Johnson, N. P., & Schleich, T. (1974) *Biochemistry 13*, 981–987.
Laws, W. R., & Shore, J. D. (1978) *J. Biol. Chem. 253*, 8593–8597.
Laws, W. R., & Shore, J. D. (1979) *J. Biol. Chem. 254*, 2582–2584.
Longworth, J. W. (1971) in *Excited States of Proteins and Nucleic Acids* (Steiner, R. F., & Weinryb, I., Eds.) pp 318–484, Plenum Press, New York.
Luisi, P. L., & Favilla, R. (1970) *Eur. J. Biochem. 17*, 91–94.
Miles, D. W., & Urry, D. W. (1968) *J. Biol. Chem. 243*, 4181–4188.
Miles, D. W., Robins, M. J., Robins, R. K., & Eyring, H. (1969) *Proc. Natl. Acad. Sci. U.S.A. 62*, 22–29.
Parker, C. A. (1968) *Photochemistry of Solutions*, Elsevier Publishing Co., Amsterdam.
Purkey, R. M., & Galley, W. C. (1970) *Biochemistry 9*, 3569–3575.
Ross, J. B. A., Rousslang, K. W., & Kwiram, A. L. (1980) *Biochemistry 19*, 876–883.
Rossman, M. G., Liljas, A., Brändén, C.-I., & Banaszak, L. (1975) *Enzymes, 3rd Ed. 11*, 61–102.
Subramanian, S., & Ross, P. D. (1977) *Biochem. Biophys. Res. Commun. 78*, 461–466.
Subramanian, S., & Ross, P. D. (1978) *Biochemistry 17*, 2193–2197.
Subramanian, S., & Ross, P. D. (1979) *J. Biol. Chem. 254*, 7827–7830.
Taniguchi, S., Theorell, H., & Åkeson, Å. (1967) *Acta Chem. Scand. 21*, 1903–1920.
Theorell, H., & Yonetani, T. (1964) *Arch. Biochem. Biophys. 106*, 252–258.
Theorell, H., & Tatemoto, K. (1971) *Arch. Biochem. Biophys. 142*, 69–82.
Voelter, W., Records, R., Bunnenburg, E., & Djerassi, C. (1968) *J. Am. Chem. Soc. 90*, 6163.
Weinryb, I., & Steiner, R. F. (1971) in *Excited States of Proteins and Nucleic Acids* (Steiner, R. F., & Weinryb, I., Eds.) pp 319–484, Plenum Press, New York.
Wetlaufer, D. B. (1962) *Adv. Protein Chem. 17*, 303–390.
Witholt, B., & Brand, L. (1968) *Rev. Sci. Instrum. 39*, 1271–1278.

Use of Resonance Energy Transfer To Monitor Membrane Fusion†

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ABSTRACT: An assay for vesicle–vesicle fusion involving resonance energy transfer between *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl), the energy donor, and rhodamine, the energy acceptor, has been developed. The two fluorophores are coupled to the free amino group of phosphatidylethanolamine to provide analogues which can be incorporated into a lipid vesicle bilayer. When both fluorescent lipids are in phosphatidylserine vesicles at appropriate surface densities (ratio of fluorescent lipid to total lipid), efficient energy transfer is observed. When such vesicles are fused with a population of pure phosphatidylserine vesicles by the addition of calcium, the two probes mix with the other lipids present to form a new membrane. This mixing reduces the surface density of the energy acceptor resulting in a decreased efficiency of resonance energy transfer which is measured experimentally. These changes in transfer efficiency allow kinetic and quantitative measurements of the fusion process. Using this system, we have studied the ability of phosphatidylcholine, phosphatidylserine, and phosphatidylcholine–phosphatidylserine (1:1) vesicles to fuse with cultured fibroblasts. Under the conditions employed, the majority of the cellular uptake of vesicle lipid could be attributed to the adsorption of intact vesicles to the cell surface regardless of the composition of the vesicle bilayer.

Membrane fusion is involved in a variety of important biological processes, and therefore various attempts have been made to elucidate the mechanism(s) of fusion. In particular, the use of unilamellar lipid vesicles for this purpose has received wide attention (Miller & Racker, 1976; Papahadjopoulos et al., 1976, 1977, 1979; Newton et al., 1978; Pagano & Weinstein, 1978; Portis et al., 1979). Although it is clear that vesicle–vesicle fusion does occur under certain conditions (Maeda & Ohnishi, 1974; Miller & Racker, 1976; Hoekstra et al., 1979; Wilschut & Papahadjopoulos, 1979), the procedures used to detect such fusion suffer from low sensitivity or an experimental design which precludes their use in more complex systems. Consequently, an alternative method(s) by which membrane fusion can be measured which is both sensitive and versatile is needed.

Several recent reports (Keller et al., 1977; Gibson & Loew, 1979; Deamer & Uster, 1980) suggest that membrane fusion in a wide variety of systems might be studied by using resonance energy transfer. This approach relies upon the interactions which occur between two fluorophores if the emission band of one, the energy donor, overlaps with the excitation band of the second, the energy acceptor, and the two probes exist in close physical proximity (Forster, 1949; Fung & Stryer, 1978; Stryer, 1978). When these conditions are met, the energy from a photon absorbed by the energy donor can be transferred to the energy acceptor which will then fluoresce as though it had been excited directly. Since the efficiency of fluorescence energy transfer between two given fluorophores is dependent upon their spatial separation (Fung & Stryer, 1978), this technique provides a means by which lipid mixing

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during membrane fusion can be followed. Vanderwerf & Ullman (1980) have recently shown that this method can, in fact, be utilized to detect fusion between small unilamellar lipid vesicles.

