Rapid determination by centrifugal ultrafiltration of inter-mixed micellar/vesicular (non-lecithin-associated) bile salt concentrations in model bile: influence of Donnan equilibrium effects

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Abstract We have developed the technique of rapid (<2 h) centrifugal ultrafiltration to measure the inter-mixed micellar/vesicular (non-lecithin-associated) bile salt (BS) concentrations (IMC) of individual BS in model biles. This methodology uses a centrifugal concentrator with a reinforced membrane, through which a small fraction (<15% total volume) of model biles was ultrafiltered by low centrifugal forces (1500 *g*, 5–60 min). Total and individual BS concentrations in the filtrate were measured by high performance liquid chromatography. However, non-filterable anions, in this case BS/lecithin/cholesterol mixed micelles and unilamellar vesicles, induce an asymmetric distribution of ions across the membrane. Therefore, BS concentrations in the filtrate exceeded the true IMC, which was estimated taking into account Donnan equilibrium effects. To confirm the hypothesis that a correction for Donnan forces was necessary, distributions of BS and another filterable anion, chloride, were measured in systems containing the non-filterable polyanion dextran sulfate as an analogue for non-filterable polyanionic mixed micelles and vesicles. An asymmetric distribution of the monovalent anions chloride and BS monomers as well as polyvalent simple BS micelles was indeed present during centrifugal ultrafiltration. This new methodology was validated by comparing IMC values with those obtained by modified equilibrium dialysis also corrected for Donnan equilibrium effects (Donovan, J. M., et al. 1991. *J. Lipid Res.* **32:** 1501–1512). Centrifugal ultrafiltration, which utilizes <1 ml of bile, determines the composition in the IMC necessary to separate micelles and vesicles of native biles by techniques that involve dilution of bile such as gel filtration chromatography.—**Donovan, J. M., and A. A. Jackson.** Rapid determination by centrifugal ultrafiltration of inter-mixed micellar/vesicular (non-lecithin-associated) bile salt concentrations in model bile: influence of Donnan equilibrium effects. *J. Lipid Res.* 1993. **34:** 1121–1129.

Supplementary key words vesicles • micelles • cholesterol

In model and native biles, cholesterol is solubilized in at least three types of lipid aggregates that coexist pathophysiologically: simple micelles of bile salts (BS) and cholesterol, mixed micelles of BS, lecithin and cholesterol, and in cholesterol-supersaturated bile, BS/lecithin/cholesterol vesicles (1). An understanding of the respective roles of micelles and vesicles in cholesterol crystallization and gallstone dissolution in the gallbladder and cholesterol absorption from gallbladder and upper small intestine requires accurate separation of these lipid aggregates, as well as subsequent determination of their compositions and properties (2). In turn, accurate separation of cholesterol-carrying lipid aggregates in bile requires a knowledge of equilibria between mixed micelles, vesicles, and their common intermediates, the monomer and simple micellar concentrations of non-lecithin-associated BS. We have suggested earlier (3) that the appropriate terminology for this concentration is the inter-mixed micellar/intervesicular (non-lecithin-associated) BS concentration (IMC) (3).

In an earlier paper (3), we described modified equilibrium dialysis, a new method for determining correct values for the IMC in model bile. This method includes an appreciable correction for Donnan equilibrium effects (especially in concentrated biles) induced by non-dialyzable negatively charged mixed micelles and vesicles[2], which previous equilibrium dialysis determinations of IMC values did not take into account (8–10). However, because

Abbreviations: BS, bile salt; IMC, inter-mixed micellar/vesicular bile salt concentration; TC, sodium taurocholate; EYL, egg yolk lecithin; HPLC, high performance liquid chromatography; TDC, sodium taurodeoxycholate; MWCO, molecular weight cut off.

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[2]Although at physiological biliary lipid compositions only a small fraction of BS is associated with vesicles as compared with mixed micelles (4), BS do bind to vesicles (4–7). Therefore we have included BS/lecithin/cholesterol vesicles as non-membrane permeable species.

of multiple changes of dialysant, the modified method requires approximately 2 days to determine the IMC and is cumbersome for application to native bile. Ideally, the IMC should be measured "on-line" to facilitate separation of biliary lipid aggregates. Therefore, we have devised a much more rapid technique, centrifugal ultrafiltration, to measure the IMC in hours, using a much smaller volume of bile (<1 ml vs. 10 ml for modified equilibrium dialysis), and have validated this technique against modified equilibrium dialysis[3]. By directly testing previously made assumptions for asymmetry of monomeric and simple micellar BS across a dialysis membrane, we also obtain insights into the Donnan effects induced by native bile across biliary and gallbladder epithelia.

METHODS

Materials

Taurocholate (TC) (Sigma, St. Louis, MO) was purified (12). Other BS (Calbiochem-Behring, La Jolla, CA, and Sigma), grade I egg yolk lecithin (EYL) (Lipid Products, South Nutfield, UK), cholesterol (Nu-Chek Prep, Elysian, MN), [1-^{14}C]palmitoyl-2-stearoyl-*sn*-glycerophosphocholine and [^{3}H]cholesterol (New England Nuclear, Boston, MA) were used as received. By high performance liquid chromatography (HPLC) (13) (Beckman Instruments, Wakefield, MA), BS purity with respect to other conjugates was >95%, and by thin-layer chromatography (butanol–acetic acid–water 10:1:1 v/v/v), BS were >99% pure. Cholesterol was >99% pure by gas–liquid chromatography. Purity of EYL (>99%) was confirmed by thin-layer chromatography ($CHCl_3$–MeOH–H_2O, 65:35:4, v/v/v), as was radiochemical purity (>99%) of the ^{14}C-labeled lecithin (New England Nuclear). NaCl (Mallinckrodt, Paris, KY) was roasted for 3 h at 500°C to oxidize and remove organic impurities. All other chemicals were of ACS quality or highest reagent grade purity. $^{3}H_2O$ was obtained from New England Nuclear. Pyrex glassware was alkali-washed overnight in EtOH–2 M KOH (1:1, v/v), followed by a 24-h acid washing in 2 M HNO_3, and thorough rinsing in filtered, deionized, and glass-distilled water.

