Temperature Dependence of Divalent Cation Induced Fusion of Phosphatidylserine Liposomes: Evaluation of the Kinetic Rate Constants†

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ABSTRACT: The effect of temperature and divalent cation binding (Ca^{2+}, Sr^{2+}, Ba^{2+}) on the kinetic rate constants of aggregation and fusion of large phosphatidylserine liposomes is measured for the first time. Fusion is monitored by the Tb^{3+}/dipicolinate assay. Fusion rate constants increase with temperature (15–35 °C) in a roughly linear fashion. These rate constants are not otherwise sensitive to whether the temperature is above or below the phase transition temperature of the Ba^{2+} or Sr^{2+} complex of phosphatidylserine, as measured by differential scanning calorimetry. Hence, the isothermal transition of the acyl chains from liquid-crystalline to gel phase induced by the cations is not the driving force of the initial fusion event. The aggregation rate constants increase with temperature, and it is the temperature dependence of the energetics of close approach of the liposomes which underlies this increase. On the other hand, the aggregation becomes more reversible at higher temperatures, which has also been observed with monovalent cation induced liposome aggregation where there is no fusion. Calculations on several cases show that the potential energy minimum holding the liposome dimer aggregates together is ~5–6 kT deep. This result implies that the aggregation step is highly reversible; i.e., if fusion were not occurring, no stable aggregates would form.

The fusion of unilamellar liposomes among themselves (Nir et al., 1983a; Bentz et al., 1983a,b) or to planar bilayers (Düzgüneş & Ohki, 1981; Cohen et al., 1982) involves an aggregation step followed by the fusion event. Uncoupling the kinetics of these two steps is necessary to determine which physical forces cause two liposomes to aggregate and which cause them to fuse. The mechanisms of these events are contained in the kinetic rate constants of the reaction

$$V_1 + V_1 \underset{D_{11}}{\overset{C_{11}}{\rightleftharpoons}} V_2 \overset{f_{11}}{\rightharpoonup} F_2 \qquad (1)$$

where V_1 denotes the liposome, V_2 denotes the dimer aggregate, and F_2 denotes the fused doublet, i.e., when the bilayers have merged and the encapsulated contents of the liposomes have mixed. Recently, we have established the theoretical and experimental methodology necessary to obtain these rate constants (Wilschut et al., 1980, 1981; Nir et al., 1980b, 1982, 1983a; Bentz et al., 1983a,b). Under conditions of aggregation and fusion, there will be higher order reactions occurring; however, monitoring the fusion at early times can yield the kinetic rate constants of this first reaction (Bentz et al., 1983a). Here we use this methodology to extract these primary rate constants for Ca^{2+}-, Ba^{2+}-, and Sr^{2+}-induced fusion of phosphatidylserine liposomes (PS LUV,[1] diameter ~0.1 μm).

The dependence of the fusion rate constant (f_{11}) on the lipid composition, electrolyte content, and temperature is a direct measure of the molecular events involved in the destabilization of the apposed bilayers and the resealing of the fused doublet. The aggregation rate constant (C_{11}) reflects the balance of forces which mediates close apposition (Nir & Bentz, 1978; Parsegian et al., 1979; Bentz & Nir, 1981b). The dissociation rate constant (D_{11}), together with the value of C_{11}, can be used to estimate the depth of the potential energy minimum which holds the liposomes together in the aggregated state (Bentz & Nir, 1981a,b). Obtaining the rate constants of liposome aggregation by light scattering or absorbance requires very careful consideration of the interference effects and the geometry of the aggregate (Kerker, 1969; Nir et al., 1980a, 1981, 1983a; Bentz & Nir, 1981a,b). This is especially difficult when aggregation is accompanied by liposome fusion. Fortunately, with calibrated fusion assays (Nir et al., 1983a), liposomes which fuse can now provide one of the best experimental systems for testing and refining the theoretical models of aggregation. For this work, we have used the Tb/dipicolinate fusion assay which monitors the mixing of aqueous contents of the liposomes (Wilschut & Papahadjopoulos, 1979; Wilschut et al., 1980, 1983; Düzgüneş et al., 1981a,b; Bentz et al., 1983a,b).

The molecular mechanisms which underlie liposomal fusion have proven difficult to establish (Nir et al., 1983a). Studies using multilamellar liposome dispersions (MLV) have shown the morphologies accessible to the lipids (Hui et al., 1981, 1983; Verkleij, 1984) and the forces between average bilayers in the structure (Rand, 1981); however, the application of these data to the interaction between two unilamellar liposomes or biological vesicles is not simple. One such equilibrium property of PS MLV which has been considered germane to the driving

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[1] Abbreviations: DPA, dipicolinic acid; EDTA, ethylenediaminetetraacetate; LUV, large unilamellar vesicle(s) (diameter ~100 nm); MLV, multilamellar vesicle(s); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SUV, small unilamellar vesicle(s) (diameter ~30 nm); TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

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force for liposomal fusion is the shift in the gel–liquid-crystalline phase transition temperature, T_c, induced by divalent cations (Papahadjopoulos et al., 1977). Briefly [see Düzgüneş & Papahadjopoulos (1983) for a review], it was proposed that fusion would occur when the liposomes and divalent cations were incubated at a temperature above the T_c of the liposomes in Na^+ buffer alone and below the T_c of the lipid/metal ion complex as measured at equilibrium. Thus, initially the acyl chains of the liposome bilayer are in the fluid or liquid-crystalline phase, and upon divalent cation binding and liposome aggregation, the metal ion/lipid complexes induce the crystallization of the acyl chains. This phase transition was proposed as the driving force for the fusion reaction. Some studies attempted to test this point further (Sun et al., 1978; Vistnes & Puskin, 1981), but the results were inconclusive because they used Ca^{2+} and PS liposomes where the ionotropic T_c is above 100 °C, and whether fusion occurred above this temperature could not be tested. A recent study has used Sr^{2+} and Ba^{2+} with PS liposomes, whose ionotropic T_c's are in the range of 20–30 °C, and it was shown that the overall rate of fusion monotonically increased with temperature well above the ionotropic T_c (Düzgüneş et al., 1984). Therefore, the liposomes showed fusion at temperatures where the acyl chains were in the fluid phase throughout the reaction process. Consequently, the ionotropic phase transition temperature shift is clearly not necessary to induce fusion.

The following question still remains: What is the temperature dependence of the fusion reaction per se? While the overall rate of fusion of PS LUV with Ba^{2+} and Sr^{2+} (Düzgüneş et al., 1984) and with Ca^{2+} (Wilschut et al., 1985) increases with temperature, these kinetics are dominated by the aggregation rates. In this work, we have examined this problem, and the answer is that all of the rate constants (f_{11}, C_{11}, *and* D_{11}) increase with temperature. The fusion rate constants increase by 3–10-fold per 10 °C, and the aggregation rate constants increase by 1.5–2-fold per 10 °C.

