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N-NBD-L-α-DILAUROYLPHOSPHATIDYLETHANOLAMINE

A NEW FLUORESCENT PROBE TO STUDY SPONTANEOUS LIPID TRANSFER *

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Migration of the fluorescent phospholipid *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-L-α-dilauroylphosphatidylethanolamine between small sonicated egg phosphatidylcholine vesicles was studied by use of the fluorescence resonance energy transfer method. Contrary to the results of lipid transfer experiments reported for acyl chain NBD-labeled phospholipids (Nichols, J.W. and Pagano, R.E. (1982) Biochemistry 21, 1720–1728), the migration kinetics of *N*-NBD-DLPE had to be described by a sum of two exponential functions. The fast component ($t_{1/2} \approx 38$ min) was assigned to lipid transfer via soluble monomers and the slow component ($t_{1/2} \approx 400$ min) to transbilayer motion. A reversible four-stage process is suggested as a kinetic model. Mathematical treatment of this scheme is given yielding an analytical expression for the time dependence of NBD emission intensity. The use of *N*-NBD-DLPE in the resonance energy transfer measurements offers the advantage of simple chemical synthesis of the fluorescent probe and leads to additional information on transbilayer motion which was not available with the NBD-labeled lipids used so far.

Introduction

Mutual interaction of lipid membranes occurs in two different ways. The first one requires direct physical membrane contact leading to fusion of lipid regions and volumes trapped by the membranes. This type of interaction is responsible for many cell biological processes like transport of macromolecules through plasma membranes by exo- and endocytosis, fertilization or pathological cell fusion [1]. Moreover, it is discussed as a possible mechanism for the vesicle-mediated uptake of drugs and pharmaceutica by cells [2]. The second, indirect way, known as transfer of amphipathic molecules like phospholipids or cholesterol through the aqueous phase is important for membrane assembly and control of membrane composition. In view of its importance for cell biological processes, as well as for medical applications, the investigation of membrane communication has been the subject of various biophysical publications. An elegant and sensitive method to study lipid transfer between vesicles makes use of resonance transfer of excitation energy between fluorescent groups covalently linked to the membranes [3,4]. In a typical experiment, NBD as the energy donor group is attached to the hydrophobic part of the migrating phospholipid molecules, while tetramethylrhodamine as energy acceptor is linked to the free amino group of nonmigrating DPPE. Both types of labeled phospholipids are incorpo-

* Dedicated to Professor O.E. Polansky on the occasion of his 65th birthday.

Abbreviations: DLPE, L-α-dilauroylphosphatidylethanolamine; DPPE, L-α-dipalmitoylphosphatidylethanolamine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; NBD, 7-nitro-2,1,3-benzoxadiazol-4-yl; DOPC, dioleoylphosphatidylcholine.

rated in amounts of 1–2 mol% in donor vesicles. The NBD emission intensity is strongly quenched by effective energy transfer to neighbouring tetramethylrhodamine groups. Upon addition of unlabeled acceptor vesicles, NBD-containing lipids migrate from donor to acceptor membranes and begin to exhibit full fluorescence intensity. Thus, the main contribution to the increase in fluorescence intensity arises from those NBD-labeled lipids which are incorporated into the acceptor vesicles, while a relatively small intensity decresae is induced in the donor vesicles. In their experiments, Nichols and Pagano [3,4] made use of a special class of molecules with the energy donor group covalently attached to a C_6- or C_{12}-chain in the hydrophobic part of phospholipids (C_6-, C_{12}-NBD lipids). The advantage of many of these compounds can be seen in their optimal migration rate which is high enough to circumvent the application of stopped-flow techniques and short enough to avoid artefacts due to instrumental fluctuations during long-time measurements. On the other hand, a three-step procedure is necessary to synthesize the lipid analogues which makes their application difficult from the chemical point of view. For this reason, we feel that it would be useful to make available a different type of fluorescent lipid for the resonance, energy transfer measurements by linking NBD with the free amino group of PE. So far, the *N*-NBD analogues of PE derived from egg PC [5] and from DOPC [6] have been described as the only representatives of this class of labeled lipids. The compounds proved to be unsuitable for migration studies due to their extremely low transfer rates between lipid vesicles, and for this reason headgroup-labeled PE derivatives are generally regarded as compounds which do not undergo spontaneous transfer between membranes. However, taking into account the influence of fatty acid chain length on the transfer rate of lipids [7,8], we expect reasonably high migration rates for PE molecules with short acyl chains. This idea prompted us to react NBD chloride with commercially available DLPE and use the product for resonance energy transfer measurements instead of C_6 or C_{12}-NBD lipids. While changes in NBD emission intensity upon transfer of the C_6- and C_{12}-NBD lipids could be described by a single exponential function, the corresponding results for the migration of the new compound *N*-NBD-DLPE were more complicated. A detailed analysis of the kinetic data is given in the present study.

