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METABOLISM OF 3β-HYDROXY-5α- AND 3β-HYDROXY-5β-PREGNAN-20-ONE BY LEAF HOMOGENATES

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Key Word Index—*Cheiranthus cheiri*; *Nerium oleander*; *Strophanthus kombé*; *Digitalis purpurea*; *Corchorus capsularis*; 3β-hydroxy-5α-pregnan-20-one; 3β-hydroxy-5β-pregnan-20-one; 5α-pregnane-3,20-dione; 5α-pregnane-3β,20β-diol; 5β-pregnane-3,20-dione; 5β-pregnane-3β,20α-diol; 5α-pregnane-3β,20α-diol; 20β-hydroxy-5α-pregnan-3-one; progesterone.

Abstract—Leaf homogenates of *Cheiranthus cheiri*, *Nerium oleander*, *Strophanthus kombé*, *Digitalis purpurea*, and *Corchorus capsularis* were examined for their ability to metabolize 3β-hydroxy-5α- and 3β-hydroxy-5β-pregnan-20-one-[16,17-^{3}H]. All leaf homogenates were able to metabolize 3β-hydroxy-5α-pregnan-20-one to 5α-pregnane-3,20-dione and 5α-pregnane-3β,20β-diol. After a 2 hr incubation, *C. cheiri* converted 80% of the extractable radioactive material to 5α-pregnane-3β,20β-diol. *N. oleander* transformed 42% to 5α-pregnane-3,20-dione. Lesser amounts of both metabolites were formed from 3β-hydroxy-5α-pregnan-20-one by the other tissues. In addition, small amounts of progesterone, 20β-hydroxy-5α-pregnan-3-one, and 5α-pregnane-3β,20α-diol were produced from 3β-hydroxy-5α-pregnan-20-one by various tissues. Little of the 3β-hydroxy-5β-pregnan-20-one was metabolized. After a 2 hr incubation, the highest conversion of 3β-hydroxy-5β-pregnan-20-one to 5β-pregnane-3β,20β-dione was achieved with *S. kombé*. Only *D. purpurea* was able to transform 3β-hydroxy-5β-pregnan-20-one into 5β-pregnane-3β,20β-diol. No other metabolites of 3β-hydroxy-5β-pregnan-20-one were detected.

INTRODUCTION

A number of studies have recently been published on the metabolism of various pregnane and pregnene derivatives by plants and plant tissue cultures. Graves and Smith [1] first reported that cell cultures of *Digitalis purpurea*, *D. lanata*, and *Nicotiana tabacum* metabolized pregnenolone to progesterone, while *D. lanata* also yielded 5α-pregnane-3,20-dione. Progesterone was readily metabolized by these and several other tissue cultures to 5α-pregnane-3,20-dione and 3β-hydroxy-5α-pregnan-20-one [1]. In addition, *Rosa* sp. tissue cultures also formed 20α-hydroxy-pregn-4-en-3-one and 20β-hydroxy-pregn-4-en-3-one from progesterone [1]. Pregnenolone may be metabolized by two pathways, one involving reduction at C-20 to give the corresponding 20α- or 20β- derivatives, and the other involving reduction of the B ring to give 5α-pregnane-3,20-dione which is subsequently metabolized to 3β-hydroxy-5α-pregnan-20-one. Microsomes from *Dioscorea deltoidea* and *Cheiranthus cheiri* suspension cultures can readily convert progesterone to 5α-pregnane-3,20-dione [2]. The system requires NADPH but does not utilize NADH [2].

Callus cultures of *Sophora angustifolia* have been reported by Furuya *et al.* [3] to convert pregnenolone and progesterone to 3β-hydroxy-5α-pregnan-20-one and 3β-palmitoxy-5α-pregnan-20-one. These authors [4] also found that *N. tabacum* cultures metabolized progesterone to 3β-palmitoxy-5α-pregnan-20-one.

