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UPTAKE OF BILE ACIDS BY ISOLATED RAT HEPATOCYTES*

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Abstract—Hepatic uptake of five common bile acids was examined in isolated rat hepatocytes. Taurocholic acid (TCA), glycocholic acid (GCA), cholic acid (CA), deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA) were studied. Uptake was extremely rapid and was linear for at least 45 sec for all bile acids tested at substrate concentrations from 1 to 400 μM. Both nonsaturable binding to the cell surface membrane and the initial rate of uptake (V_0) of the dihydroxy bile acids (DCA and CDCA) were about ten times greater than those of the trihydroxy bile acids (TCA, GCA and CA) which correlates with the higher lipophilicity of the dihydroxy bile acids. Kinetic analysis demonstrated that uptake of these bile acids was due both to a saturable process and a linear process. The apparent diffusion constant (D_{app}) of the unsaturable process for the dihydroxy bile acids was also ten times greater than that for the trihydroxy bile acids. After correction for the nonsaturable binding to the cell membrane and linear entry, the K_m and V_{max} for the uptake was determined. Conjugation with taurine decreased the K_m of CA but not the V_{max}, while glycine conjugation did not alter either parameter, suggesting that conjugation with taurine may increase its affinity for the transport system. The trihydroxy bile acids have a higher affinity but a lower transporting capacity for the saturable process than the dihydroxy bile acids. *In vivo* hepatic extraction appears to be more dependent on the affinity of the bile acid for the transport system than the capacity at which it can be transported.

Hepatic uptake of bile acids plays an important role in the enterohepatic circulation of bile acids and involves three important processes: (1) translocation from plasma to liver across the sinusoidal membrane of the hepatocytes, (2) translocation across the canalicular membrane into bile, and (3) reabsorption from the distal half of the ileum. Investigators have demonstrated that the hepatic uptake of bile acids is a carrier-mediated process, which follows Michaelis–Menten kinetics [1–6]. The efficiency of both hepatic uptake and intestinal absorption of bile acids is dependent on the chemical structure of the bile acid such as the number of hydroxyl groups and if it is conjugated with glycine or taurine [7–9]. Previously, we compared the area under the plasma concentration time curve (AUC) of five common bile acids after administration into the femoral and portal veins of rats [10] and demonstrated that conjugation has a more important effect on hepatic extraction of bile acids *in vivo* than the number of hydroxyl groups.

The recent advent of a technique for preparing viable, isolated hepatic parenchymal cells provides a suitable model for the kinetic study of the hepatic uptake process largely separated from biliary excretion. This model has been used to characterize the hepatic-uptake kinetics of taurocholic acid and cholic acid [6, 11, 12] as well as a number of organic compounds [13–16].

The purpose of the present study was to determine more directly the effect of conjugation and position of hydroxyl groups on the hepatic uptake of bile acids by comparing their kinetics of uptake into isolated rat hepatocytes. The bile acids examined were: taurocholic acid (TCA), glycocholic acid (GCA), cholic acid (CA), deoxycholic acid (DCA), and chenodeoxycholic acid (CDCA).

MATERIALS AND METHODS

The bile acids used in this study were [^{3}H]-taurocholic acid (3.39 Ci/mmole), [^{3}H]-glycocholic acid (3.0 Ci/mmole), [^{3}H]-cholic acid (14 Ci/mmole), [^{3}H]-deoxycholic acid (4 Ci/mmole), and [^{14}C]-chenodeoxycholic acid (50.0 mCi/mmole). All these bile acids were purchased from the New England Nuclear Corp. (Boston, MA) and found to be at least 98–99 per cent pure by thin-layer chromatography. [^{14}C]-Dextran (1.043 mCi/ml) was also purchased from the New England Nuclear Corp. Nonlabeled bile acids and carbonylcyanide-*m*-chlorophenyl hydrazone (CCP) were purchased from CalBiochem (San Diego, CA). Collagenase (type II) was purchased from the Worthington Biochemical Corp. (Freehold, NJ). All other chemicals were commercially available and of analytical grade.

