Purification and Characterization of Hamster Liver Microsomal 7α-Hydroxycholesterol Dehydrogenase

SIMILARITY TO TYPE I 11β-HYROXysteroid DEHYDROGENASE

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While studying the bile acid synthetic pathway of hamsters, we discovered an NADP+-dependent liver microsomal 7α-hydroxycholesterol dehydrogenase (7α-HCD) activity that was not observed in rat liver microsomal fractions. The hamster liver microsomal 7α-HCD was purified to homogeneity using 2',5'-ADP and cholic acid-agarose affinity chromatography. 7α-HCD displayed a molecular weight of approximately 34,000 on SDS-polyacrylamide gel electrophoresis; it is an intrinsic membrane protein of the hamster liver endoplasmic reticulum and exists as a multimeric aggregate in pure form. Partial N-terminal amino acid sequence analysis showed that 7α-HCD had high sequence similarity to human 11β-hydroxysteroid dehydrogenase (11β-HSD; 24/30 amino acid identity). The Kₘ values for corticosterone and 7α-hydroxycholesterol were 1.2 and 1.9 μM, respectively, for purified 7α-HCD; both reactions displayed identical Vₘₐₓ values (approximately 170 nmol/min/mg of protein). The IC₅₀ of carbenoxolone, a competitive inhibitor of 11β-HSD, was 75 nm for 7α-hydroxycholesterol dehydrogenation and 210 nm for corticosterone dehydrogenation. The tissue-specific expression in hamster was as follows: adrenal > liver > kidney > testis ≈ brain > lung.

Microsomal 7α-HCD is uniquely expressed in hamster liver and to some extent in human liver but not in rat liver. Western blot analysis with two antibodies elicited against an N-terminal peptide of the human 11β-HSD and purified hamster liver 7α-HCD, respectively, suggested the presence of multiple forms of 7α-HCD in hamster liver, most likely due to the existence of a family of 11β-HSD proteins. Since 7α-cholesterol is a potent inhibitor of cholesterol 7α-hydroxylase, alternative mechanisms for regulation of bile acid synthesis may exist in human and hamster liver due to production of this metabolite and its potential as an oxysterol.

The first regulated step in bile acid synthesis is hydroxylation of cholesterol to 7α-hydroxycholesterol by cholesterol 7α-hydroxylase, the apparent rate limiting reaction in this pathway (1). Oxidation of the 3β-ol group to an oxo group and rearrangement of the unsaturated bond at Δ⁷-position to Δ⁴ followed by hydroxylation at either the C-12 or C-27 position converts the sterol to its active form essential for digestion of fatty nutrients. Recently, an alternate pathway to bile acids has been elucidated involving first 27-hydroxylation of cholesterol and subsequent oxidation at position C-3 and hydroxylation at position C-7 eventually leading to chenodeoxycholic acid (2).

A unique hamster liver microsomal 7α-hydroxycholesterol dehydrogenase (7α-HCD) was discovered in our laboratory during the development of an HPLC method for assay of cholesterol 7α-hydroxylase activity (3, 4). The oxidized metabolite produced by 7α-HCD, 7-oxocholesterol, is a bioactive sterol and potent competitive inhibitor of cholesterol 7α-hydroxylase activity (approximately Kᵢ for 7-oxocholesterol of 7 μM versus approximately Kᵢ for exogenous cholesterol of 100 μM; Ref. 5). Although oxysterol derivatives are formed during chemical autoxidation of cholesterol, the presence of 7-oxo-bile acids observed in the blood of infants suggests that a unique metabolic pathway leading to 7-oxo-bile acids may exist in humans and other mammals (6). Individuals with the rare inherited disease cerebroretinal xanthomatosis who lack cholesterol 27-hydroxylase activity display significant levels of 7-oxocholesterol in their blood (7). In addition, chronic salt loading of hypertensive baboons (8) or rhesus monkeys with hemorrhagic fever (9) also result in significant blood levels of 7-oxocholesterol, in addition to 7α- and 7β-hydroxycholesterol. Interestingly, intravenous infusion of 7-oxocholesterol into rats decreased bile acid secretion and increased cholesterol 7α-hydroxylase enzyme activity, protein content, and mRNA levels (10) to compensate for the lack of bile acid production. Therefore, hepatic 7α-HCD and its products may participate in the regulation of bile acid synthesis.

