Mechanism of Poly(ethylene glycol)-Induced Lipid Transfer between Phosphatidylcholine Large Unilamellar Vesicles: A Fluorescent Probe Study†

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ABSTRACT: Experiments were performed to assess three possible mechanisms of poly(ethylene glycol) (PEG) induced rapid lipid transfer between large unilamellar vesicles composed of dioleoylphosphatidylcholine: (1) transfer between aggregated vesicles, (2) transfer through an aqueous medium of lowered dielectric constant, and (3) transfer via a PEG carrier. The results showed that close contact between vesicles as a result of PEG dehydration was largely responsible for the rapid lipid transfer observed in the presence of PEG. The rate and extent of lipid transfer were also examined at 10 wt % PEG and analyzed in terms of a two-state model especially developed to account for the initial rate of lipid transfer as followed by the fluorescence lifetime of DPHpPC as a fluorescent lipid probe. Analysis revealed that two rate processes were involved in DPHpPC transfer between bilayers, both in the absence and presence of PEG. Since the maximum extent of transfer was 50%, transbilayer diffusion of DPHpPC seemed not to contribute to either process. The fast process in the presence of PEG was identified as due to rapid interbilayer monomer diffusion between closely apposed vesicles, and, in the absence of PEG, as due to monomer diffusion through the aqueous phase. The origin of the slow process, in both cases, remains obscure. The Arrhenius activation energies (and entropies) for the initial rates at temperatures from 10 to 48 °C were 15.3 ± 0.3 kcal/mol (−26.3 ± 0.2 eu) and 10.6 ± 0.5 kcal/mol (−16.1 ± 0.3 eu) in the absence and presence of PEG, respectively. The slow process was invariant with temperature. From these studies, we propose that PEG enhances the rate of intervesicle lipid transfer by forcing dehydration and aggregation of vesicle bilayers, thereby altering the properties of the aqueous phase between membranes.

There has been increasing interests in recent years in the mechanism of membrane fusion induced by poly(ethylene glycol) (PEG)[1] (Morgan et al., 1983; Boni et al., 1984; MacDonald, 1985; Parente & Lentz, 1986b). Several studies have used lipid transfer assays and turbidity measurements to monitor PEG-induced fusion (McDonald, 1985; Parente & Lentz, 1986a; Boni & Hui, 1987; Yamazaki et al., 1989). However, lipid transfer does not necessarily reflect fusion. It has been shown that vesicles composed of pure phosphatidylcholine did not fuse in the presence of PEG even though extensive lipid transfer occurred between vesicles (Burgess et al., 1991a). Incorporation of small amounts of amphipathic compounds in phosphatidylcholine vesicles was sufficient to allow fusion in the presence of PEG. It is evident that we must distinguish between structural perturbations that support lipid transfer and others that support fusion. Nonetheless, an understanding of the mechanism of PEG-induced lipid transfer should provide important insight into the behavior of closely juxtaposed bilayers, and this, in turn, is important for understanding the fusion process.

Extensive studies have been carried out to investigate the mechanism of lipid transfer (Jones & Thompson, 1990; Fullington et al., 1990; Wimley & Thompson, 1990). Transfer appears to occur primarily via diffusion of lipid monomers through the aqueous medium, but may involve, at higher lipid concentrations, direct transfer between transiently interacting vesicles. Despite this information, little is known about the mechanism of PEG-induced lipid transfer. It has been shown from zeta potential measurements that PEG is excluded from the water space directly adjacent to the membrane surface (Arnold et al., 1990). This means that PEG can not induce lipid transfer by directly perturbing the lipid bilayer but must exert its influence by an indirect mechanism. The mechanism of this indirect effect is unknown. One possible mechanism that comes to mind is one in which PEG simply causes aggregation of vesicles, with transfer being more likely between aggregated vesicles simply because of their juxtaposition. Transfer could be further enhanced by alteration of the properties of the thin layer of water between adjacent bilayers. A second possibility is that PEG so alters the properties of the bulk solution that transfer through the aqueous environment becomes more favorable and more rapid. Finally, PEG could act as a carrier for phospholipids, thereby increasing the rate of the apparent rate-limiting step, dissociation of a phospholipid molecule from the bilayer into water. The purpose of this report is to distinguish between these various possibilities.

1-Palmitoyl-2-[[[2-[4-(phenyl-*trans*-1,3,5-hexatrienyl)-phenyl]ethyl]oxy]carbonyl]-3-*sn*-phosphatidylcholine (DPHpPC)[1] has been used in this laboratory as a relatively nonperturbing fluorescent lipid whose fluorescence anisotropy probes membrane order and whose fluorescence lifetime can monitor local changes in lipid concentration due either to lipid transfer or to phase separation (Parente & Lentz, 1986a,b). Our earlier applications of this probe to monitor fusion (Parente & Lentz, 1986b) treated the data in a way that was valid only in the limit of extensive mixing of lipids between fusing vesicles. In the current report, we develop a two-state model for the lipid transfer process that should be valid both during the initial phase of transfer and near the completion

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[1] Abbreviations: PEG, poly(ethylene glycol); EDTA, ethylenediaminetetraacetic acid, tetrasodium salt; TES, *N*-[tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid; DOPC, 1,2-dioleoyl-3-*sn*-phosphatidylcholine; DPHpPC, 1-palmitoyl-2-[[[2-[4-(phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]oxy]carbonyl]-3-*sn*-phosphatidylcholine; LUVET, large unilamellar vesicles made by a rapid extrusion technique.

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of the process. Using this model, we have been able to analyze measurements of probe fluorescence lifetime to obtain rate constants for relatively slow intervesicle probe transfer in the presence of 10 wt % PEG. We show elsewhere that this concentration of PEG does not induce fusion of large unilamellar vesicles but does induce lipid transfer between vesicles (Burgess et al., 1991a). The temperature dependence of the rate constants reported here provides a measure of how interbilayer water structure in the presence of PEG differs from the structure of bulk water into which a lipid must diffuse during the dissociation step of the transfer process.

