Molecular species composition of inter-mixed micellar/vesicular bile salt concentrations in model bile: dependence upon hydrophilic–hydrophobic balance

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Abstract We systematically studied the bile salt (BS) species composition of the intermixed micellar/vesicular (non-lecithin-associated) BS concentrations (IMC) of model biles using physiological biliary compositions prepared with mixtures of 12 common BS of humans. BS distributions in the IMC, which was composed of monomers plus simple micelles, were determined by rapid centrifugal ultrafiltration and/or modified equilibrium dialysis as functions of total lipid concentration, BS/lecithin ratio, cholesterol content, pH, and the weighted hydrophobic index of BS species. IMC values increased from 3 to 9.5 mM with increases in total lipid concentration and BS/lecithin ratio, but decreased appreciably as the overall BS composition of physiological BS mixtures became more hydrophobic. However, IMC values were not altered by increases in cholesterol content (0–10%) that induced a phase transition from a one-phase micellar system to a two-phase system of micelles and vesicles. As pH values were decreased (8 to 5), with partial protonation but not precipitation of glycine-conjugated BS (pK'a ≈ 4.3 by titration), IMC values decreased slightly. For all model biles studied, IMC values of BS mixtures were markedly smaller than those previously found for model biles composed with taurocholate (Donovan, J. M. et al. 1991. *J. Lipid Res.* **32:** 1501–1512). Hydrophilic BS were preferentially distributed in the IMC, whereas hydrophobic BS were preferentially associated with lecithin in mixed micelles and vesicles. Hence, BS composition, in addition to total lipid composition and BS/lecithin ratio, is a critical determinant of the relative and absolute concentrations of the BS species in the inter-mixed micellar/vesicular bile salt concentration.—**Donovan, J. M., A. A. Jackson, and M. C. Carey.** Molecular species composition of inter-mixed micellar/vesicular bile salt concentrations in model bile: dependence upon hydrophilic–hydrophobic balance. *J. Lipid Res.* 1993. **34:** 1131–1140.

Supplementary key words vesicles • micelles • cholesterol • critical micellar concentration • hydrophobicity

Understanding equilibria among biliary lipid aggregates is crucial for interpreting the physical chemistry of native bile. However, previous investigations have been largely limited to model biles containing a single bile salt (BS) (reviewed in (1, 2)), whereas only isolated studies have focused on the physical chemistry of BS mixtures (3–5). However, human bile contains a complex and variable mixture of 12 common BS (6), the relative composition of which varies in gallstone disease (7) and as a consequence of bile acid therapy (8). The critical micellar concentration (CMC) (2) as well as many other physical chemical properties of individual BS, including equilibrium cholesterol solubilities, vary widely (9, 10). These physical–chemical properties are in part related to the hydrophobic–hydrophilic balance of individual BS, i.e., their relative affinities for water and hydrocarbon as inferred by reverse phase high performance liquid chromatography (HPLC) (11). Based on the weighted average of BS retention times by HPLC, a quantitative measure of the overall hydrophobicity of BS mixtures has been developed (12).

Depending upon total lipid concentration and relative lipid composition, BS within the organs of the enterohepatic circulation may exist as BS monomers, simple

Abbreviations: IMC, inter-mixed micellar/vesicular bile salt concentration; BS, bile salt; HPLC, high performance liquid chromatography; EYL, egg yolk lecithin; CMC, critical micellar concentration; TC, sodium taurocholate; GC, sodium glycocholate; TUDC, sodium tauroursodeoxycholate; GUDC, sodium glycoursodeoxycholate; TCDC, sodium taurochenodeoxycholate; GCDC, sodium glycochenodeoxycholate; TDC, sodium taurodeoxycholate; GDC, sodium glycodeoxycholate; TLC-S, sodium taurolithocholate-sulfate; GLC-S, sodium glycolithocholate-sulfate; TLC, sodium taurolithocholate; GLC, sodium glycolithocholate.

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micelles of BS and cholesterol, mixed micelles of BS, lecithin and cholesterol, and in cholesterol-supersaturated bile, unilamellar and multilamellar BS/lecithin/cholesterol vesicles (6). Knowledge of the inter-mixed micellar/vesicular (non-lecithin-associated) BS concentration (IMC) is required to accurately separate biliary lipid aggregates by methods that involve dilution of bile, such as gel filtration chromatography (13). We previously demonstrated in model biles containing the single BS taurocholate (TC) (14) that IMC values vary substantially with changes in total lipid concentration and BS/lecithin ratio within the physiological range. We hypothesized that the correct concentration of the monomeric and simple micellar BS fraction that comprises the IMC would also depend upon the BS species present.

In the present study, we have used the recently developed methods of modified equilibrium dialysis (14) and rapid centrifugal ultrafiltration (15) to systematically examine the BS composition of the IMC of model biles as functions of a range of physical-chemical conditions of pathophysiological importance.[2] Furthermore, determination of the distribution of BS species among the lipid aggregates of bile allows insights into the pathophysiological importance of alterations in biliary BS composition, particularly with respect to BS-membrane interactions and phase equilibria.

