Binding of bile acids by glutathione S-transferases from rat liver

Hajime Takikawa, Yuichi Sugiyama, and Neil Kaplowitz[1]

Liver Research Laboratory, Medical and Research Services, Wadsworth Veterans Administration Hospital Center and the University of California, Los Angeles, CA 90073

Abstract Binding of bile acids and their sulfates and glucuronides by purified GSH S-transferases from rat liver was studied by 1-anilino-8-naphthalenesulfonate fluorescence inhibition, flow dialysis, and equilibrium dialysis. In addition, corticosterone and sulfobromophthalein (BSP) binding were studied by equilibrium and flow dialysis. Transferases Y_aY_a and Y_aY_c had comparable affinity for lithocholic ($K_d \approx 0.2$ μM), glycochenodeoxycholic ($K_d \approx 60$ μM), and cholic acid ($K_d \approx 60$ μM), and BSP ($K_d \approx 0.09$ μM). Y_aY_c had one and Y_aY_a had two high affinity binding sites for these ligands. Transferases containing the Y_b subunit had two binding sites for these bile acids, although binding affinity for lithocholic acid ($K_d \approx 4$ μM) was lower than that of transferases with Y_a subunit, and binding affinities for the other bile acids were comparable to the Y_a family. Sulfated bile acids were bound with higher affinity and glucuronidated bile acids with lower affinity by Y_aY_a and Y_aY_c than the respective parent bile acids. In the presence of GSH, binding of lithocholate by Y_aY_c was unchanged and binding by Y_bY_b' was inhibited. Conversely, GSH inhibited the binding of cholic acid by Y_aY_c but had less effect on binding by Y_bY_b'. Cholic acid did not inhibit the binding of lithocholic acid by Y_aY_a. — **Takikawa, H., Y. Sugiyama, and N. Kaplowitz.** Binding of bile acids by glutathione S-transferases from rat liver. *J. Lipid Res.* 1986. **27:** 955–966.

Supplementary key words 1-anilino-8-naphthalenesulfonate displacement • flow dialysis • equilibrium dialysis • corticosterone • sulfobromophthalein (BSP) • bile acid sulfate • bile acid glucuronide • ligandin

The process by which bile acids are translocated intracellularly from sinusoidal to canalicular membrane of the hepatocyte is poorly understood. Bile acid binding by cytosolic proteins has been proposed as having an important role in both intracellular transport of bile acids and protection from potential toxic effects of bile acids. The cytosolic GSH S-transferases (1–6) and newly identified bile acid binders (6, 7) have been considered the major intracellular binders of bile acids. Among the various GSH S-transferases, lithocholic acid has been reported to bind preferentially to transferases having the Y_a subunit (3, 5), and our previous results also showed that lithocholic acid bound to Y_aY_c with higher affinity than to Y_bY_b (6). However, there have been conflicting reports as to the binding of cholic acid by the transferases. Hayes, Strange, and Percy-Robb (4) reported that cholic acid bound to transferases with Y_a or Y_c subunits, while no binding was detected by transferases with Y_b subunits. On the other hand, Pattinson (8) reported that enzymatic activity of only transferases with Y_b subunits was inhibited by cholic acid. Vessey and Zakim (9) reported that the enzymatic activity of both Y_aY_c and transferases with Y_b subunit were inhibited by cholic acid. More recently Hayes and Chalmers (10) reported that bile acids, such as cholic acid, inhibit various Y_b subunit-containing molecular forms of GSH S-transferase. Despite these conflicting reports, no systematic study of the binding of various bile acids by transferases, including binding stoichiometry, has been reported.

Maruyama et al. (11, 12) recently presented evidence that bile acids and steroids are selectively bound by Y_b subunit-containing transferases; they indicated that the true physical and binding properties of these proteins were revealed after extensive dialysis of hepatic cytosol. In contrast to others, they identified GSH S-transferases with Y_b subunits as predominantly anionic enzymes distinct from cationic forms with Y_a and/or Y_c subunits. They proposed that the ordinary cationic character of the Y_b subunit-containing enzymes is an artifact caused by bound endogenous ligand which can be removed by dialysis.

In a subsequent communication, Homma and Listowsky (13) indicated that the anionic forms of Y_b-containing transferases are converted to more basic forms at elevated temperatures (25–30°C) or at pH values above 9.0, presumably due to removal of bound GSH under

---

Abbreviations: ANS, 1-anilino-8-naphthalenesulfonate; BSP, sulfobromophthalein; SDS, sodium dodecyl sulfate; CDNB, 1-chloro-2,4-dinitrobenzene.

[1]Address reprint requests to Dr. Neil Kaplowitz, Liver Research Lab., W151N, Wadsworth VA Medical Center, Los Angeles, CA 90073.

these conditions. Addition of GSH was reported to restore the anionic forms.

In the present study, we report a systematic assessment of the specificity and the stoichiometry of binding of bile acids by GSH S-transferases. We also report the effect of sulfation and glucuronidation of bile acids on binding affinity, the effect of pH on bile acid binding to transferases, and comparison with the binding of sulfobromophthalein (BSP) and corticosterone. Moreover, we have carefully checked the effect of extensive dialysis and the presence of GSH on the behavior of the GSH S-transferases in ion exchange chromatography and chromatofocusing and their binding properties.

METHODS

Purification of GSH S-transferases from rat liver

Livers from male Sprague-Dawley rats weighing ca. 250 g were perfused in situ with 0.01 M sodium phosphate, 0.25 M sucrose buffer, pH 7.4, and removed. Homogenates (33% w/v) were prepared in the same buffer and the supernatant (cytosol) was harvested after centrifugation at 100,000 *g* for 60 min (6). Cytosol was applied to a column of Sephacryl S-200, Superfine (Pharmacia, 5 × 97 cm) and eluted with 0.01 M sodium phosphate, pH 7.4. Pooled fractions containing GSH S-transferases from several columns were concentrated by ultrafiltration using a PM-10 membrane (Amicon), applied to a Sephadex G-75 Superfine column (5 × 96 cm) and eluted with 0.01 M sodium phosphate buffer. Purified Y fraction (GSH S-transferase peak) was dialyzed against 0.01 M Tris-HCl (pH 7.8) for 40 hr and applied to an octyl-GSH affinity gel column (1.5 × 24 cm) which was prepared according to the method of Mannervik and Guthenberg (14). After washing with 0.01 M Tris-HCl, pH 7.8, and subsequently with the same buffer containing 0.2 M NaCl, GSH S-transferases were eluted with the same buffer containing 0.2 M NaCl and 5 mM octyl-GSH. Pooled transferases from affinity chromatography were dialyzed three times against 0.01 M sodium phosphate buffer (pH 7.4) and separated by chromatofocusing according to the method of Mannervik and Jenson (15). Protein was applied to a PBE-118 (Pharmacia Fine Chemicals, Uppsala, Sweden) column which was equilibrated with 25 mM triethylamine-HCl (pH 10.5) and eluted with 1:80 diluted Pharmalyte-HCl (pH 8.0). In some cases, commercially available affinity-purified rat liver GSH S-transferase (Sigma Chemical Co., St. Louis, MO) was directly purified by chromatofocusing. GSH S-transferase activity was measured with 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. An aliquot of each fraction was mixed with 3 ml of 0.1 M potassium phosphate buffer (pH 6.5) containing 0.1 mM CDNB and 2.67 mM GSH, and the reaction was monitored by A_{340} at 37°C. Each peak with CDNB activity was pooled, transferase activity for various substrates was determined, and homogeneity was confirmed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Transferase activities for CDNB, 1,2-dichloro-4-nitrobenzene (DCNB), 1,2-epoxy-3-(*p*-nitrophenoxy)propane, sulfobromophthalein, *trans*-4-phenyl-3-buten-2-one, ethacrynic acid were assayed according to the method of Habig, Pabst, and Jakoby (16). SDS polyacrylamide gel electrophoresis was performed using 12.5% gel according to the method of Laemmli (17). The enzymes were identified by their elution position and dimeric subunit composition and have been designated according to Mannervik and Jenssen (15) as Y_aY_a, Y_aY_c, Y_bY_b, Y_bY_b', $Y_b'Y_b'$. These proteins recently have been given the alternative designations of transferase 1-1, 1-2, 3-3, 3-4, 4-4, respectively (18). We have been unsuccessful in obtaining a pure Y_cY_c transferase protein from this procedure.

