Studies on the formation of C_7-oxygenated cholesterol and β-sitosterol metabolites in cell-free preparations of rat liver

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Abstract The microsomal fraction and the 18,000 *g* supernatant fluid obtained from livers from normal rats, cholestyramine-treated rats, or from rats with a bile fistula have been used to compare the 7α-hydroxylation of [4-^{14}C]cholesterol and β-[4-^{14}C]sitosterol (24α-ethyl-cholesterol). It was not possible to increase the specific formation of 7α-hydroxy-β-sitosterol above 0.05% with any of the preparations. This conversion was less than 1% of that found for cholesterol. The inhibitory effect of added 7-oxo- and 7β-hydroxy-β-sitosterol on the 7α-hydroxylation of cholesterol was found to be much less than that of the corresponding cholesterol compounds. 7α-Hydroxy-β-sitosterol was without effect. It is concluded that the activity of the cholesterol 7α-hydroxylase is dependent upon the structure of the steroid side chain.

Supplementary key words 7α-hydroxylation · cholestyramine · diurnal variations · bile fistula · phenobarbital · inhibition by C_7-oxygenated sterols · interconversion of 7β-hydroxy- and 7-oxosterols · epoxides · hydroxyalkylated Sephadex LH-20 · thin-layer chromatography · gas–liquid chromatography–mass spectrometry

In the presence of NADPH and oxygen the liver microsomal fractions from different species have the ability to 7α-hydroxylate different steroids, including their glucuronide and sulfate derivatives (1). The enzyme(s) that hydroxylates steroid hormones appears to differ from the enzyme that uses cholesterol as a substrate, although similarities exist (2).

The 7α-hydroxylation of cholesterol is considered to be the rate-limiting step in bile acid biosynthesis (3). However, the 3β-hydroxy-Δ^5-structure does not seem to be a prerequisite for this hydroxylation. Thus, cholestanol is converted efficiently into its 7α-hydroxy derivative by rat liver microsomes (4). Furthermore, Bell et al. (5) found that coprostanol is metabolized in high yields to normal bile acids in the rat, indicating that coprostanol can be 7α-hydroxylated in this animal.

Results obtained from other studies in vivo seem to indicate that the 7α-hydroxylating systems in the liver do not discriminate against sterols with a side-chain structure different from that of cholesterol. Thus, Subbiah (6) has reported preliminary data that show that ^{14}C-labeled hydroxylated 24α-ethyl substituted coprostanoic acids are present in the bile from rats given β-[4-^{14}C]sitosterol (24α-ethyl-cholesterol). Salen, Ahrens, and Grundy (7) have made the observation that radioactive normal 5β-cholanoic acids can be isolated from patients given ^{3}H-labeled β-sitosterol in yields similar to those obtained when [4-^{14}C]cholesterol is used as a precursor.

To obtain more direct data on the structural requirements of the 7α-hydroxylating enzyme system in bile acid biosynthesis, we have compared the metabolism of cholesterol and β-sitosterol in cell-free preparations of rat livers. Our interest in the metabolic fate of β-sitosterol has also been stimulated by the recommended use of this substance in human sterol balance studies (8).

MATERIALS AND METHODS

Solvents

Analytical grade solvents and reagents were used; they were purchased from E. Merck A.G., Darmstadt, West Germany unless otherwise stated. They were used without further purification except for dioxane and diethyl ether. Dioxane was purified by filtering it through Al_2O_3

Abbreviations: Systematic names of the sterols referred to in this text by trivial names are as follows: cholesterol, 5-cholesten-3β-ol; β-sitosterol, 24α-ethyl-5-cholesten-3β-ol; stigmasterol, 24α-ethyl-5,22-cholestadien-3β-ol; campesterol, 24α-methyl-5-cholesten-3β-ol; cholestanol, 5α-cholestan-3β-ol; β-sitostanol, 24α-ethyl-5α-cholestan-3β-ol; coprostanol, 5β-cholestan-3β-ol. Compounds referred to as α- or β-epoxides denote 5α,6α-epoxycholestan-3β-ol and 5β,6β-epoxycholestan-3β-ol, respectively, or the corresponding derivatives of 24α-ethylcholestan derivatives. Compounds referred to as C_{27} or C_{29} compounds denote derivatives of cholesterol or β-sitosterol, respectively. TMS ether, trimethylsilyl ether; HA-Sephadex LH-20, hydroxyalkylated Sephadex LH-20; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry.

(Woelm, Eschwege, West Germany), activity grade I, and it was stored over Al_2O_3. Diethyl ether was distilled over $LiAlH_4$ immediately before use.

Substrates

[4-^{14}C]Cholesterol (specific radioactivity, 55–61 mCi/mmole) and 24α-ethyl-4-[4-^{14}C]cholesten-3-one (specific radioactivity, 58 mCi/mmole) were purchased from the Radiochemical Centre, Amersham, England. [4-^{14}C]Cholesterol, 50 μCi, was acetylated and the ester was purified on a column containing 3 g of Adsorbosil, 100–140 mesh, coated with 25% $AgNO_3$ (Applied Science Laboratories, State College, Pa.). Continuous elution with benzene–heptane 4:96 (v/v) gave a major peak of material (after about 200 ml) that was further purified by preparative TLC on $AgNO_3$-impregnated silica gel (see below). After mild saponification (9) of the cholesteryl acetate fraction, the resulting sterol was purified by reversed-phase chromatography on a 50-ml column of HA-Sephadex LH-20 (see below).

