Epimerization of the four 3,7-dihydroxy bile acid epimers by human fecal microorganisms in anaerobic mixed cultures and in feces[1]

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Abstract The conversion of 3,7-dihydroxy bile acids by anaerobic mixed cultures of intestinal microorganisms was studied in fecal samples from eight healthy adult males. Incubations using substrate chenodeoxycholic acid (CDCA) and ursodeoxycholic acid (UDCA) were performed simultaneously in separate microbial suspensions from the same fecal samples. A time course study was done on four samples, chosen randomly from the eight. In the incubation of CDCA, substrate CDCA always decreased rapidly in amount; UDCA increased in amount, as did 3β,7β-dihydroxy-5β-cholanoic acid (3β,7β) and 3β,7α-dihydroxy-5β-cholanoic acid (3β,7α). In the incubation of UDCA, UDCA gradually decreased in amount; (3β,7β), CDCA, and (3β,7α) increased gradually in amount. All reactions involved four epimers. After 48–72 hr UDCA was predominant and the reactions appeared to have reached equilibrium. In cultures from all eight samples, after 72–96 hr, a predominance of β-hydroxy configurations at 7-position and α-hydroxy configurations at 3-position was observed. To compare these bile acid compositions to those in feces, an in vivo study using nine subjects was carried out. Concurrent with the collection of feces, transit time of food through the gut was measured. In samples from five subjects, in which amounts of lithocholic acid (LCA) was small, four 3,7-dihydroxy epimers were found. In samples from the other four, however, CDCA, the predominant epimer in bile, had apparently been converted to LCA by 7-dehydroxylation, and four epimers were not always found. In contrast to the incubation study, UDCA was not always the predominant 3,7-dihydroxy epimer in the fecal study. This may have been due to the transit times, which averaged 26.4 ± 8.9 SD hr, being much shorter than the time it took for the incubation reactions to reach equilibrium.—**Setoguchi, T., S. Higashi, S. Tateno, K. Yahiro, and T. Katsuki.** Epimerization of the four 3,7-dihydroxy bile acid epimers by human fecal microorganisms in anaerobic mixed cultures and in feces. *J. Lipid Res.* 1984. **25:** 1246–1256.

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The physiology of bile acids is linked to the digestion and absorption of fat and is characterized by enterohepatic circulation. Terminal ileal absorption of glycine- and taurine-conjugated bile acids is essential to human physiology. During the enterohepatic cycle, peptide bond and hydroxy groups are vulnerable, especially in the large intestine, to microbial attack, but the steroid nucleus is essentially invulnerable. Some of the converted bile acids are absorbed in the process and influence the bile acid circulation. Chenodeoxycholic acid (CDCA) and ursodeoxycholic acid (UDCA) are now used as therapeutic agents for the dissolution of cholesterol gallstones (1, 2). The mutual transformation of these two acids has been reported (via 7-ketolithocholic acid) in mixed cultures and single cultures of intestinal microorganisms (3–14). Recently, human intestinal microbial conversion of bile acids has been studied intensively. The conversion patterns in mixed cultures of intestinal flora may exemplify the in vivo conversion process as a whole. In this study, therefore, we report the conversion patterns of 3,7-dihydroxy bile acids in anaerobic mixed cultures of human intestinal flora and, for comparison, the bile acid compositions in human fecal samples.

MATERIALS AND METHODS

Subjects

Eight subjects were used in the incubation study and nine in the fecal study. Three of the subjects used in

Abbreviations: GLC, gas–liquid chromatography; $(R)R_T$, (relative) retention time; TLC, thin-layer chromatography; LCA, lithocholic acid; 3β-LCA, 3β-hydroxy epimer of LCA; CDCA, (3α,7α), chenodeoxycholic acid; UDCA, (3α,7β), ursodeoxycholic acid; 7-KLCA, 7-ketolithocholic acid; (3β,7α), 3β,7α-dihydroxy-5β-cholanoic acid; (3β,7β), 3β,7β-dihydroxy-5β-cholanoic acid. Trivial names of bile acids and their systematic counterparts (in the text, bile acids are usually referred to by trivial names): lithocholic acid, 3α-hydroxy-5β-cholanoic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; ursodeoxycholic acid, 3α,7β-dihydroxy-5β-cholanoic acid; 7-ketolithocholic acid, 3α-hydroxy-7-keto-5β-cholanoic acid.

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the incubation study were also used in the fecal study. All were healthy Japanese adult males between the ages of 22 and 36 on an ordinary Japanese diet. None showed any evidence of hepatic or gastrointestinal disorders, and none had received antibiotics for several months.

Bile acids as substrates and authentic agents

Chenodeoxycholic acid (CDCA) of more than 99% purity was donated by Eisai Co., Ltd., Tokyo. Ursodeoxycholic acid (UDCA) of more than 99% purity and 3β,7β-dihydroxy-5β-cholanoic acid (3β,7β) [mp 184.5–185.5°C, $[\alpha]_D^{20°}$ + 57.4° (c, 0.5; ethanol)], and 3β,7α-dihydroxy-5β-cholanoic acid (3β,7α) [mp 196°C, $[\alpha]_D^{23°}$ + 7.3° (c, 2.0; ethanol)] were donated by Tokyo Tanabe Co., Ltd., Tokyo. The (3β,7β) and (3β,7α) were prepared by the method reported by Danielsson et al. (15). 7-Ketolithocholic acid (7-KLCA) was prepared from CDCA by CrO_3 oxidation (16).

