Conversion of 7α-hydroxycholesterol and 7α-hydroxy-β-sitosterol to 3α,7α-dihydroxy- and 3α,7α,12α-trihydroxy-5β-steroids in vitro

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Abstract The metabolism of 7α-hydroxycholesterol and 7α-hydroxy-β-sitosterol (24α-ethyl-5-cholestene-3β,7α-diol) has been compared in rat liver subcellular fractions. 7α-Hydroxy-β-sitosterol was shown to be metabolized in the same manner as 7α-hydroxycholesterol. Thus, the following C_{29} metabolites have been identified: 24α-ethyl-7α-hydroxy-4-cholesten-3-one, 24α-ethyl-7α,12α-dihydroxy-4-cholesten-3-one, 24α-ethyl-7α-hydroxy-5β-cholestan-3-one, 24α-ethyl-5β-cholestane-3α,7α-diol, 24α-ethyl-7α,12α-dihydroxy-5β-cholestan-3-one, and 24α-ethyl-5β-cholestane-3α,7α,12α-triol. The C_{29} compounds were generally less efficient substrates. The most pronounced difference was noted for the Δ^4-3-oxosteroid 5β-reductase. Thus, 7α-hydroxy-4-cholesten-3-one was three to four times as efficiently reduced as the C_{29} analog. The oxidation of the 3β,7α-dihydroxy-Δ^5-steroid to the 7α-hydroxy-Δ^4-3-oxosteroid, the 12α-hydroxylation of the 7α-hydroxy-Δ^4-3-oxosteroid, and the reduction of the 7α-hydroxy-5β-3-oxosteroid to the 3α,7α-dihydroxy-5β-steroid occurred in up to two times better yields for the C_{27} steroids.

Supplementary key words Δ^5-3β-hydroxysteroid dehydrogenase · Δ^5-3-oxosteroid isomerase · Δ^4-3-oxosteroid 12α-hydroxylase · Δ^4-3-oxosteroid 5β-reductase · 3α-hydroxysteroid dehydrogenase · hydroxyalkylated Sephadex LH-20 · thin-layer chromatography · gas–liquid chromatography–mass spectrometry

The conversion of the steroid nucleus of cholesterol during the biosynthesis of bile acids in rat liver (for recent reviews see Refs. 1 and 2) starts with 7α-hydroxylation in the endoplasmic reticulum followed by an NAD-dependent oxidation to 7α-hydroxy-4-cholesten-3-one. This compound may either undergo an NADPH-dependent 12α-hydroxylation followed by NADPH-supported saturation of the 4–5 double bond and reduction of the 3-oxo group to a 3α-hydroxy substituent (3) or undergo the two last-mentioned reactions directly leading to the formation of 5β-cholestane-3α,7α-diol (4). The rate-limiting step in the conversion of cholesterol is the 7α-hydroxylation (5), but the reduction of the double bond and the 12α-hydroxylation are also of importance (4, 6). In a previous study it was found that β-sitosterol was at least 100 times less efficient a substrate than cholesterol for the microsomal 7α-hydroxylase, indicating that the steroid side chain structure may be of importance for this enzyme (7). To investigate if the further transformations in the steroid nucleus were also affected by the side chain structure, the metabolic fates of 7α-hydroxycholesterol and 7α-hydroxy-β-sitosterol in rat liver subcellular fractions have been compared.

METHODS

Solvents

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

Substrates

Labeled and unlabeled C_7-oxygenated derivatives of cholesterol and β-sitosterol were synthesized and purified as previously described (7). Labeled and unlabeled 7α-hydroxy-4-cholesten-3-one, 7α,12α-dihydroxy-4-cholesten-3-one, and 5β-cholestane-3α,7α-diol, as well as the 24α-ethyl-substituted analogs, were prepared in vitro as described in Experimental Procedures. 7α-Hydroxy-4-

Abbreviations: Systematic names of the steroids referred to in the text by trivial names are as follows: cholesterol, 5-cholesten-3β-ol; β-sitosterol, 24α-ethyl-5-cholesten-3β-ol; 7α-hydroxycholesterol, 5-cholestene-3β,7α-diol; 7α-hydroxy-β-sitosterol, 24α-ethyl-5-cholestene-3β,7α-diol. Compounds referred to as C_{27} or C_{29} compounds denote derivatives of cholesterol and β-sitosterol, respectively. LC, liquid chromatography; TCV, total column volume; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry; TMS ether, trimethylsilyl ether.

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TABLE 1. Chromatographic properties of 3,7- and 3,7,12-oxygenated steroids

Steroid	LC System[a]			TLC System[b]			GLC[c]	
	A	B	C	1	2	3	t_R[d,e]	C_{29}/C_{27}[f]
	% TCV			R_f[e]				
5-Cholestene-3β,7α-diol	85	110		0.25(0.26)			2.08(3.21)	1.54
3β-Hydroxy-5-cholesten-7-one	115			0.45(0.47)			3.95(6.16)	1.56
7α-Hydroxy-4-cholesten-3-one	90	60	370	0.51(0.53)	0.78(0.80)		2.66(4.09)	1.54
7α-Hydroxy-5α-cholestan-3-one				0.69			2.26	
7α-Hydroxy-5β-cholestan-3-one	135			0.80(0.81)		0.51	2.46(3.75)	1.53
5α-Cholestane-3α,7α-diol				0.29			2.07	
5β-Cholestane-3α,7α-diol	110			0.37(0.39)	0.66	0.16	2.16(3.33)	1.54
7α,12α-Dihydroxy-4-cholesten-3-one	55		200	0.15	0.45(0.51)		3.07(4.53)	1.48
7α,12α-Dihydroxy-5α-cholestan-3-one				0.26	0.63		2.54	
7α,12α-Dihydroxy-5β-cholestan-3-one	70		270	0.40	0.76(0.81)		2.80(4.17)	1.49
5β-Cholestane-3α,7α,12α-triol	65		270	0.08	0.23		2.25(3.33)	1.48
4,6-Cholestadien-3-one	230			0.88(0.89)	0.91		2.53(3.98)	1.57

