# Bile acid formation in man: metabolism of 7α-hydroxy-4-cholesten-3-one in bile fistula patients

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**Abstract** 7α-Hydroxy-4-cholesten-3-one is thought to be an intermediate in human bile acid synthesis. This conclusion is based on in vivo experiments in animals and on in vitro studies in which homogenates of animal and human livers were used. To further establish that this compound is an intermediate in human bile acid synthesis, its metabolism was studied in subjects with complete bile fistulas. After administration of $^3$H-labeled 7α-hydroxy-4-cholesten-3-one by single intravenous injection, approximately 85% of the administered isotope was recovered in the bile during the first 12 hr. More than 96% of the radioactivity recovered in the bile was identified as either chenodeoxycholic acid or cholic acid, with only a trace amount of the radioactivity present as neutral sterols. This study gives support to the hypothesis that 7α-hydroxy-4-cholesten-3-one is a natural intermediate in human bile acid synthesis.

**Supplementary key words** cholic acid · chenodeoxycholic acid

THE MAJOR PATHWAY for human bile acid synthesis is postulated to follow the reaction sequence shown in Fig. 1. These reactions are based on studies using rats with bile fistulas (1–6) and liver homogenates from rat (1–4), guinea pig (7), and man (8). The initial steps in the synthesis of cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) and chenodeoxycholic acid (3α,-7α-dihydroxy-5β-cholan-24-oic acid) are thought to be identical through the formation of the intermediate 7α-hydroxy-4-cholesten-3-one. The pathway is then assumed to bifurcate depending upon whether this intermediate undergoes 12α-hydroxylation. If hydroxylated, this substrate is channeled into cholic acid synthesis; if it is not hydroxylated it is shunted into chenodeoxycholic acid synthesis. The present investigation was undertaken to determine if 7α-hydroxy-4-cholesten-3-one administered intravenously is converted into cholic acid and chenodeoxycholic acid in man.

Presented in part at the annual meeting of the American Federation for Clinical Research held at Atlantic City, N.J., 30 April 1972.

## METHODS

### Synthesis and labeling

7α-Hydroxy-4-cholesten-3-one was prepared using the method of Björkhem et al. (5). Identification of the synthetic product was done as follows. The melting point of this compound crystallized from methanol–water was 180°C; this was comparable to that reported by Björkhem et al. (5) and Danielsson (6). The characteristic ultraviolet absorption maximum at 244 nm for $\Delta^4$-3-keto steroids (9) was observed ($\epsilon$ = 16,000; reported $\epsilon$, 16,000 [4]). Mass spectroscopy, using an RMU6-D Hitachi–Perkin-Elmer instrument (Perkin-Elmer Corp., Norwalk, Conn.), disclosed a molecular ion of $m/e$ 400, which corresponds to the calculated molecular weight of the compound.

Labeling was carried out using tritium enolic exchange on aluminum oxide chromatography (10). After repeated recrystallization, the [2ξ,4-$^3$H]7α-hydroxy-4-cholesten-3-one had a specific activity of 1.2 μCi/mg. Radiopurity was demonstrated as follows. 5 mg of unlabeled 7α-hydroxy-4-cholesten-3-one was added to some of the tritiated material (450,000 dpm). This mixture was chromatographed on alumina (neutral, activity grade III) using 30% ethyl acetate in benzene as the

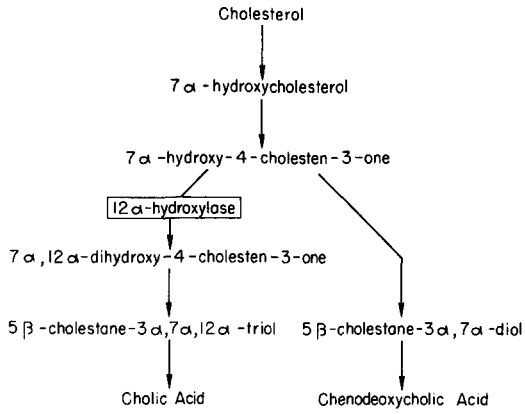


FIG. 1. Diagramatic representation of bile acid synthesis from cholesterol. The proposed pathways are shown to branch at the point of 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one.

moving phase. The eluant was collected and single coincident peaks were obtained for both the radioactivity and absorbance at 244 nm. The purity of the labeled compound was found to be 98% on thin-layer chromatography using benzene–ethyl acetate 3:7 (v/v) as the developing solvent.

### Patients

Two patients who had T-tube bile fistulas were studied after informed consent had been obtained. They were judged to have complete external bile fistulas by (*a*) the absence of fecal urobilinogen (less than 5 mg/100 g stool) and (*b*) the absence of secondary bile salts in their bile. Bile was collected in a container on the floor, and presumably the siphoning effect of the bile in the T tube accounted for the completeness of the fistula. Both patients were studied approximately 15–20 days after placement of the T tube when they were eating a regular diet and when their liver function tests were normal. These two patients were studied after single injections (10.2 and 7.9 μCi, respectively) of [2ξ,4-$^3$H]-7α-hydroxy-4-cholesten-3-one, which was dissolved in sterile absolute ethanol and injected intravenously.