Male Sprague–Dawley rats (250–300 g) were used as liver donors. Hepatocytes were prepared by the procedures of Berry and Friend [2] as modified by Baur *et al.* [1] and described in detail previously [13, 14]. Preparations were considered suitable when the following criteria were fulfilled: trypan blue exclusion was greater than 90 per cent, respiratory stimulation by addition of succinate was less than 20 per cent, and respiratory control by the addition of

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an uncoupler of oxidative phosphorylation (CCP) was greater than 50 per cent [13].

To determine the initial velocity of uptake (V_0) of the bile acids, isolated hepatocytes (1.5–2.5 mg protein/ml) were incubated with 30–50 nCi/ml of ^{3}H- or ^{14}C-labeled bile acids and various amounts of nonlabeled bile acids (sodium salt). After a 5-min preincubation at 37°, 0.1 ml of one of the bile acids was added to 2.5 ml of cell suspension in oxygenated incubation buffer to yield final bile acid concentrations of 1–400 μM. Duplicate samples of the cell suspension were removed at 15, 30, 45, and 60 sec after addition of substrate and placed in 400 μl polyethylene tubes previously layered with 50 μl of 3 M KOH and 50 μl of silicone oil (density, 1.05 g/ml; Aldrich Chemical Co., Milwaukee, WI). The samples were then centrifuged for 5 sec in a rapid accelerating table-top microfuge (Beckman Instruments, Fullerton, CA). The cells, being more dense (density, 1.10–1.14 g/ml) than the silicone oil, move through the oil into the KOH within 1–2 sec. The volume of adherent medium passing through the oil

