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Tauroconjugation of Cholic Acid Stimulates 7α-Dehydroxylation by Fecal Bacteria

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We examined the effect of the type of cholic acid conjugation (taurine-conjugated, glycine-conjugated, or unconjugated cholic acid) on cholic acid 7α-dehydroxylation by intestinal flora. Cholic acid 7α-dehydroxylation in fecal cultures, in cultures of a defined limited flora consisting of a mixture of seven bacterial species isolated from the intestinal tract, and in a binary culture of a 7α-dehydroxylating *Clostridium* species plus a cholic acid-deconjugating *Bacteroides* species was studied. We found that tauroconjugation of cholic acid significantly ($P < 0.05$) increased bacterial 7α-dehydroxylation of cholic acid into deoxycholic acid from 34 to 55% in fecal cultures, from 45 to 60% in defined limited fecal cultures, and from 75 to 100% in binary cultures. Equimolar concentrations of free taurine did not stimulate 7α-dehydroxylation in fecal cultures or in the defined limited flora, but free taurine did stimulate 7α-dehydroxylation in the binary culture. In the binary culture of *Clostridium* species strain 9/1 plus *Bacteroides* species strain R1, the minimal flora capable of increased 7α-dehydroxylation of taurocholic acid, strain R1 deconjugated taurine and rapidly reduced it to H_2S. *Bacteroides* species strain R1 did not grow unless taurine or another appropriate reducible sulfur source was present. *Clostridium* species strain 9/1 did not grow or 7α-dehydroxylate unless H_2S or another source of reduced sulfur was present. We conclude that the increased 7α-dehydroxylation of tauroconjugated cholic acid depends on the reduction of taurine to H_2S, which is a necessary growth factor for the 7α-dehydroxylating bacteria.

Primary bile acids, predominantly cholic acid (CA) and chenodeoxycholic acid (CDCA), are excreted into the intestinal tract as glycine or taurine conjugates. The ratio of glyco- to tauroconjugated bile acids can vary substantially (17). Although usually around 3:1, the ratio can be as high as 9:1—for example, in African rural women—and as low as 0.1:1 in taurine-fed subjects (8, 21). Because taurine is the preferential molecule for bile acid conjugation in the liver, diets rich in taurine-containing food such as meat and seafood will increase tauroconjugation (8, 17).

Most bile acids are reabsorbed in the ileum as conjugates. However, during each enterohepatic cycle between 10 and 15% of the bile acids escape into the colon, where they are exhaustively converted by the intestinal flora. It is estimated that the amount of tauroconjugated bile acids that passes daily through the colon can rise more than 10-fold from less than 0.2 μmol with a much larger fraction of glycoconjugated bile acids to more than 2 μmol with almost no glycoconjugated bile acids (8). This is a consequence of the combined effects of increased bile acid production and increased tauroconjugation, as, for example, in people on a high-meat and -fat diet (17). Only 1 to 3% of the bile acids will eventually be excreted in the feces. Fecal bile acids are completely deconjugated and have undergone various other reactions such as dehydroxylation, (de)hydrogenation, and epimerization, etc. (14, 18, 23).

In this study we present data suggesting that taurine stimulates in vitro bacterial 7α-dehydroxylation of CA. Bacterial 7α-dehydroxylation of the primary bile acids CA and CDCA is the key reaction leading to the formation of the secondary bile acids deoxycholic acid (DCA) and lithocholic acid (LCA) (12). If the 7α-dehydroxylation-stimulating effect of taurine is also present in vivo in the intestinal tract, then taurine may be an important mediator in the effect of a Western diet on the bile acid metabolism of the intestinal flora. A Western-type diet, with its high meat and fat content, will increase the intestinal bile acid concentration and, by virtue of its high taurine content, will also increase the percentage of taurine-conjugated bile acids (6, 13). Studies have shown that a Western-type diet will indeed increase the fecal 7α-dehydroxylase activity (9), although the exact mechanism is still a matter of debate.

We studied bacterial bile acid metabolism first in fecal cultures and subsequently in defined mixed cultures composed of several strains of bacteria isolated from feces. Fecal cultures may show substantial variation among individuals and over time for the same individual (22). Also, because of their complexity and the often-changing equilibrium among metabolically interdependent species, fecal cultures are not very stable and are therefore difficult to work with in terms of reproducibility. Limited defined fecal cultures are more stable, and they provide a more accurate picture of actual colon conditions than do pure cultures of fecal bacteria. We finally studied the increased 7α-dehydroxylation of taurocholic acid (TCA) in the minimal flora capable of increased 7α-dehydroxylation of tauroconjugated CA. This minimal flora consisted of two strains, *Bacteroides* sp. strain R1 and *Clostridium* sp. strain 9/1.

