Influence of an 18-Hydroxyl Group on the Interaction of Oestrogens and Hydroxysteroid Oxidoreductases

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1. Partially purified 17 β -hydroxy steroid-NAD⁺ oxidoreductases, prepared from *Pseudomonas testosteroni* (EC 1.1.1.51), human term placenta (EC 1.1.1.62) and the cytoplasmic fraction of rat liver (EC 1.1.1.–) were tested for their ability to catalyse the oxidoreduction of 18-hydroxyoestradiol-17 β and 18-hydroxyoestrone. The products of incubation were identified by chromatographic procedures and by mass spectrometry. 2. The *Pseudomonas* enzyme catalysed both the oxidation of 18-hydroxyoestradiol-17 β and the reduction of 18-hydroxyoestrone; in contrast, the placental and rat liver enzymes only catalysed the reduction of 18-hydroxyoestrone. 3. These results were confirmed, by using a spectrophotometric assay; equimolar quantities of oestradiol-17 β and 18-hydroxyoestradiol-17 β were oxidized at approximately the same rate by the microbial enzyme. 4. These findings suggest that 18-hydroxyoestradiol-17 β may be a normal oestrogen metabolite. 5. The differences in ability of the mammalian and microbial enzymes to metabolize 18-hydroxylated oestrogens is explained on the basis of recognition sites with different geometrical dimensions, characteristic of the placental (Descomps & Crastes de Paulet, 1969) and the microbial steroid enzymes (Fosset & Crastes de Paulet, 1967).

The chemical properties of 18-hydroxyoestrone differ from those of oestrone by its reaction to strong alkali in forming 18-noroestrone and by the ability of its 18-hydroxyl group to influence reactions at C-17. It was found that 18-hydroxyoestrone, when reduced with NaBH₄, yielded both 18-hydroxyoestradiol-17 β (70%) and 18-hydroxyoestradiol-17 α (30%) (Findlay *et al.* 1974). Under similar conditions, oestrone is reduced almost exclusively to oestradiol-17 β . The formation of relatively more of the 17 α -epimer in the presence of an 18-hydroxyl group by chemical reduction was not found when 18-hydroxyoestrone was incubated with rat or rabbit liver slices (Findlay *et al.* 1974), despite the presence of an active 17 α -hydroxy steroid oxidoreductase in rabbit liver (Ball & Breuer, 1970). Only 18-hydroxyoestradiol-17 β was identified as a metabolite. In view of the importance of oxidoreduction reactions in steroid metabolism, it was decided to investigate whether 18-hydroxyoestrone and 18-hydroxyoestradiol-17 β could act as substrates for other 17 β -hydroxy steroid oxidoreductases. For this purpose, partially purified enzymes were prepared from *Pseudomonas testosteroni*, human term placenta and rat liver, and tested for their ability to metabolize these oestrogens. This paper presents the results of these investigations, together with an account of the preliminary purification of a soluble 17 β -hydroxy steroid-NAD(P)⁺ oxidoreductase from rat liver.

Experimental

Materials

Buffers. Tris (0.045M) and phosphate buffers were prepared as described by Gomori (1955). Sodium pyrophosphate buffer (0.167M) was prepared by dissolving 74g of sodium pyrophosphate in 1 litre of water and adjusting the pH to 9.5 by the addition of 0.167M-HCl. The buffers used in the partial purification of human placental 17 β -hydroxy steroid oxidoreductase were prepared by the method of Jarabak *et al.* (1962). They were: medium A, 10mM-potassium phosphate–20% (v/v) glycerol–1mM-EDTA–7mM- β -mercaptoethanol (final pH 7.0); medium B, 10mM-potassium phosphate–50% glycerol–5mM-EDTA–7mM- β -mercaptoethanol (final pH 7.0). All pH values were checked with a Corning model pH-meter after the required dilutions.

