Rapid Kinetics of Ca$^{2+}$-induced Fusion of Phosphatidylserine/Phosphatidylethanolamine Vesicles

THE EFFECT OF BILAYER CURVATURE ON LEAKAGE*

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We have employed both small unilamellar vesicles (SUV) and large unilamellar vesicles formed by the reverse phase evaporation technique (REV) to study the initial kinetics of membrane aggregation and fusion. Stopped flow measurements of the calcium-induced changes in the turbidity of SUV and REV, formed from 1:1 (mol/mol) mixtures of bovine phosphatidylserine (PS) and Escherichia coli phosphatidylethanolamine (PE), were used to follow particle aggregation. Simultaneous measurements of the fluorescence resonance energy transfer from N-(7-nitro-2,1,3-benzoxadiazol-4-yl) (NBD)-PE to rhodamine (Rho)-PE incorporated into the vesicle bilayers established that 1) both initial aggregation and fusion can be described as a bimolecular process and 2) the rate-limiting step of membrane fusion is aggregation. Thus fusion takes place in the microsecond time domain.

Parallel experiments, which simultaneously measured aggregation and the dequenching of encapsulated carboxyfluorescein (CF) in the presence and absence of antifluorescein antibodies in the suspension medium, established that the small unilamellar vesicles rapidly lose their contents of CF as they fuse. On the other hand, the first few cycles of fusion of the large unilamellar vesicles are nonleaky, but leakage develops within 1–2 s as the particles grow in size. Thus the results demonstrate that the SUV are poor models for the study of nonleaky fusion, while the REV must be carefully tested before unambiguous interpretation of fusion assays involving the formation of tight complexes (such as the terbium-dipicolinic assay) can be made.

NBD-PE undergoes very rapid, Ca$^{2+}$-promoted changes in quantum yield which can obscure the resonance energy transfer signals. Thus data from the NBD-PE/Rho-PE energy transfer pair must be carefully scrutinized for artifacts.

Fusion of phospholipid vesicles is currently being studied in a number of laboratories as a model for the fusion of biological membranes occurring in such processes as exocytosis and intracellular traffic between organelles (1–6). Several assays which use properties of fluorescent probes to demonstrate fusion of phospholipid vesicles to each other, to planar bilayers, or to biological structures such as cells or cell organelles have been published (7–12). These fall into two classes: those which show mixing of the membrane components (labeled phospholipids or lipid-soluble probes) or those which indicate the mixing of labeled material trapped within the vesicle lumens. Ideally, both assays need to be carried out simultaneously to demonstrate fusion, since mixing of membrane components does not necessarily imply core mixing and vice versa. For these rapid kinetic studies we have chosen the membrane mixing assay developed by Struck et al. (10). In parallel experiments, we have used vesicle-encapsulated carboxyfluorescein (7) in the presence and absence of external antifluorescein antibody to study fusion-related leakage.

Past literature has presented conflicting opinions as to whether small molecules escape from unilamellar vesicles during fusion (13–15). Our investigation of the rapid kinetics of the Ca$^{2+}$-induced fusion of small unilamellar sonicated phospholipid vesicles formed from mixtures of natural phosphatidylserine and phosphatidylethanolamine gave the result that Ca$^{2+}$ induces aggregation-dependent leakage of materials trapped in the core space of those vesicles. However, with large vesicles of the same composition, we found little leakage in the time range where initial aggregation and fusion (as monitored by membrane mixing) has taken place. These results are discussed in relation to the design of membrane fusion assays.

After a brief account of the work presented here was published (16), Wilschut et al. (17) reported findings similar to ours, basing their conclusions on hand mixing experiments. We comment on the appropriateness of the latter experimental work.

EXPERIMENTAL PROCEDURES

Materials—Bovine brain phosphatidylserine, Escherichia coli phosphatidylethanolamine, and head group-labeled NBD-1 and rhodamine-phosphatidylethanolamine dissolved in CHCl$$_3$$ were purchased from Avanti Polar Lipids (delivered on dry ice), stored at −20 °C, and used within 30 days. Affinity purified rabbit antifluorescein antibody was a gift from Dr. Pierre Henkart. Carboxyfluorescein

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1 The abbreviations used are: NBD, N-(7-nitro-2,1,3-benzoxadiazol-4-yl); PL, phospholipid; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; Rho, lissamine rhodamine; CF, carboxyfluorescein; SUV, small unilamellar vesicles; REV, large unilamellar vesicles formed by reverse phase evaporation; OD, optical density; HEPES, N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid; HEPES buffer, 10 mM HEPES, 10 mM KCl, pH 7.4; HEPES-CF, 78 mM carboxyfluorescein in HEPES buffer.
Bilayer Vesicle Fusion Kinetics

(1989) Rochester, NY) was purified by LH-20 chromatography according to Ralston et al. (18). All other chemicals were of reagent grade and used without further purification.

