Separation of 7α- and 7β-hydroxysteroid dehydrogenase activities from *Clostridium absonum* ATCC# 27555 and cellular response of this organism to bile acid inducers

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Abstract Both 7α- and 7β-hydroxysteroid dehydrogenases (HSDH) were induced by either chenodeoxy- (CDC) or deoxycholic (DC) acid in *C. absonum*. 7β-HSDH was partially purified 35-fold from CDC-induced cultures of *C. absonum* by Procion Red (PR) affinity chromatography and high performance liquid chromatography (HPLC) using a TSK 3000 SW gel filtration column. A relative molecular weight of 200 K was estimated for 7β-HSDH using Sephacryl S-300 chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the 35-fold purified 7β-HSDH showed six polypeptides in the molecular weight range of 40–50 K. Induction of cultures of *C. absonum* with CDC or DC (0.4 mM) also resulted in the differential synthesis of at least five new polypeptides with molecular weights of 94 K, 42 K, 32 K, 21 K, and 16 K. The 16 K polypeptide was induced by DC but not by CDC. SDS-PAGE of Triton X-100-solubilized membranes from these extracts revealed the presence of a new membrane-associated polypeptide of molecular weight 80 K. The 80 K polypeptide was not detected in the soluble fraction. The soluble inducible polypeptides were eliminated during purification of the 7α- and 7β-HSDH and, therefore, are not required for these enzyme activities. It is proposed that this organism synthesized 7α- and 7β-HSDH as well as a series of other proteins in response to bile acids which may, in the absence of the dehydrogenases, be toxic to *C. absonum*. The HSDH's catalyze the epimerization of chenodeoxycholic acid to ursodeoxycholic acid, which is less toxic than the chenodeoxycholic acid. The other proteins may assist the survival of the organism in a high bile acid environment by mechanisms not yet understood.—**Macdonald, I. A., B. A. White, and P. B. Hylemon.** Separation of 7α- and 7β-hydroxysteroid dehydrogenase activities from *Clostridium absonum* ATCC# 27555 and cellular response of this organism to bile acid inducers. *J. Lipid Res.* 1983. **24:** 1119–1126.

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Ursodeoxycholic acid is a naturally occurring bile acid found both in human bile (1) and feces (2) and has been shown to be formed from chenodeoxycholic acid (CDC) by the mixed fecal flora (3–5). Additionally, a number of anaerobes in pure culture including *Clostridium absonum* (6, 7), *Clostridium sp.* (8, 9), *Eubacterium aerofaciens* (10, 11), *Peptostreptococcus productus T.* (10), and an unknown gram-positive anaerobe isolated by Hirano and Masuda (12) have been shown to participate in the formation of UDC from CDC. In *C. absonum* two enzymes have been identified, a 7α- and 7β-HSDH (13), the former oxidizing CDC to 7-KLC while the latter reduces 7-KLC to UDC (6, 7). In this organism both enzymes are inducible by the substrate, CDC, and surprisingly a non-substrate, DC (13). In the case of *E. aerofaciens, Peptostreptococcus productus T.* (10, 11), and the anaerobe isolated by Hirano and Masuda (12), only a 7β-HSDH is present; as a result the epimerization of CDC to UDC in whole cell cultures depends on co-culturing either of these organisms with a 7α-HSDH-elaborating bacterium such as *B. fragilis* (14, 15) or *E. coli* (16, 17). Thus far, *C. absonum* is the only known 7α-OH-epimerizing organism in which there is evidence for the presence of bile acid-inducible enzymes. This bacterium may represent an excellent model system for studying the physiological significance of bile acid epimerization and bile acid toxicity.

It is the purpose of this communication to describe the partial purification and characterization of CDC-induced 7β-HSDH in *C. absonum* and the presence in the

Abbreviations: Systematic names of bile acids referred to in the text by their trivial names are as follows: cholic acid, 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid; chenodeoxycholic acid (CDC), 3α,7α-dihydroxy-5β-cholan-24-oic acid; deoxycholic acid (DC), 3α,12α-dihydroxy-5β-cholan-24-oic acid; ursodeoxycholic acid (UDC), 3α-7β-dihydroxy-5β-cholan-24-oic acid; 7-ketolithocholic acid (7-KLC), 3α-hydroxy-7-oxo-5β-cholan-24-oic acid. HSDH, hydroxysteroid dehydrogenase; BHI, brain heart infusion; DTT, dithiothreitol; PR, Procion Red; HPLC, high performance liquid chromatography; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; V.P.I., Virginia Polytechnic Institute; ATCC, American Type Culture Collection.

cytosol and membrane fractions of several new polypeptides which are also induced by bile acids.

