The metabolism of 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid into cholic acid: an enzyme assay using homogenates of human liver

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Summary An enzyme assay was developed to measure the conversion of the bile acid precursor, 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (THCA), into cholic acid using homogenates of human liver biopsies. The average rate of metabolism of THCA into cholic acid was found to be 3.9 ± 0.5 (±1 SD) pmoles of cholic acid formed/mg liver/minute in twelve normal liver biopsies. This assay system can be used to determine if the syndrome of neonatal cholestasis associated with a metabolic block in the conversion of THCA into cholic acid is transmitted as a genetic trait.

Supplementary key word bile acid synthesis

Recently, a new syndrome of neonatal cholestatic liver disease associated with the excretion of large amounts of the bile acid precursor, 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (THCA) was described (1,2). The metabolic defect in this condition is the result of a block in the conversion of THCA into cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) (2). Two of the patients described were from a single family, of opposite sex, and separated by a normal sibling, suggesting an autosomal recessive mode of inheritance (2). However, the parents of these two children do not have more than the normal trace amount of THCA in their bile (2). Therefore, to determine if this condition is transmitted in a genetic fashion, one will have to demonstrate a reduced capacity of the presumed heterozygote carriers to metabolize THCA into cholic acid. The purpose of this study was to develop an in vitro assay system to measure the rate of conversion of THCA into cholic acid using homogenates of biopsies from normal human liver.

Methods

Reference and radiolabeled compounds. Authentic THCA was isolated from the bile of *Alligator mississippiensis*, and labeled with tritium as described previously (2); the specific activity was 56 μCi/mg. Cholic acid was purchased from Eastman Organic Chemicals (Rochester, N. Y.) and recrystallized from acetone.

Liver biopsies were obtained from patients who had normal liver function tests (bilirubin, alkaline phosphatase and SGOT) after informed consent was obtained. A sample from each biopsy was examined by routine light microscopy and all were histologically normal. The tissue samples were obtained between 8 a.m. and 10 a.m. and placed in 10% aqueous sucrose solution (w/v) at 4°C. The specimens were then blotted, weighed, minced with a clean knife, and homogenized in 3 ml of 10% sucrose using a loose fitting Potter-Elvehjem homogenizer within 15–30 min after collection. Portions of the homogenates, equivalent to 20–80 mg of liver tissue (wet wt), were used in the incubation reactions. The incubation mixtures contained 6.5 ml of 0.1 M Tris buffer, at different pH measurements (vide infra), containing 25 mg of ATP, 5 mg of NAD, 15 mg of reduced glutathione, 4 mg of AMP, 10 mg of $Mg(NO_3)_2 \cdot 6H_2O$, 22 mg of trisodium citrate and 4 mg of Coenzyme A. ^{3}H-labeled THCA (400,000 dpm), with different amounts of unlabeled carrier, was dissolved in 10 μl of acetone and added to the incubation flasks. Incubations were carried out in room air at 37°C in a metabolic shaker (Dubnoff, Scientific Apparatus Corp.). The reactions were stopped by adding NaOH to a final concentration of 4.5 N. The solutions were then hydrolyzed in a steel bomb at 130°C for 24 hr. The hydrolysate was cooled and diluted with an equal volume of water. The pH was then adjusted to less than 1 with 12 N HCl, and the solution was extracted twice with an equal volume of ethyl acetate. After evaporation, the bile acids in the combined extracts were separated by partition chromatography on a 1 × 30 cm Celite column using 70% aqueous acetic acid (v/v) as the stationary phase and increasing percentages of benzene in petroleum ether (bp 60–70°C) as the moving phase (100 ml volumes). The percentage of the recovered radioactivity identified as cholic acid in the last quarter of the 60% benzene fraction and the first half of the 80% benzene fraction was used to calculate the amount of THCA converted into cholic acid by the homogenate. Radioactivity was measured in a Beckman model LS-250 scintillation counter using Biosolv 3 solubilizer and Flouralloy TLA counting mixture (Beckman Instruments, Fullerton, California).

