12α-Hydroxysteroid dehydrogenase from *Clostridium* group P strain C48-50 ATCC #29733: partial purification and characterization

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Abstract The growth of *Clostridium* group P strain C48-50 [an anaerobe that contains 12α-hydroxysteroid dehydrogenase (12α-HSDH) in the absence of other dehydrogenases active upon bile salts] is greatly enhanced by the addition of 2.0% D-fructose or D-glucose to the growth medium. Other sugars were less effective. The production of NADP-dependent 12α-HSDH paralleled the growth of the organism which was optimal at 72 hr. Growth (and enzyme production) were suppressed by the addition of bile salt to the medium; the order of suppression was deoxycholate $>$ chenodeoxycholate $\gg$ cholate; 1 mM of either of the dihydroxy-bile salts inhibited 96% of the growth and 100% of the enzyme production. Kinetic studies on cell-free preparations of 12α-HSDH revealed a pH optimum of 7.8 with greater linearity of NADP evolution with time occurring only at more alkaline pH values (9–10). Lineweaver–Burke plots revealed Michaelis constant (K_m) values in the range of $3-5 \times 10^{-4}$ M for deoxycholate and its glycine and taurine conjugates, while higher values were found for cholate and conjugates (K_m value for taurocholate was 3×10^{-3} M). Although there was no activity with NAD, 12α-HSDH was shown to bind onto both NAD- and NADP–Sepharose columns, with stronger binding on the latter. The enzyme was purified 20-fold by NAD–Sepharose chromatography. The molecular weight was estimated at 100,000 by Sephadex G-200 and a series of molecular weight markers. Substrate specificity studies showed that a variety of bile salts containing 12α-OH groups reacted; notably, the 3α-sulfates of cholate and deoxycholate were nonsubstrates.—**Macdonald, I. A., J. F. Jellett and D. E. Mahony.** 12α-Hydroxysteroid dehydrogenase from *Clostridium* Group P strain C48-50 #29733: partial purification and characterization. *J. Lipid Res.* 1979. **20:** 234–239.

Supplementary key words 12α-hydroxyl group oxidation · bile salt oxidoreductase

12α-HSDH (E. C. 1.1.1.–) has been described in *Clostridium perfringens* (1), *Clostridium leptum* (2), *Eubacterium lentum* (3), and crude preparations of *Pseudomonas testosteroni* (4). Mahony et al. (5) recently documented the presence of this enzyme in a number of Clostridia. One such organism (designated *Clostridium* group P, strain C48-50) was distinct from the other 12α-HSDH-containing Clostridia, both in colonial morphology and in the absence of intracellular 3α- and 7α-HSDH. In this communication, we report the effects of sugars and bile salts on the growth of *Clostridium* group P C48-50, and partial purification and characterization of NADP-dependent 12α-HSDH.

MATERIALS AND METHODS

All hydroxyl-bile salts were obtained from Calbiochem, Los Angeles, CA; ketonic bile salts were from Steraloids Inc., Wilton, NH. Bile salt sulfates were kindly donated by Dr. G. A. D. Haselwood and 5α-bile salt esters were the gift of Dr. W. Elliot. All sugars and nicotinamide adenine dinucleotide phosphate (NADP) were from Sigma Chemical Company, St. Louis, MO. Brain–heart infusion broth and cooked meat medium were from Difco Laboratories, Detroit, MI. Glycine, sodium hydroxide, sodium phosphate, EDTA, PPO, POPOP, methanol, chloroform, benzene, and acetic acid were from Canadian Laboratories, Montreal, Quebec. Molybdophosphoric acid and *p*-hydroxybenzaldehyde were from E. Merke, Darmstadt, West Germany and Eastman-Kodak, Toronto, Ontario. Partially purified 3α-HSDH was a product of Worthington Biochemical Corporation, Freehold, NJ and 7α-HSDH was prepared by us as described before (6). Sephadex G-200 and Sepharose 4B were from Pharmacia, Dorval, Quebec. Uniformly labeled D-[^{14}C]glucose (240 mCi/mmol) was a product of New England Nuclear Corp.

