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Purification and properties of a 3α-hydroxysteroid dehydrogenase of rat liver cytosol and its inhibition by anti-inflammatory drugs

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An NAD(P)-dependent 3α-hydroxysteroid dehydrogenase (EC 1.1.1.50) was purified to homogeneity from rat liver cytosol, where it is responsible for most if not all of the capacity for the oxidation of androsterone, 1-acenaphthenol and benzenedihydrodiol (*trans*-1,2-dihydroxycyclohexa-3,5-diene). The dehydrogenase has many properties (substrate specificity, pI, M_r, amino acid composition) in common with the dihydrodiol dehydrogenase (EC 1.3.1.20) purified from the same source [Vogel, Bentley, Platt & Oesch (1980) *J. Biol. Chem.* **255**, 9621–9625]. Since 3α-hydroxy steroids are by far the most efficient substrates, the enzyme is more appropriately designated a 3α-hydroxysteroid dehydrogenase. It also promotes the NAD(P)H-dependent reductions of quinones (e.g. 9,10-phenanthrenequinone, 1,4-benzoquinone), aromatic aldehydes (4-nitrobenzaldehyde) and aromatic ketones (4-nitroacetophenone). The dehydrogenase is not inhibited by dicoumarol, disulfiram, hexobarbital or pyrazole. The mechanism of the powerful inhibition of this enzyme by both non-steroidal and steroidal anti-inflammatory drugs [Penning & Talalay (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 4504–4508] was examined with several substrates. Most non-steroidal anti-inflammatory drugs are competitive inhibitors (e.g. K_i for indomethacin, 0.20 μM for 9,10-phenanthrenequinone reduction at pH 6.0, and 0.835 μM for androsterone oxidation at pH 7.0), except for salicylates, which act non-competitively (e.g. K_i for aspirin, 650 μM for androsterone oxidation). The inhibitory potency of these agents falls sharply as the pH is increased from 6 to 9. Most anti-inflammatory steroids are likewise competitive inhibitors, except for the most potent (betamethasone and dexamethasone), which act non-competitively. The enzyme is inhibited competitively by arachidonic acid and various prostaglandins.

Hydroxysteroid dehydrogenases are a family of nicotinamide nucleotide-dependent oxidoreductases that catalyse interconversions of hydroxy and carbonyl groups of steroids in a positional and sterically specific manner (Talalay, 1963). Among

Abbreviations and trivial names used: dehydrogenase, 3α-hydroxysteroid dehydrogenase (EC 1.1.1.50); dihydrodiol dehydrogenase, *trans*-1,2-dihydrobenzene-1,2-diol dehydrogenase (EC 1.3.1.20); benzenedihydrodiol, *trans*-1,2-dihydroxycyclohexa-3,5-diene; androsterone, 3α-hydroxy-5α-androstan-17-one; betamethasone, 9α-fluoro-11β,17,21-trihydroxy-16β-methylpregna-1,4-diene-3,20-dione; cortisone, 17,21-dihydroxypregn-4-ene-3,11,20-trione; cortisol, 11β,17,21-trihydroxypregn-4-ene-3,20-dione; dexamethasone, 9α-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione; prednisone, 17,21-dihydroxypregna-1,4-diene-3,11,20-trione; prednisolone, 11β,17,21-trihydroxypregna-1,4-diene-3,20-dione; triamcinolone, 9α-fluoro-11β,16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione; indomethacin, 1-(*p*-chlorobenzyl)-5-methoxy-2-methylindol-3-ylacetic acid; ibuprofen, 2-(*p*-isobutylphenyl)-propionic acid; meclofenamic acid, *N*-(2,6-dichloro-*m*-tolyl)anthranilic acid; tolmetin, 5-(4-methylbenzoyl)-1-methylpyrrol-2-ylacetic acid; oxyphenbutazone, 4-butyl-1-(*p*-hydroxyphenyl)-2-phenylpyrazolidine-3,5-dione; zomepirac, 5-(4-chlorobenzoyl)-1-methylpyrrol-2-ylacetic acid; IC_{50} value, concentration required to produce 50% inhibition of enzyme activity under specified conditions.

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these enzymes, 3α-hydroxysteroid dehydrogenase (EC 1.1.1.50) of animal tissues is unusual in that it has been implicated in a variety of apparently unrelated functions, including the hepatic reduction of dihydrocortisone to tetrahydrocortisone (Tomkins, 1956*a*,*b*) and the conversion of 5α-dihydrotestosterone into 5α-androstane-3α,17β-diol. The latter transformation may regulate androgen action in androgen-dependent tissue (Walsh & Wilson, 1976; DeKlerk *et al.*, 1979). In common with other dual nucleotide-dependent hydroxysteroid dehydrogenases, 3α-hydroxysteroid dehydrogenase promotes transhydrogenation between NADPH and NAD^+ in the presence of catalytic concentrations of steroids (Hurlock & Talalay, 1958; Talalay & Williams-Ashman, 1958*a*,*b*) and may thereby regulate nicotinamide nucleotide concentrations.

Vogel *et al.* (1980) showed that homogeneous dihydrodiol dehydrogenase (*trans*-1,2-dihydrobenzene-1,2-diol dehydrogenase, EC 1.3.1.20) from rat liver cytosol has dual nicotinamide nucleotide specificity, and oxidizes benzenedihydrodiol as well as 1-acenaphthenol and 3α-hydroxy steroids. Purified dihydrodiol dehydrogenase preparations decreased the mutagenic activity of benzo[*a*]pyrene and benz[*a*]anthracene-8,9-diol 10,11-oxide in the Ames test (Glatt *et al.*, 1979, 1982).

The present paper describes the purification, substrate specificities and inhibitor profiles of the NAD^+- and $NADP^+$-linked 3α-hydroxysteroid dehydrogenase of rat liver cytosol. We show that the 3α-hydroxysteroid dehydrogenase and dihydrodiol dehydrogenase activities are mediated by the same protein, which also promotes the oxidation and reduction of diverse xenobiotics. A noteworthy and unforeseen property of this enzyme is potent inhibition by both non-steroidal and steroidal anti-inflammatory drugs and the binding of arachidonic acid and prostaglandins (Penning & Talalay, 1983).

