Biochemical Pharmacology, Vol. 41, No. 10, pp. 1439–1447, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

CHARACTERISTICS OF CHOLESTEROL 7α-HYDROXYLASE AND 7α-HYDROXYCHOLESTEROL HYDROXYLASE ACTIVITIES OF RODENT LIVER

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(*Received* 6 *June* 1990; *accepted* 13 *November* 1990)

Abstract—A second cholesterol-derived metabolite in addition to 7α-hydroxycholesterol was observed to be produced from endogenous microsomal cholesterol in the presence of hamster liver microsomal fractions and NADPH, when analyzed by HPLC using the method of Ogishima and Okuda (*Anal Biochem* **158**: 228–232, 1986). However, only 7α-hydroxycholesterol was produced in the presence of rat hepatic microsomal protein fractions and NADPH. The second metabolite was facilely produced when endogenous 7α-hydroxycholesterol was incubated with hamster liver microsomes and NADPH, but not with rat liver microsomes. The second metabolite derived from either endogenous cholesterol or exogenous 7α-hydroxycholesterol contained three hydroxyl groups as shown by mass spectrometric analysis. After oxidation of the 3β-ol group by cholesterol oxidase, the metabolite comigrated with 7β-hydroxycholest-3-one on normal phase HPLC, but was resolved from both 7α- and 7β-hydroxycholest-3-one on reverse phase HPLC. The data indicate that the second metabolite is a hydroxylated product of 7α-hydroxycholesterol, possibly cholest-5-ene-3β,7α,12α-triol. Cholestyramine feeding increased production of both 7α-hydroxycholesterol and its metabolite from endogenous cholesterol by 3-fold in hamster liver microsomes *in vitro*. However, the direct conversion of 7α-hydroxycholesterol to the metabolite by hamster liver microsomes was not increased appreciably after cholestyramine feeding (20–30%). The hydroxylation of 7α-hydroxycholesterol was similar in characteristics to cholesterol 7α-hydroxylase activity in that it was dependent on NADPH, was inhibited by several known P450 inhibitors, and was affected by an inhibitory autobody elicited against rat hepatic NADPH:cytochrome P450 oxidoreductase. 5,6- and 7,8-Benzoflavone were poor inhibitors ($IC_{50} \simeq 1$ mM) of cholesterol 7α-hydroxylase activity in liver microsomes from cholestyramine-fed rats, but caused a striking enhancement of the 7α-hydroxylase activity of liver microsomes from untreated rats *in vitro*. In contrast, 7,8-benzoflavone inhibited cholesterol 7α-hydroxylase and 7α-hydroxycholesterol hydroxylase activities of microsomes from normal and cholestyramine-fed hamsters. However, 5,6-benzoflavone stimulated cholesterol 7α-hydroxylase activity in liver microsomes from normal and cholestyramine-fed hamsters, but inhibited 7α-hydroxycholesterol hydroxylase activity by approximately 50%. These results suggest that hepatic cholesterol 7α-hydroxylase and 7α-hydroxycholesterol hydroxylase activities apparently involve multiple forms of cytochrome P450 in untreated and cholestyramine-treated hamsters.

The major primary bile acids of all mammalian species require insertion of a 7α-hydroxyl group into the B ring of cholesterol as the rate-limiting step in their biosynthesis [1]. The introduction of this group is a key reaction in the biosynthesis of cholic acid and is believed to be a major regulatory step in the formation of chenodeoxycholic acid. Several species, however, synthesize bile acids with a 7β-hydroxyl group at this position [2, 3]. The physiological role and occurrence of this 7β-hydroxylation reaction among various species have yet to be characterized in detail.

Ogishima and Okuda [4] reported a nonisotopic HPLC method for measurement of cholesterol 7α-hydroxylase activity, using endogenous microsomal cholesterol as substrate. In principle, the procedure involves the conversion of the product, 7α-hydroxycholesterol, to 7α-hydroxy-4-cholest-3-one (7α-chol-3-one)‖ by cholesterol oxidase (EC 1.1.3.6). Cholesterol oxidase catalyzes the oxidation of the 3β-hydroxyl group of cholesterol or related sterols to a keto group by dioxygen and the isomerization of the Δ^5-bond to yield a conjugated double bond which serves as a chromophoric group (α,β-unsaturated ketone; $\varepsilon_{240\,nm} = 16{,}000\ M^{-1}\,cm^{-1}$) [5]. This enzyme does not catalyze the oxidation of hydroxyl groups at position C-7α and C-7β [6]. 7β-Hydroxycholesterol can also be oxidized by cholesterol oxidase to yield 7β-hydroxy-4-cholest-3-one (7β-chol-3-one) [5]. Measurement of 7α- and 7β-hydroxylation of cholesterol is possible, if resolution of two oxidized steroid derivatives can be achieved. In this paper, we utilized this simple and accurate assay of cholesterol 7α- and 7β-hydroxylase activities to characterize the reactions catalyzed by rat and hamster liver microsomes. A new cholesterol metabolite apparently derived from 7α-hydroxycholesterol, which we showed not to be 7β-hydroxycholesterol, was observed to be formed in the presence of hamster hepatic microsomal protein and NADPH, but not rat hepatic microsomal protein.

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‖ Abbreviations: 7α-chol-3-one, 7α-hydroxy-4-cholest-3-one; and 7β-chol-3-one, 7β-hydroxy-4-cholest-3-one.

