Biosynthesis of cholestanol: 5α-cholestan-3-one reductase of rat liver

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ABSTRACT The 3-β-hydroxysteroid dehydrogenase of rat liver which catalyzes the conversion of 5α-cholestan-3-one to 5α-cholestan-3β-ol is localized mainly in the microsomal fraction. The enzyme required NADPH as hydrogen donor and differed from the known 3-β-hydroxysteroid dehydrogenases of the C_{19} series in being inactive in the presence of NADH. The microsomal preparations did not reduce the 3-keto groups of cholest-4-en-3-one, cholest-5-en-3-one, or 5β-cholestan-3-one to the corresponding 3β-hydroxy compounds. The conversion of 5α-cholestan-3-one to 5α-cholestan-3β-ol was only slightly inhibited by the reaction product or by other monohydroxy steroids, but a strong inhibitory effect was noted with cholest-5-en-3-one, 5α-cholestane-3β,7α-diol and 5α-cholestan-7-on-3β-ol.

The microsomes, but not high speed supernatant solution, catalyzed the reverse of the cholestanone reductase reaction, namely the conversion of 5α-cholestan-3β-ol to 5α-cholestan-3-one in the presence of oxygen and an NADP-generating system.

The action of the microsomal preparations upon 5α-cholestan-3-one produced 5α-cholestan-3α-ol in addition to the 3β-epimer. The 3-α-hydroxysteroid dehydrogenase involved functioned with either NADH or NADPH as hydrogen donor. The ratio of 5α-cholestan-3β-ol to 5α-cholestan-3α-ol formed from 5α-cholestan-3-one was approximately 10:1 and was independent of the sex of the animal from which the microsomes were prepared.

KEY WORDS 5α-cholestan-3-one · cholestanone reductase · 5α-cholestan-3β-ol · biosynthesis · 5α-cholestan-3α-ol · 3-β-hydroxysteroid dehydrogenase · 3-α-hydroxysteroid dehydrogenase · microsomal enzyme · rat · liver

This is the second paper in a series entitled Studies on the Biosynthesis of 5α-Cholestan-3β-ol, of which the first is reference 1.

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography. Systematic names of the steroids referred to in the text by their trivial names are: cholestanol, 5α-cholestan-3β-ol; cholestenone, cholest-4-en-3-one; cholestanone, 5α-cholestan-3-one; allocholesterol, cholest-4-en-3β-ol; epicholestanol, 5α-cholestan-3α-ol; androstanedione, 5α-androstane-3,17-dione; androstenedione, androst-4-ene-3,17-dione; dihydrotestosterone, 5α-androstan-3-on-17β-ol; coprostanol, 5β-cholestan-3β-ol; coprostanone, 5β-cholestan-3-one; 7-ketocholestanol, 5α-cholestan-7-on-3β-ol; 7α-hydroxycholestanol, 5α-cholestane-3β,7α-diol.

THE TRANSFORMATION of cholestenone to cholestanol in mammalian tissues probably involves two steps (1). First, a microsomal 5α-reductase catalyzes the reduction of the double bond of cholestenone to form cholestanone. Next, the saturated ketone is converted to cholestanol under the influence of a 3-β-hydroxysteroid dehydrogenase, henceforth referred to as cholestanone reductase.[1]

The formation of cholestanol from cholestanone in rat liver homogenates fortified with ATP and NAD^+ had previously been demonstrated by Harold, Abraham, and Chaikoff (2) but the properties of the enzyme involved were not investigated. Yamasaki, Noda, and Shimizu (3) reported the partial purification of a soluble 3-β-hydroxysteroid dehydrogenase from rat liver which acted preferentially upon steroids of the C_{27} series. The observed conversions were small, however, and the action of the enzyme preparations on cholestanone was not investigated.

The present study deals with the enzyme activity which catalyzes the conversion of cholestanone to cholestanol. This activity is localized predominantly in the microsomal fraction of rat liver. The incubation of cholestanone with the microsomal preparations produces mainly cholestanol, but some epicholestanol, a 3α-hydroxysteroid, is also formed.

The activity which converts cholestanone to cholestanol seems to reside in a protein different from the "particulate" 3-β-hydroxysteroid dehydrogenase of the

[1] Although the enzyme described in this paper resembles the 3-β-hydroxysteroid dehydrogenases that catalyze the oxidoreduction of 3-keto or 3-hydroxysteroids of the C_{19} and C_{21} series, it seems more convenient to refer to it specifically as cholestanone reductase in order to avoid a more complicated nomenclature.

C_{19} series described by Rubin and Strecker (4). This conclusion is based upon the finding that cholestanone reductase is NADPH-specific, while the microsomal 3-β-hydroxysteroid dehydrogenases of the C_{19} series function with either NADH or NADPH as hydrogen donors. In addition, the ratio of 3α- to 3β-epimers formed by the 3-hydroxysteroid dehydrogenases of the C_{19} series depends upon the sex of the rats from which liver microsomes are obtained, while the proportion of 3α- to 3β-hydroxy isomers produced from cholestanone is not subject to a significant sex difference.

EXPERIMENTAL PROCEDURE

Labeled Compounds

5α-Cholestan-3-one-4-^{14}C was prepared from cholestanol-4-^{14}C (New England Nuclear Corp., Boston, Mass.) by dichromate oxidation (5). The saturated ketone was purified by silicic acid column chromatography (6) and was found to contain less than 0.1% of cholestanol when assayed by TLC (described below). The labeled compound was compared with a known sample of cholestanone (mp 127–129°C, α_D = +41.5°) purchased from Steraloids, Inc., Pawling, N.Y. Both compounds had identical chromatographic properties when examined by TLC or GLC (1) and were chromatographically homogeneous.

