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Multiple Forms of 7-α-Hydroxysteroid Dehydrogenase in Selected Strains of *Bacteroides fragilis*

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Multiple forms of 7-α-hydroxysteroid dehydrogenase were detected in six of nine strains of *Bacteroides fragilis*. The enzymes differed with respect to pyridine nucleotide specificity, thermal stability, divalent metal cation requirement, and elution profiles from Sephadex G-200 columns. The nicotinamide adenine dinucleotide phosphate (NADP)-dependent enzyme required divalent metal cations, preferentially Mn^{2+} (K_m, 57 μM), for maximum catalytic activity. The NADP-dependent enzyme was labile at 65 C for 10 min, whereas the nicotinamide adenine dinucleotide (NAD)-dependent enzyme was stable at 65 C for 10 min. The specific activity of both the NAD- and NADP-dependent enzymes in crude extracts increased markedly (15- and 7.5-fold, respectively) during the transition from exponential- to stationary-phase growth in glucose medium containing 0.5 mM sodium cholate. The time course of apparent enzyme induction correlated temporally with the transformation of the 7-α-hydroxy group of cholate in the culture supernatant fluid. Both NAD- and NADP-dependent 7-α-hydroxysteroid dehydrogenase activities were found to be widely, but not universally, distributed in different strains and subspecies of *B. fragilis*. No NAD- or NADP-dependent 7-α-hydroxysteroid dehydrogenase activity could be detected in *B. fragilis* subsp. *vulgatus* Virginia Polytechnic Institute (VPI) no. 4245, subsp. *thetaiotaomicron* VPI 0061-1, or subsp. *distasonis* VPI 4243.

Bacteroides fragilis (summing all of the various subspecies) accounts for approximately 20 to 25% of the total cultivable fecal microflora of man (11). The subspecies which have been isolated in the highest viable numbers from fecal samples include: subsp. *vulgatus* 1st; subsp. *thetaiotaomicron* 2nd; and subsp. *distasonis* 3rd. The other subspecies are usually found in much lower numbers (11).

The normal intestinal flora of man contains bacteria which are capable of enzymatically transforming bile acids into a variety of metabolites (1, 10). One of the main reactions carried out by these organisms is the oxidation of one or more hydroxy groups of bile acid substrates to form keto bile acids that can be detected in the feces (12, 13). Members of the genus *Bacteroides* have been reported to oxidize primarily the 7-α-hydroxy group of bile acid substrates (2). Moreover, Aries and Hill (2) reported that the 7-α-hydroxysteroid dehydrogenases assayed in crude cell extracts prepared from *B. fragilis* NCTC 9343 (ATCC 25285) and *Escherichia coli* were nicotinamide adenine dinucleotide (NAD) dependent, whereas the enzyme from *Clostridium welchii* and certain strains of the genus *Bifidobacterium* was highly nicotinamide adenine dinucleotide phosphate (NADP) dependent. More recently, MacDonald et al. (9) reported that selected strains of *E. coli* had NAD-dependent 7-α-hydroxysteroid dehydrogenase. Although bile acid dehydrogenases of various types have been detected in crude cell extracts of different intestinal bacteria, very little is known about the molecular characteristics of the purified enzymes or the mechanism of regulation of biosynthesis of these enzymes. Further, the physiological significance of these enzymatic reactions to the bacteria is nebulous. Evidence for multiple forms of 7-α-hydroxysteroid dehydrogenase in certain strains of *B. fragilis* and selected data regarding the molecular characteristics of these enzymes are presented in this communication.

MATERIALS AND METHODS

Bacterial strains and cultural conditions. Selected strains of *B. fragilis* were kindly donated by J. L. Johnson and W. E. C. Moore of the Virginia Polytechnic Institute (VPI) and State University, Anaerobe Laboratory. Stock cultures of *B. fragilis* were maintained in chopped meat medium under anaerobic conditions (5). For enzyme characterization

studies, *B. fragilis* was cultured in a chemically defined growth medium as previously described (6). Growth of bacteria was determined by measuring the culture turbidity with a Klett-Summerson colorimeter equipped with a number 66 (red) filter.

