APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1979, p. 992-1000
0099-2240/79/05-0992/09$02.00/0

Vol. 37, No. 5

Bile Salt 3α- and 12α-Hydroxysteroid Dehydrogenases from *Eubacterium lentum* and Related Organisms

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Received for publication 22 February 1979

Thirty-two strains of *Eubacterium lentum* and phenotypically similar anaerobic gram-positive bacilli were screened for intracellular bile salt 3α- and 12α-hydroxysteroid dehydrogenase (HSDHase) activities. These organisms were categorized into four groups: (A) those containing 12α-HSDHase only (10 strains), (B) those containing 3α- and 12α-HSDHase (13 strains), (C) those containing 3α-HSDHase only (2 strains), and (D) those devoid of any measurable HSDHase activity (7 strains). Of the respective four groups, 9/10, 13/13, 0/2, and 0/7 were like the neotype strain of *E. lentum* (ATCC 25559) in that they produced H_2S in a triple sugar iron agar butt, reduced nitrate to nitrite, and weakly decomposed hydrogen peroxide. The other strains were variable for nitrate reduction and activity on hydrogen peroxide, but all the organisms in the first three categories (with one exception) were H_2S producers (triple sugar iron agar butt) and all (with one exception) were designated *E. lentum*, whereas the organisms of category B were non-H_2S producers (triple sugar iron agar butt). Five of these seven were not stimulated by arginine and are designated "phenotypically similar organisms." Thin-layer chromatography of extracted spent bacterial medium of four representative strains from each group grown in the presence of cholate revealed the presence of (A) 12-oxo product, (B) 12-oxo and 3-oxo products, (C) 3-oxo product, and (D) the absence of any of these products. The 12α-HSDHase of category B organisms was unstable unless 10^{-3} M dithioerythritol was added to the buffer. With the exception of 3 out of 32 strains, there was a positive correlation between the production of measurable amounts of 12α-HSDHase and H_2S production. Growth curves and the effect of arginine on growth and the production of 3α- and 12α-HSDHase were examined in representative strains of categories A, B, and C. Both enzymes were shown to bind onto a nicotinamide adenine dinucleotide-Sepharose column and could be eluted by high-ionic-strength buffer, resulting in approximately 25-fold and 18-fold purification, respectively. Molecular weight estimations by Sephadex G-200 gave values of 205,000 and 125,000 for the 3α- and 12α-HSDHase, respectively. Purified 12α-HSDHase was investigated with respect to pH requirement, substrate specificity, and enzyme kinetics.

Eubacterium lentum, a gram-positive, anaerobic, nonmotile rod, has been isolated from normal human feces and human infections. The neotype strain of this non-saccharolytic species has been designated by Moore et al. (18) and has been reported to contain cytochromes *a*, *b*, and *c* and carbon monoxide-binding pigments (20). Growth can be greatly stimulated by arginine (19), which is degraded to ornithine and carbamyl phosphate, the latter being a source of adenosine 5'-triphosphate, ammonia, and carbon dioxide (19). Recently, *E. lentum* has been shown to degrade bile salts, by oxidizing 3α- and 12α-OH groups (15), and corticosteroids, by dehydroxylating the 21-OH group and oxidizing the 3α-OH group (4). This communication reports the presence of 3α- and/or 12α-hydroxysteroid dehydrogenase (HSDHase) activities against bile salts in a collection of *E. lentum* and the absence of these enzymes in not phenotypically similar organisms; we further report the behavior and purification of these enzymes on nicotinamide adenine dinucleotide (NAD)-Sepharose columns.

MATERIALS AND METHODS

Bacterial strains and their characterization. Strains used (Table 1) were from the Virginia Polytechnic Institute Anaerobe Laboratory, V. D. Bokkenheuser, S. M. Finegold, and the American Type Culture Collection. All strain designations in Table 1 are those of Virginia Polytechnic Institute except for 25559

TABLE 1. *3α- and 12α-HSDHase activities in E. lentum and related organisms*

Category	Strain	Source[a]	Growth stimulation[b]	H_2S	NO_3^-	Gas from H_2O_2	12α-HSDH[c]	3α-HSDH[c]	*E. lentum*
A	9104A[d]	1	3.0	+	+	+	+++	−	+
	8135A	1	3.2	+	+	+	++	−	+
	3162P	1	4.1	+	+	+	+	−	+
	9130	1	7.2	+	+	+	+	−	+
	8662C	1	5.2	+	+	+	+	−	+
	6701K_2	1	1.3	+	+	+	+++	−	?[e]
	6598A	1	2.7	+	+	+	+++	−	+
	6718E	1	3.1	+	+	+	+	−	+
	8902	1	8.8	+	+	+	+	−	+
	116	2	11.0	−	−	−	+	−	?
B	25559[d]	3	2.7	+	+	+	++	+++	+[f]
	3995	4	3.7	+	+	+	++	++	+
	3999	4	3.3	+	+	+	+++	+	+
	8056	1	3.6	+	+	+	+	++	+
	8330TA	1	3.0	+	+	+	+++	++	+
	5483	1	4.4	+	+	+	+	++	+
	8371B	1	3.3	+	+	+	+	++	+
	8123	1	2.4	+	+	+	+	++	+
	8114	1	2.2	+	+	+	+	++	+
	11450C	1	3.5	+	+	+	+++	+++	+
	7725	1	4.0	+	+	+	+	+	+
	6266C	1	5.0	+	+	+	+	+	+
	9758	1	3.0	+	+	+	+	+	+
C	3197[d]	1	3.5	+	+	−	−	+++	+
	9066	1	5.2	+	+	−	−	+	+
D	3060D[d]	1	3.0	−	+	−	−	−	?
	9262	1	1.0	−	+	−	−	−	−
	9391B	1	1.0	−	+	−	−	−	−
	7616B	1	2.0	−	+	−	−	−	?
	3219D	1	1.0	−	−	−	−	−	−
	11602	1	1.0	−	+	+	−	−	−
	9489B	1	1.0	−	+	−	−	−	−

