Comparative binding of bile acids to serum lipoproteins and albumin

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Abstract Characteristics of the binding of lithocholic acid (LC), chenodeoxycholic acid (CDC), and cholic acid to human plasma proteins were studied. Affinity of the different plasma protein fractions for the bile acids studied decreased with increased polarity of the steroid nucleus of the bile acid. Binding of LC, CDC, and cholic acid to the lipoprotein-free, albumin-rich plasma fraction was characterized by two classes of binding sites with respective K_Ds of 2, 5, and 51 μM, and of 39, 2,387, and 5,575 μM, while corresponding B_{max} values were similar for the different bile acids, at around 6 and 100 nmol/mg protein. Bile acid binding to the different lipoprotein fractions was characterized by a single population of binding sites, with a K_D ranging from 47 to 66 μM for LC, 695 to 1010 μM for CDC, and 2,511 to 2,562 μM for cholic acid. B_{max} values, at 416-913 nmol/mg protein, were similar among the different bile acids studied. Both glycine- and taurine-conjugated, as well as unconjugated LC competitively inhibited [24-^{14}C]LC binding to low density (LDL) and to high density lipoproteins (HDL) to the same extent, while the more polar LC-3-sulfate, CDC, and cholic acid were increasingly less potent in displacing LC binding from the respective lipoproteins. Furthermore, all bile acids studied shared the same lipoprotein binding site. The lipoprotein fluorescence at 330-334 nm, following excitation at 280 nm, was diminished after incubation with LC, suggesting that the bile acid masks the tryptophan residues of the protein moiety. Finally, the initial rate of uptake of 1 μM LC, in isolated hamster hepatocytes, at around 0.045 nmol · sec^{-1} · mg cell wt^{-1}, was not affected by the protein carrier. However, for the same concentration of LC, bound to either LDL or HDL, LC binding resulted in 75-77% of the total [24-^{14}C]LC nonspecifically bound to the hepatocyte, compared to 65% when bound to albumin, and 45% in the absence of protein. The studies show that, under conditions when the serum bile acid concentration exceeds the capacity of the high affinity class of albumin binding sites for bile acids, lipoproteins have similar or greater affinity to bind bile acids than does albumin. The ability of lipoproteins to increase the nonspecific association of lithocholic acid with liver cells may also facilitate bile acid association with extrahepatic tissues. As lipoproteins, in contrast to albumin, are targeted to most cells, they may play a major role in the transport of potentially toxic bile acids to peripheral cells.—**Ceryak, S., B. Bouscarel, and H. Fromm.** Comparative binding of bile acids to serum lipoproteins and albumin. *J. Lipid Res.* 1993. **34:** 1661-1674.

Supplementary key words lithocholic acid • cholic acid • chenodeoxycholic acid • HDL • LDL • carrier proteins

Bile acid transport from the intestine to the liver via the portal circulation represents an integral phase of the enterohepatic circulation (1-3). Fasting bile acid concentrations in the systemic circulation at 2-3 μM, are around 6-fold lower than those in the portal circulation, reflecting the efficient extraction of bile acids by the liver under normal physiological conditions (4). However, significant spillover of bile acids from the portal to the systemic circulation can occur, especially in hepatobiliary disorders (5, 6), including alcoholic liver cirrhosis (7, 8), chronic active hepatitis (9), extrahepatic cholestasis (8), idiopathic portal hypertension, and hepatic vein occlusion (10), resulting in 10- to 100-fold higher systemic bile acid concentrations. The majority of these bile acids consisted of amidated derivatives of chenodeoxycholic acid (CDC) and cholic acid (5, 6), while slight increases in serum lithocholic acid (LC) have also been reported (7, 11, 12). Furthermore, in patients with stagnant loop syndrome, increased serum bile acid levels were mainly attributable to increases in unconjugated bile acids, probably due to the reabsorption of large quantities of unconjugated bile acids in the small intestine of these patients (13).

In the bloodstream, transport of bile acids is facilitated by their binding to serum proteins (14). The major bile acid carrier is albumin, although a portion of bile acids has been isolated from plasma bound to lipoproteins (14-16). Under conditions of liver dysfunction and cholestasis, however, significantly increased levels of lipoprotein-bound CDC and cholic acid, as well as trace levels of LC and deoxycholic acid have been reported

Abbreviations: LC, lithocholic acid; TLC, taurolithocholate; CDC, chenodeoxycholic acid; GLC, glycolithocholate; LFAR, lipoprotein-free, albumin-rich; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins.

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(16–18). As lipoproteins, in contrast to albumin, are internalized by most tissues, they may serve to target bile acids to extrahepatic tissues. This could be a mechanism responsible for the high concentrations of these bile acids that have also been found in tissues of patients with extrahepatic cholestasis (19).

Early studies by Rudman and Kendall (14) established a positive correlation between the decreased hydroxylation of bile acids and their ability to bind to albumin. This was further corroborated in more recent studies by Roda et al. (20), in which the apparent binding constants of human serum albumin for different bile acids were quantitated. Studies by Salvioli et al. (21) extended the correlation between bile acid hydrophobicity, as evaluated by octanol–water partitioning, and protein binding to include plasma lipoproteins. In in vivo studies, Cowen et al. (22) demonstrated that 87–99% of unconjugated, amidated, and sulfated forms of LC were tightly bound to serum proteins after intravenous injection. Studies by Malavolti et al. (23) from our laboratory showed that significant amounts of LC bound to all classes of lipoproteins. Similarly, recent in vivo and in vitro studies by Hedenborg et al. (24) described the rapid distribution of CDC and cholic acid, as well as their respective derivatives, among serum lipoprotein and nonlipoprotein fractions of cirrhotic patients. Although several reports describe the relative distribution of LC, CDC, and cholic acid among the different plasma protein fractions, there are few studies to date that quantitate the comparative binding of bile acids to plasma lipoproteins and to albumin.

Detailed knowledge concerning the kinetics of bile acid binding to plasma lipoproteins and albumin appears desirable in light of the various mechanisms through which certain bile acids may express cytotoxic as well as tumorigenic properties. Specifically, it is necessary to study the comparative quantitative importance of lipoproteins and albumin as carriers of mono-, di-, and trihydroxy bile acids. The aim of the present study, therefore, was to assess the characteristics of the binding of unconjugated, amidated, and sulfated forms of LC, as well as of CDC and cholic acid, to plasma lipoproteins and albumin.

METHODS

Materials

Lithocholic acid (LC), its taurine (TLC) and glycine (GLC) conjugates, as well as chenodeoxycholic acid (CDC) and cholic acid were obtained from Steraloids (Wilton, NH) as free acids and were 98–99% pure as determined by gas-liquid chromatography. [24-^{14}C]LC (sp act 55 mCi/mmol) was purchased from Amersham Corporation (Arlington Heights, IL), and was more than 98% pure by thin-layer chromatography. Both [24-^{14}C]CDC (sp act 50 mCi/mmol) and [24-^{14}C]cholic acid (sp act 52 mCi/mmol) were purchased from DuPont NEN Research Products (Boston, MA), and were 98% and 99.9% pure, respectively, as judged by thin-layer chromatography. All other reagents used were of analytical grade available from commercial sources.

Separation of plasma lipoprotein and albumin fractions

Blood from fasting healthy human subjects and from male Golden Syrian hamsters (100–120 g body weight) was collected into sterile glass tubes containing 0.15% EDTA. Plasma was obtained after centrifugation at 300 *g* for 4 min at 4°C. Plasma lipoprotein and albumin fractions were separated by density gradient ultracentrifugation, according to the method of Redgrave, Roberts, and West (25) as previously described (26, 27). The different plasma protein fractions were removed by needle aspiration of layers corresponding to the following densities: very low density lipoproteins (VLDL), d < 1.006 g/ml; low density lipoproteins (LDL), d 1.019–1.063 g/ml; high density lipoproteins (HDL), d 1.063–1.210 g/ml; and the lipoprotein-free, albumin-rich fraction (LFAR), d > 1.210 g/ml (28). The individual lipoprotein- and albumin-containing fractions were dialyzed for 12 h at 4°C against 0.15 M NaCl, 0.01 M Tris, 0.01 M EDTA, 0.001 M NaN_3, pH 7.4.