METHODS

Materials

Sodium salts of the taurine (T-) and glycine (G-) conjugates of ursodeoxycholate (UDC), lithocholate-sulfate (LC-S), cholate (C), deoxycholate (DC), and chenodeoxycholate (CDC), and lithocholate (LC) (Calbiochem-Behring, La Jolla, CA, and Sigma Chemicals, St. Louis, MO) were >95% pure with respect to other conjugates on HPLC (16) (Beckman Instruments, Wakefield, MA), and >99% pure by thin-layer chromatography (butanol–acetic acid–water 10:1:1 v/v/v). All were used as received except TC (Sigma), which was purified (17). The purities of egg yolk lecithin (EYL) (Lipid Products, South Nutfield, UK) and cholesterol (Nu-Chek Prep, Elysian, MN), as well as other chemicals, and glassware preparation were as previously described (14).

Model bile solutions

Model biles were prepared in aqueous solution (0.15 M NaCl, 0.001 NaN_3, pH 7.4) as in our earlier paper (14). Except as noted below, pH values were not adjusted and were ≈6.3–7.2 as prepared. Systems were designed to encompass physiological BS/(BS + EYL) ratios (0.6–0.8), total lipid concentrations (1–10 g/dl), cholesterol contents (0–10 mol%, i.e., mol/100 mol lipid), and physiological BS mixtures as displayed in **Table 1.** BS mixture A, which was used for all studies except where noted, was the average BS composition of 45 biles from cholesterol gallstone patients (18). BS mixtures B–H (Table 1) were composed to correspond to biles ranging in hydrophobicity from 40% ursodeoxycholate conjugates (B) to the average of the 10 most hydrophobic biles (H) from cholesterol gallstone patients (18). BS mixture B is typical of patients undergoing cholesterol gallstone dissolution therapy with ursodeoxycholic acid (19).

For studies of the effect of pH on IMC values, model biles (BS mixture C, BS/(BS + EYL) = 0.7, 10% cholesterol, 3 g/dl) were adjusted to pH 5, 6.5, and 8 with μl quantities of 0.2 N HCl or NaOH. The hydrophobic indices of all model biles were calculated for the appropriate pH according to the method of Heuman (12).

Determination of the IMC

For the method of rapid centrifugal ultrafiltration (15), Centripor concentrators (Spectrum Medical Industries, Los Angeles, CA, molecular weight cut-off 10,000) were centrifuged briefly (15 min, 1500 *g*) with distilled water (0.5 ml). For concentrated biles (10 g/dl), concentrators with a molecular weight cut-off of 8,000 were used as described in the accompanying article (15). Model bile (0.4 ml) was centrifuged for 5–60 min at 1500 *g* (37°C), and after discarding approximately 25 μl of initial filtrate, total and individual BS concentrations in the next 20–40 μl were quantified by HPLC (16). Measured BS concentrations were multiplied by the appropriate correction factors (range 0.99–0.81), which gave identical results to those obtained by modified equilibrium dialysis (15).

For measurements by modified equilibrium dialysis, dialysis membranes (molecular weight cut-off 12,000, Spectrum Medical Industries) were used to separate 1.0 ml cells (Fisher Scientific) containing model bile and 0.15 M NaCl (1.0 ml, 0.001 M NaN_3, pH 7.4) at 37°C (14). At intervals of 2 h or greater over a 36-h period, the model bile was replaced nine times by 1.0 ml of an identical model bile solution. During the final three changes, aliquots were removed from the dialysate for HPLC determination of BS concentrations (16), from which the IMC was calculated (14).

During the time course of this study, the rapid centrifugal ultrafiltration method was developed and validated (15). Because of the facility of the rapid centrifugal ultrafiltration method in that it requires smaller volumes of bile (1 ml vs. 10 ml) and can be completed more rapidly (2 h vs. 36 h), the majority of the data described herein was determined by rapid centrifugal ultrafiltration. As BS

[2]Presented in part at the National Meeting of the American Gastroenterological Association, New Orleans, LA, May 19–22, 1991 (46).

TABLE 1. Compositions of bile salt mixtures

Bile Salt Mixtures	HI[a]	Percent of Individual Bile Salts							
		A	B	C	D	E	F	G	H
		%							
TUDC	-0.47	2	14	11	8	7	2	3	0
TLC-S	-0.45	0.5	1	0	0	0	0	1	0
GUDC	-0.43	3	26	17	13	12	5	5	2
GLC-S	-0.20	1	2	0	0	0	0	3	0
TC	0.00	11	7	11	14	10	15	10	5
GC	0.07	21	15	26	24	19	26	22	18
TCDC	0.46	11	6	7	8	10	16	9	11
TDC	0.59	7	4	5	7	5	3	5	8
GCDC	0.51	25	18	12	15	23	28	27	27
GDC	0.65	20	7	10	11	15	6	16	29
TLC	1.00	0.5	0	0	0	0	0	0	0
GLC	1.05	0.5	0	0	0	0	0	0	0
Hydrophobic Index[b]		0.34	0.02	0.07	0.14	0.23	0.26	0.29	0.43

