Adsorption of mixtures of bile salt taurine conjugates to lecithin-cholesterol membranes: implications for bile salt toxicity and cytoprotection

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Abstract Tauroursodeoxycholate (TUDC), a relatively hydrophilic bile salt, reduces disruption of cholesterol-rich membranes by more hydrophobic bile salts such as taurocholate (TC), taurochenodeoxycholate (TCDC), or taurodeoxycholate (TDC). We examined the interactions of these bile salts in adsorption to large unilamellar vesicles to determine whether TUDC may stabilize membranes by preventing adsorption of more toxic bile salts. Fractional adsorption was quantified by rapid ultrafiltration. Adsorption coefficient A_i was defined for each bile salt i as ([bound i]/[free i])/[lecithin]. Affinity of different bile salts for lecithin vesicles varied with their relative hydrophobicity, increasing in the order TUDC<TC<<TCDC≤TDC. A_i of each bile salt fell with its accumulation on membranes, reaching a minimum at bound bile salt/lecithin mole ratio (B:L) between 0.05 and 0.1, then increasing with formation of higher-affinity mixed micelles. Inclusion of cholesterol in vesicles reduced A_i of all bile salts. In heterologous binding studies at submicellar concentrations, A_i of each bile salt varied with total B:L but was independent of the specific bile salts present on the membrane. Addition of TUDC to TDC reduced binding of TDC to membranes only slightly and lowered the threshold TDC concentration associated with transition to mixed micelles. However, above this threshold, TUDC markedly altered the adsorption of TDC to lecithin-containing phases. We conclude that TUDC does not directly stabilize membranes; rather, reduced permeabilization and dissolution of cholesterol-rich membranes after addition of TUDC to TDC may result from effects on the formation and structure of simple and mixed micelles.—**Heuman, D. M., R. S. Bajaj, and Q. Lin.** Adsorption of mixtures of bile salt taurine conjugates to lecithin-cholesterol membranes: implications for bile salt toxicity and cytoprotection. *J. Lipid Res.* 1996. **37:** 562–573.

Supplementary key words vesicle • micelle • detergent • membrane • permeability • bile salt toxicity • ursodeoxycholic acid • cholestasis

Bile salts are sterol detergents that solubilize phospholipid and cholesterol in bile and products of fat digestion in the small intestine. Bile salts at high concentrations can damage cell membranes by dissolving membrane lipids and causing dissociation of integral membrane proteins (1–5). At submicellar concentrations, bile salts may disrupt membrane integrity through more subtle effects on membrane permeability and fluidity (6–8), including increased transmembrane flux of divalent cations (9–11) and altered activity of critical membrane-bound enzymes (12, 13).

Previous investigators who have examined the adsorption of bile salts to membranes (14–17) showed that binding affinity falls as bile salt accumulates on a membrane, reaching a minimum, then increasing as membranes first become permeable, then dissolve. The minimum adsorption affinity is associated with a critical mole ratio of bound bile salt to lecithin (B:L) on the order of 0.1. Adsorption affinity varies with different bile salts and falls with inclusion of cholesterol in the membrane bilayer. Most previous investigations of bile salt-membrane adsorption have used single homogeneous bile acids, generally unconjugated, and the interactions of different bile salts in membrane disruption have not been examined.

Recently we showed that taurine conjugates of certain hydrophilic bile salts such as ursodeoxycholate, which are poor detergents, may protect cholesterol:lecithin membranes against disruption by more hydrophobic bile salts (18). This finding may have important therapeutic implications because ursodeoxycholic acid has been found to protect against liver injury in a variety of cholestatic disorders, possibly by attenuating the toxicity of endogenous hydrophobic bile salts (19, 20). The

Abbreviations: TUDC, tauroursodeoxycholate (ursodeoxycholyl taurine); TC, taurocholate (cholyl taurine); TCDC, taurochenodeoxycholate (chenodeoxycholyl taurine); TDC, taurodeoxycholate (deoxycholyl taurine); C:L, cholesterol:lecithin mole ratio; B:L, bound bile salt:lecithin mole ratio; A_i, adsorption coefficient of bile salt i = ([bound i]/[free i])/[lecithin].

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mechanism of this protective effect is unknown, but several investigators have suggested that ursodeoxycholate and its conjugates may stabilize membranes against detergent disruption (7, 21).

In the current studies, we have examined the adsorption of radiolabeled hydrophobic and hydrophilic bile salt taurine conjugates to lecithin membranes at both pre-micellar and micellar concentrations, using rapid ultrafiltration. Our findings indicate that adsorption of individual bile salt taurine conjugates to membranes is a function of their hydrophilic–hydrophobic balance and, in mixed bile salt solutions, is predictably related to the ratio of total bound bile salt to lecithin in the membrane bilayer. TUDC, a relatively hydrophilic bile salt, had low affinity for membranes and had little effect on binding of TDC to intact membranes at submicellar concentrations. In contrast, at higher TDC concentrations, TUDC produced marked changes in adsorption of TDC to lecithin-containing phases (membranes and mixed micelles). We hypothesize that the protective action of TUDC results from effects on the formation and composition of mixed micelles.

MATERIALS AND METHODS

Materials

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 (22). ^{14}C- and ^{3}H-labeled taurocholate and taurine were purchased from NEN-DuPont, Boston, MA. ^{14}C- and ^{3}H-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, Hachey, and Klein (23) 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 (24). 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 and 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. (25). In some instances [^{14}C]1-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 (25); 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 autooxidation of phospholipid and sterols.

