Transbilayer Movement of Fully Ionized Taurine-Conjugated Bile Salts Depends upon Bile Salt Concentration, Hydrophobicity, and Membrane Cholesterol Content[†,‡]

Joanne M. Donovan*[,§,||] and Audrey A. Jackson[§]

Brockton/West Roxbury Department of Veterans Affairs Medical Center, 1400 VFW Parkway, Boston, Massachusetts 02132, and Department of Medicine, Harvard Medical School, Brigham and Women's Hospital, and Harvard Digestive Diseases Center, 75 Francis Street, Boston, Massachusetts 02115

Received March 17, 1997; Revised Manuscript Received June 13, 1997[⊗]

ABSTRACT: Taurine-conjugated bile salts mediate rapid transmembrane flux of divalent cations, irrespective of whether bile salts and divalent cations are initially on the same or opposite side of the membrane. We therefore hypothesized that ionized bile salts can equilibrate between membrane hemileaflets. We quantitated bile salt binding to large unilamellar egg yolk phosphatidylcholine (EYPC) ± cholesterol (Ch) vesicles under conditions in which one or both hemileaflets were initially exposed to bile salts. At unbound taurodeoxycholate (TDC) concentrations >0.2 mM, the dependence of binding on TDC concentration after 30 min was indistinguishable for vesicles prepared by either method and did not change from 30 minutes to 24 h. At unbound TDC concentrations <0.1 mM, the ratio of bound/free TDC to EYPC vesicles doubled over a single exponential time course. Equilibration times were greater for the more hydrophilic bile salts taurocholate and tauroursodeoxycholate, for EYPC/Ch vesicles, and at lower temperatures. For glycine-conjugated bile salts, time-dependent changes in binding did not occur, consistent with more rapid equilibration of the small fraction of the protonated form. We conclude that fully ionized conjugated bile salts translocate between lipid bilayer hemileaflets, in contrast to previous observations that equilibration of fully ionized unconjugated bile salts occurs at a negligible rate in small unilamellar vesicles. The rate of "flip−flop" increases with increases in intramembrane bile salt concentration and hydrophobicity but decreases with cholesterol content and lower temperature. We speculate that physiologically, even in the absence of a specific membrane transporter, bile salts can gain access to intracellular compartments and mediate increases in divalent cation flux that may underlie cytotoxicity.

Bile salts interact with membranes in diverse physiological systems of pathophysiological significance (*7*). At micellar concentrations, bile salts solubilize membranes, release intracellular enzymes, and cause cell death (*18*, *19*). Moreover, bile salts also appear to be cytotoxic at the much lower submicellar concentrations within hepatocytes (*19*, *27*, *39*, *42*, *50*). Nonetheless, the detergent properties of bile salts are central to their physiological functions: secretion of biliary lipids at the canalicular membrane (*12*, *46*), solubilization of cholesterol in bile (*5*, *8*), facilitation of lipid absorption (*9*), and as recently shown, absorption of divalent cations in the small intestine (*37*, *38*). The toxic detergent effects of bile salts can be tempered: hydrophilic bile salts such as ursodeoxycholic acid conjugates protect against the cytotoxic and disruptive effects of hydrophobic bile salts (*16*, *18*). Despite extensive knowledge of the physical chemistry of bile salt-containing biliary lipid aggregates and their ability to solubilize cholesterol (*5*, *10*, *29*), far less is known regarding the mechanism by which bile salts interact with lipid membranes (*5*, *7*) and, under differing circumstances, mediate either cytotoxicity or cytoprotection.

A major avenue of bile salt cytotoxicity has been presumed to be alterations in membrane permeability. However, we have recently demonstrated that, at the relatively low concentrations present within the hepatocyte (*42*), bile salts do not alter membrane permeability to non-ionic solutes such as water (*2*). In contrast, we found that hydrophobic bile salts increase membrane permeability to divalent cations by several orders of magnitude (*1*). Curiously, an increase in magnesium permeability was observed even under conditions when magnesium and taurine-conjugated bile salts were initially present on opposite sides of the membrane (*1*) (Jackson, A. A., Donovan, J. M., unpublished data). We hypothesized that taurine-conjugated bile salts could rapidly gain access to both membrane hemileaflets.

Previous studies suggested that un-ionized bile acids can "flip−flop", i.e., the protonated, uncharged species can redistribute across membrane bilayers rapidly, with $t_{1/2}$ values less than 1 s (*6*). Because pK_a' values of unconjugated and presumably glycine-conjugated bile salts in phospholipid bilayers approach neutral pH values (*4*), the uncharged fraction rapidly equilibrates across lipid bilayers. In contrast, NMR studies of fully ionized unconjugated bile salts did not show equilibration of species in the outer and inner membrane hemileaflets (*6*). Thus, taurine conjugates, which have pK_a' values below 1 and remain fully ionized under physi-

[†] Supported in part by research funding from the Veterans Administration, and Center Grant DK 34854 from the National Institutes of Health (U.S. Public Health Service).

[‡] Presented in part at the National Meeting of the American Gastroenterological Association, San Francisco, CA, May 19−22, 1996, and published in abstract form [Donovan, J. M., & Jackson, A. A. (1996) *Gastroenterology 110,* A896].

* Correspondence should be addressed to Brockton/West Roxbury Department of Veterans Affairs Medical Center, 1400 VFW Parkway, Boston, MA 02132. E-mail address: donovan.joanne@brockton.va.gov.

[§] Brockton/West Roxbury Department of Veterans Affairs Medical Center.

[||] Brigham and Woman's Hospital and Harvard Digestive Diseases Center.

[⊗] Abstract published in *Advance ACS Abstracts,* September 1, 1997.

S0006-2960(97)00592-8 CCC: $14.00

ological conditions, have not been believed to flip−flop across membrane bilayers.

We hypothesize that rapid translocation of fully ionized bile salts across lipid bilayers could explain our observations that bile salts facilitate transmembrane transport of divalent cations when located on opposite sides of the bilayer (*1*) (Jackson, A. A., Donovan, J. M., unpublished data). By quantitating bile salts bound to large unilamellar vesicles under conditions where one or both membrane hemileaflets were initially exposed to aqueous bile salt solutions, we demonstrate that bile salts indeed redistribute across membrane bilayers. We have examined effects of bile salt concentration, hydrophobicity, and conjugation as well as membrane composition and temperature on bile salt flip−flop. These results suggest that bile salts can undergo translocation across cell membranes via an additional pathway, flip−flop of fully ionized as well as uncharged bile salts, as well as via previously characterized high-affinity bile salt transport proteins (*30*, *45*, *48*).