MATERIALS AND METHODS

Materials. Cholesterol oxidase (from *Nocardia erythropolis*) was supplied by Boehringer Mannheim Biochemicals (Indianapolis, IN). 7α- and 7β-Hydroxycholesterol were obtained from Steraloids Inc. (Wilton, NH). Lovastatin was a gift from the Merck Co. (Rahway, NJ). Other chemicals were purchased from the Sigma Chemical Co., Inc. (St. Louis, MO) and were of analytical or HPLC grade.

Animals. Male Sprague–Dawley rats [Harlan Sprague Dawley, Inc., Indianapolis, IN; Hsd:SD, 150–180 g] and Syrian Golden hamsters [Charles River Breeding Laboratories, Inc., Wilmington, MA; Lak:LVG(SYR), 60–90 g] were fed either laboratory chow, chow containing 3% cholestyramine, or chow containing 0.1% lovastatin for at least 2 weeks *ad lib.* Liver microsomes were prepared from both species as described previously [7] and stored at −70°. Protein concentration was determined by the method of Lowry *et al.* [8] with bovine serum albumin as the standard.

Assay for cholesterol 7α-hydroxylase and 7α-hydroxycholesterol hydroxylase activities. To establish conditions under which 7α-chol-3-one was well resolved from 7β-chol-3-one, various ratios of *n*-hexane to 2-propanol were tested to resolve the 3-one derivatives using a μPorasil silica HPLC column (Waters Associates, Inc., Milford, MA). The ratio of 95:5 for *n*-hexane:2-propanol produced the best resolution. Standard curves were established to allow quantitation of metabolites with the HPLC method and account for recovery of the metabolites. The peak height responses for 7α- and 7β-chol-3-one were more than 10-fold larger than those for 7α- and 7β-hydroxycholesterol due to the marked differences in their molar absorptivities. The extraction efficiencies for 7α-chol-3-one were 83% with rat microsomes and 70% with hamster microsomes; the extraction efficiency for 7β-chol-3-one was 61% with hamster microsomes.

Microsomes were incubated at 37° for 20 min with 0.1 M potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA, 20 mM cysteamine-HCl, 5 mM $MgCl_2$, 5 mM sodium isocitrate, 0.075 units of isocitrate dehydrogenase and 0.5 mM NADPH in a final volume of 0.5 mL. When exogenous cholesterol or 7α-hydroxycholesterol were included in the reaction mixtures, they were dissolved in 2-propanol and added in 5-μL volumes to the reaction mixture. The reactions were initiated by addition of NADPH. At the end of the incubation, 60 μL of 5% (w/v) sodium cholate and 40 μL of 0.1% (v/v) cholesterol oxidase (0.24 units) dissolved in 10 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol and 1 mM dithiothreitol, were added to the reaction mixtures. This mixture was further incubated for 20 min. The reaction was terminated by adding 0.6 mL of methanol and the mixture was extracted three times with 6 mL of petroleum ether.

The extract was evaporated to dryness and the residue was reconstituted in *n*-hexane:2-propanol (95:5, v/v). The samples were analyzed by normal phase HPLC as described by Ogishima and Okuda [4] using a μPorasil silica column in a Waters RCM 8 × 10 cartridge (Waters Associates, Inc.). A portion (25%) of the residue was injected on a column equilibrated with a mixture of *n*-hexane and 2-propanol (95:5, v/v). The column was eluted with the same solvent at a rate of 2.0 mL/min. Absorbance of the effluent was monitored at 240 nm. The HPLC system consisted of a pump (Kratos Spectroflow model 400, Applied Biosystems, Inc., Foster City, CA) equipped with a UV spectrophotometer (Kratos Spectroflow model 757 absorbance detector). The amounts of 7α- and 7β-hydroxycholesterol were determined by the same HPLC method, except that these metabolites were monitored at 214 nm (data not shown). The retention times for 7α- and 7β-hydroxycholesterol, as well as the hydroxylated metabolite of 7α-hydroxycholesterol, were altered by treatment with cholesterol oxidase, demonstrating that all three compounds probably contain a 3β-ol group. When the metabolites were analyzed using reverse phase liquid chromatography, a μBondapack C18 column was used in a RCM 8 × 10 cartridge and the compounds were eluted using isocratic conditions (80% acetonitrile in water) over a 25-min period.

Inhibitors of cytochrome P450-mediated reactions were added to the reaction mixtures in 5 μL of dimethylformamide (final solvent concentration 1%). Sodium cholate was dissolved in water. The acid form of lovastatin was prepared by saponification of its lactone form in 0.1 N NaOH at 50° for 2 hr [9] and then dissolved in 0.1 M potassium phosphate buffer, pH 7.4.

Mass spectrometric analysis. Fractions containing the metabolites of interest were collected from normal phase HPLC as described above and the solvent was evaporated under a stream of nitrogen. The residue was reconstituted with 50 μL of methylene chloride:2-propanol (95:5, v/v) and analyzed by gas chromatography/mass spectrometry (GC/MS). The GC/MS analyses were performed using a Hewlett–Packard model HP5992 GC/MS (Hewlett–Packard Co., Palo Alto, CA) unit fitted with a membrane separator and a 2 m × 2 mm column packed with 3% SP-2100 on 100/120 Supelcoport (Supelco, Bellefonte, PA). GC conditions were as follows: Grade V helium was used as carrier gas at a flow rate of 30 mL/min, and analyses were performed under isothermal conditions (275°). Following 70 eV electron ionization, a quadrupole mass filter was used to scan from m/z = 600–35 at a rate of 1.2 Hz.

