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### Research Article

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# Identification and Characterization of a Bile Acid Receptor in Isolated Liver Surface Membranes

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**ABSTRACT** It is generally assumed that hepatic transport of bile acids is a carrier-mediated process. However, the basic mechanisms by which these organic anions are translocated across the liver cell surface membrane are not well understood. Since carrier-mediated transport involves binding of the transported molecule to specific receptor sites, we have investigated the possibility that bile acid receptors are present in liver surface membranes.

Isolated liver surface membranes were incubated at 4°C with [<sup>14</sup>C]cholic acid and [<sup>14</sup>C]taurocholic acid, and membrane-bound bile acid was separated from free by a rapid ultrafiltration technique through glass-fiber filters. Specific bile acid binding is rapid and reversible and represents approximately 80% of the total bile acid bound to liver surface membranes. Taurocholic acid binding is independent of the medium pH, while cholic acid binding demonstrates an optimum at pH 6.0. Analysis of equilibrium data for both cholic and taurocholic acid binding indicates that specific binding is saturable and consistent with Michaelis-Menten kinetics, while non-specific binding is nonsaturable. Apparent maximal binding capacity and dissociation constant values indicate a large capacity system of receptors that have an affinity for bile acids comparable to that of the hepatic

transport mechanism. Scatchard analysis of the saturation kinetics as well as inhibition studies suggest that bile acids bind to a single and noninteracting class of receptors. In addition, sulfobromophthalein, an organic anion that competes with bile acids for hepatic uptake, also inhibits cholic acid binding. In contrast, no inhibition was demonstrated with indocyanine green and probenecid. Specific bile acid binding is enriched and primarily located in liver surface membranes and found only in tissues involved in bile acid transport.

Specific bile acid binding is independent of Na<sup>+</sup>, Ca<sup>2+</sup>, and Mg<sup>2+</sup> and does not require metabolic energy. In addition, thiol groups and disulfide are not required for activity at the binding site. However, specific bile acid binding is markedly decreased by low concentrations of proteolytic enzymes and is also decreased by the action of neuraminidase and phospholipases A and C.

These results are consistent with the existence of a homogeneous bile acid receptor protein in liver surface membranes. The primary surface membrane location of this receptor, its binding properties, and its ligand specificity suggest that bile acid binding to this receptor may represent the initial interaction in bile acid transport across liver surface membranes.

## INTRODUCTION

A highly efficient enterohepatic circulation maintains bile acids in a small but dynamic pool (1). This recycling system allows bile acids to circulate 5 to 15 times a day and to exert their major physiological functions in the absorption of dietary lipids, regulation of cholesterol metabolism, solubilization of biliary cholesterol, and bile formation (2-7). Maintenance of an adequate bile acid pool is dependent upon active absorption by the ileum (8, 9), return to the liver in the portal blood bound to albumin (10, 11), extraction by hepatic parenchymal cells (12), and secretion against a concentration gradient into the bile canaliculi (13).

This work was presented in part at the VI Meeting of the International Association for the Study of the Liver, Acapulco, Mexico, 20-22 October 1974; at the 25th Annual Meeting of the American Association for the Study of Liver Diseases, Chicago, 29-30 October 1974; and at the Annual Meeting of the American Gastroenterological Association, San Antonio, 17-22 May 1975, and abstracted in *Gastroenterology*. 67: 778, 1974.

Dr. Accatino was a postdoctoral research fellow during the period when these studies were undertaken, and he was partially supported by funds from the Catholic University of Chile, Santiago, Chile. His present address is: Departamento de Medicina Interna, Universidad Catolica de Chile, Casilla 114-D, Santiago, Chile.

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Although intestinal mechanisms of bile acid absorption have been extensively studied (8, 9, 14), direct examination of hepatic transport steps has not been possible because of the inaccessibility of hepatic sinusoids and bile canaliculi. However, it is believed that hepatic transport of bile acids is a complex process that involves uptake from the portal plasma into the hepatocyte by an efficient, high-capacity, and saturable mechanism (12, 15, 16), rapid intracellular movement, conjugation, and excretion into the bile canaliculus by a specific, saturable process against a concentration gradient (7, 12, 13). These physiologic characteristics suggest that carrier-mediated transport is involved in bile acid translocation across hepatocyte surface membranes. Since most models of carrier-mediated transport assume that translocation involves and, in fact, begins with binding of the transported molecules (ligands) to specific receptor sites (17, 18), we have examined the possibility that specific bile acid-binding sites are present in liver surface membranes.

The present studies demonstrate that bile acid receptors are present in liver surface membranes and that the interaction of both unconjugated and conjugated bile acids with these receptors is reversible, saturable, inhibited by specific organic anions, and very sensitive to proteolytic digestion. These findings suggest that specific surface membrane proteins may be involved in the hepatic transport of bile acids.

## METHODS

**Materials.** [24-<sup>14</sup>C]Cholic acid (50–60 mCi/mmol, 99% radiochemically pure) was obtained from New England Nuclear, Boston, Mass. [24-<sup>14</sup>C]Taurocholic acid (5 mCi/mmol, >98% radiochemically pure) was obtained from ICN Chemical and Radioisotope Division, Irvine, Calif. Purity of labeled bile acids was confirmed by thin-layer chromatography (19). Unlabeled cholic, taurocholic, chenodeoxycholic, and taurochenodeoxycholic acids were obtained from Maybridge Research Chemicals, Cornwall, England, and recrystallized to achieve greater than 98% purity as determined by thin-layer chromatography. Other materials were obtained from the indicated sources: dithiothreitol (DTT),<sup>1</sup> *N*-ethylmaleimide (NEM), *p*-nitrophenol, sodium fluoride, 2,4-dinitrophenol, phospholipase A (from *Vipera russelli*), neuraminidase (from *Clostridium perfringens*), *N*-acetylneuraminic acid (sialic acid), albumin (bovine), adenosine 5'-monophosphate, adenosine 5'-triphosphate, cytochrome *c*, and ouabain: Sigma Chemical Co., St. Louis, Mo.; phospholipase C (from *Cl. perfringens*), trypsin (bovine pancreas) treated with *L*-(tosylamide 2-phenyl)ethyl chloromethyl ketone (TPCK), soybean trypsin inhibitor, and chymotrypsin (bovine pancreas): Worthington Biochemical Corp., Free-

<sup>1</sup> *Abbreviations used in this paper:* BSP, sulfobromophthalein; DTNB, 5,5'-dithiobis[2-nitrobenzoic acid]; DTT, dithiothreitol; ICG, indocyanine green;  $K_a$ , apparent dissociation constant or medium concentration yielding half-maximal binding; *N*, apparent maximal binding capacity; NEM, *N*-ethylmaleimide; TPCK-trypsin, trypsin treated with *L*-(tosylamide 2-phenyl)ethyl chloromethyl ketone.

hold, N. J.; 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB): Eastman Organic Chemical Division, Eastman Kodak Co., Rochester, N. Y.; Bromsulphalein (sulfobromophthalein, BSP) and Cardio-Green (indocyanine green, ICG): Hynson, Westcott & Dunning, Inc., Baltimore, Md.; probenecid (*p*-dipropylsulfamyl)benzoic acid): Merck & Co., Inc., New York. All chemicals were reagent grade or the highest quality available. Double distilled water was used in all assays.

