Hydrophobic Interaction of Lysophospholipids and Bile Salts at Submicellar Concentrations†

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ABSTRACT: A series of environment-sensitive, fluorescent-labeled *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-monoacylphosphatidylethanolamine (*N*-NBD-lysoPE) probes of differing acyl chain length (C_{12}–C_{18}) was used to demonstrate the hydrophobic interaction between lysophospholipids and two different bile salts at concentrations below their respective critical micelle concentrations (cmc's). Formation of submicellar aggregates in the presence of bile salt–phospholipid mixed micelles could facilitate lipid absorption in the intestine. To ensure the use of submicellar lysolipid concentrations in the experiments, the cmc of each fluorescent lysolipid probe was determined by concentration-dependent self-quenching. The cmc values obtained for the various *N*-NBD-lysoPE probes were as follows (μM): monolauroyl, $\geq$40; monomyristoyl, 4; monopalmitoyl, 0.3; monostearoyl, 0.04. Probe concentrations well below their respective cmc's were used in all experiments. The fluorescence of a solution of each lysolipid probe was monitored as the concentration of bile salt was gradually increased. The increase in fluorescence was taken as a measure of the ability of the bile salt molecules to complex with the probe molecule, thereby increasing the fluorescent yield of the lysolipid probe molecule. Determination of the cmc of the bile salts in the presence of the lysolipid probe was made in parallel with the fluorescence measurement by monitoring the increase in light scattering of the solution. Both bile salts were shown to induce maximal increases in fluorescence of the *N*-NBD-lysoPE derivatives at concentrations of bile salt well below their respective cmc values, indicating the existence of submicellar lysolipid–bile salt aggregates. Examination of the data within the framework of the Hill equation model for cooperativity indicated that between two and five molecules were a minimum estimate for the number of bile salts required to give maximal fluorescence. The affinity of the bile salt–lysolipid interaction was found to increase with the hydrophobicity of both the bile salt (TDC > cholate) and the lysolipid (C_{18} > C_{16} > C_{14} > C_{12}).

One of the major functions of bile salts is to aid in the digestion and absorption of fats in the intestinal lumen (Erlinger, 1987). Bile salt–phospholipid mixed micelles serve as a reservoir for cholesterol, fatty acid, and lysophospholipid monomers thereby increasing the efficiency of their absorption by epithelial cells lining the small intestine (Shiau, 1987). It has been demonstrated that these mixed micelles do not accelerate the absorption of lipids by an endocytic process (Hoffman, 1970; Simmonds, 1972; Wilson & Dietschy, 1972) or a collisional process between the micelles and the cell membranes (Westergaard & Dietschy, 1976). Rather, these micelles are thought to penetrate the unstirred water layer adjacent to the luminal cell membrane and serve to increase the concentration of lipid monomers at the membrane surface thereby enhancing the rate of absorption (Westergaard & Dietschy, 1974; 1976; Wilson, 1981). As might be expected, these micelles serve to facilitate the absorption of hydrophobic compounds to a much greater extent than more polar compounds since the unstirred water layer and not the membrane itself is the major cellular permeability barrier for the more hydrophobic compounds (Sallee & Dietschy, 1973). Lysophospholipid monomers then enter the cell passively and are reacylated to phospholipids within the cell interior (Tso & Balint, 1976).

Recently, we have shown that phospholipids transfer through the aqueous phase between bile salt–phospholipid mixed micelles much faster than between phospholipid bilayers (Nichols, 1988; Fullington et al., 1990). The observed rapid rate of intermicellar lipid transfer through the aqueous phase may be the predominant physiological role played by bile salt mixed micelles in facilitating lipid absorption in the intestine. Bile salts could conceivably be effecting this accelerated rate either by increasing the rate of dissociation of phospholipid from the micelles relative to the bilayers or by forming bile salt–phospholipid heterodimers or heteromultimers that shuttle the phospholipid between the micelles and the enterocytes. We have used the environment-sensitive fluorescent probe *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)monoacylphosphatidylethanolamine (*N*-NBD-lysoPE)[1] with varying chain lengths (C_{12}–C_{18}) to study the interaction of lysophospholipids and bile salts at concentrations well below their respective cmc's. The results demonstrate the bile salts and lysophospholipids form aggregates at the low aqueous concentrations expected to exist in the water phase in equilibrium with bile salt–phospholipid mixed micelles. These submicellar aggregates could greatly facilitate the absorption of lysolipids from the aqueous phase in the intestine. A preliminary report of these