For the incubation study, substrates (CDCA and UDCA) were further purified to more than 99.9% by preparative thin-layer chromatography (described below).

Methyl esters of the bile acids were prepared with methanol–concentrated H_2SO_4 100:4 (v/v). The solution was left overnight at room temperature. The ester was extracted with benzene from methanolic water that had been alkalinized with sodium bicarbonate beforehand. The benzene was washed with water until the washings became neutral. After the solvent was evaporated, the methyl ester was recrystallized from methanol that contained a small amount of water.

The crystals of both the free acids and the methyl esters were dried at 60°C in vacuo for more than 48 hr. Further drying at 80°C for 6 hr did not reduce the weight.

Mixed cultures of human intestinal microorganisms

The anaerobic incubation procedure was performed in a 162 cm × 84 cm × 76 cm anaerobic chamber (Hirasawa Works Co., Tokyo), similar to a Forma Anaerobic System, filled with 80% nitrogen, 10% CO_2, and 10% H_2. A GAM medium (Nissui Co., Tokyo) was used for the culture (17); it contained 1% peptone, 0.3% soybean peptone, 1% protease peptone, 1.35% serum powder containing hemin, 0.5% yeast extract, 0.22% beef extract, 0.12% liver extract, 0.25% KH_2PO_4, 0.3% NaCl, 0.03% L-cysteine–HCl·H_2O, 0.15% sodium thioglycolate, and 0.3% glucose. Five ml of the culture medium was dispensed into each test tube. Alkaline bile acid solutions (pH 9–10) were prepared by mixing 37 μmol of either CDCA or UDCA in 0.1 N NaOH. The bile acid solutions were added to the media. After these media were autoclaved at 123°C for 10 min, they were immediately cooled in an ice bath. They were then kept for more than 24 hr in the anaerobic chamber before inoculation. Final pH of the media was 7.2–7.3.

Sample feces were collected from each incubation study subject in polyethylene bags filled with O_2-free CO_2 gas. The feces in each bag were well mixed and fecal suspensions were prepared by tenfold dilutions of 1 g of feces with a prereduced 1/30 M phosphate buffer (pH 7.6) containing 0.05% L-cysteine–HCl·H_2O. Each suspension was centrifuged at 170 *g* for 5 min; the supernatant was recentrifuged at 2800 *g* for 10 min, and the sediment was resuspended in 10 ml of the phosphate buffer. This microbial suspension contained approximately 8×10^9/ml of intestinal bacteria. A standard volume (0.1 ml) of the fecal suspension was inoculated into the medium containing a bile acid. Final bile acid concentration was 283 nmol/ml. The seeded tubes were incubated in the anaerobic chamber at 37°C.

Time course studies were performed on four of the eight samples. Incubations were terminated at 8, 16, 24, 48, 72, and 96 hr by adding 0.5 ml of 10% KOH solution to the time-labeled tubes, which were then kept at −20°C until bile acid analysis. For analysis of the bile acids in an equilibrium state, the seeded tubes of all eight samples were checked after 72–96 hr of incubation.

Extraction of bile acids from incubation medium

A volume of the incubation medium was cooled, acidified to pH 2 with cold 6 N hydrochloric acid, and 0.1 vol of cold methanol was added. Bile acids were extracted with two volumes of cold ethyl acetate (18). Foamy flocculent precipitates were observed between the two layers. The upper clear ethyl acetate layer was removed after centrifugation. The extraction process was repeated three times, the combined ethyl acetate solutions were washed with cold water until the washings became neutral, and the ethyl acetate was evaporated to dryness. Methylation was performed as described above.

Extraction of bile acids from feces

Each freshly voided sample was well mixed in a polyethylene bag. About 30 g of feces was taken from each, diluted with an equal amount of water, and homogenized with a Waring blender. The bile acids were extracted from 0.5 g of diluted feces with 8 ml of methanol by mixing the suspension for 20 min then centrifuging. The extraction process was performed three times and the combined methanol solutions were evaporated at 60°C under N_2.

The extracted bile acids were dissolved in 2 ml of methanol. Then 0.5 ml of saturated sodium bicarbonate solution and 2 ml of water were added. Neutral lipids were extracted with 3 ml of benzene. This extraction process was performed three times.

The alkaline methanol–water was cooled on ice and acidified with 2 N HCl. Free bile acids were then extracted with 5 ml of cooled ethyl acetate. The ethyl acetate extraction was performed three times, the combined ethyl acetate extracts were washed with water until the washings became neutral, and the ethyl acetate was evaporated. Bile acids were extracted without hydrolysis or solvolysis, because thin-layer chromatography indicated that most of the bile acids were free, neither conjugated nor sulfated. Methylation was performed as described above.

Preparative thin-layer chromatography of the methyl esters of bile acids

The methyl esters of the bile acids were separated by thin-layer chromatography. The bile acids were placed on 20 × 20 cm glass plates coated with 0.25 mm of silica gel (TLC plates; silica gel 60, without fluorescent indicator, Merck, Darmstadt) and developed with benzene–acetone 70:30 (v/v). The plates were sprayed with water to visualize the bile acid spots. (Reference standards were also visualized with a 10% phosphomolybdic acid solution.) Areas of equal size were scribed around the spots and the silica gel in each area was scraped from the plate. The bile acids were extracted by immersing the silica gel in 2 ml of methanol and agitating. This process was repeated three times; the methanol was then evaporated. The bile acid methyl esters were dissolved in benzene, and the benzene was washed with water and evaporated to dryness.