EXPERIMENTAL PROCEDURES

Bile salts (Sigma, St. Louis, MO), grade I egg yolk phosphatidylcholine (EYPC,[1] Lipid Products, South Nutfield, U.K.), and cholesterol (Nu-Chek Prep, Elysian, MN) were used as received, or purified as previously described (*13*). By high-performance liquid chromatography (HPLC) (*36*) (Beckman Instruments, Wakefield, MA), bile salt purity with respect to other conjugates was >98%. Thin-layer chromatography demonstrated that bile salt and EYPC purity were >99% (*13*). All other chemicals were of highest reagent grade purity.

Vesicle Preparation. Unilamellar vesicles were prepared by coprecipitation of EYPC ± Ch from MeOH/$CHCl_3$, drying first under a stream of N_2 and then under reduced pressure, followed by resuspension in aqueous solution (150 mM NaCl, 1 mM NaN_3, pH 7.4) with or without bile salts at the appropriate final concentration (0−1.5 mM). For large unilamellar vesicles, EYPC ± Ch lipid dispersions were extruded multiple times through two 0.1-μM Nuclepore filters (Corning Costar Corp., Cambridge, MA) in a high-pressure vesicle extruder (Model HPVE-S, Sciema Technical Services, Ltd., Richmond, BC, Canada) (*20*). In the absence of bile salts, lipid concentrations were 30−60 mg/mL to allow concentrated solutions of vesicles to be added to bile salt solutions (see below). For systems prepared with bile salts, the initial concentration of EYPC ± Ch was 3 mg/mL. Hence, for systems prepared with bile salts in both membrane hemileaflets, each data point shown represents a separate preparation of vesicles.

Bile Salt/Vesicle Binding. Large unilamellar EYPC or EYPC/Ch (molar ratio 2:1) vesicles (3 mg/mL) were incubated for periods from 0.25 to 24 h at 37 °C with varying bile salt concentrations (0.1−2.0 mM). Concentrated vesicle suspensions were added to larger volumes of bile salt-containing solutions to ensure that vesicles were not exposed to bile salt concentrations significantly greater than final concentrations after membrane adsorption of bile salts. Unbound bile salt concentrations were determined by centrifugal ultrafiltration (30000 MWCO microconcentrators, Amicon Division, W. R. Grace & Co., Beverly, MA) (*13*). Total and unbound bile salt concentrations were quantified by HPLC (*36*). Incubations and centrifugal ultrafiltration were performed at 37 °C, except for selected experiments, in which both incubation and centrifugal ultrafiltration were conducted at 5 or 22 °C. Results are expressed as the means ± SD for 3−5 experiments, with duplicate measurements performed for each individual experiment.

Quasielastic Light Scattering. Vesicle size (mean hydrodynamic diameter) was measured by quasielastic light scattering (*2*, *11*). The intensity of scattered light at a 90° angle during and after incubation with bile salts was also measured as an index of the relative mass of vesicles. Essentially all the intensity of scattered light is derived from vesicles (*11*). For all bile salt concentrations shown, there was no decrease in scattered light intensity that suggested mixed micelle formation.

Determination of pK_a' Values. To exclude the possibility that the pK_a' value of EYPC-bound TDC increased sufficiently so that transmembrane migration occurred by means of the protonated form, equilibrium titrations of 6.6 mM TDC alone or with 39 mM EYPC were conducted at 22 °C with continuous magnetic stirring, as described previously (*44*). The latter conditions were chosen such that >95% of TDC was bound to EYPC. Titration with 1 M HCl was conducted from pH 10 to pH 2.

RESULTS

The basic approach was to measure bile salt binding to unilamellar vesicles under conditions that would allow initial access of bile salts to one or both hemileaflets. When bile salts are added to suspensions of large unilamellar vesicles, bile salts can partition into the outer membrane hemileaflet, but not into the inner membrane hemileaflet unless transmembrane flip−flop occurs. When aqueous bile salt solutions are added to anhydrous EYPC ± Ch lipid films, bile salts partition into all portions of the lipid membrane. The process of extrusion allows access of aqueous solution to both sides of the membrane bilayer (*20*, *28*) and produces large unilamellar vesicles that contain bile salts in both hemileaflets. The calculated internal volume of these vesicles based on vesicle diameter (*2*) was negligible (<0.001% of total volume) (*32*). Thus, entrapped aqueous bile salts did not contribute to observed binding. Moreover, the ratio of surface area in the outer hemileaflet to that in the inner hemileaflet is calculated to be 1.01. We tested the hypothesis that, for a given free bile salt concentration, approximately twice as much bile salt would be bound when both membrane hemileaflets were accessible as would when only a single membrane hemileaflet was accessible.

Bile Salt Binding to EYPC Vesicles. Figure 1 displays binding of the hydrophilic bile salt TUDC to EYPC vesicles under two conditions: (1) as bile salts were added externally to EYPC vesicles and incubated for 30 min or (2) when TUDC was present during EYPC vesicle formation by extrusion. Under both conditions, TUDC binding increased monotonically with free bile salt concentration. However, for any given free bile salt concentration, approximately twice as much TUDC was bound when both hemileaflets were accessible as when only the external hemileaflet was accessible. This is consistent with the hypothesis that transmembrane transfer of TUDC does not occur over 30 min; hence,

[1] Abbreviations: EYPC, egg yolk phosphatidylcholine; Ch, cholesterol; TDC, taurodeoxycholate; TUDC, tauroursodeoxycholate; TC, taurocholate; GDC, glycodeoxycholate; GUDC, glycoursodeoxycholate.