Materials and Methods

Phosphatidylserine from bovine brain was purchased from Avanti Polar Lipids (Birmingham, AL) and stored as a chloroform solution under argon at −40 °C. $TbCl_3$ was obtained from Alfa (Danvers, MA); dipicolinic acid (DPA, 2,6-pyridinedicarboxylic acid), *N*-[tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid (TES), and L-histidine were from Sigma; NaCl was from Mallinckrodt (Paris, KY); $CaCl_2$, $BaCl_2$, and $SrCl_2$ were from Fisher. Cholate (Calbiochem) was recrystallized twice. Water was distilled twice, the second time in an all-glass apparatus, and further purified in a Barnstead Nanopure filtration system.

Large unilamellar liposomes were prepared by reverse-phase evaporation as described previously (Wilschut et al., 1980; Düzgüneş et al., 1983a) and contained either (i) 2.5 mM $TbCl_3$ and 50 mM sodium citrate, (ii) 50 mM DPA (Na salt) and 20 mM NaCl, or (iii) 1.25 mM $TbCl_3$, 25 mM sodium citrate, 25 mM DPA, and 10 mM NaCl. All solutions were buffered with 2 mM TES and 2 mM L-histidine, pH 7.4. Unencapsulated material was eliminated by gel filtration on Sephadex G-75 (Pharmacia) using 100 mM NaCl, 2 mM TES, and 2 mM L-histidine, pH 7.4 (NaCl buffer), and 1 mM EDTA as elution buffer. For calibration of the fusion assay, a portion of the Tb liposomes (type i above) was passed through another Sephadex G-75 column equilibrated with NaCl buffer (containing no EDTA). Liposome concentrations were determined by phosphate analysis (Bartlett, 1959).

To obtain a more uniform liposome size distribution, the following procedure was used. After the reverse-phase evaporation step, the liposomes were centrifuged at 10000*g* to remove multilamellar and very large liposomes. Normally, the upper four-fifths of the supernatant is collected for successive extrusion through 0.2- and 0.1-μm Bio-Rad polycarbonate membranes (Düzgüneş et al., 1981b). Here only the upper half of the supernatant was collected for extrusion. According to dynamic light scattering, the *Z*-average diameter of these extruded liposomes was 0.12 μm, whereas the ordinary procedure yielded 0.15 μm as a *Z*-average diameter.

For differential scanning calorimetry, liposomes were prepared in NaCl buffer containing 0.1 mM EDTA, incubated with the divalent cations for 30 min, concentrated by centrifugation at 10000*g* for 20 min, and placed in aluminum calorimeter pans. Thermograms were obtained at a scan rate of 5 °C/min and a sensitivity setting of 1 mcal/s in a Perkin-Elmer DSC-2 calorimeter. Liposomes in Na^+ buffer were concentrated by centrifugation at 150000*g* for 3 h at 4 °C.

The Tb fluorescence scale was calibrated by lysing the Tb liposomes (freed of EDTA) with 0.5% (w/v) sodium cholate in the presence of 20 μM free DPA and sonicated for 5 min under argon in a bath-type sonicator. The fluorescence value obtained was set to 100%. Fluorescence and 90° light-scattering measurements were made in an SLM 4000 fluorometer which allows simultaneous monitoring of fluorescence (excitation at 276 nm and emission at 545 nm, with a Corning 3-68 cutoff filter to eliminate contribution to the signal from light scattering, which was always less than 1.5% of the maximal Tb fluorescence intensity) and light scattering (using a Corning 7-54 band-pass filter). The output of the fluorometer was recorded on an Omniscribe chart recorder, at fast chart speeds when necessary. Additional details of the fusion assay have been described elsewhere (Wilschut et al., 1980; Bentz et al., 1983b).

The chelation of Tb by DPA is prevented outside the liposomes by the presence of the divalent cations and EDTA. Ba^{2+} and Sr^{2+} are very effective in quenching Tb fluorescence in the presence of 0.1 mM EDTA (Bentz et al., 1983b). The Tb/DPA complex which forms during the fusion of Tb and DPA vesicles will then be completely dissociated if it is released into the medium containing divalent cations and EDTA or if the medium enters the liposome interior. The dissociation was measured directly by encapsulating the Tb/DPA complex and following the decrease in fluorescence (initially set at 100%) when fusion was induced by divalent cations.

The *fusion experiment* starts with equal concentrations of Tb liposomes (type i above) and DPA liposomes (type ii above) and measures the fluorescence kinetics of mixing of the vesicles' contents during fusion. The *dissociation experiment* starts with Tb/DPA liposomes (type iii above) and measures the kinetics of dissociation of the Tb/DPA complex due to its leakage and the influx of divalent cations and EDTA into the liposomes during fusion. In fact, the fluorescence intensity measured at *any* time (either in the fusion experiment or in the dissociation experiment) equals the percentage of the total amount of Tb which is complexed with DPA at that time. With both types of experiments, 0.1 mL of the liposome stock was suspended into 0.9 mL of the NaCl buffer (0.1 mM final EDTA concentration) in a quartz cuvette of 1-cm path length and stirred continuously. Divalent cations were introduced by adding an aliquot of a concentrated solution (200 mM) with a Hamilton syringe.

The experimental procedure for obtaining the primary rate constants for fusion, given in eq 1, has been described in detail previously (Bentz et al., 1983a). Briefly, a 1:1 mixture of Tb-containing and DPA-containing liposomes is mixed with

the divalent cation solution, and the resultant fluorescence curve is denoted F. This curve shows the fraction of initially encapsulated Tb which is complexed with the DPA. Since the Tb/DPA complex is rapidly broken up by EDTA and divalent cations, the complex can exist only within the fused liposomes (Bentz et al., 1983b; Wilschut et al., 1980, 1983). Thus, following fusion there will be a loss of fluorescence due to the Tb/DPA complex leaking from the liposomes and the influx of medium into the fused structures. We correct for this by using liposomes containing Tb/DPA complex at the same total lipid concentration. Inducing these liposomes to fuse with the same divalent cation concentration yields a decrease in fluorescence due to this dissociation of the Tb/DPA complex. The percent of dissociation is given by the curve denoted D. As shown before (Bentz et al., 1983a,b), the sum of $F + 0.5D$ initially equals the total percent of Tb/DPA complex formed due to the fusion experiment, i.e., the amount which would be observed if there were no leakage out of or influx of medium into the fused liposomes.

