Quantitative aspects of the interaction of bile acids with human serum albumin

A. Roda,[1] **G. Cappelleri, R. Aldini, E. Roda, and L. Barbara**

Istituto di Scienze Chimiche, Facolta di Farmacia,[2] and Clinica Medica III, Universita di Bologna, Italy

Abstract The interaction of human serum albumin with twelve bile acids (ba) has been studied by equilibrium dialysis technique using ^{3}H- and ^{14}C-labeled bile acids. The physiological bile acids studied were: cholic, chenodeoxycholic, deoxycholic, lithocholic, ursodeoxycholic, and 7-ketolithocholic acids, all in the free and conjugated (with glycine and taurine) forms. For each bile acid studied, the interaction was characterized by two classes of binding sites, the first consisting of 2–4 sites and the second of 8–30. K_1 values (liter/mol) for the different bile acids were: cholic acid, 0.3×10^4; chenodeoxycholic acid, 5.5×10^4; deoxycholic acid, 4.0×10^4; ursodeoxycholic acid, 3.8×10^4; 7-ketolithocholic acid, 1.9×10^4; lithocholic acid, 20×10^4. The affinity constant of a bile acid for albumin decreases with an increase in the number of hydroxy groups and also with the replacement of 7-hydroxy by 7-keto groups. The affinity constant is similar for glycine and taurine conjugated bile acids, but is slightly higher for unconjugated than conjugated forms.—**Roda, A., G. Cappelleri, R. Aldini, E. Roda, and L. Barbara.** Quantitative aspects of the interaction of bile acids with human serum albumin. *J. Lipid Res.* 1982. **23:** 490–495.

Supplementary key words albumin affinity constants • equilibrium dialysis • albumin fluorescence spectrum • bile acid uptake

The interaction of bile acids (BA) with plasma proteins has been known since 1957 when Rudman and Kendall (1), using an equilibrium dialysis method, reported that among the plasma proteins, the human albumin (HSA) fraction exhibits the greatest binding activity towards BA and that the affinity is reduced by the introduction of polar groups into the steroid moiety of the BA. The authors calculated the affinity constants (K) of the secondary binding sites for cholic acid only. They used the Kloz double reciprocal plot method which gives undue importance to data points calculated at low concentrations of BA. These give dramatically different values for the number of binding sites (n) and K when compared to the data derived from the Scatchard plot, in spite of a remarkably high correlation coefficient. More recently, Burke, Panvelivalla, and Tabaqchali, (2), using a steady-state gel filtration method, measured the affinity constant for cholic and taurocholic acid and found that the interaction with albumin was characterized by two classes of BA binding sites and that cholic acid is more avidly bound than its taurine conjugate. The affinity constant values of cholic acid reported by the above mentioned authors (1, 2) are not in agreement, probably due to the different methodological conditions, i.e., differences in temperature, BA, and albumin concentrations, and the type of equation used in the data processing.

The binding of plasma albumin largely determines both the levels in plasma and the rate of hepatic clearance from the blood stream of many BA-like organic anions (3–5). The emerging importance of the serum BA levels as a test of liver function (6, 7) and the exact definition of their determinants arouses new interest in the re-evaluation of this problem.

To date, few and incomplete studies are available on the mechanisms of the interaction between BA and HSA. We also know very little about the role of BA-albumin binding in hepatic BA uptake.

The availability of ^{3}H- and ^{14}C-labeled BA and the development of a sensitive and specific method, such as radioimmunoassay (RIA) (8, 9), have facilitated an accurate measurement of the affinity of BA for HSA over a wide range of molar ratios. Using an equilibrium dialysis method, we measured the affinity constant of che nodeoxycholic, cholic, deoxycholic, lithocholic, ursodeoxycholic, and 7-ketolithocholic acids, both free and conjugated with glycine and taurine, for HSA. The physical constants that govern this interaction are also reported.

Abbreviations: BA, bile acid; K, affinity constant; HSA, human serum albumin; RIA, radioimmunoassay; GLC, gas–liquid chromatography; GCA, glycocholic acid; TCA, taurocholic acid; CA, cholic acid; TCDCA, taurochenodeoxycholic acid; GCDCA, glycochenodeoxycholic acid; CDCA, chenodeoxycholic acid; LCA, lithocholic acid; GLCA, glycolithocholic acid; UDCA, ursodeoxycholic acid; 7-KLCA, 7-ketolithocholic acid; DCA, deoxycholic acid.