RESULTS

Characterization of 7α-hydroxycholesterol and its metabolite. As seen in Fig. 1A, the 7α- and 7β-hydroxycholest-3-one derivatives (7α- or 7β-chol-3-one) could be resolved easily by normal phase HPLC [4]. When reaction mixtures containing hamster liver microsomes and NADPH were extracted and analyzed by normal phase HPLC, two metabolites were observed with retention times identical to 7α- and 7β-chol-3-one. To establish their identity, the metabolites were collected separately and analyzed by GC/MS. Mass spectra for the HPLC peaks of interest are shown in Fig. 2. Spectrum A is the EI mass spectrum for the oxidation product (formed by

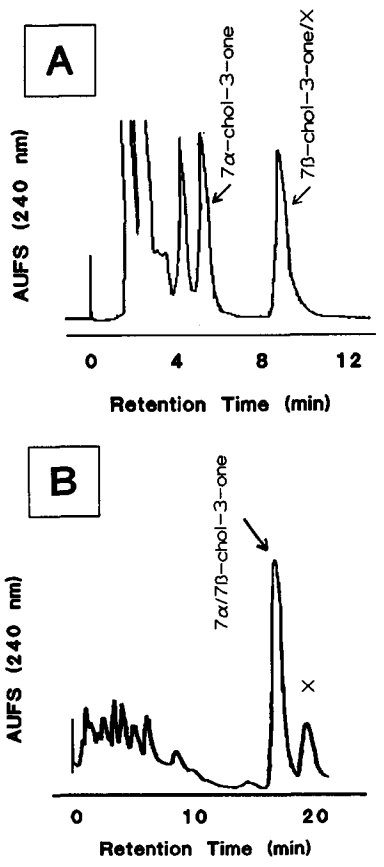

Fig. 1. Resolution of the hydroxylated metabolite of 7α-hydroxycholesterol from 7α- and 7β-hydroxycholesterol by HPLC. (A) The hydroxylated metabolite of 7α-hydroxycholesterol (X) was separated from 7α-hydroxycholesterol (7α-chol-3-one) using normal phase HPLC. (B) The hydroxylated metabolite of 7α-hydroxycholesterol was separated from 7β-hydroxycholesterol (7β-chol-3-one) using reverse phase HPLC with an isocratic mobile phase of 80% acetonitrile in water. The metabolite of 7α-hydroxycholesterol was formed during the microsomal metabolism of exogenous 7α-hydroxycholesterol and was first isolated by normal phase HPLC. The metabolites and authentic reference compounds were oxidized by cholesterol oxidase prior to analysis by HPLC.

action of cholesterol oxidase) of 7α-hydroxycholesterol, 7α-chol-3-one. The high mass peak at $m/z = 382$ represents the neutral loss of water from dehydration of the "B" ring, characteristic of alicyclic alcohols. This mass spectrum is identical to that obtained for the cholesterol oxidase-oxidized product of authentic 7α-hydroxycholesterol (data not shown). Spectrum B is the EI mass spectrum of a putative hydroxylated metabolite of 7α-hydroxycholesterol after oxidation with cholesterol oxidase. This trihydroxycholestene had a high mass peak at $m/z = 400$, again representing dehydration typical of other trihydroxycholestene derivatives. The GC retention time for the system utilized was 7.6 min for the 7α-chol-3-one and 10.0 min for the cholesterol oxidase-oxidized metabolite formed by hydroxylation of 7α-hydroxycholesterol. The mass spectra for the new metabolite of 7α-hydroxycholesterol were identical when the metabolite was obtained during metabolism of either endogenous cholesterol or exogenously added 7α-hydroxycholesterol (data not shown). From the mass spectral data, the exact position of hydroxylation could not be deduced by the techniques available to us.

To further establish conditions under which 7β-hydroxycholesterol would not cochromatograph with the new metabolite, a reverse phase HPLC system was established to separate 7β-chol-3-one from the new metabolite, denoted X (Fig. 1B). Using a μBondapak C18 column, 7β-chol-3-one was resolved from the cholesterol oxidase-oxidized metabolite formed from 7α-hydroxycholesterol using an isocratic mobile phase of 80% acetonitrile in water. When analyzed by reverse phase HPLC, no 7β-chol-3-one was observed in the fraction obtained by normal phase HPLC presumed to contain either the putative 7β-chol-3-one and/or the hydroxylated 7α-hydroxycholesterol metabolite. These results clearly demonstrate that the hydroxylated metabolite of 7α-hydroxycholesterol is formed from endogenous microsomal cholesterol or exogenous 7α-hydroxycholesterol by hamster liver microsomal protein and NADPH.

Cholesterol hydroxylase activity of rodent microsomal fractions. The formation of 7α-hydroxycholesterol in rat and hamster liver microsomes and of the hydroxylated metabolite of 7α-hydroxycholesterol in the presence of hamster liver microsomes was linear for up to 20 min. Cholesterol 7α-hydroxylase specific activity was shown to be independent of reaction volume (data not shown), and a 0.5-mL reaction volume was used for the assays.

The characteristics of the 7α-hydroxycholesterol hydroxylase reaction were established using exogenous 7α-hydroxycholesterol as substrate (Table 1). When liver microsomes were incubated with NADPH and an NADPH-regenerating system, measurable amounts of endogenous microsomal cholesterol were converted to 7α-hydroxycholesterol and the second metabolite. Little or no conversion of 7α-hydrocholesterol to the second metabolite was noted in the presence of liver microsomes from untreated hamsters alone. Upon addition of NADPH, marked conversion of 7α-hydroxycholesterol to the metabolite was noted during 20 min (approx. 65 pmol/min/mg protein). Boiling the microsomal protein prevented this reaction, indicating that formation of the second metabolite is an enzyme-mediated reaction dependent on microsomal protein and NADPH; this is characteristic of the cytochrome P450-dependent monooxygenases. These results demonstrate that while the hydroxylated metabolite of 7α-hydroxycholesterol can be formed from microsomal cholesterol, exogenously added 7α-hydroxycholesterol is directly converted to the new metabolite.

