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Epimerization of chenodeoxycholic acid to ursodeoxycholic acid by human intestinal lecithinase-lipase-negative *Clostridia*

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Abstract Six strains of lecithinase-lipase-negative *Clostridia*, isolated from human feces, were capable of oxidizing chenodeoxycholic acid to 3α-hydroxy-7-keto-5β-cholanoic acid and of epimerizing it to ursodeoxycholic acid. The identity of the reaction products was confirmed by comparing their mass spectra, obtained by combined gas–liquid chromatography–mass spectrometry, with those of authentic reference compounds. 3α-Hydroxy-7-keto-5β-cholanoic acid was reduced by growing cultures of all clostridial strains to chenodeoxycholic acid and to ursodeoxycholic acid, the latter being the preferred conversion product of most strains. However, ursodeoxycholic acid was not attacked by any of the strains. Growth kinetic experiments with three strains showed that chenodeoxycholate was transformed during the log or lag phase. No bile acid conversion could be seen during the stationary phase. While the concentration of chenodeoxycholic acid decreased and that of ursodeoxycholic acid increased tending towards plateaus, the concentration of 3α-hydroxy-7-keto-5β-cholanoic acid passed through a maximum. We propose a reaction sequence with 3α-hydroxy-7-keto-5β-cholanoic acid as an intermediate for the epimerization of chenodeoxycholic acid to ursodeoxycholic acid. This demonstration is the first using isolated bacterial strains.—**Edenharder, R., and T. Knaflic.** Epimerization of chenodeoxycholic acid to ursodeoxycholic acid by human intestinal lecithinase-lipase-negative *Clostridia*. *J. Lipid Res.* 1981. **22:** 652–658.

Supplementary key words bile acids · gas–liquid chromatography–mass spectrometry · growth kinetics and bile acid transformation

The primary human bile acids, cholic and chenodeoxycholic acid, are secreted as conjugates of glycine and taurine into the duodenum and are mostly reabsorbed in the ileum. The remaining fraction reaches the large bowel, where the bile acids are extensively metabolized by the intestinal flora. Known transformations include deconjugation of conjugated bile acids to yield free bile acids (1–10), dehydroxylation, mainly at the C_7 hydroxyl group of the steroid nucleus (1, 2, 4, 11–18), oxidation of the hydroxyl groups at C_3,C_7, and C_{12}, reduction of carbonyl moieties to either α or β hydroxyl functions (1–3, 8, 12, 14–16, 19–21), and epimerization at C_5 of bile acids with the 5β configuration (22, 23). Other reported microbial degradations include side chain metabolism (24), degradation to nonsteroidal compounds (25), introduction of carbon double bonds into the steroid nucleus (26, 27), hydrolytic cleavage of sulfate esters (28, 29), and esterification of the 3β-hydroxyl group with fatty acids (27).

Microbial epimerization of the 3α-hydroxyl group of bile acids has been demonstrated with fecal dilutions (3, 30) and with isolated strains (2, 15). Preliminary reports of 12α-hydroxyl epimerization have also appeared (5, 15). No intestinal microorganisms responsible for the epimerization of the 7α-hydroxyl group have previously been identified, although fecal bile acids with 7β-hydroxyl groups have been detected (31, 32).

More recently, 7α-epimerization was demonstrated with fecal dilutions (30, 32–35). In this communication we present evidence for 7α-hydroxyl group epimerization in chenodeoxycholic acid by isolated strains of lecithinase-lipase-negative *Clostridia* and we propose a mechanism for the epimerization process by these bacteria.

Abbreviations: The following are systematic names of bile acids referred to in the text by trivial names: cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; ursodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid; lithocholic acid, 3α-hydroxy-5β-cholanoic acid. TFA, trifluoroacetyl; HFIP, hexafluoroisopropyl; DMCS, dimethylchlorosilane; GLC, gas–liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry.

[1] This work is part of the thesis of T. Knaflic to be submitted as partial requirement for the degree of "Doktor der Medizin" to the faculty of medicine, Johannes Gutenberg Universität Mainz.