Actual compositions of BS mixtures were prepared to correspond to (A) average BS composition of 45 biles from cholesterol gallstone patients; (B), 40% ursodeoxycholate conjugates; (H), average of the 10 most hydrophobic human biles; and intermediate compositions (C–G). BS composition of human biles is from ref. 18. Abbreviations: HI, hydrophobic index; TUDC, sodium tauroursodeoxycholate; TLC-S, sodium taurolithocholate-sulfate; GUDC, sodium glycoursodeoxycholate; GLC-S, sodium glycolithocholate-sulfate; TC, sodium taurocholate; GC, sodium glycocholate; TCDC, sodium taurochenodeoxycholate; TDC, sodium taurodeoxycholate; GCDC, sodium glycochenodeoxycholate; GDC, sodium glycodeoxycholate; TLC, sodium taurolithocholate; GLC, sodium glycolithocholate.

[a]Values of individual hydrophobic indices (HI) from or by the method of Heuman (12).

[b]Hydrophobic indices of BS mixtures calculated by the method of Heuman (12).

composition and concentration in the IMC fraction as determined by either method did not differ (15), the method used for each determination is not specified herein.

Critical micellar concentration (CMC)

The CMC of BS mixture A (Table 1, 37°C, 0.15 M NaCl) was determined by the method of spectral shift of Rhodamine 6G (Aldrich Chemical Co., Milwaukee, WI) (3). The CMC was defined as the lowest BS concentration where an inflection point occurred in a plot of wavelength of maximum absorbance versus BS concentration.

Determination of pK'a values

Equilibrium titrations of BS mixture C (BS/(BS + EYL) = 1.0 and 0.7, 0% cholesterol, initial BS concentration 127 mM, initial volume = 5 ml) were conducted at 22°C with continuous magnetic stirring, as described previously (20). Solutions were inspected carefully after each addition of 1 M HCl, in order to record the onset of a Tyndall effect indicating precipitation of protonated glycine-conjugated BS. The pK'a values were calculated by a modification of the method of Back and Steenberg (21–23).

Statistical analysis

Statistical comparisons were performed using the software StatViewSE (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Influence of physical-chemical variables

Fig. 1 plots IMC values of model biles (BS/(BS + EYL) = 0.7, 10% cholesterol) composed with BS mixture A (Table 1) and with TC (data from ref. 14) as functions of total lipid concentration (1–10 g/dl). For all total lipid concentrations, IMC values of biles containing BS mixtures are substantially lower than the corresponding IMC

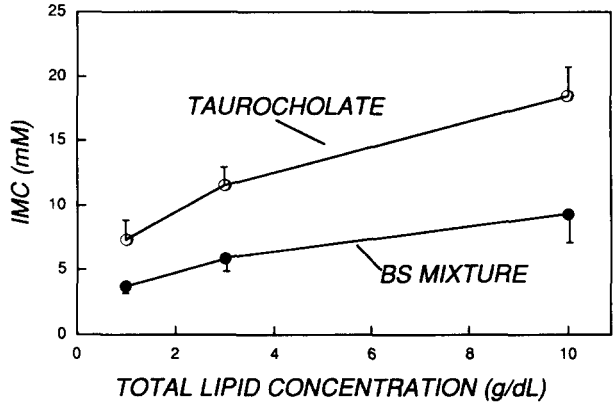

Fig. 1. Influence of total lipid concentration on the IMC of model biles composed with BS mixture A (Table 1, ●) or TC (○) (from ref. 14). Other conditions were BS/(BS + EYL) = 0.7, 10% cholesterol, 0.15 M NaCl, 37°C. Standard deviations are shown by bars.

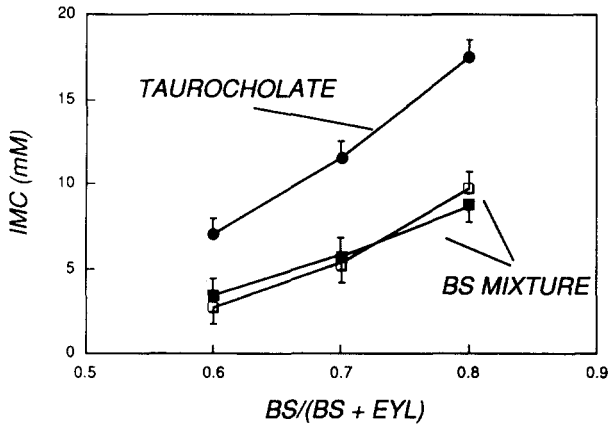

Fig. 2. Influence of BS/(BS + EYL) ratio on IMC values of model biles composed with BS mixture A (Table 1, ■) or TC (●) (from ref. 14). Conditions were 3 g/dl, 10% cholesterol, 0.15 M NaCl, 37°C. Values are also given for biles composed with 0% cholesterol (□) (BS mixture A, 3 g/dl, 0.15 M NaCl, 37°C). Standard deviations are shown by bars.

values of TC-containing biles (14). However, with TC as well as BS mixture A, IMC values increased approximately linearly with increases in total lipid concentration.

