Oestradiol-17 α dehydrogenase from chicken liver

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The NADP⁺-linked oestradiol-17 α dehydrogenase (EC 1.1.1.148) present in cell-free extracts of chicken liver was investigated with the aim of separating it from a closely related oestradiol-17 β dehydrogenase (EC 1.1.1.62) found in the same subcellular fraction. However, its chromatographic behaviour on CM-cellulose and DEAE-cellulose was almost identical with that previously reported for the latter enzyme, including resolution into two peaks on the anion-exchanger. Both peaks contained oestradiol-17 α dehydrogenase and oestradiol-17 β dehydrogenase activity. Further attempts to separate the putative enzymes by dye-ligand chromatography with the use of the dyes Procion Yellow, Reactive Red and Cibachron Blue linked to Sepharose were unsuccessful, and they behaved identically on affinity columns of adenosine 2',5'-bisphosphate-agarose and 17 β -oestradiol 3-hemisuccinate bound to Sepharose. A previous report of partial separation on Sephadex G-200 was not confirmed. Slab gel electrophoresis of enzyme preparations after affinity chromatography on adenosine 2',5'-bisphosphate-agarose revealed multiple bands in systems containing sodium dodecyl sulphate, whereas analysis by rod gel electrophoresis gave two major and one minor bands that stained coincidentally for oestradiol-17 α dehydrogenase, oestradiol-17 β dehydrogenase, epitestosterone dehydrogenase and testosterone dehydrogenase activities. Isoelectric focusing gave four enzymically active peaks that each oxidized oestradiol-17 α and -17 β . Apparent K_m values for the two forms of oestradiol-17 α dehydrogenase obtained by DEAE-cellulose chromatography were 17 and 23 μ M for oestradiol-17 α , and 8.7 and 11.0 μ M for NADP⁺. Limited kinetic studies with oestradiol-17 α and -17 β with the use of the mixed-substrate method showed that the total velocity was equal to the sum of the separate velocities. The active-site inhibitor-alkylating agent 17 β -(1-oxoprop-2-ynyl)androst-4-en-3-one did not cause time- or temperature-dependent inhibition, in contrast with the reported case of the oestradiol-17 β dehydrogenase and 20 α -hydroxysteroid dehydrogenase activities of the human placental oestradiol dehydrogenase. NADP⁺ appeared to afford some protection against inhibition. Investigation of substrate specificity with a limited range of steroids suggests that the enzyme(s) from chicken liver differs substantially from the oestradiol-17 β dehydrogenase from human placenta, and although the evidence is not conclusive it suggests the existence of one enzyme.

Oestradiol-17 α and -17 β differ only in the chirality of their substituents at C-17, yet they show marked differences in biological activity, the 17 β -epimer being the oestrogenic hormone. Both

Abbreviations used: 17 α -dihydroequilin, oestra-1,3,5(10),7-tetraene-3,17 α -diol; 17 α -dihydroequilenin, oestra-1,3,5(10),6,8-pentaene-3,17 α -diol; epitestosterone, 17 α -hydroxyandrost-4-en-3-one; oestradiol-17 α , oestra-1,3,5(10)-triene-3,17 α -diol; oestriol, oestra-1,3,5(10)-triene-3,16 α ,17 β -triol; OPA, 17 β -(1-oxoprop-2-ynyl)androst-4-en-3-one; SDS, sodium dodecyl sulphate.

compounds are reversibly oxidized to oestrone by preparations of chicken liver (Renwick & Engel, 1967), and because enzymes that readily interconvert biologically active and inactive molecules warrant further study (Engel & Groman, 1974) we have investigated the oestradiol dehydrogenases of chicken liver. Some of the properties of a highly purified NADP⁺-linked oestradiol-17 β dehydrogenase (EC 1.1.1.62) from that tissue have been described (Renwick *et al.*, 1981), and we now report attempts to purify the oestradiol-17 α dehy-

drogenase activity (EC 1.1.1.148), which is present in lesser amount.

Materials and methods

Materials

Na_2CO_3 , NaHCO_3 , $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ were of analytical grade and were bought from Riedel-de Hen (Hannover, West Germany), Mallinckrodt (St. Louis, MO, U.S.A.), Prolab (Paris, France) or Merck (Darmstadt, West Germany).

