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Bacterial Formation of ω-Muricholic Acid in Rats

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In the feces of conventional rats, the amount of ω-muricholic and hyodeoxycholic acids vary according to the diet. To understand this phenomenon, we investigated the bacterial formation of these bile acids. The present paper reports the first isolation, from conventional rat feces, of a strain of *Clostridium* group III which transforms β-muricholic acid, the main bile acid in germfree rats, into ω-muricholic acid.

According to recent studies (2, 14, 19, 20), hyodeoxycholic acid (3α,6α-dihydroxy-5β-cholanoic acid) and ω-muricholic acid (3α,6α,7β-trihydroxy-5β-cholanoic acid) are among the most abundant bile acids in the feces of the conventional male rat. These two bile acids coexist in some subjects, whereas in others only one or the other is present. These variations depend on the composition of the animal's diet (19, 20). To understand how the diet exerts the change, we need to know the mode of formation of these bile acids. The purpose of our study, therefore, was to study this process. To that end, we tried to isolate the bacteria which form these chemical species. In this work we investigated whether bacterial strains possessing a 6-isomerase exist and whether they are capable of transforming β-muricholic acid (3α,6β,7β-trihydroxy-5β-cholanoic acid), the main bile acid of the axenic rat (7), into ω-muricholic acid.

MATERIALS AND METHODS

Diet and animals. The animals used were male adult axenic and conventional Fischer rats, which received a diet of the following composition: 220 g of casein, 580 g of corn starch, 90 g of corn oil, 50 g of cellulose, and 45 g of a mineral and vitamin mixture. This diet, admixed with 90 mg of cholesterol per 100 g, was called the SNC diet. It was sterilized by autoclaving.

Surgical technique. The deviation of the bile of conventional rats towards the urinary bladder was performed by introducing a silicone catheter (Silastic A 602 101, Dow Corning) into the upper part of the bile duct. The other end of the catheter was fitted with a shoulder and inserted into the urinary bladder, where it was retained at the level of the vesical dome by a purse-string suture. The abdominal wall was closed in two planes by means of 4 × 0 silk thread and in the cutaneous plane by an intradermal overcasting. The rats recovered their initial body weight within 1 week and continued to grow. Their bile was excreted in the urine.

Biochemical methods. (i) Analysis of bile acids. Most of the methods used for bile acid analysis have been described previously (14). The composition of fecal bile acids was determined by the [4-^{14}C]cholesterol isotopic equilibrium method. In this method, the various chemical species of bile acids exhibit the same specific activity. The chemical species, after separation from the unsaponifiable fraction and methylation, were chromatographed in the presence of standards on Silica Gel G thin-layer plates by means of a chloroform-acetone-methanol mixture (70:25:5, vol/vol/vol). The distribution of the radioactivity on these chromatograms was used to establish the bile acid composition.

Gas-liquid chromatography was done according to the method of Siegfried et al. (15).

(ii) Molecules used. [4-^{14}C]cholesterol was supplied by Commissariat a l'Energie Atomique (C.E.A.) (Saclay). β-Muricholic and 3α-hydroxy-6-keto-5β-cholanoic acids were prepared according to previously described methods (18). 3α-Hydroxy-6β-[^{3}H]hydroxy-5β-cholanoic acid was prepared by reduction of 3α-hydroxy-6-keto-5β-cholanoic acid, using sodium boro-[^{3}H]hydride (specific activity, 15 Ci/mmol), supplied by C.E.A. Hyodeoxycholic acid was obtained from Steraloids (Pawling, N.Y.). ω-Muricholic acid was identified by thin-layer and gas-liquid chromatography relative to a sample of ω-muricholic acid which had been characterized by mass spectrometry of its trimethylsilyl ethers, according to the technique of Sjövall et al. (16).

(iii) Search for 6-isomerase. Fecal suspensions or bacterial cultures were used for the determination of 6-isomerase.

The bacterial cultures were either growing or resting cells. In the first case, 5 mg of bile acid was introduced into a test tube and dissolved in a minimum amount of sodium carbonate; 10 ml of nutritive broth was added to the culture tube, sterilized by autoclaving at 120°C, and then inoculated. In the second case, the bacterial cells obtained by culture (see below) were incubated for 24 h with the substrate in 0.02 M phosphate buffer, pH 7.0. The concentration of bile acid

was 0.5 mg/ml. The fecal suspensions were made in the same buffer and incubated with the substrate in the same way as the resting cells.

