Mass Action Kinetics of Phosphatidylserine Vesicle Fusion As Monitored by Coalescence of Internal Vesicle Volumes†

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ABSTRACT: The kinetics of Ca^{2+}-induced fusion of sonicated phosphatidylserine vesicles is analyzed by means of the mass action model. The results of calculations are shown to simulate the experimental results for the mixing of aqueous vesicle volumes, release of vesicle contents and for the observed increase in light scattering [Wilschut, J., Düzgünes, N., Fraley, R., & Papahadjopoulos, D. (1980) *Biochemistry* (first of three papers in this issue)]. The calculations give the distribution of vesicle sizes during the initial stages of the fusion process and an estimate for the occurrence of multiple fusion events. It is estimated that during the first few seconds from the beginning of the fusion process in the above systems only a small fraction of the material trapped will leak during each fusion event. The fraction of material which leaks per fusion event is further reduced with increased Ca^{2+} concentrations. The values of the rates of fusion which describe the above experiments suggest that the rate limiting step of the overall fusion reaction is the aggregation and close approach of vesicles to each other rather than the fusion event per se.

The recent development of a fusion assay, monitoring mixing of aqueous vesicle contents (Wilschut & Papahadjopoulos, 1979; Wilschut et al., 1980), provides a direct experimental approach to analyzing the kinetics of vesicle–vesicle fusion. This approach can be adequately applied to the fusion of phosphatidylserine (PS)[1] vesicles as induced by Ca^{2+}, by Mg^{2+} (under certain conditions) (Papahadjopoulos et al., 1975, 1977; Day et al., 1977; Sun et al., 1978), or by trivalent cations such as La^{3+} (Hammoudah et al., 1979). The present work gives estimates for the rate of Ca^{2+}-induced fusion of PS sonicated vesicles, for the leakiness during fusion events, and for the occurrence of multiple fusion events during the time course of the process.

In the attempt to determine the rate of a vesicle–vesicle fusion reaction, it has to be recognized that this process is complex and consists of several stages: vesicle aggregation and close approach, bilayer destabilization, and finally the actual fusion event per se, which results in the formation of one new vesicle compartment out of two. From the rate of vesicle aggregation and that of the overall aggregation and fusion process, it may be possible to determine the rate, or to find at least an upper-bound estimate for the duration, of the fusion event per se. With respect to vesicle aggregation, we recently demonstrated (Nir & Bentz, 1978; Nir et al., 1980; Bentz & Nir, 1980) that a theory of colloidal science, i.e., the Derjaguin–Landau–Verwey–Overbeek theory (Derjaguin & Landau, 1941; Verwey & Overbeek, 1948), can give an order of magnitude estimate for the rate of aggregation of PS vesicles under the influence of the cations Na^+, Ca^{2+}, or Mg^{2+}, whose binding to PS bilayers had been determined previously by several experimental procedures (Newton et al., 1978; Nir et al., 1978; Kurland et al., 1979a,b; Portis et al., 1979). In parallel, we have developed a procedure based on the mass action model (Bentz & Nir, 1981) which permits a kinetic analysis of particle size distribution during the time course of vesicle aggregation and fusion.

In the present work, we have applied the above model to an analysis of the rate of the overall PS vesicle fusion reaction determined experimentally. The results show that the fusion event per se can be a fast phenomenon, not rate limiting to the overall aggregation and fusion process. In addition, we present an analysis of the relative kinetics of coalescence of internal vesicle volumes and that of the release of vesicle contents as determined from the rate of increase of carboxyfluorescein fluorescence, employing the decrease of self-quenching of this dye upon dilution (Blumenthal et al., 1977). The results show that most of the material captured in the vesicles is retained during their fusion. Finally, the measurement of the intensity of scattered light after dissociation of the vesicle aggregates by addition of excess EDTA provides information about the average increase in vesicle size as a result of fusion. We have analyzed these data with the above kinetic model, which predicts the distribution of vesicle sizes at any time during the fusion process, and the experimental and calculated changes in scattering intensity are compared.

Theory

Mass Action Kinetics for Vesicle–Vesicle Fusion. With an initially monodisperse system of vesicles, we may write the initial fusion reaction step as

$$V_1 + V_1 \xrightarrow{C_{11}} F_2 \qquad (1)$$

where V_1 denotes a sonicated vesicle and F_2 a vesicle which is the product of the fusion of two sonicated vesicles. C_{11} denotes the rate constant for this reaction. Since the fusion reaction is irreversible, we do not consider the reverse reaction $F_2 \rightarrow V_1 + V_1$. Clearly, there exists an intermediate step for this reaction, i.e., the formation of the aggregated dimer before fusion. The existence of these prefusion structures will be ignored in the present analysis for two reasons. First, writing the kinetic equations in this form still provides a self-consistent set of rate equations in which the value of C_{11} is unambiguously

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[1] Abbreviations used: PS, phosphatidylserine; DPA, dipicolinic acid; CF, carboxyfluorescein; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; PC, phosphatidylcholine; SUV, small unilamellar vesicles, radius ~125 Å; EDTA, ethylenediaminetetraacetate.

