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

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Abstract We modified classic equilibrium dialysis methodology to correct for dialysant dilution and Donnan effects, and have systematically studied how variations in total lipid concentration, bile salt (taurocholate):lecithin (egg yolk) ratio, and cholesterol content influence inter-mixed micellar/vesicular (non-lecithin-associated) concentrations (IMC) of bile salts (BS) in model bile. To simulate large volumes of dialysant, the total volume (1 ml) of model bile was exchanged nine times during dialysis. When equilibrium was reached, dialysate BS concentrations plateaued, and initial and final BS concentrations in the dialysant were identical. After corrections for Donnan effects, IMC values were appreciably lower than final dialysate BS concentrations. Quasielastic light scattering was used to validate these IMC values by demonstrating that lipid particle sizes and mean scattered light intensities did not vary when model biles were diluted with aqueous BS solutions of the appropriate IMC. Micelles and vesicles were separated from cholesterol-supersaturated model bile, utilizing high performance gel chromatography with an eluant containing the IMC. Upon rechromatography of micelles and vesicles using an identical IMC, there was no net transfer of lipid between micelles and vesicles. To simulate dilution during gel filtration, model biles were diluted with 10 mM Na cholate, the prevailing literature eluant, resulting in net transfer of lipid between micelles and vesicles, the direction of which depended upon total lipid concentration and BS/lecithin ratio. Using the present methodology, we demonstrated that inter-mixed micellar/vesicular concentrations (IMC) values increased strongly (5 to 40 mM) with increases in both bile salt (BS):lecithin ratio and total lipid concentration, whereas variations in cholesterol content had no appreciable effects. For model biles with typical physiological biliary lipid compositions, IMC values exceeded the critical micellar concentration of the pure BS, implying that in cholesterol-supersaturated biles, simple BS micelles coexist with mixed BS/lecithin/cholesterol micelles and cholesterol/lecithin vesicles. We believe that this methodology allows the systematic evaluation of IMC values, with the ultimate aim of accurately separating micellar, vesicular, and potential other cholesterol-carrying particles from native bile. —**Donovan, J. M., N. Timofeyeva, and M. C. Carey.** 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.* 1991. **32:** 1501–1512.

Supplementary key words equilibrium dialysis • gel chromatography • quasielastic light scattering • Donnan equilibrium • critical micellar concentration

Cholesterol gallstone formation is a complex process, hallmarked by the precipitation of solid cholesterol monohydrate crystals from cholesterol-supersaturated bile (1). In cholesterol-unsaturated model biles, cholesterol is solubilized in two types of lipid aggregates that coexist under physiological conditions: simple micelles that contain bile salts (BS) and cholesterol, and mixed micelles that contain BS, lecithin, and cholesterol (2). In cholesterol-supersaturated bile, cholesterol is also transported in unilamellar vesicles that are composed of lecithin and cholesterol (3–6). Other metastable particles may exist in native bile (7), but are not well characterized. Despite considerable advances (8), the physical-chemical processes that lead to crystallization of cholesterol monohydrate from cholesterol-supersaturated bile are incompletely understood (1). It is believed that small unilamellar cholesterol-rich vesicles fuse to form multilamellar vesicles, and cholesterol monohydrate crystals have been observed to nucleate from these aggregates (9). Further growth of cholesterol monohydrate crystals apparently occurs from cholesterol monomers that diffuse to crystal surfaces from both supersaturated micelles and vesicles (10).

An understanding of the respective roles of micelles and vesicles in cholesterol monohydrate crystal formation re-

Abbreviations: IMC, inter-mixed micellar/vesicular bile salt concentration; BS, bile salt; EYL, egg yolk lecithin; TC, sodium taurocholate; QLS, quasielastic light scattering; CMC, critical micellar concentration; HPLC, high performance liquid chromatography.

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quires their accurate separation for structural and kinetic studies as well as for determination of relative and absolute lipid compositions (11). In cholesterol-supersaturated biles, the number or composition of phases may change over hours or days, implying that these biles are in metastable states, and not at true thermodynamic equilibria. In either thermodynamic state, biliary mixed micelles and vesicles coexist with a common intermediate, the monomer or monomer plus simple micellar concentration of BS.[2] The range of correct values for this monomer (plus micellar) concentration of BS, which we term the IMC to represent the inter-mixed micellar/vesicular (non-lecithin-associated) concentration of BS, has not been determined in an accurate or systematic manner (11). Only if the IMC is kept constant can different cholesterol "carriers" be separated unchanged by a technique such as gel chromatography that dilutes the system. When BS concentrations greater than the IMC are used, vesicles are transformed into micelles; conversely, when BS concentrations below the IMC are used, micelles are transformed into vesicles (12). Due to a lack of experimental knowledge of the IMC, previous attempts to separate micelles and vesicles by gel filtration and ultracentrifugation (5, 6, 13, 14) have had serious shortcomings (for review, see ref 11). Sömjen and Gilat (5) demonstrated that during gel filtration using the most commonly used eluant, the unconjugated BS, Na cholate, at a concentration of 10 mM, the relative proportions of micelles and vesicles were altered, since upon rechromatography of the vesicular fraction under identical conditions, 70% of the "vesicle" cholesterol eluted with the micellar fraction. Moreover, the relative lipid compositions of both micellar and vesicular phases are altered by an incorrect IMC in the eluant buffer (15).

In this work, we utilized an adaptation of the technique of equilibrium dialysis to determine IMC values of model biles. Although equilibrium dialysis had previously been used to estimate the IMC of systems containing mixed micelles and vesicles (16–18), bile was invariably diluted by water flux into the dialysant (bile solution under study) and the relative lipid composition was altered by appreciable losses of BS from dialysant to dialysate. We present herein two critical modifications of previous dialysis approaches to determine the IMC of bile, and utilize this method to examine systematically the effects of physical-chemical variables of pathophysiological relevance on the IMC of model biles[3].

METHODS

Materials

Sodium taurocholate (TC) (Calbiochem-Behring, La Jolla, CA) was purified (20, 21); grade I egg yolk lecithin (EYL) was obtained from Lipid Products (South Nutfield, UK), and cholesterol (Nu-Chek Prep, Elysian, MN) was recrystallized from hot 95% ethanol as necessary. Sodium cholate was obtained from Calbiochem-Behring. By high performance liquid chromatography (HPLC) (22) (Beckman Instruments, Wakefield, MA), TC purity was >99%. Cholesterol was >99% pure by gas-liquid chromatography. Purity of EYL was confirmed by thin-layer chromatography ($CHCl_3$–MeOH–H_2O 95:35:4, v:v:v) and HPLC of the benzoyl diacylglycerol derivatives (23). NaCl (Mallenckrodt, Paris, KY) was roasted for 3 h at 600°C to oxidize and remove organic impurities. All other chemicals were of ACS quality or highest reagent grade purity. Pyrex glassware was alkali-washed overnight in EtOH–2 M KOH 1:1 (v/v), followed by 24-h acid-washing in 4 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, and resuspension in aqueous solution (0.15 M NaCl, 0.001 M NaN_3, pH 7.2). Model biles were prepared with various BS/(BS+EYL) ratios (0.5–0.8), total lipid concentrations (1–10 g/dl), and cholesterol contents (0–10 mol%, i.e., mol/100 mol lipid). BS/EYL ratio is expressed as BS/(BS+EYL), since for a series of experiments this ratio was kept constant while cholesterol content was varied. For gel chromatography, model biles were prepared using tracer amounts of [^{14}C]*sn*-1-16:0:*sn*-2-18:1 lecithin and [^{3}H]cholesterol (New England Nuclear, Boston, MA).

