Lipid Mixing during Membrane Aggregation and Fusion: Why Fusion Assays Disagree†

Nejat Düzgüneş,*,‡,§ Theresa M. Allen,*,‡,∥ Jane Fedor,‡ and Demetrios Papahadjopoulos‡,⊥

Cancer Research Institute and Departments of Pharmaceutical Chemistry and Pharmacology, University of California, San Francisco, California 94143-0128

Received May 11, 1987; Revised Manuscript Received August 21, 1987

ABSTRACT: The kinetics of lipid mixing during membrane aggregation and fusion was monitored by two assays employing resonance energy transfer between *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) and *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine (Rh-PE). For the "probe mixing" assay, NBD-PE and Rh-PE were incorporated into separate populations of phospholipid vesicles. For the "probe dilution" assay, both probes were incorporated into one population of vesicles, and the assay monitored the dilution of the molecules into the membrane of unlabeled vesicles. The former assay was found to be very sensitive to aggregation, even when the internal aqueous contents of the vesicles did not intermix. Examples of this case were large unilamellar vesicles (LUV) composed of phosphatidylserine (PS) in the presence of Mg^{2+} and small unilamellar vesicles (SUV) composed of phosphatidylserine in the presence of high concentrations of Na^+. No lipid mixing was detected in these cases by the probe dilution assay. Under conditions where membrane fusion (defined as the intermixing of aqueous contents with concomitant membrane mixing) was observed, such as LUV (PS) in the presence of Ca^{2+}, the rate of probe mixing was faster than that of probe dilution, which in turn was faster than the rate of contents mixing. Two assays monitoring the intermixing of aqueous contents were also compared. The Tb/dipicolinic acid assay reported slower fusion rates than the 1-aminonaphthalene-3,6,8-trisulfonic acid/*N*,*N*′-*p*-xylylenebis(pyridinium bromide) assay for PS LUV undergoing fusion in the presence of Ca^{2+}. These observations point to the importance of utilizing contents mixing assays in conjunction with lipid mixing assays to obtain the rates of membrane destabilization and fusion. They also indicate that the results of probe mixing assays have to be interpreted cautiously and that such assays can be sensitive to simple aggregation.

Studies on liposome fusion have delineated the role of specific molecules such as phospholipids, glycolipids, and cytoplasmic Ca^{2+}-binding proteins and biophysical processes such as ion binding, osmotic pressure, and phase transitions in membrane fusion [reviewed by Nir et al. (1983), Düzgüneş (1985), Sundler (1985), and Hong et al. (1987)]. The development of fluorescence assays for membrane fusion has facilitated these studies and provided the means to monitor the kinetics of membrane fusion. These assays report either the coalescence of aqueous contents of membrane vesicles or the intermixing of membrane components [reviewed in Düzgüneş and Bentz (1988)].

Membrane fusion results in the communication between two aqueous compartments initially separated by the two fusing membranes and involves two distinct steps: (i) the close approach and adhesion of the membranes (aggregation) and (ii) the destabilization and merging of the lipid bilayers in the region of adhesion, with concomitant mixing of contents within the two aqueous compartments (Nir et al., 1983). In certain instances, destabilization may result in the formation of a trilaminar (i.e., a single bilayer) diaphragm between the two compartments, causing intermixing of membrane components without the intermixing of contents (Palade, 1975; Neher, 1974; Pinto da Silva & Nogueira, 1977; Melikyan et al., 1983), in a process that has been called fusion (Palade, 1975) but can also be considered as semifusion (Düzgüneş, 1985). In other cases destabilization may lead to the formation of the hexagonal H_{II} phase, with extensive leakage of contents and lipid mixing but without the coalescence of internal contents (Ellens et al., 1986).

Fluorescence assays monitoring the intermixing of aqueous contents include the Tb/DPA[1] (terbium/dipicolinic acid; Wilschut et al., 1980) and ANTS/DPX [1-aminonaphthalene-3,6,8-trisulfonate/*N*,*N*′-*p*-xylenebis(pyridinium bromide); Ellens et al., 1985] assays. The most widely used assay for the intermixing of membrane components is based on resonance energy transfer (RET) between 7-nitro-2,1,3-benzoxadiazol-4-yl (NBD) and Rhodamine (Rh) (Struck et al., 1981). The probes are covalently attached to the head group of phosphatidylethanolamine (PE). Since RET depends on the proximity of the energy donor (NBD) and energy receptor (Rh), the changes in surface densities of the probes during membrane fusion can be monitored as changes in fluorescence intensity.

There are two versions of the lipid mixing assay:

† This work was supported by Grant GM28117 from the National Institutes of Health (D.P. and N.D.), by a Grant-in-Aid from the American Heart Association with funds contributed by the California Affiliate (N.D.), by a grant from the UCSF Academic Senate (N.D.), and by grants from the Alberta Heritage Foundation for Medical Research and the Medical Research Council of Canada, MA-6487 (T.M.A.).

* Authors to whom correspondence should be addressed.

‡ Cancer Research Institute.

§ Department of Pharmaceutical Chemistry.

∥ Permanent address: Department of Pharmacology, University of Alberta, Edmonton, Alberta, Canada T6G 2H7.

⊥ Department of Pharmacology.

[1] Abbreviations: ANTS, 1-aminonaphthalene-3,6,8-trisulfonic acid; DPA, dipicolinic acid; DPX, *N*,*N*′-*p*-xylylenebis(pyridinium bromide); LUV, large unilamellar vesicles; NBD, 7-nitro-2,1,3-benzoxadiazol-4-yl; NBD-PE, phosphatidylethanolamine labeled at the amino group with NBD; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; RET, resonance energy transfer; Rh, Rhodamine; Rh-PE, phosphatidylethanolamine labeled at the amino group with lissamine Rhodamine B sulfonyl; SUV, small unilamellar vesicles.

0006-2960/87/0426-8435$01.50/0

(i) Probe dilution. Here both probes are incorporated in one population of vesicles ("labeled" vesicles), and their dilution into "unlabeled" vesicles is monitored as the increase in NBD fluorescence, resulting from decreased RET from the donor to the acceptor (Struck et al., 1981; Rosenberg et al., 1983; Eidelman et al., 1984; Ababei & Hildenbrand, 1984; Düzgüneş et al., 1985; Ellens et al., 1985; Wilschut et al., 1985a,b).

(ii) Probe mixing. In this version of the assay each probe is placed in a separate population of vesicles, and the quenching of donor fluorescence (or increase of acceptor fluorescence) is monitored (Hoekstra, 1982a,b; Wilschut et al., 1983; Silvius & Gagné, 1984a,b; Morris et al., 1985). RET pairs other than NBD and Rh have also been used for probe mixing assays (Vanderwerf & Ullman, 1980; Uster & Deamer, 1981).

Here, we have examined the kinetics of lipid mixing obtained with the two assays and found drastic differences between them. We have addressed the possibility of artifacts arising from probe exchange in the absence of bulk-phase lipid mixing. We have compared the kinetics of lipid mixing with that of contents mixing to gain insight into the membrane events accompanying fusion. We have also examined the behavior of the probes independent of their role as reporters of membrane fusion. A preliminary report of our findings has been presented (Allen & Düzgüneş, 1985).

