REDUCTION OF 3α-HYDROXY-5β-CHOL-6-EN-24-OIC ACID TO LITHOCHOLIC ACID IN RATS

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ABSTRACT

After [24-^{14}C]Δ^6-lithocholic acid was injected into the cecum of rats, [^{14}C]lithocholic acid was identified as a metabolite in feces. When the labeled Δ^6-bile acid was injected intraperitoneally into bile-fistula rats, radioactivity excreted in bile was contained most abundantly in the taurine-conjugated fraction of bile acids. In the fraction, taurine conjugate of [^{14}C]Δ^6-lithocholic acid but of neither [^{14}C]lithocholic acid nor other bile acids was found. The results showed that [24-^{14}C]Δ^6-lithocholic acid was reduced to [^{14}C]lithocholic acid by the intestinal flora but not by the liver, which, however, was capable of conjugating Δ^6-lithocholic acid with taurine.

INTRODUCTION

Much attention has recently been focussed on the metabolism of chenodeoxycholic acid (1-3) and ursodeoxycholic acid (4,5) in connection with their usefulness as cholesterol gallstone-dissolving agents.

Although the biochemical interconversion of both bile acids is well known, the mechanism for epimerization of their 7-hydroxyl group has not yet been established (6-12). Besides 7-ketolithocholic acid, Δ^6-lithocholic acid has been also suspected as an intermediate in this conversion, but no direct evidence for the occurrence of the Δ^6-lithocholic acid has been revealed. Samuelsson (13,14) has reported that such a Δ^6-derivative, 3α,12α-dihydroxy-5β-chol-6-en-24-oic acid

(Δ^6deoxycholic acid), is an intermediate in the conversion of cholic acid to deoxycholic acid. Quite recently, Malavolti et al (15) have shown that Δ^6-lithocholic acid is actually formed from chenodeoxycholic acid as a metabolite by the action of human intestinal bacteria.

We have also been studying the significance of the Δ^6-acid in the conversion of the epimeric acids. The present paper deals with our independent results on the metabolism of Δ^6-lithocholic acid in the whole rat.

MATERIALS AND METHODS

Materials

Δ^6-Lithocholic acid and [24-^{14}C]Δ^6-lithocholic acid were synthesized by a modification of the method of Kagan (16,17), using chenodeoxycholic acid and [24-^{14}C]chenodeoxycholic acid (sodium salt, 50 μCi, > 45 mCi/mmol, Amersham) mixed with a suitable amount of carrier as the starting material, respectively. Briefly, 7-ketolithocholic acid (I) was prepared from chenodeoxycholic acid by partial oxidation with CrO_3 (18). This keto acid (I) was methylated by dissolving in methanol containing conc. HCl and acetylated with acetic anhydride in pyridine. The methyl ester acetate (II) was recrystallized from acetone and water, mp 142-143°C. Bromination of the 7-keto derivative (II) with bromine gave methyl 3α-acetoxy-6α-bromo-7-oxo-5β-cholan-24-oate (III), mp 173-175°C. The bromo ester (III) was reduced with sodium borohydride to methyl 3α-acetoxy-6α-bromo-7α-hydroxy-5β-cholan-24-oate (IV), mp 152-153°C. The desired Δ^6-derivative was obtained by refluxing the above bromohydrin (IV) in acetic acid with zinc dust which was added in small portions. The excess of zinc was removed by filtration, and methyl 3α-acetoxy-5β-chol-6-en-24-oate (V) was purified by column chromatography on silica gel and recrystallized from methanol, mp 124-125°C. Δ^6-Methyl ester acetate (V) was hydrolyzed with 3% KOH in methanol to give 3α-hydroxy-5β-chol-6-en-24-oic acid (VI), mp 216-217°C.

Throughout the experiment, purification of each compound was performed through column chromatography on silica gel, and Δ^6-derivatives were treated under N_2 atmosphere when possible and not heated with alkali solution to avoid possible degradation.

^{1}H-NMR spectra of these compounds were listed in Table 1.

A new compound, tauro-Δ^6-lithocholic acid, was synthesized by a modification of the method of Tserung (19). The solid was recrystallized from ethanol-ethyl acetate as needles, mp 186-188°C. IR 1660 cm^{-1} (-CONH-).