5α-Cholestan-3β-ol-4-^{14}C (New England Nuclear Corp.) was purified by chromatography on a $AgNO_3$–silicic acid column (6). The purified product contained less than 0.1% of 5α-cholestan-3-one and less than 0.25% of cholesterol.

Cholest-5-en-3-one-4-^{14}C was prepared from cholesterol-4-^{14}C (7) and purified as described previously (1).

5α-Androstane-3,17-dione-4-^{14}C was obtained by reduction of androst-4-ene-3,17-dione-4-^{14}C (New England Nuclear Corp.) with a microsomal Δ^4-3-ketosteroid 5α-reductase preparation as described previously (1). At the end of the incubation period (30 min), the reaction products were extracted as described below and identified by TLC (see below) and by GLC on a 6 ft column packed[2] with 2% QF-1 (methyl fluoroalkyl silicone) on 100–120 mesh Gas-Chrom Q, at a column temperature of 220°C. Retention times relative to cholestane (1.56 min): 5α-androstane-3,17-dione, 4.59; androst-4-ene-3,17-dione, 6.86.

The reaction products were chromatographed on a silicic acid column (8) together with 3.0 mg of unlabeled 5α-androstane-3,17-dione and 2.0 mg of androst-4-ene-3,17-dione. 5α-Androstane-3,17-dione was eluted with 40% (v/v) ethyl ether in *n*-hexane and androst-4-ene-3,17-dione with 50% (v/v) ethyl ether in *n*-hexane. Small amounts of 5α-androstan-3α-ol-17-one followed by the 3β-epimer emerged from the column between the two major peaks. The radioactive purity of 5α-androstane-3,17-dione-4-^{14}C was confirmed as follows: (*a*) the specific activity of each column fraction remained constant throughout the androstanedione band; (*b*) when the compound was recrystallized twice from acetone–water and once from ethanol–water in the presence of unlabeled carrier, the specific activity remained unchanged (6320, 6280, and 6270 cpm/mg).

Dihydrotestosterone-4-^{14}C was prepared biosynthetically from testosterone-4-^{14}C (New England Nuclear Corp.) exactly as described above. The identity of the reaction products was confirmed by TLC (see below) and by GLC on a 6 ft column containing 2% QF-1 as the stationary phase at a column temperature of 220°C. Retention times relative to cholestane (1.56 min): testosterone, 4.04; dihydrotestosterone, 2.63. The radioactive purity of the biosynthetic dihydrotestosterone was confirmed by the finding that during cochromatography with unlabeled dihydrotestosterone on a silicic acid column, the specific radioactivity remained constant throughout the dihydrotestosterone band. The specific radioactivity of the dihydrotestosterone-4-^{14}C in the presence of unlabeled carrier remained constant during two recrystallizations from acetone–water followed by a third recrystallization from ethanol–water (693, 688, and 707 cpm/mg).

Preparation of Enzyme (*9*)

Female albino rats (200 g) of the Wistar strain were killed by cervical dislocation and their livers were removed immediately and chilled on ice. All subsequent operations were carried out at 0–5°C. Portions of liver (4 g) were homogenized in a Potter-Elvehjem homogenizer with 6 ml of a solution containing potassium phosphate buffer, pH 7.4, 0.167 M; nicotinamide, 0.075 M; sucrose, 0.25 M; and neutralized EDTA, 2.5 mM. Cellular debris and nuclei were removed by centrifugation at 500 × *g* for 5 min. The supernatant solution was centrifuged at 10,000 × *g* for 10 min to sediment the mitochondrial fraction; the microsomes were collected by centrifugation of the mitochondrial supernatant solution at 100,000 × *g* for 60 min. The microsomal fraction was resuspended in fresh homogenizing medium and again sedimented by centrifugation at 100,000 × *g*. The washing process was repeated and the washed microsomal particles were finally suspended in fresh homogenizing medium equal to the volume of the 10,000 × *g* supernatant solution. The washed microsomes were stored at −15°C and lost no activity during a storage period of 1 month. A typical preparation had a protein concentration of 5 mg/ml (10).

[2] All column packings for GLC were obtained from Applied Science Laboratories, Inc., State College, Pa.

Assay of Cholestanone Reductase Activity

The complete assay system contained in a volume of 1 ml: potassium phosphate buffer, pH 7.4, 0.167 mmole; $MgCl_2$, 11 μmoles; $NADP^+$, 1.5 μmoles; glucose-6-phosphate, 2.7 μmoles; glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 1 IU; cholestanone-4-^{14}C, 1 μmole, solubilized with 15 mg of Triton X-100,[3] and washed microsomes containing approximately 1.5 mg of protein. The radioactivity of the substrate was 1.5×10^4 cpm/μmole as determined in a Nuclear-Chicago model 702 scintillation counter (6). The incubation mixture was shaken in air at 37°C for 20 min (identical results were obtained when the gas phase was oxygen or nitrogen). Reaction products were separated and determined exactly as described previously, namely by extraction of the steroids with methylene dichloride–ethanol mixtures, TLC, and scintillation counting of the pertinent spots (1, 11). Since the specific radioactivity of the substrate is known, the radioactivity data can be expressed in terms of mμmoles of substrate converted to a given product. More than 90% of the substrate radioactivity was consistently recovered in cholestanone and cholestanol. The procedure outlined in this section is referred to henceforth as "standard assay" or "standard assay conditions." Boiled enzyme controls exhibited no detectable conversion of cholestanone to cholestanol.

