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Exchange and Flip-Flop of Dimyristoylphosphatidylcholine in Liquid-Crystalline, Gel, and Two-Component, Two-Phase Large Unilamellar Vesicles†

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Received April 3, 1989; Revised Manuscript Received September 25, 1989

ABSTRACT: The rate and extent of spontaneous exchange of dimyristoylphosphatidylcholine (DMPC) from large unilamellar vesicles (LUV) composed of either DMPC or mixtures of DMPC/distearoylphosphatidylcholine (DSPC) have been examined under equilibrium conditions. The phase state of the vesicles ranged from all-liquid-crystalline through mixed gel/liquid-crystalline to all-gel. The exchange rate of DMPC between liquid-crystalline DMPC LUV, measured between 25 and 55 °C, was found to have an Arrhenius activation energy of 24.9 ± 1.4 kcal/mol. This activation energy and the exchange rates are very similar to those obtained for the exchange of DMPC between DMPC small unilamellar vesicles (SUV). The extent of exchange of DMPC in LUV was found to be approximately 90%. This is in direct contrast to the situation in DMPC SUV where only the lipid in the outer monolayer is available for exchange. Thus, transbilayer movement (flip-flop) is substantially faster in liquid-crystalline DMPC LUV than in SUV. Desorption from gel-phase LUV has a much lower rate than gel-phase SUV with an activation energy of 31.7 ± 3.7 kcal/mol compared to 11.5 ± 2 kcal/mol reported for SUV. A defect-mediated exchange in gel-phase SUV, which is not the major pathway for exchange in LUV, is proposed on the basis of the thermodynamic parameters of the activation process. Surprisingly, the rates of DMPC exchange between DMPC/DSPC two-component LUV, measured over a wide range of compositions and temperatures, were found to exhibit very little dependence on the composition or phase configuration of the vesicles. Evidence is given for defect-mediated exchange from two-component gel phases. The fraction of DMPC exchanging between two-component vesicles indicated fast flip-flop relative to exchange at many, but not all, compositions and temperatures at or above 28 mol % DMPC. Although DMPC exchange between LUV composed of 0.1 mol % DMPC in DSPC has desorption rates equal to those at the other compositions, the flip-flop rates at this composition are at least an order of magnitude smaller.

In biological membranes, the hydrophilic and hydrophobic moieties of the component lipid and protein molecules are strictly segregated. A number of processes occur spontaneously, however, that violate this segregation. These processes include spontaneous interbilayer lipid exchange, transbilayer movement of lipids (flip-flop), insertion into the bilayer of polypeptides, membrane fusion, and bilayer permeation by small polar molecules. Dynamic or static structural defects in the membrane organization have been suggested to be the loci for these processes (Marsh et al., 1976; DeKruijff & Van Zoellen, 1978; Shaw & Thompson, 1982; Wong & Thompson, 1982; Scotto & Zakim, 1988).

† This work was supported by USPHS NIH Grant GM-14628.

0006-2960/90/0429-1296$02.50/0

Defects of this type occur as the result of thermally driven fluctuations in membrane structure or can be induced by osmotic pressure gradients (Taupin et al., 1975) or by the inclusion of components such as detergents, integral membrane proteins (Thompson & Huang, 1986), and lipid oxidation products (Shaw & Thompson, 1982). Geometric constraints can also cause structural packing defects as may be the case in gel-phase small unilamellar vesicles (SUV).[1] These structures are thought to be polyhedral, consisting of planar gel-phase domains connected by disordered defects (Blaucock & Gamble, 1979). Extensive defects in the packing of component molecules may also exist in the boundaries between phases in multicomponent, multiphase bilayers.

In order to examine the role of defects in the spontaneous interbilayer exchange and flip-flop of phospholipids, we have been studying these processes in one-component and in two-component phospholipid vesicles as a function of the temperature and the phase configuration of the vesicle bilayer. The studies reported in this paper focus on the movements of [^{3}H]dimyristoylphosphatidylcholine (DMPC)[1] in aqueous dispersions of large unilamellar vesicles composed of either DMPC or mixtures of DMPC and distearoylphosphatidylcholine (DSPC).[1] This particular binary lipid system was chosen because in aqueous dispersions it exhibits a large two-phase region on the equilibrium phase diagram as shown in Figure 5 (Knoll et al., 1981). We have restricted the study to large unilamellar vesicles (LUV)[1] because the properties of these structures closely resemble those of the multilamellar liposomes upon which the phase diagram is based (Lichtenberg et al., 1981; Hope et al., 1985; Lentz et al., 1976b). A preliminary report of this work has appeared elsewhere (Wimley & Thompson, 1989).

Experimental Procedures

Preparation of Large Unilamellar Vesicles. All lipids were obtained from Avanti Polar Lipids (Birmingham, AL) and were found by thin-layer chromatography to be more than 98% pure. Lipids in chloroform solution were placed in a large round-bottom flask and dried rapidly in a rotary evaporator warmed to 30 °C. Samples which showed any nonuniformity in lipid deposition on the flask were redissolved in chloroform and dried again to ensure complete mixing of the dried lipids. After lyophilization overnight, buffer (10 mM PIPES,[1] 50 mM KCl, 1 mM EDTA,[1] and 0.02% weight NaN_3, pH 7.0) at 60 °C was added to the flask, which was also warmed to this temperature. The flask was shaken to form a rough liposomal suspension that was then frozen (−20 °C) and thawed (45 °C) at least 5 times before preparation of LUV.

