for $[X_1']$ and $[X_2']$, respectively. When these are substituted into eq 2, one obtains

$$[R'] = 1/\left\{\phi_0 + \frac{\phi_1\alpha_1}{1 + \phi_1[R']} + \frac{\phi_2\alpha_2}{1 + \phi_2[R']}\right\} \quad (A2)$$

where

$$\phi_0 = 1 + [L']$$
$$\phi_1 = Q_1(1 + \beta_1[L'])$$
$$\phi_2 = Q_2(1 + \beta_2[L'])$$

Equation A2 may be solved iteratively to yield the value of $[R']$. Upon substitution of this value into eqs 3′ and 4′, the values of $[X_1']$ and $[X_2']$ are obtained. Then, the fractional saturation of agonist is calculated via the following transformation of eq 5:

$$y = [L'][R'](1 + Q_1\beta_1[X_1'] + Q_2\beta_2[X_2']) \quad (5')$$

Registry No. GppNHp, 34273-04-6; BTX, 23509-16-2; carbamylcholine, 462-58-8.

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Mechanism of Spontaneous, Concentration-Dependent Phospholipid Transfer between Bilayers†

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ABSTRACT: We have previously demonstrated that spontaneous phospholipid transfer between bilayer vesicles at higher vesicle concentrations is characterized not only by a first-order desorption rate but also by a second-order process dependent on vesicle concentration (Jones & Thompson, 1989b). We have extended our studies to examine the mechanism of this second-order process by investigating transfer as a function of lipid type, temperature, aqueous medium composition, and vesicle size. The results suggest a mechanism of concentration-dependent transfer in which the rate of lipid monomer desorption from vesicle bilayers is enhanced in transient vesicle–vesicle complexes.

The fact that lipids spontaneously transfer between biological and bilayer membranes in aqueous dispersion is well established [for reviews, see Sleight, (1987); Dawidowicz, (1987); and Phillips et al. (1987)]. It is generally recognized that transfer is independent of vesicle concentration in the low vesicle concentration range at which earlier studies have been

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carried out (Roseman & Thompson, 1980; Nichols & Pagano, 1981, 1982; McLean & Phillips, 1981, 1984). This observation of first-order kinetics has been generally accepted to indicate that lipid transfer occurs by way of rate-limiting monomer desorption from donor vesicles followed by rapid diffusion through the aqueous phase to acceptor vesicles. In a previous report, we examined the rate of 1-palmitoyl-2-[^{3}H]oleoylphosphatidylcholine and 1-palmitoyl-2-(10-pyrenyldecanoyl)phosphatidylcholine transfer between 1-palmitoyl-2-oleoylphosphatidylcholine vesicles over the extended concentration range 0.1–40 mM (Jones & Thompson, 1989b). In agreement with earlier studies, we found that in the concentration range below 2 mM transfer kinetics are best described by first-order process, independent of vesicle concentration. However, at acceptor lipid concentrations above 2 mM, a second-order term, proportional to acceptor concentration, must be added to the first-order desorption rate to describe adequately the kinetics of transfer. This result suggests that lipid transfer is the result of two concomitant processes, the one limited by lipid desorption from the donor vesicle and the other dependent on vesicle–vesicle interactions. At low lipid concentrations, the contribution of the second-order process to the transfer flux is negligible.

In this paper, we investigate the mechanism by which vesicle–vesicle interactions enhance lipid transfer rates by examining transfer as a function of lipid and medium composition, temperature, and vesicle size. Our results support a mechanism for concentration-dependent transfer in which the rate of monomer desorption is enhanced by interaction with a closely apposed acceptor vesicle in a transiently stable vesicle–vesicle complex. The marked enhancement of lipid transfer rates by vesicle–vesicle interactions may have important implications for the role of spontaneous lipid transfer in membrane biogenesis since this process results in large transfer fluxes at membrane bilayer concentrations equivalent to those found in many biological systems. A preliminary report of this work has appeared elsewhere (Jones & Thompson, 1989a).

Experimental Procedures

Materials. 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC),[1] dimyristoylphosphatidylcholine (DMPC),[1] 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG),[1] 1-palmitoyllysophosphatidylcholine, and 1-myristoyllysophosphatidylcholine were purchased from Avanti Polar Lipids (Birmingham, AL). 1-Palmitoyl-2-(10-pyrenyldecanoyl)phosphatidylcholine (PyrPC)[1] was purchased from KSV Biochemicals (Helsinki, Finland). [9,10-^{3}H]Oleic acid, [9,10-^{3}H]myristic acid, [1,2-^{3}H]cholesterol, and [^{14}C]cholesteryl oleate were obtained from New England Nuclear (Boston, MA). Unlabeled oleic acid was purchased from Sigma (St. Louis, MO), and unlabeled myristic acid was obtained from Nu Chek Prep (Elysian, MN). All lipids were checked for purity by TLC[1] on silica gel G plates and stored under nitrogen at 0 °C. 4-Pyrrolidinopyridine was purchased from Aldrich (Milwaukee, WI) and further purified by recrystallization from low-boiling petroleum ether. DEAE-Sephacel was obtained from Pharmacia-LKB (Piscataway, NJ). *N,N'*-Dicyclohexylcarbodiimide (DCC)[1] was purchased from Sigma.

Synthesis of Labeled Phospholipids. Tritiated oleic and myristic anhydrides were prepared from the corresponding fatty acids using DCC as described by Selinger and Lapidot (1966). 1-Palmitoyl-2-[^{3}H]oleoylphosphatidylcholine ([^{3}H]-POPC)[1] and 1-myristoyl-2-[^{3}H]myristoylphosphatidylcholine ([^{3}H]DMPC)[1] were synthesized by acylating the appropriate lysophosphatidylcholine with the corresponding anhydride employing 4-pyrrolidinopyridine according to the procedure described by Mason et al. (1981). The labeled product was purified by preparative TLC using a solvent system of $CHCl_3$/MeOH/H_2O (65/25/5). The purity of both compounds was >99%.

