24-Hydroxycholesterol is a substrate for hepatic cholesterol 7α-hydroxylase (CYP7A)

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Abstract (24S)-Hydroxycholesterol is formed from cholesterol in the brain and is important for cholesterol homeostasis in this organ. Elimination of (24S)-hydroxycholesterol has been suggested to occur in the liver but little is known about the metabolism of this oxysterol. In the present investigation, we report formation of 7α,24-dihydroxycholesterol in pig and human liver. 7α-hydroxylase activity toward both isomers of 24-hydroxycholesterol [(24S) and (24R)] was found in a partially purified and reconstituted cholesterol 7α-hydroxylase (CYP7A) enzyme fraction from pig liver microsomes. In contrast, a purified enzyme fraction of pig liver oxysterol 7α-hydroxylase with high activity toward 27-hydroxycholesterol did not show any detectable activity toward 24-hydroxycholesterol. 7α-Hydroxylation of 24-hydroxycholesterol was strongly inhibited by 7-oxocholesterol, a known inhibitor of CYP7A. Human CYP7A, recombinantly expressed in Escherichia coli and in simian COS cells, showed 7α-hydroxylase activity toward both cholesterol and the two isomers of 24-hydroxycholesterol, with a preference for the (24S)-isomer. Our results show that 24-hydroxycholesterol is metabolized by CYP7A, an enzyme previously considered to be specific for cholesterol and cholestanol and not active toward oxysterols. Because CYP7A is the rate-limiting enzyme in the major pathway of bile acid biosynthesis, the possibility is discussed that at least part of the 24-hydroxycholesterol is converted into 7α-hydroxylated bile acids by the enzymes involved in the normal biosynthesis of bile acids.—Norlin, M., A. Toll, I. Björkhem, and K. Wikvall. **24-Hydroxycholesterol is a substrate for hepatic cholesterol 7α-hydroxylase (CYP7A).** ***J. Lipid Res.*** **2000.** 41: **1629–1639.**

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(24*S*)-Hydroxycholesterol is formed from cholesterol in the brain by a brain-specific hydroxylase and is important for cholesterol homeostasis in this organ (1, 2). It was recently shown that there is a continuous net flux of (24*S*)-hydroxycholesterol over the brain–blood barrier to the systemic circulation in humans (1, 3). Because this flux was similar to the uptake of this steroid in the liver of human volunteers and because there was no renal elimination, the liver must be the main eliminator of (24*S*)-hydroxycholesterol.

In principle the liver may eliminate (24*S*)-hydroxycholesterol in unmetabolized or conjugated form through the bile or degrade it into bile acids. The flux of unmetabolized and sulfated (24*S*)-hydroxycholesterol through the intestine was measured in patients with an ileal fistula (I. Björkhem and J. E. Åkerlund, unpublished observation). This flux was less than 5% of the production of (24*S*)-hydroxycholesterol from the brain, indicating that there is a further metabolism of (24*S*)-hydroxycholesterol in the liver.

Hepatic metabolism of 24-hydroxycholesterol has been studied in mice (4). The data show that 24-hydroxycholesterol is taken up and converted to more polar metabolites in mouse liver but these metabolites were not identified.

If (24*S*)-hydroxycholesterol is converted into normal bile acids, a 7α-hydroxyl group must be introduced in the steroid nucleus. Previous work has demonstrated the existence of two cytochrome *P*-450 enzymes catalyzing 7α-hydroxylation in the different pathways of bile acid biosynthesis, one that is active toward cholesterol called the cholesterol 7α-hydroxylase (CYP7A) and another that is active toward 27-hydroxycholesterol, referred to as the oxysterol 7α-hydroxylase (5–11). A cDNA has been isolated encoding a cytochrome *P*-450 enzyme called CYP7B, which is primarily expressed in brain but also in liver and kidney (12). This enzyme catalyzes 7α-hydroxylation of 25-hydroxycholesterol and 27-hydroxycholesterol (13, 14). In view of the structural similarity between (24*S*)-hydroxycholesterol and the other side chain-hydroxylated cholesterol species, it appeared likely that

Abbreviations: δ-ALA, δ-aminolevulinic acid; CYP, cytochrome *P*-450; CYP7A, cholesterol 7α-hydroxylase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; IPTG, isopropyl β-D-thiogalactopyranoside; LXR, liver X receptor; POEL, polyoxyethylene 10 lauryl ether; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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also (24*S*)-hydroxycholesterol could be a substrate for oxysterol 7α-hydroxylase.

In contrast to CYP7A, which is found only in the liver, oxysterol 7α-hydroxylase is present in extrahepatic tissues and organs (15–18). In accordance with this, substantial amounts of 7α-hydroxylated 27-hydroxycholesterol and metabolites of this steroid are found in the circulation (5). However, significant levels of 7α-hydroxylated (24*S*)-hydroxycholesterol and its metabolites have not been found in the circulation (I. Björkhem, unpublished observation). In a study of 7α-hydroxylation of various side chain-oxidized steroids, Zhang et al. (18) found a significant 7α-hydroxylation of 27-hydroxycholesterol and 25-hydroxycholesterol in rat glial cells and neurons. 24-Hydroxycholesterol was, however, not converted under the conditions used. These findings suggest that oxysterol 7α-hydroxylase is not active toward 24-hydroxycholesterol.

The results of the present investigation show that highly purified pig liver oxysterol 7α-hydroxylase does not catalyze 7α-hydroxylation of 24-hydroxycholesterol. Surprisingly, cholesterol 7α-hydroxylase of porcine and human origin was found to be active toward 24-hydroxycholesterol.

EXPERIMENTAL PROCEDURES

Materials

DEAE-Sepharose CL6B, S-Sepharose Fast Flow, and Q-Sepharose Fast Flow were purchased from Pharmacia (Uppsala, Sweden) and hydroxylapatite (Macroprep ceramic HTP) was purchased from Bio-Rad (Hercules, CA). Octylamine-Sepharose was prepared by coupling 1,8-diamino-octane to CNBr-Sepharose 4B (Pharmacia) (19). [4-^{14}C]cholesterol (52 mCi/mmol) was obtained from Amersham International (Amersham, UK). 27-Hydroxycholesterol, which was a kind gift from L. Tökes (Syntex, Palo Alto, CA), was prepared from kryptogenin, according to the methods described by Scheer, Thompson, and Mosettig (20). 24-Hydroxycholesterol was synthesized as described (21). 3α-^{3}H-labeled racemic 24-hydroxycholesterol was prepared from unlabeled racemic 24-hydroxycholesterol by reduction of the corresponding enol ether with $NaB^{3}H_4$ as described previously (2). The material was purified by preparative thin-layer chromatography, using toluene–ethyl acetate 1:1 (v/v) as solvent and had a specific radioactivity of 200 mCi/mmol. 11β-Hydroxyprogesterone, 7-oxocholesterol (3β−hydroxy-5-cholesten-7-one), δ-aminolevulinic acid (δ-ALA), cholesterol oxidase (*Cellulomonas* species), dithiothreitol (DTT), dilauroylglycero-3-phosphorylcholine, dimethyl sulfoxide, phenylmethylsulfonyl fluoride (PMSF), polyoxyethylene 10 lauryl ether (POEL), and Triton X-100 were from Sigma (St. Louis, MO). Isopropyl β-D-thiogalactopyranoside (IPTG), lysozyme, and deoxyribonuclease I (DNase I) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). *Escherichia coli* strain DH5α was purchased from Life Technologies (Rockville, MD). Materials for cell culture media were obtained from Life Technologies and Difco (Detroit, MI). The expression vector pJL/H7α1.5 containing cDNA for human CYP7A was a generous gift from J. Y. L. Chiang (Northeastern Ohio Universities, Rootstown, OH) (22). The pSVL simian virus 40 eukaryotic expression vector containing cDNA for human CYP7A was a generous gift from K. Okuda (Miyazaki Medical College, Miyazaki, Japan) (23). Other chemicals, reagents, and materials were those used previously in our laboratories (2, 19).