Abbreviations: HSDH, hydroxysteroid dehydrogenase; NAD$^+$, nicotinamide adenine dinucleotide; NADP$^+$, nicotinamide adenine dinucleotide phosphate; EDTA, ethylenediaminetetraacetic acid; DC, deoxycholate or 3α,12α-dihydroxy-5β-cholanoate; CDC, chenodeoxycholate or 3α,7α-dihydroxy-5β-cholanoate; DTE, dithioerythritol.

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Growth and harvest of *Clostridium* group P strain C48-50

Unless otherwise designated, brain–heart infusion broth, containing 0.1% sodium thioglycolate and 2.0% D-fructose, was used for optimal growth of the organisms. These bacteria were maintained in cooked meat medium at 4°C or anaerobically on brain–heart infusion–agar plates supplemented with 2.0% D-fructose at 25°C. Cultures were incubated in cooked meat for 48 hr at 37°C prior to inoculation into 10 ml of broth and grown in an atmosphere of hydrogen and carbon dioxide provided by a Gas Pak (BBL) generator for 96 hr at 37°C. Cultures were centrifuged at 6000 *g* for 20 min at 4°C in an International B-20 centrifuge. The pellet was resuspended in 1.5 ml of 0.1 M sodium phosphate, pH 7.0, containing 10^{-3} M EDTA and the bacteria were ruptured in a French pressure cell. The cell-free preparation was centrifuged at 6000 *g* for 20 min at 4°C and the supernatant fluid was decanted from the pellet.

Assay for 12α-hydroxysteroid dehydrogenase activity

The formation of NADH was followed at 25°C at 340 nm using a Beckman spectrophotometer with a 10-in Beckman recorder. Each assay cuvette contained 1.7×10^{-3} M $NADP^+$, 0.17 M glycine/NaOH buffer (pH 9.5), 1.0×10^{-3} M glycodeoxycholate, and 100 μl of freshly prepared 12α-HSDH (final volume 3.0 ml). Enzyme produced by scaling up the medium volume from 10 ml to 200 ml was rapidly frozen in a 100-ml flask and lyophilized in a Virtis lyophilizer. The dried material was stored at −20°C, reconstituted (10 mg/ml), and 50 μl per assay was used for routine kinetic and pH dependence studies. For pH studies, 0.17 M glycylglycine was used at pH values lower than 7. A unit of 12α-HSDH is defined as sufficient enzyme to catalyze the formation of 1 μmol NADPH per min under the above described conditions.

Verification of oxidation site

Sodium cholate (10^{-4} M), under standard assay conditions, was allowed to oxidize to completion (until there was no further increment in absorbance at 340 nm). The reaction mixture (3 ml) was adjusted to pH 3 (HCl), extracted with 3 ml of diethyl ether and reconstituted to 0.3 ml. Fifty-μl aliquots were subjected to thin-layer chromatography with the following solvent systems: *a*) chloroform–methanol–acetic acid 40:4:2, *b*) chloroform–methanol–acetic acid 40:2:1, and *c*) benzene–dioxane–acetic acid 75:20:2 (all v/v/v) modified from Eneroth (7). Plates were sprayed with molybdophosphoric acid (8), or *p*-hydroxybenzaldehyde reagent (9), or anisaldehyde reagent (10). The standards (Steraloids) 7α,12α-dihydroxy-3-oxo-5β-cholanoate, 3α,12α-dihydroxy-7-oxo-5β-cholanoate, and 3α,7α-dihydroxy-12-oxo-5β-cholanoate were chromatographed in parallel. Unsprayed, as well as *p*-hydroxybenzaldehyde-sprayed, products of *Clostridium* group P strain C48-50 oxidation were eluted from the plate by scraping and washing with methanol. The eluate was subsequently tested with 3α-HSDH (*P. testosteroni*) and 7α-HSDH (*E. coli*) as described before (11).

