ISOLATED FECAL MICROORGANISMS CAPABLE OF 7 α-DEHYDROXYLATING BILE ACIDS *,‡

By BENGT E. GUSTAFSSON, M.D, TORE MIDTVEDT, M.D., AND ARNE NORMAN, M.D.

(From the Department of Germfree Research, Karolinska Institutet, Stockholm, and the Department of Clinical Chemistry, Danderyds Sjukhus, Danderyd, Sweden)

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Bile acids are transformed by microorganisms in the intestinal tract into a variety of metabolites (1–3). To some extent these metabolites are absorbed and further transformed by liver enzymes prior to excretion in the bile. Thus feces contain a complicated mixture of metabolites of the primary bile acids, cholic, and chenodeoxycholic acids. The secondary reactions include splitting of conjugates, elimination of the hydroxyl groups at C-7, and oxidation of hydroxyl groups at C-3, C-7, and C-12 to keto groups which can be reduced to both α- and β-hydroxyl groups. Besides these main reactions, others have been described in animals, such as the formation of allo compounds (4) and unsaturated derivatives (5). However, these reactions seem to be of minor quantitative importance.

The aims of the current investigations on the role of the intestinal flora in bile acid metabolism, are to isolate the microorganisms responsible for bile acid conversions and to study their in vivo activity in monoinfected exgermfree rats. This report describes in vitro alterations of chenodeoxycholate[1] by anaerobic cultures of intestinal microogranisms from rat and man and the isolation of single strains capable of 7α-dehydroxylation.

The main transformations of chenodeoxycholate in vivo are known and some of the compounds shown in Fig. 1 might be formed by intestinal microorganisms in cultures originally containing chenodeoxycholate. As shown in Table I, only seven out of the fourteen metabolites have been isolated from animals so far; the other seven metabolites may be formed in small amounts only or as short lived intermediates.

Materials and Methods

The 24-C^{14}-labeled free and conjugated bile acids were synthetized and purified as described earlier (17, 18). Sources and references to synthesis of unlabeled bile acids are given in Table I.

* Bile Acids and Steroids No. 165.

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[1] Names, numbers, and abbreviations of bile acids used in this manuscript refer to Table I.

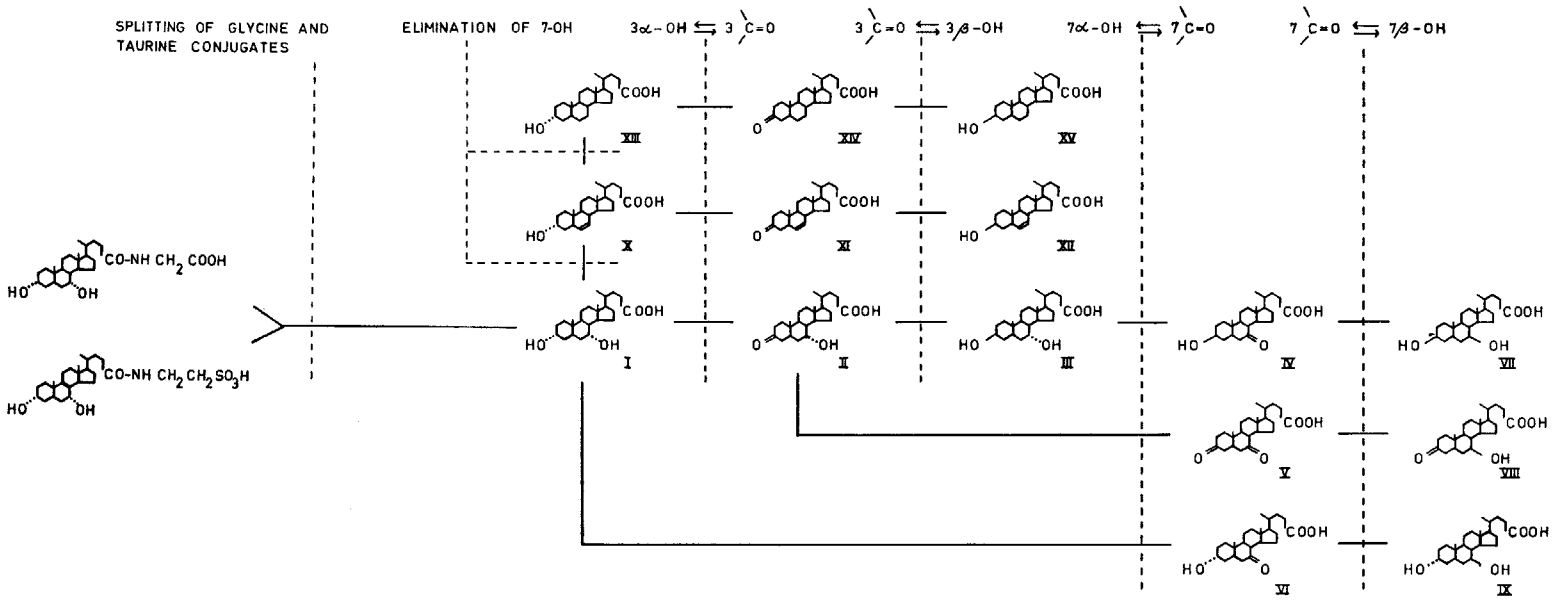

FIG. 1. The main potential metabolic transformations of chenodeoxycholic acid during its enterohepatic circulation. The formulas of identified and potential metabolites include those which may be transitory intermediates or present only in trace amounts. The scheme does not imply a specific pathway for the formation of the different metabolites in vivo.