Chemical synthesis of 7α,24-dihydroxycholesterol

A racemic mixture of 7α,24-dihydroxycholesterol was prepared from 24-hydroxycholesterol by oxidation with *tert*-butylperbenzoate as described by Stárka (24).

Purification of cytochrome *P*-450 active in 7α-hydroxylation of 27-hydroxycholesterol from pig liver

Cytochrome *P*-450 catalyzing the 7α-hydroxylation of 27-hydroxycholesterol was prepared from pig liver microsomes as previously described by Norlin and Wikvall (11). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the purified cytochrome *P*-450 fraction showed one major and two or three minor protein bands. The cytochrome *P*-450 content in the purified enzyme fraction varied between 2 and 3 nmol of cytochrome *P*-450 per mg of protein.

Purification of cytochrome *P*-450 active in 7α-hydroxylation of cholesterol from pig liver

Cytochrome *P*-450 catalyzing the 7α-hydroxylation of cholesterol was prepared from pig liver microsomes according to the methods described by Toll et al. (7). This cytochrome *P*-450 fraction showed several bands on SDS-PAGE and contained 1–2 nmol of cytochrome *P*-450 per mg of protein.

Expression of human CYP7A in *Escherichia coli*

The methods used for expression of human CYP7A in *E. coli* and purification of the recombinantly expressed protein were essentially the same as those described by Karam and Chiang (22), with the following modifications. The expression plasmid pJL/H7α1.5 containing cDNA encoding for human CYP7A was transformed into *E. coli* strain DH5α instead of TOPP3 cells. Two liters of "terrific broth" (2.4% yeast extract, 1.2% tryptone, 0.4% glycerol, 17 mM KH_2PO_4, and 72 mM KH_2PO_4) containing ampicillin at 100 mg/l was inoculated with a 6-h culture of transformed DH5α cells in Luria-Bertani (LB) broth containing ampicillin at 100 mg/l. The culture was grown at 37°C with shaking at 200 rpm until the OD_{600} reached 0.5–0.6. Protein synthesis was induced by adding IPTG and δ-ALA to a final concentration of 1 and 0.2 mM, respectively, and the *E. coli* culture was grown under good aeration at 30°C for 16 h with shaking at 150 rpm.

Purification of recombinantly expressed human CYP7A in *Escherichia coli*

The purification procedure was essentially the same as that described by Karam and Chiang (22). The harvested cells were placed on ice for 20 min and then centrifuged at 4,500 *g* for 10 min at 4°C. The pellet was homogenized in 50 ml of 100 mM potassium phosphate buffer, pH 7.4, containing 0.5% sodium cholate, 20% glycerol, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM DTT, and 0.25 mM PMSF. Lysozyme was added to a concentration of 200 μg/ml and the cell suspension was placed on ice with stirring for 1 h and then sonicated for 3 min at intervals of 15 sec. The cell lysate was centrifuged at 100,000 *g* for 1 h and the supernatant was collected. The pellet was resuspended in 20 ml of 100 mM potassium phosphate buffer (pH 7.4), 0.5% sodium cholate, 20% glycerol, 0.1 mM EDTA, 0.1 mM DTT, and 0.25 mM PMSF and centrifuged again. Twenty-five units of DNase I was added and the combined supernatants were stored on ice overnight. All buffers used in chromatography contained 20% glycerol, 0.1 mM EDTA, 0.1 mM DTT, and 0.25 mM PMSF. Phosphate buffer was used as the potassium salt. Column eluates were monitored by measuring the absorbance at 416 nm. The lysate was subjected to chromatography on an octylamine-Sepharose column (2.7 × 25 cm) equilibrated with 100 mM phosphate buffer, pH 7.4, containing 0.5% sodium cholate. Protein was eluted with equilibrating buffer. Fractions most active in 7α-hydroxylation of cholesterol

were dialyzed against 10 mM phosphate buffer, pH 7.4, containing 0.3% sodium cholate and applied to a hydroxylapatite column (2.7 × 6 cm) equilibrated with the same buffer. The column was washed with 10 mM phosphate, pH 7.4, containing 0.3% sodium cholate and then with 50 mM phosphate, pH 7.4, containing 0.3% sodium cholate. Protein was eluted with 360 mM phosphate, pH 7.4, containing 0.2% POEL. Eluted enzyme fractions were pooled and dialyzed against 100 mM phosphate buffer, pH 7.4, containing 0.2% POEL, 20% glycerol, 0.1 mM EDTA, 0.1 mM DTT, and 0.25 mM PMSF prior to assay of catalytic activity.

Expression of human CYP7A in simian COS cells

COS-M6 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. The cells were transfected with the pSVL vector containing cDNA encoding human CYP7A (23). In control experiments, COS cells were transfected with pSVL vector without the CYP7A cDNA insert. Transfection was carried out by electroporation in 0.4-cm cuvettes (GenePulser II; Bio-Rad), using a single pulse of 0.4 kV and 100 μF. In each experiment a number of 20×10^6 cells were transfected with 20 μg of DNA in a volume of 0.8 ml of phosphate-buffered saline containing calcium chloride and magnesium chloride (Dulbecco's PBS; Life Technologies). In most experiments, the cells were grown for 24 or 48 h in medium containing 5 μM 24-hydroxycholesterol (unlabeled) dissolved in dimethyl sulfoxide. After incubation, medium and cells were harvested separately. The cells were suspended in 1 ml of 50 mM Tris-acetate buffer and homogenized (sonicated for 5 sec). Cells and medium were extracted with trichloroethane–methanol 2:1 (v/v) and the organic phase was analyzed for 7α-hydroxylated metabolites as described below.

In some experiments microsomes were prepared from transfected cells (25). In these experiments the COS cells were grown in medium without 24-hydroxycholesterol and harvested 30 h after transfection. Microsomes prepared from cells transfected with pSVL vector containing CYP7A cDNA and cells transfected with vector alone were incubated as described below.