In this report, we demonstrate that fusion between a vesicle containing the fluorescent phospholipid analogues *N*-NBD-PE[1] and *N*-Rh-PE with a second membrane devoid of fluorescent lipid decreases the efficiency of energy transfer between the two fluorophores. This is because the membrane formed by the fusion process has a lower surface density (ratio of fluorescent lipid to total lipid) of *N*-Rh-PE, the energy acceptor, than does the original vesicle preparation which contained this analogue. Control experiments indicate that the observed changes in transfer efficiency between *N*-NBD-PE and *N*-Rh-PE are correlated with fusion events and not with unrelated processes such as lipid exchange (Martin & MacDonald, 1976; Duckwitz-Peterlein et al., 1977; Roseman & Thompson, 1980; Nichols & Pagano, 1981) which could also reduce the efficiency of energy transfer. The application of this method to monitor both vesicle–vesicle fusion and vesicle–cell interactions is presented.

Experimental Procedures

Materials and Routine Procedures. Phosphatidylserine (bovine brain), *N*-NBD-PE, and DOPC were purchased from Avanti Biochemicals, Birmingham, AL. Lissamine Rhodamine B sulfonyl chloride was obtained from Molecular Probes, Plano, TX. [^{14}C]DOPC was purchased from Applied Science Laboratories, State College, PA. Small unilamellar vesicles were prepared by sonication at 10 °C under an inert atmosphere of argon gas in phosphate-buffered saline containing 0.1 mM EDTA. All lipids were periodically checked for purity by thin-layer chromatography and were repurified as necessary.

Preparation of N-Rh-PE. *N*-Rh-PE was synthesized by reacting 5 mg of dioleoylphosphatidylethanolamine with a 5-fold molar excess of lissamine Rhodamine B sulfonyl chloride in 1 mL of MeOH/$CHCl_3$ (9:1) containing 15 μL of redistilled triethylamine for 2 h at 22 °C. After the reaction mixture was dried under a stream of argon gas, 1 volume each of water and acetone was added, and the sample was mixed and extracted by using 2 volumes ethyl acetate. The organic phase was washed with water, and the *N*-Rh-PE was purified by preparative thin-layer chromatography in $CHCl_3$/MeOH/acetone/acetic acid/H_2O (5:1:2:1:0.5). It should be noted that several isomers of *N*-Rh-PE were formed during the reaction corresponding to the different isomers of the Rhodamine B sulfonyl chloride present. The one most suitable for the resonance energy transfer measurements in this study had an R_{PE} (migration relative to phosphatidylethanolamine) of ~1.23 in the above solvent system.

Fluorescence Measurements. Steady-state emission and excitation spectra were obtained by using an Aminco-Bowman spectrofluorometer equipped with crossed polarizers to reduce light scattering. The excitation and emission band slits were 1 mm. Peak absorbance of samples was kept <0.1 to reduce inner filter effects. Following each measurement, vesicles were disrupted with Triton X-100 (1% final concentration). This treatment eliminated energy transfer and allowed the determination of the concentrations of *N*-NBD-PE and *N*-Rh-PE from their emission intensities by using direct excitation. All spectra were obtained at room temperature (22 °C).

Calculations of Transfer Efficiency. The efficiency of energy transfer was calculated from the fluorescence emission intensity of *N*-NBD-PE at 530 nm by using eq 1 under Results. Fluorescence intensities were recorded in the presence (F_0) and absence (F) of Triton X-100. Values obtained in the presence of Triton X-100 were corrected for sample dilution and for the effect of Triton X-100 on the quantum yield of *N*-NBD-PE.

Vesicle–Vesicle Fusion. Fusion between vesicles containing fluorescent lipid (both *N*-NBD-PE and *N*-Rh-PE) and vesicles devoid of fluorescent lipid was initiated by the addition of calcium to the indicated final concentrations. In order to determine the extent of fusion between the two vesicle populations, the efficiency of energy transfer between *N*-NBD-PE and *N*-Rh-PE was calculated before and after the addition of calcium. From the observed transfer efficiencies, the corresponding surface densities of *N*-Rh-PE (molecules of *N*-Rh-PE per molecule of lipid) were estimated by using the data shown in Figure 2. If a decrease in transfer efficiency, and thus surface density, was observed, the extent of dilution of the energy acceptor which would be required for this decrease was calculated. For example, if *N*-Rh-PE surface density was decreased from 0.01 to 0.005, then each vesicle containing the fluorescent lipids must have fused with one vesicle devoid of fluorescent lipid if it is assumed that all input vesicles participated in fusion events. Of course, these estimates would represent the minimum number of vesicles from each population which must have coalesced to form the final fusion product. Rates of fusion between vesicle mixtures were measured by following changes in fluorescence intensity at 530 nm (λ_{ex} = 450 nm) as a function of time.

The ability of distinct vesicle populations to undergo calcium-induced fusion was also assessed by their chromatographic behavior on Bio-Gel A15m (1 × 60 cm) columns before and after exposure to calcium. Fractions of 1.3 mL were collected, and each fraction was monitored for radioactivity, fluorescent lipid, and efficiency of energy transfer between fluorescent lipids as described above. The eluting buffer was phosphate-buffered saline containing 0.1 mM EDTA in all cases.

Cells and Vesicle–Cell Incubations. Monolayer cultures of Chinese hamster V79 fibroblasts were grown to confluency and harvested as previously described (Struck & Pagano, 1980). Cells (10^7/mL) were incubated with 1 mg/mL DOPC/*N*-NBD-PE/*N*-Rh-PE, 0.2 mg/mL phosphatidylserine/*N*-NBD-PE/*N*-Rh-PE, or 0.2 mg/mL phosphatidylserine/DOPC/*N*-NBD-PE/*N*-Rh-PE vesicles in the presence or absence of 5 mM Ca^{2+} as indicated. Incubations were carried out at 37 °C for 30 min. Incubations were stopped by washing the cells 3 times with 5 mL of cold phosphate-buffered saline containing 10 mM EDTA. Before the final wash, cells were transferred to new tubes. Cellular uptake of fluorescent lipid was determined by extraction of the cells after vesicle treatment as previously described (Struck & Pagano, 1980). In all cases, only a small fraction (<5%) of the applied vesicles became cell associated (Table I). The extent of vesicle–cell adsorption was estimated as described under Results.