[a] (1) Virginia Polytechnic Institute Anaerobe Laboratory; (2) kindly donated by V. D. Bokkenheuser; (3) American Type Culture Collection; (4) kindly donated by S. M. Finegold.

[b] Stimulation by 2.0% arginine.

[c] +++, >0.3 unit per 10-ml culture; ++, 0.1 to 0.3 unit per 10-ml culture; +, <0.1 unit per 10-ml culture; −, activity not measurable. Substrates: 12α-HSDHase = 7α-,12α-dihydroxy-5β-cholanoate; 3α-HSDHase = 3α-,7α-dihydroxy-5β-cholanoate.

[d] Representative strains further studied.

[e] ?, Classification is debatable.

[f] ATCC 25559 = Neotype strain of *E. lentum*.

(American Type Culture Collection) and 116 (Bokkenheuser). All strains were gram-positive rods or ovals that were obligately anaerobic, nonfermentative, nonproteolytic, and indole negative when tested by the procedures described previously (8). Inoculum was from an overnight culture in cooked-meat Phytone medium (BBL Microbiology Systems, catalog no. 11130).

Production of H_2S was measured in the butts of anaerobically prepared (8) triple sugar iron agar slants that were stabbed and incubated in an atmosphere of oxygen-free nitrogen. A darkening of the butt within 3 days indicated a positive reaction.

Gas from hydrogen peroxide was measured by adding 3% hydrogen peroxide to 72-h growth on the surface of supplemented brain heart infusion agar (8) containing 2% arginine. A positive reaction was indicated by the evolution of gas bubbles from the area of growth.

Cultures grown in indole-nitrite medium (BBL Microbiology Systems) were incubated for 3 days and were then tested for nitrite by adding reagents containing sulfanilic acid and 1,2-dimethyl-α-naphylamine. Zinc was added to all cultures that were negative for nitrite to confirm that nitrate was still present in the medium.

Culture conditions for enzyme production. Strains were grown from lyophilized cultures in 10-ml volumes of cooked-meat broth (BBL Microbiology Systems) for 48 h at 37°C and kept at 5°C as stock cultures.

From these stock cultures, strains were subcultured

into 10-ml volumes of cooked-meat broth and grown as previously described (15), with the addition of 2.0% L-arginine hydrochloride (Sigma). For detailed studies on the four representative strains, 0.1 ml of cooked-meat culture was inoculated into 10 ml of freshly autoclaved brain heart infusion broth (Difco) containing 0.01% sodium thioglycolate and either 0.75 or 2.0% L-arginine hydrochloride. The cultures were incubated anaerobically for 48 h at 37°C in a GasPak jar.

Cell disruption and preparation of cell-free supernatant fluid. Cell-free supernatant fluid was prepared as previously described (15). For detailed studies on the four representative strains, cultures were harvested at 48 h by centrifuging at 6,000 × *g* for 20 min at 4°C. The pellet was suspended in 1.0 ml of 0.1 M sodium phosphate buffer (pH 7.0) and recentrifuged. The final sediment was suspended in 1.5 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 10^{-3} M ethylenediaminetetraacetic acid and 10^{-3} M dithioerythritol.

The 1.5-ml suspension of washed cells was disrupted with a French pressure cell at 18,000 pounds and then centrifuged at 6,000 × *g* for 10 min to sediment the cell debris. The supernatant fluid was collected and assayed for 3α-, 7α-, and 12α-HSDHase activities. Enzyme preparations, similarly derived from 200-ml cultures, were rapidly frozen with liquid nitrogen, lyophilized in a VirTis lyophilizer, and stored at −20°C. Freshly reconstituted enzyme was used for further studies.

Detection of 3-oxo and 12-oxo products in spent bacterial medium. Three-milliliter volumes of spent bacterial medium were acidified to pH 3–4 (3 N HCl) and extracted with 5 ml of diethyl ether. The ether was evaporated, and the extract was reconstituted in 300 μl of methanol–water, 4:1 (vol/vol). Samples of 30 μl of reconstituted extract were plated on silica-coated plates. Solvents were: (A) chloroform–methanol–acetic acid, 20:2:1 (vol/vol/vol); (B) chloroform–methanol–acetic acid, 40:2:1 (vol/vol/vol); and (C) benzene–dioxane–acetic acid, 70:20:1 (vol/vol/vol). Plates were developed with anisaldehyde (10) or *p*-hydroxybenzaldehyde (13).