We have reported the transformation of progesterone-[4-^{14}C] to 3β-hydroxy-5α-pregnan-20-one

and 5α-pregnane-3β,20β-diol by suspension cultures of *D. deltoidea*. Both transformation products were present as conjugates in this plant tissue culture [5]. Furuya *et al.* [6] subsequently extended these observations and have shown that suspension cultures of *D. purpurea* are capable of metabolizing progesterone to 5α-pregnane-3,20-dione, 3β-hydroxy-5α-pregnan-20-one and its glucoside, 5α-pregnane-3β,20α-diol and its glucoside, 5α-pregnane-3β,20β-diol and its glucoside, 20α-hydroxy-pregn-4-en-3-one and its glucoside, and 20β-hydroxy-pregn-4-en-3-one and its glucoside. The metabolism of 3β-hydroxy-5α-pregnan-20-one-[16,17-^{3}H] by *D. deltoidea* tissue suspension cultures to 5α-pregnane-3β,20α-diol and 5α-pregnane-3β,20β-diol has recently been observed [7].

We have previously reported the conversion of pregnenolone into progesterone and 5α-pregnane-3,20-dione, and the transformation of progesterone into 5α-pregnane-3,20-dione and 3β-hydroxy-5α-pregnan-20-one by leaf homogenates of cardenolide producing plants [8].

The bioconversion of progesterone to 5α- and 5β-pregnane-3,20-dione and 3β-hydroxy-5α- and 3β-hydroxy-5β-pregnan-20-one by *D. lanata* leaves has been shown [9]. Furthermore, *D. lanata* will incorporate 5β-pregnane-3,20-dione and 3β-hydroxy-5β-pregnan-20-one into cardenolides [10]. However, most of the metabolites of pregnenolone and progesterone by leaves of cardenolide producing plants, leaf homogenates, and tissue cultures are 5α-compounds, although both pregnenolone and progesterone are incorporated into cardenolides [9–16] which are 5β derivatives.

Table 2. Metabolism of 3β-hydroxy-5β-pregnan-20-one-[16,17-^{3}H] by leaf homogenates

Leaf source	% Radioactivity recovered as 5β-Pregnane-3,20-dione	5β-Pregnane-3β,20β-diol
Control	0·32 ± 0·12	0·32 ± 0·19
Cheiranthus cheiri	1·21 ± 0·13	0·39 ± 0·06
C. cheiri, heated	0·22 ± 0·18	0·43 ± 0·22
Nerium oleander	0·76 ± 0·24	0·36 ± 0·13
Strophanthus kombé	1·28 ± 0·12	0·39 ± 0·11
Digitalis purpurea	0·80 ± 0·13	3·19 ± 0·28
Corchorus capsularis	0·87 ± 0·15	0·25 ± 0·09

Homogenates from 1 g of leaves were incubated with 1·0 μCi 3β-hydroxy-5β-pregnan-20-one-[16,17-^{3}H] for 2 hr at 30° in the presence of an NADPH generating system as described in the Experimental. Each experiment was conducted in triplicate. Each flask was extracted, and the extract co-chromatographed in 4 solvent systems, followed by liquid scintillation counting.

In order to continue our investigation on the metabolism of steroids by cardenolide producing plants, we have prepared 16,17-^{3}H labeled 3β-hydroxy-5α-pregnan-20-one and 3β-hydroxy-5β-pregnan-20-one and have examined the transformation of these substrates by various leaf homogenates.

RESULTS

Leaf homogenates of *C. cheiri*, *N. oleander*, *S. kombé*, *D. purpurea*, and *C. capsularis* were examined for their ability to metabolize 3β-hydroxy-5α- and 3β-hydroxy-5β-pregnan-20-one. The results are given in Tables 1 and 2 for the two substrates. As can be seen in Table 1, these leaf homogenates

Table 1. Metabolism of 3β-hydroxy-5α-pregnan-20-one-[16,17-^{3}H] by leaf homogenates

Leaf source	% Radioactivity recovered as 5α-Pregnane-3,20-dione	Progesterone	5α-Pregnane-3β,20β-diol	20β-Hydroxy-5α-pregnan-3-one	5α-Pregnane-3β,20α-diol
Control	0·60 ± 0·22	0·27 ± 0·12	0·45 ± 0·27	0·71 ± 0·35	0·46 ± 0·21
Cheiranthus cheiri	3·46 ± 0·54	0·91 ± 0·34	79·80 ± 6·68	0·32 ± 0·16	1·35 ± 0·43
C. cheiri, heated	0·31 ± 0·09	0·11 ± 0·09	0·42 ± 0·17	0·37 ± 0·32	0·23 ± 0·19
Nerium oleander	42·47 ± 5·56	4·16 ± 1·33	2·69 ± 0·74	7·11 ± 0·65	0·57 ± 0·26
Strophanthus kombé	6·96 ± 1·04	6·51 ± 0·73	0·72 ± 0·25	6·17 ± 2·54	0·37 ± 0·17
Digitalis purpurea	5·77 ± 1·26	2·28 ± 0·58	5·49 ± 1·12	0·83 ± 0·24	0·33 ± 0·21
Corchorus capsularis	32·15 ± 6·32	18·12 ± 4·57	4·33 ± 0·38	1·91 ± 0·11	4·69 ± 1·14

