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Characterization and localization of progesterone 5α-reductase from cell cultures of foxglove (*Digitalis lanata* EHRH)*

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Progesterone 5α-reductase, which catalyses the reduction of progesterone to 5α-pregnane-3,20-dione, was isolated and characterized from cell cultures of *Digitalis lanata* (foxglove). Optimum enzyme activity was observed at pH 7.0, and the enzyme had an apparent K_m value of 30 μM for its substrate progesterone. The enzyme needs NADPH as reductant, which could not be replaced by NADH. For NADPH, the apparent K_m value is 130 μM. The optimum temperature was 40 °C; at temperatures below 45 °C, the product 5α-pregnane-3,20-dione was reduced by a second reaction to 5α-pregnan-3β-ol-20-one. Progesterone 5α-reductase activity was not dependent on bivalent cations. In the presence of EDTA, 0.1 mM-Mn^{2+} had no influence on enzyme activity, whereas 0.1 mM-Ca^{2+}, -Co^{2+} and -Zn^{2+} decreased progesterone 5α-reductase activity. Only 0.1 mM-Mg^{2+} was slightly stimulatory. EDTA and thiol reagents such as dithiothreitol stimulate progesterone 5α-reductase activity. By means of linear sucrose gradient fractionation of the cellular membranes, progesterone 5α-reductase was found to be located in the endoplasmic reticulum.

INTRODUCTION

The biosynthesis of cardenolides has been investigated by several methods. After application of [^{14}C]-progesterone to the leaves of foxglove (*Digitalis lanata*), labelled compounds were isolated. In addition to a number of cardenolides, 5α-pregnane-3,20-dione, 5β-pregnane-3,20-dione, 5α-pregnan-3β-ol-20-one and Δ5-pregnen-3β-ol-20-one could be found (Bennett *et al.*, 1968). The importance of the 5α-pregnanes for cardenolide biosynthesis is not yet established, since no 5α-cardenolides have been found in *Digitalis lanata*. However, 5β-pregnane-3,20-dione and 5β-pregnan-3β-ol-20-one were converted into cardenolides after application to *Digitalis lanata* plants, but 5α-pregnan-3β-ol-20-one yielded neither cardenolides nor uzarigenin (Tschesche *et al.*, 1970). Leaf homogenates of *Cheiranthus cheiri*, *Digitalis purpurea*, *Strophanthus kombe* and *Corchorus olitorius* have been shown to reduce progesterone to 5α-pregnane-3,20-dione, and to small amounts of 5α-pregnan-3β-ol-20-one (Stohs & El-Olemy, 1972*a*). In these cases, no 5β-metabolites could be detected.

The biosynthetic pathway for cardenolides has also been studied using cell cultures from different plants. Graves & Smith (1967) first reported on the transformation of progesterone to 5α-pregnane-3,20-dione and 5α-pregnan-3β-ol-20-one by several suspension cultures including *Digitalis* species. The conversion of progesterone into 5α-pregnan-3β-ol-20-one and its palmitate ester has been demonstrated in suspension cultures of *Nicotiana tabacum* and *Sophora angustifolia* (Furuya *et al.*, 1971). *Dioscorea deltoidea* suspension cultures are capable of metabolizing progesterone to 5α-pregnan-3β-ol-20-one and 5α-pregnan-3β,20β-diol (Stohs & El-Olemy, 1972*b*). Furuya *et al.* (1973) examined the metabolism of progesterone in cell suspension cultures of *Digitalis purpurea*. They isolated 5α-pregnan-3β-ol-20-one, 5α-pregnane-3β,20α-diol, 5α-pregnane-3β,20β-diol, Δ4-pregnen-20α-ol-3-one, Δ4-pregnen-20β-ol-3-one and their corresponding glycosides, as well as 5α-pregnane-3,20-dione (Furuya *et al.*, 1973). Cell suspension cultures of *Digitalis purpurea* were also capable of metabolizing 5β-pregnane-3,20-dione and 5β-pregnan-3β-ol-20-one to several 5β-pregnane derivatives and their glycosides, but no 5α-derivatives or cardenolides could be detected (Hirotani & Furuya, 1975).