Gas–liquid chromatography

The bile acid methyl esters were analyzed on a 200 cm × 3 mm glass column packed with 3% QF-1 on 80–100 mesh Gas Chrom Q (Applied Science, Philadelphia, PA) at 270°C; carrier gas (N_2) 60 ml/min; detector temperature 300°C. They were also analyzed on a Shimadzu 5A gas chromatograph (Shimadzu Co., Ltd., Kyoto) with flame ionization detectors. Peak areas were calculated with a Shimadzu apparatus, chromatopac C-RIA. Bile acid levels were determined by comparison with external standards of known concentrations.

Mass spectrometry

Bile acid methyl esters were analyzed on a JEOL quadrupole mass spectrometer, JEOL QH-100 (JEOL, Ltd., Tokyo), with an attached gas chromatograph, JEOL MS-GCG05, and microcomputer data system. The glass column mentioned above was attached to the gas chromatograph. The temperature of the injector was 300°C; the GLC oven, 270°C; the interface, 270°C; the ion source, 150°C. Helium was the carrier gas at a flow rate of 50 ml/min. The operating parameters were: electron energy, 70 eV; mass range, m/z 250–m/z 500; integration time, 0.63 or 1.25 sec from m/z 250 to m/z 500.

Spectra were obtained by electron impact ionization and chemical ionization mass spectrometry. Ten continuous scans of each GLC peak were made. Each included the top of the GLC peak, i.e., the point of the highest absolute ion intensity. For each scan, after subtracting corresponding background ion intensity, relative ion intensity at each m/z was calculated as a percentage of the most abundant ion, and a mean (relative) ion intensity for each m/z calculated.

Transit time of dietary residue through the gut

In the fecal study, transit times of dietary residue were measured on two occasions by giving each subject 20 radiopaque barium sulfate-impregnated silicone markers after each meal: bar shaped markers (average weight 19.8 mg, specific gravity 1.73) after breakfast; markers cut from double tubing (average weight 42.2 mg, sp. gr. 1.35) after lunch; and markers cut from single tubing (average weight 21.0 mg, sp. gr. 1.35) after dinner. The feces from the first two defecations after the administration of the markers were collected in polyethylene bags and the markers were distinguished and counted by radiography. Transit times were calculated using the method of Cummings et al. (19, 20). Bile acid compositions of the samples were then analyzed.

RESULTS

Anaerobic incubation

After microorganisms were added to the culture media, satisfactory growth was observed. The initial pH of the media was 7.2–7.3, which decreased to 5.8–6.2 after 8 hr incubation, then gradually increased to 6.8–7.0. Turbidity increased for 24–48 hr; after 48 hr an occasional slight decrease was observed.

From extraction to methylation, percentage recoveries of CDCA and UDCA when bacteria were added to the media (without incubation of the media) were 88.7 ± 4.5 (SD) % and 87.4 ± 6.4 (SD) %, respectively; percentage recovery of CDCA when bacteria were not added was 85.1 ± 5.7 (SD) %. As for recovery of bile acids by preparative thin-layer chromatography, percentage recoveries of 0.5, 1, 2, 4, 6, 8, 20, and 44 μg of methyl CDCA were 73.6, 77.2, 75.5, 77.5, 77.8, 82.8, 85.3, and 92.1%, respectively; for methyl UDCA, 0.5, 1, 2, 4, 6, 8, 20, and 40 μg, recoveries were 75.2, 78.2, 77.5, 84.5, 82.5, 85.0, 90.3, 97.1%, respectively.

No bile acids were detected in the media or in the original microorganisms. When substrate bile acids were dissolved in the media and the media were placed in

the incubation chamber without microorganisms, there was no conversion.

Time course curve

Time course studies (see Methods) were done on four of the incubation samples (samples B, C, D, and E), chosen randomly from the eight. Incubations using substrate UDCA and substrate CDCA were performed simultaneously in separate microbial suspensions from the same fecal samples. The reactions reached near equilibrium in 48–72 hr. Two typical time course curves are shown in **Fig. 1.** In the incubation of CDCA, CDCA decreased rapidly in amount, while UDCA increased in amount, as did (3β,7β) and (3β,7α). In the incubation of UDCA, UDCA decreased gradually in amount, while (3β,7β), CDCA, and (3β,7α) increased gradually. In both reactions, after 72 hr, UDCA was predominant.

Equilibrium state analysis

As in the time course study, substrate UDCA and substrate CDCA cultures were incubated simultaneously in separate microbial suspensions from the same fecal samples. After 72–96 hr, the bile acids in the culture media were extracted and analyzed. Epimerization of 3,7-dihydroxy bile acids is shown in **Table 1.** In all eight samples there was a marked presence of UDCA; (3β,7β), CDCA, and (3β,7α) were also noted in that order. Statistically significant differences were noted between UDCA and (3β,7β) ($P < 0.001$), (3β,7β) and CDCA ($P < 0.001$), CDCA and (3β,7α) ($P < 0.001$). Ratios of 3αOH/3βOH and 7αOH/7βOH in **Table 2** and **Table 3** were calculated from the data shown in Table 1 and show that, without exception, the α-hydroxy configuration is dominant at 3-position and the β-hydroxy configuration is

