Regulation of 25- and 27-hydroxylation side chain cleavage pathways for cholic acid biosynthesis in humans, rabbits, and mice: assay of enzyme activities by high-resolution gas chromatography–mass spectrometry

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Abstract **In classic cholic acid biosynthesis, a series of ring modifications of cholesterol precede side chain cleavage and yield 5β-cholestane-3α, 7α, 12α-triol. Side chain reactions of the triol then proceed either by the mitochondrial 27-hydroxylation pathway or by the microsomal 25-hydroxylation pathway. We have developed specific and precise assay methods to measure the activities of key enzymes in both pathways, 5β-cholestane-3α, 7α, 12α-triol 25- and 27-hydroxylases and 5β-cholestane-3α, 7α, 12α, 25-tetrol 23R-, 24R-, 24S- and 27-hydroxylases. The extracts from either the mitochondrial or microsomal incubation mixtures were purified by means of a disposable silica cartridge column, derivatized into trimethylsilyl ethers, and quantified by gas chromatography–mass spectrometry with selected-ion monitoring in a high resolution mode. Compared with the addition of substrates in acetone, those in 2-hydroxypropyl-β-cyclodextrin increased mitochondrial triol 27-hydroxylase activity 132% but decreased activities of the enzymes in microsomal 25-hydroxylation pathway (triol 25-hydroxylase and 5β-cholestane-3α, 7α, 12α, 25-tetrol 23R-, 24R-, 24S- and 27-hydroxylases) 13–60% in human liver. The enzyme activities in both pathways were generally 2- to 4-times higher in mouse and rabbit livers compared with human liver. In all species, microsomal triol 25-hydroxylase activities were 4- to 11-times larger than mitochondrial triol 27-hydroxylase activities but the activities of tetrol 24S-hydroxylase were similar to triol 27-hydroxylase activities in our assay conditions. The regulation of both pathways in rabbit liver was studied after bile acid synthesis was perturbed. Cholesterol feeding up-regulated enzyme activities involved in both 25- (64–142%) and 27- (77%) hydroxylation pathways, while bile drainage up-regulated only the enzymes in the 25-hydroxylation pathway (178–371%). Using these new assays, we demonstrated that the 25- and 27-hydroxylation pathways for cholic acid biosynthesis are more active in mouse and rabbit than human livers and are separately regulated in rabbit liver.**—Honda, A., G. Salen, S. Shefer, Y. Matsuzaki, G. Xu, A. K. Batta, G. S. Tint, and N. Tanaka. **Regulation of 25- and 27-hydroxylation side chain cleavage pathways for cholic acid biosynthesis in humans, rabbits, and mice: assay of enzyme activities by high-resolution gas chromatography–mass spectrometry.** ***J. Lipid Res.*** **2000.** 41: **442–451.**

Supplementary key words bile acids • 5β-cholestane-3α, 7α, 12α-triol 27-hydroxylase • 5β-cholestane-3α, 7α, 12α-triol 25-hydroxylase • 5β-cholestane-3α, 7α, 12α, 25-tetrol 23R-hydroxylase • 5β-cholestane-3α, 7α, 12α, 25-tetrol 24R-hydroxylase • 5β-cholestane-3α, 7α, 12α, 25-tetrol 24S-hydroxylase • 5β-cholestane-3α, 7α, 12α, 25-tetrol 27-hydroxylase

In classic cholic acid biosynthesis, cholesterol is first converted to 7α-hydroxycholesterol by hepatic microsomal cholesterol 7α-hydroxylase, the rate-limiting enzyme in the pathway (1). Hepatic microsomal and cytosolic enzymes further metabolize 7α-hydroxycholesterol to 5β-cholestane-3α, 7α, 12α-triol by a series of ring modifications. The side chain of 5β-cholestane-3α, 7α, 12α-triol is then hydroxylated by either mitochondrial 27-hydroxylase (2, 3) or microsomal 25-hydroxylase (4, 5) (**Fig. 1**). In the 27-hydroxylation pathway, 5β-cholestane-3α, 7α, 12α, 27-tetrol is oxidized to 3α, 7α, 12α-trihydroxy-5β-cholestanoic acid by the same mitochondrial 27-hydroxylase (6) or cytosolic alcohol and aldehyde dehydrogenases (7, 8) and finally converted into cholic acid by cleavage of the terminal three carbons as propionic acid. In the 25-hydroxylation pathway, either C-23R, C-24R, C-24S, or C-27 position of 5β-cholestane-3α, 7α, 12α, 25-tetrol is hydroxylated by microsomal enzymes (9, 10), and the cytosolic fraction exclusively cleaves 5β-cholestane-3α, 7α, 12α, 24S, 25-pentol

Abbreviations: β-CD, 2-hydroxypropyl-β-cyclodextrin; CTX, cerebrotendinous xanthomatosis; DTT, dithiothreitol; GC, gas chromatography; MS, mass spectrometry; SIM, selected-ion monitoring; TLC, thin-layer chromatography; TMS, trimethylsilyl.

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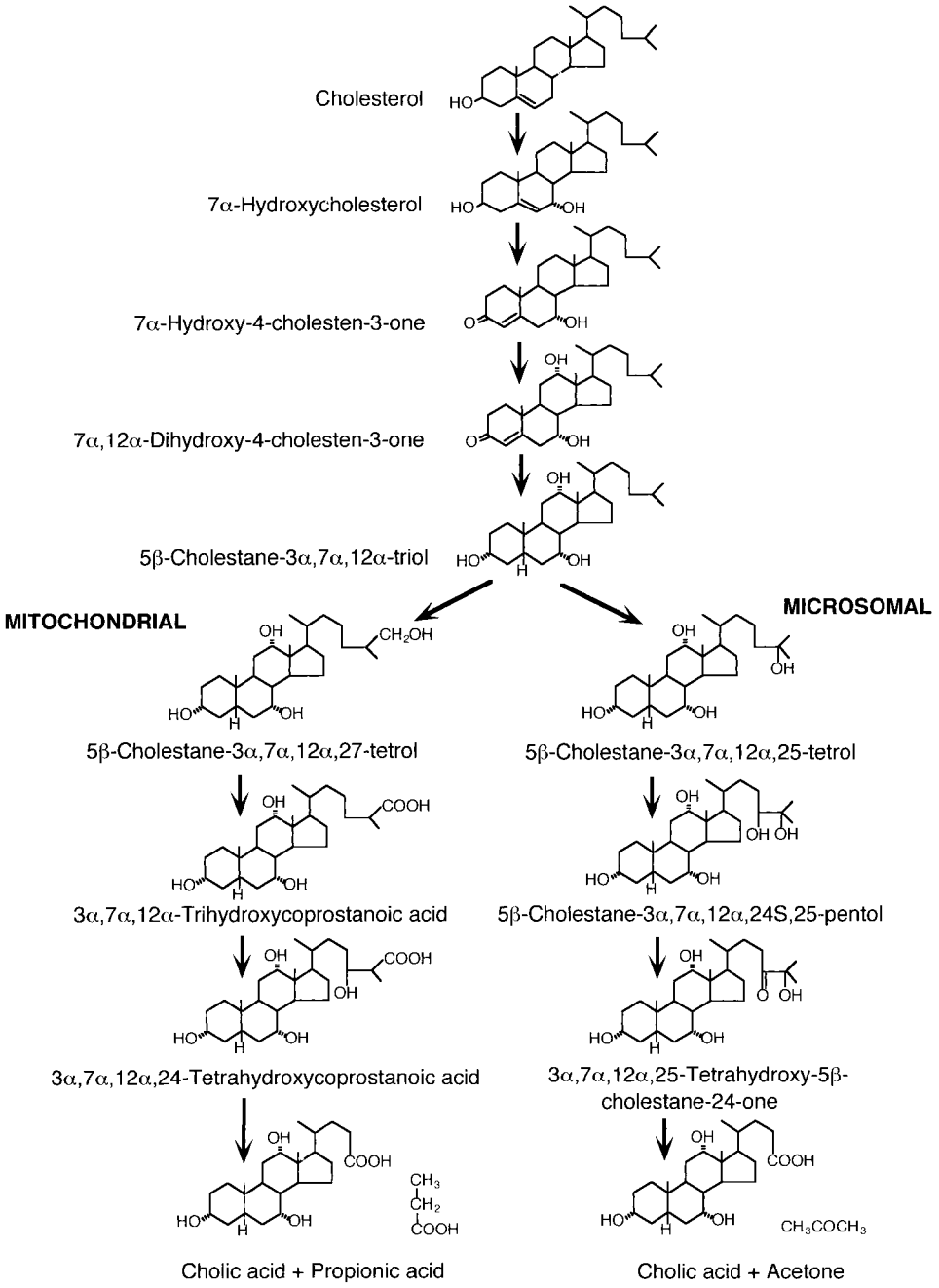

