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Fluorescence Method for Measuring the Kinetics of Fusion between Biological Membranes†

Dick Hoekstra,* Tiny de Boer, Karin Klappe, and Jan Wilschut

ABSTRACT: An assay is presented that allows continuous and sensitive monitoring of membrane fusion in both artificial *and* biological membrane systems. The method relies upon the relief of fluorescence self-quenching of octadecyl Rhodamine B chloride. When the probe is incorporated into a lipid bilayer at concentrations up to 9 mol % with respect to total lipid, the efficiency of self-quenching is proportional to its surface density. Upon fusion between membranes labeled with the probe and nonlabeled membranes, the decrease in surface density of the fluorophore results in a concomitant, proportional increase in fluorescence intensity, allowing kinetic and quantitative measurements of the fusion process. The kinetics of fusion between phospholipid vesicles monitored with this assay were found to be the same as those determined with a fusion assay based on resonance energy transfer [Struck, D. K., Hoekstra, D., & Pagano, R. E. (1981) *Biochemistry 20*, 4093–4099]. Octadecyl Rhodamine B chloride can be readily inserted into native biological membranes by addition of an ethanolic solution of the probe. Evidence is presented showing that the dilution of the fluorophore, occurring when octadecyl Rhodamine containing influenza virus is mixed with phospholipid vesicles at pH 5.0, but *not* pH 7.4, resulted from virus–vesicle fusion and was not related to processes other than fusion. Furthermore, by use of this method, the kinetics of fusion between Sendai virus and erythrocyte ghosts and virus-induced fusion of ghosts were readily revealed. Dilution of the probe was not observed upon prior treatment of fluorescently labeled Sendai virus with trypsin. Virus-induced fusion between fluorescently tagged ghosts and ghosts devoid of the probe was only observed (at 37 °C) after a low-temperature preincubation; no fluorescence development was seen during virus-induced aggregation at low temperature nor when ghosts and the virus were directly mixed at 37 °C. These results indicated that spontaneous intermembrane transfer of the fluorophore did not occur. It is our contention that this technique may be of considerable value for investigating fusion between biological membranes and, hence, provides an important tool in elucidating the mechanism of fusion in such systems.

As a crucial, intermediate step, membrane fusion is involved in a variety of biological events. It occurs in such diverse processes as intracellular transport, endocytosis (Steinman et al., 1983), and exocytosis (Gratzl et al., 1980), while enveloped viruses exploit their membrane fusion capacity to deliver their genomes into host cells for replication (White et al., 1983). Although many of the physiological functions mediated by fusion events are largely understood, the molecular mechanism by which the fusion process itself occurs is not. To gain insight into this mechanism, various model systems are used, in particular those involving phospholipid vesicles. Studies employing these simplified systems have particularly benefitted from the availability of sensitive and quantitative assays to monitor continuously the fusion reaction (Vanderwerf & Ullman, 1980; Wilschut et al., 1980; Struck et al., 1981; Uster & Deamer, 1981; Hoekstra, 1982a; MacDonald & MacDonald, 1983). Thus, by detailed kinetic analysis of the fusion process it became possible to evaluate the significance of distinct structural and physical membrane changes in relation to the mechanism of fusion (Wilschut et al., 1980, 1981, 1983; Nir et al., 1983; Hoekstra, 1982a,b; Hoekstra & Martin, 1982; Düzgünes et al., 1984).

It would appear highly advantageous to adapt these fusion assays, which monitor either the mixing of contents or the merging of the lipid bilayers, to the more complex biological

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membranes. The common procedures used to examine fusion in these systems, such as microscopic or cytochemical techniques, suffer from both accurate quantitation of the fusion events and low sensitivity; i.e., extensive fusion activity may be required before it can be detected. Unfortunately, the experimental design of these assays precludes such an application as the probe molecules, reporting the fusion event, cannot become part of the fusing membrane system by exogenous addition; i.e., their presence is required prior to the formation of the (vesicle) membrane.

In this paper, a method is presented that not only allows continuous monitoring of fusion between artificial membranes but also provides an opportunity to reveal the kinetics of fusion between biological membranes. The principle of the assay relies upon the self-quenching properties of the fluorescent dye octadecyl Rhodamine B chloride (R_{18}).[1] The probe can be readily inserted into both artificial and biological membranes. Upon fusion of R_{18}-containing membranes with membranes devoid of the probe, the surface density of the fluorophore decreases, resulting in an increase in fluorescence that, as a measure of fusion, can be monitored continuously. The versatility of this assay to monitor fusion is demonstrated in a variety of artificial and biological systems.