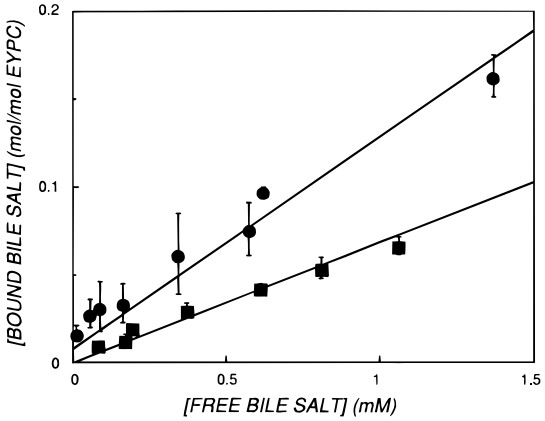

FIGURE 1: Binding of TUDC to EYPC vesicles differs depending on initial incubation conditions: 30 min exposure, external only (■), and vesicles prepared in the presence of TUDC (●). For any free bile salt concentration, bound TUDC was approximately double for vesicles prepared in the presence of TUDC as compared with vesicles that were incubated with TUDC externally. This is consistent with the hypothesis that the surface area of EYPC available for bile salt binding is 2-fold higher when both the inner and outer membrane leaflets are initially accessible. Conditions: 37 °C, 0.15 M NaCl. Error bars represent SD.

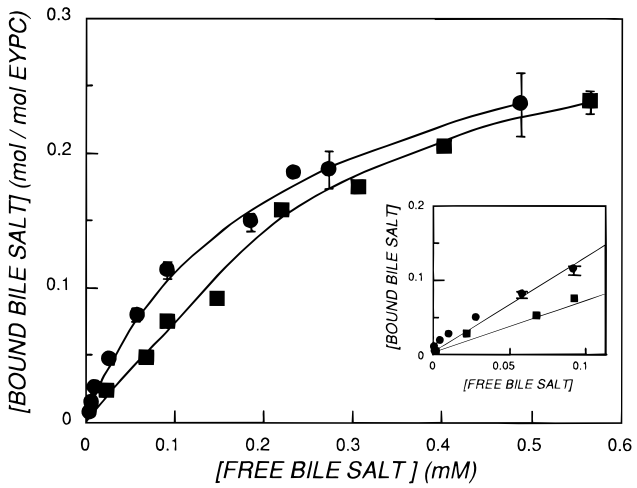

FIGURE 2: Binding of TDC to EYPC vesicles for two different initial incubation conditions: 30 min exposure, external only (■), and vesicles prepared in the presence of TDC (●). At higher free TDC concentrations (>0.3 mM), binding under both conditions converges. As the inset shows, at low free TDC concentrations (<0.1 mM), bound TDC was approximately double for vesicles prepared in the presence of TDC as compared with vesicles that were exposed to TDC externally. Conditions: 37 °C, 0.15 M NaCl. Error bars represent SD.

binding is constrained to the outer membrane hemileaflet, at least for this time period.

Figure 2 displays binding of the hydrophobic bile salt TDC to EYPC vesicles as bile salts were added externally and incubated for 30 min or when TDC was present during vesicle formation by extrusion. The distinction between binding affinity under conditions when one or both hemileaflets are accessible is less apparent for TDC than for TUDC binding to EYPC vesicles. As shown in the inset, at low (<0.1 mM) free TDC concentrations, bile salt binding was approximately 2-fold higher when both membrane hemileaflets were accessible than when TDC was added to preformed vesicles. However, at higher free TDC concentrations, binding is indistinguishable for experimental conditions under which bile salts initially have access to one or both membrane

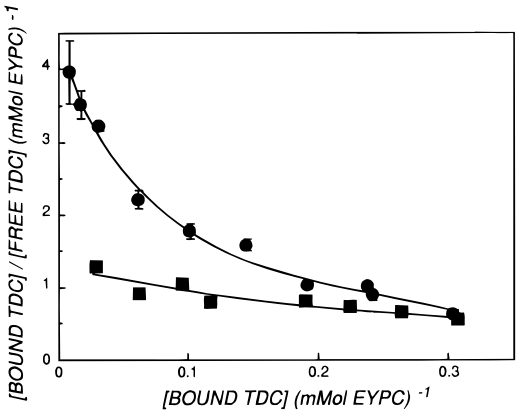

FIGURE 3: Scatchard plot of binding of TDC to EYPC vesicles for two different initial incubation conditions: 30 min exposure, external only (■), and vesicles prepared in the presence of TDC (●). At low free bile salt concentrations, binding curves diverge. Conditions: 37 °C, 0.15 M NaCl. Error bars represent the SD.

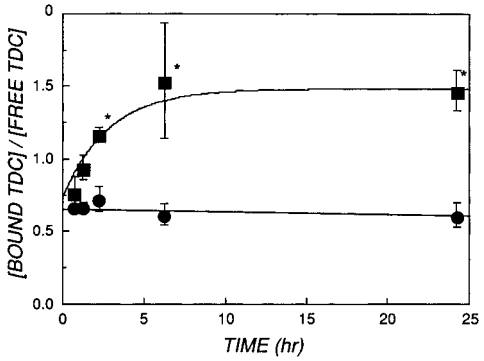

FIGURE 4: Time course of binding of TDC to EYPC vesicles for two total bile salt concentrations: 0.86 (●) and 0.25 mM (■). At the higher TDC concentration, binding does not vary over a 24-h period, suggesting the bile salts have equilibrated with both membrane hemileaflets prior to the initial time point. At the lower TDC concentration, the ratio of bound to free TDC rises to reach a plateau after several hours. Conditions: 37 °C, 0.15 M NaCl. Error bars represent SD. Asterisks denote that values significantly ($p < 0.05$) differ from the ratio of bound to free TDC at the initial time point.

hemileaflets. Quasielastic light scattering studies demonstrated that this was not due to formation of mixed micelles at aqueous concentrations up to 0.6 mM TDC, as the scattered light intensity and mean hydrodynamic diameter were unchanged (data not shown). Figure 3 displays the binding of TDC to EYPC vesicles as a Scatchard plot. The binding affinity of TDC under both conditions is identical at the highest bile salt concentrations but clearly diverges at lower TDC concentrations.