Model bile solutions

Model biles were prepared by coprecipitation of lipids from MeOH–$CHCl_3$, drying first under a stream of N_2, and then under reduced pressure, followed by resuspension in aqueous solution (0.15 M NaCl, 0.001 M NaN_3, pH 7.4). Model biles were prepared either with a single BS, TC, or taurodeoxycholate (TDC), or with a physiological mixture of 12 BS corresponding to the average composition of biles from 45 cholesterol gallstone patients (14): tauroursodeoxycholate, 2%; glycoursodeoxycholate, 3%; TC, 11%; glycocholate, 21%; taurochenodeoxycholate, 11%; TDC, 7%; glycochenodeoxycholate, 25%; glycodeoxycholate, 20%; taurolithocholate-sulfate, 0.5%; glycolithocholate-sulfate, 1%; taurolithocholate, 0.5%; and glycolithocholate, 0.5%.

Chemical analysis

BS were quantified by comparison with HPLC of individual BS with concentrations determined by dry weight (13). Correlation of peak areas with BS concentrations was linear (r > 99%) for all BS. To measure the distribution of filterable anions on both sides of the ultrafiltration membrane (see below and (3)), chloride concentrations were determined spectrophotometrically (Sigma Diagnostics).

Centrifugal ultrafiltration

Centripor concentrators (Spectrum Medical Industries, Los Angeles, CA, molecular weight cut-off 10,000, or 8,000 for total lipid concentration >7 g/dl, as discussed below) were prepared by centrifuging (15 min, 1500 *g*) with distilled water (0.5 ml), and carefully removing all water with a syringe, with scrupulous attention to the area under the filter where small droplets may be retained. Model bile (0.4 ml) was placed in the reservoir with a reinforced dialysis membrane, and inserted in a holder to retain filtrate. After centrifugation for 5–60 min at 1500 *g*, variable amounts of filtrate were produced, ranging from ≈0.3–0.4 μl/min for systems with high total lipid concentrations (10 g/dl), to ≈3 μl/min for systems with low total lipid concentrations (1 g/dl). Preliminary studies (vide infra) showed that BS concentrations increased over the first 25 μl of filtrate, due to dilution from adsorbed water in the membrane, as well as to possible membrane adsorption of BS. As BS concentrations in subsequent aliquots reached a plateau when 25 μl were ultrafiltered, this volume of initial filtrate was discarded in subsequent studies, and total and individual BS concentrations in the next 20–40 μl were determined by HPLC. As discussed below, the concentrations of BS in the filtrate exceed the concentrations in the model bile. Therefore, only a minimum volume was filtered so that the composition of the initial solution was not altered. BS concentrations were routinely measured after 2–3 centrifugations using the same membrane to confirm that a plateau in filtered BS concentration was reached. Membrane failure, which occurred during 5–10% of centrifugations, was detected either by higher ultrafiltration rates during centrifugation, or by a continuous increase in BS concentrations over sequential centrifugations.

Because the micellar zone of the ternary phase diagram

[3]Presented in part at the National Meeting of the American Gastroenterological Association, New Orleans, LA, May 19–22, 1991 (11).

of BS, lecithin, and cholesterol depends upon temperature (15), model biles were prepared and centrifuged at 37°C. IMC values of model biles composed with the single bile salt TC, or with a physiological mixture of BS with varying total lipid concentration (1–10 g/dl), BS/(BS + EYL) ratio (0.6–0.8), and cholesterol content (0–10%) were measured both by centrifugal ultrafiltration and by the validated modified equilibrium dialysis method (3). Results are expressed as means ± 1 SD for replicate measurements of at least four separate determinations.

To demonstrate filtration of monomers and simple micelles, BS solutions (TC and BS mixture, 1–30 mM) were centrifugally ultrafiltered and BS concentrations in original solutions and filtrates were measured. To determine the retention of mixed micelles by the Centripor concentrator membrane, EYL and cholesterol concentrations in the filtrate were determined for model biles (3–10 g/dl, BS/(BS + EYL), 10% cholesterol) prepared with tracer amounts of [^{14}C]lecithin and [^{3}H]cholesterol. Scintillation counting was carried out in a Beckman Scintillation Counter (Beckman Instruments).

Studies determining the distribution of chloride and BS in initial solutions and filtrates were conducted using the polyvalent anion dextran sulfate (mean mol wt 50,000, Sigma Chemicals) as an analog for polyvalent mixed micelles. The net charge of dextran sulfate was calculated from the sulfur content (17.9% by weight) and corresponded to 1.8 negative charges per glucose residue. Solutions were prepared to contain from 10 to 150 mEq sulfate residues, with varying BS concentrations (5–20 mM TC or the BS mixture).