Fig. 1. Cholic acid biosynthesis from cholesterol. In classic cholic acid biosynthetic pathway, a series of ring modifications of cholesterol precedes side chain oxidation to yield 5β-cholestane-3α, 7α, 12α-triol. Side chain cleavage of the triol proceeds either by the mitochondrial 27-hydroxylation pathway or by the microsomal 25-hydroxylation pathway.

to cholic acid by releasing the terminal three carbons as acetone (9, 11).

Although there is no direct evidence, it has been presumed that the mitochondrial 27-hydroxylation pathway is the major route for side chain cleavage in cholic acid biosynthesis in normal human (12) and rat (13). However, little is known about the regulation of the alternative microsomal 25-hydroxylation side chain cleavage pathway when bile acid synthesis is perturbed. A rare, recessive lipid storage disease, cerebrotendinous xanthomatosis (CTX), has genetically defective mitochondrial sterol 27-hydroxylase (CYP27) (14). The patients show markedly reduced production of chenodeoxycholic acid whereas cholic acid synthesis is almost normal (15). In addition, large amounts of 25-hydroxylated C_{27}-bile alcohols are excreted in bile and urine of CTX subjects (16, 17). These data suggest that in CTX virtually all cholic acid is synthesized from 5β-cholestane-3α, 7α, 12α-triol via the microsomal 25-hydroxylation pathway. However, a recent report by Rosen et al. (18) showed that mice with disrupted ste-

rol 27-hydroxylase gene (knockout model) had markedly reduced cholic acid and chenodeoxycholic acid production without accumulation of bile alcohols. They speculated that the mice could not compensate for the lack of the sterol 27-hydroxylase through the alternative 25-hydroxylation pathway because microsomal 25-hydroxylase activity was not active in mice.

We now report new precise assays for several key enzyme activities in the microsomal 25- and mitochondrial 27-hydroxylation pathways based on high-resolution gas chromatography–mass spectrometry (GC–MS). Two advantages are that the method does not require radiolabeled substrates and bile alcohol reaction products can be easily purified from the crude lipid extract of the incubation mixture by disposable silica cartridge column. By using the new assay methods, the enzyme activities were compared among human, mouse, and rabbit. In addition, the effects of cholesterol feeding and depletion of the bile acid pool on the enzyme activities were studied in rabbit liver. The results did not support the idea that the microsomal 25-hydroxylation pathway in mice was much less active than in human. Further, rabbits fed cholesterol up-regulated both 25- and 27-hydroxylation cleavage pathways, while bile drainage eliminated the bile acid pool and up-regulated only the 25-hydroxylation pathway.

MATERIALS AND METHODS

Chemicals

Sitosterol (24-ethyl-5-cholesten-3β-ol) was purchased from Sigma Chemical Co. (St. Louis, MO) and purified by recrystallization. 5β-Cholestane-3α, 7α, 12α-triol was prepared by electrolytic coupling of cholic acid with isovaleric acid according to Bergström and Krabisch (19). 5β-Cholestane-3α, 7α, 12α, 25-tetrol was synthesized from cholic acid by the method of Dayal et al. (20). 5β-Cholestane-3α, 7α, 12α, 27-tetrol was prepared by lithium aluminum hydride reduction of the methyl ester of 3α,7α,12α-trihydroxy-5β-cholestan-27-oic acid which was isolated from the bile of *Alligator mississippiensis*. 5β-Cholestane-3α, 7α, 12α, 23R, 25-pentol was isolated from bile and feces of patients with CTX (21). 5β-Cholestane-3α, 7α, 12α, 24R, 25-pentol and 5β-cholestane-3α, 7α, 12α, 24S, 25-pentol were prepared from 5β-cholestane-3α, 7α, 12α, 25-tetrol by the method of Hoshita (22). 5β-Cholestane-3α, 7α, 12α, 25, 27-pentol was a gift from Dr. T. Hoshita (Pharmaceutical Institute, Hiroshima University, Hiroshima, Japan). 2-Hydroxypropyl-β-cyclodextrin (β-CD) and Tween-80 were purchased from Pharmatec Inc. (Alachua, FL) and Fisher Scientific Co. (Springfield, NJ), respectively.

Preparation of liver specimens

Human liver specimens became available from five healthy people who died unexpectedly when no suitable recipient for liver transplantation could be found (University of Minnesota Hospital NIH contract No. 1-DK-62274). Five mice, 3 months of age, were fed a normal chow diet and were killed and livers were removed at the end of the 12-h dark phase of the cycle. In rabbit experiments, 14 New Zealand white strain rabbits weighing 2.5–3.2 kg were purchased from Hazleton Laboratories (Denver, PA). The control rabbits (n = 5) were fed normal rabbit chow for 10 days. Some rabbits (n = 5) were fed rabbit chow containing 2% cholesterol in the diet (about 3,000 mg/day) for 10 days. For other rabbits (n = 4), bile fistulas were constructed under anesthesia (ketamine 35–40 mg/kg body weight combined with xylazine 3–4 mg/kg body weight intramuscularly). During the period of bile drainage (for 7 days), 0.9% NaCl was administered intravenously, and chow and water were provided ad libitum. The rabbits were killed at the end of the 12-h dark phase of the cycle, and livers were collected. The animal protocols were approved by Subcommittee on Animal Studies at the VA Medical Center (East Orange, NJ) and Institutional Animal Care and Use Committee at the University of Medicine and Dentistry of New Jersey-New Jersey Medical School (Newark, NJ). All liver tissues were stored at −70°C until used.

