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FUSION KINETICS OF PHOSPHATIDYLCHOLINE-PHOSPHATIDIC ACID MIXED LIPID VESICLES

A PROTON NUCLEAR MAGNETIC RESONANCE STUDY

MEI-JUNE LIAO and J.H. PRESTEGARD

Chemistry Department, Yale University, New Haven, CT 06511 (U.S.A.)

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Summary

The kinetics of Ca^{2+}-induced fusion of phosphatidylcholine-phosphatidic acid vesicles has been studied using the dependence of proton nuclear magnetic resonance linewidths on vesicle size. The linewidth of the lipid acyl chain methylene resonance has been shown to be sensitive to changes in vesicle size but insensitive to vesicle aggregation. For vesicle systems with the same lipid composition, the linewidth increases in a linear fashion with vesicle radius over the range 125—300 Å. This dependence has been used to determine quantitatively fusion rates and the dependence of such rates on Ca^{2+} as well as an vesicle concentration. For vesicle concentrations in the range of $3 \cdot 10^{-6}$—10^{-5} M and Ca^{2+} concentration at a level approaching 1 : 1 with respect to phosphatidic acid, the initial fusion rates have been found to be fast, with half-times of 1—10 min. An order of reaction of 2.7 with respect to vesicle concentration has been observed. Mechanisms of vesicle fusion are discussed in view of these observations.

Introduction

Small unilamellar phospholipid vesicles composed of mixtures of phosphatidylcholine and phosphatidic acid have been shown to undergo vesicle-vesicle fusion on addition of Ca^{2+} [1]. The system is particularly interesting in that the number of fusions can be controlled by the amount of Ca^{2+} added, and in that vesicle contents are largely retained during small numbers of fusions.

Abbreviation: Mes, 2-(*N*-morpholino)ethanesulfonic acid.

Because of these properties, the mechanism of fusion for this system warrants further investigation. We present here a detailed study of the kinetics of the fusion process. The kinetics will be shown to be consistent with a slow fusion step subsequent to rapid ion binding and aggregation. The time scale of ion binding is believed to be less than 1 ms and the half-time for two vesicles aggregating at lipid concentrations used here (33 mM) can be estimated to be 10 ms on the basis of previous light-scattering work by Haynes and coworkers [2—4]. Our own work indicates that the time scale of vesicle fusion is of the order of minutes, suggesting that neither ion binding nor vesicle aggregation is the rate-limiting step [1]. Thus, it is of interest to study the kinetics of this last step directly. The light scattering or turbidity techniques used previously are not, however, easily applied to a study of this last step because scattering due to aggregation tends to obscure any subsequent changes in vesicle structure.

Ideally, one would like to have a rapid monitoring technique sensitive to changes in vesicle size but insensitive to vesicle aggregation. Magnetic resonance studies of the past several years suggest that proton nuclear magnetic resonance (^{1}H-NMR) may fulfill most of these requirements. It has long been known that small unilamellar vesicles (radius 125—150 Å) give well-resolved ^{1}H-NMR resonances for methyl and methylene protons of the constituent lipids, while resonances of the more extended multilamellar membrane structures are broadened nearly beyond detection [5—7]. Vesicle systems which vary in size in response to changes in composition have also been used to demonstrate a monotonic increase in linewidth with vesicle radius over the range 120—220 Å [8]. There remains, however, some controversy as to the origin of linewidth variation as a function of vesicle size. Several groups have contended that the linewidth reduction is a result of the rapid isotropic tumbling of the small vesicles [9—13]. Other groups have contended that the reduction is primarily due to increased mobility of lipid hydrocarbon chains in structures of small radius of curvature [14—18]. If the latter interpretation were true, ^{1}H-NMR linewidth would be dependent on vesicle size and not on aggregation, thus providing the monitor of vesicle fusion desired. Several strong points of evidence support the latter view. First, ^{1}H-NMR linewidths of small vesicles show very little dependence on viscosity (η) as opposed to the linear dependence expected for linewidths dominated by vesicle tumbling [17,18]. Second, extensive vesicle aggregation induced by lectins has been shown not to increase the linewidths of ^{13}C resonances [19]. These resonances have widths determined by much the same factors as proton resonances.

The above observations suggest that an attempt to use ^{1}H-NMR in quantitatively monitoring kinetics of vesicle fusion is warranted. We will demonstrate that linewidths are relatively insensitive to aggregation in the phosphatidylcholine-phosphatidic acid system and then establish a quantitative relationship between vesicle size and proton linewidths. A quantitative relationship between linewidth and vesicle size can be used to evaluate rate constants and orders of reaction for the fusion process. The evaluation procedure is most easily conceptualized when initial vesicles are assumed to have a single discrete size. The systems treated here actually have rather broad size distributions for both initial and final vesicles. However, when data are taken from the dependence of initial rates on vesicle concentration the assumption is not at all critical for the

order of reaction and will have a minimal effect on the rate constant. Initial rate experiments do require an ability to make repeated observations on the time scale of seconds. A stop-flow cell combined with pulse Fourier transform acquisition of data in a high-sensitivity superconducting spectrometer has been used to accomplish this task.

