Distribution of mixtures of bile salt taurine conjugates between lecithin-cholesterol vesicles and aqueous media: an empirical model

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Abstract Bile salts are surfactants that partition into phospholipid bilayers. When liposomes or membranes are exposed to mixed solutions of bile salts, the more hydrophobic bile salt species associate preferentially with the lipid bilayer. As a consequence, in the aqueous phase, the free monomeric concentration of bile salt declines and the more hydrophilic species become relatively enriched. Above a critical saturating concentration of lecithin-associated bile salt, a phase transition occurs with loss of membrane integrity and formation of mixed micelles. In this paper we present a quantitative model which, for mixed solutions of bile salt taurine conjugates, predicts the distribution of bile salt monomers between large unilamellar vesicles composed of lecithin and cholesterol and the aqueous phase. The model is based on association isotherms for individual bile salts, determined by an ultrafiltration method with empirical curve fitting, and is critically dependent upon the observation that association coefficients of each bile salt are a function of the total bound bile salt/lecithin mole ratio. Given the concentrations of individual bile salts, lecithin and cholesterol, the model permits calculation of the membrane-bound bile salt/lecithin ratio and the concentration of each bile salt remaining free as soluble monomer in the aqueous phase, as well as the overall hydrophilic–hydrophobic balance (hydrophobicity index) of the bile salts remaining free in aqueous solution. Distribution data determined empirically for a variety of mixtures of bile salt taurine conjugates and large unilamellar vesicles of varying cholesterol:lecithin ratio agree closely with predictions. This model may be of value in predicting the physical, biological and toxic properties of mixed bile salt solutions.—**Heuman, D. M.** Distribution of mixtures of bile salt taurine conjugates between lecithin-cholesterol vesicles and aqueous media: an empirical model. *J. Lipid Res.* 1997. **38:** 1217–1228.

Supplementary key words bile acids and salts • phospholipid • cholesterol • toxicity • mixed micelle

Bile salts are biological detergents critical for digestion of lipids. They also, by virtue of their surfactant effects, may disrupt cell membranes. Bile salts at monomeric concentrations can be shown to associate with model membranes by partitioning into the lipid bilayer. When the concentration of bile salts in a phospholipid bilayer reaches a critical saturating limit, a transition occurs, with loss of the barrier to permeability of water-soluble macromolecules (1–3). This transition appears to involve first formation of an intermediate hexagonal phase, followed by extraction of lipids into cylindrical, worm-like or rod-like mixed micelles (4–7). At concentrations below this threshold, bile salts can selectively increase membrane permeability to divalent cations (8), alter catalytic properties of membrane-associated enzymes (9), and alter membrane surface potential (10). These effects also may depend upon the bile salt:lecithin ratio in the lipid bilayer.

The affinity of bile salts for a phospholipid bilayer is a function of their hydrophilic–hydrophobic balance (11, 12). Bile salt hydrophilic–hydrophobic balance is determined by a number of factors, including the number of oxy groups on the sterol nucleus, the orientation of those groups, the state of conjugation and ionization of the side chain, and the presence or absence of additional nuclear substituents such as sulfate or glucuronate (13–15). Association of bile salts with the phospholipid bilayer is also influenced by the cholesterol content and specific phospholipid composition of the lamellar phase, as well as by the physical conditions in the aqueous phase such as ionic strength, acidity, specific ion composition and temperature (2, 3).

The nature of the association of bile salts with mem-

Abbreviations: C_i, overall concentration of bile salt (M); B_i, bound (lecithin-associated) concentration of bile salt i (moles bound bile salt per liter of solution); F_i, concentration of bile salt i free (non-lecithin-associated) in aqueous solution (M); L, lecithin concentration (M); A_i, association coefficient of bile salt i = $(B_i/F_i)/L$ (liters/mole); C:L, cholesterol:lecithin mole ratio; TUDC, ursodeoxycholyl taurine; TC, cholyl taurine; TCDC, chenodeoxycholyl taurine; TDC, deoxycholyl taurine; TLC, lithocholyl taurine.

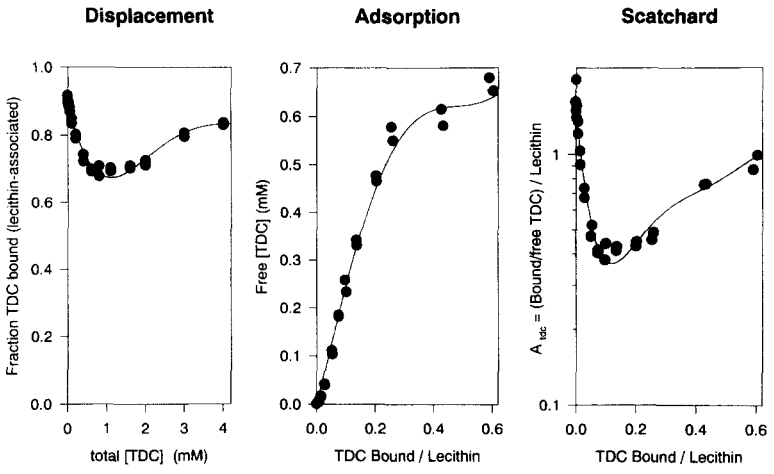

Fig. 1. A typical bile salt-membrane association isotherm. Shown are three different types of plot describing the binding of taurodeoxycholate to pure egg lecithin vesicles at [lecithin] = 0.0055 M in 0.14 M NaCl with 0.01 M Tris buffer, pH 7.4, 23°C. A) Displacement plot reveals fractional binding of TDC (B_{tdc}/C_{tdc}) to lecithin as a function of total TDC concentration C_{tdc}. B) Association plot reveals the concentration of TDC free in aqueous solution in the form of monomers and simple micelles (F_{tdc}) as a function of the ratio of bound TDC to lecithin in lecithin-containing phases (vesicles, mixed micelles). C) Scatchard plot shows the affinity of TDC for lecithin-containing phases (association coefficient A_{tdc} defined as ([bound TDC]/([free TDC])/[lecithin]) as a function of the lecithin-bound TDC concentration B_{tdc}/L. In each case, with accumulation of bile salts on vesicles, the association coefficient and fractional binding decline in a curvilinear manner. Above a critical concentration associated with a B_{tdc}/L value of approximately 0.1, transition to mixed micelles begins, and affinity coefficients thereafter increase progressively with increasing bile salt concentration.