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defined as the rate constant for the overall process of vesicle fusion. Second, as we only determine the concentration of fused vesicles from fluorescence intensities, introduction of the intermediate structure would yield merely another set of adjustable parameters.

Thus, we continue to consider the next steps of the reaction in which the following processes will occur:

$$V_1 + F_2 \xrightarrow{C_{12}} F_3$$
$$V_1 + F_3 \xrightarrow{C_{13}} F_4 \qquad (2)$$
$$F_2 + F_2 \xrightarrow{C_{22}} F_4$$

We use the notation F_i to denote the vesicle that is the fusion product of i vesicles V_1 and C_{ij} to denote the rate constant for the fusion of the species F_i with the species F_j. As long as we consider only the early stages of the fusion reaction, where the formation of cochleate structures (Papahadjopoulos et al., 1975) may be neglected, this set of reactions describes the overall fusion process.

For these reactions, we can write the mass action kinetic equations for fusion as (Boyd, 1977; Bentz & Nir, 1981)

$$\frac{d}{dt}[V_1(t)] = -2C_{11}[V_1(t)]^2 - C_{12}[V_1(t)][F_2(t)] - \ldots$$

$$\frac{d}{dt}[F_2(t)] = C_{11}[V_1(t)]^2 - C_{12}[V_1(t)][F_2(t)] - \ldots$$

and, in general

$$\frac{d}{dt}[F_i(t)] = \sum_{j=1}^{[i/2]} C_{j(i-j)}[F_j(t)][F_{i-j}(t)] - \sum_{j=1}^{M-i}(1 + \delta_{ij})C_{ij}[F_i(t)][F_j(t)] \qquad (3)$$

where $[F_i(t)]$ denotes the concentration of the species F_i at the time t and (d/dt) $[F_i(t)]$ is the time rate of change in the concentration of the species F_i. The general equation for the species F_i is explained as follows. There are two types of reactions which directly alter the concentration of the species F_i. The first type

$$F_j + F_{i-j} \xrightarrow{C_{j(i-j)}} F_i \qquad (4)$$

increases the concentration of F_i, and j runs from 1 to $[i/2]$—the largest integer less than or equal to $i/2$. The first summation accounts for these reactions, as may be seen by setting $i = 2$, so that this summation reduces to $C_{11}[F_1(t)]^2$. Note that F_1 is equivalent to V_1, and they are used interchangeably. The second reaction type

$$F_i + F_j \xrightarrow{C_{ij}} F_{i+j} \qquad (5)$$

decreases the concentration of F_i, and j runs from 1 to the largest size of a fused vesicle which has a nonzero concentration, which we simply denote by M. The term $1 + \delta_{ij}$ is defined to equal 1 when $i \neq j$ and equal to 2 when $i = j$. In this way, we account for the stoichiometry of the reaction, as may be seen by setting $i = 1$, where we obtain the equation for $(d/dt)[V_1(t)]$.

While our program is capable of giving solutions for any combination of rate constants, in this work we have equated the higher order rate constants to C_{11}. With the data on the fusion process now available, we are attempting a quantification of the kinetics, using a minimum number of parameters to fit the data. The value of C_{11} obtained from our fit will be essentially equal to an average value for the true rate constants.

Combinatorial Calculation of Tb/DPA Complex Formation during Vesicle–Vesicle Fusion. In the fusion assay that is the experimental basis to the present work, terbium is encapsulated as the $Tb(citrate)_3^{6-}$ chelation complex in one population of vesicles and the sodium salt of dipicolinic acid (DPA; pyridine-2,6-dicarboxylic acid) in another. Fusion results in the fast formation of the fluorescent $Tb(DPA)_3^{3-}$ complex and can be monitored continuously. EDTA (0.1 mM) and Ca^{2+} (>1 mM) effectively prevent the formation of the Tb/DPA complex in the external medium. [For a more extensive account of the assay, see Wilschut et al. (1980).] In the present study, DPA was encapsulated in PS vesicles at a 10-fold higher molar concentration (150 mM) than Tb (15 mM). The ratio of Tb vesicles (denoted at T vesicles) to DPA vesicles (denoted as P vesicles) was 1:1. If the total concentration of vesicles is X_0, the maximal Tb fluorescence, obtained by perfect and complete mixing of the Tb and the DPA in the vesicles, is proportional to $^1/_2 X_0$. If q is the intensity of the fluorescence due to the fusion of a P with a T vesicle, then the maximal fluorescence is

$$I_T = \tfrac{1}{2}X_0 q \qquad (6)$$

Consider a fused dimer, denoted F_2 as in eq 1. This dimer could have formed from four possible reactions:

$$P + P \rightarrow P_2$$
$$P + T \rightarrow PT$$
$$T + P \rightarrow TP$$
$$T + T \rightarrow T_2 \qquad (7)$$

Since we measure the fusion reaction by Tb/DPA complex formation, we can observe only half of the dimers which have formed, i.e., the PT and TP pairs. Hence, if all of the vesicles were to undergo just a dimerization, only 50% of the maximal fluorescence would be seen. Of course, this assumes that there is no leakage of vesicle contents. However, as explained in the next subsection, this leakage can be accounted for by using the carboxyfluorescein release data. Therefore, in the remainder of this section, we will assume that there is perfect mixing of the contents of the fusing vesicles, and we will show how to calculate the percentage of the maximal Tb/DPA complex production for a given outcome of fusion events.