There was a conspicuous difference in the 7α-hydroxylase and 7α-hydroxycholesterol hydroxylase activities between hamster and rat hepatic microsomes (Table 2). First, no 7α-hydroxycholesterol hydroxylase activity was observed with liver

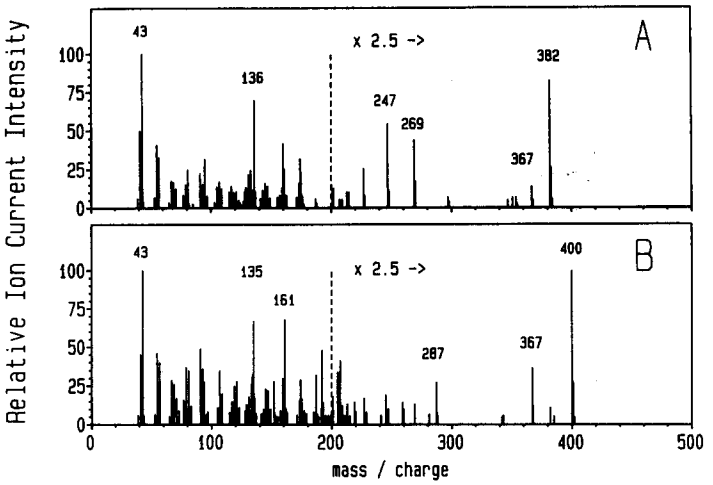

Fig. 2. Mass spectra of 7α-hydroxycholesterol and its hydroxylated metabolite. The metabolites were isolated from microsomal reaction mixtures and resolved on normal phase HPLC. The purified metabolites were collected and analyzed by mass spectrometry. (A) 7α-Hydroxycholesterol derived from endogenous cholesterol. (B) The microsomal metabolite of 7α-hydroxycholesterol. The mass spectra of the microsomal metabolite of 7α-hydroxycholesterol obtained from either endogenous cholesterol or authentic 7α-hydroxycholesterol were identical (data not shown).

Table 1. Metabolism of cholesterol and 7α-hydroxycholesterol by hamster liver microsomal fractions

Incubation mixture	Microsome preparation	Substrate or product recovered	
		7α-Chol-3-one*	7α-Hydroxycholesterol 3-one metabolite
		(nmol recovered/mL)	
	Control†	0.2 ± 0.1	0.2 ± 0.1
	Boiled control	0.3 ± 0.1	0.5 ± 0.1
NADPH	Control†	1.4 ± 0.07	1.2 ± 0.1
7α-Hydroxycholesterol	None	9.5 ± 1.4	0.2 ± 0.1
7α-Hydroxycholesterol	Control†	8.1 ± 0.8	1.4 ± 0.1
7α-Hydroxycholesterol + NADPH	Control	4.2 ± 0.5	4.2 ± 0.4
	Cholestyramine	3.5 ± 0.7	5.2 ± 0.5
	Boiled control†	8.6 ± 0.7	1.2 ± 0.1

* 7α-Hydroxycholesterol (10 nmol) and/or NADPH were added to a 1-mL reaction mixture containing 0.1 M potassium phosphate buffer, pH 7.4, 0.1 mM EDTA, 20 mM cysteamine-HCl, 5 mM $MgCl_2$, 5 mM sodium isocitrate, and 0.075 units of isocitrate dehydrogenase. When included in the reaction mixture, the final microsomal protein concentration was 3 mg/mL. The results are expressed as nmol of 7α-hydroxycholest-3-one or the 3-one derivative of the hydroxylated metabolite of 7α-hydroxycholesterol/mL ± standard deviation (N = 3). The samples were incubated for 20 min before termination of the reaction, and cholesterol oxidase was added subsequently. The oxidized 7α-hydroxycholesterol substrate and products were analyzed by normal phase HPLC as described by Ogishima and Okuda [4].

† There was no statistical difference in results using hepatic microsomes from control or cholestyramine-fed hamsters.

microsomes from either control or cholestyramine-treated rats. Second, the cholesterol 7α-hydroxylase activity was greater in rat microsomes than hamster microsomes, possibly due to the fact that 7α-hydroxycholesterol is not metabolized further in the presence of rat hepatic cytochromes P450. Addition of exogenous cholesterol had little or no effect on either activity with liver microsomes from untreated or cholestyramine-treated rodents. With hamster liver microsomes, 7α-hydroxycholesterol hydroxylase activity was concave upward with respect to the concentration of microsomal protein utilized in the reaction mixture, whereas cholesterol 7α-hydroxylase activity was convex with respect to the concentration of microsomal protein. When the concentration of microsomal protein was higher than 3 mg/0.5 mL, the activity of 7α-hydroxycholesterol hydroxylase was greater than that of cholesterol 7α-

Table 2. Characterization of hepatic, microsomal cholesterol 7α-hydroxylase and 7α-hydroxycholesterol hydroxylase activity *in vitro*

	Cholesterol 7α-hydroxylase				7α-Hydroxycholesterol hydroxylase	
	Rat		Hamster		Hamster	
Incubation system	Activity*	%	Activity	%	Activity	%
Complete†	142	100	26	100	26	100
Boiled microsomes	19	13	4	16	11	42
−NADPH	12	8	5	18	3	10
−NADPH, +NADH	29	20	5	18	3	11
+anti-rat NADPH:cytochrome P450 oxidoreductase globulin	44	31	14	54	9	34

* The results performed in duplicate are expressed as pmol/min/mg microsomal protein. The source of substrate was endogenous microsomal cholesterol.