β-[4-^{14}C]Sitosterol was prepared from 4-^{14}C-labeled 24α-ethyl-4-cholesten-3-one (100 μCi) by sodium borohydride reduction of the dienol trimethylsilyl ether derivative followed by preparative TLC as described previously (10). The isolated β-[4-^{14}C]sitosterol was acetylated and carried through the same analytical procedure as described above for [4-^{14}C]cholesteryl acetate.

Unlabeled cholesterol (ADA, Stockholm, Sweden) and β-sitosterol (Merck) were purified in the same way as the labeled sterols. HA-Sephadex LH-20 chromatography made possible the removal of contaminating campesterol and stigmasterol from β-sitosterol (11, 12).

Labeled and unlabeled C_7-oxygenated derivatives of cholesterol and β-sitosterol were prepared according to standard methods. The 7α-hydroxy and 7β-hydroxy derivatives were synthesized by oxidation with tertiary butylperbenzoate (Fluka A. G., Buchs, Switzerland) of the respective steryl acetates as described by Starka (13). After saponification as described above, purification was accomplished by chromatography on HA-Sephadex LH-20 followed by preparative TLC (see below). The 7-oxo compounds were prepared by the procedure of Atwater (14), and the products were purified on HA-Sephadex LH-20 after saponification as described above for the steryl acetates.

Hydrogenation of 2–5 μCi of [4-^{14}C]cholesteryl or β-[4-^{14}C]sitosteryl acetate with PtO_2 as catalyst afforded the respective stanyl acetates, which were purified as described above for the steryl acetates.

5α,6α-Epoxycholestan-3β-ol (Steraloids Inc., Pawling, N.Y.) was purified by chromatography on HA-Sephadex LH-20 and then by preparative TLC. Cholestane-3β,5α,6β-triol, 5-cholestene-3β,7α,12α-triol, and 5β-cholestane-3α,7α,12α-triol were a gift of Dr. H. Danielsson, Department of Chemistry, Karolinska Institutet.

All compounds were characterized by GLC–MS as described below. Radioactive compounds were analyzed by GLC combined with radioactivity detection (Barber-Colman monitoring system, model 5190) and by radioautography of thin-layer chromatoplates. By these techniques, impurities at a level of 0.1% can usually be detected.

Each substrate was chromatographed on a HA-Sephadex LH-20 column the day before the experiment to eliminate any decomposition products.

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. 0.25 M sucrose solutions were made 0.01 M with respect to β-mercaptoethylamine (15) and 0.001 M with respect to EDTA. The pH was kept at 7.0. The phosphate buffer was 0.1 M with a pH of 7.0 and was made 28 mM with regard to nicotinamide and 0.01 M with respect to β-mercaptoethylamine.

A modified Bucher medium with a pH of 7.4 was also used. It was prepared by mixing 10 ml of a 1 M solution of KH_2PO_4, 36 ml of a 1 M solution of $K_2HPO_4 \cdot 3H_2O$, 13.8 ml of a 1 M solution of nicotinamide, and 1.8 ml of a 1 M solution of $MgCl_2 \cdot 6H_2O$ and diluting the solution to a total volume of 500 ml.

Column chromatography

Hydroxyalkylated (55% substituted) Sephadex LH-20 (HA-Sephadex LH-20), 140–170 mesh, was synthesized with a mixture of C_{11}–C_{14} epoxides by the method described by Ellingboe, Nyström, and Sjövall (16). This support was used for either reversed-phase (methanol–water–dichloroethane 95:5:25 [v/v/v]) or straight-phase chromatography (heptane–chloroform 4:1 [v/v]). The temperature during the analysis was 22–24°C. The temperature dependence was most marked in straight-phase chromatography, which was carried out on columns containing 4 ml of freshly prepared solvent-equilibrated HA-Sephadex LH-20. A total of 25 fractions of 1.0 ml were collected. To elute material that might have been retained on the column, 10 ml of a 1:1 (v/v) mixture of acetone–diethyl ether was used. Straight-phase chromatography on HA-Sephadex LH-20 permitted a separation of all the compounds mentioned under Substrates except for the 7α- and 7β-hydroxy derivatives of both cholesterol and β-sitosterol for which only a partial separation was accomplished. The separation factor for the analogous C_{27} and C_{29} derivatives was approximately 1.1.

In reversed-phase chromatography, columns containing 50 ml of HA-Sephadex LH-20 were used for the initial purification of labeled and unlabeled reference sterol de-

rivatives as well as for the characterization of unknown metabolites. Fractions corresponding to 4–8% of the total bed volume were collected. Small columns (10 ml) were used for the purification of labeled substrates on the day before incubation and for the purification of crude extracts of homogenates. To elute material that might have been retained on the column, 10 ml of a 1:1 (v/v) mixture of acetone–diethyl ether was used.

Thin-layer chromatography

Ordinary 20 × 20 cm plates were used; they were coated either with a silica gel G (Merck) suspension or a suspension of 30 g of silica gel G, 5 g of $AgNO_3$, and 64 ml of concentrated ammonium hydroxide solution (17). The latter plates were developed in benzene–heptane 3:5 (v/v) for the purification of steryl acetates. For C_7-oxygenated cholesterol and β-sitosterol derivatives, the plates were developed in diethyl ether–cyclohexane 9:1 (v/v) (18). For more polar sterols, ethyl acetate was used. Separated compounds were detected by iodine vapor or by radioautography (see below). Compounds localized by nondestructive detection methods were extracted from the gel with two successive portions of 5 ml of chloroform–methanol 4:1 (v/v)/per cm^2 of gel. For analytical and identification purposes, the plates were sprayed with a solution of 70% H_2SO_4 saturated with $K_2Cr_2O_3$ and then heated at 120°C.

