Fusion of Dimyristoyllecithin Vesicles as Studied by Proton Magnetic Resonance Spectroscopy†

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ABSTRACT: The rate of fusion of vesicles prepared from commercial dimyristoyllecithin has been studied as a function of temperature using proton magnetic resonance techniques. The results indicate that for concentrated solutions fusion of small vesicles takes place *via* a two-step process which leads to a bimodal distribution of larger vesicle sizes. The results also indicate that the rate of this fusion process increases dramatically near the hydrocarbon phase transition temperature of the vesicle. The significance of the results is discussed in terms of a possible means of preparing vesicles with a predetermined size distribution and in terms of a possible means of controlling the fusion process.

The physical properties of vesicular structures prepared from aqueous phospholipid dispersions by sonication have in recent years attracted much interest from the scientific community. The structures have been characterized as closed spherical shells of 200–300 Å diameter having a single bilayer sheet forming a continuous permeability barrier to substances trapped either inside or outside the sphere (Huang, 1969). They have been of interest mainly because a bilayer in this configuration serves as a convenient model for studying the structure and permeability properties of cell membranes (Papahadjopoulos *et al.*, 1972). The vesicle is, however, also attracting attention as an interesting model for cell–cell and vesicle–cell fusions which occur in natural systems. Recent work with *Acholeplasma laidlawii* has shown the feasibility of studying fusion of a vesicle with a viable single cell organism and has in addition pointed to the utility of the vesicle as a means of introducing exogenous substances to living systems under semicontrolled conditions (Grant and McConnell, 1973).

Especially in view of the vesicle's potential as a means of introducing extrinsic substances, it is essential to understand the precise conditions, if any, which can promote vesicle–vesicle or vesicle–cell fusion. In the hope of shedding some light on the nature of these conditions, we have examined the rate of vesicle–vesicle fusion for vesicles formed from commercial dimyristoyllecithin (DML)[1] as a function of temperature and have attempted to characterize the process whereby fusion occurs.

The well-characterized properties of lipid dispersions suggest a particular temperature range which would be of interest in this study. Most aqueous dispersions exhibit a transition from a low-temperature state which has hexagonally packed lipid hydrocarbon chains to a high-temperature state having semifluid hydrocarbon chains. Some recent work on the fusion of dipalmitoyllecithin vesicles has indicated fusion behavior to vary dramatically near the dipalmitoyl phase transition, ~42° (Taupin and McConnell, 1972). Similar behavior for DML near its phase transition, 20°, will be discussed here.

The choice of a homogeneous lipid and in particular DML for this study was dictated by its very well defined set of phase transitions and the fact that they occur in an easily accessible temperature range near 20° (Hinz and Sturtevant, 1972). One must be cautioned, however, in that lipids homogeneous in hydrocarbon chain length do not occur frequently in natural systems and for this reason the behavior of DML and other homogeneous lipids may be somewhat atypical of natural systems. Moreover, there is some evidence that the properties of homogeneous lipids are sensitive to low levels (below thin-layer chromatography (tlc) detection) of impurities (Colacicco, 1972) and that one or more impurity may be obligatory for rapid vesicle fusion. These possibilities should not detract, however, from any insight this study may offer into the fusion process.

The actual process of vesicle fusion which under circumstances described here results in formation of progressively larger structures can be easily followed by monitoring the proton magnetic resonance (pmr) spectrum of the system. The pmr spectra of lipid dispersions have been described by others (Horwitz, 1972; Sheetz and Chan, 1972), but briefly, a dispersion of 300-Å vesicles at a temperature above the hydrocarbon phase transition will have narrow well-resolved lines representing ~100% of the intensity expected on the basis of DML structure. As vesicle size increases, the observable lines broaden and lose intensity until, in the limit of extended bilayers or multilayers, little or no signal can be detected under high-resolution conditions. Thus, increase in line width and decrease in intensity can be used to analyze vesicle decay. The information obtained through analysis of these data should be useful in developing systems which will easily fuse with biological membranes. The analysis will also point to the possibility that physical restraints on the fusion process can be used to manipulate the average size of a transient population of vesicles and that these vesicles can be harvested to yield vesicle dispersions homogeneous in size useful in further studies of membrane structure.

