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Bile Alcohol Metabolism in Man

CONVERSION OF
5β-CHOLESTANE-3α,7α,12α,25-TETROL TO CHOLIC ACID

Gerald Salen, S. Shefer, T. Setoguchi, and E. H. Mosbach

From the Veterans Administration Hospital, East Orange, New Jersey 07019; Department of Medicine, College of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, New Jersey 07102; and The Public Health Research Institute of the City of New York, Inc., New York 10016

Abstract To study the role of Cα-hydroxy bile alcohols as precursors of cholic acid, [G-4H]5β-cholestane-3α,7α,12α,25-tetrol was administered intravenously to two subjects with cerebrotendinous xanthomatosis (CTX) and two normal individuals. One day after pulse labeling, radioactivity was present in the cholic acid isolated from the bile and feces of the subjects with CTX and the bile of the normal individuals. In the two normal subjects, the sp act decay curves of [G-4H]-cholic acid were exponential, and no traces of [G-4H]-5β-cholestane-3α,7α,12α,25-tetrol were detected. In contrast, appreciable quantities of labeled 5β-cholestan-3α,7α,12α,25-tetrol were present in the bile and feces of the CTX subjects. The sp act vs. time curves of fecal [G-4H]5β-cholestane-3α,7α,12α,25-tetrol and [G-4H]-cholic acid showed a precursor-product relationship. Although these results suggest that 5β-cholestane-3α,7α,12α,25-tetrol may be a precursor of cholic acid in man, the possibility that Cα-hydroxy intermediates represent the normal pathway can not be excluded.

Introduction In the rare inherited sterol storage disease, cerebrotendinous xanthomatosis (CTX), bile acid production is subnormal (1) but considerable quantities of Cα bile alcohols are excreted in bile and feces (2). Two bile alcohols have been identified conclusively: 5β-cholestane-3α,7α,12α,25-tetrol and 5β-cholestan-3α,7α,12α,24α,26-pentol (2), and thus it appears that diminished bile acid synthesis in CTX results from defective oxidation of the cholesterol side chain. However, according to current views on bile acid biosynthesis, bile alcohols hydroxylated at carbon 25 are not considered precursors of bile acids (3). Fig. 1 illustrates two possible pathways for cholic acid synthesis from cholesterol. The steroid nucleus undergoes change first and the key rate-determining reaction is the transformation of cholesterol (1) to 7α-hydroxycholesterol (II) followed by oxidation of the hydroxyl group on carbon 3 to a ketone and migration of the double bond to the 4,5 position to form 7α-hydroxycholesterol-4-en-3-one (III). At this point, 7α-hydroxycholesteryl-4-en-3-one (III) is 12α-hydroxylated to yield 7α,12α-dihydroxycholesterol-4-en-3-one (IV), and this compound is reduced to 5β-cholestane-3α,7α,12α-triol (V). Side chain degradation now proceeds, and according to Staple (4), 5β-cholestane-3α,7α,12α-triol is hydroxylated at carbon 26 to form 5β-cholestane-3α,7α,12α,26-tetrol (VI), which is oxidized to 3α,7α,12α-trihydroxy-5β-cholestanolic acid and then hydroxylated at carbon 24, forming 3α,7α,12α,24α-tetrahydroxy-5β-cholestanolic acid (VII). β-Oxidation of the Coenzyme A derivative of this compound gives cholic acid (X) and propionic acid. However, the accumulation of considerable quantities of the two bile alcohols hydroxylated at carbon 25 in CTX suggested that cholic acid may be produced from 5β-cholestane-3α,7α,12α,25-tetrol and 5β-cholestane-3α,7α,12α,24α,25-pentol. In this study, the formation of bile acid from radioactive 5β-cholestane-3α,7α,12α,25-tetrol was examined in normal and CTX individuals. The results show that 5β-cholestane-3α,7α,-

*The following systematic names are given to bile acids referred to by trivial names: cholic acid, 3α,7α,12α-trihydroxy-5β-cholestanolic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholestanolic acid.
12α,25-tetrol was indeed converted into cholic acid in man.

