**3α-Hydroxyysteroid-5β-oxidoreductase in Tissue Cultures of Digitalis lanata**

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Putative intermediates of cardenolide biosynthesis, namely progesterone, pregnenolone, 5β-pregnane-3,20-dione or 5β-pregn-3β-ol-20-one, were administered to light- or dark-grown shoot cultures of *Digitalis lanata*. The unsaturated compounds were reduced to their respective 5α-pregnanes, 5β-pregnane-3,20-dione was reduced to 5β-pregn-3α-ol-20-one and 5β-pregn-3β-ol-20-one was isomerized to the respective 3α-pregnane.

Suspension cultures of *Digitalis lanata*, on the other hand, accumulated both the 3α- and the 3β-isomer of 5β-pregn-3α-ol-20-one when incubated in the presence of 5β-pregn-3β-ol-20-dione. When 5β-pregn-3α-ol-20-one was administered the cultured cells accumulated large amounts of the 3β-isomer together with small amounts of 5β-pregn-3,20-dione, which may be regarded as an intermediate during the isomerization reaction.

Cell-free, buffered extracts from light-grown shoots were shown to reduce 5β-pregn-3,20-dione almost exclusively to 5β-pregn-3α-ol-20-one when 0.05 M MgCl₂ were present in the incubation mixture. Under these conditions the formation of 5β-pregn-3β-ol-20-one was inhibited. The enzyme activity could be recovered from membrane-free supernatants. Optimum enzyme activity occurred at pH 7.0 and 42 °C. The energy of activation was 56.2 kJ/mol and the enzyme reaction was found to be NADP+-dependent. SH reagents were essential for enzyme activity. The enzyme seems to be specific for 5β-pregn-3α-ones since neither 5α-pregn-3α-ones nor Δ⁴/Δ⁵-pregnenes were reduced.

The NADP⁺:5β-pregnane 3α-hydroxysteroid-5β-oxidoreductase described here may play a role in the regulation of cardenolide biosynthesis by removing precursors, such as 5β-pregnane-3,20-dione, from the pathway.

**Introduction**

Despite many investigations we still know little about the enzymes involved in cardenolide biosynthesis. The most common method used to establish the proposed pathway (Fig. 1) was the feeding of radiolabelled compounds which were thought to be precursors of cardenolides. In 1964 Tschesche and Lilienweiss [1] already reported on the incorporation of pregn(5)en-3β-ol-20-one-glucoside in cardenolides. Later on, the hypothesis that pregnenolone might be a precursor of cardiac glycosides was supported by Tschesche and Brassat [2] and by Caspi and Lewis [3]. The conversion of pregnenolone to progesterone was demonstrated by several authors [4–6]. Progesterone was converted to 5α-pregnan-3β-20-dione, 5β-pregnan-3,20-dione, 5α-pregnan-3β-ol-20-one and pregn(5)en-3β-ol-20-one by *Digitalis lanata* plants [7]. After the application of progesterone, suspension cultures of *Digitalis purpurea* formed 5α-pregnan-3,20-dione, 5α-pregnan-3β-ol-20-one, 5α-pregnan-3β,20α-diol, pregn(4)en-20α-ol-3-one and pregn(5)en-20β-ol-3-one as well as the corresponding glucosides [8]. It is remarkable that only 5αH-derivatives could be detected although all *Digitalis* cardenolides are 5βH-configured. Actually, leaves of *Digitalis lanata* are able to transform progesterone to cardenolides [3] but the conversion of 5α-pregnenes to 5β-derivatives or vice versa was never observed [9, 10]. Hence, the role of the 5α-derivatives still has to be elucidated.