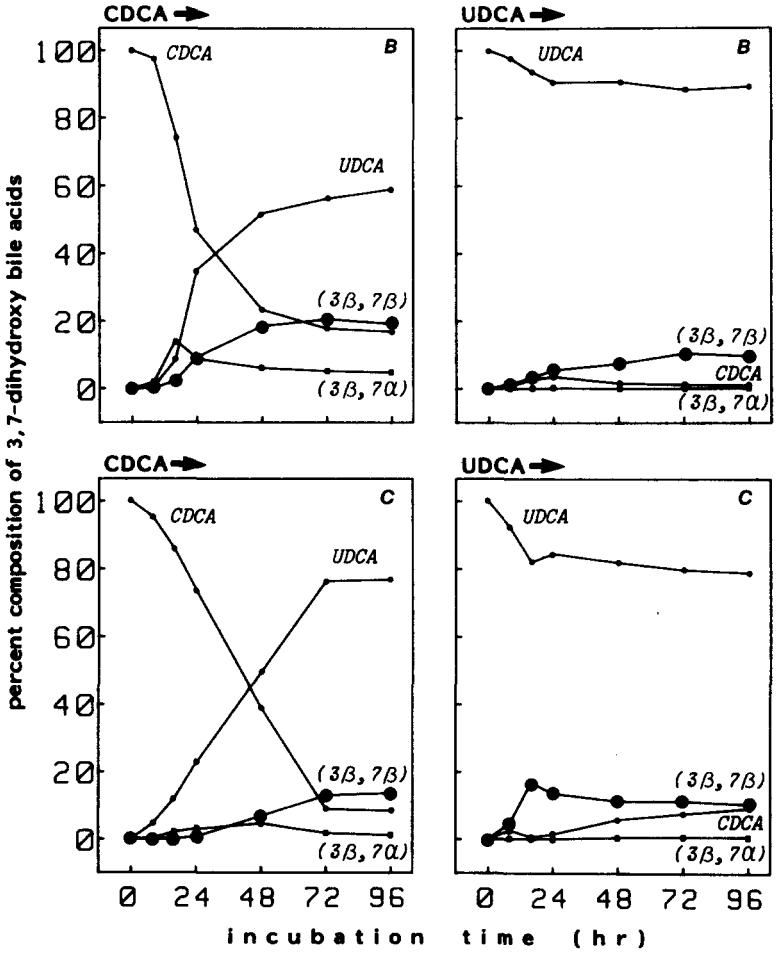

Fig. 1. Typical time course curves of mutual conversions of 3,7-dihydroxy bile acids by anaerobic mixed cultures. At time 0 fecal microorganisms from two healthy adult males (subjects B and C) were inoculated into non-selective GAM media, containing 600 μg of either CDCA or UDCA and were incubated for 8, 16, 24, 48, 72, and 96 hr. The substrates are indicated with arrows. For all four time course study samples (subjects B, C, D, and E), average recovery of 3,7-dihydroxy bile acids, calculated from 8, 16, 24, 48, and 72 hr incubations, was 411 ± 144 SD μg in substrate CDCA incubations and 447 ± 128 SD μg in substrate UDCA incubations. For 96-hr incubations, see Table 1.

TABLE 1. Conversion of CDCA and UDCA by human

Sample	Substrate CDCA: Products (3α,7α)	(3α,7β)	(3β,7α)	(3β,7β)	Total 3,7-diOH	Other Products: 7-KLCA	LCA	Sum
A	49.7[a] (12.7)[b]	265.6 (67.9)	2.7 (0.7)	73.3 (18.7)	391.3[c]	32.9	850.0	1274.2[d] [83.4][e]
B[f]	159.6 (16.8)	561.5 (59.1)	45.7 (4.8)	182.7 (19.2)	949.5	451.3	0	1400.8 [91.7]
C[f]	73.5 (8.4)	671.7 (76.8)	10.1 (1.2)	119.5 (13.7)	874.8	97.1	0	971.9 [63.6]
D[f]	36.6 (3.0)	1002.7 (82.1)	7.4 (0.6)	174.4 (14.3)	1221.1	48.9	0	1270.0 [83.1]
E[f]	16.5 (4.0)	293.9 (71.3)	6.1 (1.5)	95.9 (23.3)	412.4	230.1	296.5	939.0 [61.5]
F	55.3 (6.4)	639.4 (74.4)	12.8 (1.5)	151.7 (17.7)	859.2	261.0	62.5	1182.7 [77.4]
G	76.5 (13.4)	383.7 (67.0)	24.3 (4.3)	88.0 (15.4)	572.5	396.7	669.5	1638.7 [107.2]
H	59.0 (9.6)	454.3 (73.5)	7.9 (1.3)	96.7 (15.6)	617.9	186.9	510.8	1315.6 [86.1]

[a] Recovered real amount, nmol, corrected by an external standard.
[b] Amount of each 3,7-dihydroxy bile acid as a percentage of the total.
[c] Recovered total 3,7-dihydroxy bile acids, nmol, corrected by external standards.
[d] Total of 3,7-dihydroxy bile acids plus (7-KLCA + LCA), nmol, corrected by external standards. Small amounts of other bile acids were noted, but were not included in the sum.
[e] The sum as a percentage of the initial substrate amount (1.53 μmol).
[f] Samples on which time course studies were performed.

dominant at 7-position. 7-KLCA as well as other small amounts of keto bile acids, probable intermediates, was noted in all reactions. Although the small number of studies and wide spread of values do not permit a definitive conclusion, during the conversion process, the presence of LCA was noted more markedly in CDCA incubations than in UDCA incubations.

Transit time of dietary residue through the gut

For the fecal study, transit time of dietary residue through the gut was measured twice in nine healthy adult subjects using radiopaque markers (see Methods). The transit time was measured for comparison to the incubation time in the in vitro study. The transit times, calculated by Cummings' method, ranged from 18 to 48 hr and averaged 26.4 ± 8.9 (SD) hr (**Table 4**).