The search for metabolites was made by diluting or suspending these growing cell, resting cell, or fecal preparations in isopropyl alcohol and refluxing for 1 h. The preparation was then centrifuged, and the supernatant fluid was collected, dried, recovered by water, acidified at pH 2.0, and extracted with chloroform-methanol (2:1, vol/vol). The chloroform extract was dried, recovered in methanol, methylated, silylated, and subjected to gas-liquid chromatography according to the technique of Siegfried et al. (15). The retention times relative to the trimethylsilyl ether of methyl chenodeoxycholate, indicated in parentheses, show that a satisfactory separation was obtained between the derivative of ω-muricholic acid (1.71) and that of β-muricholic acid (1.18), as well as between the derivative of 3α,6β-dihydroxy-5β-cholanoic acid (0.98) and the derivatives of hyodeoxycholic acid (1.08) and 3α-hydroxy-6-keto-5β-cholanoic acid (1.99).

(iv) Search for 6-isomerase: tritium test. The tritium test was based on the hypothesis that the inversion of the position of the C6-hydroxyl takes place by means of a ketone intermediate. Accordingly, if this hydroxyl is tritiated, the formation of ketone is accompanied by a loss of tritium: 3α-OH,6β-O^{3}H → 3α-OH, 6-CO + ^{3}H → 3αOH, 6αOH. The incubation of conventional rat feces with the labeled bile acid (3α-hydroxy-6β[^{3}H]hydroxy-5β-cholanoic acid) showed that this hypothesis was correct and that the tritium was bound to a volatile acceptor; drying the preparation led to the disappearance of tritium. The following technique was therefore used: two counting vials received 50,000 dpm of 3α-hydroxy-6β[^{3}H]hydroxy-5β-cholanoic acid and were incubated overnight with a fecal suspension or a bacterial culture in 0.02 M phosphate buffer, pH 7.0. One of the preparations was dried and dissolved in the same volume of water as the initial volume. The other preparation was not dried. The two vials received 1 ml of Soluene 350 and were maintained at 60°C until dissolution was complete. A 10-ml amount of Instagel (Packard) was added. The preparations were left for 30 min at room temperature to eliminate chemiluminescence, after which they were counted in a liquid scintillation spectrometer equipped with an external standard device for correction of quenching. The disappearance of ^{3}H from the preparation which had been dried indicated the development of a 6-ketone. Formation of 6α-hydroxyl was confirmed by thin-layer or gas-liquid chromatography.

Bacteriological methods. The following media were used for the counts: medium B′ (13) contained, per liter, 15 g of peptone (Evans), 10 g of tryptone (Difco), 10 g of yeast autolysate (Difco), 10 g of agar, and 0.1 g of Tween 80 (Merck), pH 6.5; medium DA contained, per liter, 15 g of peptone, 10 g of tryptone, 5 g of liver extract (Paines and Byrne), and 10 g of agar, pH 8.0. This medium was admixed with a rhamnose solution and with sterile decomplemented horse serum to obtain a 1% concentration of each.

For identifying strain R6 x 76, we used medium F derived from Trypticase soy broth (BBL) modified by Aranki et al. (1), to which we added 1% (vol/vol) of a 5% cysteine·HCl solution, 1% of a 0.05% menadione solution, and 1% decomplemented horse serum. The carbohydrates whose fermentation was studied were added at 1% (wt/vol) each. Detection of hydrogen sulfide and indole was done in a culture by using medium F admixed with 0.05% glucose; the pH of the culture was measured after 7 days of incubation at 37°C. Reduction of nitrates to nitrites was investigated in culture medium F admixed with 0.05% glucose and 1% sodium nitrate. Determination of catalase was made by emulsifying in H_2O_2 the colonies obtained on medium F containing 1% agar. Medium F admixed with agar and sterile horse blood (5%, vol/vol), sterile egg yolk (1%, vol/vol), and medium F admixed with gelatin were used to detect hemolysin, hydrolysis of egg yolk, and gelatinase, respectively. The amount of volatile fatty acids was established in medium F and in medium F containing 1% glucose. The determination was made by gas-liquid chromatography according to the method of Rogosa and Love (11). All tests were made on 7-day cultures incubated at 37°C. The optical density of 48-h cultures was measured in medium D, i.e., medium DA without agar. The morphology was examined at 48 h in cultures harvested on medium DA. The resting cells were obtained by 72-h cultivation of strain R6 x 76 on 200 ml of medium DA, contained in Roux flasks. The cells from three Roux flasks were harvested in 15 ml of 0.02 M phosphate buffer, pH 7.0.

