Formation and metabolism in vitro of 5,6-epoxides of cholesterol and β-sitosterol

Leif Aringer and Peter Eneroth

Department of Chemistry, Karolinska Institutet, and the Hormone Laboratory, Department of Obstetrics and Gynecology, Karolinska Sjukhuset, S-104 01 Stockholm 60, Sweden

Abstract The formation of 5α,6α- and 5β,6β-epoxides of cholesterol and β-sitosterol in rat liver subcellular fractions has been studied. The results show that the epoxidation seems to occur only in connection with the nonspecific tissue oxidation of the sterols. The β-epoxides were formed in three- to fourfold excess over the α-epoxides. Both cholesterol epoxides were efficiently converted by a microsomal hydrolase into the 3β,5α,6β-triol. The conversion was less extensive with β-sitosterol epoxides, especially the β-epoxide. The possible biological significance in the formation of the sterol epoxides and the triols was evaluated by their ability to inhibit the microsomal cholesterol 7α-hydroxylase. Only the cholesterol epoxides and especially the β-epoxide were active in this respect.

Supplementary key words lipid peroxidation · epoxide hydrolase · cholestane-3β,5α,6β-triol · cholesterol-7α-hydroxylase · inhibition by 5,6-epoxysterols and 3β,5α,6β-trihydroxysterols · hydroxyalkylated Sephadex LH-20 · thin-layer chromatography · gas–liquid chromatography–mass spectrometry

5,6-Epoxides of cholesterol (1) and β-sitosterol (2) have been shown to be formed in rat liver homogenates. The cholesterol epoxide can be converted into the 3β,5α,6β-triol in vivo (3). This triol has been reported to lower serum cholesterol in the rabbit (4) and in the monkey (5), most likely through its inhibitory effect on the cholesterol biosynthesis (6, 7). It was therefore considered to be of interest to study the conditions governing the formation of cholesterol and β-sitosterol 5,6-epoxides, their metabolism, and their possible effects on the 7α-hydroxylation of cholesterol. The regulation of the latter reaction, which is rate limiting in bile acid formation, has also been suggested to be related to cholesterol biosynthesis (8).

MATERIALS

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 it through Al_2O_3 (Woelm, Eschwege, West Germany), activity grade I. It was stored over Al_2O_3.

Substrates

[4-^{14}C]Cholesterol (specific radioactivity, 55–61 mCi/mmole) and β-sitosterol (4-^{14}C-labeled; specific radioactivity, 61 mCi/mmole) were purchased from the Radiochemical Centre, Amersham, England. The labeled sterols were purified as previously described for [4-^{14}C]cholesterol (2). Unlabeled cholesterol (ADA, Stockholm, Sweden) and β-sitosterol (Merck) were purified in the same way as the labeled sterols.

[4-^{14}C]Cholestane-3β,5α,6β-triol and 24α-ethyl [4-^{14}C]cholestane-3β,5α,6β-triol were synthesized from [4-^{14}C]cholesterol and β-[4-^{14}C]sitosterol, which were oxidized by performic acid and hydrogen peroxide as described by Mosbach et al. (9). The respective reaction product was saponified (10) and then purified by LC system A (see Table 1).

4-^{14}C-labeled 5β,6β-epoxysterols were prepared from [4-^{14}C]cholestane-3β,5α,6β-triol or 24α-ethyl [4-^{14}C]-cholestane-3β,5α,6β-triol after conversion of the triol into the 3,6-diacetate followed by 5α-acetylation by reflux boiling in acetic acid anhydride containing *p*-toluenesulfonic acid (14). Saponification of the triacetate with KOH in dry methanol at reflux temperature for 3 hr (14) yielded the 5β,6β-epoxide. The epoxide was purified by LC

Abbreviations: Systematic names of the sterols referred to in the text by trivial names are as follows: cholesterol, 5-cholesten-3β-ol; β-sitosterol, 24α-ethyl-5-cholesten-3β-ol. Compounds referred to as α- or β-epoxides denote 5α,6α-oxidocholestan-3β-ol and 5β,6β-oxidocholestan-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; TCV, total column volume; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry; LC, liquid chromatography.

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TABLE 1. Chromatographic properties of 3,5,6-substituted sterols and their TMS ether derivatives

Sterol	LC System[b]		TLC System[c]			GLC, 3% SE-30[a] TMS Ether Analyzed[d]			GLC, 3% QF-1[a] TMS Ether Analyzed		
	A[e]	B[f]	1[g]	2[h]	3[i]	Mono-TMs	Di-TMS	Tri-TMS	Mono-TMS	Di-TMS	Tri-TMS
	% TCV		R_F			relative retention time			relative retention time		
Cholestane-3β,5α,6β-triol	76	340	0.30	0.17	0.05	5.15	3.92	2.86	7.62	4.00	2.17
24α-Ethylcholestane-3β,5α,6β-triol	84	320	0.30	0.17	0.05	8.25	6.29	4.56	11.7	6.10	3.30
Cholestane-3β,5α,6α-triol		270	0.20		0.05		4.22	3.53			
6-Oxocholestane-3β,5α-diol		270	0.69		0.31	4.86					

[a] A Pye gas chromatograph (model 104) equipped with a hydrogen flame ionization detector was used. The columns (2 m × 4 mm, SE-30, or 1.3 m × 4 mm, QF-1) contained silanized Supelcoport, 80–100 mesh, coated with either 3% SE-30 or 3% QF-1 (Supelco, Inc.). The temperatures were flash heater, 270°C, and column oven, 250°C (SE-30) 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.

[b] Liquid chromatography. The values are elution volumes as percentages of the total column volume.

[c] The figures are R_F values.

