

Effects of taurocholate on the size of mixed lipid micelles and their associations with pigment and proteins in rat bile¹

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Abstract Mixed lipid micelles were isolated from rat bile on taurocholate-equilibrated Sephadex G100 and G200 columns (5–60 mM) to study relationships between lipids and other constituents of bile. Phospholipid, cholesterol, and a bile salt peak co-eluted as mixed micelles at all taurocholate concentrations. The micellar radius, derived from the elution profile, increased progressively from ~1.6 nm to ~3.5 nm when the column taurocholate concentration was reduced from 40–60 mM to 5 mM (the physiological range for rat bile). Biliary bile pigment and bromsulphthalein, added in vivo, eluted as self-aggregates that were smaller than the lipid micelles. In contrast, on bromsulphthalein-equilibrated columns, unconjugated bromsulphthalein associated weakly with lipid micelles but this association accounted for less than 10% of the unconjugated dye in bile. No associations were found between lipid and proteins when SDS-polyacrylamide gel electrophoretic polypeptide patterns of column fractions were compared with the lipid elution profiles at different taurocholate concentrations. Two high molecular weight protein aggregates were demonstrated in bile (>222,000 M_r) by Sephadex G200 chromatography. These studies provide a reliable estimate of rat bile lipid micelle size and suggest that bile pigment, bromsulphthalein and proteins do not form strong associations with biliary mixed micelles but exist in bile predominantly as self-aggregates.—**Reuben, A., K. E. Howell, and J. L. Boyer.** Effects of taurocholate on the size of mixed lipid micelles and their associations with pigment and proteins in rat bile. *J. Lipid Res.* 1982. **23**: 1039–1052.

Supplementary key words column chromatography of bile • biliary lipid mixed micelles • bilirubin and BSP aggregates • biliary proteins

The complex nature of bile has stimulated many studies of its physicochemical properties and of the interactions that occur among its constituents. Extensive investigations have been concerned with the behavior of bile salts and bile salt-lipid micelles (1–3), but relatively little attention has been given to the relationship between the lipids, organic anions, and proteins. Early studies suggested the existence of macromolecular complexes in bile composed of lipids, pigment, and proteins (4–8) but these were overlooked thereafter, in favor of bile salt-lipid micelles. Recently it has been suggested

that the biliary secretion of many low molecular weight organic compounds is enhanced by self-aggregation or by their association with the mixed micelles in bile (9, 10). Although the formation of self-aggregates is well documented for organic anions such as bilirubin and bromsulphthalein (BSP) (10–12), the association between organic anions and biliary lipid micelles is controversial (13). In addition, the formation of lipid-protein complexes has been proposed as a possible mechanism by which biliary lipids are transported within the hepatocyte and these may also be secreted into bile (14–18). Ample precedents exist for lipid-protein associations in cell membranes (19) and serum lipoproteins (20), but biliary lipoproteins and intracellular lipoproteins destined for bile have not been definitely described.

In the present study, we have examined the interactions between biliary lipids, organic anions, and proteins in rat bile utilizing gel filtration chromatography as the major technique. Bile salt solutions were used for both column equilibration and elution in order to maintain micellar integrity. The bile salt concentration was varied to change the size of the lipid micelles, and thus their elution profiles, which enabled comparison with the elution profiles of pigments and proteins. Sucrose density gradient ultracentrifugation and transmission

Abbreviations: BSP, bromsulphthalein; HDL, high density lipoprotein; IgA, immunoglobulin A; K_{av} , approximate distribution coefficient; M_r, molecular weight; NaTC, sodium taurocholate; NaTT, sodium tetrathionate; PAS, periodic acid-Schiff; PMFS, phenylmethylsulphonyl fluoride; SD, standard deviation; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TEM, transmission electron microscopy; TLC, thin-layer chromatography; V_e, sample elution volume; VLDL, very low density lipoprotein; V₀, void volume; V_t, total volume.

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electron microscopy (TEM) were also used in a few parallel experiments.

MATERIALS AND METHODS

Collection and handling of bile samples

Fed male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing between 220 and 350 g and anesthetized with sodium pentobarbitone, were used in all the experiments. Bile was collected by drainage following biliary cannulation with polyethylene tubing (PE-10 Clay-Adams Division of Becton, Dickinson, Parsippany, NJ). The abdominal incision was closed to reduce fluid loss during bile collection and body temperature was kept at 37°C with an overhead infrared lamp. Contamination with pancreatic secretion was avoided by careful ligation of the bile duct above the level of the pancreas. All bile secreted within 15 min of completion of surgery was discarded. Sample purity was confirmed on subsequent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), by the absence of a typical pancreatic exocrine protein pattern (21).

Bile was collected at room temperature and used within 2 hr in the initial gel filtration studies. Subsequent experiments established that the biliary protein composition was consistent during 6 hr of continuous biliary secretion. Furthermore, no changes occurred in the elution profiles of lipids and pigment (apart from some loss of color absorbance) or in the electrophoretic pattern of the proteins, following at least 2 weeks storage with sodium azide at 4°C. Therefore, all the gel filtration studies, which examined the relationships between mixed lipid micelles and proteins, were performed sequentially on the same chromatography column with aliquots from a pooled sample of bile, collected from two rats for 6 hr each and used within 10 days. This allowed a direct comparison of the protein elution profiles and electrophoretic patterns from experiments with different eluant bile salt concentrations. Only fresh samples were used for ultracentrifugation and electron microscopy.

Rat bile containing bromsulphthalein (BSP; Hynson, Westcott and Dunning, Baltimore, MD) was prepared, *in vivo*, by the constant intravenous infusion of a 2 mM BSP solution (1.67 mg per ml in 0.15 M NaCl) given at a rate of 0.12 $\mu\text{mol min}^{-1}$ (100 $\mu\text{g min}^{-1}$), which is below the transport maximum for BSP (22).

Gel filtration chromatography

Thirty-six gel filtration experiments were performed in K-9 or SR-10/50J columns (Pharmacia Fine Chem-

icals, Uppsala, Sweden) containing between 19 and 39 ml of swollen Sephadex G100 (Pharmacia Fine Chemicals) pre-equilibrated at room temperature with three-column volumes of the solution subsequently used for elution. The electrolyte composition (Na^+ 125 mM, Cl^- 105 mM, K^+ 5 mM, and HCO_3^- 25 mM) (10) and bile salt concentration of the solution were chosen to mimic the physiological conditions of rat bile (23).

In all of these experiments, the void volume of the column (V_0) was first determined with blue dextran (molecular weight 2×10^6 M_r, Pharmacia Fine Chemicals) and the total volume of the column (V_t) was measured from the complete elution volume of [^{14}C]sucrose, which was mixed with the test sample prior to chromatography (0.015–0.05 μCi , in 30–100 μl water, New England Nuclear, Boston, MA). The bile samples were not otherwise modified prior to chromatography, except where explicitly stated. Bile (0.4 to 3.0 ml) (1–5% bed volume) was applied to the column for descending chromatography. The eluate fractions of 1–1.5 ml were analyzed for phospholipid, radioactivity, and one or more of the following: protein, bile pigment, cholesterol, bile salts, and BSP. To vary the size of the bile salt-lipid micelle sufficiently to change its elution profile, different bile salt concentrations were used for column equilibration and elution: 0, 5, 10, 15, 20, 30, 40, and 60 mM sodium taurocholate, (A grade, 97% pure by TLC, Calbiochem-Behring, La Jolla, CA) with two to nine replicates at each column condition. The resulting elution patterns of the lipids, proteins, and bile pigments were then compared.