The purity of the different protein fractions was assessed by SDS-PAGE according to the method of Irwin et al. (29), followed by silver staining (30). Albumin comprised more than 90% of the proteins associated with the LFAR fraction. No attempt was made to identify the other protein components of this fraction. However, distinct protein bands were observed at apparent molecular weights of 12, 14, 17, 23, 56, and 76 kDa. The total protein concentration of the different lipoprotein and LFAR fractions was determined by the method of Bradford (31), using the Bio-Rad® protein assay (Bio-Rad Laboratories, Richmond, CA). Enzymatic assays were used to measure cholesterol and triglyceride (BioDynamics, Boehringer Mannheim, Indianapolis, IN) as well as phospholipid (Nippon Shoji Kaisha Ltd., Higashi-Ko, Osaka, Japan) concentrations. The concentrations of these components were virtually identical to those described in the literature (28). Furthermore, no difference in lipoprotein particle composition was observed after bile acid binding (23, 27).

Equilibrium dialysis

Equilibrium dialysis was used to assess the binding of LC, CDC, and cholic acid to the plasma lipoprotein and albumin fractions (20, 21, 23). Stock solutions of the sodium salt of the respective bile acids were prepared fresh in the morning of each experiment and were diluted accordingly with 0.15 M NaCl, 0.01 M Tris, 0.001 M NaN_3, pH 7.6 (dialysis buffer), in order to achieve working dialysate solutions with final concentrations ranging from 0.05

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to 150 μM for LCA, and 0.1–2400 μM for both CDC and CA. The concentrations of unlabelled bile salt in the dialysate solutions were confirmed by gas–liquid chromatography (32).

Fifteen milliliters of the respective bile salt solution, containing 0.05 μCi of the sodium salt of the appropriate ^{14}C-labeled bile acid, was pipetted into 20-ml scintillation vials. Aliquots of 300 μl of each lipoprotein or LFAR fraction were pipetted into nitrocellulose dialysis bags (Spectra Por, MW cutoff, 6,000–8,000), which were suspended in the vials and incubated at 37°C for the indicated times in a shaking incubator at 140 strokes per min. Prior to use, the nitrocellulose dialysis tubing was initially boiled for 1 h in 1 mM EDTA, containing 0.1% Na_2CO_2, followed by extensive washing with H_2O and a final wash with 0.15 M NaCl.

At indicated periods of time, duplicate aliquots of the dialysand and dialysate solutions were taken for determination of ^{14}C radioactivity by scintillation counting. The respective lipoprotein- and albumin-bound ^{14}C-labeled bile salt was calculated from the difference at equilibrium between ^{14}C in the dialysis bag (free+bound bile salt = total) and ^{14}C in the dialysate (free bile salt). From the initial ^{14}C bile salt concentration, both the molar concentration of free bile salt and the moles of bile salt bound per unit of protein were calculated, as described later for the determination of the apparent binding constants.

Binding studies

Determination of apparent binding constants. Initially, experiments were carried out to determine the time of equilibration of bile salt (0.05 and 100 μM [24-^{14}C]LC) between the incubation medium and the interior of incubated dialysis bags, containing only dialysis buffer. The concentration of lipoprotein and LFAR fractions, at which the binding of LC, CDC, and cholic acid is linear, was evaluated by studying the binding of 0.05 μM of the respective ^{14}C-labeled bile salt to increasing concentrations of protein (0.005–50 mg/ml) after 4–20 h incubation. The effect of incubation time on the stability of bile salt binding to plasma lipoprotein and albumin fractions was studied, and unless otherwise indicated, a 6–18 h incubation period was used for all other binding studies.

The concentration-dependent binding of LC, CDC, and cholic acid was studied by incubating 50, 500, and 700 μg protein/ml, respectively, of the different lipoprotein fractions, and 500, 2000, and 4000 μg/ml, respectively, of the LFAR fraction, with increasing concentrations of ^{14}C-labeled LC (0.15–150 μM), CDC (0.5–2400 μM), and cholic acid (0.5–2400 μM). Saturation binding curves were derived by plotting the amount of ^{14}C-labeled bile salt bound per mg of protein on the y axis against the concentration of unbound bile salt on the x axis. The data were analyzed according to the method of Scatchard (33) by linear and nonlinear curve fitting. The maximum number of binding sites (B_{max}, nmol/mg protein) and the dissociation constant (K_D, μM) for one class of binding sites were derived from the equation of the regression line. The apparent binding constants for two classes of binding sites were determined by computer analysis using the EBDA/LIGAND program, as modified for microcomputers (34).

Competitive inhibition of LC binding to LDL and HDL. The ability of LC, TLC, GLC, LC-3-sulfate, CDC, and cholic acid to inhibit [24-^{14}C]LC binding to LDL and HDL, respectively, was studied. In these experiments, the dialysate solutions contained increasing concentrations of the different bile salts in addition to 0.05 μCi [24-^{14}C]LC. The effective concentration of the respective bile salt at which 50% of the [24-^{14}C]LC remained specifically bound (EC_{50}) to either LDL or HDL was determined by plotting the percentage of maximum [^{14}C]LC bound on the y axis, against the negative log of the respective bile acid concentration on the x axis. The dissociation constant for the competitor (K_I) was calculated from the EC_{50} using the method of Cheng and Prusoff (35) as follows: $K_I = EC_{50}/((1 + ([L]/K_D))$, where [L] is the concentration of the [24-^{14}C]LC present in the incubation medium, and K_D is the equilibrium dissociation constant for the radioligand, as determined from the saturation binding experiments.

Effect of delipidation on the release of bound LC from plasma lipoproteins and albumin. After incubation of the respective lipoprotein (50 μg protein/ml) and LFAR (500 μg/ml) fractions with 80 μM [24-^{14}C]LC, lipoprotein-bound LC was separated from unbound LC by ultracentrifugation and reflotation of the VLDL, LDL, and HDL fractions at their respective densities (28). The LC bound to the LFAR fraction was separated from the unbound LC by dialysis of the protein fraction for 5 h against dialysis buffer, with five buffer changes.

Bound [^{14}C]LC was quantitated by scintillation counting, and aliquots of the different protein fractions were delipidated with 20 volumes of chloroform–methanol 1:1 (v/v). After 3 h incubation at 4°C, the solubilized protein fractions were centrifuged at 300 *g* for 3 h at 4°C. The organic layer was gently removed, taking care not to disturb the protein pellet. The ^{14}C content of the protein pellets was determined by scintillation counting. Replicate samples of the delipidated protein fractions were used to determine protein, cholesterol, and phospholipid content, as previously described. Lipoprotein cholesterol and phospholipid were not detectable after delipidation.

Determination of tryptophan fluorescence in lipoprotein and LFAR fractions. The fluorescence of the different lipoprotein fractions was measured at 20°C, and at 310–360 nm, after excitation at 280 nm, using a Perkin-Elmer LS3 spectrofluorometer (Perkin-Elmer, Norwalk, CT) both before and after binding of 50 μM LC, as described by Roda et al. (20) and Pico and Houssier (36).

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Hepatocellular uptake of LC

Isolation of hepatocytes. Hepatocytes were isolated from male Golden Syrian hamsters (100-120 g) by perfusion of the liver with collagenase, as previously described (27, 37). Isolated hepatocytes were suspended at a final concentration of 40-50 mg wet wt/ml of Krebs-Henseleit bicarbonate buffer containing 118 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 25 mM $NaHCO_3$, pH 7.4. Prior to each experiment, the cells were incubated for 20-30 min at 37°C under constant agitation and gassing with O_2-CO_2 95:5 (v/v) to allow the cell to reach steady state. Cell viability was assessed by trypan blue exclusion, and only cell suspensions with >90% viability were used.

Effect of LDL, HDL, and albumin on the uptake of LC. Both LDL and HDL, isolated from hamster plasma (approximately 1 mg/ml), as well as hamster albumin (10 mg/ml, Research Plus, Bayonne, NJ), respectively, were incubated with 5 μCi [24-^{14}C]LC for 3 h at 37°C. [24-^{14}C]LC bound to the lipoprotein fractions was separated from unbound labeled LC by ultracentrifugal reflotation at respective densities of 1.063 g/ml and 1.210 g/ml, for LDL and HDL (28). Albumin-bound ^{14}C-labeled LC was separated from unbound labeled LC by dialysis for 5 h against dialysis buffer with five buffer changes.