Figure 4 displays the time course of TDC binding to EYPC vesicles at two different total bile salt concentrations. At a total TDC concentration of 0.86 mM, corresponding to a free TDC concentration of ≈0.2 mM, there is no change in the ratio of bound to free TDC from 15 min to 24 h (circles). Resolution at time points below 15 min is limited by the time required for physical separation of bound and free bile salts. At this initial bile salt concentration, one possibility is that transient micelle formation and re-formation of vesicles allows redistribution of bile salts in both hemileaflets. However, at a total TDC concentration of 0.25 mM, corresponding to a free TDC concentration of <0.1 mM, the ratio of bound to free TDC increases over several hours (squares). In this case, aqueous bile salt concentration

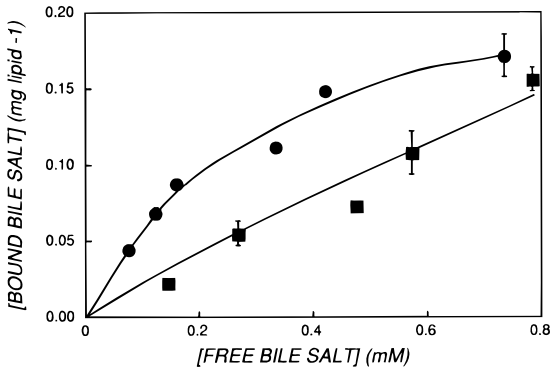

FIGURE 5: Binding of TDC to EYPC/Ch (2/1 molar ratio) vesicles differs depending on initial incubation conditions: 30 min exposure, external only (■), and vesicles prepared in the presence of TDC (●). In contrast, TDC binding to EYPC vesicles at similar free bile salt concentrations (>0.3 mM) is similar irrespective of whether one or both membrane hemileaflets are initially accessible to bile salts (see Figure 2). Conditions: 37 °C, 0.15 M NaCl. Error bars represent SD.

remains well below the concentration (≈0.6 mM) at which vesicles are solubilized as mixed micelles (*2*). The increase in bound TDC concentration over several hours is consistent with equilibration of TDC across the membrane bilayer, with binding to both membrane hemileaflets.

The ratio of bound/free TDC is predicted to increase by a factor of 2, since the membrane area available for binding increases 2-fold when both membrane hemileaflets are accessible. In Figure 4, the data are fit to

$$C_b/C_f = (C_b^{\infty}/C_f^{\infty})(2 - e^{-kt}) \quad (1)$$

where C_b and C_f denote the bound and free TDC concentrations, C_b^{∞} and C_f^{∞} denote values at infinite time, and k is the time constant. This expression, a single exponential with an increase in amplitude of a factor of 2, approximates an expression that is rigorously derived in Appendix I. The solid curve in Figure 4 shows the least squares best fit, with a value of $k = 0.4\ h^{-1}$ ($r^2 = 0.97$). The time course of TUDC binding (0.06 mol/mol EYPC, final concentration) was somewhat slower, with a value of $k = 0.25\ h^{-1}$ ($r^2 = 0.85$, data not shown).

Because of previous observations that bile salts increase membrane permeability to calcium (*1*), we examined whether the addition of calcium (8 mM $CaCl_2$) would increase the rate of TDC flip−flop. At a total TDC concentration of 0.25 mM, corresponding to a free TDC concentration of ≈0.04 mM, the rate of change of the bound/ free TDC concentration was unchanged (data not shown).

Bile Salt Binding to EYPC/Ch Vesicles. Figure 5 displays TDC binding to EYPC/Ch vesicles (molar ratio 2/1) under conditions where TDC is initially accessible to the external or both membrane hemileaflets. Binding under conditions when both membrane hemileaflets are initially accessible is substantially higher than binding to the external hemileaflet alone after 30 min. At the highest unbound TDC concentrations studied, binding affinity converged for both experimental conditions, irrespective of whether TDC is initially accessible to one or both membrane hemileaflets. Thus, the addition of cholesterol, which decreases membrane fluidity, also inhibits translocation of bile salts across the lipid bilayer.

Effect of Temperature on Flip−Flop Rate. Figure 6 displays the time dependence of bile salt binding at three temperatures: 37, 22, and 5 °C. At 37 °C, the time course of flip−flop is more rapid than the resolution of the method. However, the rate decreased at lower temperatures, and the solid lines (Figure 6, panels B and C) show fitted curves with rate constants of 0.32 and 0.13 h^{-1} at 22 and 5 °C, respectively. The dashed line (Figure 6A) shows a predicted curve for the lowest rate constant consistent with the observed data, i.e., that passes through the value at 15 min less one standard deviation.

Effect of Bile Salt Conjugation and Ionization on Flip−Flop Rate. Glycine conjugated bile salts have been postulated to flip−flop across membrane bilayers rapidly because of the fraction that is present in the protonated form (*6*). Figure 7 shows the time course of binding of TDC and glycodeoxycholate (GDC) to EYPC vesicles at pH 7.4. There was no change in GDC binding over a 24-h period, consistent with rapid partition into and equilibration across the bilayer occurring prior to the initial measurement at 15 min. Similarly, at pH 7.4 and pH 9.5, glycoursodeoxycholate (GUDC) binding was identical after 30 min and 24 h of incubation (data not shown).

Because the pK_a' of bile salts in lipid membranes is up to several pH units higher than in bulk solution, we examined the possibility that the pK_a' of EYPC-bound TDC was sufficiently high to allow flip−flop via a small but finite protonated fraction. Potentiometric titration curves of TDC were identical in the presence or absence of EYPC (data not shown), from which we infer that the pK_a' of TDC in EYPC bilayers is less than 2.5.[2] Thus, the protonated fraction of TDC at the pH value examined herein was estimated to be less than 1/30000.

Table 1 summarizes estimates for rate constants for bile salt flip−flop, together with the corresponding free bile salt concentration and the bile salt/EYPC ratio in the lipid bilayer. For taurine-conjugated bile salts, flip−flop rate depends upon the bile salt/EYPC ratio in the lipid bilayer. However, at comparable bile salt/EYPC ratios, the rate constant for TDC is only minimally faster than for taurocholate (TC) or TUDC. At comparable free bile salt concentrations, there was a distinction in binding affinity under conditions where one or both membrane hemileaflets were accessible for TUDC but not for TDC (Figure 1 vs Figure 2). Comparison of the rate constants at similar values of bound bile salt/EYPC ratios suggests that this is primarily due to the much stronger binding affinity of TDC for membrane bilayers rather than differences in flip−flop rate for comparable intramembrane bile salt concentrations. Because of rapid transmembrane equilibration of GDC and GUDC, only a lower bound for the flip−flop rate could be determined by these methods.

