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Studies on the Mechanism of Membrane Fusion: Kinetics of Calcium Ion Induced Fusion of Phosphatidylserine Vesicles Followed by a New Assay for Mixing of Aqueous Vesicle Contents†

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ABSTRACT: We describe an assay for following the mixing of aqueous contents during fusion of phospholipid vesicles. Terbium is encapsulated as the $Tb(citrate)_3^{6-}$ chelation complex in one population of vesicles, dipicolinic acid (DPA) in another. Vesicle fusion results in the formation of the fluorescent $Tb(DPA)_3^{3-}$ chelation complex. The presence of EDTA (0.1 mM) and Ca^{2+} (>1 mM) prevents the formation of the Tb/DPA complex in the external medium. We have studied the Ca^{2+}-induced fusion of small or large unilamellar vesicles (SUV or LUV, respectively) composed of phosphatidylserine (PS). In addition, vesicle aggregation was monitored by light scattering, and release of vesicle contents was followed by carboxyfluorescein (CF) fluorescence enhancement. The addition of Ca^{2+} induced an immediate enhancement in Tb fluorescence with both SUV and LUV, which occurs on the same time scale as aggregation but much faster than the release of CF. The release of contents from LUV occurs with a considerable delay. It is estimated that the initial fusion of SUV is accompanied by 10% leakage of the internal volume per fusion event; in contrast, fusion of LUV is essentially nonleaky. Massive release of vesicle contents appears to be a secondary phenomenon related to the collapse of fused vesicles. The initial rate and the extent of Tb fluorescence enhancement are markedly dependent on the Ca^{2+} concentration. Threshold Ca^{2+} concentrations are 1.2 and 2.4 mM for SUV and LUV, respectively. At saturating Ca^{2+} concentrations (>10 mM), the rate of fusion of LUV is slightly lower than that of SUV at the same vesicle concentration. At any Ca^{2+} concentration, the rates of both SUV and LUV fusion are consistent with vesicle aggregation being rate limiting. When measured at a subsaturating Ca^{2+} concentration, fusion is essentially second order over a wide range of relatively low vesicle concentrations, whereas at higher vesicle concentrations the order is decreased. This suggests that at high vesicle concentrations (and at relatively low Ca^{2+} concentrations) aggregation may proceed faster than fusion.

The molecular mechanism of biological membrane fusion has received a great deal of attention in a number of recent investigations. The majority of these studies dealt with the application of artificial membrane systems as simplified models for biological membranes. In particular, the divalent cation induced aggregation and fusion of negatively charged phospholipid bilayers have been studied extensively, since they show several characteristics that are similar to those of biological membrane fusion (Papahadjopoulos et al., 1977, 1978; Papahadjopoulos, 1978).

It has been shown previously that Ca^{2+} induces massive aggregation of sonicated phosphatidylserine (PS)[1] vesicles (Lansman & Haynes, 1975; Portis et al., 1979; Düzgüneş & Ohki, 1977), release of vesicle contents (Papahadjopoulos & Bangham, 1966; Papahadjopoulos et al., 1977; Portis et al., 1979), and formation of large cochleate structures (Papahadjopoulos et al., 1975). These structural reorganizations occur at a threshold Ca^{2+} concentration of about 1 mM; at this concentration, the binding of Ca^{2+} to PS approaches a level corresponding to the stoichiometric ratio of one Ca^{2+} per two

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[1] Abbreviations used: PS, phosphatidylserine; DPA, dipicolinic acid; CF, carboxyfluorescein; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; DSC, differential scanning calorimetry; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles.

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PS (Newton et al., 1978; Nir et al., 1978; Portis et al., 1979). In the final Ca^{2+}/PS complex, the transition temperature (T_c) is shifted to extremely high values (Jacobson & Papahadjopoulos, 1975; Newton et al., 1978), and the lamellar repeat distance becomes as short as 53 Å (Newton et al., 1978), indicating that Ca^{2+} can induce an isothermal phase transition in PS bilayers. It has been proposed that Ca^{2+} forms an anhydrous "trans" complex involving PS molecules in apposed bilayers (Papahadjopoulos et al., 1978; Portis et al., 1979). The addition of excess EDTA to a Ca^{2+}/PS complex, formed by Ca^{2+}-induced aggregation of PS sonicated vesicles, results in the formation of large unilamellar vesicles (Papahadjopoulos et al., 1975). This indicates that at some stage of the transformation process vesicle fusion must occur.

The eventual formation of an anhydrous Ca^{2+}/PS complex raises the possibility that during the initial stages of vesicle interaction Ca^{2+} can induce a close approach of two vesicles by forming, at the area of contact, a trans complex involving PS molecules in the apposing vesicles (Portis et al., 1979). Formation of this complex would then trigger the actual fusion of the two membranes. This could imply that fusion immediately follows vesicle aggregation, thus allowing mixing of the aqueous vesicle volumes before extensive release of the contents can occur. the observed massive release of vesicle contents could then result from more extensive involvement of PS molecules in a collapsed anhydrous structure rather than from the actual fusion events that occur initially. In order to test this hypothesis, we have developed an assay which monitors the mixing of aqueous vesicle contents. The method is based on the interaction between terbium ions (Tb^{3+}) and dipicolinic acid (DPA), forming the fluorescent $Tb(DPA)_3^{3-}$ complex.

Although in the present study the assay is exclusively applied to the Ca^{2+}-induced fusion of PS vesicles, it can be adapted to other vesicle systems for which fusion has been described. In any case, the new assay would meet a more rigorous criterion for fusion than the criteria that have been considered most frequently before, such as vesicle aggregation (Kremer & Wiersema, 1977), mixing of membrane components (Maeda & Ohnishi, 1974; Miller & Racker, 1976; Miller et al., 1976; Papahadjopoulos et al., 1974, 1976), release of vesicle contents (Portis et al., 1979), or increase of vesicle size and/or morphological changes (Lau & Chan, 1975; Lawaczeck et al., 1976; Kantor & Prestegard, 1978; Liao & Prestegard, 1979; Verkleij et al., 1974; Koter et al., 1978; Stollery & Vail, 1977; Papahadjopoulos et al., 1975; Day et al., 1977). Previous attempts to monitor mixing of vesicle contents have been limited mainly to methods based on enzyme reactions (Ingolia & Koshland, 1978; Holz & Stratford, 1979; Hoekstra et al., 1979). However, enzyme reactions in general may not be fast enough to adequately follow rapid vesicle fusion. An important advantage of the assay described in this paper is the extremely fast rate of the Tb/DPA complex formation, which allows kinetic analyses of vesicle fusion phenomena. A preliminary report on this work has been published (Wilschut & Papahadjopoulos, 1979).

Materials and Methods

Materials. Phosphatidylserine (PS) was purified from bovine brain (Papahadjopoulos & Miller, 1967; Papahadjopoulos et al., 1977) and stored as a chloroform solution in sealed ampules under argon at −40 °C. $TbCl{\cdot}6H_2O$ (99.9% pure) was obtained from Alfa, dipicolinic acid (DPA, pyridine-2,6-dicarboxylic acid) from Sigma, and carboxyfluorescein (CF) from Eastman Kodak (Lot No. C41). The latter compound was purified as described (Blumenthal et al., 1977). All other chemicals were purchased in the highest purity available. Water was distilled twice, the second time in an all-glass apparatus.

Vesicle Preparation. Small unilamellar vesicles (SUV) were prepared in either (a) 15 mM $TbCl_3$ and 150 mM sodium citrate, (b) 150 mM DPA (sodium salt), or (c) 100 mM CF (sodium salt). In addition, the 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. The lipid (PS) was dispersed in aqueous medium at a concentration of 10 μmol/mL and sonicated for 1 h in a bath-type sonicator (Papahadjopoulos et al., 1977) under argon at 20 °C. Subsequently, the preparations were centrifuged for 1 h at 115000*g* in a Beckman SW 50.1 rotor to remove large vesicles and/or aggregates. Usually greater than 95% of the lipid was recovered in the supernatant.