**Liver fractionation.** Male Sprague-Dawley rats, 200–250 g. (Charles River Breeding Laboratories, Inc., Boston, Mass.) allowed free access to food (Purina Rat Chow) and water were used in all experiments. Liver surface membranes were prepared according to the procedure of Neville (20) through step 12 as described by Pohl et al. (21).

The pellet from the centrifugation of the homogenate at 1,500 *g* for 10 min contained nuclei, surface membranes, and cell debris and is designated "nuclear pellet." Mitochondrial, lysosomal, and microsomal fractions were prepared from the supernate by a modification of the procedure of Evans and Gurd (22). The supernate was centrifuged at 100,000 *g* for 60 min, and the resulting pellet was dispersed in 0.25 M sucrose. This suspension was then separated into mitochondrial (8,000 *g* for 10 min), lysosomal (12,000 *g* for 10 min), and microsomal (105,000 *g* for 60 min) fractions. All procedures were performed at 0–4°C. Surface membrane and intracellular membrane fractions were washed once with both ice-cold 1 mM NaHCO<sub>3</sub> and 0.15 M NaCl to remove excess sucrose and loosely bound proteins. Fractions were then resuspended in 1 mM NaHCO<sub>3</sub> at a protein concentration of 12–15 mg per ml, stored at –70°C, and used within the following 48 h. The bile acid-binding activity was stable for at least 3 days when liver surface membranes were stored at –70°C.

**Enzyme analysis.** Glucose-6-phosphatase (EC 3.1.3.9) was measured by the method of de Duve et al. (23); 5'-nucleotidase activity (EC 3.1.3.5) by the method of Song and Bodansky (24); and (Na<sup>+</sup>,K<sup>+</sup>)-ATPase (EC 3.6.1.4) by the method of Ismail-Beigi and Edelman, after overnight freezing (25). Enzyme activities were determined by the initial rate of release of phosphate from appropriate substrates at 37°C and expressed as micromoles of phosphate released per milligram protein per hour. Cytochrome *c* oxidase activity (EC 1.9.3.1) was measured by the method of Straus (26) and expressed as micrograms of amine-hydrochloride formed per milligram protein per minute.

**Chemical and immunochemical determinations.** Protein was determined by the method of Lowry et al. (27) with bovine serum albumin as standard. Inorganic phosphorus was measured by the method of Fiske and Subba-Row (28). Sialic acid was determined by the method of Warren (29) with *N*-acetylneuraminic acid as standard.

Liver surface membranes solubilized in 1% Triton X-100 (vol/vol) (30) were tested for contaminating rat serum albumin by double immunodiffusion in agar (31), with rabbit antiserum to rat serum albumin and rat serum as a control.

**Binding assay.** The binding of radiolabeled bile acids was determined in an incubation medium that contained 66 mM monosodium phosphate/dissodium phosphate buffer (standard buffer), pH 6.0 and 7.4 when [<sup>14</sup>C]cholic acid binding was assayed and pH 7.4 when [<sup>14</sup>C]taurocholic acid binding was measured. The binding reaction was started by adding membrane fractions suspended in 1 mM NaHCO<sub>3</sub> to complete a reaction volume of 0.2 ml with a final protein concentration of 6.0–8.0 mg of protein per ml of incubation medium. The incubation mix was gently shaken in a con-

stant temperature bath at 4°C, and the binding reaction was terminated by vacuum filtration to separate membrane-bound from free <sup>14</sup>C-labeled bile acid. Glass-fiber disks (Whatman grade GF/C, W & R Balston Ltd., Maidstone, Kent, England) of 24-mm diameter, prewetted uniformly by soaking in buffer, were placed on 25-mm glass filter holders (Millipore Corp., Bedford, Mass.) fitted to an apparatus capable of subjecting three filter holders to simultaneous vacuum filtration and connected to a vacuum by a three-way stopcock. The filters were rinsed once with 1 ml of ice-cold standard buffer and freed of excess moisture by application of the vacuum. Triplicate 50-μl samples of the incubation mix were withdrawn from the assay tube and filtered rapidly at high filtration rate (2 ml/s). Filters with adsorbed membrane-[<sup>14</sup>C]bile acid complex were immediately washed four times with 1 ml of ice-cold standard buffer. Washed filters were removed from holders with the vacuum on, placed in scintillation vials, and covered with 0.3 ml of Protosol (New England Nuclear). After 12 h, 10 ml of Toluene-Omnifluor (New England Nuclear; containing 98% 2,5-diphenyloxazole and 2% *p*-bis-(*O*-methylstyryl)-benzene, 4 g per liter of Toluene) was added to each vial, and the radioactivity of the samples was determined in a Packard 2425 Tri-carb liquid scintillation spectrometer (Packard Instruments Co., Inc., Downers Grove, Ill.) at 80% efficiency. Quench was determined by automatic external standardization.

Free bile acid concentration was estimated by subtracting the total bile acid bound per sample from the total amount of bile acid present in the sample. Nonspecific interaction of bile acids with membrane fractions was determined after preincubation of the liver surface membranes at 37°C for 3 h to denature the specific bile acid binding sites. Since nonspecific binding varied in different surface membrane preparations and at different bile acid concentrations, nonspecific bile acid binding was determined in each experiment. Specific bile acid binding is total binding measured in a given experimental condition corrected for a nonspecific value determined under the same condition.

*Determination of binding constants.* Specific bile acid binding constants were determined from the plot of total bile acid binding versus free bile acid concentration according to the following equation (32):

$$b = \frac{N[c]}{K_a + [c]} + m[c]$$

In this equation, *b* is the total bile acid bound at the free bile acid concentration, [*c*]. The first term of the equation represents specific bile acid binding, where *N* (moles per milligram protein) is the maximal binding capacity of the system and *K<sub>a</sub>* (millimolar) is the apparent dissociation constant that represents the free bile acid concentration at which half-maximal binding takes place. The second term represents nonspecific binding where *m* is the slope of the least-squares straight line fitted to nonspecific binding, which appeared to be nonsaturable within the bile acid concentration range used in this study. The best-fit line for this model and the values for apparent *N*±1 SE and *K<sub>a</sub>*±1 SE were determined by a nonlinear least-squares regression program (33) on a CDC series 6000 computer (Control Data Corp., Minneapolis, Minn.).

*Tissue specificity of bile acid binding.* Liver, brain, skeletal muscle, and kidney were homogenized in 1 mM NaHCO<sub>3</sub> with a Dounce homogenizer, filtered first through two layers of cheesecloth, then four layers to remove connective

tissue, and spun at 100,000 *g* for 60 min. Human erythrocyte stroma ("ghosts") were prepared by one step hypotonic hemolysis according to Mitchell et al. (34). Resultant particulate fractions from homogenates and erythrocyte stroma were washed once with both ice-cold 1 mM NaHCO<sub>3</sub> and 0.15 M NaCl and resuspended in 1 mM NaHCO<sub>3</sub>. Aliquots of these suspensions were tested for binding activity by incubation with [<sup>14</sup>C]cholic acid (initial concentration, 2 mM) in standard buffer, pH 6.0, at 4°C for 20 min.

*Effect of medium and membrane modifications on bile acid binding.* The effect of sulfhydryl reagents and inhibitors of oxidative phosphorylation on specific cholic acid binding was determined by incubating liver surface membrane fractions with either DTT, NEM, DTNB, NaF, 2,4-dinitrophenol, or *p*-nitrophenol at the concentrations shown in Table III, in standard buffer, pH 6.0, at 4°C for 20 min. [<sup>14</sup>C]Cholic acid (1.2 mM) was added to the reaction mix, the incubation was prolonged for another 20 min at 4°C, and [<sup>14</sup>C]cholic acid binding was determined. A sodium-free medium was obtained by replacing the standard buffer with 66 mM monopotassium phosphate/dipotassium phosphate buffer, pH 6.0; the sodium concentration in standard buffer was increased by adding NaCl to the incubation medium.