branes remains incompletely understood. Schubert et al. (2) and Schubert and Schmidt (3) demonstrated that bile salt-membrane binding could be approximated using models of receptor-ligand interaction. In this formulation the nonlinearity of the resulting Scatchard plots is explained by assuming a single high affinity receptor configuration resulting from hydrophobic interaction of a bile salt with a cluster of lecithin molecules, superimposed on lower affinity or nonspecific binding. Although the thermodynamic soundness of this model has been questioned (1), it provides a good empirical description of the binding behavior observed. More recent theoretical studies such as those of Clarke (10) indicate that curves resembling multireceptor-ligand binding plots may be generated with partition of anionic surfactants into lipid bilayers, and that the distribution coefficients of the bile salts vary, in part, due to development of a charged boundary layer that repels further accumulation of the anionic surfactant. The question of how best to describe the interaction of ionic detergent molecules with lipid bilayers, whether as adsorption, ligand binding or partition, remains a controversial area of biophysical chemistry (16). In this paper we will use the terms "membrane association" and "membrane binding" interchangeably, without attempting to further dissect this difficult issue.

In previous studies we examined the association of bile salt taurine conjugates with large unilamellar vesicles (17). As illustrated in a typical bile salt distribution isotherm (**Fig. 1**), the affinity of bile salts for vesicles decreases exponentially with accumulation of bile salt on the membrane, reaching a plateau, then increasing with onset of membrane solubilization. The binding affinity of each bile salt was found to be a function of the total bound bile salt/lecithin ratio and was essentially independent of the specific bile salts accumulating on the membrane (17). This observation is consistent with recent findings of Fahey, Carey, and Donovan (18), which indicate that all of the common bile salts partition into phospholipid monolayers in a similar manner, floating as monomers near the lipid–water interface, long axis parallel to the plane of the bilayer, with their hydrated polar groups exposed to the aqueous medium and their non-polar groups interacting with the adjacent phospholipid acyl chains. Based on these findings, we have developed and validated an empirical model that accurately describes the distribution of mixtures of bile salt taurine conjugates between lecithin:cholesterol large unilamellar vesicles and the aqueous phase. This model may represent a novel means by which physical properties of bile salt-lecithin-cholesterol solutions can be estimated, and may provide new insights into the toxic and protective interactions of hydrophilic and hydrophobic bile salts with biliary lipids and cell membranes.

MATERIALS AND METHODS

Egg yolk lecithin (>99% phosphatidylcholine) and cholesterol were purchased from Sigma, St. Louis, MO and Avanti Polar Lipids, Alabaster, AL. They were dissolved in chloroform, stored in darkness at −20°C under nitrogen and used within 8 weeks of purchase. Mole weight of phosphatidylcholines averaged 730 as determined by the method of Bartlett (19). ^{14}C- and ^{3}H-labeled taurocholate and taurine were purchased from NEN-DuPont, Boston, MA. ^{14}C- and ^{3}H-labeled taurine

conjugates of ursodeoxycholate, chenodeoxycholate, deoxycholate, and lithocholate were synthesized from labeled taurine and free bile acids by a modification of the method of Tserng, Hackey, and Klein (20) and were purified to apparent homogeneity by thin-layer chromatography, using the solvent system chloroform–methanol–water–acetic acid 65:24:15:9. Purity was confirmed by high performance liquid chromatography using the method of Nakayama and Nakagaki (21). Unconjugated and conjugated bile acids were purchased from Calbiochem, La Jolla, CA. All unlabeled bile acids used were determined by gas–liquid chromatography to be >99% free of bile acid contaminants. In a limited number of experiments, we used the method of Del Vecchio et al. (22) to remove traces of surface active contaminants from each of the four commercial bile salt taurine conjugates. We found that this additional step had no effect on association of any of the bile salts with lecithin vesicles. Therefore, in the remaining studies, the commercial bile salts were used without further purification.

Large unilamellar vesicles of mean diameter 100 nm were prepared from cholesterol and egg lecithin by the method of Hope et al. (23). In some instances [^{14}C]l-palmitoyl-2-linoleoyl phosphatidylcholine or [^{3}H]cholesterol (NEN-DuPont, Boston MA) were included with the lipids. Lipids were dissolved in warm *tert*-butanol, lyophilized to dryness, and vigorously hydrated with vortex mixing in buffer A (0.14 M NaCl, 0.01 M Tris-HCl, 0.001 M sodium azide, pH 7.4). After five freeze-thaw cycles using liquid nitrogen, lipids were extruded under N_2 through paired 0.1-micron polycarbonate filters using an extrusion apparatus (Lipex Biomembranes, Vancouver, BC). Vesicles were used within 24 h of preparation. Advantages of this method of vesicle preparation for these experiments are *i*) greater than 90% of vesicles prepared by this method are unilamellar (23); thus for a given lipid concentration the membrane surface available to interact with bile salts is uniform; *ii*) the large size of the vesicles minimizes strain anomalies related to curvature, which are prominent in small unilamellar vesicles prepared by ultrasonication; *iii*) there is no possibility of residual contamination as in vesicles prepared by detergent dialysis methods; and *iv*) the rapidity of the method minimizes the risk of auto-oxidation of phospholipid and sterols.

Experimental design

Centricon 30 plastic ultrafiltration cartridges (Amicon, Waltham, MA.) were used. Previous studies have demonstrated that these filters effectively retain lecithin in the form of mixed micelles or vesicles, while allowing free passage of bile salt monomers and simple micelles with negligible binding of bile salts to the filter (17, 24). Radiolabeled bile salts in buffer A were added to the Centricon 30 filtration chamber along with known and constant amounts of vesicular lipids, and varying concentrations of unlabeled homologous and heterologous bile salts. Total volume was 1.0 ml. Samples were allowed to equilibrate at 23°C for 25 min. A 0.1-ml aliquot was obtained prior to filtration for determination of radioactivity. Samples were then subjected to gentle centrifugal ultrafiltration for 10 min at 23°C. The total ultrafiltrate volume averaged 0.1 to 0.2 ml. Aliquots of ultrafiltrate (0.1 ml) were obtained for determination of radioactivity.