The identification media were inoculated and incubated in an anaerobic glove box similar to that described by Aranki et al. (1). They were introduced into the glove box 48 h before inoculation. The inoculum was 1% (vol/vol) of a 48-h culture made in medium D.

To study the oxygen sensitivity of strain R6 x 76, the following procedure was used. Counts were made from a 48-h culture in medium D or from gnotobiotic rat feces homogenized by grinding in the glove box in ninefold their weight of medium D, using an Ultra Turrax (Janke und Kundel KG, Staufen i. Br., Germany). Tenfold dilutions were made in medium D in an anaerobic glove box, and 0.1 ml of these dilutions was inoculated on medium DA which had been poured into petri dishes and prereduced by 48 h of incubation in the anaerobic glove box. The inoculated plates were removed from the glove box and exposed to air for either 10 min or 1 h before being reintroduced into the anaerobic chamber and incubated.

Counts of other *Clostridium* cultures were made outside the glove box in medium B′, using the deep agar tube technique (10).

RESULTS

Isolation of the active strain from the feces of a conventional rat after surgery. The first step of the experiment was to obtain a group of rats producing mainly ω-muricholic acid and little or no hyodeoxycholic acid. To that end, the bile of a conventional rat was shunted to the urinary bladder by means of a bile-duct urinary bladder catheter. One month later, the feces of this rat were collected, diluted in saline, and heated at 70°C for 10 min. This

fecal solution was administered to four germfree rats through a stomach tube. These rats received SNC diet and were designated SNC-M rats. Four conventional rats were fed the same diet and were designated SNC-H rats. Table 1 shows the bile acid composition in the feces of these rats 2 months later. The two compositions differed in the high amount of ω-muricholic acid and the low amount of hyodeoxycholic acid as well as of an unknown metabolite of β-muricholic acid (located in area 4b) in SNC-M rats as compared with SNC-H rats (18). The decrease observed in area 9 (Table 1) probably corresponds to the decrease in 3α-hydroxy-6-keto-5β-cholanoic acid, but this has not been established yet.

After the axenic rats received a fecal suspension, heated at 70°C for 10 min, obtained from intact conventional rats, they produced hyodeoxycholic acid, as had the conventional rats. Thus, the results suggest that when bile was shunted from the rat intestine, the bacterial flora in this organ was modified; the bacteria involved in the formation of hyodeoxycholic acid disappeared or decreased and could not develop when the feces of conventional rats were inoculated into axenic rats.

The second step consisted of isolating *Clostridium* strains from the feces of SNC-M rats and inoculating them through a stomach tube into axenic rats. The presence of bacterial 6-isomerase was detected, by the rapid test, in the feces of these gnotobiotic rats.

TABLE 1. *Composition of fecal bile acids of SNC-H and SNC-M rats: molar percentage of the methyl esters at different areas of thin-layer chromatograms (TLC)*[a]

TLC area[b]	Standard (methyl-)	Composition (%)[c]	
		SNC-H rats	SNC-M rats
0		2.5	2.5
1	Cholate	3.7 ± 0.4	2.7 ± 0.2
2	α-Muricholate	2.9 ± 0.4	9.0 ± 0.1
3	ω-Muricholate	2.5 ± 0.3	30.8 ± 1.5
4a	β-Muricholate	2.7 ± 0.7	7.3 ± 0.5
4b[d]		12.2 ± 0.1	3.1 ± 0.2
5	Hyodeoxycholate	27.1 ± 1.7	
6		2.3 ± 0.1	1.9 ± 0.2
7	Chenodeoxycholate	1.0 ± 0.3	1.1 ± 0.1
8	Deoxycholate	9.8 ± 0.5	9.6 ± 0.2
9[e]		20.4 ± 0.7	17.1 ± 0.6
10	Lithocholate	6.4 ± 0.9	6.6 ± 0.2
11		5.3 ± 0.3	8.0 ± 0.6

[a] Silica Gel G; chloroform-acetone-methanol (70:25:5).

