Fusion of Phosphatidylcholine Bilayer Vesicles: Role of Free Fatty Acid†

Howard L. Kantor and James H. Prestegard*

ABSTRACT: The transformation of DMPC vesicles containing myristic acid to larger structures was examined by nuclear magnetic resonance techniques. The process was found to be characterized by a rate which is approximately first order in vesicle concentration. Rates, however, show a dramatic increase with increasing myristic acid content. The fatty acid is characterized as being active under conditions in which it is neutral and dispersed in the bulk bilayer phase. In addition a 1:1 correlation was noted between leakage of contents and vesicle transformation. On the basis of these observations a mechanism is postulated involving stabilization of leaks in bilayer membranes by myristic acid, and the resultant formation of activated sites for vesicle transformation. The relation to fusion in natural systems is discussed.

The transformation of synthetic lipid bilayer membranes from small vesicular structures to larger more extended structures has generated much interest because of its possible analogy to membrane fusions of importance in both molecular biology and medicine (Huang & Pagano, 1975; Poste & Allison, 1973; Poste & Papahadjopoulos, 1976; Gregoriadis et al., 1976). Some time ago we had investigated the transformation to more extended structures of one such synthetic system, dimyristoylphosphatidylcholine (DMPC)[1] vesicles containing small amounts of fatty acids. In a paper reporting results of that investigation, the necessity of having fatty acid present and the necessity of operating at or near the gel-to-liquid crystalline phase transition temperature was emphasized (Kantor & Prestegard, 1975). We hypothesized that lateral separation of a fatty acid rich phase may be a prerequisite for bilayer transformation. A hypothesis based on phase transition phenomena was deemed reasonable in view of suggestions linking lateral phase separation to fusion in other model systems (Papahadjopoulos et al., 1974, 1976a,b; Lee & Chan, 1977; Lau & Chan, 1974), and implication of a fatty acid rich phase seemed reasonable in view of proposed roles for other minor membrane components with detergent-like properties (Ahkong et al., 1974).

We nevertheless felt compelled to pursue experiments directed at testing this hypothesis. Experiments employing differential scanning calorimetry (DSC) to examine phase behavior of mixed DMPC–myristic acid preparations and nuclear magnetic resonance (NMR) experiments to characterize the distribution and physical state of myristic acid in the mixed systems are reported here.

Since that earlier publication, it has also become clear that mere transformation of lipid bilayer structures does not necessarily imply fusion (Martin & McDonald, 1976; Papahadjopoulos et al., 1976a,b). Transfer of lipid may occur by simple diffusion of individual molecules to a new structure or lipid bilayers may break and merge with other structures, but without concomitant transfer of internal contents to the newly formed entity. Both merging of bilayers and efficient retention of contents should exist in good models for biological fusion. We therefore attempt to further characterize the mechanism for transformation in the DMPC–myristic acid system through kinetic studies and studies of solute retention during transformation.

Materials and Methods

Dimyristoylphosphatidylcholine (1,2-dimyristoyl-*sn*-glycero-3-phosphorylcholine) lot no. 400488 was purchased from Calbiochem, San Diego, Calif., and was purified by alumina and silicic acid chromatography as previously described (Kantor & Prestegard, 1975). Carbon-12 myristic acid was purchased from Matheson Coleman and Bell. Carbon-13 myristic acid (MA), enriched at the carboxyl group to ~90%, was synthesized from unlabeled myristic acid using a modified Hundsdieker reaction (Cason & Walbo, 1972) followed by $^{13}CO_2$ addition to a Grignard reagent. ^2H_2O buffer for all experiments except those related to leakage of contents or changes in rate with pD consisted of 0.01 M PO_4^{3-} prepared from sodium salts and titrated to pD 7.3. NaN_3 (0.02%) was added.

Lipid mixtures were prepared in chloroform solution and dried for >10 h under vacuum at room temperature. Vesicle samples were then produced by vortex mixing the lipids with ^2H_2O buffer and bath sonicating as previously described (Kantor et al., 1977). Samples for experiments to examine the fate of vesicle contents were prepared in phosphate buffer (as described above) containing 0.5 M tetramethylammonium chloride then eluted from a Sephadex G-50 column which had been equilibrated with phosphate buffer containing 0.5 M KCl. In all cases >95% of external tetramethylammonium (TMA) was removed by this treatment.

