In vitro transformation of chenodeoxycholic acid and ursodeoxycholic acid by human intestinal flora, with particular reference to the mutual conversion between the two bile acids

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Abstract Nine fecal samples from four healthy subjects were examined for their ability to transform chenodeoxycholic acid (CDCA) and ursodeoxycholic acid (UDCA) in in vitro anaerobic broth cultures. Seven samples converted CDCA and UDCA into each other (more than 50% of CDCA was converted into UDCA while 10% or less of UDCA was converted into CDCA), and produced 7-keto-lithocholic acid and lithocholic acid equally from both acids. No alteration of the 7β-hydroxy group of UDCA was demonstrated by two fecal samples that failed to perform mutual 7-epimerization, suggesting the conversion of UDCA into lithocholic acid via CDCA. The 3α-hydroxy groups of these substrate and metabolite bile acids were invariably partially epimerized to 3β-hydroxy groups by all the fecal samples. Evidence is presented for the prevalence of these 7- and 3-epimerizing organisms among the human intestinal flora. —**Hirano, S., N. Masuda, and H. Oda.** In vitro transformation of chenodeoxycholic acid and ursodeoxycholic acid by human intestinal flora, with particular reference to the mutual conversion between the two bile acids. *J. Lipid Res.* 1981. **22:** 735–743.

Supplementary key words mutual 7-epimerization · 3α-epimerization · 7α-dehydroxylation

The microbial transformation of bile acids that takes place in the lower alimentary tract plays an important role in the in vivo metabolism of bile salts and also of cholesterol in general. Most of the transforming reactions involved can be reproduced in in vitro cultures of mixed intestinal microflora: hydrolysis of the peptide bond in conjugated bile acids, removal of the 7α-OH group, and dehydrogenation of the α-OH substituents at C-7, C-3, and C-12. The last reaction, which leads to the formation of an oxo group, is reversible and a stereospecific reduction of the oxo moiety into a β-OH group has been shown to be carried out at least at the C-3 position of various bile acids (1).

Comprehensive studies of this sort on the intestinal contents of the rat have been reported by Midtvedt and co-workers (2–4). Very few studies, however, are available for human samples (5–7), which should exhibit their own distinct features, as the components of biliary bile acids and the composition of the intestinal flora might differ markedly between man and animals.

This report is concerned with the comparative transformation of chenodeoxycholic acid (CDCA) and ursodeoxycholic acid (UDCA) by mixed microbial cultures of human feces. Of late, these two isomeric bile acids have been widely used for the treatment of gallstones (8, 9), and, because of the large amounts of the agents administered orally to the patients, the in vivo metabolism of these bile acids has become a matter of concern (10–12). In this study, emphasis is put on two epimerizing reactions, the conversion of the 7α-OH group of CDCA into a β-configuration thus yielding UDCA, and the formation of 3β-OH epimers from the series of 3α-hydroxylated bile acids. The former reaction was found to be reversible, and CDCA and UDCA were mutally converted by most of the fecal samples.

MATERIALS AND METHODS

Fecal specimens

Samples of feces were collected successively from four healthy male adults (age range 30 to 60 years)

Abbreviations: GLC, gas-liquid chromatography; MS, mass spectrometry; RRT, relative retention time; m-TMS, methyl ester trimethylsilyl ether; CDCA, chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanoic acid); UDCA, ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholanoic acid); LCA, lithocholic acid (3α-hydroxy-5β-cholanoic acid); 7-keto-LCA, 7-keto-lithocholic acid (3α-hydroxy-7-keto-5β-cholanoic acid).

on an ordinary Japanese diet. All subjects showed no evidence of hepatic disorder or gastrointestinal disease, and had received no antibiotic during the last several months prior to the study. A portion of freshly voided feces was suspended in 10 times its volume of 0.02 M phosphate buffer (pH 7.5) supplemented with 0.05% L-cysteine-HCl. After removal of coarse material by centrifugation at low speed, the suspension was used as an inoculum within 2 hr of collection.

Culture experiment

A standard volume (one loopful or 0.1 ml) of the fecal suspension was inoculated into bile acid medium in tubes or in flasks (see below), and after incubation for a specified period of time under anaerobic or aerobic conditions, the spent culture medium was analyzed for bile acids.

Bile acid medium

The basal medium consisted of polypeptone (Daigo-Eiyo Chemical Co.), 2 g; yeast extract (Difco Laboratories), 1 g; the salt solution recommended by Holdeman and Moore (13), 4 ml; and L-cysteine-HCl, 0.05 g per 100 ml of 0.02 M phosphate buffer at pH 7.5. The buffer solution was used as a diluent to maintain the pH above 7.0 during the entire period of incubation. After addition of 0.2 mM bile acid, the broth was dispensed in 4-ml quantities into 12 × 105-mm test tubes or in 40-ml quantities into 100-ml Erlenmeyer flasks, and sterilized at 120°C for 15 min. No destruction of bile acids was caused by autoclaving.

