Kinetics of Soluble Lipid Monomer Diffusion between Vesicles†

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ABSTRACT: The fluorescent phospholipid 1-acyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]phosphatidylcholine (C_{12}-NBD-PC) was used to study the kinetics of lipid transfer between phospholipid vesicles. A model based on lipid transfer resulting from the diffusion of soluble monomers was found to accurately predict the kinetics of this transfer process. From these studies, we conclude that (i) C_{12}-NBD-PC transfer between vesicles results from the diffusion of soluble monomers and not from vesicle collision, (ii) the rate at which a lipid molecule enters or leaves a bilayer is dependent upon both its molecular structure and the characteristics of the donor and acceptor bilayers, and (iii) under the appropriate conditions, either the rate of lipid association or dissociation from the bilayer or a combination of both may determine the rate of transfer.

The transfer of lipid molecules between various membranes and/or lipoproteins is a ubiquitous process of important biological significance. For example, the function and activity of phospholipids, fatty acids, cholesterol, steroid hormones, fat-soluble vitamins, and lipophilic drugs in cells and in whole organisms require their transfer between numerous bilayer and micellar lipid structures. Although little is known of the molecular mechanisms for these transfer processes in vivo, there is increasing evidence that these molecules can be passively transferred between various combinations of vesicles, lipoproteins, and cellular membranes as soluble monomers diffusing through the aqueous medium (Duckwitz-Peterlein et al., 1977; Smith & Scow, 1979; Doody et al., 1980; Duckwitz-Peterlein & Moraal, 1978; Phillips et al., 1980; Roseman & Thompson, 1980; De Cuyper et al., 1980).

In this paper, we present a kinetic model for this diffusional transfer process and test its validity by measuring the kinetics of transfer of the fluorescent phospholipid C_{12}-NBD-PC[1] between two populations of vesicles. At concentrations greater than 50 mol %, the fluorescence of C_{12}-NBD-PC molecules is more than 98% self-quenched, so that the transfer of these molecules to nonfluorescent acceptor vesicles with the concomitant development of fluorescence can be directly monitored.

The results of our measurements confirm that in the case of C_{12}-NBD-PC, lipid transfer occurs via free monomers and is not a collision-mediated process. By application of this model, we have shown that (i) the rate at which a lipid molecule enters or leaves a bilayer is dependent upon both its molecular structure and the characteristics of the donor and acceptor bilayers and (ii) under the appropriate conditions, either the rate of lipid association or dissociation from the bilayer or a combination of both may determine the rate of transfer. In addition, this model provides guidelines for distinguishing between other possible molecular mechanisms for lipid transfer in future investigations.

Kinetic Model. Nakagawa (1974) proposed a kinetic model to describe the rate of micelle association and dissociation in a surfactant solution. This model was later applied by Thilo (1977) to describe the molecular exchange of lipids between two populations of bilayer vesicles. In the Thilo model, the rates of monomer–vesicle association and dissociation are assumed to be independent of the lipid composition and size of the vesicles. We have extended this model to include the possibility that monomer association and dissociation may vary, depending on the characteristics of the lipid vesicles.

Phospholipids (P) are assumed to exist in equilibrium between the bilayer state (b) and the monomer state (m) as described by

$$P_m \underset{k_-}{\overset{k_+}{\rightleftharpoons}} P_b \qquad (1)$$

The Nakagawa model (as applied to phospholipids that spontaneously form bilayers) predicts that the rate at which a monomer escapes from the bilayer is proportional to $[P]_b$, the concentration of phospholipid in a unit volume of solution that exists in the outer leaflet of the bilayer exposed to the bulk solution, whereas the rate at which a monomer enters the bilayer is proportional to the product of the lipid monomer concentration, $[P]_m$, and the external surface area of the bilayer in a unit volume of solution. This analysis assumes that the rate of transbilayer movement ("flip-flop") of the P lipid is slow compared to its rate of intervesicular transfer. In a solution of bilayer vesicles, the external surface area equals the product of the lipid concentration of the external leaflet $[P]_b$ and the area per mole of lipid (s), such that

$$\frac{d[P]_b}{dt} = k_+ s[P]_m[P]_b - k_-[P]_b \qquad (2)$$

At equilibrium $d[P]_b/dt = 0$, and therefore

$$[P]_m = k_-/sk_+ \qquad (3)$$

In this case where there is only one lipid component, $[P]_m$ is equal to the critical bilayer concentration (cbc). The cbc is independent of the phospholipid concentration.

In a manner analogous to eq 2, the transfer of two lipid types (D and A) between donor (I) and acceptor (II) populations

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[1] Abbreviations used: DOPC, dioleoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; C_6-NBD-PC, 1-acyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-caproyl]phosphatidylcholine [manufacturer's name: 1-acyl-2-(*N*-4-nitrobenzo-2-oxa-1,3-diazole)-aminocaproyl phosphatidylcholine]; C_{12}-NBD-PC, 1-acyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]phosphatidylcholine; *t*-Boc-PC, 1-acyl-2-[12-(*N*-(*tert*-butoxycarbonyl)amino)dodecanoyl]phosphatidylcholine; pyrene-PC, 1-palmitoyl-2-pyrenedecanoylphosphatidylcholine; cbc, critical bilayer concentration; cmc, critical micelle concentration; NaCl–Hepes, 0.9% NaCl in 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4.

