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Formation of Asymmetric Phospholipid Membranes via Spontaneous Transfer of Fluorescent Lipid Analogues between Vesicle Populations†

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ABSTRACT: A method is presented for generating artificial lipid vesicles bearing an asymmetric distribution of either of the fluorescent lipid analogues 1-acyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]phosphatidylcholine or 1-acyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-phosphatidylcholine, in which the fluorescent lipid is located predominantly in either the outer or inner leaflet of the vesicle bilayer. The procedure is based on the observation that these lipid analogues undergo rapid spontaneous transfer (exchange) between vesicle populations [Nichols, J. W., & Pagano, R. E. (1981) *Biochemistry 20*, 2783–2789]. When an excess of nonfluorescent acceptor vesicles is mixed with small unilamellar vesicles containing 5 mol % fluorescent lipid, approximately 50% of the fluorescent lipid is transferred to the acceptor vesicles, whereas if fluorescent multilamellar vesicles are used, only approximately 10% of the analogues is available for transfer. These fractions of fluorescent lipid available for intervesicular transfer correspond closely to the amount of phospholipid residing in the outermost leaflet of the donor vesicles, suggesting that only fluorescent lipids present in the outer surface of the vesicles can spontaneously transfer between vesicle populations. Evidence demonstrating that the movement of the fluorescent lipid between vesicle populations is the result of a net transfer process rather than lipid exchange is also presented. A novel assay based on resonance energy transfer is described for determining the size of the exchangeable fluorescent lipid pool, a measure of the degree of asymmetry of these preparations. Finally, for demonstration of the usefulness of asymmetric vesicles in distinguishing various pathways of vesicle–cell association, preliminary results are presented on their interactions with Chinese hamster fibroblasts in vitro.

The asymmetric distribution of the various lipid classes in the plasma membranes of cells is emerging as a general feature of membrane structure [reviewed in Bergelson & Barsukov (1977), Rothman & Lenard (1977), Op den Kamp (1979), and Van Deenen (1981)]. In a number of studies, the choline-containing lipids have been found to be enriched on the external leaflet of the membrane lipid bilayer, while the acidic and amino-containing lipids are enriched on the inner half of the membrane. Several questions of general interest relating to the maintenance of this asymmetry remain to be explored. It is of interest, for example, to determine whether or not lipid asymmetry is conserved during fusion of two asymmetric membranes with one another. In addition, there exists the possibility that exchange of lipids into the plasma membrane of a cell from an exogenous source such as lipoproteins is an asymmetric process, resulting in the modification and/or maintenance of cell surface lipid asymmetry.

In principle, it should be possible to begin to address such questions using "asymmetric" lipid vesicles in which a particular lipid is restricted to the inner or outer leaflet of the vesicle bilayer. The distribution of that lipid following vesicle–vesicle fusion (Papahadjopoulos et al., 1979), vesicle–cell fusion (Pagano & Weinstein, 1978), or vesicle–cell exchange

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(Sandra & Pagano, 1979) could then be assessed by appropriate assays for lipid asymmetry. If, for example, the transbilayer arrangement of the vesicle lipids was conserved during fusion, then lipids initially restricted to the outer (or inner) leaflet of the vesicles should reside more or less exclusively on the external (or internal) leaflet of the fusion product.

In this paper, we present a method for generating artificial lipid vesicles with an asymmetric distribution of fluorescent NBD[1]–lipid analogues. Our strategy is based on the observation that certain lipids will spontaneously transfer between various combinations of vesicles, cells, and lipoproteins (Charlton et al., 1976, 1978; Galla et al., 1979; Barsukov et al., 1980; Phillips et al., 1980; Nichols & Pagano, 1981). In this report, we demonstrate that certain NBD–phospholipid analogues will readily exchange between vesicle populations and that only those fluorescent molecules residing in the outer leaflet of the vesicle bilayer are capable of undergoing such transfer. Thus, by incubating NBD-containing vesicles with nonfluorescent acceptors and subsequently separating the two populations of vesicles, it is possible to generate asymmetric vesicles in which the fluorescent lipid species is restricted largely to the inner or outer leaflet of the vesicle bilayer. A novel assay for quantifying the degree of asymmetry in these preparations on the basis of resonance energy tranfer is presented, along with our preliminary observations on the interactions of these asymmetric preparations with cells in vitro.

Experimental Procedures

Materials and Routine Procedures. NBD-chloride and oleic acid were purchased from Sigma Chemical Co. Carbonyldiimidazole was purchased from Aldrich Chemical Co. Phospholipase D (cabbage) was purchased from Boehringer-Mannheim. Radioactivity was measured with a Packard Tri-Carb liquid scintillation counter using a toluene-based counting fluid. Relative fluorescence intensity was determined by using an Aminco-Bowman spectrophotofluorometer (American Instrument Co.).

Lipids. DOPC, C_6-NBD-PC, and *N*-NBD-PE were purchased from Avanti Biochemical Corp. 1-Acyl-2-[12-[(*t*-Boc)amino]dodecanoyl]phosphatidylcholine was purchased on special order from Avanti Biochemical Corp. and used for the synthesis of C_{12}-NBD-PC as described elsewhere (Nichols & Pagano, 1981). DOPE was prepared from DOPC by using phospholipase D and ethanolamine (Comfurius & Zwaal, 1977) and was purified by silicic acid column chromatography.