Assay for NAD-dependent 3α- and 12α-hydroxysteroid dehydrogenase activities. The formation of reduced NAD was followed at 340 nm using a Beckman DBGT grating spectrophotometer and 10-inch recorder. Each assay cuvette (3.0 ml) contained 5.0×10^{-3} M NAD, 0.17 M glycine–NaOH buffer (pH 9.5), 5.0×10^{-3} M bile salt, and 50 μl of lyophilized *E. lentum* cell-free preparation (20 mg/ml) or 100 μl of freshly sonicated preparation. One unit of enzyme was defined as the amount of enzyme required to yield 1 μmol of reduced NAD per min (ϵNAD = $6.2 \times 10\ M^{-3}$). Michaelis constants (K_m values) and maximum velocities were calculated on the basis of Lineweaver-Burk plots using initial reaction velocities. A minimum of 10 concentrations of substrate over a 20-fold range was tested for each determination.

Preparation of NAD-coupled Sepharose and column chromatography of 3α- and 12α-HSDHase preparations. Approximately 200 ml of packed Sepharose 4B was thoroughly washed with doubly distilled water and activated with cyanogen bromide at 20°C as described by Cuatrecasas (5). The activated Sepharose was washed with 4 liters of 0.1 M ice-cold sodium bicarbonate (pH 9.5) and subsequently divided into two equal portions; each portion was added to a 50-ml solution of NAD (1.0 g/50 ml) and gently stirred overnight. Each Sepharose preparation was washed with 2 liters of 0.1 M ice-cold sodium bicarbonate (pH 9.5) and stored at 4°C until ready for use. The NAD-substituted Sepharose preparations were poured into a column (1 by 10 cm), and the column was equilibrated with 0.1 M phosphate buffer containing 10^{-3} M ethylenediaminetetraacetic acid and 10^{-3} M dithioerythritol. One hundred milligrams of lyophilized *E. lentum* preparation was dissolved in 1.0 ml of doubly distilled water and chromatographed on the nucleotide column. Elution was effected by increasing the ionic strength of the buffer. Fractions were collected in 1.0-ml volumes. Selected fractions were pooled, rapidly frozen, and lyophilized. The lyophilized fraction was used for molecular weight estimations, kinetic studies, and protein estimation.

Quantification of Sepharose-linked nucleotides. Washed and packed Sepharose samples (1 ml) were dried and digested with perchloric acid, and inorganic phosphate was measured (2). NAD residues were computed on the basis of 2 equivalents of phosphate per residue; unsubstituted Sepharose served as a blank.

Protein determinations. Protein was estimated according to Lowry et al. (12).

Redox potential determinations. Redox potential (Eh) values were determined using an Orion platinum redox electrode (model 96-78) attached to an Orion pH/Eh meter.

Molecular weight estimation. The molecular weight was estimated by Sephadex G-200 chromatography (1). Molecular markers (Pharmacia) were ovalbumin, alcohol dehydrogenase, aldolase, catalase, and 3α-HSDHase from *Pseudomonas testosteroni* (Sigma).

RESULTS

3α- and 12α-HSDHases in *E. lentum* and phenotypically similar organisms. The 32 strains screened could be divided into four groups: (A) those containing only 12α-HSDHase (10 strains); (B) those strains containing both 3α- and 12α-HSDHase (13 strains); (C) those containing only 3α-HSDHase (2 strains); and (D) those devoid of any measurable HSDHase (7 strains), as shown in Table 1. With the exception of 3 out of 32 strains (no. 116 and the group C organisms), there was a positive correlation between H_2S production and the presence of 12α-HSDHase. All of the organisms in groups A, B, and C, with the exception of 6701K_2, were markedly stimulated by arginine, whereas most of the strains (5/7) in group D were not responsive to arginine and grew relatively poorly in brain heart infusion broth. Of the respective four groups, 9/10, 13/13, 0/2, and 0/7 were like the *E. lentum* neotype strain ATCC 25559 in that they produced H_2S in the triple sugar iron agar

buffer, reduced nitrate to nitrite, and produced gas from H_2O_2. The other strains were variable for nitrate reduction and gas from H_2O_2, but all the organisms in the first three categories (with the exception of 116) produced H_2S in the triple sugar iron agar butt and have been designated *E. lentum*. Strain 116 appears unique, since it is negative with respect to H_2S production, nitrate reduction, and gas from H_2O_2, yet contains 12α-HSDHase. The taxonomic position of strain 116 remains questionable. Five of the seven organisms in group D were judged sufficiently unlike *E. lentum* to be grouped with them and were designated "non-*E. lentum*." The taxonomic position of strains 3060D and 7616B remains in question.

Because there was only slight evolution of gas when H_2O_2 was added to growth on the arginine slants, we questioned that this observation represented true catalase activity. Accordingly, broth cultures of representative strains (8902, 6718E, 7725, 25559, 9066, 3197, and 116) were analyzed for decomposition of H_2O_2, by using the method of Beers and Sizer (3), by E. M. Gregory, Department of Biochemistry and Nutrition, Virginia Polytechnic Institute. No breakdown of H_2O_2 was detected by crude extracts of any of these cultures (E. M. Gregory, personal communication). We are unable to explain the cause of gas evolution when H_2O_2 was added to broth on arginine slants.

