Determination of hepatic cholesterol 7α-hydroxylase activity in man

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Abstract Methods were developed to determine the activity of the microsomal enzyme cholesterol 7α-hydroxylase in human liver. The enzyme assay could be performed with as little as 20 mg of fresh liver tissue, thus making the procedure applicable to specimens obtained by percutaneous liver biopsy. Optimal assay conditions were determined and the identity and radioactive purity of the reaction product, cholest-5-ene-3β,7α-diol (7α-hydroxycholesterol) were established. Specific enzyme activity was measured in a number of patients with disorders of lipid metabolism.

The first committed step in the biosynthesis of bile acids is the conversion of cholesterol to 7α-hydroxycholesterol (1). This transformation is catalyzed by the hepatic microsomal enzyme cholesterol 7α-hydroxylase, which is believed to be rate limiting for bile acid biosynthesis in experimental animals and in man (2–7). In order to study the activity of cholesterol 7α-hydroxylase in patients with disorders of lipid metabolism, methods were needed to perform the enzyme assay with the relatively small amounts of liver tissue obtained by percutaneous liver biopsy. The present paper deals with the preparation of microsomes from 20–60 mg of liver tissue, describes optimal conditions for the in vitro assay of cholesterol 7α-hydroxylase activity, and provides proof of the identity and radioactive purity of the product of the enzymatic reaction, 7α-hydroxycholesterol (cholest-5-ene-3β,7α-diol).

METHODS

Purification of substrate

[4-^{14}C]Cholesterol (specific radioactivity, 58 mCi/mmole) was purchased from New England Nuclear Corp., Boston, Mass. The labeled sterol was purified by chromatography on a column of silicic acid (Bio-Sil, Bio-Rad Laboratories, Richmond, Calif.) in the presence of unlabeled 7α-hydroxycholesterol, 7β-hydroxycholesterol, and 7-ketocholesterol. The column was eluted with increasing amounts of peroxide-free ether in hexane (8), and cholesterol was found in the fractions eluted with 10% ether in hexane (v/v). Unlabeled cholesterol, U.S.P., was purified in an identical manner.

Preparation of 7α- and 7β-hydroxycholesterol

A mixture of the diols was obtained by the reduction of 7-ketocholesterol (Schwarz/Mann, Orangeburg, N.Y.) with sodium borohydride (9). The epimers were separated by preparative TLC and were purified further by column chromatography on deactivated silicic acid (3).

Cholest-5-ene-3β,7α-diol diacetate

This compound was prepared by a method similar to that used by Mitropoulos and Balasubramaniam (10) with some modifications as follows: 5 mg of 7α-hydroxycholesterol was added to a mixture of 1 ml of dry pyridine and 0.2 ml of freshly distilled acetic anhydride in 1 ml of benzene and left for 24 hr at 37°C. The solvents were evaporated under nitrogen, 10 ml of ice cold water was added, and the crude reaction product was extracted with hexane. The hexane layer was washed with 2 N HCl to remove excess pyridine, then with water, dried over anhydrous Na_2SO_4, and evaporated under a stream of nitrogen. Crystallization from hexane yielded 5.2 mg of diacetate of purity greater than 98% as estimated by semiquantitative TLC.

Clinical procedure

Liver tissue was obtained from 16 individuals, hospitalized at the Manhattan Veterans Administration Hospital, either by percutaneous liver biopsy (7 subjects) or at abdominal surgery (9 subjects). The patients received the regular hospital diet. All biopsies were performed at about 10 a.m.; the subjects had been fasted after the evening meal of the preceding day. Informed consent was obtained

Abbreviations: CTX, cerebrotendinous xanthomatosis; TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

from all the subjects, and their liver function and blood coagulation tests were normal. Surgical liver biopsies were performed during cholecystectomy for cholesterol cholelithiasis in three subjects, subtotal gastrectomy with vagotomy for chronic peptic ulcer disease in five subjects, and cholecystojejunostomy for relief of extrahepatic obstruction of common bile duct caused by pancreatic neoplasm in one subject. Percutaneous liver biopsies were performed with a 16-gauge Menghini needle on three subjects with cholelithiasis, three subjects with hyperlipidemia, and one subject with cerebrotendinous xanthomatosis. 20–60 mg of liver tissue was removed by percutaneous biopsy, and 44–700 mg was obtained by abdominal surgery. In no instance was significant bleeding noted. Liver histology was normal in each case. After removal, the liver tissues were immediately placed in ice-cold homogenizing solution (composition described below) and were transported to the laboratory in an ice bath within 15–20 min.

