Cyclical Oxidation–Reduction of the C_3 Position on Bile Acids Catalyzed by Rat Hepatic 3α-Hydroxysteroid Dehydrogenase

I. Studies with the Purified Enzyme, Isolated Rat Hepatocytes, and Inhibition by Indomethacin

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Abstract

We recently identified that the Y′ bile acid binders are 3α-hydroxysteroid dehydrogenases (3α-HSD). In the present studies, purified 3α-HSD catalyzed rapid ^3H loss from [3β-^3H, C_{24}-^{14}C]lithocholic and chenodeoxycholic acids without net conversion to 3-oxo bile acids under physiologic pH and redox conditions. [3β-^3H]Cholic acid was a poor substrate. The Y′ fraction of hepatic cytosol was exclusively responsible for this activity and ^3H was transferred selectively to $NADP^+$. Time-dependent ^3H loss was also seen in isolated hepatocytes. Further hydroxylation products of lithocholic and chenodeoxycholic acids lost ^3H at the same rate, whereas ^3H loss from lithocholic acid rapidly ceased, which suggests compartmentation of this bile acid in hepatocytes. Indomethacin inhibited ^3H loss from bile acids either in incubations with the pure enzyme or in isolated hepatocytes. Indomethacin did not alter the initial uptake rate of bile acids by hepatocytes, but caused a redistribution of unconjugated bile acids into the medium at early time points (2.5 and 5.0 min) and that of conjugated bile acids at later time intervals (30 min). ^3H loss from the 3β position therefore can be used to probe the interaction between bile acids and cytosolic 3α-HSD in intact cells, and indomethacin is capable of inhibiting this interaction.

Introduction

Recent studies have begun to elucidate the mechanisms of bile acid transport by both the sinusoidal and canalicular poles of hepatocytes (1–3). However, little is known about the intracellular transport of bile acids or the sites of bile acid biotransformation (4, 5). We have characterized previously the binding properties of the major bile acid binding proteins in rat liver cytosol (6–9). Three families of proteins with different molecular weights have been studied: GSH *S*-transferases (45–50-kD dimers), Y′ bile acid binders (33-kD monomers), and Z protein (14-kD monomer). Among these proteins, GSH *S*-transferases that have the Y_a subunit (ligandin) and Y′ bile acid binders exhibit the highest affinities for bile acids (6–8). Although the purified proteins bind bile acids, no direct evidence exists that bile acids actually interact with these proteins in hepatocytes. Also, the role of these proteins in vectorial translocation of bile acids from one pole of the hepatocyte to the other remains speculative.

Recently, we reported that Y′ bile acid binders are 3α-hydroxysteroid dehydrogenases (3α-HSD)[1] (10). This enzyme catalyzes the bidirectional oxidation-reduction of the 3 position (C_3) on bile acids. Under physiological redox and pH conditions, this enzyme in pure form or in hepatocytes reduced 3-oxo-cholic acid to cholic acid, but did not catalyze the net conversion of cholic to 3-oxo-cholic acid (10). However, 3α-HSD may catalyze the bidirectional equilibrium cycling of bile acids (3α-OH ⇌ 3-oxo) without net conversion of 3α-OH bile acids to 3-oxo bile acids (Fig. 1). In this report, [3β-^3H, 24-^{14}C]bile acids were used to test this hypothesis by monitoring ^3H loss from bile acids in studies with purified 3α-HSD and in isolated hepatocytes. The loss of 3β-^3H was used to probe the interaction of bile acids with cytosolic 3α-HSD in intact hepatocytes. Also, the effect of indomethacin, a competitive inhibitor of this enzyme (11, 12), was examined.

Methods

Preparation of double-labeled bile acids (Fig. 1). [3β-^3H]Bile acids were synthesized by [^3H]sodium borohydride treatment of 3-oxo bile acids (13). Solutions of 3-oxo-5β-cholanic acid (Sigma Chemical Co., St. Louis, MO), 2.2 mg in 1.2 ml methanol and 0.01 ml water; 3-oxo-7α-hydroxy-5β-cholanic acid methyl ester (gift from Dr. K. Uchida, Shionogi Research Laboratory, Osaka, Japan), 0.8 mg in 0.4 ml methanol and 0.01 ml water; and 3-oxo-7α,12α-dihydroxy-5β-cholanic acid (Calbiochem, San Diego, CA), 3.2 mg in 0.8 ml methanol and 0.08 ml water, were individually mixed with excess [^3H]sodium borohydride (500 mCi/mmol, Amersham Corp., Arlington Heights, IL) and incubated at room temperature for 90 min. After incubation, 2.5 ml of water was added to the mixture, acidified to pH 1 with 2 N HCl, and bile acids were extracted twice with diethylether. Ether extracts were washed with water and evaporated to dryness. The product with 3-oxo-7α-hydroxy-5β-cholanic acid methylester was dissolved in 0.4 ml methanol, saponified at 80°C for 1 h with 2 ml of 1 N NaOH, extracted with diethylether after acidification, and evaporated to dryness. Dried samples were applied to thin-layer chromatography (TLC) plates and developed with the solvent system of isooctane/ethyl acetate/acetic acid (50:50:0.7 vol/vol) for monohydroxy and dihydroxy

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1. *Abbreviations used in this paper:* 3α-HSD, 3α-hydroxysteroid dehydrogenases; LDH, lactic dehydrogenase; TLC, thin-layer chromatography.

