3β-Hydroxysteroid Oxidoreductase in Suspension Cultures of *Digitalis lanata* EHRH

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A 3β-hydroxysteroid oxidoreductase was isolated and characterized in the microsomes of *Digitalis lanata* cell cultures. The enzyme catalyzes the conversion of 5α-pregnane-3,20-dione to 5α-pregnan-3β-ol-20-one and requires $NAD(P)H_2$. The enzyme was found to have a pH optimum of 8.0. The reaction had an optimum incubation temperature of 25 °C with linear reduction for the first 4 h, reaching maximum enzyme activity after 7 h. Substrate kinetics for 5α-pregnane-3,20-dione and $NADPH_2$ resulted in apparent K_m-values of 18.5–20 μM for 5α-pregnane-3,20-dione and 50–120 μM for the co-substrate $NADPH_2$. In order to localize 3β-hydroxysteroid oxidoreductase differential centrifugation as well as linear sucrose density gradient centrifugation were performed. The results obtained lead to the conclusion that 3β-hydroxysteroid oxidoreductase is not associated with a single cell compartment, but consists of a major soluble part and a markedly smaller part of endoplasmic reticulum-associated activity.

Introduction

The knowledge on the enzymic background of steroid and cardenolide biosynthesis is fairly poor. Progesterone and pregnenolone are putative precursors for both pathways. The metabolism of pregnanes and various pregnane derivatives in plants and plant tissue cultures has been the subject of numerous studies in the last decades, mainly with regard to steroids and cardenolides. Steroid biotransformations were performed with various pregnane derivatives, mostly resulting in the stereospecific reduction of 3-keto- and 20-keto-groups as well as the reduction of the C_4-C_5 double bond. Benett and co-workers [1] reported on biotransformations with progesterone. When they applied ^{14}C-labelled progesterone to leaves of *Digitalis lanata,* a large variety of radioactive compounds was detected including, among others, 5α-pregnane-3,20-dione, 5β-pregnane-3,20-dione, 5α-pregnan-3β-ol-20-one and 5β-pregnan-3β-ol-20-one. Graves and Smith [2] observed that a large number of suspension cultures, including *Digitalis* species, were able to metabolize progesterone to 5α-pregnane-3,20-dione and 5α-pregnan-3β-ol-20-one. Microsomes of *Dioscorea deltoidea* and *Cheiranthus cheiri* suspension cultures were found to convert progesterone to 5α-pregnane-3,20-dione in the presence of $NADPH_2$ [3]. The reduction of progesterone to 5α-pregnane-3,20-dione and small amounts of 5α-pregnan-3β-ol-20-one by leaf homogenates of *Cheiranthus cheiri, Digitalis purpurea, Strophanthus kombé* and *Corchorus olitorius* was described by [4]; these species failed to produce 5β-derivatives. In addition, the metabolism of 3β-hydroxy-5α-pregnan-20-one and 3β-hydroxy-5β-pregnan-20-one by leaf homogenates of a number of different plants was investigated [5], this time also resulting in small amounts of 5β-derivatives. Hirotani and Furuya [6] reported on the metabolism of 5β–pregnane-3,20-dione and 3β-hydroxy-5β-pregnan-20-one by *Digitalis* suspension cultures. Although numerous investigations concerning pregnane metabolism demonstrated the production of 5α-derivatives, the significance of 5α-pregnanes in cardenolide biosynthesis is not yet known. Cardenolides isolated from intact plants are 5β-derivatives without exception. *Digitalis* tissue cultures have lost the ability to synthesize cardiac glycosides [2]. Luckner and Diettrich [7] therefore suggested that the deficiency in cardenolide biosynthesis could be based on a lack of enzymes involved in the cardenolide pathway. The feeding of precursors of cardenolides could not stimulate cardenolide and steroid production as well. The main problem is that there is little information available on the enzymes which might be involved in cardenolide biosynthesis. A

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microsomal activity of progesterone 5α-reductases found in *Cheiranthus cheiri* and *Dioscorea deltoidea* catalyzing the conversion of progesterone to one single metabolite, 5α-pregnane-3,20-dione, in the presence of $NADPH_2$ at a pH of 7.0 [3].

Progesterone 5α-reductase from *Digitalis lanata* microsomes, which converts progesterone into 5α-pregnane-3,20-dione and smaller amounts of 5α-pregnan-3β-ol-20-one without producing detectable amounts of 5β-derivatives, was recently isolated and characterized by [8]. The conversion of 5α-pregnane-3,20-dione was catalyzed by a 3β-hydroxysteroid oxidoreductase. In the present report we will describe 3β-hydroxysteroid oxidoreductase, which was found to reduce 5α-pregnane-3,20-dione to 5α-pregnan-3β-ol-20-one (Fig. 1).