Incubation procedures

Incubations with purified enzyme fractions or microsomes were carried out at 37°C for 20, 40, or 60 min. The substrates 27-hydroxycholesterol (6 μg, unlabeled), [4-^{14}C]cholesterol (0.6–12 μg), 24-hydroxycholesterol (0.1–12 μg, unlabeled), or 24-[3α-^{3}H]hydroxycholesterol (12 μg), dissolved in 25 μl of acetone were incubated with varying amounts of cytochrome *P*-450 (0.003–0.14 nmol) and 1 μmol of NADPH in a total volume of 1 ml of 50 mM Tris-acetate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA. In incubations with purified microsomal enzyme fractions, 2 U of NADPH-cytochrome *P*-450 reductase were added. Triton X-100 at a concentration of 0.05% (w/v) and 5 mM DTT were added in incubations with cholesterol and in all incubations with CYP7A purified from *E. coli* (19). Incubations with *E. coli*-expressed enzyme also contained 40 μg of dilauroylglycero-3-phosphorylcholine (22). Incubations with intact liver microsomes (0.2–1 mg) were performed as for purified microsomal enzyme fractions, except that the addition of NADPH-cytochrome *P*-450 reductase was omitted. Incubations with microsomes prepared from COS cells contained 0.5 mg of microsomal protein, 6 μg of 24-hydroxycholesterol, 2 U of NADPH-cytochrome *P*-450 reductase, 5 mM DTT, and 0.05% Triton X-100. All incubations were terminated with 5 ml of trichloroethane–methanol 2:1 (v/v).

Analysis of incubations with 27-hydroxycholesterol

As an internal recovery standard, 0.25 μg of 11β-hydroxyprogesterone was added to the terminated incubations. The organic phase was collected and evaporated with N_2. The samples were dissolved in 25 μl of acetone and 900 μl of 100 mM potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA. A second reaction was started by the addition of 0.2 units of cholesterol oxidase dissolved in 0.1 ml of the incubation buffer. The reaction mixture was incubated for 20 min at 37°C and the reaction was terminated with 5 ml of trichloroethane–methanol 2:1 (v/v). The organic phase was collected and evaporated with N_2. The samples were dissolved in 0.1 ml of mobile phase, hexane–isopropanol 88:12, and subjected to high-performance liquid chromatography (HPLC) on a 125 × 4 mm silica column (LiChrosphere Si 60, 5 μm; Merck, Rahway, NJ) at a flow rate of 0.7 ml/min and steroids with a 3-oxo-Δ^4 structure were monitored at 240 nm. The retention times were 8–9 min for 7α,27-dihydroxy-4-cholesten-3-one and 12–13 min for 11β-hydroxyprogesterone.

The amounts of product formed were calculated from a standard curve obtained from a series of incubations with known amounts of 5-cholestene-3β,7α,27-triol and cholesterol oxidase as described by Toll et al. (10).

Analysis of incubations with cholesterol

Formation of 7α-hydroxycholesterol in liver microsomes and purified fractions was analyzed with [4-^{14}C]cholesterol. After extraction of the incubations, the organic phase was collected and evaporated with N_2 and the samples were applied on silica gel thin-layer chromatography plates. The chromatoplates were developed once in a solvent system consisting of toluene–ethyl acetate 40:60 (v/v) and scanned for radioactivity with a Berthold (Bad Wildbad, Germany) Tracemaster 20 TLC scanner. Incubations with cholesterol were analyzed on the same day as the incubations were performed (19).

CYP7A activity in living COS cells and in microsomes prepared from COS cells was assayed with the endogenous cholesterol as substrate. Cell homogenate and medium or terminated microsomal incubations were extracted, the organic phase was evaporated with N_2, and the samples were incubated with cholesterol oxidase and subjected to HPLC in the same way as incubations with 27-hydroxycholesterol except that the mobile phase was hexane–isopropanol 98:2. The retention time for the enzymatically formed 7α-hydroxy-4-cholesten-3-one in this system was 16–17 min, which was identical to the retention time of the authentic reference compound.

Analysis of incubations with unlabeled 24-hydroxycholesterol

The incubations were analyzed similarly to the incubations with 27-hydroxycholesterol. As an internal recovery standard, 0.1 μg of testosterone was added to the terminated incubations. After extraction and evaporation of the organic phase the samples were incubated with cholesterol oxidase and subjected to HPLC similarly to the incubations with 27-hydroxycholesterol with hexane–isopropanol 94:6 as the mobile phase. The retention times in this system were 15–16 min for testosterone and 20–22 min for 7α,24-dihydroxy-4-cholesten-3-one (see **Fig. 1**).

Analysis of incubations with radiolabeled 24-hydroxycholesterol

The organic phase from the extraction of the incubation with 3α-^{3}H-labeled 24-hydroxycholesterol was subjected to radio-HPLC chromatography, using a Kontron (Everett, MA) instrument and a YMC-Pck ODS-A column, 250 × 4.6 mm, with methanol–water 85:15 (v/v) as effluent. Under these conditions the two isomers of 24-hydroxycholesterol as well as the two isomers of the product, 7α-hydroxylated 24-hydroxycholesterol, separated (see **Fig. 2**). In this system, the (24*R*)-isomer was found to have a longer retention time than the (24*S*)-isomer.

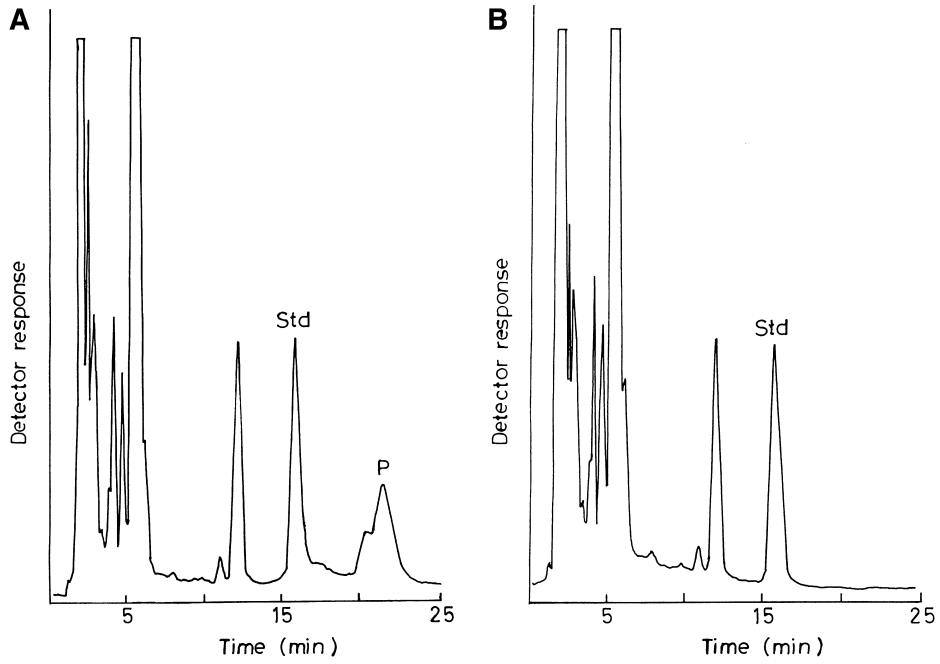

Fig. 1. HPLC analysis of extracts from incubations with unlabeled 24-hydroxycholesterol. HPLC of an extract from an incubation with unlabeled, racemic 24-hydroxycholesterol and the purified reconstituted pig liver cholesterol 7α-hydroxylase fraction (A) and the corresponding control (an incubation without NADPH-cytochrome *P*-450 reductase) (B). Two enzymatically derived products were eluted after 20–22 min. These products probably correspond to the hydroxylated products of the two isomers of 24-hydroxycholesterol. Incubations were performed and analyzed as described in Experimental Procedures.

