with the idea that the small vesicles (line width of 14 Hz) are transformed into species that have a much broader line width (>100 Hz) with little material that has intermediate line widths and, by implication, intermediate sizes (C. F. Schmidt, unpublished results). It is important at this point to note that the total computer integrated intensity of the ^{31}P signal remained constant over the time course of the transformation, so that the broad line-width species can be identified with the transformed vesicles characterized by Schullery and co-workers as single-walled vesicles of about 700-Å diameter.[1]

The qualitative analysis described above can be put on a more quantitative basis, as follows: If an NMR resonance is the sum of two or more single lines with roughly equal integrals and identical chemical shifts, but line widths that differ by a factor of five or more, then the overall line width will be primarily determined by that of the sharp component. However, the line width measured at one-fourth or one-eighth of the total peak height will reflect the broad component to a much greater degree. Explicitly, for two Lorentzian lines, the line-shape function is

$$g(y) = \frac{f_S}{\pi^2}\left(\frac{x}{x^2 + y^2}\right) + \frac{f_B}{\pi^2}\left(\frac{ax}{a^2x^2 + y^2}\right) \qquad (1)$$

Here, x is the line width of the sharp component and $y = 2(\nu - \nu_0)$, where ν is the frequency in hertz, f_S and f_B are the fractions of the total integral present as sharp and broad components, respectively, such that $f_S + f_B = 1$, and a is the broad line width divided by the sharp component half-width.

[1] The ^{31}P line width characteristic of molecules in the transformed vesicles can be estimated from the composite spectra. In addition, the results of this study and that of Schullery et al. (1980) (Figure 3) indicate that there is a population of small vesicles remaining after several days of incubation that is similar to, and possibly identical with, the original vesicles. So the broad line-width estimate was confirmed by observing the ^{31}P signal from a vesicle preparation that had been size fractionated using Sepharose Cl-2B chromatography to remove residual small vesicles. The ^{31}P line width of these vesicles was 100 Hz at 24 °C. Despite the fact that the transformed vesicles are not as homogeneous as the small vesicles, the line shape, accessed by measuring the fourth and eighth widths, was within 10% of being Lorentzian. We thank Dr. P. Felgner for preparing these vesicles.

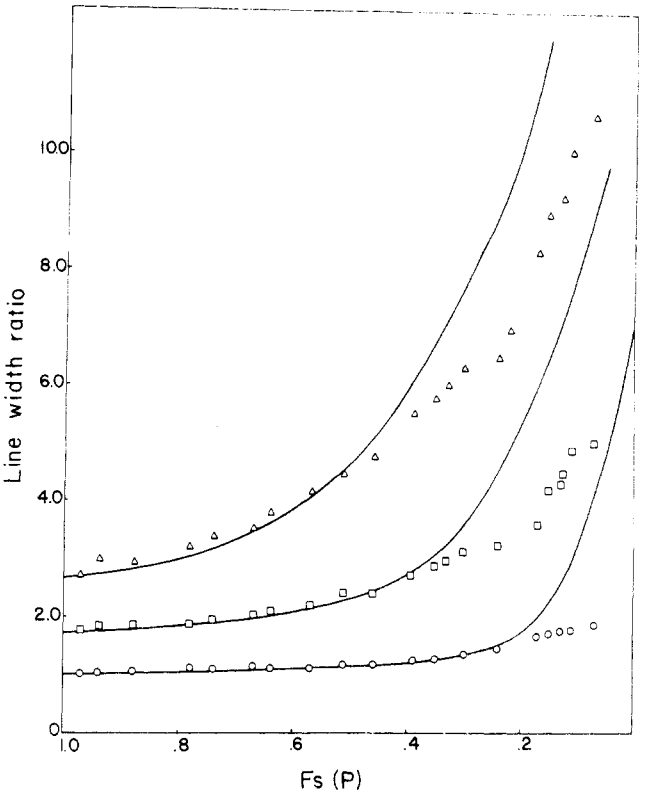

FIGURE 4: Solid lines are ^{31}P line-width ratios calculated by using eq 2 for the sum of a sharp line (14 Hz) and a broad line (100 Hz) as a function of the fraction of the total intensity present as the sharp component f_S. The line-width ratios are the widths at one-half (lower curve), one-fourth (middle curve), and one-eighth of the total composite peak height, divided by the sharp half-width. The data points are the line-width ratios measured from ^{31}P NMR spectra of the transforming vesicles at one-half (○), one-fourth (□), and one-eighth (△) of the total height as a function of the normalized peak height parameter P. The equivalence of f_S and P is discussed under Results.