MATERIALS AND METHODS

Methods

Growth and harvest of C. absonum. This was performed as described before (13) with the following modifications. Strain ATCC # 27555 or V.P.I. # 6905 was maintained in cooked meat broths at 4°C. Overnight *C. absonum* starter cultures at 37°C and in brain-heart infusion (BHI) broth were used to inoculate 100-ml volumes of freshly autoclaved and cooled BHI broth. Cultures were grown at 37°C for 2.5 hr, at which time individual bile acids were added to growing cultures. Cultures were allowed to grow another 3.5 hr before harvesting (total growth time, 6 hr). Cells were harvested by centrifuging at 6000 *g* for 20 min at 4°C. The cell pellets were suspended in 3 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM DTT.

Preparation of cell extracts. The bacteria were lysed in a French pressure cell at 10,000 lb/in^2 and broken cells were centrifuged at 100,000 *g* for 2 hr at 4°C in a Beckman preparative ultracentrifuge. The supernatant fluid was carefully decanted. Cell extracts were rapidly frozen and stored at −70°C. Freshly thawed extracts were assayed for NADP-dependent 7α- and 7β-HSDH (13) activities. Several 100-ml volumes of 4×10^{-4} M CDC-induced cultures of *C. absonum* were grown and the cell extracts were prepared as described above. This material was lyophilized (13) and the dry material was stored at −20°C and subsequently used for purification studies.

Solubilization of C. absonum membranes. The unwashed 100,000 *g* pellets (above) were frozen after preparation and subsequently thawed and solubilized by the addition of 3.0 ml of 0.1 M phosphate buffer (pH 7.0) containing 2% Triton X-100, 1.0 mM EDTA, and 1.0 mM DTT. The pellets were suspended and the suspension was allowed to stand at 0°C for 2 hr before centrifugation at 100,000 *g* for 2 hr at 4°C as described above. The supernatant fluid was decanted, assayed for 7α- and 7β-HSDH activities and proteins, and stored at −70°C.

Assay for 7α- and 7β-HSDH activities. This was performed as previously described (7, 13). Assay mixtures in a total volume of 1.0 ml were *a*) NADP-dependent 7α-HSDH, 1.0 mM cholic acid, 1.0 mM NADP, 0.3 M glycine/NaOH buffer (pH 10.5); *b*) NAD-dependent 7α-HSDH, 1.0 mM cholic acid, 5.0 mM NAD, 0.3 M glycine/NaOH buffer (pH 9.5); and *c*) NADP-dependent 7β-HSDH, 1.0 mM UDC, 1.0 mM NADP, 0.3 M glycine/NaOH buffer (pH 9.5). In all assay systems, enzyme activity was measured where initial reaction velocity was proportional to protein concentration.

Affinity chromatography purification of 7β-HSDH using Procion Red agarose. Three hundred mg of lyophilized CDC-induced *C. absonum* cell extract (50 mg of protein) was dissolved in 1.0 ml of twice-distilled water and dialyzed overnight against 20 volumes of 0.1 M phosphate, pH 7.0, (containing 1.0 mM EDTA and DTT). The dialysate was placed on a 1 × 3 cm Procion Red (PR) column (18) and equilibrated with 0.1 M phosphate (1.0 mM EDTA and DTT), pH 7.0, at room temperature. The column was allowed to stand for about 15 min before washing with about 50 ml of the same buffer. Subsequently the column was washed with 25 ml of the same buffer containing 200 mM NaCl. Lastly, the 7β-HSDH activity was eluted with 600 mM NaCl in the above buffer. The PR column was washed with 3 M NaCl (50 ml) and distilled water (50 ml), and re-equilibrated with buffer as above before the column was reused.

