Cholesterol and 27-hydroxycholesterol 7α-hydroxylation: evidence for two different enzymes

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Abstract The use of 2-hydroxypropyl-β-cyclodextrin as a vehicle for solubilizing cholesterol and 27-hydroxycholesterol has led to a study of their rates of 7α-hydroxylation in microsomal preparations from hamster liver and HepG2 cells. Addition of the vehicle alone to the cholesterol 7α-hydroxylase assay always caused a several-fold increase in activity. Preloading the vehicle with cholesterol further augmented the rate of 7α-hydroxycholesterol formation. Preloading the vehicle with 27-hydroxycholesterol or 27-hydroxycholestanol (molar ratio 1/1.2) minimally decreased cholesterol 7α-hydroxylase activity (−12%), compared with preloading with cholestanol (−50%), a known competitive inhibitor of the enzyme. Microsomes from hamster liver yielded rates of 7α,27-dihydroxcholesterol formation of 1.5 to 3.0 nmol/min per mg protein, compared with 0.3 nmol/min per mg protein for 7α-hydroxycholesterol. Although cholesterol and cholestanol had minimal effects on the rate of 7α-hydroxylation of 27-hydroxycholesterol, addition of an approximately equimolar amount of 27-hydroxycholestanol inhibited the rate of formation by 65%. Attempts to separate and identify the two C-27 sterol 7α-hydroxylases chromatographically led to the finding that Emulgen 913 selectively inactivates 7α-hydroxylation of 27-hydroxycholesterol. These results indicate that the metabolic pathway for bile acid synthesis from 27-hydroxycholesterol is not governed by cholesterol 7α-hydroxylase. **—Martin, K. O., K. Budai, and N. B. Javitt.** Cholesterol and 27-hydroxycholesterol 7α-hydroxylation: evidence for two different enzymes. *J. Lipid Res.* 1993. **34:** 581–588.

Supplementary key words 2-hydroxypropyl-β-cyclodextrin • cholestanol

Recognition that in humans both 7α-hydroxycholesterol and 27-hydroxycholesterol[2] are metabolized to cholic and chenodeoxycholic acids (1) raised the possibility that two different metabolic pathways can lead to bile acid synthesis from cholesterol.

As it is well established that cholesterol 7α-hydroxylase is the rate-limiting step for one pathway, a key question concerns the role of this enzyme in the metabolic pathway beginning with 27-hydroxycholesterol.

Previous studies of the substrate specificity of a solubilized and purified cholesterol 7α-hydroxylase (2) and of bile acid synthesis in HepG2 cells (3) both implied that cholesterol 7α-hydroxylase does not participate in the metabolic pathways beginning with 27-hydroxycholesterol. Based on these findings we began to test critically the hypothesis that different enzymes catalyze the 7α-hydroxylation of cholesterol and 27-hydroxycholesterol, using 2-hydroxypropyl-β-cyclodextrin, a molecule with a hydrophobic cavity that accommodates sterols (4). Although we have not succeeded in isolating a 27-hydroxycholesterol 7α-hydroxylase, we believe that sufficient evidence has been obtained to propose that it is different from cholesterol 7α-hydroxylase.

MATERIALS AND METHODS

Liver tissue

Syrian hamsters each weighing between 90 and 110 g were anesthetized and the abdomens were opened surgically to permit rapid chilling of the liver by perfusion of the portal vein with ice cold 0.25 M sucrose containing 10 mM Tris-HCl buffer and 1 mM EDTA, pH 7.4. The livers were removed and weighed and portions were taken for the preparation of microsomes.

In one study, three hamsters received a chow diet supplemented with 4% cholestyramine for 7 days prior to removal of the liver.

HepG2 cells were cultured as reported in detail previously (5), with the exception that the Dulbecco's Modified Eagle Medium that was used (DMEM 4500; Gibco,

Abbreviations: 2-HPBCD, 2-hydroxypropyl-β-cyclodextrin; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; TMS, trimethylsilyl.

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[2]Recent publications have chosen to use 27-hydroxycholesterol rather than the conventional name 26-hydroxycholesterol to indicate that the mitochondrial enzyme stereospecifically hydroxylates only the methyl group in position C-27. The stereochemistry is depicted in Javitt, N.B. 1990. 26-Hydroxycholesterol: synthesis, metabolism, and biologic effects. *J. Lipid Res.* **31:** 1527–1534.

Grand Island, NY) contained a higher concentration of glucose than the standard medium. After 7 days of growth the cells were harvested by scraping with a rubber policeman.