Experimental Procedures

Lipids and Lipid Vesicles. Phosphatidylserine (PS, bovine brain), dioleoylphosphatidylcholine (DOPC), *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (*N*-NBD-PE), and *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine (*N*-Rh-PE) were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). Large unilamellar vesicles (LUV) were prepared by reverse-phase evaporation in 100 mM NaCl/5 mM HEPES, pH 7.4, and sized to an average diameter of 0.1 μm by extrusion through Unipore polycarbonate filters (Bio-Rad) as described (Düzgünes et al., 1983). Small unilamellar vesicles (SUV) were prepared by sonication (Hoekstra, 1982a).

Viruses. Sendai virus (Z strain) and influenza virus (X-47) were grown in the allantoic cavity of 10-day-old embryonated chicken eggs, isolated and purified by differential centrifugation (Maeda et al., 1975), and stored in phosphate-buffered saline, pH 7.4, at −80 °C. The fusogenic F-protein of Sendai virus was inactivated (Shimizu & Ishida, 1975; Oku et al., 1982) by incubation with trypsin (10 μg/70 μg of viral protein) in 10 mM phosphate buffer, pH 7.2, for 20 min at 37 °C, followed by addition of a 2-fold excess of soybean trypsin inhibitor.

Preparation of Human Erythrocyte Ghosts. Human erythrocytes (type A^+) were obtained from the Red Cross Blood Bank and used within 5 weeks after they had been drawn. Sealed ghosts were prepared according to the method of Steck & Kant (1974) with some modifications (Hoekstra et al., 1983). Albumin-loaded ghosts were prepared by resuspending the lysed cells in 4–5 volumes of 120 mM KCl/30 mM NaCl/10 mM sodium phosphate, pH 7.4, containing 5% (w/v) bovine serum albumin. The mixture was incubated on ice for 15 min, and the ghosts were subsequently resealed at 37 °C (45 min) in the presence of 1 mM Mg^{2+}. Nonentrapped albumin was removed by repeated centrifugation (20 min, 22000*g*, 4 °C), and the final pellet was suspended in the KCl/NaCl/sodium phosphate buffer and stored on ice. The concentration of ghosts (milligrams of protein per milliliter) was determined by measuring protein (prior to addition of albumin) or lipid phosphorus after lipid extraction, assuming 674 nmol of phospholipid/mg of protein (Cohen & Solomon, 1976).

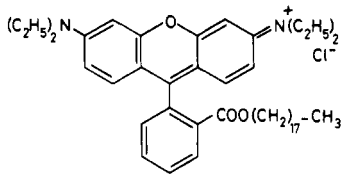

FIGURE 1: Structural formula of octadecyl Rhodamine B chloride (R_{18}).

Incorporation of Octadecyl Rhodamine B Chloride (R_{18}) into Various Membranes. Octadecyl Rhodamine B chloride (R_{18}, Figure 1), originally synthesized by Keller et al. (1977), was obtained from Molecular Probes, Inc. (Junction City, OR). A stock solution was prepared in chloroform/methanol (1:1) and stored at −20 °C. For incorporation into phospholipid vesicle bilayers, an aliquot of the stock solution was premixed with the phospholipid solutions in chloroform/methanol to give the desired lipid/fluorophore ratio. R_{18}-containing lipid vesicles were then prepared as described above. Insertion of the probe into membranes from viruses, native erythrocytes, and ghosts was accomplished as follows. A small amount of R_{18} was dried under a stream of argon gas and solubilized in ethanol. A total of 10 μL of this solution (10–20 nmol of R_{18}) was injected, under vortexing, into 1 mL of the membrane-containing incubation medium (ca. 1 mg of protein). The mixture was incubated in the dark for 1 h at room temperature. Nonincorporated R_{18} was subsequently removed by chromatography on Sephadex G-75 (1 × 25 cm), and the R_{18}-containing membrane fraction was recovered in the void volume of the column. Preliminary experiments revealed that chromatography of a membrane-free but R_{18}-containing sample resulted in a complete retention of the fluorophore on top and in the upper part of the column. To quantitatively determine the amount of R_{18} that became incorporated into the membranes, viruses and ghosts were extracted with chloroform/methanol/0.1 N HCl. An aliquot of the lipid extract (in chloroform) was taken and the fluorescence was measured. The response of the fluorometer was calibrated by using known concentrations of R_{18}. A linear relationship between the amount of R_{18} and fluorescence intensity was found up to at least 1000 pmol/mL.

Determination of Self-Quenching of R_{18} Fluorescence. Phospholipid vesicles (LUV or SUV) were prepared, containing various percentages of R_{18} with respect to total lipid. Fluorescence was determined prior to and after addition of Triton X-100 (1% v/v, final concentration), and from the ratio, the percentage of fluorescence quenching was calculated after correcting the fluorescence in the presence of detergent for sample dilution. Triton X-100 did not affect the quantum efficiency of R_{18} as determined by fluorescence measurements of a nonquenching concentration of the probe (0.1 mol %) in DOPC bilayers in the presence and absence of detergent. The efficiency of R_{18} self-quenching in the various biological membranes was determined in a similar manner. For fusion measurements, the fluorescence scale was calibrated such that the residual fluorescence of the membranes was taken as the zero level and the value obtained after addition of Triton X-100, corrected for sample dilution, as 100% (infinite dilution).