Chromatography of 7α- and 7β-HSDH by HPLC. Either 0.6 ml of 25 mg/ml solution of crude lyophilized CDC-induced 7α- and 7β-HSDH preparation or 2.0 ml of 7β-HSDH eluate from the PR column was subjected to HPLC chromatography on a 7.5 mm × 60 cm TSK 3000 SW gel filtration column (Beckman Co., Palo Alto, CA) at room temperature using a Beckman model 332 HPLC. The sample was filtered through a 0.2-μm pore diameter nylon filter prior to injection. Proteins were eluted off the column with 0.1 M phosphate buffer containing 1.0 mM EDTA, 1.0 mM DTT, and 0.1 M NaCl. The flow rate was 0.85 ml/min. Column eluate was monitored at 280 nm; fractions (0.85 ml) were collected and assayed immediately for enzyme activity.

Characterization of product from UDC, NADP, and purified 7β-HSDH. A 1.0-ml assay mixture consisting of 0.10 mM UDC, 0.10 mM NADP, and 0.30 M glycine/NaOH buffer (pH 9.5) was incubated at 25°C with 50 μl of lyophilized 7β-HSDH (20 mg/ml water) which had been purified by PR affinity chromatography as described above. The reaction was stopped after 1 hr by dropping the pH value to approximately 3.0 with a small volume of 1.0 M HCl. The assay mixture was then extracted by two sequential 2-ml volumes of ether. The combined ether extracts were evaporated to dryness, reconstituted with 50 μl of methanol–water 4:1 (v/v) and two 20-μl aliquots were subjected to TLC using chloroform–methanol–acetic acid 40:2:1 (v/v/v). Pure standards of UDC (Sigma), CDC (Sigma), and 7-KLC (Steraloids) were run in parallel and were co-chromatographed with one of the extraction aliquots. Plates were sprayed with Komarowsky's reagent as previously described (19).

Characterization of product from 7-KLC, NADPH, and purified 7β-HSDH. This was performed exactly as described above except the reaction mixture consisted of a 1.0-ml volume of 0.10 mM 7-KLC, 0.10 mM NADPH, and 0.1 M sodium phosphate buffer (pH 6.0).

Polyacrylamide gel electrophoresis. Soluble (cytosol) polypeptides and Triton X-100-solubilized polypeptides were analyzed by one-dimensional SDS-PAGE as described previously (20, 21). The above fractions from cells grown *a*) in the absence of bile acids, *b*) in the presence of 0.40 mM CDC, *c*) in the presence of 0.20 mM CDC, and *d*) in the presence of 0.40 mM DC were subjected to SDS-PAGE. Gels were stained with Coomassie blue R250 in water–methanol–acetic acid 68:25:7 (v/v/v) for 4 hr and then destained with the same solvent for approximately 24 hr.

Protein determination. Proteins were measured according to Lowry et al. (22).

Molecular weight determination of 7β-HSDH. The molecular weight of purified 7β-HSDH was estimated by passing approximately 1 unit (sufficient enzyme to produce 1 mole of NADP/min) through a 125 × 2.5 cm Sephacryl S-300 column at a flow rate of about 1.5 ml/min. Fractions of 8 ml were collected and the 7β-HSDH-active fractions were identified. The column was standardized with the following markers: thyroglobulin (monomer) 300 K; aldolase, 142 K; human serum albumin, 66.5 K, and RNase, 13.7 K, (all from Sigma Chemicals) in a similar fashion to that originally described by Andrews (23).

Materials

Strain ATCC # 27555 (or VPI # 6905) was donated by Dr. L. V. Holdeman. It was originally isolated by Dr. S. Nakamura, Kanazawa, Japan, and is available from the ATCC. Brain heart infusion (BHI) broth and cooked meat broth were products of Difco Laboratories, Detroit, MI. Chenodeoxycholic acid, ursodeoxycholic, and NAD and NADP were from Sigma Chemical, St. Louis, MO. Cholic acid was from J. T. Baker Chemicals, Phillipsburg, NJ. Immobilized Procion Red on agarose was from Pierce Chemicals, Rockford IL. Molecular weight standards for gel filtration were from Sigma Chemicals. Electrophoretically pure reagents including high and low range molecular weight standards for SDS-PAGE were obtained from Bio-Rad laboratories, Richmond, CA.

