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HIGH AND LOW AFFINITY BINDING OF [^{3}H]CHOLATE TO RAT LIVER PLASMA MEMBRANES*†

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Abstract—The transport of bile acids across sinusoidal and canalicular membranes of hepatocytes is characterized as carrier mediated. Such a carrier should specifically bind bile acids at physiological concentrations. We examined the binding of [^{3}H]cholate to rat liver plasma membranes using a microcentrifugation technique and detected high (K_D = 1.23 ± 0.44 μM, B_{max} = 21.8 ± 3.3 pmol/mg protein) and low (K_D = 1.97 ± 1.33 mM, B_{max} = 41.5± 25.3 nmol/mg protein) affinity binding sites. Maximal binding was achieved within 15–45 sec and was stable for 2 min at 37°. Binding to the high affinity site was reversible, was not Na^+ dependent or attributable to vesicular uptake, and exhibited a broad pH optimum. Binding to this site was negligible or not detected in liver mitochondrial and microsomal fractions, was saturable, and was inhibited by other bile acids. The IC_{50} values for bile acids as inhibitors of [^{3}H]cholate binding at the high affinity site were: taurocholate, 1.9 nM; glycodeoxycholate, 3.1 nM; chenodeoxycholate, 5.6 nM; taurochenodeoxycholate, 7.3 nM; glycochenodeoxycholate, 11 nM; lithocholate, 13 nM; taurodeoxycholate, 20 nM; glycocholate, 3.6 μM; and deoxycholate, 5.6 μM. [^{3}H]Cholate specific binding was inhibited by 10^{-5} M bromosulfophthalein, bilirubin and indocyanin green. These data support the hypothesis that the high affinity binding site represents a carrier which is shared by bile acids and nonbile acid organic anions.

Bile acids play a major physiological role in the transport of lipids, including dietary lipids and fat soluble vitamins, in the regulation of cholesterol biosynthesis in liver and small intestine, and in bile formation [1]. Bile acids are efficiently taken up from portal blood across the sinusoidal membrane by hepatocytes, translocated across the cell, and then transported across the canalicular membrane into bile (see Refs. 2 and 3 for review). The uptake of bile acids is specific, saturable, demonstrates competitive inhibition by other bile acids, and is energized by the Na^+ gradient generated by (Na^+, K^+)-ATPase [4–8]. The secretion of bile acids across the canalicular membrane into bile is less thoroughly characterized, but has also been shown to demonstrate saturation kinetics, competition with other bile acids, and secretion against a concentration gradient [9–13]. These observations are all consistent with a carrier-mediated transport of bile acids across sinusoidal and canalicular membranes. Based on the postulate that carrier-mediated transport requires the binding of the transported bile acid to a specific binding site, we have investigated the binding of cholic acid to specific sites in liver plasma membranes. Three reports have appeared in the literature on cholic acid binding to rat liver plasma membranes, but with conflicting results. Accatino and Simon [14] reported a single low affinity (K_D = 1.29 mM) binding site for cholic acid in rat liver plasma membranes, while Anwer *et al.* [15] reported three binding sites, a high affinity (K_D = 0.1 μM) and two low affinity sites. Bellentani *et al.* [16] recently reported that they were unable to demonstrate the existence of any specific saturable bile acid binding sites in rat liver plasma membrane. In this report, we describe the binding of [^{3}H]cholate to two sites in liver plasma membranes. Binding to the high affinity site was saturable, was inhibited by other bile acids and organic anions, and was half-saturated (K_D) at a concentration similar to the concentration of bile acids in portal blood, suggesting that this site represents a bile acid carrier.

METHODS

Animals. Female Sprague–Dawley rats weighing 200 ± 25 g were obtained from Harlan Industries, Indianapolis, IN, and were allowed to acclimate for at least 5 days before use. Food (Purina Lab Chow) and H_2O were available *ad lib.*; the animal rooms were maintained on a 12-hr automatically timed light/dark cycle.

Preparation of liver plasma membrane fractions. Liver plasma membranes were prepared by discontinuous sucrose density-gradient centrifugation by a modification of the method of Song *et al.* [17] and Boyer and Reno [18] as described by Scharschmidt *et al.* [19]. The membranes were finally suspended

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in 1.5 ml of modified Hanks' buffer, consisting of 137 mM NaCl, 5.2 mM KCl, 0.9 mM $MgSO_4$, 0.12 mM $CaCl_2$ and 10 mM Na_2HPO_4 adjusted to pH 7.5, and frozen in liquid N_2 for no longer than 4 days. Protein was determined by the method of Lowry *et al.* [20] with bovine serum albumin as standard.

Preparation of rat liver subcellular fractions. Nuclei were prepared by discontinuous sucrose density gradient ultracentrifugation of rat liver homogenate at 60,000 *g* for 1.25 hr as described by Tata [21]. The nuclei were harvested by resuspending the pellet. Mitochondria and microsomes were prepared by differential centrifugation as described by Fleischer and Kervina [22]. Livers were perfused, excised and homogenized in a Teflon–glass Potter–Elvehjem homogenizer with 15 strokes. The homogenate was centrifuged at 700 *g* for 10 min. The nuclear pellet was discarded, and the supernatant fraction was centrifuged at 5,000 *g* for 10 min. This mitochondrial pellet was then washed and centrifuged at 24,000 *g* twice for 10 min, resuspended in modified Hanks' buffer by homogenization, and stored in liquid nitrogen. The supernatant fraction from the mitochondrial pellet was centrifuged at 9,000 *g* for 20 min, and the 9,000 *g* supernatant was centrifuged twice at 105,000 *g* for 1 hr to obtain microsomes.