METHODS

Clinical. Studies were conducted in two subjects with CTX (E. E. and E. S.) and two normolipidemic individuals (J. S. and A. S.). Complete clinical descriptions and metabolic data of the CTX subjects have been published (1, 5). The patients were hospitalized at the East Orange Veterans Administration Hospital and were fed regular hospital diets during the course of this investigation.

Steroid analyses. Stool specimens were collected from both CTX subjects and were combined into 2-day pools. The stools were mixed with water, homogenized, and stored at −20°C; portions were lyophilized, and the dry powder was used for steroid analyses. A portion of each pool was analyzed for the purposes of the present study. Bile was collected by duodenal intubation after stimulation of the gall bladder with cholecystokinin (obtained from Professor Victor Mutt, Karolinska Institute, Stockholm, Sweden). The bile was either lyophilized and the dry powder used for analyses or deproteinized with ethanol and used after evaporation of the alcohol.

Extraction of bile alcohols and bile acids from bile and feces. A weighed specimen of dried bile or feces was extracted with a mixture of 300 ml of ethanol and 2 ml of concentrated NH₄OH in a Soxhlet apparatus. The ethanol extract was evaporated on a 60°C water bath under nitrogen leaving a residue that was suspended in 200 ml of water containing 1 ml of concentrated NH₄OH.

The neutral lipids (cholesterol, cholestanol, sterol precursors, and bile alcohols) were extracted from the aqueous suspension containing the conjugated bile acids with three 100-ml portions of ethyl acetate. The ethyl acetate extracts were combined, washed, and the solvent removed in a rotary evaporator. The neutral lipids were dissolved in 5 ml of ethyl acetate.

To isolate the bile acids, the remaining aqueous phase was concentrated to about 100 ml by evaporation under nitrogen. About 8 g of NaOH was added to make the solution 2 N, and the mixture was heated at 120°C in an autoclave for 3 h. The mixture was cooled in ice and acidified to pH 2 by the addition of concentrated HCl. 200 ml of peroxide-free ethyl ether was added, and the free bile acids were extracted into the ether phase; two additional extractions with 100-ml portions of ether were performed. The ether phases were pooled, washed with water, and evaporated in a rotary evaporator.

Isolation of 5β-cholestan-3α,7α,12α,25-tetrol. The ethyl acetate extract was applied to an alumina column (neutral alumina, grade V, 100-200 mesh, Calbiochem, San Diego, Calif.). Neutral sterols (cholesterol, cholestanol, and plant sterols) were eluted with 100 ml ethyl acetate. 5β-cholestane-3α,7α,12α,25-tetrol was eluted with 100 ml 5% methanol in ethyl acetate, and the pentahydroxy bile acids were eluted with 100 ml of 10% methanol in ethyl acetate. The fraction eluted with 5% methanol in ethyl acetate was obtained after evaporation of the solvent and the residue of 5β-cholestane-3α,7α,12α,25-tetrol was further purified by TLC. The bile alcohol was applied as a 6-cm band along

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TABLE I

Radioactivity and Mass of Methyl Cholate Isolated from Bile

<table>
<thead>
<tr>
<th>Subject</th>
<th>Days after pulse labeling</th>
<th>Specimen</th>
<th>Methyl cholate* (dpm)</th>
<th>Methyl cholate (mg)</th>
<th>dpm/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. S.</td>
<td>1</td>
<td>Bile</td>
<td>3,700</td>
<td>13.8</td>
<td>270</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>9,800</td>
<td>58.1</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td>3,200</td>
<td>36.8</td>
<td>90</td>
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<tr>
<td></td>
<td>4</td>
<td></td>
<td>400</td>
<td>11.2</td>
<td>30</td>
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<tr>
<td></td>
<td>8</td>
<td></td>
<td>300</td>
<td>153.0</td>
<td>2</td>
</tr>
<tr>
<td>J. S.</td>
<td>1</td>
<td>Bile</td>
<td>18,200</td>
<td>57.3</td>
<td>320</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>4,100</td>
<td>32.7</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td>1,000</td>
<td>21.2</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td></td>
<td>100</td>
<td>4.3</td>
<td>20</td>
</tr>
</tbody>
</table>

