Effect of cecum and appendix on 7α-dehydroxylation and 7β-epimerization of chenodeoxycholic acid in the rabbit[1]

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Abstract The effect of surgical removal of cecum and appendix on bile acid metabolism was studied in the rabbit. Bile acid composition of bile and intestinal contents were analyzed by gas–liquid chromatography and individual bile acids were further identified by mass spectrometry. In normal, intact rabbits (group I), the biliary bile acids consisted largely of deoxycholic acid (DA) 95.3 ± 1.0 (mean ± SE) %; cholic acid (CA) 2.3 ± 1.1% and lithocholic acid (LA) 1.0 ± 0.3% were also detected. Removal of cecum and appendix (group II) produced significant changes in biliary bile acid composition: DA fell to 58.3 ± 31.8%, CA rose to 37.7 ± 10.4%, and LA was barely detectable (0.3 ± 0.1%). Administration of chenodeoxycholic acid (CDA), 125 mg/day, produced severe hepatotoxicity, reduced food intake, and produced weight loss (group III). Biliary LA rose to 15.0 ± 1.3%, while DA was 61.3 ± 14.0% and CDA 21.8 ± 14.6% of total biliary bile acids. Feeding CDA to animals without cecum and appendix (group IV) resulted in a slight increase of LA (3.2 ± 2.2%) compared to group III, and appreciable amounts of ursodeoxycholic acid (UDA) 32.0 ± 9.8% and of 7-ketolithocholic acid (7-KLA) 3.0 ± 0.6% appeared in bile. The animals of group IV exhibited no hepatotoxicity and ate and gained weight normally. These results indicate that the microbial population of cecum and appendix is active in 7α-dehydroxylation of primary bile acids and that removal of these organs results in an increased formation of UDA by an unknown mechanism.—**Yahiro, K., T. Setoguchi, and T. Katsuki.** Effect of cecum and appendix on 7α-dehydroxylation and 7β-epimerization of chenodeoxycholic acid in the rabbit. *J. Lipid Res.* 1980. **21:** 215–222.

Supplementary key words cecectomy with appendectomy · ursodeoxycholic acid · 7-ketolithocholic acid · lithocholic acid

Feeding of chenodeoxycholic acid[3] (CDA) to rabbits produces cirrhotic and necrotic changes in the liver (1–4). It has been suggested that CDA itself is not very hepatotoxic and that the effect of oral administration of CDA is due to its conversion to lithocholic acid (LA) which is absorbed and also "recycled" via coprophagy (4, 5).

Cholic acid (CA) and small amounts of CDA are the primary bile acids in the rabbit, but appreciable amounts of 5α-cholanoic acids are also known to be present (6). In the rabbit 7α-dehydroxylation of the primary bile acids by the intestinal bacterial flora is so pronounced that deoxycholic acid (DA) (the secondary bile acid of CA) is the major biliary bile acid. LA is present in trace amounts (4, 7). In rabbit feces, however, LA may amount to 10–20% of fecal total bile acids. It seems reasonable to suggest that fecal LA arose partially via 7α-dehydroxylation of CDA and partially via 12α-dehydroxylation of DA. The 12α-dehydroxylation may be the more important pathway of LA formation in the normal rabbit since in this species CDA appears to be a minor, trace component of bile. The biliary bile acid composition of the normal rabbit indicates but does not prove that in this species LA is less readily absorbed from the large intestine than DA (4).

The administration of CDA to rabbits produces a large increase of LA in the intestine (4). The rabbit is unable to detoxify LA by sulfation (as in man) (8, 9) or by re-hydroxylation (as in the rat) (10). Apparently an appreciable proportion of the LA is absorbed and reaches the liver via the portal circulation to account for the observed toxic effects.

Abbreviations: GLC, gas–liquid chromatography; R_T, retention time; TLC, thin-layer chromatography; CA, cholic acid; DA, deoxycholic acid; LA, lithocholic acid; CDA, chenodeoxycholic acid; 7-KLA, 7-ketolithocholic acid; UDA, ursodeoxycholic acid.

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[3] The following systematic names are given to sterols and bile acids referred to by trivial names: cholesterol, cholest-5-en-3β-ol; cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; ursodeoxycholic acid, 3α,7β-dihydroxy-5β-cholanoic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid; lithocholic acid, 3α-hydroxy-5β-cholanoic acid; 7-ketolithocholic acid, 3α-hydroxy-7-keto-5β-cholanoic acid.

