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

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

Suspension cultures of *Digitalis lanata,* on the other hand, accumulated both the 3 α- and the 3 β-isomer of 5β-pregnan-3-ol-20-one when incubated in the presence of 5 β-pregnane-3,20-dione. When 5 β-pregnan-3 α-ol-20-one was administered the cultured cells accumulated large amounts of the 3 β-isomer together with small amounts of 5 β-pregnane-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 5 β-pregnane-3,20-dione almost exclusively to 5 β-pregnan-3 α-ol-20-one when 0.05 M $MgCl_2$ were present in the incubation mixture. Under these conditions the formation of 5 β-pregnan-3 β-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 NADPH-dependent. SH reagents were essential for enzyme activity. The enzyme seems to be specific for 5 β-pregnan-3-ones since neither 5 α-pregnane-3-ones nor Δ^4/Δ^5-pregnenes were reduced.

The NADPH:5 β-pregnane 3 α-hydroxysteroid-5 β-oxidoreductase described here may play a role in the regulation of cardenolide biosynthesis by removing precursors, such as 5 β-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-3 β-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 5 α-pregnane-3,20-dione, 5 β-pregnane-3,20-dione, 5 α-pregnan-3 β-ol-20-one and pregn(5)en-3 β-ol-20-one by *Digitalis lanata* plants [7]. After the application of progesterone, suspension cultures of *Digitalis purpurea* formed 5 α-pregnane-3,20-dione, 5 α-pregnan-3 β-ol-20-on, 5 α-pregnan-3 β,20 α-diol, pregn(4)en-20 α-ol-3-one and pregn(e)en-20 β-ol-3-one as well as the corresponding glucosides [8]. It is remarkable that only 5 αH-derivatives could be detected although all *Digitalis* cardenolides are 5 βH-configured. Actually, leaves of *Digitalis lanata* are able to transform progesterone to cardenolides [3] but the conversion of 5 α-pregnanes to 5 β-derivatives or *vice versa* was never observed [9, 10]. Hence, the role of the 5 α-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 Δ^5-3 β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-ketosteroid isomerase (3 β-HSD),

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which probably does not play a major regulatory role in cardenolide biosynthesis [12]. Gärtner *et al.* [13] characterized a progesterone 5 β-reductase. This enzyme, isolated from *Digitalis purpurea* seedlings, is responsible for the transformation of progesterone to 5 β-pregnane-3,20-dione. The product of this reaction was further metabolized to 5 β-pregnan-3 β-ol-20-one by the 3 β-hydroxysteroid oxidoreductase which was isolated from suspension cultures of *Digitalis lanata* [14]. The corresponding progesterone 5 α-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 3 α-hydroxysteroid-5 β-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 1 l 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 $MgCl_2$, 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 3 α-hydroxysteroid-5 β-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 5 β-pregnane-3,20-dione. The pregnane substrate was added as a solution in CH_2Cl_2 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 ice-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 CH_2Cl_2 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 mercaptoethanol) and filtered through two layers of Miracloth (Calbiochem, La Jolla, Calif. U.S.A.). After centrifugation (8000 × *g* for 30 min) the supernatant was removed and 1 M $MgCl_2$ was added to yield a final concentration of 50 mM. The solution was stirred for 20 min and then centrifuged at 49,000 × *g* for 20 min. After removal of the supernatant the pellet was resuspended in buffer containing 50 mM $MgCl_2$. 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 preincubated 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 × *g* for 20 min. The supernatant was divided into several portions each of which was incubated in a waterbath 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 × *g* for 10 min. The combined organic phases were dried over Na_2SO_4 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 β-pregnane-3 α-ol-20-one were isolated. GC-MS: 318 (M^+, 11), 300 ($M^+ - H_2O$, 39), 285 ($M^+ - H_2O - CH_3$, 10), 43 (C_2H_3O, 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 ($CDCl_3$): 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 β, ax.); 2.47 (1 H; t; H-17 α); 2.04 (3 H; s; CH_3-21); 0.85 (3 H; s; CH_3-19); 0.52 (3 H; s; CH_3-18).

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. H_2 was used as the carrier gas.