Steroids of the C_{19} series were separated on Silica Gel G plates with the solvent system ethyl acetate–benzene 1:1 (1). The following R_f values were observed: androstanedione, 0.70; 5α-androstan-3α-ol-17-one, 0.54; 5α-androstan-3β-ol-17-one, 0.47; dihydrotestosterone, 0.50; 5α-androstane-3α,17β-diol, 0.32; 5α-androstane-3β,17β-diol, 0.26.

Identification of Reaction Products

Cholestanol. An incubation was carried out with 2 μmoles of cholestanone-4-^{14}C (1.2×10^5 cpm/mg) and proportionately increased amounts of microsomes and cofactors. The steroids were extracted from the reaction mixture and chromatographed on a $AgNO_3$–silicic acid column (6) together with known steroids (Fig. 1). The identity and radioactive purity of the major reaction product, cholestanol, was established as follows: (*a*) during chromatography the radioactivity remained associated with the cholestanol, and the specific radioactivity of each column fraction throughout the cholestanol band remained constant within the precision of measurement (2210 cpm/mg); (*b*) when this biosynthetic cholestanol was oxidized with CrO_3 (5), the cholestanone formed had the same specific activity (2210 cpm/mg) as the cholestanol from which it had been prepared, and its specific radioactivity remained unchanged during silicic acid column chromatography (Fig. 2) and two recrystallizations from methanol–water (2195, 2200 cpm/mg).

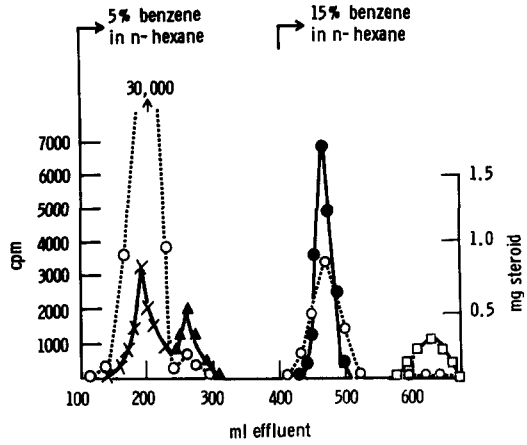

FIG. 1. Chromatography of steroids from incubation of 2 μmoles of cholestanone-4-^{14}C with rat liver microsomes. $AgNO_3$–silicic acid column, 200 × 18 mm, 25 ± 1°C, eluted with benzene–*n*-hexane mixtures. The following amounts of unlabeled carrier steroids were added: cholestanone, 2.5 mg; epicholestanol, 2.0 mg; cholestanol, 5 mg; cholesterol, 1 mg. O---O counts per minute; ×——× milligrams of cholestanone; ▲——▲ milligrams of epicholestanol; ●——● milligrams of cholestanol; □——□ milligrams of cholesterol.

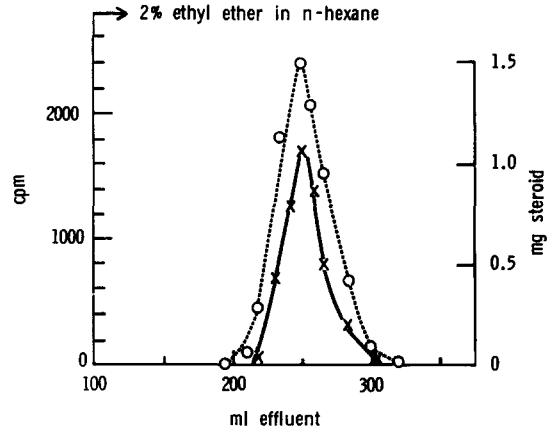

FIG. 2. Chromatogram of cholestanone prepared from biosynthetic cholestanol-4-^{14}C by CrO_3-oxidation. Silicic acid column 200 × 18 mm (8), eluted with ethyl ether–*n*-hexane mixtures. O---O counts per minute; ×——× milligrams of cholestanone.

Epicholestanol. During column chromatography of the reaction products, a small peak emerged immediately after the cholestanone; separation of the two substances was incomplete (Fig. 1). The combined material from the two peaks was rechromatographed with additional epicholestanol carrier (Steraloids, Inc., Pawling, N.Y.; mp 180–183°C, $\alpha_D = +24.5°$) on an alumina column which gave a complete separation of cholestanone and

[3] Supplied through the courtesy of Rohm & Haas Co., Philadelphia, Pa. Triton X-100 [a nonionic detergent, mixture of *p*,*t*-octyl poly(phenoxyethoxy) ethanols] was found to be a much better solubilizer for cholestanone than Tween 20. The substrate emulsion was prepared as described previously (1).

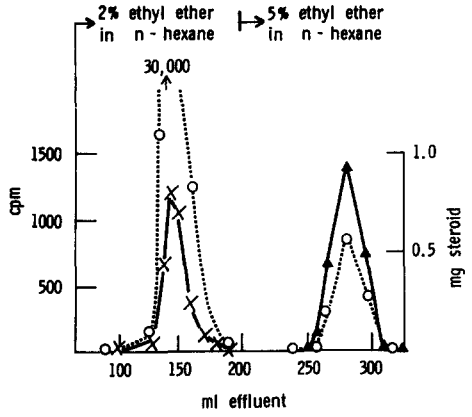

FIG. 3. Chromatogram of cholestanone plus epicholestanol peaks from Fig. 1. Column 200 × 18 mm, neutral Woelm alumina, activity grade IV, eluted with ethyl ether–*n*-hexane mixtures. O---O counts per minute; ×——× milligrams of cholestanone; ▲——▲ milligrams of epicholestanol.