Chemical analysis

EYL and cholesterol concentrations were assayed enzymatically (24, 25), or by scintillation counting (Beckman Instruments). TC was quantified enzymatically (26) or by HPLC (22). The HPLC method was initially calibrated using an internal standard (taurolithocholic acid-3-sulfate; Calbiochem-Behring). Chloride concentrations were de-

[2]Theoretically, one might consider that the aqueous monomer concentrations of lecithin and cholesterol molecules in bile would be of similar importance; however, for purposes of particle separation in biles with total lipid concentrations greater than 1 g/dl, these monomer concentrations are insignificant (approximately 10^{-10} and 10^{-8} M, respectively) compared with total concentrations of lecithin and cholesterol. In the case of BS, monomer concentrations (approximately 5 mM in the model biles in this work) are a significant fraction of the total BS concentration.

[3]Presented in part at the National Meeting of the American Gastroenterological Association, San Antonio, Texas, May 13–16, 1990 and published as an abstract (19).

termined spectrophotometrically (Sigma Chemical Co., St. Louis, MO).

Equilibrium dialysis

When model bile is dialyzed against BS-free dialysate, bile composition is altered by transfer of BS into the dialysate, as well as dilution by osmotic forces tending to draw dialysate water into bile. Because the final composition of dialysant bile is altered, the equilibrium BS concentration in the dialysate reflects the IMC of the altered bile rather than that of the initial bile. Theoretically, this problem could be avoided by dialyzing an infinitely large reservoir of dialysant bile against a very small volume of dialysate. The method described below modifies previous techniques to approximate such conditions.

As depicted schematically in **Fig. 1,** equilibrium dialysis was carried out in 1.0-ml cells (Fisher Scientific, Medford, MA), separated by Spectrapor dialysis membranes (molecular weight cutoff (MWCO) 12,000, Spectrum Medical Industries, Los Angeles, CA) that were first prepared by exhaustive washing in glass-distilled water. Dialysant model bile (1.0 ml) 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 7.2) was placed in the other. The entire apparatus was immersed in a continuously shaken water bath at 37°C. At minimal intervals of 2 h, small portions ($\approx$30 μl) of both dialysant and dialysate were removed, and the dialysant (model bile) was replaced by 1.0 ml of the initial model bile solution. The dialysant was exchanged nine times, since in preliminary experiments (see below) equilibrium was reached after approximately seven changes.[4] The dialysate BS concentration was then determined in duplicate during the final three exchanges of model bile, and corrected for Donnan equilibrium effects (see below) (27), to obtain values for the IMC. Results from one to four individual experiments (n = 6–24 determinations) are reported as IMC values ± SD.

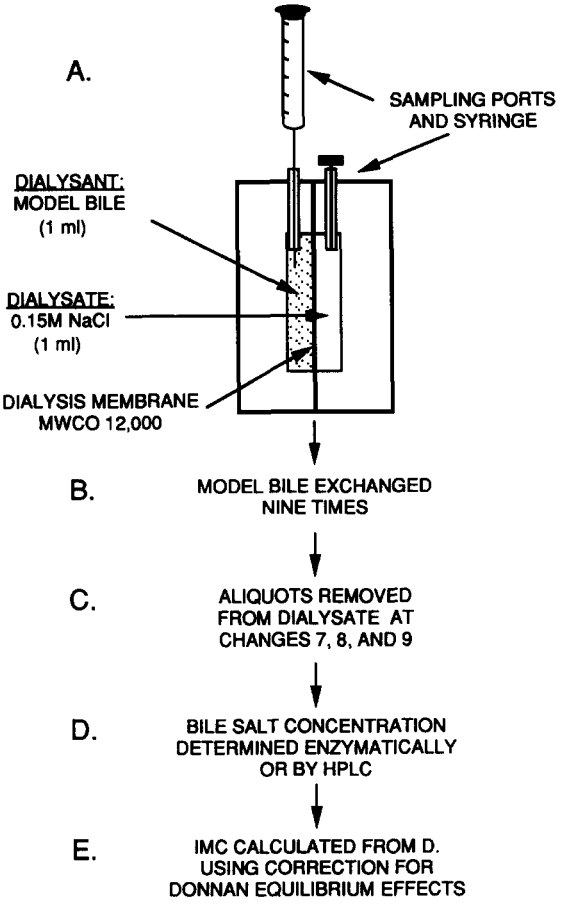

Fig. 1. Flow chart depicting steps A–E of equilibrium dialysis method for determining the IMC of model biles. Model bile (dialysant) and dialysate were placed in equilibrium dialysis chambers (1.0 ml each) separated by a dialysis membrane with molecular weight cut-off (MWCO) of 12,000. A syringe and needle were used to gain access to each chamber for periodic sampling. During incubation at 37°C, model bile was exchanged nine times at minimal intervals of 2 h. Small portions of dialysate were removed after changes 7, 8, and 9, and the BS concentration was determined by HPLC or enzymatically. The IMC was calculated from the dialysate BS concentration, following correction for Donnan equilibrium effects (see text).

Donnan equilibrium calculations

BS-containing mixed micelles are highly charged and non-diffusible (except via dissociation to monomers). Therefore Donnan equilibrium effects must be considered in calculating the distribution of any other diffusible charged species, e.g., monomeric BS and simple BS micelles, across a dialysis membrane (27). In systems with non-diffusible anions, e.g., mixed micelles, equilibrium concentrations of diffusible cations are lower in the dialysate than in the dialysant; conversely, equilibrium concentrations of diffusible anions are higher in the dialysate than in the dialysant. For systems with more than one freely diffusible monovalent anion, e.g., TC monomers and chloride anions, the ratio of their concentrations in the dialysant and dialysate are identical at equilibrium (27). For example,

$$\frac{[TC_{Monomer}]_{dialysant}}{[TC_{Monomer}]_{dialysate}} = \frac{[Cl]_{dialysant}}{[Cl]_{dialysate}} \qquad Eq.\ 1)$$

where $[Cl]_{dialysate}$, $[Cl]_{dialysant}$, $[TC_{Monomer}]_{dialysate}$, and $[TC_{Monomer}]_{dialysant}$ are the concentrations of chloride and monomeric TC in the dialysate and dialysant, respectively. Strictly, equation 1 only applies for monovalent, non-aggregating ions, i.e., for TC concentrations in the dialy-