Materials and Methods

Dimyristoyllecithin (1,2-dimyristoyl-*sn*-glycero-3-phosphorylcholine) was purchased from Calbiochem, San Diego, Calif. (lot no. 300018), and used without further purification. Samples were prepared to be 10% by weight in D_2O with 0.02% sodium azide added to retard microbial growth. Each

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[1] Abbreviation used is: DML, dimyristoyllecithin.

sample was sonicated under nitrogen at the lowest power level of a probe type sonicator (Branson Model S125 with microtip) for 5 min or until translucent. The temperature during this operation was held at 26° with an external water bath and sonication was carried out in 1–2-min intervals to minimize temperature differences between sample and bath. The vesicle solutions were then Millipore filtered (0.2-μ filter) and immediately placed in a controlled-temperature bath at the temperature desired for incubation.

Nuclear magnetic resonance (nmr) spectra were taken on a Varian A-60A spectrometer operating at an ambient temperature of 42°. Typically, samples were withdrawn from the incubation bath, transferred to the spectrometer, and run after a 2-min equilibration period. Intensities of the resulting spectra were recorded and scaled with respect to a standard manganese doped H_2O–D_2O sample run immediately following each set of lecithin samples. At 10% concentration measured intensities for most spectra are good to ±5%.

Analytical sieve chromatography on a Sepharose 2B column was used to determine initial and final vesicle size distributions. Approximately 0.8 ml of a 10% DML solution was diluted to 3 ml and applied along with a tyrosine total volume marker to a 27 × 2.6 cm column thermostated at 30°. Elution with 0.02% NaN_3 was carried out at a rate of 0.65 ml/min. Fractions were collected in 6-min intervals and analyzed by observing absorbance at 275 nm. The resulting elution profiles were corrected for nonideality (Huang and Charlton, 1972), and vesicle sizes were determined using the equation of Ackers (1967). The calibration constants used were determined for a previous but similar Sepharose column using nonenzymatic protein markers and an egg lecithin vesicle preparation of assumed size 240 Å.

Low angle X-ray diffraction and calorimetric measurements courtesy of D. M. Engelman and J. M. Sturtevant were used to characterize the final state of the decayed vesicles. X-Ray data were determined at temperatures both above and below the expected transition temperature directly on a 10% sample which had been incubated at 20° for a period of several days. For the calorimetric measurements a tenfold dilution of the sample was necessary to facilitate handling. The apparatus and techniques for both calorimetric and X-ray studies have been described previously (Hinz and Sturtevant, 1972; Engelman and Rothman, 1972).

It has been suggested that impurities present in commercial preparations or formed during sonication markedly effect vesicle fusion rate (M. Sheetz, private communication). We therefore subjected our samples to several procedures to test this assertion. A sample sonicated and incubated for a period in excess of 1 week was found to have less than 2% lysolecithin by tlc and to have more than 98% of its esterified fatty acids fully saturated and 14 carbons in length by glc. Variations in solution composition including substitution of H_2O for D_2O, the deletion of NaN_3, the addition of KCl, and the substitution of Tris buffer prior to sonication produced no qualitative change in the fusion results. However, treatment of a DML sample by silicic acid chromatography prior to dispersion, collecting only the mid 50% of the eluted DML, produced a sample which had an extremely low fusion rate at 20°. That this procedure has "purified" the sample is not absolutely certain since recombination of all fractions eluted does not restore fusion behavior and the recombinant sample does have detectable tlc impurities. The behavior nevertheless prompted us to investigate the effects of some probable contaminants one of which, myristic acid, when added to the silicic acid purified fraction restored fusion behavior. It thus seems possible that the commercial preparation contains a low level impurity necessary for fusion. Positive identification of an impurity at a level below normal tlc detection will require further study. The results presented here on the commercial DML preparation can, however, be reproduced to the extent that there is a high rate of fusion at 20° and a low rate above that temperature by adding <3% myristic acid to a silicic acid purified sample.