Preparation of liver microsomes and mitochondria

The liver specimen was weighed, minced, and homogenized with a loose-fitting Teflon pestle in 4 volumes of 3 mm Tris-HCl buffer (pH 7.4) containing 0.25 m sucrose, and 0.1 mm EDTA. The homogenate was centrifuged at 700 *g* for 10 min, and the supernatant was centrifuged at 7,000 *g* for 20 min. The 7,000 *g* pellet (mitochondrial fraction) was washed twice with homogenizing medium and suspended in 100 mm potassium phosphate buffer (pH 7.4) containing 1 mm EDTA, 5 mm DTT, 50 mm KCl, and 20% glycerol (v/v) (storage buffer). The 7,000 *g* supernatant was then centrifuged at 105,000 *g* for 90 min and the pellet (microsomal fraction) was suspended in storage buffer. Protein concentrations of the mitochondrial and microsomal fractions were determined by the method of Lowry et al. (23).

Assay of mitochondrial 5β-cholestane-3α, 7α, 12α-triol 27-hydroxylase activity

The composition of the standard reaction mixture (final volume 0.5 ml) was 100 mm potassium phosphate buffer (pH 7.4) containing 0.1 mm EDTA, 50–400 μg of mitochondrial protein, and 50 nmol of 5β-cholestane-3α, 7α, 12α-triol (final concentration 100 μm) dissolved in 12 μl of a 33% aqueous solution of β-CD. The reaction was initiated by adding a mixture of NADPH (final concentration 1.2 mm), isocitrate (final concentration 5 mm) and 0.2 unit of isocitrate dehydrogenase, and continued for 20 min at 37°C. The reaction was stopped with 2 ml of ethyl acetate. After addition of 1 μg of 5β-cholestane-3α, 7α, 12α, 25-tetrol as an internal recovery standard, bile alcohols were extracted twice with 2 ml of ethyl acetate and the extracts were evaporated to dryness under nitrogen. The residue was dissolved in 100 μl of chloroform–acetone–methanol 35:25:2 (v/v/v), and applied to a Bond Elut SI (unbonded silica, 500 mg) cartridge (Varian, Harbor City, CA) which was prewashed with 3 ml of the same solvent. After washing out sterols with 5 ml of the same solvent, bile alcohols were eluted with 3 ml of chloroform–acetone–methanol 35:25:20 (v/v/v). The eluate was evaporated to dryness and bile alcohols were converted into trimethylsilyl (TMS) ether derivatives by addition of 100 μl of TMSI-H (GL Sciences Inc., Tokyo, Japan) and incubation at 55°C for 15 min. High-resolution gas chromatography–mass spectrometry (GC-MS) with selected-ion monitoring (SIM) was performed using a JMS-SX102 instrument equipped with a data-processing system JMA DA-6000 (JEOL, Tokyo, Japan). The accelerating voltage was 10 kV, the ionization energy 70 eV, the trap current 300 μA, and the mass spectral resolution about 10,000. An Ultra Performance capillary column (25 m × 0.32 mm ID) coated with methylsilicone (Hewlett-Packard, Palo Alto, CA) was used with a flow-rate of helium carrier gas of 1.0 ml/min. The column oven was programmed to change from 100 to 265°C at 30°C/min, after a 1-min delay from the start time.

Assay of 5β-cholestane-3α, 7α, 12α-triol 25-hydroxylase activity

The reaction mixture (final volume 0.5 ml) consisted of 100 mm potassium phosphate buffer (pH 7.4) containing 0.1 mm EDTA, 50–400 μg of microsomal protein, and 25 nmol of 5β-

cholestane-3α, 7α, 12α-triol (final concentration 50 μm) dissolved in 10 μl of acetone. The incubation was started by the addition of a mixture of NADPH (final concentration 1.2 mm), glucose-6-phosphate (final concentration 3.6 mm), and 2 units of glucose-6-phosphate dehydrogenase, and continued for 20 min at 37°C. The reaction was stopped by adding 2 ml of ethyl acetate. After addition of 1 μg of 5β-cholestane-3α, 7α, 12α, 27-tetrol as an internal recovery standard, bile alcohols were extracted twice with 2 ml of ethyl acetate, purified by a Bond Elut SI cartridge, derivatized to TMS ether, and quantified by the same procedure as the triol 27-hydroxylase assay described above.

Assay of 5β-cholestane-3α, 7α, 12α, 25-tetrol 23R, 24R, 24S and 27-hydroxylase activities

The reaction mixture (final volume 0.5 ml) consisted of 100 mm potassium phosphate buffer (pH 7.4) containing 0.1 mm EDTA, 100–400 μg of microsomal protein, and 100 nmol of 5β-cholestane-3α, 7α, 12α, 25-tetrol (final concentration 200 μm) dissolved in 10 μl of 0.75% (w/v) Tween-80 solution. The incubation was started by the addition of a mixture of NADPH (final concentration 1.2 mm), glucose-6-phosphate (final concentration 3.6 mm), and 2 units of glucose-6-phosphate dehydrogenase, and continued for 20 min at 37°C. The reaction was stopped by adding 2 ml of ethyl acetate. After addition of 1 μg of 5β-cholestane-3α, 7α, 12α, 27-tetrol as an internal recovery standard, bile alcohols were extracted twice with 2 ml of ethyl acetate, purified by a Bond Elut SI cartridge, and derivatized to TMS ether by the same procedure as triol 27- and 25-hydroxylase assays described above. The column oven was programmed to change from 100 to 265°C at 30°C/min, after a 1-min delay from the start time.

Statistics

Data are reported here as the mean ± SEM. The statistical significance of differences between the results in the different groups was evaluated by the Student's two-tailed *t*-test and significance was accepted at the level of $P < 0.05$.

RESULTS

The recoveries of 5β-cholestane-3α, 7α, 12α, 25-tetrol, 5β-cholestane-3α, 7α, 12α, 27-tetrol, 5β-cholestane-3α, 7α, 12α, 24R, 25-pentol, and 5β-cholestane-3α, 7α, 12α, 24S, 25-pentol from the Bond Elut SI cartridge were checked by adding a mixture of 100 ng each of the bile alcohol, 5β-cholestane-3α, 7α, 12α triol and cholesterol to the column. The bile alcohols and sterols in the eluate were quantified by GC/SIM using sitosterol as an internal standard. The recoveries of 5β-cholestane-3α, 7α, 12α, 25-tetrol, 5β-cholestane-3α, 7α, 12α, 27-tetrol, 5β-cholestane-3α, 7α, 12α, 24R, 25-pentol, and 5β-cholestane-3α, 7α, 12α, 24S, 25-pentol were 104.2 ± 3.5% (n = 4), 98.8 ± 3.8% (n = 4), 99.0 ± 3.3% (n = 4), and 100.2 ± 2.5% (n = 4), respectively, while more than 99.9% of 5β-cholestane-3α, 7α, 12α triol and cholesterol (n = 4) were eliminated by the purification procedure.