Experimental

Materials

Livers of adult male Sprague–Dawley rats were removed, perfused via the hepatic vein with cold 10mM-potassium phosphate buffer, pH 7.0, containing 250mM-sucrose and 10mM-EDTA, and stored at −80°C.

The following materials were obtained from the stated sources: DEAE-cellulose (Whatman DE-52, preswollen microgranular form), Reeve–Angel (Clifton, NJ, U.S.A.); powdered hydroxyapatite (Bio-Gel HTP), Bio-Rad Laboratories (Richmond, CA, U.S.A.); Polybuffer Exchanger-94, Polybuffer-74/HCl for chromatofocusing and Sephadex G-100, Pharmacia (Piscataway, NJ, U.S.A.); spectroscopic-grade glycerol (J. T. Baker Chemicals, Phillipsburg, NJ, U.S.A.); enzyme-grade sucrose and $(NH_4)_2SO_4$, Schwarz/Mann (Spring Valley, NY, U.S.A.); dithiothreitol, Tris base, pyridine-4-carboxyaldehyde, menadione, dicoumarol, indomethacin and pyrazole, Sigma Chemical Co. (St. Louis, MO, U.S.A.); β-NAD^+ and $NADP^+$, Boehringer Mannheim (Indianapolis, IN, U.S.A.); disodium salts of NADH and NADPH, P-L Biochemicals (Milwaukee, WI, U.S.A.); 9,10-phenanthrenequinone, 4-nitroacetophenone, 4-nitrobenzaldehyde and 4-carboxybenzaldehyde, Fluka–Tridom Chemicals (Hauppauge, NY, U.S.A.); 1-acenaphthenol, 1,4-benzoquinone, 1,4-naphthoquinone, duroquinone (2,3,5,6-tetramethyl-1,4-benzoquinone), 4-methoxybenzaldehyde, acetophenone and propiophenone, Aldrich Chemical Co. (Milwaukee, WI, U.S.A). All quinones were recrystallized. Daunorubicin was a gift from Dr. N. R. Bachur (University of Maryland Cancer Center, Baltimore, MD, U.S.A.). Synthesis of *trans*-1,2-dihydroxycyclohexa-3,5-diene was modified from Jerina *et al.* (1970).

Meclofenamic acid and flufenamic acid were given by Dr. M. I. Gluckman and Dr. M. Black (Warner–Lambert, Morris Plains, NJ, U.S.A.). Tolmetin and zomepirac were provided by Dr. L. D. Muschek (McNeil, Spring House, PA, U.S.A.). Oxyphenbutazone was a gift from Dr. F. Clarke (Ciba–Geigy, Summit, NJ, U.S.A.).

The purity of all fatty acids, including prostaglandins (Sigma Chemical Co.), was checked by t.l.c. on Silica-GF plates (Analtech, Newark, DE, U.S.A.) with benzene/dioxan/acetic acid (20:20:1, by vol.) as solvent (Green & Samuelsson, 1964). Prostaglandin A and B concentrations were determined by u.v. spectroscopy, as were those of prostaglandins E_1 and E_2 after dehydration to prostaglandins B_1 and B_2 respectively (Crabbé, 1977; Andersen, 1969).

Enzyme assays

Initial velocities (corrected for non-enzymic rates where necessary) were determined by measuring changes in the absorption of the nicotinamide nucleotides at 340nm (ε 6270$M^{-1}\cdot cm^{-1}$) in 1.0ml systems in 1.0cm-pathlength cuvettes at 25°C. Reactions were started with enzyme.

(*a*) *Purification.* Several assay systems were used to monitor the enzyme purification. Androsterone oxidation was measured in systems containing 50μM-androsterone, 2.3mM-NAD^+ and 50mM-glycine/NaOH buffer, pH 9.0. For the oxidation of 1-acenaphthenol (500μM) and benzenedihydrodiol (1.46mM) the assay systems were similar except that the nucleotide was 2.3mM-$NADP^+$. The

reduction of 9,10-phenanthrenequinone was measured in systems containing 40 μM-quinone, 180 μM-NADPH and 100 mM-potassium phosphate buffer, pH 6.0. Assays with 4-nitroacetophenone (2.3 mM) were conducted under identical conditions. All substrates were dissolved in 20 μl of acetonitrile. One enzyme unit is the amount of enzyme that catalyses the oxidation or reduction of 1 μmol of nicotinamide nucleotide/min.

(*b*) *Inhibition.* Enzyme inhibition was measured by two methods. For oxidation of androsterone the system contained 75 μM-androsterone (in 20 μl of acetonitrile), 2.3 mM-NAD^+ and 100 mM-potassium phosphate buffer, pH 7.0. For reduction of 9,10-phenanthrenequinone the system contained 40 μM-phenanthrenequinone (in 20 μl of acetonitrile), 180 μM-NADPH and 100 mM-potassium phosphate buffer, pH 6.0. Various concentrations of inhibitors were added in 20 μl of methanol, ethanol or acetonitrile. Control velocities were determined in the presence of appropriate quantities of organic solvents. IC_{50} values were determined by linear-regression analysis of median-effect plots (Chou & Talalay, 1981). K_i values were derived from measurements of the effect of several concentrations of inhibitor on the initial velocity of the reaction in the presence of three to five fixed substrate concentrations. These results were then analysed by Dixon (1953) plots, or their transformation to a secondary plot (Segel, 1975), to give the desired dissociation constants. Maximum velocities and Michaelis constants were computed by the Wilkinson (1961) hyperbolic method.