DISCUSSION

If fully ionized bile salts cannot flip−flop across lipid bilayers, then the stoichiometry of bile salt binding to membrane lipids must depend upon whether bile salts in solution are in contact with a single (outer) membrane hemileaflet or both membrane hemileaflets. Both the

[2] An additional 2 microequiv was required to reach pH 3.0 for a system containing 33 microequiv of TDC (6.6 mM, 5 mL). To reach pH 2.5, an additional 7 microequiv was required. Since 33 microequiv of TDC were present, we estimate that to titrate to the pK_a', an additional 16.5 microequiv would be required. Hence, we conservatively estimate that the pK_a' of TDC must lie below 2.5.

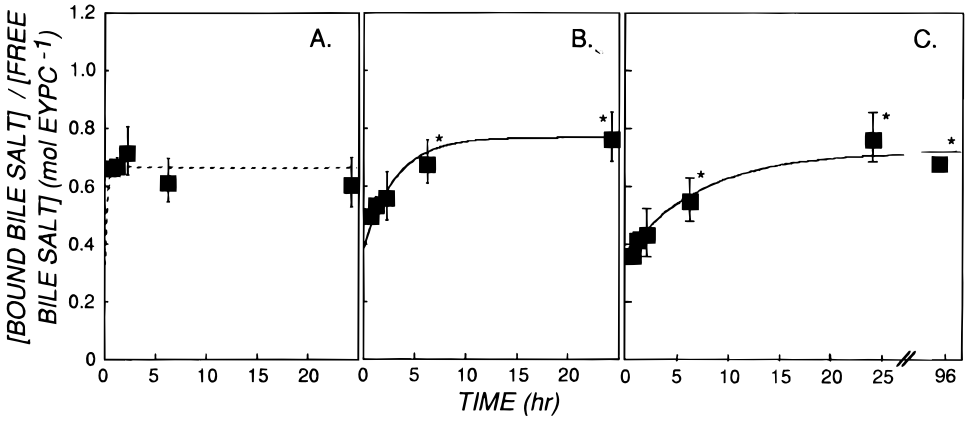

FIGURE 6: Time course of bound/free bile salt concentrations for TDC at (A) 37, (B) 22, and (C) 5 °C. Solid lines at 22 and 5°C represent single exponential fits. Dashed line at 37 °C represents a single exponential fit with intercept at $t = 0$ at one-half the final value (see text for details). Conditions: total bile salt concentration, 0.86 mM; EYPC, 3 mg/mL. Error bars represent SD. Asterisks denote that values significantly ($p < 0.05$) differ from the ratio of bound to free TDC at the initial time point.

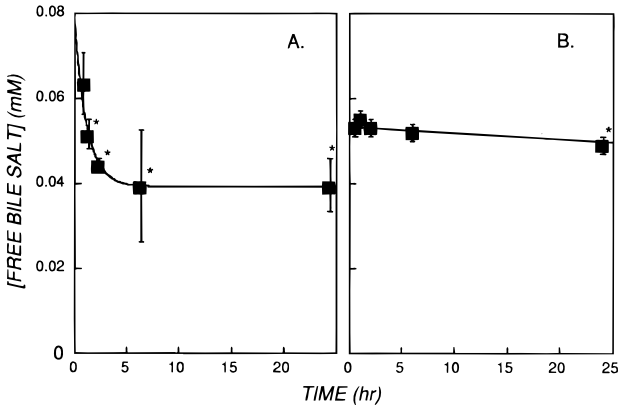

FIGURE 7: Time course of free bile salt concentration for (A) TDC and (B) GDC. GDC binding is unchanged over 24 h, consistent with rapid equilibration across the membrane bilayer. In contrast, at this low concentration, TDC equilibrates more slowly with a $t_{1/2}$ of 1.7 h for the single exponential fit shown. Conditions: total bile salt concentration, 0.22 mM; EYPC, 3 mg/mL; 37 °C. Error bars represent SD. Asterisks denote that values significantly ($p < 0.05$) differ from the free TDC at the initial time point.

Table 1: Comparison of Rate Constants for Bile Salt Flip−Flop

bile salt	vesicle composition	[free bile salt][a,b] (mM)	[bound bile salt][b] (mol/mol of EYPC)	k^c (h^{-1})
TDC	EYPC	0.04 ± 0.005	0.05 ± 0.005	0.4
TDC	EYPC	0.25 ± 0.01	0.15 ± 0.01	>4
TDC	EYPC/Ch (2/1 mol/mol)	0.4 ± 0.05	0.13 ± 0.02	0.06
TC	EYPC	0.22 ± 0.01	0.04 ± 0.005	0.3
TUDC	EYPC	0.5 ± 0.02	0.06 ± 0.005	0.25
GDC	EYPC	0.05 ± 0.005	0.04 ± 0.005	>4
GUDC	EYPC	0.19 ± 0.01	0.02 ± 0.002	>4

[a] Values are given as mean ± SD. [b] Free bile salt concentration at 24 h. [c] Rate constant derived from eq 1 (see text for details).

convergence of bile salt binding to one vs both hemileaflets at high bile salt concentrations as well as the observed increase in bile salt binding over time imply that bile salts gain access to both membrane hemileaflets. We have interpreted time-dependent changes in bile salt affinity for EYPC ± Ch vesicles as reflecting an increase in the accessible membrane area and infer from these data in combination with titration studies that fully ionized taurine conjugates undergo flip−flop. An intrinsic advantage of this experimental approach is that the system is not perturbed by addition of exogenous molecules. The dependence of the time course of bile salt/membrane binding on bile salt hydrophobicity, concentration, membrane composition, and temperature provide further support that increases in binding affinity represent membrane flip−flop.

The finding that bile salts flip−flop across membranes in a concentration-dependent fashion is not unexpected. At bile salt concentrations approaching those that solubilize membranes, bile salts must gain access to both membrane hemileaflets (*26*) in order to mediate observed increases in membrane permeability to large molecules (*1*, *40*, *41*), and ultimately, to solubilize membranes as mixed micelles. Changes in the outer membrane hemileaflet alone are predicted to be insufficient to alter membrane permeability to polar solutes, which depends on the composition of both the inner and the outer membrane hemileaflet (*31*). Indeed, osmotic water permeability can be estimated from the independent contributions of each hemileaflet (*31*). If structural changes induced by bile salts were confined to the external membrane hemileaflet, permeability would be predicted to increase by a factor of 2 at the most. However, we have previously demonstrated that bile salts increase membrane permeability to divalent cations by up to several orders of magnitude (*1*). We infer that bile salts must alter the structural organization of both inner and outer membrane hemileaflets.