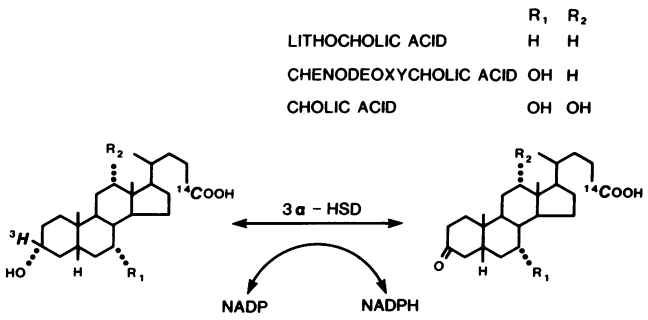

Figure 1. Equilibrium cycling of double-labeled bile acids catalyzed by 3α-HSD. ^3H loss from 3β position of bile acids is shown.

bile acids and with the solvent system of benzene/dioxane/acetic acid (55:40:2 vol/vol) for trihydroxy bile acids. The lanes corresponding to 3α-hydroxy bile acids were scraped, and bile acids were extracted with methanol and evaporated to dryness. [3β-^{3}H]Bile acids were mixed with respective [^{14}C]bile acids to establish the $^3H/^{14}C$ ratio between 5 and 10; [^{14}C]lithocholic acid (55 mCi/mmol, Amersham Corp.), [^{14}C]chenodeoxycholic acid (60 mCi/mmol, Amersham Corp.), and [^{14}C]cholic acid (52 mCi/mmol, New England Nuclear, Boston, MA) were employed for this purpose. No contamination by 3β-hydroxy [3α-^{3}H]bile acids was insured by clear separation on TLC and by the demonstration that ^3H was completely lost from the bile acids by incubation with 3α-HSD and excess $NADP^+$. The mixtures of ^3H and ^{14}C were referred to as double-labeled bile acids.

Purification of 3α-HSD. The major form of 3α-HSD (3α-HSD-I) was purified from male Sprague–Dawley rat liver cytosol, as previously reported (10). Briefly, Y' fraction (30,000–40,000 mol wt) was obtained by Sephadex G75 superfine chromatography of cytosol, and 3α-HSD was purified by sequential chromatography with Affigel blue, chromatofocusing on PBE 94 and 3'-phosphoadenosine 5'-phosphate–agarose gel. Purified protein was homogeneous on sodium dodecyl sulfate–polyacrylamide gel electrophoresis according to the method of Laemmli (14).

Kinetics of pure 3α-HSD-I with radiolabeled bile acid. The enzyme (2 μg) was incubated at 37°C in 1 ml 0.05 M sodium phosphate buffer, pH 7.0, which contained 100 μM $NADP^+$ double-labeled lithocholic acid and 20 μM unlabeled lithocholic acid. The ^3H loss at 2 min was determined at each bile acid concentration and the results expressed as nanomoles bile acid metabolized per minute. The reaction was linear for 2 min. The results were then fit by nonlinear least squares to the Michaelis–Menten equation to derive kinetic parameters.

Conversion of lithocholic and 3-oxo-5β-cholanic acid by purified 3α-HSD under normal redox conditions. [^{14}C]3-oxo-5β-Cholanic acid was prepared from [^{14}C]lithocholic acid by treatment with bacterial 3α-HSD, followed by purification on TLC. Partially purified 3α-HSD was incubated with either [^{14}C]lithocholic acid or 3-oxo-5β-cholanic acid (5 μM final concentration) in 0.05 M sodium phosphate buffer, pH 7.0, which contained a mixture of the four nicotinamide coenzymes approximating the normal redox state in the liver (15, 16): 20 or 100 μM $NADP^+$, 400 μM NADPH, 800 μM NAD^+, and 200 μM NADH. Incubation was performed at 37°C for 1–30 min. Bile acids were extracted with diethylether at pH 1, washed with water, and evaporated to dryness. Samples were analyzed on TLC with a solvent system of isooctane/ethylacetate/acetic acid (50:50:0.7 vol/vol), and lanes corresponding to respective 3α-hydroxy and 3-oxo forms were scraped and counted.

Incubation of double-labeled bile acids with 3α-HSD. Double-labeled bile acids (each 1 μM) in 0.05 M sodium phosphate buffer, pH 7.0, which contained four nicotinamide coenzymes (final volume 1 ml) were incubated with purified 3α-HSD (2 μg) at 37°C for 5–40 min. After incubation, bile acids were extracted with diethylether, washed with water, and evaporated to dryness. Radioactivity was determined by liquid scintillation spectrometry. After correction for crossover of ^{14}C into ^3H channel, $^3H/^{14}C$ ratios were calculated. In the experiments to determine the effects of indomethacin, the same incubation was performed for 20 min in the presence of 2–20 μM indomethacin.

^3H loss catalyzed by subcellular fractions. Liver homogenate (33% vol/vol in 0.01 M sodium phosphate buffer, 20% sucrose, pH 7.4) was centrifuged for 10 min at 2,500 *g* and pellet was discarded. The supernatant was centrifuged for 60 min at 100,000 *g*. The pellet was washed three times with the buffer and resuspended in it. Protein concentrations in homogenate and fractions were measured by protein assay (Bio-Rad Laboratories, Richmond, CA). Double-labeled lithocholic acid (1 μM) was incubated at 37°C for 20 min either with homogenate or fractions (0.6 mg protein) in 0.05 M sodium phosphate buffer, pH 7.0, which contained four nicotinamide coenzymes. Bile acids were extracted with butanol at pH 1, washed with water, and evaporated to dryness and radioactivity was determined.

Cytosol (100,000 *g* supernatant) was fractionated on Sephadex G75 superfine into six-pooled fractions as previously reported (17). Double-labeled lithocholic acid (1 μM) was incubated at 37°C for 10 min with 10 μl of each fraction in 0.05 M sodium phosphate, pH 7.0, which contained four nicotinamide coenzymes. Bile acids were extracted with diethylether after acidification, washed with water, and evaporated and radioactivity was determined.

High-performance liquid chromatography (HPLC) of nicotinamide coenzymes. To show the fate of ^3H lost from 3β position of bile acids, incubation mixtures at 30 min were analyzed on HPLC according to the method of Jones (16) using an Ultrasil-ODS column (10 μm, 4.6 × 25 cm; Altex Scientific, Inc., Berkeley, CA) under isocratic condition with 3.75% methanol in 0.1 M potassium phosphate buffer, pH 6, with a flow rate of 2 ml/min. Aliquots of incubation mixtures with double-labeled lithocholic acid were directly applied to HPLC. Nicotinamide coenzymes were detected either with A_{340} or A_{260} and radioactivities (^3H and ^{14}C channel) were monitored continuously with a radioactive flow detector (Radiomatic Instruments & Chemical Co., Inc., Tampa, FL).

Preparation of isolated hepatocytes. Isolated hepatocytes were prepared from male Sprague–Dawley rats according to the method of Moldeus (18) as previously reported (19). Viability was > 90% as assessed by trypan blue exclusion.