Enzyme determinations. ATPase activities were measured with a coupled enzymatic assay as described by Scharschmidt *et al.* [19]. 5'-Mononucleotidase activity, a liver plasma membrane marker, was determined by the method of Lesko *et al.* [23]. Mitochondrial contamination was determined by measurement of succinate-tetrazolium reductase activity [24]. Microsomal contamination was determined by measurement of glucose-6-phosphatase activity [25]. Alkaline phosphatase activity was determined by a modification of the method of Tietz *et al.* [26].

Binding assays. Membrane preparations were removed from liquid N_2, thawed, and diluted with modified Hanks' buffer to give a protein concentration of 0.7 to 1.2 mg/ml. The standard binding assay was carried out in 400-μl polyethylene centrifuge tubes at 37° in modified Hanks' buffer in a final volume of 200 μl. All assays were performed in triplicate or quadruplicate. All bile acids were dissolved in modified Hanks' buffer. Preliminary experiments showed that there was no significant difference between the binding constants for [^{3}H]cholate in fresh and frozen (4 days) membranes. Therefore, all assays were carried out using frozen (maximum of 4 days) membranes.

Plasma membranes at various protein concentrations (100–1000 μg protein/ml of incubation medium) were incubated with [^{3}H]cholate (1 μM) in modified Hanks' buffer, and binding was assayed. Total binding was found to be linear in the range of 300–900 μg/ml protein. Therefore, all assays were performed in a protein concentration range of 350–600 μg/ml.

Competition experiments. The binding assay was begun by the addition of 100 μl of membrane suspension (containing 70–120 μg protein) into a polyethylene tube containing 50 μl of [^{3}H]cholate (final concentration 10 nM) plus 50 μl of various concentrations of unlabelled cholate or other ligands. The tube was incubated at 37° for 1 min and then centrifuged for 2 min at 10,000 *g* in a Beckman microfuge (Beckman Instruments Inc., Irvine, CA). In preliminary experiments, an aliquot (50 μl) of supernatant was assayed by scintillation spectrometry to quantitate free ligand. Values thus obtained did not differ significantly from the total concentration since the amount bound was less than 4% of the total. Therefore, in subsequent calculations, the total concentration was used as an estimate of the free concentration. The supernatant fraction was removed carefully with a pasteur pipette drawn out to a very fine tip connected to house vacuum. The tube was cut near the pellet, and the pellet was added to 0.5 ml of tissue solubilizer, incubated for 1–2 hr at 50°, neutralized with 17 μl glacial acetic acid, and assayed for the bound ligand by scintillation spectrometry. In each experiment, correction was made for supernatant fluid trapped in the pellet by quantitating the trapped volume of ^3H_2O in control tubes in triplicate. The trapped amount in each experiment was 0.4 to 0.5% of total ^3H_2O added.

Saturation experiments. Membranes were incubated with 50 μl of varied concentrations of [^{3}H]cholate (0.1 to 10 μM) in the presence and absence of 50 μM (final concentration) unlabeled cholate. Binding assays were carried out as described above. These studies were designed to characterize binding at the high affinity site only.

Characterization of the binding. The effects of time and temperature, pH, osmolarity, and sodium dependence on the binding were investigated. Details of the methodology for each experiment are described in the table and figure legends and in Results.

Thin-layer chromatography of radioligand. TLC of [^{3}H]cholate was carried out on instant TLC sheets (Silica gel impregnated glass fiber sheets; Gelman Instrument Co., Ann Arbor, MI) developed in an *n*-butanol : acetic acid : H_2O (10 : 1 : 1, by vol.) solvent system and showed a 97% purity. After a typical binding assay in plasma membrane, microsomes, and liver homogenates, the pellet was extracted with ethanol, and the extract was analyzed by TLC. Ninety-five percent of the radioactivity in the pellet comigrated with authentic [^{3}H]cholate used as standard. These data indicate that the ligand was not metabolized under conditions of the binding assay.

Materials. [^{3}H]Cholate (16 Ci/mol), ^3H_2O and Aquassure scintillation mixture were obtained from New England Nuclear (Boston, MA). Bile acids were obtained from Calbiochem (La Jolla, CA). Tissue solubilizers used were NCS (Amersham, Arlington Heights, IL) or Soluene 350 (Packard, Downer's Grove, IL). Sucrose and other chemicals were of the best grade available. Radioactivity was analyzed by a Liquid Scintillation Spectrometer (Packard model 3310, Downers Grove, IL). Efficiencies were determined using [^{3}H]toluene as an internal standard.

Data analysis. K_D and B_{max} values from competition experiments were determined by an iterative curvilinear curve-fitting computer program by Munson and Rodbard [27] and will hereafter be referred to as LIGAND. This program is based on the math-

Table 1. Enzymatic characterization of rat liver plasma membranes (LPM)

	Homogenate	LPM	Enrichment
Succinate dehydrogenase (μmol formazan/mg·hr)	2.99 ± 0.23	5.29 ± 0.70	1.77
5′-Mononucleotidase (μmol P_i/mg·hr)	3.09 ± 0.09	55.7 ± 2.39	18.0
Glucose-6-phosphatase (μmol P_i/mg·hr)	5.85 ± 0.28	16.6 ± 1.54	2.84
Alkaline phosphatase (μmol P_i/mg·hr)	0.15 ± 0.008	1.56 ± 0.24	10.2
Mg^{2+}-ATPase (μmol P_i/mg·hr)	3.69 ± 0.26	55.0 ± 8.95	14.9
(Na^+K^+-ATPase (μmol P_i/mg·hr)	1.26 ± 0.23	48.8 ± 8.30	38.7

Each value represents the mean ± SEM of three to eleven determinations.

ematical theory developed by Feldman [28] describing any number (n) of ligands reacting with any number of classes (m) of binding sites.