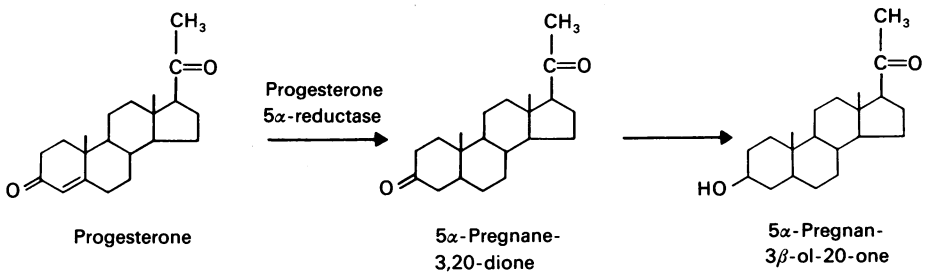

Fig. 1. Reaction catalysed by progesterone 5α-reductase

Abbreviation used: 3β-MSD, 3β-hydroxy-Δ5-steroid dehydrogenase.
* Dedicated to Professor G. Richter, on the occasion of his 60th birthday.

Microsomes isolated from *Cheiranthus cheiri* and *Dioscorea deltoidea* converted progesterone into a single metabolite, 5α-pregnane-3,20-dione, in the presence of an NADPH-regenerating system. The reaction has pH optimum at about 7 (Stohs, 1969).

Until now, nothing more has been known about the enzymes involved in the conversions described above. The progesterone 5α-reductase has now been detected in cell-free extracts of *Digitalis lanata* suspension cultures. This report describes the properties of the enzyme which catalyses the reduction of progesterone into 5α-pregnane-3,20-dione (Fig. 1).

MATERIALS AND METHODS

Cell suspension cultures

Suspension cultures of *Digitalis lanata* were cultivated as described by Petersen & Seitz (1985).

Chemicals

Progesterone, cholesterol, 5α-pregnane-3,20-dione, 5α-pregnan-3β-ol-20-one and NADPH were purchased from Sigma (Munich, Germany). Glucose 6-phosphate, glucose-6-phosphate dehydrogenase and $NADP^+$ were obtained from Boehringer (Mannheim, Germany).

Preparation of microsomal fractions

The whole procedure was performed at 0–4 °C. Cell suspensions were filtered under suction. Then the cells were homogenized in 1 ml of buffer (0.1 M-Hepes/KOH/2 mM-EDTA/0.25 M-sucrose/1 mM-dithiothreitol, pH 7.0)/g fresh weight for 2 × 15 s with an Ultra Turrax (Janke und Kunkel, Staufen, Germany). This homogenate was filtered through Miracloth and centrifuged for 20 min at 8000 ***g***. The supernatant was stirred with 1 M-$MgCl_2$ (50 μl/ml) for 20 min and then centrifuged at 49000 ***g*** for 20 min. The supernatant was discarded and the pellet was resuspended in buffer to a final protein concentration of 0.2–0.5 mg/ml. Protein concentrations were determined according to Bradford (1976). In some experiments, the microsomal fraction was prepared by centrifuging the 8000 ***g*** supernatant at 100000 ***g*** for 1 h.

Sucrose density gradient centrifugation

Crude homogenates for sucrose density gradient centrifugation were obtained by homogenizing the cells in 0.3 ml of buffer [0.1 M-Hepes/KOH (pH 7.0)/1 mM-EDTA/1 mM-dithiothreitol/10% (w/w) sucrose]/g fresh weight. The supernatant (4.5 ml) of an 8000 ***g*** centrifugation was layered on 30 ml of a linear gradient from 15–45% (w/w) sucrose. The gradient was centrifuged for 3 h in an SW 28 rotor at 110000 ***g*** and then fractionated into 1.8 ml fractions and assayed for progesterone 5α-reductase activity and for marker enzymes.

Sucrose concentrations were measured using a refractometer (Zeiss, Oberkochen, Germany).

Progesterone 5α-reductase activity

The standard assay contained 1.0–2.5 mg of microsomal protein, 5 mM-glucose 6-phosphate, 1 mM-$NADP^+$, 8.4 nkat of glucose-6-phosphate dehydrogenase/ml and 40 μM-progesterone in a final volume of 5 ml. The reaction was initiated after a 20–30 min preincubation period by the addition of progesterone. The incubation was carried out for 30 min at 47 °C, and was terminated by shaking with 10 ml of methylene dichloride. Cholesterol (200 nmol/assay) was used as an internal standard.

For the gradient fractions, the assay volume was reduced to 1 ml. The assays were incubated for 45 min at 47 °C.