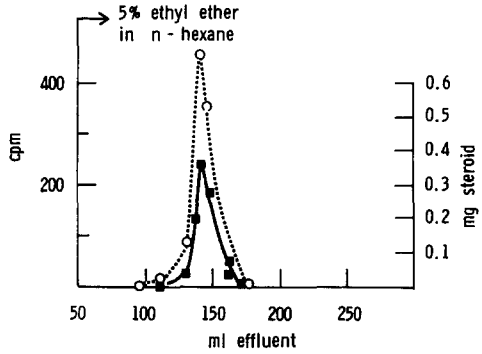

FIG. 4. Chromatogram of epicholestanol acetate prepared from epicholestanol band of Fig. 3. Column 200 × 18 mm, neutral Woelm alumina, activity grade II, eluted with ethyl ether–*n*-hexane mixtures. O——O counts per minute; ■——■ milligrams of epicholestanol acetate.

epicholestanol (Fig. 3). The identity and radioactive purity of this minor reaction product as epicholestanol was established as follows: (*a*) the radioactivity remained associated with the epicholestanol carrier, and the specific radioactivity remained constant throughout the epicholestanol band (520 cpm/μmole). The free stanol was acetylated with pyridine and acetic anhydride and chromatographed on an alumina column (Fig. 4). The specific radioactivity remained constant throughout the epicholestanol acetate band (530 cpm/μmole), and during two recrystallizations from methanol–water (516, 525 cpm/μmole).

Cholestanone from Reversal of Cholestanone Reductase Reaction. The formation of cholestanone from cholestanol by subcellular fractions of rat liver was established as follows. Cholestanol-4-^{14}C was incubated with rat liver microsomes at pH 9 in an atmosphere of oxygen for 30 min (as described in detail in Table 6), and the steroids were extracted as usual and chromatographed on a silicic acid

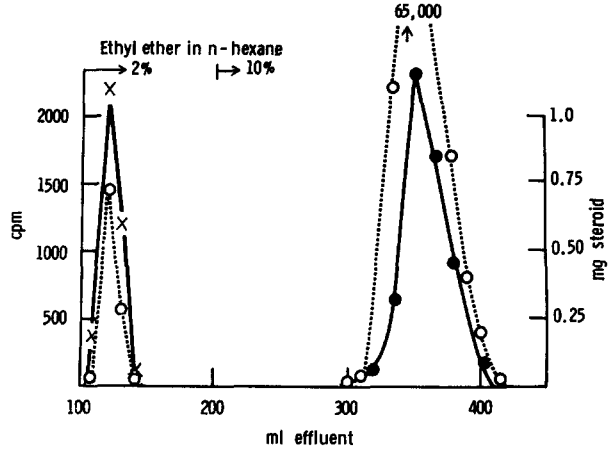

FIG. 5. Chromatography of steroids from incubation of cholestanol-4-^{14}C with rat liver microsomes in oxygen at pH 9.0. For details, see Table 6. Silicic acid column, 200 × 18 mm, eluted with ethyl ether–*n*-hexane mixtures. O------O counts per minute; ×——× milligrams of cholestanone; ●——● milligrams of cholestanol.

column (6) with 2 mg each of unlabeled cholestanone and cholestanol. The radioactivity of the cholestanone remained associated with the known carrier, and its specific radioactivity remained constant throughout the cholestanone band (1270 cpm/mg. Fig. 5). The material from the cholestanone band was reduced with $NaBH_4$ (1) and yielded a mixture of cholestanol and epicholestanol. Chromatography of this mixture on an alumina column (Fig. 6) resulted in the isolation of cholestanol with a specific activity of 1300 cpm/mg. This specific activity remained constant throughout the cholestanol band and did not change during recrystalliation of the stanol from acetone–water (1290 cpm/mg) and from ethanol–water (1300 cpm/mg).

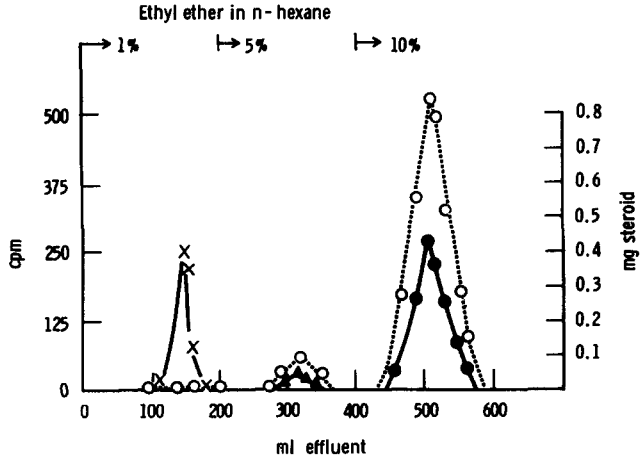

FIG. 6. Chromatography of reaction products obtained from $NaBH_4$ reduction of cholestanone-4-^{14}C band from Fig. 5. Column 200 × 18 mm, neutral Woelm alumina, activity grade IV. Eluted with ethyl ether–*n*-hexane mixtures. O------O counts per minute; ×——× milligrams of cholestanone; ●——● milligrams of cholestanol; ▲——▲ milligrams of epicholestanol.