Calculation of the IC_{50} values and the 95% confidence limits for bile acids was carried out by the logit transformation as described by Rodbard and Frazier [29]. Data from two to five experiments were combined to give a regression line with a correlation coefficient of not less than 0.86.

Statistical analysis of the binding constants at different incubation temperatures was determined by Student's *t*-test.

RESULTS

Enzyme assays. Enzyme determinations were initially carried out on both fresh and frozen membrane preparations, and the results were not significantly different. Therefore, subsequent assays were performed in previously frozen membranes. Table 1 shows the enzyme activities in liver plasma membranes in comparison with crude liver homogenate. The preparation was enriched in plasma membrane enzyme markers: 5′-mononucleotidase, (Na^+, K^+)-ATPase, Mg^{2+}-ATPase and alkaline phosphatase, There was little microsomal (glucose-6-phosphatase) or mitochondrial (succinate dehydrogenase) contamination. This membrane preparation was heterogenous consisting of both sinusoidal ((Na^+, K^+)-ATPase) and canalicular (Mg^{2+}-ATPase, 5′-mononucleotidase and alkaline phosphatase) domains of rat liver plasma membranes.

Characterization of [^{3}H]*cholate binding.* The binding of 10 nM [^{3}H]cholate to plasma membranes was carried out in the presence of unlabeled cholate at concentrations ranging from 0 to 1000 μM as shown in Fig. 1A. The specific binding was then transformed to the Scatchard plot (Fig. 1B); these data indicated the presence of two binding sites. The analysis of untransformed competition binding data by LIGAND gave a dissociation constant (K_D) and binding capacity (B_{max}) of 1.23 ± 0.44 μM and 21.8 ± 3.3 pmol/mg protein, respectively, for the

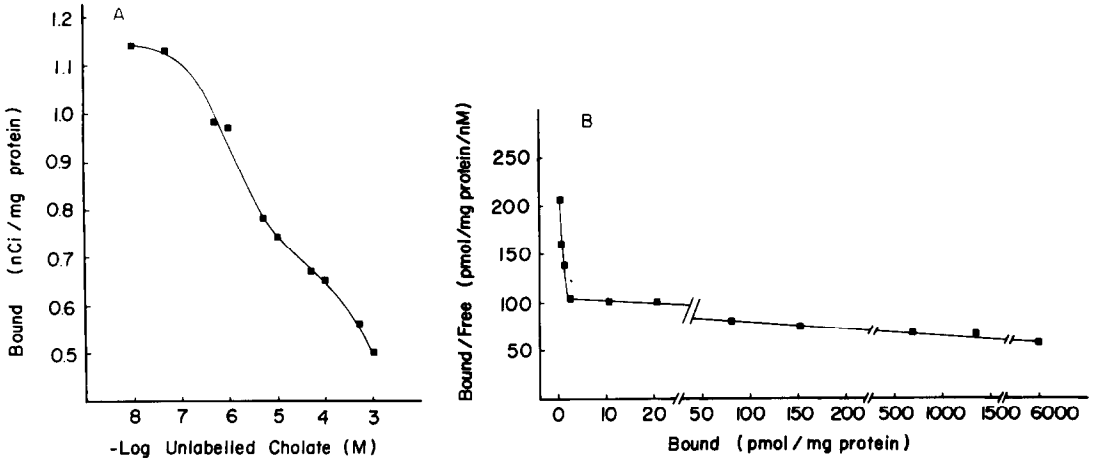

Fig. 1. Competition between [^{3}H]cholate binding and unlabeled cholate (A) and Scatchard plot of total binding uncorrected for nonspecific binding (B). K_D and B_{max} values of the high affinity site were 1.23 ± 0.44 μM and 21.8 ± 3.3 pmol/mg protein, respectively, whereas K_D and B_{max} values of the low affinity site were 1.97 ± 1.33 mM and 41.5 ± 25.3 nmol/mg protein respectively. Nonspecific binding was 15.7 ± 4.35 pmol/mg protein/nM. Binding parameters were determined by LIGAND. Membranes were incubated with 10 nM [^{3}H]cholate in the presence of 0–1000 μM unlabeled cholate, and binding was determined as described in Methods. The data represent the mean of three determinations, each carried out in triplicate. Binding constants represent the mean ± SEM of the three determinations.

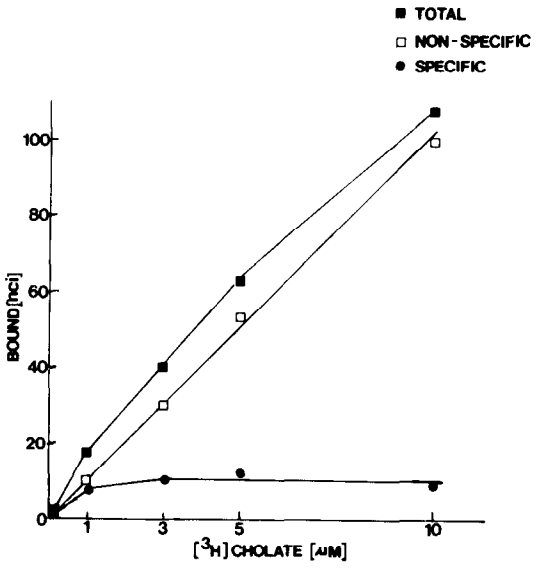

Fig. 2. Saturability of the high affinity [^{3}H]cholate binding site. Specific binding is the difference between [^{3}H]cholate binding in the presence (nonspecific binding) and absence (total binding) of 50 μM unlabeled cholate. The data represent the mean of two determinations, each carried out in triplicate.