Analysis by combined gas chromatography-mass spectrometry

The 7α-hydroxylated products corresponding to the two peaks in the HPLC chromatogram were converted into trimethylsilyl ether and analyzed by combined gas chromatography-mass spectrometry. In the latter analysis a Hewlett-Packard (Palo Alto, CA) 5973 quadrupole instrument was used, equipped with a Hewlett-Packard 19891A-102 column (25 m × 200 μm). The initial temperature was 180°C followed by a rise of 20°C/min up to 250°C and then 5°C/min up to 300°C. The temperature was kept at 300°C for 11.5 min. Under these chromatographic conditions, the (24*R*)- and (24*S*)-hydroxycholesterol did not separate and this was the case also with the corresponding 7α-hydroxylated products. In some specific experiments a DB 210/30 W column (30 m × 320 μm) was used. The temperature was kept at 140°C for 1 min, and then it was increased at 20°C/min to a final temperature of 198°C (see ref. 1). Under these chromatographic conditions the (24*R*)- and (24*S*)-isomers of 24-hydroxycholesterol separate (1). In this case the (24*S*)-isomer has the longest retention time (Fig. 5).

Other methods

NADPH-cytochrome *P*-450 reductase was prepared from pig liver microsomes as described by Yasukochi and Masters (26).

Protein concentrations in microsomal fractions and cell homogenates were determined by the method of Lowry et al. (27). The concentration in purified protein fractions was determined by measuring the absorbance at 280 nm (concentration in milligrams per milliliter equals the absorbance of protein at 280 nm). Cytochrome *P*-450 concentration in purified fractions was estimated by measuring the absorbance at 416 nm as an indication of total heme content. On the average, a 1 μM heme protein solution has an A_{416} of 0.12 (28).

SDS-PAGE was performed according to Laemmli (29) with 15% acrylamide and 0.09% bisacrylamide slab gels (15 × 10 × 0.1 cm) containing 0.1% (w/v) SDS. The gels were polymerized by addition of 0.1% (v/v) tetramethylenediamine and 0.1% (w/v) ammonium hydrogen sulfate. Electrophoresis was carried out at 100 V per slab gel for 13–14 h to obtain optimal resolution and the gels were silver stained according to the methods described by Wray et al. (30).

RESULTS

7α-Hydroxylation of cholesterol and 27-hydroxycholesterol by purified cytochrome *P*-450 fractions from pig liver

27-Hydroxycholesterol 7α-hydroxylase (oxysterol 7α-hydroxylase) and cholesterol 7α-hydroxylase were partially purified from pig liver microsomes by sodium cholate solubilization and chromatographic methods according to procedures previously described for purification of these enzymes (7, 11). The 7α-hydroxylation of cholesterol and 27-hydroxycholesterol by the two enzyme fractions was examined (**Table 1**). The oxysterol 7α-hydroxylase enzyme fraction showed high activity toward 27-hydroxycholesterol but no detectable 7α-hydroxylase activity toward cholesterol. The cholesterol 7α-hydroxylase fraction showed 7α-hydroxylase activity toward cholesterol and 27-hydroxycholesterol with the highest 7α-hydroxylase activity toward cholesterol. The specific activity toward 27-hydroxycholesterol in the cholesterol 7α-hydroxylase fraction was much lower than in the oxysterol 7α-hydroxylase fraction (Table 1).

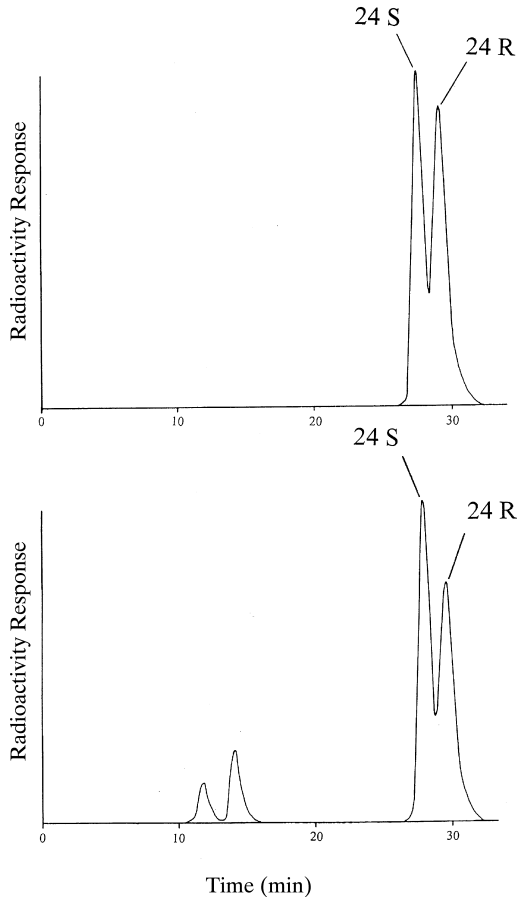

Fig. 2. Radio-HPLC analysis of extracts from incubations with ^{3}H-labeled 24-hydroxycholesterol. Radio-HPLC of an extract of incubation of racemic mixture of ^{3}H-labeled 24-hydroxycholesterol with the purified reconstituted pig liver cholesterol 7α-hydroxylase (lower chromatogram) and the corresponding control (upper chromatogram). Two enzymatically derived products were eluted after 10–15 min. As judged from the appearance in other chromatographic systems (1), the isomer of 24-hydroxycholesterol with the longest retention time is the (24*R*)-isomer.

7α-Hydroxylation of 24-hydroxycholesterol by purified cytochrome *P*-450 fractions from pig liver

The oxysterol 7α-hydroxylase and the cholesterol 7α-hydroxylase enzyme fractions were incubated with racemic 24-hydroxycholesterol to investigate the ability of these fractions to 7α-hydroxylate this steroid. Initially, the incubations with 24-hydroxycholesterol were analyzed similarly to the analysis of incubations with 27-hydroxycholesterol (where the product is converted to 7α,27-dihydroxy-4-cholesten-3-one prior to analysis), using various modifications of the HPLC mobile phase to screen for peaks that corresponded to enzymatically derived products. Because 24-hydroxycholesterol is less polar than 27-hydroxycholesterol but more polar than cholesterol, it was assumed that if 7α,24-dihydroxy-4-cholesten-3-one was formed, it would have a retention time intermediate to those of 7α,27-dihydroxy-4-cholesten-3-one and 7α-hydroxy-4-cholesten-3-one. Incubations were performed with and without NADPH-cytochrome *P*-450 reductase in order to detect if any HPLC peaks found would correspond to cytochrome *P*-450-mediated enzyme activity. In incubations with the oxysterol 7α-hydroxylase fraction, no peaks dependent on NADPH-cytochrome *P*-450 reductase were found. However, in incubations with the cholesterol 7α-hydroxylase fraction, two peaks occurred, most probably corresponding to hydroxylated products of the two isomers of 24-hydroxycholesterol. The two peaks disappeared when NADPH-cytochrome *P*-450 reductase or NADPH was omitted from the incubation mixture. As expected, the peaks increased with incubation time and with increased amount of substrate. Typical examples of HPLC chromatograms with the cholesterol 7α-hydroxylase fraction are shown in Fig. 1.

