Isotope derivative assay of microsomal cholesterol 7α-hydroxylase

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Abstract A rapid method was developed to measure cholesterol 7α-hydroxylase activity of hepatic microsomes by the direct determination of the mass of 7α-hydroxycholesterol formed. The method is based on the quantitative acetylation of the incubation mixture with [^{3}H]acetic anhydride and the separation of the biosynthetic 7α-hydroxycholesterol as its diacetate by thin-layer chromatography on alumina. Amounts of 7α-hydroxycholesterol as low as 0.1 nmole could be measured. A comparison of the proposed isotope derivative method with the previously used isotope incorporation method showed that the latter underestimated the enzyme activity by about 20%.

Supplementary key words 7α-[^{3}H,^{14}C]hydroxycholesterol diacetate · hepatic microsomes · microsomal enzyme

Until recently, the only methods available for the determination of cholesterol 7α-hydroxylase activity were isotope incorporation procedures (1, 2). These methods did not take into account the microsomal cholesterol pool available to the enzyme and did not measure directly the amount of 7α-hydroxycholesterol formed. Mitropoulos and Balasubramaniam (3) recently published a double isotope derivative dilution procedure that overcomes some of the disadvantages of the isotope incorporation method but is relatively time-consuming because it involves two radioautography steps and a preliminary isolation of the 7α-hydroxycholesterol from the other components of the reaction mixture.

We wish to report the development of a more rapid and convenient double isotope derivative procedure for the assay of cholesterol 7α-hydroxylase activity. This method is based on the nearly quantitative acetylation of the reaction mixture with labeled acetic anhydride followed by TLC separation of 7α-hydroxycholesterol diacetate from the other reaction products. The validity of the new method was verified by demonstration of the radioactive purity of 7α-hydroxycholesterol diacetate and by comparison with the previously used isotope incorporation procedure.

Systematic names of steroids referred to in the text by their trivial names are as follows: 7α-hydroxycholesterol, 5-cholestene-3β,7α-diol; 7β-hydroxycholesterol, 5-cholestene-3β,7β-diol; 7-ketocholesterol, 5-cholesten-3β-ol-7-one; 7α-hydroxycholesterol 3β-monoacetate, 3β-acetoxy-5-cholesten-7α-ol; 7β-hydroxycholesterol 3β-monoacetate, 3β-acetoxy-5-cholesten-7β-ol; 7-ketocholesterol acetate, 3β-acetoxy-5-cholesten-7-one.

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

MATERIALS

Labeled compounds

[4-^{14}C]Cholesterol (New England Nuclear Corp., Boston, Mass.) was purified by chromatography on a silicic acid column (4) (Bio-Sil; Bio-Rad Laboratories, Richmond, Calif.) in the presence of unlabeled 7α- and 7β-hydroxycholesterol. The column was eluted with increasing amounts of peroxide-free ether in hexane. The purified product came off the column with 10% ether in hexane (v/v) and contained less than 0.06% 7α-hydroxycholesterol.

7α-[4-^{14}C]Hydroxycholesterol was prepared from [4-^{14}C]cholesterol (5). It was shown to be 98–99% pure by TLC (1).

[^{3}H]Acetic anhydride (New England Nuclear), 50 mCi/mmole, was diluted with nonradioactive acetic anhydride and distilled under a stream of N_2 at 50–60°C. The specific radioactivity of each batch of [^{3}H]acetic anhydride was determined by acetylating samples of known mass of [4-^{14}C]cholesterol, isolating the doubly labeled acetylated product by TLC as described below, and determining the amount of ^{3}H incorporated into the samples.

Reference compounds

A mixture of 7α- and 7β-hydroxycholesterol was prepared by $NaBH_4$ reduction of 7-ketocholesterol (Schwarz/Mann, Orangeburg, N.Y.), and the isomers were separated by preparative TLC and column chromatography (6). 7α-Hydroxycholesterol was further purified by crystallization from methanol.

7-Ketocholesterol acetate and 7α- and 7β-hydroxycholesterol 3β-monoacetates were prepared as described pre-

viously (5) and separated by TLC using silica gel G plates and ether–benzene 1:9 as solvent system. The following R_f values were observed: 7-ketocholesterol acetate, 0.62; 7α-hydroxycholesterol 3β-monoacetate, 0.27; 7β-hydroxycholesterol 3β-monoacetate, 0.34. 7α- and 7β-Hydroxycholesterol diacetates were prepared and separated as described under Experimental Procedure.

