Reid, K. S. C., Lindley, P. F., & Thornton, J. M. (1985) *FEBS Lett. 190*, 209–213.
Ross, P. D., & Subramanian, S. (1977) *Biochem. Biophys. Res. Commun. 77*, 1217–1223.
Russu, I. M., Lin, A. K.-L. C., Yang, C.-P., & Ho, C. (1986) *Biochemistry 25*, 808–815.
Sheldrick, G. M. (1978) in *Computing in Crystallography* (Schenk, H., Olthof-Hazekamp, R., van Koningsveld, H., & Bassi, G. C., Eds.) pp 34–42, Delft Univeristy, Delft, The Netherlands.
Singh, J., & Thornton, J. M. (1985) *FEBS Lett. 191*, 1–6.
Steed, J. M., Dixon, T. A., & Klemperer, W. J. (1979) *J. Chem. Phys. 70*, 4940–4944.
Thomas, K. A., Smith, G. M., Thomas, T. B., & Feldman, R. J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4843–4847.
Votano, J. R., & Rich, A. (1985) *Biochemistry 24*, 1966–1970.
Votano, J. R., Gorecki, M., & Rich, A. (1977) *Science (Washington, D.C.) 196*, 1216–1218.
Votano, J. R., Altman, J., Wilchek, M., Gorecki, M., & Rich, A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3190–3194.
Wang, A. H.-J., & Burley, S. K. (1987a) *Acta Crystallogr., Sect. C: Cryst. Struct. Commun.* (in press).
Wang, A. H.-J., & Burley, S. K. (1987b) *Acta Crystallogr., Sect. C: Cryst. Struct. Commun. 43*, 1011–1012.
Wyckoff, R. W. G. (1969) *Crystal Structures, The Structure of Benzene Derivatives*, Interscience, New York.

Determination of Lipid Asymmetry in Human Red Cells by Resonance Energy Transfer†

Jerome Connor and Alan J. Schroit*
Department of Cell Biology, The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston, Houston, Texas 77030

Received February 13, 1987; Revised Manuscript Received April 8, 1987

ABSTRACT: This report describes the application of a resonance energy transfer assay to determine the transbilayer distribution of 7-nitro-2,1,3-benzoxadiazol-4-yl (NBD)-labeled lipid analogues. The validity of this technique was established by determining the relationship between the distance of separation of lissamine rhodamine B labeled phosphatidylethanolamine (*N*-Rho-PE) acceptor lipid and NBD-labeled donor lipid and energy transfer efficiency. By determination of the distance between probes at 50% transfer efficiency (R_0), the distance between fluorophores distributed symmetrically (outer leaflet label) and asymmetrically in artificially generated vesicles was determined. Calculation of the average distance between probes revealed a 14-Å difference between NBD-lipid and *N*-Rho-PE localized in the same leaflet and in opposing leaflets, respectively. Application of this technique to the study of the transbilayer distribution of NBD-lipid in human red blood cells (RBC) showed that exogenously supplied NBD-phosphatidylserine (NBD-PS) was selectively transported to the inner leaflet, whereas NBD-phosphatidylcholine remained in the outer leaflet. In contrast, pretreatment of the RBC with diamide (a SH cross-linking reagent) blocked the transport of NBD-PS. The absence or presence of NBD-PS in the outer leaflet was independently verified by employing "back-exchange", trinitrobenzenesulfonic acid derivatization, and decarboxylation with PS decarboxylase experiments. These control experiments yielded results which confirmed the lipid distributions determined by the resonance energy transfer assay.

It has been established that anionic phospholipids such as phosphatidylethanolamine (PE)[1] and phosphatidylserine (PS) are asymmetrically distributed in biological membranes (Rothman & Lenard, 1977; Op den Kamp, 1979). In red blood cells (RBC), the aminophospholipids preferentially reside in the inner leaflet, while the choline phospholipids are predominantly localized in the outer leaflet (Verkleij et al., 1973; Gordesky et al., 1975). Both the biogenesis and the maintenance of this phospholipid asymmetry appear to be important components of homeostasis since the translocation of lipids between leaflets has been implicated in several important biological processes. For example, translocation of PS in activated platelets (Bevers et al., 1983) and sickle RBC (Chiu et al., 1979; Lubin et al., 1981) has been shown to be important in clotting mechanisms (Bevers et al., 1982) and in the pathogenesis of sickle cell anemia (Schwartz et al., 1985; Franck et al., 1985), respectively. In addition, PS has been shown to be an important factor in macrophage recognition of both artificial (Schroit & Fidler, 1982) and biological membranes (Tanaka & Schroit, 1983; Schroit et al., 1985). Since monitoring unlabeled endogenous lipid translocation (inner to outer leaflet) is difficult, alternative methods have recently been introduced that follow the fate of exogenous lipids inserted into

† This work was supported in part by Developmental Fund Grant 175416 from The University of Texas M. D. Anderson Hospital and Tumor Institute at Houston and by National Institutes of Health Grant CA-40149.

[1] Abbreviations: DOPC, dioleoylphosphatidylcholine; HEPES saline, 145 mM NaCl, 5 mM KCl, 20 mM HEPES, and 10 mM glucose; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; LUV, large unilamellar vesicle(s); *N*-Rho-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; NBD-PC and -PS, 1-oleoyl-2-[[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]phosphatidylcholine and -phosphatidylserine, respectively; PSdC, phosphatidylserine decarboxylase; RBC, human red blood cells; SUV, small unilamellar vesicle(s); TLC, thin-layer chromatography; TNBS, trinitrobenzenesulfonic acid; Tnp, trinitrophenyl; ^{125}I-PE, *N*-[3-(3-[^{125}I]iodo-4-hydroxybenzyl)-propionyl]dipalmitoylphosphatidylethanolamine; RET, resonance energy transfer.

0006-2960/87/0426-5099$01.50/0

the outer leaflet of RBC. These systems are, however, based on the inaccessibility of the lipids to a variety of agents and do not directly determine true lipid distribution and the actual presence of PS in the inner bilayer leaflet. Examples of this are the inability to (1) "back-exchange" NBD-PS to acceptor membranes (Schroit et al., 1985), (2) reduce spin-labeled PS by ascorbate (Seigneuret & Devaux, 1984), and (3) produce [^{14}C]lyso-PS from [^{14}C]PS by phospholipase A_2 (Tilley et al., 1986) after insertion of these lipids into RBC.