MATERIALS AND METHODS

Products used. The following culture broths were used: supplemented tryptic soy broth (sTSB) consisting of tryptic soy broth (Bio-Mérieux, Charbonnières les Bains, France) supplemented with 0.0001% vitamin K (Konakion; Roche, Basel, Switzerland) and buffered with 20 mM $K_2HPO_4 \cdot 3H_2O$ (15) (Merck, Darmstadt, Germany); a medium termed TSB equivalent consisting of 1.7% tryptone (Difco, Detroit, Mich.), 0.3% Phytone (BBL, Cockeysville, Md.), 0.5% NaCl (Merck), 0.1% D-glucose (Merck), and 0.0001% vitamin K and buffered with 20 mM $K_2HPO_4 \cdot 3H_2O$, and the so-called minimal medium broth or MM broth con-

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sisting of 2% Casamino Acids (Difco), 20 mM $K_2HPO_4 \cdot 3H_2O$ (Merck), 0.1% D-glucose, 0.02% tryptophan (Merck), 3 mM NaOH, and 0.0001% vitamin K. Bile acids at a final concentration of 0.4 mM were added to the culture broth before sterilization. The autoclaved culture broth was prereduced in an anaerobic glove box for 24 h prior to use.

The sodium salts of the bile acids CA, glycocholic acid (GCA), TCA, and CDCA were from Calbiochem-Behring (La Jolla, Calif.). DCA, 3-ketocholic acid (3-ketoCA), 7-ketocholic acid (7-ketoCA), 7-ketochenodeoxycholic acid (7-ketoCDCA), 3β-hydroxy-5β-cholanoic acid, and 23-nordeoxycholic acid were from Steraloids (Wiltshire, N.H.). LCA was from Fluka (Buchs SG, Switzerland). Ursocholic acid (UCA) was prepared and purified as described by Samuelsson (19). Taurine was from Fluka, Dithiothreitol was from Janssen Chimica, Geel, Belgium, and riboflavin was from Merck.

Optical density at 650 nm (OD_{650}) of growing cultures was measured on a Bausch & Lomb Spectronic 20 photometer.

Incubation conditions. All experiments were carried out in an anaerobic glove box at 37°C (Anaerobic System; Forma Scientific, Marietta, Ohio) with an atmosphere consisting of 80% N_2, 10% H_2, and 10% CO_2.

Cultures were started by immediately transferring freshly voided human feces to the anaerobic glove box and suspending approximately 0.5 g of feces in 6 ml of sTSB. Subcultures were started every 3 days.

Isolation and identification of pure cultures of anaerobic intestinal bacteria with bile acid-transforming activity. Strains were identified by the method of Holdeman et al. (11) and with the Rapid ID 32 A system of Bio-Mérieux (Marcy-l'Etoile, France).

Bacteria whose growth was stimulated by taurine were isolated from sTSB agar plates containing 15 mM taurine and 0.05% iron-ammonium-citrate. H_2S-producing black-pigmented colonies were picked and subcultured. Two bacterial species with 7β-hydrogenating activity (*Eubacterium contortum* 3b7b and strain 60/1) were isolated on Columbia agar plates (Merck) supplemented with 8 or 15 mM taurine, 0.01% hemin, 0.0001% vitamin K, and 0.4 mM CA. The other strains, including the strains with CA-7α-dehydroxylating activity, were isolated from agar plates containing 5% horse blood. The fecal suspensions from which these bacteria were isolated had been subcultured on TCA containing sTSB.

Analytical procedures. (i) Analysis of bile acid transformations. Two milliliters of a three-day-old culture was twice extracted with 6 ml of diethyl ether after addition of 100 μg of 23-nordeoxycholic acid as an internal standard and acidification to pH 2 with 1 ml of 2N HCl. The ether extracts were collected, evaporated, and derivatized to methyl esters with diazomethane. Gas chromatography was performed on a 7 ft (ca. 2 m) 3% OV-1 column at 260°C and a 3% OV-17 column at 250°C after trimethylsilylation of the methylated bile acids with Tri-Sil (Pierce, Rockford, Ill.). The retention times of the silylated bile acids were compared with those of reference products and with the relative retention times described by Elliot et al. (4).

(ii) Analysis of sulfite levels. Sulfite levels were determined colorimetrically with the acid fuchsine-formaldehyde reagent (1). To 2 ml of culture medium, 10 ml of fuchsine reagent (40 mg of basic fuchsine in 100 ml of 2.4 M H_2SO_4) and 70 ml of distilled water were added.

After 10 min, 1 ml of 10 M Formol and water to a total volume of 100 ml were added. OD_{570} was measured after 10 min. Negative controls were uninoculated culture broth. Taurine did not interfere with sulfite measurements.