Large unilamellar vesicles (LUV) were prepared by the reverse-phase evaporation technique described before (Szoka & Papahadjopoulos, 1978), with several minor modifications. The vesicles were made in either (a) 2.5 mM $TbCl_3$ and 50 mM sodium citrate, (b) 50 mM DPA (sodium salt) and 20 mM NaCl, or (c) 50 mM CF (sodium salt). In addition, the media contained 2 mM L-histidine and 2 mM Tes adjusted to a final pH of 7.4. Phosphatidylserine (10 μmol) was dissolved in 1.0 mL of diethyl ether (distilled over sodium bisulfite) and sonicated under argon for 5 min at 20 °C with 0.3 mL of aqueous medium. The resulting emulsion was evaporated in a rotary evaporator at 30 °C under reduced pressure (350 mmHg) to remove the bulk ether until a stable gel formed. Collapse of the gel was then initiated by a brief (5–10 s) vortex mixing, and evaporation was continued at a pressure of about 150 mmHg for approximately 2 min, after which an additional 0.7 mL of the aqueous medium was added. The resulting preparation was kept under high vacuum (10 mmHg) in the evaporator for another 15–20 min to remove residual ether. The vesicles were "sized" by extrusion (Olson et al., 1979) through polycarbonate Unipore membranes (Bio-Rad) with a pore size of 0.1 μm under a nitrogen pressure of 4–5 atmospheres. Lipid recovery after extrusion was approximately 80%.

Vesicles were separated from nonencapsulated material by gel filtration on Sephadex G-75 (column size, 1.0 × 20 cm; elution buffer, 100 mM NaCl, 2 mM L-histidine, and 2 mM Tes, pH 7.4). For reasons indicated below (see Encapsulation of $TbCl_3$ and DPA in Phosphatidylserine Vesicles), 1.0 mM EDTA was included in the elution buffer with the Tb vesicles (and, for consistency, also with the DPA vesicles). With CF-containing vesicles, the EDTA concentration in the buffer was 0.1 mM. For purposes of calibration, parts of the Tb and DPA vesicle preparations were rechromatographed on Sephadex G-75 with the above elution buffer without EDTA to remove the external EDTA. In contrast to SUV, LUV are osmotically sensitive. In order to prevent leakage of vesicle contents, the osmolality of the elution buffer during gel filtration was slightly higher than that of the medium inside the vesicles. Osmolalities (determined with a vapor pressure osmometer, Wescom Instruments) of the Tb-, DPA-, and CF-containing media used for the LUV preparation were all 160–170 mosmol/kg, while that of the elution buffer was 190 mosmol/kg. As a marker for the encapsulated volume of the vesicles, in some experiments 1 mM [^{14}C]sucrose (1 mCi/mmol) was included in the media in which the vesicles (SUV or LUV) were made.

Fluorescence and Light-Scattering Measurements. Except for the experiment in Figure 1, all fluorescence and light-scattering measurements were performed with an SLM-4000

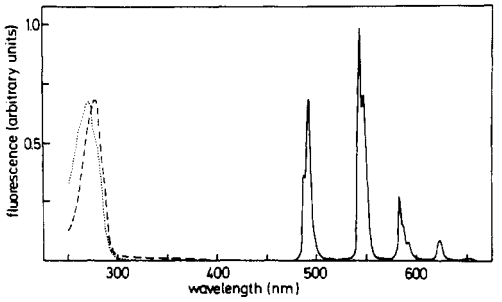

FIGURE 1: Fluorescence emission (—) and excitation (- - -) spectra of the $Tb(DPA)_3^{3-}$ complex. The solution contained 5 μM $TbCl_3$ and 50 μM DPA in 0.1 M NaCl, 2 mM L-histidine, and 2 mM Tes, pH 7.4. Excitation was at 276 nm (emission spectrum) and emission at 491 nm (excitation spectrum). Spectra were recorded in an Amin-co–Bowman SPF 500 spectrofluorometer. The figure also shows the absorbance spectrum of DPA (···); absorbance coefficients (maximum): 271 nm, 4.2×10^3 M^{-1} cm^{-1}; 276 nm, 3.4×10^3 M^{-1} cm^{-1}.

fluorometer (SLM Instruments). The instrument has a "T" format design which allowed 90° fluorescence and 90° light-scattering measurements to be performed simultaneously. The temperature of the sample holder was maintained at 25 °C, and the solution in the cuvette was continuously stirred. Unless indicated otherwise, 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. $CaCl_2$ or EDTA were added as concentrated solutions directly to the cuvette with a Hamilton syringe.

The Tb/DPA complex was excited at 276 nm; fluorescence was measured through the monochromator set at 545 nm, employing a Corning 3-68 cutoff filter to eliminate contribution to the signal from light scattering. Light scattering was measured in the second emission channel by using a Corning 7-54 band-pass filter. CF was excited at 430 nm in LUV experiments and at 493 nm with SUV. Emission was detected at >530 nm through a Corning 3-68 cutoff filter, while light scattering was measured through the monochromator set at the excitation wavelength. Complete release of CF from vesicles was obtained by addition of Triton X-100 (0.1% v/v). CF concentrations were determined by absorbance measurements ($\epsilon_{493}^{1mM} = 70$).

Other Methods. For negative staining, vesicle preparations were dialyzed against 0.1 M ammonium acetate, pH 7.0, and mounted on Formvar-coated copper grids that had been subjected to glow-discharge treatment immediately before use. The samples were stained with 1.5% ammonium molybdate as before (Olson et al., 1979).

Lipid phosphorus was determined according to Bartlett (1959). Radioactivity was measured by liquid scintillation counting. Phase transitions were detected with a Perkin-Elmer DSC-2 differential scanning calorimeter as described before (Jacobson & Papahadjopoulos, 1975; Newton et al., 1978).

Principle of the Fusion Assay. The basis of the assay is the formation of the fluorescent $Tb(DPA)_3^{3-}$ chelation complex (Grenthe, 1961; Barela & Sherry, 1976; Thomas et al., 1978). Since fluorescence is generated through an internal energy transfer from the ligand to the metal ion (Sinha, 1971; Barela & Sherry, 1976), the complex is excited at a wavelength close to the absorption maximum of DPA, while the fluorescence spectrum is characteristic for Tb (Figure 1). The fluorescence intensity of Tb on its own is very low (Horrocks et al., 1977) but is enhanced 10^4-fold by its interaction with DPA (Barela & Sherry, 1976). In the fusion assay, $TbCl_3$ is encapsulated in one population of vesicles and DPA in another. Fusion of the vesicles would then register as an increase of the fluorescence intensity due to the formation of the Tb/DPA complex. However, for practical applicability, several conditions have to be met. First, both $TbCl_3$ and DPA have to be amenable to encapsulation within phospholipid vesicles without affecting the structure of the bilayer; in addition, their rates of leakage from the vesicles should be low. Second, the formation of the Tb/DPA complex should be fast enough to permit kinetic analyses of vesicle fusion. Third, in order to eliminate the possibility that mere leakage of vesicle contents would result in fluorescence development, the composition of the external medium has to be such that formation of the complex outside the vesicles is effectively prevented.

Encapsulation of $TbCl_3$ and DPA in Phosphatidylserine Vesicles. $TbCl_3$ was encapsulated in the presence of the relatively weak chelator citrate to prevent interaction of Tb^{3+} with PS. The high coordination number of 9–10 that has been reported for Tb^{3+} (Horrocks et al., 1977) in principle permits the formation of the $Tb(citrate)_3^{6-}$ complex. The effectiveness of citrate in preventing the Tb^{3+}–PS interaction was verified by (a) determination of the temperature (T_c) of the gel to liquid crystalline phase transition of PS in the presence of $TbCl_3$ or $TbCl_3$ with excess citrate and (b) comparison of the relative encapsulation of Tb/citrate in PS vesicles with that of radioactive sucrose as a marker for the entrapped volume.

In the absence of citrate, 2.5 mM $TbCl_3$ shifted the T_c of PS multilamellar vesicles, as determined by differential scanning calorimetry, to approximately 40 °C, which is close to the value of 38 °C recently observed for the La^{3+}–PS system (Hammoudah et al., 1979). If present at a 10–20-fold excess over $TbCl_3$, citrate completely reversed this effect of Tb^{3+} on the phase transition of PS: the calorimetry scan was identical with that of a control in a 100 mM NaCl medium (results not shown). Also, DPA at pH 7.4 in concentrations as high as 150 mM had no effect on the phase transition of PS.