Isolated hamster hepatocytes were incubated at 37°C with [24-^{14}C]LC (control), or with [24-^{14}C]LC bound to LDL and HDL (10 μg protein/ml), respectively, as well as to albumin (10 and 100 μg/ml). Cell aliquots were removed at 20, 30, 40, 50, and 60 sec for determination of LC uptake by a previously described centrifugation technique (27). Briefly, cell aliquots were placed in microcentrifuge tubes containing successive layers of 50 μl of 3 N NaOH, 200 μl of an oil layer of dibutylphthalate-bis(2-ethylhexyl) phthalate 3:2 (v/v) (Kodak, Rochester, NY), and 250 μl Krebs-Henseleit bicarbonate buffer. After addition of the cell suspension, the tubes were centrifuged at 12,000 *g* for 1.5 min in a Beckman Microfuge (Beckman Instruments, Palo Alto, CA). The buffer and oil layers were gently removed by aspiration, and the tubes containing the cell pellet in NaOH were heated in a water bath at 100°C for 2 h. After neutralization of the cell suspension with HCl, the radioactivity in the cell pellets was determined by scintillation counting. In the absence of cells, the radioactivity remained in the aqueous layer, and there was no contamination of the oil layer with radioactivity.

Data were analyzed by linear curve fitting. Initial rates of LC uptake (nmol · sec^{-1} · mg cell wet wt^{-1}) were derived from the equation of the respective regression line obtained from the plot of LC uptake as a function of time.

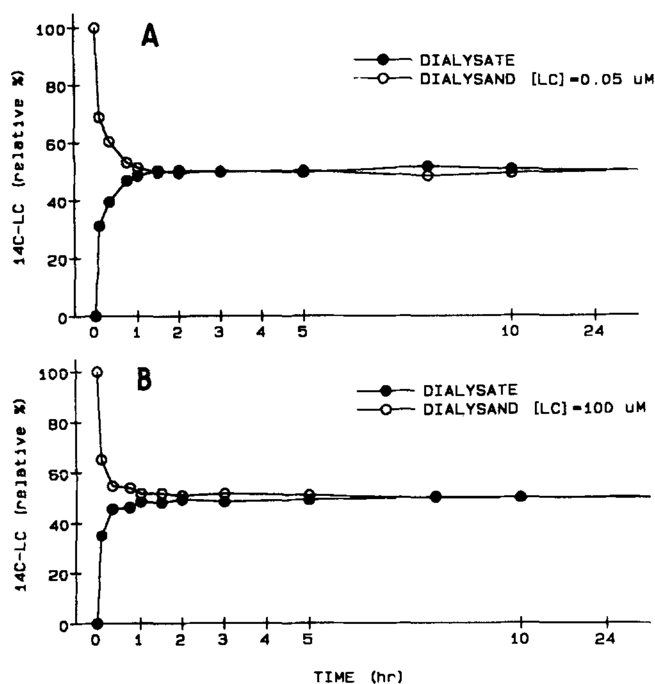

Fig. 1. Distribution of [24-^{14}C]lithocholic acid at equilibrium: 0.05 μM lithocholic acid (LC), A; 100 μM LC, B. Dialysis bags containing dialysis buffer alone were incubated with either 0.05 μM or 100 μM [24-^{14}C]LC at 37°C for time periods ranging from 5 min to 24 h.

RESULTS

Determination of equilibrium binding conditions

As shown in **Fig. 1A,** in incubation mixtures containing only dialysate buffer and 0.05 μM [24-^{14}C]LC, equilibration of the bile salt between the outside and the inside of the dialysis bags was reached in 1.5 h at 37°C. This is evidenced by a decline of radioactivity with time in the dialysate paralleled by a corresponding increase in radioactivity inside the dialysis bags, until the concentration of ^{14}C was essentially equal on both sides of the dialysis membrane. The addition of 100 μM LC in the dialysate did not significantly change the time at which equilibration was reached, which occurred in about 2.5 h (Fig. 1B). In both cases, once it was reached, equilibrium remained stable for at least 24 h. At all time points, complete recovery of the radioactivity was obtained in the combined dialysand and dialysate solutions, indicating no binding of bile salt to the nitrocellulose membranes.

Linearity and time course of bile salt binding

In order to characterize the LC, CDC, and cholic acid binding to the different plasma protein fractions, the linearity of bile salt binding to lipoprotein and LFAR fractions was evaluated by studying the binding of 0.05 μM ^{14}C-labeled LC, CDC, and cholic acid, respectively, to increasing concentrations of protein at 37°C for 4-20 h. The results of these studies define the protein concentration range used in the subsequent binding studies. In **Fig. 2A-D,** the linear regression line in each graph

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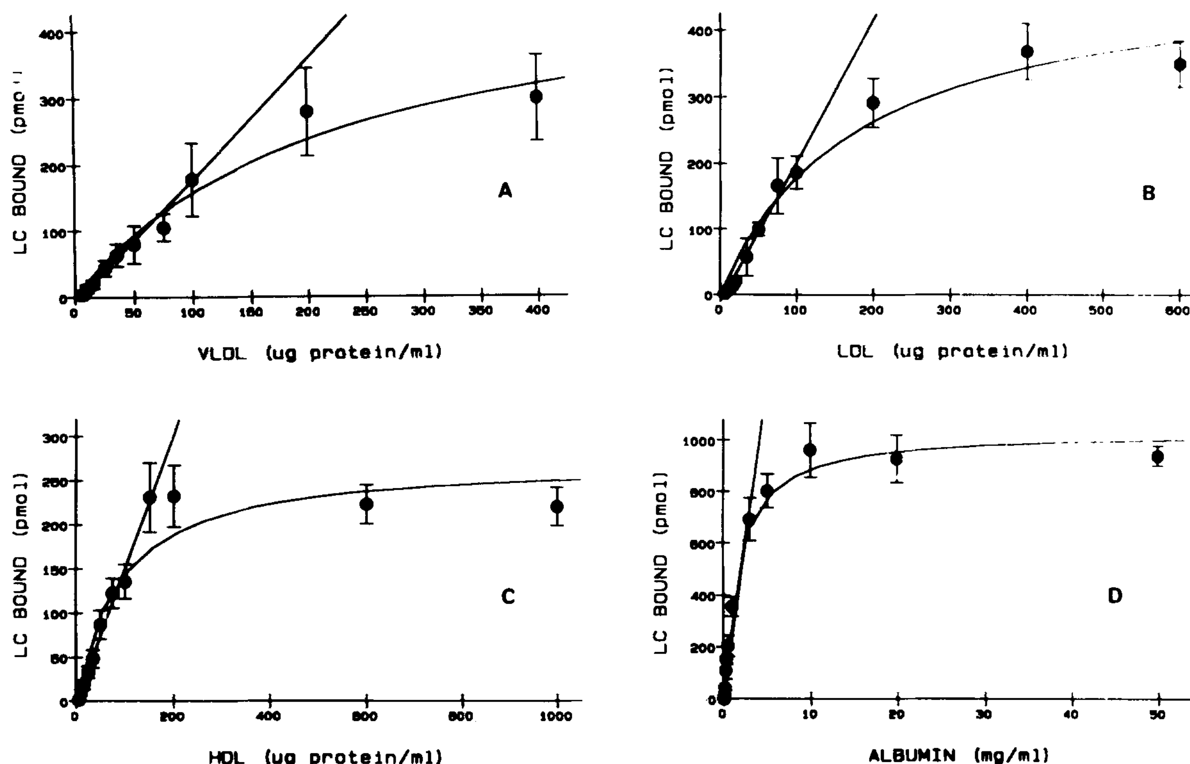

Fig. 2. Binding of [24-^{14}C]lithocholic acid ([24-^{14}C]LC) to increasing concentrations of protein from the isolated plasma fractions: VLDL, A; LDL, B; HDL, C; lipoprotein-free, albumin rich (LFAR) fraction, D. Plasma protein fractions were serially diluted to obtain protein concentrations ranging from 0.005 to 50 mg/ml. Each protein concentration was incubated with 0.05 μM [24-^{14}C]LC at 37°C for 4–20 h. Results are the mean ± SEM of six experiments performed in duplicate.

depicts the range of protein concentrations that were linearly proportional to the amount of LC bound. The upper level of this range is approximately 100 μg/ml for VLDL, LDL, and HDL, respectively, and 1 mg/ml for the LFAR fraction. Similar studies were performed with CDC and cholic acid (results not shown). For CDC and cholic acid, respectively, the upper levels of the range of protein concentrations that were linearly proportional to the amount of bound bile salt were 500 and 700 μg/ml for both LDL and HDL, and 2,000 and 4,000 μg/ml for the albumin-containing fraction.