Data analysis

Bile salt binding to nonfilterable particles (vesicles, hexagonal phase and mixed micelles) was quantified by comparing the concentration of unbound radiolabeled bile salts in the ultrafiltrate to the total concentration of radioactivity in the unfiltered sample. Consistent with the terminology of Donovan and Jackson (24), we refer to this bound fraction as lecithin-associated bile salt. Binding parameters in Scatchard analysis were determined for bound bile salt:lecithin ratios less than 0.1 by fitting data to a curve of the general form $A_i = a_1 \exp(-b_1 \times B_T/L) + a_2$, by means of the Marquardt-Levenberg algorithm using Sigmaplot for Windows 2.0 (Jandel Scientific, San Rafael, CA). The maximum bound bile salt:lecithin ratio of 0.1 was chosen for analysis of bile salt-membrane interactions because, above this value, a phase transition occurs and high affinity binding of bile salts to hexagonal phase and mixed micelles leads to an increase in the overall binding affinity (Fig. 1).

Theoretical considerations and modelling

The mathematical analysis of interaction of a single bile salt with lecithin vesicles has been discussed previously by Lichtenberg (1). In a solution containing lecithin and n different bile salts, each having different affinity constants, we will use C_i to indicate the overall molar concentration of bile salt i, B_i to represent the bound concentration of bile salt i (that is, bile salt adsorbed to lecithin), and F_i to indicate the concentration of bile salt i free in aqueous solution. Thus

$$C_i = B_i + F_i. \qquad Eq.\ 1)$$

In a solution containing n different bile salts and lecithin, the total lecithin-bound bile salt is given by

$$B_T = B_1 + B_2 + \cdots + B_n. \qquad Eq.\ 2)$$

The total bile salt free as monomer in solution is given by

$$F_T = F_1 + F_2 + \cdots + F_n \qquad Eq.\ 3)$$

and the overall concentration of bile salt in the system is

$$C_T = C_1 + C_2 + \cdots + C_n = B_T + F_T. \quad Eq.\ 4)$$

For each bile salt, we can define an association coefficient A_i as

$$A_i = (B_i/L)/F_i \quad Eq.\ 5)$$

where L indicates the molar concentration of lecithin (units of A_i = liters/mole). Empirically for each bile salt, A_i is observed to decrease progressively with accumulation of bile salts on the membrane. The value of A_i at very low bile salt concentrations is essentially a constant for a given set of physical conditions (membrane composition, temperature, ionic strength, pH); this limiting value of A_i we will term the association constant, K_i. A_i in previous studies was found to decline exponentially with increasing B_T/L but was essentially independent of the specific bile salt bound (17). Thus, for example, the decline in the association constant for taurocholate (A_{tc}) observed with accumulation of the heterologous ligands taurodeoxycholate or tauroursodeoxycholate on egg phosphatidylcholine large unilamellar vesicles was similar to that observed with accumulation of the homologous ligand taurocholate.

From equations 2 and 5 we determine that

$$B_T/L = \sum_{i=1}^{n} A_i F_i \quad Eq.\ 6)$$

Because direct measurement of free and bound bile salt concentrations is cumbersome, it is often preferable to express this key parameter in terms of the total concentration of each bile salt in the system. Thus

$$B_T/L = \sum_{i=1}^{n} C_i A_i/(A_i L + 1) \quad Eq.\ 7)$$

and

$$F_T = \sum_{i=1}^{n} C_i/(A_i L + 1). \quad Eq.\ 8)$$

Given the proportion of each bile salt in the overall solution (C_i/C_T), one can express the total concentration of bile salts (C_T) required to produce a specific value of B_T/L by the formula:

$$C_T = \frac{B_T/L}{\sum_{i=1}^{n} (C_i/C_T)\, A_i/(A_i L + 1)} \quad Eq.\ 9)$$

Under conditions in which bile salt concentrations are very low relative to lecithin concentration, K_i can be substituted for A_i. This permits direct calculation of the distribution of all bile salts between vesicles and the aqueous phase and their proportions in each phase. At higher concentrations, the interdependence of A_i and B_T/L must be taken into account.

The relationship between A_i and B_T/L must be determined empirically for each bile salt. In our studies, distribution of bile salts between vesicles and the aqueous medium has been quantified experimentally by ultrafiltration, and the resulting data have been approximated mathematically by non-linear curve fitting. Different formulas for data fitting may give very similar curves, despite the fact that they involve different assumptions and lead to different inferences regarding the nature of bile salt-membrane interaction. For the purpose of developing an empirical model suitable for predicting behavior of bile salts in the presence of lecithin and cholesterol, it is not necessary that the underlying mechanisms of interaction be known, provided that the formulas and parameters chosen for curve fitting accurately approximate the observed data. We have chosen to use the simplest formula that provides a good fit: a monoexponential of the form $A_i = a_1 \exp(-b_1 \times B_T/L) + a_2$, as used in previous studies of Schubert and associates (2, 3).

A complete mathematical solution to equations 7 and 8 is not straightforward. However, once the relationship between A_i and B_T/L is known for each bile salt and expressed numerically, one can closely approximate the solution by testing a range of possible values of B_T/L in equation 7. The method is as follows. The range of possible B_T/L values from 0 to 0.1 (the concentration of membrane bound bile salt associated with onset of membrane disruption in our earlier studies) is divided into small increments (typically 0.001 or less). For each incremental value of B_T/L, one calculates the expected A_i for each bile salt in the mixture. These expected values of A_i are then substituted in the right side of equation 7 along with C_i and L, and the expected binding of each bile salt B_i is calculated. The difference between the starting value chosen for B_T/L (left side of equation 7) and the calculated value of B_T/L based on the corresponding values of A_i (right side of equation 7) is determined. If the initial chosen value of B_T/L is not correct, then the starting value and the calculated value will not be equal. Conversely, the point at which the difference between these numbers is zero corresponds to the true B_T/L. Once B_T/L is known, it is a simple matter to calculate F_i and B_i for each bile salt in the mixture. In addition, the hydrophobicity index of bile salts in the aqueous phase can be calculated readily from individual F_i as previously described (13).[1] This approach has the ad-