Incubation

For anaerobic growth, the seeded tubes were incubated in an anaerobic jar which was then evacuated and filled with N_2. Flasks were inoculated under flushing with N_2 through the liquid phase and tightly stoppered. During incubation, samples were removed at intervals while the anaerobic atmosphere inside the flask was maintained by flushing with N_2. The N_2 gas was used after removal of residual oxygen by passing over heated copper gauze. Moderately aerobic cultivation was carried out in flasks placed in an air incubator with intermittent shaking for a short time twice a day. Highly aerobic incubation was in L-shaped tubes continuously shaken on a shaker incubator (100 oscillations/min, over 40-mm stroke).

Extraction and derivatization of bile acids

Portions (4 ml) of acidified culture samples were repeatedly extracted with ethyl acetate, and the pooled solvent phases were washed and evaporated. After methylation by an acidic methanol technique, the methylated bile acids were extracted with the same solvent but at an alkaline reaction this time. After washing and evaporation of the extract, the final residue was reconstituted in 0.5 ml of chloroform (methyl cholanoate), and 3-μl aliquots were applied to GLC (3% QF-1). Subsequently, the methylated samples were converted to fully trimethylsilylated ethers by the method of Makita and Wells (14), and the reaction mixture (methyl cholanyl trimethylsilyl ether) was injected directly into a 3% Hi Eff-8B column.

Analysis of bile acids by GLC

A Hitachi 163 gas–liquid chromatograph equipped with a flame ionization detector was used isothermally. The column was a U-shaped glass tube, 2 m × 3 mm i.d., packed with 3% QF-1 coated on Chromosorb WAW DMCS, 80–100 mesh, or with 3% Hi Eff-8B (cyclohexanedimethanol succinate) coated on 80–100 mesh Gas Chrom Q. The column was held at 260°C (QF-1) or 230°C (Hi Eff-8B), and the temperature of injector and detector was kept 50°C higher than that of the column. N_2 was used as carrier gas at a flow rate of 30–40 cm^3/min.

The individual bile acids were identified by comparative evaluation of both RRT values as methyl and methyl-TMS derivatives (cf. Fig. 2). Quantities were determined from the peak areas related to those of known amounts of methyl or methyl-TMS deoxycholate (depending upon the derivatization of the sample bile acid), and expressed as the percentage of the bile acid mixture in each sample. The total recovery of bile acids ranged from 80 to 90%.

MS analysis

The methyl-TMS derivatives of bile acids were analyzed by MS with a Hitachi M-60 gas chromatograph–mass spectrometer. The effluents from the Hi Eff-8B column were conducted into the mass spectrometer through a Ryhage-type glass helium separator. The column was operated at 240°C. The helium separator was held at 320°C, the injector at 280°C, and the ion source at 200°C. The helium inlet pressure was held at 1.2 kg/cm^2. The ionization voltage was 20 eV, and the accelerating voltage was maintained at 3.2 kV (m/e max. 1500). Spectra were recorded from m/e 0-700 in 4 sec. Background spectra, mainly derived from the stationary phase, were recorded and subtracted from the sample spectra. The mass spectra of sample bile acids were compared with those of the same derivatives of the corresponding authentic references.

