Bile acid synthesis: 7α-hydroxylation of intermediates in the sterol 27-hydroxylase metabolic pathway

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Abstract The recognition that the 7α-hydroxylation of 27-hydroxycholesterol is catalyzed by an enzyme that is different from cholesterol 7α-hydroxylase raises the question as to the number of similar enzymes that may be present in liver and subserve bile acid synthesis. Thus, both 3β-hydroxy-5-cholestenoic acid and 3β-hydroxy-5-cholenoic acid, further oxidation products derived from 27-hydroxycholesterol, are also 7α-hydroxylated during their metabolism to chenodeoxycholic acid. Using a microsomal fraction of hamster liver and competition plot analysis, we found that the 7α-hydroxylase activity for the acid substrates was approximately one-tenth that found for 27-hydroxycholesterol. Mixtures of the different substrates did not depress the total rate of 7α-hydroxylation. The evidence supports the view that these substrates share the same catalytic site on a single enzyme.—**Lee, C., K. O. Martin, and N. B. Javitt.** Bile acid synthesis: 7α-hydroxylation of intermediates in the sterol 27-hydroxylase metabolic pathway. *J. Lipid Res.* 1996. **37:** 1356–1362.

Supplementary key words microsomal 7α-hydroxylases • hamster liver • 27-hydroxycholesterol • 3β-hydroxy-5-cholestenoic acid • 3β-hydroxy-5-cholenoic acid

With the discovery that the microsomal fraction prepared from human and hamster livers contains different 7α-hydroxylase enzymes for the metabolism of cholesterol and 27-hydroxycholesterol (1), it became apparent that two pathways exist for the synthesis of chenodeoxycholic and cholic acids. In the absence of 27-hydroxycholesterol 7α-hydroxylase activity, the multifunctional mitochondrial enzyme catalyzing the side chain oxidation of cholesterol would further metabolize 27-hydroxycholesterol to 3β-hydroxy-5-cholestenoic acid. Further side chain oxidation of this C_{27} acid in peroxisomes generates 3β-hydroxy-5-cholenoic acid. In addition to 27-hydroxycholesterol, both these monohydroxy C_{27} and C_{24} acids are 7α-hydroxylated on a metabolic pathway leading to chenodeoxycholic acid synthesis.

Although none of the microsomal 7α-hydroxylases catalyzing these enzymatic activities has been purified, techniques exist (2) for determining whether the substrates share the same primary site on a single enzyme when a mixture of enzymes is present, as occurs in liver microsomes. We applied this method (2) to determine whether one or more enzymes subserves the 7α-hydroxylation of these substrates.

EXPERIMENTAL

Animals

Livers were removed from male Syrian hamsters using an approved protocol. The animals were obtained from Charles River Laboratories (Wilmington, MA) and were fed a regular rodent chow diet except that in some studies 4% cholestyramine was added to the diet for 7 days.

Enzyme assays

Microsomal fractions of hamster liver were prepared by differential ultracentrifugation using 0.25 M sucrose as described in detail previously (1). For each substrate, preliminary studies were done to establish conditions in which product formation was proportional to time and to the amounts of microsomal protein. It was found that zero order kinetics were obtained when the substrate concentration was 50 μM and the amounts of microsomal protein ranged from 50 to 100 μg. The incubation

Abbreviations: GLC-MS, gas-liquid chromatography-mass spectrometry; HPLC, high performance liquid chromatography; HECAMEG, 6-O-(N-heptylcarbamoyl)-methyl-α-D-glucopyranoside.

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period was 15 min for most studies. The total volume of the enzyme reaction mixture was 0.25 ml and the reaction was stopped by adding 50 µl glacial acetic acid. An appropriate internal standard (500 ng) was then added and the sterols or acids were extracted into ethyl acetate. The 7α-hydroxylated sterol product was analyzed by isotope ratio GLC–MS after addition of deuterated cholest-5-ene-3β,7α,27-triol (500 ng) as described in detail previously (3). The acidic metabolites were analyzed as fluorescent derivatives by HPLC using either ursodeoxycholic or 5β-cholanoic acid as an internal standard. Heat-inactivated microsomes served as the control. These assays were done in parallel under identical conditions and the values were routinely subtracted.

