Competitive inhibition of side chain oxidation of 3α,7α-dihydroxy-5β-cholestan-26-oic acid by 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid in the hamster

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Abstract 3α,7α-dihydroxy-5β-cholestan-26-oic acid (DHCA) and 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (THCA) are metabolized into chenodeoxycholic acid and cholic acid, respectively, through oxidation and cleavage of the terminal three carbons of the side chain. The present study was designed to determine if the same or different side chain oxidation systems are used by these compounds in the bile fistula hamster model. Although a single injection of [^{3}H]THCA is nearly completely metabolized into cholic acid, only about 50% is converted into cholic acid when THCA is infused at a rate of 0.083 μmol/min. The remainder is excreted in the bile unchanged indicating saturation of the side chain oxidation system. Fifty-nine ± 1.1% (±1 SEM) of a single injection of [^{3}H]DHCA is metabolized into chenodeoxycholic acid in bile fistula hamsters infused with either saline or cholic acid at a rate of 0.083 μmol/min. The remainder was excreted as several other metabolic products including cholic acid. However, when [^{3}H]DHCA was administered during an 0.083 μmol/min infusion of THCA, only 39.0 ± 4.5% of the radioactivity in bile was identified as chenodeoxycholic acid. Thus, this study indicates that DHCA and THCA share at least one of the enzymes involved in side chain oxidation.—**Cass, O. W., G. C. Williams, and R. F. Hanson.** Competitive inhibition of side chain oxidation of 3α,7α-dihydroxy-5β-cholestan-26-oic acid by 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid in the hamster. *J. Lipid Res.* 1980. **21:** 186–191.

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3α,7α-dihydroxy-5β-cholestan-26-oic acid (DHCA) and 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (THCA), are putative intermediates in the formation of chenodeoxycholic acid (1–3) and cholic acid (4–7), respectively (see **Fig. 1**). The side chain oxidation of DHCA and THCA is thought to take place via β-oxidation (2, 6, 7). However, it is not known if the same or different β-oxidation enzyme systems are involved in these reactions (see Fig. 1). The present study was designed to determine if the metabolism of DHCA into chenodeoxycholic acid is competitively inhibited during a constant intravenous infusion of THCA into bile fistula hamsters. The dosage of THCA infused was chosen to exceed the capacity of the side chain oxidation system of the hamster liver to metabolize THCA into cholic acid. Therefore, if both DHCA and THCA share the same enzyme system for side chain oxidation, the infused THCA should competitively inhibit the metabolism of a single injection of DHCA into chenodeoxycholic acid.

METHODS

Reference and labeled compounds

DHCA and THCA were isolated from the bile of the *Alligator mississippiensis* as described previously (8) and identified using mass spectroscopy (3, 8). [^{3}H]-DHCA and [^{3}H]THCA (sp act 32.6 μCi/μmol and 24.4 μCi/μmol, respectively) were prepared using the method described by Hofmann, Szczepanik, and Klein (9). Thin-layer chromatography (TLC) showed that both [^{3}H]DHCA and [^{3}H]THCA were more than 99% pure. Cholic acid and chenodeoxycholic acid were purchased from Calbiochem, La Jolla, CA and recrystallized before use. Only single spots of these compounds were observed by TLC.

Animal experiments

Male, golden Syrian hamsters, weighing 80–120 g, were anesthesized with ether and cannulas were inserted into the common bile duct and jugular vein (Silastic tubing 0.025 in. O.D., Dow Corning, Midland, MI). The bile duct and venous cannulas were fastened

Abbreviations: TLC, thin-layer chromatography; DHCA, 3α,7α-dihydroxy-5β-cholestan-26-oic acid; THCA, 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid.