Materials and Methods

Materials. Phosphatidylethanolamine (PE), prepared by transphosphatidylation of egg phosphatidylcholine (PC), bovine brain phosphatidylserine (PS), egg PC, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-PE (NBD-PE), and *N*-(lissamine Rhodamine B sulfonyl)-PE (Rh-PE) were purchased from Avanti Polar Lipids (Birmingham, AL). Dipicolinic acid (DPA) and *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) were obtained from Sigma Chemical Co. (St. Louis, MO). NaCl and diethyl ether were from Mallinckrodt (Paris, KY); $CaCl_2$, $MgCl_2$, and ethylenediaminetetraacetic acid (EDTA) were from Fisher Scientific (Fairlawn, NJ). 1-Aminonaphthalene-3,6,8-trisulfonic acid (ANTS) and *N*,*N*′-*p*-xylylenebis(pyridinium bromide) (DPX) were purchased from Molecular Probes (Eugene, OR). Water was twice distilled, the second time in an all-glass apparatus, and further purified in a Barnstead Nanopure apparatus (Barnstead Co., Boston, MA).

Preparation of Liposomes. Large unilamellar vesicles (LUV) were prepared by the reverse-phase evaporation technique as described by Szoka et al. (1980) with some modification (Düzgüneş et al., 1981, 1983) and contained the following aqueous media: (i) 2.5 mM $TbCl_3$, 50 mM sodium citrate; (ii) 50 mM sodium DPA, 20 mM NaCl; (iii) 25 mM ANTS, with sufficient NaCl to adjust the osmolality to 190 mosM; (iv) 90 mM DPX. All solutions contained 5 mM TES and were adjusted to pH 7.4. Liposomes were sized by extrusion (2×) under argon through polycarbonate membranes of 0.1-μm pore diameter (Nucleopore, Pleasanton, CA) in a Millipore filtration apparatus. Liposomes were freed of unencapsulated material by gel filtration over Sephadex G-75 (Pharmacia, Piscataway, NJ) with 100 mM NaCl/5 mM TES/1 mM EDTA as the elution buffer.

Large unilamellar vesicles to be used in the fluorescent phospholipid assays were also prepared by the reverse-phase evaporation technique using a solution comprised of 100 mM NaCl/5 mM TES/0.1 mM EDTA (pH 7.4). For the probe dilution assay, fluorescence-labeled vesicles were prepared with 0.6 mol % each NBD-PE and Rh-PE. For the probe mixing assay, one population of vesicles contained 2 mol % NBD-PE and the other, 2 mol % Rh-PE. In some experiments, the probe concentrations were varied to ascertain the effect of the probes on the apparent rate of fusion.

Small unilamellar vesicles (SUV) were prepared by sonication as described by Papahadjopoulos et al. (1972). The lipids were mixed in chloroform, dried in a rotary evaporator, suspended in 100 mM NaCl/5 mM TES/0.1 mM EDTA (pH 7.4) at a concentration of 10 μmol/mL, and sonicated for 1 h in a bath-type sonicator (Laboratory Supplies Co., Hicksville, NY) under argon. The temperature was maintained at approximately 20 °C by circulating the water in the bath. The resulting clear suspension was then centrifuged at 25 °C for 1 h at 100000*g* to eliminate any remaining large or multilamellar vesicles; the upper four-fifths of the supernatant was collected for use in the experiments.

Fluorescence Measurements. Fluorescence emission was monitored with an SLM-4000 fluorometer, and readings were recorded on a Houston Instruments chart recorder. A stirring apparatus located beneath the cuvette holder enabled constant mixing of the medium. All divalent cations were added by a Hamilton syringe while fluorescence emission was constantly monitored. Some experiments were performed on a Spex Fluorolog 2 fluorometer with a Datamate data acquisition system.

In the probe dilution method, the residual fluorescence of the labeled vesicles containing 0.6 mol % each of NBD-PE and Rh-PE was taken as 0% of maximum fluorescence. Vesicles to be used for the calibration of the fluorescence to 100% maximum fluorescence (F_{max}) contained 0.3 mol % of each of these lipids. The labeled vesicles were mixed with vesicles containing no fluorescent phospholipids (unlabeled vesicles) at a ratio of 1:1 and a total lipid concentration of 0.05 μmol/mL. Complete intermixing of all the bilayers upon fusion would be expected to result in a membrane containing 0.3 mol % each of the two fluorescent phospholipids, which was taken as the theoretical maximum fluorescence. In a variation of this method, vesicles containing 1.0, 0.5, or 0.2 mol % each of NBD-PE and Rh-PE were mixed with unlabeled vesicles at a 1:1 ratio, and the maximum fluorescence was set by using vesicles containing 0.5, 0.25, or 0.1 mol % probe molecules, respectively. The vesicles were suspended in 1 mL of 100 mM NaCl, 5 mM TES, pH 7.4, and 0.1 mM EDTA at 25 °C. NBD fluorescence measurements were made continuously, using an excitation wavelength of 450 nm and an emission wavelength of 520 nm. Aggregation of the vesicles was monitored simultaneously in the second emission channel of the fluorometer by using a Melles-Griot 450-nm band-pass filter.

For the probe mixing method, NBD-PE was incorporated in one population of vesicles and Rh-PE in a second vesicle population, at a concentration of 2 mol %. Intermixing of the lipids of the two vesicle populations leads to RET between NBD and Rh and a decrease in the NBD fluorescence signal. The two populations of vesicles were mixed in a 1:1 ratio, and the fluorescence of NBD was followed as described above.

For the ANTS/DPX assay, an ANTS solution is entrapped in one vesicle population and a DPX solution in the other (Ellens et al., 1985; Düzgüneş et al., 1985). The quenching of ANTS fluorescence by DPX (Smolarsky et al., 1977), initially encapsulated in separate populations of vesicles, is a measure of the intermixing of aqueous contents. Fusion events between the two vesicle populations lead to a decrease in fluorescence. Release of contents from the liposomes and their dilution into the medium do not result in the decrease of fluorescence, since quenching by DPX is highly concentration dependent. Leakage of quenched ANTS/DPX from liposomes

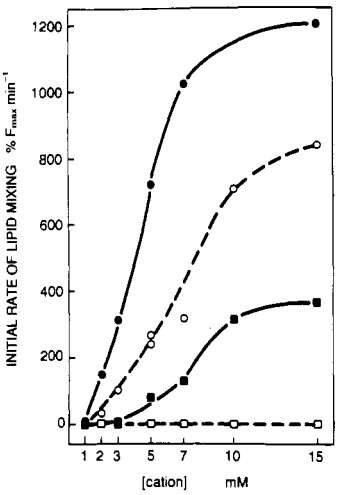

FIGURE 1: Initial rate of lipid mixing in LUV (PS) as a function of divalent cation concentration. Circles, Ca^{2+}; squares, Mg^{2+}; filled symbols, probe mixing assay; open symbols, probe dilution assay. Temperature, 25 °C; aqueous medium, 100 mM NaCl, 5 mM TES, pH 7.4, and 0.1 mM EDTA; lipid concentration, 0.05 μmol/mL.

leads to a fluorescence increase (Ellens et al., 1984). ANTS fluorescence (>530 nm) was followed by means of a Corning 3-68 cutoff filter, with the excitation wavelength at 360 nm. Vesicles containing either ANTS or DPX were mixed at a 1:1 ratio, at a final lipid concentration of 0.05 μmol/mL in the same aqueous medium used for the other assays. The initial fluorescence of the suspension was set at 100% maximum fluorescence. Other details of the assay are given in Ellens et al. (1985) and Düzgüneş and Bentz (1988).