Strain 3060D (non-*E. lentum*) could be further differentiated from the representative *E. lentum* strains of categories A, B, and C by (i) a final pH value of 6.25 ± 0.21 (two determinations) versus 8.51 ± 0.096 (four determinations); (ii) a final Eh value of 40 ± 40 mV (two determinations) versus 154 ± 20 mV (four determinations); and (iii) the absence of any oxidation product versus the presence of 12-oxo, 3- and 12-oxo, and 3-oxo products as demonstrated by thin-layer chromatography of extracted spent bacterial medium (5, 13).

Growth, arginine stimulation, and dehydrogenase production in strains 25559, 9104A, and 3197. The results are summarized in Fig. 1 and 2. Clearly the optimal arginine concentration for production of 3α- and 12α-HSDHase does not necessarily correspond to that required for optimal growth of the organisms. Only in strain 3197 was the optimum identical at 2%. For a 10-ml culture, an absorbance of 0.5 at 660 nm was approximately equivalent to a dry weight of 7 mg. A range of 200 to 500% stimulation by L-arginine hydrochloride was observed for most of the strains that were stimulated. In strain ATCC 25559, the ratio of 3α-HSDHase to 12α-HSDHase varied somewhat from one experiment to the next. In addition, repeated experiments using 10-ml culture volumes showed a threefold greater 3α-HSDHase to 12α-HSDHase ratio than we previously observed using 200-ml culture volumes (14). The relative yield of 3α-HSDHase apparently increased while that of 12α-HSDHase remained unchanged. There does not, however, appear to be any difference in the cell mass per unit volume when 10-ml cultures are compared to 200-ml cultures (15).

Behavior of 3α- and 12α-HSDHase on NAD-Sepharose columns. As shown in Fig. 3, both 3α- and 12α-HSDHase are capable of binding to NAD-Sepharose and can be eluted by high ionic strength. This can be demonstrated with enzyme preparations from strains 9104A, 25559, and 3197. Purification of the 3α- and 12α-HSDHase was approximately 25- and 18-fold, respectively. Columns were reusable after extensive washing with 0.01 M HCl and 0.05 M Na_2CO_3 and re-equilibration with buffer. The extent of substitution of NAD-Sepharose was estimated at 105 nmol per ml of packed absorbant.

Molecular weight estimations of 3α- and 12α-HSDHase. The molecular weights of purified 3α-HSDHase from strain 3197 and 12α-HSDHase from strain 9104A were estimated at 205,000 and 125,000, respectively. Both these values were higher than those published for *P. testosteroni* 3α-HSDHase (47,000) (21), *Escherichia coli* 7α-HSDHase (105,000) (16), and *Clostridium* group P 12α-HSDHase (100,000) (14) (Fig. 4).

Stability of crude and purified 12α-HSDHase from strain 9104A. When dithioerythritol was deleted from the buffer used for preparation of 9104A 12α-HSDHase, the enzyme yields were less than 20% of those obtained in the presence of dithioerythritol. The crude liquid preparation, in the presence of dithioerythritol, was stable for 4 to 6 days. The lyophilized preparation was stable for 3 months at −20°C (100% activity present) with a loss of 20% activity and 65% activity when kept at 4°C and room temperature, respectively. 12α-HSDHase, purified approximately 18-fold, lost only 5% activity when stored at −20°C for 3 months.

pH dependency, kinetic, and substrate specificity studies of 12α-HSDHase from *E. lentum* 9104A. The effect of pH value on purified 12α-HSDHase from 9104A is demonstrated in Fig. 5. A rather broad flat curve with an optimum pH range of 8 to 10.5 was observed. No activity was detected with absence of enzyme or substrate at any pH within the range studied. When the pH was measured before and after the