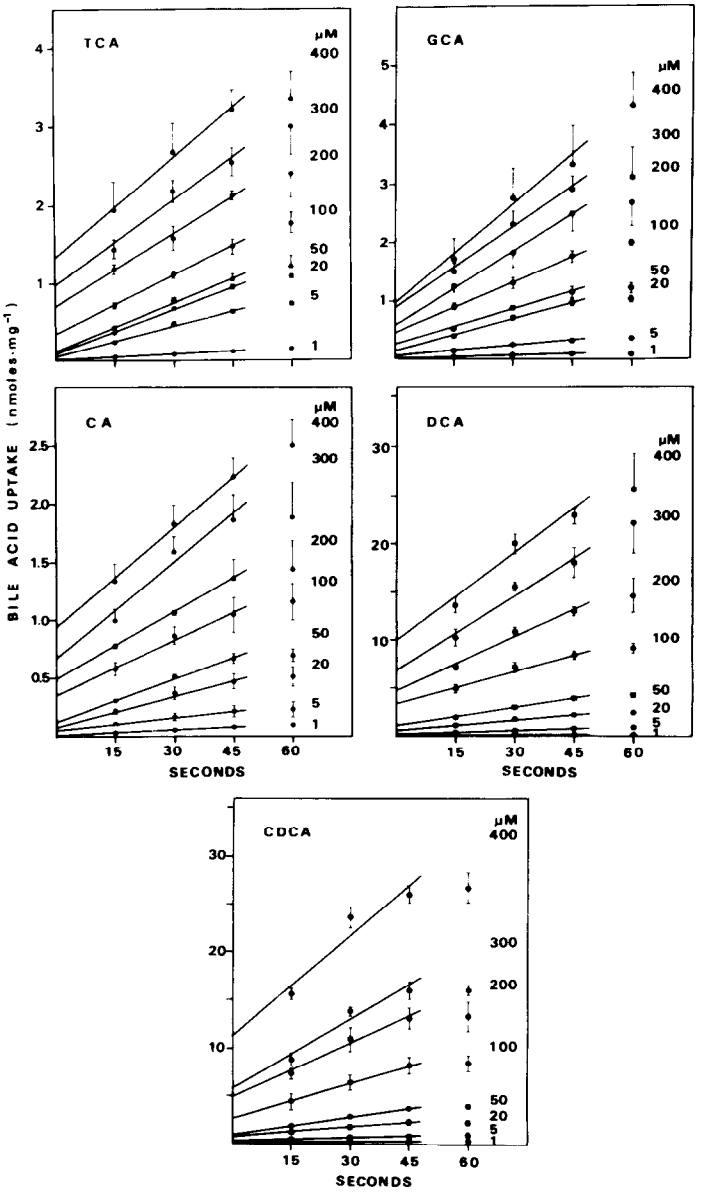

Fig. 1. Uptake of bile acids into isolated hepatocytes. ^{3}H and/or ^{14}C bile acids were added to suspensions of isolated hepatocytes to yield final concentrations of 1–400 μM. Samples of suspensions were obtained at 15, 30, 45 and 60 sec, and the amount of radioactive label contained in cellular fraction was determined. Each point is the mean ± S.E. for three to five independent experiments. Initial velocities of uptake (V_0) for each substrate concentration were determined from the slope of the least-squares regression line through 15-, 30- and 45-sec samples. Key: taurocholic acid (TCA), glycocholic acid (GCA), cholic acid (CA), deoxycholic acid (DCA), and chenodeoxycholic acid (CDCA).

Table 1. Nonsaturable binding of bile acids to plasma membrane*

Bile acids	Binding (nmoles/mg protein) Substrate concentration (μM) 20	200
Taurocholic acid (TCA)	0.09 ± 0.02	0.68 ± 0.16
Glycocholic acid (GCA)	0.21 ± 0.003	0.57 ± 0.09
Cholic acid (CA)	0.08 ± 0.015	0.44 ± 0.05
Deoxycholic acid (DCA)	0.63 ± 0.03	4.72 ± 0.44
Chenodeoxycholic acid (CDCA)	0.75 ± 0.13	4.94 ± 0.48

* Nonsaturable binding was determined from a positive y-intercept of the least-squares regression line for 45 sec in Fig. 1. Each value is the mean ± S.E. for three to five independent experiments.

with the cells was determined by incubating the cells with impermeable [^{14}C]-dextran as previously described [13].

To determine the amount of ^{3}H- or ^{14}C-labeled bile acids taken into the cells, each polyethylene tube was cut just above the oil–KOH interface, and the bottom portion of the tube containing the KOH and cells was placed in a scintillation vial. After the protein had dissolved in the KOH (about 20 hr), 75 μl of 2 N HCl and 10 ml of 3a70B scintillation fluid (Research Products International Corp., Elk Grove Village, IL) were added, and each sample was counted in a Tri-Carb liquid scintillation spectrophotometer (Packard Instruments Corp., Downers Grove, IL). The upper portion of each tube containing the cell-free incubation medium was also