[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 (8), used in methanol–water–dichloroethane 95:5:25 (v/v/v). Column dimensions, 1.7 cm² × 30 cm; flow rate, 0.3 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. System C, Lipidex-1000 (Packard) used in methanol–water–butanol–chloroform 70:30:7:3 (by vol). Column dimensions, 1.4 cm² × 30 cm; flow rate, 0.2 ml/cm²/min at 24°C.

[b] System 1, diethyl ether–cyclohexane 9:1 (v/v) (9); system 2, diethyl ether–ethyl acetate 1:1 (v/v); system 3, diethyl ether–cyclohexane 2:1 (v/v).

[c] A Pye gas chromatograph (model 104) equipped with a hydrogen flame ionization detector was used. The column (2 m × 4 mm) contained silanized Supelcoport, 80–100 mesh, coated with 3% SE-30 (Supelco, Inc.). Temperatures: flash heater, 270°C; column oven, 250°C. Carrier gas, nitrogen; gas flow rate, 480 ml/cm²/min.

[d] Retention times relative to that of 5α-cholestane.

[e] Numbers in parentheses are mobilities (R_f or t_R) for 24-ethyl-substituted analogs of the indicated C_{27} compounds.

[f] Ratios of retention times of C_{29} compounds to those of corresponding C_{27} compounds.

cholesten-3-one and 24α-ethyl-7α-hydroxy-4-cholesten-3-one were purified in TLC system 1 and LC systems A and B (**Table 1**). 7α,12α-Dihydroxy-4-cholesten-3-one and 24α-ethyl-7α,12α-dihydroxy-4-cholesten-3-one were purified by TLC system 2 and LC system C. 5β-Cholestane-3α,7α-diol and 24α-ethyl-5β-cholestane-3α,7α-diol were purified in TLC system 1 and LC system A. 7α-Hydroxy-5β-cholestan-3-one and 24α-ethyl-7α-hydroxy-4-cholesten-3-one were prepared from 5β-cholestane-3α,7α-diol and 24α-ethyl-5β-cholestane-3α,7α-diol, respectively. The latter compounds were heated under reflux with aluminum *tert*-butoxide as described by Danielsson (10). The products were purified in TLC system 3 and LC system A. 4,6-Cholestadien-3-one was prepared as described by Greenhalgh, Henbest, and Jones (11).

The following reference steroids were gifts of Dr. Ingemar Björkhem, Dr. Henry Danielsson, and Dr. Jan Gustafsson, Department of Chemistry, Karolinska Institutet: 7α-hydroxy-4-cholesten-3-one, 7α-hydroxy-5α-cholestan-3-one, 7α-hydroxy-5β-cholestan-3-one, 5α-cholestane-3α,7α-diol, 5β-cholestane-3α,7α-diol, 7α,12α-dihydroxy-4-cholesten-3-one, 7α,12α-dihydroxy-5α-cholestan-3-one, 7α,12α-dihydroxy-5β-cholestan-3-one, and 5β-cholestane-3α,7α,12α-triol. Biosynthesized C_{27} and C_{29} compounds were used in in vitro studies.

All compounds were characterized by GLC–MS as described below. Radioactive compounds were analyzed by GLC combined with radioactivity detection (Packard monitoring system, model 893) and by radioautography of thin-layer chromatoplates.

Coenzymes and cofactors

These compounds were purchased from Sigma Chemical Co., St. Louis, Mo., and were used without further purification.

Solutions for homogenization and incubation

The solutions were prepared with doubly distilled water. Unless otherwise stated, a modified Bucher medium (7), pH 7.4, a 0.1 M Tris-Cl^- buffer solution, pH 7.2, or a 0.1 M potassium phosphate buffer, pH 7.4, containing 0.125 M sucrose was used.

Protein determination

Protein was determined according to the method of Lowry et al. (12).

