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Studies on the Enzymatic Conversion of Oxygen-substituted Sterols to Cholesterol*

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SUMMARY

The intermediary role of oxygenated sterols in the conversion of cholest-7-en-3β-ol to cholesta-5,7-dien-3β-ol by rat liver homogenates is considered, assuming that an oxygen molecule may attack the double bond of cholest-7-en-3β-ol. Labeled cholestan-7α,8α-epoxy-3β-ol, cholestane-3β,7β,8α-triol, cholest-8-ene-3β,7ξ-diols, cholestane-3β,7α,8α-triol, cholestane-3β,-8α-diol-7-one, and cholest-8(14)-ene-3β,7α-diol have been synthesized. All these compounds, but cholestane-3β,7α,8α-triol, are efficiently transformed to cholesterol under oxygen atmosphere. However, they cannot be considered as obligatory intermediates in the biosynthesis of cholesterol from cholest-7-en-3β-ol since under anaerobiosis they are transformed to cholest-7-en-3β-ol. The implications of these findings and the mechanisms involved are discussed.

It is now established that the conversion of cholest-7-en-3β-ol[1] into cholesterol implies the obligatory role of cholesta-5,7-dien-3β-ol (1–3). The introduction of the double bond in Position 5 takes place through removal of 5α and 6α hydrogen atoms of cholest-7-en-3β-ol (4, 5). This process has an absolute requirement for oxygen (1, 6) while it is still doubtful which pyridine nucleotide is involved in the reaction (1, 7, 8).

It has been proposed that oxygenated intermediates are involved in the formation of cholesta-5,7-dien-3β-ol from cholest-7-en-3β-ol (9–11). The intermediary role of 5α, 6α, and 6β hydroxy derivatives of cholest-7-en-3β-ol has been rendered unlikely by Slaytor and Bloch (9) and Dewhurst and Akhtar (10). In addition Paliokas and Schroepfer (11) demonstrated that [4-^{14}C]5α,8α-epidioxy-Δ^{6}-cholesten-3β-ol is not incorporated into both cholesta-5,7-dien-3β-ol and cholesterol. The possibility that other intermediates might be formed by oxygen interaction with cholest-7-en-3β-ol was considered by us. Therefore compounds containing oxygen atoms at 7 and 8 position or both have been tested here as possible intermediates of the formation of the 5,7-diene. Such intermediates could be formed by the attack of oxygen to the double bond in Position 7.

We synthesized labeled cholestan-7α,8α-epoxy-3β-ol (*I*), cholestane-3β,7β,8α-triol (*II*) and cholest-8-ene-3β,7ξ-diols (*III*) (*IV*) (Fig. 1) and showed that they are efficiently converted to cholesterol by rat liver homogenates only under aerobic conditions, while under nitrogen they are all transformed into cholest-7-en-3β-ol. Similarly, 6α and 6β hydroxy derivatives of cholest-7-en-3β-ol (9), cholesta-7,14-dien-3β-ol,[2] cholesta-8,14-dien-3β-ol (12), cholesta-7,9(11)-dien-3β-ol (13) and cholest-8-ene-3β,6α-diol (9) are converted to cholest-7-en-3β-ol in anaerobiosis, while cholest-8(14)-en-3β-ol (14), cholest-14-en-3β-ol[2] and cholest-7-ene-3β,5α-diol (10) are not metabolized under anaerobic conditions. To elucidate further which structural changes might be involved in the formation of the seven double bond from oxygenated sterols, labeled cholestane-3β,7α,8α-triol (*V*) (Fig. 1), cholestane-3β,8α-diol-7-one (*VI*), and cholest-8(14)-ene-3β,7α-diol (*VII*) have been synthesized (Fig. 2); the last two compounds only are metabolized to cholest-7-en-3β-ol under anaerobiosis, while the first one is recovered unchanged both under aerobic and anaerobic conditions.

EXPERIMENTAL PROCEDURE

Synthesis of Radioactive Sterols

Cholest-7-en-3β-ol—Cholesta-5,7-dien-3β-ol acetate was hydrogenated in benzene using tris triphenylphosphinerhodium chloride as catalyst (15). Cholest-7-en-3β-ol acetate, m.p. 120°,

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[1] The configuration of the hydrogen at carbon 5 in the various sterols mentioned in this paper is α.

[2] Unpublished results from our laboratories.