EXPERIMENTAL PROCEDURE

Animals

Male Wistar rats weighing 200–250 g were fed ground Purina chow. Cholestyramine-treated rats received 5% cholestyramine[1] in their diet for 7 days. Treatment with phenobarbital consisted of daily intraperitoneal injections of 100 mg/kg body weight for 5 days (7).

Preparation of enzyme and assay system

Rats were killed by cervical dislocation during the circadian minimum of cholesterol 7α-hydroxylase activity (8, 9). The livers were removed immediately and chilled on ice, and microsomes were prepared as described previously (1).

A typical preparation of microsomes contained approximately 1 mg of protein per 0.1 ml of microsomal suspension. Incubations were carried out for 20 min in the assay system described previously (1), which contains in a volume of 0.5 ml: potassium phosphate buffer, pH 7.4, 70 mM; $MgCl_2$, 4.5 mM; $NADP^+$, 1.25 mM; glucose-6-phosphate, 2.5 mM; glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 2 IU; [4-^{14}C]cholesterol, 200 μM, solubilized with 0.15 mg of Cutscum;[2] and 1 mg of microsomal protein. In most experiments the substrate, [4-^{14}C]cholesterol, was preincubated for 5 min at 0°C with the microsomal preparation before the NADPH-generating system was added to start the reaction. The incubations were carried out in air at 37°C with shaking and exclusion of light. The incubation was terminated by the addition of 15 to 20 vol of dichloromethane–ethanol 5:1 (v/v) per volume of the incubation mixture. 2.5 ml of water was added, the mixture was shaken vigorously in a vortex mixer for 1 min and centrifuged, and the organic solvent layer was separated. The extraction was repeated and the organic phases were combined and evaporated under a stream of N_2 at 60°C. Under these conditions, 90–95% of the steroids was recovered from the incubation mixture. The dried steroid fraction was dissolved in benzene–methanol 4:1 and transferred quantitatively into Reactivials, capacity 0.3 ml (Pierce Chemical Co., Rockford, Ill.). The samples were evaporated under N_2 and then reevaporated twice after addition of 0.2 ml of benzene–methanol 4:1. For acetylation, 15 μl of a solution of 5% [^{3}H]acetic anhydride in dry pyridine was added to each vial containing the dried steroids. The vials were tightly stoppered and their contents mixed thoroughly. After 6 hr at 100°C, excess [^{3}H]acetic anhydride and pyridine were removed by repeated addition of benzene–methanol and evaporation under a stream of N_2 at 50–60°C. Extension of the heating period from 6 to 16 hr is possible, but it does not improve the yield of diacetate (75–95%).

The dried acetylated steroids were dissolved in 50 μl of benzene–methanol 4:1 (v/v) and were applied as a streak 1.5 cm long to alumina G plates, 0.25 mm thick (Analtech, Inc., Newark, Del.). The alumina plates were activated at 110°C for 30 min and then transferred while hot to a 40°C hot plate. The acetylated steroid samples were applied to the warm TLC plate. 30 μg of unlabeled acetylated sterols was applied to the same streak as carriers. The 7α-[^{3}H,^{14}C]hydroxycholesterol diacetate formed was separated from its 7β epimer and from the monoacetylated and unacetylated steroidal contaminants by TLC with ether–hexane 1:1 (v/v) as the solvent system. The radioactive diacetylated product was made visible by spraying the plate lightly with 3.5% phosphomolybdic acid in isopropanol (**Fig. 1**). The pertinent bands were removed from the plate by suction and placed in scintillation vials containing 0.3 ml of water and 14 ml of scintillation solution consisting of 5 g of 2,5-diphenyloxazole and 100 g of naphthalene per liter of dioxane. For radioassay the vials were counted in a Beckman LS-200B liquid scintillation counter, and suitable corrections were made for background, crossover, and efficiencies of the two labels. Losses could occur during extraction and acetylation procedures; therefore, known amounts of ^{14}C-labeled cholesterol, 7α-hydroxycholesterol, 7β-hydroxycholesterol, and 7-ketocholesterol separately and in mixtures of similar proportions as those encountered in the incubation experiments were used to determine these losses. It was found that under the conditions described above all these steroids were extracted to the same extent (90–95%) and also that the yield of formation of cholesterol acetate, 7α- and 7β-hydroxycholesterol diacetates, and 7-ketocholesterol acetate was similar (75–95%). Losses due to extraction and acetylation procedures were calculated either by comparing the counts in ^{14}C found in these acetylated steroids with the initial counts added or by using a known amount (50 nmoles) of unlabeled 7β-hydroxycholesterol, added after the incubation, as internal standard. A zero-time control was run for each experiment, and the results were corrected accordingly.

