papers and notes on methodology

Rapid (1 hour) high performance gel filtration chromatography resolves coexisting simple micelles, mixed micelles, and vesicles in bile

David E. Cohen and Martin C. Carey[1]

Department of Medicine, Harvard Medical School, Brigham and Women's Hospital, and Harvard Digestive Diseases Center, Boston, MA 02115

Abstract We describe the use and validation of Superose® 6, a high performance gel filtration medium for rapid, high resolution separation and sizing of coexisting simple micelles, mixed micelles, and vesicles in bile. We fractionated model biles (1.7-4.2 g/dl total lipid concentration, 0.15 M NaCl) composed of lecithin (L), cholesterol (Ch), and the common bile salt taurocholate (TC) using Superose® 6 gel filtration columns (1.0 cm diameter, 30 cm length, 0.5 ml model bile application, 1.0 ml fractions) pre-equilibrated and eluted with 2.5-10.0 mM TC. Lipid particle sizes were determined by quasielastic light scattering and lipid compositions by conventional analyses. In the absence of L and Ch, pure TC "biles" (32.2 mM), when eluted in the presence of 7.5 mM TC, yielded a single peak of particles (mean hydrodynamic radii, $\bar{R}_h$ values of 11-15 Å), consistent with simple TC micelles. Model biles containing L and TC ([L] = 13.8 mM, [TC] = 32.2 mM) were fractionated with baseline resolution into TC-L mixed micelles, ($\bar{R}_h$ values of 30-40 Å) and simple TC micelles. In agreement with the ternary TC-L-H_2O phase diagram (Mazer, N. A., et al. 1980. *Biochemistry.* **19:** 601-615), the proportions of simple and mixed micelles were inversely related to L concentrations ([L] = 0-32.2 mM) and correlated positively with eluant TC concentration. Superose® 6 gel fractionation of model biles "supersaturated" with Ch (TC:L:Ch molar ratio 27:63:10, total lipid concentration 3 g/dl) yielded high resolution separation of vesicles ($\bar{R}_h$ value of 320 Å) from mixed micelles of TC-L-Ch ($\bar{R}_h$ values of 40-50 Å) and simple TC micelles ($\bar{R}_h$ values of 11-15 Å). At an eluant TC concentration of 7.5 mM, Ch-rich vesicles (Ch/L molar ratio = 1.6) separated that contained 40% of total Ch, 9% of total L, and no TC, accurately reflecting predictions of the quarternary L-Ch-TC-H_2O metastable phase diagram (Mazer, N. A., and M. C. Carey. 1983 *Biochemistry.* **22:** 426-442). This suggested that a 7.5 mM TC concentration approximated the intermicellar concentration under the experimental conditions. We also fractionated an identical model bile using conventional Sephacryl® S-300, a medium generally used to study model and native biles. Compared with Superose® 6, the Sephacryl® S-300 column of equivalent size yielded particle separations with lower resolution and speed (30 h v 1 h). These studies suggest that high resolution separation and sizing of biliary lipid particles may now be achieved with sufficient rapidity to characterize the kinetics of particle interconversions in bile.—**Cohen, D. E., and M. C. Carey.** Rapid (1 hour) high performance gel filtration chromatography resolves coexisting simple micelles, mixed micelles, and vesicles in bile. *J. Lipid Res.* 1990. **31:** 2103-2112.

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Fractionation of model and native biles by use of gel filtration has assumed an important role for characterizing both sizes and compositions of biliary micelles and vesicles (1-6). In addition, gel filtration has provided a means by which cholesterol (Ch)-rich biliary vesicles and micelles may be isolated for physical-chemical studies (7-9).

Physical properties of conventional gel filtration media customarily used in fractionating biles (e.g., Sephacryl® and Sephadex®, Pharmacia-LKB, Piscataway, NJ) impose important limitations on separation time and resolution of biliary lipid aggregates. Separations on the basis of particle sizes result from sieving through pores in small gel beads (approx. range of bead diameters 10-100 μm) formed by cross-linked dextrans. Resolution of discrete lipid particle populations is hindered by the relatively broad distribution of bead sizes comprising conventional gels (40-105 μm in the case of Sephacryl® S-300). To compensate for this, low eluant flow rates are required to enhance resolution, requiring hours to days to achieve analytical and preparative separations of biliary lipid particles despite optimizing other specifications (i.e., column dimensions and volume of bile applied). Because model and native biles are frequently metastable systems (10-13), lipid dynamics may alter proportions of both micelles and vesicles during prolonged transit times through the columns.

Recently, Superose®, a gel filtration medium designed for rapid, high resolution separations of mixtures of pro-

Abbreviations: Ch, cholesterol; TC, taurocholate; L, lecithin; CMC, cricital micellar concentration; IMC, intermicellar concentration; SUV, small unilamellar vesicle; QLS, quasielastic light scattering.

[1]To whom correspondance should be addressed at: Department of Medicine, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115.

teins, was introduced by Pharmacia-LKB. Superose® is composed of small, homogeneous beads (13 ± 2 μm) of cross-linked dextrans. Using Superose® 6, we describe the fractionation of simple micelles, mixed micelles, and vesicles coexisting in model biles (10, 11, 14-16) and demonstrate that the medium yields faster, higher resolution separations than Sephacryl® S-300 under otherwise equivalent conditions.