Examination of samples as a function of temperature was accomplished by incubating in constant temperature baths for periods terminated by raising the temperature to 35–40 °C, where samples have been shown not to decay appreciably.

As the free fatty acid is ionizable, experiments were conducted at a variety of pDs. Measurements were made with a glass electrode and pD was calculated by adding 0.4 unit to the reading as described by Wang & Copeland (1973). Potassium phthalate and sodium bicarbonate buffers were used for pD <5 and ≥10, respectively.

Proton NMR spectra were used to quantitate the extent of

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[1] Abbreviations used: DMPC, dimyristoylphosphatidylcholine; PC, phosphatidylcholine; MA, myristic acid; TMA, tetramethylammonium ion; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance.

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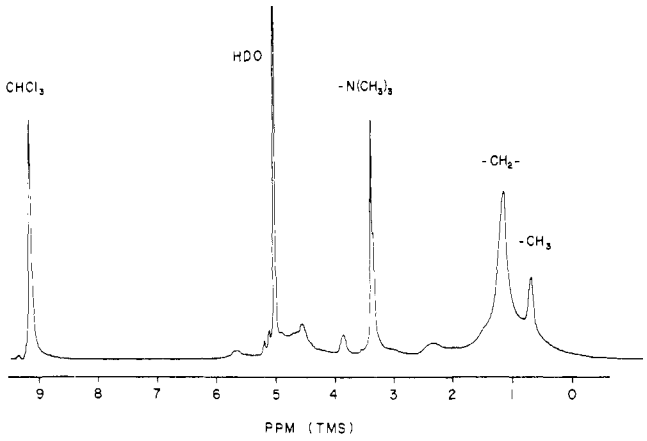

FIGURE 1: ^1H NMR spectrum of 3.3 wt % DMPC vesicles in D_2O buffer at 270 MHz.

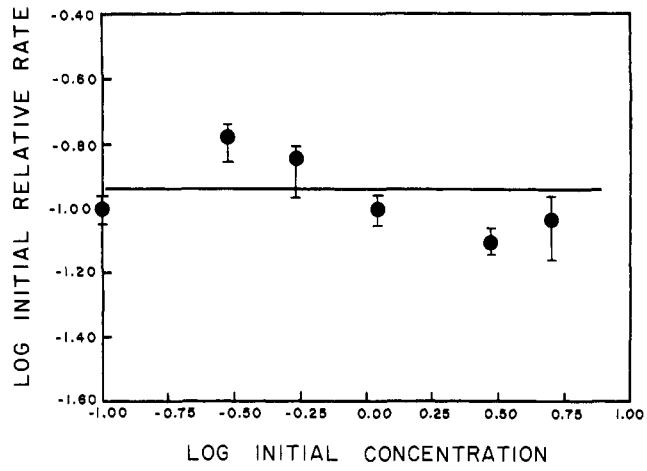

FIGURE 2: Relative initial rates of vesicle decay vs. initial DMPC concentration; 8.2 mol % MA in DMPC at 20 °C, pD 7.3. Error bars represent maximum and minimum rates based on errors in peak area determination.

vesicle transformation. These were obtained on a Bruker HX-270 spectrometer in the Fourier transform mode with the probe temperature maintained at 35 °C. Between 25 and 1000 scans were collected depending on the concentration of the sample. Intensities of spectra were standarized against a sealed coaxial tube containing 98% deuterated chloroform and chromium acetylacetonate.

^{13}C spectra were used to characterize the extent of fatty acid ionization in our preparations. Spectra were obtained at 67.9 MHz with proton decoupling. A minimum of 1000 scans were collected for each spectrum.

^{13}C-labeled myristic acid also proved a convenient label for analyzing the fatty acid distribution between vesicle and solution phases. Ultrafiltration using Millipore PSED Pellicon membranes in a 25-mm filtration cell enables separation of fractions. Vesicle samples were prepared in phosphate buffer similar to that previously described but at pD 10.0 ± 0.2. Filtration was done at 50 psi and 30 °C with continuous stirring and with at least 5 volume replacements after each reduction of volume to one-half the initial. Separation on a Sephadex G-50 column at 12 °C was also employed. A chloroform extract was prepared from both filtrate and residue. ^{13}C NMR spectra of these samples were compared with the spectra of the initial lipid mixture in ^2H_2O and chloroform.