Gas–liquid chromatography

A Pye gas chromatograph (model 104) equipped with a hydrogen flame ionization detector was used. The columns (2 m × 4 mm) contained silanized Chromosorb W, 100–120 mesh, coated with either 1.5% SE-30 or 3% QF-1 (Supelco, Inc., Bellefonte, Pa.). The carrier gas was nitrogen. The temperatures were: flash heater, 295°C; and column oven, 245°C (SE-30) or 240°C (QF-1). All compounds were analyzed as their trimethylsilyl ether (TMS) derivatives. The derivatives were redissolved in 100–500 μl of heptane, and 4 μl was injected into the column. In quantitative work 4 μl of standard compound solutions was always injected to check linearity of the detector response. Retention times are given relative to 5α-cholestane (t_R).

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; inlet system, 270°C; ion source, 290°C; energy of bombarding electrons, 22.5 eV.

Measurement of radioactivity

Either a Frieseke-Hoepfner (Erlangen-Bruch, West Germany) gas flow counter FHT 90 B or a Packard model 2009 liquid scintillation spectrometer was used. Radioactive spots on thin-layer chromatograms were localized by exposing a sheet of Agfa Gevaert Structurix D7 DW for 1–7 days. Films were developed with Agfa Gevaert G-150 and fixed by Agfa Gevaert G-334.

EXPERIMENTAL PROCEDURES

In vitro studies of the 7α-hydroxylation of cholesterol and β-sitosterol

Male Sprague-Dawley rats weighing about 150 g were purchased from a local supplier. Three or four animals were used in each experiment. Two types of cell-free preparations of rat liver were used: (*a*) the 18,000 *g*[1] supernatant fraction and (*b*) the 100,000 *g* sediment.

Supernatant fraction. Unless otherwise stated the rats were fed a diet containing 100 g of pulverized pellet food (Anticimex, Stockholm, Sweden), 50 ml of corn oil (ADA, Stockholm) 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 and weighed 175–180 g. They were killed by a blow to the head at 7 p.m. The livers were excised, cut in pieces, and rinsed free from blood by dipping them into the homogenizing medium. To 10 g of wet liver was added 40 ml of the β-mercaptoethylamine-containing sucrose solution. The mixture was homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle (the diameter difference between the inner glass wall and that of the pestle was 0.15 mm). The pooled homogenates were centrifuged at 800 *g* for 10 min. The supernatant solutions obtained were centrifuged at 18,000 *g* for 15 min, and the resulting supernatant solutions were pooled and immediately used for the incubations. To 4.25 ml of the phosphate buffer containing β-mercaptoethylamine was added 4.25 ml of the 18,000 *g* supernatant. All steps were carried out at 4°C. The incubation procedure was as follows:

1. The homogenate was transferred from an ice bath to a 37°C water bath and kept there for 2 min with shaking.
2. The incubation vessel was vibrated (on a Super-Mixer) and a stream of nitrogen was continuously blown into the vessel. After 20 sec the substrate (5–10 μg of either [4-^{14}C]cholesterol or β-[4-^{14}C]sitosterol) dissolved in 100 μl of acetone was added and the vibrating was continued for an additional 2 min.
3. The vessel was placed in the water bath, and 10 μmoles of NADPH dissolved in 250 μl of the phosphate buffer was added. Shaking was initiated and a stream of air was blown into the vessel for 30 sec. The incubation was then continued for an additional 30 min.

In experiments with presumed inhibitors of the 7α-hydroxylating system, the inhibitor was added either with or before the labeled substrate. In the former case, the compounds were added in a volume of 300 μl of acetone. In

[1] The centrifugal force, *g*, was calculated in the center of the solution.

TABLE 1. Purification by reversed-phase chromatography on hydroxyalkylated Sephadex LH-20 of extracts after incubations with [4-^{14}C]cholesterol or β-[4-^{14}C]sitosterol

[4-^{14}C]Cholesterol (6)			β-[4-^{14}C]Sitosterol (5)[a]		
Fractions Collected	Radioactivity Recovered[b]	Recovery in Pooled Fractions[b]	Fractions Collected	Radioactivity Recovered[b]	Recovery in Pooled Fractions[b]
% TCV[c]	%	%	% TCV[c]	%	%
0–55	0.6	Pool A	0–60	0.3	Pool A
55–60	0.0	79	60–65	0.0	83
60–65	0.1		65–70	0.0	
65–180	4.5	Pool B	70–200	1.4	Pool B
180–230	4.8	6	200–250	1.9	2
230–280	51.6		250–300	36.7	
280–330	31.7	Pool C	300–350	46.8	Pool C
330–380	1.7	2	350–400	5.7	2
380–430	0.2		400–450	0.2	
430–530[d]	3.2	Pool D	450–500[d]	5.5	Pool D
		5			5
Unaccounted for[e]	1.6	8	Unaccounted for[e]	1.5	8

[a] Number of experiments carried out with the 18,000 *g* supernatant fluid of rat liver homogenate.
[b] Calculated from the amount present in the lipid extract.
[c] Solvent, methanol–water–dichloroethane 95:5:25 (v/v/v); TCV, total column volume.
[d] Solvent, acetone–diethyl ether 1:1 (v/v).
[e] Mainly material not soluble in the mobile phase.

the latter case, the inhibitor was added in 200 μl of acetone according to procedures 1 and 2 above. After 2 min at 37°C, procedures 2 and 3 were repeated for the addition of [4-^{14}C]cholesterol.