Preparation of cell extracts for enzyme assays. Cells were harvested by centrifugation at 13,700 × *g* for 15 min (0 to 4 C) from culture samples taken during the stationary phase of growth (22 to 26 h), unless otherwise indicated. Cells were suspended in 0.1 M tris(hydroxymethyl)aminomethane(Tris)-hydrochloride (pH 7.2) containing 10 mM 2-mercaptoethanol (2-ME), 0.5 mM ethylenediaminetetraacetic acid, and 1 mM sodium cholate (sonication buffer). The cells in suspensions were ruptured by sonic oscillation in a test tube using a Biosonik III (probe type) sonicator for a total of three 15-s burst intervals. The broken cell suspensions were then centrifuged at 105,000 × *g* for 2 h at 0 to 4 C, and the supernatant fluid was used for 7-α-hydroxysteroid dehydrogenase assay and for further enzyme purification using ammonium sulfate fractionation and Sephadex G-200 chromatography.

Enzyme assay. The standard reaction mixture (total volume, 1.0 ml) for 7-α-hydroxysteroid dehydrogenase included 50 mM Tris-hydrochloride (pH 8.5), 5 mM 2-ME, 0.5 mM NAD or NADP, 1 mM sodium cholate, 2.5 mM $MnCl_2$, and enzyme preparation. The partially purified enzyme activities collected from pooled fractions from the Sephadex G-200 column and crude cell extracts dialyzed for 12 h in Tris-hydrochloride (pH 7.2) plus 10 mM 2-ME was shown to be absolutely dependent on NAD or NADP, sodium cholate, and cell extract. Specific activities were always determined with protein concentrations that were directly proportional to initial reaction velocities.

The enzymes were assayed spectrophotometrically, and the initial rates of reduction of pyridine nucleotide were determined in a Gilford model 2400-S recording spectrophotometer at a wavelength of 340 nm (Gilford Instrument Co., Oberlin, Ohio). Initial rates were converted to micromoles of NAD, reduced form, + H^+ or NADP, reduced form, + H^+ formed assuming a molar extinction coefficient of 6.22 × 10^3/1 mol per cm. NAD, reduced form, + H^+ and NADP, reduced form, + H^+ oxidase activity was < 0.001 IU of enzyme activity per mg of protein in crude cell extracts. Protein estimations were performed according to the method of Lowry et al. (8) by using bovine serum albumin as the protein standard. All assays were performed at 25 C, and specific activities are expressed as micromoles of substrate converted per minute per milligram of extracted protein.

Glucose concentrations were determined by using a Glucostat assay kit (Worthington Biochemical Corp., Freehold, N.J.). The concentration of sodium cholate remaining in the culture supernatant fluid was monitored by using the partially purified NAD-dependent 7-α-hydroxysteroid dehydrogenase.

Enzyme product identification. The enzymatic product was identified by adding approximately 3.5 × 10^5 counts/min of [^{14}C]cholic acid (40 Ci/M) (New England Nuclear, Boston, Mass.) to the standard reaction mixtures. A 45 to 75% $(NH_4)_2SO_4$ precipitate dialyzed 12 h in 50 mM Tris-hydrochloride (pH 7.2) buffer plus 10 mM 2-ME was used as the enzyme preparation. The reactions were stopped after 15 min of incubation by adding concentrated HCl until the reaction mixtures were approximately pH 1.0. The bile acid products were extracted by adding 1.0 ml of redistilled ethylacetate. The ethylacetate extracts were concentrated about fourfold under a stream of nitrogen gas. The concentrated extracts were then spotted on Silica gel (20 by 20 cm) thin-layer chromatography (TLC) plates (Baker-flex, J. T. Baker Chemical Co., Phillipsburg, N.J.), and the radiolabeled products were separated in a series of solvent systems, S-1, S-5, S-6, S-7, and S-11, as described by Eneroth (3). The thin-layer radiochromatograms were cut into strips, and the radiolabeled products were located by using a Packard radiochromatogram model 7201 scanner (Packard Instrument Co., Downers Grove, Ill.). The products were also located by spraying the thin-layer plates with phosphomolybdic acid and heating for 10 to 15 min at 180 C. Products were compared with known standards.

Chemicals. NAD and NADP were obtained from P-L Biochemicals, Inc., Milwaukee, Wis. All bile acid and bile salts were obtained from Calbiochem, San Diego, Calif. All other chemicals were of the highest grade commerically available.

