Bacterial 7-dehydroxylation of cholic acid and allocholic acid

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ABSTRACT An obligate anaerobic organism capable of dehydroxylating cholic acid to deoxycholic acid and allocholic acid to allodeoxycholic acid was isolated from feces of the rabbit. It was a member of the bacteroides group (Gram-variable, nonsporulating anaerobes). The growth of the organism was inhibited by neomycin, 10–20 μg/ml. The existence of this organism affords a satisfactory explanation for the development of gallstones in the cholestanol-fed rabbit and for their absence in rabbits simultaneously treated with neomycin.

SUPPLEMENTARY KEY WORDS deoxycholic acid · allodeoxycholic acid · gallstone · rabbit · anaerobe · neomycin

Rabbits receiving stock diet containing 0.25–1% of cholestanol for 3–6 wk develop gallstones (1). An important component of these stones is glycoallodeoxycholic acid (2). Stone formation in the cholestanol-fed rabbit is preventable by daily oral administration of 0.125–0.5 g of neomycin (3); the antibiotic appears to exert its action by lowering the concentration of allodeoxycholic acid in the bile (3). To explain the phenomenon several mechanisms were proposed, the most attractive of which suggests an alteration in the intestinal flora mediated by neomycin (3). Support for the role of intestinal bacteria in the pathogenesis of gallstones in the cholestanol-fed rabbit may be derived from the following observations: (*a*) in the bile fistula rabbit, cholic acid is the predominating bile acid (4); (*b*) radioactive cholic acid administered to the rabbit is converted to deoxycholic acid after repeated enterohepatic cycles (5), whereas administered deoxycholic acid is not further metabolized (6); (*c*) cholic acid is the major bile acid of the germ-free rabbit, while deoxycholic acid is absent (7); (*d*) cholic acid incubated aerobically—and, more strikingly, anaerobically—with mixed cecal flora is transformed into dehydroxylated bile acids (8); (*e*) obligate anaerobic bacterial strains from the intestine of rat and man are capable of 7α-dehydroxylation of chenodeoxycholate (9) and of cholate (10).

Thus, substantial evidence indicates that intestinal bacteria are involved in the dehydroxylation of bile acids and therefore points to their role in the pathogenesis of gallstones in the cholestanol-fed rabbit. The bacteria involved should occur in all rabbits and in substantial numbers since animals on cholestanol-rich diets uniformly produce gallstones. In a previous study coliform bacteria were excluded as major participants in the metabolic processes culminating in the formation of gallstones (3). Our interest, therefore, turned to the other large segment of the intestinal flora: the anaerobes. This approach was further stimulated by the findings of Gustafsson, Midtvedt, and Norman (9), who demonstrated that obligate anaerobes from the feces of rat and man were capable of in vitro dehydroxylation of chenodeoxycholic acid and of cholic acid (10).

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography. Systematic names of the sterols and bile acids which are referred to in the text by their trivial names are as follows: cholestanol, 5α-cholestan-3β-ol; cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid; allocholic acid, 3α,7α,12α-trihydroxy-5α-cholanoic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; allodeoxycholic acid, 3α,12α-dihydroxy-5α-cholanoic acid; 7-ketodeoxycholic acid, 3α,12α-dihydroxy-7-keto-5β-cholanoic acid.

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Bacteriological Media and Antibiotics

Dehydrated culture media were obtained from Difco Laboratories, Inc., Detroit, Mich. (Difco), or from Baltimore Biological Products, Baltimore, Md. (BBL). Constituents of media prepared in the laboratory were obtained from the following sources: Peptone S and Peptone C from Albimi Laboratories, Flushing, N. Y.; beef extract, yeast extract, peptone, agar, gelatin, and bovine serum from Difco. Gall's cysteine broth (Gall, L. S., personal communication) was used for the isolation, purification, and maintenance of the organisms, made up according to her formula (w/v): Peptone S, 1%; Peptone C, 1%; Bacto beef extract, 1%; Bacto yeast extract, 1%; glucose, 0.1%; K_2HPO_4 (anhydrous), 0.1%; KH_2PO_4 (anhydrous), 0.1%; bovine serum, 0.1%.

