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Structural Changes in Vesicle Membranes and Mixed Micelles of Various Lipid Compositions after Binding of Different Bile Salts†

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ABSTRACT: Binding equilibria of common bile salts (BS) and different mixtures of membrane lipids were correlated with BS-induced structural changes of large unilamellar vesicles, with transition of vesicles to mixed micelles (MM), and with successive transformations of MM. At very low BS concentrations, in the outer vesicle monolayer definite BS/lipid aggregates are formed, the size and BS binding strength of which depend on the BS and lipid species involved. At increasing BS concentrations, binding to the membranes is hampered, and above a critical BS content, membrane stress due to asymmetric BS binding leads to formation of transient membrane holes, as shown by inulin release from the vesicles. Independent of the BS and lipid species, membrane solubilization starts at a ratio $r = 0.3$ of bound BS/lipid. Increasing phosphatidylserine, phosphatidylethanolamine, and cholesterol contents stabilize the lecithin membrane against BS to different degrees and in different ways, whereas the destabilization by sphingomyelin is probably due to the enhancement of the membrane gel–liquid transition temperature. Conjugation of the BS with glycine or taurine has a modulating effect on membrane hole formation, rather than on lipid solubilization. Diphenylhexatriene fluorescence anisotropy indicates a BS-induced drop of the internal membrane order and its restoration during membrane solubilization. At higher concentrations ursodeoxycholate induces additional condensation, whereas the other BS cause internal disorder in the MM. Above ratios r of approximately 8:1, we found a release of BS from these MM and suggest a rodlike structure for them. The results were discussed with respect to BS/membrane interactions during lipid excretion from the liver cell.

Bile salts play an important role both in the absorption of dietary lipids from the intestine and in the excretion of cholesterol from the liver into the intestine. They are highly efficient in dissolving cholesterol with the help of lecithin to form mixed micelles, the primary species of lipid aggregates in bile. Ruetz et al. (1987) recently demonstrated that bile salt excretion from the liver cell at the canalicular surface domain is driven by a negative membrane potential and occurs via a carrier glycoprotein. Cholesterol and lecithin are probably excreted as vesicles. The mechanism and the site of solubilization of these vesicles, however, are unknown. After fusion of the vesicles with the canalicular part of the liver cell plasma membrane (cLPM),[1] bile salts may specifically extract lipids from distinct membrane areas or may solubilize vesicles after their budding into the canalicular space.

Moreover, bile salts possibly bind to membranes of cell organelles and, after excretion, especially to the outside of the cLPM and interact with several membrane lipids. As postulated for some cholestatic diseases, disarrangement of the physiologic bile salt composition or disturbance of membrane protecting mechanisms, therefore, can result in severe cellular dysfunctions.

For further elucidation of the mechanisms of membrane/bile

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[1] Abbreviations: LUV, large unilamellar vesicle; Chol, cholesterol; EYL, egg yolk lecithin; PE, phosphatidylethanolamine; EYPE, egg yolk phosphatidylethanolamine; PS, phosphatidylserine; BPS, bovine brain phosphatidylserine; Sph, sphingomyelin; BSph, bovine brain sphingomyelin; EYSph, egg yolk sphingomyelin; PSph, porcine erythrocyte sphingomyelin; [^{14}C]DPPC, [^{14}C]dipalmitoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; OG, *n*-octyl β-D-glucopyranoside; BS, bile salt; cholate, 3α,7α,12α-trihydroxy-5β-cholanic acid; GC, glycocholate; TC, taurocholate; DC, deoxycholate (3α,12α-dihydroxy-5β-cholanic acid); GDC, glycodeoxycholate; TDC, taurodeoxycholate; CDC, chenodeoxycholate (3α,7α-dihydroxy-5β-cholanic acid); GCDC, glycochenodeoxycholate; TCDC, taurochenodeoxycholate; UDC, ursodeoxycholate (3α,7β-dihydroxy-5β-cholanic acid); GUDC, glycoursodeoxycholate; TUDC, tauroursodeoxycholate; LC, lithocholate (3α-hydroxy-5β-cholanic acid); GLC, glycolithocholate; TLC, taurolithocholate; cLPM, canalicular part of the liver cell plasma membrane; ^{13}C NMR, carbon-13 nuclear magnetic resonance; ^{31}P NMR, phosphorus-31 nuclear magnetic resonance.

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salt interactions and the solubilization of bilayers to mixed micelles, we investigated the processes during the addition of common bile salts to vesicles of various lipid compositions. Equilibrium binding (Schurtenberger et al., 1985; Schubert et al., 1986, 1988) as well as changes in internal order and permeability of the membranes has been repeatedly investigated (O'Connor et al., 1985; Schubert et al., 1986) with defined combinations of bile salts and lipids. In this study, we present more detailed data of binding equilibria between bile salts and a variety of lipids as well as the consequences of bile salt binding on internal order and structural alterations of unilamellar vesicles and mixed micelles.

Materials and Methods

Vesicle Preparation. Large unilamellar vesicles (LUV) of homogeneous size were prepared according to the method of fast and controlled dialysis of mixed detergent/lipid micelles (Milsmann et al., 1978). Bile salts, cholesterol (Chol), phosphatidylserine from bovine brain (BPS), lecithin from fresh egg yolk (EYL), phosphatidylethanolamine from egg yolk (EYPE), and sphingomyelins from egg yolk (EYSph) and bovine brain (BSph) were purchased from Sigma Chemical Co., St. Louis, MO; sphingomyelin from porcine erythrocytes (PSph) was from SupelcoInc., Bellefonte, PA; *n*-octyl β-D-glucopyranoside (OG) was from Calbiochem GmbH, Frankfurt, FRG; radioactively labeled compounds were from NEN Chemicals, Dreieich, FRG. Chol and sodium cholate were recrystallized twice from methanol before use. Sphingomyelins were analyzed for their fatty acid composition by gas–liquid chromatography. The main fatty acids were 16:0 in EYSph, 18:0 and 24:1 in BSph, and 16:0, 24:0, and 24:1 in PSph. The mean chain length of fatty acids was calculated as 17.2 (EYSph) and 21.1 (BSph and PSph); the mean number of cis double bonds per molecule was calculated as 0.16 (EYSph), 0.51 (BSph), and 0.39 (PSph). Vesicles with a diameter greater than 80 nm had to be used for ultracentrifugation studies of binding equilibria. Vesicles from EYL/PE (7:3 mol/mol) and EYL/PS (7:3 mol/mol), therefore, were obtained by dialyzing mixed micelles with OG as detergent. The other vesicles were prepared with cholate as mixed micelle detergent.