TABLE I

Chemical Names and Previous Isolations of Chenodeoxycholate Metabolites

No.	Acid	Abbreviation	Reference to method of synthesis	Isolation of metabolites of chenodeoxycholate	
				Animals	Microorganisms
I	3α,7α-Dihydroxy-5β-cholanoic acid (chenodeoxycholic acid)	3α-OH, 7α-OH	*		
II	3-Keto-7α-hydroxy-5β-cholanoic acid	3-Keto, 7α-OH	(6)‡	Man (3)	
III	3β,7α-Dihydroxy-5β-cholanoic acid	3β-OH, 7α-OH	(6)‡	Man (3)	
IV	3β-Hydroxy-7-keto-5β-cholanoic acid	3β-OH, 7-keto			
V	3,7-Diketo-5β-cholanoic acid	3,7-diketo	(7)		*Alcaligenes faecalis* (8)
VI	3α-Hydroxy-7-keto-5β-cholanoic acid	3α-OH, 7-keto	(9)	Man (3, 10)	*E. coli* (7)
VII	3β,7β-Dihydroxy-5β-cholanoic acid	3β-OH, 7β-OH			
VIII	3-Keto-7β-hydroxy-5β-cholanoic acid	3-keto, 7β-OH			
IX	3α,7β-Dihydroxy-5β-cholanoic acid (ursodeoxycholic acid)	3α-OH, 7β-OH	*	Man (11)	
X	3α-Hydroxy-5β-chol-6-anoic acid	3α-OH, Δ^6	(12)§		
XI	3-Keto-5β-chol-6-anoic acid	3-keto, Δ^6			
XII	3β-Hydroxy-5β-chol-6-anoic acid	3β-OH, Δ^6			
XIII	3α-Hydroxy-5β-cholanoic acid (lithocholic acid)	3α-OH	*	Man (13) Rat (15)	Mixed anaerobic culture of fecal microorganisms (14)
XIV	3-Keto-5β-cholanoic acid	3-keto	(5)	Man (5)	*E. freundii* (16) (from lithocholic acid)
XV	3β-Hydroxy-5β-cholanoic acid	3β-OH	(5)	Man (13, 5)	*E. freundii* (16) (from lithocholic acid

Source of bile acid: *Steraloids, Ltd., Croydon, Surrey, England.

‡ Dr. H. Danielsson, Dr. P. Eneroth, Dr. J. Sjövall.

§ Dr. B. Samuelsson.

Bacteriological Procedures.—

Culture media: In preliminary experiments it was found that the microorganisms capable of 7 α-dehydroxylation were easily subcultured anaerobically in Oxoid Todd-Hewitt broth (TH broth). The medium was sterilized at 115°C for 10 min according to the manufacturer's description, and unless otherwise mentioned, this medium was used in all the experiments. In order to isolate single colonies, slants of Todd-Hewitt medium with 1.5 % agar added were used. The isolated strains were studied by conventional biochemical methods.

Sterilization of bile acids: Labeled bile acids as sodium salts dissolved in 80 % ethanol were added to thick wall centrifuge tubes (inside diameter 13 mm, height 100 mm). After evaporation, the centrifuge tubes were sterilized by autoclaving at 120°C for 20 min. No destruction of the bile acids was observed after this treatment. In order to get a homogeneous bile salt concentration, the tubes were carefully shaken after adding 4.5 ml of sterile broth.

Inoculation: When testing for bile acid transformation, 0.5 ml of a culture incubated anaerobically for 72 hr was transferred to 4.5 ml of TH broth containing the bile acid to be studied. The same dilution (1:10) was also used when inoculating the control tubes without bile acid. If not freshly made, all the media were heated in boiling water and rapidly cooled to 37°C immediately prior to the inoculation.

Incubation: The incubation took place anaerobically at 37°C for 72 hr. For anaerobic conditions the excess cotton of the tube plugs was burned off, the plugs carefully pushed a little into the tubes, and soaked with 4 to 6 drops of 10 % $NaHCO_3$ and 6 to 8 drops of 40 % pyrogallic acid. The tubes were immediately sealed with rubber stoppers.

Chemical Procedures.—

Extraction methods: All subcultures were centrifuged for 1 hr at 25,000 *g* and the supernatant decanted. The sediment was washed with $\frac{1}{15}$ M phosphate buffer, pH 7.4, and centrifuged as above. The washed sediment was extracted by refluxing in acetone. The supernatant and phosphate washing were combined, acidified with hydrochloric acid, and the labeled bile acids extracted with ether. The residue after the acetone extraction was dissolved in water, acidified, and the labeled bile acids extracted with ether. The ether extracts were evaporated, dissolved in acetone, and aliquots taken for chromatographic analysis or isotope determinations.

Chromatographic technique: The chromatographic phase systems used have been listed in Table II. Columns for reversed phase partition chromatography consisted of a 4 ml stationary phase supported on 4.5 g hydrophobic Supercel. Two ml fractions were collected and titrated with 0.02 N NaOH in methanol.

Alumina chromatography of the bile acid methyl esters was carried out with Woelm aluminum oxide grade III, eluting with increasing concentrations of benzene in hexane or ethyl acetate in benzene (19).

Thin-layer plates were prepared using a suspension of 58 ml distilled water and 30 g Kieselgel G (20) or of 58 ml distilled water, 30 g Kieselgel G, and 7 g silver nitrate (21) .The chromatoplates were activated in an oven at 110°C for 1 hr before use. Spots were visualized by spraying with concentrated sulfuric acid and charring at 250°C.

Fractionation of microbial transformation products of chenodeoxycholic acid: Preliminary fractionation of labeled metabolites of chenodeoxycholic acid was obtained with reversed phase partition chromatography on columns. Chromatography with phase systems F1 and F2 separated the metabolites into five groups (e.g. Fig. 2): Group A, 3,7-dihydroxycholanoic acids; group B, 3-hydroxy-7-ketocholanoic acids; group C, 3-keto-7-hydroxycholanoic acids; group D, 3-hydroxycholanoic acids and 3,7-diketocholanoic acid; and group E (retained in the stationary phase of F2), 3-ketocholanoic acid.