[1] Abbreviations: PS, phosphatidylserine; DOPC, dioleoylphosphatidylcholine; *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine; *N*-Rh-PE, *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine; R_{18}, octadecyl Rhodamine B chloride; LUV, large unilamellar vesicles; SUV, small unilamellar vesicles; RET, resonance energy transfer; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

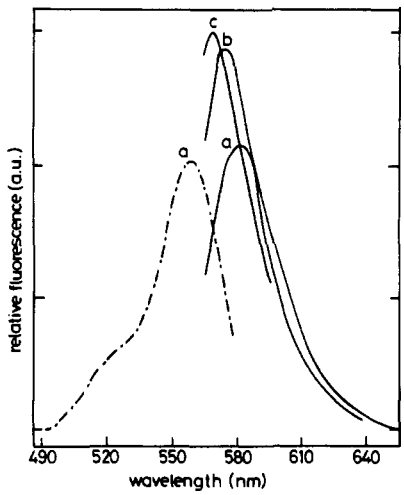

FIGURE 2: Excitation (-·-) and emission (—) spectra of R_{18}. Curves a are excitation and emission spectra of 2 mol % R_{18} in DOPC vesicles. Curves b and c show the emission spectra of R_{18} in ethanol and chloroform, respectively. R_{18}-containing viruses or ghosts, prepared as described under Experimental Procedures, gave identical spectra as those obtained in (a).

Resonance Energy Transfer Fusion Assay. Lipid vesicles (LUV) were prepared containing 0.8 mol % each of the fluorescent donor lipid (*N*-NBD-PE) and acceptor lipid (*N*-Rh-PE). Upon fusion with membranes devoid of fluorescent lipid (Struck et al., 1981; Hoekstra, 1982a), the surface density of the fluorophores and, hence, the efficiency of energy transfer will decrease. At the probe concentrations used, the decrease in energy transfer efficiency is proportionally related to the extent of fusion. The NBD fluorescence scale was calibrated as described above, except that the fluorescence obtained after addition of Triton X-100 was, in addition, corrected for the effect of the detergent on the quantum yield of NBD fluorescence (Struck et al., 1981).

Fluorescence Measurements. Continuous monitoring of NBD fluorescence (excitation and emission wavelengths of 475 and 530 nm, respectively) or R_{18} fluorescence (excitation and emission wavelengths of 560 and 590 nm, respectively) was carried out with a Perkin-Elmer MPF 43 spectrophotofluorometer, equipped with a chart recorder. The sample chamber was equipped with a magnetic stirrer, and the temperature was controlled with a thermostated circulating water bath. The final incubation volume in all measurements was 2 mL.

Results

Fluorescence Properties of R_{18}. Figure 2 shows the excitation and emission spectra of R_{18}, incorporated into unilamellar DOPC vesicles (a), and the emission spectra of the fluorophore, solubilized in ethanol (b) and chloroform (c). In the latter solvents an identical excitation spectrum as that in (a) was obtained, exhibiting a maximum at 560 nm. The emission maximum wavelength was dependent upon the environment of the probe molecules, displaying a maximum of 586, 578, and 569 nm in membranes (a), alcohol (b), and chloroform (c), respectively. Concomitantly, an increase in the relative fluorescence intensity at the emission maximum was seen with decreasing solvent polarity. Excitation and emission spectra recorded from R_{18}-labeled viruses and erythrocyte ghosts were identical with those presented in (a).

These results indicate that, when incorporated into membranes, R_{18} presumably has the rhodamine moiety oriented so that it remains in the vicinity of the lipid–water interface with the hydrocarbon tail embedded in the membrane bilayer, similarly as reported for a variety of other fluorescent amphiphiles (Derzko & Jacobson, 1980; Prendergast et al., 1981).

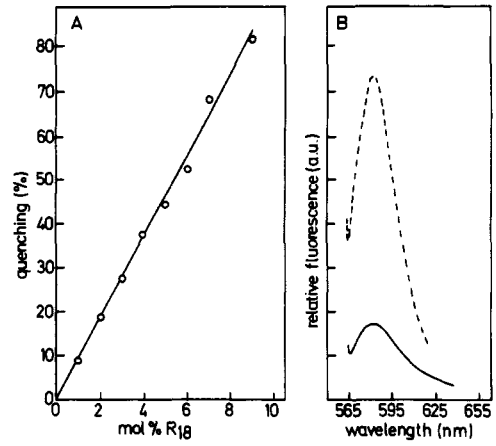

FIGURE 3: Efficiency of self-quenching of R_{18} in DOPC vesicles (A) and relief of self-quenching upon addition of Triton X-100 to R_{18}-containing Sendai virus (B). (A) SUV were prepared containing various amounts of R_{18}. The percentage of self-quenching was determined by measuring fluorescence before and after addition of Triton X-100 (1% v/v). (B) R_{18} was incorporated into Sendai virus membranes (ca. 20 nmol/mg of viral protein). Emission spectra (λ_{ex} = 560 nm) were recorded before (—) and after (---) addition of Triton X-100 (1% v/v).