Table 1. Chemical shifts of the proton resonances of bile acid derivatives

Compound	δ (ppm)								
	6β-H	cis olefin 6,7-H	3β-H	7β-H	24-CO_2Me	3α-OAc	21-Me	19-Me	18-Me
Methyl 3α-acetoxy-7-oxo-5β-cholan-24-oate (II)	——	——	4.5-4.9 (b,1H)	——	3.70 (s,3H)	2.04 (s,3H)	0.95 (d,3H)	0.72 (s,3H)	0.67 (s,3H)
Methyl 3α-acetoxy-6α-bromo-7-oxo-5β-cholan-24-oate (III)	5.25 (d,1H)	——	4.5-4.9 (b,1H)	——	3.70 (s,3H)	2.02 (s,3H)	0.92 (d,3H)	0.95 (s,3H)	0.66 (s,3H)
Methyl 3α-acetoxy-6α-bromo-7α-hydroxy-5β-cholan-24-oate (IV)	4.75 (dd,1H)	——	4.4-4.9 (b,1H)	3.90 (d,1H)	3.70 (s,3H)	2.05 (s,3H)	0.93 (d,3H)	0.99 (s,3H)	0.68 (s,3H)
Methyl 3α-acetoxy-5β-chol-6-en-24-oate (V)	——	5.53 (s,2H)	4.5-5.0 (b,1H)	——	3.70 (s,3H)	2.06 (s,3H)	0.95 (d,3H)	0.86 (s,3H)	0.70 (s,3H)

Animals and their treatment

Male Wistar strain rats of about seven weeks, weighing 220 g, were used in all experiments (Shizuoka Agric. Coop. Assoc. for Laboratory Animals, Shizuoka). They were kept at least for one week before use in an air-conditioned room (23°C, 60% in humidity) lighted for 12 h (6:00 to 18:00), fed on a commercial balanced rat diet (Clea CE-2, Nihon Clea Co., Tokyo) and water ad libitum.

In a series of experiments, rats were laparotomized under anesthesia with ether and a solution of 5 mg of [24-^{14}C]Δ^6-lithocholic acid (0.475 μCi) in 1 mL of 0.9% NaCl-50% aqueous ethanol containing 20 μL of 1 N NaOH was injected into the cecum. After the operation, the animals were kept individually in a metabolic cage and their feces and urine were separately collected for 2 days. The liver and the intestines together with their contents were excised when the animals were sacrificed after 2 days.

In another series of experiments, rats were furnished with a bile-fistula as usual, and after 3 h they were injected intraperitoneally with a solution of 2.5 mg of [24-^{14}C]Δ^6-lithocholic acid (0.172 μCi) in 0.5 mL of 0.9% NaCl-50% aqueous ethanol containing 10 μL of 1 N NaOH. The bile-fistulated animals were kept individually in a restricted cage with free access to food and water. Fistula bile was collected at selected intervals for 24 h after the administration of [24-^{14}C]Δ^6-lithocholic acid.

Analysis of bile acids

Extraction of bile acids from biological materials except bile was achieved in the usual way (20). Briefly, the liver or the intestines including their contents was homogenized in physiological saline and extracted by refluxing in 95% ethanol containing 0.5% NH_4OH. Feces were freeze-dried and extracted by refluxing in 90% ethanol, 85% ethanol, and chloroform/methanol (1:1, v/v) successively for 2 h each. The pooled extract was evaporated to dryness and neutral lipids were removed by extracting with hexane. Each extract was dissolved in 0.1 N NaOH and percolated through an Amberlite XAD-2 column. After washing the column with water, bile acids were eluted with ethanol. Hydrolysis of conjugates by alkali and solvolysis of sulfates were omitted from the procedure because it is well known that most of the fecal bile acids are present in the unconjugated form (21). Monohydroxy, dihydroxy, and trihydroxy bile acids were separated by column chromatography on silica gel, eluted with various chloroform/ethanol mixtures (22). Bile acids were separated by preparative thin-layer chromatography on a plate of silica gel H (type 60), eluted from the gel, and purified through a column of Sephadex-LH 20 (22).

Bile was extracted with hot ethanol and the filtrate was evaporated to dryness. The residue was partitioned between ethanol/water and heptane/ether (1:1:1:1, by vol). The aqueous ethanol layer containing bile acids was evaporated and the residue was fractionated into three groups according to the type of conjugation by column chromatography on PHP-GEL (23,24). Successive elution with 90%-ethanolic solutions containing 0.1 M acetic acid, 0.2 M formic acid, and 0.3 M potassium acetate gave unconjugated, glycine-conjugated, and taurine-conjugated

bile acids, respectively. Individual bile acids in each fraction were isolated by preparative thin-layer chromatography on a plate of silica gel H in a suitable solvent system.