The sterols were extracted by shaking the total assay mixture twice with 10 ml of methylene dichloride. The combined organic phases were evaporated under reduced pressure and the residue was dissolved in 1.2 ml of methylene dichloride, transferred to Eppendorf vials and dried under a stream of filtered air. The sterols were redissolved in 500 μl of 80% (v/v) acetone and 100 μl of light petroleum (b.p. 100–140 °C). The acetone phase was transferred to a new Eppendorf vial and the light petroleum phase was washed again with 500 μl of 80% (v/v) acetone. After evaporation of the combined acetone phases, the residue was dissolved in 20 μl of methylene dichloride.

Product identification and quantification

G.l.c. was performed on a Packard GC 427 using a 180 cm × 0.2 cm (internal diam.) glass column packed with 3% OV-17 on Gas-chrom Q (100–120 mesh). The temperature was raised from 190 °C to 265 °C at a rate of 3 °C/min. The carrier gas was helium.

For identification of the reaction products, g.l.c. was also performed on a Shimadzu GC-9A using a 30 m × 0.25 mm (internal diam.) fused silica capillary column (DB-1701). The temperature was 275 °C with N_2 as carrier gas.

In addition, g.c./m.s. was carried out on a Finnigan Mat 112S g.c./m.s. using a DB-1701 fused silica capillary column (30 m × 0.25 mm internal diam.) with H_2 as carrier gas. M.s. at 70 eV (rel. int.): 5α-pregnan-3β-ol-20-one, *m/z* 318 (M^+, 39), 300 ($M^+ - H_2O$, 15), 285 ($M^+ - H_2O - CH_3$, 6), 84 (68) 43 (C_2H_3O, 100); 5α-pregnane-3,20-dione, *m/z* 316 (M^+, 37), 298 ($M^+ - H_2O$, 25), 283 ($M^+ - H_2O - CH_3$, 5), 84 (66) 43 (C_2H_3O, 100).

Determination of marker enzymes

Glucan synthase I (EC 2.4.1.34) with GDP–glucose as substrate was measured according to Ingold & Seitz (1986). UDP-glucose : steroyl glucosyltransferase (EC 2.4.1.35) was determined according to Sauer & Robinson (1985). Cytochrome *c* oxidase (EC 1.9.3.1) and NADH–cytochrome *c* reductase (EC 1.6.99.3) were assayed according to Hodges & Leonard (1974), with slight modifications.

RESULTS

Product identification

The reduced products of progesterone were identified as 5α-pregnane-3,20-dione and 5α-pregnan-3β-ol-20-one. In accordance with the molecular formulae, $C_{21}H_{32}O_2$ and $C_{21}H_{34}O_2$, the mass spectra contained molecular ions at *m/z* 316 and 318. The distinction between isomeric compounds was possible by comparing the retention times of the isolated compounds with standards (see Table 1).

Thiol sensitivity

Thiol reducing agents stimulated 5α-reductase activity. Most effective were dithiothreitol and mercaptoethanol

Table 1. Retention times of different pregnanes

Pregnanes were separated by g.l.c. using a fused silica capillary column (DB-1701) at 275 °C, with N_2 as carrier gas.

Pregnane	Retention time (min)
5β-Pregnan-3β-ol-20-one	12.99
5α-Pregnan-3α-ol-20-one	13.91
5α-Pregnan-3β-ol-20-one	14.53
5β-Pregnane-3,20-dione	15.49
5α-Pregnane-3,20-dione	16.93

which caused a 3-fold increase in enzyme activity at concentrations of 1 mM. In contrast, the microsomal progesterone 5α-reductase activity from female rat hypothalamus was unaffected by dithiothreitol (Bertics & Karavolas, 1984).

Effects of metal ions and EDTA

The effects of several bivalent cations as well as EDTA on progesterone 5α-reductase activity were tested. In the presence of EDTA, there was no strict dependence of the enzyme on the tested cations at concentrations of 0.1 and 1.0 mM. $CaCl_2$, $CoCl_2$ and $ZnCl_2$ inhibited enzyme activity by more than 50%, whereas $MnCl_2$ (0.1 mM) did not influence enzyme activity. EDTA stimulated the enzyme with an optimum at 2 mM.