[4] In theory, after an infinite time, lecithin and cholesterol concentrations would become equal in dialysate and dialysant, transferred through monomer concentrations of each. However, over the duration of these experiments, transfer of these lipids is insignificant (see Results), and only equilibration of BS concentrations occurs.

sate and the IMC fraction of the dialysant that are below the critical micellar concentration (CMC) of TC (approximately 5 mM in 0.15 M NaCl (28)). For simple TC micelles of aggregation number n, the following equation applies:

$$\left[\frac{[TC_{n\text{-}mer}]_{dialysant}}{[TC_{n\text{-}mer}]_{dialysate}}\right]^n = \frac{[Cl]_{dialysant}}{[Cl]_{dialysate}} \quad Eq.\ 2)$$

where $[Cl]_{dialysate}$, $[Cl]_{dialysant}$, $[TC_{n\text{-}mer}]_{dialysate}$, and $[TC_{n\text{-}mer}]_{dialysant}$ are the concentrations of chloride and each species of simple TC micelle with aggregation number n that are present in the dialysate and dialysant, respectively. The left side of equation 2 is raised to the nth power, since n is the valence of a simple TC micelle containing n monomers.

To calculate the distribution of multivalent simple micelles in the dialysate and dialysant, the concentration of simple micelles of each aggregation number must be known. In the case of TC, aggregation occurs gradually at approximately the CMC (28), and the exact distribution of n-mers is unknown; hence, a rigorous calculation of the distribution of BS between the dialysate and dialysant is impossible. To estimate the magnitude of Donnan equilibrium effects on BS distribution, we have neglected effects of BS aggregation, and assumed that:

$$\frac{[TC_{IMC}]_{dialysant}}{[TC]_{dialysate}} = \frac{[Cl]_{dialysant}}{[Cl]_{dialysate}} \quad Eq.\ 3)$$

where $[Cl]_{dialysate}$, $[Cl]_{dialysant}$, $[TC]_{dialysate}$, and $[TC_{IMC}]_{dialysant}$ are the concentrations of chloride and TC in the dialysate and in the IMC fraction of the dialysant, respectively.

Donnan equilibrium considerations and electroneutrality further require the following conditions:

$$[Na]_{dialysate}\,[Cl]_{dialysate} = [Na]_{dialysant}\,[Cl]_{dialysant} \quad Eq.\ 4)$$

$$[Na]_{dialysate} = [Cl]_{dialysate} + [TC]_{dialysate} \quad Eq.\ 5)$$

Since after multiple changes of dialysant, $[Na]_{dialysant}$ and $[Cl]_{dialysant}$, are equal to their concentrations in the original model bile (including sodium added in the form of NaTC), and $[TC]_{dialysate}$ is a measured quantity, then $[Na]_{dialysate}$ and $[Cl]_{dialysate}$ can be calculated from equations 4 and 5. The IMC can then be calculated from equation 3 in rearranged format:

$$[TC_{IMC}]_{dialysant} = \frac{[Cl]_{dialysant}}{[Cl]_{dialysate}}\,[TC]_{dialysate} \quad Eq.\ 6)$$

We show later (see Results) that experimentally obtained values of $\frac{[Cl]_{dialysant}}{[Cl]_{dialysate}}$ agree within experimental error with values obtained by this simple theoretical model. Although use of equation 3 provides a minimum estimate of the difference between $[TC_{IMC}]_{dialysant}$ and $[TC]_{dialysate}$, we demonstrate later (see Results) that this simplified model is adequate to determine the IMC of model biles.

Quasielastic light scattering

Quasielastic light scattering (QLS) was used as previously described (29, 30). Equipment included a Spectra Physics (Mountain View, CA) model 164 argon ion laser, a 64 channel Langley Ford autocorrelator (Amherst, MA), and a thermostatically controlled sample holder. Analysis yielded mean hydrodynamic radius ($\bar{R}_h$), normalized light intensity ([intensity/concentration]/[intensity of initial solution/concentration of initial solution]), and polydispersity, a measure of the width of particle distributions about the mean (2).

To validate IMC values measured by the modified equilibrium dialysis method, model biles were diluted tenfold with solutions containing a variety of TC concentrations (0–30 mM TC, 0.15 M NaCl, 37°C, pH 7.2), spanning the range of IMC values measured by modified equilibrium dialysis. Values of $\bar{R}_h$, normalized light intensity, and polydispersity were determined immediately after dilution, and at 3, 6, 24, and 48 h. Values were similar at all times, and results obtained at 3 h are reported in this work.

High performance gel chromatography

Micelles and vesicles were isolated from model biles by high performance gel chromatography at 37°C (12) (30 ml/h, 1.0-ml fractions, Superose 6, Pharmacia, Piscataway, NJ), using the appropriate IMC in the eluant (11 mM TC, 0.15 M NaCl, 0.001 M NaN_3). EYL and cholesterol concentrations of each fraction were determined by radiochemical analysis. To verify that lipid compositions of the micelles and vesicles were unaltered when the IMC was used as the eluant in gel chromatography, fractions that contained maximum concentrations of vesicles and micelles were reapplied to the column within 1 h, and rechromatographed with a TC eluant identical to that used in the initial separation.

RESULTS

Modified equilibrium dialysis method

Fig. 2 depicts BS concentrations in the dialysant and the dialysate during nine changes of dialysant (3 g/dl, BS/(BS + EYL) = 0.6, 10% cholesterol). During approximately seven changes of dialysant, BS concentrations in the dialysate increased to reach a plateau. Initially, dialysant BS concentrations decreased, due to BS dialyzing into the dialysate and osmotic forces drawing water into the dialysant. However, with repeated exchanges of di-

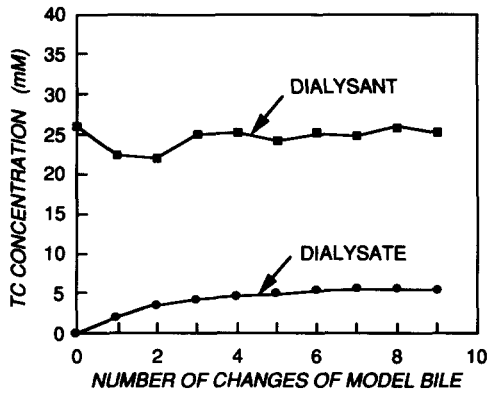

Fig. 2. BS (taurocholate, TC) concentrations in the dialysant and dialysate chambers are plotted for nine successive changes of model bile (BS/(BS+EYL)=0.6, 10% cholesterol, 3 g/dl, 0.15 M NaCl, 37°C). At the earliest time points, the BS concentration in the dialysant decreased due to BS outflow into the dialysate, as well as osmotically induced water flow into the dialysant. The BS concentration in the dialysate gradually increased, and stabilized after seven changes of model bile.