The concentrations of citrate and of DPA that can be encapsulated in LUV are limited by the osmotic sensitivity of these vesicles. For citrate (trisodium salt), the maximal concentration is approximately 50 mM; higher concentrations result in the partial release of vesicle contents when the external medium is 100 mM NaCl. On the other hand, higher concentrations can be employed with SUV, which are not osmotically sensitive. As a consequence, the actual citrate concentrations during the vesicle preparations were lower in LUV than in SUV. Therefore, the Tb to citrate molar ratio was routinely kept at 1:20 for LUV (2.5 mM $TbCl_3$, 50 mM citrate) and 1:10 for SUV (15 mM $TbCL_3$, 150 mM citrate); however, since the external citrate concentration is continuously reduced during the gel filtration after preparation of the vesicles, 1.0 mM EDTA was included in the elution buffer to prevent binding of Tb^{3+} to the outside of the vesicles.

Typical values (nmol/μmol of lipid) for the encapsulation of Tb, DPA, and CF in PS vesicles were 11.1, 210, and 221, respectively, for LUV, and 1.7, 16.3, and 9.1 for SUV. In addition, values determined for the entrapped volume of each vesicle preparation were obtained by including 1 mM [^{14}C]-sucrose (1 mCi/mmol) in the original Tb, DPA, and CF solutions. The LUV invariably trapped amounts of Tb, DPA, and CF corresponding to 4.2–4.4 μL of the original solution per μmol of PS. In each preparation, this value showed a fair correspondence to the amount of radioactive sucrose trapped, indicating that the encapsulation of the compounds was proportional to the internal volume of the vesicles. The entrapped volume of 4 μL/μmol of lipid is slightly larger than the value of 3 μL/μmol that can be calculated for vesicles with a diameter of 1000 Å, assuming an area per molecule of 70 Å^2 and a bilayer thickness of 40 Å (Papahadjopoulos & Ki-

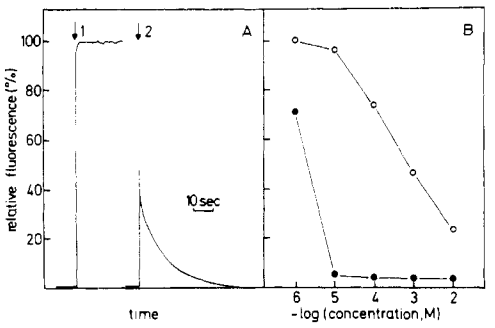

FIGURE 2: Kinetics of the Tb/DPA complex formation: effects of EDTA and Ca^{2+}. (A) Florescence profiles obtained upon injection of 20 μL of 0.05 mM $TbCl_3$ and 0.5 mM citrate, pH 7.4, into 1.0 mL of 0.01 mM DPA in 0.1 M NaCl, 2 mM L-histidine, and 2 mM Tes, pH 7.4 (arrow 1), and upon injection of 20 μL of 0.05 mM $TbCl_3$, 0.5 mM citrate, and 0.5 mM DPA, pH 7.4, into 1.0 mL of the above NaCl medium containing 0.1 mM EDTA and 2.0 mM $CaCl_2$ (arrow 2). (B) Final fluorescence intensities of 1.0 μM $TbCl_3$, 10 μM citrate, and 10 μM DPA in the above NaCl medium containing various concentrations of EDTA (●) or $CaCl_2$ (○).

melberg, 1973). SUV appeared to encapsulate [^{14}C]sucrose to an extent equivalent to 0.2–0.3 μL/μmol of PS which corresponds to a calculated average vesicle diameter of 220–250 Å. Invariably, the values for the encapsulation of Tb, DPA, and CF (1.7, 16.3, and 9.1 nmol/μmol of lipid, respectively) corresponded to a lower encapsulated volume (approximately 0.1 μL/μmol) than that for the encapsulation of sucrose. This suggests that the Tb/citrate complex, DPA, and CF are excluded from the electrostatic double-layer region adjacent to the membrane. The extent of Tb encapsulation in SUV corresponds to approximately seven Tb/citrate complexes per vesicle.

Leakage of Tb or DPA from the vesicles was measured by addition of excess DPA or Tb/citrate, respectively, to vesicle preparations in the absence of EDTA. The ratio of the fluorescence intensities before and after the addition of cholate (added to a concentration of 0.5%, w/v, in order to release all vesicle contents) was taken as a measure of the fraction of the vesicle contents that had leaked out. Leakage rates for Tb were extremely low. At 0 °C, values obtained were 2% in 24 h for SUV and 0.5% in 24 h for LUV. At 25 °C, these values were approximately 5% and 1%, respectively. DPA permeates the PS bilayer more readily than the Tb/citrate complex, particularly at room temperature. At 0 °C, the rate of leakage of DPA was approximately 10% in 24 h for SUV and 4% in 24 h for LUV. At 25 °C, these values were 50% (about 5%/h initially) and 20%, respectively. The higher rate of leakage of DPA is probably due to its more hydrophobic character than that of the Tb/citrate complex. Before use in fusion experiments, vesicles were kept at 0 °C. The very low rate of leakage of Tb is advantageous since in the fusion assay this compound is present in limiting concentrations, while DPA is in excess.

Kinetics of the Tb/DPA Complex Formation: Effects of Citrate, Ca^{2+}, and EDTA. Upon fusion of a Tb/citrate-containing vesicle with a DPA-containing vesicle, the Tb/citrate complex is expected to dissociate in favor of the formation of the fluorescent Tb/DPA complex, since DPA has a very high affinity for Tb (Grenthe, 1961). As pointed out above, this reaction should be rapid and should not occur in the external medium. Figure 2 shows a number of experiments on the kinetics of the Tb/DPA complex formation and of its dissociation in the medium in which the actual fusion assay is done. To avoid inner-filter effects, these experiments were done in dilute solution at Tb, citrate, and DPA concentrations of 1, 10, and 10 μM, respectively. Even at these low concentrations, the formation of the Tb/DPA complex from Tb/citrate and DPA is extremely fast (Figure 2A). Also, the extent of fluorescence of 1 μM Tb and 10 μM DPA was not affected by citrate in concentrations up to 0.5 mM. It should be noted that the rate of the Tb/DPA complex formation after fusion of two vesicles would be expected to be even faster than the rate registered in the above control experiment, since inside the vesicles the reactants are present at some 10^3–10^4-fold higher concentrations.

EDTA and Ca^{2+} both strongly interfere with the complex formation, through interaction with Tb and DPA, respectively. Figure 2B shows the equilibrium values of Tb fluorescence (1 μM Tb, 10 μM citrate, and 10 μM DPA) in the presence of increasing concentrations of EDTA or Ca^{2+}. It is clear that EDTA is a more potent quencher of the fluorescence than Ca^{2+}. When both EDTA (0.1 mM) and Ca^{2+} (>1 mM) are present, the fluorescence intensity is reduced to almost zero (<1% of the maximal value).

To simulate the condition of leakage of vesicle contents during or after fusion, we injected a concentrated solution of Tb, citrate, and DPA (molar ratio 1:10:10) into a medium containing 0.1 mM EDTA and 2.0 mM Ca^{2+} (Figure 2A). A very fast initial and a slow subsequent quenching of the fluorescence was observed. The intensity was reduced to 30% of the maximal value in 1 s, to 10% within 15 s, and to <1% within 1 min. With 5 mM Ca^{2+} and 0.1 mM EDTA, an even faster decrease of the fluorescence intensity was observed. Moreover, during the actual vesicle fusion, the overall concentrations of Tb, citrate, and DPA are about an order of magnitude lower than the concentrations in this control experiment. In that case, the extreme dilution occurring upon release of vesicle contents is sufficient to significantly reduce the fluorescence intensity. Besides, under these conditions, the quenching potencies of EDTA and Ca^{2+} will be even more pronounced than in the experiment of Figure 2A.