Solubilization of enzymes

Microsomal fractions of hamster liver were solubilized using Emulgen (Kao Corp. Tokyo, Japan) as described previously for the solubilization and purification of cholesterol 7α-hydroxylase (4). In addition, HECAMEG, 6-O-(N-heptylcarbamoyl)-methyl-α-D-glucopyranoside) (Vegatec, Villejuif, France), a new surfactant for the solubilization of membrane proteins, was also used according to the manufacturer's instructions. Microsomes were exposed for 30 min at 0°C to 1% HECAMEG in 0.1 M K phosphate buffer, pH 7.4, containing 1 mM EDTA and 20% glycerol. Soluble and insoluble fractions were obtained by centrifugation at 150,000 *g* for 60 min and the pellet was resuspended in the original volume of the same buffer. After the solubilization procedure, the detergents were removed from the soluble fraction on a Bio-beads SM-2 column (Bio-Rad Lab Inc. Melville, NY) using a 2-ml bed volume prior to enzyme assay.

Analysis of 3β,7α-dihydroxy-5-cholestenoic and 3β,7α-dihydroxy-5-cholenoic acids by HPLC using a fluorescent detector

Minor modifications of the procedure of Kamada, Masako, and Tsuji (5) to accommodate the micro scale of our assay were used. Addition of 50 µl 0.01% KOH in methanol to the ethyl acetate extract converted the acids to their potassium salts. After removal of the methanol in vacuo, 20 µl of a solution containing 0.05 µmol of dicyclohexyl-18-Crown-6-ether (Aldrich, Milwaukee, WI) and 0.5 µmol of 1-bromacetylpyrene (Wako Chemical Co., Osaka, Japan) in 40 µl acetonitrile was added to form the acetylpyrene esters. The reaction mixtures were placed in a heating block at 40°C for 30 min, then in an ice bath, and were diluted with 5 ml acetonitrile; 10-µl aliquots were injected onto a Supelcosil LC-18 column (4.6 mm × 5 cm, 3 µ; Supelco, Bellefonte, PA).

For HPLC analysis, a Shimadzu (Model # LC-6A) dual pump instrument was used with a Chrompack (Raritan, NJ) fluorescence detector (Cat # 57191). The excitation/emission wavelengths were 360 nm and 460 nm, respectively.

Chromatography was performed using a linear gradient with acetonitrile–methanol 2:1 (v/v) mixed with water. Gradient elution began with 25% water, the water content was decreased to 0% during the initial 25 min, and an isocratic phase of acetonitrile–methanol 2:1 lasted for 15 min. Flow rate was constant at 1.0 ml/min throughout. Using standards of each of the acids, fluorescence was found to be proportional to concentration and therefore the area of the internal standard was used to calculate the amount of each compound.

Analysis of 7α,27-dihydroxycholesterol

Isotope ratio mass spectrometry with deuterated 7α,27-dihydroxycholesterol as an internal standard was used as described in detail in our previous publication (3).

Syntheses

3β-Hydroxy-5-cholestenoic acid was prepared from the 3-acetate of 27-hydroxycholesterol by Jones oxidation; the products were extracted into ethyl acetate. After removal of the acetate, the free acid was recrystallized from aqueous methanol and was shown to be homogeneous by thin-layer chromatography on silica gel G using a solvent system of isooctane–isopropyl ether–ethyl acetate–acetic acid 4:4:2:0.5 (v/v). GLC–MS analysis of the methyl acetate ester yielded the characteristic ions at *m/z* 412 (M_o–60), 291, and 255.

The 7α- and 7β-hydroxy derivatives of 3β-hydroxy-5-cholestenoic acid were prepared from the methyl ester acetate of 3β-hydroxy-5-cholestenoic acid using copper bromide and tertiary butyl peroxybenzoate as described (6) and each of the isomers was separated and purified by thin-layer chromatography.

The 3β-hydroxy-5-cholenoic acid was purchased from Research Plus (Bayonne, NJ) and was recrystallized from aqueous dimethylformamide. The 7α- and 7β-hydroxylated derivatives were prepared from the methyl ester acetate as described above. Nafimidone (1-[2-(naphthoylmethyl]imidazole hydrochloride) was a gift from Dr. Keith Walker at Syntex Research, Palo Alto, CA.

Experimental design of active site competition

Substrate competition studies were done precisely as described (2) for determining whether catalysis of two different compounds is occurring at the same active site. For this analysis the concentrations (a_o, b_o) of the two substrates that will yield the same rate of 7α-hydroxylation as that obtained when each is incubated individually are established. Based on this information, mixtures of

the two substrates are prepared where p varies from 0 to 1 and specifies the concentrations for each substrate, $(1-p)$ a_o and pb_o, to be used in combination. The rate of 7α-hydroxylation of each substrate is then determined and the values are plotted against p.