Formation of Bilayer Vesicles—If NBD-PE or Rho-PE were to be incorporated into the vesicles, they were preincubated with the lipids in the organic solvent. For the preparation of SUV, 4.0 mg of lipid were dissolved in a 20-ml glass scintillation counting vial, followed by exposure to 3 × 10⁻⁶ Torr for 15 min to remove traces of organic solvent. The lipid was hydrated with 2.5 ml of a solution containing 16 mM HEPES, 10 mM KCl, pH 7.4 (HEPES buffer) or with 2.5 ml of HEPES-buffered 76 mM carboxylfluorescein. The suspension was mixed by vortexing and subsequently sonicated for 6 min at room temperature under argon using a flat tip probe (Heat Systems, W-350, Plainview, NY). The samples were centrifuged at 30,000 g for 10 min to remove metal particles and multimellar structures.

Large unilamellar vesicles were made by modifications of the reverse-phase evaporation technique of Stoka and Papahadjopoulos (19). 25 mg of lipid were dried under argon and dissolved in 2.5-3.0 ml of ethyl ether. One ml of HEPES buffer or of HEPES-DE was added and the sample was sealed and sonicated in a bath sonicator (Laboratory Supplies Company, Inc., Hicksville, NY) until one phase formed (about 10 min). The sample was placed in a rotary evaporator and the ether was removed while the sample was rotated. As the gel phase formed, the sample was occasionally removed from the evaporator and thoroughly mixed by vortexing. A 1.0-ml aliquot of buffer (or for CF-containing vesicles, HEPES-buffered sucrose at a concentration to match the osmotic pressure of the HEPES-DE) was added and the remaining ether was removed by connecting the rotary evaporator to a vacuum pump for approximately 30 min. The solution was then filtered first through a 0.4-μ filter followed by filtration through a 0.2-μ filter.

The CF-containing samples were chromatographed on Sephadex G-25 to remove nonincorporated CF. Osmotic pressure was maintained by eluting the columns with buffered sucrose. Absence of free CF in the chromatographed samples was demonstrated by lack of decrease of CF fluorescence (excitation/emission, 492 nm/515 nm) upon the addition of antifluorescein.

Fluorescence measurements were made in cuvettes (5 × 5 mm) in a thermostatted cell holder in a Perkin-Elmer 650-10S spectropho-

while this machine lacked the precision for good scanning measurements, it was adequate for measurements at set wavelengths as a function of time. NBD to rhodamine resonance energy transfer was measured at 460 nm/590 nm using either crossed polaroid filters or a Schott OG515 high pass filter to reduce scattering artifacts. Results with both configurations were virtually identical. No attempt was made to mix faster than within 2 s.

Stopped flow measurements were made using an Amino Chance monochromator to select excitation wavelengths and an Amino Chance stopped flow drive. Fluorescence intensity was measured at right angles to the incident beam using either a Schott OG515 high pass for fluorescence) or a 590-nm bandpass interference filter for resonance energy transfer measurements. OD was measured simultaneously at 450 nm for resonance energy transfer or 420 nm for CF. The latter wavelength was chosen to reduce the direct absorption of fluorescence which interfered with the recording of the turbidity changes as the particles aggregated. Introduction of crossed polaroids did not change the results except to reduce transmission by > 95% and fluorescence intensity by approximately 50%. Mixing configurations are detailed in the tables and figures.

Data acquisition was under the control of a PDP 11/34 computer which sampled and digitized the output from the two photomultipliers and stored the records on disks. Data analysis was performed using the curve fitting routines available on the interactive MLAB modeling program running on the NIH DEC 10 computer facility or the SAAM 27 modeling program running on the VAX 11/780 in the Laboratory of Theoretical Biology, NCI.

