Biochimica et Biophysica Acta, 384 (1975) 12—24

BBA 67437

NAD- AND NADP-DEPENDENT 7α-HYDROXYSTEROID DEHYDROGENASES FROM *BACTEROIDES FRAGILIS*

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(Received September 2nd, 1974)

Summary

Twenty strains of *Bacteroides fragilis* were screened for hydroxysteroid oxidoreductase activity in cell-free preparations. Eighteen strains were shown to contain NAD-dependent 7α-hydroxysteroid dehydrogenase. Sixteen of the strains containing the NAD-dependent enzyme also contained NADP-dependent 7α-hydroxysteroid dehydrogenase, but invariably in lesser amounts. A strain particularly rich in both 7α-hydroxysteroid dehydrogenase activities was selected for further study. Measurement of activity as a function of pH revealed a fairly sharp optimal activity range of 9.5—10.0 for the NAD-dependent enzyme and a broad flat optimal range of 7.0—9.0 for the NADP-dependent enzyme. Michaelis constants for trihydroxy-bile acids for the NAD-dependent enzyme were in the range of 0.32—0.34 mM, whereas dihydroxy-bile acids gave a K_m of 0.1 mM.

Thin-layer chromatography studies on the oxidation product of 3α,7α-dihydroxy-5β-cholanoic acid (chenodeoxycholic acid)by the dehydrogenase revealed a band corresponding to that of synthetic 3α-hydroxy, 7-keto-5β-cholanoic acid. Similarly the oxidation product of chenodeoxycholic acid by both 7α-hydroxysteroid dehydrogenase and commercially available 3α-hydroxysteroid dehydrogenase revealed a band corresponding to that of synthetic 3,7-diketo-5β-cholanoic acid. Neither of these two oxidation products could be distinguished from those by the *Escherichia coli* dehydrogenase oxidation previously reported. Disc-gel electrophoresis of a cell-free lyophilized preparation indicated one active band for NAD-dependent activity of mobility similar to that for the NADP-dependent *E. coli* enzyme. The NADP-dependent dehydrogenase was unstable and rapidly lost activity after polyacylamide disc-gel electrophoresis, ultracentrifugation, freezing on refrigeration at 4° C. No 3α- or 12α-oriented oxidoreductase activity was demonstrated in any of the strains examined.

Introduction

7α-hydroxysteroid dehydrogenase has been reported in both aerobic bacteria, such as *Escherichia coli* [1,2], and anaerobes, such as *Bacteroides* [2,3], and *Bifidobacteria* [2]. The application of the *E. coli* enzyme, in combination with the 3α-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* [4] has been useful for the individual quantification of glycine and taurine conjugates of 3α,7α-dihydroxy-5β-cholanoic acid (chenodeoxycholic acid) and 3α,12α-dihydroxy-5β-cholanoic acid [5,6] ordinarily inseparable by thin-layer chromatography [7].

Currently however, the fluorometric assay for quantification of total 7α-hydroxyl groups using *E. coli* 7α-hydroxysteroid dehydrogenase has been limited to the estimation of chenodeoxycholic acid and its conjugates, since the oxidation of 3α,7α,12α-trihydroxy-5β-cholanoic acid and conjugates, in this assay system, is incomplete [6]. In addition, 3α- and 12α-hydroxysteroid dehydrogenase activities have been described in very few strains of microorganisms and frequently in the presence of other contaminating dehydrogenase activity [2].

It is therefore, of practical interest, to investigate other species of bacteria for their dehydrogenase activity. It is the purpose of this communication to report 20 strains of *Bacteroides fragilis* which were screened for 3α-,7α- and 12α-hydroxysteroid oxidoreductase activity and to characterize in a selected strain, the NAD- and NADP-dependent 7α-hydroxysteroid dehydrogenase activities.

Materials

Todd-Hewitt broth (Bioquest Co.) to which was added 0.1% sodium thioglycollate (Fisher Chemicals) and 0.01% 3α,7α,12α-trihydroxycholanoic acid (Calbiochemicals): mixture subsequently referred to as "broth", was used for growing *B. fragilis*. Isolates of this species were obtained from clinical specimens by the staff of the Department of Medicine, Pathology Institute, Halifax, Nova Scotia. These bacteria were maintained in cooked meat medium (Difco Laboratories) at 4°C. Phenazine methosulphate, nitro blue tetrazolium, NAD^+, $NADP^+$, Coomassie brilliant blue and barbituric acid (Veronal) were obtained from Sigma Chemical Co. HCl and H_3PO_4, mono- and di-basic sodium phosphates, sucrose and acrylamide were purchased from Baker Co.; glycine, NaOH and EDTA were from Fisher Chemicals. All di- and tri-hydroxylated bile acids were from Calbiochemicals and 3α-hydroxy-7-keto-5β-cholanoic acid and 3,7-diketo-5β-cholanoic acid were from Steraloids. The keto-bile acids were twice recrystalized from methanol/water (3 : 1, v/v) at −20°C before usage for thin-layer chromatographic studies. 3α-Hydroxysteroid dehydrogenase (EC 1.1.1.50) was a product of Worthington Enzyme Co.

