Enhancement of the 7α-dehydroxylase activity of a gram-positive intestinal anaerobe by *Bacteroides* and its significance in the 7-dehydroxylation of ursodeoxycholic acid

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Abstract The 7α-dehydroxylation of chenodeoxycholic acid (CDCA) and cholic acid (CA) by a *Eubacterium lentum*-like intestinal anaerobe was specifically enhanced by the bacteroides present in mixed cultures and also by the addition to the growth medium of cell extracts from the bacteroides. The 7α-dehydroxylating organism also possessed 7α-hydroxysteroid dehydrogenase activity, and, in collaboration with a 7β-dehydrogenating organism, converted ursodeoxycholic acid (UDCA) into CDCA. Large quantities of lithocholic acids were produced from UDCA as well as CDCA in in vitro cocultures of these three kinds of microorganisms.—**Hirano, S., and N. Masuda.** Enhancement of the 7α-dehydroxylase activity of a gram-positive intestinal anaerobe by *Bacteroides* and its significance in the 7-dehydroxylation of ursodeoxycholic acid. *J. Lipid Res.* 1982. **23:** 1152–1158.

Supplementary key words 7α-dehydroxylase • 7α-hydroxysteroid dehydrogenase • 7β-hydroxysteroid dehydrogenase • 7α-dehydroxylating *Eubacterium lentum*-like organism

The removal of the 7α-hydroxy group from cholic acid and chenodeoxycholic acid is physiologically the most important of the microbial transformations of bile acids in the intestinal tract. After deconjugation, these primary bile acids are converted through this reaction (7α-dehydroxylation) into deoxycholic acid (DCA), one of the major components of biliary bile acids, and lithocholic acid (LCA), a presumptive hepatotoxic substance, respectively. Actually, more than 80% of the fecal bile acids are the products of this reaction. Our knowledge of the individual microorganisms accounting for this reaction, however, is incomplete. Despite numerous attempts, only a few organisms have so far been shown to be capable of performing 7α-dehydroxylation: four strains of *Clostridium* consisting of *C. sordellii* (1), *C. bifermentans* (1–3) and *C. leptum* (4, 5); four strains of *Bacteroides* comprising *B. fragilis* (6) and an unclassified species (7); and several strains of a presumed *Lactobacillus* species (8–10). The quantitative distribution of these organisms among the intestinal microflora was not determined and, in addition, only a feeble dehydroxylase activity was usually demonstrated in in vitro cultures of these organisms. The available data are far from sufficient for a complete understanding of the predominant in vivo reaction.

Recently, we isolated 13 intestinal anaerobes capable of 7α-dehydroxylating bile acids (11). Among them, strains b-8 and c-25, which represent one species, were recovered by plating higher dilutions of fresh human feces, thus implying their occurrence at substantial levels among the intestinal flora. Moreover, it was found during the course of isolation that the 7α-dehydroxylase activity of this species was specifically enhanced to a great extent by mixing the organism with bacteroides, which are also the predominant bacteria in the normal intestine. It was then found that ursodeoxycholic acid (UDCA), the 7β-hydroxy epimer of CDCA, was readily converted into LCA by the combined action of this activated 7α-dehydroxylation and the previously reported 7-epimerizing system (12).

This study reports these experimental data focusing mainly on the enhanced 7α-dehydroxylation, which seems to play an important role in the actual transformation of bile acids in vivo.

MATERIALS AND METHODS

Bacterial strains

All the 7α-dehydroxylating organisms used in this study were isolated in our laboratory (11). Among them,

Abbreviations: GLC, gas–liquid chromatography; MS, mass spectrometry; RRT, relative retention time; CA, cholic acid (3α,7α,12α-trihydroxy-5β-cholanoic acid); CDCA, chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanoic acid); DCA, deoxycholic acid (3α,12α-dihydroxy-5β-cholanoic acid); 7KD, 7-ketodeoxycholic acid (3α,12α-dihydroxy-7-keto-5β-cholanoic acid); 7KL, 7-ketolithocholic acid (3α-hydroxy-7-keto-5β-cholanoic acid); LCA, lithocholic acid (3α-hydroxy-5β-cholanoic acid); UDCA, ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholanoic acid).