LUV were produced by extruding the lipid suspension under N_2 pressure 10 times (100–500 psi) through two stacked 0.1-μm Nucleopore polycarbonate filters (Hope et al., 1985; Mayer et al., 1986) while keeping the lipid solution and extrusion device well above the phase transition temperature of the lipid mixture. The size distribution of all preparations was determined by using a NICOMP Model HN5-90 quasi-elastic light scatterer with a NICOMP Model 270 autocorrelator capable of size distribution analysis. Size distribution analysis on many samples showed that relatively consistent preparations can be made under very different conditions of composition, temperature, and lipid concentration. The size analysis gave distributions with an average mean diameter of 90–100 nm and an average full width at half-maximum of 20–40 nm. Occasionally, a smaller peak at 20–50 nm comprising 2–10% of the total mass was observed. This peak was usually not observed if, after the freeze–thaw cycles, the suspension of lipids was centrifuged in a table-top centrifuge for 15 min and only the resuspended pellet used in the extrusion process. This observation suggests that the small vesicle contamination is present before the extrusion process begins. The presence of 15 mol % charged lipids in the acceptor vesicles caused them to be consistently 10–20 nm smaller in diameter than the donor vesicles which lacked the charge.

Exchange Experiments. Donor vesicles contained DMPC (or DMPC and DSPC), [^{3}H]DMPC (specific activity >500 μCi/μmol), and a trace of [^{14}C]cholesteryl oleate (specific activity 50 μCi/μmol) as a nonexchangeable marker. Acceptors contained DMPC (or DMPC and DSPC) and 15 mol % dipalmitoylphosphatidylglycerol (DPPG)[1] to impart a charge on the acceptors which was utilized to separate the two vesicle types on ion-exchange columns (McLean & Phillips, 1981; Bar et al., 1986). DPPG was chosen because it mixes almost ideally with both DMPC and DSPC and has a phase transition temperature between the two other lipids (Silvius, 1982); therefore, the effect of the charged lipid on the phase state of the acceptors was minimized. After incubation (2–12 h) at the experimental temperature, acceptors and donors were mixed together to give a 10:1 acceptor to donor ratio at 1 mM total lipid concentration in 2–3-mL total volume. Control experiments performed on a few samples quenched from high temperatures gave results identical with those obtained for vesicles preequilibrated at the experimental temperature. Vesicles used in experiments at temperatures at which the vesicles were in the gel phase were cooled slowly (10 °C/h) from 55 °C and were incubated up to 12 h at the experimental temperature. At various times, aliquots of 0.1 μmol of lipid were removed and eluted over 1 cm^3 of DEAE-Sephacel ion-exchange resin with 1.2 mL of buffer after the columns were preequilibrated with 0.3 μmol of unlabeled donors. Approximately 50–90% of the donors were recovered, and 100% of the acceptors were retained on the columns. ^{3}H and ^{14}C in the eluate were determined by liquid scintillation counting in a Packard Model 460 LSC. $^3\bar{H}_d(t)$ is the normalized fraction of [^{3}H]DMPC remaining in the donors after correction for donor recovery and is given by

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

where $^3H(t)$ and $^{14}C(t)$ are the amounts of the two labels in an aliquot eluted from a column at time t and $^3H(0)$ and $^{14}C(0)$ are the amounts in an aliquot eluted from a column at time zero (10–30 s). This treatment of the data explicitly corrects the results for donor recovery.

The experimental data were fit to a single-exponential decay by a nonlinear least-squares fitting procedure which uses the simplex algorithm. An experimental uncertainty of $\sigma = 0.019$ was obtained from the residual error of several hundred data points from typical experiments fit to a single-exponential exchange. The residual errors were Gaussian around zero with no dependence on the magnitude of $^3\bar{H}_d(t)$. With the experimental uncertainty known, χ^2 values for the curve fits were calculated. Criteria for an acceptable fit were reduced χ^2 values less than 2.5 and randomly distributed residual errors.

The possibility of aggregation or fusion of vesicles was examined by using quasi-elastic light scattering, a technique very sensitive to large particle contamination of a solution of

[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EDTA, ethylenediaminetetraacetic acid; LUV, large unilamellar vesicles prepared by extrusion; SUV, small unilamellar vesicles prepared by sonication.

FIGURE 1: Illustration of exchange of monomeric lipid between donor and acceptor vesicles through the aqueous phase.

scatterers. All samples, except one, showed no aggregation or fusion at the temperatures studied. This included 1 mM DMPC incubated at 5 °C for 160 days (data not shown). LUV containing 0.1 mol % DMPC/99.9% DSPC, however, were found to aggregate extensively at temperatures ≤35 °C and to fuse below about 20 °C. Inclusion of 1.75 mol % DPPG in the 0.1% DMPC LUV was found to prevent aggregation completely at all temperatures; therefore, donor vesicles in the 0.1 mol % DMPC experiments contained 1.75% DPPG.

Lipids were periodically checked by thin-layer chromatography, especially after long- or high-temperature experiments, for the presence of degradation products. No more than 5% were ever observed, and for most experiments, it was much less.

Kinetic Analysis. Exchange of lipid molecules between vesicles at low concentrations occurs via lipid monomers or small micelles, and the rate-limiting step is the desorption of the lipid from the bilayer (Martin & MacDonald, 1976; Roseman & Thompson, 1980; Doody et al., 1980; Nichols & Pagano, 1981, 1982; De Cuyper et al., 1983; Arvinte & Hildebrand, 1984). The kinetics are illustrated in Figure 1. Assuming steady-state exchange and a single exchangeable pool (either fast or slow flip-flop relative to exchange), the rate equations can be solved to give

$$ {}^3\bar{H}_d(t) = 1 - \frac{k_2k_4D}{k_1k_3A + k_2k_4D} \times \exp\left[-\frac{f(k_1k_3A + k_2k_4D)t}{k_2D + k_3A}\right] + \frac{k_2k_4D}{k_1k_3A + k_2k_4D} \quad (2) $$

