Cofactor requirements for 7α-dehydroxylation of cholic and chenodeoxycholic acid in cell extracts of the intestinal anaerobic bacterium, *Eubacterium* species V.P.I. 12708

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Abstract The characteristics of 7α-dehydroxylase, a bile acid-biotransforming enzyme, were determined using dialyzed cell extracts of *Eubacterium* sp. V.P.I. 12708. 7α-Dehydroxylase was induced by cholic acid in this organism. Induction by cholic acid resulted in the differential synthesis of at least five new polypeptides with molecular weights of 77,000, two at 56,000, 27,000 and 23,500, as determined by both one and two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis. The relative molecular weight of 7α-dehydroxylase activity was estimated by anaerobic Bio-Gel A 1.5 M gel filtration chromatography to be 114,000. NAD^+ was the only cofactor found to consistently stimulate 7α-dehydroxylase activity in dialyzed cell extracts. The specific activity increased 4- to 6-fold with either cholic or chenodeoxycholic acid as a substrate in the presence of NAD^+. NAD^+ was also required for the reduction of the Δ^6-intermediate to deoxycholic acid. Other pyridine or flavin nucleotides were ineffective cofactors when added alone. Saturation kinetics for NAD^+ with cholic or chenodeoxycholic acid as substrates were hyperbolic, and Lineweaver-Burk plots yielded apparent K_m values of 0.13 mM and 0.06 mM, respectively. Similar kinetics were obtained with cholic acid giving an apparent K_m of 25 μM. The substrate saturation curve for chenodeoxycholic acid 7α-dehydroxylation indicated substrate inhibition at high concentrations of chenodeoxycholic acid (>50 μM). These studies show that 7α-dehydroxylase is an inducible enzyme and requires NAD^+ as a cofactor in this bacterium.—**White, B. A., A. F. Cacciapuoti, R. J. Fricke, T. R. Whitehead, E. H. Mosbach, and P. B. Hylemon.** Cofactor requirements for 7α-dehydroxylation of cholic and chenodeoxycholic acid in cell extracts of the intestinal anaerobic bacterium, *Eubacterium* species V.P.I. 12708. *J. Lipid Res.* 1981. **22:** 891–898.

Supplementary key words Δ^6-intermediate · induction of 7α-dehydroxylase · substrate saturation kinetics

The composition of bile acids in biliary secretions of man is determined by both liver biosynthetic enzymes and intestinal bacterial transformation reactions. The two most important quantitative biotransformations carried out by intestinal bacteria are deconjugation of conjugated bile acids yielding free bile acids, and the 7α-dehydroxylation of cholic acid and chenodeoxycholic acid forming deoxycholic acid and lithocholic acid, respectively. Secondary bile acids are more "toxic" than their corresponding primary bile acids and have been implicated in the etiology of a number of diseases of the gastrointestinal system (1). For example, lithocholic acid has been reported to be hepatotoxic (2), to cause hyperplasia and liver tumors (1), to enhance mutagenicity (3) and tumorgenicity (4) of known chemical carcinogens, and to cause DNA strand breaks in mouse lymphoblastoma cells (5). Kelsey and Pienta (6) have shown that lithocholic acid can transform Syrian hamster embryo cell in culture. Moreover, Low-Beer and Nutter (7) have presented evidence which suggests that deoxycholic acid may be important in increasing the cholesterol saturation of biliary bile and potentially increasing the risk of cholesterol gallstone formation.

7α-Dehydroxylation activity has been detected in members of several genera of anaerobic intestinal bacteria, including *Bacteroides* (8), *Clostridium* (9), and

Abbreviations: Systematic names of bile acids referred to in the text by their trivial names are as follows: cholic acid, 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholan-24-oic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholan-24-oic acid; lithocholic acid, 3α-hydroxy-5β-cholan-24-oic acid; Δ^6-intermediate, 3α,12α-dihydroxy-5β-6-cholen-24-oic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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Eubacterium (10). However, the levels of 7α-dehydroxylating bacteria have been reported to be in the range of only 10^4 to 10^5 per gram feces (11).

Our laboratory has been investigating 7α-dehydroxylation in the intestinal anaerobic bacterium, *Eubacterium* sp. V.P.I. 12708. White et al. (12) and Lipsky and Hylemon (13) reported that cholic acid induced 7α-dehydroxylase and NADH:flavin oxidoreductase activities in this bacterium. We previously reported (10) the assay conditions for 7α-dehydroxylase in cell extracts of *Eubacterium* sp. V.P.I. 12708. The present investigation reports on the detailed characterization of 7α-dehydroxylase activity in cell extracts with respect to cofactor requirements, substrate saturation kinetics, and characterization of reaction products. In addition, evidence will be presented that shows cholic acid causes an increase in the differential rate of synthesis of several new polypeptides.