Materials and Methods

Chemicals

NBD chloride was purchased from Fluka and *N*-NBD-ethanolamine was obtained from Molecular Probes, Plano, TX.

Lipids. Egg yolk L-α-phosphatidylcholine grade 1 was purchased from Lipid Products, South Nutfield, U.K. DLPE was from Fluka. Both lipids showed single spots on TLC with $CHCl_3$/MeOH/H_2O (65 : 24 : 4, v/v).

Methods

N-NBD-DLPE was prepared as described in Ref. 5 by mixing solutions of 100 mg DLPE (180 μmol) in 5 ml $CHCl_3$/MeOH (1 : 1, v/v) and 100 mg NBD chloride (500 μmol) in 5 ml $CHCl_3$/MeOH (1 : 1, v/v) and adding 35 μl triethylamine (25 μmol). After incubation at 25 °C for 1 h, the solvent was removed in a rotary evaporator. For purification of *N*-NBD-DLPE by medium pressure liquid chromatography [9], the reaction mixture was redissolved in 1 ml $CHCl_3$/MeOH (1 : 1, v/v), applied to a prepacked column (LiChroprep Si 60, 40–63 μm, Lobar A) and eluted with a linear gradient ranging from 100% $CHCl_3$ to 10% $CHCl_3$/90% MeOH (v/v). Unreacted dye and DLPE could be separated from the reaction product *N*-NBD-DLPE which was characterized by elementary analysis of N (7.38%; calc. 7.5%) and P (4.15%; calc. 4.3%), and by ^{1}H-NMR (90 MHz, dimethylsulfoxide-d_6): δ 8.60 (d, 1 H, NBD, $J = 9.0$ Hz), 6.51 (d, 1 H, NBD, $J = 9.0$ Hz), 1.30 (s, 4 OH, methylene), 0.94 (m, 6 H, methyl). The corresponding ^{1}H-NMR data for NBD chloride under the same conditions are: δ 8.78 (d, 1 H, $J = 7.6$ Hz), 8.11 (d, 1 H, $J = 7.6$ Hz). *N*-NBD-DLPE was isolated with a yield of 60–70%. The purified product showed a single spot on analytical TLC plates with an R_F value of 0.53 in $CHCl_3$/MeOH/H_2O (65 : 25 : 4, v/v).

N-Tetramethylrhodamine-DPPE was synthesized from tetramethylrhodamineisothiocyanate (Fluka) and DPPE in $CHCl_3$/MeOH (2 : 1, v/v) according to Ref. 10. The compound was purified

on preparative TLC plates (silica gel) in a solvent mixture of chloroform/methanol/acetone/acetic acid/water (5 : 1 : 2 : 1 : 0.5, v/v). The final product showed a single spot on analytical TLC plates ($R_F = 0.76$) with the same solvent mixture.

Concentrations of labeled phospholipids in stock solutions ($CHCl_3$/MeOH) were determined from the absorbances using molar absorption coefficients of $6.4 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ for NBD at 460 nm [5] and $9 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ for tetramethylrhodamine at 553 nm [10].

Preparation of vesicles. Small unilamellar vesicles from egg yolk L-α-phosphatidylcholine were prepared by the sonication method in 10 mM Tris-HCl (pH 7.4) using a Branson sonifier B15 for 20 min under Ar. Aggregates and multilamellar vesicles were removed by ultracentrifugation at $100\,000 \times g$ and passage over a Sepharose 4B column [11].

Transfer of N-NBD-DLPE between egg yolk L-α-phosphatidylcholine vesicles. Donor vesicles labeled with 1 mol% *N*-NBD-DLPE and 2.5 mol% *N*-tetramethylrhodamine-DPPE were mixed at zero-time with unlabeled vesicles under Ar in the fluorescence cell which was sealed afterwards with a Teflon stopcock to exclude oxygen during the measurements.

Lipid concentrations were determined by phosphate analysis [12]. Donor vesicle concentration was 0.15 mg/ml (approx. 250 μM) unless otherwise noted.