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the principal subjects of this investigation were strains b-8 and c-25, which represent one species. They were gram-positive, non-spore-forming, non-flagellated, anaerobic bacilli that did not ferment any of the 30 carbohydrates tested and did not give any of the 10 biochemical reactions tested, except for positive H_2S production. They shared these negative features with *Eubacterium lentum*, but their growth was not stimulated by arginine (13) and they were negative for nitrate reduction and catalase activity (14). They possessed both 7α-dehydroxylase and 7α-hydroxysteroid dehydrogenase activities (11) like most of the 7α-dehydroxylating organisms previously reported (1, 2, 7, 10). Strain c-24, used as an enhancing partner, was isolated from the same fecal sample as strain c-25. Strain c-24 is an anaerobic gram-negative rod producing succinic acid as a major fermentation product, and was tentatively assigned to the genus *Bacteroides*. Laboratory strains of various *Bacteroides* species and other anaerobic organisms, originally obtained from Prof. T. Imamura, Ryukyu University, were also tested for the enhancing effect. Strain b-52 was one of the 7β-dehydrogenating organisms that was isolated from human fecal samples and classified with a high degree of certainty as *Peptostreptococcus productus*, as described in detail in our previous paper (12). All of these strains were maintained on semisolid GAM agar medium (Nissui Pharmaceutical Co., Tokyo) (15) and 0.1-ml portions of an overnight culture in peptone-yeast extract-glucose broth (16) were used as inocula.

Transformation of bile acids by bacterial cultures

The basal medium was the same 2% peptone–1% yeast extract broth buffered at pH 7.5 (2% PY) as used previously for the detection of microbial 7α-dehydroxylation of bile acids (11). After addition of bile acid at a final concentration of 150 μg/ml, the medium was dispensed in 4-ml quantities in small test tubes, and used immediately after preparation and autoclaving. The test strains, singly or in combination, were grown in this medium for 2–8 days in an anaerobic jar under an atmosphere of 90% N_2 and 10% CO_2 (oxygen-free), and the spent culture medium was assayed for bile acid metabolites. Prior to assay, bacterial growth was measured by reading the percent transmittance of light at 660 nm on a photometer (%T) and the pH of the culture was checked electrometrically to insure that it was maintained at an alkaline pH value (7α-dehydroxylation can barely take place in an acidic environment) (11).

Culture supernatant and cell extract from *Bacteroides*

When indicated, the assay medium was supplemented with the following *Bacteroides* preparations: *B. ovatus* strain k-5, arbitrarily selected as one of the enhancing bacteroides, was subcultured in 150 ml of a buffered GAM broth (freshly prepared by dissolving a dehydrated commercial product in 0.02 M sodium phosphate buffer at pH 7.5) and the culture was centrifuged at 6,000 *g* for 20 min after 21 hr of anaerobic incubation (early stationary phase culture). The supernatant fluid was added to the assay medium (2.0 ml per 4.0 ml) after sterilization by passage through a 0.45-μm membrane filter. The cell sediment was washed three times in 0.02 M sodium phosphate buffer at pH 7.5, resuspended in 15 ml of the same buffer, and disrupted with a Branson Sonifier B-12 (Branson Sonic Power Co., Danburg, CT) at 80 w for 4 min. The sonicate was filter-sterilized and 0.5-ml portions were added to the assay medium. These supplemented media were deoxygenated by boiling and cooling before inoculation.

Assay of bile acids

All the procedures of extraction, derivatization, and analyses by gas–liquid chromatography and mass spectrometry were carried out in the manner described in our previous report (17). In brief, the bile acids extracted with ethyl acetate from acidified culture samples were methylated by a methanol-sulfuric acid procedure and chromatographed on a 3% QF-1 column. The individual bile acids were identified by RRT values (relative retention time, methyl deoxycholate = 1.00), and the identity was further confirmed by combined GLC–MS. For that, the methylated samples were converted into the complete trimethylsilyl ethers by the method of Makita and Wells (18) and the derivatives were subjected to MS after separation on a 3% Hi Eff-8B column. The quantity of bile acids was determined from the area of the GLC peaks compared with that of a known amount of methyl deoxycholate and expressed as percentages of the total bile acids. In each experiment, blank tubes (uninoculated medium) were processed in parallel and served as controls for the recovery of sample bile acids.