[1]The bile salt monomeric hydrophobicity index (13) is a normalized weighted average of the logarithms of bile salt retention coeffi-

TABLE 1. Association parameters from homologous and heterologous bile salt binding studies, derived by non-linear curve fitting to association isotherms as described in the text

C:L	Bile Salt	a_1	b_1	a_2
0	TUDC	68	44	59
	TC	190	51	70
	TCDC	1200	34	340
	TDC	1600	54	420
	TLC	7300	73	3900
0.25	TUDC	46	43	27
	TC	110	33	48
	TCDC	660	30	180
	TDC	500	53	210
0.50	TUDC	25	75	31
	TC	66	70	36
	TCDC	440	53	150
	TDC	500	71	200
1.00	TUDC	25	41	35
	TC	49	74	32
	TCDC	260	59	97
	TDC	200	74	82

Conditions included ionic strength 0.15 M, pH 7.4, and temperature 23°C. Vesicles were composed of egg yolk lecithin (>99% phosphatidylcholine) and cholesterol at various cholesterol:lecithin mole ratios (C:L). Data were determined by ultrafiltration after 30 min equilibration. Some of the association plots on which these parameters were based have been published previously (17). Binding parameters were derived by non-linear fitting of data to equation in the form: $A_i = a_1 \exp(-b_1 B_T/L) + a_2$ (units = liter/mole).

vantage of being essentially independent of the formula used for curve fitting. The calculations can be performed easily using a simple personal computer spreadsheet program. By repeating the calculations for different of values of C_T or L, one can examine the dependence of various parameters on these variables.

For example, consider a solution of TUDC and TCDC, each 100 μM, with 1 mM lecithin. From **Table 1,** values of a_1, b_1, and a_2 for TUDC are 68, 44, and 59; for TCDC, 1220, 34, and 340, respectively. If we assume a value for B_T/L of 0.01, we calculate values for A_{tudc} and A_{tcdc} of 103 and 1206. From equation 7, B_T/L is then calculated to be 0.064 (B_{tudc} and B_{tcdc} from equation 5 are 9 and 55 μM, respectively). This exceeds the assumed initial value of 0.01 by 0.054 and therefore is not the correct value. As the process is repeated while increasing B_T/L incrementally, the difference between the initial and calculated values of B_T/L decreases. At $B_T/L = 0.0444$, calculated values for A_{tudc} and A_{tcdc} are 68 and 610, yielding calculated B_{tudc} and B_{tcdc} of 6.4 and 38 and a value for B_T/L of 0.0444. This point, at which the initial and calculated values of B_T/L are identical, represents the true value of B_T/L. Finally, the free concentrations of the bile acids, F_{tudc} and F_{tcdc}, can be determined to be 94 and 62 μM, respectively, and from these the hydrophobicity index of the free bile salts in the aqueous phase is calculated to be −0.10.

cients determined using reverse phase HPLC. It reflects the mean hydrophilic–hydrophobic balance of bile salts in a mixed solution. It is calculated by the formula:

$$HI = \sum_{x=1}^{n} HI_x P_x$$

where HI_x is the hydrophobicity index and P_x is the proportion (mole fraction) of bile salt x in a mixture of n different bile salts. Values of HI_x for TUDC, TC, TCDC, TDC, and TLC are, respectively, −0.47, 0.0, +0.56, +0.69, and 1.00.

RESULTS

Parameters and Scatchard curves obtained by non-linear curve fitting to homologous and heterologous binding isotherms for taurine conjugates of ursodeoxycholate, cholate, chenodeoxycholate, deoxycholate, and lithocholate are shown in Table 1 and **Fig. 2.** Conditions included pH 7.4, ionic strength 0.15 M, and temperature 23°C. Association isotherms were defined for each of the bile salts using vesicles containing cholesterol and phosphatidylcholine at mole ratios of 0, 0.25, 0.5, or 1.0. From these studies it is evident that the effect of cholesterol on binding parameters is approximately log linear. Thus, for cholesterol:lecithin ratios other than those shown, association isotherms can be approximated by log linear interpolation of the individual parameters.

The ability of this model to predict the distribution of bile salts between vesicles and aqueous media was tested as shown in **Figs. 3 through 7.** We performed a series of studies using TUDC, TC, TCDC, and TDC in various combinations and proportions and large unilamellar vesicles having cholesterol:lecithin ratios of 0 or 0.5. In these studies we allowed the lecithin concentration to vary while holding constant the individual bile salt concentrations and the cholesterol:lecithin ratio. Total bile salt concentration in each study was 200 μM; lecithin concentration was increased progressively from 0 to 10 mM. For each experiment we have plotted fractional binding of each bile salt to lecithin (B_i/C_i), the total concentration of bile salts remaining free in the aqueous phase (F_T), the hydrophobicity index of free bile salts as defined by us previously (13), and the ratio of total bound bile salts to lecithin (B_T/L), all as a function of lecithin concentration. Concentrations were chosen such that the total B_T/L would not exceed 0.1. As shown, there was good agreement between predicted and measured results.