Consider a fused vesicle which is composed of k original vesicles, i.e., F_k. Of the k original vesicles which comprise this F_k vesicle, the number of T-type vesicles could be any number between zero and k. Since we assume that the vesicles are fusing randomly, it follows that the probability that an F_k vesicle is comprised of i T-type vesicles and $k - i$ P-type vesicles is given by the binomial distribution, i.e.

$$\frac{1}{2^k}\frac{k!}{(k-i)!i!}$$

We must also take into account which of the two compounds (Tb or DPA) has the smaller concentration inside the vesicle, since that is the limiting reagent for the fluorescence enhancement. Clearly, the concentrations of Tb and DPA inside of this F_k vesicle are directly proportional to their respective concentrations in the original vesicles. In the assay, the ratio of the concentration of DPA to that of Tb is 10. However, let us first consider the simpler case wherein this ratio is very high, implying that DPA is always in large excess. Then the fluorescence of the F_k vesicle will be just equal to the number of T-type vesicles which comprise it times the unit of fluorescence q. The fluorescence reaction will not occur when

there are only P- or T-type vesicles in the fused F_k vesicle. This means that 2 out of 2^k possible cases would not contribute. Thus, the fraction of fusion events which produce F_k vesicles and also result in the fluorescence reaction is

$$1 - \frac{2}{2^k} = 1 - \frac{1}{2^{k-1}}$$

If all monomers fuse to form dimers, we would have a fluorescing fraction of $^1/_2$, whereas for trimers the fraction would be $^3/_4$, and for a k-mer, where k is large, the above fraction would approach unity. With the notation of eq 6, the contribution of F_k to the fraction of the maximal fluorescence reaction is

$$k[F_k(t)](1 - 1/2^{k-1})/X_0 \tag{8}$$

Since we must treat a more complicated case, where either Tb or DPA may be the limiting reagent, it will be useful to write eq 8 in a more general form. The fraction of the maximal fluorescence in the F_k vesicles is given by

$$\sum_{i=0}^{k-1} iq \left[\frac{k!}{(k-i)!i!} \frac{F_k(t)}{2^k} \right] \Bigg/ \frac{1}{2} X_0 q \tag{9}$$

The factor in brackets is the concentration of F_k vesicles which contain i T-type vesicles. This factor times iq gives the concentration of Tb available for complex formation times the unit of fluorescence q. The fraction of the maximal fluorescence at time t is defined as

$$I(t) = \frac{2}{X_0} \sum_{k=2}^{M} \sum_{i=0}^{k-1} i \left[\frac{k!}{(k-i)!i!} \frac{[F_k(t)]}{2^k} \right] \tag{10}$$

where $\sum_{k=2}^{M}$ denotes the summation over all sizes of fused vesicles.

Now consider the case where $[Tb] \leq {}^1/_3[DPA]$. Within the fused structure, the total concentration of Tb is $i[Tb]$ while that of DPA is $(k-i)[DPA]$. Suppose, first, that $i[Tb] \leq {}^1/_3(k-i)[DPA]$. Since Tb is the limiting reagent in the case at hand, it follows that the fluorescence for this fused structure will be proportional to iq.

Now, in the other case where $i[Tb] > {}^1/_3(k-i)[DPA]$, we must treat DPA as the limiting reagent to the complexation. In order to calculate the percentage of the maximal fluorescence for this structure, we define $\alpha \equiv {}^1/_3[DPA]/[Tb]$ so that α gives the original excess of the dipicolinic acid concentration in the measurement of the maximal fluorescence. Notice that $\alpha \geq 1$. Whenever DPA is the limiting reagent in the fused structure, the fluorescence for that vesicle will be given by $(k-i)\alpha q$, since the Tb $(k-i)\alpha$ T-type vesicles will have complexed within the fused F_k vesicle.

These considerations may be summarized as follows. The fluorescence of an F_k vesicle will be proportional to iq if $i \leq (k-i)\alpha$, i.e., $i \leq k\alpha/(1+\alpha)$, and to $(k-i)\alpha q$ if $i > (k-i)\alpha$, i.e., $i > k\alpha/(1+\alpha)$. Hence, the percentage of the maximal fluorescence at time t, $I(t)$, may be written as

$$I(t) = \frac{2}{X_0} \sum_{k=2}^{M} B_k[F_k(t)] \tag{11}$$

where

$$B_k = \frac{1}{2^k} \left(\sum_{i=1}^{k'} \frac{k!}{(k-i)!i!} i + \sum_{i=k'+1}^{k-1} \frac{k!}{(k-i)!i!} (k-i)\alpha \right) \tag{12}$$

and

$$k' = \left[\frac{k\alpha}{1+\alpha} \right] \tag{13}$$

that is, k' is the largest integer less than or equal to $k\alpha/(1+\alpha)$.