Bile acids

All the bile acids used were from the same source as described in our previous papers (17).

RESULTS

Source and isolation of 7α-dehydroxylating strains b-8 and c-25

These two strains were recovered from the same fecal samples and by the same procedure as were the 7β-dehydrogenating organisms previously reported (12). In performing an anaerobic viable count of human fecal bacteria, 181 colonies were picked and, after purifica-

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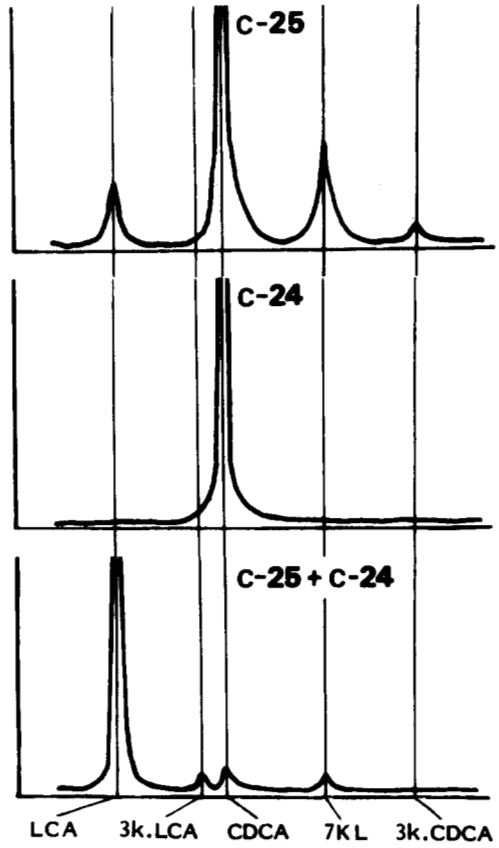

Fig. 1. Gas–liquid chromatographic separation of bile acids produced from CDCA in pure cultures of strain c-25 (a 7α-dehydroxylating bacterium) and c-24 (a *Bacteroides* strain) and in a culture of combination of the two. Bile acids extracted from 4-day cultures were separated as methyl esters on QF-1. Besides 7α-dehydroxylation (to give LCA) and 7α-dehydrogenation (7KL), strain c-25 shows a weak 3α-dehydrogenase activity (3-keto acids derived from LCA and CDCA in minor quantities). In the data given hereafter, the 3-keto acids are included in the fractions of the parent 3α-hydroxy acids.

tion by colonial reisolation, screened for bile acid-transforming activity. The isolates were divided into groups of five, and each set of five strains was cultured in a tube of 2% PY containing CDCA for 4 days under anaerobic conditions. Of the 36 mixed cultures, two showed the formation of LCA through 7α-dehydroxylation of CDCA. When the ten constituent strains from the two positive mixed cultures were examined for 7α-dehydroxylation in individual pure cultures under the same cultural conditions, strains b-8 and c-25 were found to account for the reaction, but the degree of dehydroxylation was much lower than that in the original mixed cultures. This finding led us to undertake the following investigation.

Enhancement of the 7α-dehydroxylase activity of strains b-8 and c-25 by *Bacteroides*

Strain b-8 or c-25 was combined with each of the other strains from the original mixed cultures and examined for 7α-dehydroxylation. An intense 7α-dehydroxylation resulting in 90% or more conversion was observed when the companion strains were gram-negative anaerobic bacilli, which were then provisionally identified as *Bacteroides* at the genus level. Chromatograms showing typical results are presented in **Fig. 1,** in which are compared the metabolites from CDCA produced by a pure culture of strain c-25, a pure culture of strain c-24 (a *Bacteroides* strain from the mixed culture, free of any bile acid-transforming activity), and a culture of the two strains combined. Strain c-25 showed both 7α-dehydroxylase and 7α-hydroxysteoid dehydrogenase activities, but only a small portion of CDCA was converted into LCA or a 7-keto acid. In the combined culture, 7α-dehydroxylation was strikingly enhanced, resulting in an almost quantitative conversion of CDCA into LCA.