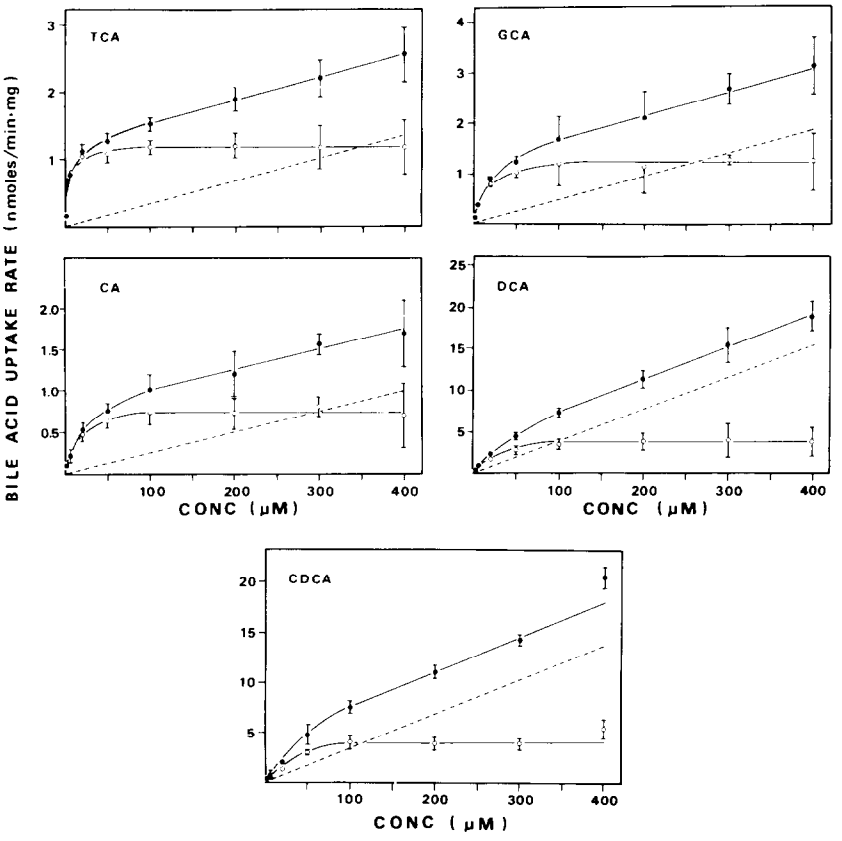

Fig. 2. Plot of initial velocities of uptake (V_0) vs bile acid concentrations. Each point is the mean ± S.E. of three to five independent experiments. The upper line of each figure represents the overall uptake velocity determined from data in Fig. 1, while the lower line represents uptake due only to the saturable component of the uptake process. The dashed line represents uptake due to the unsaturable component of the uptake process (see text). The nomenclature of each panel is the same as for Fig. 1.

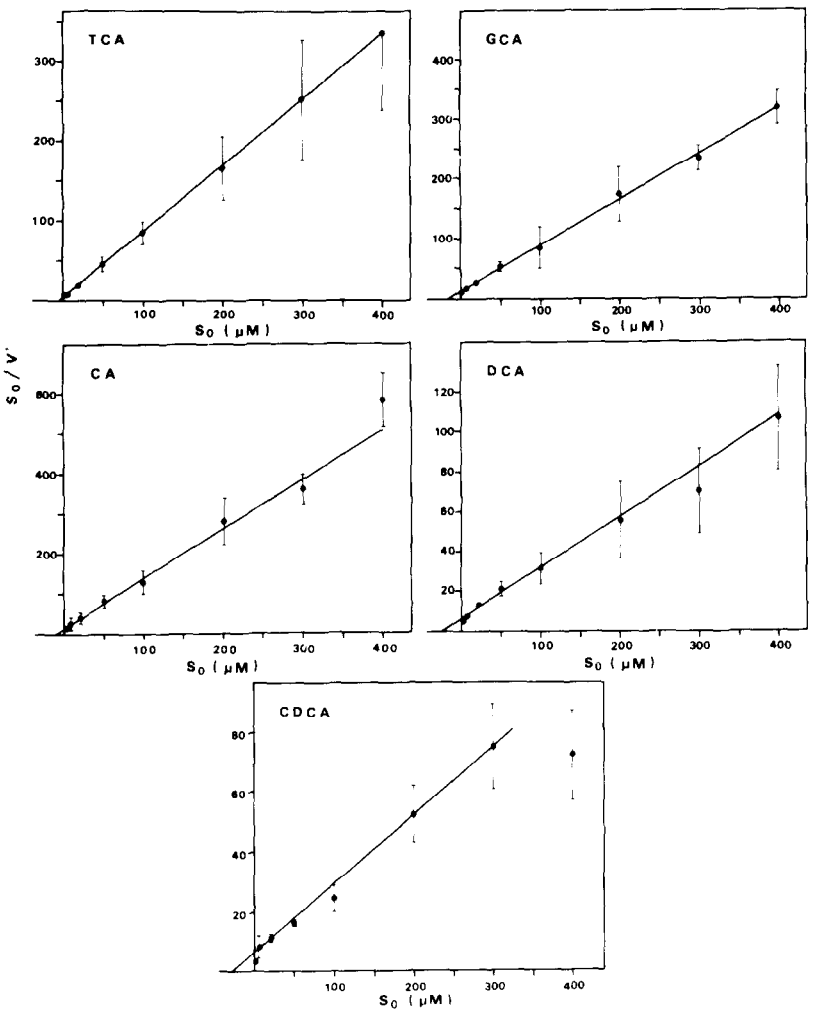

Fig. 3. Hanes–Woolf plots of the saturation curves for bile acids using the data in Fig. 2. Each point is the mean ± S.E. of three to five independent experiments. Values for V_{max} and K_m were determined from the reciprocal of the slope and negative x-intercept, respectively, from the least-squares regression line through the mean value at each substrate concentration. The nomenclature of each panel is the same as for Fig. 1.