Only four enzymes involved in the biosynthesis of putative cardenolide precursors have been described. Pilgrim [11] reported on the occurrence of a cholesterol side-chain-cleaving enzyme (SCCE) in *Digitalis* seedlings and suggested that the enzymatic formation of pregnenolone represents the metabolic bottle-neck in the cardenolide pathway. The preliminary results presented were never substantiated by a detailed characterization of the SCCE, whereas the next enzymatic steps in the putative cardenolide pathway were investigated in more detail. Pregnenolone is converted to progesterone by the Δ⁴-3β-hydroxyestroid dehydrogenase/Δ³-Δ⁴-ketosteroid isomerase (3β-HSD), 3α-Hydroxyesteroid-5β-oxidoreductase...
which probably does not play a major regulatory role in cardenolide biosynthesis [12]. Gärtner et al. [13] characterized a progesterone 5β-reductase. This enzyme, isolated from Digitalis purpurea seedlings, is responsible for the transformation of progesterone to 5β-pregnane-3,20-dione. The product of this reaction was further metabolized to 5β-pregnan-3β-ol-20-one by the 3β-hydroxysteroid oxidoreductase which was isolated from suspension cultures of Digitalis lanata [14]. The corresponding progesterone 5α-reductase, which is most probably not involved in cardenolide formation, was isolated from the microsomes of suspension-cultured Digitalis lanata cells [15].

The present paper describes and discusses the role of a 3α-hydroxysteroid-5β-oxidoreductase occurring in suspension cultures as well as dark- and light-grown shoot cultures of Digitalis lanata.

Materials and Methods

Tissue cultures

Suspension cultures of Digitalis lanata were propagated in MS medium [16] without phytohormones. The suspensions were grown in 11 shake flasks kept in the dark on gyratory shakers; they were maintained and subcultivated as described by Kreis and Reinhard [17].

Shoot cultures were initiated from axillary tips and maintained in 300 ml shake flasks in liquid medium as described elsewhere [18, 19].

Application of the precursors

The incubations were carried out under standard cultivation conditions. Shoot cultures: the putative cardenolide precursor under consideration was dissolved in DMSO (stock solution: 20 mg ml⁻¹) and 0.25 ml of the solution added aseptically to 50 ml culture medium containing about 15 g wet shoots (10 ± 1 g fresh mass). Suspension cultures: About 20 g wet mass (11 ± 1 g fresh mass) were used. Other conditions were as described for the shoot cultures.

Protein extraction

Between 3 and 6 d after transfers into fresh medium shoots of the culture strain D [19] were harvested by suction filtration and washed with double-distilled water. All subsequent procedures were carried out at 4 °C. The tissue was homogenized in a mortar with 2.5 ml per g fresh weight of buffer. Three different extraction buffers (TRIS-HCl, HEPES-KOH and MOPS-KOH, each adjusted to pH 7.5) at 2 different strengths (50 mm, 100 mm) were tested. Finally, the tissues were extracted with 0.05 M HEPES-KOH buffer pH 7.5 containing 0.25 M sucrose, 0.05 M MgCl₂, 0.003 M cysteine and 0.045 M mercaptoethanol (= Buffer I). After centrifugation (10 min at 12,000 × g) the supernatant was removed and its protein concentration determined according to Bradford [20]. Bovine serum albumin served as the protein standard.

Acetone powders were prepared from 4-day-old shoots and stored at -20 °C until further use. The powder was suspended in approximately 20 volumes of Buffer I, after which the suspension was vortexed vigorously for 1 min and finally centrifuged for 10 min at 12,000 × g. The supernatant was used for the enzyme assay.

Standard assay for 3α-hydroxysteroid-5β-oxidoreductase

The assay was performed in 1.5 ml Eppendorf cups containing the following in a total volume of 250 μl: 5 μl DMSO, 0.6 μmol NADPH (in 45 μl buffer) or NADPH-regenerating system (consisting of 0.18 μmol NADP, 2.3 μmol glucose-6-phosphate and 6.67 nkat glucose-6-phosphate dehydrogenase in 45 μl buffer), 200 μl protein extract (containing 0.3 – 0.8 mg protein ml⁻¹) and 0.32 μmol of 5β-pregnan-3,20-dione. The pregnane substrate was added as a solution in CH₂Cl₂ and the solvent evaporated prior to the addition of the other components of the assay.

The reaction was terminated by extracting the steroids with 500 μl iced cold EtOAc. Prior to the extraction 10 μl of a testosterone solution (0.1 mg ml⁻¹ EtOH) were added as the internal standard. The cups were shaken vigorously and the phase separation was facilitated by centrifugation (15 s at 12,000 × g). The upper phase was removed and evaporated at 40 °C. The residue was dissolved in 200 μl CH₂Cl₂ and centrifuged at 12,000 × g for 10 min. The supernatant was then analyzed by GC.

