Regulation of bile acid 7-dehydroxylase activity by NAD^+ and NADH in cell extracts of *Eubacterium* species V.P.I. 12708

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Abstract The 7α-dehydroxylation of primary bile acids by *Eubacterium* sp. V.P.I. 12708 required a cell extract prepared from a cholic acid-induced culture and NAD^+. NADH (0.5 mM) inhibited bile acid 7-dehydroxylase activity more than 50% when added to reaction mixtures containing NAD^+ (0.5 mM). Saturation kinetics and double reciprocal plots of NADH inhibition were consistent with negative cooperativity. 7-Dehydroxylase activity was modulated by the molar ratio of NAD^+–NADH with maximal activity at a NAD^+ mole fraction of 0.75 to 0.85. NADH stimulated 7-dehydroxylase activity (30% to 50%) at low concentration(<0.15 mM) and inhibited at higher concentrations. Reduction of the proposed Δ^6-intermediate (3α-hydroxy-5β-6-cholen-24-oic acid) to lithocholic acid required a cell extract from a cholic acid-induced culture and was stimulated by the addition of NAD^+. Reduced flavin nucleotides stimulated (32% to 62%) and NADH (0.5 mM) inhibited (78%) the reduction of the Δ^6-intermediate to lithocholic acid. 7-Dehydroxylase was highly specific for bile acid substrates and required a free C-24 carboxyl group and an unhindered 7α- or 7β-hydroxy group on the B-ring of the steroid nucleus for activity. Bile acid 7α- and 7β-dehydroxylase and Δ^6-reductase activities all co-eluted from an anaerobic high performance liquid chromatography gel filtration column. However, approximately 80% to 96% of the total units of activity were lost. A substantial portion (20% to 30%) of the total activity was recovered when material from low molecular weight (8,000 to 14,000 M_r) eluting fractions was added back to fractions containing enzyme activity. These studies show that 7-dehydroxylase is highly specific for substrates and its activity may be regulated by the NAD^+–NADH ratio in the bacterial cell.—**White, B. A., D. A. M. Paone, A. F. Cacciapuoti, R. J. Fricke, E. H. Mosbach, and P. B. Hylemon.** Regulation of bile acid 7-dehydroxylase activity by NAD^+ and NADH in cell extracts of *Eubacterium* species V.P.I. 12708. *J. Lipid. Res.* 1983. **24:** 20–27.

Supplementary key words Δ^6-intermediate • substrate specificity • HPLC chromatography

The 7α-dehydroxylation of cholic acid and chenodeoxycholic acid results in the formation of deoxycholic and lithocholic acid, respectively (1). Samuelsson (2) proposed that the initial step in 7α-dehydroxylation consists of the diaxial trans-elimination of the 7α-hydroxy group and the 6β-hydrogen followed by reduction of the proposed Δ^6-intermediate to secondary bile acids. Various intestinal anaerobic bacteria have been reported to have 7α-dehydroxylation activity (3–6); however, the enzymology of this biotransformation has been studied in detail only recently. Bile acid 7-dehydroxylase activity has been reported to be inhibited by molecular oxygen and highly labile during attempted purification procedures (7, 8).

Eubacterium sp. V.P.I. 12708 contains a cholic acid-inducible 7-dehydroxylase that is stimulated by the addition of exogenous NAD^+ (7). White, Fricke, and Hylemon (8) recently demonstrated that this organism could also 7β-dehydroxylate ursodeoxycholic acid directly to lithocholic acid. 7β-Dehydroxylation may be carried out by the same enzyme or enzyme complex that 7α-dehydroxylates cholic acid and chenodeoxycholic acid (8). The present report describes the regulation of 7-dehydroxylase activity by pyridine nucleotides, cofactor requirements for the reduction of the Δ^6-intermediate, substrate specificity studies, and the discovery of a low molecular weight stimulatory factor for 7-dehydroxylase.

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; ursodeoxycholic acid, 3α,7β-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-hydroxy-5β-6-cholen-24-oic acid; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

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METHODS AND MATERIALS

Growth of bacteria

Characteristics, growth conditions, and media for the anaerobic culturing of *Eubacterium* sp. V.P.I. 12708 have been described previously (9). Bacterial cultures, in 1- or 2-liter volumes, were induced to synthesize 7-dehydroxylase by the addition of sodium cholate (0.1 mM) at hourly intervals during logarithmic growth (7). Cell extracts were prepared anaerobically by disruption with a chilled French Pressure cell (7). The 105,000 *g* supernatant fluid was immediately dialyzed (4°C) against 1 liter of anaerobic 50 mM sodium phosphate buffer (pH 6.8) containing 12% glycerol (v/v) and 5 mM glutathione for 18 hr with one buffer change. Protein concentrations were determined by the spectrophotometric method of Kalb and Bernlohr (10).