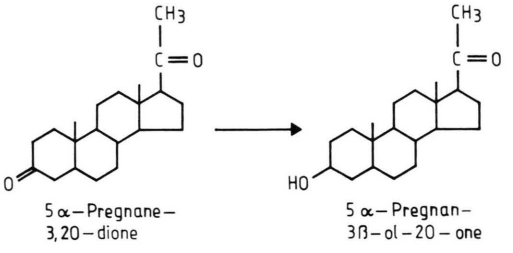

Fig. 1. Reaction catalyzed by 3β-hydroxysteroid oxidoreductase.

Materials and Methods

Cell cultures

Suspension cultures of *Digitalis lanata* were propagated in 1000 ml Erlenmeyer flasks containing 300 ml medium and subcultivated weekly as previously described [9].

Determination of fresh weight and packed cell volume

For the determination of fresh weight 2 ml of the cell suspension were filtered under suction. The packed cell volume was taken from 1 ml of the cell suspension using a modified hematokrit method [10].

Preparation of microsomes

Homogenization was performed in 0.1 M HEPES-buffer containing 0.25 M sucrose, 2 mM EDTA, 1 mM DTT, pH 8.0 (1 ml per g fresh weight). Unless otherwise stated this buffer was used throughout. All operations were carried out at 4 °C. Cells were harvested 5–6 days after onset of cultivation, dried under suction and homogenized with an Ultra Turrax homogenizer (Janke and Kunkel, Staufen im Breisgau, F.R.G.). After filtration the crude homogenate was centrifuged at $8000 \times g$ for 20 min and the pellet was discarded. $MgCl_2$ was added (final concentration 0.025 M) and the solution stirred for 20 min in order to precipitate cell membranes [11]. After centrifugation ($49{,}000 \times g$, 20 min) the microsomal pellet was washed with 1 ml buffer and afterwards resuspended in buffer to a final protein concentration of $0.2-0.7\ mg \cdot ml^{-1}$.

Standard enzyme assay

The microsomal fraction was incubated for 2 h with 40 μM 5α-pregnane-3,20-dione, dissolved in acetone, in the presence of a $NADPH_2$-regenerating system (1 mM $NADP^+$, 5 mM glucose-6-phosphate and 42 nkat glucose-6-phosphate-dehydrogenase) in a total volume of 5 ml homogenization buffer. The reaction was initiated by the addition of 5α-pregnane-3,20-dione as the substrate. Incubations were performed at 25 °C on a gyratory shaker and the reaction terminated by adding 10 ml methylene dichloride. The assays were either frozen at −18 °C or used immediately for pregnane extraction.

For the determination of the pH-optimum the microsomal pellet was resuspended in 0.1 M HEPES or 0.1 M TRIS-HCl (both containing 0.25 M sucrose, 1 mM DTT, 2 mM EDTA) and adjusted to pH-values ranging from 6.0 up to 9.0.

Extraction of pregnanes

Each assay contained 40 μM 5α-androstane-17β-ol-3-one as an internal standard. Enzyme assays were mixed for 30 s. Phase separation was facilitated by centrifugation (5 min, 7000 rpm). The organic phase was removed and collected and the water phase was re-extracted using 10 ml CH_2Cl_2. The combined organic phases were evaporated under reduced pressure. The residue was dissolved in 1.2 ml CH_2Cl_2, which was then removed in a stream of filtered air. Lipids and other membrane components were removed by additional washing

steps, which involved the addition of 0.5 ml 80% acetone and 0.1 ml petroleum benzine (b.p. 100–140 °C) to the residue. The samples were mixed for 30 s and centrifuged at 16,000 × *g*. The acetone phase was transferred to a new vial and the petroleum benzine phase washed once again with 0.5 ml 80% acetone. The combined acetone phases were evaporated in a stream of filtered air and the residue redissolved in 20 µl methylene dichloride. Samples were analyzed by gas liquid chromatography. The absolute amounts were calculated by the use of relative response factors obtained by standard mixtures of known concentrations.

Localization

Differential centrifugation. Cell homogenates were prepared as described above. The subcellular fractions were separated by centrifugation into 8000 × *g*-soluble, 20,000 × *g*-soluble and 100,000 × *g*-soluble portions and their respective insoluble parts. Fractions were diluted to a final protein content of 0.2–0.7 mg · ml^{-1} for the standard enzyme assays. Each fraction was assayed individually for enzyme activity.

Linear sucrose density gradient. Five ml of the supernatant from a 8000 × *g* centrifugation were layered on a 33.5 ml linear sucrose density gradient ranging from 15 to 45% (w/w) or 20 to 40% (w/w) sucrose in 0.1 mM HEPES, 1 mM DTT and 2 mM EDTA, pH 8.0. The gradients were centrifuged for 3 h at 27,000 rpm in a LKB ultracentrifuge (Ultrospin 70, rotor SRP 28 SA) and the fractions (2.2 ml) assayed for 5α-pregnane-3,20-dione reductase activity and marker enzymes.