Preparation of microsomes

Microsomes were prepared as described by Petersen and Seitz [21] with slight modifications. All
operations were carried out in a cold room (4 °C) or on ice. The shoots were washed with tap water, homogenized in a mortar in 1.0 ml per g fresh mass 0.05 M HEPES-KOH buffer pH 7.0 (containing 0.25 M sucrose, 0.003 M cysteine and 0.045 M mercaetoethanol) and filtered through two layers of Miracloth (Calbiochem, La Jolla, Calif. U.S.A.). After centrifugation (8000 x g for 30 min) the supernatant was removed and 1 M MgCl₂ was added to yield a final concentration of 50 mM. The solution was stirred for 20 min and then centrifuged at 49,000 x g for 20 min. After removal of the supernatant the pellet was resuspended in buffer containing 50 mM MgCl₂. The enzyme activities in the supernatant and in the resuspended pellet were determined as described above with the following modifications: the pH of the buffer was adjusted to 7.0 and the incubation was carried out at 42 °C for 1 h.

Enzyme characterization

pH Optimum: the effect of the buffer pH on the enzyme activity was examined with the following buffers: HEPES-KOH (pH 6.5 to 7.5) and TRIS-HCl (pH 7.5 to 8.5). Protein was extracted with Buffer I and the buffers exchanged using Sephadex G-25 columns. Temperature optimum: incubations were carried out at temperatures ranging from 22 to 62 °C. The protein extracts (Buffer I) were pre-incubated at the respective temperature for 5 min.

Product isolation and identification

About 46 g shoots were homogenized in a mortar in 120 ml Buffer I. The homogenate was filtered through Miracloth and centrifuged at 20,000 x g for 20 min. The supernatant was divided into several portions each of which was incubated in a water-bath at 37 °C for 5 h with an equal volume of Buffer I containing 0.18 mmol NADP⁺, 2.3 mmol glucose-6-phosphate and 6.67 μkat glucose-6-phosphate dehydrogenase. The enzyme reaction was terminated by the addition of EtOAc. The buffer phase was extracted four times, each time with 150 ml EtOAc. In order to facilitate phase separation the mixture was centrifuged each time at 4000 x g for 10 min. The combined organic phases were dried over Na₂SO₄ and the solvent then evaporated under reduced pressure. The residue was dissolved in 450 μl EtOAc and used for preparative TLC on PSC plates KG 60 F254, 1 mm (Merck, Darmstadt, F.R.G.). The solvent mixture was composed of 80 parts chloroform and 20 parts EtOAc (v/v). After development the zones containing pregnanes were scraped off and each processed independently. The pregnanes were eluted from the silica gel with 80 ml EtOAc. The organic phase was evaporated. The residue was dissolved in 450 μl EtOAc and used for a further preparative TLC using a mixture of chloroform and EtOAc (60 + 40; v/v) as the solvent. In this way about 20 mg 5 ß-pregnane-3 α-ol-20-one were isolated. GC-MS: 318 (M⁺, 11), 300 (M⁺-H₂O, 39), 285 (M⁺-H₂O-CH₃, 10), 43 (C₆H₅O, 100). IR: 3411 (m; -OH); 2960 (s; C-H); 2920 (s; C-H); 1700 (s; C=O; C-20); 1450 (m; -OH); 1040 (s; C-O-). 1H NMR (CDCl₃): spectra were measured at 250 MHz on a Bruker AC 250 spectrometer. Chemical shifts are given in ppm downfield from tetramethylsilane which was used as the internal standard: 3.58 (1 H; m; H-3 ß, ax.); 2.47 (1 H; t; H-17 α); 2.04 (3 H; s; CH₃-21); 0.85 (3 H; s; CH₃-19); 0.52 (3 H; s; CH₃-18).

Extraction of pregnanes

Pregnanes were extracted from the tissue culture material using the method of Wichtl et al. [22, 23] originally devised for the extraction of cardenolides, with the modifications introduced by Stuhlemmer et al. [19]. The extracts were analyzed by GC and TLC.