Results

The excitation and emission spectra of *N*-NBD-PE and *N*-Rh-PE incorporated into unilamellar lipid vesicles are shown in Figure 1. As can be seen, there is a significant overlap between the emission band of *N*-NBD-PE and the excitation band of *N*-Rh-PE. Consequently, energy transfer between this

[1] Abbreviations used: *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; *N*-Rh-PE, *N*-(lissamine Rhodamine B sulfonyl)dioleoylphosphatidylethanolamine; EDTA, ethylenediaminetetraacetic acid; DOPC, dioleoylphosphatidylcholine.

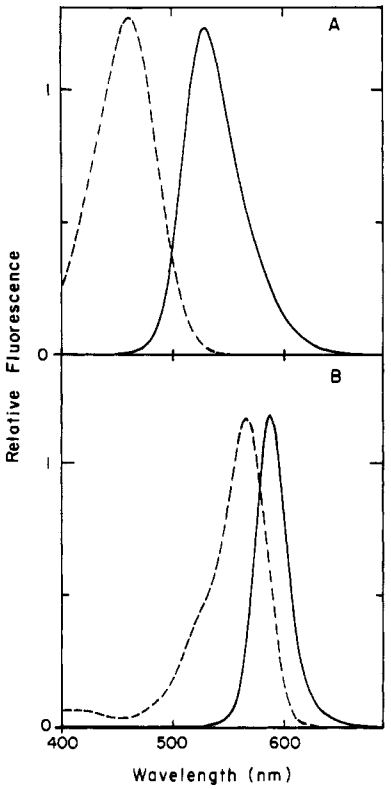

FIGURE 1: Excitation (---) and emission (—) spectra in phosphatidylserine vesicles containing 1 mol % fluorescent analogues. (A) Spectra of *N*-NBD-PE; (B) spectra of *N*-Rh-PE. Vesicles were prepared as described under Experimental Procedures.

donor–acceptor pair is expected to be relatively efficient, even at low (~0.01) surface densities (ratio of fluorescent lipid to total lipid) of the energy acceptor when the two probes are present in the same bilayer. In addition, it should be noted that there is a minimum at 450 nm in the excitation spectrum of *N*-Rh-PE which is near the excitation maximum of *N*-NBD-PE. This allows energy transfer measurements under conditions which minimize direct excitation of the energy acceptor, *N*-Rh-PE.

When a sample containing similar amounts of both *N*-NBD-PE and *N*-Rh-PE is excited at 470 nm, essentially all of the fluorescence at 530 nm comes from *N*-NBD-PE. Thus, the efficiency of energy transfer (quenching of the energy donor) in such samples is defined by the relationship (Fung & Stryer, 1978):

$$E = 1 - F/F_0 \qquad (1)$$

where F is the fluorescence at 530 nm in the presence of *N*-Rh-PE and F_0 is the fluorescence at 530 nm in the absence of *N*-Rh-PE (or presence of detergent). The dependence of transfer efficiency on the surface density of the energy acceptor is shown in Figure 2. As can be seen, at low surface densities of *N*-Rh-PE (0–0.01), the efficiency of energy transfer is nearly proportional to the ratio of *N*-Rh-PE to total lipid in the vesicle bilayer. Thus, in this range, any decrease in the surface density of the energy acceptor will result in a proportional decrease in measured transfer efficiency. The efficiency of energy transfer is independent of the surface density of the energy donor, *N*-NBD-PE, a situation similar to that found with other donor–acceptor pairs (Vanderwerf & Ullman, 1980).

To test the feasibility of using fluorescence energy transfer to detect fusion events, we employed the calcium-induced fusion of phosphatidylserine vesicles (Maeda & Ohnishi, 1974; Miller & Racker, 1976; Papahadjopoulos et al., 1976, 1977; Newton et al., 1978; Hoekstra et al., 1979; Portis et al., 1979; Wilschut & Papahadjopoulos, 1979) as a model system. When a vesicle preparation containing both *N*-NBD-PE and *N*-Rh-PE is excited at 470 nm, emission maxima at 530 and 585

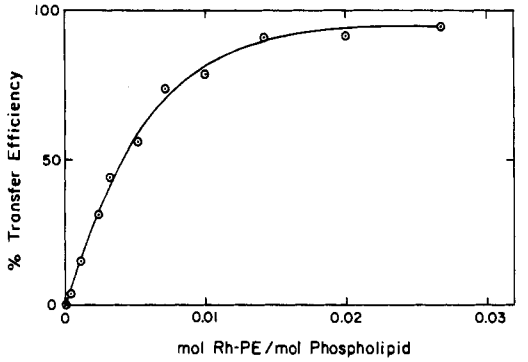

FIGURE 2: Efficiency of energy transfer as a function of the number of energy acceptors per phospholipid molecule (surface density). The molar ratio of *N*-NBD-PE to total phospholipids (1:99) was kept constant while the amount of *N*-Rh-PE was varied to give the indicated surface densities. The transfer efficiency for each vesicle preparation was determined as described in the text.