Quenching of R_{18} Fluorescence. Figure 3 demonstrates that as the surface density of R_{18} in DOPC bilayers is increased, the fluorescence becomes increasingly quenched. At surface densities ≤9 mol %, the efficiency of self-quenching is directly proportional to the ratio of R_{18} to total lipid. Therefore, in this range, any decrease in the surface density of R_{18}, occurring upon fusion of R_{18}-containing membranes with membranes devoid of the fluorophore, will result in a proportional relief of fluorescence self-quenching.

Insertion of R_{18} into Biological Membranes. When included into the incubation medium as an ethanolic solution, the fluorescent amphiphile incorporated spontaneously into biological membranes. Routinely, Sendai virus (0.9 mg/mL of protein) was incubated with ca. 18 μM R_{18}. After an incubation of 1 h at room temperature, 50–70% of the probe became membrane associated, implying that the final probe concentration may vary between 2.3 and 3.1 mol % with respect to total viral lipid (ca. 400 nmol of lipid/mg of protein; Loyter & Volsky, 1982). At this probe density (cf. Figure 3A), an efficiency of self-quenching of ca. 30% would be expected. However, the R_{18}-labeled virus preparations generally displayed a degree of quenching that varied from 60 to 75% (Figure 3B). This apparent discrepancy can be explained if it is assumed that the probe does not randomize across the membrane but, instead, is predominantly localized in the outer leaflet of the bilayer. Taking 50% of the total lipid pool of Sendai virus membranes to reside in the outer leaflet, the ratio of R_{18} to total lipid would thus range from 4.6 to 6.2 mol %. Furthermore, if it is taken into account that the molar ratio of phospholipid to cholesterol is ca. 2 (Loyter & Volsky, 1982), while the surface area per molecule of phospholipid to that of a cholesterol molecule differs by a factor of ca. 2 (Papahadjopoulos & Kimelberg, 1974), there appears to be a good agreement between the calculated surface density and the experimentally observed degree of R_{18} self-quenching in fluorophore-containing Sendai virus membranes. On the basis of the above assumption, a similar consistency, regarding the efficiency of probe incorporation vs. surface density and self-quenching, was seen for erythrocyte membranes and influenza virus, labeled with R_{18} in a similar manner as Sendai virus. Fluorescence microscopic examination of the R_{18}-labeled ghosts did not reveal a (visible) appearance of R_{18} fluorescence

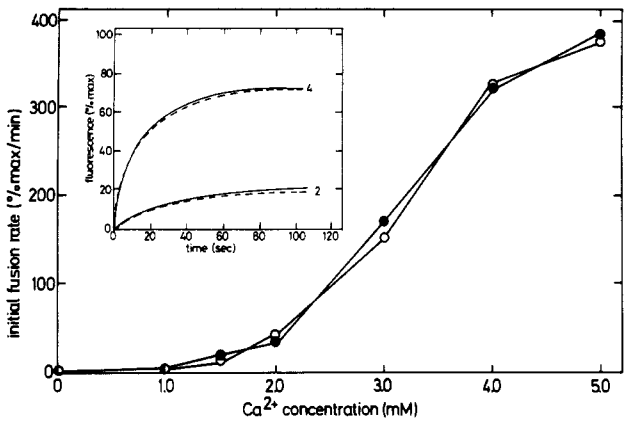

FIGURE 4: Ca^{2+}-induced fusion of PS vesicles: a comparison between RET and R_{18} assay. LUV consisting of PS/*N*-NBD-PE/*N*-Rh-PE (98.4:0.8:0.8) or PS/R_{18} (96:4) were mixed with nonlabeled PS vesicles (molar ratio 1:4) in 100 mM NaCl/5 mM HEPES, pH 7.4, at a final lipid concentration of 25 μM. The incubation temperature was 37 °C. Fusion was induced by rapid injection of Ca^{2+} into the medium, and the kinetics of lipid dilution, as revealed by an increase in NBD fluorescence (- - -, insert) or R_{18} fluorescence (—, insert), were continuously monitored. The insert depicts the development of fluorescence obtained at 4 and 2 mM Ca^{2+}. Initial rates (●, RET assay; ○, R_{18} assay) were calculated from the tangents to the curves at $t = 0$ and plotted as a function of the Ca^{2+} concentration.

