APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1989, p. 2954–2959
0099-2240/89/112954-06$02.00/0

Vol. 55, No. 11

Formation of Δ^2- and Δ^3-Cholenoic Acids from Bile Acid 3-Sulfates by a Human Intestinal *Fusobacterium* Strain

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Received 31 May 1989/Accepted 24 August 1989

We isolated two strains of an unnamed *Fusobacterium* species from human intestinal microflora, which stereospecifically transformed bile acid 3-sulfates into C-3-unsubstituted, ring A-unsaturated bile acids. Both 3α- and 3β-sulfates of 5β-bile acids were metabolized to Δ^3-5β-cholenoic acids; 3β-sulfates of 5α-bile acids were converted into a mixture of Δ^2-5α-bile acids and 3α-hydroxy-5α-bile acids, whereas 3α-sulfates of 5α-bile acids were left intact. Unsulfated bile acids were not transformed into unsaturated derivatives. These strains differ from previously isolated intestinal bacteria, which desulfated bile acid sulfates without further transformation.

It is known that bile acids, especially lithocholic acid, are partially sulfated by the human liver prior to excretion in the bile. In the large intestine these sulfoconjugates are converted by the intestinal microflora into less polar and more lipid-soluble compounds (6, 18) which are more readily reabsorbed. As a result, the intestinal microflora enhances the enterohepatic recirculation of sulfated bile acids and reduces their fecal excretion. Using gnotobiotic rats associated with specific bile acid-desulfating microorganisms, we have shown that microbial desulfation causes a substantial delay in the elimination of toxic lithocholic acid from the body (8, 21). Several intestinal steroid-desulfating microorganisms have been isolated and characterized. However, only three of them are active towards a narrow range of bile acid sulfates (10, 22, 26). These strains liberate the bile acids from their sulfate esters without transforming the bile acid nucleus.

We now report on the isolation of a new type of bile acid-desulfating bacterium which transforms 3-sulfated bile acids into compounds identified as ring A-unsaturated bile acids lacking the 3-hydroxyl group. The substrate specificity of this desulfating activity is described and possible reaction pathways are discussed.

MATERIALS AND METHODS

Bacteriology. Brucella broth and Columbia broth were purchased from GIBCO Bio-Cult Ltd. (Paisley, United Kingdom). Brain heart infusion broth was from BBL Microbiology Systems (Cockeysville, Md.). The peptone-yeast medium described by Holdeman et al. (9) was prepared with Bio-Tripcase (bioMérieux, Brussels, Belgium) and yeast extract (Difco, Detroit, Mich.). All media were enriched with 0.0005% hemin (Sigma Chemical Co., Dorset, United Kingdom) and 0.0001% vitamin K (Konakion; Roche, Basel, Switzerland) and reduced with 0.05% L-cysteine hydrochloride. Agar plates were prepared by adding 2% agar (Difco) to the liquid medium. Cultures were incubated in an anaerobic glove box at 37°C under 80% N_2–10% H_2–10% CO_2. Growth was measured by determination of the optical density of the cultures with a Bausch and Lomb Spectronic 20 spectrophotometer at 520-nm wavelength and 10-mm path length. Identification of bacterial strains was carried out according to Holdeman et al. (9). Bile acid transformations were studied in cultures in test tubes (5 ml of medium) or Erlenmeyer flasks (100 ml of medium) containing 20 to 100 μM bile acids.

Isolation procedure. Samples of fresh stools from a single healthy male individual on a Western diet were immediately transferred into the anaerobic chamber. Samples of approximately 0.5 g of fecal material were suspended in 5 ml of prereduced medium. From these suspensions, serial 10-fold dilutions were made and streaked onto agar plates. After 2 to 6 days of incubation, single colonies and mixtures of different colony types were subcultured in 5 ml of medium containing 100 μM of chenodeoxycholic acid 3-sulfate to check for bile acid-desulfating activity.

