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# Electrophoretic and immunochemical characterization of 3α-hydroxysteroid/dihydrodiol dehydrogenases of rat tissues

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The properties of 3α-hydroxysteroid/dihydrodiol dehydrogenase from Sprague–Dawley rat liver cytosol have been re-examined in light of several reports which suggest that multiple forms of the enzyme may exist in this tissue. During enzyme purification, chromatography on DE-52 cellulose and chromatofocusing columns indicated the existence of only one form of the protein. Re-chromatography of the purified enzyme by either of these techniques failed to resolve the protein into additional forms. When the purified enzyme was subjected to SDS/polyacrylamide-gel electrophoresis a single band corresponding to $M_r$ 34000 was detected. Two-dimensional gels showed one predominant protein with a pI of 5.9. Using the homogeneous enzyme as antigen, high-titre polyclonal antibody was raised in rabbits. Western-blot analysis of cytosolic proteins prepared from male and female Sprague–Dawley rat liver indicated the presence of a single immunoreactive band with an $M_r$ of 34000 in both sexes. All of the 3α-hydroxysteroid dehydrogenase activity present in rat liver cytosol could be immunotitrated with the antibody and the resulting titration curve was superimposable on the titration curve obtained with the purified enzyme. Western-blot analysis of cytosolic proteins prepared from livers of male Wistar and Fischer rats also revealed the presence of a single immunoreactive protein with an $M_r$ of 34000. These data indicate that, contrary to previous reports, only one form of the dehydrogenase may exist in liver cytosols prepared from a variety of rat strains. Although 3α-hydroxysteroid dehydrogenase activity is known to be widely distributed in male Sprague–Dawley rat tissues, Western blots indicate that only the liver, lung, testis and small intestine contain immunoreactive protein with an $M_r$ of 34000. The levels of immunoreactive protein in these tissues follow the distribution of dihydrodiol dehydrogenase.

## INTRODUCTION

The 3α-hydroxysteroid dehydrogenase of rat liver cytosol [3α-hydroxysteroid:NAD(P)$^+$ oxidoreductase (EC 1.1.1.50)] is known to catalyse the NAD(P)$^+$-linked oxidoreduction of a wide variety of steroidal and non-steroidal substrates. Steroidal reactions include the conversion of 5α-dihydrotestosterone (a potent androgen) to 3α-androstanediol (a weak androgen) (Hoff & Schreifers, 1973), the reduction of 5β-dihydrocortisone to tetrahydrocortisone (Tomkins, 1956), and the formation of 3α-hydroxycholanic acids, critical to the formation of bile acids (Ikeda, *et al.*, 1981, 1984). On this basis, the rat liver enzyme is believed to play an important regulatory role in androgen, glucocorticoid and bile acid metabolism.

In addition to steroid hormone metabolism, 3α-hydroxysteroid dehydrogenase has been shown to catalyse the NADPH-linked reduction of aromatic ketones and quinones. In rat liver cytosol the enzyme is also indistinguishable from dihydrodiol dehydrogenase [*trans*-1,2-dihydrobenzene-1,2-diol dehydrogenase (EC 1.3.1.20)] (Penning *et al.*, 1984), and readily catalyses the oxidation of benzenedihydrodiol (*trans*-1,2-dihydroxycyclohexa-3,5-diene) to catechol. By virtue of this property, the enzyme may play an important role in the detoxification of polycyclic aromatic hydrocarbons. Recent studies on the regio- and stereo-specificity of the homogeneous enzyme for *trans*-dihydrodiol metabolites of polycyclic aromatic hydrocarbons indicate that it will oxidize dihydrodiol proximate carcinogens of 5-methylchrysene, benzo[*a*]pyrene, benz[*a*]anthracene and 7,12-dimethylbenz[*a*]anthracene (Smithgall *et al.*, 1986, 1988). By oxidizing the *trans*-dihydrodiols of polycyclic aromatic hydrocarbons to less carcinogenic metabolites, the enzyme may effectively suppress the formation of *anti*-diol epoxide ultimate carcinogens.

3α-Hydroxysteroid dehydrogenase has also been the recent focus of attention since it satisfies many of the criteria expected of a target enzyme for anti-inflammatory drugs (Penning & Talalay, 1983) and catalyses the dehydrogenation of certain prostaglandins (Penning & Sharp, 1987).

Although a single isoenzymic form of the 3α-hydroxysteroid/dihydrodiol dehydrogenase was originally purified to homogeneity from rat liver cytosol by two separate groups (Vogel *et al.*, 1980; Penning *et al.*, 1984), more recent work has provided evidence for the existence of multiple forms of the enzyme (Wörner & Oesch, 1984; Ikeda *et al.*, 1984). In addition, reports describing the purification of three Y′-bile acid-binding proteins from rat liver cytosol have shown that these

Abbreviations and trivial names used: 3α-hydroxysteroid dehydrogenase, 3α-hydroxysteroid:NAD(P)$^+$ oxidoreductase (EC 1.1.1.50); dihydrodiol dehydrogenase, *trans*-1,2-dihydrobenzene-1,2-diol dehydrogenase (EC 1.3.1.20); androsterone, 3α-hydroxy-5α-androstan-17-one; benzenedihydrodiol, *trans*-1,2-dihydroxycyclohexa-3,5-diene; PAGE, polyacrylamide-gel electrophoresis.