Lipids were dissolved together with detergent in methanol. Solvent was removed completely under reduced pressure, and the dry lipid/detergent mixture was dissolved in phosphate buffer (10 mmol/L phosphate, 150 mmol/L NaCl, pH adjusted to 7.35) to yield a final lipid concentration of 17 mmol/L and a lipid/cholate ratio of 0.6 mol/mol or a lipid/OG ratio of 0.2 mol/mol. The mixed micelle solutions were dialyzed for at least 24 h against a continuous flow of phosphate buffer, by use of a commercially available dialysis apparatus (LIPOPREP, Diachema AG, Langnau, Switzerland) and a highly permeable dialysis membrane with a cutoff of 10000 daltons (Diachema). EYL/EYSph (6:4 mol/mol) vesicles were prepared at 40 °C and the other vesicles at room temperature. After dialysis, EYL/EYPE (7:3 mol/mol) oligolamellar vesicles were pressed through polycarbonate membranes with 100-nm pores (Nuclepore, Pleasanton, CA), on a suitable apparatus (EXTRUDER, Lipex, Vancouver, Canada), as described by Hope et al. (1985). The resulting unilamellar vesicles were dialyzed for an additional 12 h.

[^{3}H]Inulin was intrapped by adding the radioactive marker to the buffer used to dissolve the dry lipid–detergent mixture. Inulin not entrapped in vesicles was separated on Sepharose 4B-CL (Pharmacia Fine Chemicals AB, Uppsala, Sweden).

The formed vesicles appeared unilamellar on electron micrographs of freeze–fracture replicas and negatively stained samples. Vesicles were homogeneous in size, as determined by laser autocorrelation spectrometry (NANOSIZER, Coulter Electronics Ltd., Harpenden, U.K.).

Equilibrium Binding Studies. Binding equilibria of lipid vesicles and ^{3}H-labeled bile salts (BS) were studied with BS concentrations starting at 20 μmol/L. Lipids (total concentration 750 μmol/L) were labeled with [^{14}C]DPPC. The pH of incubations was 7.35 (for exceptions, see footnotes of Table II).

Up to BS concentrations at which the onset of membrane solubilization was detected, 2 mL of vesicle suspensions in different BS concentrations was ultracentrifuged in polycarbonate tubes (140000*g*, 210 min, 25 °C; Beckman L5-75, rotor Ti 50.3). Free BS concentrations were determined in the supernatants.

At higher BS concentrations, i.e., in the presence of mixed micelles, binding equilibria were studied by equilibrium dialysis at room temperature with highly permeable cellulose membranes (cutoff 10000 daltons, Diachema). Dialysis chambers were rotated for 4 h in a commercially available apparatus (Diachema).

At very high BS concentrations, in which the size of mixed micelles was to some extent smaller than the cutoff of the dialysis membrane, binding equilibria were determined according to a modified method first described by Hummel and Dreyer (1962). To a Sephadex G-50 column (0.9 × 25 cm; Pharmacia) equilibrated with the free BS concentration, the lipid solubilized in 1 mL of the same BS concentration was added. In the effluent, bound BS concentrations were calculated from the BS additional to free BS in the EYL fractions or from the trough in free BS concentration following the mixed micelle peak.

Determination of Vesicle Stability. BS-dependent vesicle stability was determined by ultracentrifugation. A total of 2 mL of suspensions containing LUVs of low lipid concentration (750 μmol/L) and varying BS concentrations was ultracentrifuged (140000*g*, 210 min, 25 °C). Release of radioactively labeled inulin and lecithin from vesicles was determined in the supernatants. All determinations were performed in duplicate. The results, which did not differ by more than 10%, were averaged. The observation that, during cholate-induced membrane solubilization, the appearance of [^{14}C]DPPC in the supernatants exactly coincides with a decrease in turbidity indicates that the radioactive lecithin mimicks the behavior of all glycerophospholipids. In parallel experiments, the course of the solubilization of [^{3}H]cholesterol and [^{14}C]DPPC or [^{14}C]sphingomyelin and [^{3}H]DPPC by cholate was also found to be identical.

DPH Fluorescence Anisotropy Measurements. DPH (1,6-diphenyl-1,3,5-hexatriene; Sigma) was suspended in phosphate buffer by tip sonication (2 min, 15 W, Branson Co., Danbury, CN) and mixed with vesicle suspension to yield a molar lipid/DPH ratio of 400:1. The mixture was incubated for 12 h at 30 °C in the absence of light. Thereafter, fluorescence leveled off. A total of 50 μL of DPH-labeled vesicle suspensions was mixed with 1.65 mL of bile salt solutions in phosphate buffer. Final BS concentration varied from 0 to 20 mmol/L. The lipid concentration was 150 μmol/L.

Steady-state anisotropy measurements were performed with an MPF-3 fluorescence spectrometer (Perkin-Elmer, Norwalk, CN) using polarizing filters in the excitation and emission light path. Samples were excited at 366 nm. $I_{\parallel}$ and $I_{\perp}$, fluorescence intensities parallel and perpendicular to the excitation beam, were recorded at 430 nm. Stray light was reduced by an

additional cutoff filter (390 nm). Fluorescence intensities were corrected for scattered light of unlabeled vesicle suspensions. Light scattering contribution to total $I_{\parallel}$ was less than 2%.