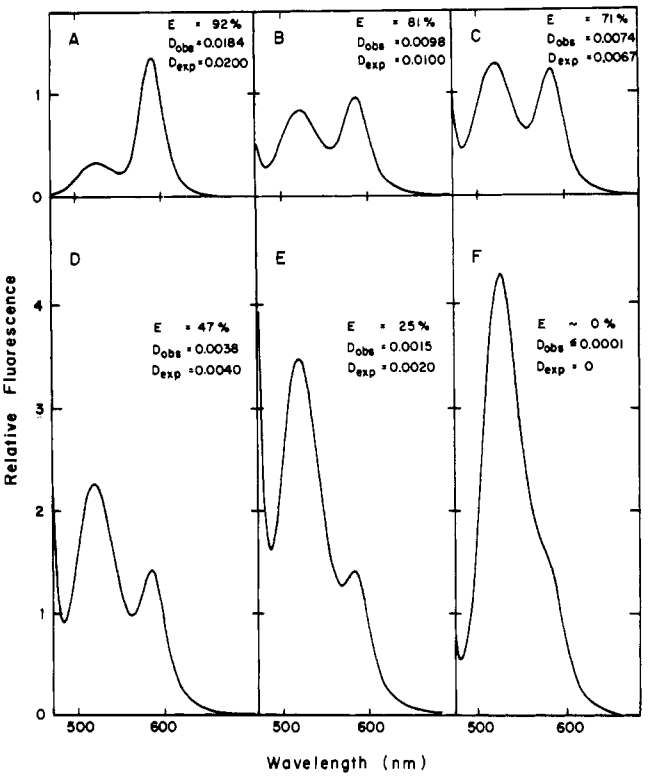

FIGURE 3: Effect of fusion between phosphatidylserine/*N*-NBD-PE/*N*-Rh-PE and pure phosphatidylserine vesicles on energy transfer. Phosphatidylserine/*N*-NBD-PE/*N*-Rh-PE (96:2:2) vesicles were mixed with varying amounts of pure phosphatidylserine vesicles, and $CaCl_2$ was added to induce fusion. The final calcium concentration was 5 mM. After 10 min at room temperature, emission spectra were obtained by exciting the samples at 450 nm as described under Experimental Procedures. The ratio of phosphatidylserine to phosphatidylserine/*N*-NBD-PE/*N*-Rh-PE vesicles was as follows: (A) 0; (B) 1; (C) 2; (D) 4; (E) 9; (F) detergent added, assumed to be ~∞. The concentration of phosphatidylserine/*N*-NBD-PE/*N*-Rh-PE vesicles was held constant at 250 μg/mL. Transfer efficiencies (E) were calculated from eq 1 and converted to observed surface densities (D_{obsd}) of *N*-Rh-PE by using the standard curve shown in Figure 2. The expected surface density (D_{exp}) of *N*-Rh-PE in samples B–E was determined by assuming complete randomization of all lipid present during the fusion process. D_{exp} for sample A is based on the ratio of *N*-Rh-PE to total lipid in the original phosphatidylserine/*N*-NBD-PE/*N*-Rh-PE vesicle preparation, while that of sample F, the detergent-solubilized mixture, was assumed to be nearly 0.

nm are observed (Figure 3A). This latter peak, characteristic for *N*-Rh-PE, arises from fluorescence energy transfer between the donor–acceptor pair. This preparation was then mixed with varying amounts of pure phosphatidylserine vesicles, and fusion was initiated by the addition of calcium. The spectral changes in accompanying fusion are 2-fold (Figure 3B–E). First, there is a marked reduction in the emission peak at 585 nm, and, in the extreme case represented by detergent solubilization of the vesicles, the shoulder seen at this wavelength is due solely to the direct excitation of *N*-Rh-PE (Figure 3F). Second, there is an increase in the fluorescence yield from *N*-NBD-PE. Together, these changes are indicative of a reduction in the efficiency of energy transfer between *N*-NBD-PE and *N*-Rh-PE. These results are consistent with fusion between the phosphatidylserine/*N*-NBD-PE/*N*-Rh-PE vesicles and the pure phosphatidylserine vesicles followed by lateral diffusion of the fluorescent lipids in the plane of the newly formed membrane. This latter process lowers the surface density of the energy acceptor and, thus, decreases the efficiency of energy transfer (see Figure 2) compared to the starting vesicles. Because it is possible to calculate energy transfer efficiencies from the spectra shown in Figure 3, estimates of the *N*-Rh-PE surface density after fusion can be obtained. The observed transfer efficiencies as well as the corresponding energy acceptor densities are given in Figure 3. As can be seen, the estimated surface densities of *N*-Rh-PE are nearly identical with those calculated from the assumption that the fusion process involved a complete randomization of all input lipid. From this, we conclude that under the conditions employed, formation of the final fusion product involves the coalescence of at least 10 of the input small, unilamellar vesicles (compare surface densities in parts A and E of Figure 3) and that essentially all of the input vesicles participate in such fusion events. It should be noted that the spectral changes shown in Figure 3 are absolutely dependent upon the addition of calcium. Finally, as expected, addition of this cation to a DOPC/*N*-NBD-PE/*N*-Rh-PE and pure DOPC vesicle mixture does not lead to changes in the efficiency of energy transfer (data not shown).

To provide further evidence that observed changes in the efficiency of energy transfer were due to vesicle–vesicle fusion, we analyzed vesicle mixtures by gel filtration both before and after the addition of calcium. In these experiments, it was necessary to reduce the phosphatidylserine content of the vesicles to 50 mol % in order to eliminate losses during the chromatography of samples after the addition of calcium. When a mixture of phosphatidylserine/phosphatidylcholine/*N*-NBD-PE/*N*-Rh-PE and phosphatidylserine/[^{14}C]phosphatidylcholine vesicles is chromatographed on Bio-Gel A15m, both vesicle populations elute at a position corresponding to small, unilamellar vesicles with a diameter of 250–500 Å (Figure 4A). The ratio of *N*-NBD-PE to *N*-Rh-PE as well as transfer efficiency is constant across the peak (Figure 4B). If this mixture is exposed to calcium prior to chromatography, both fluorescent lipids and the radiolabeled phosphatidylcholine elute at the void volume (Figure 5A). While the ratio of *N*-NBD-PE to *N*-Rh-PE across the peak is similar to that of the starting vesicles, the efficiency of energy transfer is significantly reduced for all fractions containing fluorescent lipid (Figure 5B). This analysis is consistent with vesicle–vesicle fusion and also provides further evidence that essentially all of the input vesicles are involved in the fusion process.

