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Characterization of Phospholipid Transfer between Mixed Phospholipid–Bile Salt Micelles†

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ABSTRACT: Concentration-dependent self-quenching of the fluorescent phospholipid *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (*N*-NBD-PE) was used to measure the rate of *N*-NBD-PE transfer between phosphatidylcholine–bile salt mixed micelles. In a previous study using the same technique, the rate of *N*-NBD-PE transfer between phosphatidylcholine–taurocholate mixed micelles was found to be several orders of magnitude faster than its transfer between phosphatidylcholine vesicles as a result of an increased rate of transfer through the water at low micelle concentrations and an increased rate of transfer during transient micelle collisions at higher micelle concentrations [Nichols, J. W. (1988) *Biochemistry 27*, 3925–3931]. In this study we have determined the influence of bile salt structure, incorporation of cholesterol, and temperature on the rate and mechanism of phospholipid transfer between mixed micelles. We found that both transfer pathways were a common property of mixed micelles prepared from a series of different bile salts and that the rates of transfer by both pathways increased as a function of the degree of bile salt hydrophobicity. Cholesterol incorporation into phosphatidylcholine–taurocholate mixed micelles displaced taurocholate from the micelles and resulted in an increased rate of transfer through the water and a decreased rate of transfer during micelle collisions. The temperature dependence of the transfer rates was used to calculate the activation free energy, enthalpy, and entropy for both mechanisms. The activation enthalpy was the major barrier to transfer by both mechanisms. However, the observed increase in the rate of phospholipid transfer through the water between mixed micelles relative to vesicles, and the increased rate of collision-dependent transfer between mixed micelles prepared with the more hydrophobic bile salts, are both primarily the result of increased activation entropy.

Fluorescent-labeled NBD[1] phospholipids have recently been shown to transfer rapidly between phospholipid–taurocholate mixed micelles when compared to their transfer between vesicles (Nichols, 1988). This increased rate of transfer was shown to result from an increase in the rate of phospholipid dissociation into the water phase (aqueous diffusion mechanism), which is the rate-limiting step at low concentrations of micelles, and to result predominantly from collision-dependent transfer at higher concentrations of micelles. These conclusions were based on a kinetic analysis of NBD-labeled phospholipid transfer between mixed phospholipid–taurocholate micelles in which the rate of transfer was determined from the loss of self-quenching of the fluorescent phospholipid

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[1] Abbreviations: NBD, 7-nitro-2,1,3-benzoxadiazol-4-yl; *N*-NBD-PE, *N*-(NBD)diacylphosphatidylethanolamine; *N*-NBD-DLPE, *N*-(NBD)-dilaurylphosphatidylethanolamine; *N*-NBD-DMPE, *N*-(NBD)dimyristoylphosphatidylethanolamine; *N*-NBD-DPPE, *N*-(NBD)dipalmitoylphosphatidylethanolamine; *N*-Rh-PE, *N*-(lissamine rhodamine B sulfonyl)dioleoylphosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; imc, intermicellar concentration; cmc, critical micelle concentration; HBS, HEPES-buffered saline, 0.9% NaCl in 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4.

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molecules as they exchanged with unlabeled phospholipids in acceptor micelles of the same composition.

In the present paper, we used the same technique to investigate the influence of bile salt structure, incorporation of cholesterol, and temperature on the rate and mechanism of phospholipid transfer between phospholipid–bile salt mixed micelles. We found that transfer by both the aqueous diffusion and collision-dependent transfer mechanisms is a general feature of the bile salts tested. However, the rates for both mechanisms were highly dependent on bile salt structure, varying as much as 2 orders of magnitude. A preliminary report of this work has appeared elsewhere (Fullington & Nichols, 1989).

Experimental Procedures

Materials and Routine Procedures. DOPC, dilaurylphosphatidylethanolamine, *N*-NBD-DMPE, and *N*-NBD-DPPE were purchased from Avanti Polar Lipids, Inc. Lissamine rhodamine B sulfonyl chloride was obtained from Molecular Probes, Inc. Taurocholate was purchased from Calbiochem. NBD chloride and all of the bile salts and acids with the exception of taurocholate were purchased from Sigma Chemical Co. Bile salts and acids (100 μg) produced single spots on silica gel thin-layer chromatography in chloroform/methanol/acetic acid/water, 65:25:2:4, when developed by either iodine or sulfuric acid charring. *N*-NBD-DLPE and *N*-Rh-PE were synthesized, respectively, by reacting NBD chloride with dilaurylphosphatidylethanolamine and lissamine rhodamine B sulfonyl chloride with dioleoylphosphatidylethanolamine (Struck et al., 1981). The fluorescent probes, bile salts, and phospholipids were periodically tested for purity by silica gel thin-layer chromatography and repurified as necessary. Lipids were stored at −20 °C, and bile salts were stored desiccated at 4 °C. Bile salt and cholesterol concentrations were determined by weight, and phospholipid concentrations were determined by a lipid phosphorus assay (Ames & Dubin, 1960).

Mixed Micelle and Vesicle Preparation. Mixed micelles were prepared by evaporating the solvents under nitrogen from equimolar amounts of bile salts, phospholipids and, where indicated, specified amounts of cholesterol. Following approximately 12 h of desiccation in vacuo, the dried phospholipid–bile salt mixtures were solubilized with the amount of 10 mM bile salt solution in HBS required to obtain a 1:2 molar ratio of phospholipid to bile salt and were stirred until clear. Vesicles were prepared by ethanol injection as previously described (Kremer et al., 1977; Nichols & Pagano, 1982).