alysant, the BS concentration in the dialysant stabilized and approximated values (within 2%) of the initial model bile solution. Insignificant amounts of EYL and cholesterol (<0.4% of initial model bile) were present in the dialysate chamber at the end of the experiment.[5]

As outlined above, because of Donnan equilibrium effects, measured TC concentrations in the dialysate, $[TC]_{dialysate}$, were predicted to exceed the actual IMC. **Table 1** shows the prediction of our simplified theoretical model for the ratio, $\frac{[TC_{IMC}]_{dialysant}}{[TC]_{dialysate}}$, i.e., the ratio of the IMC to the dialysate BS concentration. To obtain the IMC, final dialysate BS concentrations were multiplied by these factors, ranging from 0.79 (for 10 g/dl model bile) to 0.99 (for 1 g/dl model bile). As shown in Table 1, these factors were principally determined by total lipid concentration, and largely independent of BS/(BS+EYL) ratio and cholesterol content. Whereas the magnitude of the derived correction factors fell within experimental error for biles with total lipid concentrations of 1 g/dl, the correction factors for biles with total lipid concentrations of 3 and 10 g/dl resulted in substantial differences between dialysate BS concentrations and the IMC.

According to equation 3, the ratio $\frac{[Cl]_{dialysant}}{[Cl]_{dialysate}}$ is predicted to be equal to the theoretical value for $\frac{[TC_{IMC}]_{dialysant}}{[TC]_{dialysate}}$. Table 1 demonstrates that experimental values for $\frac{[Cl]_{dialysant}}{[Cl]_{dialysate}}$ were indeed comparable to theoretically calculated values.

[5]We estimated the maximum error introduced by transfer of lecithin and cholesterol as follows: the highest concentrations of lecithin and cholesterol in an initial model bile were 33 and 18 mM, respectively (10 g/dl, 10% cholesterol and BS/BS + EYL = 0.8, IMC = 38 mM). If 0.4% of the lecithin was transferred to the dialysate, the final concentration would be 0.14 mM, which would bind only ≈0.4 mM BS. Thus, the maximum error in the IMC would be ≈1%.

Validation of the IMC by QLS

Since dilution of model biles with a solution containing the correct IMC does not change micellar size (2), QLS was used to provide an independent validation of IMC values obtained by the equilibrium dialysis method. This technique is similar to the classical light scattering method used by Shankland (31) and Nichols (32). When model biles containing both micelles and vesicles are diluted with BS concentrations that exceed the IMC, vesicles are transformed into micelles, and both $\bar{R}_h$ values and the normalized light intensity are predicted to fall (30). On the other hand, when model biles are diluted with BS solutions in concentrations below the IMC, micelles are transformed into vesicles (18). Thus normalized light intensity rises, and since light scattered from larger vesicles dominates the QLS signal (30), R_h values remain approximately constant. However, when model biles are diluted with solutions containing BS concentrations at the IMC, neither the $\bar{R}_h$ values, polydispersity, nor normalized light intensity change appreciably (2).

Fig. 3 displays a typical experiment for a model bile containing micelles and vesicles (3 g/dl, BS/(BS+EYL)=0.7, 10% cholesterol). Here the changes in values of $\bar{R}_h$, polydispersity, and normalized light intensity (all on the vertical axes) are plotted against a range of TC diluent concentrations (0–30 mM, horizontal axis). Initial values of $\bar{R}_h$, polydispersity, and normalized light intensity for the undiluted system are shown by the horizontal arrows. When the bile was diluted with concentrations of TC that fell below the IMC as derived by modified equilibrium dialysis (to the left of the arrowheads), which in this case was approximately 11 mM, $\bar{R}_h$ fell slightly (Fig. 3a), normalized light intensity rose (Fig. 3b), and polydispersity remained unchanged (Fig. 3c). Thus, some of the micelles were transformed into vesicles. Conversely, when the system was diluted with TC concentrations that were well above the IMC (to the right of the arrowheads), $\bar{R}_h$ values attained micellar sizes (Fig. 3a), normalized light intensity fell (Fig. 3b), while polydispersity increased (Fig. 3c), consistent with the transformation of vesicles into micelles. Since $\bar{R}_h$ values and polydispersity did not change substantially with diluent concentrations below the IMC, a precise determination of the IMC by monitoring these parameters was not possible. However, under the same conditions, measurements of normalized light intensity (Fig. 3b) allowed the IMC to be determined to within 1–2 mM. In the example displayed in Fig. 3,

TABLE 1. Correction factors for calculating the IMC from dialysate BS concentrations based upon Donnan equilibrium considerations

Model Bile Composition		Correction Factors		
		$\frac{[TC_{IMC}]_{dialysant}}{[TC]_{dialysate}}$ [a] (Theoretical)		$\frac{[Cl]_{dialysant}}{[Cl]_{dialysate}}$ [b] (Experimental)
BS/(BS + EYL) Molar Ratio	Total Lipid Concentration	0% Cholesterol	10% Cholesterol	10% Cholesterol
	g/dl			
0.8	10	0.81	0.80	
0.7	10	0.79	0.82	0.81
0.6	10	0.81	0.80	
0.8	3	0.94	0.95	
0.7	3	0.93	0.94	0.92
0.6	3	0.94	0.94	
0.8	1	0.99	0.99	
0.7	1	0.98	0.98	N.D.[c]
0.6	1	0.98	0.98	

[a] $\frac{[TC_{IMC}]_{dialysant}}{[TC]_{dialysate}}$ is the theoretical ratio of the IMC to the equilibrium BS concentration in the dialysate, based upon our simplified model (equation 6). These values were used to calculate IMC values from final measured BS concentrations in the dialysate (see text for details).

[b] Analytically determined values of $\frac{[Cl]_{dialysant}}{[Cl]_{dialysate}}$ are predicted to be equal to theoretical values for $\frac{[TC_{IMC}]_{dialysant}}{[TC]_{dialysate}}$ (see text for details).

[c] N.D., not determined.

neither $\bar{R}_h$ values, normalized light intensity, nor polydispersity changed appreciably upon dilution when the diluent solution contained an IMC of 11 mM TC.

Comparison of modified equilibrium dialysis and QLS methods for determining the IMC

To provide independent validation of the modified equilibrium dialysis method of measuring the IMC (with Donnan corrections), IMC values of a series of model biles (3 and 10 g/dl, BS/(BS+EYL)=0.5–0.8, 0 and 10% cholesterol, 0.15 M NaCl, 37°C) were measured both by equilibrium dialysis and by QLS. As shown in **Fig. 4,** there was a close correlation between IMC values measured by both methods, since the line of identity was not significantly different from the least squares fit to the data (slope = 1.04, r = 0.98, 95% confidence limits 0.90–1.22). When corrections for Donnan effects were omitted, the least square fit yielded a line with slope 1.47 (95% confidence limits 1.25–1.68). The strong agreement between IMC values derived by QLS and modified equilibrium dialysis suggests that the theoretically derived Donnan correction factors are appropriate within experimental error.