Due to the small sample volumes the standard enzyme assay for 5α-pregnane-3,20-dione was modified. The total volume of the assay was 1 ml instead of 5 ml. The concentration of the $NADPH_2$-regenerating system and the substrate concentration were the same as in the standard assay (s.a.), as were incubation time and temperature. The reaction was terminated with 2 ml methylene dichloride and pregnanes were extracted as described above.

Identification of pregnanes

All pregnanes were identified and quantified by gas liquid chromatography. The separations were carried out on a Packard GC 427 gas chromatograph equipped with a hydrogen/synthetic air flame ionization detector. One microliter extract in methylene dichloride was injected and analyzed on a column (180 cm · 0.2 cm i.d.) packed with 3% silicone OV-17 (Serva, Heidelberg) on gas-chrome Q (0.125–0.15). The column temperature was 230 °C. Helium was used as the carrier gas. 5α-Pregnan-3β-ol-20-one was identified as the sole reaction product by co-chromatography with commercially available pregnanes, comparing relative retention times.

The product 5α-pregnan-3β-ol-20-one was additionally identified by GC-MS.

Determination of marker enzymes

NADH-dependent cytochrome *c* oxidase (mitochondria) was assayed according to [12].

The assays for the antimycin-insensitive NAD(P)H cytochrome *c* reductase activity (endoplasmic reticulum) were performed according to [13].

UDPG-steroyl-glucosyltransferase activity (plasma membrane) was assayed according to [14].

Enzyme assays for glucansynthetase I activity with GDP-glucose as the substrate (GS I–G; Golgi apparatus) were performed according to [15].

Analytical techniques

The protein concentration was determined using the method of Bradford [16] with bovine serum albumin as the standard. The sucrose concentration of the gradient fractions was determined with an Abbé refractometer.

Results

The activity of a 3β-hydroxysteroid oxidoreductase was detected in the supernatant of a 8000 × *g* centrifugation as well as in the microsomal fraction of *Digitalis lanata* cell cultures. Pregnanes were extracted from the enzyme assay by several washing steps and afterwards separated and identified by gas liquid chromatography. The metabolite obtained in the enzyme assay was identified by comparing its retention time with those of authentic pregnanes (5α/β-pregnan-3α-ol-20-one, 5α/β-pregnan-3β-ol-20-one). It was found to be 5α-pregnan-3β-ol-20-one. No 5β-pregnan-3β-ol-20-one was formed *in vitro*. These results were confirmed by GC-MS analysis.

In order to determine the optimum physiological conditions for the enzyme activity a cell culture characterization was carried out. Different growth parameters as well as enzyme activity were examined in their dependence on the time of culture (Fig. 2). 3β-Hydroxysteroid oxidoreductase showed maximum activity between the 5th and 6th day after inoculation. This coincides with the maximum of growth of the cell culture. Additionally, the protein content of the 8000 × *g* supernatant during cell cultivation was measured. The resulting data revealed a linear increase in the protein concentration up to day four with a continuous decline in the subsequent period of cultivation.

Characterization of 3β-hydroxysteroid oxidoreductase

Time course of the reaction. 3β-Hydroxysteroid oxidoreductase exhibited a linear reduction rate for the first 4 h of the incubation period. Beyond 4 to 5 h, product formation slowed down, stopping completely after 7 h (Fig. 3).

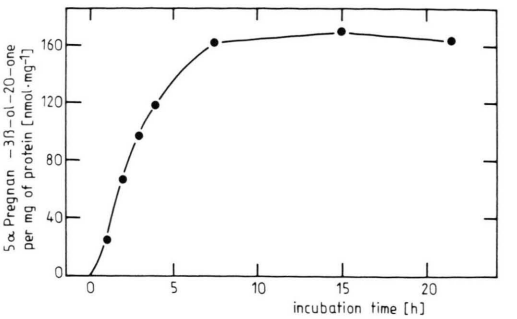

Fig. 3. Time course of reaction. The protein concentration was in the range of 0.25 to 0.5 mg · ml^{-1}.