Fluorescence shifts caused by photoisomerization of DPH (Shinitzky & Barenholz, 1974) and an increase of sample temperature by irradiation were reduced by limiting exposure periods to excitation (no longer than 5 s). Temperature was held constant at 26 ± 0.5 °C, as recorded by a thermistor probe (Hellma GmbH, Müllheim, FRG) in the measured sample. Fluorescence measurements were performed 10 min after vesicles were mixed with detergent.

The steady-state fluorescence anisotropy r_s was determined as

$$r_s = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$$

The structural order parameter S_{DPH} was calculated according to van Blitterswijk et al. (1981).

Results

Stability of Vesicles against BS. In titration experiments, after sedimentation of remaining larger aggregates, the release of [^{3}H]inulin from preloaded vesicles and the solubilization of membrane lipids, mimicked by [^{14}C]DPPC, into mixed micelles were determined in the supernatants. In earlier studies (Schubert et al., 1986), we demonstrated that the primary part of inulin release is due to formation of transient and rapid-resealing membrane holes after BS addition, rather than to continuous permeation through a permanently disturbed membrane.

The stabilizing effect of cholesterol (Chol), gradually substituted for egg yolk lecithin (EYL), against the trihydroxy BS cholate, taurocholate (TC), and glycocholate (GC) is shown in Figure 1. Except for GC, which, in a narrow concentration range, induces inulin release from vesicles independent of their Chol content, the formation of membrane holes as well as membrane solubilization is hampered by Chol. Concentration ranges of BS between onset and completion of the membrane effects as well as concentrations which induce half-maximal effects increase with the Chol content of the membranes. Interestingly enough, in striking contrast to the GC effects, TC-induced formation of membrane holes at increasing Chol content is markedly delayed.

The release and solubilization curves show the same sigmoid character in nearly all investigated combinations of BS and membranes of different lipid composition. The inflection points of the curves roughly coincide with the half-maximal appearance of [^{3}H]inulin or [^{14}C]lecithin in the supernatants (for exceptions, see below).

In addition to Figure 1, BS concentrations that lead to half-maximal inulin release from vesicles or half-maximal solubilization of the vesicle membranes into mixed micelles are presented in Table I. Chol has a negligible membrane stabilizing effect against BS with two α-oriented hydroxy groups, i.e., deoxycholate (DC) and chenodeoxycholate (CDC) (Table IA). With increasing Chol content, essentially the same membrane perturbing BS concentrations are required.

The membrane effects of the stereoisomeric bile salts CDC and ursodeoxycholate (UDC) and their corresponding conjugates differ markedly. Higher concentrations of ursodeoxycholates with 3α,7β-dihydroxy configuration are needed to disturb pure EYL and Chol-containing membranes. Moreover, the solubilization of 20 and 30 mol % Chol containing membranes into smaller mixed micelles is incomplete. After ultracentrifugation of the slightly turbid suspensions, only part of the lipid was found in the supernatants, even at high concentrations of UDC and its conjugates (Table IA).

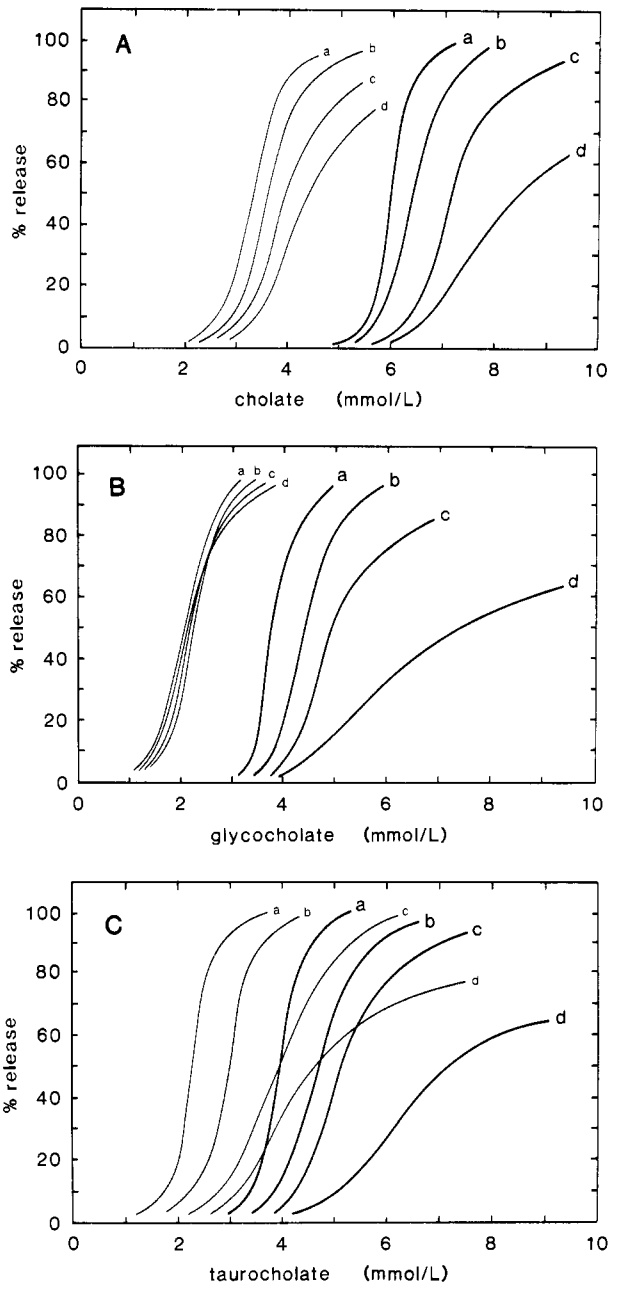

FIGURE 1: Inulin and lecithin release from cholesterol-containing vesicles induced by (A) cholate, (B) glycocholate, or (C) taurocholate. Total lipid concentration, 750 μmol/L. (a) Pure EYL; (b) EYL/Chol, 9:1 (mol/mol); (c) EYL/Chol, 8:2 (mol/mol); (d) EYL/Chol, 7:3 (mol/mol). (Thinner lines and smaller letters) [^{3}H]Inulin release; (thicker lines and larger letters) [^{14}C]DPPC release.