Thin-layer chromatography

20 × 20 cm plates coated with silica gel G were used in the systems described in Table 1. Separated compounds were detected by iodine vapor or by radioautography (see below). Compounds were extracted from the gel with 5 ml of chloroform–methanol 4:1 (v/v) per cm² of gel followed by 5 ml of methanol/cm² of gel. For identification purposes

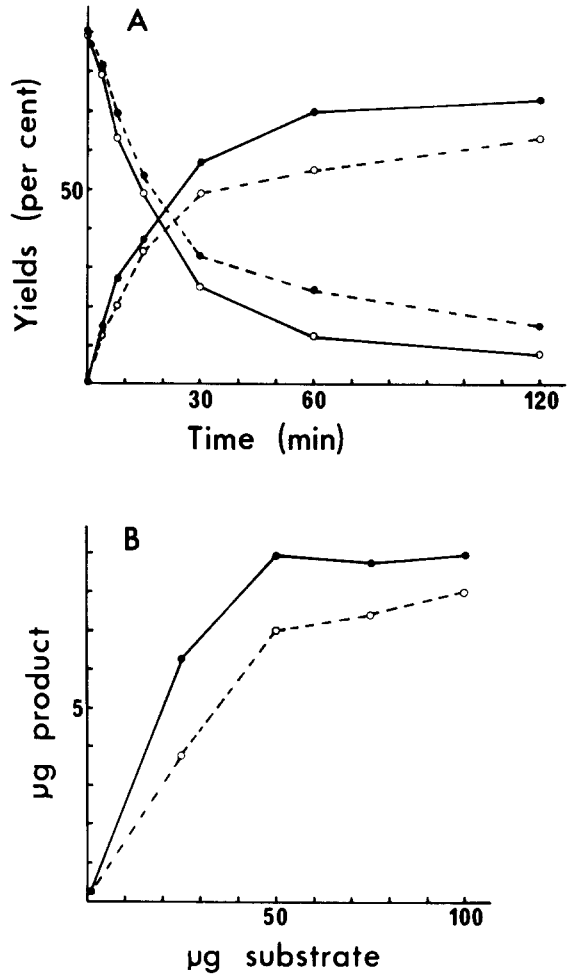

Fig. 1. Effects of time (*A*) and substrate concentration (*B*) on conversion of 4-^{14}C-labeled 7α-hydroxycholesterol and 7α-hydroxy-β-sitosterol to 7α-hydroxy-4-cholesten-3-one and 24α-ethyl-7α-hydroxy-4-cholesten-3-one, respectively. Incubations were made with a rat liver 100,000 *g* sediment fraction fortified with NAD. 1 μg of substrate was added in *A*. Protein content in each incubation flask was 7 mg (*A*) and 2 mg (*B*). Each circle represents the mean of four experiments. O—O, 7α-hydroxycholesterol; ●- - -●, 7α-hydroxy-β-sitosterol; ●—●, 7α-hydroxy-4-cholesten-3-one; O- - -O, 24α-ethyl-7α-hydroxy-4-cholesten-3-one.

the plates were sprayed with a solution of 70% H_2SO_4 saturated with $K_2Cr_2O_7$ and then heated at 120°C.

Gas–liquid chromatography–mass spectrometry

This type of analysis was carried out with an LKB-9000 instrument equipped with a 1.5% SE-30 column (2 m × 3 mm) operated at 250°C, with helium as carrier gas (flash heater, 270°C; molecular separator, 280°C; ion source, 290°C; energy of bombarding electrons, 22.5 eV).

Measurement of radioactivity

Either a Frieseke-Hoephner (Erlangen Bruch, West Germany) gas flow counter FHT 90 B or a Packard model 2009 liquid scintillation spectrometer was used. At least 2000 cpm above the background was counted. Radioactive spots on thin-layer chromatograms were localized by exposing a sheet of Agfa Gevaert Osray M3 for 1–14 days. In GLC analysis with radioactivity detection, at least 4000 dpm was injected.

EXPERIMENTAL PROCEDURES

Male rats of the Sprague-Dawley strain (weight 150–210 g) were used. They were fed a standard pellet food. The animals (three to five in each experiment) were killed by a blow to the head at 9 a.m. The livers were excised, cut in pieces, and rinsed free of blood by dipping them into the homogenizing medium. The different subcellular fractions were prepared as described below (*A–C*). These steps were carried out at 4°C. Substrates were in each case added in 100–300 μl of acetone with concomitant vibration of the mixture as described previously (7). Incubations were terminated by dropwise addition of the homogenate and washings (2 × 10 ml of 0.9% NaCl) from a separatory funnel into 60 ml of chloroform–methanol 2:1 (v/v) with stirring. The procedure was completed for each homogenate within 1.5–2 min. The homogenate was repeatedly extracted with the same solvent until recovery of the radioactivity was complete. The combined chloroform phases were taken to dryness and then purified by LC and TLC (see Table 1).

For control experiments, cell-free preparations prepared as described in *A–C* were heated to 80°C, kept at that temperature for 15–20 min, and then centrifuged at 3200 *g* for 10 min. The supernate was used for the incubations as described in *A–C*. 1 μg of substrate was used.

A. Conversion of 7α-hydroxycholesterol and 7α-hydroxy-β-sitosterol in a rat liver 100,000 *g* sediment fraction fortified with NAD

To 10 g of wet liver was added 40 ml of the Bucher medium. The mixture was homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle (the difference between the diameter of the inner glass wall and that of the pestle was 0.15 mm). The pooled homogenates were centrifuged at 800 *g* for 10 min, and the resulting supernatant fluids were then centrifuged twice at 20,000 *g* for 15 min. The 20,000 *g* supernates were pooled and then centrifuged at 100,000 *g* for 60 min. A microsomal pellet obtained from 2 g of wet liver was suspended in 10 ml of the Bucher medium and was then homogenized with the Teflon pestle. Unless otherwise stated, 10 ml of this homogenate was incubated for 30 min at 37°C with 1–100 μg of 4-^{14}C-labeled 7α-hydroxycholesterol or 7α-hydroxy-β-sitosterol in the presence of 4 μmoles of NAD. For the studies on the effects of substrate concentration, the homogenate was diluted 1:4 with the homogenizing medium and then 10 ml was used for the incubation.