Incubation of double-labeled bile acids with isolated hepatocytes. Double-labeled bile acids (5 μM) were incubated with isolated hepatocytes (2×10^6 cells/ml) in preoxygenated Krebs–Henseleit buffer with 25 mM Hepes, pH 7.4, at 37°C in room air in an orbital shaker. Some incubations were done in the presence of indomethacin (50 or 100 μM). At various times, aliquots (0.5 ml) of the suspension were taken and cells were separated from the media by centrifugation (Eppendorf Microfuge; Brinkmann Instruments Inc., Westbury, NY; 5 s). Cells were treated with 1 N potassium hydroxide at room temperature for 24 h. Media and treated cell suspensions were acidified to pH 1 with 2 N HCl, extracted with butanol, and washed with water. Aliquots of butanol layer were evaporated and counted. The remainder of butanol extracts from cells were evaporated and further analyzed on TLC.

A portion of the evaporated extracts was directly analyzed on TLC with a solvent system of chloroform/methanol/acetic acid/water (65:24:15:9 vol/vol) with appropriate standards before deconjugation of amide bonds. Most samples were treated with cholylglycine hydrolase (Sigma Chemical Co.) and extracted with ether, as previously described (13). Deconjugated samples were dissolved in methanol, applied on TLC plates, and developed with isooctane/ethyl acetate/acetic acid (10:10:2 vol/vol). Bile acid standards were visualized with 10% phosphomolybdic acid in ethanol followed by heating at 120°C. Sample lanes were scraped every 5–7 mm and radioactivity was determined.

To exclude the possibility of cell damage by indomethacin, the leakage of lactic dehydrogenase (LDH) into media was checked after 30 min incubation of hepatocytes (2×10^6 cells/ml) in the absence and presence of 100 μM indomethacin. Cells were separated from media and resuspended in equal volume of buffer and 10% Triton X-100. The

media were used directly for LDH assay using the Sigma diagnostic kit (Sigma Chemical Co.). Indomethacin did not cause increased LDH release (10% for both indomethacin and controls).

To exclude the possibility that ^3H loss might occur due to catalysis by enzyme and coenzymes in media released by cell lysis, the media separated after 30 min incubation was incubated with double-labeled lithocholic acid (2 μM in final) for an additional 30 min. Media were then processed as described above, and the $^3H/^{14}C$ ratios determined. No significant loss of ^3H from lithocholic acid occurred under this condition.

Incubation of [^{14}C]lithocholic acid with isolated hepatocytes. To examine the extent of sulfation and glucuronidation of lithocholic acid, [^{14}C]lithocholic acid was incubated with isolated hepatocytes under the same conditions as those with double-labeled bile acids. Extraction of bile acids was performed with Sep Pak C_{18} cartridges (Waters Instruments, Inc., Rochester, MN) to avoid the solvolysis of sulfate esters during the extraction of bile acids under acid conditions. Media and KOH-treated cells were mixed with 1 M sodium phosphate buffer and pH was adjusted to eight. Samples were applied to prewashed cartridges and washed with water, and bile acids were eluted with methanol (13). Evaporated samples were treated with cholylglycine hydrolase. Unconjugated bile acids were extracted with Sep Pak C_{18} cartridges and analyzed on TLC. Sulfate and glucuronide of lithocholic acid migrate slower than all the unconjugated bile acids on TLC with the solvent system of isooctane/ethyl acetate/acetic acid (10:10:2 vol/vol). These bile acids were prepared as previously reported (8, 9, 13).

Effects of indomethacin on the initial uptake of bile acids by isolated hepatocytes. Initial uptake of [^{14}C]chenodeoxycholic and [^3H]taurocholic acid (5 Ci/mmol, New England Nuclear) (each 5 μM) by isolated hepatocytes (2×10^6 cells/ml in Krebs–Henseleit, 25 mM Hepes, pH 7.4) was examined in the absence and presence of 100 μM indomethacin added at time zero. Incubation was performed at 37°C for 15, 30, 45, and 60 s, and cells were separated from media by rapid filtration onto a filter (0.45 μm Millipore/HAWP-25; Continental Water Systems, Bedford, MA), followed by washing with buffer. Radioactivity in cells was then determined. To estimate the binding of bile acids to cell surfaces, control experiments were performed at 4°C for 60 s. In case of taurocholic acid, the effect of preincubation of cells with 100 μM indomethacin for 30 min before determining bile acid uptake was also examined.

Results

Conversion of lithocholic and 3-oxo-5β-cholanic acid by purified 3α-HSD under normal redox conditions. 3-Oxo-5β-cholanic acid was rapidly reduced to lithocholic acid by purified 3α-HSD under physiological conditions; however, negligible conversion of lithocholic acid to its 3-oxo form was detected (Fig. 2). After 30 min incubations of radiolabeled lithocholic or 3-oxo-5β-cholanic acids with isolated hepatocytes, no 3-oxo-5β-cholanic acid was detected either in cells or medium (data not shown).

Incubation of double-labeled bile acids with 3α-HSD. Time-dependent ^3H loss from the 3β position of lithocholic and chenodeoxycholic acid was catalyzed by 3α-HSD under physiological redox conditions (Fig. 3). This reaction did not occur in the absence of 3α-HSD or nicotinamide coenzymes. No ^3H loss from cholic acid was observed under this condition.

To exclude an isotope effect, we determined the Michaelis constant (K_m) for ^3H loss from lithocholic acid catalyzed by pure 3α-HSD in the presence of $NADP^+$. The K_m was 1.8 μM and maximum velocity (V_{max}) 2.6 μmol/min per mg, which agrees closely with the K_m of 2.6 μM and V_{max} of 2.9 μmol/min per mg for unlabeled bile acid determined by the rate of reduction of $NADP^+$ (10). Thus, the enzyme does not distinguish between labeled and unlabeled bile acid.

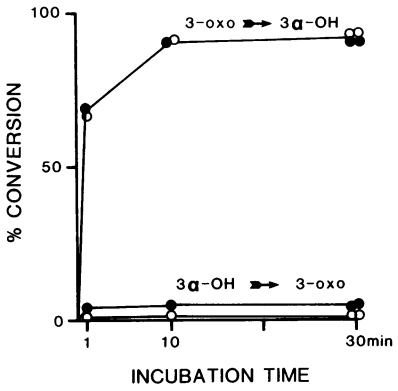

Figure 2. Metabolism of lithocholic and 3-oxo-5β-cholanic acid (5 μM) catalyzed by 3α-HSD. Incubation was performed at 37°C with four nicotinamide coenzymes in 0.05 M sodium phosphate buffer, pH 7.0: $NADP^+$ 20 μM (●) or 100 μM (○), NADPH 400 μM, NAD^+ 800 μM, and NADH 200 μM. Separation of 3α-hydroxy and 3-oxo forms was performed by TLC.