Fecal bile acid analysis

Amounts and compositions of the four 3,7-dihydroxy-5β-cholanoic acid epimers in the 18 samples from the nine fecal study subjects (two samples for each) are shown in Table 4. In samples from five subjects, the amounts of LCA were smaller than those of the 3,7-dihydroxy bile acids. In samples from the other four, the amounts were larger.

On the preparative TLC plates, the (3β,7α) ester tended to be separate, but the UDCA ester overlapped almost all of the (3β,7β) ester, and both esters overlapped the DCA ester, which in turn overlapped the CDCA ester. By splitting a co-chromatographed DCA ester spot at the center, the CDCA ester could be separated from the overlapping UDCA and (3β,7β) esters. Except in cases where unusually large amounts of DCA obscured their presence and necessitated the repetition of TLC, the overlapping epimers could then be separated by GLC. Other 3,12-dihydroxy epimers did not affect the identification and measurement.

In samples where the amounts of DCA were large, amounts of LCA were also large. In all samples amounts of LCA were greater than those of the 3β-epimers of LCA. Keto (oxo) bile acids were observed in all samples, mainly 7-ketolithocholic acid, 3α-hydroxy-12-oxo-5β-

TABLE 2. Ratios of 3α OH/3β OH and 7α OH/7β OH in bile acids at equilibrium: CDCA 96 hr incubation

Sample	(3α,7α)/(3β,7α)	(3α,7β)/(3β,7β)	(3α,7α)/(3α,7β)	(3β,7α)/(3β,7β)
A	18.4[a]	3.6	0.19	0.04
B	3.5	3.1	0.28	0.25
C	7.3	5.6	0.11	0.08
D	5.0	5.8	0.04	0.04
E	2.7	3.1	0.06	0.06
F	4.3	4.2	0.09	0.08
G	3.1	4.4	0.20	0.28
H	7.5	4.7	0.13	0.08

[a] Calculated from the data in Table 1.

fecal microorganisms in 96-hr anaerobic mixed cultures

Substrate UDCA							
Products				Total 3,7-diOH	Other Products		Sum
(3α,7α)	(3α,7β)	(3β,7α)	(3β,7β)		7-KLCA	LCA	
28.0 (3.6)	605.5 (77.9)	6.6 (0.9)	137.2 (17.7)	777.3	195.7	322.3	1295.3 [84.8]
16.7 (1.0)	1433.8 (89.1)	2.5 (0.2)	156.4 (9.7)	1609.4	97.9	0	1707.3 [111.7]
77.2 (9.4)	648.1 (79.1)	6.6 (0.8)	87.8 (10.7)	819.7	31.1	0	850.8 [55.7]
18.0 (2.6)	585.6 (84.4)	3.9 (0.6)	86.3 (12.4)	693.8	142.9	0	836.7 [54.8]
49.2 (12.5)	269.1 (68.2)	21.2 (5.4)	55.3 (14.0)	394.8	120.4	381.2	896.4 [58.7]
39.4 (5.3)	594.4 (80.6)	10.3 (1.4)	93.0 (12.6)	737.1	55.6	0	792.7 [51.9]
54.1 (8.8)	474.9 (77.4)	13.3 (2.2)	71.1 (11.6)	613.4	130.0	49.4	792.8 [51.9]
61.2 (11.5)	396.0 (74.4)	12.1 (2.3)	63.2 (11.9)	532.5	111.2	175.6	819.3 [53.6]

cholanoic acid, and 3-oxo-12α-hydroxy-5β-cholanoic acid. 5α-Cholanoic acids were not searched for extensively.

In samples where amounts of CDCA were small, a portion of the homogenized feces was doubly diluted; 0.5 g of the suspension was put into each of four tubes, and 0.5, 1.0, 2.0, and 5.0 mg of CDCA was added. After extraction and methylation, percentage recoveries of CDCA were 95.2 ± 1.7 (SD) %. In samples where amounts of LCA were small, a similar procedure was followed, and recoveries of LCA ranged from 87 to 95%.

Fecal amounts in grams and bile acid amounts in μmoles are also shown in Table 4 (amounts given for samples I-2, K-1, and L-2, are for approximately 2 days; all others are for approximately 1 day).

Identification of bile acids

The methyl esters of biological CDCA, UDCA, (3β,7α), (3β,7β), 7-KLCA, and others were separated by preparative TLC (see Materials and Methods), and the esters were co-chromatographed with authentic esters. Identical relative R_f and R_T values for the 3,7-dihydroxy epimers were observed, as shown in **Table 5.**

Positive identification was also established by GLC-quadrupole mass spectrometry (**Fig. 2**). In both the incubation and fecal studies the bar graphs of electron impact mass spectra of methyl esters of biological CDCA, UDCA, (3β,7α), and (3β,7β) were identical, line for line, with the fragmentation pattern of their authentic counterparts. The base ion was m/z 273 [M − (115 + 18)] for the (3β,7α) ester and m/z 388 (M − 18) for the (3β,7β) ester. Except in the CDCA ester, the molecular ion (M^+) was always detected at m/z 406. The ion at m/z 273 was more typical of the CDCA than of the UDCA ester.

In ammonia chemical ionization mass spectra, at a source temperature of 150°C, the base ion was usually m/z 424 (M + 18). The ions at m/z 406 (M^+) and 371 [M − (2 × 18) + 1] were always observed.