Several facts are apparent from examination of these figures. For a given total concentration C_T and individual concentrations C_i of a mixture of bile salts, B_T/L is maximal and association coefficients are minimal when

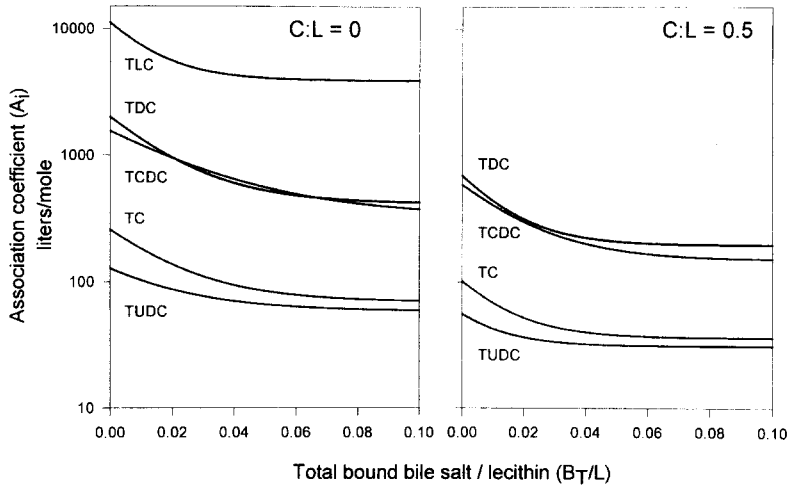

Fig. 2. Dependence of association coefficient on B_T/L. Idealized Scatchard plots for five different bile salt taurine conjugates at cholesterol:lecithin mole ratios (C:L) of 0 (left) or 0.5 (right). Curves were generated from association parameters in Table 1 and are shown on a logarithmic plot for comparative purposes. Note that the current model is confined to $B_T/L < 0.1$; above this value, increases in apparent binding affinity (A_i) reflect membrane disruption and formation of hexagonal and mixed micellar phases.

lecithin concentration is near zero. As the lecithin concentration is increased, distribution of bile salts into membranes causes the free bile salt concentration to fall progressively. The declining free bile salt concentration causes B_T/L, with which it is at equilibrium, to decline, and association coefficients therefore rise. If multiple bile salts of differing hydrophobicity are present, the fractional binding of the more hydrophobic bile salts is much greater than that of the more hydrophilic bile salts. Thus there is a progressive increase in the proportion of hydrophilic bile salts remaining free as soluble monomers in the aqueous phase, and as a consequence, the hydrophobicity index of bile salt monomers in solution declines progressively as the lecithin concentration increases. Conversely, at low lecithin concentrations, the hydrophobic species are highly en-

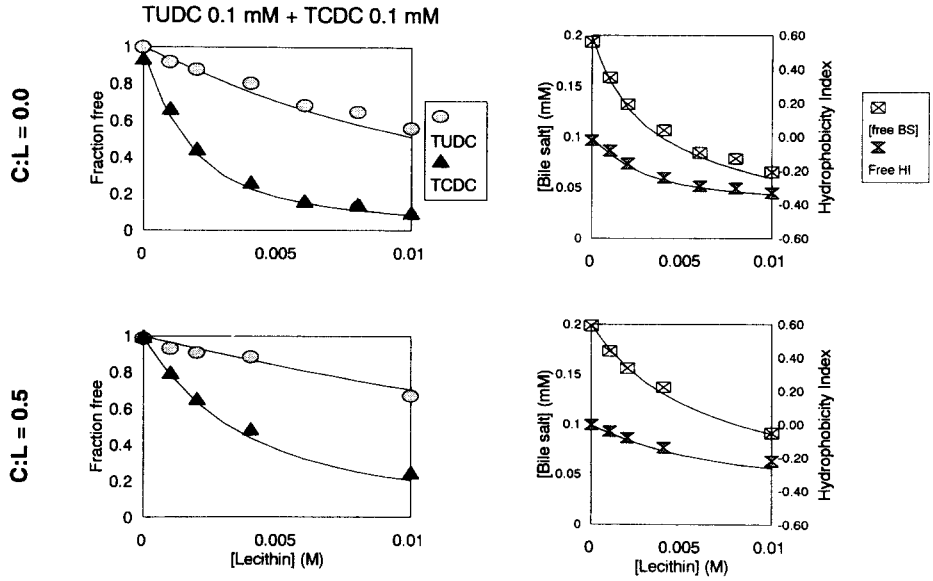

Fig. 3. Validation of the computer model of bile salt:vesicle binding. Bile salt proportions used = TUDC:TCDC 1:1. Lines indicate predictions of the model; symbols signify individual experimental observations. Figures show the effect of allowing lecithin concentration to vary, while holding constant the individual and total bile salt concentrations (C_i and C_T) and the cholesterol:lecithin ratio. Conditions included total bile salt concentration C_T = 0.2 mM, 0.14 M NaCl with 0.01 M Tris, pH 7 4, 23°C. In each group of four the graphs on the left indicate concentrations of individual bile salts remaining free (non-lecithin associated) in aqueous solution as a function of increasing lecithin concentration; the graphs on the right show the total free bile salt concentration and the overall hydrophobicity index of bile salts remaining free in aqueous solution. Upper graphs in each group of four were obtained in the absence of cholesterol; lower graphs were obtained at a cholesterol:lecithin mole ratio (C:L) of 0.5.

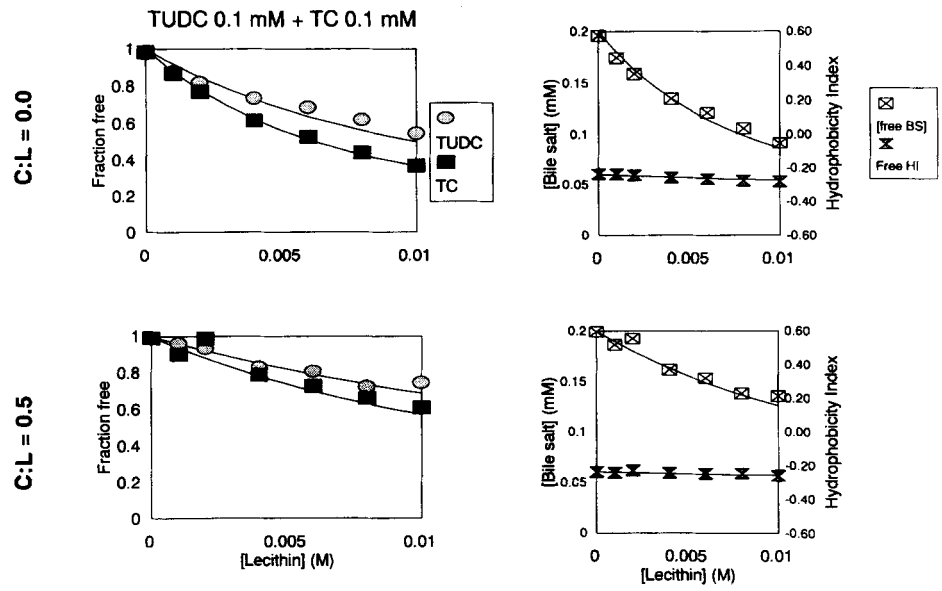

Fig. 4. Validation of the computer model of bile salt:vesicle binding. Bile salt proportions used = TUDC:TC 1:1. Other conditions are as described in legend to Fig. 3.

riched in membranes as compared to the hydrophilic species. As lecithin concentrations increase to very high levels, the majority of all bile salts become membrane bound, B_i approaches C_i, and the proportions of different bile salts in the membrane come to approximate their overall proportions in the mixture.