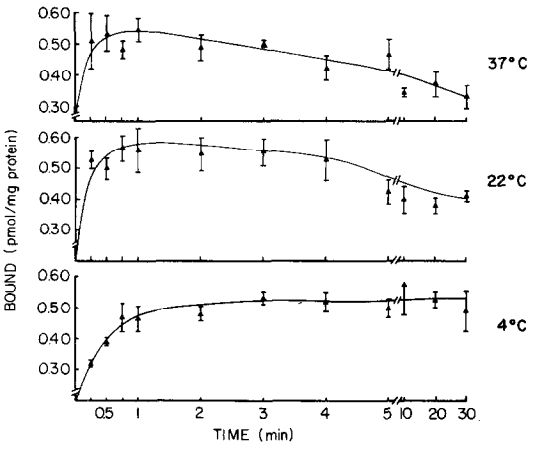

Fig. 3. Effects of time and temperature on the total binding of 10 nM [^{3}H]cholate. The data represent the mean ± SEM of four determinations, each carried out in triplicate or quadruplicate.

high affinity site and a K_D and B_{max} of 1.97 ± 1.33 mM and 41.5 ± 25.3 nmol/mg protein, respectively, for the low affinity site.

The specific binding of [^{3}H]cholate to the high affinity site was saturable (Fig. 2). Specific binding constituted only about 10% of the total binding. We could not demonstrate saturability of the low affinity site, however, since cholate is an ionic detergent which forms micelles and solubilized the membrane at concentrations above 2–3 mM.

Effect of time and temperature. Total binding of 10 nM [^{3}H]cholate to plasma membranes at 4°, 22° and 37° reached a maximum within 15–45 sec (Fig. 3). The maximal number of binding sites (B_{max}, both low and high affinity sites) calculated from the competition experiment between [^{3}H]cholate binding (10 nM) and various concentrations of unlabeled cholate (0–1000 μM) was decreased at 22° but, because of the variability, there was no statistically significant difference at 22° and 37° (Table 2). Binding assays in subsequent experiments were incubated at 37° for 1 min.

When [^{3}H]cholate (10 nM) was incubated with membranes until equilibrium was attained (1 min, 37°) followed by addition of an excess (1 mM) of unlabeled cholate and binding assayed, dissociation was found to be essentially complete within 10 sec (data not shown). These results indicated that binding of [^{3}H]cholate to plasma membranes was reversible and was not attributable to covalent binding.

Effect of Na^+ *and osmolarity.* The specific binding of 10 nM [^{3}H]cholate to plasma membranes in the presence or absence of Na^+ in the incubation medium demonstrated Na^+ independence, since binding in the presence and absence of Na^+ was not significantly different (Table 3). To determine if the binding involved uptake of cholate into vesicles, the specific binding of 10 nM [^{3}H]cholate to plasma membranes was measured in the presence of a range of concentrations of sucrose in the incubation medium. Since increased osmotic activity of the incubation medium would decrease vesicle volume, a decrease in [^{3}H]cholate associated with the pellet would be evidence supporting vesicular uptake. There were no significant differences, however, in specific binding over the wide range (0–250 mM) of sucrose concentrations tested (Table 3). These data indicate that the binding was unlikely to be a vesicular uptake phenomenon. These results agree well with the report by Scharschmidt and Keeffe [30] that the

Table 2. Effect of incubation temperature on [^{3}H]cholate binding constants

	Dissociation constant		Binding capacity	
Incubation temperature	High affinity (μM)	Low affinity (mM)	High affinity (pmol/mg protein)	Low affinity (nmol/mg protein)
37°	1.23 ± 0.44	1.97 ± 1.33	21.8 ± 3.3	41.5 ± 25.3
22°	0.34 ± 0.15	0.22 ± 0.05	12.6 ± 9.0	3.87 ± 1.76

Each number represents the mean ± SE of three to four determinations of each of three membrane preparations. Binding parameters were determined by LIGAND. Nonspecific binding determined by LIGAND was 19.4 ± 3.6 pmol/mg protein/nM and 15.7 ± 4.4 pmol/mg protein/nM at 22 and 37° respectively.

Table 3. Effect of Na^+ and osmolarity on specific binding of [^{3}H]cholate to liver plasma membranes

	Binding (pmol/mg protein)
Na^+	
Concentration (mM)	
Tris Na^+	
0 157	0.33 ± 0.04
157 0	0.29 ± 0.03
Osmolarity	
Sucrose concentration (mM)	
0	0.32 ± 0.05
125	0.27 ± 0.03
250	0.28 ± 0.02

Membranes (70–100 μg protein) were incubated with 10 nM [^{3}H]cholate and buffer containing the indicated concentrations of Tris or Na^+ (Na^+ requirement experiments) or sucrose (osmolarity experiments) in the presence and absence of 1 mM unlabeled cholate. Specific binding was calculated as the difference between total binding (binding in the absence of 1 mM unlabeled cholate) and nonspecific binding (binding in the presence of 1 mM unlabeled cholate). Values are the mean ± SE of three to four experiments each carried out in triplicate or quadruplicate.

membranes prepared by the present method contain very few vesicles.