Methods

Growth and harvest of Bacteroides fragilis

Strains of *B. fragilis* were subcultured from 48-h cooked meat cultures to

10 ml of freshly boiled and cooled broth. After 24 h incubation at 37°C, such cultures were inoculated into 200-ml volumes of freshly autoclaved broth for incubation at 37°C. Unless otherwise designated, the cultures were harvested at 48 h by centrifuging at 6000 × *g* for 20 min at 4°C in an International B-20 centrifuge. The pellet was resuspended in 10 μl of 0.1 M sodium phosphate buffer (pH 7.0) and recentrifuged. The final sediment was resuspended in 15 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 10^{-3} M EDTA.

Extraction and preparation of 7α-hydroxysteroid dehydrogenase

The 15-ml volume of washed cells described above was sonicated with a Fisher ultrasonic probe at 100 W for a period of 4 min and then centrifuged at 6000 × *g* for 10 min to sediment the cell debris. The supernatant fluid was collected and assayed for 3α-, 7α- and 12α-oxidoreductase activities.

The enzyme sonicate from Strain 18 (see Table I) was ultracentrifuged at 100 000 × *g* for 2 h in a Beckman Model L-2 ultracentrifuge. Supernatant fluid was collected and assayed for enzyme activity both as a function of protein concentration and pH. Freshly ultracentrifuged enzyme was frozen with liquid N_2 and lyophilized in a Virtis lyophilizer. Lyophilized material was stored at −20°C and reconstituted to its original volume for the kinetic determination of Michaelis constants (K_m) and maximal velocities (V) for several substrates.

Assay for 7α-hydroxysteroid dehydrogenase activity

The formation of NADH was followed at 25°C at 340 nm using a Beckman spectrophotometer with a 10-inch Beckman recorder. Each assay cuvette contained, for routine screening of enzyme activity, $1.7 \cdot 10^{-3}$ M NAD^+ or $1.7 \cdot 10^{-3}$ M $NADP^+$, 0.17 M glycine NaOH buffer (pH 9.5), $1.0 \cdot 10^{-3}$ M bile acid and 100 μl of crude *B. fragilis* sonicate. Kinetic studies on Strain 18 were performed using 45 μl of ultracentrifuged enzyme preparation (approx. 0.07 unit) or 45 μl of lyophilized and reconstituted preparation. 1 unit of enzyme is defined as the amount of enzyme required to yield 1 μmol of NADH per min under the above described conditions. An estimated extinction coefficient of $6.2 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ was used for NADH [8].

Preparation of E. coli 7α-hydroxysteroid dehydrogenase

This was performed with Strain 23 *E. coli* as previously described [6].

Thin-layer chromatography of oxidation products

$1.0 \cdot 10^{-3}$ M chenodeoxycholic acid underwent oxidation for 1 h in the presence of 0.30 units of (a) 7α-hydroxysteroid dehydrogenase from *B. fragilis* strain 18, (b) 7α-hydroxysteroid dehydrogenase from *E. coli*, (c) 7α-hydroxysteroid dehydrogenase from *B. fragilis* Strain 18 and 3α-hydroxysteroid dehydrogenase from *P. testosteroni* and (d) 7α-hydroxysteroid dehydrogenase from *E. coli* and 3α-hydroxysteroid dehydrogenase from *P. testosteroni*. Standard conditions were employed except the NAD^+ concentration was $3.4 \cdot 10^{-3}$ M. The reaction mixture was then acidified with 0.2 ml conc. HCl and twice extracted with two 3-ml vol. of redistilled *n*-butanol. Low-speed centrifugation (600 × *g*) for 10 min aided phase separation. The combined top phases of each reaction mixture were reduced to dryness under a stream of N_2 and the residue

reconstituted into 0.5 ml methanol/water (4 : 1, v/v). 30 μl of reconstituted extract and 20 μl bile acid solutions (10 mg/ml) were chromatographed on thin-layer silica plates with trimethyl pentane/ethylacetate/acetic acid (10 : 10 : 4, by vol.). (Solvent system was a modified Eneroth system [9].) Plates were dried, sprayed with concentrated H_2SO_4 and charred at 200°C for 30 min.

Complete oxidation studies

Both di- and trihydroxysubstrates at cuvette concentrations $5 \cdot 10^{-5}$ M were allowed to undergo oxidation in the presence of 45 μl of lyophilized and reconstituted enzyme (about 0.15 units) and $3.4 \cdot 10^{-3}$ M NAD^+ (reaction volume 3.0 ml). The total change in absorbance (enzyme blank subtracted) directly quantified the total amount of bile acid in the cuvette.