In each experiment, an approximate distribution coefficient, K_{av} (24), was calculated with the formula $K_{av} = V_e - V_0 / V_t - V_0$ for both phospholipids and pigments, using the appropriate elution volume (V_e) and column parameters, V_0 and V_t , defined above. High molecular weight substances elute rapidly from the column and therefore have low elution volumes with a low K_{av} , whereas the converse is true for substances of low molecular weight. Molecular size calibration of the columns was made with globular protein standards, comprising proteins in the molecular weight range 13,700–222,000 (Pharmacia Fine Chemicals). Calibration measurements were made in the presence (20 mM NaTC) and absence of bile salts with similar results.

To examine the behavior of an exogenous organic anion, bile from rats infused with BSP was submitted to gel filtration in two concentrations of sodium taurocholate (5 and 30 mM), selected because clear-cut differences in the mixed lipid micelle elution pattern had already been obtained. Control chromatograms of unconjugated BSP alone were also obtained on 30 mM NaTC columns. In addition, rat bile containing BSP added *in vivo* was chromatographed on columns that

were pre-equilibrated and eluted with 30 mM NaTC-electrolyte solutions containing 3 or 6 mM unconjugated BSP.

Three additional column chromatography experiments were undertaken to analyze protein behavior in greater detail. In the first of these experiments using Sephadex G100, calcium (1.95 mM), magnesium (1.15 mM), and phosphate (0.0625 mM) were added to a 30 mM NaTC eluant, to assess the effects of these other major inorganic salts (at the concentrations found in rat bile) on the peptide patterns in column fractions. Next, a G200 Sephadex column (105 cm long, 68 ml bed volume) was used to increase the resolution of protein and lipid elution profiles in the presence (30 mM NaTC) and absence of bile salts. In the final column experiment, following G100 gel filtration of bile with a bile salt-free eluant, the void volume fractions were pooled and lyophilized. The dry sample was dissolved in 2 ml of 30 mM NaTC electrolyte solution and resubmitted to gel filtration on G100 Sephadex using 30 mM NaTC eluants, in order to determine if the additional protein that appeared in the void volume when bile salts were absent would elute later in the columns, when the mixed lipid micelles were reconstituted.

Ultracentrifugation

The relationship between mixed lipid micelles and organic anions was also examined in three ultracentrifugation experiments, as previously described (10). A continuous sucrose density gradient (5–30% w/v), in 5, 15, or 30 mM sodium taurocholate-electrolyte solution, was prepared in 3½ in × 9/16 in cellulose nitrate centrifuge tubes (Beckman Instruments Inc., Spinco Division, Palo Alto, CA). [¹⁴C]Taurocholate (Tauro [Carbonyl-¹⁴C]cholic acid sodium salt 0.625–1.25 μCi, Amersham Corporation, The Radiochemical Center, Amersham, England) was added in 25-μl aliquots to 0.5-ml samples of fresh bile, in order to label the bile salts. Samples were layered on to each gradient prior to spinning in an SW41 rotor at 40,000 rpm and 16°C, in a Beckman L5-65 ultracentrifuge. After centrifugation for 16, 60, or 90 hr, eighteen 0.68-ml fractions were collected from the top of each tube using a Buchler Auto-densi-flow fractionator (Buchler, Fort Lee, NJ, Instrument Division of Nuclear-Chicago) and assayed for phospholipid, [¹⁴C]taurocholate radioactivity, and bile pigment concentration.

Electron microscopy

Samples of undiluted fresh whole bile and peak phospholipid and pigment column fractions were examined by TEM in a Siemens Elmiskop 102, after negative staining with 3% phosphotungstic acid, buffered to pH 7.4.

Analytical techniques

Phospholipids were estimated by measuring total phosphorus with the method of Bartlett (25). Total bile salt concentration was determined using a 3α-hydroxysteroid dehydrogenase assay (26) with NaTC standards (hydroxysteroid dehydrogenase was obtained from Worthington-Millipore Corporation, Freehold, NJ) and cholesterol was measured by gas-liquid chromatography (27) using a Hewlett-Packard 5830A gas chromatograph (Hewlett-Packard, Avondale, PA) with a 6 ft × ¼ in × 4 mm I.D. column of 3% OV17 on Chromasorb WHP 100/120 mesh; injection and detector temperatures 300°C, column temperature 275°C, hydrogen and nitrogen flow rates of 45 ml min⁻¹ and air flow of 300 ml min⁻¹.

Bilirubin was measured as total bile pigment by absorption spectrophotometry at 400 nm, close to the absorption maximum (410–420 nm) obtained with both diluted whole rat bile (fresh and stored) and column eluate fractions containing bile pigment but no phospholipid or cholesterol. In each case, an identical absorption spectrum was found for bile pigment. Total BSP in bile and column fractions was quantified by the method of Seligson, Marino, and Dodson (28). Unconjugated and conjugated BSP were separated by TLC and identified using a combination of ultraviolet light fluorescence and ninhydrin or ammonia staining (29). ¹⁴C Radioactivity was measured using liquid scintillation counting with external standardization for quench correction (Beckman LS 7,000).

An approximate elution profile for biliary proteins was obtained using the Coomassie Blue binding reaction (30) (BioRad protein assay, BioRad Laboratories, Richmond, CA), as it is a rapid sensitive test. In common with other dyes (31, 32), Coomassie Blue reacts with bile salts in the concentrations found in bile and those used in the gel filtration experiments. Therefore, appropriate aliquots of the eluants used for column chromatography were added to the standard curve protein samples (bovine gamma globulin, Sigma Chemical Co., St. Louis, MO). Proteins were examined more specifically by SDS-PAGE in a linear 1.5-mm-thick 5–16% acrylamide gradient slab (pH 8.9), modified from the method of Maizel (33). The proteins in whole bile were either precipitated with 90% cold acetone or lyophilized. Single or pooled sequential pairs of column fractions (1.3 to 1.7 ml) were also lyophilized. All samples were solubilized in 180 μl of a medium containing 100 μl Tris-phosphate buffer, pH 6.7 (33), 5.6 mM EDTA, and 2.2% SDS (final concentrations), in both reducing (dithiothreitol and iodoacetamide, 55.6 mM each) and non-reducing conditions. Sucrose (0.33 M) was included to increase sample density, together with bromphenol

blue as an electrophoretic marker. Half of this solution (90 μ l) was applied to the 3% acrylamide stacking gel (pH 6.7) and electrophoresis was carried out at room temperature for \sim 15 hr at \sim 25 mA constant current using a pH 8.9 Tris-glycine buffer (33). All samples were solubilized completely except for fractions from bile salt-free columns. The addition of sodium taurocholate before lyophilization (final concentration 20 mM) solved this problem. The polypeptide bands were detected by staining with Coomassie Brilliant Blue, periodic acid-Schiff reagent (34, 35), or silver nitrate (36). As a check on the stability of the biliary proteins, these were also examined after collection of bile directly into chilled acetone or into tubes containing various proteolytic inhibitors: Trasylol, 3,000 KIU ml⁻¹; leupepsin and pepstatin, each 1 μ g ml⁻¹; and phenylmethylsulphonyl fluoride, PMSF, 0.1 mM with sodium tetrathionate, NaTT, 5 mM. Standard molecular weight markers were run simultaneously as well as preparations of rat lipoprotein apoproteins (VLDL, HDL, and serum proteins of density > HDL), for comparison with the proteins in bile and elution fractions.