Enzymatic hydrolysis

Taurine-conjugated bile acids were hydrolyzed enzymatically at 37°C for 20 h in an incubation system consisting of sodium acetate buffer (pH 5.6, 25 μmol), bile acid (~0.4 mg), 2-mercaptoethanol (3 mg), ethylenediamine tetraacetic acid (disodium salt, 15 mg), cholylglycine hydrolase (Sigma C-3636, 0.8 mg) in a total volume of 2.6 mL (25,26). Free bile acids were extracted with ether and analyzed as described above.

Measurement of radioactivity, ^{1}H-NMR, and IR

Samples were dissolved in 10 mL of ACS II (Amersham Co.) and radioactivity was counted in a liquid scintillation spectrometer (Aloka, model LSC 651). Quenching was corrected by automatic external standardization. Thin-layer plates were scanned for radioactivity on a thin-layer autoradioscanner (Aloka, model TRM-1B). The ^{1}H-NMR spectra were obtained on a Hitachi R-900 spectrometer using $CDCl_3$ as the solvent and Me_4Si as the internal standard. The infrared spectra were recorded on a Hitachi EPI-G 31 spectrometer.

RESULTS AND DISCUSSION

Since the epimerization at C-7 of chenodeoxycholic acid and ursodeoxycholic acid is likely to occur by the action of intestinal bacteria, which are abundant in the cecum (27), where the enterohepatic circulation is considerably inactive, [24-^{14}C]Δ^6-lithocholic acid was injected as a postulated intermediate into the cecum of three rats. Recovery of the radioactivity two days after the injection is shown in Table 2.

When the radioactive substances recovered from the small intestine were chromatographed on a 15% $AgNO_3$-pretreated plate of silica gel, they remained on the origin. Since all the common bile acids in rats could run and be completely separated each other by our solvent system employed, the radioactive substances remained on the origin were

suspected as some metabolites of [^{14}C]Δ^6-lithocholic acid including conjugates. However, further characterization could not be made, because of no effective recovery of the ^{14}C-compounds from the plate. The liver and the large intestine including its contents had only a little radioactivity.

Table 2. Radioactivity recovered after the administration of [24-^{14}C]Δ^6-lithocholic acid in the cecum

	% Recovery[a]						
			Large intestine		Feces		
Rat no.	Liver	Small intestine	Cecum	Other part	Day 1	Day 2	Total[b]
Rat 1	0.7	9.3	0.9	0.7	46.7	10.0	68.3
Rat 2	0.7	8.3	2.0	1.3	44.7	16.1	73.1
Rat 3	1.1	13.9	5.9	2.7	44.5	18.7	86.8
Mean	0.8	10.5	2.9	1.6	45.3	14.9	76.1

[a] Based on the injected radioactivity.
[b] Urine contained scarcely any radioactivity.

Since the radioactivity was found mainly in the feces as expected, the two-day pool of fecal extract was analyzed. Monohydroxy, dihydroxy, and trihydroxy bile acid fractions were separated by column chromatography, being checked with the aid of thin-layer chromatography. These fractions contained 39.8%, 2.3%, and 1.7% of the total radioactivity in the pooled extract, respectively. A fraction with a considerable amount of radioactivity was left behind on the column

after the complete elution of trihydroxy bile acids. Although the fecal bile acids are mainly present in the unconjugated form, there was a possibility that the fraction contained the conjugated metabolites together with some decomposed products of the labile Δ^6-derivatives. In this study, however, characterization of this fraction was not carried out further. When each fraction corresponding to either dihydroxy or trihydroxy bile acids was chromatographed on a thin-layer plate in solvent system 2 (see the note in Table 3) and was scanned for radioactivity, no peaks were detected in the radioscannogram. Moreover, scarcely any radioactivity was found in the zone corresponding to chenodeoxycholic acid and ursodeoxycholic acid, when the extract from the adsorbent scraped off the plate was assayed in a liquid scintillation spectrometer. The results indicate that no detectable hydration of [^{14}C]Δ^6-lithocholic acid to either chenodeoxycholic acid or ursodeoxycholic acid took place in the rat *in vivo*.

In contrast with these results, the monohydroxy bile acid fraction from rat 1 and 2 gave a distinct peak on radioscanning, when thin-layer chromatographed in solvent system 2. Radioactive substances re-extracted from the adsorbent corresponding to the peak gave two peaks in the radioscannogram after argentation chromatography on a thin-layer plate predeveloped in 15% $AgNO_3$. The one peak showed the same Rf value with that of unchanged Δ^6-lithocholic acid and the other with that of lithocholic acid. The radioactive substance with the Rf value identical with that of lithocholic acid was re-extracted and converted to its methyl ester, a half portion of which was acetylated

further. Rf values of the metabolite and its derivatives were listed in Table 3.