[1] Address reprint requests to Dr. Aldo Roda, Istituto di Scienze Chimiche, Universita di Bologna, Via S. Donato, 15, 40100 Bologna, Italy.

[2] A. Roda.

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MATERIALS AND METHODS

Reagents

Unlabeled bile acids were supplied by Calbiochem, La Jolla, CA. They proved to be more than 98% pure by GLC. Labeled BA were supplied by the Radiochemical Centre, Amersham, United Kingdom, and by New England Nuclear Corp., Boston, MA. ^{3}H-7-Ketolithocholic acid was generously supplied by Dr. H. Fromm (Gastroenterology and Nutrition Unit, University of Pittsburgh, PA). BA were purified by thin-layer chromatography a few days before the experiment using propionic acid–isoamyl acetate–water–N-propanol 3:4:1:2 (v/v/v/v) as the solvent system. Crystalline HSA, purchased from Sigma, London (essentially fatty acid free: less than 0.005%), was used without further purification.

Procedure

Equilibrium dialysis was performed in a cellulose sac washed with distilled water and then with phosphate buffer, 0.1 M, pH 7.2. The sac was stored in the same buffer at 4°C until used. Five-ml 1% HSA solutions were dialyzed against 25 ml of buffer containing BA. The initial concentration of the BA ranged from 1 to 8,000 μM. A corresponding labeled BA (50,000 dpm/ml) was added (1 ml). All measurements were performed in triplicate. A cellulose sac containing the same buffer served as the control. The systems were equilibrated by mechanical shaking for 36 hr. Repeated recoveries indicated that adsorption by sacs was statistically insignificant.

The concentration of BA in the outer solution (free fraction) and in the inner solution (bound + free fraction) allowed us to calculate the bound BA. We measured the concentration of radioactivity (dpm/ml) using the liquid scintillation technique. Radioactivity was measured in Unisolve® (Koch-Light Lab. Ltd., England) using a liquid scintillation counter (ISOCAP 300, Searle Analytic Inc., now Tracor Analytic, Elk Village, IL). The BA concentration was also measured using a specific radioimmunoassay developed in this laboratory (8, 9), which allows the measurement of BA in solution at levels as low as 1 μM.

A close agreement ($r > 0.98$) of values was observed when we compared the equilibrium concentration obtained by isotopic dilution with that obtained directly by RIA.[3] The coefficient of variation between the two methods was less than 5%. We calculated the equilibrium concentration using both methods. Only the data derived from dialysis that yielded recoveries of labeled BA within 95–105% were used.

[3] Roda, A. Unpublished results.

HSA fluorescence was measured with a Perkin Elmer spectrophotofluorometer using a 285 mm wavelength exciting light and the fluorescence spectrum recorded from 300 to 450 mm.

Expression of the data

From the concentration of radioactivity and the initial concentration of BA we calculated the molar concentration (A) of unbound BA and the moles of BA bound per mole of HSA (r). The apparent association constants were derived from the Scatchard method by plotting experimentally derived values from r/A vs. r. It appears to be the most appropriate method when more than one class of sites are involved in the interaction. For all the bile acids assessed, experimental data were interpolated with a curve, assuming two classes of binding sites. The curve was derived from the equation:

$$r = [n_1k_1A/(1 + K_1A)] + [n_2K_2A/(1 + K_2A)]$$

where n_1 and n_2 represent the number of primary and secondary binding sites, respectively, and K_1 and K_2 are the corresponding association constants.

RESULTS

Experimental conditions

All measurements were carried out at 37°C ± 0.2°C in phosphate buffer 0.1 M, pH 7.2, ionic strength 0.15. The equilibrium conditions were assessed measuring the molar ratio of BA vs. HSA at different times. Equilibrium was reached after 36 hr, and a similar trend was observed for all the BA assessed.