Preliminary Stopped Flow Experiments—Initial experiments established that hand or stopped flow rapid mixing of CF-containing vesicles against isomotic buffered sucrose produced no release of CF. Thus when argon in a 20-ml glass scintillation counting vial was passed over the solutions of stopped flow mixing. Also, binding of Ca²⁺, which occurs when the mixing time (20), did not induce a leaky process which proceeded faster than aggregation, whereas stopped flow mixing with Triton X-100 (final concentration 0.1%) produced release too fast to measure. Preliminary experiments established that 0.33 mg/ml antifluorescein would quench the fluorescence of 3 μM CF. This reaction took place within the mixing time of the stopped flow (< 3 ms). Mixing both types of vesicles against hypotonic media did induce rapid leakage, measurable by stopped flow. No appreciable energy transfer was seen when PC vesicles labeled with NBD-PE and Rho-PE were aggregated with Ca²⁺ in the presence of calelectrin, a protein which aggregates PC vesicles (21). This shows that fusion is required for energy transfer to occur.

RESULTS

Kinetcs of Vesicle Aggregation—In a series of previous papers we have demonstrated that the progress of the dimmerization and multimerization of both small unilamellar vesicles (20, 22, 27) and larger biological vesicular structures (23-26) can be measured by the increase in optical density. This can be described as the sum of two second order processes:

\[ \Delta OD = A_1 \cdot \frac{k_1 t}{1 + k_1 t} + A_2 \cdot \frac{k_2}{1 + k_2 t} \]  

where \( A_1 \) and \( A_2 \) are the amplitudes and \( k_1 \) and \( k_2 \) are the apparent rate constants of the fast and slow reactions at a given vesicle concentration. We previously demonstrated that the fast process represents the aggregation of monomer vesicles into dimers, which would be the earliest contact-mediated interactions between particles (23, 27). We have no theoretical justification for the slower process, but arbitrarily ascribe it to the formation of larger multimeric aggregates.

Mixtures of PS:PE (1:1, mol/mol) were chosen over pure PS for these studies since it has been demonstrated that this mixture is less likely to form open structures during fusion (28) and that the mixture has a slightly lower \( k_0 \) for Ca²⁺-initiated aggregation and fusion than pure PS (20). Fig. 1, A and B (lower curves), shows typical changes in OD as a function of time for small unilamellar vesicles and large unilamellar vesicles, respectively. Equation 1 gives an excellent fit to the data for the first several seconds when the aggregates are predominantly dimers and oligomers; however, it fails at > 200 s as larger aggregates are formed. The data is not well fit by alternative models such as single or double exponential processes.

A second order process should be linearly dependent on substrate concentration. As has been previously demonstrated for Ca²⁺-promoted aggregation of PS vesicles (20) and pH-dependent aggregation of PE vesicles (22), the rates for optical density change are linear with lipid concentration with a fitted intercept of approximately zero (Fig. 2).

If we presume a vesicle diameter of 250 Å and a surface area per PL of 60 Å², an apparent rate constant (\( k_{app} \)) of 1.64 \( \times 10^5 \) M⁻¹ s⁻¹ for the fast reaction was calculated from the slope of Fig. 2A and the relation (20):

\[ k_{app} = \frac{N_{PL}}{2} \cdot \frac{k_1}{k_2} \]  

where \( N_{PL} \) is the number of phospholipid molecules per vesicle and \( k_1 \) is the initial rate at the molar phospholipid concentration [PL].

Assuming a value of \( 6.8 \times 10^4 \) M⁻¹ s⁻¹ for the diffusion-limited rate of vesicle collisions (20, 25, 26) gives a success rate for formation of a stable dimer of ~1/400 collisions or about the same as that reported for PS SUV at 10-fold higher calcium concentration (20).

Similar data was generated for the REV (Fig. 2B). Assuming

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2 All PDP 11/34 programs and documentation may be obtained from C. C. Gibson, DRS, BEIB, Bldg. 13, Room 3W13, NIH, Bethesda, MD 20205.

3 T. C. Südhof and S. J. Morris, unpublished data.

4 S. J. Morris, unpublished data.
an average diameter of 1500 Å for the REV, a similar calculation yielded a $k_{app}$ of $7.92 \times 10^7$ m$^{-1}$ s$^{-1}$ and a success rate of $-1/85$ collisions.

**Kinetics of Membrane Fusion**—Mixing of the lipid bilayers has been measured during fusion of unilamellar vesicles by resonance energy transfer (28-31) from NBD-PE to Rho-PE (10, 32, 33). When these probes are incorporated into the PS:PE vesicles, stopped flow mixing with Ca$^{2+}$ produces an initial increase in fluorescence at 590 nm which is 10-100-fold faster than aggregation (Fig. 1, insets). This process was fit to a single exponential and subtracted from the data before applying Equation 1 (see discussion below).