In other experiments, affinity-purified transferases were dialyzed on separate occasions against: *1)* 0.01 M sodium-phosphate (pH 7.4) for 22 hr; *2)* the same buffer containing 0.1 mM phenylmethyl sulfonyl chloride for 36 hr; and *3)* 0.01 M Tris-HCl (pH 8.0) for 36 hr as performed by Maruyama et al. (11, 12). Then group separation of transferases was performed by chromatofocusing as described above.

In addition, the same purification steps without the affinity chromatography were performed to exclude an effect of the affinity step on the chromatofocusing pattern.

Group separation of transferases with DEAE Sephacel column chromatography. Y fraction of rat cytosol was dialyzed and separated on a DEAE Sephacel column according to the method of Maruyama, Arias, and Listowsky (11). Y fraction purified by sequential Sephacryl S-200 and Sephadex G-75 superfine column chromatography was dialyzed with 0.01 M sodium phosphate, 0.1 mM phenylmethyl sulfonyl chloride, pH 7.4, and 0.01 M Tris-HCl, pH 8.1. Dialyzed protein was applied to a DEAE Sephacel column (1 × 17.5 cm) which was equilibrated with 0.01 M Tris-HCl (pH 8.1). After elution of the cationic transferases by the same buffer, an anionic transferase was eluted by a linear NaCl gradient in the same buffer (up to 0.1 M) followed by the same buffer containing 1 M NaCl.

In addition, cytosol was freshly prepared at 4°C and dialyzed at 4°C for 16 hr against 0.01 M Tris-HCl, pH 8, containing 1 mM EDTA and 1 mM thioglycerol according to Homma and Listowsky (13), and 1.0 ml was applied to a 1 × 9 cm column of DEAE-Sephacel and eluted with the same buffer at 4°C with or without 1 mM GSH in the buffer. The column was washed successively with 0.3 M and 2 M NaCl. Fractions were assayed for GSH S-transferase activity with CDNB as above.

Chromatography of transferase Y_bY_b' on DEAE-Sephacel as above. Purified transferase Y_bY_b' was applied to DEAE Sephacel (1 × 10 cm) eluted at 4°C with 0.01 M Tris-HCl,

pH 8.0, containing 1 mM EDTA and 1 mM thioglycerol either alone, following incubation with 1 mM GSH at 4°C for 1 hr, or with 1 mM GSH in the eluting buffer. The column was then washed with the same buffer containing 0.3 M NaCl. Fractions were assayed for GSH S-transferase activity using CDNB as above.

Binding of bile acids by 1-anilino-8-naphthalene sulfonate (ANS) fluorescence inhibition technique (6, 19)

Binding of sulfated and glucuronidated bile acids. Sulfated and glucuronidated bile acids were synthesized as previously reported (20, 21). To cuvettes containing 2 ml of 0.01 M sodium phosphate buffer (pH 7.4) and purified transferases (Y_aY_a or Y_aY_c, final concentration 0.6 μM), varying amounts of ANS were added and fluorescence was determined at 480 nm during excitation at 400 nm at room temperature using a Hitachi MPF-4 spectrofluorometer. The same titrations were performed in the absence and presence of 3 μM lithocholic acid, 3 μM lithocholic acid-3-sulfate, and 15 μM lithocholic acid-3-glucuronide. Results were expressed as Scatchard plots (22). Binding of bile acids and their sulfates and glucuronides to Y_aY_a and Y_aY_c was determined by varying the bile acid concentrations at constant ANS concentration (15 μM). K_i values were calculated by nonlinear least squares method using Michaelis-Menten equation as described in our previous report (19).

Briefly, the inhibition curve was simulated by the following equation using various K_i values so as to find the K_i value that best fits the data:

$$I_t = \left\{ P - C_b - \frac{C_b \cdot K_c}{(C_t - C_b)} \right\} \cdot \left\{ 1 + \frac{K_i - (C_t - C_b)}{C_b \cdot K_c} \right\}$$

where I_t and C_t are the total concentration of the inhibitor and ANS, C_b is the bound concentration of ANS, P is the protein concentration, and K_c is the dissociation constant of ANS (19).

Effect of pH on binding of ANS and bile acids by GSH S-transferases. ANS binding to five forms of transferases (Y_aY_a, Y_aY_c, Y_cY_c, Y_bY_b, Y_bY_b'), was determined as mentioned above. Y_cY_c was kindly supplied by Dr. T. D. Boyer, UCSF. The effect of pH changes on ANS binding was determined using 0.01 M sodium phosphate buffer, pH 6.0, 7.0, and 8.0. For binding of lithocholic, chenodeoxycholic, cholic, and taurocholic acids, increasing concentrations of these bile acids were added to the cuvette containing transferases (0.17–0.6 μM) and ANS (15 μM) at pH 6.0, 7.0, and 8.0, and K_i values were calculated (19).

Binding of bile acids by flow dialysis. Binding of lithocholic, glycochenodeoxycholic, and cholic acids was examined by flow dialysis at room temperature (23, 24). A Spectrapor 2 membrane (Spectrom Medical Industries, Los Angeles, CA) was placed between the upper and lower chambers. As a control study, 0.35–0.5 ml of 0.01 M phosphate buffer (pH 7.4) containing labeled bile acid, i.e., [^{14}C]lithocholic acid (59 mCi/mmol), [^{3}H]glycochenodeoxycholic acid (2.3 Ci/mmol), [^{3}H]cholic acid (25 Ci/mmol) (New England Nuclear) was placed in the upper chamber. Binding of these labels to the chamber or membrane was negligible in the presence of protein (>99% recovery). In the absence of protein, only lithocholic acid showed binding to the chamber (42%). The lower chamber was eluted with the same buffer (flow rate, 30 ml/hr) and the outflow was collected every 2 min and radioactivity of each fraction was counted. Equilibrium of diffusion of bile acids was achieved within 6 min. Next, the same volume of buffer containing purified transferase and labeled bile acid was placed in the upper chamber. Protein concentrations used were 7.7–14.8 μM for lithocholic acid binding, 14.3–66 μM for glycochenodeoxycholic acid binding, and 48–182 μM for cholic acid binding. The lower chamber was perfused with the same buffer and flow rate as above and the outflow was collected every 2 min. Unlabeled bile acids were added stepwise to the upper chamber every 8 min and the radioactivity of each fraction was counted.

Fig. 1 shows an example of flow dialysis. Free fraction (f) at each bile concentration was calculated as follows:

$$f = \frac{\text{transfer ratio (protein)}}{\text{transfer ratio (buffer)}}$$

where transfer ratio is the radioactivity filtered per 2 min divided by the radioactivity in upper chamber. The upper chamber contained either binding protein or buffer as control. The transfer ratio in buffer controls did not change from the beginning to the end of each experiment as shown in Fig. 1.

The free and bound concentrations of bile acids were calculated as follows:

$$C_f = C_t \cdot f$$
$$C_b = C_t - C_f$$

where, C_t, C_b, C_f are total, bound, and free concentrations of bile acid, respectively. Results were expressed as Scatchard plots, and number of binding sites (n) and K_d values were calculated from the untransformed data by nonlinear least squares using the following equation:

$$C_b = \frac{n_1(P) \cdot C_f}{K_{d1} + C_f} \left(+ \frac{n_2(P) \cdot C_f}{K_{d2} + C_f} \right)$$

where P is protein concentration.

The equation in parentheses was only used for the binding of lithocholic acid to Y_aY_c, in which high and low affinity binding sites were revealed by the Scatchard plot.