The methyl esters of biological 3β-hydroxy-7-oxo-5β-cholanoic acid, 3-oxo-7α-hydroxy-5β-cholanoic acid, 3-

TABLE 3. Ratios of 3α OH/3β OH and 7α OH/7β OH in bile acids at equilibrium: UDCA 96 hr incubation

Sample	(3α,7α)/(3β,7α)	(3α,7β)/(3β,7β)	(3α,7α)/(3α,7β)	(3β,7α)/(3β,7β)
A	4.2[a]	4.4	0.05	0.05
B	6.8	9.2	0.01	0.02
C	11.6	7.4	0.12	0.08
D	4.6	6.8	0.03	0.05
E	2.3	4.9	0.18	0.38
F	3.8	6.4	0.07	0.11
G	4.1	6.7	0.11	0.19
H	5.1	6.3	0.15	0.19

[a] Calculated from the data in Table 1.

TABLE 4. Amounts and compositions of fecal bile acids from healthy adult Japanese males

Sample	Transit Time	Fecal Amount (g)	3-monoOH Bile Acids		3,7-diOH Bile Acids				3,12-diOH Bile Acids[a]	3,7,12-triOH Bile Acids[b]	Keto Bile Acids[c]
			LCA	3β-LCA	(3α,7α)	(3α,7β)	(3β,7α)	(3β,7β)			
I-1	19.3	300.5	2.10	—[d]	433 (62.9)	177 (25.7)	44.0 (6.4)	33.9 (4.9)	19.7	890	26.7
-2	48.0	210.0	8.29	—	141 (40.2)	179 (51.0)	11.5 (3.3)	19.6 (5.6)	48.2	1080	38.0
A-1	18.4	307.3	31.0	—	367 (73.8)	100 (20.1)	20.0 (4.0)	10.4 (2.1)	278	968	14.0
-2	19.3	231.2	122	8.96	391 (83.7)	62.5 (13.4)	9.35 (2.0)	3.96 (0.9)	467	471	18.8
B-1	19.8	240.0	8.27	—	999 (81.3)	185 (15.1)	35.9 (2.9)	8.19 (0.7)	1.13	1730	11.5
-2	24.1	120.1	22.9	—	225 (47.4)	189 (39.8)	26.8 (5.6)	33.9 (7.1)	t[e]	1140	27.4
J-1	20.4	120.6	12.9	5.27	276 (70.4)	95.9 (24.4)	11.9 (3.0)	8.44 (2.2)	0.52	433	6.65
-2	25.4	59.7	27.6	6.37	145 (67.4)	55.8 (26.0)	9.15 (4.3)	5.04 (2.3)	17.9	265	4.37
C-1	20.9	140.0	69.6	4.92	158 (72.5)	54.1 (24.8)	4.13 (1.9)	1.64 (0.8)	186	210	7.69
-2	24.6	165.0	120	9.73	74.0 (56.8)	48.5 (37.2)	3.03 (2.3)	4.82 (3.7)	236	130	37.6
K-1	46.5	314.2	476	59.9	290 (87.9)	27.5 (8.3)	12.3 (3.7)	—	1740	360	60.8
-2	30.7	230.5	320	41.7	7.94 (33.6)	15.0 (63.5)	0.69 (2.9)	—	802	—	24.4
L-1	21.4	139.5	133	24.3	11.4 (100)	t	t	—	181	—	19.2
-2	41.5	149.8	207	40.5	17.2 (88.5)	1.72 (8.8)	0.52 (2.7)	—	339	—	41.6
M-1	22.5	304.3	133	23.3	29.8 (31.5)	52.6 (55.6)	1.48 (1.6)	10.7 (11.3)	524	188	10.9
-2	22.3	268.4	208	27.1	5.36 (32.9)	9.62 (59.1)	—	1.30 (7.9)	290	5.16	12.7
N-1	24.6	89.0	185	20.8	12.9 (71.4)	4.94 (27.4)	—	0.22 (1.2)	315	t	30.0
-2	26.3	100.6	225	27.4	23.9 (59.5)	16.3 (40.5)	t	—	406	17.2	12.7

Transit time was measured and bile acids were analyzed on two occasions for each subject. Bile acid amounts are given in μmol. 3,7-diOH (dihydroxy) bile acid amounts are also expressed (in parentheses) as a percentage of the total 3,7-diOH bile acids. Small amounts of unlisted bile acids were observed. Fecal amounts are for approximately 1 day, except for I-2, K-1, and L-2, which are for approximately 2 days. Transit times were calculated using Cummings' method.

[a] Primarily deoxycholic acid.

[b] Primarily cholic acid.

[c] Primarily 7-ketolithocholic acid, 7-ketodeoxycholic acid, 3-keto-12α-hydroxy-5β-cholanoic acid, and 3α-hydroxy-12-keto-5β-cholanoic acid.

[d] Not detected.

[e] Trace amounts (bile acid amounts were corrected by external standards).

TABLE 5. RR_f and RR_T values in TLC and GLC

Bile Acids	RR_f[a]	RR_T[b]
Methyl chenodeoxycholate (3α,7α-diOH)		
Authentic	1.00	1.00
In vitro[c]	1.01	1.00
In vivo[d]	1.00	1.00
Methyl ursodeoxycholate (3α,7β-diOH)		
Authentic	1.20	1.06
In vitro	1.20	1.06
In vivo	1.19	1.05
Methyl ester of 3β,7β-dihydroxy-5β-cholanoic acid		
Authentic	1.16	0.96
In vitro	1.15	0.97
In vivo	1.14	0.97
Methyl ester of 3β,7α-dihydroxy-5β-cholanoic acid		
Authentic	1.37	0.85
In vitro	1.39	0.86
In vivo	1.37	0.86

[a] R_f values are relative to that of methyl chenodeoxycholate. RR_f value of methyl deoxycholate: 1.06.