Disappearance of glucose in spent bacterial medium from a culture grown in the presence of D-[^{14}C] glucose

Strain C48-50 was grown as described above in the presence of 2.0% glucose. In addition, 5 mCi of D-[U-^{14}C]glucose was included. Twenty-five μl of spent bacterial medium was counted in a Nuclear Chicago Mark II liquid scintillation counter using a full ^{14}C window. Additionally, 25 μl of cell-free preparation and cell debris (solubilized by 1.0 ml of perchloric acid after French pressing and centrifugation), harvested at 96 hr, were counted similarly. All samples were counted in duplicate in glass vials containing 10 ml of toluene–Triton scintillation mixture consisting of 5.87 g of PPO, 98 mg of POPOP, 360 ml of Triton X-100, and 3 liters of redistilled toluene. Total glucose concentration in the spent bacterial medium and uninoculated (sterile) control medium was measured by the glucose oxidase-based method of Trinder (12) using a Technicon AA2 autoanalyzer.

Synthesis of NAD- and NADP-substituted Sepharose 4B and affinity chromatography of 12α-HSDH

Approximately 200 ml of packed Sepharose 4B was thoroughly washed with doubly distilled water and activated by cyanogen bromide at 20°C as described by Cuatracasas (13). The activated Sepharose was washed with 4 liters of 0.1 M ice-cold sodium bicarbonate, pH 9.5, and was subsequently divided into two equal portions; a portion each was added to 50-ml solutions of NAD (1 g/50 ml) and NADP (1.2 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- and NADP-substituted Sepharose preparations were poured into a column 1 × 10 cm and the column was equilibrated with 0.1 M sodium phosphate buffer containing 10^{-3} M EDTA and 10^{-3} M dithioerythritol (DTE). Ten mg (approximately 10 units of 12α-HSDH) of lyophilized material was dissolved in 1.0 ml of doubly distilled water and chromatographed on the nucleotide column. Elution

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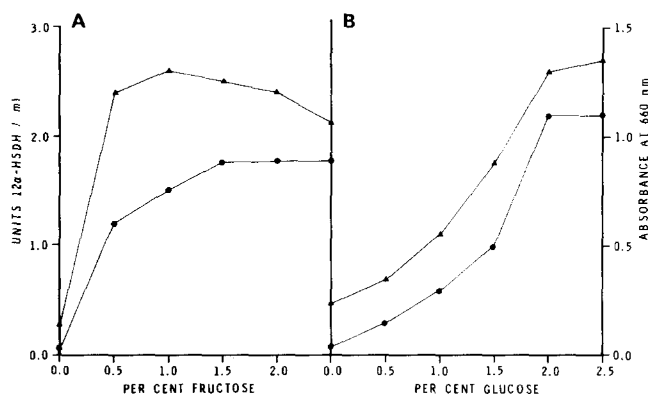

Fig. 1. Effect of fructose (*A*) and glucose (*B*) on the growth of C48-50 (▲ —— ▲) and the production of NADP-dependent 12α-HSDH in cell-free preparations (● —— ●). (Substrate GDC). Cultures were grown for 96 hr.

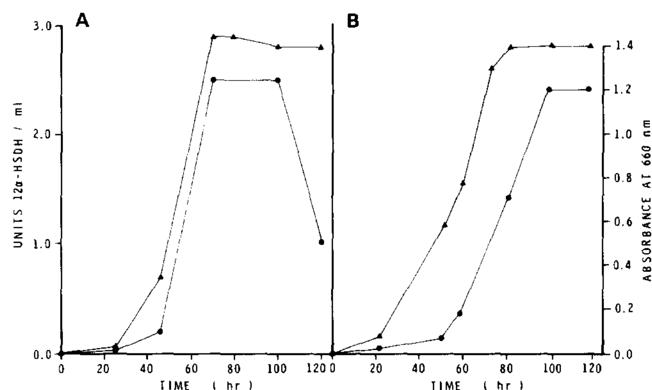

Fig. 2. Time course curve for the growth of C48-50 (▲ —— ▲) and production of 12α-HSDH (*A*) in the presence of 2% fructose and (*B*) in the presence of 2% glucose. (Substrate GDC).

was effected by increasing the ionic strength of the buffer. Fractions were collected in 1.0-ml volumes. Selected fractions were rapidly frozen and lyophilized, and the dried material was stored at −20°C for molecular weight estimations, kinetic studies, and protein determinations. The extent of substitution of nucleotides per ml packed Sepharose was estimated by phosphate determination (14).