For a given value of f_S, the new peak height, $g(y = 0)$, can be calculated, and y, the new line width, can be determined at $g(y) = {}^1/_2g(0)$, ${}^1/_4g(0)$, and ${}^1/_8g(0)$ as a multiple of x, the original sharp half-width. The solid lines in Figure 4 are the calculated, normalized to x, half-, quarter-, and eighth-widths for the sum of a sharp resonance and a line 7 times broader ($a = 7$)[1] as a function of the fraction of the sharp line. It is apparent from Figure 4 that the half-width of the composite line is not sensitive to the presence of the broad line until the broad line is ~90% of the signal, but that the eighth-width reflects the broad line at a much lower f_B. The theoretical curves can be compared with the experimental line-width ratios by defining a normalized peak height parameter, P, such that

$$P(t) = \frac{h(t) - h(\infty)}{h(0) - h(\infty)} \qquad (2)$$

Here, $h(\infty)$ is the extrapolated final peak height. For the sum of two Lorentzian lines, if $h(0) = 1$, then $h(\infty) = 1/a$, $h(t) = f_S + f_B/a$, and $P(t) = f_S$ from eq 2. For more than one broad component, it can be shown that $P = f_S$ only if the broad components have equal integrals, but that it is a good first approximation otherwise. The main conclusion that can be drawn from Figure 4, then, is that for about the first three quarters of the transformation the composite line shape can be described fairly well as the sum of the original narrow resonance (14 Hz) and a single broad (100-Hz)[1] line. This result justifies the assumptions made in eq 1 and 2. In order to fit the observed line-width ratios at longer times, it is necessary to invoke the presence of components with line

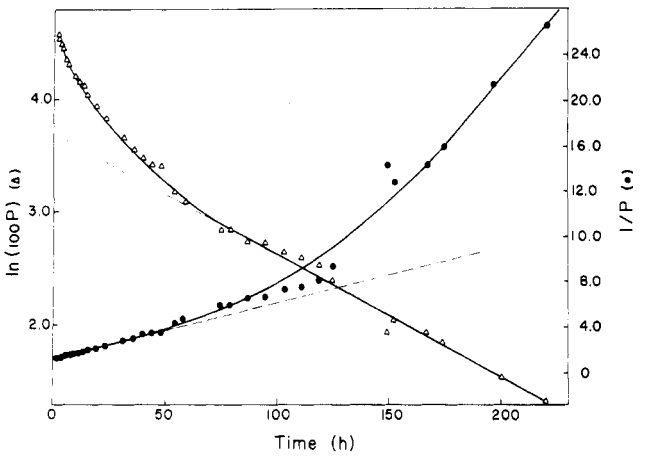

FIGURE 5: Plots of the time dependence of the natural logarithm (Δ; right oridinate) and the reciprocal (○; left ordinate) of the ^{31}P normalized peak height parameter P. The dashed lines indicate the linear portions of both curves.

widths intermediate between these two extremes. For example, the observed values for the eight-width ratio for $P < 0.3$ are in good agreement with those calculated for a single broad line with $a = 5$. This example also illustrates the sensitivity of the fitting procedure.

This analysis indicates the complexity of the dipalmitoylphosphatidylcholine vesicle size transformation. Further evidence for this can be seen in the kinetic plots of the normalized small vesicle peak height parameter P shown in Figure 5. For about the first 2 days of transformation ($P > 0.3$), the reciprocals of P, but not the logarithms, show a linear dependence on time, whereas after that the reverse appears to be the case and ln P has a roughly linear time dependence. However, any assertion about changes in the kinetics in this region is speculative, because P is no longer a well-defined parameter.

