CHENODEOXYCHOLIC ACID SYNTHESIS IN THE HAMSTER: A METABOLIC PATHWAY VIA 3β, 7α-DIHYDROXY-5-CHOLEN-24-OIC ACID

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ABSTRACT

The quantitative significance of the metabolism of 3β, 7α-dihydroxy-5-cholen-24-oic acid to chenodeoxycholic acid was evaluated in the hamster. A precursor-product relationship was established in this species by the finding that intravenous administration to an animal previously given cholesterol-4-^{14}C caused a significant reduction in the specific activity of chenodeoxycholic acid. Administration of 12.9 μmole of the precursor was followed by a 10-fold increase in chenodeoxycholic acid excretion although the predominant excretory pathway was via biliary excretion as a monosulfate. The data indicate that synthesis of bile acid from cholesterol via the intermediate 3β, 7α-dihydroxy-5-cholen-24-oic acid can be a quantitatively important pathway.

We have recently reported that 3β-hydroxy-5-cholen-24-oic acid is metabolized for the most part to chenodeoxycholic[1] acid and to a minor extent to lithocholic acid (1). These findings in the hamster are similar to our observations in man (2). However, in the hamster it is known that lithocholic acid can be metabolized to chenodeoxycholic acid (3), but no similar pathway could be demonstrated in man (4). It was found that 3β, 7α-dihydroxy-5-cholen-24-oic acid is metabolized to chenodeoxycholic acid in the carp (5) and rat (6) and further reported that this acid-labile bile acid is normally present in bile but is lost during the usual procedures for bile acid analysis (7, 8). To further evaluate this

[1] The following trivial names: cholesterol, 5-cholestene-3β-ol; 26-hydroxycholesterol, 5-cholestene-3β, 26-diol; chenodeoxycholic acid, 3α, 7α-dihydroxy-5β-cholan-24-oic acid; cholic acid, 3α, 7α, 12α-trihydroxy-5β-cholan-24-oic acid.

pathway for chenodeoxycholic acid synthesis, we have done quantitative studies of the metabolism and excretion of 3β,7α-dihydroxy-5-cholen-24-oic acid in the hamster, a species with primary bile acids identical to those in primates.

METHODS AND MATERIALS

Synthesis of 3β,7α-dihydroxy-5-cholen-24-oic acid was from methyl-3β-acetoxy-5-cholen-24-oate according to the method reported by Yamaga (5). Methyl-3β-acetoxy-5-cholen-24-oate (400 mg) was dissolved in freshly distilled carbon tetrachloride (15 ml) and N-bromosuccinimide (250 mg) was added. The mixture was refluxed under ultraviolet irradiation for 8 minutes, cooled, and filtered. The filtrate was stirred at room temperature for 2 hours after the addition of 5 g neutral alumina (activity grade I). After 2 hours the alumina was filtered off and the solvent was evaporated in vacuo. The residue was hydrolyzed in ethanolic 2N potassium hydroxide at room temperature for 24 hours. The hydrolysate was acidified to pH 2.2 with dilute HCl and the precipitate that formed was extracted with ethyl acetate. Analysis by TLC (solvent system, isooctane-ethyl acetate-acetic acid, 20:40:1 v/v) indicated that further purification was needed; this was accomplished on silica gel plates coated with rhodamine 6 G. The material on the plates was visualized by irradiation with ultraviolet light. The acid thus obtained was further purified by recrystallization from ethyl acetate, m.p. 212°C; reported (5) m.p. 206-210°C.

Total bile acids in bile were estimated using 3α-hydroxysteroid dehydrogenase (Worthington, Biochem., Freehold, NJ). Enzymatic hydrolysis was done using cholylglycine hydrolase (Sigma Chem. Co., St. Louis, MO). Both methods have been reported in detail previously (9,10).

Alkaline hydrolysis was done using 1.25N NaOH as described previously (3). However, acidification of the hydrolysate for extraction of the bile acids was done with 1N HCl and to a pH of not less than 2.2.

Bile acids from bile were analyzed by GLC after addition of an internal standard, 3α,7α-dihydroxy-12-oxo-5β-cholan-24-oic acid (Steraloids, Wilton, NH), alkaline hydrolysis, acidification as described above, and extraction with ethyl acetate. Both the diacetate (pyridine/acetic anhydride) and the di-trimethyl silyl ether (Tri-sil/BT, Pierce, Rockford, IL) derivatives of the methyl esters were prepared. It was found that the diacetate derivative of methyl-3β,7α-dihydroxy-5-cholen-24-oate decomposed on the column but that the trimethyl silyl derivative gave a single peak with a retention time of 16.95 minutes after injection onto a 6-ft glass column (2 mm I.D.) packed with 3% SP-2250 on 100/120 Supelcoport (Supelco. Inc., Bellefonte, PA) kept isothermal at 250°C.

