Transbilayer and Interbilayer Phospholipid Exchange in Dimyristoylphosphatidylcholine/Dimyristoylphosphatidylethanolamine Large Unilamellar Vesicles†

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ABSTRACT: The rates of spontaneous interbilayer and transbilayer exchange of [^{3}H]dimyristoylphosphatidylcholine ([^{3}H]DMPC) were examined in DMPC and DMPC/dimyristoylphosphatidylethanolamine (DMPE) large unilamellar vesicles in the liquid-crystalline-, gel-, and mixed-phase states. DMPC desorption rates from either gel or liquid-crystalline phases containing DMPE are very similar to the corresponding rates from pure DMPC gel or liquid-crystalline phases. This is not the case for DMPC desorption from distearoylphosphatidylcholine (DSPC)-containing gel phases, where the desorption rates are significantly faster than from a pure DMPC gel phase [Wimley, W. C., & Thompson, T. E. (1990) *Biochemistry 29*, 1296–1303]. We proposed that the DMPC/DSPC behavior results from packing defects in gel phases composed of both DMPC and DSPC molecules because of the four-carbon difference in the acyl chain lengths of the two species. The present results strongly support this hypothesis because no such anomalous behavior is observed in DMPC/DMPE, which is similar to DMPC/DSPC in phase behavior but does not have the chain length difference. The inclusion of 10–30 mol % DMPE in DMPC bilayers was also found to have a significant effect on the rate of transbilayer movement (flip-flop) of [^{3}H]DMPC in the liquid-crystalline phase. Between 10 and 30 mol % DMPE, flip-flop of DMPC is slowed by at least 10-fold relative to flip-flop in DMPC bilayers, and the entropy and enthalpy of flip-flop activation are both substantially decreased. These results are not consistent with lipid headgroup dehydration as the major energetic barrier to flip-flop but rather suggest that the spontaneous formation of "fluctuation defects" and the energetics of a lipid passing through or being part of such a defect may be the limiting factors. A mechanistic model is proposed. Finally, some predictions based on the model are made and experimentally tested for the related process of bilayer permeation by protons. The permeation results were found to be entirely consistent with the model.

Many processes occur in biological membranes that may be the result of defects in the packing of the component molecules of the bilayer. These include interbilayer and transbilayer lipid exchange (Wimley & Thompson, 1990b), insertion of polypeptides into bilayers (Scotto & Zakim, 1988), membrane fusion (Wong & Thompson, 1982; Ellens et al., 1985, 1986), bilayer permeation by polar compounds (Papahadjopoulos et al., 1973; Cruzeiro-Hansson et al., 1989), and the activation or control of membrane proteins (Lichtenberg et al., 1986). The types of defects which may be important are static structural defects resulting from lateral domain interfaces and protein–lipid boundaries or local defects resulting from the inclusion of especially long or short hydrophobic molecules within the bilayer. In addition, lateral density fluctuations can lead to transient defects in lipid packing (Freire & Biltonen, 1978; Cruzeiro-Hansson et al., 1989) that could be important in the function of biological membranes. We have studied transbilayer and interbilayer lipid exchange at various temperatures and compositions in dimyristoylphosphatidylcholine (DMPC)[1] and DMPC/distearoylphosphatidylcholine (DSPC)[1] large unilamellar vesicles in order to evaluate the effects of some of these types of defects on these processes (Wimley & Thompson, 1990b). In the work presented here, we have extended these studies to DMPC/dimyristoylphosphatidylethanolamine (DMPE)[1] large unilamellar vesicles. In this system, both transbilayer and interbilayer lipid exchange rates can be measured in the same experiments over a wide range of conditions.

In a previous paper, we have shown that the exchange of [^{3}H]DMPC between gel phases composed of DMPC and DSPC is faster than the rates in a pure DMPC gel phase. We proposed that this results from defects in the packing of the lipid molecules resulting from the four-carbon difference in the acyl chain lengths of the two lipid species (Wimley & Thompson, 1990b). In the experiments presented here, we have examined this hypothesis by performing similar experiments in large unilamellar vesicles of the mixture DMPC/DMPE. This binary mixture is very similar to DMPC/DSPC in phase behavior, but the two lipid species do not have an inherent acyl chain length mismatch.

In addition to static defects in gel phase bilayers, transient fluctuation defects may occur in the liquid-crystalline phase and may affect bilayer processes. For instance, the transbilayer movement of lipids (flip-flop) has been proposed to occur via such defects (Taupin, 1975; Homan & Pownall, 1987). It has also been proposed, however, that for lipid flip-flop to occur the headgroup must be dehydrated prior to transbilayer movement (Redelmeier et al., 1990) and that the activation energy for flip-flop is mainly that of dehydration. We have shown previously that bilayer curvature can have significant

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[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; DMPG, dimyristoylphosphatidylglycerol; DSPC, distearoylphosphatidylcholine; LUV, large (100-nm diameter) unilamellar vesicle(s) prepared by extrusion; OLV, oligolamellar (350–700-nm diameter) vesicle(s); PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EDTA, ethylenediaminetetraacetic acid.

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effects on lipid flip-flop (Wimley & Thompson, 1990b), which is consistent with a mechanism involving fluctuation defects. To further investigate the mechanism of flip-flop and the possible role of dynamic fluctuation defects, we have measured the rate of flip-flop of [^{3}H]DMPC as a function of temperature and composition in mixtures of DMPC and DMPE between 10 and 30 mol % DMPE. The results suggest that fluctuation defects are important in the transbilayer movement of lipid molecules and that small, biologically reasonable alterations in bilayer composition can have significant effects on the rate of spontaneous transbilayer lipid diffusion.

Part of this work has appeared elsewhere (Wimley & Thompson, 1990a).