100,000 g sediment. Unless otherwise stated the rats used for these experiments were not manipulated prior to the day of the experiment. In some experiments the rats were given daily intraperitoneal injections of 1.0 ml of saline containing phenobarbital (100 mg/kg); this treatment lasted for 6 days. In other rats, bile was drained through a bile fistula. After the operation the animals were given free access to food and water, and they were killed after 3–6 days. 20% homogenates of livers were prepared as described above with the exception that β-mercaptoethylamine was omitted from the sucrose solution. The 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 17 ml of phosphate buffer not containing β-mercaptoethylamine and was then homogenized with the Teflon pestle. 10 ml of this homogenate was used for each incubation. The incubation procedure was that described using the 18,000 *g* supernatant.

In vitro studies on the conversion of 7β-hydroxycholesterol and 7β-hydroxy-β-sitosterol into their 7-oxo derivatives

Three 200-g male rats of the Sprague-Dawley strain were used in each experiment; the animals did not receive any special treatment. The microsomal fraction was isolated as described above, with the exception that both the homogenization and the incubation were carried out in the modified Bucher medium. Incubations were carried out as described for the 18,000 *g* supernatant but with added NADP$^+$ as coenzyme (19) (one μmole of NADP$^+$ dissolved in 250 μl of the Bucher medium).

In vitro studies on the conversion of 7-oxocholesterol and 7-oxo-β-sitosterol into the 7β-hydroxy derivatives

Three 200-g male rats of the Sprague-Dawley strain (no pretreatment) were used in each experiment. 20% homogenates of livers in the Bucher medium were prepared and then centrifuged at 800 *g* for 10 min. The supernatant solution was then centrifuged twice at 20,000 *g* for 15 min. For incubations, 6.0 ml of the 20,000 *g* supernatant was mixed with 4.0 ml of Bucher medium. The incubation procedure was that described under (*a*) for the supernatant fraction; no coenzyme was added.

Extraction and work-up procedure

Incubations were terminated by dropwise addition of the homogenate to 60 ml of chloroform–methanol 2:1 (v/v) while stirring. 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 applied to the HA-Sephadex LH-20 column as described above. The fractions obtained were pooled and subjected to preparative TLC with radioautographic detection. After elution of the me-

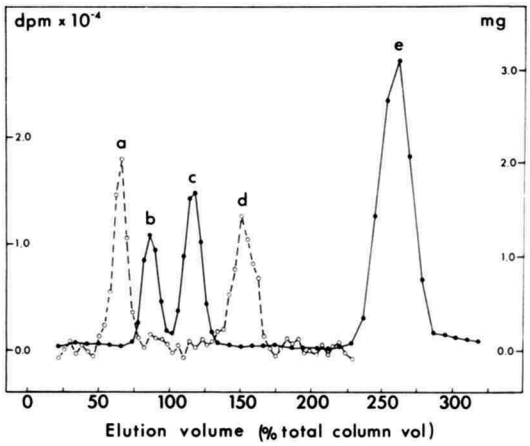

Fig. 1. Chromatography of reference steroids on a 50-ml hydroxyalkylated Sephadex LH-20 column (30 cm × 1.67 cm²) at 22°C. Solvent, methanol–water–dichloroethane 95:5:25 (v/v/v); flow rate, 0.27 ml/min/cm². Solid lines represent 4-^{14}C-labeled compounds. Broken lines represent unlabeled compounds. Reference compounds: *a*, 5β-cholestane-3α,7α,12α-triol; *b*, 7α-hydroxycholesterol; *c*, 7-oxocholesterol; *d*, 5α,6α-epoxycholestan-3β-ol; *e*, cholesterol.

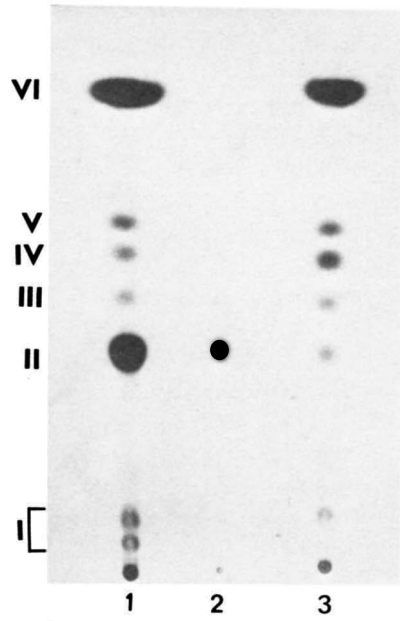

Fig. 2. Radioautographic recording of a thin-layer chromatogram of pool B metabolites (see Table 1). 1, metabolites from an incubation with [4-^{14}C]cholesterol; 2, synthetic 7α-[4-^{14}C]hydroxycholesterol; 3, metabolites from an incubation with β-[4-^{14}C]sitosterol. Compounds were subsequently identified as cholestanetriols (I), 7α-hydroxy (II), 7β-hydroxy (III), 7-oxo (IV) and the 5ξ,6ξ-epoxy (V) derivatives of cholesterol and β-sitosterol, respectively. VI is cholesterol and β-sitosterol.

tabolites, aliquots were taken for radioactivity determination. Yields were calculated on the basis of the amounts of labeled substrate added to the incubation mixture.