[^{3}H]DOPC (≥140 mCi/mmol) was synthesized by the acylation of L-α-glycerophosphorylcholine with [^{3}H]oleic acid imidazolide by an adaptation of the method of Boss et al. (1975). The imidazolide was prepared by reacting carbonyldiimidazole with [9,10-$^3H_2$]oleic acid (New England Nuclear) and unlabeled oleic acid in benzene for 30 min at 22 °C. A 2.5–4-fold molar excess of the imidazolide was then combined with glycerophosphorylcholine, the benzene removed by evaporation under a stream of argon gas, and the mixture heated with mixing to 70 °C for 1 h. The reaction was then allowed to proceed overnight at 50 °C. An excess of unlabeled oleic acid imidazolide was then added and the mixture further heated to 70 °C for 1 h and overnight at 50 °C. All reaction steps at elevated temperature were carried out under vacuum. The reaction was terminated by using an acidic Folch extraction procedure. The lipid phase was then washed and purified by thin-layer chromatography in chloroform–methanol–28% ammonium hydroxide (65:35:5). [1-^{14}C]DOPC (100 mCi/mmol) was purchased from Applied Science Laboratories and similarly purified.

Br-PC was prepared by bromination of DOPC essentially as described by Dawidowicz & Rothman (1976). *N*-Rh-PE was prepared as previously described (Struck et al., 1981).

Lipids were stored at −20 °C, periodically monitored for purity by thin-layer chromatography, and purified when necessary. Except for isotopically labeled lipids, the concentrations of all lipid stock solutions were determined by a modified lipid phosphorus procedure (Ames & Dubin, 1960).

Vesicle Preparation. Vesicles were prepared by ethanol injection (Kremer et al., 1977) as follows. LUV were made from Br-PC, DOPC, or DOPC/DOPE (1:1 w/w) containing [^{3}H]DOPC as a marker for lipid concentration. In a typical LUV preparation, approximately 40 μmol of lipid was dried down under a stream of argon gas and further dried under vacuum. The lipids were then dissolved in 1 mL of ethanol and injected with stirring into 13 mL of 50 mM KCl or HCMF. When Br-PC or 1:1 DOPC/DOPE was used, a slight warming of the alcohol solution was required to effect complete solution of the lipids. The resulting vesicle preparation was dialyzed overnight at 4 °C against several changes of buffer. The formation of large vesicles was confirmed by their exclusion from Sepharose 4B (Pharmacia Fine Chemicals) or Bio-Gel A15m (Bio-Rad Laboratories) columns and by negative staining electron microscopy of the suspension which revealed large, predominantly unilamellar vesicles, ≥1000 Å in diameter. SUV were made from DOPC, 5 mol % C_6-NBD-PC or C_{12}-NBD-PC, and [^{14}C]DOPC as a marker for lipid concentration. In a typical SUV preparation, approximately 5 μmol of lipids (dried as described for LUV) was dissolved in 1 mL of ethanol and injected into 13 mL of 50 mM KCl or HCMF with stirring. The preparation was dialyzed at 4 °C overnight against several changes of buffer before use. Chromatographic behavior on Sepharose 4B or Bio-Gel A15m columns and examination of negatively stained preparations by electron microscopy indicated that the majority of the vesicles present in these preparations were unilamellar, with diameters of ≃300 Å. For some experiments, SUV were also prepared from the above lipids by ultrasonication as previously described (Huang, 1969).

Where necessary, suspensions of vesicles were concentrated by dialysis at 4 °C against a 20% (w/v) solution of Aquacide III (Calbiochem-Behring Corp.) in 50 mM KCl or HCMF with rapid stirring. After sufficient concentration, the vesicle suspensions were further dialyzed overnight at 4 °C against several changes of buffer before use. Final vesicle concentrations were determined by scintillation counting.

Preparation of Asymmetric SUV with a Fluorescent Lipid Enriched on the Inner Leaflet of the Vesicle Bilayer by Lipid Exchange. Fluorescent donor (SUV) and nonfluorescent acceptor (LUV) vesicles were prepared as described above. Donor and acceptor vesicles were then mixed in the ratio 1:10

[1] Abbreviations used: Br-PC, dibromostearoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; [^{14}C]DOPC, dioleoyl[1-^{14}C]phosphatidylcholine; [^{3}H]DOPC, di[9,10-$^3H_2$]oleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; EDTA, ethylenediaminetetraacetic acid; HCMF, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered, calcium- and magnesium-free Puck's saline, pH 7.4; LUV, large unilamellar vesicles; SUV, small unilamellar vesicles; NBD, 4-nitro-2,1,3-benzoxadiazole; *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; C_6-NBD-PC, 1-acyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]phosphatidylcholine; C_{12}-NBD-PC, 1-acyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-dodecanoyl]phosphatidylcholine; PBS, phosphate-buffered saline, pH 7.4; PC, phosphatidylcholine; *N*-Rh-PE, *N*-(lissamine rhodamine B sulfonyl)dioleoylphosphatidylethanolamine; *t*-Boc, *tert*-butoxycarbonyl.

(w/w), and the mixture was incubated (exchange incubation) for 10 min at 22 °C for the C_6-NBD-PC analogue or 60 min at 37 °C for the C_{12}-NBD-PC analogue. Donor vesicles were then separated from acceptor vesicles by high-speed centrifugation (90 min at 2×10^5 g, 4 °C). Following centrifugation, the supernatant containing the donor SUV was removed from the centrifuge tubes, taking care not to disturb the pellet containing the majority of the LUV. The SUV donors so obtained were then incubated with a fresh aliquot of LUV acceptors and centrifuged a second time. These centrifugation steps were repeated a total of 2–4 times. After separation and recovery of the vesicles from the final incubation, the SUV preparations were concentrated and dialyzed before use. Lipid concentrations for vesicle preparations containing radiolabeled lipid were determined by scintillation counting.

Preparation of Asymmetric SUV with a Fluorescent Lipid Restricted to the Outer Leaflet of the Vesicle Bilayer by Lipid Exchange. Outer leaflet labeled vesicles were prepared in a similar fashion, except that the starting SUV were nonfluorescent and were made from [^{14}C]DOPC only, while the LUV contained the fluorescent NBD–lipid analogue. Only a single exchange incubation was required, and the separation of SUV from LUV was carried out as above.