[d] 250 μl of the supernate formed from a preequilibrated mixture of 2.0 ml of pyridine, 2.5 ml of hexamethyldisilazane, and 0.5 ml of trimethylchlorosilane was added to 50 μg of the substrate. The conversion to the 3β,6α- and 3β,6β-di-TMS ether derivatives of the trihydroxysterols and the 3β-TMS ether derivative of the 6-oxosterol was completed within 3 hr. Small amounts (about 5 μg) of the 3β-TMS ether derivative of the 3β,5α,6β-trihydroxysterols could be demonstrated after 5 min. The tri-TMS ether derivatives had to be prepared with trimethylbromosilane in the preequilibrated reagent (11) instead of trimethylchlorosilane. The di-TMS ether derivatives or the parent triols were used as substrates. The conversion was almost complete (95–97%) after 72 hr.

[e] 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 (12), used in methanol–water–dichloroethane 95:5:25 (v/v/v). Column dimensions, 1.7 cm² × 50 cm; flow rate, 0.3 ml/cm²/min at 24°C.

[f] Sephadex LH-20 used in dichloromethane. Column dimensions, 1.0 cm² × 20 cm; flow rate, 0.5 ml/cm²/min at 24°C. 5β-Cholestane-3α,7α,12α-triol was eluted at 190% TCV.

[g] Ethyl acetate, silica gel G. Mobilities of 5β-cholestane-3α,7α,12α-triol and 5-cholestene-3β,7α,12α-triol were 0.46 and 0.27, respectively.

[h] Silica gel G. Plates were developed first with diethyl ether–cyclohexane 9:1 (v/v) then with ethyl acetate–diethyl ether 3:1 (v/v). The first solvent was allowed to rise 20 cm and the second 6 cm. Mobilities of 5β-cholestane-3α,7α,12α-triol and 5-cholestene-3β,7α,12α-triol were 0.19 and 0.15, respectively (relative to the first front).

[i] Diethyl ether–cyclohexane 9:1 (v/v); silica gel G (2, 13).

systems A and B and TLC system 3, followed by TLC system 4 (see **Tables 1** and **2**); after saponification, pure 5β,6β-epoxide and 5α,6α-epoxide were obtained in the proportion 40:1.

4-^{14}C-labeled 5α,6α-epoxycholestan-3β-ol and 24α-ethyl-5α,6α-epoxycholestan-3β-ol were obtained after treatment of [4-^{14}C]cholesterol and β-[4-^{14}C]sitosterol with monoperphthalic acid as described by Fieser and Rajagopalan (16) and Fieser and Fieser (17). The products were purified by LC systems A and B followed by TLC system 4 (Tables 1 and 2). The ratio of 5α,6α-epoxide to 5β,6β-epoxide obtained after saponification was 9:1.

Unlabeled 3β,5α,6β-trihydroxysterols and epoxysterols were synthesized and purified as described above for the 4-^{14}C-labeled compounds. Cholestane-3β,5α,6α-triol was obtained after oxidation of cholesterol with osmium tetroxide as described by Prelog and Tagmann (18). It was purified in TLC system 1. 6-Oxocholestane-3β,5α-diol was purchased from Steraloids Inc., Pawling, N.Y. 5β-Cholestane-3α,7α,12α-triol, 5-cholestene-3β,7α,12α-triol, and 26-hydroxycholesterol were gifts from Dr. H. Danielsson and Dr. I. Björkhem, Department of Chemistry, Karolinska Institutet. 7α-Hydroxycholesterol, 7β-hydroxycholesterol, and 7-oxocholesterol were synthesized and purified as described previously (2).

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 on 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 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 with regard to β-mercaptoethylamine were used. The pH was 7.0.

Protein determination

Protein was determined according to the method of Lowry et al. (19).

Thin-layer chromatography

Ordinary 20 × 20 cm plates were used in the systems described in Tables 1 and 2. Separated compounds were detected by iodine vapor or by radioautography (see below). Compounds were extracted from the gel with 5 ml

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TABLE 2. Chromatographic properties of sterol epoxides and their acetate and TMS ether derivatives

Sterol Epoxide	Derivative Analyzed by LC[a]	Derivative Analyzed by TLC[b]			Derivative Analyzed by GLC[c]		
	Parent Compound	Parent Compound[d]	Acetate[e]	TMS Ether[f]	Parent Compound	Acetate	TMS Ether
	% TCV		*R_F*		*relative retention time*		
5α,6α-Epoxycholestan-3β-ol	150	0.48	0.26	0.85	2.58	3.71	2.95
5β,6β-Epoxycholestan-3β-ol	140	0.50	0.35	0.67	2.46[g]	3.21[h]	2.85[h]
24α-Ethyl-5α,6α-epoxycholestan-3β-ol	170	0.48	0.27	0.85	4.14	5.95	4.73
24α-Ethyl-5β,6β-epoxycholestan-3β-ol	160	0.50	0.36	0.67	3.96[g]	5.16[h]	4.59[h]

[a] LC (liquid chromatography) system A. See Table 1, footnote *e*. Values are elution volumes as percentages of the total column volume.
[b] The figures are R_F values.
[c] 3% SE-30. Conditions as in Table 1, footnote *a*. The values are retention times calculated relative to that of 5α-cholestane.
[d] TLC system 3: diethyl ether–cyclohexane 9:1 (v/v); silica gel G (2, 13).
[e] TLC system 4: benzene–heptane 6:1 (v/v). Plates were prepared with a suspension of 30 g of silica gel G, 5 g of $AgNO_3$, and 64 ml of concentrated ammonium hydroxide solution (15). Mobilities of the acetates of 7-oxocholesterol and 26-hydroxycholesterol were 0.16 and 0.43, respectively.
[f] TLC system 5: heptane–hexamethyldisilazane 48:2 (v/v); silica gel G. Plates were prerun in pure hexamethyldisilazane and dried at 40°C for 1 hr. The trimethylsilyl ether derivatives were then applied dissolved in heptane. Mobility of the trimethylsilyl ether derivatives of 26-hydroxycholesterol was 0.91.
[g] Decomposing; figure denotes major peak seen.
[h] Slight decomposition.

of chloroform–methanol 2:1 (v/v) per cm^2 of gel followed by 5 ml of methanol per cm^2 of gel. For 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–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 1000 cpm above the background were counted. Radioactive spots on thin-layer chromatograms were localized by exposing a sheet of Agfa Gevaert Osray M3 for 1–14 days.