[b] Retention times (R_T) are relative to that of methyl chenodeoxycholate on a 3% QF-1 column. RR_T value of methyl deoxycholate: 0.85.

[c] Bile acid obtained from anaerobic cultures.

[d] Bile acid obtained from fecal samples. For available data on these bile acids, see references 21 and 22.

oxo-7β-hydroxy-5β-cholanoic acid, four 3,12-dihydroxy-5β-cholanoic acid epimers, 3-oxo-12α-hydroxy-5β-cholanoic acid, and 3α-hydroxy-12-oxo-5β-cholanoic acid were shown to exist by a combination of TLC, GLC, and quadrupole mass spectrometry. The methyl esters of LCA and its 3β-epimer were likewise demonstrated.

DISCUSSION

Before the authors demonstrated in previous experiments (7, 8) the interconversion of chenodeoxycholic acid and ursodeoxycholic acid and the reduction of 7-ketolithocholic acid to these bile acids in anaerobic mixed cultures of total fecal microorganisms, the interconversion of the bile acids had not been presented clearly and the conversion of CDCA to UDCA, at least in part, had been supposed to be a hepatic reaction (3, 5, 6, 23, 24); in several investigations 7-ketolithocholic acid had been shown to be converted by the intestinal microorganisms only to chenodeoxycholic acid (25, 26). Now there is no doubt about the mutual epimerization of 3,7-dihydroxy bile acids by microbial conversions. The precise mechanism of the microbial reaction, however, cannot be deduced with certainty from our experiments or the experiments of others, although many reports have described in vitro epimerization mechanisms in intestinal bacteria (7–14).

As mentioned above, 7-KLCA is reduced to CDCA and UDCA by the intestinal microorganisms; in this experiment, the presence of 7-KLCA, was observed in every conversion, but it did not consistently appear as an intermediate peak in the time course studies. Fedorowski et al. (9) speculate that both 7-KLCA and the unsaturated intermediate, Δ^6- or Δ^7-lithocholenic acid, act as intermediates in the conversion reaction.

The medium used in the incubation study was nonselective. Each species has its own nutritional requirements and its own growth curve. Thus, in anaerobic mixed cultures like ours, different microorganisms are not in the same growth phase: some may be in a lag phase, some may be in a stationary phase, and some may be in a phase of decline. Nevertheless, the mixed culture is a useful tool for the analysis of human intestinal conversions of 3,7-dihydroxy bile acids.

In the time course study, all four samples reached a near equilibrium state in 48–72 hr, and all reactions involved four 3,7-dihydroxy epimers. The final α/β OH ratios in the CDCA reactions and the UDCA reactions were roughly similar. Fedorowski et al. (9) have shown that 7-dehydroxylation is more typical of CDCA than UDCA. Their view is supported by this experiment.

The predominancy of the β-hydroxy configuration at 7-position observed in the incubation study is in contrast to the results obtained by Fromm et al. (5, 6). They have maintained that the liver converts 7-KLCA mainly to CDCA. It is significant to note that the predominance of the β-hydroxy configuration at 7-position and of the α-hydroxy configuration at 3-position was without exception in our incubation study. Although these conversions of intestinal flora appear chaotic, they are not chaotic, but orderly.

A UDCA (3α,7β-dihydroxy epimer) predominance has never been reported in the bile of normal human subjects (23, 24). Kurozumi et al. (27) found that a UDCA predominance sometimes occurs in the bile of bears. The main purpose of the fecal study was to find whether it occurs in human feces. Of the eighteen samples examined, CDCA was the dominant epimer in fourteen, and UDCA in four. In the incubation samples, reactions took 48–72 hr to reach equilibrium; the transit times of the radiopaque markers through the gut ranged from 18.4 to 48.0 hr and averaged only 26.4 hr. Although the data obtained by these two studies do not permit a definite conclusion, the epimerization of CDCA and UDCA to yield a mixture of all four 3,7-dihydroxy epimers appears to be a frequent occurrence. Had the transit times been longer, UDCA might have been predominant in more cases.