As shown in Table 2, $MgCl_2$ (1 mM) in the presence of EDTA (2 mM) appears to stimulate enzyme activity. If EDTA was omitted from the assay mixture, activity declined to 65% of the standard assay. In the absence of EDTA, the addition of 0.1 mM-$MgCl_2$ was associated with an increase in enzyme activity, but this activity was still less than the activity under standard conditions. The addition of higher $MgCl_2$ concentrations was associated with a decrease in enzyme activity. Although the addition of 0.1 mM-$MgCl_2$ increased enzyme activity, it was not added to the standard enzyme assay, since it also enhanced 5α-pregnan-3β-ol-20-one accumulation.

Table 2. Effects of $MgCl_2$, EDTA and dithiothreitol on progesterone 5α-reductase activity

The standard assay mixture contained 40 μM-progesterone, 5 mM-glucose 6-phosphate, 1 mM-$NADP^+$, 8.4 nkat of glucose-6-phosphate dehydrogenase/ml and 1.0–2.5 mg of microsomal protein, in a final volume of 5 ml. The buffer used for standard assays consisted of 0.1 M-Hepes/KOH, pH 7.0, 0.25 M-sucrose, 2 mM-EDTA and 1 mM-dithiothreitol. Incubations were carried out as described in the Materials and methods section.

Conditions	Activity (% of standard)
Standard	100
$MgCl_2$ added (1 mM)	126
EDTA omitted	65
EDTA omitted and:	
$MgCl_2$ added (0.1 mM)	81
$MgCl_2$ added (1 mM)	54
$MgCl_2$ added (5 mM)	16
Dithiothreitol omitted	28

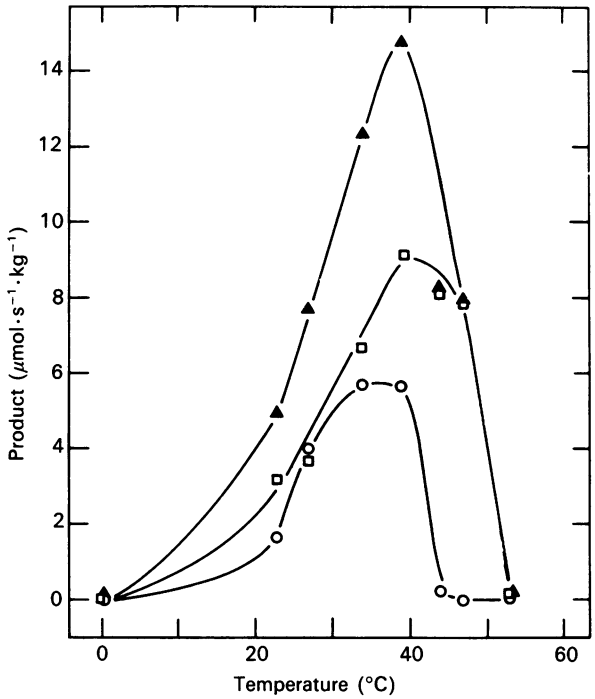

Fig. 2. Effect of temperature on the 5α-reduction of progesterone

The enzyme was assayed by the standard procedure and incubated at various temperatures. Products: ○, 5α-pregnan-3β-ol-20-one; □, 5α-pregnane-3,20-dione; ▲, sum of reaction products.

Effect of pH

Progesterone 5α-reductase exhibited high activity over a pH range of 6.3–7.5, with an optimum at pH 7.0. The incubation mixtures were adjusted to various pH values with Hepes buffer (pH 6.0–7.5) or Tris buffer (pH 7.5–8.5). For other Δ4-steroid 5α-reductases in animal cells, slightly alkaline or slightly acidic pH optima have been reported (Frederiksen & Wilson, 1971; Cheng & Karavolas, 1975; Hudson, 1981; Scheer & Robaire, 1983).

Temperature dependence and reaction kinetics

The effect of the incubation temperature on progesterone 5α-reductase activity is shown in Fig. 2. The enzyme exhibited maximum activity at 40 °C. Depending on the temperature, the product 5α-pregnane-3,20-dione is subsequently metabolized to varying degrees to 5α-pregnan-3β-ol-20-one. At temperatures above 45 °C, no 5α-pregnan-3β-ol-20-one could be detected after 30 min of incubation.