Preparation of microsomes

Syrian hamster liver was homogenized in chilled 0.25 M sucrose (4 ml/g of liver) using a Potter-Elvehjem homogenizer. Sequential centrifugation at 800 *g* for 10 min and 20,000 *g* for 20 min, respectively, removed nuclei, cell debris, and mitochondria. The microsomal pellet, obtained by another centrifugation at 100,000 *g* for 60 min, was washed once in 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM EDTA, and was resuspended in the same buffer; the suspension was then divided into aliquots that were kept at −80°C. No loss of activity for either cholesterol 7α-hydroxylase or 27-hydroxycholesterol 7α-hydroxylase was noted to occur in samples stored frozen for several months.

Harvested HepG2 cells were suspended in 0.25 M sucrose and disrupted by nitrogen cavitation using a Parr bomb (6), a technique particularly useful for cells in culture (7). Microsomal fractions were then prepared as described above. In some studies the pellet consisted of the whole particulate fraction, obtained by centrifugation at 330,000 *g* for 30 min after initial removal of cell debris and nuclei by centrifugation at 1000 *g* for 10 min. The pellet was suspended in 0.1 M potassium phosphate (pH 7.4) containing 1 mM EDTA.

Microsomal fractions of human liver were a gift from Dr. David Swinney, Syntex Research (Palo Alto, CA). Periodic assay of cholesterol 7α-hydroxylase activity indicated no loss of activity in the frozen state over a 12-month period.

Enzyme assays

Cholesterol 7α-hydroxylase. We recently reported the usefulness of 2-hydroxypropyl-β-cyclodextrin (2-HPBCD, mol wt = 1500) as a vehicle for solubilizing sterols for both cell culture and in vivo studies (4) and we have now extended these studies to its use in the assay of cholesterol 7α-hydroxylase. For these studies the vehicle was purchased as a 45% sterile solution from Pharmatec, Alachua, FL.

The usual assay mixture consisted of 50 μg microsomal protein in 0.25 ml 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA and 1 mM NADPH. After the studies that indicated the usefulness of 2-HPBCD preloaded with cholesterol for obtaining maximum enzyme activities, the standard assay mixture contained 5 μg of cholesterol in 5 μl of 2-HPBCD, giving final concentrations of 52 μM and 6 mM, respectively.

The assay tubes containing all components except NADPH were prepared in an ice bath. The tubes were then prewarmed to 37°C for 3 min prior to the addition of NADPH to initiate the enzymatic reaction. Zero-min controls were always run in parallel by the addition of 5 N NaOH at 3 min followed by chilling and the addition of NADPH. Zero-min values ranged from 0.1 to 0.8 ng of 7α-hydroxycholesterol. The rate of product formation was found to be directly proportional to time for as long as 15 min. For routine purposes the incubation was terminated at 10 min by adding 20 μl of 5 N NaOH.

[^{2}H]7α-hydroxycholesterol was then added to the assay mixture, the sterols were extracted with ethyl acetate, and the fraction containing 7α-hydroxycholesterol was obtained by TLC on silica gel with a solvent system of $CHCl_3$–CH_3OH 95:5 (v/v).

27-Hydroxycholesterol 7α-hydroxylase assay. Development of this assay paralleled the techniques used for the validation of the cholesterol 7α-hydroxylase assay. The usual assay mixture contained 5 μg of stereospecific 27-hydroxycholesterol (25R,26-hydroxycholesterol; Research Plus, Bayonne, NJ) in 5 μl of 2-HPBCD. Attempts to use acetone as a vehicle yielded much lower estimates of enzyme activity. Endogenous 27-hydroxycholesterol was not detected in the microsomal fractions analyzed at 0 min (minimum detectable amount 0.2 ng). At the end of the 10-min incubation period [^{2}H]7α,27-dihydroxycholesterol prepared from [^{2}H]27-hydroxycholesterol (8) by a known procedure (9) was added and the TLC fraction containing the 7α-hydroxylated product was obtained as described above.

As part of these studies the effect of the following compounds on enzyme activities was determined: cholestanol; deoxycholic acid (Steraloids, Wilton, NH); 27-hydroxycholestanol, prepared by palladium-catalyzed hydrogenation of stereospecific 27-hydroxycholesterol; 3β-hydroxy-5-cholestenoic acid prepared by chromate oxidation of 27-hydroxycholesterol-3-acetate; and Emulgen 913 (Kao Corp., Tokyo, Japan). All the compounds were dissolved in 45% 2-HPBCD (1–2 mg/ml) and then diluted with buffer to obtain the appropriate concentration for evaluating their effects on the 7α-hydroxylase assays.