Experimental Procedures

Vesicle Preparation. All lipids were obtained from Avanti Polar Lipids (Birmingham, AL). Purity was periodically confirmed by thin-layer chromatography. [^{3}H]DMPC (66 Ci/mol) was prepared by the method of Jones and Thompson (1989), and [^{14}C]cholesteryl oleate (56.6 Ci/mol) was obtained from New England Nuclear (Boston, MA). Except where noted, the buffer used was 10 mM PIPES,[1] 50 mM KCl, 1 mM EDTA,[1] and 0.02% NaN_3, pH 7.0.

Large 100-nm diameter unilamellar donor vesicles (LUV)[1] were prepared as described in Wimley and Thompson (1990b). The size and unilamellar nature of LUV preparations were confirmed by quasi-elastic light scattering and negative-stain electron microscopy. Oligolamellar vesicle (OLV)[1] acceptors of 350–700-nm diameter were prepared by extruding MLV solutions 20 times or more through three stacked 0.4-μm Nucleopore polycarbonate filters at a temperature just above the phase transition temperature of the lipids. Vesicles prepared in this way are known to be oligolamellar with between 30 and 60% of the total lipid in the outer bilayer and the remainder in the several inner lamellae (Mayer et al., 1986). The resulting vesicles had size distributions which were very broad and heterogeneous centered around 300–700-nm diameter. A significant amount of small ($\leq$300 nm) and large ($\geq$1 μm) vesicles were also present. Less heterogeneous solutions were obtained by pelleting the vesicles twice for 15–30 min at 9000g in a Beckman Model L3-40 ultracentrifuge to remove vesicles smaller than about 350 nm (which remain in the supernatant), followed by a table-top centrifugation for 2–10 min at 20–30g to remove vesicles larger than about 1 μm. These procedures produced OLV acceptor vesicles of between 350 and 700 nm with mean diameters of about 400 nm and with significantly narrower size distributions.

Large unilamellar vesicles with entrapped fluorescein isothiocyanate labeled dextran, MW 40 000 (FD-40), were prepared as in Wimley and Thompson (1990b) except that 20 mg/mL FD-40 was included in the buffer and the freeze–thaw process was repeated 20 times. The vesicle solution, at 75 mM, was extruded through 0.1-μm filters as described above to produce 100-nm unilamellar vesicles. Most of the untrapped FD-40 (<2% was entrapped) was removed by passing the solution through a Sephacryl S300 molecular sieve column and collecting the vesicles which eluted in the void volume. The vesicle solution was diluted to 100 μM and titrated to pH 6.0 followed by equilibration for 24 h.

Exchange Experiments. In order to measure the exchange of radioactive lipids between bilayers, a method of separating acceptors and donors is required. One commonly used method is to include 10–15 mol % of a charged lipid in one of the populations and use small ion-exchange columns to separate the vesicle types (McLean & Phillips, 1981, 1984; Bar et al., 1986; Wimley & Thompson, 1990b). As a result, donors and acceptors must have different compositions, and certain conditions must be true before any experimental results can be properly interpreted. A much better experimental protocol is described here for the first time which utilizes donors and acceptors of different sizes but of essentially identical composition. Donors were 100-nm diameter large unilamellar vesicles as before, but the acceptors were the 350–700-nm diameter oligolamellar vesicles described above.

In these experiments, donors contained DMPC or DMPC/DMPE, [^{3}H]DMPC, and a trace of [^{14}C]cholesteryl oleate as a nonexchangeable marker. Acceptors contained DMPC or DMPC/DMPE. In addition, all vesicles contained 1.75 mol % dimyristoylphosphatidylglycerol (DMPG)[1] to impart a slight charge on the vesicles to prevent aggregation that occurred otherwise at low temperatures. The effect of this small amount of DMPG was negligible because it has the same acyl chains as the other components and is very similar to DMPC in its phase behavior (Silvius, 1982). For the sake of simplicity, experiments discussed in this work will be referred to as 0, 10, 20, and 30 mol % DMPE in DMPC when, in fact, 1.75 mol % of the DMPC has been replaced by DMPG.

In each experiment, acceptors and donors were incubated separately at the experimental temperature and then were mixed to give 0.7 mM total concentration and a 50:1 acceptor:donor ratio. At various times, an aliquot containing 0.1 μmol of lipid was removed and was centrifuged for 4 min at 120000g in a Beckman Airfuge ultracentrifuge. The supernatant, which contained 50–80% of the donors and $\leq$7% of the acceptors, was removed, and the ^{3}H and ^{14}C were determined by liquid scintillation counting. The data are expressed

$$^3\bar{H}_d(t) = \frac{^3H(t)/^{14}C(t)}{^3H(0)/^{14}C(0)} \quad (1)$$

where $^3\bar{H}_d(t)$ is the normalized fraction of [^{3}H]DMPC remaining in the donors at time t, $^3H(t)$ and $^{14}C(t)$ are the amounts of the labels recovered at time t, and $^3H(0)$ and $^{14}C(0)$ are the amounts recovered at time = 0 (10–30 s). The data are thus explicitly corrected for the donor recovery for each point and normalized to 1.0 at time = 0. The data analysis is described below.

The large acceptor vesicles used in these experiments had a tendency to settle slowly out of solution if left unstirred. Therefore, the sample vials were rotated in a circulating water bath at an angle of about 45° at approximately 20 rpm. This was done with a small piece of glass tumbling in the bottom of the vial, and as a result, the vesicles remained in suspension indefinitely.