† The complete system including NADPH, an NADPH-regenerating system, and liver microsomes from cholestyramine-treated animals as described in Materials and Methods.

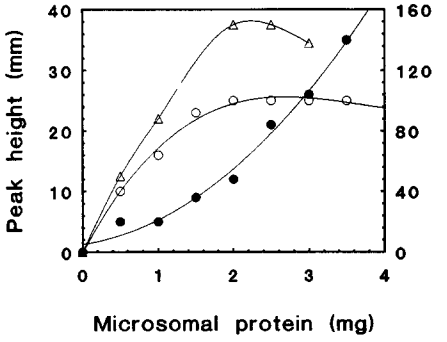

Fig. 3. Dependence of the rate of formation of 7α-hydroxycholesterol and the metabolite of 7α-hydroxycholesterol on hepatic microsomal protein concentration. Liver microsomes from untreated hamsters [(○) 7α-chol-3-one; (●) hydroxylated metabolite of 7α-hydroxycholesterol; left axis] and untreated rats [(△) right axis] were used. Duplicate measurements of each protein concentration were obtained.

hydroxylase (Fig. 3). This nonlinear response to protein concentration might be expected if the second metabolite was derived from 7α-hydroxycholesterol. In the rat, 7α-hydroxylase activity was initially linear with respect to the amount of microsomal protein up to 3 mg/mL; no further hydroxylation of 7α-hydroxycholesterol could be detected at any protein concentration utilized.

When microsomes from cholestyramine-fed animals were utilized, omission of NADPH from the incubation mixture decreased the two hydroxylase activities by over 80% (Table 2). NADH was only 10% as effective as NADPH and denaturing the microsomal protein by boiling led to little or no activity (Table 2). Anti-NADPH:cytochrome *c* (P450) oxidoreductase globulin inhibited both reactions with hamster microsomes, suggesting that cholesterol 7α-hydroxylase activity in rat or hamster and 7α-hydroxycholesterol hydroxylase activity in hamster were dependent on NADPH:cytochrome P450 oxidoreductase and, most probably, on cytochrome P450 as well (Table 2). In our hands, the anti-rat NADPH:cytochrome *c* oxidoreductase globulin was not as effective in inhibiting the hydroxylase and NADPH:cytochrome *c* oxidoreductase activities of hamster liver microsomal fractions as it was in inhibiting the corresponding rat activities (data not shown).

The activities of cholesterol 7α-hydroxylase in rat liver microsomes were induced 5- to 6-fold from 24 ± 3 to 134 ± 18 pmol/min/mg protein by cholestyramine feeding. The 7α-hydroxycholesterol hydroxylase activity was too low to quantitate in microsomes from either untreated or cholestyramine-fed rats (Table 3). The specific activities of cholesterol 7α-hydroxylation and 7α-hydroxycholesterol hydroxylase of hamsters were both increased 2.5 to 3-fold by cholestyramine feeding when endogenous microsomal cholesterol was utilized as substrate. However, cholestyramine administration caused only a slight increase (20–30%) in the 7α-hydroxycholesterol hydroxylase activity when exogenous 7α-hydroxycholesterol was used as substrate (Table 1). Administration of lovastatin to rodents had little or no effect on these activities. Both hepatic microsomal b_5 and P450 content decreased slightly in cholestyramine- and lovastatin-fed hamsters, but no significant difference was seen in normal and cholestyramine-fed rats (Table 3).

Effects of various agents on microsomal cholesterol 7α-hydroxylase and 7α-hydroxycholesterol activities. Addition of sodium cholate, lovastatin and several known inhibitors of cytochrome P450 to microsomal incubation mixtures *in vitro* altered the relative activities of cholesterol 7α-hydroxylase and 7α-hydroxycholesterol hydroxylase in liver microsomes from cholestyramine-fed rats in a concentration-dependent manner. The IC_{50} values for each compound are given in Table 4. Miconazole was a potent inhibitor of 7α-hydroxylase activity (IC_{50} = 1.3 μM), whereas lovastatin elicited moderate inhibition (IC_{50} = 0.6 mM). 7,8-Benzoflavone, 5,6-benzoflavone, metyrapone and sodium cholate were

Table 3. Effects of cholestyramine and lovastatin treatment on microsomal cholesterol 7α-hydroxylase and 7α-hydroxycholesterol hydroxylase activity

Group	Animal		Cholesterol 7α-hydroxylase* (pmol/min/mg protein)	7α-Hydroxycholesterol hydroxylase* (pmol/min/mg protein)	Cytochrome b_5 (nmol/mg protein)	Cytochrome P450 (nmol/mg protein)
Control	Rat	(N = 3)	24 ± 3	ND†	0.54 ± 0.04	0.59 ± 0.30
	Hamster	(N = 6)	9 ± 3	10 ± 2	0.48 ± 0.03	1.15 ± 0.09
Cholestyramine	Rat	(N = 3)	134 ± 18‡	ND	0.52 ± 0.04	0.61 ± 0.07
	Hamster	(N = 6)	31 ± 11‡	26 ± 10§	0.42 ± 0.08‡	0.86 ± 0.01‡
Lovastatin	Hamster	(N = 6)	8 ± 3	7 ± 1§	0.41 ± 0.03‡	0.89 ± 0.12‡

* Values are averages ± SD; the number of animal liver microsomal preparations studied is shown in parentheses.
† ND, not detected.
‡ $P \leq 0.05$ relative to control.
§ $P \leq 0.01$ relative to control.