RESULTS

For the study of the intracellular localization of cholestanone reductase, a 10% homogenate of rat liver was separated centrifugally into mitochondria, microsomes, and high speed supernatant solution. It was found that cholestanone reductase activity resided predominantly in the microsomal fraction (Table 1). The data summarized in this table were obtained with $MgCl_2$ and NADPH as the sole cofactors; preliminary studies had shown that of the cofactors originally present in a Bucher homogenate (12) only NADPH, or an NADPH-generating system, was required.

Microsomes prepared from livers of mature female rats were more active than those of male animals of equal weight or those of immature females (50 g), but these differences usually did not exceed 10–20%. Some cholestanone reductase activity was found to be present in microsomes prepared from rat adrenals, intestinal mucosa, brain, and kidneys, but the activities observed were 10–20 times lower than that of liver on a per milligram of protein basis.

The relationship between reaction rate and enzyme concentration is illustrated in Fig. 7. Proportionality was observed at the lower end of the curve, i.e., when the protein concentration was varied from 0.4 to 1.7 mg of protein per ml. In the standard assay system, containing about 1.5 mg of protein per ml, the rate of reduction of cholestanone was nearly linear during the first 30 min (Fig. 8). A 20 min incubation period was adopted, therefore, as representative of the initial reaction rate. The effect of substrate concentration on reaction rate is illustrated in Fig. 9. Under standard assay conditions, the rate increased with substrate concentration up to 0.5 μmole/ml.

The effect of pH on reaction rate is shown in Fig. 10. The rate of cholestanone reduction was maximal between pH 7 and pH 7.5. When Tris buffer [tris (hydroxymethyl)amino methane] was used instead of phosphate buffer, the pH optimum of the system was unchanged.

The data summarized in Table 2 indicate that the reduction of cholestanone to cholestanol has an absolute requirement for NADPH. In contrast, the conversion of

TABLE 1 INTRACELLULAR LOCALIZATION OF CHOLESTANONE REDUCTASE

	Cholestanol Formed
	mμmoles/mg protein
Whole homogenate	29
Mitochondria	15
Microsomes	102
Final supernatant solution	2
Mitochondria + final supernatant solution	10
Microsomes + final supernatant solution	44
Microsomes + mitochondria	45
Microsomes + mitochondria + final supernatant solution	27

Tissue fractions were prepared as described in Experimental Procedure. Standard assay conditions (0.5–1.25 mg of protein and 1 μmole of cholestanone per vessel, total volume 1 ml; incubation 20 min).

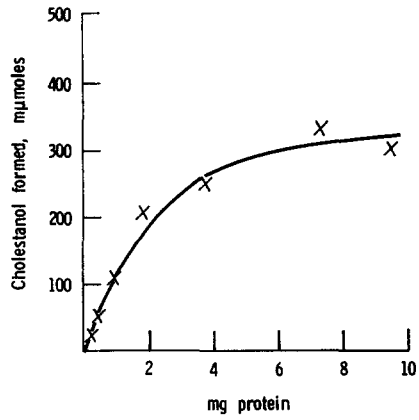

FIG. 7. Effect of increasing amounts of microsomes on the rate of reduction of cholestanone. Standard assay conditions (1 μmole of substrate; 20 min incubation).

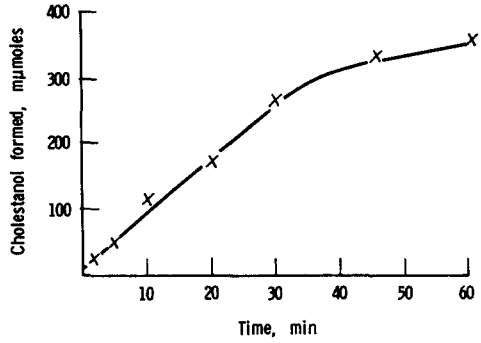

FIG. 8. Time course of enzymatic reduction of cholestanone. Standard assay conditions (substrate, 1 μmole).

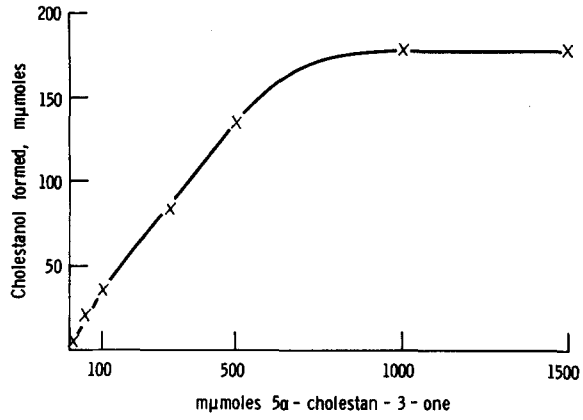

FIG. 9. Effect of substrate concentration on the rate of reduction of cholestanone. Standard assay conditions (substrate, 1 μmole; volume, 1 ml).