Since reversed phase partition chromatography does not give a good separation of α- and

β-isomers, alumina chromatography was used for further separation of each group. The labeled metabolites isolated in this way were identified by thin-layer cochromatography with appropriate standards in different systems. Phase systems S12 and S15 gave separations which could be predicted from "polarity" considerations. Phase system S10 gave good separation of 7α- and 7β-hydroxy isomers and caused derivatives with ketogroups to be relatively more adsorbed than the corresponding derivatives with hydroxyl groups. Silver nitrate–containing plates gave good separations between hydroxycholanoic acids and their corresponding Δ^6-unsaturated derivatives, the latter being retarded.

Isotope determinations: The isotope content in fluid samples was determined by plating aliquots and counting with a Frieseke-Hoepfner methane gas-flow counter. After thin-layer chromatography (TLC), radioactive spots were located by autoradiography (5). For quantitation, the optical density of the film at 550 mμ was measured (22, 5).

TABLE II

Chromatographic Systems

Phase system	Moving phase	Milliliters	Stationary phase	Milliliters
F1 (23)	Methanol:water	165:135	Chloroform:heptane	45:5
F2 (23)	Methanol:water	180:120	Chloroform:heptane	45:5
S10 (20)	Trimethylpentane:isopropyl alcohol:acetic acid	60:20:0.5		
S12 (20)	Trimethyl pentane:ethyl acetate:acetic acid	5:25:0.2		
S15 (20)	Trimethylpentane:ethyl acetate:acetic acid	20:20:0.2		
I (24)	*n*-Butanol:water:acetic acid	50:5:5		

RESULTS

Isolation of Single Strains—For the isolation of single strains of bacteria capable of 7α-dehydroxylation of chenodeoxycholic acid the following procedures were used (see also Fig. 3).

1. Fresh feces were suspended 1:10 in TH broth and incubated anaerobically for 72 hr at 37°C.

2. Anaerobic incubation of subculture 1 in TH broth at 37°C three times for 72 hr each. The ability of this subculture to form lithocholic acid was checked as described, by inoculating 0.5 ml in 4.5 ml TH broth containing C^{14}-labeled chenodeoxycholic acid and incubating for 72 hr at 37°C.

3. Anaerobic incubation at 37°C for 120 hr.

4. Heating of subculture 3 under strict anaerobic conditions at 70°C for 20 min.

5. Inoculation of the heated material in step 4 in TH broth containing 40 % ox bile sterilized by filtration through a Millipore type GS filter. Anaerobic incubation for 72 hr at 37°C.

6. Incubation of subculture 5 in TH broth for 72 hr at 37°C. Check of lithocholic acid formation.

7. Subculture 6 was spread on TH slants and incubated anaerobically for 120 hr at 37°C. Colonies with a slightly greyish color and smooth and glistening

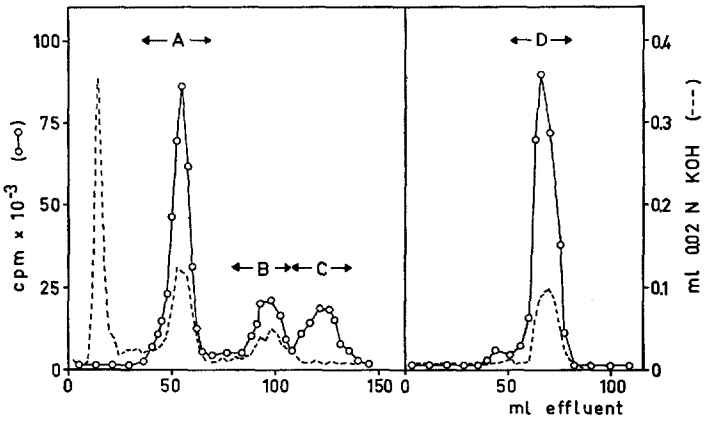

FIG. 2. Chenodeoxycholate metabolites produced by strain II. Group column chromatographic separation of labeled metabolites formed in broth cultures originally containing 100 μM chenodeoxycholate-24-C^{14}. Left curve, phase system F1. Right curve, phase system F2. Rechromatography of labeled products retained in the column after the chromatography shown in the left part. Titrations of reference substances (3 α-OH, 7 α-OH) (3 α-OH, 7-keto) and (3 α-OH) (-----) refer to left-hand ordinate; isotope determinations (O-O-O) refer to right-hand ordinate.

surface were inoculated in TH broth for 72 hr at 37°C. Check of ability of colonies to form lithocholic acid.

8. The lithocholic acid–producing strains were labeled and cultured in TH broth and on TH agar slants consecutively several times to ensure that the strains were pure.

With the procedures described four strains, labeled I, II, III, and V were isolated from one sample of rat feces (25). Three strains, labeled VI, VII, and VIII were isolated from another rat. One strain (K. D.) was isolated from a sample of feces from a male, 27 years of age, from outside the institute. He had no history of intestinal disease and had not received any antibacterial therapy during the past year.

The subcultures isolated in step 2 were labeled fecal subculture A and the subcultures isolated in step 6 were labeled fecal subculture B. Fecal subculture A

has been subcultured for more than 100 times with the 7α-dehydroxylating power intact. Microscopic examination revealed that among these subcultures there was a gradually increasing prevalence of Gram-positive cocci and Gram-positive rods. The gram-negative rods later disappeared and no growth of these bacteria could be found in the culture when it was incubated anaerobically on blood agar plates.

Preliminary investigations of the heat resistance had demonstrated that the microorganisms capable of 7α-dehydroxylation survived the heating of a fecal sample to 80°C for 5 min. and the more specific isolation procedures consisted of exposing the cultures to heat and bile.