Bile acid analysis. Unless stated otherwise, the bile acids and steroid sulfates were obtained from Steraloids (Wilton, N.H.). Taurine and glycine conjugates of lithocholic, chenodeoxycholic, deoxycholic, and cholic acid were purchased from Maybridge (Launceston, United Kingdom). The preparation of allobile acids and of bile acid sulfates was described previously (20, 22). A mixture of Δ^2- and Δ^3-cholenoic acids was prepared by application of the alkaline solvolysis procedure (22) to bile acid 3-sulfates, a treatment known to result in the formation of these two isomers (19). For that purpose a few milligrams of a bile acid sulfate was dissolved in 1 ml of 20% (wt/vol) KOH in ethylene glycol and heated at 210°C for 20 min. After cooling and addition of 1 ml of water, the solution was heated at 120°C for 15 min to hydrolyze possible bile acid glycol esters. The reaction mixture was diluted with 5 ml of a solution of 10% KH_2PO_4 and 10% NaCl in water, followed by 1 ml of 6 N HCL. The bile acids were then extracted with diethyl ether and methylated with ethereal diazomethane. On gas-liquid chromatography (GLC) of their methyl esters or methyl ester acetates, the cholenoic acid isomers formed a double peak on a 3% OV-1 column (100-120 GasChrom Q) at 268°C and were clearly separated on a capillary Ultra-2 column (Packard, Warrenville, Ill.) under a programmed temperature gradient from 87 to 240°C at 30°C/min and from 240 to 270°C at 2°C/min. The Δ^2- and Δ^3-isomers were separated as their methyl esters by preparative thin-layer chromatography on $AgNO_3$-impregnated silica gel with benzene-acetone (85:15, vol/vol) (2). In this way, GLC-pure Δ^2- and Δ^3-cholenoic acid methyl esters were obtained from the 3-sulfates of lithocholic (5β-H), allolithocholic (5α-H), deoxycholic (5β-H, 12α-ol), chenodeoxycholic (5β-H, 7α-ol), and allochenode-

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oxycholic (5α-H, 7α-ol) acids. The structure of these products was confirmed by GLC-mass spectrometry of their methyl esters and methyl trimethylsilyl ethers on a single-focusing AEI MS-12 mass spectrometer (Associated Electrical Industries Ltd., Manchester, United Kingdom) coupled to a Pye-Unicam series chromatograph. All the compounds exhibited the major ions expected on the basis of the known behavior of the saturated cholanoic acid derivatives. Monounsaturation was recognized primarily by the mass of the nuclear ion fragment appearing at two mass units less than that of the saturated compound. Distinction between Δ^2- and Δ^3-compounds was based on the diagnostically significant retro-Diels-Alder ions (nucleus + side chain − 54; nucleus − 54) which are characteristic of a Δ^2-configuration (2–4). It must be pointed out that the Δ^3-12α-hydroxy-5β-cholenoic acid methyl ester obtained from Steraloids actually was a mixture of Δ^2- and Δ^3-isomers which could be purified as described above. Our findings confirmed similar observations made by Child et al. (3), who were, however, unable to separate both compounds. In contrast to the chemical preparation of cholenoic acids, which always resulted in the formation of a mixture of Δ^2- and Δ^3-isomers, the microbially produced cholenoic acids presented as a single chromatographic peak with mobility and mass spectrum identical to those of one of the synthetic cholenoic acid isomers.

Microbial metabolites of sulfated bile acids were analyzed as described previously (22). Briefly, 5 ml of culture medium was desalted on a reverse-phase octadecylsilane-bonded cartridge (J. T. Baker Chemical Co., Phillipsburg, N.J.); bile acids were then eluted with methanol. After addition of 50 μg of 23-nordeoxycholic acid as internal standard, the eluate was divided into two equal parts and evaporated to dryness. To determine the amount of desulfated bile acids, one part was dissolved in 5 ml of a solution of 10% KH_2PO_4 and 10% NaCl in water, and the desulfated bile acids were extracted twice with 8 ml of diethyl ether. The extract was evaporated and the bile acids were derivatized to methyl ester trimethylsilyl ethers or to methyl ester acetates for analysis by GLC and combined GLC-mass spectrometry. To determine the total amount of bile acids, the other part was solvolyzed at 37°C overnight in acidic methanol-acetone. The solvolysate was neutralized with 25% NH_4OH and evaporated to dryness. After addition of 5 ml of a solution of 20% NaCl in water and 1 ml of 1 N HCl, the bile acids were extracted with diethyl ether and further analyzed as described above.