Fig. 2 displays IMC values of model biles (3 g/dl) containing either mixture A (solid squares, 10% cholesterol) or TC (solid circles, data from ref. 14) as functions of increases in BS/(BS + EYL) ratio. For biles containing either TC or BS mixture A, IMC values increased as BS/(BS + EYL) ratios increased (0.6–0.8). Fig. 2 also shows that IMC values of micellar biles (3 g/dl) composed without cholesterol (open squares) were comparable to metastable model biles composed with 10 mol% cholesterol that contained supersaturated micelles and vesicles (24). Thus, as was found for TC (14), the IMC of model biles composed with BS mixtures in the physiological range was influenced neither by micellar cholesterol content nor by the coexistence of vesicles in addition to micelles.

The CMC of BS mixture A was 1.2 mM (37°C, 0.15 M NaCl), similar to the value (1.4 mM) that Staggers and colleagues (20) obtained for a BS mixture of identical hydrophobic index (0.34). For total lipid concentrations and BS/(BS + EYL) ratios in the physiological range (Figs. 1 and 2), the IMC always exceeded the CMC. Thus, in biles with physiological BS/EYL compositions, both simple and mixed micelles coexist irrespective of the presence of vesicles (24, 25).

At solution pH values approaching their pK'a values, negatively charged glycine-conjugated BS become partially protonated, and consequently more hydrophobic (12). **Fig. 3a** displays the titration curve of a simple micellar solution of BS mixture C in the absence of EYL; Fig. 3b displays the titration curve for BS mixture C with a BS/(BS + EYL) ratio of 0.7 (0% cholesterol), which contains simple and mixed micelles. In the pure BS system (Fig. 3a) titration of glycine-conjugated BS began at point W, at pH ≈8, and precipitation began at pH 3.61. This value for BS mixture C with a hydrophobic index of 0.07 is slightly lower than the value of 3.83 reported by Staggers and colleagues (20) for a BS mixture with similar proportions of taurine conjugates (33%) but with a higher hydrophobic index (0.34). Titration was complete at pH ≈3.1 (Z). For the EYL-containing system, titration began at pH ≈8.0 (W), and at pH 4.05 (Y), a Tyndall effect indicated precipitation of insoluble protonated glycine-conjugated BS. Titration was complete at pH ≈3.0, and the calculated equivalence based on the moles of glycine-conjugated BS and moles of HCl was 97%. The pK'a value of BS mixture C in the absence of EYL was calculated to be 4.3, and increased to 4.5 in the EYL-containing system, values similar to those of Staggers and

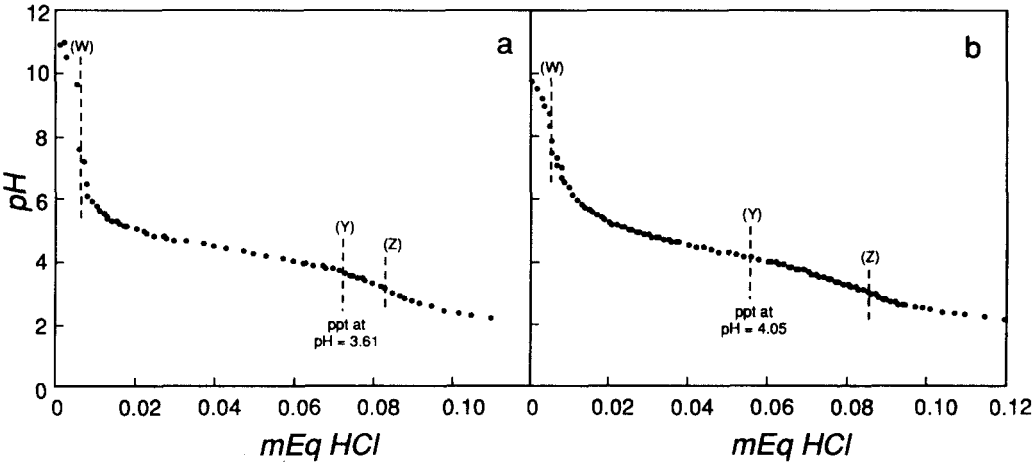

Fig. 3. pH-titration curves of BS mixture C (Table 1) in solutions (0.15 M NaCl, 22°C) containing either (a) simple micelles, i.e., without EYL or (b) simple and mixed micelles, i.e., at a BS/(BS + EYL) ratio of 0.70. Glycine-conjugated BS are titrated from inflection point (W) to inflection point (Z), with the onset of precipitation of protonated BS (Tyndall effect) occurring at a pH corresponding to point (Y) (see text).

colleagues (20) for a pure BS mixture and a mixed micellar system containing a mixture of BS, fatty acids and traces of mono-, di-, and triacylglycerols. As has previously been shown for monomeric and micellar unconjugated BS (26), pK'a values of glycine-conjugated BS in simple or mixed micelles exceeded pK'a values observed for monomeric BS concentrations (23).