B. Conversion of 7α-hydroxy-4-cholesten-3-one and 24α-ethyl-7α-hydroxy-4-cholesten-3-one in a rat liver 100,000 *g* sediment fraction fortified with NADPH

To 10 g of wet liver was added 40 ml of the Tris-Cl^- buffer. A microsomal pellet was prepared as described in *A*. A fraction of this pellet, corresponding to 2 g of wet liver, was suspended in 10 ml of the Tris-Cl^- buffer, homogenized, and centrifuged at 100,000 *g* for another 60 min. The sediment was suspended in another 10 ml of Tris-Cl^- buffer, and the mixture was incubated for 20 min at 37°C with 0.5–20 μg of 4-^{14}C-labeled 7α-hydroxy-4-cholesten-3-one or the 24α-ethyl-substituted analog in the presence of 12 μmoles of NADPH.

C. Conversion of 7α-hydroxy-4-cholesten-3-one 7α,12α-dihydroxy-4-cholesten-3-one, and the 24α-ethyl-substituted analogs in a rat liver 100,000 *g* supernatant fraction fortified with NADPH

To 10 g of wet liver was added 20 ml of the potassium buffer. The mixture was homogenized and centrifuged at 800 *g* for 10 min as described in *A*. The 800 *g* supernatant fraction was centrifuged at 20,000 *g* for 15 min, and the resulting supernate was centrifuged twice at 100,000 *g* for 60 min. Unless otherwise stated, 1 ml of the 100,000 *g* supernate and 9 ml of the phosphate buffer were incubated at 37°C for 20 min with 1–150 μg of 4-^{14}C-labeled 3-oxosteroid in the presence of 12 μmoles of NADPH.

RESULTS[2]

Conversion of 7α-hydroxycholesterol and 7α-hydroxy-β-sitosterol in a rat liver 100,000 *g* sediment fraction fortified with NAD

Purification of extracts from incubations with 7α-hydroxy[4-^{14}C]cholesterol and 7α-hydroxy-β-[4-^{14}C]sitosterol was accomplished by LC system A (Table 1). Fractions eluted between 65 and 140% TCV (C_{27} metabolites) or between 70 and 160% TCV (C_{29} metabolites) (7, 13), which contained 93–98% of the radioactivity eluted from the column, were subjected to analysis in TLC system 1 followed by radioautographic detection. Both substrates gave rise to two major compounds that showed chromatographic properties similar to those of 7α-hydroxycholesterol (compound I) and 7α-hydroxy-4-cholesten-3-one (compound II) (for R_f values see Table 1). Extraction of the relevant TLC zones followed by trimethysilylation of the dried extract and GLC analysis with radioactivity detection revealed one predominant labeled peak for each of the extracted zones. The retention times for the C_{27} compounds confirmed the preliminary identification made by TLC. The C_{29} metabolites were eluted 1.54 times later than the corresponding C_{27} compounds (see Table 1). The mass spectra of the C_{27} and C_{29} TMS ether derivatives of compound I showed the features previously reported (7) for the TMS ether derivatives of 7α-hydroxycholesterol and 7α-hydroxy-β-sitosterol. The mass spectrum of the TMS ether derivative of C_{27} compound II was identical with that of the authentic TMS ether derivative of 7α-hydroxy-4-cholesten-3-one, with prominent peaks at *m/e* 472 (M), 25%; 457, 53%; 382, 100%; 367, 16%; 349, 10%; 338, 8%; 269, 43%; and 174, 96%. The corresponding C_{29} metabolite yielded the same fragmentation pattern and differed from the C_{27} metabolite only in that the side chain-containing fragments were found 28 mass units above those found in the mass spectrum of the C_{27} compound. Prominent peaks were detected at *m/e* 500 (M), 23%; 485, 49%; 410, 100%; 395, 12%; 377, 10%; 366, 9%; 269, 53%; and 174, 97%.

In quantitative work the overall recovery of ^{14}C after LC and TLC was found to be 80–90%. The conversion of 7α-hydroxycholesterol and 7α-hydroxy-β-sitosterol into the respective 7α-hydroxy-Δ^4-3-oxo derivatives differed somewhat with regard to both incubation time and substrate concentration (**Fig. 1**). The conversion was less than 0.1% in control experiments.

Conversion of 7α-hydroxy-4-cholesten-3-one and 24α-ethyl-7α-hydroxy-4-cholesten-3-one in a rat liver 100,000 *g* sediment fraction fortified with NADPH

After purification of the incubation extracts in LC system C (Table 1), fractions eluted between 140 and 260% TCV (C_{27} metabolites) or between 170 and 310% TCV (C_{29} metabolites) were subjected to further purification in TLC system 2. One major labeled spot was obtained with both C_{27} and C_{29} metabolites (R_f = 0.45 and 0.51, respectively). In the former case the compound showed a chromatographic behavior equal to that of 7α,12α-dihydroxy-4-cholesten-3-one (Table 1). Extraction of the appropriate TLC zones followed by trimethylsilylation of the dried extract and subsequent GLC analysis with radioactivity detection revealed only one major labeled peak. The retention time (t_R) for the TMS ether derivative of the C_{27} metabolite was that of the TMS ether derivative of authentic 7α,12α-dihydroxy-4-cholesten-3-one. The corresponding derivative of the C_{29} metabolite yielded a t_R value of 4.53, i.e., 1.48 times the t_R value for the analogous C_{27} metabolite (see Table 1). GLC–MS analysis of the TMS ether derivative of the C_{27} metabolite confirmed the chromatographically suggested identity. Prominent peaks were found at *m/e* 560 (M), 1%; 545, 4%; 470, 7%; 455, 3%;