EXPERIMENTAL PROCEDURES

A: Studies on the formation of 5,6-epoxysterols and 3β,5α,6β-trihydroxysterols from cholesterol and β-sitosterol

Male rats of the Sprague-Dawley strain (weight 180–200 g) were fed a standard pellet food. The animals (three or four 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. To 10 g of wet liver was added 40 ml of the sucrose solution containing EDTA and β-mercaptoethylamine. 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 500 *g* for 10 min. The supernatant solution was used for the preparation of a 4500 *g* sediment and an 18,000 *g* supernatant fraction. All steps were carried out at 4°C.

4500 g sediment. The 500 *g* rat liver supernatant fraction was centrifuged at 4500 *g* for 20 min. The supernate was discarded and the sediment was resuspended in the same amount of sucrose solution as the discarded supernatant. The suspension was centrifuged twice and the sediment was resuspended each time as described above. To 4.25 ml of the phosphate buffer containing β-mercaptoethylamine was added 4.25 ml of the suspension, which contained about 3 mg of protein per ml.

18,000 g supernate. The 500 *g* rat liver supernatant fraction was centrifuged at 18,000 *g* for 15 min. The supernate was decanted and 4.25 ml was added to 4.25 ml of the phosphate buffer. In some experiments the 18,000 *g* supernatant fraction was heated to 80°C, kept at that temperature for 15–20 min, and then centrifuged at 3200 *g* for 10 min. To 4.25 ml of the phosphate buffer was added 4.25 ml of the supernate, which contained about 10 mg of protein per ml.

The homogenates prepared as described above were incubated for 30 min at 37°C with 10 μg of [4-^{14}C]cholesterol or β-[4-^{14}C]sitosterol in the presence of 10 μmoles of NADPH or 10 μmoles of NADH. In some experiments, 12 μmoles of NADP, 1 unit of isocitric dehydrogenase in

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50% glycerol (Sigma, type IV), 38 μmoles of isocitric acid, and 0.09 μmole of $MnCl_2 \cdot 4H_2O$ were added. The substrates, 5–10 μg purified in LC system A (see Tables 1 and 2) on the day before the experiment, were added in 100 μl of acetone under nitrogen with concomitant vibration of the mixture as described previously (2). In some experiments the homogenates were preincubated at 50°C for 5 min immediately before they were incubated at 37°C.

Incubations were terminated by dropwise addition of the homogenate to 60 ml of chloroform–methanol 2:1 (v/v) with 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 purified in LC system A. The fraction eluted between 60 and 200% TCV (or between 65 and 230% TCV when β-sitosterol was used as the substrate) was subjected to radioautographic detection after separation in TLC system 3. Metabolites that migrated similarly to or higher than the 7α-hydroxylated sterols (R_F 0.30) were cut out and eluted from the gel. The gel below the position of the 7α-hydroxylated sterols was left to dry and then developed in ethyl acetate–diethyl ether 3:1 (v/v) to separate trihydroxy sterols (see Table 1). These metabolites were eluted from the gel and quantified. The 5,6-epoxysterols were separated into a 5α,6α- and a 5β,6β-epoxide fraction with TLC system 5 (Table 2) and then quantified.

B: Studies on the formation of 3β,5α,6β-trihydroxysterols from 5,6-epoxides of cholesterol and β-sitosterol

A 500 *g* rat liver supernatant fraction prepared as described in A was used for the preparation of four different subcellular fractions, namely, a 4500 *g* sediment, an 18,000 *g* supernatant, a 100,000 *g* sediment, and a 100,000 *g* supernatant fraction.

4500 g *sediment and 18,000* g *supernate.* These fractions were prepared as described in A.

100,000 g *sediment.* The 500 *g* rat liver supernatant fraction was centrifuged twice at 18,000 *g* for 15 min. The 18,000 *g* supernate was 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 sucrose solution, homogenized with the Teflon pestle, and centrifuged at 100,000 *g* for another 60 min. The washed microsomal pellet was suspended in another 10 ml of sucrose solution and homogenized. To 4.25 ml of the phosphate buffer was added 4.25 ml of the homogenate, which contained about 2 mg of protein per ml.

100,000 g *supernate.* The 500 *g* rat liver supernatant fraction was centrifuged twice at 18,000 *g* for 15 min. The 18,000 *g* supernate was centrifuged twice at 100,000 *g* for 60 min. To 4.25 ml of the phosphate buffer was added 4.25 ml of the 100,000 *g* supernate, which contained about 7 mg of protein per ml.

The homogenates prepared as described above were incubated for 30 min at 37°C with 1–100 μg of 4-^{14}C-labeled 5,6-epoxysterols dissolved in 100–300 μl of acetone. The incubation procedure was that described previously (2). In some experiments, 10 μmoles of NADPH or 10 μmoles of NADH was added to the homogenate. Cell-free preparations that had been heated at 80°C for 20 min as described in A and buffer solutions lacking tissue components were used for control experiments.

The incubations were terminated and extracted as described in A. The combined chloroform phases were taken to dryness and then purified in LC system B (see Table 1). The fraction containing the 3β,5α,6β-trihydroxysterols, eluted at about 250–400% TCV, was then subjected to chromatography in TLC system 1. After radioactive detection, these metabolites were analyzed as their trimethylsilyl ether derivatives by GLC, GLC–MS, and GLC with radioactivity detection. Metabolites less polar than the 3β,5α,6β-trihydroxysterols in LC system B were analyzed in TLC system 3 and by GLC.