RESULTS

Partial purification of 7-α-hydroxysteroid dehydrogenase by Sephadex G-200 chromatography. Crude cell extracts of *B. fragilis* subsp. *thetaiotaomicron* NCTC 10852 (VPI 5482) dialyzed 12 h in 50 mM Tris-hydrochloride (pH 7.2) plus 10 mM 2-ME showed both NAD- and NADP-dependent 7-α-hydroxysteroid dehydrogenase activity. To determine the nature of these activities, a 45 to 75% saturated $(NH_4)_2SO_4$ precipitate suspended in 0.1 M Tris-hydrochloride (pH 7.2) containing 10 mM 2-ME was applied to a Sephadex G-200 column (2.6 by 100 cm) that had been previously equilibrated with 10 mM potassium phosphate buffer (pH 7.2). Enzymes were eluted with 10 mM potassium phosphate buffer (pH 7.2). Enzyme activities (both NAD and NADP dependent) were assayed in the various fractions, and the total units of each activity per 5-ml fraction was determined. There were at least two peaks of 7-α-hydroxysteroid dehydrogenase activity, one apparently specific for NAD and the other apparently specific for NADP (Fig. 1). Although not shown, fractions 28 to 31 had relatively low but only NADP-dependent enzyme activity, whereas fractions 59 to 62 had only NAD-dependent activity. These results suggest that these two forms of 7-α-hydroxysteroid dehydrogenase are specific for pyridine nucleotide. The NAD-dependent enzyme had a molecular

weight of approximately 80,000 ± 5,000 and the NADP-dependent enzyme 127,000 ± 10,000. Fractions having relatively high levels of NAD- and NADP-dependent 7-α-hydroxysteroid dehydrogenase activities were pooled (bars, Fig. 1) and used for further enzyme characterization. The pooled fractions showed 17- and 11-fold purification over crude cell extracts for NADP- and NAD-dependent activities, respectively. The substrate specificity of NAD- and NADP-dependent activities are shown in Table 1. Both enzyme forms used free bile acids as well as conjugated bile "salts," providing the steroid had a 7-α-hydroxy group. These data suggest that both the NAD- and NADP-dependent enzymes are specific for the 7-α-hydroxy group. The pH optimum for both enzyme forms was between 8.5 and 9.0 for the oxidative reaction (Fig. 2).

Radiolabeled enzyme product from both the NAD- and NADP-dependent reactions was separated using TLC techniques in a series of solvent systems (see Materials and Methods). The data in Fig. 3 show the separation of ^{14}C-labeled bile acid products using solvent system S-6 (3) for both the NAD- and NADP-dependent enzyme reactions. These results, as well as those obtained in other solvent systems, showed that both the NAD- and NADP-dependent enzyme generated the same steroid product. In additional experiments, these results were substantiated by scraping both NAD- and NADP-dependent steroid enzyme products from TLC plates into a test tube, extracting the

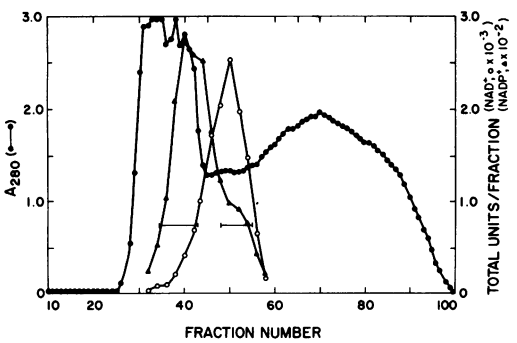

FIG. 1. *Partial separation of NAD- and NADP-dependent forms of 7-α-hydroxysteroid dehydrogenase from B. fragilis subsp. thetaiotamicron NCTC 10852 by Sephadex G-200 chromatography. A 15-ml sample of a 45 to 75% saturated $(NH_4)_2SO_4$ precipitate suspended in 0.1 M Tris-hydrochloride (pH 7.2) buffer + 10 mM 2-ME containing 20 mg of protein per ml was applied to the column. Fractions (5 ml) were collected and assayed for absorption at 280 nm (●) and NAD (○) and NADP (▲) dependent 7-α-hydroxysteroid dehydrogenase activity.*