It is worth noting that if $k \leq \alpha + 1$, then $k' = k - 1$ and the second summation in the definition of B_k drops out. This leaves

$$B_k = \frac{1}{2^k} \sum_{i=1}^{k-1} \frac{k!}{(k-i)!i!} i = \frac{k}{2^k}(2^{k-1} - 1) \tag{14}$$

which is identical with the first case treated, i.e., a large excess of DPA.

Calculating the percentage of the maximal fluorescence as a function of time requires only the concentrations of the various fused vesicle sizes, i.e., the values of $F_k(t)$. It may be of further interest to note that all of these equations remain valid if $[Tb] > {}^1/_3[DPA]$ provided $\alpha \equiv 3[Tb]/[DPA] > 1$.

Estimation of Tb/DPA Production from Tb/DPA Fluorescence and CF Leakage. The release of CF from the vesicles following the addition of Ca^{2+} is an indication that leakage of other encapsulated materials, such as Tb, DPA, and Tb/DPA, also occurs. This means that the measured Tb fluorescence intensity is an underestimation of the total amount of the fluorescing Tb/DPA complex produced by the fusion of Tb with DPA-containing vesicles. In the following equations, we show how to estimate the total amount of the Tb/DPA complex produced, i.e., the fraction of maximal fluorescence, $I(t)$, as given in eq 11.

It is assumed that the release of CF equals the release of other trapped materials, i.e., it is a measure for the release of vesicle contents in the fusion assay. In addition, it is assumed that no release occurs from vesicles which do not fuse. The latter assumption is supported by the fact that no detectable leakage occurs during a period of several minutes before the addition of Ca^{2+} or after the addition of EDTA to the medium. For simplicity, we will further assume that the leakage occurs mainly during the fusion events. With these assumptions, a rough estimate for the quantity $I(t)$ is given by

$$I_M(t) + {}^1/_2 L(t) < I(t) < I_M(t) + L(t) \tag{15}$$

in which $L(t)$ is the measured fraction of the maximal CF release and $I_M(t)$ is the measured fraction of maximal Tb/DPA fluorescence. The origin of this relation is simple. In the early stage of fusion, when there are only monomers and fused dimers, the amount of Tb/DPA complex which has leaked into the medium is proportional to half the concentration of dimers, since only half of them contain the complex. The fraction of CF leakage at this stage is equal to the dimer concentration times the same proportionality constant. So ${}^1/_2 L(t)$ gives the fraction of Tb/DPA complex which has leaked, and $I(t) = I_M(t) + {}^1/_2 L(t)$. At an advanced stage of fusion, when almost all the vesicles have undergone at least one fusion event, then $L(t)$ becomes equal to the fraction of the originally encapsulated CF which has leaked from each of the vesicles. Hence, $L(t)$ is equal to the fraction of Tb/DPA complex which has leaked into the medium, and $I(t) = I_M(t) + L(t)$. At intermediate stages, the relationship between $L(t)$ and Tb leakage must be calculated according to a model which relates leakage to the order of the fused structure.

We choose to make the simplest assumption about the relationship between leakage and fusion: the amount of material which has leaked from a fused vesicle is proportional to the number of original vesicles in it. That is, each original vesicle now comprising the fused F_k vesicle releases a fraction, β, of its encapsulated volume by leakage into the medium. The

fraction of the maximal CF release by k-mers is $\beta k[F_k(t)]/X_0$ where X_0 is the total concentration of original vesicles, and the fraction of the maximal CF release, $L(t)$, is given by

$$L(t) = \sum_{k=2}^{M} \beta k[F_k(t)]/X_0 \quad (16)$$

But since the total amount of lipid is conserved during fusion

$$X_0 = \sum_{k=2}^{M} k[F_k(t)] + [V_1(t)] \quad (17)$$

hence

$$L(t) = \beta(X_0 - [V_1(t)])/X_0 = \beta(1 - [V_1(t)]/X_0) \quad (18)$$

Equation 18 simply states that the measurable leakage, L, is given by the leakage from each vesicle times the fraction of original vesicles which have fused at least once.

Now, let us consider the loss of Tb/DPA fluorescence due to leakage. In an F_k vesicle with i T vesicles and $(k - i)$P vesicles, the amount of Tb and DPA which remains encapsulated is $i(1 - \beta)$[Tb] and $(k - i)(1 - \beta)$[DPA], respectively. Hence, regardless of which reagent is concentration limiting to the Tb fluorescence, the measured fraction of the maximal Tb fluorescence, $I_M(t)$, is less than $I(t)$ due to leakage and

$$I_M(t) = (1 - \beta)I(t) \quad (19)$$

Combining eq 18 and 19 gives the relation between the measurable fraction of the maximal Tb fluorescence, $I_M(t)$, and the fraction of maximal Tb/DPA produced, $I(t)$, as

$$I(t) = I_M(t)/\left\{1 - \frac{L(t)}{1 - [V_1(t)]/X_0}\right\} \quad (20)$$

where $L(t)$ is the measured fraction of the maximal CF release at time t.

