Cholesterol 7 α -Hydroxylase in Rat Liver Microsomal Preparations

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Subcellular fractions containing microsomes prepared from rat livers homogenized in the absence of EDTA catalysed the oxidation of cholesterol to 7 α -hydroxycholesterol, 7-oxocholesterol, 7 β -hydroxycholesterol and 5 α -cholestane-3 β ,5,6 β -triol. These reactions required native protein, molecular oxygen and NADPH. It is suggested that these compounds are formed by a peroxidation analogous to the peroxidation of fatty acids catalysed by liver microsomal preparations. Incubations of [4-¹⁴C]cholesterol with microsomal preparations from rat liver homogenized in the presence of EDTA gave 7 α -hydroxy[¹⁴C]cholesterol as the main product. This reaction required molecular oxygen and NADPH, and was inhibited by CO. The mass of 7 α -hydroxycholesterol formed during the incubation was measured by a double-isotope-derivative dilution procedure. This procedure was used to assay the activity of cholesterol 7 α -hydroxylase and to measure low concentrations of endogenous 7 α -hydroxycholesterol in liver.

The introduction of the 7 α -hydroxyl group into the ring system of cholesterol is thought to be the first step in the sequence leading from cholesterol to cholic acid (Bergström, 1959). Cholesterol 7 α -hydroxylase, the enzyme catalysing this step, is confined to liver microsomal preparations and requires NADPH and molecular O₂ to function. Because of the inhibitory effect of CO on the activity of 7 α -hydroxylase, cytochrome *P*-450 has been implicated as the terminal oxidase for this hydroxylation (Boyd *et al.*, 1969). Circumstantial evidence accumulated during the past few years indicates that 7 α -hydroxylation may be rate-limiting in cholic acid formation. Hence, by studying the effect of various physiological factors on the activity of 7 α -hydroxylase, it may be possible to obtain information about the regulation of bile acid formation in the liver.

Current methods for assaying the activity of 7 α -hydroxylase are based on measuring the incorporation into 7 α -hydroxycholesterol (cholest-5-ene-3 β ,7 α -diol) of radioactive cholesterol added to a suspension of liver microsomal preparations and incubated in the presence of a suitable medium. Such methods may give erroneous results for the activity of 7 α -hydroxylase because liver microsomal preparations contain appreciable amounts of cholesterol. It is possible that only a portion of this endogenous cholesterol acts as substrate for the enzyme and that the proportion of the endogenous cholesterol that is metabolically active varies under different physiological conditions. It is also possible that the added radioactive cholesterol mixes with the endogenous cholesterol to different extents, depending on the way in which it is added. These considerations have led us to develop a more direct method for measuring the

activity of cholesterol 7 α -hydroxylase in rat liver microsomal preparations.

Cholesterol in aqueous suspension is susceptible to attack by O₂ with the formation of 7-oxocholesterol (3 β -hydroxycholest-5-en-7-one), 7 β -hydroxycholesterol (cholest-5-ene-3 β ,7 β -diol), 7 α -hydroxycholesterol and other products. Since this process is enhanced in the presence of a microsomal preparation containing NADPH, 7 α -hydroxycholesterol can be formed from cholesterol by peroxidative reactions, as well as by the action of cholesterol 7 α -hydroxylase. This presents a problem in the assay of cholesterol 7 α -hydroxylase.

In this paper we describe conditions for the assay *in vitro* of the activity of 7 α -hydroxylase in liver microsomal preparations. These conditions minimize NADPH-induced peroxidation of cholesterol to 7 α -hydroxycholesterol and non-physiological products including 7-oxocholesterol, 7 β -hydroxycholesterol and 5 α -cholestane-3 β ,5,6 β -triol. The product of cholesterol 7 α -hydroxylase activity is measured by a double-isotope-derivative dilution procedure (Peterson & Eilers, 1965). This assay procedure has been used to measure the activity of the enzyme and the steady-state concentration of 7 α -hydroxycholesterol in liver subcellular fractions.

Experimental

Animals and their treatment

For all normal purposes male rats of the Wistar strain, weighing 150-175 g and fed on M.R.C. Diet no. 18 (Bruce & Parkes, 1946), were used. Rats

treated with cholestyramine resin were fed on a diet containing 5% (w/v) cholestyramine for 7 days.