Binding of BA with HSA

Experimental data for the binding of the studied BA with HSA are shown in **Fig. 1.** The results, analyzed by the Scatchard procedure by plotting r/A vs. r, indicated that more than one binding class must be implicated, since the relationship is markedly curvilinear. The analysis of the curves is compatible with at least two classes of BA binding sites. The first part of the curve is a straight line with the intercept at the abscissa ranging from 2 to 4. More than one mole of BA is therefore bound per mole albumin in the first class while many moles are more weakly bound in the second class.

The results obtained in terms of affinity constants and number of binding sites are shown in **Table 1.** Lithocholic acid, a monohydroxy BA, has the greatest affinity with HSA, while taurocholic acid has the lowest. The data indicate that the introduction of hydroxy groups into the steroid ring reduces the affinity of BA for HSA. Furthermore, the conjugation with glycine or taurine

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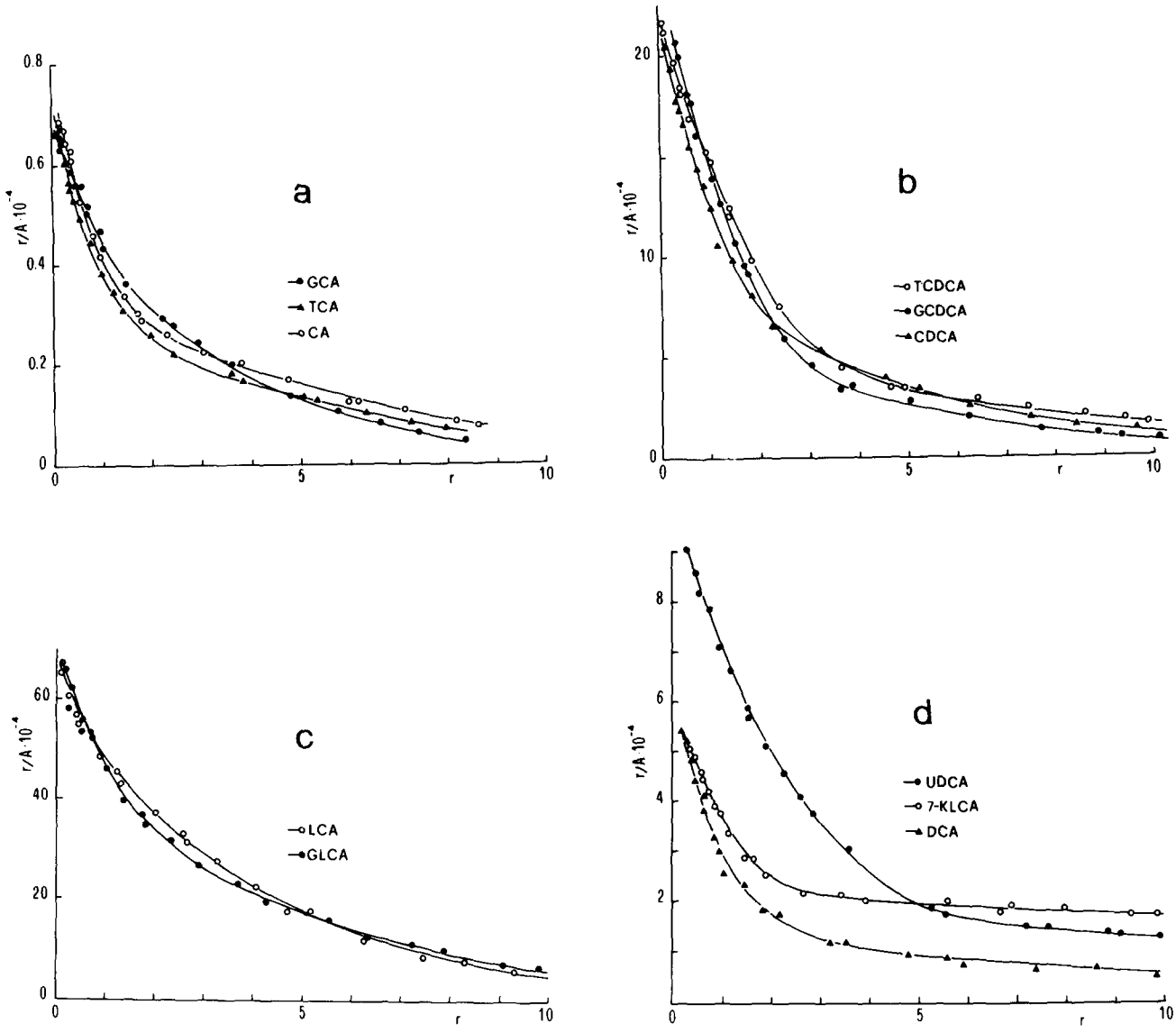