Results

Identification of [^{3}H]cholic acid formed from ^{3}H-labeled THCA. The identity of [^{3}H]cholic acid formed during the incubation of ^{3}H-labeled THCA was shown by column partition chromatography on Celite and by

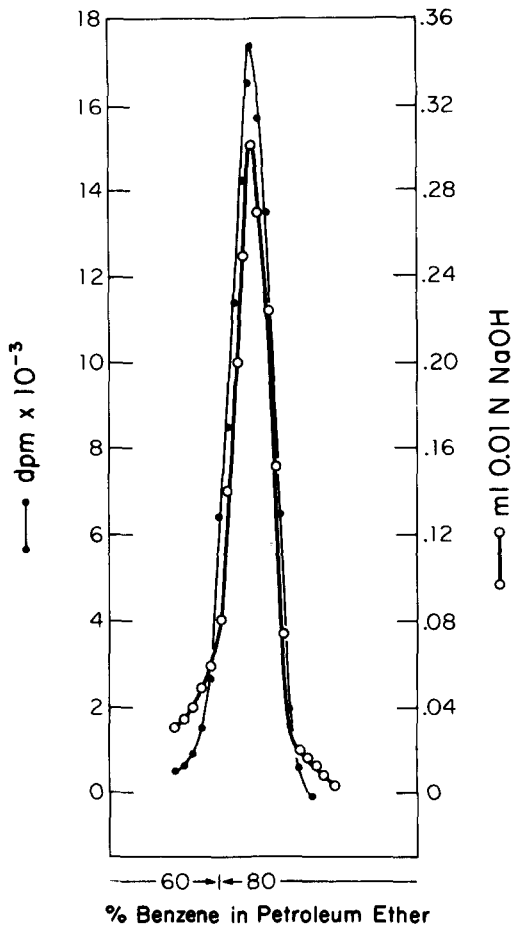

Fig. 1. Distribution of radioactivity (● —— ●) and added cholic acid (○ —— ○) using Celite column partition chromatography after incubating ^{3}H-labeled THCA with human liver.

recrystallization with authentic cholic acid. After hydrolysis and extraction of an incubation reaction, 15 mg of unlabeled cholic acid was added and the mixture was chromatographed on a Celite column as described above. As shown in **Fig. 1,** single coincident peaks were observed for the radioactivity and the mass of cholic acid, which was identified by titration using 0.01 N NaOH. In a separate experiment, 2.25

TABLE 1. Recrystallization of [^{3}H]cholic acid isolated after incubating human liver homogenates with ^{3}H-labeled THCA

Number	Solvent	Weight	Specific Activity
		mg	*dpm/mg*
1[a]		100	2250
2	ethyl acetate	85	2127
3	ethyl acetate	74	2094
4	acetone	65	2012
5	acetone	50	2069

[a] 2.25 × 10^5 dpm of radioactivity was added to 100 mg of unlabeled cholic acid.

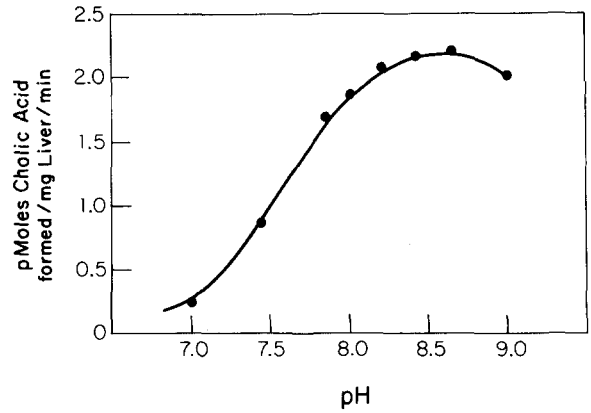

Fig. 2. Effect of pH on the rate of conversion of THCA to cholic acid. Each incubation mixture contained 50 mg of liver tissue with a substrate concentration of 1μm. The reactions were stopped after 30 min.