Methods

Vesicle preparation. Vesicles were prepared by bath sonication using lipids dispersed in a ^2H_2O solution (p^{2}H 6.4) containing 100 mM NaCl at a total lipid concentration of 10% (w/v) and a phosphatidylcholine to phosphatidic acid ratio of 2 : 1. Phosphatidylcholine was isolated from egg yolks and phosphatidic acid was prepared from the phosphatidylcholine by the action of phospholipase D. Detailed procedures for vesicle preparation and isolation of materials have been described previously [1].

Vesicles for the study of line width dependence on vesicle size were obtained by using the dependence of fusion on Ca^{2+} level to regulate size. Fusion of small sonicated vesicles was induced by adding an equal volume of Ca^{2+}-containing solution with a Ca^{2+} concentration sufficient to achieve a Ca^{2+} to phosphatidic acid ratio ranging from 0.2 to 1. The resulting solution was incubated at 37°C for 30 min and the added Ca^{2+} then sequestered by addition of an equal molar amount of EDTA. The resulting vesicles were sized using analytical gel permeation chromatography on Sepharose 2B. The details of size determination procedures have again been given previously [1].

Vesicle solutions for the kinetics studies were prepared by dilution of a stock solution of vesicles at 10% (w/v) total lipid prepared as described above. Ca^{2+} solutions for induction of fusion were prepared in the same NaCl/^2H_2O solution at Ca^{2+} concentrations sufficient to yield an 0.8 : 1 Ca^{2+} to phosphatidic acid ratio when mixed with an equal volume of vesicle solution.

NMR spectroscopy. NMR spectra for the determination of line width dependence on vesicle size were obtained on a Perkin Elmer R32 spectrometer equipped with a TT7 Nicolet Fourier Transform accessory. The spectra were run at 35 ± 1°C.

For the kinetic studies spectra were obtained on a Bruker HX270 spectrometer operating in the pulse Fourier transform mode and using quadrature detection. Temperatures were held at 35 ± 2°C. Successive spectra for kinetic analysis were accumulated in an automated mode using standard Nicolet Technology software for the BNC12 spectrometer computer. In a typical experiment one free induction decay resulting from a 90° pulse, requiring 0.4 s and using 4096 data points was accumulated for each spectrum. 30 such free induction decays were stored directly on disk at 10-s intervals. These data were exponentially weighted and transformed to the frequency domain prior to analysis.

The stop-flow cell used for mixing and observation in the spectrometer was of a simple design. It consisted of a 'Y' junction constructed of 1 mm (inner diameter) Teflon tubing mounted at the top of a standard 5 mm NMR tube. Each of the inlet tubes were connected to 50 cm of similar tubing coiled and situated in a glass cylinder attached to the top of the spinner turbine that holds

the NMR tube. The Teflon tubes hold vesicle and Ca^{2+} solutions in a region of high magnetic field prior to mixing and the glass cylinder acts as an insulator to maintain sample temperature for the brief period of time between sample insertion and the beginning of observation. In our superconducting magnet, field strength in the region just above the spinner turbine is close enough to observation field to polarize spins nearly to their observation condition. Before placing the loaded cell into the spectrometer, the cell was incubated for 30 min in a thermostatically controlled cylinder to bring it to probe temperature (37°C). The sample was mixed using parallel-driven syringes within 2 min of lowering the cell into the spectrometer probe and data accumulation was started within 1 s of mixing.

Data analysis. In most cases data can be reduced to resonance amplitudes and widths measured directly from plotted spectra. For analysis of the relationship of vesicle size to line width, however, we attempted to extract more meaningful parameters. Spectra were digitized and transferred to a PDP 11/45 computer. The experimental data were then simulated with a program which sums Lorenzian lines of given frequency, width and intensity. A search for the best set of frequencies, widths and intensities was carried out by searching for a minimum in χ^2 using a program based on modified gradient search procedure similar to that outlined by Bevington [20].

