Effects of Submicellar Bile Salt Concentrations on Biological Membrane Permeability to Low Molecular Weight Non-Ionic Solutes†

Ariela Albalak,‡,§ Mark L. Zeidel,⊥ Stephen D. Zucker,‡,§ Audrey A. Jackson,|| and Joanne M. Donovan*,‡,§,⊥

Department of Medicine, Harvard Medical School, Brigham and Women's Hospital, and Harvard Digestive Diseases Center, 75 Francis Street, Boston, Massachusetts 02115, Brockton/West Roxbury Department of Veterans Affairs Medical Center, 1400 VFW Parkway, Boston, Massachusetts 02132, and Laboratory of Epithelial Cell Biology, Renal-Electrolyte Division, University of Pittsburgh Medical Center and Oakland Department of Veterans Affairs Medical Center, Pittsburgh, Pennsylvania 15213

Received February 28, 1996; Revised Manuscript Received April 15, 1996⊗

ABSTRACT: Bile salts have been hypothesized to mediate cytotoxicity by increasing membrane permeability to aqueous solutes. We examined whether submicellar bile salt concentrations affect model and native membrane permeability to small uncharged molecules such as water, urea, and ammonia. Osmotic water permeability (P_f) and urea permeability were measured in large unilamellar vesicles composed with egg yolk phosphatidylcholine (EYPC) ± cholesterol (Ch) or rat liver microsomal membranes by monitoring self-quenching of entrapped carboxyfluorescein (CF). Ammonia permeability was determined utilizing the pH dependence of CF fluorescence. Submicellar bile salt concentrations did not significantly alter P_f of EYPC ± Ch or rat liver microsomal membranes. At taurodeoxycholate (TDC) or tauroursodeoxycholate concentrations approaching those that solubilized membrane lipids, CF leakage occurred from vesicles, but P_f remained unchanged. Higher bile salt concentrations (0.5−2 mM TDC) did not alter P_f of equimolar EYPC/Ch membranes. The activation energy for transmembrane water flux was unchanged (12.1 ± 1.2 kcal/mol for EYPC) despite the presence of bile salts in one or both membrane hemileaflets, suggesting strongly that bile salts do not form transmembrane pores that facilitate water flux. Furthermore, submicellar bile salt concentrations did not increase membrane permeability to urea or ammonia. We conclude that at submicellar concentrations, bile salts do not form nonselective convective channels that facilitate transmembrane transport of small uncharged molecules. These results suggest that bile salt-mediated transport of specific substrates, rather than nonselective enhancement of membrane permeability, underlies bile salt cytotoxicity for enterocytes and hepatocytes.

Bile salts are physiological detergents that interact with biological membranes in the hepatocyte, at the canalicular membrane, and in the intestine. Under pathophysiological conditions, bile salts are cytotoxic (Heuman et al., 1991; Lowe & Coleman, 1981; Schmucker et al., 1990; Yousef et al., 1987) and have been hypothesized to mediate hepatocyte damage in cholestatic liver diseases of diverse etiologies. Although the association of bile salts with phosphatidylcholine and cholesterol to form micelles has been well characterized (Cabral & Small, 1989; Carey & Small, 1978; Mazer et al., 1980), interactions of bile salts with lipid bilayers have received relatively scant attention (Schubert et al., 1983, 1986; Schubert & Schmidt, 1988, Small & Bourgès, 1966; Small et al., 1966a,b).

At concentrations approaching the critical micellar concentration (CMC),[1] bile salts have been shown to induce *transient* increases in membrane permeability for molecules ranging in size from the disaccharide raffinose to dextrans of 70000 Da (Schubert et al., 1983,1986; Schubert & Schmidt, 1988). However, subsequent work concluded that these findings represented an artifact of transient high local bile salt concentrations during mixing (Schubert et al., 1991). Bile salt-enhanced transmembrane flux of large molecules such as peptides has been reported, apparently independent of membrane solubilization (Gordon et al., 1985; Shao & Mitra, 1992). Bile salts also have been observed to increase membrane permeability to small ions, including calcium (Donovan et al., 1994; Maenz & Forsyth, 1984; Oelberg et al., 1988; Zimniak et al., 1991), protons (Zhao & Hirst, 1990a,b), sodium (Accatino & Gavilan, 1988), and ferrous ions (Sanyal et al., 1991,1992). Nonetheless, the physical chemical mechanisms by which physiological solutes traverse lipid bilayers in the presence of bile salts are unclear.

One hypothesis of how bile salts mediate effects on membrane permeability is that bile salts aggregate as dimers or larger structures within membranes, with formation of

† Supported in part by research funding from the Veterans Administration, and Research Grants DK 43955, DK 48217, and DK 02047, and Center Grant DK 34854 from the National Institutes of Health (U.S. Public Health Service).

* Correspondence should be addressed to this author at Brockton/West Roxbury Department of Veterans Affairs Medical Center, 1400 VFW Parkway, Boston, MA 02132.

‡ Harvard Medical School.

§ Brigham and Women's Hospital and Harvard Digestive Diseases Center.

|| Brockton/West Roxbury Department of Veterans Affairs Medical Center.

⊥ University of Pittsburgh Medical Center and Oakland Department of Veterans Affairs Medical Center.

⊗ Abstract published in *Advance ACS Abstracts,* June 1, 1996.

[1] Abbreviations: P_f, osmotic water permeability; EYPC, egg yolk phosphatidylcholine; Ch, cholesterol; CF, carboxyfluorescein; TDC, taurodeoxycholate; TUDC, tauroursodeoxycholate; CMC, critical micellar concentration; SDS, sodium dodecyl sulfate; GDC, glycodeoxycholate; P_{NH3}, ammonia permeability; P_{urea}, urea permeability.

S0006-2960(96)00497-7 CCC: $12.00

membrane pores of increasing size, and consequent disruption of membrane barrier function (Carey, 1985). These postulated aggregates have been termed "reverse micelles" in that their *hydrophilic* surfaces would be apposed, rather than their hydrophobic surfaces as in surfactant micelles that form in aqueous solution. However, experimental evidence that bile salts form reverse micelles has been limited to systems containing long chain alcohols or organic solvents (Fontell, 1972; Vadnere & Lindenbaum, 1982). It remains unproven whether bile salts can aggregate to form reverse micelles within lipid bilayers and thus, to create a functional hydrophilic domain within the membrane core.

We hypothesized that, if bile salts self-associate to form a hydrophilic milieu within the hydrophobic core of lipid bilayers, the rate at which small uncharged hydrophilic solutes traverse the bilayer would increase and, conversely, that lack of an increase in membrane permeability would imply that bile salts do not create a hydrophilic domain within biological membranes. Using fluorescent probes to measure intravesicular volume and pH, we have systematically studied the effect of bile salts on large unilamellar vesicle permeability to water and to the non-ionic species urea and ammonia.[2] We speculated that the presence of bile salts in both membrane hemileaflets may be required to effect changes in membrane permeability. Because fully ionized bile salts are believed to equilibrate, or "flip-flop", between the inner and outer membrane hemileaflets rather slowly ($t_{1/2}$ > 1 h) (Cabral et al., 1987; Kamp et al., 1993), we compared systems in which bile salts were added to either or both membrane hemileaflets. We demonstrate that the presence of hydrophobic or hydrophilic bile salts in one or both membrane hemileaflets does not increase membrane permeability to any of these uncharged molecules and infer that bile salts do not form nonselective membrane channels. These results suggest that bile salt cytotoxicity for enterocytes and hepatocytes may result from bile salt-induced transport of specific solutes rather than by formation of nonselective pores.

