7-Hydroxylation of 3-oxygenated C_{27}-, C_{28}-, and C_{29}-steroids in rat liver 18,000 *g* supernate

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Abstract Structurally closely related steroids have been tested as substrates for the NADPH-dependent cholesterol- and cholestanol-7α-hydroxylase(s) considered to be the rate-limiting enzyme(s) in bile acid biosynthesis. Of the steroids tested, 5-cholesten-3α-ol, 5α-cholestan-3α-ol, 5β-cholestan-3α-ol, 5β-cholestan-3β-ol, 4-cholesten-3α-ol, 4-cholesten-3β-ol, 5α-cholestan-3-one, 5β-cholestan-3-one, 24α-methylcholesterol and the 24α-ethyl derivatives of cholestanol, 5β-cholestan-3α-ol, 5β-cholestan-3β-ol, and 4-cholesten-3-one, only 4-cholesten-3β-ol was 7α-hydroxylated to a significant extent (approximately ⅕ of the conversion of exogenous cholesterol). This suggests that the 7α-hydroxylase(s) is sensitive to the structure of the side chain, and that it requires a rather flat steroid nucleus (Δ^4-, Δ^5-, or 5α-steroid) and an equatorial or quasiequatorial hydroxyl group at C_3. The nature of the 7α-hydroxylation is discussed and the importance of the β-side of the steroid molecule is emphasized. Minute amounts of the 7β-hydroxy derivatives were formed from 4-cholesten-3β-ol, 5β-cholestan-3α-ol, 24α-ethyl-5β-cholestan-3α-ol and, probably, from 5β-cholestan-3β-ol and 24α-ethyl-5β-cholestan-3β-ol.

Supplementary key words cholesterol · effects of the potassium ion on 7α-hydroxylation · 7β-hydroxylation · lipid peroxidation · thin-layer chromatography · gas–liquid chromatography–mass spectrometry

In a previous study it was found that the plant sterol, β-sitosterol (24α-ethylcholesterol), could not be specifically 7α-hydroxylated by rat liver microsomes fortified by NADPH (1). These results agree with those of Boyd et al. (2) who presented evidence indicating that the 7α-hydroxylase is dependent upon the structure of the side chain. Shefer, Hauser, and Mosbach (3) and Aringer and Eneroth (1) have shown that cholestanol is efficiently converted to the 7α-hydroxy derivative, suggesting that the structure of the steroid nucleus is not of the same importance for the 7α-hydroxylase enzyme as is the side chain. The present study was undertaken to explore whether sterols other than cholesterol and cholestanol may be 7α-hydroxylated in vitro.

MATERIALS

Solvents

All solvents and reagents were analytical grade and purchased from Merck AG, Darmstadt, West Germany unless otherwise stated. They were used without further purification unless otherwise stated [except for dioxane which was purified by filtering it through Al_2O_3 (Woelm, Eschwege, West Germany) activity grade I. It was stored over Al_2O_3].

Substrates

4-^{14}C-Labeled compounds were prepared as follows. 5α-Cholestan-3β-ol was formed by H_2/PtO_2 reduction in ethyl acetate–acetic acid 98:2 (v/v) from [4-^{14}C]-cholesterol (The Radiochemical Centre, Amersham, England, sp act 55–61 mCi/mmol). 5α-Cholestan-3-one was prepared from 5α-cholestan-3β-ol by oxidation with chromic acid in sulfuric acid and acetone (4). The product was purified in TLC system 1 (**Table 1**). 5α-Cholestan-3α-ol was made by sodium borohydride reduction of 5α-cholestan-3-one in methanol, yielding 5α-cholestan-3α-ol and 5α-cholestan-3β-ol in the proportions 1:7 (TLC system 1). 4-Cholestan-3-one was prepared by oxidation of 4-^{14}C-labeled cholesterol with cholesterol oxidase (Boehringer, Mannheim, West Germany) as described by Richmond (7). Subsequent workup and purification in TLC system 1 (Table 1) yielded pure 4-cholesten-3-one. This

Abbreviations: systematic names of the steroids referred to in the text by trivial names are as follows: cholesterol, 5-cholesten-3β-ol; cholestanol, 5α-cholestan-3β-ol; and β-sitosterol, 24α-ethyl-5-cholesten-3β-ol. Other abbreviations are LC, liquid chromatography; TLC, thin-layer chromatography; TLC–RD, thin-layer chromatography followed by radioactivity detection; GLC, gas–liquid chromatography; GLC–RD, gas–liquid chromatography combined with radioactivity detection; GLC–MS, gas–liquid chromatography–mass spectrometry; TMS, trimethylsilyl ether; t_R, retention time relative to that of 5α-cholestane; TCV, total column volume.

TABLE 1. Chromatographic properties of 3-oxygenated steroids

Steroid	LC System[a]		TLC System[b]			GLC[c]	
	A	B	1	2	3	3% SP-2100	3% QF-1
	% TCV		R_f			t_R	
5-Cholesten-3α-ol	250		0.43	0.44	0.60	1.65	1.50
5-Cholesten-3β-ol	260	70	0.30	0.35	0.50	2.05	1.98
4-Cholesten-3α-ol	225	57	0.45	0.46	0.62	1.71	
4-Cholesten-3β-ol	240	66	0.34	0.39	0.55	1.98	
5α-Cholestan-3α-ol	275		0.42	0.45	0.61	1.71	1.59
5α-Cholestan-3β-ol	270		0.27	0.32	0.47	2.11	2.09
5β-Cholestan-3α-ol	230		0.39	0.40	0.55	1.71	1.64
5β-Cholestan-3β-ol	275		0.38	0.42	0.59	1.68	1.56
4-Cholesten-3-one	230	45	0.47	0.52	0.69	2.40	7.90[d]
5α-Cholestan-3-one	315		0.69	0.69	0.81	1.94	5.22[d]
5β-Cholestan-3-one	285		0.75	0.75	0.84	1.77	4.82[d]
24α-Methyl-5-cholesten-3β-ol	280		0.30	0.35	0.50	2.62	2.56
24α-Ethyl-5α-cholestan-3β-ol	310		0.27	0.33	0.48	3.34	
24α-Ethyl-5β-cholestan-3α-ol	265		0.39	0.41	0.56	2.68	
24α-Ethyl-5β-cholestan-3β-ol	315		0.38	0.43	0.60	2.64	
24α-Ethyl-4-cholesten-3-one	265		0.47	0.52	0.69	3.70	

[a] System A, hydroxyalkylated 55% substituted Sephadex LH-20, 140–170 mesh synthesized with a mixture of C_{11}–C_{14} epoxides as described by Ellingboe, Nyström, and Sjövall (5) used in methanol–water–dichloroethane 95:5:25 (v/v/v). Column dimensions, 0.75 cm² × 13 cm; flow rate, 0.5 ml/cm²/min at 24°C. System B, Sephadex LH-20 used in dichloromethane–benzene 2:1 (v/v). Column dimensions, 1.0 cm² × 20 cm; flow rate, 0.5 ml/cm²/min at 24°C. TCV = total column volume.