Proton Permeation of Bilayers. Proton permeation was assayed by measuring the pH-sensitive fluorescence of fluorescein isothiocyanate labeled dextran, MW 40 000 (FD-40), trapped within large unilamellar vesicles. Vesicle solutions of 100 μM lipid at pH 6.0 and vesicle-free solutions at pH 8.0 were first temperature-equilibrated in an SLM Model 4800 fluorescence spectrometer. At time = 0, equal volumes of the two were mixed to bring the pH of the external medium to 7.0. The fluorescence of the entrapped FD-40 was monitored as the pH 6.0 vesicles equilibrated with the pH 7.0 medium via proton or hydroxyl movement across the bilayers. Excitation was at 493 nm (slits 0.5 nm), and emission was at 518 nm (slits 4 nm). Between pH 6 and 7, the fluorescence intensity of FD-40 increases by a factor of 3.5 $\pm$ 0.6 and is linear with pH. The data can be expressed in the form

$$\frac{F(t) - F(0)}{F(\infty) - F(0)} = 1 - e^{-kt} \quad (2)$$

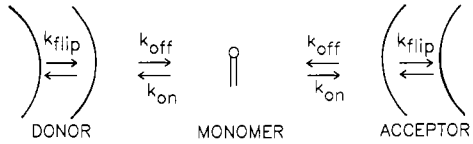

FIGURE 1: Illustration of the mechanism of equilibrium exchange of monomeric lipid molecules between donor and acceptor vesicles through the aqueous phase. k_{off}, k_{on}, and k_{flip} are the desorption (off), adsorption (on), and flip-flop rate constants, respectively.

where $F(t)$, $F(\infty)$, and $F(0)$ are the fluorescence intensities at time t, at infinite time, and at time = 0, respectively. The first-order "rate constant" k is an empirical value from which the halftime of equilibration is obtained.

Kinetic Analysis. In Figure 1, the mechanism of lipid exchange between vesicles is given. The mathematics describing the exchange kinetics and data analysis are described in detail in the Appendix. Briefly, one can write the differential equations which describe this mechanism with one equation for each of the five pools of lipid. Assuming that flip-flop is either very slow or that it is faster than or equal to desorption, the differentials can be solved analytically (Wimley & Thompson, 1990b; Wimley, 1990). In many of the experiments to be discussed, flip-flop is somewhat slower than desorption, and thus a different approach is required. Numerical integration of the differential equations was combined with a nonlinear least-squares curve-fitting procedure to allow the simultaneous determination of k_{flip} and k_{off} from kinetic data without requiring that the analytical solution to the differentials be known. See the Appendix for details.

To perform the curve fitting, the value of the base line or infinite time fractional exchange must be known. In more than 80 exchange experiments with DMPC and DMPC/DSPC LUV utilizing this (data not shown) and other methods (Wimley & Thompson, 1990b), the exchangeable fraction of [^{3}H]DMPC from LUV was found to be 90 ± 4%. Therefore, a nonexchangeable fraction of [^{3}H]DMPC of 10% was assumed for all of the DMPC/DMPE experiments in which it could not be measured directly. In addition, the 1–7% of acceptors which are not removed during the separation of donors and acceptors also add to the measured base line. As a result, the base-line value used in all of the DMPC/DMPE experiments was set equal to the 10% nonexchangeable fraction plus the 1–7% acceptor "leakage" which was measured separately for each preparation of acceptors. In experiments between 24 and 30 °C, the base-line values were experimentally determined (data not shown) and were found to agree well with this assumed base line.

Results

A phase diagram taken from Silvius (1986) for multilamellar vesicles of the binary lipid mixture DMPC/DMPE is given in Figure 2. Like DMPC/DSPC, it has a large gel–liquid-crystalline-phase coexistence region with all-gel phases below it and a liquid-crystalline phase above it. A series of exchange experiments were carried out at 10, 20, and 30 mol % DMPE in DMPC from 15 to 55 °C above, within, and below the two-phase coexistence region of the phase diagram. Above 30 mol % DMPE, homogeneous, unilamellar vesicles are difficult to prepare and are not stable with respect to aggregation and fusion at low temperatures. Therefore, experiments were not done above 30 mol % DMPE.

Typical results for [^{3}H]DMPC exchange between DMPC/DMPE vesicles at 50 °C are shown in Figure 3. In exchange experiments such as these, the rate of transbilayer lipid movement (flip-flop) affects the intervesicular exchange

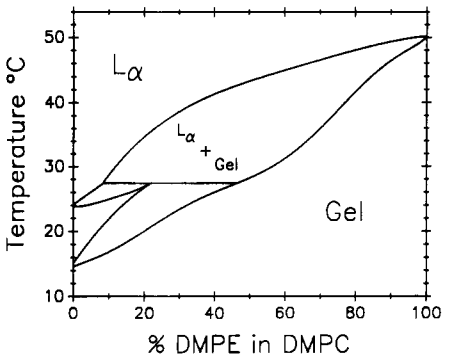

FIGURE 2: Equilibrium phase diagram of the binary system DMPC/DMPE taken from Silvius (1986). This phase diagram was constructed by using differential scanning calorimetry of multilamellar vesicle dispersions. Curvature- or size-dependent thermotropic effects are significant in vesicles smaller than about 40 nm in diameter (Lichtenberg et al., 1981; Slater et al., 1983; Lentz et al., 1976a,b; Wimley & Thompson, 1990b). The LUV used in the experiments presented here, however, are significantly larger (100 nm) and thus are very similar to MLV in thermotropic phase behavior (Lichtenberg et al., 1981; Slater et al., 1983; Hope et al., 1985; Wimley & Thompson, 1990b). Consequently, it is appropriate to apply phase diagrams obtained with multilamellar dispersions to LUV bilayers if interbilayer interactions do not affect phase behavior as would be the case in the experiments presented here with 0–30 mol % DMPE in DMPC.

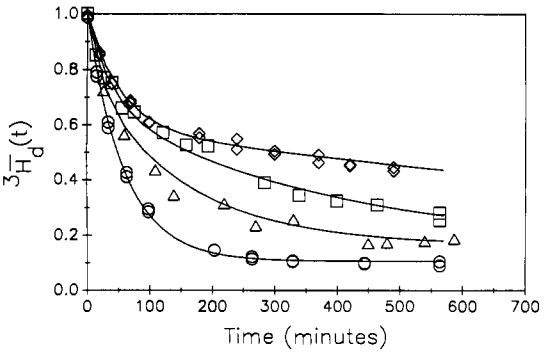

FIGURE 3: Examples of [^{3}H]DMPC exchange between vesicles of 0–30 mol % DMPE in DMPC at 50 °C. The mole percents DMPE for the four sets of data are (top to bottom) 30% (◇), 20% (□), 10% (▽), and 0% (○). Donor vesicles, containing [^{3}H]DMPC and [^{14}C]-cholesteryl oleate as a nonexchangeable marker, were mixed with unlabeled acceptors, and the equilibrium exchange of the [^{3}H]DMPC was monitored as a function of time. $^3\bar{H}_d(t)$ is the normalized fraction of [^{3}H]DMPC remaining in the donor vesicles at time = t (see eq 1 in the text). The experimental details are given under Experimental Procedures. The lines are the result of curve fitting. The 100% DMPC experiments can be fit by a single-exponential equation while the others require the use of the numerical-integration/curve-fitting procedure because the flip-flop rates are slower than exchange (see Appendix). The desorption rates are very similar among all of these experiments.