Nicotinamide nucleotides, Nitro Blue Tetrazolium, phenazine methosulphate, steroid substrates, adenosine 2',5'-bisphosphate-agarose, all protein standards and all reagents used in electrophoresis were bought from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

DEAE-cellulose and CM-cellulose (DE-52 and CM-52) from W. and R. Balston (Maidstone, Kent, U.K.) were used in accordance with the manufacturer's instructions. Sepharoses 2B and 4B were bought from Pharmacia Fine Chemicals (Uppsala, Sweden). Ampholines were obtained from LKB Produkter (Bromma, Sweden).

Buffers

The following buffers were used: A, 50mM-sodium phosphate, pH 7.2; B, 25mM-sodium phosphate, pH 7.2, containing 50% (v/v) glycerol; C, 5mM-sodium phosphate, pH 6.5; D, 10mM-Tris/HCl, pH 7.5; E, 5mM-sodium phosphate, pH 7.2; F, 10mM-sodium phosphate, pH 7.2.

Tissue

Fresh chicken liver was bought from or given by Fairday Poultry Processors (Henderson, Auckland, New Zealand) and processed within a few hours of slaughter.

T.l.c.

Purity of steroid substrates and tentative identification of steroid products of enzyme-catalysed reactions were ascertained by t.l.c. in diethyl ether/benzene (2:1, v/v), diethyl ether/benzene (1:2, v/v) and diethyl ether/ethyl acetate (2:1, v/v) as described (Renwick *et al.*, 1981).

Affinity chromatography

Procion Yellow, Reactive Red and Cibachron Blue dyes were conjugated to Sepharose 2B by Dr. G. K. Scott, using the method reported by Travis *et al.* (1976).

Columns were equilibrated in buffer E and run under starting conditions before the application of a linear 0–0.5M-KCl gradient in the same buffer.

17 β -Oestradiol-3-Sepharose was prepared with the use of non-radioactive oestradiol 3-hemi-

succinate (Cuatrecasas, 1970). The affinity column was equilibrated in buffer E and eluted under starting conditions before the application of 0.1M-KCl in buffer E.

The amount of oestradiol bound per ml of Sepharose 4B was determined by hydrolysis of the affinity matrix in 1M-NaOH for 16h at 37°C followed by ether extraction and quantification by Brown's (1955) modification of the Kober reaction.

Electrophoresis

Polyacrylamide-gel electrophoresis was carried out in slabs and rods. Slab gel electrophoresis was performed in 10% (w/v) gels of 0.5mm thickness at pH 8.8 by the Laemmli (1970) procedure. Separation was obtained at room temperature at 150V (25mA), until the tracking dye (Bromophenol Blue) was about 1.0cm below the stacking gel. The voltage was then increased to 200V (25–30mA), and electrophoresis was continued until the tracking dye just left the bottom of the plate. Both SDS-containing and non-SDS-containing systems were used. Proteins were located with Coomassie Brilliant Blue dye.

Rod gel electrophoresis was performed in 5% gels (w/v) at pH 8.8 and stained for protein or steroid dehydrogenase activity as described previously (Renwick *et al.*, 1981).

Isoelectric focusing

Isoelectric focusing was carried out in an LKB B100-1 column at 7°C with Ampholines in the range pH 5–7. Glycerol was used as a stabilizing agent. The power was initially set at 1.0W, but after 5h running time it was increased to 1.5–2.0W for 24h.

Fractions (0.7ml) were then collected for determination of pH, enzyme and protein content as described previously (Renwick *et al.*, 1981). Ampholines were removed by dialysis before measurement of enzyme activity.

Gel filtration

A column (2.5cm $\times$ 120cm) of Sephadex G-200 was equilibrated in buffer F and samples (5ml) were applied from below followed by a 5ml wash. Ascending chromatography was carried out at 10.5ml/h, a Pharmacia pump being used. Fractions (10ml) were collected.

Enzyme assays and units of activity

Steroid dehydrogenase assays were performed as described by Renwick *et al.* (1981) with 0.4 μ mol of steroid unless otherwise stated. One unit of enzyme activity is defined as the formation of 1 μ mol of NADPH/min under the conditions of assay. All activities are expressed in munits.