Fluorescence measurements were performed on a Perkin-Elmer LS 5 spectrofluorometer at 25 °C in a thermostatically controlled sample holder. NBD was excited at 475 nm. For transfer kinetics the fluorescence intensity was monitored at the maximum (530 nm). In order to compensate for instrumental fluctuations during the measurements, emission intensities were calibrated with respect to a standard probe ($1 \cdot 10^{-6}$ M NBD-ethanolamine in ethanol). Emission intensities were insensitive to gentle manual shaking of the sample which means that artefacts due to vesicle agregation and settling of fluorescent materials were insignificant. The correlation between fluorescence intensities F and concentrations C of *N*-NBD-DLPE in donor (D) and acceptor (A) vesicles is given by

$$F = f_D C \tag{1a}$$

$$F = f_A C \tag{1b}$$

according to Ref. 4. Proportionality contants f_D and f_A were determined from calibration plots obtained from samples of donor and acceptor vesicles labeled with varying small quantities of *N*-NBD-DLPE.

Kinetic calculations. Changes of fluorescence intensities with time were analysed by fitting the experimental data with the function

$$F(t) = a - \sum_{i=1}^{m} b_i e^{-\gamma_i t} \qquad (m = 1, 2) \tag{2}$$

by a nonlinear least-squares method [13]. The quality of the fit was assessed by comparing experimental and calculated data points and by the residuals.

Equilibrium emission intensities. The emission intensities F_{eq} at equilibrium give information on the amount of fluorescent lipids available for transfer. The following two cases have to be considered:

(1) All NBD-labeled lipid molecules undergo transfer. The equilibrium concentrations of the fluorescent lipid in donor and acceptor vesicles, C^{D}_{eq1} and C^{A}_{eq1}, are given by

$$C^{D}_{eq1} = \frac{1}{1+n} C_T \tag{3a}$$

$$C^{A}_{eq1} = \frac{n}{1+n} C_T \tag{3b}$$

n is the ratio of acceptor to donor vesicle concentration and C_T is the total concentration of *N*-NBD-DLPE. By use of Eqn. 1, we can calculate the equilibrium emission intensity

$$F_{eq1} = \frac{1}{1+n} f_D C_T + \frac{n}{1+n} f_A C_T \tag{4}$$

(2) Only NBD-labeled lipids incorporated in the outer leaflet of donor vesicles are available for transfer. Assuming a distribution of 2/3 of the migrating lipid in the outer and 1/3 in the inner leaflet [14] the concentrations are:

$$C^{D}_{eq2} = \tfrac{1}{3} C_T + \tfrac{2}{3} \frac{1}{1+n} C_T \tag{5a}$$

$$C^{A}_{eq2} = \tfrac{2}{3} \frac{n}{1+n} C_T \tag{5b}$$

The equilibrium emission intensity for this case is:

$$F_{eq2} = \tfrac{2}{3}F_{eq1} + \tfrac{1}{3}f_D C_T \quad (6)$$

Eqns. 4 and 6 were normalized with respect to $F_A = f_A C_T$, the maximal possible emission intensity:

$$F'_{eq1} = \left(\frac{F_{eq1}}{f_A C_T}\right) \times 100[\%]$$

$$= \frac{1}{1+n}\left(\frac{f_D}{f_A} + n\right) \times 100[\%] \quad (7a)$$

$$F'_{eq2} = \left(\tfrac{2}{3}F'_{eq1} + \tfrac{1}{3}\frac{f_D}{f_A}\right) \times 100[\%] \quad (7b)$$

Results

Preparation and purification of N-NBD-DLPE

N-NBD-DLPE was prepared by standard methods [5]. However, the purification of the reaction product was carried out in a more efficient way than described [5], by medium pressure liquid chromatography [9]. The separation of the reaction mixture into three main components is demonstrated in Fig. 1.

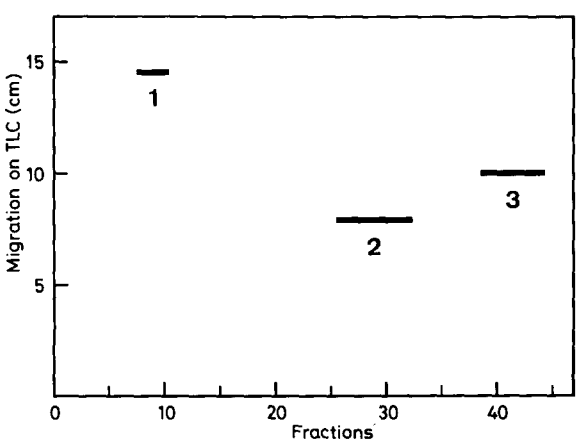


Fig. 1. Two-dimensional chromatography of the reaction mixture of NBD chloride and DLPE. (1) Unreacted dye; (2) *N*-NBD-DLPE; (3) unreacted DLPE (stained with ninhydrin). Bands 1 and 2 were yellow and could not be stained with ninhydrin.