kinetics by altering the exchangeability of the inner monolayer lipids. When flip-flop rates are faster than or equal to intervesicular exchange, the apparent kinetics of exchange are monoexponential with exchangeable fractions of 90% or more (Wimley & Thompson, 1990b). If, however, flip-flop is slower than exchange, then nonexponential kinetics are observed. Exchangeable fractions under these conditions are still 90% or more. When flip-flop is very slow compared to exchange, the kinetics of exchange again become monoexponential with an exchangeable fraction of around 50%. Only the 100% DMPC experiment in Figure 3 can be fit with a single exponential with almost all of the lipid exchangeable, indicating flip-flop is faster than or equal to exchange. Above 30 °C, in the 10, 20, and 30 mol % DMPE experiments, on the other hand, the kinetics are not simple exponentials because flip-flop

rates are somewhat slower than exchange. Analysis of these experiments required the use of the numerical integration/curve-fitting procedure described in the Appendix. Under these conditions, both off-rates and flip-flop rates can be determined in the same experiments. Below 30 °C, most of the experiments for 10, 20, and 30 mol % DMPE in DMPC appear to be single exponential with exchangeable fractions of about 90%, again indicating fast flip-flop relative to exchange. The experiments in Figure 3 indicate the rate of flip-flop of [^{3}H]DMPC in liquid-crystalline-phase bilayers depends very strongly on the composition of the vesicles, decreasing with increasing DMPE content. The off-rate for [^{3}H]DMPC is approximately the same for all of these experiments.

DMPC Desorption and Flip-Flop in DMPC/DMPE LUV. Panels A–C of Figure 4 are Arrhenius plots of desorption (open symbols) and flip-flop rates (filled symbols) of [^{3}H]DMPC in DMPC/DMPE vesicles with 10, 20, and 30 mol % DMPE. As a reference, the solid lines on the plots represent the [^{3}H]DMPC exchange rate data for pure DMPC liquid-crystalline and gel phases (Wimley & Thompson, 1990b). The dashed curves represent the predicted desorption rates in the two-phase region of the phase diagram. Where two experimental points are given for a single temperature, both flip-flop and desorption can be measured, and where only a single point is given, the exchange is apparently single exponential and only k_{off} can be determined.

The off-rates of [^{3}H]DMPC from DMPC/DMPE liquid-crystalline-phase LUV do not display a significant dependence on composition. In the two-phase and gel-phase regions, the desorption rates reflect, semiquantitatively, the phase structure of the vesicles. This is in contrast to the lack of such a dependence with DMPC/DSPC vesicles (Wimley & Thompson, 1990b). This is an important observation, and a detailed discussion will be given later.

The rates of [^{3}H]DMPC flip-flop in DMPC/DMPE vesicles shown in Figure 4A–C display an exponential dependence on DMPE concentration in the range 10–30 mol % even though the majority of the lipid molecules in the bilayer at these compositions are still DMPC. Between 0 and 30 mol % DMPE, at 50 °C, the flip-flop rate of [^{3}H]DMPC decreases at least 10-fold. The Arrhenius activation energies are 24.2 ± 2 kcal/mol for 10 mol %, 19.2 ± 3 kcal/mol for 20 mol %, and 14.9 ± 3 kcal/mol for 30 mol % DMPE in DMPC. These differences are notable in that they suggest significant alterations in bilayer structure or dynamics even at these relatively low concentrations of DMPE.