Homogenates from 1 g of leaves were incubated with 1·0 μCi 3β-hydroxy-5α-pregnan-20-one-[16,17-^{3}H] for 2 hr at 30° in the presence of an NADPH generating system. Incubation mixtures were extracted and assayed as described in the Experimental. Each expt was conducted in triplicate, and each value represents the mean with the standard deviation.

in 2 hr were able to metabolize 3β-hydroxy-5α-pregnan-20-one to a number of products, namely, 5α-pregnane-3,20-dione, progesterone (4-pregnen-3,20-dione), 5α-pregnane-3β,20β-diol, 20β-hydroxy-5α-pregnan-3-one and small amounts of 5α-pregnane-3β,20α-diol.

C. cheiri was metabolically most active, converting 80% of the extractable radioactivity from 3β-hydroxy-5α-pregnan-20-one-[16,17-^{3}H] to 5α-pregnane-3β,20β-diol. The *C. cheiri* metabolized only very small amounts of this substrate to the other metabolites. No products were formed when a heat treated homogenate was used.

N. oleander was able to transform *ca* 42% of the 3β-hydroxy-5α-pregnan-20-one into 5α-pregnane-3,20-dione, with only small amounts of the other products being formed. *N. oleander* leaf homogenates did yield the highest conversion to 20β-hydroxy-5α-pregnan-3-one, which amounted to 7% of the recovered radioactivity.

Under our experimental conditions, leaf homogenates of *C capsularis* produced the greatest amounts of progesterone and 5α-pregnane-3β,20α-diol from the 3β-hydroxy-5α-pregnan-20-one-[16,17-^{3}H], consisting of 18 and 4·7%, respectively, of the recovered radioactivity.

D. purpurea leaf homogenates metabolized the 3β-hydroxy-5α-pregnan-20-one to the least extent, with *ca* 5·5% of both 5α-pregnane-3,20-dione and 5α-pregnane-3β,20β-diol being formed.

Table 3. Co-crystallization of metabolites from 3β-hydroxy-5α-pregnan-20-one to constant specific activity

Metabolite	Solvent	cpm/mg
5α-Pregnane-3,20-dione	Chloroform–hexane	2512 ± 58
	Chloroform–methanol–water	2467 ± 63
	95% Ethanol	2609 ± 72
5α-Pregnane-3β,20β-diol	Chloroform–hexane	1937 ± 73
	Chloroform–methanol–water	2004 ± 38
	95% Ethanol	1987 ± 52
Progesterone	Chloroform–hexane	897 ± 26
	Chloroform–methanol–water	836 ± 31
	Methanol–ether	856 ± 27

5α-Pregnane-3,20-dione, 5α-pregnane-3β,20β-diol, and progesterone were isolated by preparative TLC following incubation of leaf homogenates with 5α-pregnan-3β-ol-20-one-[16,17-^{3}H]. Each was recrystallized following the addition of the corresponding non-radioactive reference material. Each value is the mean with the standard deviation for 3 determinations.

When 3β-hydroxy-5β-pregnan-20-one-[16,17-^{3}H] was employed as the substrate, little metabolism occurred (Table 2). Only *D. purpurea* was able to convert it to 5β-pregnane-3β,20β-diol, with only 3% of the substrate being transformed in 2 hr. *C. cheiri* and *S. kombé* gave the greatest metabolism to 5β-pregnane-3,20-dione, with a maximum conversion of less than 1·3% in 2 hr. No other products were detected using this substrate.

All products were identified by co-chromatography in 4 different solvent systems. In addition, due to the large amounts of 5α-pregnane-3,20-dione, 5α-pregnane-3β,20β-diol, and progesterone formed, these products were isolated by PLC and co-crystallized to constant specific activity. The results and solvents used are given in Table 3.