Compared to the configuration of the hydroxy groups, their number has a major influence on the BS effects in vesicle membranes. On the one hand, cholate and its conjugates as 3α,7α,12α-trihydroxy BS induce half-maximal release and solubilization at approximately a 5-fold concentration of the α,α-dihydroxy BS. On the other, the 3α-monohydroxy BS lithocholate (LC) and its conjugates induce no increase of inulin permeation or solubilization of the membranes up to their limit of solubility (considerably below 0.1 mmol/L at 25 °C).

Conjugation of BS with glycine or taurine generally results in a slight enhancement of their capacity to perturb membranes of different lipid compositions (see Table I, Figure 1). The only exception is glycoursodeoxycholate (GUDC), which causes membrane effects at higher concentrations than the

Table I: Bile Salt Concentrations (mmol/L) Inducing Half-Maximal Permeation (p) of Inulin and Solubilization (s) of Membrane Lipids into Mixed Micelles[a]

(A) Effects of Dihydroxy Bile Salts on Cholesterol- (Chol) Containing Egg Yolk Lecithin (EYL) Membranes

			mol % Chol in EYL membranes		
bile salt		pure EYL	10	20	30
DC	p	0.65	0.65	0.65	0.7
	s	1.2	1.3	1.3	1.3
GDC	p	0.43	0.50	0.55	0.60
	s	0.85	0.90	0.90	1.0
TDC	p	0.45	0.60	0.60	0.65
	s	0.85	0.90	0.90	1.0
CDC	p	0.62	0.71	0.67	0.68
	s	1.2	1.4	1.4	1.4
GCDC	p	0.46	0.56	0.57	0.61
	s	0.95	1.1	1.1	1.2
TCDC	p	0.47	0.51	0.57	0.61
	s	1.1	1.0	1.0	1.2
UDC	p	2.0	2.5	2.6	3.6
	s	4.4	4.8	5.4	5.4 (22%)
GUDC	p	2.6	3.6	4.2	5.1
	s	4.8	6.1	6.7 (50%)	8.0 (30%)
TUDC	p	2.2	2.7	2.8	2.9
	s	4.0	4.2	4.3 (30%)	4.4 (12%)

(B) Effects of Trihydroxy Bile Salts on EYL Membranes Containing Egg Yolk Phosphatidylethanolamine (EYPE)

			mol % EYPE in EYL membranes		
bile salt		pure EYL	20	50	70
cholate	p	3.2	3.8	4.9	6.9
	s	6.0	6.5	8.9	10.4
GC	p	2.3	3.0	4.0	5.0
	s	3.5	4.8	6.8	8.6
TC	p	2.3	2.8	3.6	4.5
	s	4.1	4.3	6.5	8.1

(C) Effects of Trihydroxy Bile Salts on EYL Membranes Containing Bovine Brain Phosphatidylserine (BPS)

		mol % BPS in EYL membranes			
bile salt		20	50	70	100
cholate	p	4.0	4.4	3.2	3.3
	s	6.5	7.0	6.6	6.8
GC	p	2.8	3.2	2.0	3.8
	s	4.3	5.3	4.8	7.3
TC	p	2.9	3.4	2.8	6.2
	s	3.9	6.8	6.2	8.0

(D) Effects of Trihydroxy Bile Salts on EYL Membranes Containing Egg Yolk Sphingomyelin (EYSph)

		mol % EYSph in EYL membranes			
bile salt		5	15	30	40
cholate	p	2.6	2.3	1.9	0.9
	s	4.8	4.5	4.1	3.6
GC	p	2.1	1.7	1.3	1.1
	s	3.5	3.4	3.1	3.7
TC	p	2.1	1.6	1.2	0.9
	s	3.6	3.3	2.8	3.7

[a] Total lipid concentrations, 750 μmol/L. Percent values in parentheses represent amounts of inulin or lipid obtained in supernatants at high BS concentrations (see text). If no percentage is cited, permeation and solubilization reach 100%.

nonconjugated UDC (see Table IA).

Partial substitution of EYL for other phospholipids effects membrane stability against cholate and its conjugates differently (Table IB–D). Compared to pure EYL, egg yolk phosphatidylethanolamine (EYPE) clearly stabilizes the membrane, whereas increasing egg yolk sphingomyelin (EYSph) content up to 30 mol % successively destabilizes against bile salt attack.

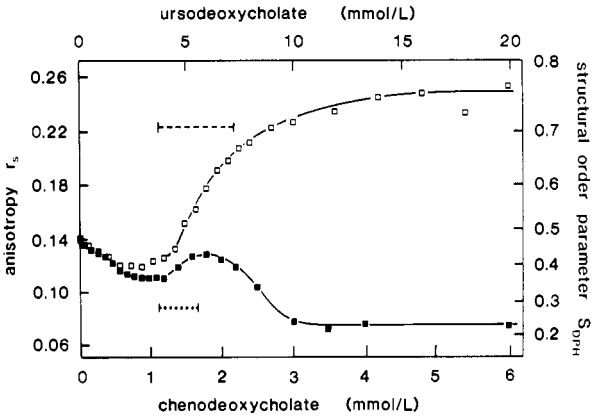

FIGURE 2: Changes in steady-state fluorescence anisotropy r_s of DPH and structure order parameters, S_{DPH}, in vesicle membranes and mixed micelles of EYL/Chol, 7:3 (mol/mol), at increasing content of (□) ursodeoxycholate or (■) chenodeoxycholate. (Dashed horizontal bar) UDC-induced vesicle solubilization; (dotted horizontal bar) CDC-induced vesicle solubilization.