Since glycine-conjugated bile salts rapidly translocate across the membrane as their protonated neutral species (*6*), the time dependence of binding of glycine-conjugated species serves as an internal control for our inference that membrane binding reflects available surface area. The lack of time-dependent changes in binding affinity of GUDC or GDC implies either that flip−flop occurs prior to the initial time point (15 min) or over a time scale much longer than 24 h. The latter is inconsistent with knowledge of the rapid flip−flop of other un-ionized species (*21*). Furthermore, the strong temperature dependence of flip−flop of taurine conjugates species is also consistent with relatively high energy intermediates.

For identical free bile salt concentrations, flip−flop is much faster for TDC than for TUDC (Figures 1 and 2). This is not unexpected given the much lower binding affinity of TUDC for lipid bilayers than TDC (*16*, *17*). However, Table 1 shows that, for comparable intramembrane bile salt mole

fractions, TDC flip−flop is only marginally faster than TUDC and TC. This would be consistent with differences in the interactions of their respective steroid nuclei with the acyl chains of phosphatidylcholine (*15*). As inferred from surface balance studies, the ursodeoxycholate steroid moiety "floats" more superficially on a phosphatidylcholine monolayer. A more peripheral location within the bilayer would be predicted to decrease the tendency for flip−flop.

Independent methods of measurements of bile salt flip−flop across membrane bilayers have been limited because of the inability to distinguish bile salts in one or both membrane hemileaflets. Hence, we were required to use an indirect method of observing bile salt flip−flop. Cabral and Small (*6*) took advantage of differences in the NMR behavior in the inner and outer hemileaflets to measure flip−flop rates of fully ionized unconjugated bile salts but were unable to observe bile salt migration from the outer to the inner bilayer over a 24-h period. There were several differences in experimental conditions as compared with the present work. The maximum bile salt/EYPC molar ratio studied by Cabral and Small was ≈0.08 (*6*), a value below the maximum used in the present studies (≈0.2). Moreover, NMR determinations of flip−flop rate were possible precisely because of differences in the NMR shifts and, hence, chemical potentials of phospholipid molecules in the highly convex outer monolayer and the extremely concave inner monolayer (*6*). Because membrane curvature significantly alters the chemical potential of membrane lipids (*34*), it is reasonable to infer that flip−flop rates may differ for the large unilamellar vesicles studied herein and small, highly curved sonicated vesicles. Because the radius of curvature of the plasma membrane and other intracellular organelles is larger than that of small unilamellar vesicles, large unilamellar vesicles are more likely to reflect properties of physiological membranes.

Membrane curvature clearly affects membrane permeability for a number of solutes. For example, Kamp and colleagues demonstrated that proton transport across the bilayer of small unilamellar vesicles occurred over approximately 10 min (*21*). However, in large unilamellar vesicles, pH gradients dissipate at least an order of magnitude faster (*25, 33*). Similarly, transmembrane flip−flop of fatty acids and their analogues as well as porphyrins differs in small and large unilamellar vesicles (*22*−*24*). Differences in the nature of membrane defects have been suggested for large as opposed to small unilamellar vesicles (*47*). Thus, there is ample precedent that both membrane permeability and the propensity for transmembrane flip−flop differ substantially for the highly curved small unilamellar vesicles used elsewhere (*6*) as compared to the large unilamellar vesicles used herein.

An alternative possibility to flip−flop across membranes is transient reorganization of membrane lipids into mixed micelles. With re-formation of vesicles, bile salts would be redistributed into both hemileaflets. At the highest total bile salt concentrations examined (0.86 mM, Figure 4), this is an alternative explanation. However, both the dependence of bile salt binding on temperature at the same total concentration (Figure 6) and the observations of a time-dependent increase in bile salt binding at total concentrations well below those that solubilize membranes (maximum TDC concentration 0.25 mM, Figure 4) make a transient phase transition unlikely.

The mechanism by which bile salts flip−flop across lipid bilayers is unclear. Schubert and colleagues (*41*) suggested that after the initial adsorption of bile salts, transbilayer reorganization occurs. From surface balance studies of unconjugated dihydroxy bile salts (*15*), we estimate that, at a bile salt/EYPC ratio of 0.1, the surface area of the outer hemileaflet would increase by approximately 9%. The known ability of bile acids to condense phospholipid monolayers (*15, 43*) is more than counterbalanced by the increase in absolute number of molecules in the hemileaflet. Disparity in surface area creates mechanical stress that reduces the $t_{1/2}$ for flip−flop of zwitterionic phosphatidylcholine to a few minutes (*35*). Schubert and colleagues suggest that, when cholesterol is present in the bilayer, rapid transmembrane flip−flop of cholesterol allows relaxation of mechanical stress induced by bile salts (*41*), a phenomenon that could in part account for the much slower rate of flip−flop observed for EYPC/cholesterol bilayers (see Table 1).

Rare formation of membrane defects could also allow equilibration of lipids in the outer and inner membrane hemileaflet. Wimley and Thompson (*47*) demonstrated that transbilayer movement of zwitterionic dimyristoylphosphatidylcholine had a $t_{1/2}$ of ≈ 1 h, and suggested that flip−flop may occur through spontaneous formation of "fluctuation defects". We and others have demonstrated that, at concentrations approaching those that solubilize membrane lipids, bile salts induce formation of large membrane defects that allow convective transport of large molecules such as carboxyfluorescein, inulin, and dextrans (*1, 16*). These defects are of a size to allow complete equilibration of entrapped molecules with the outer aqueous solution (*1*). We speculate that the rare formation of these defects could allow rapid lateral diffusion with equilibration of outer and inner membrane hemileaflets.