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[1] Abbreviations: NBD, 7-nitro-2,1,3-benzoxadiazol-4-yl; *N*-NBD-lysoPE, *N*-(NBD)monoacylphosphatidylethanolamine; *N*-NBD-LPE, *N*-(NBD)monolauroylphosphatidylethanolamine; *N*-NBD-MPE, *N*-(NBD)monomyristoylphosphatidylethanolamine; *N*-NBD-PPE, *N*-(NBD)monopalmitoylphosphatidylethanolamine; *N*-NBD-SPE, *N*-(NBD)monostearoylphosphatidylethanolamine; cmc, critical micelle concentration; HBS, HEPES-buffered saline [150 mM NaCl, 0.1 mM Na_2EDTA, 0.04% NaN_3, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 25 °C]; TBS, Tris-buffered saline [150 mM NaCl, 0.1 mM Na_2EDTA, 0.04% NaN_3, 10 mM tris(hydroxymethyl)-aminomethane, pH 8.5, 25 °C]; TDC, taurodeoxycholate.

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findings has appeared previously (Shoemaker & Nichols, 1990).

Experimental Procedures

Materials and Routine Procedures. The sodium salts of TDC and cholate were purchased from Sigma Chemical Co., analyzed for purity by silica gel thin-layer chromatography (TLC), and stored desiccated at room temperature. Both bile salts appeared as a single spot when 100 μg was run in chloroform/methanol/acetic acid/water, 65/25/2/4. Monoacylphosphatidylethanolamines were purchased from Avanti Polar Lipids, Inc., and *N*-NBD-labeled monolauroylPE (LPE), monomyristoylPE (MPE), monopalmitoylPE (PPE), and monostearoylPE (SPE) were prepared and purified by a previously established method (Struck et al., 1981). For one series of experiments *N*-NBD-LPE was prepared from *N*-NBD-dilauroylPE with phospholipase A_2 (Kates, 1975). Purity of the final products was assessed by TLC and was always greater than 99%. Phospholipids were stored at −20 °C, and concentrations were determined by the method of Ames and Dubin (1960). NBD-Cl, HEPES, Trizma base, and sodium azide were purchased from Sigma Chemical Co. Sodium chloride was from J. T. Baker, Inc. Solutions of TDC were prepared in HEPES-buffered saline [HBS; 150 mM NaCl, 0.1 mM Na_2EDTA, 0.04% NaN_3, 10 mM HEPES, pH 7.4, 25 °C] and of cholate in Tris-buffered saline [TBS; 150 mM NaCl, 0.1 mM Na_2EDTA, 0.04% NaN_3, 10 mM Tris, pH 8.5, 25 °C]. Since the pK_a for TDC and cholate are 2.0 and 5.0, respectively (Small, 1971), the pH of the two buffer solutions were chosen to ensure complete ionization of the respective bile salts. By having the two bile salts completely ionized, we have effectively measured the cmc for the pure bile salt and not the cmc for a subpopulation of the ionized bile salt that existed in equilibrium with the un-ionized form. At constant ionic strength and temperature, varying the pH in a region where only the ionized form of the bile salt exists has very little effect on the value for the cmc.

Fluorescence and Light Scattering Measurements. Experiments were performed on an SLM 8000C spectrofluorometer. Solutions in the thermostatically controlled (25 °C) water-jacketed cuvette were stirred with a magnetic stirrer. Fluorescence was monitored with excitation at 475 nm (16-nm slit) and emission at 530 nm (16 nm), and light scattering at 90° was measured at 450 nm (16 nm). Data for both fluorescence and light scattering of a given sample were monitored for sequential 25-s time periods without the sample being removed from the instrument. In the cholate experiments it was necessary to first perform fluorescence measurements in the absence of the *N*-NBD-lysoPE probes to enable us to subtract the background signal (light scattering spillover) that became significant at the higher bile salt concentrations in the cholate/TBS solutions. Data were collected and analyzed on an IBM-AT-compatible computer with curve fitting performed by a nonlinear least-squares regression analysis (Marquardt, 1963). For illustrative purposes the fluorescence data in Figures 2–4 were normalized by subtracting any background fluorescence of the probe in the absence of bile salt (F_o) from the measured value (F) and dividing by the maximal fluorescence obtained at the highest concentration of bile salt (F_{max}) minus the background fluorescence $[(F - F_o)/(F_{max} - F_o)]$. This was necessary to better compare the data from all the experiments since the different probes were present at different concentrations and necessitated the use of several different gains.