Effect of pH. The specific binding of 50 nM [^{3}H]cholate was shown to be optimal at pH 6.5–7.5 (Table 4). However, specific binding was not decreased by more than 15% in the pH 5.5–9.5 range. Nonspecific binding increased significantly at lower pH (data not shown), presumably due to the decreased ionization (the pK_a of cholic acid is 5) and increased lipid solubility of cholic acid.

Binding of [^{3}H]cholate to liver subcellular fractions. Studies characterizing the binding of 10 nM [^{3}H]cholate in the presence of 0–1000 μM unlabeled cholate and analyzed by LIGAND showed that the high affinity site was present in homogenate, plasma membrane, and nuclei (Table 5). The significance of the presence of the high affinity site in nuclei is not known (but may suggest the existence of a bile acid or organic anion carrier in this organelle). The low affinity site was present in all liver subcellular fractions excluding nuclei.

Competition for [^{3}H]cholate binding by other bile acids. These studies were designed to examine the high affinity binding site only, since this site appeared most likely to represent a bile acid carrier. The binding of [^{3}H]cholate in the presence of 50 μM unlabeled cholate was defined as the nonspecific binding for the high affinity site. The Scatchard plot of specific binding of 10 nM [^{3}H]cholate to plasma membranes in the presence of unlabeled cholate concentrations varying from 0 to 50 μM gave a linear curve (Fig. 4A) indicating that there was only one single binding site in this range. The K_D and B_{max}, calculated by LIGAND, were 5.0 ± 0.45 μM and 263 ± 25 pmol/mg protein respectively. The higher B_{max} than that obtained previously when both high affinity and low affinity sites were analyzed simultaneously indicated that the unlabeled cholate concentration of 50 μM used as blank also displaced [^{3}H]cholate bound to some of the low affinity sites, thus including these sites as the high affinity site. We did not consider using a lower concentration of unlabeled cholate as blank since this would not have saturated the high affinity site. The Hill plot from this same data (Fig. 4B) yielded a straight line with the slope (n_H) (calculated by least squares linear regression) of 0.88 which is sufficiently close to unity to indicate that there was no significant negative cooperativity. These data indicate that this experimental model was valid for estimating IC_{50} values at

Table 4. Effect of pH on [^{3}H]cholate specific binding

pH	% Control
5.5	95.6
6.5	99.8
8.5	90.5
9.5	85.7
Control (pH 7.5)	100

Membranes (70 μg protein) were incubated with 50 nM [^{3}H]cholate in buffer at various pH in the presence and absence of 1.0 mM unlabeled cholate. The binding assays were performed as described in Methods. Specific binding was measured as the difference between total binding (binding in the absence of 1.0 mM unlabeled cholate) and nonspecific binding (binding in the presence of 1.0 mM unlabeled cholate). Results are expressed as percent control where control refers to the specific binding in modified Hanks' buffer, pH 7.5; control binding was 0.26 pmol/mg protein.

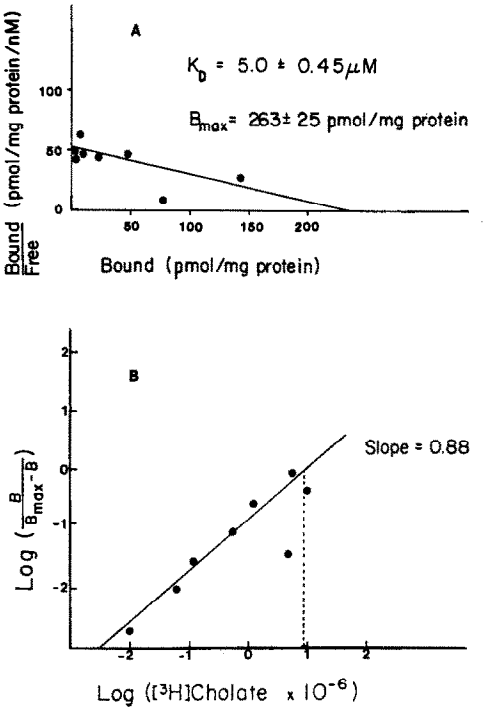

Fig. 4. Scatchard analysis (A) and Hill plot (B) of binding of 10 nM [^{3}H]cholate in the presence of 0–50 μM unlabeled cholate, i.e., binding to the high affinity site. The data represent the mean of two determinations, each carried out in triplicate.

Table 5. Binding constants of [^{3}H]cholate to liver subcellular fractions

Fraction	Binding constants			
	High affinity		Low affinity	
	K_D (μM)	B_{max} (pmol/mg protein)	K_D (mM)	B_{max} (nmol/mg protein)
Homogenate	3.80	62.80	3.92	6.44
Mitochondria			0.79	28.3
Microsome			0.16	7.32
Nuclei	4.38	352.2		
Plasma membrane	1.23	21.80	1.97	41.50

Each experiment was carried out in quadruplicate. Binding was determined by incubating 10 nM [^{3}H]cholate in the presence of 0–1000 μM unlabeled cholate. Binding parameters were determined by LIGAND.

the high affinity cholate binding site. The concentration of bile acids necessary to reduce specific binding of 10 nM [^{3}H]cholate by 50% (IC_{50}) determined by logit transformation [29] is presented in Table 6. Figure 5 shows the competition of [^{3}H]cholate binding to the high affinity site by unlabeled cholate or other bile acids in the concentration range from 0 to 50 μM. All bile acids studied were able to compete with cholate for this binding site. The order of potencies of the bile acids as inhibitors of [^{3}H]cholate binding was taurocholate = glycodeoxycholate = chenodeoxycholate = taurochenodeoxycholate $\geq$ glycochenodeoxycholate = lithocholate = taurodeoxycholate $\gg$ cholate = glycocholate = deoxycholate.