TABLE 1. *Substrate specificity of NAD- and NADP-dependent forms of 7-α-hydroxysteroid dehydrogenase*[a]

Steroid[b]	Relative V_{max}	
	NAD-dependent activity	NADP-dependent activity
Cholate	79	100
Chenodeoxycholate	100	89
Taurocholate	63	89
Taurochenodeoxycholate	91	79
Glycocholate	79	91
Glycochenodeoxycholate	96	89
Deoxycholate[c]	8	7
Glycodeoxycholate	<1	<1
Taurodeoxycholate	<1	<1
Lithocholate[d]	<1	<1
Glycolithocholate[d]	<1	<1
Taurolithocholate[d]	<1	<1

[a] The reaction was initiated by the addition of 227 μg of partially purified enzyme from pooled fractions from a Sephadex G-200 column. The specific activities of these fractions were 1.28 and 0.313 IU of enzyme activity per mg of extracted protein for the NAD- and NADP-dependent enzyme activities, respectively.

[b] Each steroid was assayed at a concentration of 1.0 mM under standard reaction conditions.

[c] This steroid was found to be contaminated with small amounts of cholic acid by TLC analysis.

[d] Steroid was dissolved in 95% ethanol.

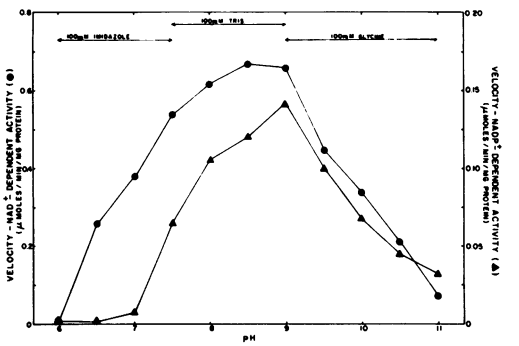

FIG. 2. *Effect of pH on catalysis of 7-α-hydroxysteroid dehydrogenase. The reaction for both NAD- and NADP-dependent enzyme activities was initiated by the addition of approximately 227 μg of partially purified enzyme preparation.*

mixed product(s), and rechromatographing in a series of solvent systems. In each solvent system, the product(s) moved as a single peak (Fig. 3D), showing that they were indeed identical. In all solvent systems tested, the product moved with R_f values similar to those reported for 7-ketodeoxycholate (3).

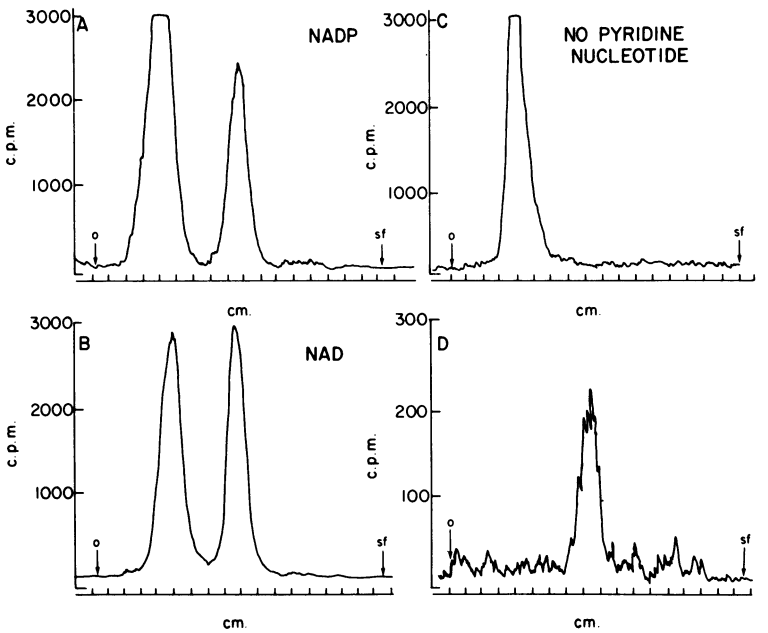

FIG. 3. *Thin-layer chromatographic separation of ^{14}C-labeled cholic acid and enzymatic product of NAD- and NADP-dependent 7-α-hydroxysteroid dehydrogenase in solvent S-6 (3). Radiolabeled enzyme products of both NADP-(A) and NAD-(B) dependent enzyme forms were mixed and rechromatographed (D). No product was formed in the absence of pyridine nucleotide (C). Arrows indicate the origin (O) and solvent front (sf).*