It may be argued that different fatty acid residues of sphingomyelin (Sph), which lead to unequal length of the side chains, may contribute to the effect of EYSph in EYL membranes. Consequently, in parallel experiments, we used 30 mol % sphingomyelin from bovine brain (BSph) or porcine erythrocytes (PSph) instead of EYSph. Compared to EYSph, BSph and PSph have an enhanced mean length and a different degree of saturation of the fatty acid residues (see Materials and Methods). Both natural mixtures of Sph induce less pronounced destabilization effects than EYSph. Cholate and TC concentrations required for half-maximal inulin release or membrane solubilization were approximately 10% higher for PSph- and 20% higher for BSph-containing vesicles, whereas GC induced essentially the same effects in vesicles with the three different Sph (Schubert and Troschel, data not presented).

The effects of bovine phosphatidylserine (BPS) are not proportional to its content in EYL membranes. Stability increases to a maximum at 50 mol % BPS. After a drop at 70 mol %, pure PS membranes show enhanced stability, particularly against the conjugates of cholate. The stronger acidic TC induces nonsigmoid, nearly linear, release and solubilization curves at narrow concentrations (figures not presented).

Fluorescence Anisotropy Measurements. The internal order of various mixtures of membranes and BS was studied with DPH as a fluorescence probe. Figure 2 show the changes in the steady-state fluorescence anisotropy r_s of EYL membranes containing 30 mol % Chol at the addition of CDC and UDC.

The changes in r_s for all investigated lipid compositions are similar to the effect of CDC at the addition of other BS with two or three α-oriented hydroxy groups, i.e., cholate (Schubert et al., 1986), DC, and their conjugates. Initial r_s values are 0.14 for membranes containing EYL/Chol, 7:3 (mol/mol), (see Figure 2) and 0.095 for EYL/EYSph, 7:3 (mol/mol). Pure EYL membranes and membranes containing 30 mol % EYPE or BPS substituted for EYL have similar r_s values (~0.06). Initial r_s values decrease to a shallow minimum in the region of BS concentrations leading to inulin release (see Table I). An increase of r_s coincides with the onset of membrane solubilization, and r_s attains a maximum immediately after completion of solubilization. Continued addition of α,α-dihydroxy BS leads to a local disorder in mixed micelles and to lower limiting r_s values (ca. 0.07 or 0.045) if the lipid moiety of the mixed micelles is composed of EYL/Chol, 7:3

Table II: Equilibrium Binding Data of Bile Salts and Vesicles[a]

bile salt	lipid composition (mol/mol)	K_a (mol^{-1})	n	b	l	$[BS]_l$ (mol/L)	s	$[BS]_s$ (mol/L)	m	$[BS]_m$ (mol/L)
UDC[b]	EYL	9.5×10^3	0.031	16.1	0.01	150×10^{-6}	0.25	3.5×10^{-3}	4	31×10^{-3}
CDC	EYL	12.2×10^3	0.17	2.9	0.07	130×10^{-6}	0.36	1.1×10^{-3}	7	13×10^{-3}
cholate	EYL/PS, 7:3	2.1×10^3	0.126	4.0	0.05	300×10^{-6}	0.3	4.5×10^{-3}	8	26×10^{-3}
cholate	EYL/PE, 7:3	2.0×10^3	0.08	6.3	0.03	250×10^{-6}	0.3	5.0×10^{-3}	8	26×10^{-3}
cholate[c]	EYL/Chol, 7:3	2.8×10^3	0.042	11.9	0.014	180×10^{-6}	0.3	6.0×10^{-3}	7	18×10^{-3}
cholate[c]	EYL/Sph, 7:3	3.7×10^3	0.123	4.1	0.04	180×10^{-6}	0.27	2.5×10^{-3}	8	21×10^{-3}
cholate[d]	EYL	4.8×10^3	0.087	5.8	0.03	200×10^{-6}	0.3	4.0×10^{-3}	10	25×10^{-3}

[a] K_a, association constant; l, n, s, and m (mol/mol), definite values of the ratio r of bound bile salt/lipid; l, limit of the linear part; n, calculated initial binding site; b ($=1/2n$), lipids per bile salt in the outer monolayer at the initial binding site (see text); s, onset of membrane solubilization into mixed micelles; m, maximal ratio of bile salts bound to lipids in mixed micelles; $[BS]_l$, $[BS]_s$, and $[BS]_m$, bile salt concentrations to achieve binding ratios l, s, or m; lipid concentration in all samples, 750 μmol/L. [b] Since UDC solutions above 7 mmol/L gelatinize at pH 7.35 after several hours, equilibrium binding at higher concentrations was performed at pH 7.5. [c] Data from Schubert et al. (1988). [d] Data from Schubert et al. (1986).

(mol/mol), or mixtures of phospholipids.

Anisotropy characteristics at binding of UDC to membrane lipids show essential differences after the onset of solubilization. r_s increases beyond the initial value without a subsequent drop at higher BS content (see Figure 2). Also using pure EYL membranes, we found a corresponding increase of r_s (up to 0.16) after their total solubilization.

The effects of lithocholate and its glycoconjugate and tauroconjugate were investigated with EYL membranes containing 30 mol % Chol. Up to the limits of solubility (ca. 30 μmol/L for LC and GLC and 100 μmol/L for TLC), these three α-monohydroxy BS induce a decrease in r_s of approximately 10%.