Determination of N-NBD-lysoPE Critical Micelle Concentrations. Stock solutions of each of the *N*-NBD-lysoPE probes were prepared and their respective concentrations increased in HBS while the fluorescence was monitored. Concentration-dependent self-quenching of the NBD fluorophore was used as an indicator of the cmc (Nichols & Pagano, 1981; Nichols, 1985). For the measurements made with *N*-NBD-LPE and *N*-NBD-MPE the fluorescence was corrected for inner filtering according to

$$F_{corr} \approx F_{obs} \text{ antilog } [(OD_{ex} + OD_{em})/2] \qquad (1)$$

where F_{corr} is the absorbance-corrected fluorescence, F_{obs} is the fluorescence measured, and OD_{ex} and OD_{em} are the optical densities of the fluorescent samples at 475 and 530 nm, respectively (Lakowicz, 1983).

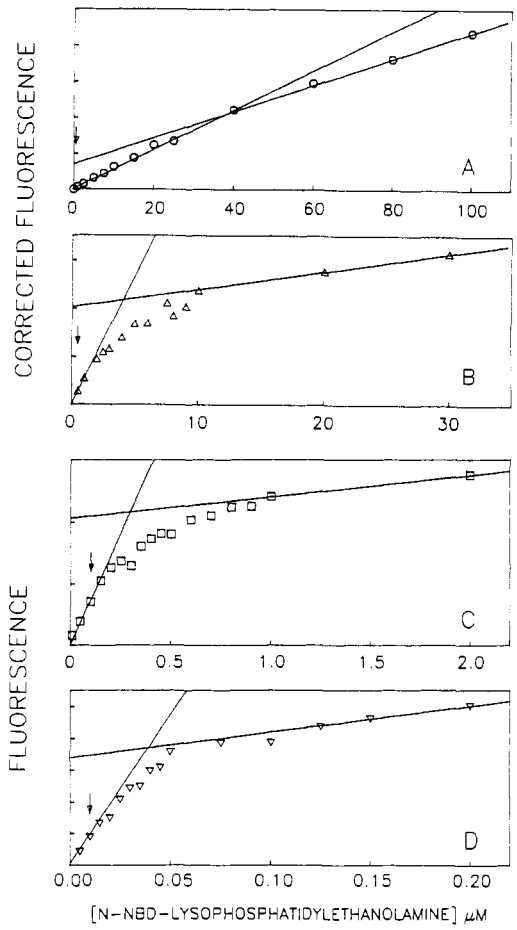

FIGURE 1: Measurement of the critical micelle concentrations of *N*-NBD-lysoPE derivatives. Fluorescence (excitation 475 nm, slit 16 nm; emission 530 nm, slit 16 nm) was measured on solutions of increasing concentrations of (A) *N*-NBD-LPE, (B) *N*-NBD-MPE, (C) *N*-NBD-PPE, or (D) *N*-NBD-SPE added to a series of cuvettes. Fluorescence in (A) and (B) was corrected for inner filtering as described under Experimental Procedures. The concentrations of these probes chosen for subsequent experiments, indicated by the arrows, were in the range where the self-quenching, i.e., micelle formation, was negligible.

Results

Prior to performing experiments on the interaction between bile salts and the *N*-NBC-lysoPE's, it was important to ensure the lysophospholipids were used at concentrations well below their cmc's where the monomer species predominated. The cmc for each of the probes was determined, and the results are shown in Figure 1. cmc determinations were done in HBS, and we obtained the following concentration values for the different probes (μM): *N*-NBD-LPE, ≤40; *N*-NBD-MPE, 4; *N*-NBD-PPE, 0.3; *N*-NBD-SPE, 0.04. To ensure that the monomer species of lysolipid predominated in our experimental