A number of genes encoding proteins that oxidize hydroxyl groups of sterols have been identified to date (11, 12). For example, unique 3β-, 11β-, and 17β-hydroxysteroid dehydrogenases have been described; each possesses a unique stereochemical reaction, pyridine nucleotide specificity, and tissue-specific localization. These short chain alcohol dehydrogenases found in the liver are thought to be involved in either the processing or termination of function of various bioactive steroids, such as bile acids, dehydroepiandrosterone, 5-ene-androsten-3β,17β-diol, androstenedione, cortisol, corticosterone, 17α-hydroxyprogrenolone, pregnenolone, and progesterone. However, no enzyme with 7α-hydroxysteroid dehydrogenase activity has been described to date.

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The abbreviations used are: 7α-HCD, 7α-hydroxycholesterol dehydrogenase; C₁₂E₉, dodecyl nonaoxyethylene glycol monooether; DE52, DEAE-cellulose; HPLC, high performance liquid chromatography; HSD, hydroxysteroid dehydrogenase; IC₅₀, concentration causing 50% inhibition; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylene glycol 8000; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

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Since 7-oxocholesterol apparently could be formed by an enzyme-mediated process, we purified this enzyme to homogeneity from hamster liver to further characterize it. Using the purified enzyme, we analyzed the N-terminal amino acid sequence of the protein, studied its substrate specificity, and elicited a polyclonal antibody to assess the tissue- and species-specific expression of 7α-HCD. Our results suggest that this enzyme, which exhibits high substrate specificity for corticosterone, cortisol, and 7α-hydroxycholesterol in the hamster liver (and possibly humans), may be a member of the 11β-hydroxysteroid dehydrogenase family. However, either it is not expressed in rat liver, or the rat 11β-HSD does not catalyze oxidation of 7α- or 7β-hydroxysteroids.

**EXPERIMENTAL PROCEDURES**

**Materials**—7α-Hydroxycholesterol and 7-oxocholesterol were obtained from Steraloids Inc. (Wilton, NH), DEAE-cellulose (DE52) was obtained from Whatman. 2',5'-ADP-agarose, cholic acid-agarose, dodecyl nonaoyxyethylglycine monoester (C12E9), and other chemicals were purchased from Sigma in analytical or HPLC grade. An antibody fraction was obtained from ImmunoChem Research, Inc., Hamilton, MT) and injected into a rabbit (Myrtles, Inc., Thompson Station, TN, 1–2 kg) were fed laboratory chow ad libitum. Male New Zealand White rabbits (Myrtles, Inc., Thompson Station, TN, 1–2 kg) were fed laboratory chow ad libitum.

Liver microsomal fractions in 10% glycerol were stored at –70 ºC. Purified enzyme preparations were obtained from F. P. Guengerich (Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN).

**Animals**—Male Syrian hamsters (Charles River Breeding Laboratories, Inc., Wilmington, MA; LabLKG(SYR), 60–90 g) and male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN; Hsd:Sprague-Dawley, 180–250 g) were fed laboratory chow ad libitum. Liver microsomal fractions were prepared from hamsters, rat, human liver, and other tissues as described previously (13) and assayed immediately.

Liver microsomal fractions in 10% glycerol were stored at –70 ºC for up to 3 months without loss of activity. Protein concentration was determined by the method of Lowry et al. (14) using bovine serum albumin as the standard. Male New Zealand White rabbits (Myrtles, Inc., Thompson Station, TN, 1–2 kg) were fed laboratory chow ad libitum during antibody production.

**HPLC Assay of 7α-Hydroxycholesterol Dehydrogenase Activity**—Microsomal protein fractions were incubated at 37 ºC for 5 min with 0.1 mM potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA and 20 mM cysteamine-HCl in a final volume of 1 ml. After the addition of NADPH at a concentration of 0.5 mM, the reaction mixture was added in 2-propanol to the reaction mixture at a final concentration of 60 μM to initiate the reaction. The solvent concentration was always less than 1% 2-propanol and had no effect on the enzyme activity. The reaction was terminated by adding an equal volume of methanol, and the reaction mixture was extracted three times with 5 ml of petroleum ether. The extract was analyzed by normal phase HPLC as described previously (9).

**Fluorometric Assay of 7α-Hydroxycholesterol Dehydrogenase Activity**—Purified enzyme preparations were incubated at 37 ºC in a final volume of 2 ml with the same buffer used in the HPLC assay method. The reaction was initiated by adding exogenous 7α-hydroxycholesterol or other steroids in 2-propanol. Enzyme activity was monitored by measuring the production of NADPH at λ excitation = 340 nm and λ emission = 460 nm for 2 min using a model 50B luminescence spectrometer LS 50B (Perkin-Elmer). The reaction mixtures containing NADP+ and 7α-hydroxycholesterol alone were first monitored as control rates. Reaction rates were calculated before and after the addition of purified 7α-HCD to give enzymatic rates of NADP+ reduction. For the rate measurements to obtain Michaelis-Menten parameters, the results were analyzed by nonlinear regression analysis of data obtained from triplicate assays at five different substrate concentrations.