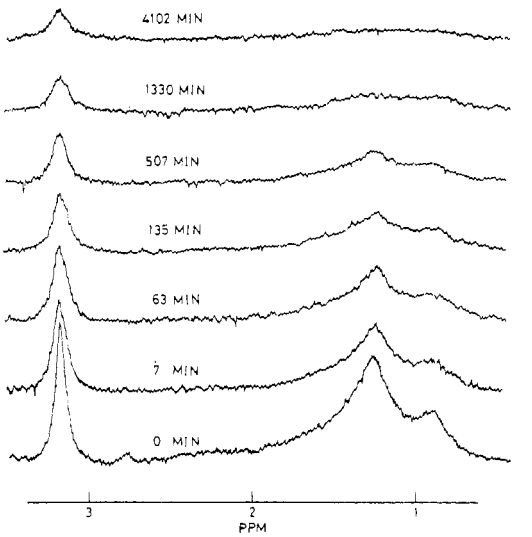

FIGURE 1: Pmr spectra of a 10% solution of DML incubated at 20.0° and removed periodically to record the spectra.

Results

A freshly sonicated 10% solution of commercial DML is a translucent preparation of very low viscosity. In an uncontrolled laboratory environment (*i.e.*, temperatures ranging from 20 to 28°) departure from this state is frequently observed in that the sample becomes more opalescent and more viscous, apparently as a function of time. Examination of pmr spectra of such a sample confirms the fact that a very substantial change in the nature of the vesicle suspension has taken place.

Intensity Measurements. The most apparent change in the pmr spectrum is a loss in the integrated intensity of the resolvable resonances. This behavior as observed during incubation at 20° is illustrated in Figure 1. The initial spectrum shows that the relative intensities of the three well-resolved peaks are not far from those expected on the basis of the structure of DML. For the choline methyl (3.2 ppm), the methylene (1.3 ppm), and the terminal methyl (0.9 ppm) relative intensities are approximately 1:3.0:0.6. This is the expected appearance for a spectrum of small diameter vesicles in suspension (Sheetz and Chan, 1972). As time progresses, intensities of the resolved peaks drop in favor of some unresolved components in the base line, with the methylene having a slightly greater rate of decrease. The intensities seem to reach a limiting value after several hours which is about 25% of the original. The final intensity ratios are difficult to measure given the quality of the data, but they do not differ greatly from the initial ratios. Without substantial changes in intensity ratios the residual intensity could be interpreted either as failure of 25% of the original vesicles to collapse to a larger structure, or as the actual spectrum of the large structure characteristic of the final state.

The functional dependence of the intensity drop with time is of some interest in that a simple vesicle–vesicle fusion might

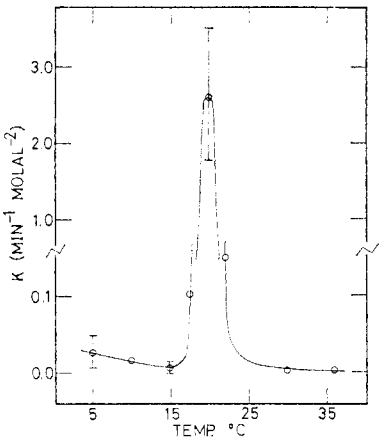

FIGURE 2: Third-order rate constants determined on 10% DML samples.

be expected to follow second-order kinetics and intensity should drop as the reciprocal of time. Although it is difficult to quantitate the data in Figure 1 because of the rather arbitrary division of intensity into resolvable and unresolved components, it is still clear that the data do not show a $1/t$ dependence but, instead, display a dependence characteristic of an order of reaction greater than two. Using the data on choline methyl intensity (those data are least sensitive to small changes in vesicle size) and assuming the residual choline intensity of 30% to be characteristic of the product, best fit is in fact obtained with a third-order equation. The correlation coefficient for this fit is 0.99 as compared to 0.96 for fit to a second-order equation. Inclusion of data on other peaks or assuming the intensity of the final state to be less than 30% tends to make the apparent order even higher.