RESULTS

As shown in **Table 1,** the specific activities of both 7α- and 7β-HSDH can be greatly enhanced if either CDC or DC are added to growing cultures. Lower specific activities were associated with the Triton X-100-solubilized membrane fraction. The soluble polypeptide profiles from induced and uninduced cultures of *C. absonum* were analyzed by SDS slab gel electrophoresis (**Fig. 1a and b**). At least five bands were markedly enhanced in the induced preparation. They were a very large band, molecular weight 94 K, and a series of smaller bands of molecular weights 42 K, 32 K, 21 K, and 16 K, the last being induced by DC but not by CDC. The Triton X-100-solubilized proteins prepared from corresponding membrane fractions were also analyzed by SDS-PAGE. A new inducible polypeptide was observed at 80 K and a more intense band at 94 K in solubilized membranes from induced cells. The ratio of 80 K band intensity/94 K band intensity was somewhat higher in cells induced by CDC than in cells induced by DC. (compare row C with row A in Fig. 1b).

To determine if any of the observed bands shown in Fig. 1 corresponded to either the 7α- or 7β-HSDH activities, we chromatographed a sample of soluble cell extract on a HPLC-TSK 3000 gel filtration column, assayed fractions, and subsequently analyzed polypeptide profiles by SDS-PAGE. The data in **Fig. 2** show the absorbance (280 nm) scan of CDC-induced cell extract and the levels of 7α- and 7β-HSDH per fraction. Clearly, the 7α- and 7β-HSDH activities can be partially separated, but low amounts of 7α-HSDH are present in the peak fractions of 7β-HSDH. (A similar enzyme separation can be achieved with Sephacryl S-200 or S-300, data not shown). Rather distinct and reproduc-

TABLE 1. Induction of 7α- and 7β-HSDH by chenodeoxycholic acid and deoxycholic acid

Inducer	NADP Dep. 7α-HSDH (Cholic Acid)	NAD Dep. 7α-HSDH (Cholic Acid)	NADP Dep. 7β-HSDH (UDC)
		specific activity[a]	
None	0.024	0.005	0.00
4.0×10^{-4} M CDC	0.24	0.059	0.13
2.0×10^{-4} M CDC	0.069	0.020	0.035
4.0×10^{-4} M DC	0.12	0.019	0.09

[a] Specific activities are expressed as μmol of NAD(P)H per min per mg protein.