Fig. 1. Scatchard plot of data obtained by equilibrium dialyses of BA and HSA. Each point represents the mean values of three experiments. a) GCA, glycocholic acid; TCA, taurocholic acid; CA, cholic acid. b) TCDCA: taurochenodeoxycholic acid; GCDCA: glycochenodeoxycholic acid; CDCA: chenodeoxycholic acid. c) LCA: lithocholic acid; GLCA: glycolithocholic acid. d) UDCA: ursodeoxycholic acid; 7-KLCA: 7-ketolithocholic acid; DCA: deoxycholic acid.

slightly reduces the affinity constants compared to the respective free BA.

Among the free dihydroxy BA, the affinity constant for ursodeoxycholic acid was 30% lower than that for both chenodeoxycholic and deoxycholic acid. The replacement of a 7-hydroxy group with a 7-keto group significantly reduces the affinity of a BA for albumin.

The relationship between albumin concentration and the percent of BA bound is shown in **Fig. 2.** Lowering the HSA concentration decreases the percent of glycolithocholic, glycocholic, and glycochenodeoxycholic acid bound when present in the medium at a high level (100 μM). At low BA concentrations (2 μM), only the percent of glycocholic acid bound was affected by reducing HSA concentration.

Albumin fluorescence

Since the fluorescence spectrum of albumin has been reported to be sensitive to conformational changes in the protein (10, 11), we have performed an additional set of experiments measuring the fluorescence spectra of HSA with and without all the above BA.

The experiments were set up using phosphate buffer (pH 7.2, 0.1 M) solutions with HSA (1 mg/100 ml), containing the BA at concentrations ranging from 4 to 400 μM. We excited with light at a wavelength of 285

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TABLE 1. Equilibrium constants and binding sites for bile acids vs. human serum albumin

Bile Acid	K_1 Liter/mol	No. of Sites/mol	K_2 Liter/mol	No. of Sites/mol
Glycolithocholic acid	19.6×10^4	3.2	4.1×10^4	9.5
Glycochenodeoxycholic acid	4.9×10^4	3.3	0.24×10^4	12.5
Glycodeoxycholic acid	3.5×10^4	2.0	0.08×10^4	19.0
Glycocholic acid	0.26×10^4	2.9	0.04×10^4	8.7
Lithocholic acid	20×10^4	3.1	3.9×10^4	10
Chenodeoxycholic acid	5.5×10^4	3.4	0.31×10^4	14.5
Deoxycholic acid	4.0×10^4	2.1	0.07×10^4	15.0
Ursodeoxycholic acid	3.8×10^4	2.8	0.08×10^4	29.0
Cholic acid	0.33×10^4	2.8	0.03×10^4	12.0
Taurochenodeoxycholic acid	4.5×10^4	3.2	0.20×10^4	14.0
Taurocholic acid	0.18×10^4	2.3	0.03×10^4	10.0
7-ketolithocholic acid	1.9×10^4	2.0	0.09×10^4	30

mm and examined the fluorescence spectrum from 300 to 450 mm. The fluorescence spectra are shown in **Fig. 3.**

At the maximum peak of fluorescence (340–345 mm), 7-ketolithocholic acid was the only BA that induced a significant quenching. The quenching was concentration-dependent and was present up to the lowest levels of BA, i.e., 4 μM. No variations were observed for the other BA, except for lithocholic acid which showed about 15% quenching at the highest concentration.

DISCUSSION

The data reported in the present investigation confirm that the BA are bound to HSA. The values of the association constants are quite similar to those of most steroids (12, 13) and organic anions (14), but lower than those of fatty acids (15).

The binding may be described in terms of two classes of binding sites. The primary class of sites shows values ranging from 2 to 4 for all the BA; the secondary class, working at a much higher concentration, shows values ranging from 9 to 30.