From the mass-action kinetic model using eq 1 together with higher order reactions (e.g., $V_1 + F_2 \rightleftharpoons V_1F_2 \rightharpoonup F_3$), we have shown (Bentz et al., 1983a) that these corrected fusion curves for a 1:1 initial mixture of Tb- and DPA-containing liposomes can be accurately approximated by the equation[2]

$$F_{cor} \equiv F + 0.5D = 100A(t)\mathcal{F}(t) \qquad (2)$$

where

$$A(t) = (1 + 4\hat{C}_{11}X_0t)^{1/4} - 1$$

$$\mathcal{F}(t) = 1 + \frac{\exp(-\hat{f}_{11}t) - 1}{\hat{f}_{11}t}$$

$$\hat{C}_{11} = C_{11}/(1 + K_r)$$

$$\hat{f}_{11} = f_{11}(1 + K_r)$$

$$K_r = D_{11}/f_{11}$$

and X_0 is the liposome concentration. We note that $\mathcal{F}(0) = 0$, $\mathcal{F}(\infty) = 1$ and that eq 2 is an adequate substitute for the exact numerically integrated solutions when $\hat{C}_{11}X_0t \leq 0.5$.

When the lipid concentration is very low, so that the overall fusion reaction is aggregation rate limiting (i.e., $\hat{C}_{11}X_0t \ll 1 \ll \hat{f}_{11}t$), then $\mathcal{F} \sim 1$ and the corrected fusion curve can be fitted by $A(t)$ which yields the value of $\hat{C}_{11}$. At higher lipid concentrations, the overall fusion reaction will become increasingly rate limited by the fusion reaction, per se, and the value of f_{11} can be obtained from these data. Here we have always used at least three different lipid concentrations, and often four, to verify the fitting of $\hat{C}_{11}$ and $\hat{f}_{11}$ since eq 2 must hold for each lipid concentration.

If it is the case that $D_{11} \ll f_{11}$, then this procedure would yield the values of the true rate constants, i.e., $\hat{C}_{11} = C_{11}$ and $\hat{f}_{11} = f_{11}$ since $K_r = D_{11}/f_{11} \ll 1$. While each combination of C_{11}, f_{11}, and D_{11} yields a unique mathematical curve for F_{cor}, we have shown that using a 1:1 mixture of Tb to DPA liposomes can only yield reliable estimates for $\hat{C}_{11}$ and $\hat{f}_{11}$ due to the experimental uncertainty, which is only ±1% (Bentz et al., 1983a). In order to fit the value of D_{11}, and hence the true values of C_{11} and f_{11}, it is sufficient to use a 1:9 mixture of Tb to DPA liposomes at a high lipid concentration (Bentz et al., 1983a). This experiment will yield the value of K_r, and hence, with the known values of $\hat{C}_{11}$ and $\hat{f}_{11}$, one obtains the values for each of the primary rate constants C_{11}, D_{11}, and f_{11}. For fitting these data, we have estimated that there are 80000 PS molecules per liposome assuming a 0.1-μm diameter liposome with a 50-Å bilayer thickness and 70 Å^2 per PS head group (Bentz et al., 1983a).

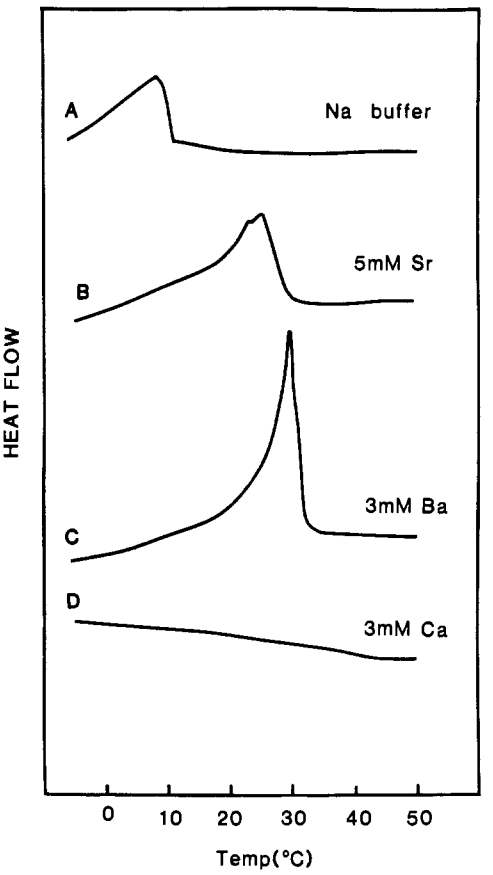

FIGURE 1: Endothermic gel–liquid-crystalline phase transitions for PS LUV using differential scanning calorimetry. In scan A, the liposomes are incubated in Na^+ buffer alone, and the gel–liquid-crystalline transition occurs between −5 and 12 °C with a peak temperature $T_c \sim 6$ °C. Scan B was obtained from the PS LUV incubated in Na^+ buffer plus 5 mM Sr^{2+} with $T_c \sim 24.5$ °C. Scan C shows the effect of 3 mM Ba^{2+} plus Na^+ buffer, where $T_c \sim 29$ °C. Finally, in scan D, it is seen that 3 mM Ca^{2+} shifts the T_c above 50 °C. Actually, the T_c exceeds 100 °C in this case (Portis et al., 1979).

RESULTS

Figure 1 shows the phase behavior of the PS LUV in the absence and presence of the divalent cations. In 100 mM Na^+, pH 7.4, the liposomes show a broad endothermic transition between −5 and 12 °C with a peak temperature, T_c, of 6 °C (scan A). In the presence of the divalent cations, the liposomes have fused to multilamellar equilibrium structures. In 5 mM Sr^{2+}, the T_c is shifted to 24.5 °C (scan B); in 3 mM Ba^{2+}, the T_c is shifted to 29 °C (scan C); in 3 mM Ca^{2+}, the T_c is shifted to above 100 °C [scan D and see Portis et al., (1979)]. Previously, it was found for 10 mM Sr^{2+} and 10 mM Ba^{2+} that

[2] More refined corrections are made when the fusion kinetics are analyzed (Nir et al., 1980b; Bentz et al., 1983a,b). When we denote the fusion signal corrected for complex dissociation by F_{cor}, then the observed fusion signal, F, and the observed dissociation signal, D, rigorously yield $F_{cor} = F + \delta D$, where the value of δ depends upon the primary rate constants. Initially, $\delta = 0.5$ as explained in Bentz et al. (1983a), and in time $0.5 \leq \delta \leq 1.0$. For the quantitative analyses presented in this paper, δ has been computed via numerical integration of the mass-action kinetic equations (Bentz et al., 1983a). However, for all practical purposes, since we only deal with the initial kinetics, taking $\delta = 0.5$ does not entail any serious error.