In a separate set of experiments, incubations were performed with 3α-^{3}H-labeled racemic 24-hydroxycholesterol. Figure 2 shows a chromatogram obtained in the analysis of an incubation of this material with a reconstituted cholesterol 7α-hydroxylase system, using radio-HPLC. Two radioactive products were obtained with a retention time as expected for 7α-hydroxylated products of the (24*R*)- and (24*S*)-isomers of 24-hydroxycholesterol. The rate of hydroxylation of the (24*R*)-isomer appeared to be somewhat higher than that of the (24*S*)-isomer. Material corresponding to the two HPLC peaks was converted into trimethylsilyl ether and analyzed by combined gas chromatography-mass spectrometry. Material from the two peaks gave identical mass spectra, which were identical to those of synthetic 7α-hydroxylated (24*R*)- and (24*S*)-hydroxycholesterol (**Fig. 3**). Characteristic peaks were seen at m/z 634 (M), 591 (M-43), 544 (M-90, base peak), 501 (M-90-43), 454 (M-2x90), and 145 (loss of TmSiO + C_{24} – C_{27}).

TABLE 1. Catalytic properties of the purified cytochrome *P*-450 fractions from pig liver

	7α-Hydroxylation		
	Cholesterol	27-Hydroxycholesterol	24-Hydroxycholesterol
	pmol/nmol P-450/min		
Microsomes	3 (1–5)	620 ± 112	8 (7–9)
Oxysterol 7α-hydroxylase fraction	⩽0.01	7,113 ± 2,652	⩽5
Cholesterol 7α-hydroxylase fraction	1,042 ± 189	264 ± 49	328 ± 79

The table shows the catalytic properties of pig liver microsomes and of the two purified enzyme fractions from pig liver. Incubations were performed with 15 μM substrate as described in Experimental Procedures. The data are given as the means of two to five experiments with standard deviation or range.

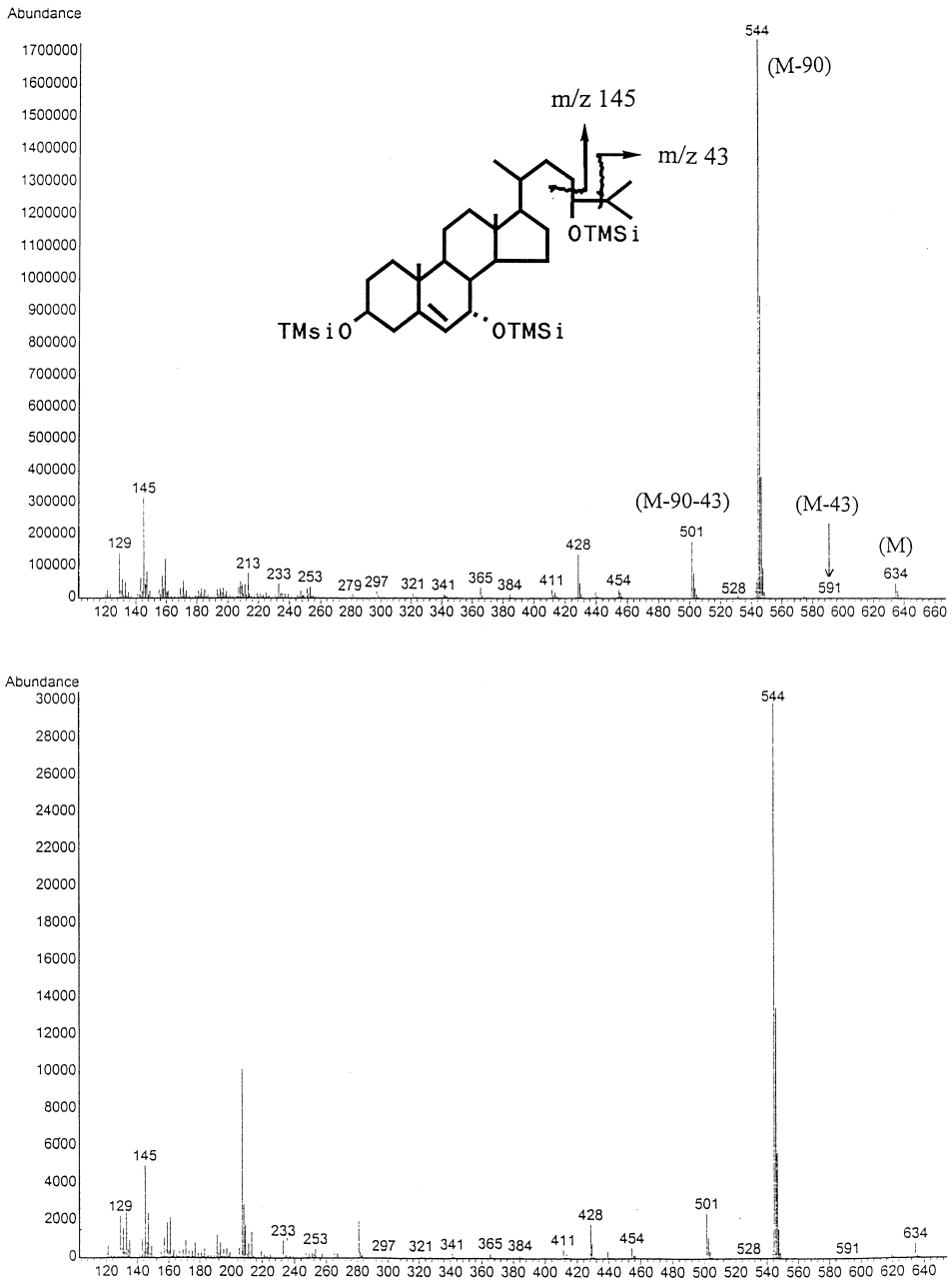

Fig. 3. Mass spectrum of product isolated from incubations with 24-hydroxycholesterol. Mass spectrum of trimethylsilyl ether of synthetic 7α,24-hydroxycholesterol (upper spectrum) and product isolated from an incubation of 24-hydroxycholesterol with the reconstituted pig liver cholesterol 7α-hydroxylase (lower spectrum).

The specific 7α-hydroxylase activity toward 24-hydroxycholesterol in the cholesterol 7α-hydroxylase fraction was estimated by using the standard curve for formation of 7α,27-dihydroxy-4-cholesten-3-one. The highest 7α-hydroxylase activity found toward 24-hydroxycholesterol was 460 pmol/nmol *P*-450 × min (Table 1).

Effects of 7-oxocholesterol on the 7α-hydroxylase activities toward cholesterol, 24-hydroxycholesterol, and 27-hydroxycholesterol

To obtain further information concerning the 7α-hydroxylase activity toward 24-hydroxycholesterol and to investigate its possible relation to the CYP7A enzyme, the cholesterol 7α-hydroxylase fraction was incubated in the presence of 7-oxocholesterol, which is known to be a strong inhibitor of CYP7A (31, 32). As expected, 7-oxocholesterol efficiently inhibited cholesterol 7α-hydroxylation (**Fig. 4**). 7-Oxocholesterol was found to be an effective inhibitor also of the 7α-hydroxylase activity toward 24-hydroxycholesterol. A concentration of 3 μM inhibited the formation of 7α,24-dihydroxycholesterol by 70%. Formation of 7α,27-dihydroxycholesterol by the oxysterol 7α-hydroxylase fraction was, however, not inhibited by 7-oxocholesterol (Fig. 4).