Table 3. Rf values of the metabolite of [24-^{14}C]Δ^6-lithocholic acid and its derivatives

Compound	Rf[a]		
	Acid	Methyl ester	Methyl ester acetate
Radioactive metabolite	0.44	0.54	0.71
Lithocholic acid	0.44	0.54	0.71
Δ^6-Lithocholic acid	0.34	0.43	0.68

[a] Values on 15% $AgNO_3$-pretreated plate of silica gel developed in solvent system 2 (benzene/dioxane/acetic acid = 75:20:2, by vol).

The results indicate that the metabolite is lithocholic acid. The ratio of the radioactivity distributed in lithocholic acid and Δ^6-lithocholic acid was about 1:1 as estimated based on the peak area of the thin-layer radioscannogram. This shows that the reduction of Δ^6-lithocholic acid to lithocholic acid occurred to a considerable extent in rat 1 and 2. However, when the fecal extract from rat 3 was analyzed in the same way, scarcely any radioactive lithocholic acid was detected. The disparity in the results may be due to the difference in the state of the intestinal flora among the rats.

Our findings are consistent with the observation *in vitro* by White *et al* (28), who reported NAD-linked reduction of Δ^6-lithocholic acid by a cell extract of *Eubacterium* species V.P.I. 12708.

To elucidate whether the liver participates in the Δ^6-reduction or not, [24-^{14}C]Δ^6-lithocholic acid was injected intraperitoneally into two bile-fistula rats. Radioactivity excreted in bile amounted to, on an average, 64.4% (61.3 and 67.4%) of the total within 3 h and 76.4% (75.7 and 77.0%) during 24 h. Bile acids extracted from the 3-h portion of bile were fractionated into three groups according to the type of conjugation. Radioactivity was found most abundantly in the taurine-conjugated fraction and only a little in the other fractions as shown in Table 4.

When a portion of the taurine-conjugated fraction from each of rats was chromatographed on a thin-layer plate, only a single peak appeared on radioscanning. The Rf value of the radioactive metabolite coincided with that of tauro-Δ^6-lithocholic acid as shown in Table 5. Enzymatic hydrolysis of another portion of the sample gave a radioactive compound having the same Rf value as Δ^6-lithocholic acid (Table 5).

Table 4. Distribution of radioactivity in different conjugation types of bile acids

Rat no.	% Radioactivity		
	Unconjugated	Glycine-conjugated	Taurine-conjugated
Rat 4	2.3[a] (7.0)[b]	1.1 (3.4)	29.4 (89.6)
Rat 5	3.4 (8.8)	0.6 (1.6)	34.7 (89.7)
Mean	2.9 (7.9)	0.9 (2.5)	32.1 (89.7)

[a]Based on the injected radioactivity.
[b]Percentage of the radioactivity recovered in bile.

Table 5. Rf values of the biliary metabolite of [24-^{14}C]Δ^6-lithocholic acid and authentic standards

Sample	Rf[a] System 1[b]	Rf[a] System 2[c]
[^{14}C]Metabolite in bile		
Taurine-conjugated fraction	0.42	
Hydrolyzed taurine-conjugated fraction[d]		0.34
Standards		
Tauro-Δ^6-lithocholic acid	0.43	
Taurolithocholic acid	0.53	
Δ^6-lithocholic acid		0.34
Lithocholic acid		0.44

[a] Values on 15% $AgNO_3$-pretreated plate of silica gel.
[b] Isopropylalcohol/chloroform/acetic acid/water (30:30:4:1, by vol).
[c] See the note in Table 3.
[d] Enzymatic hydrolysis with cholylglycine hydrolase.

The results indicate that the radioactive compound in the biliary taurine-conjugated fraction was tauro-Δ^6-lithocholic acid. No radioactive bile acids, including lithocholic acid, other than Δ^6-lithocholic acid could be detected on thin-layer plates in any fractions of conjugation types.

The findings suggest that rat liver is capable of neither hydrogenating nor hydrating the Δ^6-double bond of Δ^6-lithocholic acid under the experimental conditions employed. It is of interest to note that Δ^6-lithocholic acid was not susceptible to any metabolic changes

in the steroid nucleus but could be a substrate for conjugation with taurine.

Although Malavolti et al (29) reported briefly that human liver had the capability of converting Δ^5-lithocholic acid to ursodeoxycholic, chenodeoxychoic, 7-ketolithocholic, and lithocholic acids, our present results in rats have shown that no hepatic biotransformation of Δ^6-lithocholic acid occurrs except for conjugation mainly with taurine. The discrepancy might be due to the difference in species.