Competition for [^{3}H]cholate binding by other organic anions. The inhibition of 10 nM [^{3}H]cholate specific binding by non-bile acid organic anions (10^{-5} M) is presented in Table 7. The order of potency of these organic anions for inhibition of [^{3}H]cholate binding was indocyanin green > bromosulfophthalein > bilirubin $\gg$ morphine glucuronide.

DISCUSSION

The binding of [^{3}H]cholate to liver plasma membranes was characterized as binding to two distinct sites, a relatively high affinity site (K_D = 1.23 μM; B_{max} = 21.8 pmol/mg protein) and a low affinity, high capacity site (K_D = 1.97 mM, B_{max} = 41.5 nmol/mg protein) (Fig. 1). The rate of association was extremely rapid, with maximal binding obtained within 45 sec, even at 4° (Fig. 3). Likewise, dissociation of [^{3}H]cholate and membranes was essentially complete within 10 sec. The rapid rates of association and dissociation between [^{3}H]cholate and membranes precluded estimation of the equilibrium binding constant (K_D) as a ratio of the rate constants. The extremely rapid rate of dissociation also necessitated quantitation of the receptor–ligand complex under conditions that minimized dissociation, i.e. minimized the total time the receptor–ligand complex was exposed to concentrations of free ligand significantly lower than that present at equilibrium. In the microcentrifugation technique used in the present studies, the ligand–receptor complex was never exposed to ligand-free medium. Although centrifugation is the best method for receptor–ligand studies with rapid dissociation rates, it has the disadvantage of higher levels of nonspecific binding owing to sequestered radio-ligand [31]. ^3H_2O was used as a tracer to correct for the volume (about 0.8 μl) of medium trapped in the pellet; nevertheless nonspecific binding accounted for approximately 90% of the total binding (Fig. 2). The planar amphiphathic nature of cholic acid [1] probably also con-

Table 6. Concentration of bile acids necessary to reduce specific binding of [^{3}H]cholate to membranes by 50% (IC_{50})

Bile acid	IC_{50} (95% CL) (μM)
Taurocholate	0.0019 (0.00085–0.0042)
Glycodeoxycholate	0.0031 (0.00014–0.066)
Chenodeoxycholate	0.0056 (0.0018–0.018)
Taurochenodeoxycholate	0.0073 (0.0012–0.027)
Glycochenodeoxycholate	0.011 (0.002–0.063)
Lithocholate	0.013 (0.0074–0.022)
Taurodeoxycholate	0.020 (0.0024–0.16)
Glycocholate	3.6 (1.1–11)
Deoxycholate	5.6 (3.2–10)

Data represent the IC_{50} (95% confidence limits) determined from the logit transformation of the percentage inhibition of specific binding derived from data shown in Fig. 5.

Table 7. Inhibition of [^{3}H]cholate specific binding to the high affinity site by organic anions

Organic anion (10^{-5} M)	% Inhibition
Indocyanin green	100.0
Bromosulfophthalein	65.0
Bilirubin	40.8
Morphine glucuronide	12.4

Membranes (70 μg) were incubated with 10 nM [^{3}H]cholate in buffer in the presence of a 10 μM concentration of the organic anions. Specific binding is the difference between binding in the presence (nonspecific binding) and absence (total binding) of 50 μM unlabeled cholate; specific binding was 0.33 pmol/mg protein. The data represent the mean of two determinations, each carried out in triplicate.

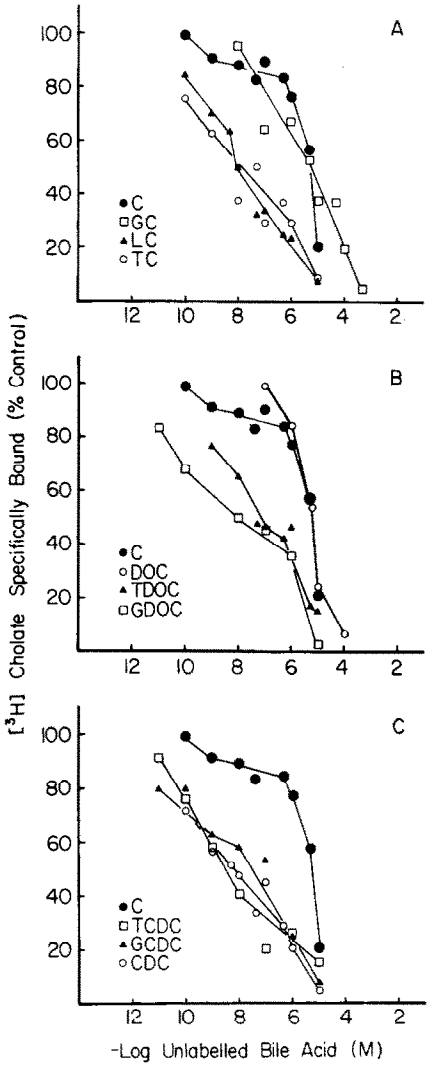

Fig. 5. Competition between binding of 10 nM [³H]cholate to the high affinity site and unlabeled bile acids (0–50 μM). The ordinate represents the percent of maximal specific [³H]cholate binding (% control) which is the binding in the absence of competitor. Specific binding is the difference between binding in the presence (nonspecific binding) and absence (total binding) of 50 μM unlabeled cholate. The data represent the mean of two to five determinations, each carried out in triplicate. Abbreviations: C, cholate, GC, glycocholate; LC, lithocholate; TC, taurocholate; DOC, deoxycholate, TDOC, taurodeoxycholate; GDOC, glycodeoxycholate; TCDC, taurochenodeoxycholate; GCDC, glycochenodeoxycholate; and CDC, chenodeoxycholate.