[2] The mass spectra of the TMS ethers of the following compounds have been examined by the reviewers and 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 60, Sweden: 7α-hydroxy-4-cholesten-3-one, 7α,12α-dihydroxy-4-cholesten-3-one, 7α-hydroxy-5β-cholestan-3-one, 5β-cholestane-3α,7α-diol, 5β-cholestane-3α,7α,12α-triol, and the 24α-ethyl-substituted analogs.

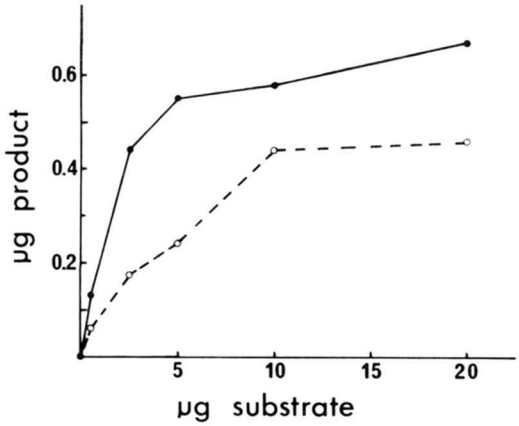

Fig. 2. Effects of substrate concentration on formation of 7α,12α-dihydroxy-4-cholesten-3-one (solid line) and 24α-ethyl-7α,12α-dihydroxy-4-cholesten-3-one (broken line) during incubations of 4-^{14}C-labeled 7α-hydroxy-4-cholesten-3-one and 24α-ethyl-7α-hydroxy-4-cholesten-3-one, respectively, with a rat liver 100,000 *g* sediment fraction fortified with NADPH. Protein content in each incubation flask was 4 mg. Each circle represents the mean of two experiments.

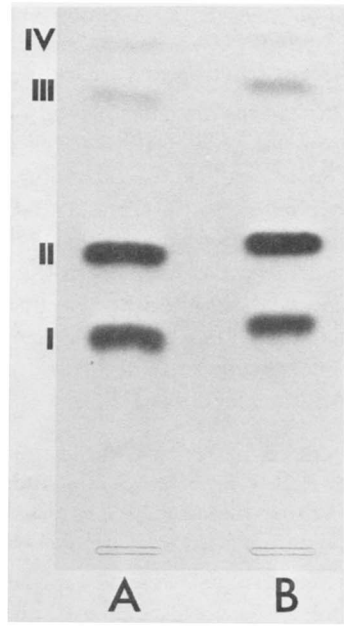

Fig. 3. Radioautographic recording after TLC analysis of metabolites formed from 4-^{14}C-labeled 7α-hydroxy-4-cholesten-3-one (*A*) and 24α-ethyl-7α-hydroxy-4-cholesten-3-one (*B*). Incubations were made with a rat liver 100,000 *g* supernatant fraction fortified with NADPH. Compounds I–IV were subsequently identified as 5β-cholestane-3α,7α-diol (I), 7α-hydroxy-4-cholesten-3-one (II), 7α-hydroxy-5β-cholestan-3-one (III), and 4,6-cholestadien-3-one (IV), or the analogous 24α-ethyl-substituted derivatives.

380, 98%; 357, 13%; 347, 7%; 295, 12%; and 267, 100%. The fragmentation pattern of the analogous C_{29} metabolite was almost the same and differed from the C_{27} metabolite only in that the side chain-containing fragments were found 28 mass units above those found in the mass spectrum of the C_{27} compound. Prominent peaks were detected at *m/e* 588 (M), 1%; 573, 4%; 498, 6%; 483, 4%; 408, 100%; 375, 7%; 357, 11%; 295, 13%; and 267, 94%.

Fractions eluted between 260 and 450% TCV (C_{27} metabolites) or between 310 and 550% TCV (C_{29} metabolites) in LC system C (see Table 1) were analyzed by TLC (system 1), GLC with radioactivity detection, and GLC–MS and were found to contain the unchanged 7α-hydroxy-Δ^4-3-oxo steroids. No attempts were made to identify C_{27} metabolites that were eluted either before 140% TCV or after 450% TCV or C_{29} metabolites eluted before 170% TCV or after 550% TCV. The yields of labeled compounds in these fractions amounted to 11–25% (C_{27}) and 8–25% (C_{29}) of the total radioactivity eluted from the column.

In quantitative work the overall recovery of ^{14}C after LC and TLC was found to be 75–90%. The conversion of 7α-hydroxy-4-cholesten-3-one to 7α,12α-dihydroxy-4-cholesten-3-one was 1.3–2.5 times that of the C_{29} derivative (**Fig. 2**). The conversion was less than 0.4% in control experiments.