Furthermore, the binding of 0.05 μM LC to the different plasma protein fractions was determined at designated times, ranging from 5 min to 24 h. The results of this study indicate that the binding of LC to the different protein fractions reaches equilibrium in less than 3 h, and remains stable for up to 24 h (results not shown). Under the same conditions, no significant difference in CDC and cholic acid binding was observed after incubations of 4 and 20 h.

Characteristics of LC, CDC, and cholic acid binding to lipoproteins and to albumin

As concentrations of LC, CDC, and cholic acid used in these studies were well below their respective critical micellar concentrations (38), the soluble bile salts were monomeric. The binding of 0.05–150 μM [24-^{14}C]LC to 50 μg/ml of VLDL, LDL, and HDL, as well as to 500 μg/ml of the LFAR fraction was studied. Binding saturation curves and Scatchard plots are depicted in **Fig. 3A–D.** The binding of LC to VLDL, LDL, and HDL was characterized by a single class of binding sites with a respective dissociation constant (K_D) of 66, 47, and 49 μM (Fig. 3A–C and **Table 1**). The maximum number of LC binding sites (B_{max}), determined by Scatchard analysis, was 931 nmol/mg protein for VLDL (Fig. 3A and Table 1), 716 nmol/mg protein for LDL (Fig. 3B and Table 1), and 790 nmol/mg protein for HDL (Fig. 3C and Table 1). LC binding to the LFAR fraction was characterized by two classes of binding sites with respective K_D values of 2 and 39 μM and B_{max} values of 6 and 98 nmol/mg protein (Fig. 3D and Table 1).

The saturation curves and Scatchard plots depicting the binding of 0.05–2,400 μM [24-^{14}C]CDC to 500 μg/ml LDL and HDL, as well as to 2,000 μg/ml of the LFAR fraction are illustrated in **Fig. 4A–C.** CDC binding to LDL and to HDL was characterized by one class of binding sites, with apparent K_D values of 1,010 and 695 μM, respectively, and corresponding B_{max} values of 668 and 560 nmol/mg protein (Fig. 4A, B; Table 1). CDC binding

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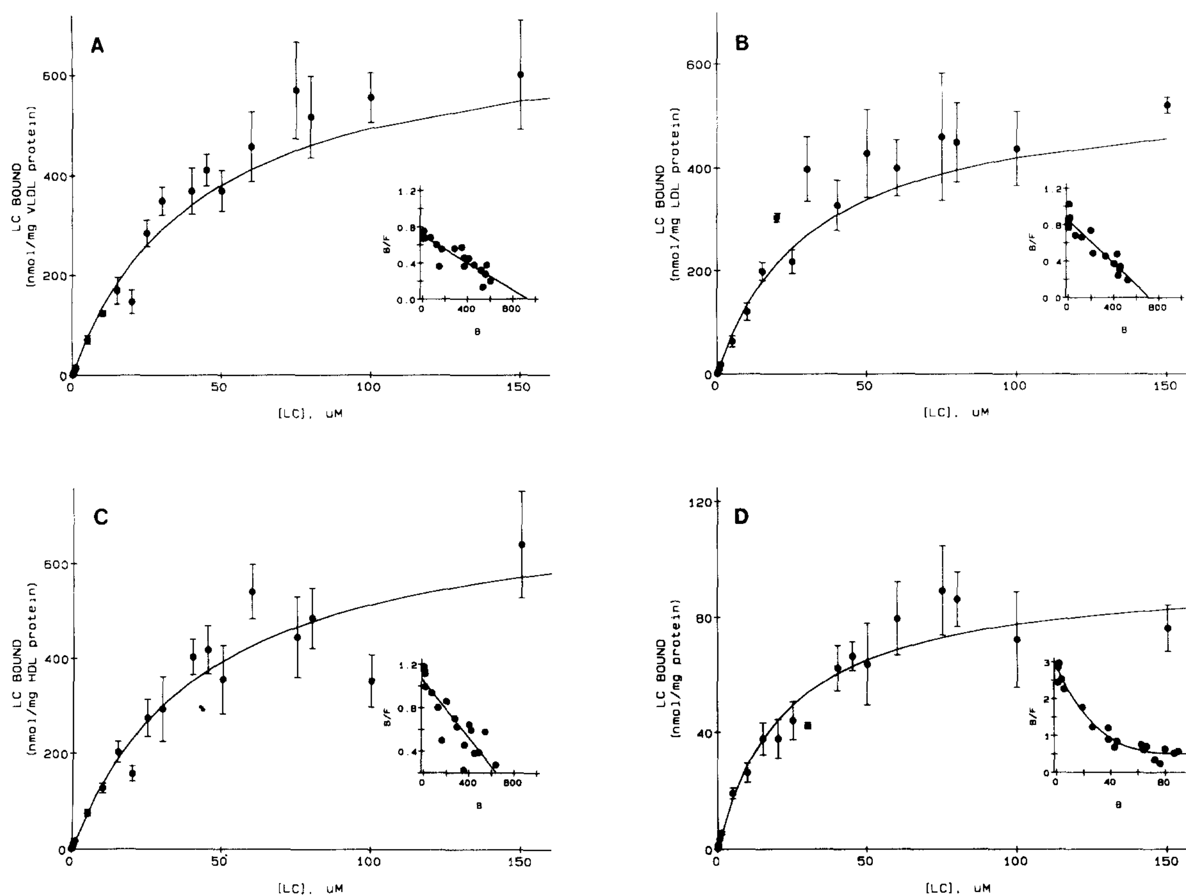

Fig. 3. Saturation curve and Scatchard plot (inset). Binding of increasing concentrations of lithocholic acid to: VLDL, A; LDL, B; HDL, C; lipoprotein-free, albumin rich (LFAR) fraction, D. Protein concentration of the lipoprotein and LFAR fractions was adjusted to 50 and 500 μg/ml, respectively, which is in the range at which binding of lithocholic acid (LC) to the protein is linear. Aliquots of the different fractions were incubated at 37°C for 6-18 h in dialysis buffer containing increasing concentrations (0.15-150 μM) of [24-^{14}C]LC. Results are the mean ± SEM of 11 experiments performed in duplicate.

to the LFAR fraction was characterized by two classes of binding sites. The respective K_D and B_{max} values for each class were 5 and 2,387 μM, and 6 and 111 nmol/mg protein (Fig. 4C and Table 1).

Fig. 5A-C depicts the saturation curves and Scatchard plots of the binding of cholic acid to 700 μg/ml LDL and HDL, and to 4,000 μg/ml of the LFAR fraction. As with the other bile salts studied, cholic acid binding to lipoproteins was characterized by one class of binding sites, with K_D values of 2,562 and 2,511 for LDL and HDL, respectively (Fig. 5A, B; Table 1). The respective B_{max} values were 539 and 416 nmol/mg protein. Cholic acid binding to the LFAR fraction was also characterized by two classes of binding sites, with K_D values of 51 and 5,575 μM, and B_{max} values of 5 and 89 nmol/mg protein, respectively (Fig. 5C; Table 1).