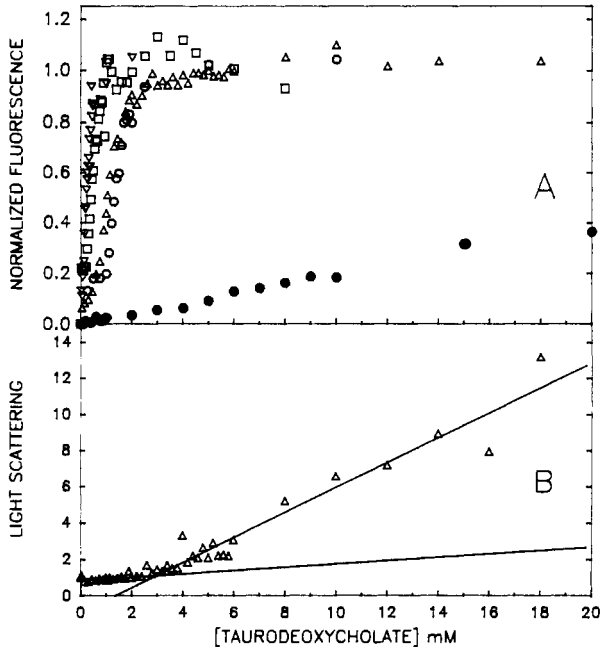

FIGURE 2: Sequential measurement of fluorescence and light scattering from samples containing *N*-NBD-lysoPE and increasing concentrations of sodium taurodeoxycholate (TDC). (A) Fluorescence (excitation 475 nm, slit 16 nm; emission 530 nm, slit 16 nm) was normalized (see Experimental Procedures) for better comparison of the data obtained with the differing concentrations of probes. Symbol definitions are as follows: (○) *N*-NBD-LPE, 0.5 μM; (△) *N*-NBD-MPE, 0.5 μM; (□) *N*-NBD-PPE, 0.1 μM; (▽) *N*-NBD-SPE, 0.01 μM; (●) NBD-Cl, 0.5 μM. (B) Light scattering (90° at 450 nm, 16-nm slits) data were fit with a linear regression analysis for the data above and below 2.5 mM. Light scattering data obtained for *N*-NBD-MPE are representative of the data obtained with the other three probes.

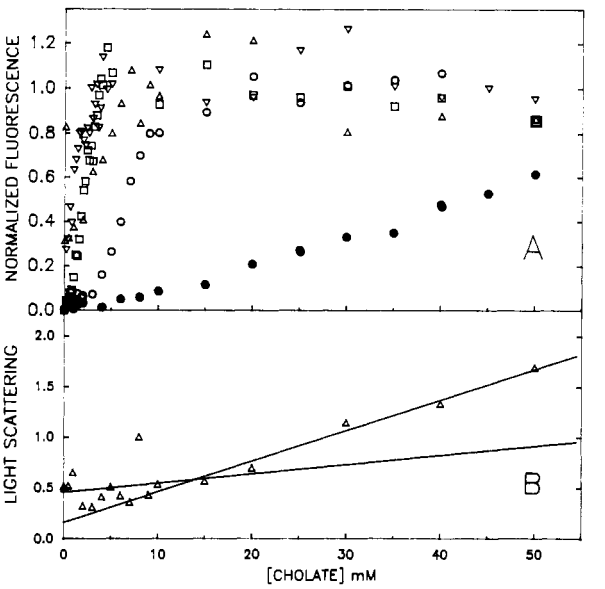

FIGURE 3: Sequential measurement of fluorescence and light scattering from samples containing *N*-NBD-lysoPE and increasing concentrations of sodium cholate. (A) Fluorescence (excitation 475 nm, slit 16 nm; emission 530 nm, slit, 16 nm) was normalized by subtracting any background fluorescence of the probe in the absence of cholate from the measured value and dividing by the maximal fluorescence obtained at the highest concentration of cholate. Symbol definitions are as follows: (○) *N*-NBD-LPE, 0.5 μM; (△) *N*-NBD-MPE, 0.5 μM; (□) *N*-NBD-PPE, 0.1 μM; (▽) *N*-NBD-SPE, 0.01 μM; (●) NBD-Cl, 0.5 μM. (B) Light scattering (90° at 450 nm, 16-nm slits) data were fit with a linear regression analysis for the data above and below 10 mM. Light scattering data obtained for *N*-NBD-MPE are representative of the data obtained with the other three probes.

solutions, we used the following concentrations of the probes in subsequent experiments (μM): *N*-NBD-LPE, 0.5; *N*-NBD-MPE, 0.5; *N*-NBD-PPE, 0.1; *N*-NBD-SPE 0.01.