Standard Curves for Energy-Transfer Measurements. The energy-transfer assay for determination of the exchangeable pool size of the NBD–lipid analogues, a measure of their asymmetry in the vesicle preparations, is described in detail under Results and Discussion. The assay requires the use of two standard curves which were generated as follows. *Standard curve I* is the relative fluorescence at 530 nm (λ_{ex} = 470 nm) vs. micrograms of NBD-PC present in the vesicles. The relative fluorescence of known, unquenched amounts of C_6-NBD-PC in 2% Triton X-100 was measured and corrected for the lower efficiency ($\simeq$50%) of NBD fluorescence in Triton as compared to its fluorescence in DOPC vesicles at unquenched concentrations. For determination of this correction factor, DOPC SUV containing unquenched concentrations of C_6-NBD-PC (0.1 and 0.3 mol %; Nichols & Pagano, 1981) were made by ethanol injection and dialyzed, and their relative fluorescence (λ_{ex} 470 nm; λ_{em} 530 nm) was determined in the presence and absence of 2% Triton X-100. After correction for volume change due to the addition of detergent, the fluorescence of each sample was found to be reduced 1.95–2.00-fold. *Standard curve II* is the relative fluorescence at 600 nm (λ_{ex} 470 nm) vs. micrograms of NBD-PC in the vesicles. This curve was constructed by adding varying amounts of C_6-NBD-PC SUV (up to 2 mol %) to DOPC SUV and determining the fluorescence which developed at 600 nm (λ_{ex} 470 nm) as a result of the spontaneous transfer of the fluorescent lipid from the self-quenched NBD-PC vesicles into the DOPC vesicles. The amount of NBD-PC present in the DOPC SUV was determined from standard curve I after reading the fluorescence of each sample at 530 nm (λ_{ex} 470 nm). The fluorescence at 600 nm was then plotted as a function of the amount of NBD-PC present. Both standard curves were linear over the range of interest.

Cells. Monolayer cultures of Chinese hamster V79 fibroblasts (Ford & Yerganian, 1958) were grown to confluency in Eagle's minimal essential medium supplemented with 12% horse serum in a water-saturated atmosphere of 5% CO_2 in air. Single cell suspensions were prepared by trypsinization as described (Struck & Pagano, 1980).

Vesicle–Cell Incubations and Assays for Lipid Uptake. Cell pellets containing (5–10) $\times 10^6$ cells were resuspended in 1 mL of vesicle suspension at various concentrations and incubated for 30–60 min at 2 or 37 °C. Cell suspensions were washed 3 times in cold HCMF, the last wash being carried out in a new tube. The cells were then resuspended in buffer and divided into aliquots for fluorescence microscopy and lipid extraction. Vesicle-derived lipid present in these extracts was quantified by measurements of fluorescence and/or radioactivity.

Microscopy. Fluorescence microscopy was performed with a Zeiss Universal microscopy equipped with epiillumination for fluorescence. The light source was the 457.9-nm line from an argon–krypton gas laser (Control Laser Corp.) which was defocused and attenuated for observation and photography.

Results and Discussion

Acyl Chain Labeled NBD–Phospholipid Analogues Readily Transfer between Vesicles. When SUV containing C_6-NBD-PC and *N*-Rh-PE (donor vesicles) are mixed with LUV composed of DOPC (acceptor vesicles), transfer of C_6-NBD-PC from the donor to acceptor vesicles is observed (Figure 1). In contrast, neither *N*-Rh-PE nor the LUV marker lipid ([^{14}C]DOPC) is transferred between the two vesicle populations. Similar results were obtained by using C_{12}-NBD-PC in place of C_6-NBD-PC. In the case of C_{12}-NBD-PC, incubations were carried out at 37 °C to obtain rapid transfer of the fluorescent lipid. From these results, it is clear that only certain lipids are readily transferrable and that this process most likely represents mass transfer rather than a one-for-one exchange of lipid monomers between vesicle types (also, see below).

Exchangeable Pool of NBD–Lipids Corresponds to the Fluorescent Lipids in the External Leaflet of the Vesicle Bilayer. Because of the rapid kinetics of the transfer of NBD–lipids between vesicles and the fact that "flip-flop" of phospholipids across artificial bilayers is usually slow (Johnson et al., 1975; Rothman & Dawidowicz, 1975), it seemed that by adjusting the radio of donor to acceptor vesicles, it should be possible to remove all of the outer leaflet NBD–lipid from the donor bilayer without affecting the pool residing on the inner leaflet, assuming that the mole fraction of NBD–lipid is small ($\leq$5%). To test this possibility, we took advantage of the efficient resonance energy transfer which occurs between an NBD–lipid and *N*-Rh-PE present in the same vesicle.

The energy-transfer method is based on the fact that when two fluorophores, an energy donor and an energy acceptor, are in close physical proximity, direct excitation of the donor results in efficient transfer of energy to the acceptor species which then fluoresces. In the present study, NBD–lipid analogues (λ_{ex}^{max} 470 nm; λ_{em}^{max} 525 nm) served as the fluorescent donor species, while *N*-Rh-PE (λ_{ex}max 560 nm; λ_{em}max 580 nm) was the fluorescent acceptor. The spectra of these fluorescent lipid derivatives are given elsewhere (Struck et al., 1981). However, it should be noted that (i) there is substantial overlap between the emission band of NBD and the excitation band of rhodamine and (ii) excitation of rhodamine at 470 nm is minimal. Therefore, this pair of fluorophores can be used effectively in energy-transfer measurements.

An example of the energy-transfer phenomenon is presented in Figure 2 in which excitation spectra were obtained while sample fluorescence was monitored at 600 nm. When DOPC vesicles containing 1 mol % *N*-Rh-PE are used, the excitation spectrum shown in Figure 2a (upper trace) is obtained in which a single maximum at about 560 nm is seen. However, when DOPC vesicles containing *both* *N*-Rh-PE and NBD-PC are used, the spectrum given in Figure 2b (upper trace) is obtained.