Calorimetric experiments were used to investigate possible phase separations in the mixed lipid preparation. The differential scanning calorimeter used for these experiments was designed by Privalov (Privalov et al., 1975). The procedures used have been described previously (Mabrey & Sturtevant, 1977). In order to specify onset and completion temperature for the phase diagram, the sharply ascending and descending portions of the curves were extrapolated back to baseline.

Results

Order of Reaction. We have previously shown that the behavior of the DMPC–MA system can be described as a redistribution of lipid between small vesicles and structures with more extended bilayers. At this time we would like to present a more thorough set of data on the kinetics of this process. The decrease in small vesicle population can be monitored using the intensity of the narrow residual lines in the choline region of the ^1H NMR spectrum at 3.3 ppm from Me_4Si as seen in Figure 1. The broad choline resonance from more extended structures is assumed to contribute only in the form of a rise in apparent baseline. Variations in choline methyl intensity are paralleled by changes in fatty ester methylene and terminal methyl peaks. Intensities of methylene resonances are affected less by contributions from extended structures and have been applied as a monitor of structural change in some experiments.

The time course of vesicle transformation is known to be complex, having a very rapid initial rate and a much slower rate at long times. Since it is possible that more than one process is involved, and since rapid fusion processes are potentially more useful as models for biological fusion, we will concentrate on initial rates in our analysis. As in our previous work, transformation is only rapid near the phase transition (20 °C) in the presence of fatty acid. Only data *under* these conditions will be considered.

An order of reaction can be extracted from a plot of relative initial rates vs. initial concentration of small vesicles. We have determined initial rates from decreases in NMR intensity using the first two to three points obtained as a function of incubation time at 20 °C. It is important to note that, when the relative initial rate is derived from fractional changes in concentration with time, a line of zero slope indicates the presence of a first-order process. Figure 2, which shows data for an 8.2 mol % myristic acid sample, demonstrates a reaction order of 1.0 ± 0.3.

An attempt was made to construct such a plot for a 13.9 mol % sample. Results indicate an order of reaction less than 2; however, transformation is so rapid at this fatty acid level that the second measured point is near completion of the rapid transformation phase. In the limit when only a single nonzero-time measurement is possible, initial rates become unreliable and insensitive to changes in relative concentration regardless of reaction order. At 8.2 mol % fatty acid the second point is in all cases at 50% or less completion of the rapid phase and a reaction order can be detected with confidence. Any proposed transformation mechanism must be consistent with this observed order.

Leakage of Contents. We have postulated that classical fusion entails the retention of vesicle contents in the fused product. An investigation of the fate of vesicle contents in the DMPC system was initiated by examining the leakage of a large impermeable ion, TMA, during vesicle transformation.

A series of spectra from such an experiment is shown in Figure 3. Note that in the initial spectrum (A) the resonance from TMA inside the vesicles (3.13 ppm) is shifted upfield from that of external TMA. The assignment of these peaks was corroborated by the addition of $Fe(CN)_6^{3-}$ (spectrum B).

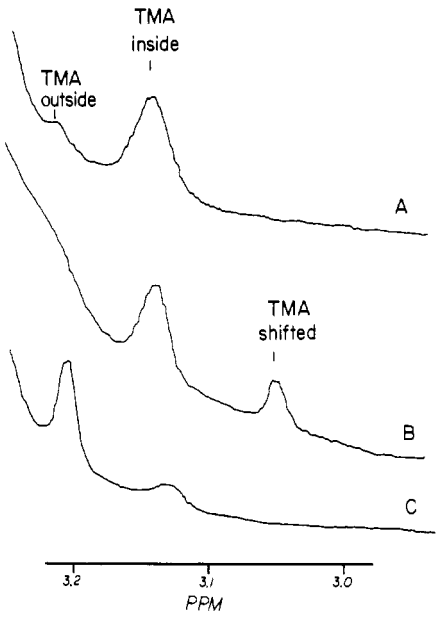

FIGURE 3: Spectra of TMA showing leakage during vesicle transformation. (A) MA (8.2 mol %) in DMPC vesicles prepared in TMA containing D_2O buffer and eluted from a Sephadex G-50 column. (B) Aliquot of sample A with 20 mM $Fe(CN)_6^{3-}$ shift reagent. (C) Sample A incubated at 20 °C for 90 min.