Immediately before use, two drops of sterile cysteine bicarbonate solution and one drop of sterile 10% sodium bicarbonate were added per 10 ml of medium. Cysteine bicarbonate solution consisted (w/v) of 10% L-cysteine hydrochloride (Calbiochem or Merck) and 13% sodium bicarbonate in distilled water. The solution was covered with a 1:1 mixture of paraffin and vaseline and autoclaved. In some experiments Gall's broth was enriched with 2% gelatin. "Deep agar" tubes were prepared by adding 0.75% agar to cysteine-free Gall's broth.

Fermentation reactions were done partly in Cysteine Trypticase Agar with phenol red indicator and Taxos Carbohydrate Discs (BBL) and partly in 1% peptone in water with 1% carbohydrate and phenol red indicator. Motility Sulfide Medium (Difco) was used to test for H_2S production, and proteolytic properties were examined in Todd–Hewitt broth (Difco) with 5% gelatin. Nuclease production was assayed on deoxyribonuclease plates (Difco). PathoTec strips (Warner-Chilcott, Morris Plains, N. J.) were employed to test for cytochrome oxidase, phenylalanine deaminase, urease, and lysine decarboxylase. Nitrate reduction was studied in 1% peptone in water containing 0.1% KNO_3. Neomycin sulfate was obtained from the Eli Lilly Co., Indianapolis, Ind.

Dehydroxylation of bile acids was studied in Todd–Hewitt broth (Difco) or, when carbohydrates were involved, in sugar-free broth. Prior to autoclaving, Todd–Hewitt broth was enriched with 1 g of L-cysteine hydrochloride and 2 g of sodium bicarbonate per liter. Sugar-free broth consisted (w/v) of: Trypticase (BBL), 2%; NaCl, 0.25%; K_2HPO_4 (anhydrous), 0.15%; Na_2SO_3, 0.02%; sodium thioglycolate, 0.06%; and L-cysteine hydrochloride (dissolved in NaOH), 0.04%. The final pH of the medium was 7.5. To test the fermenting abilities of the organisms we added appropriate carbohydrates to the medium, which was then autoclaved at 115°C for 15 min.

Bile Acids

Cholic acid-24-^{14}C. The commercial product (Nuclear Research Chemicals, Orlando, Fla.) 3.97 mg, 50 μc, was purified, if necessary, by preparative TLC on (0.5 mm thick) Silica Gel G in the upper phase of toluene–acetic acid–water 5:5:1 by volume (10). The purified product contained less than 0.5% deoxycholic acid.

Allocholic acid-^3H. Allocholic acid was obtained from the bile of the giant salamander (12) and exposed for 2 wk to 3 mc of $^3H_2$ gas (13) at New England Nuclear Corp., Boston, Mass. The product was treated with 5% methanolic KOH for 2 hr, extracted in ethyl acetate, and dissolved in methanol. A 10 mg sample was purified by preparative TLC on Silica Gel PF plates (Brinkmann) in benzene–isopropanol–acetic acid 30:10:1 (11). The purified sample contained less than 0.1% allodeoxycholic acid.

Allodeoxycholic acid. The acid was isolated from gallstones of rabbits fed a diet containing 1% cholestanol for 4 wk (2).

Methyl 3,12-diketo-5α-cholanoate. The compound was prepared from methyl allodeoxycholate by oxidation with Kiliani reagent (2). Additional reference compounds were obtained from Mann Research Laboratories and Steraloids Inc., Pawling, N. Y.