Results

It is first essential to demonstrate that proton NMR spectra respond to changes in vesicle size in preference to changes in state of aggregation. 270 MHz ^{1}H-NMR spectra of a phosphatidylcholine : phosphatidic acid (2 : 1) preparation at 37°C are presented in Fig. 1a—c. Resonances from the choline methyl, from acyl chain methylenes and from chain-terminal methyls are clearly resolved at 3.3, 1.3 and 0.9 ppm, respectively. Adding Ca^{2+} to a 1 : 1 Ca^{2+} to phosphatidic acid ratio produces substantial broadening of all resonances, but results in little change in integrated area (Fig. 1a and b). Under these conditions aggregation is known to occur and can be visually observed as a large increase in sample turbidity. Vesicle fusion to structures approximately twice the initial diameter is also known to occur. It is noteworthy that the methylene broadens more on a percent basis than the other resonances; a fact which is more easily explained on the basis of chain mobility changes with vesicle size than on the basis of a change in overall tumbling rate with aggregation. EDTA added to a concentration equal to that of the Ca^{2+} in solution has been demonstrated to reverse aggregation [3,4]. EDTA added to the sample in Fig. 1b, however, produces little change in spectral appearance (Fig. 1c). Thus line widths change as a function of vesicle size but not state of aggregation.

Insensitivity to aggregation can also be demonstrated by running the above experiment at 10°C instead of 37°C. Fusion is much slower at 20°C and is minimal at 10°C but aggregation, as judged by turbidity change, is similar over this temperature range. Spectra before and after Ca^{2+} addition at 10°C are observed to be similar as illustrated in Fig. 1d—f.

A more quantitiative description of linewidth changes as a function of vesicle size would be of considerable value in analyzing motional changes of hydro-

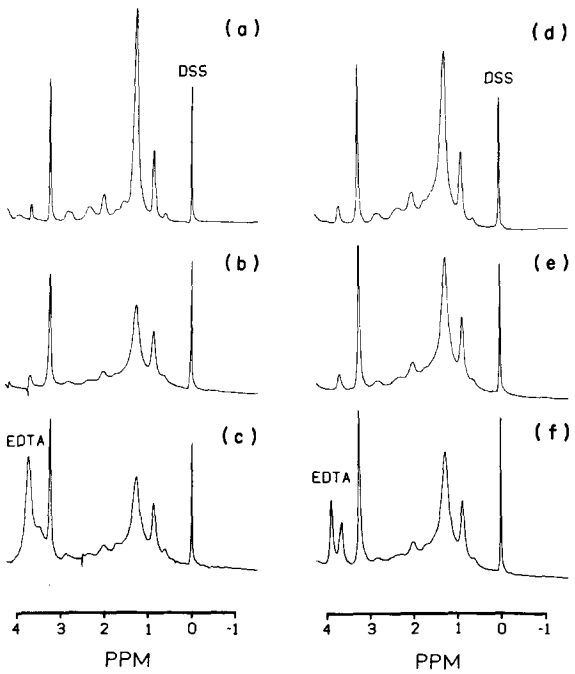

Fig. 1. 270 MHz ^{1}H-NMR spectra of 8% phosphatidylcholine : phosphatidic acid (2 : 1) vesicles at 37° C (a—c) and at 10° C (d—f). The initial vesicle preparation (a) was mixed with an equal volume of Ca^{2+}-containing solution at Ca^{2+} to phosphatidic acid ratio of 1 : 1 and the mixture was incubated for 1 h at 37° C (b). This was followed by addition of an equal molar amount of EDTA (c). The conditions were similar for (d—f) except that the mixture was incubated in the presence of Ca^{2+} at 10° C (e). TMS, trimethylsilane; DSS, 2,2-dimethyl-2-silapentane-5-sulfonic acid.

carbon chain segments in the vesicle bilayer. It may also allow a logical assessment of the most appropriate parameter to monitor in kinetic experiments to follow. A more quantitative description is, however, not easily obtained. Resonances overlap extensively in the region of interest; methyl (0.9 ppm), bulk methylenes (1.3 ppm) and methylenes α to carboxyl groups or double bonds (2.1 ppm). Moreover, there is no reason to believe that even in a homogeneous vesicle population all bulk methylenes would yield Lorenzian line components of the same widths. In a single lipid molecule, this methylene resonance represents 10—12 methylenes distributed over the length of the esterified fatty acid chain. Each of these could have a different degree of mobility and a different linewidth [21]. As a first approximation we have, in the past, simulated such spectra using two Lorenzian components for the bulk methylenes [8]. Following this procedure, we have fitted the region of the spectrum between 0 and 2.3 ppm using four Lorenzian lines; two for the bulk methylene, one for the methyl and one for the methylenes α to carboxyls or double bonds. Since a very extensive set of data had been acquired at 90 MHz, these data were used in the fitting process. Field dependence of linewidths has been examined at low fields and found to be negligible [22]. We therefore expect results to be applicable to data collected at 270 MHz.