MATERIALS AND METHODS

Bile salts (Sigma, St. Louis, MO), grade I egg yolk phosphatidylcholine (EYPC, Lipid Products, South Nutfield, U.K.), and cholesterol (Nu-Chek Prep, Elysian, MN) were used as received. Sodium dodecyl sulfate (SDS) and 5(6)-carboxyfluorescein (CF) were obtained from Molecular Probes (Junction City, OR) and (Sigma Chemicals (St. Louis, MO), respectively. High-performance liquid chromatography (HPLC) (Rossi et al., 1987) (Beckman Instruments, Wakefield, MA) demonstrated that bile salt purity with respect to other conjugates was >98%. By thin-layer chromatography, bile salt purity and EYPC purity were >99% (Donovan & Jackson, 1993). All other chemicals were of highest reagent grade purity. Pyrex glassware was alkali washed in EtOH−2 M KOH (1:1, v/v), followed by 24 h acid washing in 2M HNO_3 and thorough rinsing in filtered, deionized, and glass-distilled water.

Vesicle Preparation. Unilamellar vesicles were prepared by co-precipitation of EYPC ± Ch (molar ratio 1:1) 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, 10 mM HEPES, pH 7.4) containing CF (1−10 mM). The lipid dispersion was extruded multiple times through two 0.1 μM Nucleopore filters in a high-pressure vesicle extruder (model HPVE-S, Sciema Technical Services, Ltd., Richmond, BC, Canada) (MacDonald et al., 1991). Mean vesicle diameter was confirmed by quasielastic light scattering to be 1050 ± 30 Å (Cohen et al., 1990; Donovan et al., 1991), a size sufficiently large as to obviate curvature effects. Vesicles were separated from extravesicular CF by rapid gel filtration chromatography (Pharmacia HR10/30 Superose 6, Pharmacia−LKB, Piscataway, NJ) and elution with 150 mM NaCl, 1 mM NaN_3, 10 mM HEPES, pH 7.4. In selected experiments, vesicles were resuspended and extruded in the presence of TDC or TUDC (0.1−0.25 mM). The intermicellar/intervesicular bile salt concentration was measured by centrifugal ultrafiltration (Donovan & Jackson, 1993) and used as the eluant during gel chromatography to ensure that vesicles were continuously exposed to bile salts and, hence, would contain bile salts in both the inner and outer membrane hemileaflets.

Liver microsomal membranes were isolated from fasted male Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, MA), as previously described (Zucker et al., 1994). Livers were resected and homogenized in cold 0.25 M sucrose, 10 mM Tris-HCl, 1 mM EDTA (pH 8.0) containing 10 mM CF, followed by centrifugation at 41 000*g* for 10 min at 4 °C. Extravesicular CF was removed by serial washes (80 000*g* × 25 min) until the supernatant appeared clear, generally after 5−6 cycles. Pelleted microsomes were resuspended in sucrose buffer (pH 7.4). Thus, microsomes were estimated to be >99% free of endogenous bile salts. Membrane orientation was maintained as shown by intact latency (97%) by mannose-6-phosphatase assay. All microsomal preparations were stored on ice and utilized within 6 h of preparation, over which time no significant change in latency was demonstrated. Microsomal diameter was determined by quasielastic light scattering and confirmed by transmission electron microscopy to be approximately 1650 Å. Protein was quantified by the Bio-Rad assay (Bio-Rad Laboratories, Hercules CA).

Bile Salt/Vesicle Binding. Large unilamellar EYPC or equimolar EYPC/Ch vesicles (1 mg/mL) were incubated for ~24 h at 37 °C with varying TDC or TUDC concentrations (0.1−2.0 mM). The aqueous concentration of bile salts was determined by centrifugal ultrafiltration at 37 °C (Donovan & Jackson, 1993), with individual bile salt species quantified by HPLC (Rossi et al., 1987). Binding of TDC to EYPC vesicles was also measured under conditions used to induce osmotic water flux (200 mM sucrose, 150 mM NaCl).

Quasielastic Light Scattering. Vesicle size (mean hydrodynamic diameter) was determined by quasielastic light scattering as previously described (Cohen et al., 1990; Donovan et al., 1991). Vesicle size and intensity of scattered light at a 90° angle during and after incubation with bile salts were also measured. Because vesicles are much larger than micelles, virtually all scattered light intensity is derived from vesicles (Cohen et al., 1990). Hence, scattered light

[2] Presented in part at the National Meeting of the American Gastroenterological Association, Boston, MA, May 19−22, 1993, and published in abstract form [Donovan, J. M., Albalak, A., Jackson, A. A., Zucker, S., & Zeidel, M. L. (1993) *Gastroenterology 104,* A896; Zucker, S. D., Yeon, H. B., Donovan, J. M., Zeidel, M. L., & Gollan, J. L. (1993) *Gastroenterology 104,* A1025].

intensity can be utilized as an estimate of the relative mass of vesicles during solubilization.

Osmotic Water Permeability. Initial experiments examined the effect of incubation time of bile salts and vesicles on membrane permeability. Vesicles were mixed with bile salts and, at various intervals, subjected to an osmotic gradient using stopped-flow techniques (*vida infra*). Because preliminary studies demonstrated that longer incubation times did not influence measured permeabilities, in most subsequent permeability studies with externally added bile salts, vesicles were preincubated with bile salts for 5 min at 37 °C. In selected experiments at higher bile salt concentrations, water permeability was measured after shorter times of incubation with bile salts, as noted below. Solutions were prepared by addition of a small volume (0.01−0.02 mL) of vesicles to a larger volume (∼3 mL) of buffer composed with the appropriate final bile salt concentration and sufficient anti-fluorescein antibody (Harris et al., 1990) to quench extravesicular CF. Thus, vesicles were never exposed to bile salt concentrations significantly greater than the stated final concentration. Moreover, due to the low final lipid concentration (∼0.01 mg/mL), the unbound bile salt concentration approximated the total bile salt concentration. For permeability studies with bile salts incorporated into both membrane hemileaflets, the appropriate aqueous bile salt concentration was always present following resuspension (*vida supra)*.

Osmotic water permeability (P_f) was determined in EYPC ± Ch vesicles or microsomes containing 10 mM CF by monitoring time-dependent changes in CF self-quenching by stopped-flow fluorimetry. Solution osmolality was determined by freezing point depression (Osmomette, Precision Systems, Natick, MA). Extravesicular osmotic pressure was increased abruptly to 400−500 mOsm by mixing CF-containing vesicles with an equal volume of solution containing 200−400 mM sucrose, 150 mM NaCl, and the appropriate bile salt concentration (1 mM NaN_3, 10 mM HEPES, pH 7.4) in a stopped-flow apparatus (SF.17MV, Applied Photophysics, Leatherhead, U.K.) (Harris et al., 1990; Zeidel et al., 1992a). Fluorescence was monitored at an excitation wavelength of 490 ± 1.5 nm, using an emission wavelength >515 nm, and temperature was maintained at 37 °C by a circulating water bath, except as noted. Control experiments demonstrated that the excitation and emission spectra of CF were unchanged by bile salt concentrations studied. The abrupt increase in extravesicular osmolality leads to water efflux, thereby increasing the intravesicular CF concentration and resulting in self-quenching of fluorescence. Preliminary experiments demonstrated that total fluorescence was linearly dependent upon intravesicular volume (Grossman et al., 1992; Zeidel et al., 1992a,c). Calculations of P_f were performed using MathCAD software (MathSoft, Cambridge, MA) (Grossman et al., 1992; Zeidel et al., 1992a,c). Water permeability is defined by:

$$dV(t)/dt = (P_f)(SAV)(MVW)[(C_{in}/V(t)) - C_{out}]$$

where $V(t)$ is the relative intravesicular volume as a function of time, P_f is the osmotic water permeability in cm/s, SAV is the vesicle surface area to volume ratio, MVW is the molar volume of water (18 cm^3/mol), and C_{in} and C_{out} are the initial concentrations of total solute inside and outside the vesicle, respectively. Results are expressed as the mean ± SD of two to four series of experiments, with each experiment representing the mean of five to ten individual stopped-flow injections. Comparisons between experiments were made by Student's *t*-test, with values of $p > 0.05$ considered nonsignificant.