Table 4. IC_{50} values of some compounds on hamster and rat liver microsomal cholesterol 7α-hydroxylase and 7α-hydroxycholesterol hydroxylase activities *in vitro*

Compounds	IC_{50}* (M)			
	Cholestyramine-fed animal		Untreated animal	
	Cholesterol 7α-hydroxylase	7α-Hydroxycholesterol hydroxylase	Cholesterol 7α-hydroxylase	7α-Hydroxycholesterol hydroxylase
		Rat		
Miconazole	1.3×10^{-6}	—†	1.2×10^{-5}	—
5,6-Benzoflavone	6.3×10^{-3}	—	Stimulated‡	—
7,8-Benzoflavone	2.5×10^{-3}	—	Stimulated	—
Metyrapone	4.0×10^{-3}	—	2.0×10^{-3}	—
Sodium cholate	1.6×10^{-3}	—	5.7×10^{-3}	—
Lovastatin	6.0×10^{-4}	—	2.6×10^{-4}	—
		Hamster		
Miconazole	6.6×10^{-5}	5.0×10^{-5}	ND§	ND
5,6-Naphthoflavone	Stimulated	8.7×10^{-4}	Stimulated	1.9×10^{-3}
7,8-Naphthoflavone	3.5×10^{-4}	1.7×10^{-3}	1.6×10^{-4}	8.9×10^{-4}
Metyrapone	8.8×10^{-3}	3.2×10^{-4}	Stimulated	1.4×10^{-3}
Sodium cholate	5.8×10^{-3}	3.2×10^{-4}	1.7×10^{-3}	4.8×10^{-4}
Lovastatin	1.2×10^{-3}	6.1×10^{-4}	Stimulated	5.0×10^{-4}

* IC_{50}, molar concentration necessary to inhibit hydroxylase activity by 50%. The substrate was endogenous microsomal cholesterol.
† Little or no activity detected.
‡ Stimulated, activity increased in the presence of this compound.
§ ND, not determined.

poor inhibitors ($IC_{50} \geq 1$ mM). Most of these compounds affected the activity of 7α-hydroxylase in control rat microsomes in an identical manner, except for 5,6- and 7,8-benzoflavone (Table 4).

The behaviors of these compounds with hamster microsomes were similar to those seen in rat liver microsomes (Table 4); miconazole inhibited both cholesterol 7α-hydroxylase and 7α-hydroxycholesterol hydroxylase activities of hamster liver microsomes although higher concentrations were needed to attain 50% inhibition (Fig. 4). Miconazole inhibited the cholesterol 7α-hydroxylase activity of liver microsomes of cholestyramine-fed rats most effectively, elicited moderate inhibition on 7α-hydroxycholesterol hydroxylase activity of liver microsomes of cholestyramine-fed hamsters, and was a poorer inhibitor of cholesterol 7α-hydroxylase activity of liver microsomes of cholestyramine-fed hamsters.

Addition of either 5,6- or 7,8-benzoflavone caused an enhancement of the cholesterol 7α-hydroxylase activity of liver microsomes from untreated rats, i.e. 7.0- and 2.0-fold increases, respectively (Fig. 5). However, these flavonoid derivatives had an inhibitory effect on the hepatic microsomal cholesterol 7α-hydroxylase activities of cholestyramine-treated rats. 5,6- and 7,8-Benzoflavone had very different effects on cholesterol 7α-hydroxylase activity in hamster liver microsomes compared to rat in both untreated and cholestyramine-fed animals. Compared to the rat, 7,8-benzoflavone inhibited cholesterol 7α-hydroxylase activity of liver microsomes of both untreated and cholestyramine-fed

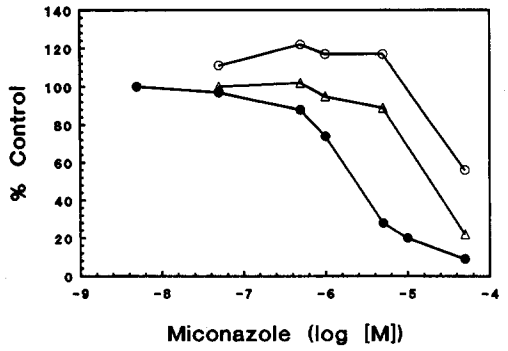

Fig. 4. Effect of miconazole on cholestyramine-fed rat and hamster hepatic microsomal cholesterol 7α-hydroxylase and 7α-hydroxycholesterol hydroxylase activities *in vitro*. The results are the average of triplicate determinations performed with hamster [7α-chol-3-one (○); hydroxylated metabolite of 7α-hydroxycholesterol (△)] or rat [7α-chol-3-one (●)] liver microsomes. The standard deviations for the values shown were less than 10% of the average value in all cases. Control activities for rat and hamster liver microsomes were 140 and 26 pmol 7α-hydroxycholesterol formed/min/mg protein and 25 pmol of the metabolite of 7α-hydroxycholesterol formed/min/mg protein for hamster liver microsomes.