MATERIALS AND METHODS

Growth of bacteria

Characteristics, growth conditions, and medium for the anaerobic culturing of *Eubacterium* sp. V.P.I. 12708 have been described previously (12). Bacterial cultures in 1- to 3-liter volumes were induced to synthesize 7α-dehydroxylase by the addition of 0.1 mM sodium cholate at hourly intervals during logarithmic growth. The characteristics of the induction process and the preparation of the cell extracts were described previously (12). Cell extracts were dialyzed anaerobically (4°C) against 1 liter of 50 mM sodium phosphate buffer (pH 6.8) containing 12% (vol/vol) glycerol and 5 mM glutathione for 18 hr with one buffer change. Dialyzed cell extracts were stored under argon gas atmosphere at −70°C for up to 4 weeks without loss of 7α-dehydroxylase activity. Cell extract protein concentrations were determined by the method of Kalb and Bernlohr (14).

Quantitative enzyme assay for 7α-dehydroxylase

Enzymatic 7α-dehydroxylation of [^{14}C]carboxyl-labeled cholic or chenodeoxycholic acid was followed by measuring the rate of secondary bile acid formation using a radiochromatographic assay procedure (11). Unless otherwise indicated, the standard reaction mixture contained (total volume 1.0 ml): 25 mM sodium acetate-morpholinopropane-sulfonate (MOPS) buffer (pH 7.5), 104 μM [^{14}C]cholic acid or [^{14}C]chenodeoxycholic acid (0.2 μCi/reaction mixture), 0.5 mM NAD^+, 0.2 mM $FADH_2$, and cell extract (1 to 2 mg). Assays were initiated by the addition of enzyme and incubated anaerobically (37°C) under 100% argon for 2 min. Reduced flavin nucleotides were prepared as described by Feighner and Hylemon (15). Enzyme activity was stopped by the addition of 1.0 ml of 0.5 N HCl to the assay mixtures. The acidified mixtures were extracted and bile acid products were quantitated as described previously (11). The specific activity of 7α-dehydroxylase was linear with time up to 4 min and with protein (0.5 to 2.5 mg).

Identification of 7α-dehydroxylation products

The products of 7α-dehydroxylation were characterized and identified by three independent methods. First, products were subjected to thin-layer chromatography in several solvent systems (11). In addition, the product of cholic acid 7α-dehydroxylation and a pure standard of 3α,12α-dihydroxy-Δ^6-5β-cholen-24-oic acid were analyzed by argentation chromatography on thin-layer plates predeveloped with 5% $AgNO_3$. Next, bile acid products of 7α-dehydroxylation were individually treated with stereospecific 3α-hydroxy, 7α-hydroxy, and 12α-hydroxysteroid dehydrogenases and the derivatives were chromatographed as described previously (11). Finally, bile acid methyl esters (16) were subjected to direct probe mass spectral analysis (Finnigan 4000 61 10 Data System). Mass spectra were obtained using the following conditions; source temperature, 250°C; ionization voltage, 75 eV; probe temperature at which the metabolites were volatilized, 200–250°C.

Anaerobic gel filtration column chromatography

Anaerobic gel filtration chromatography of cell extracts was carried out on a column (1.6 × 73 cm) of Bio-Gel A 1.5 M at 4°C (15). The column was washed with anaerobic 50 mM sodium acetate-MOPS buffer (pH 6.8) containing 12% (vol/vol) glycerol and 5 mM glutathione. Protein was eluted with the same buffer and fractions (4 ml) were collected by hand in stoppered tubes under an argon gas atmosphere. Fractions were assayed for NADH:flavin oxidoreductase activity as described by Lipsky and Hylemon (13) and for cholic acid 7α-dehydroxylase activity. The column was calibrated with NADH:flavin oxidoreductase (260,000) from *Eubacterium* sp. V.P.I. 12708, catalase (240,000), aldolase (158,000), bovine serum albumin (68,000), and blue dextran 2000.

Polyacrylamide gel electrophoresis

Soluble polypeptides of *Eubacterium* sp. V.P.I. 12708 grown in the presence or absence of sodium cholate were analyzed by one- and two-dimensional SDS-PAGE. Unlabeled soluble polypeptides were analyzed

using a modified second dimension originally described by O'Farrell (17). Modifications were in slab gel thickness (0.75 mm) and the concentration of polyacrylamide in the exponential gradient (7 to 20%). Gels were stained with 0.25% Coomassie brilliant blue R-250 in methanol–acetic acid–water 25:7:68 for 4 hr and then destained with the same solvent mixture for approximately 24 hr. For two-dimensional SDS-PAGE, induced and uninduced cultures (5 ml volume) were labeled with 200 μCi of [^{35}S]methionine during exponential phase growth. Soluble polypeptides were then subjected to isoelectric focusing in 2.5 × 130 mm glass tubing as described by O'Farrell (17). Following equilibration, the focusing gel, which contained approximately 100,000 cpm, was electrophoresed in the modified second dimension described above. The slab gels were dried and subjected to radioautography for 7 days using Kodak X-OMAT R X-ray film.