Competitive inhibition of LC binding by TLC, GLC, LC-3-sulfate, CDC, and cholic acid

The ability of TLC, GLC, LC-3-sulfate, CDC, and cholic acid to competitively inhibit [24-^{14}C]LC binding to both LDL and HDL was studied. The competition binding profiles for TLC, GLC, and LC-3-sulfate are depicted in **Fig. 6A, B,** while those for CDC and cholic acid are depicted in **Fig. 7A, B.**

TABLE 1. Constants of the binding of LC, CDC, and cholic acid to plasma lipoproteins and albumin

	B_{max}			K_D		
	LC	CDC	Cholic	LC	CDC	Cholic
	nmol/mg protein			μM		
VLDL	931	n.d.	n.d.	66	n.d.	n.d.
LDL	716	668	539	47	1,010	2,562
HDL	790	560	416	49	695	2,511
Albumin						
Class 1	6	6	5	2	5	51
Class 2	98	111	89	39	2,387	5,575

Mean data are shown in Figs. 3A-D, 4A-C, and 5A-C. Maximum numbers of binding sites (B_{max}) and dissociation constants (K_D) were determined by Scatchard analysis of the values of 11 experiments for LC and 4 experiments each for CDC and cholic acid. For experimental details, see legends of Figs. 3, 4, and 5. Abbreviations used: n.d., not determined.

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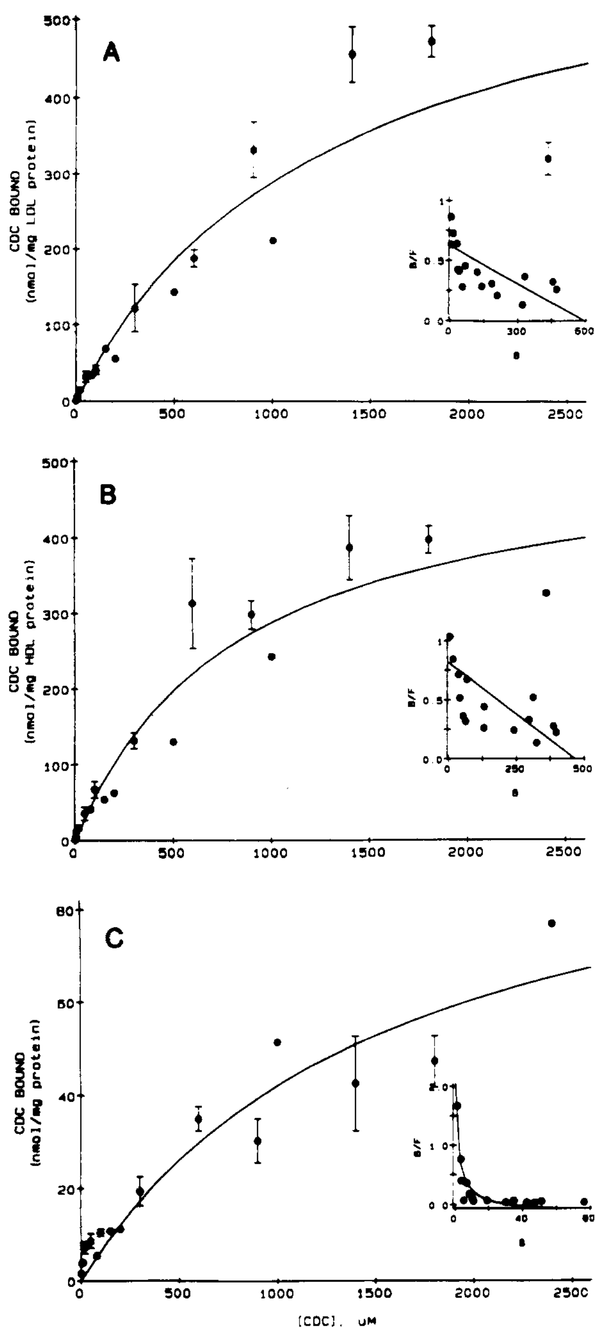

Fig. 4. Saturation binding curve and Scatchard plot (inset). Binding of increasing concentrations of chenodeoxycholic acid (CDC) to: LDL, A; HDL, B; lipoprotein-free, albumin-rich (LFAR) fraction, C. Protein concentration of the lipoprotein and LFAR fractions was adjusted to 500 and 2,000 μg/ml, respectively, which is in the range at which binding of CDC to the protein is linear. Aliquots of the different fractions were incubated at 37°C for 6–18 h in dialysis buffer containing increasing concentrations (0.5–2400 μM) of [24-^{14}C]CDC. Results are the mean of four experiments, performed in duplicate.

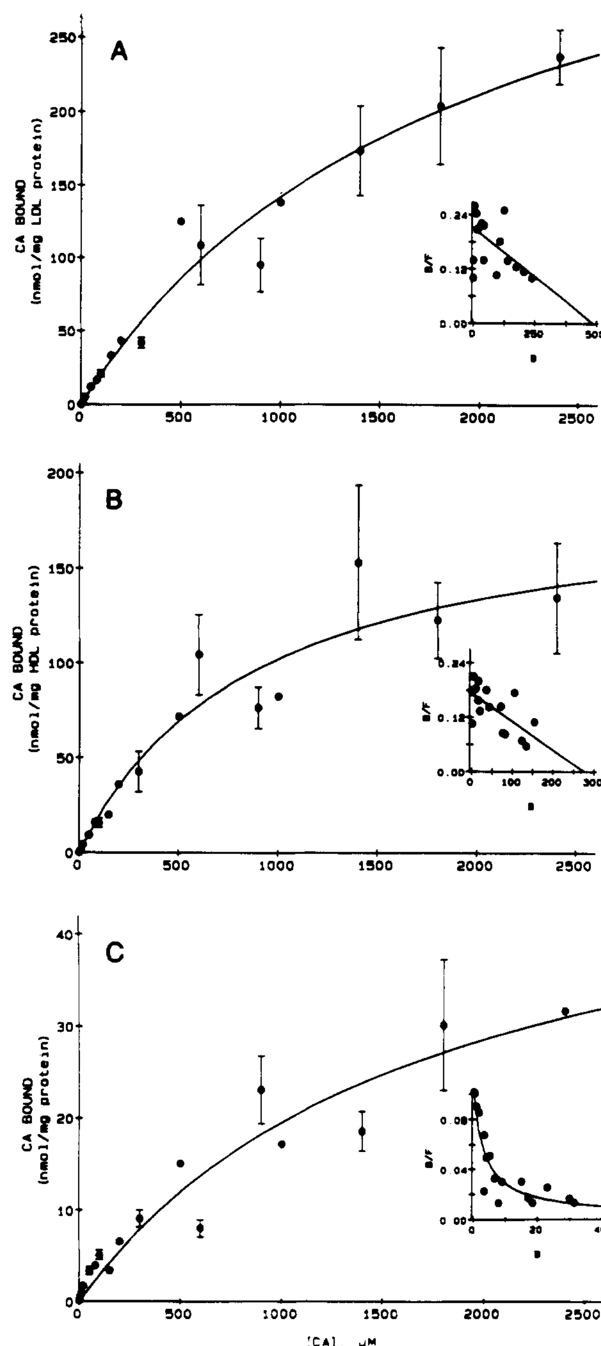

Fig. 5. Saturation binding curve and Scatchard plot (inset). Binding of increasing concentrations of cholic acid to: LDL, A; HDL, B; lipoprotein-free, albumin-rich (LFAR) fraction, C. Protein concentration of the lipoprotein and LFAR fractions was adjusted to 700 and 4,000 μg/ml, respectively, which is in the range at which binding of cholic acid to the protein is linear. Aliquots of the different fractions were incubated at 37°C for 6–18 h in dialysis buffer containing increasing concentrations (0.5–2400 μM) of [24-^{14}C]cholic acid. Results are the mean of four experiments, performed in duplicate.