Preparation of liver cell fractions and incubations

The rats were killed by cervical dislocation at about 8.00 a.m. on the day of the experiment. The livers were removed immediately, chilled in ice and perfused with ice-cold 0.25M-sucrose to remove contaminating haemoglobin. The perfused livers were then minced in a stainless-steel mincer and homogenized in 4vol. of ice-cold 0.1M-potassium phosphate buffer (pH 7.4) containing nicotinamide (30mM) and either MgCl_2 (5mM) or EDTA (1mM). The homogenate was centrifuged at 800g for 10min and the supernatant obtained was centrifuged at 15000g for 15min. The supernatant from this centrifugation (S_{15} fraction) was centrifuged at 104000g for 60min to give a supernatant fraction (S_{104} fraction) and a microsomal pellet. The microsomal fraction was prepared by suspending the microsomal pellet in 0.1M-potassium phosphate buffer (pH 7.4) containing nicotinamide (30mM) and MgCl_2 (5mM).

The standard incubation mixture contained 3ml of the S_{15} fraction (20–24mg of protein) and 2ml of potassium phosphate buffer (pH 7.4) containing nicotinamide (30mM) and MgCl_2 (5mM). For some experiments the microsomal fraction from 3ml of the S_{15} fraction was incubated. In these experiments the standard incubation mixture contained, in a final volume of 5ml, 0.1M-potassium phosphate buffer (pH 7.4), 30mM-nicotinamide, 5mM- MgCl_2 , 1mM- NADP^+ , 10mM-glucose 6-phosphate and 1 unit of glucose 6-phosphate dehydrogenase (1 μmol of NADPH formed/min). In every case the substrate cholesterol, labelled or unlabelled, was added to the incubation mixture as a solution in acetone or as a suspension in Tween 80. The incubations were carried out with air as the gas phase (unless specified otherwise) at 37°C with constant shaking. The incubation was terminated by adding 20vol. of chloroform-methanol (2:1, v/v) and 1ml of 0.25M- H_2SO_4 . The mixture was shaken vigorously and the precipitated protein removed by filtration. The filtrate was washed with 0.2vol. of 0.9% NaCl and the phases were allowed to separate. The aq.-methanol phase contained no radioactivity and was discarded. The lower chloroform phase was evaporated to dryness under N_2 and the products of incubation were separated by t.l.c. on Kieselgel H with diethyl ether as solvent, without prior equilibration, at 5°C. The radioactive bands were located by radioautography.

The areas of the plate corresponding to the bands of blackening on the radioautogram were scraped off the plate and eluted in chloroform-methanol (2:1, v/v). Samples were taken simultaneously for radioassay and for measurement of mass when required.

Acetylation and assay of 7 α -hydroxycholesterol

For the acetylation of 7 α -hydroxycholesterol the samples were dried in small conical tubes. To each tube was added 20 μl of dried pyridine and 20 μl of a 10% (w/v) solution of freshly distilled [^3H]acetic anhydride in benzene. The tubes were tightly stoppered and the contents thoroughly mixed. After 36h at 37°C the excess of [^3H]acetic anhydride and pyridine were removed by distillation at 50–60°C under a stream of N_2 .

The [^3H , ^{14}C]cholest-5-ene-3 β ,7 α -diol diacetate formed was separated from the monoacetylated and unacetylated steroid and from other radioactive contaminants by t.l.c. on Kieselgel H, first with benzene-ethyl acetate (10:1, v/v) as the solvent system, and then rechromatographed with benzene-ethyl acetate (5:1, v/v) as the solvent system. The radioactive diacetylated product, located on the t.l.c. plate on each occasion by radioautography, was finally eluted from the silica gel and the $^3\text{H}/^{14}\text{C}$ ratio was determined.

The net amount (T) of 7 α -hydroxycholesterol synthesized during an incubation was calculated from the equation $T = \{[(AR) - B]/S\} - Z \mu\text{mol}$, where A is the amount of 7 α -hydroxy [^{14}C]cholesterol (d.p.m.) formed during the incubation, R is the $^3\text{H}/^{14}\text{C}$ ratio in the purified diacetoxy compound, S is the specific radioactivity of the [^3H]acetic anhydride (d.p.m./ μmol), B is the amount of ^3H introduced by subjecting a sample to the same procedures as the microsomal 7 α -hydroxycholesterol (d.p.m.) and Z is the amount of 7 α -hydroxycholesterol in the incubation mixture (μmol) at zero time.