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proteins all display 3α-hydroxysteroid dehydrogenase activity and are distinct from glutathione *S*-transferase (Stolz *et al.*, 1987). Because of the potential importance of the dehydrogenase in polycyclic aromatic hydrocarbon and prostaglandin metabolism, we have re-examined the issue of isoenzymic forms using chromatographic, electrophoretic and immunochemical approaches. Our experiments support the original conclusion that one major form of the dehydrogenase exists in Sprague–Dawley rat liver cytosol.

## MATERIALS AND METHODS

Electrophoretic reagents, nitrocellulose membranes, goat anti-(rabbit IgG)–horseradish peroxidase conjugate and 4-chloro-1-naphthol were obtained from Bio-Rad (Richmond, CA, U.S.A.). Hydrogen peroxide (30%, v/v) was purchased from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Tween-20 and Freund's adjuvant were products of Sigma Chemical Co. (St. Louis, MO, U.S.A.). Androsterone (3α-hydroxy-5α-androstan-17-one) was purchased from Steraloids (Wilton, NH, U.S.A.). Benzenedihydrodiol was synthesized as previously described (Smithgall & Penning, 1986). Fischer, Sprague–Dawley and Wistar rats were purchased from Charles River (Wilmington, DE, U.S.A.).

3α-Hydroxysteroid dehydrogenase was purified according to the published procedure (Penning *et al.*, 1984) to a final specific activity of 2.0 μmol of androsterone oxidized/min per mg of protein and 0.2 μmol of benzenedihydrodiol oxidized/min per mg under standard assay conditions (see below). Homogeneous 3β-hydroxysteroid dehydrogenase of rat liver cytosol was a generous gift from Dr. Paul Talalay (Johns Hopkins University School of Medicine, Baltimore, MD, U.S.A.).

### Assay for 3α-hydroxysteroid/dihydrodiol dehydrogenase activity

Assay systems (1.0 ml) contained either 75 μM-androsterone, 2.3 mM-$NAD^+$, 100 mM-potassium phosphate (pH 7.0) plus 4% (v/v) acetonitrile or 1.0 mM-benzenedihydrodiol, 2.3 mM-$NADP^+$, 100 mM-potassium phosphate (pH 7.0) plus 4% (v/v) methanol. Reactions were initiated by the addition of enzyme and followed by measuring the change in nicotinamide nucleotide absorbance at 340 nm and 25 °C.

### Preparation of antiserum against 3α-hydroxysteroid/dihydrodiol dehydrogenase

Adult female New Zealand White rabbits were immunized with a single dose of purified 3α-hydroxysteroid dehydrogenase (400 μg) in Freund's complete adjuvant by multiple intradermal injections. Blood (30 ml) was collected 4 weeks later from the marginal ear vein and the serum prepared as described by Vaitukalis (1981). Antibodies for the dehydrogenase were detected by Western-blot analysis as described below. Rabbits were bled every 2 weeks thereafter, and showed high titres of antibodies even after 51 weeks.

### Western-blot analysis

Relative antibody titres and specificity were determined by immunoblotting (Towbin *et al.*, 1979). Purified enzyme or cytosolic protein was subjected to discontinuous SDS/polyacrylamide-gel electrophoresis (PAGE) using 5% stacking and 12% separating gels (80 mm × 50 mm × 0.75 mm), as described by Laemmli (1970). Following electrophoresis, proteins were electrotransferred to nitrocellulose membranes using a Bio-Rad Trans-blot apparatus according to the manufacturer's instructions (Bio-Rad, Richmond, CA, U.S.A.). Uniform efficient transfer was observed after 3 h at 60 V. Following transfer, membranes were washed in TBS (50 mM-Tris/HCl, pH 7.5, containing 0.4 M-NaCl) plus 0.05% Tween-20, and then incubated for 1 h in TBS plus 3% (w/v) Carnation Instant Milk to reduce background antibody binding (Johnson *et al.*, 1984). The membrane was then incubated for 1 h with various dilutions of the antiserum in TBS plus 3% milk, washed with TBS/Tween, and incubated with goat anti-(rabbit IgG)–horseradish peroxidase conjugate diluted 1:1000 with TBS plus 3% milk. Bands were developed by addition of 4-chloro-1-naphthol and $H_2O_2$.

### Preparation of cytosolic fractions of rat tissues

All rat tissues were homogenized in 50 mM-Tris/HCl (pH 8.6) containing 250 mM-sucrose, 1 mM-EDTA and 1 mM-dithiothreitol (3 ml of buffer per g of tissue), and the homogenates were centrifuged at 10000 ***g*** for 30 min. The resulting post-mitochondrial supernatant was then subjected to ultracentrifugation (100000 ***g*** for 60 min), and the resulting cytosolic fraction was used directly for blotting experiments.