When this first, very fast transient of fluorescence change is removed, the energy transfer data is seen empirically to fit well to Equation 1 with the fluorescence changes having almost the same rate constants as the OD changes (Fig. 1). Thus for both types of vesicles formed from this lipid mixture, it would seem that the rate-limiting step for fusion (intermixing of membrane lipids) is initial aggregation. For any given Ca$^{2+}$ concentration, the $k$ values and the amplitudes for dimerization and higher order aggregates are proportional to the vesicle concentration (20, 27). The relative amplitudes of the OD changes are due to the size of the particles (20, 23, 24). The relative amplitudes of the fluorescence changes and NBD-PE to rhodamine-PE fluorescence resonance energy transfer during calcium-promoted fusion of PS:PE (1:1) vesicles. Fluorescent labels were incorporated into REV and SUV at ~1 probe/100 lipid molecules. For each vesicle size, equal concentrations of NBD- and rhodamine-containing vesicles were mixed together and then subjected to stopped flow mixing against a buffered calcium chloride solution. Final lipid and calcium concentrations were 25 µg/ml and 3.0 mM for Experiment A and 54 µg/ml and 5 mM for Experiment B. $A_1$, $k_1$, $A_2$, and $k_2$, as defined in Equation 1, were calculate by a four-parameter fit to the data of Fig. 1.

**TABLE I**

<table>
<thead>
<tr>
<th>Experiment and mixing configuration</th>
<th>Measurement</th>
<th>$A_1$</th>
<th>$k_1$</th>
<th>$A_2$</th>
<th>$k_2$</th>
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<tr>
<td>A. NBD- and rhodamine-labeled SUV</td>
<td>ΔOD</td>
<td>0.010</td>
<td>2.35</td>
<td>0.030</td>
<td>5.10</td>
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<tr>
<td></td>
<td>ΔFL</td>
<td>55.6</td>
<td>2.51</td>
<td>43.2</td>
<td>5.02</td>
</tr>
<tr>
<td>B. NBD- and rhodamine-labeled REV</td>
<td>ΔOD</td>
<td>0.004</td>
<td>0.519</td>
<td>0.029</td>
<td>4.05</td>
</tr>
<tr>
<td></td>
<td>ΔFL</td>
<td>47.1</td>
<td>0.680</td>
<td>52.9</td>
<td>9.66</td>
</tr>
</tbody>
</table>

*Units for amplitude changes are OD$_{sec}$ and percentage of total fluorescence (FL) change. Since increasing the Ca$^{2+}$ concentration did not increase the total fluorescence amplitude, implying complete randomization of the probe molecules, the latter was calculated as $A_1/(A_1 + A_2)$, etc.
cence changes are due to the randomization of the probe molecules and the relative change in the efficiency of the energy transfer reaction (29–31).

**Kinetics of Contact-induced Leakage**—Incorporation of carboxyfluorescein into unilamellar liposomes at concentrations high enough to produce self-quenching of the fluorophore has been used to assess leakage induced by crystal → liquid crystal phase transitions as well as the incorporation of proteins into the lipid bilayer (34–36). In the work under consideration here, dequenching (relief of self-quenching) of CF induced by Ca

+ could result either from induced leakage of the membranes or from the expansion of the fluorophore due to the increase in volume expected to result from nonleaky fusion. The latter effect would be greatly enhanced if the CF-containing vesicle fused with an empty vesicle; the former should be reduced or abolished by anti-fluorescein in the suspension medium, as noted above.

For the SUV, release of carboxyfluorescein as measured by dequenching has approximately the same time course as aggregation (Table II, Experiments A and B). Rates and amplitudes of the OD changes are almost identical for the same lipid and Ca

+ concentrations in the presence and absence of antibody. They are also approximately the same for approximately the same lipid concentrations regardless of whether the vesicles contained only CF or were a mixture of CF and empty vesicles. For CF-containing SUV in the absence of empty vesicles, the increase in CF fluorescence shows two second order processes with similar rates for ΔFl and ΔOD. However, if anti-fluorescein was placed in the external buffer of this experiment, the amplitude of the fluorescence dequenching was reduced to zero.

In order to test for transfer of CF between vesicle compartments, CF-SUV were mixed with empty SUV (Fig. 3A and Table II, Experiment B). In this case, the amplitude of both phases of fluorescence change was reduced ~10-fold in the presence of antibody, while the rates did not change appreciably. The persistence of small amplitude phases of CF dequenching in the presence of antibody suggests that a small percentage of the initial and subsequent SUV contacts result in nonleaky transfer of CF between vesicle compartments. However, the bulk of fluorescence dequenching is due to leakage.