* The radiopurity and isotope stability of [G-3H]methyl chololate were confirmed by a single crystallization of each specimen from ethanol: water (9:1, vol/vol).

with a reference standard of authentic 5β-cholestan-3α,7α,12α,25-tetrol (2, 6) to 20 x 20-cm glass plates coated with a 0.25-mm thick layer of silica gel G (Analtech, Inc., Newark, Del.), and the plates were developed in chloroform: acetone : methanol, 35:25:5 (vol/vol). The band corresponding to authentic 5β-cholestan-3α,7α,12α,25-tetrol (Rf = 0.46) was removed from the plate and eluted with methanol after visualization with I2 vapor. The methanol was evaporated, and the cholestanetetrol was redissolved in exactly 4 ml of methanol containing 100 μg of 5α-cholestan as an internal standard. 3 ml was taken for radioactivity assay and the remainder for quantitation by GLC.

Isolation and purification of cholic acid. The free bile acids isolated from the feces or bile were applied to 20 x 20-cm glass plates coated with a 0.25-mm layer of silica gel G along with a reference standard of cholic acid. The plates were developed in glacial acetic acid : diisopropylether : isooctane, 35:25:50 (vol/vol). Cholic acid (Rf = 0.35) was eluted with methanol after visualizing the band with I2 vapor. The solvent was evaporated and the residue was dissolved in 5 ml of methanol containing 0.5 ml concentrated H2SO4 to form the methyl ester. The next morning 5 ml of water was added and methyl cholate was extracted with 20 ml of ethyl ether: benzene, 2:1 (vol/vol). The benzene-ethyl ether layer was collected and washed to neutrality with an aqueous solution of saturated NaHCO3 and water and then was evaporated. Trace amounts of methyl allocholate were removed by thin-layer chromatography (TLC) after the methyl cholate fraction was applied to glass plates coated with 0.25-mm thick layers of Silica Gel G and developed with methanol : acetone : chloroform, 5:25:70 (vol/vol). The Rf of methyl allocholate is 0.35, and the Rf of methyl cholate is 0.40. Methyl cholate was eluted with methanol after visualization with I2 vapor. The solvent was evaporated and the residue was dissolved in exactly 5 ml of methanol containing 100 μg of 5α-cholestan as an internal standard. 4 ml was taken for radioactivity assay and the remainder for gas-liquid chromatography (GLC).

GLC. 5β-cholestan-3α,7α,12α,25-tetrol and methyl cholate were quantitated as trimethylsilyl derivatives using 5α-cholestan as an internal standard and authentic 5β-cholestan-3α,7α,12α,25-tetrol and methyl cholate as external standards. Aliquots containing 2-10 μg of 5β-cholestan-3α,7α,12α,25-tetrol or methyl cholate were injected into 6-ft glass columns packed with 3% QF-1 on Gas Chrom Q (100-120 mesh, Applied Science Labs, Inc., State College, Pa.). The instrument was a Hewlett-Packard Model 7610 A (Hewlett-Packard Co., Palo Alto, Calif.) equipped with a flame ionization detector and electronic integrator. The following operating conditions were employed: column temperature, 230°C; detector, 250°C; and flash heater, 250°C.

Radioactivity measurements. Radioactivity was measured in a Beckman LS-200 B liquid scintillation spectrometer (Beckman Instruments, Inc., Fullerton, Calif.). Appropriate corrections were made for quench and background. The counting efficiency for tritium was 51%.

Preparation of [G-3H]5β-cholestan-3α,7α,12α,25-tetrol. 5β-cholestan-3α,7α,12α,25-tetrol (6) was tritium labeled by the Wilzbach technique at New England Nuclear, Boston, Mass. (7). The radioactive material (5 mg) was diluted with pure, unlabeled tetrol (5 mg) and purified by chromatography on alumina and then recrystallized from ethyl acetate to constant specific radioactivity. The radio purity of this material was monitored by repeated TLC and found to be 98% cholestanetetrol. The purified labeled tetrol (20 μCi/mg) was dissolved in ethanol to give a solution containing 2 μCi/ml. Each patient received 2 μCi of labeled tetrol dispersed in 150 ml of 0.9% NaCl solution by intravenous injection.