Enzyme inhibition

In experiments with OPA, the buffer, water, enzyme and NADP⁺ were incubated in a cuvette for 4 min at 37°C followed by the addition of OPA and initiation of the reaction by steroid substrate 1 min later.

Purification of enzyme

All steps were carried out between 0 and 4°C. Fractions containing enzyme were concentrated by dialysis against flakes of poly(ethylene glycol) (Carbowax 20M) before further dialysis against the appropriate buffer.

Centrifugation. Centrifugation was carried out in a Sorvall RC-5B instrument with GSA and SS-34 rotors.

Step 1: preparation of cell-free extract. Excess fat was removed from fresh chicken liver, and 130 g of tissue was chopped and homogenized in a Sorvall Omni-Mixer with buffer A and a tissue/buffer ratio of 1:3 (w/v). The homogenate was centrifuged at 16500g for 45 min, and the pellet was resuspended in an equal volume of buffer, homogenized and centrifuged as before. The supernatant fractions were combined.

Step 2: first (NH₄)₂SO₄ precipitation. Crystalline (NH₄)₂SO₄ (260 g/l) was added to the combined supernatant fractions and the pH of the solution was maintained at 7.2 by the dropwise addition of 3M-NH₃. Stirring was continued for at least 30 min after dissolution of the salt, and the precipitate that formed was collected by centrifugation at 16500g for 45 min. The pellet was discarded.

Step 3: second (NH₄)₂SO₄ precipitation. The supernatant fraction obtained after step 2 was similarly treated with the same salt (200 g/l), and the precipitate obtained was resuspended in buffer B, stored at -20°C and used as the source of enzyme in the steps that follow.

Step 4: CM-cellulose chromatography. Samples, usually 150 ml of the second (NH₄)₂SO₄ precipitate in buffer B, were dialysed for 8 h against buffer C, concentrated by dialysis against flakes of poly(ethylene glycol) and centrifuged at 27000g for 5 min before application to a column (2.5 cm $\times$ 40 cm) of CM-cellulose equilibrated in the same buffer. Chromatography was carried out under starting conditions before application of 0.5M-KCl in buffer C.

Fractions containing enzyme activity, which was eluted in the front, were combined and stored in 50% (v/v) glycerol at -20°C if not used immediately.

Step 5: DEAE-cellulose chromatography. The preparation from step 4 was dialysed against buffer D, concentrated and applied to a column (2.5 cm $\times$ 40 cm) of DEAE-cellulose equilibrated in

the same buffer. Elution was begun under starting conditions with 250 ml of buffer followed by 250 ml of 0.1M-KCl in buffer D. Fractions containing enzyme activity were combined and stored in 50% (v/v) glycerol at -20°C.

Step 6: adenosine 2',5'-bisphosphate-agarose chromatography. The preparations from step 5 were dialysed against buffer E and concentrated before each was applied separately to a column (0.9 cm $\times$ 8 cm) of adenosine 2',5'-bisphosphate-agarose equilibrated in the same buffer. Elution was begun under starting conditions with 50 ml of buffer before application of a linear 0-0.25 mM-NADP⁺ gradient in buffer E. Fractions containing enzyme activity were stored in 50% (v/v) glycerol at -20°C if not used immediately.

Results and discussion

Purification of oestradiol-17 α dehydrogenase present in cell-free extracts of chicken liver

Although it is known that cell-free extracts of chicken liver contain oestradiol-17 α dehydrogenase activity (Renwick & Engel, 1967), its spectrophotometric determination in crude homogenates is frequently not possible until after salt fractionation because of its low concentration in the starting material. The method of purification is summarized in Table 1.

When applied to columns of CM-cellulose and eluted under starting conditions, the oestradiol dehydrogenase activity appeared in the front, whereas most of the pigment was bound to the cation-exchanger and was eluted in 0.5M-KCl. The elution profile was virtually identical with that described for the oestradiol-17 β dehydrogenase, and further chromatography of the active fractions on DEAE-cellulose at pH 7.5 gave two peaks of activity similar to those reported for that enzyme (Renwick *et al.*, 1981), although different conditions were employed. The first peak, A, emerged with the front; the second, B, was eluted with a band of pigment in 0.1M-KCl. The active fractions from each peak were pooled separately (both contained oestradiol-17 α dehydrogenase and oestradiol 17 β -dehydrogenase activities) and were chromatographed on small columns of adenosine 2',5'-bisphosphate-agarose. Peak A was largely bound but could be eluted in a gradient of NADP⁺ (0-0.25 mM); peak B gave a more complex elution pattern on a similar column; about 60% of the activity (designated peak C) was unbound and appeared in the void volume; the remainder (peak D) was eluted in a gradient of NADP⁺ (0-0.25 mM). Both peaks contained oestradiol-17 α dehydrogenase and oestradiol-17 β dehydrogenase activities.