### Protein determinations

Protein concentrations were determined either by the method of Lowry *et al.* (1951) or Bradford (1976) using crystalline bovine serum albumin (Armour Pharmaceuticals, Kanakee, IL, U.S.A.) as standard.

## RESULTS

### Chromatographic resolution of 3α-hydroxysteroid/dihydrodiol dehydrogenase

The observations of Ikeda *et al.* (1984) and Stolz *et al.* (1987), which suggest that DE-52 cellulose column chromatography and chromatofocusing can both resolve 3α-hydroxysteroid dehydrogenase into multiple forms, prompted a re-examination of the chromatographic behaviour of our 3α-hydroxysteroid dehydrogenase preparation. DE-52 cellulose column chromatography and chromatofocusing profiles obtained in one of our typical enzyme purifications (Figs. 1*a* and *b*) indicate the presence of only one form of the enzyme. Although the DE-52 cellulose chromatography was performed on a 40–75% $(NH_4)_2SO_4$ fraction of rat liver cytosol, previous measurements indicated that all the enzyme activity is fractionated in this range (Penning *et al.*, 1984). The chromatofocusing column differs from that described by Stolz *et al.* (1987) in that our procedure focused in a narrower range (pH 7.0–5.0). Although our procedure was optimized for the resolution of multiple forms of the enzyme between pI 6.0 and 5.0, only one species of the dehydrogenase was detected in this procedure. Rechromatography of electrophoretically homogeneous enzyme resulted in single coincident peaks of enzyme activity and protein (Figs. 1*c* and *d*). This rechromatography was performed on enzyme that was purified 6 months earlier. These observations indicate that neither rechromatography nor aging of the enzyme results in the generation of multiple forms.