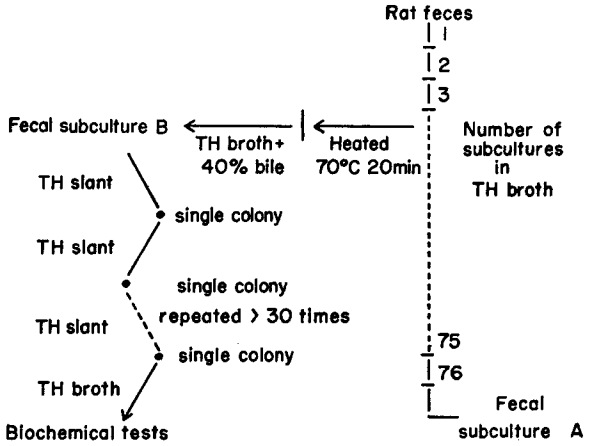

FIG. 3. Isolation of single bacterial strains capable of 7α-dehydroxylation.

Microscopic examination of the subcultures after the heating and growth on bile-containing medium (fecal subculture B) demonstrated Gram-positive rods of various lengths and sizes. No growth was found when the culture was incubated aerobically. The conversion of chenodeoxycholic acid to lithcholic acid was found to be of the same magnitude in fecal subculture B as in fecal subculture A, thus indicating that the microorganisms with this ability had survived the heating and exposure to bile.

Before using the single strains isolated in step 8 of the isolation procedures in further experiments, they were subcultured at least three times on TH agar slants and each time the new inoculum was taken from a single colony. The strains have been repeatedly cultivated on TH agar slants and in TH broth, and at the present time more than thirty such transfers have been made without any changes in the characteristics of the strains. By random choice, strain II of the isolated strains was selected for the further chemical identification of the bile acid metabolites.

Characterization of the Single Strains.—All eight strains appeared to be mainly identical. They were all strictly anerobic, non-motile, moderately thermoresistant Gram-positive rods. On microscopic examination young cultures (24 to 48 hr) showed slender, Gram-positive rods with a length of about 3 to 8μ, occurring singly or in pairs and as short chains with an angled appearance. In older cultures many of the rods became Gram-negative or granulated and seemed to be partly autolyzed. After 48 hr or longer of incubation all the strains produced a greyish white sediment which was slightly adherent to the bottom of the tubes. The strains also grew well in Difco brain-liver-heart medium and Difco thioglycollate medium, but the sediments formed in those media were not found to be adherent to the bottom of the tubes.

The strains were cultivated on TH agar slants, and minute and transparent colonies could be seen after 36 to 42 hr of incubation. After 4 to 5 days of incubation the diameter of the colonies was 3 to 4 mm, the colonies had a slightly greyish color, and showed no signs of pseudopodial growth. Except for strain K. D., the surfaces of the colonies were smooth and glistening. Strain K. D. showed intermediate to rough colonies. The microscopic appearance of the bacteria from a surface colony was the same as that of the bacteria grown in TH broth except for a tendency toward parallel grouping. The colonies grown on brain-liver-heart and thioglycollate agar slants showed the same morphological characteristics as those on TH agar slants. The growth on thioglycollate was, however, somewhat slower. On microscopic examination no apparent difference was observed among the bacteria grown on the three different media. The strains have been cultivated in various media including those specially developed to induce spore formation. Terminal swellings of the bacterial bodies resembling spores could be demonstrated (Fig. 4 *a*). These swellings, however, were Gram-positive and did not take spore stains. More seldom, central thickenings of the bacterial bodies were found. These thickenings were seen especially after incubation for some days in thioglycollate medium (Fig. 4 *b*). In older colonies the parts of the bacteria on each side of the thickenings were Gram-variable and poorly stained. When these older colonies were reincubated for a short time in fresh media the swellings disappeared.

Fecal samples could be heated to 80°C for 5 min without the loss of the 7α-dehydroxylating microorganisms, but the three strains, II, VII, and K. D., did not survive the same heating after having been cultivated in TH broth for 72 hr. Cultivated in the same way they survived 70°C for 20 min.

The strains, II, VII, and K. D., were used in fermentation studies. Using sugar-free thioglycollate medium as a base the following carbohydrates (1 % w/v) were fermented with the production of acid and gas: xylose, glucose, fructose, and galactose. The final pH of the medium in the case of the fermentable carbohydrates was 5.30–5.60 after 72 hr of incubation at 37°C. Strains II and VII also fermented arabinose and sucrose, whereas strain K. D. fermented

maltose as well. Rhamnose, mannose, cellobiose, lactose, melibiose, trehalose, raffinose, melezitose, inulin, adenitol, inositol, salicin, and esculin were not fermented. In addition, sorbitol and mannitol were slowly fermented by all three strains tested; the final pH was found to be about 6.00–6.20 after the inoculation. Strain K. D. showed the same slight fermentation of dulcitol also; strains II and VII did not ferment this substance.

All the strains produced H_2S in Difco motility sulfide medium. Indole and skatole were not formed, nor was gelatin liquefied. Nitrate was not reduced to nitrite. The strains were catalase- and oxidase-negative and they did not

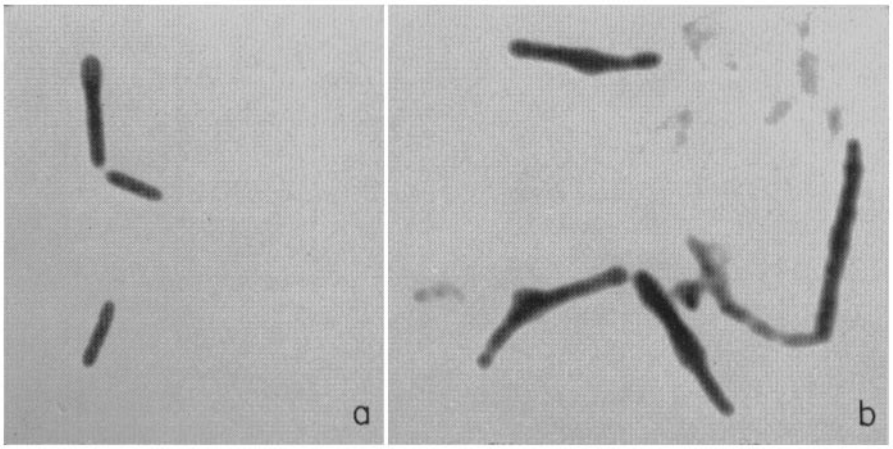

FIG. 4. Cultures showing terminal swelling (*a*) and central thickening (*b*) of the bacteria. Gram stain. × 4800.

produce acetylmethylcarbinol. They were not able to grown in Koser's citrate medium and they grew on Løffler serum medium without liquefaction of the medium. No coagulation occurred when the strains were cultivated in litmus milk medium and Crossley milk medium. The strains were found to be apathogenic for conventional mice and could be injected intraperitoneally without causing any reactions in the test animals.