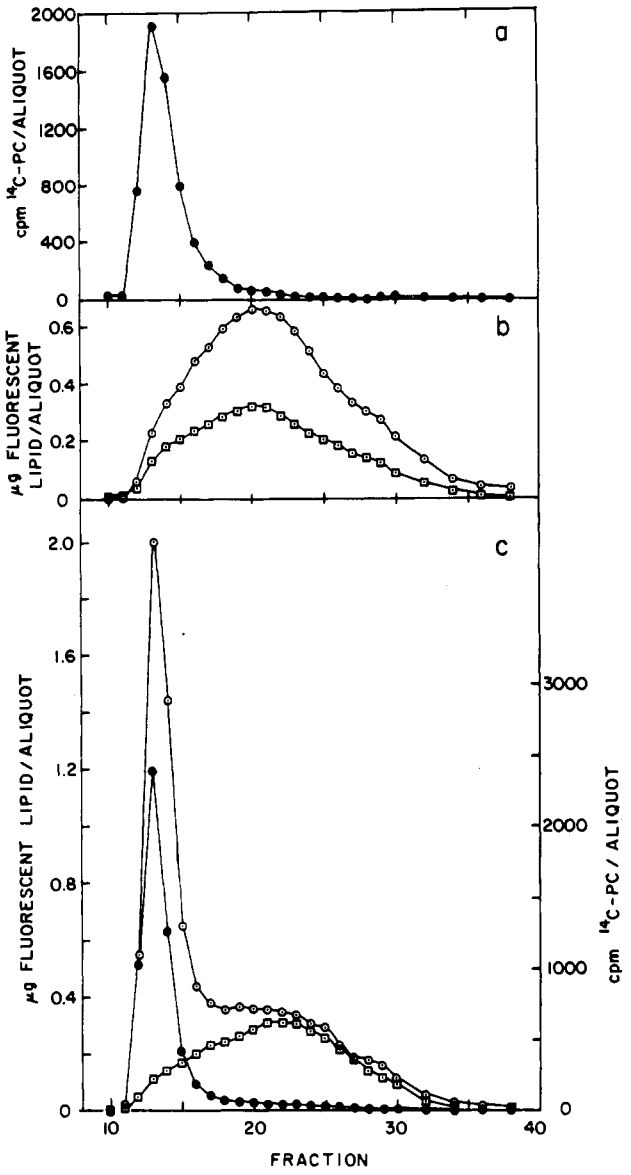

FIGURE 1: Intervesicular transfer of C_6-NBD-PC between large (LUV) and small (SUV) unilamellar vesicle populations. LUV were formed from [^{14}C]DOPC and SUV from DOPC/C_6-NBD-PC/*N*-Rh-PE (97:2:1). Elution profiles were obtained on a 1 × 56 cm Bio-Gel A15m column. Sample size = 1.0 mL; 1.3 mL/fraction. Buffer: PBS + 0.1 mM EDTA, pH 7.4. (a) LUV (12 mg) alone; (b) SUV (1 mg) alone; (c) mixture of LUV (12 mg) and SUV (1 mg) after 30 min at 22 °C. (●) [^{14}C]DOPC; (○) C_6-NBD-PC; (□) *N*-Rh-PE.

As can be seen, a second peak corresponding to the excitation maximum of NBD now appears in the spectrum due to energy transfer from NBD to rhodamine with resulting rhodamine fluorescence. Figure 2b (lower trace) is the excitation spectrum taken for the same amount of NBD-PC present in DOPC vesicles in the absence of *N*-Rh-PE. As can be seen, there is an excitation peak at 470 nm because the emission spectrum of NBD slightly overlaps that of *N*-Rh-PE. No significant contribution to the observed fluorescence at 600 nm is seen with DOPC vesicles containing no fluorescent analogues (Figure 2a, lower trace). The fluorescence due to energy transfer can be calculated from the excitation peak height (λ_{em} 600 nm) at 470 nm (Figure 2b, upper trace) after correcting for contributions due to NBD emission at 600 nm and to the direct excitation of rhodamine at 470 nm. Thus, the energy transfer (ET) is given by the relation

$$ET = h_1 - h_2 - h_3 \quad (1)$$

where h_1, h_2, and h_3 are the excitation peak (λ_{em} 600 nm)

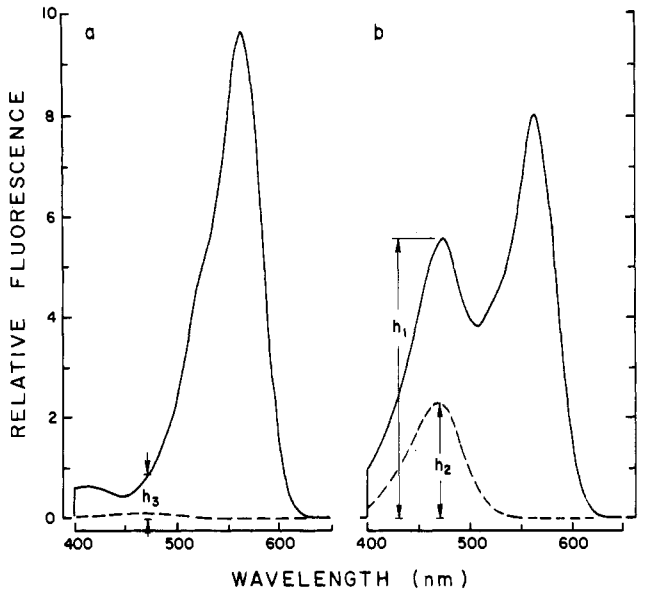

FIGURE 2: Spectral analysis for energy-transfer measurements. Excitation spectra were obtained while sample emission at 600 nm was being monitored. Spectra were taken for the following SUV preparations: (a) (upper trace) DOPC/*N*-Rh-PE (99:1), (lower trace) DOPC; (b) (upper trace) DOPC/*N*-Rh-PE/C_6-NBD-PC (97.3:1.0:1.7), (lower trace) DOPC/C_6-NBD-PC(98.3:1.7). h_1, h_2, and h_3 represent the peak heights at 470 nm required for calculation of the degree of energy transfer from NBD to rhodamine fluorophores (see text for details).

heights at 470 nm for DOPC/*N*-Rh-PE/NBD-PC, DOPC/NBD-PC, and DOPC/*N*-Rh-PE vesicles, respectively.