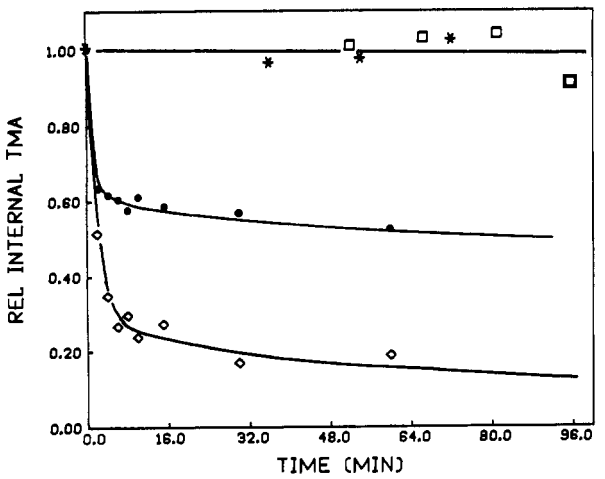

FIGURE 4: Loss of TMA from vesicles as a function of time at 13 (*), 20 (◊), and 30 °C (□) for DMPC-myristic acid vesicles. (●) Corresponds to relative vesicle concentration after incubation at 20 °C. Samples were 2% (w/v) lipid composed of DMPC and 9 mol % myristic acid.

DMPC vesicles are impermeable to this shift reagent, which therefore interacts solely with external TMA shifting the peak at 3.21 ppm upfield. When the vesicle system containing 8.2 mol % myristic acid is incubated at 20 °C, a decrease in the internal TMA peak is noted, as illustrated in spectrum C. This loss represents a substantial increase in permeability as compared with the sample incubated at 13 and 30 °C as shown in Figure 4.

Maximum leakage at 20 °C for fatty acid containing vesicles suggests a direct association with vesicle transformation. Figure 4 also illustrates the decrease in small vesicle population as determined by NMR intensity as a function of time. Note the qualitative similarity in time dependencies for leakage and transformation.

Because leakage of ions at lipid phase transitions in nonfusing systems has been reported (Marsh et al., 1976; Inoue, 1974; Blok et al., 1976), it is important to compare leakage

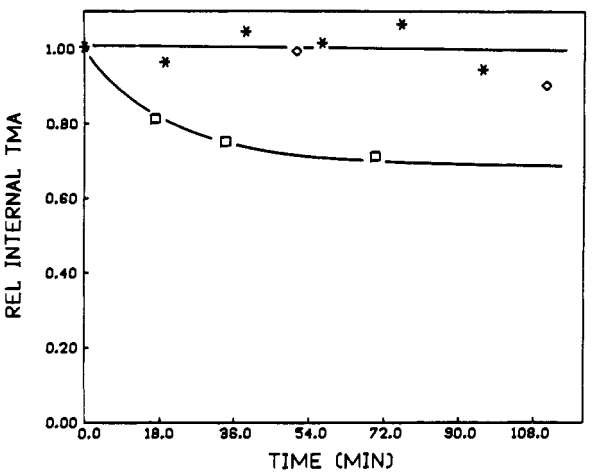

FIGURE 5: Internal TMA vs. time for DMPC vesicles incubated at 13 (*), 20 (□), and 30 °C (◊). Samples were 2% (w/v) DMPC.

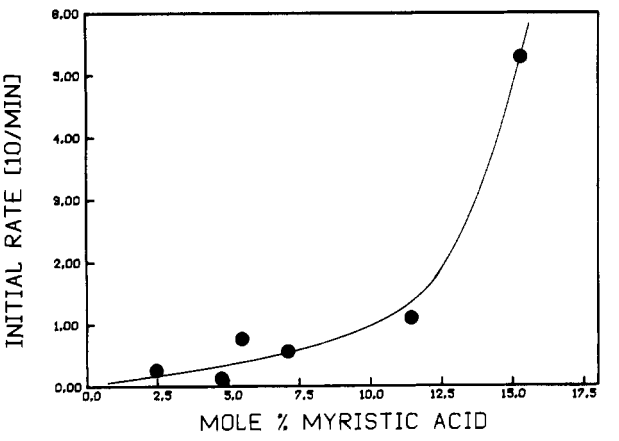

FIGURE 6: Initial relative rate vs. mol % MA; 0.4 wt % lipid, 20 °C.

rates in fatty acid containing DMPC vesicles with those in pure DMPC vesicles, which are known not to fuse appreciably. Data for pure vesicles was obtained at 13, 20, and 30 °C. A plot of the results over a 2-h period, as seen in Figure 5, illustrates negligible TMA leakage at 13 and 30 °C, but a considerable leakage at 20 °C. At longer times (10 h) total leakage at 20 and 30 °C becomes comparable. Note that at short times both the pure and the fatty acid containing samples show maximum permeability at 20 °C. If the data in Figure 4 is corrected for leakage occurring independently of transformation, by assuming comparable permeability for unfused vesicles with and without free fatty acid, an approximate 1:1 correlation of leakage and vesicle transformation is observed.