Binding of lithocholic acid by Y_aY_a and the effect of cholic acid studied by equilibrium dialysis. Binding of lithocholic acid to Y_aY_a was also measured by equilibrium dialysis at 4°C.

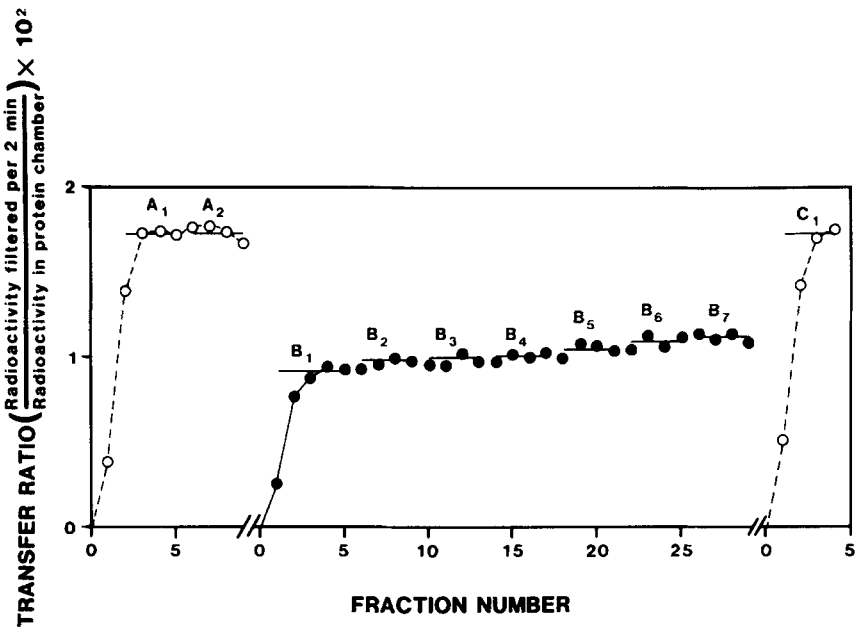

Fig. 1. Transfer ratios of [^{3}H]glycochenodeoxycholic acid (GCDCA) from the upper to lower chamber by flow dialysis. Flow dialysis was performed with 0.01 M phosphate buffer, pH 7.4 (A and C) or with 14.3 μM of Y_aY_a (B) in the upper chamber. In the experiment with protein, varying concentrations of GCDCA were titrated into the protein chamber. Flow rate for the lower chamber was 30 ml/hr and every 2 ml was collected and the radioactivity was counted. A_1, B_1, and C_1 were with 0.2 μCi of ^{3}H-labeled GCDCA (0.2 μM). GCDCA was added to the protein compartment to achieve the following concentrations: 40 μM for A_2, 2 μM for B_2 and B_3, 4 μM for B_4 and B_5, 10 μM for B_6 and B_7. Transfer ratio of 1% corresponds to 2,400 cpm of transferred radioactivity.

Using cells of 2 × 1.1 cm, a tracer amount of [^{14}C]lithocholic acid (0.014 μCi) with various concentrations of unlabeled lithocholic acid (0.2 to 4 μM) was added to the protein compartment (Y_aY_a concentration, 1.0 μM). After equilibrium was reached (48 hr), radioactivity on both sides of the Spectrapor 2 membrane was determined. Lithocholic acid showed negligible binding to the cell and membrane (>95% recovery). The result was expressed as a Scatchard plot. When the effect of cholic acid on the binding of lithocholic acid to Y_aY_a was examined, cholic acid was initially added to the protein compartment to achieve a concentration of 50 μM. The effect of cholic acid was also checked by adding cholic acid to achieve a concentration of 50–500 μM in the protein compartment containing 0.22 μM [^{14}C]lithocholic acid.

Effect of GSH on binding of lithocholic acid and cholic acid by Y_aY_c and Y_bY_b'. Equilibrium dialysis at 4°C was performed as above with Y_aY_c (1.0 μM) or Y_bY_b' (11 μM) in the presence or absence of GSH (0.5 mM) with labeled and varying unlabeled lithocholic acids. Flow dialysis was performed as above with Y_aY_c (81 μM) or Y_bY_b (60 μM) in the presence or absence of GSH (0.5 mM) with labeled and varying unlabeled cholic acids. In these experiments flow dialysis was performed at 4°C to avoid possible effects of room temperature on the proteins.

Binding of sulfobromophthalein (BSP) by Y_aY_a and Y_aY_c studied by equilibrium dialysis. Various amounts of BSP (0.2–10 μM) were added to 1 μM Y_aY_a and Y_aY_c. After equilibrium was reached (48 hr) at 4°C, BSP concentrations on both sides of the membrane were measured by an Aminco DW-2 double beam spectrophotometer (A_{650}–A_{580}) after alkalinization with 10 N NaOH.

Binding of corticosterone by transferases. Binding of [^{3}H]corticosterone (0.1 μCi, 465 Ci/mmol, New England Nuclear) to transferases Y_aY_a, Y_aY_c, Y_bY_b, Y_bY_b' and the acidic transferase was examined both by flow dialysis and equilibrium dialysis (final protein concentrations 7–15 μM). K_d was approximated using a very low concentration of corticosterone (7 nM) ($K_d >> C_f$) were C_f is the unbound corticosterone concentration; then

$$C_b = \frac{n(P) \cdot C_f}{K_d + C_f} \text{ simplifies to } C_b = \frac{n(P) \cdot C_f}{K_d}$$

Since C_b (bound concentration), C_f, P (protein concentration), and n (one binding site assumed) were known, K_d could be estimated. Recovery of corticosterone was greater than 96% in the absence of protein.

Effect of lithocholic and cholic acid on enzyme activity of Y_aY_a transferase

To examine the effect of bile acids on transferase activity, CDNB activity of Y_aY_a in the absence and presence of bile acids was assayed at various concentrations of CDNB and GSH. CDNB activities were examined at a constant concentration of CDNB (0.1 mM) while GSH

concentrations were varied (0.04–2 mM) or at a constant concentration of GSH (6.67 mM) while CDNB concentrations were varied (0.02–1 mM). Each experiment was performed in the absence and presence of 0.2 μM lithocholic acid or 100 μM cholic acid.

Protein concentrations

To define the protein concentration of various transferases used for binding studies, amino acid composition was measured on aliquots in duplicate as previously described (6).

RESULTS

Purification of GSH S-transferases

GSH S-transferases were purified from rat liver cytosol or from commercially available affinity-purified rat liver enzyme. Group separation of transferases was performed by chromatofocusing. Five peaks of transferase activity correspond to Y_aY_a, Y_aY_c, Y_bY_b, Y_bY_b', $Y_b'Y_b'$, as reported by Mannervik and Jensson (15). An anionic transferase, eluted with 0.025 M sodium acetate buffer, pH 6, containing 1 M NaCl, accounted for less than 5% of the total enzyme activity. The chromatofocusing pattern of the affinity-purified commercial material was nearly the same as the proteins starting from liver cytosol with the exception that it lacked a $Y_b'Y_b'$ peak.

No effect of dialysis of the protein using two different buffers to remove endogenous ligands according to the method of Maruyama et al. (11, 12) could be recognized in the elution pattern from chromatofocusing. The same chromatofocusing pattern was observed when the purification was performed without the octyl-GSH affinity step by directly applying cytosol or Y fraction after dialysis. In DEAE Sephacel chromatography of the Y fraction (**Fig. 2**), the bulk of transferase activity eluted in the void volume and only about 10% of the total activity, which is assumed to be anionic transferase, bound to the column and eluted with the NaCl gradient.

When cytosol was chromatographed on DEAE-Sephacel using the conditions of Homma and Listowsky (13), even with preincubation with GSH (1 mM) and GSH in the buffer, most (>90%) of the enzyme activity (>95% recovery) was eluted in the void volume (not shown). In addition, under these conditions, chromatography of Y_bY_b' on DEAE-Sephacel with or without GSH (1 mM) preincubation or GSH (1 mM) in the eluting buffer revealed >95% recovery of the added enzyme activity in the void volume and no detectable enzyme activity eluted when the column was washed with increasing salt concentration (not shown).