TMS ether derivatives of bile alcohols were well separated by capillary gas chromatography. Retention times were: 5β-cholestane-3α, 7α, 12α, 25-tetrol (13.7 min), 5β-cholestane-3α, 7α, 12α, 27-tetrol (14.5 min), 5β-cholestane-3α, 7α, 12α, 23R, 25-pentol (16.4 min), 5β-cholestane-3α, 7α, 12α, 24R, 25-pentol (17.0 min), 5β-cholestane-3α, 7α, 12α, 24S, 25-pentol (17.2 min), and 5β-cholestane-3α, 7α, 12α, 25, 27-pentol (18.4 min). The major frag-

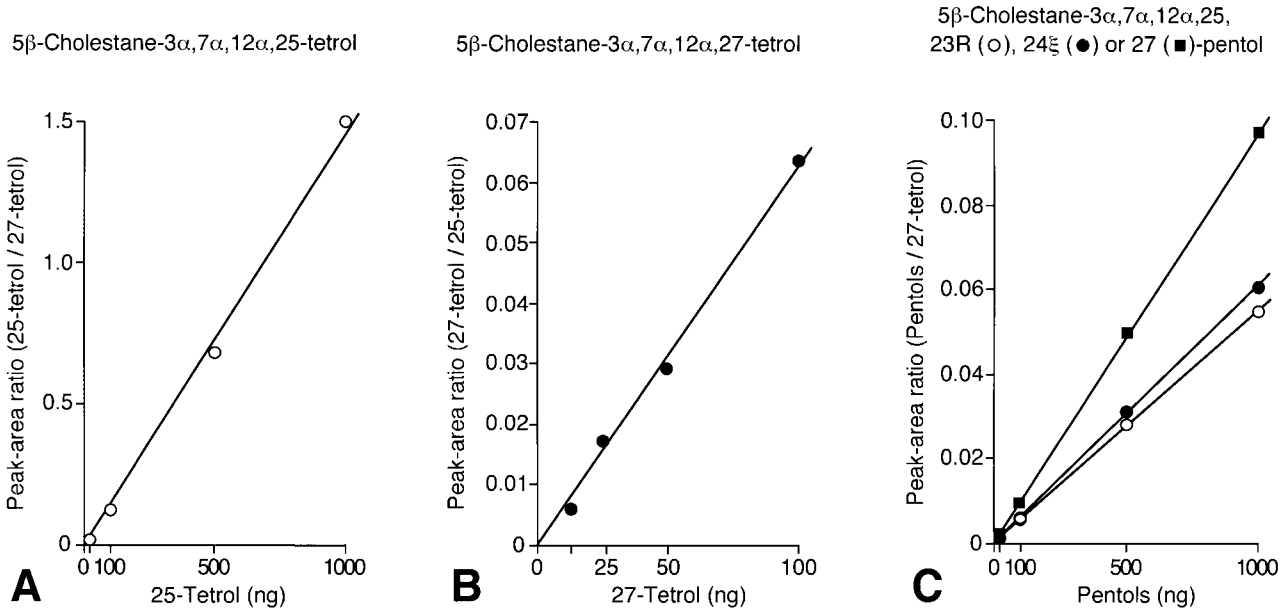

Fig. 2. Calibration curves for formation of (A) 5β-cholestane-3α, 7α, 12α, 25-tetrol, (B) 5β-cholestane-3α, 7α, 12α, 27-tetrol, and (C) 5β-cholestanepentols. Varying amounts of the bile alcohols were mixed with 1 μg of 5β-cholestane-3α, 7α, 12α, 25-tetrol (for B) or 5β-cholestane-3α, 7α, 12α, 27-tetrol (for A and C) as an internal recovery standard, converted into TMS derivatives and determined by high-resolution GC–SIM. The linearities were checked by simple linear regression, and the lines satisfied the relationships x = 663.44y + 22.03 (n = 4; r = 0.998; $P < 0.005$) for 5β-cholestane-3α, 7α, 12α, 25-tetrol; x = 1507.46y + 4.04 (n = 4; r = 0.996; $P < 0.005$) for 5β-cholestane-3α, 7α, 12α, 27-tetrol, x = 18289.32y − 4.22 (n = 4; r = 1.000; $P < 0.0001$) for 5β-cholestane-3α, 7α, 12α, 23R, 25-pentol; x = 16503.95y − 3.89 (n = 4; r = 1.000; $P < 0.0001$) for 5β-cholestane-3α, 7α, 12α, 24ξ, 25-pentol; and x = 10314.59y − 4.49 (n = 4; r = 1.000; $P < 0.0001$) for 5β-cholestane-3α, 7α, 12α, 25, 27-pentol.

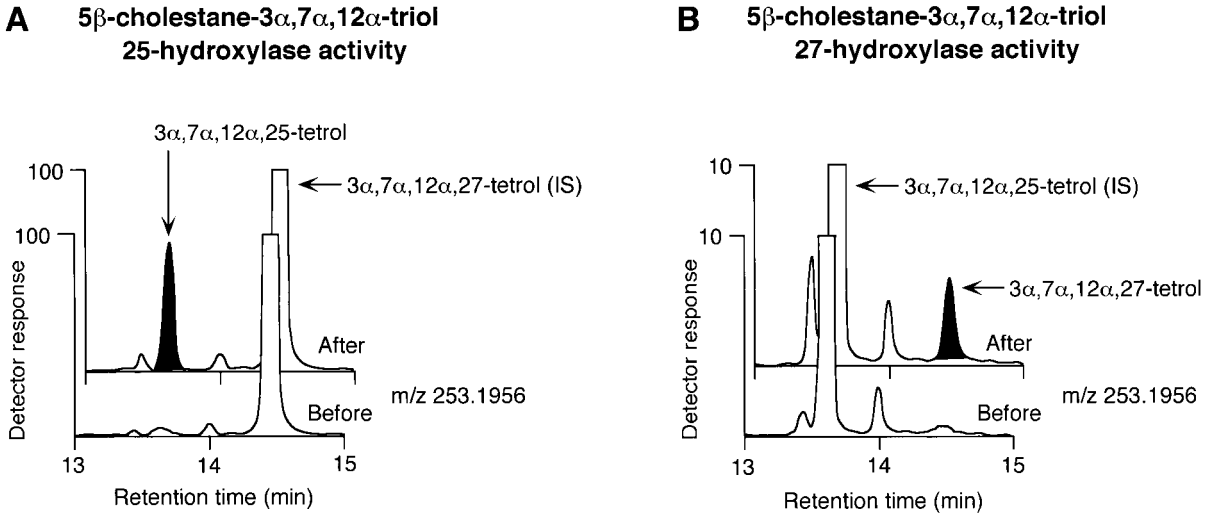

Fig. 3. Representative high-resolution GC–SIM chromatograms of standard assay mixture for (A) microsomal 5β-cholestane-3α, 7α, 12α-triol 25-hydroxylase and (B) mitochondrial 5β-cholestane-3α, 7α, 12α-triol 27-hydroxylase in control human liver. Before, without incubation (0 time); After, after 20 min incubation; IS, internal standard.