Fluorescence and Light-Scattering Measurements. Light-scattering and slow fluorescent transients ($t_{1/2} > 2$ s) were measured on a Perkin-Elmer MPF-44E fluorescence spectrophotometer. Solutions in the cuvette were stirred continuously with a magnetic stirrer, and the temperature was controlled with a circulating water bath. Inner filtering was minimized by using concentrations of *N*-NBD-PE that resulted in less than 0.2 absorbance at the maximum excitation wavelength. Analogue output from the Perkin-Elmer fluorometer was digitized and stored on an IBM-XT. Fast fluorescence transients ($t_{1/2} < 2$ s) were measured on an SLM 8000C spectrofluorometer equipped with the SLM stopped-flow accessory. Temperature was controlled with a circulating water bath, and the digital output was stored on an IBM-AT-compatible computer.

Measurement of the Intermicellar Concentration of Bile Salts. Before a series of transfer experiments was begun, the intermicellar concentration (imc) of the water-soluble bile salt in equilibrium with the phospholipid–bile salt mixed micelles was determined as described previously (Nichols, 1988). The imc of the nonfluorescent acceptor micelles was determined by injecting an aliquot of the micellar solution into a cuvette containing an estimate of the imc for that bile salt and monitoring any changes in the intensity of scattered light at 90° occurring after the initial shift. If the bile salt in solution partitions into the micelles, the micelles will decrease in size (Mazer et al., 1980), resulting in a decrease in light scattering. The opposite happens if the bile salt partitions out of the micelles. The concentration of bile salt in solution was systematically varied until no changes in light scattering were observed following micelle addition. This concentration of bile salt in solution was considered to be at equilibrium with the mixed micelles and was functionally defined as the imc.

An analogous procedure was used to measure the imc for the fluorescent donor mixed micelles. Donor micelles were injected into different concentrations of the appropriate bile salt to find the concentration that resulted in no fluorescence change following the initial change after injection. An increase in fluorescence indicated that the bile salt in solution was partitioning into the mixed micelles and reducing the self-quenching interaction of the *N*-NBD-PE. A fluorescence decrease reflected bile salts leaving the micelle. The concentration of the bile salt solution was systematically varied to obtain no change in fluorescence which reflected equilibrium of the bile salt in solution with that contained in micelles, that is, the imc. The imc values measured by these two techniques for a given pair of donor and acceptor micelles never differed by more than 7%. In those cases where they differed by more than 0.1 mM, the imc measured for the donors was used in the fluorescence transfer rate experiments.

Measurement of N-NBD-PE Transfer between Micelles. The method for using the concentration-dependent self-quenching of micellar *N*-NBD-PE fluorescence to measure its transfer from donor to acceptor micelles has been described previously (Nichols, 1988). In the experiments presented here, donor micelles were prepared with 20 mol % of the phospholipid replaced with *N*-NBD-PE. At this mole fraction the *N*-NBD-PE fluorescence was self-quenched by approximately 50%. Mixing these donor micelles with acceptor micelles not containing *N*-NBD-PE resulted in an increase in fluorescence as the *N*-NBD-PE equilibrated with the acceptor micelles. A nonlinear least-squares fitting method using the Marquardt algorithm (Marquardt, 1963) was used to obtain the parameters of a single-exponential equation that gave the best fit to the experimental data. For all of the fluorescent traces obtained, a single-exponential equation was sufficient to describe the data accurately; that is, additional exponential terms did not significantly improve the goodness of fit.

Kinetic Analysis. Given a first-order exponential equation, the initial rate of fluorescence change ($\Delta F_{t=0}/\Delta t$) is equal to the apparent first-order rate constant (k_{app}) times the total change in fluorescence following equilibration (ΔF_{eq}). The initial rate of fluorescence change was calculated in this way and then divided by the maximum change in fluorescence to obtain the initial fractional rate of change of maximum fluorescence. ΔF_{max} occurs when the fluorescent donor micelles are mixed with an excess of nonfluorescent acceptor micelles such that the extent of self-quenching remaining at equilibrium is insignificant. The fractional rate of fluorescence change can then be used to calculate the fractional rate of *N*-NBD-PE mass transfer from donors to acceptors from the following equation that is derived in the Appendix.

$$R = \frac{(\Delta[D]_2)_{t=0}}{[D]_T \Delta t} = \frac{(1 + \Delta F_{max}/F_0)}{(2 + \Delta F_{max}/F_0)} \frac{\Delta F_{t=0}}{\Delta F_{max} \Delta t} \quad (1)$$

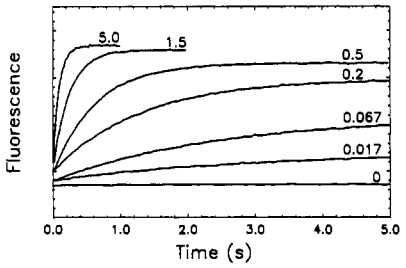

FIGURE 1: Fluorescent traces reflecting the rapid transfer of *N*-NBD-DMPE between DOPC–chenodeoxycholate mixed micelles. Fluorescence (excitation 475 nm; emission 530 nm) was recorded on the SLM 8000C spectrofluorometer following mixing in the stopped-flow device. Stock donor micelles (*N*-NBD-PE:DOPC: chenodeoxycholate, 2:8:20 [mM]) and stock acceptor micelles (DOPC–chenodeoxycholate, 10:20 [mM]) were diluted into 1.7 mM chenodeoxycholate in HBS (the previously determined imc for both) to obtain the desired concentrations in the observation chamber following the mixing of equal volumes. The final concentration of donor micelle DOPC was constant for all traces at 0.033 mM. The final concentrations of acceptor DOPC were varied and resulted in different traces. The numbers above each trace refer to the final concentrations of acceptor micelle DOPC in mM. Temperature was 25 °C.

R is the initial fractional rate of *N*-NBD-PE transfer, $(\Delta[D]_2)_{t=0}$ is the initial change of bulk solution concentration of *N*-NBD-PE in the acceptor micelles, $[D]_T$ is the total bulk solution concentration of *N*-NBD-PE, $\Delta F_{t=0}$ is the initial rate of fluorescence change, ΔF_{max} is the maximum fluorescence change, F_0 is the fluorescence at $t = 0$, and Δt is the elapsed time from $t = 0$.