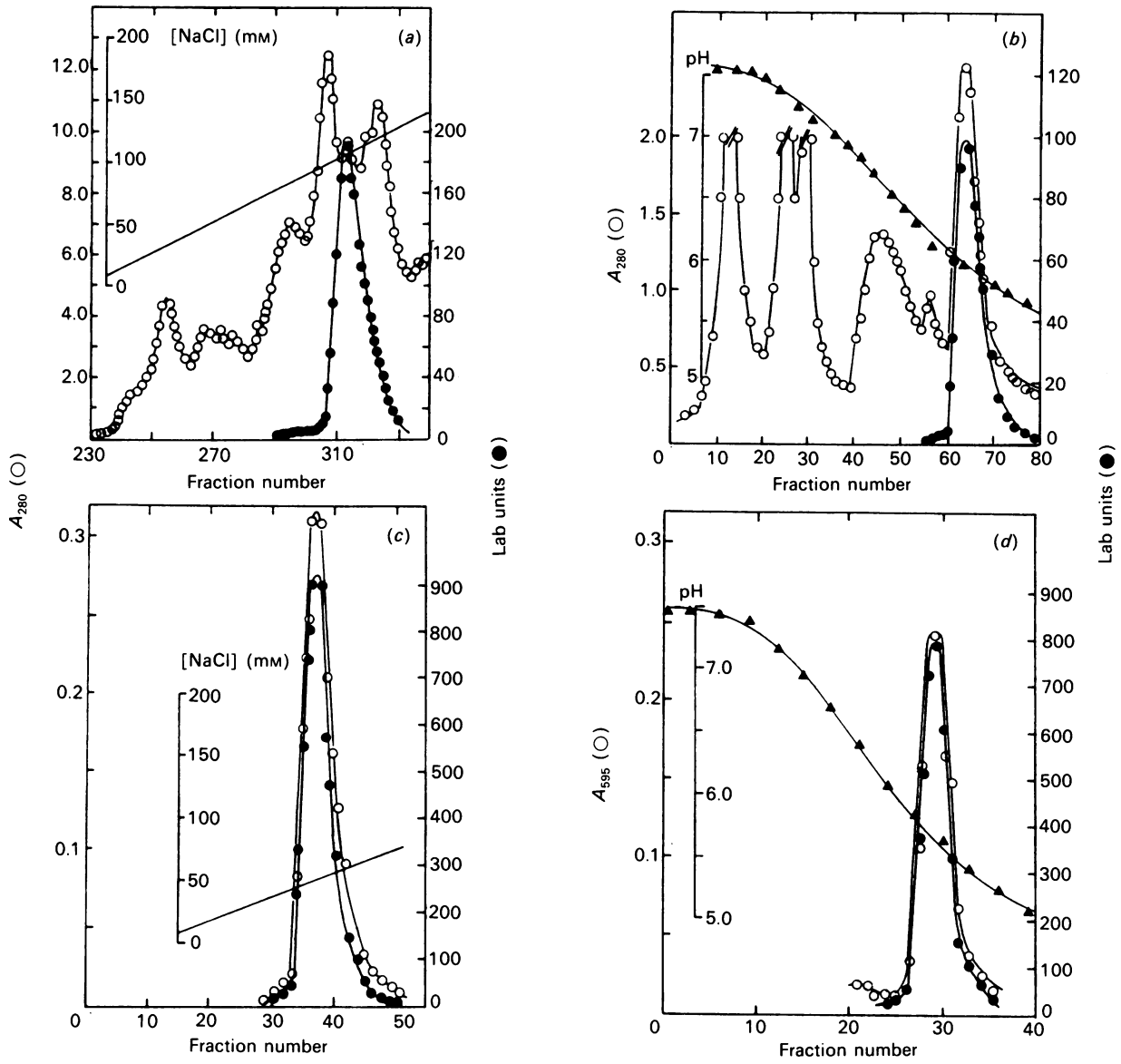


**Fig. 1. Chromatography of 3α-hydroxysteroid/dihydrodiol dehydrogenase during routine purification on DE-52 cellulose (*a*) and chromatofocusing columns (*b*), and re-chromatography of electrophoretically homogeneous enzyme on DE-52 cellulose (*c*) and chromatofocusing columns (*d*)**

The 40–75% $(NH_4)_2SO_4$ fraction (670 ml) obtained from 50 male Sprague–Dawley rat livers was applied to a DE-52 cellulose column (70 cm × 5 cm) equilibrated in 10 mM-Tris/HCl (pH 8.6), 1 mM-EDTA and 1 mM-2-mercaptoethanol. The column was eluted with a linear gradient of 0–250 mM-NaCl in the running buffer. The $A_{280}$ of each fraction (16.5 ml) was measured and aliquots of each fraction were assayed for 3α-hydroxysteroid dehydrogenase activity using androsterone as substrate (*a*). Peak fractions from the DE-52 cellulose column were chromatographed on a hydroxyapatite column as described by Penning *et al.* (1984), which resulted in a single peak of activity. Active fractions were pooled, concentrated and applied to a chromatofocusing column packed with Polybuffer Exchanger (PBE-94) and pre-equilibrated with 25 mM-imidazole/HCl (pH 7.5), 20% (v/v) glycerol, 1 mM-EDTA and 1 mM-2-mercaptoethanol. The column was eluted with a linear pH gradient (7.0–5.0). The $A_{280}$ and enzymic activity in each fraction (5.9 ml) was measured (*b*). Electrophoretically homogeneous enzyme (500 μg) was applied to a DE-52 cellulose column (2 ml) which was equilibrated and eluted as described above. Fractions (0.25 ml) were collected and their $A_{280}$ and enzyme activity were measured (*c*). Electrophoretically homogeneous enzyme (500 μg) was applied to a chromatofocusing column (2 ml), which was equilibrated and eluted as described. Fractions (0.725 ml) were collected and aliquots were removed for either protein determination by the method of Bradford (1976) ($A_{595}$) or enzyme activity (*d*). Enzyme activity in each case is measured in lab units. 1 Lab unit is equal to the oxidation of 159 pmol of androsterone/min and corresponds to an absorbance change of 0.001 *A* units at 340 nm.

## Electrophoretic criteria for the homogeneity of rat liver 3α-hydroxysteroid/dihydrodiol dehydrogenase

When 3α-hydroxysteroid/dihydrodiol dehydrogenase purified according to the published procedure (Penning *et al.*, 1984) was subjected to SDS/PAGE a single band of $M_r$ 34000 was observed even when the gel was overloaded (Fig. 2). Two-dimensional gel electrophoresis of the purified enzyme (isoelectrofocusing followed by SDS/PAGE) gave one predominant spot which