The magnitude of these effects can be dramatic. For example, from the data shown in Fig. 3, in an equimolar mixture of tauroursodeoxycholate and taurochenodeoxycholate, each C_i = 100 μM, addition of 2 mM lecithin decreases the free concentration of TCDC to 42 μM while decreasing free TUDC only to 87 μM; thus the mole ratio of TUDC:TCDC free as monomers in the aqueous phase increases from 1 to greater than 2. Cor-

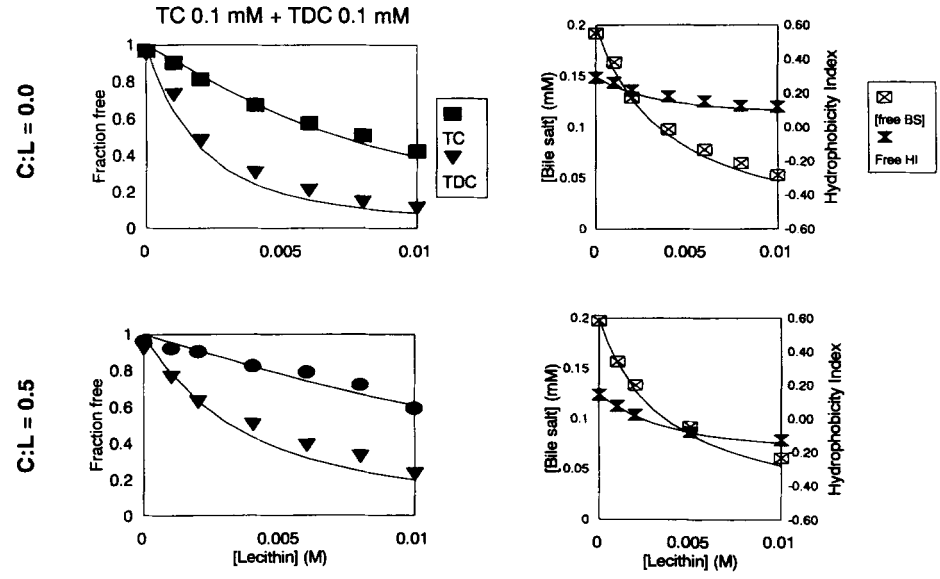

Fig. 5. Validation of the computer model of bile salt:vesicle binding. Bile salt proportions used = TC:TDC 1:1. Other conditions are as described in legend to Fig. 3.

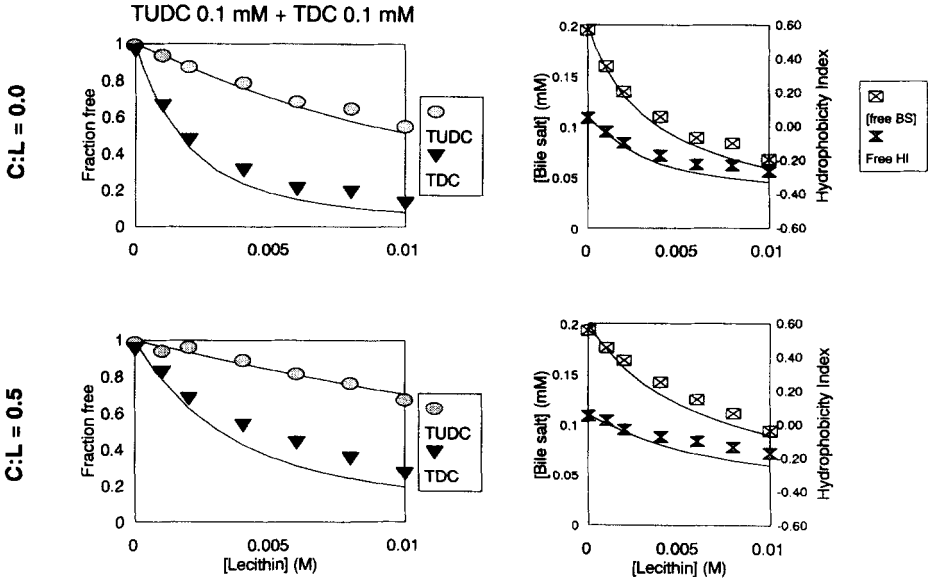

Fig. 6. Validation of computer model of bile salt:vesicle binding. Bile salt proportions used = TUDC:TDC 1:1. Other conditions are as described in legend to Fig. 3.

respondingly, there is a marked decline in the hydrophobicity index of non-lecithin-associated aqueous bile salts from −0.005 to −0.17. Further increasing L to 10 mM causes TUDC to decline further to 51 μM while TDC falls to 8 μM, a mole ratio of aqueous TUDC:TCDC of 6.4, corresponding to a hydrophobicity index of −0.34. TCDC represents a disproportionate fraction of the lecithin-bound bile salt; this disproportion is greatest at lecithin concentrations near zero, where 89% of the lecithin-associated bound bile salt is TCDC, falling to 82% when L = 2 mM and 65% when L = 10 mM. B_T/L decreases from 0.058 to 0.036 to 0.014 as [L] is increased progressively from 1 nM to 2 mM to 10 mM.