Solubilization of microsomal cholesterol using 2-HPBCD

As part of the validation of the cholesterol 7α-hydroxylase assay using 2-HPBCD, the effect on enzyme activity of removing endogenous cholesterol from the microsomes and then reconstituting them was evaluated. For this purpose 2.0-ml aliquots of hamster liver microsome suspension (500 μg of protein, 12.1 μg total cholesterol) in 0.1 M potassium phosphate buffer with 1 mM EDTA, pH 7.4, were placed in centrifuge tubes and were maintained at either 4°C or 37°C (two five-tube sets at each temperature). To one set at each temperature, 2-HPBCD was added to a final concentration of 30 mM. At 10, 30, 60, 120 min, and 24 h, one tube from each set was removed and immediately centrifuged in a chilled rotor at 4°C for 40 min at 150,000 *g* to recover the

microsomal pellet. The pellets, resuspended in their original volume, and the supernates were assayed for 7α-hydroxylase activities, protein content, and the amount of cholesterol before and after saponification.

Isotope ratio mass spectrometry

Using a Hewlett-Packard GLC–MS (Model #5890-5970) and a fused silica column (CP-sil 19 CB, 0.25 mm i.d., 25 m length; Chrompack, Raritan, NJ), the appropriate TLC fraction was injected in the splitless mode as the TMS ether with temperature programming from 260 to 270°C at 0.3°C/min and a column head pressure of 5 psi.

For quantifying 7α-hydroxycholesterol the detector was programmed in the simultaneous ion monitoring mode for *m/z* 456 and *m/z* 461 and the amount of endogenous 7α-hydroxycholesterol was calculated from the respective peak areas. The retention time of 7α-hydroxycholesterol TMS was 12.5 min and that of the 7β-hydroxycholesterol epimer was 14.2 min.

For quantifying 7α,27-dihydroxycholesterol the peak areas of *m/z* 544 (product) and 548 (standard), respectively, were determined. The retention time of the TMS ether was 22.8 min.

Determination of cholesterol

Cholesterol was quantified by GLC analysis using 5β-cholestan-3β-ol as an internal standard. A Shimadzu Model 14A instrument with a flame detector was used and samples were injected in the split mode (1:100) onto a fused silica column (CP-sil 5, 0.25 mm i.d., 50 m length; Chrompack, Raritan, NJ). The initial temperature was 245°C and programming was at 0.5°C/min with a head pressure of 3 kg/cm². Under these conditions the retention time of cholesterol was 10.9 min and that of the internal standard was 10.1 min. Linearity of response was found over a range of 0.02 to 2.0 μg injected.

Determination of protein

The Pierce (Rockford, IL) BCA Protein Assay Reagent based on the biuret reaction was used to measure protein.

RESULTS

Validation of enzyme assays

Linearity of product formation. As shown in **Fig. 1,** the amount of product formed at 10 min was proportional to the amount of microsomal protein, which ranged from 25 to 100 μg. In most studies each assay contained approximately 50 μg of microsomal protein and, as shown in **Fig. 2,** the rate of product formation was linear for at least 10 min. When the microsomes were placed in boiling water for 15 min prior to the addition of substrates and

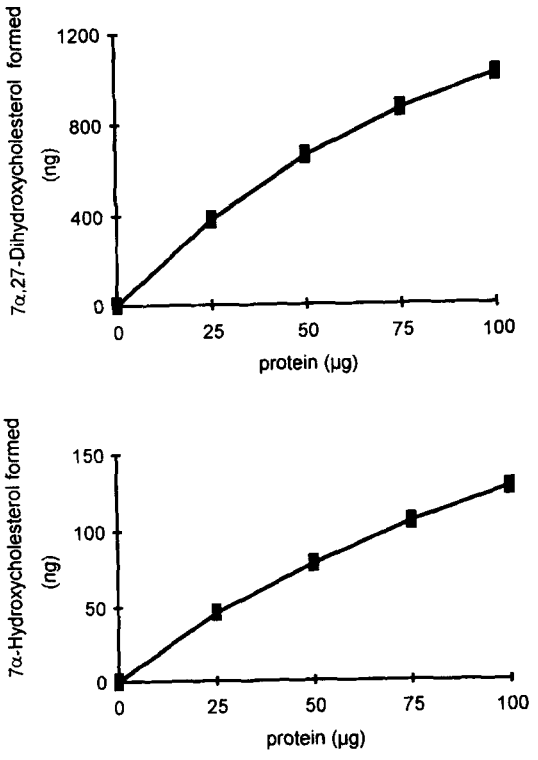

Fig. 1. Relationship of product formation to microsomal protein. To establish standard assay conditions, microsomal protein was varied over a range of 25 to 100 μg. As shown in the upper panel, the amount of 7α,27-dihydroxycholesterol that was formed was proportional to the amount of microsomal protein. Total incubation time was 10 min. As depicted in the bottom panel a similar relationship was found for 7α-hydroxycholesterol formation using the same assay conditions.

cofactors, no additional product was formed during the 10-min period of incubation.