Quantitative enzyme assay for 7-dehydroxylase and reduction of 3α-hydroxy-5β-6-cholen-24-oic acid

Enzymatic 7-dehydroxylation of [^{14}C]carboxyl-labeled cholic acid, chenodeoxycholic acid, [11,12^{3}H (N)]ursodeoxycholic acid, or the reduction of [^{14}C]carboxyl-labeled 3α-hydroxy-5β-6-cholen-24-oic acid was followed by measuring the rate of secondary bile acid formation using a radiochromatographic assay procedure (11). Unless otherwise noted, the standard cell extract reaction mixture contained (1.0 ml total volume): 25 mM sodium acetate–morpholine–propane–sulfonate (MOPS) buffer (pH 7.5), 104 μM radiolabeled bile acid substrate, 0.5 mM NAD^+, and cell extract (1 to 2 mg protein). Assays were initiated by the addition of cell extract and were incubated anaerobically (37°C) under an argon atmosphere for 2 min. The reaction was stopped by acidification. The acidified reaction mixtures (pH 1 to 2) were extracted with ethyl acetate and the bile acid products were quantitated as described previously (11). Lithocholic acid was resolved from the Δ^6-intermediate by argentation chromatography on thin-layer plates predeveloped in 10% $AgNO_3$. Care must be taken to count the silica gel samples rapidly as the $AgNO_3$ appears to react with the scintillation cocktail to form a very strong color quench within 20 min. Argentation chromatography was performed under subdued lighting. Reduced flavin nucleotides were prepared using molecular hydrogen and a palladium catalyst as described by Feighner and Hylemon (12). 7-Dehydroxylation and the reduction of Δ^6-intermediate were linear with time up to 4 min and the rates were linear with protein (0.5 to 2.5 mg protein).

Anaerobic high performance lipid chromatography

Anaerobic high performance liquid chromatography (HPLC) of induced cell extracts was carried out with a Beckman Model 332 HPLC using an Altex Spherogel TSK 3000 column (0.75 cm × 60 cm) and precolumn (0.75 cm × 10 cm) at room temperature. The column was washed with anaerobic 50 mM sodium phosphate buffer (pH 6.8) containing 12% glycerol (v/v), 0.1 M NaCl, and 5 mM glutathione at a flow rate of 0.85 ml/min. Protein was eluted with the same buffer and fractions were collected (4°C) by hand at 1-min intervals in stoppered tubes under an argon atmosphere. Fractions were assayed immediately for cholic acid, chenodeoxycholic acid, and ursodeoxycholic acid 7-dehydroxylase activity as well as the reduction of the Δ^6-intermediate. The column was calibrated with aldolase (158,000), bovine serum albumin-dimer (136,000), bovine serum albumin-monomer (68,000), ovalbumin (45,000), chymotrypsinogen (25,000) and cytochrome C (12,500).

Polyacrylamide gel electrophoresis

Partially purified (HPLC) polypeptides from induced and uninduced cell extracts were analyzed by SDS-PAGE as previously described (7). Polypeptide profiles were visualized by Coomassie blue staining (7).

Substrate specificity of 7-dehydroxylase

Substrate specificity of 7-dehydroxylase was determined by individually adding steroids (100 μM) to reaction mixtures under standard assay conditions. Enzyme activity was terminated after 5 min by the addition of 0.5 ml of dilute (0.5 N) HCl. Steroids were extracted into ethyl acetate and separated on Silica Gel G plates. Unconjugated bile acids, bile alcohols, glycochenodeoxycholic acid, and glycolithocholic acid were separated in solvent systems described by Eneroth (13). Mono-unsaturated bile acids were separated on Silica Gel G plates treated with 10% $AgNO_3$ using solvent systems described by Eneroth (13). Taurine-conjugated bile acids were separated in solvent systems described by Macdonald et al. (14). Glycocholic acid and glycodeoxycholic acid were separated in solvent systems described by Aries and Hill (15). All steroids were detected by spraying with phosphomolybdic acid followed by heating for 10–15 min at 180°C. Positive activity was defined as the formation of the appropriate 7-dehydroxylated product in extracts prepared from a cholic acid-induced culture following the addition of NAD^+. Negative controls included assays performed with cell extracts prepared from uninduced cultures as well as cell extracts prepared from induced cultures minus NAD^+.