It is also important to note that, when experiments on pure systems were conducted at 20 °C in the presence of $Fe(CN)_6^{3-}$, the internal TMA peak simply decreased in area, rather than displaying a change in chemical shift as a function of time. A likely explanation for the absence of a shift is that at this critical temperature vesicles form breaks of sufficient lifetime and size to completely equilibrate internal and external environments. If, at 20 °C, bilayers remained intact, but simply became more permeable to molecules in general, $Fe(CN)_6^{3-}$ would slowly diffuse into the vesicle as TMA diffused out. Such an increase in internal $Fe(CN)_6^{3-}$ concentration would result in a time dependent change in chemical shift of the TMA resonance.

Fatty Acid Dependence. Due to the profound effect of fatty acid on the rates of vesicle transformation, it is clear that we must give this minor but important constituent further con-

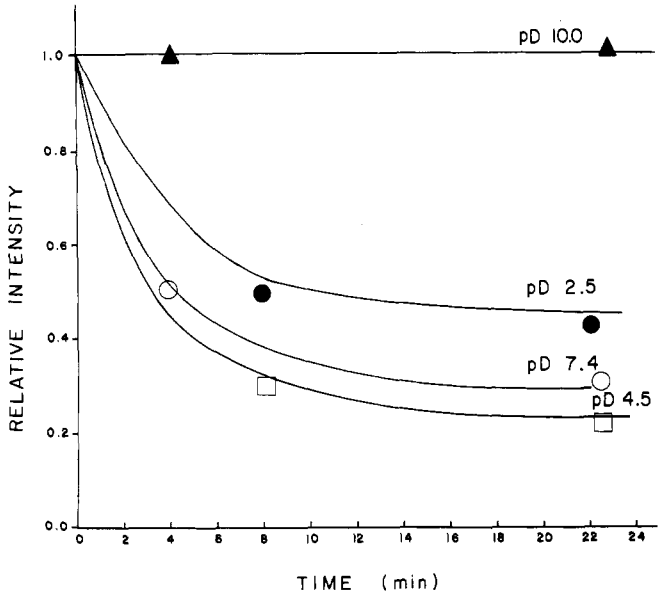

FIGURE 7: Vesicle concentration vs. time at several pD values. MA (10.9 mol %) in DMPC, 1.3 wt % lipid, 20 °C.

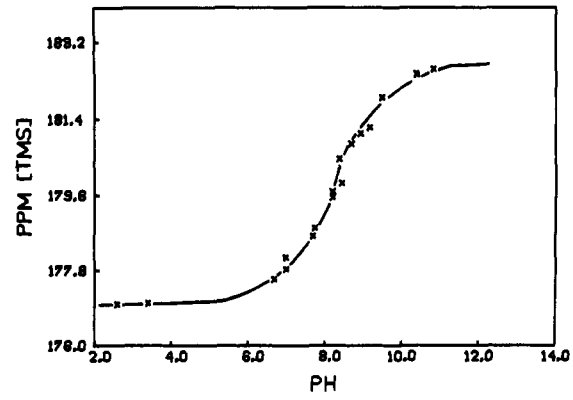

FIGURE 8: ^{13}C chemical shift of carbonyl in myristic acid; egg PC vesicles as a function of pH. 7.1% (w/v) lipid containing 16 mol % [1-^{13}C]myristic acid in D_2O without buffer. Change in ionic strength did not significantly alter the pK.

