Regulation of bile acid synthesis. Measurement of cholesterol 7α-hydroxylase activity in rat liver microsomal preparations in the absence of endogenous cholesterol

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Summary A rapid procedure was developed to measure hepatic cholesterol 7α-hydroxylase activity in the absence of endogenous microsomal cholesterol. This method involves the preparation of an acetone powder from the microsomal fraction of rat liver that retains its cholesterol 7α-hydroxylase activity and contains virtually no endogenous cholesterol. The enzyme activity is measured in the presence of labeled exogenous cholesterol as the only substrate source, and can be expressed in terms of picomoles of product formed when a simple isotope incorporation procedure is employed. Optimal assay conditions were determined and the reproducibility of the acetone powder cholesterol 7α-hydroxylase assay was established. A comparison of the proposed method with the previously used double isotope derivative procedure showed comparable enzyme activities in control rats and both methods exhibited an increase in the rate of hydroxylation after cholestyramine treatment and a decrease following cholic acid treatment. In contrast, the acetone powder assay did not show any change in cholesterol 7α-hydroxylase activity during cholesterol feeding. These findings suggest that bile acid feeding influences the amount of active cholesterol 7α-hydroxylase present in the liver whereas cholesterol feeding does not.—**Shefer, S., F. W. Cheng, S. Hauser, A. K. Batta,** and **G. Salen.** Regulation of bile acid synthesis. Measurement of cholesterol 7α-hydroxylase activity in rat liver microsomal preparations in the absence of endogenous cholesterol. *J. Lipid Res.* 1981. **22:** 532–536.

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The 7α-hydroxylation of cholesterol is the major rate-limiting step in the conversion of cholesterol into bile acids (1, 2). The enzyme system cholesterol 7α-hydroxylase (EC 1.14), which catalyzes this reaction, plays a major role in the control of primary bile acid synthesis. This enzyme has been studied extensively in recent years. It is located in the smooth endoplasmic reticulum and is dependent on cytochrome P-450 and on NADPH-cytochrome P-450 reductase (3–7). Several methods have been employed for the determination of cholesterol 7α-hydroxylase activity. The isotope incorporation procedure (1, 8) determines the percentage of label incorporated into 7α-hydroxycholesterol from a radioactive exogenous cholesterol substrate. This method does not take into account the dilution of the exogenous cholesterol by the endogenous microsomal cholesterol pool available to the enzyme. Thus, under these conditions, cholesterol 7α-hydroxylase activity is underestimated, depending on the size of the pre-existing microsomal cholesterol pool. On the other hand, the double isotope derivative dilution techniques (9, 10) and the mass fragmentographic procedure (11) measure directly the mass of the biosynthetic 7α-hydroxycholesterol with high accuracy and are not affected by the specific radioactivity of the substrate pool. However, both techniques are complex, time consuming, and require special equipment not widely available. Moreover, in the case of cholesterol feeding where an increase in the size of the substrate pool is noted (12), there is always the possibility that the observed increase in enzyme activity is due to expansion of the substrate pool by dietary cholesterol rather than to increased amount of active cholesterol 7α-hydroxylase in the liver.

In order to overcome these difficulties, a rapid assay system was developed that determines the activity of cholesterol 7α-hydroxylase in the absence of endogenous cholesterol. This method involves the preparation of an acetone powder from rat liver microsomes that eliminates virtually all endogenous cholesterol and utilizes labeled exogenous cholesterol as the only substrate source. The amount of 7α-hydroxycholesterol formed during the incubation can be accurately calculated from the specific radioactivity of the substrate in the incubation medium and the radioactivity recovered in the product. This technique was used to test the effect of cholic acid, cholestyramine, and cholesterol feeding on the activity of cholesterol 7α-hydroxylase.

MATERIALS

Purification of substrate

[4-^{14}C]Cholesterol (New England Nuclear Corp., Boston, MA, sp act 3×10^6 dpm/μmol) was purified before use by thin-layer chromatography on a 0.25-mm-thick silica gel G plate (Analtech, Inc., Newark,

Abbreviations: The following systematic names are given to compounds referred to by abbreviation of trivial names: EDTA, ethylenediamine tetraacetic acid; Cutscum, isooctylphenoxypolyoxyethelene ethanol; cholic acid, 3α, 7α, 12α,-trihydroxy-5β-cholanoic acid; 7α-hydroxycholesterol, 5-cholestene-3β, 7α-diol; 7β-hydroxycholesterol, 5-cholestene-3β,7β-diol.

DE) developed with ether at 5°C. Markers of cholesterol, 7α- and 7β-hydroxycholesterol were applied along the sides of the plate and made visible with spray reagent that consisted of 3.5% phosphomolybdic acid in isopropanol. The corresponding cholesterol band, $R_f = 0.88$ (8), was removed from the plate, eluted with acetone, and dried under N_2 at 40°C. The purified product contained less than 0.06% 7α-hydroxycholesterol.