The effects of EDTA, Ca<sup>2+</sup>, and Mg<sup>2+</sup> concentrations on specific binding were determined by incubating liver surface membranes with 10 mM EDTA, CaCl<sub>2</sub>, or MgCl<sub>2</sub> in 0.1 M Tris-maleate buffer, pH 6.0. Bile acid binding assayed in Tris-maleate buffer did not differ from that measured in standard buffer, pH 6.0. After 20 min at 4°C, [<sup>14</sup>C]cholic acid was added to the medium, the incubation was continued for another 20 min at 4°C, and binding was determined.

To characterize the chemical nature of the liver surface membrane receptors, the effect of enzymatic treatment of surface membranes on bile acid binding was examined. Membrane aliquots were preincubated with different concentrations of TPCK-trypsin, chymotrypsin, phospholipases A and C, and neuraminidase at 37°C for 20 min under the various conditions described in Table IV, after which [<sup>14</sup>C]cholic acid binding was assayed at 4°C.

*Statistical analysis.* Variances of best-fit lines obtained by nonlinear least-squares regression were compared by an *F* test. Significance of differences among estimated parameters was determined by a two-sample *t* test or a *Z* score (35). *P* values equal to or less than 0.05 were considered significant.

## RESULTS

*Liver surface membrane fractions.* It has previously been demonstrated that preparation of partially purified liver surface membranes according to Neville (20) separates a heterogeneous fraction of surface membranes enriched in bile canaliculi (21). This fraction yields 1.6±0.2 mg of membrane protein per g of wet weight of liver and contains 13.2 and 16.7% of total 5'-nucleotidase and (Na<sup>+</sup>, K<sup>+</sup>)-ATPase activities, respectively (Table I). Furthermore, both 5'-nucleotidase and (Na<sup>+</sup>, K<sup>+</sup>)-ATPase, which are considered markers of the surface membrane (36), demonstrate 18-fold enrichment in activity in the surface membrane fraction relative to homogenate. On the other hand, activity of glucose-6-phosphatase and cytochrome *c* oxidase, markers of the microsomal and mitochondrial fractions, respectively, represents less than

TABLE I  
Enzymatic Activity of Normal Rat Liver

Enzyme	Homogenate sp act	Partially purified surface membranes		
		Sp act	RSA*	Recovery†
				%
5'-Nucleotidase	5.0±0.5§	91.4±3.7§	18	13.2±1.5
(Na <sup>+</sup> , K <sup>+</sup> )-ATPase	2.0±0.4§	36.1±5.2§	18	16.7±0.7
Glucose-6-phosphatase	5.6±0.4§	6.0±1.4§	1	0.8±0.3
Cytochrome c oxidase	75.3±5.5	144.1±15.4	2	1.6±0.2

Enzymatic activity was determined as described in Methods, within 48 h of membrane preparation. Values are means±SEM.

\* RSA, relative specific activity (membrane/homogenate).

† Recovery, percent of total homogenate enzymatic activity present in the surface membrane fraction.

§ Micromoles of phosphate per milligram protein per hour.

|| Micrograms of amine-hydrochloride per milligram protein per minute.

2% of their total activities in homogenate and shows little if any enrichment in the surface membrane fraction.

Serum albumin, a soluble protein that binds bile acids, is a potential contaminant of the surface membrane preparation. However, no precipitation line was formed between solubilized liver surface membranes and rabbit antiserum to rat serum albumin in a double immunodiffusion assay in agar, indicating no detectable albumin contamination of the liver surface membrane fraction.

**Bile acid binding assay.** The binding of labeled bile acids to liver surface membrane fractions was determined by a rapid ultrafiltration technique using glass-fiber filters. Preliminary studies demonstrated that these filters retain more than 98% of membrane protein and bind less than 0.4% of the labeled bile acid after four washes (Fig. 1). On the other hand, when [<sup>14</sup>C]cholic acid is equilibrated with liver surface membranes before application to the filter, approximately 40% of the radioactivity remains on the filters after four 1-ml washes. This quantity, which is not reduced by further washing, will be referred to as "total binding." In contrast, only 3% of [<sup>14</sup>C]cholic acid is retained when it is equilibrated with liver surface membranes previously incubated at 37°C for 3 h to denature specific bile acid binding sites. This is referred to as "nonspecific binding." Specific bile acid binding, which is the difference between total and nonspecific binding, remains constant during the washing procedure, indicating that the equilibrium of the binding reaction is unaltered.

These studies demonstrate that specific membrane receptor-bile acid complexes are retained on glass-fiber disks during ultrafiltration. Adequate washing of the membrane receptor-bile acid complexes retained by the filters is necessary to decrease background radioactivity and nonspecifically bound bile acid, in order to obtain both sensitivity and reproducibility.

**Nonspecific binding.** Bile acid binding to liver surface membranes is susceptible to heat when incubated at 37°C at pH 7.4 (Fig. 2). Loss of binding activity determined after preincubation of surface membranes for various times demonstrates first-order decay kinetics for 2 h. Further incubation does not result in additional loss of binding activity, suggesting that residual binding is nonspecific, a conclusion that was confirmed by the non-

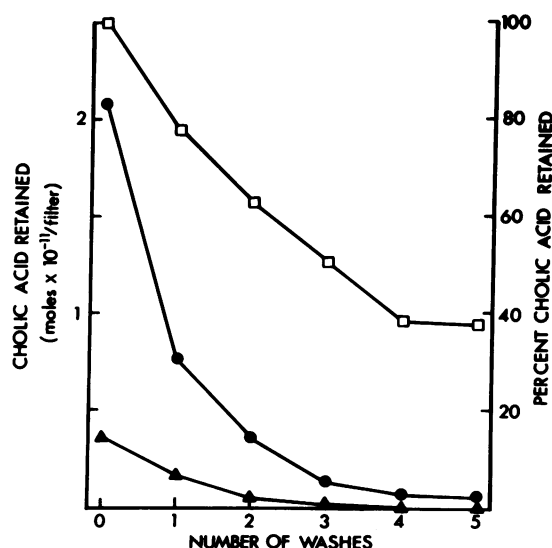


FIGURE 1 Membrane-bound and free cholic acid retained on GF/C filters as a function of the number of 1-ml washes. Total binding (□) and nonspecific binding (●) of [<sup>14</sup>C]cholic acid (initial concentration, 0.5 μM) were determined in standard buffer, pH 6.0, as described in Methods. Cholic acid adsorbed to GF/C filters (▲) was determined by filtration of aliquots of incubation medium in the absence of surface membranes. Filters were removed from the holders after the number of washes indicated.