[b] System 1, benzene–ethyl acetate 7:1 (v/v). System 2, diethyl ether–cyclohexane 2:1 (v/v). System 3, diethyl ether–cyclohexane 3:2 (v/v). Precoated Silica gel 60 plates (20 × 20 cm Merck) were used. The solvent was allowed to rise 10 cm twice and then 20 cm in system 3.

[c] A Pye gas chromatograph (model 104) equipped with a hydrogen flame ionization detector was used. The columns (2 m × 4 mm, SP 2100, or 1.3 m × 4 mm, QF-1) contained Supelcoport (100–120 mesh, SP-2100, 80–100 mesh, QF-1) coated either with 3% SP 2100 or 3% QF-1 (Supelco Inc. Bellefonte PA). The temperatures were flash heater, 270°C; and column oven, 255°C (SP 2100) or 225°C (QF-1). Carrier gas was nitrogen and gas flow rate was 480 ml/cm²/min. The values are retention times relative to that of 5α-cholestane. All compounds containing a hydroxyl group were analyzed as their TMS ethers (6).

[d] Column oven temperature was 240°C.

compound was used for the synthesis of 5-cholesten-3α-ol via the dienol trimethylsilyl ether derivative as described previously (8). The product was purified in TLC system 1. Reduction of 4-cholesten-3-one with sodium borohydride in isopropanol afforded a mixture of 4-cholesten-3α-ol and 4-cholesten-3β-ol in the proportion 1:10. The products were purified in TLC system 1 and LC system B (Table 1). Hydrogenation in acetic acid of 4-cholesten-3-one with HBr and palladium oxide (Fluka AG, Switzerland) as catalysts (9) yielded 5β-cholestan-3-one and 5α-cholestan-3-one in the proportions 8:1. The products were purified in TLC system 2. 5β-Cholestan-3α-ol was made by sodium borohydride reduction of 5β-cholestan-3-one in methanol followed by TLC purification in system 3. The ratio between 5β-cholestan-3α-ol and 5β-cholestan-3β-ol was 5:1. Better yields of 5β-cholestan-3β-ol were obtained by hydrogenation of 5β-cholestan-3-one for 4 hr with H_2 and PtO_2 (3 mg) in 2 ml of acetic acid containing 0.3% HBr (10). This afforded, after workup and TLC purification (system 3), 82% 5β-cholestan-3β-ol and 4% 5β-cholestan-3α-ol.

[6-^{3}H]24α-Methyl-5-cholesten-3β-ol (sp act 250–350 mCi/mmol) was prepared by NaB_3H_4 reduction of 3β-acetoxy-24α-methyl-5-cholesten-6-one as described by Samuelsson (11). 3β-Acetoxy-24α-methyl-5-cholesten-6-one was synthesized from 24α-methyl-5-cholesten-3β-ol according to the procedure of Mauthner and Suida (12) as modified by Dodson and Riegel (13). 24α-Methyl-5-cholesten-3β-ol was prepared from a plant sterol mixture (β-sitosterol, Fluka AG, Switzerland) by recycling chromatography on lipophilic Sephadex (14). [4-^{14}C]3β-Hydroxy-5-androsten-17-one (50–60 mCi/mmol) was purchased from NEN Chemicals GmbH, West Germany.

Unlabeled cholesterol was purchased from ADA, Stockholm, Sweden. Unlabeled 5α-cholestan-3α-ol, 5α-cholestan-3β-ol, 5β-cholestan-3α-ol, 5β-cholestan-3β-ol, 5α-cholestan-3-one, and 5β-cholestan-3-one were purchased from Steraloids, Inc., Pawling, NY. Unlabeled 5-cholesten-3α-ol, 4-cholesten-3-one, 4-cholesten-3α-ol, and 4-cholesten-3β-ol were prepared from cholesterol as described for the labeled substrates. Labeled and unlabeled 24α-ethyl-substituted

5α-cholestan-3β-ol, 5β-cholestan-3α-ol, 5β-cholestan-3β-ol, and 4-cholesten-3-one were prepared from β-[4-^{14}C]sitosterol (58 mCi/mmol, The Radiochemical Centre, Amersham, England) and β-sitosterol (Merck), respectively, as described above. β-Sitosterol was purified as described previously (1).

Each substrate was purified in LC system A or B 1–3 days before it was used for an in vitro experiment. The purity was checked by radioautography after TLC analysis, GLC combined with radioactivity detection, and GLC–MS.

Reference compounds

5α-Cholestane-3α,7α-diol (15), 5α-cholestane-3β-7α-diol (15), 5β-cholestane-3α,7α-diol (16), 7α-hydroxy-4-cholesten-3-one (16), 7β-hydroxy-4-cholesten-3-one (17, 18), 7α-hydroxy-5α-cholestan-3-one (15), 7α-hydroxy-5β-cholestan-3-one (16), 5-cholestene-3β,7α,12α-triol (19), and 5α-cholestane-3β,7α,12α-triol (15) were gifts of Dr. Ingemar Björkhem, Department of Clinical Chemistry, Huddinge Sjukhus, Huddinge, Sweden and Drs. Henry Danielsson, Jan Gustafsson, and Kjell Wikvall, Department of Pharmaceutical Biochemistry, University of Uppsala, Uppsala, Sweden. These compounds had been synthesized as described in the respective references.

C_7-Oxygenated derivatives of cholesterol and 24α-methyl-5-cholesten-3β-ol were prepared as described previously (1). C_7-Oxygenated derivatives of 5-cholesten-3α-ol were obtained after incubation of this steroid with soybean lipoxidase and linoleic acid as described by Johansson (20). Mild saponification (21) and reduction of 3β-acetoxy-5α-cholestan-7-one (Steraloids Inc.) with sodium borohydride in methanol afforded the C_7-oxygenated derivatives of cholestanol. 5β-Cholestane-3β,7α-diol was prepared by reduction of 7α-hydroxy-5β-cholestan-3-one with lithium aluminum hydride in diethyl ether. 4-Cholestene-3α,7α-diol and 4-cholestene-3β,7α-diol were obtained after reduction of 7α-hydroxy-4-cholesten-3-one with sodium borohydride in isopropanol. 5α-Cholestane-3,7-dione, 5β-cholestane-3,7-dione, and 5β-cholestane-3,7,12-trione were all obtained by chromic acid oxidation (4) of the corresponding 3,7-diols or 3,7,12-triol. 5β-Cholestane-3α,7β-diol was prepared by reduction of 5β-cholestane-3,7-dione with lithium aluminum hydride in diethyl ether. 3β,7α-Dihydroxy-5-androsten-17-one and 3β,7β-dihydroxy-5-androsten-17-one were gifts of Dr. G. Johansson, Department of Surgery, Karolinska Sjukhuset, Stockholm. These compounds had been synthesized as described in references 20 and 22.

The chromatographic properties of all reference steroids are described in **Table 2.** Each reference compound was analyzed by GLC–MS. The compounds that contained a hydroxyl group were analyzed as TMS ethers.

Solutions for homogenization and incubation

The solutions were prepared with doubly distilled water. Unless otherwise stated, a 0.25 M sucrose solution containing 0.01 M β-mercaptoethylamine and 0.001 M EDTA, and a 0.1 M phosphate buffer made 0.028 M with regard to nicotinamide and 0.01 M in β-mercaptoethylamine concentration were used. The pH was 7.0.