[1] Questran, Mead Johnson & Co., Evansville, Ind.

[2] Cutscum is a detergent sold by Fisher Scientific Co. It is isooctylphenoxypolyoxyethylene ethanol. The use of acetone or Tween 80 as solubilizers for the substrate yielded the same results as those obtained with Cutscum.

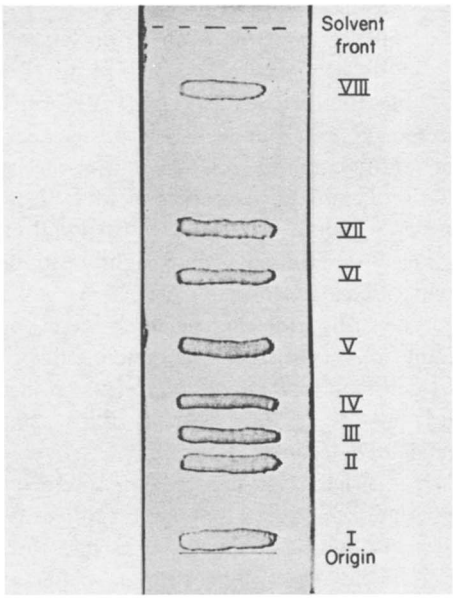

Fig. 1. Thin-layer chromatogram of the acetylated sterols obtained from an incubation of [4-^{14}C]cholesterol with rat liver microsomes. Samples were applied as streaks 1.5 cm long on 0.25-mm-thick alumina G (Analtech) plates and developed with ether–*n*-hexane 1:1 (v/v). R_f values of the reference compounds used were: I, 7-ketocholesterol and 7α- and 7β-hydroxycholesterol, 0.03; II, 7α-hydroxycholesterol 3β-monoacetate, 0.15; III, cholesterol, 0.20; IV, 7β-hydroxycholesterol 3β-monoacetate, 0.26; V, 7-ketocholesterol acetate, 0.37; VI, 7β-hydroxycholesterol diacetate, 0.51; VII, 7α-hydroxycholesterol diacetate, 0.61; and VIII, cholesterol acetate, 0.88.

RESULTS

Identification of 7α-hydroxycholesterol diacetate

The contents of 10 incubation tubes were extracted and acetylated with [^{3}H]acetic anhydride. 7α-[^{3}H,4-^{14}C]Hydroxycholesterol diacetate was separated from the other reaction products by preparative TLC on alumina as described in Experimental Procedure (see also Fig. 1) and was eluted from the plate with acetone. After dilution with unlabeled known 7α-hydroxycholesterol diacetate carrier, the ^{3}H/^{14}C ratio and the specific radioactivity were determined by scintillation counting and GLC (for GLC conditions, see legend to Fig. 2). The 7α-[^{3}H,4-^{14}C]hydroxycholesterol diacetate was chromatographed on a column of Bio-Sil and eluted with increasing amounts of ether in hexane. The pure compound came off the column with 5% ether in hexane (v/v). The specific radioactivities remained constant throughout the 7α-hydroxycholesterol diacetate band (**Fig. 2**). The column fractions containing the pure diacetate were combined and the specific radioactivity was determined. The specific radioactivity (with respect to both ^{14}C and ^{3}H) as well as the ^{3}H/^{14}C ratio remained unchanged after two crystallizations from methanol (**Table 1**).

TABLE 1. Identification of biosynthetic 7α-hydroxycholesterol (diacetate)

[^{3}H,4-^{14}C]7α-Hydroxycholesterol Diacetate	Specific Radioactivity		^{3}H/^{14}C
	^{14}C	^{3}H	
	dpm/mg		
From preparative TLC	929	23,200	24.9
From column chromatography	909	22,900	25.2
After 1st crystallization from methanol	963	23,800	24.7
After 2nd crystallization from methanol	882	22,100	25.1

7α-Hydroxycholesterol diacetate obtained from a large scale incubation was diluted with unlabeled carrier and separated from the other components of the incubation mixture by preparative TLC (Fig. 1). After elution from the plate, the compound was subjected to column chromatography (Fig. 2) and was crystallized twice from methanol. During all of these operations, the specific radioactivity with respect to ^{14}C and ^{3}H remained constant, as did the ^{3}H/^{14}C ratio.