Liposome Preparation. Small unilamellar vesicles (SUV)[1] were prepared by sonication of liposomes of the appropriate lipid mixture using a probe sonicator as described by Barenholz et al. (1977). Unless otherwise noted, the vesicles were prepared in 10 mM Pipes (pH 7.0), 25 mM KCl, 0.5 mM EDTA,[1] and 0.02% NaN_3.

Large unilamellar vesicles (LUV)[1] were prepared in the same buffer by filter extrusion of liposomes using a high-pressure extrusion apparatus obtained from Lipex Biomembranes Inc. (Vancouver, BC). This method is described in detail by Hope et al. (1985). All vesicles were incubated overnight at room temperature prior to experiments. Phospholipid phosphate concentrations were determined by using the assay described by Bartlett (1959).

Charged Vesicle Assay. Lipid transfer was monitored by using the charged vesicle assay described in detail by Jones and Thompson (1989b) which is based on methods originally developed by Hellings et al. (1974). This assay employs two vesicle populations, negatively charged donor and neutral acceptor vesicles. The lipid transfer rate is determined by monitoring the movement of label from the donor to the acceptor vesicles as a function of time. Unless otherwise noted, vesicle compositions were as follows. Donors were POPC small unilamellar vesicles which contained 15 mol % POPG, a trace amount of a tritium-labeled lipid of which the transfer rate was determined, and a trace of [^{14}C]cholesteryl oleate which served as a nonexchangeable marker. In experiments reported earlier, we showed the charge on the donor vesicle to have no effect on the transfer process (Jones & Thompson, 1989b). POPC SUV were used as acceptors. In each case, a given concentration of donor and neutral acceptor vesicles was initially equilibrated at the appropriate temperature prior to mixing. Subsequently, the vesicles were mixed to give a total volume of 1.25 mL. At time intervals, a 0.05-mL aliquot was placed on a minicolumn containing 0.8 mL of DEAE-Sephacel which had been preequilibrated with 0.3 μmol of acceptor vesicles. The neutral acceptor vesicles were recovered by elution with 1.2 mL of Pipes buffer. This procedure ensured high acceptor recoveries (90–95%). Donor leakage was less than 5%. The vesicle dispersions were not stirred during transfer, since stirring has never been found by us to affect transfer. Following experiments, lipids were extracted with chloroform/methanol and analyzed by TLC. No breakdown products of any of the lipids were detected.

The movement of label from donors to acceptors due to spontaneous transfer is given by

$$[^3H]_{ST} = [^3H]_t - \{[^3H]_0[^{14}C]_t/[^{14}C]_0\}$$

Here $[^3H]_0/[^{14}C]_0$ is the initial ratio of the two labels in the donor vesicles. This equation corrects the observed net tritium transfer for leakage of donor vesicles through the ion-exchange

[1] Abbreviations: POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; PyrPC, 1-palmitoyl-2-(10-pyrenyldecanoyl)phosphatidylcholine; DMPC, 1,2-dimyristoylphosphatidylcholine; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol; DOPE, 1,2-dioleoylphosphatidylethanolamine; [^{3}H]POPC, 1-palmitoyl-2-[^{3}H]oleoylphosphatidylcholine; [^{3}H]DMPC, 1-myristoyl-2-[^{3}H]myristoylphosphatidylcholine; SUV, small unilamellar vesicle(s); LUV, large unilamellar vesicle(s); TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid; Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid); DEAE, diethylaminoethyl; NBD, 7-nitro-2,1,3-benzoxadiazol-4-yl; DCC, dicyclohexylcarbodiimide.

column or transfer due to donor–acceptor fusion. The corrected percent transfer is given by normalizing the calculated spontaneous transfer for acceptor recovery (Jones & Thompson, 1989b):

$$X(t) = [^3H]_{ST}/0.91$$

PyrPC Transfer. All excimer/monomer intensity measurements (E/M) were carried out on an SLM–Aminco (Urbana, IL) Model 4800 spectrofluorometer. Temperature was regulated to ±0.1 °C. The excitation wavelength was 346 nm (2-nm slit width). Monomer and excimer emissions were monitored at 378 nm (1-nm slit width) and 470 nm (1-nm slit width), respectively, using two monochromators. All samples were flushed and sealed under argon in gas-tight cuvettes to eliminate artifacts arising from either oxygen quenching or lipid peroxidation.

PyrPC transfer between vesicles was determined as follows. A given concentration of POPC vesicles containing 4 mol % PyrPC was equilibrated at 37 °C. After initial measurements were taken, unlabeled acceptor vesicles were added to give a total volume of 3 mL, and the decrease in E/M was monitored as a function of time. Pure donor vesicles were used as a control. Detailed descriptions of the experimental conditions are given when results are presented. Light scattering was always negligible. Analysis of lipids following experiments yielded no evidence of breakdown products. The principles underlying the use of pyrene lipids in transfer studies have been described previously (Roseman & Thompson, 1980; Correa-Freire et al., 1982; Jones & Thompson, 1989b).

Kinetic Analysis

The development of the kinetic model used in this analysis is presented in detail by Jones and Thompson (1989b). The differential equation for the initial transfer rate between donor and acceptor vesicles is

$$\frac{d[N_d]}{dt} = \frac{k_1k_2[N_d][D]}{k_2[D] + k_3[A]} - k_1[N_d] - k_c[N_d][A] \quad (1)$$

where [D] and [A] are the donor and acceptor vesicle concentrations, respectively, $[N_d]$ is the concentration of lipid in donor vesicles, k_1 is the dissociation rate constant from donor vesicles; k_2 and k_3 are association rate constants for donor and acceptor vesicles, respectively, and k_c is the second-order rate constant. In all experiments, transfer was monitored between liquid-crystalline vesicles of like geometric configuration. Thus, net lipid transfer from donors is assumed to be compensated for by back-transfer from acceptors. Therefore, [D] and [A] are treated as constants in the subsequent formulations, and k_2 is set equal to k_3.