Equilibrium Binding of Bile Salts to Vesicles. Binding equilibria were determined by ultracentrifugation, equilibrium dialysis, and gel chromatography (see Materials and Methods). A Scatchard plot for a wide range of CDC concentrations comprising the binding to pure EYL in vesicles and mixed micelles is presented in Figure 3A. The slope, which decreases up to a CDC concentration of 1.1 mmol/L (enlarged version in Figure 3B) and a ratio of bound cholate to membrane lipid of $r = 0.36$, is consistent with the binding of CDC to intact vesicles without detectable membrane solubilization.

The binding of cholate to intact vesicles is biphasic. The Scatchard relationship is essentially linear up to a limiting ratio l. An apparent maximal binding constant K_a may be calculated from the initial linear part with a distribution-free calculation method (Crabbe, 1985).

Extrapolation to the abscissa gives the number n of BS/membrane lipids in the initial bilayer binding site. Assuming that, in a first stage, BS at low concentrations bind exclusively to the outer membrane leaflet and that the rates of BS transbilayer movement (flip flop) are low (several hours; Cabral et al., 1987) compared to the time required to separate bound from free BS in the supernatant (~30 min), half the reciprocal n then gives the initial maximal number b of lipids associated with one BS molecule in the outer monolayer (see Table II).

We investigated the binding behavior of different BS to a variety of lipid compositions in LUVs and, after their solubilization at higher bile salt concentrations, to mixed micelles. The data for equilibrium binding measurements are summarized in Table II.

The Scatchard representations have a similar form in all investigated binding equilibria of different BS and lipid compositions. The deviation from linearity in the decreasing part of the curve above a limiting ratio l suggests that binding to lipid vesicles at higher BS concentrations is hampered. Above the onset of membrane solubilization at a molar ratio s of bound BS/total lipid, the shape of all plots indicates cooperative binding of BS to lipids in mixed micelles up to a maximal saturation ratio m. Enhancement of BS concentrations above the corresponding $[BS]_m$ leads to a decrease in the size of the mixed micelles (less than 10000 daltons), which then, to an increasing extent, permeate the dialysis membrane. Equilibrium binding of $[BS]_m$-exceeding concentrations, therefore, was determined by gel chromatography on Sephadex G-50 (see Materials and Methods). A decrease of r and $r/[BS]_{free}$ values with increasing BS concentrations suggests that [BS] above m are released from mixed micelles.

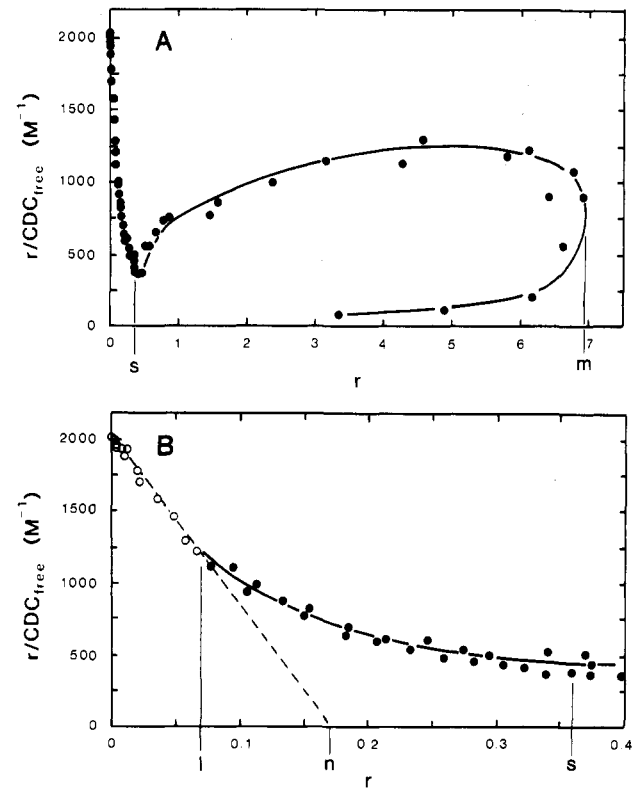

FIGURE 3: Scatchard plots of CDC/EYL binding equilibria starting from EYL vesicles (mean vesicle diameter, 80 nm; total EYL concentration, 750 μmol/L). (A) CDC concentration range up to 50 mmol/L; (B) enlarged version of a CDC concentration range up to 1.3 mmol/L. (r) Ratio of bound bile salt/lipid: (l) limit of the initial linear part; (n) extrapolated initial binding site; (s) onset of vesicle solubilization; (m) maximal ratio of bile salts/lipid in mixed micelles. Open circles represent binding data used to calculate initial binding of CDC to EYL vesicles.

Discussion

Our study reports on the different binding behavior of bile salts (BS) to various lipid compositions and the alterations induced in bilayers and mixed micelles. Large unilamellar

vesicles (LUV) are an excellent model for studying membrane disturbances induced by asymmetric bile salt binding. Moreover, with LUV, the transitions of BS/lipid mixed bilayers to mixed micelles can be easily correlated with the binding data.

In a ^{13}C NMR study, Cabral et al. (1987) determined very low rates of BS transbilayer movement (flip flop) of several hours at low concentrations and a deprotonated state of BS. On the basis of these findings, BS, after addition to unilamellar vesicles, are absorbed exclusively to the outer membrane monolayer. Consequently, the linear part of the Scatchard plots for our binding data indicates a definite initial aggregation behavior of BS to lipids in one monolayer. This "initial membrane binding site" of BS can be described by the binding strength between the BS and the lipids (quantified by the association constant K_a) and the number b of lipids per bile salt in this intramembranous aggregate. From the sterical prospective, low b values point to a tendency of BS toward self-aggregation in the membrane, whereas a condensing of membrane lipids around BS monomers may be concluded from high b values.

Our study shows that the initial binding strength of each dihydroxy BS, chenodeoxycholate (CDC) and ursodeoxycholate (UDC), to egg yolk lecithin (EYL) vesicles is essentially stronger than that of the more hydrophilic trihydroxy BS cholate. Moreover, we found the highest number of lipid molecules forming the initial binding site of UDC.