Fig. 4 displays the effect of pH on IMC values of model bile with the same BS mixture (C, BS/(BS + EYL) = 0.7, 10% cholesterol). Between pH values of 8 and 6.5, where pK'a values imply that greater than 99.98 and 99% of the glycine conjugates would be dissociated, neither the calculated hydrophobic index (0.07), nor the IMC changed. However, at pH 5.0, where approximately 20% of the glycine conjugates are protonated but soluble in mixed micelles (22, 23), the hydrophobic index increased to 0.13, and the IMC fell slightly. Clearly, variations in pH within the range (6.0–7.5) that is physiologically observed in bile (27) do not affect the IMC appreciably.

Influence of BS composition

Since the hydrophobic index of native biles can vary either pathophysiologically or with pharmacological manipulations, the IMC was measured for systems containing BS mixtures of varying hydrophobicity. **Fig. 5** displays the IMC as a function of the hydrophobic index (0.02–0.43) for several different model biles (BS mixtures A–H in Table 1, 3 g/dl, 10% cholesterol) at two different BS/(BS + EYL) values (= 0.7 and 0.8). For both BS/(BS + EYL) ratios, variations in the hydrophobicity of the BS mixture appreciably influenced the IMC, which decreased as the overall hydrophobic index of the mixture increased (values from Table 1). For comparison, a model bile composed with the single BS TC (hydrophobic index

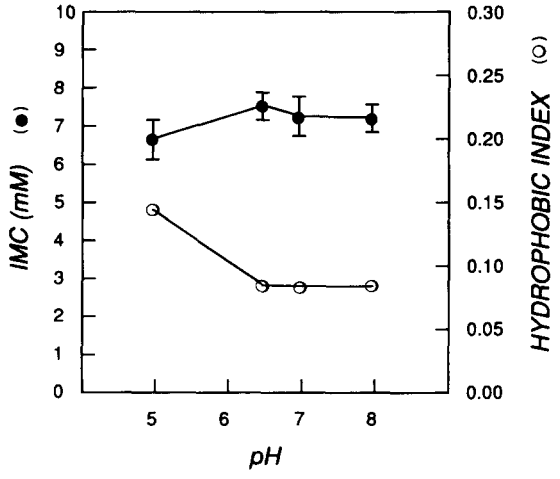

Fig. 4. Influence of pH on IMC values (●, left hand axis) of model biles composed with BS mixture C (Table 1) at natural pH (6.9) and titrated with HCl or NaOH to pH values of 5.0, 6.5, and 8.0. Conditions were 3 g/dl, 10% cholesterol, 0.15 M NaCl, 37°C. The overall hydrophobic index (○, right hand axis) was calculated by the method of Heuman (12).

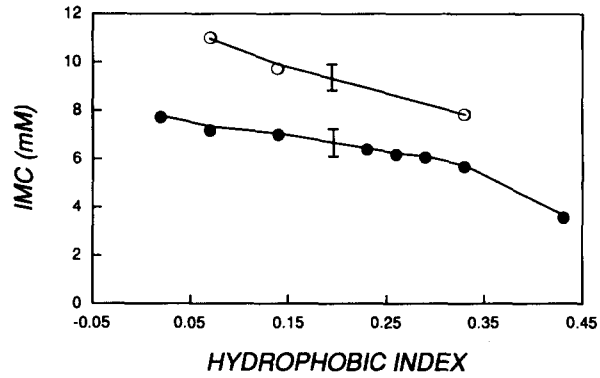

Fig. 5. Dependence of the IMC on hydrophobicity of the BS mixture as expressed by hydrophobic index (12) on the abscissa for model biles composed with BS mixtures A–H (Table 1) and with BS/(BS + EYL = 0.7 (●) or 0.8 (○). Other conditions were 3 g/dl, 10% cholesterol, 0.15 M NaCl, 37°C. Average standard deviation is shown by the bar.

0.00, BS/(BS + EYL) = 0.7), had an IMC of 11 mM (14), a value substantially exceeding that of model bile B, which has a nearly identical hydrophobic index (0.02). Thus, BS mixtures have IMC values that are lower than individual BS for the same overall hydrophobic index as conventionally determined by HPLC (12).

Species of BS in the IMC fraction

Fig. 6 depicts the percentage of each BS in the total bile and in the IMC fraction, i.e., present as simple and mixed micelles, for the eight most abundant BS arranged in order of increasing hydrophobicity (mean of 22 biles with BS mixture A, total lipid composition 1–10 g/dl, BS/(BS + EYL) ratio = 0.6–0.8, 0–10 mol% cholesterol). For the four most hydrophilic BS (ursodeoxycholate and cholate conjugates), the mol% BS in the IMC fraction was significantly greater than that in the overall bile ($P < 0.05$ for each BS). Conversely, for the four most hydrophobic BS (deoxycholate and chenodeoxycholate conjugates), the mol% BS in the IMC fraction was significantly less than that in the overall bile ($P < 0.05$). Consequently, the relative BS composition of monomers and simple micelles in the IMC had an appreciably lower hydrophobic index (0.20) than the overall BS composition of the bile (0.34). By inference, the hydrophobic index of BS in the mixed micelles is higher (0.38) than that of overall BS composition. There was no correlation between the relative percentage of BS in the IMC fraction and variations in total lipid concentration, BS/(BS + EYL) ratio or cholesterol content (all $P > 0.1$).