Integration of eq 1 under these conditions yields

$$[N_d]_t = [N_d]_0 \exp\left[\left(\frac{k_1[D]}{[D] + [A]} - k_1 - k_c[A]\right)t\right] \quad (2)$$

where $[N_d]_0$ = initial concentration of donor lipid. Data are expressed as the fractional transfer of lipid from donor vesicles. Thus, $[N_d]_0 = 1$. The fraction of lipid remaining in the donors at time t is given by $[N_d]_t = 1 - [X(t)/X(\infty)]$ where $X(t)$ is the fraction transferred at time t and $X(\infty)$ is the fraction available for transfer. Unless otherwise noted, $X(\infty)$ is set equal to 0.67. This value is obtained by assuming two-thirds of the lipid is present on the outer monolayer and transbilayer movement is slow relative to desorption. This has been shown to be the case for phospholipid transfer from POPC SUV (Homan & Pownall, 1988).

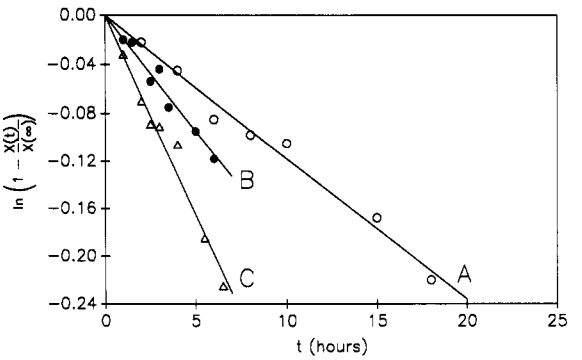

FIGURE 1: [^{3}H]POPC transfer from POPC SUV for 0.01 mM donors at 30 °C. Acceptor concentrations are (A) 0.5; (B) 10.0, and (C) 20.0 mM. Points represent averages obtained from triplicate experiments consisting of at least eight data points.

Table I: Rate Constants for [^{3}H]POPC and [^{3}H]DMPC Transfer at 30 °C

lipid	k_1[a]	k_c[b] (h^{-1} mM^{-1})	k_1/k_c (mM)
POPC	0.0087 ± 0.0009	0.00086 ± 0.00008	13 ± 3
DMPC	0.41 ± 0.05	0.013 ± 0.003	31 ± 6

[a] k_1 values were determined by using 0.01 mM donors and 0.5 mM acceptors. Values represent averages obtained from triplicate experiments consisting of at least six data points. [b] k_c values were determined by using 0.01 mM donors and 10 or 20 mM acceptors. Values are averages obtained from triplicate experiments consisting of at least six data points.

From eq 2, the slope of a log plot of $[N_d]_t$ vs time is given by

$$\text{slope} = \frac{k_1[D]}{[D] + [A]} - k_1 - k_c[A] \quad (3)$$

At dilute lipid concentrations, the term $k_c[A]$ is negligible relative to the other terms in eq 3. Thus, k_1 is given by

$$-\text{slope}\ \frac{[D] + [A]}{[A]}$$

Values for k_c were calculated by substitution of k_1 values determined at dilute lipid concentration into eq 3. The value of $k_c[A]$ was at least 20-fold lower than $[k_1[D]/([D] + [A]) - k_1$ under these conditions. Kinetic data were fitted by an iterative nonlinear least-squares analysis (Johnson & Frasier, 1985) on a Control Data Corp. Cyber 730 computer.

Results

Figure 1 illustrates [^{3}H]POPC transfer between SUV at 30 °C as a function of concentration. In Table I, the values of k_1, the first-order desorption rate constant, and k_c, the second-order rate constant, are given for DMPC and POPC transfer from POPC small unilamellar vesicles at 30 °C. It is apparent that both k_1 and k_c are about 2 orders of magnitude larger for DMPC than for POPC transfer at this temperature. The ratios of k_1 to k_c are, however, within a factor of 3 for the two systems. The somewhat larger value of the k_1/k_c for DMPC suggests that the second-order process is less efficient relative to desorption for this lipid compared to POPC.

Temperature Dependence of k_1. The temperature dependence of k_1 for POPC transfer from POPC SUV is shown as an Arrhenius plot in Figure 2. The activation energy for this first-order process was determined following the analysis developed by Nichols (1985). This treatment is based on a theoretical description of amphiphile monomer–micelle dissociation developed by Aniansson et al. (1976) from Kramers'

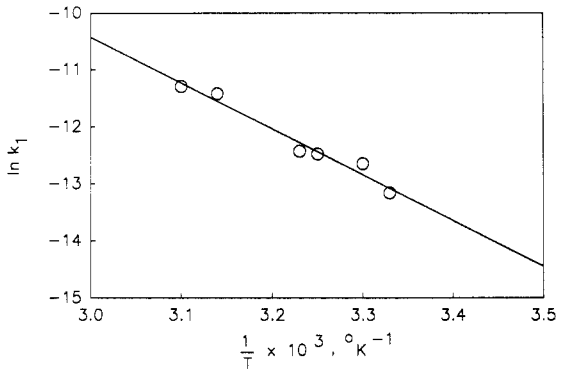

FIGURE 2: Arrhenius plot of k_1 values. Points represent averages of at least duplicate experiments consisting of at least eight data points.