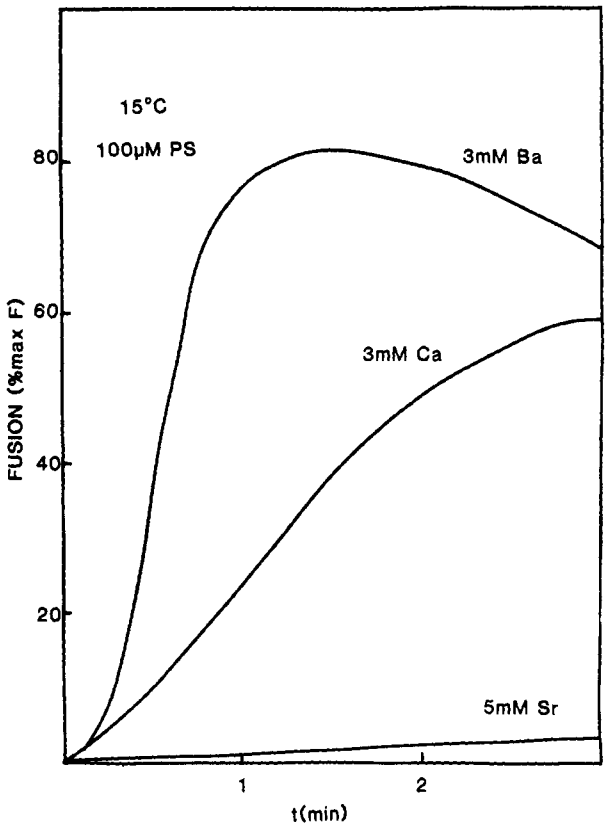

FIGURE 2: Fusion of PS LUV at 15 °C with 3 mM Ba^{2+}, 3 mM Ca^{2+}, and 5 mM Sr^{2+}, plus Na^+ buffer. For all cases, 50 μM Tb liposomes and 50 μM DPA liposomes were mixed to yield 100 μM total lipid. Fusion is given by the percent of maximal fluorescence which would be achieved if all of the Tb were chelated by the DPA.

the respective T_c values are 27 and 31.5 °C (Düzgüneş et al., 1984). Hence, the value of T_c is fairly insensitive to the amount of divalent cation added once there is enough to induce aggregation and fusion. Thus, at 35 °C, the fusion induced by Ba^{2+} and Sr^{2+} [see Figure 3 and Düzgüneş et al. (1984)] occurs with liposomes whose acyl chains remain in the liquid-crystalline state throughout the process.

Figure 2 shows the fusion of the PS LUV (100 μM lipid) at 15 °C as induced by 3 mM Ba^{2+}, 3 mM Ca^{2+}, and 5 mM Sr^{2+} in 100 mM Na^+ buffer. It is noteworthy that with 3 mM Ba^{2+} over 80% of the encapsulated Tb^{3+} has complexed with the DPA inside of the fusing liposomes within 1–2 min. If all of the liposomes underwent two rounds of fusion (yielding fused triplets) and there were no dissociation of complex, then we would expect only 75% maximum fluorescence, since only three-fourths of the fused triplets would contain both Tb and DPA, while the other fourth would contain only Tb or DPA (Wilschut et al., 1980; Nir et al., 1980b). Since in reality the fusion process follows mass-action kinetics, we know that even after 2 min there are significant concentrations of unfused monomers as well as many higher order fusion products (fused quadruplets, quintuplets, etc.). In fact, by use of the aggregation rate constant derived below (cf. Table I) for the case of Ba^{2+}, the average aggregate size (regardless of the extent to which the liposomes within the aggregate have fused together) is about 4.3 after 1 min and about 7 after 2 min [see eq 10 in Bentz & Nir (1981a)]. As we will show below, the aggregation rate constants for each of the divalent cation cases are not too different. As a result, the lack of fusion with Sr^{2+} is due only to its very small fusion rate constant at this temperature.

These data, especially for Ba^{2+} and Ca^{2+}, suggest that there is very little Tb/DPA complex dissociation occurring initially. Figure 3 shows the dissociation of the preencapsulated Tb/DPA complex induced by 3 mM Ba^{2+} as a function of temperature. Clearly, as the temperature is lowered, the dissociation of complex, due to both the leakage of complex into the medium and the influx of medium into the fused liposomes, also occurs more slowly. The sharp decrease in the dissociation reaction between 25 and 15 °C is due to decreases in both the aggregation and fusion rates as we will see below. The data for Ca^{2+} and Sr^{2+} show a similar temperature dependence.

The stars on the curves in Figure 3 show the times at which the fusion curves (see Figure 2) reach their maximal values. From Figure 2, we see that at 15 °C the fusion signal for 3 mM Ba^{2+} reaches a maximum at about 1.5 min and it declines thereafter. Although the liposomes continue to fuse, producing more Tb/DPA complex, there is a net loss in the amount of Tb/DPA complex due to leakage and the influx of medium. The fusion curves for 3 mM Ba^{2+} at 25 and 35 °C are not shown, but the stars show that once the dissociation process reaches 30–40% the additional fusion reactions cannot compensate for the loss of fluorescence due to complex dissociation.

In Figure 4 we show how the fusion and dissociation curves can be combined to obtain the total fusion signal that would occur if there were no Tb/DPA complex dissociation, i.e., if the internal contents of the liposomes merged without mixing with the external medium. The case shown is 3 mM Ba^{2+} with 2 μM PS at 25 °C. The signal obtained from the fusion experiment is denoted F, and the signal from the dissociation experiment is denoted D. As explained previously (Bentz et al., 1983a,b), during the initial stages one can simply take the sum of $F + 0.5D$ to give the total fluorescence signal that would be obtained in the fusion experiment if there were no Tb/DPA complex dissociation occurring.[2] This summation is shown by the closed circles (●) in the figure. The dashed line shows the fit to these data after assuming that the aggregation reaction is completely rate limiting and that the aggregation rate constant $\hat{C}_{11} = 2.8 \times 10^7$ M^{-1} s^{-1}.

When the aggregation is completely rate limiting, the fusion data corrected for dissociation depend only upon the product of the liposome or lipid concentration, denoted X_0, and the time t (Bentz et al., 1983a; Ellens et al., 1984). Because the fluorescence curves are normalized by the lipid concentration in each case, the aggregation kinetics depend on the first, not the second, power of the lipid concentration. Figure 5 shows the corrected fluorescence data in symbols for the fusion induced by 3 mM Ca^{2+} at 15 °C plotted vs. X_0t (in units of micromolar-minute) for 10, 50, and 100 μM total lipid concentration. For a given value of X_0t, we find that there is less fluorescence as the lipid concentration increases. This proves that the fusion reaction per se is inhibiting the overall process in this case; otherwise, all of the data would lie on the same curve. The solid lines show the theoretical curves for $\hat{C}_{11} = 8 \times 10^6$ M^{-1} s^{-1} and $\hat{f}_{11} = 0.05$ s^{-1}. The dashed line shows the curve for purely aggregation rate-limiting fusion with the same $\hat{C}_{11}$ value.