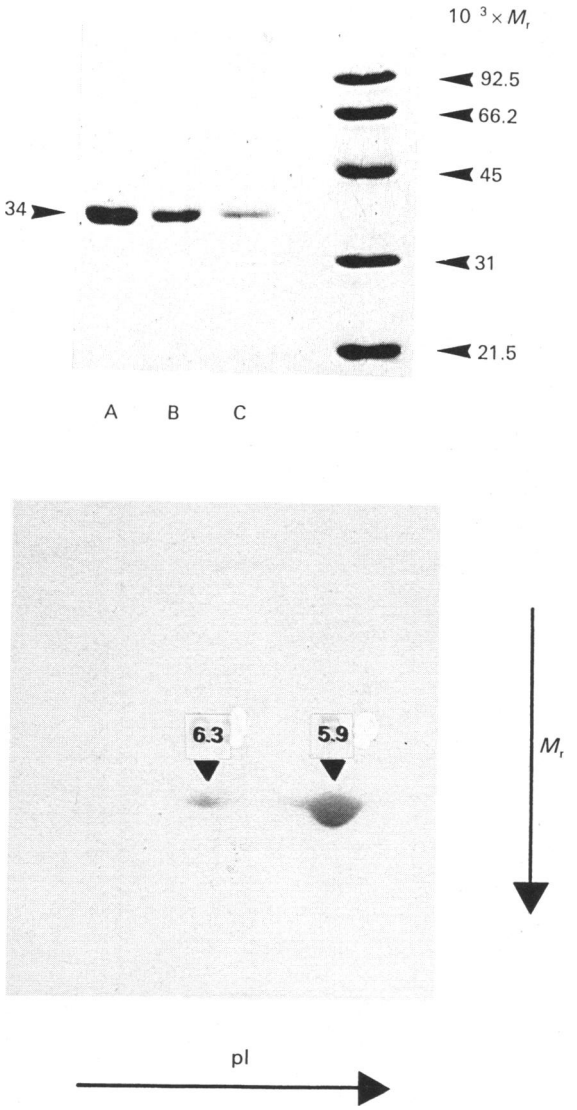


**Fig. 2. SDS/PAGE and 2-dimensional gel electrophoresis of 3α-hydroxysteroid/dihydrodiol dehydrogenase**

Increasing amounts of the purified enzyme were subjected to 5%/12% discontinuous SDS/PAGE and stained with Coomassie Blue (top panel): lane A (5 μg); lane B (2.5 μg); lane C (1.25 μg); molecular mass standards are also shown. Alternatively, purified enzyme (2.5 μg) was subjected to 2-dimensional electrophoresis (focusing followed by SDS/PAGE) according to the procedure of O'Farrell (1975) and visualized with Coomassie Blue (bottom panel).

accounted for > 90% of the protein on the gel when quantified by densitometry (Fig. 2). These results are in contrast with those recently reported by Wörner & Oesch (1984), which suggested that the 3α-hydroxysteroid dehydrogenase displayed charge heterogeneity and gave rise to five bands on focusing gels that varied in pI from 5.6 to 5.1. The two-dimensional gel shown in Fig. 2 focused down to a pI of 5.0. From the scale shown isoforms with isoelectric points between 5.6 and 5.1 would have been clearly resolved. Only that portion of the gel which stained for protein is shown.

### Immunochemical characterization of 3α-hydroxysteroid/dihydrodiol dehydrogenase in Sprague–Dawley rat liver cytosol with high-titre polyclonal antibody

Female rabbits immunized with a single dose of purified 3α-hydroxysteroid dehydrogenase responded with high titres of anti-(3α-hydroxysteroid dehydrogenase) antibodies within 4 weeks. Purified 3α-hydroxysteroid dehydrogenase (250 ng) was detectable by immunoblotting with antiserum dilutions as high as 1:30000. Western-blot analysis of total cytosolic proteins prepared from either male or female Sprague–Dawley rat liver cytosol led to the detection of a single band corresponding to an $M_r$ of 34000 (Fig. 3). The data indicate that rat liver cytosol contains only one protein species that is immunoreactive with anti-(3α-hydroxysteroid dehydrogenase) antibodies. No bands were observed when pre-immune serum was used as primary antibody, and no cross-reactivity was seen between antiserum and purified 3β-hydroxysteroid dehydrogenase (results not shown). This report represents the first description of a high-titre monospecific antibody for a mammalian 3α-hydroxysteroid dehydrogenase.

### Immunotitration of 3α-hydroxysteroid dehydrogenase

Using the undiluted antiserum to 3α-hydroxysteroid dehydrogenase, all of the enzyme activity present in rat liver cytosol or the purified protein could be immunotitrated (Fig. 4). The two immunotitration curves are superimposable and both are sigmoidal in shape. This suggests that only one protein capable of catalysing the oxidation of androsterone is present in either the cytosol or purified preparation. Using the immunotitration curve obtained with the purified enzyme, it is possible to calculate the percentage of the cytosolic protein represented by 3α-hydroxysteroid dehydrogenase. This calculation indicates that the dehydrogenase comprises about 1–1.5% of the soluble protein and agrees with previous estimates (Penning & Talalay, 1983).

The observation that undiluted antiserum is required to immunotitrate enzyme activity, while antiserum can be diluted considerably for Western-blot analysis, suggests that only a small proportion of the antibodies within the polyclonal are directed toward the active site of the dehydrogenase. This also suggests that many antibody–antigen complexes form which permit the enzyme to remain catalytically active. It has been possible to increase the sensitivity of the immunotitration procedures by either purifying the IgG component of the antiserum by $(NH_4)_2SO_4$ precipitation (Herbert *et al.*, 1973) and/or by using suspensions of *Staphylococcus aureus* (which contain Protein A) to act as co-precipitant (Kessler, 1981). Together these procedures increased the sensitivity of the immunotitration procedure by approx. 20-fold (results not shown).

### Detection of immunoreactive 3α-hydroxysteroid dehydrogenase in livers of different rat strains

Ikeda *et al.* (1984) showed the presence of multiple forms of 3α-hydroxysteroid dehydrogenase in Wistar rat liver, whereas our work has concentrated on the enzyme present in the liver of Sprague–Dawley rats. It is conceivable that the differences reported may be explained by variation between rat strains. To address