Preparation of 3α,12α-dihydroxy-5β-6-cholen-24-oic acid

The unsaturated dihydroxycholanoic acid 3α,12α-dihydroxy-5β-6-cholen-24-oic acid ("Δ^6-acid") was synthesized exactly as described by Kagan and Jacques (18). The starting material was ethyl 3α,12α-dihydroxy-7-keto-5β-cholan-24-oate (mp 148°C) a gift from A. F. Hofmann. This keto ester (17 g) was refluxed for 2 hr with acetic anhydride and glacial acetic acid, 90 ml of each, and the 3α,12α-diacetoxy-ester was not isolated but dissolved in 120 ml of glacial acetic acid. To this solution was added a solution of 5.82 g of bromine in 36 ml of acetic acid and the reaction mixture was allowed to stand overnight. The product was extracted with ether and was crystalline when the ether was evaporated. Recrystallization from methoxyethanol–water 5:1 gave 17.4 g (80%) of colorless plates (mp 133–135°C) of 3α,12α-diacetoxy-7-keto-6α-bromo-5β-cholan-24-oic acid.

Fifteen grams of the bromo ester above was reduced with 4 g of $NaBH_4$ in 500 ml of methanol by standing at room temperature overnight. Water was then added and the reduced compound was extracted into ether. The residue, after evaporation of the ether, was recrystallized from methanol–water 5:1 yielding 11.5 g (77%) of needles (mp 164–166°C) of ethyl 3α,12α-diacetoxy-6α-bromo-7α-hydroxy-5β-cholan-24-oate.

Eight hundred fifty-five mg of the above compound was refluxed with 800 mg of Zn dust in acetic acid for 2 hr. The acid was evaporated and the residue was taken up in ether–benzene. The organic solvent was evaporated and the residue was hydrolyzed in 3% metanolic KOH overnight at room temperature. The solution was then diluted with water, acidified with 1 N HCl, and extracted with ether. The ether was dried and evaporated, and the residue was crystallized from dioxane leaving colorless needles (185 mg) of 3α,12α-dihydroxy-5β-6-cholan-24-oic acid of mp 161°C, yield 26.7%. Synthesis of the "Δ^6-acid" by this method yields exclusively the Δ^6-*cis* compound (19).

Chemicals and enzymes

[24-^{14}C]Cholic acid (50 mCi/mmol) and [24-^{14}C]-chenodeoxycholic acid (50 mCi/mmol) were purchased from Amersham Corp. [^{35}S]Methionine (985 Ci/mmol) was purchased from New England Nuclear. 3α-Hydroxysteroid dehydrogenase was obtained from Worthington Biochemicals. 7α-Hydroxysteroid dehydrogenase and 12α-hydroxysteroid dehydrogenases were isolated as described previously (20, 21). Molecular weight standards for column chromatography were Combithek Calibration Proteins II from Boehringer Mannheim. Electrophoretically pure reagents including high and low range molecular weight standards for SDS-PAGE were obtained from Bio-Rad Laboratories. The pyridine nucleotides were purchased from PL-Biochemicals Inc. and flavin nucleotides and *Torula* yeast glucose-6-phosphate dehydrogenase from Sigma Chemical Co. Bile acids and bile salts were purchased from Calbiochem. All other chemicals were of the highest grade commercially available.

RESULTS

Identification of 7α-dehydroxylation reaction products

The relative mobilities of bile acid standards and the isolated products of cholic and chenodeoxycholic acid 7α-dehydroxylation in solvent systems S-1, S-6, and S-6 $AgNO_3$ of Eneroth (22) were determined as previously described (11). The respective products of cholic acid and chenodeoxycholic acid 7α-dehydroxylation co-migrated with standards of deoxycholic and lithocholic acid. Enzymatic treatment of the isolated bile acid products with stereospecific hydroxysteroid dehydrogenases yielded the expected derivatives of deoxycholic and lithocholic acid. The unknown product of cholic acid yielded the expected 3-keto and 12-keto derivatives of deoxycholic acid. The 7α-hydroxysteroid dehydrogenase had no effect on this product indicating the absence of a 7α-hydroxy group. Similar results were obtained with the unknown product of chenodeoxycholic acid 7α-dehydroxylation and its derivatives, indicating that the product is lithocholic acid.