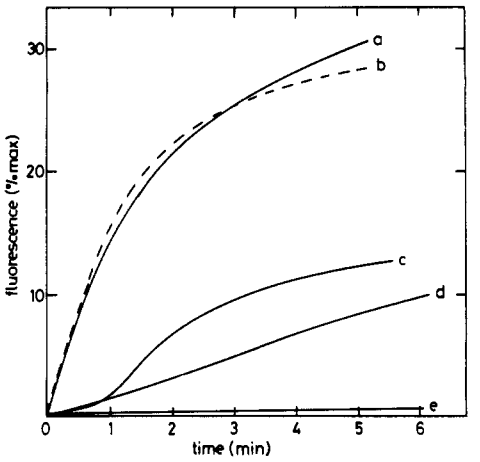

FIGURE 5: Fusion between (i) LUV and influenza virus and (ii) Sendai virus and erythrocyte membranes and (iii) Sendai virus induced fusion of erythrocyte ghosts. The kinetics of fusion were monitored by the RET assay (b) or R_{18} assay (a, c–e). (a) R_{18} was inserted into the membrane of influenza virus as described under Experimental Procedures. Viral phospholipid (12 nmol) was mixed with 12 nmol of pure PS vesicles (LUV) in 140 mM NaCl/10 mM sodium citrate, pH 5.0 at 37 °C. (b) Influenza virus (12 nmol of phospholipid) was rapidly injected into the same medium as in (a), containing 12 nmol of PS/*N*-NBD-PE/*N*-Rh-PE LUV, and fusion was monitored as a function of time. (c) R_{18}-containing Sendai virus (25 μg of protein), prepared as described under Experimental Procedures, was suspended in 120 mM KCl/30 mM NaCl/10 mM sodium phosphate, pH 7.4 at 37 °C. Fusion with ghosts was initiated by rapid injection of 130 μg of ghost protein into the medium. (d) Albumin-containing ghosts were labeled with R_{18}. Equal amounts (30 μg of protein each) of R_{18}-containing and nonlabeled ghosts were mixed with Sendai virus (50 μg of protein) in a final incubation volume of 200 μL; the same buffer as in (c) was used. The mixture was incubated at 4 °C for 15 min. Prewarmed buffer was added subsequently, raising the temperature to 37 °C and the incubation volume to 2 mL, and fusion was monitored in the fluorometer. (e) Same as (c) and (d), except that the R_{18}-containing Sendai virus in (c) had been pretreated with trypsin (see Experimental Procedures) and the ghost–virus incubation at 4 °C in (d) was omitted and, instead, was done at 37 °C.

patches but, rather, showed a smooth peripheral ring of fluorescence (not shown), as expected for a random distribution of the probe in the, presumably, outer leaflet (see above) of the membrane.

Transfer of R_{18} between Labeled and Nonlabeled Membranes. In principle, transfer of R_{18} from labeled into nonlabeled membranes can occur through a number of different processes, and three potential mechanisms may be involved: (i) spontaneous diffusion through the aqueous phase, (ii) transfer between aggregated membranes, or (iii) fusion. Various control experiments (not shown) excluded the possibility that fluorophore dilution was accomplished by spontaneous transfer, involving the passive transfer of these molecules between labeled and nonlabeled membranes as soluble monomers diffusing through the aqueous medium. Thus, when a mixture of erythrocytes or ghosts, of which 25% were labeled with R_{18}, was incubated for 1 h at 37 °C, still 25% of the population was labeled after termination of the incubation. Similarly, when R_{18}-labeled DOPC or PS vesicles were mixed with nonlabeled DOPC or PS vesicles, respectively, no increase in fluorescence was seen. Addition of 10 mM Mg^{2+}, which induces massive aggregation of PS LUV, but not fusion, did not result in a significant increase in fluorescence during a period of 5 min. After longer incubation times (5–10 min) some dilution of the R_{18} probe became apparent, but the maximal extent of fluorescence development remained low (ca. 5% after 10 min and remaining constant for at least 3 h), indicating that contact-mediated transfer of the fluorophore was virtually negligible (see also below). However, when various Ca^{2+} concentrations were injected into a medium containing R_{18}-labeled and nonlabeled PS LUV (ratio 1:4), a rapid development of fluorescence was seen (Figure 4), its rate increasing with increasing Ca^{2+} concentrations.

The results suggested that the increase in fluorescence observed upon addition of Ca^{2+}, but not Mg^{2+}, was accomplished by a dilution of the probe as a result of Ca^{2+}-induced fusion of the vesicles. This suggestion was corroborated by experiments showing that when *N*-NBD-PE and *N*-Rh-PE containing PS LUV and nonlabeled PS LUV were mixed in identical proportions as above and fusion was monitored by the RET fusion assay, similar rates of lipid dilution were measured compared to those obtained with the R_{18} assay (Figure 4). As shown in the insert of Figure 4, the overall kinetics of Ca^{2+}-induced fusion of PS vesicles, monitored by either the RET or the R_{18} assay, were essentially indistinguishable.