The exact number of weaker sites cannot be accurately defined, due to the limitation of the analytical procedure. In addition, at higher BA concentrations, above the critical micellar concentration, the accuracy and precision of the measurement is reduced by the association of the BA molecules themselves (16).

Among the BA, lithocholic acid, both free and conjugated, appears to be the most tightly bound to albumin. Such unusually high affinity of lithocholic acid to albumin compared to the other BA has also been documented by others (17). They reported that lithocholic acid spontaneously binds to bovine serum albumin, perhaps by a peptide link with the ϵ-NH_2 groups of the lysine, even if existence of electrostatic interactions between the positively charged ϵ-amino group of lysine and the negatively charged $-$carboxy group of the BA seems more likely. It is possible that the two above-mentioned forces represent the primary force responsible for lithocholic and albumin interaction. The binding is further stabilized by a secondary force, represented by a hydrophobic interaction and Van de Waal's forces.

Moreover, the affinity of the BA for albumin is reduced by an increase in the polarity of the steroid ring, as reported (10) for digoxin and other steroids (12). The introduction of additional OH groups, into the steroid ring, destabilized the bond due to the hydrogen bonds formed with water. In fact, dihydroxy bile acids had an affinity constant about 10-fold lower than the monohydroxy BA; furthermore, the 3,7,12-OH cholanoic acid, cholic acid, is the least tightly bound BA to albumin.

The 3,7,12-hydroxy groups are on the same side of the steroid ring, so that the moiety is divided into two parts, a lipophilic and a hydrophilic one. Since the interaction with the protein is lipophilic, it is not surprising that the K_2 values are relatively dependent on the number of hydroxy groups. Teresi and Luck (18) also found an increase in the binding with chain length for fatty acid

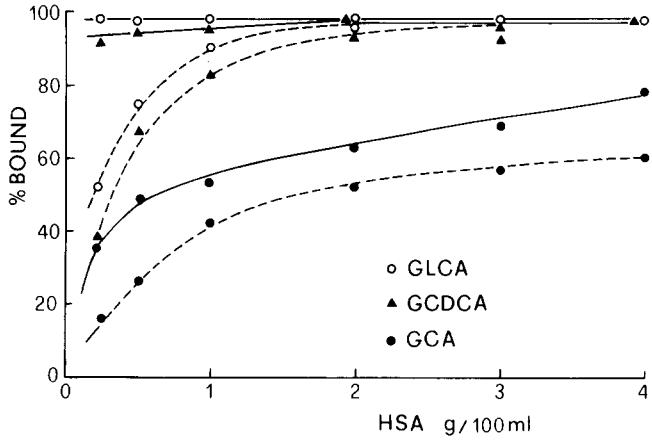

Fig. 2. Relationship between the percentage of three representative BA bound to HSA and the concentration of HSA. Solid lines, initial BA concentration, 2μmol/L; dotted lines, initial BA concentration, 100 μmol/L. For BA symbols, see Fig. 1.

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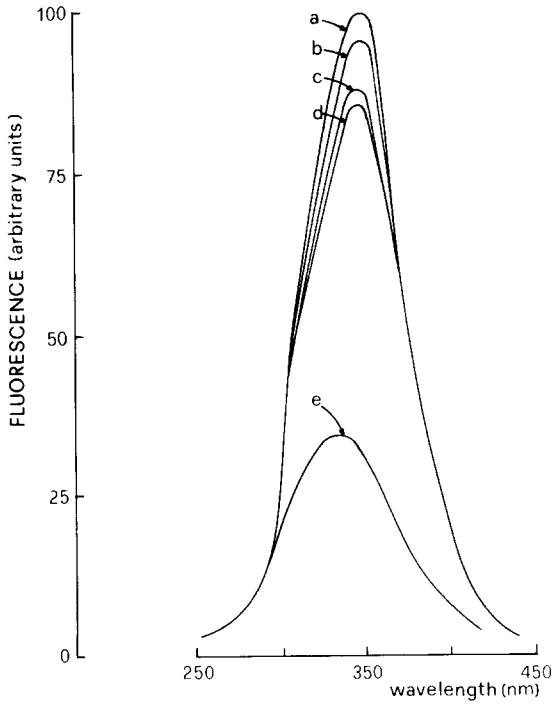

Fig. 3. Fluorescence spectra of HSA (0.2%) with and without BA at a concentration of 4, 40, and 400 μM. a) HSA, 0.2%; b) HSA, 0.2% + 7-KLCA, 4 μM c) HSA, 0.2% + LCA, 400 μM; d) HSA, 0.2% + 7-KLCA, 40 μM: e) HSA, 0.2% + 7-KLCA, 400 μM. For BA symbols, see Fig. 1.

anions, from acetate through caprylate. Markus and Karush (19) reported that the binding of alkyl sulfates was increased by an increase in the size of the non-polar part of the moiety.