Table 1. *Purification of oestradiol-17 α dehydrogenase activity from chicken liver*

Cell-free extracts were prepared from about 130 g of fresh chicken liver and fractionated as described in the text. Oestradiol-17 α dehydrogenase activity was determined by the rate of formation of NADPH at 340 nm. The results below were obtained from 130 g of tissue.

Fraction	Volume (ml)	Total protein (mg)	Total activity (munits)	Specific activity (munits/mg)	Purification (fold)	Yield (%)
Cell-free extract	464					
2nd (NH ₄) ₂ SO ₄ ppt. after dialysis and concentration	41	3538	10097	2.9	1.0	100
After CM-cellulose, dialysis and concentration	25	1713	8280	4.8	1.7	82
After DEAE-cellulose						
Peak A	64	333	1817	5.5	1.9	18
Peak B	40	504	1896	3.8	1.3	19
Peaks A + B						37
After adenosine 2',5'-bisphosphate-agarose						
Peak A	5.8	2.9	600	206.9	71.3	5.9
Peak C	3.3	106	340	3.2	1.1	3.4
Peak D	2.2	2.6	520	200	69	5.2
Peaks A + C + D						14.5

Affinity chromatography

It has been reported (Renwick *et al.*, 1979) and confirmed in the present experiments that affinity chromatography on adenosine 2',5'-bisphosphate-Sepharose of preparations of chicken liver that contained oestradiol-17 α dehydrogenase, oestradiol-17 β dehydrogenase, epitestosterone dehydrogenase and testosterone dehydrogenase activities effected purification but no resolution into constituent enzymes. Similar results were obtained with the predominantly hydrophobic ligand *p*-(phenoxypropoxy)aniline, and further attempts to separate the oestradiol-17 α dehydrogenase from oestradiol-17 β dehydrogenase activity on dye-ligand columns with the dyes Procion Yellow, Reactive Red and Cibachron Blue were unsuccessful under the conditions used. The active fractions were eluted unresolved at the tail of the void volume.

Although is it possible that our enzyme preparations consisted of at least two protein molecules that resembled each other so closely that they behaved identically on ion-exchange, adenosine 2',5'-bisphosphate-agarose and dye-ligand chromatography, the evidence was beginning to favour the existence of one enzyme capable of oxidizing the epimeric oestradiols to the common product, oestrone. We therefore attempted to separate the putative activities by linking the steroid substrates to Sepharose. The high cost of reagents precluded the synthesis of a column with bound oestradiol-17 α as well as one with bound oestradiol-17 β , so we linked 17 β -oestradiol 3-hemisuccinate to Sepharose 4B. Hydrolysis of the matrix after use and determination of oestradiol showed that 0.36 μ mol

had been bound/ml of Sepharose, compared with the reported value of 0.5 μ mol/ml (Cuatrecasas, 1970). The enzymes were not separated in these experiments.

Gel filtration

Renwick & Engel (1967) reported that they had achieved a partial separation of the oestradiol-17 α dehydrogenase and oestradiol-17 β dehydrogenase by gel filtration on a long column of Sephadex G-200, but repetition of their work with purer preparations of higher specific activity failed to confirm the results. Re-examination of their data supports the view that they were most probably observing fine precipitation, which is frequently encountered when determining oestradiol-17 α dehydrogenase and oestradiol-17 β dehydrogenase activities in low protein concentrations, and they did not include albumin in their assay mixtures. A similar phenomenon occurs with active preparations of oestradiol-17 β dehydrogenase, where the product, oestrone, may be thrown out of aqueous solution because of its low solubility. Hence the need to check the identity of the reaction product(s) from time to time, especially when working with steroid-transforming enzymes.