Similarly solutions of $5 \cdot 10^{-5}$ M 3α,7α-dihydroxy-5β-cholanoyl glycine were subjected to sequential complete oxidation by: (a) *B. fragilis* 7α-hydroxysteroid dehydrogenase and then *P. testosteroni* 3α-hydroxysteroid dehydrogenase [10], (b) *P. testosteroni* 3α-hydroxysteroid dehydrogenase and then *B. fragilis* 7α-hydroxysteroid dehydrogenase. In addition, *E. coli* 7α-hydroxysteroid dehydrogenase was tested sequentially with *B. fragilis* 7α-hydroxysteroid dehydrogenase in either order. Approx. 0.15 units of dehydrogenase was used in all cases. All stock solutions of substrates for kinetic studies of *B. fragilis* 7α-hydroxysteroid dehydrogenase were standardized by complete oxidation studies with this enzyme.

Polyacrylamide disc-gel electrophoresis

Disc gel electrophoresis with 5% acrylamide gels has been previously described [1]: gel buffer was that of Gabriel (System II) [11] except Soln 13, which was adjusted to 4 g barbital and 0.44 g Tris/l (pH 7.0). Gels were stained for protein by Coomassie brilliant blue [12] and for enzyme activity by 5-min incubation in the dark with $1.7 \cdot 10^{-3}$ M NAD^+ (or $NADP^+$), 0.17 M glycine/NaOH buffer (pH 9.5), 10 M 3α,7α-dihydroxy-5β-cholanoyl glycine, 0.1 mg/ml phenazine methosulphate and 0.4 mg/ml nitro blue tetrazolium [1,13]. Coomassie brilliant blue-stained gels were de-stained in 10% trichloroacetic acid and "activity stained" gels were de-stained with 7% acetic acid.

Assay for protein concentration

Protein determination of 7α-hydroxysteroid dehydrogenase preparations was performed according to Lowry et al. [14] with crystalline bovine serum albumin (Sigma) as standard.

Results

A survey of hydroxysteroid dehydrogenase activities in 20 strains of B. fragilis

As shown in Table I, *B. fragilis* Strains 5 and 18 contained more than 1.25 units/ml crude preparation of NAD-dependent 7α-hydroxysteroid dehydrogenase. This corresponded to values in excess of 220 units/g dry wt or 2000 units/10^{10} cells. Strain 5 was subcultured into forms 5W and 5G on the basis of colony appearance (white and gray, respectively) but are considered as a variation in colony form of the same strain. The average value for the remaining

TABLE I

7α-HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN *B. FRAGILIS*

Strain No.	NAD-dependent dehydrogenase (units/ml)			NADP-dependent dehydrogenase (units/ml)		
	GDC*	GCDC*	GLC*	GDC	GCDC	GLC
1	0	0.17	0	0	0.068	0
2	0	0.63	0	0	0.015	0
3	0	0.27	0	0	0.039	0
4	0	0.58	0	0	0	0
5W,5G**	0	1.64,1.50	0	0	0.19,0.19	0
6	0	0.20	0	0	0.087	0
7	0	0.14	0	0	0.048	0
8	0	0.14	0	0	0.053	0
9	0	0	0	0	0	0
10	0	0	0	0	0	0
11	0	0.14	0	0	0.029	0
12	0	0.42	0	0	0.140	0
13	0	0.14	0	0	0.029	0
14	0	0.20	0	0	0.039	0
15	0	0.19	0	0	0.019	0
16	0	0.19	0	0	0	0
17	0	0.17	0	0	0.026	0
18***	0	1.40	0	0	0.95	0
19	0	0.77	0	0	0.068	0
20	0	0.18	0	0	0.053	0
Average	0	0.37	0	0	0.097	0

* GDC 3α-, 12α-dihydroxy-5β-cholanoic acid; GCDC 3α-, 7α-dihydroxy-5β-cholanoic acid; GLC 3α-hydroxy-5β-cholanoic acid.

** Strains containing NAD-dependent dehydrogenase activity in yields in excess of 20 units/200 ml culture.

*** Strain presently studied.

18 strains was 0.26 units/ml (416 units/10^{10} organisms) approx. 0.2 the yield of enzyme obtained from Strains 5 and 18. Measurable but relatively low amounts of NADP-dependent activities were present in 16 out of the 20 strains of *Bacteroides* (average 0.097 units/ml or 155 units/10^{10} organisms). There was a great deal of variation in the relative amounts of the two enzymes from one strain to another at a fixed harvest time (48 h).

At variance with the observations of Aries and Hill [2] no NAD- or NADP-dependent 3α- or 12α-hydroxysteroid dehydrogenase was found in any of the organisms tested.

The yield of enzyme extracted for a single strain of organisms was reproducible for the NAD-dependent enzyme but rather variable for the less stable NADP-dependent enzyme.