In selected experiments to examine the effect of an increase rather than a decrease in vesicle volume on measured values of P_f, vesicles were exposed to a solution with lower osmolality (75 mM NaCl, 10 mM HEPES, pH 7.4). Osmotic water permeability of rat liver microsomes also was determined in the presence or absence of 1 mM HgCl or 1 mM *p*-CMBS (*p*-chloromercuribenzenesulfonate), agents that are known to inhibit water flux through protein water channels (Zeidel et al., 1992b).

Urea Permeability. EYPC vesicles prepared as described above were incubated for several hours with buffer containing 150 mM urea, 10 mM CF, 150 mM NaCl, 1 mM NaN_3, and 10 mM HEPES at pH 7.4. In the stopped-flow apparatus, vesicles were exposed abruptly to a solution containing 150 mM sucrose, 10 mM CF, 150 mM NaCl, 1 mM NaN_3 and 10 mM HEPES at pH 7.4, creating an abrupt transmembrane urea gradient. Diffusion of urea across the lipid bilayer induces an osmotic gradient that results in water efflux and CF self-quenching. Since transmembrane water flux is much more rapid than urea flux, the time course of fluorescence change reflects membrane urea permeability (Grossman et al., 1992; Lande et al., 1994; Priver et al., 1993). Urea permeability (P_{urea}) was calculated as

$$P_{urea} = (J_{urea})/[(SA)(\Delta c)]$$

where J_{urea} is the flux of urea across the membrane, SA is the surface area of the vesicle, and Δc is the difference in concentration of urea across the membrane (Grossman et al., 1992; Lande et al., 1994; Priver et al., 1993).

Ammonia Permeability. EYPC vesicles were prepared containing 0.1 mM CF, 150 mM NaCl, 1 mM NaN_3, and 10 mM HEPES at pH 7.4. At this concentration, CF self-quenching is minimal, and fluorescence intensity strongly depends upon pH. After equilibration at pH 6.8 for several minutes, vesicles were exposed abruptly to the same buffer that additionally contained 10 mM NH_4Cl. As NH_3 enters the intravesicular aqueous compartment and is protonated, intravesicular pH rises, and fluorescence increases. From the time course of the fluorescence changes, the permeability to ammonia was determined in a manner analogous to that for urea (Lande et al., 1994; Priver et al., 1993).

RESULTS

Bile Salt Binding to Large Unilamellar Vesicles. To interpret membrane permeability in light of the relative mole fraction of bile salts bound to the membrane, the partitioning of TDC and TUDC between large unilamellar EYPC ± Ch vesicles and the aqueous solution was examined. Figure 1 shows the dependence of membrane bound bile salt on the free aqueous concentration of TDC or TUDC for systems containing 1 mg of vesicles/mL. With increasing bile salt concentrations, bile salt/membrane binding increased monotonically, without any discontinuity that would suggest a phase transition or bile salt self-association within the membrane. Moreover, bile salt affinity for vesicles was not altered by sucrose concentrations used to induce osmotic water flux (200 mM sucrose, 150 mM NaCl; data not shown).

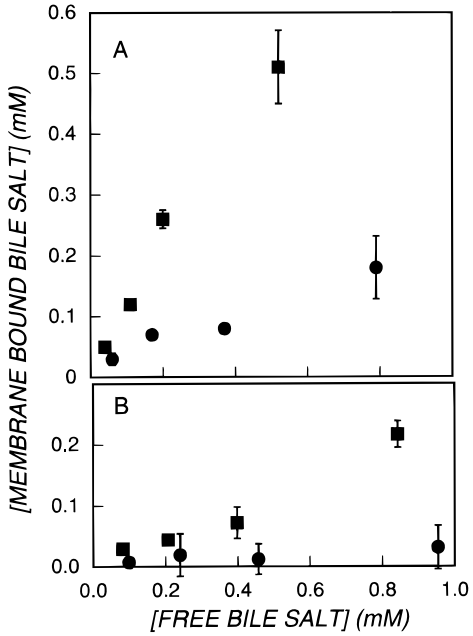

FIGURE 1: Binding of TDC (■) and TUDC (●) to (A) EYPC and (B) equimolar EYPC/Ch large unilamellar vesicles (1 mg of EYPC ± Ch/mL, 37 °C, 150 mM NaCl, pH 7.4) as determined by centrifugal ultrafiltration and HPLC measurement of unbound bile salt. At any unbound bile salt concentration, substantially more TDC than TUDC is bound to EYPC ± Ch vesicles. As compared to vesicles composed with EYPC alone, addition of Ch decreases membrane binding for both bile salts. Error bars represent SD, for $n = 4-8$.

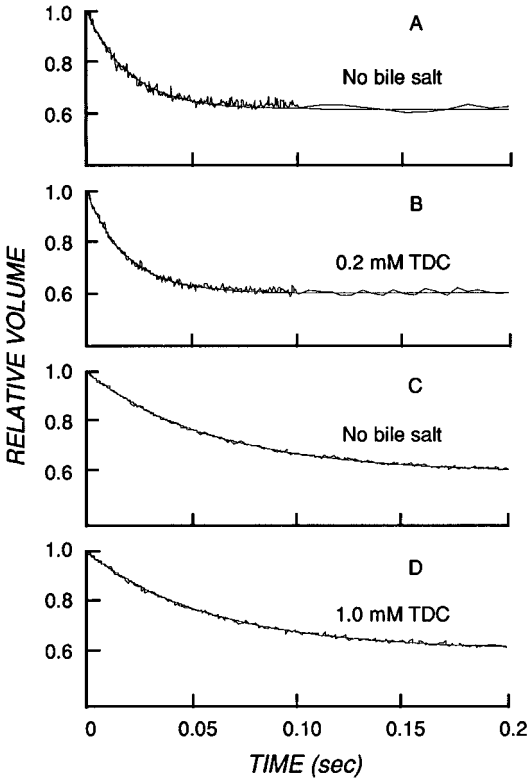

FIGURE 2: Effect of TDC on the time course of volume change of large unilamellar vesicles containing 10 mM CF upon exposure to an osmotic gradient inducing water efflux. Representative data (average of five to ten determinations) are displayed for (A) control EYPC and (C) equimolar EYPC/Ch vesicles as well as (B) EYPC and (D) EYPC/Ch vesicles that were preincubated with 0.25 and 1.0 mM TDC, respectively. A fitted single exponential curve is shown for each. Although the time course is more prolonged for EYPC/Ch vesicles, addition of TDC has no effect on the rate of volume change.

As has been observed previously (Bajaj & Heuman, 1993), TDC has a higher affinity for EYPC than does TUDC, and addition of Ch decreases the membrane binding affinity of both bile salts.

Effects of Bile Salts on Osmotic Water Flow. Figure 2 displays the time dependence of fluorescence after an increase in external osmotic pressure abruptly induces water efflux from the vesicles. As the vesicles shrink, intravesicular CF concentration increases and self-quenching occurs; hence, water efflux decreases fluorescence. From the difference between the initial and final osmolalities, the relative vesicle volume can be calculated and used to transform fluorescence to relative volume, as indicated on the vertical axis. Figures 2A and B show that the time course of water efflux from EYPC vesicles is not altered by the presence of 0.2 mM TDC. P_f was calculated to be 0.012 ± 0.003 and 0.011 ± 0.003 cm/s with and without 0.2 mM TDC, respectively. Similarly, Figures 2C and D demonstrate that the time course of water efflux from equimolar EYPC/Ch vesicles was unchanged by 1.0 mM TDC. P_f was 0.004 ± 0.001 cm/s for equimolar EYPC/PC vesicles at 37 °C, both with and without 1.0 mM TDC. QLS experiments in the absence of sucrose demonstrated that vesicles remained intact at these bile salt concentrations, with no appreciable change in size. Although P_f was lower for EYPC/Ch vesicles than for EYPC vesicles, P_f did not differ in in the presence of TDC. Thus, even at concentrations exceeding those estimated to be present under pathophysiological intracellular conditions (see Discussion), bile salts did not affect P_f of EYPC ± Ch bilayers.