where k_1 and k_4 are the desorption rates, k_2 and k_3 are the on-rates, ${}^3\bar{H}_d(t)$ is the normalized fraction of [^{3}H]DMPC remaining in the donors at time t, A and D are the concentrations of the acceptor and donor vesicles, respectively, and f is the fraction of exchangeable lipid which is on the outer surface of the vesicle (i.e., that part of the exchangeable fraction which is available for exchange at any instant in time). Thus, for unilamellar vesicles, $f = 0.5$ for fast flip-flop (both monolayers exchangeable) and 1.0 for slow flip-flop (only the outer monolayer exchangeable). The on-rates (k_2, k_3) are assumed to be equal because the donors and acceptors are very similar in size and composition. This assumption was found to be approximately true even for DMPC transferring from liquid-crystalline DMPC into gel-phase DSPC vesicles at 35 and 28 °C (data not shown). Considering the 10:1 acceptor:donor ratio used in these experiments and assuming that the desorption rates from acceptors and donors are equal, eq 1 can be reduced to

$$ {}^3\bar{H}_d(t) = [1 - {}^3H(\infty)]e^{-fk_1t} + {}^3H(\infty) \quad (3) $$

where ${}^3H_d(\infty)$ is ${}^3\bar{H}_d(t)$ extrapolated to infinite time and k_1 is the desorption rate of DMPC from the donor vesicles. This single-exponential decay equation is fit to the experimental data as described under Experimental Procedures. ${}^3H_d(\infty)$ and k_1 are treated as variables and are determined from the curve fit. Uncertainties in the parameters are estimated by the method of Bevington (1967). The exchange of DMPC between gel-phase LUV is too slow to be followed to completion, and therefore a direct determination of ${}^3H_d(\infty)$ could not be made. In this case, k_1 was calculated assuming a base line consistent with both fast flip-flop and slow flip-flop. Because of the multiplication of k_1 by f in eq 2, this choice of an assumed base line had only a small effect on the calculated value of k_1 and the average of the two values was used. The uncertainties associated with the gel-phase rate constants include this additional uncertainty.

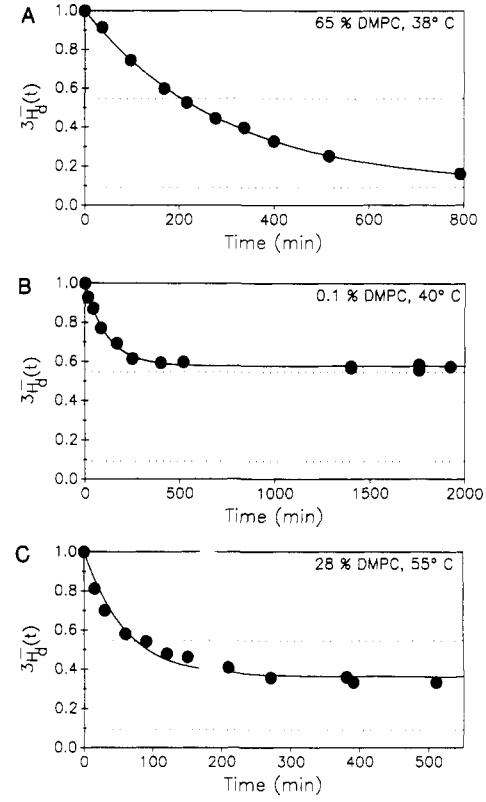

FIGURE 2: Typical experimental data illustrating the three types of results obtained. The points are the experimental data, and the solid curves are a single-exponential decay function (eq 3) fit to the data. Each experiment is 1 mM total lipid, 10:1 acceptor:donor ratio, and 2–3-mL total volume. The horizontal dotted lines are the predicted base-line values for complete exchange (0.0909) and for exchange from the outer monolayer only (0.5455). The composition of the vesicles and the temperature of each experiment are given in the upper right corner. (A) Exchange of [^{3}H]DMPC from donor vesicles for a sample which exhibited almost complete exchange with a single rate constant. Most of the experiments were qualitatively similar to (A). (B) Exchange of [^{3}H]DMPC in one of the 0.1% DMPC, 98.15% DSPC, and 1.75% DPPG experiments which exhibited exchange from the outer monolayer only (see text). (C) The worst of the non-single-exponential experiments. Note that the base line falls between the two predicted base lines and that the residual errors are quite nonrandom.

The exchangeable fraction of lipid can be calculated for experiments in which ${}^3H_d(\infty)$ is determined experimentally by

$$ \text{fraction exchangeable} = \frac{1 - {}^3H_d(\infty)}{A/(A + D)} \quad (4) $$

where the fraction exchangeable is the fraction of lipid which is available for exchange independent of the A:D ratio. $A/(A + D)$ is the expected value of $1 - {}^3H_d(\infty)$ for complete equilibrium exchange.

Results

Typical data for DMPC exchange between LUV are shown in Figure 2. Figure 2A shows a typical exchange experiment in which essentially all of the [^{3}H]DMPC exchanges with a single rate (DMPC/DSPC, 65:35 at 38 °C). Figure 2B shows an experiment which displayed exchange from the outer monolayer only (DMPC/DSPC/DPPG, 0.1:98.15:1.75 at 40

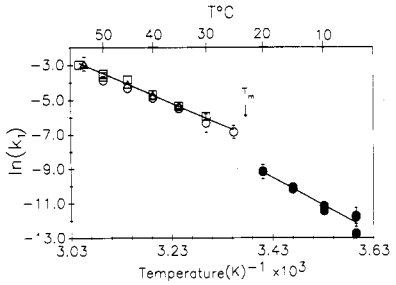

FIGURE 3: Arrhenius plot of DMPC desorption rates from DMPC LUV. Each point represents the result of one exchange experiment, and each has a calculated uncertainty. A few are shown; many are smaller than the symbols. Different symbols represent different vesicle preparations used in similar experiments. The two distinct lines represent sets of experiments performed above and below the phase transition temperature (T_m) of 23 °C. Each distinguishable point below T_m actually contains two identical experiments run simultaneously. At various times, aliquots were eluted over an ion-exchange column as described in the text, and the ^3H and ^{14}C in the donors were determined. A total of 12–20 points were taken for each experiment.