MATERIALS AND METHODS

Materials

The sodium salt of taurocholate (TC) (Calbiochem, San Diego, CA) was recrystallized to achieve greater than 99% purity (10). [^{3}H(G)]TC (sp act 8.1 Ci/mmol), [24-^{14}C]TC (sp act 46.4 mCi/mmol), [4-^{14}C]Ch (sp act 60.0 mCi/mmol), and 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycerophosphocholine (lecithin,L) (sp act 57.5 mCi/mmol) were obtained from New England Nuclear (Boston, MA) and were of the highest radiopurities available. Grade I egg lecithin (Lipid Products, South Nutfield, Surrey, U.K.) was >99% pure by high performance liquid chromatography (17) and thin-layer chromatography (200 μg application). Cholesterol was obtained from Nu-Check-Prep (Elysian, MN) and found to be 99% pure by gas-liquid chromatography. All other chemicals and solvents were ACS or reagent quality (Fisher Scientific Co., Medford, MA). ACS grade sodium chloride was roasted at 600°C for 4 h in a muffle furnace to oxidize and remove organic impurities. Pyrex brand glassware was acid-washed for 24 h (1 M HNO_3) followed by 24 h alkali-alcohol washing (EtOH-2 M KOH 1:1, v:v) and rinsed thoroughly with purified H_2O prior to drying. H_2O was filtered, ion-exchanged, and glass-distilled (Corning Glass Works, Corning, NY).

Model biles and small unilamellar vesicles (SUVs)

After coprecipitation from stock solutions ($CHCl_3$-MeOH 1:1, v:v) (10), dried L/TC/Ch films were resuspended in aqueous buffer (0.15 M NaCl, pH ~ 6-7) that included 3.0 mM NaN_3 as an antimicrobial agent. Final total lipid concentrations ranged from 1.7 to 4.2 g/dl (32.2 mM TC, 0-32.2 mM L, 0-5.1 mM Ch). Prior to gel filtration, model biles were equilibrated at 20°C for at least 24 h with periodic vortex mixing under an atmosphere of argon. SUVs of pure L were prepared by sonication in 0.15 M NaCl, 3.0 mM NaN_3 as described elsewhere (13).

Gel filtration systems

Conventional gel filtration. Sephacryl® S-300 (Pharmacia-LKB, Piscataway, NJ) with molecular mass fractionation in the range of 10,000 to 1.5 × 10^6 daltons and bead diameter ranging from 40 to 105 μm (average 70 μm) was packed according to manufacturer's specifications in a Pharmacia-LKB C10/40 gel filtration column. A 1.0 cm × 36 cm gel bed was chosen in order to simulate the prepacked Superose® 6 column (see below). As determined in preliminary experiments by quasielastic light scattering and lipid analysis (see below), a Pharmacia-LKB P-1 peristaltic pump delivering eluant with a flow rate of 1.0 ml/h yielded the best resolution of biliary lipid particles. Columns were calibrated using blue dextran as a void volume (V_o) marker and acetone as a total volume (V_t) marker.

Superose® gel filtration. A prepacked and factory-calibrated (i.e., with specified V_o and V_t) Pharmacia HR10/30 Superose® 6 column contained a 1.0 cm × 30 cm gel bed with molecular mass fractionation in the range of 5,000 to 5 × 10^6 daltons and 13 ± 2 μm bead diameters. A Pharmacia P-500 pump in continuous mode was employed to deliver eluant at a flow rate of 30 ml/h.[2]

Column pre-equilibration and model bile fractionation

Both Sephacryl® S-300 and Superose® 6 columns were pre-equilibrated with 50 ml (equivalent to two column volumes) of 2.5, 5.0, 7.5, or 10.0 mM TC, 0.15 M NaCl, and 3.0 mM NaN_3 at 20°C and eluted with the same solution. Equal volumes of model biles (0.5 ml) were applied to both columns and 1.0-ml fractions were collected using a Pharmacia-LKB Frac-100 fraction collector. Lipid analysis of fractions from the column (see below) revealed that recoveries uniformly exceeded 90% and were close to 100% in most experiments.

Analytical procedures

Quasielastic light scattering (QLS). QLS measurements of model biles and individual column fractions were performed on a home-built apparatus (12) at a scattering angle of 90° and sample temperature of 20°C. Mean diffusion coefficients ($\bar{D}$) of particles in solution were derived by cumulants analysis of intensity autocorrelation functions (18). The $\bar{D}$ value of each particle population was then translated into the mean hydrodynamic radius ($\bar{R}_h$) by the Stokes-Einstein relationship utilizing the viscosity of the solvent (11).

Lipid analysis. Concentrations of TC were determined by the 3α-hydroxysteriod dehydrogenase method (10) and by liquid scintillation counting using a Beckman (Fullerton, CA) model LS-5000 TD scintillation counter and a hydrofluor scintillant (National Diagnostics, Highland Park, NJ). We determined L concentrations by both phosphorus analysis (10) and liquid scintillation counting and Ch concentrations were assayed by gas-liquid chromatography (Shimadzu model GC9a, Kyoto, Japan) (19) and liquid scintillation counting.

[2]Although the Pharmacia P-500 pump was designed specifically for use with Superose® 6 and related columns, standard HPLC pumps may be used for this purpose.

RESULTS

Separation of simple micelles, mixed micelles, and L-rich vesicles

Fig. 1 shows the influence of eluant TC concentration on the Superose® 6 elution profile of a model bile ([TC] = 32.2 mM, [L] = 13.8 mM) predicted to contain coexisting simple and mixed micelles (14). At an eluant TC concentration of 10.0 mM (Fig. 1A), a single peak of TC and L ($\bar{R}_h$ values of 30–35 Å), consistent with mixed micelles was observed within the included volume. However, no unequivocal simple micellar peak was identified although there is a suggestion of such at 24 ml. Fig. 1B demonstrates that for an eluant TC concentration of 7.5 mM, a mixed micellar peak ($\bar{R}_h$ values of 30–35 Å) was again observed in the included volume. In addition, a peak of pure TC in excess of the eluant TC concentration (7.5 mM) eluted at V_t and by QLS this column fraction gave $\bar{R}_h$ values of 11–15 Å, consistent with simple TC micelles. When the eluant TC concentration was reduced to 5.0 mM (Fig. 1C), the mixed micellar peak eluted earlier (elution volumes of 17–18 ml compared with 19–20 ml in Figs. 1A and 1B), consistent with larger mixed micelles (measured $\bar{R}_h$ values of 40–45 Å by QLS) and the simple micellar peak eluted later, consistent with smaller simple micelles. It is clear from these results that, as the proportion TC eluting as simple micelles near V_t increased, the proportion of TC in mixed micelles descreased, representing a shift of TC from mixed micelles to simple micelles. Fig. 1D demonstrates that when the eluant TC concentration was further reduced to 2.5 mM, a peak of L with a measured $\bar{R}_h$ value of 250 Å containing concentrations of TC equal to the eluant concentration was observed at V_o and was consistent with unilamellar vesicles (20). A prominent pure TC peak was also present and eluted slightly later than V_t which further confirmed that the simple micellar peak was shifted slightly to smaller micellar sizes. These data illustrate the small concentration dependence of simple micellar sizes in the case of TC (21) compared with the large concentration dependence of mixed TC-L micellar sizes (14).