DISCUSSION

Our results clearly demonstrate that 3β-hydroxy-5α-pregnan-20-one can readily serve as substrate for the steroid metabolizing enzymes in leaf homogenates of a number of cardenolide producing plants. However, 3β-hydroxy-5β-pregnan-20-one is an extremely poor substrate under identical conditions. Cardenolides are 5β-derivatives. If cardenolide biosynthesis is a major steroid metabolic route in these plants, one would expect the 3β-hydroxy-5β-pregnan-20-one to constitute a better substrate than the corresponding 5α-isomer. Such is not the case.

Our present *in vitro* results support previous *in vivo* [9] and *in vitro* [8] observations, and conclusively demonstrate that 5α-pregnane derivatives are preferentially metabolized over 5β-pregnane analogs.

EXPERIMENTAL

Homogenates. Plants of *Cheiranthus cheiri*, *Digitalis purpurea*, *Strophanthus kombé*, *Nerium oleander*, and *Corchorus olitorius* were greenhouse-grown for 6–8 months. Homogenates (*ca* 30%) were prepared as previously described, in a buffer solution containing 0·25 M sucrose, 0·05 M tris chloride (pH 7·4), 0·05 M $MgCl_2$, 0·045 M mercaptoethanol, 0·003 M cysteine HCl, and 1 mg/ml bovine serum albumin fraction V [8].

Radioisotopes. 3β-Hydroxy-5α-pregnan-20-one-[16,17-^{3}H] and 3β-hydroxy-5β-pregnan-20-one-[16,17-^{3}H] were prepared from the corresponding Δ^{16} compounds by catalytic reduction of the double bond with tritium gas. Crude tritiated products

were purified on Si gel H plates developed with CH_2Cl_2–MeOH (96:5·5), and both products had a radiochemical purity greater than 98%. The sp act of the 5α-compound was *ca* 1·6 Ci/mmol and that of the 5β-compound was *ca* 2·1 Ci/mmol.

Incubations. Homogenate equivalent to 1 g leaves was incubated with 1·0 μCi 3β-hydroxy-5α- or 3β-hydroxy-5β-pregnan-20-one-[16,17-^{3}H] in 5·0 ml homogenization buffer which also contained 1·5 mg $NADP^+$, 7·0 mg glucose-6-phosphate and 2·5 units glucose-6-phosphate dehydrogenase. Substrate was added to all experimental and control flasks in 0·10 ml 70% EtOH. Control flasks contained homogenization buffer without homogenate to give a final 5·0 ml vol. Heated *C. cheiri* control flasks contained homogenate that had been heated at 100° for 10 min. All incubations were conducted at 30° on a waterbath shaker for 2 hr, aerating with 95% O_2–5% CO_2. Each reaction mixture was immediately extracted with EtOAc–HOAc (100:1). The organic phase was dried over dry sodium sulphate, and evaporated to dryness under vacuum. The efficiency of extraction of radioactivity for both substrates was *ca* 70–90%.

Metabolite identification. *Ca* 5000–10000 cpm from each of the extracts was co-chromatographed with reference standards on Si gel H (Brinkmann) plates which were divided into 2 cm wide columns, and developed in one of the following solvent systems: CH_2Cl_2–CH_3OH (96:5·5); $CHCl_3$–Me_2CO (9:1); $CHCl_3$–MeOH–H_2O (188:12:1); or heptane–EtOAc (5:2, developed 7×). Reference standards were located by exposure of the plates to I_2 vapors. Radioactivity associated with each reference standard, as well as the remainder of each column, was determined by liquid scintillation counting employing the methods previously described [8]. The results for the studies involving the metabolism of 3β-hydroxy-5α-pregnan-20-one-[16,17-^{3}H] are given in Table 1, while the identical studies utilizing 3β-hydroxy-5β-pregnan-20-one-[16,17-^{3}H] are given in Table 2. Each reaction flask was set up in triplicate, and each extract was co-chromatographed in the four solvent systems. Each value in Tables 1 and 2 represent the mean of 12 values with the standard deviation. The metabolites from 3β-hydroxy-5α-pregnan-20-one corresponding to 5α-pregnane-3,20-dione, progesterone, and 5α-pregnane-3β,20β-diol were isolated from pooled extracts by PLC on Si gel $PF_{254+366}$ plates with CH_2Cl_2–MeOH (96:5·5). Each metabolite was co-crystallized to constant sp act after the addition of authentic nonradioactive material to the metabolite. The solvents employed and the results are given in Table 3. Each value represents the sp act in cpm/mg of triplicate samples following each recrystallization.

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