Protein concentrations

These were determined either by the method of Lowry *et al.* (1951) or by that of Bradford (1976), with bovine serum albumin as standard.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis was performed with 3%/9% double-stack gels as described by Davies (1964) and Ornstein (1964). Protein bands were stained with 0.01% Coomassie Brilliant Blue. Enzyme activity (purple bands) was detected by incubating the gels in 100 mM-potassium phosphate buffer, pH 7.0, containing either 0.5 mM-androsterone or 5 mM-1-acenaphthenol as substrate, 1.58 mM-$NADP^+$, 0.32 mM-Nitro Blue Tetrazolium and 0.08 mM-phenazine methosulphate in the dark at room temperature (Schultz *et al.*, 1977). Sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis was done by the procedure of Laemmli (1970).

Purification of 3α-hydroxysteroid dehydrogenase

Our method of purification of 3α-hydroxysteroid dehydrogenase differed considerably from the procedure described for rat liver cytoplasmic dihydrodiol dehydrogenase (Vogel *et al.*, 1980). All operations were at 4°C.

Step 1: fractionation of rat liver cytosol. Forty frozen livers were thawed and homogenized in 3 vol. of 50 mM-Tris/HCl buffer, pH 8.6, 250 mM-sucrose, 1 mM-EDTA and 1 mM-dithiothreitol in a Waring Blendor (four 15 s pulses) at 4°C. The homogenate was centrifuged at 10000*g* for 30 min, and the microsomal fraction was precipitated from the supernatant fraction by the addition of 0.2 vol. of 100 mM-$CaCl_2$. The pH was adjusted to 8.6, and the clear cytosol was obtained by centrifugation at 10000*g* for 30 min. $(NH_4)_2SO_4$ fractionation of the cytosol (40–75% saturation) precipitated the dehydrogenase, which was dissolved in a minimal volume of 10 mM-Tris/HCl buffer, pH 8.6, containing 1 mM-EDTA and 1 mM-dithiothreitol, and dialysed overnight against three 6-litre batches of the same buffer.

Step 2: DEAE-cellulose chromatography. The dialysed material (445 ml) was applied to a DEAE-cellulose column (5 cm × 65 cm) equilibrated with 10 mM-Tris/HCl buffer, pH 8.6, containing 1 mM-EDTA and 1 mM-dithiothreitol. The column was washed with equilibration buffer, and the 3α-hydroxysteroid dehydrogenase was eluted with a linear gradient of 10–250 mM-NaCl (6 litres) dissolved in the equilibration buffer. 3α-Hydroxysteroid dehydrogenase was eluted in the region 80–100 mM-NaCl. The active fractions were pooled (300 ml) and dialysed against 10 mM-potassium phosphate buffer, pH 7.0, containing 1 mM-dithiothreitol and 20% (v/v) glycerol (three 6-litre batches), which concentrated the volume to 169 ml.

Step 3: hydroxyapatite chromatography. The dialysed protein was applied to a hydroxyapatite column (2.6 cm × 52 cm) equilibrated with 10 mM-potassium phosphate buffer, pH 7.0, containing 1 mM-dithiothreitol and 20% glycerol. The column was developed with a linear concentration gradient (1500 ml) of 10–200 mM-potassium phosphate buffer, pH 7.0. The 3α-hydroxysteroid dehydrogenase activity was eluted in the region of 100–120 mM buffer. The active fractions (200 ml) were dialysed against 10 mM-potassium phosphate buffer, pH 7.0, containing 1 mM-dithiothreitol and 20% glycerol, and concentrated by batch elution from a second hydroxyapatite column (2 cm × 4.8 cm) equilibrated with dialysis buffer (final volume 43 ml).

Step 4: chromatofocusing. The deep-red concentrated 3α-hydroxysteroid dehydrogenase preparation was dialysed against 25 mM-imidazole/HCl buffer, pH 7.4, containing 1 mM-EDTA, 0.5 mM-dithiothreitol and 20% glycerol (three 6-litre batches). The sample was then applied to a chromatofocusing column (2 cm × 19 cm) packed with Polybuffer exchanger PBE-94 and equilibrat-

ed with 25mM-imidazole/HCl buffer, pH7.4, containing 1mM-EDTA, 0.5mM-dithiothreitol and 20% glycerol. A linear pH gradient from 7 to 5 was then generated through the column by elution with 14 bed volumes of Polybuffer 74/HCl, pH5.0, containing 1mM-EDTA, 0.5mM-dithiothreitol and 20% glycerol. The 3α-hydroxysteroid dehydrogenase, which was eluted at a pI of 5.8, was concentrated by ultrafiltration, and dialysed against 50mM-potassium phosphate buffer, pH7.0, containing 1mM-EDTA, 1mM-dithiothreitol and 20% glycerol (final volume 13ml).

Step 5: Sephadex G-100. The concentrated enzyme (13ml) was eluted from a Sephadex G-100 column (2.6cm × 90cm), equilibrated with 50mM-potassium phosphate buffer, pH7.0, containing 1mM-EDTA, 1mM-dithiothreitol and 20% glycerol, as a sharp symmetrical protein peak at 1.6–1.9 void volumes (90% recovery). The peak contained activity towards androsterone, 1-acenaphthenol, 4-nitroacetophenone, 9,10-phenanthrenequinone and benzenedihydrodiol. The active fractions (72ml) were concentrated by ultrafiltration to 12ml, dialysed against 20mM-potassium phosphate buffer, pH7.0, containing 1mM-EDTA, 1mM-dithiothreitol and 30% glycerol, and 0.5ml portions were stored at −80°C.

Results and discussion

Properties and identity of 3α-hydroxysteroid dehydrogenase from rat liver cytosol

The 3α-hydroxysteroid dehydrogenase was purified to homogeneity (see the Experimental section and Table 1) with an overall yield of 24%. The increase in specific activity (150-fold) for androsterone oxidation over the 40–75%-satn.-$(NH_4)_2SO_4$ fraction indicates that the enzyme is a major component of the cytosolic protein. Thus 20–30mg of purified enzyme was obtained from 40 livers.

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate gave a single band (M_r 34000), and gel filtration on Sephadex G-100 showed a single symmetrical protein and enzyme activity peak (M_r 33000). The purified 3α-hydroxysteroid dehydrogenase is therefore a homogeneous monomer.