The 30% lower affinity constant value for ursodeoxycholic acid with respect to its 7 -epimer may be due in part to differences in the ratio of the number of moles of ionized bile salt to undissociated bile acid as reported by Igimi and Carey (20). Conjugation with glycine or taurine proved to reduce the affinity of the BA for albumin to a limited extent.

These findings are in agreement with Rudman and Kendall (1) but not with Burke et al. (2) who reported that cholic acid is about 10-fold more avidly bound to albumin than its taurine conjugated form. Methodological factors, such as buffer anion competition, electrostatic interactions between buffer and albumin, ionic strength, the type of albumin used, and differences in temperature may explain the differences reported between the two investigators.

The affinity constant of 7-ketolithocholic acid is 50% lower than other disubstituted BA; the introduction of a keto group in the steroid ring modified the stereochemistry of the moiety to a significant extent. The keto group, in fact, is not on the same side as the hydroxy groups, but on the lipophilic part of the steroid. The primary interaction with HSA is still due to electrostatic forces between the carboxylic group of the BA and NH_3^+ lysine group of the protein.

The interaction of the remaining part of the BA moiety and the protein has not yet been fully elucidated. The severe quenching induced by 7-ketolithocholic acid, as observed by fluorescence spectra, may suggest a major conformational change in the albumin. The same phenomenon was also observed with a similar BA, 3-hydroxy, 12 ketocholanic acid.[3]

Physiological implications

In physiological conditions, bile acids are present in serum at concentrations ranging from 1 μM (fasting) to 10 μM (postprandial state), with a molar ratio to albumin not higher than 0.02; therefore, the first class of binding sites adequately characterizes the interaction of the BA with albumin, at least under normal conditions.

From the K values, it is possible to calculate the percent of unbound BA at a known initial BA concentration: 20% of cholic acid, while 1.5% of chenodeoxycholic and 0.5% lithocholic are unbound when present at an initial concentration in the medium of 1 μM for the BA and 400 μM for albumin. Previous studies (2, 5) showed that organic anions, such as sulfobromophthalein and BA in an unbound state, are taken up by the liver faster than the corresponding HSA complexes.

However, quite recently (21) evidence has been presented that the uptake of fatty acids is mediated by a receptor on the liver cell surface. The existence of a receptor for albumin on the hepatocytes may account for the efficient hepatic extraction of those molecules which are most tightly bound to the albumin (22). More recently, Forker and Luxon (23), using a perfused rat liver model, reported that the uptake of taurocholate depends not only on the free BA fraction but also on the interaction between HSA and the cell surface. Another factor to be considered is the possible competition for the albumin binding sites among the BA and other anions in the serum.

Recent reports (24) from our laboratory show that patients with unconjugated hyperbilirubinemia have a higher hepatic [^{14}C]glycocholic acid uptake; these same patients have low cholyl conjugates, but normal chenodeoxycholyl conjugated serum fasting levels. This may be accounted for by an easier displacement of cholic than chenodeoxycholic acid from albumin by unconjugated bilirubin due to its weaker binding to albumin. Furthermore, hypoalbuminemia should be expected to influence the binding of BA, and therefore, their liver uptake, as it has been shown for other albumin bound anions with K_1 values similar to the BA (5).

Since cholic acid has the lowest K_1 value, this BA may be the most affected by hypoalbuminemia. Our data are

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in agreement with this hypothesis. At a physiological (2 μM) concentration of BA, lowering the protein concentration resulted in a decrease in the bound fraction for glycocholic acid, and, to a very limited extent, for conjugated dihydroxy BA (Fig. 3).

The present data suggest an important role for BA albumin binding in the overall liver uptake process.

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