### Analytical methods

All solvents were reagent grade or were distilled prior to use. Individual bile samples were collected each 30–60 min and diluted with an equal volume of ethanol; the fractions were stored at 3°C. Hydrolysis of the bile acids was carried out in 4.5 N NaOH in a steel bomb at 130°C for 24 hr. The hydrolysate was diluted with an equal volume of water, the pH was adjusted to 1 with 12 N HCl, and the solution was then extracted with diethyl ether. After evaporation of the ether extracts, the bile acids were separated by partition chromatography on Celite (11) as described previously (12). Methylation of bile acids was done using diazomethane.

Radioactivity was measured in a Beckman model LS-250 scintillation counter using Biosolv 3 solubilizer and Fluoralloy TLA counting mixture (Beckman Instruments, Fullerton, Calif.).

## RESULTS

### Formation of bile acids from [2ξ,4-$^3$H]7α-hydroxy-4-cholesten-3-one

There was rapid excretion of the radioactivity in the bile following a single intravenous injection of labeled 7α-hydroxy-4-cholesten-3-one. Both patients studied excreted approximately 85% of the administered radioactivity during the first 12 hr of the study.

Aliquots of each bile sample collected from both patients during the first 12 hr after injection were combined and extracted twice with an equal volume of petroleum ether (bp 60–70°C) to determine if any of the administered radioactivity was excreted as neutral sterols. The petroleum ether extracts were washed with water and counted. There were similar results for both patients. Only 0.5% of the radioactivity in the combined sample was extracted with petroleum ether. As a control, 300,000 dpm of [2ξ,4-$^3$H]7α-hydroxy-4-cholesten-3-one was added to a 50% solution of nonradioactive bile in ethanol and extracted in a similar fashion with petroleum ether. Over 94% of the added radioactivity was present in the first petroleum ether extract.

The remaining combined aliquots of the bile excreted during the first 12 hr were evaporated and hydrolyzed. After hydrolysis a separate extraction with petroleum ether was carried out and no radioactivity above background was found in the extract. After acidification (pH $< 1$) the bile acids were extracted with diethyl ether and chromatographed by partition chromatography on Celite columns (1 × 30 cm), using 100-ml volumes for the moving phase. The eluant was collected in fractions and counted. As shown in Fig. 2, there were two major peaks of radioactivity, which were located in the 40% and 80% benzene fractions. These two peaks accounted for over 96% of the radioactivity placed on the column. The two smaller peaks in the 20% and 60% benzene fractions were not further identified.

The material eluted in the 40% and 80% benzene fractions was identified as chenodeoxycholic and cholic acid, respectively, by reverse isotope dilution as shown in Table 1. The specific activities of the recrystallized bile acids and methyl ester derivatives were constant after the first recrystallization.

## DISCUSSION

The commonly accepted criteria that must be satisfied before a compound can be considered an intermediate

TABLE 1. Recrystallization of the material in the 40% (chenodeoxycholic acid) and 80% (cholic acid) benzene fractions from patients 1 and 2

| Patient | Cholic Acid | | | | Chenodeoxycholic Acid | | | |
|---|---|---|---|---|---|---|---|---|
| | Radioactivity Added[a] | Solvent | Wt | Sp Act | Radioactivity Added[a] | Solvent | Wt | Sp Act |
| | *dpm* | | *mg* | *dpm/mg* | | | *mg* | *dpm/mg* |
| 1 | 54,000 | Ethyl acetate | 70.5 | 643 | 98,000 | Ethyl acetate–heptane | 76.3 | 743 |
| | | Ethyl acetate | 50.0 | 526 | | Ethyl acetate–heptane | 50.8 | 950 |
| | | Acetone–water | 36.8 | 528 | | Ethyl acetate | 46.1 | 957 |
| | | Methyl cholate | | | | Methyl chenodeoxycholate | | |
| | | Methanol–water | 15.2 | 527 | | Benzene–heptane | 36.2 | 974 |
| 2 | 165,000 | Ethyl acetate | 85.3 | 1625 | 700,000 | Ethyl acetate–heptane | 80.8 | 6050 |
| | | Ethyl acetate | 72.0 | 1635 | | Ethyl acetate–heptane | 7.13 | 6654 |
| | | Acetone–water | 52.6 | 1582 | | Ethyl acetate | 56.1 | 6614 |
| | | Methyl cholate | | | | Methyl chenodeoxycholate | | |
| | | Methanol–water | 28.7 | 1610 | | Benzene–heptane | 31.2 | 6903 |

[a] The radioactive material was added to 100 mg of unlabeled authentic bile acid and recrystallized using the solvents indicated.