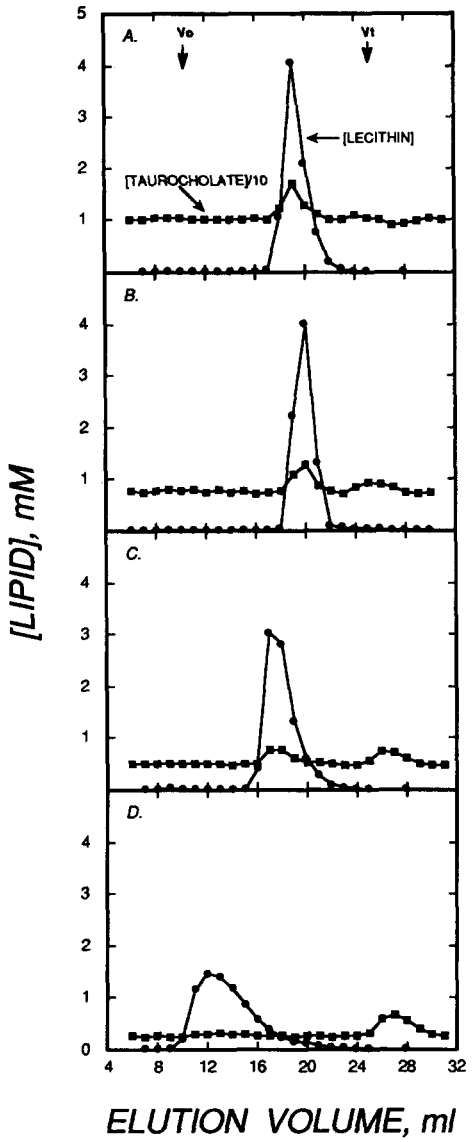

Fig. 1. Superose® 6 gel filtration of a mixed micellar solution of taurocholate, TC, (32.2 mM) and lecithin, L, (13.8 mM) with eluant (0.15 NaCl, 3.0 mM NaN_3, pH ~ 6–7) containing TC concentrations of (A) 10 mM (B) 7.5 mM (C) 5.0 mM, and (D) 2.5 mM at 20°C. Lipid concentrations of fractions displayed are for TC ÷ 10 (■) and L (●) plotted as functions of elution volume. Void V_o and total volumes V_t, respectively, are indicated by arrows.

To define the elution behavior of preformed SUVs added to gel filtration columns pre-equilibrated and eluted with TC, we show in **Fig. 2** the influence of eluant TC concentration on the gel filtration profile of SUVs of pure L ($\bar{R}_h$ value of 250 Å) employing the Superose® 6 column. After elution with 10.0 mM TC (Fig. 2A), peaks of L and TC appeared in the included volume with $\bar{R}_h$ values of 30–35 Å, identical in sizes to the mixed micelles in Fig. 1A. While the TC content of these mixed micelles exceeded 10.0 mM, a TC deficit which formed a trough appeared in the fractions eluting near V_t (Fig. 2A). Figs. 2B and 2C show that, in the presence of 7.5 and 5.0 mM TC, SUVs were also dissolved into mixed micelles giving $\bar{R}_h$ values of 30–35 Å and $\bar{R}_h$ values of 40–45 Å, respectively, paralleling the elution volumes for mixed micelles in Figs. 1B and 1C. Again, the sizes of the TC peaks in Fig. 2B and 2C were of identical magnitude to the TC troughs near V_t. Using 2.5 mM TC in the eluant (Fig. 2D), the L peak eluted at V_o with $\bar{R}_h$ value of 250 Å indicating that SUVs were not dissolved to form mixed micelles but TC in slight excess of the eluant concentration co-eluted with the SUV peak. In all cases, when TC containing mixed particles shifted to larger sizes (Fig. 2), the trough in TC concentration (inverse of the mixed micellar TC) moved to higher elution volumes reflecting a shift toward smaller simple micellar sizes.

To provide direct evidence for a transition from simple micelles plus mixed micelles to pure mixed micelles with