× 10^5 dpm isolated from the cholic acid fraction as described above was added to 100 mg of cholic acid and recrystallized. The specific activity of [^{3}H]-cholic acid was constant within the precision of the measurement (estimated at ±5%) (see **Table 1**).

Properties of the assay system. The relationship between the reaction rate and the pH of the incubation medium is shown in **Fig. 2**. The optimal pH was approximately 8.5. As shown in **Fig. 3** the rate of the reaction was linear for the first hour, after which the rate decreased. In subsequent experiments, incubations were carried out at pH 8.5 for 30 min. The relationship between the amount of liver tissue and the rate of cholic acid formation is shown in **Fig. 4**. As shown, the formation of cholic acid from THCA was linear from less than 20 mg to more than 80 mg of

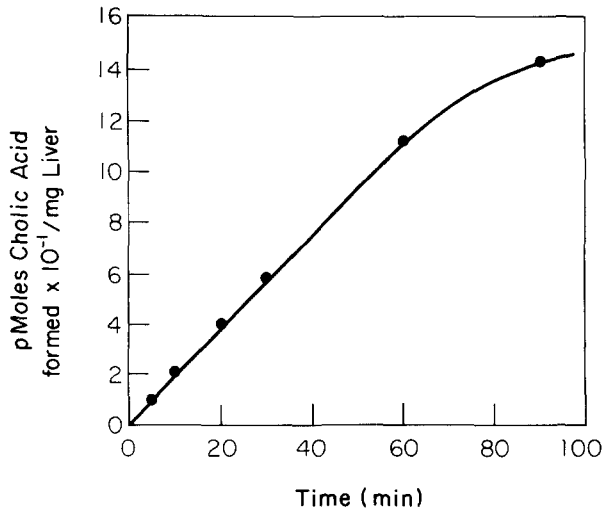

Fig. 3. Effect of time on the conversion of THCA to cholic acid. Each incubation mixture contained 50 mg of liver tissue with a substrate concentration of 0.5μM. The reactions were carried out at pH 8.5 for variable lengths of time.

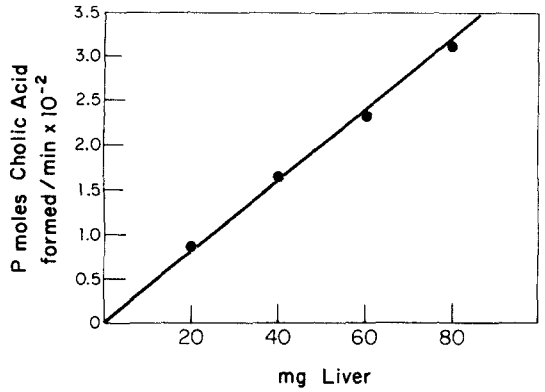

Fig. 4. Effect of increasing amounts of liver tissue on the rate of conversion of THCA to cholic acid. The substrate concentration was 1μM.

liver tissue. The effect of the substrate concentration on the initial velocity of the reaction is shown by a reciprocal (Lineweaver-Burk) plot in **Fig. 5.** A straight line was observed with an apparent K_m of 2.0 μM. Therefore, in the assay system, the concentration of THCA used in the incubation flasks was 10 μM to ensure that the enzyme system was saturated.

The activity of this enzyme system was measured in 12 subjects who had a variety of clinical disorders. The results are presented in **Table 2**. The average activity of this enzyme system was 3.9 pmoles of cholic acid formed/mg liver/minute.