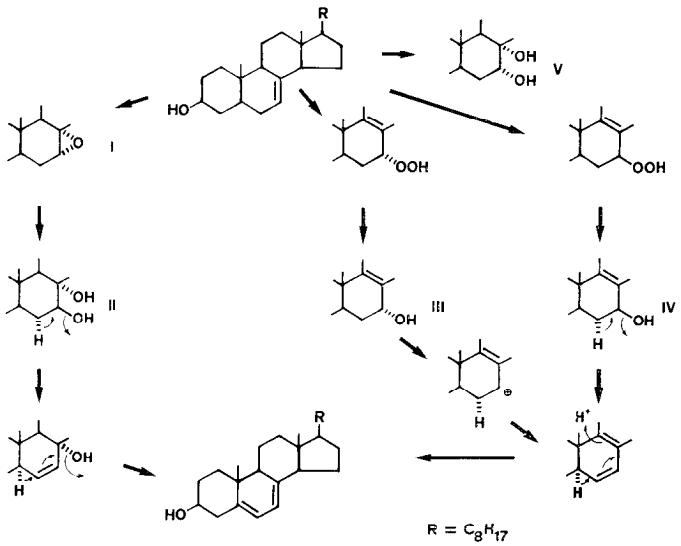

FIG. 1. Hypothetical pathways of transformation of cholest-7-en-3β-ol to cholesta-5,7-dien-3β-ol.

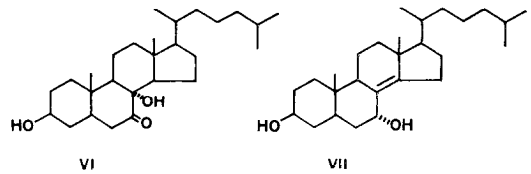

FIG. 2.

$[\alpha]_D^{20}$ +4.1° (c = 1 in chloroform) was saponified by refluxing 1 hour with 5% methanolic potassium hydroxide. The free sterol was isolated by ether extraction of the cooled and diluted solution and crystallized from methanol, m.p. 121–123°, $[\alpha]_D^{20}$ +6.3° (c = 1 in chloroform); literature m.p. 122–123°, $[\alpha]_D^{20}$ +6.5° (16).

The acetate derivative of this sterol showed a single peak by gas-liquid chromatography with a relative retention time of 5.09 and the mass spectrum showed the expected molecular ion at *m/e* 428 and a fragmentation pattern in agreement with the literature (17). The isomerization of the double bond from 7 to 8 and 8(14) position or both is excluded since, under the same conditions, cholest-8-en-3β-ol and cholest-8(14)-en-3β-ol acetates showed a relative retention time of 4.76 and 4.56, respectively.

Cholest-7-en-3-one—The foregoing material was oxidized with chromic acid in acetone according to the Jones procedure (18). Purification of the material by Silica Gel G-Celite (50:50; v/v) column chromatography yielded a white solid which showed a single spot on Silica Gel HF thin layer chromatography either with chloroform or with benzene-ethyl acetate (90:10; v/v). Crystallization from methanol yielded pure cholest-7-en-3-one, m.p. 145–146°, $[\alpha]_D^{20}$ +24.9° (c = 1 in chloroform); literature m.p. 146–148°, $[\alpha]_D^{20}$ +24.7° (19).

[2,4-$^3H_4$]Cholest-7-en-3β-ol—Cholest-7-en-3-one was labeled at Positions 2 and 4 by exchange with tritiated water (0.5 Ci per ml) on basic alumina (20). The tritiated ketone by reduction with lithium aluminum hydride in anhydrous ethyl ether and crystallization from methanol yielded [2,4-^{3}H$_4$]cholest-7-en-3β-ol, m.p. 122–123°, $[\alpha]_D^{20}$ +6.4° (c = 1 in chloroform). The product showed a single spot with an R_F value identical to that of standard cholest-7-en-3β-ol by thin layer argentation chromatography (21), which contained virtually all the radioactivity (98.8%).

A portion of the labeled sterol was acetylated with acetic anhydride in pyridine. No change of molar radioactivity was observed after acetylation. Gas-liquid chromatography analysis of the acetate showed a single peak with a retention time of 5.09 corresponding to cholest-7-en-3β-ol acetate.

The specific activities of the various preparations of cholest-7-en-3β-ol are reported in the following sections.

[2,4-$^3H_4$]Cholestan-7α,8α-epoxy-3β-ol—In essence the reaction described by Fieser and Goto (22) for the preparation of cholestan-7α,8α-epoxy-3β-ol acetate was utilized, except that metachloroperoxybenzoic acid was used instead of monoperphthalic acid. [2,4-^{3}H$_4$]Cholest-7-en-3β-ol (specific activity 23.2 μCi per μmole) was allowed to stand at 0°. To avoid further transformation of the epoxide, the reaction was stopped after 12 hours, when only part of the cholest-7-en-3β-ol was transformed (22). The product was isolated by partition between ether and 5% sodium hydroxide and evaporation of the ether extract after washing with water and drying over sodium sulfate. The ether extract was subjected to basic alumina thin layer chromatography with chloroform-methanol (97:3; v/v) which separated the epoxide from monoene sterols. The spot corresponding to the monoene sterols was extracted and acetylated: the resulting product gave a single peak in gas-liquid chromatography having the retention time of cholest-7-en-3β-ol. The epoxide after crystallization from petroleum ether yielded a white solid (specific activity 23.2 μCi per μmole), m.p. 118–120°, $[\alpha]_D^{20}$ −8.2° (c = 1 in chloroform).