So that the possibility that only lipids present in the external leaflet of the vesicle bilayer are available for exchange between vesicle populations could be tested, the following experiment was carried out. Three types of donor vesicles were first prepared: (i) multilamellar vesicles formed from DOPC/*N*-Rh-PE/C_6-NBD-PC (98:1:1), (ii) SUV formed from DOPC/*N*-Rh-PE/C_6-NBD-PC (98:1:1), and (iii) SUV made from DOPC/*N*-Rh-PE/*N*-NBD-PE (98:1:1). The fluorescence due to energy transfer observed for each of these preparations was then quantified as described above. Next, increasing amounts of SUV acceptors formed from DOPC were added, and the decrease in energy transfer resulting from intervesicular exchange of C_6-NBD-PC between donor and acceptor vesicles (see Figure 1) was determined (Figure 3). When donor vesicles were multilamellar, the maximal reduction in energy transfer was only about 10% of the control values, regardless of the amount of acceptors used. This value of 10% corresponds closely to the size of the exchangeable lipid pool in multilamellar vesicles determined by using phospholipid exchange proteins (DiCorleto & Zilversmit, 1977) and to the amount of lipid present in the external monolayer of a multilamellar vesicle population as determined by titration with UO_2^{2+} (Bangham et al., 1967). When SUV donors containing C_6-NBD-PC were used, the level of energy transfer was reduced to nearly 50% of that present in the starting vesicles upon addition of increasing amounts of SUV acceptors. This value is in good agreement with estimates of the fraction of lipid residing in the external leaflet of an SUV (60–70% of total) determined both by physical methods (Bystrov et al., 1971; Huang et al., 1974) and with phospholipid exchange proteins (Johnson et al., 1975; Rothman & Dawidowicz, 1975; Sandra & Pagano, 1979). The finding of a somewhat smaller exchangeable pool for C_6-NBD-PC than expected (50% vs. 60–70%) might be due to the presence of slightly larger vesicles and/or to the possibility that in a multicomponent SUV, each

Table I: Recovery of Vesicles following Ultracentrifugation[a]

vesicle type	composition	buffer	no.[b]	recovery[c] (%)
SUV	DOPC	HCMF or 50 mM KCl	1	96
SUV	DOPC/C_6-NBD-PC (or C_{12}-NBD-PC) (95:5)	HCMF or 50 mM KCl	1	95
			1	90
LUV	DOPC/DOPE (1:1)	50 mM KCl	1	13.5
			2	6.1
			3	3.6
			4	2.1
LUV	DOPC	50 mM KCl	1	75
LUV	Br-PC	HCMF or 50 mM KCl	1	<2

[a] SUV and LUV of the indicated lipid compositions containing trace amounts of [^{3}H]DOPC and [^{14}C]DOPC, respectively (see Experimental Procedures), were prepared. Each vesicle preparation was then centrifuged at (2×10^5)g for 90 min, and recovery was calculated by (a/b) × 100, where a = cpm/vol supernatant after centrifugation and b = cpm/vol vesicle suspension prior to centrifugation. In the case of the DOPC/DOPE (1:1) LUV, the LUV pellet was resuspended in fresh 50 mM KCl after each of four consecutive centrifugations. [b] Cumulative number of centrifugations [90 min at (2×10^5)g]. [c] In supernatant.

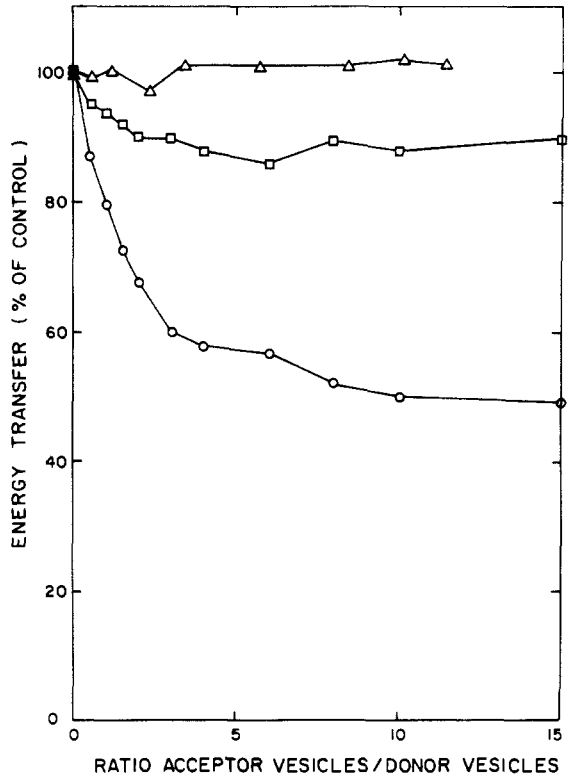

FIGURE 3: The exchangeable pool of NBD–lipids resides in the external leaflet of the vesicle bilayer. The energy transfer in rhodamine- and NBD-containing donor vesicles was measured as a function of added DOPC SUV acceptors. Loss of energy transfer resulted when the NBD analogues exchanged into the DOPC acceptor vesicle and were no longer in close proximity to rhodamine. Donor vesicles were multilamellar (□) or SUV (○) made from DOPC/*N*-Rh-PE/C_6-NBD-PC (98:1:1) or SUV (△) made from DOPC/*N*-Rh-PE/*N*-NBD-PE (98:1:1).

lipid species may not distribute at random (Litman, 1973; Lentz & Litman, 1978) across the bilayer. Finally, it should be noted that in control experiments using donor SUV containing the nonexchangeable polar head group labeled analogue *N*-NBD-PE (Struck & Pagano, 1980), no diminution in energy transfer was seen regardless of the amount of acceptors used. Thus, from the similarity of the data in Figure 3 to previous estimates of the amount of externally disposed lipid in vesicle bilayers, we conclude that *the exchangeable pool of NBD–lipids corresponds only to fluorescent lipids present in the external leaflet of the vesicle bilayer, while the nonexchangeable lipids correspond to the pool of NBD–lipids residing in the internal leaflet.* This conclusion is also supported by previous studies from this laboratory using isotopically asymmetric SUV in which it was shown that the outer, but not inner, leaflet lipids of the vesicle can participate in the exchange of lipids between vesicles and cells (Sandra & Pagano, 1979).