Indomethacin, in a concentration-dependent fashion, inhibited ^3H loss from lithocholic and chenodeoxycholic acid catalyzed by 3α-HSD. ID_{50} values for this inhibition were between 5–10 μM (Fig. 4).

^3H loss catalyzed by various subcellular fractions. ^3H loss was only catalyzed by the cytosolic fraction of liver homogenate and was not catalyzed by the mixed particulate fraction (mitochondria and microsomes) contained in 100,000 *g* pellet (Table I). Among various cytosolic fractions only Y′ fraction catalyzed this reaction (Table II). The presence of some activity in Y fraction is due to the cross-contamination of 3α-HSD in the crude Y fraction. We have previously shown that 3α-HSD elutes as a peak corresponding to the Y′ fraction with some crossover to the Y fraction (10).

HPLC of nicotinamide coenzymes. The transfer of the ^3H lost from 3β position to a specific cofactor was examined by HPLC (Fig. 5). Elution of ^3H corresponded exclusively to NADPH (52% of the total ^3H of the incubation was recovered with NADPH and the rest remained on the bile acid). No ^{14}C corresponding to lithocholic acid was detected in the eluants because lithocholic acid remained bound to the column under this condition. [^3H]Water, which would elute at the void volume, was not detected. This ^3H transfer reaction did not occur

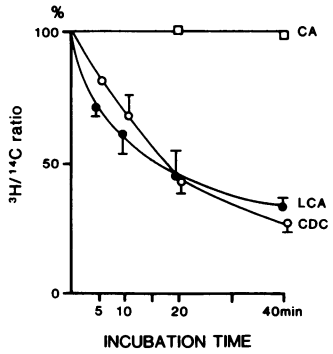

Figure 3. ^3H loss from double-labeled bile acids catalyzed by purified 3α-HSD. In this and subsequent figures 100% represents the starting $^3H/^{14}C$ ratio. Incubation was performed at 37°C with four nicotinamide coenzymes in 0.05 M potassium phosphate buffer, pH 7.0. Concentrations of $NADP^+$, NADPH, NAD^+, and NADH were 100, 400, 800, and 200 μM, respectively. Final concentration of each bile acid was 1 μM. Values are means±SD from three experiments. LCA, lithocholic acid; CDC, chenodeoxycholic acid; and CA, cholic acid.

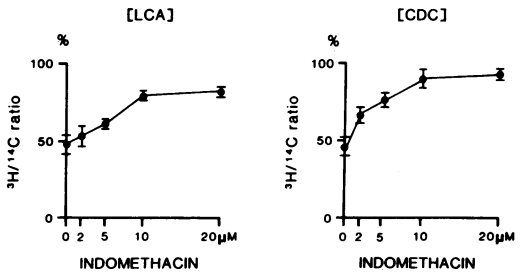

Figure 4. Effect of indomethacin on ^3H loss from double-labeled bile acids by purified 3α-HSD. Conditions are the same as in Fig. 3. Incubation was performed for 20 min. Values are means±SD, $n = 3$, expressed as percentage of the starting ratio.

in the absence of 3α-HSD (Fig. 5) or coenzymes (data not shown).

Incubation of double-labeled bile acids with isolated hepatocytes. In incubations of cells with the three double-labeled bile acids, time-dependent ^3H loss was observed from the bile acids extracted from both cells and medium (Fig. 6). ^3H loss was most rapid with chenodeoxycholic acid and slowest with cholic acid. The rate of ^3H loss occurred more slowly from the bile acids in media than in cells. ^{14}C, representing bile acids, was mainly distributed in cells (Table III). Distribution of radioactivity in cells plateaued after 5 min incubation with either chenodeoxycholic or cholic acid and after 20 min with lithocholic acid.

^3H loss in hepatocytes was inhibited by indomethacin (Fig. 7). Also, indomethacin altered the distribution of [^{14}C]bile acids between cells and media with significantly more radioactivity associated with the media (Table IV).

Further hydroxylation of bile acids in hepatocytes and their $^3H/^{14}C$ ratios were assessed by TLC of extracts of the cells and media (Fig. 8). Time-dependent hydroxylation of lithocholic and chenodeoxycholic acids occurred up to 30 min (Fig. 9). The decrease of $^3H/^{14}C$ ratios in lithocholic acid metabolites differed among mono-, di-, and trihydroxy metabolites. The loss of ^3H from di- and trihydroxy bile acid metabolites of lithocholic acid proceeded in parallel with incubation time, whereas $^3H/^{14}C$ ratio of original monohydroxy bile acid (lithocholic acid) plateaued after 5 min incubation.

Sep pak C_{18} extraction and TLC identification revealed that only a small part (< 10%) of the remaining lithocholic acid both in cells or media was sulfated or metabolized to a

Table I. ^3H Loss from [^3H, ^{14}C]Lithocholic Acid Catalyzed by Subcellular Fractions

	$^3H/^{14}C$ ratio
	%
Homogenate	56.5
Cytosol	32.5
Pellet (100,000 *g*)	96.7

1 μM lithocholic acid was incubated at 37°C for 20 min with 0.6 mg protein from each fraction. Bile acids were extracted with butanol at pH 1, and $^3H/^{14}C$ ratio was calculated. Values are means of duplicate reactions.

Table II. ^3H Loss from [^3H, ^{14}C]Lithocholic Acid by Various Hepatic Cytosolic Fractions

Fraction	Approximate molecular weight	$^3H/^{14}C$ ratio
		%
X	80,000	104
Albumin	65,000	100
Y	50,000	89
Y′	35,000	77
Unknown	20,000	104
Z	14,000	104

Gel filtration of cytosol was performed on Sephadex G75 superfine, and eluates were divided into six fractions according to protein peaks, as previously reported (17). 1 μM lithocholic acid was incubated for 10 min with 10 μl of each fraction.

glucuronidated form (data not shown). Indomethacin had minimal effects on the further hydroxylation of bile acids (Fig. 10). ^3H loss from both di- and trihydroxy bile acids derived from incubations with chenodeoxycholic acid was inhibited by indomethacin. However, in incubations with lithocholic acid, indomethacin inhibited ^3H loss from di- and trihydroxy metabolites, but not from unchanged lithocholic acid (Fig. 10).