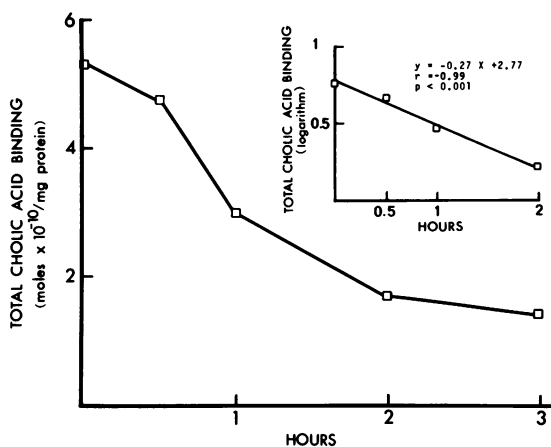


FIGURE 2 Effect of preincubation of liver surface membranes at 37°C on cholic acid binding. Liver surface membrane fractions suspended in 1 mM NaHCO<sub>3</sub> were incubated at 37°C for the time period indicated. [<sup>14</sup>C]Cholic acid was added (initial concentration, 10 μM), and total binding determined in standard buffer, pH 6.0, as described in Methods. Values were not corrected for nonspecific binding.

saturable nature of bile acid binding to these membranes (Fig. 6).

Temperature-dependent loss of bile acid binding to liver surface membranes was not prevented by EDTA, DTT, CaCl<sub>2</sub>, or MgCl<sub>2</sub>; however, incubation at 4°C

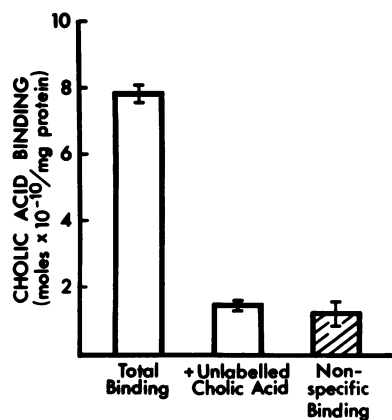


FIGURE 3 Reversibility of specific cholic acid binding to liver surface membranes. Membrane fractions were incubated with [<sup>14</sup>C]cholic acid (initial concentration, 20 μM) in standard buffer, pH 6.0, at 4°C for 20 min, and total cholic acid binding (first open column) was determined. Parallel reactions were performed in which a 200-fold excess of unlabeled cholic acid was added to the mixture after a preliminary incubation at 4°C for 20 min, and the incubation prolonged another 20 min (second open column). Nonspecific binding (dashed column) was determined by using membrane fractions preincubated at 37°C for 3 h. Bile acid binding was measured as described in Methods. Bars indicate means ± SEM.

prevented loss of binding capacity. Therefore, binding reactions were performed at 4°C. In addition, thin-layer chromatography of bile acids extracted with butanol from the incubation mix showed that binding does not result in detectable chemical changes of cholic and taurocholic acids.

In contrast to specific binding, nonspecifically bound [<sup>14</sup>C]cholic acid is not displaced by unlabeled bile acid. At equilibrium, the addition of a large excess of unlabeled cholic acid should release specifically bound [<sup>14</sup>C]cholic acid. As shown in Fig. 3, addition of a 200-fold excess of unlabeled cholic acid releases approximately 80% of the membrane-bound [<sup>14</sup>C]cholic acid. These studies demonstrate that nonreversible binding to liver surface membranes and binding to liver surface membranes preincubated at 37°C for 3 h are not significantly different and that nonspecific binding represents approximately 20% of total [<sup>14</sup>C]cholic acid bound to liver surface membrane fractions.

*Time-course of specific cholic acid binding to liver surface membranes.* Binding of [<sup>14</sup>C]cholic acid to liver surface membranes was measured after incubating aliquots of surface membranes with [<sup>14</sup>C]cholic acid at 4°C for different periods of times. As shown in Fig. 4, equilibrium is reached in 15 min and is stable for at least 60 min, indicating that bile acid-binding sites are unaltered under these conditions, in contrast to incubation at 37°C.

*Effect of pH on binding.* The effect of hydrogen ion concentration upon the interaction of [<sup>14</sup>C]cholic acid and its taurine conjugate [<sup>14</sup>C]taurocholic acid with liver surface membranes was examined to determine optimum conditions. As shown in Fig. 5, specific binding of cholic acid (pK = 4.98) is markedly influenced by the hydrogen ion concentration in the medium with a defined optimum at pH 6.0. Since the concentration of [<sup>14</sup>C]-

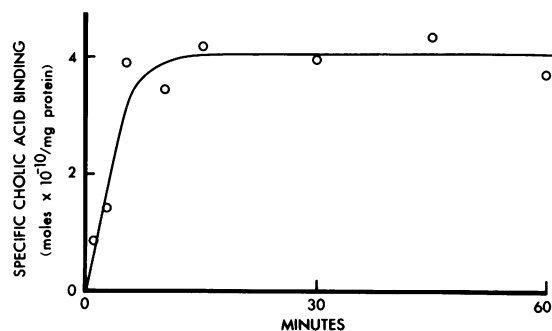


FIGURE 4 Time-course of specific cholic acid binding to liver surface membranes. Membrane fractions were incubated with [<sup>14</sup>C]cholic acid (initial concentration, 10 μM) in standard buffer, pH 6.0, for the time period indicated. Specific cholic acid binding was determined as described in Methods.

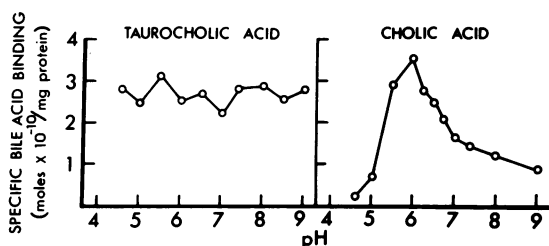


FIGURE 5 Effect of pH on specific bile acid binding to liver surface membranes. Membrane fractions were incubated with [ $^{14}\text{C}$ ]taurocholic acid (initial concentration, 20  $\mu\text{M}$ ) or [ $^{14}\text{C}$ ]cholic acid (initial concentration, 10  $\mu\text{M}$ ) in standard buffer at the pH indicated, at 4°C for 20 min. Specific bile acid binding was determined as described in Methods.

cholic acid (10  $\mu\text{M}$ ) used in this experiment is far from saturating, the data reflect primarily changes in the affinity of the receptors for cholic acid. However, because the solubility of cholic acid is decreased at acid pH, low binding activity at pH lower than 6.0 may result from precipitation of cholic acid (37). In addition, since the relative concentration of unionized cholic acid changes from approximately 10% at pH 6.0 to practically 0% at pH 9.0 (37), it is likely that the decrease of cholic acid binding with increasing pH is related to preferential binding of the unionized form of cholic acid to liver surface membranes. This suggests that hydrophobic interactions are probably more important than electro-

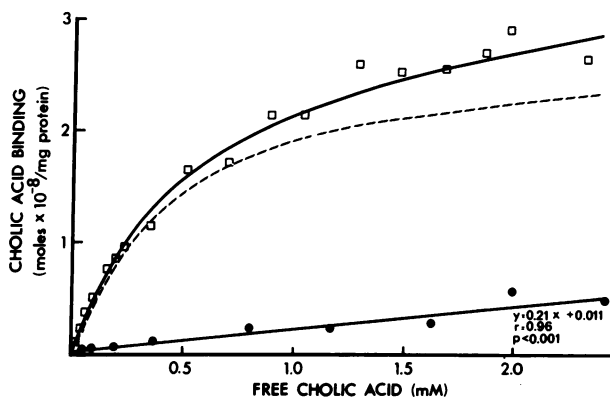


FIGURE 6 Binding of taurocholic acid to liver surface membranes as a function of cholic acid concentration. Membranes were incubated in standard buffer, pH 6.0, containing varying concentration of [ $^{14}\text{C}$ ]cholic acid. Total binding ( $\square$ ) and nonspecific binding ( $\bullet$ ) were determined as described in Methods. The best-fit line for total cholic acid binding was identified by nonlinear least-squares regression, and that for nonspecific binding by linear least-squares regression. Specific cholic acid binding (---) is the calculated difference between total and nonspecific binding. Binding constants  $\pm\text{SE}$  for specific cholic acid binding are:  $N = 28.44 \pm 0.62$  nmol per mg membrane protein, and  $K_d = 0.46 \pm 0.06$  mM.

static interactions in the binding process of this bile acid to membrane receptors. In contrast, specific binding of taurocholic acid ( $\text{pK} = 1.8$ ), which is almost completely ionized in this range of pH, is independent of pH in the range examined.