When screening large numbers of fecal isolates for bile acid-desulfating activity, only desulfated bile acids were determined. Thus the analysis was substantially shortened by omitting the time-consuming desalting and solvolysis procedure.

Determination of microbial transformation products from unconjugated unsulfated bile acids and from taurine- or glycine-conjugated bile acids was carried out as described previously (22).

RESULTS

Isolation. Cultures of mixed human fecal microflora on brucella, Columbia, or brain heart infusion broth transformed chenodeoxycholic acid 3-sulfate into variable amounts of chenodeoxycholic acid, isochenodeoxycholic acid, ursodeoxycholic acid, lithocholic acid, isolithocholic acid, and two unidentified unsulfated metabolites. GLC on a QF-1 column at 240°C of the methyl ester acetates of the latter two products showed retention times of 0.46 and 0.57 relative to 23-nordeoxycholic acid methyl ester acetate. Mixtures of the same metabolites were still found in cultures after heating of 10% fecal suspensions at 80°C for 10 min or after treatment with equal volumes of pure ethanol for 45 min to select for spore-forming microorganisms. The bile acid-desulfating activity, however, could not be maintained in subsequent cultures, and no bile acid-desulfating isolates were obtained in this way.

Positive isolates were obtained only after direct plating of untreated fresh fecal suspensions on brain heart infusion or brucella agar. Cultures from single colonies and mixtures of different colonies were screened for production of unsulfated metabolites from chenodeoxycholic acid 3-sulfate. Only 3 of 365 cultures tested metabolized the substrate and produced the two above-mentioned unidentified compounds without other bile acid metabolites. Two cultures in brucella broth were further purified by sequential plating and culturing single colonies. During this procedure the production of one of the unidentified products (retention time, 0.57) disappeared. Two microorganisms obtained in pure cultures, termed strains H35 and H83, produced only one unsulfated metabolite (retention time, 0.46), which was identified as 7α-hydroxy-Δ^3-5β-cholenoic acid.

Identification. Strains H35 and H83 grown in brucella broth appeared as nonsporing, nonmotile, gram-negative straight rods (0.5 to 1 μm by 5 to 8 μm), occurring singly and often in short chains. Colonies after 3 days of incubation on brucella agar plates were 0.5 to 0.7 mm in diameter, round, with irregular margins and surface, low convex, colorless, and transparent. Growth did not occur unless under strictly anaerobic conditions. Only low cell densities (optical density, <0.15) developed on commercially available complex media (e.g., brucella broth, brain heart infusion) or on the peptone-yeast medium of Holdeman et al. (9); strain H83 grew slightly more densely than did strain H35. Growth was not stimulated by the addition of 0.02% Tween-80, 0.2% formate plus 0.2% fumarate, 20 mM lactate or pyruvate, 0.3% threonine, or 0.1% arginine, taurine, or $NaNO_3$. Bile (2% desiccated ox bile) inhibited growth. Sugars were not fermented, nor did they stimulate growth. Both strains produced ammonia, butyrate, and acetate. No propionate was formed from threonine or lactate, and pyruvate was not metabolized. Both strains were negative for esculine hydrolase, starch hydrolase, catalase, lecithinase, urease, reduction of nitrate, and production of indole. H_2S was not produced.

Because of their strictly anaerobic character and their gram-negative, nonsporing, rod-shaped cells, the strains belong to the family *Bacteroidaceae*. Because of their production of butyrate as a major fatty acid, they were tentatively classified in the genus *Fusobacterium*. However, they do not correspond to any of the species described in *Bergey's Manual* (15) or in the *Anaerobe Laboratory Manual* (9). Since morphological characteristics and biochemical tests revealed no obvious differences between strain H35 and H83, both isolates most probably belong to the same unidentified species and were termed *Fusobacterium* sp. strains H35 and H83.