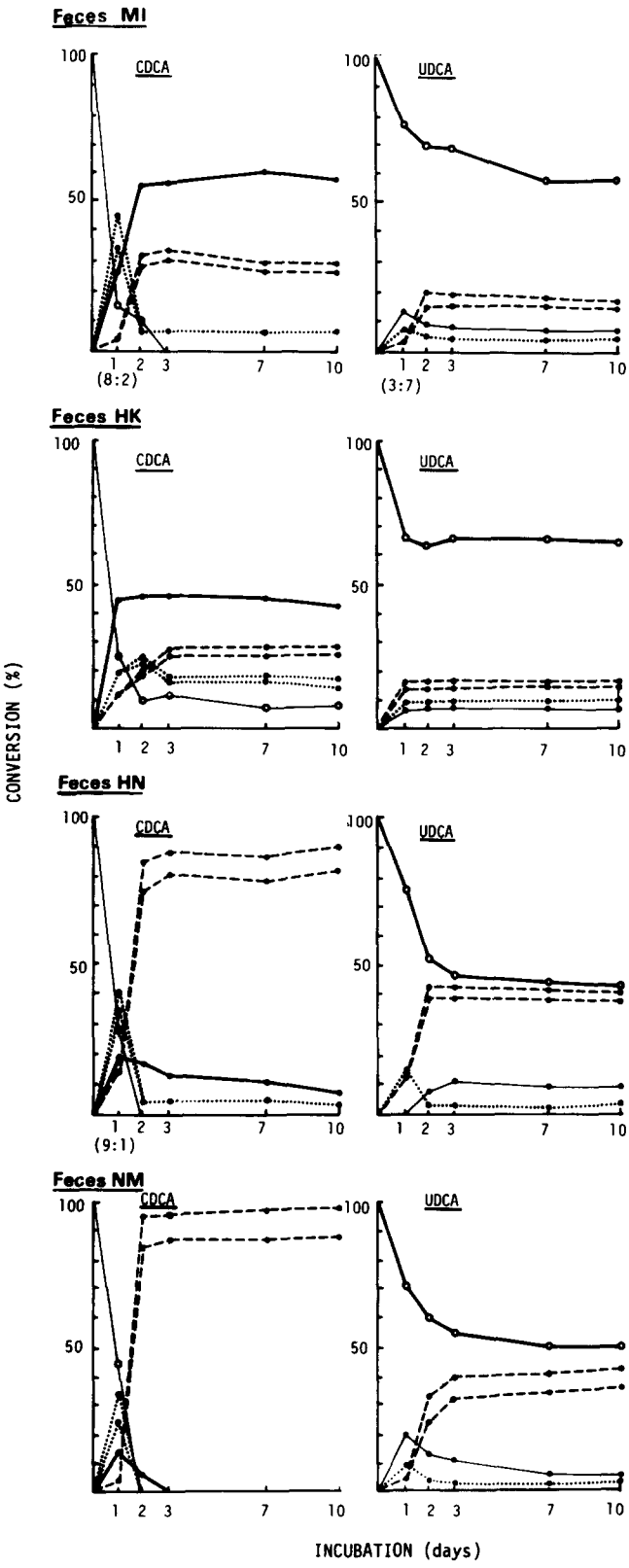

Fig. 1. Changes in percentage composition of bile acid metabolites produced from CDCA and UDCA by cultures of feces MI, HK, HN, and NM during 10 days of anaerobic incubation. Flasks containing 40 ml of bile acid medium were inoculated with a portion of fecal suspension and samples were removed for assay at specified times during incubation under N_2. Bile acids were quantified by separation of methyl cholanoates on QF-1 as for percentage composition. (——), CDCA; (——), UDCA; (– – –), LCA; (· · · · ·), 7-keto-LCA. Where double lines are shown, the lower represents the percentage of 3α-OH acid, and the upper represents the sum of percentages for 3α- and 3B-OH isomers. Figures in parentheses under the day of incubation indicate the ratio of CDCA to 3β-UDCA, both of which are shown as CDCA in the figures.

Bile acids

The following bile acids were used as substrates: CDCA was kindly supplied by Tokyo Tanabe Pharmaceutical Co., Ltd., Tokyo; UDCA was a gift of Dr. T. Setoguchi, Miyazaki Medical College; 7-keto lithocholic acid was purchased from Gaschro-Kogyo Co., Ltd., Tokyo. No impurities were detected in these preparations by GLC.

RESULTS

Transformation of CDCA and UDCA by cultures of human fecal bacteria

The type and yields of the metabolites from CDCA and UDCA were compared in cultures of four fecal samples (**Fig. 1**). With all the samples, UDCA was metabolized at a slower rate and to a lesser extent than CDCA. No unchanged CDCA was left in the culture by the 2nd or 3rd day of incubation, whereas UDCA was metabolized by less than 50% within the same period and little increase in consumption was observed on prolonged incubation. LCA (the 7-dehydroxylated product) and 7-keto-LCA (7-dehydrogenated) were formed from both CDCA and UDCA. In contrast to 7-keto-LCA, which appeared in most cases as a transient intermediate in the early phase of incubation, LCA progressively increased in concentration to reach a plateau after 2 or 3 days of incubation. The final yield of LCA varied significantly with different fecal samples; feces HN and NM caused an almost quantitative conversion of both CDCA and UDCA into LCA while the conversion by feces MI and HK accounted for only 20–30% of the substrates consumed.