placed in a scintillation vial containing 10 ml of 3a70B and counted as described above. No significant radioactivity was detectable in the oil phase that separated the cells from the incubation medium. The amount of bile acid in each pellet fraction was calculated after correction for the adherent fluid, and results were expressed as nanomoles of bile acid per milligram of cellular protein. Protein was determined according to Lowry *et al.* [17] with bovine serum albumin as the standard.

RESULTS

The rate of uptake of bile acids into hepatocytes at substrate concentrations from 1 to 400 μM is shown in Fig. 1. The uptake process was extremely rapid and was linear for at least 45 sec for all bile acids examined. Extrapolation of these lines to zero time yielded a positive intercept. A plot of this value with the corresponding substrate concentration yielded a straight line for all the bile acids, suggesting nonsaturable, nonspecific binding such as adsorption to the cell surface. The zero time intercepts of a low and high concentration of each bile acid (20 and 200 μM) are listed in Table 1. A remarkable difference was revealed between dihydroxy and trihydroxy bile acids, suggesting that dihydroxy bile acids have a higher affinity for the plasma membrane than the trihydroxy bile acids. The rate of uptake of the dihydroxy bile acids (DCA and CDCA) was about ten times greater than that of trihydroxy bile acids (TCA, GCA and CA).

The initial rate of uptake (V_0), calculated from the slope of the increase in bile acid concentration in the cells with time (as in Fig. 1), was plotted against each substrate concentration (Fig. 2). A nonlinear curve suggesting saturation of the uptake process was obtained for all bile acids examined. Since the rate of uptake was linear with substrate concentrations above 100 μM for all bile acids, the overall

Table 2. Kinetic parameters of bile acids*

Bile acids	K_m (μM)	V_{max} (nmoles·min^{-1}·mg^{-1})
Taurocholic acid (TCA)	3.69	1.21
Glycocholic acid (GCA)	14.7	1.30
Cholic acid (CA)	13.1	0.83
Deoxycholic acid (DCA)	24.6	4.00
Chenodeoxycholic acid (CDCA)	32.8	4.35

* Values for K_m and V_{max} were determined from the negative x-intercept and slope, respectively, from the least-squares regression line through the mean value at each substrate concentration in the Hanes–Woolf plots shown in Fig. 3.

uptake process was considered to be a combination of a saturable process and a linear one as reported by Anwer *et al.* [11, 12] for TCA and CA. To separate the relative contributions of each of these processes, the apparent diffusion constants (D_{app}) were calculated from the slopes of the linear portion of each curve above 100 μM. Rate constants of 3.4, 4.8, 2.6, 38.4, and 34.1 pmoles/mg of protein per min per μM was obtained for TCA, GCA, CA, DCA and CDCA respectively. D_{app} values for dihydroxy bile acids were ten times greater than those of trihydroxy bile acids. Uptake due to passive diffusion at each substrate concentration was then calculated by multiplying D_{app} by the substrate concentration. This value was then subtracted from the overall uptake rate at each substrate concentration to yield the initial velocity of uptake of the saturable component. A Hanes–Woolf plot of these data yielded a straight line for the uptake of all bile acids examined (Fig. 3). The kinetic parameters, K_m and V_{max}, were determined from these plots and are listed in Table 2. Both the K_m and V_{max} for dihydroxy bile acids (DCA and CDCA) were greater than those of trihydroxy bile acids (TCA, GCA and CA). While conjugation of cholic acid with glycine did not alter the kinetic parameters, conjugation with taurine decreased the K_m.