ments of bile alcohols generated by GC-MS in the electron ionization (EI) mode are described previously (24). The calibration curves for the bile alcohols were established by focusing the multiple ion detector on *m/z* 253.1956 for 5β-cholestane-3α, 7α, 12α, 25-tetrol and 5β-cholestane-3α, 7α, 12α, 27-tetrol, *m/z* 307.2425 for 5β-cholestane-3α, 7α, 12α, 23R, 25-pentol, *m/z* 321.2582 for 5β-cholestane-3α, 7α, 12α, 24R, 25-pentol and 5β-cholestane-3α, 7α, 12α, 24S, 25-pentol, and *m/z* 349.2895 for 5β-cholestane-3α, 7α, 12α, 25, 27-pentol (**Fig. 2**). Good linearities were obtained at least up to 100 ng for 5β-cholestane-3α, 7α, 12α, 27-tetrol and up to 1000 ng for the other bile alcohols. **Figure 3** and **Fig. 4** show representative chromatograms obtained by the analysis of standard incubation mixture from a control human liver at zero time (before) and at 20 min (after). The peaks of 5β-cholestane-3α, 7α, 12α, 25-tetrol (Fig. 3A) and 5β-cholestane-3α, 7α, 12α, 27-tetrol (Fig. 3B) at 20 min incubation corresponded to about 3.9 ng (9.0 pmol) and 220 pg (0.5 pmol), respectively. 5β-Cholestane-3α, 7α, 12α, 23R, 25-pentol, 5β-cholestane-3α, 7α, 12α, 24R, 25-pentol, 5β-cholestane-3α, 7α, 12α, 24S, 25-pentol, and 5β-cholestane-3α, 7α, 12α, 25, 27-pentol peaks shown after incubation (Fig. 4) corresponded to about 9.0 ng (20 pmol), 448 pg (0.96 pmol), 536 pg (1.2 pmol), and 240 pg (0.53 pmol), respectively.

Optimal conditions for the determination of the enzyme activities were established by using a control human liver. The enzymatic reactions were linear with up to 400 μg of either microsomal or mitochondrial proteins (**Fig. 5A** and **Fig. 6A**) and with time up to 20 min (Figs. 5B and 6B). The effects of 5β-cholestane-3α, 7α, 12α-triol concentration on the activities of microsomal triol 25-hydroxylase and mitochondrial triol 27-hydroxylase are shown in Fig. 5C. The highest 25-hydroxylase activity was observed when 50 μm of the substrate was added to the incubation mixture while optimal substrate concentration for 27-hydroxylase

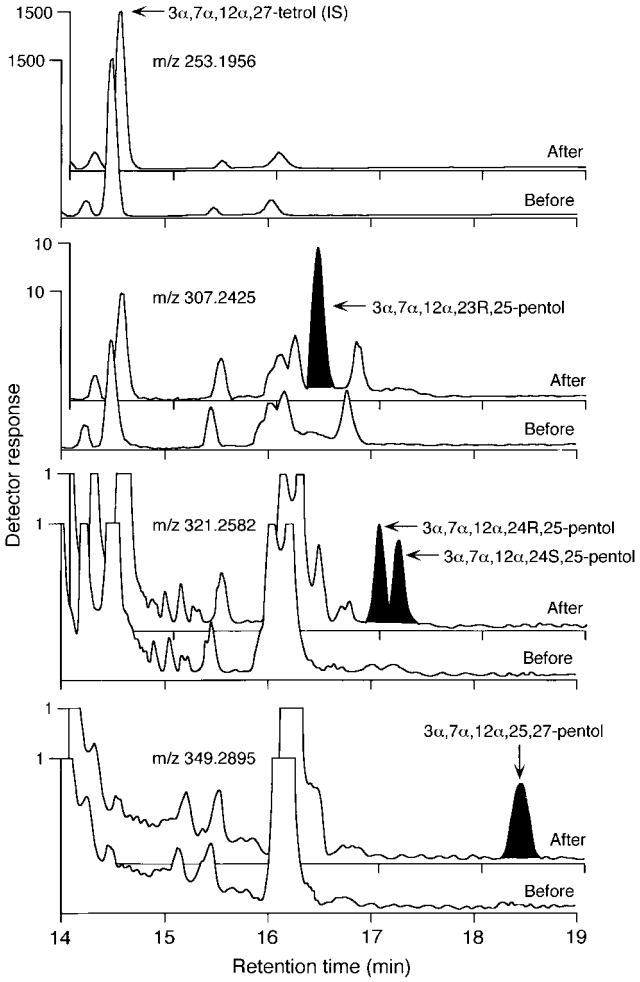

Fig. 4. Representative high-resolution GC–SIM chromatograms of standard assay mixture for microsomal 5β-cholestane-3α, 7α, 12α, 25-tetrol 23R, 24R, 24S, and 27-hydroxylases in control human liver. Before, without incubation (0 time); After, after 20 min incubation; IS, internal standard.