Analysis of microbial taurine metabolism. A flask containing 150 ml of sTSB equivalent was inoculated with 15 ml of a 24-h culture of strain R1 on sTSB. After addition of 8 mM [^{35}S]taurine (7.3 μCi/mmol; Amersham International, Amersham, United Kingdom), the flask was incubated in a 37°C water bath and flushed with 10% H_2–90% N_2 (15 to 25 ml/min). The effluent gas mixture was conducted through a cold trap retaining the methane- and ethanethiol and subsequently through a series of three consecutive wash bottles that contained 1 N NaOH to bind H_2S. Samples of the culture and the wash fluid were taken at regular intervals. At the end of the experiment, the culture was acidified with 6 N H_2SO_4 to free any remaining volatile fatty acids. After addition of 8 ml of scintillation fluid (Lumagel; Lumac Systems Inc., Titusville, Fla.), the samples were counted twice for 5 min each time in a Packard Tri-Carb Scintillation counter (Packard, Warrenville, Ill.).

Statistical analysis of DCA formation by fecal cultures in culture broth supplemented with differently conjugated forms of CA. A repeated-measurement model was fitted to the difference of DCA formation by fecal cultures in TCA-supplemented versus CA-supplemented sTSB. The program BMDP5V of the statistical package BMDP (3) was chosen to accommodate for the missing data. Only subcultures 2, 3, 4, 9, and 12 could be considered, since the incorporation of all subcultures resulted in a failure to estimate the parameters. This program assumes a Gaussian structure of the data at each subculture; an unstructured covariance matrix provided the best estimates. The P values are based on an asymptotic z test. The choice of this program was dictated by the nature and number of data available. Because DCA formation in any subculture was to some extent dependent on the DCA formation in the preceding culture and because not all subcultures were analyzed, no statistical procedure other than the one we used was powerful enough to calculate the statistical significance of our experiments.

RESULTS

Effect of type of conjugation on CA metabolism in complete fecal cultures. (i) Primary fecal cultures. Approximately 0.5-g amounts of freshly voided human feces, from four different sources (subjects 1, 2, 3, and 4), were incubated in 6 ml of sTSB with either 0.4 mM TCA, 0.4 mM GCA, or 0.4 mM nonconjugated CA supplemented with equimolar concentrations of free taurine or glycine or not supplemented. After 3 days of incubation, the bile acids were extracted and analyzed. No significant differences in the metabolization of differently conjugated forms of CA by fecal cultures from the same source were found, but significant differences ($P < 0.05$) in the respective amounts of CA metabolites for fecal cultures from different sources did exist. For two of our sources, we repeatedly ($n = 5$) found—irrespective of the type of conjugation—that CA was primarily 7α-dehydroxylated into DCA (DCA levels as a fraction of total CA were 61.8% ± 6.2% and 77.7% ± 4.4%, respectively, for subjects 1 and 2). Fecal cultures from the two other sources—again irrespective of the type of CA conjugation—exhibited significantly lower levels ($P < 0.01$) of 7α-dehydroxylation (DCA levels were 35.2% ± 3.6% of total bile acids for subjects 3 and 4). Formation of 3-ketoCA was, however, significantly increased ($P < 0.01$) in fecal cultures from subjects 3 and 4 (31.3% ± 3%) in comparison with those from subjects 1 and 2 (7.6% ± 10.8%). Minor amounts (<10%) of UCA, 7-ketoCA, and free CA were found in cultures from all four sources.

These metabolic profiles resemble those reported for the 7α-dehydroxylation of CDCA by Hirano et al. (10) and Setoguchi et al. (20).

(ii) Subcultures of primary fecal cultures. Primary fecal cultures were repeatedly subcultured in sTSB that always contained the same CA conjugate. After incubation, bile acids were extracted and analyzed. We found that repeated subculturing induced changes in the metabolization pattern until more or less stable metabolization patterns were reached from the fourth subculture onwards.

Fecal cultures that were subcultured in sTSB supplemented with tauroconjugated CA transformed on average more CA into DCA (55% ± 21%) than did fecal cultures subcultured in sTSB supplemented with free CA with or without equimolar concentrations of taurine or glycine (34% ± 22%) or fecal cultures subcultured in sTSB supplemented with glycoconjugated CA (35% ± 21%) (Fig. 1).

The difference in DCA formation between TCA-supplemented and GCA-supplemented sTSB cultures as a function of the sequence number of the subculture could be expressed by the following equation, which showed a reasonable fit: DCA formation from TCA − DCA formation from GCA = $-4.54 + 12.425 \cdot \ln$(subculture sequence no.). The standard error of the estimate and the corresponding P value are 0.147 and $P < 0.0001$, respectively, for the first parameter and 0.392 and $P < 0.0001$, respectively, for the second, indicating that the differences in DCA formation between TCA- and GCA-supplemented broths were statistically significant.