We examined the possibility that higher bile salt concentrations would increase P_f. Because preliminary experiments demonstrated that a substantial fraction of CF had leaked out of the vesicles during the initial (5 min) incubation period with TDC concentrations ≥0.5 mM, we examined the behavior of vesicles immediately after exposure to bile salts. Figure 3 displays a representative series of curves recorded after EYPC vesicles were incubated with 0.7 mM TDC for varying periods of time. Similar results were obtained with 0.6 and 0.8 mM TDC (data not shown). The vertical axis displays fluorescence, whereas the time after abrupt exposure of vesicles to increased osmolality is shown on the horizontal axis. The curve at the top of Figure 3 was recorded after a 30 s incubation with TDC. At successive intervals over the ensuing 5 min, vesicle aliquots were exposed to an osmotic gradient, with the change in fluorescence displayed by individual curves in descending order. With increasing incubation time with TDC, CF leaked from the vesicles and was quenched by anti-fluorescein antibody. Hence, relative fluorescence values at $t = 0$ (y-intercepts, Figure 3) decrease with progressively longer TDC incubation times, and each successive curve is displaced downward with a diminished amplitude, corresponding to less intravesicular CF. However, as shown in Figure 4A, P_f is not altered by increasing time of previous incubation with TDC. Figure 4B displays the amplitudes of the fluorescence curves (closed circles) at various times after incubation with 0.7 mM TDC. In this figure, the ordinate represents the time of incubation with bile salt, rather than the time after osmotic shock, as was shown in Figure 3. The amplitude of fluorescence decreases exponentially with incubation time (rate constant of 176 s^{-1};

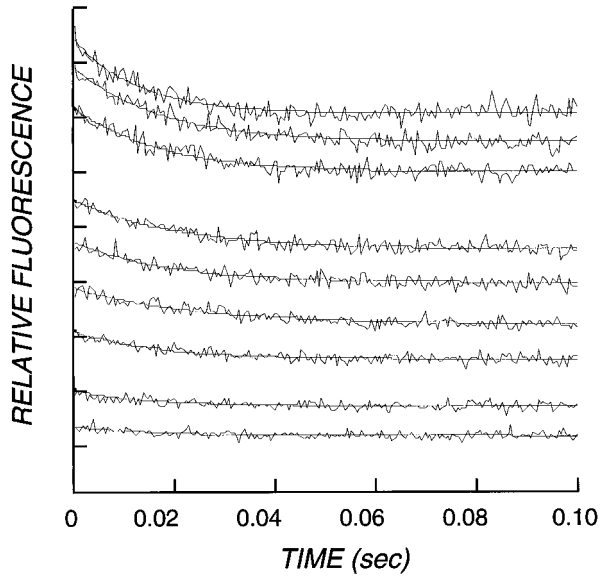

FIGURE 3: Fluorescence changes in vesicles containing 10 mM CF preincubated with 0.7 mM TDC and exposed to an osmotic gradient. The *x*-axis represents the time after exposure of vesicles to an osmotic gradient inducing water efflux and a concomitant quenching of intravesicular CF fluorescence. From top to bottom, curves represent approximately 30, 45, 60, 90, 120, 150, 210, 270, and 330 s of incubation with TDC. Each curve represents a separate determination of water permeability, after increasing duration of incubation with TDC. Immediately after exposure to TDC, absolute fluorescence is greatest, and the amplitude of the curve is greatest. With increasing time of exposure to TDC, absolute fluorescence decreases as CF leaks from the vesicles and is quenched by anti-CF antibody present in the external solution. The amplitude of each curve also decreases, as the total quantity of intravesicular CF declines. Despite a concentration of TDC high enough to cause leakage of CF, the time course of fluorescence remains constant, indicating that the rate of water flux across intact vesicles is unchanged. Individual fitted single exponential curves are also displayed.

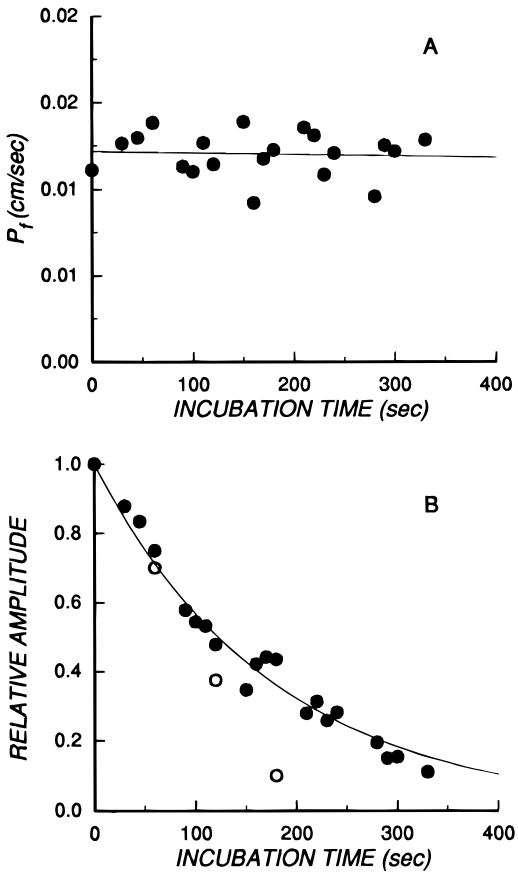

FIGURE 4: Effect of duration of incubation of EYPC vesicles (~0.01 mg/mL) with 0.7 mM TDC on (A) osmotic water permeability (P_f) and (B) amplitude of exponential decay of fluorescence change (●). Also shown is the time dependence of the intensity of scattered light at 90° (○). The decrease in relative amplitude reflects a decrease in total intravesicular CF as vesicles undergo solubilization into mixed micelles, as demonstrated by the decrease in scattered light intensity and confirmed by quasielastic light scattering determinations of mean hydrodynamic radius. However, P_f of the remaining intact vesicles is not altered by 0.7 mM TDC.

$r^2 > 0.98$ for single exponential fit). The intensity of scattered light declines with a comparable time course (open circles), suggesting that bile salts are solubilizing vesicles as mixed micelles, which was confirmed by QLS measurements of hydrodynamic diameter. Nonetheless, P_f of residual vesicles did not change during incubation with TDC sufficient to result in solubilization of >90% of vesicles. We conclude that even at bile salt concentrations high enough to solubilize vesicles, P_f remains unaltered.

Because of previous reports that bile salts cause transient increases in membrane permeability (Schubert et al., 1983, 1986; Schubert & Schmidt, 1988), CF-containing vesicles were simultaneously exposed to submicellar bile salt concentration (0.25 mM TDC) and an osmotic gradient to induce water flux. Again, P_f did not differ significantly from control measurements (data not displayed).

Values of P_f in EYPC (Figure 5A) and equimolar EYPC/Ch (Figure 5B) vesicles were determined over a range of bile salt hydrophobicities and concentrations. Neither TUDC, glycodeoxycholate (GDC), taurocholate nor the very hydrophobic bile salt taurolithocholate, significantly changed water permeability at concentrations up to the point where CF leakage occurred from EYPC vesicles ($p > 0.05$ for all comparisons with control). As was observed for EYPC vesicles, neither TDC nor TUDC at any concentration altered P_f of EYPC/Ch vesicles (Figure 5B). Bile salts also caused leakage of CF from equimolar EYPC/Ch vesicles, but at higher bile salt concentrations than with EYPC vesicles. Because of the possibility that changes in permeability occurred only when bile salts were present in both membrane hemileaflets, we examined vesicles prepared in the presence of varying concentrations of TDC and TUDC (aqueous concentration, 0.01−0.25 mM). Despite incorporation of bile salts into both membrane hemileaflets, P_f was unchanged from control values. Under all conditions shown in Figure 5, QLS demonstrated that vesicles remained intact, with no appreciable change in size. Thus, even with bile salt in both hemileaflets of the bilayer, increases in water permeability could not be demonstrated at bile salt concentrations approaching those at which vesicles are transformed into mixed micelles.