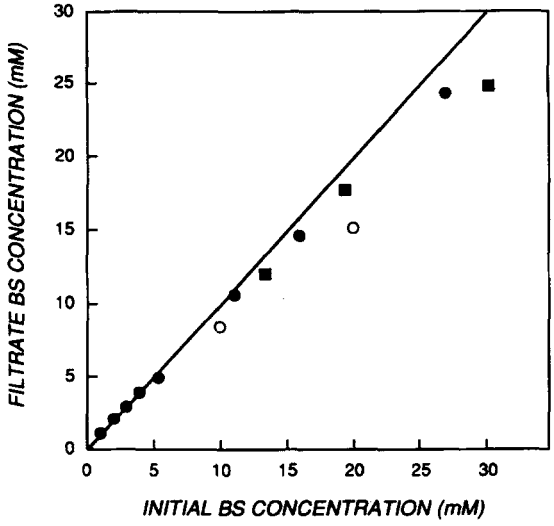

Fig. 1. Ultrafiltration of pure BS solutions composed with either TC (●) or the BS mixture (■) with 8000 (○) or 10,000 (●, ■) MWCO membranes. There is minor retention of simple micelles up to ≈20 mM, but above this concentration, BS retention by the membrane increases markedly.

Equilibrium dialysis

Equilibrium dialysis was carried out as described previously (3). In brief, 1.0 ml cells (Fisher Scientific) were separated by Spectrapor dialysis membranes (molecular weight cutoff 12,000, Spectrum Medical Industries) that were first prepared by exhaustive washing in glass-distilled water. The entire apparatus was immersed in a continuously shaken water bath at 37°C. Model bile (1.0 ml, dialysant) was placed in one side of the equilibrium dialysis cell, and dialysate (1.0 ml, 0.15 M NaCl, 0.001 M NaN_3, pH 7.4) was placed in the other. At intervals of 2 h or greater over a 36-h period, the model bile solution (dialysant) was replaced repeatedly by 1.0 ml of an identical (original) model bile solution. Dialysate BS concentrations were determined at equilibrium for duplicate samples (30 μl) during the final three changes of model bile. The IMC was calculated from the equilibrium BS concentrations using corrections for Donnan equilibrium effects, as described previously (3).

RESULTS

Validation of centrifugal ultrafiltration

To demonstrate that monomers and simple micelles passed freely through the membrane, pure BS solutions (TC or BS mixture) were subjected to centrifugal ultrafiltration. As **Fig. 1** demonstrates, BS filtrate concentrations were only slightly lower than the initial solutions up to 20 mM BS, i.e., spanning the range of observed IMC values in model biles of physiological composition (16). Retention of simple micelles was similar for TC (closed circles) as compared with a mixture of BS (squares), both for the 8,000 (open circles) and 10,000 (closed symbols) molecular weight cut-off (MWCO) membranes. At BS concentrations above 20 mM (Fig. 1) appreciably more simple micelles were retained by the membrane. We discuss below how this minor retention of BS affects accurate measurement of the IMC. For BS mixtures, filtrate BS composition was identical to BS composition in the initial solution (± 6%, $P > 0.2$), implying that hydrophobic BS were not selectively retained.

Fig. 2 displays BS concentrations in aliquots of filtrate obtained sequentially with the same filtration unit for three different model biles (composed with TC/(TC + EYL) = 0.8, 0% cholesterol; BS mixture, BS/(BS + EYL) = 0.8, 10% cholesterol; and TDC/(TDC + EYL) = 0.7, 10% cholesterol; all 3 g/dl). As discussed in the accompanying article (16), IMC values depend greatly on the BS composition of the model bile. However, for all three model biles, after approximately 25 μl of filtrate had passed through the membrane, BS concentrations reached a plateau. When $^{3}H_2O$ was used to wash the filters, approximately 6–8 μl remained adsorbed to the membrane

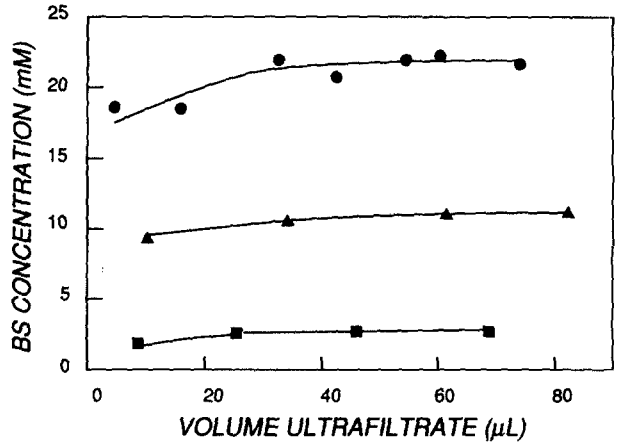

Fig. 2. BS concentration in ultrafiltrate of model biles with TC (●), BS mixtures (▲), or pure TDC (■), as functions of the volume of ultrafiltrate produced. After approximately 25 μl of ultrafiltrate was produced, BS concentrations reached a constant value (see text for details). Other conditions were TC/(TC + EYL) and BS/(BS + EYL) = 0.7, TDC/(TDC + EYL) = 0.8, 3 g/dl, 10% cholesterol, 37°C.

despite removal of all visible water. Thus, the admixture of adsorbed water from the membrane diluted the initial aliquot of ultrafiltrate.

During centrifugation of systems containing simple and mixed micelles as well as vesicles (4) (BS/(BS + EYL) = 0.7–0.8, 10% cholesterol, 3–7 g/dl), no detectable EYL or cholesterol (<0.1% of values in initial model bile) was present in the filtrate using membranes with MWCO 8,000 or 10,000. However, for 10 g/dl BS mixtures, up to 10% of total EYL was ultrafiltered by a 10,000 MWCO membrane, but <0.1% by an 8,000 MWCO membrane. Ultrafiltration of mixed micelles by the 10,000 MWCO membranes was accompanied by a continuing rise in BS concentration in the filtrate, which illustrated the importance of measuring several sequential filtrate BS concentrations as shown in Fig. 2. Therefore, 8,000 MWCO membranes were used in all experiments where total lipid concentrations exceeded 7 g/dl.