Preparation of liver microsomes

Temperature was maintained between 0 and 5°C during preparation of the microsomes, either by means of an ice bath or by working in a cold room (4°C). The liver tissue was blotted dry on sterile gauze, weighed, and put into a 15-ml centrifuge tube (Corning no. 8441) in the amount of homogenizing medium necessary to obtain a solution of 4–10% concentration (w/v). The homogenizing medium contained: sucrose, 0.3 M; nicotinamide, 0.075 M; EDTA, 0.002 M; and mercaptoethanol, 0.02 M. Samples of liver tissue greater than 100 mg (obtained by surgical biopsy) were gently chopped with a sterile blade or extruded through a tissue press (Harvard Apparatus Co., Millis, Mass.) before homogenization. The tissues were homogenized in the same centrifuge tube with a loose-fitting Teflon pestle (radial clearance, 0.5 mm) operated at 450 rpm for 10–15 sec. The entire homogenizing procedure was timed to avoid excessive cellular disruption.

The whole homogenate was centrifuged at 11,000 *g* for 10 min to sediment nuclei, cell debris, and the mitochondrial fraction. The supernatant solution was transferred to a polycarbonate tube (IEC no. 2804),[1] cut to reduce the volume from 4 to 3 ml, and centrifuged at 100,000 *g* for 60 min. The sedimented microsomes remained in the centrifuge tube after decantation of the supernatant solution. They were suspended in 1 ml of homogenizing medium, homogenized for 10 sec with a Teflon pestle (radial clearance, 0.5 mm), and centrifuged again as before (100,000 *g* for 60 min). The supernatant solution was decanted and the sedimented microsomal pellet was homogenized in the same centrifuge tube with a volume of homogenizing medium to give a solution of known concentration (4–10%). These microsomal suspensions were used for the assay of cholesterol 7α-hydroxylase; the microsomal protein concentration was determined by the method of Lowry et al. (11). A typical preparation had a concentration of 1–3 mg of protein/ml. The procedure described above, performed with a single transfer of the 11,000 *g* supernate into the polycarbonate centrifuge tube (used for both centrifugation and homogenization of the microsomes), permitted the isolation of 25–30 mg of microsomes/g of liver tissue (wet wt).

Assay of cholesterol 7α-hydroxylase activity

Incubations of the microsomal enzyme with [4-^{14}C]cholesterol were performed using a procedure similar to that previously described for the rat liver enzyme (3). Some modifications were made with respect to incubation time and substrate/protein ratio. The incubations were carried out in 15-ml culture tubes.

The substrate consisted of [4-^{14}C]cholesterol (6×10^5 dpm, 5×10^{-6} M) and unlabeled cholesterol (100 μM) solubilized with 0.75 mg of Cutscum (isooctylphenoxypolyethylene-ethanol, Fisher Scientific Co., Fair Lawn, N.J.). The substrate (in solution in cyclohexane) and the detergent (in acetone solution) were mixed. The organic solvents were evaporated under nitrogen, and the residue was "solubilized" by vigorous mixing in 0.15 ml of homogenizing medium. The assay system contained, in a volume of 0.5 ml, 0.15 ml of an NADPH-generating system with the following composition: potassium phosphate buffer, pH 7.4, 70 mM; $MgCl_2$, 4.5 mM; NADP, 1.25 mM (buffered to pH 7.4); glucose-6-phosphate, 2.5 mM (buffered to pH 7.4); glucose-6-phosphate dehydrogenase, 5 enzyme units. The NADPH-generating system was incubated for 5 min at 37°C before starting the cholesterol 7α-hydroxylase determination. The NADPH-generating system (0.15 ml) was added to the "solubilized" substrate, and the assay was started by adding 0.2 ml of microsomal solution (containing 0.1–0.3 mg of microsomes). The incubation time was 20 min at 37°C in air, care being taken to avoid unnecessary exposure to light. A boiled enzyme control was run with each experiment. The reaction was stopped by the addition of 15 vol (7.5 ml) of dichloromethane–ethanol 5:1 (v/v); then 2 ml of water was added, and the assay tubes were shaken for 2 min on a Vortex mixer and centrifuged at 2000 rpm in a clinical centrifuge (IEC no. 803).