TABLE 2 PYRIDINE NUCLEOTIDE REQUIREMENT OF 3α- AND 3β-HYDROXYSTEROID DEHYDROGENASES*

Pyridine Nucleotide Present	3β-Hydroxy Compound Formed from 1 μmole of:			3α-Hydroxy Compound Formed from 1 μmole of:		
	Cholestanone	Androstanedione	Dihydrotestosterone	Cholestanone	Androstanedione	Dihydrotestosterone
	mμmoles					
NADPH-generating system†	220	43	19	22	433	423
NADPH, 1.5 mM	181	48	17	18	330	339
NADH, 1.5 mM	3	83	12	25	303	453

* Standard assay conditions, except as indicated.
† Consisting of NADP, 1.5 mM; glucose-6-phosphate, 2.7 mM; glucose-6-phosphate dehydrogenase 1 IU/ml.

TABLE 3 SEX DIFFERENCES IN ACTIVITY OF MICROSOMAL 3-HYDROXYSTEROID DEHYDROGENASES*

Substrate (1 μmole)	Sex of Rats	3α-Hydroxy Compound Formed	3β-Hydroxy Compound Formed	Ratio of β/α Compound
		mμmoles		
Cholestanone	F	16	234	14.7
	M	13	195	15.0
Androstanedione	F	287	33	0.12
	M	275	250	0.91

* Standard assay conditions. See Experimental Procedure.

3-ketosteroids of the C_{19} series, androstanedione and dihydrotestosterone, to the 3β-hydroxy compounds proceeded with either NADH or NADPH. The data listed in the right-hand part of Table 2 show that the microsomal preparations were 5–20 times more active in reducing the C_{19} 3-ketosteroids to the 3α-hydroxy compounds than to the corresponding 3β-epimers. In contrast, cholestanone, in the presence of NADPH, was predominantly reduced to the 3β-hydroxy epimer; the proportion of cholestanol to epicholestanol was about 10:1. The formation of epicholestanol proceeded to the same extent with either NADH or NADPH.

Table 3 summarizes data illustrating that the proportions of 3α- and 3β-hydroxy isomers produced during the reduction of cholestanone by microsomes are not subject to a sex difference: the ratio of cholestanol to epicholestanol formed was approximately 15 regardless of the sex of the rats whose livers were used in the preparation of the microsomes. These results are in contrast to the enzymatic reduction of androstanedione, a C_{19} steroid, by the same preparations: although microsomes from either male or female rats produced approximately the same amounts of 5α-androstan-3α-ol-17-one under standard conditions, the formation of the 3β-epimer, 5α-androstan-3β-ol-17-one, proceeded at a much lower rate with microsomes from female rats than with microsomes from male animals. Consequently the ratio of 3β- to 3α-epimer observed was approximately 0.1 in females and 0.9 in males.

The inhibition of the microsomal cholestanone reductase by 3-ketosteroids and by sterols is illustrated in Table 4. The reaction product, cholestanol, and other monohydroxysteroids of the C_{27} series were only slightly inhibitory. Pronounced inhibition was observed with sterols having 7-oxygen functions, namely 7-ketocholestanol and 7α-hydroxycholestanol, and with cholest-5-en-3-one. Saturated 3-ketosteroids, as well as Δ^4-3-keto-

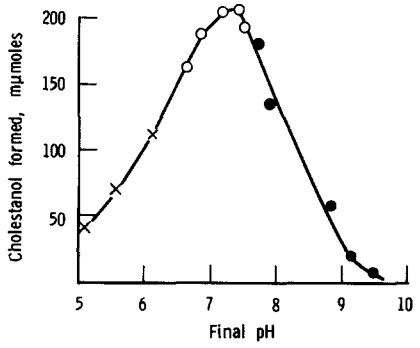

FIG. 10. Effect of pH on cholestanone reductase activity. Standard assay conditions (substrate, 1 μmole) with phosphate, Tris, acetate, or glycine buffers of different pH values.
×, 0.167 M sodium acetate buffer; ○, 0.167 M potassium phosphate or Tris buffers; ●, 0.167 M glycine–NaOH buffer.

TABLE 4 INHIBITION OF CHOLESTANONE REDUCTASE BY STEROIDS OF THE C_{19} AND C_{27} SERIES*

Inhibitor	Cholestanol Formed	Inhibition
	mμmoles	%
None	237	—
Cholestanol	204	14
Epicholestanol	188	20
Coprostanol	192	19
Cholesterol	213	10
7-Ketocholestanol	88	63
7α-Hydroxycholestanol	45	81
Coprostanone	163	31
Cholest-4-en-3-one	150	37
Cholest-5-en-3-one	81	66
Androstanedione	152	36
Dihydrostestosterone	159	33
Androstenedione	207	13
Testosterone	188	21

* Standard assay conditions (1 ml total volume). Concentration of substrate (cholestanone) and of inhibitors was 1 mM. The inhibitors were solubilized together with the substrate in the presence of Triton X-100.

steroids of the C_{19} series, produced moderate inhibitory effects.

Table 5 summarizes data on the specificity of the cholestanone reductase. Of the four C_{27} 3-ketosteroids tested only cholestanone was appreciably reduced.

Experiments dealing with the reversal of the cholestanone reductase action (i.e. the conversion of cholestanol to cholestanone) are summarized in Table 6. Microsomes incubated in oxygen catalyzed this reaction in the presence of an NADP-generating system at an alkaline pH. The activity was localized predominantly in the microsomal fraction of rat liver; the high speed supernatant solution was about 30 times less active on a per milligram of protein basis under the conditions employed.