Experimental design

Centricon 30 plastic ultrafiltration cartridges (Amicon, Waltham, MA) were used. 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 for varying times (usually 30 min) at 23 degrees centigrade. A 0.1-ml aliquot was obtained prior to filtration for determination of radioactivity. Samples were then subjected to gentle centrifugal ultrafiltration for 10 minutes at 23°C, and 0.1-ml aliquots of ultrafiltrate were obtained for determination of radioactivity. In preliminary studies, we noted a small amount of nonspecific adsorption of bile salts to the plastic ultrafiltration chambers (less than 10%). This was apparent only at very low bile salt concentrations (less than 10 micromolar) and was eliminated by pre-rinsing the filtrate receptacle with a high bile acid concentration (100 mM deoxycholate in buffer A), and by determining the pre-filtration bile salt concentration after equilibration of the solution with the cartridge and membrane. Also, in preliminary experiments, bile salt adsorption to membranes was noted to be rapid and bound fraction did not change between 15 and 240 min; therefore, subsequent experiments were performed after 30 min incubation.

Data analysis

Bile salt binding to nonfilterable particles (vesicles 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 (26), we refer to this bound fraction as lecithin-associated bile salt. Corrections for Gibbs-Donnan effects, as described by Donovan and Jackson (26), were not required as these effects are negligible at the low bound bile acid concentrations used in these studies (less than 5 millimolar). Binding data have been presented in three different forms: displacement plots (total bile acid concentration vs. fraction bound), Scatchard plots (concentration bound vs. bound/unbound ratio, each normalized for the lecithin concentration), and affinity plots (unbound bile acid concentration vs. bile salt/lecithin ratio in the lecithin-associated phases). Binding parameters in Scatchard analysis were determined by fitting data to a curve of the general form $A_i = \times \exp(-y\,B/L) + z$ by means of the Marquardt-Levenberg algorithm using Sigmaplot for Windows 2.0 (Jandel Scientific, San Rafael, CA).

RESULTS

In the absence of vesicles, concentrations of labeled bile salts were unaffected by filtration over the range of concentrations up to 10 mM. This shows that neither bile salt monomers nor simple bile salt micelles were retained by the ultrafiltration membranes, and that nonspecific adsorption of bile acids to the ultrafiltration membranes was negligible, consistent with the findings of Donovan et al. (26). When large unilamellar vesicles using labeled lecithin and/or cholesterol were subjected to centrifugal ultrafiltration, neither lecithin nor cholesterol was detected in the filtrate, indicating that vesicles were retained completely. Addition of bile salts at concentrations that caused partial dissolution of vesicles also caused no labeled lecithin or cholesterol to appear in the filtrate; thus, large mixed micelles also were retained completely over the range of bile acid concentrations used.

After addition of vesicles, bile salt concentrations in the filtrate dropped significantly relative to the unfiltered samples, indicating adsorption to the membranes. Displacement plots indicating the fractional binding of four bile salt taurine conjugates to large unilamellar vesicles of egg lecithin ([L] = 5.6 mM) as a function of bile salt concentration are shown in **Fig. 1.** In each case as bile salts accumulated on the membrane, the fractional binding decreased, reaching a minimum before again increasing. Fractional binding of each bile salt studied was reduced in a concentration-dependent manner by inclusion of cholesterol in vesicles.

The same data can be represented by Scatchard plots as shown in **Fig. 2.** In this type of graph, the horizontal axis displays the ratio of bile salt to lecithin in lecithin-containing phases (vesicles and mixed micelles). The vertical axis displays the ratio (Bound BA/Free BA)/Lecithin, which is the adsorption coefficient A_i of the bile salt i for the membrane (units = $mole^{-3}$). The value of this coefficient extrapolated to bound bile salt/lecithin mole ratio of zero is a measure of the intrinsic affinity of the bile salt for the membrane, which we will designate by the affinity constant K_i. A graph of this form indicates how binding affinity declines as bile salt accumulates on the membrane, and can be analyzed mathematically to determine binding parameters. At a bile salt-lecithin ratio of between 0.05 and 0.1, a minimum is reached, above which affinity again increases. The reasons for this transition include increasing membrane permeability to bile salts (thus allowing interaction with both inner and outer leaflets) and formation of higher affinity hexagonal and mixed micellar phases. The B:L ratio associated with minimum value of A_i we

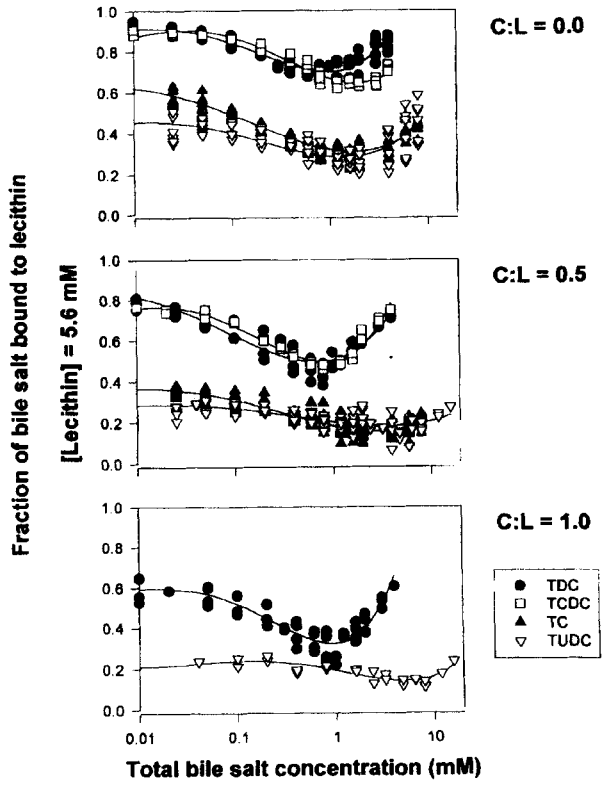

Fig. 1. Adsorption of four bile salt taurine conjugates to lecithin. The vertical axis indicates proportion of bile salt that is present in non-filterable, lecithin-associated forms (vesicles and/or mixed micelles); the total bile salt concentration (mM) is shown on the horizontal axis. Studies shown were performed at a lecithin concentration of 5.6 mM at cholesterol:lecithin mole ratios of 0.0 (top), 0.5 (center), or 1.0 (bottom). Other conditions included ionic strength 0.15 M (exclusive of added bile salts), pH 7.4, 23°C. TUDC, tauroursodeoxycholate; TC, taurocholate; TCDC, taurochenodeoxycholate; TDC, taurodeoxycholate. Curves shown were fitted by fourth order linear regression.