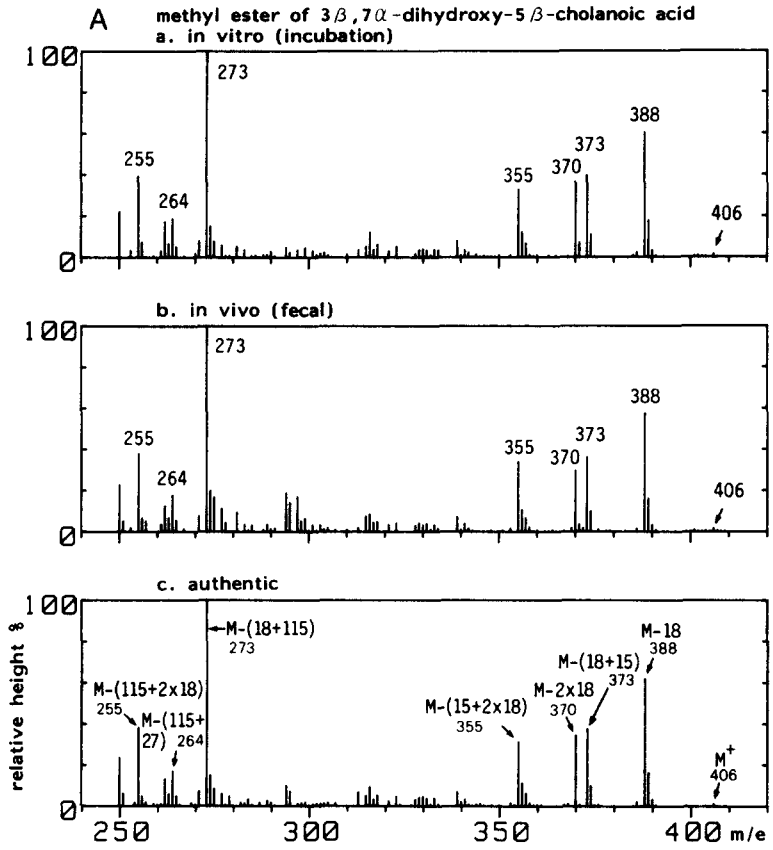

Fig. 2. Bar graphs of electron impact mass spectra of methyl esters of authentic, in vivo (fecal), and in vitro 3β,7α-dihydroxy-5β-cholanoic acid (A) and 3β,7β-dihydroxy-5β-cholanoic acid (B). In vitro esters were obtained from mixed anaerobic cultures of intestinal microorganisms.

The average transit time in our fecal study was less than half as long as that of Caucasian subjects in other studies (28, 29). In Cummings' studies (19, 20), markers were administered once a day for 3 consecutive days. Because many of our subjects passed the markers within 24 hr, we could not employ the same method. We administered markers three times in 1 day, once after each meal.

A second purpose of the fecal study was to confirm the existence of the four 3,7-dihydroxy epimers in feces. In the fecal samples containing small amounts of LCA, four epimers were conclusively identified. In the others, CDCA, the predominant epimer in bile, had apparently been converted to LCA by 7-dehydroxylation, and in some cases only three epimers were identified.

In the incubation study amounts of LCA in three out of the eight samples were too small to measure, but faint LCA peaks were noted in all GLC profiles. At first we attributed the small amounts of LCA to some problem in the culture media. In the fecal study, however, amounts of LCA in the samples from five of the nine subjects were also very small, corroborating the findings of the incubation study.

In experiments using anaerobic mixed cultures, Fromm et al. (5, 6, 11), Fedorowski et al. (9), and Higashi, Setoguchi, and Katsuki (7, 8) separated and identified only two 3,7-dihydroxy-5β-cholanoic acid epimers. In in vivo studies also, only two epimers have been separated and identified (11). Hirano, Masuda, and Oda (10), using a combination of two gas chromatographic columns and different bile acid derivatives, were able to suggest that, in some samples, four 3,7-dihydroxy epimers did exist, but their measurement and identification were not conclusive.

We propose that in anaerobic mixed cultures of intestinal microorganisms and in the in vivo microbial process the epimerization of the four 3,7-dihydroxy bile acids always occurs. In cultures with a single or a limited number of strains, conversions involving only two or three epimers may be possible. In subjects in vivo, if much LCA is produced, the conversion of CDCA to other epimers may be small. However, any experiment dealing with the epimerization of 3,7-dihydroxy bile acids should take into account that the process includes four epimers. Moreover, results from previous experiments should be reinterpreted in this light.

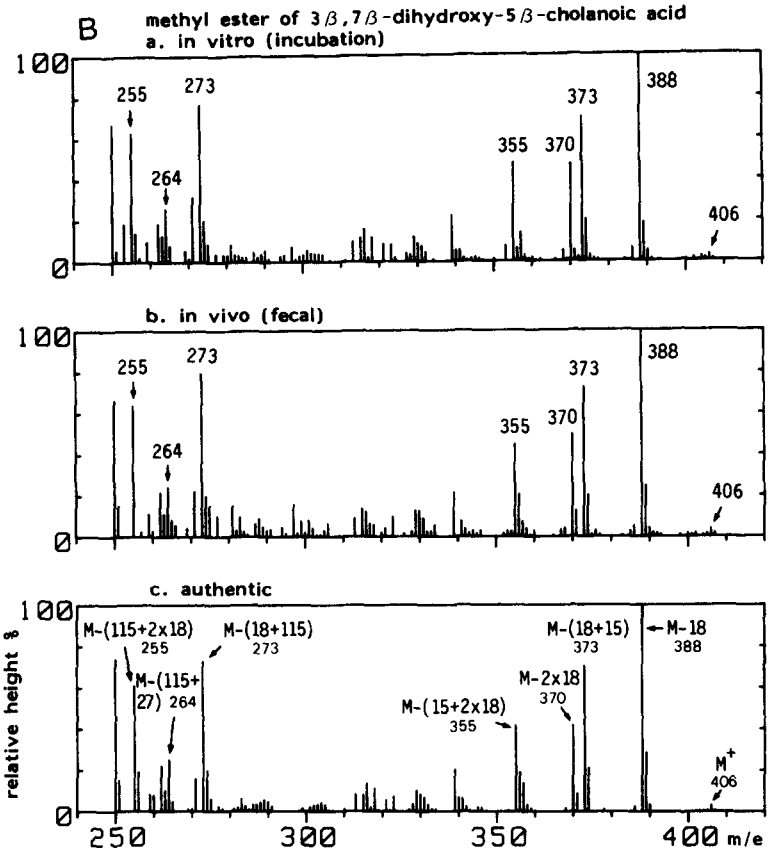

Fig. 2. *Continued*

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