Materials and Methods

Materials

Chloroform stock solutions of 1,2-dioleoyl-3-*sn*-phosphatidylcholine (DOPC)[1] and DPHpPC[1] were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). Lipids were verified to be greater than 98% pure by thin-layer chromatography on Analtech (Newark, DL) GHL silicic acid plates developed in a 65:25:4 (v/v/v) $CHCl_3/CH_3OH/H_2O$ mixture and stained with iodine vapors. DPHpPC was also viewed under near-UV light. Poly(ethylene glycol) (PEG)[1] (average molecular weight 8000) was obtained from Fisher Scientific (lot 874229) and was purified by a procedure described elsewhere (Lentz et al., 1991). *N*-[Tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid (TES)[1] was purchased from Calbiochem (LaJolla, CA). Other chemicals were reagent grade or of the highest quality available. Buffer solutions were filtered prior to use through a Nalgene disposable filter (Nalgene Company, Rochester, NY) to remove dust which might interfere with the fluorescence measurements. Dialysis tubing of 12000 to 14000 and 50000 molecular cutoffs were obtained from Spectrum Medical Industries, Inc. (Los Angeles, CA). Filters with pore sizes of 0.1 and 0.05 μm were purchased from Nucleopore Corporation (Pleasanton, CA). ^{14}C-radiolabeled PEG and ^3H_2O were obtained from Amersham Corporation (Arlington Heights, IL). The delrin rod for constructing the dialysis cells was purchased from the Golden Rule Company (Burlington, NC).

Methods

Vesicle Preparation. Large unilamellar extrusion vesicles (LUVET)[1] were prepared by the method of Hope et al. (1985). Dried lipid samples were suspended at a temperature above the gel–liquid-crystalline phase transition in an appropriate amount of buffer (100 mM NaCl, 2 mM TES, 1 mM EDTA, pH 7.4) to reach concentrations of 2.5 and 0.25 mM for probe-free and probe-rich vesicles, respectively. The lipid was allowed to equilibrate and fully hydrate above its phase transition for approximately 30 min, with agitation. The resulting multilamellar vesicles were then forced repeatedly (seven times) through a 0.1-μm polycarbonate filter (Nucleopore, Pleasanton, CA) above their phase transition under an argon pressure of approximately 200 psi. This procedure yielded a fairly homogeneous, stable population of unilamellar vesicles with an average diameter of 1300 Å (Burgess et al., 1991a). The concentrations of all vesicle samples were determined by phosphate analysis using a modification of the procedure of Chen et al. (1956).

Fluorescence Lifetime Measurements and the Lipid Transfer Assay. All fluorescence measurements were made on an SLM 48000 spectrofluorometer (SLM-Aminco, Urbana, IL) as described in detail elsewhere (Burgess et al., 1991a; Burgess & Lentz, 1991). Calibration curves were generated for 0 wt % and 10 wt % PEG at a lipid concentration of 0.25 mM by measuring the DPHpPC lifetime at three different lipid/probe ratios, as described by Burgess and Lentz (1991). Once the calibration curves were established for a particular lipid system, probe-containing (donor; lipid/probe = 10) vesicles and probe-free (acceptor) vesicles were mixed and added to buffer solution with or without 10% PEG, resulting in a final phospholipid concentration of 0.025 and 0.25 mM for the donor and acceptor vesicles, respectively. After transfer to a cuvette, the first measurement of lifetime was made immediately and thereafter lipid transfer kinetics were monitored by measuring the phase shift fluorescence lifetime at a modulation frequency of 30 MHz (averaged over 50 data points) for up to 130 h. We have shown previously (Barrow & Lentz, 1985; Parente & Lentz, 1986a,b) that the average fluorescence lifetime of DPH is well approximated by the 30 MHz phase lifetime. The phase shift of an isochronal reference fluorophore at 23 °C [DPH in heptane; $\tau = 6.75$ ns, 2×10^{-1} M; Barrow and Lentz (1983)] was measured before and after the kinetic experiment to determine the average phase angle of the reference (there was no change over the time period of the experiment). The sample phase angles were converted to lifetimes by the method of Spencer and Weber (1969).

Dialysis Experiments. Three dialysis experiments were designed to distinguish between the three possible mechanisms of lipid transfer induced by PEG, as mentioned above. Experiment I was performed by using 12000–14000 molecular cutoff dialysis membrane. Samples of probe-containing and probe-free vesicles (50 μL each) were delivered into dialysis bags containing 100 μL of buffer (100 mM NaCl, 2 mM TES, 1 mM EDTA, pH 7.4) to reach a final concentration of 0.025 mM for donor and 0.25 mM for acceptor vesicles. The bags were dialyzed for varying lengths of time against 5-mL volumes of buffer containing 10 wt % PEG, emptied into a cuvette, rinsed three times with 300 μL of buffer, and then diluted into a cuvette to reach a final volume of 1.7 mL. Phase angle data were collected immediately after the sample was loaded into the cuvette.

Experiments II and III were performed in a specially designed dialysis chamber. Two subchambers made of delrin were separated by a membrane filter, with a sample port located at the top and a magnetic stir bar at the bottom of each chamber. Membrane filters had molecular cutoff pore sizes of 12000–14000 Da for experiment II. In experiment III, filters having a pore size of 0.05 μm were used. A total of 1.5 mL of 2.5 mM probe-free vesicles was delivered into one side of the chamber, and 1.5 mL of 0.25 mM probe-containing vesicles was delivered into the other side. PEG-containing buffer was delivered into each side of the chamber to reach a final volume of 3 mL and 10% (w/v) PEG concentration and incubated at 48 °C during the experiment. Samples (50 μL) were extracted from the chamber every 10 min and diluted into buffer (final volume of 1.7 mL) in fluorescence cuvettes for phase angle measurements to follow probe transfer between the vesicles in the different chambers.