Chemicals and enzymes

24-[^{14}C]Cholic acid (50 mCi/mmol), 24-[^{14}C]-chenodeoxycholic acid (50 mCi/mmol), and [11, 12-^{3}H (N)]ursodeoxycholic acid (37 Ci/mmol) were purchased

TABLE 1. Effect of oxidized and reduced pyridine nucleotide analogues on cholic acid 7α-dehydroxylase activity in cell extracts of *Eubacterium* sp. V.P.I. 12708

Pyridine Nucleotide(s) Added[a]	Specific Activity[b]
None added	149 ± 51
NAD^+	704 ± 21
3-Acetylpyridine adenine dinucleotide	252 ± 5
Nicotinamide hypoxanthine dinucleotide	252 ± 51
3-Acetylpyridine hypoxanthine dinucleotide	208 ± 58
3-Pyridinealdehyde adenine dinucleotide	94 ± 43
Thionicotinamide adenine dinucleotide	328 ± 23
Nicotinamide mononucleotide	202 ± 30
NAD^+ + NADH	356 ± 7
NAD^+ + 3-acetylpyridine adenine dinucleotide (reduced)	646 ± 33
NAD^+ + nicotinamide hypoxanthine dinucleotide (reduced)	608 ± 79

[a] Final concentrations of pyridine nucleotides were 0.5 mM.
[b] Units are nmoles of deoxycholic acid formed per hr per mg protein.

from New England Nuclear. 24-[^{14}C]-3-Hydroxy-5β-6-cholen-24-oic acid (0.14 nCi/μmol) was synthesized chemically from chenodeoxycholic acid as described previously (7). Radiolabeled bile acids were greater than 99% pure as determined by thin-layer chromatography. The following bile acids and alcohols were obtained from Steraloids, Inc: methyl-3α,7α,12α-trihydroxy-5β-cholan-24-oate, mp 154–156°C; 3α,6α-dihydroxy-5β-cholan-24-oic acid, mp 203–205°C; 3α,6β-dihydroxy-5β-cholan-24-oic acid, mp 210–212°C; 3α,6α,7α-trihydroxy-5β-cholan-24-oic acid, mp 188–189°C; 3α,6β,7β-trihydroxy-5β-cholan-24-oic acid, mp 226–228°C; 5β-cholane-3α,7α,12α-24-tetrol, mp 225–227°C; 3β-hydroxy-5-cholen-24-oic acid, mp 232–234°C; 3α,12α-dihydroxy-5β-8-cholen-24-oic acid, mp 170–173°C; and 3α-hydroxy-12-keto-5β-9-cholen-24-oic acid, mp 113.5–114.5°C. N-(3α,7α-Dihydroxy-5β-cholan-24-oyl) glycine, mp 108–112°C; N-(3α,7α,12α-trihydroxy-5β-cholan-24-oyl) taurine, mp 197–202°C; N-(3α,7α-dihydroxy-5β-cholan-24-oyl) taurine, mp 148–151°C; cholic acid, mp 204–206°C; chenodeoxycholic acid, mp 143–146°C; and 3α,7α-dihydroxy-12-keto-5β-cholan-24-oic acid, mp 227–228°C were purchased from Calbiochem. N-(3α,7α,12α-Trihydroxy-5β-cholan-24-oyl) glycine, mp 132–135°C and ursodeoxycholic acid, mp 198–200°C were obtained from Sigma Chemical Co. 5β-Cholestane-3α,7α-diol, mp 83–85°C was chemically synthesized as described by Bergstrom and Krabisch (16) and 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid, mp 180–182°C was chemically synthesized as described by Batta et al. (17). All steroids were shown to be pure by thin-layer chromatography.

Molecular weight standards for column chromatography were Combitlek 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 P. L. Biochemicals Inc. and flavin nucleotides and *Torula* yeast glucose-6-phosphate dehydrogenase were from Sigma Chemical Co. All other chemicals were the highest grade commercially available.

RESULTS

Effect of pyridine nucleotides on 7-dehydrogenase activity

We reported previously that maximal 7-dehydroxylase activity required the addition of NAD^+ to anaerobically dialyzed cell extracts (7). Moreover, preliminary data indicated that NADH may inhibit 7-dehydroxylase activity (7). Therefore, the specificity of this enzyme for pyridine nucleotides and the possible mechanism of NADH inhibition was investigated further. First, analogues of NAD^+ and NADH were individually added to cell extracts and 7-dehydroxylase specific activity was determined. The results presented in **Table 1** show that replacement of the amino group of the adenine moiety with a hydroxy group and/or the amide side chain of the pyridine ring with acetyl, aldehyde, or thiol group substantially reduced the ability of pyridine nucleotides to alter 7-dehydroxylase activity.

Saturation kinetics were determined for NAD^+ in the presence or absence of selected constant concentrations of NADH. Saturation kinetics for NAD^+ in the absence of NADH were hyperbolic; however, when 0.5 mM NADH was included in reaction mixtures the saturation kinetics were biphasic (**Fig. 1**). Atkinson (18) has emphasized that the shape of the curve depends largely on the scale of the abscissa employed. In the scale used for Fig. 1, there is an apparent cooperative interaction between NAD^+ and NADH. Double reciprocal plots for several different NADH concentrations were biphasic (**Fig. 2**). Similar concave downward double reciprocal plots have been reported by Levitzki and Koshland (19) to be consistent with negative cooperativity. Additional studies showed that the inhibitory effect of NADH was reduced substantially (50%) by the presence of 0.1 M sodium chloride (**Table 2**).