RESULTS

Conversion of 5β-cholestan-3α,7α,12α,25-tetrol into cholic acid

Normal man. About 2 μCi of [G-3H]5β-cholestan-3α,7α,12α,25-tetrol was injected intravenously into two normolipidemic men, and samples of intestinal bile were obtained over the next 9 days. Cholic acid was isolated and purified from each bile specimen. The results are presented in Table I and Fig. 2. In both individuals, the cholic acid was labeled with tritium which indicated that [G-3H]5β-cholestan-3α,7α,12α,25-tetrol had been converted to the bile acid. The highest sp act was found 1 day after intravenous pulse labeling showing that the bile alcohol was transformed rapidly into cholic acid. The subsequent exponential decay curve ruled out continuous formation of cholic acid from a slowly turning over pool of bile alcohol, a finding supported by the absence of mass or radioactivity corresponding to 5β-cholestan-3α,7α,12α,25-tetrol in the bile. As expected, no radioactivity was detected in chenodeoxycholic acid isolated from these subjects.

CTX. After the intravenous injection of 2 μCi of [G-3H]5β-cholestan-3α,7α,12α,25-tetrol to two individu-

*Only cholestnanetetrol was purified from these specimens; the cholesthanepentol fractions contained several pentahydroxy bile alcohols including 5β-cholestan-3α,7α,12α,24α,25-pentol, 5β-cholestan-3α,7α,12α,23δ,25-pentol, and 5β-cholestan-3α,7α,12α,22δ,25-pentol. The role of these bile alcohols as precursors of cholic acid remains to be established and is currently under investigation.

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als with CTX, one sample of intestinal bile was obtained the following day, and specimens of feces were collected for the ensuing 10 days. 5β-cholestan-3α,7α,12α,25-tetrol and cholic acid were isolated and purified from each specimen and the results are presented in Table II and Fig. 3A and B. 1 day after intravenous pulse-labeling, tritium was present in 5β-cholestan-3α,7α,12α,25-tetrol and cholic acid from both bile and feces. Unlike in the normal individuals, appreciable quantities of 5β-cholestan-3α,7α,12α,25-tetrol were detected in both bile and feces. The difference in the sp act of biliary and fecal [G-3H]5β-cholestan-3α,7α,12α,25-tetrol might be expected since biliary [G-3H]5β-cholestanetetrol sp act represents the average value of a 10-min collection of bile while fecal [G-3H]5β-cholestanetetrol reflects the average value found in a 2-day stool collection plus the time lag necessary to transit the intestine. The sp act of cholic acid from the bile of the CTX subjects 1 day after pulse labeling was one-sixth as high as in the normolipidemic subjects even though all individuals received the same dose of [G-3H]5β-cholestan-3α,7α,12α,25-tetrol. In the feces, the maximum sp act of 5β-cholestan-3α,7α,12α,25-tetrol was reached on the 3rd day and then declined rapidly, whereas the sp act of fecal cholic acid rose more slowly and did not reach a maximum until the 5th day before declining. The presence of tritium in all cholic acid fractions plus the intersecting sp act decay curves indicate that cholic acid was formed from 5β-cholestan-3α,7α,12α,25-tetrol. Fur-
Table II

Radioactivity and Mass of 5β-Cholesterol-3α,7α,12α,25-Tetrol and Methyl Cholate Isolated from Bile and Feces*