Using these assay conditions the K_m and V_{max} for cholesterol 7α-hydroxylase were found to be 16.4 μM and 397 pmol/mg per min, respectively (**Fig. 3**), and those for 27-hydroxycholesterol 7α-hydroxylase, 24.8 μM and 2278 pmol/mg per min, respectively (Fig. 3).

Effect of 2-hydroxypropyl-β-cyclodextrin on cholesterol 7α-hydroxylase activities. Both 2-HPBCD alone and 2-HPBCD preloaded with cholesterol were added to the cholesterol 7α-hydroxylase assay. As shown in **Table 1,** the presence of 2-HPBCD alone in the assay mixture increased the rate of product formation. The rate was further increased when the 2-HPBCD contained cholesterol. Cholesterol 7α-hydroxylase activity was significantly greater in microsomes obtained from animals that had been maintained on a diet including cholestyramine. This difference in catalytic activity was maintained when the assay mixture contained 2-HPBCD.

Effect of depletion of microsomal cholesterol on cholesterol 7α-hydroxylase activity. It was found that 2-HPBCD rapidly solubilized microsomal cholesterol at 37°C. As shown in **Fig. 4,** 81% of microsomal cholesterol (9.8 μg) was ex-

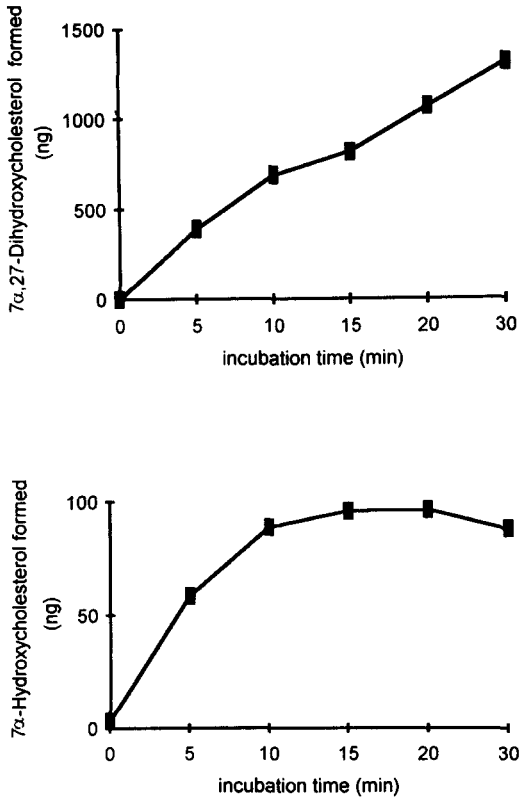

Fig. 2. Linearity of product formation with time. Based on the data illustrated in Fig. 1, the standard assay contained approximately 50 μg of protein. Both 7α,27-dihydroxycholesterol formation (upper panel) and cholesterol 7α-hydroxycholesterol formation (lower panel) were proportional to time for at least 10 min.

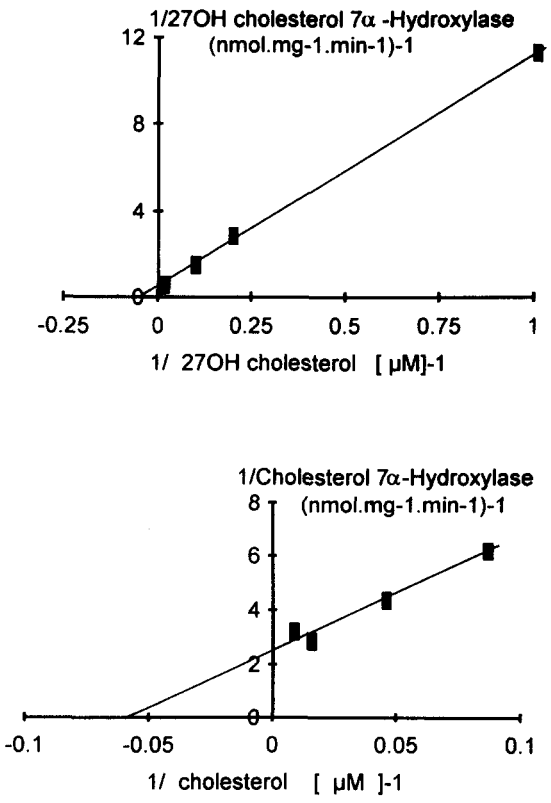

Fig. 3. Lineweaver-Burk plots for determination of enzyme kinetics. Using the standard assay conditions, 27-hydroxycholesterol 7α-hydroxylase was found to have a K_m of 24.8 μM and a V_{max} of 2278 pmol/mg per min (upper panel). Cholesterol 7α-hydroxylase was found to have a K_m of 16.4 μM and a V_{max} of 397 pmol/mg per min.