In man, administered CDA can be converted to ursodeoxycholic acid (UDA) and this 7β-epimerization can be quite pronounced in some (but not all) patients (11). According to the postulation by Salen et al. (11), the 7β-epimerization of CDA to UDA may be defined as another detoxification mechanism because CDA is not converted to toxic LA but to UDA. Apparently this reaction does not occur in CDA-fed, intact rabbits to a significant extent.

In the present experiments we set out to investigate the contribution of the microbial flora of cecum and appendix to 7α-dehydroxylation in the rabbit. We found that removal of these organs greatly decreased the formation of LA from administered CDA and in addition observed the appearance of UDA and 7-ketolithocholic acid (7-KLA). In these animals, CDA feedings did not produce the toxic symptoms observed in intact rabbits.

MATERIALS AND METHODS

Bile acids

Chenodeoxycholic acid (of more than 99% purity by GLC) was obtained from Eisai Co., Ltd., Tokyo. Ursodeoxycholic acid (of more than 99% purity by GLC) was obtained from Tokyo Tanabe Co., Ltd., Tokyo. 7-ketolithocholic acid was prepared from chenodeoxycholic acid by CrO_3 oxidation (12), and material of 99% purity (by GLC) was obtained after recrystallization from benzene. Methyl esters of the bile acids were prepared with acidic methanol, methanol–conc. sulfuric acid 25:1 (v/v) (13).

Animals

Male rabbits (New Zealand White), weighing between 2.5–3.5 kg, were used throughout. The animals were housed in individual metabolic cages and maintained on a diet of Nippon CLEA CR-2 Rabbit Chow pellets ad libitum. All animals were acclimated to this food for at least 3 weeks before experimentation. The food intake was monitored daily, and water was given ad libitum.

Feeding of chenodeoxycholic acid

Chenodeoxycholic acid (125 mg/day) was given orally for 3 weeks in divided doses at 9 AM and at 5 PM. The bile acid was wrapped in a vegetable leaf and hand-fed to the rabbits. Usually the animals ate the entire dose of CDA. In some animals CDA was administered by gavage.

Surgical removal of cecum and appendix

The rabbits were starved for a period of 12 hr prior to the operation. Under general anesthesia with sodium nembutal (30–50 mg/kg), the cecum and appendix were removed except for about three segments of the cecum, which were necessary to maintain passage of the intestinal contents. Fluid was infused intravenously and 200 mg of synthetic penicillin was injected intramuscularly for the first two postoperative days. Generally, the rabbits began to eat pellets by the third postoperative day, and then began to gain weight gradually. After successful operations, the rabbits showed entirely normal growth and eating habits. No experiments were scheduled for at least 3 weeks after the operation.

Experimental design

The rabbits were divided into four groups as in **Table 1.** They were acclimated to the regimen for 3 weeks, followed by the 3-week experimental period. Groups I and II were control groups for comparison with groups III and IV which received 125 mg/day of CDA. At the end of the 3-week experimental period the animals were killed; gallbladder bile, and

TABLE 1. Effect of removal of the cecum and appendix on biliary bile acid composition[a] in the rabbit

Group	No. Rabbits	Lithocholic Acid	Deoxycholic Acid	Chenodeoxycholic Acid	Ursodeoxycholic Acid	7-Ketolithocholic Acid	Cholic Acid
I Normal	5	1.0 ± 0.3[b]	95.3 ± 1.0	1.4 ± 0.2	n.d.[c]	n.d.	2.3 ± 1.1
II Cecectomy with appendectomy	6	0.3 ± 0.1 $P < 0.05$[d]	58.3 ± 13.0 $P < 0.02$	1.4 ± 0.9	2.6 ± 2.6	n.d.	37.7 ± 10.4 $P < 0.01$
III Control + CDA 125 mg/day	4	15.0 ± 1.3[b]	61.3 ± 14.0	21.8 ± 14.6	n.d.	n.d.	3.2 ± 1.4
IV Cecectomy with appendectomy + CDA 125 mg/day	4	3.2 ± 2.2 $P < 0.005$[e]	12.8 ± 8.8 $P < 0.05$	36.9 ± 14.9	32.0 ± 9.8 $P < 0.02$	3.0 ± 0.6 $P < 0.005$	11.9 ± 6.8