Fig. 7 displays the relationship between the hydrophobic index of the IMC and the hydrophobic index of total BS in each of the model biles in Table 1 (3 g/dl, BS/(BS + EYL) = 0.7, 10% cholesterol). There is a positive linear correlation between the hydrophobic index of the IMC and that of the total BS, with a slope of 0.94 (least squares fit, $r = 0.92$), and an intercept on the abscissa of −0.14.

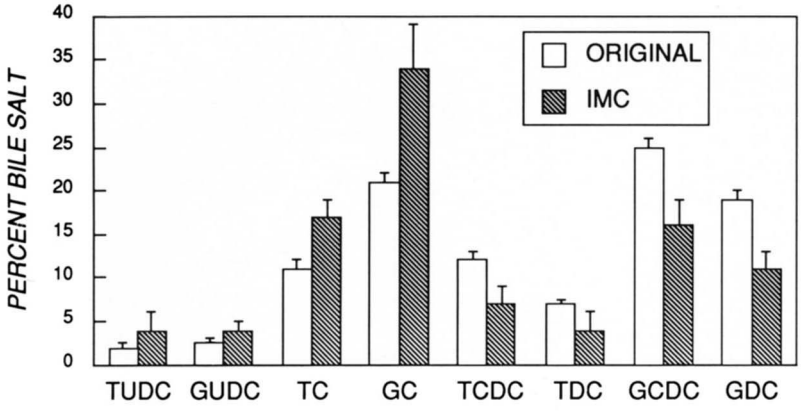

Fig. 6. Relative BS compositions of the IMC fraction (monomeric plus simple BS micelles) and whole bile arranged in order of increasing hydrophobicity for the eight most abundant BS in the mixture (abscissa). Values for percent BS are averages for all biles composed with model bile A in Table 1 (total lipid concentration 1–10 g/dl, BS/(BS + EYL) = 0.6–0.8, cholesterol = 0–10%). Standard deviations are shown by bars.

Therefore, on average, BS in the IMC have a hydrophobic index that is 0.14 units more hydrophilic than the corresponding overall biles. This relationship appears to hold over a wide range of model biles containing BS compositions of pathophysiological relevance.

Fig. 8 shows the percentage enrichment of BS in the IMC fraction, i.e., the ratio of mol% in the IMC to mol% in whole bile, as a function of BS hydrophobicity. Each point represents the relative enrichment of each BS species, i.e., the ratio of mol% in the IMC fraction to mol% in whole bile for 22 model biles composed with BS mixture A (Table 1). As a close approximation, the relative enrichment of individual BS was related linearly to the hydrophobicity of the BS (least squares fit with $r = 0.85$). Within the concentration ranges studied, there were no statistically significant trends in the dependence of the degree of enrichment on total lipid concentration or BS/lecithin ratio.[3]

DISCUSSION

The present work extends our previous observations that the IMC depends upon total lipid concentration and BS/lecithin ratio, but not on cholesterol content (14). BS species composition as measured by hydrophobic index (12) additionally determines the total concentration of BS present as monomers and simple micelles. In contrast, variations in pH within the physiological range do not significantly alter the IMC, since potentiometric titration demonstrates that only small proportions of glycine-conjugated BS are protonated even at the most acidic pH values observed in gallbladder bile (27).

In addition to confirming that the IMC depends upon the individual molecular species of BS as suggested previously (28, 29), the present work demonstrates that the composition of the IMC is not identical to the overall biliary BS composition, but the relative proportions of BS in the IMC differ substantially from that in whole bile (Figs. 5 and 6). These results have relevance both for the accurate separation of biliary lipid aggregates and for pathophysiological effects of BS mixtures in organs of the enterohepatic circulation. The data demonstrate that BS partitioning between biliary lipid aggregates is an extremely complex phenomenon that awaits further quantitative study.

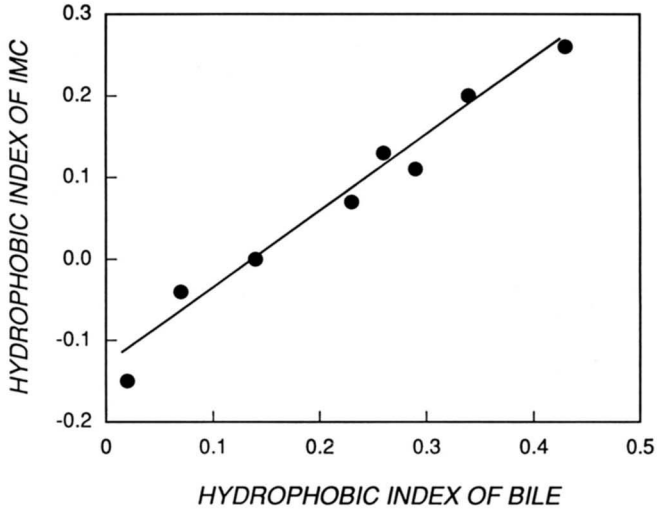

Fig. 7. Linear dependence of the hydrophobic index of the IMC fraction (monomeric plus simple BS micelles) on the hydrophobic index of BS in whole bile (model biles A–H, Table 1, total lipid concentration 3 g/dl, BS/(BS + EYL) = 0.7, cholesterol = 10%). Note that the relationship extrapolates to a value of −0.14, which shows that for all biles the BS composition of the IMC was more hydrophilic by 0.14 units than that of the overall BS composition.