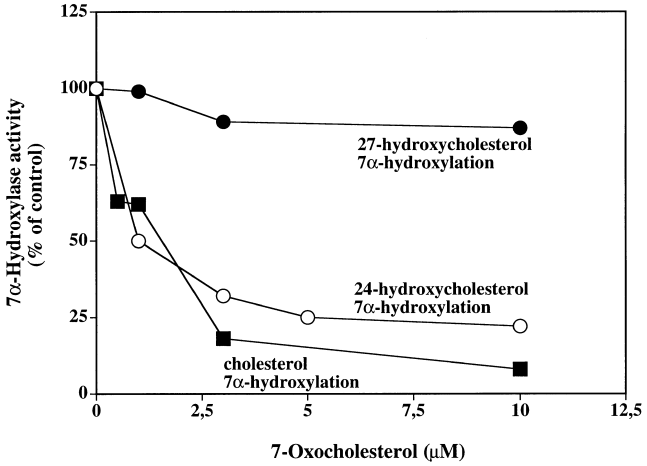

Fig. 4. Effect of 7-oxocholesterol on the 7α-hydroxylation of 27-hydroxycholesterol, cholesterol, and 24-hydroxycholesterol. Effect of different amounts of 7-oxocholesterol on the rate of 7α-hydroxylation of 27-hydroxycholesterol (solid circles) by the oxysterol 7α-hydroxylase fraction and the 7α-hydroxylation of cholesterol (solid squares) and 24-hydroxycholesterol (open circles) by the cholesterol 7α-hydroxylase fraction. Incubations were performed with 15 μM substrate as described in Experimental Procedures, except for the addition of 7-oxocholesterol. 100% of control represents the activity without addition of inhibitor.

7α-Hydroxylation of 24-hydroxycholesterol by human CYP7A recombinantly expressed in *Escherichia coli*

Because the studies with purified enzyme fractions from pig liver indicated that the enzyme responsible for 7α-hydroxylation of 24-hydroxycholesterol might be the CYP7A, we found it of interest to test this hypothesis in experiments with recombinantly expressed human CYP7A. *Escherichia coli* cells harboring an expression plasmid containing the human CYP7A gene were grown in the presence of IPTG and δ-ALA to induce protein synthesis (22). The CYP7A protein obtained in this way was purified by chromatography on octylamine-Sepharose and hydroxylapatite. The 7α-hydroxylase activity of the recombinantly expressed CYP7A was analyzed in a reconstituted system with NADPH-cytochrome *P*-450 reductase, Triton X-100, DTT, dilauroylglycero-3-phosphorylcholine, and NADPH as described in Experimental Procedures. The purified CYP7A showed 7α-hydroxylase activity toward both cholesterol and 24-hydroxycholesterol. Incubations with recombinantly expressed enzyme were performed both with unlabeled and radiolabeled 24-hydroxycholesterol. Formation of 7α-hydroxylated product was verified by combined gas chromatography-mass spectrometry. The specific activity toward 24-hydroxycholesterol was about a third of the activity toward cholesterol (**Table 2**). Recombinantly expressed human CYP7A 7α-hydroxylated both isomers of 24-hydroxycholesterol with the highest activity toward the (24*S*)-isomer (**Fig. 5B**). Most incubations were performed with 5 to 15 μM substrate but formation of 7α-hydroxylated metabolite was detectable with as little as 0.25 μM 24-hydroxycholesterol. With this concentration about 40% of the substrate was converted into product. Control incubations without NADPH-cytochrome *P*-450 reductase or with solubilized protein from DH5α cells without the pJL/H7α1.5 vector showed no detectable formation of product.

TABLE 2. 7α-Hydroxylation by human CYP7A recombinantly expressed in *Escherichia coli*

	7α-Hydroxylase Activity
	pmol/nmol P-*450/min*
Cholesterol	13,200 ± 2,100
24-Hydroxycholesterol	4,200 ± 1,400

The table shows the 7α-hydroxylation by CYP7A recombinantly expressed in *E. coli*. Incubations were performed as described in Experimental Procedures. The data are given as the means of four experiments with standard deviation.

The apparent K_m value for the 7α-hydroxylation of 24-hydroxycholesterol by *E. coli*-expressed human CYP7A, determined from double-reciprocal plots, was found to be 6 μM ($r = 0.85$). Construction of Eadie-Hofstee plots from the data gave a K_m value of 5 μM. A K_m of 3 μM was obtained for cholesterol 7α-hydroxylation, which is similar to the value (7 μM) reported by Karam and Chiang (22).

It is of interest that the human cholesterol 7α-hydroxylase had a higher activity toward the naturally occurring (24*S*)-isomer than toward the (24*R*)-isomer, which seems to be in contrast to the porcine cholesterol 7α-hydroxylase. To further substantiate that the enzyme from the two different species have different specificities toward the two isomers of 24-hydroxycholesterol, an extract of an incubation of a racemic mixture of 24-hydroxycholesterol with the purified reconstituted pig liver cholesterol 7α-hydroxylase was subjected to gas chromatography under the same conditions as in the analysis of the incubation with the recombinant human CYP7A enzyme. From the results obtained (Fig. 5), it is evident that the porcine and human enzyme differ in specificity toward the two isomers.

7α-Hydroxylation of 24-hydroxycholesterol by human full-length CYP7A recombinantly expressed in COS cells

Because the expression vector used for *E. coli* expression experiments contains a cDNA that encodes a truncated form of the enzyme (22), we wanted to compare the catalytic properties of this protein with the properties of full-length CYP7A. To exclude the possibility that 7α-hydroxylation of 24-hydroxycholesterol was catalyzed only by truncated CYP7A, experiments were performed with full-length human CYP7A transiently expressed in simian COS cells. Transfection of COS cells with the pSVL vector containing CYP7A cDNA and incubation with the endogenous cholesterol, present in the COS cells, and added 24-hydroxycholesterol for 24 or 48 h resulted in formation of both 7α-hydroxycholesterol and 7α,24-dihydroxycholesterol (**Table 3**). The amount of 7α,24-dihydroxycholesterol formed was about a fifth of the amount of 7α-hydroxycholesterol. The 7α-hydroxylase activity toward cholesterol was assayed with endogenous substrate only because previous investigations have indicated that addition of exogenous cholesterol to COS cells transfected with CYP7A cDNA does not further in-