[a] Percent of total bile acids. Unknown bile acids (a few percent of total bile acids) were detected but not added to the sum.
[b] Mean ± standard error of the mean.
[c] Not detectable.
[d] Significantly different from group I by paired t-test.
[e] Significantly different from group III by paired t-test.

contents of stomach, small intestine, cecum and appendix (or the contents of the cecal stump), and colon contents were collected and weighed. Liver sections were taken for histologic examination. Pertinent aliquots of intestinal contents or bile were analyzed immediately after the rabbits were killed. Other aliquots were stored at −20°C and processed as required.

Analytical methods

Bile acids of bile and intestinal contents: preparation of methyl esters. Bile was extracted by the addition of 20 volumes of methanol. The precipitate was centrifuged off and discarded. The solvent was evaporated on a water bath under a stream of N_2. For gastrointestinal contents, about 1–2 g of each sample was lyophilized and extracted with 120 ml ethanol in a Soxhlet apparatus for 6 hr. For alkaline hydrolysis, a 10% NaOH aqueous solution was used at 122°C for 3 hr. After acidification with 4 N HCl, ethyl acetate–methanol 20:1 (v/v) was used for extracting sterols and bile acids. The ethyl acetate layer was washed with water, dried over sodium sulfate, and evaporated. Methylation of the free bile acids was accomplished by an overnight reaction with acidic methanol, methanol–conc. sulfuric acid, 25:1 (v/v) at room temperature. The methyl esters were extracted with benzene from the aqueous methanol solution after the pH had first been adjusted to 8–9 with $NaHCO_3$ (13).

Aliquots of the methyl esters were taken for estimation by gas–liquid chromatography. An external recovery standard containing cholesterol and taurine conjugates of lithocholic, deoxycholic, and cholic acids was used to correct for losses during the procedure. By omitting a solvolysis step, the total LA may have been underestimated in all animals, although it has been reported that the rabbit is unable to modify LA by sulfation (4).

Gas–liquid chromatography was used to determine the concentration of biliary cholesterol, lithocholic acid, 7-ketolithocholic acid, deoxycholic acid, chenodeoxycholic acid, ursodeoxycholic acid, trihydroxy bile acids, and others. The methyl esters were estimated on a 200 cm × 3 mm glass column packed with 3% QF-1 on 80–100 mesh Gas Chrom Q (Applied Science Laboratories, Inc., State College, PA) at 260°C. The bile acid methyl esters and cholesterol were estimated by comparison with their respective external standards of known concentration. Routine estimation of bile acids and cholesterol by GLC was possible without derivatization of the hydroxyl groups.

Preparative thin layer chromatography. The methyl esters of bile acids were isolated by TLC on 20 × 20 cm glass plates coated with 0.25 mm thick layers of silica gel (TLC plates silica gel 60, without fluorescent indicator, Merck, Darmstadt). The plates were developed with benzene–acetone 70:30 (v/v). Appropriate standards were applied along the edges of each plate, the spots were visualized with phosphomolybdic acid, and the pertinent bands were scraped from the plate; the bile acids were eluted from the silica gel with methanol. The methanol was evaporated and the bile acids were dissolved in ethyl acetate. The ethyl acetate was washed three times with water, and was evaporated.

Mass spectrometry. Mass spectra of the bile acid methyl esters and their TMS derivatives were obtained with a LKB-Shimadzu GLC-Mass apparatus, GC-MS 9000, with a computer system, PAC-300 DG. TMS derivatives were prepared with the reagent bis-trimethylsilyl dimethylamine–TMS imidazole–acetonitrile 5:1:5 (v/v), at 60°C for 20 min (14). The samples were injected into a glass column, 100 cm × 3 mm ID, packed with 3% OV-1 on 80–100 mesh Gas Chrom Q. Injection port temperature was 320°C; column temperature, 270°C; and separator temperature, 330°C. The flow of carrier gas (He) was 40 ml/min. Ion source temperature, emission current, electron energy, and accelerating voltage were 270°C, 60 A, 20 eV, and 3.5 KV, respectively. Scan speed was 8; chart speed was 20 cm/sec; the slit was 0.2/0.05.