Throughout the purification the enzyme fractions maintained a constant ratio of specific activities for the following reactions: the oxidations of androsterone by NAD^+, of 1-acenaphthenol by $NADP^+$ and of benzenedihydrodiol by $NADP^+$, and the reduction of 4-nitroacetophenone by NADPH, suggesting that all of these reactions are catalysed by the same protein. The chromatographic profiles at each step of the purification and the activities in peak fractions suggest that this NAD(P)H-linked oxidoreductase

Table 1. *Purification of 3α-hydroxysteroid dehydrogenase of rat liver cytosol*

Enzyme activities were determined as described in the Experimental section with androsterone (A), 1-acenaphthenol (B) and 4-nitroacetophenone (C) as substrates. Enzyme activity with benzenedihydrodiol was measured on peak fractions only. The ratio of activity for androsterone to that for benzenedihydrodiol was 3:1 in these peak fractions, and remained constant during the purification. The yields are given in terms of the 40–75%-satn.-$(NH_4)_2SO_4$ fraction.

Purification step	Volume (ml)	Total protein (mg)	Androsterone (A)				1-Acenaphthenol (B)				4-Nitroacetophenone (C)				Proportions of specific activities A:B:C
			Total activity (μmol/min)	Specific activity (μmol/min per mg)	Purification factor	Yield (%)	Total activity (μmol/min)	Specific activity (μmol/min per mg)	Purification factor	Yield (%)	Total activity (μmol/min)	Specific activity (μmol/min per mg)	Purification factor	Yield (%)	
Cytosol	1520	20064	402	0.020	1		54.4	0.0027	1		338	0.0168	1		1:0.14:0.84
$(NH_4)_2SO_4$ (40–75% satn.)	447	7150	254	0.036	1.8	100	79.0	0.0111	4.1	100	313	0.0433	2.6	100	1:0.30:1.2
DEAE-cellulose	300	1290	177	0.137	6.85	70	34.5	0.0267	9.9	44	258	0.199	11.8	82	1:0.20:1.4
Hydroxyapatite	186	287	132	0.462	23	52	22.3	0.0776	28.8	28	150	0.518	30.8	48	1:0.17:1.1
Chromatofocusing	110	35	112	3.21	160	44	18.4	0.524	194	23	108	3.09	184	35	1:0.16:0.96
Sephadex G-100	71.5	26.2	83.7	3.18	160	33	13.4	0.511	189	17	90.6	3.46	205	29	1:0.16:0.96
Final concentration	11.0	26.0	82.9	3.19	160	33	17.7	0.680	252	22	75.5	2.9	173	24	1:0.21:0.90

accounts for most of the 3α-hydroxysteroid dehydrogenase, benzenedihydrodiol dehydrogenase and ketone reductase activities of rat hepatic cytosol.

Several observations indicate that the purified 3α-hydroxysteroid dehydrogenase is identical with the dihydrodiol dehydrogenase described by Vogel *et al.* (1980). The two proteins are monomers with M_r 33000–34000 with similar pI values (5.8–6.3), amino acid compositions (not shown) and substrate specificities, i.e. ability to oxidize androsterone, benzenedihydrodiol and 1-acenaphthenol, which is a relatively specific substrate for the dihydrodiol dehydrogenase (Vogel *et al.*, 1980). Furthermore, in zymographic experiments, in which the oxidation of androsterone by NAD^+ or of 1-acenaphthenol by $NADP^+$ was linked to Nitro Blue Tetrazolium, polyacrylamide-gel electrophoretograms of the purified enzyme showed a single protein band that stained for both enzyme activities.

Nucleotide and substrate specificity

The 3α-hydroxysteroid dehydrogenase is a versatile oxidoreductase that reacts at comparable rates with both nicotinamide nucleotides. Measurement of bimolecular rate constants ($V_{max.}/K_m$) for a number of substrates indicates that the reactions are more efficient in the presence of NADPH and $NADP^+$ and that in most cases this reflects the lowering of the K_m for the co-substrates by the nucleotides (Table 2).

Bimolecular rate constants also indicate that the enzyme displays a distinct preference for the reduction of quinones and 3-oxo steroids over aromatic aldehydes and ketones, whereas 3α-hydroxy steroids are overwhelmingly more efficiently oxidized than are 1-acenaphthenol or benzenedihydrodiol. The enzyme is most appropriately designated 3α-hydroxysteroid dehydrogenase rather than dihydrodiol dehydrogenase, since the $V_{max.}/K_m$ values for the oxidation of androsterone at pH9.0 with either nicotinamide nucleotide are 150–375 times larger than those for 1-acenaphthenol and benzenedihydrodiol (Table 2).

The enzyme reduces a series of biologically important 3-oxo steroids of the androstane (C_{19}), pregnane (C_{21}) and cholane (C_{24}) series, in which the A/B ring fusion may be *cis* or *trans*. Further details of the steroid specificity have been reported (Mukharji, 1982).

The dehydrogenase also reduces a number of quinones in addition to 9,10-phenanthrenequinone (Table 3). In general, unhindered quinones are the

Table 2. *Kinetic constants of 3α-hydroxysteroid dehydrogenase for various substrates with both nicotinamide nucleotides*

Reduction reactions were monitored in 1 ml systems containing 100 mM-potassium phosphate buffer, pH 6.0, 180 μM-NADH or -NADPH and 40 μl of acetonitrile, which was used to dissolve 5α-androstane-3,17-dione (2–20 μM), 9,10-phenanthrenequinone (2–20 μM), 4-nitrobenzaldehyde (15–900 μM) or 4-nitroacetophenone (70–1050 μM). Oxidation reactions were also monitored in 1 ml systems containing 50 mM-glycine/NaOH buffer, pH 9.0, or 100 mM-potassium phosphate buffer, pH 7.0, 2.3 mM-NAD^+ or -$NADP^+$ and 40 μl of acetonitrile, which was used to dissolve androsterone (7.5–75 μM), 1-acenaphthenol (210–1400 μM) or benzenedihydrodiol (73–1460 μM). In all cases reactions were started by the addition of enzyme and the change in the absorption at 340 nm was measured at 25°C. $V_{max.}$ and K_m were computed by the Wilkinson hyperbolic method (Wilkinson, 1961).