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MATERIAL AND METHODS

Bacterial strains, media, and culture conditions

Lecithinase-lipase-negative *Clostridia,* isolated from feces of patients with large bowel cancer, were obtained from Dr. W. Döll, Institut für Hygiene und Mikrobiologie der Universität Würzburg (D-8700 Würzburg). These strains were originally grown from spores, surviving 10 min of heating at 80°C. Stock cultures were maintained in a cooked meat medium (Oxoid[2], CM 81), supplemented with Schaedler broth (Oxoid[2], CM 497) and 1 ml/liter of 0.1% resazurine solution, pH 7.6. Unless otherwise stated, this medium containing 0.25 mM sodium chenodeoxycholate, ursodeoxycholate, or 3α-hydroxy-7-keto-5β-cholanoate was used for transformation experiments. To account for the possible necessity of enzyme induction, two serial bacterial transfers were performed; both tests were incubated anaerobically at 37°C until the late stationary phase. Bile acid transformation was then checked with selected samples.

All media used were prereduced and anaerobically sterilized. Bacteria were transferred as described by Holdeman and Moore (36), with the modification that a multiple gas supply head with glass Pasteur pipets was used in a sterile hood (CEAG-Schirp, model DF600)[3]. In general, screw-cap vials with additional butyl rubber stoppers were used for bacterial cultivation.

Chemicals

Sodium chenodeoxycholate was purchased from Calbiochem, Giessen; ursodeoxycholic acid and 3α-hydroxy-7-keto-5β-cholanoic acid were from Steraloids, Wilton NH. Trifluoroacetic anhydride and hexafluoroisopropanol were supplied by Merck-Schuchardt, München.

[2] Oxoid Deutschland, D-4230 Wesel, Poppelbaumstrasse 18-20.
[3] CEAG-Schirp, Reinraumtechnik, D-4711 Bork (Westfalen), Industriegelände

TABLE 1. Transformation of chenodeoxycholate to 3α-hydroxy-7-keto-5β-cholanoate and ursodeoxycholate by lecithinase-lipase-negative *Clostridia*

Label of Clostridial Strains	Percent of Total Bile Acids Present in the Stationary Phase		
	3α,7α	3α,7β	3α,7-keto
25.11.c	57	23	20
226/7A	49	5	46
226/7B	36	16	48
228/7	19	14	67
42/50	40	32	28
304/6	25	60	15

3α,7α, chenodeoxycholic acid; 3α,7β, ursodeoxycholic acid; 3α,7-keto, 3α-hydroxy-7-keto-5β-cholanoic acid.

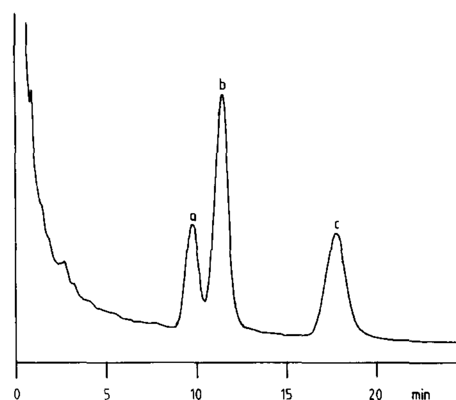

Fig. 1. Transformation of chenodeoxycholate by strain 42/50, a lecithinase-lipase-negative *Clostridium.* Bile acids were analyzed by gas–liquid chromatography as described in Materials and Methods. (a), Chenodeoxycholic acid; (b), ursodeoxycholic acid; (c), 3α-hydroxy-7-keto-5β-cholanoic acid. Structures were verified by GLC–MS as shown in Figs. 2 and 3.

Extraction of bile acids, gas–liquid chromatography, and combined gas–liquid chromatography–mass spectrometry

Bacterial suspensions of about 10 ml were acidified with 4 N HCl to pH 1–2 and centrifuged at 5000 *g*. The dried sediments were extracted twice with 3 ml of acetone at 50°C. The aqueous supernatants were decanted and extracted three times with 1.5 volumes of ethyl ether. The pooled ether extracts were then washed twice with 0.5 volumes of water, and the combined water washings were reextracted once with ether. The pooled ether phases were evaporated to dryness under reduced pressure and the acetone extracts were added and evaporated.