**Equilibrium studies.** The effect of increasing concentrations of free bile acid on binding to liver surface membranes is shown in Fig. 6 for [ $^{14}\text{C}$ ]cholic acid at its optimum pH 6.0. Total cholic acid bound to surface membranes increases in a nonlinear fashion with increasing free cholic acid concentrations; in contrast, nonspecific cholic acid binding increases linearly and does not demonstrate saturability within the range of concentrations used in this study. Hence, the calculated difference between total and nonspecific binding values (specific cholic acid binding) increases in a hyperbolic fashion consistent with a saturation of a limited number of bile acid binding sites. Binding constants could be identified by a nonlinear least-squares analysis of the rectangular hyperbola. Apparent maximal binding capacity  $N$  and dissociation constant  $K_d$  for cholic acid binding at pH 6.0 are  $28.44 \pm 0.62$  nmol per mg protein and  $0.46 \pm 0.06$  mM, respectively.

To determine whether homogeneous or heterogeneous cholic acid-binding sites are present in liver surface membranes, the ratio of specifically bound to free cholic acid was plotted against the amount bound per milligram protein, according to Scatchard (38) (Fig. 7). Linearization of the data by this method also gives estimates of  $K_d$ , which is derived from the slope of the line, and

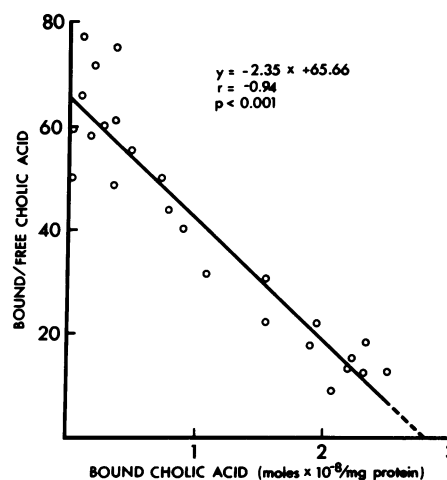


FIGURE 7 Modified Scatchard analysis of specific cholic acid binding to liver surface membranes. Plotted data correspond to specific cholic acid binding values determined from binding kinetics described in Fig. 6. The best-fit linear function was determined by least-squares regression. The value of  $N$ , estimated by extrapolating the line to the abscissa, is 27.9 nmol per mg membrane protein. The value of  $K_d$ , estimated as  $1/\text{slope}$ , is 0.43 mM.

$N$ , which is estimated from the extrapolation of the line to the abscissa. The data best fit one straight line ( $P < 0.001$ ) and thus suggest that a single class of noninteracting binding sites is involved. In addition, the apparent  $K_d$  of 0.43 mM and  $N$  of 27.90 nmol per mg protein obtained by this method agree well with those estimated above.

To determine whether unconjugated and conjugated bile acids interact similarly with the surface membrane receptors, we determined cholic and taurocholic acid binding kinetics at physiological pH. The relationship between the free concentration of taurocholic acid and binding to liver surface membranes is shown in Fig. 8. Specific taurocholic acid binding demonstrates saturation kinetics similar to that previously shown for cholic acid binding, and nonspecific binding is nonsaturable in the range of concentrations examined. Furthermore, Scatchard analysis of specific taurocholic acid binding again demonstrated a single class of noninteracting sites. The binding constants of specific cholic acid and taurocholic acid binding at pH 7.4 are compared in Table II. At this pH,  $N$  and  $K_d$  values are similar for specific cholic acid and taurocholic acid binding. In addition,  $K_d$  values indicate that membrane receptors are saturated at cholic acid and taurocholic acid concentrations below their critical micellar concentrations (37).

*Inhibition of specific cholic acid binding by bile acids and other organic anions.* To examine the ligand specificity of the surface membrane bile acid-binding sites, we incubated liver surface membranes with [ $^{14}$ C]cholic

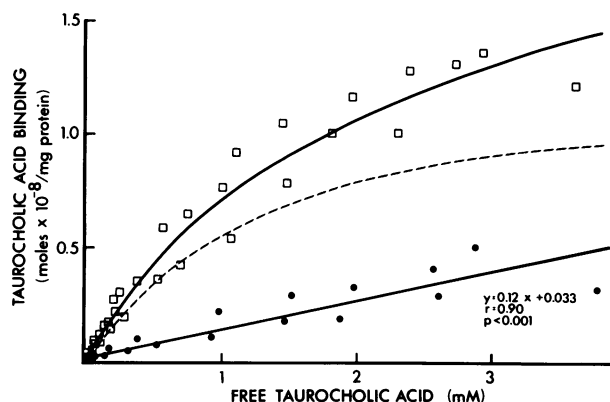


FIGURE 8 Binding of taurocholic acid to liver surface membranes as a function of taurocholic acid concentration. Membranes were incubated in standard buffer, pH 7.4, containing varying concentration of [ $^{14}$ C]taurocholic acid. Total binding ( $\square$ ) and nonspecific binding ( $\bullet$ ) were determined as described in Methods. The best-fit line for total taurocholic acid binding was identified by nonlinear least-squares regression, and that for nonspecific binding by linear least-squares regression. Specific taurocholic acid binding (---) is the calculated difference between total and nonspecific binding.

TABLE II  
Apparent Binding Constants for Specific Cholic and Taurocholic Acid Binding to Liver Surface Membranes

Bile acid	Maximal binding capacity ( $N$ )	Dissociation constant ( $K_d$ )
	nmol/mg membrane protein	mM
Cholic acid	$17.97 \pm 2.31$	$1.29 \pm 0.33$
Taurocholic acid	$14.08 \pm 1.47$	$1.39 \pm 0.26$
$P$ value	NS	NS

Kinetics of cholic and taurocholic acid binding to liver surface membranes was performed at pH 7.4, as described in Fig. 8. Binding constants  $\pm$ SE were estimated by a nonlinear least-squares regression analysis of the kinetic data.

acid, at the optimum pH 6.0, in the presence of unlabeled bile acids or unlabeled organic anions (Fig. 9). We choose 0.7 and 1.2 mM of unlabeled inhibitor to study inhibition because at higher concentrations of unlabeled bile acids in the presence of a constant and higher than  $K_d$  concentration of [ $^{14}$ C]cholic acid, precipitation occurred and micelles were formed.