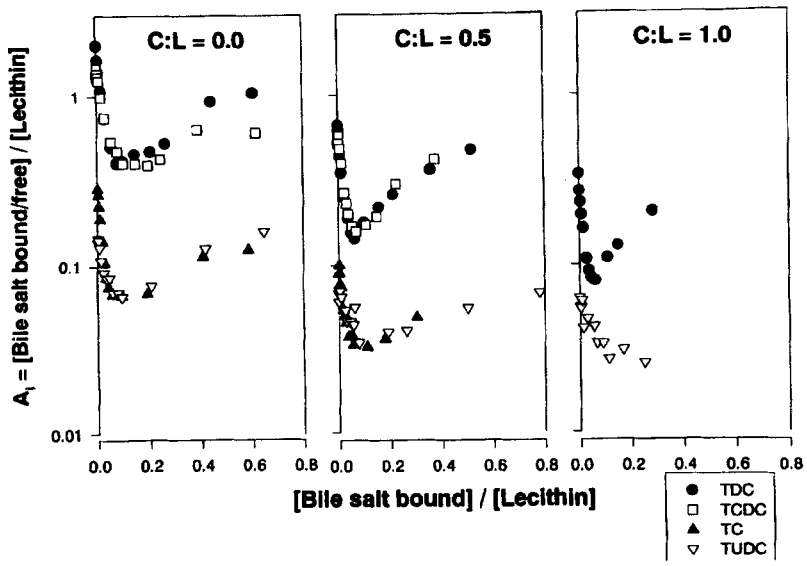

Fig. 2. Data from studies summarized in Fig. 1 have been displayed here as Scatchard plots; individual points are means of 2–8 determinations. The vertical axis shows the adsorption coefficient A_i, defined as the ratio of the bound (lecithin-associated) to free (non-lecithin-associated) bile salt fractions divided by the lecithin concentration (units mmole^{-1}). The horizontal axis indicates the mole ratio of bile salt to lecithin in the non-filterable, lecithin-associated form (membranes and mixed micelles). Conditions and abbreviations are as described in Fig. 1.

will refer to as the transition threshold. Unlike the displacement plots shown in Fig. 1, Scatchard plots for relatively dilute lipid systems at bile salt concentrations below the micellar threshold are essentially independent of the lecithin concentration used.

An adsorption isotherm plot of the same data is shown in **Fig. 3.** Here the free, non-lecithin-associated bile acid concentration is shown on the horizontal axis and the

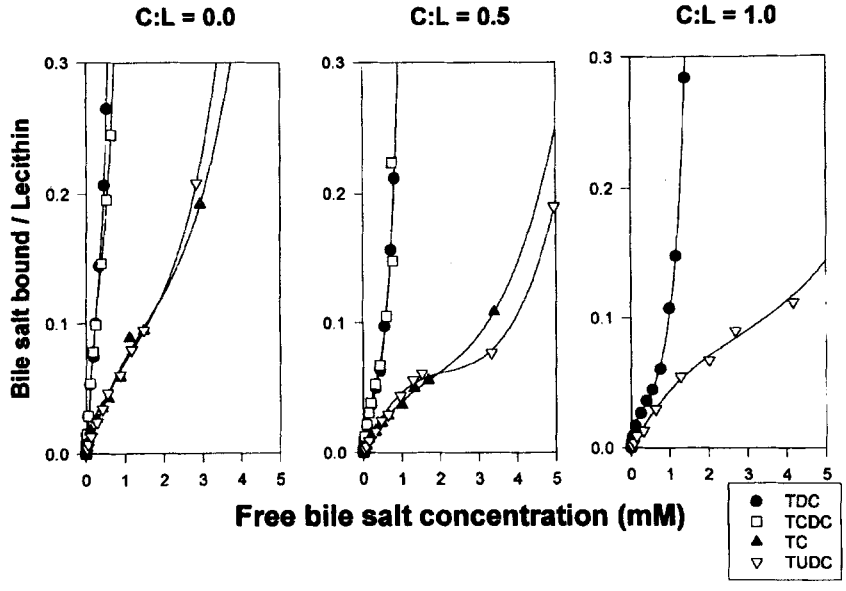

Fig. 3. Same data as Figs. 1 and 2, presented as adsorption plots. The mole ratio of bound bile salt to lecithin in membranes and mixed micelles is shown on the vertical axis; the horizontal axis indicates the corresponding free, non-lecithin-associated bile salt concentration. Cholesterol:lecithin mole ratios were 0 (left), 0.5 (center), and 1.0 (right). Other conditions and abbreviations are as described in Fig. 1. Data are means of 2–8 determinations; lines were fitted by 4th order linear regression. Note the increase in slope at cholesterol:lecithin mole ratio of approximately 0.1.

bound bile salt/lecithin mole ratio in the lecithin-containing phases (vesicles and mixed micelles) is shown on the vertical axis. In a plot of this sort, the slope at each point is the affinity coefficient A_i and the slope at the origin is the affinity constant K_i. Plots of this form are conceptually useful because they demonstrate how adsorption of bile salts to lecithin is dependent upon the bile salt concentration free (non-lecithin-associated) in the aqueous phase. At submicellar concentrations, the free bile salt is presumed to consist solely of monomers in solution and is equivalent to the bile acid monomer activity. At higher concentrations, both monomers and simple micelles may coexist in this phase (27). An increase in slope of the affinity plot, invariably noted at B:L between 0.05 and 0.1, indicates the free bile salt concentration associated with the onset of the transition to hexagonal and micellar phases. In the absence of lecithin, more hydrophobic bile salts with higher affinity for membranes (TDC and TCDC) achieved this threshold at free aqueous concentrations that are relatively low (on the order of 0.2 mM), whereas higher concentrations of TC (1.2 mM) and TUDC (1.6 mM) were required because of the lower affinity of these bile salts for membranes. The threshold values increased progressively with inclusion of cholesterol in the membrane.