Examination of the ratio of oestradiol-17 α dehydrogenase and oestradiol-17 β dehydrogenase activities throughout purification showed that they remained fairly constant at about 1:3–5. A constant ratio was reported by Renwick *et al.* (1981) during the purification of the oestradiol-17 β dehydrogenase from the same tissue but with a different procedure. It was originally reported (Renwick & Engel, 1967) that no oestradiol-17 α

dehydrogenase was found in their first $(\text{NH}_4)_2\text{SO}_4$ precipitate, which suggested the existence of two distinct enzymes in the same subcellular fraction of chicken liver. However, these authors determined activities at 25°C, and measurements of similar preparations at 37°C reveal oestradiol-17 α dehydrogenase activity.

Effect of pH

The pH optimum of the NADP⁺-linked oxidation of oestradiol-17 α was 9.5 for the activity present in peaks A and B separated on DEAE-cellulose. The activity profiles were very similar to those reported by Renwick & Engel (1967) for oestradiol-17 α dehydrogenase and oestradiol-17 β dehydrogenase activities at 25°C and by Renwick *et al.* (1981) for the latter at 37°C.

Kinetic constants and substrate specificity

Kinetic data were obtained for peaks A and B separated by chromatography on DEAE-cellulose. Apparent Michaelis constants and maximum velocities were determined graphically by the method of Lineweaver & Burk (1934), and are shown in Table 2. Although they differ a little from those first reported for the oestradiol-17 α dehydrogenase from chicken liver (Renwick & Engel, 1967), the substrate concentration and assay temperature were higher, albumin was included in the assay system and the enzyme activity had been resolved into two discrete peaks.

Given the possible existence of two closely related but distinct oestradiol dehydrogenases, the rates of oxidation of both oestradiol epimers were compared when the two substrates were present separately by using the method of mixed substrates advocated by Dixon & Webb (1979). The observed individual velocities with oestradiol-17 α and oestradiol-17 β were 6.7 munits/mg and 35 munits/mg respectively and the mixed substrate velocity was 41.4 munits/mg, which suggests the

presence of two different enzymes. Attempts to determine the mixed-substrate velocity with mixtures of both substrates at equal relative concentrations were frustrated by increasing turbidity in the assay system, which was probably caused by precipitation of the oxidation product, oestrone. This limitation may be obviated by the use of a chemical or radiochemical method for measuring enzyme activity and so permit more-rigorous mixed-substrate studies such as those reported by Houslay *et al.* (1974) for human brain monoamine oxidase.

Inhibition of oestradiol-17 α dehydrogenase

The simultaneous inactivation of both the oestradiol-17 β dehydrogenase and 20-hydroxy-steroid dehydrogenase activities of the human placental oestradiol dehydrogenase was reported by Tobias *et al.* (1982) with active-site-directed 17 β -substituted progestin analogues, and we examined the effects of OPA on the oestradiol dehydrogenase activities of chicken liver. In the concentrations used (10–100 mM) OPA did not produce time- or concentration-dependent inhibition, in contrast with the placental enzyme.

When oestradiol-17 β and testosterone were substrates a constant inhibition of 50% was obtained; these compounds are identical in rings B, C and D. Oxidation of their epimers, oestradiol-17 α and epitestosterone, which vary only in the α -orientation of the hydroxy group at C-17, showed inhibition that ranged from 30% at low concentrations of OPA (10–25 mM) to 60% when the inhibitor was present at 50–100 mM. If NADP⁺ was omitted from the assay mixtures only 15–25% activity was obtained for all four enzymic transformations, which suggests greater inhibition by OPA in the absence of NADP⁺.

The limited availability of the inhibitor and problems associated with the water-solubility of the unconjugated steroid substrates used in these experiments precluded more-detailed study.

Table 2. Apparent values for K_m and V for oestradiol-17 α dehydrogenase and oestradiol-17 β dehydrogenase activities at 37°C. NADP⁺-linked oxidation of oestradiol-17 α dehydrogenase and oestradiol-17 β dehydrogenase activities were carried out under standard conditions of assay at 37°C (Renwick *et al.*, 1981) with preparations of peaks A and B obtained after chromatography on DEAE-cellulose. Assays were done in triplicate at each substrate concentration, and apparent values for K_m and V were derived from double-reciprocal plots; mean values are given.