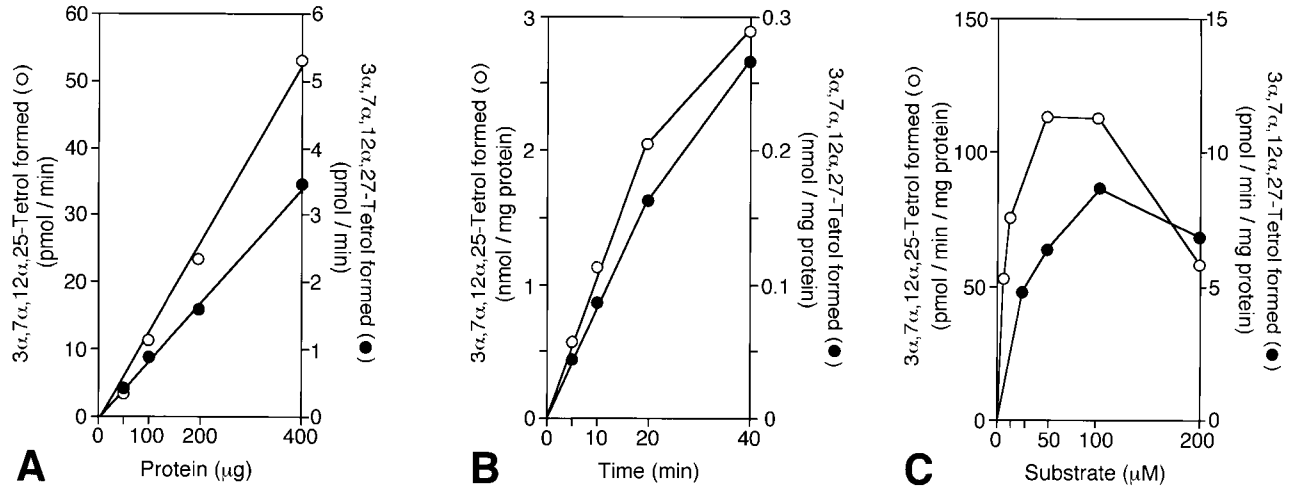

Fig. 5. Determination of optimal conditions for assay of microsomal 5β-cholestane-3α, 7α, 12α-triol 25-hydroxylase and mitochondrial 5β-cholestane-3α, 7α, 12α-triol 27-hydroxylase in human liver. Microsomal and mitochondrial fractions from control human liver were prepared and assayed as described under Materials and Methods with the following changes: (A) amount of microsomal or mitochondrial protein was varied; (B) time of incubation was varied; (C) concentration of 5β-cholestane-3α, 7α, 12α-triol was varied.

was found at 100 μm. Figure 6C shows the effects of 5β-cholestane-3α, 7α, 12α, 25-tetrol concentration on the activities of microsomal tetrol 23R, 24R, 24S, and 27-hydroxylases. Increasing enzyme activities were observed up to 200 μm of substrate for the tetrol hydroxylases.

Table 1 shows the effects of acetone, β-CD, and Tween-80 as substrate vehicles on enzyme activities in human liver. Compared with the addition of substrates dissolved in acetone, those in β-CD increased mitochondrial 5β-cholestane-3α, 7α, 12α-triol 27-hydroxylase activity 132% but decreased activities of the enzymes in microsomal 25-hydroxylation pathway 13–60%. Tween-80 raised 5β-cholestane-3α, 7α, 12α-triol 27-hydroxylase and 5β-cholestane-3α, 7α, 12α, 25-tetrol 23R-hydroxylase activities 55% and 96%, respectively, but reduced 5β-cholestane-3α, 7α, 12α-triol 25-hydroxylase activity 47%.

Table 2 compares the enzyme activities in human, mouse, and rabbit livers. In mice and rabbits, enzyme activities involved in both mitochondrial 27-hydroxylation and microsomal 25-hydroxylation pathways were generally 2- to 4-times higher than those in humans. In all species, microsomal 5β-cholestane-3α, 7α, 12α-triol 25-hydroxylase activities were 4- to 11-times greater than mitochondrial 5β-cholestane-3α, 7α, 12α-triol 27-hydroxylase activities. In human and rabbit, the activities of microsomal 5β-cholestane-3α, 7α, 12α, 25-tetrol 24S-hydroxylase were not significantly different from mitochondrial triol 27-hydroxylase. However, tetrol 24S-hydroxylase activity was

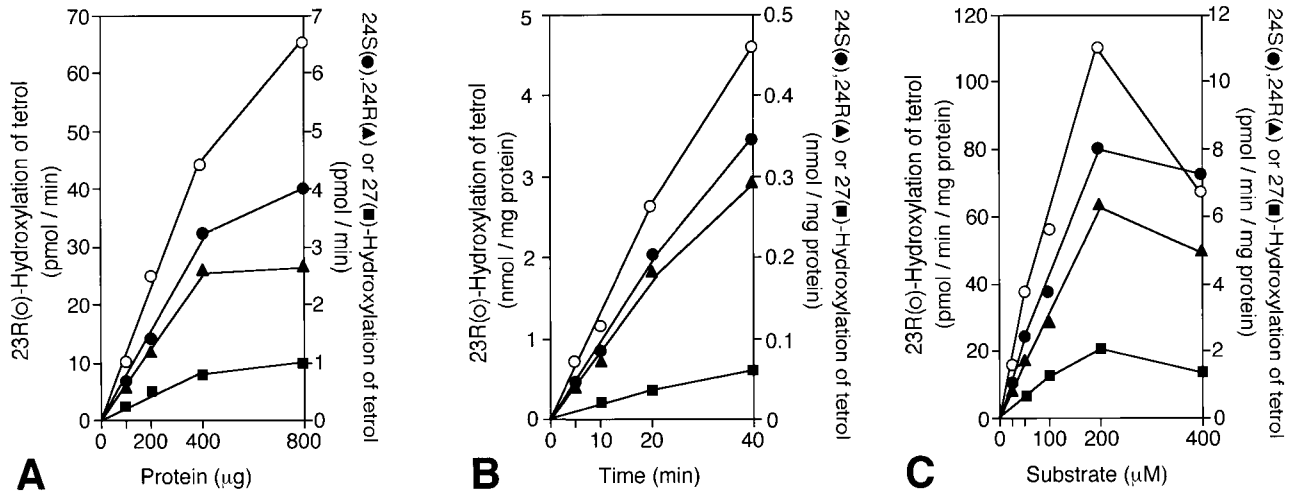

Fig. 6. Determination of optimal conditions for assay of microsomal 5β-cholestane-3α, 7α, 12α, 25-tetrol 23R, 24R, 24S, and 27-hydroxylases in human liver. Microsomal fraction from control human liver was prepared and assayed as described under Materials and Methods with the following changes: (A) amount of microsomal protein was varied; (B) time of incubation was varied; (C) concentration of 5β-cholestane-3α, 7α, 12α, 25-tetrol was varied.

TABLE 1. Effects of acetone, β-cyclodextrin, and Tween-80 on 5β-cholestane-3α,7α,12α-triol and 5β-cholestane-3α,7α,12α,25-tetrol hydroxylase activities in human liver

Enzyme	Effects of Substrate Vehicles on Enzyme Activities		
	Acetone	β-Cyclodextrin	Tween-80
	pmol/min/mg protein		
5β-Cholestane-3α,7α,12α-triol			
25-Hydroxylase	112 (100%)	97 (87%)	59 (53%)
27-Hydroxylase	3.7 (100%)	8.6 (232%)	5.7 (155%)
5β-Cholestane-3α,7α,12α,25-tetrol			
23R-Hydroxylase	75 (100%)	47 (63%)	147 (196%)
24R-Hydroxylase	16 (100%)	7.4 (46%)	15 (94%)
24S-Hydroxylase	18 (100%)	10 (56%)	18 (100%)
27-Hydroxylase	5.5 (100%)	2.2 (40%)	5.0 (91%)

The final concentrations of acetone, β-cyclodextrin, and Tween-80 in incubation mixtures were 2% (v/v), 0.8% (w/v), and 0.015% (w/v), respectively.

significantly lower than triol 27-hydroxylase activity in mouse ($P < 0.05$).