In this study, we have developed a system which monitors the distribution of exogenously added fluorescent analogues of PS and PC by employing the NBD/rhodamine resonance energy transfer (RET) system characterized by Struck et al. (1981). As predicted by Fung and Stryer (1978), we demonstrate in a model membrane system that NBD-labeled lipid displays very different energy transfer profiles dependent upon its transbilayer distribution relative to a rhodamine-labeled acceptor molecule. Thus, strong RET is observed when both fluorophores are in the same leaflet whereas significant decreases in RET efficiency occur when identical amounts of both probes are asymmetrically distributed. By extending this model system to erythrocytes, we show that exogenously supplied NBD-PS translocates from the outer to inner leaflet and adopts a preferential asymmetric distribution in the RBC membrane.

Experimental Procedures

Materials and Routine Procedures. DOPC, NBD-PC, and *N*-Rho-PE were purchased from Avanti Polar Lipids (Birmingham, AL). NBD-PS was synthesized from NBD-PC by phospholipase D catalyzed base exchange in the presence of L-serine (Comfurius & Zwaal, 1977) and purified by thin-layer chromatography (TLC). ^{125}I-Labeled PE was prepared as previously described (Schroit, 1982). All lipids were purified/analyzed by TLC on heat-activated silica gel 60 thin-layer plates (Merck) using $CHCl_3$/methanol/acetone/acetic acid/water (5:1:2:1:0.5). PSdC was prepared by sonication of *Escherichia coli* JA 200 pLC8-47 (obtained from Dr. Barbara Bachmann, Yale University Gene Bank, New Haven, CT), an *E. coli* strain bearing the hybrid plasmid CO1E1-psd$^+$ (Clarke & Carbon, 1976) for the overproduction of PSdC (Tayhach et al., 1979) as described previously (Denkins & Schroit, 1986). PSdC activity was determined by its ability to decarboxylate dispersions of NBD-PS at 37 °C as assessed by the appearance of NBD-PE on thin-layer plates and was found to be ~300 units/mg of protein (1 unit = 1 nmol of NBD-PS decarboxylated per minute at 37 °C). Human red blood cells (RBC) in heparinized saline were obtained by venipuncture, pelleted by centrifugation, and washed 3 times with 10 volumes of HEPES saline. The cells were then labeled with ^{51}Cr, washed, and resuspended to 2×10^7 RBC/mL. Diamide (Sigma)-treated RBC were prepared by incubating the ^{51}Cr-labeled RBC in the presence of 5 mM diamide for 30 min at 37 °C, followed by three washes with HEPES saline. Steady-state fluorescence was measured at 535 nm (λ_{ex} 468 nm with a 468-nm band-pass filter) for NBD-labeled lipids and at 595 nm (λ_{ex} 535 nm) for *N*-Rho-PE with a Farrand MK II spectrophotofluorometer at room temperatures using 10-nm slits. Lipid concentrations were determined according to Ames and Dubin (1960).

Preparation of Symmetric Vesicles. (A) Vesicles Containing N-Rho-PE and NBD-PC in Both Leaflets. DOPC LUV containing the indicated amounts of symmetrically distributed *N*-Rho-PE and NBD-PC and trace amounts of ^{125}I-PE (to facilitate monitoring of vesicle lipid concentrations) were prepared by resuspending mixed dried lipids in HEPES saline to 1 mg of lipid/mL, followed by extrusion through 0.1-μm polycarbonate filters (Hope et al., 1983).

(B) Vesicles Containing N-Rho-PE and NBD-PC in the Outer Leaflets. Insertion of the fluorescent lipids into the outer leaflet was accomplished by injecting, under constant mixing, appropriate amounts of NBD-PC in ethanol (final ethanol concentration <1%) to preformed DOPC/^{125}I-PE LUV (1 mg of lipid/mL). The suspension was dialyzed against 4 L of HEPES saline for 24 h and the procedure repeated with *N*-Rho-PE. After additional dialysis, aliquots of the vesicles were analyzed for incorporation of NBD-PC and *N*-Rho-PE by quantifying both NBD and rhodamine fluorescence in the presence of 1% Triton X-100 by comparison to standard curves generated from known amounts of NBD-PC and *N*-Rho-PE. The presence of NBD-PC exclusively in the outer leaflet was confirmed by the ability to transfer all of the NBD-lipid to an acceptor SUV population.

Preparation of Asymmetric Vesicles. LUV with a preferential distribution of NBD-PC in the inner leaflet and *N*-Rho-PE in the outer leaflet were prepared as follows: LUV with a symmetric distribution of NBD-PC were made from DOPC/NBD-PC as described above. These donor LUV were then mixed with a 5-fold excess (w/w) of a homogeneous population of acceptor DOPC SUV which had been presized on Bio-Gel A-15M. Following a 24-h incubation at 4 °C, the mixed vesicle populations were separated by chromatography on Bio-Gel A-15M (1.1 × 45 cm). Fractions were collected and monitored for fluorescence and radiation by scintillation counting. The LUV (containing NBD-PC and ^{125}I-PE) eluted with the void volume, while the SUV (containing NBD-PC exchanged from the outer leaflet of the donor LUV) eluted as a distinct, included peak. The presence of NBD-PC only in the inner leaflet was verified by two methods. First, analysis of NBD-PC distribution in the excluded (LUV) and included (SUV) peaks revealed that the fluorescent lipid was distributed equally between both populations. Second, another attempt to remove NBD-PC from the preexchanged LUV to back-exchange to a fresh population of acceptor vesicles containing only *N*-Rho-PE (1 mol %) failed to quench the NBD-PC (λ_{ex} 468 nm; λ_{em} 535 nm), nor was *N*-Rho-PE fluorescence (λ_{em} 595 nm) sensitized by NBD-PC emission. If any NBD-PC had remained on the outer leaflet of the LUV, its exchange into the *N*-Rho-PE-containing vesicles would have been detected. After adjustment of the LUV lipid concentration (based on ^{125}I-PE), *N*-Rho-PE was added to the outer leaflet by ethanol injection as described above. The presence of NBD-PC exclusively in the inner leaflet was confirmed by the inability to transfer the NBD-lipid to an acceptor SUV population.