The rate of progesterone 5α-reduction at 47 °C proceeds linearly up to 90 min. The amount of 5α-pregnan-3β-ol-20-one produced is low at this temperature and is only detectable at incubation times over 30 min. Based on these observations, standard enzyme assays were carried out at 47 °C for 30 min, although this was not the temperature optimum of the enzyme. These incubation conditions provided sufficient product for g.c. analysis and minimal conversion of 5α-pregnane-3,20-dione to 5α-pregnan-3β-ol-20-one.

Table 3. Pyridine nucleotide requirements of progesterone 5α-reductase

Cofactors of additions were 1 mM-NADPH or -NADH or a regenerating system consisting of 5 mM-glucose 6-phosphate, 1 mM-$NADP^+$ and 8.4 nkat of glucose-6-phosphate dehydrogenase/ml. Other conditions were as described in the Materials and methods section.

Cofactor added	Specific activity (μkat/kg)	Relative activity (%)
None	0.2	4
NADPH	5.7	100
NADPH regenerating system	5.3	92
NADH	0	0

Substrate and cofactor requirements

Table 3 indicates that either NADPH or NADPH-regenerating system can provide the necessary reducing equivalents for progesterone 5α-reductase activity. In the standard assay, NADPH was supplied by a regenerating system consisting of glucose 6-phosphate, glucose-6-phosphate dehydrogenase and $NADP^+$. No stimulation of the enzyme activity compared with the control was observed when NADH was added to the incubation mixture. Progesterone 5α-reductase was saturated at concentrations of 1 mM-NADPH (Fig. 3). From a Hanes plot, an apparent K_m value for NADPH of 130 μM was calculated (Fig. 4).

The optimum progesterone concentration was 40 μM. At higher concentrations, slight substrate inhibition was observed. The K_m value for progesterone, calculated from a Hanes plot, was 30 μM.

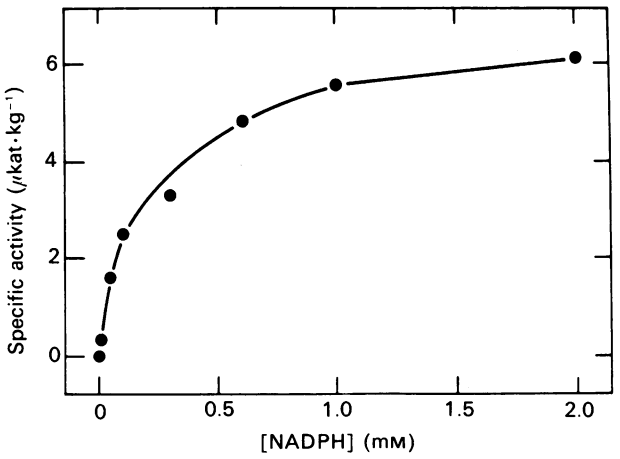

Fig. 3. Effect of NADPH concentrations on progesterone 5α-reductase

NADPH was applied via a regenerating system consisting of 5 mM-glucose 6-phosphate, 8.4 nkat of glucose-6-phosphate dehydrogenase/ml and various $NADP^+$ concentrations. The other incubation conditions were as described in the Materials and methods section

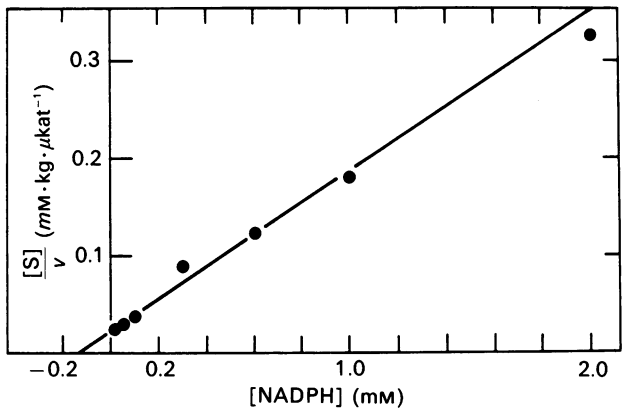

Fig. 4. Hanes plot for progesterone 5α-reductase at various concentrations of NADPH

The incubation conditions were as described in the legend to Fig. 3. A K_m value of 130 μM was determined for NADPH.