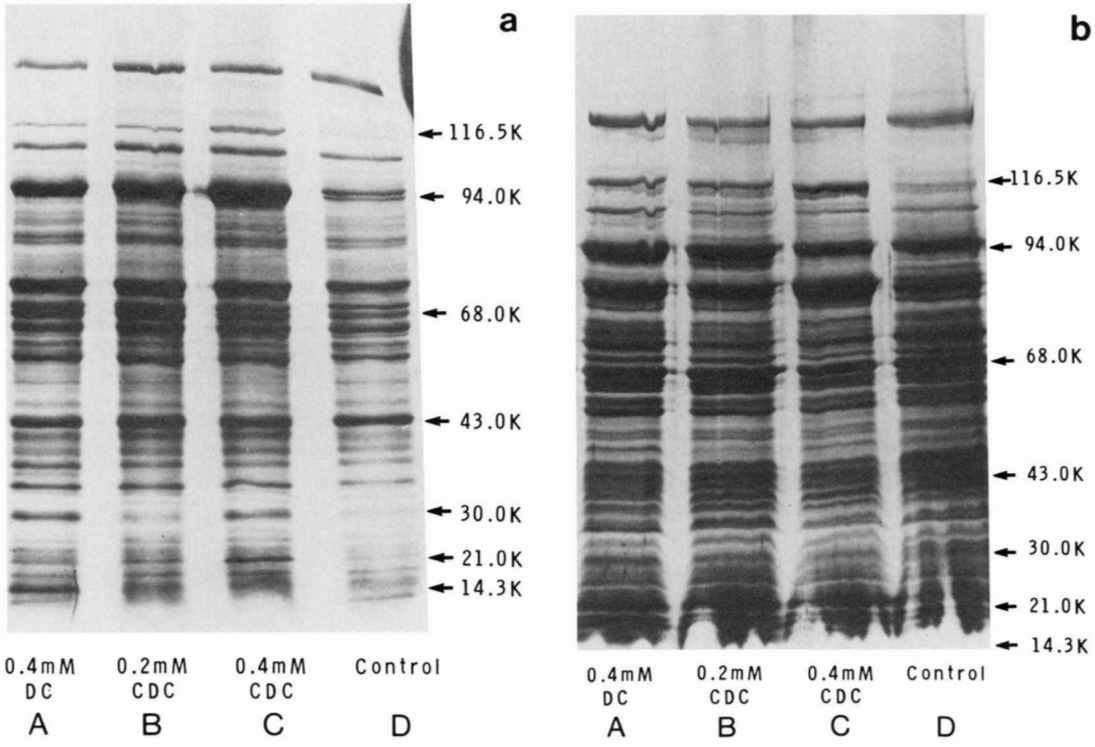

Fig. 1. a), Soluble polypeptide profile of cell extracts prepared from *C. absonum* grown with A) 0.4 mM DC, B) 0.2 mM CDC, C) 0.4 mM CDC, and D) no bile acids. Approximately 50 μg of protein was added per well. Molecular weights of high and low range standards are given on the right. Comparison reveals enhanced synthesis of polypeptides at molecular weights of 94 K, 42 K, 32 K, 21 K, and 16 K by the addition of CDC or DC to the medium. b), Triton X-100-solubilized polypeptides from membranes of *C. absonum*. Series corresponds to that in Fig. 1a. Approximately 50 μg of protein was added per well. Comparison reveals enhanced synthesis of a new polypeptide at molecular weight of 80 K by the addition of CDC or DC in the medium.

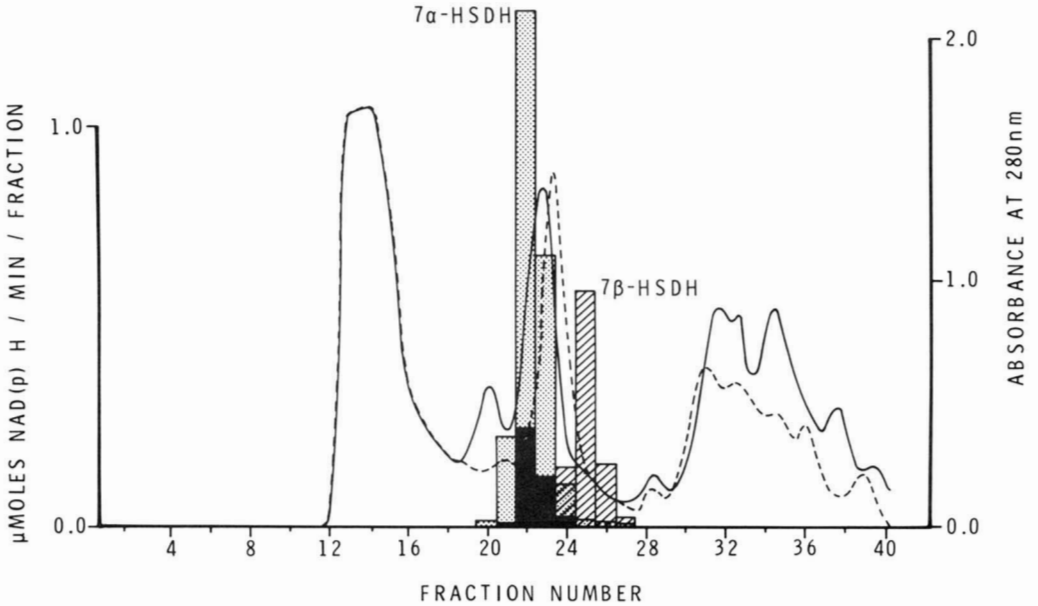

Fig. 2. Elution profile of 7α- and 7β-HSDH activities by HPLC-TSK 3000 gel filtration column. Approximately 10 mg of protein was injected and eluted at 0.85 ml/min. Dotted bars depict NADP-dependent 7α-HSDH; hatched bars depict NADP-dependent 7β-HSDH, and solid bars depict NAD-dependent 7α-HSDH. "Unbroken" profile represents absorbance at 280 nm of CDC induced (active) cell extract; "broken" profile represents that for the uninduced cell extract.