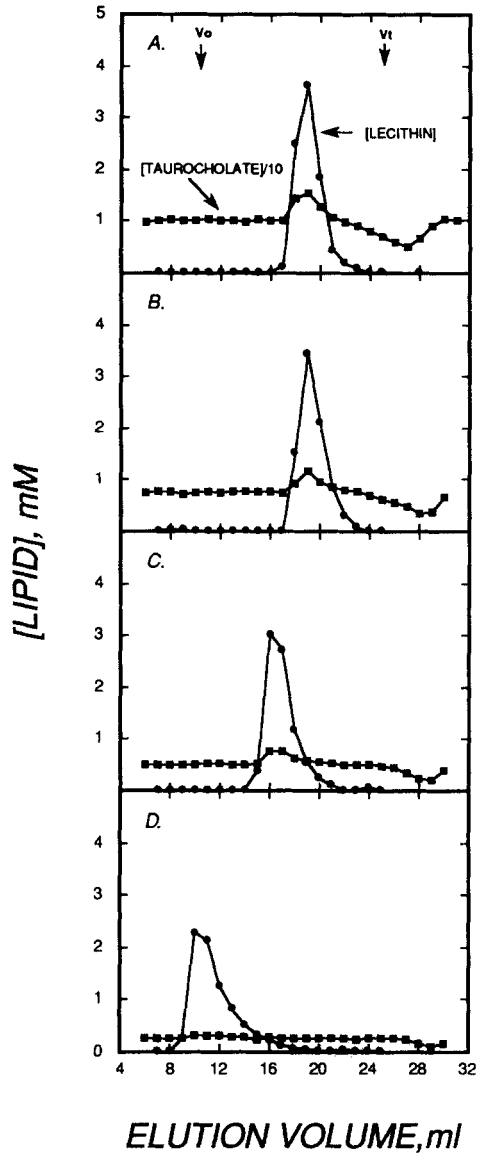

Fig. 2. Superose® 6 gel filtration of sonicated L vesicles ([L] = 13.2 mM) with eluant (0.15 M NaCl, 3.0 mM NaN_3, pH ~ 6-7) containing TC concentrations of (A) 10 mM (B) 7.5 mM (C) 5.0 mM, and (D) 2.5 mM at 20°C. Lipid concentrations of fractions displayed are for TC ÷ 10 (■) and L (●) plotted as functions of elution volume. Void V_o and total volumes V_t, respectively, are indicated by arrows.

increasing biliary L content (14, 15), we fractionated a series of TC-L solutions containing a fixed TC concentration with increasing L concentrations all plotting within the micellar zone of the TC-L-H_2O phase diagram (14). In **Fig. 3A**, the Superose® 6 gel filtration profile of a pure TC micellar solution (32.2 mM) eluted with a TC concentration of 7.5 mM (concentration at V_o) demonstating a simple micellar peak ($\bar{R}_h$ values of 11-15 Å) at V_t. Fig. 3 (B, C and D) depicts gel filtration profiles with the same eluant, for a TC concentration of 32.2 mM and increasing L concentration in the model bile solution. In all cases, a mixed micellar peak of L and TC ($\bar{R}_h$ values of 30-35 Å) eluted in the included volume and coexisted with TC simple micelles eluting at approximately V_t. With progressive increases in L concentration, the proportion of TC eluting in the mixed micellar peak increased, whereas the proportion of simple micelles decreased (Figs. 3B-D). In addition, the simple micellar peak moved slightly to higher elution volumes. With L contents of 21.5 mM and 32.2 mM, the simple micellar peak disappeared at V_t (Figs. 3E, F) and, in fact, a small trough appeared in the position of V_t (25-28 ml) for the highest L concentration. Although the proportions varied (Figs. 3A-F), the elution volumes and QLS sizes of mixed micelles ($\bar{R}_h$ values of 30-35 Å) did not change appreciably whereas elution volumes of simple micelles increased slightly with added L. This decrease in simple micellar sizes was too small to be resolved by QLS (22).

Separation of simple micelles, mixed micelles, and Ch-rich vesicles

To demonstrate that Superose® 6 resolves coexisting vesicles, mixed micelles, and simple micelles in a supersaturated model bile (TC:L:Ch molar ratio 63:27:10, 3 g/dl, 0.15 M NaCl, 20°C, equilibration time 48 h), we used a series of eluant TC concentrations as displayed in **Fig. 4**. Fig. 4A plots the elution profiles of all three biliary lipids with an eluant TC concentration of 10.0 mM. Large particles, whose QLS sizes ($\bar{R}_h$ value of 320 Å) were consistent with vesicles, eluted at V_o. These were separated by five baseline fractions from mixed micelles of TC-L-Ch ($\bar{R}_h$ value of 40 Å) that were included within the gel. Fig. 4B demonstrates the results of fractionating the same model bile with 7.5 mM TC eluant. Again, vesicles ($\bar{R}_h$ value of 320 Å) eluated at V_o with mixed TC-L-Ch micelles ($\bar{R}_h$ value of 40 Å) included within the gel. In contrast to Fig. 4A, a discrete peak of TC simple micelles ($\bar{R}_h$ values of 11-15 Å) eluted at volumes slightly in excess of V_t. Fig. 4C shows that when the eluant TC concentration was decreased to 5.0 mM, vesicles eluting at V_o were not resolved from large TC-L-Ch mixed micelles which eluted at 16-17 ml (compared with 18-19 ml in Figs. 4A and 4B). Since the larger vesicles contaminated micellar fractions and contributed disproportionately to the scattered light intensity, mixed micellar sizes could not be determined in the mixtures by QLS (12). Compared with Fig. 4B, the magnitude of the mixed micellar peak decreased whereas the magnitude of the simple micellar peak increased and simple micellar sizes became smaller as inferred from increasing elution volumes. Fig. 4D shows that, with an eluant concentration of 2.5 mM TC, the simple micellar peak increased in magnitude, the mixed micellar TC peak disappeared, and a near complete transformation of mixed micelles into vesicles occurred.