An additional remarkable reaction was common to all fecal samples. Large quantities of CDCA, occasionally over half of it, were converted into a compound eluting at the same rate as UDCA, and conversely, the substrate UDCA was also transformed into a compound with the same elution rate as CDCA, although there was less than 10% conversion (mutual 7-epimerization). In highly 7-dehydroxylating cultures (HN and NM), once the epimerization conversion products were produced they were then reduced

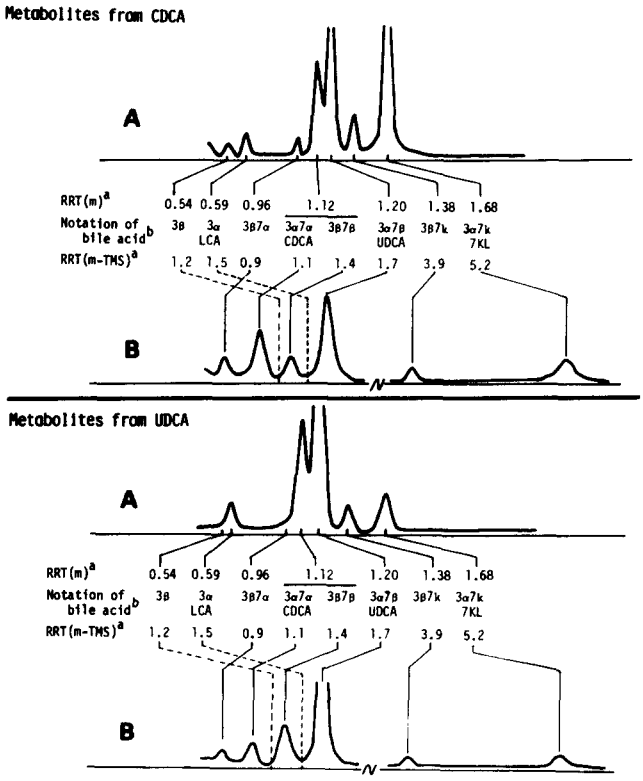

Fig. 2. Gas–liquid chromatograms comparing the separation of methyl cholanoates on QF-1 (A) and the separation of the methyl cholanyl trimethylsilyl ethers on Hi Eff-8B (B). Samples: 1-day cultures of feces MI in the presence of CDCA (top) or UDCA (bottom) (cf. Fig. 1).. [a]RRT(m), retention time relative to that of methyl deoxycholate; RRT(m-TMS), retention time relative to that of the trimethylsilyl ether of methyl deoxycholate. [b]Expressed by substituents in 5β-cholanoate: α,α-OH; β,β-OH; and k, keto group.

in quantity, with a corresponding increase in LCA. In these samples it appears that the dehydroxylation at C-7 takes preference over 7-epimerization.

Besides these reactions, all concerned with the 7-OH group, most of the substrate and metabolite bile acids were attended by a minor peak eluting immediately before it (cf. Fig. 2). The only exception was UDCA, the front peak of which was not detected by chromatography of the methyl cholanoates on QF-1, since it emerges in the tail of the peak of CDCA, because of the closeness of elution times as will be described below. These front peaks were always produced in small quantity and their quantities were roughly proportional to the amounts of the corresponding major acids. In reference to the published RRT values (15, 16), it seems very likely that these minor products represent the 3β-hydroxy epimers derived from the respective 3α-hydroxylated bile acids. It is known that the conversion of an equatorial 3α-OH group into an axial 3β-configuration results in the shortening of the elution time on QF-1 (17).

Identification of metabolites by GLC and MS

The GLC of methylated samples on QF-1 is in current use for the detection of a wide range of bile acids in biological materials (18, 19), but a disadvantage is the similarity of the elution rates of the methyl esters of CDCA and 3β-epimerized UDCA (RRT 1.12 and 1.10, respectively). Consequently, in the human fecal cultures where interconversion between CDCA and UDCA takes place together with the 3α-epimerization of the respective bile acids, the peak eluting at RRT 1.12 could represent CDCA or 3β-UDCA or both. The distinction was achieved by GLC of m-TMS derivatives on Hi Eff-8B column (**Fig. 2**). The methyl cholanoates eluting at RRT 1.12 on QF-1 were separated by this procedure into two peaks with RRT of 1.1 (CDCA) and 1.4 (3β-UDCA). As can be seen from the figure, a large portion of CDCA was converted into UDCA by fecal cultures, and parts of the CDCA remaining unconsumed and the UDCA produced were epimerized at their 3α-OH groups, thus giving rise to the appearance of four stereoisomeric 3,7-diols: 3α,7α-, 3β,7α-, 3α,7β-, and 3β,7β-dihydroxy-5β-cholanoates. The substrate UDCA was also converted into CDCA, although the conversion was less extensive than the reverse reaction, followed by the 3α-epimerization of both acids, thus yielding the same four stereoisomers.