Generation of Asymmetric Vesicles on a Preparative Scale by Spontaneous Transfer of NBD–Lipid Analogues between Vesicle Populations. While separation of LUV and SUV can be achieved by gel-filtration chromatography for analytical purposes (Figure 1), complete separation and good recovery of SUV with this method are difficult on a preparative scale. For this reason, we sought to develop an alternative method for separation of vesicle populations on the basis of their sedimentation properties. Conditions were sought under which virtually all vesicles of one type would sediment upon centrifugation, while vesicles of a second type would remain in the supernatant. Table I summarizes our results using SUV and LUV of different lipid compositions and in two different buffer systems. As can be seen, SUV formed from DOPC, DOPC/C_6-NBD-PC (95:5), or DOPC/C_{12}-NBD-PC (95:5) were almost completely recovered in the supernatant following centrifugation. This finding is in agreement with others (Huang, 1969; Barenholz et al., 1977) who have demonstrated that prolonged centrifugation of sonicated vesicle preparations under similar conditions results in sedimentation of contaminating multilamellar and larger single-walled vesicles, but not of the smaller limiting-diameter SUV. Three types of LUV acceptors were tested for their ability to sediment under similar conditions so that essentially complete separation from the SUV donors could be achieved. LUV composed solely of DOPC were unsuitable for this purpose since only about 25% was recovered in the lipid pellet under the conditions employed (Table I). However, LUV composed of Br-PC (Fox et al., 1970; Dawidowicz & Rothman, 1976) or DOPC/DOPE (1:1) proved to be satisfactory in this respect. In the case of 1:1 DOPC/DOPE, however, it was necessary to pellet and wash the LUV several times to obtain a preparation in which "contamination" of the high-speed supernatant was <2%. The large unilamellar nature of these vesicles was confirmed by negative-staining electron microscopy, and the results reported in this paper were independent of whether LUV formed from Br-PC or 1:1 DOPC/DOPE were used.

When [^{14}C]DOPC/C_6-NBD-PC (95:5) donor SUV were subjected to two successive incubations with [^{3}H]DOPC/DOPE (1:1) acceptor LUV, a large fraction of the C_6-NBD-PC initially present in the nonsedimentable donor vesicles was recovered in the LUV pellet after high-speed centrifugation. As can be seen from the data in Table II, the donor SUV recovered after the second centrifugation had a ratio of C_6-NBD-PC/[^{14}C]DOPC which was approximately one-half that

Table II: Recovery of Donor (NBD-PC Containing) Vesicles at Various Stages of Incubation with Acceptor Vesicles[a]

SUV preparation	max contamination by LUV acceptor vesicles (mg)	donor vesicles (mg)	NBD fluorescence (RFU)[b]	NBD fluorescence/ mg of donor vesicles	recovery of donor vesicles (%)
starting donor vesicles	-	1.0	1.05	1.05	100
high-speed supernatant after first incubation	$\lesssim$0.1	0.88	0.59	0.67	88
high-speed supernatant after second incubation	$\lesssim$0.10	0.68	0.31	0.46	68

[a] Donor SUV containing [^{14}C]DOPC/C_6-NBD-PC (95:5) were incubated twice with acceptor LUV containing [^{3}H]DOPC/[^{3}H]DOPE (1:1) for 10 min at 22 °C. The vesicles were separated after each incubation by ultracentrifugation, and the SUV supernatant was recovered (see Experimental Procedures). NBD fluorescence of SUV aliquots was monitored at 530 nm (λ_{ex} 470 nm) and SUV concentrations were determined by scintillation counting. Recovery of donor vesicles was determined as for Table I. [b] Relative fluorescence units.

Table III: Asymmetry Data for SUV Donors Containing NBD-PC Analogues[a]

analogue in SUV donors	no. incubations with LUV acceptors	distribution of NBD-lipid in 100 μg of recovered SUV: total NBD (μg)	inner leaflet (nonexchangeable) (μg)	outer leaflet (exchangeable) (μg)	% NBD-lipid exchanged
C_6-NBD-PC	0	5.23	1.86	3.37	64
	2	2.20	2.00	0.20	9
C_{12}-NBD-PC[b]	0	5.00	1.89	3.11	62
	2	2.02	1.84	0.18	9