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can be described by eq 4–7, where $k_{D_+}^{I}$ and $k_{D_+}^{II}$ are the

$$\frac{d[D]_I}{dt} = k_{D_+}^{I}[D]_m(s_D[D]_I + s_A[A]_I) - k_{D_-}^{I}[D]_I \quad (4)$$

$$\frac{d[D]_{II}}{dt} = k_{D_+}^{II}[D]_m(s_D[D]_{II} + s_A[A]_{II}) - k_{D_-}^{II}[D]_{II} \quad (5)$$

$$\frac{d[A]_I}{dt} = k_{A_+}^{I}[A]_m(s_D[D]_I + s_A[A]_I) - k_{A_-}^{I}[A]_I \quad (6)$$

$$\frac{d[A]_{II}}{dt} = k_{A_+}^{II}[A]_m(s_D[D]_{II} + s_A[A]_{II}) - k_{A_-}^{II}[A]_{II} \quad (7)$$

on-rate constants of the D lipid into vesicle populations I and II, respectively. $k_{D_-}^{I}$ and $k_{D_-}^{II}$ are the corresponding off-rate constants. The rate constants for the A lipid are analogous. $[D]_m$ and $[A]_m$ are the free monomer lipid concentrations, and $[D]_I$, $[D]_{II}$, $[A]_I$, and $[A]_{II}$ are the concentrations of the D and A lipids in the subscripted vesicle populations exposed to the bulk solution. s_A and s_D are the surface areas per mole of A or D lipid, respectively.

In the experiments described in this paper the rate of transfer of only one of the two lipids is monitored. Since lipid intermixing between the two vesicle populations is likely to alter the properties of the bilayer and, as a result, change the magnitude of the corresponding rate constants, we therefore measured only the initial rate of transfer and made no attempt to describe the kinetics as lipid intermixing approaches equilibrium.

Upon mixing population I vesicles with population II vesicles, the concentration of D in the free monomer state, $[D]_m$, will change to a new steady-state equilibrium value which reflects the total concentrations of A and D lipids exposed to the bulk solution. The rate at which $[D]_m$ approaches this new steady-state equilibrium is given by

$$\frac{d[D]_m}{dt} = \frac{-d[D]_I}{dt} - \frac{d[D]_{II}}{dt} \quad (8)$$

Substituting eq 4 and 5

$$\frac{d[D]_m}{dt} = k_{D_-}^{I}[D]_I - k_{D_+}^{I}[D]_m(s_D[D]_I + s_A[A]_I) + k_{D_-}^{II}[D]_{II} - k_{D_+}^{II}[D]_m(s_D[D]_{II} + s_A[A]_{II}) \quad (9)$$

For total D and A lipid concentrations in excess of their soluble monomer concentrations, the transfer of lipid required for $[D]_m$ to reach its new steady-state equilibrium value will be small in relation to the total concentration of A and D lipids. Therefore, eq 9 can be integrated assuming that $[D]_I$, $[A]_I$, $[D]_{II}$, and $[A]_{II}$ are constant. Letting $[D]_m = [D]_m^0$ at $t = 0$

$$[D]_m = \frac{k_{D_-}^{I}[D]_I + k_{D_-}^{II}[D]_{II}}{k_{D_+}^{I}(s_D[D]_I + s_A[A]_I) + k_{D_+}^{II}(s_D[D]_{II} + s_A[A]_{II})} + \left[[D]_m^0 - \frac{k_{D_-}^{I}[D]_I + k_{D_-}^{II}[D]_{II}}{k_{D_+}^{I}(s_D[D]_I + s_A[A]_I) + k_{D_+}^{II}(s_D[D]_{II} + s_A[A]_{II})}\right] \times \exp[-k_{D_+}^{I}(s_D[D]_I + s_A[A]_I) - k_{D_+}^{II}(s_D[D]_{II} + s_A[A]_{II})]t \quad (10)$$

The half-time for $[D]_m$ to reach steady-state equilibrium is therefore

$$t_{1/2} = \frac{0.693}{k_{D_+}^{I}(s_D[D]_I + s_A[A]_I) + k_{D_+}^{II}(s_D[D]_{II} + s_A[A]_{II})} \quad (11)$$

As the exponential term in eq 10 approaches zero, $[D]_m$ approaches its steady-state equilibrium value.

$$[D]_m = \frac{k_{D_-}^{I}[D]_I + k_{D_-}^{II}[D]_{II}}{k_{D_+}^{I}(s_D[D]_I + s_A[A]_I) + k_{D_+}^{II}(s_D[D]_{II} + s_A[A]_{II})} \quad (12)$$

If initially only population I contains the fluorescent probe, D, eq 5 can be reduced to

$$\frac{d[D]_{II}}{dt} = k_{D_+}^{II}s_A[D]_m[A]_{II} \quad (13)$$

for the initial transfer rate. Under the assumption that the free monomer concentration is at steady-state equilibrium during the initial rate measurements, eq 12 can be substituted into eq 13 such that

$$R = \frac{d[D]_{II}}{dt} = \frac{k_{D_+}^{II}k_{D_-}^{I}[D]_I[A]_{II}}{k_{D_+}^{I}\left(\frac{s_D}{s_A}[D]_I + [A]_I\right) + k_{D_+}^{II}[A]_{II}} \quad (14)$$

R is the initial rate of transfer of C_{12}-NBD-PC molecules from population I to population II.

Inverting eq 14 and assuming $s_D/s_A = 1$ give

$$\frac{1}{R} = \left(\frac{k_{D_+}^{I}}{k_{D_+}^{II}k_{D_-}^{I}}\right)\left(\frac{1}{X_D^I}\right)\left(\frac{1}{[A]_{II}}\right) + \left(\frac{1}{k_{D_-}^{I}}\right)\left(\frac{1}{[D]_I}\right) \quad (15)$$

X_D^I is the mole fraction of D in vesicle population I.