Substrate	Sample	Apparent K_m (μM)	V (nmol of substrate/min per mg of protein)
Oestradiol-17 α (3.3–67.5 μM)	Peak A	17.0	5.5
	Peak B	23.0	6.7
Oestradiol-17 β (3.3–133 μM)	Peak A	25.0	35
NADP ⁺ (1.6–16.6 μM)	Peak A	8.7	8.1
	Peak B	11.0	5.2

Substrate specificity

The rates of oxidation of several 17-hydroxy steroids relative to that of oestradiol-17 β , the most reactive substrate, are given in Table 3. Limited supplies of chromatographically pure compounds precluded determination of values for K_m and V in most cases, and a detailed examination of substrate specificity of the sort reported for the oestradiol-17 β dehydrogenase of human placenta by Langer *et al.* (1959). However, some tentative observations may be made. It was confirmed that oestradiol-17 β was the most reactive substrate in the presence of NADP $^+$. We detected no activity with NAD $^+$ and either oestradiol epimer, unlike a previous report (Renwick *et al.*, 1981) where we found a very low concentration (approx. 1%) of NAD $^+$ -linked activity, but the modes of preparation of the enzyme differed substantially. We also found that the preparation used in the present experiments oxidized oestradiol-17 α and the C $_{19}$ steroids epitestosterone and testosterone at significantly higher rates than those reported previously.

In view of our earlier findings we were not surprised to find that oestriol, an oestrogen with a third hydroxy group in the 16 α -position, did not react, nor did the ring-B-unsaturated 17 α -oestrogens 17 α -dihydroequilin and 17 α -dihydroequilenin, an unexpected result in the light of earlier observations on the specificity of the oestradiol-17 β dehydrogenase of human placenta (Langer *et al.*, 1959). This group found that dihydroequilin and dihydroxyequilenin (ring-B-unsaturated oestrogens with a 17 β -oriented hydroxy group) showed increased reactivity.

It is also of interest that the 5 β -androstane-3 α ,17 α -diol was not oxidized, whereas the closely related compound 5 α -androstane-3 β -androstane-3 β ,17 α -diol gave a rate almost one-half that of oestradiol-17 β .

Electrophoresis in polyacrylamide gels

Rod gel electrophoresis of peak A material after chromatography on adenosine 2',5'-bisphosphate-agarose revealed two major and one minor bands that stained coincidentally for oestradiol-17 α dehydrogenase, oestradiol-17 β dehydrogenase, epitestosterone dehydrogenase and testosterone dehydrogenase activities (Fig. 1) as well as protein. These results were identical with those reported for the oestradiol-17 β dehydrogenase of chicken liver (Renwick *et al.*, 1981), although the mode of preparation of that enzyme was substantially different and it was examined in discontinuous systems in 4.8 and 7.5% gels, pH 8.3–8.4, whereas 5% gels were used in the present experiments at pH 8.8.

Peak D was resolved into six bands, five of which were present in the blank that contained no steroid substrate. The bands were probably caused by an NADP $^+$:polyol oxidoreductase of the sort reported by Velle & Engel (1964).

Slab gel electrophoresis of fractions containing enzyme activity gave the following results. Peak A, after affinity chromatography on adenosine 2',5'-bisphosphate-agarose, showed only one band (R_m 0.06) in a non-SDS-containing system, in contrast with electrophoresis in an SDS-containing system, where major bands were detected at M_r 69000, 50000, 31000, 23000, 19000 and 14000. Those at M_r 23000 and 19000 were most prominent. Peak D gave two bands (R_m 0.17 and 0.25) in a non-SDS-containing system, whereas SDS/polyacrylamide-gel electrophoresis revealed bands at M_r 66000, 42000, 35000, 23000, 19000 and 14000.

The different results obtained by rod and slab gel electrophoresis probably reflect the different percentages of gel employed (5% in the former and 10% in the latter) and the better resolving power of the slab system.

Table 3. *Substrate specificity of oestradiol-17 α dehydrogenase and oestradiol-17 β dehydrogenase activities*

The substrate specificity of the oestradiol-17 α dehydrogenase and oestradiol-17 β dehydrogenase activities was examined with the limited range and quantities of chromatographically pure substrates available and samples of enzyme present in peak A after DEAE-cellulose chromatography. All assays were carried out at 37°C in duplicate (the recordings were virtually identical) against controls without steroid. Mean values are given. The rates given are relative to that with oestradiol-17 β .