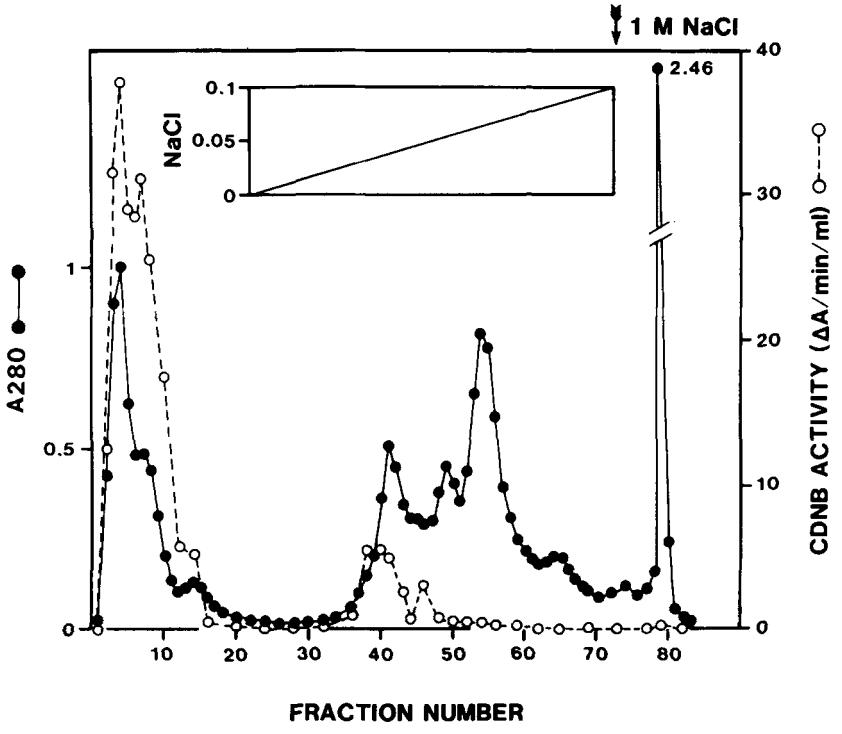

Fig. 2. Chromatography of dialyzed rat Y fraction on a DEAE-Sephacel: 6 ml of sample (25 mg of protein) was applied after extensive dialysis of the Y fraction (GSH S-transferase peak) obtained from Sephadex G-75 superfine chromatography of cytosol. The column (1 × 17.5 cm) was eluted with 50 ml of 0.01 M Tris–HCl, pH 8.1, followed by 125 ml of the same buffer with NaCl gradient (0–0.1 M) and a final wash with 1 M NaCl in the same buffer. Flow rate was 10 ml/hr and 2.5-ml fractions were collected. A_{280} (●) and CDNB activity (○) of each fraction were measured.

Binding of the sulfated and glucuronidated bile acids by Y_aY_a and Y_aY_c

Binding of the sulfated and glucuronidated bile acids to transferases was examined by the ANS fluorescence inhibition technique. Lithocholic acid and its sulfate and glucuronide competitively inhibited the ANS fluorescence due to the binding of ANS to Y_aY_b and Y_aY_c (not shown). Cholic acid also competitively displaced the binding of ANS by Y_aY_a (not shown). Various bile acids and their sulfates and glucuronides decreased the bound ANS fluorescence (**Fig. 3**). Dissociation constants (K_d) obtained from Fig. 3 are listed in **Table 1.** K_d values for binding of various bile acids to Y_aY_a and Y_aY_c were almost the same. In general. sulfated bile acids were bound with higher affinity by Y_aY_a and Y_aY_c than their parent bile acids, and glucuronidated bile acids were bound with lower affinity.

Effect of pH on the binding of ANS and bile acids by transferases

ANS binding by Y_aY_c and Y_bY_b' at different pH is shown in **Fig. 4** and the summary of F_{max} and K_d values for ANS binding by five transferases is listed in **Table 2.** In the case of Y_aY_a and Y_aY_c, K_d values differed at various pH conditions while F_{max} values remained almost constant. On the other hand, K_d values for ANS binding to Y_bY_b and Y_bY_b' remained almost constant at different pH while F_{max} values differed at various pHs.

K_d values for binding of various bile acids to Y_aY_a and Y_aY_c at pH 6–8 determined by ANS displacement method are listed in **Table 3.** Lithocholic acid (1 μM) competitively inhibited ANS binding to Y_aY_c at each pH (not shown). pH had little effect on lithocholate binding but did affect the other bile acids (higher affinity at pH 7 or 8). In the case of Y_cY_c, Y_bY_b, and Y_bY_b', ANS fluores-

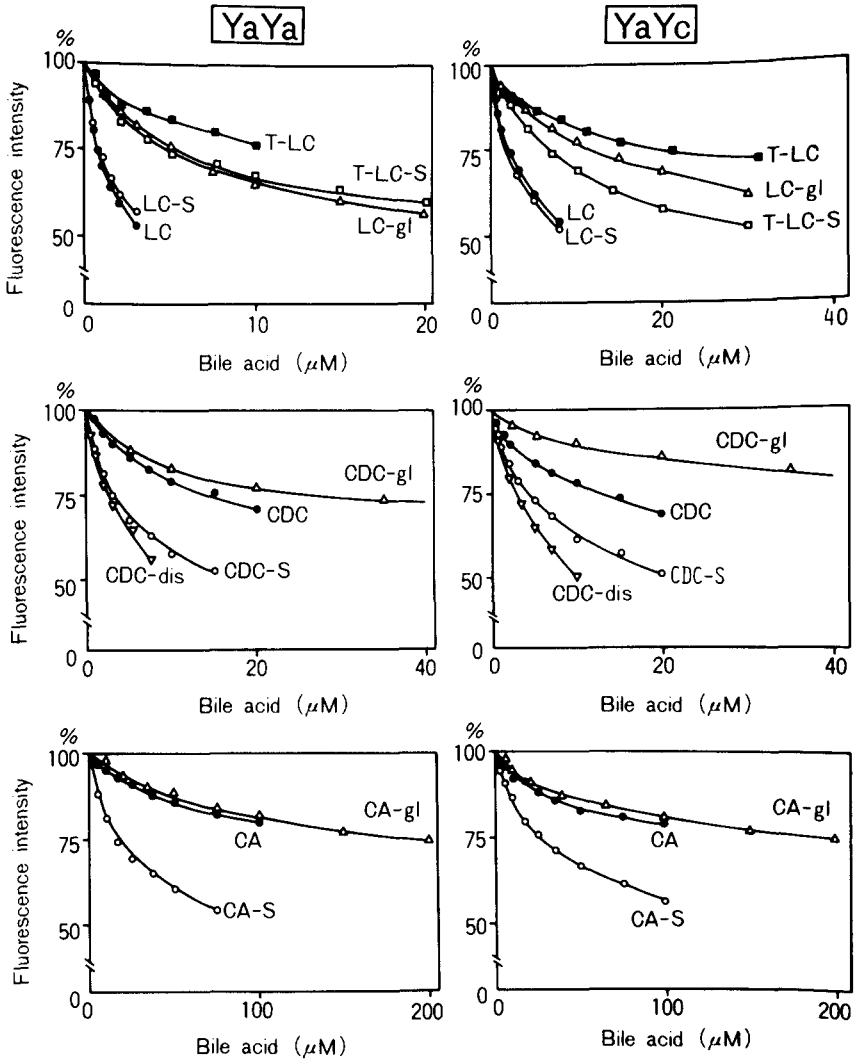

Fig. 3. Inhibition of the fluorescence of ANS (15 μM) bound to Y_aY_a and Y_aY_c (0.6 μM) by various bile acids. The fluorescence was determined at 480 nm during excitation at 400 nm in 0.01 M phosphate buffer, pH 7.4, at room temperature. Abbreviations: LC, lithocholic acid; CDC, chenodeoxycholic acid; CA, cholic acid. T-, tauro-conjugate; -S, -sulfate; -gl, -glucuronide.