An example of an experimental spectrum and the best-fit simulation is given in Fig. 2. Relative intensities are in all cases representative of occurrence of functionally distinct groups in phospholipid preparations. In Fig. 3, linewidths

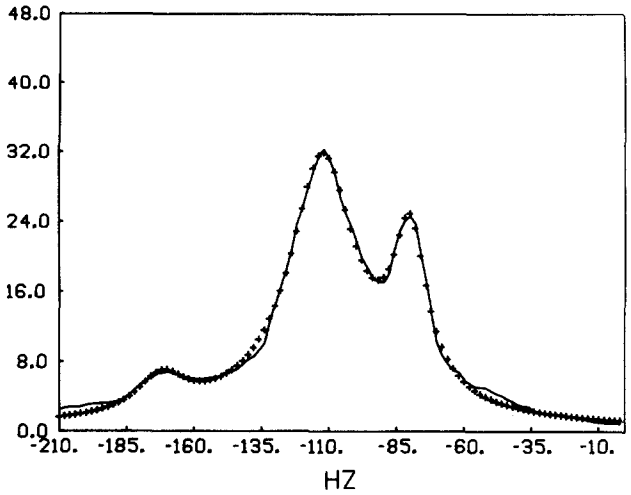

Fig. 2. Lorentzian line fitting of an ^{1}H-NMR spectrum of phosphatidylcholine : phosphatidic acid (2 : 1) vesicles at 90 MHz and 35° C. ———, the observed spectrum which was digitized to 100 data points in the region of 0—2.3 ppm down field from TMS. (+), the simulated curve using components of 26 Hz and 60 Hz width in a ratio of 2.6 to 1 for the methylene.

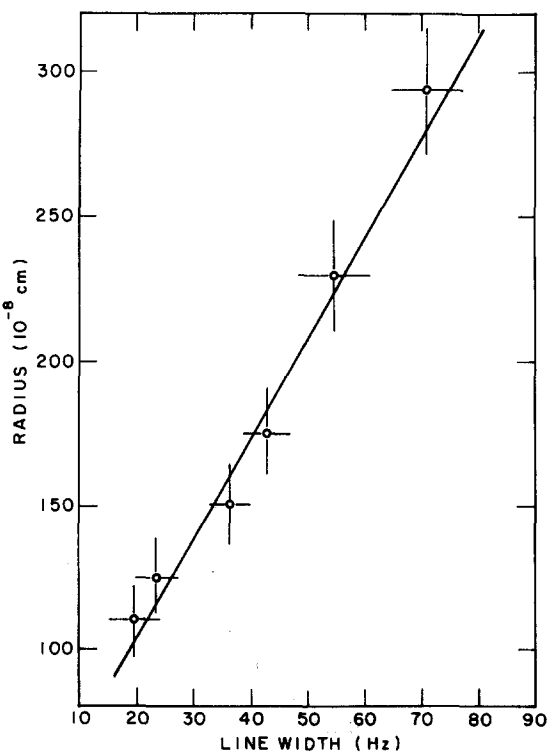

Fig. 3. 90 MHz ^{1}H-NMR linewidths of methylene resonances vs. vesicle radius for phosphatidylcholine : phosphatidic acid (2 : 1) vesicles (35° C).

for methylenes extracted by such fits are reported for vesicles of various size produced by adding different stoichiometric amounts of Ca^{2+}. For the methylene resonance the weighted average of the linewidths for the two-component lines is reported. Sizes, or radii, are determined from elution volumes in Sepharose 2B columns.

Widths increase in a near linear fashion with vesicle size. It is noteworthy that the slopes of the plots are similar to those for a series of phosphatidylcholine-phosphatidylethanolamine vesicles studied previously, in which size was varied by changing composition [8]. A potential direct effect of composition on linewidth presented an obstacle to interpretation of the previous data [23]. No such complications exist for the phosphatidylcholine-phosphatidic acid system and individual widths could contribute to a motional analysis of the hydrocarbon region.

For our purposes it is, however, most important that the linear variation observed is large enough (slope 3.6 Å/Hz for the methylenes) to be used as a monitor of size changes during rapid fusion processes. In cases where the relationship between spectral parameters and size can be accepted as an empirical one, parameters more easily accessible than linewidth may be appropriate. Spectra in Fig. 1, for example, indicate a large change in methyl to methylene amplitude ratio. This ratio may therefore provide a suitable parameter in some cases. In cases where a single sample is to be observed as a function of time, amplitude of a single resonance may also be an adequate monitor. Since no molecules are destroyed during fusion, the area of a properly fit resonance must remain constant. Amplitudes and widths of Lorenzian lines are inversely related under these circumstances and thus provide equivalent information. We will use methylene amplitude in the following analysis of fusion kinetics.