Compared to the 7α-hydroxy group of CDC and other common BS, the separation of the steroid moiety of UDC into hydrophilic and hydrophobic hemispheres is less pronounced. A self-aggregation of UDC molecules, therefore, is less probable than hydrophilic and hydrophobic contact with the surrounding lipids. CDC, by contrast, may form dimers oriented perpendicular to membrane surface and with the hydroxy groups facing each other (Schubert et al., 1986), in a similar manner as deoxycholate (DC) (Saito et al., 1983), whereas cholate is probably placed flat on the membrane surface, neither forming dimers nor inducing extensive lipid aggregation (Ulmius et al., 1982; Schubert et al., 1986).

Between a limiting binding ratio l and the onset of membrane solubilization at a ratio s, binding constants change with increasing BS content of the membrane. This deviation from linearity in the Scatchard plot coincides with an approximately linear relationship between r (=$[BS]_{bound}$/lipid) and $[BS]_{free}$. A corresponding constant equilibrium partition coefficient was first found by Schurtenberger et al. (1985) for BS binding to EYL vesicles. Our binding data corroborate a constant coefficient in all investigated combinations of BS and lipid mixtures. The accompanying experiments, however, clearly show that this model is limited to severely disturbed membranes.

Apparently, membrane disturbances start when BS in the outer monolayer are forced to share increasingly more lipids involved in BS binding. Consequently, enhanced self-aggregation of the BS molecules and changing orientations of lipids and BS are favored. Furthermore, the increasing differences in the number of molecules between the two membrane leaflets result, at a limiting membrane stress, in sudden membrane foldover (Schubert et al., 1986) during which membrane pores are transiently formed.

In earlier ^{31}P NMR studies we demonstrated that the bilayer signal is preserved at a BS/lipid ratio of $r = 0.28$ (Schubert et al., 1986). An additional narrow signal could be correlated with the appearance of smaller vesicles. Vesiculations are obviously caused by a decreasing lipid distance, which is demonstrated by DPH fluorescence anisotropy. Membrane defects and changes in membrane elasticity can serve as starting points for budding off of the membrane or fusion to multilamellar aggregates. Near a ratio r of 0.3, larger membrane holes are probably stabilized by the BS-induced difference in the areas of the head and chain moieties in the membrane (Petrov et al., 1980). This critical ratio is virtually independent of the head groups of the lipids and the hydrophilic moieties of the BS. In vesicles with increasing BS amount, therefore, hydrophilic interactions of BS with lipids gradually are replaced by contact of the hydrophobic hemisphere of the BS steroid backbone and the hydrocarbon part of the phospholipids.

Moreover, similar BS contents at the onset of membrane solubilization suggest a limiting structure of intact bilayers. Above a BS/lipid ratio of approximately 1:3, membrane holes, in which the inner edge is primarily formed by BS, begin to connect, and the arising islands leave the residual membrane. The solubilized bilayerlike aggregates are well characterized as BS/lipid mixed disk micelles, in which the outer edge is formed primarily by BS (Small, 1967; Mazer et al., 1976).

In DPH fluorescence anisotropy measurements, the fluorescent lifetimes of approximately 10 ns are nearly independent of a changing cone angle of DPH motion (Kawato et al., 1978). Therefore, even in mixed micelles of small size (6 nm, 30 000 daltons; Müller, 1981) with a corresponding rotational correlation time of 25 ns, steady-state fluorescence anisotropy can be correlated with internal order (Jähnig, 1979; van Blitterswijk et al., 1981).

In the concentration range of lipid solubilization, all investigated BS induce a restoration of the internal order of the lipid aggregates, a finding which supports the suggestion that BS-poor mixed micelles are dislike membrane fragments (Small, 1967; Mazer et al., 1976). Continued addition of UDC induces an ordering effect, whereas the common BS with 7α-hydroxy configuration lead to local disorder in mixed micelles during their first transformation from disks to ellipsoids above a molar BS/lipid ratio of 2:1 (Müller, 1981).

We found a second mixed micelle transition that results in a release of bound BS. The critical BS concentrations including this transition depend on the BS and lipid species involved, whereas the maximal BS/lipid ratio in mixed micelles is similar and virtually independent of the lipid composition or the hydroxylation degree of the 7α-hydroxy BS. A primarily hydrophobic contact between BS and lipids, therefore, is also indicated in these mixed micelles. Our studies show that approximately 10 000 daltons is a critical aggregate size for maximal BS binding. The composition of the aggregates can be calculated as 16–20 BS and 2–3 lipid molecules. The striking change of the aggregation behavior at this limit favors a discrete arrangement of paired phospholipids. We, therefore, suggest a model of mixed micelles with a maximal BS/lipid ratio of approximately 8:1, in which two phospholipids are oriented with their hydrocarbon tails toward each other. Two rings, each consisting of 8 BS molecules, are placed one on top of the other around this rod. Thereby, the hydrophobic hemispheres of their steroid backbones are in contact with the hydrophobic acyl chain core. Contact of additional BS to lipids then forces disaggregation of the lipid pair. Phospholipid monomers, however, obviously lose their bound BS molecules.