The identity of these four isomers was confirmed by MS of the m-TMS derivatives (**Fig. 3**). The mass spectrum of the RRT 1.7 compound (presumed UDCA) from a culture originally containing CDCA showed the fragmentation pattern characteristic of the m-TMS of authentic UDCA, and the fragmentation pattern of the RRT 1.1 compound (presumed CDCA) formed from UDCA by fecal cultures was indistinguishable from that of the methyl-TMS of authentic CDCA. When compared as methyl-TMS derivatives, CDCA exhibited the base peak at m/e 370 (M − 2 × 90(trimethylsilanol)) and no line at m/e 460 (m − 90) and m/e 535 (M − 15 (methyl group)); while UDCA gave the base peak at m/e 460, a large fragment at m/e 370, and a weak ion at m/e 535. These fragmentation reactions are in good agreement with the published data by Sjövall, Eneroth, and Ryhage (20).

The RRT 1.4 and 0.9 compounds, the methyl-TMS derivatives of presumed 3β-epimers from UDCA and CDCA, respectively, exhibited fragmentation patterns similar to those of the respective 3α-substituted analogues. Although some differences were noted in the relative intensities of the major fragments, it remains to be established whether the differences were sufficient to determine the isomeric configurations at C-3. This stands out in sharp contrast to the marked differences in the mass spectral pat-

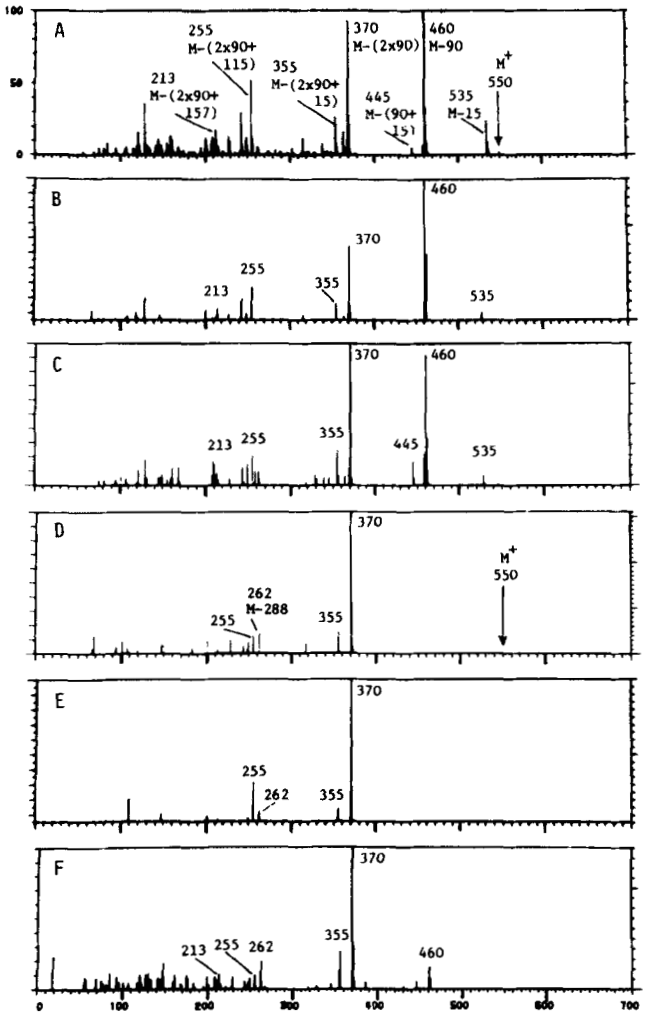

Fig. 3. Mass spectra of 7α- and 3α-epimerization products. The methyl-TMS derivatives separated on Hi Eff-8B were analyzed by MS (cf. Fig. 2). A, RRT 1.7 compound (presumed UDCA) formed in cultures grown in the presence of CDCA; B, authentic UDCA; C, RRT 1.4 compound (presumed 3β-epimer of UDCA) in cultures containing UDCA; D, RRT 1.1 compound (presumed CDCA) formed in cultures grown in the presence of UDCA; E, authentic CDCA; F. RRT 0.9 compound (presumed 3β-epimer of CDCA) in cultures containing CDCA. Lost fragments: 15, CH_3; 90, $(CH_3)_3$-SiOH; 115, C20–C24; 157, C15–C17.C20–C24; 288, A-C6.

terns of the 7-hydroxy stereoisomers mentioned above. For establishment of the stereospecific configuration of 3-hydroxy substituents, the GLC behavior of methyl silanates on the Hi Eff-8B column seems to be more reliable, although Sjövall (19) discouraged the use of this phase for the separation of silyl ethers of bile acids.