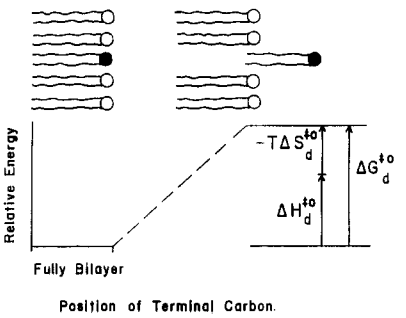

FIGURE 3: Energy diagram for formation of the activated complex for monomer transfer.

theory of reaction rates (Kramers, 1940). According to this model, the off-rate constant is given by the relationship:

$$k_1 = (D_m/l_b)^2 \exp(-\Delta G_d^{\ddagger o}/RT) \quad (4)$$

where D_m is the diffusion constant for the exiting monomer in the activated state, $\Delta G_d^{\ddagger o}$ is the maximum energy barrier to monomer dissociation, and l_b is the width of the barrier that is RT energy units below its maximum. R and T are the gas constant and absolute temperature, respectively. l_b^2/D_m is the time required for diffusional motion over the distance l_b, and $\exp(-\Delta G_d^{\ddagger o}/RT)$ is the relative probability that a monomer resides in the region of length l_b or within RT energy units of G. If it is assumed that the free energy of dissociation is a linear function of the length of monomer exposed to the bulk solution, then the ratio of l_b to the total length of the monomer, $l = 21$ Å for POPC (Hauser et al., 1981), is equal to the ratio $RT/\Delta G_d^{\ddagger o}$. With this assumption, a value of approximately 0.7 Å is obtained for l_b. D_m, the diffusion constant of the exiting monomer, is essentially that of the free monomer in solution, as shown in Figure 3. A value of $D_m = 5 \times 10^{-6}$ cm^2 s^{-1} was chosen as a reasonable estimate for the POPC molecule.

At constant pressure, $\Delta G_d^{\ddagger o} = \Delta H_d^{\ddagger o} - T\Delta S_d^{\ddagger o}$. Substitution into eq 4 yields

$$\ln k_1 = \frac{-\Delta H_d^{\ddagger o}}{RT} + \frac{\Delta S_d^{\ddagger o}}{R} + \ln (D_m/l_b) \quad (5)$$

The values computed from the data in Figure 1 using eq 5 are $\Delta H_d^{\ddagger o} = 15.3 \pm 0.6$ kcal mol^{-1}, $\Delta S_d^{\ddagger o} = 25.1 \pm 1.8$ cal mol^{-1} K^{-1}, and, at 37 °C, $\Delta G_d^{\ddagger o} = 23.1 \pm 0.1$ kcal mol^{-1}.

Temperature Dependence of the Second-Order Transfer Process. Since k_c was observed to be proportional to k_1, the activation energy specific for the second-order process was determined by analyzing the k_1/k_c ratio for [^{3}H]POPC transfer as a function of temperature. However, since collision frequency increases with increasing temperature, the k_1/k_c ratios must be normalized for a constant relative collision frequency, as described in Table II, to determine the effect of temperature on the efficiency of the second-order transfer process. Table II gives this normalized ratio for the indicated temperatures. It is apparent that this ratio is independent of temperature over the range examined. For this to be the case, k_c must have the same temperature dependence as k_1. Thus, the activation energies for both the first- and second-order processes are essentially the same.

Table II: Temperature Dependence of the k_1/k_c Ratio[a]

T (°C)	k_1/k_c[b]	$(k_1/k_c)_N$[c]
30	12.8 ± 2.3	8.3 ± 1.5
34	18.3 ± 4.8	12.9 ± 3.4
37	9.8 ± 2.9	7.4 ± 2.2
45	9.8 ± 2.3	8.8 ± 2.1
50	9.3 ± 1.8	9.3 ± 1.8

[a] Values represent averages obtained from at least triplicate experiments consisting of at least eight data points. [b] k_1[D] = 0.1 mM; [A] = 0.5 mM; k_c[D] = 0.1 mM; [A] = 10 or 20 mM. [c] Collision frequency is given by $f = 10^3\pi N_A(D_A + D_D)R_{AD}/V$ where N_A is the number of acceptor vesicles, D_A and D_D are diffusion coefficients for acceptor and donor vesicles, respectively, R_{AD} is the combined radii of the donor and acceptor vesicles, and V is the volume. The only variation with temperature occurs in the diffusion coefficient: $D = kT/6\pi\eta R$. Thus, all the values shown in this column were normalized for collision frequency relative to that at 50 °C according to the changes in viscosity and absolute temperature.

Table III: [^{3}H]POPC Transfer at 45 °C as a Function of Vesicle Size[a]

vesicle diameter (nm)	k_1 (h^{-1})	k_1/k_c (mM)	$(k_1/k_c)_N$[c]
20	0.041 ± 0.003	9.8 ± 2.3	9.8 ± 2.3
100	0.017 ± 0.001	189 ± 42	5.7 ± 1.4

[a] Values represent averages obtained from at least duplicate experiments consisting of at least 10 data points. [b] k_1[D] = 0.01 mM; [A] = 0.5 mM; k_c[D] = 0.01 mM; [A] = 50, 80, or 100 mM. k_c values were determined from duplicates of experiments at each of these concentrations. Thus, six experiments were used to calculate k_c. k_1 values were determined from triplicate experiments. [c] The k_1/k_c ratios were normalized for a constant collision frequency in the following manner. In the collision frequency equation $f = 10^3\pi N_A(D_A + D_D)R_{AD}/V$, the difference in frequency with vesicle size is given by the relative number of acceptor vesicles since the contributions from radii and diffusion coefficients cancel. Thus, the k_1/k_c ratio for the 100-nm vesicles was normalized relative to that for the 20-nm vesicles by calculating the relative number of vesicles at a given lipid concentration. The number of vesicles was determined from the formula for the area of a sphere $A = 4\pi r^2$, with 80 Å^2 used as the area of the POPC molecule.