	$C_{27}H_{46}O_2$	
Calculated:	C 80.54	H 11.52
Found:	C 80.51	H 11.50

The crystallized epoxide on basic alumina thin layer chromatography showed a single spot which contained 99.4% of the radioactivity. A portion of the product after acetylation and crystallization from methanol yielded [2,4-^{3}H$_4$]cholestan-7α,8α-epoxy-3β-ol acetate (specific activity 23.2 μCi per μmole) m.p. 84–85° (low melting form) (22), $[\alpha]_D^{20}$ +7.3° (c = 1 in chloroform). Under our crystallization conditions we never obtained the high melting form reported by Fieser and Goto (22). The mass spectrum of the acetate showed the expected molecular ion at *m/e* 444.

[2,4-$^3H_4$]Cholestane-3β,7α,8α-triol—[2,4-^{3}H$_4$]Cholest-7-en-3β-ol acetate (specific activity 8.67 μCi per μmole) oxidized with osmium tetroxide as previously described (15), yielded a mixture of [2,4-^{3}H$_4$]cholestane-3β,7α,8α-triol and [2,4-^{3}H$_4$]cholestane-3β,7β,8β-triol 3β-acetates (m.p. 155–157°). Several crystallizations from diethyl ether-heptane allowed the separation of crystalline [2,4-^{3}H$_4$]cholestane-3β,7α,8α-triol 3β-acetate (specific activity 8.67 μCi per μmole), m.p. 172–174°, $[\alpha]_D^{20}$ −21.7° (c = 1 in chloroform). The α configuration was assigned to the vicinal hydroxy groups based on the known tendency for rear approach of the reagent to the molecule (23). To confirm this assignment and to prove the purity of the triol the compound was treated with hydrochloric acid in dioxane. A quantitative transformation of the radioactive cholestane-3β,7α,8α-triol 3β-acetate to cholestan-3β-ol-7-one acetate was observed, in agreement with the quantitative transformation of ergostane-3β,7α,8α-triol to ergostan-3β-ol-7-one reported by Meakins and Stephenson (24). This reaction occurs via a *trans*-elimination of the tertiary hydroxy group followed by ketonization and requires an intramolecular 1,2 hydride shift as we previously observed in the acid treatment of cholestane-3β,7α,8α-triol 3β-acetate labeled with tritium at Position 7 which gave rise to

cholestan-3β-ol-7-one 3β-acetate labeled at Position 8 (15). On the other hand both the two isomers mixture and the mother liquors of crystallization under the same conditions yielded a mixture containing cholestan-3β-ol-7-one and cholesta-7,9(11)-dien-3β-ol acetates, in agreement with the transformation of 5β-lumistane-3β,7ξ,8ξ-triol into 5β-lumistan-3β-ol-7-one, and 5β-lumista-8,14-dien-3β-ol observed by Castells *et al.* (25).

The [2,4-$^3H_4$]cholestane-3β,7α,8α-triol 3β-acetate was saponified with lithium aluminum hydride in diethyl ether. The excess reagent was destroyed by careful addition of methanol followed by water, the product was extracted into ether and the ether extract was dried over sodium sulfate. Evaporation of the ether solution gave a crude product which was purified by thin layer chromatography in chloroform-methanol (95:5; v/v). The desired product was isolated by extraction of the silica gel with ether. Crystallization from methanol yielded [2,4-$^3H_4$]-cholestane-3β,7α,8α-triol (23) (specific activity 8.67 μCi per μmole) m.p. 210–212°, $[\alpha]_D^{20}$ −19.5° (c = 1 in chloroform); literature m.p. 213–214°, $[\alpha]_D^{22}$ −20.7° ± 3 (19).

$C_{27}H_{48}O_3$		
Calculated:	C 77.09	H 11.50
Found:	C 76.94	H 11.53

Thin layer chromatography on Silica Gel HF (solvent benzene-ethyl acetate 50:50; v/v) showed a single component which contained 98.4% of the total radioactivity.