It is apparent from this study that the various bile acids inhibit [ $^{14}$ C]cholic acid binding, suggesting that they interact with the same binding sites. In contrast

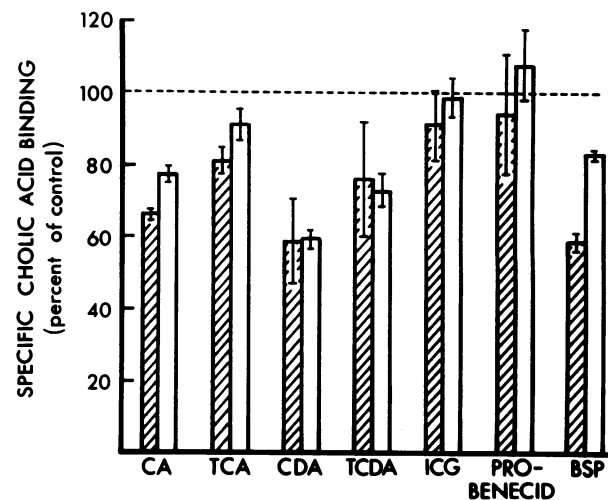


FIGURE 9 Inhibition of specific [ $^{14}$ C]cholic acid binding to liver surface membranes by various bile acids and organic anions. Surface membranes were incubated in standard buffer, pH 6.0, with either 0.7 mM (dashed column) or 1.1 mM [ $^{14}$ C]cholic acid (open column) in the presence of 0.6 mM unlabeled bile acids or unlabeled organic anions. Specific [ $^{14}$ C]cholic acid binding was determined as described in Methods. Bars represent means  $\pm$ SEM of those different experiments done in triplicate. CA, cholic acid; TCA, taurocholic acid; CDA, chenodeoxycholic acid; TCDA, taurochenodeoxycholic acid.



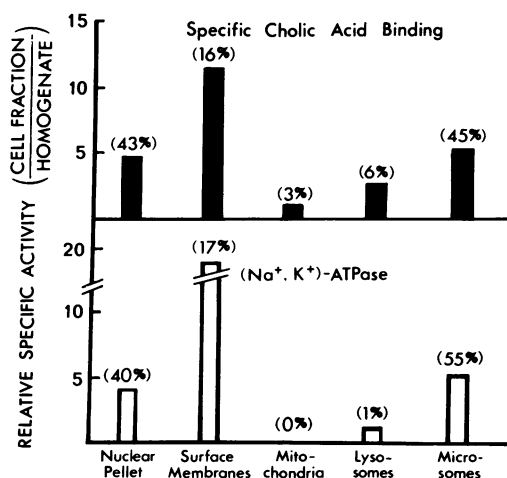


FIGURE 10 Relative specific cholic acid binding and (Na<sup>+</sup>, K<sup>+</sup>)-ATPase activities in liver subcellular fractions. Aliquots of liver homogenate, "nuclear pellet," surface membranes, and mitochondrial, lysosomal, and microsomal fractions were assayed for both binding of cholic acid (initial concentration, 1 mM) and (Na<sup>+</sup>, K<sup>+</sup>)-ATPase activity, as described in Methods. The values in parentheses represent percent recovery of specific cholic acid binding and (Na<sup>+</sup>, K<sup>+</sup>)-ATPase activity in each fraction.

ICG and probenecid, which are organic anions transported by the liver into bile (39-41), do not inhibit [<sup>14</sup>C]-cholic acid binding to surface membranes. However, BSP, an organic anion which has been shown to compete for hepatic uptake with bile acids (42, 43), inhibits [<sup>14</sup>C]cholic acid binding.

**Subcellular bile acid binding.** Since it was postulated that bile acids are transported across liver surface membranes by a carrier-mediated process, the subcellular distribution of the bile acid-binding process was examined in order to determine the primary subcellular location of specific bile acid-binding sites. Cell fractionation and surface membrane preparation were followed by measuring (Na<sup>+</sup>, K<sup>+</sup>)-ATPase activity. The low-speed "nuclear pellet," which is the first step in surface membrane isolation, demonstrates a fivefold increase in specific cholic acid binding relative to homogenate (Fig. 10). Further purification of the surface membranes increases binding 12-fold. In contrast, intracellular membrane fractions rich in mitochondria, lysosomes, and microsomes show less relative cholic acid binding than surface membranes. The distribution of (Na<sup>+</sup>, K<sup>+</sup>)-ATPase parallels relative cholic acid binding activity in subcellular fractions. Moreover, 16% of total specific cholic acid binding is recovered in the surface membrane fraction (Fig. 10), a value comparable to that for 5'-nucleotidase (13%) and (Na<sup>+</sup>, K<sup>+</sup>)-ATPase (17%) (Table I). Thus, specific bile acid binding increases with liver surface membrane purification and its subcellular distri-

bution corresponds with that of other markers of the surface membrane, providing evidence that location of the bile acid-binding process is in the surface membranes.

**Tissue specificity of specific bile acid binding.** Specific [<sup>14</sup>C]cholic acid binding was determined at a saturating concentration of this bile acid in tissues not known to transport bile acids and compared to liver and kidney binding. Neither brain or muscle homogenates demonstrate significant specific cholic acid binding (Table III). Similarly, red blood cell stroma ("ghosts") do not specifically bind cholic acid. In contrast, homogenate from kidney, an organ which has a tubular carrier-mediated transport mechanism for bile acids (44), demonstrates similar binding activity to that of liver homogenate. Thus, bile acid binding appears to be a property limited to tissues capable of bile acid transport.

**Effect of medium and membrane modification on bile acid binding.** Specific cholic acid binding appeared to be independent of the ionic composition of the incubation medium. As shown in Table IV, replacement of standard buffer with 0.1 M Tris-maleate buffer does not modify cholic acid binding. In addition, specific cholic acid binding is also independent of Na<sup>+</sup>, Ca<sup>2+</sup>, and Mg<sup>2+</sup> in the incubation medium.

Various sulfhydryl reagents and inhibitors of oxidative phosphorylation do not modify specific cholic acid binding to liver surface membranes, suggesting that thiol groups and disulfide are not involved at the active site for bile acid binding and that the binding reaction is independent of metabolic energy. In contrast, proteolytic digestion of liver surface membranes markedly reduces specific bile acid binding. As shown in Table V, cholic acid binding is extremely sensitive to low concentrations to TPCK-trypsin or chymotrypsin. This

TABLE III  
Specific Cholic Acid Binding to Various Tissues

Tissue	Specific cholic acid binding <i>nmol/mg protein</i>
Liver homogenate (5)	1.60 ± 0.63
Liver surface membranes (3)	23.4 ± 0.85
Erythrocyte stroma ("ghosts") (3)	0
Skeletal muscle homogenate (2)	0
Brain homogenate (3)	0.15 ± 0.10
Kidney homogenate (2)	1.38 ± 0.25

Aliquots of liver, skeletal muscle, brain and kidney homogenates, liver surface membranes, and erythrocyte stroma (6 mg of protein per ml) were incubated with [<sup>14</sup>C]cholic acid (initial concentration, 2 mM) in standard buffer, pH 6.0, for 20 min at 4°C. Specific cholic acid binding was determined as described under Methods. Values are means ± SEM. The numbers of experiments are shown in parentheses.

marked reduction in binding is certainly due to the proteolytic action of these enzymes since trypsin in the presence of soybean trypsin inhibitor does not modify cholic acid binding to liver surface membranes. Furthermore, although modest but definite digestion of membrane protein by 5  $\mu\text{g}/\text{ml}$  of trypsin (11.2%) and chymotrypsin (7.2%) was measured in the ultrafiltrate, loss of binding activity was far out of proportion to proteolysis.