RESULTS

Food intake and weight change

Change of food intake and body weight during the 3-week experimental period for all animals fed CDA are shown in **Figs. 1** and **2.** The data indicate that the symptoms of CDA toxicity (reduced food intake and weight loss) in the normal, intact rabbits of group III, are absent in the animals of group IV (cecum and appendix removed). It was pointed out above (Methods section) that weight gain and food intake of cecectomized animals on stock diet (group II) did not differ from those of intact controls on the same diet (group I).

Biliary bile acid composition

Table 1 lists the biliary bile acid composition of all four groups of rabbits studied. The bile of intact animals on stock diet (group I) contained largely DA (95.3%) and small percentages of CA (2.3%), CDA (1.4%), and LA (1.0%). Removal of cecum and appendix (group II) caused a significant decrease in the percentage of DA and LA, no change in CDA, and a large increase in biliary CA (Table 1). Sham-operated rabbits (n = 3) (laparotomy only, under the same condition as group II) showed biliary bile acid values similar to those of group I: DA, 97.4 ± 1.4 SE%; CA, 1.5 ± 0.7%; LA, 1.0 ± 0.5%.

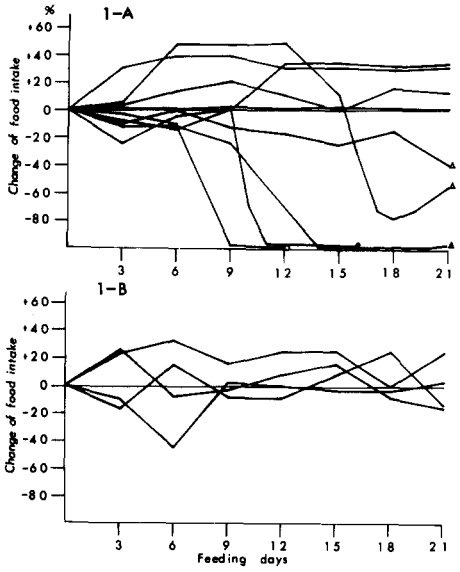

Fig. 1. Changes of food intake of control and operated (cecectomy with appendectomy) rabbits, groups III and IV. The rabbits were fed CDA, 125 mg per day. (1-A): Five of nine intact control animals showed severe depression of food intake. One animal (*) died on the 16th day, one animal (○) was killed on day 12 due to severe toxicity, and three animals (△ and ▲) also exhibited severe toxic symptoms but were alive until the scheduled date of killing (day 21). These five animals were not included in the statistical analysis of the bile acid composition; they were given CDA by gavage, and their bile acid composition was similar to that of the less affected rabbits. (1-B): All four of the operated animals of group IV survived and had normal eating habits.

The administration of CDA to intact rabbits (group III) caused marked changes in biliary bile acid composition. LA and CDA increased, while the proportions of CA and DA decreased. In contrast, in cecectomized, CDA-fed rabbits (group IV), there was only a slight increase in LA but UDA was now a significant biliary constituent, and relatively minor proportions of 7-KLA also were detected.

Bile acid composition of cecal contents in controls and cecectomized animals fed CDA

Gas–liquid chromatograms of the bile acids found in cecal contents of individual rabbits of groups III and IV are shown in **Fig. 3.** Recovery of added glycine conjugate of DC (5, 10 and 100 mg to 1 g of lyophilized feces) was 74–93% in whole procedures. Administration of CDA to the control animals induced a large increase of LA in the cecal contents (control group). LA and DA were the major bile acids. A small amount of CDA and several unknown bile acids were observed in these animals. UDA was not detected in these rabbits; however, small amounts of 7-KLA were found in two rabbits.

After removal of most of the cecum and appendix, administration of CDA did not increase LA in the remaining section of cecum. Furthermore, UDA and 7-KLA as well as CDA were observed in appreciable quantities in all of the operated animals.

Identification of ursodeoxycholic acid

The methyl ester of biological UDA was separated by preparative TLC (see Materials and Methods) and the ester was cochromatographed with authentic methyl UDA. Identical relative R_f and R_T values were observed as shown in **Table 2.**

Positive identification of the ursodeoxycholic acid was also estabished by GLC–mass spectrometry (**Fig. 4**). The mass spectrum of the TMS ether of the methyl ester of purified biological UDA was identical line for line with the fragmentation pattern of the TMS ether of authentic methyl UDA. The molecular ion (M^+) was detected at m/e 550; lines at m/e 535 ($M\text{-}CH_3$; 550-15), and m/e 460 [$M\text{-}OSi(CH_3)_3H$; 550-90] are more typical of the 7β-epimer than of the 7α-epimer, as reported previously by others (11).