Fig. 5 compares the Superose® 6 (Fig. 5A) and Sephacryl® S-300 (Fig. 5B) gel filtration profiles of the model

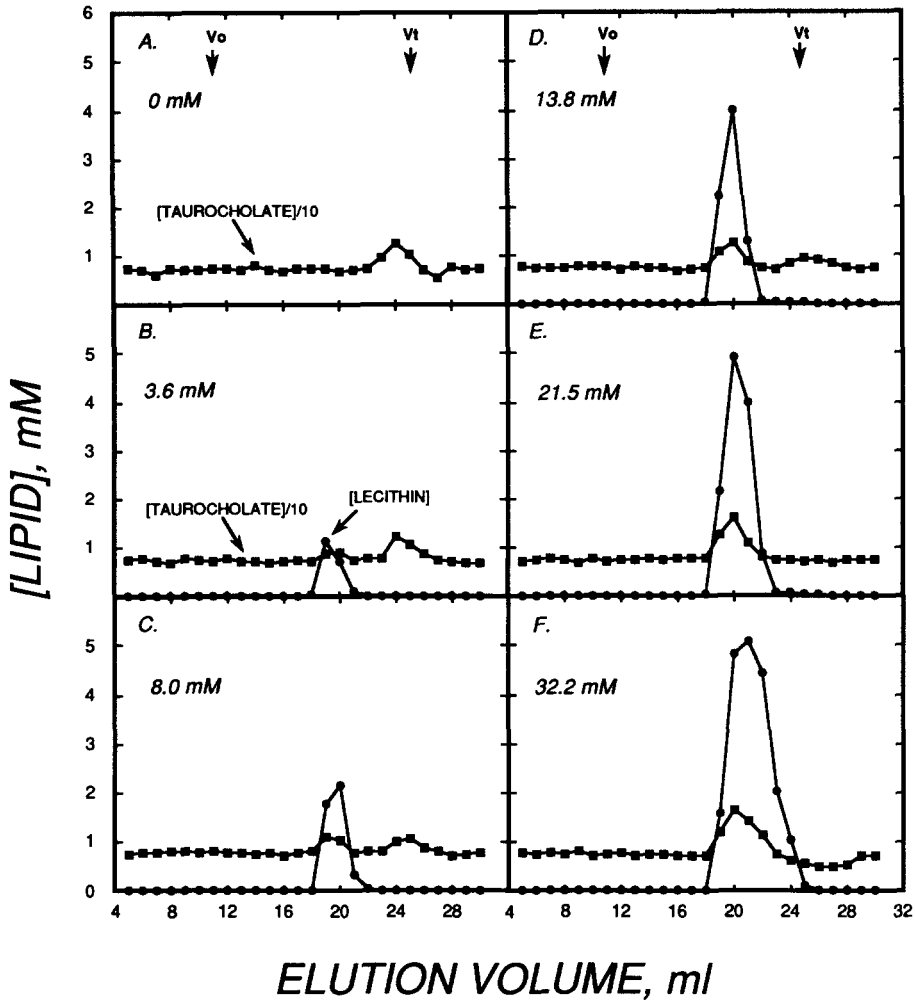

Fig. 3. Superose® 6 gel filtration of model biles containing both TC and mixed L-TC micelles. Lipid concentrations of fractions displayed are for TC ÷ 10 (■) and L (●) plotted as functions of elution volume. TC concentration of model biles was held constant at 32.2 mM, and L concentrations were varied as follows: (A) 0 mM, (B) 3.6 mM, (C) 8.0 mM, (D) 13.8 mM, (E) 21.5 mM, (F) 32.2 mM. Other conditions were 0.15 M NaCl, 3.0 mM NaN_3, 20°C pH ~ 6-7. To preserve micellar integrity, columns were pre-equilibrated and eluted with an approximate intermicellar concentration (IMC) of 7.5 mM TC.[5] Void V_o and total volumes V_t, respectively, are indicated by arrows.

bile used in Fig. 4 eluted with a 7.5 mM TC concentration. Fig. 5A displays the results with Superose® 6 gel filtration where vesicles with QLS sizes ($\bar{R}_h$ value of 320 Å) and Ch/L molar ratio (1.6) were separated by five baseline fractions from the included mixed TC-L-Ch micelles ($\bar{R}_h$ value of 40 Å) and simple TC micelles ($\bar{R}_h$ values of 11-15 Å). When compared with the unfractionated model bile, the vesicle peak contained 40% Ch and 9% L with no TC in excess of the eluting concentration. The remaining biliary lipids were distributed in simple micelles (i.e., no Ch and L were detected) and mixed micelles. In Fig. 5B, the Sephacryl® S-300 elution profile shows several important differences compared with the Superose® 6 profile (Fig. 5A). *1*) Although Sephacryl S-300 supported higher flow rates, a low flow rate (1 ml/h) was crucial to achieve complete resolution of vesicles and mixed micelles as assessed by QLS (22). Therefore, the total separation time for "high resolution" by Sephacryl® S-300 was 30 h compared with 1 h for Superose® 6. *2*) Despite this low flow rate, vesicles and mixed micelles were less well resolved with Sephacryl® S-300 compared to Superose® 6. For example, only one baseline fraction was observed at an elution volume of 17 ml (Fig. 5B) compared with five baseline fractions with Superose® 6 (Fig 5A). *3*) Compared with the sharp simple micellar peak at V_t using Superose® 6 (Fig. 5A), the simple micellar profile was flattened with Sephacryl® S-300 (Fig 5B). Likewise TC in the mixed micellar peak was higher with Sephacryl® S-300, all suggesting that inter-particle shifts of TC occurred during the prolonged elution time. Further, after Sephacryl® S-300 fractionation, simple micelles could not be detected in any V_t fraction by QLS analysis, presumably due to higher dilution of the particles.