TABLE 1. Effect of cofactors on 7α-dehydroxylation activity in cell extracts of *Eubacterium* species V.P.I. 12708

Cofactor(s)[b]	7α-Dehydroxylation Specific Activity[a]	
	Cholic Acid[c]	Chenodeoxycholic Acid[c]
None	100.7 ± 44.5	139.2 ± 48.3
NAD^+	628.2 ± 107.1	503.4 ± 137.3
$NAD^+ + FADH_2$	627.2 ± 178.1	521.2 ± 138.3
$NAD^+ + FMNH_2$	623.8 ± 119.7	477.8 ± 62.9
$NAD^+ + FAD$	764.3 ± 30.6	625.8 ± 62.9
$NAD^+ + FMN$	744.6 ± 34.0	569.4 ± 32.4
$NADP^+$	137.2 ± 16.7	218.9 ± 32.7
NADH	38.7 ± 43.1	34.4 ± 6.6
NADPH	29.2 ± 35.6	79.7 ± 22.8
$NAD^+ + NADH + FADH_2$	88.8 ± 20.4	231.1 ± 112.4
$FADH_2$	111.7 ± 6.6	189.8 ± 90.8
$FMNH_2$	103.5 ± 6.8	223.8 ± 50.6
FAD	158.6 ± 8.3	244.0 ± 83.0
FMN	88.6 ± 2.9	181.1 ± 90.0

[a] Units are nmoles of deoxycholic or lithocholic acid formed per hr per mg protein.
[b] Final concentrations of pyridine and flavin nucleotides were 0.5 and 0.2 mM, respectively.
[c] Final concentration of substrate was 104 μM.

Bile acid products were also esterified with methanol and subjected to mass spectral analysis. The major fragment ions at m/e of 388, 370, 273, and 255 as well as the minor ions, conformed to those of the standard deoxycholic acid. Similar results were obtained with the product of chenodeoxycholic acid 7α-dehydroxylation confirming its identity as lithocholic acid.

Cofactor requirements for 7α-dehydroxylation

The effects of various pyridine and flavin nucleotides on the specific activity of 7α-dehydroxylation was determined with either cholic or chenodeoxycholic acid as the substrate. As shown in **Table 1,** specific activity values of approximately 100 nmol hr^{-1} per mg $protein^{-1}$ were obtained in assays lacking exogenous cofactors. The addition of NAD^+ to reaction mixtures increased the specific activities 4- to 6-fold. In general, similar specific activities were obtained when either reduced or oxidized flavin nucleotides were added to assays in combination with NAD^+. However, NAD^+ was the only cofactor giving high specific activities when added alone to the assays. The flavin nucleotides or $NADP^+$ when added alone were not effective cofactors. NADH and NADPH (0.5 mM) inhibited 7α-dehydroxylation of cholic acid by approximately 62% and 71%, respectively; the inhibitions for chenodeoxycholic acid were 75% and 42%, respectively.

An unexpected finding was that NAD^+ stimulated 7α-dehydroxylation activity without any detectable reduction. Spectrophotometric assays were monitored using anaerobic cuvettes (3-ml volume) at 340 nm (37°C) using a Beckman Model 35 Recording Spectrophotometer. Assay mixtures contained standard components and concentrations with [^{14}C]cholic acid as the substrate, except that $FADH_2$ was omitted. The assays, which were run for time periods of up to 6 min, revealed no detectable reduction of NAD^+. However, analysis of the same reaction mixtures by the standard radiochromatographic methods showed that between 31% and 41% of the cholic acid substrates had been 7α-dehydroxylated.

Reduction of the Δ^6-intermediate

In additional studies, the reduction of the chemically synthesized Δ^6-intermediate to deoxycholic acid was shown to require an induced cell extract and NAD^+ (**Fig. 1**). The individual addition of $FADH_2$ did not cause any apparent increase in the reduction of the Δ^6-intermediate. Moreover, under standard assay conditions argentation chromatography of bile acid products has failed to detect any accumulation of the Δ^6-intermediate.