C: Studies on the effects of 5,6-epoxysterols and 3β,5α,6β-trihydroxysterols on the 7α-hydroxylation of cholesterol

In these experiments, 150-g male rats (three or four in each experiment) were fed a diet containing 100 g pulverized pellet food, 50 ml of corn oil, and 5 g of cholestyramine (2) 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 p.m. A rat liver 18,000 *g* supernatant fraction was prepared in the sucrose solution containing β-mercaptoethylamine and EDTA as described in A.

The homogenates were incubated with 5 μg of [4-^{14}C]cholesterol at 37°C for 30 min in the presence of 10 μmoles of NADPH. The presumed inhibitors of the 7α-hydroxylation of cholesterol were dissolved in 300 μl of acetone and added to the homogenate 2 min before the addition of the substrate. The incubation procedure was that described previously (2).

The incubations were terminated and extracted as described in A. The combined chloroform phases were taken to dryness and then purified in LC system A. The fraction eluted between 65 and 180% TCV was further purified in TLC system 1. After radioautographic detection, the metabolites were eluted and aliquots were taken for radioactivity measurement.

RESULTS

Identification of 5,6-epoxysterols and 3β,5α,6β-trihydroxysterols formed from cholesterol and β-sitosterol

The sterol epoxide fraction obtained after the initial column and TLC purification (LC system A and TLC

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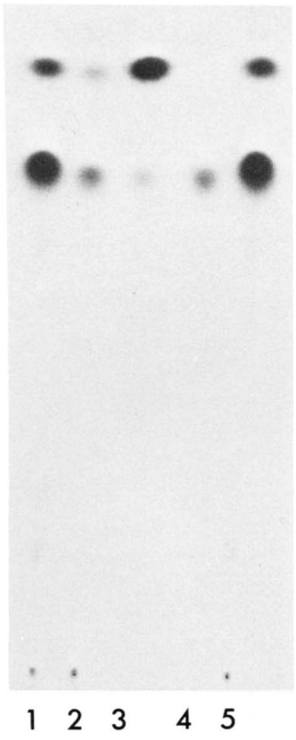

Fig. 1. TLC of TMS ethers of 5α,6α- and 5β,6β-epoxysterols on a hexamethyldisilazane-pretreated (see Table 2) silica gel G plate. Solvent system: heptane–hexamethyldisilazane 48:2 (v/v). Position 1, epoxide fraction isolated from incubations with [4-^{14}C]cholesterol and iron-supplemented rat liver microsomes in the absence of EDTA; position 2, same as in position 1 but with EDTA present during incubation; position 3, authentic 5α,6α-epoxycholestane-3β-trimethylsilyl ether; position 4, authentic 5β,6β-epoxycholestane-3β-trimethylsilyl ether; position 5, same as in position 1 except that β-[4-^{14}C]sitosterol was used as substrate.

system 3, Tables 1 and 2) could be further resolved by TLC systems 4 and 5 (see Table 2 and **Fig. 1**). The compounds with mobilities in TLC system 5 identical with those of 5α,6α- and 5β,6β-epoxides of cholesterol or β-sitosterol were analyzed by GLC and GLC–MS after extraction from the appropriate TLC zones. Retention times and mass spectral data were the same as those of the respective reference compounds. The mass spectrum of the TMS derivative of the isolated 5β,6β-epoxide of cholesterol is shown in **Fig. 2.** An almost identical spectrum was obtained with the TMS derivative of cholesterol 5α,6α-epoxide, and the same fragmentation pattern was seen with the TMS derivatives of the β-sitosterol metabolites.

To quantify the amount of epoxide formed from cholesterol and β-sitosterol under different conditions, the possible conversion of each epoxide into the 3β,5α,6β-trihydroxysterol had to be studied. The triol isolated from incubations with an 18,000 *g* supernatant or a microsomal fraction, using labeled sterols in the former case and sterol 5,6-epoxides in the latter case as substrates, showed the same chromatographic properties as those of the 3β,5α,6β-trihydroxysterol (see Table 1). The 3β,5α,6α-trihydroxysterol was not formed according to the TLC data. Stepwise trimethylsilylation of the triol formed from the 5,6-epoxysterols did not in any case reveal with subsequent GLC analysis the presence of a stereoisomer of the 3β,5α,6β-triol. Similarly, mass spectrometric scanning of the entire peak formed by each trimethylsilyl ether derivative did not indicate the presence of any other triol but the 3β,5α,6β compound. The mass spectra of the tri-TMS ether derivatives of cholestane-3β,5α,6β-triol and 24α-ethylcholestane-3β,5α,6β-triol are shown in **Fig. 3.**

Studies on the conversion of 5α,6α- and 5β,6β-epoxides of cholesterol and β-sitosterol into the corresponding 3β,5α,6β-trihydroxysterols

The hydrolysis of the 5,6-epoxides of cholesterol in different rat liver subcellular fractions was studied to deter-