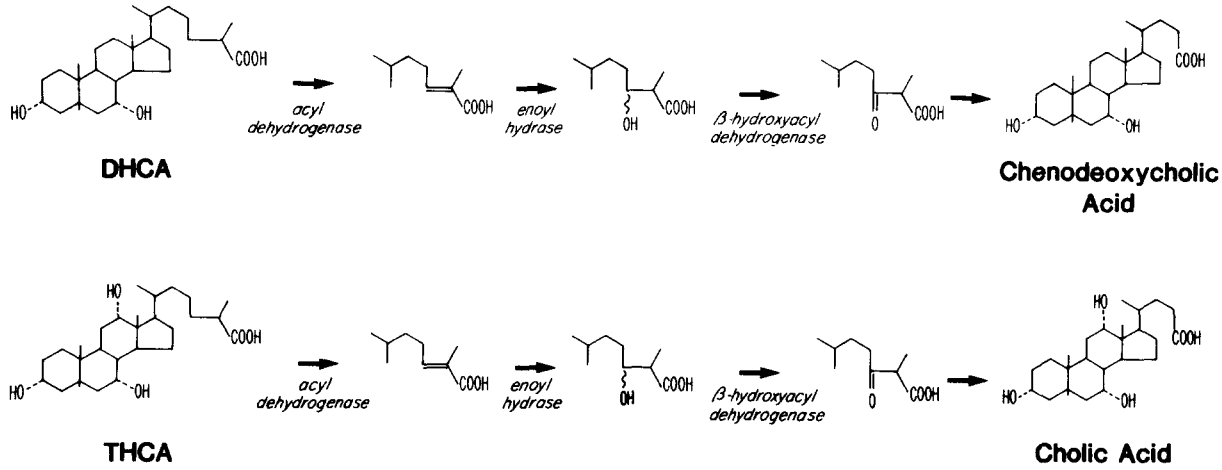

Fig. 1. Proposed pathways of side chain oxidation of DHCA and THCA via β-oxidation.

to a harness which was implanted subcutaneously on the backs of the hamsters. The animals were kept in restraining cages and allowed to recover overnight during which time the intravenous cannula was infused with 0.9% NaCl at a rate of 0.73 ml/hr. Approximately 18 hr after surgery the hamsters were given either bolus intravenous injections of [^{3}H]THCA or [^{3}H]DHCA, or intravenous injections of THCA (4 hamsters) or cholic acid (4 hamsters) at a rate of 0.083 μmol/min (5 μmol/hr at a concentration of 3.12 mM) for 2 hours. At that point, an intravenous bolus injection (0.5 ml over 10 min) of 1.5 μCi (46.0 nmol) of [^{3}H]DHCA was administered to each hamster. All bile acids were dissolved in saline (pH 7.4) containing albumin at a concentration of 2.5 g/dl. The THCA infusion was continued at the same rate for 4 more hours during which the bile was collected in hourly fractions.

Bile acid analysis

Aliquots of the bile samples were counted for radioactivity in a Beckman model LS-250 liquid scintillation counter using a Fluoralloy TLA counting mixture (Beckman Inst., Fullerton, CA). The remainder of the bile samples were hydrolyzed in 4.5 N NaOH at 115°C for 4 hr in a steel bomb. After acidification, the bile acids were extracted twice with diethyl ether, and the ether was evaporated under a stream of nitrogen. Aliquots were applied to TLC plates (Silica Gel G, 0.025 mm, Brinkman Inst.) developed with ethyl acetate–isooctane–acetic acid 10:10:2 (v/v). The plates were scraped into twenty equal sections and counted as described previously (10).

Experimental design

The experimental design of the study was as follows. Unlabeled THCA was infused at a sufficient rate to insure that some of the THCA was excreted unchanged in the bile. It was assumed that at that point at least part of the enzymatic system involved in the metabolism of THCA into cholic acid was saturated. If DHCA and THCA share this same enzyme system, there should be competitive inhibition between these two bile acids with the enzyme system, and the fraction of the injected [^{3}H]DHCA metabolized into chenodeoxycholic acid should be reduced compared to a similar injection of [^{3}H]DHCA into hamsters receiving cholic acid. Cholic acid was used as a control bile acid that also requires hepatic uptake, intracellular transport, and conjugation but not side chain oxidation before secretion into bile. If DHCA and THCA do not share the same enzyme system, the fraction of [^{3}H]DHCA metabolized into chenodeoxycholic acid should not be reduced during an infusion of THCA.