RESULTS

Whole bile was subjected to gel filtration and lipid, pigment, and protein peaks were resolved. Each of the individual elution profiles was highly reproducible at a given eluant bile salt concentration but showed great variation when different eluant bile salt concentrations were used. In the results shown below, data are first given from which the estimates of bile salt-lipid micelle size are calculated. Evidence is then presented that suggests that organic anions (such as bile pigment and BSP) and most of the biliary proteins are present in bile as aggregates and do not form strong associations with lipid micelles in the physiological concentrations of bile salts used in these studies.

Bile salt-lipid mixed micelles

Under all conditions tested, all the phospholipids and cholesterol in bile co-eluted from the columns in a single peak as shown by the examples in **Fig. 1**. A bile salt peak also co-eluted with the phospholipids and chole-

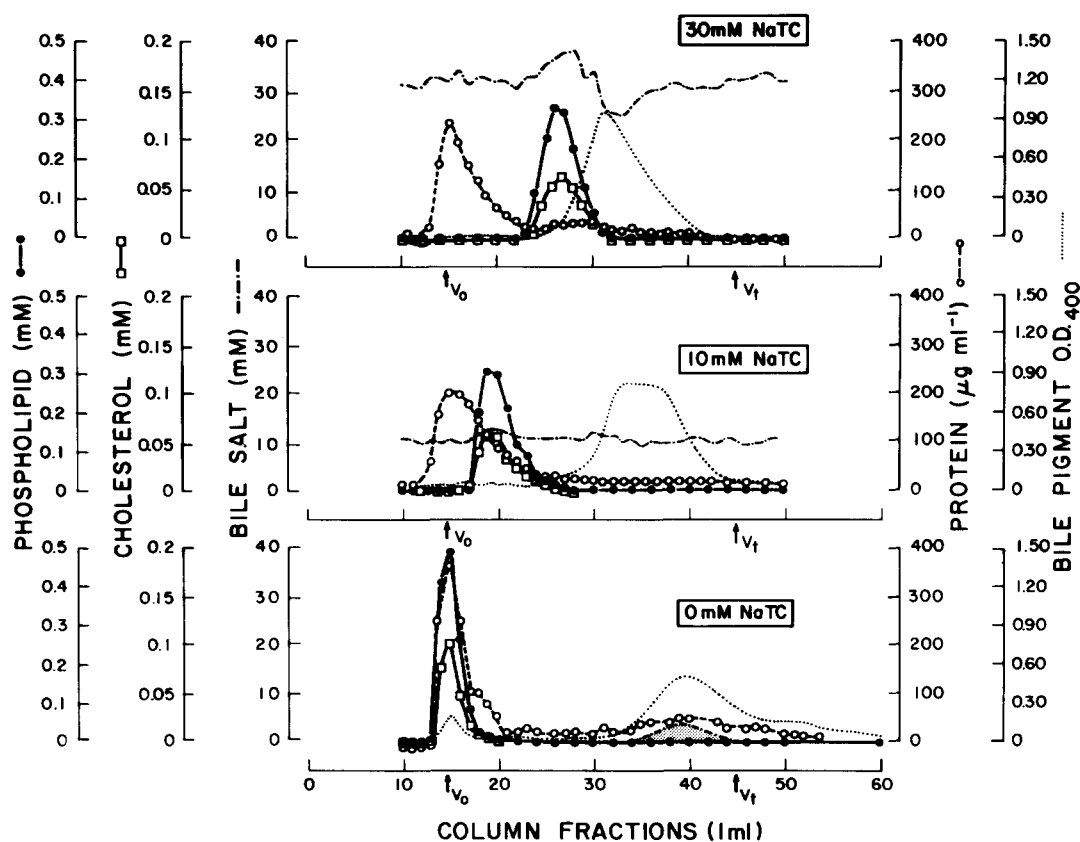


Fig. 1. Sephadex G100 gel filtration chromatography of rat bile. Samples (1.0 ml) from a common pool of bile containing 16 mM bile salts, 1.6 mM phospholipid, and 0.29 mM cholesterol were applied to columns that had been pre-equilibrated and were subsequently eluted, at 0.31 ml min⁻¹, with bile salt-electrolyte solutions containing 30 mM (top), 10 mM (middle), or 0 mM (bottom) sodium taurocholate (NaTC). Phospholipid (●—●), cholesterol (□—□), bile salt (— · —), protein (○—○), and bile pigment (· · · · ·) concentrations are shown on the ordinate. Cholesterol and phospholipid were only detected in the micelle fractions. The position of the phospholipid peak was used as a marker of the elution volume of the mixed lipid micelles in bile. The bile salt peak on a 0 mM NaTC column is shown by the shaded area. Column fractions (ml), including void volume (V_0) and total volume (V_t), are shown on the abscissa.

terol, unless sodium taurocholate-free eluants were used. The co-migration of these three components indicates that bile salt-phospholipid-cholesterol mixed micelles have been isolated. The approximate distribution coefficient (K_{av}) for these micelles (calculated from the elution volume of the phospholipid peaks) varied linearly with the bile salt concentration used for elution, between 5 and 40 mM NaTC, but did not increase with higher concentrations, as shown in Fig. 2. Thus, there was an inverse relationship between the intermicellar bile salt concentration and the micelle K_{av} and hence, the apparent micellar radius. The latter ranged from ~ 1.6 nm (K_{av} 0.47 ± 0.05 SD $n = 7$, 40–60 mM NaTC) to ~ 3.5 nm (K_{av} 0.07 ± 0.03 SD $n = 4$, 5 mM NaTC). Size estimates were made by comparison with globular protein standards.

The bile salt concentration in the mixed micelle peak, and consequently the micellar bile salt:lecithin ratio, varied directly with the NaTC concentration used for column elution. At eluant bile salt concentrations greater than those of the bile samples (~ 15 mM), it appeared that bile salts were induced into the mixed lipid micelles, as suggested by the high peak bile salt concentrations (Fig. 1, top). Bile salts dissociated completely from phospholipid and cholesterol when bile salt-free eluants were used (Fig. 1, bottom), resulting in a substantial increase in size of the phospholipid-cholesterol complexes, i.e., liposomes were produced that were excluded by both Sephadex G100 and G200 gels and thus appeared in the void volume.

Bile pigment and BSP

When the columns were equilibrated and eluted with high bile salt concentrations (30–60 mM NaTC), the bile pigment eluted soon after the phospholipid peak, presumably in aggregated form (Fig. 1, top) since, according to molecular weight, monomers (585 M_r) and dimers should elute at the total volume of a G100 Sephadex column (V_t). In contrast to the lipids, the pigment peak was broad, probably indicating that a range of multimers was present, because at high bile salt concentrations adsorption to the gel was not detected (Fig. 1, top). When the bile salt concentration was progressively lowered, elution of the bile pigment peak was delayed (Fig. 2) and adsorption to both gel and proteins became noticeable. This was most marked in bile salt-free conditions (Fig. 1, bottom), when much of the pigment eluted with the total column volume (as indicated by the elution profile of [14 C]sucrose) and “trailing” on the column was pronounced. In addition, up to 13% of the pigment was excluded from the gel and appeared in the void volume together with the lipids and proteins. The relationship between the distribution coefficient (K_{av}) of the major pigment peak and bile salt concentration was less exact than that for the lipids and, in