Thus, by three independent methods (TLC, GLC, and mass spectrometry) it is conclusively proven that UDA was present in the bile of our group IV animals. The presence of UDA in the cecal contents was established similarly.

Identification of 7-ketolithocholic acid

The methyl ester of biological 7-KLA was separated by preparative TLC and the ester was cochromatographed with authentic methyl 7-KLA. Identical relative R_f and R_T values were observed (Table 2).

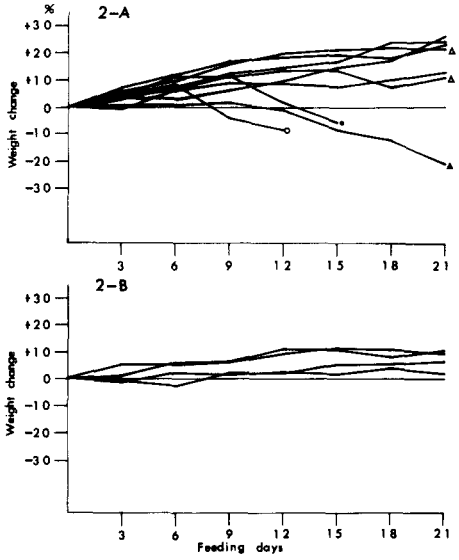

Fig. 2. Weight changes of controls (group III) and operated (cecectomy with appendectomy) rabbits (group IV). Rabbits were fed CDA, 125 mg per day. (2-A): In the intact control group, three of the nine animals suffered a 10–20% weight loss. The symbols in Fig. 2 correspond to the animals with the same symbols in Fig. 1. (2-B): The operated animals (group IV) showed no weight loss.