The organic solvent layer was removed and evaporated to dryness under N_2 on a 50°C water bath. This extraction procedure was found to permit the recovery of 85–90% of the labeled sterols. The sterol fraction was dissolved in $CHCl_3$ and applied to silica gel G plates, layer thickness 0.25 mm (type JEAB, Analtech, Newark, Del.). Unlabeled 7α- and 7β-hydroxycholesterol and 7-ketocholesterol (30 μg of each) were applied at the same spot.

[1] International Equipment Co., Needham Heights, Mass.

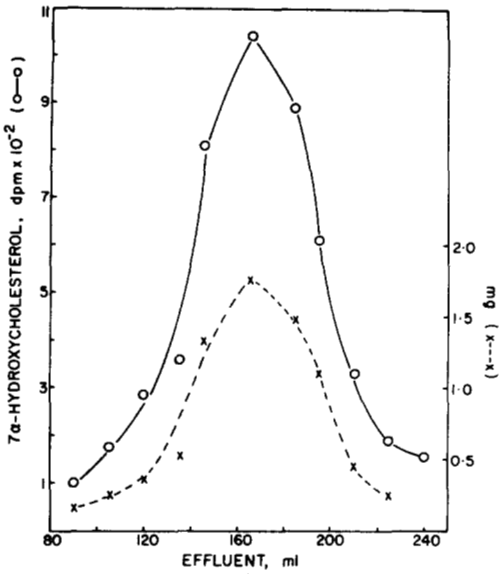

Fig. 1. Column chromatography of biosynthetic 7α-hydroxycholesterol obtained by large-scale incubation. Column fractions were assayed for 7α-hydroxycholesterol by GLC and for ^{14}C by scintillation counting.

The plates were developed with peroxide-free ether at 5°C, dried, and sprayed with a 0.2% (w/v) solution of 2′,7′-dichlorofluorescein in methanol. The spots were made visible under UV illumination (366 nm). The spots corresponding to the positions of 7α-hydroxycholesterol and cholesterol were removed from the plate by suction, transferred into liquid scintillation vials containing 0.4% butylbenzoxazolyl thiophene (BBOT) in toluene, and counted in a liquid scintillation counter (Beckman LS-200B), with suitable corrections for background and quenching. Enzyme activity was expressed as dpm incorporated into 7α-hydroxycholesterol per mg protein per 20 min.

The results were corrected for the nonenzymatic oxidation of cholesterol by subtracting the values obtained for the boiled enzyme blanks (usually 20–30% of the experimental sample). Losses during the extraction procedure were calculated on the basis of initial radioactivity of the cholesterol and the total counts recovered after extraction.

TABLE 1. Identification of biosynthetic 7α-hydroxycholesterol obtained from incubation of human hepatic microsomes with [4-^{14}C]cholesterol[a]

	Specific Radioactivity
	dpm/μmole
7α-[4-^{14}C]Hydroxycholesterol (from preparative TLC)	1418
7α-[4-^{14}C]Hydroxycholesterol (from partition column)	1549
7α-[4-^{14}C]Hydroxycholesterol (after crystallization from acetone–water)	1582
[4-^{14}C]Cholest-5-ene-3β,7α-diol diacetate	1475

[a] Biosynthetic 7α-[4-^{14}C]hydroxycholesterol obtained from a large-scale incubation of microsomes with [4-^{14}C]cholesterol was separated and purified as described in Results.