Dependence of Transfer on Bilayer Curvature. In order to examine the effect of bilayer curvature on the first- and second-order transfer processes, values of k_1 and k_c were determined for POPC transfer from large unilamellar POPC vesicles at 45 °C. The average diameter of these vesicles was 100 nm as determined by quasi-elastic light scattering. For transfer between LUV, the second-order rate constant must be normalized to a constant collision frequency since the number of vesicles and hence collision frequency is much lower for LUV than for SUV at the same lipid concentration. Results are presented in Table III. For this system, the rate of transbilayer movement relative to the desorption rate is not known. The results in Table III were calculated by assuming transbilayer movement is negligible, and thus a value of 0.5 for $X(\infty)$, the fraction of lipid available for transfer, was used for these vesicles. The k_1 value is slightly less for LUV than for SUV at 45 °C. The k_1/k_c values given in column 3 ratio suggest that for a given lipid concentration, the second-order process is much less efficient for the large vesicles than for SUV. However, when this ratio is normalized to a constant

Table IV: PyrPC Transfer from POPC at 37 °C as a Function of Ionic Composition[a]

salt	k_1 (h^{-1})	k_1/k_c (mM)[b]
25 mM KCl	0.023 ± 0.003	15 ± 4
100 mM KCl	0.015 ± 0.002	13 ± 3
200 mM KCl	0.012 ± 0.002	9.2 ± 3
500 mM KCl	0.0081 ± 0.0009	11 ± 4
1 M KCl	0.0052 ± 0.0006	8.8 ± 3
1 M LiCl	0.0061 ± 0.0006	9.6 ± 3

[a] Values represent averages obtained from duplicate experiments consisting of at least eight data points. [b] k_1[D] = 0.05 mM; [A] = 1.0 mM; k_c[D] = 0.05 mM; [A] = 20 mM.

Table V: [^{3}H]Cholesterol Transfer from POPC at 37 °C[a,b]

[A]	k_1 (min^{-1})	$t_{1/2}$ (min)	$X(\infty)$
1	0.017 ± 0.002	41 ± 4	0.14 ± 0.02
10	0.015 ± 0.002	47 ± 6	0.18 ± 0.02
20	0.018 ± 0.001	39 ± 4	0.18 ± 0.01
40	0.016 ± 0.002	45 ± 7	0.16 ± 0.02

[a] Values represent averages obtained from duplicate experiments consisting of at least 10 data points. [b] [D] = 0.01 mM in all cases.

collision frequency, as presented in Table III, column 4, the second-order transfer process appears to be slightly more efficient per vesicle collision for the larger vesicles. It is clear that the concentration-dependent transfer process is not a consequence of the strained molecular packing conditions known to exist in SUV.

Effect of Aqueous Medium Salt Concentration on Transfer. First-order lipid transfer rates for a number of systems have been shown to be dependent on the salt concentration in the aqueous medium (De Cuyper et al., 1983). To ascertain whether the second-order transfer process was similarly dependent on salt concentration, k_c and k_1 values were measured for PyrPC transfer from POPC SUV at different KCl concentrations. The PyrPC transfer assay was employed because the ion-exchange minicolumns are inefficient at relatively high salt concentrations. Results are given in Table IV. The k_1 value decreases with increasing KCl concentration. This phenomenon arises from the "salting out" of the lipid from the aqueous phase as described by De Cuyper et al. (1983). The k_c values also decrease with increasing ionic strength. However, the ratio of the first- and second-order rate constants is independent of ionic strength within experimental error. The rate constants were also determined for 1 M LiCl. In this case, the ratio of the constants agrees within experimental error with that obtained for 1 M KCl.

Cholesterol Transfer. Although cholesterol transfer is not the subject of this report, limited data obtained for this biological membrane lipid are included because of the contrast they provide when compared to the phospholipid transfer results. Table V gives rate constants and fractional exchange for the transfer of [1,2-^{3}H]cholesterol from POPC at 37 °C as a function of vesicle concentration. The nonexchangeable fraction was previously observed by Bar et al. (1986) and Nemecz and Schroeder (1988) and presumably reflects the complex phase structure of cholesterol-containing vesicles. It is evident that over this concentration range the observed rate constants are independent of concentration. The half-time and fractional transfer values are in general agreement with those reported by Bar et al. (1986) for cholesterol transfer from POPC at low mole percent cholesterol.

Discussion

An activation energy diagram for the desorption of [^{3}H]POPC at 37 °C is given in Figure 3. The rate of transfer is limited by the rate of formation of the activated complex illustrated as a lipid monomer which has moved normal to the plane of the bilayer with only the terminal carbons remaining in it. The $\Delta G_d^{\ddagger\circ}$ of activation and the relative magnitudes of the $\Delta H_d^{\ddagger\circ}$ and $T\Delta S_d^{\ddagger\circ}$ are shown. The enthalpic contribution to the free energy has been previously discussed by Nichols (1985) in terms of a cavity theory of solvation. The disruption of lipid–lipid van der Waals and electrostatic head-group interactions to form the bilayer cavity necessary for lipid desorption acts to confer a positive enthalpy on the process. Also, water–water hydrogen bonds must be broken to accommodate the lipid acyl chain region in the aqueous phase. This process is likewise enthalpically unfavorable. Unfavorable entropic forces in the formation of the transition state arise principally from hydrophobic interactions of the lipid acyl chain region with the surrounding water (Tanford, 1980). However, a favorable entropic contribution should arise from the disruption of bilayer packing constraints concomitant with formation of the relatively disordered transition state.