tributes significantly to nonspecific binding since the hydrophobic side of cholic acid can readily interact nonspecifically with hydrophobic areas of the plasma membrane.

The present results agree in several respects to those of other investigators who have characterized the binding of cholate to liver plasma membranes. Accatino and Simon [14] used a filtration method to describe a single class of binding sites with a K_D and B_{max} of 1.29 mM and 18 nmol/mg protein, respectively, when [¹⁴C]cholate (60 mCi/mmol) was used as the ligand. The binding constants of the low affinity site in the present studies are in good agreement with those of Accatino and Simon [14]. Anwer *et al.* [15] used a centrifugation technique to describe a high affinity site (K_D = 0.1 μM, B_{max} = 9.4 pmol/mg protein) as well as a medium (K_D = 0.4 mM, B_{max} = 9.5 nmol/mg protein) and low (K_D = 5 mM, B_{max} = 320 nmol/mg protein) affinity site when [¹⁴C]cholate was used as the ligand. The binding parameters for the high and medium affinity sites obtained by Anwer *et al.* [15] agree reasonably well with those described here; differences in the actual K_D and B_{max} values may be due to the fact that these workers used a manual curve-fitting procedure whereas we used a computer curve-fitting method [27]. However, the present data clearly do not agree with Bellentani *et al.* [16]. These authors used [¹⁴C]cholic acid at pH 6.0 in the presence of ethanol and saw no saturable, specific binding. Their failure to see any specific binding may be due to the presence of ethanol which lowers specific binding as shown by Bellentani *et al.* [16] and unpublished data from our own laboratory.

Anwer *et al.* [15] also reported a 50% decrease in B_{max} of the high affinity site at 10, 20 and 27° relative to that at 37°. We found no statistically significant difference in B_{max} of the high affinity site at 22 vs 37° (Table 2) although there was a trend toward a decreased value at 22°. Temperature changes in the range of 0–37° will strongly affect rates of association and dissociation but only rarely have an effect on binding capacity [31]. The reason for the tendency for lower B_{max} at lower temperatures is not known; however, the data suggest that additional binding sites became exposed with increasing membrane fluidity at the higher temperature.

Specific binding of [³H]cholate was not attributable to vesicular uptake, was independent of Na^+, showed a broad pH optimum, and was saturable. The K_D of the high affinity site is in the same concentration range as the concentration of bile acids present normally in the rat portal blood (60 μM) [32] and is similar to the K_m for cholate uptake characterized in isolated hepatocytes (13 μM) [33]. Furthermore, all bile acids studied competed effectively with [³H]cholate for binding to the high affinity site (Table 6, Fig. 5). These data are all consistent with the hypothesis that the high affinity site represents a bile acid carrier in plasma membrane.

Uptake of cholate by isolated hepatocytes has been shown to occur by both an Na^+-dependent and an Na^+-independent carrier system [34], whereas in the present study binding of cholate to plasma membranes was independent of Na^+. Anwer and Hegner [34] demonstrated that Na^+ did not alter the apparent K_m for cholate uptake into hepatocytes but rather stimulated V_{max}, suggesting that Na^+ did not influence the binding of cholate to the carrier but stimulated translocation of the carrier across the membrane. Thus, the observation that the binding of [³H]cholate to plasma membranes is Na^+ independent is not in conflict with Na^+-dependent cholate transport.

All bile acids examined competed for [³H]cholate binding in membranes. Since their inhibition curves were generally parallel, it is reasonable to assume that this inhibition was of a competitive nature. The

IC_{50} values for the bile acids examined ranged from about 2 nM for taurocholate to 5 μM for deoxycholate. Conjugation of cholate with taurine thus increased the affinity of cholate for the binding site by almost three orders of magnitude. In contrast, conjugation of cholate with glycine had a minimal effect on the affinity. These effects are comparable in direction but not in magnitude to the effect of cholate conjugation on the K_m for uptake of the bile acids into isolated hepatocytes; conjugation with taurine decreased the K_m for cholate from 13.1 to 3.7 μM whereas conjugation with glycine had no effect on the K_m [33]. Anwer and Hegner [34] also demonstrated that conjugation of cholate with taurine decreased the K_m for Na^+-dependent uptake of bile acid into hepatocytes from 58 to 15 μM.