Validation of IMC values by gel chromatography

A model bile containing both micelles and vesicles (3 g/dl, BS/(BS+EYL)=0.7, 10% cholesterol) was subjected to gel chromatography, utilizing its measured IMC value (11 mM TC, 37°C). **Fig. 5a** depicts the separation of micelles and vesicles, as well as their relative EYL and cholesterol concentrations. After isolation, fractions containing either micelles or vesicles were rechromatographed using the same concentration of TC in the eluant. Despite considerable dilution, there was no transformation of vesicles into micelles (absence of micellar peak in Fig. 5b), and the cholesterol/EYL ratio of the eluted vesicles was 1.3, a value identical to that of the vesicles applied to the column. Further, when the micellar peak was rechromatographed (Fig. 5c), none of the micellar lipid was found in any fraction in which vesicles previously eluted. Thus, the IMC measured by the present technique, which includes both monomeric BS and simple BS micelles, completely preserves vesicle and mixed micelle integrity during particle separation by gel chromatography.

Artifacts introduced by the use of 10 mM Na cholate as the IMC

To evaluate systematic errors in relative concentrations and compositions of micelles and vesicles introduced by the literature use of 10 mM Na cholate for separation of micelles and vesicles from bile (11), we also diluted a series of model biles (BS/(BS+EYL)=0.6–0.8, 10% cholesterol, 1–10 g/dl, pH 7.2) with 10 mM Na cholate, and measured $\bar{R}_h$ values, normalized light intensity, and polydispersity. **Fig. 6** shows that for all model biles with total lipid con-

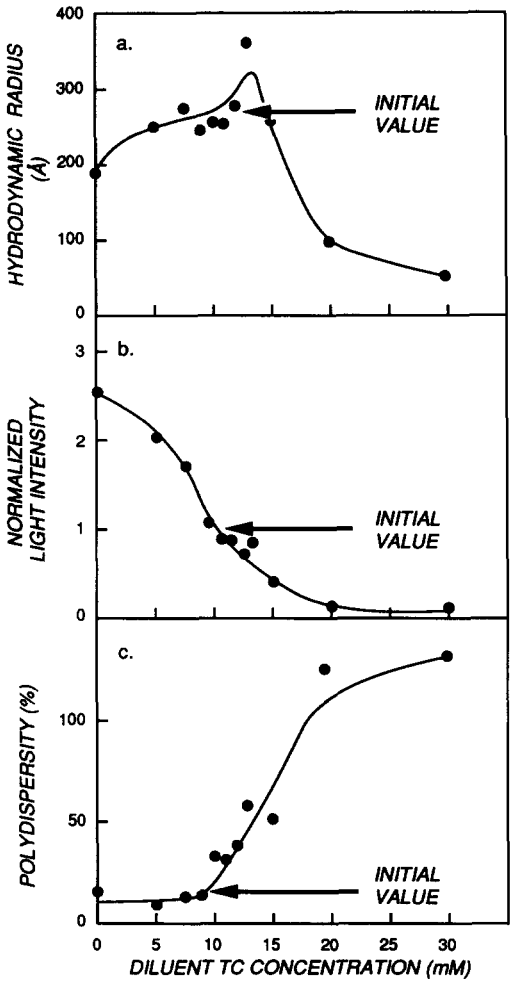

Fig. 3. Final mean hydrodynamic radius (R_h value) (Fig. 3a), normalized light intensity (Fig. 3b) and polydispersity (Fig. 3c) obtained by QLS when a cholesterol-supersaturated model bile (BS/(BS+EYL)= 0.7, 10% cholesterol, 3 g/dl, 0.15 M NaCl, 37°C, 3 h after dilution) was diluted tenfold with TC concentrations that varied from 0 to 30 mM (abscissa). Horizontal arrows indicate the respective values obtained for the original undiluted model bile. Using a diluent concentration of approximately 11 mM TC, neither $\bar{R}_h$ values, normalized light intensity, nor polydispersity changed appreciably upon dilution (further described in text).

centrations 3, 7, or 10 g/dl, the normalized light intensity increased after dilution with 10 mM Na cholate; we thus infer that for these total lipid concentrations, micelles were transformed into vesicles. In contrast, in the case of model biles with a total lipid concentration of 1 g/dl and a BS/(BS+EYL) ratio of 0.6, the normalized light intensity decreased (Fig. 6); hence, dilution with 10 mM Na cholate resulted in net transformation of vesicles into micelles for this dilute model bile. For model biles with total lipid concentrations of 1 g/dl and BS/(BS+EYL) ratios of 0.7 and 0.8, the normalized light intensity remained approximately constant following dilution with 10 mM Na cholate; hence for these particular biles only, 10 mM Na cholate approximated the IMC.

Influence of physical-chemical variables on IMC values

As displayed in **Fig. 7,** IMC values of model biles containing only micelles (3 g/dl, 0% cholesterol, 37°C) increased with increasing BS/(BS+EYL) ratio. Model systems containing micelles plus vesicles (3 g/dl, 10% cholesterol) showed an identical increase as displayed by the broken line (Fig. 7), with values similar to those in the absence of cholesterol. At physiological lipid compositions (BS/(BS+EYL)≈0.7), the IMC exceeded the CMC of TC (approximately 5 mM), suggesting that in native biles, both simple and mixed micelles coexist with vesicles.

Fig. 8 plots the dramatic increases in IMC values of model biles with increases in total lipid concentrations for BS/(BS+EYL) ratios spanning the physiological range (BS/(BS+EYL)=0.6, 0.7, and 0.8, 10% cholesterol). Most notable were the increases with higher BS/(BS+EYL) ratios, e.g., 0.8. Of importance is that for all total lipid concentrations above 1 g/dl, the IMC exceeded the CMC of TC, which approximates 5 mM (28). In comparison, the IMC in the absence of lecithin (BS/(BS+EYL)=1.0) increased linearly with increases in total lipid concentration, since all BS in solution are physically in the form of monomers or monomers plus simple micelles.

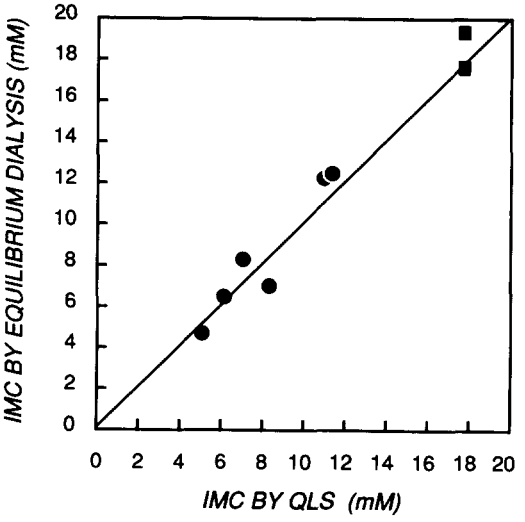

Fig. 4. Comparison of IMC values determined by equilibrium dialysis (corrected for Donnan equilibrium effects) and by QLS (see text) for model biles of total lipid concentration 3 g/dl (●) and 10 g/dl (■). The solid line depicts the line of identity between the two methods. The least squares fit of the data had a slope of 1.04 (95% confidence limits 0.90–1.22). If final BS concentrations in the dialysate were not corrected for Donnan equilibrium effects, IMC values would have been approximately 7% and 25% higher for biles with total lipid concentrations of 3 and 10 g/dl, respectively, and the slope of the least squares fit of the data would have been 1.47 (95% confidence limits 1.25–1.68).