Lipid/RBC Incubations and Quantification of Lipid Uptake. ^{51}Cr-Labeled RBC (2×10^7 cells/mL) were added with mixing to appropriate concentrations of NBD-PC or NBD-PS in ethanol (1:100 ethanol/aqueous). The RBC/lipid suspension was incubated at 37 °C for 30 min with gentle agitation and then washed twice in HEPES saline. These NBD-containing RBC were then added to appropriate concentrations of *N*-Rho-PE in ethanol. The suspension was again incubated for 30 min at 37 °C and washed. Lipid uptake in the presence of 1% Triton X-100 was determined by measuring the relative NBD and rhodamine fluorescence in aliquots of the RBC both before and after washing.

Resonance Energy Transfer in Model Membranes. Resonance energy transfer efficiency (E) of the various vesicle populations was determined by assessing the degree of donor (NBD-lipid) quenching. Briefly, vesicles (20 μM DOPC)

containing constant concentrations of NBD-PC (1 mol %) and increasing concentrations of *N*-Rho-PE (0.25–3.0 mol %) or constant concentrations of *N*-Rho-PE (1 mol%) and increasing concentrations of NBD-PC (0.25–3.0 mol %) were prepared, and the relative NBD-PC fluorescence was determined in the absence and presence of 1% Triton X-100 [corrected for sample dilution and detergent-mediated quenching effects (Tanaka & Schroit, 1983)].

Percent E was calculated by using the equation:

$$E = (1 - F/F_\tau) \times 100 \quad (1)$$

where the relative fluorescence intensity is measured in intact vesicles (F) and after the addition of detergent (F_τ) (Fung & Stryer, 1978; Struck et al., 1981).

Resonance Energy Transfer in RBC. RBC containing either 1 mol % NBD-PC or 1 mol % NBD-PS (based on 3×10^{-9} mol of lipid phosphate/2×10^7 RBC) were measured for energy transfer efficiency after the addition of varying concentrations of *N*-Rho-PE (0.25% to 3.0 mol %) using the same technique outlined for the model membranes. Emission spectra of the RBC were obtained from RBC containing 1% NBD-lipids and 2% *N*-Rho-PE by excitation at 468 nm and scanning the emission from 500 to 620 nm at a 10 nm/min scan rate. Blank spectra were obtained by scanning control RBC populations containing identical amounts of *N*-Rho-PE only. Data acquisition and spectra subtraction were controlled by an Apple IIe computer that sampled and digitized data from the fluorometer at 0.3-nm intervals.

Determination of NBD-PS Asymmetry in RBC with PSdC. To determine the fraction of NBD-PS localized in the external leaflet of the cells, washed NBD-PS-treated ^{51}Cr-labeled RBC were incubated with PSdC for 1 h at 37 °C [2.5×10^8 RBC (175 μg of PSdC)$^{-1}$ mL^{-1}]. The cells were then washed 3 times with ice-cold phosphate-buffered saline and lysed with 5 mM phosphate buffer, pH 8.0. The lysate was centrifuged at 15000*g*, and the pellets were extracted with $CHCl_3$/MeOH/0.1 N HCl (2:2:1.8). The organic phase was dried, and the fluorescent lipids were separated by TLC. The regions corresponding to NBD-PS (lipid inaccessible to PSdC; lipid residing in the inner leaflet) and NBD-PE (decarboxylated NBD-PS; lipid residing in the outer leaflet) were scraped, extracted, and quantified by fluorescence. PSdC did not induce cell lysis since ^{51}Cr release (<3%) was identical with that of control cells not incubated with PSdC.

Determination of NBD-PS Asymmetry in RBC with TNBS. NBD-PS localized in the outer leaflet of the RBC was derivatized with TNBS by using the nonpermeable conditions described by Gordesky et al. (1975). Briefly, 1.4×10^9 ^{51}Cr-labeled RBC were resuspended in 10 mL of isotonic buffer containing 120 mM $NaHCO_3$, 40 mM NaCl, and 1.5 mM TNBS, pH 8.6, for 90 min at 20 °C. Unreacted TNBS was then removed by washing the cells in the same buffer without TNBS. The cells were then lysed with 12 mM $NaHCO_3$ and 4 mM NaCl and centrifuged at 15000*g*. The hemoglobin was collected, and the pellets were extracted with $CHCl_3$/MeOH/0.1 N HCl (2:2:1.8). The organic phase was dried, and known amounts of phospholipid (as determined by P_i analysis) were separated by TLC. The region corresponding to NBD-PS (unreacted inner leaflet lipid) was scraped, and quantified by fluorescence using an appropriately generated standard curve, and normalized to the amount of phospholipid spotted. Tnp-NBD-PS could not be directly determined since Tnp derivatization quenched the probes' fluorescence below the limit of reliable fluorescence measurements.

The extent of TNBS penetration was estimated by ^{51}Cr release and by assessing the amount of Tnp-hemoglobin formed by using the nonpenetrating conditions described above, as compared to the amount of Tnp-hemoglobin found in RBC labeled with TNBS in hypotonic buffer (12 mM $NaHCO_3$, 4 mM NaCl, and 1.5 mM TNBS). Tnp-hemoglobin was quantified by measuring the absorbance of acidified acetone-precipitated, acid-hydrolyzed, ether-extracted Tnp-amino acids at 340 nm as described previously (Gordesky et al., 1975). The results of these assays indicated that TNBS treatment did not induce cell lysis (^{51}Cr release <3% and equal to control nontreated cells) nor did it permeate the membrane (<3% Tnp-hemoglobin formed compared to the amount of Tnp-hemoglobin obtained by using lysed controls).