Thin-layer chromatography

Unless otherwise stated, precoated Silica gel 60 plates (Merck) were used in the systems described in Tables 1 and 2. Separated compounds were detected and extracted from the gel as described previously (1).

Gas–liquid chromatography–mass spectrometry

This type of analysis was carried out with an LKB 9000 instrument equipped either with a 1.5% SE-30 column (2 m × 3 mm) or with a 3% QF-1 column (3.5 m × 3 mm) operated at 250°C and 240°C, respectively. Helium was used as carrier gas. Other temperatures were flash heater, 270°C; molecular separator, 280°C; and ion source, 290°C. The energy of the bombarding electrons was 22.5 eV.

Measurement of radioactivity

^{14}C- and ^{3}H-labeled compounds were localized and measured as described previously (23).

EXPERIMENTAL PROCEDURES

Standard procedure

Male rats, 150 g, (3 or 4 in each experiment) were fed a diet containing 100 g of pulverized pellet food (Anticimex, Stockholm, Sweden), 50 ml of corn oil (ADA) and 5 g of cholestyramine (Cuemid, Merck, Sharp and Dohme, West Point, PA) for 5–6 days. On the day of the experiment the rats had usually gained 25–30 g. They were killed by a blow to the head at 7 PM. A rat liver 18,000 *g* supernatant fraction was prepared in the sucrose solution containing β-mercaptoethylamine and EDTA as described previously (1). A 4.25 ml portion of the 18,000 *g* supernate and 4.25 ml of the phosphate buffer were used for the incubations with 18,000 *g* supernate. Incubations were made with 5–200 μg of 4-^{14}C-labeled substrate in the presence of 10 μmol of NADPH for 30 min at 37°C unless otherwise stated. The substrates

TABLE 2. Chromatographic properties of di- and trioxygenated reference steroids

Steroid	TLC[a] System	TLC[a] R_f	TLC[a] System	TLC[a] R_f	GLC[b] SP-2100	GLC[b] QF-1
4-Cholestene-3α,7α-diol	4	0.09	5	0.30	2.04	
4-Cholestene-3β,7α-diol	4	0.22			2.08	
5-Cholestene-3α,7α-diol	4	0.08	5	0.33	2.17	
5-Cholestene-3α,7β-diol	4	0.27			2.15	
5-Cholestene-3β,7α-diol	4	0.20	5	0.47	2.04	1.83
5-Cholestene-3β,7β-diol	4	0.29	5	0.55	2.76	2.47
24α-Methyl-5-cholestene-3β,7α-diol	4	0.21			2.57	
24α-Methyl-5-cholestene-3β,7β-diol	4	0.30			3.47	
5α-Cholestane-3α,7α-diol	4	0.22			2.04	1.80
5α-Cholestane-3β,7α-diol	4	0.22	5	0.50	2.14	2.00
5α-Cholestane-3β,7β-diol	4	0.18	5	0.45	3.02	2.80
5β-Cholestane-3α,7α-diol	4	0.25			2.12	1.88
5β-Cholestane-3α,7β-diol	4	0.26			2.35	
5β-Cholestane-3β,7α-diol	4	0.35			1.94	1.66
7α-Hydroxy-4-cholesten-3-one	4	0.39			2.65	
24α-Ethyl-7α-hydroxy-4-cholesten-3-one	4	0.41			4.08	
7β-Hydroxy-4-cholesten-3-one	4	0.37			3.42	
3α-Hydroxy-5-cholesten-7-one	4	0.32			3.18	
3β-Hydroxy-5-cholesten-7-one	4	0.34	5	0.69	3.79	
24α-Methyl-3β-hydroxy-5-cholesten-7-one	4	0.35			4.81	
3β-Hydroxy-5α-cholestan-7-one	4	0.33	5	0.67	3.40	
7α-Hydroxy-5α-cholestan-3-one	3	0.38			2.18	
7α-Hydroxy-5β-cholestan-3-one	3	0.47			2.39	
5α-Cholestane-3,7-dione			2	0.32	2.92	
5β-Cholestane-3,7-dione	1	0.36	2	0.37	2.53	
5-Cholestene-3β,7α,12α-triol	4	0.03	6	0.20	2.21	
5α-Cholestane-3β,7α,12α-triol			6	0.25	2.17	
5α-Cholestane-3,7,12-trione			7	0.41	4.02	
3β,7α-Dihydroxy-5-androsten-17-one			8	0.19	0.56	
3β,7β-Dihydroxy-5-androsten-17-one			8	0.23	0.71	

[a] Systems 2 and 3, see Table 1; system 4, diethylether–cyclohexane 9:1 (v/v); system 5, ethyl acetate–cyclohexane 4:1 (v/v); system 6, ethyl acetate; system 7, benzene–ethyl acetate 7:3 (v/v); system 8, trimethylpentane-*n*-butyl acetate–acetone 2:2:1 (v/v/v) (20). In system 8 the solvents were allowed to rise 20 cm three times. Precoated Silica gel 60 plates (Merck) were used, except in system 4. In this case handmade (20 × 20 cm) plates coated with Silica gel G (Merck) were utilized.

[b] See Table 1 footnote *c*.

were added dissolved in 300 μl of acetone under nitrogen and with concomitant vibration of the mixture as described previously (1).

Experiments with iron-supplemented microsomes

Liver microsomes were prepared from the cholestyramine-treated rats as described by Mitton, Scholan, and Boyd (24). Microsomes equivalent to 1 g of wet liver, suspended in 4.25 ml of 154 mM KCl solution and 4.25 ml of 0.1 M Tris buffer, pH 7.4, containing 2 mM EDTA were used for each incubation. The incubation procedure was that described above. ADP and $FeSO_4$ were added immediately after the addition of 10 μmol of NADPH so that the concentrations were 1 mM and 0.2 mM, respectively.

Extraction and workup procedure

All incubations were terminated and extracted with chloroform–methanol 2:1 (v/v) as described previously (23). The extracts were purified by liquid chromatography in system A (see Table 1). Three fractions were collected as follows: fraction I was collected between 0 and 60% TCV except when extracts from the incubations with the C_{29}-steroids were purified. In these latter cases fraction I was collected between 0 and 65% TCV. Fraction II was collected from the end of fraction I to either 140% TCV (experiments with 5α-cholestan-3α-ol, 5β-cholestan-3α-ol, and 5β-cholestan-3β-ol), 150% TCV (cholestanol), 160% TCV (24α-ethyl-5β-cholestan-3α-ol and 24α-ethyl-5β-cholestan-3β-ol), 170% TCV (24α-ethyl-5α-cholestan-3β-ol), 180% TCV (cholesterol, 5-cholesten-3α-ol, 4-cholesten-3α-ol, 4-cholesten-3β-ol, and 4-cholesten-3-one), 200% TCV (5α-cholestan-3-one, 5β-cholesten-3-one, and 24α-methyl-5-cholesten-3β-ol), or 210% TCV (experiments with 24α-ethyl-4-cholesten-3-one). Fraction III was collected from the end of fraction II to 400% TCV.