tracted within 10 min, the earliest time point tested. GLC analysis of the solubilized cholesterol before and after saponification showed that the concentration did not change, indicating that the solubilized fraction contained only free cholesterol. In contrast, the cholesteryl ester concentration of the microsomes rose from 6% before solubilization to 21% afterward.

As shown in **Fig. 5,** microsomes depleted of their cholesterol retained their cholesterol 7α-hydroxylase activity equivalent to that of nonextracted microsomes upon addition of a sufficient amount of substrate.

Microsomal 27-hydroxycholesterol 7α-hydroxylase activity. The methods used in this study did not detect either 27-hydroxycholesterol or 7α,27-dihydroxycholesterol in the microsomal fraction of hamster liver (lower limit of detection 5 ng).

The addition of 1 μg (2.5 nmol) of 27-hydroxycholesterol dissolved in 5 μl (67 μmol) of acetone to the standard

TABLE 1. Effect of 2-hydroxypropyl-β-cyclodextrin on cholesterol 7α-hydroxylase activity

	Cholesterol 7α-Hydroxylase Activity		
	Additions to Assay Mixture		
Hamster Microsomes	None	1.5 μmol 2-HPBCD[a]	1.5 μmol 2-HPBCD + 13 nmol Chol
	pmol/min/mg protein		
Control (n = 3)	20.5 ± 3.2	146 ± 17	328 ± 24
Cholestyramine-fed (n = 3)	33.0 ± 5.2[b]	267 ± 20[c]	574 ± 49[c]
Ratio Control/cholestyr.	1.61	1.82	1.75

Values given as mean ± standard error; n, number of animals.
[a]Five μl of 45% 2-HPBCD added to 250 μl assay mixture; final concentration = 6 mM.
[b]$P < 0.05$.
[c]$P < 0.01$.

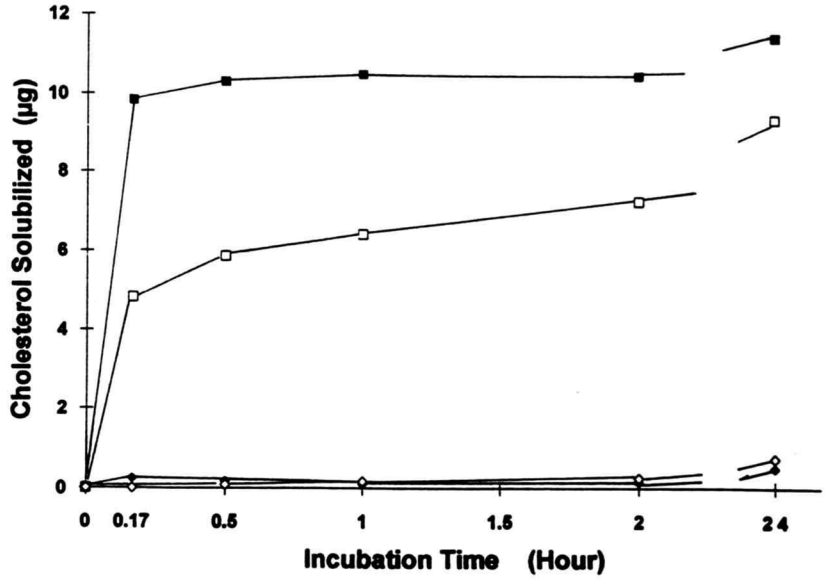

Fig. 4. Solubilization of microsomal cholesterol with 2-hydroxypropyl-β-cyclodextrin. Microsomal suspensions, each containing 12.1 µg of cholesterol, were incubated with buffer alone or buffer containing 0.1 ml of the vehicle for intervals ranging from 10 min to 24 h at 4° and 37°C. Virtually no cholesterol was solubilized in the absence of 2-HBPCD. In contrast, 9.1 µg, or 81%, of the total microsomal cholesterol was solubilized within 10 min at 37°C in the presence of the vehicle. Open and filled boxes = buffer + vehicle at 4° and 37°C, respectively; open and filled diamonds = buffer alone at 4° and 37°C, respectively.

assay mixture yielded 28 ng (67 pmol) of the 7α-hydroxylated product, which was much less than the 224 ng (536 pmol) found when the same amount of 27-hydroxycholesterol was dissolved in 5 µl (1.5 µmol) of 2-HPBCD. When 2-HPBCD was added to the assay mixture containing the acetone-dissolved substrate, the yield of product increased to that obtained with 2-HPBCD alone. Based on these findings, 2-HPBCD was used as the vehicle in all subsequent studies.