In view of these data, the methylated bile acid eluting at 1.12 in Fig. 1 (provisionally designated as CDCA) was re-examined, and the breakdowns for selected samples are included in Fig. 1. The 1.12 peaks tested invariably consisted of both components, CDCA and 3β-UDCA.

Investigation of successive fecal samples from the same subjects

Following the previous experiment, fecal samples were collected successively from the same group of subjects. Three freshly voided fecal specimens were incubated with CDCA and UDCA and also with 7-keto-LCA, a hypothetical intermediate of 7-epimerization (**Fig. 4**). Feces MI and NM converted CDCA and UDCA into each other, and the same metabolites including LCA were obtained from both 3,7-diols and also from 7-keto-LCA. Feces HK, unlike the previous

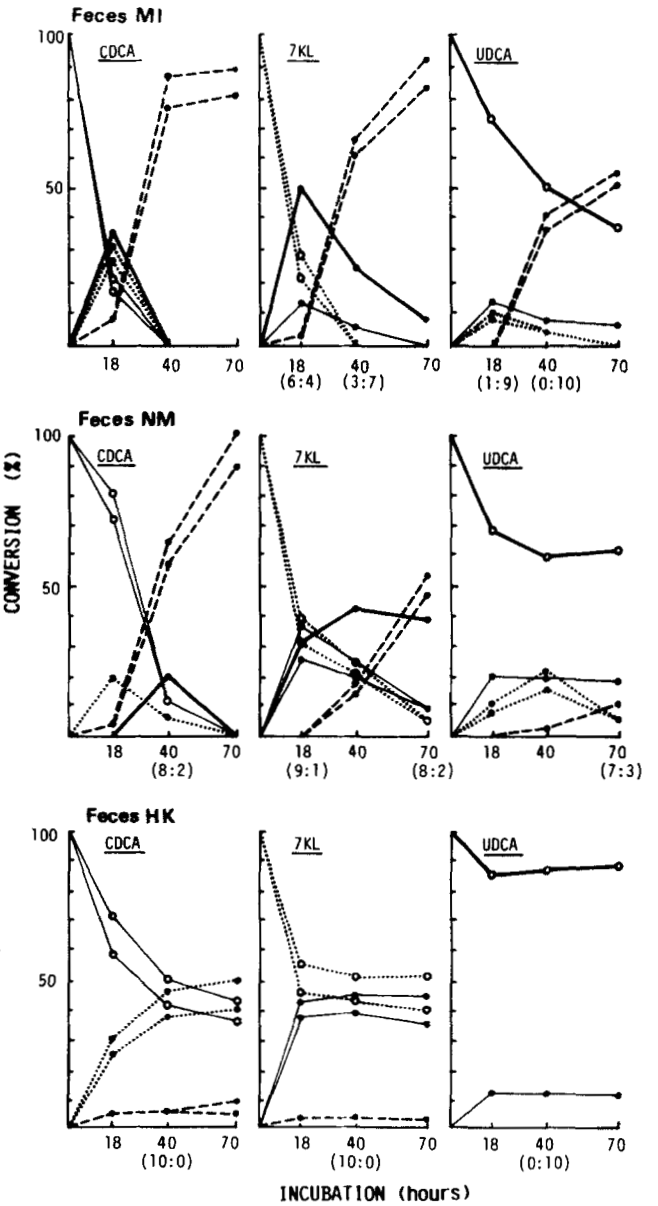

Fig. 4. Changes in percentage composition of bile acid metabolites produced from CDCA, 7-keto-LCA (7KL) and UDCA by cultures of feces MI, NM, and HK during 70 hr of anaerobic incubation. For procedures and legends, see Fig. 1.

sample (Fig. 1), was unable to interconvert CDCA and UDCA and, presumably relating to this failure, produced neither 7-keto-LCA nor LCA from UDCA (3β-epimer was the only product from UDCA by this specimen); however, it converted CDCA and 7-keto-LCA into each other through reversible 7α-dehydrogenation and produced small amounts of LCA from both acids. New specimens from MI and HK were re-examined, resulting in similar findings.

From this series of experiments it may be concluded that the intestinal flora of normal persons usually include the microorganisms responsible for the mutual conversion between the 7α- and 7β-OH groups, and the occasional lack of this activity results in the decreased metabolism of the 7β-OH group in UDCA. The formation of 3β-epimers was effected by each sample, and all the substrate bile acids and their 7-hydroxy metabolites underwent this reaction.