Similar experiments were performed by using a mixture of phosphatidylserine/phosphatidylcholine/*N*-NBD-PE/*N*-Rh-PE and pure phosphatidylcholine vesicles (data not shown).

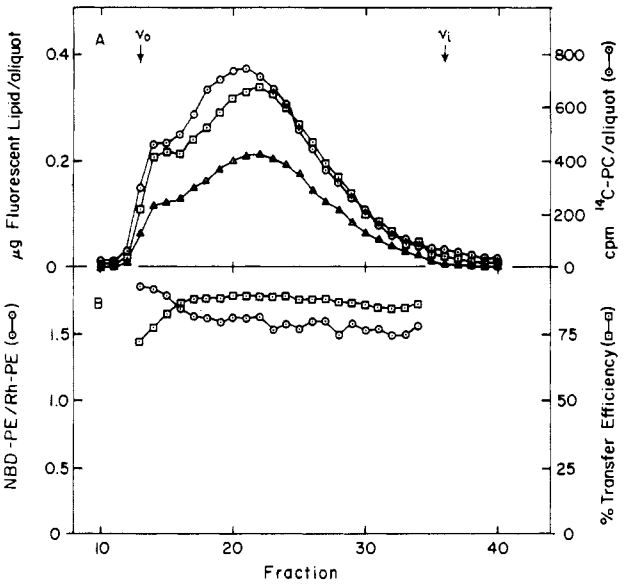

FIGURE 4: Analysis of vesicle mixtures by gel filtration prior to calcium-induced fusion. Phosphatidylserine/phosphatidylcholine/*N*-NBD-PE/*N*-Rh-PE (50:47:2:1) vesicles were mixed with phosphatidylserine/[^{14}C]phosphatidylcholine (1:1) vesicles and chromatographed on Bio-Gel A15m as described under Experimental Procedures. (A) Elution profile of [^{14}C]phosphatidylcholine (○), *N*-NBD-PE (□), and *N*-Rh-PE (△); (B) ratio of *N*-NBD-PE to *N*-Rh-PE (○) and transfer efficiency (□) for fractions containing vesicle lipid. All other conditions were as described under Experimental Procedures.

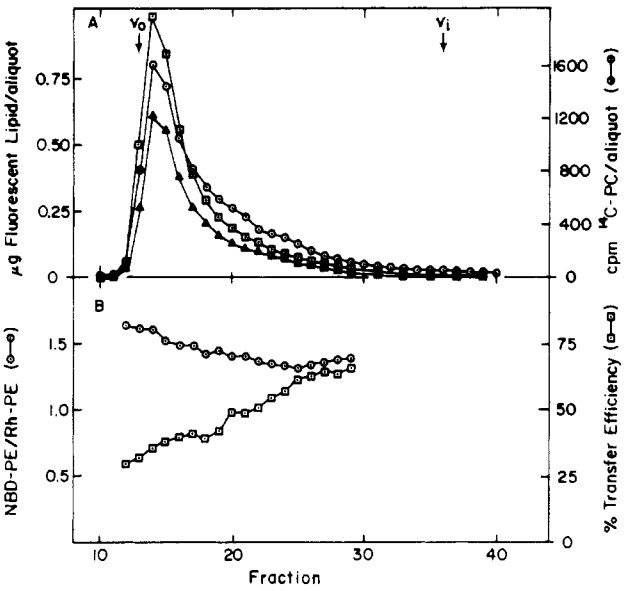

FIGURE 5: Analysis of vesicle mixtures by gel filtration after calcium-induced fusion. A vesicle mixture as described in Figure 4 was subjected to calcium-induced fusion and then analyzed by chromatography on Bio-Gel A15m. Fusion was stopped after 60 min at 37 °C by the addition of sufficient EDTA to chelate the calcium (20 mM) present in the reaction mixture. (A) Elution profile of [^{14}C]-phosphatidylcholine (○), *N*-NBD-PE (□), and *N*-Rh-PE (△); (B) ratio of *N*-NBD-PE to *N*-Rh-PE (○) and transfer efficiency (□) for fractions containing vesicle lipid.

In the absence of calcium, both populations cochromatograph on Bio-Gel A15m. However, after the addition of calcium, virtually all of the fluorescent lipid elutes at the void volume of the column while the behavior of the phosphatidylcholine vesicles is unaltered. In addition, the efficiency of energy transfer is nearly identical for both samples and is constant for all fractions containing fluorescent lipid. Thus, there appears to be no significant interaction between the two vesicle

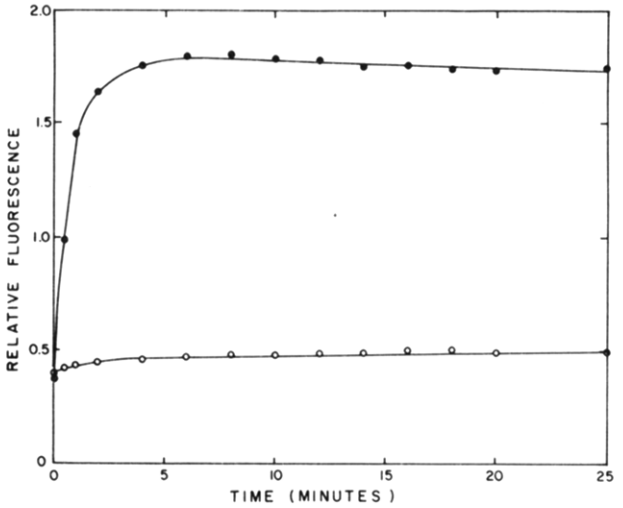

FIGURE 6: Ability of fused phosphatidylserine vesicles (cochleate lipid cylinders) to undergo subsequent calcium-induced fusion with small, unilamellar vesicles. Pure phosphatidylserine vesicles were preincubated for 10 min at room temperature in the presence or absence of 2 mM calcium. At zero time, aliquots of phosphatidylserine/*N*-NBD-PE/*N*-Rh-PE vesicles and calcium chloride were added in that order. Fusion between the vesicle populations was monitored by following the increase in emission at 530 nm as described in Figure 3. (●) Preincubation in the absence of calcium; (○) preincubation in the presence of calcium.

populations. This result demonstrates that an assay based on resonance energy transfer can be used to analyze the ability of distinct vesicle (or membrane) populations to fuse with each other.