Kinetic Models for Phospholipid Transfer between Micelles. For each of the different micelle compositions tested, the dependence of the initial rates (R) on the acceptor concentration of DOPC was measured. These data were used to determine which combination of the following three transfer models best described the initial rate kinetics.

aqueous diffusion

$$R = k_{dis}[L]_2/([L]_1 + [L]_2)$$

bimolecular collision

$$R = k_{bi}[L]_2$$

termolecular collision

$$R = k_{ter}([L]_2)^2$$

$[L]_1$ and $[L]_2$ are the bulk solution concentrations of DOPC in the donor and acceptor micelle populations, respectively; k_{dis}, k_{bi}, and k_{ter} ae the aqueous dissociation, bimolecular collision, and termolecular collision rate constants, respectively.

The initial rate data were fit by a nonlinear least-squares method (Marquardt, 1963) first to each of the three models alone, second to the three combinations of two models, and third to all three models combined to determine which combination of equations gave the best fit to the rate data. The F_χ test was used to test whether the addition of a given term significantly improved the goodness of fit [see p 200 in Bevington (1969)].

RESULTS

Measurement of N-NBD-PE Transfer between Mixed Micelles. *N*-NBD-DMPE transfer between mixed micelles was measured by recording the increase in fluorescence after donor micelles containing *N*-NBD-DMPE were injected into a stirred cuvette containing the desired concentration of acceptor micelles [see Nichols (1988) for complete description]. Some of the mixed micelles tested in this study transferred *N*-NBD-DMPE with half-times of less than 2 s, and a stopped-flow mixing device was used to measure their transfer rates. Figure 1 depicts a series of fluorescence traces resulting from the mixing of donor and acceptor DOPC–chenodeoxycholate mixed micelles with the stopped-flow device. As the concentration of acceptor micelles (expressed as DOPC concentration) was increased from zero to 5 mM with the donor micelle concentration held constant, both the equilibrium fluorescence and the rate of *N*-NBD-DMPE equilibration increased. The initial rates of fluorescence change were calculated, converted to the fractional initial rate of total *N*-NBD-DMPE transfer by using eq 1, and plotted against receptor DOPC concentration in Figure 2.

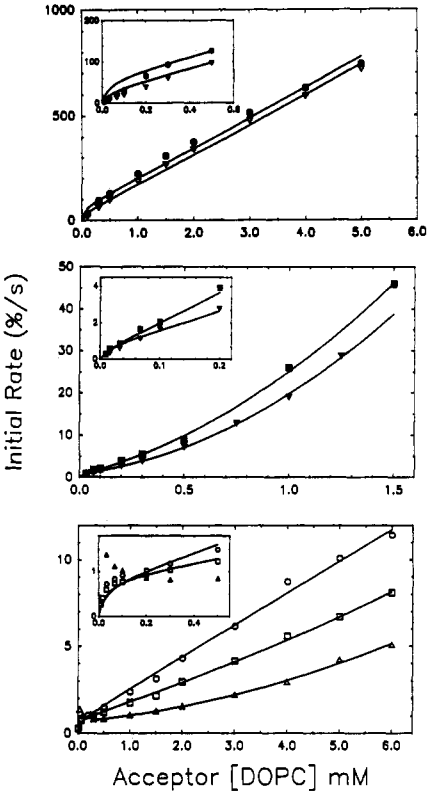

FIGURE 2: Initial rates of *N*-NBD-DMPE transfer (percent per second) between DOPC–bile salt mixed micelles versus the concentration of acceptor micelle DOPC. Rate measurements were made following the procedure outlined in Figure 1 for chenodeoxycholate and deoxycholate mixed micelles and were made in a similar manner previously described for micelles with slower transfer rates [see Nichols (1988)]. Initial rates were calculated from fluorescence data by using the equation presented under Experimental Procedures. The data were fitted with curves obtained by adding the equations describing the aqueous diffusion and collisional components of transfer listed under Experimental Procedures. Donor micelles were composed of 2:8:20 mM *N*-NBD-DMPE:DOPC:bile salt, and acceptor micelles consisted of only 10:20 mM DOPC:bile salt. Symbols refer to the type of bile salt; (▽) chenodeoxycholate; (●) deoxycholate; (■) taurodeoxycholate; (▼) glycodeoxycholate; (○) cholate; (□) taurocholate; (△) glycocholate. The graph inserts expand data at low concentrations. All experiments were carried out at 25 °C.

Equation 1 predicts that the initial fractional rate of maximum fluorescence change is equal to the initial fractional rate of maximum probe transfer from donors to acceptors (R) when corrected by the factor $(1 + \Delta F_{max}/F_0)/(2 + \Delta F_{max}/F_0)$. Since the ratio $\Delta F_{max}/F_0$ (maximum fluorescence change following the equilibration with excess acceptor micelles/initial fluorescence of the donor micelles) is dependent on the mole fraction of probe in the donor micelles, we tested this relationship by comparing the transfer rates of *N*-NBD-DMPE:DOPC:taurocholate donor micelles containing 20, 15, and 10 mol % of the DOPC replaced with *N*-NBD-DMPE. We found for a wide range of acceptor micelle concentrations

Table I: Comparison of Rate Constants and imc Values for Different Bile Salts (25 °C)

bile salt	OH position			imc (mM)	$t_{1/2}$[a] (s)	k_{dis}[b] (s^{-1})	k_{bi}[c] (M^{-1} s^{-1})	k_{ter}[d] (M^{-2} s^{-1})	cmc[e] (mM)	HI[f]
deoxycholate	3α,		12α	1.6	0.189	58.0	144	*g*	3.0	+0.72
chenodeoxycholate	3α,	7α		1.7	0.236	29.0	143	*g*	4.0	+0.59
taurodeoxycholate	3α,		12α	1.0	1.42	0.853	12.2	12.0	2.4	+0.59
glycodeoxycholate	3α,		12α	1.0	2.33	1.18	5.54	13.0	2.0	+0.65
cholate	3α,	7α,	12α	5.9	18.6	0.728	1.84	*g*	11.0	+0.13
taurocholate	3α,	7α,	12α	4.8	25.5	0.850	0.953	0.0441	6.0	+0.00
glycocholate	3α,	7α,	12α	5.0	42.3	*h*	0.268	0.0790	10.0	+0.07