Rate constants obtained by fitting data to a third-order rate law provide a convenient means of making quantitative comparison of rates under different environmental conditions. As one might expect, rates of reaction vary most drastically in the hydrocarbon phase transition region (*ca.* 20°). The third-order rate constants obtained from data taken at temperatures near 20° and controlled to ±0.5° are summarized in Figure 2. At low temperatures rates of decay are low, half-times being on the order of several days. At and near 20° the rates increase by more than two orders of magnitude, half-times reaching the order of 30 min; above 25° rates are unusually slow, half-times being much greater than 1 week. The increase in rate near 20° is quite dramatic; so much so that the apparent decay as a function of time of a sample in an uncontrolled laboratory environment is often more correctly described as decay as a function of the number of room temperature fluctuations which bring sample temperatures into a narrow rapid decay region about 20°.

The rates illustrated in Figure 2 are not highly reproducible and error bars in the figure show the range of values obtained when several runs at the same temperature were made. Errors in absolute rate could result from some variation in initial sample condition, possibly fatty acid content. To provide some assurance of reliability and for comparative purposes all runs were made in conjunction with a run on part of the sample held at 20°.

Low-temperature samples pose a special problem in that they must be brought through the rapid decay region to a higher temperature in order to observe their pmr spectrum. During the time spent in this region some decomposition will naturally occur. To minimize these contributions to decay in the 10° sample, a fresh aliquot was withdrawn from a reservoir

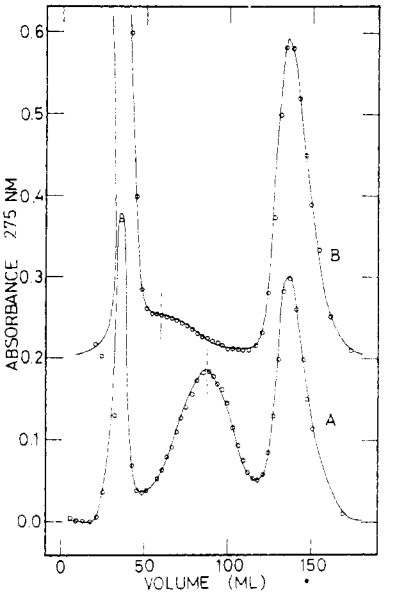

FIGURE 3: Elution profiles of DML samples on a Sepharose 2B column: (A) DML vesicles maintained above 25°; (B) DML vesicles incubated 10 min at 20°.

held at the incubation temperature for each pmr measurement. In this and other low-temperature runs an attempt was also made to correct for decomposition during measurement using the 20° rate constant and an estimate of time spent in the transition region.

Line-Width Measurements. In addition to changes in line intensities, line widths are observed to change as a function of time. Their changes are small but even more abrupt than those just discussed. Using the choline peak as an example, the initial width of 4.5 Hz increases rapidly to a value of 6.0 Hz in the first 10 min, then suffers a very slow rate of increase over the several hours that intensity decays. The width during this second period never exceeds a value of more than twice the original line width. Again the initial width is characteristic of a collection of small vesicles. The increase in width which occurs in the first few minutes suggests an initial phase of reaction in which particle size increases slightly. The period that follows, in which line width increases but does not broaden to extinction as it loses intensity, suggests a slow conversion of the slightly enlarged vesicles to an extensive form having a very broad undetectable spectrum.

Analytical Sieve Chromatography Results. The suggested change in particle size which occurs in the first 10 min can be examined further by subjecting a sample to analytical sieve chromatography on a Sepharose 2B column. The results are shown in Figure 3. Profile A is from a run on a freshly sonicated preparation which was not allowed to drop below 25°. It shows three peaks: the peak at 50 ml is from a few particles of size beyond the exclusion limit of the column and marks the void volume; the peak at 136 ml is from a tyrosine marker which indicates total volume; and the third broad, but well-resolved, peak represents the majority of the lecithin vesicles. The elution volume of the broad peak, 86 ml, indicates that the vesicles have an average size of 290 Å. Profile B is the result of a run on a sample incubated at 20° for 10 min before filtering and applying it to the column. It is obvious that few 290 Å diameter particles remain and that there is a substantial increase in the fraction of particles in excess of 1000 Å diameter (the approximate exclusion limit of the column). There is also a peak at 60 ml indicating a vesicle population of 500 Å diameter. At first glance this peak appears insignificant compared

to the large particle peak. However, if one considers the fact that large particles scatter considerably more light, the absorbance of the 500-Å peak is more compatible with the percentage of particles one would expect to remain in vesicular form, given the rate constants in Figure 2. Thus gel filtration seems to confirm an initial small increase in vesicle size (to something around 500 Å diameter) accompanied or followed by a collapse to a much larger structure (to something in excess of 1000 Å diameter).