The effects of cholesterol feeding and bile fistula on the hepatic enzyme activities were studied in rabbits. As shown in **Table 3**, cholesterol feeding significantly up-regulated 5β-cholestane-3α, 7α, 12α-triol 25- (69%) and 27-hydroxylase activities (77%). In contrast, bile fistula significantly up-regulated microsomal 25-hydroxylase activity (178%) but significant up-regulation was not observed in mitochondrial 27-hydroxylase activity. Microsomal 5β-cholestane-3α, 7α, 12α, 25-tetrol 23R, 24R, 24S and 27-hydroxylase activities were coordinately up-regulated with microsomal 25-hydroxylase activity by cholesterol (64–142%) and bile fistula treatments (188–371%).

DISCUSSION

The activities of 5β-cholestane-3α, 7α, 12α-triol 27-hydroxylase (2, 3), 25-hydroxylase (11, 25), and 5β-cholestane-3α, 7α, 12α, 25-tetrol 23R, 24R, 24S and 27-hydroxylases (10) have generally been assayed by isotope incorporation methods using radioactive substrates. Formed bile alcohol products in the incubation mixtures were separated by thin-layer chromatography (TLC) and radioactivities were measured. The methods are simple but the separation of each radioactive bile alcohol by TLC was not always precise because hydroxy groups can be introduced in a number of different positions in the side chain of 5β-cholestane-3α, 7α, 12α-triol, such that a mixture of C-23R, C-24R, C-24S, C-25, C-26 and C-27 tetrols can be formed (1). To overcome the above disadvantage, Oftebro et al. (26) developed a GC-SIM method for assaying 5β-cholestane-3α, 7α, 12α-triol 25- and 27-hydroxylase activities. Although the method detected the masses of 5β-cholestane-3α, 7α, 12α, 25-tetrol and 5β-cholestane-3α, 7α, 12α, 27-tetrol, which are usually more reliable than radioactivities, their separation by low-resolution GC-SIM was not sufficient for accurate quantification. Theoretically, the present high-resolution GC-SIM method is more specific and reliable than an ordinary low-resolution GC-SIM. In addition, capillary GC column conditions completely resolved 5β-cholestane-3α, 7α, 12α, 25- and 27-tetrols, and the separation of 5β-cholestanepentols was also complete enough for quantification.

TABLE 2. Activities of 5β-cholestane-3α,7α,12α-triol and 5β-cholestane-3α,7α,12α,25-tetrol hydroxylases in human, mouse, and rabbit liver

Enzyme	Human (n = 5)	Mouse (n = 5)	Rabbit (n = 5)
	pmol/min/mg protein		
5β-Cholestane-3α,7α,12α-triol			
25-Hydroxylase	34 ± 10	144 ± 18[a]	144 ± 22[b]
27-Hydroxylase	7.8 ± 1.8	16 ± 1[b]	13 ± 1[a]
5β-Cholestane-3α,7α,12α, 25-tetrol			
23R-Hydroxylase	22 ± 12	196 ± 31[a]	51 ± 11
24R-Hydroxylase	3.2 ± 1.3	119 ± 15[a]	45 ± 9[b]
24S-Hydroxylase	4.3 ± 1.6	10 ± 2[c]	10 ± 2
27-Hydroxylase	1.6 ± 0.4	1.4 ± 0.2	2.5 ± 0.1

[a] $P < 0.001$ vs. human.
[b] $P < 0.01$ vs. human.
[c] $P < 0.05$ vs. human.

TABLE 3. Effects of cholesterol feeding and bile fistula on 5β-cholestane-3α,7α,12α-triol and 5β-cholestane-3α,7α, 12α,25-tetrol hydroxylase activities in rabbit liver

Enzyme	Control (n = 5)	Cholesterol[a] (n = 5)	Bile Fistula[a] (n = 4)
	pmol/min/mg protein		
5β-Cholestane-3α,7α,12α-triol			
25-Hydroxylase	144 ± 22	243 ± 29[b]	400 ± 55[d,e]
27-Hydroxylase	13 ± 1	23 ± 3[b]	17 ± 4
5β-Cholestane-3α,7α, 12α,25-tetrol			
23R-Hydroxylase	51 ± 11	95 ± 13[b]	179 ± 42[b]
24R-Hydroxylase	45 ± 9	109 ± 14[d]	212 ± 38[d,e]
24S-Hydroxylase	10 ± 2	24 ± 3[c]	46 ± 8[d,e]
27-Hydroxylase	2.5 ± 0.1	4.1 ± 0.5[b]	7.2 ± 0.8[d,e]

[a] Treatment with 2% cholesterol in chow for 10 days or bile fistula for 7 days.
[b] $P < 0.05$ vs. control.
[c] $P < 0.01$ vs. control.
[d] $P < 0.005$ vs. control.
[e] $P < 0.05$ vs. cholesterol.

As convenient internal recovery standards, we used 1 μg of 5β-cholestane-3α, 7α, 12α, 25-tetrol for quantification of mitochondrial 5β-cholestane-3α, 7α, 12α, 27-tetrol and 1 μg of 5β-cholestane-3α, 7α, 12α, 27-tetrol for microsomal 5β-cholestane-3α, 7α, 12α, 25-tetrol and 5β-cholestanepentols. The purification of the bile alcohols from the lipid extract was facilitated by a disposable silica cartridge column. The 5β-cholestanetetrols and 5β-cholestanepentols were almost completely recovered while more than 99.9% of 5β-cholestane-3α, 7α, 12α triol and cholesterol were eliminated. Isolated microsomal and mitochondrial fractions contained small amounts of endogenous 5β-cholestane-3α, 7α, 12α, 27-tetrol and 5β-cholestane-3α, 7α, 12α, 25-tetrol, respectively. However, these quantities were less than 1% of the added internal standards.

β-CD is a cyclic oligosaccharide of seven glucopyranose units and has been used to deliver hydrophobic drugs to target sites both in vitro and in vivo (27, 28). It was reported that the addition of cholesterol dissolved in β-CD markedly raised cholesterol 27-hydroxylase activity in rat liver mitochondria compared with that in acetone (29). Our results showed that β-CD also increased mitochondrial 5β-cholestane-3α, 7α, 12α triol 27-hydroxylase activity, but diminished microsomal 5β-cholestane-3α, 7α, 12α triol 25-hydroxylase and 5β-cholestane-3α, 7α, 12α, 25-tetrol 23R, 24R, 24S and 27-hydroxylase activities in human liver (Table 1).