A was determined from the amount of ^{14}C eluted from the 7 α -hydroxycholesterol band on the chromatogram of the extracts of the incubation mixture. B was determined from the amount of ^3H incorporated into samples of 7 α -hydroxy [^{14}C]cholesterol of known specific radioactivity subjected to all the procedures to which the microsomal samples were subjected and allowing for the amount of ^3H due to the mass of 7 α -hydroxy [^{14}C]cholesterol in the sample. For the determination of Z a sample of 7 α -hydroxy [^{14}C]cholesterol of known specific radioactivity was added to the incubation mixture, the sample was extracted at zero time and the fraction of 7 α -hydroxycholesterol obtained was taken through the same steps as the incubated sample. Z was determined from the amount of ^3H incorporated into the sample, allowing for the amount of ^3H due to the blank (B) and for the amount of ^3H due to the mass of 7 α -hydroxy [^{14}C]cholesterol added to the sample originally.

Cholesterol in the subcellular fractions was determined by the method of Clark *et al.* (1968). Protein was determined by the biuret method. Cytochrome P-450 was determined from the CO difference

spectrum of the reduced pigment (Omura & Sato, 1964). Wherever suitable the results are expressed as means values $\pm$ S.D.

Radioassay

Radioactivity was assayed in a Beckman LS-250 liquid-scintillation spectrometer. The energy spectrum was corrected for quenching by an automatic gain control and this correction was found to be reliable over the range of quenching encountered in the present experiments. Counting rates were corrected for quenching by the external-standard channels-ratio method.

Materials

NADP⁺, NAD⁺, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were obtained from C.F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Haematoporphyrin dihydrochloride and β -mercaptoethylamine were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Kieselgel H was obtained from E. Merck A.G., Darmstadt, Germany. Alumina type H was obtained from Laporte Industries Ltd., London W.1, U.K. Cholestyramine resin (Cuemid) was obtained from Merck, Sharp and Dohme, Division of Merck and Co. Inc., West Point, Pa., U.S.A. All other chemicals were obtained from British Drug Houses Ltd., Poole, Dorset, U.K. Solvents used were commercial AnalaR-grade preparations. Benzene was dried over sodium wire and redistilled. Pyridine was redistilled over NaOH. Diethyl ether was purified on a column of alumina type H before use. Radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

[³H]Acetic anhydride (100mCi/mmol), used for acetylation of 7 α -hydroxycholesterol, was diluted with non-radioactive acetic anhydride, and a 10% (w/v) solution in benzene was distilled twice under a stream of N₂ at 50–60°C before each acetylation. The specific radioactivity of each batch of [³H]acetic anhydride was determined by acetylating samples of known mass of [4-¹⁴C]cholesterol, isolating the doubly labelled acetylated product and determining the amount of ³H incorporated into the samples. [4-¹⁴C]Cholesterol (60mCi/mmol) was diluted to the desired specific radioactivity and purified by t.l.c. on Kieselgel H with ether at 5°C (R_F 0.66) and with benzene-ethyl acetate (3:7, v/v; R_F 0.54) before it was added to the incubation medium.

7 α -Hydroxycholesterol was synthesized from cholesterol (Lythgoe & Trippett, 1959). Cholesterol in pyridine was photo-oxygenated in the presence of haematoporphyrin dihydrochloride (Nickon & Bagli, 1959) and the 3 β -hydroxy-5 α -cholest-6-ene-5-hydroperoxide formed was isolated from the reaction

mixture and recrystallized from diethyl ether-methanol (m.p. 146–149°C). A suspension of 3 β -hydroxy-5 α -cholest-6-ene-5-hydroperoxide in chloroform, left at room temperature for 24h with intermittent shaking, isomerized to 3 β -hydroxycholest-5-ene-7 α -hydroperoxide (m.p. 154–155°C). This hydroperoxide in methanol was then reduced with sodium borohydride to 7 α -hydroxycholesterol, which was recrystallized several times from aq. methanol to give fine needles of m.p. 184–187°C. 7 α -Hydroxy[4-¹⁴C]-cholesterol was synthesized from [4-¹⁴C]cholesterol essentially by the above procedure, but the purification of the intermediate compounds and the final product was by t.l.c. on Kieselgel H.