**Purification of 7α-Hydroxycholesterol Dehydrogenase**—The microsomal fraction from hamster liver was suspended at 6.3 mg/ml in 20 mM potassium phosphate buffer, pH 7.4, containing 10% (v/v) glycerol and 0.5 mM EDTA (elution buffer). The detergent C12E9 was then added to obtain a final concentration of 0.3%. The suspension was stirred for 20 min at room temperature while protein was sedimented at 135,000 × g for 20 min. Polyethylene glycol 8000 (PEG) was added to the supernatant to attain a final concentration of 8%, the suspension was stirred for 10 min, and the mixture was sedimented at 10,000 × g for 10 min. The supernatant was dialyzed with the original buffer overnight and then applied to a DE52-cellulose column (1 × 22 cm) equilibrated with same buffer used for solubilization of protein from the microsomal membrane. The active fractions that were not retained by the DE52 matrix were pooled and applied to a 2',5'-ADP-agarose column (1 × 6 mm). After the enzyme bound to the column, the column was eluted first by 20 column volumes of 50 mM NaCl in elution buffer and then by gradient elution from 0 to 0.5 mM NAD+ in 10 column volumes of elution buffer to remove proteins not bound by the 2',5'-ADP matrix. Finally, the 7α-HCD activity was eluted by gradient elution from 0–0.2 mM NADP+ in 10 column volumes of elution buffer containing 0.03% C12E9. The active fractions from 2',5'-ADP affinity column were pooled and applied to a cholic acid-agarose affinity column (1 × 6 mm). 7α-HCD activity was eluted by a cholic acid gradient from 0.2 to 0.8% (v/v) in elution buffer consisting of 20 mM potassium phosphate buffer, pH 7.4, containing 0.5 mM EDTA, 10% (v/v) glycerol, and 0.03% (v/v) C12E9. All procedures were performed at 4 ºC.

The purity of each fraction was determined by 12% SDS-PAGE using a Bio-Rad Mini Protein II apparatus. After fixing the protein with 10% ethanol and 5% acetic acid, the gel was soaked in a solution containing 3.4 mM K2Cr2O7 and 3.2 mM HNO3, followed by staining with 12 mM silver nitrate. Protein bands were visualized with 0.28 mM sodium carbonate and 1.85% parafomaldehyde.

**Partial N-terminal Amino Acid Sequence Analysis**—Purified enzyme from cholic acid affinity chromatography was subjected to 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane using transfer buffer, pH 11, containing 10 mM CAPS and 10% (v/v) methanol. Transferred protein was stained with 0.2% Coomassie Blue followed by destaining with 30% methanol and 10% acetic acid. The proteins of interest were manually cut from the membrane and sequenced using an Applied Biosystems 470A gas phase protein sequencer equipped with a 120A phenylthiohydantoin analyzer (Applied Biosystems, Foster City, CA) in the Protein Chemistry Core Laboratory (Department of Biochemistry and Molecular Biology, University of Louisville). The repetitive yield at each cycle was approximately 94%, and the protein sequencing was replicated five times with nearly perfect identity of sequence.

**Production of Antibody**—Purified enzyme preparations from the 2',5'-ADP-agarose affinity column were subjected to 12% SDS-PAGE. After staining with 0.1% Coomassie Blue dye, the protein of interest was excised and extracted with 0.1 M glycine-HCl buffer, pH 8.0, containing 0.1% SDS. After dialysis against 5 mM NH4CO3 and 0.05% SDS, the lyophilized protein was precipitated with 90% acetone containing 1 mM HCl, and the pellet was rinsed with chilled acetone prior to air drying. Antigen was mixed with RIBI emulsion (RIBI Immunocor, Inc., Hamilton, MT) and injected into a rabbit at multiple sites on the back. A second inoculation at 14 days was made with 5 mg of antigen mixed with 0.7 ml of Freund’s Incomplete Adjuvant (Life Technologies, Inc.). Blood was obtained biweekly to determine specific antibody content. After clotting, the serum was heated and stored in the presence of 50 μM phenylmethylsulfonyl fluoride at –70 ºC. Antibody against 7α-HCD protein was detected by an enzyme-linked immunosorbent assay method.

**Western Blot Analysis**—Purified enzyme or microsomal protein samples were subjected to 12% SDS-PAGE. After transfer of protein to a nitrocellulose membrane, the membrane was blocked in a solution of 20 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM NaCl and 0.5% Tween 20 (0.1% TTBS) and washed with 0.05% TTBS. The membrane was incubated with a 1:2000 to 1:4000 dilution of the primary antibody, and after several washes the membrane was incubated with a 1:5000 dilution of second goat anti-rabbit globulin conjugated with horseshadish peroxidase. Protein-immunoglobulin complex formation was detected by enhanced chemiluminescent light emission of luminol oxidation by horseradish peroxidase using Renaissance Western blotting chemiluminescence reagents (DuPont).