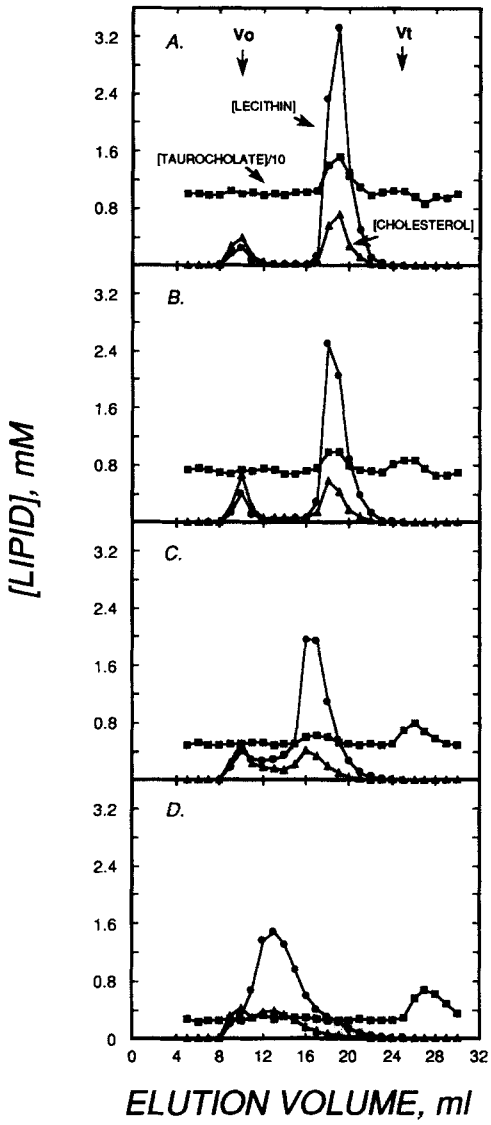

Fig. 4. Superose® 6 gel filtration analysis of a Ch supersaturated model bile (molar TC/L/Ch ratio 63:27:10, 3 g/dl total lipid concentration) with eluant (0.15 M NaCl, 3.0 mM NaN_3) containing TC concentrations of (A) 10 mM (B) 7.5 mM (C) 5.0 mM, and (D) 2.5 mM at 20°C, pH ~ 6-7. Lipid concentrations of fractions displayed are for TC ÷ 10 (■) and L (●) plotted as functions of elution volume. Void V_o and total volumes V_t, respectively, are indicated by arrows.

DISCUSSION

Rapid, high resolution separation of simple micelles, mixed micelles, and vesicles from bile is a pressing need, and we have herein demonstrated that this aim is achieved using Superose® gel filtration technology. Although similar gel filtration methodologies (TSK G6000PW and G5000PW gel exclusion columns, Toyo Soda, Tokyo, Japan) have been used for separating lipid vesicles and viruses (23) and for sizing bile salt-L mixed micelles (24), Superose® has not been used for the fractionation of two classes of micelles and a population of vesicles in the same biliary system.

Ultracentrifugation techniques for fractionation of biliary vesicles and micelles provide separations as rapid as 2 h (25) and, unlike gel filtration, require no added bile salt to preserve micellar integrity. However, to form a gradient that resolves vesicles and micelles, as much as 33% (w/v) metrizamide was added to biles in one study (26). This additive may potentially influence the phase relations and physical-chemical properties of isolated biliary vesicles and micelles. Further, complete resolution of micelles and vesicles for accurate phase equilibria determinations has not been reported using ultracentrifugation techniques nor has resolution of simple from mixed micelles (25, 26).

The coexistence of simple and mixed micelles at intermediate and high bile salt: lecithin ratios (in the range for human biles) was predicted by QLS (14) and nuclear magnetic resonance (NMR) (15), and subsequently verified in model biles by equilibrium dialysis (16). Nevertheless, we demonstrated here the unequivocal "visual" separation of simple and mixed micelles by gel filtration and characterized their composition and sizes. Resolution of simple and mixed micelles by Superose® 6 gel filtration depends critically on eluant TC concentration. It is likely that in Fig. 1A, a discrete simple micellar peak was barely perceptible because the simple micellar concentration in the original sample closely matched the eluting TC concentration (10 mM). This TC concentration is appreciably in excess of the critical micellar concentration (CMC) ~ 3-4 mM (concensus value in 0.15 M NaCl at 25°C) (27) and thereby represents a concentration of simple micelles and TC monomers. As the eluting TC concentration was decreased from 7.5 mM (Fig. 1B) to 5.0 mM (Fig. 1C), the elution volume of TC-L mixed micelles decreased from 19-20 ml to 17-18 ml corresponding to mixed micellar growth from $\bar{R}_h$ values of 30 to 45 Å. This was consistent with the loss of monomers of TC from mixed micelles to eluant (14). Because 7.5 mM TC allowed the resolution of simple micelles and mixed micelles while preserving mixed micellar sizes compared to those in the unfractionated model bile ($\bar{R}_h$ value of 30 Å), the intermicellar concentration (IMC) (6, 14, 16, 28) perforce lies closer to 7.5 mM than to 5.0 mM or 10 mM (Fig. 1).[3] As demonstrated in Fig. 1D, mixed micelles were transformed into vesicles due to extensive loss of TC from the micelles to the eluant which contained a submicellar TC concentration of 2.5 mM (20).

[3]The "IMC value" of 7.5 mM TC referred to in this paper should not be interpreted as the true IMC value. The true IMC values of model and presumably native biles varies with total lipid concentration, bile salt species, temperature, Ch content, ionic strength, and bile salt/L molar ratio (16, 28). Knowledge of the true IMC value for an individual model bile would allow quantitatively precise separations of vesicles, mixed micelles, and simple micelles. Our purpose was to demonstrate that Superose® 6 columns may be used to achieve separations of these particle populations with speed and precision. Compared to the other eluant TC concentrations that we studied (2.5, 5.0, and 10 mM), 7.5 mM produced the most accurate particle separations for the particular purposes of this work and under the physical-chemical conditions used (Figs. 1, 3-5).