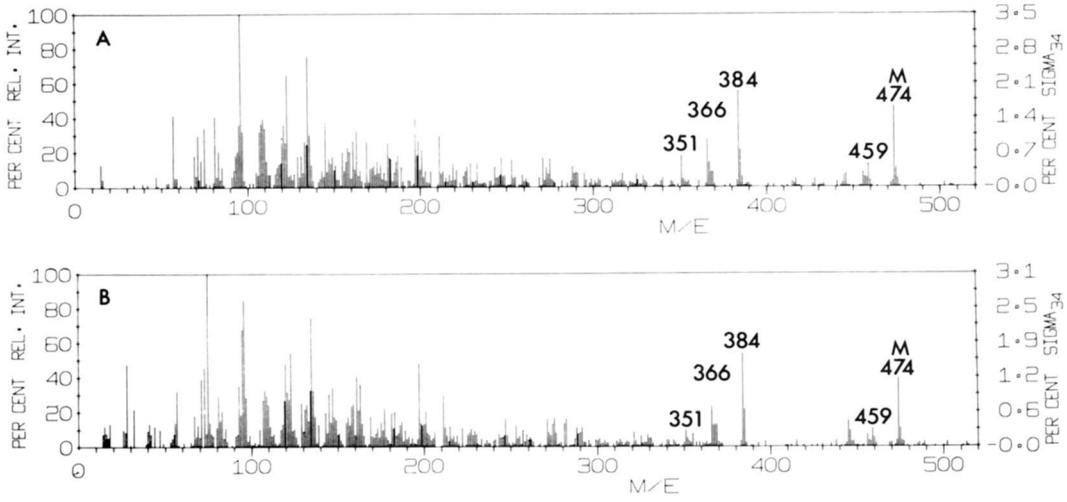

Fig. 2. *A*, GLC–MS analysis of the TMS ether derivative of 5β,6β-epoxycholestan-3β-ol isolated (see Fig. 1 and text) from an incubation of [4-^{14}C]-cholesterol with a rat liver 18,000 *g* supernatant fraction fortified with NADPH. *B*, GLC–MS analysis of the TMS ether derivative of authentic 5β,6β-epoxycholestan-3β-ol.

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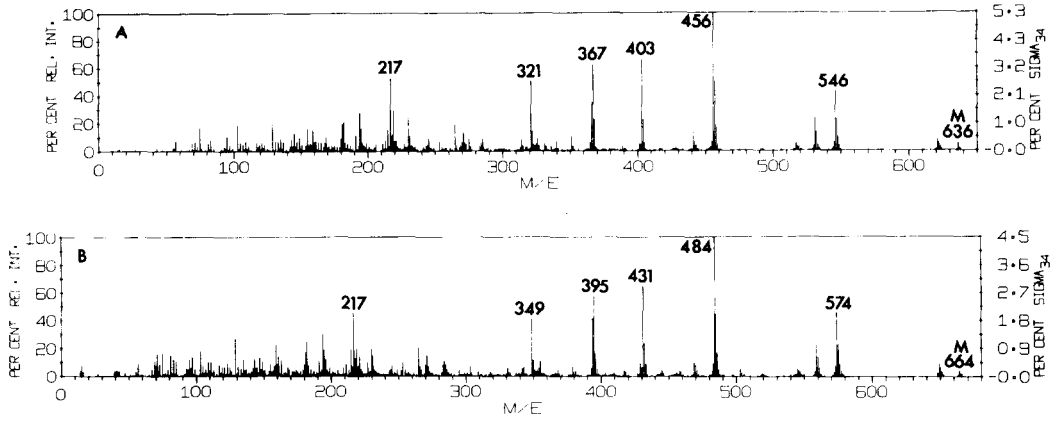

Fig. 3. GLC–MS analyses of the tri-TMS ether derivatives (see Table 1) of cholestane-3β,5α,6β-triol (*A*) and 24α-ethylcholestane-3β,5α,6β-triol (*B*) isolated from incubations with rat liver microsomes and 4-^{14}C-labeled 5β,6β-epoxycholestan-3β-ol or 24α-ethyl-5β,6β-epoxycholestan-3β-ol, respectively.

mine the location of the enzyme. The results are presented in **Table 3.** Both 5,6-epoxides could be converted in high yields to the 3β,5α,6β-triol by all fractions containing microsomes or mitochondria, whereas the soluble fraction almost lacked hydrolytic activity. Small amounts of other metabolites were also formed in the different fractions. One of these had chromatographic properties (Table 1) identical with those of 6-oxocholestane-3β,5α-diol. The yield of this compound was 1–10% of the amount of cholestane-3β,5α,6β-triol formed.

The epoxide hydrolase activity in the 4500 *g* sediment fraction could at least in part be explained by the presence of endoplasmic reticulum. Parallel incubations with 10 μg of [4-^{14}C]cholesterol were therefore made and yielded 0.5% 7α-hydroxycholesterol, 0.1% 7β-hydroxycholesterol, and 0.3% 26-hydroxy[4-^{14}C]cholesterol, indicating the presence of considerable amounts of microsomal 7α-hydroxylase. Further work on the hydrolysis of epoxysterols was carried out with the 100,000 *g* sediment fraction, which produced less than 0.02% 26-hydroxycholesterol from [4-^{14}C]cholesterol, indicating the presence of only small amounts of the mitochondrial 26-hydroxylase (20).

The addition of NADPH and NADH to washed microsomes did not increase the formation of the 3β,5α,6β-trihydroxysterol even when the sterol epoxide was preadded in amounts of 100 μg. The effects of pH and time on the reaction are shown in **Fig. 4,** *A* and *B*. In these experiments, buffer controls yielded less than a 1% conversion. The results presented in Fig. 4*C* show the substrate specificity of the 5,6-epoxysterol hydrolase(s). The procedural losses in these experiments were less than 15% as measured by radioactivity recoveries.

The 5,6-epoxysterol hydrolase could be inhibited by the C_7-oxygenated sterols formed from cholesterol and β-sitosterol at the same time as the epoxides. Thus, using 100 μg of preadded (2) 7β-hydroxycholesterol or 7-oxocholesterol and an 18,000 *g* supernatant fraction, the conversion

TABLE 3. Percentage conversion of 4-^{14}C-labeled 5α,6α-epoxycholestan-3β-ol and 5β,6β-epoxycholestan-3β-ol into cholestane-3β,5α,6β-triol after incubations with rat liver subcellular fractions

Cell-free Preparation Used[b]	Coenzyme Added	Substrate Added[a]		
		1 μg of α-Epoxide	1 μg of β-Epoxide	1 μg of α-Epoxide + 100 μg of Preadded Unlabeled α-Epoxide
4500 *g* sediment	0	39	38	
18,000 *g* supernate	0	54		
18,000 *g* supernate	NADPH	53	58	
100,000 *g* sediment	0	59	59	8
100,000 *g* sediment	NADH	62		7
100,000 *g* sediment	NADPH	62	55	7
100,000 *g* sediment preheated at 80°C	0	1	1	
100,000 *g* supernate	0	0	1	

[a] Values are means of two experiments.
[b] The amounts of protein in each incubation flask were: 4500 *g* sediment, 13.5 mg; 18,000 *g* supernate, 44 mg; 100,000 *g* sediment, 8.5 mg; 100,000 *g* supernate, 26 mg.