[2,4-$^3H_4$]Cholestane-3β,8α-diol-7-one—[2,4-$^3H_4$]Cholestane-3β,8α-diol-7-one acetate, m.p. 161–163°, $[\alpha]_D^{20}$ −10.5° (c = 1 in chloroform) was obtained from pure [2,4-$^3H_4$]cholestane-3β,7α,8α-triol 3β-acetate (specific activity 18.6 μCi per μmole) by using the procedure described by Davey *et al.* (26) for the preparation of cholestan-8α-ol-7-one from cholestane-7α,8α-diol. Saponification of the acetate with 3% methanolic KOH and crystallization from methanol yielded [2,4-$^3H_4$]cholestane-3β,8α-diol-7-one (specific activity 18.6 μCi per μmole), m.p. 205–206° $[\alpha]_D^{20}$ −0.65° (c = 1 in chloroform).

$C_{27}H_{46}O_3$		
Calculated:	C 77.46	H 11.08
Found:	C 77.49	H 11.09

Thin layer chromatography on Silica Gel HF with chloroform-methanol (99:1; v/v) showed only one spot which contained 99.2% of the radioactivity of the chromatographed material. The mass spectrum of the 3β-acetate showed the expected molecular ion at *m/e* 460.

[2,4-$^3H_4$]Cholestane-3β,7β,8α-triol—[2,4-$^3H_4$]Cholestane-3β,8α-diol-7-one acetate (specific activity 8.67 μCi per μmole) was reduced by using the procedure described by Davey *et al.* (26) for the reduction of cholestan-8α-ol-7-one. The mixture of the expected cholestane-3β,7α,8α-triol and cholestane-3β,7β,8α-triol was chromatographed on Silica Gel G-Celite (50:50; v/v) column. The fractions eluted with benzene-ethyl ether (90:10; v/v) contained 3β,7α,8α-triol, m.p. and mixed m.p. 210–212°, benzene-ether (80:20; v/v) eluted cholestane-3β,7β,8α-triol (specific activity 8.67 μCi per μmole) m.p. 170–172°.

$C_{27}H_{48}O_3$		
Calculated:	C 77.09	H 11.50
Found:	C 76.83	H 11.48

Thin layer chromatography on Silica Gel HF (solvent chloroform-methanol, 95:5; v/v) of 3β,7β,8α-triol showed a single component which contained 98.8% of the total radioactivity. No radioactivity corresponding to 3β,7α,8α-triol was found.

[2,4-$^3H_4$]Cholest-8(14)-ene-3β,7α-diol—[2,4-$^3H_4$]Cholest-7-en-3β-ol (specific activity 18.6 μCi per μmole) was oxidized with selenium dioxide in benzene and acetic acid according to Fieser *et al.* (23). The [2,4-$^3H_4$]cholest-8(14)-ene-3β,7α-diol diacetate obtained in this way was reduced with lithium aluminum hydride in ethyl ether. Extraction and crystallization from methanol yielded [2,4-$^3H_4$]cholest-8(14)-ene-3β,7α-diol (specific activity 18.6 μCi per μmole); m.p. 156–157°, $[\alpha]_D^{20}$ −20.5°; literature m.p. 157–158°, $[\alpha]_D^{20}$ −21° (23).

$C_{27}H_{46}O_2$		
Calculated:	C 80.54	H 11.52
Found:	C 80.35	H 11.48

The chemical purity and radiopurity of the compound was checked by thin layer chromatography on Silica Gel HF (solvent chloroform-methanol 97:3; v/v). The single component observed upon this analysis contained 98.8% of the total radioactivity. Oxidation of the compound with CrO_3 in pyridine yielded a single diketone, cholest-8(14)-ene-3,7-dione, m.p. 129–130°, $[\alpha]_D^{20}$ −29.3° (c = 1 in chloroform), λ_{max} 262 ε 10.400 ir (nujol) 1718, 1695, 1668, 1595 cm^{-1}; the compound showed a relative retention time of 9.33 and its mass spectrum showed the expected molecular ion at *m/e* 398. The presence of cholest-8-ene-3,7-dione was excluded from the ultraviolet spectrum, the relative retention time and mass spectrum as reported in the following paragraphs.

[2,4-$^3H_4$]Cholest-8-en-3β-ol-7-one 3β-acetate—Thionyl chloride was added at 0° to a solution of [2,4-$^3H_4$]cholestane-3β,8α-diol-7-one 3β-acetate (specific activity 16.7 μCi per μmole) in pyridine. After standing for 12 hours at 0° the solution was poured into crushed ice and extracted four times with ethyl ether. The ethereal layer was washed with 5% hydrochloric acid and 5% $KHCO_3$ solution. The labeled material was purified by chromatography on a column of Silica Gel G-Celite (50:50; v/v). Fractions eluted with benzene-hexane (10:90; v/v) were pooled. Crystallization from methanol of the residue yielded [2,4-$^3H_4$]-cholest-8-en-3β-ol-7-one acetate (specific activity 16.7 μCi per μmole), m.p. 158–160°, $[\alpha]_D^{20}$ −32.3° (c = 1 in chloroform), λ_{max} 253, ε 10.000; literature m.p. 154–157°, $[\alpha]_D^{20}$ −34°, λ_{max} 252.5 ε 10.000 (27).