Discussion

This study was undertaken to determine if the conversion of 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (THCA) into cholic acid can be measured using quantities of human liver that can be obtained from percutaneous liver biopsies. As shown in Table 2,

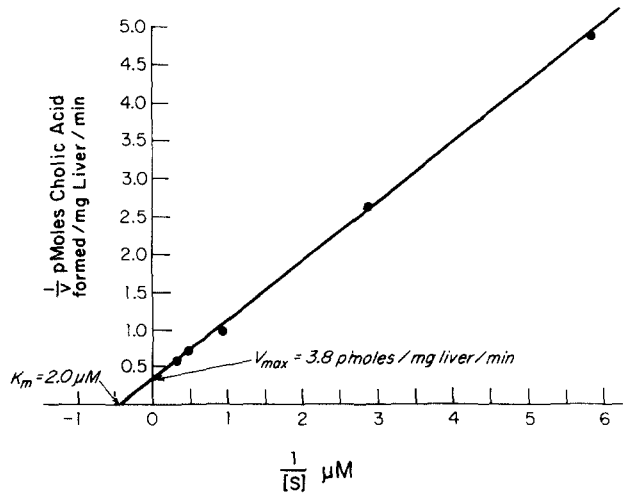

Fig. 5. Relationship between initial velocity (v) of the reaction THCA cholic acid and the substrate concentration(s). Each incubation mixture contained 50 mg of liver tissue.

TABLE 2. Enzyme activity (THCA → cholic acid) in normal human liver[a]

Patient	Age	Weight of liver tissue (mg)	Diagnosis	Enzyme activity pmoles cholic acid formed/mg liver/minute.
1	48	184	Peptic ulcer	4.0
2	54	486	Hysterectomy	3.0
3[b]	62	60	Peptic ulcer	3.6
4	78	350	Left hemicolectomy	4.0
5	64	480	Right hemicolectomy	4.0
6	48	293	Right hemicolectomy	4.0
7	60	196	Right hemicolectomy	4.0
8	40	274	Peptic ulcer	3.8
9	38	143	Peptic ulcer	4.0
10	35	108	Cholecystectomy	5.1
11	48	204	Peptic ulcer	3.8
12[b]	16	25	Lymphoma	3.5
				Average 3.9 ± 0.5[c]

[a] Assay conditions were: 0.1M Tris buffer, pH 8.5; 50 mg of homogenized liver tissue except in patients 3 and 12 where the entire specimen was used; incubated at 37°C for 30 min with 400,000 dpm of ^{3}H-labeled THCA at a concentration 10 μM.
[b] Needle biopsy.
[c] Standard deviation.

the rate of conversion of THCA into cholic acid is defined within a reasonably narrow range using homogenates containing 25–60 mg of liver tissue.

Previous studies in rats have demonstrated that the conversion of THCA to cholic acid takes place in mitochondria and have suggested that a heat stable factor present in the 105,000 *g* supernatant fraction is required for optimal activity of this enzyme system (3). Therefore, in the present study, whole liver homogenates were used to simplify the assay procedure.

The metabolism of THCA into cholic acid is thought to take place through β-oxidation (4) and therefore involves several enzymatic steps. Fig. 5 shows that this enzyme system appears to be saturable; however, which step in this reaction is rate limiting is unknown. It should be pointed out that the K_m and V_{max} observed in this study are probably only estimates of the true K_m and V_{max} of the overall reaction.

THCA exists naturally in either the 25-D or the 25-L stereoisomeric form and the bile of *Alligator mississippiensis* is known to contain 25-L-THCA (5), the isomer used in the present study. When administered intravenously into a bile fistula rat, the labeled compound used in this investigation is rapidly and almost completely converted into cholic acid (2). Which stereoisomer is present in man is not known. However, in a previous study, we demonstrated that man can metabolize a racemic mixture of 25-D-and 25-L-3α,7α-dihydroxy-5β-cholestan-26-oic acid (DHCA) into chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-oic acid) (6). Therefore it is

likely that man can also metabolize both 25-D-and 25-L-THCA into cholic acid.

The condition of neonatal cholestasis associated with a metabolic block in the conversion of THCA to cholic acid is thought to be inherited as an autosomal recessive trait (2). Thus, the maximal rate of conversion of THCA into cholic acid in the livers of the heterozygote carriers should be only half that found in normal livers. However, before the assay system described in this report can be used to determine if this syndrome is inherited, additional normal subjects, who are matched as to age and sex of the possible heterozygote carriers, will have to be tested.

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