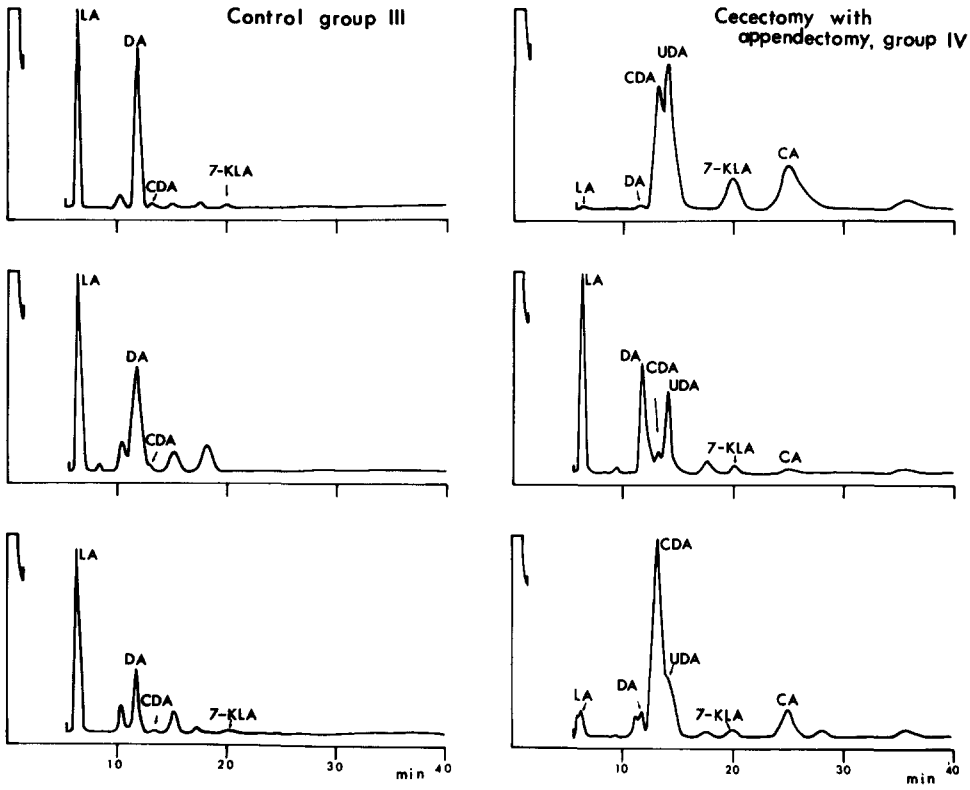

Fig. 3. Gas–liquid chromatograms of bile acids of cecal contents of individual rabbits of groups III and IV. The chromatograms on the left represent the bile acid patterns of cecal contents of three intact rabbits fed CDA. The chromatograms on the right illustrate the bile acid patterns of material isolated from the cecal stump. Note uniformly large LA peak in the animals of group III and the appearance of UDA and 7-KLA in the rabbits of group IV. Abbreviations in this figure: LA, methyl lithocholate; DA, methyl deoxycholate; CDA, methyl chenodeoxycholate; UDA, methyl ursodeoxycholate; 7-KLA, methyl 7-ketolithocholate; CA, methyl cholate.

Positive identification of the 7-KLA was also established by GLC–mass spectrometry (**Fig. 5**). The mass spectrum of the methyl ester of purified biological 7-KLA was identical line for line with the fragmentation pattern of authentic methyl 7-KLA. Molecular ion (M^+) was detected at m/e 404. Loss of water was observed at m/e 386 ($M-H_2O$; 404-18) and m/e 253 [M-(115 + 2 × 18)]. An ion at m/e 194, valuable aid for the identification of 7-keto derivatives, was observed at about 70% intensity (15).

Thus, the correspondence obtained by three independent methods (TLC, GLC, and mass spectrometry) conclusively proves that 7-KLA was present in the bile of our rabbits. The presence of 7-KLA in the cecal contents was demonstrated in a similar manner.

DISCUSSION

It is now well established that oral administration of chenodeoxycholic acid induces dissolution of cholesterol gallstones in man (16, 17). However, the administration of CDA to man still remains controversial because hepatotoxicity was developed in various animal species treated with this bile acid. Thistle and Hofmann (17) reported that about one-quarter of the patients ingesting CDA had transiently elevated

TABLE 2. Relative R_f and R_T values in TLC and GLC

Bile Acid	R_f[a]	Relative R_f[b]	R_T[c]	Relative R_T[d]
			min	
Methyl ursodeoxycholate				
Authentic	0.46	1.18	14.38	1.05
Biological	0.46	1.18	14.33	1.05
Methyl 7-ketolithocholate				
Authentic	0.60	1.54	20.66	1.51
Biological	0.60	1.54	20.60	1.51

[a] R_f values of bile acid methyl esters. Solvent system was benzene–acetone 70:30 (v/v).

[b] R_f values were given relative to that of methyl chenodeoxycholate.

[c] Retention times of methyl ester derivatives on 3% QF-1. Column temperatures 260°C.

[d] Retention times were given relative to that of methyl chenodeoxycholate.