In order to diminish concentration effects, 7-dehydroxylase reaction velocity was determined while varying the mole fraction NAD^+ [$NAD^+/(NAD^+ + NADH)$] when the sum of NAD^+ and NADH was held constant at 0.16 mM and 0.3 mM. Interestingly, the curves show optimal 7-dehydroxylase activity at a mole fraction NAD^+ of 0.75 to 0.85 (**Fig. 3**). The V_{max} estimated from saturation kinetics for NAD^+ in the absence of NADH is approximately 1000 nmol/hr per mg protein, whereas the maximal activities at NAD^+ mole fraction of 0.75

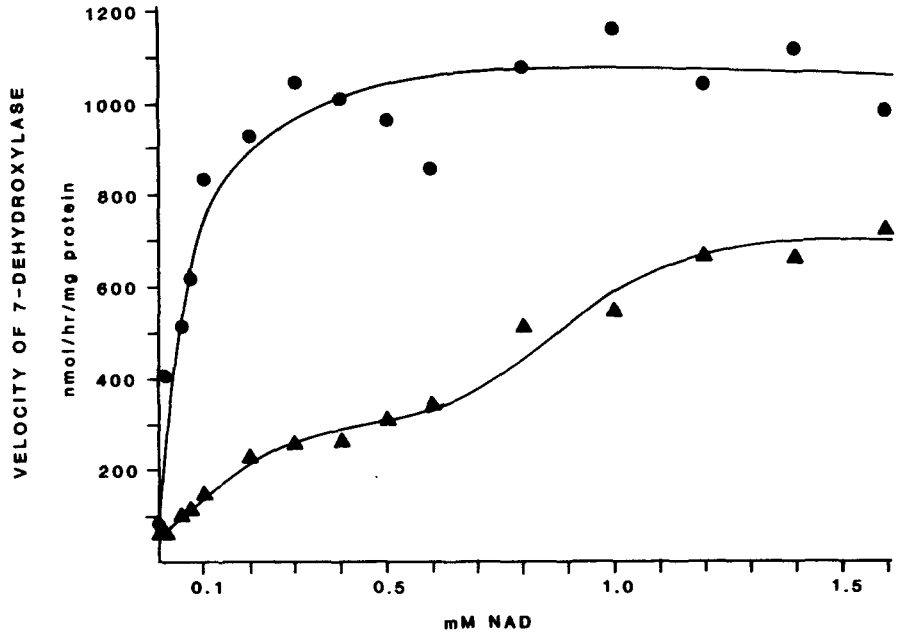

Fig. 1. The effect of NAD^+ concentration on bile acid 7-dehydroxylase activity in the absence (●) and presence (▲) of 0.5 mM NADH using cholic acid as (104 μM) as the substrate. Assays contained 1 mg of cell extract protein and all other standard assay components described in Methods and Materials. 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-PDH). The G-6-PDH 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* sp. V.P.I. 12708. The G-6-PDH system did not inhibit 7-dehydroxylase activity at standard assay conditions of NAD^+ and primary bile acid.

to 0.85 are 1300 to 1500 nmol/hr per mg protein. Surprisingly, when these reaction mixtures were monitored spectrophotometrically under anaerobic conditions at 340 nm (37°C) a cholic acid, but not deoxycholic acid, dependent increase in the oxidation of NADH was detected at an NAD^+ mole fraction of 0.8 (0.06 mM NADH and 0.24 mM NAD^+).

Cofactor requirements for the reduction of the Δ^6-intermediate to lithocholic acid

The effect of various pyridine and flavin nucleotides on the rate of [^{14}C]-3α-hydroxy-5β-6-cholen-24-oic acid reduction to lithocholic acid was determined (**Table 3**). The addition of NAD^+ increased the specific activity approximately twofold. Surprisingly, both $FADH_2$ and $FMNH_2$ stimulated this reduction to rates similar to primary bile acid 7α-dehydroxylation when added to reaction mixtures containing NAD^+. Stimulation by flavin nucleotides was not detected when either cholic acid, chenodeoxycholic acid, or ursodeoxycholic acid was used as the bile acid substrate (7, 8). The addition of FAD or FMN to reaction mixtures with or without NAD^+ inhibited slightly the reduction of the Δ^6-intermediate. NADH (0.5 mM) inhibited (78%) the conversion of the Δ^6-intermediate to lithocholic acid when added to reaction mixtures containing NAD^+ and $FADH_2$. We have not detected hydration of the [^{14}C]-Δ^6-intermediate to either chenodeoxycholic acid or ursodeoxycholic acid under our standard assay conditions.