Control experiments

Cell-free preparations that had been heated at 80°C for 10 min were used throughout. Incubations were performed without the addition of the proper coenzyme whenever applicable. In addition, incubations were carried out in buffer solutions lacking tissue components.

RESULTS

Identification and recoveries of metabolites formed from cholesterol and β-sitosterol

The recovery of radioactive substances by the extraction procedure was 85–103% (mean 91%) in all incubations with 18,000 *g* supernates and the microsomal fractions, regardless of whether labeled cholesterol or β-sitosterol had been used as a substrate. The weight of the dried extracts was 23–35 mg. The subsequent purification on HA-Sephadex LH-20 columns afforded a nearly complete recovery of all labeled compounds (see Table 1).

The precision of the methodology, including the TLC purification, was checked by studying the overall recovery of 7α-[4-^{14}C]hydroxycholesterol added to each of eight incubation flasks containing the 18,000 *g* supernate. The amount of radioactivity used was 20–60 nCi, i.e., corresponding to a 4% conversion of cholesterol when added to the homogenate in amounts between 0.5 and 1.5 μCi. The mean recovery was found to be 86% (74–100%).

Purification of the crude extract on HA-Sephadex LH-20 in the reversed-phase type of solvent system yielded four major fractions (pools A–D, Table 1). The polarity of the compounds in the respective fractions are defined by the elution pattern shown in Fig. 1 for C_{27} steroids. The analogous C_{29} compounds are retained longer (separation factor about 1.15), which explains the difference in the pooling of fractions as shown in Table 1.

Pool B was subjected to TLC with radioautographic detection. Fig. 2 shows such an analysis carried out with pool B from parallel incubations of [4-^{14}C]cholesterol and β-[4-^{14}C]sitosterol with the 18,000 *g* supernatant fluid fortified with NADPH. As can be seen from the figure, the metabolite pattern was qualitatively the same, except for one spot close to the origin, which appeared only in extracts from incubations with cholesterol. The same results were also obtained when 4-^{14}C-labeled cholesterol or β-sitosterol was incubated with a microsomal preparation. Experiments with microsomes or the 18,000 *g* supernatant fluid from rats receiving a cholestyramine-containing diet, from rats with a bile fistula, or from rats killed in the morning or in the evening, gave results compatible with those shown in Fig. 2.

The polar compounds located close to the origin (I, Fig. 2) were extracted from the gel, and pools of such extracts were analyzed by TLC in ethyl acetate. Three major metabolites of cholesterol were then separated; one had the same mobility as cholestane-3β,5α,6β-triol and another had the mobility of 5β-cholestane-3α,7α,12α-triol. The

third compound had a mobility of 0.75 relative to that of cholestane-3β,5α,6β-triol and a mobility of 0.54 relative to that of 5β-cholestane-3α,7α,12α-triol, suggesting that it might be identical with 5-cholestene-3β,7α,12α-triol (20). The corresponding radioautographic analysis of pooled β-sitosterol metabolites revealed only one major compound that had a mobility equal to that of cholestane-3β,5α,6β-triol. No further experiments were done to identify the polar metabolites.

Spots corresponding to positions II–V in Fig. 2 were compounds with mobilities equivalent to those of 7α-hydroxy-, 7β-hydroxy-, and 7-oxocholesterol, and 5α,6α-epoxycholestan-3β-ol, respectively. After extraction of the appropriate TLC zones, individual pools of compounds II–V that had been obtained from incubations with cholesterol were converted into TMS ether derivatives and analyzed by GLC–MS. Mass spectra of 7α- and 7β-hydroxycholesterol TMS ethers (t_R = 2.05 and 2.73, respectively, on the SE-30 column; t_R = 1.65 and 2.18, respectively, on the QF-1 column) were identical with those of metabolites II and III and were characterized by the pronounced base peak at *m/e* 456. Except for the molecular ion at *m/e* 546 (3% relative intensity), no other marked ions were seen in the high mass ends of the spectra. The mass spectra of the 7-oxocholesterol TMS ether and of metabolite IV (t_R = 3.72 on the SE-30 column; t_R = 9.15 on the QF-1 column) were identical with those published by Brooks, Horning, and Young (21). The mass spectrum of the TMS ether of the epoxy compound(s) (t_R = 2.67 on the SE-30 column) showed the same fragmentation pattern as that of the TMS ether of the synthetic 5α,6α-epoxycholestan-3β-ol with characteristic peaks at *m/e* 474, 459, 456, 445, 384, 369, 366, 356, 351, 341, and 329 in the high mass end of the spectrum and with a base peak at *m/e* 96. A similar mass spectrum was obtained by Gray, Lawry, and Brooks (22) using an ionizing voltage of 70 eV.