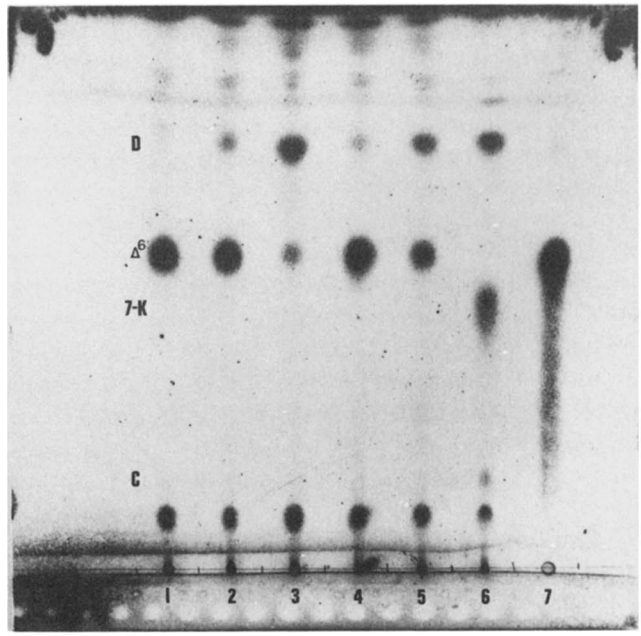

Fig. 1. Reduction of the Δ^6-intermediate (Δ^6) to deoxycholic acid (D) by induced cell extracts of *Eubacterium* sp. V.P.I. 12708. Lane 1, uninduced cell extracts containing 0.5 mM NAD^+; lane 2, induced cell extracts, no additions; lane 3, induced cell extract + 0.5 mM NAD^+; lane 4, induced cell extract + $FADH_2$; lane 5, induced cell extract + 0.5 mM NAD^+ and 0.2 mM $FADH_2$; lane 6, induced cell extract containing cholic acid (C) + 0.5 mM NAD^+ (7-K is 3α,12α-dihydroxy-7-keto-5β-cholanoic acid); and lane 7, Δ^6-standard. The reactions were terminated after 5 min and bile acid products were extracted and chromatographed on 5% $AgNO_3$-treated TLC plates. Bile acids were detected by spraying thin-layer plates with phosphomolybdic acid and heating for 10–15 min at 180°C.

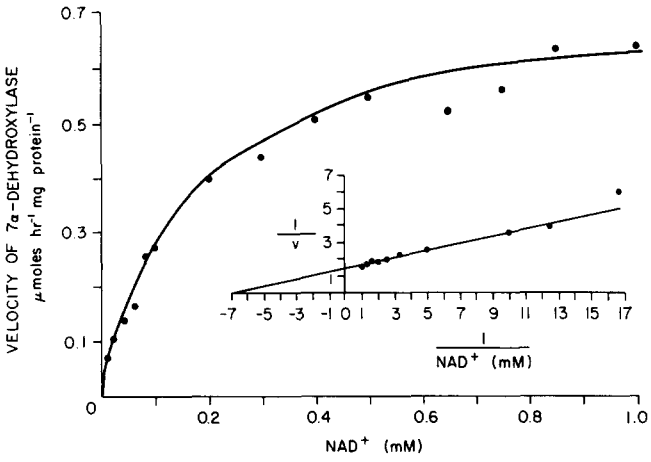

Fig. 2. The effect of NAD^+ concentration on 7α-dehydroxylation activity using cholic acid (104 μM) as the substrate. Inset shows double reciprocal plot of the data. Assays contained 1 mg of extract protein and all other standard assay components described in Materials and Methods. The assays also contained a NADPH-regenerating system consisting of 0.1 mM NADPH, 5 mM glucose-6-phosphate, and 2.5 units of NADPH-specific glucose-6-phosphate dehydrogenase (G-6-P DH). The G-6-P DH system was employed to inhibit the activity of $NADP^+$-dependent 7α-hydroxysteroid dehydrogenase which is a competing enzyme for primary bile acid substrates in extracts of *Eubacterium* species V.P.I. 12708. The G-6-P DH system did not inhibit 7α-dehydroxylation activity at standard assay concentrations of NAD^+ and primary bile acid.

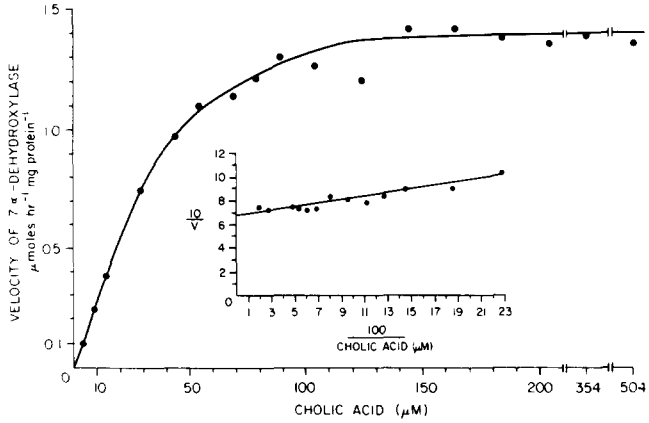

Fig. 4. The effect of cholic acid concentration on 7α-dehydroxylation in the presence of 1 mM NAD^+. Inset shows double reciprocal plot of the data. Assay conditions were as described in Fig. 2.

Saturation kinetics

Saturation kinetics of 7α-dehydroxylation were determined for NAD^+, cholic acid, and chenodeoxycholic acid. The saturation curves for NAD^+ with either cholic acid (**Fig. 2**) or chenodeoxycholic acid (**Fig. 3**) as the substrate were hyperbolic, and double reciprocal plots yielded apparent K_m values of 0.13 mM and 0.06 mM NAD^+, respectively. The substrate saturation kinetics for cholic acid (**Fig. 4**) gave an apparent K_m of 25 μM. In contrast, the substrate saturation kinetics for chenodeoxycholic acid (**Fig. 5**) were different with substrate concentrations above 50 μM showing inhibition. The curve was hyperbolic at substrate concentrations below 50 μM, but gradually declined between the concentrations of 50 μM to 204 μM. Marked inhibition occurred at concentrations above 204 μM.