Time courses were then examined, with CDCA and CA as substrates (**Fig. 2**). Pure cultures of strain c-25 consumed only small portions of both acids even after prolonged incubation for 7 days. When strain c-25 was co-cultured with strain c-24, both primary bile acids

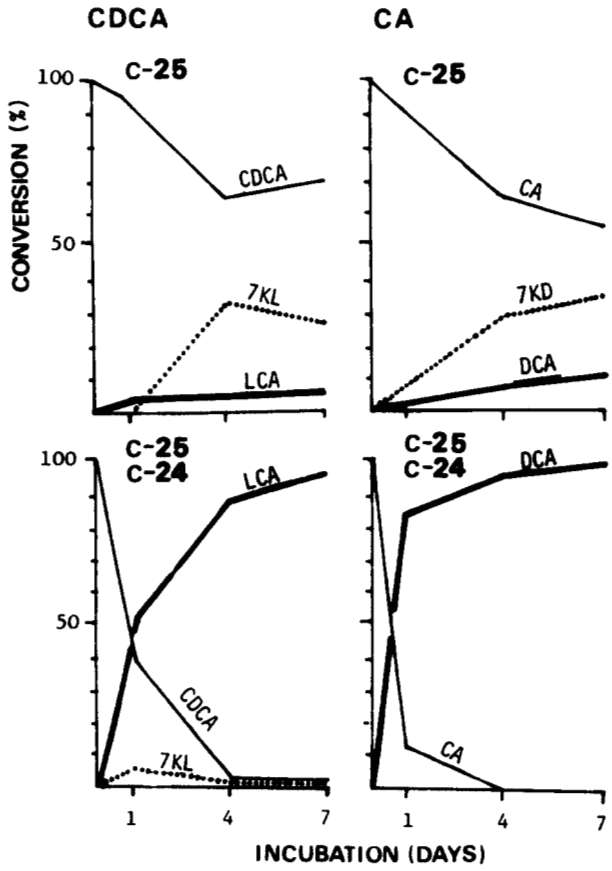

Fig. 2. Time course of transformation of CDCA and CA by strain c-25, singly or in combination with strain c-24 (a *Bacteroides* strain). Changes in percentage composition of individual bile acids were followed during the course of anaerobic incubation for 7 days.

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were completely catabolized through 7α-dehydroxylation by the 4th day of incubation.

Comparative enhancing effect of *Bacteroides* and non-bacteroides strains

The stimulatory effect of various laboratory strains on the 7α-dehydroxylation by strain c-25 was compared (**Table 1**). An almost complete conversion of CDCA into LCA was achieved with any of the *Bacteroides* strains, regardless of their own bile acid-transforming ability, whereas non-bacteroides anaerobes and *Escherichia coli* exerted little or no influence on the 7α-dehydroxylation of CDCA by c-25.

Enhancement of 7α-dehydroxylation by the culture supernatant and disrupted cell extract from a *Bacteroides* strain

To gain some insight into the mechanism of enhancement, strain c-25 was grown in the presence of the culture supernatant or cell extract from a *Bacteroides* strain (**Table 2**). A laboratory strain of *B. ovatus*, k-5, that showed similar enhancing ability (see Table 1) was used. Specific enhancement of 7α-dehydroxylation (but not of 7α-dehydrogenation) was caused by both preparations. Enhancement by the cell extract in particular was comparable to that induced by co-culturing with living bacteroides. It thus seems likely that an unidentified metabolite(s) synthesized by the *Bacteroides* strain, which is mostly cell-bound but partly released into the medium (presumably through cell lysis), is responsible for the enhancement. No increase in bacterial growth (%T) was observed by the addition of these preparations to the growth medium.