Table I: Thermodynamic Parameters of Activation of DMPC Desorbing from DMPC LUV and SUV

vesicle type	E_a	$-T\Delta S^\ddagger$	$\Delta H^\ddagger$	$\Delta G^\ddagger$
L_α DMPC SUV[a]	20 ± 4	+3.9 ± 2	19.6 ± 4	23.5 ± 5
L_α DMPC LUV	24.9 ± 1.4	−0.4 ± 2.0	24.3 ± 1.4	23.9 ± 4.7
gel DMPC SUV	11.5 ± 2	+13.5 ± 3	11 ± 2	24.5 ± 6
gel DMPC LUV	31.7 ± 3.7	−6.3 ± 4.0	31.1 ± 3.7	24.8 ± 6.5

[a] Thermodynamic parameters (kilocalories per mole) of the activation process at 23 °C calculated from Arrhenius plot data for LUV (this study, Figure 3) and for SUV (McLean & Phillips 1984; M. Schläppi, unpublished results) calculated according to Homan and Pownall (1988). Uncertainties with two significant digits were calculated from the data; the others are estimates.

°C). Also shown are the curve fits of a single-exponential decay (eq 3) to the data. The desorption rate from the donor vesicles and the exchangeable fraction of DMPC were determined from such fits. Figure 2C shows data from one of the few experiments in which the [^{3}H]DMPC did not exchange with a single-exponential decay (DMPC/DSPC, 28:72 at 55 °C). From such an experiment, only the initial rate of exchange can be determined, assuming most of the initial exchange occurs from the outer monolayer. Values obtained from these non-single-exponential experiments were always given a much higher uncertainty than the others.

Exchange of DMPC between DMPC LUV. A series of experiments were performed using 100-nm-diameter large unilamellar vesicles composed of liquid-crystalline DMPC in the temperature range between 55 and 25 °C and also using gel-phase DMPC LUV between 20 and 5 °C. All 21 of the liquid-crystalline-phase experiments gave data qualitatively similar to Figure 2A. Values of k_1 are presented as open symbols in an Arrhenius plot in Figure 3. The plot is linear with $r^2 = 0.968$. The magnitude and temperature dependence of k_1 are similar to values reported for liquid-crystalline-phase DMPC small unilamellar vesicles (McLean & Phillips, 1984; M. Schläppi, unpublished results) with an Arrhenius activation energy of 24.9 ± 1.4 kcal/mol. The free energy, enthalpy, and entropy of activation for desorption of DMPC from liquid-crystalline LUV and SUV are listed in Table I. The fraction of exchangeable DMPC for all the liquid-crystalline-phase experiments was found to be 89.6 ± 7.2%. This signifies that approximately 10% of the [^{3}H]DMPC was not available for exchange over the time course of these experiments. This nonexchangeable fraction of [^{3}H]DMPC is independent of temperature as can be seen in Figure 4.

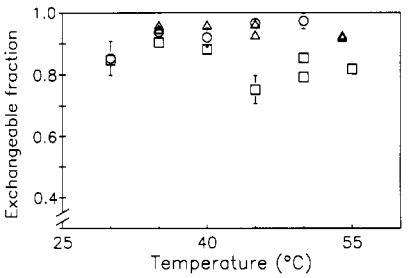

FIGURE 4: Exchangeable fractions of DMPC in liquid-crystalline-phase DMPC LUV as a function of temperature. The exchangeable fractions are calculated from the extrapolated base-line values determined in the curve-fitting procedure in the same experiments as Figure 3. The different symbols represent the same experiments here as they do in Figure 3. A few representative error bars are included. The gel-phase experiments had kinetics which were very slow and could not be followed long enough for a determination of the exchangeable fraction.

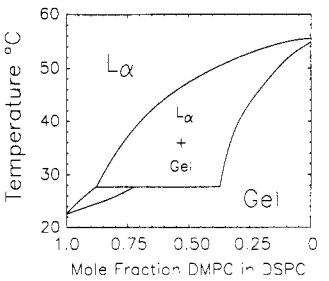

FIGURE 5: Phase diagram of the binary system DMPC/DSPC taken from Knoll et al. (1981). This phase diagram was constructed from a compilation of the literature on this system as well as small-angle neutron-scattering results. Liquid-crystalline and gel phases coexist in the region marked L_α + gel.

The filled circles in Figure 3 are data for the exchange of DMPC between gel-phase large unilamellar vesicles. The Arrhenius activation energy is 31.7 ± 3.7 kcal/mol, which is almost 3-fold higher than the value of 11 ± 2 kcal/mol reported for gel-phase SUV (McLean & Phillips, 1984; Schläppi, unpublished results). The free energy, enthalpy, and entropy of activation for desorption from gel-phase SUV and LUV are listed in Table I.

Exchange of DMPC between DMPC/DSPC LUV. A phase diagram for this system is presented in Figure 5 (Knoll et al., 1981). It displays a two-phase region where a DSPC-enriched gel phase and a DMPC-enriched liquid-crystalline phase coexist over a wide range of temperatures and compositions. The DMPC desorption rate and exchangeable fraction were measured for six compositions in the range 0.1–75 mol % DMPC in DSPC at temperatures above, within, and below the two-phase region. The results of these experiments are presented in Figures 6 and 7. Above ~33 °C, k_1 is equal to that from pure liquid-crystalline-phase DMPC LUV within experimental error in all cases despite large differences in the composition and phase configuration of the vesicles. At temperatures below about 33 °C, k_1 decreases below the pure DMPC liquid-crystalline values in a manner apparently independent of composition or phase configuration. The two-component gel-phase values of k_1, however, are consistently higher than the rates predicted by extrapolating to this temperature regime the data for DMPC exchange between pure DMPC gel-phase LUV (Figure 3).