The final step of the procedure for obtaining the primary rate constants is measuring the corrected fusion data for a 1 to 9 mixture of Tb-containing to DPA-containing liposomes (see Materials and Methods). Figure 6 shows some of the data and the corresponding fitted curves. For 3 mM Ca^{2+} at 25 °C, we find that $D_{11}/f_{11} \sim 0$; with 3 mM Ba^{2+} at 35 °C, $D_{11}/f_{11} \sim 2$; and with 5 mM Sr^{2+} at 35 °C, $D_{11}/f_{11} \sim 1$. As explained in Bentz et al. (1983a), $\hat{K} = \hat{f}_{11}/\hat{C}_{11}X_0$ must be in the range of 2–10 in order to clearly discern the value of D_{11}/f_{11}. For 3 mM Ca^{2+} at 25 °C (□), clearly $D_{11} \ll f_{11}$; thus, C_{11} and f_{11} are equal to the estimates of $\hat{C}_{11}$ and $\hat{f}_{11}$. For 5

Table I: Aggregation and Fusion Rate Constants for PS LUV

divalent cation	T (°C)	f_{11}[a] (s^{-1})	C_{11}[a] (M^{-1} s^{-1})	D_{11}[a] (s^{-1})
3 mM Ca^{2+}	15	$(5.0 \pm 2.0) \times 10^{-2}$	$(1.0 \pm 0.5) \times 10^{7}$	0[b]
	25	$(3.0 \pm 1.0) \times 10^{-1}$	$(2.0 \pm 1.0) \times 10^{7}$	0[b]
	35	≥ 1[c]	$(3.5 \pm 0.5) \times 10^{7}$	[c]
3 mM Ba^{2+}	15	$(3.0 \pm 1.0) \times 10^{-2}$	$(3.5 \pm 1.0) \times 10^{7}$	0[b]
	25	$(1.0 \pm 0.5) \times 10^{-1}$	$(5.0 \pm 2.0) \times 10^{7}$	$(1.0 \pm 0.5) \times 10^{-1}$
	35	$(2.0 \pm 1.0) \times 10^{-1}$	$(1.5 \pm 0.5) \times 10^{8}$	$(4.0 \pm 2.0) \times 10^{-1}$
5 mM Sr^{2+}	15	$(1.0 \pm 0.5) \times 10^{-3}$	$>5 \times 10^{6}$[d]	[d]
	25	$(1.0 \pm 0.5) \times 10^{-2}$	$>2 \times 10^{7}$[d]	[d]
	35	$(5.0 \pm 3.0) \times 10^{-2}$	$(7.0 \pm 3.0) \times 10^{7}$	$(5.0 \pm 0.3) \times 10^{-2}$

[a] The uncertainties in parameter estimates reflect the fitting of the theoretical curves to the data. The experimental fluorescence curves are reproducible to ±1% (Bentz et al., 1983a,b; Wilschut et al., 1980, 1981). [b] The value of 0 implies only that $D_{11} << f_{11}$; i.e., the aggregation is essentially irreversible. [c] In this case, f_{11} was so large that it would have required in excess of 500 μM PS to obtain D_{11}, an experiment which is not feasible without rapid mixing techniques. If D_{11} is appreciable (i.e., ≥ 0.5 s^{-1}, which is unlikely), then C_{11} would be larger and f_{11} somewhat smaller. Rigorously, the values shown only represent $\hat{f}_{11}$ and $\hat{C}_{11}$. [d] The fusion rate constants here are so small compared with aggregation that further estimates of C_{11} and D_{11} cannot be made except by using lipid concentrations below 1 μM, where the total fluorescence intensity is too small to be reliably measured. When the overall kinetics are completely fusion rate limiting, fluorescence curves do not depend on lipid concentration and only f_{11} is obtained directly (Bentz et al., 1983a,b).

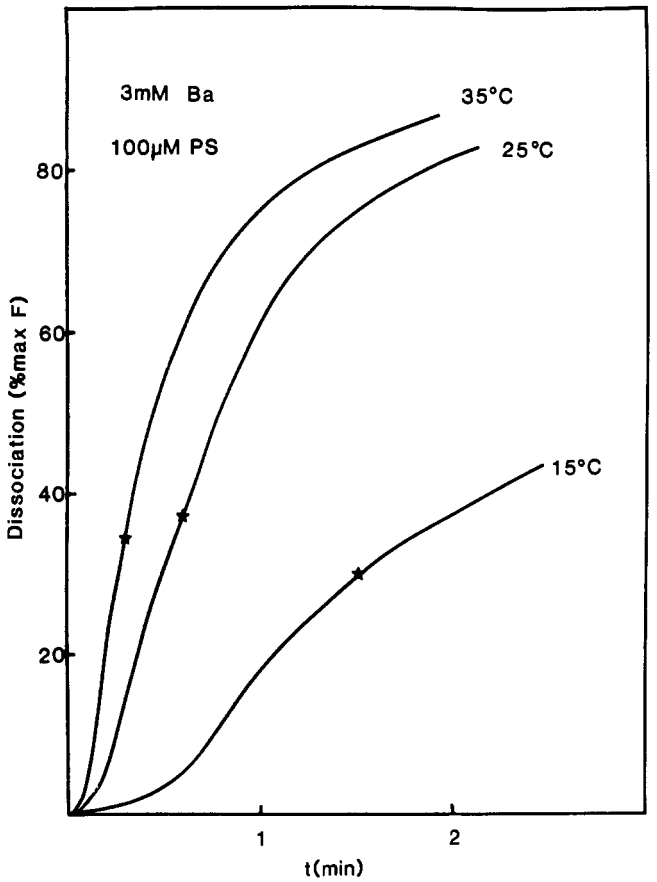

FIGURE 3: Tb/DPA dissociation for 100 μM Tb/DPA liposomes induced to fuse by 3 mM Ba^{2+} at 15, 25, and 35 °C. The dissociation is due to the Tb/DPA complex mixing with the external medium through leakage from the liposomes and the influx of medium into the intraliposomal compartments. The stars show the times at which the corresponding fusion signals (see Figure 2) reach their maximal values.

mM Sr^{2+} at 35 °C (▲), $D_{11}/f_{11} \simeq 1$, and since $\hat{C}_{11} = 3.5 \times 10^{7}$ M^{-1} s^{-1} and $\hat{f}_{11} = 0.09$ s^{-1}, $C_{11} = 7 \times 10^{7}$ M^{-1} s^{-1} and $D_{11} = f_{11} \cong 0.05$ s^{-1} (see eq 2 and Table I). For 3 mM Ba^{2+} at 35 °C (●), the data are best fitted by $D_{11}/f_{11} = 2$ where $\hat{C}_{11} = 5.4 \times 10^{7}$ M^{-1} s^{-1} and $\hat{f}_{11} = 0.7$ s^{-1}; hence, $C_{11} = 1.5 \times 10^{8}$ M^{-1} s^{-1}, $f_{11} = 0.2$ s^{-1}, and $D_{11} = 0.4$ s^{-1}.