TABLE 1. Dissociation constants (μM) for binding of various bile acids and their conjugates by Y_aY_a and Y_aY_c examined by ANS fluorescence inhibition technique

Bile Acid	Y_aY_a	Y_aY_c
Lithocholic acid	0.36	0.48
Lithocholic acid-3-sulfate	0.47	0.42
Lithocholic acid-3-glucuronide	3.6	3.5
Taurolithocholic acid	6.3	5.9
Taurolithocholic acid-3-sulfate	4.1	1.8
Chenodeoxycholic acid	8.9	7.3
Chenodeoxycholic acid-3-sulfate	1.9	2.2
Chenodeoxycholic acid-3,7-disulfate	1.3	1.1
Chenodeoxycholic acid-3-glucuronide	33	49
Cholic acid	77	60
Cholic acid-3-sulfate	12	16
Cholic acid-3-glucuronide	108	97

Dissociation constants were calculated from the displacement of ANS by increasing concentrations of bile acid (see Fig. 5) using nonlinear least squares method as described in Methods. Protein concentration was 0.6 μM and ANS was 15 μM.

cence was increased by addition of bile acids at each pH as we have previously described (6).

Binding of bile acids by transferases determined by flow dialysis

Results of bile acid binding to transferases by flow dialysis are depicted as Scatchard plots (**Fig. 5**). Transferases Y_aY_a, Y_aY_c, Y_bY_b, and Y_bY_b' were examined using representative bile acids: lithocholic (Fig. 5a), glycochenodeoxycholic (Fig. 5b), and cholic acids (not shown). Binding parameters are summarized in **Table 4.** Y_aY_c exhibited one binding site for each bile acid examined. A lower affinity secondary binding site was seen only with lithocholic acid. The other three transferases exhibited two binding sites for each bile acid. The binding affinity for lithocholic acid by Y_aY_a, and Y_aY_c was more than an order of magnitude greater than that of Y_bY_b and Y_bY_b', while binding affinities for glycochenodeoxycholic and cholic acid were nearly the same for all four transferases.

Effect of GSH on binding of bile acids

Using equilibrium dialysis, lithocholic acid binding by Y_aY_c was unaffected by GSH (K_d 0.13 μM without and 0.11 μM with GSH). However, lithocholic acid binding to Y_bY_b' was affected by GSH (K_d 2.6 μM without and 9.3 μM with GSH). Using flow dialysis, GSH was found to have a large influence on the binding of cholic acid by Y_aY_c (K_d 61 μM without and >200 μM with GSH) but a much smaller effect on the binding of cholic acid by Y_bY_b' (K_d 18 μM without and 26 μM with GSH).

Binding of lithocholic acid by Y_aY_a and the effect of cholic acid

To examine whether cholic acid competitively inhibits the binding of lithocholic acid to Y_aY_a, equilibrium dialysis was performed with and without 50 μM cholic acid. Cholic acid (50 μM) showed no effect on the binding of lithocholic acid by Y_aY_a (**Fig. 6**). The effect of cholic acid on lithocholic acid binding was also examined at different concentrations of cholic acid (**Fig. 7**). There was no significant decrease of lithocholic acid binding by cholic acid.

Binding of BSP to Y_aY_a and Y_aY_c

BSP binding to Y_aY_a and Y_aY_c by equilibrium dialysis showed that both the transferases have the same affinity for BSP. However Y_aY_a had two and Y_aY_c had one binding site (**Fig. 8**).

Binding of corticosterone by the transferases

Binding of corticosterone to various transferases was examined by flow dialysis and equilibrium dialysis using a very low concentration of corticosterone (7 nM) so as to detect high affinity binding. With all the transferases studied, including the acidic transferase, K_d values were 100–200 μM. Although these values are only approximations based on one concentration of corticosterone, it was clear that no high affinity binding could be detected. No selectivity for corticosterone was exhibited by Y_aY_a or Y_aY_c versus Y_bY_b or Y_bY_b'.

Inhibition of enzyme activity of Y_aY_a by lithocholic and cholic acid

As described in Methods, the effect of bile acid on transferase Y_aY_a activity was examined by varying either GSH or CDNB in the presence or absence of bile acids. Kinetics of inhibition with respect to either CDNB or GSH was of either the mixed or noncompetitive type; the changes of V_{max} for CDNB and GSH as a result of the addition of either lithocholic or cholic acid were much greater than the changes of K_m (K_m for CDNB was 0.64, 0.88, and 0.63 mM and V_{max} was 1.8, 0.75, and 1.5 ΔA/min with no bile acid, lithocholic, or cholic acid, respectively, and K_m for GSH was 0.37, 0.35, and 0.34 mM and V_{max}

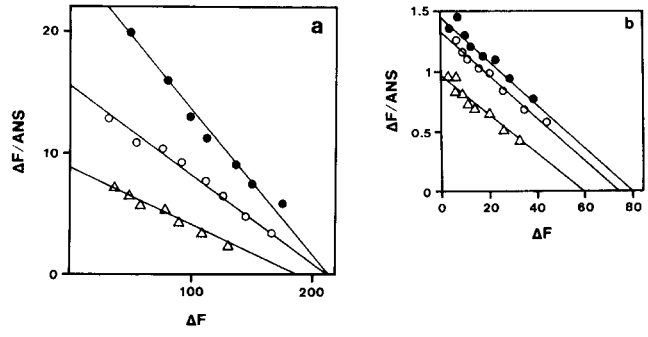

Fig. 4. Scatchard plot of ANS binding to Y_aY_a (a) and Y_bY_b' (b) at pH 6 (○), pH 7 (●), and pH 8 (Δ). ANS concentrations were varied 5 to 50 μM while protein concentration (0.6 μM) was fixed.

TABLE 2. Binding of ANS by GSH S-transferases at different pH

Transferase	pH 6		pH 7		pH 8	
	F_{max}	K_d	F_{max}	K_d	F_{max}	K_d
	U/μM	*μM*	*U/μM*	*μM*	*U/μM*	*μM*
Y_aY_a	260	9.6	220	15	200	21
Y_aY_c	130	9.8	120	14	100	21
Y_cY_c	120	27	100	34	93	40
Y_bY_b	30	24	26	36	20	26
$Y_b'Y_b'$	45	56	43	61	34	66

Increasing concentrations of ANS were added to cuvettes containing protein (0.17–0.6 μM) in 0.01 M sodium phosphate buffer at room temperature (see Fig. 6). F_{max} and K_d values were calculated by nonlinear least squares method as described in Methods.

was 0.22, 0.07, and 0.17 ΔA/min with no bile acid, lithocholic, or cholic acid, respectively).

DISCUSSION

The objective of our studies was to characterize the binding affinities and stoichiometries of various rat GSH S-transferases for bile acids. Two major classes of rat transferases have been described: Y_a and/or Y_c versus Y_b and/or Y_b' subunit compositions. Since some controversy exists about the physical properties of these two classes as well as their bile acid binding properties, at first we carefully examined the behavior of these proteins during several different purification procedures. Recently, Maruyama and Listowsky (12) reported that transferases with Y_b subunits have an acidic pI after dialysis to remove putative endogenous ligands, which they believe caused the apparent pI values to change artifactually into the cationic range. We have found that, with the same extensive dialysis of the cytosol, Y fraction from gel filtration of cytosol, or the pooled GSH S-transferases from affinity chromatography, the transferases with Y_b subunits consistently eluted as cationic proteins (apparent pI 9.2–8.7) on chromatofocusing. The actual pI values, however, will be somewhat lower since chromatofocusing yields more basic values. The anionic transferase eluted by 0.025 M acetate buffer (pH 6.0) containing 1 M NaCl, which was also shown to be a dimer of Y_b subunit by SDS gel, had only about 5% of total enzyme activity. We also used DEAE-Sephacel chromatography of extensively dialyzed Y fraction according to the conditions of Maruyama et al. (11, 12), but 90% of the transferase was eluted in the void volume, and therefore only a small proportion of the transferase activity behaved as acidic proteins.