Growth of bacteria and extraction of the 7α-hydroxysteroid dehydrogenase

Similar to the 7α-hydroxysteroid dehydrogenase activity in *E. coli*, the corresponding enzymes in *B. fragilis* could not be induced by the inclusion of cholic acid into the growth medium. As in an earlier investigation [2], cholic acid was included in the growth medium for the routine screening of strains of

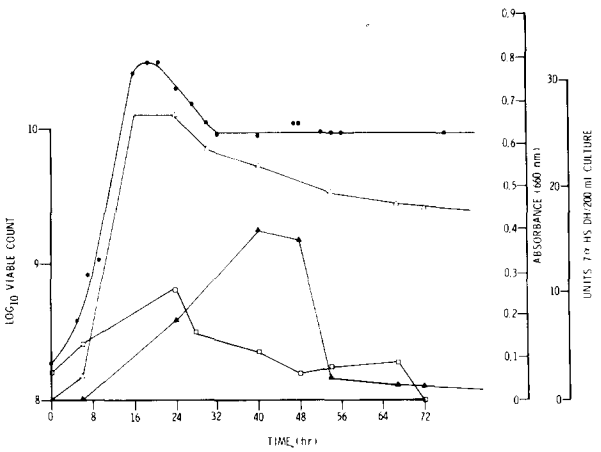


Fig. 1. Growth curve for a 200-ml culture of strain 18 *B. fragilis*. log viable cell counts (□), absorbance at 660 nm (●), total units NAD-dependent 7α-hydroxysteroid dehydrogenase (△), total units NADP-dependent 7α-hydroxysteroid dehydrogenase (▲) with respect to time.

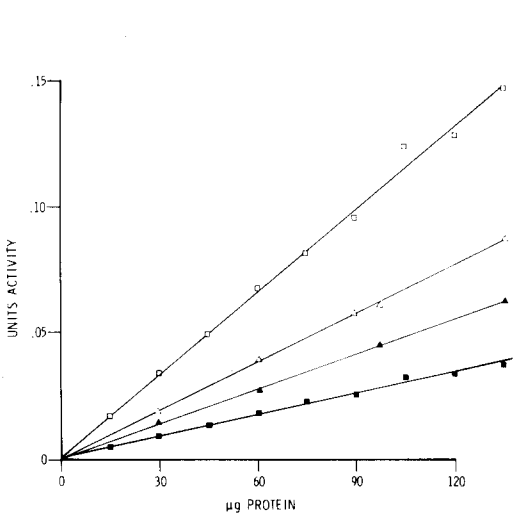
organisms. However, no difference in rates of growth or in yield of either of the two enzymes was detected when 3α,7α,12α-trihydroxycholanoic acid was omitted or 3α,7α-dihydroxycholanoic acid was included. Two strains, 18 and 12, were tested in this respect at harvest times of 6 and 18 h. At 6 h (Fig. 1) no trace of bile acid could be measured in the sonicated bacteria when these compounds had been initially included in the medium. The relative yields of NAD-dependent and NADP-dependent 7α-hydroxysteroid dehydrogenase activities were highly dependent on the time of harvest of the organisms. This is demonstrated in Fig. 1 for Strain 18. The NAD-dependent enzyme activity reached its highest yields earlier (t = 20—24 h) while the NADP-dependent activity was found in optimal yields substantially later (t = 40—48 h) in the growth curve. Although the exact times of peak yields of the enzymes were slightly variable from one experiment to the next, the harvest time for optimal yield of NADP-dependent enzyme was reproducibly more than twice the time for optimal yield of NAD-dependent enzyme.

Effect of ultracentrifugation and pH value on NAD- and NADP-dependent activities

Both the NAD- and NADP-dependent oxidoreductases of Strain 18 gave a linear relationship when activity was plotted against the total protein in the cuvette up to and beyond 120 μg protein (Fig. 2). Ultracentrifugation effected a 1.7 fold purification for the NAD-dependent enzyme while a loss in specific activity of the NADP-dependent enzyme on ultracentrifugation was found to occur (Fig. 2). Variation of pH revealed a relatively sharp optimal activity range between pH 9.5 and 10.0 for the NAD-dependent enzyme and a broad flat optimal range for pH 7.0—9.0 for the NADP-dependent enzyme (Fig. 3).

Stabilities

The relative stabilities of the two enzymes when subjected to routine procedures is seen in Table II. The NAD-dependent enzyme did not appear to



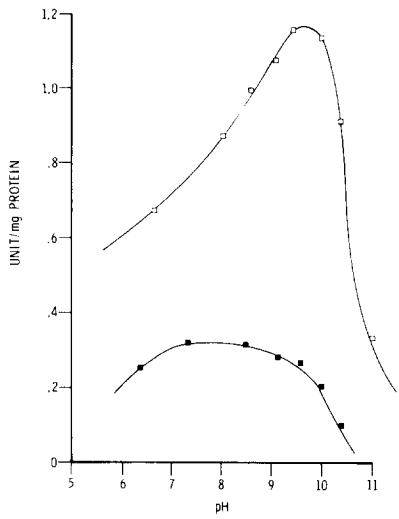


Fig. 2. Units of 7α-hydroxysteroid dehydrogenase versus µg protein in the assay cuvette. △, Crude NAD-dependent dehydrogenase activity; □, ultracentrifuged NAD-dependent dehydrogenase; (▲), crude NADP-dependent dehydrogenase activity; ■, ultracentrifuged NADP-dependent dehydrogenase activity.