Bile acids were analyzed as TFA-HFIP derivatives (37, 38) by gas–liquid chromatography. A Varian model 1700 gas chromatograph equipped with dual flame ionization detectors and silanized glass columns, 1.8 m long and 3 mm i.d., packed with 3% QF-1 on acid-washed, DMCS-treated Chromosorb W, 100–200 mesh, was used for all determinations. The operating conditions were: injection temperature 240°C, column temperature 230°C, detector temperature 300°C, nitrogen flow-rate 30 ml/min.

For GLC–MS analysis, a Varian model 1700 gas chromatograph with a Biemann-Watson two-stage helium separator combined with a Varian MAT model CH 7A mass spectrometer and a Varian Spectro System 100 data system were used. Nitrogen was replaced by helium. The operating conditions were: separator temperature 220°C, accelerating voltage

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3 kV, electron energy 70 eV, trap current 1000 μA, and ion source temperature 220°C.

RESULTS

Identification of chenodeoxycholate metabolites

In screening strains of lecithinase-lipase-negative *Clostridia* for their ability to act on chenodeoxycholate, we found six strains that produced two reaction products with chromatographic properties identical to those of ursodeoxycholic acid and 3α-hydroxy-7-keto-5β-cholanoic acid (**Table 1, Fig. 1**). Evidence for bacterial epimerization and oxidation of the 7α-hydroxyl group of chenodeoxycholic acid was obtained by GLC–MS. **Figs. 2** and **3** prove the structural identity of transformation product b, Fig. 1, with authentic ursodeoxycholic acid, and of compound c, Fig. 1, with 3α-hydroxy-7-keto-5β-cholanoic