This theory of lipid solvation accounts well for the dependence of lipid transfer rates on a variety of factors such as lipid structure and vesicle phase properties. For example, transfer of a lipid with longer acyl chains necessitates the formation of a larger water cavity that results in a higher activation energy. Thus, if the activation barrier is a linear function of acyl chain length, transfer rates should show an exponential dependence on chain length due to the exponential dependence of rates on activation energies. This prediction has been well borne out experimentally (Ferrell et al., 1985). Similarly, increased packing constraints in gel-phase vesicles should signficantly increase the activation enthalpy and thus cause slower off-rates. This has likewise been observed experimentally (McLean & Phillips, 1984).

The details of the energy diagram shown in Figure 3 are similar to those described by McLean and Phillips (1984) for the transfer of DMPC between small unilamellar vesicles. In both cases, the $\Delta G_d^{\ddagger\circ}$ of activation arises from both unfavorable enthalpic and net unfavorable entropic terms. This type of energy diagram lies in contradistinction to that observed by Nichols (1985) for the transfer of phosphatidylcholines labeled in the acyl chain with NBD[1] between small DOPC vesicles and by Wimley and Thompson (1989) for the transfer of DMPC between large unilamellar vesicles, 100-nm diameter. In these two cases, the activation energy is dominated by the enthalpic term with a slightly favorable entropic contribution. The presence of the relatively polar fluorescent group in the acyl chain region of NBD-PC certainly results in more favorable hydrophilic interactions with water than those obtained for diacyl lipids. Hence, the unfavorable contribution to the entropy is significantly reduced for these lipids. The more uniform lipid packing in the large vesicles examined by Wimley and Thompson likely results in a greater entropy gain during transition-state formation that is sufficient to offset the negative entropy arising from lipid–water interactions. These comparative studies reveal that the specific forces involved in transition-state formation for the lipid monomer transfer are complex functions of the system in question.

Comparison of the first- and second-order rate constants for the transfer of POPC and DMPC from POPC vesicles at 30 °C demonstrates that the second-order rate constant is roughly proportional to the first-order constant. This result precludes a model in which the second-order process reflects lateral diffusion of lipid molecule within a transient fusion complex of two vesicles occurring upon collision, as suggested by Gurd (1960). If this were the case, the second-order rate constant would be equivalent for these lipids, since all phos-

pholipids in liquid-crystalline vesicles have very similar lateral diffusion coefficients (Quo & Wade, 1979). Thus, the contribution of the second-order process to the flux of the relatively fast-moving DMPC would have been negligible. The failure to observe significant transfer of the nonexchangeable marker cholesteryl oleate also argues against the mechanism suggested by Gurd (1960). The observed rough proportionality between second-order and first-order constants is, however consistent, with a model in which the second-order process also occurs via monomer transfer between vesicles.

The data in Tables I and II lead to the conclusion that the second-order rate constant has essentially the same temperature dependence as the first-order desorption constant and hence the same activation parameters. This observation is also consistent with the idea that the second-order process is also essentially desorption and that no significant activation energy is to be associated with the vesicle–vesicle interactions evident in the second-order process.

This result strongly supports the conclusion that the interacting vesicles do not enter into physical contact with the concomitant dehydration of the vesicle surfaces (Rand & Parsegian, 1989). For phosphatidylcholine SUV approaches of less than approximately 15 Å, a large repulsive energy barrier must be overcome (Lis et al., 1983). This barrier arises principally from the work required to remove polarized water molecules from the vesicle surface. Thus, the formation of vesicle–vesicle complexes of this type would incur a very large activation energy. This, however, is not observed.

The half-time of transfer at a given concentration can be used in conjunction with the number of acceptor vesicles to determine a collision efficiency defined as the number of lipids transferred per collision. The number of acceptor vesicles, N_A, is determined at a given lipid concentration from the vesicle radius, the cross-sectional area of a lipid molecule, and the fraction of lipid in the outer monolayer. At 50 mM concentration, using 10 nm for vesicle radius and 80 Å^2 for the POPC cross-sectional area, N_A is 2.62×10^{14} vesicles/mL under the assumption that two-thirds of the lipid is in the outer monolayer. The collision frequency, f, is calculated from N_A and the diffusion coefficient as described in Table II, footnote *b*. At 37 °C, for 50 mM acceptor lipid, f is 1.08×10^6 collisions s^{-1} mL^{-1}.

The number of lipids transferred by the second-order process per unit time at a given concentration is calculated from the k_c value and the number of lipids in a donor vesicle. Using the above value for the POPC cross-sectional area, it is found that a 20-nm diameter vesicle contains 1540 lipid molecules in the outer monolayer. With a second-order half-time of 8.7 h at 37 °C, it is determined that 770 lipid molecules are transferred in 8.7 h. Therefore, when the above value of collision frequency is used, the collision efficiency is 2.3×10^{-8} lipids/collision, or 43.9×10^6 collisions are required to desorb one lipid molecule. This result indicates that the second-order transfer process is not limited by vesicle collisions and is in fact highly inefficient.

The results obtained in this study are consistent with a model in which the rate of monomer desorption from one vesicle is enhanced by interaction with a closely apposed vesicle (Jones & Thompson, 1989b). The two vesicles in the complex are not in physical contact but are separated by a water-filled gap. The dimension of the gap is dictated by the minimum in the interaction energy function of the two vesicles (Lis et al., 1983). Thus, the effective activation energy associated with transition-state formation is deceased by monomer–acceptor vesicle interactions as depicted in Figure 4. The rate of transfer by