Gas chromatography (GC)

Pregnane-containing extracts were analyzed on an HP 5890 gas chromatograph connected to a 3393 A integrator (Hewlett-Packard, Waldbronn, F.R.G.) using a column with cross-bonded 14% cyanopropylphenyl–86% methylpolysiloxane (Rtx-1701, 30 m, 0.32 i.d.; Restek Corp., Bellefonte, PA, U.S.A.). The oven temperature was raised from 150 °C at a rate of 30 °C min⁻¹, then kept at 230 °C for 10 min, after which the temperature was raised to 270 °C at a rate of 30 °C min⁻¹. After 12 min at 270 °C the analysis was stopped. The injection volume was 2 μl, injector and detector temperature were set to 280 °C. H₂ was used as the carrier gas.

Thin layer chromatography

In addition to GC, pregnanes and pregnenes were identified by their Rf values and colour reactions in
TLC on silica gel using a mixture of 20 parts ethyl acetate and 80 parts chloroform (v/v) as the developing solvent system. Steroid spots were made visible by spraying the TLC plates with a mixture of 0.5 ml methoxybenzaldehyde, 10 ml acetic acid, 85 ml methanol and 5 ml sulfuric acid.

Results
Biotransformation of pregnanes and pregnenes in vivo

5β-pregnan-3β-ol-20-one, 5β-pregnan-3α-ol-20-one, 5β-pregnan-3,20-dione, progesterone (pregn-4-ene-3,20-dione) and pregnenolone (pregn-5-en-3β-ol-20-one) (for structures see Fig. 1 and 2) were administered to suspension cultures, dark-grown shoot cultures and light-grown shoot cultures of Digitalis lanata. Free pregnanes and pregnenes were analyzed by gas chromatography. Controls, which were incubated in the absence of the respective precursors, contained no detectable amounts of free pregnanes and pregnenes.

Suspension cultures

When administered exogenously to suspension-cultured cells 5β-pregnane-3,20-dione was consumed rapidly and 5β-pregnan-3β-ol-20-one and 5β-pregnan-3α-β-ol-20-one accumulated to levels of 9700 and 8300 nmol g⁻¹, respectively. In order to trace the back reactions, the 3β-hydroxy- and the 3α-hydroxy-pregnane were fed to the cultured cells. Both substrates were converted into their corresponding 3-hydroxy isomer, probably via the intermediate formation of 5β-pregnane-3,20-dione. Actually, this compound accumulated to considerable levels only in the experiments with 5β-pregnan-3α-ol-20-one but not in those with the 3β-isomer, indicating that 5β-pregnan-3,20-dione 3β-reduction is the preferred reaction in suspension-cultured cells (Table 1a). Free pregnanes could not be detected after the administration of exogenous progesterone or pregnenolone.

Fig. 1. Hypothetical pathway leading from cholesterol to cardiac glycosides.
Table I. Transformations of 5β-pregnanes and pregnenes in *Digitalis lanata* tissue cultures. Products formed are given as nmol g⁻¹ d.w.

<table>
<thead>
<tr>
<th></th>
<th>5β-pregnane-3,20-dione</th>
<th>5β-pregnan-3β-ol-20-one</th>
<th>5β-pregnan-3α-ol-20-one</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>a) Suspension culture</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>substrate</td>
<td>9700</td>
<td>8300</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>2400</td>
<td>7200</td>
<td></td>
</tr>
<tr>
<td>5000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>b) Dark-grown shoot cultures</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>substrate</td>
<td>40</td>
<td>230</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td></td>
<td>230</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td></td>
<td></td>
<td>substrate</td>
</tr>
<tr>
<td><strong>c) Light-grown shoot cultures</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>substrate</td>
<td>traces</td>
<td>510</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td></td>
<td>470</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td></td>
<td></td>
<td>substrate</td>
</tr>
</tbody>
</table>

Dark-grown shoot cultures

When administered to dark-grown shoots 5β-pregnane-3,20-dione was converted to 5β-pregnan-3β-ol-20-one and its 3α-isomer. In contrast to suspension-cultured cells, they accumulated neither 5β-pregnan-3,20-dione nor 5β-pregnan-3β-ol-20-one after the administration of 5β-pregnan-3α-ol-20-one (Table I b). Administration of progesterone or pregnenolone resulted in the accumulation of 5α-pregnan-3,20-dione (335 nmol g⁻¹ d.w.) and 5α-pregnan-3β-ol-20-one (1680 nmol g⁻¹ d.w.), respectively. In summary, 3α-reduction seems to be the preferred reaction.