Identical results were found when DCA formation in TCA-supplemented sTSB was compared with DCA formation in sTSB supplemented with GCA or free CA with or without taurine or glycine.

Effect of type of conjugation on CA 7α-dehydroxylation in cultures of a limited, defined mixed intestinal flora. To increase the reproducibility of our experiments and analyze the effect of changes in the dehydroxylation level on the composition of the intestinal flora, a limited mixed flora was reconstituted with pure cultures of the following bacteria that had been

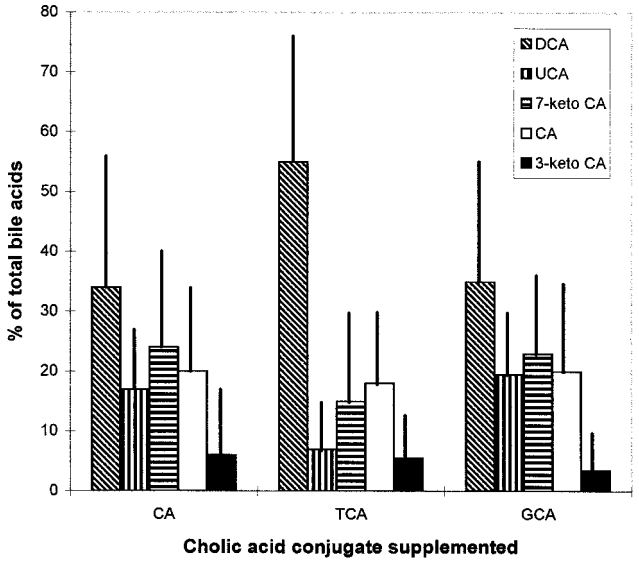

FIG. 1. Levels of CA metabolites (means ± standard deviations [error bars], $n = 37$) in 3-day-old cultures of complete fecal flora subcultured in sTSB with either 0.4 mM free CA with or without 0.4 mM taurine or glycine (CA), 0.4 mM TCA, or 0.4 mM GCA.

isolated from fecal material. Two *Clostridium* species (termed 9/1 and 10b) that both dehydroxylated CA, reduced 7-ketoCA (30 to 65% after 3 days), deconjugated GCA but not TCA, and did not metabolize UCA or CDCA were used. Two strains with bile acid-7β-hydrogenating activity were used. The first strain, identified as *E. contortum* 3b7b, possessed CA-, UCA-, CDCA-, ursochenodeoxyocholic acid (UCDCA)-, and LCA-3α-epimerizing activity in addition to 7β-hydrogenating 7-ketoCA and 7-ketoCDCA. No bile acid-deconjugating activity was observed. The second strain, termed 60/1, was a gram-negative unidentified bacillus that 7β-hydrogenated 7-ketoCA and 7-ketoCDCA and deconjugated TCA, GCA, taurochenodeoxycholic acid (TCDCA), and glycochenodeoxycholic acid (GCDCA). Also used were a *Fusobacterium* species strain termed R14 with 7-hydroxysteroid-dehydrogenating and bile acid-deconjugating activity, a strain termed 60/5 with an *Actinomyces*-like morphology that possessed TCA- and GCA-deconjugating activity but was not stimulated by taurine, and a taurine-stimulated nonfermenting *Bacteroides* species strain termed R1 that deconjugated tauroconjugated bile acids and desulfated estrone sulfate and resembled an intestinal steroid-desulfating *Bacteroides* strain described earlier (26).

After 3 days of incubation of this limited defined flora in a sTSB supplemented with differently conjugated forms of CA, we found a significantly higher level of DCA formation ($P < 0.05$) in 0.4 mM TCA-supplemented sTSB than in 0.4 mM CA- or 0.4 mM GCA-supplemented sTSB (Table 1). Substituting the taurine-stimulated *Bacteroides* strain R1 with its rapid deconjugating activity (100% deconjugation after 4 h in pure cultures) with another taurine-stimulated strain which deconjugated 100% of the TCA after 24 h (*Peptococcus niger* H4 [see reference 25]) led to a reduction of DCA formation from 60 to 26% ($P < 0.01$). Eliminating *Bacteroides* strain R1 from the mixed culture led to rapid TCA deconjugation (100% deconjugation after 5 h) by the unidentified strain termed 60/5; strain 60/5 was, however, not stimulated by taurine, and the 7α-dehydroxylating activity was reduced from 60 to 30% ($P < 0.01$).