To obtain enough material to carry out the identifications of β-sitosterol metabolites II–V, 10 incubations each with 1–2 mg of β-sitosterol added to 50 ml of the 18,000 *g* supernatant were performed. Subsequent work-up and purification yielded fractions that were subjected to GLC–MS analysis after trimethylsilylation. β-Sitosterol metabolites II–IV could thus be identified as the 7α- and 7β-hydroxy- and the 7-oxo derivatives (t_R = 3.09, 4.16, and 5.72, respectively, on the SE-30 column; t_R = 2.48, 3.29, and 14.1, respectively, on the QF-1 column) by comparison with synthetic compounds. The mass spectra showed side chain-containing fragments as peaks 28 mass units above those recorded for the corresponding cholesterol derivatives. The fragmentation pattern noted in the mass spectrum of the TMS ether of β-sitosterol metabolite V (t_R = 4.17 on the SE-30 column) differed slightly from that of the TMS ether of 5α,6α-epoxycholestan-3β-ol, suggesting that it might have been a mixture of α- and β-epoxides. The homogeneity of metabolites II–V of cholesterol and β-sitosterol was further checked by GLC with combined mass and radioactivity detection. All metabolites gave rise to a single radioactive peak and appeared pure by these criteria. To exclude the presence of 26-hydroxycholesterol or 26-hydroxy-β-sitosterol in the pools of epoxycholesterol and epoxy-β-sitosterol, respectively, these pools were chromatographed on an HA-Sephadex LH-20 column. 26-Hydroxycholesterol is eluted well ahead of 5α,6α-epoxycholestan-3β-ol in the solvent system used in the analysis shown in Fig. 1. Only minute amounts of a compound with chromatographic properties of 26-hydroxycholesterol and 26-hydroxy-β-sitosterol, respectively, could be traced.

TLC and GLC analyses of pool C and of compounds retained on the HA-Sephadex LH-20 column after elution with 4.3–4.5 total bed volumes (see Table 1) of mobile phase showed the presence of unchanged sterols and small amounts of less polar compounds, possibly ester derivatives. These were not further characterized.

Effect of diurnal variation, cholestyramine, and phenobarbital treatment on sterol conversion

In agreement with the findings made by other investigators, we found that the in vitro 7α-hydroxylation of cholesterol in the rat varied between day and night (23, 24); it was increased by dietary cholestyramine treatment (4) and was virtually unaffected by phenobarbital treatment (25). Efficiency of conversion was increased in bile fistula rats (3).

Microsomes from rats not pretreated and killed in the morning had a 0.5–1.0% conversion; those from rats killed in the evening had a 1.0–2.0% conversion; those from animals pretreated with cholestyramine and killed in the evening had a 1.5–4.5% conversion, and those from bile fistula rats had a 4.0–8.0% conversion. Parallel incubations carried out with β-[4-^{14}C]sitosterol showed that the formation of 7α-hydroxy-β-sitosterol did not change with the condition of the rats. Representative values for the yields of cholesterol and β-sitosterol metabolites in the 18,000 *g* supernatant fluid from livers of rats pretreated with cholestyramine and killed at 7 p.m. are shown in Table 2. The pattern of metabolites formed from β-sitosterol was similar to that caused by autoxidation.

Evaluation of the autoxidation of β-sitosterol

When EDTA was omitted from the 18,000 *g* supernatant (2) the yields of all β-sitosterol metabolites were increased 2–5 times and it was not possible to decide whether or not the small amounts of 7α-hydroxy-β-sitosterol formed during the standard incubation procedure could have been formed through the action of a specific 7α-hydroxylase or by autoxidation.

TABLE 2. Yields of metabolites from incubation of 18,000 *g* supernatant fractions with [4-^{14}C]cholesterol and β-[4-^{14}C]sitosterol in the presence of NADPH

[4-^{14}C]Cholesterol (5)[a]			β-[4-^{14}C]Sitosterol (5)[a]		
	Percentage Conversion			Percentage Conversion	
Metabolite Formed	18,000 *g* Supernate	Buffer Control	Metabolite Formed	18,000 *g* Supernate	Buffer Control
7α-Hydroxycholesterol	3.0[b] (1.9–4.4)[c]	0.1 (0.1–0.2)	7α-Hydroxy-β-sitosterol	0.1 (0.1–0.2)	0.1 (0.1–0.2)
7β-Hydroxycholesterol	0.2 (0.1–0.4)	0.1 (0.1–0.2)	7β-Hydroxy-β-sitosterol	0.2 (0.1–0.2)	0.1 (0.1–0.2)
7-Oxocholesterol	0.3 (0.1–0.5)	0.2 (0.1–0.3)	7-Oxo-β-sitosterol	0.4 (0.1–0.8)	0.1 (0.1–0.3)
5ξ,6ξ-Epoxycholestan-3β-ol	0.2 (0.1–0.2)	0.3 (0.2–0.4)	24α-Ethyl-5ξ,6ξ-epoxycholestan-3β-ol	0.3 (0.1–0.6)	0.3 (0.1–0.4)

[a] Number of experiments.
[b] Seven experiments.
[c] Figures within parentheses denote the range.

Since cholestanol is not readily autoxidized, we compared the degree of 7α-hydroxylation of cholestanol and β-sitostanol in a 18,000 *g* supernatant fluid. Whereas a compound with the TLC mobility of 7α-hydroxycholestanol (4) was formed in yields of 4–5%, only a 0.02–0.05% yield of the tentatively identified 7α-hydroxy-β-sitostanol was found in two experiments.

Effect of added sterols on 7α-hydroxylation of cholesterol and β-sitosterol

Feeding the animals a diet containing 2.5 g of β-sitosterol, 60 g of triolein, and 100 g of pulverized pellets for 10 days to increase the content of liver β-sitosterol (26) did not improve significantly the 7α-hydroxylation of β-sitosterol in vitro.

Incubation with increasing amounts of β-sitosterol did not increase the yields of 7α-hydroxy-β-sitosterol. To compare the distribution of cholesterol and β-sitosterol after incubation with the 18,000 *g* supernate, the reaction mixture was separated into a microsomal pellet and a 100,000 *g* supernatant fluid. Subsequent GLC analysis showed that β-sitosterol, added in trace amounts or in amounts comparable with those of endogenous cholesterol present in the 18,000 *g* supernatant fluid, was associated with the microsomes to the same extent (75%) as different amounts of added [4-^{14}C]cholesterol and endogenous cholesterol. When the same experiment was carried out without tissue components, it was found that as much as 20–55% of the labeled sterols could not be transferred from the incubation flask into the centrifugation tube. The corresponding figures in experiments with the 18,000 *g* supernatant fluid were 0–2%.