In the Tb/DPA method, a solution of terbium citrate is entrapped in one population of vesicles and the Na salt of DPA is entrapped in a second vesicle population (Wilschut et al., 1980). Fusion events leading to intermixing of aqueous contents entrapped within the two vesicle populations result in an increase in fluorescence signal due to formation of a fluorescent Tb/DPA complex. Leakage of the Tb/DPA complex from liposomes (or entry of the medium into the internal aqueous space) leads to a decrease in fluorescence signal as a result of its interaction with Ca^{2+} and EDTA. For the Tb/DPA assay, Tb-containing and DPA-containing vesicles were mixed at a 1:1 ratio; the excitation wavelength was set at 276 nm and the emission at 545 nm was followed by using the Corning cutoff filter transmitting wavelengths above 530 nm. One hundred percent Tb fluorescence was determined in the absence of EDTA after 25 μM Tb vesicles were lysed with 0.5% (w/v) sodium cholate or 0.8 mM $C_{12}E_8$ (octaethylene glycol dodecyl ether; Calbiochem) in the presence of excess free DPA (20 μM) and sonicated for 5 min. Details of the assay are presented in Wilschut et al. (1980) and Düzgüneş and Bentz (1988).

RESULTS

Probe Mixing vs Probe Dilution during Membrane Fusion. The aggregation and fusion of LUV (PS) in the presence of divalent cations have been studied thoroughly with the use of the Tb/DPA assay and freeze–fracture electron microscopy (Wilschut et al., 1980, 1981, 1985b; Düzgüneş et al., 1984; Bentz & Düzgüneş, 1985). The probe mixing and probe dilution assays were therefore compared initially with this system. The initial rates of lipid mixing are plotted as a function of Ca^{2+} or Mg^{2+} concentrations in Figure 1. The rates obtained with the probe mixing assay in the presence of Ca^{2+} (filled circles) were about 3-fold higher than that obtained with the probe dilution assay (open circles). The difference was less pronounced at high Ca^{2+} concentrations.

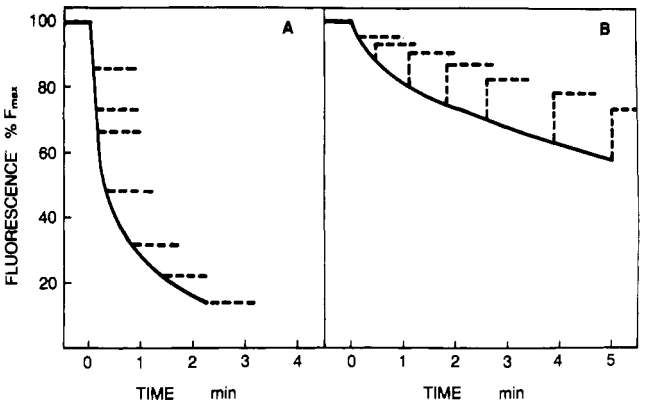

FIGURE 2: Time course of lipid mixing in LUV (PS) detected by the probe mixing assay. Either 3 mM Ca^{2+} (A) or 5 mM Mg^{2+} (B) was added at $t = 0$, and the process was stopped by adding a 2-fold molar excess of EDTA (dashed lines). Lipid concentration, 0.05 μmol/mL.

A crucial test for the reliability of the lipid mixing assays was to examine probe mixing and probe dilution in the presence of Mg^{2+}, which induces aggregation, but not fusion, of LUV (PS), as revealed by the Tb/DPA assay and electron microscopy (Wilschut et al., 1981). Although the probe dilution assay did not report any lipid mixing (open squares), the probe mixing assay indicated rapid and extensive lipid mixing (filled squares, Figure 1). It appears that the latter assay was not only reporting fusion, as in the case of Ca^{2+}, but also aggregation. This may be expected, since the approach of the membrane surfaces closer than about 100 Å could result in RET between the donor and acceptor fluorophores, as pointed out by Gibson and Loew (1979). On the other hand, the probe dilution assay was insensitive to the aggregation of the vesicles, since both probes are in the same membrane. This observation also indicated that no significant probe transfer occurred from the labeled vesicles to the unlabeled ones during aggregation.

In the probe mixing assay, NBD fluorescence decreases as a result of RET to Rh-PE if the fluorophores are in close proximity. If the proximity arises from interbilayer apposition, the fluorescence should return to the original (100%) level when the vesicles are deaggregated. If RET occurs because of membrane mixing or probe transfer between the membranes, deaggregation should not alter the level of fluorescence. Figure 2 shows the time course of fluorescence decrease for LUV (PS) in the presence of 3 mM Ca^{2+} (A) or 5 mM Mg^{2+} (B). EDTA was added to the vesicles at various times after the addition of the divalent cations. In the case of Ca^{2+}, EDTA addition arrested the fluorescence at the level obtained during fusion. Thus, the fluorescence time course (solid line) reflects the extent of probe intermixing during Ca^{2+}-induced fusion of LUV (PS). In the case of Mg^{2+}, however, the fluorescence level is partially reversible upon EDTA addition (Figure 2B). Therefore, the decrease in fluorescence signal in this case was arising partially from the aggregation of the vesicles. Since Mg^{2+}-induced aggregation of LUV (PS) is reversible and does not lead to fusion (Nir et al., 1981; Wilschut et al., 1981), the observation that EDTA addition did not completely restore the original fluorescence suggests that some lipid mixing or probe exchange between vesicles occurred in the aggregated state.

Similar results were obtained upon EDTA addition to SUV (PS) fusing in the presence of 2 mM Ca^{2+} or 7 mM Mg^{2+} (Figure 3). The observation that the fluorescence signal is arrested immediately by EDTA in the case of Ca^{2+} is in agreement with earlier interpretations that the vesicles fuse

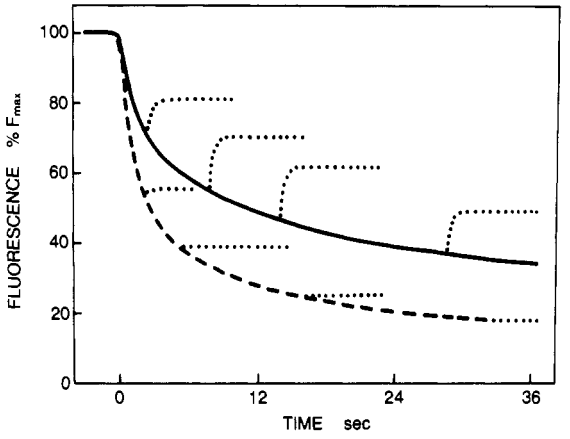

FIGURE 3: Lipid mixing during fusion of SUV (PS) induced by 2 mM Ca^{2+} (dashed line) or 7 mM Mg^{2+} (solid line) and monitored by the probe mixing assay. A 2-fold molar excess of EDTA was added at various times as indicated by the dotted lines. Lipid concentration, 0.05 μmol/mL.

as soon as they aggregate at the vesicle concentration employed in these experiments (Wilschut et al., 1980; Nir et al., 1980). SUV (PS) do undergo fusion in the presence of Mg^{2+}, unlike LUV (Wilschut et al., 1981). However, the fact that EDTA partially reverses the probe mixing signal indicates that some of the vesicles had aggregated but not fused. This observation corroborates our earlier conclusion, based on light scattering and the Tb/DPA assay, that fusion is delayed with respect to aggregation in this system (Wilschut et al., 1981).