Experimental Procedures

Phosphatidylserine (PS; from bovine brain) small unilamellar vesicles (SUV) were prepared in either (a) 15 mM $TbCl_3$ and 150 mM sodium citrate, (b) 150 mM dipicolinic acid (DPA, sodium salt), or (c) 100 mM carboxyfluorescein (CF, sodium salt). In addition, these media contained 2 mM L-histidine and 2 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes) adjusted to a final pH of 7.4. PS was sonicated in aqueous medium at a concentration of 10 μmol/mL for 1 h in a bath-type sonicator under argon at 20 °C. Subsequently, the preparations were centrifuged for 1 h at 115000g in a Beckman SW 50.1 rotor. The supernatant containing more than 95% of the lipid material was used as the vesicle preparation. Nonencapsulated material was separated from the vesicles by gel filtration on Sephadex G-75. The elution buffer was 100 mM NaCl, 2 mM L-histidine, 2 mM Tes, and 1.0 mM EDTA, pH 7.4. The amounts of the above compounds encapsulated in the vesicles, determined as described (Wilschut et al., 1980), were 1.7 (Tb), 16.3 (DPA), and 9.1 (CF) nmol/μmol of lipid.

Fluorescence and light-scattering measurements were performed simultaneously with an SLM-4000 fluorometer (SLM Instruments, IL) equipped with two emission channels. The temperature of the sample was maintained at 25 °C, and the solution was stirred magnetically. Measurements were carried out in a final volume of 1.0 mL of 100 mM NaCl, 2 mM L-histidine, 2 mM Tes, and 0.1 mM EDTA, pH 7.4, at a total lipid concentration of 50 μM. In the fusion measurements, the ratio of Tb to DPA vesicles was 1:1, 25 μM each. To initiate the fusion reaction, $CaCl_2$ was added as a 100 mM solution directly to the cuvette with a Hamilton syringe (final concentration 1.5 mM or 1.25 mM, as noted). Termination of the fusion reaction was achieved by addition of 30 μL of a 100 mM EDTA solution, that was pretitrated with NaOH to compensate for the release of hydrogen ions accompanying binding of Ca^{2+} to EDTA at pH 7.4. The Tb/DPA complex was excited at 276 nm; fluorescence was measured at 545 nm. A Corning 3-68 cutoff filter was placed between the sample and the monochromator to prevent second-order light scattering from disturbing the fluorescence signal. Light scattering was measured in the second emission channel with a Corning 7-54 band-pass filter. CF was excited at 493 nm, while emission was detected at >530 nm through a Corning 3-68 cutoff filter. Complete release of CF from the vesicles was obtained by addition of Triton X-100 (0.1% v/v). A more extensive description of the above methodology as well as the sources of the various chemicals used can be found in Wilschut et al. (1980).

Results

Fusion was induced in a 1:1 mixture of Tb and DPA vesicles by introduction of $CaCl_2$. The time development of the Tb fluorescence is presented in column 2 of Table I. The Tb fluorescence is expressed as a percentage of the maximal value; i.e., it represents the fraction of the total amount of Tb present that has become associated with DPA.

The fluorescence of the Tb/DPA complex is reduced from its potential value (expected from perfect mixing) by the release, during or after fusion, of a fraction of the vesicle contents into the external EDTA- and Ca^{2+}-containing medium, which will result in quenching of the fluorescence. Therefore, we measured the release of vesicle contents in a separate experiment, under otherwise identical conditions, employing the relief of self-quenching of carboxyfluorescein (CF) after dilution (Blumenthal et al., 1977). The results are shown in column 3 of Table I. Column 4 of Table I presents the experimental Tb fluorescence intensities after correction for the release of vesicle contents (see Theoretical Section on leakage, eq 20).

The next step in the procedure was to take the original concentration of vesicles (in units of mol of vesicles/L) and to choose a rate constant, C_{11}, which gives an approximate match to the experimental total percent of fluorescence at an initial time (column 4 of Table I) with the calculated value expected at this time by using eq 3–14. The calculated values for the percentage of the maximal Tb fluorescence shown in column 5 are obtained from solving the mass action kinetic equation, eq 3, by using the above rate constant. We have not attempted to optimize the fit to the data.

From the value of C_{11}, we can also calculate the fraction of the original dispersion of vesicles which has undergone at least one fusion event, which is simply one minus the fraction of vesicles which remains in the original sonicated state. These values are shown in column 6 of Table I.

The results in columns 4 and 5 of Table I indicate that the calculated levels of Tb/DPA complex formation simulate reasonably well the experimental results, once they have been corrected for leakage. As time goes on, the calculated values progressively overestimate the experimental values. This is expected because the calculations have not taken into account entry of Ca^{2+} and/or EDTA into the fused vesicles, which could result in quenching of the Tb fluorescence. This possibility is supported by the fact that the degree of this overestimation is larger in Table I(B), in the presence of 1.5 mM Ca^{2+}, than in Table I(A), where Ca^{2+} concentration in the medium is 1.25 mM.