Cholest-5-ene-3 β ,7 α -diol diacetate was prepared by acetylation of 7 α -hydroxycholesterol. A portion (30mg) of 7 α -hydroxycholesterol was dissolved in 2ml of pyridine and allowed to react with 1ml of acetic anhydride for 24h at 37°C. After evaporation of the solvents the oily residue was triturated with methanol, from which it was crystallized to yield transparent needles of cholest-5-ene-3 β ,7 α -diol diacetate (m.p. 122–123°C). 7-Oxocholesterol and 7 β -hydroxycholesterol were synthesized by the method of Fieser *et al.* (1949).

Results

When [4-¹⁴C]cholesterol was incubated in the presence of NADPH, with the microsomal fraction of rat liver initially homogenized in the absence of EDTA, more than 10% of the radioactivity was incorporated into products more polar than cholesterol. A typical radioautogram from a chromatogram of the extracts of an incubation mixture is shown in Plate 1(a). The main product was 7-oxocholesterol, but there was some incorporation of radioactivity into 5 α -cholestane-3 β ,5,6 β -triol, 7 β -hydroxycholesterol and 7 α -hydroxycholesterol. When the microsomal preparations were inactivated by heating them at 100°C for 10min before incubation, the formation of all products, including 7-oxocholesterol, was markedly diminished (Table 1). A similar diminution was observed when NADPH was excluded from the incubation medium. Under the conditions used for these experiments, CO failed to inhibit the formation of 7 α -hydroxycholesterol (Table 2).

It has been reported by Scholan & Boyd (1968) that thiol compounds such as β -mercaptoethylamine completely inhibit the NADPH-induced microsomal autoxidation of cholesterol and that they activate 7 α -hydroxylase. In agreement with their findings, β -mercaptoethylamine added to the incubation mixture decreased the formation of products such as 7-oxocholesterol, 7 β -hydroxycholesterol and 5 α -cholestane-3 β ,5,6 β -triol, but there was no increased

Table 1. *Incorporation of radioactivity into 7 α -hydroxycholesterol and other steroids during incubations of [4-¹⁴C]cholesterol with various subcellular fractions*

The subcellular fractions were prepared from rat livers homogenized in the absence of EDTA. Boiled microsomal fraction and boiled S₁₀₄ fraction were obtained by heating at 100°C for 10 min. All samples contained 6.8 mg of microsomal protein and were incubated for 1 h. Other experimental details are given in the Experimental section.

Subcellular fraction	Incorporation of radioactivity (% of [¹⁴ C]cholesterol)			
	7 α -Hydroxycholesterol	7-Oxocholesterol	7 β -Hydroxycholesterol	5 α -Cholestane- 3 β ,5,6 β -triol
Microsomal fraction	1.3	4.6	1.9	4.5
Microsomal fraction without NADPH*	0.6	1.2	0.8	1.2
Boiled microsomal fraction	0.4	0.6	0.3	1.1
S ₁₅ fraction	2.4	5.1	2.2	5.0
Microsomal fraction + boiled S ₁₀₄ fraction	2.0	5.8	2.0	4.9

* NADP⁺, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were omitted from the incubation mixture.

Table 2. *Requirement for oxygen and effect of CO on the incorporation of radioactivity into 7 α -hydroxycholesterol and into other steroids formed during incubation of [4-¹⁴C]cholesterol with rat liver microsomal fraction*

The microsomal fraction was obtained from liver homogenates prepared without EDTA. All samples contained 7.3 mg of microsomal protein, were gassed with the appropriate mixture before the addition of [4-¹⁴C]cholesterol and were incubated for 1 h. Other experimental details are given in the Experimental section.

Gas mixture	Incorporation of radioactivity (% of [¹⁴ C]cholesterol)			
	7 α -Hydroxycholesterol	7-Oxocholesterol	7 β -Hydroxycholesterol	5 α -Cholestane- 3 β ,5,6 β -triol
N ₂ + O ₂ (9:1, v/v)	1.5	5.5	2.2	4.4
N ₂ (100%)	0.5	1.0	0.7	1.1
CO + O ₂ (9:1, v/v)	1.8	6.0	2.2	4.6

formation of 7 α -hydroxycholesterol (Fig. 1). Moreover, in incubations in the presence of β -mercaptoethylamine (10 mM) CO had only a slight inhibitory effect on the formation of 7 α -hydroxycholesterol. These findings prompted us to look for conditions in which the enzymic formation of 7 α -hydroxycholesterol is even greater than in the presence of β -mercaptoethylamine, and in which the formation of non-physiological peroxidation products of cholesterol is minimized.