Fig. 3. Units of 7α-hydroxysteroid dehydrogenase activity per mg protein versus pH value. □, NAD-dependent dehydrogenase; ■ NADP-dependent 7α-dehydrogenase. In each case enzyme was the ultracentrifuged preparation and substrate 3α,7α-dihydroxycholanoyl glycine (GCDC).

lose any measurable activity upon freezing and thawing, ultracentrifugation and lyophilization whereas the NADP-dependent enzyme was extremely unstable under the same conditions. No significant NADP-dependent 7α-hydroxysteroid dehydrogenase activity was found in the resuspended pellet after ultracentrifugation.

Kinetic and substrate specificity studies

Maximal velocities (V) and Michaelis constants (K_m) for *B. fragilis* Strain 18 NAD-dependent 7α-hydroxysteroid dehydrogenase are summarized in Table III. The K_m values for dihydroxy-bile acids are approx. 1/3 those for the trihydroxy bile acid with very little difference within each group. No kinetic studies were performed on the NADP-dependent enzyme. Both enzymes were

TABLE II

RELATIVE STABILITIES OF NAD- AND NADP-DEPENDENT 7α-HYDROXYSTEROID DEHYDROGENASES

Treatment of enzyme	NAD-dependent dehydrogenase (units/ml)	NADP-dependent dehydrogenase (units/ml)
Crude (t = 48 h)	1.3	0.95
Ultracentrifuged	1.3	0.31
Lyophilized	1.3	0.20
Crude, frozen and thawed	1.3	0.20
Lyophilized, frozen and thawed	1.3	0.08

TABLE III

MAXIMAL VELOCITIES AND MICHAELIS CONSTANTS FOR SUBSTRATES OF NAD-DEPENDENT 7α-HYDROXYSTEROID DEHYDROGENASE

All solutions standardized by complete oxidation in the presence of *Bacteroides* dehydrogenase and spectrophotometric estimation of NADH [8]. Maximal velocities expressed in μmol NADH/min per μg protein. All kinetic experiments performed at 25°C with a substrate concentration range of 0.01—1 mM.

Variable substrate	V	K_m (mM)
3α,7α,12α-Trihydroxy-5β-cholanoic acid	8.5	0.34
3α,7α,12α-Trihydroxy-5β-cholanoyl glycine	10	0.33
3α,7α,12α-Trihydroxy-5β-cholanoyl taurine	10	0.32
3α,7α-Dihydroxy-5β-cholanoic acid	9.1	0.10
3α,7α-Dihydroxy-5β-cholanoyl glycine	13	0.10
3α,7α-Dihydroxy-5β-cholanoyl taurine	12	0.10
NAD^+ *	9.1	0.45

* Substrate 3α,7α-dihydroxy-5β-cholanoic acid (chenodeoxycholic acid).

inactive against purified [1] 3α,7β-dihydroxycholanoic acid indicating the absence of any 7β-hydroxysteroid dehydrogenase activities . Only bile acids containing the 7α-hydroxyl group were substrates.