Table I: Kinetics of Ca^{2+}-Induced Fusion of Sonicated PS Vesicles and Production of Tb/DPA Complex

time (s)	Tb fluorescence, % of max exptl, $I_M(t)$	CF leakage, % of max, $L(t)$	Tb fluorescence, % of max exptl cor for leakage,[a] $I(t)$	Tb fluorescence, % of max calcd[b]	calcd % of fused vesicles[c]
		(A) 1.25 mM Ca^{2+} (0.1 mM EDTA)			
6	5.0	3.4	7	8	15
12	10.0	5.4	14	15	29
18	14.9	7.6	20	20	36
24	19.6	8.6	24	25	44
30	24.2	10.3	30	29	50
60	36.6	15.0	46	45	69
120	39.7	23.5	56	62	83
		(B) 1.5 mM Ca^{2+} (0.1 mM EDTA)			
3	12.5	3.2	14	16	31
6	25.0	6.0	29	28	48
12	40.0	11.1	48	43	67
18	44.9	13.9	55	53	77
24	46.6	16.0	58	60	82
30	46.9	18.0	59	66	86
60	43.6	28.5	63	80	94

[a] The values in this column are obtained from the experimental quantities in the second and third columns of the table by using eq 20. Note that a rough estimate can be obtained by using eq 15. [b] The values in this column are calculated by solving for the set of kinetic equations, eq 3, and by using eq 9–14. [c] The values in this column indicate the percent of lipid in a fused state; e.g., when the value in Table I (A) is 83, the percent of vesicles in a monomer state is 17.

Table II: Distribution of Vesicle Sizes during Ca^{2+}-Induced Fusion of Sonicated PS Vesicles in 1.5 mM Ca^{2+}

k[a]	radius (Å)	% of initial no. of particles at time t:[b] $[F_k(t)]/X_0$ $t = 6$ s	$t = 30$ s
1	125	52	14
2	167	18	12
3	199	3	5
4	227	0.9	4
5	250	0.14	2
10	344	7×10^{-5}	0.1
20	477	10^{-11}	3×10^{-4}

[a] k represents the order of fusion: $k = 1$, nonfused; $k = 2$, one fusion or dimer, etc. [b] The numbers in these columns indicate the percent of vesicles in a given fusion state relative to the initial number of vesicles. The percent of lipid in a given fusion state k is obtained by multiplication with k.

Table III: Light Scattering Intensity at 90° after Termination of Ca^{2+}-Induced Aggregation and Fusion of Sonicated PS Vesicles by Addition of EDTA[a]

Ca^{2+} (mM)	time (s)	intensities relative to dispersed state exptl	calcd	calcd % of fused vesicles
2.0	9	4.1	4.6	91
1.5	5	2.1	1.9	44
	10	2.7	2.5	62
	25	4.7	3.6	83
	60	5.9	5.1	94
1.25	64	4.6	2.8	70

[a] The calculations employ eq (8, 1, 11), (8, 1, 12), (8, 1, 20), and (8, 1, 21) in Kerker (1969) for the scattering from hollow spheres. The external radius of a sonicated vesicle was taken as 125 Å, and the bilayer thickness is 40 Å. For fused vesicles, the bilayer thickness was taken as 45 Å to account for expected thickening following calcium binding. The radii are calculated by assuming conservation of lipid during fusion (see Table II for values of the radii). The calculations employed a value of $\lambda = 2075$ Å for the wavelength in the medium. The value of λ in air is 2760 Å.

The reaction rates which give the above fit to the experimental fusion reaction of PS vesicles are very sensitive to Ca^{2+} concentrations, the values of C_{11} are 5.8×10^6 and 1.3×10^6 M^{-1} s^{-1} in the presence of 1.4 and 1.15 mM Ca^{2+}, respectively, which corresponds to 1.5 and 1.25 mM Ca^{2+} in the presence of 0.1 mM EDTA.

Table II shows the calculated distribution of vesicles in the presence of 1.5 mM Ca^{2+} at two times. The second column gives the radii of vesicles. In our calculations, we have assumed a spherical shape with an initial radius of 125 Å for the sonicated vesicles. During the fusion process, the amount of lipid is assumed to be conserved. The third and fourth columns indicate what is the percent of particles in a given fusion state. As noted in connection with eq 3–5, the quantity M is the largest size allowed in a particular run of the program. The program can allow $M = 100$ or above, but in practice $M = 32$ has been used. As is indicated in Table II, the calculated fraction of particles of size $k = 20$ is negligibly small for the times of interest. Hence, even the use of $M = 20$ could suffice.