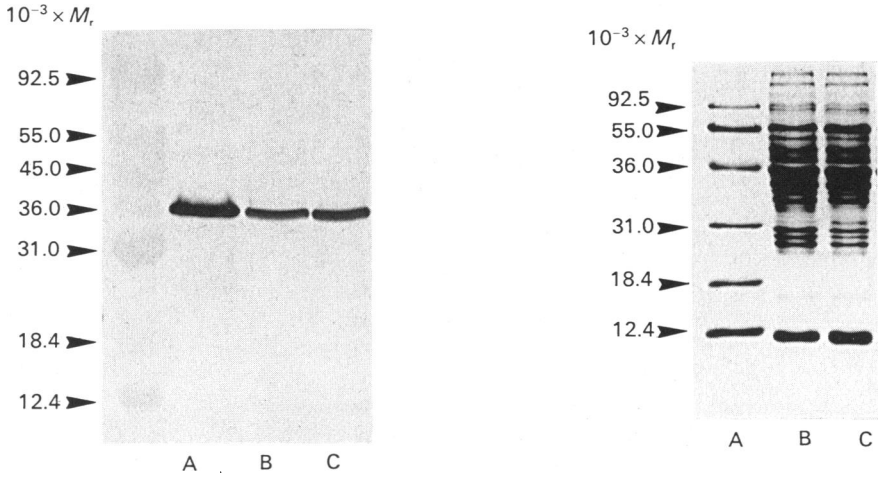


**Fig. 3. Western-blot analysis of male and female Sprague–Dawley rat liver cytosol with polyclonal antibody for 3α-hydroxysteroid/dihydrodiol dehydrogenase**

Purified enzyme (2.0 μg) and male and female Sprague–Dawley rat liver cytosolic proteins (50 μg) were subjected to discontinuous SDS/PAGE, transferred to nitrocellulose membranes and reacted with antiserum diluted 1:500. The antibody–antigen complexes were visualized as described under Materials and methods. Immunoblots are shown in the left panel: lane A (purified enzyme); lane B (male rat liver cytosol); and lane C (female rat liver cytosol). Positions of molecular mass standards are also shown. Gels stained for protein are shown in the right-hand panel: lane A (molecular mass standards); lane B (50 μg of male liver cytosol); and lane C (50 μg of female liver cytosol).

this issue Western-blot analysis was performed on total cytosolic proteins prepared from male Wistar and Fischer rat livers. In both strains the antisera raised against the 3α-hydroxysteroid dehydrogenase, lung, testis, small rat liver cross-reacted with a single protein band which corresponded in $M_r$ to 34000 (Fig 5). This pattern of immunoreactivity may suggest that only a single enzyme species is present in the cytosol prepared from livers from these two rat strains.

### Detection of immunoreactive 3α-hydroxysteroid dehydrogenase in extrahepatic rat tissues

Previous studies have established that the following extrahepatic rat tissues contain indomethacin-sensitive 3α-hydroxysteroid dehydrogenase, lung, testis, small intestine, prostate, heart, spleen, seminal vesicle and brain (Smithgall & Penning, 1985; Penning *et al.*, 1985). To determine whether or not these extrahepatic enzymes were antigenically related to the rat liver dehydrogenase, cytosols from all of these tissues were subjected to Western-blot analysis. Surprisingly, immunoreactive protein ($M_r$ 34000) was only detected in small intestine, lung and testis (Fig. 6). Interestingly, the level of immunoreactive protein detected in these tissues followed the tissue levels of dihydrodiol dehydrogenase: liver > lung > small intestine > testis (Ivins & Penning, 1987).

## DISCUSSION

Following the original description of the purification of 3α-hydroxysteroid/dihydrodiol dehydrogenase to apparent homogeneity (Vogel *et al.*, 1980; Penning *et al.*, 1984), several reports have appeared suggesting that multiple isoenzymic forms may exist in rat liver cytosol (Wörner & Oesch, 1984; Ikeda *et al.*, 1984; Boutin, 1986; Stolz *et al.*, 1987). This paper describes chromatographic, electrophoretic and immunochemical evidence for the existence of only one major form of the enzyme in Sprague–Dawley rat liver cytosol. Ion-exchange and chromatofocusing methods failed to resolve more than one form of the enzyme either during the initial purification of the enzyme or after prolonged storage of the purified enzyme. Further characterization of the purified dehydrogenase by a variety of electrophoretic methods (SDS/PAGE and 2-dimensional gel electrophoresis) also provides evidence for the existence of only one form of the enzyme (pI 5.9, $M_r$ 34000).

Immunochemical analysis also supports the existence of a single form of 3α-hydroxysteroid/dihydrodiol dehydrogenase in rat liver cytosol. Using the antibodies raised against the purified enzyme, Western blots revealed the presence of a single immunoreactive protein in both male and female rat liver cytosol with an $M_r$ of 34000. Although it could be argued that isoenzymic forms may have similar $M_r$ but different pI values, immunotitration of enzyme activity catalysed by rat liver cytosol and the purified enzyme gave smooth, superimposable curves. It is unlikely that isoenzymes would have identical immunoreactivity and share common immunotitration curves.