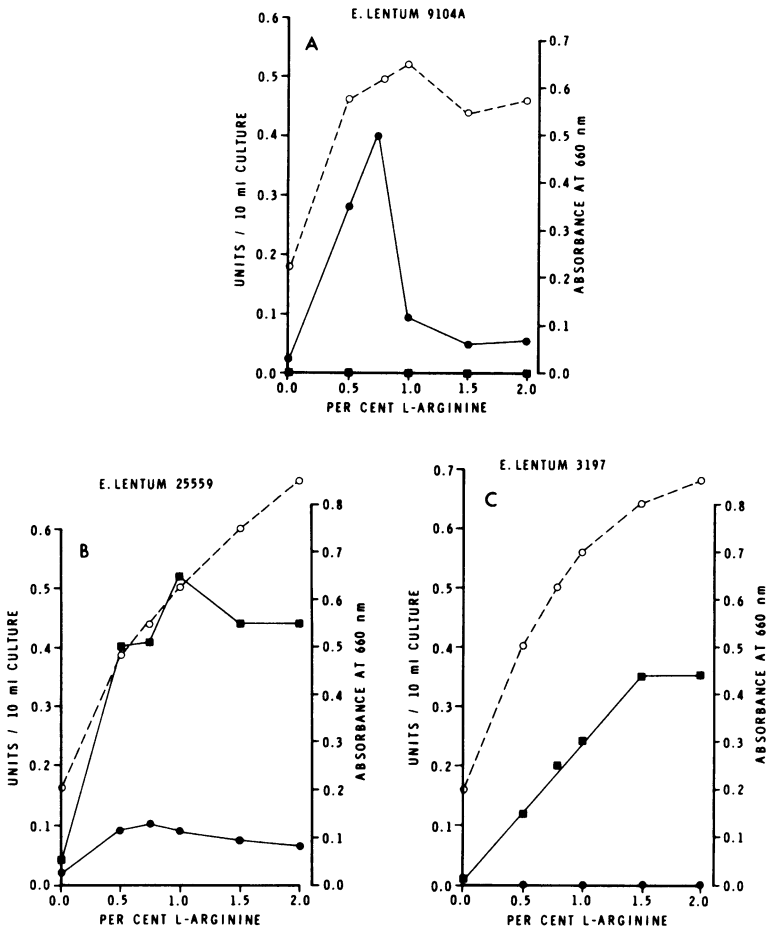

FIG. 1. *Effect of* L*-arginine hydrochloride on growth* (○) *measured as absorbance of culture at 660 nm, and on production of 3α-HSDHase* (■) *(substrate, 3α-,7α-dehydroxy-5β-cholanoate) and 12α-HSDHase* (●) *(substrate, 7α-,12α-dihydroxy-5β-cholanoate) in strains (A) 9104A, (B) 25559, and (C) 3197. The cells were harvested at 48 h, when the stationary phase had been reached.*

reaction, the change was invariably less than 0.1 pH unit. Kinetic data for a limited number of substrates are presented in Table 2.

In addition to those compounds listed in Table 2, allocholate, allodeoxycholate, 5β-cholan-3α,7α,12α-tetrol, and taurine conjugates of cholate and deoxycholate were all substrates. Notably, the three sulfates of cholate and deoxycholate were non-substrates; no reactivity was measurable with chenodeoxy- or ursodeoxycholate. The total absence of 7α-HSDHase was confirmed in strains 9104A, 25559, 3197, 6266C, and 9066 by three findings. (i) On complete oxidation of chenodeoxycholate, only one equivalent of reduced NAD was evolved by 3α-HSDHase-containing organisms and none by organisms containing only 12α-HSDHase. (ii) No significant 7α-OH bioconversion was demonstrable in spent bacterial medium (13). (iii) No evidence of 7-oxo product was observed on thin-layer chromatography with anisaldehyde (10) or *p*-hydroxybenzaldehyde (13) spray reagents.

DISCUSSION

This investigation further increases the earlier evidence (15) that suggests that there are two separate HSDHases, at least one of which is found in all *E. lentum* strains, and that the 12α-HSDHase is the more labile of the two. With the possible exception of strains 116, 3060D, and 7616B, it appears that one can differentiate between *E. lentum* and phenotypically similar organisms on the basis of the presence of 3α- and/or 12α-HSDHase in the former and absence in the latter. The stimulation of growth by L-arginine hydrochloride and the pH and Eh values of spent bacterial medium are also of assistance in differentiating these groups. Among the three