The material eluted in LC fraction II (which contained the 7-oxygenated derivatives of the respective

substrates) was further purified in TLC system 4 (see Table 2) except the metabolites from 5α-cholestan-3-one and 5β-cholestan-3-one which were purified in LC system 3 (see Table 1).

The material eluted in LC fraction III (which contained the 3-monooxygenated steroids, see Table 1) was further purified in TLC system 1 or 3. The former system was used for extracts from incubations with unsaturated substrates.

The metabolites were extracted from the gel and the amount of radioactivity was determined. Unless otherwise stated, the TMS ether derivatives were prepared (6) and the metabolites were analyzed by GLC–RD and GLC–MS.

Standard procedure for identification of metabolites

A metabolite was considered as identified when the following criteria were fulfilled. *1*) When the TLC and GLC mobilities were the same as for the reference compounds (see Tables 1 and 2). *2*) When the mass spectrum was identical to that of the authentic reference compound when GLC–MS analysis was performed with an SE-30 column.

Metabolites formed from the 5α- and 5β-saturated steroids were also oxidized with CrO_3 and the resulting oxoderivatives were identified with TLC, GLC–RD, and GLC–MS as described above. They were analyzed without prior derivatization.

The mass spectra of all identified metabolites, reference compounds, and substrates can be made available through the WHO collection of mass spectral data for steroids, Dr. Jan Sjövall, Department of Chemistry, Karolinska Institutet S-104 01 Stockholm, Sweden.

RESULTS

The identifications described below were made with material from incubations with 18,000 *g* supernate fractions.

Identification of metabolites of cholesterol, 5-cholesten-3α-ol, and 24α-methyl-5-cholesten-3β-ol

The 7α-hydroxy-, 7β-hydroxy-, and 7-oxoderivatives of these steroids were identified according to the standard procedure. [4-^{14}C]5-Cholestene-3β,7α,12α-triol could be identified from LC fractions I and II after incubations with cholesterol. It was purified and identified as described below for the cholestanol metabolite 5α-cholestane-3β,7α,12α-triol.

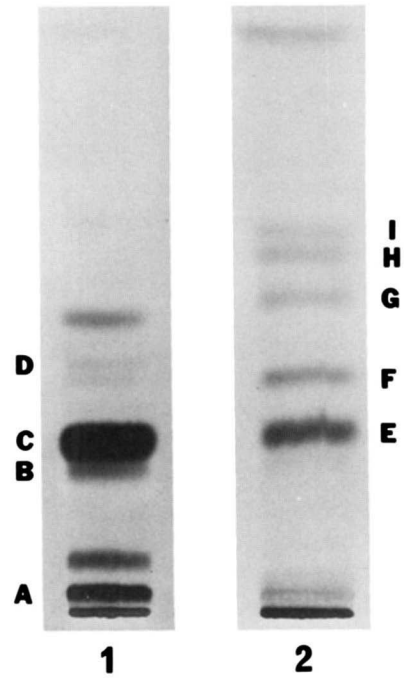

Fig. 1. TLC analysis (system 4, Table 2) with radioautographic detection of compounds eluted in LC fraction II (see Experimental Procedures). Incubations were made with rat liver 18,000 *g* supernate and [4-^{14}C]cholestanol (1) or [4-^{14}C]4-cholesten-3β-ol (2). The metabolites were subsequently identified as follows: *A*, 5α-cholestane-3β,7α,12α-triol; *B*, 5α-cholestane-3β,7β-diol; *C*, 5α-cholestane-3β,7α-diol; *D*, 3β-hydroxy-5α-cholestan-7-one; *E*, 4-cholestene-3β,7α-diol and 5α-cholestane-3β,7α-diol; *F*, 4-cholestene-3β,7β-diol; *G–I*, see text.

Identification of metabolites from cholestanol and 24α-ethyl-5α-cholestan-3β-ol

Metabolites A and B (**Fig. 1**) from cholestanol were extracted from the gel and rechromatographed in TLC system 6 and 5, respectively. They were identified as 5α-cholestane-3β,7α,12α-triol (A) and 5α-cholestane-3β,7β-diol (B) according to the standard procedure. 5α-Cholestane-3β,7α,12α-triol could also be isolated from LC fraction I which was further purified on a 50-ml hydroxyalkylated Sephadex LH-20 column (Lipidex 5000, Packard Instruments, Downers Grove, IL) in the solvent system methanol–water–dichloroethane 95:5:25 (v/v/v) followed by TLC–RD in system 6 of the metabolites eluted at 60–85% TCV. Compound C was identified as 5α-cholestane-3β,7α-diol and compound D as 3β-hydroxy-5α-cholestan-7-one.

When analyzed by TLC–RD in system 4 (Table 2) 24α-ethyl-5α-cholestan-3β-ol afforded radioactive spots at $R_f = 0.20$ (H), $R_f = 0.24$ (I), and $R_f = 0.35$ (J), i.e., slightly above those found for 5α-cholestane-3β,7β-diol ($R_f = 0.18$), 5α-cholestane-3β,7α-diol ($R_f = 0.22$), and 3β-hydroxy-5α-cholestane-7-one (R_f

= 0.33). Metabolites H and I were extracted from the gel and rechromatographed in TLC system 5. Each metabolite afforded one single radioactive spot (R_f = 0.48 and R_f = 0.53, respectively). The R_f values for 5α-cholestane-3β,7β-diol and 5α-cholestane-3β,7α-diol were 0.45 and 0.50 in this TLC system. In this (Table 1) and in other studies (1, 23, 25, 27), C_{29}-steroids oxygenated in the A and B rings have had R_f values identical to or slightly higher than those of analogous C_{27}-steroids. Metabolites H–J are therefore suggested to be identical with 24α-ethyl-5α-cholestane-3β,7β-diol (H), 24α-ethyl-5α-cholestane-3β,7α-diol (I), and 24α-ethyl-3β-hydroxy-5α-cholestan-7-one (J). However, the yields of metabolites H–J were very low and, due to lack of material, no further attempts were made to identify these compounds.

Identification of metabolites from 5α-cholestan-3α-ol, 5β-cholestan-3α-ol, and 5β-cholestan-3β-ol

The C_7-oxygenated metabolites identified from these substrates were 5α-cholestane-3β,7α-ol from incubations with 5α-cholestan-3α-ol and 5β-cholestane-3α,7β-diol from incubations with 5β-cholestan-3α-ol. These metabolites were identified according to the standard procedure (see Experimental Procedures and Table 2). The presence of 5α-cholestane-3β,7α-diol after incubations with 5α-cholestan-3α-ol might be explained by the presence of 5α-cholestan-3β-ol in LC fraction III (see **Table 3**). This latter steroid had probably been formed from 5α-cholestan-3-one which was also present in LC fraction III. 5α-Cholestane-3α,7α-diol, 5β-cholestane-3α,7α-diol, and 5β-cholestane-3β,7α-diol could not be identified. If these compounds were formed, the yields must have been less than 0.02–0.03%.