In order to further investigate the reaction order, as well as other variables which influence the small vesicle size transformation, we have used ^1H instead of ^{31}P NMR. The chief advantage of ^1H NMR is the higher sensitivity of protons, which allows lower concentrations to be observed in shorter periods of time. That it is not necessary to spin or decouple the samples also helps to reduce the number of instrumental variables. The principal disadvantage of using ^1H NMR for phospholipids below their phase transition is that the signals from the different proton resonances overlap. Only the composite peak height and line width, measured from the base line of a 10-kHz-wide spectrum, can be determined unambiguously, so that line-shape or kinetic analyses are not meaningful. As the transformation proceeds, the fatty acid methylene proton resonance apparently broadens beyond detection, while the choline methyl peak simply broadens. That this is the case has been previously suggested by Lichtenberg and co-workers (Lichtenberg et al., 1975; Figure 5) on the basis of continuous-wave 220-MHz spectra of different size vesicle preparations, and was confirmed here by following the transformation of sonicated vesicles prepared from $N(CD_3)_3$–dipalmitoylphosphatidylcholine. The spectra (not shown) for this sample at 23 °C show the loss with time of nearly all of the phospholipid ^1H signal, except for a small, relatively sharp peak, assigned to the choline NCH_2 proton resonance.

The time dependence of the composite proton parameters is similar to that observed for the phosphate ^{31}P resonance; i.e., the line width increases by about a factor of two in a roughly linear fashion, while the peak height decreases in an exponential manner by a factor of four or more. However,

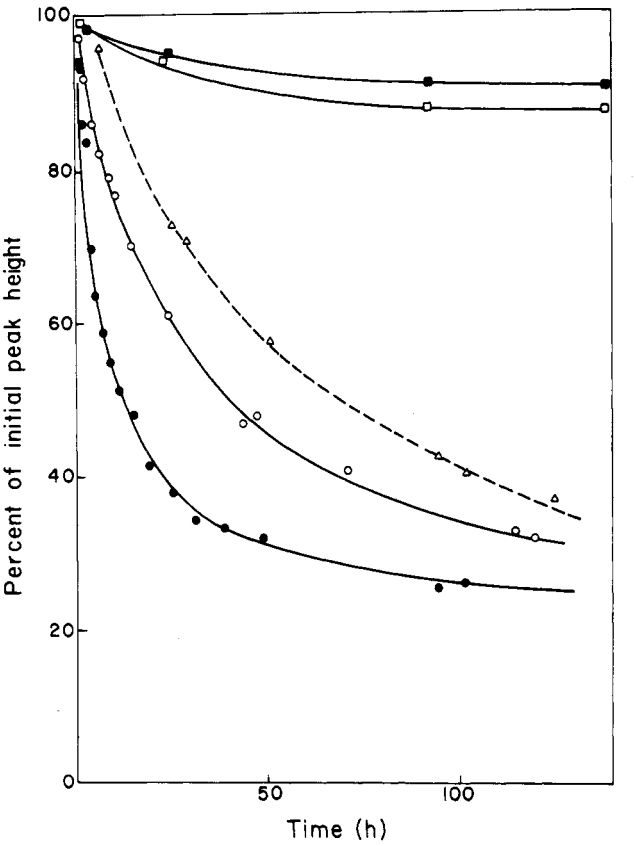

FIGURE 6: Comparison of the time dependence of the choline methyl proton relative peak height $[100h(t)/h(0)]$ or dipalmitoylphosphatidylcholine vesicles under the following conditions: (●) a 53 mM sample kept at room temperature; (○) a 13 mM sample prepared by dilution of the 53 mM sample and kept at room temperature; (Δ) an identical 13 mM sample that was heated to 45 °C for nine different 15-min periods during the first 25 h of incubation at room temperature, and two additional periods between 25 and 53 h. The sample was then kept at room temperature without heating: (□) a 13 mM sample which had 1.2 mM Pr^{3+} added at 45 °C and was then cooled to room temperature; (■) a 25 mM sample prepared with a French pressure cell.

a comparision between the time courses of the peak height parameter P for choline methyl protons, phosphate ^{31}P, and fatty acid methylene protons (for the deuterated phosphatidylcholine) for three different samples with concentrations near 40 mM (data not shown) indicates that the apparent transformation rate depends on both the nuclei observed and their position within the molecule. This could be due to different broadening mechanisms, or a differential effect of changing vesicle curvature on different motional time scales and amplitudes. However, the transformation rate also varied among different sonicated vesicle preparations. In some cases, this could be correlated with the initial average vesicle size (see below). In other cases, it could not. It seems likely that, in addition to the method of measurement, the absolute rate depends on the detailed history of a sample and its preparation.