Fig. 3 displays IMC values determined by centrifugal ultrafiltration and modified equilibrium dialysis for biles composed with TC (circles) or the BS mixture (squares). IMC values were comparable when measured by both methods, and the least squares fit of the data (slope = 0.92 and r = 0.95) did not differ significantly from the line of identity. Moreover, individual BS concentrations in the IMC of the BS mixture (data not displayed) were identical (± 2%) by each method (n = 9 for modified equilibrium dialysis and n = 12 for centrifugal ultrafiltration, $P > 0.1$ for all BS). Therefore, both the BS composition and concentration in the IMC as measured by rapid centrifugal ultrafiltration were comparable to those obtained by the modified equilibrium dialysis method (3).

Donnan equilibrium effects: theoretical considerations

BS in mixed micelles and vesicles are negatively charged non-dialyzable and non-filterable anions. Such "fixed" anions induce an asymmetrical distribution of dialyzable ions across a dialysis membrane, known as the Donnan equilibrium effect (17). The concentration of dialyzable anions in the dialysate exceeds the concentration of dialyzable anions in the dialysant, whereas cation concentration in the dialysant exceeds that of the dialysate.

An analogous situation holds during centrifugal ultrafiltration of systems containing non-filterable anions. In the initial solution, the concentration of filterable anions (in the case of model bile, chloride and BS monomers and simple micelles) is less than the concentration of filterable cations (sodium). To achieve electroneutrality in the filtrate, the total filtered anion concentration must equal the total filtered cation concentration. The concentration of filtered anions must exceed the concentration of filterable anions in the initial solution, and conversely, the concentration of filtered cations must be less than their concentration in the initial solution. Therefore, during centrifugal ultrafiltration, the measured BS concentration in the filtrate is expected to exceed the true IMC, similar to the case during modified equilibrium dialysis where the BS concentration in the dialysate exceeded the IMC (3). We hypothesized that during centrifugal ultrafiltration, monovalent anions would distribute asymmetrically as predicted by Donnan equilibrium effects. Further, we hypothesized that distribution of BS monomers and simple micelles would approximate that of chloride anions, producing a pseudo-Donnan effect, despite the fact that BS simple micelles are polyvalent, and thus the Donnan

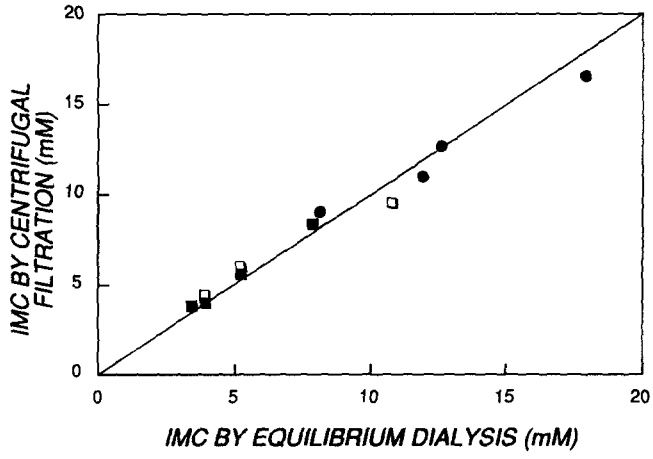

Fig. 3. Comparison of IMC values of model biles containing TC (●) or a BS mixture (■, □) determined by modified equilibrium dialysis and centrifugal ultrafiltration with 8000 (□) or 10000 (●, ■) MWCO units, both corrected for Donnan equilibrium effects. The line of identity (solid line) between the two methods is not significantly different from the least squares fit (slope of 0.92, r = 0.95).

equilibrium conditions derived for monovalent anions would not apply strictly.[4] We tested both hypotheses experimentally by measuring chloride and BS concentrations in systems containing the non-filterable polyanion dextran sulfate as an analog for non-filterable polyanionic mixed micelles.

According to classical Donnan equilibrium theory (17), the ratio of anions and cations in the initial solution and filtrate can be expressed by the following equation:

$$\frac{[Na^+_{filtrate}]}{[Na^+_{initial}]} = \frac{[Cl^-_{initial}]}{[Cl^-_{filtrate}]} \qquad Eq.\ 1)$$

where $[Na^+_{initial}]$ and $[Na^+_{filtrate}]$ refer to sodium concentrations in the initial model bile and in the filtrate, respectively, and $[Cl^-_{initial}]$ and $[Cl^-_{filtrate}]$ refer to chloride concentrations in the initial model bile and in the filtrate, respectively. Since the ratio of any other filterable anion must also be equal to the ratio of chloride anions (17), the BS concentration in the IMC can be related to the BS concentration in the filtrate by the expression:

$$[BS_{IMC}] = \underbrace{\frac{[Cl^-_{initial}]}{[Cl^-_{filtrate}]}}_{\text{Correction factor (C.F.)}} \times [BS_{filtrate}] \qquad Eq.\ 2)$$

where $[BS_{IMC}]$ and $[BS_{filtrate}]$ refer to the BS monomeric and simple micelle concentrations in the initial model bile (defined as the IMC) and in the filtrate, respectively. Implicit in equation 2 is the assumption that BS simple micelles, which have a net negative charge equal to their aggregation number less bound sodium cations, are distributed identically to monovalent ions. In fact, Donnan theory predicts (17) that

$$\frac{[Cl^-_{initial}]}{[Cl^-_{filtrate}]} = \left(\frac{[BS_{IMC}]}{[BS_{filtrate}]}\right)^{1/n} \qquad Eq.\ 3)$$

where n is the net simple micellar charge. We later show experimentally that despite this assumption, the IMC can be accurately estimated (vide infra).