The difference between the probe mixing and probe dilution methods was especially pronounced in the case of LUV (PS/PE), which fuse at a considerably slower rate than LUV (PS), and in the case of LUV (PS/PC), which aggregate but do not fuse in the presence of Ca^{2+} or Mg^{2+} (Düzgüneş et al., 1981; Uster & Deamer, 1981). The initial rates of lipid mixing for LUV (PS/PE) induced by various concentrations of divalent cation are shown in Figure 4. At lower Ca^{2+} concentrations (below 10 mM), the probe mixing rates (solid circles) were about 15 times higher than the probe dilution rates (empty circles) and at 15 mM Ca^{2+} about 10 times higher. For Mg^{2+}, the probe mixing rate was about 90-fold higher at 5 mM (solid squares) and about 80-fold higher at 15 mM than the probe dilution rate (open squares). The differential rates being higher at the lower divalent cation concentrations may be attributed to fusion being slow relative to aggregation under these ionic conditions, since the probe mixing method appears to be highly sensitive to aggregation. The latter conclusion was strengthened by observations on Ca^{2+}- or Mg^{2+}-induced aggregation of LUV (PS/PC) discussed below.

Our earlier studies utilizing the Tb/DPA assay had shown that LUV composed of PS and PC (1:1) aggregated but did not fuse in the presence of Ca^{2+} or Mg^{2+} (Düzgüneş et al., 1981). Figure 5 shows that the probe mixing method was very sensitive to the aggregation of these vesicles, whereas the probe dilution method indicated a negligible rate of lipid mixing at high Ca^{2+} concentrations. Another interesting feature of the aggregation of LUV (PS/PC) was that the initial rate of probe mixing did not exhibit a saturation behavior, as was the case with LUV (PS) and LUV (PS/PE). It should be noted, however, that the rates obtained with PS/PC vesicles were more than an order of magnitude slower than with PS/PE vesicles at the same divalent cation concentration. This difference in the initial rate of probe mixing could be due to a faster rate of aggregation for LUV (PS/PE), a faster redistribution of probes as a result of fusion, or a closer approach

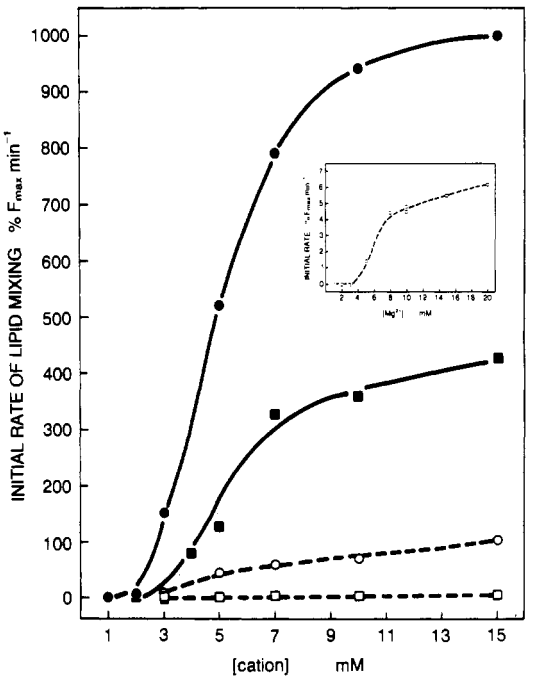

FIGURE 4: Initial rate of lipid mixing during the fusion of LUV (PS/PE) induced by various concentrations of Ca^{2+} (circles) or Mg^{2+} (squares) and monitored by the probe mixing assay (solid symbols) or the probe dilution assay (open symbols). Inset: The initial rate of fusion as a function of Mg^{2+} concentration, at an expanded scale.

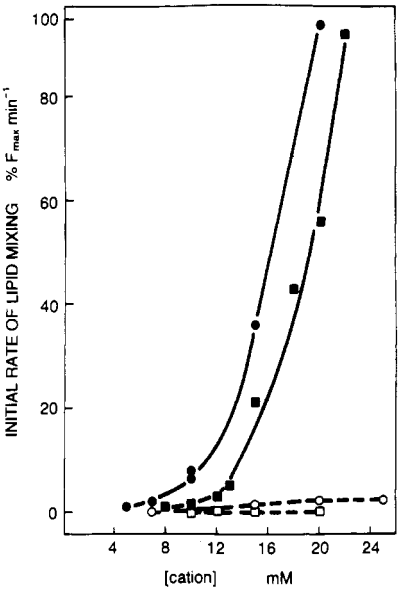

FIGURE 5: Initial rate of lipid mixing during aggregation of LUV (PS/PC) induced by Ca^{2+} or Mg^{2+}. The description of the symbols is the same as in Figure 4.

of the vesicle surfaces in the aggregated state.

Calculations of the interaction energy vs intermembrane distance indicate that SUV probably aggregate in the primary minimum, since their secondary minimum is not deep enough to sustain stable aggregation, while LUV may aggregate in the secondary minimum (Nir et al., 1981, 1983). It was of interest, therefore, to investigate the behavior of the fluorescence probes under conditions where SUV aggregate but do not fuse. Such conditions are obtained with SUV (PS) in the presence of high concentrations of Na^+ (Day et al., 1980; Bentz et al., 1983). The initial rates of lipid mixing during aggregation of SUV (PS) is shown as a function of Na^+ concentration in Figure 6. The probe dilution assay indicated that probe molecules did not transfer from labeled to unlabeled

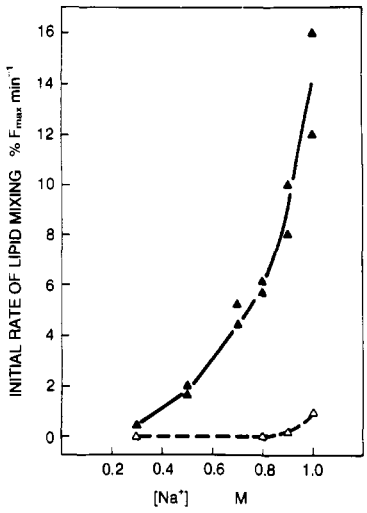

FIGURE 6: Initial rate of lipid mixing during aggregation of SUV (PS) in the presence of NaCl as monitored by the probe mixing assay (filled symbols) or the probe dilution assay (open symbols).