ible differences in the 280 nm HPLC scan profile can be seen between induced and uninduced cell extracts, presumably reflecting qualitative differences in the proteins synthesized. As seen in Fig. 2, the uninduced cell extract profile largely lacked a peak in fraction 20, showed some "downshifting" of a peak approximating the 7α-HSDH active region, and showed some subtle differences in the low molecular weight area (fractions 30–40). Fractions 21–22 (containing 7α-HSDH activity) were analyzed by SDS-PAGE. Surprisingly, no inducible polypeptides at 94 K, 42 K, 32 K, 21 K, and 16 K were associated with the 7α- or 7β-HSDH fractions. However, identification of bands corresponding to the enzymes could not be clearly made (**Fig. 3**).

When crude induced cell extracts were subjected to PR affinity chromatography and then eluted with 600 mM NaCl, approximately 9-fold purification of 7β-HSDH was achieved. The yield of 7β-HSDH was near quantitative while the 7α-HSDH could be eliminated (**Fig. 4** and **Table 2**). Chromatography of cell extracts on HPLC-TSK 3000 column subsequent to the PR affinity column gave a 35-fold purification of 7β-HSDH (Fig. 4 and Table 2). When 35-fold purified 7β-HSDH was subjected to SDS-PAGE, approximately six visible bands in the molecular weight range of 40–60 K were visible on Coomassie blue staining (**Fig. 5**). None of the series of bands of molecular weights 94 K, 42 K, 32 K, 21 K, and 16 K (or the 200 K band) observed in crude induced preparations, were present in 35-fold purified 7β-HSDH. A molecular weight estimation of 7β-HSDH by gel filtration revealed a molecular weight of approximately 200 K. This result in the light of SDS-PAGE studies of PR-HPLC-purified 7β-HSDH suggests that this enzyme may exist as a tetramer.

When PR-affinity chromatography-purified 7β-HSDH was tested with UDC as substrate and NADP at pH 9.5, clearly only one reaction product was visible on TLC. This product co-chromatographed with and was identical on inspection (visible and UV) to 7-KLC (19). Likewise, 7-KLC was converted to UDC when incubated at pH 6.0 with NADPH in the presence of this enzyme preparation. No measurable CDC was produced, confirming the absence of 7α-HSDH after PR affinity chromatography.

DISCUSSION

Preparation of a 7β-HSDH free of 7α-HSDH contamination represents an important step in the use of 7β-HSDH for quantification of 7β-OH-containing bile acids similar to the quantification of 7α-OH-containing bile acids by 7α-HSDH (24). (However, there are now two other sources of 7β-HSDH without 7α-HSDH com-