Calibration of Extent of Fluorescence during Fusion. For fusion measurements, the Tb fluorescence scale was calibrated as follows. In the absence of EDTA, 20 μM DPA was added to Tb vesicles (at a concentration identical with the concentration of Tb vesicles present in the fusion assay, i.e., routinely 25 nmol of lipid/mL). Subsequently, cholate (0.5% w/v) was added to release the contents from the vesicles. DPA addition to the intact vesicles invariably showed a greater than 95% latency of the Tb in the vesicles. The fluorescence observed after cholate addition was taken as the maximal (100%) value. In order to check whether this calibration procedure accurately relates to the level of fluorescence that would be obtained after complete mixing of the vesicle contents, we prepared LUV containing a 1:1 mixture of the Tb and DPA solutions that are routinely encapsulated separately in LUV. As expected, these vesicles showed a high level of Tb fluorescence which was not changed by addition of EDTA to the external medium. Addition of cholate to the vesicles (50 μM lipid) in the absence of EDTA resulted in a decrease of the fluorescence intensity due to dilution. Subsequent addition of 20 μM DPA brought the fluorescence intensity back to exactly the level that was seen before the cholate addition. This indicates that the fluorescence intensity of a certain amount of Tb, together with an excess of DPA, present at millimolar concentrations inside a vesicle is identical with its fluorescence intensity after release from the vesicle (to about 10^5-fold lower concentrations) in the presence of 20 μM DPA. Therefore, the fluorescence intensity measured at any time during the fusion assay represents, as a percentage, the amount of Tb that is associated

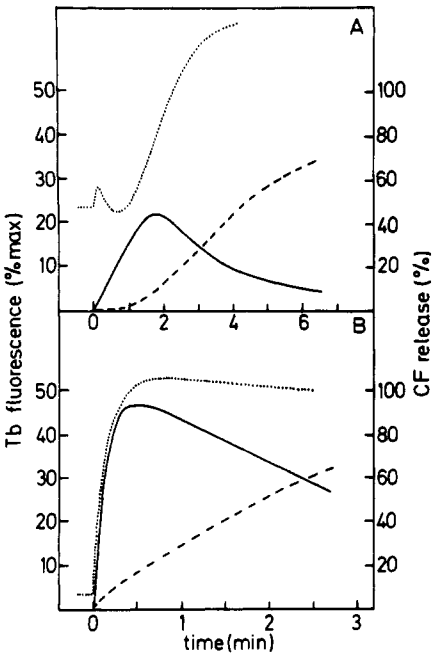

FIGURE 3: Ca^{2+}-induced aggregation and fusion of phosphatidylserine vesicles, LUV (A) and SUV (B). $CaCl_2$ was introduced as a 0.1 M solution into a 1:1 mixture of Tb and DPA vesicles at a total lipid concentration of 0.05 mM. Final $CaCl_2$ concentrations (not corrected for the presence of 0.1 mM EDTA in the medium): LUV, 3.0 mM; SUV, 1.5 mM. Tb fluorescence (—) is presented as a percentage of the maximal value, determined as described in the text. Light scattering (···) was monitored simultaneously with Tb fluorescence, and CF release (---) was measured in separate experiments. A 100% release of CF corresponds to the fluorescence intensity after addition of Triton X-100 (0.1% v/v) to the vesicles; 0% release corresponds to the residual fluorescence due to CF inside the vesicles (approximately 5% and 10% of the maximal value for SUV and LUV, respectively).

with DPA relative to the total amount of Tb present.

Results

Aggregation and Fusion of Vesicles. The time course of the fluorescence intensity that is obtained when Ca^{2+} is added to a 1:1 mixture of Tb and DPA vesicles in the presence of 0.1 M NaCl is shown in Figure 3. In the absence of Ca^{2+}, the fluorescence remained constant at a very low level. After the addition of $CaCl_2$ to the vesicle suspension, the Tb fluorescence intensity increased sharply until a maximum was reached which was followed by a relatively slow decrease. These results indicate that Ca^{2+} induces an immediate mixing of vesicle contents. The rate of development and the extent of Tb fluorescence were markedly dependent on the Ca^{2+} concentration and the vesicle concentration, as will be discussed below.

Vesicle aggregation was monitored simultaneously in these experiments by following light scattering. It is clear from the results obtained with SUV (Figure 3B) that aggregation proceeds at a similar time scale as fusion. Scattering curves obtained with LUV (Figure 3A) had a strikingly different appearance. Before the addition of Ca^{2+}, the scattering intensity was several-fold higher than that of SUV at the same lipid concentration. Addition of Ca^{2+} to LUV produced a fast but limited increase in the scattering. After a subsequent decrease, the scattering increased dramatically. We interpret this time sequence as follows. The initial increase and subsequent decrease represent the aggregation of the vesicles. The small magnitudes of these changes relate to the large initial size of the vesicles. It can be predicted from theoretical considerations implicating interference effects (S. Nir, personal communication) that aggregation and/or fusion of hollow spheres with a starting diameter on the order of 1000 Å may result initially in a small increase and subsequently in a decrease of the light scattering at 276 nm. An additional factor, which in later stages of the aggregation and fusion process could contribute to the observed decrease in scattering, is a clearing of the light path due to flotation or precipitation of lipid aggregates, which occurs even in a stirred solution. The dramatic subsequent increase in the scattering is superimposed on the continuing decrease and presumably represents a reorganization within the vesicle aggregates. This reorganization is subsequent to fusion (the secondary increase starts when the Tb fluorescence has almost reached its maximum) but coincides with massive release of vesicle contents (see below). Therefore, the reorganization probably involves a collapse of the internal aqueous space of fused vesicles, as a first stage in the formation of cochleate cylinders (Papahadjopoulos et al., 1975). Such a collapse, which would occur as a result of Ca^{2+} entry into the vesicles, is likely to profoundly change the scattering properties of the vesicle aggregates, since the PS molecules become more extensively involved in a tightly packed anhydrous Ca^{2+}/PS complex (Portis et al., 1979) with an entirely different refractive index than that of the original material.

Similar reorganization processes are probably occuring with SUV as well. However, these changes cannot be distinguished in terms of scattering data from the initial aggregation. Since the starting diameter of the vesicles is very small, aggregation and fusion both result in a more pronounced initial increase in scattering than with LUV. This could mask the secondary increase caused by the collapse of the vesicles.

Kinetics of Fusion and Release of Contents. The eventual decrease in the Tb fluorescence intensity (Figure 3) in all likelihood is caused by the release of vesicle contents and dissociation of the Tb/DPA complex in the external medium. Because of the initially high Tb fluorescence levels that are attained, the release of vesicle contents appears to be delayed or to be occurring at a slower rate than vesicle fusion. Therefore, it was of interest to study the release of vesicle contents in a more direct way. This was done in separate experiments, under conditions otherwise identical with those in the fusion assay, employing the enhancement of fluorescence intensity that occurs upon dilution of carboxyfluorescein (CF) initially encapsulated at high self-quenching concentrations in the vesicles (Weinstein et al., 1977; Blumenthal et al., 1977; Portis et al., 1979). As shown in Figure 3A, the CF release from LUV after addition of $CaCl_2$ is clearly delayed with respect to fusion, indicating that at least the initial fusion events are essentially nonleaky. The massive release of CF that occurs after the lag period would appear to be the result of the collapse of the vesicles rather than of the fusion per se. With SUV (Figure 3B), the CF release starts immediately after Ca^{2+} addition. Therefore, it seems that fusion of the small vesicles, in contrast to that of LUV, is accompanied by a significant leakage of contents.