We employed the increase in fluorescence of the *N*-NBD-lysoPE monomers as an indication of bile salt–lysophospholipid association. The quantum yield of the NBD probe increases as a function of the hydrophobicity of its microenvironment (Monti et al., 1977). The increase in the light scattering of the same solution was used to monitor the degree of micellization. The concentration of Na-TDC was varied at the constant submicellar concentration of lysoPE probe noted above, and the fluorescence and light scattering of each solution were recorded (Figures 2 and 4A). In all four *N*-NBD-lysoPE derivatives examined, increasing the TDC concentration from 0 to 2 mM resulted in the maximal level of fluorescence attained. This result was surprising due to the fact that the process of micellization, as indicated by the onset of the increase in light scattering, did not begin until the concentration of TDC was greater than 2 mM [however, there was the anticipated slight upward slope due to the concentration dependence of the light scattering signal below 2 mM attributable to the increasing concentration of bile salt (Kratohvil, 1983)]. The increase in fluorescence was also markedly influenced by the acyl chain length of the probe. As the chain was lengthened, the increased hydrophobicity initiated the aggregation process at correspondingly lower concentrations of bile salt. When the unconjugated NBD-Cl fluorophore was present in the TDC solutions, no significant increase in fluorescence occurred until the cmc value was reached. The marked difference in behavior of NBD depending on whether or not it was attached to a lysophospholipid makes it unlikely the interaction between the TDC and the *N*-NBD-lysoPE's was due to a specific association between the NBD moiety and the bile salt. Also, the hydrocarbon chain dependence of the concentration of TDC that gave half-maximal fluorescence (Figure 4A) indicates the increase in fluorescence was not simply due to a general solvent effect on the quantum yield of the fluorophore resulting from changes in the refractive index or dielectric constant of the solution.

A completely analogous series of experiments was carried out with the bile salt sodium cholate in TBS at pH 8.5. As is apparent in Figure 3, the maximum fluorescence for each of the different chain length lysophospholipid probes was again achieved at concentrations of bile salt well below the cmc determined for cholate by light scattering. The onset of micellization was similarly mimicked by the increase in the fluorescence of the unconjugated fluorophore, NBD-Cl, indicating that a micelle was required to induce this increase in quantum yield. To determine if the concentration of probe influenced the interaction between bile salt and lysolipid, the concentration of *N*-NBD-MPE was lowered from 500 to 50 nM. This produced results that were indistinguishable from the data obtained for the higher concentration of probe (data not shown); i.e., the concentration of cholate at which half-maximal fluorescence was obtained remained unchanged. Therefore, it appeared that, given any finite concentration of lysolipid, the same fraction of that lysolipid would be complexed with cholate at a given cholate concentration.

The dependence of the cholate–lysolipid interaction was greatly influenced by hydrophobic forces. Once more the greater the hydrophobicity of the lysolipid probe, the lower the concentration of bile salt required to initiate the aggregation (Figure 4B). A comparison of the data obtained with both bile salts indicated that the hydrophobicity of the bile salt also plays a role in the interaction. The bile salt with the lower hydrophobic index, cholate (Heuman, 1989), both complexed with the lysolipids and formed micelles at a higher

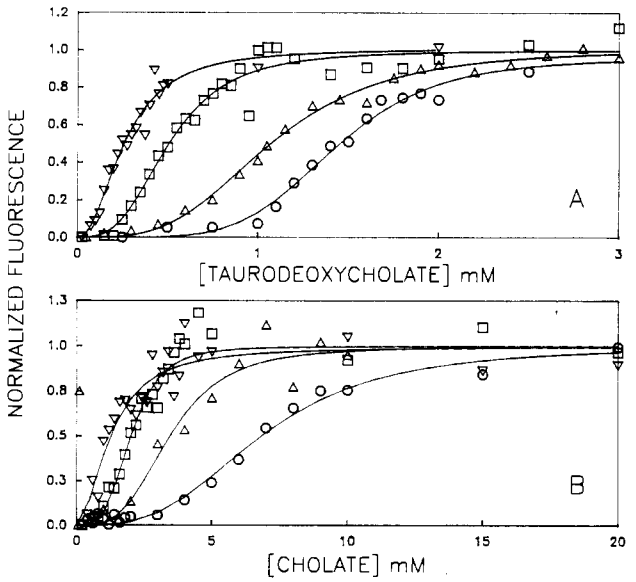

FIGURE 4: Measurement of normalized fluorescence of *N*-NBD-lysoPE derivatives in increasing concentrations of (A) taurodeoxycholate and (B) cholate. Same data as that in Figures 2 and 3 but on an expanded abscissa. Symbol definitions are as follows: (○) *N*-NBD-LPE, 0.5 μM; (△) *N*-NBD-MPE, 0.5 μM; (□) *N*-NBD-PPE, 0.1 μM; (▽) *N*-NBD-SPE, 0.01 μM. Solid curves are the best fit of the data generated by the Hill equation employing the derived constants n_{app} and K' listed in Table I.