Protein determinations

Protein was estimated according to Lowry et al. (15).

Molecular weight estimation

The molecular weight was estimated by Sephadex G-200 chromatography (16). Molecular markers (Pharmacia) were ovalbumin, alcohol dehydrogenase, aldolase, catalase, and 3α-HSDH from *P. testosteroni* (Sigma).

RESULTS

Effect of sugars and bile salts on the growth of *Clostridium* group P strain C48-50 and the production of 12α-HSDH

Both glucose and fructose significantly enhanced the growth of this Clostridium (**Figs. 1** and **2**). Although the effect appeared to be more rapid in the case of fructose, both sugars gave optimal growth and enzyme yield at 2%. The production of 12α-HSDH in all cases appeared to parallel the growth, and was enhanced by 2% fructose or glucose by approximately a factor of 15. Under optimal conditions, approximately 3 units of enzyme can be obtained from a 10-ml culture. This yield is a factor of 5 less than the 7α-HSDH obtainable from strain 23 *E. coli* (6) but surpasses the yield of 12α-HSDH from the same organism grown in unsupplemented medium (5) by more than a factor of 10. Ribose and mannose were only partially effective in enhancing the growth and enzyme yield. Other sugars, including galactose, sucrose, and lactose, and the sugar derivatives gluconic acid and glucosamine were totally ineffective in enhancing either the growth of C48-50 or the enzyme yield.

Enzymatic assay (12) reveals the disappearance of nearly ⅔ of the glucose during a culture period of 144 hr (**Table 1**), yet a negligible amount of ^{14}C label is lost. Less than 1% of label appears to be associated with the biomass (t = 96 hr). It is also evident that the pH drop during the 144-hr growth was less than 0.5 pH unit. Trace amounts of acetic acid in spent bacterial medium were evident from gas–liquid chromatography.[1] No carbon dioxide appeared to be evolved.

In contrast to *Clostridium perfringens* (1), the addition of 10^{-3} M cholate to the medium slightly depressed the growth of C48-50 and markedly depressed the enzyme production; the addition of 10^{-3} M 3α,12α-dihydroxy-5β- and 3α,7α-dihydroxy-5β-cholanoates (DC and CDC) completely depressed the growth and

[1] Macdonald, I. A., J. F. Jellett, and D. E. Mahony. Unpublished observation.

TABLE 1. Disappearance of glucose in spent bacterial medium on growth of C48-50 in the presence of 2.0% [U-^{14}C]glucose

Time During Growth[a]	% Glucose in Spent Bacterial Medium	% Disappearance of Glucose (glucose oxidase)	% ^{14}C in Spent Bacterial Medium	Absorbance (660 nm) of Culture	pH
0[b]	2.05	0	100	0	7.3
24	1.99	2.9	100	0.31	7.3
48	1.65	19.5	99	1.60	7.2
72	1.12	45.4	97	1.80	7.1
96	0.89	56.5	99	1.80	7.0
144	0.70	65.8	97	1.70	7.0

[a] See Methods and Fig. 1*B*.
[b] Sterile control.