Bile acid-desulfating activity. Cultures of *Fusobacterium* sp. strains H39 and H83 on brucella broth showed no differences in bile acid-desulfating activity. Desulfation was limited to C-24 bile acids and was specific for sulfate esters at the 3-hydroxyl group (Table 1). The 3-sulfates of C-22 or C-23 bile acids, of neutral steroids (androstanes and sterols such as cholesterol, coprostanol, epicoprostanol, and cholestanol), and of steroids with aromatic A-ring (estrone,

TABLE 1. Transformants of bile acid sulfates by cultures of *Fusobacterium* sp. strains H35 and H83

Substrate[a]	Configuration of:		Transformation products[a,b]
	C-5 hydrogen	Sulfate group	
LCA 3-sulfate	5β	3α	Δ^3-5β-CE (>95)
IsoLCA 3-sulfate	5β	3β	Δ^3-5β-CE (>95)
AlloLCA 3-sulfate	5α	3α	
IsoalloLCA 3-sulfate	5α	3β	Δ^2-5α-CE (46) +
Iso-Δ^5-CE 3-sulfate	Δ^5	3β	alloLCA (54)
CDCA 3-sulfate	5β	3α	Δ^3-7α-ol-5β-CE (48)
CDCA 7-sulfate	5β	7α	
IsoCDCA 3-sulfate	5β	3β	Δ^3-7α-ol-5β-CE (<5)
IsoCDCA 7-sulfate	5β	7α	
AlloCDCA 3-sulfate	5α	3α	
AlloCDCA 7-sulfate	5α	7α	
IsoalloCDCA 3-sulfate	5α	3β	Δ^2-7α-ol-5α-CE (30)
IsoalloCDCA 7-sulfate	5α	7α	+ alloCDCA (15)
DCA 3-sulfate	5β	3α	Δ^3-12α-ol-5β-CE (62)
DCA 12-sulfate	5β	12α	
CA 3-sulfate	5β	3α	
CA 7-sulfate	5β	7α	
CA 12-sulfate	5β	12α	

[a] LCA, Lithocholic acid; CE, cholenoic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; CA, cholic acid. The prefix "allo" refers to a 5α-steroid nucleus; the prefix "iso" refers to a β-orientation of the 3-hydroxyl group.

[b] The amount of products formed by strains H35 and H83 showed no significant differences. The values in parentheses are mean percentages of two determinations for each strain.

estradiol) were not desulfated (data not shown), nor were C-24 bile acids sulfated at C-7 or C-12.

The most striking feature of the bile acid-desulfating activity by the *Fusobacterium* strains was the formation of C-3-unsubstituted, ring A-unsaturated bile acids. Cholenoic acids were the sole transformation products of 5β-bile acid sulfates, whereas transformation products of 5α-bile acid sulfates included 3α-hydroxy-5α-bile acids in addition to the 5α-cholenoic acids. The position of the double bond formed was determined by the configuration at C-5. In general, in 5α-bile acids the double bond was formed between C-2 and C-3 (Δ^2-5α-cholenoic acids); in 5β-bile acids the double bond formed between C-3 and C-4 (Δ^3-5β-cholenoic acids). The orientation of the 3-sulfate group on the bile acid molecule had no influence on the position of the double bond in 5β-bile acids, as both 3α- and 3β-sulfates were converted to the same Δ^3-bile acid. On the other hand, in the 5α series (allobile acids) only 3β-sulfates were transformed, whereas 3α-sulfates were left intact. It should be noted that the 3α-hydroxy-5α-bile acids additionally formed from 5α-bile acid 3β-sulfates had an inverted 3α-hydroxyl group.

Bile acid 3-sulfates with a 7- and/or 12-hydroxyl group were apparently less easily transformed: in contrast to monohydroxy bile acid 3-sulfates, dihydroxy bile acid 3-sulfates (deoxy- and chenodeoxycholic acids) were only partially converted, and trihydroxy bile acid 3-sulfates (cholic acid) were not. Addition to the culture medium of monohydroxy bile acid sulfates did not enhance the desulfating activity towards the 3-sulfates of polyhydroxy bile acids, nor did it induce desulfating activity towards the allobile acid 3-sulfates.