Our preliminary studies of fusion kinetics indicated that, for 2 : 1 phosphatidylcholine-phosphatidic acid vesicles, addition of Ca^{2+} to a 1 : 1 phosphatidic acid to Ca^{2+} ratio resulted in a rapid fusion process that was complete in 30 min [1]. This process appears to be biphasic, with a more rapid process accounting for 80% of the total change in methylene amplitude in 5—10 min. This more rapid process is more interesting as an analog of natural fusions. In addition to characterizing its rate, an order of reaction with respect to vesicle concentration would be particularly valuable. Orders of reaction are best determined from a study of initial rates as a function of vesicle concentration. Since one would like to determine initial rates during the first 20% of vesicle decays, sequential spectra must be acquired at very short time intervals. The stop-flow cell described in Methods was employed to achieve this goal.

Fig. 4 shows an example in which fusion in a 120 mM sample of 2 : 1 phosphatidylcholine-phosphatidic acid vesicles was initiated by mixing with an equal volume of 32 mM Ca^{2+} solution. Spectra at 270 MHz were obtained every 10 s but only some of the plots are shown. Methylene amplitude is seen to decrease to 50% of its initial value in 4 min, while its weight average linewidth increases from 35 to 55 Hz. The change in line width is appropriate for most vesicles to have undergone one fusion. The change in amplitude is slightly greater than expected, perhaps indicating some formation of multilamellar products and perhaps reflecting phase separation of the phosphatidic acid-Ca^{2+}

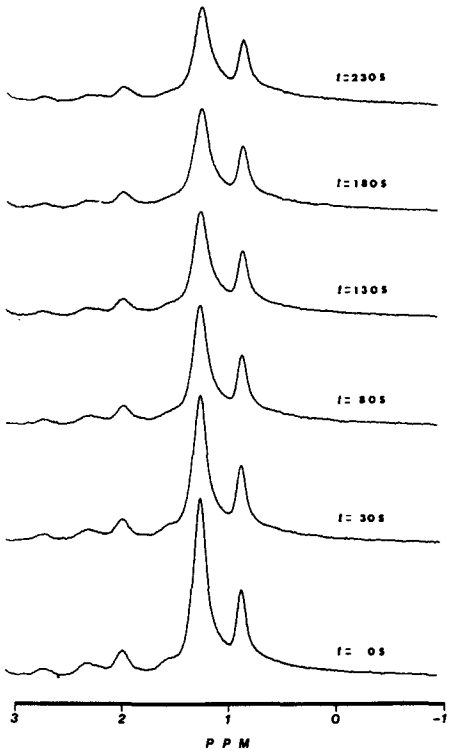

Fig. 4. 270 MHz ^{1}H-NMR spectra versus time for 120 mM phosphatidylcholine : phosphatidic acid (2 : 1) vesicles on addition of 32 mM Ca^{2+} solution (37° C).

gel-state complex [24]. Both multilayers and gel-state lipids in vesicles give rise to broad resonances which appear as a base-line rise when narrow spectral regions are fit. Loss of spectral area is less than 5% if the full spectral width is fit.

In a study of reaction order, one might consider Ca^{2+} concentration dependence as well as vesicle concentration dependence. A dramatic dependence of size distribution of fusion products on Ca^{2+} concentration has already been noted [1]. Virtually no change in size takes place below a Ca^{2+} to phosphatidic acid ratio of 0.3 : 1. The average size of fusion product increases above this ratio until it reaches a plateau above a ratio of 1 : 1. One might also expect a dependence of fusion rate on Ca^{2+} concentration in this range. This is indeed observed as indicated in Fig. 5. A series of vesicle samples all at 150 mM total lipid were mixed with solutions of varying Ca^{2+} content. Methylene amplitude is plotted as a function of time. Curves plateau at different levels consistent with differences in size distributions of fusion products. Rates as taken from the initial slope of each curve are also seen to vary dramatically from 5 mM to 10 mM Ca^{2+} (Fig. 6). Beyond 15 mM Ca^{2+} initial rates as well as size distributions converge on a single value.

Rate dependence on vesicle concentration for simple processes could be studied at any fixed Ca^{2+} concentration. In our system the situation is more complicated in that the final size distribution and, probably, rate depend more