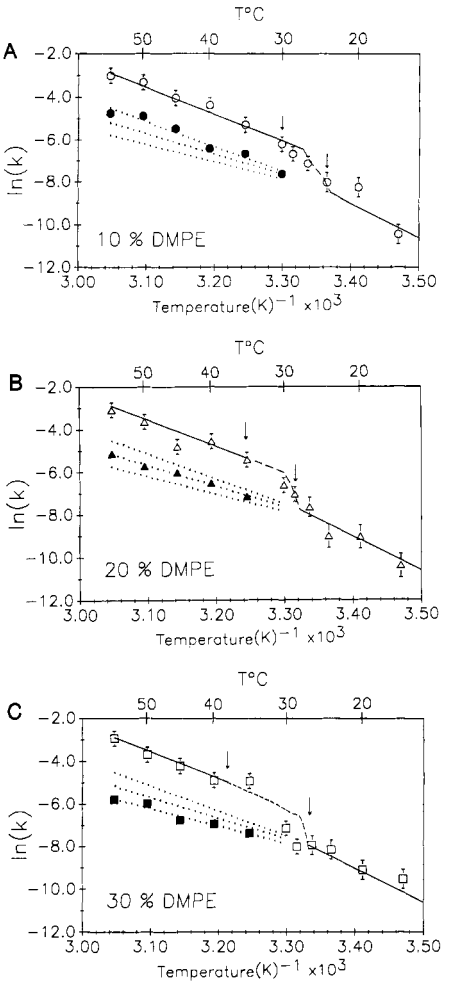

FIGURE 4: Arrhenius plots of [^{3}H]DMPC desorption and flip-flop in DMPC/DMPE vesicles. Open symbols represent desorption rates, and filled symbols represent flip-flop rates. The solid linear portions of the upper curve represent the desorption rates of [^{3}H]DMPC in pure DMPC LUV (Wimley & Thompson, 1990b) in the liquid-crystalline and gel phases. The arrows represent the onset and completion temperature of the phase transition of the mixture. The dashed lines between the arrows represent the expected behavior in the two-phase region calculated by extrapolation of the pure phase data. All temperatures with two experimental points represent conditions under which both desorption and flip-flop could be measured. For the others, only desorption could be measured because flip-flop was faster than or equal to exchange. The three dotted lines represent the best fit to the flip-flop rates for the 10, 20, and 30 mol % DMPE experiments (top to bottom). Each point is the average of 2–6 experiments except for 30% DMPE at 30 and 50 °C where the points represent more than 20 separate experiments. The uncertainties in the desorption rates were estimated from the scatter in the data. Uncertainties in the flip-flop rates are similar. (A) Experiments with 88.25:10:1.75 DMPC/DMPE/DMPG referred to in the text as 10% DMPE. (B) Experiments with 78.25:20:1.75 DMPC/DMPE/DMPG referred to in the text as 20% DMPE. (C) Experiments with 68.25:30:1.75 DMPC/DMPE/DMPG referred to in the text as 30% DMPE.

Discussion

DMPC Desorption. In our previous work (Wimley & Thompson, 1990b), we observed that, in DMPC/DSPC mixtures in the gel phase, the desorption of DMPC is faster than from a pure DMPC gel phase at the same temperature. We proposed that this is the result of the four-carbon acyl chain length difference between DMPC and DSPC which results in the DMPC molecules being more disordered in a DSPC-containing gel phase than in a pure DMPC gel phase. In the work presented here, we have tested this hypothesis by examining DMPC desorption in DMPC/DMPE mixtures. The phase diagram for DMPC/DMPE is very similar to DMPC/DSPC, but the acyl chain lengths are the same for DMPC and DMPE. Thus, one would expect the desorption rates of DMPC from DMPC/DMPE gel phases to be the same as for DMPC gel phases because packing defects of the type proposed above do not exist.

In liquid-crystalline-phase DMPC/DMPE mixtures, the desorption rates and activation energies are indistinguishable from those in pure DMPC bilayers (Figure 4A–C). This was also found to be true for DMPC/DSPC mixtures (Wimley & Thompson, 1990b). Thus, it appears that, in the liquid-crystalline phase, acyl chain or headgroup differences do not significantly alter the molecular interactions experienced by exchanging DMPC molecules. These results agree with those of Gardam et al. (1989), who found nearly ideal mixing, in the liquid-crystalline phase, of a wide variety of fluorescent phospho- and sphingolipid analogues.

In bilayers containing both gel and liquid-crystalline phases, the intervesicular exchange kinetics will reflect the inherent desorption rates from the two phases, the fraction of the ex-

Table I: Thermodynamic Parameters of DMPC Flip-Flop Activation in DMPC/DMPE Vesicles[a]

composition	$\Delta H^{\ddagger}$ (kcal/mol)[a]	$-T\Delta S^{\ddagger}$ (kcal/mol)	$\Delta G^{\ddagger}$ (kcal/mol)[b]
0% DMPE[c]	28.0 ± 3	−6.25 ± 5	22.77 ± 0.45
10% DMPE	23.50 ± 1.90	−1.31 ± 2.10	22.19 ± 0.40
20% DMPE	18.58 ± 3.02	+4.03 ± 3.22	22.61 ± 0.40
30% DMPE	14.26 ± 2.53	+8.88 ± 2.73	23.03 ± 0.40

[a] Thermodynamic parameters calculated from Arrhenius plot data at 50 °C by using the method described in Homan and Pownall (1988). Uncertainties are calculated from the uncertainties in the Arrhenius plot data shown in Figure 4A–C. [b] Free energies are calculated from the flip-flop rate constants at 50 °C (Homan & Pownall, 1988), and the uncertainties are estimated from the uncertainties in the rate constants. [c] The 0% DMPE values are estimated by extrapolating the 10, 20, and 30% data back to 0% DMPE. Flip-flop rates in pure DMPC LUV are not measurable because they are similar to or faster than desorption and thus are kinetically invisible in exchange experiments.

changing lipid in each of the phases, and the relative rate of exchange between phases in the same bilayer or the rate of fluctuations between the gel- and liquid-crystalline-phase states. If the exchange between phases in the same bilayer is slow with respect to intervesicular exchange, then intervesicular exchange will be characterized by two distinct lipid exchange processes, one from each of the two phases. In contrast to this, intervesicular exchange in mixed-phase vesicles with fast exchange between phases in the same bilayer will be characterized by a single kinetic process, and the apparent intervesicular exchange rate will equal the rates from each phase multiplied by the time-average fraction of the exchanging lipid in each phase.

In DMPC/DSPC mixed-phase and gel-phase bilayers, the kinetics are not consistent with either fast or slow interphase exchange (Wimley & Thompson, 1990b). This is because the rate of DMPC desorption from DSPC-containing gel phases is faster than from a pure DMPC gel phase and, in mixed-phase bilayers, is similar to the rates of DMPC desorption from liquid-crystalline phases. As a result of the relatively fast gel-phase desorption rates, the temperature dependence of DMPC desorption in DMPC/DSPC vesicles does not reflect the expected behavior calculated from the pure phase data and from the phase diagram.

In most of the DMPC/DMPE, mixed-phase experiments, the exchange of DMPC is apparently single exponential with most of the DMPC in the exchangeable pool (data not shown). As a result, exchange between phases in the same bilayer or phase-state fluctuations must be faster than intervesicular exchange ($t_{1/2} \leq 5$ h at 30 °C). In panels A–C of Figure 4 are shown the expected (assuming fast interphase exchange) and observed DMPC desorption rates from DMPC/DMPE vesicles in the liquid-crystalline, mixed-phase, and gel-phase states. In this case, the observed results reflect the expected behavior, and thus the desorption rates of DMPC from DMPC/DMPE gel phases must be very similar to the rates from a pure DMPC gel phase. As a result, these experiments strengthen the hypothesis proposed in the previous work that a mismatch in the length of the acyl chains can lead to defects in lipid packing in gel-phase bilayers (Wimley & Thompson, 1990b).