A partially purified precipitate [the 45 to 75% saturated $(NH_4)_2SO_4$ fraction] of crude cell extracts dialyzed 12 h in 50 mM Tris-hydrochloride (pH 8.0) buffer containing 10 mM 2-ME and 1 mM ethylenediaminetetraacetic acid lost all detectable NADP-dependent 7-α-hydroxysteroid dehydrogenase activity. However, the addition of certain divalent metal cations to the enzymatic reaction mixture restored greater than 90% of the initial activity of this enzyme. Manganese ions were the most effective in this regard (Table 2). Moreover, metal cation saturation kinetics showed a very high binding affinity for Mn^{2+} (K_m, 57 μM). The NAD-dependent enzyme lost <10% of its initial activity when dialyzed 12 h under these same conditions.

The NADP-dependent enzyme activity decreased rapidly (>70% of initial activity) when incubated at 65 C for 10 min at pH 7.2 or 8.0, whereas the NAD-dependent enzyme activity was completely stable to thermal denaturation at 65 C under these same experimental conditions (Fig. 4). In additional experiments, the NAD-dependent enzyme activity was shown to be stable to heating at 75 C for 30 min if bile acid substrates were present in the incubation mixture.

The specific activities of both NAD- and NADP-dependent 7-α-hydroxysteroid dehydrogenase assayed in crude cell extracts of culture samples taken at points indicated by the numbered arrows throughout the different growth stages of *B. fragilis* subsp. *thetaiotaomicron* NCTC 10852 are shown in Fig. 5. The data show that the specific activities of both NAD- and

TABLE 2. *Metal ion requirement for activity of the NADP-dependent form of 7-α-hydroxysteroid dehydrogenase*[a]

Cation chloride	Concn (mM)	Sp act[b]	K_m values[c]
No divalent metal cation added		<0.001	
Mg^{2+}	2.5	0.004	1.7
Mn^{2+}	2.5	0.024	0.057
Ca^{2+}	2.5	<0.001	
Ba^{2+}	2.5	<0.001	
Co^{2+}	2.5	<0.001	

[a] Partially purified $(NH_4)SO_4$ precipitate (45 to 75% saturation dialyzed 12 h in Tris-hydrochloride [pH 8.0] + 10 mM 2-ME + 1 mM ethylenediaminetetraacetic acid) was used as the enzyme preparation for these experiments.

[b] Reported as international units of enzyme activity per milligram of protein.

[c] Values were determined from Lineweaver-Burk plots of metal cation saturation kinetics.

NADP-dependent 7-α-hydroxysteroid dehydrogenase increased during the stationary phase of growth. Furthermore, the increase in specific activity was greater (three- to fourfold) when 0.5 mM sodium cholate was included in the growth medium (Fig. 5). It has not yet been determined if the increase in specific activities of 7-α-hydroxysteroid dehydrogenase in the presence of 0.5 mM sodium cholate is the result of an increase in the differential rates of enzyme synthesis or a stabilization of these enzymes by cholic acid. However, the increase in specific activity of these enzymes did not occur until the concentration of glucose was lower than 6 to 8 mM. Further, the pH of the culture supernatant fluid decreased gradually from 7.2 at time 0 to a pH of 5.0 at 14 h and remained at this level until the experiment was concluded. Changes in the cultural pH, rates of glucose utilization, and growth curves were essentially identical when cells were cultured in the presence or absence of 0.5 mM sodium cholate. The data presented in Fig. 5 showed that the 7-α-hydroxy group of sodium cholate was not altered until cultures of *B. fragilis* were in the stationary phase of growth. The bacterial alteration of the 7-α-hydroxy group of sodium cholate corresponded very closely with the temporal increase in the specific acitivities of both the NAD- and NADP-dependent 7-α-hydroxysteroid dehydrogenase. In other experiments, ^{14}C-labeled cholic acid was added to growing cultures of *B. fragilis*, and at points throughout the different growth stages samples were taken and acidified to pH 1.0, and bile acid products were extracted in ethylacetate. The radiolabeled products were separated and identified by TLC techniques in a series of solvents described in Materials and Methods. The results showed that the product generated by cell cultures under these growth conditions was exclusively 7-ketodeoxycholate and identical to the product generated by the partially purified 7-α-hydroxysteroid dehydrogenase enzymes. Deoxycholate is not generated under these experimental conditions. In this regard, Aries and Hill (2) reported that 7-α-dehydroxylase activity was produced only by bacteria grown in a medium with a final pH above 6.5.