Transformation of CDCA by dilutions of human feces

To estimate the levels of viable bacteria catalyzing the respective bile acid transformations, serially diluted fecal samples were incubated with CDCA (**Fig. 5**). The proportion of 7α-dehydroxylating and 7α-dehydrogenating conversions of CDCA varied depending upon the dilution of inoculum; dehydroxylation was efficiently effected by concentrated inocula while dehydrogenation was still evident with higher dilutions, in accord with the published data with rat specimens (2). 7α-Epimerization was caused by all dilutions and large portions of the epimer were produced even at the highest dilutions tested, indicating the prevalence of the causative organisms at a concentration of more than 10^6 units per g feces. In addition, all dilutions effected 3α-epimerization.

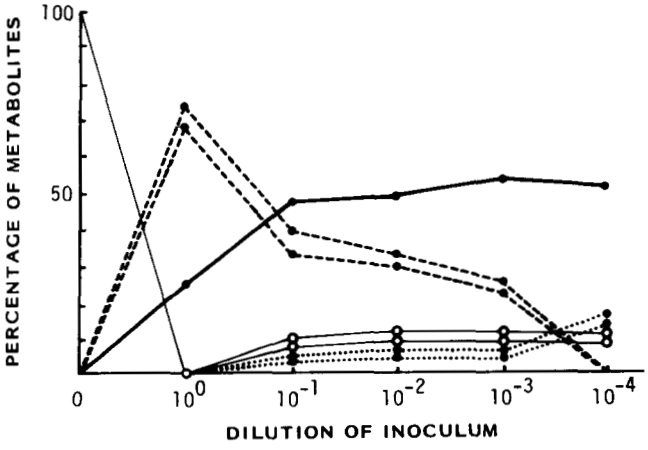

Fig. 5. Bile acid metabolites formed from CDCA by cultures of decimal dilutions of human feces. An aliquot (0.1 ml) of each dilution was grown anaerobically for 4 days in 4 ml of broth containing CDCA in tubes. Bile acids were separated as methyl esters and quantified as percentages of the total bile acids. For legends, see Fig. 1.

Effects of heating, neomycin, and aeration

To provide some insight into the nature of the microorganisms accounting for the individual bile acid reactions, the following investigations were attempted. A 1:10 suspension of a mixture of fecal samples from three subjects was heated at 80°C for 20 min prior to inoculation into tubes of bile acid medium. The resulting culture was unable to 7α-dehydroxylate CDCA but retained 7α-dehydrogenation, 7α-epimerization, and 3α-epimerization unimpaired, although the onset of these reactions was somewhat delayed. The same fecal suspension was grown in the presence of neomycin at a final concentration of 150 μg/ml. The culture consumed CDCA at a rate similar to that of the control culture, but did not perform 7α-dehydroxylation and showed significantly suppressed 7α-epimerization. 7α-Dehydrogenation and 3α-epimerization were unaffected and, as a result, CDCA was exclusively, if not entirely, metabolized through 7α-dehydrogenation to give a large quantity of 7-keto-LCA, a small portion of which was epimerized at its 3α-OH group.

A fecal sample (HN of Fig. 1) was incubated with either CDCA or UDCA under intermittent aeration. 7-Dehydroxylation was barely demonstrated; interconversion between CDCA and UDCA was observed in the early phase of incubation; both CDCA and UDCA were extensively converted into the same mono- and dioxo products; and 3α-epimerization was left intact. Under more aerobic conditions (continuous aeration) on the other hand, neither 7- nor 3-epimerization was carried out, and UDCA was oxidized only at C-3 to give a small amount of 3-oxo acid (no 3β-epimer produced), whereas CDCA was oxidized at both C-7 and C-3 giving rise to a large quantity of 7-keto-LCA and a small amount of 3-oxo product. These findings suggest that 7-epimerization is a prerequisite to the oxidation of the 7β-OH group of UDCA and, furthermore, that both 7- and 3-epimerization are essentially of an anaerobic nature, even though they are not so sensitive to the effect of oxygen.

DISCUSSION

Evidence has been presented in this study for the occurrence of mutual conversion between CDCA and UDCA in in vitro cultures of human intestinal microflora. Confirmative identity of the conversion products

was established from the combined data of GLC and MS. This 7-epimerizing interconversion was demonstrable with most of the fecal samples and even with the higher dilutions of these samples, suggesting the prevalence of the causative organisms among the normal intestinal flora. Moreover, the organisms were shown to be relatively heat-resistant and did not require extreme anaerobic conditions for growth. This is in sharp contrast to the 7α-dehydroxylation that was completely abolished by moderate heating and intermittent aeration of the culture (21). The epimerizing activity, however, was markedly suppressed by the addition of neomycin to the growth medium and completely inhibited in a highly aerated system. Some neomycin-sensitive bacteria might be concerned with the conversion which is essentially of an anaerobic nature. In view of these attitudes, attempts are being continued in our laboratory to isolate pure cultures responsible for this phenomenon.