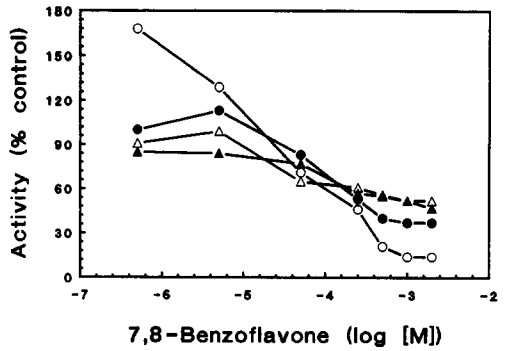

Fig. 6. Effect of 7,8-benzoflavone on cholestyramine-fed and normal hamster hepatic microsomal cholesterol 7α-hydroxylase and 7α-hydroxycholesterol hydroxylase activities *in vitro*. Cholesterol 7α-hydroxylase activities of liver microsomes from untreated [(○) control activity, 9 ± 3 pmol/min/mg protein] or cholestyramine-fed [(●) control activity, 31 ± 11 pmol/min/mg protein] hamsters and 7α-hydroxycholesterol hydroxylase activities of liver microsomes from untreated [(△) control activity, 10 ± 2 pmol/min/mg protein] and from cholestyramine-fed [(▲) control activity, 26 ± 10 pmol/min/mg protein] hamsters were measured in duplicate. Values are the averages of duplicate measurements which did not vary by more than 10%.

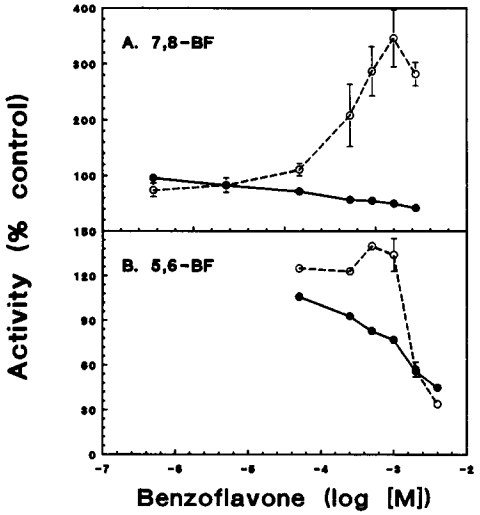

Fig. 5. Effects of 5,6- and 7,8-benzoflavone on cholestyramine-fed and normal rat hepatic microsomal cholesterol 7α-hydroxylase activities *in vitro*. The cholesterol 7α-hydroxylase activity values shown are averages ± SD for three preparations measured in duplicate with liver microsomes from normal [(○) control activity, 24 pmol/min/mg protein] and cholestyramine-fed [(●) control activity, 133 pmol/min/mg protein] rats. (A) Inhibition by 7,8-benzoflavone. (B) Inhibition by 5,6-benzoflavone.

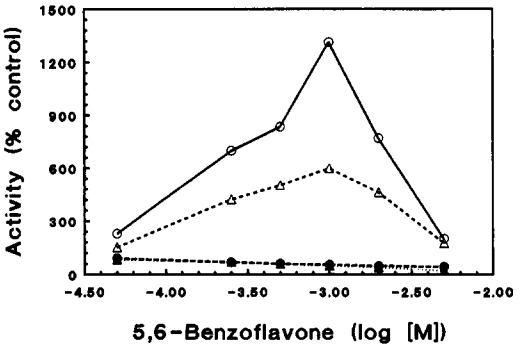

Fig. 7. Effect of 5,6-benzoflavone on normal and cholestyramine-fed hamster hepatic microsomal cholesterol 7α-hydroxylase and 7α-hydroxycholesterol hydroxylase activities *in vitro*. Cholesterol 7α-hydroxylase activities of liver microsomes from untreated [(○) control activity, 9 ± 3 pmol/min/mg protein] or cholestyramine-fed [(△) control activity, 31 ± 11 pmol/min/mg protein] hamsters and 7α-hydroxycholesterol hydroxylase activities of liver microsomes from untreated [(●) control activity, 10 ± 2 pmol/min/mg protein] and from cholestyramine-fed [(▲) control activity, 26 ± 10 pmol/min/mg protein] hamsters were measured in duplicate. Values are the averages of duplicate measurements which did not vary by more than 10%.

hamsters (Fig. 6). However, 5,6-benzoflavone stimulated hepatic cholesterol 7α-hydroxylase activity in both normal and cholestyramine-fed hamsters (Fig. 7). This effect was somewhat greater in hepatic microsomes from untreated animals than in cholestyramine-fed animals. In contrast, 5,6- and 7,8-benzoflavone were poor inhibitors of 7α-hydroxycholesterol hydroxylase in normal and cholestyramine-fed hamster liver microsomes; IC_{50} values ranged from 0.87 to 1.7 mM (Table 4 and Figs. 6 and 7).