DISCUSSION

We have previously determined the effects of both conjugation and the number and position of hydroxyl groups on bile acids on their extraction by the liver (E_h) by comparing the area under the plasma concentration time curve (AUC) after administration of five common bile acids into the femoral and portal veins of rats. The results indicated that conjugation has a more important effect on *in vivo* hepatic extraction of bile acids than the number of hydroxyl groups [10]. In this study, we examined further the characteristics of hepatic uptake of these five common bile acids by comparing their uptake into isolated parenchymal cell preparations.

The uptake of cholic and taurocholic acid into isolated hepatocytes is known to occur rapidly [6, 11, 12, 18]. These results were confirmed in the present study and extended to three other bile acids. The uptake of these five bile acids was linear for at least 45 sec. In comparison to the trihydroxy bile acids (CA, TCA and GCA), the overall uptake rate for the dihydroxy bile acids (DCA and CDCA) was about ten-fold greater (Fig. 1). This is due to a number of factors. The 10-fold larger apparent diffusion constant (D_{app}) for the dihydroxy bile acids is probably due to their greater lipophilicity. CDCA and DCA also had a much higher zero time positive intercept (Fig. 1), apparently indicating a higher adsorption to the plasma membrane. Thus, the higher adsorption of the dihydroxy bile acids to the plasma membrane may also be due to their higher lipophilicity. Anwer *et al.* [19] reported the existence of three different sites for the binding of bile acids to rat liver plasma membranes, one with low, one with medium, and one with high affinity for CA. The low and medium affinity sites were due to nonspecific binding with plasma membrane lipids, while the high affinity binding site was postulated to be the carrier for CA transport. The kinetic parameters for the saturable uptake process for the dihydroxy bile acids were also markedly different (Table 2). Both K_m and V_{max} of CDCA and DCA are larger than those of the trihydroxy bile acids. Thus, the dihydroxy bile acids have a lower affinity and a higher transporting capacity for the saturable process than do the trihydroxy bile acids.

Conjugation of the bile acid with glycine or taurine had less of an effect on the uptake of bile acids into isolated hepatocytes than the position of the hydroxyl groups on the steroid nucleus. The overall uptake of conjugated cholic acid was greater than for cholic acid (Fig. 2). Its conjugation with taurine, the most abundant bile acid in rats, decreased the K_m but conjugation with glycine did not. While conjugation had no consistent effect on the K_m, it increased the V_{max} with both glycine and taurine conjugation (Table 2). This suggests that the taurine conjugation may increase the affinity for the transporting system of the bile acids.

Since under normal physiological conditions, the total concentration of bile acids in the portal blood is about 60 μM [20], the saturable uptake process probably plays an important role in the hepatic uptake of bile acids and the unsaturable uptake process would be relatively insignificant. However, once the blood concentration of bile acids is elevated above 100 μM as in disease, the latter process could be quantitatively important.

Previous *in vivo* experiments in this laboratory have demonstrated that TCA has the highest hepatic extraction (E_h) (about 80 per cent) followed by GCA (65 per cent), CA (55 per cent) and CDCA (55 per cent), with DCA exhibiting the lowest E_h (40 per

cent) [10]. The data presented in this manuscript demonstrate that both the nonsaturable binding to the cell surface and the uptake rate (V_0) for the dihydroxyl bile acids (DCA and CDCA) are about 10-fold greater than those of the trihydroxy bile acids (TCA, GCA and CA). This correlates with the higher lipophilicity of the dihydroxy bile acids. While the hepatocytes have a lower capacity to transport the trihydroxy bile acids, they have a higher affinity for them than the dihydroxy bile acids. The relatively good inverse correlation between E_h and K_m suggests that the affinity of the bile acid for the carrier may be one of the most important factors in determining the hepatic extraction of bile acids. However, the K_m and binding to albumin undoubtedly have some effect. Anwer *et al.* [18], for example, have reported that the presence of albumin decreases the uptake rate of TCA and CA by isolated rat hepatocytes due to the decrease of effective free fraction of these bile acids. Trihydroxy bile acids have a lower affinity for plasma albumin than dihydroxy bile acids [21, 22]. Thus, it appears that the affinity of the bile acids for the carrier is the major determinant of the extraction of bile acids by the liver.

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