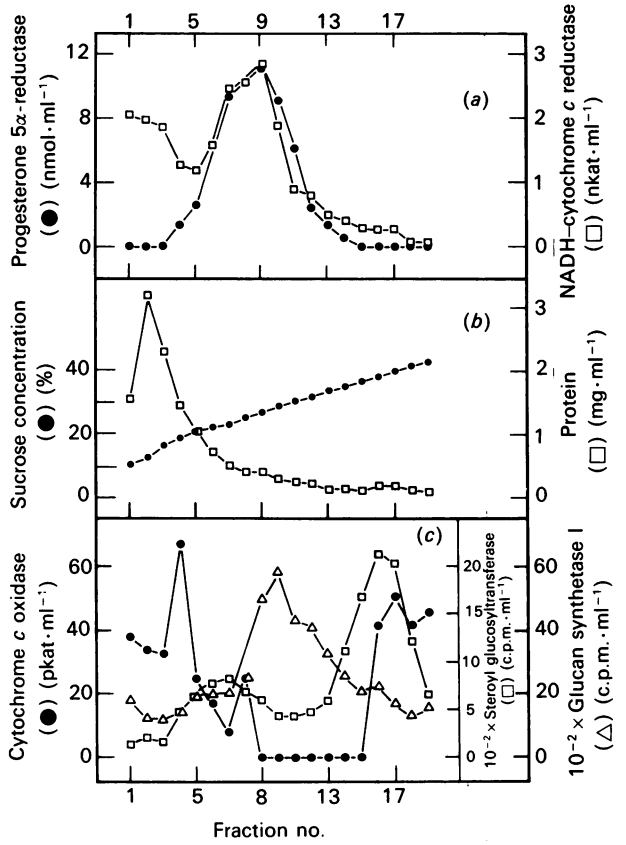

Fig. 5. Sucrose density gradient fractionation of progesterone 5α-reductase and different marker enzymes

Linear gradients from 15–45% (w/w) sucrose were centrifuged for 3 h in a SW 28 rotor at 110000 *g*. The supernatant (4.5 ml) of a 8000 *g* centrifugation from crude cell preparations was layered on the gradient. (*a*) Distribution of progesterone 5α-reductase and NADH–cytochrome *c* reductase as an endoplasmic reticulum marker enzyme. (*b*) Distribution pattern of protein. (*c*) Distribution of the marker enzymes glucan synthase I, cytochrome *c* oxidase and steroyl glucosyltransferase

Localization of progesterone 5α-reductase

The 5α-reductase seems to be located in the microsomal fraction (100000 *g* pellet or $MgCl_2$ precipitate) as shown by differential centrifugation. In the crude homogenate, as well as in supernatants, only very small amounts of enzyme activity could be detected. This may be due to a soluble inhibitor, so the possibility cannot be ruled out that at least one part of the enzyme activity is not membrane-bound.

In order to get more detailed information on the localization of the enzyme, crude homogenates of *Digitalis lanata* cells were centrifuged on linear sucrose gradients. After the fractionation of the gradients, the activities of several marker enzymes and of progesterone 5α-reductase were determined in the separate fractions. As shown in Fig. 5(*a*), the distribution of NADH–cytochrome *c* reductase, a marker enzyme for the endoplasmic reticulum, corresponds closely with the distribution of progesterone 5α-reductase activity in the gradient. The maximum activities for both enzymes were found at a sucrose density between 1.09 and 1.11 g/cm^3. The marker enzymes for the mitochondria (cytochrome *c* oxidase), the Golgi apparatus (glucan synthase I), and the plasma membrane (steroyl glucosyltransferase) showed a different distribution and other maxima (Figs. 5*b* and 5*c*). Thus the membrane-bound progesterone 5α-reductase is located in the endoplasmic reticulum.

DISCUSSION

An assay for progesterone 5α-reductase *in vitro* has been established for microsomal preparations from suspension cultures of *Digitalis lanata*. The enzyme catalyses the reduction of progesterone to 5α-pregnane-3,20-dione. The temperature optimum for the 5α-reductase at 40 °C is lower than that reported for the enzyme system of 3β-hydroxy-Δ5-steroid dehydrogenase and Δ5/Δ4-oxosteroid isomerase (3β-HSD) from *Digitalis lanata* at 50 °C (Seidel, 1988). 3β-HSD catalyses the reaction from pregnenolone to progesterone, which is also thought to be part of the postulated biosynthetic pathway of cardenolides.