METHODS

Bacteriology

Isolation of Anaerobic Bacteria. Pellets of rabbit feces were ground in Todd–Hewitt broth. The suspension was diluted in decimal steps in Todd–Hewitt broth to 10^{-12}, and 1 ml aliquots from each dilution were transferred to 9 ml of Todd–Hewitt broth or Gall's cysteine broth. The tubes were incubated anaerobically at 37°C for 7 days. Obligate anaerobes free from aerobic bacteria were usually obtained in dilutions from 10^{-7} to 10^{-10}. The anaerobes were purified by repeated isolations of colonies from deep agar or from anaerobically incubated blood agar plates.

Anaerobic Techniques. Liquid media and deep agars were regenerated by heating to 100°C for 30 min immediately before use. Anaerobic conditions were established in test tubes by the pyrogallic acid–sodium bicarbonate method (9). Larger volumes of broth cultures were placed in Erlenmeyer flasks and covered with a 2–3 cm layer of sterile mineral oil. Plates were placed in jars with Gaspak (BBL). Incubation was done at 37°C.

Dehydroxylation of Bile Acids

Solutions of sodium cholate-^{14}C (500 μg/ml) and allocholate-^3H (500 μg/ml) were prepared; 0.5 ml aliquots

were transferred to 9.5 ml of Todd–Hewitt broth to give a final concentration of 25 μg/ml of labeled sodium cholate or allocholate, respectively. The mixtures were autoclaved at 121°C for 15 min, cooled, and checked for sterility. In the dehydroxylation experiments, six tubes were inoculated with approximately 0.1 ml of a 3- to 5-day old broth culture. Two uninoculated tubes served as control for the stability of the bile acids. The tubes were incubated anaerobically at 37°C for 7 days. If bacteriological controls showed absence of contaminants at the end of the incubation period, the tubes with bile acid were placed in boiling water for 30 min, cooled, and centrifuged at 20,000 *g* for 30 min. The supernatant fluid was recovered for bile acid analysis. The cells contained negligible amounts of radioactivity and were discarded.

Extraction of Bile Acids. The recovered supernatant fluid was acidified to pH 1 with dilute HCl in an ice-bath. The bile acids were extracted with peroxide-free diethyl ether containing 10% absolute methanol. The organic layer was washed with water and evaporated under a stream of air at temperatures below 60°C. The residue containing labeled bile acids was dissolved in a small volume of methanol.

Measurement of 7-Dehydroxylation Reaction. The labeled bile acid methyl esters were separated by TLC as described below. After development the plates were dried and sprayed with 2′,7′-dichlorofluorescein; the spots were observed under long-wave UV radiation. The pertinent spots were identified by comparison with reference compounds and then transferred by a suction device (14) to a scintillation vial. 4 ml of ethanol was added to each vial. To elute the bile acid esters from the silica gel, we heated the sealed vials at 60°C for 30 min. Scintillation fluid (0.6% 2,5-diphenyloxazole in toluene) was then added and the samples were counted in a Beckman model 200B scintillation counter with corrections for background and quenching.

Chromatography

TLC. Free bile acids were chromatographed on Silica Gel G in the upper phase of the mixture toluene–acetic acid–water 5:5:1 or trimethylpentane–isopropanol–acetic acid 60:20:0.5 (11).

The methyl esters of the bile acids were prepared by addition of concentrated H_2SO_4 to the methanol solution of the free bile acids to give a 1% solution (v/v) of H_2SO_4 in methanol. The solution was left at room temperature overnight. The esters were extracted with ether and chromatographed on Silica Gel G in acetone–benzene 3:7 (11).

GLC. The methyl esters of the bile acids were chromatographed on 180 cm × 4 mm glass columns containing 3% QF-1 (methyl fluoroalkyl silicone) on Gas-Chrom Q, 100–120 mesh (Applied Science Corp., State College, Pa.) at 270°C (2). The instrument used was a Barber-Colman Selecta 5000 gas chromatograph with flame ionization detectors.

Identification of Allodexycholic Acid

Allodeoxycholic acid-^{3}H extracted from cultures incubated with allocholic acid-^{3}H was identified as follows.