Conversion of 7α-hydroxy-4-cholesten-3-one and 24α-ethyl-4-cholesten-3-one in a rat liver 100,000 *g* supernatant fraction fortified with NADPH

After purification of the crude extracts in LC system A (Table 1), the fractions obtained between 65 and 305% TCV (C_{27} metabolites) and between 70 and 355% TCV (C_{29} metabolites) (7, 13), which contained 96–99% of the radioactivity eluted from the column, were further purified in TLC system 1 (Table 1). The C_{27} and C_{29} metabolite patterns after radioautographic detection looked very much the same (**Fig. 3**). Compounds II and IV were found to be 7α-hydroxy-4-cholesten-3-one and 4,6-cholestadien-3-one, respectively, or the 24α-ethyl-substituted analogs as evidenced by GLC and GLC–MS. Control experiments revealed that 4,6-cholestadien-3-one was nonenzymatically formed.

The TLC mobilities of compounds I and III were the same as, and the TLC mobilities of C_{29} metabolites I and III were close to, those noted for 5β-cholestane-3α,7α-diol and 7α-hydroxy-5β-cholestan-3-one, respectively. Extraction of the appropriate TLC zones followed by trimethysilylation and GLC analysis with radioactivity detection yielded a single labeled peak in each instance. The retention times were those expected (Table 1) on the basis of the TLC data, the C_{29} metabolites being eluted 1.53–1.54 times later than the analogous C_{27} compounds. The mass spectra of the TMS ether derivatives of C_{27} metabolites I and III were identical with those of the TMS ether derivatives of authentic 5β-cholestane-3α,7α-diol and 7α-hydroxy-5β-cholestan-3-one, respectively. Prominent peaks were detected at *m/e* 458 (M − 90), 2%; 368, 100%; 353, 31%; 260, 41%; 255, 30%; 247, 16%; 201, 20%; and 147,

TABLE 2. Conversion of 4-^{14}C-labeled 7α-hydroxy-5β-cholestan-3-one and 24α-ethyl-7α-hydroxy-5β-cholestan-3-one to 5β-cholestane-3α,7α-diol and 24α-ethyl-5β-cholestane-3α,7α-diol, respectively, after incubations with a rat liver 100,000 *g* supernatant fraction fortified with NADPH

Substrate Added[a]	Incubation Time	Yield[b]	
		3α,7α-Diol	3-Oxo-7α-ol
	min	%	
C_{27}	4	50	22
C_{29}	4	25	49
C_{27}	20	57	18
C_{29}	20	48	27

[a] To each incubation flask containing 31 mg of protein was added 9 μg of substrate. C_{27} denotes the 5β-cholestane derivative and C_{29} denotes the 24α-ethyl-5β-cholestane derivative.

[b] 3α,7α-Diol means the 3α,7α-dihydroxy-5β-derivative and 3-oxo-7α-ol indicates the 7α-hydroxy-5β-3-oxosteroid. Each value is the mean of two experiments.

36% in the mass spectrum of C_{27} compound I and at *m/e* 474 (M), 1%; 459, 15%; 384, 100%; 369, 32%; 351, 54%; 314, 33%; 299, 9%; and 271, 42% in the mass spectrum of C_{27} compound III. The TMS ether derivatives of the corresponding C_{29} metabolites yielded the same fragmentation pattern as the C_{27} analogs with the exception that the side chain-containing fragments were found 28 mass units above the corresponding fragments in the mass spectrum of the C_{27} derivative. Prominent peaks were detected at *m/e* 486 (M −90), 3%; 396, 100%; 381, 39%; 288, 37%; 275, 16%, 255, 32%; 201, 27%; and 147, 46% in the mass spectrum of C_{29} compound I and at *m/e* 502 (M), 2%; 487, 14%; 412, 100%; 397, 27%; 379, 44%; 342, 26%; 299, 9%; and 271, 51% in the mass spectrum of C_{29} compound III.

In quantitative work the overall recovery of ^{14}C after LC and TLC was found to be 80–90% irrespective of whether a C_{27} or a C_{29} compound was used as substrate. The conversion of 7α-hydroxy-4-cholesten-3-one and 24α-ethyl-7α-hydroxy-4-cholesten-3-one to 5β-saturated steroids (7α-hydroxy-5β-3-oxosteroid and 3α,7α-dihydroxy-5β-steroid) was studied as a function of time and substrate concentration (**Fig. 4**). The reduction of the double bond, quantitated as the sum of 5β-saturated steroids, was far more effective (three to four times) for the C_{27} than for the C_{29} compound when large amounts (100 and 150 μg) of substrate were used. In control experiments the yields of 5β-saturated metabolites were less than 1%. It is apparent from the figure that when the 7α-hydroxy-5β-3-oxosteroids had been formed they were efficiently converted to the 3α,7α-dihydroxy-5β-steroids.

The reduction of the 3-oxo group was also studied with 7α-hydroxy-5β-cholestan-3-one and 24α-ethyl-7α-hydroxy-5β-cholestan-3-one as substrates (**Table 2**). The substrate concentration chosen corresponded to the amount of 24α-ethyl-7α-hydroxy-5β-cholestan-3-one formed during incubation with 24α-ethyl-7α-hydroxy-4-cholesten-3-one at substrate saturation. Control experiments showed less than 1% conversion. It is apparent that the reduction of the C_{27} compound occurs at a rate exceeding that for the reduction of the C_{29} analog. The difference, however, was less evident after 20 min of incubation.