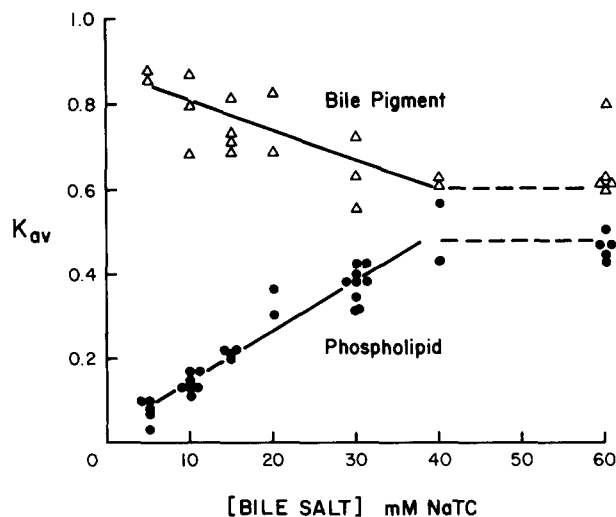


Fig. 2. Effect of bile salt concentration on the elution behavior of bile pigment and lipid micelles of rat bile, on Sephadex G100 gel filtration chromatography. K_{av} values of bile pigment (Δ) and phospholipid (\bullet), obtained by Sephadex G100 gel filtration chromatography of rat bile, are plotted on the ordinate against the NaTC concentration in mM on the abscissa. The least squares linear regression curves for bile pigment ($y = 0.84 - 0.0062x$, $n = 17$, $r = 0.72$, $P < 0.001$) and phospholipid ($y = 0.27 + 0.012x$, $n = 29$, $r = 0.97$, $P < 0.001$) are shown by the solid lines, for NaTC concentrations between 5 and 40 mM inclusive.

contrast, showed regression in the opposite direction (Fig. 2). In other words, while the lipid micelles diminished in size with the increase in bile salt concentration, the pigment aggregates increased in size.

Chromatography of bile containing BSP (added in vivo) gave a two-component elution profile in 30 mM NaTC on columns that were not equilibrated with BSP (Fig. 3), suggesting the presence of two molecular weight populations of the dye. An early peak or shoulder (as in Fig. 3), representing $\sim 20\%$ of the BSP in bile, coincided with the elution volume of unconjugated BSP (chromatographed without bile). This was confirmed by TLC of the column fractions. As with the bile pigment, the elution of unconjugated BSP, prior to the total column volume, suggested that unconjugated BSP eluted in an aggregated form. The later peak, eluting in the total column volume (together with [14 C]sucrose) was composed of BSP conjugates on TLC (Fig. 3). In 5 mM NaTC, a single elution peak of BSP was seen that extended beyond the total column volume, indicating that adsorption to the gel had now occurred at the lower bile salt concentration. When the bile containing BSP was chromatographed on columns equilibrated with 30 mM NaTC and either 3 mM or 6 mM unconjugated BSP, a small peak of BSP co-eluted with the lipid micelles. Allowing for the differences in molar concentrations of bile salts and BSP in the column eluants, the association between BSP and the mixed micelles appeared to be less than one-third of that of bile salts for

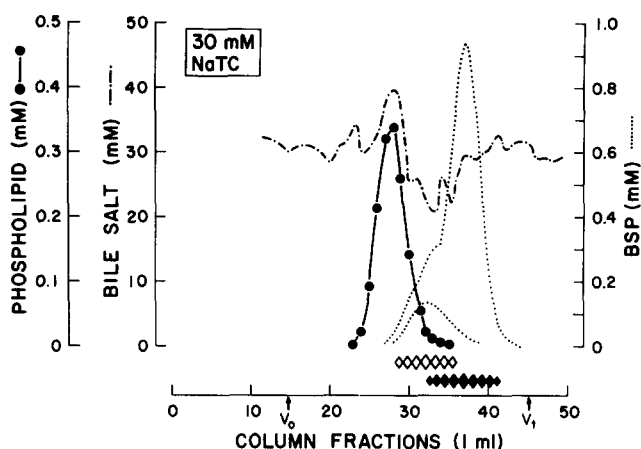


Fig. 3. Sephadex G100 gel filtration chromatography of rat bile containing bromsulphthalein (BSP). One ml of bile, containing $4.6 \mu\text{mol}$ of BSP, added *in vivo*, was applied to a column that had been pre-equilibrated and was subsequently eluted at 0.30 ml min^{-1} , with a bile salt-electrolyte solution containing 30 mM NaTC but no BSP. Phospholipid, bile salt, and BSP concentrations are shown on the ordinate; cholesterol (as in Fig. 1) is omitted for clarity. The elution profile of unconjugated BSP, run separately, is shown as a small BSP curve with a single peak in fraction #32. TLC results for unconjugated and conjugated BSP in column fractions are shown schematically on the abscissa by the open and closed diamonds, respectively. In addition to the column fractions, void volume (V_0) and total column volume (V_t) are shown.

the phospholipids and cholesterol and accounts for less than $\sim 10\%$ of the unconjugated BSP in bile.

Sedimentation profiles of bile lipids, pigment, and pure bile salt micelles, after 60 hr centrifugation in sucrose gradients containing 5, 15, and 30 mM NaTC are illustrated in Fig. 4. At each bile salt concentration, the lipids sedimented to a higher sucrose density (1.043 to 1.047 gm/ml) than either the pigment or pure bile salt micelles. This separation occurred if centrifugation was performed for only 19 hr, or continued for 90 hr. Density equilibrium was not reached for any component, as previously reported by Scharschmidt and Schmid in similar experiments (see Fig. 3 of Reference 10). The density difference between the mixed lipid micelle and the bile pigment increased as the bile salt concentration was reduced. This reflects a change in the density of the pigment aggregate (Fig. 4) since no significant change in the density of the mixed lipid micelle could be detected in 5, 15, or 30 mM NaTC.

Transmission electron microscopy of negatively stained images of bile and gel filtration fractions showed two populations of particles, whose size varied considerably in all samples examined (16–33 nm and 44–83 nm). Neither particle could be identified as lipid or pigment aggregates, however, because particles of both size ranges were seen in either the pure lipid or pigment peaks from the columns. In addition, the size of these particles was 10–20 times larger (by TEM) than the

estimates for either the lipid micelles or pigment aggregates, given by column chromatography.

Proteins

Most of the biliary proteins eluted in the void volume of Sephadex G100 columns (Fig. 1), indicating that they were in a high molecular weight form, exceeding M_r 60,000–70,000. Two secondary “false” peaks were also seen, due to the reaction between bile salts and the Coomassie Blue protein reagent. The first of these peaks is due to the bile salt peak of the mixed lipid micelles (Fig. 1, top), while the second peak appears in the total volume of the column and, as noted earlier, is due to bile salts that have dissociated from the bile sample at low column bile salt concentrations (Fig. 1, bottom).