Chromatography. The biosynthetic product had the same R_f values upon TLC and the same retention times during GLC as a known sample. Methyl 3β,12α-dihydroxy-5α-cholanoate and methyl 3α,7α-dihydroxy-5α-cholanoate, which have longer retention times than methyl allodeoxycholate (15), were not detected.

Oxidation. After a large-scale incubation the contents of 10 tubes were combined and the bile acid methyl esters, obtained as described above, were chromatographed (2) on a 4-g column of Woelm alumina, activity grade IV, in the presence of 3.6 mg of methyl allodeoxycholate carrier. Methyl allodeoxycholate was eluted with 45% ethyl acetate in benzene, and the specific activity of this compound remained constant throughout the methyl allodeoxycholate band (56,400 cpm/mg). The methyl allodeoxycholate fractions were combined, evaporated to dryness, and diluted with an additional quantity of carrier to a specific activity of 35,800 cpm/mg. This material was oxidized with Kiliani reagent (2) to methyl 3,12-diketo-5α-cholanoate and chromatographed on a 4-g column of Woelm alumina, activity grade II. The diketo compound was eluted with 5% ethyl acetate in benzene and the specific activity remained constant throughout the diketocholanoate band (36,200 cpm/mg).

IR. After an additional large-scale incubation 20 tubes containing a total of 5 mg of allocholic acid-^{3}H were incubated for 7 days at 37°C in Todd–Hewitt broth as described above. Methyl allodeoxycholate-^{3}H was isolated (without addition of unlabeled carrier) by preparative TLC on 2-mm thick Silica Gel G in acetone–benzene 35:65. The methyl allodeoxycholate band was located by spraying a 2 cm section of the plate with 2′,7′-dichlorofluorescein, scraped off the plate, and placed in a small column. Methyl allodeoxycholate was eluted with ethyl acetate, the solvent was evaporated, and the solid residue (0.37 mg) was made into a pellet with 45 mg of KBr. The IR spectrum, obtained in a Perkin–Elmer model 421 spectrophotometer, was identical with that of an authentic sample of methyl allodeoxycholate (2).

The identification of methyl deoxycholate obtained from cultures incubated with cholic acid-24-^{14}C was carried out in an analogous manner.

RESULTS

Characteristics of Isolated Organisms

Among the obligate anaerobic organisms isolated from the rabbit feces, strain FA 1/146 was of particular interest.

Morphology. The organism was a highly pleomorphic, Gram-variable, nonmotile, noncapsulated, nonsporulating rod. Even in cultures incubated for 2–3 days at 37°C in Todd–Hewitt broth the organisms varied from short and plump to elongated and slender rods that could be straight or slightly curved. The ends were pointed, rounded, or club-shaped. Occasionally the bacteria terminated in spherical masses up to 10 times the diameter of the rod. The Gram stain was taken up irregularly: some bacteria retained the Gram stain whereas others were Gram-negative except for one or two Gram-positive dots.

Growth Condition. After 24–48 hr, FA 1/146 grew with uniform turbidity in liquid media incubated at 25–42°C. It tolerated 2.5% sodium cholate in Todd–Hewitt broth, produced extremely slimy growth along the wall or at the bottom of tubes with 5% sodium cholate, and was completely inhibited by 10% sodium cholate (0.25 M). It was inhibited by 6% NaCl (1 M).

Biochemical Reactions. Dextrose, mannitol, dulcitol, sorbitol, maltose, fructose, xylose, and arabinose were promptly fermented with production of acid and gas. Lactose and salicin were fermented slowly with production of acid only. The organism failed to ferment raffinose, inositol, sucrose, rhamnose, and glycerol within 7 days. The methyl red test was positive and the nitrate reduction test weakly positive. Small amounts of H_2S were produced. The organism did not produce indole, urease, catalase, cytochrome oxidase, phenylalanine deaminase, or lysine decarboxylase. It failed to digest gelatin or deoxyribonucleic acid. Sheep erythrocytes were not hemolyzed.