Fig. 11 (top) demonstrates the effect of indomethacin (100 μM) on the $^3H/^{14}C$ ratio during the early time of incubation of double-labeled chenodeoxycholic acid with isolated hepatocytes. After only 2.5 min incubation, significant ^3H loss was observed both in cells and media, both of which were inhibited by indomethacin. Table V demonstrates the hydroxylation and conjugation of chenodeoxycholate and its metabolites in cells and media in the absence and presense of 100 μM indomethacin. At these early time points, indomethacin also demonstrated an effect on the distribution of bile acids in cells and media. As early as 2.5 min, most bile acids had already been conjugated with taurine. However, significantly more unconjugated bile acid was recovered in the medium in the presence of indomethacin.

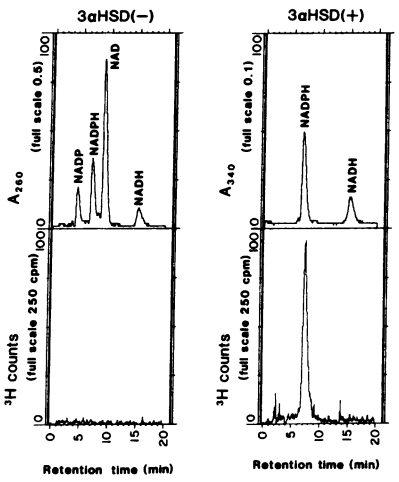

Figure 5. HPLC showing the transfer of ^3H from the 3β position of lithocholic acid to NADPH. Incubation was performed at 37°C for 30 min with 5 μM [^3H], [^{14}C]lithocholic acid, 7 μg enzyme, and mixture of four coenzymes at pH 7. Column: Ultrasil-ODS (10 μm, 4.6 × 25 cm; Altex Scientific, Inc.). Solvent: 3.75% methanol in 0.1 M potassium phosphate, pH 6 (2 ml/min). The conditions on the left and right were identical other than the absence or presence of enzyme.

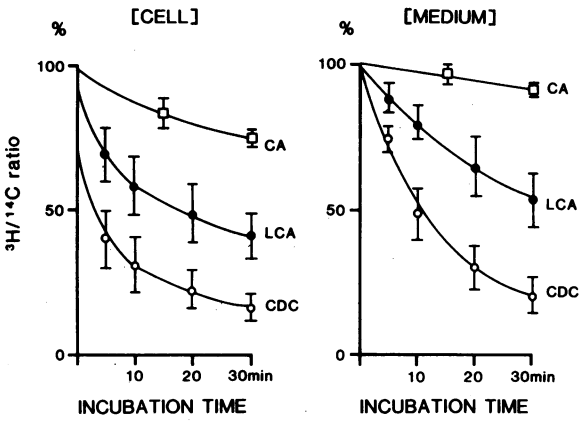

Figure 6. ^3H loss from double-labeled bile acids incubated with isolated hepatocytes. Incubation was performed at 37°C with 5 μM bile acids, 2×10^6 cells/ml in Krebs–Henseleit buffer, 25 mM Hepes, pH 7.4. Media and KOH-treated cells were extracted with butanol at pH 1 and aliquots of butanol layer were counted for ^3H and ^{14}C. Values are means±SD of six experiments from three different cell preparations.

Fig. 11 (bottom) shows the $^3H/^{14}C$ ratio of taurochenodeoxycholic acid in cells during incubation with dual-labeled chenodeoxycholic acid. Taurochenodeoxycholic acid was the major metabolite in cells, representing about half of the total radioactive bile acids (cells plus media). Within 2.5 min, ^3H loss from taurochenodeoxycholic acid rapidly occurred in hepatocytes and was inhibited by indomethacin. Thus, indomethacin not only inhibited ^3H loss from bile acid in the medium (mostly unconjugated chenodeoxycholate), but also inhibited ^3H loss from bile acids that were inside the hepatocytes.

Effects of indomethacin on the initial rate of uptake of bile acids by isolated hepatocytes. As shown in Table VI, indomethacin (100 μM) had no effects on the initial uptake of chenodeoxycholic or taurocholic acids. Also, preincubation of cells with indomethacin did not affect the initial rate of uptake of taurocholic acid.

Table III. ^{14}C Bile Acids in Media during Incubation of Double-labeled Bile Acids with Isolated Hepatocytes

Bile acids	Incubation time	Percentage of total ^{14}C in media
	min	
Lithocholic acid	5	8.1±0.4
	10	8.3±1.1
	20	11.4±0.8
	30	12.7±2.7
Chenodeoxycholic acid	5	21.4±3.7
	10	17.4±3.7
	20	20.7±4.2
	30	24.7±2.5
Cholic acid	15	36.7±7.4
	30	30.3±4.9

Data are expressed as percentage of total ^{14}C in the incubation mixture (cells plus media) recovered in the media at various times. Results are means±SD for experiments with three cell preparations.

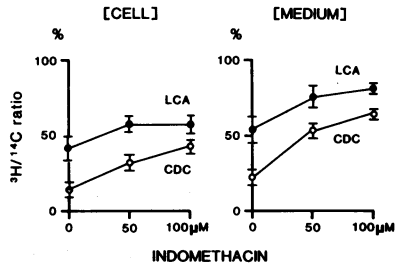

Figure 7. Effect of indomethacin on ^3H loss from double-labeled bile acids in isolated hepatocytes. Incubation was performed for 30 min under the same conditions as in Fig. 6. Values are means±SD of four experiments from two different cell preparations.

Discussion

We have previously demonstrated that the 33-kD Y′ bile acid binders are 3α-HSD (10). Since these cytosolic proteins can either bind or metabolize bile acids, it is of great interest to examine their possible role in intracellular bile acid transport. Although bile acids also bind to two other classes of cytosolic proteins (6–9), there is reason to believe that binding to the Y′ proteins may be the most important interaction. Although GSH *S*-transferases bind bile acids with comparable affinity as 3α-HSD (7), in the presence of physiologic concentrations of GSH, binding of some bile acids is markedly inhibited (8). Z protein also binds bile acids, but would be expected to preferentially bind fatty acids with much higher affinity (9). Indeed, the work of others using bile acid affinity probes demonstrates preferential labeling of a 33-kD cytosolic protein in intact hepatocytes (20, 21).