Chemicals. Oestrone, oestradiol-17 β and 17-epioestrinol [1,3,5(10)-oestratriene-3,16 β ,17 β -triol] were the recrystallized products of commercial preparations, purchased from Schering A.G., Berlin, Germany. 18-Hydroxyoestrone [3,18-dihydroxy-1,3,5(10)-oestratrien-17-one] was generously given by Professor Sir D. H. R. Barton (preparation by the method of Baldwin *et al.*, 1968). 18-Hydroxyoestradiol-17 α [1,3,5(10)-oestratriene-3,17 α ,18-triol] and 18-hydroxyoestradiol-17 β [1,3,5(10)-oestratriene-3,17 β ,18-triol] were prepared by reduction of 18-hydroxyoestrone with NaBH₄ in methanol and purified in paper- and thin-layer-chromatographic systems as described previously (Findlay *et al.*

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1974). [4-¹⁴C]Oestradiol-17 β (specific radioactivity 50mCi/mmol) was purchased from New England Nuclear Corp., Boston, Mass., U.S.A., and purified by chromatography in a Bush B₁ paper-chromatographic system (Bush, 1952).

Dry bovine serum albumin was purchased from Behringwerke A.G., Marburg/Lahn, Germany. The nicotinamidenucleotides were supplied by Boehringer, Mannheim, Germany. All other reagents were the products of E. Merck A.G., Darmstadt, Germany.

The scintillation fluid contained 0.25g of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 4g of 2,5-diphenyloxazole in 1 litre of dry sulphur-free toluene.

Methods

Human term placentae were obtained immediately after delivery and chilled on ice during transport. The villi were rapidly dissected away from major vessels, connective tissue and foetal membranes ready for homogenization. The microbial enzyme, 3(or 17) β -hydroxy steroid-NAD(P)⁺ oxidoreductase (EC 1.1.1.51) from *Ps. testosteronei*, was a commercial preparation purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Liver was obtained from male Sprague-Dawley rats which had been starved the night before use. They had previously been maintained on a commercial diet (cf. Findlay *et al.*, 1974).

Determination of protein. Protein was determined by the method of Lowry *et al.* (1951). Protein concentrations were expressed in mg/ml and were read from a standard curve, obtained with bovine serum albumin.

(NH₄)₂SO₄ precipitation. Solid (NH₄)₂SO₄ was added to the cytosol fractions to the required saturation (Green & Hughes, 1955) and the pH values maintained at about 7.3 by the addition of 0.1M-NH₃; the mixtures were stirred for 15min at 2°C and then centrifuged at 12000g_{av.} for 10min.

Enzyme preparations. (a) *Ps. testosteronei*. A cell-free supernatant of the dried cells was prepared by the method of Ainsworth & Ryan (1970). Dried cells (50mg) were ground in a mortar with 50mg of alumina (II-III grade; Merck) and 5.0ml of ice-cold Tris buffer (0.045M; pH7.2) containing 1mM-EDTA. The resultant suspension was centrifuged at 2600g_{av.} for 15min at 2°C; the supernatant was retained, made up to 10ml with Tris buffer and then stored at 2°C. The protein concentration was 0.24mg/ml. No losses in activity were detected over a period of 3 months.

(b) Human placenta. The preparation of 17 β -hydroxy steroid-NAD(P)⁺ oxidoreductase from human placenta involved an (NH₄)₂SO₄ fraction of the cytosol prepared as described by Jarabak *et al.* (1962). A portion (75g) of villus tissue was homogenized with 150ml of medium A for 15s in a 1000ml cup of the Waring blender at 2-5°C. The homogenate was centrifuged for 30min at 10000g_{av.}, the super-

natant decanted and the residue discarded. (NH₄)₂SO₄ was added to the cytosol to a saturation of 50%. After centrifugation, the supernatant was discarded and the precipitate was dissolved in medium B such that 0.3ml was equivalent to 1g of tissue, giving a protein concentration of 38.5mg/ml. The enzyme was stored in this medium at 2°C without loss of activity.