Figure 4 shows in greater detail the effect of cholesterol on the adsorption of TDC to vesicles. It is apparent that with increasing membrane cholesterol, the binding affinity of TDC for lecithin was reduced in a concentration-dependent manner. Of note, the B:L ratio associated with the transition threshold was between 0.05 and 0.1 and was similar in the presence and absence of cholesterol. The free TDC concentration required to produce a bound bile salt:lecithin mole ratio of 0.1 increased from 180 micromolar in the absence of cholesterol to approximately 550 and 920 micromolar, respectively, when cholesterol was included at cholesterol:lecithin mole ratios of 0.5 or 1.0.

Figure 5 contains Scatchard plots that illustrate the results of homologous and heterologous displacement studies. In these studies, we examined the distribution of labeled TUDC, TC, or TDC between membranes and the aqueous phase in response to changes in the concentration of unlabeled TUDC, TC, or TDC. The adsorption coefficient A_i for each bile acid is shown on the vertical axis as a function of the ratio of bound bile acid to lecithin, on the horizontal axis. The upper figures show data obtained with cholesterol-free vesicles; the lower figures refer to vesicles with cholesterol:lecithin mole ratio of 0.5. For each of the three bile acids studied, the adsorption coefficients of individual bile salts declined as a function of the total bound bile salt/lecithin ratio, but were essentially independent of the specific bile salt accumulating on the membrane. Thus, although

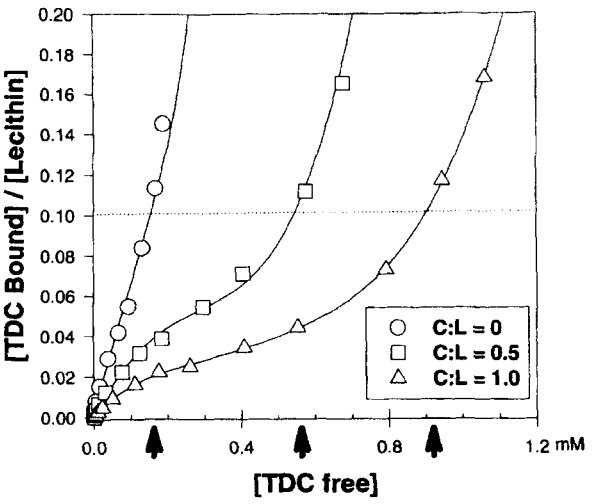

Fig. 4. Adsorption isotherm showing association of TDC with lecithin (membranes and mixed micelles) as a function of increasing membrane cholesterol:lecithin mole ratios (C:L) of 0, 0.5, and 1.0. Each point represents the mean of 4–8 determinations. The vertical axis indicates the mole ratio of bound TDC to lecithin in lecithin-containing vesicles and mixed micelles (B/L). The horizontal axis indicates the concentration of free (non-lecithin-associated) TDC. Lines were fitted by 4th order regression. Dotted line marks a B/L ratio of 0.1, corresponding approximately to the inflection point marking the onset of transition from vesicles to higher affinity phases (hexagonal, mixed micellar). The free bile acid concentrations associated with this ratio for each condition are marked by arrows.

TUDC and TC had a lower affinity for membranes than TDC, the effect of bound TUDC or TC on the heterologous displacement of TDC was identical to the effect of bound homologous TDC. A good empirical fit was obtained to curves of the form $A_i = \times \bullet \exp^{(-y \bullet B/L)} + z$, as in the previous report of Schubert and Schmidt (16) and Schmidt et al. (17); calculated binding parameters are shown.

Figure 6 shows the ratios of the adsorption coefficients for TUDC/TC, TDC/TC, and TDC/TUDC in heterologous binding studies, as a function of B:L. The lines shown were obtained by calculating the predicted ratios of adsorption coefficients over the same range of B:L values, using the parameters derived from curve fitting in Fig. 5. Differences in adsorption coefficients are maximal at low bile salt concentrations. For each bile salt, as B:L increases, affinity of binding of TUDC converges toward that of the more hydrophobic bile salts, and affinity of TC converges slightly toward TDC.

Figure 7 indicates the effect of added TDC on binding of TDC to lecithin, over a range of bile salt concentrations. In these studies TUDC was added to TDC at a fixed ratio of 3:1. In previous studies, addition of TUDC to TDC at this ratio was found to reduce membrane disruption as compared to TDC alone, both in cholesterol-rich vesicles and in living cells. Data are shown for vesicles having cholesterol:lecithin ratios of 0, 0.5, or 1.0.