Relative molecular weight determination of 7α-dehydroxylase

Cell extracts containing 7α-dehydroxylase activity were chromatographed anaerobically on a Bio-Gel A 1.5 M gel filtration column as described in Materials and Methods. The 7α-dehydroxylase activity eluted as a single symmetrical peak (**Fig. 6**) with maximum activity in fraction 12. The relative molecular weight of 7α-dehydroxylase was estimated from three independent experiments to be 114,000. NADH:flavin oxidoreductase activity, which is also induced by cholic acid in this bacterium (13), eluted in fractions 8–10 with a peak in fraction 9. Although 7α-dehydroxylase

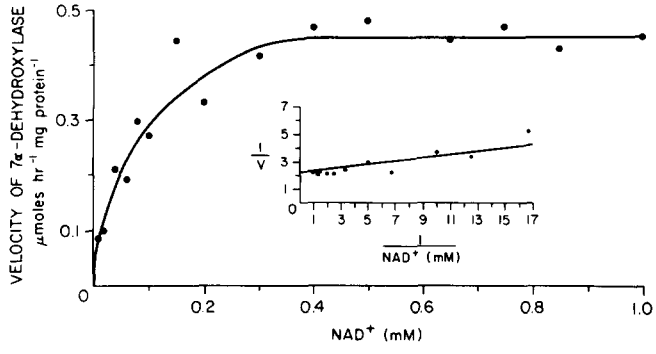

Fig. 3. The effect of NAD^+ concentration on 7α-dehydroxylation activity using chenodeoxycholic acid (104 μM) as the substrate. Inset shows double reciprocal plot of the data. Assay conditions were as described in Fig. 2.

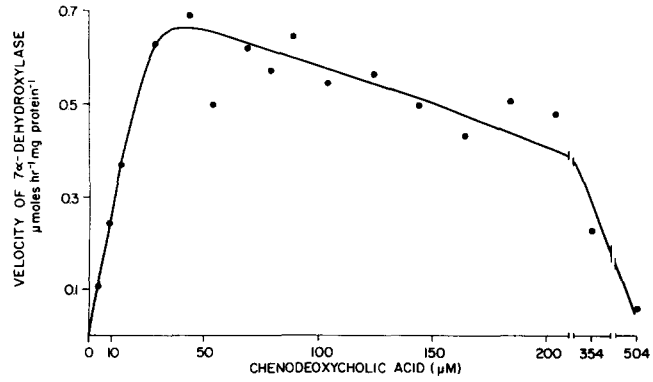

Fig. 5. The effect of chenodeoxycholic acid concentration on 7α-dehydroxylation activity in the presence of 1 mM NAD^+. Assay conditions were as described in Fig. 2.

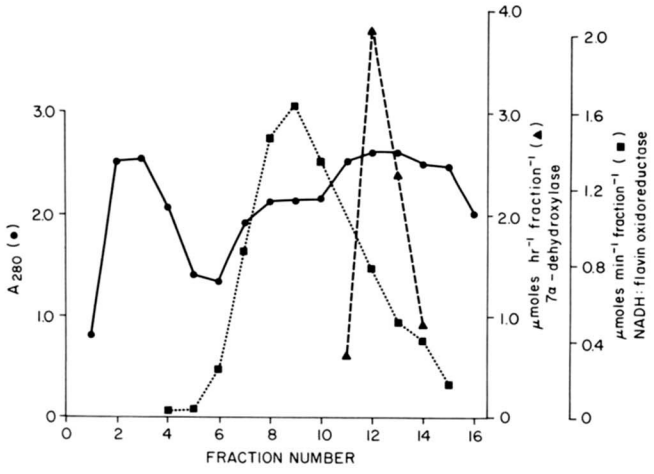

Fig. 6. Anaerobic Bio-Gel A 1.5-M column chromatography of cell extract 7α-dehydroxylase activity from *Eubacterium* species V.P.I. 12708. 7α-Dehydroxylase and NADH:flavin oxidoreductase activities were assayed as described in Materials and Methods.

activity is stable in frozen cell extracts, the activity was highly labile during column chromatography. Approximately 90% of the original activity added to the column was lost during the elution. Similar lability was evident during attempts to purify the enzyme by ion exchange or affinity chromatography.