Effects of SDS. We examined P_f in the presence of SDS, an anionic detergent with a single 12-carbon length hydrophobic group, whose structure differs greatly from the steroid ring and carboxylic acid side chain structure of bile salts. When EYPC vesicles were preincubated with 0.35 mM SDS, P_f did not increase significantly (data not shown). At an SDS concentration of 3.5 mM, somewhat above the CMC (1.4 mM at 100 mM NaCl, 25 °C; Phillips, 1955), gradual leakage of CF could be observed, similar to studies with TDC shown in Figures 3 and 4, but again P_f was not signficantly increased from control values. Thus, another ionic detergent did not increase osmotic membrane permeability to water.

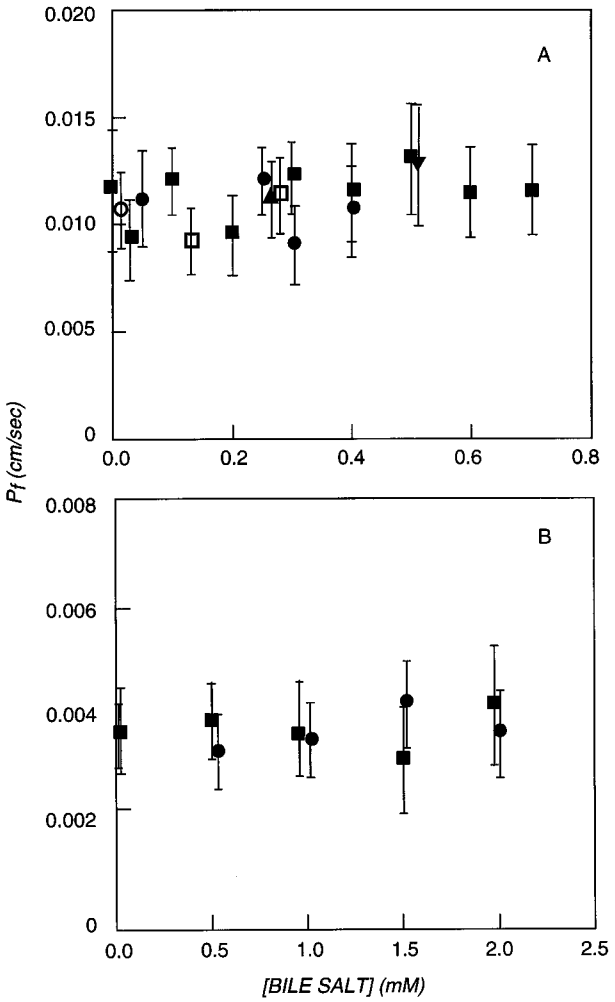

FIGURE 5: Osmotic water permeability (P_f) for (A) EYPC and (B) equimolar EYPC/Ch large unilamellar vesicles (~0.01 mg/mL) in the presence of various concentrations of (■) TDC, (●) TUDC, (▲) a 1/1 molar mixture of TDC and TUDC, (□) GDC, (▼) taurocholate, and (○) TLC. Bile salt concentrations were limited by leakage of CF (TDC, GDC) or by the limits of solubility (TLC). All studies were performed at 37 °C. Error bars represent SD of two to five separate experiments, and all differences between experimental means and control experiments in the absence of bile salts were not signficant.

Activation Energy of Transmembrane Water Flux. From measurements of water permeability at various temperatures (10−45 °C), the activation energy of osmotic water flow through EYPC vesicles was determined. Figure 6 demonstrates that the dependence of the natural logarithm of P_f on $1/T$ (K^{-1}) was linear under all conditions studied: without bile salts, in the presence of 0.25 mM TDC added externally, or in the presence of an aqueous concentration of 0.09 mM TDC during formation and isolation of the vesicles. From the slope of the linear dependence of P_f on $1/T$, the activation energy was calculated and is shown in Table 1. Without bile salts, the activation energy for EYPC bilayers was 12.2 ± 1.3 kcal/mol. The activation energies of water efflux did not differ significantly in the presence of externally added TDC, TUDC, or GDC or when TDC was incorporated into both membrane hemileaflets ($p > 0.05$ for all bile salts as compared with control). Thus, externally added bile salts do not appear to create a transmembrane pore with a low activation energy that allows for a nonselective increase in bilayer permeability.

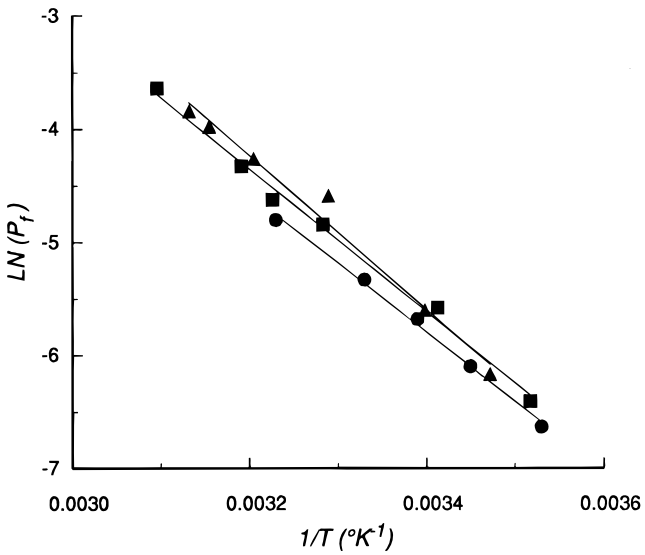

FIGURE 6: Arrhenius plot of the natural logarithm of the osmotic water permeability (P_f) as a function of the reciprocal of temperature (K^{-1}) for ~0.01 mg of EYPC vesicles/mL alone (●), with 0.25 mM TDC added externally (■), and with 0.09 mM TDC incorporated into both membrane hemileaflets (▲). The activation energy (E_a) can be determined from the slope, which is equal to $-E_a/RT$, where R is the gas constant (see Table 1). Neither the absolute value of P_f nor E_a is altered by TDC.

Table 1: Effects of Bile Salt Hydrophobicity and Hemileaflet Position on Activation Energy of Osmotic Water Permeability of EYPC Large Unilamellar Vesicles[a]

	bile salt concentration (mM)	method of preparation	activation energy (kcal/mol)	r^2
control	0.00		12.2 ± 1.3	0.99
TDC	0.09	incorporated in both hemileaflets	13.1 ± 1.5	0.99
TDC	0.25	added externally	13.5 ± 2.9	0.98
GDC	0.25	added externally	13.6 ± 2.4	0.99
TUDC	0.25	added externally	13.8 ± 2.4	0.98

[a] Conditions: 0.15 mM NaCl, pH 7.4. All bile salt concentrations are below the CMC (Cabral & Small, 1989).

Ammonia and Urea Permeabilities. Ammonia, as an uncharged species rather than the protonated ammonium ion, rapidly traverses the membrane and transiently raises intravesicular pH as protons are utilized to convert ammonia to ammonium ion, resulting in increased fluorescence. Figures 7A and B display the time course of fluorescence after ammonium chloride is added to 0.1 mM CF-containing EYPC vesicles in the presence or absence of 0.25 mM TDC. The ammonia permeability of EYPC vesicles (P_{NH3}) was not changed by 0.25 mM TDC (Figure 7B), GDC, TUDC, or cholate (data not displayed).

Urea permeability (P_{urea}) was also unchanged by the presence of TDC, either added externally (0.25 mM, as shown in Figure 7C and D) or incorporated into both membrane hemileaflets (aqueous concentration 0.05−0.1 mM; data not shown). Thus, bile salts did not alter membrane permeability to two additional low molecular weight uncharged species.