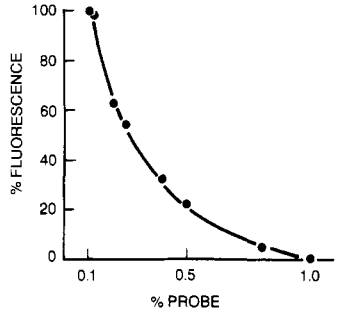

FIGURE 7: Relative fluorescence of LUV (PS) containing the indicated mole percentages of NBD-PE and Rh-PE. Fluorescence was excited at 450 nm and detected at 530 nm. The total amount of NBD in the fluorometer cell was kept constant while the mole percentage was varied. For example, twice as many vesicles containing 0.5% probe were added as those containing 1% probe, while the total vesicle concentration was maintained at 0.05 μmol/mL by adding unlabeled vesicles.

vesicles even when the vesicles aggregated in a primary minimum (open triangles). However, the probe mixing assay indicated that the probes could sense aggregation when they were placed in separate populations of vesicles (filled triangles).

Under conditions where SUV do fuse, such as in the presence of Ca^{2+} or Mg^{2+}, the probe mixing assay also reported faster initial rates of fusion than the probe dilution assay (data not shown).

Effect of Probe Concentration. Figure 7 shows the change in NBD fluorescence as the percentage of both probes in the membrane is varied. For these measurements, vesicles containing different amounts of probe were prepared. The total amount of NBD-PE plus Rh-PE in the fluorometer cell was kept constant (0.05 μM) between vesicles of different probe concentration by varying the ratio of labeled/unlabeled vesicles and keeping the overall vesicle concentration at 50 μM lipid. The fluorescence of vesicles containing 0.1 mol % of each probe was set to 100%, and that of vesicles containing 1 mol % of each probe was taken as 0%. It is clear that the plot is not linear, in contrast to earlier studies where the NBD-PE concentration in the membrane was kept constant and the Rh-PE concentration was varied (Struck et al., 1981). The present method of determining the change in relative fluorescence is a closer approximation to the changes in fluorescence obtained during membrane fusion. Although the overall plot is not linear, segments of the curve between, for example, 0.4% and 0.2% probe may be considered close to linear for the purposes of the probe dilution assay. The data in Figure 7 yield an R_0 value (distance of 50% energy transfer between donor and acceptor probes) of 65 Å (Düzgüneş & Bentz, 1988).

The probe dilution assay was performed with PS vesicles (LUV) containing different probe concentrations. In one set of experiments, the fluorescence of vesicles containing 0.5 mol % of each probe was set to 0% maximum fluorescence, and that of vesicles with 0.25 mol % probe was set to 100%. The nonlinearity of the fluorescence vs percent probe curve is not significant when these limited probe ranges are employed to calibrate the assay. A similar series of experiments were performed with vesicles containing 1 mol % probe (set to 0% maximum fluorescence) and 0.5 mol % probe (set at 100% maximum fluorescence). The latter vesicles produced slightly faster fusion curves than the former, and the difference increased at higher Ca^{2+} concentrations (data not shown). However, these differences were too small to explain the differences between the probe mixing and probe dilution assays, which employ different probe concentrations.

The effect of probe concentration on the apparent rate of lipid mixing was also investigated for the probe mixing assay. The initial rates and time courses of fusion of PS vesicles containing either 1 or 2 mol % of each probe were very similar (data not shown). This observation indicates that the higher rates of lipid mixing obtained with the probe mixing assay are not the result of higher probe concentrations (2 mol % vs 0.6 mol % for probe dilution) employed for these experiments. Furthermore, the increase of the concentration of probe molecules from 1 to 2 mol % does not appear to change the properties of the vesicles with respect to aggregation and lipid mixing.

Effects of Ion Binding and Aggregation on NBD Fluorescence. It is important to ascertain the degree to which divalent cations affect the fluorescence of the probes used for the fusion assays for an unambiguous interpretation of changes in fluorescence as lipid mixing. When NBD-PE and Rh-PE were incorporated in separate populations of PC vesicles at 1 mol %, the addition of Ca^{2+} did not have any effect on the fluorescence (data not shown). A similar result was obtained by Hoekstra (1982a). The decrease in fluorescence reported by Parente and Lentz (1986) may be ascribed to the use of 5 mol % NBD-PE in dipentadecanoyl-PC LUV. When the probes were in PS vesicles, Ca^{2+} addition affected the fluorescence differently depending on the concentration of probes. Figure 8 shows the time course of fluorescence of the vesicles used for calibrating F_{max} in the presence of Ca^{2+} and in the absence of any unlabeled vesicles. The dotted line represents the fluorescence of vesicles containing 0.1 mol % of each probe, the dashed line vesicles with 0.25 mol % probe, and the solid line vesicles with 0.5 mol % probe. This dependence of Ca^{2+}-induced fluorescence change on the probe concentration may be a reflection of the local concentration of NBD-PE and Rh-PE. It appears that Ca^{2+} binding to PS increases NBD-PE fluorescence, but the presence of sufficiently high concentrations of Rh-PE reduces the fluorescence due to RET. The increase in NBD fluorescence is probably the result of the change in dielectric constant near the membrane/water interface, arising from Ca^{2+} binding to PS and the partial dehydration of the head group. The above results indicate that changes in NBD fluorescence in the presence of Ca^{2+} are not due to the interaction of Ca^{2+} directly with the probe [cf. Morris et al. (1985) and Parente and Lentz (1986)].

Aggregation of vesicles also affected the fluorescence signal observed during the probe dilution assay. When LUV (PS) were incubated in the presence of Ca^{2+}, the fluorescence first

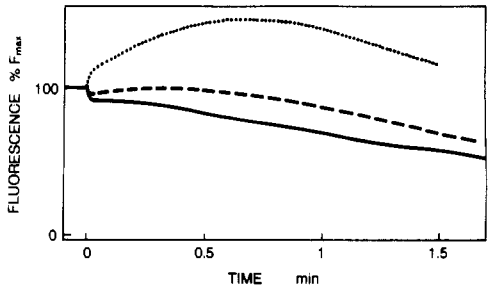

FIGURE 8: Time course of fluorescence of LUV (PS) containing 0.1 (dotted line), 0.25 (dashed line), or 0.5 (solid line) mol % NBD-PE and Rh-PE in the presence of 5 mM Ca^{2+} but without any unlabeled vesicles.