At temperatures below 45 °C, the product of the 5α-reductase is subsequently reduced enzymically to 5α-pregnan-3β-ol-20-one. The properties of that enzyme activity are currently under investigation in our laboratory. It is not yet clear whether the two reductions are catalysed by one or two enzymes. The differences in temperature dependence hint at two separate enzymes. In the rat hypothalamus, steroid 5α-reductase and 3α-hydroxysteroid oxidoreductase can be partially separated by subcellular fractionation (Krause & Karavolas, 1980).

As with other Δ4-steroid 5α-reductases in rat liver (McGuire *et al.*, 1960), rat prostate (Frederiksen & Wilson, 1971), human skin (Voigt *et al.*, 1970) and rat hypothalamus (Cheng & Karavolas, 1975), the progesterone 5α-reductase from *Digitalis lanata* requires NADPH. Other plant reductases such as cucurbitacin B Δ23-reductase (Schabort & Potgieter, 1968) and 12-oxophytodienoic acid reductase (Vick & Zimmerman, 1986) prefer NADPH but can also use NADH. The apparent K_m values for progesterone 5α-reductase are 30 μM for progesterone and 130 μM for NADPH. They are of the same order of magnitude as the K_m values reported from the 3β-HSD (12.5 μM for pregnenolone and 82 μM for NAD^+) in suspension cultures of *Digitalis lanata* (Seidel, 1988). Progesterone 5α-reductase from rat hypothalamus shows a higher substrate affinity for progesterone and exhibits an apparent K_m of 0.113 μM in the microsomal fraction (Bertics & Karavolas, 1984).

The activity of 5α-reductase is increased by a factor of about three by the addition of thiol reagents such as dithiothreitol or mercaptoethanol. This stimulation by thiol reagents and the strong inhibition by *p*-hydroxymercuribenzoate (S. Wendroth & H. U. Seitz, unpublished work) indicate the participation of thiol groups.

Membrane-bound progesterone 5α-reductase is located on the endoplasmic reticulum as demonstrated by the sucrose density fractionation of cellular membranes. The distribution of the progesterone 5α-reductase was almost identical with that of the marker enzyme for the endoplasmic reticulum, NADH–cytochrome *c* reductase. The subcellular localization of progesterone 5α-reductase in rat hypothalamus has been investigated by Krause & Karavolas (1980); after differential centrifugation, 5α-reductase activity was enriched in the 105000 *g* pellet, suggesting a microsomal localization. The Δ4-steroid 5α-reductase with testosterone as the substrate has been found in microsomal subcellular fractions as well as in the nuclear fractions. This has been demonstrated for rat epididymal cells (Robaire *et al.*, 1977), human hyperplastic prostatic tissue (Hudson, 1981) and rat prostate (Frederiksen & Wilson, 1971). In higher plants, reductases are often soluble enzymes, as reported for 12-oxophytodienoic acid reductase (Vick & Zimmerman, 1986), hydroxyphenylpyruvate reductase (Petersen & Alfermann, 1988), tropinone reductase (Dräger *et al.*, 1988) and D-ribose-5-phosphate reductase (Negm & Marlow, 1985). However, some reductases are membrane-bound enzymes. 3-Hydroxy-3-methylglutaryl-CoA reductase was localized in the mitochondrial fractions of fresh sweet potato roots (Suzuki & Uritani, 1976), in sliced potato tuber microsomes (Kondo & Oba, 1986) and in a variety of membrane fractions in radish seedlings (Bach *et al.*, 1986).

The function of the progesterone 5α-reductase remains to be investigated. If the enzyme is part of the biosynthetic pathway of cardenolides, an isomerase would have to convert the 5α-derivatives to 5β-derivatives. On the other hand, the 5α-reductase could be a part of other biosynthetic pathways. In elucidating these possibilities, a knowledge of its substrate specificity would be of great advantage. In rat liver at least five enzymes have been found which perform 5α-reduction on Δ4-3-oxosteroids (McGuire & Tomkins, 1960; McGuire *et al.*, 1960). In contrast with the rat liver enzymes, rat prostate (Frederikson & Wilson, 1971) and rat hypothalamus (Cheng & Karavolas, 1975) probably contain only a single 5α-reductase enzyme with a broad specificity for Δ4-3-oxosteroids. Progesterone and 20α-dihydroprogesterone are more reactive substrates than testosterone. Rat liver microsomes also contain several 5α-reductases (Golf & Graef, 1978). In order to obtain more detailed information on the properties of the progesterone 5α-reductase in *Digitalis lanata*, the enzyme needs to be solubilized and purified.

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