[a] $t_{1/2}$ was calculated from the apparent rate constant at 1.0 mM acceptor [DOPC]. [b] Dissociation rate constant. [c] Bimolecular collisional rate constant. [d] Termolecular collisional rate constant. [e] cmc measured by surface tension at 0.15 M NaCl (Roda et al., 1983). [f] HI is the hydrophobicity index obtained from reverse-phase high-pressure liquid chromatography at pH 7.4 (Heuman, 1989). [g] Inclusion of the term containing these rate constants did not significantly improve the fit of the data. [h] The rate data for DOPC–glycocholate micelles are inconsistent with the model for aqueous diffusion.

(0.5–5.0 mM DOPC) that the calculated values of *R* were not dependent on the mole fractions of probe in the donor micelles (data not shown). Donor micelles with 20 mol % *N*-NBD-PE were used routinely in subsequent experiments since they provided the optimum compromise between minimizing the mole fraction of probe and maximizing the degree of self-quenching in the donor micelles.

Effect of Bile Salt Structure. Figure 2 illustrates the dependence of the initial rate (*R*) of *N*-NBD-DMPE transfer on the concentration of acceptor micelles expressed as DOPC concentration. The initial rate data for each bile salt were fitted to the combination of kinetic models (aqueous diffusion, bi- or termolecular collision; see Experimental Procedures) that best described the acquired data. The kinetic modeling indicated that, with the exception of glycocholate, all of the bile salt–phospholipid mixed micelles exhibited kinetics consistent with an aqueous diffusion mechanism at low micelle concentrations and a collisional mechanism at high micelle concentrations. The rate constants obtained from the initial rate plots are presented in Table I for comparison. Mixed micelles prepared from glycocholate exhibited unusual kinetic behavior at low concentrations (Figure 2) which was inconsistent with any of the three models. However, at higher micelle concentrations, the kinetics were consistent with transfer by a combination of bi- and termolecular collisions.

The half-times of *N*-NBD-DMPE transfer calculated at 1.0 mM ranged from 0.19 to 42.3 s—a difference of over 2 orders of magnitude—depending on bile salt structure. The bile salts tested varied in three major structural respects: conjugation of the side chain with taurine or glycine and the number and position of hydroxyl groups located on the steroid nucleus. Conjugation significantly lowered the rate of *N*-NBD-DMPE transfer. Taurine conjugation of cholate increased the half-time for transfer by a factor of 1.4 and glycine conjugation by a factor of 2.3. A qualitatively similar effect was observed for the deoxycholate conjugates. The half-time for transfer was 7.5 times longer for micelles prepared with taurodeoxycholate and 12.3 times longer for micelles prepared with glycodeoxycholate relative to those prepared with deoxycholate (Table I).

A breakdown of the rate constants (Table I) provides insight into the specifics of how conjugation affects the transfer of phospholipid between mixed micelles. Taurine conjugation of cholate had relatively small effects on the dissociation rate constant (k_{dis}). In contrast, the collisional rate constants, specifically the bimolecular collisional rate constants, were lower for taurocholate and glycocholate than for cholate mixed micelles. The micelles prepared from deoxycholate conjugates also had lower bimolecular collisional rate constants relative to those prepared from deoxycholate. Both the dissociation and collision-dependent rate constants were much lower for taurodeoxycholate and glycodeoxycholate than for deoxycholate micelles.

The effect of the 7α-hydroxyl group can be seen by comparison of the transfer kinetics of cholate and its conjugates glycocholate and taurocholate with deoxycholate and its analogous conjugates glycodeoxycholate and taurodeoxycholate. The half-time for *N*-NBD-DMPE transfer between micelles prepared from cholate was over 100 times slower than for deoxycholate, and the half-times for both glycocholate and taurocholate mixed micelles were 18 times slower. The rate constants were similarly affected. All of the rate constants were much lower for cholate than for deoxycholate mixed micelles. Although the dissociation rate constants remained relatively unchanged, the collisional rate constants were at least 12 times smaller for the mixed micelles prepared with taurine and glycine conjugates of cholate than for those prepared with conjugates of deoxycholate. A comparison of the phospholipid transfer rate between mixed micelles prepared from cholate with that for micelles prepared from chenodeoxycholate illustrates that removal of the 12α-hydroxyl also results in a large decrease in the half-time (18.6 to 0.24 s) reflected in large increases in the dissociation and collisional rate constants (Table I).

Whereas the number of hydroxyl groups greatly altered the rate of transfer of probe between micelles, the position of the hydroxyl groups on the steroid nucleus did not significantly affect the transfer. Neither the half-times nor the rate constants for the dihydroxy bile salts chenodeoxycholate (hydroxyls in the 3α- and 7α-positions) and deoxycholate (hydroxyls in the 3α- and 12α-positions) differed appreciably.

Effect of Cholesterol. The effect of cholesterol incorporation into DOPC–taurocholate mixed micelles on the rate of *N*-NBD-PE transfer was determined for two different NBD probes (*N*-NBD-DLPE and *N*-NBD-DPPE). At low concentrations of mixed micelles, *N*-NBD-DLPE—the shorter-chained probe—transfers primarily by aqueous diffusion and therefore can be used to accurately measure the effect of cholesterol on the dissociation rate constant without interference from collision-dependent terms. On the other hand, transfer by aqueous diffusion of *N*-NBD-DPPE is insignificant relative to the collision-dependent transfer, and therefore this probe was used to determine the effect of cholesterol incorporation on transfer resulting from mixed micelle collisions.