As can be seen in Fig. 3B and Table II, the results for the REV are more complex. The amplitudes and rates for optical density changes in the presence or absence of the antibody are similar. However, there is no interpretable fast phase of fluorescence increase corresponding to that seen with SUV samples mixed in the absence of antibody. Fig. 3B shows the typical result; almost no initial change in fluorescence, while optical density shows a rapid, second order increase. Release of CF is seen within 1–2 s, but with other than second order kinetics. The addition of antifluorescein completely abolishes the CF fluorescence changes; no phase of CF dequenching with a greatly diminished amplitude, which would correspond to dilution into a nonleaky compartment, is detected. CF dequenching eventually appears if insufficient antibody is added to quench all the fluorescein present in the sample.

Although fusion of SUV was leaky, experiments in the presence of antifluorescein demonstrated a nonleaky component; we would have expected some dequenching of CF when CF-REV were mixed with empty REV in the presence of the antibody, due to dilution of CF into a larger vesicle volume (Fig. 3B and Table II, Experiment D). However, the concentration of CF used in the REV was purposely set very high (78 mM) to detect any leakage. Thus the expected 3-fold dilution upon fusion of a filled REV with an empty REV would produce only a 2-fold increase in fluorescence of relatively low amplitude (34). The low signal-to-noise ratio in the REV experiment may have precluded the observation of this small nonleaky component of fusion seen with SUV. Using a lower CF concentration would have produced a larger relative change in signal; however, this would have been within the present noise of the fluorescence channel. Dequenching due to dilution upon fusion is being pursued as a method to examine volume changes using improved optics and data processing to improve signal-to-noise ratios.

**DISCUSSION**

**Aggregation As the Rate-limiting Step of Membrane Fusion**—We have previously shown that the aggregation of vesicular structures, which appears to be one continuous reaction in hand mixing experiments, can be resolved by stopped flow techniques into two processes: dimerization and the formation of multimers. The present communication demonstrates that this type of analysis can be extended to membrane fusion events.

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**Table II**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>A1</th>
<th>k1</th>
<th>A2</th>
<th>k2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. CF-SUV (6.7 μg/ml)</td>
<td>0.003</td>
<td>0.002</td>
<td>2.10</td>
<td>1.41</td>
</tr>
<tr>
<td>B. CF-SUV + control SUV (20 μg/ml)</td>
<td>0.012</td>
<td>0.010</td>
<td>4.77</td>
<td>5.69</td>
</tr>
<tr>
<td>C. CF-REV (15 μg/ml)</td>
<td>0.012</td>
<td>0.015</td>
<td>3.65</td>
<td>1.28</td>
</tr>
<tr>
<td>D. CR-REV + control REV (30 μg/ml)</td>
<td>0.007</td>
<td>0.011</td>
<td>3.96</td>
<td>4.11</td>
</tr>
</tbody>
</table>

*Fluorescence data could not be fit to Equation 1.
Antibody, when present, was premixed with the CaCl solution to a conical in the presence of large unilamellar vesicles in the presence of fluorescence curves in mixed with empty vesicles at a ratio of approximately 1:2, and stopped by estimating the initial slope of the hand mixing experiments. Our results for PS:PE (1:1) vesicles are quite similar and the differences in the SUV results may be explained by differences in lipid mixes. However, Papahadjopoulos and collaborators (13, 28) found little differences between PS and PS:PE (1:1) for Ca²⁺ threshold and measurable rates of fusion for hand mixing experiments. We would suspect that the error in estimates of initial rates made by estimating the initial slope of the hand mixing experiments may bias the results calculated by the mass action model. Another example of such distortion is the assertion that aggregation will be rate-limiting only at very dilute vesicle concentrations; Nir et al. (38) measured fusion rates which were 3-fold slower than aggregation at lipid concentrations of ~80 μg/ml for SUV. Our data shows that aggregation is the rate-limiting step in the fusion process for PS:PE (1:1) vesicles at lipid concentrations up to 120 μg/ml.

**Tight Fusion of REV**—The relief of self-quenching of CF seen during the fast phase with SUV indicates that CF leaks rapidly out of these vesicles upon Ca²⁺-induced fusion. The evidence for leakage is strengthened by the use of the antibody (Fig. 3 and Table I). A similar conclusion was recently inferred from hand mixing experiments (17). While this type of analysis may lead to valid conclusions, from the experimental conditions presented in Ref. 17 (40-60 μg lipid, 2.0-3.0 mM Ca²⁺, 25°C) the values noted above for k_app would predict half-times for initial events of 0.5-2.0 s. These half-times could not be resolved by hand mixing. A similar criticism can be leveled against Hoekstra's (probably valid) conclusion that membrane and core contents mix at the same rates during SUV fusion (14).