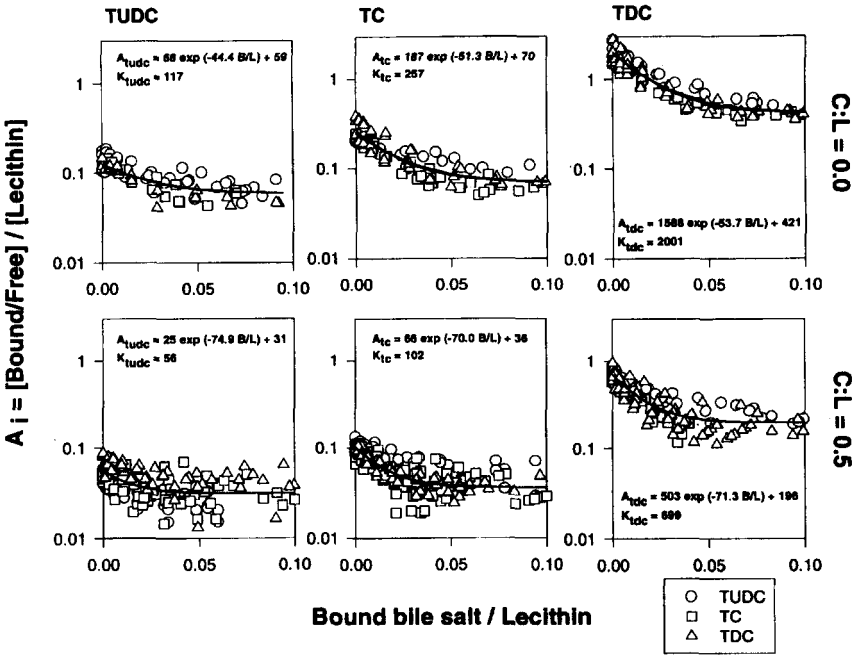

Fig. 5. Scatchard plots showing heterologous and homologous displacement of TUDC, TC, and TDC from vesicles. Shown is displacement of trace concentrations of radiolabeled TUDC (left), TC (center), or TDC (left) by progressively increasing concentrations of unlabeled TUDC (○), TC (□), or TDC (△). In each figure, the vertical axis shows on a logarithmic scale the adsorption coefficient, defined as (fraction bound/fraction free) ÷ [lecithin]. The horizontal axis indicates the mole ratio of bound bile salt to lecithin (B/L). Upper figures indicate results with vesicles composed of lecithin only; lower figures are results with cholesterol-containing vesicles (cholesterol:lecithin mole ratio = 0.5). Data are displayed for concentrations below the micellar threshold (bound bile salt/lecithin < 0.1) and have been fitted empirically to curves of the form $A_i = x \bullet \exp^{(y \bullet B/L)} + z$. K_i = calculated A_i at $B/L \rightarrow 0$.

The overall Scatchard binding plots are shown in the upper figures; the pre-micellar portion of the plot is shown in the lower figures. It is apparent that addition of TUDC to TDC in a 3-fold mole excess produced only a slight decrease in adsorption of TDC to vesicles, consistent with its markedly lower adsorption coefficients. Added TUDC also slightly lowered the transition threshold TDC bound/lecithin ratio. In contrast, above the transition threshold, TUDC markedly altered the adsorption characteristics of TDC. Over a narrow range of concentrations above the threshold, TUDC increased adsorption of TDC to lecithin. At higher concentrations, corresponding to those at which protective effects have been noted previously (18), TUDC reduced the apparent affinity of TDC binding.

DISCUSSION

Bile salts are secreted actively into biliary canaliculi and achieve high concentrations in bile, on the order of 10 to 20 mM (28). At high rates of canalicular secretion, bile salts can cause cholestasis and hepatocellular necrosis. Several lines of evidence suggest that bile salt-induced cholestasis results, at least in part, from detergent disruption of the canalicular membrane (29, 30). Toxic bile salt infusions lead to secretion into bile of canalicular membrane structural components, including proteins such as alkaline phosphatase and lipids such as sphingomyelin (1, 31). Toxicity of individual bile salts in vivo parallels their relative detergency (32). Rats that are genetically deficient in the multiple organic anion canalicular transporter required for biliary secretion of glycosulfolithocholate are resistant to the toxic effects of this bile acid (33). Alternatively, other studies suggest that bile salts may produce cell injury via effects on intracellular membranes, including those of the endoplasmic reticulum (34–36) or mitochondria (12, 13, 37, 38). In some of the latter studies, protective effects of ursodeoxycholate have been attributed to membrane stabilization.

In our previous work (18) a direct protective action of TUDC against disruption and dissolution of membranes by more toxic bile salts was confirmed in model membrane vesicles composed solely of egg lecithin and cholesterol. Changes in vesicle permeability were determined by quantifying release of [^{3}H]inulin, and dissolution of membrane lipids was monitored by noting changes in turbidity. Disruption of membranes by bile salts increased with increasing relative bile salt hydro-