The determination of chemical and radiopurity of the compound, by thin layer chromatography on Silica Gel HF (solvent chloroform-methanol 97:3; v/v) indicated the presence of a single spot which contained 99% of the radioactivity. The mass spectrum showed the expected molecular ion at *m/e* 442. Saponification of the acetate with methanolic KOH yielded [2,4-$^3H_4$]-cholest-8-en-3β-ol-7-one, m.p. 112–113°, $[\alpha]_D^{20}$ −20.5° (c = 1 in chloroform); literature m.p. 114–116°, $[\alpha]_D^{20}$ −22° ± 2 in dioxane (28).

[2,4-$^3H_4$]Cholest-8-ene-3β,7ξ-diols—[2,4-$^3H_4$]Cholest-8-en-3β-ol-7-one 3β-acetate (specific activity 16.7 μCi per μmole) was reduced with lithium aluminum hydride in ethyl ether. After 12 hours stirring at room temperature, the mixture was extracted as usual. The analysis of the product on thin layer chromatography showed the presence of two spots of similar polarity which, however, could be eluted separately (diol A and diol B). The mass spectrum of both the radioactive diols showed the expected molecular ion at *m/e* 402. Oxidation with CrO_3 in pyridine performed separately on the two diols yielded in both cases a single diketone having a relative retention time of 10.37. Exactly the same diketone was obtained by oxidizing pure cholest-8-en-3β-ol-7-one under the same conditions. After crystallization the compound showed m.p. 138–139°, λ_{max} 253 ε 10.000.

The mass spectrum of the compound showed the expected molecular ion at *m/e* 398. A characteristic peak at *m/e* 192 arising from the cleavage of the 8,14 and 11,12 bonds was present. Since the cholest-8(14)-en-3,7-dione, described above, showed different ultraviolet spectrum and gas-liquid chromatography retention time and the peak at *m/e* 192 was absent from its mass spectrum it was possible to exclude the presence of an 8(14) ene system in the cholest-8-ene-3,7-dione and corresponding diols.

Incubation Procedures

Sprague-Dawley rats, 120 to 140 g body weight, were decapitated and their livers perfused with cold buffer, quickly excised and collected in ice cold 0.1 M phosphate buffer, pH 7.4. The following part of the procedure was performed in the cold up to the incubation step. Liver pulp was prepared by passing the livers through a stainless steel press (29) and collected in a graduated cylinder. Phosphate buffer, enriched with 0.006 M $MgCl_2$ and 0.03 M nicotinamide, according to Bucher and McGarrahan (30) was added to make the ratio of tissue to buffer 1:2.5 (v/v). The homogenate was prepared by means of a glass homogenizer, clearance of 0.5 mm, giving only 5 to 6 strokes. The homogenate was centrifuged at 1,000 × *g* for 10 min. The supernatant was centrifuged at 10,000 × *g* for 20 min to give a supernatant fraction which, filtered through a gauze was used for all the incubations. The average protein content for the homogenate was determined according to Lowry *et al.* (31) and was 20 to 25 mg per ml.

The labeled precursor sterols were dissolved in a small amount of acetone; Tween 80 and phosphate buffer were added (4 mg of Tween 80 per ml) and the acetone evaporated under a stream of nitrogen to obtain a solution containing 20 to 40 μg per ml of labeled precursor. This solution, 0.25 ml, was pipetted into the side arm of the incubation flask which contained 10 ml of the homogenate plus 1.75 ml of Tween 80 in buffer (4 mg per ml). In trap experiments 200 μg of unlabeled cholest-7-en-3β-ol were dissolved in the 1.75 ml of Tween solution. The flasks used for aerobic experiments were flushed with oxygen and tightly sealed with a stopper. For anaerobic experiments, to ensure a strict nitrogen atmosphere, a maximum of six flasks, maintained at 4° and attached to the side arm of a tube connected with a gas burette filled with nitrogen, were shaken and alternatively evacuated and filled with nitrogen five times, and maintained in a closed nitrogen atmosphere under slight pressure throughout the incubation. All the flasks, both under nitrogen and oxygen were transferred to a Dubnoff incubator and shaken for 15 min at 37° before adding the substrate, which was then poured into the main well of the flasks and incubated for an additional 60 min. In each experiment parallel incubations of labeled cholest-7-en-3β-ol were run both under nitrogen and oxygen as a control of the anaerobiosis and of the homogenate activity, respectively. The reaction was stopped by adding 10 ml of 2 N KOH in absolute ethanol and the mixture was saponified at 50° for 2 hours. The unsaponifiable fraction was extracted with petroleum ether (boiling point 60–80°), washed with water and dried over anhydrous sodium sulfate. The solvent was filtered and evaporated in vacuum. A portion of the residue was used to determine the radioactivity recovered in the unsaponifiable fraction. Separation and purification of cholesterol and cholest-7-en-3β-ol was performed as indicated in the following sections.