Another test for our analysis arises from predicting the relative change in light scattering at the given wavelength due to increase in the size of vesicles. We employ a formula given by Kerker (1969) for the scattered intensity due to a shell structure [see also Oster & Riley (1952) and Chong & Colbow (1976)]. In our calculations, we have assumed a spherical shape, and the program gives the radii and distribution of vesicle sizes as illustrated in Table II. It has to be emphasized that using expressions for simple Rayleigh scattering, i.e., considering the vesicles as small solid spheres, would have given unrealistically large relative scattering intensities.

The program calculates the relative scattered intensity at an angle θ and at a time t from the equation

$$I(\theta,\lambda,t) = \sum_{k=1}^{M} I_k(\theta,\lambda)[F_k(t)]/X_0 \qquad (21)$$

where $I_k(\theta,\lambda)$ is the scattered intensity of a k-mer (i.e., a vesicle resulting from $k-1$ fusion events) at a scattering angle θ and at a given wavelength λ, relative to that of a monomer of a shell structure. The quantities $[F_k(t)]$ have the same meaning as in eq 3 and are the concentrations of fused k-mers at time t; likewise, X_0 is the concentration of monomers, i.e., the dispersed sonicated vesicles at the beginning of the fusion experiment.

In Table III the calculated values for the intensity of scattered light at 276 mM and 90° relative to the dispersed state as well as the corresponding experimental values are shown. The latter data were obtained by determination of the relative increase in light scattering after termination of the

fusion reaction by addition of excess EDTA (see Experimental Procedures). It is clear that there is a good correspondence.

Discussion

Rate-Limiting Step in Vesicle–Vesicle Fusion: Close Approach. An obvious prerequisite for the fusion of vesicles is their close approach, or aggregation. In the framework of the DLVO theory (Derjaguin & Landau, 1941; Verwey & Overbeek, 1948), PS vesicles are mutually attracted by van der Waals interactions and repelled by electrostatic interactions, due to their net negative surface charge. Thus, cations which have large binding capacity to PS are effective in causing their charge neutralization. For instance, Ca^{2+} is more effective in charge neutralization than Mg^{2+} (Newton et al., 1978; Nir et al., 1978; Nir & Bentz, 1978). Calculations of electrostatic interactions between PS vesicles have been described in detail in Nir & Bentz (1978) and Nir et al. (1980), who supplemented the equations of Wiese & Healy (1970) by explicit consideration of the binding of cations such as Ca^{2+}, Mg^{2+}, and Na^{2+}. Details of van der Waals interactions between PS vesicles are given in Nir (1977), Nir & Andersen (1977), and Nir & Bentz (1978). Several recent studies emphasized that at short distances of separation between lipid surfaces (of the order of 5–10 diameters of water molecules) there are strong repulsive forces which can be termed as structural forces (LeNeveu et al., 1977; Mitchell et al., 1978; Van Megen & Snook, 1979). However, as recently summarized by Overbeek (1980), there is still a good deal of argument about the magnitude and the range of these structural forces. For sonicated vesicles, in particular, their magnitude has not been assessed.

The results of Nir & Bentz (1978) and Nir et al. (1980) demonstrated that the present theory is capable of predicting the threshold concentrations of Ca^{2+}, Mg^{2+}, and Na^{2+} required for fast aggregation of PS vesicles. More recently, Bentz & Nir (1980, 1981) demonstrated that the kinetics of sodium-induced aggregation of PS vesicles (Day et al., 1980) can be simulated by the mass action model with the rate $\bar{C}_{11}$ calculated by the Smoluchowski (1917) and Fuchs (1934) expression with potential barrier given as the sum of attractive van der Waals interactions and repulsive electrostatic interactions. Thus, we are confident that our calculated rates for aggregation, $\bar{C}_{11}$, are reliable, at least within an order of magnitude. The calculated value of the rate of aggregation, $\bar{C}_{11}$, for sonicated PS vesicles in the presence of 100 mM NaCl and 1.15 mM Ca^{2+} is 2×10^6 M^{-1} s^{-1}. In comparison, the value obtained in the present work for the average rate of the overall fusion reaction of sonicated PS vesicles in 1.25 mM Ca^{2+} and 0.1 mM EDTA (see Table I, footnote *a*) is 1.3×10^6 M^{-1} s^{-1}. Likewise, with 1.4 mM Ca^{2+}, the estimated rate constant for fusion is 6×10^6 M^{-1} s^{-1} while the rate constant for aggregation is calculated to be 4×10^6 M^{-1} s^{-1}. These two rates are essentially equal given the uncertainties in the binding constant of Ca^{2+} (Nir et al., 1978) and in the fusion data. The conclusion we reach is that there is no significant delay due to the fusion reaction per se. Hence, from data currently available, we may suggest that the close approach of vesicles to each other is the rate-limiting step in Ca^{2+}-induced fusion of sonicated PS vesicles. This statement is, of course, limited to situations where the fusion reaction can occur, as is the case in the experiments of Wilschut et al. (1980), who chose a proper temperature. As pointed out (Papahadjopoulos et al., 1977), little fusion of PS vesicles occurs below the phase transition temperature of the lipid (in the presence of the buffer), which means that under these conditions the delay in the overall fusion reaction due to the fusion event per se would be very long, whereas no significant delay in aggregation is to be expected, in accord with the above observation.