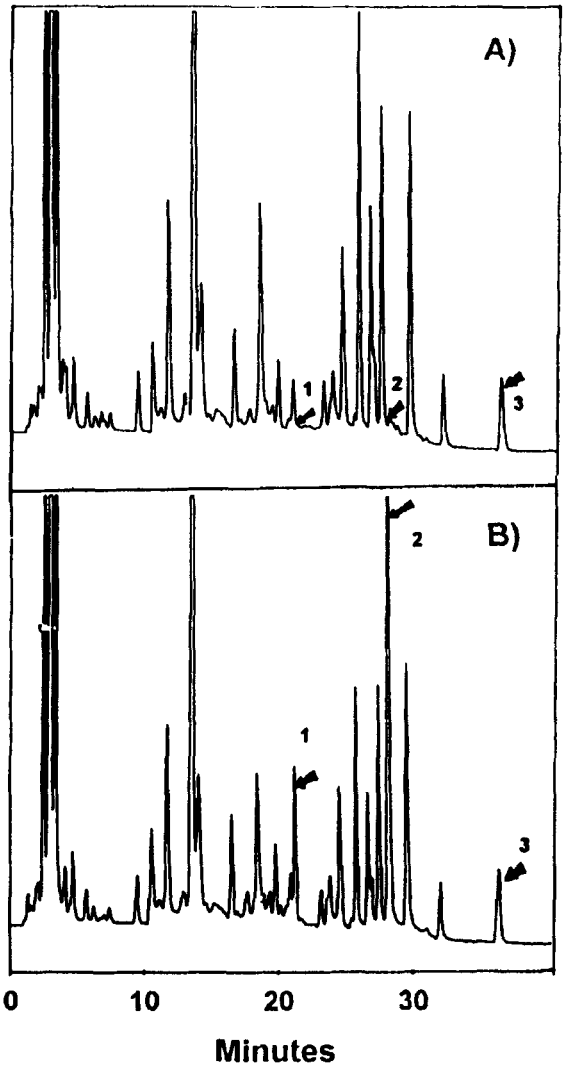

Fig. 1. HPLC analysis of acetylpyrene derivatives in the ethyl acetate extract of microsomes from hamster liver incubated with and without added 3β-hydroxy-5-cholestenoic acid. Panel B (lower) indicates the retention time of the metabolite, 3β,7α-dihydroxy-5-cholestenoic acid (1), together with the substrate, 3β-hydroxy-5-cholestenoic acid (2), and the internal standard, 5β-cholanoic acid (3). Panel A (upper) shows the fluorescent peaks obtained from the ethyl acetate extract of incubated control hamster liver microsomes. As indicated by the arrows in the top panel, although numerous fluorescent peaks are present, they do not have the same retention times as that of 3β-hydroxy-5-cholestenoic acid and the 7α-hydroxy metabolite, respectively.