Complete oxidation studies

Complete oxidation studies for both di- and trihydroxy substrates were

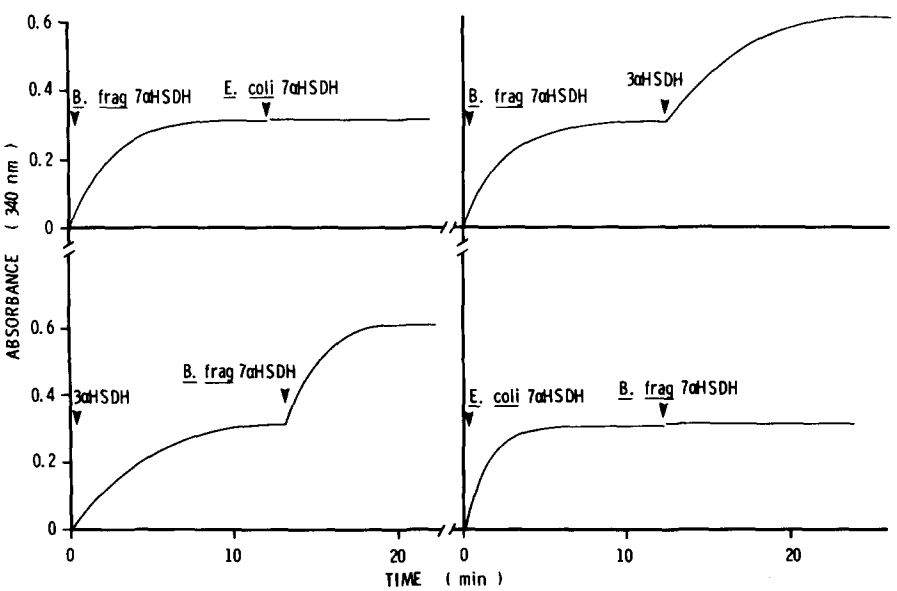


Fig. 4. Sequential tandem oxidations of a solution of $5 \cdot 10^{-5}$ M chenodeoxycholic acid. A, with *B. fragilis* 7α-hydroxysteroid dehydrogenase and then *E. coli* 7α-hydroxysteroid dehydrogenase; B, with *B. fragilis* 7α-hydroxysteroid dehydrogenase and then 3α-hydroxysteroid dehydrogenase; C, with 3α-hydroxysteroid dehydrogenase and then *B. fragilis* 7α-hydroxysteroid dehydrogenase; D, with *E. coli* 7α-hydroxysteroid dehydrogenase and then *B. fragilis* 7α-hydroxysteroid dehydrogenase. Approx. 0.15 units of enzyme were used in all cases. Absorbance changes were monitored on a 10-inch Beckman recorder at 0.1 inch/min.

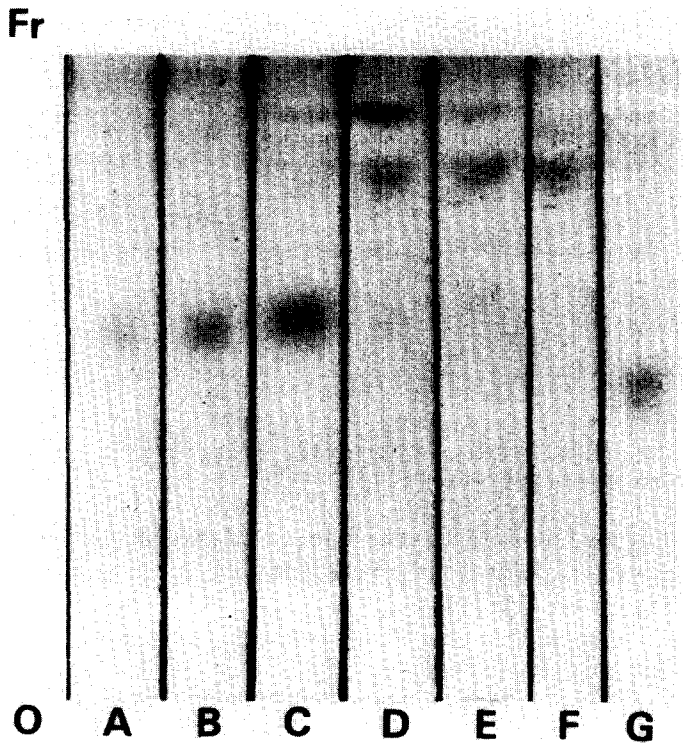


Fig. 5. Thin-layer chromatography of oxidation products of chenodeoxycholic acid by 7α-hydroxysteroid dehydrogenase activity, by 3α- and 7α-hydroxysteroid dehydrogenase activities and unoxidized chenodeoxycholic acid. Bile acids enumerated in order of origin ○: A, 3α-hydroxy-7-keto-5β-cholanoic acid from *B. fragalis* NAD-dependent 7α-hydroxysteroid dehydrogenase oxidation of chenodeoxycholic acid; B, 3α-hydroxy-7-keto-5β-cholanoic acid from *E. coli* 7α-hydroxysteroid dehydrogenase oxidation of chenodeoxycholic acid; C, commercial 3α-hydroxy-7-keto-5β-cholanoic acid; D, 3,7-diketo-5β-cholanoic acids from oxidation by both *P. testosteroni* 3α-hydroxysteroid dehydrogenase and *B. fragilis* NAD-dependent 7α-hydroxysteroid dehydrogenase; E, 3,7-diketo-5β-cholanoic acids from oxidation by both 3α-chenodeoxycholic acid and *E. coli* 7α-hydroxysteroid dehydrogenase; F, commercial 3,7-diketo-5β-cholanoic acid; G, unoxidized chenodeoxycholic acid. Minor spots are attributed to impurities or incompletion of oxidation.

useful for the accurate standardization of stock bile acid solutions. Both di- and trihydroxy substrates at concentrations $1.0 \cdot 10^{-4}$ or $5 \cdot 10^{-5}$ M underwent complete oxidation within 30 min (see Methods). Sequential 3α- and 7α-hydroxysteroid dehydrogenase oxidation of $5 \cdot 10^{-5}$ M chenodeoxycholic acid, each enzyme gave a stoichiometric yield of NADH regardless of the order of oxidation. (Fig. 4a and c). When *E. coli* 7α-hydroxysteroid dehydrogenase was substituted for 3α-hydroxysteroid dehydrogenase, only one equivalent of NADH could be obtained (Figs. 4b and d).