Table I: Kinetic Constants[a] Derived from Fit of Fluorescence Data to Hill Equation

	taurodeoxycholate			cholate		
	n_{app}	K' (mM)	K^*_s (mM)	n_{app}	K' (mM)	K^*_s (mM)
N-NBD-LPE	5.11	6.20	1.43	3.12	401	6.83
N-NBD-MPE	3.38	1.29	1.08	3.38	66.2	3.46
N-NBD-PPE	3.08	0.125	0.509	3.73	13.5	2.01
N-NBD-SPE	2.16	0.0552	0.262	1.89	1.56	1.26

[a] n_{app} = the minimum estimate of the number of substrate binding sites per lysolipid molecule required to give maximum fluorescence assuming a high degree of cooperativity in the system; K' = a constant comprising the interaction factors a, b, c, ... i and the intrinsic dissociation constant K_s; $K^*_s = K'^{1/n_{app}}$, the concentration of bile salt that gives half-maximal fluorescence.

concentration than TDC. Therefore, with both bile salts examined there appeared to be a hydrophobic interaction with each of the *N*-NBD-lysoPE probes when both the probe and the bile salt were present below their respective cmc's.

In an attempt to quantitate the interaction between the lysophospholipid and bile salt monomers, we fit our data (Figure 4 and Table I) to theoretical curves generated from the Hill equation, the empirically derived form of the Adair–Pauling simple sequential interaction model (Segel, 1975), modified as

$$(F - F_o)/(F_{max} - F_o) = [S]^n/(K' + [S]^n) \quad (2)$$

where F equals measured fluorescence, F_o equals the background fluorescence in the absence of bile salt, F_{max} equals maximal fluorescence obtained, [S] is the bile salt concentration in millimolar, and n is the minimum number of substrate (bile salt) binding sites. The constant, K', is composed of the intrinsic dissociation constant, K_s, as well as the interaction factors a, b, c, etc. that serve to provide the cooperative behavior of the system. Since the Hill equation was originated to explain protein–ligand interaction, the formalism includes at least n identical binding sites whose initial affinities, K_s, are increased by some interaction factor upon the binding of substrate to another site on the molecule, i.e.

$$K' = K_s^n(a^{n-1}b^{n-2}c^{n-3}...i^1) \quad (3)$$

In the analysis of our results we treated the observed increase in fluorescence analogously with substrate binding or reaction velocity. The fluorescence increased as one or more bile salt molecules interacted with the *N*-NBD-lysoPE. The sigmoidicity of the fluorescence could result from cooperative behavior of bile salt binding, a nonlinear effect of bile salt binding on the fluorescence yield, or some combination of the two. In the fluorescence analogy to the Hill equation (eq 2), each bile salt that binds is assumed to increase the fluorescence yield of the *N*-NBD-lysoPE by $1/n$ of the maximum fluorescence. The maximum fluorescence was achieved when n bile salt molecules were bound per *N*-NBD-lysoPE monomer. In our system the cooperativity may not be sufficient to allow the Adair–Pauling simple sequential interaction model to be reduced to the Hill equation (Segel, 1975). Therefore one must substitute the value n_{app} for n in the Hill equation and note that n_{app} will be less than the actual number of binding sites. In other words, our system behaves in each case as if there were n_{app} binding sites with strong cooperativity, but the data could equally well be represented by more binding sites with the degree of cooperativity decreasing proportionately with the increase in the number of binding sites above the value of n_{app}.

One can see from the generally good agreement between the data and the theoretical curves (Figure 4) that the association process between lysophospholipids and bile salts can be accurately described by a cooperative process with the minimum estimate of the number of bile salts associating with lysolipid varying between 2 and 5 (Table I). Most of the lysolipids required at least three bile salt molecules to explain the sigmoidicity. The least hydrophobic lysolipid, *N*-NBD-LPE, required a mimimun of five TDC molecules, however, and the most hydrophobic, *N*-NBD-SPE, required a minimum of two of either of the bile salt monomers to form the mixed complex. It should also be pointed out that the more hydrophobic the lysolipid derivative, the higher the affinity for both of the bile salts studied.