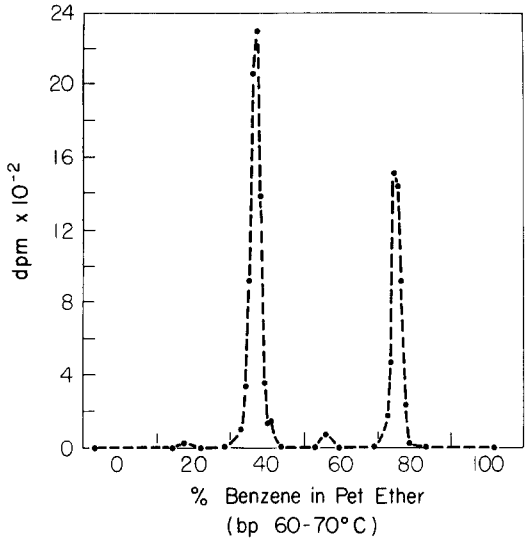


FIG. 2. Chromatographic separation of radioactivity in bile after administration of $^3$H-labeled 7$\alpha$-hydroxy-4-cholesten-3-one. Aliquots of the bile collected during the first 12 hr after administration were combined, hydrolyzed, and chromatographed on a Celite partition column.

in bile synthesis are (*1*) isolation and identification of the proposed intermediate from liver tissue or bile, (*2*) conversion of isotopically labeled cholesterol into the intermediate in vivo, and (*3*) rapid and efficient metabolism of the synthetic labeled intermediate into bile acids in vivo.

Based on studies using bile fistula rats (5, 6) and liver homogenates from rat (4, 13, 14) and man (8), 7$\alpha$-hydroxy-4-cholesten-3-one has been assumed to be an intermediate in human bile acid synthesis. The present study demonstrated that 7$\alpha$-hydroxy-4-cholesten-3-one administered intravenously is rapidly and efficiently metabolized to cholic and chenodeoxycholic acid by the human liver. Therefore, this study provides additional evidence that 7$\alpha$-hydroxy-4-cholesten-3-one is an intermediate in human bile acid synthesis. However, final proof that this compound is a precursor in bile acid synthesis must await its isolation from liver tissue or bile and the demonstration that it can be formed from cholesterol in vivo.

This study was supported by grant AM 15077-01, by a grant (RR-400) from the General Clinical Research Centers Program of the Division of Research Resources, National Institutes of Health, by the Robert Pollock Memorial Research Fund, and by the Atomic Energy Commission.

*Manuscript received 24 July 1972; accepted 21 September 1972.*

## REFERENCES

1. Danielsson, H., and T. T. Tchen. 1968. Steroid metabolism. *In* Metabolic Pathways. 3rd ed. D. M. Greenberg, editor. Academic Press, New York. 141–149.
2. Elliott, W. H., and P. M. Hyde. 1971. Metabolic pathways of bile acid synthesis. *Amer. J. Med.* **51:** 568–579.
3. Lindstedt, S. 1970. Catabolism of cholesterol by way of bile acids. *In* Atherosclerosis: Proceedings of the Second International Symposium. R. J. Jones, editor. Springer-Verlag, New York. 262–271.
4. Einarsson, K. 1968. On the properties of the 12$\alpha$-hydroxylase in cholic acid biosynthesis. *Eur. J. Biochem.* **5:** 101–108.
5. Björkhem, I., H. Danielsson, C. Issidorides, and A. Kallner. 1965. On the synthesis and metabolism of cholest-4-en-7$\alpha$-ol-3-one. *Acta Chem. Scand.* **19:** 2151–2154.
6. Danielsson, H. 1961. Synthesis and metabolism of $\Delta^4$-cholestene-7$\alpha$-ol-3-one. *Acta Chem. Scand.* **15:** 242–248.
7. Björkhem, I., H. Danielsson, and K. Einarsson. 1967. On the conversion of cholesterol to 5$\beta$-cholestane-3$\alpha$,7$\alpha$-diol in guinea pig liver homogenates. *Eur. J. Biochem.* **2:** 294–302.
8. Björkhem, I., H. Danielsson, K. Einarsson, and G. Johansson. 1968. Formation of bile acids in man: conversion of cholesterol into 5$\beta$-cholestane-3$\alpha$,7$\alpha$,12$\alpha$-triol in liver homogenates. *J. Clin. Invest.* **47:** 1573–1582.
9. Fieser, L. F., and M. Fieser. 1959. Steroids. Reinhold Publishing, New York. 15.

10. Hofmann, A. F., P. A. Szczepanik, and P. D. Klein. 1968. Rapid preparation of tritium-labeled bile acids by enolic exchange on basic alumina containing tritiated water. *J. Lipid Res.* **9:** 707–713.

11. Mosbach, E. H., C. Zomzely, and F. E. Kendall. 1954. Separation of bile acids by column-partition chromatography. *Arch. Biochem. Biophys.* **48:** 95–101.

12. Hanson, R. F., and G. Williams. 1971. The isolation and identification of 3$\alpha$,7$\alpha$-dihydroxy-5$\beta$-cholestan-26-oic acid from human bile. *Biochem. J.* **121:** 863–864.

13. Johansson, G. 1970. Effect of cholestyramine and diet on hydroxylations in the biosynthesis and metabolism of bile acids. *Eur. J. Biochem.* **17:** 292–295.

14. Danielsson, H., K. Einarsson, and G. Johansson. 1967. Effect of biliary drainage on individual reactions in the conversion of cholesterol to taurocholic acid. *Eur. J. Biochem.* **2:** 44–49.