SDS-PAGE of the proteins in bile, without disulfide bond reduction, showed approximately 14 polypeptide

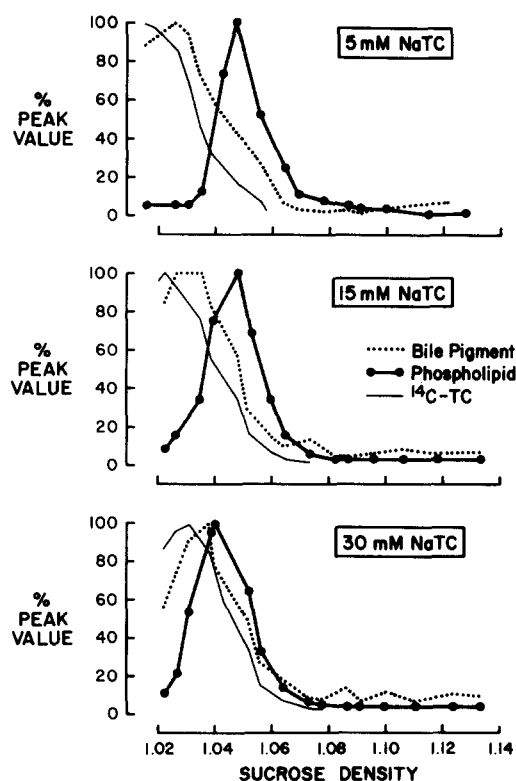


Fig. 4. Sucrose density gradient ultracentrifugation of rat bile. Aliquots (0.5 ml) of rat bile, containing ^{14}C -labeled NaTC, added *in vitro*, were layered over 11.7 ml, 5–30% (w/v) continuous sucrose gradients and centrifuged at 16°C at 40,000 rpm for 60 hr with a Beckman L5-65 ultracentrifuge in an SW41 rotor. The gradients were prepared by dissolving sucrose in bile salt-electrolyte solutions containing 5 mM (top), 15 mM (middle), and 30 mM (bottom) NaTC. Bile pigment, phospholipid, and ^{14}C -radioactivity are expressed on the ordinate as percent of peak values. Sucrose density (g ml^{-1} at 15°C) of the 0.68 ml gradient fractions, is shown on the abscissa. Peak values (top to bottom): a, bile pigment: 0.295; 0.340; 0.405 O.D. units at 400 nm; b, phospholipid: 0.38; 0.32; $0.28 \mu\text{mol/fraction}$; c, ^{14}C -radioactivity: 155,475; 155,475; 156,142 dpm/fraction.

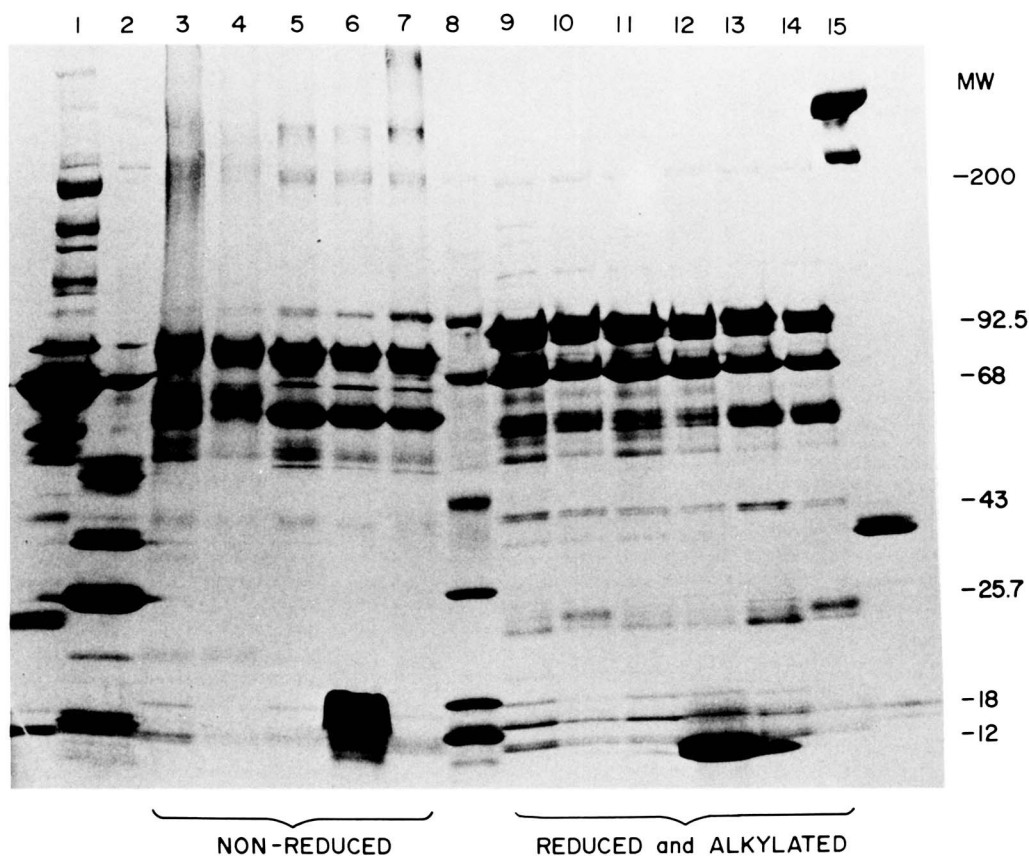


Fig. 5. SDS-PAGE of the proteins of rat bile. SDS-PAGE (33) of the proteins from 167- μ l samples of lyophilized fresh rat bile, or bile collected in the presence of various proteolytic inhibitors or into chilled acetone (see key below). All the samples were obtained from two animals. Both nonreducing (lanes 3 to 7 inclusive) and reducing conditions (lanes 1, 2 and 8–15, inclusive) were used during protein solubilization, prior to electrophoresis. SDS-PAGE of rat serum apolipoproteins (see key) is also shown, together with standard molecular weight markers (lane 8): myosin (M_r 200,000), phosphorylase b (M_r 92,500), bovine serum albumin (M_r 68,000), ovalbumin (M_r 43,000), cytochrome c (M_r 12,300), (Bethesda Research Laboratories Inc., Rockville, MD).

bands on Coomassie Blue staining (**Fig. 5**, lanes 3–7 inclusive, and **Table 1**). When disulfide bond reduction and alkylation were included in the method, over 25 distinct polypeptide bands were detected (**Fig. 5**, lanes 9–14 inclusive) and gel penetration by the proteins was complete. Fifteen to seventeen of these bands were also PAS positive but neither PAS nor silver nitrate staining detected additional polypeptides. The biliary polypeptide spectrum was unaltered by collecting the bile in tubes containing various proteolytic inhibitors, chilled acetone (**Fig. 5**), or pancreatic secretions (not shown). Molecular weights ranged from \sim 10,000 to \sim 300,000. Trace amounts of three smaller polypeptides (M_r \sim 5000– \sim 10,000) were occasionally seen when the gel was heavily overloaded. However, no similarities were seen between rat serum apolipoproteins and biliary proteins. The polypeptide patterns of the Sephadex G100 column fractions collectively included all the bands that were seen in whole bile (**Fig. 5** and **Fig. 6**), suggesting that no qualitative loss of proteins occurred during chro-

TABLE 1. Key to lanes in **Fig. 5**

Source	Lane Number	Sample
Rat serum lipoproteins	1	$d > 1.20$
	2	$1.063 < d < 1.20$ (HDL)
	15	$d < 1.006$ (VLDL)
Rat bile (nonreducing conditions)	3	rat #1, cold acetone
	4	rat #2, leupepsin + pepstatin
	5	rat #1, PMSF + NaTT
	6	rat #1, Trasylol
	7	rat #1, fresh bile
Rat bile (reducing conditions)	9	rat #1, cold acetone
	10	rat #2, leupepsin + pepstatin
	11	rat #1, PMSF + NaTT
	12	rat #1, Trasylol
	13	rat #1, fresh bile
	14	rat #2, fresh bile
Molecular weight markers	8	