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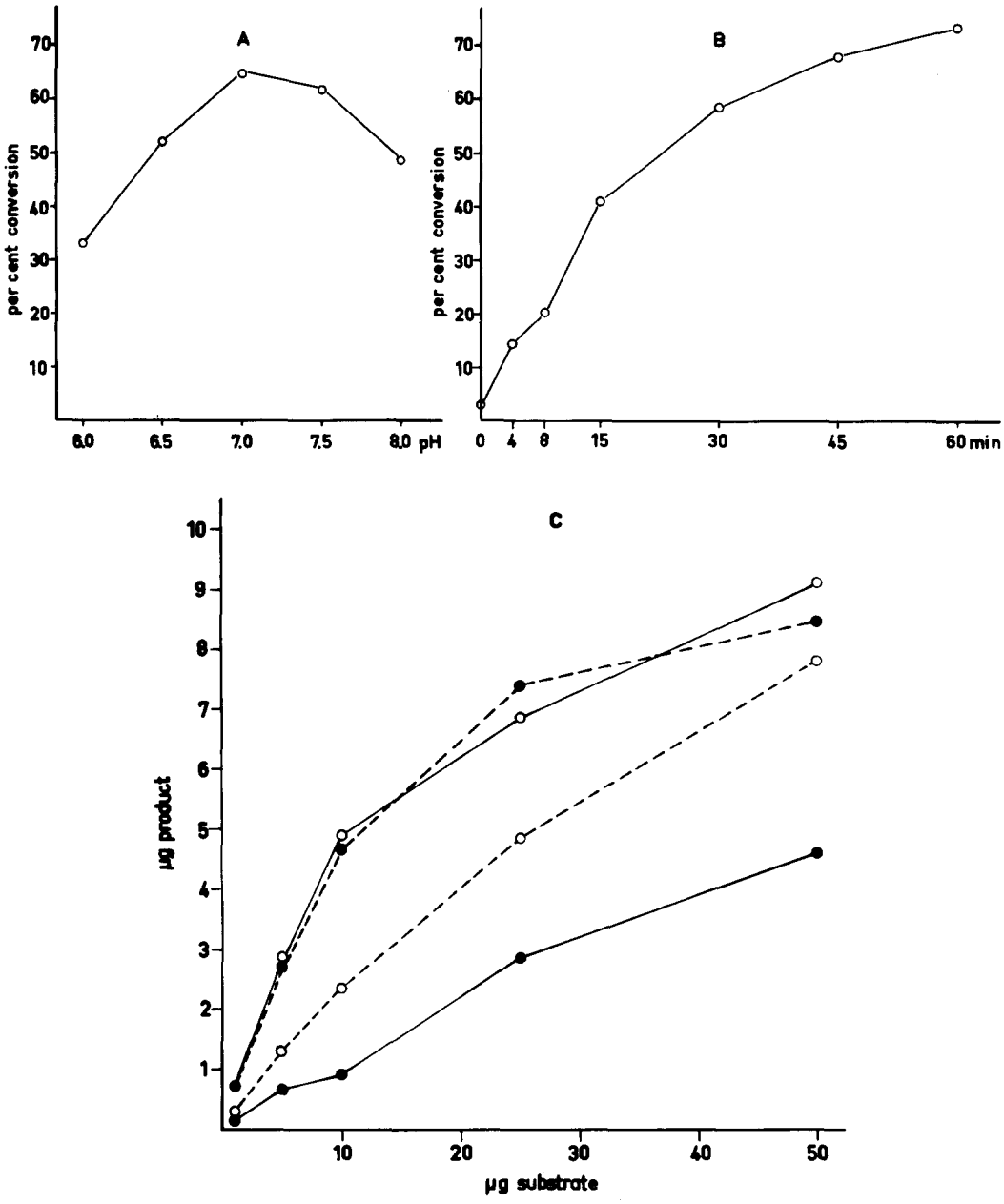

Fig. 4. Effects of pH (*A*), time (*B*), and substrate concentration (*C*) on the conversion of 5,6-epoxysterols to 3β,5α,6β-trihydroxysterols. Incubations were performed with a 100,000 *g* rat liver sediment fraction (see Experimental Procedures). The protein content in each incubation flask was 8.5 mg. Except in (*C*), 1 μg of substrate was added. ○—○, 5α,6α-epoxycholestan-3β-ol; ●- - -●, 5β,6β-epoxycholestan-3β-ol; ○- - -○, 24α-ethyl-5α,6α-epoxycholestan-3β-ol; ●—●, 24α-ethyl-5β,6β-epoxycholestan-3β-ol.

of a mixture of 0.1 μg of 5α,6α-epoxide and 0.4 μg of 5β,6β-epoxide of cholesterol to cholestane-3β,5α,6β-triol decreased from 81% to 28 and 38%, respectively. The corresponding amounts obtained in experiments with 100 μg of preadded 7β-hydroxy-β-sitosterol and 7-oxo-β-sitosterol were 65 and 62%, respectively.

Studies on the formation of 5,6-epoxysterols from cholesterol and β-sitosterol

As seen in **Table 4,** the formation of sterol epoxides closely followed that of the classical autoxidation products, i.e., 7-oxosterols and 7β-hydroxysterols. Recoveries of 4-^{14}C-labeled metabolites were 89% after HA-Sephadex LH-20 chromatography. Final recoveries of C_7-oxygenated metabolites were of the same order as reported previously (2), and the figures for α- and β-epoxysterols were almost the same, ranging from 71 to 79%.