The time course of transformation of litho-, isolitho-, and isoallolithocholic acid 3-sulfate showed that the conversion was completed within 3 days after inoculation of the cultures (Fig. 1). In comparison, cheno- and isoallochenodeoxycholic

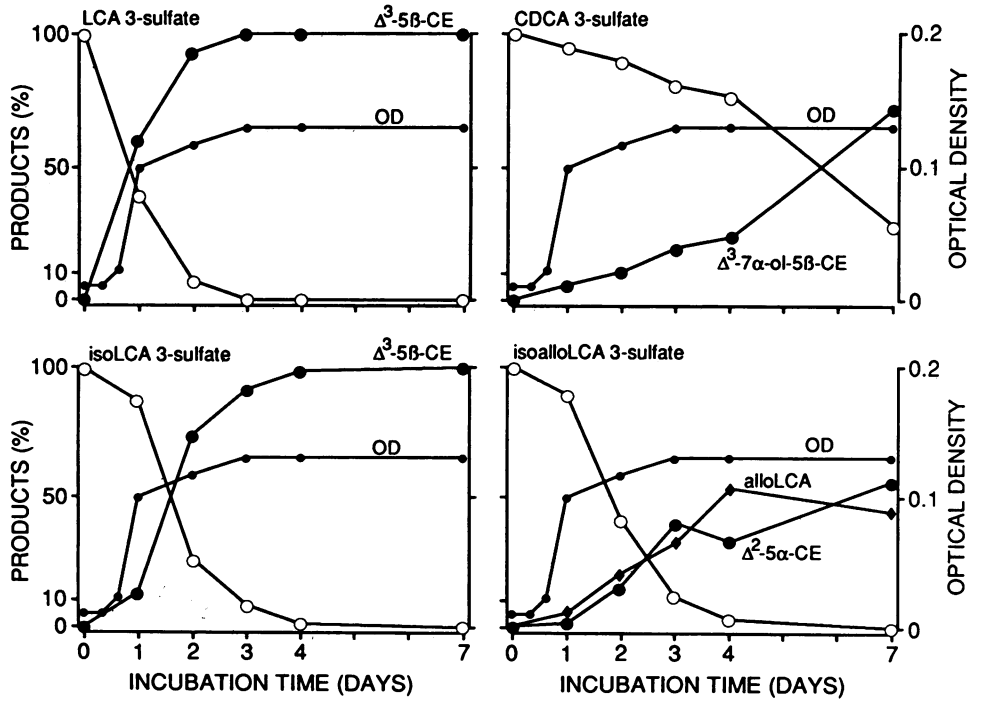

FIG. 1. Time course of bile acid sulfate transformations in growing cultures of *Fusobacterium* sp. strain H35. Growth was measured as optical density (OD) of cultures at 520 nm.

TABLE 2. Transformations of free bile acids by cultures of *Fusobacterium* sp. strains H35 and H83

Bile acids[a]	Configuration at:		Transformation products[b] by:	
	C-3	C-5	Strain H35	Strain H83
LCA; CDCA; DCA; CA	3α-ol	5β-H		
IsoLCA; isoCDCA	3β-ol	5β-H		
AlloLCA; alloCDCA	3α-ol	5α-H		
IsoalloLCA; isoalloCDCA	3β-ol	5α-H		
3-KetoLCA	3-keto	5β-H	3α-ol, 5β (2)	3α-ol, 5β (8)
3-KetoCDCA	3-keto	5β-H	3α,7α-diol, 5β (34)	3α,7α-diol, 5β (88)
3-Keto-alloCDCA	3-keto	5α-H	3α-ol, 5α (49)	3α-ol, 5α (97)
3-Keto-7α-ol-Δ^4-CE	3-keto	Δ^4		

[a] For abbreviations of bile acids, see Table 1, footnote *a*.
[b] Cholanoic acids with indicated configuration of hydroxyl groups and C-5 hydrogen. Values in parentheses are mean percentages of two determinations.

acid 3-sulfate were desulfated more slowly, and desulfation continued until at least 7 days after inoculation. During transformation of 5β-bile acid 3-sulfates into the respective Δ^3-cholenoic acids, no formation of unsulfated 3-hydroxy-5β-bile acids was observed. In contrast, the formation of Δ^2-cholenoic acids and of 3α-hydroxy-5α-bile acids from 3β-sulfated 5α-bile acids proceeded synchronously. Whether one of the two products was a reaction intermediate could not be derived from these data.