Neomycin Sensitivity Test. Multiplication of FA 1/146 was inhibited by 10–20 μg of neomycin/ml of medium; this is the neomycin sensitivity of strains of *Escherichia coli*.

Dehydroxylation Reactions

Cholic Acid. Since, during the initial stages of the study, allocholic acid was in short supply, sodium cholate-24-^{14}C was used to establish suitable conditions for conversion. Table 1, which contains the results of a typical experiment, shows that most of the 7-dehydroxylation took place within the first 4 days of incubation. At that time the culture had usually reached the stationary phase. Conversion continued at a low rate at least up to the 7th day, and a few experiments, not shown in the table, indicated that even greater conversion could be obtained on prolonged incubation. Approximately 33% of the cholate was converted at the end of 7 days, whatever the initial concentration of cholate.

Keto derivatives of bile acid were produced during the early phases of the incubation (Table 2). By the 7th day approximately 75% of these compounds had disappeared. When FA 1/146 was incubated with 6 μg of sodium cholate/ml of medium rather than 25 μg/ml as shown in Table 2, 90% of the keto derivatives disappeared by the 7th day. Otherwise, the figures were similar. In systems with cholic acid concentrations of 100 μg/ml or 400 μg/ml the maximum concentrations of the keto derivatives observed were 12 and 4%, respectively. They occurred early in the incubation and were reduced by the 7th day.

Allocholic Acid. Table 3 summarizes the data obtained when sodium allocholate-^{3}H was incubated at 37°C for 7 days with culture FA 1/146 in Todd–Hewitt broth or

TABLE 1 EFFECT OF CONCENTRATION AND INCUBATION TIME ON 7-DEHYDROXYLATION OF SODIUM CHOLATE

Cholate in TH Broth	Days of Incubation			
	1	2	4	7
μg/ml	*% conversion to deoxycholate*			
6	0	24	31	39
25	5	23	37	35
100	2	6	27	33
400	1	2	30	31

TABLE 2 FORMATION OF 7-KETODEOXYCHOLIC ACID AND "MONOKETO-MONOHYDROXY" ACIDS FROM SODIUM CHOLATE*

Incubation Period	Sodium Cholate Converted to		
	Deoxycholic Acid	7-Ketodeoxycholic Acid	"Monoketo-monohydroxy" Acids†
days	*percentage*		
1	5	8	0
2	23	19	17
4	36	5	7
7	35	5	4

* Concentration of sodium cholate, 25 μg/ml.
† A mixture of 3α-hydroxy-12-keto-5β-cholanoic acid and 12α-hydroxy-3-keto-5β-cholanoic acid.

TABLE 3 CONVERSION OF ALLOCHOLATE TO ALLODEOXYCHOLATE*

Broth Medium	No. of Experiments	% Conversion of Allocholate to Allodeoxycholate
Todd–Hewitt	4	6.2 (4.5–8.2)†
Sugar-free	4	3.2 (1.9–5.7)†

* 7 days' incubation at 37°C. Concentration of sodium allocholate-^{3}H: 25 μg/ml.
† Range.

sugar-free broth. The percentage of conversion to allodeoxycholate was considerably lower than for cholate. Nevertheless, the formation of allodeoxycholic acid from allocholic acid was clearly demonstrated. The 7-dehydroxylation reaction proceeded more readily in Todd–Hewitt broth than in the sugar-free medium.

DISCUSSION

In a previous paper (3) it was concluded that if bacteria were involved in the pathogenesis of gallstone formation in the cholestanol-fed rabbit they should be capable of removing the 7α-hydroxyl group of allocholic acid, be sensitive to neomycin, and occur in all rabbits in substantial numbers. The organism described in the present paper, FA 1/146, meets these specifications.