The fraction of exchangeable DMPC has a complex behavior in the two-component LUV. At temperatures below 50 °C (45 °C in one case) and DMPC ≥28 mol %, a single-exponential decay was observed with an exchangeable fraction of 91.2 ± 7.2%. The experiments done on 0.1 mol % DMPC LUV between 55 and 25 °C exhibited a single-

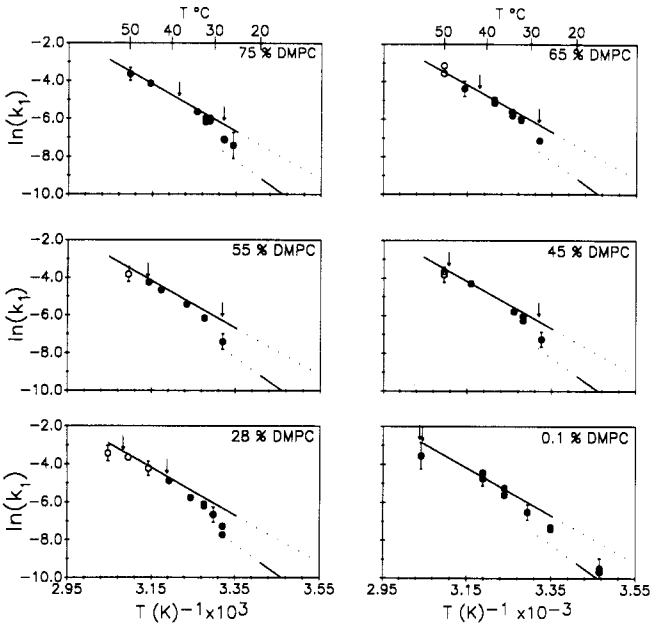

FIGURE 6: Arrhenius plots of ln k_1 vs T^{-1} for DMPC exchanging between DMPC/DSPC LUV at different compositions. The mole percent DMPC is given in each panel. The remainder of the lipid is DSPC. The 0.1% DMPC samples contained an additional 1.75% DPPG to prevent aggregation. The arrows denote the onset and completion temperatures of the phase transition as determined from the equilibrium-phase diagram (Figure 5). The solid lines are taken directly from Figure 3 and represent the values of ln k_1 for DMPC exchanging between pure liquid-crystalline or gel-phase DMPC LUV. The closed symbols represent experiments which displayed single-exponential kinetics. The open symbols represent experiments with more complex kinetics which had to be deconvoluted (see text). A few represenative error bars are included.

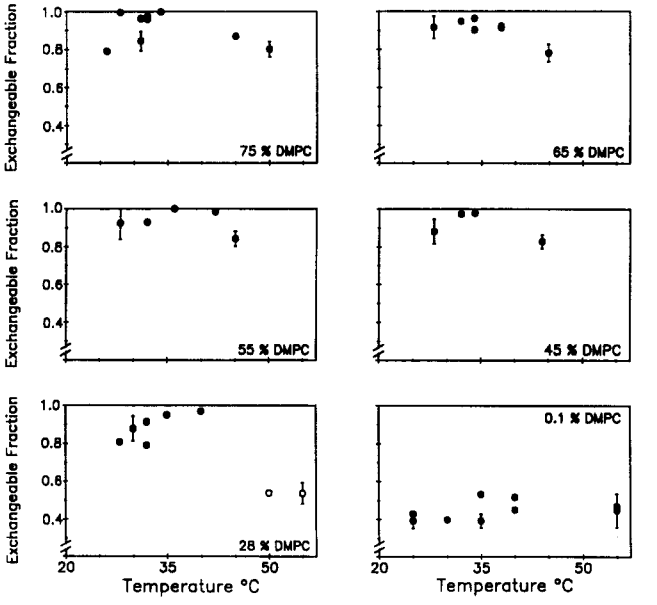

FIGURE 7: Exchangeable fraction of [^{3}H]DMPC in DMPC/DSPC LUV determined in the same experiments as the data in Figure 6. The mole percent DMPC is given in each panel. Solid symbols represent experiments in which DMPC exchanged with a single rate constant. Open symbols represent nonexponential experiments in which the rate and fraction of the *fastest process only* were determined. A few representative error bars are included.

exponential exchange rate; the exchangeable fraction of DMPC was only 45.1 ± 5.1%. Experiments at temperatures ≥50 °C (45 °C in one case) and DMPC between 28 and 65 mol % displayed kinetics which were apparently not single-exponential decays. The non-single-exponential behavior appears to correlate with temperature and composition but not phase structure. The most sensitive criterion for determining nonexponential behavior is the form of the residual error plot. This plot for the data shown in Figure 2C, one of the non-single-exponential experiments, showed the residual errors to be very nonrandom. Only 8 of 52 experiments were of this type. In the two non-single-exponential experiments which could be partially deconvoluted (DMPC/DSPC, 28:72, at 50 and 55 °C), the fraction exchanging via the faster rate was found to be approximately 50%, and the rates were the same as DMPC desorption from liquid-crystalline DMPC LUV. Only the initial rate of exchange could be determined from the other non-single-exponential experiments, assuming most of the initial exchange is from the outer monolayer only. The uncertainty in this assumption was taken into account when calculating the uncertanty in the rate constants determined in these few experiments.