The addition of NADH or NADPH to the 4500 *g* sediment or to the 18,000 *g* supernatant fraction did not stimulate, and heating the 18,000 *g* supernatant fraction to 80°C for 20 min prior to the incubation did not inhibit, the formation of either the 5α,6α- or the 5β,6β-epoxide. Using microsomes preheated at 50°C and supplemented with 100,000 *g* supernatant fluid as described for squalene

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TABLE 4. Yields of metabolites from [4-^{14}C]cholesterol and β-[4-^{14}C]sitosterol after incubations with rat liver subcellular fractions

Cell-free Preparation Used	Coenzyme Added	Percentage Conversion[a] Metabolites Formed from [4-^{14}C]Cholesterol[b] 7α-OH	7β-OH + 7-Oxo	Epoxides	3β,5α,6β-OH	β/α[c]	Metabolites Formed from β-[4-^{14}C]Sitosterol[b] 7α-OH	7β-OH + 7-Oxo	Epoxides	3β,5α,6β-OH	β/α[c]
4500 *g* sediment	0	0.02	0.08	0.04		3.5	0.03	0.08	0.04		2.8
4500 *g* sediment	NADH	0.18	0.12	0.07	0.02	3.4					
4500 *g* sediment	NADPH regenerating system	0.23	0.08	0.06	0.02	3.7	0.07	0.12	0.09		3.3
18,000 *g* supernate	0	0.25	0.11	0.09	0.03	3.3					
18,000 *g* supernate	NADH	0.32	0.09	0.08	0.04	3.3					
18,000 *g* supernate	NADPH	1.41	0.04	0.02	0.02	3.6	0.05	0.07	0.03	0.01	3.3
18,000 *g* supernate preheated at 50°C	NADPH	0.03	0.04	0.03	0.01	3.4	0.05	0.06	0.03	0.02	3.2
18,000 *g* supernate preheated at 80°C	0	0.14	0.45	0.25	0.06	3.9	0.08	0.34	0.24	0.04	3.9
100,000 *g* sediment preheated at 50°C[d]	NADPH	0.05	0.11	0.09	0.04	3.3					
100,000 *g* sediment, iron added[e]	NADPH	0.25	0.76	0.41	0.16	3.8	0.29	0.88	0.32	0.24	3.4
100,000 *g* sediment, iron added, EDTA omitted	NADPH	1.85	5.26	3.77	1.25	3.5	1.95	6.56	3.86	0.32	3.5
Soybean lipoxidase[f]	0	5.15	12.1	5.01	0.09	3.7	7.34	17.4	6.53	0.14	3.7
Air oxidation[g]	0	0.07	0.10	0.21	0.04	3.6					

[a] Each value is the mean of at least three experiments.

[b] 7α-OH, 7β-OH, and 7-oxo denote the 7α-hydroxy, 7β-hydroxy, and 7-oxo derivatives of cholesterol and β-sitosterol, respectively. 3β,5α,6β-OH refers to cholestane-3β,5α,6β-triol and 24α-ethylcholestane-3β,5α,6β-triol, respectively. The term epoxides refers to both the 5α,6α-epoxide and the 5β,6β-epoxide of cholesterol and β-sitosterol, respectively.

[c] β/α is the ratio between the yields of the isomeric epoxides. This ratio was never less than 2.6 and never exceeded 4.4 in any individual experiment.

[d] Liver microsomes and a soluble fraction from 200-g male rats of the Sprague-Dawley strain were prepared in a 0.25 M sucrose solution as described by Yamamoto and Bloch (21). Microsomes equivalent to 1 g of wet liver were suspended in 4.25 ml of a 0.05 M potassium phosphate buffer, pH 7.5, containing 0.125 M sucrose, and heated at 50°C for 5 min; then, after addition of 4.25 ml of the soluble fraction, they were immediately incubated as described in Experimental Procedures.

[e] Liver microsomes from 200-g male rats of the Sprague-Dawley strain were prepared as described by Mitton, Scholan, and Boyd (1). 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. Incubation procedure was that described under Experimental Procedures. ADP and $FeSO_4$ were added immediately after the addition of 10 μmoles of NADPH so that the concentrations were 1 mM and 0.2 mM, respectively.

[f] Labeled compounds were incubated with soybean lipoxidase and linoleic acid at 30°C for 30 min as described by Johansson (22).

[g] Labeled compounds were kept dry at 24°C for 3 days and then analyzed as described in Experimental Procedures.

epoxidase (21) did not change the ratio between β-epoxide and α-epoxide yields, nor did preheating of the 18,000 *g* supernate to 50°C. The ratio was similar both to that obtained with ferrous ion-supplemented microsomes known to exhibit NADPH-dependent lipid peroxidase activity (22) and to that found with soybean lipoxidase. Air autoxidation of labeled cholesterol and β-sitosterol also produced a β- to α-epoxide ratio of the same order of magnitude.

Studies on the effects of 5,6-epoxysterols and 3β,5α,6β-trihydroxysterols on the 7α-hydroxylation of cholesterol

To test the significance of the formation of 5,6-epoxysterols and 3β,5α,6β-trihydroxysterols, the effects of these compounds on the 7α-hydroxylation of [4-^{14}C]cholesterol in vitro were studied. The results are presented in **Table 5.** Of the compounds tested, only the 5,6-epoxides of cholesterol were able to significantly interfere with the cholesterol 7α-hydroxylase. The inhibitory potency of the 5β,6β-epoxide was most pronounced. 50 μg of 7-oxocholesterol, which was used as a "control inhibitor" in each set of experiments, caused a reduction in the conversion of cholesterol to 7α-hydroxycholesterol from about 3% to 0.4% (0.2–0.6%).

DISCUSSION

The formation and metabolism of cholesterol 5,6-epoxides has attracted attention in studies on hypercholesterolemia (23), hypertension (23), and carcinogenesis (24). In the latter studies, Black and Douglas (24) demonstrated the formation of cholesterol α-epoxide upon ultraviolet radiation of hairless skin in mice. In general, however, limitations in the methodology used have excluded a distinc-

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tion between the α- and β-epoxides. Our approach to this problem is based on our finding that the 5α,6α- and 5β,6β-epoxides of cholesterol can easily be separated as the acetate derivatives on $Ag(NH_2)^+$-containing silica gel plates or by running the trimethylsilyl ether derivatives on hexamethyldisilazane-treated silica gel plates after a preliminary purification by liquid chromatography and TLC. Although the trimethylsilyl ether derivatives were hydrolyzed to some extent (1–5%), this TLC system was less sensitive to overloading and gave a more pronounced separation than that using the acetate derivatives and was therefore preferred in quantitative work.