(c) Rat liver. Liver tissue was freed from connective tissue and fat and washed in ice-cold 0.25M-sucrose. The tissue was chopped with fine scissors in 0.25M-sucrose (10%, w/v; pH7.2) and then homogenized by 20 strokes of a cell homogenizer (Colora Messtechnik, Lorch/Württemberg, Germany) at 10 strokes/min. The homogenate was centrifuged for 20min at 23000g_{av.} in a refrigerated centrifuge. The supernatant obtained was centrifuged in a Beckman-Spinco model L-2 centrifuge for 1h at 105500g_{av.}. The supernatant fraction thus obtained was transferred to fresh centrifuge tubes and re-centrifuged for 1h at 105000g_{av.}. Some of the thin yellow liquid layer, floating on top of the centrifuge tubes, was removed with a Pasteur pipette before the cytosol fraction was subjected to (NH₄)₂SO₄ fractionation. The precipitates formed after saturation with (NH₄)₂SO₄ were collected after centrifugation and redissolved in a minimum quantity (2-3ml) of sodium pyrophosphate buffer (0.017M; pH9.5). These sediments were then dialysed against 5 litres of the same buffer for approx. 16h at 2°C. Further precipitation occurred in the 40-80%-satd. (NH₄)₂SO₄ fraction during dialysis; the precipitate was removed by centrifuging for 10min at 12000g_{av.}. The dialysed fractions and portions of the cytosol fraction were stored in convenient quantities at 2°C and at -18°C.

Assay of 17 β -hydroxy steroid oxidoreductase activity in rat liver during preparation. The rate of oxidation of [4-¹⁴C]oestradiol-17 β by preparations of the rat liver enzyme were assayed in duplicate. Unlabelled oestradiol-17 β was added to the labelled substrate to give 94000d.p.m., equivalent to 36.8nmol in 0.1ml of ethanol. To 0.1ml of this solution propylene glycol (two drops) was added, and the ethanol was evaporated off under N₂ in a water bath at 40°C. To the residue was added 2.0ml of sodium pyrophosphate buffer (0.017M; pH9.5) and 0.2ml of NAD⁺ (3 μ mol). The contents were preincubated for 1-2min at 37°C before addition of 20-50 μ l (100-400 μ g of protein) portions of the enzyme preparation. Incubations were carried out for 1h at 37°C in air. The contents were extracted with 2 $\times$ 2vol. of freshly distilled ether or dichloromethane; the pooled solvent extract was washed with water (2 $\times$ 2ml) and then evaporated to dryness under N₂ or *in vacuo* at 40°C. The residues were chromatographed on silica gel G in chloroform-acetone (9:1, v/v) with standards in neighbouring lanes. Standard oestrone (*R_F* value, $\pm$ s.d., 0.44 $\pm$ 0.03; *n* = 6) and oestradiol-17 β (*R_F* value, $\pm$ s.d., 0.29 $\pm$ 0.03;

$n = 6$) were located with Folin-Ciocalteu reagent (Mitchell & Davies, 1954). No other metabolites were found. The areas of gel, corresponding to oestrone and oestradiol-17 β , were carefully transferred to counting vials containing 0.3 ml of methanol, and left for 15 min. Then 12 ml of scintillation fluid was added, the contents were well shaken and the vials were counted for radioactivity in a Nuclear-Chicago mark II liquid-scintillation spectrometer. The activity of the enzyme (in nmol/h) was calculated from the relationship:

$$\frac{a \times 36.8}{b}$$

where a is d.p.m. in the 'oestrone' area, b is d.p.m. in the oestradiol-17 β area and 36.8 denotes the nmol of substrate used. Total recoveries of incubated radioactivity averaged $72.2\% \pm 4.7$ ($n = 40$). One unit of enzyme activity was defined as the amount necessary to catalyse the oxidation of 1 nmol of oestradiol-17 β /h under standard assay conditions. Specific activity was defined as units/mg of protein.