Polyacrylamide gel electrophoresis

The soluble polypeptide profiles from induced (cholic acid) and uninduced cultures of *Eubacterium* sp. V.P.I. 12708 were analyzed by both one- and two-dimensional PAGE. At least three new polypeptides, at molecular weights of 77,000, 27,000, and 23,500 were detected when this organism was grown in the presence of cholic acid (**Fig. 7**). When [^{35}S]methionine-labeled polypeptides from induced (**Fig. 8A**) and uninduced (Fig. 8b) cultures were analyzed by two-dimensional SDS-PAGE, at least five new polypeptides were observed. The three new polypeptides observed by one-dimensional SDS-PAGE were detected in the two-dimensional SDS-PAGE profile, as well as two additional polypeptides at a molecular weight of 56,000 but with different pI values. None of these polypeptides was detected in uninduced cultures of this bacterium.

DISCUSSION

7α-Dehydroxylation of bile acids is a unique reaction in steroid biochemistry involving both a putative dehydration and reductive step. The proposed reaction mechanism for 7α-dehydroxylation of cholic acid was elucidated by Samuelsson (23) using whole animals and doubly labeled (^{3}H and ^{14}C) cholic acid. The initial step in the reaction is believed to proceed by a diaxial transelimination of water resulting in the release of the 7α-hydroxy group and the 6β-hydrogen. The resulting Δ^6-intermediate is subsequently reduced by transhydrogenation at the 6α and 7β position to yield deoxycholic acid. However, the enzyme can reduce the Δ^6-*cis* intermediate (Fig. 1). Therefore, the exact mechanism of 7α-dehydroxylation by this bacterium is not yet clear. Ferrari, Scolastico, and Beretta (24) presented data to support this mechanism by demonstrating the specific reduction of the Δ^6-intermediate in vitro by cell extracts of a 7α-dehydroxylating strain of *Clostridium bifermentans*. In this regard, we have shown that the reduction of the Δ^6-intermediate required cell extracts prepared from cultures of *Eubacterium* sp. V.P.I. 12708 grown in the presence of cholic acid and the addition of NAD^+ to the reaction mixture.

The cofactor requirement for 7α-dehydroxylation should be viewed and interpreted in conjunction with Samuelsson's (23) proposed reaction mechanism. A priori, one might predict a requirement for reduced cofactors for the conversion of the Δ^6-intermediate to deoxycholic acid (15). In this regard, the

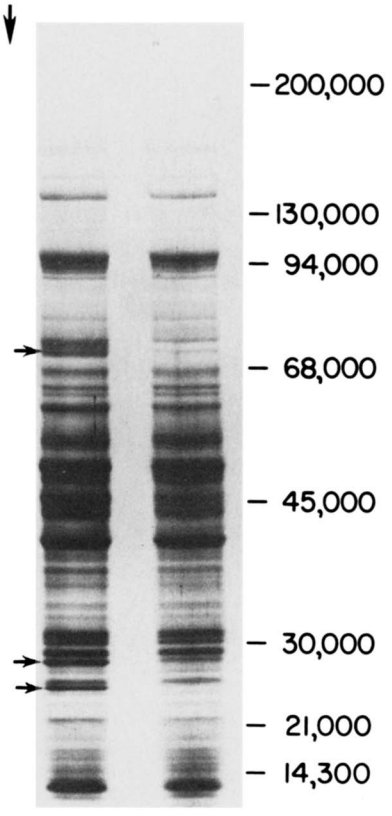

Fig. 7. Soluble polypeptide profiles of cholic acid-induced (left) and uninduced (right) cultures of *Eubacterium* species V.P.I. 12708 analyzed by exponential SDS-PAGE slabs. Approximately 25 μg of protein was added per well. Molecular weights of high and low range standards are given on the right. Arrows indicate the synthesis of new polypeptides at molecular weights of 77,000, 27,000, and 23,500.