Although our experimental data conform nicely to analysis by the Hill equation, there are differences between our system and this treatment of protein–ligand interaction that should be emphasized. First of all, it should be pointed out that the nature of the hydrophobic interaction between the bile salt and the lysolipid bears little resemblance to the traditional protein–ligand interaction for which the Hill equation was derived. It is highly unlikely that the sequential bile salt molecules interacting with lysolipid will have identical affinities, K_s, that are modified by successive interaction factors. Nevertheless, since there are no constraints placed on the values of K_s or a, b, c, etc., the affinity of the lysolipid in our system for each additional bile salt (K_1, K_2, etc.) can be accurately represented by the product of two numbers ($aK_s = K_1$; $bK_s = K_2$; etc.), and the model was therefore capable of approximating our data very well. Second, the Hill equation was originally derived to describe binding interactions that displayed a marked degree of cooperativity, and it is well-known that self-association of bile salts displays only a mild degree of cooperativity (albeit in the absence of added lysolipid) in comparison with the micellization of other detergents (Mukerjee et al., 1984). An additional caveat that we must point out was that while we attempted to minimize the possibility of self-association of the lysolipids by working well below the cmc for the different probes, we have no independent evidence that there was only one probe molecule present in the aggregates that were formed.

The Hill analysis does provide a framework within which to present our data, however, and we feel it is useful as a preliminary model for the interaction process we are presenting. Unfortunately at this early stage of characterization of the system we have no idea of the actual stoichiometry of the aggregates for a given concentration of bile salt. It must also be kept in mind that the information gleaned from the Hill equation does not allow one to dissect any absolute constants for a system. The n_{app} value is a *minimum* number for the bile salts binding to a lysolipid molecule, and the K' represents a *lumped* constant that includes all of the equilibrium constants for each of the bile salts bound. An additional constant, K^*_s, that is equivalent to the concentration of bile salt that produces half-maximal fluorescence of the lysolipid probe can be derived from the first two constants (the nth root of K'), and as a first approximation we will employ it to represent the concentration of bile salt at which half of the lysolipid probe is complexed.

Discussion

The major finding of the current study was that *N*-NBD-lysoPE derivatives were capable of binding to bile salts at concentrations well below their respective cmc's. This is the first time that this submicellar molecular interaction between lysophospholipids and bile salts has been demonstrated experimentally. These submicellar aggregates may be important intermediates in the physiological mechanism of phospholipid absorption in the intestine. Previous studies in similar model systems have inferred the interaction of bile salt monomers with cholesterol (Chijiiwa & Nagai, 1989) and bilirubin IXα (Carey & Koretsky, 1979); however, the physiological significance of these interactions in native bile has yet to be demonstrated. Up to this point in time lysophospholipid molecules have been assumed to exist in bile salt solutions as monomers or dissolved within bile salt micelles containing ten or more bile salt monomers (Patton, 1981; Kratohvil et al., 1986). The existence of smaller aggregates of lysolipid and bile salts would effectively increase the concentration of lysolipid in the water phase (nonmicellar) available for absorption by the epithelial cells lining the small intestine.

The values for the cmc's of the *N*-NBD-lysoPE derivatives obtained in this study (Figure 1) were roughly 20-fold lower than those found previously for other native lysolipids of equivalent chain length but containing a choline headgroup (Stafford et al., 1989). The explanation for the difference in cmc value can be attributed to the addition of the hydrophobic fluorophore to the lysolipid (Nichols, 1985) and the removal of a positive charge by the attachment of the NBD group to the lysophospholipid headgroup (Chattopadhyay & London, 1988). The presence of the aromatic NBD moiety undoubtedly contributed additional hydrophobicity to the compounds, and therefore, one would expect the lower cmc value. Nonetheless, we observed the expected dependence of the cmc's on carbon chain length of the various *N*-NBD-lysoPE's; i.e., the value of the cmc decreased by an order of magnitude as the length of the hydrocarbon chain was increased by two methylene groups (Tanford, 1980). Therefore, while altering somewhat the concentration at which micelles were formed, the NBD group was not inducing anomalous micelle formation. Also, the pH at which the experiments were performed would maintain the majority of the PE in the anionic form, making it unlikely that there was substantial hydrogen bond mediated dimerization of the protonated and deprotonated species (Smith & Tanford, 1973).