DMPC Flip-Flop. The addition of 10–30 mol % DMPE to DMPC bilayers causes a significant decrease in the rate of DMPC transbilayer movement in the liquid-crystalline phase. Table I gives the thermodynamic parameters of flip-flop activation that describe the differences between a DMPC molecule in the "ground state" (i.e., fully imbedded in the bilayer) and in the highest free energy state encountered during transbilayer movement. The effect of DMPE is to decrease $\Delta H^{\ddagger}$ and increase $-T\Delta S^{\ddagger}$, leading to a net increase in $\Delta G^{\ddagger}$.

From these data, one can obtain information about the activated- or highest free energy state of a lipid molecule undergoing flip-flop. This state must occur when the polar headgroup enters or is in the hydrophobic core of the bilayer. First, it is important to note that these data suggest that changes in hydration or dehydration of the PC polar headgroup are not responsible for the effect of DMPE on DMPC flip-flop. Hydration changes would be expected to change $\Delta H^{\ddagger}$ and $\Delta G^{\ddagger}$ in the same direction, which is not the case. Instead, the decreased enthalpy may signify that disruption of lipid–lipid interactions and/or the exposure of the polar headgroup to the hydrocarbon core are decreased in the activated state by DMPE. The more negative entropy suggests several possibilities: There can be an increase in the exposure of the hydrophobic groups to water in the activated state, although one would not expect this to be accompanied by a large decrease in $\Delta H^{\ddagger}$. There can also be an increase in order of the lipid molecules in the activated state with increasing DMPE. This will eliminate a favorable free energy contributed by the decrease in order gained by attaining the activated state. A similar situation was encountered previously for DMPC desorption from DMPC gel-phase small sonicated vesicles (SUV), where the high degree of disorder in the ground state causes a significant loss of "favorable" entropy (Wimley & Thompson, 1990b). The more unfavorable entropy can also result from a decrease in the lifetime or frequency of occurrence of the fluctuations or defects that may be responsible for flip-flop as discussed below. In conclusion, these thermodynamic parameters suggest that the activated state of DMPC molecules undergoing flip-flop is more ordered, and possibly occurs less frequently in the presence of 10–30 mol % DMPE than in pure DMPC.

The fact that the addition of DMPE has little effect on DMPC desorption rates suggests that interactions between DMPC molecules are not greatly affected by DMPE in the bilayer. Thus, the effect of DMPE on DMPC flip-flop must be on some general bilayer property such as the amplitude or frequency of bilayer fluctuations or the nature of the structures thus formed. In this case, the highest free energy state for flip-flop is likely to be the defect state, and thus the thermodynamic parameters of activation describe the energetic differences between the defect state and the average bilayer configuration. PE molecules are considered to have a different molecular "shape" than PC because of the smaller headgroup and the fewer waters associated with it (Gruner et al., 1985). It may be that in mixtures of DMPC and DMPE the different lipids can be arranged in a fluctuation defect so as to make it more ordered than in DMPC alone. In addition, DMPE may also alter bilayer dynamics so as to alter the frequency of bilayer fluctuations. The thermodynamics of DMPC flip-flop in this system are the basis for the following mechanistic model for flip-flop: The loci for lipid flip-flop in DMPC liquid-crystalline bilayers are spontaneous fluctuation defects. These defects are modulated by DMPE so as to make them less disordered (and possibly less frequent). These effects may be due, in part, to better packing of DMPE in the putative fluctation defects or complementary packing of DMPE with DMPC due to its smaller headgroup and different molecular shape. Although the involvement of spontaneous defects in flip-flop is not a new idea, these studies are the first in which a description of the putative defects and their modulation by bilayer composition has been made.

Proton Permeation in DMPC and DMPC/DMPE LUV. A testable prediction of this hypothesis is that bilayer permea-

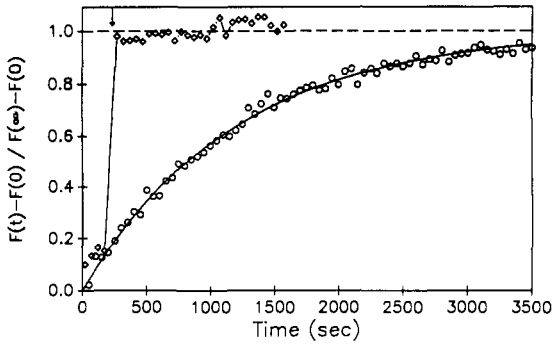

FIGURE 5: Typical pH equilibration experiment. LUV with entrapped fluorescein isothiocyanate labeled dextran 40 000 Da (FD-40) were prepared as described earlier. Vesicles were equilibrated at pH 6 and at 100 μM total concentration in a SLM Model 4800 fluorescence spectrometer. At time = 0, the external pH was increased to 7.0 by adding an equal volume of a vesicle-free solution at pH 8, and the fluorescence of the entrapped FD-40 was monitored. An instantaneous rise in fluorescence of about 60% of the total change occurred probably due to untrapped FD-40. The remaining, time-dependent rise in fluorescence (represented by circles) occurred as an exponential process in all cases as the vesicle interiors came to pH equilibrium with the external medium via proton conduction across the bilayer. This example is for 70:30 DMPC/DMPE LUV. In a separate experiment, 0.02% v/v of the nonionic detergent Triton-X100 (a subsolubilizing amount) was added at the time indicated by the arrow. This caused the fluorescence change to be instantaneous (represented by triangles), demonstrating that bilayer permeability is, in fact, responsible for the slow increase in fluorescence.