TABLE 5 SPECIFICITY OF CHOLESTANONE REDUCTASE*

Substrate	Reduction Product	
		mμmoles
5α-Cholestan-3-one-4-^{14}C	Cholestanol	253
Cholest-4-en-3-one-4-^{14}C	Allocholesterol†	2
Cholest-5-en-3-one-4-^{14}C ‡	Cholesterol	1
5β-Cholestan-3-one	Coprostanol§	<20

* Standard assay conditions (1 μmole of substrate, total volume 1 ml). See Experimental Procedure. Preparation of ^{14}C-labeled substrates and analysis of reaction products has been described previously (1).

† Since Triton X-100 was used as the solubilizing agent, the microsomal cholestanone 5α-reductase was inactivated (1), and cholestanone was not formed.

‡ Partially converted to cholest-4-en-3-one by a microsomal isomerase (unpublished observations from this laboratory).

§ Reaction mixture analyzed by GLC on a 6-ft column packed with 1% QF-1 on 100–120 mesh Gas-Chrom Q, at a column temperature of 210°C. Retention times relative to cholestane (1.74 min): coprostanol, 2.68; cholesterol, 3.04, coprostanone, 6.64. Formation of 20 mμmoles of coprostanol (2% conversion) would have been detected.

TABLE 6 CONVERSION OF CHOLESTANOL TO CHOLESTANONE BY SUBCELLULAR FRACTIONS OF RAT LIVER

The complete assay system contained in a volume of 1 ml: potassium phosphate buffer, 0.167 mmole, pH 7.4; or glycine–NaOH buffer, 0.167 mmole, of different pH values, as indicated; NADP, 1.5 μmoles; potassium pyruvate, 9 μmoles; lactate dehydrogenase, 23.5 enzyme units (C. F. Boehringer and Sons); cholestanol-4-^{14}C, 1 μmole, solubilized with 15 mg of Triton X-100; and 1.5 mg of microsomal protein or 15 mg of soluble protein derived from the high speed supernatant solution; incubated in oxygen at 37°C for 30 min.

Tissue Fraction	Initial pH	Cholestanone Formed*
		mμmoles
Microsomes	7.4	6
	9.0	24
	9.8	16
	10.6	0
High speed supernatant solution	7.4	0
	9.8	6

* Corrected for boiled enzyme controls, which were of the order of 4 mμmoles of cholestanone or less.

TABLE 7 INHIBITION OF CHOLESTANONE REDUCTASE BY *p*-CHLOROMERCURIBENZOATE WITH CHOLESTANONE AS SUBSTRATE

Additional Compounds Present	Cholestanone Formed
	mμmoles
None*	263
GSH, 10 mM	265
CMB†, 0.1 μM	234
CMB, 1 μM	244
CMB, 10 μM	188
CMB, 100 μM	40
GSH, 10 mM, plus CMB, 100 μM‡	272
CMB, 100 μM, then GSH 10 μM§	60

* Standard assay conditions. See Experimental Procedure.

† CMB, *p*-chloromercuribenzoate.

‡ GSH and CMB added simultaneously.

§ CMB added first, GSH added at end of 10 min, and incubation continued for an additional 10 min.

The data summarized in Table 7 indicate that the microsomal cholestanone reductase was inhibited by *p*-chloromercuribenzoate (CMB). Eighty-five per cent inhibition was observed with 100 μM CMB, and 28% inhibition with a 10 μM concentration of the inhibitor. The inhibitory effect of CMB was prevented by the simultaneous addition of GSH. However, when the inhibitor was added first, the subsequent addition of GSH did not reverse the inactivation of the enzyme.

DISCUSSION

The biosynthesis of cholestanol from cholesterol in rat liver probably involves cholest-5-en-3-one, cholest-4-en-3-one, and cholestanone as intermediates (13). The present study, in conjunction with the data published previously (1), has shown that the enzymes that catalyze the conversion of cholest-4-en-3-one to cholestanone, and of cholestanone to cholestanol, are localized in the microsomal fraction. Since the microsomes also contain a Δ^4-3-ketosteroid isomerase that catalyzes the transformation of cholest-5-en-3-one to cholest-4-en-3-one (unpublished observations in this laboratory), it is now known that three of the four steps involved in the biosynthesis of cholestanol are carried out by microsomal enzymes. It remains to be demonstrated whether the initial step in this reaction sequence, i.e., the action of a 3-β-hydroxysteroid dehydrogenase on cholesterol to yield cholest-5-en-3-one, is also associated with the particulate fraction. The conversion of cholestanol to cholestanone by a microsomal preparation, observed in the present study, suggests the possibility that cholesterol oxidase activity may likewise be found in the microsomal fraction of rat liver.

The findings reported here differ in certain respects from earlier data published by others. Tombropoulos, Werbin, and Chaikoff (14) found that the activities involved in the transformation of cholestenone to cholestanol were present to about equal extents in the microsomal and mitochondrial fractions of rat liver, but they did not attempt to study cholestenone 5α-reductase and cholestanone reductase separately. We pointed out previously that the low over-all conversion rates reported by these authors were probably due to the low solubility of the intermediate cholestanone in the incubation medium (1). Yamasaki et al. (3) observed that the 3-keto groups of cholest-4-en-3-one and of cholest-5-en-3-one were reduced by a soluble enzyme from rat liver in the presence of NADH, presumably to the corresponding 3β-hydroxy compounds, allocholesterol and cholesterol. However, the reaction rates (determined by following the oxidation of NADH spectrophotometrically) were very low and the reaction products were not identified. Although it is well known that high speed supernatant solution of rat liver contains 3-β-hydroxysteroid dehydrogenases that catalyze the reduction of 3-ketosteroids of the C_{19}, C_{21}, and cardenolide series (15), we were unable to demonstrate the presence of cholestanone reductase activity in such preparations.