TABLE 1. Transformation of CDCA by strain c-25, in pure culture and in cocultures with various anaerobic strains

Companion Strain		% of CDCA[a] Converted to LCA	7KL
None		10	21
None		6	10
None		8	17
None		11	6
Clostridium perfringens WK47	(None[b])	17	35
Peptococcus micros 5462	(None)	10	21
Propionibacterium acnes 1592	(None)	36	25
Bifidobacterium adolescentis 1565	(None)	11	29
Fusobacterium russii 1101	(None)	17	55
Eubacterium aerofaciens 1585	(None)	22	11
Escherichia coli C	(–2H)	13	49
Bacteroides fragilis 2536	(–2H)	82	17
Bacteroides fragilis N-5	(–2H)	92	5
Bacteroides ovatus C-1-7	(–2H)	99	1
Bacteroides ovatus K-5	(None)	100	0
Bacteroides thetaiotaomicron Y-3-3	(None)	97	0
Bacteroides thetaiotaomicron 550	(–2H)	100	0
Bacteroides vulgatus Y-2-2	(None)	97	0

[a] Four-day cultures of strain c-25 grown singly or in combination with another organism in the presence of CDCA were assayed for bile acids, and the percentage of major metabolites formed through 7α-dehydroxylation (LCA) and 7α-dehydrogenation (7KL) was determined.

[b] Bile acid-transforming activity of companion strains: None, no demonstrable activity; –2H, 7α-dehydrogenation.

TABLE 2. Enhancing effect of culture supernatant and cell extract from *Bacteroides* strain k-5 on the 7α-dehydroxylation of CDCA and CA by strain c-25

Addition[a]	% of CDCA[b] Converted to LCA	7KL	% of CA Converted to DCA	7KD
None	12	33	33	26
Bacteroides:				
Culture supern.	28	29	63	17
Cell extract	99	1	90	5
Living cells	99	0	99	1

[a] Two ml of culture supernatant or 0.5 ml of a sonicated cell extract of *B. ovatus* strain k-5 was added to 4 ml of 2% PY containing 150 μg of CDCA or CA per ml.

[b] After 4 days of anaerobic incubation with strain c-25, the spent culture media were assayed for bile acids; 7α-dehydroxylation (LCA or DCA) and 7α-dehydrogenation (7KL or 7KD) products are given as percentage conversions. All figures are the averages from triplicate tests.

Effect of bacteroides on 7α-dehydroxylating strains of *Clostridium*

Eight active clostridial strains, isolated and maintained in our laboratory (11), were examined for stimulation by bacteroides (by co-culturing). The strong and consistent stimulation shown for the *E. lentum*-like b-8 and c-25 strains was never observed with these clostridial organisms.

Conversion of ursodeoxycholic acid into lithocholic acid

In a previous study (12), we reported on the interconversion between UDCA (7β-OH) and CDCA (7α-OH) by the combined action of two kinds of microorganisms active for 7α- and 7β-hydroxysteroid dehydrogenase, respectively. As strain c-25 shows 7α-hydroxysteroid dehydrogenase activity besides 7α-dehydroxylation, it should be expected that c-25, when co-cultured with a 7β-hydroxysteroid dehydrogenase-positive bacterium, might convert UDCA into CDCA and 7α-dehydroxylate the resulting CDCA into LCA. The following experiments were attempted to confirm this assumption and to examine the effect of the enhancing *Bacteroides* on the overall reaction. Strains c-25 (7α-dehydrogenating and 7α-dehydroxylating), b-52 (7β-dehydrogenating), and k-5 (stimulating the 7α-dehydroxylase activity of c-25) were cultured singly or in

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TABLE 3. Comparative transformation of CDCA and UDCA by strains c-25, b-52, and k-5, singly or in combination[a]