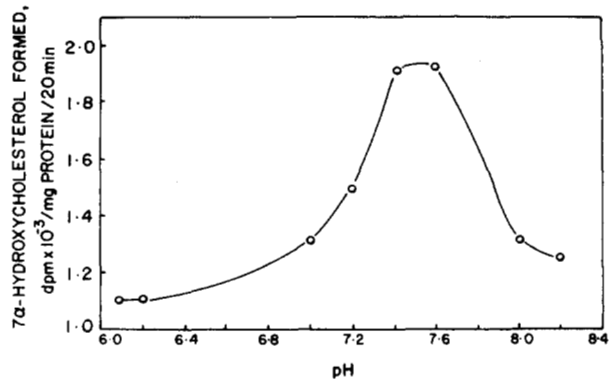

Fig. 2. Effect of pH on microsomal cholesterol 7α-hydroxylase activity of human liver. Standard assay conditions except for pH of assay system (microsomal protein, 0.2 mg/tube).

RESULTS

Identification of biosynthetic 7α-hydroxycholesterol

The labeled 7α-[4-^{14}C]hydroxycholesterol formed during the incubation of the microsomal fraction of human liver tissue with [4-^{14}C]cholesterol was identified as follows. The assay system was scaled up by a factor of 10, and the reaction mixture was extracted as described in the experimental section. The sterols were separated by preparative TLC on 2-mm-thick silica gel G plates (Analtech) with ether at 5°C. The following R_F values were obtained: cholesterol, 0.89; 7α-hydroxycholesterol, 0.30; 7β-hydroxycholesterol, 0.41; 7-ketocholesterol, 0.58. The 7α-hydroxycholesterol band was removed from the plate and eluted from the silica gel with ether–chloroform 1:1 (v/v). The solvent was evaporated under nitrogen; the residue was diluted with unlabeled 7α-hydroxycholesterol, and its specific radioactivity was determined by scintillation counting and GLC (Table 1).

The 7α-hydroxycholesterol was then chromatographed on a column of silicic acid (Bio-Rad Laboratories) deacti-

TABLE 2. Intracellular localization of cholesterol 7α-hydroxylase

	7α-Hydroxycholesterol Formed
	dpm/mg protein/20 min
Mitochondria	334
Microsomes	1457
11,000 *g* supernatant solution[a]	1309
100,000 *g* supernatant solution	0

[a] Calculated per milligram of microsomal protein in the 11,000 *g* supernatant solution.

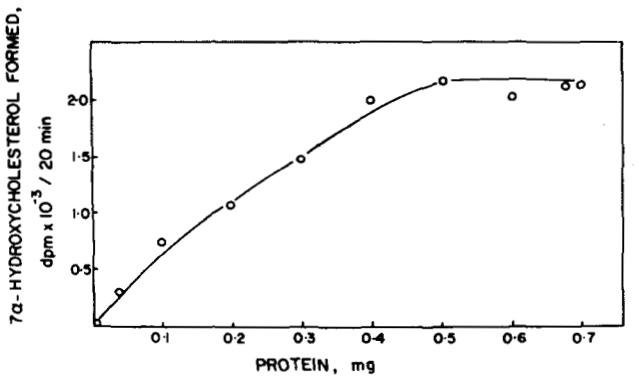

Fig. 3. Effect of increasing amounts of microsomal protein on the rate of 7α-hydroxycholesterol formation. Standard assay conditions except for protein concentration.