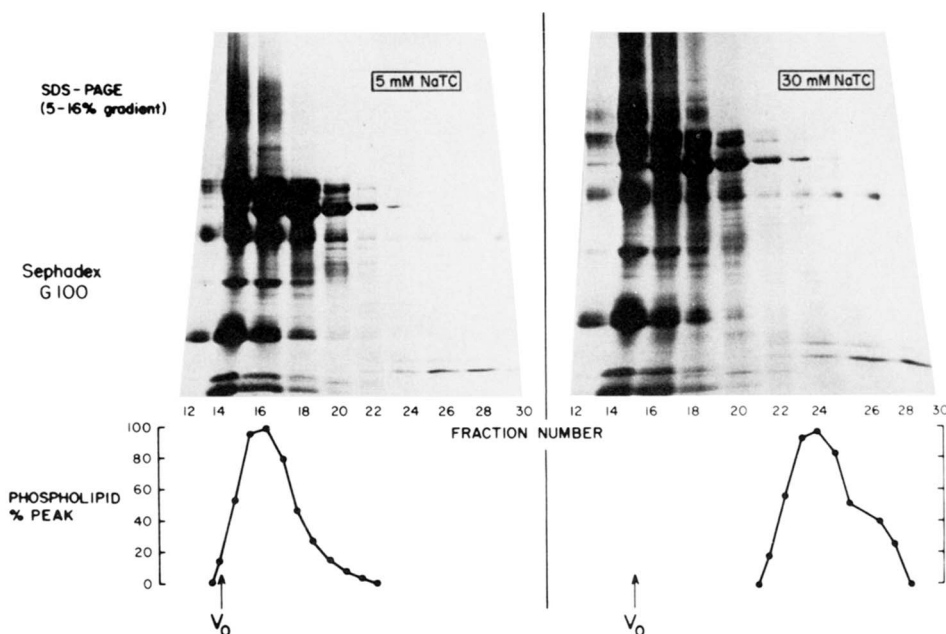


Fig. 6. SDS-PAGE of the proteins of rat bile after Sephadex G100 gel filtration chromatography: effect of bile salt concentrations on the elution behavior of the proteins and the relation with the lipid micelles. Column chromatography of 1.5 ml of rat bile was performed (as in Fig. 1) with eluants containing 5 or 30 mM NaTC. The proteins from sequential pairs of column fractions were submitted to SDS-PAGE, after lyophilization and solubilization in reducing conditions. In each experiment, the elution of the mixed lipid micelles is shown below by the phospholipid profiles, while the electrophoretic patterns of corresponding pairs of column fractions are shown above. The phospholipid concentration of individual fractions is expressed on the ordinate as percent of peak values for ease of comparison. Bile salt and cholesterol profiles are not shown but are similar to those seen in Fig. 1. V_0 , 14 and 15 ml; V_r , 45 and 44 ml; of 5 and 30 mM NaTC columns, respectively.

matography. A dense spectrum of bands was seen in the void volume fractions (10 to 20 inclusive), and some intermediate and small molecular weight proteins separated later (fractions 22–30). These data confirm the results of the total protein assay (Fig. 1) which indicated that most of the biliary protein was excluded by the Sephadex G100 gel. There was no discernable change in this pattern, and no movement of any individual band when the eluant bile salt concentration was reduced progressively from 40 to 5 mM NaTC, despite the marked change in the peak elution fraction of the mixed lipid micelle (Fig. 6). Many small and intermediate molecular weight polypeptides were found in the void volume, even when disulfide bond reduction was omitted or when physiological levels of Ca^{2+} , Mg^{2+} , and phosphate were present in the eluant solution (not shown). This suggests that these smaller proteins are aggregated with those of higher molecular weight.

The extent of this protein aggregation was examined further on Sephadex G200, which has a higher exclusion limit than G100. In contrast to the single void volume protein peak on Sephadex G100 (Fig. 1), two high molecular weight peaks were seen on Sephadex G200, in the presence of bile salts (Fig. 7). SDS-PAGE showed that in the first peak (Fig. 7, void volume fractions 19–24), the molecular weights of the main component bands were: $\sim 88,800$, $\sim 52,500$, $\sim 23,400$, and

$22,500$. These molecular weights correspond closely with those of the components of secretory IgA (secretory piece, heavy chain, and light chains, respectively (37)), which has been shown to be a major component of rat bile (38). A wide spectrum of bands was seen in the second peak (Fig. 7, fractions 25–33) but these have not been identified further in this study. The molecular weights ranged from $\sim 12,000$ to over 200,000, many of which were seen in bile, even under nonreducing conditions (Fig. 5).

Following Sephadex G200 gel filtration in the absence of bile salts (Fig. 8), there was a marked increase in void volume proteins, associated with a reduction in the height of the second peak. The most noticeable change in the corresponding SDS-PAGE pattern was a shift of most of the M_r 88,800 polypeptide band into the void volume together with minor components of other bands.

The tendency for biliary proteins to associate with biliary lipids when bile salt-free eluants were used, was also examined in a final experiment (see Methods). Following gel filtration of bile on Sephadex G100 without bile salts, the void volume (fractions 10–20 inclusive), which contained all the biliary phospholipid and cholesterol, was concentrated and submitted to repeat gel filtration in 30 mM NaTC. Several proteins, which were present in the original void volume fractions, now