Although the present work is limited to a study of small unilamellar vesicles, the conclusion that the mutual close approach of vesicles is the rate-limiting step of the overall fusion reaction is also supported by results obained with larger unilamellar vesicles (Wilschut et al., 1980). The binding of Ca^{2+} to the larger vesicles is essentially identical with its binding to small vesicles (C. Newton, personal communication). By using the same binding constant of Ca^{2+} to PS, we find that even with 2 mM Ca^{2+} the value of $\bar{C}_{11}$ would be of the order of 10^5 M^{-1} s^{-1} for vesicles of radii 300–500 Å. This value is much lower than the value for vesicles of a radius of 125 Å in 1.15 mM Ca^{2+}. Hence, larger Ca^{2+} concentrations are required in order to increase the aggregation rates of larger vesicles. Our calculations indicate that in the presence of 5 mM Ca^{2+} the values of $\bar{C}_{11}$ are of the order of 10^6–10^7 M^{-1} s^{-1} for vesicles of radii 300–500 Å, i.e., similar to the value of $\bar{C}_{11}$ in the presence of 1.5 mM Ca^{2+} for the small vesicles. We note that at the same vesicle concentration the rate of fusion of the larger vesicles in the presence of 5 mM Ca^{2+} is approximately the same as the rate of fusion of the small vesicles in the presence of 1.75 mM Ca^{2+} (Wilschut et al., 1980).

The fact that for a given Ca^{2+} concentration the value of $\bar{C}_{11}$ decreases with vesicle radius is readily understood by recalling that the potential barrier, V, acting between two vesicles is approximately proportional to the vesicle radius (Wiese & Healy, 1970), whereas the value of $\bar{C}_{11}$ is approximately proportional to $\exp(-V/kT)$ (Verwey & Overbeek, 1948; Nir et al., 1980), where k is the Boltzmann constant and T is the absolute temperature.

Leakage vs. Fusion. It had been shown previously that Ca^{2+} induces massive aggregation of PS vesicles, release of vesicle contents, and eventually formation of large, collapsed, cylindrical structures, called cochleates (Papahadjopoulos et al., 1977; Portis et al., 1979). In these final structures, the Ca^{2+}/PS ratio is 0.5, while the lamellar repeat distance has become very short (53 Å), indicating almost complete absence of water between the bilayers (Newton et al., 1978; Portis et al., 1979). Yet, the formation of the cochleates is presumed to result from the fusion of the original vesicles. Recently, Ginsberg (1978) questioned the significance of the Ca^{2+}/PS system as a model for membrane fusion and suggested that divalent cations cause mere lysis of PS vesicles rather than their fusion. Nir & Pangborn (1979) opposed this suggestion, and argued that the release of vesicle contents is nearly second order with respect to the vesicle concentration (Portis et al., 1979), indicating the dependency of the leakage on vesicle–vesicle contact. Finally, Wilschut et al. (1980) demonstrated that Ca^{2+} induces very rapid fusion of PS vesicles and that the release of vesicle contents is largely a secondary phenomenon—related to the collapse of the vesicles following their fusion. In these latter studies, it was estimated that the actual fusion of small unilamellar vesicles is accompanied by the release of only 10% of the encapsulated volume. Our present analysis strongly supports this conclusion. For example, in Table I(B), we see that after 12 s about 67% of the vesicles have undergone at least one fusion event while only 11% leakage is observed. After 30 s, when 82% of the total vesicle content is still retained, only 14% of the vesicles are left in a nonfused state, while by that time a significant proportion of the vesicles has undergone multiple fusion events (Table II). It is of interest to note that if we denote 100% fusion as the hypothetical situation where all the vesicles have

fused once (e.g., 100 fusion events per 200 vesicles) then in the latter case there is 119% fusion (i.e., 119 fusion events per 200 vesicles) whereas the leakage is 18%. The results in Table I as well as other results of Wilschut et al. (1980) indicate that with larger Ca^{2+} concentrations less leakage occurs for the same degree of fusion. In this respect, it should be mentioned that the retention of contents during Ca^{2+}-induced vesicle fusion has also been demonstrated by Liao & Prestegard (1979) for vesicles composed of a mixture of phosphatidic acid and phosphatidylcholine (PC), and by Hoekstra et al. (1979) for PS/PC vesicles. It is noteworthy that leakage of vesicle contents can never be completely ruled out during fusion events. The exchange of material between the medium and the interior of the vesicles can be expected whenever the internal volume of the combined compartment differs from the sum of the internal volumes of the fusing vesicles. When spherical vesicles fuse to form a larger spherical vesicle, the fused vesicle has a larger internal volume than the sum of the internal volumes of the constituent vesicles. For example, for the system treated here, let V denote the internal volume of the original vesicles. Then a fused dimer has an internal volume of nearly $4V$, while a fused decamer has an internal volume of over $40V$. However, with PS vesicles in these later stages, which require more experimental elucidation, the vesicular structure collapses, and internal volume is lost.

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