Substrate	Concentration (μ M)	Relative rate of oxidation (oestradiol-17 β = 100)
5 α -Androstane-3 β ,17 α -diol	57	49
5 β -Androstane-3 α ,17 α -diol	57	0
Epitestosterone	59	60
Testosterone	59	27
Oestradiol-17 α	61	41
Oestriol	67	0
17 α -Dihydroequilin	67	0
17 α -Dihydroequilenin	67	0

Fig. 1. Polyacrylamide rod gel electrophoresis of native enzyme preparations

Samples of peak A material obtained after chromatography on adenosine 2',5'-bisphosphate-agarose were examined by rod gel electrophoresis in 5% (w/v) gels at pH 8.8 and were stained for oestradiol-17 α dehydrogenase, oestradiol-17 β dehydrogenase, testosterone dehydrogenase and epitestosterone dehydrogenase activity as previously described (Renwick *et al.*, 1981). The designations α , β , e and t respectively refer to the substrates oestradiol-17 α , oestradiol-17 β , epitestosterone and testosterone used in these experiments. No activity was detected in controls where NADP⁺ or steroid substrate was omitted.

Isoelectric focusing

Isoelectric focusing of peak A material after anion-exchange chromatography revealed four peaks that were enzymatically active. All four peaks oxidized oestradiol-17 α and -17 β . The isoelectric points were pH 6.5, 6.3, 6.2 and 6.1, compared with 6.9, 6.8 and 6.0 obtained by isoelectric focusing in gel of the oestradiol-17 β dehydrogenase that was prepared and examined under different conditions (Renwick *et al.*, 1981). These findings recall earlier work by Hasnain & Williamson (1974, 1975), who reported the incom-

plete separation of the soluble oestradiol-17 α dehydrogenase and oestradiol-17 β dehydrogenase of rabbit liver and subsequently described the purification and properties of multiple forms of the enzyme from that tissue (Hasnain & Williamson, 1977), although they did not report reactivity towards oestradiol-17 β in the last-mentioned publication.

General comments

Henderson & Warren (1984) have described the purification and characterization of an oestradiol-17 α dehydrogenase and an oestradiol-17 β dehydrogenase from the microsomal fraction of horse placenta, where more-detailed investigation of their substrate specificities may show that the preferred substrates are ring-B-unsaturated oestrogens. In our experiments we have been unable to resolve the oestradiol dehydrogenase activities of chicken liver into constitutive enzymes despite the application of a wide range of separation methods. But it is possible, even if two discrete proteins exist, that their closely related structures precluded resolution by the methods employed. Preliminary kinetic evidence obtained by using the quantitative mixed-substrate procedure supports the idea of the existence of two separate enzymes. In contrast, all the results from chromatographic, electrophoretic and isoelectric focusing experiments favour the view that both activities are properties of the same protein, and it is probable that related hydroxysteroid dehydrogenase functions reflect the substrate specificity of the enzyme, although this has yet to be proved. The resolving power of high-pressure liquid chromatography may answer the question if the enzyme activities are stable to the analytical conditions.

If separate enzymes do not exist, how can one explain the oxidation of the oestradiol epimers in this tissue? One possible transformation is an epimerization by way of an enzyme-bound 16,17-enediol, without the formation of oestrone, but there is no evidence for such a mechanism, and it has been shown irrefutably that oestrone is the common product of the enzyme-catalysed oxidation of both oestradiols (Renwick & Engel, 1967).

The third possibility is the concept of dual enzyme activity at a single active site. There are precedents. Examples of the phenomenon in steroid-transforming enzymes have been discussed with particular reference to the oestradiol-17 β dehydrogenase and 20 α -hydroxysteroid dehydrogenase activities of the human placental oestradiol dehydrogenase (Tobias *et al.*, 1982), and Lau *et al.* (1982a,b) have reported the purification and characterization of multiple forms of a 3(17) α -hydroxysteroid dehydrogenase from female rabbit kidney and compared these activities with those

from rabbit liver. However, the existence of a hydroxysteroid dehydrogenase that reversibly oxidizes stereoisomeric substrates to a common product is considerably more intriguing, especially with regard to the nature of the active site.