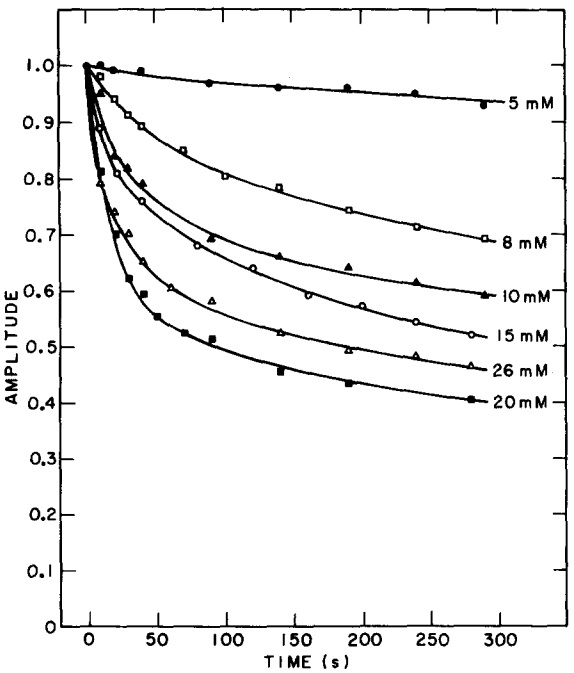

Fig. 5. Absolute intensities of methylene resonances vs. time for 75 mM phosphatidylcholine : phosphatidic acid (2 : 1) vesicles in the presence of various Ca^{2+} concentrations. Data were taken at 270 MHz; 37° C.

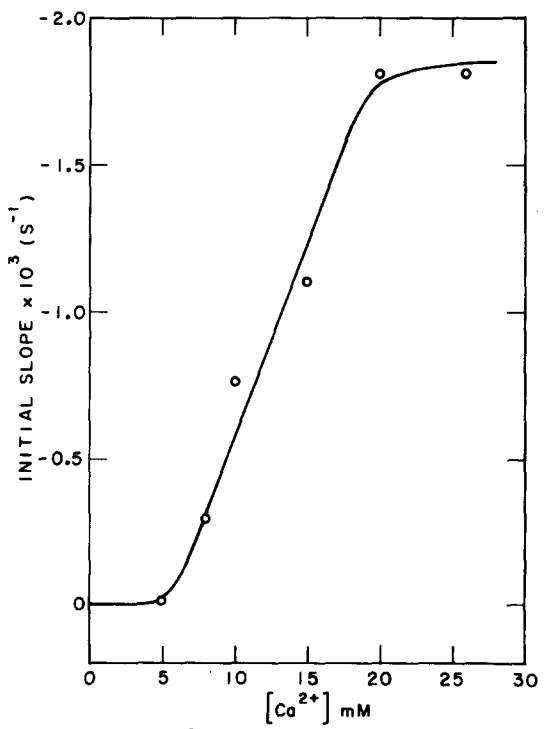

Fig. 6. Effect of Ca^{2+} addition on the change in the initial slopes of the curves in Fig. 5.

on phosphatidic acid to Ca^{2+} ratio than on Ca^{2+} concentration. This suggests that it is the phosphatidic acid-Ca^{2+} complex concentration and not just Ca^{2+} concentration that should be held constant. Ca^{2+} to phosphatidic acid ratios above 0.8 : 1 (above 20 mM for samples such as those used for data in Fig. 6) offer an advantage in that rates and product distributions remain relatively independent of fraction of phosphatidic acid complex above this point. A further complication exists, however, in that ratios of Ca^{2+} to phosphatidic acid much above 0.8 : 1 promote some formation of larger, possibly multilamellar products. In the interest of emphasizing two vesicle fusions in our data we have therefore chosen to run at a fixed Ca^{2+} to phosphatidic acid ratio of 0.8 : 1 rather than at higher levels. The binding of Ca^{2+} to phosphatidic acid is known to be tight, so that more than 90% of the Ca^{2+} should be associated even in the samples of the highest dilution [25]. The binding process is also known to be rapid enough (1 ms) to prevent a direct influence of Ca^{2+} concentration on any rate process we can observe [2].

The time course of fusion as a function of vesicle concentration at fixed Ca^{2+} to phosphatidic acid ratio is shown in Fig. 7. The rates can be seen to increase in a monotonic fashion as a function of vesicle concentration over the range from 20 mM to 70 mM in total lipid. The rates are rapid throughout the range approaching completion of the initial rapid decay phase in 5 min as previously observed.