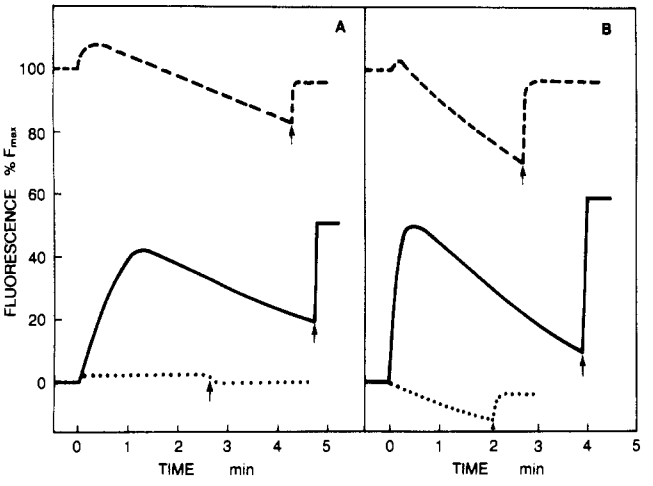

FIGURE 9: Time course of lipid mixing during Ca^{2+}-induced fusion of LUV (PS), followed by the probe dilution assay (solid lines): (A) 3 mM and (B) 5 mM Ca^{2+}. The dashed lines show the change in fluorescence of the vesicles containing 0.3 mol % of each probe, used for calibrating F_{max}, in the absence of unlabeled vesicles. The dotted lines show the fluorescence of vesicles containing 0.6 mol % of each probe, in the absence of unlabeled vesicles. A 2-fold excess of EDTA was added at the times indicated by the arrows.

increased and then declined (Figure 9, solid lines). The onset of the decline was more rapid for higher Ca^{2+} concentrations. The subsequent addition of EDTA to stop the aggregation and fusion reactions (arrows) caused an increase in fluorescence. A corresponding decrease in fluorescence was observed with vesicles containing 0.3 mol % probe, used for calibrating the 100% fluorescence, in the absence of any unlabeled vesicles (Figure 9, dashed lines). Again, EDTA addition caused an increase in fluorescence due to the dispersion of vesicles. Thus, the actual extent of fusion in extensively aggregating systems is given by the level of fluorescence attained after the removal of Ca^{2+} and the dispersal of the vesicles. The decline in fluorescence could be the result of several factors: (i) extensive aggregation of the vesicles and the reduction in the number of particles passing through the light path in the fluorometer cell; (ii) lateral phase separation of the probes from the Ca^{2+}/PS complexes, causing an increase in RET; and (iii) inner filter effects due to increases in sample turbidity.

Coalescence of Aqueous Contents vs Lipid Mixing. Figure 10 shows the time course of lipid mixing and of aqueous contents mixing during fusion of LUV (PS) induced by 2 mM Ca^{2+}. Both probe dilution and probe mixing assays revealed faster kinetics compared to the contents mixing assays. The ANTS/DPX assay detected consistently faster fusion rates than the Tb/DPA assay (Figure 11). In contrast, fusion of cardiolipin/dioleoyl-PC LUV by divalent cations monitored by the Tb/DPA assay appeared faster than when monitored by the ANTS/DPX assay (Düzgüneş et al., 1988). The rate

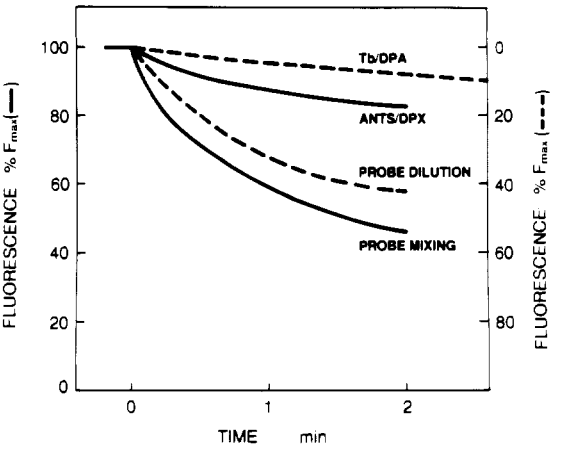

FIGURE 10: Time course of intermixing of contents and lipid mixing during fusion of LUV (PS) induced by 2 mM Ca^{2+}. Contents mixing was assayed by either the Tb/DPA or the ANTS/DPX assay. Lipid mixing was monitored by either the probe dilution or the probe mixing assay. The fluorescence scale for the solid lines is on the left and that for the dashed lines is on the right. Fusion results in an increase of the fluorescence in the Tb/DPA and probe dilution assays and a decrease in the ANTS/DPX and probe mixing assays.

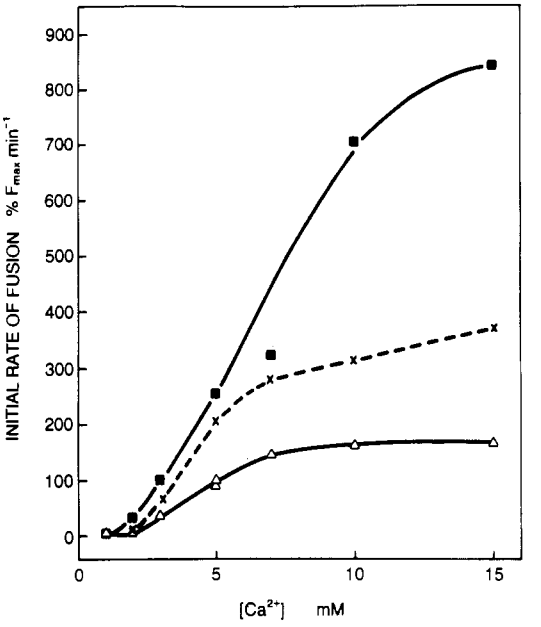

FIGURE 11: Initial rate of fusion of LUV (PS) in the presence of varying concentrations of Ca^{2+} as detected by the probe mixing assay (squares), the ANTS/DPX assay (×), or the Tb/DPA assay (triangles).

and time course of leakage (or dissociation) of Tb/DPA or of ANTS/DPX were similar, indicating that the difference between the fusion assays could not be attributed to differences in leakage rates.

The difference in fusion rates obtained by the Tb/DPA and the ANTS/DPX assays could theoretically be due to several factors including the following: (i) different size distributions of the vesicle populations; (ii) different rates of reaction of the two components of the assay; and (iii) the different interaction of the encapsulated compounds with the vesicle membrane. These three factors are unlikely to have affected the results for the following reasons: The size distributions of the vesicles were ascertained to be very similar, by means of dynamic light scattering measurements. Although the reaction mechanisms are different (chelation for the Tb/DPA reaction and collisional quenching for the ANTS/DPX reaction), the rates of these reactions are very rapid, especially at the concentrations found in the vesicle interior, and thus would not be expected