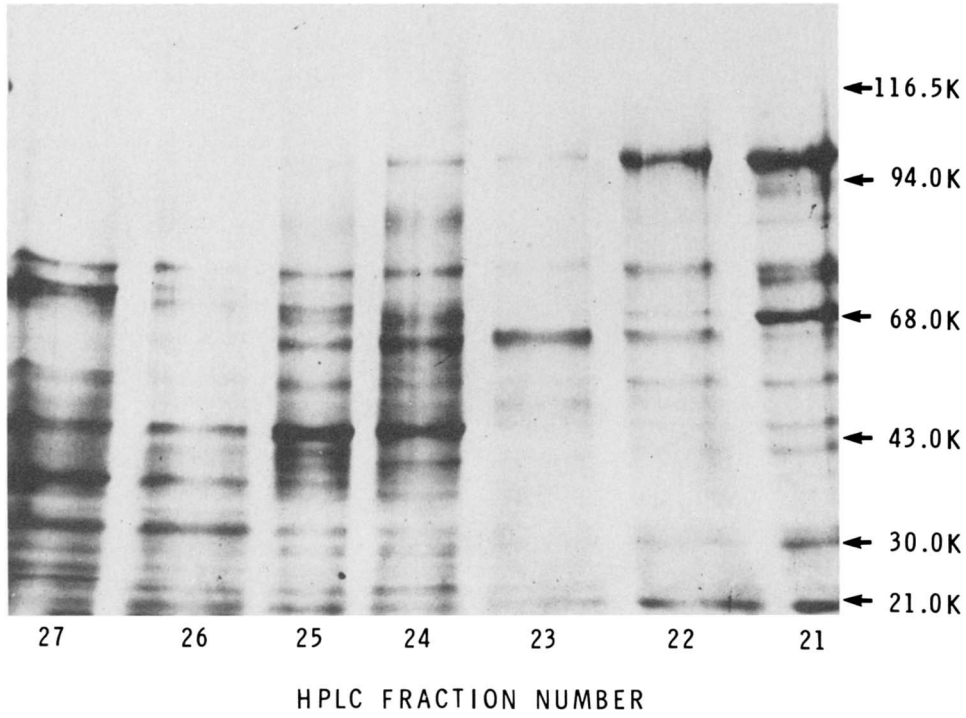

Fig. 3. Polypeptide profile of 7α- and 7β-HSDH-active fraction (fraction numbers correspond to those of Fig. 2). Approximately 50 μg of protein was added per well.

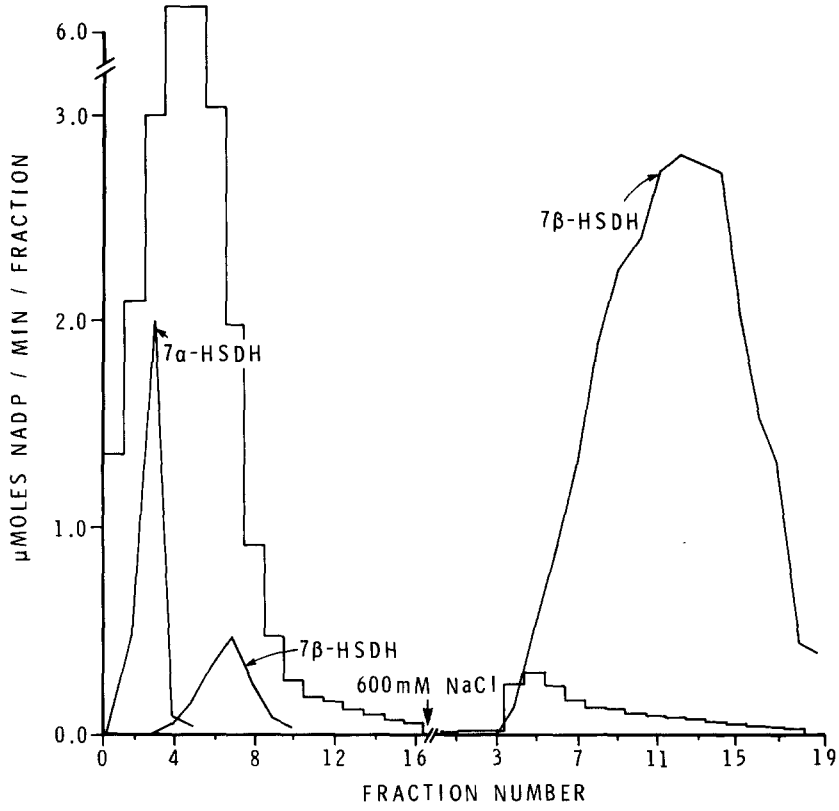

Fig. 4. Procion Red affinity chromatography purification of 7β-HSDH from crude cell extracts of *C. absonum*.

ponent but these microbial enzymes are of low specific activity and unstable (10, 11).) A portion of the NADP-dependent 7α-HSDH activity can be eluted from the PR column by 3.0 M NaCl, but this enzyme has not been further characterized.[1] The fate of the NAD-dependent 7α-HSDH (which cannot be recovered from the PR column by 3.0 M NaCl elution), is not known; however, a previous study has shown that this enzyme is very unstable (13). Although the NAD- and NADP-dependent components of 7α-HSDH co-chromatograph on HPLC, the relative instability of the former suggests they may be two separate entities. The 7β-HSDH enzyme is clearly distinct from either form of the 7α-HSDH enzymes. The relatively poor yield of 7β-HSDH on purification using both PR chromatography and HPLC suggests that this enzyme may become less stable on purification and further purification may be difficult for this reason.