Thin Layer Chromatography

This was carried out using four types of layers (0.25 mm thickness): Silica Gel HF, Silica Gel G impregnated with 30% silver nitrate, neutral alumina Woelm impregnated with 15% silver nitrate and basic alumina Woelm. The bands corresponding to the sterols were identified through the use of authentic standards and detected by ultraviolet light either directly or with 0.05% Rhodamine 6G. The spots were sucked off the plates and the sterols were eluted by means of appropriate solvents.

Gas-Liquid Chromatography

This was carried out using a Perkin-Elmer 881 gas chromatograph equipped with a flame ionization detector and glass sylanized columns 2 m long, packed with PhSi 3% on sylanized Chromosorb W (100 to 200 mesh). The column temperature was maintained at 220°–240°, the nitrogen flow rate was 40 ml per min. Acetate derivatives of the sterols were used for gas-liquid chromatography determinations and the relative retention times were measured against standard cholestane.

Radioactivity Determinations

Scintillation counting was carried out by means of a Packard liquid scintillation spectrometer, 3000 series. The labeled sterol samples were dissolved in 10 ml of the liquid scintillation fluid (4 g of 2,5-diphenyloxazole, 100 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene, 1000 ml of toluene) and counted as long as necessary to ensure accurate statistics. The calculation of the absolute counts (disintegrations per min) was determined by measuring the efficiency of a [^{3}H-]toluene internal standard added to the samples after they had been counted. The average efficiency was 35% for the ^{3}H samples.

Mass Spectrometry

An LKB 9000 gas-liquid chromatography-mass spectrometer was used for the analysis of the sterols as acetate derivatives. The working conditions were reported elsewhere (17).

Ultraviolet Spectrometry

Ultraviolet spectrometry was performed with a Perkin-Elmer 137 spectrometer using ethanol solutions of sterols.

Purification of Cholesterol as Dibromide

Aliquots of the unsaponifiable fraction of each incubation (100,000 to 250,000 dpm) were diluted with 50 mg of unlabeled cholesterol and dissolved in 0.5 ml of ethyl ether. A diluted solution of bromine (0.5 g) and sodium acetate (50 mg) in acetic acid (10 ml) was added drop by drop until an orange color persisted. The solution was cooled at 0° and the dibromide precipitate was filtered and washed with acetic acid. Free cholesterol was recovered from the dibromide by zinc reduction in ethyl ether containing acetic acid. Bromination and reduction were repeated to ensure complete purification of cholesterol. The recovered cholesterol was crystallized from methanol to constant specific activity.

In control experiments, labeled cholest-7-en-3β-ol (about 200,000 dpm) was diluted with 50 mg of unlabeled cholesterol which was purified as described above. No radioactivity was recovered in the crystallized sample.

Purification of Cholest-7-en-3β-ol

Aliquots of the unsaponifiable material were added to 2 mg of unlabeled cholest-7-en-3β-ol. When cholestan-7α,8α-epoxy-3β-ol was used as precursor, the mixture was subjected to thin layer chromatography on basic alumina in benzene-ethyl ether (30:70; v/v) to ensure separation of cholest-7-en-3β-ol and cholesterol from the epoxide. When cholest-8-ene-3β,7ξ-diols,

cholest-8(14)-ene-3β,7α-diol, cholestane-3β,7α,8α-triol, and cholestane-3β,7β,8α-triol were used as precursors, the unsaponifiable fraction, plus 2 mg of unlabeled cholest-7-en-3β-ol was subjected to thin layer chromatography on Silica Gel HF in chloroform-methanol (95:5; v/v) to separate cholest-7-en-3β-ol and cholesterol from the more oxygenated compounds. The fractions corresponding to cholest-7-en-3β-ol plus cholesterol eluted from the plates of either basic alumina or Silica Gel HF were separated further by thin layer chromatography on neutral alumina-$AgNO_3$. The developing solvent was chloroform-methanol (95:5; v/v). The spot corresponding to cholest-7-en-3β-ol was eluted and, after gas-liquid chromatography control of its purity, was diluted with 30 mg of unlabeled cholest-7-en-3β-ol; no change in the molar radioactivity was observed during recrystallization.

The spot corresponding to cholesterol was also eluted and, in some experiments, further purified through bromination, as described above.