Fusion of Biological Membranes. To test the R_{18} assay in a system involving a biological membrane, we first examined fusion between influenza viruses and PS vesicles. To compare the R_{18} assay with the RET assay, influenza virus was first mixed with PS vesicles containing *N*-NBD-PE and *N*-Rh-PE. At pH 7.4, no significant dilution of the fluorophores was observed, which is consistent with the inability of influenza virus to fuse at neutral pH (Maeda et al., 1981a,b; White et al., 1982). At pH 5.0, influenza virus fused rapidly with the PS vesicles, as revealed by the rapid increase of NBD fluorescence (Figure 5, curve b). Next, the same experiment was carried out with influenza virus that had been labeled with R_{18} and with nonlabeled PS LUV. As shown in Figure 5, curve a, the kinetics of fluorescence development were very similar to those obtained with the RET assay. The initial fusion rates were 18.2 (RET) and 18.1% max/min (R_{18}). In conjunction with the fact that the RET assay is not affected by pH changes (Blumenthal et al., 1983; Eidelman et al., 1984), the similarity in fusion kinetics would argue against pH-induced alterations of the molecular properties of R_{18} such that spontaneous transfer might occur at low pH. Furthermore, no dilution of fluorophore was seen (not shown) when

R_{18}-containing DOPC vesicles were incubated with vesicles devoid of the probe at various pH values (pH 4.0–9.0).

Next, we examined the fusion between Sendai virus and erythrocyte membranes, utilizing the R_{18} assay. Sendai virus was labeled with the fluorophore and subsequently incubated with erythrocyte ghosts at 4 °C. Although the viruses bind avidly to the membranes under these conditions, concomitant dilution of R_{18} was not detected (not shown). When the temperature was raised to 37 °C or when labeled Sendai virus was directly mixed with ghosts at 37 °C (Figure 5, curve c), a gradual relief of self-quenching was seen during the first minute, followed by a subsequent, faster rate of relief. Interestingly, the kinetics of relief of self-quenching were very similar, irrespective of whether viruses and ghosts were mixed directly at 37 °C or had been preincubated at 4 °C, indicating that fusion between the viral membrane and the biological target membrane per se does not require binding at low temperature. As a control experiment, R_{18}-labeled Sendai virus was treated with trypsin, which is known to digest the F-protein, thereby abolishing its fusion activity (Shimizu & Ishida, 1975; Oku et al., 1982). As shown in Figure 5 (curve e), incubation of trypsinized R_{18}-labeled virus with the ghosts did not result in fluorescence development, consistent with the inability of trypsinized viruses to fuse. Finally, when investigating the fusion properties of Sendai virus with ghosts and liposomes as a function of pH (D. Hoekstra et al., unpublished observations), we observed that both the rate and extent of fusion increased by more than 10-fold when raising the pH from 4.5 to 8.0, in agreement with results reported by Haywood & Boyer (1982), while the extent of binding of R_{18}-labeled virus at low temperature was independent of the pH. These results further support the reliability of the R_{18} assay, both at neutral and at low pH.

An accompanying event in virus–cell fusion is the occurrence of virus-mediated cell–cell fusion, provided that viruses and cells are preincubated at 4 °C to allow virus-induced cell aggregation. Indeed, when ghosts, labeled with R_{18}, were mixed with nonlabeled ghosts in a 1:1 ratio, incubated with Sendai virus at 4 °C, and subsequently diluted with prewarmed medium (37 °C), a gradual increase in fluorescence was seen as a function of time (Figure 5, curve d). By contrast, when the 4 °C preincubation step was omitted (Figure 5, curve e), no apparent decrease in R_{18} surface density could be detected, indicating that fusion had not taken place. These results were readily confirmed by fluorescence microscopic examination of the preparations (not shown).

Quantitation of the Fusion Process. The results in Figures 4 (insert) and 5 were expressed as a percentage of the fluorescence that would be obtained upon infinite dilution of the fluorescent amphiphile. Since the conditions of labeling were taken such that the surface density of R_{18} never exceeded a value of 8 mol %, dilution of the probe will result in a concomitant development of fluorescence, which is directly proportional to the extent of intermixing of nonlabeled and labeled membranes (cf. Figure 3A). Thus, when a labeled membrane fraction and a nonlabeled membrane fraction are mixed in a ratio of 1:1 (Figure 5a,b,d) or 1:4 (Figure 4), complete randomization would result in a fluorescent signal corresponding to 50 and 80%, respectively, of the signal at infinite dilution. It can then be calculated that at 4 mM Ca^{2+} ca. 85% and at 2 mM Ca^{2+} ca. 25% fusion of PS LUV had taken place after 1.5 min (Figure 4), while interaction between influenza virus and PS vesicles (Figure 5a,b) resulted in ca. 60% fusion after 5 min, as determined with either the RET or R_{18} assay. (It should be noted that the size of an influenza virus particle is about the same as that of the LUV used here.)