The effect of several variables on the vesicle transformation time course for a single preparation, as monitored by using the relative choline methyl proton peak height, is shown in Figure 6. The rate of transformation can be made slower by (1) decreasing the phospholipid concentration, (2) adding Pr^{3+} to the external volume, or (3) increasing the temperature above the phase transition. In the Pr^{3+} experiment, it was also observed that the turbidity increase normally seen upon cooling to room temperature was greatly decreased. Both effects are presumably due to the electrostatic repulsion between vesicles created by the binding of the Pr^{3+} ions. In the temperature

experiment, the sample temperature was periodically increased to 45 °C for 15 min and then recooled to 23 °C (see Figure 6 legend). The turbidity of the dispersion decreased within a minute after reheating to values slightly higher than those observed before cooling. Upon being recooled, the sample became turbid again over a period of about 0.5 h, similar to the original sample (see, e.g., Figure 1). This sample, after 5 days at room temperature (except for the periods indicated), was spectroscopically identical with an aliquot kept at room temperature throughout. In another experiment, the transformation of two aliquots, one of which was taken to 23 °C within 0.5 h of sonication, the other which was kept at 45 °C for 24 h prior to cooling, was monitored and found to be identical (data not shown).

The effect of increasing the initial average size, as indicated by the initial out/in ratio of the vesicles, was also investigated. Vesicles prepared with a French pressure cell (Barenholz et al., 1979; Lichtenberg et al., 1981), with an initial out/in ratio of 1.6, transformed to a much smaller extent than sonicated vesicles (Figure 6). The extent of transformation of a preparation of vesicles with an initial out/in ratio of 1.8, due to incomplete sonication, was intermediate between these two extremes. In all three cases, the final out/in ratio was 1.3 ± 0.1. The effect of vesicle size on the transformation rate cannot be assessed adequately at this time, because of the small overall peak height changes.

Discussion

Schullery and co-workers (1980) showed that the slow transformation of small dipalmitoylphosphatidylcholine vesicles yields unilamellar vesicles of an average diameter of 700 Å at room temperature. Vesicles of this size require the combined phospholipid of 12–18 small vesicles. The ^{31}P line-shape analysis summarized in Figure 4 suggests a model in which the small vesicles transform into the product vesicles without producing a significant number of intermediate-size vesicles during the first 2 days of incubation. Such a model has previously been suggested to explain differential scanning calorimetry data (Suurkuusk et al., 1976). The temperature scanning NMR experiment shown in Figure 6 establishes an approximate correlation between the isothermal and the scanning modes of observation, so that the NMR and the calorimetric data can be compared. A more serious problem in interpreting these data is the relation of the measured parameters, both NMR line widths and transition temperatures and enthalpies, to the vesicle radius of curvature. We have recently shown that intermediate-sized unilamellar dipalmitoylphosphatidylcholine vesicles of about 600 Å average diameter can be prepared by the French press method (Lichtenberg et al., 1981). These vesicles exhibit a broad thermotropic transition centered between 37 and 41 °C, with heat-capacity contributions at both extremes. The ^{31}P NMR line width (35 Hz) of these vesicles at 24 °C is also intermediate between those of the sonicated and the fractionated transformed vesicles. Both of these results indicate that the intermediate-size vesicles are not present in large amounts in the transforming vesicle system. The functional form of the heat-capacity and line-width dependence on radius and the physical interpretations of these changes are the subject of ongoing studies.

Additional evidence for the "small to large" transformational model can be derived from the results of other types of experiments. Sedimentation velocity Schlieren patterns for distearoylphosphatidylcholine vesicles after 24 h of transformation at 40 °C, where the temperature relative to that of the transition is about the same as in these studies, and at 21 °C, showed essentially only two well-defined peaks, one for the original vesicles and one corresponding to considerably larger vesicles (Larrabee, 1979). Gel permeation chromatography using Sepharose CL-2B also showed a bimodal distribution of lipid phosphorus (Schullery et al., 1980). It should be noted that electron microscopy gives ambiguous results with regard to the question of two populations.