RESULTS AND DISCUSSION

All of the labeled substrates were tested for nonspecific labeling of cholesterol or cholest-7-en-3β-ol. Amounts equivalent to those used in the true experiments were incubated either with buffer solution or with boiled liver enzymes. The samples were processed in parallel with the actual experiment and no radioactivity was recovered in the purified cholesterol or cholest-7-en-3β-ol. All the experiments reported in the tables were carried out at least twice and each assay was made in duplicate. Conversion values did not differ by more than 3%. The figures quoted represent individual assays. In anaerobiosis the conversion of the tested sterols was of the same order as for cholest-7-en-3β-ol, that is, no more than 2% and, for this reason, conversions of this order of magnitude were not considered to be significant.

Up to now, only two possible mechanisms of the transformation of cholest-7-en-3β-ol have been postulated: (*a*) a direct *cis*-elimination of 5α and 6α hydrogen atoms with involvement of an enzyme-oxygen complex (10); (*b*) an hydroxylation either of Position 5 or Position 6, followed by dehydration (32). Other possible pathways, involving first an oxygen attack at Position 7 with the formation of an oxygenated sterol may be considered. Such sterol should be further transformed into cholesta-5,7-dien-3β-ol.

It has been shown that molecular oxygen in a biological system can attack a double bond, with the formation of (*a*) an epoxide, as in the case of both squalene (33, 34) and aromatic hydrocarbons (35) by rat liver microsomes; (*b*) a *cis* diol containing two atoms of atmospheric oxygen, as in the oxidative metabolism of toluene by *Pseudomonas putida* (36); (*c*) an hydroperoxy derivative such as 15-hydroperoxy-8,11,13-eicosatrienoic acid formed from 8,11,14-eicosatrienoic acid by soy bean lipoxidase (37).

These considerations may be applied to the possible oxygen attack of the double bond of cholest-7-en-3β-ol: some molecular species which may originate by each possible mechanism are reported (Fig. 1).

Labeled Compounds *I*, *III*, and *IV* have been incubated with rat liver homogenates both under aerobic and anaerobic conditions. Some possible pathways of anaerobic transformation of these compounds are described in Fig. 1. The known elimination of 5α and 6α hydrogens (4, 5, 38) was taken into consideration.

The β-epoxide and 7α,8β-diol have not been tested since the 1–3 sterical hindrance between the 8β position and the C18 and C19 methyl groups renders rather unlikely their formation in a biological system. The formation of Compounds *III* and *IV* has been supposed to derive from reductive cleavage of the corresponding hydroperoxy derivative (39).

Should the metabolism of Compound *V* proceed through an initial *trans* elimination of water, this might happen only between the 6β hydrogen and the αOH. However, since other authors have proved that this hydrogen is retained during cholesterol formation (4, 5, 40) triol *V* was not tested at first.

The failure of the tested compounds to be transformed into cholesterol under anaerobiosis (Table I) excludes their obligatory intermediary role in the biosynthesis of cholesterol from cholest-7-en-3β-ol. On the other hand, Compounds *I*, *III*, and *IV* are

TABLE I

Aerobic and anaerobic conversion of cholestan-7α,8α-epoxy-3β-ol and cholest-8-ene-3β,7ξ-diols by rat liver homogenates[a]

Experiment	Substrate	μg	μCi/μmole	Gas phase	Unsaponifiable total radioactivity	Cholest-7-en-3β-ol radioactivity	Cholesterol radioactivity
					%	%	%
1	Cholestan-7α,8α-epoxy-3β-ol (*I*)	5	23.2	N_2	89.6	72.5	2.10
1	Cholestan-7α,8α-epoxy-3β-ol (*I*)[b]	5	23.2	N_2	87.2	67.5	0.79
1	Cholest-7-en-3β-ol	5	11.0	N_2	79.7		2.60
1	Cholestan-7α,8α-epoxy-3β-ol (*I*)	5	23.2	O_2	98.9		45.7
1	Cholest-7-en-3β-ol	5	11.0	O_2	73.9		58.0
2	Cholest-8-ene-3β,7ξ-diol (diol A) (*III* or *IV*)	3	16.7	N_2	61.2	43.6	2.20
2	Cholest-8-ene-3β,7ξ-diol (diol A) (*III* or *IV*)[b]	3	16.7	N_2	63.8		0.10
2	Cholest-7-en-3β-ol	5	24.0	N_2	78.0		3.10
2	Cholest-8-ene-3β,7ξ-diol (diol A) (*III* or *IV*)	3	16.7	O_2	62.0		48.7
2	Cholest-7-en-3β-ol	5	24.0	O_2	81.2		75.0
3	Cholest-8-ene-3β,7ξ-diol (diol B) (*III* or *IV*)	6	16.7	N_2	65.2	40.9	0.47
3	Cholest-8-ene-3β,7ξ-diol (diol B) (*III* or *IV*)[b]	6	16.7	N_2	61.1	56.8	0.40
3	Cholest-7-en-3β-ol	5	24.0	N_2	89.0		0.70
3	Cholest-8-ene-3β,7ξ-diol (diol B) (*III* or *IV*)	6	16.7	O_2	53.6		36.3
3	Cholest-7-en-3β-ol	5	24.0	O_2	82.3		49.2

[a] The incubation conditions are reported in details under "Experimental Procedure."