What then are the reasons for the results observed in the different laboratories? First, several of the reports that have appeared (Wörner & Oesch, 1984; Ikeda *et al.*, 1984; Boutin, 1986; Stolz *et al.*, 1987) used different purification procedures to that originally described (Penning *et al.*, 1984). Secondly, charge heterogeneity has only been detected upon isoelectrofocusing or chromatofocusing and it is possible that additional forms may arise as an artifact of these focusing procedures. For example, Kaplowitz's group has separated the three forms of Y′-bile acid-binding protein using a chromatofocusing column in the pH range 7–4 (Stolz *et al.*, 1987). It is noteworthy that in this procedure a sharper focusing gradient was used than that used in our purification procedure. In our experience 3α-

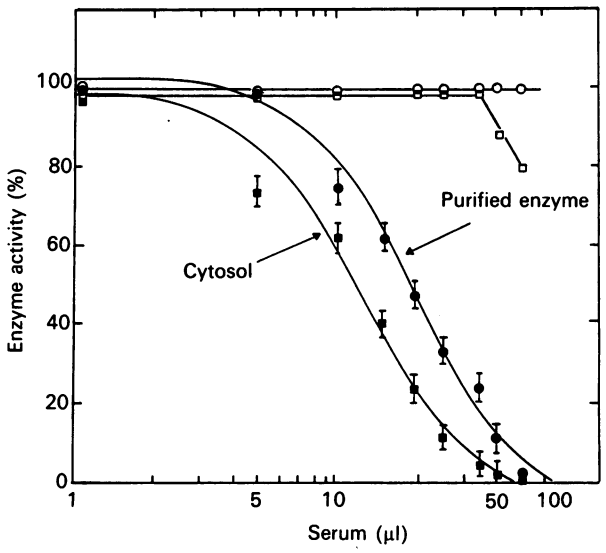


**Fig. 4. Immunotitration of 3α-hydroxysteroid dehydrogenase activity of purified enzyme and rat liver cytosol**

Purified enzyme (4.86 μg) or cytosol (60 μg) was incubated for 18 h in the presence of increasing amounts of antiserum in 10 mM-potassium phosphate (pH 7.4) containing 0.15 M-KCl, 1 mM-EDTA and 1 mM-2-mercaptoethanol. Following centrifugation, the supernatant was assayed for enzyme activity using androsterone as substrate: (●), activity remaining in purified enzyme; (■), activity remaining in cytosol. An identical experiment was performed in which the pre-immune serum was substituted for the antiserum: (○), activity remaining in purified enzyme after treatment with pre-immune serum; (□), activity remaining in cytosol after treatment with pre-immune serum.

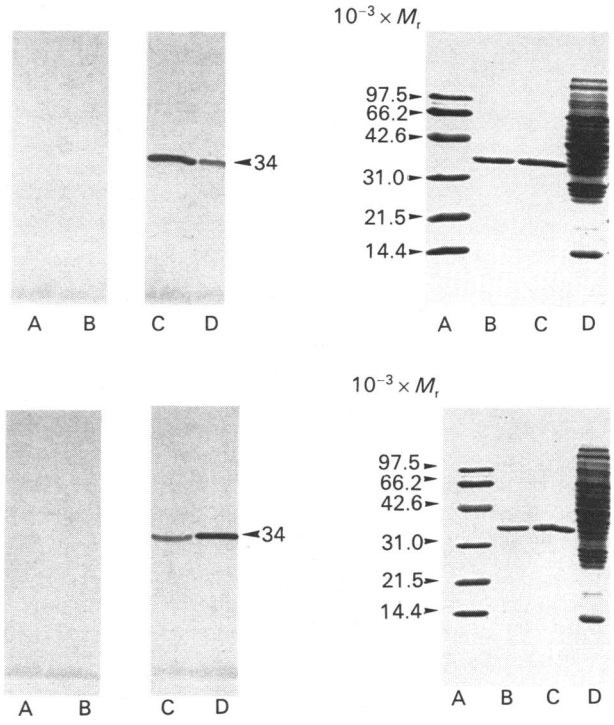


**Fig. 5. Western-blot analysis of cytosolic proteins from male Wistar and male Fischer rat liver**

Purified enzyme (0.2 μg) and either male Wistar or male Fischer rat liver cytosolic proteins (50 μg) were subjected to discontinuous SDS/PAGE, transferred to nitrocellulose membranes and reacted with antiserum diluted 1:1000. The antigen–antibody complexes were visualized as described under Materials and methods. Western blots of Wistar male rat liver cytosol (top left-hand panel): lanes A and C (purified enzyme); lanes B and D (cytosolic protein). Lanes A and B were treated with pre-immune serum and lanes C and D were treated with antisera. Gels were also stained for protein (top right-hand panel): lane A (molecular mass standards); lanes B and C (1.25 and 2.5 μg of purified enzyme respectively); and lane D (50 μg of cytosolic protein from Wistar rat liver). Western blots of Fischer male rat liver cytosol (bottom left-hand panel): lanes A and C (purified enzyme); lanes B and D (cytosolic protein). Lanes A and B were treated with pre-immune serum and lanes C and D were treated with antisera. Gels were also stained for protein (bottom right-hand panel): lane A (molecular mass standards); lanes B and C (1.25 and 2.5 μg of purified enzyme respectively); and lane D (50 μg of cytosolic protein from Fischer rat liver).

hydroxysteroid dehydrogenase becomes unstable if maintained at pH 5 for any length of time and this could contribute to the results obtained by Stolz *et al.* (1987). Following their initial report of a homogeneous preparation, Wörner & Oesch (1984) detected five forms of the enzyme upon isoelectrofocusing. It is of interest that the focusing studies of Stolz *et al.* (1987) and Wörner & Oesch (1984) report the existence of one major form of the enzyme and several minor forms of lower pI. In each case the pI of the major form is closest to that originally described for the dehydrogenase (Penning *et al.*, 1984). This suggests that during focusing minor forms may be derived from the major form by either oxidation of cysteine or deamidation of glutamine and asparagine to yield more acidic proteins (Righetti & Drysdale, 1976).