<table>
<thead>
<tr>
<th>Subject</th>
<th>Days after labeling</th>
<th>Specimen</th>
<th>5β-Cholesterol-3α,7α,12α,25-Tetrol</th>
<th>Methyl cholate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>dpm/mg</td>
<td>mg</td>
</tr>
<tr>
<td>E. E.</td>
<td>1</td>
<td>Bile</td>
<td>1,700</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Feces</td>
<td>60</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Feces</td>
<td>620</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Feces</td>
<td>140</td>
<td>2.7†</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Feces</td>
<td>220</td>
<td>5.2†</td>
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<tr>
<td></td>
<td>9</td>
<td>Feces</td>
<td>10</td>
<td>0.8</td>
</tr>
<tr>
<td>E. S.</td>
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<td>Bile</td>
<td>1,300</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Feces</td>
<td>320</td>
<td>5.0†</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Feces</td>
<td>6,400</td>
<td>5.4†</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Feces</td>
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<td></td>
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<td>Feces</td>
<td>60</td>
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</tr>
<tr>
<td></td>
<td>9</td>
<td>Feces</td>
<td>20</td>
<td>1.7†</td>
</tr>
</tbody>
</table>

* Both [3H]5β-cholesterol-3α,7α,12α,25-tetrol and [3H]methyl cholate were isolated from specimens of bile and feces after intravenous pulse labeling with 2 μCi of [3H]5β-cholesterol-3α,7α,12α,25-tetrol. When sufficient mass was available, the radiopurity and isotopic stability of [3H]cholestane-3α,7α,12α,25-tetrol and [3H]methyl cholate were confirmed by crystallization. [3H]methyl cholate was crystallized from ethanol:water (9:1, vol/vol) and [3H]5β-cholesterol-3α,7α,12α,25-tetrol from ethyl acetate.

† Crystallized material had same sp act as the material eluted from the thin-layer plate.

Thermore, analysis of the sp act time curves shows that the cholic acid curve intersects with the cholestanetetrol curve at its maximum sp act and suggests a precursor-product relationship (8). This finding implies that a large portion of the cholic acid was formed from 5β-cholestanetetrol in these patients.

Discussion

The results of this investigation clearly demonstrate that cholic acid was formed from 5β-cholestanetetrol in both normal and CTX individuals. In the former group, the maximum radioactivity was detected in the cholic acid 1 day after labeling with [3H]5β-cholestanetetrol. This observation indicated that the side chain of the labeled C5 hydroxy tetrol was oxidized to a carboxylic acid. In the CTX subjects not only was radioactivity from [3H]5β-cholestanetetrol detected in cholic acid isolated from bile and feces, but the sp act vs. time curves of fecal 5β-cholestanetetrol and cholic acid suggested a precursor-product relationship. The theoretical considerations underlying this relationship have been published in a recent review by Reiner (8). As we have previously noted, the precursor-product relationship indicates that 5β-cholestanetetrol was a major precursor of cholic acid in these subjects.

These findings have at least two important implications. First, as concerns CTX, they support our hypothesis that the genetic abnormality in this disease results in impaired bile acid synthesis, specifically, incomplete degradation of the cholesterol side chain. The demonstration of delayed conversion of 5β-cholestanetetrol into cholic acid as compared with normal lipidemic subjects coupled with the persistence of 5β-cholestanetetrol and 5β-cholestanetetrol-pentol in bile and feces suggests that the cholic acid synthetic pathway is blocked distal to the formation of these bile alcohols. Presumably, there is a diminished capacity of an enzyme that catalyzes the oxidation of either the tetrol or pentol (2). However, an alternative explanation that might account for the accumulation of these bile alcohols is that 26-hydroxylation of 5β-cholestanetetrol is subnormal. Although this study clearly shows that cholic acid is produced from 5β-cholestanetetrol, the normal pathway might still involve 5β-cholestanetetrol-pentol which is formed from the 26-hydroxylation of 5β-cholestanetetrol-pentol by hepatic mitochondria (9). Thus the accumulation of C5-hydroxy bile alcohols in CTX might represent side products rather than normal cholic acid intermediates. However, an observation in favor of C5-hydroxy bile alcohols as intermediates of cholic acid in man is the recent demonstration by Björkhem, Gustafsson, Johansson and Persson (9) that 5β-cholestanetetrol-pentol was preferentially formed from 5β-cholestanetetrol-pentol by human hepatic microsomes. Only trace amounts of the C5-hydroxy derivative 5β-cholestanetetrol or other cholestanetetrols were formed. In contrast, these investigators showed that human liver mitochondria could 26-hydroxylate a variety of bile alcohols including
5β-cholestane-3α,7α,12α-triol. Furthermore, the microsomal system acting on 5β-cholestanetriol was much more active in producing 5β-cholestane-3α,7α,12α,25-tetrol. Consequently, the production of 3α-hydroxy bile alcohols by human liver microsomes appears to be considerably more specific than the comparable system in the rat where incubation of 5β-cholestane-3α,7α,12α-triol gives a mixture of 5β-cholestanol tetrols. However, even in the rat, the formation of 5β-cholestane-3α,7α,12α,25-tetrol predominates (10).