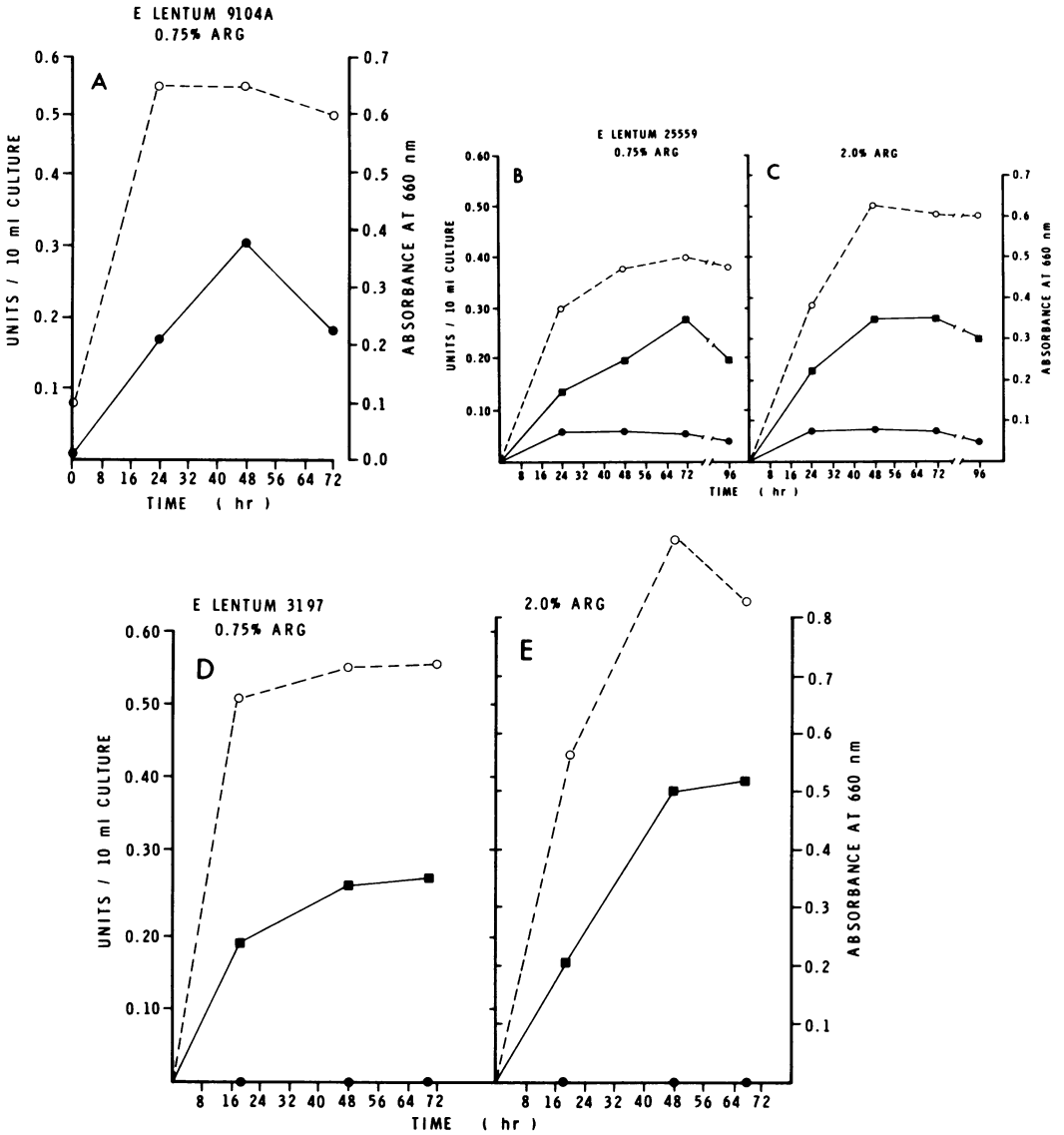

FIG. 2. *Growth and production of enzymes for (A) strain 9104A in the presence of 0.75% L-arginine hydrochloride; (B) and (C) strain 25559 in the presence of 0.75% and 2.0% arginine hydrochloride; and (D) and (E) strain 3197 in the presence of 0.75% and 2.0% arginine hydrochloride.* (○) *Absorbance of culture at 660 nm;* (■) *3α-HSDHase (substrate, 3α-,7α-dihydroxy-5β-cholanoate);* (●) *12α-HSDHase (substrate, 7α-,12α-dihydroxy-5β-cholanoate).*

representative strains whose growth and enzyme production are stimulated by L-arginine hydrochloride, there are distinct differences in the optimal arginine concentration required for enzyme production. The production of 12α-HSDHase in 9104A appears to parallel the amount of growth up to an arginine concentration of 0.75%; the mechanism of loss of activity at higher arginine concentrations is not understood.

Unfortunately, the interactions of 3α- and 12α-HSDHase with NAD-substituted Sepharose are not specific. A number of unrelated dehydrogenases have also been shown to interact with nucleotide-substituted Sepharose and are similarly eluted by high ionic strength (11). The nonspecificity of any of the above-named systems resides in (i) the biological interaction of nucleotides with proteins other than dehydrogenases and (ii) the weak ion exchange capacity of nu-