Formation of cholenoic acids from sulfate-free bile acids with a 3α- or 3β-hydroxyl group, added to the culture medium, was not observed (Table 2), not even in the presence of bile acid sulfates acting as possible enzyme inducers. 3-Keto bile acids were hydrogenated into the corresponding 3α-hydroxy bile acids, showing 3α-hydroxy steroid dehydrogenase activity, but were not transformed into cholenoic acids. Taurine-conjugated bile acids were deconjugated; glycine-conjugated bile acids were not.

DISCUSSION

The two *Fusobacterium* strains described in this paper transformed bile acid 3-sulfates into Δ^2- or Δ^3-bile acids by removal of the 3-sulfate group. Both 3α- and 3β-sulfates of 5β-bile acids were specifically converted into Δ^3-5β-cholenoic acids, whereas 3β-sulfates of 5α-bile acids were metabolized to a mixture of Δ^2-5α-cholenoic acids and 3α-hydroxy bile acids; 5α-bile acid 3α-sulfates were left intact (Fig. 2). In this respect, these strains were different from all other bile acid-desulfating bacteria described earlier which showed similar substrate specificities, but which did not further transform the desulfated bile acids (10, 22, 26).

Palmer first reported the transformation of lithocholic acid sulfate by human fecal cultures into an unsaturated bile acid which was tentatively identified as Δ^2- or Δ^3-cholenoic acid (19). The same bile acid was demonstrated earlier ("compound B") in feces and bile from patients after oral administration of [^{14}C]lithocholic acid (16). Kelsey et al. (11, 13) demonstrated that human fecal cultures formed Δ^3-cholenoic acid but not the Δ^2-isomer from lithocholic acid sulfate. These and other studies (1, 17) also showed that Δ^3-cholenoic acid was the major product of lithocholic acid sulfate metabolism by human fecal cultures, while lithocholic and isolithocholic acid were minor products. Our data agree with these studies. In addition, we demonstrated that the *Fusobacterium* strains in pure cultures formed Δ^3-cholenoic acid as the sole product from lithocholic acid sulfate. Borriello and Owen (1) reported on the metabolism of lithocholic acid sulfate into a mixture of Δ^3-cholenoic acid, isolithocholic acid, and cholanoic acid by pure strains of the genus *Clostridium* (20 of 37 strains tested). Desulfation yields were not mentioned. Since these strains were not available for further study, we examined under identical experimental conditions 24 ATCC and NCIB type strains of the *Clostridium* species mentioned. In this unpublished study we failed, however, to detect any degradation of lithocholic acid sulfate or its 3β- or 5α-isomers. It should be noted, however, that bile acid sulfates hydrolyze spontaneously at low pH whereas cholenoic acids are easily generated at high pH, conditions which may lead to erroneous results and, hence, must be avoided in growing cultures as well as during bile acid sulfate analysis.

The mechanism of bile acid desulfation by the *Fusobacterium* strains has yet to be elucidated. The Δ^3-cholenoic