Spectrophotometric assay for 17 β -hydroxy steroid oxidoreductase activity in preparations from *Ps. testosteroni* and human term placenta. The method described by Jarabak *et al.* (1962) was used. Spectrophotometric assays were carried out in quartz cuvettes of 1.0 cm light-path in a Beckman DB spectrophotometer, in which the cell compartment was maintained at a temperature of $30 \pm 0.5^\circ\text{C}$. The reaction cuvettes contained, in a final volume of 3.13 ml, 440 μmol of sodium pyrophosphate buffer, pH 9.5, 25 mg of bovine serum albumin [0.5 ml of a 5% (w/v) solution], 0.15 μmol of steroid substrate in 0.025 ml of 95% (v/v) ethanol, 3.0 μmol of NAD $^+$ and the appropriate quantities of enzyme. The final pH was 9.5. Measurements at 340 nm were taken against a blank cuvette, from which the steroid substrate was omitted. Control assays showed that the reaction was strictly dependent on both nicotinamide nucleotide and steroid. Readings were taken every 30 s for 5–10 min after addition of the enzyme.

Assays for the oxidation or reduction of 18-hydroxylated oestrogens by different preparations of 17 β -hydroxy steroid oxidoreductase activity. Duplicate assays were performed as follows.

(a) Reduction: the steroid ketone (40 μg) was dissolved in two drops of methanol in a Kober tube, to which was then added 2.0 ml of phosphate buffer (0.1 M; pH 6.2), 0.2 ml of nicotinamide (4.9 μmol), 0.2 ml of NADH (1.2 μmol) and 0.1–0.2 ml of enzyme preparation. Incubations were carried out at 40°C for 90 min in air. The contents were extracted twice with 5 ml of freshly distilled ether, and the ether extracts were washed with water (2×1 ml), before being evaporated to dryness under N_2 .

(b) Oxidation: the same procedure as (a) was used to assay the oxidation of the steroid alcohols, except

that phosphate buffer and NADH were replaced by sodium pyrophosphate buffer (0.167 M; pH 9.5) and NAD $^+$ (1.2 μmol) respectively).

The residues of the ether extracts were chromatographed on formamide-impregnated paper (Schleicher und Schüll 2043b), developed in monochlorobenzene-ethyl acetate (3:1, v/v). Neighbouring lanes contained test substances which were located by Folin-Ciocalteu reagent (Mitchell & Davies, 1954). After drying, the paper strips were eluted with methanol and the oestrogens measured by u.v. spectroscopy in ethanol at 280 nm against the equivalent blanks (cf. Findlay *et al.*, 1974).

Further identification of the 18-hydroxylated oestrogens was carried out as described by Findlay & Breuer (1972), by t.l.c. and g.l.c. and finally by mass spectrometry.

Results

17 β -Hydroxy steroid oxidoreductase activity in rat liver

(NH_4) $_2\text{SO}_4$ fractionation. Fractionation of preparation I (Table 1) revealed a distribution of activity between the 30–60% and the 60–90%-satn. sediments of the cytosol, with a higher specific activity in the latter fraction. In subsequent fractionations, approx. 60% of the total activity of the cytosol was present in the sediment formed at 40–80% saturation, but the specific activity was only 1.4-fold above that in the cytosol. The dialysed 40–80%-satn. sediment was used in studies with 18-hydroxylated oestrogens on the day of its preparation.

Adsorption by calcium phosphate gel and stability of the activity. Preliminary studies indicated that the activity was removed from solution by the addition of activated calcium phosphate gel at a concentration of 2 mg of gel/mg of protein. In a subsequent attempt, a portion (37.2 mg of protein) of the 40–80%-satn. fraction (preparation III) was subjected to calcium phosphate adsorption; no significant increase in specific activity was achieved (Table 1).

The activity of the various preparations was assayed at various time-intervals after keeping at 2°C and at -18°C . A decline in the specific activity of the cytosol preparation was noted after 24 h at 2°C (Table 2). After 5 days, approx. 50% of the initial activity remained. Storage at -18°C appeared to decrease the rate of inactivation of the (NH_4) $_2\text{SO}_4$ preparations.

Cofactor specificity. The activity in the 40–80%-satn. fraction was found to be NAD $^+$ -dependent under the assay conditions used. In the absence of cofactor, the oxidation of [4- ^{14}C]oestradiol-17 β was barely detectable, whereas NADP $^+$ produced only 55% of the activity found with NAD $^+$. The presence of both cofactors did not significantly increase the specific activity above that found with NAD $^+$ alone.