RESULTS

Metabolism of THCA

Following a single bolus intravenous injection of 1.5 μCi of [^{3}H]THCA (61.4 nmol) into a bile fistula hamster, approximately 85% of the injected radioactivity was recovered in the bile in the first 4 hours. After hydrolysis, an aliquot was applied to a TLC plate developed with ethyl acetate–isooctane–acetic acid 10:10:2 (v/v). The plate was divided into twenty equal sections which were scraped off and counted. Ninety percent of the radioactivity was located in the cholic acid fraction and the remainder was in the THCA fraction. Another hamster was infused with [^{3}H]THCA which was diluted with unlabeled THCA (final sp act, 0.090 μCi/μmol) at a rate of 0.083 μmol/min for 4 hours. The bile was collected hourly and

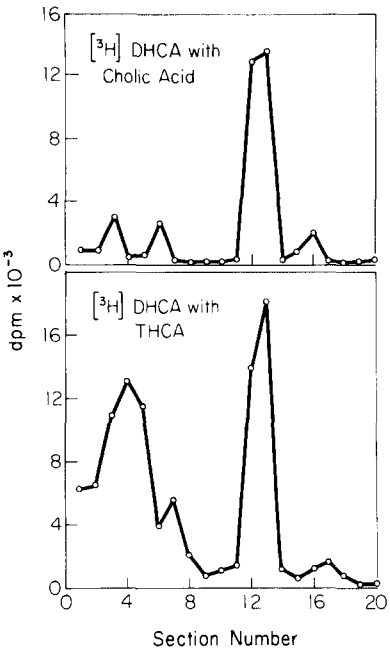

Fig. 2. Representative examples of the distribution of the radioactivity in bile determined by thin-layer chromatography after the intravenous injection of [^{3}H]DHCA into hamsters infused with either cholic acid or THCA. Controls: cholic acid—section 3; THCA—section 6; chenodeoxycholic acid—sections 12, 13; DHCA—sections 16, 17. The plates were divided into twenty equal sections which were counted. The solvent was ethyl acetate–isooctane–acetic acid 10:10:2 (v/v).

analyzed as described above. Seventy-three percent of the radioactivity was recovered in the 4-hour period and an average of 57.7 ± 3.6 (±1 SD) of the infused THCA was excreted in the bile unchanged; the remainder was excreted as cholic acid.

Metabolism of [^{3}H]DHCA

[^{3}H]DHCA was administered intravenously by single injection to two bile fistula hamsters. Eighty-five and 90% of the injected radioactivity was recovered in the bile within 4 hours of the injection. The hourly bile samples from each hamster were combined, hydrolyzed, and chromatographed on TLC. Fifty-nine percent and 61% of the radioactivity was present in the fractions from the two hamsters that corresponded to chenodeoxycholic acid. Three other smaller peaks of radioactivity were also observed that corresponded to DHCA, THCA, and cholic acid.

Metabolism of [^{3}H]DHCA during an infusion of THCA and cholic acid

Four hamsters were infused intravenously with unlabeled THCA at a rate of 0.083 μmol/min and four hamsters were infused at an equal rate with cholic acid for 2 hours. All eight animals were then given single intravenous injections of 1.5 μCi of [^{3}H]DHCA after which the infusion of THCA or cholic acid was continued at the same rate for 4 additional hours. The cumulative excretion of radioactivity over the 4-hour period averaged 82.3 ± 3.2% (±1 SEM) for the THCA-infused hamsters and 80.1 ± 2.8% for the cholic acid-infused hamsters. The hourly bile samples were combined from each hamster and hydrolyzed. Representative TLC chromatograms are shown in **Fig. 2.** There was a significant reduction in the percentage of the injected DHCA metabolized into chenodeoxycholic acid in the hamsters infused with THCA compared to the hamsters infused with cholic acid. Fifty-nine ± 1.1% (±1 SEM) of the radioactivity excreted in the bile of the hamsters infused with cholic acid was in the chenodeoxycholic acid fraction. However, only 35.5 ± 4.4% of the radioactivity in the bile of the hamsters infused with THCA was in the chenodeoxycholic acid fraction.

The combined radioactivity in the sections of the TLC plates corresponding to chenodeoxycholic acid was added to 500 μmoles of unlabeled authentic chenodeoxycholic acid and recrystallized. The specific activities remained constant through four crystallizations (1510, 1407, 1382, 1489 dpm/μmol) using ethyl acetate–petroleum ether as solvents.