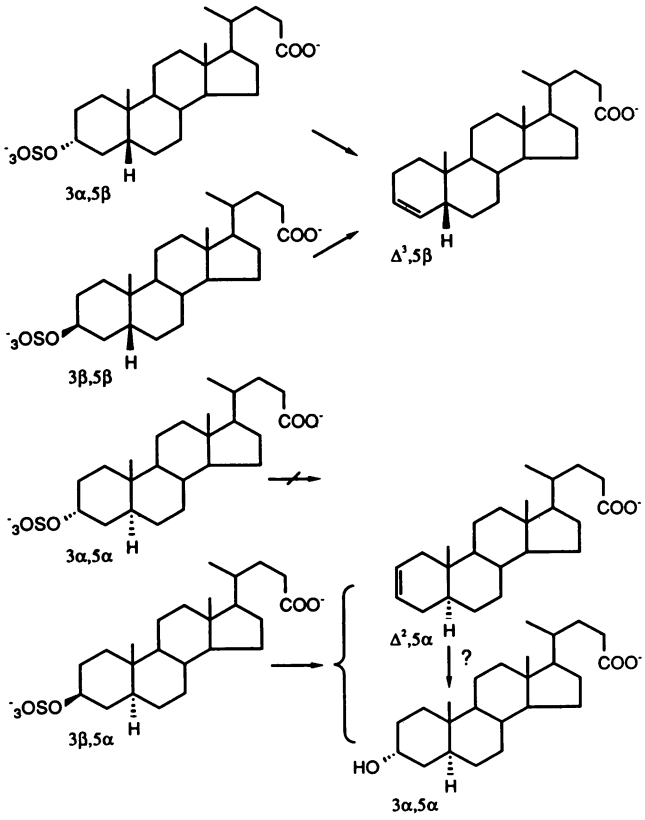

FIG. 2. Metabolism of bile acid sulfates by *Fusobacterium* sp. strains H35 and H83.

acids may be formed in one single step involving a lyase enzyme removing an H_2SO_4 molecule and leaving a double bond in the bile acid nucleus. Analogously, a double bond between C-6 and C-7 is formed by removal of an H_2O molecule during bacterial 7α-dehydroxylation of primary bile acids (5, 14, 23). It is not known, however, whether the bile acid sulfates are nucleotidylated prior to removal of the H_2SO_4 moiety, nor whether additional polyunsaturated intermediates labilizing the leaving group are formed, as Coleman et al. (5) recently proposed for bile acid 7-dehydroxylation. On the other hand, in the case of 7-dehydroxylation the Δ^6-double bond is quickly hydrogenated, whereas the Δ^3-cholenoic acids are end products. As a result, the bile acid desulfation reaction is electroneutral, in contrast to dehydroxylation which is reductive. The pathway of 5α-bile acid 3β-sulfate metabolism into Δ^2-5α-cholenoic acids and 3α-hydroxy-5α-bile acids seems more complicated. The 3α-hydroxy-5α-bile acids most probably are not reaction intermediates since unsulfated bile acids were not dehydrated into cholenoic acids. Hence, the 3-hydroxy bile acid may be formed either by hydration of the Δ^2-cholenoic acid or directly from the sulfate ester by the involvement of a sulfohydrolase enzyme cleaving the C-O bond of the C-O-S linkage and inverting the 3-hydroxyl group. Cleavage of the C-O bond with subsequent inversion of configuration is a common feature of the alkyl sulfatases found in soil microorganisms (7). On the other hand, the bile acid-desulfating intestinal bacteria reported earlier (10, 22, 26) did not invert the configuration at C-3, and therefore most probably cleaved the O-S bond of the sulfate ester.

Little is known about the occurrence and the clinical significance of the microbial formation of cholenoic acids in vivo. The observation in bile after administration of [^{14}C]lithocholic acid of a [^{14}C]cholenoic acid (19) suggests that this product, formed in the intestines by the microflora, enters the enterohepatic circulation and probably is not metabolized by the liver. Tanida et al. (24, 25) identified Δ^3-cholenoic acid (up to 10% of total fecal bile acids) in stools of healthy subjects and patients with adenomatous polyps of the large bowel. On the other hand, increased transformation of lithocholic acid sulfate into cholenoic acid was observed in fecal cultures from persons on a high-beef-fat diet (high risk for contracting colon cancer) as compared with persons on a vegetarian/low-beef diet (low risk) (12). Both studies, however, dealt with insufficient data to draw definite conclusions. The fact that this unsubstituted bile acid was not reported by other investigators might be due to its very high chromatographic mobility, by which it may not be recognized as a bile acid, and/or to the lack of a commercially available reference product. More careful examination of fecal bile acids for the occurrence of cholenoic acids will be necessary.

ACKNOWLEDGMENT

This work was supported by a grant from the Belgian National Fund for Medical Research (NFGWO).

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