The recent report by Boutin (1986) describing the purification of 3α-hydroxysteroid dehydrogenase from female Sprague–Dawley rat liver also requires comment. This study attributes the inability to obtain large quantities of the purified enzyme and the higher specific activity observed to the fact that the purification originally described (Penning *et al.*, 1984) permitted the co-purification of several proteins of great similarity. Although the suggestion was made that some of these contaminating proteins represented isoenzymic forms of the dehydrogenase, no direct evidence for the existence of these isoenzymes was given. Our study shows that male and female rat liver cytosols contain only one form of immunoreactive 3α-hydroxysteroid/dihydrodiol dehydrogenase. Moreover, the amount of immunoreactive enzyme present in these cytosols appears to be similar, suggesting that livers from either sex should yield comparable amounts of enzyme (i.e. 1 mg of enzyme/rat liver). Based on these results considerably more enzyme should have been purified by Boutin (1986) from female rat liver, which suggests that the low yield observed may be related to a loss of > 90% of the enzyme during the chromatofocusing step.

Another report that describes multiple forms of the dehydrogenase was recently published by Ikeda *et al.* (1984). They isolated two forms by DEAE-cellulose chromatography. When these forms were subjected to

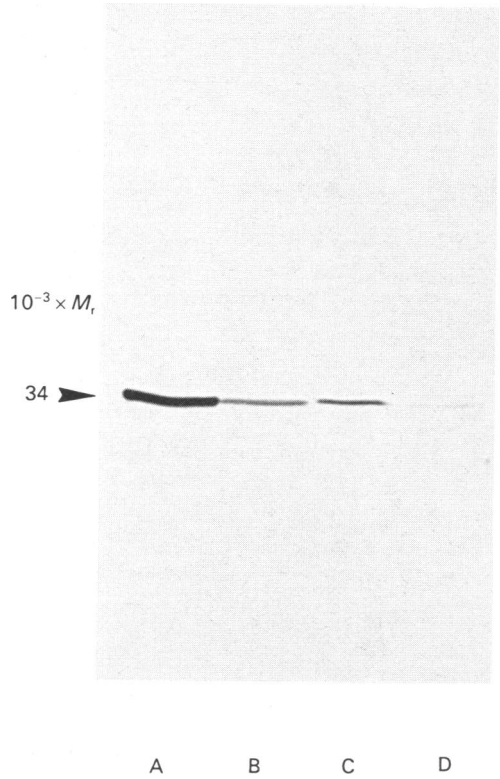


**Fig. 6. Detection of immunoreactive 3α-hydroxysteroid/dihydrodiol dehydrogenase in male Sprague–Dawley rat tissues**

Cytosolic proteins prepared from liver, small intestine, lung and testis of male Sprague–Dawley rats were subjected to SDS/PAGE and electrotransferred to nitrocellulose membranes for Western-blot analysis as described under Materials and methods. Lane A (100 μg of liver cytosol); lane B (100 μg of lung cytosol); lane C (100 μg of small intestine cytosol); and lane D (100 μg of testis cytosol).

subsequent CM-Sephadex chromatography each form gave rise to three additional isoenzymes. It should be emphasized that these multiple forms were isolated from male Wistar rats. Although these six enzymes were chromatographically resolved and thoroughly characterized in terms of their substrate specificity, no functional differences were observed between the various forms. A striking feature of the present work is that even in Wistar and Fischer rats the antibody for 3α-hydroxysteroid dehydrogenase detected only one protein band ($M_r$ 34000) in the livers of each of these strains. Although the existence of isoenzymes of 3α-hydroxysteroid dehydrogenase has been proposed by several laboratories, unless different properties are assigned to these isoenzymes the possibility will exist that they merely represent artifacts of the purification procedure employed.

A novel feature of the work described here was the production of the high-titre, monospecific, polyclonal antibody for 3α-hydroxysteroid/dihydrodiol dehydrogenase. Using this antibody a cDNA library constructed from a poly(A)$^+$RNA preparation of Sprague–Dawley rat liver was screened in the expression vector λ-gt11 (Isaacson *et al.*, 1988). The ability of the antibody to detect only two identical positive clones among the 300000 plaques screened, not only highlights the monospecificity of the antibody, but provides further evidence for the existence of only one gene product. If several isoforms of the enzyme exist it would be predicted that the polyclonal antibody, which contains multiple antigenic recognition sites, would detect more than two identical clones.

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