Reaction and substrate	Nucleotide	pH	K_m (μM)	$V_{max.}$ (μmol/min per mg)	$10^2 \times V_{max.}/K_m$	Ratio of $V_{max.}/K_m$ for NADPH/NADH or $NADP^+/NAD^+$
Reduction						
5α-Androstane-3,17-dione	NADH	6.0	16.3±3.6	1.4±0.16	8.59	6.98
5α-Androstane-3,17-dione	NADPH	6.0	1.9±0.47	1.14±0.07	60.0	
9,10-Phenanthrenequinone	NADH	6.0	4.88±0.74	3.74±0.22	76.6	5.27
9,10-Phenanthrenequinone	NADPH	6.0	1.42±0.23	5.74±0.18	404	
4-Nitrobenzaldehyde	NADH	6.0	301±35.6	2.2±0.10	0.731	7.32
4-Nitrobenzaldehyde	NADPH	6.0	99±14.0	5.3±0.23	5.35	
4-Nitroacetophenone	NADH	6.0	2180±626	2.8±0.72	0.129	7.36
4-Nitroacetophenone	NADPH	6.0	474±71	4.5±0.73	0.949	
Oxidation						
Androsterone	NAD^+	7.0	60±13.0	3.13±0.43	5.22	3.14
Androsterone	$NADP^+$	7.0	6.1±1.2	1.0±0.14	16.4	
Androsterone	NAD^+	9.0	30.0±3.2	1.62±0.08	5.40	1.39
Androsterone	$NADP^+$	9.0	11.7±2.3	0.88±0.05	7.52	
1-Acenaphthenol	NAD^+	9.0	1800±500	0.26±0.05	0.0144	2.81
1-Acenaphthenol	$NADP^+$	9.0	4200±1000	1.7±0.34	0.0405	
Benzenedihydrodiol	NAD^+	9.0	1054±280	0.157±0.025	0.0149	3.18
Benzenedihydrodiol	$NADP^+$	9.0	1164±197	0.546±0.054	0.0469	

Table 3. *Substrate specificity of 3α-hydroxysteroid dehydrogenase for quinones, aldehydes and ketones*
The reduction rates were determined with 180 μM-NADPH at pH 6.0 as described in the legend to Table 2 and in the Experimental section. All substrates were dissolved in 40 μl of acetonitrile. Abbreviation: N.D., not detectable (<0.005 μmol/min per mg).

Substrate	Concentration (μM)	Specific activity (μmol/min per mg)
9,10-Phenanthrenequinone	40	4.0
1,4-Benzoquinone	40	1.19
1,4-Naphthoquinone	40	0.080
Duroquinone	40	0.026
Menadione	40	0.026
4-Nitrobenzaldehyde	1000	3.4
4-Carboxybenzaldehyde	1000	N.D.
4-Methoxybenzaldehyde	1000	N.D.
Pyridine-4-carboxyaldehyde	1000	N.D.
4-Nitroacetophenone	1000	2.88
Acetophenone	1000	0.008
Propiophenone	1000	N.D.

most efficient electron acceptors. For example, the rate of reduction of 1,4-benzoquinone is much higher than those of duroquinone (2,3,5,6-tetramethyl-1,4-benzoquinone) and menadione (2-methyl-1,4-naphthoquinone).

The 3α-hydroxysteroid dehydrogenase reduces aromatic aldehydes in preference to aromatic ketones. The ability of these compounds to serve as substrates is extremely sensitive to the electron-withdrawing or -donating effects of substituents. Powerful electron withdrawal appears to be necessary for substrate activity, as exemplified by 4-nitrobenzaldehyde. Replacement of the *p*-nitro group by a less electronegative (4-carboxy) or an electron-donating (4-methoxy) group destroys substrate activity. This may also explain the slightly more rapid reduction of 4-nitrobenzaldehyde than of 4-nitroacetophenone, since the α-methyl group of *p*-nitroacetophenone is electron-donating.

Inhibitor specificity and identity of 3α-hydroxysteroid dehydrogenase

The purified 3α-hydroxysteroid dehydrogenase differs from other soluble rat liver NAD(P)H-dependent carbonyl oxidoreductases in its sensitivity to inhibitors. Since ethanol and 3β-hydroxy steroids are not substrates, and the enzyme is not significantly inhibited by pyrazole (10% inhibition at 400 μM), it is clearly distinct from rat liver alcohol dehydrogenase (EC 1.1.1.1) (Jörnvall & Markovič, 1972; Markovič *et al.*, 1972). Although the 3α-hydroxysteroid dehydrogenase reduces a number of quinones, it may be distinguished from the flavoprotein quinone reductase (EC 1.6.99.2) by its low pI of 5.8 and lack of inhibition by dicoumarol (Ernster *et al.*, 1962; Märki & Martius, 1960). Although the 3α-hydroxysteroid dehydrogenase of rat hepatic cytosol resembles the NADPH-linked aldehyde reductase of rat liver (Felsted *et al.*, 1974*a*,*b*) (pI 5.8 and M_r 34000), it is not identical with this enzyme. Thus the liver 3α-hydroxysteroid dehydrogenase has dual nucleotide specificity, is not inhibited by barbiturates and does not reduce daunorubicin or 4-carboxybenzaldehyde. Insensitivity to disulfiram also distinguishes this enzyme from aldehyde dehydrogenases.