It has been very difficult to design experiments that can trace the movement of bile acids in intact hepatocytes and to determine the physiologic importance of the interaction of bile acids with cytosolic proteins. It is not even certain that bile acids enter cytosol as opposed to intracellular transfer by lateral diffusion in membranes or within vesicles. Having identified that the 33-kD Y′ bile acid binders are 3α-HSD (10), we now could take advantage of its enzymatic properties to probe these issues.

Table IV. Effect of Indomethacin on the Distribution of ^{14}C Bile Acids in Media after 30 min Incubation of Double-labeled Bile Acids with Isolated Hepatocytes

	Indomethacin concentration (μM)		
Bile acids	0	50	100
	%	%	%
Lithocholic acid	12.7±2.7	28.5±1.8*	40.3±4.5‡
Chenodeoxycholic acid	24.7±2.5	40.8±4.9§	50.7±3.7‡

Results are means±SD of percentage of total ^{14}C radioactivity present in media for six experiments. Comparisons were by analysis of variance.
* $P < 0.001$ vs. control.
‡ $P < 0.01$ 100 μM vs. 50 μM.
§ $P < 0.01$ vs. control.

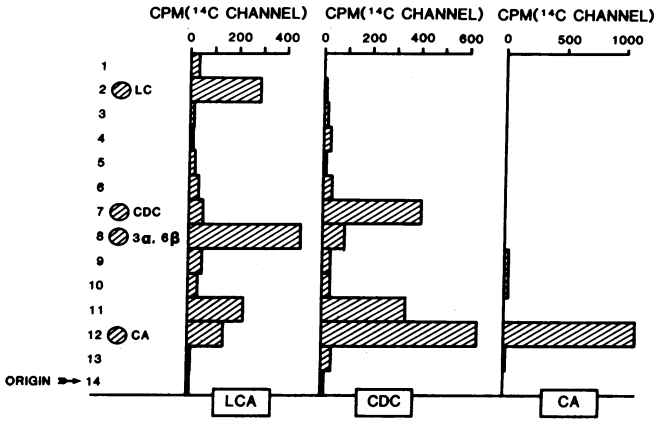

Figure 8. TLC showing the hydroxylation pattern of bile acids by isolated hepatocytes (30 min incubation). Bile acids in cells were extracted, treated with cholylglycine hydrolase, and aliquots in methanol were applied onto a Silica gel plate (20 × 20 cm). Every 7 mm of the lanes were scraped and counted. Solvent: Isooctane/ethyl acetate/acetic acid (10:10:2 vol/vol).

We previously demonstrated that pure 3α-HSD with a physiologic mixture of cofactors and the enzyme in isolated hepatocytes rapidly reduced 3-oxo-cholic acid, but did not convert cholic acid to 3-oxo-cholic acid (10). We have extended this observation to a higher affinity substrate for the enzyme, lithocholic acid, in this report. Thus, due to its cofactor preference and pH optimum, the enzyme functions as a reductase and not a dehydrogenase in terms of net reactions. However, in the presence of a mixture of oxidized and reduced cofactors, it is expected that bidirectional, cyclic oxidation–reduction of the C_3 position would occur without net conversion to 3-oxo bile acid. Therefore, we prepared [3β-^{3}H]bile acids to

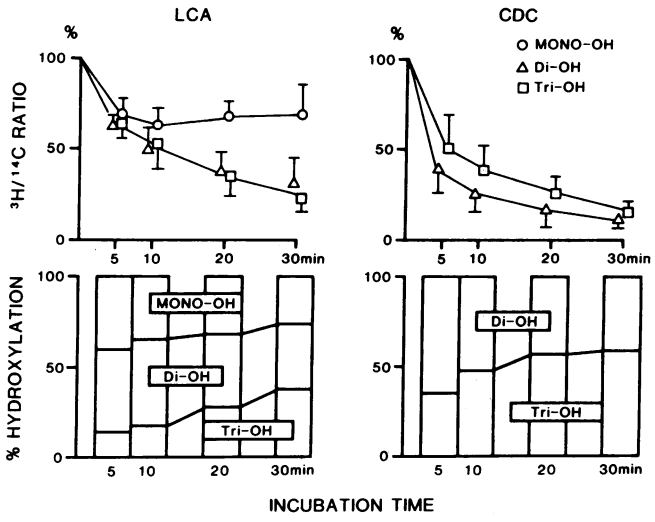

Figure 9. ^{3}H/^{14}C ratio and hydroxylation of double-labeled bile acids in hepatocytes. Butanol extracts of cells were separated on TLC, the area corresponding to mono-, di-, and trihydroxy bile acids were scraped and counted (Fig. 8). Percentages of hydroxylation of bile acids were calculated from ^{14}C radioactivities. Values are means±SD of three pooled butanol extracts of cells from three different cell preparations corresponding to Fig. 6.

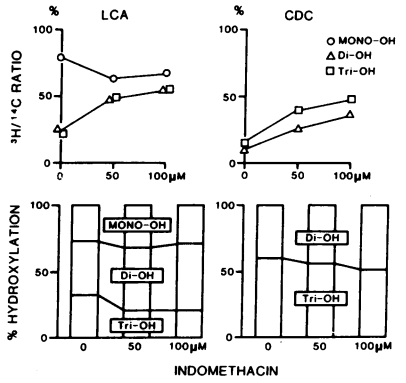

Figure 10. Effect of indomethacin on ^{3}H/^{14}C ratio and hydroxylation of double-labeled bile acids in hepatocytes (30 min incubation). Values are means of two pooled butanol extracts of cells from two different cell preparations corresponding to Fig. 7.

test this hypothesis. ^{3}H loss from lithocholic and chenodeoxycholic acids occurred in the presence of a mixture of cofactors and pure 3α-HSD.