Cholesterol incorporation into the DOPC–taurocholate mixed micelles increases the IMC for taurocholate (Table II). However, cholesterol addition has little effect on the rate of *N*-NBD-DLPE dissociation from micelles to the water phase until 2 mM has been added. At 2 mM cholesterol the dissociation rate increases roughly 33%. Two millimolar cholesterol is equivalent to 6.2 mol % of the total lipid, which is close to the saturation level of cholesterol for a similar mixture of

Table II: Effect of Cholesterol on the Dissociation Rate of *N*-NBD-DLPE from DOPC–Taurocholate Mixed Micelles[a]

cholesterol (mM)	imc (mM)	$t_{1/2}$[b] (s)	k_{dis} (s^{-1})
0	4.4	18.3	0.038
1.0	4.8	18.0	0.039
1.5	5.0	17.9	0.039
2.0	5.4	13.4	0.052

[a] Micelles were prepared to contain 10 mM DOPC, 20 mM taurocholate, and the stated concentrations of cholesterol. Data are the average of two experiments. [b] $t_{1/2} = \ln 2/k_{dis}$ and represents the maximal rate of transfer of *N*-NBD-DLPE via aqueous diffusion.

Table III: Effect of Cholesterol on the Rate of Collision-Dependent Transfer of *N*-NBD-DPPE between DOPC–Taurocholate Mixed Micelles[a]

cholesterol (mM)	imc (mM)	$t_{1/2}$[b] (s)	k_{bi} (M^{-1} s^{-1})	k_{ter} (M^{-2} s^{-1})	k_{app}^{col}[c] (s^{-1})
0	4.4	53.3	7.77	625	1.3×10^{-2}
1.0	4.7	90.7	4.48	410	0.8×10^{-2}
2.0	5.1	139	2.70	415	0.5×10^{-2}

[a] Micelles were prepared in a solution containing 10 mM DOPC, 20 mM taurocholate, and the stated concentrations of cholesterol. Data are the average of two experiments. [b] $t_{1/2} = \ln 2/k_{app}^{col}$ and represents the maximal rate of transfer of *N*-NDB-DPPE via collisional transfer. [c] k_{app}^{col} is equal to the apparent rate constant calculated for the rate of transfer of *N*-NBD-DPPE at 1.5 mM acceptor micelle DOPC.

micelles prepared from egg lecithin and taurocholate (Mazer & Carey, 1983).

The effect of cholesterol on the collision-dependent transfer of *N*-NBD-DPPE is shown in Table III. The rate of collision-dependent transfer decreases by a factor of 2.6 as the cholesterol content approaches cholesterol saturation at 2 mM (6.2 mol %).

In summary, cholesterol addition tends to increase the rate at which phospholipids dissociate into the water phase but to decrease the rate of transfer during transient micellar collisions.

Effect of Temperature. *N*-NBD-DLPE was also used to compare the temperature dependence from 10 to 30 °C of the dissociation rate constant—the rate-limiting step of the aqueous diffusion pathway—between DOPC–taurocholate micelles and DOPC vesicles. Arrhenius plots of the data are shown in Figure 3A. Activation energies were calculated from the Arrhenius plots and are presented in Table IV. The dissociation rate constant for micelles is 3 orders of magnitude greater than that for vesicles. The activation free energy for *N*-NBD-DLPE transfer between vesicles is 3.8 kcal/mol greater than for transfer between micelles at 25 °C. The activation enthalpy for dissociation from the vesicles is only 1 kcal/mol greater than that for micelles, whereas the activation entropy favors dissociation from micelles relative to vesicles by 2.8 kcal/mol.

The longer chained probe, *N*-NBD-DPPE, was used to examine the collision-dependent transfer between micelles as explained above. Arrhenius plots in Figure 3B illustrate the

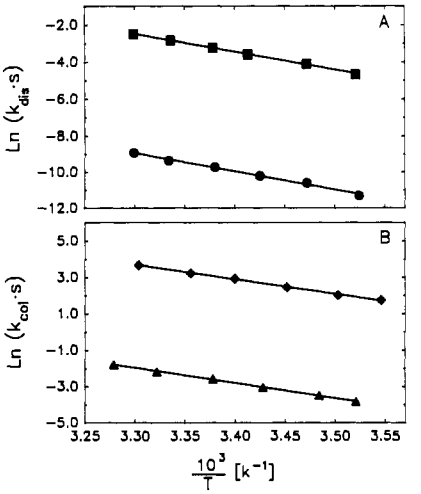

FIGURE 3: Arrhenius plots of *N*-NBD-PE transfer by the aqueous diffusion and the collision-dependent mechanisms. (A) *N*-NBD-DLPE was used to measure phospholipid transfer by aqueous diffusion. Low acceptor micelle concentrations of 0.033 mM were used for taurocholate (■) micelles so that probe transfer resulted from aqueous diffusion only. Data for dissociation of the probe from DOPC vesicles (●) are shown for comparison. Activation energies for the dissociation rate constants (k_{dis}) were calculated by using the model described by Aniansson et al. (1976) and are presented in Table IV. (B) *N*-NBD-DPPE was used to measure collision-dependent transfer as explained under Results. Acceptor micelle concentrations were sufficiently high [5.0 mM for chenodeoxycholate (◆) micelles and 6.0 mM for taurocholate (▲) micelles] to ensure that probe transfer occurred predominantly through transient micelle collisions. Activation energies were calculated from the apparent first-order collision constant (k_{col}) by using the Eyring (1935) activated complex theory and are presented in Table V.

temperature dependence from 10 to 32 °C of phospholipid transfer between DOPC–chenodeoxycholate mixed micelles and between DOPC–taurocholate mixed micelles. Activation energies calculated from these data are presented in Table V. The collisional rate constant for the chenodeoxycholate mixed micelles was larger than that for taurocholate mixed micelles by a factor of 290. Differences in the activation free energies were primarily due to differences in the activation entropies; 2.9 kcal/mol of the 3.4 kcal/mol difference in activation free energy was due to entropy.