In Table I, we have assembled the rate constants obtained for the aggregation and fusion of these PS LUV induced by

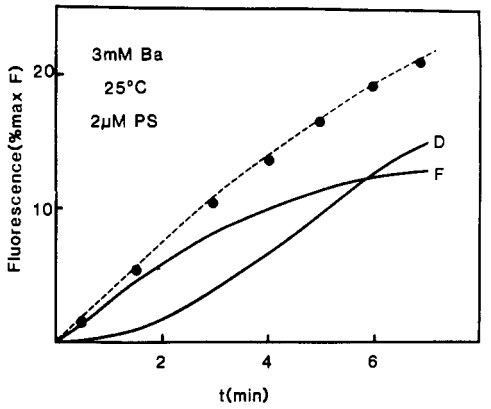

FIGURE 4: Fusion curve, denoted F, and dissociation curve, denoted D, for PS LUV with 3 mM Ba^{2+} at 25 °C. The total lipid concentration is 2 μM, i.e., 1 μM each of Tb liposomes and DPA liposomes for the fusion experiment and 2 μM Tb/DPA liposomes for the dissociation experiment. The solid circles show the fusion data corrected for dissociation,[2] i.e., $F_{cor} = F + 0.5D$. The dashed line is the theoretical curve predicted from eq 2 with $\hat{C}_{11} = 2.8 \times 10^{7}$ M^{-1} s^{-1} and $X_0 = 2.5 \times 10^{-11}$ M. This is an aggregation rate-limiting case; i.e., $\mathcal{F} = 1$ in eq 2, and we can specify only that $f_{11} \geq 0.07$ s^{-1}. Data taken at higher lipid concentrations, up to 100 μM, fix the value of $f_{11} \simeq 0.1$ s^{-1}; see Table I.

3 mM Ca^{2+}, 3 mM Ba^{2+}, and 5 mM Sr^{2+} at each of the three temperatures studied.

Discussion

The study has shown how each of the primary rate constants for liposome aggregation and fusion (i.e., C_{11}, D_{11}, and f_{11}) can be directly obtained from the overall fusion kinetics. By rigorously separating aggregation from fusion and quantifying these rate constants, we can begin to establish the mechanisms by which each process is occurring. Our primary result is that *all* of these rate constants increase with temperature. Our discussion will elucidate the implications of this finding.

First we will illustrate the meaning of the values of the rate constants calculated for these systems. Starting from the monodisperse state of the liposomes, the time required for 10% of the liposomes to dimerize is $0.052/C_{11}X_0$ if the aggregation is irreversible, where X_0 is the molar concentration of liposomes (Bentz et al., 1981a). With 50 μM PS and assuming 80 000 PS molecules per liposome (Materials and Methods), X_0 =

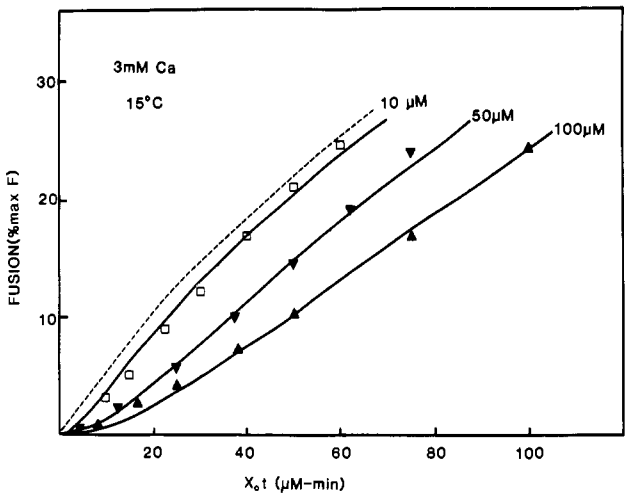

FIGURE 5: Fitting of $\hat{C}_{11}$ and $\hat{f}_{11}$ for the PS LUV induced to fuse by 3 mM Ca^{2+} at 15 °C. These fusion data are corrected for dissociation in all cases and are shown (□) for 10 μM lipid, (▼) for 50 μM lipid, and (▲) for 100 μM lipid. The solid lines show the theoretical curves from eq 2 for $\hat{C}_{11} = 8 \times 10^6$ M^{-1} s^{-1}, $\hat{f}_{11} = 0.05$ s^{-1}, and X_0 equal to the total lipid concentration divided by 80 000 (PS molecules/liposome). The dashed line shows the theoretical curve for low lipid concentration, i.e., purely aggregation rate limiting, for the same value of $\hat{C}_{11}$. Note that these data are plotted against the product of lipid concentration and time in the units of micromolar-minutes. For the calculations, X_0 is taken as the liposome concentration since the units of $\hat{C}_{11}$ are M^{-1} (of liposomes) s^{-1}.

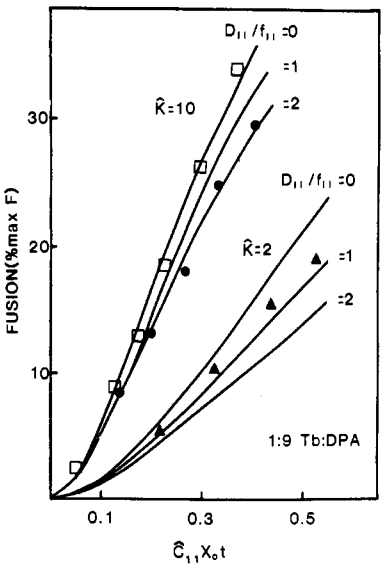

FIGURE 6: Fitting of the values of C_{11}, D_{11}, and f_{11} with a 100 μM sample of the 1:9 Tb:DPA liposome mixture. The fusion signals, corrected for dissociation, are plotted for 3 mM Ca^{2+} at 25 °C (□), 3 mM Ba^{2+} at 35 °C (●), and 5 mM Sr^{2+} at 35 °C (▲) vs. $\hat{C}_{11}X_0 t$ where t is the time (in seconds), X_0 is the liposome concentration (100 μM/80 000 = 1.25×10^{-9} M), and $\hat{C}_{11}$ is obtained from the 1:1 Tb:DPA liposome system as described in Figure 5.

6.25×10^{-10} M. For 3 mM Ba^{2+} at 25 °C, $C_{11} = 5 \times 10^7$ M^{-1} s^{-1}; hence, the time required for 10% dimerization is 1.6 s. If we imagine now that all of the liposomes are initially aggregated to the dimer state, then the time required for 10% of those dimers to fuse is $0.105/f_{11}$ if there is no disaggregation (Nir et al., 1982; Bentz et al., 1983a). From the same initial state, the time required for 10% of the dimers to dissociate is $0.105/D_{11}$ if there is no fusion (Bentz & Nir, 1981a,b). For 3 mM Ba^{2+} at 25 °C, $f_{11} = 0.1$ s^{-1}; thus, in 1.05 s 10% of these dimers will fuse. Obviously, when all three processes occur simultaneously, these simple equations can provide only rough estimates for the relevant time scale for each event.