Microsomal preparations from rat liver that was homogenized in the presence of 1 mM-EDTA and incubated with [4-¹⁴C]cholesterol produced 7 α -hydroxycholesterol as the main product (Plate 1b). The percentage incorporation of added radioactive cholesterol into the 7 α -hydroxycholesterol fraction (Table 3) was much higher than in similar incubations

with microsomal fraction from liver homogenized in the absence of EDTA. The absolute requirement for O₂ and NADPH for the formation of 7 α -hydroxycholesterol is shown in Table 3. In incubations with N₂ as the gas phase, or in aerobic incubations with NADPH excluded, the amount of 7 α -hydroxy[¹⁴C]cholesterol formed from added [4-¹⁴C]cholesterol was negligible, as was also the case with heat-inactivated microsomal preparations. The requirement for NADPH could be replaced by the soluble fraction of the cell (S₁₀₄). In agreement with the findings of other workers (Shefer *et al.*, 1968; Scholan & Boyd, 1968; Wada *et al.*, 1969; Gielen, 1969), CO present in the gas phase inhibited the formation of 7 α -hydroxycholesterol.

7 α -Hydroxycholesterol enzymically produced from several incubations was pooled, mixed with 20 mg of

EXPLANATION OF PLATE I

(a) Radioautograph of a thin-layer chromatogram of the extracts from an incubation of [4-¹⁴C]cholesterol with the microsomal fraction of rat liver homogenized in the absence of EDTA. The solvent used was ether at 5°C. The positions of reference compounds on the chromatogram are shown to the right of the radioautograph. (b) Radioautographs of thin-layer chromatograms of the extracts from incubations of [4-¹⁴C]cholesterol with the microsomal fraction from rat liver homogenized in the presence of EDTA. The solvent was ether at 5°C. (i) [4-¹⁴C]cholesterol incubated with microsomal fraction in the presence of NADPH; (ii) [4-¹⁴C]cholesterol incubated with microsomal fraction in the presence of NADPH and NAD⁺. The positions of reference compounds on the chromatograms are shown to the right of the radioautographs.

authentic 7 α -hydroxycholesterol and crystallized to constant specific radioactivity from aq. methanol. The specific radioactivity of the crystals remained constant at 2385 c.p.m./mg during two recrystallizations and was the same as the calculated specific radioactivity of the original mixture. A portion (12.35 mg) of radioactive 7 α -hydroxycholesterol

obtained after the second recrystallization was acetylated. The specific radioactivity of the diacetate, expressed in c.p.m./ μ mol, was similar to that of the radioactive 7 α -hydroxycholesterol (945 and 959 c.p.m./ μ mol respectively).

The second step in the sequence leading from cholesterol to cholic acid is the formation of 7 α -hydroxycholest-4-en-3-one, a step catalysed by a microsomal enzyme and requiring NAD⁺ as cofactor (Berséus *et al.*, 1969). The enzyme, 12 α -hydroxylase, that introduces the 12 α -hydroxyl group into the ring system requires O₂ and NADPH to function (Suzuki *et al.*, 1968). Hence, the addition of NAD⁺ (0.3 mM) to the standard incubation mixture allowed the reaction to proceed further, to the formation of 7 α -hydroxycholest-4-en-3-one and 7 α ,12 α -dihydroxycholest-4-en-3-one. The addition of increasing amounts of 7 α -hydroxycholesterol to the incubation medium increased the amount of ¹⁴C incorporated into 7 α -hydroxycholesterol and diminished the incorporation of radioactivity into 7 α -hydroxycholest-4-en-3-one and into 7 α ,12 α -dihydroxycholest-4-en-3-one (Table 4). However, 7 α -hydroxycholesterol added at a concentration of 100 μ M inhibited its own synthesis.

The sensitivity of the acetylation method for the determination of 7 α -hydroxycholesterol was evaluated by acetylating known amounts of the steroid and determining it on the basis of the known specific radioactivity of the acetic anhydride. The curve of observed versus expected values was linear over the range determined (Fig. 2). The accuracy of the procedure was further assessed by recoveries obtained when 7 α -hydroxycholesterol was added in known increments to microsomal suspensions. The recovery curve (Fig. 2) had the same slope as the standard curve but was shifted by 1.0 μ g on the ordinate, indicating the presence of endogenous 7 α -hydroxycholesterol in microsomal preparations.