At very low lecithin concentrations the quantitative effect of binding on free bile salt concentrations is negligible and $C_i \rightarrow F_i$. Thus if we set [L] in our model to very low values (here 10^{-12} M), we can determine the relationship between B_T/L and free bile salt concentra-

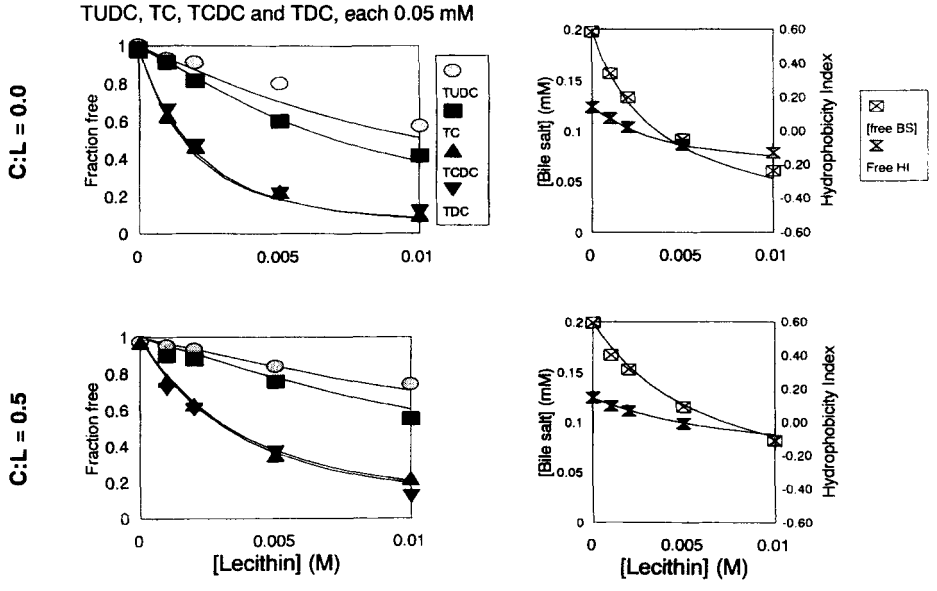

Fig. 7. Validation of computer model of bile salt:vesicle binding. Bile salt proportions used = TUDC:TC:TCDC:TDC 1:1:1:1. Other conditions are as described in legend to Fig. 3.

TABLE 2. Predicted total bile salt concentrations C_T associated with bound bile salt/lecithin mole ratio (B_T/L) of 0.1, under conditions of temperature 23°C, NaCl 0.14 M, Tris 0.01 M, pH 7.4

C:L	Bile Salts and Ratios	Predicted Total Bile Salt Concentration C_T (μM) to Yield $B_T/L = 0.1$		
		A: [L] = 10^{-12} M	B: [L] = 2×10^{-3} M	C: [L] = 10×10^{-3} M
0	TUDC	1670	1870	2670
	TC	1410	1610	2410
	TCDC	260	460	1260
	TDC	230	430	1230
	TLC	25	220	1020
	TDC + TUDC, 1:1	410	700	1690
	TDC + TUDC, 1:3	660	1020	2070
	TC + TCDC, 1:1	440	720	1660
	TUDC + TC + TCDC + TDC, 1:1:1:1	430	710	1670
0.5	TUDC	3220	3420	4220
	TC	2770	2970	3770
	TCDC	660	860	1660
	TDC	510	710	1510
	TLC	77	280	1080
	TDC + TUDC, 1:1	880	1180	2220
	TDC + TUDC, 1:3	1380	1750	2910
	TC + TCDC, 1:1	1060	1330	2300
	TUDC + TC + TCDC + TDC, 1:1:1:1	960	1250	2260

At very low lecithin concentrations, the mass of bound bile sale is negligible and C_T and C_i are equivalent to F_T and F_i, respectively.

tions. This may be a particularly useful approach when exploring the interactions of bile salts with multiple membranes of differing composition, especially when the area of membrane available to interact with bile salt is not well defined (for example, in a living cell).

If a specific concentration of membrane-bound bile salt is associated with a specific biological, physical, or physiological effect, then the model also can be used to estimate the total bile salt concentration C_T associated with this critical B_T/L ratio, provided that the concentrations of bile salts, lecithin, and cholesterol are known. This may be an extremely important application of the model. An example of this application is shown in **Table 2,** in which we have predicted the concentrations of bile salts required to produce $B_T/L = 0.1$ (a value that may correspond to the threshold for disruption of the membrane permeability barrier (17, 25, 26)). As demonstrated in column A, setting [L] = 1 picomolar, the model predicts that for solutions of a single bile salt, the free concentration of TUDC (F_{tudc}) required to produce $B_T/L = 0.1$ is 1.44 mM, whereas with TC, TCDC, or TDC this value of B_T/L will occur at 1.25, 0.26, or 0.23 respectively. Because binding of bile salts to lecithin bilayers lowers the free bile salt concentration and renders the mixture of free bile salts remaining in the aqueous phase more hydrophilic, the concentration C_T of a mixture of bile salts of defined proportions C_i/C_T that is required to produce $B_T/L = 0.1$ will increase as lipid concentration increases, and this effect will be most dramatic for the more hydrophobic bile salts. For example, for an equimolar mixture of TUDC and TDC, the total bile salt concentration required to produce $B_T/L = 0.1$ is predicted to increase 4-fold from 410 μM to 704 μM to 1688 μM as [L] is increased progressively from 1 pM to 2 mM to 10 mM. For TUDC alone the corresponding values are 1672, 1872, and 2672 μM, an increase of only 1.6-fold; in contrast for TDC alone, the corresponding values are 233, 433, and 1233, an increase of 5.3-fold.

DISCUSSION

In this paper we present a novel approach by which the distribution of mixtures of bile salts between membranes and aqueous media can be determined mathematically. The approach uses bile salt-membrane association isotherms determined using rapid ultrafiltration and analyzed using non-linear curve fitting, as described previously by Schubert and colleagues (2, 3). The subsequent development of an empirical model was made possible by our observation that, as ionized bile salts accumulate on a lipid bilayer, the resulting decline in bile salt association coefficients is a function of the total bound bile salt/lecithin mole ratio, but is essentially independent of the specific bile salts present on the membrane (17). Fractional binding data for various bile salt mixtures determined empirically show good agreement with the predictions of the model.