Light-grown shoot cultures

The conversion of pregnanes and pregnenes to their 3α-hydroxy derivatives was even more pronounced in light-grown shoots, where only traces of 5β-pregnan-3β-ol-20-one could be detected after the administration of 5β-pregnan-3,20-dione (Table I c). As in the experiments with dark-grown shoots administration of pregnenolone and progesterone caused the accumulation of 5α-pregnan-3,20-dione (63 nmol g⁻¹ d.w.) and 5α-pregnan-3β-ol-20-one (465 nmol g⁻¹ d.w.), respectively. In these experiments 5β-pregnanes could not be detected,
although green shoot cultures are capable of producing the 5β-configured cardenolides [19].

**Characterization of the 3α-hydroxysteroid-5β-oxidoreductase**

Since cardenolide-producing tissue (green shoots) and cardenolide-competent tissue (white shoots) differed considerably from the non-producing/non-competent tissue (suspension-cultured cells) with regard to the accumulation of 3α-pregnanes we became interested in the enzyme catalyzing their formation. Protein extracts obtained from the *Digitalis lanata* shoot culture D [19] were used in the characterization of the 3α-hydroxysteroid 5β-oxidoreductase.

**Optimization of enzyme extraction and assay conditions**

The effect of the extraction buffer on the enzyme activity was tested. At lower buffer strengths the enzyme activity was higher. Similar levels of enzyme activity could be measured in HEPES-KOH and TRIS-HCl buffers, whereas only weak enzyme activity was detected in MOPS-KOH buffer (Table II).

The standard substrate (5β-pregnane-3,20-dione) was dissolved or suspended in various solvents. Maximal enzyme activity was measured when dimethyl sulphoxide (DMSO) was used. In other solvents, such as acetone, ethyl acetate, ethylene glycol, Buffer I and methanol, enzyme activities were much lower, reaching only 75%, 62%, 43%, 42% and 11%, respectively, of the DMSO control.

Optimal enzyme activity was observed at pH 7.0, with about 80% of the maximal activity at pH 6.5 and 7.5.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Concentration [mM]</th>
<th>Rel. enzyme activity [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIS-HCl</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>TRIS-HCl</td>
<td>100</td>
<td>74</td>
</tr>
<tr>
<td>HEPES-KOH</td>
<td>50</td>
<td>105</td>
</tr>
<tr>
<td>HEPES-KOH</td>
<td>100</td>
<td>87</td>
</tr>
<tr>
<td>MOPS-KOH</td>
<td>50</td>
<td>41</td>
</tr>
<tr>
<td>MOPS-KOH</td>
<td>100</td>
<td>18</td>
</tr>
</tbody>
</table>

The enzyme activity was maximal at 42 °C with a sharp decrease at higher temperatures. This might be due to protein denaturation. The energy of activation was 56.2 kJ/mol.

Under standard incubation conditions (37 °C, Buffer I) the conversion of 5β-pregnane-3,20-dione to 5β-pregnane-3α-ol-20-one was linear for 1 h when the extracts were adjusted to protein concentrations between 0.2 and 0.8 mg per ml.

When the enzyme extract was kept at 25 °C for 24 h the enzyme activity dropped to 40% of the control. However, the extracts could be stored for 24 h at −20 °C or +4 °C without any obvious loss in enzyme activity.

**Characterization and localization of the 3α-hydroxysteroid-5β-oxidoreductase**

The effect of the bivalent cation Mg2+ on the 3α-hydroxysteroid-5β-oxidoreductase activity had to be tested since the formation of the by-product 5β-pregnan-3β-ol-20-one was inhibited by high concentrations of MgCl2 [14]. Actually, MgCl2 is required for optimal 3α-reductase activity since the highest conversion rates could be observed with MgCl2 at 0.05 to 0.1 mM.