Fig. 1. Effect of β -mercaptoethylamine on the incorporation of [4-¹⁴C]cholesterol into 7 α -hydroxycholesterol and other steroids

Microsomal fractions from liver homogenized in the absence of EDTA were incubated for 60 min with [4-¹⁴C]cholesterol in the presence of variable concentrations of the thiol. ○, Radioactivity incorporated into 7 α -hydroxycholesterol; ●, total radioactivity incorporated into 7-oxocholesterol, 7 β -hydroxycholesterol and 5 α -cholestane-3 β ,5,6 β -triol.

Table 3. Requirement for NADPH and oxygen and inhibition by CO of the 7 α -hydroxylation of cholesterol by microsomal fractions prepared from rat liver homogenized in the presence of EDTA

All samples contained 7.8 mg of microsomal protein and were incubated for 1 h. Samples incubated with gas phase other than air were gassed with the appropriate mixture before the addition of [4-¹⁴C]cholesterol. Other experimental details are given in the Experimental section.

Conditions	% of added [4- ¹⁴ C]cholesterol incorporated into 7 α -hydroxycholesterol
Standard incubation mixture	5.00
Boiled microsomal fraction	0.45
Without NADPH*	0.43
Standard incubation mixture	
N ₂ + O ₂ (9:1, v/v)	4.97
N ₂ (100%)	0.50
CO + O ₂ (9:1, v/v)	1.00

* NADP⁺, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were omitted from the incubation mixture.

Table 4. Incorporation of radioactivity into the fractions of 7α -hydroxycholesterol, 7α -hydroxycholest-4-en-3-one and $7\alpha,12\alpha$ -dihydroxycholest-4-en-3-one on incubation of $[4-^{14}\text{C}]$ cholesterol with rat liver microsomal fraction in the presence of various concentrations of 7α -hydroxycholesterol

The standard incubation mixture contained 5.6mg of microsomal protein and included NAD^+ (0.3mM). $[4-^{14}\text{C}]$ Cholesterol and 7α -hydroxycholesterol were added as a suspension in Tween 80. All flasks contained 4.8mg of Tween 80 and they were incubated for 30min.

7α -Hydroxycholesterol added (μM)	Incorporation of radioactivity (% of $[^{14}\text{C}]$ cholesterol)		
	7α -Hydroxycholesterol	7α -Hydroxy- cholest-4-en-3-one	$7\alpha,12\alpha$ -Dihydroxy- cholest-4-en-3-one
0	2.16	2.56	0.79
2.5	2.32	2.56	0.66
5.0	2.68	2.60	0.55
10.0	2.93	2.47	0.50
25.0	3.43	1.55	0.38
100.0	3.10	0.82	0.23

Fig. 2. Standard curves of 7α -hydroxycholesterol assayed with the acetylation method in the absence (○) and in the presence (●) of liver microsomal fraction

For experimental details see the text.

To confirm the identity of the endogenous 7α -hydroxycholesterol present in microsomal preparations, 7α -hydroxy $[4-^{14}\text{C}]$ cholesterol ($0.08\mu\text{g}$) of known specific radioactivity was added to 1ml of microsomal preparation and the sample was subjected to the procedure described for incubated samples. After the final $^3\text{H}/^{14}\text{C}$ ratio was measured, the

$[^3\text{H},4-^{14}\text{C}]$ cholest-5-ene- $3\beta,7\alpha$ -diol diacetate ($3.47\mu\text{g}$) was mixed with unlabelled diacetate and crystallized to constant specific radioactivity. During all five recrystallizations the $^3\text{H}/^{14}\text{C}$ ratio of the steroid remained more or less constant (Table 5).

With the conditions of incubation described above and the acetylation method for measuring the 7α -hydroxycholesterol produced, the rate of synthesis of 7α -hydroxycholesterol in liver microsomal fraction was found to be $1.33 \pm 0.11 \text{ nmol/30min}$ per mg of protein (four determinations). The concentration of endogenous 7α -hydroxycholesterol was $0.09 \pm 0.006 \text{ nmol/mg}$ of microsomal protein (three determinations). The rate of synthesis of 7α -hydroxycholesterol in liver microsomal fraction from rats treated with cholestyramine was $2.82 \pm 0.51 \text{ nmol/30min}$ per mg of protein (four determinations) and the concentration of endogenous 7α -hydroxycholesterol was $0.35 \pm 0.006 \text{ nmol/mg}$ of microsomal protein (three determinations).