TABLE 1. CA metabolites in 3-day-old cultures of defined limited fecal flora subcultured in sTSB supplemented with free CA with or without taurine or glycine, TCA, or GCA

sTSB supplement	Amt of indicated metabolite (mean ± SD, $n = 37$) as % of total CA added			
	DCA	UCA	7-ketoCA	CA
0.4 mM CA with or without 0.4 mM taurine or glycine	45 ± 15	46 ± 12	2 ± 1	7 ± 5
0.4 mM TCA	60 ± 14	32 ± 13	2 ± 4	6 ± 9
0.4 mM GCA	44 ± 14	47 ± 9	2 ± 3	7 ± 5

Quantitation of the species that constituted the limited fecal flora in cultures that had been repeatedly subcultured in sTSB supplemented with TCA versus sTSB supplemented with GCA or free CA with or without taurine or glycine revealed that all the species that composed the limited defined flora were still present and that there were no significant differences in their respective numbers in the differently supplemented sTSB cultures.

Effect of type of conjugation on CA 7α-dehydroxylation in mixed cultures of *Bacteroides* sp. strain R1 and *Clostridium* sp. strain 9/1. The minimal fecal flora capable of increased 7α-dehydroxylation of TCA consisted of two strains, *Bacteroides* sp. strain R1 and *Clostridium* sp. strain 9/1. *Clostridium* sp. strain 9/1 deconjugated GCA and 7α-dehydroxylated free CA but could not deconjugate or dehydroxylate TCA. *Bacteroides* sp. strain R1 deconjugated TCA but showed no other metabolic activity towards TCA, GCA, or CA. In TSB equivalent supplemented with 0.4 mM bile acid, TCA was 100% dehydroxylated to DCA by 9/1 plus R1, free CA was 75% dehydroxylated ($P < 0.01$), and GCA was only 50% dehydroxylated ($P < 0.01$) (Fig. 2).

The time course of formation of DCA from TCA revealed that deconjugation of TCA was 100% after 5 h and 7α-dehy-

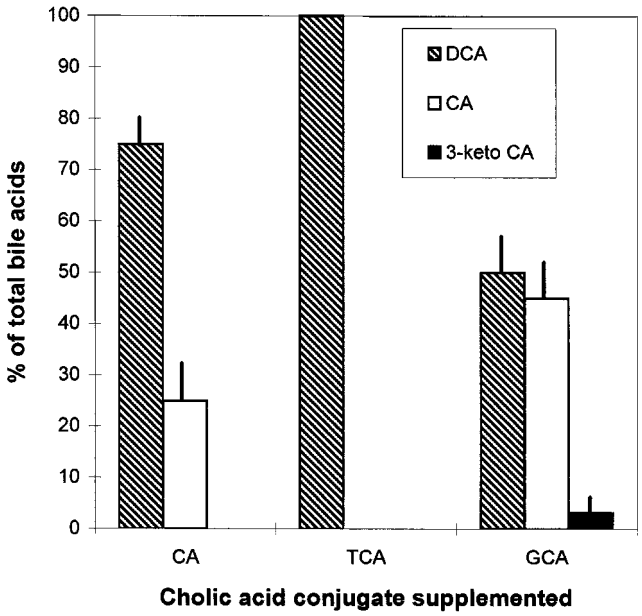

FIG. 2. Levels of CA metabolites (means ± standard deviations [error bars], $n = 37$) in 3-day-old mixed cultures of *Bacteroides* sp. strain R1 plus *Clostridium* sp. strain 9/1 in TSB equivalent broth supplemented with either 0.4 mM free CA with or without 0.4 mM taurine or glycine (CA), 0.4 mM TCA, or 0.4 mM GCA.

TABLE 2. CA metabolites in 3-day-old binary cultures of *Bacteroides* sp. strain R1 plus *Clostridium* sp. strain 9/1 in MM

MM supplement	Amt of indicated metabolite (mean ± SD, $n = 5$) as % of total CA added		
	DCA	7-ketoCA	CA
0.4 mM TCA	27 ± 9	61 ± 9	12 ± 8
0.4 mM TCA + 0.1% yeast extract	95 ± 3	3 ± 12	2 ± 9
0.4 mM TCA + 0.4 mM taurine	30 ± 8	50 ± 5	20 ± 7
0.4 mM CA	9 ± 2	50 ± 4	41 ± 4
0.4 mM CA + 0.1% yeast extract	30 ± 9	20 ± 7	50 ± 7
0.4 mM CA + 0.4 mM taurine	25 ± 6	51 ± 6	24 ± 5
0.4 mM CA + 0.4 mM taurine + 0.1% yeast extract	95 ± 5	3 ± 7	2 ± 4

droxylation started only after complete deconjugation of TCA. During the exponential growth phase of the R1-plus-9/1 culture, we found a transiently high concentration of 7-ketoCA (maximal concentration, 50% of the total amount of bile acids added after 18 h of incubation) that had completely disappeared after 30 h of incubation.