Strain	Bile Acid	Incubation	Bile Acid Metabolites			
			LCA	CDCA	UDCA	7KL
		days		%		
c-25	CDCA	4	7	86		8
	UDCA	4			100	
k-5	CDCA	4		100		
	UDCA	4			100	
c-25 +k-5	CDCA	4	100			
	UDCA	4			100	
b-52	CDCA	4		100		
	UDCA	4			94	6
b-52 +c-25	CDCA	2	2	90	7	1
		4	6	69	21	3
		8	8	24	60	8
	UDCA	2		3	97	
		4		6	88	6
		8		13	78	8
b-52 +c-25 +k-5	CDCA	2	24	47	26	2
		4	45	32	20	3
		8	65	4	28	3
	UDCA	2	1	4	90	5
		4	26	8	62	4
		8	71	2	23	4

[a] The test strains were grown individually or in combination in 2% PY containing CDCA or UDCA for the indicated incubation periods. Bile acids extracted from the spent culture medium were quantified as to percentage composition.

combination in 2% PY containing either CDCA or UDCA. The percentage compositions of the metabolites after 2–8 days of anaerobic incubation are summarized in **Table 3.** Strain c-25 converted a small portion of CDCA into LCA and 7KL by itself, and quantitatively dehydroxylated CDCA when co-cultured with strain k-5, the same findings as described above. No transformation of UDCA was demonstrated by c-25 or/and k-5. Strain b-52 oxidized the 7β-OH group of UDCA to an oxo group, 7KL, without any effect on the 7α-OH group in CDCA. By mixing c-25 and b-52, CDCA and UDCA were mutually converted into each other accompanying the appearance of small amounts of 7KL as an intermediate. The conversions favored the 7β-hydroxy acid (13% conversion of UDCA into CDCA as against 60% conversion in the opposite direction, on the 8th day of incubation), and, although a small amount of LCA was formed from CDCA by the 7α-dehydroxylase activity of c-25, no LCA was detected from UDCA, presumably because of the low concentration of the CDCA converted from UDCA. When strain k-5 was added to the mixed cultures, large quantities of LCA (60–70% conversion) were derived equally from both CDCA and UDCA. This result shows that the epimerizing conversion of UDCA (given as a substrate) into CDCA was largely facilitated by the removal of the resulting CDCA from the medium by the enhanced 7α-dehydroxylation and that the UDCA derived from substrate CDCA was reversed to the original CDCA as CDCA was removed from the medium by 7α-dehydroxylation. A comparison of the metabolism of CDCA and UDCA by the combination of strains c-25, b-52, and k-5 was reinvestigated (**Fig. 3**). Similar findings were obtained, although the rate and extent of LCA formation varied to some extent from one experiment to another, presumably because of the difficulty in securing a consistent relative growth of the microorganisms under study.

DISCUSSION

Added to our previous investigation (12), this study indicates again the importance of the cooperative reactions by different microorganisms for understanding the microbial transformation of bile acids in the intestine.

Among the thirteen 7α-dehydroxylating organisms previously reported (11), one species of a gram-positive intestinal anaerobe comprising strains b-8 and c-25 was unique for its enhanced 7α-dehydroxylase activity in the presence of bacteroides. Two of the 181 colony-forming units from the fecal flora of the donor were of this species. This prevalence, taken together with the intense enhancement of their 7α-dehydroxylase activity by species of *Bacteroides*, which are also prevalent among the intestinal microflora, helps explain the extensive 7α-dehydroxylating catabolism of biliary bile acids in the in-

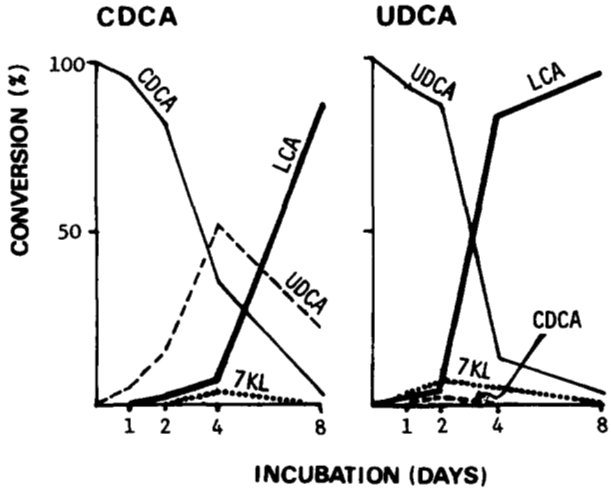

Fig. 3. Time course of transformation of CDCA and UDCA by mixed culture of strains c-25, b-52, and k-5. Experimental and analytical procedures are as described in the footnote to Table 3.