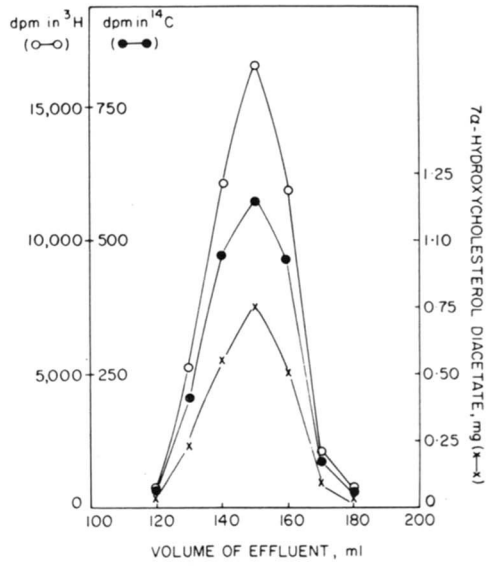

Fig. 2. Column chromatography of biosynthetic 7α-hydroxycholesterol as its diacetate derivative obtained from a large-scale incubation experiment. Column fractions were assayed for ^{14}C and ^{3}H by scintillation counting in a Beckman LS-200B scintillation counter and for mass of 7α-hydroxycholesterol diacetate by GLC in a Hewlett-Packard model 7610A gas chromatograph. A 180 cm × 3 mm glass column, packed with 3% QF-1 on 80–100 mesh Gas-Chrom Q, was used at a column temperature of 240°C. Retention time of 7α-hydroxycholesterol diacetate, 4.5 min (5α-cholestane, internal standard, 0.7 min).

The efficiency and sensitivity of the determination of 7α-hydroxycholesterol as its diacetate derivative were evaluated by measuring the recovery of known amounts of 7α-hydroxycholesterol from standard incubation mixtures. There was good agreement between the observed and the expected values over the range determined (**Fig. 3**). The values found were slightly higher than the amounts of 7α-hydroxycholesterol recovered in the absence of microsomes, indicating the presence of endogenous microsomal 7α-hydroxycholesterol (about 0.1 nmole/mg of protein). The method was further validated by measuring the

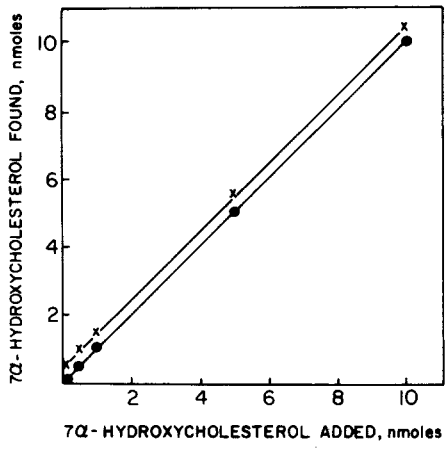

Fig. 3. Recovery of 7α-hydroxycholesterol from rat liver microsomes. Known amounts of 7α-[4-^{14}C]hydroxycholesterol (0–10 nmoles) were added to standard incubation mixtures containing 4.25 mg of microsomal protein (×). The identical mixture containing no microsomes was run in parallel (●). The amounts of 7α-hydroxycholesterol in the solutions were determined by the method described in Experimental Procedure with a precision of ±5%. It can be seen that the original solution of microsomes contained approximately 0.5 nmole of 7α-hydroxycholesterol per 4.25 mg of microsomal protein.

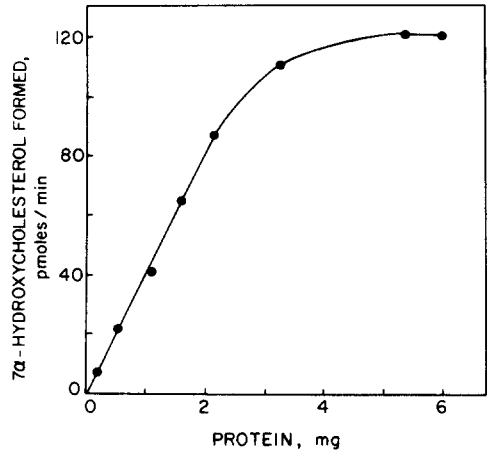

Fig. 4. Effect of increasing amounts of microsomal protein on the rate of 7α-hydroxycholesterol formation; standard assay conditions except for protein concentration.

amounts of biosynthetic 7α-hydroxycholesterol formed in incubation mixtures containing increasing amounts of microsomal protein (**Fig. 4**). The rate of formation of 7α-hydroxycholesterol was linear with respect to protein concentration up to 4 mg of protein/ml.