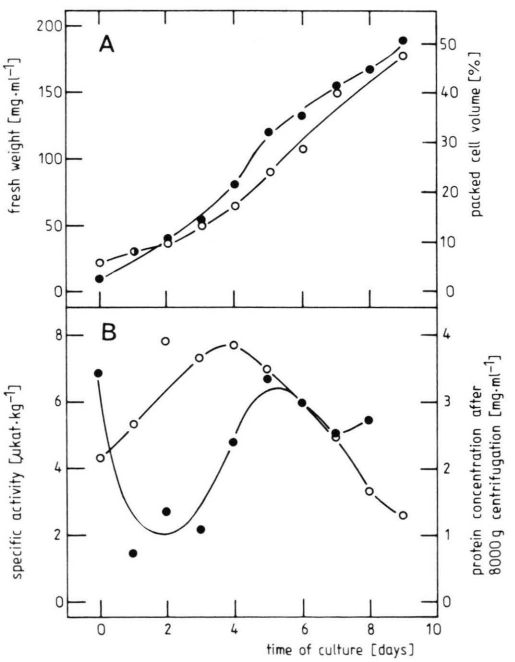

Fig. 2. Characterization of a cell culture of *Digitalis lanata* in a time period of 10 days. The following parameters were determined: A: ○—○ fresh weight, ●—● packed cell volume. B: ●—● specific activity of 3β-hydroxysteroid oxidoreductase and protein concentration in the supernatant of a 8000 × *g* centrifugation ○—○.

Effect of pH. The effect of the pH on the reduction of 5α-pregnane-3,20-dione was tested with HEPES/KOH buffer and TRIS/HCl buffer in the range of pH 6.0–9.0. 3β-Hydroxysteroid oxidoreductase showed a pH-optimum at 8.0 (data not shown).

Effect of temperature. Enzyme assays were incubated at different temperatures ranging from 0 °C to 60 °C. The temperature optimum obtained was 25 °C. An increase in the temperature to 27 °C resulted in a distinct reduction of 3β-hydroxysteroid oxidoreductase activity. At higher temperatures enzyme activity was reduced further, resulting in 10–20% of maximum activity at temperatures above 40 °C (Fig. 4).

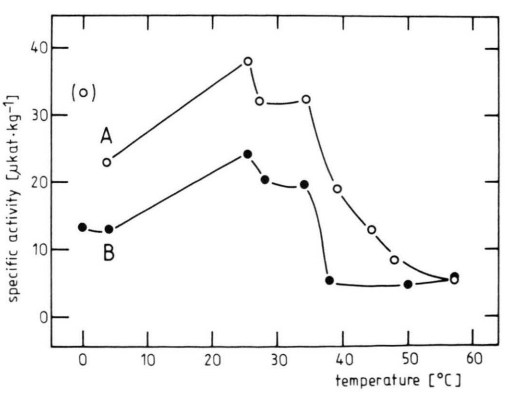

Fig. 4. Effect of temperature on the activity of 3β-hydroxysteroid oxidoreductase. The results of two independent experiments (A and B) are shown.

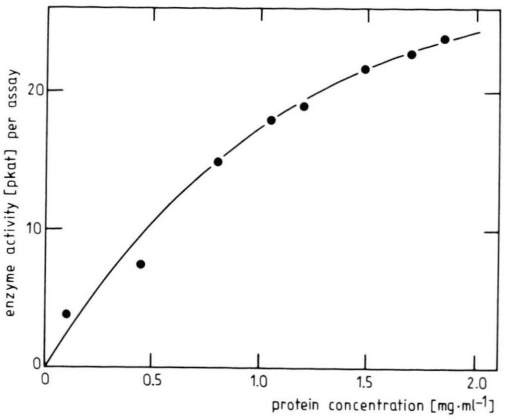

Fig. 5. Effect of protein concentration on the reduction of 5α-pregnane-3,20-dione.

Effect of protein concentration in the assay. Fig. 5 shows the dependence of 3β-hydroxysteroid oxidoreductase activity on the protein concentration. Reduction appeared to be linear up to 0.75 mg protein content per ml final volume. From 0.75 to 2 mg · ml^{-1} no constant values could be obtained. For this reason standard assays contained 0.2–0.5 mg · ml^{-1} of protein.

Effect of bivalent cations. The effect of bivalent cations on 3β-hydroxysteroid oxidoreductase activity was investigated by adding Ca^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+} or Zn^{2+} as chlorides (data not shown). In this case $NADPH_2$ at a concentration of 1 mM was used in place of the $NADPH_2$-regenerating system, since glucose-6-phosphate dehydrogenase is said to be inhibited by some bivalent cations. Enzyme activity could not be stimulated by any of the cations added. On the contrary, all of the cations tested inhibited oxidoreductase activity to a greater or lesser degree, without exception. The loss of activity amounted to 14 to 39% of the activity found in the control. The inhibitory effect of $MgCl_2$ on enzyme activity was quite important with regard to $MgCl_2$ precipitation during microsomal preparation. The microsomal pellet therefore had to be washed carefully with buffer before being resuspended for the enzyme assays.