The extent to which fusion is leaky can be estimated from the ratio of the initial rate of fusion to that of the initial rate of release. In this respect, it should be emphasized that the rate of increase of Tb fluorescence is an underestimate of the actual rate of fusion since (a) any release of vesicle contents and/or entry of Ca^{2+} and EDTA into the vesicles will reduce the Tb fluorescence and (b) during the first round of fusion (assuming binary interactions) in a 1:1 mixture of Tb vesicles and DPA vesicles only 50% of the total number of fusion events will result in the formation of the Tb/DPA complex. We have experimentally verified this by lowering the proportion of Tb

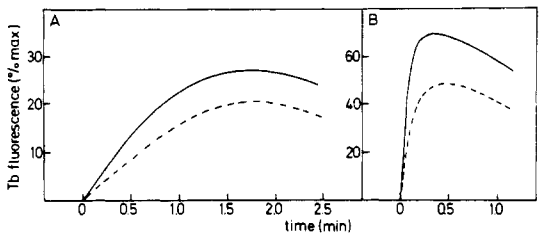

FIGURE 4: Tb fluorescence development during fusion in a 1:9 mixture of Tb and DPA vesicles, LUV (A) and SUV (B). Lipid concentration was 0.05 mM. $CaCl_2$ concentrations: LUV, 3.0 mM; SUV, 1.5 mM. For comparison, the curves obtained with the 1:1 mixture of Tb and DPA vesicles at the same total lipid concentration are shown (---, also see Figure 3). The 100% Tb fluorescence value in either mixture represents the maximal intensity that can be obtained with the amount of Tb present.

vesicles in the mixture from 50% to 10% (Figure 4). As expected, in the 1:9 mixture, an increased rate of Tb fluorescence development as well as an increased maximal fluorescence level was observed. For SUV (at 1.5 mM Ca^{2+}), the ratio of the rate of fluorescence increase in the 1:9 mixture to that in the 1:1 mixture was approximately 2.0; for LUV (at 3 mM Ca^{2+}), the ratio was 1.6. Both ratios are fairly close to the expected value of 1.8, but tended to decrease with increasing Ca^{2+} or vesicle concentrations, suggesting that at relatively high rates of fusion the initial rate of the Tb fluorescence increase is influenced to a certain degree by multiplicity of fusion events. A separate paper by Nir et al. (1980) presents an extensive theoretical analysis of these aspects of the fusion assay.

If we now compare (Figure 3B) the initial rate of CF release from SUV at 1.5 mM Ca^{2+} (0.8%/s) with the corrected rate of mixing of vesicle contents (10%/s, i.e., twice the rate of the observed Tb fluorescence increase), it appears that during the early time period the rate of release is approximately 10% of the rate of fusion. This indicates that any early fusion event between SUV is accompanied by the release of about 10% of their encapsulated volume (further evidence to corroborate this conclusion is given in section 2 under Appendix).

Termination of the Fusion Reaction with EDTA. In order to eliminate the possibility that the Tb/DPA complex formation was occurring outside the vesicles due to release of vesicle contents at transiently high local concentrations, we studied the effects of terminating the fusion reaction by addition of excess EDTA. Any fluorescent complex present within the aggregates but outside the vesicles would be quenched after dissociation of the vesicles in the presence of EDTA. On the other hand, EDTA should have no effect if the fluorescent complex is in the interior of fused vesicles. The degree to which the Tb fluorescence intensity could be fixed by addition of EDTA varied with the type of vesicle used, the stage of the fusion process, the vesicle concentration, and the Ca^{2+} concentration. Typical results on the effects of EDTA are shown in Figure 5. The addition of EDTA early after the addition of Ca^{2+} results in complete fixation of the Tb fluorescence level with both SUV and LUV Figure 5A,B). An important conclusion that can be drawn from this observation is that at least initially the Tb/DPA reaction is occurring exclusively within the vesicles. Addition of EDTA at later times results in an increasingly pronounced drop in the fluorescence intensity, particularly with LUV (Figure 5A,B). Increasing the Ca^{2+} concentration (Figure 5C,D; see also Figure 6) or the vesicle concentration (results not shown) has similar effects.

The decrease of Tb fluorescence upon addition of EDTA can be due to either (a) quenching of the fluorescence resulting

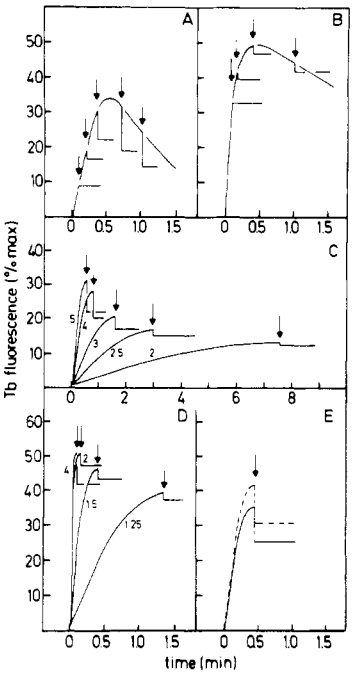

FIGURE 5: Termination of the fusion process by addition of excess EDTA. (A) and (B) panels each show a compilation of a series of experiments in which EDTA was added (to a 2-fold molar excess over Ca^{2+}) at several time intervals (see arrows) after the initiation of the fusion by Ca^{2+}. (A) LUV; initial Ca^{2+} concentration, 5.0 mM. (B) SUV; initial Ca^{2+} concentration, 1.5 mM. In (C) and (D) (LUV and SUV, respectively), fusion was initiated by various Ca^{2+} concentrations (millimolar concentrations are indicated) after which a 2-fold molar excess of EDTA was added at the peak of the Tb fluorescence intensity (see arrows). In (E), LUV fusion was initiated by addition of a mixture of EDTA and $CaCl_2$ (pH 7.4) to concentrations of 10 and 20 mM, respectively, (—), or by addition of 10 mM $CaCl_2$ alone (---). In either case, EDTA was added (see arrow) to a 2-fold molar excess over the (net) Ca^{2+} concentration. In all cases, the lipid concentration was 0.05 mM and the ratio of Tb to DPA vesicles 1:1. $CaCl_2$ was added as a 0.1 M solution and EDTA as a 0.1 M (A–D) or 0.2 M (E) solution. EDTA solutions were pretitrated with NaOH to compensate for the release of H^+ ions accompanying binding of Ca^{2+} to EDTA at pH 7.4.

from complex formation within the aggregate but outside the vesicles, or (b) an artifact such as entry of EDTA into the vesicles and/or disruption of part of the vesicles induced by EDTA. On the basis of additional evidence presented in section 1 under Appendix, we conclude that EDTA does induce a disruption of vesicles and partial release of vesicle contents and that Tb/DPA complex formation is not likely to occur outside the vesicles.

Ca^{2+} Concentration Dependence of Fusion. The initial rate as well as the apparent extent of fusion is markedly dependent on the Ca^{2+} concentration in the medium. Figure 6A,B shows the actual fluorescence profiles obtained at various Ca^{2+} concentrations with LUV and SUV, respectively. In Figure 6C,D, the initial rates of the Tb fluorescence increase are plotted against the Ca^{2+} concentration. In addition to the data obtained at a lipid concentration of 50 nmol/mL (closed circles), these latter figures also present data obtained at a lipid concentration of 10 nmol/mL for SUV and 200 nmol/mL for LUV (open circles). At these latter concentrations, the number of vesicles in each sample is the same for both types of vesicles (the number of lipid molecules per vesicle is approximately 4000 in 250-Å SUV and 80 000 in 1000-Å LUV).

For fusion of SUV, an apparent threshold concentration of 1.2 mM Ca^{2+} was observed; for LUV, the threshold appeared to be 2.4 mM. These values were obtained by extrapolation to zero rate of the steeply increasing part of the curve relating