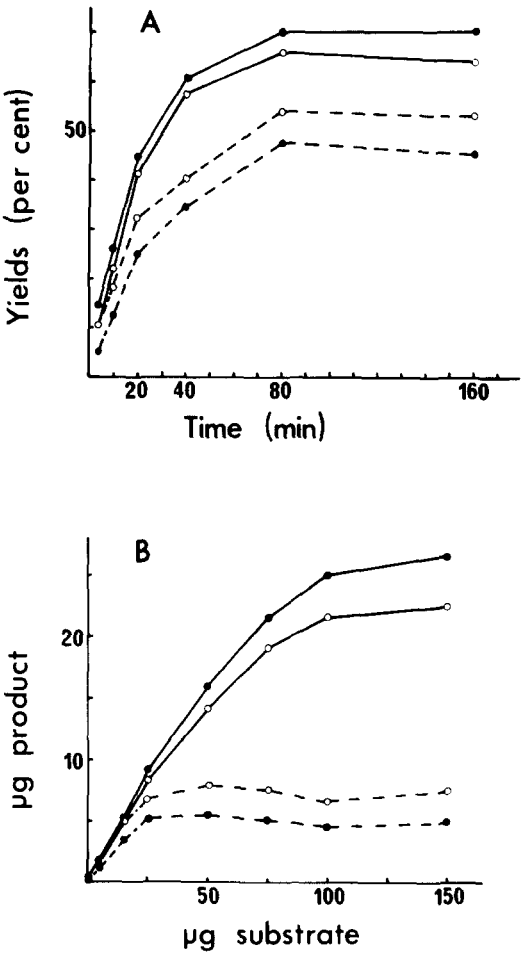

Fig. 4. Effects of time (*A*) and substrate concentration (*B*) on conversion of 7α-hydroxy-4-[4-^{14}C]cholesten-3-one and 24α-ethyl-7α-hydroxy-4-[4-^{14}C]cholesten-3-one to 5β-saturated steroids. Incubations were made with a rat liver 100,000 *g* supernatant fraction fortified with NADPH. 1 μg of substrate was added in *A*. Protein content in each incubation flask was 24 mg (*A*) and 26 mg (*B*). Each circle represents the mean of two experiments. ●—●, sum of 7α-hydroxy-5β-cholestan-3-one and 5β-cholestane-3α,7α-diol; O- - -O, sum of 24α-ethyl-7α-hydroxy-5β-cholestan-3-one and 24α-ethyl-5β-cholestane-3α,7α-diol; O—O, 5β-cholestane-3α,7α-diol; ●- - -●, 24α-ethyl-5β-cholestane-3α,7α-diol.

Conversion of 7α,12α-dihydroxy-4-cholesten-3-one and 24α-ethyl-7α,12α-dihydroxy-4-cholesten-3-one in a rat liver 100,000 *g* supernatant fraction fortified with NADPH

The reduction of 1 μg amounts of 7α,12α-dihydroxy-4-cholesten-3-one and the analogous C_{29} derivative during 20 min of incubation was studied after purification of the crude extracts in LC system C. Fractions eluted between

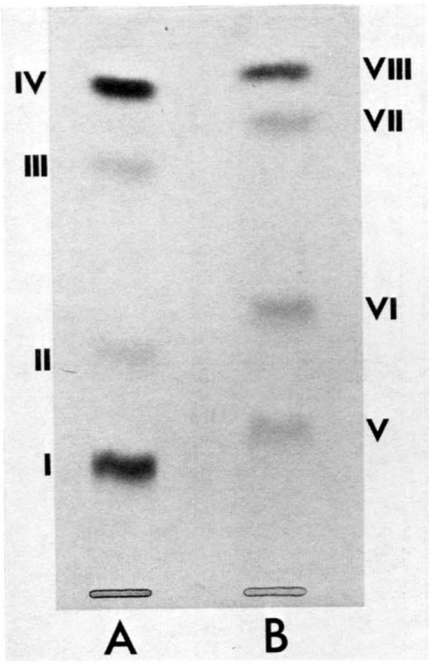

Fig. 5. Radioautographic recording after TLC analysis of metabolites formed from 4-^{14}C-labeled 7α,12α-dihydroxy-4-cholesten-3-one (*A*) and 24α-ethyl-7α,12α-dihydroxy-4-cholesten-3-one (*B*) after incubation with a rat liver 100,000 *g* supernatant fraction fortified with NADPH. TLC system 2 (see Table 1). I–III and V–VII were subsequently identified as 5β-cholestane-3α,7α,12α-triol (I), 7α,12α-dihydroxy-4-cholesten-3-one (II), 7α,12α-dihydroxy-5β-cholestan-3-one (III), 24α-ethyl-5β-cholestane-3α,7α,12α-triol (V), 24α-ethyl-7α,12α-dihydroxy-4-cholesten-3-one (VI), and 24α-ethyl-7α-hydroxy-5β-cholestan-3-one (VII). Compounds IV and VIII were suggested to have a 12α-hydroxy-$\Delta^{4,6}$-3-oxo structure.