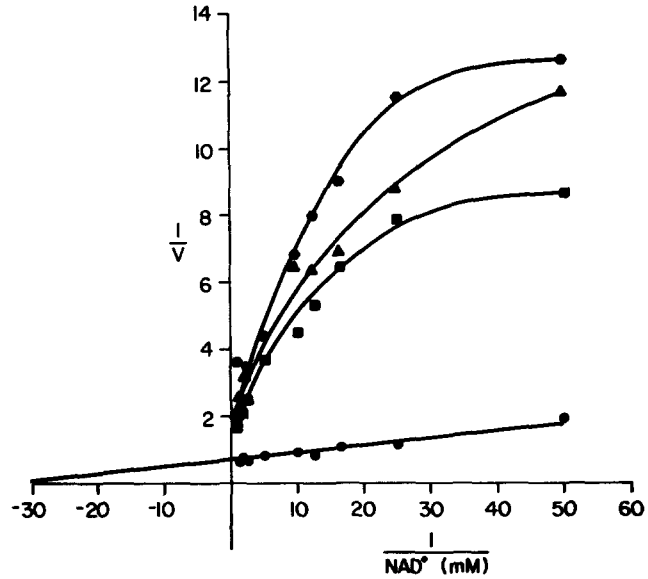

Fig. 2. Double reciprocal plot of the rate of 7-dehydroxylation using cholic acid (104 μM) as the substrate. NAD^+ concentrations were varied in the presence of 0.5 mM NADH (■), 0.75 mM NADH (▲), and 1.0 mM NADH (●), and in the absence of NADH (●). Assays conditions were as described in Fig. 1.

TABLE 2. Effect of 0.1 M NaCl addition of the regulation of 7α-dehydroxylase activity by pyridine nucleotides

Addition[a]	Specific Activity[b]
None	214 ± 48
NAD^+	810 ± 107
NAD^+ + NADH	169 ± 10
NAD^+ + NaCl	656 ± 31
NAD^+ + NADH + NaCl	493 ± 46

[a] Final concentrations of pyridine nucleotides were 0.5 mM.
[b] Units are nmoles of deoxycholic acid formed per hr per mg protein.

Bile acid substrate specificity

The bile acid substrate specificity of 7-dehydroxylase was determined as described in Methods and Materials. Cholic acid, chenodeoxycholic acid, 3α,7α-dihydroxy-12-keto-5β-cholan-24-oic acid, and ursodeoxycholic acid were 7-dehydroxylated under the conditions described. The following steroids were not 7-dehydroxylated: methyl-3α,7α,12α-trihydroxy-5β-cholan-24-oate; N-(3α,7α,12α-trihydroxy-5β-cholan-24-oyl) glycine; N-(3α,7α-dihydroxy-5β-cholan-24-oyl) glycine; N-(3α,7α,12α-trihydroxy-5β-cholan-24-oyl)taurine; N-(3α,7α-dihdyroxy-5β-cholan-24-oyl) taurine; 3α,6α-dihydroxy-5β-cholan-24-oic, 3α,6α,7α-trihydroxy-5β-cholan-24-oic acid; 3α,6β-dihydroxy-5β-cholan-24-oic acid; 3α,6β,7β-trihydroxy-5β-cholan-24-oic acid; 5β-cholane-3α,7α,12α,24-tetrol, 5β-cholestane-3α,7α-diol, and 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid. The data indicate that 7-dehydroxylase requires a free C-24 carboxyl group and an unhindered 7α- or 7β-hydroxy group on the B-ring of the steroid nucleus for activity. In addition, there was no detectable reduction of the following mono-unsaturated bile acids: 3β-hydroxy-5-cholen-24-oic acid; 3α,12α-dihydroxy-5β-8-cholen-24-oic acid, and 3α-hydroxy-12-keto-5β-9-cholen-24-oic acid.

Anaerobic gel filtration chromatography using HPLC

Dialyzed cell extracts containing 7-dehydroxylase activity were chromatographed anaerobically on an Altex TSK 3000 gel filtration column as described in Methods and Materials. Chenodeoxycholic acid, ursodeoxycholic acid 7-dehydroxylase activities, and Δ^6-reductase activity all co-eluted as a single symmetrical peak (**Fig. 4**). 7-Dehydroxylase activity was labile during HPLC, as approximately 80% to 96% of the original units were lost during the chromatography. Similar lability was previously reported in earlier attempts at purification of 7-dehydroxylase (7). However, it was discovered that if materials in HPLC fractions 28–34 were individually added back to pooled fractions 21–24 containing 7-dehydroxylase activity (4% of total units), a substantial