Water Content Measurement. In order to measure the rate of water loss from the dialysis bag during experiment I described above, the same experimental procedure described above was used except that the normal buffer was replaced by one containing a small amount of ^3H_2O. A sample (20 μL) was removed from the bag every 2 min during the first 10 min and every 10 min during the rest of the experiment (140-min duration), diluted into scintillation solution, and counted with a LKB 1211 Rackbeta liquid scintillation counter (Wallac, Finland). The data were corrected for counting efficiency and normalized to obtain the percent of total water content removed from the bag as a function of time.

Analysis of Kinetic Experiments

Kinetic Model. The kinetic analysis for this study is based on a two-state model that assumed that lipid transfer occurred

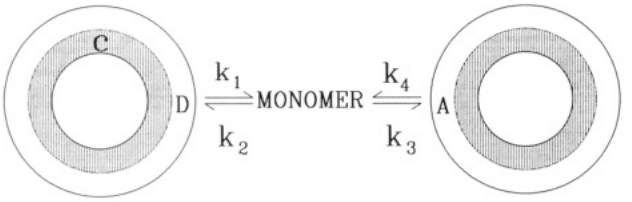

FIGURE 1: Schematic drawing of the two-state model for lipid transfer. Population D represents probe molecules in the outer leaflet of probe-rich donor vesicles and population A represents probe molecules in probe-poor acceptor vesicles. C represents a lipid population unavailable on the time scale of this experiment. k_1 is the off-rate constant of lipid dissociation from donor vesicles and is the rate constant observed in our measurements.

between two vesicle probe environments, a probe-rich donor environment, D, and a probe-poor acceptor environment, A. Probe molecules in the probe-rich population (low lipid–probe ratio) would have a low fluorescence lifetime due to the tendency of DPHpPC to form dimers (Lentz & Burgess, 1989), while probe molecules in the probe-poor population (high lipid–probe ratio) would exist mainly as monomers, which have a longer fluorescence lifetime (Lentz & Burgess, 1989). Our initial experiments made it clear that only half of the initial probe-rich donor environment participated in the transfer process, i.e., transbilayer migration of DPHpPC did not contribute to the observed kinetics of transfer. This meant that a third unchanging or constant probe-rich environment, C, had to be taken into account in our analysis (see Figure 1). Since, under our conditions, aggregated vesicles still have a water region between their membrane bilayers, our analysis follows closely the analysis of Nichols and Pagano (1981), who assumed that transfer proceeded via dissociation of a monomer into an aqueous environment followed by reassociation of the monomer with either the donor or acceptor vesicles (see Figure 1). This model has been treated in detail by several authors, including Nichols and Pagano (1981), Storch and Kleinfeld (1986), and Jones and Thompson (1989). Starting with eq 10 of Jones and Thompson and recognizing that, with respect to the transfer process, our donor and acceptor vesicles are essentially identical (probe on-rates and off-rates should be equal for the different environments), we obtain for the time dependence of the number of moles of probe molecules associated with the donor population, $N_D(t)$

$$\frac{d[N_D]}{dt} = \frac{k[D][N_D]_0}{[D] + [A]} - k[N_D] \tag{1}$$

where $[N_D]_0 = N_A(t) + N_D(t)$ is the initial number of moles of probe associated with the donor population and [D] and [A] are the molar concentrations of lipid in donor and acceptor vesicles, respectively (since donor and acceptor vesicles were of the same size and had the same number of molecules per vesicle). The solution of this differential equation has the single-exponential form

$$N_D(t) = [N_D]_0 \frac{[D]}{[D] + [A]} \left(1 + \frac{[A]}{[D]} e^{-kt}\right) \tag{2}$$

in which $k = k_1$ is the rate constant for loss of probe from the outer leaflet of a donor vesicle membrane. The value of k was adjusted to obtain a fit to the data. For experiments either at high temperatures or in the presence of PEG, a single exponential of the form of eq 2 did not provide a good fit to the entire time course of lipid transfer and only the initial time points were used in the fitting procedure (see Results).

Treatment of Lifetime Data. The observed fluorescence lifetime of DPHpPC in the mixed population system under study is the average of the lifetime contributions from the three environments described above. The time-resolved decay of fluorescence intensity $F(t)$ for such a multicomponent system can be expressed as

$$F(t) = \sum_i \alpha_i e^{-t/\tau_i} \tag{3}$$

where τ_i are the individual decay times and α_i the associated preexponential factors whose sum is normalized to 1. For a multiexponential decay of fluorescence, the average lifetime can be calculated as

$$\langle \tau \rangle = \sum_i f_i \tau_i = f_D \tau_D + f_A \tau_A + f_C \tau_C \tag{4}$$

where τ_i is the lifetime of component i and f_i is the fractional intensity contributed by the i th component. For the particular system under study, the lifetime is averaged over three membrane compartments, donor (D), acceptor (A), and constant (C) compartments, as shown. The fractional intensity from the ith component is related to the preexponential factors

$$f_i = \frac{\alpha_i \tau_i}{\sum_i \alpha_i \tau_i} \tag{5}$$

The transfer of lipid molecules from donor to acceptor vesicles can, therefore, be quantitatively followed by resolving the time dependence of the observed lifetime into the time dependencies of fractional intensities contributed by individual species. The fractional intensities can be related to the desired quantities $N_D(t)$, $N_A(t)$, and N_C in the following way: the ratio of any two fractional intensities can be used to obtain the ratio of the corresponding N_i, according to the expression

$$f_A/f_D = \alpha_A \tau_A / \alpha_D \tau_D = N_A F_A / N_D F_D \tag{6}$$

where F_i is the molar fluorescence intensity of probe in the ith environment. This relationship is also applicable to the ratio of population C relative to D, etc.