140 and 570% TCV (C_{27} metabolites) or between 160 and 730% TCV (C_{29} metabolites), which contained 93–95% of the radioactivity eluted from the column were pooled and subjected to TLC analysis (**Fig. 5**). Subsequent extraction of the appropriate TLC zones followed by trimethysilylation, GLC analysis with radioactivity detection, and GLC–MS analysis of the C_{27} metabolites I–III (see Fig. 5) confirmed the preliminary identification of compound I as 5β-cholestane-3α,7α,12α-triol, of compound II as 7α,12α-dihydroxy-4-cholesten-3-one, and of compound III as 7α,12α-dihydroxy-5β-cholestan-3-one. The corresponding C_{29} compounds (V–VII, Fig. 5) appeared at 1.48–1.49 times the t_R of the C_{27} metabolites. GLC–MS analysis confirmed that the C_{29} metabolites were analogous to the C_{27} metabolites. Thus, the mass spectrum of the TMS ether of compound I showed prominent peaks in the high end of the spectrum at *m/e* 546 (M − 90), 2%; 456, 16%; 366, 49%; 351, 12%; 343, 16%; and 253, 100%; and the mass spectrum of the TMS ether of compound V showed prominent peaks in the high end of the spectrum at *m/e* 574 (M − 90), 1%; 484, 15%; 394, 37%; 379, 7%; 343, 22%; and 253, 100%.

Compounds IV and VIII were also formed in similar yields in control experiments. Their chromatographic behavior suggested that they were formed by the elimination of water from the respective 7α,12α-dihydroxy-Δ^4-3-oxosteroid. GLC–MS analysis (t_R = 3.26 and 4.99 for the C_{27} and the C_{29} steroid, respectively) yielded tentative molecular ions at *m/e* 380 (C_{27}) and 408 (C_{29}). These ions probably arise by loss of trimethylsilanol from a 12α-hydroxy-$\Delta^{4,6}$-3-oxo structure. The base peaks seen in both cases were recorded at *m/e* 267, i.e., loss of trimethylsilanol plus the side chain. No further attempts were made to identify these compounds.

In quantitative work the overall recovery of ^{14}C after LC and TLC was found to be 75–80%. 1 μg of 7α,12α-dihydroxy-4-[4-^{14}C]cholesten-3-one yielded the 7α,12α-dihydroxy-5β-3-oxo derivative in 8% yield and the 3α,7α,12α-trihydroxy-5β-derivative in 26% yield. The corresponding figures for the C_{29} metabolites were 14 and 15%, respectively. Control experiments showed less than 1% conversion into each metabolite.

DISCUSSION

The identification of the metabolites formed from 7α-hydroxy-β-sitosterol have in part been based on comparisons with chromatographic data obtained with synthetic C_{27} steroids (Table 1). In every case where a comparison of synthetic C_{27} sterol and C_{29} sterol derivatives substituted in the steroid nucleus has been made, the t_R values have shown a reasonably constant ratio for analogous C_{27} and C_{29} compounds, and the R_f values on TLC have been almost the same (Table 1 and Refs. 7 and 13). This seems not to be true for 12α-substituted derivatives, in which case increased R_f values and slightly decreased t_R values were obtained for the C_{29} metabolites. The identifications made, however, rely primarily on GLC–MS data in combination with GLC with radioactivity detection. The mass spectrometric fragmentation patterns for C_{27} and C_{29} steroids substituted in the steroid nucleus have never differed by more than the day-to-day variations obtained for a C_{27} derivative.

It is apparent from the data presented in this paper that 7α-hydroxy-cholesterol and 7α-hydroxy-β-sitosterol undergo the same transformations in those rat liver subcellular fractions that convert 7α-hydroxysteroids into 3α,7α-dihydroxy-5β- and 3α,7α,12α-trihydroxy-5β-steroids. The C_{27} compound is, however, in all instances more or less the preferred substrate.

The conversion of 24α-ethyl-7α-hydroxy-4-cholesten-3-one to 24α-ethyl-7α-hydroxy-5β-cholestan-3-one was markedly less (one-third to one-fourth as efficient) than that found with the C_{27} substrate when large amounts of

substrate were added, indicating that the reduction of the double bond was hindered by the 24α-ethyl substituent in the side chain. Studies on the reduction of 7α-hydroxy-5β-cholestan-3-one and the 24α-ethyl-substituted analog in amounts that corresponded to the highest levels ever formed during incubations with 24α-ethyl-7α-hydroxy-4-cholesten-3-one for 20 min showed that the ratio between 24α-ethyl-5β-cholestane-3α,7α-diol and the C_{27} analog was 0.8. This indicates that the comparatively limited formation of 24α-ethyl-5β-cholestane-3α,7α-diol (Fig. 4 *B*) from 24α-ethyl-7α-hydroxy-4-cholesten-3-one is mainly explained by a rather strict substrate structure requirement of the Δ^4-3-oxosteroid 5β-reductase, as also suggested by Berséus (4).

The microsomal 12α-hydroxylation, previously shown to be influenced by the number and positions of substituents in the A and B rings of the steroid substrate (14), was obviously hindered when 24α-ethyl-7α-hydroxy-4-cholesten-3-one was used as a substrate. This hindrance as well as those noted for the other C_{29} metabolites studied in this investigation is, however, much less spectacular than with the microsomal 7α-hydroxylase (7), which emphasizes the specificity of the cholesterol 7α-hydroxylase in bile acid biosynthesis.

In the rat liver, 7α-hydroxy-β-sitosterol is formed only by nonspecific tissue autoxidation (7, 13). It is clear from the present study that the conversion of this compound in vitro is comparatively hindered. The net conversion of β-sitosterol into bile acids by the same route as cholesterol must therefore be considered comparably limited.

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