Fig. 4 schematically displays the relevant ionic species in the initial model bile and the filtrate, along with expressions of initial concentrations in terms of the correction factor (C.F.), derived from equation 2. Applying the condition of electroneutrality to both the initial solution and the filtrate, we obtain the following expressions:

$$[BS_{initial}] + [Cl^-_{initial}] = [Na^+_{initial}] \qquad Eq.\ 4)$$

$$[BS_{filtrate}] + [Cl^-_{filtrate}] = [Na^+_{filtrate}] \qquad Eq.\ 5)$$

where $[BS_{initial}]$ is equal to the sum of $[BS_{IMC}]$ and $[BS_{lecithin\text{-}associated}]$. By substituting for $[Cl^-_{initial}]$ and $[Na^+_{initial}]$, we obtain:

$$[BS_{initial}] + [Cl^-_{filtrate}] \times [C.F.] = [Na^+_{filtrate}]/[C.F.] \qquad Eq.\ 6)$$

Equations 5 and 6 can then be solved for the correction factor in terms of the measurable quantity $[Cl^-_{filtrate}]$:

$$C.F. = \frac{-[BS_{initial}] + \sqrt{[BS_{initial}]^2 + 4([Cl^-_{filtrate}] + [BS_{filtrate}]) \times [Cl^-_{filtrate}]}}{2\,[Cl^-_{filtrate}]} \qquad Eq.\ 7)$$

or in terms of the known quantity [NaCl], the initial NaCl concentration:

$$C.F. = \frac{[BS_{filtrate}] + \sqrt{[BS_{filtrate}]^2 + 4[NaCl] \times ([NaCl] + [BS_{initial}])}}{2\,([NaCl] + [BS_{initial}])} \qquad Eq.\ 8)$$

Of note, under conditions where $[BS_{initial}]$ equals $[BS_{IMC}]$, i.e., there are no mixed micelles or vesicles, as well as for conditions of very high NaCl concentrations, equations 7 and 8 simplify to a value of one for the correction factor.

Fig. 5A displays the magnitude of the correction factor as calculated from equation 8 and the expressions in Fig. 4 as functions of the non-diffusible anion concentration (= $[BS_{lecithin\text{-}associated}]$) for three different initial NaCl values. The bottom horizontal axis depicts the concentration of non-filterable anions, whereas the top axis depicts the concentration of non-filterable (mixed micellar and vesicular) BS in a model bile of typical physiological composition (TC/(TC + EYL) = 0.7, 10% cholesterol, IMC values from ref. 3). Although the correction factor is not significantly different from unity for systems of 1 g/dl (≈0.99), it becomes substantial (≈0.78) for concentrations of 10 g/dl as are found in gallbladder bile. In theory, increasing ionic strength decreases the asymmetrical distribution of ions, even within the limited range from 0.10 to 0.25 M.

Fig. 5B displays correction factors for systems containing 0.15 M NaCl, with three different values of the IMC from 0 to 40 mM, again as functions of the concentration of non-diffusible anion concentration, i.e., $[BS_{lecithin\text{-}associated}]$. As shown in Fig. 5B, the correction factor depends upon the

[4]The latter assumption was made for the calculation of Donnan equilibrium effects in modified equilibrium dialysis, and was indirectly confirmed by the fact that values of the IMC were used to correctly separate micelles and vesicles by gel filtration chromatography (3).

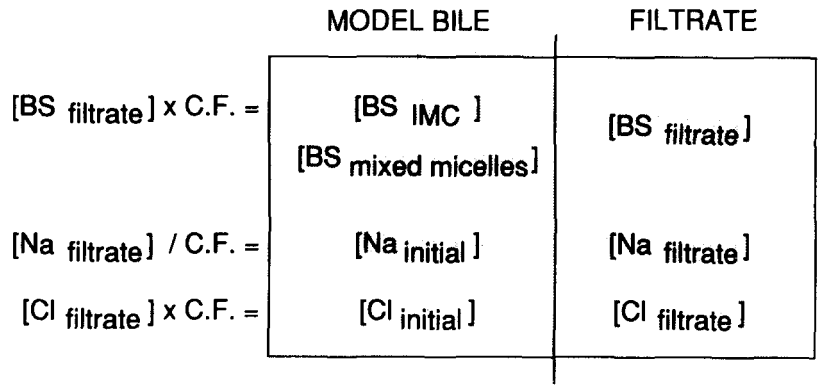

Fig. 4. Schematic diagram of the ionic species present in the model bile and in the filtrate, and the equations relating these concentrations (see text for details).

IMC as well as the NaCl concentration, as BS anions (and their sodium counterions) increase the effective ionic strength.

Donnan equilibrium effects: experimental considerations

To test the theoretical approach described above, filtrate BS concentrations were directly measured in the presence of known concentrations of non-filterable anions. As the non-filterable anion concentration in BS/EYL systems depends on the IMC, we studied systems containing BS and the polyvalent anion dextran sulfate. In this way, correction factors required to calculate the IMC from filtrate BS concentration could be determined experimentally and compared with values derived from theoretical considerations outlined above. **Fig. 6a** displays values of actual $[Cl^-_{initial}]/[Cl^-_{filtrate}]$ ratios as functions of theoretically calculated correction factors predicted by Donnan equilibrium considerations. Despite the rapid time course of ultrafiltration, the distribution of chloride anions during rapid centrifugal ultrafiltration conforms to Donnan equilibrium predictions. Fig. 6b displays values of $[BS_{initial}]/[BS_{filtrate}]$ ratios, which also agree with the distribution of chloride anions predicted by Donnan theory, both for membranes with 10,000 (squares) and 8,000 (circles) MWCO membranes for TC (closed squares) and for a mixture of BS (open symbols). Thus, in the presence of non-filterable anions, BS concentrations in the ultrafiltrate exceed those in the initial solution.