[a] Uniformly labeled donor SUV containing DOPC and various NBD-lipid analogues (95:5) were prepared as described (see Experimental Procedures), incubated under appropriate conditions twice with a 10-fold excess of acceptor DOPC/DOPE (1:1) LUV or Br-PC LUV, and separated from acceptor LUV as described in Table II. For determination of the size of the exchangeable pool of NBD-lipid in the unknown samples, a known amount of the sample (determined from radioactivity measurements) was added to a known amount of DOPC/*N*-Rh-PE SUV, the excitation spectrum (λ_{em} 600 nm) was taken, and the amount of energy transfer was determined by using eq 1. The correction for NBD fluorescence emission at 600 nm in the absence of rhodamine (h_2 in eq 1) was made by measuring the NBD fluorescence in the sample (λ_{ex} 470 nm; λ_{em} 530 nm) and using standard curves I and II (see Materials and Methods). From this value of energy transfer in the unknown sample, the amount of NBD-lipid which distributed into the rhodamine-containing vesicles could then be calculated from a standard curve relating the amount of energy transfer seen in a series of SUV containing 1 mol % *N*-Rh-PE and variable amounts of C_6-NBD-PC/DOPC. Under the assumption that the equilibrium distribution of the NBD-lipid between the unknown SUV sample and the DOPC/*N*-Rh-PE SUV simply reflects the amounts of each vesicle present (see text), the total amount of NBD-lipid in the exchangeable pool could then be calculated. In the case of equal amounts of the unknown sample and the rhodamine-containing vesicles, for example, the exchangeable pool size would be twice the amount of NBD found associated with the DOPC/*N*-Rh-PE vesicles. [b] Because the half-time for equilibration of C_{12}-NBD-PC ($t_{1/2} \simeq$ 10 min, 22 °C) between vesicle populations was much longer than that for C_6-NBD-PC ($t_{1/2} \simeq$ s, 22 °C) (unpublished observations), incubations with C_{12}-NBD-PC were carried out at 37 °C for 60 min to assure complete equilibration of the probe between vesicle populations.

of the starting SUV preparation. The overall yield of SUV in this type of experiment is ~70% based on [^{14}C]DOPC recovery), with losses due primarily to the deliberate discarding of the supernatant closest to the LUV pellet and to concentrating the vesicles at various stages in the experiment. The data in Table II (and Figure 1c) also demonstrate that the loss of C_6-NBD-PC from donor to acceptor vesicles is not accompanied by a simultaneous movement of nonfluorescent, isotopically labeled lipids in the opposite direction. Thus we conclude that the NBD–lipid analogues move via a mass transfer rather than a one-for-one exchange of lipid monomers between vesicles. Finally, it should be noted that while there is a substantial loss of the NBD–lipid present in donor vesicles upon incubation with appropriate acceptors, the amount of NBD–lipid present initially is only 5% of the total vesicle lipid. Therefore a net loss of 50–60% of the fluorescent species does not represent a gross change in the total amount of lipid present in the vesicles.

Quantitative Determination of NBD–Lipid Asymmetry in Vesicles by Resonance Energy Transfer. The degree of NBD–lipid asymmetry in vesicles can be determined by using resonance energy transfer by mixing vesicles containing an exchangeable NBD–lipid with DOPC/*N*-Rh-PE SUV. Any NBD–lipid present in the external leaflet of the former preparation will equilibrate between the two vesicle populations according to the relative amounts of the two types present (see Figure 3). Because of this equilibration, energy transfer between NBD and rhodamine will result, with the level of energy transfer being a linear function of the mole fraction of NBD–lipid present in the *N*-Rh-PE containing vesicles (unpublished data). This is due to the fact that the efficiency of energy transfer is independent of the surface density (moles of donor per mole of total lipid) of the energy donor (Fung & Stryer, 1978). Since the amounts of lipid present in each vesicle type are known quantities, it is then possible to estimate the exchangeable pool of NBD–lipid in the mixture from the measured fluorescence energy transfer.

Typical spectra of such mixtures are shown in Figure 4. In Figure 4a, DOPC/C_6-NBD-PC and DOPC/*N*-Rh-PE vesicles were present, while in Figure 4b, a similar preparation of DOPC/C_6-NBD-PC vesicles which had previously been incubated and separated from LUV acceptors (Table II) were mixed with DOPC/*N*-Rh-PE vesicles. While the absolute amounts of lipid present in the C_6-NBD-PC containing vesicles were the same in both cases, the amount of energy transfer (excitation peak height at 470 nm; λ_{obsd} 600 nm) observed in Figure 4a was considerably greater than that observed in Figure 4b. After correction for NBD emission at 600 nm and the direct excitation of rhodamine by 470-nm light, this difference is even more pronounced. Thus, incubating DOPC/C_6-NBD-PC SUV with LUV acceptors markedly reduces the size of the exchangeable pool of NBD-PC in the recovered SUV.

Table III summarizes typical data obtained by using the resonance energy-transfer assay on SUV preparations containing DOPC and C_6- or C_{12}-NBD-PC. In each case, the

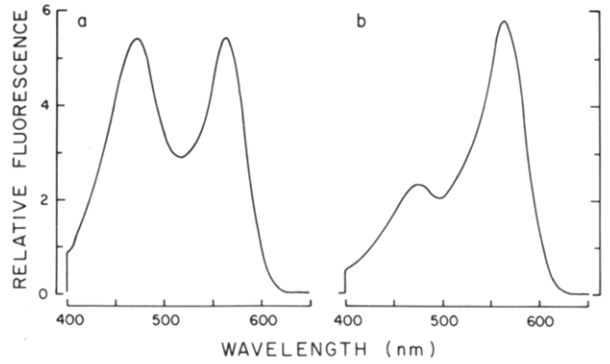

FIGURE 4: Use of resonance energy transfer to assay the pool size of exchangeable NBD–lipids in vesicles. Excitation spectra (λ_{em} 600 nm) were taken of 100 μg of DOPC/*N*-Rh-PE vesicles (99:1) which were mixed with 100 μg of (a) DOPC/C_6-NBD-PC (95:5) SUV or (b) DOPC/C_6-NBD-PC (95:5) SUV which had first been incubated with and separated from an excess of LUV. Note that while the same amounts of unknown samples were present in both (a) and (b), the levels of energy transfer (a measure of the quantity of exchangeable NBD-PC present) are very different.

exchangeable pool of NBD–lipid was substantially reduced compared to the starting vesicles, following incubations with LUV acceptors. Since the exchangeable pool represents lipid present in the external leaflet of the SUV (see above), this results in a significant asymmetry in the distribution of the NBD–lipid remaining in the recovered SUV. Thus, by use of these methods, vesicles with a nearly absolute asymmetry in NBD–lipid can be generated.