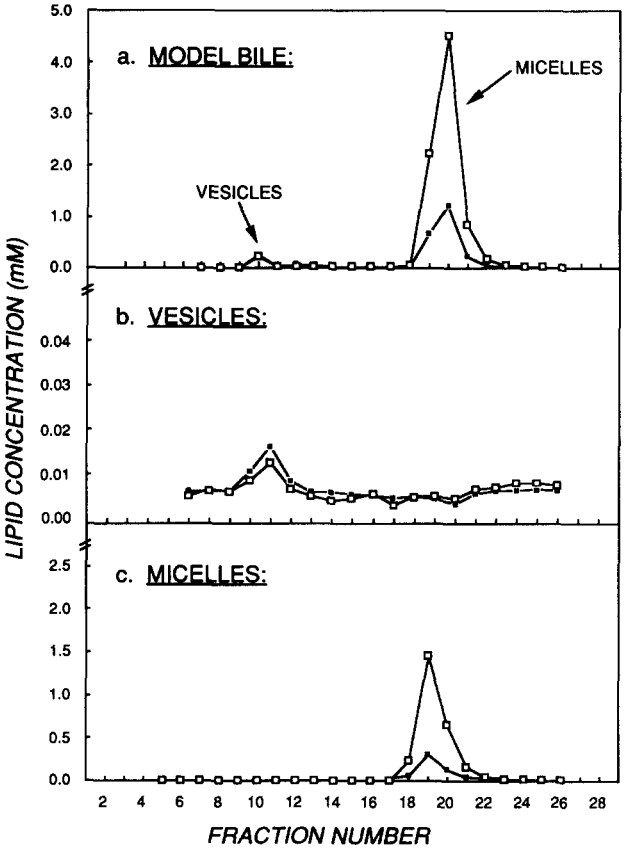

Fig. 5. Gel filtration of model bile (BS/(BS+EYL)=0.7, 10% cholesterol, 3 g/dl, 0.15 M NaCl, 37°C, pH 7.2) using the correct IMC as eluant (11 mM TC). The ordinate shows the cholesterol (closed symbols) and EYL (open symbols) concentrations (mM). Scales are not identical in order to encompass dilutions during rechromatography. a: Initial chromatography yielded two elution peaks, which were confirmed by QLS to have $\bar{R}_h$ values consistent with vesicles (380 Å) and mixed micelles (≤40 Å). b: Upon rechromatography of the vesicle peak under identical conditions, vesicles eluted at the same position as in (a), and no lipid eluted at the position of the micellar peak as in Fig. 5a. c: Rechromatography of the micellar peak under the same conditions demonstrated that no lipid eluted at the position of the vesicle peak as in (a).

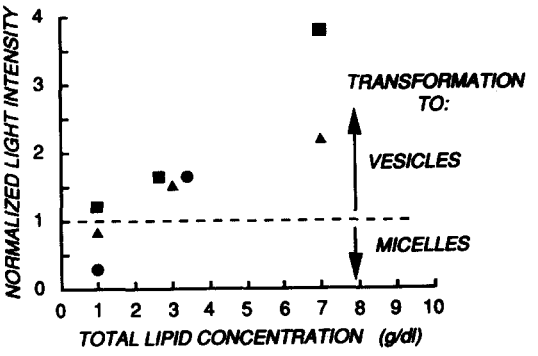

Fig. 6. Influences of tenfold dilution with 10 mM Na cholate on normalized light intensity of model biles (1–10 g/dl, 10% cholesterol, 0.15 M NaCl, 37°C, pH 7.2) for three different BS/(BS+EYL) ratios: 0.8 (■); 0.7 (▲); and 0.6 (●). When the relative proportions of vesicles and micelles remained constant upon dilution, the normalized light intensity was 1.0. However, 10 mM Na cholate dilution of a model bile with a BS/(BS+EYL) ratio of 0.6, and 1 g/dl total lipid concentration resulted in a fall in the normalized light intensity because vesicles were transformed into micelles; this indicated that 10 mM Na cholate exceeded the IMC of this model bile. Dilution with 10 mM Na cholate increased the normalized light intensity of the other model biles containing total lipid concentrations greater than 1 g/dl. Irrespective of BS/(BS+EYL) ratio in these biles, micelles were transformed into vesicles, indicating that the Na cholate concentration was less than the appropriate IMC values. Only in the cases of model biles with BS/(BS+EYL) ratios of 0.8 and 0.7 and 1 g/dl total lipid concentration did the normalized light intensity remain fairly constant, indicating that 10 mM Na cholate approximated the IMC of these systems.

Fig. 9 extends the observations in Fig. 7: IMC values of three different model biles (3 g/dl, BS/(BS+EYL)= 0.5–0.7) were essentially independent of molar cholesterol content (0–10%). Despite the fact that the micellar phase boundary was traversed as cholesterol content increased from 0 to 10 mol% (33), IMC values remained approximately constant but differed for each BS/(BS+EYL) ratio, decreasing in the rank order 0.7>0.6>0.5. Thus, the IMC of model bile solutions does not change when cholesterol-rich vesicles are present in addition to micelles.

DISCUSSION

In these studies we have developed and validated a modified equilibrium dialysis method for measuring the IMC of model biles. Furthermore, we have critically compared this method with an independent technique, QLS, and have shown that IMC values by both techniques are identical within experimental error. Importantly, we have demonstrated that these IMC values are the experimentally relevant quantities that must be utilized for accurate separation of vesicles and micelles from model bile using

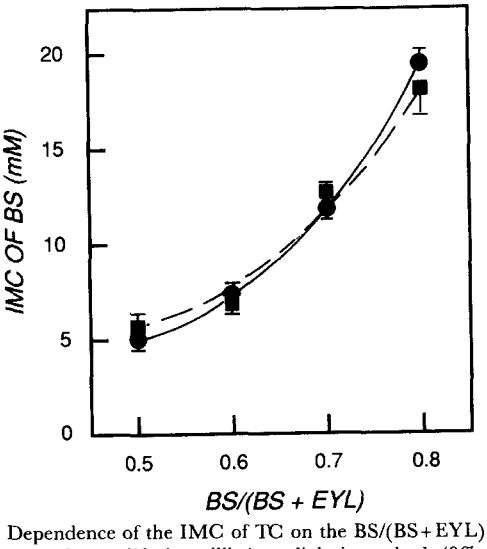

Fig. 7. Dependence of the IMC of TC on the BS/(BS+EYL) ratio as determined by the modified equilibrium dialysis method: (0% (●) and 10% (■) cholesterol, 3 g/dl, 0.15 M NaCl, 37°C, pH 7.2). Standard deviations are shown by the bars.