Experimental Procedures

Materials and Routine Procedures. DOPC, DMPC, DPPC, and [^{3}H]DOPC were prepared as previously described (Huang & Pagano, 1975). NBD chloride was purchased from Sigma Chemical Co. Phospholipase A_2 (hog pancreas) and phospholipase D (cabbage) were products of Boehringer Mannheim. Lipids were stored at −20 °C, periodically monitored for purity by thin-layer chromatography, and purified when necessary. The concentrations of all phospholipids were determined by a modified Chen method (Ames & Dubin, 1960). Relative fluorescence intensity was determined by using an American Instrument Co. spectrophotofluorometer.

Fluorescent Lipids. C_6-NBD-PC was purchased from Avanti Biochemical Corp. *t*-Boc-PC was synthesized on special order by Avanti Biochemical Corp. C_{12}-NBD-PC was synthesized from *t*-Boc-PC by the following procedure developed by Dr. D. K. Struck. *t*-Boc-PC was deblocked by treatment with 6% anhydrous HCl in glacial acetic acid at room temperature for 2 h, extracted, and reacted at room temperature for 2 h with a 4-fold molar excess of NBD chloride in ethyl acetate/methanol (5:1) containing 1–2 μL of triethylamine/mL of solvent. The reaction mixture was extracted and the lipid phase washed and purified by silicic acid column chromatography. The resulting product gave a single spot by thin-layer chromatography in chloroform/methanol/water (65:25:4) and was fluorescent, phosphate positive, ninhydrin negative, and sensitive to digestion with phospholipase A_2.

Preparation of Vesicles. Small unilamellar acceptor vesicles were prepared by the method of Batzri & Korn (1973) as

modified by Kremer et al. (1977a). A solution of 200 μL of absolute ethanol containing 4 mg of lipid (DOPC, DMPC, DPPC) was injected into 4 mL of NaCl–Hepes followed by overnight dialysis. DOPC solutions were injected at room temperature and dialyzed at 2 °C. Injection and dialysis of DMPC and DPPC solutions were carried out at 50 °C. Vesicles containing C_{12}-NBD-PC and mixtures of C_{12}-NBD-PC and DOPC were prepared by injecting 20–30 μg of lipid in 50 μL of absolute ethanol into 4 mL of NaCl–Hepes at room temperature followed by overnight dialysis at 2 °C. The size and vesicular nature of donor and acceptor material were confirmed by negative-staining electron microscopy, and, in the case of the acceptors, their elution profiles on Sepharose 4B and Bio-Gel A15m columns were also monitored. Due to the small amounts of C_{12}-NBD-PC available and its absorption to Sepharose 4B and Bio-Gel A15m, elution profiles of the donor vesicles could not be obtained. In the absence of further proof, we cautiously refer to the C_{12}-NBD-PC dispersions as vesicles. C_{12}-NBD-PC content of the donor vesicles was assayed by reading the fluorescence of Triton X-100 solubilized vesicles and comparing it to a standard curve. NBD fluorescence was monitored at 540 nm (λ_{ex} = 475 nm) by using crossed Glan prism polarizer filters in the light path.

Concentration Dependence of NBD-PC Fluorescence. A total of 32 μg each of C_6-NBD-PC and C_{12}-NBD-PC were dried separately under argon, desiccated under vacuum overnight to remove residual solvents, suspended in NaCl–Hepes, vortex mixed, and serially diluted. Relative fluorescence was read as previously described. The lipid suspensions were allowed to equilibrate for several hours before fluorescence readings were made. No subsequent changes in fluorescence were observed after 1-day incubation at 2 °C.

Determination of Quenching of C_{12}-NBD-PC in Vesicles. Vesicles containing 10 nmol of total lipid/mL were prepared as above containing varying percentages of C_{12}-NBD-PC and [^{3}H]DOPC and dialyzed overnight. The final concentration of [^{3}H]DOPC was determined by scintillation counting and of C_{12}-NBD-PC by fluorescence assay of Triton X-100 solubilized vesicles. The percentage of quenched fluorescence (% Q) is calculated from the relative fluorescence of unquenched C_{12}-NBD-PC (F_U) in the presence of Triton X-100 and quenched molecules (F_Q) in vesicles by the relation

$$\% Q = \left[\frac{F_U - F_Q}{F_U}\right] \times 100 \qquad (16)$$

Relative fluorescence was corrected for the increased quantum efficiency of C_{12}-NBD-PC in DOPC vesicles compared to Triton X-100 micelles (factor of 1.54).

Measurement of Rates of Transfer. Quenched vesicles (C_{12}-NBD-PC alone or 1:1 C_{12}-NBD-PC/DOPC) were allowed to equilibrate in a fluorescence cuvette followed by addition of approximately 50-fold excess of acceptor vesicles (DOPC, DMPC, or DPPC). The vesicles were well mixed, and the transfer of C_{12}-NBD-PC molecules from the quenched donor vesicles to the unquenched acceptor vesicles was determined by the increase of fluorescence intensity continuously monitored on a chart recorder. The number of moles transferred was calculated from standard curves derived at 20 and 49 °C by using DOPC vesicles containing unquenched concentrations of C_{12}-NBD-PC. The fluorescence quantum yield for C_{12}-NBD-PC is higher at 20 °C than at 49 °C but at a given temperature is not significantly different when incorporated into DOPC, DMPC, or DPPC vesicles. Extraction and thin-layer chromatography of donor and acceptor vesicle lipids following the rate measurements indicated no detectable breakdown products. Preliminary experiments using sonicated instead of ethanol-injected donor and acceptor vesicles resulted in similar rates of transfer.