Metabolites other than those identified above were also found in LC fraction II. In an attempt to identify 5α-cholestane-3α,7β-diol and 5β-cholestane-3β,7β-diol (which were not available as authentic reference compounds), each unknown metabolite of 5α-cholestan-3α-ol and 5β-cholestan-3β-ol isolated from LC fraction II and formed in more than 0.02% yield was oxidized with chromic acid (4) and then analyzed by TLC–RD in TLC systems 1 and 2. No radioactive compounds with TLC mobilities as 5α-cholestane-3,7-dione were formed from metabolites of 5α-cholestan-3α-ol. One radioactive spot (at t_R = 0.26) from incubations with 5β-cholestan-3β-ol gave rise, after oxidation, to one single spot at R_f = 0.36 in TLC system 1 and at R_f = 0.37 in TLC system 2, which were the R_f values found for 5β-cholestane-3,7-dione in the two TLC systems. This suggests that the metabolite of 5β-cholestan-3β-ol with R_f = 0.26 was identical with 5β-cholestane-3β,7β-diol.

Identification of metabolites from 24α-ethyl-5β-cholestan-3α-ol and 24α-ethyl-5β-cholestan-3β-ol

The metabolite pattern from these steroids after TLC–RD (system 4) was very similar to that found after incubations with 5β-cholestan-3β-ol and 5β-cholestan-3α-ol. Thus 24α-ethyl-5β-cholestan-3β-ol afforded a metabolite at R_f = 0.26 (system 4), i.e., the R_f value of the tentatively identified 5β-cholestane-3β,7β-diol. Chromic acid oxidation (4) followed by TLC–RD in systems 1 and 2 afforded in each system one major radioactive spot with an R_f value 0.01 units above that of 5β-cholestane-3,7-dione. 24α-Ethyl-5β-cholestan-3α-ol was metabolized to a compound with t_R = 0.27 which, after extraction from the gel followed by GLC–RD analysis, gave rise to a single radioactive peak at t_R = 3.56, i.e., 1.52 times the t_R value for 5β-cholestane-3α,7β-diol. In previous studies (23, 27) the ratio between the t_R values on SP-2100 columns for analogous C_{29}- and C_{27}-steroids have ranged between 1.52 and 1.58 for steroids oxygenated in the A and B rings. The R_f values after TLC analysis have been identical or slightly higher for the C_{29}-steroids (1, 23, 25, 27, and Table 1). Thus the 24α-ethyl-5β-cholestan-3β-ol and 24α-ethyl-5β-cholestan-3α-ol metabolites were tentatively identified as 24α-ethyl-5β-cholestane-3β,7β-diol and 24α-ethyl-5β-cholestane-3α,7β-diol, respectively. GLC–MS analysis confirmed the preliminary identification of the latter compound, yielding a mass spectrum that differed from that of the C_{27} analog only in that side chain-containing fragments were found 28 mass units above those found in the mass spectrum of the C_{27} compound.

To exclude the formation of 7α-hydroxylated metabolites of 24α-ethyl-5β-cholestan-3β-ol and 24α-ethyl-5β-cholestan-3α-ol, each radioactive metabolite of these compounds formed in yields above 0.02% was oxidized with chromic acid (4) and then analyzed in TLC system 2. No radioactive spots were seen at the t_R value of 5β-cholestane-3,7-dione or immediately above (t_R = 0.37–0.40).

Identification of metabolites from 5α-cholestan-3-one and 5β-cholestan-3-one

These substrates were found to be efficiently converted into the 3-hydroxy derivatives. The metabolites identified from LC fraction II were those isolated also from incubations with 5α-cholestan-3α-ol and 5α-cholestan-3β-ol or 5β-cholestan-3α-ol and 5β-cholestan-3β-ol. The presence of the 3-oxo-7α-hydroxy metabolites could not be confirmed. If these metabolites were present the yields could not have exceeded 0.02–0.03%. Since 7α-hydroxylated 5α-cholestan-3-

TABLE 3. Formation of C_7-oxygenated metabolites of 3-oxygenated C_{27}-, C_{28}-, and C_{29}-steroids

Substrate[a]	Compound Identified[b]	Identification from LC Fraction No.[c]	18,000 *g* Supernate[d]	Iron-Supplemented Microsomes[e]
			Yields (%)	
5-Cholesten-3β-ol	5-Cholestene-3β,7α,12α-triol	I + II	0.59	0.03
	5-Cholestene-3β,7α-diol	II	2.84	2.23
	5-Cholestene-3β,7β-diol	II	0.14	2.52
	3β-Hydroxy-5-cholesten-7-one	II	0.19	4.91
24α-Methyl-5-cholesten-3β-ol	24α-Methyl-5-cholestene-3β,7α-diol	II	0.07	1.85
	24α-Methyl-5-cholestene-3β,7β-diol	II	0.04	1.83
	24α-Methyl-3β-hydroxy-5-cholesten-7-one	II	0.21	2.57
5-Cholesten-3α-ol	5-Cholestene-3α,7α-diol	II	0.16	3.50
	5-Cholestene-3α,7β-diol	II	0.25	4.00
	3α-Hydroxy-5-cholesten-7-one	II	0.28	16.90
4-Cholesten-3α-ol	5α-Cholestane-3β,7α-diol	II	0.21	
	4-Cholesten-3α-ol	III	47	
	4-Cholesten-3-one	III	6.3	
	5α-Cholestan-3β-ol	III	9.9	
4-Cholesten-3β-ol	4-Cholestene-3β,7α-diol	II	0.58	≤0.05
	4-Cholestene-3β,7β-diol	II	0.19	≤0.05
	5α-Cholestane-3β,7α-diol	II	0.23	≤0.05
	4-Cholesten-3β-ol	III	70	36
	4-Cholesten-3-one	III	0.9	43
	5α-Cholestan-3β-ol	III	2.3	0.7
5α-Cholestan-3β-ol	5α-Cholestane-3β,7α,12α-triol	I + II	1.32	<0.01
	5α-Cholestane-3β,7α-diol	II	3.95	0.06
	5α-Cholestane-3β,7β-diol	II	0.12	<0.01
	3β-Hydroxy-5α-cholestan-7-one	II	0.05	≤0.02
24α-Ethyl-5α-cholestan-3β-ol	24α-Ethyl-5α-cholestane-3β,7α-diol[f]	II	0.02	
	24α-Ethyl-5α-cholestane-3β,7β-diol[f]	II	0.01	
	24α-Ethyl-3β-hydroxy-5α-cholestan-7-one[f]	II	0.01	
5α-Cholestan-3α-ol	5α-Cholestane-3β,7α-diol	II	0.17	
	5α-Cholestan-3α-ol	III	81	
	5α-Cholestan-3-one	III	0.3	
	5α-Cholestan-3β-ol	III	3.8	
5β-Cholestan-3α-ol	5β-Cholestane-3α,7β-diol	II	0.04	0.03
24α-Ethyl-5β-cholestan-3α-ol	24α-ethyl-5β-cholestane-3α,7β-diol	II	0.09	
5β-Cholestan-3β-ol	5β-Cholestane-3β,7β-diol[f]	II	0.04	
24α-Ethyl-5β-cholestan-3β-ol	24α-Ethyl-5β-cholestane-3β,7β-diol[f]	II	0.04	
4-Cholesten-3-one	5α-Cholestane-3β,7α,12α-triol	I + II	1.16	≤0.05
	5α-Cholestane-3β,7α-diol	II	1.91	≤0.05
	5α-Cholestane-3β,7β-diol	II	0.10	≤0.05
	4-Cholesten-3-one	III	6.4	71
	4-Cholesten-3β-ol	III	1.9	0.6
	5α-Cholestan-3-one	III	4.5	6.3
	5α-Cholestan-3α-ol	III	6.3	0.6
	5α-Cholestan-3β-ol	III	42	4.0
5α-Cholestan-3-one	5α-Cholestane-3β,7α,12α-triol	I + II	1.25	
	5α-Cholestane-3β,7α-diol	II	5.40	
	5α-Cholestane-3β,7β-diol	II	0.21	
	5α-Cholestan-3-one	III	8.3	
	5α-Cholestan-3α-ol	III	11.5	
	5α-Cholestan-3β-ol	III	41	

[a] 5–10 μg of substrate was used in each case.