DISCUSSION

We have modified the nonisotopic HPLC method of Ogishima and Okuda [4] to measure hepatic microsomal cholesterol 7α-hydroxylase and 7α-hydroxycholesterol hydroxylase activities using endogenous cholesterol as substrate for both reactions. We observed that the 7α-hydroxycholesterol hydroxylase activity was unique for hamster hepatic microsomes; little or none of this activity was observed with liver microsomal protein from control or cholestyramine-treated rats. Using GS/MS, we have demonstrated that this metabolite is a hydroxylated metabolite of 7α-hydroxycholesterol, although the position of hydroxylation could not be ascertained by the instrumentation available. Einarsson and coworkers [10, 11] have described the formation of cholest-5-ene-3β,7α,12α-triol and its subsequent conversion to cholic acid in the rat. However, we could not demonstrate significant formation of such a metabolite from 7α-hydroxycholesterol in reaction mixtures containing rat hepatic microsomal protein and NADPH. The mass spectra for this metabolite obtained from reaction mixtures containing hamster liver microsomes and NADPH had an M-18 high mass peak, but the relative intensities of M-18, M-36, M-48 mass peaks differed. Further studies are in progress to identify the position of hydroxylation on the new metabolite. The 7α-hydroxycholesterol hydroxylase reaction was also shown to utilize exogenous 7α-hydroxycholesterol as a substrate, since the products were identical as determined by GC/MS and HPLC when either endogenous cholesterol or exogenous 7α-hydroxycholesterol was used as a source of substrate. The affinity and turnover of the hydroxylase for 7α-hydroxycholesterol must be high to convert the limited amounts of endogenously formed 7α-hydroxycholesterol to the new metabolite at the rate observed *in vitro* (Table 2, Fig. 3). These results suggest pronounced differences in the 7α-hydroxycholesterol hydroxylase activity in hamsters which may allow use of 7α-hydroxycholesterol hydroxylation to form bile salts, without requisite 3β-ol oxidation occurring first as described for rat liver [12].

Using this HPLC method, we compared the effects of various stimulatory and inhibitory agents on these two enzyme activities in hamster hepatic microsomes. The 7α-hydroxycholesterol hydroxylase reaction was similar in characteristics to cholesterol 7α-hydroxylase activity in that it was NADPH-dependent, was affected by known inhibitors of cytochrome P450, and was inhibited by anti-rat hepatic NADPH:cytochrome P450 oxidoreductase globulin. These results suggest that both hydroxylase activities are catalyzed by cytochrome P450. The differential effects of known inhibitors of cytochrome P450, such as 5,6- and 7,8-benzoflavone, on cholesterol 7α-hydroxylase and 7α-hydroxycholesterol hydroxylase in hamster liver microsomes further suggest that the two hydroxylase activities in hamsters are the result of at least two distinct forms of the enzyme.

Like others, we found induction of cholesterol 7α-hydroxylase activity by cholestyramine feeding. It has been suggested that cholestyramine interferes with the enterohepatic circulation of bile acids by interrupting feedback inhibition of cholesterol 7α-hydroxylation. However, other investigators [13] and our own results show that the IC_{50} value for sodium cholate in inhibiting cholesterol 7α-hydroxylase activity was in the millimolar range, possibly precluding direct inhibition of 7α-hydroxylase activity by the bile acid as a regulatory mechanism *in vivo*. A delay of at least 24 hr has been noted for the rise in cholesterol 7α-hydroxylase activity that occurs after interruption of the enterohepatic circulation of bile salts [14, 15]. Wada *et al.* have suggested that a specific form of P450 may be enhanced during biliary drainage [16]; our experiments verified Wada's report. Changes in 7α-hydroxylase activity due to circadian rhythm or cholestyramine feeding have been correlated with changes in protein and mRNA levels for a specific form of cytochrome P450 [17, 18]. Recently, Sundseth and Waxman [19] have demonstrated short-term (feeding precursors to cholesterol), mid-term (diurnal variation), and long-term regulation (bile acid negative feedback) of the cholesterol 7α-hydroxylase enzyme. These changes most likely are regulated at the pretranslational level. In cholestyramine-fed hamsters, total content of cytochromes b_5 and P450 decreased slightly in liver microsomes, while 7α-hydroxylase activity increased. In rat, a similar trend was noted, although statistical significance was not attained in our studies.

The differential effects of 5,6- and 7,8-benzoflavone on cholestyramine-fed and normal rat liver 7α-hydroxylase activity suggest that a different form of P450 was induced during cholestyramine feeding. Similar inhibitory and stimulatory effects of these flavonoids have been shown for other substrates of cytochrome P450 [20]. Chiang *et al.* [21] have purified two cytochromes P450 catalyzing cholesterol 7α-hydroxylation from cholestyramine-treated female rats which are similar in activity and physical properties. These two forms of cholesterol 7α-hydroxylase, while not completely characterized biochemically, may represent the two forms of cytochrome P450, constitutive and cholestyramine-inducible, seen in our studies with male Sprague–Dawley rats. However, the constitutive form may be another hepatic cytochrome P450 possessing cholesterol 7α-hydroxylase activity, which may explain the identification of only one inducible cholesterol 7α-hydroxylase gene in rats [17, 22]. The 7α-hydroxycholesterol hydroxylase enzyme appears to be distinct from either of those involved in cholesterol 7α-hydroxylase activities. Further studies will be required to deduce the amino acid sequences and regulation of these P450s. Our findings that cholestyramine markedly increased the rate of formation of 7α-hydroxycholesterol and its metabolite suggest that 7α-hydroxycholesterol hydroxylation may be involved in the metabolism of cholesterol into unique bile acids or by a different metabolic pathway in the hamster liver.

The existence of other bile salts like 7β-hydroxy derivatives or the metabolite we have described in hamster liver has been considered to have beneficial effects *in vivo* [23, 24]. Since the bile acid metabolism

in hamsters is more similar to that of humans than rats, we are pursuing other experiments in the hamster to determine the potential of our findings and its possible relevance to human pathophysiology during cholestasis.

Acknowledgements—This study was supported in part by grants from the U.S. Public Health Service (ESO4244 RAP), from the University of Louisville School of Medicine, and from the Division of Gastroenterology, Department of Medicine, University of Louisville School of Medicine. Wu Song is a Visiting Fellow from the Department of Pharmacology, China Pharmaceutical University, Nanjing 210009, People's Republic of China. The authors wish to thank Chem Jiaming for her excellent technical assistance.

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