Inhibition by non-steroidal anti-inflammatory agents

The finding that human brain carbonyl reductase is weakly inhibited by indomethacin (IC_{50} 100 μM) (Wermuth, 1981) led us to examine its effect on the 3α-hydroxysteroid dehydrogenase. To our surprise, indomethacin potently inhibited all the reactions promoted by the 3α-hydroxysteroid dehydrogenase, with an IC_{50} value of 0.735 μM for the reduction of 9,10-phenanthrenequinone by NADPH at pH 6.0, and 3.33 μM for the oxidation of androsterone by NAD^+ at pH 7.0 (Penning & Talalay, 1983). Dixon plots indicate that indomethacin is a powerful competitive inhibitor of the reduction of 9,10-phenanthrenequinone (K_i 0.20 μM) and of 5α-androstane-3,17-dione (K_i 0.20 μM) and the oxidation of androsterone (K_i 0.835 μM) (Fig. 1). Other major types of non-steroidal anti-inflammatory agents are also competitive inhibitors of these enzymic reactions, with K_i values ranging from 0.835 to 131 μM for the oxidation of androsterone (Table 4). Salicylates and acetaminophen are much weaker and non-competitive inhibitors of the dehydrogenase (Fig. 2 and Table 4).

We emphasize that the inhibition of rat liver 3α-hydroxysteroid dehydrogenase by non-steroidal anti-inflammatory agents appears to be a specific phenomenon rather than a reflection of the lipophilicity of these drugs. Thus two other enzymes that utilize the same substrates (3-oxo steroids), namely the 3β-hydroxysteroid dehydrogenase of rat liver and the 3α-hydroxysteroid dehydrogenase of *Pseudomonas testosteroni*, are unaffected by indomethacin (Mukharji, 1982).

The inhibitory potency of non-steroidal anti-inflammatory carboxylic acids is profoundly pH-dependent (Fig. 3). Thus the IC_{50} values for the inhibition of androsterone oxidation by indomethacin may vary from 3.0 μM at pH 7.0 to 45.0 μM at pH 9.0. These pH effects appear not to be related to the pK_a values of the carboxy groups of the various drugs, which (with few exceptions) lie between 3 and 5.

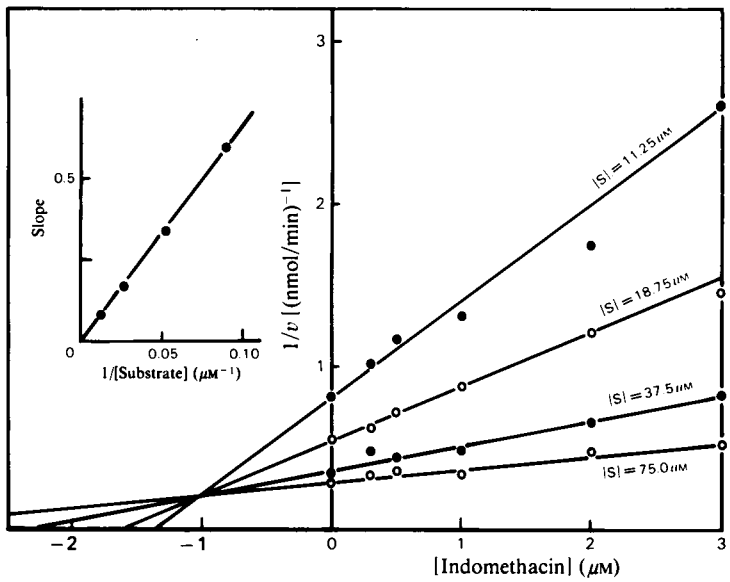

Fig. 1. *Competitive inhibition by indomethacin of the oxidation of androsterone by 3α-hydroxysteroid dehydrogenase*
Initial rates of oxidation were measured at pH 7.0 in the presence of the indicated fixed concentrations of androsterone (11.25–75 μM), 2.3 mM-NAD^+ and varied concentrations of indomethacin (0–4.0 μM). The results were plotted in accordance with Dixon (1953). The inset shows a secondary plot of slopes of the Dixon plots with respect to the reciprocal of the androsterone concentration, from which the K_i value was obtained (Segel, 1975).

Table 4. *Inhibition constants (K_i) and mechanisms of inhibition of 3α-hydroxysteroid dehydrogenase by non-steroidal anti-inflammatory agents*
The K_m and $V_{max.}$ values for 9,10-phenanthrenequinone (concentration range 0–20 μM, 180 μM-NADPH, pH 6.0), 5α-androstane-3,17-dione (concentration range 0–20 μM, 180 μM-NADPH, pH 6.0) and androsterone (concentration range 0–75 μM, 2.3 mM-NAD^+, pH 7.0) were determined in the presence of increasing amounts of inhibitor. Assays were conducted as described in the legend to Table 2. Dixon (1953) plots were constructed for at least four substrate concentrations, and the slope of each line was used to construct a secondary plot (Segel, 1975) from which K_i values were computed. In all cases the lines were drawn by linear-regression analyses. Abbreviations: C, competitive; NC, non-competitive. —, Not determined.

	K_i (μM)		
Inhibitor	9,10-Phenanthrenequinone reduction	5α-Androstane-3,17-dione reduction	Androsterone oxidation
Indomethacin	0.2 (C)	0.2 (C)	0.835 (C)
Meclofenamic acid	0.2 (C)	0.2 (C)	1.10 (C)
Tolmetin	—	—	29.0 (C)
Zomepirac	—	—	37.0 (C)
Ibuprofen	75.0 (C)	80.0 (C)	131 (C)
Oxyphenbutazone	70.0 (C)	100 (C)	104 (C)
Salicylate	420 (NC)	750 (NC)	115 (NC)
Aspirin	—	—	650 (NC)
Acetaminophen	—	—	2300 (NC)

The low micromolar concentrations of indomethacin (0.6–3.0 μM) required to produce inhibition of the 3α-hydroxysteroid dehydrogenase are comparable with those reported by Vane *et al.* (1982) for the inhibition of sheep seminal-vesicle cyclo-oxygenase (IC_{50} 0.15 μM) and by Taylor & Salata (1976) for the inhibition of bovine seminal-vesicle cyclo-oxygenase (IC_{50} 10.5 μM). We have presented evidence (Penning & Talalay, 1983) suggesting that the inhibition of 3α-hydroxysteroid