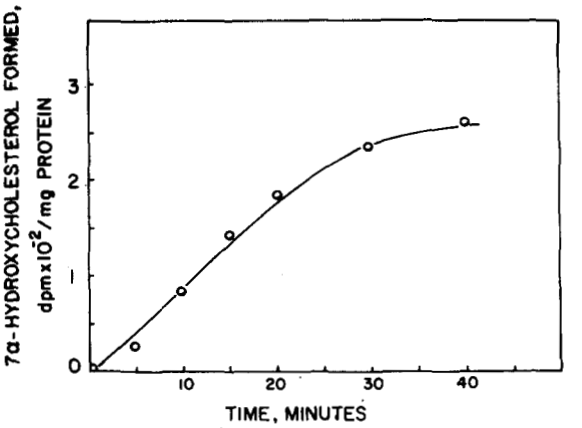

Fig. 4. Time course of enzymatic formation of 7α-hydroxycholesterol. Standard assay conditions except for incubation time (microsomal protein, 0.3 mg/tube).

vated with 17% H_2O (w/v) and eluted with increasing proportions of ether in benzene. 7α-Hydroxycholesterol was obtained with 30% ether in benzene. The specific radioactivity remained constant throughout the 7α-hydroxycholesterol band, within the precision of measurement estimated as ± 7% (Fig. 1). The column fractions that contained 7α-hydroxycholesterol were combined; the solvent was evaporated under nitrogen, and the specific radioactivity of the material eluted from the column (6.7 mg) was determined. This specific radioactivity remained unchanged after crystallization from acetone–water. Acetylation with acetic anhydride as described in the experimental section yielded 5.2 mg of cholest-5-ene-3β,7α-diol diacetate with no significant change in specific radioactivity (Table 1).

Intracellular localization of cholesterol 7α-hydroxylase

A 10% homogenate of human liver tissue (200 mg wet wt) was separated by centrifugation into mitochondria, 11,000 *g* supernatant solution, microsomes, and 100,000 *g* supernatant solution. It was found that the 7α-hydroxylation of cholesterol was catalyzed predominantly by the microsomal fraction and the 11,000 *g* supernatant solution (Table 2).

Properties of the microsomal assay system

Optimal reaction rates were obtained when the pH of the microsomal system was 7.4–7.6 (Fig. 2). The relationship between reaction rate and enzyme concentration is illustrated in Fig. 3. The rate of formation of the 7α-hydroxycholesterol was linear with respect to protein concentration up to 1 mg of protein/ml (0.5 mg of protein/tube in the standard assay system). The rate of formation of 7α-hydroxycholesterol was proportional to incubation time during the first 30 min of incubation (Fig. 4), and a reaction time of 20 min was chosen to assure optimal assay conditions. The effects of changes in substrate concentration on the reaction rate are presented in Fig. 5. The enzyme appeared to be saturated with the substrate when the cholesterol concentration corresponded to 0.34 μmole of cholesterol/mg of microsomal protein. In the standard assay system (containing approximately 0.2 mg of microsomal protein), a substrate concentration of 100 μM was used to ensure saturation of the enzyme.

A number of different materials were added to the assay system to avoid autoxidation. Boiled 100,000 *g* supernatant solution added to the microsomal suspension partially prevented the nonenzymatic formation of 7α-hydroxycholesterol, in accordance with the observations made by Mitton, Scholan, and Boyd (12), but it produced no significant increase in cholesterol 7α-hydroxylase activity. Mercaptoethylamine added to the assay system had no favorable effect on enzyme activity, nor did it reduce nonenzymatic formation of 7α-hydroxycholesterol.

Table 3 illustrates the results obtained when the activities of fresh and frozen liver or microsomes were compared. All the determinations were made using a single

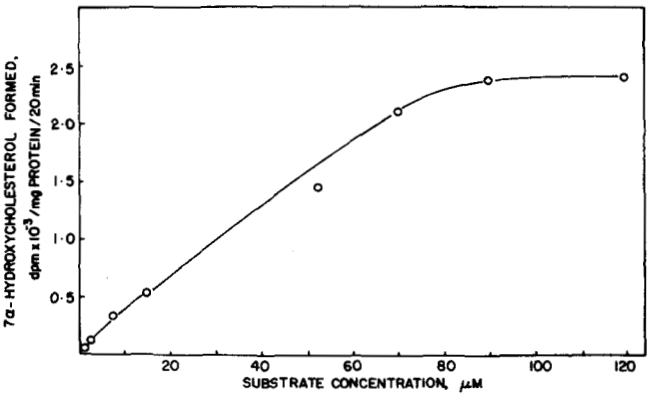

Fig. 5. Effect of substrate concentration ([4-^{14}C]cholesterol) on microsomal cholesterol 7α-hydroxylase activity. Standard assay conditions except for substrate concentration (microsomal protein, 0.25 mg/tube).