Calorimetric and X-Ray Measurements. Calorimetric and X-ray diffraction data indicate that the large structures formed on prolonged incubation at 20° are not the typical multilayer or myelin forms produced when unsonicated lecithin is dispersed in water.

The calorimetric data on 10% decayed samples range from runs showing near-normal transition temperatures and enthalpies (23.8° and 5 kcal/mol, respectively) but anomalously broad transitions (1.4°) to runs which are anomalous in all the parameters.

The X-ray diffraction patterns of a 10% sample are also atypical in that even at a temperature below the phase transition temperature, no evidence of the sharp low angle Bragg reflections found in lamellar phases (Levine, 1972) is found. The X-ray patterns do, however, show three broad low angle bands with spacing and intensity variation characteristic of the electron density profile of a bilayer (Engelman, 1971). The pattern also shows a pair of sharp reflections at higher angles indicative of a highly ordered hydrocarbon phase. These reflections broaden and move to lower angles when the sample is raised above the phase transition temperature. These data, both calorimetric and X-ray, therefore point toward an ultimate decay of the initial vesicle preparation to a state having extended bilayer structures in some nonrepeating array. An extremely large vesicular structure is a possibility.

Discussion

Temperature Dependence of Reaction Rate. Perhaps the most significant aspect of the data presented above is the dramatic variation of reaction rate with temperature. An understanding of this variation is significant in that it offers a potential means of controlling the time or compositional course of vesicle–vesicle or vesicle–cell fusion at least for commercial preparations. We have repeatedly stated that the dramatic increase in rate occurs near the hydrocarbon phase transition. For multilayers and presumably extended bilayers this transition temperature in DML is 23.7°. Closer examination shows that the rate does not reach maximum exactly at this transition temperature, but some 4 or 5° lower, at 19–20°.

That the maximum in fusion rate occurs near 19° is intriguing since this temperature lies midway between the pretransition temperature (13.5°) attributed by Ladbrooke and Chapman (1969) to head group melting and the main transition temperature (23.7°) associated with hydrocarbon melting of an extended bilayer system. This occurrence is appealing in that a well-defined active form exists: a vesicle with melted head groups and frozen hydrocarbons chains. There are, however, several facts which suggest that this correlation is not a meaningful one. First, the reaction does not proceed with an appreciable rate at 15°, a temperature well within the pretransition–main transition region. And second, since we are dealing with an irreversible vesicle fusion, there is really no reason to expect any correlation with properties more appropriate to the final extended bilayer state. There is in fact evidence for the fact that the pre- and main transition temperatures cited are inappropriate for vesicles (J. M. Sturtevant, private communication).

Our vesicle preparation, for example, shows only transitions at 24 and 19°. The significance of the fact that our fusion rate maximum occurs at the 19° transition will require further study.

Variation of Vesicle Size Distribution. Another interesting aspect of the data is the apparent variation of vesicle size with time. Both pmr line-width data and gel filtration data indicate that the increase in size does not occur in a continuous manner. Pmr data show an almost immediate increase of 25% in line width with a further increase of something less than 75% over a very long time period. Published spectra of vesicles having different sizes indicate that line width should be primarily dependent on vesicle size and in particular not dependent on solution viscosity (Sheetz and Chan, 1972). Examination of the spectra published by Sheetz and Chan indicate that a broadening of the choline peak by a factor of 2 would correspond to an increase of vesicle size to something on the order of 900 Å. Our data therefore show a small increase in vesicle size to occur in the first phase of the decomposition but fail to show an appreciable population of vesicles in the 1000-Å range at any point in the fusion process. It is possible that a distorted vesicle in the 1000-Å range would have an anomalous spectrum with both broad and sharp components, but this possibility seems unlikely in view of the rapid diffusion rates for lecithin molecules within bilayers (Devaux and McConnell, 1972) and probable averaging of nmr parameters on this time scale. It thus seems likely that decay in the second phase proceeds directly to a size much greater than 1000 Å.