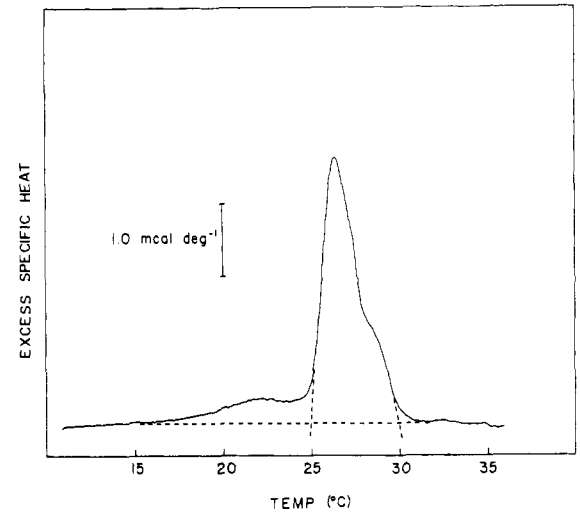

FIGURE 9: Calorimetric scan of DMPC + 8.2 mol % MA multilamellar dispersion. Dashed lines represent extrapolation performed to yield beginning and end of transiton.

sideration. We will first examine the dependence of rate of transformation on fatty acid content. Figure 6 shows initial relative rates determined from NMR spectra for samples incubated at 20 °C with free fatty acid varying between 2 and 15 mol % of total lipid. Note that the rates change in a slow linear fashion between 2 and 11 mol %, but thereafter a sharp increase appears. As rates also vary dramatically with temperature, it is important to verify that this apparent dependence on % fatty acid is not a secondary effect due to a shift in the temperature maximum. Transformation maxima were determined for 3 and 15 mol % myristic acid samples. Variations were insignificant compared with the breadth of the curves. The change in transformation rate with concentration must therefore be more directly related to the properties of the fatty acid in the vesicle system.

pH Dependence. One important property of a fatty acid is its state of ionization. Ionization as a function of pH may contribute to an alteration in rate via vesicle-vesicle electrostatic repulsion or via a more subtle change in fatty acid activity. A plot of residual vesicle concentration vs. time at several pD values is presented in Figure 7. The rates of transformation do not change appreciably when the pD is lowered from 7.4 to 4.5, and only a modest change occurs at 2.5, possibly owing to some lipid degradation which causes an alteration in vesicle properties. However, at pD 10.0 transformation is absent. That the major change in activity occurs between 7.4 and 10.0 may seem surprising in view of solution pK_a's for carboxylic acids; however, examination of ^{13}C spectra of 1-^{13}C enriched myristic acid in egg-yolk phosphatidylcholine vesicles at a series of pH values clearly shows the effective pK_a in a membrane environment to be 8.4. Data are presented in Figure 8. The 6-ppm upfield shift at low pH is typical of protonating a carboxyl group. The MA is therefore significantly charged only above pH 8, and we can conclude that vesicle-vesicle repulsion is unlikely to be an important factor in determining transformation rates under physiologic conditions.

Fatty Acid Distribution. It is important to know whether fatty acid manifests its properties as an entity in itself or as an integral part of the DMPC membrane system. It is easy to confirm that fatty acid is completely localized within the bilayer by separation of vesicle and solution fractions by molecular filtration or column techniques and spectroscopic determination of the fatty acid content of each. The 1-^{13}C-labeled myristic acid used in the previous experiment gives a well-resolved ^{13}C NMR resonance at 178 ppm (Me_4Si) in vesicle preparations. This resonance could not be observed in the solution fraction or in its chloroform-extracted concentrate indicating that at least 90% of the myristic acid is vesicle bound under conditions used in our experiments.

Though localized within a vesicle, it is conceivable that the fatty acid would manifest its properties as a laterally separated lipid phase. Differential scanning calorimetry has been employed to determine whether phase separations exist in other lipid systems (Mabrey & Sturtevant, 1976; Papahadjopoulos et al., 1977; Boggs et al., 1977). The calorimetric analysis of DMPC-MA vesicles per se could not be undertaken because of the transformation induced upon heating through the phase transition (Kantor et al., 1977); therefore DMPC-MA multilayers were observed. A typical calorimetric scan is illustrated in Figure 9. Note that the transition peak is at 26.6 °C, and an upfield shoulder occurs near 30 °C. Similar asymmetry in the transition profile is predicted for even miscible systems and has been seen in several mixed lipid preparations (Mabrey & Sturtevant, 1976, 1977; Eliasz et al., 1976). A phase diagram produced from a plot of transition range for several fatty acid concentrations appears in Figure 10. Of special significance is the lack of a region exhibiting isothermal melting over a wide range of fatty acid concentrations.