Unlabeled compounds and reagents

Cholesterol, U.S.P. used in the diet, was recrystallized from ethanol and used without further purification. A mixture of 7α- and 7β-hydroxycholesterol used as reference compounds was prepared by $NaBH_4$ reduction of 7-ketocholesterol (Schwarz/Mann, Orangeburg, NY) (13). The isomers were separated by preparative thin-layer and column chromatography (14). NADPH and cholic acid were purchased from Calbiochem, LaJolla, CA, cholestyramine from Mead Johnson and Co., Evansville, IN, and Cutscum from Fisher Scientific Co., Springfield, NJ. Acetone used in the preparation of the acetone powder was spectroquality (Matheson Coleman and Bell, Elk Grove Village, IL).

EXPERIMENTAL PROCEDURE

Animals

Male Wistar rats weighing 200–250 g were kept in individual cages and fed ground Purina chow supplemented with 5% corn oil. Their weights and food intake were recorded during the experimental period. When required, cholesterol (2%), cholic acid (1%), or cholestyramine (5%) were added to this diet (8, 15). Four rats in each group were studied along with a control group and were fed the experimental diets for 7 or 14 days.

Preparation of acetone powder and assay system

Male Wistar rats were killed at 10:00 AM by cervical dislocation. The livers were removed immediately and chilled on ice, and microsomes were prepared as described previously (9). The microsomal pellet was washed with 0.02 M potassium phosphate buffer (pH 7.4) containing EDTA (1 mM), and was resuspended in the same buffer solution, in a volume of 1 ml for each 2 g of fresh liver used. Two ml of this microsomal suspension was added dropwise with stirring to 80 ml of acetone at −10°C (16, 17). When the suspension had settled, the precipitate was collected by filtration using a Büchner funnel with #1 Whatman filter paper, washed with 40 ml of cold acetone followed by 40 ml of cold ether and dried in a vacuum desiccator at 0–4°C. All subsequent operations were carried out at 0–4°C. The dry powder was ground with a glass mortar and pestle and stored in a desiccator (18). This preparation retained its activity for at least 4 weeks. Before use, an aliquot of a known weight was homogenized in 0.25 M sucrose solution containing nicotinamide (30 mM) and EDTA (1 mM).

Incubations of the acetone powder preparations with [4-^{14}C]cholesterol were performed using a procedure similar to that previously described for the microsomal enzyme (8). The incubation mixture consisted of 100 mM phosphate buffer, pH 7.4, 5 mM $MgCl_2$, and 10 mM NADPH. The substrate, [4-^{14}C]-cholesterol (100 nmol, sp act 3×10^3 dpm/nmol) was added to the incubation mixture as a suspension with 0.15 mg Cutscum. In the standard assay system (final volume, 0.70 ml), the acetone powder used contained 0.3–0.5 mg of protein. All enzyme assays were carried out for 20 min with air as the gas phase at 37°C with constant shaking, care being taken to avoid unnecessary exposure to light. A boiled enzyme control was run with each experiment. The incubation was terminated by adding 20 volumes of dicholomethane–ethanol 5:1 (v/v), and the sterols were extracted after the addition of 3 ml of water. The organic solvent layer was removed and evaporated to dryness under N_2 at 40°C. In order to determine the amount of 7α-hydroxycholesterol formed during the incubation, the isotope incorporation procedure (8) was employed as follows. The sterol fraction was dissolved in 40 μl of acetone and applied to a 0.25-mm-thick silica gel G plate (Analtech, Inc., Newark, DE) together with unlabeled 7α-hydroxycholesterol, 7β-hydroxycholesterol, and cholesterol as markers (30 μg of each). The plates were developed with ether at 5°C, dried, and the spots were made visible by spraying lightly with 3.5% phosphomolybdic acid in isopropanol. The average R_f values observed were: 7α-hydroxycholesterol, 0.35; 7β-hydroxycholesterol, 0.47; cholesterol, 0.88. The pertinent spots were removed by suction, transferred to scintillation vials containing 0.5% (w/v) 2,5-diphenyloxazole in toluene and counted in a Beckman LS-200B liquid scintillation counter (Beckman Instruments, Fullerton, CA). Since the specific radioactivity of the substrate was known, the radioactivity data could be expressed in terms of pmol of 7α-hydroxycholesterol formed per mg protein per min. The results were corrected for the non-enzymatic oxidation of cholesterol by substracting values obtained for the boiled enzyme blanks (usually 25–30% of the experimental sample). Losses during the extraction procedure were calculated on the basis of

TABLE 1. Comparison of cholesterol 7α-hydroxylase activity in rat liver microsomes with their corresponding acetone powder preparations.