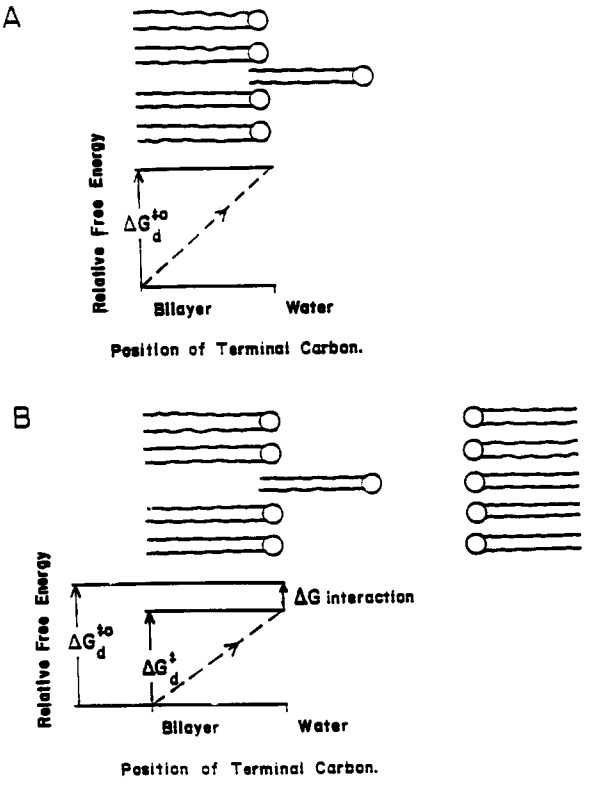

FIGURE 4: Energy diagram illustrating the effect of acceptor vesicle interaction on monomer activation energy. In panel A, the free energy associated with monomer desorption is illustrated in like manner as in Figure 3. In panel B, the decrease in activation energy upon apposition of an acceptor vesicle is illustrated with $\Delta G_{interaction}$ = the attractive energy imparted to the monomer by interaction with the acceptor vesicle. The vesicle–vesicle separation distance is assumed to be approximately that at which repulsive and attractive forces are equal (approximately 15 Å).

this interactive process is limited by the rate of formation of this lower energy transition state. The rate, however, depends on the properties of the lipid monomer in the same way as it does in the first-order process. This model also accounts for the observed correlation of the first- and second-order rate constants.

The decrease in activation energy for monomer desorption by interaction with acceptor vesicles can be calculated at a given acceptor concentration from the apparent rate constant (k_c[A]) according to the theoretical relationship between off-rate and $\Delta G_d^{\ddagger o}$ given by eq 4.

At 37 °C, the value of $\Delta G_d^{\ddagger o}$ for the first-order process was determined to be 23.1 kcal/mol with a half-time of 46 h. At 50 mM acceptor lipid concentration with a k_c value of 0.0013 h^{-1} mM^{-1}, the $\Delta G_d^{\ddagger o}$ value is 22.1 kcal/mol with an apparent half-time of 7.6 h. Thus, only approximately 1 kcal/mol difference in activation free energy is sufficient to yield an off-rate difference of approximately a factor of 5.

The force–distance figures for phosphatidylcholine vesicle–vesicle interactions have been calculated by Lis et al. (1983). At a distance greater than that at which short-range repulsive forces dominate the interaction, there exists a region in which the vesicles experience a net attractive van der Waals interaction. Vesicles may form a transient vesicle–vesicle complex at this separation distance. Thus, if an acceptor vesicle is apposed to a donor in such a complex, the formation of the activated state of the desorbing phospholipid monomer is enhanced by the attractive force between the apposing vesicle and desorbing monomer.

An approximate expression for the energy of interaction between a vesicle and a lipid monomer at a given distance from

the vesicle has been developed by Dr. V. A. Parsegian (personal communication). It is based on the treatment described in Parsegian (1975):

$$E = Hb/6d \quad (6)$$

where H is the Hamaker constant, b is the radius of a lipid molecule, and d is the distance of a monomer from an acceptor vesicle. When a value of 8.2×10^{-14} erg for H (Lis et al., 1983) and a reasonable value of $b = 6.5$ Å are used, energy values range from 0.085 kcal/mol for $d = 15$ Å to 1.3 kcal/mol for $d = 1$ Å. These energies are in rough agreement with the excess energy of about 1 kcal/mol calculated above.

Another potential consideration important in evaluating vesicle–vesicle interaction effects on monomer transfer concerns the relative statistical probability of lipid adsorption by donor and acceptor vesicles. The model presented thus far assumes once a lipid monomer completely escapes from the surface of a donor vesicle, the lipid subsequently diffuses and associates with donor and acceptor vesicles according to the [A]/[D] ratio. However, by a random diffusion process, the lipid monomer is most likely to associate with the vesicle closest to it. This vesicle would typically be the donor vesicle from which it desorbed. Thus, under dilute vesicle conditions, the measured off-rate may be an underestimate due to preferential monomer reassociation with its donor vesicle. At higher concentrations where the second-order process contributes significantly to transfer, it may be that monomer adsorption by a closely apposed acceptor vesicle becomes more favorable. Therefore, k_c values reflect monomer–vesicle interactions due to factors inherent in the diffusion process. The probabilities associated with escape from initial conditions in a random walk processes in solution have been modeled by Chandrasekhar (1943).

The hydration barrier surrounding vesicles is predicted to be stronger with increasing vesicle size due to the more planar surface of contact (Lis et al., 1983). Thus, it might be expected that k_c would be smaller for large vesicles than for smaller ones. However, comparison of the k_1/k_c ratio, normalized for collision frequency, for the transfer of [^{3}H]POPC between 20- and 100-nm diameter vesicles (Table III) indicates that the second-order process is slightly more efficient for the larger vesicles per vesicle collision. Other factors, however, must be considered which may cause transfer during the lifetime of the transient vesicle–vesicle complex to be more efficient in larger vesicles. The larger the vesicle, the greater the area of apposition of the vesicles in the complex. In addition, larger vesicles are generally more deformable, which could function to increase the area of apposition and the stability of the complex. Also, because of the dependence of the diffusion constant on size, larger vesicles diffuse away from each other more slowly than smaller vesicles and thus further increase the probability of transfer of monomers during contact. Thus, our results must be considered as a first approximation regarding the effect of vesicle size on the second-order transfer process.