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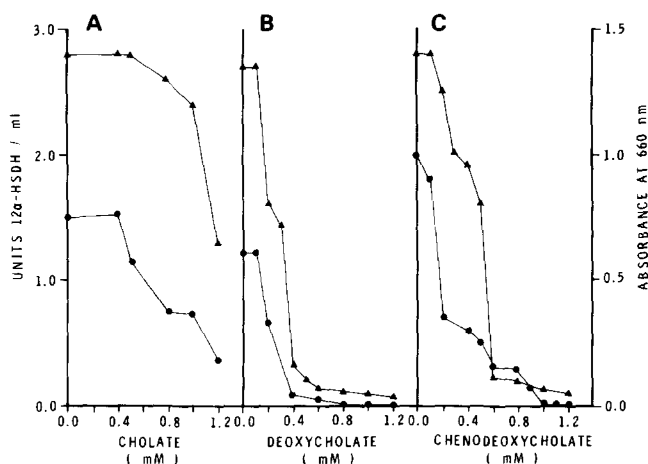

Fig. 3. Effect of (A) cholate, (B) deoxycholate and (C) chenodeoxycholate on the growth of C48-50 (▲——▲) and the production of 12α-HSDH (●——●). (Substrate GDC). Cultures were grown for 96 hr.

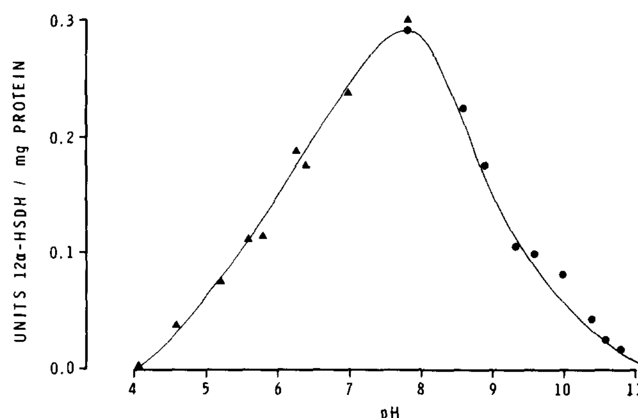

Fig. 4. Effect of pH value on 12α-HSDH activity (substrate GDC). Buffers: Glycyl-glycine/NaOH (▲——▲) and glycine/NaOH (●——●).

enzyme production (**Fig. 3**), with the former being somewhat more inhibitory than the latter at lower concentrations.

Effect of pH and substrate specificity pattern

Investigation into the effect of pH on enzyme activity revealed an optimum at pH 7.8 (**Fig. 4**). The enzyme activity was linear for 10 min at alkaline pH values (9–10.5), but rapidly lost linearity with time at pH values less than 9, even though the initial velocity was higher at neutrality. Michaelis constants and maximum velocities computed by Lineweaver–Burke plots are shown in **Table 2.** The K_m values for the glyco- and tauro-conjugates are very similar to the K_m value for DC. However, the glyco- and tauro-conjugates of cholate display considerably higher K_m values than free cholate, sodium glycocholate being the substrate with the highest K_m value of the group (2.5×10^{-3} M). 7α,12α-Dihydroxy-5β-cholanoate displayed the lowest K_m value (6.7×10^{-6} M) of any of the substrates studied in detail. Other substrates included the methyl esters of cholate and DC, 3α,12α-dihydroxy-7-oxo-5β-cholanoate, 7α,12α-dihydroxy-3-oxo-5β-cholanoate, and 5β-cholan-3α,7α,12α,24-tetrol. Notably, the three sulfates of cholate and DC were nonsubstrates. As expected, a variety of bile salts devoid of a 12α-OH group, including CDC and 3α, 6α-dihydroxy-5β-cholanoate (UDC), were nonsubstrates. There was only a minor trace of NAD-dependent activity in any of the cell-free preparations.

Thin-layer chromatographic verification of product identity

Thin-layer chromatography by three solvent systems revealed the formation of 3α,7α-dihydroxy-12-oxo-5β-cholanoate from cholate both in a cell-free reaction mixture containing cholate and NADP (see Methods) and in spent bacterial medium. No other major degradation product could be found. This product could be readily distinguished from 3α,12α-dihydroxy-7-oxo-5β-cholanoate by *a*) the color with *p*-hydroxybenzaldehyde (10) or anisaldehyde spray reagents (9), and *b*) the reactivity of the isolated product with both 3α- and 7α-HSDH (forward reaction). As previously described, 3α,7α-dihydroxy-12-oxo-5β-cholanoate can be readily distinguished from 7α,12α-dihydroxy-3-oxo-5β-cholanoate (but not from 3α,12α-dihydroxy-7-oxo-5β-cholanoate) by mobility in all three solvent systems.