We believe that the data presented here allow a clearer distinction between the aggregation and size transformation of sonicated vesicles than has been made previously. Aggregation, as monitored by the turbidity change upon cooling below the transition, for the concentrations and ionic conditions used here, is a relatively rapid process and can be reversed by increasing the temperature. On the other hand, size transformation, as monitored by changes in the NMR spectra at 24 °C, is relatively slow for pure dipalmitoylphosphatidylcholine vesicles sonicated above T_m. But while these two processes take place on very different time scales, there are two experiments which support the hypothesis (Petersen & Chan, 1978) that aggregation is a necessary prerequisite of transformation. One is that Pr^{3+} ions, when added to the external volume, greatly reduce the extent of both aggregation and transformation. However, if the transformation proceeded independently of aggregation, e.g., via lipid transfer or collision of nonaggregated vesicles, then Pr^{3+} might also affect the rate of such a process. Stronger evidence is provided by the experiment in which the vesicles were periodically heated above the transition. The transformation rate is considerably slower than that predicted if only the time that the vesicles spent above the transition is important. If one includes an additional 0.5 h for each heating period, during which the vesicles are below the transition but are not transforming because they are not yet reaggregated, then there is good agreement between the predicted rate and that observed for an identical sample kept at 23 °C.

Using the analysis given above, and the kinetic results, it is possible to propose a tentative mechanism for transformation. The disappearance of the small vesicles, as followed by the ^{31}P peak height parameter P, shows apparent second-order kinetics for the first two-thirds of the transformation. This is in agreement with Larrabee's (1979) results for distearoylphosphatidylcholine vesicles, obtained by using the sedimentation velocity Schlieren pattern areas of the small vesicle peak. The concentration dependence of the transformation rate, measured by using ^1H NMR, provides additional evidence that first-order lipid transfer mechanisms (Martin & MacDonald, 1976) do not contribute significantly to the transformation in the rather high concentration dispersions used in these studies. However, the simplest fusion mechanism consistent with second-order kinetics (Lawaczeck, 1978) is not compatible with the small to large transformation model proposed here. With this mechanism, two small vesicles collide and fuse with a finite probability. Subsequent fusion of small vesicles with this product vesicle would have a somewhat lower probability, and so on until the probability approaches zero. This mechanism would be expected to produce significant amounts of intermediate-sized vesicles. Instead, we would suggest a modification of the activated vesicle model proposed by Kantor & Prestegard (1978) for fatty acid induced fusion, as follows:

$$S + S \xrightarrow{k_1} S_2^*$$

$$S_2^* + S \xrightarrow{k_2} S_3^*$$

$$\vdots \qquad\qquad \vdots$$

$$S_{n-1}^* + S \xrightarrow{k_{n-1}} S_n$$

where $n = 12–18$. Here, the production of the initial activated vesicles, S_2^*, is concurrent with the fusion of two small vesicles, S. As before, this step would have to be much slower than the subsequent steps, so that low steady-state concentrations of the activated vesicles, S_i^*, will be reached quickly. This mechanism gives apparent second-order kinetics. It is worth noting that after two vesicles fuse, if there is a tendency for the product vesicle to become spherical, then there would be a large mass imbalance between the outer and inner monolayers of the vesicle bilayer. This instability provides a physical mechanism for the activation process and subsequent propagation steps. In addition, it is tempting to speculate that the rapid propagation is related to the aggregation of the vesicles. That is, once transformation is initiated within an aggregate, it does not stop at intermediate-sized vesicles because of the proximity of other unfused vesicles within the closely packed aggregate. We note that implicit in this mechanism is the idea that any vesicle transformation involving fusion is driven by the packing strains induced because of the very small radius of curvature of the vesicle when the vesicle is taken from the liquid-crystalline state to the gel state (Lichtenberg & Schmidt, 1981). Termination of fusion in the gel state will then occur when the packing strains are relieved by the formation of fusion products with larger radii of curvature. The fact that the larger unilamellar vesicles produced by the French pressure cell method do not transform to any great extent provides support for this idea (Lichtenberg et al., 1981).

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