Discussion

We have compared the kinetic behavior of *N*-NBD-PE transfer between phospholipid–bile salt mixed micelles for a range of bile salts differing in side-chain conjugation and the number and position of hydroxyl groups. With the exception of glycocholate, the kinetic behavior of all of the bile salts tested conformed to the model proposed earlier (Nichols, 1988), in which phospholipid transfer occurs predominantly by aqueous diffusion at low micelle concentrations and predominantly by transient bi- and termolecular collisions at high micelle concentrations. The unusual kinetic behavior observed

Table IV: Activation Energies for the Dissociation of *N*-NBD-DLPE from Micelles and Vesicles (25 °C)[a]

probe	particle	k_{dis} (s^{-1})	$\Delta G_{dis}^{\ddagger\circ}$ (kcal/mol)	$\Delta H_{dis}^{\ddagger\circ}$ (kcal/mol)	$T\Delta S_{dis}^{\ddagger\circ}$ (kcal/mol)
N-NBD-DLPE	DOPC–taurocholate micelles	$4.8 \times 10^{-2} \pm 0.04 \times 10^{-2}$	16.8 ±0.02	19.2 ±0.037	2.4 ±0.37
N-NBD-DLPE	DOPC vesicles	$7.4 \times 10^{-5} \pm 0.6 \times 10^{-5}$	20.6 ±0.05	20.2 ±0.80	−0.4 ±0.8

[a] Activation energies were calculated from the model for amphiphile monomer–micelle interactions described by Aniansson et al. (1976): $k_{dis} = [D_m/(l_b)^2] \exp(-\Delta G^{\ddagger\circ}/RT)$. At constant pressure $\Delta G^{\ddagger\circ} = \Delta H^{\ddagger\circ} - T\Delta S^{\ddagger\circ}$, and thus, $\ln k_{dis} = -\Delta H^{\ddagger\circ}/RT + \Delta S^{\ddagger\circ}/R + \ln[D_m/(l_b)^2]$. R and T have their usual meanings, D_m is the diffusion constant for the exiting monomer (assumed to be 5×10^{-6} cm^2/s), and l_b is the width of the barrier that is RT energy units below its maximum (assumed to be 0.7 Å). Values of $\Delta H^{\ddagger\circ}$ and $\Delta S^{\ddagger\circ}$ were obtained respectively from the slopes and y intercepts of the Arrhenius plots in Figure 3A. Standard deviations (±) of activation energies were calculated on the basis of standard deviations of slopes and y intercepts and predicted rate constants.

Table V: Activation Energies for the Collision-Dependent Transfer of *N*-NBD-DPPE between DOPC–Taurocholate Micelles and between DOPC–Chenodeoxycholate Micelles (25 °C)[a]

probe	particle	k_{col}[b] (s^{-1})	$\Delta G^{*\circ}_{col}$ (kcal/mol)	$\Delta H^{*\circ}_{col}$ (kcal/mol)	$T\Delta S^{*\circ}_{col}$ (kcal/mol)
N-NBD-DPPE	DOPC–taurocholate micelles	0.088 ±0.0006	18.9 ±0.01	16.0 ±0.14	−2.8 ±0.14
N-NBD-DPPE	DOPC–chenodeoxycholate micelles	25.6 ±0.34	15.5 ±0.02	15.4 ±0.15	0.1 ±0.15

[a] Activation energies were calculated from the Eyring activated complex theory (Eyring, 1935): $k_{col} = [\chi RT/N_A h] \exp(-\Delta G^{*\circ}/RT)$. At constant pressure, $\Delta G^{*\circ} = \Delta H^{*\circ} - T\Delta S^{*\circ}$, and thus $\ln k_{col} = -\Delta H^{*\circ}/RT + \Delta S^{*\circ}/R + \ln(\chi RT/N_A h)$. R and T have their usual meanings, N_A is the Avogadro constant, h is the Planck constant, and χ is the transmission coefficient (assumed to be 1.0). Values of $\Delta H^{*\circ}$ and $\Delta S^{*\circ}$ were calculated, respectively, from the slopes and y intercepts of the Arrhenius plots in Figure 3B. Linear regression analysis was used to obtain the slopes and y intercepts of the plots in Figure 3B. Standard deviations (±) of activation energies were calculated on the basis of standard deviations of slopes and y intercepts and predicted rate constants. [b] Apparent first-order rate constant for collision-dependent transfer at 6 mM DOPC concentration for taurocholate, 5 mM for chenodeoxycholate.

for low concentrations of glycocholate mixed micelles may be attributable to a nonhomogeneous mixture of micellar aggregates.

The mixed micelles resulting in the fastest transfer rate (DOPC–deoxycholate) had a half-time at 1 mM DOPC more than 200 times shorter than the slowest rate (DOPC–glycocholate). The derived dissociation and collision-dependent rate constants vary in the same order and are roughly correlated with the degree of hydrophobicity of the bile salt as determined by retention times on a reversed-phase HPLC column run at pH 7.4 in 0.15 NaCl (Table I; Heuman, 1989). However, since the hydrophobicity index does not predict the large decrease in intermicellar transfer rate observed as a result of taurine and glycine conjugation of deoxycholate, bile salt hydrophobicity alone is not adequate to predict the transfer properties of their mixed micelles.