Ionotropic Shift of T_c and Fusion. The hypothesis that the ionotropic shift of the gel–liquid-crystalline phase transition temperature drives the fusion reaction (cf. the introduction) was shown to be inadequate by Düzgüneş et al. (1984). However, since we know that the overall fusion reaction is largely aggregation rate limiting under similar experimental conditions (Bentz et al., 1983a; Nir et al., 1982), it *was* conceivable that the fusion rate constant did decrease above the induced T_c while the aggregation rate increased, such that there was still a net increase in the overall fusion rate. Now from Table I we know that this is not the case. The value of f_{11} for Ba^{2+} and Sr^{2+} increases with temperature through the range of the induced T_c. The increase of f_{11} for Ca^{2+} with temperature is also on the order of 3–10-fold per 10 °C in this range. Hence, at least for Ba^{2+} and Sr^{2+}, the initial fusion kinetics of two liposomes do not depend on the induction of an isothermal phase transition by the cations. The fusion is rapid at 35 °C where the divalent cation/PS complex is always in the liquid-crystalline state.

Fusogenic Capacities of Divalent Cations. We have shown that the fusion rate constant f_{11} for PS SUV depends largely upon the amount of divalent cation bound per PS head group (Bentz et al., 1983b). Further, it was shown that for equal amounts of bound divalent cation the value of f_{11}, which is the fusogenic capacity, decreases in the sequence $Ca^{2+} > Ba^{2+} > Sr^{2+} > Mg^{2+}$. This study focused on the threshold for fusion by using high Na^+ and Li^+ concentrations to maintain very rapid aggregation kinetics and low amounts of bound divalent cation. Therefore, the values of f_{11} were quite small, on the order of 0.003 s^{-1}. In Düzgüneş et al. (1983b), we extended this approach to PS LUV using high Mg^{2+} concentrations (5–15 mM) to elicit rapid aggregation kinetics since Mg^{2+} will not fuse PS LUV below 30 °C (Wilschut et al., 1981, 1985). Here again we found that at the fusion threshold, i.e., f_{11} ~0.003 s^{-1}, the fusogenic capacities of the divalent cations decrease in the same sequence, $Ca^{2+} > Ba^{2+} > Sr^{2+}$.

In this study, where the amounts of bound divalent cation are large and close to maximal values, we find the same sequence. Ca^{2+} is more fusogenic than Ba^{2+}. In fact, with 3 mM Ca^{2+}, where there is less Ca^{2+} bound per PS head group than with 3 mM Ba^{2+},[3] the fusion rate constant for Ca^{2+} is still larger than with 3 mM Ba^{2+}. Likewise, f_{11} for Sr^{2+} is smaller than that of Ba^{2+} even though its bulk concentration is greater. This result was somewhat anticipated by our previous work (Düzgüneş et al., 1983b) where it was shown that much more Sr^{2+} needed to be bound to the PS LUV than the SUV in order to initiate the fusion reaction, whereas for Ca^{2+} and Ba^{2+} the respective amounts did not depend on size.

The effect of liposome size on the fusion rate constant is also apparent in this study in terms of the size distribution of

[3] The values of bound cation per PS are obtained from the binding constants and equations described in Bentz (1981) and Bentz et al. (1983b). The most important point to recognize for these calculations is that they are valid only for isolated (unaggregated) vesicles. When the vesicles aggregate, the amount of bound divalent cation will increase by 20–30% simply due to the close approach of the charged surfaces (Bentz, 1982). In addition, there is substantial evidence that during and/or after fusion there is a new, stronger binding complex between (at least) Ca^{2+} and PS (Ekerdt & Papahadjopoulos, 1982). Thus, for these liposomes, we can calculate that the amount of bound divalent cation per PS head group for the respective bulk concentrations is 0.36 for Ca^{2+}, 0.39 for Ba^{2+}, and 0.39 for Sr^{2+}. These values use the binding constants obtained by McLaughlin et al. (1981) at 25 °C. The temperature dependence of binding appears to be negligible for Ca^{2+} but is not yet known for the other divalent cations.

the LUV (Olson et al., 1979). The liposome preparation method employed here (see Materials and Methods) yielded a Z-average diameter of 0.12 μm, as determined by dynamic light scattering, as compared to 0.15 μm for liposomes prepared according to the previous procedure. The fusion rate constant found here for 3 mM Ca^{2+} at 25 °C is 0.15 s^{-1}, whereas our previously published value for f_{11} for 5 mM Ca^{2+} with PS LUV of the larger Z-average size is 0.08 s^{-1} (Nir et al., 1982; Bentz et al., 1983a).

This difference is insignificant compared to the value of f_{11} for PS SUV in 2 mM Ca^{2+} which is 5 s^{-1}; however, it is important to recognize that the rate constants reported for liposomes do represent average values appropriate to the liposome size distribution. Clearly, the liposome size and the amount of bound divalent cation are crucial variables for prescribing the aggregation and fusion rate constants. Within the size distribution of the LUV used here, the amount of bound divalent cation should not vary much between the smaller and larger sizes within the distribution (Bentz, 1981, 1982). However, as we have seen here, the effect of reducing the average size on the rate constants is not insignificant. With the emergence of more refined techniques for determining the size distribution of the liposomes, it will become possible to further examine how each size within the distribution contributes to the overall average kinetic rate constants.

Mechanism of Aggregation. The DLVO (Derjaguin–Landau–Verwey–Overbeek) theory (Verwey & Overbeek, 1948) has held a central position in efforts to relate the kinetics of particle aggregation to the physical forces between the particles. The theory states that

$$C_{11} = \frac{4kTN}{3\eta W} \equiv \frac{C_{11}^{D}}{W} \tag{3}$$

where kT is the product of Boltzmann's constant and the absolute temperature, N is Avogadro's number, and η is the viscosity of the medium. C_{11}^{D} is the classical Smoluchowski diffusion-controlled aggregation rate constant (equal to 3.7 $\times 10^9$ M^{-1} s^{-1} at 25 °C), and W is a function which depends upon the free energy of interaction between two particles and the hydrodynamic coupling between the particles; i.e., as the particles approach, the effective viscosity of the intervening medium increases somewhat (Nir & Bentz, 1978; Nir et al., 1983a). W^{-1} can be thought of as the fraction of particles with sufficient kinetic energy to overcome the energy barrier between the two particles.

Calculating W from first principles is not yet possible since it requires knowing the free energy of interactions up to particle contact and the current electrostatic and van der Waals theories cannot be reliably used when the particles are closer than 20–30 Å (Ninham, 1981). Testing more sophisticated theories will require the experimental measurement of W as we have done here; i.e., for 3 mM Ca^{2+} and 25 °C, $C_{11} \sim 2 \times 10^7$ M^{-1} s^{-1} and $C_{11}^{D} = 3.7 \times 10^9$ M^{-1} s^{-1}; hence, $W \sim 185$.