[b] Unlabeled cholest-7-en-3β-ol, 200 μg, was added to the incubation mixture.

TABLE II

Aerobic and anaerobic conversion of cholestane-3β,7β,8α-triol, cholestane-3β,7α,8α-triol, cholestane-3β,8α-diol-7-one and cholest-8(14)-ene-3β,7α-diol by rat liver homogenates[a]

Substrate	μg	μCi/μmole	Gas phase	Unsaponifiable total radioactivity	Cholest-7-en-3β-ol radioactivity	Cholesterol radioactivity
				%	%	%
Cholestane-3β,7β,8α-triol (*II*)	5	16.7	N_2	87.3	55.6	0.90
Cholestane-3β,7α,8α-triol (*V*)	13	8.70	N_2	58.6	0.30	0.20
Cholestane-3β,8α-diol-7-one (*VI*)	19	18.7	N_2	81.6	29.4	0.90
Cholest-8(14)-ene-3β,7α-diol (*VII*)	5	18.7	N_2	100	59.0	0.40
Cholest-7-en-3β-ol	5	11.0	N_2	94.7		2.50
Cholestane-3β,7β,8α-triol (*II*)	5	16.7	O_2	78.0		51.0
Cholestane-3β,7α,8α-triol (*V*)	13	8.70	O_2	71.8		0.10
Cholestane-3β,8α-diol-7-one (*VI*)	19	18.7	O_2	78.9		48.0
Cholest-8(14)-ene-3β,7α-diol (*VII*)	5	18.7	O_2	81.7		66.1
Cholest-7-en-3β-ol	5	11.0	O_2	87.7		75.2

[a] The incubation conditions are reported in details under "Experimental Procedure."

efficiently transformed into cholest-7-en-3β-ol under the same experimental conditions (Table I).

This fact prompted us to test other sterol structures with oxygenated functions at Positions 7 and 8 and, eventually a double bond in 8(14) position. For this reason, labeled cholestane-3β,7β,8α-triol (*II*), cholestane-3β,7α,8α-triol (*V*), cholestane-3β,8α-diol-7-one (*VI*), and cholest-8(14)-ene-3β,7α-diol (*VII*) were incubated with rat liver homogenates and the results are reported (Table II). No conversion of triol (*V*) was observed either under aerobic or anaerobic conditions, while the dihydroxy ketone *VI* was efficiently transformed into cholest-7-en-3β-ol in anaerobiosis. These data allow us to exclude that the 7α hydroxy group of *V* is oxidized to a keto group as shown by Slaytor and Bloch (9) for cholest-7-ene-3β,6β-diol and cholest-7-ene-3β,6α-diol incubated with rat liver homogenates under anaerobiosis.

It was not attempted to demonstrate the metabolic pathways of the tested compounds. However, the results on the conversion of Compounds *III*, *IV*, and *VII* (Table II) demonstrate that rat liver homogenates are able to metabolize allylic alcohols containing oxygenated functions in Position 7. Moreover, cholest-7-en-3β-ol is the final product of the anaerobic metabolism of allylic alcohols containing oxygenated functions either at Position 6 (with a double bond in 7) (9) or, as suggested by Huntoon and Schroepfer (40), at position 15 (with a double bond in position 8(14)). A simple hypothesis for the conversion of Compounds *II* and *VI* would involve their transformation into the allylic alcohols *III* and *IV*, or *VII*. A *cis*-elimination of water may be postulated in this case both for triol *II* and dihydroxy ketone *VI*. A reaction of this type has been recently reported to occur in the biological conversion of ergost-7-ene-3β,5α-diol to ergosterol.[3] It may be postulated that the further tranformation of the allylic alcohols *III* and *IV* occur either via a concerted mechanism or through the intermediary formation of cholest-8-en-3β-ol which is converted afterwards to cholest-7-en-3β-ol (42). The last hypothesis is not valid in the case of Compound *VII*. In fact, it is known that cholest-8(14)-en-3β-ol is not converted to cholest-7-en-3β-ol by rat liver homogenates under anaerobiosis (14).

The oxygenated sterols which we incubated do not appear among the intermediates described in the biosynthesis of cholesterol. Nevertheless, the enzymes of rat liver homogenates are able to transform them. The results obtained demonstrate that either these sterols are precursors of cholest-7-en-3β-ol or that the system presents very little specificity for sterol structures.

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