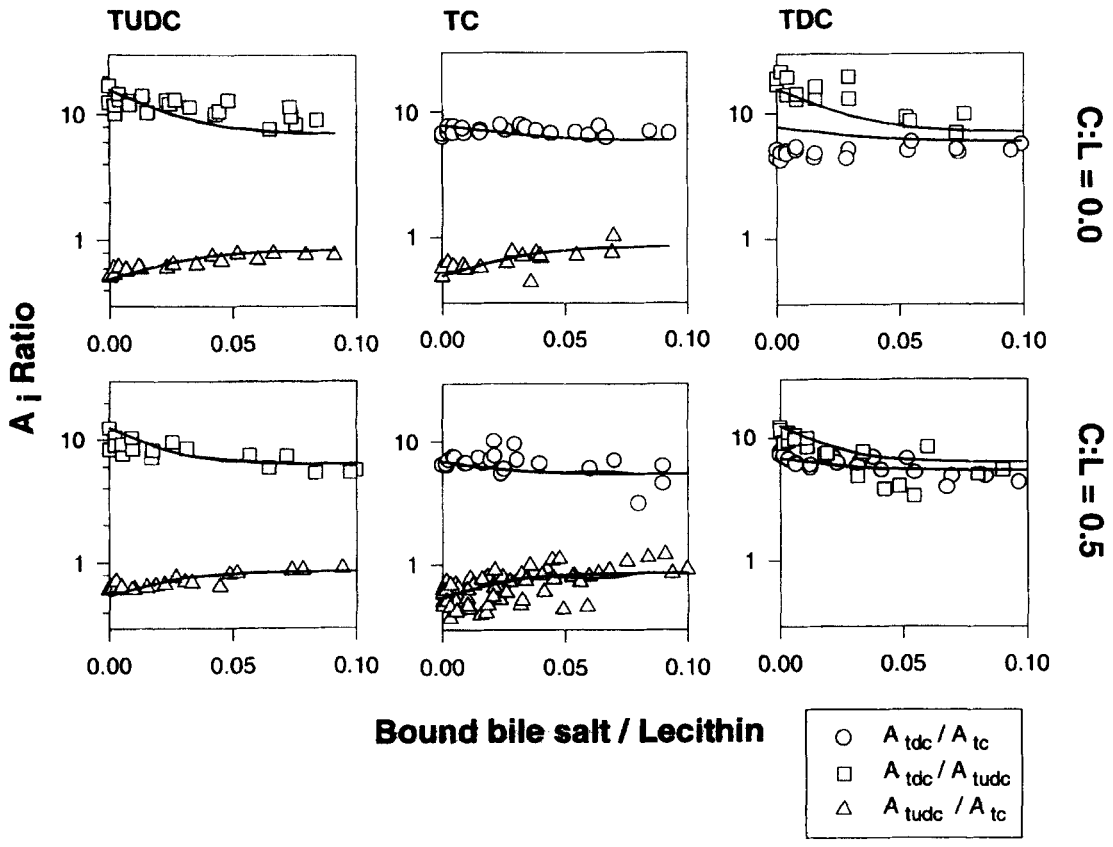

Fig. 6. Ratios of adsorption coefficients in heterologous binding studies, as a function of the total bound bile salt/lecithin ratio. ○ = A_{tdc}/A_{tc}; □ = A_{tudc}/A_{tudc}; △ = A_{tudc}/A_{tc}. Trace amounts of two different labeled bile salts (^3H and ^{14}C) were studied simultaneously in each experiment, along with increasing concentrations of unlabeled TUDC (left), TC (center), or TDC (right). Studies in upper figures were performed using lecithin vesicles containing no cholesterol; lower figures indicate results from studies using vesicles prepared with a cholesterol:lecithin mole ratio of 0.5.

phobicity. For a given bile acid concentration, increasing total lipid concentration or inclusion of cholesterol in vesicles reduced membrane disruption in a concentration-dependent manner. At C:L mole ratios less than 0.2, addition of TUDC to submaximally toxic concentrations of TDC caused additional disruption of vesicles. In contrast, at C:L ratios of 0.5 or greater, TUDC added to TDC was protective, reducing inulin release and preventing clearing of turbidity in a concentration-dependent manner. Protection was demonstrable within minutes and persisted for days. Protection was greater with TUDC than with GUDC and was only minimal with unconjugated UDC. Certain other hydrophilic bile salt taurine conjugates (hyodeoxycholic, β-muricholic, murideoxycholic, 7-keto lithocholic acids) also shared the protective effect of ursodeoxycholate. However, protection was not purely a function of hydrophilicity. Thus the very hydrophilic taurine conjugates of ursocholic and dehydrocholic acids, which are poor micelle formers, exhibited no protection against TDC in these studies.

In the current paper, we have studied the adsorption of bile salts to lecithin bilayer membranes at concentrations both below and above the membrane-mixed micellar transition threshold. We considered two general hypotheses to explain the protective action of ursodeoxycholate against membrane solubilization. First, ursodeoxycholate could physically insert into the bilayer and stabilize membranes against disruption, possibly by presenting a barrier to the insertion of more toxic bile salts. Such a membrane stabilizing effect has been suggested by electron paramagnetic resonance studies of the effects of ursodeoxycholate on mitochondrial membranes (18). Second, ursodeoxycholate could prevent membrane dissolution by interacting with more hydrophobic bile salts to alter the structure and the lipid carrying capacity of mixed micelles. Our findings strongly favor the second hypothesis. In mixed solutions of bile salt taurine conjugates, the coefficients for adsorption of each bile salt to membranes declined in a uniform and mathematically predictable manner with the accumulation of any bile salt on the lipid bilayer. TUDC, a hydrophilic bile salt, exhibited Ai which at low bile salt concentrations was 12-fold lower than that of