[3]Theoretically, as lecithin concentration approaches zero, the % of each BS in the IMC must approach that in whole bile, and conversely at high lecithin concentrations, the differences between the IMC and whole bile would be magnified. However, over the physiological range of BS and EYL concentrations, the enrichment was not a function of total lipid concentration.

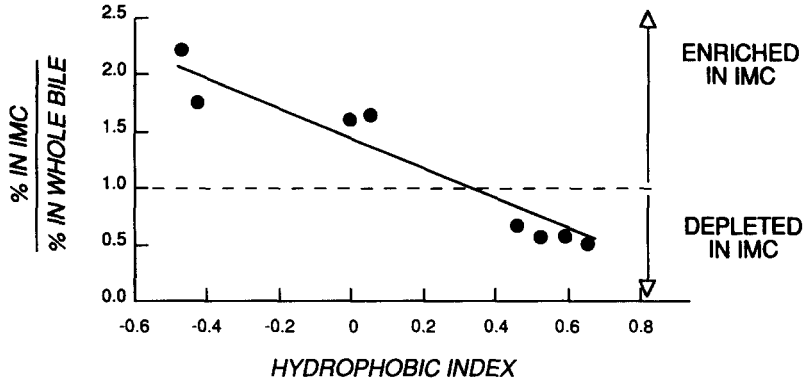

Fig. 8. Relative enrichment (values >1) or depletion (values <1) of BS in the IMC fraction for the eight most abundant BS arranged in order of increasing hydrophobicity. Biles (n = 21) were all composed with BS mixture A (Table 1). The solid line is the least squares fit to the data (r = 0.85). Values greater than 1.0 (above the horizontal dashed line) indicate that the respective BS is relatively enriched in the IMC, whereas values less than 1.0 (below the horizontal dashed line) indicate that the respective BS is depleted in the IMC.

Accurate separation of biliary lipid aggregates

It is believed that detergent properties of biliary lipid systems depend on the monomeric BS concentration, which in an ideal solution is equal to the thermodynamic activity, i.e., concentration of each BS as inferred from classical detergent systems (30). Using the Sephadex bead method of Ammon and Walter (31) to measure monomeric BS concentrations, Lee and colleagues (32) demonstrated that simple and mixed micellar systems with the same IMC values have identical BS monomer concentrations, despite wide variations in lecithin content. At concentrations above the CMC where simple BS micelles were present, the monomer BS concentration exceeded the CMC (32). Although in theory, this monomer concentration of BS could be used to separate micelles and vesicles by gel chromatography (13), solutions of BS monomers alone cannot exist experimentally at concentrations exceeding the CMC, because an aqueous solution of monomers would aggregate to form simple micelles. Therefore, the IMC, corresponding to monomeric plus simple micellar BS concentrations, is the appropriate value for separation of biliary lipid aggregates using techniques that involve dilution such as gel filtration chromatography (13).

As we showed previously for TC (14), IMC values vary widely over a physiologically relevant range of total lipid concentrations and BS/(BS + EYL) ratios. The present data further demonstrate that IMC values also depend on the BS hydrophobic index, over a range of values that span normal BS compositions and those observed in gallstone disease (18, 33) and during bile acid therapy (8). Therefore, as shown by the range of observed hydrophobic indices of the IMC (Fig. 7), a single mixture of BS cannot be used as the appropriate eluant for gel chromatographic separation of lipid aggregates of native biles. Further, since IMC values of model biles are considerably more hydrophilic than the overall BS composition of the bile (see Fig. 7), an eluant containing a BS mixture with the same composition as the native bile would not separate biliary lipid aggregates without altering their relative lipid composition and other physical-chemical properties.

Variations in BS composition also have significance for accurately separating micelles and vesicles from bile. Donovan and Jackson (34) have observed that during gel chromatography of model biles, altering the BS composition of the eluant from the more hydrophilic composition of the IMC to the overall composition of the bile can result in complete dissolution of vesicles. Stone and colleagues (35) have also observed the importance of the correct BS species in the eluant for separating micelles and vesicles from model biles. Therefore, even the relatively small decreases in the IMC (Fig. 5) of biles as functions of varying hydrophobic indices may have enormous importance for accurately separating micelles and vesicles from biles without perturbing their compositions and properties.

Pathophysiological implications

At IMC values greater than the CMC, including all model biles studied herein, simple BS micelles coexist with mixed micelles, as has previously been shown for model biles containing TC and EYL (25). Furthermore, for cholesterol-supersaturated biles above the micellar phase limit, simple BS micelles coexist with mixed micelles and vesicles. However, as the model biles studied herein span the physiological range, this work suggests that simple BS micelles (but not necessarily vesicles) always coexist with mixed micelles in human bile.