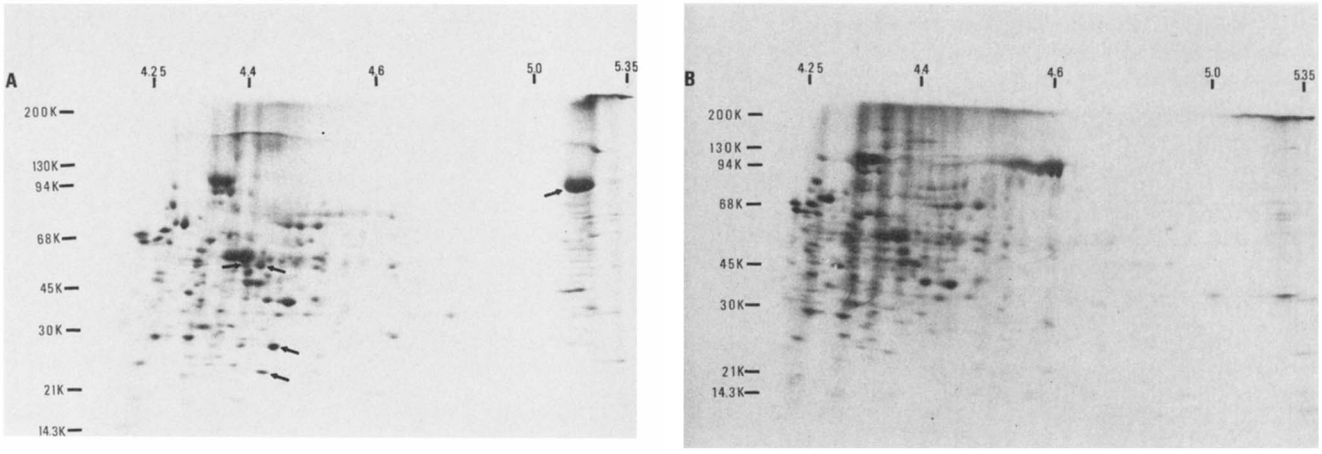

Fig. 8. Radioautographs of [^{35}S]methionine-labeled polypeptide-patterns from cholic acid induced (A) and uninduced (B) cultures of *Eubacterium* species V.P.I. 12708 as analyzed by two-dimensional SDS-PAGE. The pH range of the isoelectric focusing dimension is given on the axis. The molecular weight range is given on the ordinate. Arrows indicate the synthesis of new polypeptides at molecular weights of 77,000, two at 56,000, 27,000 and 23,500.

requirement for NAD^+ was very surprising. NAD^+ was required for the 7α-dehydroxylation of cholic acid and chenodeoxycholic acid, and for the reduction of the Δ^6-intermediate in reaction mixtures lacking exogenous electron donors. Moreover, the elimination of glutathione from reaction mixtures did not change the requirement for NAD^+ nor detectably alter 7α-dehydroxylase activity. Saturation kinetics for NAD^+ were not sigmoid, suggesting that NAD^+ does not act as a classical effector molecule during 7α-dehydroxylation. However, the results of the present study do not rule out the possibility that NAD^+ might be metabolized in some manner during the biotransformation. Additional studies are in progress to elucidate the role of NAD^+ in the 7α-dehydroxylation reaction.

Several interesting questions are raised by the proposed mechanism for 7α-dehydroxylation. For instance, are the dehydration and the reductive steps separate reactions catalyzed by two distinct separable enzymes? Alternatively, does an enzyme complex or only a single enzyme catalyze the 7α-dehydroxylation of bile acids? We found that under our standard assay conditions, the Δ^6-intermediate does not detectably accumulate. Furthermore, both 7α-dehydroxylase and the Δ^6-intermediate reduction activities eluted from gel filtration columns as a single symmetrical peak.[3] These results suggest that a single enzyme or enzyme complex may catalyze 7α-dehydroxylation reaction.

We previously reported the rapid induction (>90-fold in 0.2 cell generation) of 7α-dehydroxylase and NADH:flavin oxidoreductase activities in this bacterium (12). The induction was highly specific, requiring a free C-24 carboxyl group and an unhindered 7α-hydroxy group on the B ring of the steroid nucleus. Analysis of whole cell polypeptides by two-dimensional SDS-PAGE from induced and uninduced extracts revealed the presence of at least five new polypeptides in induced cell extracts. Although it is not known which of these polypeptides form 7α-dehydroxylase, it will be interesting to determine if any are associated with the cytoplasmic membrane.

Attempts to demonstrate uptake of [^{14}C]cholic acid in this bacterium have failed to show significant accumulation (<1 nmol min^{-1} per mg $protein^{-1}$). Therefore, the mechanism of induction has not yet been elucidated. However, Saier (25) and Dills et al. (26) have recently reviewed inductive enzyme systems in bacteria that appear to require an exogenous inducer. One model proposes the presence of a constitutive transmembrane receptor protein that has at least two binding sites with different specificities. The intracellular binding site has an affinity for an activator or repressor protein. The extracellular binding of an inducer molecule is proposed to produce an allosteric change in the transmembrane receptor protein that alters the affinity for the activator or repressor molecule. This event is hypothesized to change the internal equilibrium between transmembrane receptor and putative DNA binding sites resulting in a change in gene expression. It is not known if the synthesis of 7α-dehydroxylase is regulated in this manner; however, it is worth noting the profound effect of cholic acid on the synthesis of new polypeptides in this organism.

The physiological significance of 7α-dehydroxylation to the bacterium is not clear. Nevertheless, the reductive nature of this biotransformation might

[3] Hylemon, P. B. Unpublished data.

suggest that the bacterium may use this reaction as a mechanism to dispose of electrons generated by fermentative metabolism. It is also possible that secondary bile acids may be sufficiently toxic to other intestinal bacteria so as to provide a selective advantage for 7α-dehydroxylating bacteria. Further purification and characterization of this enzyme and the isolation of specific 7α-dehydroxylase negative mutant bacteria are required to clarify the mechanism and significance of 7α-dehydroxylation to intestinal anaerobic bacteria.

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