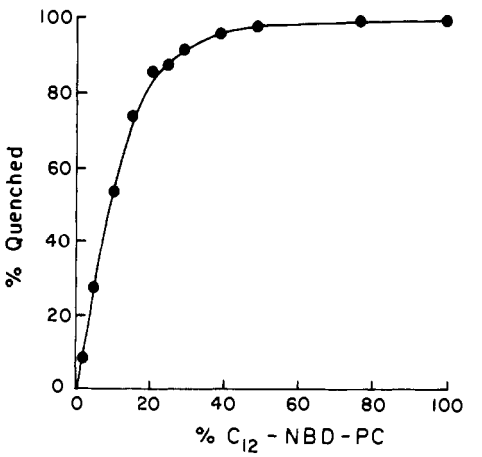

FIGURE 1: Concentration dependent quenching of C_{12}-NBD-PC in DOPC vesicles. See Experimental Procedures for details.

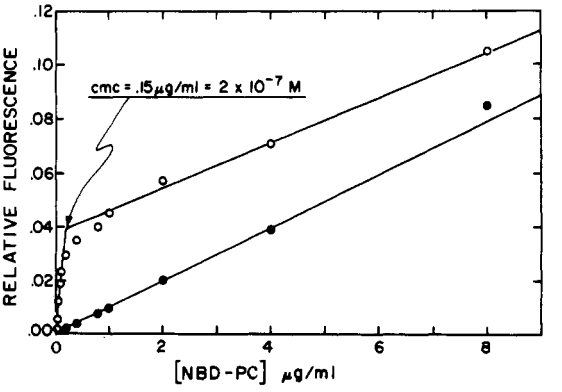

FIGURE 2: Measurement of critical bilayer concentration. See Experimental Procedures for details. (○) C_6-NBD-PC; (●) C_{12}-NBD-PC.

Results

Quenching of C_{12}-NBD-PC Fluorescence. Figure 1 demonstrates that as the percentage of C_{12}-NBD-PC incorporated into DOPC vesicles is increased, the fluorescence is increasingly quenched so that pure C_{12}-NBD-PC vesicles are greater than 98% quenched. Since C_{12}-NBD-PC/DOPC (1:1) vesicles are also almost entirely quenched, transfer rate studies using these and pure C_{12}-NBD-PC vesicles could be compared.

Measurement of Critical Bilayer Concentration. The plot in Figure 2 of relative fluorescence vs. concentration of fluorescent lipids indicates a change in slope for C_6-NBD-PC in the region of 2 × 10^{-7} M. We interpret this region of changing slope to represent the concentration at which these molecules begin to form self-quenching bilayers. At concentrations below 2 × 10^{-7} M, these molecules exist as soluble monomers with relatively high quantum efficiency whereas at higher concentrations they exist in self-quenched bilayer vesicles. This region of change will be referred to as the critical bilayer concentration (cbc), analogous to the critical micelle concentration (cmc) (Smith & Tanford, 1972). No change in slope was detected for C_{12}-NBD-PC, indicating that the cbc is less than 10^{-8} M, the limit of detection for these measurements.

Tests of Kinetic Model. Initial dequenching of C_6-NBD-PC fluorescence upon addition of excess acceptor vesicles occurs too rapidly to be measured without a rapid mixing and recording device. Therefore we chose to measure C_{12}-NBD-PC

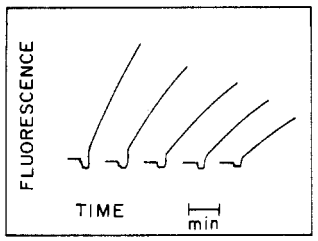

FIGURE 3: Series of initial transfer rate measurements. Donor vesicles consisting of 100% C_{12}-NBD-PC in 1.9 mL of NaCl–Hepes were equilibrated at 49 °C in an Aminco Bowman spectrophotofluorometer. Transfer was initiated by the addition of DMPC acceptor vesicles. The cuvette was stirred and fluorescence recorded. The final concentration of DMPC was 8.8×10^{-5} M; final concentrations of C_{12}-NBD-PC were varied from left to right: 1.87, 1.25, 0.94, 0.75, and 0.63×10^{-6} M.

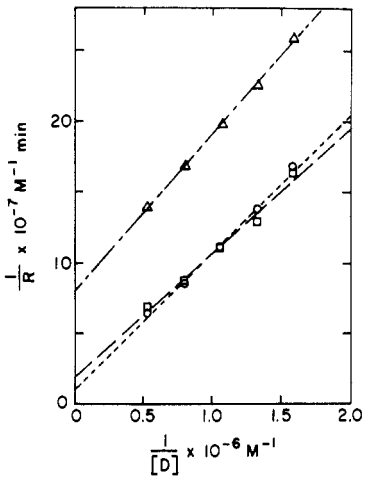

FIGURE 4: Double-reciprocal plot of initial rate vs. donor concentration at 20 °C with acceptor concentration constant. Donor vesicles were C_{12}-NBD-PC. The three different acceptor vesicles used were (△) DPPC, (□) DMPC, and (○) DOPC. Acceptor vesicle concentrations were the following: DPPC, 6.7×10^{-5} M; DMPC, 8.8×10^{-5} M; DOPC, 5.4×10^{-5} M. Slopes and intercepts were calculated and lines projected by linear regression.

transfer since its initial rate of dequenching is on a time scale that can easily be measured with standard equipment. A series of curves demonstrating the increase in fluorescence as excess DMPC vesicles are added to C_{12}-NBD-PC vesicles is shown in Figure 3. Straight lines were drawn through the initial portion of the curves in order to approximate the initial rates of transfer of C_{12}-NBD-PC into the DMPC vesicles. Less than 5% of the total C_{12}-NBD-PC in the donors was transferred to acceptors for the initial rate measurements.