The radioactivity in the section of the plates corresponding to the cholic acid control was combined and added to 100 μmoles of unlabeled authentic cholic acid. The mixture was then chromatographed on a Celite partition column (11) 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. The cholic acid fraction of this column was collected in 6-ml fractions, evaporated, and aliquots were counted for radioactivity and titrated for mass of cholic acid using 0.01 N NaOH (phenol red as the indicator). As shown in **Fig. 3,** the titration peak for cholic acid and the peak of radioactivity coincided. The material in this peak was crystallized and the specific activities remained constant through four crystallizations (256, 246, 239, 253 dpm/μmol) using ethyl acetate as the solvent.

The material in the fraction of the plates corresponding to the THCA control was also examined. The radioactivity was added to 30 μmoles of unlabeled authentic THCA and chromatographed on Celite as described above. As shown in **Fig. 4,** some of the radioactivity corresponded to the mass of THCA identified by titration. However, a significant fraction of the radioactivity had a mobility slightly less polar than

THCA. These peaks of radioactivity were not further characterized.

DISCUSSION

The side chain oxidation of DHCA and THCA in the formation of chenodeoxycholic acid and cholic acid, respectively, is thought to involve β-oxidation as shown in Fig. 1. Evidence favoring this hypothesis is that the conversion of these C_{27} compounds into C_{24} bile acids has been demonstrated to take place in the mitochondria (2, 6, 7) and that 24-hydroxylated intermediates are formed in the process (2, 7). Additionally, we demonstrated that patients with Zellweger's syndrome, an inherited disease with abnormalities in mitochondrial structure and function, have metabolic defects in bile acid synthesis which result in the accumulation of DHCA, THCA and 3α,7α,12α,24ξ-tetrahydroxy-5β-cholestan-26-oic acid (varanic acid) (see Fig. 1) (12). The present study was designed to determine if DHCA and THCA share the same side chain oxidation system. The bile fistula hamster model was used in this study because the bile acid pattern in this species is similar to that found in man (13) and therefore the metabolic pathway for bile acid synthesis in the hamster and man are presumed to be similar.

The experimental design of the study involved infusing THCA at a rate sufficient to saturate the metabolic system for converting THCA into cholic acid. A tracer amount of [^{3}H]DHCA was then injected and the THCA infusion continued. If DHCA and

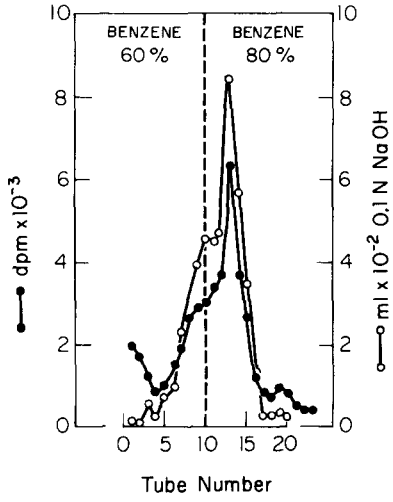

Fig. 3. Chromatographic distribution of the radioactivity isolated in the cholic acid fraction of TLC and added to authentic unlabeled cholic acid. Titration ○——○; radioactivity ●——●.

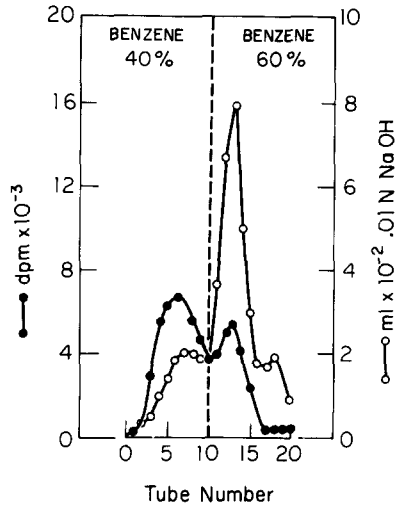

Fig. 4. Chromatographic distribution of the radioactivity isolated from the THCA fraction on TLC and added to unlabeled authentic THCA. Titration ○——○; radioactivity ●——●.