Treatment of liver surface membranes with either phospholipase C or phospholipase A in Tris-maleate buffer results in a 60 and 40% reduction, respectively, in specific cholic acid binding. That the reduction in binding is due to lipolysis is supported by the observation that phospholipase C did not reduce binding in phosphate buffer and phospholipase A required  $\text{Ca}^{2+}$  in the medium to be effective (45). In addition, treatment of the isolated membranes with neuraminidase decreases specific cholic acid binding by 40–60%. In con-

TABLE IV  
*Effect of Sulfhydryl Reagents, Metabolic Inhibitors, and Various Medium Conditions on Specific Cholic Acid Binding to Liver Surface Membranes*

Experimental condition	Specific binding % of control
Standard buffer, no additions	100.0
+ DTT, 1 mM	99.6
+ DTNB, 1 mM	115.8
+ NEM, 0.02 mM	98.3
+ NaF, 10 mM	106.7
+ 2,4-Dinitrophenol, 0.25 mM	95.9
+ <i>p</i> -Nitrophenol, 3 mM	119.4
Tris-maleate buffer, no additions	105.6
+ EDTA, 10 mM	99.5
+ $\text{CaCl}_2$ , 10 mM	106.7
+ $\text{MgCl}_2$ , 10 mM	93.8
Sodium concentration: $\text{Na}^+$ -free medium	99.3
150 mM	112.6

Liver surface membrane aliquots (6 mg of protein per ml) were incubated for 20 min at 4°C in either standard buffer, pH 6.0, in the presence of the sulfhydryl reagents and metabolic inhibitors indicated, or in 0.1 M Tris-maleate buffer, pH 6.0, in the presence of EDTA,  $\text{Ca}^{2+}$ , and  $\text{Mg}^{+2}$ . To obtain a  $\text{Na}^+$ -free medium the standard buffer was replaced with 66 mM monopotassium phosphate/dipotassium phosphate buffer, pH 6.0, and NaCl was added to the incubation medium to obtain 150 mM  $\text{Na}^+$  concentration. After preliminary incubation, [ $^{14}\text{C}$ ]cholic acid was added to the medium (initial concentration, 1.2 mM) and the incubation was continued for 20 min at 4°C. A control assay was performed in standard buffer, without additions, under the same incubation conditions. Specific cholic acid binding was determined as described in Methods. Results are expressed as percent of control specific binding activity.

TABLE V  
*Effect of Enzymatic Digestion of Liver Surface Membranes on Specific Cholic Acid Binding*

Enzymatic digestion	Specific binding % of control
TPCK-trypsin,* in standard buffer	
0.5 $\mu\text{g}/\text{ml}$	25.9
5 $\mu\text{g}/\text{ml}$	5.5
10 $\mu\text{g}/\text{ml}$	0.8
5 $\mu\text{g}/\text{ml}$ + soybean trypsin inhibitor, 15 $\mu\text{g}/\text{ml}$	101.0
Chymotrypsin, in standard buffer	
5 $\mu\text{g}/\text{ml}$	10.6
50 $\mu\text{g}/\text{ml}$	6.5
Phospholipase C, 200 $\mu\text{g}/\text{ml}$ , in standard buffer	91.5
Phospholipase C, in Tris-maleate buffer	
60 $\mu\text{g}/\text{ml}$	37.0
200 $\mu\text{g}/\text{ml}$	40.8
Phospholipase A, in Tris-maleate buffer + 10 mM $\text{CaCl}_2$	
30 $\mu\text{g}/\text{ml}$	60.6
200 $\mu\text{g}/\text{ml}$	60.5
Neuraminidase, 100 $\mu\text{g}/\text{ml}$ , in standard buffer†	105.2
Neuraminidase, in Tris-maleate buffer	
100 $\mu\text{g}/\text{ml}$	60.3
200 $\mu\text{g}/\text{ml}$	40.5

Liver surface membrane aliquots (12 mg of protein per ml) were incubated for 20 min at 37°C in 10 mM standard or Tris-maleate buffer, pH 7.4, with the various enzymes at the concentration indicated. The digested membrane suspensions were diluted with 1 vol of 0.26 M standard or Tris-maleate buffer, pH 6.0, and incubated for 20 min at 4°C in the presence of 1.2 mM [ $^{14}\text{C}$ ]cholic acid. Control assays were performed under the same buffer and incubation conditions without enzyme additions. Specific cholic acid binding was determined as described in Methods. Results are expressed as percent of control specific binding activity.

\* Contains no chymotryptic activity according to manufacturer.

† Membrane-bound sialic acid was not released by neuraminidase after incubation in standard buffer, pH 7.4, for 20 min at 4°C.

trast, no effect is apparent in phosphate buffer, a condition that does not result in release of sialic acid from the membrane. In addition, since these experiments were performed with a saturating concentration of [ $^{14}\text{C}$ ]cholic acid (1.2 mM), data reflect primarily changes in the concentration of binding sites in liver surface membranes. Protein retention after treatment of membranes with phospholipases A (30  $\mu\text{g}/\text{ml}$ ) and neuraminidase (200  $\mu\text{g}/\text{ml}$ ) was higher than 99% of the protein initially present in the incubation mix. These results suggest that the effect of neuraminidase and phospholipases on cholic acid binding is not due to contamination with proteolytic enzymes or due to solubilization of receptors and subsequent loss during ultrafiltration, but rather to the specific effect of these enzymes. Thus, bile acids appear to bind specifically to a protein located in the surface membrane, which probably requires sialic acid and phospholipids for optimal binding activity.

## DISCUSSION

In the past few years binding studies have provided a way to identify membrane-bound receptors for a variety

of molecules that either initiate a metabolic function at the surface membrane of the cell (hormones) (46, 47) or are transported into or out of the cell across the surface membrane, presumably by carrier-mediated processes (17, 18). In models of carrier-mediated transport the receptor is believed to represent the recognition site in the "carrier" structure, and it is generally assumed that binding of the ligand to the receptor is the first step in translocation (17). Therefore, direct measurement of the ligand-receptor complex allows the quantitation of this first step and the study of the site, properties, and mechanisms of the primary interaction without having to observe the final effect of that interaction in the whole system. However, since separation methods of receptor identification depend only on the binding function of the receptor and, in fact, may measure any interaction of the ligand with the membrane, different requirements must be satisfied before this interaction can be attributed to the hypothesized carrier-mediated transport process. For example, the binding must be reversible, demonstrate saturability, and be primarily located in the surface membrane of the cell; must not involve chemical transformation of the ligand; must exhibit an affinity of the receptor for the ligand comparable to that of the transport process; and must demonstrate inhibition by related compounds reflecting the specificity of the transport mechanism (18, 47-49).

A rapid and sensitive ultrafiltration technique was developed by using glass-fiber disks, high filtration rates, and small volumes of buffer to wash the filters, which permitted us to measure bile acid binding to isolated membranes without disturbing the equilibrium of the binding reaction. However, this method may measure nonspecific interactions of labeled bile acid with membranes as well as specific binding. To estimate nonspecific binding we took advantage of the observation that incubation of surface membrane fractions at 37°C for 3 h reduces total bile acid binding to approximately 20%. In contrast to specific binding, residual binding is nonsaturable and not displaced by an excess of unlabeled bile acid. Since similar characteristics have been described for nonspecific interactions of other ligands with isolated membranes (50, 51), we assumed that it represents nonspecific bile acid binding.

As expected from the steady-state kinetics demonstrated for hepatic bile acid transport (13, 15, 16), specific binding was rapid and freely reversible. Furthermore, complex formation did not result in detectable chemical alterations of the bile acid molecule. Thus, these studies indicate that this interaction is an equilibrium process involving association and dissociation between the bile acid and the receptor and does not involve an enzymatic reaction.