Fig. 7 compares experimental filtrate BS concentrations in systems containing dextran sulfate with the actual BS concentrations in the initial solution. BS filtrate concentrations (closed symbols) consistently exceeded BS concentrations in the initial solution (slope by least squares fit = 1.2). In contrast, corrected values (open symbols) more closely approximated the line of identity with the initial BS concentrations. Despite the polyvalent nature of BS simple micelles, the asymmetrical distribution of BS (Fig. 6b) approximated the distribution for monovalent anions predicted by Donnan theory, and allowed the IMC to be calculated. As Figs. 6 and 7 demonstrate, polyvalent BS micelles are distributed in a pseudo-Donnan distribution, and a correction is necessary to accurately measure the IMC.

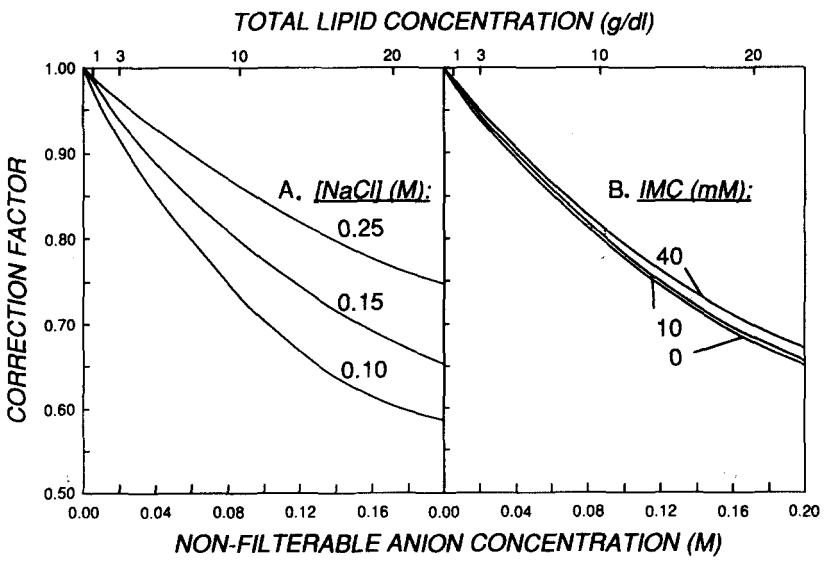

Fig. 5. Dependence of theoretical values of the correction factor on non-filterable anion concentrations for (A) varying values of [NaCl] (0–0.25); and (B) varying values of the IMC (0–40 mM) as calculated from equations 6 and 7. The total lipid concentration for model biles with BS/(BS + EYL) = 0.7 corresponding to the non-filterable anion concentrations shown is displayed on the top horizontal axis.

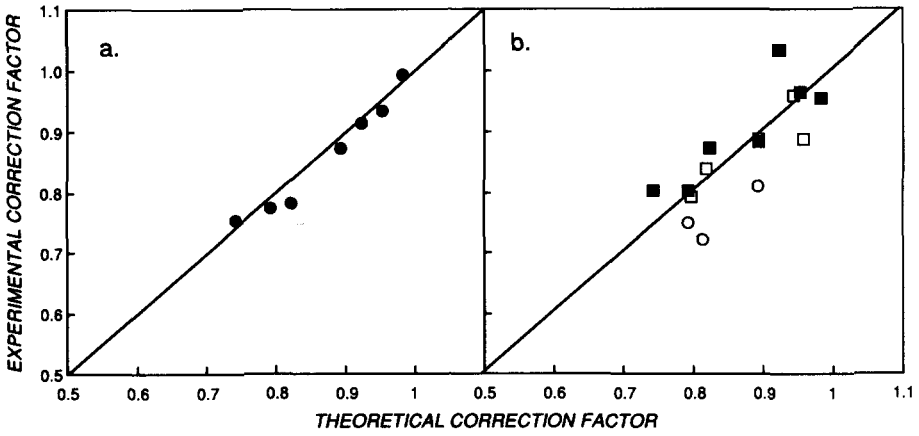

Fig. 6. Comparison of theoretical values with experimental values of the correction factor obtained by using dextran sulfate as a surrogate non-filterable anion for (a) chloride distribution (●) and (b) BS distribution of TC (■) and the BS mixture (□, ○) for 10,000 (■, □) and 8,000 (○) MWCO membranes. Theoretical values were calculated on the assumptions that Donnan equilibrium effects are present for centrifugal ultrafiltration, and that the ratio of BS monomers and simple micelles in the initial system and in the filtrate is identical to that of chloride. The least squares fits are shown and are not significantly different from the line of identity (not shown).

DISCUSSION

We have developed a rapid centrifugal method for measuring the IMC of model biles, and validated it by comparison to the technique of modified equilibrium dialysis as previously established in our laboratory (3). In contrast to modified equilibrium dialysis, which is limited by substantial expenditure of time and large volumes of bile, IMC values of model bile can be measured by the centrifugal ultrafiltration method in less than 2 h, thus allowing measurement of the IMC during the time frame wherein phase changes occur in metastable model or native biles (18). This method has the further advantage over modified equilibrium dialysis of not being subject to osmotically induced water shifts that may occur during long dialysis times.