Purification of 12α-HSDH with NAD- and NADP–Sepharose 4B columns

As shown in **Fig. 5,** 12α-HSDH was found to bind to both NAD- and NADP-substituted Sepharose 4B (equilibrated with 0.10 M phosphate buffer, pH 7). In the case of the NAD-substituted column, the binding was rather weak, as the enzyme was readily eluted with 0.02 M phosphate buffer, pH 7. However, in the case of the NADP column, the enzyme was eluted using 0.2 M phosphate buffer. The yields were 50% and

TABLE 2. Some kinetic parameters of *Clostridium* Group P C48-50 12α-HSDH

5β-Cholanoate	K_m[a]	V_{max}[b]
3α,12α-Dihydroxy-	0.40	0.14
3α,12α-Dihydroxy-glycine amide	0.30	0.10
3α,12α-Dihydroxy-taurine amide	0.50	0.09
3α,7α,12α-Trihydroxy-	0.67	0.12
3α,7α,12α-Trihydroxy-glycine amide	2.5	0.12
3α,7α,12α-Trihydroxy-taurine amide	1.0	0.09
7α,12α-Dihydroxy-	0.0067	0.04

[a] mM.
[b] Units 12α-HSDH/mg protein (crude preparation at pH 9.5).

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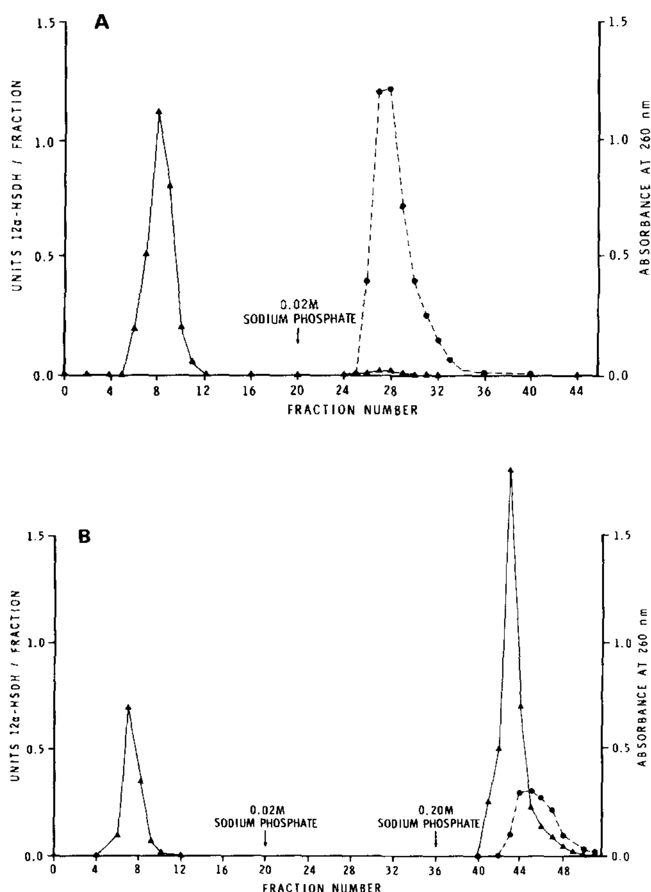

Fig. 5. Behavior of 12α-HSDH on (*A*) NAD–sepharose-4B column (1 × 10 cm) and NADP–Sepharose-4B column (1 × 10 cm); absorbance at 260 nm (▲——▲) and units 12α-HSDH (●——●). Fraction size, 1.0 ml.

12%, respectively, while the purifications were 20-fold and 3-fold, respectively. Unfortunately, on both the NAD and NADP columns, a very high percentage of the protein was capable of binding to the immobilized nucleotide. However, the columns could be neutralized once after rapid washing with 0.005 M HCl, 0.1 M sodium carbonate, and water and re-equilibrating with 0.01 phosphate, pH 7.0. The extent of substitution of nucleotide was estimated at 105 nmol NAD and 84 nmol NADP per ml packed absorbent. The molecular weight of NAD–Sepharose-4B-purified 12α-HSDH was estimated to be 100,000 by Sephadex G-200 molecular sieving.