**RESULTS**

**Purification of 7α-Hydroxycholesterol Dehydrogenase**—Since we noted that an abundant source of enzyme activity was the hamster liver microsomal protein fraction (4), we embarked upon the purification of the enzyme responsible for 7α-HCD activity from that tissue fraction. We ascertained the concentration-dependent action for several detergents in solubilizing 7α-HCD from microsomal membrane. Solubilization with C12E9 yielded the highest amount of 7α-HCD activity released among the detergents tested (data not shown). Polyethylene glycol 8000 (PEG, 8%) was chosen to precipitate nonspecific protein, since higher concentrations of PEG did not precipitate additional protein and interfered with the binding of 7α-HCD.
in subsequent chromatography steps. 7α-HCD was soluble at all PEG concentrations tested, while other proteins such as NADPH:cytochrome P450 oxidoreductase, cytochrome b₅, and cytochrome P450 were precipitated at 8% PEG.

DE52 column chromatography was used to separate tightly bound proteins from 7α-HCD (data not shown). 7α-HCD activity eluted quickly from the column with a slightly brown-colored protein; i.e. DE52 matrix did not bind 7α-HCD activity, but a large amount of unrelated protein was retained on the column. Since 7α-HCD was potently inhibited by 2',5'-AMP (4), we subsequently utilized 2',5'-ADP affinity chromatography with a NADP⁺ gradient to elute the enzyme activity of interest. To remove minor contaminants, the eluate from the 2',5'-ADP affinity column containing 7α-HCD was subjected to cholic acid-agarose affinity chromatography. Table I shows the purification steps leading to nearly homogeneous protein, and Fig. 1 shows the analysis of the various purified fractions by 12% SDS-PAGE. There were always two proteins in the active fractions, which migrated as approximately 34-kDa proteins.

**Determination of the N-terminal Sequence of Purified 7α-HCD**—In order to determine the N-terminal sequence of 7α-HCD, purified 7α-HCD prepared from the cholic acid affinity column was transferred to a polyvinylidene difluoride membrane and sequenced as described under “Experimental Procedures.” Both the upper and lower bands shown in Fig. 1 were sequenced; the results are shown in Table II. These sequences have high sequence similarity to human type I 11β-hydroxysteroid dehydrogenase (24/30 identical amino acid residues in the N termini), indicating that 7α-HCD may belong to the 11β-HSD family. Using various proteinases, we generated a peptide of human 11β-HSD with the N-terminal amino acid sequence was similar to human 11β-HSD (30/34 identical amino acid residues in the N termini), indicating that the tissue-specific expression patterns of 7α-HCD are similar (Fig. 2).

7α-HCD are similar (Fig. 2). However, in hamster liver there were two bands detected by both antibodies. The apparent content of each band was different for the two antibodies against either 7α-HCD or 11β-HSD, suggesting that 7α-HCD may be immunologically related to human 11β-HSD and that it is apparently expressed at higher levels in the liver than in the adrenal of hamster. N-terminal sequence analysis of the two bands showed that both protein species had identical N termini, also supporting the hypothesis that two forms of 11β-HSD may exist in hamster liver microsomes.

**TABLE I** Purification table for hamster liver microsomal 7α-HCD

| Fractions | Total activity | Activity recovered | Protein amount | Protein recovered | Specific activity |
|-----------|---------------|--------------------|----------------|------------------|------------------|
|           | nmol/min      | %                  | mg             | %                | nmol/min/mg      |
| Microsome | 320           | 100                | 560            | 100              | 0.58             |
| C₅₋₇E₅₀ | 1120          | 350                | 340            | 61               | 3.30             |
| PEG       | 640           | 200                | 130            | 22               | 5.10             |
| DE52      | 530           | 160                | 36             | 6                | 15               |
| 2',5'-ADP | 260           | 81                 | 2              | 0.4              | 130              |

**TABLE II** N-terminal amino acid sequence of purified hamster hepatic 7α-HCD

| Areas of homology are underlined. |
|----------------------------------|
| Top band  | HFMKYLLP1LVLVL----KEEFRPEM |
| Bottom band | HFMKYLLP1LVLVLFLAYYYYY |
| Hamster  | HFMKYLLP1LVLVLFLAYYYY??KEEFRPEM (consensus) |
| Human    | MAFMKYLLP1LGLFLMAYYYYYSANEERPEM (Refs. 17 and 18) |

**FIG. 1.** SDS-PAGE analysis of purification fractions of 7α-hydroxycholesterol dehydrogenase. After electrophoretic separation of the proteins on 12% SDS-PAGE, the gels were stained with AgNO₃, as described under “Experimental Procedures.” Lane 1, 40 μg of hamster liver microsomal protein; lane 2, 8 μg of protein from the DE₅₂ column; lane 3, 5 μg of protein from the 2',5'-ADP-agarose affinity column; lane 4, 2.2 μg of protein from the cholic acid-agarose affinity column.