[b] To explain the formation of 7-oxygenated metabolites with configurations at C_3 and C_5 other than those of the substrates, formations of monooxygenated steroids from the substrates have been presented in some cases.

[c] See Experimental Procedures.

[d] Each figure represents the mean of 3–6 experiments. Each incubation flask contained 38–47 mg of protein (30).

[e] Each figure represents the mean of 2–3 experiments.

[f] Tentatively identified, see text.

one and 5β-cholestan-3-one might have been reduced to the 3-hydroxylated derivatives, attempts were made to isolate 5α-cholestane-3α,7α-diol, 5β-cholestane-3α,7α-diol, and 5β-cholestane-3β,7α-diol. These compounds could not be identified. If they were formed the yields were less than 0.03%.

Identification of metabolites from 4-cholesten-3-one and 24α-ethyl-4-cholesten-3-one

TLC–RD analysis of LC fraction II from incubations with 4-cholesten-3-one afforded three major radioactive spots at $R_f = 0.03$ (A), $R_f = 0.22$ (B), and $R_f = 0.40$ (C). Metabolite A was identified as 5α-cholestane-3β, 7α,12α-triol as described under metabolites from cholestanol (see above), and metabolite B was found to be identical to 5α-cholestane-3β,7α-diol according to the standard procedure. GLC–RD analysis of the material eluted from position C afforded two radioactive spots at $t_R = 1.90$ and $t_R = 2.40$. No further attempts were made to identify compound C. Smaller amounts of radioactivity were found at $R_f = 0.18$. GLC–RD analysis of this radioactive material yielded radioactive peaks at $t_R = 2.65$ and $t_R = 3.02$. The radioactive material with the t_R value of 3.02 was identified as 5α-cholestane-3β,7β-diol according to the standard procedure. 7α-Hydroxy-4-cholesten-3-one and 7β-hydroxy-4-cholesten-3-one could not be identified. If these compounds were formed the yields must have been less than 0.03–0.04%. Since 7α-hydroxy-4-cholesten-3-one has been reported to be converted to 5β-cholestane-3α,7α-diol by rat liver microsomes (28) and 100,000 *g* supernatant fractions (27), attempts were made to isolate this latter compound. However, its presence could not be confirmed. If present the yields could not have exceeded 0.01%.

Incubations with 24α-ethyl-4-cholestan-3-one did not give rise to detectable amounts of 24α-ethyl-7α-hydroxy-4-cholesten-3-one. Neither was it possible to demonstrate the presence of compounds with the expected chromatographic properties of 24α-ethyl-5α-cholestane-3β,7α-diol and 24α-ethyl-5α-cholestane-3β,7α,12α-triol, i.e., compounds which after TLC analysis in both systems 4 and 5 had R_f values that were identical to or 0.00–0.03 units above those of the C_{27} analogs. In fact no compounds with even similar R_f values (less than 0.05 units below or less than 0.15 units above those of the C_{27} analogs) could be detected. If these 7α-hydroxylated C_{29} steroids were formed the yields of each one must have been less than 0.03%.

Only small amounts of 4-cholesten-3-one were present in LC fraction III from incubates with this steroid. The major labeled compound isolated from this fraction was 5α-cholestan-3β-ol. 5α-Cholestan-3α-ol, 4-cholesten-3β-ol, and 5α-cholestan-3-one were also found in this fraction (see Table 3). The metabolites isolated from LC fraction III from incubates with 24α-ethyl-4-cholesten-3-one were the 24α-ethyl-substituted analogs of those formed from 4-cholesten-3-one.

Identification of metabolites from 4-cholesten-3α-ol and 4-cholesten-3β-ol

When analyzed with TLC–RD, fraction II from incubations with 4-cholesten-3α-ol afforded two major radioactive spots at $R_f = 0.35$ (A) and $R_f = 0.40$ (B). GLC–RD analysis of the material eluted from positions A and B yielded one radioactive peak at $t_R = 1.98$ from position A and three radioactive peaks at $t_R = 1.90$, 2.02, and 2.40 from position B. No further attempts were made to identify these compounds. Smaller amounts of radioactivity were found at $R_f = 0.22$. This radioactive material was identified as 7α-hydroxycholestanol according to the standard procedure. A compound with the same chromatographic properties as 4-cholestene-3α,7α-diol could not be found. If this compound was present, its formation from 4-^{14}C-labeled 4-cholesten-3α-ol was less than 0.01%.

The metabolite pattern from incubations with 4-cholesten-3β-ol is presented in Fig. 1. Metabolites E–I were analyzed by GLC–RD on an SP-2100 column with each yielding only one prominent peak at the following respective t_R values: 2.10 (metabolite E), 2.76 (metabolite F), 2.59 (metabolite G), 2.80 (metabolite H), and 2.24 (metabolite I). Oxidation of the allylic alcohol group with MnO_2 in chloroform (29) yielded 7α-hydroxy-4-cholesten-3-one (50–80% yield) and 7β-hydroxy-4-cholesten-3-one (80–90% yield), from compounds E and F, respectively. Repeated oxidation with MnO_2 of the material with unchanged TLC mobility after MnO_2 oxidation did not increase the yields of the 3-oxo-Δ^4-compounds by more than a few percent. Compounds E and F were thus found to have contained 4-cholestene-3β,7α-diol and 4-cholestene-3β,7β-diol, respectively. The part of the material eluted from position E that was not oxidized by MnO_2 was subsequently identified (standard procedure) as 5α-cholestane-3β,7α-diol.

The GLC–MS analysis was run on a QF-1 column to separate this compound from contaminating endogenous 5-cholestene-3β,7α-diol. In attempts to identify compounds G, H, and I, these were reduced with sodium borohydride in isopropanol. The reaction products did not show a TLC mobility similar to that of 7α- or 7β-hydroxylated 4-cholesten-3β-ol. No further attempts were made to identify compounds G–I.