The 3α-hydroxysteroid-5β-oxidoreductase from green *Digitalis lanata* shoots accepted only 5β-steroids such as 5β-pregnane-3,20-dione, digi-toxigenone and 5β-androstan-3,17-dione as substrates, whereas derivatives with a 5α-configuration (like 5α-pregnane-3,20-dione) or a double bond at position 4 or 5 (like progesterone, testosteron or pregn(5)en-3,20-dione) were not converted.

The dependence of the cofactors NADPH and NADH was tested with equimolar concentrations of either compound in the assay. NADH could substitute for NADPH to some extent (about 30%). In the incubation mixtures which contained NADH 5β-pregnan-3β-ol-20-one formation was increased. With NAD+ or NADP+ in the incubation mixture 5β-pregnan-3α-ol-20-one was enzymatically oxidized to 5β-pregnan-3α-ol-20-one, but only in the absence of Mg2+.

The major part of the 3α-hydroxysteroid-5β-oxidoreductase was found to be soluble. After MgCl2 precipitation, only about 10% of the total 3α-reductase activity was recovered from the membrane fraction (Fig. 3).
Fig. 3. Localization of the 3α-hydroxysteroid-5β-oxidoreductase.

3α-Hydroxysteroid-5β-oxidoreductase activity in different cultures

The activity of the 3α-reductase in cardenolide-producing and non-producing tissue cultures was analyzed to see if there is a correlation between the activity of the enzyme and the capability for cardenolide formation (Table III). The highest enzyme activities were found in the light- or dark-grown shoot cultures (134–274 μkat kg⁻¹ protein) whereas only weak enzyme activities were measured in suspension cultures (42 μkat kg⁻¹) or young leaves (53 μkat kg⁻¹).

Discussion

Hirotani and Furuya [24] carried out a series of bioconversion experiments in which they administered different pregnanes to cultured Digitalis purpurea cells. They found that 5β-pregnan-3β-ol-20-one is converted to 5β-pregnan-3,20-dione, 5β-pregnan-3α-ol-20-one and 5β-pregnan-3β,20β-diol. In addition, 5β-pregnan-3,20-dione was reduced to 5β-pregnan-3α-ol-20-one and 5β-pregnan-3β-ol-20-one. However, cardenolide formation was not observed although radiolabelled 5β-pregnanes have been shown to be incorporated into cardenolides [25]. In similar experiments using Nerium oleander cell cultures 5β-pregnan-3β-ol-20-one was isomerized to its 3α-isomer via 5β-pregnan-3,20-dione; 5β-pregnan-3α-ol-20-one was ultimately conjugated to glucose [26]. Again, as in the experiments of Hirotani and Furuya [24], no cardenolides were formed. The results we obtained with Digitalis lanata cell suspension cultures are consistent with these previous findings. In the present study, not only pregnenes but also 5β-pregnanes were fed to both dark-grown heterotrophic shoot cultures and light-grown mixotrophic shoot cultures of Digitalis lanata. In contrast to cell suspension cultures, where the feeding of 5β-pregnan-3,20-dione caused the accumulation of 5β-pregnan-3α-ol-20-one and 5β-pregnan-3β-ol-20-one, only the 3α-isomer was accumulated in the shoot cultures. At present, it cannot be ruled out that 5β-pregnan-3β-ol-20-one was actually formed but conjugated immediately to glucose, other sugars or fatty acids. Interestingly, a part of the 5β-pregnan precursor is channelled into the cardenolide pathway since its administration to Digitalis lanata shoot cultures caused a 2- to 3-fold increase in their cardenolide content, but only in the light-grown, not in the dark-grown shoots (not documented here). Since the three culture types investigated here differed considerably in terms of their ability to accumulate 3α-pregnanes, we became interested in the enzymatic reactions involved in the formation of 5β-pregnan-3α-ol-20-one.