The rates of ^{3}H loss from lithocholic and chenodeoxycholic acids catalyzed by the pure enzyme were comparable, whereas ^{3}H loss from cholic acid was negligible. These results are consistent with our previous determination of the K_m values for these bile acids. The enzyme has nearly identical affinity for either lithocholic or chenodeoxycholic acids (10), and ^{3}H loss from these bile acids also was comparable. In contrast, the affinity for cholic acid was previously shown to be 50-fold less (10), which accounts for the minimal ^{3}H loss seen in the current experiments.

^{3}H loss from 3β position of bile acids has been reported to proceed during the enterohepatic circulation of bile acids in rats (22) and humans (23). However, the contribution of the

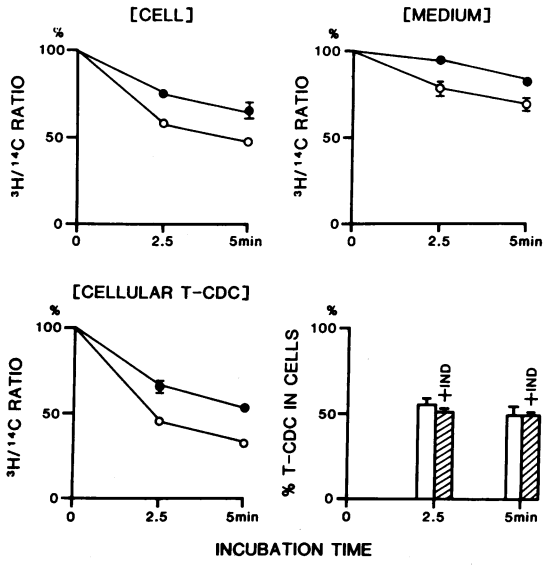

Figure 11. Effect of indomethacin on ^{3}H loss from double-labeled chenodeoxycholic acid at early time of incubation with isolated hepatocytes (*top*). [^{3}H, ^{14}C]Chenodeoxycholic acid (5 μM) was incubated in the absence (○) and presence (●) of 100 μM indomethacin, and ^{3}H/^{14}C ratios in cell and medium extracts were calculated. Means±SD, n = 4. Effect of indomethacin on ^{3}H loss from taurochenodeoxycholic acid extracted from cells incubated with double-labeled chenodeoxycholic acid (*bottom left*). Bottom right figure shows the percent total radioactivity (cells plus media) recovered as cellular taurochenodeoxycholic acid.

Table V. Hydroxylation and Amino Acid Conjugation of Double-labeled Chenodeoxycholic Acid Incubated with Isolated Hepatocytes in the Absence and Presence of 100 μM Indomethacin

	2.5 min		2.5 min plus indomethacin	
	Cells	Media	Cells	Media
Unconjugated				
Di-OH	5±1	11±0.5	5±0.8	18±0.7*
Tri-OH	2±0.3	1±0.1	2±0.6	1±0.1
Glycine conjugate				
Di-OH	0.6±0.2	0.3±0.2	0.2±0.1	0.4±0.2
Tri-OH	1±0.5	0.2±0.1	0.7±0.1	0.2±0.1
Taurine conjugate				
Di-OH	55±4	4±0.9	51±2	4±0.3
Tri-OH	18±5	2±0.2	16±2	2±0.2
Total	80.8±1.1	19.2±1.1	73.6±3.8‡	26.4±3.8‡
	5 min		5 min plus indomethacin	
	Cells	Media	Cells	Media
Unconjugated				
Di-OH	2±1	8±0.6	5±0.9‡	17±1*
Tri-OH	2±0.2	1±0.3	1±0.3	1±0.2
Glycine conjugate				
Di-OH	0.7±0.5	0.4±0.1	0.5±0.2	0.4±0.1
Tri-OH	2±0.7	0.5±0.2	1±0.6	0.3±0.1
Taurine conjugate				
Di-OH	49±5	5±0.8	49±2	6±2
Tri-OH	23±7	7±1	13±3	6±3
Total	78.4±1.5	21.6±1.5	69.2±0.2*	30.8±0.2*

Results represent percentage of total ^{14}C radioactivity recovered in each form. Values are means±SD obtained from three experiments. * $P < 0.01$ and ‡$P < 0.05$ vs. corresponding data without indomethacin (unpaired t test).

Table VI. Initial Uptake of Bile Acids by Isolated Hepatocytes in the Absence and Presence of Indomethacin

Bile acids	Indomethacin	Initial uptake
	μM	nmol/10^6 cells/min
Chenodeoxycholic acid	0	0.50±0.07
	100	0.51±0.09
Taurocholic acid	0	0.23±0.02
	100	0.24±0.08
	100*	0.23±0.06

The results are means±SD for four cell preparations. Incubations conained 2×10^6 cells/ml and 5 μM bile acid.
* Preincubation for 30 min.

liver versus bacterial metabolism during the enterohepatic circulation of bile acids could not be discerned from these earlier studies (22, 23).

It was of interest to examine the fate of ^3H lost from the bile acids in the presence of the mixture of cofactors. After incubation with the pure enzyme and a mixture of cofactors, ^3H was recovered only in NADPH. This finding is consistent with and extends earlier studies of Berseus and Bjorkhem (24) indicating that ^3H of NADPH is transferred to the 3β position of the steroid ring upon incubation of crude 3α-HSD with 3-oxo bile acid. Interestingly, most of the ^3H loss in cell incubations was recovered as [^3H]water in the media (not shown), which indicates that ^3H of NADPH undergoes further metabolism.

To use this reaction as a probe for bile acid entry into cytosol, it was necessary to confirm that the ^3H loss was specifically catalyzed by 3α-HSD. This was demonstrated using particulate fractions and various pooled fractions from the gel filtration of cytosol. The results indicated that only the cytosolic 3α-HSD in the Y′ fraction catalyzed ^3H loss.

When double-labeled bile acids were incubated with hepatocytes, ^3H loss was observed. This was most extensive with chenodeoxycholic acid and as expected least extensive with cholic acid. The greater ^3H loss from cholic acid in cell incubations as compared with the pure enzyme studies most likely is due to the accumulation of bile acids in cell cytosol at greater concentration than in the starting media. Thus, the cholic acid concentration may approach the K_m under these conditions.