Thin layer chromatography

In addition to GC, pregnanes and pregnenes were identified by their R_f 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

5 β-pregnan-3 β-ol-20-one, 5 β-pregnan-3 α-ol-20-one, 5 β-pregnane-3,20-dione, progesterone (pregn-4-ene-3,20-dione) and pregnenolone (pregn-5-en-3 β-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 5 β-pregnane-3,20-dione was consumed rapidly and 5 β-pregnan-3 β-ol-20-one and 5 β-pregnan-3 α-β-ol-20-one accumulated to levels of 9700 and 8300 nmol g^{-1}, respectively. In order to trace the back reactions, the 3 β-hydroxy- and the 3 α-hydroxy-pregnane were fed to the cultured cells. Both substrates were converted into their corresponding 3-hydroxy isomer, probably *via* the intermediate formation of 5 β-pregnane-3,20-dione. Actually, this compound accumulated to considerable levels only in the experiments with 5 β-pregnan-3 α-ol-20-one but not in those with the 3 β-isomer, indicating that 5 β-pregnane-3,20-dione 3 β-reduction is the preferred reaction in suspension-cultured cells (Table I a). Free pregnanes could not be detected after the administration of exogenous progesterone or pregnenolone.

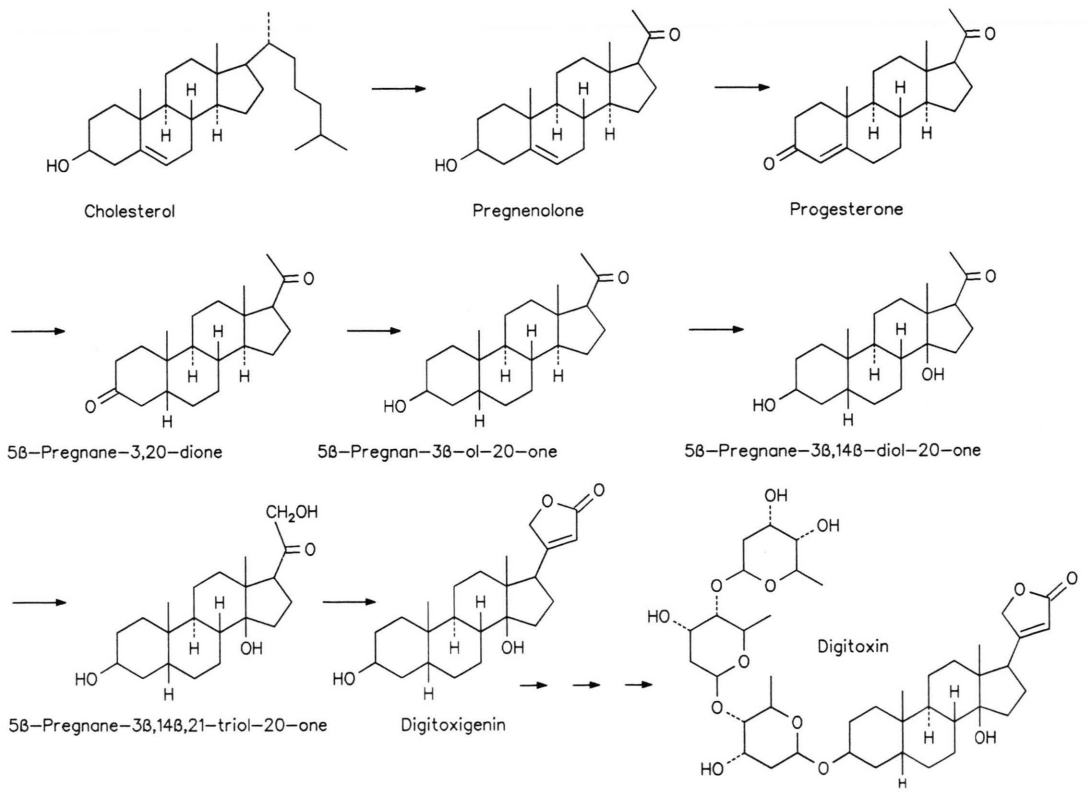

Fig. 1. Hypothetical pathway leading from cholesterol to cardiac glycosides.

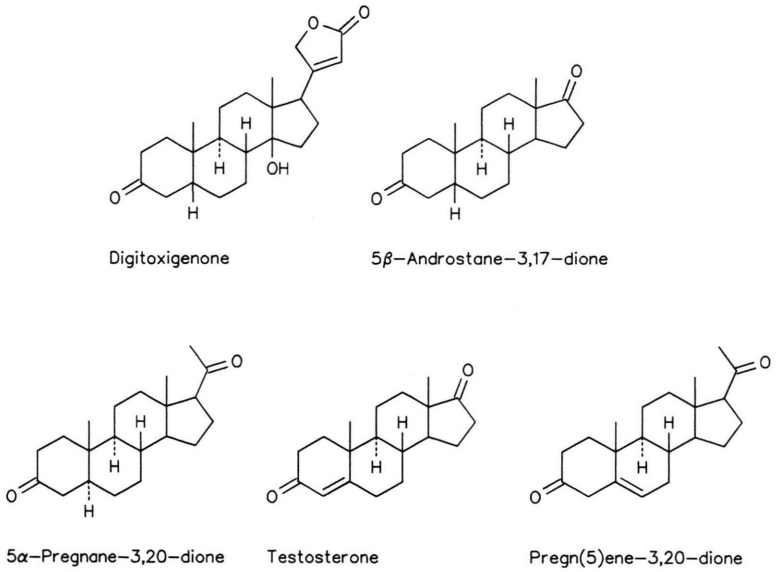

Fig. 2. Structures of the steroid compounds mentioned in the text (see also Fig. 1).