Rat #	7α-Hydroxycholesterol Formed	
	Microsomes[a]	Acetone Powder[b]
	pmol/mg protein/min	
1	11.5	10.4
2	14.3	12.9
3	12.9	11.6
4	14.6	13.2
5	13.7	12.1
Mean	13.4 ± 1.2	12.0 ± 1.1

[a] Standard assay conditions were employed (See Experimental Procedure). Enzyme activity was determined by the double isotope derivative method (10).

[b] Standard assay conditions were employed (See Experimental Procedure). Enzyme activity was determined by the isotope incorporation procedure (8).

initial radioactivity of the cholesterol and the total counts recovered after extraction. In order to check the reproducibility of this method, the same microsomal suspension was divided into eight aliquots, acetone powder was prepared from each aliquot, and the enzyme activities were measured and compared. When microsomal suspensions were used as the enzyme source (0.3–0.5 mg protein/tube), the same incubation conditions were employed but the double isotope derivative procedure (10) was used. The reaction product, [4-^{14}C] 7α-hydroxycholesterol, was acetylated by [^{3}H]acetic anhydride, isolated as its diacetate derivative, and quantitated on the basis of the [^{3}H]acetic anhydride specific activity (10).

Cholesterol determination in acetone powder and microsomal preparations

Known aliquots of microsomal and acetone powder suspensions were refluxed for 3 hr with 25% KOH in 95% ethanol (w/v). After the addition of an equal volume of water, the cholesterol was extracted three times with twice the volume of hexane and the combined hexane extracts were washed to neutral pH and evaporated under a stream of nitrogen at 40°C. The mass of cholesterol was determined by gas–liquid chromatography using a 180 cm × 4 mm glass column packed with 3% QF-1 on 80–100 mesh Gas-Chrom Q at a column temperature of 245°C (19) (Hewlett-Packard, Palo Alto, CA, model 7610 gas chromatograph).

RESULTS AND DISCUSSION

Cholesterol 7α-hydroxylase activity of rat liver microsomes and their corresponding acetone powder preparations is shown in **Table 1.** Both preparations exhibited comparable enzyme activities (13.4 ± 1.2 pmol/mg protein/min as compared to 12.0 ± 1.1), which indicates that the activity of cholesterol 7α-hydroxylase was retained following acetone treatment. Cholesterol 7α-hydroxylase activity of eight individual acetone powder preparations obtained from the same microsomal suspension ranged from 11.6 to 13.1 pmol/mg protein/min (average 12.2 ± 0.57). The small standard deviation demonstrates the precision and reproducibility of this method.

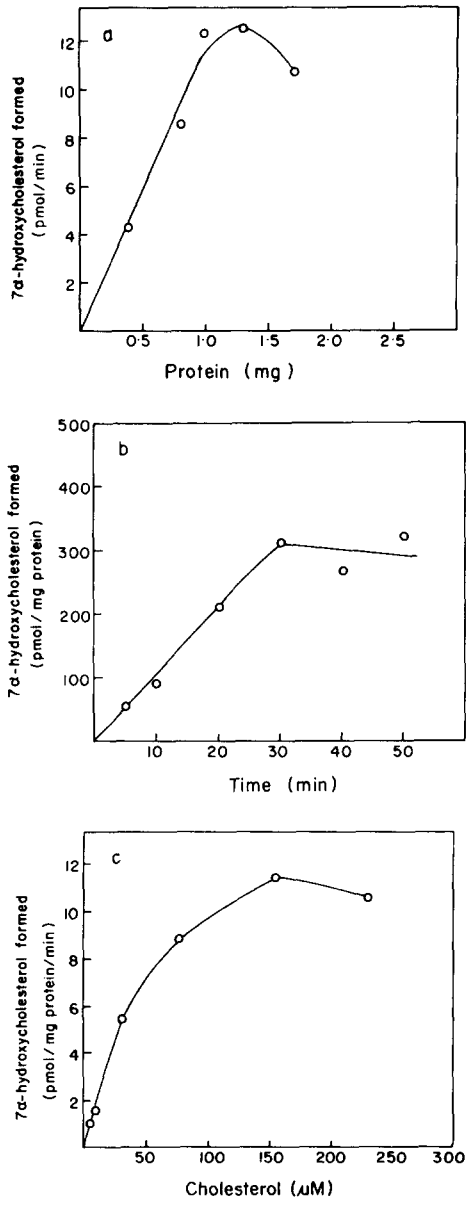

Fig. 1. Effect of enzyme concentration (a), time (b), and substrate concentration (c) on cholesterol 7α-hydroxylase activity in acetone powder prepared from hepatic microsomes of normal rats. Standard assay conditions were employed, except for enzyme concentration in (a), incubation time in (b), and substrate concentration in (c).