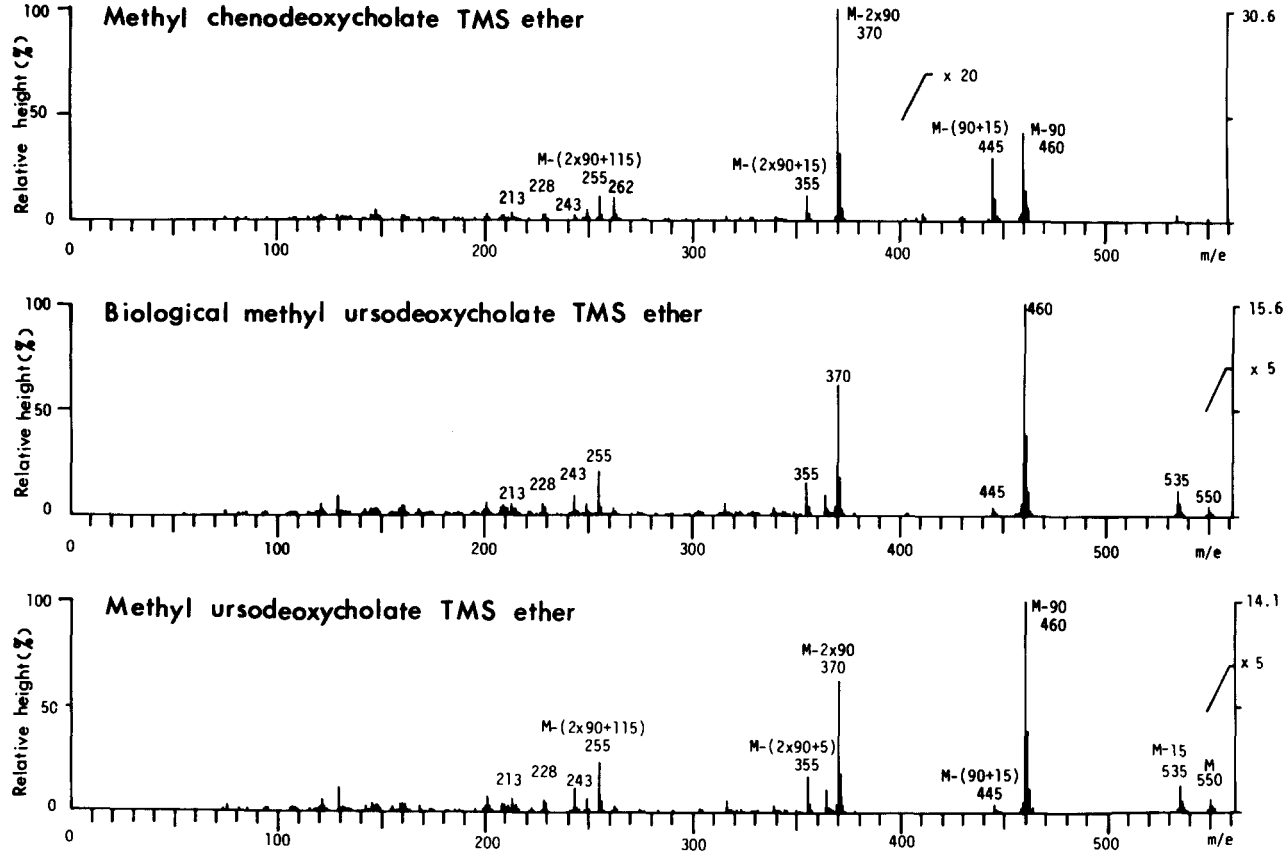

Fig. 4. Mass spectra of the TMS-ether derivatives of authentic methyl chenodeoxycholate, biological methyl ursodeoxycholate, and authentic methyl ursodeoxycholate.