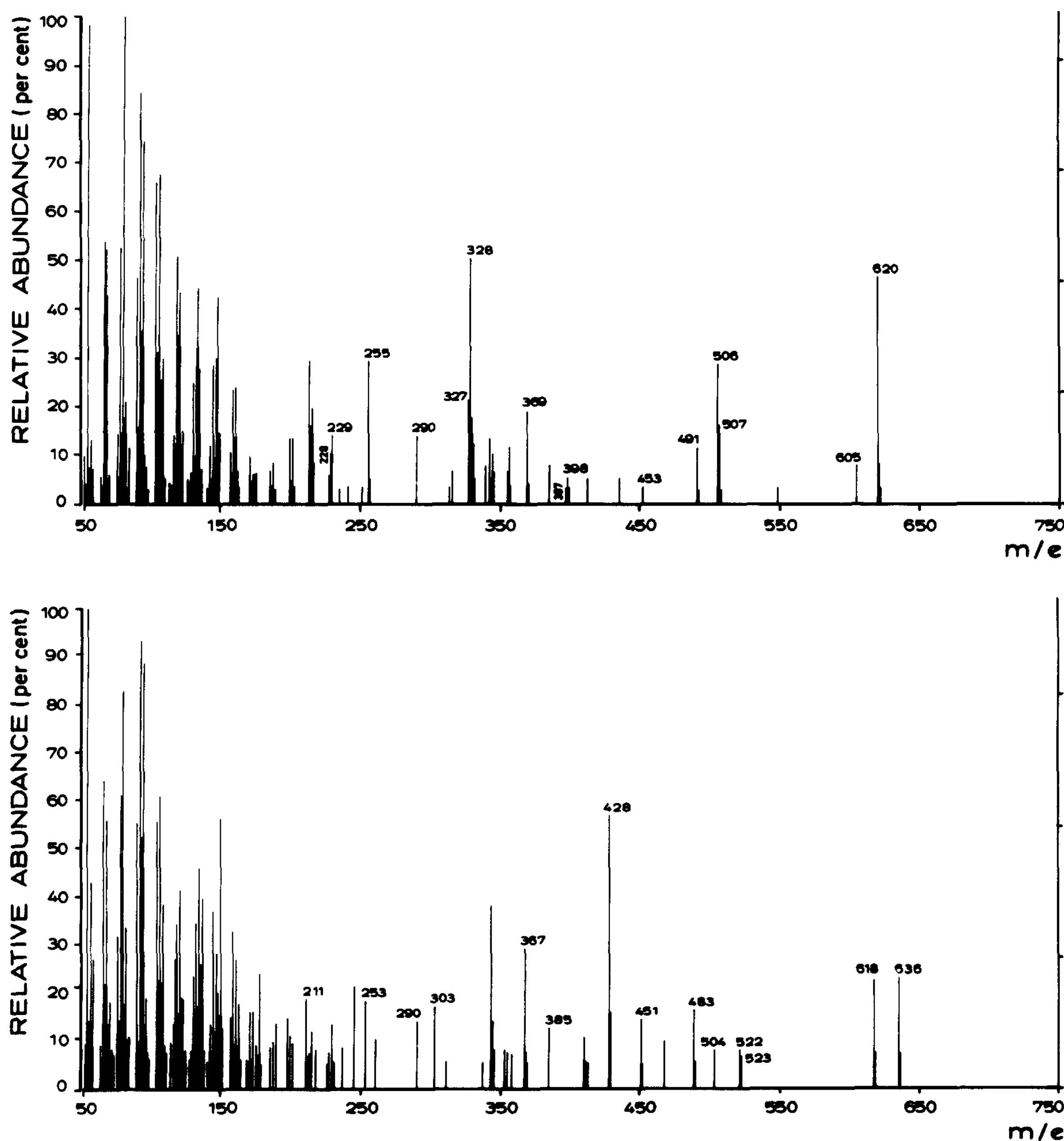

Fig. 2. Mass spectra of chenodeoxycholate metabolites from lecithinase-lipase-negative *Clostridium* 42/50. Bile acids were analyzed as trifluoroacetates-hexafluoroisopropylesters (TFA-HFIP) by GLC–MS as described in Materials and Methods. The upper part of the figure shows the mass spectrum of compound b, Fig. 1, the lower part shows that of compound c, Fig. 1.

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acid. No other transformation products could be detected.

Growth kinetics and chenodeoxycholate transformation

Correlation between chenodeoxycholate transformation and the different bacterial growth stages was tested in strains 226/7B, 228/7, and 42/50, which grew well in a defined medium containing glucose, amino acids, and vitamins (16), and in Schaedler broth. **Fig. 4** shows that chenodeoxycholate transformation by strain 42/50, growing in Schaedler broth, started in the lag phase and continued during the log phase. No bile acid conversion could be observed during the stationary phase. In the early log phase levels of both reaction products, 3α-hydroxy-7-keto-5β-cholanoic acid and ursodeoxycholic acid, increased. During the late log phase, however, forma-