Following Parente and Lentz (1986a), the local concentration of probe in one of the exchanging environments is expressed as a lipid/probe ratio

$$LP_D(t) = \frac{[D]}{N_D(t)} \tag{7}$$

where [D] is the molar concentration of lipid in donor vesicles. Similar expressions occur for all three environments.

Probe Calibration Curves. The key to the method presented here is that the fluorescence lifetime of DPHpPC is sensitive to its concentration in the bilayer (Parente & Lentz, 1986a). As described earlier, calibration curves of DPHpPC lifetime as a function of lipid–probe molar ratio must be established in each concentration of PEG. An example is shown in Figure 2. We have previously shown these calibration curves to be reasonably well described by the empirical relation

$$\tau_D = c_1 - (c_1 - c_2) e^{-c_3(LP_D - c_4)} \tag{8}$$

where c_1, c_2, c_3, and c_4 are constants for any system, as defined in Figure 2, and LP_i is the lipid–probe molar ratio in the local environment of interest.

Another calibration is necessary to obtain the molar fluorescence intensity of probe needed in eq 6. Normally, this would be proportional to the probe lifetime, but, because of its peculiar photophysical properties, this is not the case for DPHpPC (Lentz & Burgess, 1989). Experimentally, the ratio of molar fluorescence to lifetime, F/τ, can be expressed as a function of lipid–probe ratio (see Figure 3) and fit to an empirical relation similar to that used for the lifetime:

$$F/\tau = c_5 - (c_5 - c_6) e^{-c_7(LP - c_8)} \tag{9}$$

where c_5, c_6, and c_7 are constants that must be obtained for each PEG concentration and whose physical meaning is explained in the legend to Figure 3. This relationship allows the

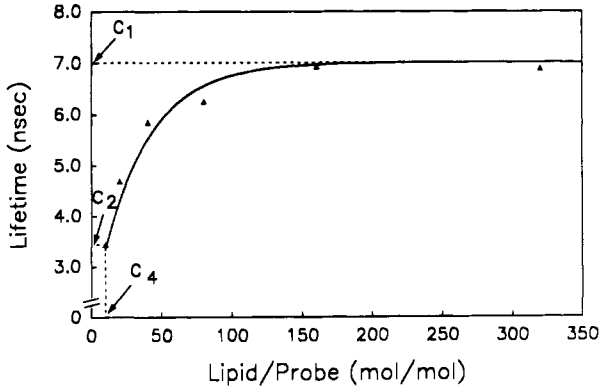

FIGURE 2: Dependence of DPH_pPC fluorescence lifetime on the surface concentration of probe in DOPC LUVET. The lipid concentration was 0.25 mM, and the vesicles contained different concentrations of probe. The phase-shift-derived lifetime recorded at a 30-MHz modulation frequency is plotted as a function of the lipid/probe molar ratio. This calibration curve of DPH_pPC probe could be well described by the empirical relation given in eq 8 under Methods, in which the adjustable parameters are c_1, the lifetime at the highest (limiting), and c_2 the lifetime at the lowest (initial), lipid/probe ratio examined, c_3, the exponential rate of lifetime change with lipid/probe ratio, and c_4, the lowest lipid/probe ratio examined (at which c_2 was observed).

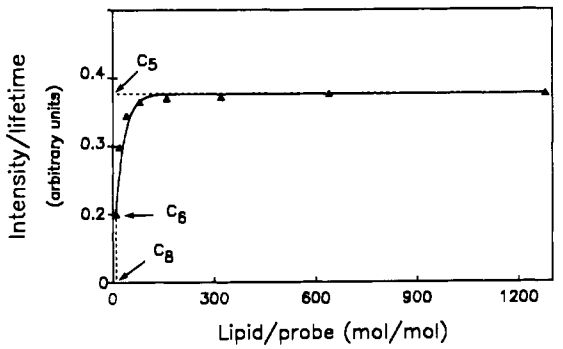

FIGURE 3: Dependence of the ratio of DPHpPC molar fluorescence intensity (arbitrary units) to fluorescence lifetime on the surface concentration of probe in DOPC LUVET. The lipid concentration was 0.25 mM, and the vesicles contained different amounts of DPHpPC. This calibration curve could be well described by the empirical relation given in eq 9, in which the adjustable parameters are c_5, the F/τ ratio at the highest (limiting), and c_6, the lowest (initial), lipid/probe ratio examined, c_7, the exponential rate of F/τ change with lipid/probe ratio, and c_8, the lowest lipid/probe ratio examined (at which c_6 was observed).

molar fluorescence for a particular environment to be calculated from the probe lifetime in that environment.

Summary of the Calculation of Observed Lifetime. At time zero in our experimental setup, the probe-rich donor population (environment D) has a 10:1 lipid/probe ratio, and the probe-poor acceptor population (population A) contains no probe. At any later time t, the lipid/probe ratio of population D will have changed due to lipid transfer, and the lipid/probe ratio of population A will have changed from infinity to a lower but still large value. To a reasonable approximation, the lifetime of probe in this environment will remain constant, since its lipid/probe ratio remains greater than 100:1 (see the calibration curve in Figure 2). The lipid/probe ratio in the unchanging environment (C) will remain at 10:1. The observed lifetime during the time course of the experiment is the average of the lifetimes contributed by these species. Combining eqs 4, 7, and 8 and using the definitions of constants given in the legend to Figure 3, we obtain for the observed average lifetime

$$\langle\tau\rangle = c_1 - (c_1 - c_2)(f_C + f_D e^{-c_3([D]/N_D(t) - c_4)}) \quad (10)$$