Effect of yeast extract and taurine on 7α-dehydroxylation of free and conjugated CA in mixed cultures of 9/1 plus R1. Addition of 0.4 mM free taurine to the semidefined TSB equivalent broth improved 7α-dehydroxylation of CA significantly from 75% ± 5% to 95% ± 4% ($P < 0.05$). Addition of 0.1% yeast extract also improved 7α-dehydroxylation of CA to 95% ± 5%. Higher concentrations of taurine or yeast extract did not further improve 7α-dehydroxylation. Addition of yeast extract or free taurine had no significant effect on formation of DCA from GCA.

To examine more closely the effect of taurine and yeast extract on 7α-dehydroxylation of free or conjugated CA, we switched to a defined MM broth that contained no vitamins and no more than 0.1% sugars. In MM broth, TCA was only 27% dehydroxylated and CA was dehydroxylated only 9% ($P < 0.01$) (Table 2); GCA was 2% dehydroxylated by 9/1 plus R1 ($P < 0.01$). Addition of 0.1% yeast extract, however, significantly increased 7α-dehydroxylation of TCA to 95% and of CA and GCA to 30% ($P < 0.05$). Addition of 0.4 mM taurine to the MM did not affect 7α-dehydroxylation of TCA but significantly increased 7α-dehydroxylation of CA to 25% ($P < 0.05$). 7α-Dehydroxylation of GCA was not affected by the addition of 0.4 mM taurine. Addition of 0.1% yeast extract plus 0.4 mM taurine significantly increased 7α-dehydroxylation of CA to 95% and of GCA to 30% ($P < 0.05$).

We subsequently examined what components of yeast extract were responsible for the 7α-dehydroxylation-stimulating effect. Of all the compounds tested (glucose, ribose, galactose, and fructose [each 15 mM]; coenzyme A [CoA] [2.5 μM]; pantothenic acid, riboflavin, thiamine, pyridoxine, folic acid, nicotine amide, and *p*-aminobenzoic acid [each 0.3 μM]; choline [1 mM]; and flavin mononucleotide [20 mM]), only riboflavin, folic acid, and pantothenic acid (0.3 μM each) stimulated 7α-dehydroxylation of TCA from 27% to 60, 60, and 70%, respectively, and, if all three compounds were added together, to 95% ($P < 0.01$) (Fig. 3).

If neither riboflavin nor pantothenic acid nor folic acid was added to the MM broth culture of 9/1 plus R1, there was high-level formation of 7-ketoCA from TCA, from CA, and from CA plus taurine (Fig. 3). However, if riboflavin, pantothenic acid, and folic acid were added together to the MM, there was a reduction of 7-ketoCA formation and increased formation of DCA from CA, CA plus taurine, and TCA. The

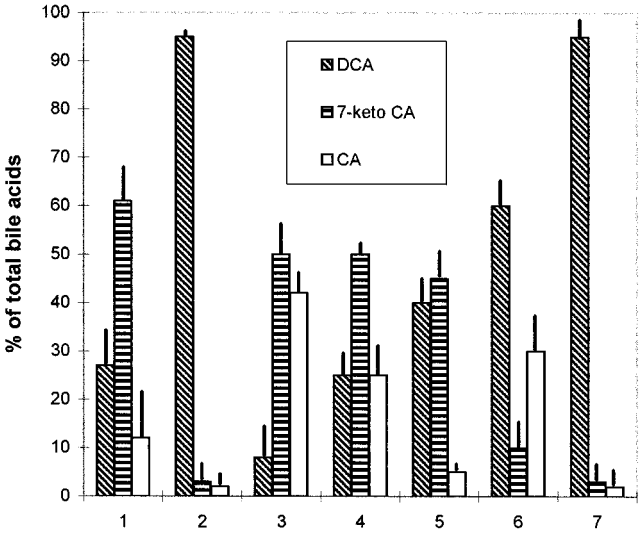

FIG. 3. Levels of CA metabolites (means ± standard deviations [error bars], $n = 4$) in 3-day-old mixed cultures of *Bacteroides* sp. strain R1 plus *Clostridium* sp. strain 9/1 in MM supplemented with 0.4 mM TCA (bar set 1), 0.4 mM TCA plus riboflavin, folic acid, and pantothenate (each 0.3 μM) (set 2), 0.4 mM CA (set 3), 0.4 mM CA plus 0.4 mM taurine (set 4), 0.4 mM CA plus 0.4 mM taurine plus 0.3 μM riboflavin plus 0.3 μM folic acid (set 5), 0.4 mM CA plus 0.4 mM taurine plus 0.3 μM pantothenic acid (set 6), or 0.4 mM CA plus 0.4 mM taurine plus 0.3 μM pantothenic acid plus 0.3 μM riboflavin plus 0.3 μM folic acid (set 7).

reduced formation of 7-ketoCA was clearly linked to the pantothenic acid and not to the addition of riboflavin or folic acid. Increasing concentrations of pantothenic acid in MM broth plus 0.4 mM CA reduced the formation of 7-ketoCA from 52% in the absence of pantothenic acid to less than 10% in the presence of 3.5 μM pantothenic acid.