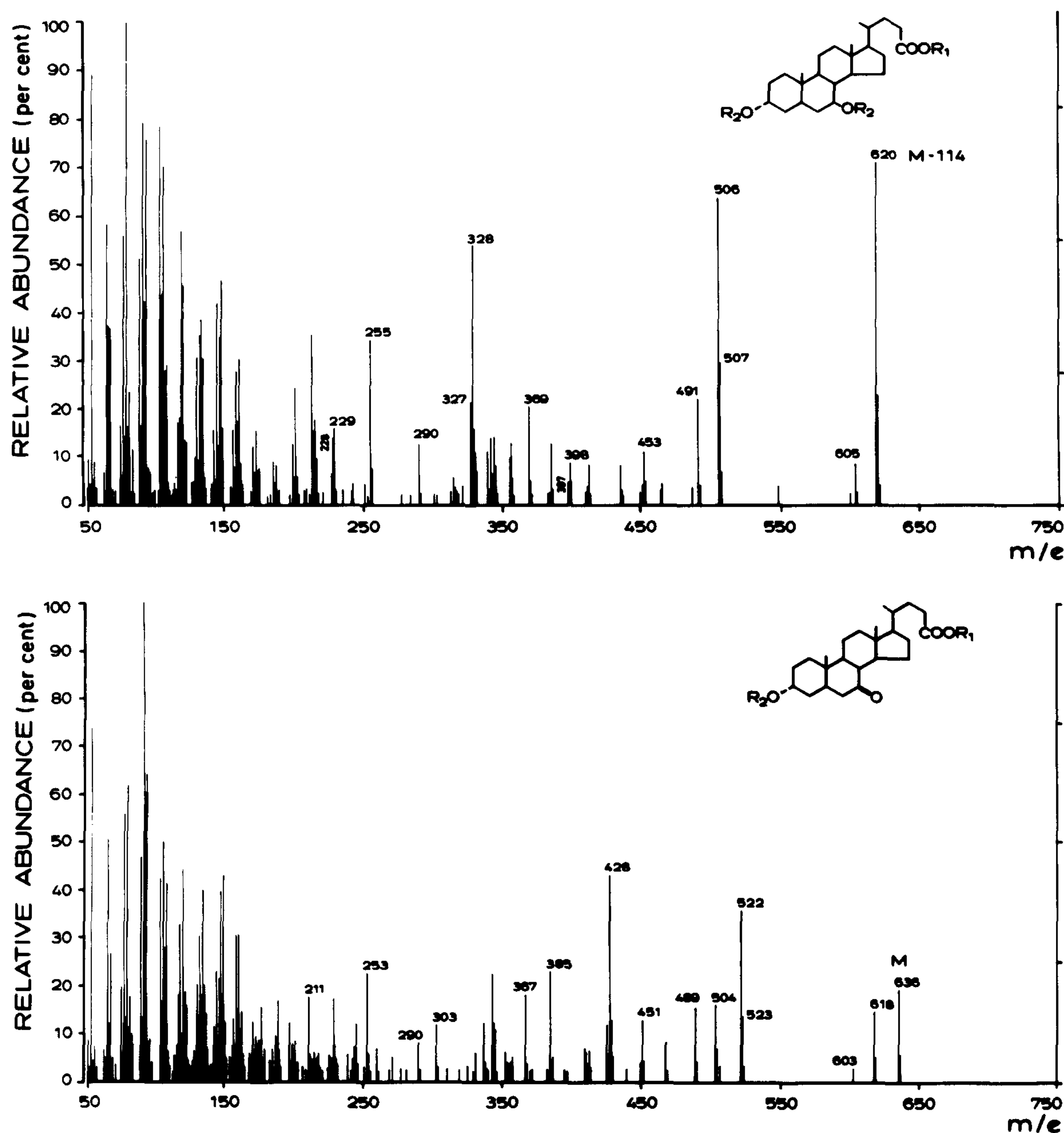

Fig. 3. Mass spectra of trifluoroacetyl-hexafluoroisopropyl derivatives of authentic ursodeoxycholic acid and 3α-hydroxy-7-keto-5β-cholanoic acid. The upper part of the figure shows the mass spectrum of the TFA-HFIP derivative of ursodeoxycholic acid ($R_1 = CH(CF_3)_2$; $R_2 = CF_3CO$); the lower part shows that of the TFA-HFIP derivative of 3α-hydroxy-7-keto-5β-cholanoic acid. Spectra were also recorded by GLC–MS. The corresponding spectra of Figs. 2 and 3 differ only in the intensities of fragment ions, which is common for spectra recorded during GLC separation. A detailed interpretation of these spectra is given elsewhere (38).