TABLE 3. Cholesterol 7α-hydroxylase activities of fresh and frozen human liver

	7α-Hydroxycholesterol	Loss of Activity
	dpm/mg protein/20 min	%
Fresh liver microsomes	2470	0
Microsomes from frozen liver tissue[a]	2110	14.6
Microsomes frozen 9 days	1915	22.5
Microsomes frozen 100 days[b]	669	73

[a] Microsomes prepared from liver stored at −20°C for 9 days.
[b] The microsomal solution was frozen and thawed two times.

piece of liver tissue. These data show that it should be feasible to store frozen liver tissue or microsomes for as long as 1 wk before performing the enzyme assay.

Clinical application of the cholesterol 7α-hydroxylase assay

The levels of cholesterol 7α-hydroxylase activity in five patients with chronic peptic ulcer disease serving as controls and six patients with cholesterol cholelithiasis are shown in Table 4. The difference of the means was statistically significant; the enzyme activity in the control group was about twice as much as that observed in the gallstone group ($P < 0.01$). Table 4 also shows values obtained from five subjects with different abnormalities of lipid metabolism. The highest activity of cholesterol 7α-hydroxylase was detected in the patient with cerebrotendinous xanthomatosis, an inherited disease characterized by tendon and tissue xanthomata and unusually low plasma cholesterol concentrations (13). In contrast, cholesterol 7α-hydroxylase was exceedingly low in the subject with obstructive jaundice, who manifested elevations of hepatic and plasma cholesterol concentrations. In one subject with type II hyperbetalipoproteinemia and xanthomatosis, the enzyme activity was lower than normal, whereas in two subjects with type V hyperbetalipoproteinemia with chylomicrons, cholesterol 7α-hydroxylase activity did not differ from normal.

TABLE 4. Cholesterol 7α-hydroxylase activity in human liver

Patient	Sex	Age	Diagnosis	Wet Wt of Liver Tissue	7α-Hydroxycholesterol
				mg	*dpm/mg protein/20 min*
F.B.	M	57	Peptic ulcer	120	2710
J.R.	M	69	Peptic ulcer	330	2320
J.R.	M	44	Peptic ulcer	540	1476
J.T.	M	60	Peptic ulcer	200	2310
R.E.	M	42	Peptic ulcer	380	2460
					2255 ± 208[a]
K.S.	F	26	Cholelithiasis	40[b]	902
J.T.	M	68	Cholelithiasis	120	796
R.P.	M	54	Cholelithiasis	50[b]	830
A.S.	M	26	Cholelithiasis	700	1524
A.D.	M	66	Cholelithiasis	110	830
J.G.	M	21	Cholelithiasis	65	819
					950 ± 116[a]
J.C.	M	35	CTX[c]	50[b]	3340
P.C.	M	42	Type II hyperlipoproteinemia	47[b]	785
J.W.	M	64	Obstructive jaundice secondary to pancreatic neoplasm	44[d]	104
C.D.	M	26	Type V hyperlipoproteinemia with chylomicrons	20[b]	2320
W.G.	M	28	Type V hyperlipoproteinemia with chylomicrons	33[b]	2290

[a] Mean ± SE.
[b] Percutaneous liver biopsy.
[c] Cerebrotendinous xanthomatosis.
[d] Liver biopsy consistent with extrahepatic biliary obstruction.

DISCUSSION

The results of the present investigation demonstrate that the activity of the hepatic microsomal enzyme cholesterol 7α-hydroxylase can be determined in the small quantities (20–60 mg) of liver tissue obtained by percutaneous biopsy. The feasibility of the method is ascribed to the stability of the enzyme in the relatively dilute solutions of cellular and subcellular fractions used during the preparation of the microsomes.