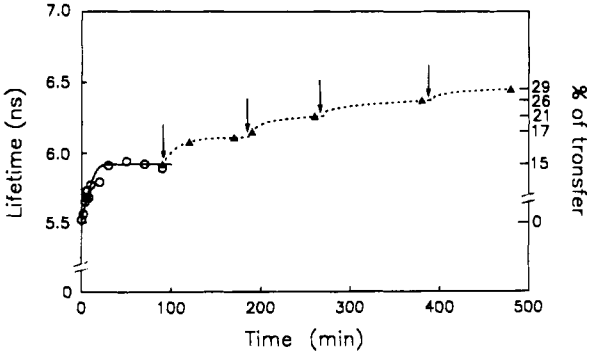

FIGURE 4: Typical data from experiment I. Observed fluorescence lifetimes of DPHpPC (left ordinate) and resulting percents of total transfer (right ordinate) are plotted as a function of time of dialysis against 10 wt % PEG. Open circles represent lifetime data for the initial dialysis after addition of the dialysis bag to PEG; the filled triangles represent measurements made after the rehydrated sample was taken out of the dialysis bag, suspended by sonication, replaced in the dialysis bag, and again subjected to PEG treatment (at the arrows) as described under Methods. Vesicle concentrations were 0.025 and 0.25 mM for donor and acceptor vesicles, respectively. The lipid/DPHpPC ratio in donor vesicles was 25:1 at time zero.

In this expression, $N_D(t)$ should be understood to be given by the kinetic expressions in eq 2. The time-dependent fractional intensities, $f_D(t)$ and $f_C(t)$, were obtained by combining eqs 5 and 6, using eq 2 for $N_D(t)$ and eqs 8 and 9 to obtain the lifetime and molar fluorescence of DPHpPC in the different vesicle compartments. In using eq 10 to fit the observed lipid transfer-related lifetime changes, the rate constant in eq 2 (k) was adjusted to minimize the sum of squared residuals between calculated and observed lifetimes using the Simplex nonlinear curve-fitting algorithm (Caceci & Cacheris, 1984). Estimates of the errors in the kinetic parameters were made in the nonlinear curve-fitting routine (Caceci & Cacheris, 1984) using the gradient matrix of the χ^2 surface as outlined by Bevington (1978).

RESULTS

Dialysis Experiments. The purpose of these experiments was to distinguish between three possible mechanisms to explain enhanced rates of lipid transfer in the presence of PEG: (1) diffusion through a minimal water space between aggregated vesicles; (2) diffusion through bulk water of decreased dielectric constant; and (3) facilitated diffusion through bulk water with PEG acting as a carrier. Figure 4 shows a typical dialysis experiment of type I, as explained under Methods. Without the assumption of any model for the lipid transfer process, this experiment can be characterized in terms of the initial rate of transfer and the final extent of transfer. Figure 4 shows that an initial stage of fairly rapid transfer was followed by a level period during which transfer was not detectable. The level period was reached in roughly 30 min, with essentially no additional transfer occurring for the rest of the 100–120-min time course of our experiment. The average extent of transfer after 30 min from triplicate experiments, each containing at least 10 data points, is recorded in Table I. To test whether transfer was truly complete after 30 min, these same samples were allowed to incubate for a total of 48 h, after which they were checked for a further lifetime change due to a lipid transfer. The results, shown in Table I, demonstrate that transfer did not stop completely after the initial rapid change but continued at a much slower rate.

After the dialysis bag was first placed in PEG solution, it was observed to shrink rapidly due to water loss into the PEG solution of low water activity. In order to determine the extent

Table I: Lipid Transfer of DOPC SUVET Vesicles in Dialysis Experiments[a]

expt no.	lipid exchange (% after 120 min)	lipid exchange (% after 48 h)
experiment I	15.3 ± 0.3[b]	30 ± 3
experiment II	2.1 ± 0.5	3.8 ± 1
experiment III	2.2 ± 0.1	3.7 ± 1

[a] Uncertainties with two significant digits were calculated from the data; the others are estimates. [b] Value taken at 100 min.

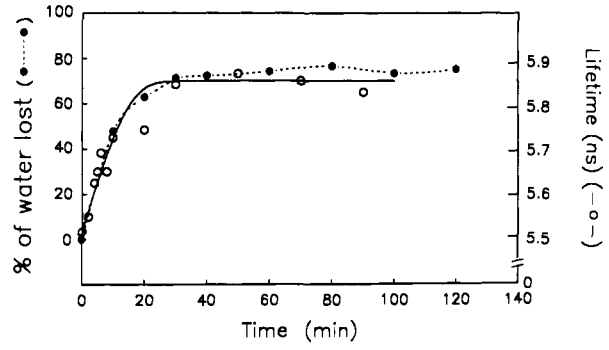

FIGURE 5: Water loss from dialysis bag treated with 10 wt% PEG. Filled circles show the fraction of bag contents lost (in percentage) during the time of dialysis in experiment I. For reference, the change in lifetime reflective of lipid transfer is also shown in open circles.

to which the initial rapid rate of lifetime change might have been limited by the rate of water removal from the bag as opposed to the rate of lipid transfer, we followed the exit of ^3H_2O from the dialysis bag during experiments such as shown in Figure 4. Figure 5 shows the time course of water leakage as obtained from such an experiment. For comparison, the data from the initial portion of the experiment shown in Figure 4 are also shown in Figure 5 to demonstrate that the time courses of probe lifetime change and of dehydration of dialysis bag contents paralleled each other. Presumably, the role of transfer of DPHpPC between vesicles was limited by the rate of dehydration of the vesicle sample in the bag. This made it impossible to obtain meaningful estimates of the rate of lipid transfer from the initial rates of lifetime change observed in dialysis experiments.