Metabolites of Chenodeoxycholate Formed by Strain II.—Fig. 2 shows the group chromatographic separation with phase systems F1 and F2 of the labeled metabolites extracted from broth cultures of strain II originally containing 100 μM of labeled chenodeoxycholate. The isolated groups *A* to *E* all contained labeled compounds. Alumina chromatography did not result in further separation. TLC of the labeled compounds in different groups (Fig. 5) revealed the following compounds: *A*, unchanged chenodeoxycholic acid; *B*, (3α-OH, 7-keto); *C*, (3-keto, 7α-OH); *D*, (3α-OH); and *E*, (3-keto). In some experiments,

group *D* contained a compound with the mobility of (3,7-diketo) and group *E* a compound more hydrophobic than (3-keto). Thus strain II contains enzymes catalyzing the removal of the 7α-hydroxyl group and the oxidation of the hydroxyl group at C-3 and C-7 to keto groups.

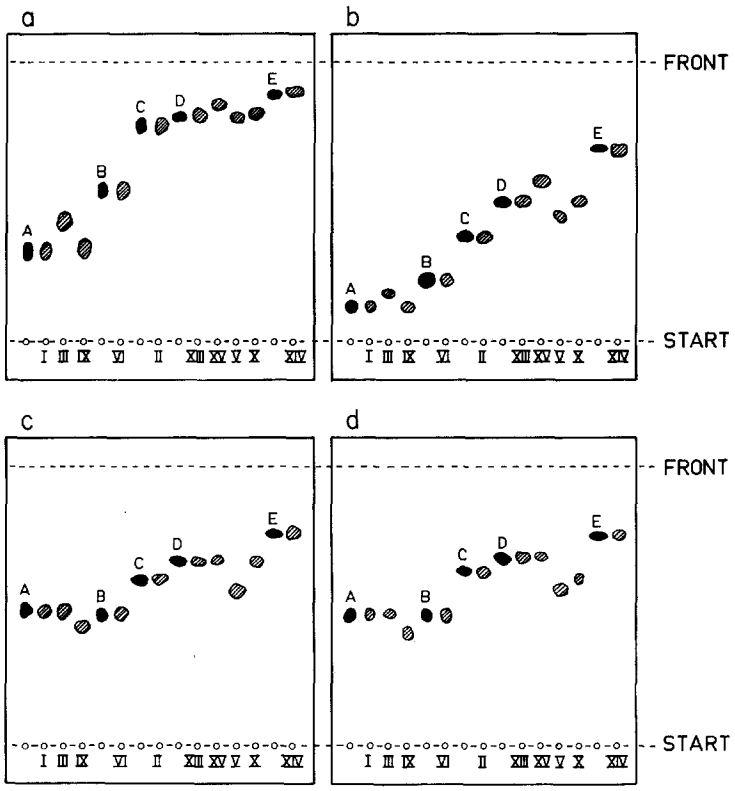

FIG. 5. Chenodeoxycholate metabolites formed by strain II. Thin-layer chromatography of groups A to E (see Fig. 2) and standards. Chromatoplate *a*, *b*, *c*-Kieselgel G; *d*-Kieselgel G containing silver nitrate. Phase systems: *a*-S12, *b*-S15, *c*, *d*-S10. Reference standards, numbers refer to Table I. The darkened areas represent radioactive metabolites detected by autoradiography and the cross-hatching represents standards detected by spraying with sulfuric acid.

Metabolites of Chenodeoxycholate Formed by Fecal Subculture B.—Fractionation was performed as described above. The same metabolites found in cultures of strain II were separated, i.e. four major compounds with the TLC mobilities of (3α-OH, 7-keto), (3-keto, 7α-OH), (3α-OH), and (3-keto) and two minor compounds, one with the TLC mobility of (3,7-diketo) and one more hydrophobic than (3-keto).

Metabolites of Chenodeoxycholate Formed by Fecal Subculture A.—Labeled metabolites formed in broth cultures of fecal subculture A originally containing 100 μM labeled chenodeoxycholate were found in groups *A* to *E*. Labeled compounds in groups *B* and *C* showed the TLC behaviors of (3α-OH, 7-keto) and (3-keto, 7α-OH), respectively. Group *E* contained two labeled compounds, one with a mobility of (3-keto) and one moving slightly faster than (3-keto). Alumina chromatography of labeled compounds in group *A* showed two labeled compounds, *A*1 and *A*2 (Fig. 6). *A*1 and *A*2 had the TLC mobilities of unchanged chenodeoxycholate and (3β-OH, 7α-OH), respectively (Fig. 7). Alumina chromatography of labeled compounds in group *D* (Fig. 8) showed two

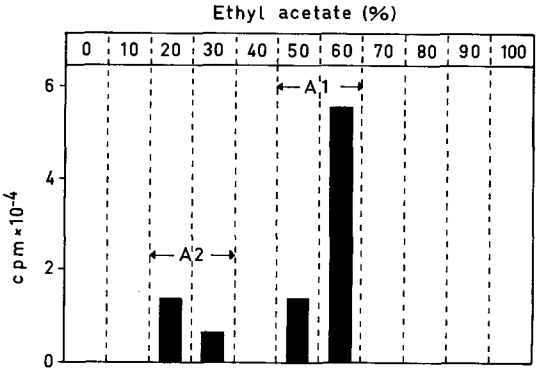

FIG. 6. Labeled metabolites from fecal subculture A. Alumina chromatography of labeled compounds in group A.

compounds, *D*1 and *D*2, with the TLC mobilities of (3α-OH) and (3β-OH), respectively (Fig. 7). In addition to enzymes performing the reactions observed with strain II and fecal subculture B, fecal subculture A contained enzymes capable of reducing the keto group at C-3 to a 3β-hydroxyl group.