The addition of cholesterol or β-sitosterol prior to the addition of labeled cholesterol did not change the yields of 7α-hydroxycholesterol nor did it change the proportion of labeled sterol present in the microsomes after incubation and subsequent centrifugation.

Simultaneous addition of unlabeled sterol and [4-^{14}C]cholesterol to the 18,000 *g* homogenate led to the same distribution of ^{14}C between the microsomes and the 100,000 *g* supernatant fluid as in the previous experiments. The yields of 7α-hydroxycholesterol decreased from 4.5% (no unlabeled sterol added) to 3.0% (50 μg of cholesterol added) and 2.3% (50 μg of β-sitosterol added), respectively.

To study the specificity of the product inhibition of the cholesterol 7α-hydroxylase, the 18,000 *g* supernatant fluid was incubated with [4-^{14}C]cholesterol after the addition of 7α-hydroxycholesterol or 7α-hydroxy-β-sitosterol. The effect of the corresponding 7β-hydroxy and 7-oxo compounds was studied in the same way. The results are shown in Table 3. It can be seen from the table that 7α-hydroxy-β-sitosterol does not inhibit the 7α-hydroxylation of cholesterol, whereas 7β-hydroxy- and 7-oxo-β-sitosterol inhibit this reaction to the same extent as 7α-hydroxycholesterol but much less than 7β-hydroxy- and 7-oxocholesterol. The inhibiting effect of 7α-hydroxycholesterol cannot be attributed to its conversion to the 7-oxo derivative since this conversion was found to be only 3%.

Interconversion of C_7-oxygenated cholesterol and β-sitosterol derivatives

From the data presented in Table 3 it can be seen that the reduction of 7-oxo-β-sitosterol to 7β-hydroxy-β-sitosterol is not of the same magnitude as that of 7-oxocholesterol. The reverse reaction, the oxidation of the 7β-hydroxy compounds, appears to occur to the same extent with the cholesterol and β-sitosterol derivatives. To see if this oxidation was related to the microsomal system described by Björkhem, Einarsson, and Johansson (19), the 7β-hydroxysterols were incubated with microsomes fortified with NADP$^+$. The cholesterol and β-sitosterol derivatives were converted to the same extent as in the 18,000 *g* supernate, fortified with NADPH, and no discrimination against the β-sitosterol compound was noted. When the reduction of 7-oxosterols into 7β-hydroxysterols was studied in a 20,000 *g* supernate (19), the ratio between the

TABLE 3. Percentage conversion of [4-^{14}C]cholesterol to 7α-[4-^{14}C]hydroxycholesterol in the presence of C_7-oxygenated sterols

Amount Added	Compound Added					
	7α-Hydroxysterol		7β-Hydroxysterol		7-Oxosterol	
	C_{27}[a]	C_{29}[b]	C_{27}	C_{29}	C_{27}	C_{29}
μg	Experiment set 1[c]		Experiment set 2		Experiment set 3	
0	2.3	2.3	4.0	4.0	4.1	4.1
12.5					2.5	
50	1.3 (3)[d]	2.9	0.7 (20)[d]	2.3 (21)[d]	0.6 (49)[e]	2.5 (38)[e]
200	0.2 (3)[d]	2.6 (2)[d]	0.2 (12)[d]	1.6 (12)[d]	0.1 (40)[e]	1.3 (32)[e]
			Experiment set 4			
0	4.4	4.4	4.4	4.4	4.4	4.4
100	2.4	4.2	0.2 (16)[d]	2.2 (15)[d]	0.3 (48)[e]	1.7 (34)[e]

[a] Denotes cholesterol derivative.
[b] Denotes β-sitosterol derivative.
[c] Each experimental set was carried out in duplicate.
[d] Percentage of added compound converted to the corresponding 7-oxo compound at the end of the incubation.
[e] Percentage of added compound converted to the corresponding 7β-hydroxy compound at the end of the incubation.

percentage conversion of the cholesterol and the β-sitosterol derivatives was found to be 1.3, i.e., the same as in the 18,000 g supernatant fluid.

DISCUSSION

The conditions chosen for the study of the substrate specificity of the rat liver cholesterol 7α-hydroxylase were essentially those described by Mitton, Scholan, and Boyd (15). In a detailed study these authors showed the importance of a brief tissue homogenization, a rapid centrifugation step, and a moderate incubation time. They also demonstrated the stimulating and stabilizing effect of adding β-mercaptoethylamine to the 18,000 g supernate and confirmed the finding of Johansson (2) that EDTA inhibits the formation of autoxidation products, presumably by an effect on microsomal lipid peroxidase. We have found that the combined addition of β-mercaptoethylamine and EDTA to the homogenizing medium limits the autoxidation of the sterol added to the incubation and improves the reproducibility of the assay of the 7α-hydroxylase.

The manner of addition of the sterol substrate or inhibitor also affects the reproducibility of the results within an experimental set. We have found that the addition of the sterol in acetone under nitrogen with concomitant vibration of the homogenate accomplishes the desired reproducibility. The use of Tween 80 for the addition of substrate in our hands did not limit autoxidation to the extent reported by Van Cantfort (27) and did not improve reproducibility.