In male hamsters selected at random, 27-hydroxycholesterol 7α-hydroxylase activity was always found to be significantly greater than that of cholesterol 7α-hydroxylase. When litter-mates of the same age and sex were maintained on an identical chow diet with and without cholestyramine (**Table 2**), 27-hydroxycholesterol 7α-hydroxylase activity remained significantly greater ($P < 0.001$), in part attributable to the proportional increase in the activities of both enzymes when the diet contained the resin.

In contrast, in HepG2 cells the 27-hydroxycholesterol 7α-hydroxylase activity was much lower than that of cholesterol 7α-hydroxylase.

Mitochondrial preparations from hamster and HepG2 cells demonstrated the ability to 27-hydroxylate cholesterol but had no detectable activity of either 27-hydroxycholesterol 7α-hydroxylase or cholesterol 7α-hydroxylase (data not shown).

Microsomal cholesterol 7α-hydroxylase and 27-hydroxycholesterol-7α-hydroxylase activities and the effect of other compounds

As shown in **Table 3,** addition of an approximately equimolar amount of cholestanol (5 µg cholestanol/5 µg exogenous cholesterol + 1.2 µg endogenous cholesterol) decreased cholesterol 7α-hydroxylase activity by 50% but had only a minor effect on 27-hydroxycholesterol 7α-hydroxylase activity. In contrast, 7α-hydroxylation of 27-hydroxycholesterol decreased by 65% in the presence of 27-hydroxycholestanol.

The addition of cholestanol, 3β-hydroxy-5-cholestenoic acid, deoxycholic acid, or an additional amount of cholesterol did not decrease activity more than 16%.

Depleting microsomes of their cholesterol as described above had essentially no effect on 27-hydroxycholesterol 7α-hydroxylase activity (data not shown).

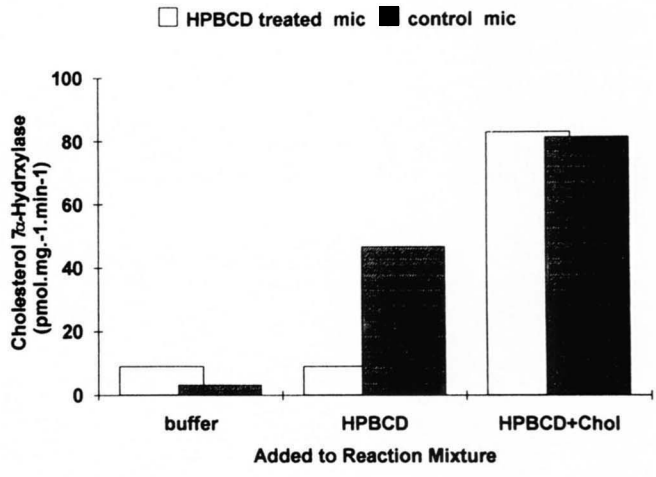

Fig. 5. Effect of depletion and reconstitution of microsomal cholesterol on cholesterol 7α-hydroxylase activity. Microsomes depleted of their cholesterol by the addition of 2-HPBCD (open squares) were then reconstituted with an equivalent amount of cholesterol that was preloaded into the vehicle. Enzyme activity was restored to the same level as that found in nondepleted microsomes (hatched squares).

TABLE 2. Cholesterol and 27-hydroxycholesterol 7α-hydroxylase in hepatic tissue

Microsomes	7α-Hydroxylase Activities		
	Cholesterol	27OH-Cholesterol	Ratio 27OH/Chol
	pmol/min/mg protein		
Hamster			
Random male (n = 6)	294 ± 31	2398 ± 407	8.2
Control (n = 3)[a]	328 ± 14	1585 ± 235	4.8
Cholestyramine (n = 3)[a]	574 ± 28	2480 ± 158	4.3
Human (n = 6)	6.7 ± 3.9	54 ± 16	8.0
HepG2 (n = 2)	102, 149	14, 16	0.1
HepG2 (n = 2)[b]	87, 91	3.4, 5.4	0.05

Values given as mean ± standard error; n, number of animals.
[a]Male litter mates on cholesterol-free chow diet without or with cholestyramine.
[b]Whole particulate fraction.