Table 1. *Purification of a 17 β -hydroxy steroid oxidoreductase from the cytosol of rat liver*

The enzyme preparations were incubated with [4-¹⁴C]oestradiol-17 β as described in the Experimental section and the formation of [4-¹⁴C]oestrone was measured. Further details are given in the text.

Preparation	Fraction	Total protein (mg)	Total activity (units)	Yield (%)	Specific activity (units/mg of protein)
I	105 500 _g _{av.} supernatant	307	4310	100	14.1
	(NH ₄) ₂ SO ₄ precipitates:				
	0–30%-satn.	16.8	74	1.7	4.4
	30–60%-satn.	106	1240	29	11.7
	60–90%-satn.	56.8	1060	24	18.7
II	105 500 _g _{av.} supernatant	387	4690	100	11.8
	(NH ₄) ₂ SO ₄ precipitates:				
	0–40%-satn.	78.4	436	9	5.5
	40–80%-satn.	174	2970	63	17.3
III	105 500 _g _{av.} supernatant	330	4160	100	12.6
	(NH ₄) ₂ SO ₄ precipitate:				
	0–40%-satn.	17.3	249	6	3.6
	40–80%-satn.	175	2660	56	15.2
	Ca ₃ (PO ₄) ₂ adsorption:				
	Starting material	37.6	572	100	15.2
	Supernatant after adsorption	26.2	332	58	12.7
	Ca ₃ (PO ₄) ₂ gel:				
	Wash I with 0.017 M-sodium pyrophosphate buffer (pH 9.5)	6.4	110	19	17.1
	Wash II with 0.167 M-sodium pyrophosphate buffer (pH 9.5)	3.0	28	5	9.2

Table 2. *Stability of various preparations of 17 β -hydroxy steroid oxidoreductase activity from rat liver*

The enzyme preparations were incubated with [4-¹⁴C]oestradiol-17 β as described in the Experimental section and the formation of [4-¹⁴C]oestrone was measured. Further details are given in the text.

Preparation	Fraction	Specific activity (units/mg of protein)				
		Days at 2°C				Days at –18°C
		0	1	5	18	
I	105 500 _g _{av.} supernatant	18.2	14.1	9.4	8.9	10.2
	30–60%-satn.-(NH ₄) ₂ SO ₄ precipitate	—	11.7	5.9	5.6	7.9
	60–90%-satn.-(NH ₄) ₂ SO ₄ precipitate	—	18.7	11.3	9.4	10.2
II	40–80%-satn.-(NH ₄) ₂ SO ₄ precipitate	—	17.3	—	—	8.1

Reaction of the 18-hydroxylated oestrogens with the enzyme preparations

It was found that NAD-dependent 17 β -hydroxy steroid oxidoreductase from *Ps. testosteroni* catalysed both the oxidation of 18-hydroxyoestradiol-17 β and the reduction of 18-hydroxyoestrone (Table 3). The preparations from human term placenta and rat liver catalysed the reduction of 18-hydroxyoestrone but not the oxidation of 18-hydroxyoestradiol-17 β . All the enzyme preparations oxidized oestradiol-17 β and reduced oestrone. No other products, such as hydroxylated derivatives or 18-noroestrone, were observed in experiments with 18-hydroxylated oestro-

gens and the enzyme preparations. As expected, 18-hydroxyoestradiol-17 α was not oxidized by the microbial enzyme.

The transformation of the substrates indicated that, when the reactions took place, the extent of transformation of 18-hydroxylated substrates was approximately equal to that for oestrone or oestradiol-17 β for each enzyme preparation (Table 3).