The process of micelle formation by bile salt aggregation is one that has received much attention due to its physiological relevance (Kratohvil & DelliColli, 1968; Oakenfull & Fisher, 1977; Chang & Cardinal, 1978a,b; Roda et al., 1983; Kratohvil et al., 1983, 1986). Our experiments were carried out at concentrations of *N*-NBD-lysoPE's well below their respective cmc's in an attempt to limit our experiments to monomer–monomer interaction at the lowest bile salt concentrations. The concentrations of the various probes used (10–500 nM) were at least 5000-fold lower than the cmc's for each of the bile salts studied. Such low phospholipid concentrations are unlikely to have had any effect on the value of the bile salt cmc determined by light scattering inasmuch as we obtained values close to those obtained previously for purified bile salt solutions using similar or independent methods (Kratohvil et al., 1983; Roda et al., 1983). Nevertheless, the effect of bile salt contaminants on the self-aggregation of bile salts below their cmc has been documented (Kratohvil et al., 1983), and in our experiments the *N*-NBD-lysoPE could be effecting similar behavior of the bile salts. Therefore, in our experiments, it could be that the lysophospholipid serves a "seeding" function for the cooperative association of successive bile salts. The light scattering sensitivity would not be adequate, however, to detect the growth of this small number of complexes in the bile salt solution.

The maximal fluorescence obtained upon addition of bile salt to the *N*-NBD-lysoPE's of varying chain length always occurred at bile salt concentrations well below their respective cmc values. It is important to note also that the fluorescence did not increase further as the bile salt concentrations were raised above their cmc values. This finding was in sharp contrast to previous results obtained with fluorescent probes as monitors of bile salt aggregation (Paul et al., 1979; Fisher & Oakenfull, 1979; Narayanan et al., 1980). In fact, our results with the NBD-Cl probe unattached to the lipid moiety mimicked the results of these previous studies. The difference in the results obtained with our series of lysolipid probes undoubtedly lies primarily in the fact that the previous fluorescent probes did not have long methylene chains to facilitate hydrophobic interaction with the bile salts. One probe with a 10-carbon acyl chain [*N*-(10-aminodecyl)-5-(dimethylamino)-1-naphthalenesulfonamide] did in fact begin to show an indication of submicellar bile salt probe association (Narayanan et al., 1980).

In previous work from this laboratory, bile salts were shown to increase the rate of phospholipid transfer through the aqueous phase between phospholipid vesicles (Nichols, 1986) and between bile salt–phospholipid mixed micelles (Nichols, 1988). In the latter case a kinetic analysis was used to measure transfer through the aqueous phase between the micelles that was 200–6000 times faster than that between phospholipid vesicles in the absence of bile salt (Nichols, 1988; Fullington et al., 1990). Although no direct measurements have been made of the coexistence of lysolipid–bile salt heterodimers or heteromultimers with mixed micelles, the data in this study predict their existence. For example, given the aqueous intermicellar concentration (IMC) of TDC that exists in equilibrium with a 1:2 molar ratio of dioleoylphosphatidylcholine–TDC micelles (1.0 mM; Fullington et al., 1990), it is apparent by examination of the data in Figure 4A that virtually all the water-soluble *N*-NBD-PPE and *N*-NBD-SPE would be associated with one or more TDC molecules. The less hydrophobic probe, *N*-NBD-MPE, would be approximately 50% associated with TDC, and the least hydrophobic *N*-NBD-LPE would be 10% associated. These statements should be true for all *N*-NBD-lysoPE concentrations that are well below their cmc's inasmuch as varying the probe con-

centration 10-fold had no effect on the cooperative binding of cholate to *N*-NBD-MPE. Thus, these *N*-NBD-lysoPE–TDC aggregates would be expected to coexist with TDC–lysolipid mixed micelles. A parallel analysis yields the same conclusion for the interaction of cholate with the *N*-NBD-lysoPE's.

The evidence presented here demonstrates the existence of heterodimers or heteromultimers at bile salt and lysolipid concentrations predicted to coexist in the intermicellar water phase under physiological conditions in the intestine. Thus, one would predict that these submicellar aggregates facilitate the absorption of lysophospholipids by bile salts in the intestine. Since aqueous transfer as defined by kinetic analysis is also thought to be responsible for the absorption of other lipids in the intestine, similar lipid–bile salt complexes would also be expected to play a significant physiological role in facilitating the transfer of these lipids from micelle to enterocyte. However, the existence of these small lysolipid–bile salt aggregates in equilibrium with mixed micelles does not constitute proof that they will increase the rate of transfer of lysolipid from the micelles to the enterocyte membrane. If these aggregates are unable to interact with the micelles and/or cell surface and increase the rate of dissociation and/or association, they will not affect the net rate of lysolipid transfer. Additional studies will be required to determine whether the formation of these lysolipid–bile salt aggregates is involved in the facilitation of lipid absorption in the intestine.

Registry No. Sodium salts of TDC, 1180-95-6; sodium salts of cholate, 361-09-1.

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