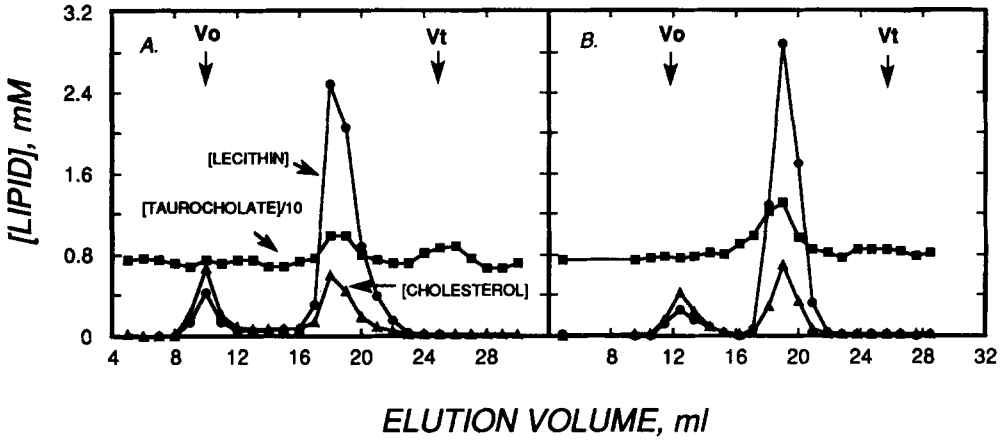

Fig. 5. Fractionation of Ch supersaturated model bile (molar TC:L:Ch ratio; 63:27:10, 3 g/dl total lipid concentration) employing (A) Superose® 6 (flow rate 30 ml/h) and (B) Sephacryl® S-300 (flow rate 1 ml/h) Lipid concentrations of fractions indicated are TC ÷ 10 (■), L (●), and Ch (▲) plotted as functions of elution volume. Other conditions were 0.15 M NaCl, 3.0 mM NaN_3, 20°C, pH ~ 6–7. To preserve micellar integrity, columns were pre-equilibrated and eluted with 7.5 mM TC the approximate IMC.[5] V_o and V_t are indicated by arrows.

As demonstrated in Fig. 2A-C, SUVs were dissolved by simple TC micelles in the eluant to form mixed micelles with $\bar{R}_h$ value of 30 Å in both 10 and 7.5 mM TC (Figs. 5A and 5B, respectively) and $\bar{R}_h$ value of 45 Å at 5.0 mM TC (Fig. 5C). Because bile salts have high solubilities in membranes (13, 20, 29), TC presumably partitioned preferentially into L vesicles when they were introduced into the Superose® 6 column. Provided total and relative compositions of all lipids (i.e., mixed micelles plus eluant TC) lie within the single phase micellar zone of the TC-L-H_2O phase diagram (29), mixed micellar systems form when eluant TC concentrations equal or exceed the CMC. Hence, the formation of micelles in the presence of 5.0 mM TC (Fig. 2C) but not in the presence of 2.5 mM TC (Fig. 2D) suggests that the true CMC of TC lies between 2.5 and 5.0 mM under these conditions (27). The "inverted peak" or trough of TC concentrations at approximately V_t (Fig. 2A-D) most likely reflects the concentration of TC that interacted with SUVs and indicates that TC in the Superose® 6 column did not re-equilibrate following TC-L interactions.

In **Fig. 6**, we plot on the ternary TC-L-H_2O phase diagram (14) the relative lipid compositions of the systems studied in Figs. 3A-F. The phase diagram predicts that simple and mixed micelles coexist in varying proportions within region I. Whereas this is supported indirectly by observations using QLS (14) and NMR (15), the Superose® 6 gel filtration data in Fig. 3A-D ([L] = 0–13.8 mM) provides direct proof of the coexistence theory. As the lecithin concentrations were further increased from 13.8 mM to 21.5 mM with TC concentration held constant, the relative lipid compositions fall within the mixed micellar zone (region II) where only mixed micelles are present. This is demonstrated in this work by the complete disappearance of the simple micellar peak at V_t in Fig. 3E. At the highest lecithin concentration (32.2 mM), which was equivalent to the mixed micellar TC concentration, no perceptible shift of the mixed micellar peak was observed (Fig. 3F) presumably because the mixed micellar growth as verified by QLS was small ($\bar{R}_h$ values increased from 30 to 40 Å) (14).

As indicated in **Fig. 7**, the relative composition of the TC-L-Ch-H_2O model bile used in this study plots within region B of the appropriate quaternary phase diagram. While at equilibrium, the phases indicated in Fig. 7 include Ch monohydrate crystals; we verified in the metastable state

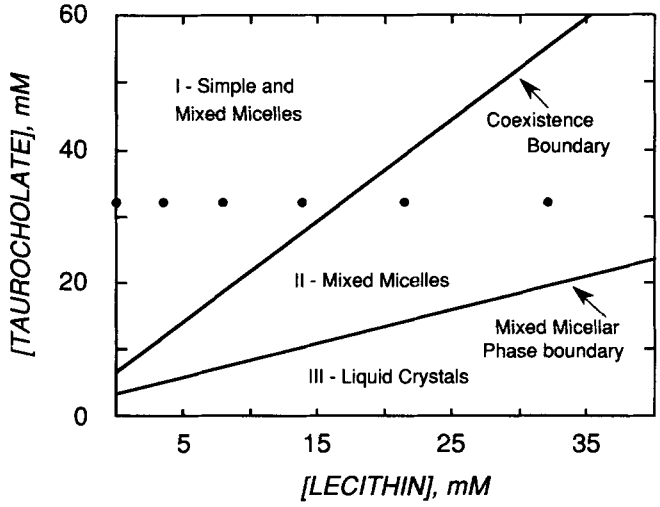

Fig. 6. Ternary TC-L-H_2O phase diagram (0.15 M NaCl, 20°C, 1 atm) in rectangular format. Region I contains simple TC micelles coexisting with mixed TC-L micelles; region II contains only mixed micelles pseudo-separated from region I by a coexistence boundary. The mixed micellar phase boundary separates region II from region III. The latter is represented here as a simplification of a complex area that contains liquid crystalline particles which coexist with mixed micelles and monomeric TC (13). Plotted as closed symbols are compositions of model biles in this work fractionated by Superose® 6 gel filtration chromatography in Fig. 3. (Phase diagram adapted from reference 14.)