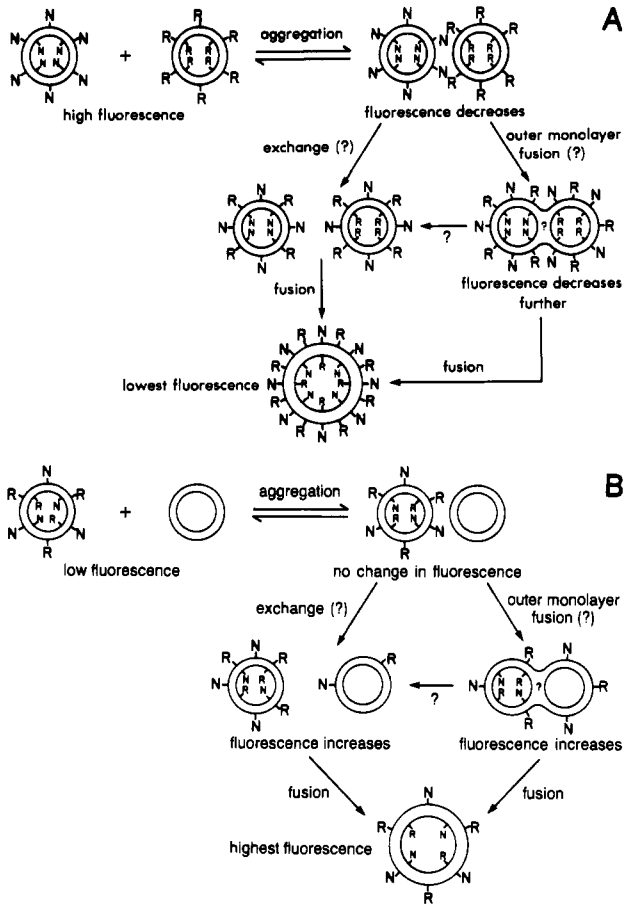

FIGURE 12: Summary of the behavior of lipid probes and of the corresponding fluorescence in the probe mixing assay (A) and the probe dilution assay (B).

to contribute to the different rates of contents mixing. Although more ANTS/DPX binds to preformed SUV (PS) than Tb/DPA, binding of either complex to LUV (PS) is negligible (J. Bentz, D. Alford, and N. Düzgüneş, manuscript in preparation). To test the possibility that the encapsulated compounds may affect the rate of fusion, the probe dilution assay was performed with vesicles encapsulating either Tb or ANTS. These compounds were shown not to affect NBD fluorescence. Tb-containing vesicles exhibited slightly faster lipid mixing rates than the ANTS-containing vesicles at 2 mM Ca^{2+}, but at higher concentrations the time courses were indistinguishable (data not shown). Thus, it appears that Tb and ANTS do not affect the lipid mixing rate to an appreciable extent. It is possible that terbium citrate or DPX interacts to a limited extent, but differentially, with negatively charged lipids, affecting their mobility or response to divalent cations, and thus their participation in the fusion reaction. These possibilities are being investigated.

Discussion

Our results and interpretations are summarized in Figure 12. We have found that the probe mixing assay (Figure 12A) is extremely sensitive to vesicle aggregation and intermembrane contact in the absence of intermixing of aqueous contents. Aggregation may be accompanied by probe exchange, as indicated by our observation that EDTA can only partially reverse Mg^{2+}-induced aggregation of LUV (PS) or Ca^{2+}-induced aggregation of LUV (PS/PC). Lipid exchange during a reversibly aggregated state has also been observed in the case of acid-induced aggregation of SUV (dimyristoyl-PE) labeled with bimane-PC and NBD-PE (Pryor et al., 1985). Our observations may also be attributed to the mixing of lipids located only in the outer monolayers of the aggregated vesicles (semifusion). Under conditions where the aqueous contents intermix, such as LUV (PS) or LUV (PS/PE) in the presence of Ca^{2+}, the probe mixing assay yieds a higher rate of lipid mixing than the probe dilution assay. This finding may be attributed to the fact that some of the fluorescence quenching in the former arises from aggregation, exchange, and possibly fusion of outer monolayers. The finding that the rate of probe mixing was the same for vesicles containing either 1 or 2 mol % probe molecules suggests that the probes do not affect the aggregation and fusion characteristics of the membranes. Nevertheless, it will be of interest to investigate whether the interaction of the probe molecules in apposed membranes induces or enhances fusion (detected by the contents mixing assays) following aggregation mediated by divalent cations. This work is in progress in our laboratory.

The probe dilution assay is generally insensitive to the mere aggregation of vesicles, even in cases where the membranes are thought to approach each other to a distance within the primary minimum of the intermembrane interaction energy, such as SUV (PS) in the presence of high Na^+ concentrations. Probe dilution does not appear to take place during aggregation, such as with LUV (PS) and Mg^{2+}. Similar observations were made by Rosenberg et al. (1983) and Wilschut et al. (1985b). However, massive aggregation of vesicles does result in a decrease of fluorescence (Figure 9), most likely because of the reduction in the number of particles (vesicles) crossing the light beam. The decrease is mostly reversible when EDTA is added, bringing the fluorescence signal back to a level that would have been obtained if massive aggregation, lateral phase separation, or inner filter effects had not occurred.

Under some conditions, the probe dilution assay reports lipid mixing in the absence of any intermixing of contents. Examples of this phenomenon are LUV (PG) in the presence of Mg^{2+} (Rosenberg et al., 1983) and LUV (PE/cholesteryl hemisuccinate) in the presence of low concentrations of Ca^{2+} or Mg^{2+} (Ellens et al., 1985). Since the assay is not sensitive to the mere aggregation of vesicles, lipid mixing observed in these cases most likely reflects semifusion. The higher rate of probe dilution compared to aqueous contents mixing observed with LUV (PS) in the presence of Ca^{2+} suggests that the outer monolayers of the vesicles begin to intermix before bilayer destabilization that leads to membrane fusion (in the sense of complete bilayer intermixing). Thus, the destabilization leading to lipid mixing between two membranes is not the same as the destabilization leading to communication between the two aqueous compartments. Wilschut et al. (1985b) have found that the rate constants for aggregation obtained with the probe dilution assay are higher than those obtained with the Tb/DPA assay and have concluded that reversible aggregation events occur which result in lipid mixing but not in contents mixing, a process depicted in the middle row of Figure 12 with a left arrow. They also suggest that the formation of a fusion intermediate does not have to result in membrane fusion. Lipid mixing does not have to impair bilayer integrity as depicted in Figure 12. It could be a 1-for-1 lipid exchange at points of close contact; the vesicles could then dissociate. The intermediate shown in Figure 12 is most likely irreversible. Since the rate constants of fusion and aggregation increase in parallel as the Ca^{2+} concentration increases, Wilschut et al. (1985b) have proposed that it is the interaction of Ca^{2+} with the vesicles during aggregation that confers fusion susceptibility onto the vesicles. However, it is also possible to interpret the results in terms of structural changes in the bilayer affecting both the aggregation and fusion steps

(Wilschut et al., 1985a). Not in all liposome systems is lipid mixing faster than contents mixing. For example, SUV (PS) or LUV (cardiolipin/dioleoyl-PC) fusing in the presence of Ca^{2+} at 25 °C exhibit very similar rates of lipid mixing and aqueous contents mixing (Hoekstra, 1982b; Wilschut et al., 1983, 1985a). In these cases, the destabilization processes leading to lipid mixing and contents mixing apparently proceed at the same rate.

Our results with the probe mixing assay in comparison with the probe dilution assay indicate that experiments utilizing the former assay have to be interpreted cautiously, since aggregated vesicles not intermixing their outer monolayers would still result in the decrease in NBD fluorescence due to resonance energy transfer across the interbilayer space [cf. Silvius and Gagné (1984a,b) and Bondeson and Sundler (1985)]. Furthermore, even in systems that do undergo fusion, rate constants of fusion could be overestimated [cf. Morris et al. (1985)].