To minimize the effect of micelle size on the transfer rates, the ratio of bile salt to phospholipid was kept high (total bile salt, 20 mM; total phospholipid, 10 mM). Bile salt rich mixed micelles approach the size of simple micelles, and their size is only slightly dependent on the bile salt to phospholipid ratio in the micelle (Mazer et al., 1980; Muller, 1984). However, because the imc values for the different bile salts were not the same, the ratio of phospholipid to bile salt in the micelle varied from a high of 0.71 (cholate) to a low of 0.53 for taurodeoxycholate and glycodeoxycholate. Therefore, the mixed micelle sizes were tested directly by HPLC molecular sieve chromatography on a Beckman TSK 5000 PW column that was equilibrated with HBS containing the appropriate bile salt at its imc. This technique will be described in detail in a future paper, but the results indicated that the radius of the largest mixed micelles (DOPC–chenodeoxycholate) was only 12% larger than that of the smallest (DOPC–taurodeoxycholate) under the conditions used for the transfer experiments (unpublished observations), and the transfer rate did not correlate with the micelle size. Although size may be a major determinant of the intermicellar transfer rates, the small size differences measured between the mixed micelles presented in Table I are unlikely to be the major determinant of the large differences in transfer rates measured in these experiments.

It is interesting to note that the imc values of the mixed micelles are not directly correlated with the rates of phospholipid transfer (Table I). Hydroxyl addition increases the imc and decreases the phospholipid transfer rates, whereas conjugation of the di- and trihydroxy bile salts decreases both the imc and the transfer rate. Glycine and taurine conjugation of both deoxycholate and cholate also lowers the cmc (Kratohvil et al., 1986; Roda et al., 1983) as well as the imc (Table I), which is inconsistent with the components of simple micelles and mixed micelles associating solely on the basis of hydrophobicity. The lower imc and cmc values measured for the conjugated relative to the unconjugated bile salts indicate that other interactions (e.g., electrostatic, hydrogen bonding, or steric constraints) between the bile salts and/or phospholipids are involved in the formation of simple and mixed micelles.

Incorporation of cholesterol into DOPC–taurocholate mixed micelles decreases the rate of collision-dependent transfer and increases the rate of phospholipid dissociation into the water phase (Tables II and III). The increase in phospholipid dissociation is only seen at the limit of cholesterol solubility (2 mM). The observed increase in the imc for taurocholate with increasing mole fractions of cholesterol suggests that the more hydrophobic cholesterol molecules are displacing the taurocholate molecules from the mixed micelles. An increase in effective viscosity is also observed when cholesterol is incorporated into the micelle interior of egg lecithin–taurocholate mixed micelles (Matsuzaki et al., 1989), which may be partially responsible for the decreased rate of collision-dependent transfer observed in these studies.

To gain more insight into the influence of bile salt structure on the aqueous diffusion and collision-dependent transfer pathways, we measured the temperature dependence and calculated the activation energies for the rate constants for both processes.

The activation free energy for lipid dissociation from micelles or vesicles is defined according to the Aniansson et al. (1976) model in which the activation free energy is the difference in the free energy of a lipid fully inserted in the micelle or vesicle (ground state) and its free energy when all but a small portion is partitioned into the aqueous phase (activated state). The free energy required to move a lipid molecule from a nonpolar phase into an aqueous phase has been interpreted to arise from the summation of the energy required to move a suitably sized cavity from the nonpolar to aqueous phase with the energy change resulting from solute–solvent interactions (Jenks, 1969; Pierotti, 1971). Analogously, the free energy of activation, which has been shown to be greater than the equilibrium free energy of transfer (Doody et al., 1980; McLean & Phillips, 1984; Nichols, 1985), results from the activated state where the lipid molecule is almost completely removed. In this state the nonpolar and aqueous cavities coexist, and therefore the free energy required to transfer to the activated state exceeds the energy required to transfer the molecule completely from one phase to the other at equilibrium.

According to the Aniansson model, the activation free energy for *N*-NBD-DLPE dissociation from micelles is 16.8 kcal/mol versus 20.6 kcal/mol for vesicles. The major component of the free energy of activation for both micelles and vesicles is activation enthalpy. This is consistent with activation energies measured for the dissociation of several different lipid molecules from vesicles (Doody et al., 1980; Pownall et al., 1983; McLean & Phillips, 1984; Nichols, 1985, 1986). Changes in entropy during the activation step are small relative to the enthalpic changes required. In other words, reduction in the van der Waals, electrostatic, and hydrogen-bonding interactions as the probe goes from the ground to the activated

state as opposed to increases in the structure of the lipid aggregate and surrounding water provides the major barrier to lipid dissociation into the water phase.

It is interesting that although the major energy barrier to dissociation from micelles and vesicles is enthalpic, the major difference between the two arises from differences in the entropy of activation. This is consistent with the observation by Gahwiller et al. (1977) that mixed micelles relative to vesicles have decreased anisotropy and a decreased rate of lateral diffusion of lipophilic probes, as well as the observation of Matsuzaki et al. (1989) that the effective viscosity of egg lecithin–taurocholate micelles is greater than that of egg lecithin micelles. Accordingly, the entropy of phospholipids in the ground state is expected to be lower in micelles (higher free energy). Thus, the change in entropy as the phospholipid goes from the ground to the activated state would be more positive for micelles than for vesicles, favoring dissociation from the micelles.

The Eyring activated complex theory (Eyring, 1935) was used to calculate and compare the activation free energies for collision-dependent transfer of *N*-NBD-DPPE between mixed micelles which result in fast (DOPC–chenodeoxycholate) and slow (DOPC–taurocholate) transfer rates. The Eyring theory can be used to describe the reaction of two micelles colliding to form a transiently fused product (activated complex) that results in phospholipid transfer.

The majority of the activation free energy for both the fast and slow transferring populations of mixed micelles is attributed to the enthalpy of activation. This increased enthalpy arises from the energy required to dehydrate the interacting surfaces and overcome the electrostatic, hydrogen-bonding, and van der Waals interactions between bile salts and phospholipids that oppose the close apposition and fusion of the two micelles. Even though the enthalpies of activation for collision are large for both populations of mixed micelles, the majority of the difference between the two results from changes in the entropy of activation. In other words, the mixed micelles with the more hydrophobic chenodeoxycholate molecules on their surface tend to structure the surrounding water molecules more than for those with the less hydrophobic taurocholate molecules. Increased entropy favors the formation of the fused complex to reduce the surface area of exposed lipids. Presumably, since there is no detectable growth in the average micelle size, this fused complex is unstable and short-lived.