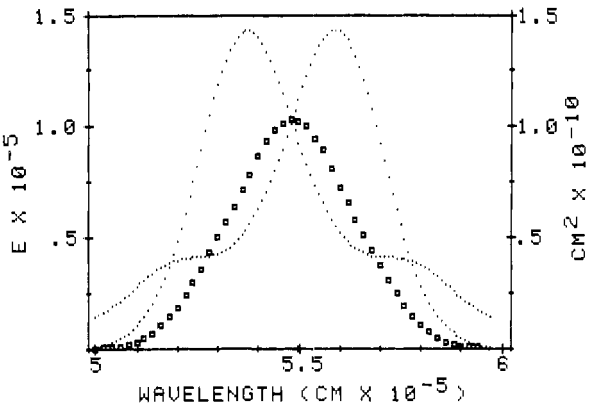

FIGURE 1: Determination of the spectral overlap integral for NBD-PC/*N*-Rho-PE. The absorption spectra of *N*-Rho-PE in ethanol (1 μM) were determined with a Gilford Response spectrophotometer and plotted as the extinction coefficient (E) vs. wavelength. The "spiegelkurve" of *N*-Rho-PE adsorption was obtained by plotting $E(2\lambda_0 - \lambda)$ where λ_0 (5.46 cm^{-5}) is the average wavelength of the absorption (5.59 cm^{-5}) and emission (5.32 cm^{-5}) maxima of *N*-Rho-PE and NBC-PC, respectively [see Forster (1948)]. To obtain J, the product of the two peaks (■) was integrated.

RESULTS

Determination of Probe Separation in Model Membranes by Resonance Energy Transfer Efficiency. The efficiency of energy transfer has been shown to be proportional to the inverse of the sixth power of the distance between the fluorophores (Fung & Stryer, 1978). In theory, separation distances of about 50–75 Å, with resolution approaching 1 Å, can be determined by employing efficient donor/acceptor pairs with a known R_0 value (the distance between the donor and acceptor at which 50% transfer efficiency is observed). Thus, if the R_0 for a particular donor/acceptor pair is known, the actual distance between the fluorophores (R) can be calculated from the measured transfer efficiencies by the equation derived from Fung and Stryer (1978):

$$R = [(0.5/E)^{1/6}]R_0 \quad (2)$$

where only the steady-state fluorescence intensity of the energy donor in the absence (f_0) and presence of acceptors (f) need be measured. R_0 (in angstroms) is given by

$$R_0 = (JK^2Q_0n^{-4})^{1/6}(9.79 \times 10^3) \quad (3)$$

where the random orientation factor $K^2 = {}^2/_3$, the refractive index of the medium $n = 1.5$ (Fung & Stryer, 1978), and Q_0, the quantum yield of the energy donor, was estimated[2] to be

[2] It is known that the quantum yield of NBD-labeled lipids in a hydrophobic environment is high, although definitive values have not been published. We have therefore elected to use a relatively high value of 0.75. It should be noted, however, that since Q has only minimal effects on R_0, Q values of 0.9 through 0.6 result in R_0 changes of <3 Å, with corresponding changes in R < 0.3 Å and net changes in distance between the symmetric and asymmetric vesicles <1 Å.

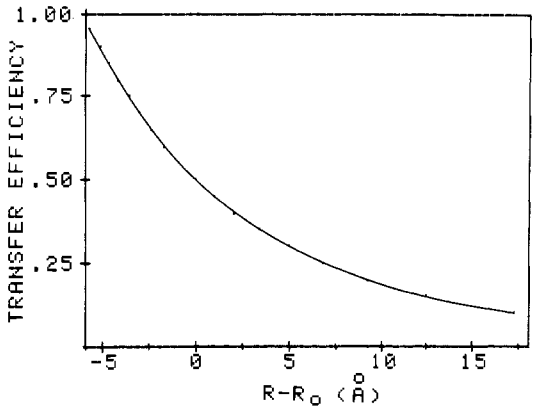

FIGURE 2: Calculated distance of probe separation from R_0 as a function of RET efficiency. The change in distance from R_0 (55.8) was calculated from eq 3.

0.75. J was determined from the integral of the spectral overlap (Figure 1) of *N*-Rho-PE absorption and NBD-PC emission as described by Forster (1948) and was found to be 3.473×10^{-13} cm^3/M, resulting in a calculated R_0 of 55.8 Å. By use of eq 2, the predicted deviation of R from R_0 can be calculated as a function of transfer efficiency (Figure 2). Thus, changes in energy transfer efficiency determined in model membrane systems can be correlated with the actual distance between probes.

To test the feasibility of employing RET to detect lipids distributed asymmetrically in a bilayer membrane, vesicles containing increasing amounts of *N*-Rho-PE and constant amounts NBD-PC in both leaflets, in the outer leaflet, and in opposing leaflets were prepared (Figure 3a). As can be seen, the transfer efficiency (the ability of *N*-Rho-PE to quench NBD-PC) depended on the density of the energy acceptor in all three systems. When NBD-PC was mixed uniformly throughout the bilayer, increasing amounts of *N*-Rho-PE resulted in a concomitant increase in RET efficiency, achieving more than 90% efficiency at 2 mol %. Similarly, when the two fluorophores occupied a single leaflet (both NBD-PC and *N*-Rho-PE in the outer leaflet), the *N*-Rho-PE was still capable of strong quenching of NBD-PC emission, analogous to the quenching obtained with vesicles containing the homogeneously distributed probes. In contrast, the extent of energy transfer was significantly lower in the vesicles containing asymmetrically distributed (NBD-PC and *N*-Rho-PE in opposing leaflets) fluorescent lipids (Figure 3a). At 2 mol % *N*-Rho-PE, <25% energy transfer efficiency was observed. We conclude, therefore, that the change in distance created by moving the energy donor from an adjacent position in the same leaflet to opposed leaflets of the same membrane results in a measurable loss of energy transfer to the energy acceptor.

The relationship of transfer efficiency to donor probe density in vesicles containing symmetrically and asymmetrically distributed probes was determined in vesicles containing increasing amounts of NBD-PC (0.25–3 mol %) with a constant amount of *N*-Rho-PE (1 mol %). As can be seen in Figure 3b, increasing concentrations of NBD-PC had no effect on energy transfer in both the symmetric and asymmetric vesicles. Thus, while the relative sensitized *N*-Rho-PE fluorescence increased with increasing NBD-PC concentration, the actual quenching of NBD-PC fluorescence remained constant, indicating that resonance energy transfer in both vesicle populations is dependent only on the concentration of the energy acceptor.