Samples treated with PEG in a dialysis bag failed to approach an expected asymptotic value for the extent of lipid transfer even after very long incubation times. It was uncertain why the rate of lipid transfer was so slowed after the initial dehydration of the vesicle sample in the dialysis bag. In order to explore further this phenomenon, the dialysis bag was removed from PEG and 200 μL of buffer was addd to the dialysis bag after 90 min in order to suspend the dehydrated vesicles. The resulting vesicle suspension was then delivered to a glass tube, sonicated at low power in a Branson 1200 Sonicator bath (Danburg, CT) for 10 min to break up aggregates, put back into the dialysis bag, and dialyzed further against 10 wt % PEG buffer. Phase angle data were collected as described previously. This procedure was repeated several times to test for further lipid transfer, with the results being presented in Figure 4. It is clear that repeated cycles of rehydration and dehydration enhanced the rate of lipid transfer and allowed the extent of transfer to approach 30% after only 8 h as opposed to the 48 h required to approach this same limit in a continually dehydrated state.

The results of experiment I have shown that direct contact of 10 wt % PEG with vesicles was not necessary to observe an increase in the rate of lipid transfer. A similar experiment has been reported by MacDonald (1985), who concluded that the action of PEG must be due only to its ability to dehydrate and aggregate membranes. MacDonald measured the refractive index to conclude that PEG did not pass through the dialysis bag. We have used [^{14}C]PEG to determine that less than 1% of PEG could pass through the bag in 48 h. However, neither ours nor MacDonald's observations can absolutely rule out the possibility that a small amount of PEG might traverse the dialysis membrane and act as a lipid carrier. Others have suggested that the action of PEG on membranes is due to its ability to alter the dielectric and polar properties of water (Arnold et al., 1985). In order to test for these possibilities, we designed two other dialysis experiments, as outlined under Methods. In experiments II and III, donor and acceptor vesicle populations were separated by molecular filtration membranes with pore sizes of 12 000–14 000 Da or 0.05 μm, respectively. Like the dialysis bag used in experiment I, the 12 000-Da membrane did not pass significant amounts of PEG, while the 0.05-μm membrane was highly permeable to PEG. In these experiments, roughly only a tenth as much lipid transfer was observed after 120 min as in experiment I (Table I), and this situation was not substantially altered after 48 h (Table I). An experiment similar in concept to experiment II was performed with the dialysis bags used in experiment I, with essentially the same result obtained with the dialysis chamber. The results of experiments II and III rule out a role for PEG as a phospholipid carrier during lipid transfer. However, these results do not rule out a small contribution of lipid transfer through the aqueous phase, the process that apparently accounts for the bulk of lipid transfer in the absence of PEG (Nichols & Pagano, 1982).

Kinetic Analysis of PEG-Mediated Lipid Transfer. In order to obtain more insight into the mechanism of PEG-mediated lipid transfer, we performed experiments to determine the rates and activation energies of transfer for DOPC LUV in the absence and presence of 10 wt % PEG at 10, 22, 37, and 48 °C as described under Methods. Triplicate experiments consisting of at least 30 data points with independent samples were performed. Typical data in the absence (frame A) and presence (frame B) of 10 wt % PEG at 37 °C are shown in Figure 6. While data are recorded in Figure 6 for up to 30 h, experiments were typically carried on for 120 h. Lifetimes in these experiments as well as data obtained at other temperatures approached an asymptotic limit of roughly 6 ns. Transfer of 50% of the lipid would correspond to a lifetime of 6.2 ns. The extent of lipid transfer at 10, 22, 37, and 48 °C in the absence and presence of 10% (w/v) PEG always clearly leveled off between 40 and 50% but never exceeded 50%.

It appeared from plots such as shown in Figure 6 that the data would be difficult to fit over the *entire time course* with a single exponential, an observation that is made even more clear by the semilogarithmic plots given in the inserts to Figure 6. When these data were fit to the kinetic model described under Methods (eq 10), a single-exponential expression with rate constant k (eq 2) gave a reasonable fit with reduced χ^2 values near 1 only for experiments conducted at low temperature and in the absence of PEG. Complete time courses obtained at higher temperatures and larger PEG concentrations were increasingly less well described by a single-exponential model. However, the *initial transfer process* could be described reasonably well by a single-exponential rise, at least up to a lifetime of about 4.0–4.5 ns (~20% lipid transfer). Initial transfer is the process most often monitored in previously published reports of lipid transfer rates. The initial rates obtained by fitting this part of the data are summarized in

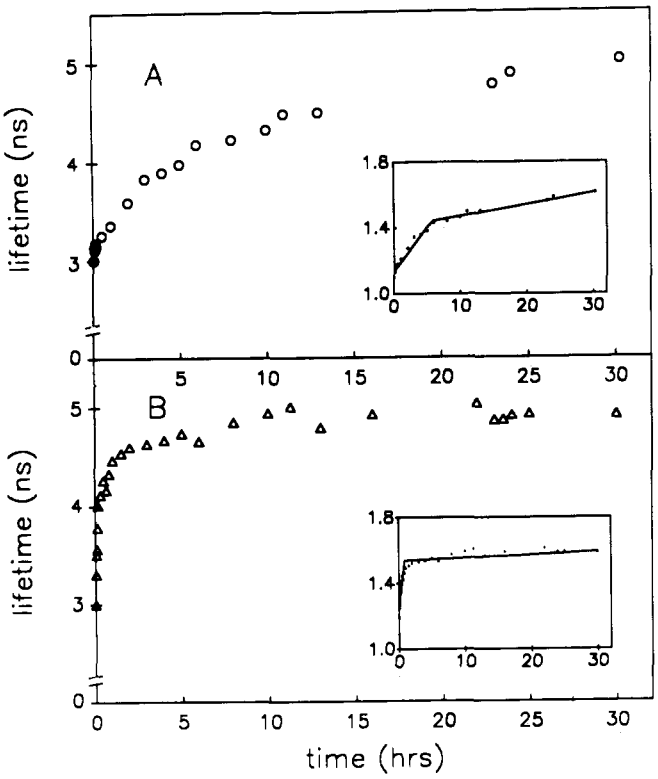

FIGURE 6: Typical kinetic data for lipid transfer between DOPC LUVET in the absence (panel A) and presence (panel B) of 10 wt % PEG at 37 °C. DPHpPC fluorescence lifetime in nanoseconds, indicative of lipid transfer, is plotted versus time in hours. Inserts shown in each panel show the data in semilogarithmic form (ln τ versus time), along with lines obtained by linear regression to the semilogarithmic data.