We have demonstrated that both 5α and 5β bile acids can be dehydroxylated by the same organism, but that during the in vitro incubation the dehydroxylation of the 5α bile acid (allocholic acid) is less extensive than that of the corresponding 5β isomer (cholic acid). In the intestinal tract of the rabbit this difference may be less pronounced since the gallstones formed in cholestanol-fed rabbits contain similar proportions of allodeoxycholic acid (approximately 50% of dry wt) and deoxycholic acid (approximately 40%) (3). It is to be expected, of course, that relative reaction rates in pure culture would differ from those in intestinal contents. In addition, there is reason to assume that the 7α-dehydroxylation of bile acids can be carried out by other, as yet unidentified strains of intestinal microorganisms (16), or that certain microorganisms can catalyze the dehydroxylation in combination, even though they are inactive individually in pure culture. Too little is known concerning relative rates of intestinal absorption of 5α and 5β bile acids to estimate the contribution of this factor in regulating the composition of the bile acid mixture that eventually reaches the gall bladder.

FA 1/146 was inhibited by only 10–20 μg of neomycin/ml of culture medium and could therefore be expected to be suppressed in animals receiving neomycin orally. In the cholestanol-fed rabbit, neomycin would reduce the relative proportions of both allodeoxycholic and deoxycholic acids in bile, with a corresponding increase of their trihydroxylated precursors, thus preventing the formation of the gallstones. It is interesting that complete suppression of gallstone formation in the cholestanol-fed rabbit required the daily administration of 500 mg of neomycin in drinking water; gallstones were still found occasionally in rabbits receiving 125 mg of neomycin daily (3). Assuming that the intestinal contents of the rabbit weight 100–200 g, that neomycin is poorly absorbed from the intestinal tract (17, 18), that it is not inactivated there, and that it is distributed evenly in the intestinal contents, the concentration of neomycin would be about 2500–5000 μg/ml, or about 125–250 times the amount required to suppress growth of FA 1/146 in vitro. It is possible that other strains capable of dehydroxylating bile acids are more resistant to neomycin than FA 1/146. In fact, neomycin plates have been used as selective media for bacteroides (19). It could also be that the organisms in their natural environment are more resistant to neomycin than indicated by laboratory investigations. The additional possibility, that neomycin inhibits gallstone formation by a preferential sequestering effect (3) upon dihydroxycholanoic acids (5α and 5β), cannot be excluded at present.

Morphology, staining properties, and gaseous requirements tend to place FA 1/146 in the group of unclassified bacteroides (20) often referred to as Gram-variable, nonsporulating anaerobes (GVNSA). Smith (21) found that species of bacteroides were the only organisms present in the stomach and all segments of the small intestine and that they formed more than 99% of the flora in cecum and feces. The prevalence of this class of organisms in rabbits should probably have incriminated them at an earlier date in the dehydroxylation of bile acids. There is little to suggest that FA 1/146 should be considered a lactobacillus. Furthermore, lactobacilli are exceedingly rare in rabbits (19, 21) and they are eliminated rapidly after the administration of neomycin in drinking water.

FA 1/146 is very similar to strain II of Midtvedt (22) and Midtvedt and Norman (10). The strain, isolated from the rat, was made available to us through the courtesy of these authors. Although they tentatively classified it as a lactobacillus, we found that the two organisms showed only minor differences in their action on carbohydrates. However, under standard conditions (Todd–Hewitt broth, 37°C, 7 days' incubation, bile acid concentration 0.025 mg/ml), strain II converted more than 80% of cholic acid to deoxycholic acid while culture FA 1/146 converted 30–40% of the compound. This difference was not related to differences in growth rates since, at the end of the incubation period, FA 1/146 consistently produced greater turbidity in the culture medium than did strain II.

It is interesting to note that culture FA 1/146, with the lower rate of dehydroxylation, originated from the rabbit, whose predominant bile acid is deoxycholic acid. This species does not possess the ability to rehydroxylate deoxycholic acid to cholic acid. In contrast, Midtvedt's strain II was isolated from the rat, in which the predominant bile acid is cholic acid. In this species the liver converts deoxycholic acid to cholic acid. One may speculate that differences in cholic acid concentrations in the intestinal tract of animals from which the bacteria originated may contribute to the observed differences in dehydroxylation rates.

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