Effect of sulfhydryl reducing agents. The influence of varying concentrations of dithiothreitol (DTT), mercaptoethanol, mercaptoethanol/cystein and Na-ascorbate on oxidoreductase activity was investigated and compared to a control with no sulfhydryl reducing agents added. DTT (1 mM, 10 mM) and mercaptoethanol (1 mM) were found to increase enzyme activity, whereas higher concentrations of mercaptoethanol (10 mM) as well as mercaptoethanol/cystein and Na-ascorbate (1 mM, 10 mM) resulted in a loss of enzyme activity of up to 50%. 1 mM DTT caused the largest increase in activity, namely 175% of the activity of the control, whereas with 10 mM DTT only 135% of the activity of the control was reached. Standard enzyme assays were performed adding 1 mM DTT to the buffer.

Substrate dependence. Substrate affinity and K_m-values of 3β-hydroxysteroid oxidoreductase for the substrate 5α-pregnane-3,20-dione and for $NADPH_2$ were determined. 5α-Pregnane-3,20-dione was added to the enzyme assays in concentrations of 0–120 μM without causing enzyme inhibition (Fig. 6). The data were evaluated in a Hanes-plot, and the apparent K_m-value was calculated to be 18.5 μM–20 μM.

3β-Hydroxysteroid oxidoreductase was found to be $NAD(P)H_2$-dependent with both $NADPH_2$ and $NADH_2$ being able to provide the necessary reduction equivalents. Standard enzyme assays were performed with a $NADPH_2$-regenerating system containing glucose-6-phosphate, $NADP^+$ and glucose-6-phosphate dehydrogenase in order to maintain a constant $NADPH_2$ concentration during incubation. To determine the incubation time at which $NADPH_2$ was totally oxidized, a short time course of enzyme activity with concentrations of 1 mM $NADPH_2$ per assay was established. If re-

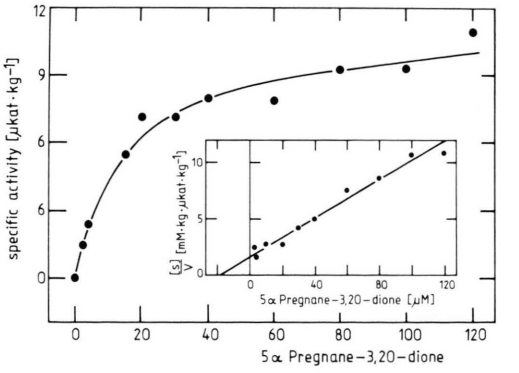

Fig. 6. Effect of 5α-pregnane-3,20-dione concentrations on 3β-hydroxysteroid oxidoreductase. The inset shows the Hanes-plot which was used to calculate the apparent K_m-value for the substrate 5α-pregnane-3,20-dione.

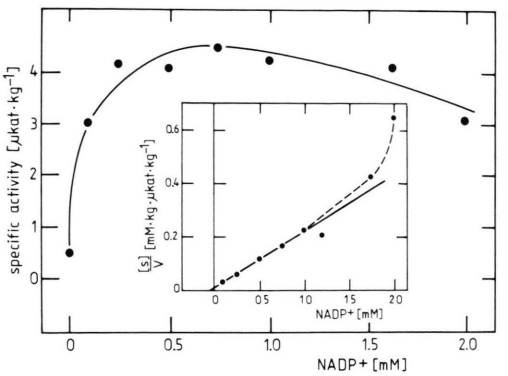

Fig. 7. Effect of $NADPH_2$-concentration on 3β-hydroxysteroid oxidoreductase. The enzyme assays were performed with a $NADPH_2$-regenerating system, containing 5 mM glucose-6-phosphate, 42 nkat glucose-6-phosphate-dehydrogenase and the varying concentrations of $NADP^+$. The inset shows the Hanes-plot which was used to determine the apparent K_m-value for $NADPH_2$.

duced $NADPH_2$ was applied an optimum incubation time of 1 h was observed. Fig. 7 shows the effect of increasing $NADPH_2$ concentrations on the enzyme activity. Concentrations above 1 mM lead to distinct enzyme inhibition. The K_m-value for $NADPH_2$ in the range between 57 μM and 120 μM was determined by a Hanes-plot from Fig. 7.

Localization of 3β-hydroxysteroid oxidoreductase

In a previous paper we described an exclusive association of a 5α-progesterone reductase with microsomes [8]. The enzyme reduces progesterone to 5α-pregnane-3,20-dione which is further converted to 5α-pregnan-3β-ol-20-one by the microsomal fraction. For this reason 3β-hydroxysteroid oxidoreductase was prevailingly characterized in the microsomal fraction.

Differential centrifugation at $8000 \times g$, $20{,}000 \times g$ and $100{,}000 \times g$, resulting in six different fractions, revealed that the 3β-hydroxysteroid oxidoreductase showed high activity in soluble fractions (supernatant) as well as in membrane fractions (pellet). According to Fig. 8 (upper part), which gives information on the total enzyme activity in the different fractions, the major part of the oxidoreductase seemed to be soluble. When specific enzyme activity was analyzed (Fig. 8, lower part), 3β-hydroxysteroid oxidoreductase was found to be accumulated in the microsomal pellet.