Membrane composition is likely to modulate bile salt flip−flop rates, as we observed experimentally for increases in cholesterol content. In the case of cholesterol, decreased membrane fluidity may be responsible in part for the decrease in flip−flop rate for cholesterol-containing membranes (Figure 5, Table 1). Membrane cholesterol is likely to prevent bile salt flip−flop at key physiological sites. For instance, the large net flux of bile salts across the cholesterol-rich canalicular membrane is mediated by ATP-requiring proteins (*30*), and flip−flop of bile salts back into the inner canalicular membrane would be extremely slow. In contrast, we speculate that at the endoplasmic reticulum membrane, which contains negligible amounts of cholesterol (*14*), conjugated bile salts may not require a specific "flippase". In the absence of the ileal bile acid transporter, bile acid malabsorption occurs (*49*), but residual absorption of conjugated bile salts may be mediated by non-protein mechanisms (*3*) that include rapid flip−flop of protonated glycine and unconjugated bile salts or slow flip−flop of taurine conjugates. The relatively high cholesterol content of intestinal brush border membranes may limit the quantitative importance of bile salt flip−flop. Nonetheless, even limited flip−flop may allow bile salts to mediate divalent cation absorption (*37, 38*).

Thus, fully ionized bile salts flip−flop across physiological membranes, albeit at a much slower rate than for glycine or unconjugated bile salts. We purposefully examined the effects of bile salts over a concentration range from micromolar concentrations likely to be present intracellularly (*42*)

to micellar concentrations present in the intestine. The goal of the present study is to determine the physicochemical processes that may occur under physiological conditions. As recently estimated by Setchell and colleagues (*42*), physiological hepatic bile salt concentrations are in the low micromolar range and increase during cholestasis. Since bile salt toxicity is observed at concentrations higher than physiological, we hypothesize that, within the range between physiological low micromolar concentrations and higher concentrations found in cholestasis, bile salts begin to interact with membranes and alter their fundamental barrier function. We speculate that the ability of fully ionized bile salts to gain access to both membrane hemileaflets may contribute to bile salt toxicity by creating transmembrane pathways that increase divalent cation permeability (*1*).

ACKNOWLEDGMENT

The authors appreciate stimulating and helpful discussion with Ariela Albalak, Ph.D.

APPENDIX I: MATHEMATICAL MODEL OF BILE SALT FLIP−FLOP

The time dependence of binding can be modeled by assuming that the available surface area of EYPC increases by a factor of 2 from time zero to infinite time. This assumes that the rate of flip−flop is slow as compared with the rate of bile salt partitioning into the outer membrane hemileaflet. This assumption is justified by rapid equilibration of other amphiphiles with membrane vesicles (*23*, *51*) and by our observation that GDC and GUDC binding is unchanged over a 24-h period (Figure 7). The rate of transmembrane transport in either direction is assumed to be first order with respect to the bile salt concentration in that hemileaflet. Thus, the net rate of accumulation of bile salts in the inner leaflet is

$$\frac{dC_{in}}{dt} = k_{flip}(C_{out} - C_{in}) \quad (2)$$

where C_{in} and C_{out} represent the concentration of bile salts in the inner and outer hemileaflet, respectively, and k_{flip} is the rate constant.

We assume that initially bile salts are only bound to the outer leaflet

$$K_{eq} = \frac{C_{out}}{C_f\left(\frac{[EYPC]}{2}\right)} \quad (3)$$

where K_{eq} is the equilibrium binding constant for bile salt binding to EYPC membranes, C_f is the free bile salt concentration, and [EYPC] is the total EYPC concentration. The effective concentration of EYPC is one-half of the total, since only the outer hemileaflet is assumed to be accessible.

Using the equations for mass balance:

$$C_f = C_t - C_{out} - C_{in} \quad \text{and} \quad C_b = C_{out} + C_{in} \quad (4)$$

where C_b is the total bound bile salt concentration, and rearranging, we obtain the expression

$$\frac{dC_{in}}{dt} = k_{flip}\left[\frac{(C_t - C_{in})}{1 + \frac{K \times EYPC}{2}}\frac{K \times EYPC}{2} - C_{in}\right] \quad (5)$$

Integration from $t = 0$, at which time $C_{in} = 0$, to time t yields an expression for C_{in}:

$$C_{in} = \frac{C_t K \times EYPC}{2(1 + K \times EYPC)}\left[1 - \exp\left[-2\frac{(1 + K \times EYPC)}{(2 + K \times EYPC)}k_{flip}t\right]\right] \quad (6)$$

substituting $\alpha = K \times EYPC = C_b^\infty/C_f^\infty$ where C_b^∞ and C_f^∞ denote the ratio at equilibrium, we obtain

$$\frac{C_b}{C_f} = \frac{(2 + \alpha) - \exp\left(-\frac{2(1 + \alpha)}{(2 + \alpha)}k_{flip}t\right)}{\frac{(2 + \alpha)}{\alpha} + \exp\left(-\frac{2(1 + \alpha)}{(2 + \alpha)}k_{flip}t\right)} \quad (7)$$

At $t = 0$, this simplifies to $1/2(C_b^\infty/C_f^\infty)$, and at $t = \infty$, this expression simplifies to C_b^∞/C_f^∞.

For typical values of C_b^∞/C_f^∞, the value of k_{flip} is within 15% of the value of k from eq 1. Hence, values derived from curves fitted to the simpler expression shown in eq 1 are used through this work.