It is apparent from the results that equal amounts of 5,6-epoxides of cholesterol and β-sitosterol are formed under conditions favoring autoxidation, i.e., the rat liver subcellular fractions exhibited an enzyme activity generating lipid peroxides (25) that produce a sterol metabolite pattern similar to that formed by soybean peroxidase (22). Thus, in rat liver subcellular fractions the net yield of sterol epoxides increased when the yield of C_7-oxygenated sterols increased. None of the coenzymes added could markedly alter the formation of either the 5α,6α- or the 5β,6β-epoxide from cholesterol or β-sitosterol. In an attempt to demonstrate the presence of a specific sterol 5,6-epoxidase, we copied the conditions used by others in studies on squalene epoxidases (21). The metabolite pattern then obtained was the same as that obtained under conditions of autoxidation. The lipid peroxidase system is stimulated by ferrous ion and NADPH, is insensitive to carbon monoxide (22), and is counteracted by vitamin E (26). It seems similar to some hydrocarbon epoxidases (27–30) and steroid epoxidases (31, 32), and it may be suggested that these are related to the lipid peroxidase.

Although we could not demonstrate the presence of a specific sterol 5,6-epoxidase in rat liver, the specific occurrence of cholesterol 5α,6α-epoxide in human serum (23) implies that such an enzyme may occur, possibly outside the liver.

The rat liver 5,6-epoxysterol hydrolase is similar in its action to that reported to hydrolyze isomeric cholestane 2,3-epoxides to the 2α,3β-diol only (33). Thus, cholesterol and β-sitosterol 5,6-epoxides were hydrolyzed by a microsomal hydrolase exclusively to the 3β,5α,6β-triol. The enzyme, like those hydrolyzing steroid 16,17-epoxysteroids (31) and 2,3-epoxysteroids (33), did not demand NADPH or NADH. It may be that the 5,6-epoxysterol hydrolase, in analogy with a hepatic epoxide hydrase (34), utilizes water (35) for the opening of the epoxide.

The 3β,5α,6β-triol formed was converted into a derivative with chromatographic properties identical with those of 6-oxocholestane-3β,5α-diol. This suggests that the 6-oxocholestane-3β,5α-diol isolated from rat feces after the oral administration of [4-^{14}C]cholestane-3β,5α,6β-triol (36) may at least in part be of hepatic origin.

Since the formation and hydrolysis of 5,6-epoxysterols seem to occur in the endoplasmic reticulum of the rat liver where the rate-limiting step in bile acid biosynthesis, i.e., the 7α-hydroxylation of cholesterol, takes place, we studied the effects of 5,6-epoxysterols and of 3β,5α,6β-trihydroxysterols on this reaction. The 5,6-epoxides of cholesterol, but not the trihydroxy derivative or the analogous derivatives of β-sitosterol, were found to inhibit the 7α-hydroxylation of cholesterol in an 18,000 *g* supernatant fraction. The lack of effect of the 5,6-epoxy derivatives of β-sitosterol might be explained only in part by a limited penetration of the C_{29} epoxides into the microsomes because they are converted by a 100,000 *g* sediment fraction into the 3β,5α,6β-triol.

From our present study and a previous one (2) on the in vitro inhibition of cholesterol 7α-hydroxylase, it seems as if the enzyme is more easily inhibited by the steroids carrying a β-oriented substituent. For instance, 5β,6β-epoxycholestan-3β-ol is three to four times as potent an inhibitor as the 5α,6α-isomer, and 7β-hydroxycholesterol is several times more inhibitory than is 7α-hydroxycholesterol. The nature of the inhibition may differ, however, between the $C_{5,6}$- and the C_7-substituted steroids, because only the 7β-hydroxy and 7-oxo derivatives of β-sitosterol (2) and not the 5,6-epoxides were inhibitory.

Although the amounts of each sterol derivative needed for inhibition in vitro exceed those that may normally be formed in vivo, the sum of autoxidation products formed may affect the 7α-hydroxylase in pathological states, for instance in vitamin E deficiency (25). The inhibitory effects of 7β-hydroxycholesterol and 7-oxocholesterol on the epoxide hydrolase will lead to an accumulation of 5,6-epoxysterols and thus to a prolonged inhibition of the cholesterol 7α-hydroxylase.

TABLE 5. Percentage conversion of [4-^{14}C]cholesterol to 7α-hydroxy[4-^{14}C]cholesterol in the presence of preadded 5,6-oxygenated sterols

Amount Added	Compound Added[a]					
	α-Epoxide-C_{27}	β-Epoxide-C_{27}[b]	α-Epoxide-C_{29}	β-Epoxide-C_{29}	3β,5α,6β-C_{27}	3β,5α,6β-C_{29}
μg						
0	3.4	3.4	2.7	2.7	3.8	3.8
25		1.9				
50	2.7	0.9			3.8	3.4
100	1.7	0.4	2.5	2.6	3.8	4.3
200	1.3	0.3	2.5	2.3	3.6	3.9

[a] Each value is the mean of two experiments. α-Epoxide-C_{27} and β-epoxide-C_{27} denote 5α,6α-epoxycholestan-3β-ol and 5β,6β-epoxycholestan-3β-ol, respectively; 3β,5α,6β-C_{27} denotes cholestane-3β,5α,6β-triol; and C_{29} denotes 24α-ethyl-substituted derivatives.

[b] In this set of experiments the conversion was 0.5% when 25 μg of 7-oxocholesterol was preadded.

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