Effect of Substrate Concentration on Formation of Chenodeoxycholate metabolites.—Five initial concentrations of chenodeoxycholate were used with fecal subcultures A and B strain II. Metabolites of labeled chenodeoxycholate were separated by TLC, using phase systems S12 and S15, and located by autoradiography. The per cent distribution of labeled products was calculated from densiometric determinations of the film. The results are shown in Table III. In broth cultures originally containing 10 μM chenodeoxycholate, most of the isolated metabolites lacked hydroxyl or keto groups at C-7. In cultures originally containing 100 μM or more of chenodeoxycholate, only a minor fraction of the isolated metabolites lacked substituents at C-7. The major fraction consisted of monoketomonohydroxy derivatives. At higher concentrations, the amount of (3α-OH) formed was fairly constant. The ability to remove

the 7-hydroxyl group was about the same in fecal subcultures A and B, and in cultures of strain II. In contrast, fecal subculture A was able to reduce the 3-keto group to the 3β-hydroxyl group while fecal subculture B and strain II

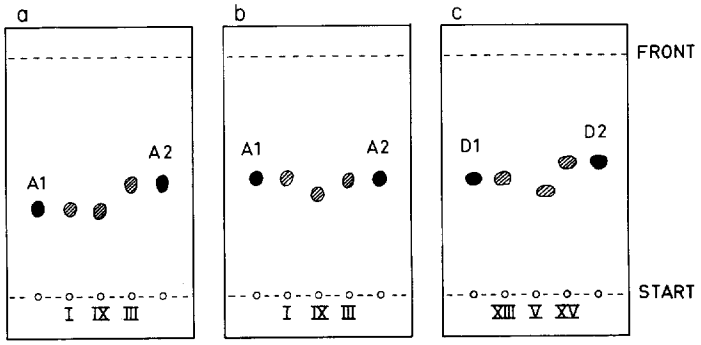

FIG. 7. Labeled metabolites form fecal subculture A. Thin-layer chromatography on Kieselgel G of fractions A1 and A2 (Fig. 5), D1 and D2 (Fig. 6), and standards. For explanation see Fig. 4. Phase systems, *a*-S12, *b*-S10, *c*-S15.

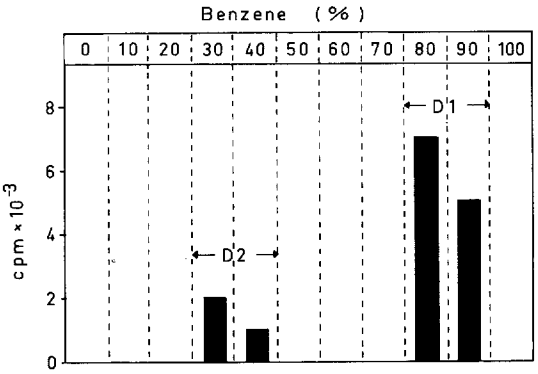

FIG. 8. Labeled metabolites from fecal subculture A. Alumina chromatography of labeled compounds in group D.

could not. Fecal subculture A contained (3β-OH, 7α-OH) and (3β-OH) at all substrate concentrations; however, these cultures contained low amounts of (3-keto, 7α-OH). Fecal subculture A also showed a much greater ability to oxidize the hydroxyl group at C-7 than did fecal subculture B or strain II.

Transformation of Chenodeoxycholate by Single Strains.—Seven out of the eight isolated single strains of bacteria with the ability to transform chenodeoxycholate to lithocholic acid, were tested for this ability after 3 days of incubation in TH broth. The original broth contained either 10 μM or 100 μM labeled

TABLE III

Concentration of Chenodeoxycholate Metabolites in 72 Hr Broth Cultures of Strain II and Fecal Subcultures A and B

Broth inoculated with	Original concentration of chenodeoxycholate in broth	Unchanged chenodeoxycholate after incubation with	Metabolites with th TLC mobilities of*						
			3β-OH, 7α-OH	3α-OH, 7-keto	3-keto, 7α-OH	3,7-diketo	3α-OH	3β-OH	3-keto
	μM	μM	μM	μM	μM	μM	μM	μM	μM
Fecal subculture A	10	0.1	Trace	0	0	0	4.5	2.5	2.9
		0.1	Trace	0.3	0	0	4.8	2.6	2.2
	100	33	9	36	1	Trace	12	5	4
		23	8	31	2	0	19	7	10
	200	70	18	80	0	0	18	10	4
		70	18	84	0	0	14	6	8
	400	144	40	168	0	0	24	12	12
		140	36	164	0	0	28	12	20
	600	282	24	228	0	0	36	18	12
		248	36	276	0	0	24	12	4
Fecal subculture B	10	0.8	0	0.2	0.1	0	8.8	0	0.1
		0.4	0	0.1	0.1	0	9.3	0	0.1
	100	67	0	12	5	Trace	15	0	7
		64	0	9	6	Trace	20	0	7
	200	148	0	22	10	0	20	0	Trace
		140	0	30	6	0	24	0	Trace
	400	308	0	44	16	0	32	0	0
		300	0	60	12	0	28	0	0
	600	510	0	56	12	0	30	0	0
		528	0	24	12	0	36	0	0
Strain II	10	0.7	0	0.4	0	0	7.9	0	1.0
		0.5	0	0.4	0	0	8.1	0	1.0
	100	49	0	18	12	2	14	0	5
		43	0	25	13	1	15	0	3
	200	126	0	40	18	Trace	14	0	2
		128	0	36	18	Trace	16	0	2
	400	304	0	52	24	0	20	0	Trace
		276	0	84	20	0	20	0	Trace
	600	462	0	84	36	0	18	0	0
		486	0	72	24	0	18	0	0

