The *in Vitro* Catabolism of Cholesterol: A Comparison of the Formation of Cholest-4-en-7α-ol-3-one and 5β-Cholestan-7α-ol-3-one from Cholesterol in Rat Liver*

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ABSTRACT: The enzymatic conversion of cholesterol to 7α-hydroxycholest-4-en-3-one and 7α-hydroxy-5β-cholestan-3-one has been studied using a preparation from rat liver *in vitro*. The data demonstrate that rat liver is able to metabolize cholesterol to 7α-hydroxycholest-4-en-3-one but not to 7α-hydroxy-5β-cholestan-3-one. It is suggested that 7α-hydroxycholest-4-en-3-one is probably an intermediate on the cholesterol to bile acid metabolic pathway. The results also provide indirect evidence that epimerization of the 3β-hydroxyl group of cholesterol to the α configuration may occur *via* ketone formation at position C-3 and, furthermore, that the Δ^5 double bond of cholesterol may be isomerized to the Δ^4 position before being reduced. A tentative scheme of the early stages in bile acid formation from cholesterol in rat liver has been presented.

During the conversion of cholesterol to bile acids chemical changes taking place in the nucleus of the former compound must inevitably include saturation of the Δ^5 double bond and epimerization of the 3β-hydroxyl group to the 3α position. It is not known with certainty at what point in the biochemical sequence these chemical reactions occur. Evidence has been presented (Bergström *et al.*, 1960; Mendelsohn *et al.*, 1965a) that 7α hydroxylation of cholesterol is an early stage in the cholesterol to bile acid metabolic pathway. Thus, it is probable that saturation of the Δ^5 double bond and epimerization of the 3β-hydroxyl group take place after the formation of 7α-hydroxycholesterol.

In a recent report, Green and Samuelsson (1964) have shown that during the biosynthesis of bile acids from cholesterol epimerization of the 3β-hydroxyl group involved the formation of a 3-keto steroid compound. Their findings also indicated that the Δ^5 double bond was isomerized to the Δ^4 position before being reduced. Therefore, in the further metabolism of 7α-hydroxycholesterol a possible intermediate might be cholest-4-en-7α-ol-3-one. However, since it is not known whether reduction of the Δ^4 double bond takes place before or after epimerization of the 3β-hydroxyl group, another intermediate may be postulated, namely, 5β-cholestan-7α-ol-3-one. The present communication compares the enzymatic conversion of cholesterol to cholest-4-en-7α-ol-3-one and 5β-cholestan-7α-ol-3-one by a rat liver preparation *in vitro*.

Experimental Procedure

All solvents were redistilled before use. Melting points were determined on a Kofler block and are uncorrected.

The synthesis of cholest-4-en-7α-ol-3-one was performed in three stages.

A. Formation of Cholesta-4,6-dien-3-one 6α,7α-Epoxide. Monoperphthalic acid was prepared as described by Fieser (1957). Five grams of cholesta-4,6-dien-3-one were dissolved in 300 ml of chloroform, mixed with 75 ml of dry diethyl ether containing 60–70 mg/ml of monoperphthalic acid, and allowed to stand 48 hr at room temperature. After pouring the mixture into 700 ml of 10% sodium bicarbonate, the organic layer was separated, washed with water, dried over sodium sulfate, and the solvent was removed *in vacuo*. Repeated crystallization of the residue from methanol gave 520 mg of cholest-4-en-3-one 6α,7α-epoxide, mp 138.5–139° [lit. (Nickon and Bagli, 1961) mp 138.5–139°].

B. Reduction of the Epoxy Ketone. The epoxide was dissolved in 20 ml of tetrahydrofuran (freshly distilled over sodium), 400 mg of lithium aluminum hydride was added, and the mixture was refluxed for 4 hr. Excess reagent was decomposed with 20 ml of ethyl acetate in diethyl ether (10% v/v of ethyl acetate in dry diethyl ether) and the mixture poured into 30 ml of an aqueous saturated solution of sodium potassium tartrate. The product was extracted six times with 50 ml of chloroform and the combined chloroform extracts were evaporated to dryness *in vacuo*. At this stage no attempt was made to crystallize and separate the mixed diols since the final stage of oxidation could be carried out just as effectively on the crude mixture.