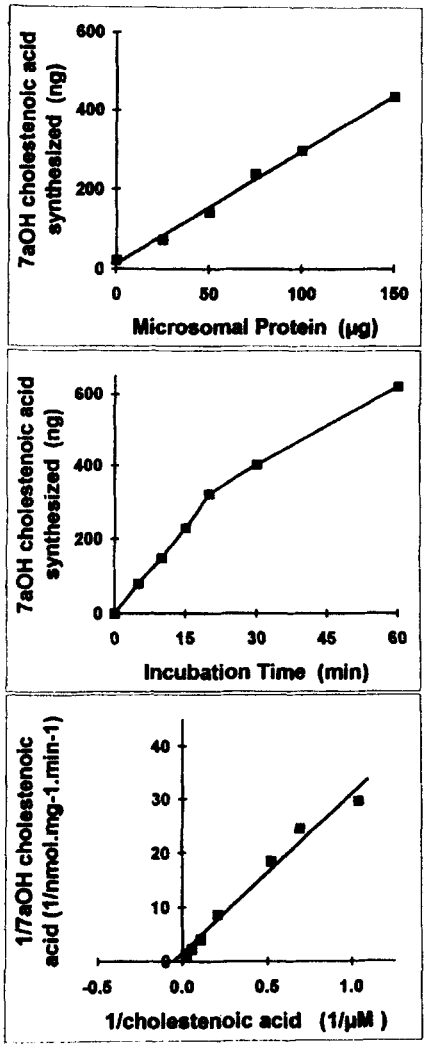

Fig. 2. Assay conditions for 7α-hydroxylation of 3β,7α-dihydroxy-5-cholestenoic acid by hamster liver microsomes. Hamster liver microsomes (100 µg protein) were incubated with 3β-hydroxy-5-cholestenoic acid at 37°C × 15 min and the rate of 7α-hydroxylation was determined by HPLC. Lineweaver-Burk analysis (bottom panel) indicated a K_m of 18 µM and a V_{max} of 604 pmol · mg^{-1} · min^{-1}. The middle panel indicates that product formation was proportional to time (microsomal protein = 100 µg). The top panel depicts the relationship of product formation to the amount of microsomal protein.

RESULTS

Figure 1 indicates the HPLC chromatograms obtained from hamster liver microsomes incubated without (upper panel A) and with 3β-hydroxy-5-cholestenoic acid (lower panel B). Although it is known that many fluorescent peaks occur using the method as described (4), none were found at the retention times for the substrate (peak # 2, 26 min) and its 7α-hydroxylated product (peak # 1, 20.1 min). A 7β-hydroxy metabolite (19.3 min) was not detected. Heat-inactivated mi-

TABLE 1. 7α-Hydroxylase activities in hamster liver microsomes on intermediates of bile acid synthesis

Substrate	7α-Hydroxylase Activity	
	Control	Cholestyramine-fed
	pmol · min⁻¹ · mg⁻¹	
Cholesterol	315 ± 17	574 ± 28[a]
27-Hydroxycholesterol	2035 ± 281	2321 ± 194
3β-Hydroxy-5-cholestenoic acid	227 ± 10	327 ± 32
3β-Hydroxy-5-cholenoic acid	378 ± 41	475 ± 28

Values are expressed as mean ± SEM. Number of animals used: controls, n = 3; cholestyramine-fed, n = 4.
[a] $P < 0.01$.

crosomes incubated with the substrates did not generate peaks corresponding to the 7α-hydroxylated metabolites (figure not shown).

For the 7α-hydroxylated product of 3β-hydroxy-5-cholenoic acid, small adjacent peaks interfered with integration causing significant inaccuracies when small product peaks were present.

Figure 2 indicates that the conditions established to assay 7α-hydroxylase activity for the C_{27} acid provided rates that were proportional to time, substrate concentration, and microsomal protein. The same conditions were also suitable to assay the C_{24} acid (Rt = 21 min) and the 7α-hydroxylated product (Rt = 13.5 min) (data not shown). As these conditions do not differ significantly from those previously found for the 7α-hydroxylation of 27-hydroxycholesterol, all the substrates could be studied under the same conditions.

Table 1 indicates the rate of 7α-hydroxylation of each of the substrates using a 50 μM concentration. Although higher 7α-hydroxylase activities were found in microsomes prepared from the livers of animals that were given cholestyramine, statistical significance at the 1% level was obtained only with cholesterol as the substrate. The rate of 7α-hydroxylation of 27-hydroxycholesterol was more than 5-fold greater than that with either of the acidic substrates.

Table 2 indicates the effect of cholesterol, 27-hydroxycholesterol, the two acid intermediates, and nafimidone on the rate of 7α-hydroxylation of 3β-hydroxy-5-cholestenoic and 3β-hydroxy-5-cholenoic acids. Adding an equimolar amount of 27-hydroxycholesterol decreased the rate of 7α-hydroxylation of the C_{27} acid by 57% and that of the C_{24} acid by 29%. Addition of an equimolar amount of the C_{27} acid significantly inhibited 7α-hydroxylation of 27-hydroxycholesterol but had no apparent effect on the 7α-hydroxylation of the C_{24} acid. Nafimidone at a concentration of 50 μM caused more than 95% inhibition of the 7α-hydroxylation of 27-hydroxycholesterol and the acidic substrates but had no effect on the 7α-hydroxylation of cholesterol.

To gain further insight in the mechanism of the competitive inhibition, mixtures of the C_{27} acid and 27-hydroxycholesterol in various ratios were prepared and the rates of 7α-hydroxylation of each substrate were determined. As shown in **Fig. 3,** 0.45 μM 27-hydroxycholesterol and 45 μM C_{27} acid, each incubated alone, gave the same rate of 7α-hydroxylation. Eight different mixtures of the two substrates, prepared as described above and ranging from $p = 0.2$ to $p = 0.8$, also yielded equal total rates of 7α-hydroxylation. Thus, essentially a straight line was obtained when the total rates were plotted against p.