Equation 15 predicts that the inverse of the initial rate of transfer is linearly dependent on the two additive terms representing the effect of acceptor and donor vesicle concentrations, respectively. This model predicts that when either donor or acceptor vesicle concentration is varied while the other is held constant, a double-reciprocal plot of rate vs. concentration results in a straight line with a slope equal to the constant of the appropriate term. The constants for the remaining term can be determined from the y intercept in either case. If both donor and acceptor vesicle concentrations are increased proportionately, the double-reciprocal plot will result in a straight line with a y intercept of 0 that can be predicted from the known vesicle concentration and the previously determined constants.

Figures 4–6 present the data from experiments performed at 20 °C testing these predictions. In Figure 4, the inverse of the initial transfer rate ($1/R$) was plotted against the inverse of the donor vesicle concentration ($1/[D]$). The acceptor concentration was held constant. For each different acceptor, the relationship is linear, as predicted. The slope of each line is the inverse of the off-rate from the donor vesicle ($k_{D_-}^{I}$) and is approximately the same for each different acceptor molecule (see Table I), as would be expected since the donor vesicles are identical in each case. The intercept when $1/[D]$ is 0 equals the first term in eq 15. Since [A] and $k_{D_-}^{I}$ are known, the ratio of the on-rates into vesicle populations I and II ($k_{D_+}^{I}/k_{D_+}^{II}$) can be calculated (see Table I). Since $k_{D_+}^{I}$ is the same for each case, the relative magnitudes of the C_{12}-NBD-PC on-rates into the three acceptors are $k_{D_+}^{DOPC} > k_{D_+}^{DMPC} > k_{D_+}^{DPPC}$.

Table I: Calculated Rate Constants for Donor and Acceptor Vesicles[a]

temp (°C)	acceptor	$k_{D_-}^{I}$ (min^{-1})[b]	$k_{D_-}^{I}$ (min^{-1})[c]	$k_{D_+}^{I}/k_{D_+}^{II}$[d]	$k_{D_+}^{I}/k_{D_+}^{II}$[e]
20	DMPC	0.0188	0.0200	18.7	22.2
	DPPC	0.0148	0.0120	46.7	27.8
	DOPC	0.0170	0.0155	5.58	2.29
49	DMPC	0.222	0.205	3.16	1.88
	DPPC	0.197	0.168	18.4	12.8
	DOPC	0.183	0.180	3.32	4.18

[a] Donor vesicles (population I) were composed of C_{12}-NBD-PC, and acceptor vesicles (population II) were composed of either DMPC, DPPC, or DOPC. Constants at 20 °C were calculated from the data plotted in Figures 4–6. Values at 49 °C were calculated from similar plots of data obtained at this temperature by using the same vesicle preparations. Concentrations of externally exposed lipids were estimated by multiplying the total concentrations by 0.6, the ratio of outer leaflet to total lipid measured for small unilamellar vesicles (Johnson et al., 1975; Rothman & Dawidowicz, 1975; DiCorleto & Zilversmit, 1977). [b] Constant terms derived from the slope of the double-reciprocal plots of R vs. [D] (Figure 4). [c] Constant terms derived from the y intercept of the double-reciprocal plots of R vs. [A] (Figure 5). [d] Constant terms derived from the y intercept of the double-reciprocal plots of R vs. [D] (Figure 4). [e] Constant terms derived from the slope of the double-reciprocal plots of R vs. [A] (Figure 5).

This comparison of on-rate constants depends on the assumption that the ratio of outer leaflet to total lipid is the same for each type of acceptor vesicle. Kremer et al. (1977a) found that DMPC and DPPC vesicles prepared by ethanol injection above their phase-transition temperatures had equal external radii within experimental error as measured by light scattering. Although size measurements for these vesicles were not made at temperatures below their phase transitions, studies from other laboratories (Barrow & Lentz, 1980; Schullery et al., 1980) using sonicated DPPC vesicles indicate that incubation for many hours or days below the phase-transition temperature results in significant fusion with concomitantly increased vesicle diameter but that fusion is insignificant for incubations of only a few hours as used in the present study. They also found no evidence of aggregation. In light of these studies, it is reasonable to assume that the three populations of acceptor vesicles are composed of small unilamellar vesicles with similar average diameters both above and below their phase transitions. We therefore conclude from the data in Figure 4 that the rate of transfer of C_{12}-NBD-PC between vesicles is dependent upon the composition of the acceptor bilayer.

Since gel filtration profiles could not be obtained for C_{12}-NBD-PC dispersions, our only evidence for vesicle rather than micelle formation comes from electron microscopy. However, even if this lipid was present as micelles, this would only result in altering the fraction of total lipid in population I that is exposed to the external solution (see footnotes for Table I). This would decrease the values of $k_{D_-}^{I}$ and increase the values of $k_{D_+}^{I}/k_{D_+}^{II}$ by a factor of 1.67. These corrections would not alter the conclusions drawn from these data.