In experiments in which $[4-^{14}\text{C}]$ cholesterol was incubated under the conditions already described for the microsomal fraction, the specific radioactivity of the 7α -hydroxycholesterol ($6215 \pm 387 \text{ d.p.m./nmol}$; five determinations) formed during a 30-min incubation was found to be lower than that of the added $[4-^{14}\text{C}]$ cholesterol ($110000 \text{ d.p.m./nmol}$) and higher than that of the total cholesterol in the incubation mixture ($4118 \pm 313 \text{ d.p.m./nmol}$; five determinations). This suggests that not all the microsomal cholesterol is accessible to cholesterol 7α -hydroxylase. If so, the activity of cholesterol 7α -hydroxylase assayed by the isotope-incorporation method is likely to be influenced by the size of the metabolically active cholesterol pool in the microsomal fraction. Moreover, it was found that the percentage incorporation of $[4-^{14}\text{C}]$ cholesterol into 7α -hydroxycholesterol was

Table 5. Identification of 7 α -hydroxycholesterol present in rat liver microsomal fraction

7 α -Hydroxy[4- 14 C]cholesterol of known specific radioactivity was added to 1 ml of microsomal fraction. Lipids were extracted and the 7 α -hydroxycholesterol fraction was isolated by t.l.c. The steroid was acetylated with [3 H]acetic anhydride and the 3 H/ 14 C ratio was measured. The acetylated product was mixed with 23mg of cholest-5-ene-3 β ,7 α -diol diacetate and the 3 H/ 14 C ratio was measured during repeated recrystallizations.

No. of crystallizations	3 H Radioactivity (d.p.m.)	14 C Radioactivity (d.p.m.)	3 H/ 14 C ratio
Original mixture	69 500	5500	12.6
1	39 240	2696	14.6
2	26 760	1940	13.8
3	22 900	1590	14.4
4	9530	714	13.3
5	3992	291	13.7

Table 6. Effect of the form in which [4- 14 C]cholesterol is added to the incubation medium on the results obtained by the isotope-incorporation method for measuring the activity of cholesterol 7 α -hydroxylase

Rat liver microsomal fraction (5.6mg of microsomal protein per flask) was incubated for 30min with [4- 14 C]cholesterol added as a solution in 30 μ l of acetone or as a suspension in 4.8mg of Tween 80.

Mode of addition of cholesterol	Percentage of [4- 14 C]cholesterol incorporated into 7 α -hydroxycholesterol	7 α -Hydroxycholesterol assayed by the acetylation method (μ g)
Tween suspension	2.0	3.0
Acetone solution	1.0	3.1

twice as much when the [14 C]cholesterol was added to the incubation mixture as a suspension in Tween 80 as when it was added as a solution in acetone. The net synthesis of 7 α -hydroxycholesterol as assayed by the acetylation method was the same in both cases (Table 6).

Discussion

Studies *in vitro* from several laboratories (Mendelsohn *et al.*, 1965; Danielsson & Einarsson, 1964; Mitton & Boyd, 1965) have shown the conversion of cholesterol into 7 α -hydroxycholesterol by subcellular fractions containing microsomes. However, work *in vitro* has been complicated by the ease with which cholesterol can form oxidation products on exposure to oxygen. A large number of autoxidation products of cholesterol, including 7 α -hydroxycholesterol, have been shown to be produced non-enzymically under various conditions (Van Lier & Smith, 1970). The formation *in vitro* of 7 α -hydroxycholesterol from cholesterol is accompanied by other oxidation products, including 7-oxocholesterol, 7 β -hydroxycholesterol and 5 α -cholestane-3 β ,5,6 β -triol (Danielsson & Einarsson, 1964; Scholan & Boyd, 1968; Gielen *et al.*, 1968). Since these last three compounds are not converted into normal bile

acids *in vivo* or *in vitro*, they presumably have no physiological significance.