In conclusion, Δ^6-lithocholic acid was reduced to lithocholic acid during the enterohepatic circulation in rats. The intestinal flora but not the liver may be responsible for the reduction.

REFERENCES

1 Thistle J L and Schoenfield L J. GASTROENTEROLOGY 61, 488 (1971).
2 Thistle J L and Hofmann A F. N ENGL J MED 289, 655 (1973).
3 Schoenfield L J, Lachin J M, et al. The Steering Committee, The National Cooperative Gallstone Study Group. ANN INTERN MED 95, 257 (1981).
4 Tokyo Cooperative Gallstone Study Group. GASTROENTEROLOGY 78, 542 (1980).
5 Makino I, Shinozaki K, Yoshino K, and Nakagawa S. JPN J GASTROENTEROL 72, 690 (1975).
6 Fedorowski T, Salen G, Tint G S, and Mosbach E H. GASTROENTEROLOGY 77, 1068 (1979).
7 Sarva R P, Fromm H, Carlson G L, Mendelow L, and Ceryak S. GASTROENTEROLOGY 78, 1252 (1980).
8 Edenhender R and Knaflic T. J LIPID RES 22, 652 (1981).
9 Sutherland J D and Macdonald I A. J LIPID RES 23, 726 (1982).
10 Macdonald I A and Roach P D. BIOCHIM BIOPHYS ACTA 665, 262 (1981).
11 Macdonald I A, Hutchison D M, and Forrest T P. J LIPID RES 22, 458 (1981).
12 Hirano S, Masuda N, and Oda H. J LIPID RES 22, 735 (1981).
13 Samuelsson B. J BIOL CHEM 235, 361 (1960).
14 Matkovis B and Samuelsson B. ACTA CHEM SCAND 16, 683 (1962).

15 Malavolti M, Fromm H, Cohen B, and Ceryak S. J BIOL CHEM 260, 11011 (1985).
16 Kagan H B and Rajagopalan J. BULL SOC CHIM FR 871 (1960).
17 Hsia S L. In: The Bile Acids (Nair P P and Kritchevsky D, Editors), Plenum Press, New York (1971), pp 95-120, and references therein.
18 Gallagher T F and Long W P. J BIOL CHEM 147, 131 (1943).
19 Tserung K -Y. Hachey D L, and Klein P D. J LIPID RES 18, 404 (1977).
20 a) Ayaki Y, Tsuma-Date T, Endo S, and Ogura M. STEROIDS 38, 495 (1981). b) Bradlow H L. STEROIDS 30, 581 (1977).
21 Madsen D, Beaver M, Chang L, B -Kardoss E, and Wostmann B. J LIPID RES 17, 107 (1976).
22 Ogura M, Goto M, and Ayaki Y. J BIOCHEM 83, 527 (1978).
23 Goto J. Hasegawa M. Kato H, and Nambara T. CLIN CHIM ACTA 87, 141 (1978).
24 Kitayama S, Ogura Y, and Ogura M. BIOL CHEM HOPPE-SEYLER 366, 737 (1985).
25 Yamamoto T. J YONAGO MED ASSOC 28, 114 (1977) (in Japanese).
26 Yamamoto T, Ikawa S, Takita M, and Ogura M. J YONAGO MED ASSOC 28, 126 (1977) (in Japanese).
27 Yahiro K. Setoguchi T, and Katsuki T. J LIPID RES 21, 215 (1980).
28 White B A, Paone D A M, Cacciapuoti A F, Fricke R J, Mosbach E H, and Hylemon P B. J LIPID RES 24, 20 (1983).
29 Malavolti M, Fromm H, Mosbach E H, and Ceryak S. HEPATOLOGY 4, 1034 (abstr) (1984).

APPENDIX

Trivial names used: Δ^6-lithocholic acid, 3α-hydroxy-5β-chol-6-en-24-oic acid; lithocholic acid, 3α-hydroxy-5β-cholan-24-oic acid; 7-ketolithocholic acid, 3α-hydroxy-7-oxo-5β-cholan-24-oic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholan-24-oic acid; ursodeoxycholic acid, 3α,7β-dihydroxy-5β-cholan-24-oic acid; tauro-Δ^6-lithocholic acid, 3α-hydroxy-5β-chol-6-en-24-oic acid N-(2-sulfoethyl)-amide; taurolithocholic acid, 3α-hydroxy-5β-cholan-24-oic acid N-(2-sulfoethyl)-amide.