These data suggest that the major energy barrier to collision-dependent transfer between micelles results from the energy required to remove water from the interface and to overcome the electrostatic repulsion between the opposing micelle surfaces, whereas the major difference between the rates observed for mixed micelles prepared from different bile salts results from the degree to which bile salts on the micelle surface structure the surrounding water molecules. Bile salts that produce a more hydrophobic micellar surface structure the water more and increase the rate of collision-dependent transfer.

From the previous discussion of the temperature dependence studies, one would predict that the correlation of the rate of phospholipid aqueous diffusion with bile salt hydrophobicity is the result of the induction of increased phospholipid structure (reduction of rotational and lateral movement) in the mixed micelle as a function of bile salt hydrophobicity. From the analogous correlation of the collision-dependent phospholipid transfer with bile salt hydrophobicity, one would predict that as the bile salt hydrophobicity is increased the water structure on the surface of the micelle is increased as well. Thus, the increased transfer rates measured for mixed micelles prepared with the more hydrophobic bile salts result primarily from the increased structural order of the micelle and the surrounding water.

These observations and predictions form the working hypotheses for additional studies into the structure of mixed phospholipid bile salt micelles.

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Appendix

In a previous publication (Nichols, 1988), the initial increase in fluorescence observed following the mixing of donor micelles containing *N*-NBD-PE at self-quenching concentrations with unlabeled acceptor micelles was related to the mass transfer of *N*-NBD-DMPE from donor to acceptor micelles by expressing the initial fluorescence change and the initial mass transfer as a fraction of the maximum change in each [eq 14 from Nichols (1988)]:

$$\Delta F_{t=0}/\Delta F_{max} = (\Delta[D]_2)_{t=0}/[D]_T$$

This relationship was derived by calculating the change in fluorescence following a discrete initial transfer of probe from donors to acceptors and is only true for the condition where either the quenching constant or mole fraction of probe to total donor lipids is large. A more general solution of the relationship can be obtained by integrating fluorescence (F) with respect to the concentration of probe transferred from donors to acceptors ($[D]_2$). The Stern–Volmer equation can be used to express the fluorescence yield (f) as a function of the mole fraction of probe in either donors or acceptors (Nichols, 1988), and thus the total fluorescence (F) can be expressed as the sum of the fluorescence emitted from the donors and the acceptors

$$F = \frac{[D]_1 f_{max}}{1 + K[D]_1/[L]_1} + \frac{[D]_2 f_{max}}{1 + K[D]_2/[L]_2} \quad (A1)$$

where the subscripts 1 and 2 refer to the donor and acceptor micelles, respectively, [D] is the bulk solution concentration of micellar *N*-NBD-PE, [L] is the bulk solution concentration of total phospholipid in the micelles, f_{max} is the maximum fluorescence yield for *N*-NBD-PE contained in the micelles, and K is the quenching constant. Since the probe is only slightly soluble in water, the total concentration of *N*-NBD-PE ($[D]_T$) is equal to the sum of that contained in the donors and acceptors.

$$[D]_T \approx [D]_1 + [D]_2 \quad (A2)$$

Combining eqs A1 and A2 and taking the derivative of F with respect to $[D]_2$ yield

$$\frac{dF}{d[D]_2} = \frac{f_{max}([L]_2)^2}{([L]_2 + K[D]_2)^2} - \frac{f_{max}([L]_1)^2}{([L]_1 + K[D]_T - K[D]_2)^2}$$

Thus, for the initial condition where $[D]_2 = 0$ at $t = 0$, the initial change of fluorescence, $(\Delta F)_{t=0}$, is related to the initial transfer of probe into the acceptor population $(\Delta[D]_2)_{t=0}$ by

$$(\Delta F)_{t=0} = f_{max}\left(1 - \frac{([L]_1)^2}{([L]_1 + K[D]_T)^2}\right)(\Delta[D]_2)_{t=0} \quad (A3)$$

The maximum change in fluorescence (ΔF_{max}) is obtained by subtracting the maximum fluorescence (F_{max}) from the initial fluorescence (F_0). The initial fluorescence occurs when $[D]_1 = [D]_T$ and $[D]_2 = 0$; thus, from eq A1

$$F_0 = \frac{[D]_T f_{max}}{1 + K[D]_T/[L]_1} \quad \text{(A4)}$$

The maximum fluorescence occurs when $[L]_2 \gg [D]_T$, $[D]_2 \approx [D]_T$, and $[D]_1 \approx 0$. Thus, from eq A1

$$F_{max} = [D]_T f_{max} \quad \text{(A5)}$$

Therefore, the maximum change in fluorescence is

$$\Delta F_{max} = F_{max} - F_0 = \frac{([D]_T)^2 f_{max} K}{[L]_1 + K[D]_T} \quad \text{(A6)}$$

Dividing eq A3 by eq A6 yields

$$\frac{(\Delta[D]_2)_{t=0}}{[D]_T} = \frac{1 + K[D]_T/[L]_1}{2 + K[D]_T/[L]_1} \frac{\Delta F_{t=0}}{\Delta F_{max}} \quad \text{(A7)}$$

This more general relationship indicates that the initial fractional change of the maximum fluorescence is not necessarily equal to, but is proportional to, the initial fractional amount of total probe transferred for a given mole ratio of probe to lipid in the donor micelles. Since dividing eq A6 by eq A4 gives

$$\Delta F_{max}/F_0 = K[D]_T/[L]_1 \quad \text{(A8)}$$

the proportionality factor can be calculated for a given donor micelle from the fluorescence data.

Registry No. DOPC, 10015-85-7; cholesterol, 57-88-5; deoxycholate, 83-44-3; chenodeoxycholate, 474-25-9; taurodeoxycholate, 516-50-7; glycodeoxycholate, 360-65-6; cholate, 81-25-4; taurocholate, 81-24-3; glycocholate, 475-31-0.

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