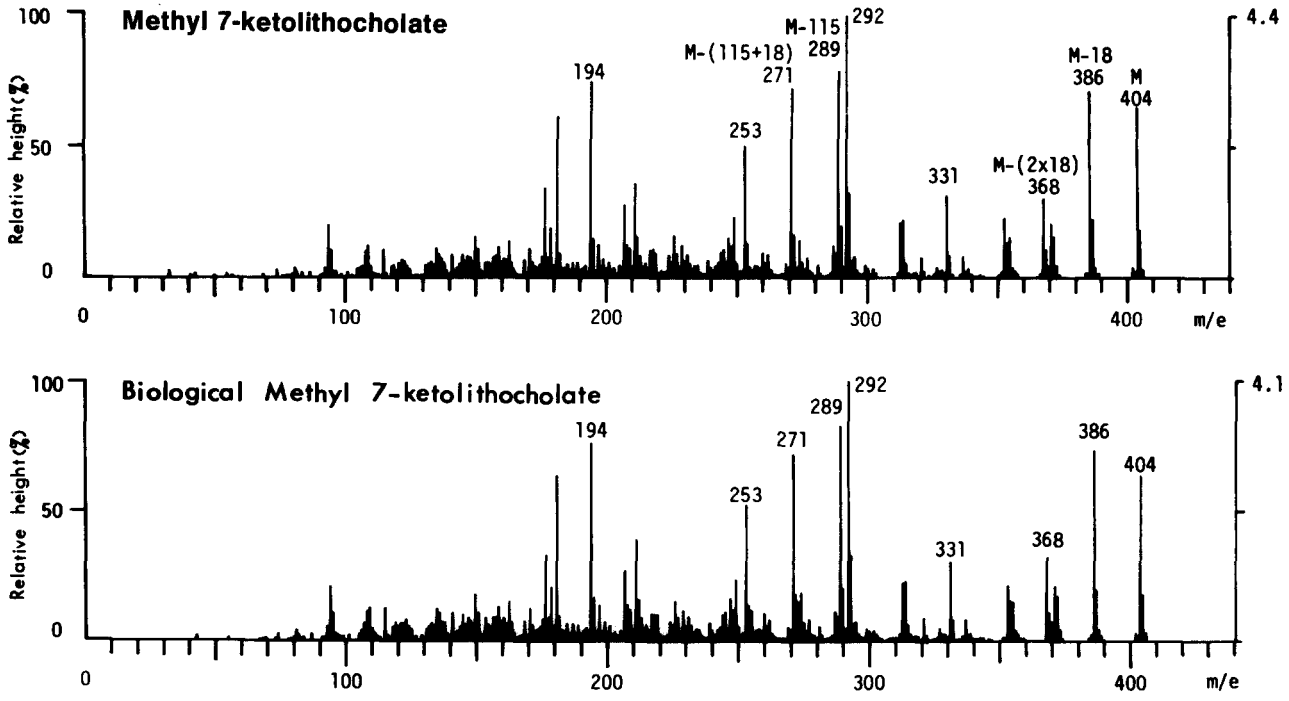

Fig. 5. Mass spectra of authentic methyl 7-ketolithocholate and biological methyl 7-ketolithocholate.

serum glutamic oxaloacetic transaminase levels, but no abnormalities in other liver function tests. In contrast, feeding of CDA produces hepatic injuries in the rhesus monkey (18–20), baboon (21, 22), and rabbit (4). It has been suggested that the severity of the liver damage is related to the percentage of LA in bile, and histological changes are indeed similar to those described for LA toxicity (4, 5).

In the rabbit, the toxic reaction induced by the CDA administration is so severe that after prolonged feeding the animals develop jaundice, ascites, and many die (4). This species seems to lack most or all of the detoxification pathways for LA described for other species, namely re-hydroxylation (10), sulfation (8, 9), or 7β-epimerization (11). This situation is similar to that in the rhesus monkey where CDA produces considerable liver damage, presumably partly because this species, like the rabbit, cannot sulfate LA to an appreciable extent (20).

The biliary LA found in the rabbits not fed CDA (groups I and II) may be derived from endogenous CDA, via 7α-dehydroxylation, or from DA via 12α-dehydroxylation by the intestinal microflora. These organisms must be heavily concentrated in the cecum since removal of this organ (group II) caused a considerable decrease in the percentage of biliary DA with concomitant increases in CA. Presumably some of this newly synthesized CA was able to escape bacterial degradation and was reabsorbed unchanged.

Administration of CDA to intact rabbits (group III) led to the appearance of CDA in bile (21.8%), but the predominant biliary bile acid was still DA (61.3%). Removal of the cecum (group IV) led to a further increase of biliary CDA (36.9%) which became the predominant bile acid but UDA was now present in almost equal proportions (32%) and DA was reduced to 12.8%. It should be stressed again that in the animals of group IV (CDA and cecectomy) biliary LA was only 3% and the liver was histologically nearly normal compared to the severe hepatotoxicity observed in intact animals fed CDA (group III).[4]

The composition of cecal bile acids was similar to that of the bile in all groups although the ratio of secondary to primary bile acids increased in the former. In the CDA-fed control animals (group III) the predominant cecal bile acids were LA and DA as expected. However, in the CDA-fed cecectomized rabbits (group IV) appreciable amounts not only of the administered CDA appeared but considerable proportions of UDA and smaller percentages of 7-KLA were also detected.

The mechanism of formation of UDA cannot be deduced from the present experimental design. It cannot be stated with certainty that the UDA was derived exclusively by bacterial action on CDA via 7-KLA: a hepatic pathway seems less likely, however, since it has been reported that 7-ketodeoxycholic acid was not changed to the 7-hydroxy derivative following a single passage through rabbit liver (23). Thus it is possible that removal of the cecum favored the predominance of microorganisms capable either of a direct inversion of the 7α-hydroxy group of CDA, or of other microorganisms capable of reducing the 7-keto group of bile acids to the 7β-hydroxy configuration (24).

Further studies will be required to establish the mechanism of UDA formation in CDA-fed, cecectomized animals.

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