[b] Chemical species in areas 1, 2, 3, 4a, 5, 7, 8, and 10 are given in column 2; chemical species in areas 0, 6, 9, and 11 are either unknown or incompletely separated.

[c] SNC-H, Conventional rats fed SNC diet. SNC-M, Germfree rats which received a dilution of feces obtained from a conventional rat that had been operated upon (see text); these rats were also fed SNC diet. Data are average percentages for four rats ± standard error of the mean.

[d] Area 4b contains an unknown bacterial metabolite of β-muricholate (17).

[e] Area 9 contains several keto acid methyl esters including methyl 3α-hydroxy-6-keto-5β-cholanoate.

The last step consisted of determining the presence of 6-isomerase in the cultures of these strains. One strain, R6 x 76, was found positive.

Morphological and physiological characters of the active strain. Strain R6 x 76 appeared in the form of nonmotile gram-positive rods, 0.3 by 1.5 μm, which were nonsporulating in the culture broth and often grouped in palisade heaps (notably in the digestive tract of gnotobiotic rats) and which sometimes exhibited median swellings.

Growth in liquid medium was poor, the maximum optical density reaching only 0.20 at 620 nm. The optical density was only 0.10 upon omitting either the serum or the rhamnose, and it was 0.05 in the absence of both ingredients. The strain developed in a nutritive broth only in an anaerobic glove box, but serial cultures outside the chamber could be obtained by using medium D admixed with 0.2% agar, or medium DA previously boiled for 30 min, and a heavy inoculum. The surface colonies (culture in an anaerobic glove box) were transparent, rhizoid, and flat, with a diameter of 1 to 3 mm. The colonies were large, compact, and rhizoid in deep agar. The strain was sensitive to molecular oxygen. When the strain was exposed to air for 10 min, the survival rate was 100%, but after 60 min, it was only 0.001%. Palladium chloride had an inhibitory effect; when the cell counts were made in an anaerobic glove box on medium DA admixed with 0.03% palladium chloride, 200-fold fewer colonies were obtained than with medium DA alone, and the size of the colonies was at least 2-fold smaller.

The strain produced gas abundantly and reduced safranine within 24 h, which indicated production of hydrogen.

In the presence of glucose, maltose, raffinose, L-arabinose, or rhamnose, the pH of the medium decreased from 6.8 to 6.2–6.0 after 7 days of incubation at 37°C. In the presence of melezitose, sucrose, lactose, trehalose, melibiose, cellobiose, mannose, galactose, fructose, sorbose, ribose, xylose, sorbitol, mannitol, glycerol, or salicin or in the absence of carbohydrates, the culture was less dense, gas was still produced, and the pH did not change.

The volatile fatty acid concentrations in the cultures with and without glucose were, respectively, 2.5 and 1.6 mg of total volatile fatty acids per ml, 1.8 and 1.2 mg of acetic acid per ml, 0.4 and 0.2 mg of propionic acid per ml, and traces of C4, iso-C4, C5, and iso-C5 acids.

The strain did not produce either hydrogen sulfide or indole, did not reduce nitrates to nitrites, and did not possess catalase, hemolysin, or gelatinase; the egg yolk reaction was negative.

The strain did not become established alone in the digestive tract of axenic rats, even after six successive inoculations by stomach tube with a great number of cells. It did become established after a previous establishment of another strain such as a *Veillonella* strain or another *Clostridium* strain, P1. In the latter case, strain R6 x 76 formed swelling terminal spores, whereas strain P1 formed swelling subterminal spores. In vitro, these spores have been very rarely observed. When a 1:100 suspension of feces from rats associated with strain R6 x 76 and strain P1 of *Clostridium* was heated at 70°C for 10 min and administered three times per os to axenic rats, strain R6 x 76 developed. On the other hand, if this heated suspension was inoculated into medium D in an anaerobic glove box, only strain P1 developed, indicating that the spores of strain R6 x 76 did not germinate in vitro but did so in vivo.

In conclusion, strain R6 x 76 belongs to the genus *Clostridium*, group III, as defined in *Bergey's Manual* (17). It does not belong to any of the species described. However, it is closely related to *C. sporosphaeroides* except for H_2S production.