There are several reports on hydroxysteroid oxidoreductases in animal tissues. In rat liver 3α- and 3β-hydroxysteroid oxidoreductases could be identified which accepted both 5α- and 5β-configurated steroids. The respective enzymes were found to be soluble or membrane-associated [27]. The dependence on reductants of these enzymes is very different; in general the cytosolic ones prefer NADPH [28], whereas the membrane-bound enzymes accept NADH and NADPH almost equally [29, 30]. In contrast to recent findings by Gärtner and Seitz [31],
who reported that the 3α-hydroxysteroid-5β-reductase of Digitalis purpurea prefers NADH as the co-substrate, NADPH is clearly preferred by the Digitalis lanata enzyme described here. In addition, we found that the 3α-hydroxysteroid-5β-reductase does not accept 5α-steroids or Δ4/Δ3-unsaturated pregnenes like progesterone or pregn(5)en-3,20-dione, whereas 5β-steroids other than 5β-pregnane-3,20-dione, like 5β-androstan-3,17-dione and digitoxigenone, were converted to their respective 3α-derivatives. Further comparisons cannot be drawn, since the 3α-hydroxysteroid-5β-reductase of Digitalis purpurea has not yet been characterized in detail. The Digitalis lanata enzyme exhibits maximal activity at pH 7.0 and 42 °C and thus resembles the cytosolic, NADPH-dependent 3α-hydroxysteroid-5α-oxidoreductase from rat hypothalamus, which works optimally between pH 6–10 and at a temperature of 45 °C [32], and the cytosolic 3α-hydroxysteroid-5α-oxidoreductase from rat prostate, which prefers NADPH as the co-substrate and requires temperatures between 45 and 47.5 °C and a pH of 7.4 for maximal activity.

5β-Pregnan-3-ones may be reduced to 3β-hydroxypregnanes involving a 3β-hydroxysteroid-5β-oxidoreductase. This enzyme is operative in Digitalis lanata cell cultures [31]. From preliminary inhibition studies with crude enzyme preparations it is deduced that the formation of 3α- and 3β-hydroxy-5β-pregnanes, respectively, are catalyzed by two different stereospecific oxidoreductases [31]. This assumption is substantiated by our finding that the 3β-hydroxysteroid-5β-oxidoreductase is strongly inhibited by Mg2+, whereas the 3α-hydroxysteroid-5β-oxidoreductase is not.

All Digitalis cardenolides are 3β-OH-configured. The results we obtained in the cell-free system indicate that epimerization of the 3-hydroxy group may occur at different stages of the proposed cardenolide pathway provided that free genins are available. It has been shown in several studies that digitoxigenin is epimerized by tissue cultures of cardenolide-producing plants very efficiently to epi-digitoxigenin, its 3α-isomer [26, 33, 34]. On the other hand, 3α-digitoxigenin and its biotransformation products have not yet been detected as indigenous compounds. In addition, digitoxigenin does not seem to be a precursor of cardenolide digitoxosides [35]. Hence, we suppose that cardenolide-specific sugars may be attached to the cardenolide precursor at an early pregan stage of biosynthesis involving the action of stereospecific glycosyltransferases. It may be assumed that the 3α-hydroxysteroid-5β-oxidoreductase removes putative genin precursors at various stages of the pathway. In such a way the 3α-hydroxysteroid-5β-oxidoreductase may contribute to the regulation of the cardenolide pathway. 3α-Hydroxysteroid-5β-oxidoreductase is very active in shoot cultures, which may be one reason for the low amounts of cardenolides accumulating in these tissues. In dark-grown shoots, which do not form cardiac glycosides, the 3α-hydroxysteroid-5β-oxidoreductase is as active as in the light-grown shoots. Cell suspension cultures, on the other hand, show only weak activity. Thus, it may be assumed that the 3α-hydroxysteroid-5β-oxidoreductase is correlated with morphological differentiation rather than with the expression of the cardenolide pathway.

Cardenolide biosynthesis seems to be regulated by different mechanisms operative at various points of the pathway. Digitalis shoot cultures, in which cardenolide formation can be triggered by light, seem to be well suited for studies into the regulation of the cardenolide pathway. In order to understand the formation of cardenolides and the regulation of the pathway our main focus should be on the sequence of modifications suggested by the putative pathway. In addition, other aspects like the removal of possible precursors from the pathway or the conjugation of pregnanes to cardenolide-specific sugars at an early stage of biosynthesis should also be considered.

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