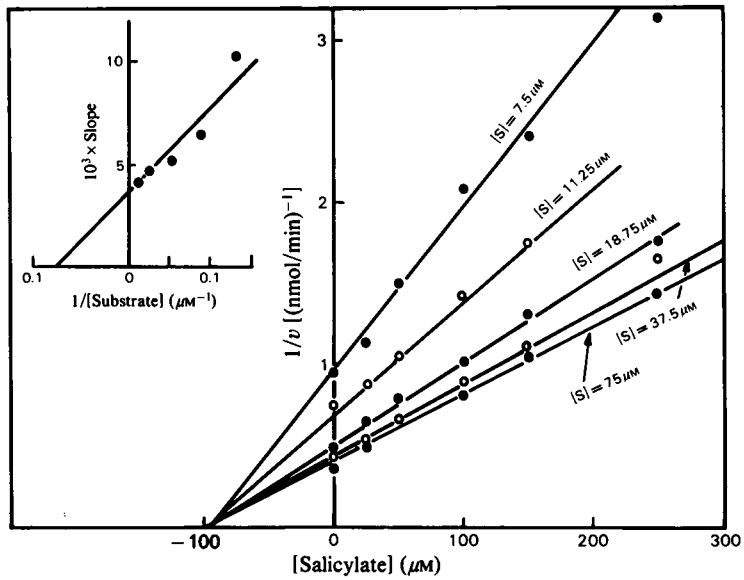

Fig. 2. *Non-competitive inhibition by salicylate of the oxidation of androsterone by 3α-hydroxysteroid dehydrogenase*
Initial rates of oxidation were measured at pH 7.0 in the presence of fixed concentrations of androsterone (7.5–75 μM), 2.3 mM-NAD^+ and varied concentrations of sodium salicylate (0–250 μM). The results were plotted in accordance with Dixon (1953). The inset shows a secondary plot of the slope of the Dixon plots with respect to the reciprocal of the androsterone concentration, from which the K_i value was obtained (Segel, 1975).

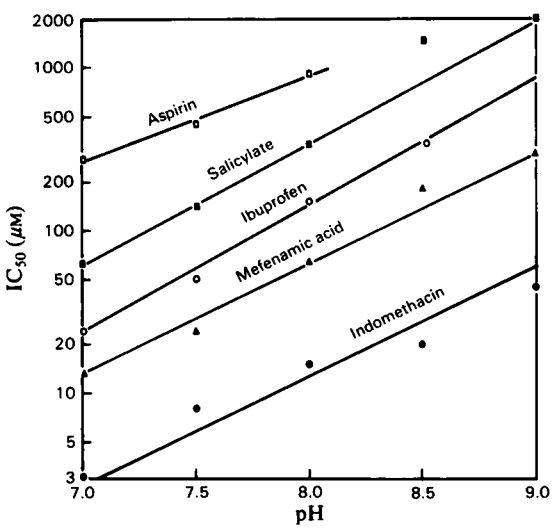

Fig. 3. *Effect of pH on the inhibition by non-steroidal anti-inflammatory agents of the oxidation of androsterone by 3α-hydroxysteroid dehydrogenase*
Initial rates of oxidation were measured with 75 μM-androsterone in the presence of 2.3 mM-NAD^+ at pH 7.0, 7.5, 8.0, 8.5 and 9.0. The uninhibited velocity at each pH value was determined, and measurements were made with various concentrations of indomethacin, mefenamic acid, ibuprofen, sodium salicylate and aspirin. From such measurements, the IC_{50} value for each inhibitor was found at each pH value. Plots of the log IC_{50} values with respect to pH are shown. Potassium phosphate (100 mM) buffer was used for measurements at pH 7–8, and Tris/HCl (100 mM) buffer was used for the higher pH values.

Table 5. *Inhibition of 3α-hydroxysteroid dehydrogenase by anti-inflammatory steroids*
The K_i values were determined as described in the legend of Table 4. Abbreviations: C, competitive; NC, non-competitive.

Inhibitor	K_i for androsterone oxidation (μM)	Kinetic nature of inhibition
Betamethasone	4.5	NC
Dexamethasone	10.0	NC
6α-Methylprednisolone	7.5	C
Prednisone	17.5	C
Prednisolone	17.5	C
Cortisone	285	C
Cortisol	190	C

dehydrogenase by non-steroidal anti-inflammatory drugs fulfils many of the same criteria as were proposed by Vane *et al.* (1982) for ascribing the mechanism of action of these agents to their inhibition of cyclo-oxygenase. We have also shown that the inhibitory activities of the non-steroidal anti-inflammatory agents on the dehydrogenase are correlated with their anti-inflammatory potencies (Table 4, and Penning & Talalay, 1983). Furthermore, concentrations of these agents which block cyclo-oxygenase may also affect androgen and corticosteroid metabolism by their action on 3α-hydroxysteroid dehydrogenase. Similarly, the dehydrogenation of proximate carcinogens to non-carcinogenic catechols may also be modified by the inhibition of the dihydrodiol dehydrogenase function of the enzyme.