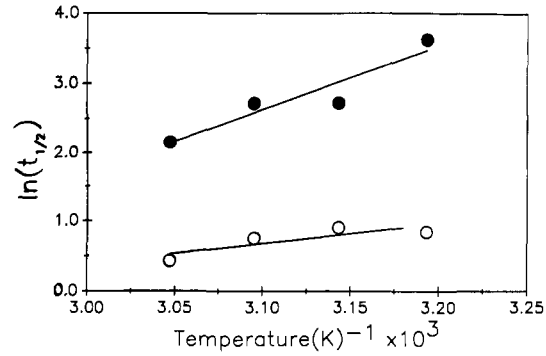

FIGURE 6: Temperature dependence of proton permeability. Logarithm of the pH equilibration half-time (in minutes) as a function of inverse temperature between 40 and 55 °C for 100% DMPC LUV (open circles) and 70:30 DMPC/DMPE LUV (filled circles). All experiments had time-dependent fluorescence increases which were simple exponentials from which the half-times were obtained. Each point is the average of two to four individual experiments like the one shown in Figure 5.

bility (which should also be influenced by fluctuation defects) will be affected by DMPE in a similar way to flip-flop. One can also predict, however, that the temperature dependence of permeability should be *greater* at higher DMPE concentrations because the improved lipid packing (increased order) in the putative fluctuation defects will lead to a higher activation energy. The reason flip-flop and permeation activation energies are affected differently by defect properties is because a lipid molecule undergoing flip-flop is *part of* the defect while the permeating molecule must pass through it. These predictions were tested by measuring the permeability of protons across 100% DMPC and 70:30 DMPC/DMPE bilayers between 40 and 55 °C. The LUV, prepared as described earlier, contained entrapped fluorescein isothiocyanate–dextran, MW 40 000 (FD-40), which has pH-sensitive fluorescence properties. A large molecule such as FD-40 will remain entrapped within the vesicles and was used in these experiments as a probe of the pH of the vesicle interiors. A typical experiment is shown in Figure 5, and all of the results are given in Figure 6. As predicted, proton permeabilities in DMPC/DMPE bilayers are 4–20-fold lower than in pure DMPC and have a significantly steeper temperature dependence.

Both the permeability and flip-flop experiments presented here indicate that lipid headgroup dehydration is probably not the major energy barrier to lipid flip-flop in this system. Instead, the energetics of spontaneous defect formation and the energetics of a lipid molecule existing in or passing through such a defect seem to be the major factors.

Although DMPC and DMPE are not representative biological lipids, the forces and effects observed in this model system may be applicable to biological membranes. It may be that one reason for the large number of lipid types which exist in all biological membranes is to fulfill the requirement for lipids of various shapes which are needed to achieve the proper balance of bilayer stability and instability, or the proper modulation of bilayer fluctuations. The phosphatidylethanolamines found in biological systems tend to have long acyl chains to be highly unsaturated. As a result, the shape imbalance between *biological* PE and PC is far greater than between DMPC and DMPE. By variation of the relative amount of these and other lipids, a cell might alter the overall molecular packing and dynamics in the bilayer, leading to a desired change in some important process such as flip-flop, interbilayer lipid exchange, enzyme activity, or lateral domain organization.

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APPENDIX

Under most experimental conditions, the spontaneous exchange of lipid molecules between bilayers occurs via lipid monomers or small micelles in the aqueous phase (Martin & MacDonald, 1976; Roseman & Thompson, 1980; Doody et al., 1980; Nichols & Pagano, 1981, 1982; Massey et al., 1982; De Cuyper et al., 1983; Arvinte & Hildenbrand, 1984). The exchange mechanism is illustrated in Figure 1 where k_{flip} is the rate of transbilayer diffusion or flip-flop in the donor and acceptor vesicles, k_{off} is the lipid off-rate or desorption rate from donor and acceptor vesicles, and k_{on} is the on-rate for a monomeric lipid in the aqueous phase adsorbing to a donor or acceptor vesicle.

The differential equations describing this mechanism can be written, with one equation for each separate pool of lipid:

$$\frac{d[^3H_{do}]}{dt} = k_{flip}[^3H_{di}] - k_{flip}[^3H_{do}] - k_{off}[^3H_{do}] + k_{on}[^3H_{mon}][D] \quad (3)$$

$$\frac{d[^3H_{di}]}{dt} = k_{flip}[^3H_{do}] - k_{flip}[^3H_{di}] \quad (4)$$

$$\frac{d[^3H_{mon}]}{dt} = k_{off}[^3H_{do}] + k_{off}[^3H_{ao}] - k_{on}[^3H_{mon}][D] - k_{on}[^3H_{mon}][A] \quad (5)$$

$$\frac{d[^3H_{ao}]}{dt} = k_{flip}[^3H_{ai}] - k_{flip}[^3H_{ao}] - k_{off}[^3H_{ao}] + k_{on}[^3H_{mon}][A] \quad (6)$$

$$\frac{d[^3H_{ai}]}{dt} = k_{flip}[^3H_{ao}] - k_{flip}[^3H_{ai}] \quad (7)$$

where $[^3H_{xy}]$ represents the [^{3}H]DMPC concentration in each kinetic pool and the subscript letters d and a represent the donor and acceptor vesicles, respectively, while the subscript letters i and o represent the inner and outer monolayers of the donor or acceptor vesicles, respectively. The subscript mon refers to monomeric lipid in the aqueous phase, and [D] and [A] are the concentration of the donor and acceptor vesicles, respectively. The rate constants are defined above. When flip-flop rates are neither very slow nor faster than or equal to desorption, an analytical solution to the differential eq 3–7 does not exist, except as an extremely complex set of equations and then only under certain conditions (Arvinte & Hildenbrand, 1984; Storch & Kleinfeld, 1986). A solution to this difficulty is to incorporate numerical integration of these differentials into a nonlinear least-squares curve-fitting procedure where the error at each experimental point, for each iteration, is the square of the difference between that point and the value obtained by numerically integrating the differential equations above to that time using the current values of k_{off} and k_{flip}.