Table I shows the average distance of separation between probes distributed in symmetric (NBD-PC outside; *N*-Rho-PE outside) and asymmetric (NBD-PC inside; *N*-Rho-PE outside) vesicles. It can be seen that within the range of reliable fluorescence measurements ($1 - F/F_\tau = \sim 0.30$–0.9), R was 52 ± 2 and 67 ± 5 Å in the symmetric and asymmetric vesicles, respectively. With these data, the difference in average probe distance between the outer and inner leaflets was determined to be approximately 14 Å (Table I).

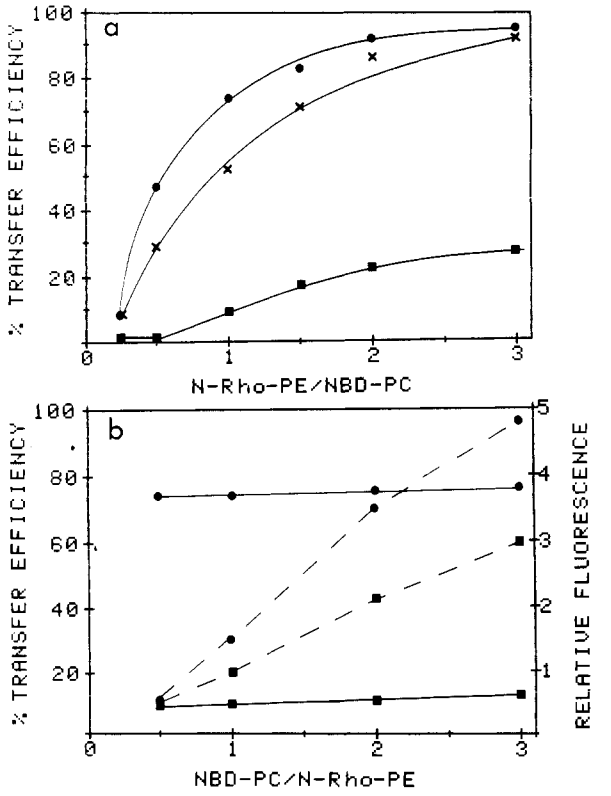

FIGURE 3: Resonance energy transfer efficiency in model membranes. Symmetric and asymmetric DOPC LUV (1 mM) were prepared from (a) 1 mol % NBD-PC and varying amounts of *N*-Rho-PE (0.25–3.0 mol %) or (b) 1 mol % *N*-Rho-PE and varying amounts of NBD-PC (0.25–3.0 mol %). A 20-μL sample was added to 1 mL of HEPES saline and measured for NBD-PC fluorescence (λ_{ex} 468 nm; λ_{em} 535 nm) in the absence and presence of 1% Triton X-100. (a) Resonance energy transfer efficiency (—) was determined according to eq 1 and plotted as a function of *N*-Rho-PE/NBD-PC. (b) Sensitized *N*-Rho-PE fluorescence (---) (λ_{ex} 468 nm; λ_{em} 595 nm) was measured and plotted as a function of NBD-PC/*N*-Rho-PE. Vesicles containing both probes in both leaflets (●), in the outer leaflet only (×), and in opposing leaflets (■) with *N*-Rho-PE and NBD-PC in the outer and inner leaflets, respectively.

Table I: Distance between Probes in Symmetric and Asymmetric Vesicles Based on RET Efficiency[a]

N-Rho-PE (mol %)	E_{sym}[b] (×10^{-2})	E_{asym}[b] (×10^{-2})	R_{sym}[c] (Å)	R_{asym}[c] (Å)	$R_{asym} - R_{sym}$ (Å)
1.00	0.52	0.09	55.4	74.3	18.8
1.50	0.71	0.17	52.6	66.8	14.2
2.00	0.86	0.22	51.0	64.0	13.0
3.00	0.92	0.27	50.4	61.8	11.4
			av distance 52.4	66.7	14.4

[a] Symmetric (*N*-Rho-PE outside; NBD-PC outside) and asymmetric (*N*-Rho-PE outside; NBD-PC inside) vesicles containing 1 mol % NBD-PC and increasing amounts of *N*-Rho-PE were prepared as described under Experimental Procedures. [b] E was obtained from data presented in Figure 3a. [c] R, the distance between probes in the symmetric (R_{sym}) and asymmetric (R_{asym}) vesicles, was calculated by using eq 2 and an R_0 value of 55.8 Å.

Resonance Energy Transfer Efficiency in RBC. The results with model membranes suggested that it would be possible to determine the transbilayer distribution of *N*-Rho-PE and NBD-labeled lipids in RBC. Thus, if RBC contain both the

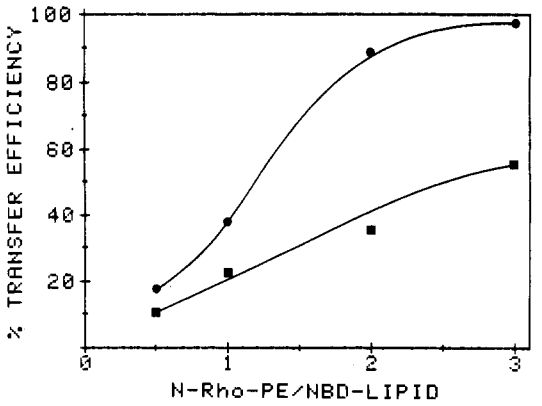

FIGURE 4: Resonance energy transfer in RBC. RBC (2×10^7 cells/mL) were incubated for 30 min at 37 °C with NBD-PC (●) or NBD-PS (■) [35 ng (10 μL of ethanol)$^{-1}$ (mL of RBC)$^{-1}$] to yield a final fluorophore concentration of 1 mol %. The cells were then washed and incubated with varying amounts of *N*-Rho-PE [23–280 ng (10 μL of ethanol)$^{-1}$ (mL of RBC)$^{-1}$]. After being washed, the RBC were adjusted to 2×10^7/mL, and both NBD (λ_{ex} 468 nm; λ_{em} 535 nm) and rhodamine fluorescence (λ_{ex} 535 nm; λ_{em} 595 nm) was measured in the absence and presence of 1% Triton X-100. Resonance energy transfer efficiency was determined according to eq 1.