The distribution of both the NAD- and NADP-dependent 7-α-hydroxysteroid dehydrogenase activities in different strains and subspecies of *B. fragilis* is shown in Table 3. Strains 5482, 0038-1, and 3524 had relatively high specific activities of the NAD-dependent 7-α-hydroxysteroid dehydrogenase; strains 25285, 2393, and 2302 had relatively low specific activities, and strains 4245, 4243, and 0061-1 had no

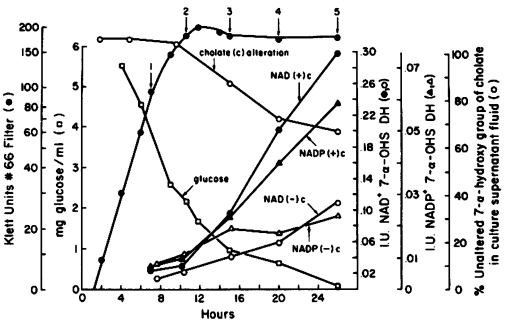

FIG. 5. *Regulation of NAD- and NADP-dependent 7-α-hydroxysteroid dehydrogenase activities and sodium cholate conversion during the different growth stages of B. fragilis subsp. thetaiotaomicron NCTC 10852. Samples were taken at points indicated by numbered arrows in glucose medium containing 0.5 mM sodium cholate (+)c or in a parallel experiment in glucose medium without sodium cholate (−)c. Cells (1.5 g [wet weight]) from each culture sample were suspended in 2.0 ml of sonication buffer, and cell extracts were prepared and assayed for NAD-(●;○) and NADP-(▲;△) dependent activities. Glucose (□) concentration and alteration of the 7-α-hydroxy group of sodium cholate (○) was assayed in the culture supernatant fluid as described in Materials and Methods.*

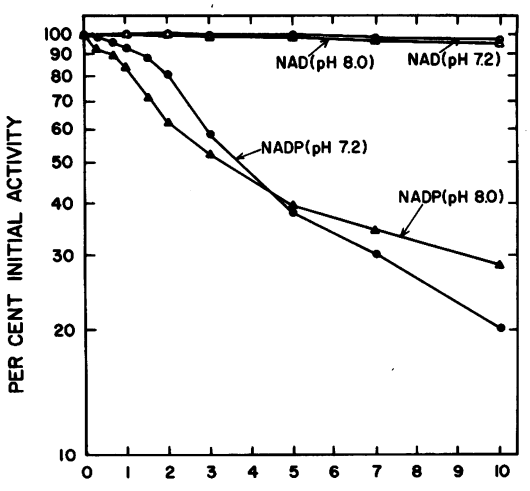

FIG. 4. *Thermal inactivation of NADP-dependent 7-α-hydroxysteroid dehydrogenase. Crude cell extracts of B. fragilis subsp. thetaiotaomicron NCTC 10852 dialyzed 12 h in 50 mM Tris-hydrochloride (pH 7.2 or 8.0) + 10 mM 2-ME were heated at 65 C at either pH 7.2 or 8.0 for a time course of 10 min in the absence of substrates. Numerous samples were taken and assayed for NAD- and NADP-dependent activity. The initial activities of the NAD- and NADP-dependent enzymes were 0.110 and 0.016 IU, respectively, in a 4-ml sample containing approximately 10.8 mg of protein per ml.*

TABLE 3. *Distribution of 7-α-hydroxysteroid dehydrogenase in different deoxyribonucleic acid homology groups of B. fragilis*[a]