The 7α-dehydroxylation-stimulating effect of taurine could also be obtained by supplementing the culture medium with the following sulfur-containing compounds at 0.4 mM: cysteine, cystine, cysteamine, homocysteine, homocystine, thioglycolate, thiosulfate, sulfite, and Na_2S. Sulfate (0.4 mM) did not stimulate 7α-dehydroxylation.

Effect of riboflavin, pantothenic acid, folic acid, and taurine on the growth and metabolic activity of strain 9/1 and strain R1 in the presence of free or conjugated CA. There was no growth of R1 in the MM broth if no suitable sulfur-containing compound was added. Sulfur-containing compounds that supported growth of R1 in the MM broth were taurine, sulfite, and thiosulfate. Addition of 0.1 mM taurine led to an OD_{650} of 0.3 with a maximal OD_{650} of 0.9 at a taurine concentration of 0.6 mM. Sulfate, cysteine, cystine, cysteamine, homocysteine, homocystine, thioglycolate, Na_2S, or methionine did not stimulate growth. All the appropriate sulfur sources were within 4 to 6 h of inoculation of the R1 culture completely reduced to H_2S. During the reduction of taurine to H_2S by R1, a transient accumulation of sulfite (maximal concentration = 0.05 mM) was observed. Incubation of R1 with [^{35}S]taurine showed that more than 99.9% of the labeled sulfur was recovered as H_2S during the exponential growth phase of R1. No other volatile sulfur-containing metabolites produced by R1 from taurine could be detected. No remaining label was detected in the R1 cells at the end of the exponential growth phase.

Growth of 9/1 in the MM was very scarce ($OD_{650} < 0.02$) unless a suitable sulfur source and 0.1% yeast extract were added. MM broth supplemented with 0.4 mM cysteine plus 0.1% yeast extract led to an OD_{650} of 0.27. Yeast extract could

also be substituted by 0.3 μM riboflavin plus 0.3 μM pantothenic acid plus 0.3 μM folic acid. We found that if 9/1 was grown in the presence of R1, the concentration of pantothenic acid needed for optimal growth could be lowered from 0.3 to 0.015 μM. The 7α-dehydroxylation activity of R1 plus 9/1 in MM was, however, reduced at 0.015 compared with 0.3 μM pantothenic acid (42% DCA from CA plus 0.4 mM taurine plus 0.015 μM pantothenic acid versus 60% DCA for 0.3 μM pantothenic acid).

Sulfur-containing compounds other than taurine that also stimulated growth of 9/1 in the MM were cysteine, cystine, cysteamine, homocysteine, homocystine, thioglycolate, and Na_2S. Sulfate was not an appropriate sulfur source, nor was sulfite or thiosulfate. Sulfite inhibited growth of 9/1 at concentrations above 0.1 mM.

Metabolic interaction between 9/1 and R1. When in the binary culture of 9/1 plus R1, R1 was substituted with *P. niger* H4 (a strain that like R1 reduces free taurine to H_2S but unlike R1 deconjugates TCA slowly, i.e., 100% deconjugation after 24 h versus 5 h for R1), we found no increased DCA production from TCA in comparison with production from CA or GCA in the TSB equivalent broth. *Bacteroides* strain 60/5 deconjugated TCA rapidly like R1 but did not reduce taurine to H_2S. We found that the combination of 60/5 plus 9/1 did not 7α-dehydroxylate CA or TCA unless cysteine was added. Addition of 0.4 mM cysteine to the combination of 60/5 plus 9/1 in the TSB equivalent broth led to a 7α-dehydroxylation of CA of only 40%, significantly different from the 75% for the R1-plus-9/1 culture ($P < 0.05$).

DISCUSSION

Our results are consistent with a speculative hypothesis that one of the factors regulating the intestinal 7α-dehydroxylation of CA may be the diet-dependent level of tauroconjugation of CA. Increased tauroconjugation of bile could possibly be one of the mechanisms causing the rapid changes in fecal 7α-dehydroxylase activity observed to occur in subjects changing from a low-meat and -fat diet to one high in meat and fat (7). However, the relative effect of increased bile acid synthesis versus increased tauroconjugation of bile acids on the level of 7α-dehydroxylase activity remains to be established.