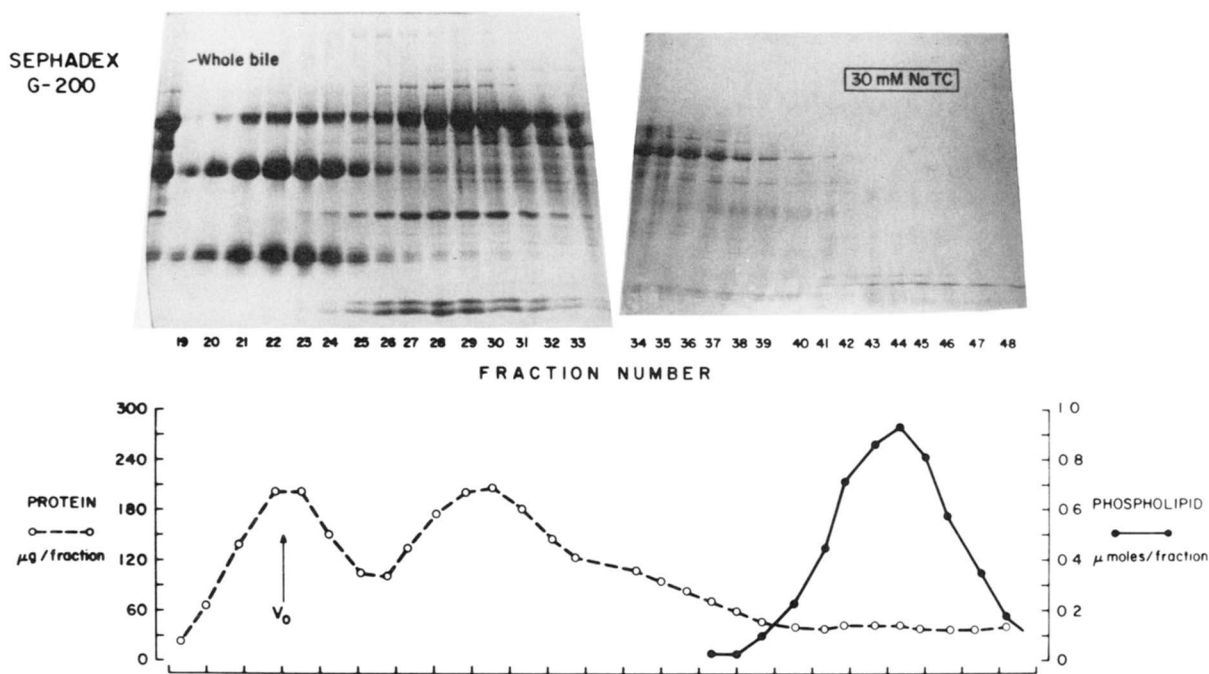


Fig. 7. Elution profiles of the proteins and lipid micelles of rat bile, on Sephadex G200 gel filtration chromatography with a bile salt-containing eluant. Column chromatography of 3 ml of rat bile eluted at 2.2 ml/hr with a bile salt-electrolyte solution containing 30 mM NaTC. The total protein and lipid micelle elution profiles (marked by the phospholipid peak) are shown below and the SDS-PAGE patterns of the proteins of whole bile and single column fractions are shown above. Bile salt and cholesterol profiles are not shown but are similar to those seen in Fig. 1. The total protein and phospholipid content, of 1.5-ml fractions, is expressed on the ordinate in μg and μmol , respectively. Note: two high molecular weight protein peaks are seen (fractions 22 and 30, respectively) V_0 22 ml, V_t 60 ml.

eluted within the column volume. This suggests that these proteins were trapped by phospholipid-cholesterol liposomes formed when bile salts were omitted. These proteins were then released when the liposomes reformed into micelles when bile salts were again added.

DISCUSSION

Gel filtration chromatography, which has been the major analytical technique in our study, was first used to measure pure bile salt micelle size almost 20 years ago (39). Although this technique had also been used to study the physical composition of bile (40), the importance of pre-equilibrating the column to minimize adsorption of bile salts to the gel (41) and thus prevent micelle disruption, was only rarely appreciated (39, 42). Consequently, a cholesterol-phospholipid aggregate was often considered to be the dominant macromolecular complex in bile (8, 40, 43, 44) until the critical role of bile salts was recognized (32, 45–47). In the present study, the co-elution of cholesterol, phospholipid, and bile salts in all experiments, except when bile salt-free eluants were used, is good evidence for the isolation of intact taurocholate-lecithin-cholesterol micelles. The size of these biliary micelles, in isotonic electrolyte so-

lutions, has been estimated here at between ~ 1.6 and ~ 3.5 nm Stokes radius.

Although the measurement of molecular radius by gel filtration is strictly only accurate for globular proteins and polysaccharides (24, 48), the estimates of micelle size in the current study agree remarkably with values obtained recently using the nonperturbing techniques of quasi-elastic light scattering (49, 50) and X ray small angle scattering (51) with model solutions of bile salt-lecithin mixtures (with and without cholesterol). These in vitro studies with model systems and a variety of bile salt species also predict the close inverse relationship that we found in rat bile between micellar size and eluant bile salt concentrations. The finding, that the intermicellar NaTC concentration during gel filtration determines the bile salt:lecithin ratio in the mixed micelles, is supported by the results of equilibrium dialysis studies of bile (52). An increase in this ratio is associated with a reduction in micelle size in our studies and in those with model micellar solutions (49–51). At concentrations of bile salts commonly found in rat bile (20 mM), the micelle size is estimated at ~ 2.8 nm in our study, close to the prediction from model systems.

The present study gives reliable estimates of rat biliary mixed micelle size probably because these “bile-salt-rich” micelles (i.e., having a bile salt:lecithin ratio

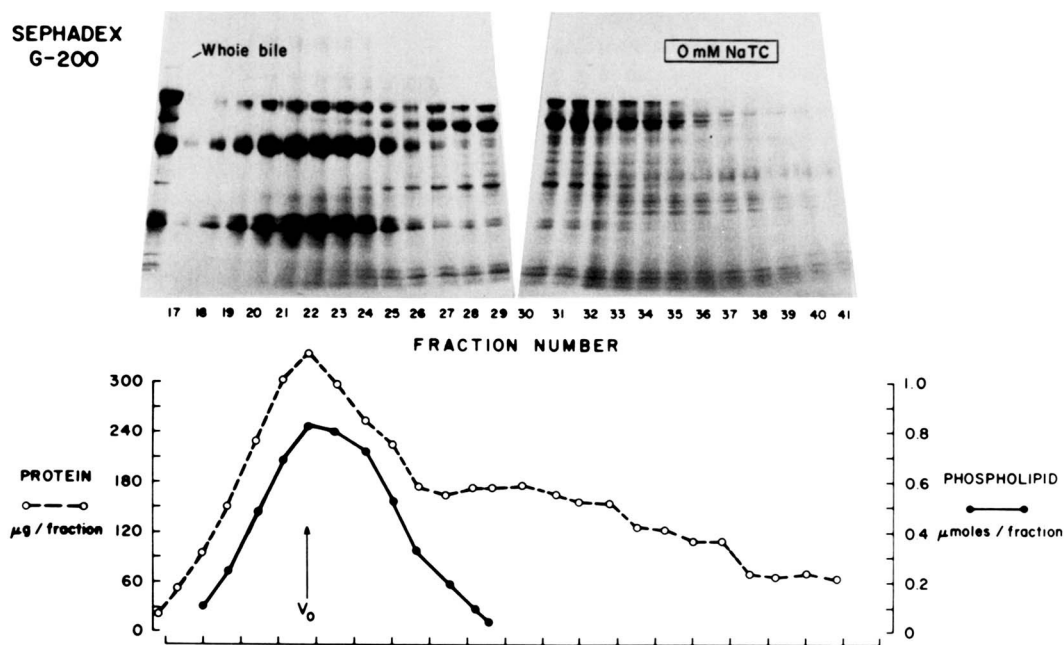


Fig. 8. Elution profiles of the proteins and lipids of rat bile, on Sephadex G200 gel filtration chromatography, with a bile salt-free eluant. Apart from the omission of bile salt from the eluant, the experimental details are the same as in Fig. 7. Note: There is an increase in the void volume protein peak (fraction 22), compared with Fig. 7, associated with a shift of M_r 88,800 polypeptide band into the void volume.

greater than 2:1) are spherical (51) and their size and shape are not influenced by dilution (51). Moreover, rat bile is unsaturated with cholesterol, contains only three lipid components, and taurocholate is the major bile salt present. When samples of greater complexity than rat bile are examined, it may be necessary to include other components of the intermicellar solution (as well as bile salt) for column equilibration and elution; for example, micelle-free ultrafiltrates of postprandial intestinal fluid have been used as eluants for the chromatography of micelles during fat digestion (53). It should also be noted that the elution behavior of lipids during column chromatography is not always as simple as in the present study. If different sterol phases are used, a progressive decrease in micelle size with increasing bile salt concentration is not always found, as Feldman and Borgstrom have shown using taurodeoxycholate (54). Furthermore, when sample concentrations of cholesterol approach saturation in bile salt-cholesterol mixtures, phase separation may occur during column chromatography (55). While column chromatography cannot replace precise thermodynamic methods such as X ray or laser light scattering for measuring micelle size, it can give reliable estimates especially in complex biological fluids, such as bile, in which the other components present can interfere with the more sophisticated techniques.