These data demonstrate further that partitioning of individual BS among monomers, simple micelles, mixed micelles and vesicles depends upon the hydrophobicity of each BS. This is to be expected because the hydrophobic index as measured by HPLC reflects the partitioning of individual BS between hydrocarbon and methanolic-aqueous phases, and depends on the precise separation conditions used (12). Differences between the affinity of BS for lecithin-containing aggregates and the hydrophobic index may reflect subtle distinctions in the packing of lecithin in mixed micelles versus the packing of hydrocarbon chains in a reversed phase HPLC column. The discrepancy in IMC values for BS mixtures of similar overall hydrophobic index most likely reflects these limitations.

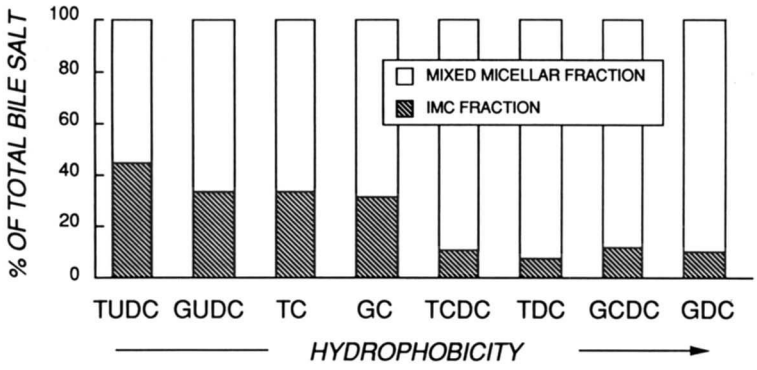

Fig. 9. Distribution of BS in the IMC (monomer plus simple micellar fraction) and in the mixed micelles plus vesicles of a model bile of typical physiological composition containing BS mixture A in Table 1 (total lipid concentration 3 g/dl, BS/(BS + EYL) = 0.7, 10% cholesterol) as calculated from the IMC and overall BS composition and concentration. See text for discussion.

In fact, preliminary data from our laboratory (36) suggests that other "more physiological" methods of determining hydrophobicity may more accurately correlate with the IMC of BS mixtures and individual BS.

From the experimentally derived composition of the IMC and the initial BS composition of the bile, the relative amount of BS present as monomers and simple micelles (the IMC) or in mixed micelles and vesicles can be calculated. **Fig. 9** demonstrates that for a model bile of typical physiological composition (BS mixture A, 3 g/dl, BS/(BS + EYL) ratio = 0.7, 10% cholesterol), approximately 45% of TUDC, the most hydrophilic BS in the mixture, is present as monomers and simple micelles in the IMC. In contrast, in the same system, greater than 90% of hydrophobic BS such deoxycholate and chenodeoxycholate conjugates are associated with lecithin, either as mixed micelles or vesicles. The propensity of hydrophilic BS for the IMC fraction rather than association with lecithin in mixed micelles and vesicles correlates with the relative inability of hydrophilic BS to dissolve lecithin vesicles (37, 38), and suggests a possible physical-chemical basis for their lack of cytotoxicity (39, 40).

Because biles of identical IMC values (BS concentration and composition) have identical monomer BS concentrations (32) and identical values for BS thermodynamic activity (30), their interactions with physiological membranes apparently depend upon the IMC composition. Indeed, the monomeric and/or simple micellar BS fraction in bile has been suggested to have specific physiological roles (41, 42). Higuchi and colleagues (41) found that cholesterol dissolution rates in model biles correlated with the monomer plus simple micellar BS concentration rather than total or mixed micellar BS concentration. The absorption of iron has been shown to be greatly enhanced by monomeric but not micellar TC concentrations (42). However, the insensitivity of the IMC to cholesterol content suggests that enhanced resistance to BS dissolution in cholesterol-enriched membranes (38) is due to intrinsic properties of these membranes rather than alterations in the IMC.

In conclusion, we have found that the major determinants of the IMC in bile include total lipid concentration, BS/lecithin ratio, and BS hydrophobicity, but not cholesterol content or pH within the physiological range. The BS composition of the IMC is more hydrophilic than the overall BS composition of biles. Conversely, the BS composition of mixed micelles and vesicles is more hydrophobic than the overall BS composition of biles. Consequently, to separate micelles and vesicles in native bile, the IMC must be measured in order to tailor the gel chromatography eluant specifically for each bile. We speculate (43) that IMC enrichment with hydrophilic BS species may provide one physical-chemical basis for cytoprotective effects of ursodeoxycholic acid on hepatocytes (44) and gastric mucosa (45). Furthermore, the composition of the IMC is a true biliary thermodynamic quantity that has been heretofore inadequately explored in bile, and may therefore critically influence many other pathophysiological processes such as cholesterol monohydrate dissolution, crystallization, and absorption of lipid from both gallbladder and small intestine.

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