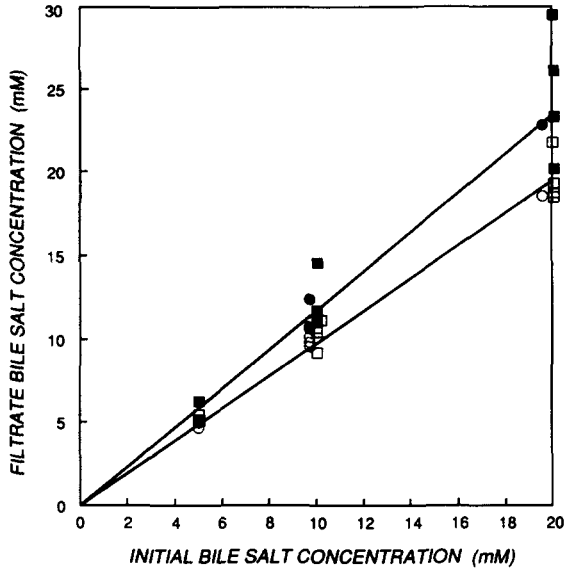

Fig. 7. Comparison of measured filtrate BS concentrations (●, ■) with values corrected for Donnan equilibrium effects (○, □). Systems were composed with dextran sulfate (50–150 mM) and either TC (●, ○) or the BS mixture (■, □). The least squares fit for the uncorrected values (closed symbols) has a slope of 1.24, consistent with the observation that ultrafiltrate BS concentrations exceeded those in the initial solution by from 5 to 50%. However, the least squares fit for the corrected values (open symbols) has a slope of 0.98, demonstrating that corrected ultrafiltrate concentrations accurately measure the IMC of biles.

Essentially, this method depends upon the separation by size of simple micelles from mixed micelles and larger aggregates. The size of the membrane pores is crucial, as the smallest mixed micelles have diameters approaching the size of the pores in the 10,000 MWCO membranes (25 Å, personal communication, 1992, Spectrum Industries). Therefore, for biles of high total lipid concentrations with high mixed micellar BS/(BS + EYL) ratios, 8,000 MWCO membranes were used to discriminate between simple and mixed micelles. As the BS/(BS + EYL) ratio in the mixed micelles increases, mixed micellar size also decreases (19), requiring a smaller MWCO to retain mixed micelles. A similar technique has been used to separate small mixed micelles from multilamellar vesicles, using a membrane with a much higher molecular weight cut-off of 100,000 (20).

The strongest evidence that the assumptions outlined above allow calculation of the IMC lies in experiments using dextran sulfate as an analog polyanion. These experiments demonstrated that despite minor retention of simple micelles by the membrane (Fig. 1), IMC values so calculated approximated the actual BS monomer plus simple micellar concentration in the original system (Fig. 7). As the ratio of the IMC to lecithin-associated BS does

not vary widely through the physiological range of biles, two countervailing effects appear to compensate for each other. As the IMC increases, simple micelles are increasingly retained by the membrane (thus decreasing the ratio of BS concentration in the filtrate to the true IMC), but the Donnan effect increases (thus increasing the ratio of BS concentration in the filtrate to the true IMC). The magnitude of both effects increases as total lipid concentration increases, concomitant with increasing concentrations of both simple and mixed micelles (3). As shown in Figs. 6 and 7, the net effect is that the experimental distribution of BS approximates the theoretical distribution of monovalent anions.

Importantly, this method does not alter the composition of the bile studied. In theory, removal of small volumes of aqueous phase containing the IMC (<15% of total volume) should not alter the relative composition of micelles and vesicles in the model bile since they remain in equilibrium with the IMC (2). However, for reasons of electroneutrality discussed above, the BS concentration in the filtrate exceeds the true IMC. It can be calculated that even after removal of 15% of the volume containing [$BS_{filtrate}$], the BS/(BS + EYL) ratio of mixed micelles and vesicles is altered by only 1%. Therefore, as shown in Fig. 2, the composition of mixed micelles and vesicles in the initial model bile is not significantly altered by the process of centrifugal ultrafiltration with removal of <15% of volume.

The eventual goal of these studies is to measure the IMC of native biles that contain mucins and other proteins that may bind BS. In preliminary studies in native biles (11), ultrafiltration is somewhat slowed as compared with model biles. However, the IMC of native biles and identically composed (with respect to lipid composition) model biles were comparable (11), suggesting that the multicomponent complex mixtures present in native bile act similarly to model systems. Because of their low absolute anion concentrations (<5 mg/ml), Donnan effects of non-filterable mucin and proteins are negligible (<1–2%) at biliary concentrations. Values obtained by modified equilibrium dialysis measurements of the IMC and calculated using theoretical values for the correction factor have been demonstrated to be the correct values for gel filtration chromatography of micelles and vesicles without net transfer of lipids between particles (3).

By measuring the asymmetrical distribution of chloride, this experimental approach can also be used to assess rapidly the net negative charge of other charged macromolecules. This work provides a frame of reference for considering the interactions of anions such as BS and unconjugated bilirubin with the physiological polyanion, biliary mucin (21).

The strong Donnan effects generated by BS are believed to be a major driving force for diffusion of calcium into bile (22). In the present work, the Donnan forces exhibited by bile are comparable to results reported for calcium distribution in the presence of BS as measured by equilibrium dialysis (23). The relationship reported by Dawes, Moore, and Rege (24):

$$C.F. = 1/(1 + 0.0025[BS]) \quad \textit{Eq. 9)}$$

gives approximately the same values of the correction factor as displayed in Fig. 5A for a NaCl concentration of 0.15 M. However, this simplified equation fails to take into account the effects of NaCl concentration or the relative magnitude of the IMC on the correction factor as shown in Fig. 5.

In conclusion, we have developed a non-invasive, rapid centrifugal method with corrections for Donnan equilibrium effects that accurately measures the IMC of bile. We believe that this methodology will facilitate investigation of rigorously derived IMC values for BS concentration and composition (16), which can then be used to separate the cholesterol-carriers of native and model biles. The ability to rapidly measure the IMC will allow analysis of biliary micelles and vesicles during cholesterol crystal formation, and give further insights into the earliest stages of cholesterol gallstone pathogenesis.