* Cf abbreviations in Table I.

chenodeoxycholate. Labeled metabolites were analyzed as described above. The results are shown in Table IV. In cultures originally containing 10 μM of chenodeoxycholate, most of the chenodeoxycholate was converted into (3α-OH) and (3-keto); i.e., had lost its substituents at C-7. All strains grown in broth originally containing 100 μM of chenodeoxycholate were shown to be

TABLE IV

Transformation of Chenodeoxycholate by Single Strains Capable of 7α-dehydroxylation

Strain source	Strain No.	Original chenodeoxycholate concentration	Unchanged chenodeoxycholate remaining in broth after incubation	Per cent of metabolites with the TLC mobilities of						
				3β-OH, 7α-OH	3α-OH, 7-keto	3-keto, 7α-OH	3,7-diketo	3α-OH	3β-OH	3-keto
		μM	%							
Rat	II	10	17	0	1	0	0	80	0	2
		100	51	0	5	10	Trace	29	0	5
Rat	III	10	20	0	9	2	Trace	63	0	6
		100	41	0	10	12	Trace	29	0	8
Rat	V	10	19	0	4	1	0	73	0	3
		100	61	0	8	8	0	21	0	2
Rat	VI	10	3	0	1	1	Trace	94	0	1
		100	62	0	16	7	Trace	14	0	1
Rat	VII	10	3	0	1	0	0	90	0	6
		100	52	0	32	8	0	7	0	1
Rat	VIII	10	3	0	3	0	0	89	0	5
		100	74	0	9	8	0	9	0	0
Human	K.D.	10	21	0	3	4	0	69	0	3
		100	82	0	1	4	0	14	0	0

The percentages given are the means of two experiments.

able to oxidize the hydroxyl group at C-3 or C-7. These reactions led to the formation of large amounts of monoketomonohydroxy derivatives, but in none of the experiments were significant amounts of the diketo derivatives isolated. Furthermore none of the strains was able to reduce the keto groups of C-3 and C-7 to the corresponding β-hydroxy derivatives, as (3β-OH, 7α-OH) and (3α-OH, 7β-OH) were not isolated.

The ability of strain II to transform chenodeoxycholate was tested repeatedly during a period of 6 months. It was found that the amounts of metabolites and

the ratio between the different metabolites varied greatly from experiment to experiment. The results in Table IV, therefore, indicate the types of reactions that different strains can perform, but do not permit conclusions regarding the relative quantitative importance or interrelations of these reactions.

Location of 7-dehydroxylated Metabolites.—In these experiments, incubations were done in broth containing 10 μM of chenodeoxycholate. After 72 hr, the cultures were centrifuged, and the isotope extracted both from the supernatant broth and the sedimented microorganisms. The distribution of the isotope is summarized in Table V. Most of the metabolites had to be extracted from the sediment with acetone in the case of fecal subculture A, but were present in the

TABLE V

Distribution of Chenodeoxycholate-24-C^{14} Metabolites between Supernatant and Sediment (Acetone-Extractable)

Broth containing 10 μM of chenodeoxycholate inoculated with	No. of experiments	Distribution of isotope in			
		Supernatant and buffer washings		Acetone extract of sediment	
		Mean	Range	Mean	Range
		%	%	%	%
Fecal subculture A	20	42	14–55	58	45–86
Fecal subculture B	10	90	82–94	10	6–18
Strain II	10	97	95–99	3	1–5

supernatant with fecal subculture B or strain II. TLC of the labeled metabolites showed that the elimination of the 7-hydroxyl group of chenodeoxycholate had been practically complete in all these experiments.

Hydrolysis of Conjugated Bile Acids.—TH broth containing 10 μ and 500 μM of 24-C^{14}-labeled glycocholate or taurocholate was inoculated with fecal subculture A, B, or strain II. After 72 hr, the broth was analyzed for unconjugated bile acids by TLC using phase system I. Complete hydrolysis occurred with fecal subculture A, whereas no hydrolysis of either taurocholate or glycocholate occurred with fecal subculture B or strain II.

DISCUSSION

Isolation and Classification of Single Strains Capable of 7α-Dehydroxylation

The isolated seven strains do not conform to any of the species described in Bergey's Manual (26) or by Prévot (27), and with the data available at present, it is impossible to give an exact classification. On the basis of staining characteristics, anaerobic condition of growth, elevated thermoresistance, other physiological characteristics, and especially the demonstration of sporelike

structures, the isolated strains most nearly resemble the members of the genus *Clostridium*. But, as recently summarized by Brzin (28) it is well known that a large degree of pleomorphism with bacterial forms resembling those described in our strains might be found in other genera of Gram-positive rods. The pleomorphism is considered to be a response of the bacteria to unfavorable environmental factors. In our strains the unusual forms especially occurred when they were cultivated in thioglycollate medium. Therefore, if the sporelike structures found in our strains are not true spores, but only pleomorphic forms of the bacteria, other species such as those in the tribe *Lactobacilleae*, genus *Corynebacterium*, and genus *Actinomyces* also have to be considered for the final classification.

Portman et al. (29) have isolated a pure culture of a microorganism from rat feces capable of performing the conversion of cholate to deoxycholate. Unfortunately their microorganism lost its ability after three transfers and they were not able to repeat their isolation. They described their strain as a Gram-negative rod, which was able to grow aerobically and to ferment lactose and utilize citrate. Our strains are strictly anaerobic Gram-positive rods and they do not ferment citrate or lactose. Thus, their strain differs in so many essential characteristics from our strains that it can not have been the same as ours.