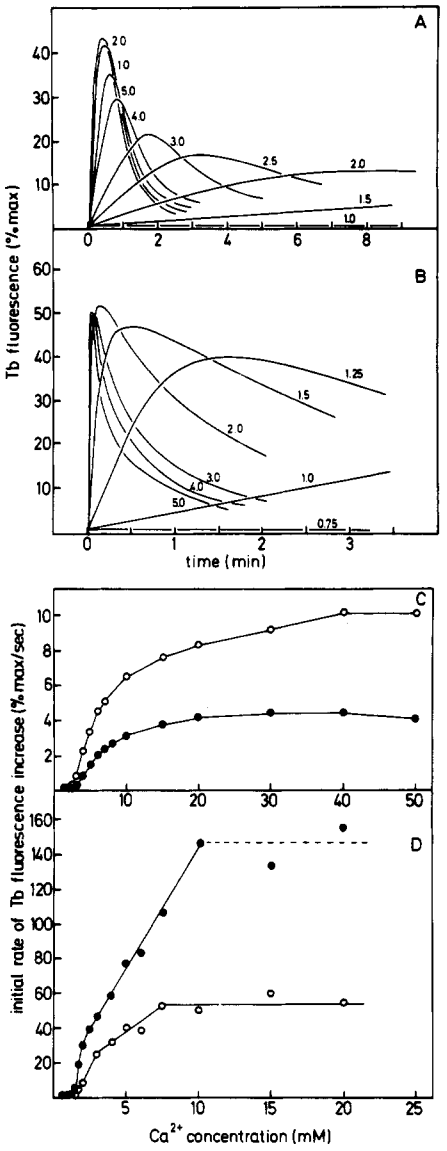

FIGURE 6: Depencence of PS vesicle fusion on the Ca^{2+} concentration. $CaCl_2$ was injected as a 0.1 or 0.2 M solution into a 1:1 mixture of Tb and DPA vesicles at a total lipid concentration of 0.05 mM. Final millimolar Ca^{2+} concentrations (not corrected for the presence of 0.1 mM EDTA in the medium) are indicated: (A) LUV and (B) SUV. Initial rates of the Tb fluorescence increase were determined from the tangents to the fluorescence curves and are plotted in (C) (LUV) and (D) (SUV). At the higher rates of fusion, fluorescence was recorded at very high chart speeds. Lipid concentration: 0.05 (●), 0.2 (LUV, panel C), and 0.01 mM (SUV, panel D) (○).

the initial rates of fusion to the Ca^{2+} concentration (Figure 6C,D). The Ca^{2+} threshold concentrations as defined above were essentially independent of the vesicle concentration. It should be emphasized that at Ca^{2+} concentrations slightly below threshold some fusion reaction could be observed (Figure 6A,B), which was obviously more pronounced, in terms of absolute rate, at higher vesicle concentrations. Therefore, it is difficult to define an absolute threshold value (i.e., a concentration below which there is no fusion whatsoever). Our definition of the threshold concentration is a kinetic one and permits an unambiguous comparison between SUV and LUV.

The initial rate of fusion increased with increasing Ca^{2+} concentration (up to 10 mM; Figure 6C,D), but at higher Ca^{2+} concentrations an apparent saturation was observed. Under those conditions, fusion rates were extremely high, but only in the case of SUV at a concentration of 50 μM were the limits of the instrumentation reached. At the lower vesicle concentration, SUV fusion could be quantitated fairly accurately. The rate of SUV fusion at saturating Ca^{2+} concentrations appeared to be about 5-fold higher than the maximal rate of fusion of LUV at the same vesicle concentration. It should be noted that the initial rates of Tb fluorescence increase may be influenced to a certain extent by multiplicity of fusion events as discussed above (see also Figure 4). However, the resulting uncertainty in the degree to which the rate of the Tb fluorescence increase represents the actual rate of fusion is limited to a factor of maximally two. In any case, as discussed above, the rates of the Tb fluorescence increase are underestimates of the actual rates of fusion.

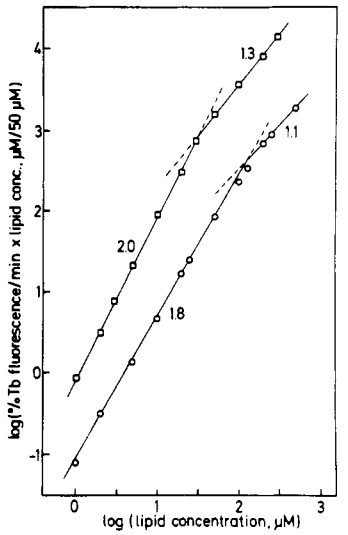

FIGURE 7: Dependence of PS vesicle fusion on the vesicle concentration. Fusion was initiated by addition of $CaCl_2$ as a 0.1 M solution to a 1:1 mixture of Tb and DPA vesicles at various lipid concentrations. In all cases, the fluorescence scale was calibrated to the amount of Tb present. Initial rates of Tb fluorescence increase (presented as percent of maximal fluorescence per min) were then corrected for the actual lipid concentration, relative to 50 μM, and plotted in a double-logarithmic manner against the lipid concentration. (○) LUV; Ca^{2+} concentration, 5.0 mM. (□) SUV; Ca^{2+} concentration, 2.0 mM.

Vesicle Concentration Dependence of Fusion. Mixing of the contents of initially separate vesicles is dependent on vesicle–vesicle interaction and is therefore expected to be second order with respect to the vesicle concentration. A lower than second-order rate (if observed) cannot be the result of a contribution of single vesicles but must imply that the frequency of vesicle–vesicle collisions is not the limiting factor for the rate of mixing of vesicle contents.

In Figure 7, the log of the initial rate of the Tb fluorescence increase at a fixed Ca^{2+} concentration is plotted against the log of the vesicle concentration. At the lower vesicle concentrations, the order of SUV fusion was exactly 2.0 and that of LUV fusion 1.8, indicating that the rate of vesicle aggregation was the major determinant of the rate of fusion. At higher vesicle concentrations, the order of the fusion reaction decreased to 1.3 for SUV and 1.1 for LUV. Therefore, at relatively high vesicle concentrations, the fusion per se is rate limiting and appears to be delayed with respect to aggregation. It should be noted that these measurements were done at a subsaturating Ca^{2+} concentration, and much higher fusion rates can be obtained at higher Ca^{2+} concentrations. This implies that the rates of fusion observed at the higher vesicle concentrations in the experiments of Figure 7 are dependent on the Ca^{2+} concentration despite the fact that fusion is delayed with respect to aggregation.

Electron Microscopy. We have used negative-stain electron microscopy to monitor the increase in vesicle size during the

early stages of Ca^{2+}-induced fusion. Before addition of Ca^{2+}, vesicle diameters were approximately 250 Å for SUV and 1000 Å for LUV (results not shown). To vesicle suspensions of 0.2 mM total lipid concentration (at 25 °C) was added $CaCl_2$ to a final concentration of 1.5 mM (SUV) and 5 mM (LUV). Fusion was stopped by addition of EDTA (as in Figure 5) at various time intervals after Ca^{2+}. At the earliest time interval (7 s for SUV and 20 s for LUV), the samples contained a high proportion of vesicles 5–10 times larger than the original. After 30–60 s following addition of Ca^{2+}, practically all the material was in the form of large vesicles. In parallel experiments with the Tb fusion assay, the maximum yield of fluorescence was reached at 7 s (for SUV) and 20 s (for LUV) after the addition of Ca^{2+}. The morphological observations, therefore, indicate that the Tb fluorescence increase is accompanied by a substantial increase in the vesicle size.

Discussion

The assay described in the present paper meets a rigorous criterion for vesicle–vesicle fusion, i.e., mixing of the internal aqueous compartments of the vesicles. It relies on the formation of the fluorescent Tb/DPA complex upon mixing of the vesicle contents and allows continuous monitoring of fusion processes. The most important advantage of the assay is the fast rate of Tb/DPA complex formation, which makes the method particularly suitable for kinetic analyses of vesicle fusion. Formation of the Tb/DPA complex is reversible, which implies that under the experimental conditions the reaction is prevented from occurring outside the vesicle and also that fluorescence quenching occurs upon release of Tb/DPA complex into the external medium. Although this could pose a disadvantage in that the extent of fusion based on the maximal value of Tb fluorescence can be an underestimate, corrections can be made conveniently by independent determination of the release of vesicle contents (Nir et al., 1980; see also Figure 3).

Assay systems described previously for following the mixing of vesicle contents have involved the use of a Ca^{2+}-sensitive dye (arsenazo III) or the use of enzyme reactions. The arsenazo system (Dunham et al., 1977) required extensive corrections for release of vesicle contents since the reaction could not be prevented from occurring in the external medium. The most thoroughly studied method based on an enzyme reaction is the rather complicated luciferase system (Ingolia & Koshland, 1978; Holz & Stratford, 1979). Unlike the arsenazo system, this method in principle does permit semicontinuous monitoring. However, accurate quantitation is extremely difficult. In addition, reported values for the extents of vesicle fusion as monitored by the luciferase assay are low, and conflicting conclusions have been reached as to the leakiness of the fusion (Ingolia & Koshland, 1978; Holz & Stratford, 1979). Comparison of these data with our results is hampered by differences in the type and lipid composition of the vesicles used. Recently, an elegant and simple fusion assay has been described by Hoekstra et al. (1979) which involves the fluorescence development due to the degradation by trypsin within vesicles of an intramolecularly quenched fluorogenic substrate in the presence of a trypsin inhibitor in the external medium. Fusion can be continuously monitored and accurately quantitated; however, the assay may not be particularly suitable for following very fast kinetics of fusion since an enzyme reaction is involved.