TABLE 2. Effect of different dietary conditions on rat hepatic cholesterol 7α-hydroxylase activity: comparison of two methods[a]

Treatment	Previous Method[b]		Proposed Method[c]	
	7α-Hydroxycholesterol Formed	Total Cholesterol in Microsomes	7α-Hydroxycholesterol Formed	Total Cholesterol in Acetone Powder
	pmol/mg protein/min	*nmol/mg protein*	*pmol/mg protein/min*	*nmol/mg protein*
None	12.8 ± 1.0[d]	50.3 ± 10.5[d]	11.5 ± 0.95[d]	0.42 ± 0.12[d]
Cholestyramine, 5% of diet[e]	21.4 ± 1.8	47.5 ± 7.9	19.9 ± 1.7	0.38 ± 0.08
Cholic acid, 1% of diet[e]	5.8 ± 0.35	51.9 ± 12.1	5.5 ± 0.48	0.47 ± 0.13
Cholesterol, 2% of diet[e]	20.5 ± 1.7	68.7 ± 17.6	11.7 ± 0.90	0.45 ± 0.15
Cholesterol, 2% of diet[f]	19.3 ± 1.5	74.8 ± 19.2	11.4 ± 0.92	0.41 ± 0.09

[a] Standard assay conditions were employed (See Experimental Procedure).
[b] Double isotope derivative method (microsomal fraction).
[c] Acetone powder assay.
[d] The data in the table represent the average of four animals for each group (mean ± S.D.).
[e] The diet was fed for 7 days.
[f] The diet was fed for 14 days.

Optimal assay conditions were determined and are illustrated in **Figs. 1a, b** and **c.** The rate of formation of 7α-hydroxycholesterol was linear with respect to protein concentration up to 1 mg of protein/tube (Fig. 1a). The rate of reaction was proportional to the incubation time during the first 30 min (Fig. 1b) and therefore a reaction time of 20 min was chosen for the standard assay system to assure optimal assay conditions. The pH optimum was at pH 7.0–7.4. This enzyme appeared to be saturated when the cholesterol concentration reached 154 μM (Fig. 1c). Tween-80 (0.15 mg/tube) could replace Cutscum as a solubilizing agent for cholesterol substrate and was found to yield comparable enzyme activities.

A comparison of the proposed acetone powder assay with the previously used double isotope derivative method (10) is illustrated in **Table 2.** Dietary regimens which might affect hepatic cholesterol content were selected. As previously noted (8, 12, 20), cholestyramine treatment greatly enhanced cholesterol 7α-hydroxylase activity in both methods, which is consistent with the fact that it interrupts the enterohepatic circulation by binding and promoting the intestinal loss of bile acids. Similarly, both methods demonstrated inhibition of cholesterol 7α-hydroxylase activity by cholic acid treatment (8, 12, 20) and indicated that this bile acid controls its synthesis by regulating the formation of 7α-hydroxycholesterol.

On the other hand, the two methods did not agree as to the effect of cholesterol feeding on the rate of cholesterol 7α-hydroxylase activity. While the double isotope derivative method (12, 21, 22) had indicated an almost twofold increase in enzyme activity in microsomes from cholesterol-fed rats, the proposed acetone powder assay did not demonstrate any change in activity. As shown in the table, acetone treatment effectively removed cholesterol from the microsomes. The cholesterol content of the acetone powder prepared from hepatic microsomes of rats treated with cholestyramine, cholic acid, or cholesterol was similar to that of rats fed the control diet and was as low as 0.38–0.47 nmol per mg of protein. In contrast, the cholesterol content of the different microsomal preparations varied. Cholesterol feeding resulted in a significant increase in the microsomal cholesterol content, which appeared to be further enhanced by the additional 7 days of feeding. The difference between the effects of cholesterol feeding measured in microsomes and in acetone powder strongly indicates that in microsomes cholesterol 7α-hydroxylase does not operate at substrate saturation (23). Cholesterol feeding leads to increase in available substrate (12) and therefore more 7α-hydroxycholesterol produced per time unit per mg of protein. The overall effect is that cholesterol feeding leads to increased bile acid synthesis in the rat, which has also been shown in balance experiments in whole animals (24).

In summary, a method has been developed that measures the amount of active cholesterol 7α-hydroxylase present in the liver in the absence of endogenous cholesterol. This method can be rapidly performed, is not affected by the pre-existing microsomal cholesterol pool, and accurately measures cholesterol 7α-hydroxylase activity under conditions where hepatic cholesterol content is varied. Moreover, the specificity of the enzyme can be unequivocally determined in the absence of endogenous cholesterol. Our findings demonstrate that cholic acid influences the amount of hepatic cholesterol 7α-hydroxylase whereas cholesterol feeding does not.

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