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testine. It should be noted here that this particular 7α-dehydroxylating species is related to *Eubacterium lentum*, an intestinal species which has been attracting our attention of late because of its various steroid-metabolizing activities (19–25).

The mechanism of the enhancement remains unclear. Bokkenheuser et al. (22) observed similar stimulation of the corticoid 21-dehydroxylase activity of *E. lentum* in mixed culture with *E. coli* and attributed the role of *E. coli* to the creation of a lower redox potential in the growth medium required for reductive dehydroxylation to proceed. This physical interpretation, however, does not explain why only the b-8 and c-25 type of bacterium and none of the other 7α-dehydroxylating organisms receives this stimulation or why the stimulation is so exclusively effected by *Bacteroides* species. This specific relationship between the stimulating and the stimulated organisms strongly suggests that certain particular metabolites of the bacteroides are specifically involved in the 7α-dehydroxylation by the b-8 and c-25 organism. The finding of this study that the enhancement was reproduced by the addition to the growth medium of cell extracts from the bacteroides supports this assumption, although fractionation of an effective metabolite has not succeeded yet.

Both CDCA and its 7β-epimer (UDCA) are now widely used as effective gallstone-dissolving agents, and the question of which of the two is superior is hotly debated (26–29). The matter of concern is the formation of LCA, a known hepatotoxic metabolite (30), from these 3,7-diols administered orally in large quantities. In contrast to the well-characterized 7α-dehydroxylation that transforms CDCA into LCA, little is known about the bioconversion of UDCA into LCA. A fundamental problem is whether the 7β-OH group in UDCA is eliminated directly (7β-dehydroxylation) or indirectly after conversion into a 7α-OH group (by ordinary 7α-dehydroxylation).

Recently, microbial epimerization between CDCA and UDCA has been suggested by its occurrence in in vitro cultures of mixed fecal flora (12, 31). The microorganisms accounting for the reaction have been reported by several workers. Macdonald, Hutchison, and Forrest (32) demonstrated the epimerization of the 7-hydroxy group in CDCA and CA by *Clostridium absonum* which elaborates both 7α- and 7β-hydroxysteroid dehydrogenases, and Edenharder and Knaflic (33) observed similar phenomenon by intestinal lecithinase-lipase-negative clostridia. As described above, we also demonstrated a 7-epimerization of primary bile acids by microbial pure cultures (12). In contrast to the clostridial strains, which elaborated both 7α- and 7β-hydroxysteroid dehydrogenases and epimerized the 7-hydroxy group by itself, the b-52 organism showed only 7β-hydroxysteroid dehydrogenase activity. The epimerizing conversion was caused by the synergistic action of this organism with another organism elaborating 7α-hydroxysteroid dehydrogenase. The b-52 organism was tentatively classified as *Peptostreptococcus productus* and was recovered from human feces at a high frequency of about 1% of the total fecal count (12), corresponding to the intestinal levels of this species reported by Moore and Holdeman (34) and Finegold, Attenberg, and Sutter (35). Such high concentrations of the 7β-dehydrogenating organism and also of a 7α-dehydrogenating bacterium (7α-hydroxysteroid dehydrogenase activity is widespread in many predominant intestinal species) may facilitate interconversion of UDCA and CDCA in the intestinal tract. An indirect process may be a more plausible explanation for 7-dehydroxylation of UDCA. This was suggested by the in vitro experiment in this report. Under the cooperation of strains b-52, c-25, and k-5, large quantities of LCA were formed from both UDCA and CDCA, presumably through the reaction sequence as follows : UDCA (by 7β-hydroxysteroid dehydrogenase of b-52 ⇌ 7KL (by 7α-hydroxysteroid dehydrogenase of c-25) ⇌ CDCA (by the 7α-dehydroxylase activity of c-25 enhanced by the *Bacteroides* k-5) → LCA.

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