C. Selective Oxidation of the Allylic Hydroxyl Group

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[1] The abbreviations used are: ATP, adenosine 5′-triphosphate; NAD, nicotinamide-adenine dinucleotide; nmr, nuclear magnetic resonance; plc, preparative layer chromatography; tlc, thin layer chromatography.

at Position C-3. The white solid residue was dissolved in 70 ml of dry chloroform and 7 g of finely powdered manganese dioxide (Mancera *et al.*, 1953) added. The mixture was stirred for 12–18 hr at room temperature after which time it was filtered through two thicknesses of Whatman No. 42 filter paper. The manganese dioxide was extracted six times with warm, dry chloroform and the combined extracts and filtrate were evaporated to dryness *in vacuo*. The residue was repeatedly recrystallized from methanol–water. The yield of cholest-4-en-7α-ol-3-one was 125 mg: mp 183–184° [lit. (Danielsson, 1961a) mp 183–184°]. The acetate prepared in the usual manner melted at 144–145° [lit. (Danielsson, 1961a) 144–145°]. Nmr and mass spectograph analyses of the sample were consistent with the structure cholest-4-en-7α-ol-3-one.

5β-Cholestan-7α-ol-3-one. Benzene and acetone were dried and freshly distilled before use. 5β-Cholestane-3α- (450 mg) and -7α-diol (Mendelsohn *et al.*, 1965b) dissolved in 5 ml of benzene was refluxed for 5 hr with 500 mg of aluminum isopropoxide in 10 ml of benzene and 7.5 ml of acetone. After acidifying the mixture with 2 N sulfuric acid the benzene layer was washed first with water, then with 1% sodium carbonate, and finally with water again. The organic layer was dried *in vacuo* and the residue was freed from mesityl oxide by the addition of 1 ml of freshly distilled xylene followed by evaporation under reduced pressure using a high vacuum pump. The residue was taken up in acetone and the ketone partially purified using the technique of plc (plc plates 20 × 40 cm, 1.0-mm thick silica gel, Kieselgel DS-5, Camag, Switzerland) with benzene–ethyl acetate (2:1) as solvent. After locating the ketone band on the plates with iodine it was eluted off the silica gel with methanol and the dried residue recrystallized twice with methanol–water. The yield of 5β-cholestan-7α-ol-3-one was 75 mg: mp 119–120° [lit. (Danielsson, 1961b) mp 121°]. The unreacted 5β-cholestane-3α,7α-diol was eluted off the silica gel with acetone and reutilized to form more ketone. Both ketones gave a single spot when subjected to tlc in three different solvent systems.

Cholesterol-4-^{14}C (specific activity 54.5 μc/mg) was obtained from the Radiochemical Centre, Amersham, England. The radioactive cholesterol was purified by elution with benzene through a neutral alumina column (activity grade 111) immediately prior to its use as substrate in the incubations.

Fractionation of the rat liver homogenates and incubation conditions were carried out as described previously (Mendelsohn and Staple, 1963), with the following modifications: (a) The homogenate (33% w/v) was centrifuged at 600*g* for 10 min to remove nuclei, cell debris, and unbroken cells, and the supernatant suspension was then centrifuged at 30,000*g* for 20 min. The supernatant from the latter centrifugation was used as the source of enzyme for the incubations. (b) When cholest-4-en-7α-ol-3-one was employed as trapping agent it became necessary to increase the amount of Tween 80 per flask from 4 mg to about 10 mg in order to obtain complete emulsification of the ketone. (c) Incubations with cholest-4-en-7α-ol-3-one were conducted aerobically with constant mechanical shaking for 30 min instead of 1 hr.