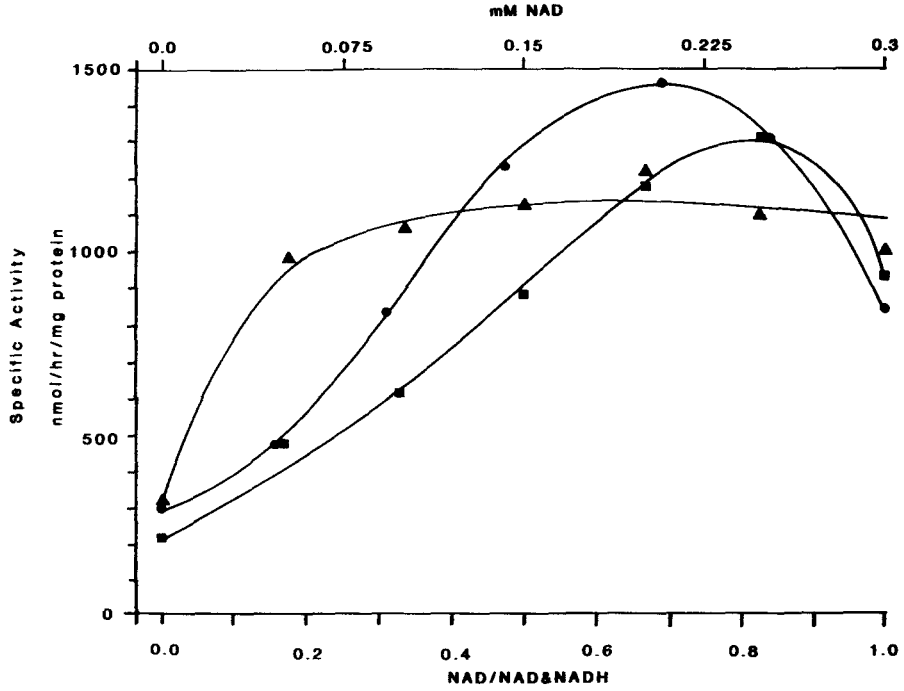

Fig. 3. The effect of NAD^+ concentration (Δ) and NAD^+ mole fraction (●, ■) on 7-dehydroxylase activity using cholic acid (104 μM) as the substrate. The total pyridine nucleotide pools were held constant (●, 0.16 mM; ■, 0.3 mM) in determinations of NAD^+ mole fraction (i.e., at mole fraction of 0.5, both NAD^+ and NADH were 0.15 mM). Assay conditions were as described in Fig. 1.

TABLE 3. Cofactor requirements for the reduction of 3α-hydroxy-5β-6-cholen-24-oic acid to lithocholic acid by cell extracts of *Eubacterium* sp. V.P.I. 12708

Cofactors Added	Specific Activity[a]
mM	
None	260 ± 3
NAD^+ (0.5)	460 ± 71
NAD^+ (0.5) + $FADH_2$ (0.2)	605 ± 26
NAD^+ (0.5) + $FMNH_2$ (0.2)	743 ± 193
NAD^+ (0.5) + FAD (0.2)	231 ± 93
NAD^+ (0.5) + FMN (0.2)	279 ± 108
NAD^+ (0.5) + $FADH_2$ (0.2) + NADH (0.5)	132 ± 5
$NADP^+$ (0.5)	231 ± 63
NADPH (0.5)	249 ± 19
NADH (0.5)	74 ± 2
$FADH_2$ (0.2)	219 ± 17
$FMNH_2$ (0.2)	273 ± 8
FAD (0.2)	94 ± 1
FMN (0.2)	166 ± 74

[a] Units are nmoles of lithocholic acid formed per hr per mg protein.

amount of the total units (22%) could be recovered (**Table 4**). The nondialyzable low molecular weight (8,000–12,000 M_r) stimulatory material was also present in uninduced cell extracts.

SDS-PAGE of HPLC column fractions

Our laboratory previously reported an increase in the differential synthesis of at least five new polypeptides at molecular weights of 77,000, two at 56,000 with different pI values, 27,000 and 23,5000 when *Eubacterium* sp. V.P.I. 12708 was grown in the presence of cholic acid (7). However, it is not clear which of these polypeptides may comprise the 7-dehydroxylase. Therefore,

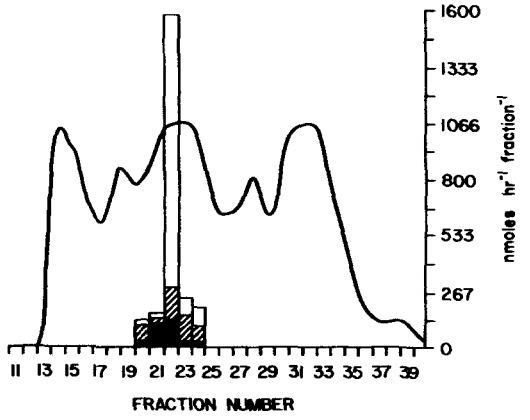

Fig. 4. Anaerobic TSK 3000 HPLC of extracts from *Eubacterium* sp. V.P.I. 12708. Sample characteristics were as follows: 1.0 ml of cell extract containing 14.3 mg of protein and a total of 8,580 units 7-α-dehydroxylase activity was applied to the column and eluted as described in Methods and Materials. The solid line represents the A_{280}. Chenodeoxycholic acid 7α-dehydroxylase activity (open bars), ursodeoxycholic acid 7β-dehydroxylase activity (solid bars), and the reduction of the Δ^6-intermediate (shaded bars) were assayed as described in Methods and Materials.