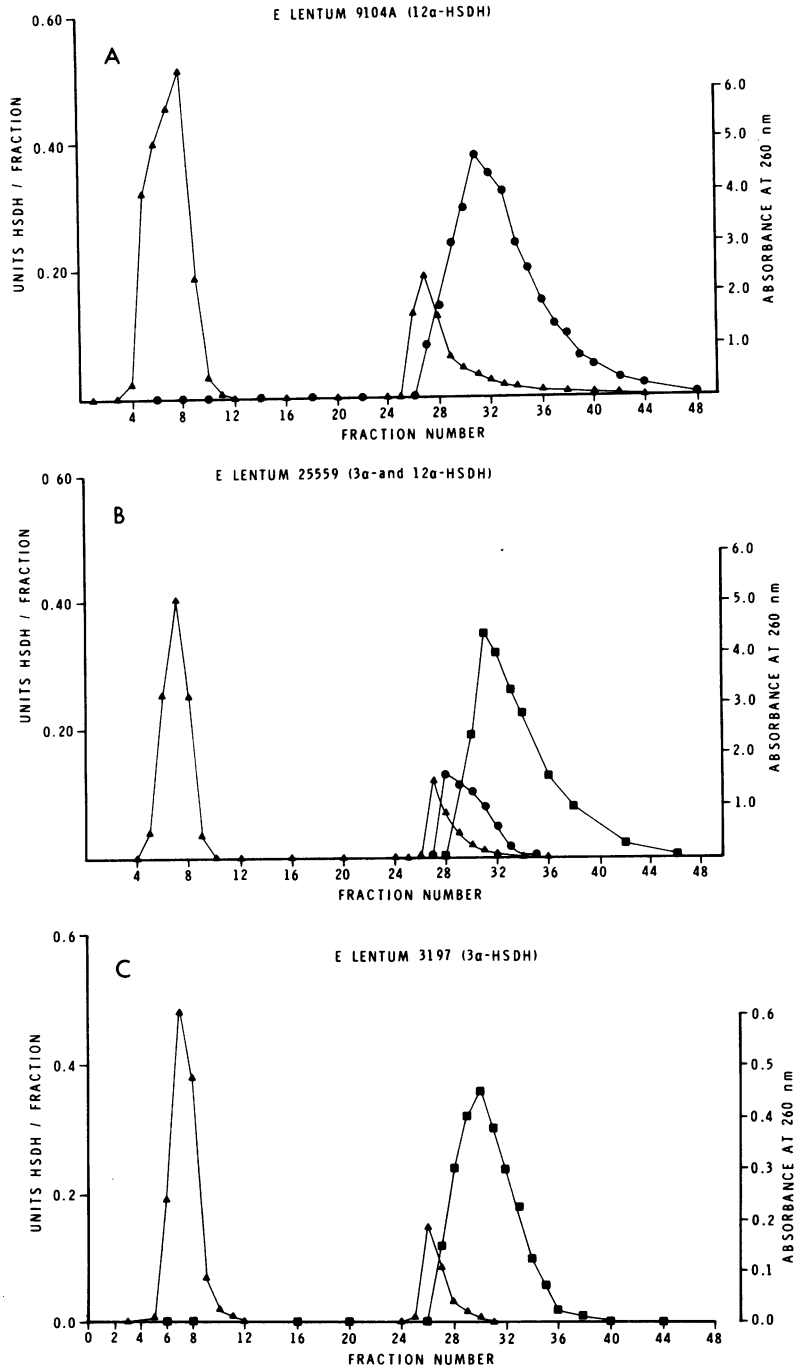

FIG. 3. *NAD-Sepharose 4B chromatography of (A) 12α-HSDHase from E. lentum 9104A, (B) 3α- and 12α-HSDHase from E. lentum 25559, and (C) 3α-HSDHase from E. lentum 3197.* (▲) *Absorbance at 260 nm;* (■) *3α-HSDHase (substrate, 3α-,7α-dihydroxy-5β-cholanoate);* (●) *12α-HSDHase (substrate, 7α-,12α-dihydroxy-5β-cholanoate). Samples of 100 mg of crude lyophilized cell-free preparation from the three respective strains were chromatographed. Elution was achieved by 0.2 M sodium phosphate buffer (pH 7.0).*

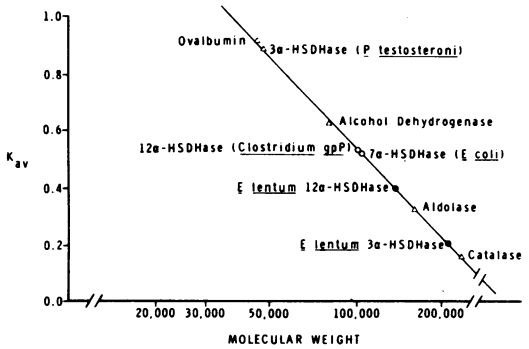

FIG. 4. *K_{av}, $(V_e - V_0)/(V_t - V_0)$, where V_e, V_t, and V_0 are the elution volume, total volume, and void volume of a Sephadex G200 column, is plotted against molecular weight (1) for the standards ovalbumin, P. testosteroni 3α-HSDHase, alcohol dehydrogenase, aldolase, and catalase (△). (●) E. lentum 3α-HSDHase and 12α-HSDHase; (○) E. coli 7α-HSDHase (16) and Clostridium group P 12α-HSDHase (14) presented for comparison. All values were the average of two determinations.*

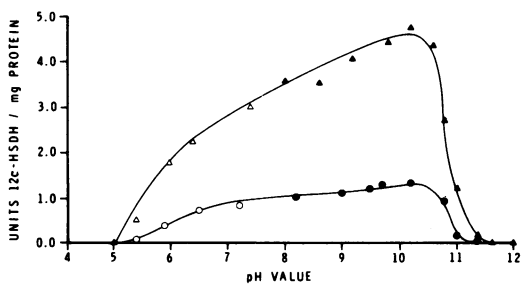

FIG. 5. *Effect of pH value on purified 12α-HSDHase from E. lentum 9104A. Substrates, 7α-,12α-dihydroxy-5β-cholanoate (○) and 3α-,7α-,12α-trihydroxy-5β-cholanoate-methyl ester (△). Open symbols: 1.0 M glycyl-glycine–NaOH; closed symbols: 1.0 M glycine–NaOH buffer.*

cleotide-substituted absorbants.

The effect of pH value on purified 12α-HSDHase from strain 9104A is similar to that demonstrated for crude 12α-HSDHase from strain 25559 (15). The relatively higher reaction velocities of the methylated bile salts and 5β-cholan-3α,7α,12α,24-tetrol compared to underivatized bile salts may reflect the preference of the enzyme for less polar nonamphipathic steroids. The lack of reactivity with 3-sulfates (similar to that of *E. coli* 7α-HSDHase [7]) and the consistently higher K_m values for conjugated bile salt compared to the corresponding free bile salt (Table 2) also support this thesis.

Current investigation focuses on the 3α-HSDHase from *E. lentum* 3197 and the possible use of this enzyme as a substitute for *P. testosteroni* 3α-HSDHase in the quantification of bile salts (6, 9). The properties of *E. lentum* 9104A 12α-HSDHase compared to 12α-HSDHase from *Clostridium* group P C48-50 (14, 17), particularly with respect to quantification of 12α-OH groups, are similarly under investigation.