Optimal binding of cholic acid at pH 6.0 may be due to changes in membrane lipids, receptor conformation

and stability, or changes in the degree of ionization of the bile acid molecule. Since taurocholic acid binding is unaltered over the pH range examined, the first two possibilities appear unlikely. On the other hand, the degree of cholic acid ionization and solubility are markedly decreased below pH 6.0 because of its relatively high pK (4.98); in contrast, taurocholic acid has a very low pK (1.8), and it is almost completely ionized in this pH range (37).

Analysis of the relationship between free concentration of bile acid and specific binding indicates that both cholic and taurocholic acid binding demonstrate saturability and is consistent with Michaelis-Menten kinetics. This suggests a finite number of receptors, in agreement with the first-order kinetics previously shown for bile acid transport (13, 15, 16). In addition, analysis of binding data using a Scatchard plot suggests that cholic acid as well as taurocholic acid bind to a single and homogeneous class of receptors that do not interact with each other.

Hepatic removal of bile acids from plasma is remarkably efficient (12) and more efficient than the extraction of other organic anions (52). Although half-saturation constant values ( $K_s$ ) for biliary secretion of bile acids have not been determined,  $K_s$  for taurocholic acid uptake in isolated perfused rat liver has been reported to be 0.7 mM (16), a value not too different from the apparent  $K_s = 1.39$  mM for specific taurocholic acid binding to liver surface membranes. Furthermore, estimated  $K_s$  values are below reported values for cholic and taurocholic acid critical micellar concentrations (37), indicating that these bile acids interact with the receptor as monomers rather than as polymolecular aggregates.

Steady-state maximal secretory rate of bile acids exceeds the usual demands imposed by the enterohepatic load of bile acids (7, 12, 13) and, in addition, is greater than maximal secretory rates of other organic anions (53, 54). Furthermore, studies on the uptake of bile acids by rat and dog liver have shown that the maximal rate of taurocholic acid uptake is 5-10 times higher than the maximal secretory rate into the bile (15, 16), demonstrating the enormous capacity of the bile acid transport process. In agreement with these observations, apparent maximal capacity values for cholic and taurocholic acid binding of 17.97 and 14.08 nmol per mg of membrane protein at pH 7.4, respectively, indicate a high concentration of bile acid receptors in isolated liver surface membranes.

Unlabeled cholic and chenodeoxycholic acid and their taurine conjugates were found to inhibit [ $^{14}$ C]cholic acid binding to liver surface membranes, which suggests that these bile acids interact with the same class of receptors. In contrast, ICG and probenecid, two organic anions transported by the liver into bile, do not inhibit cholic acid binding. Furthermore, ICG has been shown

not to interfere with bile acid transport (55, 56). BSP was found to inhibit [<sup>14</sup>C]cholic acid binding in agreement with the observation that this organic anion competes with bile acids for uptake by the liver (42, 43) and supporting the view that bile acids and BSP may share in part a common transport step (43, 54, 57). Therefore, these studies suggest that bile acid surface membrane receptors have dissociation constants and ligand specificities comparable to those previously shown for hepatic bile acid transport and support the hypothesis that bile acid binding to these receptors may represent the initial interaction in the transport process.

It is generally assumed that the "carrier" for bile acid transport is localized in the surface membrane of the hepatocyte (7, 15, 16, 43, 57). Accordingly, bile acid binding activity increases with purification of liver surface membranes. Moreover, bile acid-binding activity copurifies with the established surface membrane marker enzyme (Na<sup>+</sup>, K<sup>+</sup>)-ATPase. However, approximately 50% of both binding and (Na<sup>+</sup>, K<sup>+</sup>)-ATPase activity is recovered in the microsomal fraction, an observation consistent with recent cell fractionation studies demonstrating that at least 70% of surface membranes do not sediment in the first step of purification, most of which are recovered later in the microsomal fraction (58). Thus, the data suggest that bile acid receptors are primarily located in liver surface membranes and are consistent with their postulated role in bile acid transport.

Absence of binding activity in skeletal muscle and red blood cell "ghosts" and low activity in brain homogenate, tissues not known to transport bile acids, strongly suggest that specific bile acid-binding sites are limited to tissues which have a defined transport mechanism for bile acids. On the other hand, high binding activity in kidney homogenate, similar to that measured in liver homogenate, might be related to the fact that kidney has a tubular bile acid-reabsorptive transport mechanism which is probably carrier mediated and active (44). In addition, many transport functions in kidney and liver demonstrate similar characteristics (59).

Specific bile acid binding is independent of Na<sup>+</sup>, Ca<sup>2+</sup>, and Mg<sup>2+</sup> and does not require metabolic energy since NaF, 2,4-dinitrophenol, and *p*-nitrophenol do not modify the binding process. In addition, since DTT, NEM, and DTNB do not affect binding, thiols and disulfide are probably not required for activity at the receptor site.

The marked sensitivity of bile acid receptors to trypsin and chymotrypsin resembles the effects of these enzymes on other surface membrane receptors (51, 60-63). In addition, after neuraminidase treatment to release membrane sialic acid, specific bile acid binding was also decreased. Therefore, these studies suggest that the bile acid receptor in liver surface membranes is a glycoprotein.

Specific bile acid binding was decreased by phospholipase A and C digestion of liver surface membrane phospholipids. Although phospholipase A modifies the apolar region of phospholipids whereas phospholipase C modifies the polar head groups, similar inhibition of binding activity was observed. These effects may result from alterations of the lipid environment of the receptor; however, it is difficult to differentiate between specific effects of the enzymes and secondary effects due to products of the reaction.

The hepatocyte has a defined polarity that is represented by regional specialization of the surface membrane in areas for uptake and excretion (64). Although it has been postulated that both bile acid uptake and excretion are membrane-carrier mediated processes (7, 15, 16, 57), the inaccessibility of hepatic sinusoids and bile canaliculi has prevented accurate determinations of transport kinetics across both the sinusoidal and canalicular portions of the hepatocyte surface membrane. Furthermore, Neville's as well as other methods of surface membrane isolation separate a heterogeneous population of liver surface membranes, relatively enriched in bile canaliculi but also containing sinusoidal membranes (21, 58). Therefore, it is not possible to determine whether the bile acid receptor is preferentially located in the sinusoidal or canalicular membrane or whether it is present in both areas of the hepatocyte surface membrane. Although the capacity for hepatic uptake of organic anions is many times greater than the maximal rate of biliary excretion (15, 16, 65), this does not necessarily imply that different carrier-mediated processes are involved. Indeed, hepatic transport of BSP shows  $K_m$  values for uptake and biliary excretion in the same order of magnitude (0.72 and 0.2-0.4 mM, respectively) (56, 65). Thus, both processes might be mediated by "carriers" with similar or identical kinetic properties. If receptors are equally distributed in both sinusoidal and canalicular portions of the hepatocyte surface membrane, the different uptake and excretory capacities might be explained by the fact that the canalicular membrane has been estimated to represent only 13% of the total surface membrane area (66).

Binding studies represent a fruitful way to identify membrane-bound receptors and investigate the transport function of the liver. Further study of the initial interactions of bile acids with isolated liver surface membranes may provide insight into the basic mechanisms of bile acid transport by the liver and its role in the pathogenesis of cholestatic syndromes.

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