Isolation of Labeled Cholest-4-en-7α-ol-3-one and 5β-Cholestan-7α-ol-3-one. At the conclusion of the incubation period, 40 ml of 95% ethanol was poured into each flask. The contents of ten flasks were combined to which was added 10 mg of carrier ketone. After removal of the protein precipitate by centrifugation, it was washed once with an equal volume of acetone and the combined supernatant solutions were dried *in vacuo* at 37°. Methanol (50 ml) was added to dissolve the residue followed by 60 ml of water and the mixture was extracted four times with 40 ml of petroleum ether (bp 30–60°). The combined petroleum ether extracts were evaporated under reduced pressure, the residue was dissolved in a mixture of methanol–acetone (1:1) and further purified using the technique of multiple development plc. Solvents were chosen which gave the most effective separation between the respective ketones and cholesterol in order to minimize the chances of contamination with unreacted labeled cholesterol.

With respect to cholest-4-en-7α-ol-3-one, the dissolved residue was adsorbed onto the plates (0.5–1.0-mm thick silica gel; Kieselgel-DS-5, Camag, Switzerland) in the form of a thin band. As reference substance a spot of pure ketone was supplied on either side of the band. After developing the plates three times in the same solvent (isooctane–isopropyl ether–acetic acid (50:25:25)), the ketone was located by viewing under ultraviolet light. The ketone was eluted from the silica gel with methanol–acetone (1:1) and rerun three times in benzene–ethyl acetate (2:1). After elution from the silica gel, the dry residue was recrystallized from methanol–water to constant specific activity. The material obtained from the second crystallization was diluted with nonradioactive ketone and again crystallized to constant specific activity. The behavior of this material on tlc in 3 different chromatographic systems (benzene–isopropyl ether–acetic acid (30:10:1); benzene–ethyl acetate (2:1); isooctane–isopropyl ether–acetic acid (50:25:25)) was then compared with that of authentic cholest-4-en-7α-ol-3-one. After identification of the ketone under ultraviolet light, the bands were eluted from the silica gel with methanol–acetone (1:1), plated, and dried, and their radioactivity was assayed. In all instances the material behaved exactly like pure cholest-4-en-7α-ol-3-one and, furthermore, radioactivity was detected only in those areas occupied by the material and nowhere else on the chromatogram. This compound was further characterized by its melting point, mixture melting point behavior, and comparison of its infrared spectrum with that of authentic material.

In the experiments with 5β-cholestan-7α-ol-3-one, the petroleum ether residue from the incubation was chromatographed three times in benzene–acetic acid (95:5). The position of the ketone was located by staining a small portion of the plate with anisaldehyde reagent (Neher and Wettstein, 1951). After elution of the unstained material from the silica gel with methanol, it was evaporated to dryness and crystallized from

methanol–water. Because of the low specific activities found, the total amount of material was rechromatographed in benzene–acetic acid (95:5), eluted from the silica gel with methanol, evaporated *in vacuo*, and the residue recrystallized from methanol–water. Assay of radioactivity and further characterization of this ketone was carried out as described above.

Radioactivity of all samples was measured at infinite thinness in a windowless gas-flow counter. Counting was continued until the error was less than ±3%.

Results and Discussion

Recent studies on the degradation of cholesterol to bile acids have demonstrated: (a) that modifications in the nucleus precede oxidation of the side chain, and (b), of the nuclear changes taking place, inversion of the 3β-hydroxyl group and saturation of the Δ^5 double bond occurs after the introduction of an hydroxyl group at position C-7 (Bergström *et al.*, 1960).