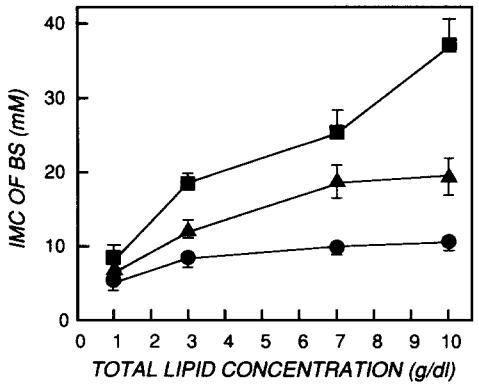

Fig. 8. Dependence of the IMC of TC on variations (1–10 g/dl) in total lipid concentration (10% cholesterol, 0.15 M NaCl, 37°C, pH 7.2), as determined by the modified equilibrium dialysis method, for three BS/(BS+EYL) ratios: 0.8 (■); 0.7 (▲); and 0.6 (●). Standard deviations are shown by the bars.

gel filtration. Although these studies were limited to model biles composed with a single BS, we hypothesize that alterations in these physical-chemical variables in native biles will alter IMC values in a similar fashion.

Although repeated exchanges of the dialysant are laborious and require a large volume of bile, this modification of previous dialysis methods is necessary to control the final composition of the dialysant. For model biles used in this study, BS in the IMC composed up to one third of total BS. If the dialysant was allowed to equilibrate with an equal volume of dialysate, a significant fraction of the total BS concentration would be transferred to the dialysate. For example, in a model bile with 3 g/dl total lipid concentration, 10% cholesterol, and BS/(BS+EYL) ratio=0.7, the initial BS concentration was 34 mM, and the IMC was 11 mM. During dialysis against an equal volume of dialysate, the BS concentration of the model bile would fall to approximately 28 mM, a significantly different value from the initial bile. Therefore, this modified equilibrium dialysis method allows the determination of the IMC of a model or native bile with an intended concentration. In contrast, classic equilibrium dialysis alone determines the IMC of a model bile that has an altered concentration and composition compared with the original model bile.

We have further modified previous equilibrium dialysis techniques by proposing a simplified model to correct for Donnan equilibrium effects caused by the presence of highly charged mixed micelles. As demonstrated by experimental values of $\frac{[Cl]_{dialysant}}{[Cl]_{dialysate}}$, diffusible anions were distributed asymmetrically across the dialysis membrane. Although the simplified model that we have proposed using equation 3 neglects the effects of formation of simple micelles on the distribution of BS across the membrane, this model provides theoretical values of $\frac{[Cl]_{dialysant}}{[Cl]_{dialysate}}$ that agree with experimental values. Due to our lack of knowledge of the concentration of simple micelles of each aggregation number, exact values for the distribution of TC micelles across the dialysis membrane cannot be rigorously calculated. Since the net charge on TC simple micelles appears as an exponent in equation 2, consideration of TC self-aggregation would increase the relative asymmetry of TC distribution across the membrane. However, Na counterions bind in part to simple micelles, decreasing their net charge, and hence the exponent in equation 2. Na counterion binding to mixed micelles decreases the net concentration of non-diffusible anions, and further diminishes the asymmetry of BS distribution across the dialysis membrane. Rigorous calculation of these effects requires a knowledge of Na binding to mixed micelles, as well as simple micelles of each aggregation number, but currently such information is unavailable.

Although we cannot theoretically calculate precise correction factors for the modified equilibrium dialysis method, Table 1 clearly demonstrates that for concentrated biles, e.g., 3 and 10 g/dl, the correction factor is appreciable, reaching approximately 20% of the IMC, whereas for dilute biles of 1 g/dl, the correction factor does not exceed experimental error. We have verified by two independent methods, QLS and gel filtration, that IMC values obtained by our simplified model are correct within experimental error for all total lipid concentrations examined (1–10 g/dl). Therefore, we believe that the estimate of the correction factors for Donnan equilibrium

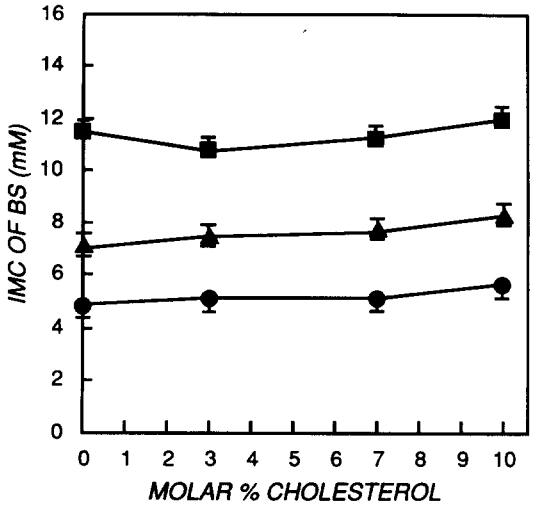

Fig. 9. Invariance of the IMC of TC in model biles with molar cholesterol content that increased from 0 to 10% (3 g/dl, 0.15 M NaCl, 37°C, pH 7.2), as determined by the modified equilibrium dialysis method. The three BS/(BS+EYL) ratios shown have distinctly different IMC values: 0.7 (■); 0.6 (▲); and 0.5 (●). Standard deviations are shown by the bars.

effects is adequate, and that this modified equilibrium dialysis method offers significant advances over previous attempts to measure IMC values systematically.

The technique described herein is limited by the substantial expenditure of time required to measure the IMC. Many native and cholesterol-supersaturated model biles are non-equilibrium systems, in that further changes are thermodynamically possible with the systems reaching equilibrium only when they contain cholesterol monohydrate crystals. As this phase transition occurs, the IMC may change as well. From the phase diagram it can be predicted that as cholesterol crystals form, the micellar phase in equilibrium with the vesicular phase and cholesterol crystals will have a higher BS/(BS+EYL) ratio than the metastable system containing only micelles and vesicles. This implies that the IMC of cholesterol-supersaturated bile will increase as equilibrium is approached. Because of the potential importance of this variable, especially in highly metastable systems, we have developed and are currently comparing with the present method a technique that measures the IMC of biles much more rapidly (34).