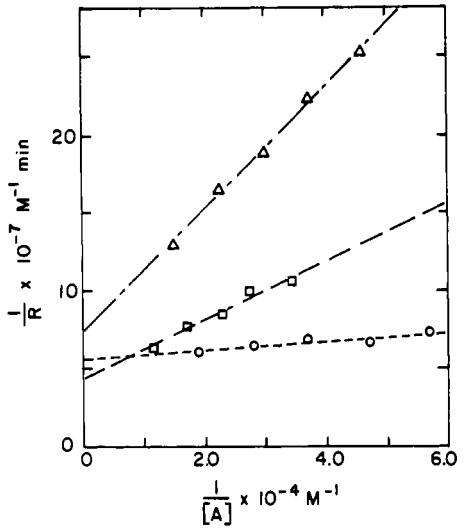

FIGURE 5: Double-reciprocal plot of initial rate vs. acceptor concentration at 20 °C, with donor concentration constant. Donor vesicles were C_{12}-NBD-PC at a constant concentration of 1.87×10^{-6} M. The concentrations of three different vesicle populations were varied as shown: (△) DPPC, (□) DMPC, (○) DOPC. Slopes and intercepts were calculated and lines projected by linear regression.

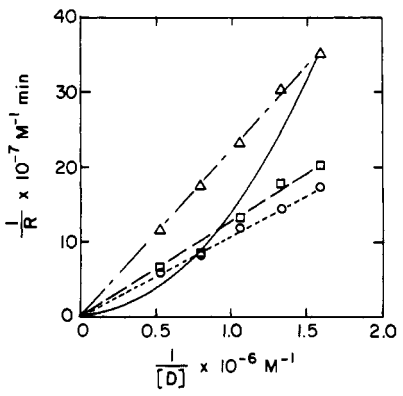

FIGURE 6: Double-reciprocal plot of initial rate vs. donor concentration at 20 °C, with the ratio of donor to acceptor concentration constant. Donor vesicles were C_{12}-NBD-PC. Initial rates for three different acceptors were measured and plotted: (△) DPPC, (□) DMPC, (○) DOPC. Acceptor concentrations were increased in proportion to the donor concentrations so that [A]/[D] was constant at 35 for DPPC, 47 for DMPC, and 29 for DOPC. The straight lines were predicted from eq 15 by using the constants derived from the slopes of the lines in Figures 4 and 5. The solid line is the expected result for a collision-mediated, bimolecular reaction.

In Figure 5, $1/R$ is plotted against the inverse of the acceptor vesicle concentration ($1/[A]$) for the same three acceptors while the donor vesicle concentration is held constant. The value for $k_{D_-}{}^{I}$ was calculated from the intercept, and this value was used to calculate $k_{D_+}{}^{I}/k_{D_+}{}^{II}$ from the slope (see Table I). It should be noted that values for $k_{D_-}{}^{I}$ and $k_{D_+}{}^{I}/k_{D_+}{}^{II}$ can be calculated independently from separate experiments, varying either the donor (Figure 4) or the acceptor (Figure 5) concentration. All of the values of $k_{D_-}{}^{I}$ for each acceptor are equal within experimental error, and the relative magnitudes of $k_{D_+}{}^{II}$ are similar to those calculated from the intercepts in Figure 4.

In Figure 6, $1/R$ is plotted vs. $1/[D]$ while the ratio [A]/[D] is held constant. The measured rates are represented by the data points, while the lines were predicted from eq 15 by using the constants calculated from the slopes in Figures 4 and 5. The relationships for all the acceptor types are linear and are accurately predicted by eq 15.

The simplest model for lipid transfer that is dependent on vesicle collision is $R = k[A][D]$ or, inverted, $1/R = 1/(k[A][D])$. The expected results in the experiments presented in Figures 4 and 5 for this collision model would be a linear dependence on inverse concentration with a y intercept of 0. The y intercepts are not 0 and therefore the data of Figures 4 and 5 do not support the collision-mediated model. The data of Figure 6 are even stronger evidence against this model. The solid line describes the expected result for collision-mediated transfer and is inconsistent with the data. Fusion of vesicles via a mechanism other than a simple bimolecular reaction can be excluded since fusion of a C_{12}-NBD-PC vesicle with a phospholipid acceptor vesicle would result in only a 50% dilution of C_{12}-NBD-PC and produce only 1–2% dequenching. Therefore, we conclude that the transfer of C_{12}-NBD-PC molecules is not dependent on vesicle collision or fusion but instead occurs via the diffusion of soluble monomers.

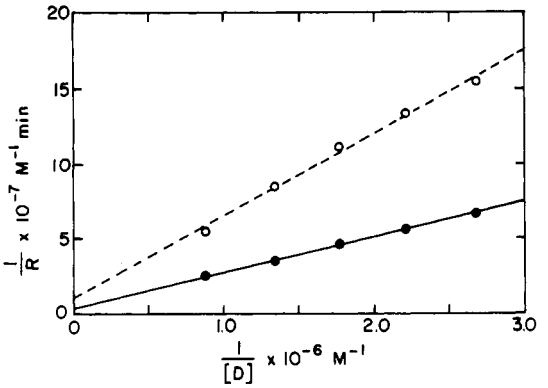

FIGURE 7: Double-reciprocal plot of initial rate vs. donor concentration at 25 °C. Acceptor vesicles were DOPC at a final concentration of 6.4×10^{-5} M. Donor vesicles were prepared from 100% C_{12}-NBD-PC (○) and 50% C_{12}-NBD-PC + 50% DOPC (●). Slopes were calculated by linear regression to obtain $k_{D_-}{}^{100\%} = 0.030$ min^{-1} and $k_{D_-}{}^{50\%} = 0.070$ min^{-1}.