Concerning the mechanism of epimerization of the 3β-hydroxyl group, this could theoretically occur in one of two ways: (1) Replacement of the hydroxyl group at position C-3 with inversion by attack of a second hydroxyl from the opposite side. (2) Oxidation of the 3β-hydroxyl group to a ketone followed by reduction to an alcohol with opposite configuration. Rosenfeld and Hellman (1961), after administration of 3α-tritiocholesterol to a patient with a bile fistula, were unable to isolate radioactive chenodeoxycholic acid and cholic acid from the bile. They concluded that cholesterol was converted to bile acids *via* a ketone intermediate. The fate of tritium during the conversion of cholesterol-3α-^{3}H-4-^{14}C and -4β-^{3}H-^{14}C to bile acids in the bile fistula rat was investigated by Green and Samuelsson (1964). Their results indicated *inter alia:* (a) that a 3-keto steroid was involved in the epimerization at position C-3, and (b) that the Δ^5 double bond was isomerized to the Δ^4 position before being reduced. It is of interest that a similar mechanism for the epimerization of cardiotonic steroids at position C-3 in rat liver has recently been proposed (Repke and Samuels, 1964).

Yamasaki *et al.* (1959a,b) incubated 7α-hydroxycholesterol with a cell-free preparation from rat liver, and on the basis of an increase in the optical density at 240 mμ which occurred during the incubation period, they proposed that 7α-hydroxycholesterol was being oxidized to a postulated metabolite, cholest-4-en-7α-ol-3-one. These workers also suggested that cholest-4-en-7α-ol-3-one was formed as an intermediate during the conversion of 7α-hydroxycholesterol to 5β-cholestan-3α,7α-diol. Danielsson (1961a) injected tritium-labeled cholest-4-en-7α-ol-3-one into a bile fistula rat and found that it was rapidly excreted in the bile as bile acids. Part of the radioactivity was present in chenodeoxycholic acid and cholic acid but several unidentified acids were also formed. However, no definite conclusion was drawn from this experiment as to whether cholest-4-en-7α-ol-3-one was an intermediate in bile acid metabolism in the rat or not.

The results presented in Table I demonstrate that rat liver is able to convert cholesterol to cholest-4-en-7α-ol-3-one *in vitro*. This finding lends considerable support to the contention that cholest-4-en-7α-ol-3-one is an intermediate in the conversion of cholesterol to bile acids. Our data also confirm those of Green and Samuelsson (1964) that during the formation of bile acids from cholesterol the Δ^5 double bond is probably isomerized to the Δ^4 position before being reduced.

With respect to the metabolism of 5β-cholestan-7α-ol-3-one, Danielsson (1961b) found that this compound was rapidly converted into bile acids when administered intraperitoneally to a bile fistula rat. Again,

TABLE I: Melting Points and Specific Activities of Cholest-4-en-7α-ol-3-one Isolated from the Incubation Mixtures.

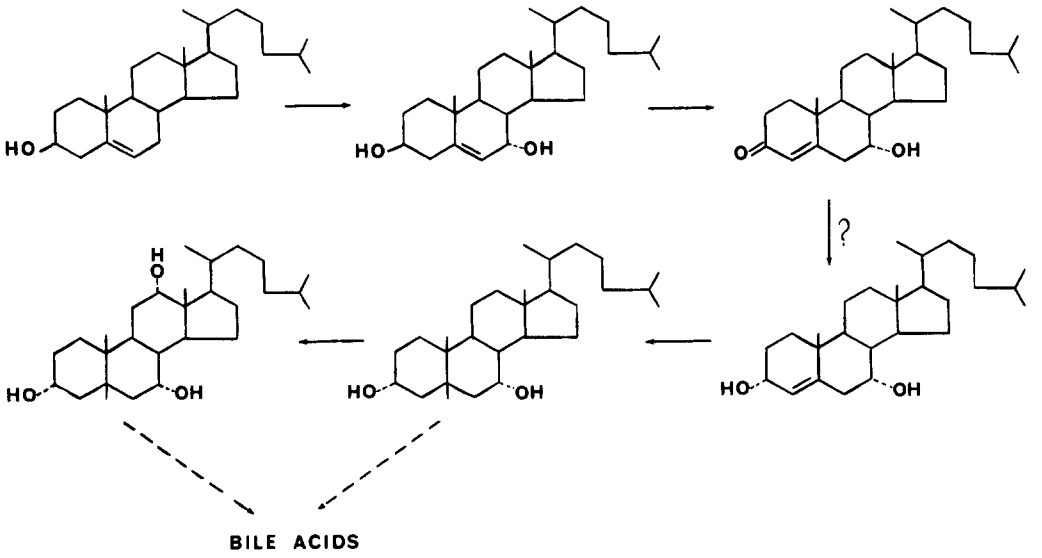
Recrystallization	Weight (mg)	Melting Point (°C)	Specific Activity (cpm/mg)
1. Methanol–water	6.5	180	140
2. Acetone–water	3.2[a]	181	143
3. Methanol–water	4.3	182.5–183	69
4. Petroleum ether (bp 80–100°)	2.7	182.5–183	71