Rat liver microsomes converted cholestanone to cholestanol (a 3β-hydroxy compound) and to epicholestanol (the 3α-hydroxy epimer) with the proportion of the former greatly predominating. The activities involved differed in their requirement for pyridine nucleotides: the 3-α-hydroxysteroid dehydrogenase catalyzed the reduction of cholestanone to epicholestanol in the presence of either NADH or NADPH; the 3β-hydroxy epimer was formed only with NADPH (Table 2).

The transformation of cholestanone to a mixture of 3α- and 3β-hydroxy epimers is analogous to the reduction of saturated C_{19} and C_{21} 3-ketosteroids under the influence of 3-α- and 3-β-hydroxysteroid dehydrogenases. It seems unlikely, however, that the microsomal enzymes that act upon cholestanone are identical with the known steroid dehydrogenases (16). This conclusion is based upon the findings that: (*a*) the formation of cholestanol took place with NADPH, but not with NADH, as the hydrogen donor, while the C_{19} 3-β-hydroxysteroid dehydrogenases were active with either NADH or NADPH (Table 2); (*b*) the ratio of 3β- to 3α-epimers formed from cholestanone was independent of the sex of the animal from which the microsomes were prepared, while the ratio of 3β- to 3α-hydroxy epimers formed from androstanedione was 0.1 in females and 0.9 in males (Table 3). These observations with androstanedione are thus in agreement with the results of Rubin and Strecker (4), who studied the 3-hydroxysteroid dehydrogenases associated with acetone-insoluble fractions of rat liver homogenates. More detailed studies of the nature of the microsomal steroid dehydrogenases await the development of methods for the solubilization and purification of these enzymes.

Cholestanone reductase activity was only moderately inhibited by the reaction product cholestanol, and by the other monohydroxysterols tested (Table 4). Stanols with oxygen functions in the 7-position were far more inhibitory. While the 7-oxygenated Δ^5-sterols may normally be found in liver [they are thought to be intermediates in bile acid formation (17)], it is less likely that the corresponding stanols are present in appreciable concentrations. Consequently, the biological significance of this observation remains uncertain. Cholestanone reductase, like cholestenone 5α-reductase, was strongly inhibited by cholest-5-en-3-one, but other 3-ketosteroids of the C_{27} or C_{19} series were far less inhibitory. The effect of cholest-5-en-3-one is difficult to evaluate since this compound tends to form hydroperoxides in the presence of oxygen (18). Conceivably, such peroxides may produce an inactivation of the microsomal enzymes.

On the basis of the data so far available, cholestanone reductase appears to act specifically on cholestanone. Under the conditions employed cholest-4-en-3-one, cholest-5-en-3-one, and coprostanone were not reduced (Table 5). This conclusion must be tentative until additional substrates can be tested and the microsomal enzyme can be obtained in a more highly purified state.

The conversion of cholestanol to cholestanone observed in the present study (Table 6) is not considered proof that the action of the microsomal cholestanone reductase is reversible, since a different microsomal enzyme might be involved in catalyzing this reaction. However, the soluble 3-hydroxysteroid dehydrogenases of the C_{19} series have been shown to act reversibly (15) with the equilibrium in favor of the reduced product (19).

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References

1. Shefer, S., S. Hauser, and E. H. Mosbach. *J. Biol. Chem.* **241:** 946, 1966.
2. Harold, F. M., S. Abraham, and I. L. Chaikoff. *J. Biol. Chem.* **221:** 435, 1956.
3. Yamasaki, K., F. Noda, and K. Shimizu. *J. Biochem. (Tokyo)* **46:** 747, 1959.
4. Rubin, B. L., and H. J. Strecker. *Endocrinology* **69:** 257, 1961.
5. Sato, J., and N. Ikekawa. *J. Org. Chem.* **24:** 1367, 1959.
6. Shefer, S., S. Milch, and E. H. Mosbach. *J. Biol. Chem.* **239:** 1731, 1964.
7. Fieser, L. F. *Org. Syn.* **4:** 195, 1963.

8. Hirsch, J., and E. H. Ahrens, Jr. *J. Biol. Chem.* **233:** 311, 1958.
9. Schneider, W. C., and G. H. Hogeboom. *J. Biol. Chem.* **183:** 123, 1950.
10. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. *J. Biol. Chem.* **193:** 265, 1951.
11. Chattopadhyay, D. P., and E. H. Mosbach. *Anal. Biochem.* **10:** 435, 1965.
12. Frantz, I. D., Jr., and N. L. R. Bucher. *J. Biol. Chem.* **206:** 471, 1954.
13. Shefer, S., S. Milch, and E. H. Mosbach. *J. Lipid Res.* **6:** 33, 1965.
14. Tombropoulos, E. G., H. Werbin, and I. L. Chaikoff. *Proc. Soc. Exptl. Biol. Med.* **110:** 331, 1962.
15. Repke, K., and L. T. Samuels. *Biochemistry* **3:** 685, 1964.
16. Dorfman, R. I., and F. Ungar. *Metabolism of Steroid Hormones*. Academic Press, New York, 1965, p. 398.
17. Danielsson, H. *Acta Chem. Scand.* **15:** 242, 1961.
18. Nickon, A., and W. L. Mendelson. *J. Org. Chem.* **30:** 2087, 1965.
19. Boyer, J., D. N. Baron, and P. Talalay. *Biochemistry* **4:** 1825, 1965.