Such iterative calculations can be simplified by redefining the $[^3H_{xy}]$ terms as $[^3H_{xy}(t)] - [^3H_{xy}(\infty)]$. In other words, redefine the $[^3H_{xy}]$ terms as the "distance" from the equilibrium (infinite time) value. Because $[^3H_{mon}(t)] - [^3H_{mon}(\infty)] = 0$, one can simplify eq 3 to

$$\frac{d[^3H_{do}]}{dt} = k_{flip}[^3H_{di}] - k_{flip}[^3H_{do}] - k_{off}[^3H_{do}] \quad (8)$$

which eliminates eq 5–7 from the numerical integration. One can now write

$$^3H_d(t) = \int_0^t \frac{d[^3H_{do}]}{dt}\,dt + \int_0^t \frac{d[^3H_{di}]}{dt}\,dt + {}^3H_d(\infty) \quad (9)$$

where $^3H_d(t)$ is the normalized fraction of [^{3}H]DMPC remaining in the donors at time = t and $^3H(\infty)$ is the normalized fraction remaining at infinite time. The only unknowns are k_{off}, k_{flip}, and $^3H(\infty)$. Equation 9 describes the exchange and was used in the numerical integration/curve-fitting procedure described above. The value of $^3H_d(\infty)$ is estimated as described earlier, and the two rate constants are the variable parameters. The sum of the square of the residual errors is minimized to obtain the best fit. In this way, k_{off} and k_{flip} can both be determined from a single experiment. Simulations of exchange reactions were done to demonstrate the correctness of this analysis (data not shown). Also, simulations were done with up to 3σ of added Gaussian noise to demonstrate the conditions under which both rate constants can be experimentally determined; k_{flip} must be greater than about $0.01k_{off}$ and must be less than $0.4k_{off}$. Outside of this range, the data do not allow the differentiation between fast or very slow flip-flop and intermediate values.

Registry No. DMPC, 18194-24-6; DMPE, 20255-95-2; H^+, 12408-02-5.

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Degradation of Dilauroylphosphatidylcholine by Phospholipase A_2 in Monolayers Containing Glycosphingolipids†

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ABSTRACT: The ability of phospholipase A_2 from porcine pancreas to degrade all of the available dilauroylphosphatidylcholine in mixed monolayers with galactocerebroside, sulfatide, or ganglioside GM1 was investigated at different constant surface pressures. Under the conditions used the interfacial glycosphingolipid composition was continuously enriched as the enzyme action proceeded. The total percentage of phospholipid degradation depends on the surface pressure and on the type of glycosphingolipid. The presence of sulfatide activates the enzyme while galactocerebroside and ganglioside GM1 are inhibitory. The extent of phospholipid hydrolysis is independent of the effect of glycosphingolipids on the enzyme velocity. This is so when the latter is measured either in conditions of constant glycosphingolipid composition and zero-order kinetics [Bianco, I. D., Fidelio, G. D., & Maggio, B. (1989) *Biochem. J. 258*, 95–99] or under variable surface composition as in the present work. The modulation of phospholipase A_2 activity by glycosphingolipids operates at two independent levels. One controls the rate of enzyme activity, and the other modulates the total extent of substrate degradation. This depends on the initial interaction of the enzyme with the interface. The glycosphingolipid effect on the activity is different depending on whether the enzyme has access to the substrate from the subphase or is already adsorbed to the lipid interface.

The intermolecular organization and lipid composition have a profound influence on the ability of phospholipases to degrade the phospholipid substrate (Dawson, 1969; Pieroni & Verger, 1979; Brokmann, 1984; Laboda et al., 1986; Verger & Pieroni, 1986; Bell & Biltonen, 1989a,b; Jain, 1990). We have recently shown that neutral and negatively charged GSLs[1] in mixed monolayers with dlPC and dlPA modify remarkably the activity of porcine pancreatic PLA_2 and *Clostridium perfringens* phospholipase C (Bianco et al., 1989, 1990). Conditions of zero-order kinetics were employed in those studies by supplying pure substrate from a reservoir to replace the phospholipid degraded according to the technique introduced by Verger and de Haas (1973). The enzyme velocity depends critically on the surface pressure and the proportions and the type of GSLs (Bianco et al., 1989, 1990). Sulf activates both phospholipases irrespective of its proportions in the film while GalCer leads to inhibition or activation depending on its molar fraction. The presence of gangliosides in relatively low proportions in the monolayer inhibits reversibly the enzyme activity. GSLs (below their critical micellar concentration but still in a molar ratio above 10:1 with respect to the enzyme) or their constituent carbohydrate moieties in the subphase do not affect the catalytic activity of PLA_2 or PLC against films of pure phospholipid (Bianco et al., 1989, 1990).

During our previous studies the question arose as to whether an enzyme with a modified rate of activity could nevertheless degrade all of the substrate available as the proportion of GSLs increased in the monolayer. We have addressed this question with films kept at constant surface pressure but whose composition becomes continuously enriched in GSLs. We found that the total extent of dlPC degradation depends on the surface pressure and on the type of GSLs but is independent of the initial rate of enzyme activity. The modulation by GSLs of the PLA_2 activity operates at two different levels, apparently independent of each other; one is related to the velocity and the other to the extent of enzyme action. The effect of GSLs depends on whether the enzyme has access to the substrate from the subphase or is already adsorbed to the interface.

Materials and Methods

Porcine pancreas PLA_2 and dlPC were from Sigma Chemical Co. (St. Louis, MO). Over 95% of the protein ran as a

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[1] Abbreviations: dlPC (dilauroylphosphatidylcholine), didodecanoyl-*sn*-glycero-3-phosphocholine; dlPA (dilauroylphosphatidic acid), didodecanoyl-*sn*-glycero-3-phosphoric acid; dpPC (dipalmitoylphosphatidylcholine), dihexadecanoyl-*sn*-glycero-3-phosphocholine; PLA_2, porcine pancreas phospholipase A_2; Cer (ceramide), *N*-acylsphingosine; GalCer (galactocerebroside), Galβ1–1′Cer; Sulf (sulfatide), Gal(3-sulfate)β1–1′Cer; GM1 (monosialoganglioside), Galβ1–3GalNAcβ1–4Gal(3–2αNeuAc)1–4Glcβ1–1′Cer; GSLs, glycosphingolipids.

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