Further confirmation of these results was obtained by using the spectrophotometric assay (Fig. 1). Equimolar quantities of both oestradiol-17 β and 18-hydroxyoestradiol-17 β were oxidized at approximately the same rates by the microbial enzyme. In

Table 3. Reaction of 18-hydroxylated oestrogens with 17 β -hydroxy steroid oxidoreductases prepared from various sources

Portions of enzyme protein (*Ps. testosteroni*, 48 μ g; placenta, 770 μ g; rat liver, 2300 μ g) were incubated with 40 μ g of oestrogen and 4.9 μ mol of nicotinamide together with either 0.1 M-phosphate buffer, pH 6.0, containing 1.2 μ mol of NADH for reduction, or with 0.167 M-sodium pyrophosphate buffer, pH 9.5, containing 1.2 μ mol of NAD⁺ for oxidation; total volume 2.6 ml. Incubations in duplicate were carried out at 40°C for 90 min in air. The oestrogens were assayed by u.v. spectroscopy after initial chromatography. '% Formation' was calculated on the basis of the total amount of steroid recovered. Further details are given in the text.

Source of enzyme	Substrate	Identified product	% Formation
<i>Ps. testosteroni</i>	18-Hydroxyoestrone	18-Hydroxyoestradiol-17 β	70
	18-Hydroxyoestradiol-17 β	18-Hydroxyoestrone	75
	18-Hydroxyoestradiol-17 α	None	—
	Oestrone	Oestradiol-17 β	55
	Oestradiol	Oestrone	70
Human term placenta	18-Hydroxyoestrone	18-Hydroxyoestradiol-17 β	70
	18-Hydroxyoestradiol-17 β	None	—
	Oestrone	Oestradiol-17 β	65
	Oestradiol-17 β	Oestrone	90
Rat liver	18-Hydroxyoestrone	18-Hydroxyoestradiol-17 β	10
	18-Hydroxyoestradiol-17 β	None	—
	Oestrone	Oestradiol-17 β	15
	Oestradiol-17 β	Oestrone	55

Fig. 1. Spectrophotometric assay of 17 β -hydroxy steroid oxidoreductase activity

(a) Portions (24 μ g of protein) of an enzyme preparation from *Ps. testosteroni* and (b) portions (400 μ g of protein) of an enzyme preparation from human placenta were assayed with oestradiol-17 β (○) or 18-hydroxyoestradiol-17 β (Δ) as substrates. The incubation mixture consisted of 440 μ mol of sodium pyrophosphate buffer, pH 9.5, 25 mg of bovine serum albumin, 3.0 μ mol of NAD⁺, 0.15 μ mol of substrate and the enzyme, total volume 3.13 ml, final pH 9.5. Each value represents a mean of duplicate determinations.

contrast, NADH production was detected when oestradiol-17 β was the steroid substrate for the placental enzyme, but not with 18-hydroxyoestradiol-17 β as substrate. It was also found that 16-epi-oestradiol did

not act as steroid substrate for the microbial enzyme or for the placental enzyme in this system. The enzyme preparation from rat liver had too little activity for this assay.

Discussion

17 β -Hydroxy steroid-NAD(P)⁺ oxidoreductase activity in rat liver

The cytosol (105 500g_{av}. supernatant) fraction of rat liver was found to contain a 17 β -hydroxy steroid oxidoreductase activity, catalysing the oxidoreduction of oestradiol-17 β and oestrone. This activity was precipitated by (NH₄)₂SO₄ (40–80% satn.) and proved to be NAD⁺- rather than NADP⁺-dependent. Attempts to further purify the enzyme by calcium phosphate gel adsorption did not succeed in increasing the specific activity.

Earlier unpublished observations (C. Mittermayer & H. Breuer) also demonstrated that the major portion of soluble 17 β -hydroxy-C₁₈-steroid oxidoreductase from rat liver was precipitated at 40–80% satn. of (NH₄)₂SO₄. In contrast with the oestrogen-metabolizing enzyme, the 17 β -hydroxy-C₁₉ steroid-NAD⁺ oxidoreductase catalysing testosterone oxidation was located almost exclusively in the microsomal fraction of rat liver, whereas the NADP-dependent activity was in the soluble fraction (Aoshima & Kochakian, 1963). It is possible that different oxidases exist for oestrogens and androgens, although this may depend on the species; thus soluble and microsomal 17 β -hydroxy steroid-NAD⁺ oxidoreductases of human liver appear to oxidize both oestradiol-17 β and testosterone without any substrate specificity (Littmann *et al.*, 1971).