DISCUSSION

The marked enhancement of the growth of C48-50 by glucose and fructose (and, to a lesser degree, by other sugars) is not explicable solely on the basis of acidic fermentation of these compounds.

Although this organism has been referred to as "afermentative" (5), the results summarized in Table 1 strongly suggest that a "neutral" fermentation process may be taking place. The pronounced lag in the glucose response curve (Fig. 1*B*) compared to the fructose response curve (Fig. 1*A*) may be a metabolic effect or glucose may be converted to fructose (by an isomerase) which is subsequently metabolized. The lack of significant ^{14}C uptake suggests that carbon dioxide is not being evolved and subsequently is fixed intracellularly. A lack of calcium carbonate formation on the addition of excess calcium chloride to the spent bacterial medium negates the presence of significant amounts of carbonate in this medium.[1] The presence of neutral products such as glycerol and glycol remains to be investigated.

From the practical point of view, the turbidity of the fructose- or glucose-fed culture approached that of *E. coli* grown aerobically and surpassed that of any of the HSDH-containing anaerobes studied to date (1–3, 5, 17). The sensitivity of this organism to bile salts was similar to that noted in *Eubacterium lentum* ATCC 25559 (3) with greater tolerance for cholate than either of the dihydroxy bile salts. As noted in the case of *E. lentum* (3), the concentration of deoxycholate in the normal human stool (18) would be sufficient to repress populations of such organisms (and their enzymes) to very low levels.

The pH optimum, 7.8, was lower than that of any of the other microbial HSDH activities studied to date; but at this pH value, the activity was linear only briefly and rapidly fell with time; the equilibrium at pH 7.8 allowed the reaction to go to approximately one-third completion. Kinetic studies (pH 9.5) revealed that conjugation of DC with either glycine or taurine did not appreciably alter the affinity of the substrate for the enzyme, but conjugation of cholate increased the K_m value one order of magnitude. In contrast with the 12α-HSDH in *C. leptum* (2), there was not an appreciable drop in V_{max} values when the 3α,7α,12α-trihydroxy cholanoates are compared to the 3α,12α-dihydroxy cholanoates.

The use of pyridine nucleotide columns for chromatography of various dehydrogenases was pioneered by Lowe and Dean (20). Unfortunately, a great deal of nonspecific binding occurs, partly due to weak ionic exchange capacity (thus the term "affinity chromatography" may be questionable in this case). However, in some cases (particularly with L-threonine dehydrogenase), Lowe and Dean (20) succeeded in a very dramatic purification even though all the elution procedures involved an increase in the ionic strength of the buffer. The NAD-substituted Sepharose gave reproducibly good results in the purifica-

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tion of C48-50 12α-HSDH, whereas both the degree of purification and the enzyme yield were relatively poor in the case of NADP-substituted Sepharose. Because of a great deal of nonspecific binding of proteins to the nucleotide columns, the capacity of the column was limited; the 1 × 10 cm column was overloaded when more than 20 mg of lyophilized preparation (about 20 units of enzyme) was applied. Disc gel electrophoresis revealed the presence of several bands not associated with 12α-HSDH activity; thus heterogeneity existed in the 20-fold purified preparation. Purified enzyme was stable for 3–4 weeks when lyophilized and stored at −20°C. This preparation may be used for the quantification of 12α-OH groups but, for routine purposes, ammonium sulfate-fractionated enzyme or crude preparations can also be used.[1]

A culture of this unique organism has been donated to the American Type Culture Collection (ATCC #29733).

The authors are grateful to Dr. T. Hindmarsh of the Nova Scotia Pathology Institute for the estimations of glucose in spent bacterial medium. This work is supported by grant MA-5075 from the Medical Research Council of Canada.

Received 28 March 1978; accepted 1 August 1978.

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