Our results demonstrate that fusion is one of the earliest events during Ca^{2+}-induced interaction of PS vesicles. Previous studies had shown that Ca^{2+} induces aggregation of SUV (PS), release of vesicle contents, and eventually formation of large cochleate cylinders (Papahadjopoulos et al., 1975, 1977; Portis et al., 1979). It now appears that fusion occurs at a similar time scale as the initial aggregation and clearly precedes the release of vesicle contents. Two important conclusions can be drawn from this observation. First, fusion does not require initial rupture or lysis of the vesicles as has been proposed by Ginsberg (1978). The close contact between the vesicles induced by Ca^{2+} through the formation of an anhydrous interbilayer complex and a possible concomitant isothermal phase transition (Portis et al., 1979) appear to be sufficient to trigger the immediate fusion of the two membranes and the mixing of the internal volumes. Second, the fusion event per se is a relatively nonleaky process (Figure 3). Thus, mechanistically, the Ca^{2+}/PS system would appear to be an almost ideal model for membrane fusion. Other systems with only low leakage during fusion have also been described recently (Liao & Prestegard, 1979). With respect to the mechanism of fusion, two points should be made. First, the aggregation of the vesicles is not sufficient to induce fusion: Mg^{2+} induces aggregation of LUV (PS), but there is neither fusion nor release of vesicle contents (J. Wilschut, N. Düzgüneş, W. J. Vail, and D. Papahadjopoulos, unpublished experiments). This is presumably related to the inability of Mg^{2+} to induce the formation of an anhydrous trans complex between apposed vesicles (Portis et al., 1979). Therefore, the complete removal of water through the formation of the trans complex would appear to provide the necessary close approach of the bilayers that initiates the actual fusion. Second, the release of vesicle contents appears to be related to the subsequent collapse of the fused vesicles (Figure 3). This collapse may be the result of the entry of Ca^{2+} into the vesicles and its binding to the inner-monolayer lipid. However, it is not clear how Ca^{2+} enters the internal vesicle compartment during a relatively nonleaky fusion event. Although the present study does not provide the experimental data that are necessary to answer this question, the following points can be made. A limited release of vesicle contents does not necessarily imply a similarly limited entry of external medium. In addition, since the Ca^{2+} concentration at the vesicle surface is extremely high, even a limited leakiness of the fusion event could result in a relatively large influx of Ca^{2+}. Furthermore, it is conceivable that the Ca^{2+} ions that are initially involved in the area of tight contact between the vesicles are transferred to the inner vesicle compartment as a result of the molecular rearrangements occurring during an otherwise nonleaky fusion event. Thermodynamically, such a mechanism would be feasible, since it would lead to a reduction of the Ca^{2+} concentration gradient across the membrane that may be considered as the driving force for fusion (Papahadjopoulos et al., 1977).

The above conclusion that fusion is one of the earliest events during Ca^{2+}-induced PS vesicle interaction prompts the question as to whether vesicle aggregation is rate limiting to the overall fusion process. PS vesicle aggregation is governed by repulsive electrostatic and attractive van der Waals forces (Nir & Bentz, 1978). In the absence of Ca^{2+}, electrostatic repulsion will keep the vesicles apart. Addition of Ca^{2+} leads to a lowering of the surface potential due to binding of Ca^{2+} to the vesicles. Aggregation starts to occur at Ca^{2+} concentrations corresponding to a Ca^{2+}/PS ratio at the vesicle surface of approximately 0.4 (Portis et al., 1979). Consequently, at that ratio, there is still electrostatic repulsion which reduces the potential rate of vesicle aggregation. The binding of Ca^{2+} to the vesicles can account for almost complete charge neutralization only when the bulk Ca^{2+} has reached concentrations of several tens millimolar (Nir & Bentz, 1978; Portis et al.,

1979). Therefore, our observation that the initial rate of fusion is dependent on Ca^{2+} concentration in a wide range (up to at least 10 mM) is entirely consistent with vesicle aggregation being rate limiting. Our data obtained at very high Ca^{2+} concentrations find a precedent in the work of Lansman & Haynes (1975). These investigators determined the rate of SUV (PS) dimerization in the presence of 50 mM Ca^{2+} and reported an apparent rate constant of 1.7×10^7 M^{-1} s^{-1}, assuming a vesicle diameter of 250 Å. This rate constant appeared to be about two orders of magnitude lower than the rate constant for diffusion-controlled aggregation that can be calculated from the theory of Smoluchowski (1917) for the coagulation of uncharged colloids. Calculations by Nir & Bentz (1978) demonstrated that the lower rate of aggregation of PS vesicles is due to a residual surface potential that is present even at 50 mM Ca^{2+}. From our data on the rate of increase of Tb fluorescence at saturating Ca^{2+} concentrations (Figure 7), the apparent rate constants can be calculated by using the following equation (Lansman & Haynes, 1975):

$$k_{app} = \frac{\alpha N}{2(1 - \alpha)Ct}$$

in which N represents the number of lipid molecules per vesicle, C the lipid concentration, and α the degree of progress of the reaction at time t. We consider $\alpha = 1$ to correspond to one complete round of fusion, i.e., 50% Tb fluorescence. When the initial fusion rates for SUV (4000 lipid molecules per vesicle) are 150% and 55% maximum Tb fluorescence per s at 50 and 10 μM lipid, respectively, while those for LUV (80 000 lipid molecules per vesicle) are 10% and 4.4% maximum Tb fluorescence per s at 200 and 50 μM lipid, respectively, values for k_{app} of 1.3×10^8–2.4×10^8 M^{-1} s^{-1} for SUV and 4.4×10^7–7.8×10^7 M^{-1} s^{-1} for LUV are obtained. These values are higher or at least of the same order of magnitude as the k_{app} for aggregation reported by Lansman & Haynes (1975). Our value for SUV even approaches to within about an order of magnitude the maximally possible, diffusion-controlled, rate of aggregation. Therefore, it can be concluded that under our experimental conditions vesicle aggregation was rate limiting to the overall fusion process. Nir et al. (1980) reach the same conclusion by analysis of fusion data obtained at subsaturating rather than saturating Ca^{2+} concentrations. Likewise, the results in Figure 7, also obtained at a subsaturating Ca^{2+} concentration, indicate that the frequency of vesicle–vesicle collision determines the rate of fusion in a wide vesicle concentration range. Only at very high vesicle concentrations does fusion appear to be delayed with respect to aggregation. Since under these conditions the actual rates of fusion are considerably lower than the rates at very high Ca^{2+} concentrations (Figure 6), it follows that although the vesicles are already aggregated their rate of fusion is dependent on the Ca^{2+}/PS ratio at the vesicle surface.

The present studies on fusion of LUV (approximately 1000-Å vesicles) clearly demonstrate that phospholipid vesicle fusion, which has been studied up to now mainly with SUV preparations, does not require a strongly curved, "strained", vesicle bilayer. Yet, a number of differences between SUV and LUV fusion were observed. First, the leakiness of the fusion event (Figure 3): The greater initial leakiness of SUV than LUV fusion can be explained by the release of a quantum of internal vesicle volume per fusion event. Such a quantum could be expected to represent a significant proportion of the internal volume of a small vesicle, whereas it would be negligible relative to the contents of a large vesicle. Second, the Ca^{2+} threshold concentrations (Figure 6): The difference in this respect between SUV and LUV is not due to a different Ca^{2+}/PS ratio at the vesicle surface, since the binding of Ca^{2+} to LUV is identical with its binding to SUV (C. Newton, unpublished experiments). It could be the result of a difference in the potential barrier between two vesicles. The height of this barrier is approximately proportional to the diameter of the vesicles (Nir et al., 1980). Thus, with LUV, a lower surface charge density is required to achieve substantial aggregation than with SUV. Third, the rate constant of fusion at saturating Ca^{2+} concentrations: The diffusion-controlled rate of aggregation of uncharged particles is independent of particle size (Smoluchowski, 1917; Lansman & Haynes, 1975). Consequently, the 3–5-fold lower rate constant of LUV fusion relative to that of SUV fusion (see above) cannot be attributed to the lower rate of diffusion of the larger vesicles. However, similar to the discrepancy in Ca^{2+} threshold concentration, the difference in the rate constant at very high Ca^{2+} levels can, at least in part, be explained by a difference in the residual potential barrier of the LUV and the SUV. In addition, it cannot be excluded that SUV have, intrinsically, a higher tendency to fuse than LUV, probably because of differences in molecular packing.