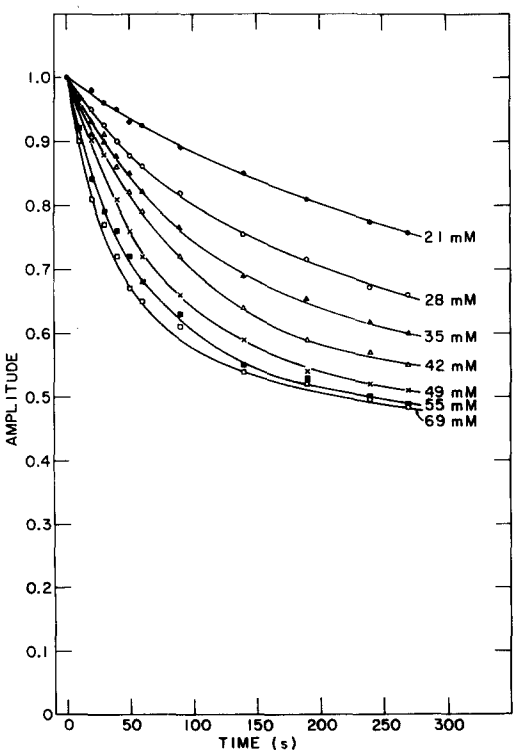

Fig. 7. Absolute intensities of methylene resonances vs. time for phosphatidylcholine : phosphatidic acid (2 : 1) vesicles at various total lipid concentrations and in the presence of Ca^{2+} at a Ca^{2+} to phosphatidic acid ratio of 0.8 : 1. Data were taken at 270 MHz; 37° C.

Discussion

The overall rate of vesicle fusion is of interest as a means of evaluating the efficiency of lipid vesicles in transferring lipid components to other membranes and in transferring internal contents across cell membranes. Rates and orders of reaction are also of interest in comparing model to natural systems. The rates as depicted in Figs. 5 and 7 are obviously quite great, with half-times of 1—10 min. High rates of fusion are also characteristic of natural systems. For example, Ca^{2+}-induced fusion of vesicles from isolated myoblast plasma membranes has been observed to reach maximum within 1 min [26].

The order of reaction with respect to vesicle concentration or Ca^{2+} concentration can be useful in establishing a mechanism of interaction. Although a measured order of reaction cannot be used to prove a mechanism, it can be used to screen proposed mechanisms. Any proposed mechanism must be consistent with observed order. For example, a mechanism for lipid transfer involving diffusion of individual molecules between vesicles predicts a first-order reaction with respect to vesicle concentration [27,28]. A measured order of reaction higher than this can eliminate such a possibility.

An analysis of the initial slopes of the plots in Figs. 5 and 7 can yield both rate constants and orders of reaction for fusion in the phosphatidylcholine-phosphatidic acid system. The early changes in these plots can be assumed to be the result of two vesicle fusion. Since we know the spectral parameters appropriate for both the initial vesicle and the two vesicle fusion product (a vesicle of radius $\sqrt{2}$ larger), we can easily convert amplitude changes to vesicle concentration changes. The methylene amplitude at any time in the initial region can be expressed as follows:

$$A(t) = a_1\nu_1 + a_2\nu_2 \tag{1}$$

where a_1 is the amplitude appropriate for a one molar sample of vesicles of the small initial size, a_2 is the amplitude appropriate for a one molar sample of vesicles of the larger product size, and ν_1 and ν_2 are the molar vesicle concentrations. Since we know the initial concentration (ν_i) and the initial amplitude, A_i, it is convenient to express observed amplitude change as a fraction of the initial value, $F(t)$.

$$F(t) = \frac{A(t)}{A_i} = 1 - \frac{(a_1 - a_2)\,\nu_2}{a_1\nu_i} \tag{2}$$

The rate of fractional change, which is easily extracted from the plots in Figs. 5 and 7, is then:

$$\frac{dF(t)}{dt} = -\frac{(a_1 - a_2)}{a_1\nu_i}\frac{d\nu_2}{dt} \tag{3}$$

The rate of change in product concentration $d\nu_2/dt$, can be expressed in terms of a rate law dependent on vesicle concentration, Ca^{2+} concentration and other factors. This is easily illustrated for the case where all factors other than vesicle concentration are held constant and included in a rate constant, k. The

order of reaction with respect to vesicle concentration is then represented by the single exponent, m.

$$\frac{d\nu_2}{dt} = k\nu_1^m \tag{4}$$

Near time zero, $\nu_1 \approx \nu_i$. Therefore,

$$\frac{dF(t)}{dt} = -\frac{(a_1 - a_2)}{a_1} k\nu_i^{m-1} \tag{5}$$

It is clear that a plot of log $(dF(t)/dt)$ vs. log ν_i will produce a plot of slope $(m-1)$, yielding the order of reaction -1. The intercept in combination with the known value of $(a_1 - a_2)/a_1$ can yield the rate constant k.

A similar analysis could be followed in extracting an order of reaction with respect to Ca^{2+} concentration. The Ca^{2+}-dependent data in Fig. 5, however, produce a highly non-linear plot as indicated in Fig. 6. The dependence on Ca^{2+} concentration is obviously more complex than that which might be presented as a simple product-type rate law. At low Ca^{2+} levels a very high order would be obtained, indicating a high level of cooperativity. At higher Ca^{2+} levels, saturation of its effect is observed. Similar Ca^{2+} dependence has been observed in studies of aggregation at low Ca^{2+} to phosphatidic acid ratios, and has been interpreted in terms of a required surface charge neutralization prior to the aggregation step [2]. The observation of 1 : 1 phosphatidic acid to Ca^{2+} complexes suggests that saturation of the negative phosphatidic acid sites may be the reason for the plateau in rates at higher Ca^{2+} levels. The data presented here may simply reflect the fact that aggregation moderated by the Ca^{2+}-phosphatidic acid complex is a prerequisite to fusion.