Lithocholate, a 3α-monohydroxy bile acid, and chenodeoxycholate, a 3α,7α-dihydroxy bile acid, were about 100 and 300 times more potent, respectively, than cholate, whereas deoxycholate, a 3α,12α-dihydroxy bile acid was about equipotent to cholate in displacing [^{3}H]cholate. These data suggest that the 7α-hydroxy group increases whereas the 12α-hydroxy group decreases affinity for the putative carrier. Conjugation of deoxycholate, which has a high IC_{50}, with glycine or taurine decreased its IC_{50} by 1100- or 300-fold, respectively, whereas conjugation of chenodeoxycholate, which already has a relatively low IC_{50}, with taurine or glycine had little effect on the IC_{50}. Thus, the bile acids analyzed fall into one of two general classes with respect to their ability to compete with [^{3}H]cholate for binding to the high affinity site: those with IC_{50} values ranging from 2 to 20 nM (taurocholate, tauro- and glycodeoxycholate, chenodeoxycholate and its taurine and glycine conjugates and lithocholate) and those with IC_{50} values in the μM range (glycocholate, cholate and deoxycholate). The high affinity of taurocholate for the binding site shown in the present studies indicates that it would be a highly suitable ligand for further characterization of the putative carrier. The high affinity should permit characterization of association and/or dissociation rate constants, and its decreased lipid solubility should decrease nonspecific binding.

Under the conditions of the present experiments, the IC_{50} values determined for the various bile acids provide good estimates of their K_i values. Thus, the concentration of the radioligand (L) used (10 nM [^{3}H]cholate) is 100-fold less than the K_D (1.23 μM) so that $IC_{50} \cong K_i$ according to the following equation described by Cheng and Prusoff [35] where

$$K_i = IC_{50}/(1 + [L]/K_D).$$

The physiologic significance of the high affinity binding site of several of the bile acids, notably taurocholate where $K_i = 2$ nM, for the proposed carrier is not known, but the data suggest some intriguing possibilities. These bile acids may serve some regulatory function at the proposed carrier at concentrations much lower than those at which they serve as substrates for transport, e.g. the apparent K_m for taurocholate uptake in female rat hepatocytes is 38 μM [36]. It has been well established that taurocholate infusion increases the transport maximum of bromosulfophthalein (BSP) in the liver [37, 38]. Marinovic *et al.* [39] showed that taurocholate increases both the V_{max} of dibromosulfophthalein hepatic uptake, suggesting a facilitation of the uptake process, and the K_m suggesting competition for a common site. Forker and Gibson [40] have proposed an allosteric model in which the organic anion carrier (i.e. for BSP) can react with taurocholate to produce a carrier that transports BSP with different parameters. The high affinity binding of taurocholate may, therefore, represent an allosteric interaction site at the carrier. However, the actual free concentrations of the bile acids at the carrier in the hepatocyte and in portal blood are not known because of micelle formation and the binding to plasma and cellular proteins. Alternatively therefore, the high affinity binding site may be appropriate for actual free concentrations of the bile acids. Finally, the high affinity binding site may not represent a bile acid carrier, but instead a protein of as yet unknown function. Further studies using radiolabeled ligands such as taurocholate are clearly needed to investigate these sites further.

Various nonbile acid organic anions (bilirubin, BSP, indocyanin green) were also shown to inhibit [^{3}H]cholate binding to the high affinity site. These data are consistent with mounting evidence that the uptake of bile acids and other organic anions into hepatocytes is shared by at least two multispecific transport systems. Anwer and Hegner [34] showed that BSP inhibited the Na^+-independent uptake of bile salts in isolated hepatocytes while Laperche *et al.* [41] showed that BSP is taken up into hepatocytes by two separate carriers, one of which is inhibited by, and presumably shared with, taurocholate. BSP inhibits cholate uptake in isolated hepatocytes [8] and inhibits both the Na^+-dependent and Na^+-independent uptake of taurocholate in liver plasma membrane vesicles [42]. Kurz and colleagues [43, 44] have developed bile acid photoaffinity probes and identified two polypeptides with apparent molecular weights of 48,000 and 54,000 which appear to transport a wide variety of organic anions including taurocholate, cholate, bilirubin, BSP and indocyanine green. Hugentobler and Meier [45] have recently described a sulfate hydroxyl exchange system in sinusoidal (basolateral) rat liver plasma membrane vesicles which is competitively inhibited by cholate and BSP. Finally, we have described the specific binding of [^{3}H]estradiol-17β-(β-D-glucuronide) ($E_2$17G) to two sites in rat liver plasma membranes; cholate and taurocholate competed with [^{3}H]$E_2$17G for the high affinity site only, whereas BSP competed for binding at both sites*. Morphine glucuronide, which had little effect on the binding of [^{3}H]cholate (Table 7), also had no effect on the binding of [^{3}H]$E_2$17G or on the uptake of [^{3}H]$E_2$17G into isolated hepatocytes†. Morphine glucuronide is also taken up very poorly by isolated hepatocytes [46] and the isolated perfused liver [47]. Taken together, these data suggest that the high affinity [^{3}H]cholate binding site described in the present studies rep-

* A. Takacs and M. Vore, *Molec. Pharmac.* in press.

† K. Brouwer, S. Durham and M. Vore, *Molec. Pharmac.* in press.

resents a carrier which is shared by the bile acids and nonbile acids such as BSP and $E_2$17G.

In summary, we have characterized high affinity and low affinity [^{3}H]cholate binding sites in rat liver plasma membranes. Binding to the high affinity site was shown to be reversible, saturable, and inhibited by the major bile acids and some organic anions. Furthermore, the K_D of the high affinity site was in the physiologic concentration range of bile acids. These characteristics support the hypothesis that the high affinity binding site represents a bile acid carrier which is shared with other organic anions. Further studies characterizing this binding site may provide useful insight into the regulation of bile acid transport, the generation of bile flow, and the pathologic basis of cholestasis.

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