Water Flux in Hepatic Microsomes. Preliminary experiments in which CF-containing microsomes were exposed to increments of hyperosmolar media demonstrated that fluorescence intensity was linearly related to microsomal volume. At 37 °C, P_f of microsomes in the absence of bile salts was

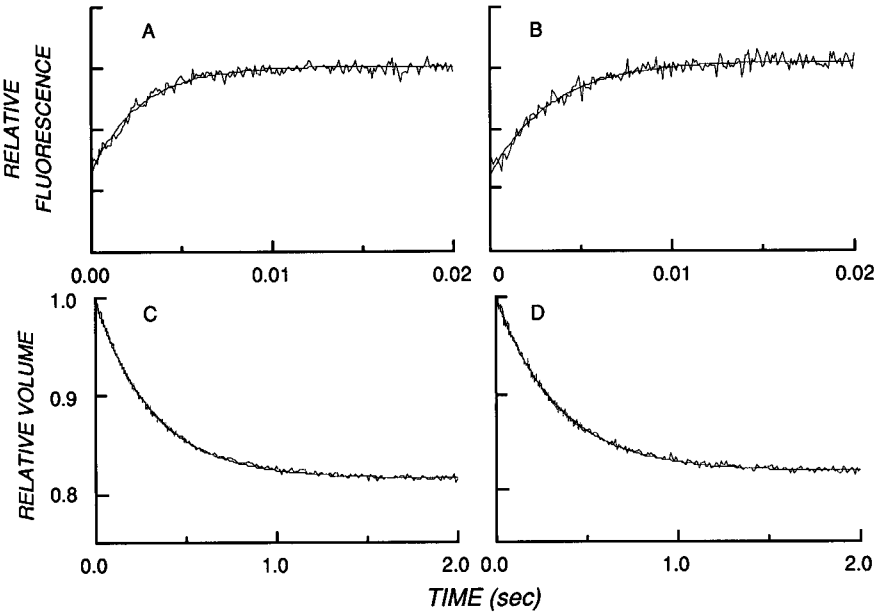

FIGURE 7: Time course of change in fluorescence of vesicles containing 0.1 mM CF at pH 6.8 as ammonia is added externally in the form of 10 mM NH_4Cl for (A) EYPC vesicles alone and (B) after preincubation with 0.25 mM TDC. NH_3 permeability was calculated to be (6.5 $\pm$ 2.0) $\times$ 10^{-2} cm/s in the presence of 0.25 mM TDC and (8.7 $\pm$ 2.5) $\times$ 10^{-2} cm/s in the absence of TDC. The time course of the change in relative volume of 10 mM CF-containing EYPC vesicles exposed to a urea gradient (C) in the absence of bile salts and (D) with 0.25 mM TDC. Urea permeability was calculated to be (5.4 $\pm$ 0.5) $\times$ 10^{-6} cm/s in the presence of 0.25 mM TDC and (5.0 $\pm$ 0.5) $\times$ 10^{-6} cm/s in the absence of TDC.

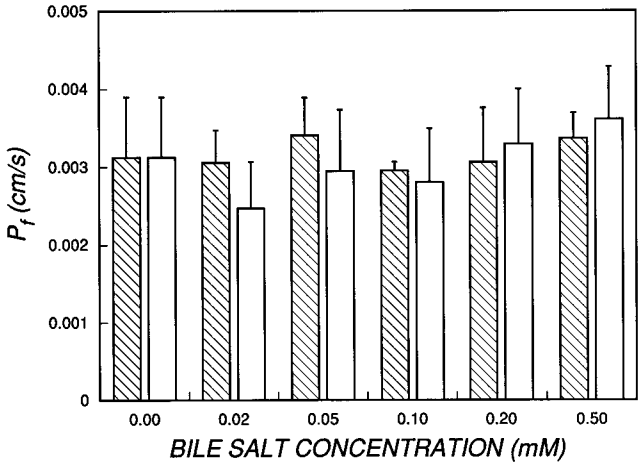

FIGURE 8: Effect of various concentrations of TDC (filled bars) and TUDC (open bars) on P_f of rat liver microsomal membranes at 37 °C. Neither bile salt significantly alters P_f of rat liver microsomal membranes. Error bars represent SD.

0.0039 $\pm$ 0.0005 cm/s, a value similar to that for EYPC + Ch vesicles. After preincubation with TDC or TUDC at concentrations up to 0.5 mM, microsomal P_f was not changed significantly from control values, as shown in Figure 8. Thus, as was observed for model EYPC $\pm$ Ch membranes, bile salts did not induce changes in water permeability in native microsomal membranes.

From an Arrhenius plot, we calculated the activation energy of P_f of microsomes to be 15.7 $\pm$ 0.6 kcal/mol (data not shown). Both values of P_f and the activation energy are consistent with water flux occurring primarily through the lipid bilayer, rather than via protein water channels (see Discussion). Moreover, the absence of any change in P_f after microsomes were incubated with known water channel inhibitors such as HgCl and *p*-CMBS (data not shown) (Zeidel et al., 1992a) implies that protein water channels are absent from hepatocyte endoplasmic reticulum.

DISCUSSION

We have systematically tested the hypothesis that at submicellar concentrations bile salts can form hydrophilic channels within the hydrophobic core of lipid bilayers and allow small aqueous solutes to traverse the membrane rapidly. Despite the use of techniques that can measure modest (>150% of control values) increases in water permeability, we were unable to demonstrate that bile salts facilitate convective water flow in biological membranes. Although membrane permeabilities of ammonia and urea differ substantially from that of water, with P_{NH3} an order of magnitude greater than P_f and P_{urea} three orders of magnitude less than P_f, bile salts did not increase membrane permeability to either of these solutes.

The studies described herein depend upon CF as a marker for intravesicular volume or pH. Because bile salts facilitate CF leakage from vesicle at concentrations approaching those at which membrane solubilization occurs (O'Connor et al., 1985; Walde et al., 1987), we are limited in our observations to vesicles that contain CF. Two mechanisms of CF release have been characterized for other synthetic surfactants (Nagawa & Regen, 1992), neither of which can be differentiated by the present studies: (1) bile salts induce complete release of CF from a fraction of vesicles, and (2) bile salts induce partial release of CF from the entire vesicle population. The possibility remains that larger membrane defects could simultaneously increase membrane permeability to water as well as other larger molecules. Therefore, at higher bile salt concentrations that induce CF leakage from large unilamellar vesicles, we limit our conclusions to the observation that vesicles that still contain CF do not display any change in water permeability. Indeed, it is likely that alterations in membrane structure at concentrations near membrane solubilization are responsible for the transient increases in permeability to larger molecules observed by some groups (Scharschmidt & Schmid, 1978; Schubert et

al., 1986, 1991). In the present study, vesicles were exposed to bile salt concentrations as close to final concentrations as possible, as we and others (Walde et al., 1987) have observed different degrees of CF release when concentrated bile salts are added to vesicles. We believe that the data shown here clearly exclude physiologically important increases in convective water flow at bile salt concentrations likely to be found within the hepatocyte.

Water Permeability through Membranes. The mechanism of water permeability through lipid membranes has been the subject of much interest (Finkelstein, 1987; Haines, 1994). The generally accepted model is that water molecules partition into the lipid bilayer and diffuse across the hydrocarbon core. The activation energy of this process is determined by the high-energy state of a water molecule within the hydrocarbon core, which is presumably the most unfavorable energy state during transmembrane water transfer. On the basis of on this model, a high (>10 kcal/mol) activation energy has been predicted and confirmed experimentally (Finkelstein, 1987).

According to this model, increased water permeability could occur by several mechanisms: (1) creation of a low-resistance pathway, a hydrophilic channel, through the membrane, (2) enhancement of partitioning of water into the hydrocarbon core of the membrane, or (3) increased diffusion through the hydrocarbon core. The minimum size of such a hydrophilic channel is a single unhydrated water molecule in diameter, approximately 1.8 Å, and is the size proposed for the low-resistance pathway for transmembrane water flux through CHIP28 (Jung et al., 1994), a protein in the aquaporin group. We infer that the absence of an increase in P_f or a decrease in the activation energy precludes formation of hydrophilic channels of any size by bile salts. Alternatively, if the hydrophilic hydroxyl groups directly interact with the hydrocarbon acyl chains, then the activation energy for transfer of a water molecule from the aqueous environment to the hydrocarbon membrane core would be reduced. Both the absence of a change in P_f or the activation energy and the evidence from surface balance studies that the hydrophilic surfaces of bile acids do not interact with the hydrocarbon chains of membrane lipids (Fahey et al., 1995) argue against this possibility. Moreover, the lack of effect of another anionic detergent, SDS, on osmotic water permeability suggests that at concentrations well below those that solubilize membranes, amphiphiles do not stabilize transient pores or channels in pure phospholipid membranes (Haines, 1994).