Finally, it should be noted that the procedure used to generate SUV in which the inner leaflet is enriched with an NBD–lipid can be adapted to produce SUV with an opposite asymmetry. In one such experiment, the starting SUV were nonfluorescent DOPC, while the LUV contained 20 mol % C_6-NBD-PC. Following isolation of the SUV fraction, the resonance energy transfer assay indicated that essentially 100% of the NBD–lipid that had been transferred to the SUV was present in the exchangeable, outer leaflet pool of the vesicles.

Incubation of Asymmetric Vesicles with Cells. Small unilamellar vesicles can interact with mammalian cells in a number of ways including vesicle–cell adsorption, fusion, endocytosis, and lipid transfer [reviewed in Pagano & Weinstein (1978)]. The mechanism which predominates under a given set of conditions depends on a number of factors including cell type, temperature, and vesicle lipid composition. Previous studies from this laboratory have shown that at 2 °C, SUV comprised of DOPC or DOPC/DOPE interact with Chinese hamster fibroblasts by the process of lipid exchange (Pagano & Huang, 1975; Sandra & Pagano, 1979). Unlike other mechanisms of vesicle–cell interaction, lipid exchange involves the transfer of vesicle lipids, but not trapped contents, to the recipient cells. Recently we extended these observations on vesicle–cell lipid exchange by using DOPC vesicles containing C_6-NBD-PC or -PE (Struck & Pagano, 1980). Examination of cells treated at 2 °C by fluorescence microscopy showed a strong fluorescent ring associated with the cell periphery, suggesting that the majority of the NBD–lipid was confined to the plasma membrane of the recipient cells.

In the present study, we incubated "uniform" and "asymmetric" DOPC/C_6-NBD-PC vesicles with cells at 2 °C, subsequently examined the cells by fluorescence microscopy, and quantified the degree of NBD–lipid uptake by the cells. Cells treated with uniformly labeled vesicles showed bright peripheral ring staining as previously seen (Struck & Pagano, 1980), while cells treated with asymmetric vesicles were nonfluorescent, even though the total amount of vesicle lipid

Table IV: Uptake of C_6-NBD-PC by Chinese Hamster Fibroblasts at 2 °C from Uniform and Asymmetric Vesicles[a]

	μg of C_6-NBD-PC in exchangeable (outer leaflet) pool of 100-μg DOPC vesicles	cellular uptake (ng of C_6-NBD-PC/10^7 cells)
uniform	2.49	77
asymmetric	0.27	8
uniform/asymmetric	9.22	9.63

[a] Uniform DOPC/C_6-NBD-PC (95:5) and asymmetric USV were prepared, and the distribution of C_6-NBD-PC on the inner and outer leaflets of the vesicles was determined by using resonance energy transfer (see Table III and Results and Discussion). The uptake of the fluorescent lipid by Chinese hamster fibroblasts following a 60-min vesicle–cell incubation at 2 °C (96 μg of lipid and 10^7 cells/mL) was then determined as described under Experimental Procedures.

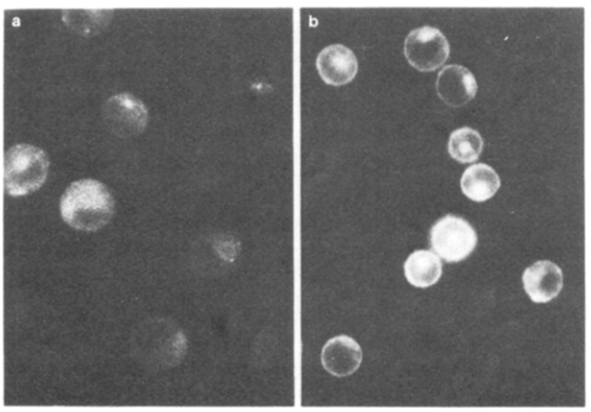

FIGURE 5: Fluorescence photomicrographs of Chinese hamster fibroblasts treated with DOPC/C_6-NBD-PC (95:5) SUV for 30 min at 37 °C. In (a), NBD–lipid was restricted to the inner leaflet of the vesicle bilayer (see text), while in (b), "uniform" vesicles in which the NBD–lipid was present on both leaflets of the vesicle bilayer were used. Incubation conditions: 5×10^6 cells and 2 μg of vesicle lipid/mL.

present in each incubation was the same (96 μg/mL). The quantitative results of this experiment are summarized in Table IV where it is seen that the amount of C_6-NBD-PC from uniform vesicles which became cell associated was about 10 times greater than the uptake from asymmetric vesicles. These results are consistent with our earier findings using isotopically asymmetric vesicles (Sandra & Pagano, 1979) which showed that at 2 °C, only those lipids in the outer leaflet of the vesicle bilayer participate in vesicle–cell exchange, with essentially no concomitant vesicle–cell adsorption.

The use of asymmetric vesicles with cells at 37 °C may prove to be useful in distinguishing vesicle–cell adsorption from other mechanisms of vesicle uptake. Fluorescence micrographs of Chinese hamster fibroblasts incubated with uniform or asymmetric vesicles at 37 °C (2 μg of vesicle lipid/mL) are shown in Figure 5. When uniform vesicles were used, the cells showed a strong peripheral ring fluorescence as well as internal fluorescence (Figure 5b), whereas when the cells were incubated with an identical concentration of asymmetric vesicles, only internal fluorescence was seen (figure 5a). Since adsorption of intact, inner leaflet labeled vesicles at the cell periphery should give rise to fluorescent ring stained cells, its absence excludes the possibility of vesicle–cell adsorption under these incubation conditions. It should be noted, however, that at higher lipid concentrations (≥20 μg of lipid/mL of cell suspension), such asymmetric vesicles gave increasing amounts of ring staining, suggesting that vesicle–cell adsorption is a

concentration-dependent process (R. E. Pagano et al., unpublished observations).

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