The optimal conditions for the assay of cholesterol 7α-hydroxylase in human liver microsomes were similar to those previously reported for the rat liver enzyme (3). The identity and radioactive purity of the reaction product, 7α-hydroxycholesterol, were demonstrated by standard procedures. Nevertheless, it must be stressed that 7α-hydroxycholesterol, and other oxidation products of cholesterol, can arise from nonenzymatic processes. For this reason, great care must be taken to minimize the nonenzymatic oxidation of cholesterol before, during, and after the incubation step. If these precautions are not observed, the boiled enzyme controls usually contain concentrations of 7α-[^{14}C]hydroxycholesterol of the same order of magnitude as the experimental samples. This results in large errors when the 7α-[^{14}C]hydroxycholesterol content of the tube containing boiled enzyme is subtracted from that of the experimental tube.

It is hoped that the enzyme assay described in this paper will be useful in estimating relative rates of bile acid synthesis in patients with disorders of sterol metabolism and in assessing the effect of treatment. A number of assumptions are involved: first, that cholesterol 7α-hydroxylase is the rate-limiting enzyme of bile acid biosynthesis in man. There now exists a considerable body of evidence

that this is the case in a number of laboratory animals, namely, the rat or the rabbit (5, 14), but analogous studies in human subjects are lacking. Second, that under the conditions of the assay the amount of enzyme is rate limiting. This is necessary if the activity of the enzyme is to serve as a measure of its concentration in the tissue. Third, that the enzyme is distributed uniformly throughout the liver. This point requires further investigation. In any case, it cannot be expected that histologically abnormal areas will have the same specific enzyme activity as adjacent normal areas. It is not known whether cholesterol 7α-hydroxylase activity of human liver undergoes cyclic changes as does the rat liver enzyme. In the present study, in order to minimize the effect of a potential diurnal variation, all biopsies were obtained at approximately 10 a.m. after the subjects had been fasted from 6 p.m. of the previous day. Clearly, to make valid comparisons of enzyme activity, adherence to a uniform experimental design is essential.

According to recent findings by Balasubramaniam, Mitropoulos, and Myant (15), the real substrate pool of the cholesterol 7α-hydroxylase and the contribution of exogenous cholesterol to this pool are difficult to establish. Therefore, we expressed the enzyme activities as dpm in 7α-hydroxycholesterol formed per mg protein per 20 min incubation time. Since the incubation conditions were identical in each case, and since there was no significant change in the microsomal cholesterol of the different patients,[2] it was possible to demonstrate differences in cholesterol 7α-hydroxylase activity among subjects with different disorders of sterol metabolism. For example, enzyme activity was about 50% lower in a group of six patients with cholesterol gallstones in comparison with a control group of five patients with peptic ulcer. This suggests that cholesterol cholelithiasis is associated with a relative reduction of bile acid synthesis (and, as previously reported, with a relative increase of hepatic cholesterol synthesis [16]).

Obviously, much additional work is needed before it can be stated that the formation of lithogenic bile is a consequence of these enzyme abnormalities.

It is hoped that the techniques reported in this paper coupled with the assay of hepatic HMG CoA reductase activity, which can be measured simultaneously on the same microsomal preparation, will permit the rapid evaluation of the relative rates of cholesterol biosynthesis and catabolism. Unlike the more laborious sterol balance and isotope kinetic techniques, a biopsy can be analyzed within 36 hr. Such information will be useful in defining cholesterol and bile acid production rates in different lipid disorders and provide a more rational means of evaluating lipid lowering therapy.

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[2] In three patients with peptic ulcer, hepatic microsomal total cholesterol averaged 74.9 nmoles/mg of microsomal protein (range 66.8–81.7); in three patients with cholesterol gallstones, hepatic microsomal total cholesterol averaged 72.5 nmoles/mg of microsomal protein (range 64.9–80.8). Nicolau, G., S. Shefer, and E. H. Mosbach. Unpublished observations.

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