**FIG. 2.** 7α-HCD protein expression correlates with enzyme activity in various hamster tissues. Microsomal protein of each tissue (0.3 mg) was used to determine enzyme activity using the normal phase HPLC method described under “Experimental Procedures.” The results are the average of duplicate assays. Microsomal protein from each tissue (20 μg) was subjected to 12% SDS-PAGE, and Western blot analysis was performed as described under “Experimental Procedures” using antibodies to human type I 11β-HSD and hamster 7α-HCD.
7α-HCD, an 11β-Hydroxysteroid Dehydrogenase

Fig. 3. 7α-HCD is expressed in hamster and human liver. Liver microsomal protein preparations (20 μg) were subjected to 12% SDS-PAGE and Western blot analysis as described under "Experimental Procedures." Microsomal protein from each liver sample (0.3 mg) was used to assay 7α-HCD activity with the normal phase HPLC method described under "Experimental Procedures" using antibodies to human type I 11β-HSD and hamster 7α-HCD. Lane 1, 40 μg of hamster liver microsomal protein; lane 2, 8 μg of protein from the DE52 eluate; lane 3, 5 μg of protein from a 2’,5’-ADP-agarose column; lane 4, 2.2 μg of protein from a cholic acid-agarose column.

Characterization of Purified 7α-Hydroxycholesterol Dehydrogenase—The absolute absorbance spectrum of the purified enzyme fraction (240–700 nm) revealed a single wavelength maximum at 278 nm (data not shown), indicating that the enzyme lacks chromophores, such as flavin or heme. When the purified 7α-HCD from 2’,5’-ADP-agarose affinity chromatography was analyzed by 4–30% nondenaturing gradient PAGE, there were two protein bands corresponding to apparent molecular masses of 440 and 240 kDa, respectively (data not shown). These results indicate that 7α-HCD exists as aggregates in the absence of detergent, a characteristic of other intrinsic membrane proteins. Since no reverse reaction could be measured under any conditions employed (data not shown), purified 7α-HCD apparently could not readily catalyze the reduction of oxo groups at either position 7 or 11 of C27 sterols, respectively. This conclusion was confirmed for 7α-hydroxy- and 7-oxocholesterol by HPLC analysis in a separate experiment at an NADPH/NADP+ concentration of 50 and 50 μM, respectively (data not shown).

Substrate Specificity—Since the literature suggests that 7-oxocholesterol is formed nonenzymatically (15), the goals of our studies were to determine which hydroxysterols are substrates and whether 7α-hydroxycholesterol may be an endogenous substrate of 7α-HCD. The purified enzyme obtained from 2’,5’-ADP affinity chromatography was used to perform substrate specificity studies, since the enzyme obtained from cholic acid affinity column chromatography displayed lower specific activity. Since 7α-HCD uses NADPH+ as a cofactor to oxidize the hydroxyl group to an oxo group in the sterol substrate, we utilized a fluorometric method to monitor the formation of NADPH to facilitate these studies; with pure protein, the rate of 7-oxocholesterol production was stoichiometric with NADP+ reduction (data not shown).

In this study, the concentrations of steroids and nucleotides utilized were 50 and 500 μM, respectively. All of the concentrations were above the concentrations of endogenous substrate normally required for maximal hydroxysteroid dehydrogenase activity (11, 12). As shown in Table III, the first three steroids were C27 sterol derivatives. Among them, 7α-hydroxycholesterol gave the highest rate of oxidation. However, the oxidation rate for 7β-hydroxycholesterol was almost 70% of the rate for 7α-hydroxycholesterol. Interestingly, cholesterol also could be oxidized, i.e. at approximately 15% of the rate seen for 7α-hydroxycholesterol. The major product observed by HPLC comigrated with authentic 3-oxocholesterol (data not shown).

These data suggest that some C27 hydroxysteroids are substrates for 7α-HCD and that oxidation at the 7-position is not absolutely stereospecific.