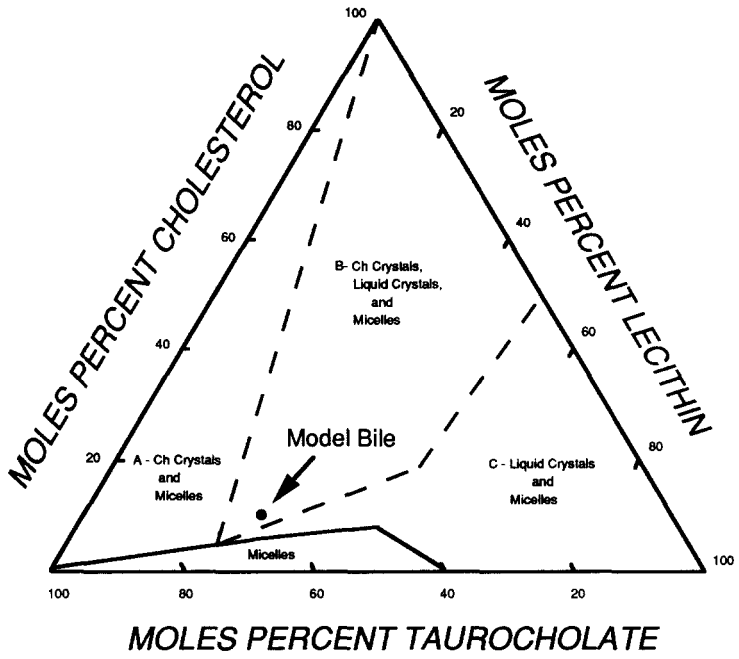

Fig. 7. Triangular cut of the equilibrium TC-L-Ch-H_2O phase diagram (3 g/dl total lipid concentration, 0.15 M NaCl, 20°C, 1 atm). Solid lines indicate phase boundaries that are known with certainty whereas dashed lines are approximate (10) as described in the text. The composition indicated as "model bile" (molar TC:L:Ch ratio 63:27:10) was fractionated into Ch-rich vesicles, mixed micelles, and simple micelles by Superose® 6 and Sephacryl® S-300 gel filtration as displayed in Fig. 5. (Phase diagram adapted from reference 10.)

that only micelles and vesicles coexist in this system (4, 10, 11). In the cholesterol-free systems that contained simple plus mixed micelles and L-TC vesicles (Figs. 1 and 3), 7.5 mM TC provided the closest approximation to the IMC. Similarly, in the system containing Ch, this TC concentration provided complete resolution of L-Ch vesicles, mixed TC-L-Ch micelles, and simple TC micelles (Fig. 4B).[3] Further, as demonstrated in Figs. 5A and 5B, the mixed micellar peak contained 60% of the total Ch in close agreement with micellar Ch solubility (Fig. 7) at the L/TC molar ratio (0.4) used in this study (10). We have calculated on the basis of the coexistence theory (11) that simple micelles should solubilize less than 2% of the total Ch in our model bile. Presumably because this Ch content was distributed over several column fractions (e.g., Figs. 2A and B), it was not detectable in our chemical assays.

The marked improvement in particle separations using Superose® 6 gel when compared to conventional gel filtration media (Fig. 5), was due to the ability of the high performance gel to support high eluant flow rates and to increase resolution, presumably by virtue of the small uniform bead sizes. To counteract decreases in resolution due to broad distributions of large particle sizes in conventional gels, lower eluant flow rates are required. However, due to the very long separation time incurred with Sephacryl® S-300, diffusion times of biliary lipid particles increase (compare Fig. 5A with Fig. 5B). This results in broader peaks occupying more elution fractions and may explain the flattening of the simple micellar peak as well as increase in size of the TC component of mixed micelles. The simple micellar peak in Fig. 5B may have broadened during the 30-h transit time in the column to the extent that TC concentrations in fractions comprising the mixed micellar peak were artifactually increased. This possibility is supported by the observation that the eluted TC concentration failed to return to the 7.5 mM baseline value even at the highest elution volumes (Fig. 5B). This suggests that either simple micelles were distributed over many elution fractions or that, within the Sephacryl S-300 column, TC from simple micelles redistributed into mixed micelles.

In contrast to simple micelles, peak broadening did not occur with vesicles or mixed micelles during Superose® 6 (Fig. 5A) or Sephacryl® S-300 (Fig. 5B) chromatography. These larger particles clearly become less entrapped within the gel and have shorter transit times through the column. Therefore, better resolution is achieved at the lower flow rate (1 ml/h) required to compensate for the distribution of bead sizes (40–105 μm) in the case Sephacryl® S-300. The very large reduction in separation times achieved with Superose® 6 is most likely the result of homogeneous bead sizes (13 $\pm$ 2 μm) combined with high flow rates (30 ml/h) that serve to limit peak broadening.

We believe that the methodology described in this work for rapid, high resolution fractionation of simple micelles, mixed micelles, and vesicles based on Superose® 6 gel filtration technology will facilitate further studies of model and native bile systems. Recent experiments in our own laboratory have already demonstrated that Superose® 6 may be used to fractionate hepatic bile from the prairie dog (22), and affiliates of our group have successfully fractionated concentrated human gallbladder biles with this technology (30). The high resolution achieved using Superose® 6 chromatography allows for isolation of micelles and/or vesicles with virtually no cross-contamination of the particle populations. For

preparative purposes, such complete particle separation may be exploited to obtain large quanities of micelles or vesicles from the same bile. More importantly, rapid particle separation by gel filteration may provide a means to characterize not only micelle and vesicle sizes in model and native biles but their dynamic interconversions. Equilibration times of hours to days are observed in native biles after experimental collection (12, 26) and in physiologically relevant model systems (13, 31). Therefore, in contrast to the long elution times required to achieve lipid particle separation by conventional gel filtration, the speed of Superose® 6 methodology may provide the opportunity to isolate micelles and vesicles at discrete time points during both equilibration and/or interconversion processes and may allow metastable intermediary particles to be isolated and identified (13).

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