energy acceptor and the energy donor in the outer leaflet, relatively strong RET should be observed. In contrast, if the probes localize in opposing leaflets, relatively weak energy transfer should be observed. Figure 4 shows RET measurements in intact RBC prelabeled for 30 min with a constant amount of NBD-PC or NBD-PS (1 mol %) and subsequently treated with increasing amounts of *N*-Rho-PE. As in the model membranes, increasing concentrations of the energy acceptor resulted in increased energy transfer efficiency. Distinct differences in the extent of energy transfer were observed, however, in cells labeled with NBD-PC and NBD-PS. NBD-PC-treated RBC display a quenching profile similar to the symmetrical and "outer leaflet only" curves obtained in the model membrane systems (Figure 3), suggesting that the two fluorophores remain in close proximity in the outer leaflet. In contrast, NBD-PS-treated RBC display much lower energy transfer, with a greater than 60% decrease in fluorescence quenching compared to the NBD-PC. On the basis of vesicle data, these results indicate that the NBD-PS is no longer in close proximity to *N*-Rho-PE. Since *N*-Rho-PE must remain in the outer leaflet, these results strongly suggest that NBD-PS transversed the outer leaflet, where energy transfer is strong, to the inner leaflet, resulting in increased distance from the energy acceptor with a concomitant release in NBD quenching.

Emission spectra of RBC containing *N*-Rho-PE (2 mol %) and NBD-PC or NBD-PS (1 mol %) are shown in Figure 5. As expected from the data presented in Figure 4, RBC containing NBD-PC (Figure 5a) exhibit a small NBD peak and a large rhodamine peak, indicating efficient energy transfer. In contrast, RBC containing NBD-PS (Figure 5b) display strong NBD emission with a lower rhodamine peak. Control NBD-PS spectra were obtained from RBC pretreated with 5 mM diamide, a disulfide cross-linking reagent that inhibits lipid translocation in RBC (Daleke & Huestis, 1985). These spectra (Figure 5c) are essentially identical with the spectra obtained by using NBD-PC (Figure 5a) and indicate that NBD-PS did not translocate under these conditions.

Several independent control experiments were performed to verify that the observed decrease in RET of NBD-PS-treated RBC was indeed due to translocation of the probe to the cells' inner leaflet. These experiments are based on observations that NBD-PS localized in the outer leaflet of model membranes (1) is removable from the membrane by back-exchange (Tanaka & Schroit, 1986), (2) can be derivatized to Tnp-NBD-PS by TNBS (Schroit et al., 1985; Denkins & Schroit, 1986), and (3) can be decarboxylated to NBD-PE by PSdC (Denkins & Schroit, 1986). The results of these experiments together with the corresponding RET data are presented in Table II. First, it can be seen that almost all of the NBD-PC could be removed from the RBC by back-exchange into SUV, as was the NBD-PS from diamide-treated RBC. The TNBS and PSdC data show that the fraction of NBD-PS that back-exchanged to the SUV was indeed in the outer leaflet, since 94% and 91% of the lipid was converted to Tnp-NBD-PS and NBD-PE, respectively. In contrast, NBD-PS was relatively inaccessible to back-exchange, TNBS and PSdC in cells not treated with diamide. Only ~10% of the lipid could be transferred to SUV, with 5% and 20% of the lipid converted to Tnp-NBD-PS and NBD-PE, respectively.

Discussion

Our results indicate that RET can be employed as a technique to determine the asymmetric distribution of fluorescent lipid probes in synthetic model membrane systems. The method has an advantage in that the average distance of separation between the energy donors and acceptors can be rapidly determined for probes with known R_0 values simply by measuring the extent of donor fluorescence quenching. Since transfer efficiency is dependent upon the concentration of the energy acceptor, determination of the sidedness of the energy donor with respect to the acceptor must be determined at constant acceptor concentrations. The intensity of the measured fluorescence is dependent, however, upon quenching of the donor fluorescence by the energy acceptor. Thus, ac-

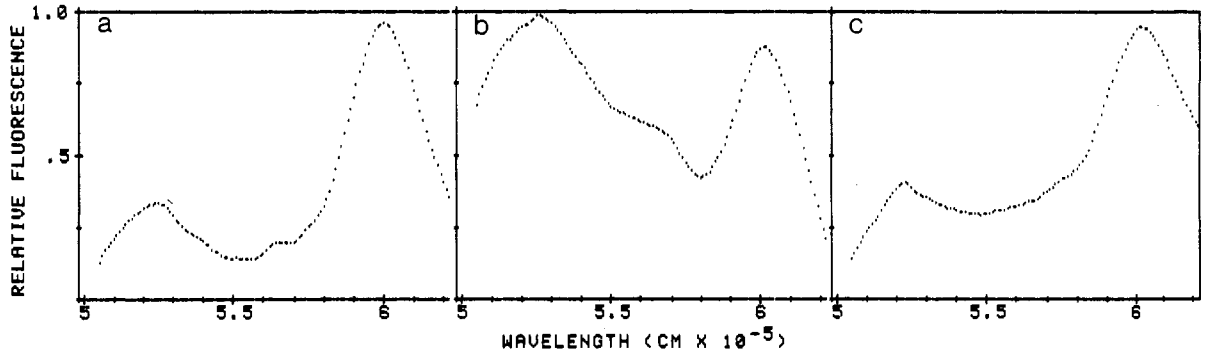

FIGURE 5: Fluorescence emission spectra of RBC. RBC (2×10^7 cells/mL), containing 1% NBD-PC (28 ng/mL RBC) or 1% NBD-PS (28 ng/mL RBC), and 2% *N*-Rho-PE (94 ng/mL RBC) were prepared as described for Figure 4. The cells were excited at 468 nm and scanned for fluorescence emission from 500 to 620 nm. Spectra were collected as described under Experimental Procedures. (a) NBD-PC-treated RBC; (b) NBD-PS-treated RBC; and (c) NBD-PS-treated RBC pretreated with diamide (5 mM).