Experiments identical with those carried out at 20 °C (Figures 4–6) were also performed at 49 °C, and the results are presented in Table I. At 49 °C, as at 20 °C, the experimental data fit the predictions of the kinetic model, and we therefore conclude that the diffusion of soluble monomers is the predominant mode of C_{12}-NBD-PC transfer at 49 °C as well as 20 °C. The values for $k_{D_-}{}^{I}$ at 49 °C are approximately 1 order of magnitude greater than $k_{D_-}{}^{I}$ measured at 20 °C. At 49 °C, ordering of the on-rate constants has been changed to $k_{D_+}{}^{DMPC} > k_{D_+}{}^{DOPC} > k_{D_+}{}^{DPPC}$ as compared to $k_{D_+}{}^{DOPC} > k_{D_+}{}^{DMPC} > k_{D_+}{}^{DPPC}$ at 20 °C.

Dependence of Off-Rate Constant on Vesicle Composition. Since vesicles composed of 50 mol % C_{12}-NBD-PC are essentially completely self-quenched, as are vesicles containing exclusively C_{12}-NBD-PC (Figure 1), the transfer rates from each type of vesicle can be measured and compared. The double-reciprocal plot of rate vs. donor concentration for the 100% and 50% C_{12}-NBD-PC vesicles is shown in Figure 7. The off-rate for the 100% vesicles ($k_{D_-}{}^{100\%} = 0.030$ min^{-1}) is about half that for the 50% vesicles ($k_{D_-}{}^{50\%} = 0.070$ min^{-1}). These results indicate that the rate at which C_{12}-NBD-PC molecules leave the donor vesicle is dependent on its composition.

Discussion

Tests of Kinetic Model. The experiments presented in Figures 4–6 demonstrate that the proposed model for lipid transfer based on the diffusion of soluble monomers accurately predicts the dependence of initial transfer rates on initial acceptor and donor concentrations. For a model that is generally applicable, independent on- and off-rate constants for both donor and acceptor vesicle compositions must be included as

well as a description of the dependence of the soluble lipid monomer concentration on both the independent rate constants and the donor and acceptor concentrations. From the data presented, we conclude that the rate at which a lipid molecule leaves or enters a bilayer vesicle is dependent upon both its own structure and characteristics of both the donor and acceptor vesicles.

Rate of Free Monomer Equilibration. The diffusion-mediated transfer model derived above is dependent upon the assumption that the free monomer concentrations reach a steady-state equilibrium rapidly compared to the time of the initial transfer rate measurements. The half-time for the free monomers to reach steady-state equilibrium after the mixing of two vesicle populations is described in eq 11 as a function of the initial on-rate constants and concentrations of the two vesicle populations. Since the experimental data fit the predictions of the kinetic model for the dependence of initial transfer rates on initial donor and acceptor concentrations, we conclude that the assumption that free monomers reach steady-state equilibrium rapidly in relation to the net transfer process is correct. Using the rate constants obtained from the kinetic data, we can predict the maximum half-time for monomer equilibration from eq 11. As an example, consider the case for the fastest transfer rate presented in Figure 3 in which the concentration of C_{12}-NBD-PC is 1.87×10^{-6} M and the concentration of DMPC is 8.8×10^{-5} M. Substituting the values for $k_{D_-}^{I} = 0.222$ (Table I) and the upper limit estimate of the cbc for C_{12}-NBD-PC of 10^{-8} M (Figure 2) into eq 3, we can estimate $k_{D_+}^{I} > 2.22 \times 10^7$ M^{-1} min^{-1}. Since $k_{D_+}^{I}/k_{D_+}^{II} = 1.88$ (Table I), $k_{D_+}^{II} > 1.18 \times 10^7$ M^{-1} min^{-1}. When these constant values are substituted into eq 11, the half-time for monomer equilibration for this case is predicted to be less than 38.5 ms.

Relationship of the Critical Bilayer Concentration to the On- and Off-Rate Constants. The critical bilayer concentration (cbc) measured for C_6-NBD-PC (2×10^{-7} M) is very similar to that measured by Galla et al. (1979) for pyrene-PC (1.5×10^{-7} M). Both of these cbc measurements are significantly greater than that measured by Smith & Tanford (1972) for DPPC molecules (4.6×10^{-10} M). Presumably the bulky fluorescent group attached to the acyl chains of these molecules decreases their stability in the bilayer state. For the NBD analogues used in the present study, increasing the length of the acyl chain containing the NBD fluorophore from 6 to 12 carbon atoms (C_6- to C_{12}-NBD-PC) decreases the cbc to a value less than can be measured by our technique ($<10^{-8}$ M). We have measured the transfer of C_6-NBD-PC molecules from quenched donor vesicles to unquenched acceptors and found it to be much faster than C_{12}-NBD-PC transfer. This suggests a correlation of cbc with the NBD-PC off-rate, although this is not a necessary prediction of our kinetic model. Since the cbc is equal to the ratio of the off- and on-rate constants, it is possible for the cbc to decrease while the off-rate remains constant. In this light, it is interesting to note that the cbc projected for DMPC, 10^{-8} M (Thilo, 1977), is similar in magnitude to that measured for C_{12}-NBD-PC, although the transfer rate for DMPC is approximately 2 orders of magnitude slower than that for C_{12}-NBD-PC as determined from the data of Martin & MacDonald (1976). There appears to be no simple direct relationship between the cbc and off-rate constant. A complete understanding of the cbc to the on- and off-rate constants is dependent upon independent measurements of all three of the constants under similar conditions.