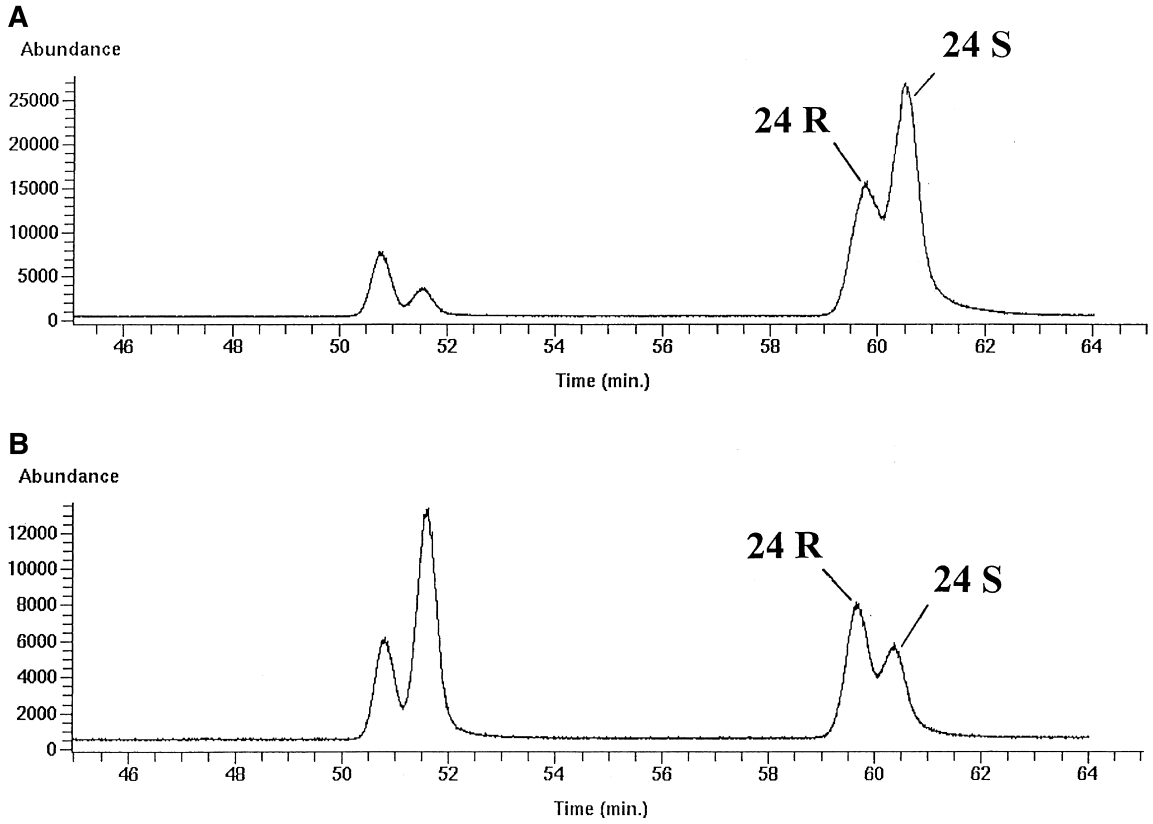

Fig. 5. Gas chromatography analysis of extracts from incubations with 24-hydroxycholesterol. Gas chromatography of trimethylsilyl ether of extract of incubations of racemic 24-hydroxycholesterol and reconstituted pig liver cholesterol 7α-hydroxylase (A) and human cholesterol 7α-hydroxylase, recombinantly expressed in *E. coli* (B). In this system the substrate has a longer retention time than the product, and the (24*S*)-isomer has a longer retention time than the (24*R*)-isomer.

crease the 7α-hydroxylase activity (10). Control experiments with COS cells transfected with pSVL vector without the CYP7A cDNA did not result in production of any detectable amounts of 7α-hydroxycholesterol or 7α,24-dihydroxycholesterol. It may be noted that whereas 7α-hydroxycholesterol was found both within the cells and in the cell medium of transfected cells, 7α,24-dihydroxycholesterol was found exclusively in the cell medium (Table 3). This is in agreement with reported findings that side chain-hydroxylated cholesterol derivatives are more easily secreted from some cells (33). The presence of 7α-hydroxycholesterol and 7α,24-dihydroxycholesterol in the medium of transfected cells was confirmed by combined gas chromatography-mass spectrometry.

Experiments were also performed with microsomes from COS cells grown in the absence of 24-hydroxycholesterol.

TABLE 3. 7α-Hydroxylated metabolites formed by COS cells expressing human CYP7A

	Control Cells[a]		Cells Transfected with CYP7A cDNA	
	Cell Homogenate	Cell Medium	Cell Homogenate	Cell Medium
	pmol/mg cell protein/24 h			
7α-Hydroxy-4-cholesten-3-one	⩽30	⩽30	637 (410–878)	633 (582–700)
7α,24-Dihydroxy-4-cholesten-3-one	⩽20	⩽20	⩽20	255 (193–309)

The table shows the 7α-hydroxylated metabolites formed by COS cells transfected with pSVL vector containing full-length CYP7A cDNA. Transfection, incubation, and analysis were performed as described in Experimental Procedures. After transfection, cells were cultured on 100-mm plates with medium containing 5 μM 24-hydroxycholesterol. After incubation for 24 or 48 h, cells and medium from each plate were harvested separately. The samples were extracted and incubated with cholesterol oxidase. All samples were analyzed for 7α-hydroxylated products by HPLC. Initially, the formation of 7α,24-dihydroxy-4-cholesten-3-one was analyzed using hexane–isopropanol 94:6 as the mobile phase. The eluate from this chromatography was collected and rechromatographed with hexane–isopropanol 98:2 to analyze the formation of 7α-hydroxy-4-cholesten-3-one. The data given are the means of three experiments with the range shown in parentheses.

[a] COS cells transfected with pSVL vector without the CYP7A cDNA insert.

TABLE 4. 7α-Hydroxylated metabolites formed in microsomes prepared from COS cells expressing human CYP7A

Microsomes from Cells Transfected with	Incubation Time	7α-Hydroxy-4-cholesten-3-one	7α,24-Dihydroxy-4-cholesten-3-one
	min	*pmol/mg protein*	*pmol/mg protein*
CYP7A cDNA	0	1,695	≤10
	60	2,441	168
Vector without insert	0	≤25	≤10
	60	≤25	≤10

The table shows the 7α-hydroxylated metabolites formed in incubations with microsomes prepared from COS cells transfected with pSVL vector containing full-length CYP7A cDNA. Transfection, preparation of microsomes, incubation, and analysis were performed as described in Experimental Procedures. The cells were harvested 30 h posttransfection and microsomes were prepared from cells transfected with vector containing CYP7A cDNA and from cells transfected with vector alone. Microsomal protein (0.5 mg) was incubated for either 0 or 60 min and the samples were incubated with cholesterol oxidase and analyzed for 7α-hydroxylated products by HPLC. Initially, the formation of 7α,24-dihydroxy-4-cholesten-3-one was analyzed using hexane–isopropanol 94:6 as the mobile phase. The eluate from this chromotography was collected and rechromatographed with hexane–isopropanol 98:2 to analyze the formation of 7α-hydroxy-4-cholesten-3-one.

Incubation of 24-hydroxycholesterol (added) and cholesterol (endogenous) for 60 min with microsomes prepared from cells transfected with pSVL vector containing CYP7A cDNA resulted in formation of both 7α-hydroxycholesterol and 7α,24-dihydroxycholesterol (**Table 4**). Control incubations terminated after 0 min contained substantial amounts of 7α-hydroxycholesterol already formed prior to incubation (i.e., during maintenance of transfected cells for 30 h prior to harvest). The actual formation of 7α-hydroxycholesterol during the 60-min incubation was 746 pmol/mg protein compared with 168 pmol/mg for 7α,24-dihydroxycholesterol. No 7α-hydroxylated products could be detected in incubations with microsomes from cells transfected with vector without the CYP7A insert (Table 4).