The second class of compounds studied was C24 bile acids. For these molecules, the hydroxyl group at the 3α-position alone was oxidized at a rate similar to that for cholesterol. A substrate with a hydroxyl group at the 7α-position was only about ¼ as good as a substrate containing only a 3α-hydroxyl group, such as cholesterol or lithocholate. If the substrate had a hydroxyl group at the 12α-position, the rate was only about ¼ of that of a 3α-hydroxyl group alone. No oxidation was de-
detected when the substrate contained a hydroxyl group at the 6α-position. Substrates with two hydroxyl groups at the 6α- and 12α-positions were not significantly oxidized. These data suggest that 7α-HCD poorly oxidizes the 3α-hydroxyl group of bile acid derivatives; hydroxyl groups at the 6α-, 7α-, and 12α-positions cannot be oxidized and also diminish dehydrogenation at the 3α-position of bile acids.

The third group of compounds tested were C21 and C19 steroid hormones. Hydrocortisone is the natural substrate of 11β-HSD in humans, and corticosterone is the natural substrate of 11β-HSD in rodents. Since 7α-HCD was obtained from hamster liver, it was not surprising that 7α-HCD oxidized corticosterone more rapidly than hydrocortisone (Table III). The enzyme can also oxidize 11-dehydrocorticosterone-21-ol to some extent but could not oxidize 11-dehydrocorticosterone-17-ol. This indicates that oxidation is position-specific for additional hydroxyl groups on 11-ol derivatives. The fact that 4-pregnene-11α-ol-3,20-dione was not oxidized indicates that oxidation at carbon 11 is also stereospecific (i.e. 11α versus 11β configuration). These data strongly suggest that 7α-HCD is a member of the 11β-HSD family. Compared with cholesterol and lithocholic acid, dehydroepiandrosterone, a 3β-hydroxy C19 sterol, was oxidized at a very low rate, demonstrating again that 7α-HCD cannot easily oxidize hydroxyl groups at the 3β-position for C19 steroids as is the case for 3β-HSD.

The last group of compounds shown in Table III normally are metabolized by dehydrogenases that utilize NAD⁺ instead of NADP⁺ to oxidize sterol substrate. Dehydroepiandrosterone is the natural substrate for 3β-HSD, while 5-androstene-3β,17β-diol is the natural substrate for 17β-HSD. Both use NAD⁺ as an oxidation cofactor. 7α-HCD did not oxidize either substrate with NAD⁺. This demonstrates that 7α-HCD does not catalyze reactions identical to those of the 3β- or 17β-HSD families. 7α-HCD oxidizes hydroxyl groups at the 7-position using NAD⁺ as cofactor but not at the 3-position of 7α-hydroxycholesterol. We previously showed that NAD⁺ can serve as an oxidizing substrate for this enzyme (4), but the \( K_m \) for NAD⁺ is 70-fold larger than that for NADP⁺ and the \( V_{max} \) is \( 1/5 \) as large, respectively. We also have performed experiments suggesting that other 7α-hydroxylated steroids, such as 7α-hydroxydehydroepiandrosterone, are also substrates for this enzyme. These experiments confirmed the conclusion that 7α-HCD is distinct from 3β-hydroxy-Δ⁵-C21-steroid oxidoreductase.

**Michaelis-Menten Kinetic Constants for Selected Substrates of 7α-HCD**—To determine whether 7α-hydroxycholesterol might be a physiological substrate for this enzyme, steady-state kinetic parameters for NADP⁺ reduction were determined. Since these reactions displayed simple Michaelis-Menten kinetics, the \( K_m \) and \( V_{max} \) of these sterols for 7α-HCD were determined with enzyme obtained from the 2,5'-ADP agarose column (Table IV). The \( K_m \) of 7α- and 7β-hydroxycholesterol was nearly identical to that of corticosterone, and the \( V_{max} \) values were also identical, indicating that both classes of sterols are metabolized at nearly identical maximal rates of substrate oxidation. When the \( V_{max} / K_m \) values were considered, corticosterone was found to be a slightly better substrate (approximately 1.8-fold) than 7α-hydroxycholesterol.

**Effect of an 11β-HCD Inhibitor on 7α-HCD Activity**—To confirm the results of the kinetic study, an inhibition experiment using carbenoxolone, a specific competitive inhibitor of 11β-HSD (16), was performed. In these experiments, the sterol substrate concentration was 4 \( \mu \)M, a concentration approximately equal to the \( K_m \) for both 7α-hydroxycholesterol and corticosterone. The concentration of NADP⁺ and purified 7α-HCD used were 0.5 \( \mu \)M and 19 \( \mu \)g/ml, respectively. The results are shown in Fig. 4. The IC₅₀ of carbenoxolone for 7α-hydroxycholesterol oxidation was approximately 75 nM, and for corticosterone oxidation it was approximately 210 nM. Although carbenoxolone is a slightly more potent inhibitor of 7α-HCD activity and the \( V_{max} / K_m \) parameters for corticosterone and 7α-hydroxycholesterol vary by 1.8-fold, 7α-hydroxycholesterol may serve as a natural substrate for the 7α-HCD found in the hamster liver microsomal fraction.