Table I. Transformations of 5 β-pregnanes and pregnenes in *Digitalis lanata* tissue cultures. Products formed are given as nmol g^{-1} d.w.

a) Suspension culture

5 β-pregnane-3,20-dione	5 β-pregnan-3 β-ol-20-one	5 β-pregnan-3 α-ol-20-one
substrate	9700	8300
–	substrate	7200
5000	2400	substrate

b) Dark-grown shoot cultures

5 β-pregnane-3,20-dione	5 β-pregnan-3 β-ol-20-one	5 β-pregnan-3 α-ol-20-one
substrate	40	230
–	substrate	230
–	–	substrate

c) Light-grown shoot cultures

5 β-pregnane-3,20-dione	5 β-pregnan-3 β-ol-20-one	5 β-pregnan-3 α-ol-20-one
substrate	traces	510
traces	substrate	470
–	–	substrate

Dark-grown shoot cultures

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

Light-grown shoot cultures

The conversion of pregnanes and pregnenes to their 3 α-hydroxy derivatives was even more pronounced in light-grown shoots, where only traces of 5 β-pregnan-3 β-ol-20-one could be detected after the administration of 5 β-pregnane-3,20-dione (Table I c). As in the experiments with dark-grown shoots administration of pregnenolone and progesterone caused the accumulation of 5 α-pregnane-3,20-dione (63 nmol g^{-1} d.w.) and 5 α-pregnan-3 β-ol-20-one (465 nmol g^{-1} d.w.), respectively. In these experiments 5 β-pregnanes could not be detected,

although green shoot cultures are capable of producing the 5 β-configured cardenolides [19].

Characterization of the 3 α-hydroxysteroid-5 β-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 3 α-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 3 α-hydroxysteroid 5 β-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 (5 β-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 II. Influence of different buffers on the activity of the 3 α-hydroxysteroid-5 β-oxidoreductase of *Digitalis lanata.*

Buffer	Concentration [mM]	Rel. enzyme activity [%]
TRIS-HCl	50	100
TRIS-HCl	100	74
HEPES-KOH	50	105
HEPES-KOH	100	87
MOPS-KOH	50	41
MOPS-KOH	100	18

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 5 β-pregnane-3,20-dione to 5 β-pregnan-3 α-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 3 α-hydroxysteroid-5 β-oxidoreductase

The effect of the bivalent cation Mg^{2+} on the 3 α-hydroxysteroid-5 β-oxidoreductase activity had to be tested since the formation of the by-product 5 β-pregnan-3 β-ol-20-one was inhibited by high concentrations of $MgCl_2$ [14]. Actually, $MgCl_2$ is required for optimal 3 α-reductase activity since the highest conversion rates could be observed with $MgCl_2$ at 0.05 to 0.1 mM.

The 3 α-hydroxysteroid-5 β-oxidoreductase from green *Digitalis lanata* shoots accepted only 5 β-steroids such as 5 β-pregnane-3,20-dione, digitoxigenone and 5 β-androstan-3,17-dione as substrates, whereas derivatives with a 5 α-configuration (like 5 α-pregnane-3,20-dione) or a double bond at position 4 or 5 (like progesterone, testosterone 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 5 β-pregnan-3 β-ol-20-on formation was increased. With NAD^+ or $NADP^+$ in the incubation mixture 5 β-pregnan-3 α-ol-20-one was enzymatically oxidized to 5 β-pregnane-3,20-dione, but only in the absence of Mg^{2+}.

The major part of the 3 α-hydroxysteroid-5 β-oxidoreductase was found to be soluble. After $MgCl_2$ precipitation, only about 10% of the total 3 α-reductase activity was recovered from the membrane fraction (Fig. 3).