More recently, an elimination of the 7α-hydroxyl group in cholic acid has been described in cultures of an aerobic soil microorganism, *Corynebacterium simplex* (30) (*Arthrobacter simplex* according to Bergey's Manual). An extensive transformation of cholic acid was also demonstrated. However, the types of metabolites formed were different from those formed in cultures of our strains. From a bacteriological point of view, the complete failure of growth under aerobic conditions, the fermentation of carbohydrates to both acid and gas, and the absence of catalase in our strains indicate that they are quite different from this soil microorganism.

Transformation of Bile Acids by Microorganisms.—In the present investigation the metabolism of chenodeoxycholate by isolated intestinal microorganisms has been described. Preliminary results with cholic acid indicate that this compound is also 7α-dehydroxylated, but both cholic acid and the resultant deoxycholic acid are further transformed by the subcultures and the isolated strains into a complex mixture of metabolites. Studies on the transformation of cholic acid are going on.

The predominant microbial transformation of cholic and deoxycholic acid in vivo involves the elimination of the 7α-hydroxyl group. The reaction mechanisms involved in the formation of deoxycholic acid in vivo have been studied by Samuelsson (12) using cholic acid stereospecifically labeled with tritium. It was established that the elimination of the hydroxyl group at C-7 starts with a diaxial transelimination of water (6β-H, 7α-OH) yielding a

Δ^6-unsaturated compound which is later hydrated. It is reasonable to assume that 7α-dehydroxylation proceeds also in the isolated intestinal microorganisms by the mechanism described above. Preliminary results indicate that a similar 7α-dehydroxylation of cholic acid also occurs with these strains. As no unsaturated derivatives of chenodeoxycholate were observed, its intermediate formation cannot be excluded as it could easily escape by rapid reduction. The rapid reduction of Δ^6-compounds is suggested by the fact that these compounds have never been isolated from animals.

It has been shown previously (14) that lithocholic acid formed from chenodeoxycholic acid in trypticase soy broth cultures of mixed anaerobic intestinal microorganisms, was only recoverable by acetone extraction of the sediment after centrifugation of the broth. The results with fecal subculture A were in agreement with these findings. However, lithocholic acid formed in single strain was almost quantitatively recovered from the supernatant broth. This indicates that lithocholic acid formed in fecal subculture A is taken up or adsorbed onto microorganisms, either living or dead, other than those responsible for its formation.

Conjugated bile acids are almost entirely split by microbial enzymes prior to their excretion in feces (31). The hydrolysis of taurine and glycine conjugates can be performed in vitro by several species of clostridia and enterococci (32). The microorganisms capable of splitting conjugates in fecal subculture A did not survive the treatment with 70°C for 20 min and treatment with 40 % bile. In agreement with this fecal subculture B and the strains capable of 7α-dehydroxylation could not split the conjugates.

During the enterohepatic circulation of bile acids, there is interconversion between compounds containing 3- and 7-keto groups and their corresponding α- and β-hydroxyl derivatives. A dehydrogenase has been isolated from *Escherichia freundii* (16) which catalyzed both dehydrogenation of the 3α-hydroxyl group, and reduction of the keto group yielding both α- and β-hydroxyl groups. Several strains of *Escherichia coli* (7) perform the reversible dehydrogenation of the 7α-hydroxyl group, but in this case reduction of the 7-keto group apparently yields only the 7α-isomers (12). The reduction in vivo of the 7-keto group to the 7β-hydroxyl group may not be due to microorganisms since liver enzymes can catalyze this reaction (33, 34).

In this investigation, the single strains capable of 7α-dehydroxylation could all oxidize the hydroxyl groups at C-3 and C-7. The two monoketomonohydroxy derivatives were isolated, but only trace amounts of a possible diketo derivative were found. In vivo, the amount of diketo derivatives in rat and man is very small (3, 35). It is not known whether the dehydrogenation of the 3α- and 7-α hydroxyl group occurring in TH broth cultures of strain II is reversible or not, but no derivatives with 3β- or 7β-hydroxyl groups were isolated. However, organisms in fecal subculture A were able to form derivatives with 3β-hydroxyl

groups. Isolation of single strains from fecal subculture A has yielded several different strains with the ability to oxidize the 7α-hydroxyl group, but so far no strains have been found capable of forming derivatives with a 3-keto group or a 3β-hydroxyl group.

The metabolism of bile acids by aerobic soil microorganisms such as *Streptomyces* (36), *Nocardia* (37), *Proactinomyces* (38), and *Corynebacterium* (30) has been described. In addition to some of the reactions shown in Fig. 1, these soil microorganisms can also form α-, β-unsaturated keto groups and norcholanic acids. It is not known whether these microorganisms can grow in animal intestinal contents, and if so, whether they are able to produce these metabolites in vivo. Formation of norcholanic acids in the present in vitro experiments was unlikely since no significant loss of isotope was observed.

SUMMARY

Strains of microorganisms capable of 7α-dehydroxylation of chenodeoxycholate were isolated from rat and human feces. All the strains were strictly anaerobic, non-motile, moderately thermoresistant Gram-positive rods. They showed some saccharolytic properties with the production of both acid and gas. They were H_2S-positive but indole-, skatole-, citrate-, catalase-, and oxidase-negative.

The isolated strains capable of 7α-dehydroxylation of chenodeoxycholate were also able to oxidize the hydroxyl groups at C-3 and C-7 to keto groups. The following metabolites were isolated: 3-keto-7α-hydroxy-5β-cholanoic acid, 3α-hydroxy-7-keto-5β-cholanoic acid, 3α-hydroxy-5β-cholanoic acid, and 3-keto-5β-cholanoic acid. The isolated strains did not have the enzymes necessary for hydrolyzing conjugated bile acids.

In mixed anaerobic cultures of fecal microorganisms, extensive reduction of the 3-keto group to the 3β-hydroxyl group occurred. The microorganism(s) responsible for this reaction have as yet not been isolated.

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