Bile is a heterogeneous aqueous solution of inorganic salts, organic anions, bile salts, lipids, and proteins (56–58). Theoretically, various physicochemical interactions

could occur between these components. In practice, the present results show that, apart from the presence of bile salt-lipid micelles, there is a marked tendency towards self-aggregation among the other major constituents of rat bile, i.e., bile pigment and proteins, and that this self-aggregation is greater than the tendency to form mixed complexes between them, since the aggregates clearly survive elution from the column. The absence of mixed complexes in column eluants conflicts with previous reports claiming that macromolecular aggregates between lipid, pigment and/or proteins exist in bile, although we cannot exclude the idea that weak associations of pigment or protein and lipid micelles may have been disrupted by the Sephadex gel and non-equilibrium conditions.

The present study provides several reasons for the confusion that has arisen concerning the size of the biliary micelle and its associations with pigment or protein. As emphasized previously (2), most estimates of biliary micelle size and weight have been affected by serious artifacts introduced by the techniques that have been used. In studies in which ultracentrifugation was used to isolate the biliary micelle, bile specimens were often diluted more than tenfold (6, 7), which disrupts micelles (44); or treated with high solute concentrations used to form density gradients (59), which may alter micelle size (3, 49, 50), mobility (60), and structure (61). Furthermore, because the existence of self-aggregates of bilirubin was not considered, the observation that bilirubin

(M_r 585) sedimented in the ultracentrifuge, was taken as evidence for its participation in mixed macromolecular complexes (6, 7, 59). Our results show that the sedimentation of bile pigment in the ultracentrifuge also depends on the bile salt concentration. For this reason, the lipid micelles were sometimes mistakenly identified by the position of the pigment peak (59) when the bile salt concentration was high.

In the present studies, the use of a wide range of sodium taurocholate concentrations for column equilibration and elution during gel filtration not only maintained micelle integrity but also varied its size, and hence its elution profile, enabling comparison with the corresponding elution profiles of pigment and proteins. By choosing an appropriate eluant bile salt concentration, this technique can therefore be used to separate the individual components of bile. In addition, the presence of bile salts in all but the lowest concentration (5 mM) effectively reduces organic anion adsorption to the Sephadex gel.

The hypothesis that organic anions are incorporated within lipid micelles in bile has been proposed (10) as an explanation for the enhanced biliary secretion of conjugated bilirubin and BSP that is induced by the infusion of micelle-forming bile salts (9). The present results suggest that only weak interactions occur between mixed lipid micelles and BSP or bilirubin, which are easily disrupted during gel filtration. The affinity of unconjugated BSP for the micelles appears to be less than one-third as strong as that between bile salts and the other lipid components of mixed micelles under similar experimental conditions. These *in vitro* findings are supported indirectly by several physiological studies that show that bile lipid secretion, presumably in micellar form, can be altered by various cholagogues without influencing organic anion transport appreciably (13, 62, 63). On the other hand, the presence in bile of self-aggregates of bilirubin (10, 11) and BSP (10, 12) has been confirmed. Moreover, these aggregates survive the nonequilibrium concentrations of gel filtration, reflecting the high affinity of these compounds for self-association. The small size of these aggregates is probably the result of partial dissolution due to dilution during gel filtration (11). It is also probable that there is some "decoration" of bile salt micelles by bile pigment, as suggested by Carey and Koretsky (11) because the λ_{max} of bile pigment showed a wavelength shift compared with pure bile pigment aggregates (11) in both diluted whole bile and column eluate fractions which contained pigment but no cholesterol or phospholipid.

Bile salts also influenced the size of the anion aggregates during gel filtration and ultracentrifugation, although the mechanism has not been explored in our study. However, the phenomenon is well documented

(11, 12) and provides an alternative interpretation for the data of Scharschmidt and Schmid (10). In the high bile salt concentrations used in their study (40–60 mM NaTC), the mixed lipid micelles and pigment aggregates would have been sufficiently similar to behave identically during ultrafiltration and ultracentrifugation (see Figs. 2 and 4), giving the impression that they were associated and that mixed complexes were present. A distinction in the size of micelles and of pigment aggregates is only readily apparent when the bile salt concentration is reduced but remains within the physiological range. Such coincidence in the mobility of pigment and lipid aggregates in bile could also account for the results in other studies (4, 6, 7, 42, 59, 64) in which "complexes" of bilirubin and lipids have been described. Therefore, it remains to be determined whether conjugated bilirubin, which is weakly lipophilic, forms physiologically important associations with lipid micelles.

The use of electron microscopy for visualization of negatively stained images of micelles is unsatisfactory because dehydration and distortion are inevitable during sample preparation. Characteristic 100 nm cigar-shaped particles, which were identified previously (65), are now considered to be artifacts (66). All estimates of micelle size obtained by electron microscopy grossly exceed those given by other techniques, even with model solutions (49). Although in the present study two populations of particles were seen reproducibly in whole bile as reported by Carey (67), both populations were also found in the same relative proportions in column fractions containing either lipid-free pigment aggregates or mixed lipid micelles, respectively. The identity of the particles seen with TEM, therefore, remains in doubt, as does the validity of this technique for characterizing bile micelles.

Bile contains a large heterogeneous mixture of proteins (68–71), whose origin and mechanism of secretion are currently the subject of much interest. In many of the earliest studies using paper electrophoresis, it has been shown that false positive dye-binding reactions were responsible for the identification of lipoproteins in bile (31, 32). In our study, SDS-PAGE was utilized for the investigation of lipid-protein interactions because it allowed the protein composition of all gel filtration fractions to be displayed simultaneously and compared with the corresponding elution profiles of the lipids and pigments. Each of the polypeptides, which were in the bile that was submitted to gel filtration, were detected in the column fractions; however, no relationship was noted between any of them and the lipids or pigment, when physiological concentrations of bile salts were used. It should be noted that, while serum lipoproteins are not immutable entities, similar structures,

if present in bile, would be expected to survive the non-equilibrium conditions of gel filtration. We therefore conclude that lipoprotein-like complexes do not normally exist in rat bile, at least with the overwhelming majority of biliary proteins, although the presence of complexes involving trace amounts of proteins (beyond the sensitivity of these techniques) cannot be excluded.

The use of SDS-PAGE following Sephadex G200 column chromatography has shown that there are at least two high molecular weight protein fractions in rat bile. Although specific identification of individual proteins was not the main purpose of the present study, the heavier of these fractions (seen in the void volume) can be reasonably explained as secretory IgA, while the second major protein peak ($M_r > 222,000$) has not been described previously and is an aggregate of at least nine bands. We do not yet know if this association has functional significance or whether it is experimentally induced.

The apparent association of lipids and proteins, when bile salt-free columns were used, probably represents protein interaction with liposomes, since the latter are formed by micelle disruption in these conditions (49, 71). This phenomenon may account for the lipid-protein complexes that have been isolated from dialyzed human bile in some studies (15, 16, 18). However, the possibility that lipid-protein associations occur in other species cannot be excluded by our studies with rat bile, especially in human gall bladder bile (72), where the concentration of constituents, cholesterol saturation, and protein composition are so different (73). Studies involving other species are clearly indicated.

In conclusion, our study has given a reliable estimate of size of rat biliary mixed lipid micelles that is consistent with data obtained using model micellar solutions and measurement by more precise thermodynamic techniques. The importance of the intermicellar bile salt concentration in determining micelle size and the elution of organic anion aggregates has also been established in native bile. Thus, this method may prove to be useful for separating the major components of bile from each other, for characterizing micelles, and for isolating them for further analysis. Finally, it appears that the bile pigment, BSP, and most of the proteins in rat bile exist in aggregated form but do not show high affinity interactions with the lipid micelles when isolated on bile salt equilibrated columns. ■

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