DISCUSSION

Cholesterol 7α-hydroxylase has been regarded to have a high specificity toward cholesterol and its 5α-saturated analog cholestanol and little or no enzymatic activity has been reported toward side chain-modified steroids. In the present work it is conclusively shown that both pig and human cholesterol 7α-hydroxylase are active toward 24-hydroxycholesterol. The finding that 7-oxocholesterol, a known inhibitor of cholesterol 7α-hydroxylase (31, 32), inhibited the formation of 7α,24-dihydroxycholesterol from 24-hydroxycholesterol by the cholesterol 7α-hydroxylase fraction suggested that cholesterol 7α-hydroxylase is the enzyme active toward 24-hydroxycholesterol. Formation of 7α,27-dihydroxycholesterol from 27-hydroxycholesterol by the oxysterol 7α-hydroxylase fraction was not inhibited by this compound. The experiments with human CYP7A recombinantly expressed in *E. coli* and in simian COS cells clearly demonstrated 7α-hydroxylation of 24-hydroxycholesterol by this enzyme. An efficient 7α-hydroxylation was seen in experiments with COS cells grown in the presence of 24-hydroxycholesterol, added to the medium immediately after transfection. The reason for maintaining the cells in the presence of 24-hydroxycholesterol was that COS cells, like all mammalian cells, contain large amounts of endogenous cholesterol (34) competing with added substrate. This experimental design allows 24-hydroxycholesterol to be exposed to the expressed CYP7A for the same period of time as endogenous cholesterol. Microsomes prepared from transfected COS cells, maintained without 24-hydroxycholesterol in the medium, also efficiently 7α-hydroxylated 24-hydroxycholesterol. In this experiment, the enzyme was exposed to endogenous cholesterol for 31 h but for only 60 min to 24-hydroxycholesterol. The results show that most of the 7α-hydroxycholesterol detected in the microsomal fraction was formed prior to the 60-min incubation (see Table 4). The actual formation of 7α-hydroxycholesterol during the incubation was about four times higher than the formation of 7α,24-dihydroxycholesterol. This relative ratio is in full agreement with the ratios obtained with *E. coli*-expressed enzyme and with purified porcine cholesterol 7α-hydroxylase. It is possible that the specific 7α-hydroxylase activities could be higher, as the 7α-hydroxycholesterol present in the microsomes may inhibit the CYP7A-mediated 7α-hydroxylation of both substrates (32).

It is noteworthy that the substrate used in the present assay was a mixture of the (24*S*)- and (24*R*)-isomers, whereas the naturally occurring isomer is the (24*S*)-isomer (1). In pig liver there was some preference for the (24*R*)-isomer whereas recombinantly expressed human CYP7A showed highest activity toward the (24*S*)-isomer.

In the experiments with recombinantly expressed human CYP7A, conversion of 24-hydroxycholesterol into 7α-hydroxylated metabolite was observed with substrate concentrations as low as 0.25 μM. This concentration should be within the physiological concentration range. Although the level of 24-hydroxycholesterol in the human liver cell is not established, plasma concentrations of 24-hydroxycholesterol are reported to be about 0.07 μg/ml in adults with elevated levels 5-fold or higher in children (1, 3). A physiological role for CYP7A-mediated 7α-hydroxylation of 24-hydroxycholesterol is also supported by the results obtained with COS cells, where 24-hydroxycholesterol is efficiently 7α-hydroxylated by living, transfected cells in an environment where large amounts of endogenous cholesterol (34), competing

with 24-hydroxycholesterol for the active site of the enzyme, is present.

The present results exclude that oxysterol 7α-hydroxylase in pig liver is important for 7α-hydroxylation of 24-hydroxycholesterol. The results do not exclude, however, that in addition to cholesterol 7α-hydroxylase there may be another hitherto unrecognized cytochrome *P*-450 capable of catalyzing this reaction. The ratio between the rate of 7α-hydroxylation of cholesterol and the rate of 7α-hydroxylation of 24-hydroxycholesterol appeared to be lower in the crude microsomal fraction of pig liver than in the purified reconstituted cholesterol 7α-hydroxylase fraction. It should be emphasized that in incubations with microsomes the enzyme is exposed to a mixture of endogenous cholesterol and the added cholesterol or 24-hydroxycholesterol. Because of this problem it is difficult to accurately measure the activity in microsomes. It is therefore not possible to draw any conclusions concerning involvement of more than one enzyme in the 7α-hydroxylation of 24-hydroxycholesterol from these experiments. In fact, after submission of the present manuscript, a paper by Li-Hawkins et al. (35) reporting the cloning of a novel 24-hydroxycholesterol 7α-hydroxylase in mouse and human, was published. This enzyme, designated CYP39A1, was reported to be specific for 24-hydroxycholesterol. The results of Li-Hawkins et al. (35) do not contradict the data in the present article. At this stage it is not possible to conclude which hydroxylase plays the major role in 24-hydroxycholesterol 7α-hydroxylation.

The importance of the position of the hydroxyl group for the substrate specificity of oxysterol metabolizing enzymes is notable. An oxygen in the ultimate or penultimate position of the side chain seems to be a prerequisite for catalysis by the oxysterol 7α-hydroxylase responsible for 7α-hydroxylation of 27-hydroxycholesterol and 25-hydroxycholesterol. A hydroxyl group at position 24, which is closer to the steroid nucleus, blocks the reaction. With respect to the CYP7A, a hydroxyl group in the 24-position does not prevent enzymatic catalysis. Presence of an ethyl group in the 24-position of the steroid side chain is known to block or markedly reduce the rate of the reaction, however (36, 37).

The pig liver cholesterol 7α-hydroxylase fraction used showed 7α-hydroxylase activity also toward 27-hydroxycholesterol, which could be due to the presence of some oxysterol 7α-hydroxylase. Another explanation could be that CYP7A is active also toward this oxysterol. In fact, we found that recombinantly expressed CYP7A is able to 7α-hydroxylate 27-hydroxycholesterol (M. Norlin and K. Wikvall, unpublished observation). The 7α-hydroxylation of other oxysterols by CYP7A is currently under investigation.

It is of interest that the rate of 7α-hydroxylation of 24-hydroxycholesterol in crude liver microsomes was almost two orders of magnitude lower than the rate of 7α-hydroxylation of 27-hydroxycholesterol. Preliminary experiments have shown a much longer half-life for (24*S*)-hydroxycholesterol than for 27-hydroxycholesterol in the human circulation (I. Björkhem, S. Meany, D. Lütjohann, A. Sakinis, and Å. Wennmalm, unpublished observation). The long half-life for (24*S*)-hydroxycholesterol may be due to the combination of a relatively low rate of hepatic 7α-hydroxylation and absence of an extrahepatic 7α-hydroxylation. In view of the low rate of 7α-hydroxylation of 24-hydroxycholesterol in microsomes, the possibility should be considered that there is a metabolism of (24*S*)-hydroxycholesterol in the liver prior to the 7α-hydroxylation step. It is also possible that, in addition to 7α-hydroxylation, alternative metabolic conversion of 24-hydroxycholesterol into yet unidentified metabolites may exist in the liver or other tissues. The 7α,24-dihydroxycholesterol formed may be further converted into bile acids by the enzymes involved in bile acid biosynthesis. Studies to investigate this possibility are in progress.

Besides being a means of elimination of (24*S*)-hydroxycholesterol from the body, it is possible that 24-hydroxycholesterol 7α-hydroxylation may play other, yet unknown, physiological roles. In this connection it might be mentioned that 24-hydroxycholesterol has been reported to be a ligand for the LXR receptor (38), which plays a role in the regulation of the metabolism of several important lipids, including cholesterol and bile acids.

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