[a] The 3.2 mg from the second crystallization was diluted with 3.2 mg of nonradioactive cholest-4-en-7α-ol-3-one to give a total weight of 6.4 mg which was again recrystallized from methanol–water. Conditions of incubation: Ten flasks were used for each incubation. Each flask contained 50,000 cpm of cholesterol-4-^{14}C, about 10 mg of Tween 80, 0.25 mg of cholest-4-en-7α-ol-3-one as trapping agent, 5 ml of phosphate buffer, pH 7.4, ATP (25 mg), NAD (5 mg), trisodium citrate dihydrate (22 mg), glutathione (15 mg), EDTA (10 mg), and 6 ml of enzyme equivalent to 2 g of rat liver. Incubation was at 37° for 30 min.

TABLE II: Melting Points and Specific Activities of 5β-Cholestan-7α-ol-3-one Isolated from the Incubation Mixtures.[a]

Recrystallization	Weight (mg)	Melting Point (°C)	Specific Activity (cpm/mg)
1. Methanol–water	4.9	120	4
2. Methanol–water	2.1	120	4

[a] Conditions of incubation: Ten flasks were used for each incubation. Each flask contained 50,000 cpm of cholesterol-4-^{14}C, 4 mg of Tween 80, and 0.25 mg of 5β-cholestan-7α-ol-3-one as trapping agent. The remainder of the contents were the same as described in Table I. Incubation at 37° was for 1 hr.



SCHEME I

it was not possible to conclude from this type of experiment whether or not 5β-cholestan-7α-ol-3-one was a true intermediate in the bile acid metabolic pathway. The data presented here (see Table II) show that 5β-cholestan-7α-ol-3-one incorporated virtually no radioactivity from cholesterol *in vitro*. We have previously demonstrated that rat liver is able to transform cholesterol into 5β-cholestane-3α,7α-diol (Mendelsohn *et al.*, 1965b) and 5β-cholestane-3α,7α,12α-triol (Mendelsohn and Staple, 1963). Since both the latter compounds incorporate significant amounts of radioactivity from cholesterol and further along the postulated bile acid metabolic pathway than 5β-cholestan-7α-ol-3-one, it appears, therefore, that under the experimental conditions described above, 5β-cholestan-7α-ol-3-one cannot be considered as an intermediate in the conversion of cholesterol to bile acids. A further point arising out of our results concerns that stage at which saturation of the Δ^4 double bond occurs. The fact that no radioactivity was observed in 5β-cholestan-7α-ol-3-one indicates that reduction of the 3-keto group to the corresponding α alcohol does not take place in the saturated keto steroid. Rather, it is probable that inversion of the 3β-hydroxyl group to the α configuration occurs just prior to or simultaneously with reduction of the Δ^4 double bond. If reduction of the 3-keto group takes place just before saturation of the Δ^4 double bond then the following compound should theoretically be formed, cholest-4-ene-3α,7α-diol. However, it might not be feasible to detect the latter as an intermediate if the reductions occur simultaneously. These possibilities are being currently investigated.