Osmotic water permeability is inversely proportional to membrane fluidity over a wide range of membrane compositions (Lande et al., 1995). Cholesterol decreases osmotic water permeability (see Figure 2; Finkelstein, 1987), possibly by decreasing membrane fluidity and inhibiting the lateral diffusion required to create transient pores or defects in the lattice structure. Although bile salts increase membrane fluidity slightly (O'Connor et al., 1985; J.M. Donovan, A. Albalak, A. A. Jackson, and M. L. Zeidel, unpublished data), we previously found that an increase of this modest degree was only associated with a 10%−20% increase in permeability (Lande et al., 1995).

Comparison of P_f in the Presence of Bile Salts and Aquaporins. In certain physiological membranes such as the erythrocyte membrane, transmembrane water flux occurs via a recently characterized class of proteins, termed the aquaporins. These protein water channels, such as CHIP28, allow single-file movement of water and increase membrane permeability by several orders of magnitude (Zeidel et al., 1992b). In contrast to our observations with bile salts, the activation energy of transmembrane water transport via aquaporins decreases to values of 3−4 kcal (Zeidel et al., 1992b).

We can compare the permeability of a bile salt molecule in an EYPC vesicle with the calculated permeability of a CHIP28 molecule. On the basis of measurements of osmotic water permeability of reconstituted proteoliposomes containing a known number of protein molecules, the permeability of a single molecule of CHIP28 has been calculated to be 1.17×10^{-13} cm^3/s (Zeidel et al., 1992b). The number of bile salt molecules per vesicle can be calculated from the bile salt binding to EYPC (Figure 1), the mean hydrodynamic radii, and the average surface area of EYPC, taken as 70 Å^2. Under experimental conditions studied (0.4 mM TDC), approximately 15 000 bile salt molecules are associated with each vesicle. Making the assumption that four bile salt molecules form a hydrophilic channel with the same permeability as a CHIP28 molecule, then the permeability of the vesicle would be

$$P_f = (\text{permeability of each tetramer}) \times (\text{number of tetramers})/(\text{surface area of a vesicle})$$

$$= (1.17 \times 10^{-13}\ \text{cm}^3/\text{s}) \times (15\,000/4)/(3.46 \times 10^{-10}\ \text{cm}^2) = 1.25\ \text{cm/s}$$

This value is ∼100-fold larger than the observed value of P_f at the highest bile salt concentrations studied. It should be noted that this calculation depends on the association number assumed for bile salt aggregates within the membrane. If eight bile salts are required to form a hydrophilic channel, then the calculated permeability would be 0.62 cm/s, a value 50-fold greater that the observed permeability. We conclude that the sensitivity of the technique used is sufficient to observe relatively modest, e.g., ≈2-fold, increases in water permeability and that bile salts do not form hydrophilic channels extending through lipid bilayers that are even one water molecule in diameter.

Pathophysiological Correlations. We purposefully examined the effects of bile salts over a concentration range from micromolar concentrations likely to be present intracellularly to micellar concentrations present in the intestine. Although intracelluar bile salt concentrations have not been directly measured, normal values are likely to be in the low micromolar range, based on bile salt binding affinity to cytosolic bile acid binding proteins (Sugiyama et al., 1983) and the canalicular bile acid transporter (Büchler et al., 1994), and bile salt-induced activation of the phosphatidylcholine transfer protein (Cohen et al., 1994). Bile salt hydrophobicity ranged from the extremely hydrophobic and cytotoxic monohydroxy bile salt taurolithocholate to the hydrophilic, cytoprotective TUDC. Despite previous observations that TDC increases proton permeability of brush border membranes at low concentrations (0.1 mM) (Zhao & Hirst, 1990a) water permeability was not altered in model or native membranes. In agreement with the present findings, Heuman and Bajaj (1994) did not find that bile salts induced leakage of inulin at concentrations below those that caused vesicle solubilization.

The negligible alterations in permeability to non-ionic species contrast with our observations that at similar concentrations, hydrophobic bile salts enhance membrane permeability to calcium and magnesium by several orders of magnitude (Donovan & Jackson, 1994; Donovan et al., 1994). Others have also observed increases in calcium permeability both *in vivo* and *in vitro* (Combettes et al., 1988; Oelberg et al., 1987; Spivey et al., 1993; Zimniak et al., 1991). We infer that any aggregation of bile salts that may occur within the membrane hemileaflet has specific functional implications and does not facilitate transport nonspecifically.

The position of bile salts within lipid bilayers most likely influences the nature of their interactions with aqueous solutes. Although X-ray diffraction studies suggest that the long axis of the steroid nucleus parallels the phospholipid acyl chains (Bourgès et al., 1967), NMR determinations of order parameters are more consistent with bile salts lying flat on the surface of the bilayer (Ulmius et al., 1982). More recent models of bile salt−phosphatidylcholine mixed micelles also support that bile salts are oriented parallel to the aqueous interface (Cohen et al., 1991; Hjelm et al., 1992; Nichols, 1990). We recently have demonstrated that in mixed monomolecular layers with phosphatidylcholine and cholesterol, bile acids are oriented with their sterol nuclei parallel to the interface (Fahey et al., 1995). Furthermore, surface balance studies also demonstrate that the fully ionized bile salt lithocholate is oriented with the long axis of the sterol nuclei parallel to the interface in a bile acid−phosphatidylcholine mixed monolayer (Bogle et al., 1995). These studies support our conclusion that bile salts do not self-associate perpendicular to the membrane to create a hydrophobic environment, or reverse micelle, within the membrane, and constitute a low-resistance pathway for osmotic water flux across the membrane.

In conclusion, even the most hydrophobic bile salts do not create reverse micelles within biological membranes that nonspecifically enhance membrane permeability to non-ionic species. In contrast, we have observed that under similar conditions, bile salts dramatically enhance membrane permeability to calcium and magnesium (Donovan & Jackson, 1994; Donovan et al., 1994). We infer that specific transport of physiologically important species such as divalent cations may underlie bile salt cytotoxic effects on enterocytes and hepatocytes.

ACKNOWLEDGMENT

We are indebted to Martin C. Carey, M.D., D.Sc., for many helpful and critical discussions and Howard Yeon for expert technical assistance.