The first-order off-rate for PyrPC transfer from POPC SUV decreases with increasing KCl concentration. A similar result was previously observed for DMPC transfer by De Cuyper et al. (1983). This effect presumably arises from less favorable water–lipid interactions at higher salt concentrations that decrease the aqueous phase solubility of lipid monomers. This "salting out" effect by hydrophobic interactions at high salt concentration has been discussed by Tanford (1980). The independence of k_1/k_c for PyrPC transfer on KCl concentration shows the salt dependence for both k_1 and k_c to be the same. This result strongly supports the model in which desorption is enhanced in the vesicle–vesicle complex.

In sharp contrast to the phosphatidylcholine transfer results, the kinetics of transfer of a trace amount of [1,2-^{3}H]cholesterol from POPC vesicles, examined at 37 °C and acceptor concentrations ranging from 1 to 40 mM, showed no dependence of cholesterol transfer rate on vesicle concentration. This result would seem to argue against vesicle–vesicle interactive transfer arising solely from probability considerations of monomer association with acceptor vesicles. If this were the case, all lipid species escaping from a donor vesicle should interact similarly with a closely apposed acceptor. Alternatively, this result is consistent with the conclusion that specific forces between monomers and acceptor vesicles lead to enhancement of transfer rates. It is quite reasonable that cholesterol interacts less efficiently with an apposed vesicle than does phosphatidylcholine. This molecule has a shorter molecular axis than that for phospholipids. Thus, a cholesterol molecule in the transition state would sense an apposing vesicle more weakly than the phosphatidylcholines examined in this study, based on the distance dependence of van der Waals forces. Also, monomer–vesicle head-group interactions may contribute significantly to the attractive force, and this force component is obviously significantly reduced for cholesterol. It may be that molecular length considerations also account for the greater efficiency of the vesicle–vesicle interactive transfer process for POPC than for DMPC. In like manner to cholesterol, the shorter DMPC monomer would interact less efficiently than POPC with an acceptor vesicle in the transition state. Thus, the relative efficiency of the second-order transfer process may be expected to be highly dependent on monomer properties.

Steck and co-workers (Steck et al., 1988) observed complex kinetics for the transfer of cholesterol from erythrocytes to various acceptors. This is in contrast to the simple first-order kinetics we report for the transfer of this molecule between POPC vesicles. They proposed that transfer in their system involves the formation of a transient collisional complex between donor and acceptor. Two alternative transfer mechanisms based on this idea are discussed by them. In the simplest of these, which they found inadequate to describe their cholesterol transfer data, there is a first-order desorption process and an additional transfer process that occurs in the transient donor–acceptor vesicle complex. In this scheme, there are then two parallel transfer processes. Transfer in the complex will always be second order regardless of the efficiency of the collisions. Our data for both phospholipid and cholesterol transfer are consistent with this mechanism (Jones & Thompson, 1989a,b); however, it is clear that cholesterol transfer in the donor–acceptor complex is negligible in our system. Furthermore, the temperature dependence of the second-order phosphoplipid transfer rate leads us to propose that transfer in the transient donor–acceptor complex is simply an enhancement of the intrinsic desorption process caused by interaction with a closely opposed vesicle, as discussed above.

The alternative mechanism discussed by Steck and co-workers (Steck et al., 1988), and found sufficient to handle their data, requires the formation of an "activated" cholesterol in a donor vesicle prior to collision with an acceptor. This "activated" cholesterol is then transferred in the collisional complex to the acceptor. Thus, two sequential process are involved. Transfer based on this mechanism will be first order as long as the collision rate is not limiting. The rate equation describing this process developed by Steck and co-workers is the same as that given by Ferrell et al. (1985) to describe the "caged" monomer transfer of several phosphatidylcholines from

small vesicles to human erythrocytes. This model is not compatible with our phospholipid transfer data.

Several other studies in the literature have demonstrated lipid transfer to be dependent on concentration under a variety of experimental conditions (Jonas & Maine, 1979; Petrie & Jonas, 1984; Mütsch et al., 1986; Nichols, 1988). Nichols and Pagano (1981) have shown that concentration-dependent transfer can arise in the absence of vesicle interactions if monomer association rates are different for donor and acceptor vesicles. It is possible that in some of these cases, the concentration-dependent transfer may have been due to this effect. Nichols (1988) demonstrated that transfer of NBD-phosphatidylcholine between a variety of mixed phospholipid–bile acid micelles depends on micelle concentration. The results in this case suggested a model in which transfer occurs upon formation of transient collisional complexes; however, higher order terms representing complexes larger than dimers are necessary to describe adequately the transfer kinetics. Thus, the mechanism of the concentration-dependent process in this case is clearly different from that described in our study. Burgess et al. (1989) examined the transfer of a variety of phospholipids between LUV in the presence of poly(ethylene glycol) (PEG). At PEG concentrations lower than that necessary to induce vesicle fusion, transfer from the vesicle outer monolayer was enhanced. PEG acts to dehydrate the vesicle surface, thus promoting closer vesicle approach. It seems possible that in the PEG concentration range where transfer was enhanced in the absence of fusion, a process similar to that described in our study may have been operative.

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Registry No. POPC, 6753-55-5; DMPC, 13699-48-4; POPG, 81490-05-3; [^{3}H]POPC, 124580-43-4; [^{3}H]DMPC, 124511-76-8; [9,10-^{3}H]oleic acid, 636-34-0; [9,10-^{3}H]myristic acid, 124511-74-6; [9,10-^{3}H]myristic anhydride, 124511-75-7; [9,10-^{3}H]oleic anhydride, 98360-02-2; cholesteryl oleate, 303-43-5; cholesterol, 57-88-5; 1-palmitoyllysophosphatidylcholine, 17364-16-8; 1-myristoyllysophosphatidylcholine, 13699-45-1.

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