Added in Proof

Silvius et al. (1987) have described novel fluorescent phospholipids with certain advantages over NBD-PE and Rh-PE in monitoring lipid mixing.

Acknowledgments

We thank Drs. Joe Bentz, Paul Meers (UCSF), and Shlomo Nir (Hebrew University of Jerusalem) for helpful discussions and Rose Antonucci for help with the preparation of the manuscript.

References

Ababei, L., & Hildenbrand, K. (1984) *Chem. Phys. Lipids 35*, 39–48.

Allen, T. M., & Düzgüneş, N. (1985) *Biophys. J. 47*, 169a.

Bentz, J., & Düzgüneş, N. (1985) *Biochemistry 24*, 5436–5443.

Bentz, J., Düzgüneş, N., & Nir, S. (1983) *Biochemistry 22*, 3320–3330.

Bondeson, J., & Sundler, R. (1985) *FEBS Lett. 190*, 283–287.

Day, E. P., Kwok, A. Y. W., Hark, S. K., Ho, J. T., Vail, W. J., Bentz, J., & Nir, S. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4026–4029.

Düzgüneş, N. (1985) *Subcell. Biochem. 11*, 195–286.

Düzgüneş N., & Bentz, J. (1988) in *Spectroscopic Membrane Probes* (Loew, L. M., Ed.) CRC, Boca Raton, FL (in press).

Düzgüneş, N., Wilschut, J., Fraley, R., & Papahadjopoulos, D. (1981) *Biochim. Biophys. Acta 642*, 182–195.

Düzgüneş, N., Wilschut, J., Hong, K., Fraley, R., Perry, C., Friend, D. S., James, T. L., & Papahadjopoulos, D. (1983) *Biochim. Biophys. Acta 732*, 289–299.

Düzgüneş, N., Paiement, J., Freeman, K. B., Lopez, N. G., Wilschut, J., & Papahadjopoulos, D. (1984) *Biochemistry 23*, 3486–3494.

Düzgüneş, N., Straubinger, R. M., Baldwin, P. A., Friend, D. S., & Papahadjopoulos, D. (1985) *Biochemistry 24*, 3091–3098.

Düzgüneş, N., Allen, T. M., Fedor, J., & Papahadjopoulos, D. (1988) in *Molecular Mechanisms of Membrane Fusion* (Ohki, S., Doyle, D., Flanagan, T., Hui, S. W., & Mayhew, E., Eds.) Plenum, New York (in press).

Eidelman, O., Schlegel, R., Tralka, T. S., & Blumenthal, R. (1984) *J. Biol. Chem. 259*, 4622–4628.

Ellens, H., Bentz, J., & Szoka, F. C. (1984) *Biochemistry 23*, 1532–1538.

Ellens, H., Bentz, J., & Szoka, F. C. (1985) *Biochemistry 24*, 3099–3106.

Ellens, H., Bentz, J., & Szoka, F. C. (1986) *Biochemistry 25*, 285–294.

Gibson, G. A., & Loew, L. M. (1979) *Biochem. Biophys. Res. Commun. 88*, 141–147.

Hoekstra, D. (1982a) *Biochemistry 21*, 2833–2840.

Hoekstra, D. (1982b) *Biochim. Biophys. Acta 692*, 171–175.

Hong, K., Düzgüneş, N., Meers, P., & Papahadjopoulos, D. (1987) in *Cell Fusion* (Sowers, A. E., Ed.) pp 269–284, Plenum, New York.

Melikyan, G. B., Abidor, I. G., Chernomordik, L. V., & Chailakhyan, L. M. (1983) *Biochim. Biophys. Acta 730*, 394–398.

Morris, S. J., Gibson, C. C., Smith, P. D., Greif, P. C., Stirk, C. W., Bradley, D., Haynes, D. H., & Blumenthal, R. (1985) *J. Biol. Chem. 260*, 4122–4127.

Neher, E. (1974) *Biochim. Biophys. Acta 373*, 327–336.

Nir, S., Bentz, J., & Wilschut, J. (1980) *Biochemistry 19*, 6030–6036.

Nir, S., Bentz, J., & Düzgüneş, N. (1981) *J. Colloid Interface Sci. 84*, 266–269.

Nir, S., Bentz, J., Wilschut, J., & Düzgüneş, N. (1983) *Prog. Surface Sci. 13*, 1–124.

Ohki, S., Düzgüneş, N., & Leonards, K. (1982) *Biochemistry 21*, 2127–2133.

Palade, G. E. (1975) *Science (Washington, D.C.) 189*, 347–358.

Papahadjopoulos, D., Nir, S., & Ohki, S. (1972) *Biochim. Biophys. Acta 282*, 277–292.

Parente, R. A., & Lentz, B. R. (1985) *Biochemistry 25*, 1021–1026.

Pinto da Silva, P., & Nogueira, M. L. (1977) *J. Cell Biol. 73*, 161–181.

Pryor, C., Bridge, M., & Loew, L. M. (1985) *Biochemistry 24*, 2203–2209.

Rosenberg, J., Düzgüneş, N., & Kayalar, C. (1983) *Biochim. Biophys. Acta 735*, 173–180.

Silvius, J. R., & Gagné, J. (1984a) *Biochemistry 23*, 3232–3240.

Silvius, J. R., & Gagné, J. (1984b) *Biochemistry 23*, 3241–3247.

Silvius, J. R., Leventis, R., Brown, P. M., & Zuckerman, M. (1987) *Biochemistry 26*, 4279–4287.

Smolarsky, M., Teitelbaum, D., Sela, M., & Gitler, C. (1977) *J. Immunol. Methods 15*, 255–265.

Struck, D. K., Hoekstra, D., & Pagano, R. E. (1981) *Biochemistry 20*, 4093–4099.

Sundler, R. (1984) *Biomembranes 12*, 563–583.

Szoka, F., Olson, F., Heath, T., Vail, W., Mayhew, E., & Papahadjopoulos, D. (1980) *Biochim. Biophys. Acta 601*, 559–571.

Uster, P. S., & Deamer, D. W. (1981) *Arch. Biochem. Biophys. 209*, 385–395.

Vanderwerf, P., & Ullman, E. F. (1980) *Biochim. Biophys. Acta 596*, 302–314.

Wilschut, J., Düzgüneş, N., Fraley, R., & Papahadjopoulos, D. (1980) *Biochemistry 19*, 6011–6021.

Wilschut, J., Düzgüneş, N., & Papahadjopoulos, D. (1981) *Biochemistry 20*, 3126–3133.

Wilschut, J., Düzgüneş, N., Hong, K., Hoekstra, D., & Papahadjopoulos, D. (1983) *Biochim. Biophys. Acta 734*, 309–318.

Wilschut, J., Nir, S., Scholma, J., & Hoekstra, D. (1985a) *Biochemistry 24*, 4630–4636.

Wilschut, J., Scholma, J., Bental, M., Hoekstra, D., & Nir, S. (1985b) *Biochim. Biophys. Acta 821*, 45–55.