**DISCUSSION**

Purified 7α-HCD had an apparent molecular mass of 34 kDa and preferred NADP⁺ as cofactor, like the liver-specific type I

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**Table IV**

| Substrates              | \( K_m^{a} \) \( \mu \)M | \( V_{max}^{a} \) nmol/min/mg | \( V_{max} / K_m \) |
|------------------------|--------------------------|-------------------------------|-------------------|
| 7α-Hydroxycholesterol  | 1.9 ± 0.5                | 160 ± 25                      | 82 ± 12           |
| 7β-Hydroxycholesterol  | 1.8 ± 0.4                | 140 ± 23                      | 77 ± 9            |
| Corticosterone         | 1.2 ± 0.4                | 180 ± 29                      | 153 ± 30          |

* The results are means of duplicate assays at five different concentrations ± S.D.
* Statistically different from corticosterone (p < 0.02).

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* J. Fitzpatrick, X. D. Lei, and R. A. Prough, unpublished results.
11β-HSD. NAD$^+$ was about 50% as effective as a cofactor at a much higher concentration than needed for NADP$^+$ (4). Partial N-terminal amino acid sequence analysis demonstrated that 7α-HCD has high sequence similarity to human and rat type I 11β-HSD in the N-terminal region, indicating that it may be related to type I 11β-HSD. The 7α-HCD dehydrogenase reaction appeared to be irreversible for the purified hamster enzyme, which was also observed by others for purified rat liver type I 11β-HSD (17). In contrast to purified rat liver type I 11β-HSD, membrane-bound (18) and expressed recombiant rat liver type I 11β-HSD (17, 19) have both reductase and dehydrogenase activity. The reason that purified 11β-HSD and 7α-HCD did not display reductase activity is unknown. Substrate specificity studies demonstrate that 7α-HCD has substrates in common with 11β-HSD but not with 3β-HSD or 17β-HSD. 7α-HCD has high affinity for 7α-hydroxycholesterol and corticosterone, indicating that both steroids may serve as endogenous substrate in vivo. Other bile acids and cholesterol derivatives could be oxidized at the 3β-ol position, albeit at much lower rates of oxidation than 7α-hydroxycholesterol.

Experiments designed to determine the species-specific expression of 7α-HCD using a polyclonal antibody demonstrated that 7α-HCD was expressed in hamster and to some extent in human microsomes but not in rat liver microsomes. The tissue-specific expression of 7α-HCD was as follows: adrenal ≥ liver > kidney > testis ≥ brain > lung, which is similar to the pattern of expression of type I 11β-HSD. These data indicate that 7α-HCD may also participate in adrenal steroid hormone metabolism. The most surprising finding of this study is that the 7-oxocholesterol formation is catalyzed by a steroid dehydrogenase similar to 11β-HSD type I. Two 11β-HSD forms have been identified. Type I 11β-HSD preferentially uses NADP$^+$ and is present mainly in liver. It may be responsible for inactivating glucocorticoid hormones, i.e. cortisol or corticosterone, in that tissue.

The type II 11β-HSD enzyme utilizes NAD$^+$ instead of NADP$^+$ as a cofactor and is present in rabbit kidney cortical collecting duct cells (20), sheep kidney (21), human placenta (22), and many human fetal tissues (23). Agarwal et al. (24) isolated a sheep kidney cDNA clone encoding Type II 11β-HSD by expression screening using Xenopus oocytes. The cDNA (1.8 kilobases) encodes a protein of 427 amino acid residues (Mr = 46,700). Based on predicted amino acid sequence, it was only 20% sequence similar to the type I 11β-HSD; regions that had identity included the putative pyridine nucleotide- and steroid-binding regions observed in many short chain alcohol dehydrogenases. The expressed enzyme functions as an NAD$^+$-dependent 11β-dehydrogenase with apparent Km values of 15 nM for cortisol and 0.7 nM for corticosterone and displayed no detectable reductase activity. The cDNA hybridized to a 1.9-kilobase mRNA species in kidney and adrenal and was detectable in colon. There was no detectable hybridization of the cDNA probe to mRNA from liver, lung, testis, ovary, heart, stomach, small intestine, or skin. The corresponding gene in humans is a candidate for the syndrome of apparent mineralocorticoid excess. In humans, the cortisol metabolite cortisone is excreted into the urine (25), but in rats about 90% of corticosterone metabolite 11-dehydrocorticosterone is recovered in bile (26), reflecting primarily hepatic metabolism.