Table 2 illustrates the activity of cholesterol 7α-hydroxylase measured by the previously described isotope incorporation method (1) and the proposed isotope derivative method. Rats under different types of treatment were chosen in order to obtain a large range of enzyme activities for comparison. Both methods clearly detected the increases in 7α-hydroxylase activity produced by administration of cholestyramine or phenobarbital. Enzyme activities determined by the isotope incorporation method were about 20% lower than those measured with the proposed isotope derivative method.

DISCUSSION

The use of the isotope incorporation method for the determination of cholesterol 7α-hydroxylase involved the assumption that the added labeled cholesterol equilibrated very rapidly with the endogenous unlabeled microsomal cholesterol. Furthermore, it did not take into account that the endogenous cholesterol pool amounted to an appreciable proportion of the total cholesterol present. For example, in our studies we added a relatively large excess of labeled cholesterol to the microsomes and did not correct for endogenous (microsomal) cholesterol in calculating cholesol 7α-hydroxylase activity (1). Others have added only tracer amounts of cholesterol to the microsomes and have assumed that the total cholesterol in the incubation mixture is available to the enzyme (10). Mitropoulos and Balasubramaniam (3) provided evidence that not all of the endogenous microsomal cholesterol pool was available to the enzyme, although their observations were difficult to interpret because even tracer amounts of labeled cholesterol added to the microsomal suspension did not necessarily mix completely with unlabeled, endogenous material. They did demonstrate that the assay of cholesterol 7α-hydroxylase could be improved by measuring the amount of 7α-hydroxycholesterol directly by an isotope derivative procedure. Their published method was relatively time-consuming because it involved two radioautographic steps for the detection of 7α-hydroxycholesterol (and its diacetate) on TLC plates.

The simplified isotope derivative procedure described in this paper does not require radioautography, so measurements of enzyme activity can be obtained within a period of 24 hr. The proposed method is based upon the finding that (*a*) the incubation mixture containing cholesterol, 7α-hydroxycholesterol, and autoxidation products can be acetylated nearly quantitatively with [^{3}H]acetic anhydride

TABLE 2. Cholesterol 7α-hydroxylase activity of rat liver microsomes determined by two methods

Treatment	7α-Hydroxycholesterol Formed	
	Previous Method[a]	Proposed Method[b]
	pmoles/mg protein/min	
None	12.1	14.6
Cholestyramine, 5% of diet, 7 days	32.5	40.7
Phenobarbital, 100 mg/kg i.p., 5 days	100.9	123.2

[a] Isotope incorporation method (1).
[b] Isotope derivative method.

in pyridine at 100°C, and (*b*) the diacetate of 7α-hydroxycholesterol can be separated from other acetylated sterols by TLC on alumina G with ether–hexane as the developing solvent. The improved accuracy of the present procedure is due to proper corrections for losses during the extraction and acetylation steps. This is done by either determining the recovery of ^{14}C in added substrate cholesterol or adding a known amount of 7β-hydroxycholesterol as an internal standard (in experiments in which unlabeled cholesterol is used as substrate of the 7α-hydroxylase).

The sensitivity of the method is such that 0.1 nmole of 7α-hydroxycholesterol can be detected. This sensitivity enabled us to obtain an estimate of the concentration of endogenous 7α-hydroxycholesterol present in the microsomes (Fig. 3, which illustrates the recovery of added 7α-hydroxycholesterol in the presence and absence of microsomes). When a microsomal suspension containing 4.25 mg of microsomal protein was analyzed, approximately 0.5 nmole of 7α-hydroxycholesterol was detected. This corresponds to about 0.1 nmole of endogenous 7α-hydroxycholesterol per milligram of microsomal protein, and this value is in good agreement with that reported by Mitropoulos and Balasubramaniam (3). The sensitivity of the present procedure can be increased further, if desired, by employing [^3H]acetic anhydride of greater specific activity, which is available commercially. This increase in sensitivity should make it possible to determine the activity of cholesterol 7α-hydroxylase in the small amounts of microsomal protein (0.2–0.5 mg) obtained during percutaneous needle biopsy.

Comparison of the proposed method with the isotope incorporation procedure (Table 2) using hepatic microsomes of control rats and of animals treated with cholestyramine or phenobarbital indicates that the new method yields enzyme activities that are approximately 20% greater.

In general, the underestimation of cholesterol 7α-hydroxylase activity by the isotope incorporation method does not appear to be serious when comparison is made between groups of animals in which enzyme activities differ 3- to 10-fold. However, the proposed method may be useful in detecting more subtle changes in enzyme activity, for example, those produced by short-term cholesterol feeding (11).

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