REFERENCES

1. Albalak, A., Jackson, A. A., and Donovan, J. M. (1997) in *Bile Acids in Gastroenterology* (Hofmann, A. F., Paumgartner, G., and Stiehl, A., Eds.) pp 256−266, Kluwer Academic Publishers, Dordecht, The Netherlands.
2. Albalak, A., Zeidel, M. L., Zucker, S., Jackson, A. A., and Donovan, J. M. (1996) *Biochemistry 35*, 7936−7945.
3. Amelsberg, A., Schteingart, C. D., Ton-Nu, H. T., and Hofmann, A. F. (1996) *Gastroenterology 110*, 1098−1106.
4. Cabral, D. J., Hamilton, J. A., and Small, D. M. (1986) *J. Lipid Res. 27*, 334−343.
5. Cabral, D. J., and Small, D. M. (1989) in *Handbook of Physiology—The Gastrointestinal System III, Section 6* (Schultz, S. G., Forte, J. G., and Rauner, B. B., Eds.) pp 621−662, American Physiology Society, Waverly Press, Baltimore, MD.
6. Cabral, D. J., Small, D. M., Lilly, H. S., and Hamilton, J. A. (1987) *Biochemistry 26*, 1801−1804.
7. Carey, M. C. (1985) in *Sterols and Bile Acids* (Danielsson, H., and Sjövall, J., Eds.) pp 345−403, Elsevier, Amsterdam.
8. Carey, M. C. (1988) in *Bile Acids in Health and Disease* (Northfield, T. C., Jazrawi, R. P., and Zentler-Munro, P. L., Eds.) pp 61−82, Kluwer Academic, Dordrecht, The Netherlands.
9. Carey, M. C., and Hernell, O. (1992) *Sem. Gastrol. Dis. 3*, 189−208.
10. Carey, M. C., and Small, D. M. (1978) *J. Clin. Invest. 61*, 998−1026.
11. Cohen, D. E., Fisch, M. R., and Carey, M. C. (1990) *Hepatology 12*, 113S−121S.
12. Cohen, D. E., Leighton, L. S., and Carey, M. C. (1992) *Am. J. Physiol. 263*, G386−G395.
13. Donovan, J. M., and Jackson, A. A. (1993) *J. Lipid Res. 34*, 1121−1129.
14. Evans, W. H., and Hardison, W. G. M. (1985) *Biochem. J. 232*, 33−36.
15. Fahey, D. A., Carey, M. C., and Donovan, J. M. (1995) *Biochemistry 34*, 10886−10897.
16. Heuman, D. M., and Bajaj, R. (1994) *Gastroenterology 106*, 1333−1341.
17. Heuman, D. M., Bajaj, R. S., and Lin, Q. A. (1996) *J. Lipid Res. 37*, 562−573.

18. Heuman, D. M., Hylemon, P. B., Pandak, W. M., and Vlahcevic, Z. R. (1991) *Hepatology 14*, 920−926.
19. Heuman, D. M., Mills, A. S., McCall, J., Hylemon, P. B., Pandak, W. M., and Vlahcevic, Z. R. (1991) *Gastroenterology 100*, 203−211.
20. Hope, M. J., Bally, M. B., Webb, G., and Cullis, P. R. (1985) *Biochim. Biophys. Acta 812*, 55−65.
21. Kamp, F., Westerhoff, H. V., and Hamilton, J. A. (1993) *Biochemistry 89*, 11074−11086.
22. Kamp, F., Zakim, D., Zhang, F. L., Noy, N., and Hamilton, J. A. (1995) *Biochemistry 34*, 11928−11937.
23. Kleinfeld, A. M., and Storch, J. (1993) *Biochemistry 32*, 2053−2061.
24. Kuzelova, K., and Brault, D. (1995) *Biochemistry 33*, 9447−9459.
25. Lande, M. B., Donovan, J. M., and Zeidel, M. L. (1995) *J. Gen. Physiol. 106*, 67−84.
26. Lasch, J. (1995) *Biochim. Biophys. Acta 1241*, 269−292.
27. Lowe, P. J., and Coleman, R. (1981) *Biochim. Biophys. Acta 640*, 55−65.
28. MacDonald, R. C., MacDonald, R. I., Menco, B. P. M., Takeshita, K., Subbarao, N. K., and Hu, L. R. (1991) *Biochim. Biophys. Acta 1061*, 297−303.
29. Mazer, N. A., Benedek, G. B., and Carey, M. C. (1980) *Biochemistry 19*, 601−615.
30. Meier, P. J. (1995) *Am. J. Physiol. 32*, G801−G812.
31. Negrete, H. O., Rivers, R. L., Gough, A. H., Colombini, M., and Zeidel, M. L. (1996) *J. Biol. Chem. 271*, 11627−11630.
32. New, R. R. C. (1990) *Liposomes, A Practical Approach*, Oxford University Press, Oxford.
33. Priver, N. A., Rabon, E. C., and Zeidel, M. L. (1993) *Biochemistry 32*, 2459−2468.
34. Qiu, R. Z., and Macdonald, R. C. (1994) *Biochim. Biophys. Acta 1191*, 343−353.
35. Raphael, R. M., and Waugh, R. E. (1996) *Biophys. J. 71*, 1374−1388.
36. Rossi, S. S., Converse, J. L., and Hofmann, A. F. (1987) *J. Lipid Res. 28*, 589−595.
37. Sanyal, A. J., Hirsch, J. I., and Moore, E. W. (1994) *Am. J. Physiol. 266*, G318−G323.
38. Sanyal, A. J., Hirsch, J. I., and Moore, E. W. (1994) *Gastroenterology 106*, 866−874.
39. Schmucker, D. L., Ohta, M., Kanai, S., Sato, Y., and Kitani, K. (1990) *Hepatology 12*, 1216−1221.
40. Schubert, R., Beyer, K., Wolburg, H., and Schmidt, K. H. (1986) *Biochemistry 25*, 5263−5269.
41. Schubert, R., and Schmidt, K.-H. (1988) *Biochemistry 27*, 8787−8794.
42. Setchell, K. D. R., Rodrigues, C. M. P., Clerici, C., Solinas, A., Morelli, A., Gartung, C., and Boyer, J. (1997) *Gastroenterology 112*, 226−235.
43. Small, D. M. (1971) in *The Bile Acids, Vol. I* (Nair, P. P., and Kritchevsky, D., Eds.) pp 249−356, Plenum Press, New York.
44. Staggers, J., Hernell, O., Stafford, R. J., and Carey, M. C. (1990) *Biochemistry 29*, 2028−2040.
45. Stieger, B., Oneill, B., and Meier, P. J. (1992) *Biochem. J. 284*, 67−74.
46. Verkade, H. J., Vonk, R. J., and Kuipers, F. (1995) *Hepatology 21*, 1174−1189.
47. Wimley, W. C., and Thompson, T. E. (1991) *Biochemistry 30*, 1702−1709.
48. Wong, M. H., Oelkers, P., Craddock, A. L., and Dawson, P. A. (1994) *J. Biol. Chem. 269*, 1340−1347.
49. Wong, M. H., Oelkers, P., and Dawson, P. A. (1995) *J. Biol. Chem. 270*, 27228−27234.
50. Yousef, I. M., Barnwell, S., Gratton, F., Tuchweber, B., Weber, A., and Roy, C. C. (1987) *Am. J. Physiol. 252*, G84−G91.
51. Zucker, S. D., Storch, J., Zeidel, M. L., and Gollan, J. L. (1992) *Biochemistry 31*, 3184−3192.

BI9705927