Table II: Comparison of RET Asymmetry Determinations with Direct Assay of Lipid Distribution by External Probes[a]

RBC	RET[c] -Triton	RET[c] +Triton	RET[c] E (%)	back-exchange[d] (% lipid removed)	TNBS[e] (% Tnp-NBD-PS)	PSdC[f] (% NBD-PE)
NBD-PC	6	54	89	85–90		
NBD-PC + diamide[b]	8	52	85	80–85		
NBD-PS	35	55	36	5–10	5	20
NBD-PS + diamide[b]	4	56	93	90–95	94	91

[a] RBC (2×10^7 cells/mL) containing 1 mol % NBD-PC or NBD-PS and 2 mol % *N*-Rho-PE were prepared as described for Figure 4. [b] RBC (2×10^7 cells/mL) were incubated with 5 mM diamide for 30 min at 37 °C, washed, and subsequently labeled with the fluorescent lipids as described. [c] The relative fluorescence (arbitrary units) of the RBC was read in the absence and presence of 1% Triton X-100. Readings obtained in the presence of detergent were corrected for sample dilution and detergent quenching. E was calculated from eq 1. [d] RBC (2×10^7 cells/mL) labeled with NBD-lipid were incubated with 1 mg of DOPC SUV for 30 min at 20 °C. The fluorescence of the initial cell suspension and of the washed vesicle-treated cells was determined in the presence of Triton X-100. The fraction of lipid removed (lipid in the outer leaflet) was calculated as follows: fraction of NBD-lipid remaining = 1 − (fluorescence of washed cells/fluorescence of unwashed cells). [e] NBD-PS-treated RBC were labeled with TNBS for 90 min at 20 °C. The cells were washed and lysed, and the amount of underivatized NBD-PS remaining was determined. The fraction of Tnp-NBD-PS formed (lipid in the outer leaflet) was calculated as follows: Tnp-NBD-PS = 1 − (NBD-PS remaining after TNBS treatment/total NBD-PS incorporated). [f] NBD-PS-treated RBC were incubated with PSdC for 60 min at 37 °C. The cells were washed and extracted, and the amounts of NBD-PE formed and of residual NBD-PS were assayed as described under Experimental Procedures. The fraction of NBD-PS in the outer leaflet (NBD-PE formed by decarboxylation) was calculated as follows: 1 − [NBD-PE/(NBD-PE + NBD-PS)].

curate assessments of distance can be obtained only when E is in the range of ~85–15% which corresponds to marginal levels of donor fluorescence (F) and very small differences in F/F_0, respectively. For donor/acceptor pairs with R_0 values of 55 Å, deviation from this distance can be detected in the range of about −5 Å (at E = 85%) to +12 Å (at E = 15%) (see Figure 2).

Table I shows the calculated distances between fluorophores localized in the same (outer leaflet only) and opposing (asymmetric) leaflets of artificially generated vesicles. Estimates of probe separation in vesicles containing from 1 to 3 mol % *N*-Rho-PE revealed average distances of separation of 52.4 and 66.7 Å in the symmetric and asymmetric vesicles, respectively. By subtraction, it appears that the net difference in probe distance between the two populations is 14.3 Å. Although we have not directly determined probe depth within the bilayer, the distances obtained are consistent with the lipid analogues being present in the outer and inner monolayer exclusively. Thus, given a monolayer diameter of 16 Å (Mason & Huang, 1978) and a net difference in probe position between the symmetric and asymmetric vesicles of 14 Å, it would appear that the NBD reporter group resides within the hydrophobic core of the leaflet at an apparent depth of 9 Å. This assumption is inconsistent with the recent observations of Chattopadhyay and London (1987), who suggested that the NBD fluorophore of both acyl chain labeled and headgroup-labeled NBD lipids resides at the lipid/water interface. Although we cannot reconcile our results with their observations, it is certain that our values reflect differences in probe distance based on vesicles containing *N*-Rho-PE and NBD-PC exclusively in the outer and inner leaflets. That these vesicles were indeed asymmetric was independently verified by (1) the ability to remove only half of the NBD-PC present in the initial symmetric vesicles and (2) the inability to remove by back-exchange any NBD-PC in the final asymmetric preparation. We therefore conclude that the differences in resonance energy transfer efficiency between NBD-PC in the outer and inner bilayer leaflet and *N*-Rho-PE assess NBD-lipid asymmetry accurately.

Experiments employing the RET system in intact RBC yielded results analogous to those obtained with synthetic model membranes. RBC containing *N*-Rho-PE and NBD-PC exhibited consistently high levels of energy transfer, whereas RBC treated with NBD-PS resulted in relatively low transfer efficiency. The lack of efficient energy transfer in the NBD-PS-treated RBC could be attributed to translocation of the exogenously inserted lipid to the inner leaflet, or conceivably to sequestration of the NBD-PS to selective sites within the outer leaflet. Although the latter is possible, several control experiments ruled this out. First, fluorescence measurements, in the absence and presence of Triton X-100, of RBC containing only the NBD-lipids did not reveal self-quenching of the NBD [see Tanaka and Schroit (1983)], indicating that the lipid did not aggregate. Second, independent control experiments employing back-exchange, TNBS derivatization, and decarboxylation by PSdC (Table II) showed the lipid was not in the outer leaflet.

Recent data have indicated that treatment of RBC with the disulfide cross-linker diamide inhibits shape changes in RBC consistent with PS translocation (Daleke & Huestis, 1985). Control experiments (Table II) using diamide-treated RBC showed that, indeed, most of the lipid was restricted to the outer leaflet of the cells. Thus, TNBS derivatized and PSdC decarboxylated 94% and 91% of the NBD-PS, respectively. In addition, emission scans revealed spectra essentially identical with those obtained by using NBD-PC-treated RBC. On the basis of these results, we conclude that NBD-PS translocation in RBC results in a distance-dependent decrease in NBD quenching efficiency which can be determined by the RET assay.