The extent of competition of the respective ligands is similar, indicating that all the bile salts studied bind to the same LDL and HDL binding sites as does [24-^{14}C]LC. Furthermore, the respective bile salts competed for 10–90% of LC specific binding over an 81-fold concentration range. This is characteristic for reversible ligand-binding site interactions obeying the law of mass action, and supports the existence of a single lipoprotein binding

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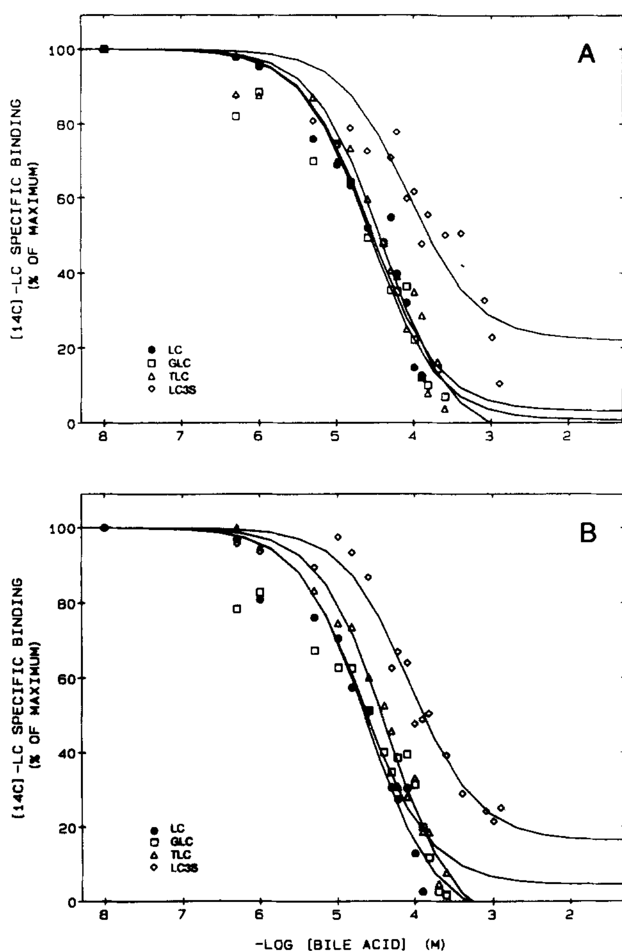

Fig. 6. Competitive inhibition of [24-^{14}C]lithocholic acid ([24-^{14}C]LC) binding to lipoproteins by GLC, TLC, and LC-3-sulfate (LC3S). LDL, A; HDL, B. Aliquots of plasma LDL and HDL fractions (50 μg protein/ml) were incubated at 37°C for 6-18 h in dialysis buffer containing 0.05 μCi [24-^{14}C]LC, with increasing concentrations of TLC, GLC, and LC-3-sulfate, respectively. Results are the mean of three experiments, performed in triplicate. Error bars were omitted for clarity of presentation.

site for these bile salts (39).

The dissociation constants (K_I) for the different bile acids to displace [24-^{14}C]LC were calculated from the EC_{50} values depicted in Fig. 6A, B and 7A, B, and are shown in **Table 2.** For both LDL and HDL, the respective K_I for LC-3-sulfate, CDC, and cholic acid is significantly higher ($P < 0.05$) than that for LC. The relative potency of the bile salts to compete for both LDL and HDL binding is as follows: LC = GLC = TLC > LC-3-sulfate ≥ CDC > cholic acid.

Partial characterization of the nature of LC binding to lipoproteins

To assess the amount of LC bound to lipid (in the case of lipoproteins) or to solvent-accessible hydrophobic protein regions, the different plasma protein fractions were delipidated after binding of 80 μM LC. After delipidation, the nmoles of LC bound per mg of protein to VLDL, LDL, and HDL were decreased by 75.2, 66.2, and 55.6%, respectively. The delipidation process also removed 77.0% of LC bound to albumin, presumably by disruption of hydrophobic associations within the protein's secondary structure (results not shown).

The nature of the binding of LC to human and bovine serum albumin has previously been studied by Roda et al. (20), and Pico and Houssier (36), respectively, using fluorescent techniques. Therefore, the fluorescence of the lipoprotein fractions in the presence and absence of bound LC was measured in order to determine the nature of bile salt binding to human serum lipoproteins. The fluorescence spectra used in these experiments correspond specifically to that of tryptophan and, to a lesser extent,

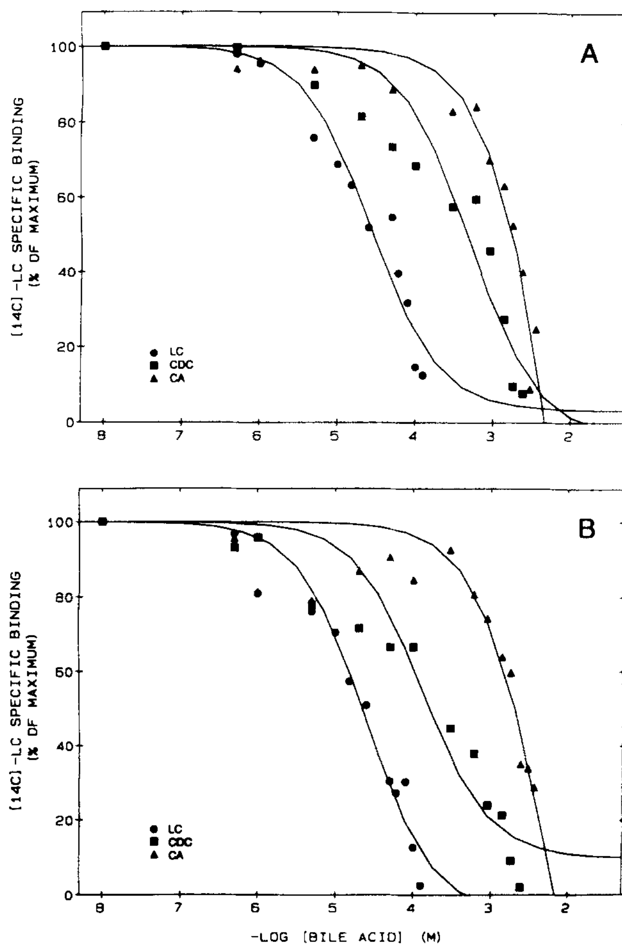

Fig. 7. Competitive inhibition of [24-^{14}C]lithocholic acid ([24-^{14}C]LC) binding to lipoproteins by CDC and cholic acid. LDL, A; HDL, B. Aliquots of plasma LDL and HDL fractions (500 and 700 μg protein/ml, respectively) were incubated at 37°C for 6-18 h in dialysis buffer containing 0.05 μCi [24-^{14}C]LC, with increasing concentrations of CDC and cholic acid, respectively. Results are the mean of three experiments, performed in triplicate. Error bars were omitted for clarity of presentation.

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TABLE 2. Dissociation constants of the inhibition of [24-^{14}C]LC binding to LDL and to HDL by different bile salts

	K_I	
	LDL	HDL
	μM	
LC	21.4 ± 3.5	21.2 ± 4.9
GLC	28.3 ± 8.3	25.0 ± 7.6
TLC	34.0 ± 6.9	36.2 ± 4.2
LC3S	290.3 ± 32.8[a]	140.6 ± 34.8[a]
CDC	429.6 ± 186.2[a]	191.5 ± 71.7[a]
Cholic	1,785.6 ± 218.4[a]	2,112.1 ± 135.6[a]

The dissociation constants (K_I) of the respective bile acids are derived from the EC_{50} values obtained from Figs. 6A, B and 7A, B. Results are expressed as the means ± the SEM of three experiments performed in triplicate. Abbreviations used: LC3S, LC-3-sulfate.

[a]Denotes significantly different K_I compared to LC ($P < 0.05$).

to that of tyrosine. The fluorescence of VLDL, LDL, and HDL decreased by 31, 33, and 23%, respectively, when measured after incubation of the lipoproteins with LC at concentrations that corresponded to their respective K_D values (45–70 μM; **Fig. 8**).

Effect of LDL, HDL, and albumin on the hepatocellular uptake of LC

In order to assess the effect of the protein carrier on the hepatocellular uptake of LC, [24-^{14}C]LC was incubated with isolated hamster hepatocytes either alone or bound to hamster LDL, HDL (10 μg protein/ml), and albumin (10 and 100 μg/ml), respectively. LC concentration was 1 μM in all experiments, except in those with 10 μg/ml albumin, in which, due to the 10-fold lower number of LC binding sites, it was 0.1 μM.