Glycyrrhetinic acid, a component of licorice, is a cyclic triterpene whose fused ring structure resembles that of glucocorticoids. This acid has been linked to a syndrome similar to mineralocorticoid excess in a Dutch population (16). The observation that glycyrrhetinic acid can decrease blood cholesterol levels (27) and increase bile secretion (28) indicates that the product of 7α-HCD might diminish the formation of 7-oxocholesterol and thus activate cholesterol 7α-hydroxylase. This effect would enhance flux of the steroids through the bile acid synthesis pathway and possibly lower blood cholesterol. 7α-HCD may also catalyze formation of a yet unknown hormone as a side product of the bile acid biosynthetic pathway in hamsters and possibly in humans.

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REFERENCES
1. Carey, M. C. & Cahalane, M. J. (1988) in The Liver: Biology and Pathobiology (Arias, I. M., Jakoby, W. B., Popper, H., Schachter, D. & Shafritz, D. A., eds) pp. 573–616, Raven Press, New York
2. Alexson, M. & Sjovall, J. (1990) J Steroid Biochem 36, 631–640
3. Song, W., Pierce, W. M., Jr., Prough, R. A. & Redinger, R. N. (1991) Biochem Pharmacol 44, 1439–1447
4. Song, W., Pierce, W. M., Jr., Saeki, Y., Redinger, R. N. & Prough, R. A. (1996) Arch Biochem Biophys 329, 272–292
5. Cantfor, J. V. (1972) Life Sci 11, 773–780
6. Strandvik, B., Wahlen, E. & Wikstrom, S. A. (1994) Scand J Clin Lab Invest 54, 1–10
7. Bjorkhem, I. (1986) Anal Biochem 154, 497–501
8. Gontcharov, V. N., Simarina, A. J., Jefremova, S. K., Schon, R. & Schubert, K. (1975) Endocrinology 64, 213–216
9. Gontcharov, N. P., Wehrberger, K. & Schubert, K. (1971) J Steroid Biochem 2, 389–391
10. Breuer, O., Sudjana-Sugiaman, E., Eggertsen, G., Chiang, J. Y. & Bjorkhem, I. (1993) Eur J Biochem 215, 705–710
11. Labrie, F., Simard, J., Lun-Thia, Y., Pelletier, G., Belghmi, K. & Belanger, A. (1994) Baillieres Clin Endocrinol Metab 8, 451–474
12. Monder, C. & Lakshmi, V. (1988) Steroids 52, 515–528
13. Remmer, H., Greim, H., Schenkm, J. B. & Estabrook, R. W. (1967) Methods Enzymol 10, 703–708
14. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J Biol Chem 193, 265–275
15. Smith, L. L. (1981) Cholesterol Autoxidation, Plenum Publishing Corp., New York
16. Stewart, P. M., Wallace, A. M., Valentinio, R., Durt, D., Shackleton, C. H. & Edwards, C. R. W. (1987) Lancet 2, 821–824
17. Monder, C. & White, P. C. (1993) Vitam Horm 47, 187–271
18. Koerner, D. R. (1969) Biochim Biophys Acta 176, 377–382
19. Agarwal, A. K., Tusie-Luna, M. T., Monder, C. & White, P. C. (1990) Mol Endocrinol 4, 1827–1832
20. Rusvai, E. & Naray-Fejes-Toth, A. (1993) J Biol Chem 268, 10717–10720
21. Yang, K. & Yu, M. (1994) J Steroid Biochem Mol Biol 49, 245–250
22. Brown, R. W., Chapman, K. E., Edwards, C. R. & Seckl, J. R. (1993) Endocrinology 132, 2614–2621
23. Stewart, P. M., Murry, B. A. & Mason, J. I. (1994) J Clin Endocrinol Metab 79, 1529–1532
24. Agarwal, A. K., Mune, T., Monder, C. & White, P. C. (1994) J Biol Chem 269, 25965–25962
25. Petersen, R. E., Wyngaarden, J. B., Guerra, S. L., Brodie, B. B. & Bunim, J. J. (1955) J Clin Invest 34, 1779–1794
26. Gustafson, J. A. & Gustafsson, S. A. (1974) Eur J Biochem 44, 225–233
27. Evdokimova, N. I. & Kamilov, I. K. (1967) in Farmakologii Alkaloidov i Glikozidov (Kamilov, I. K., ed) pp. 222–226, Fan, Tashkent, Uzbekistan
28. Ichikawa, H. (1968) Gifu Daigaku Igakubu Kyyo 15, 810–812 (abstr.)