Engel (1966) postulated that the best model for the active site of the human placental oestradiol-17 β dehydrogenase that accounted for all the available data was a hydrophobic pocket for the steroid substrate in the enzyme protein. Subsequently Descompes & Crastes de Paulet (1969) proposed a hypothetical binding site that envisaged a slot in the enzyme in which the steroid substrate could enter from one side and cofactor from the other. Substrate and cofactor were so positioned that the 17 α -hydrogen of the steroid became the 4-pro-*S*-hydrogen of the reduced cofactor. This model is consistent with a Random Bi Bi mechanism for the reaction proposed by Betz (1971) on the basis of equilibrium rate exchange, but it is more difficult to reconcile with the present situation, where one is considering the reversible transformation of two epimers and a common product.

George *et al.* (1973) have argued that, if a close fit of oxidized cofactor with steroid alcohol is of over-riding importance in the enzymic transfer of hydrogen in the enzyme-catalysed oxidation of the epimeric oestradiols, then they should interact with opposite sides of the nicotinamide ring, but they do not; the oestradiol-17 α dehydrogenase and oestradiol-17 β hydrogenase activities of chicken liver transfer the 4-pro-*S*-hydrogen to NADP⁺. Because George *et al.* (1973) believed that two separate enzymes were involved they concluded that one was 'just a little clumsy' in selecting an awkward mode of catalysis. If both reactions occur at the same active site then the mechanism warrants closer inspection.

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References

- Betz, G. (1971) *J. Biol. Chem.* **240**, 2063–2068
- Brown, J. B. (1955) *Biochem. J.* **60**, 185–193
- Cuatrecasas, P. (1970) *J. Biol. Chem.* **245**, 3059–3065
- Descompes, B. & Crastes de Paulet, A. (1969) *Bull. Soc. Chim. Biol.* **51**, 1591–1611
- Dixon, M. & Webb, E. C. (1979) *Enzymes*, 3rd edn., pp. 72–75, Longman, London
- Engel, L. L. (1966) *Proc. Int. Congr. Hormonal Steroids 2nd (Int. Congr. Ser. Excerpta Med. no. 132)*, pp. 52–59
- Engel, L. L. & Groman, E. V. (1974) *Recent Prog. Horm. Res.* **30**, 139–169
- George, J. M., Orr, J. C., Renwick, A. G. C., Carter, P. & Engel, L. L. (1973) *Bio-org. Chem.* **2**, 140–144
- Hasnain, S. & Williamson, D. G. (1974) *Can. J. Biochem.* **52**, 120–125
- Hasnain, S. & Williamson, D. G. (1975) *Biochem. J.* **147**, 457–461
- Hasnain, S. & Williamson, D. G. (1977) *Biochem. J.* **161**, 279–283
- Henderson, L. L. & Warren, J. C. (1984) *Biochemistry* **23**, 486–491
- Houslay, M. D., Garrett, N. J. & Tipton, K. F. (1974) *Biochem. Pharmacol.* **23**, 1937–1944
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Langer, L. J., Alexander, J. A. & Engel, L. L. (1959) *J. Biol. Chem.* **234**, 2609–2614
- Lau, P. C. K., Layne, D. S. & Williamson, D. G. (1982a) *J. Biol. Chem.* **257**, 9444–9449
- Lau, P. C. K., Layne, D. S. & Williamson, D. G. (1982b) *J. Biol. Chem.* **257**, 9450–9456
- Lineweaver, H. & Burk, D. J. (1934) *J. Am. Chem. Soc.* **56**, 658–666
- Renwick, A. G. C. & Engel, L. L. (1967) *Biochim. Biophys. Acta* **146**, 336–348
- Renwick, A. G. C., Chambers, S. M. & Willcox, P. (1979) *Biochem. J.* **177**, 401–408
- Renwick, A. G. C., Choong, Y. S., Chambers, S. M. & Brown, C. R. (1981) *J. Biol. Chem.* **256**, 1881–1887
- Tobias, B., Covey, D. F. & Strickler, R. C. (1982) *J. Biol. Chem.* **257**, 2783–2786
- Travis, J., Bowen, J., Tewksbury, D., Johnson, D. & Pannell, R. (1976) *Biochem. J.* **157**, 301–306
- Velle, W. & Engel, L. L. (1964) *Endocrinology (Baltimore)* **74**, 429–439