UDCA, the 7β-hydroxy epimer of CDCA, has been detected in human bile usually in trace amounts (22) but occasionally in considerable concentrations (23–25). Of late, an increased level of biliary UDCA has been reported in patients receiving CDCA for the treatment of gallstones (10). As for the biosynthesis of UDCA, it seems beyond doubt that CDCA is a precursor of UDCA, but two questions remain to be answered: whether the epimerization of the 7α-OH group to a β-position takes place directly or via a 7-oxo group, and whether the reaction(s) is carried out in the liver or in the intestine or in both. Although none of the possibilities can be excluded at present, the most common concept, mainly deduced from the results of in vivo studies, is that UDCA might be formed in the liver by a stereospecific reduction of 7-keto-LCA that is a microbial product of CDCA in the intestine (26–29). Much less information concerning the catabolism of UDCA is available. UDCA is generally considered unable to serve as a substrate for bacterial 7α-dehydroxylation (30) and 7α-dehydrogenation (31, 32), and also to be a poor substrate for liver enzymes (29, 33).

The investigations reported here clearly demonstrated that the entire process of the conversion of CDCA into UDCA, and also the reverse reaction, could be carried out purely through microbial actions without participation of any liver function. The intervention of 7-keto-LCA as an intermediate of the mutual conversions seemed very likely for the following reasons. 7-keto-LCA was invariably produced from either of the 7α- and 7β-hydroxy bile acids concurrently with the respective 7-epimers, and the substrate 7-keto-LCA was converted not only into CDCA but also into UDCA in so far as there was mutual conversion between these two epimeric bile acids.

Recently, Fedorowski et al. (7) reported findings similar to ours, i.e., the interconversion between CDCA and UDCA by human fecal cultures; but they found no 7-keto-LCA in the cultures and considered a direct conversion between these bile acids by intestinal bacteria. Gordon et al. (34) demonstrated the conversion of UDCA and 7-keto-LCA into CDCA in static cecal loops of the rat but they did not detect the reverse reaction from CDCA into UDCA.

The equilibrium of the mutual 7-epimerization, possibly via a 7-keto acid, favored the 7β-hydroxy acid and, as a result, only small portions of UDCA were epimerized to CDCA, which is more susceptible to various microbial enzymic reactions. This accounts for the decreased consumption of UDCA as compared with the rapid catabolism of CDCA. Occasionally, when there was a lack of 7-epimerization, UDCA was affected only at its 3α-OH substituent to give a small amount of the 3β-hydroxy product.

7α-Dehydroxylation of CDCA giving rise to LCA was demonstrated more or less by all the fecal samples tested. LCA was also formed from UDCA by the active feces as far as the interconversion between UDCA and CDCA existed. The finding suggests the possibility that the LCA was formed by the ordinary 7α-dehydroxylation after UDCA had been converted into CDCA. Makino and Nakagawa (11) observed the increased formation of LCA in the intestines of patients under UDCA therapy and assumed the 7β-dehydroxylation of UDCA by colon bacteria. Fedorowski et al. (7, 12) found that smaller amounts of LCA were produced from UDCA than from CDCA and attributed the difference to the stereospecificity of bacterial 7-dehydroxylases. No intestinal organisms, however, have been found capable of directly removing the 7β-OH group of UDCA, and the indirect pathway suggested above seems to be more plausible.

Various bile acids with a 3β-OH group have been detected as minor components of feces of man and animals, although their physiological significance is unknown (35–37), and the microbial formation of these 3β-epimers from α-equivalents has been shown in in vitro cultures of intestinal contents from the rat (2, 3). In this study, the epimerizing conversion was effected by highly diluted cultures of every fecal sample and all the 3α-hydroxylated bile acids were epimerized en bloc, indicating that the activity is widely distributed among the normal intestinal flora and has no specificity for the substrates. It is worthy of note that the conversion, although taking place so universally, does not exceed 10–15%, irrespective of the kind of bile acid and of fecal sample. As for

the causative organisms, a variety of intestinal bacteria including *Escherichia freundii* (38), *Clostridium perfringens, Bacillus cereus, Eubacterium* species (1), and anaerobic streptococci (39) have been listed. We have also isolated similar active strains of *C. perfringens* and *E. lentum* from human feces.[1] These bacteriological observations adequately explain the ubiquitous appearance of 3β-hydroxy bile acids in vivo as well as in fecal cultures. Concerning the reaction sequence, the intervention of a 3-oxo product as an intermediate has been postulated (38). These 3-oxo acids might have been produced in the present investigations only in undetectable amounts because of the anaerobic conditions of the culture medium.

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