Table II: Single-Exponential Fit of Initial Rate Data

sample	temp (°C)	k_{in}[a] (10^{-4} s^{-1})	χ^2[b]
no PEG	10	0.008 ± 0.0002	0.36
	22	0.035 ● 0.0012	2.51
	37	0.115 ± 0.0027	0.60
	48	0.216 ± 0.0067	1.41
PEG	10	0.210 ± 0.0050	0.61
	22	0.363 ± 0.0060	0.48
	37	1.083 ± 0.0028	4.42
	48	1.805 ± 0.0058	4.11

[a] k values were obtained by fitting data from three independent time-course experiments (each with at least 27 data points) according to eq 10. The uncertainties in k were estimated from the parameter matrix at the minimum of the χ^2 space (Bevington, 1978). [b] χ^2 is the goodness of fit parameter (reduced) resulting from the fits to initial data points.

Table II, along with an indication of the goodness of fit. A two-exponential decay model produced a good fit to the entire range of data, with χ^2 value of 0.4–1.96 both in the presence and absence of PEG (fits not shown). The exponential constants for the slow rise in lifetime in the presence of PEG at different temperatures were at least one and often two orders of magnitude smaller than were the constants for the fast process and were similar to the apparent rates obtained by fitting the entire time course observed in the absence of PEG.

It has been shown recently that spontaneous phospholipid transfer between bilayer vesicles at higher vesicle concentrations is characterized not only by first-order desorption and diffusion through the aqueous medium but also by a second-order process dependent on vesicle concentration (Jones & Thompson, 1989, 1990; Wimley & Thompson 1990). The second-order contribution to lipid transfer has been proposed

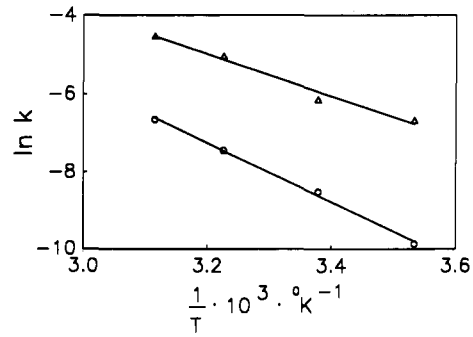

FIGURE 7: Arrhenius plots of ln k_{in} vs $1000*T^{-1}$ for lipid transfer between DOPC LUVET. Individual kinetic constants (tabulated in Table II) represent exponential constants from the initial portions of time courses such as shown in Figure 6 Kinetic constants were obtained by fitting lifetime time course data from three separate experiments according to eq 10. Error estimates for the kinetic constants are given in Table II.

to reflect vesicle–vesicle interactions (Roseman & Thompson, 1980; Nichols & Pagano, 1981, 1982; McLean & Phillips, 1981, 1984; Jones & Thompson, 1990), which apparently become significant only at high lipid concentrations (on the order of 10 mM). Most of our experiments were performed in the concentration range of 0.1–0.25 mM, and results were indistinguishable for experiments done at acceptor vesicle concentrations as high as 2 mM. This rules out the possibility that second-order transfer contributed significantly to the observed nonexponential behavior.

In order to get an estimate of the activation energy of transfer, initial rates of probe transfer obtained at 10, 22, 37, and 48 °C are plotted according to the Arrhenius formalism in Figure 7. A value of 10.6 ± 0.5 kcal/mol was obtained from the slope of the curve in Figure 7 for activation energy in the presence of 10 wt % PEG. The activation energy for the lipid transfer process in the absence of PEG was 15.3 ± 0.3 kcal/mol. The entropy term [calculated from the intercept of the Arrhenius line as described by Nichols (1985)] was −26.3 ± 0.2 eu in the absence of PEG and −16.1 ± 0.3 eu in the presence of PEG. The resulting activation free energy was 23 and 15 kcal/mol in the absence and presence of PEG, respectively.

Discussion

Mechanism of Enhanced Rate of Lipid Transfer Induced by PEG. Two laboratories have shown that the rate or extent of lipid transfer could be enhanced by PEG indirectly [i.e., without direct contact between PEG and lipid bilayers; MacDonald (1985); Yamazaki et al. (1990)]. Although this experiment demonstrates a rate enhancement reflecting dehydration and aggregation of lipid vesicles, there could be substantial contributions from PEG-induced alteration in the properties of water or from small quantities of PEG acting as a carrier of lipid molecules in the aqueous phase. The purpose of the dialysis experiments described in this study was to compare and contrast the contributions to PEG-induced lipid transfer from these three mechanisms. The results of our dialysis experiments demonstrate clearly that the main contribution to the rapid lipid transfer in the presence of PEG derives from direct contact between vesicles caused by the dehydrating influence of PEG. The equivalence of the results obtained in experiments II and III also make it clear that PEG does not promote lipid transfer through a carrier mechanism. That transfer occurred at all in experiments II and III means that transfer via diffusion through the aqueous medium does contribute to lipid transfer in the presence of PEG. However,

the rate of transfer through the aqueous medium in the presence of 10 wt % PEG was less than 15% of the rate due to contact between dehydrated contacting vesicles (Table I).

Transbilayer Self-Diffusion of DPHpPC. The fact that no more than 50% of the probe-rich lipid environment transferred lipid to the probe-poor environment over the 120-h time course of our kinetic experiments indicates that DPHpPC in the inner leaflet of the donor vesicle population was not available for exchange on the time scale of our experiment. Thus, transbilayer movement (flip-flop) of DPHpPC appears to be very slow in DOPC LUVET. This might reflect rigidity of the DPHpPC molecule due to the presence of the DPH moiety located in one chain of this phospholipid molecule.