Thin-layer chromatography of oxidation products of chenodeoxycholic acid

As seen in Fig. 5, the oxidation product formed from chenodeoxycholic acid by NAD-dependent 7α-hydroxysteroid dehydrogenase from Strain 18 *B. fragilis* could not be distinguished by thin-layer chromatography from the

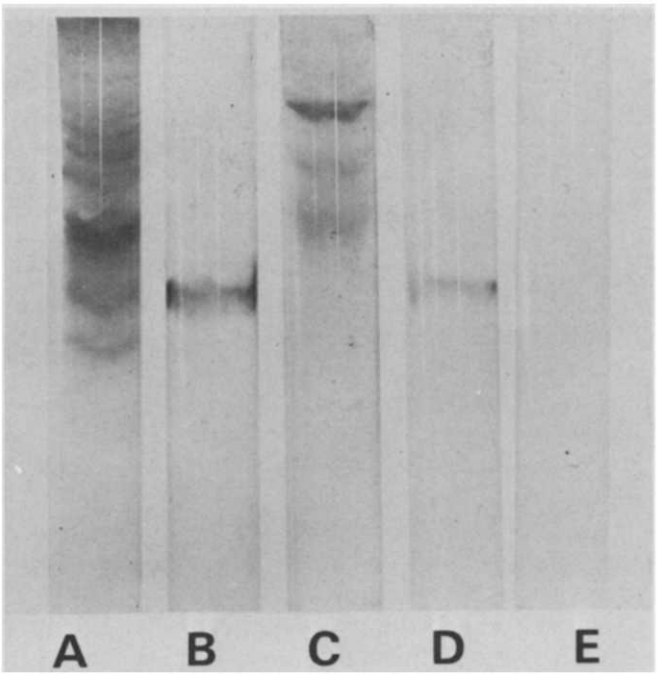


Fig. 6. Polyacrylamide disc-gel electrophoresis of 7α-hydroxysteroid dehydrogenases. (A) *E. coli* preparation (20 μl or approx. 0.6 unit enzyme) stained for proteins. (B) Stained for activity. (C) *B. fragilis* preparation (50 μl or approx. 0.07 units enzyme) stained for proteins. (D) Stained for NAD-dependent activity. (E) Stained for NADP-dependent activity. (Substrate was 3α,7α-dihydroxy-5β-cholanoyl glycine in all cases.)

analogous *E. coli* 7α-hydroxysteroid dehydrogenase oxidation product and corresponded to commercial 3α-hydroxy-7-ketocholanoic acid. Similarly the oxidation product by both *B. fragilis* 7α-hydroxysteroid dehydrogenase and *P. testosteroni* 3α-hydroxysteroid dehydrogenase of chenodeoxycholic acid behaved identically on thin-layer chromatography as the oxidation product by *E. coli* 7α-hydroxysteroid dehydrogenase and *P. testosteroni* 3α-hydroxysteroid dehydrogenase and corresponded to commercial 3,7-diketocholanoic acid.

Polyacrylamide disc-gel electrophoresis

Disc-gel electrophoretic studies with ultracentrifuged enzyme (Fig. 6) indicate only one active band for *B. fragilis* NAD-dependent 7α-hydroxysteroid dehydrogenase activity (Fig. 6d) which has similar motility to that for *E. coli* NAD-dependent 7α-hydroxysteroid dehydrogenase (Fig. 6b). Unfortunately the *B. fragilis* NADP-dependent 7α-hydroxysteroid dehydrogenase activity could not be visualized on electrophoresis (Fig. 6e).

Discussion

The NAD- and NADP-dependent enzymes appear to be distinctly separate entities with different specific activity ranges, pH/activity profiles, stabilities and appearance on polyacrylamide disc-gel electrophoresis. Their relative

abundance was highly variable between different strains of the same organism; the time requirements for optimal yield in the growth curve were quite distinct. NAD-dependent 7α-hydroxysteroid dehydrogenase appears to be associated with the growth phase of the organism whereas the NADP-dependent 7α-hydroxysteroid dehydrogenase appears to be associated with the "death" phase of the curve. No measurable NADP-dependent activity can be observed at t = 6 h while NAD-dependent activity is reproducibly present at the same harvest time (Fig. 1). It is not yet established whether the NADP-dependent 7α-hydroxysteroid dehydrogenase is, in fact, synthesized later or merely released from either an inactive or membrane-bound form upon lysis of the cells. If the latter hypothesis is true, the rather labile NADP-dependent enzyme may be partially deactivated when detached from a membrane by sonication.

Similar to the distribution of 7α-hydroxysteroid dehydrogenase in *E. coli* [6], there is a wide variety in 7α-hydroxysteroid dehydrogenase content in 20 different strains of *B. fragilis* for both enzyme systems. The average yield of NAD-dependent enzyme (560 units/10^{10} organisms) for 20 strains of *B. fragilis* (Table IV) is over 10 times the value previously reported for a single strain of *Bacteroides* [2] but only about one third that reported for 25 strains of *E. coli* studied under similar conditions [6]. In general, *B. fragilis* are more difficult and time consuming to grow than *E. coli* and the population of viable cells/unit volume medium tends to fall with aging of the culture more dramatically with *B. fragilis*. This aging process appears to be associated with lysis and the presence of NADP-dependent 7α-hydroxysteroid dehydrogenase in cell-free *B. fragilis* preparations.