Effect of Temperature and Chain Length on the Acceptor Vesicle On-Rate Constant. At 49 °C, DOPC, DMPC, and DPPC are above their phase-transition temperatures and are in a fluid state. From the values of the donor to acceptor on-rate ratios presented in Table I, the order of on-rate constants can be seen to be $k_{D_+}^{DMPC} > k_{D_+}^{DOPC} > k_{D_+}^{DPPC}$. Since they are all in the same phase state, this ordering presumably results from other physical properties of the vesicles. At 20 °C, the order changes to $k_{D_+}^{DOPC} > k_{D_+}^{DMPC} > k_{D_+}^{DPPC}$. At this temperature, both DMPC and DPPC vesicles are in the gel phase, while DOPC vesicles remain fluid. Although we have not examined other lipids as yet, we tentatively conclude that for vesicles in the same phase state, the on-rate constants tend to increase with decreased acyl chain length and increased degree of unsaturation.

The value of the donor to acceptor on-rate ratio for DOPC acceptors is essentially the same at 20 °C as at 49 °C, indicating that this shift in temperature has the same effect on the on-rates of C_{12}-NBD-PC vesicles as for DOPC vesicles. For DMPC and DPPC acceptors, this ratio increases severalfold as the temperature is decreased from 49 to 20 °C, indicating that the acceptor on-rates have decreased in relation to that of the C_{12}-NBD-PC donors. One interpretation of these results is that C_{12}-NBD-PC and DOPC vesicles are fluid at both 20 °C and 49 °C. Since DPPC and DMPC vesicles are in a gel state at 20 °C and fluid at 49 °C, a reduction of the on-rate into the gel-phase acceptor vesicles would result in an increased donor to acceptor on-rate ratio. A complete understanding of the effects of acyl chain length and phase state on the on- and off-rate constants will require a more detailed study of their temperature dependence.

Independence of Transfer Rate on Acceptor Concentration Is Not a Necessary Condition for Diffusion-Mediated Lipid Transfer. In several previous studies where rates of transfer of lipid molecules between various combinations of vesicles, lipoproteins, and cellular membranes have been measured, conditions have been selected such that the transfer rate is dependent on the donor vesicle concentration and independent of the acceptor vesicle concentration. This rate independence of acceptor vesicle concentration is inconsistent with a bimolecular vesicle collision-mediated transfer model and consistent with the diffusion-mediated model and therefore has been commonly used to distinguish between these two possible mechanisms (Roseman & Thompson, 1980; Doody et al., 1980; Charlton et al., 1976, 1978; Lawaczeck, 1978). However, it should be stressed that transfer rate independence of acceptor vesicle concentration is not a *necessary* condition for diffusion-mediated transfer. With eq 15 as a guide, the dependence of the transfer rate on acceptor vesicle concentration can be seen to be minimal under conditions where the acceptor vesicle concentration [A] is in excess of the donor vesicle concentration [D] or the on-rate into the acceptor vesicle ($k_{D_+}^{II}$) is greater than the on-rate into the donor vesicle ($k_{D_+}^{I}$). Experiments presented herein and by Duckwitz-Peterlein et al. (1977) are examples of cases where initial transfer rates were not independent of acceptor vesicle concentration but are nevertheless consistent with the diffusion-mediated model.

Relevance to Biological Lipid Transfer. Previous studies have measured the rates of spontaneous, non-protein-catalyzed, intervesicular transfer of phospholipids by measuring shifts in transition temperature (Martin & MacDonald, 1976; Duckwitz-Peterlein et al., 1977; Kremer et al., 1977b) or by measuring the loss of pyrene-PC eximer formation (Roseman & Thompson, 1980; Galla et al., 1979). Neither of the above techniques nor the C_{12}-NBD-PC dequenching technique presented herein necessarily reflects the rates of spontaneous transfer of naturally occurring phospholipids. In the case of

the fluorescent lipids, the fluorophore may result in significant alterations in the intervesicular transfer rate as compared to diacyl phospholipids. On the other hand, the calorimetric technique requires substantial transfer of phospholipids before reliable measurements can be made, and as a result, it is not possible to distinguish between intervesicular phospholipid transfer, interbilayer transfer, or vesicle rearrangement as the rate-limiting process being measured [see Roseman & Thompson (1980) for a detailed discussion of these techniques]. Although the absolute rates of diffusional transfer of naturally occurring phospholipids are still in question, we hope that our studies with C_{12}-NBD-PC will be useful in understanding the diffusion-mediated transfer process for lipids in general.

Our kinetic model provides a possible mechanism for cellular membrane regulation of the type and amount of lipid that is incorporated via the diffusion of soluble monomers. In our model, as opposed to those previously proposed, the properties of the acceptor membrane can significantly affect the transfer of lipid monomers via diffusion. In this study, the degree of acceptor membrane "regulation" was limited to changes in phospholipid composition and phase state whereas in the case of cellular membranes, regulation may result from lipid-specific on- and off-rate catalysts. For example, one mechanism of action for phospholipid exchange proteins that has been postulated is a weakening of lipid–lipid or lipid–protein interactions in cellular membranes which would result in an increased off-rate and decreased on-rate for donor and acceptor membranes (Wirtz & Zilversmit, 1969). In addition, a membrane with low on-rate and high off-rate constants for lipids in general could conceivably selectively incorporate desirable lipids with the aid of specific on-rate catalysts. This model provides the framework for future investigations into the possible regulation of free monomer diffusion-mediated transfer of lipids between biological membranes.

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