Interaction of 18-hydroxylated oestrogens and hydroxy steroid oxidoreductases

The microbial (*Ps. testosteroni*) and mammalian (human placenta and rat liver) 17 β -hydroxy steroid oxidoreductase activities differed with respect to their ability to oxidize 18-hydroxyoestradiol-17 β . The microbial enzyme oxidized oestradiol-17 β and 18-hydroxyoestradiol-17 β at approximately the same rate, whereas the mammalian enzymes only oxidized oestradiol-17 β . On the other hand, all the partially purified enzyme preparations were capable of reducing 18-hydroxyoestrone to 18-hydroxyoestradiol-17 β . These results suggest differences between the geometry of the active sites of the microbial and mammalian enzymes.

Studies have been undertaken to define the geometrical dimensions of the 'recognition site' of 17 β -hydroxy steroid oxidoreductase from *Ps. testosteroni* (Fosset & Crastes de Paulet, 1967) and human placenta (Descomps & Crastes de Paulet, 1969) by measuring K_m and V_{max} values of many steroid substrates for the purified enzymes. As a result of their studies, these authors propose recognition sites in the enzyme proteins with different geometrical dimensions characteristic for the placental and microbial enzymes. There are distinguishing differences be-

tween the dimensions of these sites (Figs. 2 and 3); however, of greater interest was the finding that substitution on the β face of the steroid molecule did not restrict its interaction with and metabolism by the microbial enzyme (Fosset & Crastes de Paulet, 1967). In contrast, substitution on the β face resulted in decreased binding with and metabolism by the placental enzyme (Descomps & Crastes de Paulet, 1969). Both enzyme activities were restricted by α substituents on the substrate molecule. Access of the steroid to the microbial enzyme is believed to occur via the α face, whereas a lateral shift in the plane of the molecule is thought to occur during access to the recognition site of the placental enzyme.

The addition of a hydroxyl group at C-18 is a substitution on the β face of the oestrogen molecule. It

Fig. 2. A possible geometrical shape for the recognition site from the 17 β -hydroxy steroid oxidoreductase of *P. testosteroni*

The catalytic site is denoted by the position of the cofactor. The drawing is adapted from the two-dimensional drawings of Fosset & Crastes de Paulet (1967).

Fig. 3. A possible geometrical shape of the recognition site from the 17 β -hydroxy steroid oxidoreductase of human placenta

The catalytic site is denoted by the position of the cofactor. The drawing is adapted from the two-dimensional drawings of Descomps & Crastes de Paulet (1969).

follows therefore that both 18-hydroxyoestrone and 18-hydroxyoestradiol-17 β can be metabolized by the microbial 17 β -hydroxy steroid oxidoreductase. However, this substitution restricts access of the substrate to the site of the placental enzyme in the presence of a 17 β -hydroxyl group (18-hydroxyoestradiol-17 β) but not in the presence of a 17-oxo group (18-hydroxyoestrone). In the presence of a 17 β -hydroxyl group, slight changes in the positions of the 17 β - and 18-hydroxyl groups may occur as a result of the formation of a hydrogen bridge (Findlay *et al.*, 1974); this could be sufficient to increase the dimensions of the β face of the molecule beyond those acceptable by the recognition site of the placental enzyme. It appears that reduction of 18-hydroxyoestrone to 18-hydroxyoestradiol-17 β is not hindered by the product.

Details of the recognition site of the rat liver enzyme await purification of the protein and a study of its kinetics; the results in the present paper indicate that the properties of its recognition site are similar to the placental enzyme site with respect to the influence of the 18-hydroxyl group on substrate-enzyme interaction.

The inability of placental 17 β -hydroxy steroid oxidoreductase to catalyse the oxidation of 18-hydroxyoestradiol-17 β , together with its ability to reduce 18-hydroxyoestrone, raise the possibility of finding 18-hydroxyoestradiol-17 β as an endogenous metabolite, particularly as 18-hydroxyoestrone has already been isolated from human pregnancy urine (Loke *et al.*, 1959).

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