Spontaneous transfer of N-NBD-DLPE between lipid vesicles

The interpretation of kinetic data for transfer of lipid molecules between vesicles is based upon a model which was originally proposed by Nakagawa [15] to describe micelle association and dissociation, and which was applied by Thilo [16] and by Nichols and Pagano [3,4] to the migration process of lipid molecules through the aqueous phase. Phospholipids (P) are assumed to exist in equilibrium between the bilayer (b) and monomer state (m). Spontaneous transfer of NBD-labeled lipids between donor (D) and acceptor (A) vesicles prepared by the same method is described in Scheme I for the special case of identical matrix lipids.

Scheme I.

$$(P_b)_D \underset{k_+}{\overset{k_-}{\rightleftharpoons}} P_m \underset{k_-}{\overset{k_+}{\rightleftharpoons}} (P_b)_A$$

k_- and k_+ are rate constants for exit and entry of monomers of P from and into bilayers. The following features are characteristic for this model: firstly, the migration process can be described by a single exponential function with the time constant $1/k_-$, i.e., the exit rate of the probe from donor vesicles is the rate-determining step and secondly, the transfer rate is independent on vesicle concentration.

In the following, we present our results on the transfer of *N*-NBD-DLPE between small unilamellar vesicles from egg yolk L-α-phosphatidylcholine. The time-course of NBD emission intensity after mixing the two vesicle populations is depicted in Fig. 2. Proportional increase in concentrations of donor and acceptor vesicles from $2 \cdot 10^{-6}$ M to $2 \cdot 10^{-4}$ M had no influence on the profile of the curve which is strong evidence that a collision-mediated mechanism can be excluded and *N*-NBD-DLPE migrates as a soluble monomer through the aqueous phase. Contrary to the results reported by Nichols and Pagano [3,4] on the migration of acyl chain NBD-labeled lipids, a sum of two exponential functions (Eqn. 1, $m = 2$) had to be applied to obtain satisfactory fits of the data.

In the experiment with C_6- and C_{12}-NBD-lipids, only the probes incorporated in the outer membrane leaflet were available for spontaneous

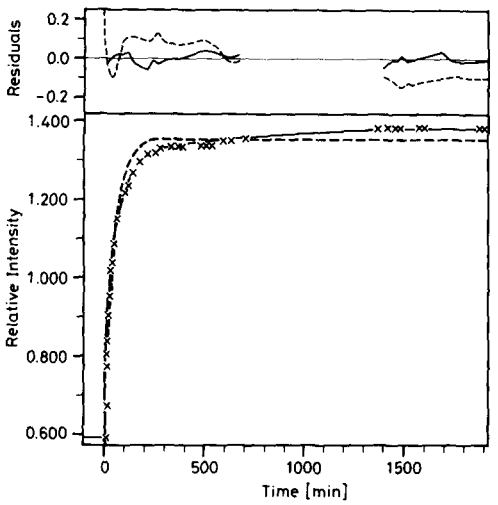


Fig. 2. Time-dependence of NBD-emission intensity (×) in a 1:1 mixture of donor and acceptor vesicles with a total lipid concentration of 250 μM. Donor vesicles were egg yolk L-α-phosphatidylcholine small unilamellar vesicles containing 1 mol% *N*-NBD-DLPE and 2.5 mol% *N*-tetramethylrhodamine-DPPE. Acceptor vesicles were unlabeled egg yolk L-α-phosphatidylcholine small unilamellar vesicles. The function given in Eqn. 2 was fitted by a nonlinear least-squares procedure to the experimental data. The single exponential fit is given by the dashed line, and the double exponential fit by the solid line. The corresponding residuals are shown in the upper pannel.

transfer [4]. The situation is quite different for vesicles labeled with *N*-NBD-DLPE. As shown in Table I, all the probe molecules can be transferred. This means that not only lipid transfer has to be taken into account but in addition transverse mo-

TABLE I

EQUILIBRIUM EMISSION INTENSITIES IN % (± MAXIMUM ERRORS)

n is the ratio of acceptor to donor vesicle concentration. F_{eq} is the experimentally determined emission intensity at infinite time (2000 min) in % of maximal possible value. F'_{eq1} was calculated from Eqn. 7a with the assumption that all *N*-NBD-DLPE molecules are available for transfer. F'_{eq2} was calculated from Eqn. 7b with the assumption that only *N*-NBD-DLPE molecules incorporated in the outer leaflet of donor vesicles are available for transfer.