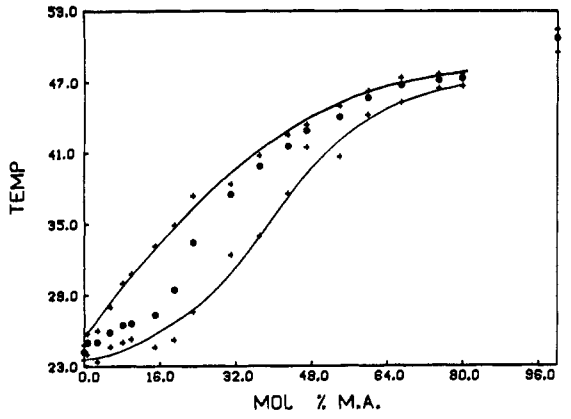

FIGURE 10: Phase diagrams of DMPC–MA multilamellar dispersion. Data derived from calorimetric scans as in Figure 8. (+) Represent beginning and end of transition; (●) indicate temperature of transition peak. Aqueous suspension of pure myristic acid crystals was examined to obtain 100 mol % data.

Discussion

We believe the above results can be used to elucidate at a molecular level some of the conditions which may be necessary for the transformation of bilayer structures. Whether the transformation observed relates to true fusion or not has been the subject of much discussion, with order of reaction playing a prominant role. Although one cannot use an order of reaction to prove a mechanism, it is important that any proposed mechanism be consistent with the observed order. Our results indicate an approximate first order process for vesicle transformation in the DMPC–MA system at 20 °C. This observation is in agreement with the work of other researchers on similar but not identical systems (Martin & MacDonald, 1976). A first-order reaction is in fact inconsistent with the simplest fusion mechanism in which rate is limited by vesilcuar collision.

Some authors have chosen to propose transformation mechanisms for this system involving diffusion of individual lipid molecules. Although this is consistent with the observed reaction order and can, when all vesicles present little or no barrier to lipid addition, give rise to rapid transformation for DMPC vesicles, we find a number of facts difficult to rationalize on the basis of this mechanism. First, no significant number of smaller or intermediate sized vesicles are observed during transformation (Kantor & Prestegard, 1975), and second, fatty acid and temperature drastically affect rates under conditions which would not be expected to significantly induce increases in free lipid concentrations.

There are many schemes which can give low apparent reaction orders. One class involves the rate-limiting activation of a single vesicle as a site of lipid transfer. Such a mechanism offers a possible site of action for free fatty acid and, as we shall show, formation of such a site at the bilayer phase transition temperature is a reasonable hypothesis. Appreciable rates can be reached if transfer is by merging of bilayers, and intermediate size vesicles will not exist in a substantial proportion if the site remains active over multiple vesicle–vesicle interactions.

A specific example of such a mechanism may be outlined as follows:

$$A \xrightarrow{k_1} A^* \quad (1)$$

$$A + A \xrightarrow{k_2} A^* \quad (2)$$

$$A^* + A^* \xrightarrow{k_3} A^* \quad (3)$$

where A* and A are the activated and nonactivated structures, respectively. The rate equations can be written:

$$\frac{d[A^*]}{dt} = k_1[A] - k_3[A^*]^2 \quad (4)$$

$$\frac{-d[A]}{dt} = k_1[A] + k_2[A][A^*] \quad (5)$$

If we assume reaction 1 proceeds at a much slower rate than the other two processes, the second term in eq 4 will ensure that A* rapidly approaches a low steady-state concentration. Because we monitor only processes leading to a change in vesicle size, we will be most sensitive to the second term in eq 5. To the extent that [A*] remains constant over time, the reaction will appear to be first order. Rates studied as a function of initial concentration will show an additional dependence on [A] which cannot exceed $[A]^{1/2}$. Numerical solution of eq 4 and 5 results in reasonable agreement with experiment at 2% (w/v) lipid containing 9 mol % myristic acid, with rate constants $k_1 = 8.0 \times 10^{-2}$ min^{-1} and $k_2 = k_3 = 11.0$ M^{-1} min^{-1}. Plots of vesicle concentration vs. time for several initial concentrations, followed by graphic determination of initial rates over the time periods used in our experiments, show an apparent order of 1.5.

Retention of vesicle contents during transformation can characterize some aspects of the activated site. Our results suggest a 1:1 loss of entrapped TMA ion with vesicle transformation when incubated at the phase transition of DMPC vesicles. Loss also decreases at higher temperatures in concert with cessation of bilayer transformation. The implication is that a leak or increase in permeability is coincident with activation at the phase transition. The results also show minimal mixing of TMA and external shift reagent ($Fe(CN)_6^{3-}$) inside nontransformed structures. The leak must therefore be sufficiently large and long lived to completely equilibrate internal and external contents. As an example, a break of area 100 Å^2, certainly large enough to pass TMA ions, would be required to remain open for at least 10^{-5} s.