**REV show no appreciable leakage during initial fusion; however, this eventually appears within 1-2 s. The relief of self-quenching of CF in REV can be interpreted in two ways: 1) initial contact and fusion is nonleaky (after a few cycles of fusion, the REV become leaky, perhaps through the formation of open structures); 2) aggregation of large unilamellar vesicles (REV) does not lead to intermediate fusion. Leaksiness develops as the vesicles are converted from aggregates to fused structures. The second model is refuted by the resonance energy transfer experiments which show that mixing of membrane components for both types of vesicles occur with essentially the same kinetics as aggregation (Figs. 1 and 2 and Table I). The results also agree with the observations of Wilschut et al. (13), who have suggested that the fused structures collapse and open as they grow larger.

**Effect of Membrane Curvature**—It is interesting to note that the mechanical force of the SUV seems to be induced by calcium-mediated vesicle-vesicle contact. One might expect that SUV would be leaky due to their very high radius of curvature, leading to defects in the packing of the phospholipids. However, CF encapsulated in both neutral and charged phospholipid vesicles leaks out very slowly. In one experiment PS:PE SUV and REV showed negligible leakage of CF (encapsulated at 78 mM) after 8 days storage at 0°C. Warming either the fresh or 8-day-old vesicles to 30°C for 2 h also did not induce release.

**Effect of Calcium on NBD**—As noted above there is a process in the fluorescence data which runs 10-100-fold faster than aggregation itself. This can be seen when NBD-containing vesicles are mixed against Ca²⁺; it is absent when Rh-containing vesicles are tested. Since it runs 10-100-fold faster than aggregation, we ascribe this change to direct interactions of Ca²⁺ with the probe molecules within the plane of a given membrane (39, 40). It quickly becomes obvious that the NBD-Ca²⁺ interactions cannot be described as a simple exponential process. Fitting and subtracting an exponential does not completely remove this artifact. Although the slopes of rate/lipid concentration plots are similar (Fig. 2), the values for the fluorescence rates are almost always higher by 15-20% than the values for optical density, usually becoming progressively worse as the lipid concentration decreases. The fitting strategy sometimes fails completely (in Fig. 24 this occurred below 40 μg/ml lipid). This requires that careful adjustment of experimental conditions be made so that removal of this.
artifact does not become an arduous problem. It would also suggest that other energy transfer pairs may be better experimental probes. Ideal pairs have high quantum yields, very high extinction coefficients and large overlap between donor emission and acceptor excitation (25–27). We have tried dansyl-PE/Rho-PE and fluorescein-PE/Rho-PE. The former was not suitable due to the low quantum yield and extinction coefficient of the donor. The latter pair also gave poor results due to the sensitivity of the donor to pH (33) and the large overlap of donor and acceptor excitation spectra.

Relevance to Core Mixing Assays—Core mixing assays which depend upon collisional quenching, or the proximity of donor and acceptor in solution would fail if fusion of vesicles were leaky, since the trapped materials would rapidly dissipate into the large volume of surrounding medium. However, assays which are based upon the formation of a complex with a high association constant, such as the formation of a terbium-dicyclohexylphosphoric acid complex (8, 13), could be little affected since formation of the complex could take place adjacent to the external surface of the membrane. Thus, our observations may explain some of the conflicting results emanating from various laboratories (12–15).

The leakage of small unilamellar vesicle SUV demonstrated here suggests that they are poor candidates for core fusion assays. An assay based on membrane mixing would be less ambiguous. The initial tight fusion of the REV makes them better candidates for core mixing studies. However, we would point out that in our experiments the time course of the development of CF release was quite variable from one preparation of REV to the next, although all preparations showed lag times of no longer than 1–2 s. This result disagrees with the studies of Wilschut et al. (17), based on hand mixing experiments, which report leakage developing after 50–100 s. It would seem that careful kinetic studies would require stopped flow analysis since leakage can develop within the mixing time for hand mixing experiments (2–3 s), making it impossible to assign the leakage seen to the initial fusion event or subsequent interactions.

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REFERENCES
29. Förster, T. (1951) Fluoreszenz organischer Verbindungen, p. 85, Vandenhoeck und Ruprecht, Gottingen, Germany