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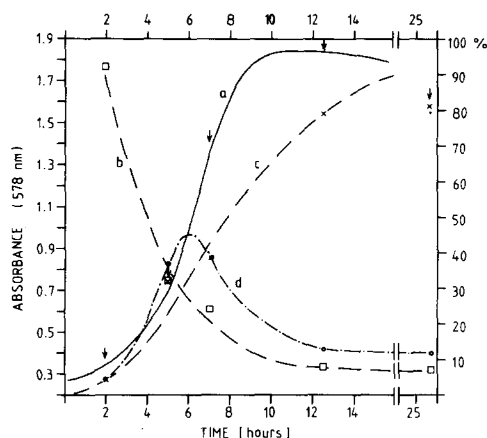

Fig. 4. Chenodeoxycholate transformation, ursodeoxycholate formation, and 3α-hydroxy-7-keto-5β-cholanoate conversion during different growth stages of strain 42/50, a lecithinase-lipase-negative *Clostridium*. The bacteria were cultivated in a 100-ml flask, with a side arm for measuring turbidity, in about 80 ml of Schaedler broth containing 0.25 mM sodium chenodeoxycholate. Growth was followed by measuring culture turbidity at 578 nm with an Eppendorf photometer (a). Ten-ml samples were taken at the times indicated by arrows and analyzed for bile acids as described above. (b), Chenodeoxycholic acid; (c), ursodeoxycholic acid; (d), 3α-hydroxy-7-keto-5β-cholanoic acid.

tion of ursodeoxycholic acid increased continuously, tending toward a plateau, but 3α-hydroxy-7-keto-5β-cholanoic acid concentration decreased. With strains 226/7B and 228/7, similar conversion patterns could be observed in Schaedler broth, but the steady state was shifted to the lag phase. Chenodeoxycholate was partially converted to 3α-hydroxy-7-keto-5β-cholanoate and ursodeoxycholate before growth started. In the defined medium, chenodeoxycholate transformation by all strains had already terminated during the lag phase, which was about three times longer than in Schaedler broth.

Transformation of ursodeoxycholate and of 3α-hydroxy-7-keto-5β-cholanoate

Possible reversibility of the epimerization reaction was assessed by providing ursodeoxycholate to the six active clostridial strains. No reverse reaction to chenodeoxycholate was observed under these experimental conditions. Doing the same with 3α-hydroxy-7-keto-5β-cholanoate, the postulated intermediate of chenodeoxycholate epimerization to ursodeoxycholate, showed that this substance was converted to both chenodeoxycholate and ursodeoxycholate by all strains (**Table 2**). The strains differed in their relative capacities to reduce the keto group to α and β hydroxyl functions, but ursodeoxycholate was the preferred reaction product of most strains.

DISCUSSION

A wide variety of bile acid biotransformations is known to be catalyzed by human intestinal bacteria (1). Whereas reductive sequences predominate in vivo, in vitro bile acid metabolism by isolated strains and highly diluted fecal samples is primarily oxidative (1–3, 8, 12, 14–16, 19–21). Reductive 7α-dehydroxylation of cholic and chenodeoxycholic acids, rarely found among isolated strains (1, 11–18, 41), is strongly favored in vivo. Constraints inherent to the strictly reducing anaerobic environment of the human colon are thought to be responsible (18). It is tempting to speculate that one of these constraints is the presence of an unknown chemical cofactor, perhaps a plasmalogen (39), since an intermediate with a double bond in the steroid nucleus has to be hydrogenated as is the case in cholesterol conversion to coprostanol. Such a cofactor might explain certain experimental discrepancies in vitro as well (15, 16)[4]. We could find no formation of lithocholic acid, the end product of reductive 7α-dehydroxylation of chenodeoxycholic acid, with our clostridial strains, but found strains that were able to transform chenodeoxycholate into 3α-hydroxy-7-keto-5β-cholanoate and ursodeoxycholate. Oxidation of only the 7α-hydroxyl group is in agreement with the well-documented wide distribution of 7α-dehydrogenating activity among members of *Bacteroides, Bifidobacterium, Clostridium, Eubacterium, Veillonella,* and *Escherichia coli* (1–3, 8, 12, 16, 19–21), and the inability of nuclear dehydrogenating *Clostridia,* a subgroup of lecithinase-lipase-negative *Clostridia,* to oxidize the 3α-hydroxyl function (40).