We anticipate that this approach to empirical modeling of bile salt-membrane interaction may be useful

both conceptually and practically. To the extent that a specific physicochemical or biological effect of bile salts is attributable to the total density of bile salts in the lipid bilayer, this model would be expected to predict how that specific effect will vary with variations in the proportions and concentrations of different bile salts, lecithin, and cholesterol. For example, it is well known that addition of lecithin to bile salt solutions protects against cytotoxicity (27–30). In vitro, the fractional disruption of model membranes (liposomes) exposed to bile salt solutions declines with increasing concentrations of liposomal lipid (25). We and others (17, 25, 26, 31) noted that loss of the liposomal permeability barrier occurs when B_T/L exceeds approximately 0.1 (0.2 in the outer leaflet, assuming bile salts below this threshold do not significantly cross the membrane over the 30-min time course of these studies). If this is a general phenomenon, then for any mixture of bile salt taurine conjugates, we would predict that C_T values obtained by setting $B_T/L = 0.1$ in the model should correspond to the threshold of vesicle disruption for that mixture of bile salts. Furthermore, we would expect this C_T will decline predictably with increasing lecithin concentration and increasing cholesterol:lecithin ratio, as illustrated in Table 2. Studies to test this hypothesis currently are in progress.

The association of bile salts with phospholipids may introduce important artefacts in many types of experiments, leading to significant methodological errors. For example, in studies of bile salt transport, parameters such as K_M typically are expressed as functions of the total bile salt concentration. This approach generally assumes that the total bile salt concentration and bile salt monomer activity in aqueous solution are similar. As our model clearly shows, in the presence of phospholipids, this assumption is invalid for any of the common bile salts and is particularly misleading for the more hydrophobic species. For example, in the presence of 10 mM lecithin, a total TDC concentration of 100 μM is associated with a free TDC concentration of only 7 μM. As it is the free aqueous bile salt monomer which is at equilibrium with binding sites on transport or receptor proteins, failure to appreciate the magnitude of the association of bile salts with lecithin and other membrane lipids may lead to systematic underestimation of the affinity of a hydrophobic bile salt for transport proteins or other receptors.

Within a cell, bile salts may adsorb to a variety of membranes (plasma membranes, nuclear envelope, endoplasmic reticulum, Golgi, mitochondria) containing different lipid and protein constituents. Many toxic and physiological effects of bile salts may result from concentration of bile salts in critical membrane microenvironments. Physiological evidence for partition of bile salts into intracellular membranes comes from the studies of Crawford et al. (32). These investigators found that the rapidity with which bile salts traverse the liver from sinusoid to canaliculus is inversely proportional to their relative hydrophobicity, indicating that bile salts within the living hepatocyte distribute into hydrophobic lipid environments. Many physiological properties of bile salts are quantitatively proportional to their relative hydrophobicity (33–36), suggesting that interaction of bile salts with hydrophobic environments such as membranes may be a key determinant of their biological activity.

Biological membranes contain a variety of phospholipids other than lecithin, and in addition are rich in proteins. The effect of these components on bile salt partition into the lipid bilayer and on the disruptive effects of bile salts has not been studied systematically. Schubert and Schmidt (3) found that substitution of phosphatidylethanolamine or phosphatidylserine for phosphatidylcholine in vesicles reduced adsorption of cholic acid, while sphingomyelin had only a small effect. The effects of membrane proteins have not been explored. Although further studies clearly are needed, it may be possible using the approach outlined here to establish in vitro binding isotherms that quantify the relationship between the monomer activities of individual bile salts in the cytoplasm and the concentrations of bile salts in various membranes within a cell. An understanding of this relationship clearly will be of value in elucidating the regulatory and toxic effects of bile salts on cellular processes.

Our model has been developed using only common taurine-conjugated bile salts, which have pK_as on the order of zero and remain fully ionized at all biologically relevant pH values. The binding of glycine conjugated and unconjugated bile salts to membranes is more complex because their pK_as are higher, averaging 3.9 and 5.0, respectively (37). As a consequence, significant amounts of the nonionic acid species may be present even at neutral pH. The acid species is much more hydrophobic than the corresponding salt, less water-soluble, and exhibits much higher affinity for membranes, but unlike the ionized species its partition into membranes does not alter membrane surface electrical potential. Preliminary studies with cholic acid and its taurine and glycine conjugates indicate that at pH 7.4 or above, association coefficients and binding isotherms of all three forms are similar, but that marked deviation is observed as pH is reduced further (D.M. Heuman, unpublished data). Even at neutral pH, a significant fraction of unconjugated bile salt that binds to membranes is in the protonated acid form. It is well known that non-ionized bile acids are poor detergents, and it is likely that unconjugated bile acids can accumulate in membranes to relatively high concentrations without causing mixed micelles to form. This fact may help to

explain why higher bound bile salt/lecithin ratios have been required to trigger mixed micelle formation in previous studies using unconjugated bile salts (2, 38). More extensive studies, therefore, are required before this binding model can be generalized to non-taurine-conjugated bile salts.

While this model should apply for most of the common bile salt taurine conjugates, some modifications of bile salt side chain or nucleus may be expected to cause significant deviations. Bulky polar substituents such as glucuronate or sulfate esterified to the bile salt nucleus would be expected to attenuate membrane binding and dramatically alter physical properties, as confirmed by Donovan, Yousef, and Carey (14). This should be kept in mind in interpreting studies that use bile salt analogs to study bile salt-membrane interactions (39, 40). In addition, bile salts that lack 3-oxo substituents may insert into membranes oriented perpendicularly rather than parallel to the plane of the bilayer, and such a difference in orientation would be expected to alter the effects of the bile salt on membrane condensation.

In summary, we have developed and validated a quantitative model that empirically describes the association of mixtures of bile salt taurine conjugates with model membranes composed of cholesterol and lecithin. We hypothesize that many of the physical and biological properties of mixed bile salt solutions may be predicted using this model. Future studies are needed to generalize the model to a broader spectrum of bile salts and lipids, to extend its predictions beyond the threshold concentration associated with lipid bilayer disruption, and to evaluate its applicability to elucidation of the toxic and physiological properties of bile salts in living systems.

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