One intriguing finding is the different stability of binary mixtures of EYL with other phospholipids or cholesterol against BS (see Table I). A direct correlation betwen internal order and stability of the membrane, as suggested by O'Connor et al. (1985), is valid only for Chol-containing EYL mem-

branes. The reduced stability of EYL/Sph vesicles with enhanced structural order and increased stability of EYL/PS and EYL/PE vesicles, in spite of an internal order similar to that of pure EYL vesicles, fail to substantiate this suggestion. Moreover, on the basis of binding data of cholate to 30 mol % Sph- or Chol-containing EYL vesicles, we assumed that the stability of membranes against cholate depends primarily on the number of lipids involved in the initial binding site (Schubert et al., 1988). This assumption, however, does not apply to PE- and PS-containing vesicles. The different K_a values point to a head-group or hydration-dependent difference in the absorption of low BS concentrations to various membranes, in PS-containing mixtures probably to an initial electrostatic repulsion. Electrostatic effects may also contribute to the discontinuous course of membrane stabilization against BS at increasing PS content (see Table IC). The induced effects in membranes of other lipids at elevated BS content, however, are probably influenced by interactions deeper in the membrane. The condensing effect of Chol (Demel & de Kruijff, 1976) can lead to reduced BS insertion into the membrane. In addition, after BS absorption to vesicles, the membrane stress induced by asymmetric distribution of molecules between the two bilayers can be rapidly compensated by high flip-flop rates of Chol (Lange et al., 1977) to the inner leaflet. The membrane foldover as well as BS redistribution to the vesicle interior, therefore, can be delayed.

Compared to that of pure EYL, the low initial BS binding strength in membranes containing egg yolk phosphatidylethanolamine (EYPE) is probably due to differences in the hydration of EYL and EYPE (Rand, 1981; Lis et al., 1982). The reason for the membrane stabilizing effect of EYPE at elevated BS content was clearly demonstrated by Madden and Cullis (1981). On the basis of ^{31}P NMR measurements, they suggested that the inverted cone shape of absorbed detergents compensates the bilayer destabilizations initially induced by the conical shape of EYPE.

In Sph-containing EYL membranes, different acyl chain length and degree of saturation modify the BS effects. Essential for the reduced membrane stability, however, are hydrogen bonds that increase the gel–liquid transition temperature T_m of the membrane (Shipley et al., 1974). At about 25 °C, as determined by DPH fluorescence anisotropy, a mixture of EYL/EYSph, 6:4, is in the middle of its broad main transition, whereas the main transition of a 7:3 mixture is just completed (Schubert and Troschel, unpublished results). This then suggests enhanced BS-induced membrane damage in the liquid crystal phase near the transition to the gel phase, whereas phase separations of sphingomyelins and lecithins at the liquid–gel transition (Untracht & Shipley, 1977) hamper BS-induced membrane conformation changes.

Our data may contribute to the discussion on the stability of the canalicular part of the liver cell plasma membrane (cLPM) against BS and on the mechanism of lipid excretion into the bile. An essentially enhanced lipid/protein ratio in the cLPM, compared to that in the basolateral part of the liver cell plasma membrane (blLPM) (Meier et al., 1984), suggests that, in the cLPM, a considerable part of the membrane lipids is less involved in interactions with membrane proteins and may form free liquid crystal phases.

Similar to other eukaryotic cell plasma membranes (Op den Kamp, 1979), PS is probably restricted to the cytoplasmic monolayer of the cLPM. Interactions of BS with PS, therefore, are plausible only after severe membrane damage or pathologic situations, when BS binding capacity of specific cytosolic proteins is exceeded (Sugiyama et al., 1983). In contrast, elevated amounts of PE and Chol in the lecithin matrix of the outer cLPM leaflet, which is exposed to high BS concentrations in the luminal space of the canaliculi, are able to hamper BS binding to the membrane and to delay the formation of membrane holes.

Sphingomyelin is not suitable for stabilization of membranes against BS, as was suggested by Coleman et al. (1980) for erythrocytes and Kremmer et al. (1976) for the cLPM. High Sph contents in the cLPM (Meier et al., 1984), however, may locally enhance the membrane melting temperature and, through interaction with BS, may be evolved into local destabilizations during fusion or budding events for lipid excretion.

Lecithin and Chol are probably delivered from the liver cell by the action of a microtubular system (Gregory et al., 1978). Our data, however, do not support fusion of cytosolic vesicles with the cLPM and subsequent selective solubilization of lipids by BS (Yousef & Fischer, 1975). On the one hand, at BS concentrations far below membrane solubilization, the membrane would become permeable, which would result in severe cell damage. On the other, we could detect no specific solubilization of lipids from membranes by any of the investigated BS. The more plausible lipid excretion mechanism, therefore, is a rapid transport of Chol- and lecithin-enriched vesicles through the cLPM.

Excreted lecithin, which of all investigated glycerophospholipids requires the lowest BS concentration to form mixed micelles, has different functions. In addition to Chol solubilization, it reduces, by cooperative binding, the free concentration of BS that otherwise would bind to the cLPM.

Conjugation of the BS carboxy groups with glycine or taurine before excretion from the liver cell may have an essentially different influence on membrane hole formation; it, however, does not appreciably enhance the capacity of BS for solubilization of membrane lipids. This is valid for all investigated common BS. Decreased BS protonation by conjugation may be much more important and may reduce BS redistribution by flip flop into the liver and epithelial cells from the bile. A mixture of BS with different degrees of hydroxylation seems to balance the total and rapid solubilization of excreted lipid as well as the limited action onto the cLPM. In contrast to the excreted lipid, the lipids of the cLPM probably have to be protected against BS attack by proteins or sugar surfaces. Even a small moiety of membrane-bound BS can decrease the internal order, which subsequently may influence the activity of integral proteins. The monohydroxy bile salts LC, TLC, and GLC do not induce essential leakiness of membranes up to their limit of solubility. Their cholestatic effect, therefore, may partially be explained by membrane disordering and an excessive counterregulation by enhanced Chol content.

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Registry No. Chol, 57-88-5; GC, 475-31-0; TC, 81-24-3; DC, 83-44-3; GDC, 360-65-6; TDC, 516-50-7; CDC, 474-25-9; GCDC, 640-79-9; TCDC, 516-35-8; UDC, 128-13-2; GUDC, 64480-66-6; TUDC, 14605-22-2; LC, 434-13-9; GLC, 474-74-8; TLC, 516-90-5; cholate, 81-25-4.

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