An authentic sample of 3-sulfoxy-7α-hydroxy-5-cholen-24-oic acid was synthesized following the reported method (11) with some modifications. Sulfur trioxide -triethylamine complex (0.92 mg, 5.1×10^{-3} mmol) was added to a solution of 3β, 7α-dihydroxy-5-cholen-24-oic acid (2 mg, 5.1×10^{-3} mmol) in dimethyl formamide (50 μl). The reaction mixture was kept at 40°C for 4 hours, after which it was diluted with diethyl ether (1 ml) and ammonium hydroxide solution (0.5 ml) and vortexed. The ammonium hydroxide layer was separated and washed with methylene chloride to remove unreacted sulfating agent and was then evaporated to dryness to yield the diammonium salt of 3-sulfoxy-7-hydroxy-5-cholen-24-oic acid. We assume that, under the conditions, only the 3-sulfate ester was formed since the 3-position is known to be more reactive and only one product was detected by TLC analysis. An infrared spectrum of the sulfate ester in KBr showed peaks at 3420 (broad, -OH), 1570 (C=O), 1220, 1060, and 975 ($-OSO_3H$) cm^{-1}.

Thin layer chromatography (TLC) plates of silica gel (Analtech, Inc., Newark, Del.) (0.25 mm thick) were used. Spots on the plates were detected with phosphomolybdic acid reagent or Lifshutz reagent (12).

Radioactivity was counted using a Beckman (cpm - 100) liquid scintillation counter and scintillation liquid "Hydrofluor" (National Diagnostics, Somerville, NJ).

Hamsters were prepared with bile duct fistula and an indwelling intravenous cannula as described in detail previously (13).

In the first study 10 μCi of 4-^{14}C cholesterol (New England Nuclear, Boston, Mass.) dissolved in 10% human serum albumin (0.8 ml) was given intravenously to a hamster. After 18 hours 3β, 7α-dihydroxy-5-cholen-24-oic acid (0.48 mg) in 8.4% sodium bicarbonate (0.5 ml) was infused. The rate of infusion was 1.2 μg/min for the initial 30 minutes and then 6.0 μg/min for the next 20 minutes. Bile samples were collected initially at 30-minute intervals and then overnight.

In another study, after bile drainage for 24 hours, the animal was injected with 3β, 7α-dihydroxy-5-cholen-24-oic acid (5.0 mg) that was dissolved in a mixture of (0.1 ml) 8.4% sodium bicarbonate and (0.5 ml) 5% dextrose in 0.9% sodium chloride solution and given over a period of 2 minutes. Bile samples were collected in tared tubes and the volume was determined gravimetrically.

RESULTS

Table 1 summarizes the results obtained following infusion of 3β, 7α-dihydroxy-5-cholen-24-oic acid to a hamster that had received an intravenous injection of 4-^{14}C cholesterol 18 hours previously. The initial

infusion at 1.2 μg/min caused a prompt reduction in the specific activity of chenodeoxycholic acid, which then returned toward pre-infusion specific activity during the overnight collection of bile. No significant change occurred in the specific activity of cholic acid during the infusion.

TABLE 1

Effect of 3β, 7α-dihydroxy-5-cholen-24-oic acid infusion on the specific activity of chenodeoxycholic and cholic acids

Bile #	Time[a] Min	Bile Flow μl/hr	Total Bile Acid Concentration μM	Specific Activity Chenodeoxycholic dpm/μmole	Specific Activity Cholic dpm/μmole
1	39	578	0.84	25.5×10^4	12.0×10^4
2[b]	30	604	0.93	2.13×10^4	12.0×10^4
3[b]	30	650	1.11	2.13×10^4	11.8×10^4
4	1040	540	2.00	15×10^4	11.4×10^4

[a] Bile collections begun sequentially 18 hours after injection of 4-^{14}C cholesterol

[b] 3β, 7α-dihydroxy-5-cholen-24-oic acid infusion at 1.2 μg/min x 30 min, then increased to 6.0 μg/min x 20 min

Table 2 summarizes the effect of an injection of 12.9 μmole of 3β, 7α-dihydroxy-5-cholen-24-oic acid given over a 2-minute interval on bile flow and bile acid excretion in a hamster. Chenodeoxycholic acid excretion rose from 0.46 to a maximum of 5.02 nanomole/min and could account for the entire increase in bile acid excretion that was determined enzymatically with steroid dehydrogenase.

However, after 5 hours of bile collection only 5.8% of the injected amount could be accounted for by calculating the increment in bile acid excretion from control levels.