The data on vesicle concentration dependence (Fig. 7) show better agreement with a product-type rate law. When plotted as log of the initial rate vs. log of vesicle concentration (Fig. 8), the data yield a reasonably linear plot in the range of vesicle concentration from $3.5 \cdot 10^{-6}$ M to $9.3 \cdot 10^{-6}$ M. Concentrations given here are calculated assuming 6000 lipid molecules per vesicle of initial size. The slope indicates that fusion order with respect to vesicle concentration is 2.7. The rate constant is $7.6 \cdot 10^6\ s^{-1} \cdot M^{-1.7}$. Close scrutiny of the data shows some departure from linearity at high and low concentrations. The order would appear somewhat higher than 2.7 at low vesicle levels and somewhat lower than this at high vesicle levels.

The order of reaction determined above can be used to rule out diffusion of individual lipid molecules as a means of vesicle growth [27]. It can also be used to rule out mechanisms similar to the one we proposed for the dimyristoylphosphatidylcholine-myristic acid system in which the rate-limiting step is activation of a vesicle which remains active for several fusions with either non-activated or other activated vesicles. This mechanism predicts an order of 1.5 [29]. Orders of reaction of 2 or greater have been observed in the transformation of other vesicle systems. For example, Martin and MacDonald have observed a second-order step in growth of dimyristoylphosphatidylcholine vesicles containing a few percent brain gangliosides and have used it to support the existence of a bivesicular step in aggregation or fusion [28].

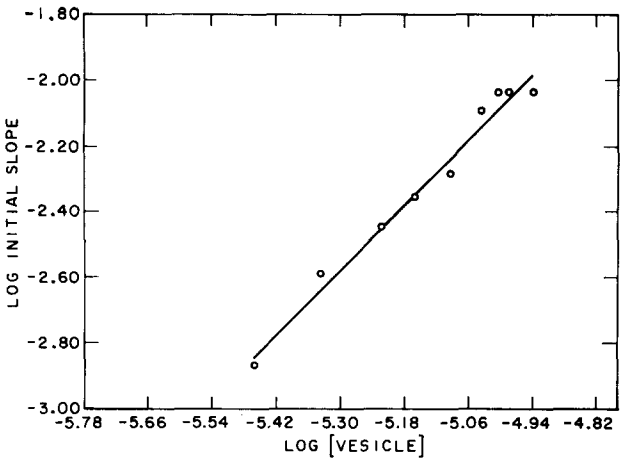

Fig. 8. Logarithm of vesicle concentration vs. logarithm of initial slopes of the curves in Fig. 7.

The dependence on Ca^{2+} and the order of 2.7 with respect of vesicle concentration observed here are consistent with the following mechanism. Firstly, Ca^{2+} binds to phosphatidic acid in amounts at least sufficient to neutralize surface charge and to form a site for subsequent vesicle interaction. This process has been studied in a 1 : 1 dimyristoyl phosphatidylcholine-dimyristoyl phosphatidic acid system and is found to have a rate constant of $1.9 \cdot 10^7\ M^{-1} \cdot s^{-1}$ [2]. Secondly, vesicles aggregate; initially only as two-vesicle complexes, but eventually with higher aggregates as well. The two-vesicle aggregation has also been studied and found to have a rate constant of $1 \cdot 10^7\ M^{-1} \cdot s^{-1}$ [2]. And thirdly, a transformation leading to fusion takes place. Unless binding and aggregation exhibited unusual concentration dependencies under our conditions, the first two steps would be too fast for the process we observe. Ca^{2+} binding and vesicle aggregation would reach an equilibrium state long before the fusion step. If we assume that the aggregation equilibrium lies well toward the monomer side and assume that two-vesicle fusions occur equally well in all sizes of aggregates or are favored in higher aggregates we would predict an order of reaction of 2 or greater. The precise value would be dependent on reactivities of various sized aggregates and aggregate formation constants. If aggregation is far advanced, all vesicles would be in contact and apparent order would decrease toward 1. The observed departure from linearity toward a lower slope at high vesicle concentrations could be an indication of this.

Agreement of predictions with observations does not rule out other possible mechanisms, but the proposed mechanism provides a viable working hypothesis. Verification of such a mechanism and elucidation of the molecular details of the vesicle merging process remain a topic for further investigation.

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