In LC fraction III, both the 4-cholesten-3α-ol and

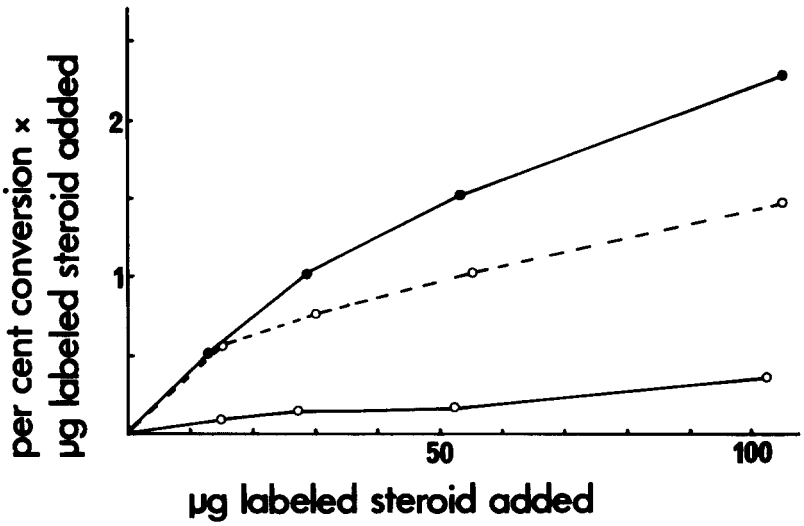

Fig. 2. Effects of substrate concentration on the 7α-hydroxylation of cholesterol ○ – – – ○, cholestanol ● —— ●, and 4-cholesten-3β-ol ○ —— ○, during incubations with rat liver 18,000 *g* supernatant fractions. Protein content in each incubation flask was about 40 mg.

the 4-cholesten-3β-ol experiments afforded 4-cholesten-3-one as well as all monooxygenated steroids formed from incubations with this steroid. The major part of the radioactivity eluted in fraction III, however, represented unchanged 4-cholesten-3α-ol and 4-cholesten-3β-ol, respectively.

Influence of autoxidation on the metabolite pattern of C_{27}-steroids

Ferrous ion-supplemented microsomes—known to exhibit NADPH-dependent lipid peroxidase activity (20, 24)—markedly increased the yields of C_7-oxygenated metabolites of cholesterol, 5-cholesten-3α-ol and 24α-methyl-5-cholesten-3β-ol. To see if these microsomes could also accomplish the formation of 7-hydroxylated derivatives of cholestanol, 4-cholesten-3β-ol and 5β-cholestan-3α-ol, these substrates were also incubated with iron-supplemented microsomes. The yields of 7α-hydroxylated cholestanol and 4-cholesten-3β-ol were very low when compared to those found after incubations with 18,000 *g* supernates whereas the formation of 7β-hydroxylated 5β-cholestan-3α-ol was not affected (Table 3).

Quantitative studies

The overall recoveries of radioactivity after extraction, LC, and TLC ranged between 70 and 90%, except when 18,000 *g* supernates had been incubated with 4-cholesten-3-one, 24α-ethyl-4-cholesten-3-one, or 5β-cholestan-3-one. In these latter cases the recoveries ranged between 50 and 60%.

The extent of 7α-hydroxylation of cholesterol, cholestanol, and 4-cholesten-3β-ol added in different concentrations to an 18,000 *g* rat liver supernatant fraction is shown in **Fig. 2.** The yields of labeled 4-cholesten-3β,7α-diol were much lower than those of 7α-hydroxycholesterol and 7α-hydroxycholestanol. The 7α-hydroxylation of cholesterol and cholestanol was also studied in parallel experiments with 10 µg of each substrate incubated according to the standard procedure. The average yields (nine experiments) were 2.73 ± 0.62 SD and 3.60 ± 1.32 SD for 5-cholestene-3β,7α-diol and 5α-cholestane-3β,7α-diol, respectively. This difference was significant ($P < 0.05$, *t* test). To explore whether cholesterol and cholestanol were 7α-hydroxylated by the same enzyme, the extent of 7α-hydroxylation with increasing ion strength of the potassium phosphate buffer was also investigated (**Table 4**). As reported by Björkhem and Danielsson (31) the formation of 5-cholestene-3β,7α-diol decreased when the molarity of the potassium phosphate buffer was increased. A parallel drop was noted in the 7α-hydroxylation of cholestanol. 7α-Hydroxylation of 3β-hydroxy-5-androsten-17-one remained constant as reported (31).

The comparison between the extent of 7α-hydroxylations made above did not take further metabolism of the derivative initially formed into consideration. However, as can be seen in Table 3, the amount of 5-cholestene-3β,7α,12α-triol or 5α-cholestane-3β,7α,12α-triol isolated from LC fractions I and II after incubations (18,000 *g* supernates) with cholesterol and cholestanol were consistently found to be approximately one-fifth and one-third of the amount of 5-

TABLE 4. Effects of the concentration of potassium phosphate buffer on 7-oxygenation of 5-cholesten-3β-ol, 5α-cholestan-3β-ol, and 3β-hydroxy-5-androsten-17-one

Buffer Concentration	Formation of Labeled 7-Oxygenated Derivatives[a]									
	5-Cholesten-3β-ol				5α-Cholestan-3β-ol				3β-Hydroxy-5-androsten-17-one[b]	
	7α-OH	7α,12α-diOH	7β-OH	7-oxo	7α-OH	7α,12α-diOH	7β-OH	7-oxo	7α-OH	7β-OH
	% Yield									
0.05 M	2.4	0.5	0.08	0.14	4.3	1.9	0.21	0.02	2.7	2.5
0.25 M	1.3	0.1	0.07	0.09	2.4	0.5	0.10	0.01	2.3	1.7
0.45 M	0.6	0.0	0.04	0.06	1.5	0.1	0.06	0.01	2.3	2.5

[a] Incubations were made with 5 μg of substrate and the standard preparation of rat liver 18,000 *g* supernate. 7α-OH, 7α,12α-diOH, 7β-OH, and 7-oxo denote the 7α-hydroxy, 7α,12α-dihydroxy, 7β-hydroxy, and 7-oxoderivatives of 5-cholesten-3β-ol, 5α-cholestan-3β-ol, and 3β-hydroxy-5-androsten-17-one respectively. Each figure represents the mean of two experiments.

[b] The crude extracts from incubations with this steroid were purified on 7-ml Lipidex 1000 (Packard Instruments) columns used in water–methanol–butanol–chloroform 55:45:7:3 (by vol.). Fractions eluted at 150–300% TCV were analyzed by TLC–RD in system 8 (see Table 2). Radioactive spots with a mobility as 7α- and 7β-hydroxylated 3β-hydroxy-5-androsten-17-one were eluted from the gel and quantitated by GLC–RD on a SP-2100 column after preparation of the TMS ether derivatives (6).

cholestene-3β,7α-diol and 5α-cholestane-3β,7α-diol, respectively.

Since 5-cholestene-3β,7α-diol is known to be converted to 5-cholestene-3β,7β-diol and 3β-hydroxy-5-cholesten-7-one by rat liver 18,000 *g* supernate (1, 32), 1 μg of biosynthesized 4-^{14}C-labeled 5α-cholestane-3β,7α-diol was incubated (after purification in TLC system 4) with a rat liver 18,000 *g* supernatant fraction as described above for cholestanol. The yields of 5α-cholestane-3β,7β-diol, 3β-hydroxy-5α-cholestan-7-one, and 5α-cholestane-3β,7α,12α-triol were 0.6, 0.2, and 42% respectively.