More detailed information concerning the localization of the 3β-hydroxysteroid oxidoreductase was obtained by means of linear sucrose density gradients. Marker enzymes for the membrane systems, plasma membrane (sterosyl-glucosyl-transferase), endoplasmic reticulum (cytochrome *c* reductase), Golgi apparatus (glucansynthetase I) and mitochondria (cytochrome *c* oxidase) were examined in the different gradient fractions and compared to the distribution pattern of 3β-hydroxysteroid oxidoreductase. The results are presented in Fig. 9. The greatest part of the 3β-hydroxysteroid oxidoreductase does not enter the gradient, but is found in the overlay. It can therefore be characterized as soluble. A distinctly smaller part of the enzyme activity seems to be associated with the endoplasmic reticulum, according to the distribution patterns of cytochrome *c* reductase and the oxidoreductase.

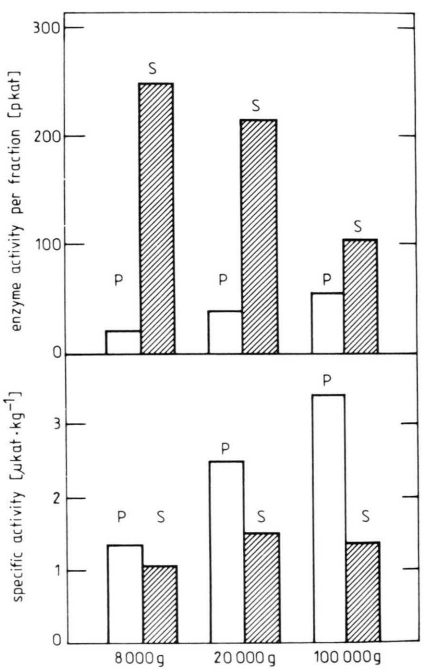

Fig. 8. Localization of 3β-hydroxysteroid oxidoreductase by a differential centrifugation. Enzyme activity per fraction (top) as well as specific activity (bottom) is shown for the different fractions. P = pellet, S = supernatant.

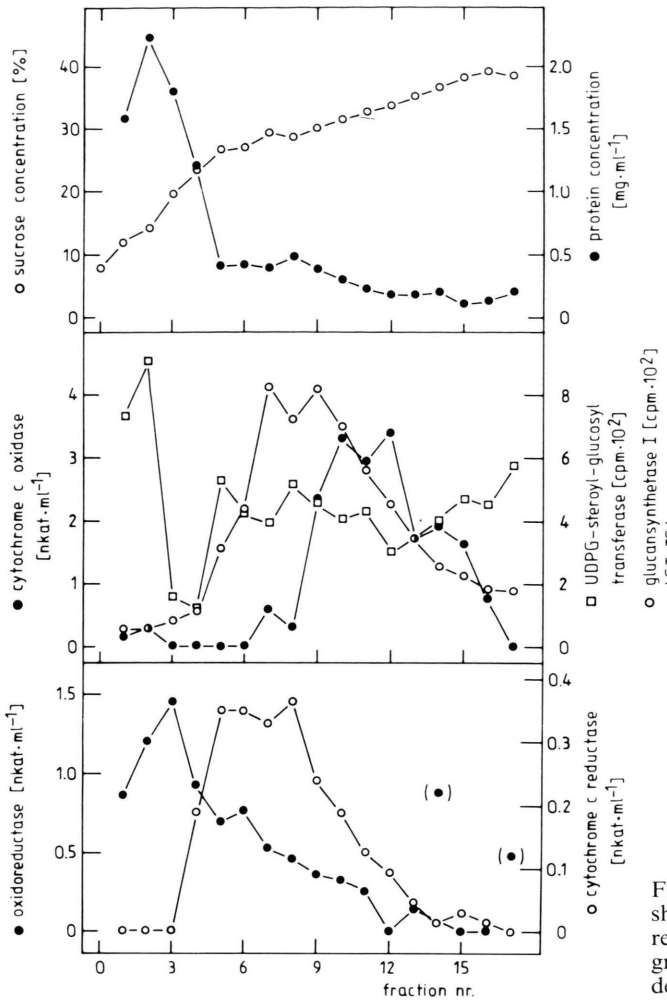

Fig. 9. Linear sucrose density gradient fractionation, showing the distribution of 3β-hydroxysteroid oxidoreductase and the different marker enzymes in the gradient. Enzyme activities were determined as described in Materials and Methods.