Effect of Emulgen 913 on sterol 7α-hydroxylase activities

Attempts to solubilize and separate the two 7α-hydroxylase activities by HPLC (10) were not successful. Further analysis of this phenomenon led to the finding that small amounts of Emulgen 913 added to the assay mixture selectively affected 7α-hydroxylation of 27-hydroxycholesterol. At a concentration of 0.2% detergent (**Fig. 6**), which is less than that necessary for solubilization of cytochrome P450 enzymes, only 30% activity remained, and the activity did not increase after removal of the detergent. Cholesterol 7α-hydroxylase activity did not fall below 70% of the initial activity.

DISCUSSION

The use of 2-HPBCD in the assay of cholesterol 7α-hydroxylase activity developed from our need to find a vehicle to study the 7α-hydroxylation of 27-hydroxycholesterol. Our previously published studies (4) indicated that, in comparison with other vehicles, the sterol remained in solution within the cavity of the cyclodextrin rather than precipitating from solution after dilution in aqueous media.

Cyclodextrins are known to have catalytic properties (11) that are attributed to their capacity to modify the interface between surfaces. The nonpolar interior of the molecule is host to many lipophilic compounds, which are then presented to the aqueous milieu in which the cyclodextrin is soluble. The findings in this study are consistent with the concept that the vehicle alters the availability of cholesterol to the active site of the enzyme. It is beyond the scope of this study to define further the nature of the interaction. Thus, in addition to substrate availability, one needs also to evaluate the rate of removal of product from the enzyme surface and the change in the configuration of the enzyme.

Accepting that cholestyramine induces an increase in the amount of cholesterol 7α-hydroxylase, our data indicate that the presence of 2-HPBCD in the assay mixture does not affect the magnitude of the increase compared with that in the appropriate control. The major value of including 2-HPBCD in the assay mixture is to aid in mea-

TABLE 3. Effect of various steroids on 7α-hydroxylase activities

Compound	7α-Hydroxylase Activities	
	Cholesterol	27-Hydroxycholesterol
	%	
None (n = 12)	100 ± 5.0	100 ± 4.0
Cholesterol (n = 6)	N/A	90 ± 4.3
27OH-cholesterol (n = 6)	92 ± 4.9	N/A
Cholestanol (n = 6)	50 ± 2.5[a]	89 ± 9.1[a]
27OH-cholestanol (n = 6)	80 ± 6.3	32 ± 5.7[a]
Cholestanoic acid (n = 6)	101 ± 4.9	90 ± 2.3
Deoxycholic acid (n = 6)	99 ± 7.0	99 ± 2.2

Values given as mean ± standard deviation; n, number of animals.
[a]$P < 0.001$.

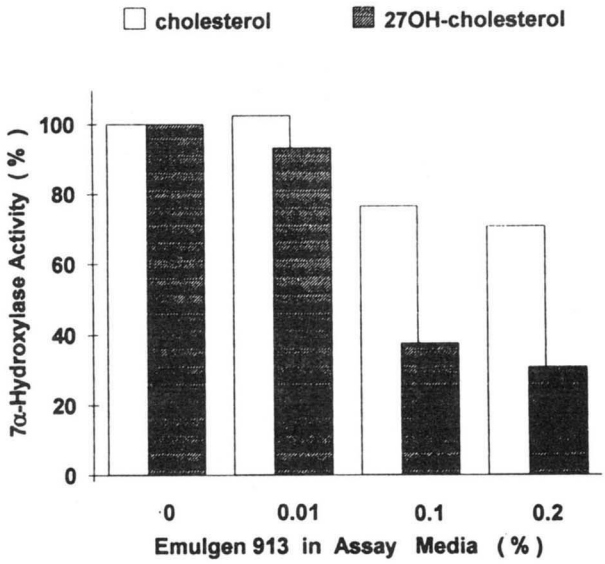

Fig. 6. Selective inhibition of 27-hydroxycholesterol 7α-hydroxylase activity by Emulgen 913. Emulgen was added to the standard assay mixture as indicated. At a concentration of 0.2% the activity of 27-hydroxycholesterol 7α-hydroxylase was 30% of control (hatched squares), compared with 75% for cholesterol 7α-hydroxylase (open squares).

suring low levels of activity such as exist in HepG2 cells and probably also when only small amounts of liver are available, as after percutaneous liver biopsy.