Discussion

Thermodynamics of DMPC Desorption from DMPC Bilayers. A desorbing lipid molecule is considered to be in an "activated" state when it is near the free energy maximum of the desorption reaction coordinate which begins with the lipid fully imbedded in the bilayer and ends with it as a monomer in the aqueous phase. As the lipid moves into this activated state, the result of thermal or other fluctuations, the hydrocarbon chains are partially removed from the hydrophobic environment and exposed to the aqueous phase. This causes a large positive change in the enthalpy and a large negative change in the entropy, due to the hydrophobic effect, relative to the lipid molecule fully imbedded in the bilayer. These effects are counteracted, in part, by the positive entropy change associated with increased acyl chain disorder as the lipid leaves the bilayer. This effect will be especially important for desorption from gel-phase LUV bilayers where the acyl chains are highly ordered in the ground state. A further positive change in entropy occurs when the lipid fully enters the aqueous phase as a result of the increase in the degrees of translational and rotational freedom associated with leaving the anisotropic bilayer and also as a result of an additional increase in the acyl chain disorder. The free energy maximum along the reaction coordinate, which is the free energy barrier to desorption, will occur when the unfavorable entropy and enthalpy changes associated with transferring the lipid into the aqueous phase are maximized, but before the lipid completely detaches from the bilayer (McLean & Phillips, 1984). The activated state, then, occurs when the lipid is almost completely in the aqueous phase with a small part of its acyl chains anchoring it to the bilayer. Consequently, the activated state of an exchanging lipid will depend very little on the properties of the bilayer from which it is exchanging. Changes in the free energy, enthalpy, and entropy of activation will mainly reflect changes in the ground state. Thus, examination of these parameters should provide information about the local interactions in the bilayer experienced by the exchanging lipid molecules.

Table I lists the thermodynamic parameters of the activation process for DMPC exchanging between liquid-crystalline and gel-phase DMPC LUV (this study) and SUV (McLean & Phillips, 1984; M. Schläppi, unpublished results) calculated from Arrhenius plot data (Homan & Pownall, 1988) extrapolated to 23 °C. In liquid-crystalline-phase LUV, $\Delta G^{\ddagger}$ is completely dominated by $\Delta H^{\ddagger}$ while liquid-crystalline SUV have a +2–5 kcal/mol contribution to $\Delta G^{\ddagger}$ from $-T\Delta S^{\ddagger}$ and a $\Delta H^{\ddagger}$ which is 3–5 kcal/mol smaller. The differences in entropy and enthalpy between liquid-crystalline SUV and LUV

observed here, however, are close to the experimental uncertainties, and, therefore, their significance is limited.

In contrast, analysis of the thermodynamics of the activation process in the gel phase reveals large, significant differences between LUV and SUV. SUV are highly curved bilayers that cannot completely adopt the rigid, planar gel phase and thus are more disordered than planar gel-phase bilayers (Sheetz & Chan, 1972; Lentz et al., 1976a; Chrzeszczyk et al., 1977; Parente & Lentz, 1984). The disordered regions are probably localized in liquidlike defects between planar gel-phase domains (Blaurock & Gamble, 1979). More nearly planar LUV bilayers can adopt the gel phase without requiring extensive defect structures. We suggest that the exchanging lipids in DMPC gel-phase SUV are the molecules existing in the defect regions while in LUV the exchanging molecules are in the intact gel-phase bilayer. Thus, lipid molecules desorbing from the more ordered gel-phase LUV bilayer are expected to have a larger $\Delta H^{\ddagger}$ and a smaller or negative $-T\Delta S^{\ddagger}$ compared to lipid molecules desorbing from the disordered defects of DMPC gel-phase SUV. These predictions are well supported by the gel-phase data in Table I. The $-T\Delta S^{\ddagger}$ contribution to $\Delta G^{\ddagger}$ for DMPC desorbing from gel-phase LUV is -6.3 ± 2.0 kcal/mol, almost 19 kcal/mol less than $-T\Delta S^{\ddagger}$ for SUV, which is $+13.5 \pm 3$ kcal/mol. $\Delta H^{\ddagger}$ for LUV in the gel phase is almost a factor of 3 larger (31.1 ± 3.7 compared to 11 ± 2 kcal/mol) than $\Delta H^{\ddagger}$ for SUV. It can be seen in Table I that the lipid molecules exchanging from gel-phase SUV experience a 9–13 kcal/mol smaller $\Delta H^{\ddagger}$ and a 10–13 kcal/mol larger, more positive $-T\Delta S^{\ddagger}$ than liquid-crystalline-phase LUV or SUV. This suggests that SUV gel-phase defects are much more disordered than even the liquid-crystalline phase.