Previous studies (Papahadjopoulos et al., 1975) have shown that "cochleate lipid cylinders" are the predominant product of the calcium-induced fusion of small, unilamellar phosphatidylserine vesicles. We have used fluorescence energy transfer to determine if this product can, itself, fuse with small unilamellar vesicles. In this experiment, pure phosphatidylserine vesicles were first incubated (10 min at 22 °C) with calcium until "fusion", as assessed by light scattering, was maximal. Then, phosphatidylserine/*N*-NBD-PE/*N*-Rh-PE vesicles were added to the incubation mixture. As can be seen in Figure 6, there was virtually no increase in fluorescence at 530 nm, suggesting that fusion between the two populations did not occur. This result also argues against calcium-induced exchange of fluorescent lipid between membranes composed of phosphatidylserine. Control experiments indicated that fusion could easily be detected when the two unilamellar vesicle preparations were premixed followed by the addition of calcium (Figure 6).

While resonance energy transfer can be used to study vesicle–vesicle interactions, this technique is particularly well suited for investigating the mechanisms of vesicle–cell interaction because of the high sensitivity of fluorescence measurements. Resonance energy transfer provides, in theory, a method by which vesicle–cell fusion and vesicle–cell adsorption–endocytosis can be distinguished. When vesicles containing *N*-NBD-PE and *N*-Rh-PE are incubated with cells, any fluorescent lipid molecules introduced into cells by fusion should freely intermix with lipid present in cellular membranes (initially the plasma membrane). This intermixing would effectively reduce the surface density of *N*-Rh-PE compared to the starting vesicles, resulting in a lower efficiency of energy transfer. However, if cellular uptake of fluorescent lipid is due to the adsorption or endocytosis of intact vesicles, there should be no reduction in energy transfer efficiency. On the basis of these criteria, we have studied vesicle–cell interactions by using resonance energy transfer.

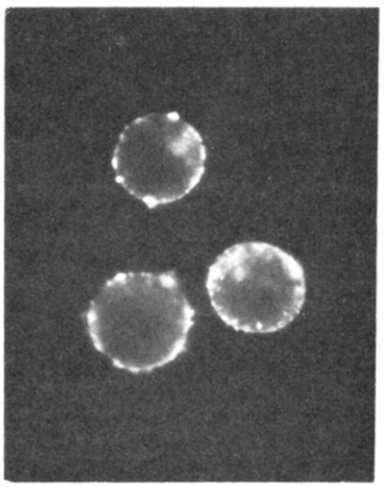

FIGURE 7: Fluorescence micrograph of V79 fibroblasts after vesicle treatment. Cells were incubated with phosphatidylserine/*N*-NBD-PE/*N*-Rh-PE (99:0.5:0.5) vesicles and washed as described under Experimental Procedures. Cells were then examined immediately with a Zeiss Universal microscope equipped with epiillumination for fluorescence as previously described (Struck & Pagano, 1980).

When phosphatidylserine/*N*-NBD-PE/*N*-Rh-PE (99:0.5:0.5) vesicles are incubated with V79 fibroblasts at 37 °C in the presence of 5 mM calcium, significant amounts of fluorescent lipid become cell associated and cannot be removed by repeated washings. Fluorescence emission spectra (λ_{ex} = 450 nm) of the starting vesicle preparation (similar to Figure 3A) and the washed, vesicle-treated cells (not shown) are essentially identical. Addition of detergent to both samples results in nearly identical enhancement of fluorescence at 530 nm from *N*-NBD-PE. Qualitatively similar results were obtained by using phosphatidylcholine/phosphatidylserine/*N*-NBD-PE/*N*-Rh-PE (49:50:0.5:0.5) or phosphatidylcholine/*N*-NBD-PE/*N*-Rh-PE (99:0.5:0.5) vesicles. Thus, the efficiency of energy transfer between cell-associated *N*-NBD-PE and *N*-Rh-PE is comparable to that seen in the starting vesicle preparation. This suggests that the vesicle-derived lipids are not able to freely intermix with those present in cellular membranes. From this observation, we conclude that the predominant mode of vesicle–cell interaction involves either the endocytosis of intact vesicles or the adsorption of vesicles at the cell surface. Fluorescence microscopy reveals that a large portion of the fluorescent lipid is present as aggregates at the cell surface (Figure 7), favoring the latter possibility.

It is possible to estimate the contribution of vesicle–cell adsorption to the total cellular uptake of vesicle lipid by using this system if one assumes that the cell-associated *N*-NBD-PE exists in two distinct pools. One pool, residing in intact vesicles, will be quenched by the energy acceptor, *N*-Rh-PE, to the same extent as in the starting vesicles. The second pool, which is free to diffuse in cellular membranes, would be largely relieved from such quenching since the surface density of *N*-Rh-PE would be significantly reduced because of intermixing of cellular and vesicle lipids. Although this model is not strictly correct, it does provide a means by which the maximal contribution of vesicle–cell adsorption to total vesicle uptake can be estimated. When two such distinct pools of *N*-NBD-PE exist, the fraction of *N*-NBD-PE present in intact vesicles should be equal to the ratio of the net increase in fluorescence from *N*-NBD-PE upon detergent solubilization of the vesicle-treated cells to the net increase in fluorescence from *N*-NBD-PE upon detergent solubilization of the starting vesicles after normalization to the same amount of *N*-NBD-PE