Discussion

3β-Hydroxysteroid oxidoreductase activity was characterized *in vitro* using the microsomal fraction of *Digitalis lanata* suspension cultures for the enzyme assays. The enzyme catalyzes the reduction of 5α-pregnane-3,20-dione to 5α-pregnan-3β-ol-20-one. Feeding experiments with cell cultures from plants which usually accumulate cardenolides provided the first hints for the existence of a 3β-hydroxysteroid oxidoreductase. In most cases, pregnenolone or progesterone were applied and converted to 5α/5β-pregnan-3β-ol-20-one [17, 18]. In mammalian tissues similar reactions were found with regard to steroid biosynthesis, transforming for example progesterone, testosterone and 5α-dihydrotestosterone [19–21]. Some of the mammalian enzymes involved in steroid biosynthesis have already been characterized, especially 5α/5β-reductases [19, 22, 23] and 3α/3β-hydroxysteroid oxidoreductases [23–25]. 3α-Hydroxysteroid oxidoreductases have also been characterized in microorganisms, for example in *Brevibacterium fuscum* [26].

5α/5β-Reductases catalyze the reduction of the C_4-C_5 double bonds of unsaturated steroids producing 5α/5β-dihydrosteroids. The same reaction is catalyzed by progesterone 5α-reductase in plants [8].

3α/3β-Hydroxysteroid oxidoreductases catalyze the hydroxylation of C_3 of steroids. These

reactions are equivalent to the reduction catalyzed by the 3β-hydroxysteroid oxidoreductase characterized in the present paper in the microsomes of *Digitalis lanata* suspension cultures. To date, no 3α/β-hydroxysteroid oxidoreductase has been described or characterized in detail in plants.

As reported above, 5α-progesterone reductase and 3β-hydroxysteroid oxidoreductase in microsomes of *Digitalis lanata* seem to be associated exclusively with one another. Similar observations were reported for the enzymes catalyzing the transformation of testosterone to androstandiol in microsomal preparations of human skin [21] and preparations of rat epididymis [27].

3β-Hydroxysteroid oxidoreductase was found to have a pH-optimum at 8.0. Comparisons of pH-optima from 3α/β-hydroxysteroid oxidoreductases and 5α/β-reductases of different mammalian organisms revealed a large variety of different pH-optima, ranging even in rat liver from 5.5 [19] to 8.0 [22]. With regard to plant enzymes possibly involved in cardenolide biosynthesis, only Δ5-3β-hydroxysteroid dehydrogenase/-Δ5/Δ4-ketosteroid isomerase [28] and progesterone 5α-reductase have been characterized in greater detail. The pH-optimum for Δ5-3β-hydroxysteroid dehydrogenase was found to be 8.0 [28], the pH-optimum for progesterone 5α-reductase 7.0 [3, 8].

The highest 3β-hydroxysteroid oxidoreductase activity was found at an incubation temperature of 25 °C, dropping markedly at 27 °C and higher temperatures. Progesterone 5α-reductase showed a temperature optimum at 40 °C [8]. At temperatures below 45 °C the resulting product 5α-pregnane-3,20-dione was subsequently reduced to 5α-pregnan-3β-ol-20-one, indicating the presence of 3β-hydroxysteroid oxidoreductase. Investigations of 3α/3β-hydroxysteroid oxidoreductases in mammalian organisms, for example in human epididymis, revealed maximum enzyme activity at a temperature of 46 °C [23]. 7α-Hydroxysteroid dehydrogenase of *Brevibacterium fuscum* [26] showed maximum activity between 25 °C and 30 °C.

According to the time course obtained for 5α-pregnane-3,20-dione reduction, 3β-hydroxysteroid oxidoreductase activity was linear for the first 4 h, reaching a plateau after 7 h of incubation time with no further product accumulation. Seidel [28] reported that Δ5-3β-hydroxysteroid dehydrogenase activity remained linear for the first 2 h of incubation. Experiments with microsomes from *Cheiranthus cheiri* and *Dioscorea deltoidea* ascertained that the reduction of progesterone to 5α-pregnane-3,20-dione reached maximum conversion after 3 h without producing any further metabolites [3]. An interesting situation is described for the microsomes of the human skin. Maximum conversion of testosterone was already observed after 20 min of incubation. Two enzymic reactions were triggered successively, namely testosterone 5α-reductase converting testosterone to 5α-dihydrotestosterone and 3α-hydroxysteroid reductase using 5α-dihydrotestosterone as the substrate. The first 10 min of incubation showed a linear increase of 5α-dihydrotestosterone resulting from testosterone 5α-reductase activity. The lack of androstandiol ensured that 3α-hydroxysteroid dehydrogenase was not active at that time. After 10 min a linear increase in 3α-hydroxysteroid dehydrogenase activity could be observed, converting the intermediate dihydrotestosterone to androstandiol [21]. A similar system is described for the microsomes of *Digitalis lanata* suspension cultures involving the two enzymes progesterone 5α-reductase and 3β-hydroxysteroid oxidoreductase [8]. Shortly after the initiation of the reaction 5α-pregnane-3,20-dione was found as a reaction product, but further incubation time resulted in the metabolite 5α-pregnan-3β-ol-20-one. The two reactions could almost be separated by taking advantage of the different temperature optima of the two enzymes.