In the case of fusion between R_{18}-labeled Sendai virus and erythrocyte membranes (Figure 5, curve c), the percent maximum fluorescence is directly proportional to the extent of fusion. If all viruses, added to the incubation mixture, would fuse and if the R_{18} probe, upon fusion, would be exclusively inserted into the outer leaflet of the ghost bilayer, the surface density of R_{18} with respect to ghost membrane phospholipid alone would be ca. 0.5 mol %, since 25 μg of viral protein corresponds to ca. 0.3 nmol of R_{18} (see above), while 130 μg of ghost protein is equivalent with ca. 50 nmol of outer leaflet phospholipid (Cohen & Solomon, 1976). Binding studies between viruses and ghosts, which can also be done by utilizing the R_{18} probe, have revealed that only 30% of the total viral dose becomes cell associated, which implies that the final concentration of R_{18}, inserted into the outer leaflet of the ghost membrane as a result of fusion, will be less than 0.2 mol % with respect to total phospholipid. Thus, fusion between R_{18}-labeled viruses and ghost membranes will essentially lead to infinite dilution of the probe. Evidence supporting this conclusion was obtained when we determined the fraction of R_{18}-containing Sendai virus that fused with ghosts during a 1-h incubation at 37 °C, after prior incubation on ice and removal of nonbound virus (not shown; D. Hoekstra et al., unpublished observations). By measuring fluorescence before and after addition of detergent, we found that ca. 40% of the initial cell-associated virus fraction had fused during this time interval, which is consistent with results previously reported by others (Lyles & Landsberger, 1979; Maeda et al., 1981b), using different techniques.

On the basis of the R_{18} assay, ca. 20% fusion had taken place between erythrocyte ghosts as induced by Sendai virus (Figure 5, curve d) after a time interval of 6 min. Since the ghosts contained albumin, large swollen polyghosts (Sekiguchi et al., 1981) could be seen in the fluorescence microscope and a rough estimate of the extent of fusion indicated a fair agreement between both methods.

Discussion

We have presented a fluorescence assay for continuous and sensitive monitoring of membrane fusion, which is applicable in both artificial and biological systems. The principle of the assay relies upon the efficient self-quenching of the fluorophore rhodamine, conjugated to a saturated C_{18} hydrocarbon chain. Upon dilution of the probe during fusion between labeled and nonlabeled membranes, the time-dependent enhancement of fluorescence intensity can be followed continuously and is proportionally related to the extent of fusion, provided that the surface density of the probe is kept below 9 mol %. Up to this concentration the extent of self-quenching is linearly dependent on the surface density.

The virtue of R_{18} is that the probe is readily inserted into biological membranes by exogenous addition of an ethanolic solution of the fluorophore. Presumably, membrane labeling is accomplished by a spontaneous transfer of R_{18} between micelles and the acceptor membrane. The spectral properties of the probe (Figures 2 and 3B) indicated that, after membrane insertion, (i) the bulk of the dye is present as monomers and dimers and that (ii) the localization of the probe is such that the rhodamine head group probably resides at the lipid–water interface while the hydrocarbon moiety is anchored in the hydrophobic interior of the membrane. The former conclusion (i) was reached from observations indicating that the peak of maximal absorption of R_{18} was the same, irrespective of whether the probe was solubilized in organic solvents or incorporated into liposomes or viral membranes. Upon aggre-

gation of probe molecules in the lateral plane of the bilayer, or upon binding of micelles to the bilayers, a shift of the absorption maximum to longer wavelengths would have been expected (Sims et al., 1974; Edidin, 1981). Furthermore, the microscopic appearance of the fluorophore, inserted into erythrocyte membranes, was consistent with a random distribution of the probe in the bilayer.

Once inserted into the membrane, the probe did not dissociate from the membranes either by a spontaneous transfer of free monomers through the aqueous phase or by a collision-mediated transfer process. This conclusion can be inferred from observations demonstrating a lack of fluorescence increase when (i) labeled DOPC or PS LUV were incubated with nonlabeled acceptor vesicles, in either the absence or presence of Mg^{2+}, (ii) R_{18}-containing Sendai virus was incubated with erythrocyte membranes at 4 °C, when extensive virus–membrane binding, but no fusion, takes place, (iii) aggregation of R_{18}-labeled and nonlabeled erythrocytes or ghosts was induced by Sendai virus at low temperature, (iv) R_{18}-labeled and nonlabeled ghosts were mixed at 37 °C, in the presence of Sendai virus, without prior incubation at 4 °C, and (v) R_{18}-labeled influenza virus was incubated with phospholipid vesicles at neutral pH. Furthermore, results obtained when the fusion of R_{18}-labeled Sendai virus with ghosts and phospholipid vesicles was characterized (D. Hoekstra et al., unpublished observations) revealed that the rate of R_{18} fluorescence development was dependent on both the acceptor membrane concentration and composition, which also argues against a mechanism involving spontaneous diffusion of R_{18} monomers through the aqueous phase (Pownall et al., 1982; Frank et al., 1983). Rather, the results provided evidence for probe dilution being due to fusion, supporting the feasibility of R_{18} as a valuable tool to detect fusion events in biological systems. The reliability of the R_{18} assay was further indicated by the close similarity of the kinetics of lipid dilution, monitored by the R_{18} assay and the well-established RET assay (Struck et al., 1981; Hoekstra, 1982a; Hoekstra & Martin, 1982; Blumenthal et al., 1983; Eidelman et al., 1984; Connor et al., 1984) during fusion of phospholipid vesicles among themselves (Figure 4) or with influenza virus (Figure 5). Furthermore, the characteristics of Sendai virus induced fusion of ghosts and virus–ghost fusion (Figure 5), when the R_{18} assay was employed, are fully compatible with data collected by other techniques.