TABLE 4. Stimulation of bile acid 7-dehydroxylase activity by low molecular weight material

HPLC Fraction No.[a]	Total Units of 7-Dehydroxylase Stimulatory Activity[b]
28	5
29	260
30	754
31	1499
32	1475
33	1236
34	129

[a] A total of 27,000 units 7-dehydroxylase activity (27 mg protein) was applied to an anaerobic HPLC gel filtration column. The only detectable 7-dehydroxylase activity was found in fractions 21–24 (Fig. 4), which contained approximately 4% of the total units.

[b] The assay for 7-dehydroxylase stimulatory activity contained in a total volume of 1.0 ml: 25 mM sodium acetate-MOPS buffer (pH 7.5), 150 μl pooled fractions 21–24, 200 μl of individual HPLC fraction, [^{14}C]cholic acid (104 μM), and NAD^+ (0.5 mM).

we fractionated cholic acid induced and uninduced cell extracts by TSK 3000 HPLC. The polypeptide profile of the most active fraction (22, Fig. 4) from induced extracts was compared to the corresponding fraction of uninduced extract by one-dimensional slab SDS-PAGE. As shown in **Fig. 5,** the induced polypeptides at molecular weights of 77,000, 56,000, and 27,000 are present in TSK 3000 fraction 22 of the induced cell extract; however, the polypeptide at 23,500 was undetectable. None of these polypeptides were detected in uninduced cell extracts.

DISCUSSION

Results described in this study suggest that the activity of bile acid 7-dehydroxylase may be regulated by the NAD^+-NADH ratio in the bacterial cell. In kinetic terms, an enzyme may be considered an allosteric protein if it shows a sigmoidal dependence of velocity on the substrate concentration in the presence or absence of an effector molecule. 7-Dehydroxylase exhibits this type of response when NAD^+ is titrated in the presence of set concentrations of NADH (Fig. 1). Levitzki and Koshland (19) have shown that a downward concave double reciprocal plot, as shown in Fig. 2, is indicative of negative cooperativity. The differential salt effect on NADH inhibition of 7-dehydroxylase activity (Table 2) is also suggestive of an allosterically regulated enzyme. The role of NAD^+ in 7-dehydroxylation remains unclear. We have not been able to detect any net reduction or metabolism of NAD^+ associated with 7-dehydroxylation. Alternatively, we feel that NAD^+ may act only as an activator of 7-dehydroxylase as reported for nitrite reductase of *Escherichia coli* (20), lipoyl dehydrogenase (21), and the esterase activity of horse liver aldehyde