The mechanism of Ca^{2+}-induced PS vesicle fusion, as discussed above, adds considerably to the significance of this system as a model for biological membrane fusion. Yet, there are at least two major discrepancies between PS vesicle fusion and biological fusion phenomena, i.e., the requirement of a relatively high Ca^{2+} concentration and the requirement of a high PS content of the bilayer. These discrepancies have been discussed in detail previously (Papahadjopoulos, 1977, 1978; Papahadjopoulos et al., 1978; Portis et al., 1979). In addition, the following remarks on other factors possibly participating in membrane fusion can now be made: (1) In the presence of phosphate, the threshold Ca^{2+} concentration is reduced and the rates of fusion are considerably enhanced (Fraley et al., 1980). (2) The Ca^{2+}-binding protein synexin (Creutz et al., 1978) also increases the rate of fusion of phospholipid vesicles (Düzgüneş et al., 1980a; K. Hong, N. Düzgüneş, and D. Papahadjopoulos, unpublished experiments). (3) In contrast to phosphatidylcholine, phosphatidylethanolamine can replace a major proportion of the PS in the bilayer without affecting the ability of the vesicles to fuse (Düzgüneş et al., 1980b); this is in agreement with recent results reported by Kolber & Haynes (1979) on the possible role of phosphatidylethanolamine in modulating membrane contact.

Acknowledgments

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Appendix

(*1*) *Effects of EDTA on Fusion.* The following experimental observations give additional support to our conclusion that addition of EDTA following multiple fusion events tends to disrupt the vesicles and releases part of their contents:

(A) In all cases where after EDTA addition a decrease of the Tb fluorescence was observed, a comparable increase could

be seen in corresponding CF release measurements (not shown). This result eliminates the possibility that the major effect of EDTA is by entry into the vesicles and favors the mechanism of EDTA-induced partial disruption of the vesicles.

(B) The extent of EDTA-induced bilayer disruption would conceivably increase with the size of the vesicles and the multiplicity of fusion sites within the aggregate, whereas the extent of Tb/DPA complex formation outside the vesicles, if occurring, would increase with the ratio of extravesicular to intravesicular volume in the aggregate and thus with the number of vesicles per aggregate. Invariably, Tb fluorescence intensities can be fixed almost completely when the vesicles in the aggregate, irrespective of their number, are relatively small: with SUV the Tb fluorescence can be consistently fixed better than with LUV (Figure 5). After Mg^{2+}-induced fusion of SUV, which results in an only limited size increase (J. Wilschut, N. Düzgüneş, S. Nir, and D. Papahadjopoulos, unpublished experiments), Tb fluorescence levels can be fixed by EDTA almost completely even after a prolonged period of time; the same holds for SUV fusion induced by 1 mM Ca^{2+} in a 5 mM NaCl medium (Wilschut et al., unpublished experiments).

(C) When fusion is induced in the presence of a high EDTA concentration (10 mM) with excess Ca^{2+}, the relative drop in the Tb fluorescence after addition of more EDTA is exactly the same as in the corresponding experiment done in the absence of a high initial EDTA concentration (Figure 5E). This indicates that the decrease in the Tb fluorescence after addition of EDTA is not due to quenching of Tb/DPA complex formed outside the vesicles, since the high initial EDTA concentration in the medium would have prevented, at least partially, such complex formation. In fact, the EDTA initially present does affect to some extent the Tb/DPA complex formation inside the vesicles presumably through entry during the fusion process, as is clear from the lower maximal Tb fluorescence level reached.

(D) LUV (PS) aggregate in the presence of mixtures of Ca^{2+} and Mg^{2+}. At the proper ratio of the two cations there is negligible Tb fluorescence development, but essentially complete release of contents, which is delayed with respect to aggregation (Wilschut et al., unpublished experiments). This observation clearly demonstrates that release of vesicle contents within an aggregate does not result in Tb/DPA complex formation. Therefore, the reduction of Tb fluorescence caused by EDTA could not be due to its interference with the Tb/DPA reaction outside the vesicles.

(*2*) *Release of Contents Associated with a Fusion Event.* Under Kinetics of Fusion and Release of Contents it was concluded that an early fusion event between SUV is accompanied by the release of about 10% of their encapsulated volume. This conclusion was essentially confirmed by the results of a fusion experiment carried out in a medium containing 5 mM rather than 100 mM NaCl.

In 5 mM NaCl the threshold Ca^{2+} concentration required to induce SUV (PS) fusion is lower than in 100 mM NaCl (Düzgüneş et al., 1980c). When fusion was induced in this medium with 1 mM Ca^{2+} (slightly above the threshold), collapse of the vesicles appeared to be prevented; after an initial increase the Tb fluorescence remained constant at a level of 40%. Under these conditions the release of CF was very limited and leveled off to a plateau value of 10% maximal CF fluorescence. In this experiment, rather than initial rates, the eventual stable situation demonstrated that the fusion had occurred without much release of vesicle contents. Although it is not quite clear why under these conditions the fusion and collapse process is interrupted, it is apparent that 1 mM Ca^{2+} is not sufficient to induce fusion beyond a limited size increase or cause the collapse of the vesicles.

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Studies on the Mechanism of Membrane Fusion: Role of Phosphate in Promoting Calcium Ion Induced Fusion of Phospholipid Vesicles†

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ABSTRACT: The role of phosphate in enhancing the calcium-induced fusion of phosphatidylserine (PS) vesicles has been examined by using the new fluorescent (terbium/dipicolinic acid) assay described by Wilschut et al. (1980) [Wilschut, J., Düzgüneş, N., Fraley, R., & Papahajopoulos, D. (1980) *Biochemistry* (preceding paper in this issue)]. In the presence of physiological levels of phosphate, the calcium concentration required for fusion of PS vesicles was lowered significantly (3–4-fold), and the rate of vesicle fusion was increased dramatically (up to 1000-fold). The fusion of PS vesicles by calcium and phosphate is shown to be specific and critically dependent on temperature, pH, ion concentrations, and the composition of the calcium phosphate crystalline phase present during the incubation. The results indicate that a significant enhancement in vesicle fusion occurs only when calcium phosphate precipitation is initiated in the presence of PS vesicles, suggesting that crystal nucleation on the vesicle surface is a prerequisite for fusion. Calcium and phosphate were shown to promote phospholipid phase separations and vesicle fusion under conditions (e.g., mixtures of PS and phosphatidylcholine) in which calcium alone is ineffective, indicating that formation of PS calcium phosphate complexes may facilitate the molecular segregation of PS into distinct domains. These experiments underline the important role that phosphate may play in calcium-mediated fusion phenomena in biological membranes.

The fusion of biological membranes is of fundamental importance in diverse cellular processes such as fertilization (Gwatkin, 1976), viral infection (Murayama & Okada, 1965), myoblast differentiation (Buckingham, 1977), trichocyst discharge in paramecia (Matt et al., 1978), and neurotransmitter release from presynaptic vesicles (Kelly et al., 1979). The involvement of Ca^{2+} and its interaction with acidic phospholipids have been implicated in a critical regulatory role in these membrane fusion phenomena (Poste & Allison, 1973; Douglas, 1975). In this respect, investigations of the interaction of Ca^{2+} with acidic phospholipids in well-defined model membrane systems (Papahadjopoulos et al., 1976; Hauser et al., 1977; Ohnishi & Ito, 1974) are particularly useful in extending our understanding of the mechanism(s) of membrane fusion.

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