In this report, we systematically studied the regulations of 5β-cholestane-3α, 7α, 12α-triol 25- and 27-hydroxylase and 5β-cholestane-3α, 7α, 12α, 25-tetrol 23R, 24R, 24S and 27-hydroxylase activities in rabbit liver. Hydroxylations of C-27 position of cholesterol and 5β-cholestane-3α, 7α, 12α-triol are believed to be catalyzed by the same mitochondrial enzyme, sterol 27-hydroxylase (CYP27) (3). It was reported that cholic acid or cholestyramine feeding did not affect 27-hydroxylation of 5β-cholestane-3α, 7α, 12α-triol in rabbits (30). **Figure 7** shows the effects of cholesterol feeding and bile drainage on cholesterol 27-hydroxylase and 7α-hydroxylase activities in rabbit liver, and which were reported previously by Xu et al. (31–34). The results clearly demonstrated that bile drainage had no effect while cholesterol feeding stimulated 27-hydroxylation of cholesterol in rabbits. The present results again showed that 5β-cholestane-3α, 7α, 12α-triol 27-hydroxylase activities were not increased by bile drainage but were significantly up-regulated (+77%) by cholesterol feeding (Table 3). The results and the effect of β-CD on both 27-hydroxylations support the contention that there is a single 27-hydroxylase enzyme (sterol 27-hydroxylase) that catalyzes C-27 hydroxylation of both cholesterol and 5β-cholestane-3α, 7α, 12α-triol.

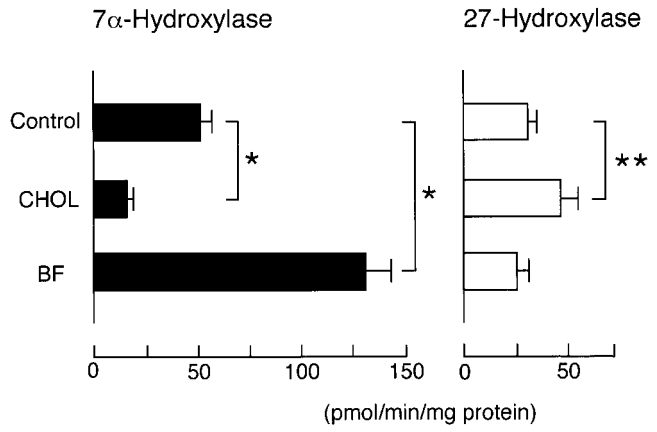

Fig. 7. Effects of cholesterol feeding and bile fistula on cholesterol 7α-hydroxylase and cholesterol 27-hydroxylase activities in rabbit liver. Data are shown as mean and SEM. Control, chow fed for 10 days (n = 5). CHOL, 2% cholesterol in chow fed for 10 days (n = 5). BF, chow-fed rabbits with bile fistula for 7 days (n = 5). * $P < 0.001$, ** $P < 0.05$. These results were reported previously by Xu et al. (31–34).

In contrast to C-27-hydroxylation, 5β-cholestane-3α, 7α, 12α-triol 25-hydroxylase seems to be a different enzyme from cholesterol 25-hydroxylase. Cholesterol 25-hydroxylase cDNAs were recently cloned by Lund et al. (35). Unlike most other sterol hydroxylases, cholesterol 25-hydroxylase was not a cytochrome P450, while 5β-cholestane-3α, 7α, 12α-triol 25-hydroxylase is believed to be cytochrome P450 (CYP3A4) (36). To the best of our knowledge, there is no report about enzyme activities in the side chain 25-hydroxylation pathway in rabbits, and data on side chain hydroxylation activities in other mammals are also limited. In rats, 25-hydroxylation of 5β-cholestane-3α, 7α, 12α-triol was not affected by cholestyramine, bile fistula, or starvation (37). In addition, Ahlberg et al. (25) reported that the enzyme activity was normal in hyperlipidemic humans and was unaffected by chenodeoxycholic acid treatment in patients with cholesterol gallstones (38). Unlike these previous reports, our results showed that 25-hydroxylase activity was significantly up-regulated by bile drainage (bile acid depletion) and cholesterol feeding in rabbits. It is not clear whether the inconsistency is due to different species or methods used. Regulation of 5β-cholestane-3α, 7α, 12α, 25-tetrol 23R, 24R, 24S and 27-hydroxylase activities were studied in rats by Cheng et al. (10). Phenobarbital administration stimulated the formation of all four pentols studied while fasting had no significant effect on these hydroxylations. The present data showed that 23R, 24R, 24S, and 27-hydroxylations of 5β-cholestane-3α, 7α, 12α, 25-tetrol were also coordinately up-regulated with 25-hydroxylase by both bile acid depletion and cholesterol feeding in rabbits.

It is well established that cholesterol 7α-hydroxylase activity is up-regulated by bile drainage (32, 33) while the activity is down-regulated by expansion of the bile acid pool during cholesterol feeding in rabbits (32, 34) (Fig. 7). Therefore, in rabbits, depletion of the bile acid pool by bile fistula drainage up-regulates the microsomal cholesterol 7α-hydroxylase and 25-hydroxylation pathways (5β-cholestane-3α, 7α, 12α-triol 25-hydroxylase and 5β-cholestane-3α, 7α, 12α, 25-tetrol 23R, 24R, 24S and 27-hydroxylases) but does not stimulate mitochondrial sterol 27-hydroxylase. In distinction, cholesterol feeding down-regulates cholesterol 7α-hydroxylase but up-regulates the sterol 27-hydroxylase and 25-hydroxylation pathways. Thus, the side chain microsomal 25-hydroxylation path-

way is separately regulated from cholesterol 7α-hydroxylase and sterol 27-hydroxylase. In rabbits with bile fistulas, 98% of the newly synthesized bile acids is cholic acid (33). Therefore, the regulation of the 25-hydroxylation pathway may be more important in rabbits than in other mammals.

Defective mitochondrial sterol 27-hydroxylase causes CTX in human (14). However, sterol 27-hydroxylase knockout mice did not show CTX-related pathological or biochemical abnormalities including the increased formation of 25-hydroxylated C_{27}-bile alcohols and cholestanol, but the production of both cholic and chenodeoxycholic acids was markedly reduced (18). CTX patients have almost normal cholic acid production (15) and it appears to be synthesized from 5β-cholestane-3α, 7α, 12α-triol via the 25-hydroxylation pathway (9, 11). Therefore, it was suspected that compared with humans, the 25-hydroxylation pathway was not active in mice (18). However, these data showed that both 5β-cholestane-3α, 7α, 12α-triol 25-hydroxylase and 5β-cholestane-3α, 7α, 12α, 25-tetrol 24S-hydroxylase activities were much higher in mouse than in human livers (Table 2). Thus, the results do not support the idea that the 25-hydroxylation pathway in mice was not as active as that in human.

In summary, we have developed improved precise assays for the activities of hepatic 5β-cholestane-3α, 7α, 12α-triol 25- and 27-hydroxylases and 5β-cholestane-3α, 7α, 12α, 25-tetrol 23R, 24R, 24S and 27-hydroxylases by high-resolution GC-SIM. The enzyme activities in mice and rabbits were generally higher than those in humans. When bile acid synthesis was perturbed by cholesterol feeding or bile drainage, cholesterol 7α-hydroxylase, sterol 27-hydroxylase, and the 25-hydroxylation pathway (5β-cholestane-3α, 7α, 12α-triol 25-hydroxylase and 5β-cholestane-3α, 7α, 12α, 25-tetrol 23R, 24R, 24S and 27-hydroxylases) were independently regulated in rabbits.

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