We considered it important to define the molecular forms of these enzymes which we used to study the binding of bile acids to avoid potential discrepancies due to the effects noted by Maruyama et al. (11, 12) and Homma and Listowsky (13). Homma and Listowsky (13) have found that the acidic transferases containing Y_b subunits are transformed into cationic forms under high pH or temperature but revert to acidic forms in the presence of GSH. However, we found no effect of GSH on the distribution of acidic or cationic forms and GSH did not cause the cationic Y_bY_b' preparation to revert to an acidic form. Since we could not demonstrate the effects they have described, it remains uncertain whether undefined experimental conditions account for the differences and whether the acidic forms described by these investigators have different binding properties for bile acids.

Transferases Y_aY_a and Y_aY_c had almost the same binding affinities for various bile acid conjugates. K_d values for lithocholic, taurolithocholic, chenodeoxycholic, and cholic acid binding by Y_aY_c, obtained using the ANS technique, were in good agreement with our previous report (6). K_d values for lithocholic acid and cholic acid binding by Y_aY_a and Y_aY_c using the ANS technique were also in good agreement with the results we obtained by flow dialysis (Table 4). Both Y_aY_a and Y_aY_c had higher affinity for sulfated bile acids than corresponding parent bile acid and lower affinity for glucuronidated bile acids than parent bile acid.

The binding affinity of Y_aY_a and Y_aY_c for ANS was highest at pH 6 and the lowest at pH 8, while no significant change of the affinity of Y_cY_c, Y_bY_b, and Y_bY_b' for ANS was observed at various pH conditions (Table 2). The binding affinity for lithocholic acid by Y_aY_a and Y_aY_c was highest at pH 6 and lowest at pH 8 while the affinities for chenodeoxycholic, cholic, and taurocholic acids by Y_aY_a and Y_aY_c was lowest at pH 6 and highest at pH 8. These results differ from those of Boyer et al. (25); they studied inhibition of the enzyme activity of Y_aY_a, Y_aY_c, and Y_cY_c by chenodeoxycholic acid and found that inhibition was greater at pH 6 than at pH 8 for Y_aY_c and Y_cY_c, and that Y_aY_a was inhibited equally at both pH 6 and 8. In contrast we determined binding by the competitive displacement of ANS and found

TABLE 3. Dissociation constants (μM) for binding of bile acids by Y_aY_a and Y_aY_c at different pH

Bile Acids	Y_aY_a			Y_aY_c		
	pH 6	pH 7	pH 8	pH 6	pH 7	pH 8
Lithocholic acid	0.11	0.32	0.42	0.11	0.33	0.27
Chenodeoxycholic acid	15	6.2	5.1	16	6.4	4.3
Cholic acid	170	83	52	120	60	32
Taurocholic acid	190	150	79	180	86	47

Increasing amounts of bile acid were added to the mixture of 15 μM ANS and 0.17–0.6 μM protein in 0.01 M sodium phosphate buffer, pH 7.4 at room temperature. K_i values were obtained from the displacement curve of ANS fluorescence (see Fig. 7) by nonlinear least squares method as described in Methods.

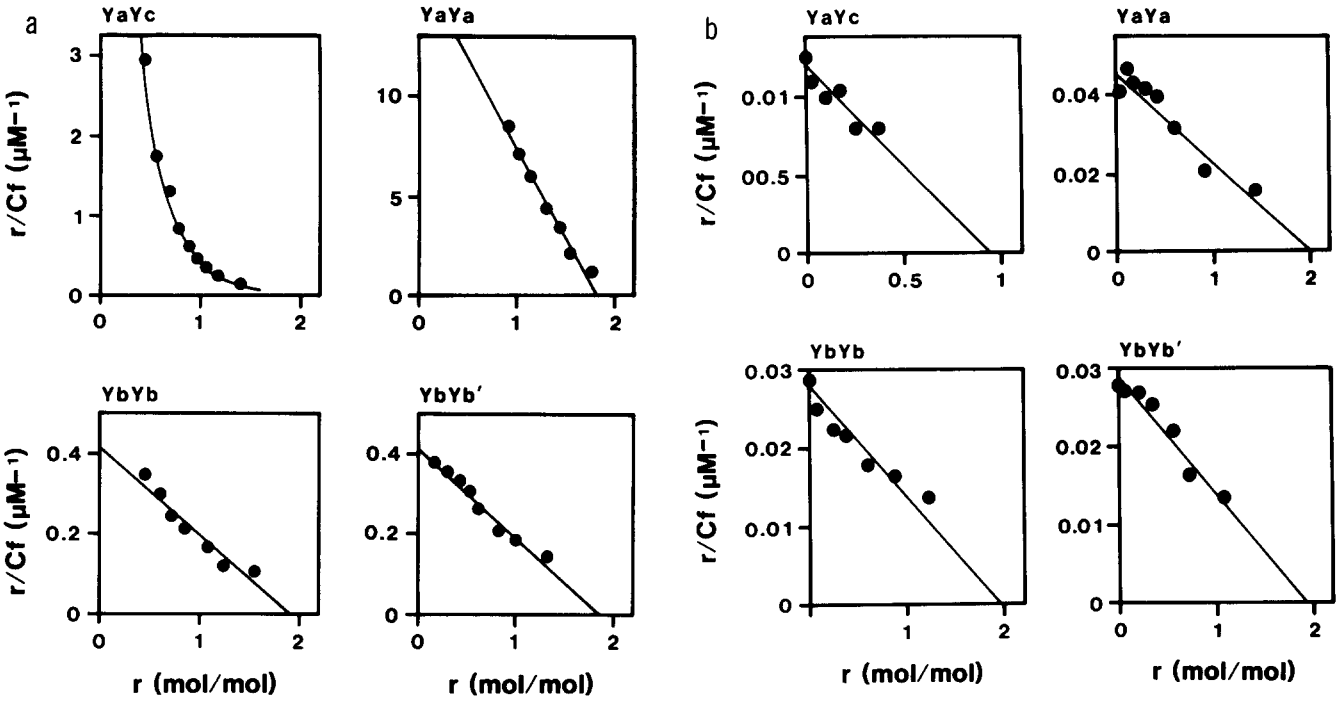

Fig. 5. Scatchard plots of the binding of lithocholic (a) and glycochenodeoxycholic (b) to various GSH S-transferases determined by flow dialysis. Flow rate was 30 ml/hr and every 2 ml was collected. Protein concentrations were 7.7–14.8 μM for lithocholic acid binding and 14.3–66 μM for glycochenodeoxycholic acid binding in 0.01 M sodium phosphate buffer, pH 7.4. r Represents the bound concentration of bile acid per μmol of protein and C_f is the free bile acid concentration.

greater bile acid binding affinity at higher pH. This apparent discrepancy may be due to the multiplicity of sites on these proteins and the possibility that the enzymatic substrate and nonsubstrate ligand binding sites are distinct.

The affinity of Y_aY_a and Y_aY_c for lithocholic acid was more than an order of magnitude greater than that of Y_bY_b and Y_bY_b'. This result agrees with the report of Hayes et al. (5) that only transferases having Y_a subunit bind lithocholic acid with high affinity. Two high affinity binding sites for lithocholic acid on Y_aY_a were observed whereas Y_aY_c had only one high affinity binding site comparable to Y_aY_a. In addition, Y_aY_c exhibited a low affinity binding site for lithocholic acid with an affinity two orders of magnitude lower than that of the high affinity site. The stoichiometry of the binding of BSP by the Y_a group was similar to that of lithocholic acid, i.e., Y_aY_a had two and Y_aY_c had one high affinity binding site and the K_d value for this high affinity site on both proteins was the same. We have previously shown that bilirubin and lithocholic acid compete for the same binding site on Y_aY_c (19). Since Kamisaka et al. (26) have shown that BSP competes with high affinity binding of bilirubin by Y_aY_c, it is reasonable to assume that BSP shares the same site as bilirubin and lithocholic acid.