An effect of the fluorescent probe on intermembrane interactions and/or the fusion process per se can be excluded. The similarity of the kinetics of fusion between various membranes (Figures 4 and 5), when monitored by different assays, argues against such a possibility. In addition, we observed no differences between R_{18}-labeled and nonlabeled erythrocytes with respect to hemolytic fragility or tendency toward clumping. Also, the kinetics of hemolysis, induced by R_{18}-labeled Sendai virus, were not significantly different from the kinetics observed when nonlabeled Sendai virus was incubated with erythrocytes (D. Hoekstra et al., unpublished observations). Further investigations are required to establish whether biological functions other than fusion and hemolysis could be affected by the fluorophore.

The strong association of the fluorophore with the membrane, after insertion, can conceivably be explained by observations reported by others (Frank et al., 1983; Pownell et al., 1982; Nichols & Pagano, 1983), indicating that the tendency of exogenously incorporated acyl or alkyl derivatives to leave the bilayer decreases with increasing chain length. The relatively long hydrocarbon chain (C_{18}) of R_{18} would thus prevent its spontaneous dissociation from the membrane. In addition, it is possible that interactions between the rhodamine head group and phospholipid head groups and/or probe–protein interactions may also contribute to its nonexchangeable behavior after membrane insertion. This was suggested by observations that when ghosts were labeled with the C_{18} derivative of fluorescein, dodecanoylaminofluorescein, a rapid spontaneous redistribution of the fluorophore was seen when labeled and nonlabeled ghosts were mixed and examined in the fluorescence microscope. It is also possible that micelles of this particular probe persistently adhered to the erythrocyte membranes, which may have led to the observed exchange. In any case, these observations have frustrated attempts to use the fluorescein derivative in conjunction with R_{18} as a suitable energy-transfer couple (Keller et al., 1977). Besides this complication, the relatively poor overlap between the emission and excitation spectra of the fluorescent donor and acceptor, respectively, gave rise to a rather low (ca. 30%) efficiency of energy transfer, compared to the efficiency between *N*-NBD-PE and *N*-Rh-PE (ca. 90%).

In spite of the availability of efficient energy-transfer couples, it is still rather complicated to establish fusion between Sendai virus and *N*-NBD-PE- and *N*-Rh-PE-containing phospholipid vesicles. This is due to the relatively high background fluorescence of the fluorophore-containing vesicles in relation to the extremely low fraction of the total vesicle population ultimately fusing with the viruses (Haywood & Boyer, 1982; Hsu et al., 1983). Not only can this difficulty now be overcome by insertion of the "fusion-reporting" molecules into the viral membrane but, in addition, this approach offers a much greater flexibility in studying membrane fusion, allowing the continuous and sensitive monitoring of the kinetics of fusion in both artificial *and* biological systems. An additional advantage of the technique presented here is that it is much faster and easier to perform than previous assays that, in the case of virus–target membrane interaction, are based on the use of radioisotopes (Haywood & Boyer, 1982; White et al., 1983) and electron spin probes (Maeda et al., 1975, 1981a,b; Lyles & Landsberger, 1979) or involve the use of indirect techniques such as hemolysis and infectivity (White et al., 1983). None of these assays permit continuous monitoring of the fusion process, while accurate quantitation of the extent of fusion is difficult, if possible at all. By use of the R_{18} assay, fusion can be directly monitored without the need of separating fused and nonfused membranes, while the extent of fusion can be readily determined.

Finally, the R_{18} assay should be widely applicable, since there seems to be no obvious limitation preventing incorporation of the probe into biological membranes other than those reported in this paper. It would thus be possible, for example, to monitor the fusion kinetics of chromaffin granules among themselves or with plasma membrane vesicles of chromaffin cells (Bental et al., 1984). An intriguing possibility would be the registration of intracellular fusion phenomena, occurring after internalization of R_{18}-containing plasma membrane during endocytosis or upon fusion from within between R_{18}-labeled influenza viruses and endosomal membranes.

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