TABLE 2. *Some kinetic parameters of E. lentum 9104A 12α-HSDH*

5β-Cholanoate	Prepn	K_m (M)	V_{max}[a]
3α,7α,12α-Trihydroxy-	Purified	5.9×10^{-5}	0.30
3α-7α-12α-Trihydroxy-methyl ester	Crude	4.0×10^{-5}	
3α,7α,12α-Trihydroxy-methyl ester	Purified	3.0×10^{-5}	0.52
3α,7α,12α-Trihydroxy-glycine	Purified	2.5×10^{-4}	0.20
3α,12α-Dihydroxy-	Purified	2.8×10^{-5}	0.22
3α,12α-Dihydroxy-glycine	Crude	1.6×10^{-4}	
3α,12α-Dihydroxy-glycine	Purified	1.7×10^{-4}	0.18
3α,12α-Dihydroxy-	Purified	3.0×10^{-5}	0.24

[a] Units of 12α-HSDH per milligram of protein (partially purified preparation at pH 9.5).

ACKNOWLEDGMENT

We acknowledge the assistance of Barbara Rao in critical review of this manuscript.

LITERATURE CITED

1. **Andrews, P.** 1965. The gel filtration behavior of protein related to their molecular weights over a wide range. Biochem. J. **96**:595–606.
2. **Bartlett, G. R.** 1959. Phosphorus assay in column chromatography. J. Biol. Chem. **234**:466–468.
3. **Beers, R. F., Jr., and I. W. Sizer.** 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. **195**:133–140.
4. **Bokkenheuser, V. D., J. Winter, P. Dehazya, and W. G. Kelly.** 1977. Isolation and characterization of human fecal bacteria capable of 21-dehydroxylating corticoids. Appl. Environ. Microbiol. **34**:571–575.
5. **Cuatrecasas, P.** 1970. Protein purification by affinity chromatography derivatization of agarose and polyacrylamide beads. J. Biol. Chem. **245**:3059–3065.
6. **Engert, R., and M. D. Turner.** 1973. Problems in the measurement of bile acids with 3α-hydroxysteroid dehydrogenase. Anal. Biochem. **51**:399–407.
7. **Haslewood, E. S., and G. A. D. Haslewood.** 1977. The specificity of 7α-hydroxysteroid dehydrogenase from *Escherichia coli*. Biochem. J. **157**:207–210.
8. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore (ed.).** 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute Anaerobe Laboratory, Blacksburg.
9. **Iwata, T., and K. Yamasake.** 1964. Enzymatic determination and thin-layer chromatography of bile acids in blood. J. Biochem. **56**:424–431.
10. **Kritchevsky, D., D. S. Martak, and G. H. Rothblat.** 1963. Detection of bile acids in thin layer chromatography. Anal. Biochem. **5**:388–392.
11. **Low, C. R., and P. G. G. Dean.** 1971. Affinity chromatography of enzymes on insolubilized cofactors. FEBS Lett. **14**:313–316.
12. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.

13. **Macdonald, I. A.** 1977. Detection of bile salts with Komarowsky's reagent and group specific dehydrogenases. J. Chromatogr. **136**:348–352.
14. **Macdonald, I. A., J. F. Jellett, and D. E. Mahony.** 1979. 12α-Hydroxysteroid dehydrogenase from *Clostridium* group P strain C48-50, ATCC 29733: partial purification and characterization. J. Lipid Res. **20**:234–239.
15. **Macdonald, I. A., D. E. Mahony, J. F. Jellett, and C. E. Meier.** 1977. NAD-dependent 3α- and 12α-hydroxysteroid dehydrogenase activities from *Eubacterium lentum* ATCC 25559. Biochim. Biophys. Acta **489**:466–476.
16. **Macdonald, I. A., C. N. Williams, and D. E. Mahony.** 1976. Behavior of 3α- and 7α-hydroxysteroid dehydrogenases on chenodeoxycholate-substituted sepharose. Steroids **28**:25–30.
17. **Mahony, D. E., C. E. Meier, I. A. Macdonald, and L. V. Holdeman.** 1977. Bile salt degradation by nonfermentative clostridia. Appl. Environ. Microbiol. **34**:419–423.
18. **Moore, W. E. C., E. P. Cato, and L. V. Holdeman.** 1971. *Eubacterium lentum* (Eggerth) Prevot 1938: emendation of description and designation of neotype strain. Int. J. Syst. Bacteriol. **21**:299–303.
19. **Sperry, J. F., and T. D. Wilkins.** 1976. Arginine, a growth-limiting factor for *Eubacterium lentum*. J. Bacteriol. **127**:780–784.
20. **Sperry, J. F., and T. D. Wilkins.** 1976. Cytochrome spectrum of an obligate anaerobe, *Eubacterium lentum*. J. Bacteriol. **125**:905–909.
21. **Squire, P. G., S. Delin, and J. Porath.** 1964. Physical and chemical characterization of hydroxysteroid dehydrogenases from *Pseudomonas testosteroni*. Biochim. Biophys. Acta **89**:409–421.