Within the micellar zone of the aqueous BS/EYL/cholesterol equilibrium phase diagram, two micellar regions are divided by a pseudo-phase limit that separates a BS-rich region in which simple micelles coexist with mixed BS/EYL micelles (the coexistence region), from an EYL rich-region in which mixed micelles are present (2). The boundary between these regions is believed to occur at a BS/(BS+EYL) value of approximately 0.6 (2, 35). As shown in Fig. 7, between BS/(BS+EYL) values of 0.5 and 0.6, the IMC falls below the CMC of TC. Since simple micelles can exist only when the IMC exceeds the CMC, our data corroborate previous estimates of the extent of the pseudo-phase limit and, hence, the coexistence region (2). Moreover, at physiological BS/EYL ratios (approximately BS/(BS+EYL)=0.7), cholesterol contents, and at total lipid concentrations similar to those found in gallbladder bile, the IMC exceeds the CMC. Therefore, simple BS micelles coexist both with BS/EYL/cholesterol mixed micelles and with cholesterol-rich vesicles under most physiological conditions.

Comparison of present results with literature IMC values

In 1970, Shankland (31) postulated that an appreciable monomeric BS concentration coexisted with mixed micellar solutions. Using classical light scattering techniques, he estimated the IMC of Na cholate/EYL solutions, and also observed an increase in the IMC at higher BS/(BS+EYL) ratios. Several investigators since have used equilibrium dialysis to estimate BS concentrations present in equilibrium with systems containing vesicles and/or micelles (17, 18, 36, 37). However, in all of these studies, model biles were allowed to equilibrate with a considerably larger volume of dialysate; hence, the total lipid concentration and the BS/(BS+EYL) ratio of model biles could not be varied independently, and the authors were unable to study IMC values while a single variable of physiological interest was altered systematically (17, 18, 36, 37). The IMC as measured in these literature studies has been termed the non-mixed micellar BS concentration (17), and was known to contain both monomers and simple (pure BS) micelles. Mazer, Benedek, and Carey (2) postulated the existence of a range of intermicellar BS concentrations to explain variations in micellar $\bar{R}_h$ values, and from their QLS data estimated IMC values that fell both below and above the CMC. **Fig. 10** shows plots of literature IMC values of model biles composed with TC, determined by several authors using equilibrium dialysis (17, 36, 38) and light scattering methods (2, 32), plotted as functions of their BS/(BS+EYL) compositions. Although these data are quite scattered, there is a clear trend, as we also found, toward higher IMC values at higher BS/(BS+EYL) ratios. Since our results demonstrate a strong effect of total lipid concentration on the IMC, we have stratified literature values according to total lipid compositions. Each zone in Fig. 10 delineates a range of total lipid concentrations, within which IMC values vary relatively little for a given BS/(BS+EYL) ratio. Our data for a total lipid concentration of 3 g/dl (open symbols, Fig. 10) closely approximate the literature data, plotting on the boundary between literature values for 2–3 and 3–5 g/dl total lipid

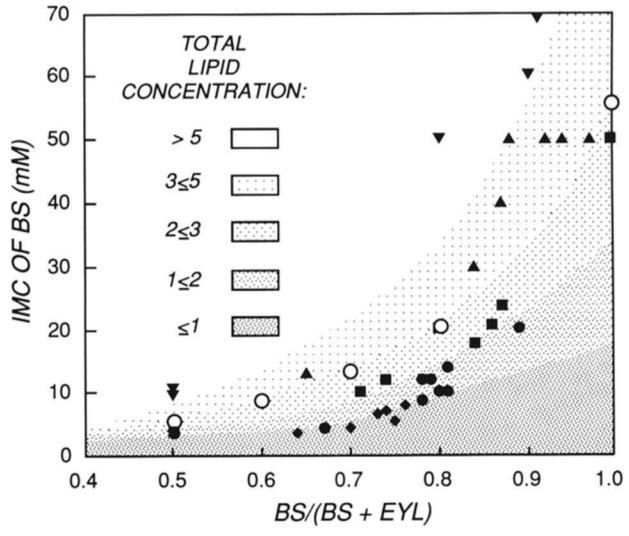

Fig. 10. Literature estimates of IMC values of model biles composed with TC plotted as functions of BS/(BS+EYL) ratio and stratified by total lipid concentration (g/dl): (♦) <1; (●) 1≤2; (■) 2≤3; (▲) 3≤5; (▼) >5. Values were determined by classic equilibrium dialysis (17, 36, 38) or by light scattering (2, 32). Shown for comparison are the IMC values derived in the present work (○) for a total lipid concentration of 3 g/dl at various BS/(BS+EYL) ratios. When the BS/(BS+EYL) ratio is 1.0, all BS in solution are monomers and simple micelles; thus, the total BS concentration is included as an IMC value.

concentration (Fig. 10). Therefore, over a broad range of relative and total lipid concentrations, only two variables, the BS/(BS+EYL) ratio and total lipid concentration, account for much of the dispersion in the wide ranges observed for IMC values of model biles composed with TC.

Pathophysiological implications

From the broad variations in IMC values presented in this work, it is clear that use of an arbitrary IMC value for separation of micelles and vesicles from bile is inadequate. Although a single concentration (10 mM) of an unconjugated BS, Na cholate, was chosen by others (5, 15, 39–41) in an attempt to minimize systematic variation in the separation of micelles and vesicles, our results (Fig. 6) suggest that experimental errors introduced by the use of 10 mM Na cholate are not uniform. Using ^{1}H-NMR to estimate the proportion of lecithin in vesicles and micelles, Groen and colleagues (42) also inferred that for dilute model biles, vesicular lecithin was greatly underestimated by the use of 10 mM Na cholate during gel filtration. We conclude from the present work that by using an eluant containing 10 mM Na cholate, separation of vesicles and micelles from model biles with total lipid concentrations similar to gallbladder bile may overestimate the proportion of vesicular lipids, whereas analogous separations from model biles of similar total lipid concentrations to hepatic bile may systematically underestimate the proportion of vesicular lipids.

Since the experimentally relevant IMC value must separate micelles and vesicles without altering their relative concentrations or compositions, we have verified that with our IMC values both micelles and vesicles can be rechromatographed with essentially no transformation between micelles and vesicles or vice versa (Fig. 5). Depending upon the composition of the model bile, this IMC value may be either above or below the CMC; hence, the IMC may contain both BS monomers and simple micelles, or BS monomers alone.

In summary, we have developed and validated a method for systematically measuring IMC values of model biles, and have utilized this method to determine the major physical-chemical variables of physiological significance that determine the IMC of model bile systems containing the single BS, TC. We conclude that *1*) the correct IMC of model biles (and by inference native biles) must be utilized to separate accurately micelles and vesicles; and *2*) the major determinants of the IMC include total lipid concentration and BS/(BS+EYL) ratio, but not cholesterol content. We show elsewhere that the relative composition of different BS in bile composed of a mixture of BS also influences both the magnitude of the IMC as well as the relative BS composition of the IMC (19, 43). We have demonstrated recently that the present method can also be employed to study the determinants of the IMC in human biles (Donovan, J. M., A. A. Jackson, N. Timofeyeva, and M. C. Carey, unpublished observations). We believe that these IMC values when used to accurately separate the cholesterol carriers of native biles should afford new insights into the nucleation phenomenon that is such a crucial step in the pathogenesis of cholesterol gallstone formation.

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