n	F_{eq}	F'_{eq1}	F'_{eq2}
1	60±4	62±2	50±2
2	73±4	75±2	58±2
10	93±4	93±2	70±2

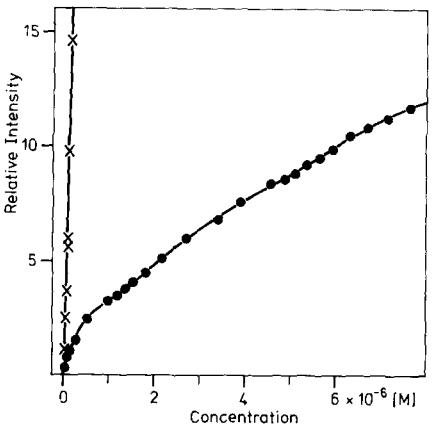


Fig. 3. Plot of emission intensity of an aqueous solution of *N*-NBD-ethanolamine (×) and an aqueous suspension of *N*-NBD-DLPE (●) vs. concentration. $\lambda_{exc} = 475$ nm, $\lambda_{em} = 535$ nm.

tion of the fluorescent probe across the bilayers contributes to the overall process.

Critical bilayer concentration of N-NBD-DLPE

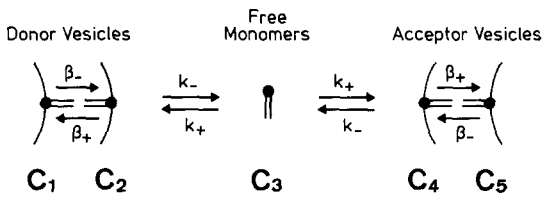
An important parameter is the solubility of the lipid monomer, i.e., the critical bilayer concentration. Attempts to determine this parameter for NBD-labeled lipids have been described in [3.4]. From breaks in plots of NBD emission intensity vs. concentration, the regions of aggregation and fluorescence self-quenching have been determined. In our experience, the reliability of the method suffered from inner filter effects. We, therefore, used a different procedure. In Fig. 3, fluorescence emission intensities of *N*-NBD-ethanolamine and of *N*-NBD-DLPE were compared. We may assume that inner filter effects are comparable for both samples and that differences in fluorescence intensities are solely due to self-quenching in lipid aggregates. The two curves in Fig. 3 differ at concentrations as low as the detection limit of the method (approx. 10^{-8} M). This means that the critical bilayer concentration of *N*-NBD-DLPE is lower than 10^{-8} M.

Discussion

In order to explain the results in Table I and the biexponential growth of emission intensity in Fig. 2, we have to extend the model presented in

Scheme I. For the case of identical donor and acceptor matrices, we obtain the sequence of transbilayer motion and lipid transfer as shown in Scheme II.

Scheme II.



The concentrations of *N*-NBD-DLPE in the different kinetic pools is given by C_1–C_5. β_- and β_+ are rate constants for the transverse lipid motion. According to Scheme II, migration of *N*-NBD-DLPE can be treated as a reversible four-stage reaction. In analogy to the Nakagawa model [15,16], we assume that the exit rate of the labeled lipid is dependent on its concentration in the bilayer state, while the entry of monomers into the bilayers is proportional to the product of monomer concentration C_3 and the total bilayer surface area in a unit volume. The latter is proportional to the concentration of lipid molecules in the bilayer state and is represented by the parameters D and A for donor and acceptor vesicles. D and A are proportional to C_D and C_A, the concentrations of donor and acceptor matrices, when the influence of the small amounts of NBD- and tetramethylrhodamine-labeled lipids on the vesicle surface areas and on lipid concentrations of donor and acceptor vesicles is neglected [3,4]. Therefore, migration of *N*-NBD-DLPE according to Scheme II can be described by the following system of differential equations:

$$\frac{dC_1}{dt} = -\beta_- C_1 + \beta_+ C_2 \tag{8a}$$

$$\frac{dC_2}{dt} = \beta_- C_1 - \beta_+ C_2 - k_- C_2 + k_+ D C_3 \tag{8b}$$

$$\frac{dC_3}{dt} = k_- C_2 - k_+ D C_3 - k_+ A C_3 + k_- C_4 \tag{8c}$$

$$\frac{dC_4}{dt} = k_+ A C_3 - k_- C_4 - \beta_+ C_4 + \beta_- C_5 \tag{8d}$$

$$\frac{dC_5}{dt} = \beta_+ C_4 - \beta_- C_5 \tag{8e}$$

According to Ref. 4, the calculation can be simplified by the assumption that for the monomer pool, the steady-state equilibrium is reached fast compared to lipid migration. With $dC_3/dt = 0$ we obtain:

$$C_3 = \frac{k_-}{(A+D)k_+} C_2 + \frac{k_-}{(A+D)k_+} C_4 \tag{9}$$

After eliminating C_3, Eqns. 8a–e were solved by the operator method [17] (see Appendix). The NBD emission intensity $F(t)$ (fig. 2) is the sum of contributions from kinetic pools 1, 2, 4 and 5:

$$F(t) = f_D(C_1 + C_2) + f_A(C_4 + C_5) \tag{10}$$

where f_D and f_A are calibration factors (see Materials and Methods, Eqn. 1). From Eqn. 10 and the solutions of Eqn. 8a–e, we obtain Eqn. 11 for the increase of fluorescence intensity:

$$F(t) = \alpha - \alpha_1 e^{-(\beta_+ + k_-)t} - \alpha_2 e^{-\beta_- t} \tag{11}$$

It is reasonable to assume an asymmetric distribution of *N*-NBD-DLPE in the donor vesicles. In small sonicated vesicles, a ratio of about 2 : 1 for the number of lipid molecules in the outer and inner monolayer is generally accepted [14,18]. Concentrations are given in moles of lipid per unit volume of bulk solution (see Refs. 3 and 4) which leads to:

$$C_2 = 2C_1 \tag{12}$$

and, from equilibrium conditions:

$$\beta_- = 2\beta_+ \tag{13}$$

From a comparison of Eqns. 2 and 11 and by use of Eqn. 13, we can calculate the rate constants β_-, β_+ and k_- from the experimentally determined γ values:

$$\beta_- = \gamma_2 \tag{14a}$$

$$\beta_+ = \gamma_2/2 \tag{14b}$$

$$k_- = \gamma_1 - \gamma_2/2 \tag{14c}$$

In Table IIA, the results for γ are listed, as well as for the rate constants β_-, β_+ and k_-. Preexponentials α calculated from Eqns. A-6a–c were in

TABLE II

RESULTS FOR SPONTANEOUS TRANSFER OF *N*-NBD-DLPE BETWEEN SMALL UNILAMELLAR VESICLES FROM EGG YOLK L-α-PHOSPHATIDYLCHOLINE FOR TWO DIFFERENT RATIOS, n, OF ACCEPTOR TO DONOR VESICLE CONCENTRATIONS

(A) Experimental γ values (Eqn. 2) and calculated rate constants (Eqn. 14) in h^{-1}. Bracketed values show half-times [a] in min. Single standard deviations are quoted for all values.

	$n=1$	$n=2$
γ_1	1.46 ± 0.06	1.104 ± 0.06
γ_2	0.09 ± 0.03	0.102 ± 0.03
k_+	1.11 ± 0.06	1.05 ± 0.06
	(37 ± 2)	(39 ± 2.5)
β_-	0.09 ± 0.03	0.102 ± 0.03
	(460 ± 170)	(410 ± 170)
β_+	0.045 ± 0.015	0.053 ± 0.015
	(900 ± 340)	(780 ± 340)

(B) Preexponentials [b]

	$n=1$	$n=2$
a	1.397 ± 0.01	1.778 ± 0.01
α	1.409 ± 0.03	1.766 ± 0.03
β_1	0.490 ± 0.01	0.746 ± 0.01
α_1	0.492 ± 0.03	0.736 ± 0.03
β_2	0.310 ± 0.01	0.440 ± 0.01
α_2	0.295 ± 0.03	0.420 ± 0.03

[a] Half-time, $t_{1/2} = \ln 2/\text{rate constant}$.

[b] a, b_1, b_2 are experimental preexponential values obtained by a nonlinear least-squares fit, Eqn. 2. α, α_1 and α_2 are the corresponding calculated values (Eqn. A-6a–c). Single standard deviations for a, β_1, β_2 and maximum errors for α, α_1, α_2 are quoted.

close agreement with the experimentally determined values for a, b_1 and b_2 (Table IIB), which is strong evidence for the validity of the kinetic model in Scheme II.

The value of $\beta_- = 0.09$ h^{-1} for transbilayer motion is in the range reported by Kornberg and McConnell [19] for flip-flop processes of spin-labeled PC in small unilamellar vesicles from egg yolk L-α-phosphatidylcholine. The difference in transversal mobility of C_6- and C_{12}-NBD lipids [4] on one hand and *N*-NBD-DLPE on the other could be due to changes in properties of the polar headgroup leading to less hydrophobic behavior upon modification of the primary amino group. Similar reasons seem to be responsible for the differences in flip-flop rates for PC ($t_{1/2} \approx 11$ days) [20] and spin-labeled PC ($t_{1/2} = 6.5$ h) [19].