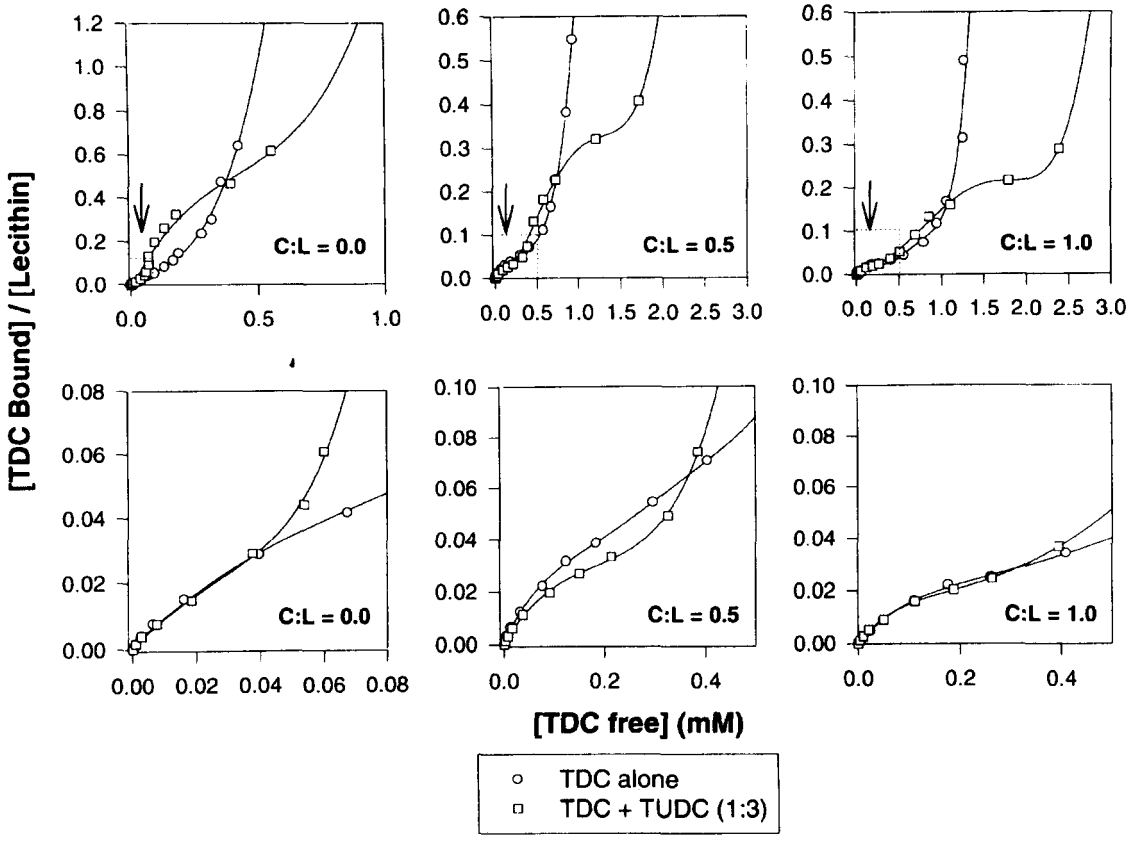

Fig. 7. Adsorption plots showing effect of added TUDC (mole ratio TUDC:TDC = 3:1) on the adsorption of TDC to lecithin-associated phases. The vertical axis indicates adsorption coefficient of TDC; the horizontal axis the ratio of bound TDC to lecithin. Studies were performed using vesicles with cholesterol:lecithin mole ratio of 0 (left), 0.5 (center), or 1.0 (right). Upper figures show adsorption over a range of concentrations at which membranes undergo dissolution to form mixed micelles; data from the premicellar range (arrows) have been expanded in the lower figures to show the effect of TUDC on binding of TDC to membranes. Data are means of 2–8 determinations; lines were fitted by 4th order linear regression.

TDC and 2-fold lower than that of TC. Addition of TUDC caused only a slight reduction in adsorption of TDC and lowered the TDC bound:lecithin ratio associated with minimum TDC adsorption coefficient (corresponding to the onset of membrane permeabilization and solubilization). This finding indicates that a direct exclusionary or membrane stabilizing effect of TUDC is unlikely.

In our previous studies we noted that addition of TUDC to TDC not only inhibited release of [^{3}H]inulin from the lumen of vesicles but also partially prevented clearing of turbidity. This indicates that TUDC can reduce the micellar solubilization of membrane lipids. The current data suggest that this effect of TUDC must result from effects on the micellar phase that increase the ratio of toxic bile salts to lecithin in micelles, either by altering mixed micellar structure or by expanding the ratio of simple to mixed micelles. This hypothesis also would explain why strongly hydrophilic non-micelle-forming bile salts fail to protect against membrane dissolution by TDC. The complexities of the adsorption isotherm in the micellar range, which result from coexistence of monomers, simple micelles, mixed micelles, vesicles and possibly an additional hexagonal phase (39), preclude firm conclusions regarding the precise effects of TUDC on these different phases in the current study.

Work of Mazer (40), Egelhaaf and Schurtenberger (41), Pedersen, Egelhaaf, and Schurtenberger (42), Long et al. (43), and Long, Kaler, and Lee (44) using quasielastic and neutron light scattering has demonstrated that bile salt-lecithin mixed micelles have an elongated rod-like or worm-like tubular structure. Most working models of these micelles propose that phospholipids are arranged radially, with bile salts at the surface acting as wedges between the phospholipid head groups. The transition from membrane to mixed micelle probably begins when insertion of bile salts into the membrane outer leaflet reaches a critical threshold level necessary for the transition from a planar to a cylindrical (hexagonal) arrangement. Consistent with this model,

recent studies of Fahey, Carey, and Donovan (45) suggest that all of the common 3-hydroxylated bile salts insert horizontally at the surface of lipid bilayers with their polar hydroxyl and acidic groups exposed to the aqueous medium and their hydrophobic surface interacting only with the outermost carbons of phospholipid acyl chains. Because all bile salts are of similar size, the wedge effects of different membrane bound bile salts would be expected to be similar. Consistent with this hypothesis, the minimum binding affinity associated with onset of mixed micelle formation always was observed at a bound bile salt/lecithin ratio in the range of 0.1, suggesting that a ratio of bile salt to lecithin in the membrane outer leaflet of between 1:5 and 1:10 introduces sufficient strain to force the transition to a cylindrical arrangement.

Differences in affinity of different bile salts for adsorption to membranes are related to the contiguous hydrophobic surface available for hydrophobic interaction with membrane lipids. Data of Roda et al. (46, 47) have shown that the axial orientation of the 7β-hydroxy group of ursodeoxycholate markedly reduces the hydrophobic surface. In the current studies TUDC was found to have a very low affinity for membranes which was even less than that of TC, a trihydroxy bile salt. This is consistent with previous estimates of relative hydrophobicity using reverse phase HPLC, but differs from findings with octanol:water partition (47–49). A variety of different solvent partition and reverse phase chromatographic methods have been used to evaluate bile salt hydrophobic–hydrophilic balance, often with conflicting results (47, 48). All previous methods have the disadvantage of requiring the use of organic solvents. In contrast, the affinity of bile salts for lipid bilayers can be determined readily by the methods used here in the complete absence of organic solvents, and may prove to be a more accurate and biologically relevant index of bile salt hydrophobicity.