Lithocholic and chenodeoxycholic acids undergo further hydroxylation within hepatocytes. We separated mono-, di-, and trihydroxy metabolites on TLC, but did not specifically identify them. However, it is known that chenodeoxycholate is converted mainly to α- and β-muricholic acids (trihydroxy bile acids) (25), and lithocholic acid is converted to 3α,6β-dihydroxy-5β-cholanic acid (dihydroxy) as well as α- and β-muricholic acids (26). The rate of ^3H loss from the trihydroxy metabolites of chenodeoxycholic acid were the same as the starting compound. However, since hydroxylation occurred very rapidly, it is not possible to determine if ^3H loss preceded or followed hydroxylation. Also, muricholic acid standards were not available so that we could not determine if it is a low-affinity substrate for 3α-HSD, like cholic acid, or a high-affinity substrate for 3α-HSD, like chenodeoxycholic acid.

The results of incubation of cells with lithocholic acid were of considerable interest. ^3H loss was less extensive than with chenodeoxycholic acid in contrast to the findings with pure enzyme. The di- and trihydroxy metabolites of lithocholic acid exhibited continuous and extensive ^3H loss over the 30-min incubation, whereas the parent bile acid lost proportionally far less ^3H, having rapidly reached a plateau by 5 min. The cessation of ^3H loss from lithocholic acid could not be accounted for by sulfation or glucuronidation of the 3α-OH position. These findings strongly suggest that lithocholic acid is compartmentalized in cells so that a fraction of it is sequestered in a site(s) not accessible to 3α-HSD or microsomal hydroxylation. Candidates for such a locus include the high-affinity binding site on the Y_a subunit of GSH *S*-transferases in cytosol or undefined, perhaps, nonspecific membrane sites. The apparent irreversibility of the sequestration of lithocholic acid from 5 to 30 min is against binding to GSH *S*-transferase, as the explanation.

Penning and co-workers (11, 12) have discovered that non-steroidal anti-inflammatory drugs are potent competi-

tive inhibitors of the steroid substrate site on 3α-HSD. Although the pharmacologic significance of this interaction remains uncertain, we took advantage of this knowledge to assess the effect of indomethacin on the ^3H loss from bile acids. Indomethacin that inhibited this reaction was catalyzed both by the pure enzyme and in hepatocytes. ID_{50} concentrations of indomethacin for the pure enzyme were < 10 μM. Indomethacin appeared to be less potent in cells, but this is probably due to both higher local bile acid concentrations accumulating in the cells (competition) and further metabolism of indomethacin (27, 28). Although indomethacin exerts other effects, such as inhibition of cyclooxygenase, it is unlikely that this could be a factor in its acute effect on bile acid metabolism. Moreover, the hydroxylation and conjugation of bile acids in hepatocytes was only minimally altered by indomethacin. However, we cannot exclude the possibility that indomethacin affects bile acid metabolism by mechanisms other than inhibition of 3α-HSD.

There are several important features of the inhibition of 3α-HSD in cells incubated with indomethacin. The distribution of bile acids between cells and medium was dramatically affected by indomethacin after 30 min incubation. Thus, in the presence of 100 μM indomethacin, bile acid (all conjugated) was shifted from the cells to the medium by more than twofold at 30 min, and this effect was also dose dependent (Table IV). In cell incubations with chenodeoxycholate for 2.5 or 5 min in the presence of indomethacin, more bile acid was found in the media mainly in the form of unconjugated chenodeoxycholate (Table V). This finding raised a number of possibilities other than a specific effect of indomethacin on 3α-HSD.

Indomethacin might inhibit ^3H loss by interfering with uptake of bile acid. Initial rates of uptake of chenodeoxycholate and taurocholate were unaffected by co-incubation or preincubation with indomethacin. Moreover, when double-labeled chenodeoxycholate was examined over the 1st 5 min of incubation with cells, the rate of amino acid conjugation was no different in the presence or absence of indomethacin. Taurochenodeoxycholate production was unaffected by indomethacin despite a significant inhibition of ^3H loss from this metabolite in cells over the 1st 5 min. Also, at the end of the 30-min period of incubation, all the bile acids were conjugated (not shown). Thus, inhibition of ^3H loss could not be attributed to an effect on bile acid uptake. Indomethacin inhibited ^3H loss from bile acids that had been taken up by the cells.

Our results strongly suggest that the competitive inhibition of 3α-HSD by indomethacin is causally related to the decreased amounts of bile acid recovered in the cells. Since initial rates of bile acid uptake were unaffected by indomethacin, inhibition of binding to 3α-HSD may have displaced bile acids into the medium. In isolated cells that have lost polarity, it is not possible to distinguish reflux (back diffusion) vs. excretion. However, an effect on the bidirectional steps in net uptake rather than excretion is suggested by the selective retention of unconjugated bile acids in the media at early time points in the presence of indomethacin. Bile acids are conjugated before canalicular excretion. Because most of the redistributed bile acid at early time points was unconjugated, it is likely that it was regurgitated by a pathway other than the usual excretory pathway. According to this hypothesis, upon entry into the cytosol the bile acid might bind to 3α-HSD, minimizing reflux from the cell. In the presence of indomethacin, binding to 3α-HSD in the cytosol would be inhibited as reflected in inhibition of ^3H loss and, thus, bile acid would reflux back into the medium. Also, indomethacin might affect other bile acid binding sites in cytosol such as the GSH *S*-transferases. Indomethacin has been shown to inhibit these enzymes (29). However, its potency in inhibiting GSH *S*-transferase activity (29) and bile acid binding by the transferases is more than an order of magnitude less than that for 3α-HSD (Takikawa, H., A. Stolz, and N. Kaplowitz, unpublished observations).

We have shown that ^3H loss from the 3β position can specifically probe the interaction of bile acids with cytosolic 3α-HSD in the intact hepatocyte. These results indicate that bile acids enter cytosol extensively. Although conjugation also may occur in the cytosol (20, 30, 31), our results indicate that binding to 3α-HSD in cytosol is a major determinant of the distribution of bile acids between hepatocytes and medium. Indomethacin inhibits the interaction of bile acids with 3α-HSD and therefore can be used to assess the physiologic importance of binding by 3α-HSD in net bile acid uptake by cells. The accompanying manuscript extends this work to the isolated perfused rat liver. In this more physiologic model, the extent of the interaction between bile acids and 3α-HSD during vectorial transit through the liver and the effect of indomethacin on bile acid extraction and excretion can be assessed.

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