Inhibition by anti-inflammatory steroids

Since the steroidal anti-inflammatory drugs are analogues of 5β-dihydrocortisone, a steroid substrate for the enzyme, it was not surprising that these compounds are potent inhibitors of the dehydrogenase. The order of potency as measured by K_i values is betamethasone > dexamethasone > 6α-methylprednisolone, prednisolone and prednisone > cortisol and cortisone (Table 5). All the steroids examined are competitive inhibitors

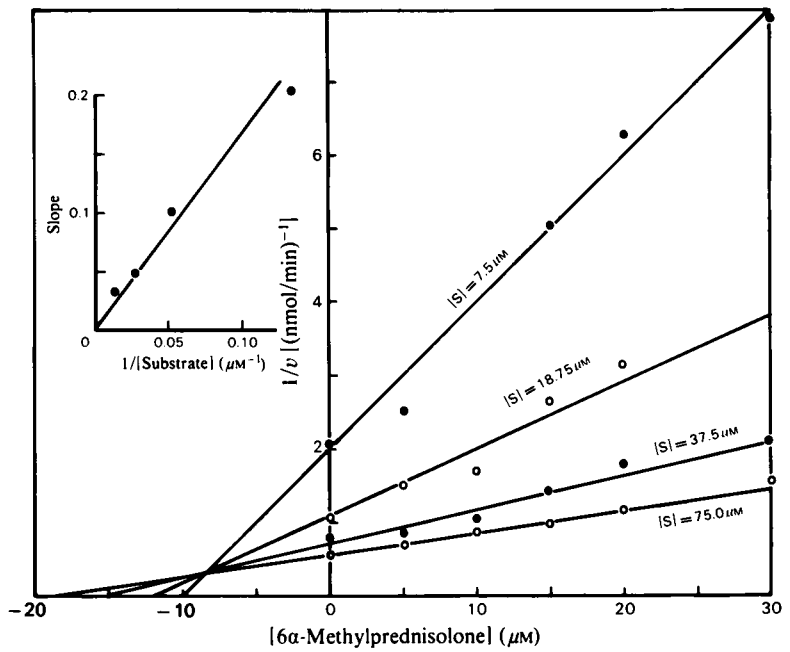

Fig. 4. *Competitive inhibition by 6α-methylprednisolone of the oxidation of androsterone by 3α-hydroxysteroid dehydrogenase*

Initial rates of oxidation were measured at pH 7.0 in the presence of fixed concentrations of androsterone (7.5–75 μM), 2.3 mM-NAD^+ and varied concentrations of 6α-methylprednisolone (0–30 μM). The results were plotted in accordance with Dixon (1953). The inset shows a secondary plot of the slope of the Dixon plots with respect to the reciprocal of the androsterone concentration, from which the K_i value was obtained (Segel, 1975).

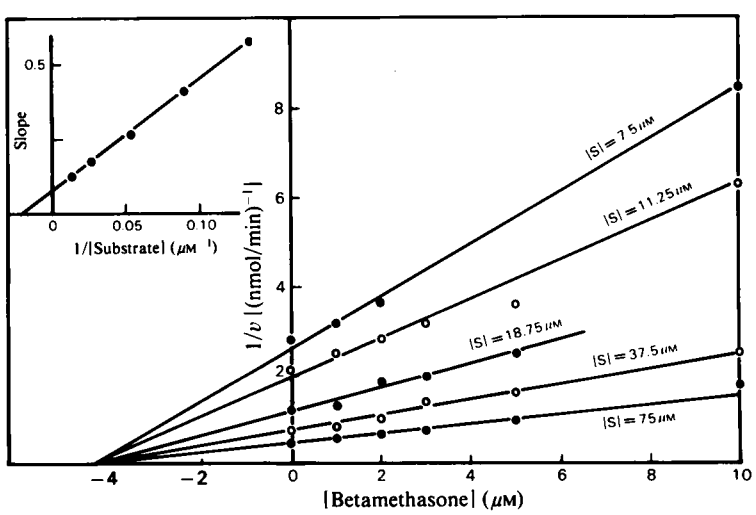

Fig. 5. *Non-competitive inhibition by betamethasone of the oxidation of androsterone by 3α-hydroxysteroid dehydrogenase*

The initial rates of oxidation were measured at pH 7.0 in the presence of fixed concentrations of androsterone (7.5–75 μM), 2–3 mM-NAD^+ and varied concentrations of betamethasone (0–10 μM). The results were plotted in accordance with Dixon (1953). The inset shows a secondary plot of the slope of the Dixon plots with respect to the reciprocal of the androsterone concentration, from which the K_i value was obtained (Segel, 1975).

Table 6. *Inhibition of 3α-hydroxysteroid dehydrogenase by arachidonic acid and prostaglandins*
The IC_{50} values of fatty acids were determined in the presence of 40 μM-9,10-phenanthrenequinone and 180 μM-NADPH at pH 6.0, or 75 μM-androsterone and 2.3 mM-NAD^+ at pH 7.0, from dose–response curves, which where analysed by median-effect plots (Chou & Talalay, 1981). The K_i values for these fatty acids were determined as described in the legend of Table 4. Abbreviation: C, competitive inhibition. —, Not determined.

Fatty acid or prostaglandin	IC_{50} (μM) 9,10-Phenanthrenequinone reduction	IC_{50} (μM) Androsterone oxidation	K_i for androsterone oxidation (μM)	Kinetic nature of inhibition
Arachidonic acid	9.0	15.0	6.0	C
Prostaglandin A_1	6.5	10.5	3.1	C
Prostaglandin B_1	2.5	8.0	0.8	C
Prostaglandin E_1	8.2	33.0	7.5	C
Prostaglandin $F_{1\alpha}$	13.0	50.0	12.0	C
Prostaglandin A_2	1.0	4.5	—	
Prostaglandin B_2	6.6	20.0	—	
Prostaglandin E_2	8.0	18.0	—	
Prostaglandin $F_{2\alpha}$	23.0	105	—	
Thromboxane B_2	15.0	60.0	—	
Prostacyclin	300	300	—	

(Table 5 and Fig. 4), except for the most potent compounds, namely betamethasone (Fig. 5) and dexamethasone, which inhibit non-competitively.

Inhibition by arachidonic acid and by prostaglandins

The enzyme possesses arachidonic acid- and prostanoid-binding sites. Examination of a series of commercial prostaglandins indicated that their order of inhibitory potency was related to their lipophilicity: prostaglandin B > prostaglandin A > prostaglandin E > prostaglandin F. Under the conditions of the assay, the prostaglandins inhibited the reduction of 9,10-phenanthrenequinone more potently than they did the oxidation of androsterone. Dixon plots of the inhibition of androsterone oxidation by prostaglandins (of the 1-series) indicate that these fatty acids are powerful competitive inhibitors. A summary of the inhibitory potencies is given in Table 6.

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