Table 3 indicates the enzyme activities that were obtained before and after solubilization of membrane-bound cholesterol 7α-hydroxylase and 27-hydroxycholesterol 7α-hydroxylase. Because of the almost complete loss of activity of the latter enzyme using either Emulgen or HECAMEG, no further attempt was made to separate the two enzymes by column chromatography.

TABLE 2. Microsomal 7α-hydroxylase activities of hamster liver: competitive effects of cholesterol metabolites and nafimidone

Competitor[b]	Substrates[a]			
	Cholesterol	27OH-Chol.	C_{27} Acid	C_{24} Acid
	7α-hydroxylase activities (%)			
None	100	100	100	100
Cholesterol	-	90	103	104
27-Hydroxycholesterol	92	-	43	71
3βOH-5-cholestenoic acid	101	64	-	98
3βOH-5-cholenoic acid	100	102	71	-
Nafimidone	93	4	3	4

Values given as mean of two separate experiments, each analyzed with duplicates.
[a] Substrates: cholesterol; 27OH-chol., 27-hydroxycholesterol; C_{27} acid, 3β-hydroxy-5-cholestenoic acid; C_{24} acid, 3β-hydroxy-5-cholenoic acid.
[b] Each competitor was added in an equimolar concentration to the substrate.

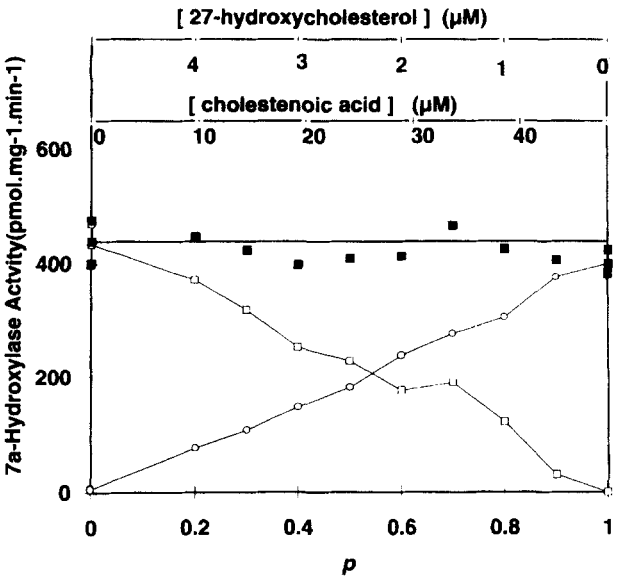

Fig. 3. Competition plot analysis of the rates of 7α-hydroxylation of 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid. 7α-Hydroxylation of 27-hydroxycholesterol (open circle) and 3β-hydroxy-5-cholestenoic acid (open square) at concentrations $(1-p) \times 0.45$ μM and $p \times 45$ μM, respectively, where p was varied from 0 to 1. The total rate of 7α-hydroxylation remained constant (closed squares) whereas the individual rates varied in proportion to their amounts in each mixture.

DISCUSSION

Although it was known that the 7α-hydroxylation of cholesterol is catalyzed by an enzyme that has very restricted substrate specificity (7), the concept that other 7α-hydroxylases may also participate in bile acid synthesis did not come into focus until the recognition that chenodeoxycholic and cholic acids could be derived from intermediates that are generated by the initial side chain oxidation of cholesterol (8, 9). The finding that a different 7α-hydroxylase present in the microsomal fraction of hamster and human liver (1, 10) and in other species in which both activities have been determined (11–13) in addition to the existence of activity in fibroblasts (14) confirmed the independence of this pathway from that beginning with the 7α-hydroxylation of cholesterol.

In the present study we specifically addressed the question as to how many different 7α-hydroxylases may be present in the microsomal fraction of hamster liver that subserves bile acid synthesis. It is known that 3β-hydroxy-5-cholestenoic acid and 3β-hydroxy-5-cholenoic acid (15) circulate in plasma and that each of these monohydroxy bile acids is further metabolized to chenodeoxycholic acid. Thus, a 7α-hydroxylation step must occur in vivo but its relationship to the 7α-hydroxylation of 27-hydroxycholesterol has not been defined.