Nonetheless, the current DLVO theory does predict several properties which are verified here for the first time. First, C_{11} increases with temperature, as may be expected since raising the temperature increases the number of collisions; however, the rate of increase of C_{11} with temperature exceeds that of C_{11}^{D}, which yields the temperature dependence of W. Earlier estimates of the temperature dependence of W, on the basis of long-range interactions (>20 Å), showed only a small decrease in W (Nir et al., 1980a) whereas these data imply a stronger decrease. Predictably, the short-range interactions are important. Second, W increases with the electrostatic surface potential of the liposomes. Since the binding constants of the cations to the PS head groups decrease in the sequence $Ba^{2+} > Sr^{2+} > Ca^{2+}$, we expect the values of C_{11} to decrease in the same sequence under otherwise equivalent conditions. This does occur. For Sr^{2+} at and below 25 °C, the fusion step was completely rate limiting, and we can only estimate a lower bound for C_{11}.

Aggregation Reversibility. Earlier attempts to show that C_{11} increases with temperature used the Na^+-induced aggregation of PS SUV, where there is no fusion. However, less aggregation was observed with increasing temperature (Nir et al., 1980a; Day et al., 1980). The explanation is that the dissociation rate constant D_{11} increased with temperature rather dramatically (Bentz & Nir, 1981a,b). This same behavior was recently noted for PC SUV (Wong & Thompson, 1982). Here we have found the same result even when there is fusion: D_{11} increases with temperature.

We have shown previously that the ratio C_{11}/D_{11} will give an estimate for the value of the depth of the energy minimum (u) holding the dimer together (Bentz & Nir, 1981a,b; Shoup & Szabo, 1982) specifically

$$\frac{C_{11}}{D_{11}} = \frac{4\pi}{3}(2a)^3(N \times 10^{-3}) \exp(-u/kT)\ M^{-1} \tag{4}$$

where a is the liposome radius (in centimeters). For these liposomes with $2a \simeq 1 \times 10^{-5}$ cm, the preexponential term equals 2.5×10^6. For Ba^{2+} at 25 and 35 °C and Sr^{2+} at 35 °C, we can make this estimate and find that the value of u is $\sim -(5.5 \pm 1)$ kT for all these cases. This minimum is not very deep, and were it not for the fact that most of the dimers are fusing before they can dissociate, the overall aggregation would be decreasing with temperature just as in previous studies with Na^+. For the other cases, we cannot estimate the well depth since the aggregation is essentially irreversible, i.e., $D_{11}/f_{11} \ll 1$.

Fusion and Leakage vs. Collapse. Central to our analysis of fusion kinetics is correcting the Tb/DPA fusion signal for the loss of fluorescence due to complex dissociation via leakage or influx of medium. In this way, we obtain a fluorescence signal which can be directly related to the number of fusion events and, hence, the fusion kinetics. However, the very existence of leakage during liposome fusion has colored the perception of the fusion event itself. It is now well-known that the kinetics of leakage, relative to the kinetics of mixing of aqueous contents, depend not only upon liposome size and composition, as well as the cations (Wilschut et al., 1983), but also upon the other environmental parameters, such as ionic strength (Nir et al., 1983b) and, as shown here, temperature. If the liposomes only lysed after contact, the corrected fluorescence signal could be used to obtain the rate constants; only in this case the rate constants would have to be *labeled* destabilization or lysis rate constants (Bentz et al., 1983a; Ellens et al., 1984, 1985). The critical parameter for discriminating whether one is looking at a lytic event, wherein most of the internal contents are lost before they can mix, or a fusion event, used in the only biologically relevant context wherein the internal contents are mixed and initially largely protected from the external environment, is the leakage or dissociation per fused liposome, defined simply as the percent leakage divided by the percent of the liposomes which have undergone fusion. This parameter, like the fusion rate constant itself, must depend on the physical state of the bilayer and can inform us on the molecular mechanisms of fusion.

As we have stated before (Bentz et al., 1983a), we can envisage several mechanisms leading to loss of contents: a burst of part of the contents during the fusion event; an am-

bient leakage from fused structures which do not reseal; and/or a collapse of the fused structures entailing a complete release. The additional mechanism of ambient leakage from isolated liposomes, after the addition of Ca^{2+} but before the intermembrane contact, is of minor, if any, consequence for the PS liposomes examined. In fact, even liposomes composed of PE and cholesteryl hemisuccinate, which leak after protonation of the cholesteryl hemisuccinate, do so only after intermembrane contact (Ellens et al., 1984, 1985). In any event, it is possible to examine the relevance of these different leakage mechanisms by using the kinetic analysis described here.

In Bentz et al. (1983a), the fusion kinetics of PS SUV in 2 mM Ca^{2+} and PS LUV in 5 mM Ca^{2+} were analyzed with 100 mM Na^+ buffer in both cases. It was found that for the SUV there is an initial release of contents which occurred as a burst of about 8% of encapsulated contents, carboxyfluorescein in that case, per fused liposome. As long as there were only fused doublets and triplets in the system, this release of contents upon fusion is constant with time, indicating that the SUV reseal after fusion. Eventually, as the aggregation and fusion products get larger, the structures collapse and evolve to the anhydrous cochleates (Papahadjopoulos et al., 1975; Portis et al., 1979). With PS LUV, initially there is no leakage per fused liposome, but in time this value increases (Wilschut et al., 1980, 1983; Bentz et al., 1983a). Subsequent analysis showed that the kinetics of leakage can be simulated quite well by assuming that the fused doublets and triplets did not sustain an ambient leakage but rather suffered collapse (J. Bentz, unpublished results). The rate of this collapse is at least 50 times slower than the fusion rate; hence, the fused doublets and triplets retain their integrity for quite a long time relative to the time required for fusion to occur.

The data presented here support this picture of LUV fusion. With each of the divalent cations, and at all temperatures, the Tb/DPA complex dissociation per fused liposome is initially zero. In time, this value increases, and it increases more rapidly at the higher temperatures. It is not yet possible to unambiguously state that collapse is the unique mechanism leading to release of contents for the PS LUV. Although collapse does eventually occur, as with the SUV, this may be due to larger fused aggregate structures, which are basically irrelevant to the analysis of the mechanism of fusion of two liposomes.

Conclusions

It is clear now how the fusion of liposomes can be independently controlled through either aggregation rate constants or the fusion rate constant. This and previous work has shown the necessity of clearly distinguishing between the different steps of the overall fusion event: aggregation and reversibility, bilayer destabilization, lipid mixing, mixing of aqueous contents, leakage, and collapse. Each of these steps, except perhaps collapse, occurs in cellular fusion, and the kinetic analysis described here together with complementary aqueous contents and lipid mixing assays will be needed to sort out the various levels of control, including protein mediation, which cells use to direct fusion.

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Registry No. Ca, 7440-70-2; Ba, 7440-39-3; Sr, 7440-24-6.

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