3β-Hydroxysteroid oxidoreductase was found to be NAD(P)H_2-dependent, showing equivalent activity with either $NADPH_2$ or $NADH_2$. The apparent K_m-value for $NADPH_2$ ranged from 57 μM to 120 μM. 3α-/3β-Hydroxysteroid dehydrogenases investigated by [23] were also able to use both $NADPH_2$ and $NADH_2$, but preferred $NADPH_2$ to $NADH_2$. Slightly different results were obtained for 3α-/3β-hydroxysteroid dehydrogenases in rat liver microsomes [25]. 3α-Hydroxysteroid dehydrogenases preferred $NADPH_2$ to $NADH_2$. 3β-Hydroxysteroid dehydrogenases showed higher affinity to $NADH_2$. 5α-Reductases, for example progesterone 5α-reductase from microsomes of *Digitalis lanata* [8] and a progesterone reductase from microsomes of *Cheiranthus cheiri* and *Dioscorea deltoidea* [33] as well as testosterone

5α-reductase from rat prostate [29] and human skin microsomes [21], were found to require $NADPH_2$ but could not use $NADH_2$. Progesterone 5α-reductase from microsomes of *Digitalis lanata* [8] had an apparent K_m-value for $NADPH_2$ of 130 μM.

3β-Hydroxysteroid oxidoreductase from microsomes of *Digitalis lanata* exhibited an apparent K_m-value between 18.5 μM and 20 μM for 5α-pregnane-2,30-dione, and 5α-progesterone reductase from microsomes of *Digitalis lanata* had a K_m-value for progesterone of around 30 μM [8]. 3α- and 3β-Hydroxysteroid dehydrogenases from human epididymis showed an apparent K_m-value for their substrates of 80 μM [23].

Investigations concerning the localization of 3β-hydroxysteroid oxidoreductase showed that the enzyme activity could be divided in two different parts, namely a soluble and a membrane-bound enzyme activity. According to the data obtained by linear sucrose density gradient centrifugation, the major part of the enzyme activity turned out to be soluble, whereas a distinctly smaller part seemed to be associated with the endoplasmic reticulum. At least two different 3α-hydroxysteroid oxidoreductase activities were found in rat hypothalamus, exhibiting different subcellular localization and cofactor requirements [24]. Most of the $NADPH_2$-dependent 3α-hydroxysteroid oxidoreductase activity was thought to be located in the cytosol, whereas the $NADH_2$-dependent 3α-hydroxysteroid oxidoreductase was enriched in the plasma membrane. From the soluble fraction of rat liver homogenates a 3α-hydroxysteroid dehydrogenase was isolated, which preferred $NADPH_2$ to $NADH_2$ [22]. Björkhem and co-workers [25] reported on different 3α- and 3β-hydroxysteroid dehydrogenases of rat liver microsomes, being membrane-bound. In the case of 5α/β-reductases, the results obtained for their location are slightly different. Progesterone 5α-reductase from *Digitalis lanata* suspension cultures [8] was associated exclusively with the endoplasmic reticulum. Rat epididymal Δ^4-steroid 5α-reductase converting testosterone to dihydrotestosterone was characterized as a membrane-bound enzyme found in both nuclear and microsomal fractions [20]. McGuire and Tomkins reported on 5β-reductases in the soluble fraction of rat liver [30] and a variety of 5α-reductases [31] in the microsomal fraction of rat liver. Additionally, in rat liver a Δ^4-ketosteroid 5β-reductase was found to be soluble [22].

The most interesting question with regard to cardenolide biosynthesis is the function of 3β-hydroxysteroid oxidoreductase; it must remain unanswered at the moment. Assuming that the enzyme is part of the biosynthetic pathway of cardenolides, the existence of an isomerase converting 5α-derivatives into 5β-derivatives has to be postulated. Taking into account that 3β-hydroxysteroid oxidoreductase is involved in other parts of steroid biosynthesis this would explain its lack of ability to form 5β-derivatives and its maximum activity during the growth phase of the cell culture where a lot of new membrane material has to be formed. Further knowledge on the interrelation between 5α-progesterone reductase and 3β-hydroxysteroid oxidoreductase is necessary to ensure that both enzyme reactions found do not result from the activity of one single enzyme protein. It is also of particular interest to detect an isomerase which forms 5β-derivatives or an enzyme which is able to synthesize 5β-derivatives of pregnanes from progesterone.

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