THCA did not compete for the same side chain oxidation enzymes there would be no difference in the metabolism of [^{3}H]DHCA in the THCA-infused or control hamsters. If competition occurred, a reduced fraction of the injected [^{3}H]DHCA would be metabolized into chenodeoxycholic acid. Because the infused THCA may have also saturated other cellular processes besides the side chain oxidation system such as hepatocyte uptake, intracellular transport, conjugation, and excretion, the control hamsters were infused at an equal rate with cholic acid, a bile acid with a similar chemical structure but one that would not compete for the side chain oxidation enzymes. However, if THCA and DHCA do not use the same uptake, transport, conjugation, and excretion systems as cholic acid, and if the infused THCA saturated this alternate transport system, instead of the side chain oxidation system, the biliary excretion rate of [^{3}H]radioactivity after a bolus injection of [^{3}H]DHCA would be reduced. This was not observed. Of the injected radioactivity, 82.3% ± 3.2% (±SEM) was excreted in the bile in the 4-hour period during an infusion of THCA, and 80.1 ± 2.8% (±1 SEM) was excreted during an infusion of cholic acid. These rates of excretion are not significantly different from that observed when [^{3}H]DHCA was given alone. Thus, it appears that only the side chain oxidation enzyme system was saturated during the infusion of THCA and not the uptake, transport, conjugation, and excretion systems. Therefore, any differences in the metabolism of [^{3}H]DHCA in the THCA- and cholic acid-infused hamsters should be due only to the influence of THCA on side chain oxidation.

When [^{3}H]THCA was administered as a tracer injection, approximately 90% was metabolized into cholic acid. However, when THCA was infused at a rate of 0.083 μmol/min, only about 50% was converted into cholic acid. Thus, this infusion rate of THCA or cholic acid was used in subsequent experiments.

The metabolism of [^{3}H]DHCA was then studied, and tracer amounts of this compound were administered by intravenous injection during infusions of saline or cholic acid at a rate of 0.083 μmol/min. About 60% of the radioactivity appearing in bile was identified as chenodeoxycholic acid during both the saline and cholic acid infusions. This indicates that cholic acid does not suppress the conversion of DHCA into chenodeoxycholic acid, which is in agreement with our earlier finding that taurocholate does not influence the conversion of THCA into cholic acid in the rat (14). However, there was a significant reduction in the metabolism of DHCA into chenodeoxycholic acid and an increase in the excretion of other labeled compounds, presumably intermediates formed during side chain oxidation, in the THCA-infused hamsters (see Fig. 2). Thus, competition exists between DHCA and THCA for at least one of the side chain oxidation enzymes.

Which enzymatic step(s) are shared by these two compounds was not determined. However, if the side chain oxidation of DHCA and THCA involves β-oxidation, one might speculate that the point of competition is prior to the introduction of the hydroxyl group at position 24 of the side chain. The 24-hydroxylation of DHCA and THCA probably involves an acyl dehydrogenase and an enoyl hydrase (2, 6, 7) (see Fig. 1). Previous studies of the β-oxidation of fatty acids have shown that several acyl dehydrogenase enzymes exist, with relative specificities depending upon the chain length of the fatty acid substrates (15). However, only a single enoyl hydrase has been found that has broad specificity for many different fatty acids (16). Therefore, if the side chain oxidation of DHCA and THCA involves a similar β-oxidation enzyme system, the most likely enzyme that is shared by these two compounds would be an enoyl hydrase involved in the hydroxylation at position 24 of the side chain.

In an earlier study, we described two children, a brother and sister, who had a metabolic defect in the metabolism of THCA into cholic acid (8). Neither DHCA nor varanic acid was present in these two children. Since varanic acid was not present, the enzymatic defect must have been located before the formation of this compound. If a single enoyl hydrase is used in both the DHCA and THCA pathways, a deficiency of this enzyme would have led to an accumulation of both DHCA and THCA. Thus, we postulated that the enzyme deficient in these two patients was an acyl dehydrogenase specific for THCA.

Fig. 3 shows that some of the radioactivity excreted in the bile following an injection of [^{3}H]DHCA was cholic acid. The formation of cholic acid from DHCA probably occurs via 12α-hydroxylation of DHCA to form THCA (see Fig. 4). This finding is in agreement with our previous study which showed that a small amount of [^{3}H]DHCA administered by intravenous injection into humans is metabolized into cholic acid (1).

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