Exchange between Two-Component DMPC/DSPC LUV. The kinetics of lipid exchange between two-component, two-phase vesicles can be a complex function of the desorption rates from each of the two phases, the flip-flop rates in the phases, and the rates of intravesicular exchange between the phases. In almost all of the two-component, two-phase experiments presented here, however, the kinetics are simple, single-exponential decays with exchangeable fractions of approximately 90% of the total lipid. (All of the non-single-exponential experiments occurred near or within the liquid-crystalline-phase region of the phase diagram.) This result suggests that either intravesicular exchange between gel and liquid-crystalline domains is fast relative to desorption from the liquid-crystalline phase or that the desorption rates of DMPC from the DSPC-enriched gel phase and DMPC-enriched liquid-crystalline phase are similar. The data in Figures 6 and 7 indicate that the latter is probably correct because the desorption rates of DMPC from the two-component vesicles display no dependence on the presence or absence of the liquid-crystalline phase. Above ~33 °C, all the values of k_1 agree within experimental error with the values for liquid-crystalline-phase pure DMPC LUV. This is true even for compositions at which 33 °C is well into the gel-phase region of the phase diagram. It is possible that, in the vesicles studied here, DMPC and DSPC experience a greater immiscibility than that predicted by the equilibrium-phase diagram and that DMPC-enriched liquid-crystalline domains exist at temperatures below the solidus line of the phase diagram. The behavior of the 0.1 mol % DMPC samples, however, indicates that this is not the case. These 0.1% DMPC vesicles were actually DSPC vesicles in which a trace of [^{3}H]DMPC was included. Vesicles such as these containing a 1000:1 or higher ratio of DSPC to DMPC almost certainly do not have phase-separated, liquid-crystalline domains of DMPC, and yet the rate constants are very similar to the other compositions (Figure 6). Also, the 0.1 mol % DMPC samples had flip-flop rates very much slower than all the other samples. Phase separation of DMPC would most probably have caused the flip-flop rates as well as the desorption rates to be similar among all the samples. Thus, it appears that the desorption rates of DMPC from a DMPC-enriched liquid-crystalline phase and DSPC-enriched gel phase are very similar at temperatures above ~33 °C. Below ~33 °C, the desorption rates decrease below the values for the pure DMPC liquid-crystalline phase vesicles but remain consistently higher than the values predicted by extrapolation of the pure DMPC gel-phase data, despite the fact that many of those experiments are with gel-phase vesicles. Perhaps these observations are the result of accelerated exchange from defects due to poor packing of the much shorter DMPC molecule in a DSPC-containing gel phase. This is not unexpected in light of the large difference in acyl chain length and phase transition temperature. There is some evidence of gel-phase immiscibility of DMPC and DSPC (Knoll et al., 1981).

As shown in the Arrhenius plots, the enthalpy and entropy of activation are similar for two-component gel-phase vesicles and liquid-crystalline vesicles above ~33 °C composed of either DMPC or DMPC/DSPC. Below ~33 °C, the curvature of the Arrhenius plots indicates an apparent temperature-dependent increase in the activation energy and activation enthalpy for all of the two-component vesicles. This may be the result of a reorganization of the gel-phase packing, resulting in increased lipid–lipid interactions.

The hypothesis given in the introduction concerning phase boundary defects and desorption is not supported by the experiments presented here. The data in Figure 6 display no detectable increase in k_1 ($\Delta k_1 < 30\%$) over the rate expected from the pure DMPC exchange in any of the experiments performed on two-component, two-phase vesicles. In fact, as stated above, DMPC exchange between two-component bilayers displays little or no dependence on composition or phase state at any temperature.

Exchangeable Fractions and Flip-Flop in One-Component DMPC LUV. The fraction of DMPC which exchanges between DMPC LUV with a single rate constant is approximately 90% despite the fact that almost 50% of the lipid is contained in the inner monolayer of the membranes. Therefore, the rate of flip-flop must be equal to or greater than the rate of intervesicular exchange. This observation is in sharp contrast to the slow flip-flop observed in DMPC SUV in exchange experiments (McLean & Phillips, 1984; M. Schläppi, unpublished results), by NMR (De Kruijf & Van Zoellen, 1978), and by other techniques (Bayerl et al., 1988). Table II lists maximum and minimum values for DMPC flip-flop halftimes as determined experimentally and obtained from the literature. In exchange experiments very similar to those reported here, using SUV (McLean & Phillips, 1984; M. Schläppi, unpublished results), very slow flip-flop rates were observed. The lower limit for flip-flop half-times in these experiments corresponds to the upper end of the range given in Table II. The LUV flip-flop half-times in Table II are only upper limits and could be much smaller; therefore, under similar conditions, the flip-flop of DMPC in liquid-crystalline LUV is a minimum of 10-fold faster than in SUV and possibly much more.

The headgroup packing in liquid-crystalline SUV is strained in both monolayer leaflets, but especially in the inner monolayer (Chrzeszczyk et al., 1977; Watts et al., 1978; Mason & Huang, 1978; Cornell et al., 1980) where the area per lipid headgroup approaches the value found in planar gel-phase

Table II: Values of DMPC Flip-Flop Half-Times in Various Bilayers

	exchange $t_{1/2}$ (h) DMPC LUV	
	T = 30 °C	T = 50 °C
L_α DMPC LUV[a]	9.6	0.74
	flip-flop $t_{1/2}$ (h) estimates	
	T = 30 °C	T = 50 °C
L_α DMPC SUV[b]	≈10–90	≈1–7
L_α DMPC LUV	≤9	≤0.7
65% DMPC LUV	≤9[c]	≈0.7–1.0[d]
28% DMPC LUV	≤9[e]	≈0.7–1.0[d]
0.1% DMPC LUV	>100[f]	>7[e]

[a] Measured for liquid-crystalline DMPC LUV in this study. [b] Estimates from De Kruijff and Van Zoellen (1978), McLean and Phillips (1984), Schläppi (unpublished results), and Bayerl et al. (1988). [c] Conditions are near the solidus line of the phase diagram but still within the two-phase region. [d] Liquid-crystalline-phase vesicles. [e] Conditions not far below the solidus line of the phase diagram; therefore, a small amount of liquid-crystalline phase may exist. [f] Gel-phase vesicles.

bilayers (Cornell et al., 1980). There is, then, an extra energy barrier to flip-flop in SUV due to the difficulty of adding lipids to the already closely packed inner monolayer. This condition does not exist in more nearly planar LUV bilayers or biological membranes. The observation that DMPC desorption rates are similar between SUV and LUV in the liquid-crystalline phase suggests that the lipid packing in the outer monolayer does not present a significant energy barrier to removal of lipid molecules from that leaflet. In order to maintain the mass distribution between the two monolayers, the transbilayer movement from the inside to the outside monolayer is almost certainly limited by the slow movement in the other direction.