Table I: Fraction of Cellular Uptake as Adherent Vesicles[a]

vesicle type	no. of expt	vesicle lipid/10^7 cells (μg)	% adherence
DOPC/*N*-NBD-PE/ *N*-Rh-PE	3	4.2	82
		5.7	75
		4.5	86
phosphatidylserine/ *N*-NBD-PE/*N*-Rh-PE	3	8.1	72
		7.0	91
		4.2	80
phosphatidylserine/ DOPC/*N*-NBD-PE/ *N*-Rh-PE	5	3.6	70
		5.7	85
		4.9	88
		6.1	82
		3.7	76

[a] V79 fibroblasts (10^7/mL) were incubated with either 1 mg/mL DOPC/*N*-NBD-PE/*N*-Rh-PE (99:0.5:0.5), 200 μg/mL phosphatidylserine/*N*-NBD-PE/*N*-Rh-PE (99:0.5:0.5), or 200 μg/mL phosphatidylserine/DOPC/*N*-NBD-PE/*N*-Rh-PE (50:49:0.5:0.5) vesicles for 30 min at 37 °C in the presence of 5 mM Ca^{2+}. After extensive washings, the fraction of *cell-associated* vesicle lipid present as adherent vesicles was estimated as described in the text.

in the two samples (this ratio is termed the enhancement ratio). That this relationship should exist is illustrated by the following experiment. A series of mixtures were prepared from stock DOPC/*N*-NBD-PE/*N*-Rh-PE, DOPC/*N*-NBD-PE, and DOPC/*N*-Rh-PE vesicle preparations. In these mixtures, the ratio of *N*-NBD-PE to *N*-Rh-PE and the absolute amount of *N*-NBD-PE were held constant, but the fraction of the total *N*-NBD-PE which was present in the DOPC/*N*-NBD-PE/*N*-Rh-PE vesicles was varied from 0 to 10. This fraction would be analogous to the pool of cell-associated *N*-NBD-PE residing in intact vesicles and, thus, quenched by *N*-Rh-PE. The remainder of the *N*-NBD-PE exists in DOPC/*N*-NBD-PE vesicles and would not be subject to such quenching, being equivalent to the pool of cell-associated *N*-NBD-PE free to diffuse in cellular membranes. Emission spectra of each mixture (λ_{ex} = 450 nm) were obtained in the presence and absence of detergent. The net enhancement of fluorescence at 530 nm (from *N*-NBD-PE) due to the addition of detergent was then calculated. The ratio of this value to that obtained with the sample containing only DOPC/*N*-NBD-PE/*N*-Rh-PE vesicles was then plotted as a function of total *N*-NBD-PE present in the DOPC/*N*-NBD-PE/*N*-Rh-PE vesicles for each mixture. This relationship was found to be linear with a slope of 1.0 (data not shown). Thus, according to our simplified model, when the relative enhancements of *N*-NBD-PE fluorescence obtained by detergent solubilization of vesicle-treated cells and the starting vesicles are equal (enhancement ratio = 1.0), 100% of the cellular uptake of vesicle lipid is due to vesicle–cell adsorption. If no enhancement was observed upon detergent treatment of the cells (ratio = 0), then stable adsorption did not occur. For enhancement ratios between 0 and 1, the fraction of intact vesicles adsorbed to the cell surface would be equal to the enhancement ratio. By use of this type of analysis, the predominant mode of vesicle–cell interaction appears to involve the adsorption of intact vesicles at the surface of the cell for all vesicle types tested (Table I).

Discussion

In this report, we have described an assay for vesicle–vesicle fusion which is based on resonance energy transfer. The fluorescent lipid analogues *N*-NBD-PE and *N*-Rh-PE serve as the energy donor and energy acceptor, respectively. Efficient energy transfer between these fluorescent lipids is observed in unilamellar lipid vesicles containing ~1 energy acceptor per 100 phospholipid molecules. Upon fusion of these vesicles with a second vesicle population containing no fluorescent lipid, the efficiency of energy transfer is reduced since lateral diffusion of the energy acceptor in the plane of the newly formed membrane effectively lowers its surface density. Since one can calculate acceptor surface densities from measured transfer efficiencies, it is possible to estimate the extent to which the energy acceptor is diluted during fusion and, thus, the extent of fusion between vesicle populations. Such estimates, in general, are not possible with assays which measure the mixing of intravesicular compartments during fusion because of the often rapid leakage of vesicle contents during this process (Portis et al., 1979; Wilschut & Papahadjopoulos, 1979). In fact, it has recently been shown that calcium-induced leakage of vesicle contents can occur without significant vesicle–vesicle fusion (Vanderwerf & Ullman, 1980). Thus, assays of the type presented here and elsewhere (Keller et al., 1977; Deamer & Uster, 1980; Vanderwerf & Ullman, 1980) should prove complementary to those used in previous studies on membrane fusion.

Several control experiments demonstrated that the observed changes in the efficiency of resonance energy transfer require membrane fusion and were not the result of unrelated processes such as lipid exchange or lipid transfer. (i) Simply mixing vesicles containing both *N*-NBD-PE and *N*-Rh-PE with a second vesicle population devoid of fluorescent lipid did not lead to changes in transfer efficiency. Such changes were only observed after the addition of calcium which is required for fusion between phosphatidylserine-containing vesicles (Maeda & Ohnishi, 1974; Miller & Racker, 1976; Papahadjopoulos et al., 1976, 1977; Newton et al., 1978; Hoekstra et al., 1979; Portis et al., 1979; Wilschut & Papahadjopoulos, 1979). (ii) Upon addition of calcium to mixtures of phosphatidylserine/*N*-NBD-PE/*N*-Rh-PE and pure phosphatidylcholine vesicles, there was an immediate increase in turbidity although no change in the efficiency of resonance energy transfer was observed. This was due, presumably, to the fact that only the phosphatidylserine-containing vesicles present in the incubation mixture had undergone fusion, a fact confirmed by gel filtration. Fusion between the phosphatidylserine/*N*-NBD-PE/*N*-Rh-PE vesicles in this mixture would not decrease the surface density of the energy acceptor, and, thus, no change in transfer efficiency would be expected. (iii) Addition of calcium (or cadmium) to mixtures of small unilamellar vesicles composed of DOPC/*N*-NBD-PE and DOPC/*N*-Rh-PE suspended in 20% (w/v) poly(ethylene glycol) caused extensive vesicle aggregation but did not result in resonance energy transfer between the two fluorophores (unpublished observations). The results in (i), (ii), and (iii) suggest that while phospholipid exchange between lipid vesicles is known to occur (Martin & MacDonald, 1976; Duckwitz-Peterlein et al., 1977; Roseman & Thompson, 1980; Nichols & Pagano, 1981), this process does not contribute significantly to the observed reduction in transfer efficiency reported here. (ii) and (iii) suggest that neither calcium alone nor calcium bound to the vesicle surface has an appreciable effect on energy transfer between *N*-NBD-PE and *N*-Rh-PE. Thus, we conclude that this assay is capable of determining the ability of two distinct vesicle populations to fuse with each other.