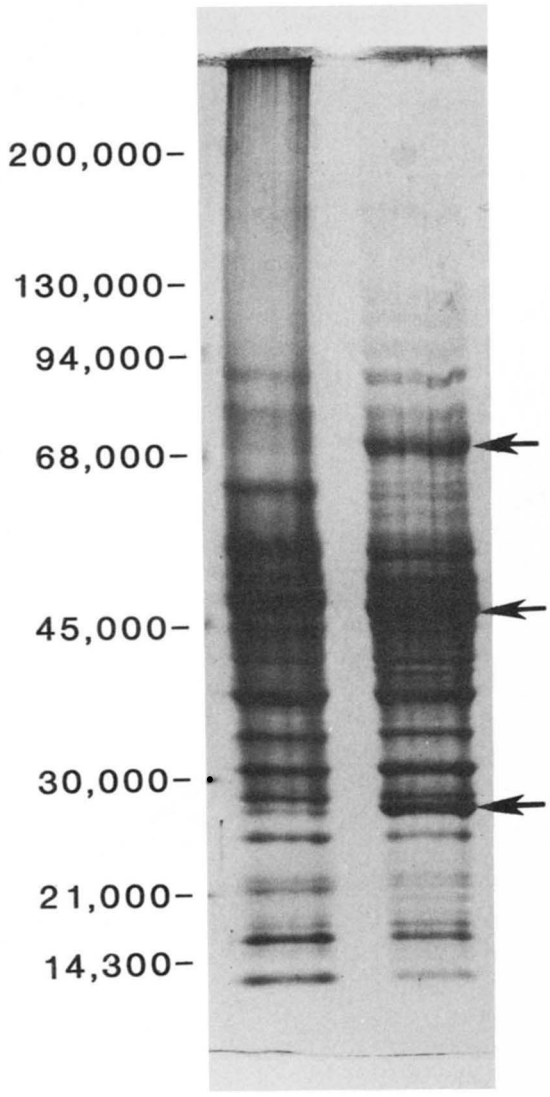

Fig. 5. Partially purified polypeptide profiles (Fraction 22) from cholic acid induced (right) and uninduced (left) cultures of *Eubacterium* sp. V.P.I. 12708 analyzed by exponential SDS-PAGE slab gels. Molecular weights of high and low range standards are given on the left. Arrows indicate cholic acid induced-polypeptides at molecular weights of 77,000, 55,000, and 27,000.

dehydrogenase (22). 7-Dehydroxylase activity appears to be sensitive to regulation by the NAD^+–NADH ratio, such that optimal 7-dehydroxylase activity is seen at an approximate NAD^+ mole fraction of 0.75 to 0.85 (Fig. 3). However, the kinetic curves are dissimilar to ones reported for pyruvate dehydrogenase complex of *E. coli* (23) and *Rickettsia prowazeki* (24).

Our data also show that NADH, an inhibitor of 7-dehydroxylase at concentrations greater than 0.15 mM, is stimulatory to 7-dehydroxylation at lower concentrations (Fig. 3). The unexpected stimulatory effect of NADH at low concentrations, and inhibitory effect at high concentrations may be similar to the enzyme reduction states reported for nitrite reductase of *E. coli* (25) and lipoyl dehydrogenase (26). In these enzyme systems, NADH reduces the enzyme to a catalytically active 2-electron form. This active form may be further reduced by NADH to a 4-electron catalytically inactive enzyme. NAD^+ in these systems acts as an activator, presumably by protecting the enzyme from over-reduction by NADH (25, 26). Whether or not 7-dehydroxylase is regulated in this manner is unknown. Purification of this enzyme and characterization of cholic acid-dependent oxidation of NADH will be required to determine if NADH donates electrons directly to 7-dehydroxylase.

The reduction of the Δ^6-intermediate was stimulated by NAD^+ and inhibited by NADH. The stimulation by reduced flavin nucleotides was unexpected because this effect was not observed with primary bile acid substrates (7, 8). The nature of the relationship between reduced flavin nucleotides and 7-dehydroxylase is unclear at present. Lipsky and Hylemon (27) have reported the induction of NADH:flavin oxidoreductase by cholic acid in this *Eubacterium* species. Although an interaction between 7-dehydroxylase and NADH:flavin oxidoreductase may be implied, we have found no direct evidence for this contention. It is interesting to note that NADH:flavin oxidoreductase oxidizes NADH, which inhibits 7-dehydroxylase, and forms NAD^+ and $FADH_2$, two cofactors that stimulate the reduction of the Δ^6-intermediate (Table 3). However, NADH:flavin oxidoreductase may simply be supplying electrons via reduced flavins to some as yet unidentified electron carrier, which then donates electrons for the reduction of the Δ^6-intermediate. We speculate that the low molecular weight stimulatory factor might function in this manner.

Several interesting questions are raised by our results. For instance, are 7α- and 7β-dehydroxylase activities and the reduction of the Δ^6-intermediate carried out by the same enzyme or enzyme complex? Also, is 7-dehydroxylase a single subunit enzyme with separate binding sites for NAD^+ and NADH, or a multisubunit enzyme with both catalytic and regulatory subunits? In regard to the first question, several lines of evidence suggest that all of the 7-dehydroxylase activities are carried out by the same enzyme or enzyme complex. First, 7α- and 7β-dehydroxylation and the reduction of the Δ^6-intermediate are all induced by cholic acid, all three activities are stimulated by the addition of NAD^+ to reaction mixtures, and are inhibited by NADH (7, 8). Thermal inactivation studies show each activity to be equally sensitive to heating (45°C) (8). Finally, all three activities co-eluted using gel filtration (7, 8) and HPLC gel filtration chromatography as a single symmetrical peak (Fig. 4). Although co-elution using these methods is con-

sistent with a single enzyme, they do not prove that all three activities reside in the same protein; however, analysis of partially purified polypeptides (Fig. 5) from induced and uninduced cell extracts revealed the presence of the induced polypeptides in fraction 22, at molecular weights of 77,000, 55,000, and 27,000, but not at 23,500. It is not known which of these polypeptides form 7-dehydroxylase, but it appears as if the induced polypeptide at 23,500 is not essential for catalytic activity. Future experiments are directed towards characterization of the low molecular weight stimulatory factor so that further purification of 7-dehydroxylase and the isolation of mutant strains of bacteria deficient in 7-dehydroxylase might be possible.

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