The 10% of DMPC which is nonexchangeable over the course of the experiments probably arises from several sources. The 100-nm extrusion vesicles used in these studies are predominantly unilamellar, but some small amount of oligolamellar structures may exist. The inner lipid of such structures would necessarily exchange more slowly than the outer bilayer lipid and therefore might appear as nonexchangeable. Also, as stated above, the inner monolayer of very small vesicles is nonexchangeable, and a small amount of small vesicles may also exist in the LUV preparations used.

Exchangeable Fractions and Flip-Flop in Two-Component LUV. The exchangeable fraction of DMPC in most of the experiments with two-component vesicles at DMPC concentrations ≥28 mol % was approximately 90%, indicating rapid flip-flop. The 0.1 mol % DMPC experiments, however, had exchangeable fractions of only 45.1 ± 5.1%, both above and below the phase transition temperature of the vesicles, while exchanging with a single exponential (Figure 2B). This result signifies very slow flip-flop with exchange from the outer monolayer only.

The experiments having non-single-exponential kinetics probably indicate intermediate flip-flop rates. They occurred at $T \geq 50$ °C (45 °C in one case) and DMPC between 28 and 65 mol %. Although the experiments do not provide quantitative information, the flip-flop rates, under the conditions, where they affect the kinetics, appear to decrease relative to the desorption rate with increasing temperature or decreasing DMPC content.

Flip-flop rates of phospholipids depend strongly on the headgroup of the lipid and only weakly on the acyl chain (Homan & Pownall, 1988) because the barrier to flip-flop is the passage of the hydrophilic headgroup and any associated water through the hydrophobic core region of the bilayer. An increase in the average thickness of the acyl chain region of a bilayer will increase the energy barrier that must be overcome and thus will decrease the flip-flop rate. The reason flip-flop rates appear to decrease relative to exchange at $T \geq 50$ °C and DMPC contents between 28 and 65 mol % may be that under these conditions the very fluid, all-liquid-crystalline, DSPC-containing bilayers have an average thickness which is large enough to produce a detectable decrease in flip-flop. One would expect slower flip-flop in gel-phase domains also, but the data in Figure 7 demonstrate that this is not the case. Most of the two-phase and gel-phase experiments have exchangeable fractions of approximately 90%, indicating rapid flip-flop in both gel and liquid-crystalline phases. The poor packing of gel-phase DMPC and gel-phase DSPC might produce defects which allow an increase in the rate of flip-flop in a manner analogous to that proposed above for the gel-phase desorption rates from two-component vesicles.

Although acceleration of DMPC desorption occurs in the 0.1% DMPC experiments in the gel phase, acceleration of flip-flop rates over the expected slow values does not. This may be the result of differences in the nature of the defects in the 0.1% samples compared to the others. In most of the other experiments, the concentration of DMPC in the gel phase is at least 50 times higher than 0.1 mol %; therefore, the defects may involve a number of DMPC molecules and may be large enough (or may span the bilayer) to allow rapid flip-flop. In the 0.1% DMPC vesicles, the defects probably result from a lone DMPC molecule surrounded by gel-phase DSPC, and this may be enough to allow more rapid desorption but not rapid flip-flop.

Registry No. DMPC, 13699-48-4; DSPC, 4539-70-2.

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Gating Kinetics of pH-Activated Membrane Fusion of Vesicular Stomatitis Virus with Cells: Stopped-Flow Measurements by Dequenching of Octadecylrhodamine Fluorescence

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Received July 18, 1989; Revised Manuscript Received October 11, 1989

ABSTRACT: To identify the initial stages of membrane fusion induced by vesicular stomatitis virus, we performed stopped-flow kinetic measurements with fluorescently labeled virus attached to human erythrocyte ghosts that contained symmetric bilayer distributions of phospholipids. Fusion was monitored spectrofluorometrically using an assay based on mixing of the lipid fluorophore octadecylrhodamine. At 37 °C and pH values near the threshold for fusion, a lag phase of 2 s was observed. The lag time decreased steeply as the pH decreased, while the initial rate of fusion showed the reverse functional dependence on pH. The observed rapid fluorescence changes resulted from fusion of virus bound to the target, and the time lags were not due to association-dissociation reactions between virus and target. For a given pH value, the temperature dependence of the lag time was similar to that of the initial rate of fusion. The results were fitted to a multistate model similar to that resulting from ion channel gating kinetics. The model allows testing of hypotheses concerning the role of cooperativity and conformational changes in viral spike glycoprotein-mediated membrane fusion.

It is well established that spike glycoproteins on the viral membrane surface are responsible for the ability of enveloped viruses to invade host cells (White et al., 1983; Ohnishi, 1988). Vesicular stomatitis virus (VSV),[1] which normally first binds to the cell surface and then enters via the endocytic pathway, can be made to fuse to the plasma membrane by briefly lowering the pH of the medium after the virus has attached to the cell (White et al., 1981; Matlin et al., 1982; Blumenthal et al., 1987; Puri et al., 1988). A glycoprotein (G protein, M_r 60 000) is the sole membrane-spanning protein in VSV, of which about 1200 copies are expressed per virion (Thomas et al., 1985). A low pH (<6.6)-induced conformational change of this protein is thought to trigger the fusion event (Crimmins et al., 1983; Blumenthal, 1988).

To gain insight into the molecular mechanism of action of the viral spike glycoproteins, we examine the kinetics of lipid mixing as a result of fusion of membranes of virus and target. Over recent years, the octadecylrhodamine (R18) fluorescence dequenching assay has become a frequent means of monitoring membrane fusion (Hoekstra et al., 1984). One particularly useful aspect of this assay is its ability to facilitate the

[1] Abbreviations: VSV, vesicular stomatitis virus; R18, octadecylrhodamine B chloride; PBS, phosphate-buffered saline; MES, 4-morpholinoethanesulfonic acid; SAAM, simulation analysis and modeling.