Clearly, it is difficult to estimate to what extent changes in RET efficiency between outer leaflet (NBD-PC and diamide-treated NBD-PS RBC) and inner leaflet (NBD-PS RBC) probes reflect upon actual distance differences. This is mainly due to an obvious difference in the quantum yield of the NBD in RBC which results in different R_0 values (eq 3), and because only about 80% of the NBD-PS translocates to the inner leaflet (based on back-exchange and formation of NBD-PE by PSdC, Table II) under the experimental conditions used here. Rough estimates can be obtained, however, (1) by comparing the quantum yield of NBD-lipid in a synthetic bilayer membrane with its quantum yield in RBC and (2) by assuming that 20% of the observed RET is the result of lipid that resides in the outer leaflet at distance R_1 (obtained from diamide-treated NBD-PS or NBD-PC RBC). The relative quantum yield of NBD-PS in RBC compared to synthetic vesicles has been previously determined to be 0.75 (Tanaka & Schroit, 1983). By substituting Q_0 (0.75) in eq 3 with $0.75Q_0$, R_0 becomes 53.2 Å in this system. If 20% of E in these RBC (not treated with diamide) originated in the outer leaflet at an average E of 0.93, then the corrected E (E_c) can be obtained from the relationship $E_c = (E_1 - 0.2E_2)/0.8$ where E_1 and E_2 are the energy transfer efficiencies of untreated and diamide-treated RBC, respec-

tively. After correction of E from the data in Table II (E_c for NBD-PS RBC = 21.8), the average distance of separation between *N*-Rho-PE and NBD-PS in RBC and in control, diamide-treated RBC (obtained from eq 2) is 61.1 and 48.0 Å, respectively. By subtraction, it appears that the net difference in probe distance between NBD-PS in the outer and inner leaflets of RBC is about 13 Å, a distance in very good agreement with the distances obtained in the artificially generated vesicle system.

In conclusion, RET measurements may provide a sensitive method by which the translocation or distribution (Uster & Pagano, 1986) of appropriately labeled lipids can be monitored in cells. In addition, this technique should facilitate accurate real-time kinetic measurements of lipid "flip-flop" in different cell types by simultaneously measuring the NBD emission of control and experimental cell populations with a dual-beam apparatus. Initial experiments employing such real-time assays have been encouraging.

References

Ames, B. N., & Dubin, D. T. (1960) *J. Biol. Chem. 235*, 769–775.

Bevers, E. M., Comfurius, P., Van Rijn, J. L. M. L., Hemker, H. C., & Zwaal, R. F. A. (1982) *Eur. J. Biochem. 122*, 429–436.

Bevers, E. M., Comfurius, P., & Zwaal, R. F. A. (1983) *Biochim. Biophys. Acta 736*, 57–66.

Chattopadhyay, A., & London, E. (1987) *Biochemistry 26*, 39–45.

Chiu, D., Lubin, B., & Shohet, S. B. (1979) *Br. J. Haematol. 41*, 233–234.

Clarke, L., & Carbon, J. (1976) *Cell (Cambridge, Mass.) 9*, 91–99.

Comfurius, P., & Zwaal, R. F. A. (1977) *Biochim. Biophys. Acta 488*, 36–42.

Daleke, D. L., & Huestis, W. H. (1985) *Biochemistry 24*, 5406–5416.

Denkins, Y., & Schroit, A. J. (1986) *Biochim. Biophys. Acta 862*, 343–351.

Forster, T. (1948) *Ann. Phys. (Leipzig) 2*, 55–75.

Franck, P. F. H., Bevers, E. M., Lubin, B. H., Comfurius, P., Chiu, D. T.-Y., Op den Kamp, J. A. F., Zwaal, R. F. A., van Deenan, L. L. M., & Roelofsen, B.(1985) *J. Clin. Invest. 75*, 183–190.

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

Gordesky, S. E., Marinetti, G. V., & Love, R. (1975) *J. Membr. Biol. 20*, 111–132.

Hope, M. J., Walker, D. C., & Cullis, P. R. (1983) *Biochem. Biophys. Res. Commun. 110*, 15–22.

Lubin, B., Chiu, D., Bastacky, B., Roelofsen, B., & Van Deenan, L. L. M. (1981) *J. Clin. Invest. 67*, 1643–1649.

Mason, J. T., & Huang, C. (1978) *Ann. N.Y. Acad. Sci. 308*, 29–49.

Op den Kamp, J. A. F. (1979) *Annu. Rev. Biochem. 48*, 47–71.

Rothman, J. E., & Lenard, J. (1977) *Science (Washington, D.C.) 195*, 743–753.

Schroit, A. J. (1982) *Biochemistry 21*, 5323–5328.

Schroit, A. J., & Fidler, I. J. (1982) *Cancer Res. 42*, 161–167.

Schroit, A. J., Madsen, J., & Tanaka, Y. (1985) *J. Biol. Chem. 260*, 5131–5138.

Schwartz, R. S., Tanaka, Y., Fidler, I. J., Chiu, D., Lubin, B., & Schroit, A. J. (1985) *J. Clin. Invest. 75*, 1965–1973.

Seigneuret, M., & Devaux, P. F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3751–3755.

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

Tanaka, Y., & Schroit, A. J. (1983) *J. Biol. Chem. 258*, 11335–11343.

Tanaka, Y., & Schroit, A. J. (1986) *Biochemistry 25*, 2141–2148.

Tayhach, R. J., Hawrot, E., Stare, M., & Kennedy, E. P. (1979) *J. Biol. Chem. 254*, 627–633.

Tilley, L., Cribier, S., Roelofsen, B., Op den Kamp, J. A. F., & Van Deenan, L. L. M. (1986) *FEBS Lett. 194*, 21–27.

Uster, P. S., & Pagano, R. E. (1986) *J. Cell Biol. 103*, 1221–1234.

Verkleij, A. J., Zwaal, R. F. A., Roelofsen, B., Comfurius, P., Kastelijn, D., & Van Deenan, L. L. M. (1973) *Biochim. Biophys. Acta 323*, 178–193.