REFERENCES

Accatino, L., & Gavilan, P. (1988) *Hepatology 8*, 898−903.
Bajaj, R., & Heuman, D. M. (1993) in *Bile Acids and the Hepatobiliary System* (Paumgartner, G., Stiehl, A., & Gerok, W., Eds.) pp 197−202, Kluwer Academic Publishers, Dordrecht.
Bogle, M. A., Leonard, M. R., Carey, M. C., & Donovan, J. M. (1995) *Gastroenterology 108*, A1037.
Bourgès, M., Small, D. M., & Dervichian, D. G. (1967) *Biochim. Biophys. Acta 144*, 189−201.
Büchler, M., Böhme, M., Ortlepp, H., & Keppler, D. (1994) *Eur. J. Biochem. 224*, 345−352.
Cabral, D. J., & Small, D. M. (1989) in *Handbook of Physiology: The Gastrointestinal System III, Section 6* (Schultz, S. G., Forte, J. G., & Rauner, B. B., Eds.) pp 621−662, American Physiology Society, Waverly Press, Baltimore, MD.
Cabral, D. J., Small, D. M., Lilly, H. S., & Hamilton, J. A. (1987) *Biochemistry 26*, 1801−1804.
Carey, M. C. (1985) in *Sterols and Bile Acids* (Danielsson, H., & Sjövall, J., Eds.) pp 345−403, Elsevier, Amsterdam.
Carey, M. C., & Small, D. M. (1978) *J. Clin. Invest. 61*, 998−1026.
Cohen, D. E., Chamberlain, R. A., Thurston, G. M., Benedek, G. B., & Carey, M. C. (1991) in *Bile Acids As Therapeutic Agents* (Paumgartner, G., Stiehl, A., & Gerok, W., Eds.) pp 147−150, Kluwer Academic, Lancaster, U.K.
Cohen, D. E., Fisch, M. R., & Carey, M. C. (1990) *Hepatology 12*, 113S-121S.
Cohen, D. E., Leonard, M. R., & Carey, M. C. (1994) *Biochemistry 33*, 9975−9980.
Combettes, L., Dumont, M., Berthon, B., Erlinger, S., & Claret, M. (1988) *FEBS Lett. 227*, 161−166.
Donovan, J. M., & Jackson, A. A. (1993) *J. Lipid Res. 34*, 1121−1129.
Donovan, J. M., & Jackson, A. A. (1994) *Hepatology 20*, 641.
Donovan, J. M., Timofeyeva, N., & Carey, M. C. (1991) *J. Lipid Res. 32*, 1501−1512.
Donovan, J. M., Jackson, A. A., & Zeidel, M. L. (1994) *Gastroenterology 106*, A885.
Fahey, D. A., Carey, M. C., & Donovan, J. M. (1995) *Biochemistry 34*, 10886−10897.
Finkelstein, A. (1987) *Water Movement through Lipid Bilayers, Pores, and Plasma Membranes*, John Wiley & Sons, New York.
Fontell, K. (1972) *Kolloid. Z. Z. Polym. 250*, 825−835.
Gordon, G. S., Moses, A. C., Silver, R. D., Flier, J. S., & Carey, M. C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7419−7423.
Grossman, E. B., Harris, H. W., Star, R. A., & Zeidel, M. L. (1992) *Am. J. Physiol. 262*, C1109−C1118.
Haines, T. H. (1994) *FEBS Lett. 346*, 115−122.
Harris, H. W., Kikeri, D., Janoshazi, A., Solomon, A. K., & Zeidel, M. L. (1990) *Am. J. Physiol. 259*, F366-F371.
Heuman, D. M., & Bajaj, R. (1994) *Gastroenterology 106*, 1333−1341.
Heuman, D. M., Mills, A. S., McCall, J., Hylemon, P. B., Pandak, W. M., & Vlahcevic, Z. R. (1991) *Gastroenterology 100*, 203−211.
Hjelm, R. P., Thigurajan, P., & Alkan, H. (1992) *J. Phys. Chem. 96*, 8653−8661.
Jung, J. S., Preston, G. M., Smith, B. L., Guggino, W. B., & Agre, P. (1994) *J. Biol. Chem. 269*, 14648−14654.
Kamp, F., Westerhoff, H. V., & Hamilton, J. A. (1993) *Biochemistry 89*, 11074−11086.
Lande, M. B., Donovan, J. M., & Zeidel, M. L. (1995) *J. Gen. Physiol. 106*, 67−84.
Lande, M. B., Priver, N. A., & Zeidel, M. L. (1994) *Am. J. Physiol. 267*, C367−74.
Lowe, P. J., & Coleman, R. (1981) *Biochim. Biophys. Acta 640*, 55−65.
MacDonald, R. C., MacDonald, R. I., Menco, B. P. M., Takeshita, K., Subbarao, N. K., & Hu, L. R. (1991) *Biochim. Biophys. Acta 1061*, 297−303.
Maenz, D. D., & Forsyth, G. W. (1984) *Digestion 30*, 138−50.
Mazer, N. A., Benedek, G. B., & Carey, M. C. (1980) *Biochemistry 19*, 601−615.
Nagawa, Y., & Regen, S. L. (1992) *J. Am. Chem. Soc. 114*, 1668−1672.
Nichols, J. W. (1990) *Biochemistry 29*, 4600−4606.
O'Connor, C. J., Wallace, R. G., Iwamoto, K., Taguchi, T., & Sunamoto, J. (1985) *Biochim. Biophys. Acta 817*, 95−102.
Oelberg, D. G., Dubinsky, W. P., Sackman, J. W., Wang, L. B., Adcock, E. W., & Lester, R. (1987) *Hepatology 7*, 245−252.
Oelberg, D. G., Wang, L. B., Sackman, J. W., Adcock, E. W., Lester, R., & Dubinsky, W. P. (1988) *Biochim. Biophys. Acta 937*, 289−299.
Phillips, J. N. (1955) *Trans. Faraday Soc. 51*, 561.
Priver, N. A., Rabon, E. C., & Zeidel, M. L. (1993) *Biochemistry 32*, 2459−2468.
Rossi, S. S., Converse, J. L., & Hofmann, A. F. (1987) *J. Lipid Res. 28*, 589−595.

Sanyal, A. J., Hirsch, J. I., & Moore, E. W. (1992) *Gastroenterology 102*, 1997−2005.
Sanyal, A. J., Shiffman, M. L., Hirsch, J. I., & Moore, E. W. (1991) *Gastroenterology 101*, 382−389.
Scharschmidt, B. F., & Schmid, R. (1978) *J. Clin. Invest. 62*, 1122−1131.
Schmucker, D. L., Ohta, M., Kanai, S., Sato, Y., & Kitani, K. (1990) *Hepatology 12*, 1216−1221.
Schubert, R., & Schmidt, K.-H. (1988) *Biochemistry 27*, 8787−8794.
Schubert, R., Jaroni, H., Schoelmerich, J., & Schmidt, K. H. (1983) *Digestion 28*, 181−190.
Schubert, R., Beyer, K., Wolburg, H., & Schmidt, K. H. (1986) *Biochemistry 25*, 5263−5269.
Schubert, R., Wolburg, H., Schmidt, K. H., & Roth, H. J. (1991) *Chem Phys Lipids 58*, 121−129.
Shao, Z. Z., & Mitra, A. K. (1992) *Pharm. Res. 9*, 1184−1189.
Small, D. M., & Bourgès, M. (1966) *Mol. Cryst. 1*, 541−561.
Small, D. M., Bourgès, M., & Dervichian, D. G. (1966a) *Nature 211*, 816−818.
Small, D. M., Bourgès, M. C., & Dervichian, D. G. (1966b) *Biochim. Biophys. Acta 125*, 563−580.
Spivey, J. R., Bronk, S. F., & Gores, G. J. (1993) *J. Clin. Invest. 92*, 17−24.
Sugiyama, Y., Yamada, T., & Kaplowitz, N. (1983) *J. Biol. Chem. 258*, 3602.
Ulmius, J., Lindblom, G., Wennerström, H., Johansson, L. B.-Å., Fontell, K., Söderman, O., & Arvidson, G. (1982) *Biochemistry 21*, 1553−1560.
Vadnere, M., & Lindenbaum, S. (1982) *J. Pharm. Sci. 71*, 881−883.
Walde, P., Sunamoto, J., & O'Connor, C. J. (1987) *Biochim. Biophys. Acta 905*, 30−38.
Yousef, I. M., Barnwell, S., Gratton, F., Tuchweber, B., Weber, A., & Roy, C. C. (1987) *Am. J Physiol. 252*, G84−G91.
Zeidel, M. L., Albalak, A., Grossman, E., & Carruthers, A. (1992a) *Biochemistry 31*, 589−596.
Zeidel, M. L., Ambudkar, S. V., Smith, B. L., & Agre, P. (1992b) *Biochemistry 31*, 7436−7440.
Zeidel, M. L., Hammond, T. G., & Harris, H. W. (1992c) *Am. J. Physiol. 263*, F62-F67.
Zhao, D. L., & Hirst, B. H. (1990a) *Dig. Dis. Sci. 35*, 589−595.
Zhao, D. L., & Hirst, B. H. (1990b) *Digestion 47*, 200−207.
Zimniak, P., Little, J. M., Radominska, A., Oelberg, D. G., Anwer, M. S., & Lester, R. (1991) *Biochemistry 30*, 8598−8604.
Zucker, S. D., Goessling, W., Zeidel, M. L., & Gollan, J. L. (1994) *J. Biol. Chem. 269*, 19262−19270.

BI960497I