Increased leakage in homogeneous phosphatidylcholine bilayers and the coincidence of this increase with the gel to liquid crystalline phase transition have been noted for other nontransforming systems (Marsh et al., 1976; Inoue, 1974; Blok et al., 1976). One investigator has proposed that the increased leakage arises from weak molecular interactions in the interfacial region between gel and liquid crystalline phases (Marsh et al., 1976). Although this suggestion relates to nonfusion systems, such a proposal could account for some of the changes noted here.

In addition, it has recently been observed that Ca^{2+} induced phosphatidylserine vesicle fusion is associated with an increase in permeability to ions (Papahadjopoulos et al., 1977). The Ca^{2+} is thought to induce a phase transition which triggers the fusion. Such a proposal is quite analogous to the increased transformation and leakage noted at the phase transition of fatty acid containing DMPC vesicles.

Quantitative comparison of transforming with nontransforming DMPC vesicle systems may elucidate the mechanism in question. Experiments performed show the frequency of such leaks in nontransforming vesicles to be one-fourth that of the transforming system. We believe the difference in leakage rate to be unimportant compared with at least an order of magnitude difference in vesicle transformation rates. Thus the formation of breaks at the phase transition is a property of both DMPC systems. A break does not necessarily result in transformation but may well be a prerequisite.

Sufficient conditions must be intimately involved with the

molecular properties of the fatty acid present. This is most dramatically illustrated by the dependence of transformation rate on fatty acid content. The abrupt increase at 15 mol %, and the temperature dependence of the transformation, point to the involvement of a separated phase of distinct fatty acid–lipid composition in promoting vesicle transformation. Our fatty acid localization experiments and the fact that transformation occurs at low rather than high pH indicate that any separation must be within the membrane rather than as a solution active species.

Calorimetric studies were undertaken to investigate this possibility. Separation of phases of significantly different composition requires a large flat region in the solidus curve of the phase diagram, which is notably absent in Figure 10. Thus a bulk separated phase high in fatty acid does not exist in this multilamellar preparation.

It is, however, plausible that the abrupt dependence of vesicle transformation on fatty acid content relates not to bulk phase behavior, but only to broken or activated vesicles which are directly involved in the process. The most important characteristic of a broken intermediate is the necessary exposure of a bilayer edge. This phenomenon is normally unfavorable thermodynamically and the necessity for fatty acid in our system may be related to stabilization of this edge.

Tanford has attempted to quantitate free energy differences between various micellar structures (Tanford, 1972, 1973). An important implication is that two chain amphiphiles such as phosphatidylcholine (PC) can minimize hydrophobic contacts effectively in a bilayer structure without undue headgroup repulsion, while single chain amphiphiles in general cannot. It is therefore reasonable for fatty acid rather than PC to prefer the more micellar character of the bilayer edge. Localization of fatty acid at the edge may provide either a suitable site for lipid transfer or merely stabilize an open growing extended bilayer. In the latter case, the abrupt rise in transformation rate at 15 mol % may be related to the edge to surface ratio of the activated species. An appealing aspect of this proposal is that if a broken vesicle grows by merging of bilayers the percentage of the total fatty acid in the bilayer which must localize at the edge decreases as the structure grows. Thus intermediate and large structures would be increasingly prone to vesicle transformation, accounting for our lack of observation of intermediate size structures.

Fatty acid at this growing edge may be either charged or uncharged, as it represents a small fraction of the total fatty acid. Inhibition at high pH need not entail a different distribution but may simply reflect repulsion between a charged edge and a charged vesicle surface.

The relevance of the aforementioned observations to fusion is an important consideration. The significant aspects of fusion as it occurs in natural systems or in potential medical applications may involve either efficient transfer of vesicle contents, or transfer of lipid itself. Our system, and many other membrane systems, have been demonstrated to leak internal contents, and therefore, offer little opportunity to study solute transfer. However, the system does exhibit lipid transfer, and, if our conclusions are correct, transfer by merging of bilayers. The role of phase transitions and substances such as free fatty acid in stimulating this merging can contribute to a general picture of prerequisites for transfer of lipid and possibly merging of bilayers during vesicle–membrane fusion.

Acknowledgments

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