Enzyme activity of strain R6 x 76. Strain R6 x 76 did not deconjugate rat bile acids. The isomerase was inactive on tauro-β-muricholic acid. Thus, the feces of an axenic rat associated with a *Veillonella* strain and *Clostridium* strain R6 x 76 exhibited the same bile acid composition as that of the axenic rat, although a suspension of the feces of the former gave a positive tritium test, indicating the presence of isomerase. Likewise, in vitro cultures of strain R6 x 76 were inactive on an extract of axenic rat bile containing tauro-β-muricholic acid.

The cultures of *Clostridium* R6 x 76 modified various substrates similarly regardless of whether growing cells or resting cells were used. The results were the same as those obtained in vitro with the feces of rats associated with *Veillonella* and *Clostridium* R6 x 76. The results are summarized in Table 2.

DISCUSSION

Although the microbial flora of the digestive tract of the conventional rat almost completely transforms the bile acids synthesized by the hepatocyte (14), only a few bacteria exhibiting these transformations have been isolated from the intestines or feces of this animal. Bacteria deconjugating the bile acids or forming ketones at the expense of hydroxyls in 3α and 7α have been obtained (3, 9, 12). The eight strains of strictly anaerobic *Lactobacillaceae* possessing a 7α-dehydroxylase, which were isolated by Gustafsson et al. (5), possess the same characters and probably belong to the same species. Neither Midvedt and Norman (9) nor Dickinson et al. (3) have succeeded in detecting this enzyme in other bacterial strains isolated from rats. No strain modifying 6β- and 7β-hydroxyls has been isolated from rats. However, deoxycholic, lithocholic, hyodeoxycholic, and ω-muricholic acids are the most abundant chemical species in conventional rat feces. The fact that the active bacterial strains have not been isolated clearly shows how difficult this particular type of study is, as is the study of the gastrointestinal flora in general. The formation of ω-muricholic acid is very active in SNC-M rats. The bacterial flora of these rats is more simple than that of conventional rats, since only the thermoresistant species are present. However, the isolation of a bacterial strain producing this metabolite proved to be very difficult and has only been accomplished through a simple biochemical test allowing rapid detection of the active strain. The sensitivity of this bacterial strain to molecular oxygen, its poor growth in culture media, the impossibility of implanting it alone in the axenic rat, and the necessary presence of other bacteria which deconjugate the tauro-β-muricholic acid to permit the isomerase to exert its action clearly show the difficulty in isolating such a strain. The above-mentioned properties are probably frequent in the bacteria of the digestive tract; for instance, the strain isolated by Gustafsson et al. (6), possessing a 7α-dehydroxylase, only exerts its action if the cholic or chenodeoxycholic acid is liberated from its bond with taurine.

Bile acids are usually ranked as primary, secondary, and tertiary bile acids:primary bile acids

TABLE 2. *Enzymatic activity of Clostridium R6 × 76*

Substrates[a]	Result
Tauro-β-muricholic acid	Tauro-β-muricholic acid
β-Muricholic acid (3α,6β,7β-TriOH)	ω-Muricholic acid (3α,6α,7β-triOH)
3α,6β-diOH	Hyodeoxycholic acid (3α,6α-diOH)
3α-OH-6-keto	Hyodeoxycholic acid (3α,6α-diOH)
Hyodeoxycholic acid (3α,6α-diOH)	Hyodeoxycholic acid (3α,6α-diOH)

[a] These substrates were incubated with growing cells, resting cells, or fecal suspensions obtained from rats associated with *Clostridium* R6 × 76. The technique used is described in the text.

are biosynthesized by the hepatocyte; secondary bile acids are primary bile acids which have been transformed by bacterial enzymes; tertiary bile acids are secondary bile acids which have been transformed by the hepatocyte into chemical species which are different from primary bile acids. Detection in the digestive tract of the conventional rat of a bacterium which transforms β-muricholic acid into ω-muricholic acid shows that the latter is not necessarily a tertiary bile acid as assumed by Madsen et al. (8). These authors based their statement on the fact that the rat hepatocyte possesses a 7β-hydroxylase and transforms hyodeoxycholic acid, a bacterial metabolite (4, 17) and consequently a secondary bile acid, into ω-muricholic acid, then called a tertiary bile acid. The transformation described suggests that ω-muricholic acid may be considered a secondary bile acid arising from the transformation, by a bacterial 6-isomerase, of β-muricholic acid, a bile acid synthesized by the hepatocyte and consequently a primary one.

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