In developing 2-HPBCD as a vehicle for the study of 7α-hydroxylation of 27-hydroxycholesterol we found that the rate of hydroxylation was much lower when the substrate was dissolved in acetone than when 2-hydroxypropyl-β-cyclodextrin was the vehicle. Because the addition of the latter vehicle to the assay mixture containing acetone increased the activity to that found when the cyclodextrin was used alone, the lower activity in the presence of acetone was not attributable to enzyme inactivation. Because, as we established previously using 27-hydroxycholesterol (4) and is generally known, sterols dissolved in the usual organic vehicles will precipitate slowly after dilution in the aqueous medium at a rate that depends in part on their concentration and their solubility in each of the phases, we propose that the mechanism of solubilization of the substrate accounts for the major difference in the enzyme activities.

The first experimental evidence that two independently regulated metabolic pathways for bile acid synthesis may exist can be derived from the report by Ogishima, Deguchi, and Okuda (2) that highly purified cholesterol 7α-hydroxylase from rat liver does not catalyze the 7α-hydroxylation of 3β-hydroxy-5-cholenoic acid. Because this monohydroxy bile acid, when administered to hamsters and rats (12), is metabolized to chenodeoxycholic acid, it is reasonable to test the hypothesis that its 7α-hydroxylation is catalyzed by an enzyme different from cholesterol 7α-hydroxylase.

Further support for the hypothesis was obtained when it was found that 27-hydroxycholesterol added to HepG2 cells, which synthesize mostly chenodeoxycholic and cholic acids (3), was metabolized to 3β-hydroxy-5-cholenoic acid. By contrast, when 27-hydroxycholesterol is administered intravenously to humans (1), it is metabolized mostly to the 7α-hydroxylated bile acids.

To further test the hypothesis, it was essential to develop reliable assays for comparing cholesterol and 27-hydroxycholesterol 7α-hydroxylase activities.

Based on these assays we have obtained considerable data indicating that the two enzymes are different. Thus, no increase occurs in the rate of 7α-hydroxylation of 27-hydroxycholesterol when the microsomes are depleted of endogenous cholesterol and the decrease that occurs on addition of exogenous cholesterol or cholestanol is much less than that expected from an inhibitor on a molar basis. The findings are in accord with the data indicating that the activities of the two enzymes are independent in different tissues. Thus, in both HepG2 cells and human microsomes, the activity of 27-hydroxycholesterol 7α-hydroxylase activity is lower compared with that of cholesterol 7α-hydroxylase, in contrast to hamster liver where the activity is much higher. The apparent similarity in the effect on the enzyme activities induced by cholestyramine may be related to the relatively high rate of cholesterol 7α-hydroxylation in control animals on a chow diet since others (personal communication, David Swinney, Syntex Research), using 2-HPBCD, inform us that when the same study was done in hamsters fed a diet that included cholesterol, the increase in cholesterol 7α-hydroxylase activity after cholestyramine treatment was several-fold greater than that in 27-hydroxycholesterol 7α-hydroxylase activity.

Attempts to separate the two enzymes have not been successful thus far because of the marked inhibitory effect of Emulgen 913 on 27-hydroxycholesterol 7α-hydroxylase activity. Thus, a 0.2% concentration of Emulgen, which is insufficient for solubilization, decreased activity by 70%.

In addition to cholesterol, other endogenous steroids with a ring configuration identical to that of cholesterol and that are capable of being 7α-hydroxylated are 3β-hydroxy-5-cholenoic acid, 3β-hydroxy-5-cholestenoic acid, and cholest-5-ene-3β,27-diol (27-hydroxycholesterol). In contrast to the nonpolar side-chain of cholesterol, the side-chains of these compounds all contain hydroxyl groups that markedly alter their orientation and provide a rationale for believing that their affinity for cholesterol 7α-hydroxylase is markedly altered. The rationale is further supported by the knowledge that neither 7α-hydroxylation of 3β-hydroxy-5-cholenoic acid (2) nor that of 3β-hydroxy-5-cholestenoic acid is catalyzed by cholesterol 7α-hydroxylase (13). Our findings with 27-hydroxycholesterol as a substrate support the knowledge that

polarization of the side chain decreases or eliminates the likelihood that the steroid is a substrate for cholesterol 7α-hydroxylase.

Because the steroid ring configuration of all these compounds is the same, it is likely that considerable homology will be found in the primary site of the enzymes that catalyze 7α-hydroxylation. Perhaps when the primary structures of all the enzymes and the genes regulating their synthesis are known, new principles governing the regulation of P-450 enzymes will emerge, but for the present, we believe our data fully support the concept that hepatic cholesterol 7α-hydroxylase has a very narrow substrate specificity (14, 15) and that other steroids are 7α-hydroxylated by different enzymes.

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