TABLE 2

Effect of 3β, 7α-dihydroxy-5-cholen-24-oic acid injection on chenodeoxycholic and total bile acid excretion in bile

Bile #	Min	Bile Flow μl/min	Total Bile Acid Concentration[a] μM[a]	Total Bile Acid Excretion Rate nmole/min	Chenodeoxycholic Acid Excretion Rate nmole/min	Percentage of Chenodeoxycholic Acid in Bile[c]
1	30	3.06	0.86	2.58	0.38	15.0
2	30	3.33	0.86	2.86	----	----
3	25	3.12	0.86	2.68	0.46	17.2
4[b]	30	2.37	0.87	2.05	1.08	53.0
5	30	3.37	1.96	6.59	4.19	63.7
6	45	3.17	1.80	5.61	3.61	64.5
7	30	3.73	1.80	6.72	4.08	60.8
8	30	3.67	1.60	5.86	3.71	63.4
9	30	3.20	2.12	6.78	3.90	57.6
10	30	3.80	2.31	8.77	5.02	57.3
11	23	3.63	1.80	6.54	3.84	58.8

a Determined by enzymatic method
b 12.9 μmole 3β, 7α-dihydroxy-5-cholen-24-oic acid injected
c Determined by GLC

Thin layer analysis of the biles collected before and after injection indicated the appearance of a new compound with an Rf less than that of the injected compound or chenodeoxycholic and cholic acid (Table 3).

Eluates of the new compound were analyzed using steroid dehydrogenase, cholylglycine hydrolase, and hydrolysis in 1.25N NaOH. No change occurred in the Rf of the compound following these procedures and no generation of NADH could be detected. Finally, the Rf of the eluate was compared with that of a synthetic sample of the ester sulfate of 3β, 7α-dihydroxy-5-cholen-24-oate using three different solvent systems (Table 3). No significant differences were found.

TABLE 3

Property	Synthetic	Metabolite from Bile
1. Hydrolysis 1.25N NaOH	No change	No change
2. Cholylglycine hydrolase	No change	No change
3. Steroid dehydrogenase	No NADH	No NADH
4. TLC[b]	0.59[e]	0.59
5. TLC[c]	0.76	0.76
6. TLC[d]	0.78	0.78

[a] No change in Rf
[b] Chloroform: methanol: acetic acid: water (65:24:15:9 v/v)
[c] n-butanol: acetic acid: water (10:1:1 v/v)
[d] Ethylacetate: n-butanol: acetic acid: water (40:30:15:15 v/v)
[e] Rf

DISCUSSION

Although Yamasaki and co-workers reported a pathway to chenodeoxycholic acid via 7α-hydroxylation of 3β-hydroxy-5-cholen-24-oic acid, the possible significance has received relatively little attention. Our findings in the hamster confirm and extend the concept initiated by the

previous studies.

From a quantitative point of view, an injection of 12.9 μmole of 3β, 7α-dihydroxy-5-cholen-24-oic acid was followed by an approximately 10-fold increase in the excretion rate of chenodeoxycholic acid. At an excretion rate of 4 nanomole/min, the daily amount of chenodeoxycholic acid would be 2.3 mg ($4 \times 1440 \times 0.393 \times 10^{-3}$), a significant fraction of an estimated pool size of 10 mg (14). However, there is considerable evidence that 7α-hydroxylation occurs at much earlier steps in bile acid synthesis from cholesterol (15), thus making it unlikely that 3β-hydroxy-5-cholen-24-oic acid is a major route.

Prehaps the major significance of the "Yamasaki" pathway is in the prevention of cholestasis that could occur by excessive synthesis and excretion of 3β-hydroxy-5-cholen-24-oate and other monohydroxy bile acids (13). Thus, unlike 3β-hydroxy-5-cholen-24-oate, injection of the 7α derivative does not cause a decrease in bile flow (cholestasis). Evidence exists that during fetal and neonatal life relatively large amounts of 26-hydroxycholesterol and 3β-hydroxy-5-cholen-24-oate can be found in biological fluids (16). The amounts that occur are, in part, dependent on the rate of 7α-hydroxylation. Our studies indicate that in the hamster and perhaps in humans 7α-hydroxylation of the monohydroxy bile acid can be followed by further metabolism to chenodeoxycholic acid.

Because of the relatively large amount of 3β, 7α-dihydroxy-5-cholen-24-oic acid that was injected, we were able to identify a sulfate meta-

bolite. Identification of the metabolite is based on our having synthetically prepared a sulfate ester, as characterized by IR spectrometry, that has the same Rf in several solvent systems. Both the synthetic and natural compounds are Lifshutz positive and are unaffected by alkaline hydrolysis or by the enzyme steroid dehydrogenase. It is clear, however, that a hiatus exists, as pointed out by Yamasaki, in our ability to analyze for this derivative. Thus the sulfatase enzymes available to us did not cleave either the synthetic or the natural compound, and the acid conditions necessary for chemical solvolysis cause decomposition because of the allylic alcohol type structure. Although resolution of the various sulfate esters may be achieved by HPLC, a satisfactory method for solvolysis would be most helpful.

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