To facilitate the analysis of large numbers of samples, we chose to prepare derivatives using 1-bromoacetylpyrene (4). This fluorescent reagent forms a stable carboxylic acid ester under mild conditions in the presence of Crown ether catalysts, obviating the need for both methylation and esterification. Sensitivity is greater than usually obtained by GLC using a flame detector and the peaks can be collected and their chemical identity further verified by other techniques, if necessary. However, because of the presence of trace amounts of many other organic acids, unidentified fluorescent peaks are obtained and the challenge is to find suitable columns and elution conditions that provide unique retention times for the compounds of interest. Although we have not yet fully achieved this goal, the conditions that have been developed permit the recovery and detection of as little as 0.1 pmole of 3β,7α-dihydroxy-5-cholestenoic acid and its metabolite from biologic material.

Using these methods we established that the microsomal fraction of hamster liver can 7α-hydroxylate both 3β-hydroxy-5-cholestenoic acid and 3β-hydroxy-5-cholenoic acid in addition to 27-hydroxycholesterol.

The competitive inhibition studies using 27-hydroxycholesterol and the C_{27} acid as substrates conform precisely to that characteristic for substrates that share a

TABLE 3. Solubilization of microsomal P450 enzymes in Emulgen and HECAMEG: effect on 7α-hydroxylase activities

Microsomes	Chol 7α	27OH 7α	Chol 7α	27OH 7α
	pmol · min⁻¹ · mg protein⁻¹		*% activity*	
Pre solubilization (3	175 ± 23	1494 ± 201	100	100
Post solubilization				
Insoluble protein				
Emulgen (3)	4.0 ± 2	13.0 ± 2	2	0.9
HECAMEG (2)	9.0	10.0	5	0.7
Soluble protein[a]				
Emulgen (3)	79 ± 12	174 ± 4	55	12
HECAMEG (2)	21	133		9

Number of studies in parentheses. Values given as mean ± SD.
[a]Detergent removed prior to enzyme assay (see Methods).

common active site of an enzyme (2). Thus, eight different mixtures of the two substrates in the molar amounts indicated did not cause any significant change in the total rate of 7α-hydroxylation. Although the method was validated using hexokinase and galactokinase, it is particularly useful for cytochrome P450 enzymes, as they are known to change substrate specificities during their purification.

With respect to the C_{24} acid, the same strategy gave anomalous results, probably because of the shortened side chain. Thus, a recent study of the active site of rat and human cytochrome P450 2E1 using a variety of alcohols and carboxylic acids that are known to be competitive inhibitors of N-nitrosodimethylamine demethylation (16) showed that the K_i values ranged from 22 to 20,100 μM. It was concluded that although all the substrates fit the active site in the pocket of the enzyme, the length of the molecule and the negative charge on the side chain greatly modify access to the channel and thus determine the concentration needed to obtain evidence for competitive inhibition. Because of the insolubility of 3β-hydroxy-5-cholenoic acid it was not possible to achieve a concentration that may have given evidence for competitive inhibition.

Although it would be of interest to study the substrate specificity of a purified 27-hydroxycholesterol 7α-hydroxylase, the information might not be applicable to the focus of this study. Thus purified P450 enzymes are known to change substrate specificities (17) and other microsomal enzymes that might catalyze 7α-hydroxylation of these substrates would be lost in the purification procedure. Thus our findings, using the whole microsomal fraction and a validated technique for distinguishing between one or more catalytic sites, support the view that a single enzyme determines the 7α-hydroxylation step in the metabolic pathway beginning with the 27-hydroxylation of cholesterol.

The findings with nafimidone (18) appear selective because of the absence of an effect on cholesterol 7α-hydroxylase, implying that if a separate enzyme does exist for the 7α-hydroxylation of 3β-hydroxy-5-cholenoic acid, it is very similar to that catalyzing the same step for 27-hydroxycholesterol and the C_{27} acid.

With respect to the overproduction of monohydroxy bile acids, which can induce a cholestatic syndrome (18), our findings lead to the hypothesis that inhibition of this single microsomal 7α-hydroxylase would result in increased production of 3β-hydroxy-5-cholenoic acid. Further studies of the determinants that regulate activity of this enzyme can give insights on the pathogenesis of drug-induced cholestatic syndromes.

This work was supported by Grant DK32995 from the National Institute of Diabetes, Digestive, and Kidney Diseases, National Institutes of Health. We thank Mrs. Suzanne Javitt for her editorial assistance in preparing this manuscript. C. L. was supported by a Summer Student Research Program (T35 DK-07421) from the National Institutes of Health.

Manuscript received 29 November 1995 and in revised form 8 March 1996.

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