Our findings with respect to binding stoichiometry of lithocholic acid and BSP determined by flow and equilibrium dialysis, indicate two high affinity sites on Y_aY_a and one high affinity site on Y_aY_c. These results are in agreement with the stoichiometries for bilirubin binding by these two proteins reported by Bhargava et al. (27). However, the same laboratory has more recently reported only one binding site for both Y_aY_a and Y_aY_c (11). In both cases they used the same technique, circular dichroism. Boyer et al. (28), using a fluorescence quench-

TABLE 4. Binding of bile acids by GSH S-transferases examined by flow dialysis

	Lithocholic Acid		Glycochenodeoxycholic Acid		Cholic Acid	
Protein	n	K_d	n	K_d	n	K_d
Y_aY_a	1.98[a] (1.72	0.15[a] 0.13)[b]	2.14[a]	43[a]	1.77	46
Y_aY_c	n_1 0.78 n_2 1.10	K_{d1} 0.17 K_{d2} 11	0.94	79	1.04	79
Y_bY_b	1.91	4.6	1.98	71	1.85[a]	27[a]
Y_bY_b'	1.83[a]	3.9[a]	1.94[a]	66[a]	1.71[a]	24[a]

Flow dialysis was performed with flow rate of 30 ml/hr at room temperature and the outflow was collected every 2 min. The unbound fraction was calculated by the transfer rate of labeled bile acid in the presence and absence of protein. K_d and n were obtained by fitting the data to the Michaelis-Menton equation using a nonlinear least squares method (see Methods for experimental details); K_d, dissociation constant (μM); n, number of binding sites.

[a] Mean values of two different experiments.

[b] Result from equilibrium dialysis.

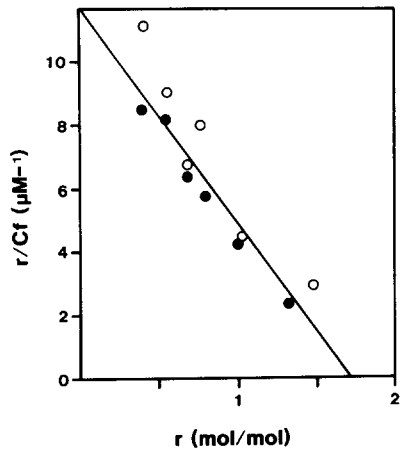

Fig. 6. Scatchard plot of the binding of lithocholic acid by Y_aY_a (1 μM) determined by equilibrium dialysis in the absence (●) and presence (○) of 50 μM cholic acid. The initial concentration of lithocholic acid was varied from 0.5 μM to 4.5 μM. Dialysis was performed at 4°C for 48 hr. Calculated binding parameters are n = 1.63, K_d = 0.14 μM without cholic acid, and n = 1.71, k_d = 0.13 μM with cholic acid.

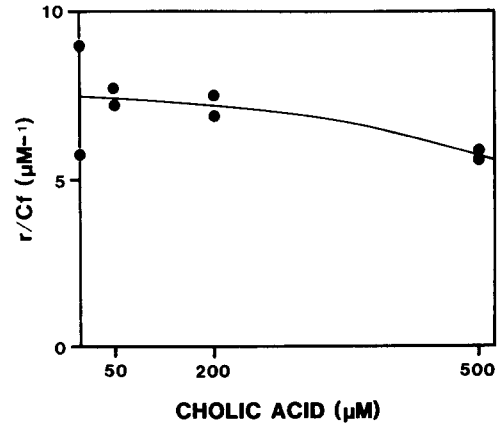

Fig. 7. Effect of cholic acid on the binding of lithocholic acid by Y_aY_a. Binding of lithocholic acid was measured by equilibrium dialysis at 4°C for 48 hr. The concentrations of Y_aY_a (1 μM) and [^{14}C]lithocholic acid (0.22 μM) were held constant and various concentrations of cholic acid were initally added to the protein compartment. The final equilibrium cholic acid concentration is given on the x-axis.

ing technique, reported one binding site for bilirubin on Y_aY_a. The reasons for discrepancies with respect to binding stoichiometries of Y_aY_a are not certain but most likely reflect the different techniques employed. Our approach has been to determine directly the bound and free concentration, which we believe to be the least ambiguous approach. The indirect techniques used by other groups rely on physical changes that may be obscured by conformational changes in the protein due to binding of ligandin to one Y_a subunit masking changes reflecting binding to the second Y_a subunit.

In contrast to the marked differences in the binding affinity for lithocholic acid by Y_a versus Y_b subunits, the binding affinities for glycochenodeoxycholic and cholic acid by the Y_a group (Y_aY_a and Y_aY_c) and the Y_b group (Y_bY_b and Y_bY_b') were nearly the same and all the proteins except Y_aY_c exhibited two binding sites for these bile acids.

The effect of GSH on bile acid binding by the transferases is of some interest. Maruyama et al. (11, 12) have demonstrated that GSH inhibits binding of Y_aY_c in cholic acid-affinity chromatography but not that of Y_b-subunit proteins. Our results with cholic acid binding in the presence or absence of GSH are consistent with their findings. GSH had less effect on the binding affinity of Y_bY_b' for cholic acid compared to Y_aY_c. Thus, although we have not shown an effect of GSH on the charge properties of the Y_b subunit-containing proteins, we have confirmed in a direct fashion that GSH influences the binding of cholic acid. However, this is not necessarily a protein-dependent selective effect of GSH. Thus, in contrast to cholic acid, lithocholate binding by Y_bY_b' was inhibited by the presence of GSH whereas binding by Y_aY_c was not. Clearly the effect of GSH on cholate binding explains the selective purification of Y_b proteins in cholic acid affinity chromatography. However, the physiologic significance and mechanism of the effect of GSH on bile acid binding by the transferases remains to be more fully defined.

Cholic acid at a concentration near its K_d value (50 μM) did not inhibit the binding of lithocholic acid to Y_aY_a (Fig. 6). Cholic acid (50–500 μM) also did not significantly affect the binding of lithocholic acid (0.22 μM, near K_d value) by Y_aY_a. These results suggest that the binding sites for lithocholic and cholic acid on the Y_a subunit are distinct. The lack of effect of GSH on litho-

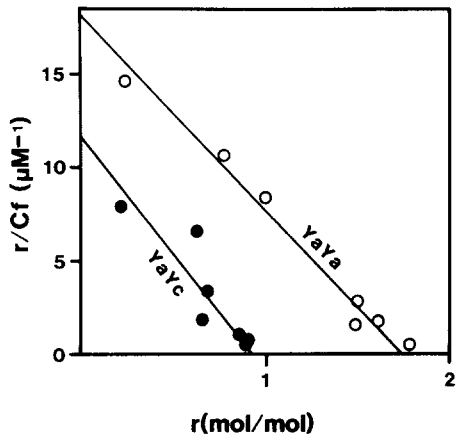

Fig. 8. Scatchard plot of the binding of BSP by Y_aY_a and Y_aY_c. Binding of BSP was determined by equilibrium dialysis at 4°C for 48 hr. Protein concentration was 1 μM and initial BSP concentration varied from 0.2 μM to 10 μM. BSP concentrations on both sides of the chamber were measured spectrophotometrically (A_{650}–A_{580}) after alkalinization. Binding parameters for BSP were n = 1.73, K_d = 0.095 μM for Y_aY_a and n = 0.92, K_d = 0.079 μM for Y_aY_c.

cholate binding by Y_aY_c in contrast to its inhibition of cholate binding by Y_aY_c is consistent with this view. Furthermore, considering that the kinetics of inhibition of transferases by these bile acids with respect to CDNB or GSH were not purely competitive and that the inhibition of ANS fluorescence bound to Y_aY_a by CDNB or GSH was not competitive, distinct binding sites may exist on Y_aY_a with spceificity for lithocholic acid (or bilirubin), cholic acid, CDNB, and GSH.