LC uptake was linear up to 60 sec, as depicted in **Fig. 9.** Initial uptake rates of 1 μM LC (mean ± SEM) were not significantly different whether LC was incubated alone or bound to 10 μg/ml LDL or HDL, or to 100 μg/ml albumin (0.045 ± 0.010, 0.043 ± 0.006, 0.040 ± 0.005, and 0.052 ± 0.012 nmol · sec^{-1} · mg cell wet wt^{-1}, respectively. Initial uptake rate of 0.1 μM LC bound to 10 μg/ml albumin was significantly lower ($P < 0.02$) at 0.003 ± 0.004 nmol · sec^{-1} · mg cell wet wt^{-1}.

Uptake values extrapolated to time 0 represent nonspecific cell-associated LC. When cells were incubated with LC alone, nonspecific LC binding represented 41% of total LC (Fig. 9). When LC was bound to either 10 μg/ml LDL or HDL, nonspecific binding increased by approximately 100% to 75–77% of total LC (Fig. 9). Nonspecific binding of the same concentration of LC was 60% when bound to 100 μg/ml albumin, which was significantly less than when LC was bound to 10 μg/ml albumin (74%, Fig. 9).

DISCUSSION

The results of the present study indicate that the binding of LC, CDC, and cholic acid to the LFAR fraction of plasma is characterized by two classes of binding sites, with respective apparent K_D values of 2, 5, and 51 μM for

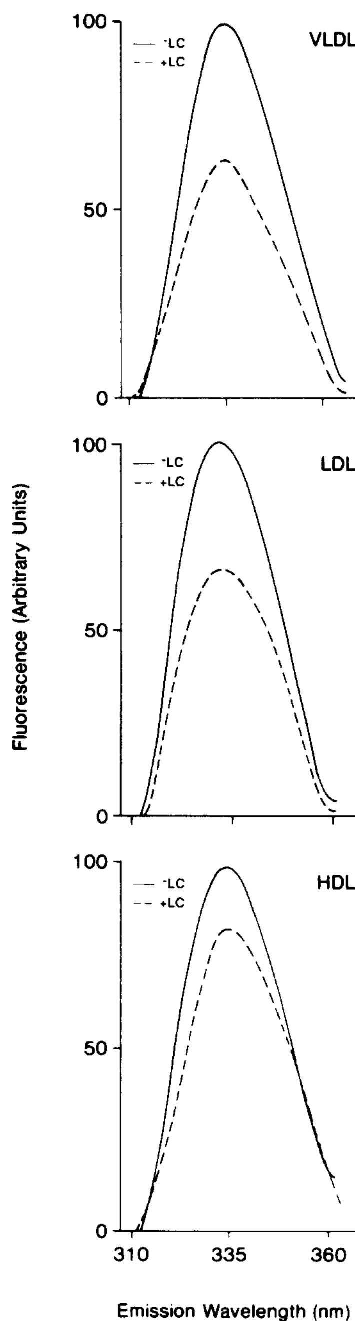

Fig. 8. Fluorescence emission spectra of human plasma VLDL, LDL, and HDL in the presence and absence of lithocholic acid (LC). Fluorescence of the lipoprotein fractions was measured at 20°C, and at 310–360 nm after excitation at 280 nm, after incubation in dialysis buffer alone, or with the addition of 50 μM LC for 10 h at 37°C.

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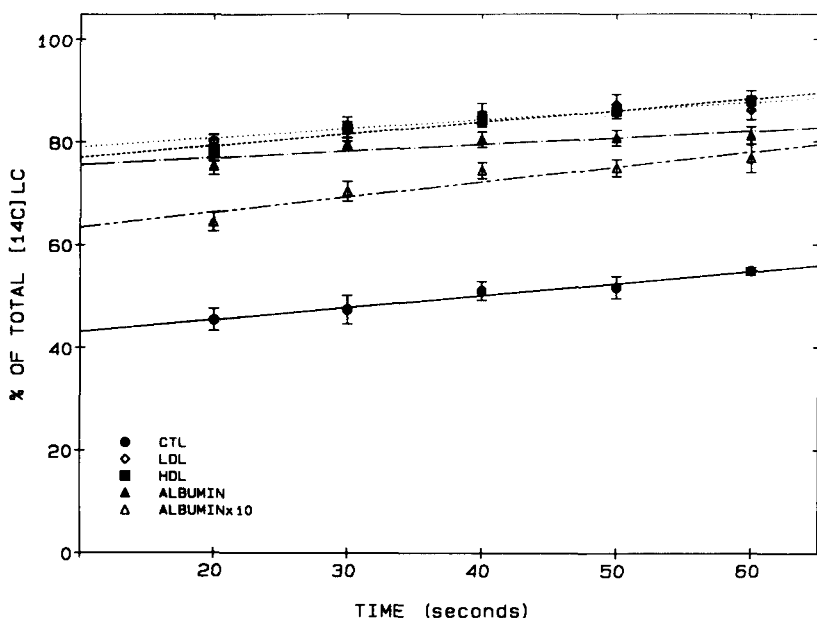

Fig. 9. Hepatocellular uptake of lithocholic acid (LC) by isolated hamster hepatocytes. Isolated hamster hepatocytes were incubated at 37°C with [24-^{14}C]LC (control, CTL), or with [24-^{14}C]LC bound to LDL and HDL (10 μg protein/ml), respectively, as well as to albumin (10 μg/ml, ALBUMIN; or 100 μg/ml, ALBUMIN × 10). LC concentration was 1 μM.

the high affinity site and 39, 2,387, and 5,575 μM for the low affinity site. This finding suggests a significant decrease in affinity with an increase in the number of hydroxyl groups of the bile acid, as previously shown by other investigators, using purified human serum albumin (14, 20). The B_{max} was similar for all three bile acids studied, with a value of about 6 nmol/mg protein for the class 1 sites and about 100 nmol/mg protein for the class 2 sites. These data are in agreement with those reported by Roda et al. (20), using a 1% human serum albumin solution in phosphate buffer; however, the B_{max} values reported in the present study are slightly lower than those reported by Roda et al. As the LFAR fraction does not consist entirely of albumin, calculation of B_{max} could be slightly underestimated as it is based on the total protein in this fraction. While the K_D values for LC are in agreement with those of 5 and 26 μM reported by Roda et al. (20), the K_D values for CDC and cholic acid found in the present study differ from those of 18 and 322 μM and 303 and 3,333 μM, respectively. Although photolabile derivatives of taurocholic acid were shown by Kramer et al. (40) to bind only to albumin in the LFAR fraction of human serum, one cannot rule out the possibility that the more tightly bound CDC may also bind to other serum proteins of this fraction. Furthermore, protein-protein interactions, protein concentrations, buffer composition, or the presence of endogenous bile acids may also explain these slight differences. In the present study, LFAR protein concentrations used to study CDC and cholic acid binding were 2,000 μg/ml and 4,000 μg/ml, respectively, compared to 10 mg/ml in the studies of Roda et al. (20).

This study is also the first to present quantitative data showing that LC, CDC, and cholic acid bind to the different lipoprotein classes with a maximum number of binding sites of 416–931 nmol/mg protein, and a dissociation constant (K_D) ranging from 47 to 66 μM for LC, 695 to 1010 μM for CDC, and 2,511 to 2,562 μM for cholic acid. The lipoproteins expressed about 5–9 times more bile salt-binding sites per mg protein than did the albumin-containing plasma LFAR fraction, while there was no difference in B_{max} values for LC, CDC, and cholic acid. The affinity of both LDL and HDL significantly decreased with increasing hydroxylation of the bile acids studied. While the in vitro data of Salvioli et al. (21) concerning the relative binding behavior of plasma protein fractions for different bile acids would suggest the presence of a positive correlation between the hydrophobicity of a bile acid and its ability to bind lipoproteins, several in vivo studies have concluded that the relative hydrophilicity of a bile acid is a determinant for its increased binding affinity for lipoproteins (15, 18, 24). The results of the present study clearly indicate that, with increasing polarity, bile acids decrease in their affinity for lipoproteins. However, in the bloodstream, the binding behavior of bile acids would be dictated by the comparative dissociation constants of their binding to lipoproteins and albumin. For example, while LDL, HDL, and albumin have lower affinities for cholic acid than for the less polar bile acids studied, these lipoproteins have a slightly higher affinity for cholic acid than does the low affinity class of albumin binding sites. Therefore, cholic acid would be more likely to bind to serum lipoproteins than to albumin once the capacity of the high affinity class of albumin binding sites is exceeded.