In the present work, microsomal fractions prepared from rat livers homogenized in the absence of EDTA converted cholesterol into 7 α -hydroxycholesterol, 7-oxocholesterol, 7 β -hydroxycholesterol and 5 α -cholestane-3 β ,5,6 β -triol. The requirement for NADPH and native protein for the catalysis of these processes is evident from our experiments. As the requirements for the formation of 7-oxocholesterol, 7 β -hydroxycholesterol and 5 α -cholestane-3 β ,5,6 β -triol are the same as those for cholesterol 7 α -hydroxylase, it is possible that a part or all of the 7 α -hydroxycholesterol formed under these conditions is derived through the same processes as the non-physiological companions of 7 α -hydroxycholesterol. Moreover, some 7 α -hydroxycholesterol could have been formed through the enzymic reduction of 7-oxocholesterol, as was demonstrated by Danielsson (1969). The possibility that 7 α -hydroxycholesterol formed in the experiments with microsomal fractions prepared without EDTA was not the product of cholesterol 7 α -hydroxylase is supported by the failure of CO to inhibit its formation. Since 7 α -hydroxycholesterol can be formed *in vitro* by non-physiological processes, the problem of physiological and non-physiological reactions must be resolved before an

enzymic assay of cholesterol 7 α -hydroxylase can be attempted.

The conditions required for the formation of non-physiological products of cholesterol, and also of 7 α -hydroxycholesterol, are similar to those under which lipid peroxidation occurs (Wills, 1969a). The fact that anti-liperoxidative agents such as EDTA or β -mercaptoethylamine inhibited the formation only of the non-physiological metabolites of cholesterol confirms the closely related nature of these two processes (Mitton *et al.*, 1971).

Microsomal fractions from liver homogenized in the presence of EDTA are devoid of liperoxidative activity (Wills, 1969b) and do not produce the non-physiological companions of 7 α -hydroxycholesterol, whereas the percentage incorporation of radioactivity into the 7 α -hydroxycholesterol fraction was comparatively high. For the formation of 7 α -hydroxycholesterol from cholesterol under these conditions there is also a requirement for O₂ and NADPH. The inhibition of the formation of the non-physiological companions of 7 α -hydroxycholesterol, with concomitant increase in 7 α -hydroxylation of cholesterol, suggests the operation of a shared or partly shared electron-transport system for the activation of O₂ by cholesterol 7 α -hydroxylase and for cholesterol peroxidation. The inhibition of 7 α -hydroxycholesterol formation in incubations with CO in the gas phase confirms earlier results (Shefer *et al.*, 1968; Scholan & Boyd, 1968; Wada *et al.*, 1969; Gielen, 1969) implicating cytochrome P-450 as the terminal oxidase for cholesterol 7 α -hydroxylase.

Microsomal fractions from liver homogenized in the presence of EDTA on incubation with [4-¹⁴C]-cholesterol in the presence of NADPH gave 7 α -hydroxy[¹⁴C]cholesterol as the main radioactive product. The inclusion of NAD⁺ in this incubation medium allowed the reactions in the sequence between cholesterol and cholic acid to proceed further. The trapping of radioactivity in the 7 α -hydroxycholesterol fraction with concomitant decrease in the incorporation of radioactivity in the next two intermediates, in incubations in the presence of unlabelled 7 α -hydroxycholesterol, provides additional evidence that 7 α -hydroxycholesterol is an early intermediate between cholesterol and cholic acid.

The determination of the mass of 7 α -hydroxycholesterol produced during the incubation provides a direct method for assaying the activity of cholesterol 7 α -hydroxylase that lacks the disadvantages of the isotope-incorporation method used previously for assaying the activity of this enzyme. The presence of appreciable amounts of cholesterol in liver microsomal preparations, only a part of which appears to act as substrate for cholesterol 7 α -hydroxylase, could lead to erroneous estimates of the activity of the enzyme measured by the isotope-incorporation method. The double-isotope-derivative dilution pro-

cedure introduced here could, under certain conditions, provide a measure of the size of the metabolically active pool of cholesterol. Moreover, the introduction of a lipid-soluble radioactive label to the incubation medium results in labelling of the endogenous cholesterol pool to different extents, depending on the way in which the label is added. The high sensitivity of the acetylation method coupled with its reproducibility makes possible the measurement of the small changes in the amounts of 7 α -hydroxycholesterol that occur during its synthesis *in vitro*. Moreover, the acetylation method enabled us to measure the 7 α -hydroxycholesterol that accumulates in subcellular fractions of liver at very low concentrations under different physiological conditions *in vivo*.

The identification of endogenous 7 α -hydroxycholesterol present in liver microsomal preparations and its increased concentration in the microsomal fractions from the livers of cholestyramine-treated rats provides additional evidence that 7 α -hydroxycholesterol is an intermediate in cholesterol catabolism.

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