Strain no.	Deoxyribonucleic acid homology group[b]	NAD-dependent[c] activity	NADP-dependent activity	Ratio (NAD-NADP)
ATCC 25285	Subsp. *fragilis* (1)	0.0101	0.0020	4.59
VPI 2393	Subsp. *fragilis* (2)	0.0064	0.0050	1.28
VPI 4243	Subsp. *distasonis* (1)	<0.0001	<0.0001	
VPI 4245	Subsp. *vulgatus*	<0.0001	<0.0001	
VPI 0061-1	Subsp. *thetaiotaomicron* (1)	<0.0001	<0.0001	
VPI 5482	Subsp. *thetaiotaomicron* (2)	0.1208	0.0180	6.71
VPI 2302	Subsp. *thetaiotaomicron* (3)	0.0218	0.0104	2.09
VPI 0038-1	Subsp. *ovatus* (1)	0.0854	0.0222	3.88
VPI 3524	Subsp. *ovatus* (2)	0.0974	0.0129	7.55

[a] Cells of *B. fragilis* were cultured in chemically defined medium containing 30 mM glucose plus 0.5 mM sodium cholate. The cells were harvested by centrifugation at 13,700 × *g* for 15 min from culture samples taken during the stationary phase of growth (22 to 26 h) and suspended in sonication buffer. The cell suspensions were broken by sonic oscillation for a total of three 15-s bursts. The broken cell suspensions were then centrifuged at 105,000 × *g* for 2 h, and the supernatant fluid was used for enzyme assays. Crude cell extracts dialyzed 12 h in 50 mM Tris-hydrochloride (pH 7.2) + 10 mM 2-ME yielded essentially the same results.

[b] Reported by Johnson (7). Number in parentheses indicates deoxyribonucleic acid homology group.

[c] Reported as international units of enzyme activity per milligram of extract protein.

detectable NAD-dependent enzyme activity. Strains 5482, 0038-1, 3524, and 2302 had relatively high NADP-dependent 7-α-hydroxysteroid dehydrogenase activities, strains 25285 and 2393 had relatively low specific activities, and strains 4245, 4243, and 0061-1 had no detectable NADP-dependent enzyme activity.

DISCUSSION

The presence of multiple forms of 7-α-hydroxysteroid dehydrogenase activity in certain strains of *B. fragilis* was unexpected in view of the previous report of only an NAD-dependent enzyme in this organism (2). However, the NADP-dependent 7-α-hydroxysteroid dehydrogenase activity in the strain of *B. fragilis* used in the studies of Aries and Hill (2) has very low NADP-dependent activity (strain 25285, Table 3) and could have been easily undetectable if optimal cultural and enzymatic assay conditions were not employed. The NAD- and NADP-dependent 7-α-hydroxysteroid dehydrogenase demonstrated in the present investigation differed with respect to divalent metal cation requirements, thermal stability, and elution profiles from Sephadex G-200 columns. The requirement for Mn^{2+} for activity of the NADP-dependent enzyme was interesting in that there are no reported metal ion requirements for steroid dehydrogenases. Aries and Hill (2) reported that the NADP-dependent 7-α-hydroxysteroid dehydrogenase from *C. welchii* CC 49 was thermally labile and the NAD-dependent enzyme from *B. fragilis* was stable to heating (58 C for 30 min). Similar observations were made in the present study with regard to the thermal stability of the NAD- and NADP-dependent 7-α-hydroxysteroid dehydrogenases.

The physiological significance of the 7-α-hydroxysteroid dehydrogenase reaction(s) to *B. fragilis* is not yet clear. However, the fact that oxidation of the 7-α-hydroxy group occurred only after glucose was nearly depleted from the growth medium (Fig. 5) suggests that bile acids might serve as potential energy sources in the absence of fermentable carbohydrates.

The distribution of these enzymes in different deoxyribonucleic acid homology groups of *B. fragilis* was interesting in that the enzymes were found in relatively high specific activity in certain strains and relatively low specific activity in other strains (Table 3). The fact that the "low-specific-activity" strains of *B. fragilis* had enzyme levels near uninduced levels of the "higher-specific-activity" strains may indicate that these enzymes are regulated differently. Further, it was also noteworthy that neither the NAD- nor NADP-dependent enzyme was detectable in *B. fragilis* subsp. *vulgatus*, which has recently been reported to be the predominant subspecies of *B. fragilis* isolated from human fecal material (11).

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