Gel filtration studies confirm this hypothesis showing a bimodal distribution after 10-min incubation with one maximum near 500 Å diameter and the other maximum at a size in excess of the exclusion limit of the column, 1000 Å.

The reason for the two-phase reaction is not obvious, but the limiting size of the small vesicle population does suggest a possibility. The solutions we are working with are 10% lecithin by weight. Assuming an initial state having 300 Å, diameter spherical vesicles and 65 $Å^2$ per lecithin molecule (Stoeckenius and Engelman, 1969) a calculated 37% of the total solution volume would be occupied by vesicles. If the size grew to 725 Å, 100% would be occupied. The 725 Å size is likely to be an overestimate since the area per lecithin molecule in a vesicle is probably larger than the 65-$Å^2$ value obtained for a multilayer system. Thus the 500-Å particle observed may well represent the limit in which spherical vesicles become close packed. If there is a strong tendency for vesicles of intermediate size to remain spherical the relation of the close-packing limit to a restriction on growth is obvious.

The fact that longer decay periods produce structures of size much in excess of 1000 Å diameter rather than structures of intermediate size can be rationalized if beyond a certain size, vesicles can be highly convoluted structures which are more conservative of solution volume. The formation of highly convoluted forms may also explain the increase in solution viscosity.

Reaction Order. A straightforward interpretation of our observed high order of reaction is prohibited by the complexity of the DML vesicle system. First, there is uncertainty about the role of fatty acid or other possible impurities. Concentrations of impurities, if required for reaction, may not vary in direct proportion to the number of remaining vesicles and thus may lead to a reaction order unrelated to reaction molecularity. Second, there is no accepted formalism for interpreting reaction order in terms of molecularity for a

system in which reacting species can be approximated by close-packed spheres. Collisions between reactive species in such a system will not have the concentration dependence predicted on a simple diffusion model and apparent order of reaction will be skewed toward higher values. Third, there is no guarantee that vesicles are homogeneous in size. A higher reaction rate for the smaller fraction could lead to a high reaction order.

If these complications play a minor role, however, the apparent reaction order is still consistent with phase two of our reaction process. As we have analyzed our decay in terms of intensity of the choline peak, and this peak shows little change in apparent intensity with vesicle size in the 300–1000-Å range (Sheetz and Chan, 1972), we detect only the final collapse to structures in excess of 2000 Å. At the close-packed limit formation of these structures may well require simultaneous collapse of several vesicles thus yielding a high reaction order. Any distinction between this explanation and those mentioned above will require extensive study.

Implications for Further Studies. Despite the uncertainties in the description of mechanism, the results do provide data useful in future studies of vesicle–vesicle fusion or in single vesicle phenomena. In regard to single vesicle properties it would be extremely valuable to have a means of preparing vesicles of a predetermined size. Neither sonication nor methods involving swelling of films (Reeves and Dowben, 1969) or protrusion in ethanol solution (Batzri and Korn, 1973) provide a convenient means of regulating vesicle size. The apparent two-phase vesicle decay, *i.e.*, rapid growth to a close-packed sphere limit followed by a slower decay to an extended bilayer structure, on the other hand, provides a potential method of synthesizing a DML vesicle distribution of desired average size. If our hypothesis concerning the reason for a two-phase decay is correct, the size could be predetermined by the initial solution concentration *via* the close-packed sphere limit. A short incubation period would produce a bimodal distribution rich in a vesicle of closed-packed size, and purification could be reduced to separating this population from a group of structures which have a size beyond the exclusion limit of a Sepharose 2B column.

In regard to vesicle–vesicle and cell–vesicle fusion means of controlling the time or compositional course of the fusion process would be very valuable. The abrupt increase in rate of fusion at a lipid phase transition suggests that temperature and choice of vesicle lipid may be one way of controlling this process. The apparent sensitivity to impurities suggests another means deserving of further study.

Acknowledgments

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