In the literature, transfer rates of phospholipids are mainly correlated with chain lengths and mobilities of fatty acid residues and with water solubilities (critical bialyer concentration) of the migrating phospholipid. For example, addition of one methylene unit to the acyl chains of pyrene-labeled phospholipids decreased their rate of transfer between apolipoprotein-phospholipid complexes by a factor of 4–5 [7], and addition of 5 methylene units [8] to pyrene alcohols reduced their transfer rates between sonicated lipid vesicles by 500. From these data, it is conceivable that *N*-NBD-DLPE (20 methylene units) migrates much faster than *N*-NBD-DPPE (28 methylene units), which indeed is known as a practically nonmigrating compound [21].

As far as the dependence of transfer rates on the critical bilayer concentration are concerned, no clear picture can be obtained from the literature. For example, in the case of C_6- and C_{12}-NBD-lipids, transfer rates and critical bilayer concentration seem to be correlated. The critical bilayer concentration for C_6-NBD-PC ($2 \cdot 10^{-7}$ M) is much higher than for C_{12}-NBD-PC (less than $1 \cdot 10^{-8}$ M) and the half-times for transfer are $t_{1/2} = 0.78$ min and 125 min, respectively. However, other workers reported low critical bilayer concentration values of less than 10^{-8} M for fast migrating lipid molecules ($t_{1/2}$ in the range of seconds [22] and minutes [23]). There appears to be no simple relation between critical bilayer concentration and transfer rates and the fact that the critical bilayer concentration for *N*-NBD-DLPE is less than $1 \cdot 10^{-8}$ M cannot be regarded as inconsistent with the relatively low half-time of intervesicle transfer.

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Appendix

In the operator method for the solution of differential equations the differentials d/dt are

replaced by the operator P:

$$\frac{dC}{dt} = PC \text{ for } C_{(t=0)} = 0 \tag{A-1}$$

$$\frac{dC}{dt} = PC - PC^0 \text{ for } C_{(t=0)} = C^0 \neq 0 \tag{A-2}$$

By combining Eqns. A-1 and A-2 with Eqns. 8a, 8b, 8d, 8e and 9, and by rearranging the expression, we obtain the following system of four linear equations:

$$PC_1 - PC_1^0 = -\beta_- C_1 + \beta_+ C_2 \tag{A-3a}$$

$$PC_2 - PC_2^0 = \beta_- C_1 - \left(\beta_+ + \frac{A}{A+D}k_-\right)C_2 + \frac{D}{A+D}k_- C_4 \tag{A-3b}$$

$$PC_4 = \frac{A}{A+D}k_- C_2 - \left(\beta_+ + \frac{D}{A+D}k_-\right)C_4 + \beta_- C_5 \tag{A-3c}$$

$$PC_5 = \beta_+ C_4 - \beta_- C_5 \tag{A-3d}$$

The operator P is treated as constant and the system of Eqn. A = 3a–d can be solved using Cramer's rule. The determinant of the system is:

$$\Delta = P(P+\beta_- + \beta_+)[(P+\beta_-)(P+\beta_+ + k_-) - \beta_-\beta_+] \tag{A-4}$$

With the assumption that transverse motion of the probe is slow compared to intervesicular transfer ($\beta_+\beta_+ \approx 0$), Eqn. A-4 reduces to:

$$\Delta = P(P+\beta_- + \beta_-)(P+\beta_-)(P+\beta_+ + k_-) \tag{A-4a}$$

The expressions obtained for C_1, C_2, C_4 and C_5 are functions of the operator P and can be transformed to original functions by a reverse Laplace-Carson transformation using the Tables of Transforms in Ref. 17. The complete set of solutions is given in Eqns. A-5a–d:

$$C_1 = \frac{C_T k_- \beta_+}{(\beta_- + \beta_+)(k_- + \beta_+)} \frac{D}{(A+D)} + \frac{k_-(C_1^0\beta_- - C_2^0\beta_+)}{(\beta_- + \beta_+)(k_- - \beta_-)} \frac{D}{(A+D)} e^{-(\beta_- + \beta_+)t} + \frac{C_1^0\left(\frac{A}{A+D}k_- - \beta_-\right) + C_T\beta_+}{(k_- + \beta_+ - \beta_-)} e^{-\beta_- t} - \left\{\beta_+\left[C_1^0\frac{D}{A+D}k_-\beta_- + C_2^0\frac{A}{A+D}k_- \times (k_- + \beta_+ - \beta_-) - C_2^0\beta_-\beta_+\right]\right\} \times [(k_- + \beta_+)(k_- - \beta_-)(k_- + \beta_+ - \beta_-)]^{-1} \times e^{-(k_- + \beta_+)t} \tag{A-5a}$$