There is reasonable evidence (both *in vivo* and *in vitro*) to show that the following compounds are probably intermediates in the conversion of cholesterol to bile acid: 7α-hydroxycholesterol (Lindstedt, 1957; Mendelsohn *et al.*, 1965a), 5β-cholestane-3α,7α-diol (Bergström and Lindstedt, 1956; Mendelsohn *et al.*, 1965b), and 5β-cholestane-3α,7α,12α-triol (Bergström *et al.*, 1960; Mendelsohn and Staple, 1963). Taken in conjunction with the present findings the sequence of the early stages in bile acid formation from cholesterol is tentatively suggested (Scheme I).

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The Biosynthesis of Ether-Containing Phospholipids in the Slug, *Arion ater*. II. The Role of Glyceryl Ether Lipids as Plasmalogen Precursors*

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ABSTRACT: Further studies of *in vivo* plasmalogen formation in *Arion ater* have been carried out. Under a variety of environmental conditions, incorporation of [1-^{14}C]palmitic acid into plasmalogens is much lower than incorporation into diacyl and glyceryl ether phospholipids. However, this depressed incorporation into plasmalogens is observed only in experiments of relatively short duration. After periods of 2 days or longer, the vinylic ether side chain of plasmalogens does become labeled from fed radioactive palmitic acid or glyceryl ethers. Evidence is presented that the ether-linked side chain of glyceryl ether phospholipids is the direct precursor of the plasmalogen vinylic ether side chain.

Studies carried out over the past few years (Rapport and Norton, 1962; Hartree, 1964) have demonstrated plasmalogens to be major lipid constituents in many animal tissues. Even though these lipids have a wide distribution in nature, the mechanism of their biosynthesis has not been determined.

During the course of a recent study on the structurally similar glyceryl ether phospholipids of *Arion ater*, we observed that the plasmalogens appeared to be metabolically quite inert (Thompson, 1965). The glyceryl ether phospholipids and the diacyl phospholipids rapidly incorporated radioactivity from fed [1-^{14}C]-palmitic acid and [6-^{14}C]glucose. However, over the time intervals studied (1–16 hr) the plasmalogens showed a low uptake of ^{14}C, with the specific radioactivity of their aldehydogenic side chains being only 10–20% of that found for side chains of the other phospholipids.

These observations are reminiscent of the results obtained by other investigators who have studied plasmalogen biosynthesis. When fatty acid incorporation into brain lipids was examined (Debuch, 1964; Carr *et al.*, 1963) and when the uptake of possible lipid precursors by heart was measured (Keenan *et al.*, 1961), esterified fatty acids invariably appeared to be formed much more rapidly than the ether-bound plasmalogen side chain. Bauman *et al.* (1965) have reported unusually active plasmalogen biosynthesis by rapidly growing cultures of *Clostridium butyricum*. Although the final rate of plasmalogen formation was high, there was a lag period before synthesis was detected.

In the experiments reported here plasmalogen synthesis in *A. ater* has been reexamined with particular attention to possible explanations for its low rate of formation.

Experimental Section

Lipid Analyses. Methods have previously been described for isolating and analyzing the *Arion* lipids (Thompson and Hanahan, 1963; Thompson, 1965). Briefly, the lipids were extracted with chloroform–methanol 1:1 (v/v) and, after removal of nonlipid contaminants, were chromatographed on silicic acid columns at 4°. The glyceryl ethers and glyceryl vinylic ethers[1] were prepared by $LiAlH_4$ hydrogenolysis (Thompson, 1965). The $LiAlH_4$ products were purified by silicic acid column chromatography (Thompson and

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[1] The term glyceryl vinylic ether denotes those 1-*O*-ethers of glycerol in which there is unsaturation between the α- and β-carbon atoms of the hydrocarbon chain.