Origin of the Biexponential Lipid Transfer Kinetics. Most laboratories that have treated interbilayer lipid transfer have used a single-exponential model for the kinetics of the *initial portion* of the transfer process. Our *initial rate* data were also well described by a single-exponential model (Table II). It is generally accepted that the first-order rate constant for lipid transfer reflects the rate of dissociation of lipid from the bilayer into an aqueous environment (Nichols & Pagano, 1981; Nichols, 1985). Since our experiments were carried out at lipid concentrations for which second-order effects should be negligible (Jones & Thompson, 1990), the transfer we have observed in the absence of PEG is expected to occur by this mechanism. Our dialysis results show that the majority of PEG-mediated lipid transfer takes place between vesicles aggregated by the dehydrating influence of PEG. It is reasonable to suppose, then, that the rate-limiting process of the initial PEG-mediated lipid transfer is the process of dissociating lipid from the bilayer into an interbilayer aqueous region whose properties are expected to be somewhat altered relative to the properties of the bulk aqueous environment.

Beyond 20% lipid exchange, it was necessary to include a second exponential term in our kinetic expression in order to obtain a reasonable fit to the data. The physical interpretation of this second exponential is not certain, but some possibilities can be examined. First, our data rule out contributions from transbilayer lipid flip-flop (maximum transfer always $\leq$50%) or second-order collision-dependent transfer (data obtained at low lipid concentration). Second, a single-exponential behavior is expected only when the donor and acceptor vesicles are identical (Nichols & Pagano, 1981; Nichols, 1985). Our experiment observes the transfer of a fluorescent probe, DPHpPC, from a probe-rich to a probe-free population of vesicles. Although DPHpPC mimics well the properties of the phosphatidylcholine class of phospholipids, it is not expected to have physical properties indistinguishable from those of pure DOPC used to provide the membrane matrix for our experiments. Thus, DPHpPC might be expected to leave a DPHpPC-rich vesicle at a different rate than it leaves from a nearly pure DOPC vesicle. Such a mismatch between forward and backward rate constants might account for the nonexponential behavior observed in lipid transfer as it occurs in the absence of PEG.

Such an explanation could hold in the presence of PEG as well, but the situation is potentially even more complex under these conditions. In our dialysis experiments, we observed rapid DPHpPC transfer, limited by the rate of sample dehydration, up to about 15% transfer (Figures 5 and 6). Thereafter, further rapid DPHpPC transfer was achieved only after the vesicle sample was removed from the dialysis bag, rehydrated, and then again dehydrated to initiate rapid transfer (Figure 4). Similar behavior was observed when transfer was stimulated by direct vesicle contact with PEG: rapid transfer occurred up to 17–20% transfer, with slow transfer continuing until 50% transfer had been reached at very long times. These observations suggest that aggregation and close approach of bilayers encourage rapid transfer for a subpopulation of lipids in a vesicle but that dehydration inhibits the spread of this process to the other lipids in the vesicle. This should not reflect inhibition of lipid lateral diffusion by PEG, as 10% PEG actually caused a slight increase in the rate of lipid lateral diffusion (unpublished observations of J. R. Wu and K. A. Jacobson). This leads to the *hypothesis* that rapid transfer of lipid occurs over a region of close membrane contact but that dehydration leads to slow exchange of lipids between these contact regions and the other regions of the bilayer. We have observed that the slow component of interbilayer lipid transfer in the presence of PEG has a rate comparable to the overall rate of transfer in the absence of PEG, consistent with the hypothesis posed here and with the possibility that transfer in the dehydrated state would be via the aqueous phase existing between aggregated clumps of vesicles.

Properties of the Interbilayer Water Space. Within the context of our interpretation that the rate-limiting process in PEG-mediated transfer is dissociation of phospholipid into an altered interbilayer aqueous phase, our results offer insight into the nature of the interbilayer environment induced by PEG. The activation enthalpy and entropy of the transfer process were different in the presence (10.6 kcal/mol, −16.1 eu) and absence (15.3 kcal/mol, −26.3 eu) of PEG. This difference in the thermodynamic parameters for lipid transfer could reflect alterations in both the water space as well as the bilayer environment associated with PEG-induced dehydration. It is well accepted that dehydration produces more dense packing of the lipid acyl chains in a bilayer (Janiak et al., 1976; Yamazaki et al., 1989). This should make the process of removing a lipid molecule and creating a cavity in the bilayer more enthalpically unfavorable, the opposite of what is observed. On the other hand, the disruption of bulk water structure in the partially dehydrated interbilayer state would be expected to make the process of moving the nonpolar acyl chain of a lipid molecule into water less entropically unfavorable, as was observed. In addition, water molecules not tightly hydrogen bonded to other water molecules might more easily fill the cavity in the bilayer left by the vacated lipid molecule, thereby explaining the observed decrease in $\Delta H^{\ddagger\ddagger}$ in the presence of PEG. Thus, the effect of PEG on the kinetics of intervesicle lipid transfer can be rationalized in terms of an alteration in interbilayer water structure associated with dehydration by PEG. In this regard, it is especially interesting to compare with the $\Delta H^{\ddagger\ddagger}$ and $\Delta S^{\ddagger\ddagger}$ for dissociation reported by Jones and Thompson (1990). These authors reported values for *both* a single vesicle and a transient vesicle pair virtually identical with the values obtained here in the absence of PEG. This indicates that water structure is not altered by thermal contact between vesicles but that close contact induced by PEG does alter interbilayer water structure.

It has been observed in this laboratory that 10 wt % PEG does not induce fusion of DOPC LUVET (Burgess et al., 1991a) although it does reduce the interbilayer fluid space to roughly 10 Å (Burgess et al., 1991b). It will be instructive in terms of the mechanism of the fusion process to determine how these activation quantities for lipid transfer vary as the PEG concentration approaches the values necessary for fusion (20–35 wt %).

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