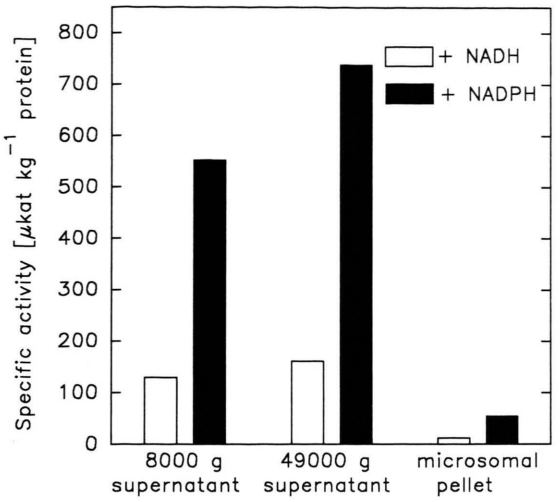

Fig. 3. Localization of the 3 α-hydroxysteroid-5 β-oxidoreductase.

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

The activity of the 3 α-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 purea* cells. They found that 5 β-pregnan-3 β-ol-20-one is converted to 5 β-pregnane-3,20-dione, 5 β-pregnan-3 α-ol-20-one and 5 β-pregnan-3 β,20 β-diol. In addition, 5 β-pregnane-3,20-dione was reduced to 5 β-pregnan-3 α-ol-20-one and 5 β-pregnan-3 β-ol-20-one. However, cardenolide formation was not observed although radiolabelled 5 β-pregnanes have been shown to be incorporated into cardenolides [25]. In similar experiments using *Nerium oleander* cell cultures 5 β-pregnan-3 β-ol-20-one was isomerized to its 3 α-isomer *via* 5 β-pregnane-3,20-dione; 5 β-pregnan-3 α-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 5 β-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 5 β-pregnane-3,20-dione caused the accumulation of 5 β-pregnan-3 α-ol-20-one and 5 β-pregnan-3 β-ol-20-one, only the 3 α-isomer was accumulated in the shoot cultures. At present, it cannot be ruled out that 5 β-pregnan-3 β-ol-20-one was actually formed but conjugated immediately to glucose, other sugars or fatty acids. Interestingly, a part of the 5 β-pregnane 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 3 α-pregnanes, we became interested in the enzymatic reactions involved in the formation of 5 β-pregnan-3 α-ol-20-one.

Table III. Cardenolides and 3 α-hydroxysteroid-5 β-oxidoreductase activity in different *Digitalis lanata* tissues.

Culture	Cardenolide content [nmol g d.w.$^{-1}$]	Specific activity [μkat kg^{-1} protein]
C	199	134
C*	48	134
C (dark)	0	157
D	631	202
F	326	167
W 1	201	277
Suspension	0	42
Young leaves	20000	53

There are several reports on hydroxysteroid oxidoreductases in animal tissues. In rat liver 3 α- and 3 β-hydroxysteroid oxidoreductases could be identified which accepted both 5 α- and 5 β-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 3 α-hydroxysteroid-5 β-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 3 α-hydroxysteroid-5 β-reductase does not accept 5 α-steroids or Δ^4/Δ^5-unsaturated pregnenes like progesterone or pregn(5)en-3,20-dione, whereas 5 β-steroids other than 5 β-pregnane-3,20-dione, like 5 β-androstan-3,17-dione and digitoxigenone, were converted to their respective 3 α-derivatives. Further comparisons cannot be drawn, since the 3 α-hydroxysteroid-5 β-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 3 α-hydroxysteroid-5 α-oxidoreductase from rat hypothalamus, which works optimally between pH 6 – 10 and at a temperature of 45 °C [32], and the cytosolic 3 α-hydroxysteroid-5 α-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.

5 β-Pregnan-3-ones may be reduced to 3 β-hydroxypregnanes involving a 3 β-hydroxysteroid-5 β-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 3 α- and 3 β-hydroxy-5 β-pregnanes, respectively, are catalyzed by two different stereospecific oxidoreductases [31]. This assumption is substantiated by our finding that the 3 β-hydroxysteroid-5 β-oxidoreductase is strongly inhibited by Mg^{2+}, whereas the 3 α-hydroxysteroid-5 β-oxidoreductase is not.

All *Digitalis* cardenolides are 3 β-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 3 α-isomer [26, 33, 34]. On the other hand, 3 α-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 pregnane stage of biosynthesis involving the action of stereospecific glycosyltransferases. It may be assumed that the 3 α-hydroxysteroid-5 β-oxidoreductase removes putative genin precursors at various stages of the pathway. In such a way the 3 α-hydroxysteroid-5 β-oxidoreductase may contribute to the regulation of the cardenolide pathway. 3 α-Hydroxysteroid-5 β-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 3 α-hydroxysteroid-5 β-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 3 α-hydroxysteroid-5 β-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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