[4] When isolating the dominant fecal flora of about 25 patients with large bowel cancer and testing the isolated strains for their bile acid transforming capacities, we found one person whose fecal flora was highly active in transforming cholic acid into deoxycholic acid. All strains active in this respect (34 out of 53) belonged to different species of *Eubacterium* and *Bacteroides.*

TABLE 2. Transformation of 3α-hydroxy-7-keto-5β-cholanoate to cheno- and ursodeoxycholate by lecithinase-lipase-negative *Clostridia*

Label of Clostridial Strains	Percent of Total Bile Acids Present in the Stationary Phase		
	3α,7-keto	3α,7α	3α,7β
25.11.c	33	0	67
226/7A	49	29	22
226/7B	21	34	45
228/7	9	8	83
42/50	29	12	59
304/6	8	18	74

3α,7-keto, 3α-hydroxy-7-keto-5β-cholanoic acid; 3α,7α, chenodeoxycholic acid; 3α,7β, ursodeoxycholic acid.

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This report is the first demonstration of 7α-hydroxyl group epimerization by isolated bacterial strains, though this reaction was recently reported in fecal dilutions (33–35). Bacterial formation of bile acids with 7β-hydroxyl groups has been found earlier in fecal dilutions (3, 30) and isolated strains (2, 15), and a preliminary report of 12α-hydroxyl group epimerization has appeared (15). Within the genus *Clostridium* there may exist species or groups able to perform hitherto unknown epimerizations: recently a *Clostridium* isolated from rat feces was shown capable of converting β-muricholic acid (3α,6β,7β-trihydroxy-5β-cholanoic acid) into ω-muricholic acid (3α,6α,7β-trihydroxy-5β-cholanoic acid) (41).

We propose, as a mechanism for the conversion of chenodeoxycholate to ursodeoxycholate by our clostridial strains, an epimerization pathway involving 3α-hydroxy-7-keto-5β-cholanoate as a free intermediate. This conclusion is drawn from the demonstration of intermediate accumulation of this ketonic acid during the epimerization process and the reduction of added 3α-hydroxy-7-keto-5β-cholanoate to chenodeoxycholate and ursodeoxycholate by growing clostridial cultures. While this conclusion agrees with the results of Azuma, Setoguchi, and Kogetsu (33), it is in contrast to the results of Fedorowski et al. (35). These authors found that labeled ursodeoxycholate was formed when stool dilutions from several subjects were incubated with [7β-^{3}H]chenodeoxycholate. This rules out a mechanism via 3α-hydroxy-7-keto-5β-cholanoate in these experiments, and points either to direct epimerization or to a mechanism proceeding via 3α-hydroxy-Δ^6-5β-cholenoate. Δ^6-Cholenoic acids, intermediates in bacterial reduction of the 7α-hydroxyl group of bile acids (42, 43), might also be intermediates in epimerization of this function, although they have hitherto not been detected in bacterial cultures or feces. We could not find any 3α-hydroxy-Δ^6-cholenoic acid in our clostridial cultures either, although enriched extracts were checked by GLC and GLC–MS. This pathway remains a hypothesis therefore.

We conclude from our results and those of Fedorowski et al. (35) that two different epimerization mechanisms for conversion of chenodeoxycholate to ursodeoxycholate can occur among different bacteria: one most probably proceeds via a 7-ketocholanoic acid and the other either by direct epimerization of the 7α-hydroxyl function or via a Δ^6-cholenoic acid as intermediate. It is also conceivable that these two different mechanisms might exist within the same bacteria, although from our experiments discrimination is not possible. 7-Ketocholanoic acids (31, 32) and bile acids with 7β-hydroxyl functions (31, 32, 44, 45) have been identified in human feces, and it seems that 7α-epimerization is pronounced in the gut, especially when conditions are less favorable for reductive 7α-dehydroxylation (32). Human fecal 7β-hydroxy bile acids may therefore be formed by bacterial epimerization of the corresponding 7α-hydroxy acids by the metabolic pathways discussed above, but may also be produced (probably in minor amounts) by hepatic reduction of bacterially-generated 7-ketocholanoic acids (46).

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