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REFERENCES

1. Carey, M. C. 1988. Lipid solubilization in bile. *In* Bile Acids in Health and Disease. T. Northfield, R. Jazrawi and P. Zentler-Munro, editors. Kluwer Academic, Dordrecht, The Netherlands. 61–82.
2. Donovan, J. M., and M. C. Carey. 1990. Separation and quantitation of cholesterol "carriers" in bile. *Hepatology.* **12:** 94S–105S.
3. Donovan, J. M., N. Timofeyeva, and M. C. Carey. 1991. Influence of total lipid concentration, bile salt:lecithin ratio, and cholesterol content on inter-mixed micellar/vesicular (non-lecithin-associated) bile salt concentrations in model bile. *J. Lipid Res.* **32:** 1501–1512.
4. Cohen, D. E., and M. C. Carey. 1990. Rapid (1 hour) high performance gel filtration chromatography resolves coexisting simple micelles, mixed micelles, and vesicles in bile. *J. Lipid Res.* **31:** 2103–2112.
5. Cohen, D. E., M. Angelico, and M. C. Carey. 1990. Structural alterations in lecithin-cholesterol vesicles following interactions with monomeric and micellar bile salts: physical-chemical basis for subselection of biliary lecithin species and aggregative states of biliary lipids during bile formation. *J. Lipid Res.* **31:** 55–70.

6. Schubert, R., and K-H. Schmidt. 1988. Structural changes in vesicle membranes and mixed micelles of various lipid compositions after binding of different bile salts. *Biochemistry.* **27:** 8787–8794.
7. Schurtenberger, P., N. Mazer, and W. Känzig. 1985. Micelle to vesicle transition in aqueous solutions of bile salt and lecithin. *J. Phys. Chem.* **89:** 1042–1049.
8. Duane, W. C. 1975. The intermicellar bile salt concentration in equilibrium with the mixed micelles of human bile. *Biochim. Biophys. Acta.* **398:** 275–286.
9. Higuchi, W. I., M. Arakawa, P. H. Lee, and S. Noro. 1987. Simple micelle-mixed micelle coexistence equilibria for the taurocholate-, taurochenodeoxycholate-, and tauroursodeoxycholate-lecithin systems. *J. Colloid Interface Sci.* **119:** 30–37.
10. Lee, P. H., W. I. Higuchi, N. A. Daabis, and S. Noro. 1985. Examination of the Sephadex G10 beads uptake method for determination of bile salt monomer concentration in taurocholate-lecithin solutions. *J. Pharm. Sci.* **74:** 880–882.
11. Donovan, J. M., A. A. Jackson, and M. C. Carey. 1991. A novel rapid method for determination of the intermicellar-intervesicular bile salt (BS) concentration (IMC) in bile: validation by a new equilibrium dialysis technique. *Gastroenterology.* **100:** A736.
12. Pope, J. L. 1967. Crystallization of sodium taurocholate. *J. Lipid Res.* **8:** 146–147.
13. Rossi, S. S., J. L. Converse, and A. F. Hofmann. 1987. High pressure liquid chromatographic analysis of conjugated bile acids in human bile: simultaneous resolution of sulfated and unsulfated lithocholyl amidates and the common conjugated bile acids. *J. Lipid Res.* **28:** 589–595.
14. Hay, D. W., M. J. Cahalane, N. Timofeyeva, and M. C. Carey. 1993. Molecular species of lecithins in human gallbladder bile. *J. Lipid Res.* **34:** 759–768.
15. Carey, M. C., and D. M. Small. 1978. The physical chemistry of cholesterol solubility in bile: relationship to gallstone formation and dissolution in man. *J. Clin. Invest.* **61:** 998–1026.
16. Donovan, J. M., A. A. Jackson, and M. C. Carey. 1993. Molecular species composition of inter-mixed micellar/vesicular bile salt concentrations in model bile: dependence upon hydrophilic-hydrophobic balance. *J. Lipid Res.* **34:** 1131–1140.
17. Tanford, C. 1961. Physical Chemistry of Macromolecules. John Wiley & Sons, Inc., New York.
18. Mazer, N. A., and M. C. Carey. 1983. Quasi-elastic light scattering studies of aqueous biliary lipid systems. Cholesterol solubilization and precipitation in model bile solutions. *Biochemistry.* **22:** 426–442.
19. Mazer, N. A., G. B. Benedek, and M. C. Carey. 1980. Quasielastic light-scattering studies of aqueous biliary lipid systems. Mixed micelle formation in bile salt-lecithin solutions. *Biochemistry.* **19:** 601–615.
20. Müller, K., and A. Schuster. 1990. Solubilization of multilamellar liposomes of egg yolk lecithin by the bile salt sodium taurodeoxycholate and the effect of cholesterol—a rapid-ultrafiltration study. *Chem. Phys. Lipids.* **52:** 111–127.
21. Smith, B. F., and J. T. LaMont. 1983. Bovine gallbladder mucin binds bilirubin in vitro. *Gastroenterology.* **85:** 707–712.
22. Rege, R. V., L. G. Dawes, and E. W. Moore. 1990. Biliary calcium secretion in the dog occurs primarily by passive convection and diffusion and is linked to bile flow. *J. Lab. Clin. Med.* **115:** 593–602.
23. Moore, E. W. 1990. Biliary calcium and gallstone formation. *Hepatology.* **12:** 206S–214S.
24. Dawes, L. G., E. W. Moore, and R. V. Rege. 1988. Gibbs-Donnan equilibrium is the major determinant of free Ca^{++} in canine bile. *Gastroenterology.* **94:** A534.