We have assessed the ability of three distinct vesicle types to fuse with the plasma membrane of intact cells by comparing the degree of quenching of *N*-NBD-PE by *N*-Rh-PE in the starting vesicles with that observed in the cells after vesicle treatment. If vesicle–cell fusion was predominant, one would expect that the quenching of cell-associated *N*-NBD-PE would have been significantly reduced. However, in all cases, cell-

associated *N*-NBD-PE was quenched to an extent similar to that present in the vesicles. From this, we conclude that the majority of the vesicle lipid transferred to cells resided in intact vesicles. Fluorescence microscopy suggests that this was due to adsorption of vesicles to the cell surface. Thus, even with vesicles which fuse efficiently with each other, apparent vesicle–cell fusion occurs only to a limited extent. However, recent studies from this laboratory suggest that the processes of vesicle–cell adsorption and fusion are strongly dependent upon vesicle concentration and that at sufficiently low concentrations, vesicle–cell fusion is the dominant mode of interaction (R. E. Pagano, O. C. Martin, and D. K. Struck, unpublished observations). Finally, it should be pointed out that the results in the present paper do not exclude a limited extent of vesicle–cell fusion which could account for the previously described vesicle-mediated delivery of biologically active molecules to the cytoplasm of intact cells (Papahadjopoulos et al., 1974; Poste & Papahadjopoulos, 1976).

Of course, similar assays for membrane fusion can be developed by using donor–acceptor pairs other than *N*-NBD-PE and *N*-Rh-PE [see Keller et al. (1977), Fung & Stryer (1978), Gibson & Loew (1979), Deamer & Uster (1980), and Vanderwerf & Ullman (1980)]. This will allow a degree of flexibility in studying membrane fusion which is not possible with most other techniques and which will hopefully prove to be useful in investigating membrane fusion in systems of greater complexity.

References

Deamer, D. W., & Uster, P. S. (1980) in *Introduction of Macromolecules into Viable Mammalian Cells*, pp 205–220, Alan R. Liss, Inc., New York.

Duckwitz-Peterlein, G., Eilenberger, G., & Overath, P. (1977) *Biochim. Biophys. Acta 469*, 311–325.

Forster, T. (1949) *Z. Naturforsch., A 4A*, 321–327.

Fung, B. K.-K., & Stryer, L. (1978) *Biochemistry 17*, 5241–5248.

Gibson, G. A., & Loew, L. M. (1979) *Biochem. Biophys. Res. Commun. 88*, 135–140.

Hoekstra, D., Yaron, A., Carmel, A., & Scherphof, G. (1979) *FEBS Lett. 106*, 140–144.

Keller, P. M., Person, S., & Snipes, W. (1977) *J. Cell Sci. 28*, 167–177.

Maeda, T., & Ohnishi, S. I. (1974) *Biochem. Biophys. Res. Commun. 60*, 1509–1512.

Martin, F. J., & MacDonald, R. C. (1976) *Biochemistry 15*, 321–327.

Miller, C., & Racker, E. (1976) *J. Membr. Biol. 26*, 319–333.

Newton, C., Pangborn, W., Nir, S., & Papahadjopoulos, D. (1978) *Biochim. Biophys. Acta 506*, 281–287.

Nichols, J. W., & Pagano, R. E. (1981) *Biochemistry 20*, 2783–2789.

Pagano, R. E., & Weinstein, J. N. (1978) *Annu. Rev. Biophys. Bioeng. 7*, 435–468.

Papahadjopoulos, D., Poste, G., & Mayhew, E. (1974) *Biochim. Biophys. Acta 363*, 404–418.

Papahadjopoulos, D., Vail, W. J., Jacobson, K., & Poste, G. (1975) *Biochim. Biophys. Acta 394*, 483–491.

Papahadjopoulos, D., Hui, S., Vail, W. J., & Poste, G. (1976) *Biochim. Biophys. Acta 448*, 245–264.

Papahadjopoulos, D., Vail, W. J., Newton, D., Nir, S., Jacobson, K., Poste, G., & Lazo, R. (1977) *Biochim. Biophys. Acta 465*, 579–598.

Papahadjopoulos, D., Poste, G., & Vail, W. J. (1979) *Methods Membr. Biol. 10*, 1–119.

Portis, A., Newton, C., Pangborn, W., & Papahadjopoulos, D. (1979) *Biochemistry 18*, 780–790.

Poste, G., & Papahadjopoulos, D. (1976) *Nature (London) 261*, 699–701.

Roseman, M., & Thompson, T. E. (1980) *Biochemistry 19*, 439–444.

Struck, D. K., & Pagano, R. E. (1980) *J. Biol. Chem. 255*, 5405–5410.

Stryer, L. (1978) *Annu. Rev. Biochem. 47*, 819–832.

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

Wilschut, J., & Papahadjopoulos, D. (1979) *Nature (London) 281*, 690–692.