DISCUSSION

Lipid peroxidase-mediated 7α-hydroxylation of cholesterol and other 3β-hydroxy-Δ^5-steroids (20) has been shown to give rise to the 7β-hydroxy derivatives in about equal amounts as the 7α-hydroxy derivatives and to even higher amounts of the 7-oxo derivatives. As can be seen from Table 3 this was also found to be the case for 24α-methyl-5-cholesten-3β-ol and 5-cholesten-3α-ol, whereas a lipid peroxidase-mediated 7-oxygenation of the Δ^4-, 5α-, and 5β-steroids tested occurred hardly at all. The results presented in Table 3 thus indicate also that the small amounts of 7α-hydroxylated 24α-methyl-5-cholesten-3β-ol and 5-cholesten-3α-ol, which were identified after incubations with rat liver 18,000 *g* supernatant fractions fortified with NADPH, actually were formed mainly through the action of nonspecific lipid peroxidase activity.

Previous reports suggesting that the plant sterol β-sitosterol cannot be 7α-hydroxylated by rat liver microsomal preparations in vitro (1, 2) indicated the dependence of the 7α-hydroxylase(s) on the structure of the steroid side chain. These findings have been confirmed in the present study. Thus 24α-methyl-5-cholesten-3β-ol did not specifically give rise to 24α-methyl-5-cholestene-3β,7α-diol.

The importance of the structure of the steroid nucleus in the microsomal 7α-hydroxylation of C_{27}-steroids has been shown in this study to be more pronounced than might have been anticipated from previous reports (3, 33). Thus, apart from cholesterol and cholestanol, 4-cholesten-3β-ol was the only compound that was found to serve as a substrate for the 7α-hydroxylase. The yields of 4-cholestene-3β,7α-diol were low compared to those obtained with cholesterol and cholestanol.

The C_{27}-steroid 7α-hydroxylase seems to require a comparatively flat molecule (Δ^4-, Δ^5- or 5α-steroid) since neither 5β-cholestan-3α-ol nor 5β-cholestan-3β-ol afforded a 7α-hydroxylated derivative. However, this requirement is not enough. The presence of a β-oriented equatorial or quasiequatorial hydroxyl group at C_3 is also needed as shown by the lack of formation of 7α-hydroxylated 5α-cholestane-3α-ol, 5-cholestene-3α-ol, or 4-cholesten-3α-ol. The presence of a 3β-hydroxyl group seems mandatory since no significant formation of 7α-hydroxy-4-cholesten-3-one, 7α-hydroxy-5α-cholestan-3-one, or 7α-hydroxy-5β-cholestan-3-one could be shown. Comparatively large amounts of 7α-hydroxylated cholestanol could be isolated from incubations with 5α-cholestan-3-one and 4-cholesten-3-one, but the conversion of these substrates into cholestanol (34, 35) at least in part explained the formation of 5α-cholestane-3β,7α-diol. The possibility that 4-cholesten-3-one was first 7α-hydroxylated and then reduced to 5α-cholestane-3β,7α-diol seems less likely since 7α-hydroxy-4-cholesten-3-one is known to be converted almost exclusively to 7α-hydroxy-5β-

cholestan-3-one and 5β-cholestane-3α,7α-diol by a 100,000 *g* supernate (27) or a microsomal fraction (28, 36) from rat liver. But these latter compounds could not be found in the present study. With 5α-cholestan-3-one an initial 7α-hydroxylation followed by a reduction of the 3-oxo group cannot be completely ruled out, although one would have expected the formation of at least detectable amounts of 5α-cholestane-3α,7α-diol from 7α-hydroxy-5α-cholestan-3-one (36).

The results on 7-hydroxylation of cholestanol and 4-cholesten-3β-ol (Table 3) indicate that the 7-hydroxy and 7-oxo derivatives of these substrates are not formed under conditions favoring autoxidation. However, the results from incubations with 5α-cholestane-3β,7α-diol demonstrate that the 7β-hydroxy derivatives can be formed from the corresponding 7α-hydroxysteroids via the 7-oxo metabolites. By contrast, a 7β-hydroxylating activity directed toward 3-hydroxysteroids of the 5β-series seemed to be present since no 7α-hydroxylation, and thus no formation via 7-oxo derivatives, could be demonstrated with these steroids (Table 3).

The effect of 7-oxygenated C_{27}- and C_{29}-steroids and the corresponding 5,6-epoxides on the cholesterol-7α-hydroxylase has been studied in previous investigations (1, 25). The results show that the β-substituted compounds, i.e., the 7β-hydroxy and the 5β,6β-oxido derivatives of cholesterol and β-sitosterol, were more potent inhibitors than the corresponding α-substituted isomers. An explanation for this might be that the substrate is attached to the 7α-hydroxylase from the β-side of the molecule and that positions 5, 6, and 7 are important for binding. As shown in this paper the enzyme requires a 3β-hydroxy group, and it should be recalled that the steroid side chain is 17β-substituted. The influence of the side chain structure seems rather pronounced since the presence of a 24α-alkyl group markedly diminishes the inhibition caused by the 5β,6β-oxido and the 7β-hydroxy derivatives (1, 25). It would be of interest to see if 3β-hydroxy-Δ^5 sterols with a 17α-oriented side chain can be converted to 7α-hydroxy metabolites.

The present report shows that the 7α-hydroxylation of both cholestanol and cholesterol was affected by increasing strength of the potassium phosphate buffer, indicating similarities in the 7α-hydroxylating systems for the two substrates. Similarities between these systems have also been reported by Cottman et al. (37). These authors found a parallelism between the 7α hydroxylation of cholestanol and cholesterol catalyzed by reconstituted systems from rat liver microsomes.

From a quantitative point of view, cholestanol appears to give better yields of the 7α-hydroxy derivative than cholesterol. This difference is enhanced if the formation of the respective 3β,7α,12α-trihydroxy metabolites is taken into consideration. This seems justified since the formation of 5-cholestene-3β,7α,12α-triol most probably proceeds via an initial 7α-hydroxylation of cholesterol (19) and since 7α-hydroxylated cholestanol has been shown to be efficiently converted to 5α-cholestane-3β,7α,12α-triol in this study.

Scallen et al. (38) have suggested that a carrier protein may take part in the 7α-hydroxylation of cholesterol. If the affinity of this protein is higher for cholestanol than for cholesterol it could explain why cholestanol is more efficiently 7α-hydroxylated than cholesterol. It is thus difficult to discuss reactions occurring in subcellular particles in such simple terms as a direct substrate–enzyme interaction, especially when the enzyme is a composite one (39). The transport or penetration of the substrate into the particle or membrane may be very important. In fact, some of the results of the present investigation illustrate this point. Thus, 5α-cholestan-3-one seems to be a far better substrate in the 7α-hydroxylation of cholestanol than cholestanol itself. It may be speculated that the oxidoreductase that reduced cholestanone to cholestanol is located in the immediate vicinity of the 7α-hydroxylase or is functionally connected to this enzyme.

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