It is important to explain the differences in the experiments between the normal and CTX subjects. In the normal subjects, cholic acid was isolated from bile rather than feces to provide as much cholic acid as possible for characterization and radioactivity assay. It is often noted that only small amounts of cholic acid remain in the feces because of bacterial transformation to deoxycholic acid during intestinal transit. In the CTX subjects, kinetic observations were made on 5β-cholestane-3α,7α,12α,25-tetrol and cholic acid isolated from the feces because: (a) very little bacterial transformations of fecal steroids are seen in these subjects (5); and (b) large amounts of both cholic acid and 5β-cholestane-3α,7α,12α,25-tetrol can be recovered from feces without sampling the enterohepatic circulation (2). However, as noted, the specific activity of biliary cholic acid was sixfold lower in the CTX subjects 1 day after pulse labeling than in the control individuals even though each subject received the same dose of [G-3H]5β-cholestane-3α,7α,12α,25-tetrol. This observation suggests that cholic acid synthesis was reduced in the CTX subjects and is consistent with earlier quantitative measurements showing low bile acid production (1).

The second important point derived from this study concerns the pathway of cholic acid biosynthesis. It is well established that cholic acid is formed from 5β-cholestane-3α,7α,12α-triol, and oxidation of the side chain was believed to proceed exclusively via 5β-cholestane-3α,7α,12α,26-tetrol and 3α,7α,12α-trihydroxy-5β-cholestanoic acid (3, 4, 11–13). Furthermore, the conversion of 5β-cholestane-3α,7α,12α-triol to 5β-cholestane-3α,7α,12α,26-tetrol has been shown to be catalyzed by mitochondrial enzymes (9). On the basis of the present findings, it seems that side chain cleavage can proceed also via 5β-cholestane-3α,7α,12α,25-tetrol and probably 5β-cholestane-3α,7α,12α,24α,25-pentol. Moreover, as shown by Björkhem et al. (9), this transformation is catalyzed specifically by microosomal rather than mitochondrial enzymes. If cholic acid biosynthesis involves Ca-hydroxy intermediates, then it is not necessary to postulate the transport of 5β-cholestane-3α,7α,12α-triol from microsomes to mitochondria. It is important to emphasize that these remarks are confined solely to the degradation of the side chain during cholic acid biosynthesis. At the present time, there is no information that cleavage of the side chain as part of chenodeoxycholic acid synthesis proceeds via the same pathway. If so, the steps would involve the 25-hydroxylation of 5β-cholestane-3α,7α-diol to yield 5β-cholestane-3α,7α,25-triol. However, the studies of Björkhem et al. (9) showed that 5β-cholestane-3α,7α-diol was considerably less 25-hydroxylated by human liver microsomes than 5β-cholestane-3α,7α,12α-triol. Therefore, there is the possibility that different pathways exist for the oxidation of the side chain for cholic acid and chenodeoxycholic acid synthesis.

ACKNOWLEDGMENTS
We are especially grateful to Dr. Thomas W. Meriwether III for referral of the CTX subjects. This work was supported in part by U. S. Public Health Service Grants HL-10894, HL-17818, and AM-05222, National Science Foundation Grant GB-31919X, and a grant from the Intellectual Products Development Research Foundation, New Rochelle, N. Y.

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