The large variations in DCA formation among subcultures derived from different primary fecal cultures and among different subcultures from the same primary fecal culture that we observed reflect the complexity of the fecal microflora and the unstable equilibrium in complex mixed floras. In cultures of the limited flora, the variations in DCA formation were much smaller and there was little or no evolution in DCA formation between the first and the last subculture, as opposed to the situation in the complete fecal cultures, where there was a clear evolution in DCA formation over the course of subsequent subcultures, probably due to changing growth equilibria among different strains.

We observed that in complete fecal cultures or limited defined fecal cultures, free CA plus taurine had no effect on 7α-dehydroxylation and only tauroconjugated CA stimulated DCA formation. This is consistent with the fact that in the colon, where the intestinal flora is most abundant, taurine is primarily bile acid-conjugated taurine because dietary taurine is reabsorbed in the small intestine. The exact mechanism whereby only bile acid-conjugated taurine induced enhanced 7α-dehydroxylation in fecal and limited fecal cultures is not completely clear. Our study of the interaction between *Bacteroides* strain R1 and *Clostridium* strain 9/1 showed that in the 9/1-plus-R1 cultures both free taurine and bile acid-conjugated taurine stimulated 7α-dehydroxylation. It may be that in fecal cultures there is intense competition for free taurine. In previous studies (26) we observed that taurine is a growth factor for several strictly anaerobic fecal bacteria. Conjugation of taurine to bile acids might limit access to taurine for most species and favor a close spatial and metabolic relationship between the bile acid-7α-dehydroxylating strain 9/1 and the bile acid-deconjugating strain R1.

The mechanism whereby tauroconjugation or free taurine in binary cultures of R1 and 9/1 leads to increased 7α-dehydroxylation of CA is clearly linked to the metabolic dependency of 9/1 on R1. The complete reduction by R1 of taurine and other oxidized forms of sulfur to H_2S and the observation that Na_2S or cysteine does not support growth of R1 suggest that the sulfur in taurine is not used for growth. The reduction of taurine to H_2S might therefore serve as an electron disposal mechanism. A similar need for taurine as a growth-stimulating factor that is completely reduced to H_2S has been demonstrated for other intestinal bacteria (24, 26). The growth stimulation of the dehydroxylating strain 9/1 by cysteine and Na_2S and the absence of a growth-stimulating effect of taurine or other oxidized forms of sulfur suggest that 9/1 needs the reduced sulfur as a growth factor.

Apart from the dependency of 9/1 on the reduced sulfur produced by R1, there exists also an intricate synchronization between the deconjugating activity of R1 and the dehydroxylating activity of 9/1. TCA is very rapidly deconjugated by R1 with a concomitant rapid release of H_2S due to the reduction of the liberated taurine. The H_2S will induce growth of 9/1 and the deconjugated CA will induce dehydroxylation activity in 9/1, but only if CA is present in a high enough concentration during the exponential growth phase of 9/1. Addition of free CA during the stationary growth phase or the substitution of R1 with an H_2S-producing strain that deconjugated TCA slowly did not lead to increased 7α-dehydroxylation of the deconjugated TCA by 9/1.

Although pantothenic acid, riboflavin, and folic acid were essential for dehydroxylation, they played no role in the increased dehydroxylation of CA observed with tauroconjugation. The observed stimulation of dehydroxylation activity in 9/1 by pantothenate and riboflavin is, however, consistent with data on the mechanism of 7α-dehydroxylation in other intestinal bacteria. Increased 7α-dehydroxylation with addition of pantothenic acid could be due to the conversion of CA to its CoA ester, which was proposed to be the initial step in the 7α-dehydroxylation in *Eubacterium* sp. strain VPI 12708 (16). However, contrary to what was reported for *Eubacterium* sp. strain VPI 12708 (2), we did not find evidence for the presence of a CA nucleotide conjugate (unpublished results) which is thought to be an intermediate in the bile acid-CoA ligation reaction. Nor did we find a stimulatory effect of CoA on the CA dehydroxylation reaction. The pantothenic acid-like factor secreted by R1 did stimulate growth of 9/1 but differed from exogenously added pantothenic acid in that it did not stimulate dehydroxylation in 9/1 as much. The stimulatory effect of riboflavin on the 7-dehydroxylation of CA, on the other hand, suggests the presence of a flavin-oxidoreductase which provides reduced flavins for the reductive steps in the reaction (5). The reason for the stimulatory effect of folic acid remains speculative.

In conclusion, we have shown that tauroconjugation of CA leads to increased DCA formation in cultures of intestinal flora. Tauroconjugation affects 7α-dehydroxylation of CA because there exists a metabolic interaction between the TCA-deconjugating strain R1 on the one hand and the CA-7α-dehydroxylating strain 9/1 on the other hand. This metabolic

interaction depends essentially on the sulfonic acid part of taurine, which is rapidly reduced by the deconjugating strain and, after reduction, serves as a source of sulfur for anabolic pathways in the dehydroxylating strain.

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