The optimal pH (9.5—10) for the NAD-dependent enzyme (Fig. 3) is similar to that described for *E. coli* [1,2] but somewhat higher than that reported by Aries and Hill [2] for the *Bacteroides* enzyme (pH 8.8—9.3). The total absence of any 3α- or 12α-oxidoreductase in any of the strains is, however, in variance with these authors. An NADP-dependent 7α-hydroxysteroid

TABLE IV

PUBLISHED AND PRESENT OBSERVATIONS ON HUMAN FAECAL *BACTEROIDES* AND *E. COLI* 7α-HYDROXYSTEROID DEHYDROGENASE ACTIVITIES

No.	Estimate	*Bacteroides*	*E. coli*	Ref.
1	Approximate number of organisms/gram wet faeces	10^{10}	10^{7}	2
2	Approximate time required to reach stationary phase	16—20 h	4—5 h	1
3	Average yield of NAD-dependent enzyme/10^{10} organisms*	560 units (20)**	1700 units (25)**	6, 1
4	Average yield of NADP-dependent enzyme/10^{10} organisms*	155 units (20)**	0 unit (10)**	***
5	Approximate number of units of total 7α-enzyme activity/ gram wet faeces	710 units	1.7 units	2, 6
6	Average K_m value (NAD-dependent dehydrogenase for trihydroxylated substrates	0.33 mM	0.93 mM	1
7	Average K_m value (NAD-dependent dehydrogenase for dihydroxylated substrates	0.10 mM	0.13 mM	1

* Data is based on viable cell count enzyme yield at t = 48 h for *B. fragilis* and t = 4.5 h for *E. coli* (1).
** The number of strains screened for dehydrogenase activity.
*** Macdonald et al., unpublished observation.

dehydrogenase in *Bacteroides* has not, to our knowledge, previously been documented. Studies performed on *Bacteroides* 7α-hydroxysteroid dehydrogenase by Aries and Hill [2] were all on cultures harvested early in stationary phase which may explain their failure to report the NADP-dependent enzyme.

The measured Michaelis constants for 7α-hydroxy-containing bile acids (Table III) indicate that the kinetic behaviour of the NAD-dependent enzyme, similar to that of *E. coli* [1], is more influenced by the extent of hydroxylation than by the state of conjugation. However, there appears to be less difference between the K_m values found in di- and trihydroxylated substrates for *B. fragilis* than for *E. coli* (Table IV). The significantly lower K_m values for *B. fragilis* enzyme than those of *E. coli* against trihydroxylated substrates may favor the application of the former for the fluorometric quantification of 7α-hydroxyl groups, particularly for trihydroxylated substrates where the *E. coli* enzyme has failed [6].

Substrate specificity studies and complete oxidation experiments with 7α- and 3α-hydroxysteroid dehydrogenase and also thin-layer chromatography of the oxidation products of chenodeoxycholic acid all confirm the group specificity of this enzyme. The absence of any 7β-oriented activity (similar to the *E. coli* enzyme) was concluded when no dehydrogenase activity was demonstrated with purified 3α,7β-dihydroxycholanoic acid.

The similar mobilities on disc gel electrophoresis (Fig. 6) observed between the *E. coli* 7α-hydroxysteroid dehydrogenase and the corresponding NAD-dependent *B. fragilis* 7α-hydroxysteroid dehydrogenase reflects similar charge/molecular weight ratios of the two proteins and perhaps a close structure relationship. Preparative disc-gel electrophoresis may be particularly useful in the purification of the latter. The apparent loss of NADP-dependent enzyme on disc-gel electrophoresis may reflect the lesser stability of this enzyme (Table II) and the lesser amount present.

Less enzyme per 200-ml culture (and from viable cell counts, per bacterial cell) is obtainable from *B. fragilis* than from *E. coli*, on the average (Table IV). Thus, the contribution of 7 keto-bile acids is likely to be greater by a single *E. coli* cell than a single *B. fragilis* cell. However, *Bacteroides*, in general are known to normally outnumber *E. coli* in faeces by as much as 1000 : 1 [2]. Thus their greater numbers and lower K_m values for trihydroxylated substrates suggest a higher overall contribution of faecal 7-keto bile acids by *Bacteroides* than by *E. coli* (Table IV).

The NAD-dependent *B. fragilis* 7α-hydroxysteroid dehydrogenase is being further studied with respect to its purification and potential application to the fluorimetric quantification of 7α-hydroxyl groups in di- and trihydroxy bile acid conjugates.

Acknowledgements

We are grateful to Miss Karen Watson for her technical assistance in this study and to the Nova Scotia Institute of Pathology for the provision of strains of *Bacteroides fragilis*. This work was supported by grant MA 5075 from the Medical Research Council of Canada.

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