To be able to measure conversions of sterol substrates below the 1% level, we found it necessary to include a column chromatographic step prior to TLC. Chromatography on hydroxyalkylated Sephadex LH-20 proved to be a rapid and efficient method for removing most of the contaminating lipids from the formed metabolites. The subsequent TLC analysis should be performed in diethyl ether–cyclohexane because the frequently used benzene–ethyl acetate solvent systems do not allow a separation of α- and β-epoxides of sterols from 7-oxosterols (15). These compounds are always formed to some extent during in vitro studies of 3β-hydroxy-Δ^5-steroids.

Using the above-mentioned conditions we have shown that neither β-sitosterol nor β-sitostanol is metabolized in vitro to a degree compatible with that noted for cholesterol or cholestanol. By pretreating the rats in ways that are known to specifically increase the 7α-hydroxylation of cholesterol such as cholestyramine treatment (4), preparing a bile-duct fistula (3), and utilizing the diurnal variation of the 7α-hydroxylase (23, 24), we hoped to notice an increased formation of 7α-hydroxy-β-sitosterol. This did not occur.

The metabolites formed from β-sitosterol, i.e., 7α-hydroxy-, 7β-hydroxy-, and 7-oxo-β-sitosterol as well as 5ξ,6ξ-epoxy-β-sitosterol and, tentatively, 24α-ethylcholestane-3β,5α,6β-triol, and their relative amounts were those suspected to be due to autoxidation in aerated aqueous solutions (28) or to a nonspecific lipid peroxidase activity (2). To avoid interference by autoxidation we compared the 7α-hydroxylation of cholestanol and β-sitostanol in stimulated animals, assuming from the data presented by Shefer, Hauser, and Mosbach (4) that the 7α-hydroxylase attacking the stanol is similar to, if not identical with, that which attacks the sterol. The results indicate that cholestanol, which (like β-sitosterol) contributes only a few percent to the endogenous sterol pool (29), is converted at least 100 times better than β-sitostanol. The lack of conversion of β-sitostanol and β-sitosterol is not likely due to a relative insolubility of the C_{29} sterols in the tissue homogenate. From our study on the distribution of added cholesterol and β-sitosterol in the tissue homogenate, it is

apparent that the two sterols behave in much the same fashion. There were similiar findings in studies on the sterol distribution in cell-free preparations of L-cell mouse fibroblasts (30). Only in the presence of a high proportion of sphingomyelin in a cellular membrane may a discrimination of β-sitosterol occur (31).

It has been reported that the specific activity of the 7α-hydroxycholesterol formed in vitro is higher than that of the total tissue cholesterol (32). This indicates that the added sterol is taken up more rapidly by the 7α-hydroxylating system than by the tissue components in general. It is therefore surprising that when we added 50–200 μg of cholesterol or β-sitosterol 2 min before the addition of [4-^{14}C]cholesterol, no effect on the formation of 7α-hydroxycholesterol was noted. The added sterols thus did not appear to saturate specific 7α-hydroxylating sites.

Adding β-sitosterol simultaneously with [4-^{14}C]cholesterol decreased the yields of 7α-hydroxycholesterol by 50%, although from the previous findings with labeled β-sitosterol it is apparent that this sterol has no affinity for the 7α-hydroxylase. The explanation then seems to be that β-sitosterol when added in amounts of 50 μg to a 10-ml homogenate coprecipitates with simultaneously added [4-^{14}C]cholesterol. It is likely that the addition of 50 μg of cholesterol decreases the formation of 7α-hydroxycholesterol for the same reason.

To avoid precipitation of [4-^{14}C]cholesterol we chose to compare the effect of C_7-oxygenated sterols on the 7α-hydroxylation of cholesterol by adding the presumed inhibitor prior to the addition of the substrate. If the effect of a preadded inhibitor nevertheless was caused by a precipitation of the substrate, one would expect compounds of similar polarity to be equally potent inhibitors. However, the slight difference in solubility between analogous cholesterol and β-sitosterol derivatives is much less than the difference found in the degree of inhibition. For instance, 7α-hydroxy-β-sitosterol had no effect under conditions where the same amounts of 7α-hydroxycholesterol caused an almost complete inhibition. Since the formation of the inhibitory 7-oxosterol was negligible with both the C_{27} and the C_{29} compounds, this does not explain the inhibitory effect of 7α-hydroxycholesterol. It is thus clear that 7α-hydroxy-β-sitosterol does not interact with the cholesterol 7α-hydroxylase. This cannot be ascribed to an inability of the compound to enter the microsomes because it has been found to be converted by a microsomal oxidoreductase to 24α-ethyl-4-cholesten-7α-ol-3-one at a rate roughly one-half of that of the corresponding cholesterol compound.[2]

The inhibitory effect of 7β-hydroxysterols cannot be explained by the conversion of these sterols to the 7-oxo compounds when the magnitude of the inhibition is related to the amounts of 7-oxosterol formed. Similarily, the inhibition brought about by the 7-oxo compounds cannot be interpreted in terms of their conversion into the 7β-hydroxysterols. Interestingly, no discrimination of 7β-hydroxy-β-sitosterol compared with 7β-hydroxycholesterol occurred upon oxidation to the 7-oxo compounds, whereas a small but persistent difference in the degree of reduction of the 7-oxo derivatives was noticed, indicating that two separate steroid C_7 oxidoreductases are present in the rat liver 18,000 *g* supernatant fluid.

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