The studies reported here used only bile salt taurine conjugates. The glycine conjugate of ursodeoxycholate has cytoprotective properties similar to, but somewhat less than, those of the taurine conjugate (50). At alkaline pH, glycine-conjugated and unconjugated bile salts adsorb to membranes with affinities similar to the corresponding taurine conjugates. However, at neutral or acidic pH their adsorption isotherms are much more complicated than those of the taurine conjugates. Whereas taurine-conjugated bile salts are strongly acidic (pK_a values on the order of zero) and remain fully ionized over the entire range of physiological pH, glycine-conjugated and unconjugated bile acids have pKa values of 3.9 and 5.0, respectively (51). Thus, even at neutral pH a small amount of the uncharged acid form is present. The uncharged bile acids are poor detergents and relatively insoluble. Moreover, they have much higher affinity for membranes than the corresponding ionized bile salts, and because their accumulation on membranes does not alter membrane charge, their effect on the adsorption isotherm differs from that of the ionized form. Studies currently are in progress to characterize the membrane adsorption isotherms and vesicle-micelle transitions of the glycine-conjugated and unconjugated bile salts as a function of pH.

Phospholipid reduces the cytotoxicity of bile salt solutions toward gastric mucosa (52), gallbladder epithelium (53), and hepatocyte canalicular membranes (54). The current studies may provide an explanation for this phenomenon. Cell plasma membranes, especially the apical membranes of cells lining the biliary tract and proximal intestine, have a high cholesterol:phospholipid ratio and therefore relatively low affinity for bile salts. If phospholipid vesicles with a low cholesterol content are also present, the adsorption of bile salts to cholesterol-poor vesicles should exceed adsorption to cholesterol-rich epithelial membranes. Dissolution of lecithin vesicles would be expected to occur at free bile salt concentrations that are insufficient to disrupt cell plasma membranes. Micelles by virtue of their high affinity for bile salts may then sequester large amounts of added bile salt with little additional increase in the free bile salt concentration. It is possible that biliary secretion of lecithin vesicles may have evolved as a mechanism to protect the biliary epithelium from toxicity of luminal bile salts. In this regard, it is noteworthy that mdr2 knockout mice, which fail to secrete phospholipid into bile, develop progressive cholestatic liver injury (55). Secretion of phospholipid also has been demonstrated in gastric epithelium and may be of importance in protecting the stomach from injury by bile salts refluxing from the duodenum (52, 56, 57).

Inclusion of cholesterol in lecithin vesicles was found to reduce binding of bile salts and would be expected to attenuate the protective effect of lecithin. Consistent with this, we found that pure lecithin vesicles were more effective than cholesterol-containing vesicles in preventing disruption of isolated canalicular plasma membranes by hydrophobic bile salts (54). This effect of cholesterol may be of great importance in the pathogenesis of gallstone disease. Patients with cholesterol gallstones secrete biliary vesicles that have an abnormally high cholesterol:lecithin ratio. Studies of O'Leary (53) show that bile salts at millimolar concentrations cause release of preformed mucin from biliary epithelium, indicative of epithelial injury, and that biliary lipids normally protect against bile salt-induced release of mucus from gallbladder epithelium. Mucus is thought to be a key factor in gallstone pathogenesis, providing the matrix in which crystal formation occurs (58). In the

prairie dog model of gallstone disease, increases in cholesterol content of bile are followed by marked increases in mucin secretion that precede formation of cholesterol crystals (59). We hypothesize that the increased cholesterol:lecithin ratio, by reducing adsorption of bile salts to biliary lecithins, allows bile salt injury to the gallbladder epithelium which is then responsible for hypersecretion of mucus.

A key finding of the current studies was the observation that affinity of bile salt taurine conjugates for a lecithin membrane depends upon the total concentration of all bile salts adsorbed to the membrane (that is, the bound bile salt/lecithin ratio) but is essentially independent of the specific bile salts bound. This finding is consistent with the model of Clarke (60), which proposes that the repulsive effect of accumulated negative charge is a major element responsible for the progressive decrease in binding affinity that accompanies adsorption of anionic surfactants to membranes. Two important consequences may be inferred. First, the adsorption behavior of any bile salt taurine conjugate may be predicted by determining either a homologous or a heterologous binding isotherm. Thus, for example, if the labeled taurine conjugate of a rare bile salt can be prepared in even microgram quantities, its adsorption behavior can be inferred by studying its displacement from membranes using common, readily available bile salts such as taurocholate or taurodeoxycholate. Second, in a mixture of bile salts, it should be possible to mathematically model the overall adsorption isotherm using equations derived from the individual bile acid adsorption isotherms. As the effects of different bile salts on membrane permeability and membrane-mixed micelle transition appear to be determined by the B/L ratio, such a model may allow one to predict for any mixture of bile salts the threshold concentration at which membrane disruption will begin. A general model of this sort would permit a quantitative distinction between simple physicochemical membrane disruptive effects of bile salts and more subtle biological effects which may be critical to cytotoxicity and cytoprotection. Attempts to develop and validate such a model currently are in progress in our laboratory.

In conclusion, we have demonstrated that adsorption of different bile salt taurine conjugates to lecithin:cholesterol model membranes is a function of their hydrophilic–hydrophobic balance. For an individual labeled bile salt, the adsorption affinity is a function of the total membrane bound bile salt and is independent of the specific bile salts present on the membrane. The affinity of tauroursodeoxycholate for membranes is an order of magnitude lower than that of TDC. Addition of TUDC to TDC displaces the latter from membranes only slightly but may displace TDC from mixed micelles. These data indicate that TUDC protects against membrane disruption by TDC via effects on formation and structure of mixed micelles rather than via stabilization of membranes.

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