Physiological concentrations of human serum apolipoproteins range from 3–12 mg/dl for apoCs of HDL and

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VLDL to 80–130 mg/dl for apoB-100 of LDL and apoA-I of HDL, respectively (41). The concentrations of serum albumin are around 40-fold higher than those of apolipoproteins, ranging from 3.5 to 4.5 g/dl (42, 43). Changes in the binding pattern of bile acids could result from changes in serum albumin or apolipoprotein (apo) concentrations. Disorders of albumin metabolism, such as hypoalbuminemia, are known to occur as a result of a variety of causes, including malnutrition, malabsorption, and cirrhosis (43). Under these conditions, serum albumin concentrations can decrease to less than 30% of their original value (43). In addition, certain hyperlipidemias can result in increased levels of serum lipoproteins and their corresponding apolipoproteins (41, 44).

Bile acids are known to compete with certain weakly acidic drugs for albumin binding sites (45, 46). Accordingly, the role of bilirubin in the kinetics of bile acid binding to serum proteins cannot be overlooked. In several in vitro studies, both conjugated (47, 48) and unconjugated (49) bilirubin was found to displace glycine- as well as taurine-conjugated cholic acid from albumin. In contrast, increasing concentrations of bilirubin did not affect the binding of glycine-conjugated CDC to serum albumin (48, 49). This may also contribute to the increased shift of cholic acid from albumin to lipoproteins observed in patients with hepatobiliary disorders (17, 18).

The ability of bile acids to competitively inhibit [24-^{14}C]LC binding to both LDL and HDL also decreased with increasing polarity of the bile acid (LC > CDC > cholic acid), in keeping with the data from the saturation binding studies, while the values calculated for K_I paralleled those calculated for K_D. Furthermore, LC binding to both LDL and to HDL was equally inhibited by unconjugated LC, and its glycine and taurine conjugates. However, LC-3-sulfate was significantly less potent than LC and its amidates in the ability to displace LC from LDL or HDL. These data are supported by other in vitro studies in which conjugation of a bile acid with glycine or taurine did not affect its affinity for albumin (14, 20). In addition, in in vivo studies of bile acid transport by lipoproteins in patients with extrahepatic cholestasis (17) as well as with liver cirrhosis and hyperbilirubinemia (18), there was no difference in the relative association of the glycine and taurine conjugates of bile acids with either serum albumin or lipoproteins. The present data are also in agreement with those of Cowen et al. (22), who reported that LC-3-sulfate demonstrated less protein binding in serum compared to that of both amidated and free LC. However, these authors reported that glycine-conjugated LC was less bound to protein than was its taurine-conjugated or free counterpart. Differences in technique and incubation times may account for this apparent discrepancy.

The amidated and sulfated derivatives of LC, as well as CDC and cholic acid, were shown to bind to the same site on LDL and HDL as did free LC, as [24-^{14}C]LC binding was displaced to the same extent by increasing concentrations of the respective bile acids. The nature of the binding of bile salts to human plasma lipoproteins may involve hydrophobic interactions with the apolipoprotein moiety. After delipidation with organic solvent of the LC-bound protein fractions, 25–45% of bound LC was non-releasable. However, it cannot be concluded that the remaining 70% is bound exclusively to lipid, since 77% of LC bound to the LFAR fraction was also removed by delipidation. Non-releasable LC binding could be due to hydrophobic interactions inaccessible to the delipidation solvent, or to non-hydrophobic binding. Indeed, studies by Nair et al. (50, 51) demonstrated that the binding of LC to liver tissue proteins appears to involve a peptide linkage with the ϵ-amino group of lysine. However, no significant difference in LC binding to serum lipoproteins was observed in a pH range 7.5–11.5. Therefore, the involvement of electrostatic interactions appears unlikely (data not shown).

Bile acid binding to human serum albumin has been shown by Scagnolari et al. (52) to involve predominantly hydrophobic interactions. Pico and Houssier (36) studied the nature of bile acid binding using fluorescence measurements and circular dichroism. These authors found that bile acid binding produced a decrease in human serum albumin fluorescence at 350 nm, which is explained by the quenching of tryptophan fluorescence by bile acids. The decrease in lipoprotein fluorescence at 310–360 nm, observed after incubation with LC, also suggests that the binding of LC to lipoproteins quenches lipoprotein fluorescence by masking the tryptophan and tyrosine residues of the protein moiety. This could be the result of hydrophobic interactions between the bile acid and the apolipoprotein. However, these data do not exclude the possibility of additional lipid–bile acid interactions, or non-hydrophobic protein–bile acid interactions.

These data are supported by other reports concerning the nature of the binding of bile acids to plasma apoproteins. In a study by Delahunty and Feldkamp (53), an increased association of endogenous glycine-conjugated cholic acid with the β-lipoprotein fraction of hyperlipidemic serum was not correlated with increased triglyceride or cholesterol content of these particles. Deoxycholic acid binding to apoB-100 of LDL was found to be rapid and specific (54, 55), while the binding of this bile acid to apoA-I and apoA-II of HDL was shown by Makino, Tanford, and Reynolds (56) to involve primarily hydrophobic interactions. In addition, studies by Donovan, Benedek, and Carey (57, 58), Middelhoff et al. (16), and Coulhon et al. (59) showed the ability of CDC and cholic acid to bind to apoA-I of HDL, disclosing that the dissociation of apoA-I from the HDL particle was concurrent with increased binding of the bile acid to the HDL particle.

The increased proportion of lipoprotein-bound bile

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acids observed during hepatobiliary disorders (17, 18) may be responsible for the increased concentrations of bile acids detected in extrahepatic tissues of patients with extrahepatic cholestasis (19), as lipoproteins, in contrast to albumin, are internalized by most cells. Results of the present study indicate that when 1 μM LC is presented to the liver cell either alone or bound to LDL, HDL, and albumin, respectively, there is no difference in the initial rate of uptake of the bile salt. However, 0.1 μM LC bound to albumin had a 10-fold lower rate of uptake. Therefore, it appears that the rate of uptake of LC is dependent on its concentration and is not affected by its protein carrier, at least for the concentrations studied in the isolated hepatocyte model. However, the nonspecific binding of LC to the liver cell was increased by about 100% to 75-77% of the total LC, when LC was bound to 10 μg/ml of either LDL or HDL. When the same concentration of LC was bound to 100 μg/ml of albumin, nonspecific binding was significantly lower than that of the lipoprotein-bound LC. However, a 10-fold lower albumin, and, consequently, LC concentration resulted in about the same degree of nonspecific binding. Some investigators have demonstrated the existence of a liver plasma membrane albumin receptor thought to facilitate the hepatic uptake of certain albumin-bound ligands (60). The enhanced nonspecific association of the lipoprotein-bound bile acid to the cell could be due to lipoproteins binding to their respective receptors, or, perhaps, to liposome binding sites that interact with phospholipid molecules (61). In this way, potentially toxic bile acids may be targeted to extrahepatic tissues.

In summary, the study indicates a similar affinity or slightly higher affinity of lipoproteins for bile salts compared to that of the low affinity site of albumin. Furthermore, lipoproteins present 5-9 times more bile salt binding sites, per mg protein, than does albumin. Lipoprotein affinity decreased with increasing polarity of the steroid nucleus of the bile acid, and was unaffected by conjugation of the bile acid with glycine or taurine. In addition, while the initial rate of uptake of the same concentration of LC was not affected by the protein carrier, hepatocellular LC association was enhanced when LC was bound to lipoproteins compared to albumin. Therefore, the binding of LC to, and transport by, plasma lipoproteins may be of considerable pathophysiological importance, if lipoproteins can serve to target bile acids to certain tissues. The deleterious effects of bile acids may not only be affected by their plasma concentrations, but also by the concentration of albumin as well as the level and composition of plasma lipoproteins.

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