$$C_2 = \frac{C_T k_- \beta_-}{(\beta_- + \beta_+)(k_- + \beta_+)} \frac{D}{(A+D)} - \frac{(C_1^0\beta_- - C_2^0\beta_+)k_-}{(\beta_- + \beta_+)(k_- - \beta_-)} \frac{D}{(A+D)} e^{-(\beta_- + \beta_+)t} + \frac{C_1^0\beta_-}{(k_- + \beta_+ - \beta_-)} e^{-\beta_- t} - \left\{\left[\frac{A}{A+D}k_-(k_- + \beta_+ - \beta_-) - \beta_-\beta_+\right] \times [C_1^0\beta_- - C_2^0(k_- + \beta_+ - \beta_-)]\right\} \times [(k_- + \beta_+)(k_- - \beta_-)(k_- + \beta_+ - \beta_-)]^{-1} \times e^{-(k_- + \beta_+)t} \tag{A-5b}$$

$$C_4 = \frac{C_T k_- \beta_-}{(\beta_- + \beta_+)(k_- + \beta_+)} \frac{A}{(A+D)} - \frac{k_-(C_1^0\beta_- - C_2^0\beta_+)}{(\beta_- + \beta_+)(k_- - \beta_-)} \frac{A}{(A+D)} e^{-(\beta_- + \beta_+)t} + \frac{k_-(C_1^0\beta_- - C_2^0 k_-)}{(k_- + \beta_+)(k_- - \beta_-)} \frac{A}{(A+D)} e^{-(k_- + \beta_+)t} \tag{A-5c}$$

$$C_5 = \frac{C_T k_- \beta_+}{(\beta_- + \beta_+)(k_- + \beta_+)} \frac{A}{(A+D)} + \frac{k^-(C_1^0\beta_- - C_2^0\beta_+)}{(\beta_- + \beta_+)(k_- - \beta_-)} \frac{A}{(A+D)} e^{-(\beta_- + \beta_+)t} - \frac{k_- C_1^0}{(k_- + \beta_+ - \beta_-)} \frac{A}{(A+D)} e^{-\beta_- t}$$

$$- \frac{k_- \beta_+ [C_1^0 \beta_- - C_2^0 (k_- + \beta_+ - \beta_-)]}{(k_- + \beta_+)(k_- - \beta_-)(k_- + \beta_+ - \beta_-)}$$

$$\times \frac{A}{(A+D)} e^{-(k_- + \beta_+)t} \quad \text{(A-5d)}$$

$$C_T = C_1^0 + C_2^0$$

The fact that the preexponential factors for the terms $\exp -(\beta_- + k_+)t$ are of equal value but opposite signs, leads to a double-exponential function for the fluorescence intensity F in Eqn. 11. From Eqn. A-5, we can also calculate the preexponential terms α (Table IIB)

$$\alpha = \frac{C_T k_-}{(k_- + \beta_+)} \frac{(f_D + n f_A)}{(1+n)} \quad \text{(A-6a)}$$

$$\alpha_1 = \frac{\frac{n}{1+n}(f_A - f_D)[C_2^0 (k_- - \beta_-) - C_1^0 \beta_-] k_-}{(k_- + \beta_+)(k_- - \beta_-)}$$

$$+ \beta_- \beta_+ \frac{C_1^0 k_- \frac{f_D + n f_A}{1+n} + f_D [C_2^0 (k_- - \beta_-) - C_1^0 \beta_-]}{(k_- + \beta_+)(k_- - \beta_-)(k_- + \beta_+ - \beta_-)}$$

(A-6b)

$$\alpha_2 = \frac{\frac{n}{1+n}(f_A - f_D) C_1^0 k_- - f_D \beta_+ C_T}{k_- + \beta_+ - \beta_-} \quad \text{(A-6c)}$$

$$n = \frac{A}{D} = \frac{C_A}{C_D}$$

Summation of the concentrations C_1, C_2, C_4 and C_5 at infinite time, yields the value

$$C_{eq} = C_T k_- / (k_- + \beta_+)$$

which can be seen as further evidence that the mathematical approach of the kinetic equations gives satisfactory results for $\beta_+ \ll k_-$.

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