Both the bile-acid-inducible bands and the 7α- and 7β-HSDH activities can be greatly suppressed by the addition of 200 μg of rifampicin to the culture at 2 hr, prior to the addition of bile acid at 2.5 hr.[1] This result implicates a de novo protein synthesis of new polypeptides. The most surprising feature of this study was that none of the immediately visible polypeptides induced by CDC or DC corresponded to the 7α- or 7β-HSDH activities on purification. White et al. (21) have also observed a similar series of cholic acid-inducible polypep-

[1] Macdonald, I. A. Unpublished observation.

TABLE 2. Purification of 7α- and 7β-HSDH by HPLC and PR affinity chromatography

Step	SA[a] 7β-HSDH (NADP)	% Yield	SA[a] 7α-HSDH (NADP)	% Yield	SA[a] 7α-HSDH (NAD)	% Yield
Crude	0.20		0.85		0.11	
HPLC	5.1	96	6.2	62	1.1	58
Crude	0.20		0.85		0.11	
PR	1.8	90	eliminated		eliminated	
HPLC	7.0	40	eliminated		eliminated	

[a] SA, specific activity expressed as μmol of NAD(P)H per mg protein.

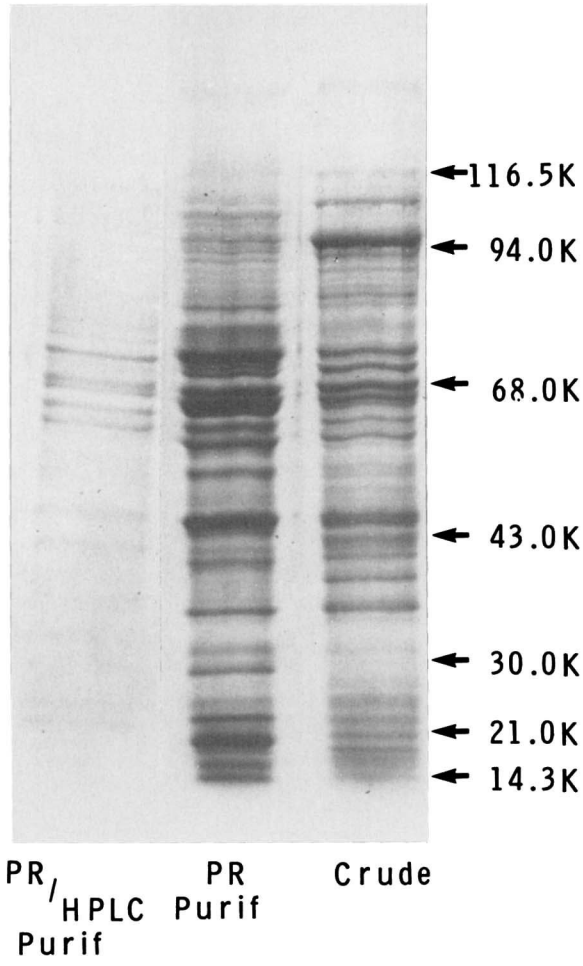

Fig. 5. Polypeptide profile of 7β-HSDH at various stages of purification.

tides in the 7-dehydroxylating bacterium *Eubacterium* sp. V.P.I. 12708. In contrast to our present results with *C. absonum*, four of the five additional polypeptides they observed as a result of induction of the organism were associated with 7-dehydroxylating activity on gel filtration chromatography. The role of bile acid-inducible polypeptides and the 7α- and the 7β-HSDH enzymes in this organism is not clear. It has been reported that UDC is less hydrophobic than CDC based on its elution from octadecylsilane (C_{18}) reverse phase HPLC chromatography (25). Therefore, UDC would be less toxic to the cell membrane than CDC. In this regard, we have observed that on BHI agar plates, cells of *C. absonum* will grow at 1 mM UDC but not at 1 mM CDC. Hence, epimerization via the 7α- and 7β- pathway may represent a detoxification process for *C. absonum* and provide a physiological explanation for the presence of these enzymes. Several possible roles of both the membrane and the cytosol polypeptides exist. Those in the membrane may act as transport or carrier proteins. Alternatively they may, in some manner, protect the cell against the detergent properties of bile acids, thus allowing this organism to survive in a high bile acid environment in which the uninduced cell could not live for a prolonged period. We are currently testing both these hypotheses.

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