Maruyama and Listowsky (12) reported that corticosterone is a good inhibitor of the binding of bilirubin to Y_bY_b as demonstrated by circular dichroism. Although they did not show the K_d value for bilirubin or the K_i value for corticosterone, we can assume that K_d for corticosterone is about 2 μM, based on the I_{50} for inhibition of the binding of bilirubin by corticosterone at about a 2:1 molar ratio of corticosterone to bilirubin from their data and the K_d for the binding of bilirubin by Y_bY_b of about 1 μM from our previous report (29). However, our results (K_d values) for corticosterone binding by various transferases, obtained by both flow dialysis and equilibrium dialysis, were more than 100 μM for all the cationic and anionic forms of the transferases. Thus, we have not confirmed the proposed selective binding of bile acids and steroids by the Y_b group. However, since we could not reproduce their purification procedure and used different procedures including chromatofocusing, it remains uncertain whether the difference in purification methods could account for the different results.

In summary, we have found that, except for the case of lithocholic acid, all bile acids are bound equally by both the Y_a and Y_b groups in the absence of GSH. Y_aY_a, Y_bY_b, Y_bY_b' exhibit two comparable binding sites for di- and trihydroxy bile acids. Y_aY_c exhibits a single site for these bile acids. Lithocholic acid seems to bind to a unique high affinity site on the Y_a subunit shared by bilirubin and probably BSP. GSH affects bile acid binding in a complex fashion. Inhibition by GSH was greater for cholic acid binding by transferase with the Y_a compared to the Y_b-subunit, whereas inhibition by GSH of lithocholate binding by transferase with the Y_b subunit occurred without inhibition of binding to the Y_a subunit.

This work was supported by VA Medical Research Funds and NIH Grant AM-30312.

Manuscript received 18 December 1985.

REFERENCES

1. Tipping, E., B. Ketterer, L. Christodoulides, and G. Enderby. 1976. The non-covalent binding of small molecules by ligandin. *Eur. J. Biochem.* **67:** 583–590.
2. Strange, B. C., R. Cramb, J. D. Hayes, and I. W. Percy-Robb. 1977. Partial purification of two lithocholic acid-binding proteins from rat liver 100,000 *g* supernatants. *Biochem. J.* **165:** 425–429.
3. Hayes, J. D., R. C. Strange, and I. W. Percy-Robb. 1979. Identification of two lithocholic acid-binding proteins. *Biochem. J.* **181:** 699–788.
4. Hayes, J. D., R. C. Strange, and I. W. Percy-Robb. 1980. Cholic acid binding by glutahtione S-transferases from rat liver cytosol. *Biochem. J.* **185:** 83–87.
5. Hayes, J. D., R. C. Strange, and I. W. Percy-Robb. 1981. A study of the structure of the Y_aY_a and Y_aY_c glutathione S-transferases from rat liver cytosol. *Biochem. J.* **197:** 491–502.
6. Sugiyama, Y., T. Yamada, and N. Kaplowitz. 1983. Newly identified bile acid binders in rat liver cytosol. Purification and comparison with glutathione S-transferases. *J. Biol. Chem.* **258:** 3602–3607.
7. Stolz, A., Y. Sugiyama, J. Kuhlenkamp, and N. Kaplowitz. 1984. Identification and purification of a 36-kDa bile acid binder in human hepatic cytosol. *FEBS Lett.* **177:** 31–35.
8. Pattinson, N. R. 1981. Isolation of two cholic acid-binding proteins from rat liver cytosol using affinity chromatography. *Biochim. Biophys. Acta.* **667:** 70–76.
9. Vessey, D. A., and D. Zakim. 1981. Inhibition of glutathione S-transferase by bile acids. *Biochem. J.* **197:** 321–325.
10. Hayes, J. D., and J. Chalmers. 1983. Bile acid inhibition of basic and neutral glutathione S-transferases in rat liver. *Biochem. J.* **215:** 581–588.
11. Maruyama, H., I. M. Arias, and I. Listowsky. 1984. Distinctions between the multiple cationic forms of rat liver glutathione S-transferase. *J. Biol. Chem.* **259:** 12444–12448.
12. Maruyama, H., and I. Listowsky. 1984. Preferential binding of steroids by anionic forms of rat glutathione S-transferase. *J. Biol. Chem.* **259:** 12449–12455.
13. Homma, H., and I. Listowsky. 1985. Temperature dependent redistribution among the multiple forms of rat Y_b-glutathione-S-transferase. *Biochem. Biophys. Res. Commun.* **127:** 425–432.
14. Mannervik, B., and C. Guthenberg. 1981. Glutathione transferase (human placenta). *Methods Enzymol.* **77:** 231–235.
15. Mannervik, B., and H. Jensson. 1982. Binary combinations of four protein subunits with different catalytic specificities explain the relationship between six basic glutathione S-transferases in rat liver cytosol. *J. Biol. Chem.* **257:** 9909–9912.
16. Habig, W. H., M. J. Pabst, and W. B. Jakoby. 1974. Glutathione S-transferase. The first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* **249:** 7130–7139.
17. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680–685.
18. Jakoby, W. B., B. Ketterer, and B. Mannervik. 1984. Glutathione transferase: nomenclature. *Biochem. Pharmacol.* **33:** 2539–2540.
19. Sugiyama, Y., A. Stolz, M. Sugimoto, and N. Kaplowitz. 1984. Evidence for a common high affinity binding site on glutathione S-transferase B for lithocholic acid and bilirubin. *J. Lipid Res.* **25:** 1177–1183.
20. Takikawa, H., H. Otsuka, T. Beppu, Y. Seyama, and T. Yamakawa. 1982. Quantitative determination of bile acid glucuronides in serum by mass fragmentography. *J. Biochem.* **92:** 985–998.
21. Murata, N., T. Beppu, H. Takikawa, H. Otsuka, T. Kasama, and T. Seyama. 1983. Determination of sulfated and non-sulfated bile acids in serum by mass fragmentography. *Steroids.* **42:** 575–592.
22. Scatchard, G. 1949. The attractions of proteins for small molecules and ions. *Ann. NY Acad. Sci.* **51:** 660–672.
23. Colowick, S. P., and F. C. Womack. 1969. Binding of

diffusible molecules by macromolecules: rapid measurement by rate of dialysis. *J. Biol. Chem.* **244:** 774–777.
24. Igari, Y., Y. Sugiyama, S. Awazu, and M. Hanano. 1981. Interspecies difference in drug protein binding-temperature and protein concentration dependency: effect on calculation of effective protein fraction. *J. Pharm. Sci.* **70:** 1049–1053.
25. Boyer, T. D., D. A. Vessey, C. Holcomb, and N. Saley. 1984. Studies of the relationship between the catalytic activity and binding of non-substrate ligands by the glutathione S-transferases. *Biochem. J.* **217:** 179–185.
26. Kamisaka, K., I. Listowsky, Z. Getmaitan, and I. M. Arias. 1975. Interaction of bilirubin and other ligands with ligandin. *Biochemistry.* **14:** 2175–2180.
27. Bhargava, M. M., N. Ohmi, I. Listowsky, and I. W. Arias. 1980. Structural, catalytic, binding, and immunological properties associated with each of the two subunits of rat liver ligandin. *J. Biol. Chem.* **255:** 718–723.
28. Boyer, T. D., W. C. Kenny, and D. Zakim. 1983. Structural, functional and hybridization studies of the glutathione S-transferases of rat liver. *Biochem. Pharmacol.* **32:** 1843–1850.
29. Sugiyama, Y., M. Sugimoto, A. Stolz, and N. Kaplowitz. 1984. Comparison of the binding affinities of five forms of rat glutathione S-transferases for bilirubin, sulfobromophthalein and hematin. *Biochem. Pharmacol.* **33:** 3511–3513.