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PREGNENOLONE AND PROGESTERONE METABOLISM BY CARDENOLIDE PRODUCING PLANTS

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Key Word Index—*Cheiranthus cheiri*; *Digitalis purpurea*; *Strophanthus kombé*; *Corchorus olitorius*; pregnenolene; progesterone; 5α-pregnan-3,20-dione; cardenolides.

Abstract—Leaf homogenates of *Cheiranthus cheiri* and *Digitalis purpurea* rapidly metabolize pregnenolone to progesterone and 5α-pregnan-3,20-dione. Under identical conditions *Strophanthus kombé* yielded only small amounts of progesterone. Leaf homogenates of *C. cheiri*, *D. purpurea*, *S. kombé* and *Corchorus olitorius* metabolized progesterone to 5α-pregnan-3,20-dione, with small amounts of 5α-pregnan-3β-ol, 20-one. With both substrates, *C. cheiri* was most active. No 5β metabolites of either pregnenolone or progesterone could be detected.

INTRODUCTION

ALTHOUGH pregnenolone and progesterone have been shown to be incorporated into cardenolides by various plants,[1-5] little is known of the intervening metabolic steps. *Digitalis lanata* leaves will convert 5β-pregnan-3β,14β-diol-one into cardenolides, but Δ^5-14-desoxydigitoxigenin and 14-anhydrodigitoxigenin are not incorporated.[6] Pregnenolone is metabolized to 5β-pregnan-3,20-dione as well as cardenolides.[2] *D. lanata* leaves treated with progesterone-4-^{14}C in 5 weeks will give rise to 5α-pregnan-3,20-dione, 5β-pregnan-3,20-dione, 5α-pregnan-3β-ol-20-one, 5β-pregnan-3β-ol-20-one, and several cardenolides.[4] Approximately 6 times as much radioactivity is found in 5α- as compared to 5β-pregnan-3,20-dione, and approximately 800 times as much radioactivity in the 5α- as compared to the 5β-pregnan-3β-ol-20-one.[4] The authors discount the significance of these 5α-isomers in cardenolide biosynthesis.[4] *D. purpurea* and other tissue suspension cultures on incubation with progesterone for 2 weeks yielded 5α-pregnan-3,20-dione and 5α-pregnan-3β-ol-20-one.[7] No 14α-hydroxyprogesterone[8] or deoxycorticosterone[9] is incorporated into digitoxigenin by intact *D. lanata* leaves. A direct replacement of the 16β-hydrogen of pregnenolone by a hydroxyl group has been shown to occur in the biosynthesis gitoxigenin in *D. purpurea* plants.[10] Both 5β-pregnan-3,20-dione and 5β-pregnan-3β-ol-20-one are incorporated into the cardenolides of *D. lanata*, while 5β-pregnan-3β,12β-diol-20-one is a good precursor of digoxigenin.[11] 5β-Δ^{14}-Pregnan-3β-ol-20-one is not a precursor of digitoxigenin.[11]

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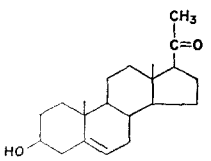
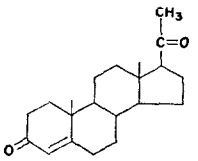
In nearly all of the preceeding studies, radioactive precursors were administered to intact plants for periods of from 2 to 9 weeks. We have investigated the ability of leaf homogenates from cardenolide producing plants to metabolize pregnenolone-7-^{3}H and progesterone-4-^{14}C during a 2-hr incubation period.

RESULTS

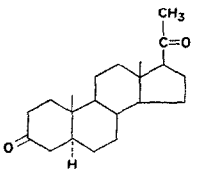
Leaf homogenates, corresponding to 1 g of leaves from cardenolide producing plants, were incubated for 2 hr with either 1 μCi 7-^{3}H-pregnenolone (I) or 0·10 μCi 4-^{14}C-progesterone (II) in a buffered medium containing an NADPH generating system. The reaction mixtures were extracted, dried, and examined by TLC, followed by liquid scintillation counting of appropriate fractions.



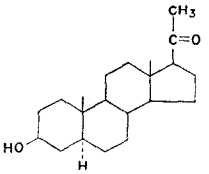
(I) Pregnenolone



(II) Progesterone



(III) 5α-Pregnan-3,20-dione



(IV) 5α-Pregnan-3β-ol, 20-one

The results from incubations with 7-^{3}H-pregnenolone are given in Table 1. At the end of 2 hr *Cheiranthus cheiri* had biotransformed approximately 70% of this substrate. Over 50% was present as ^{3}H-progesterone (II) and the remainder as ^{3}H-5α-pregnan-3,20-dione (III). Leaf homogenates of *Digitalis purpurea* yielded approximately 30% ^{3}H-progesterone from 7-^{3}H-pregnenolone with very little ^{3}H-5α-pregnan-3,20-dione being formed. *Strophanthus kombé* in 2 hr produced less than 10% ^{3}H-progesterone and no ^{3}H-5α-pregnan-3,20-dione as compared to control flasks devoid of leaf homogenate.

TABLE 1. PREGNENOLONE-7-^{3}H METABOLISM BY LEAF HOMOGENATES

Leaf source	% Radioactivity present in Progesterone	% Radioactivity present in 5α-Pregnan-3-20-dione
Control	3·56 ± 0·47	2·90 ± 0·13
Cheiranthus cheiri	52·30 ± 2·59	17·80 ± 1·60
Digitalis purpurea	31·87 ± 1·93	4·85 ± 0·41
Strophanthus kombé	11·04 ± 0·43	2·92 ± 0·23

The homogenate from 1 g of leaves was incubated for 2 hr with 1·0 μCi pregnenolone-7-^{3}H. The extraction and assay procedure is as described in the Experimental. Each value represents the average of 3–4 determinations with the standard deviation.

The two metabolites from 7-^{3}H-pregnenolone were isolated by preparative TLC on thick silica gel H plates, and removed from the silica gel by Soxhlet extraction. Each was co-crystallized with the corresponding non-radioactive compound to constant specific activity from the solvents given in Table 2, thereby confirming the identity of the two metabolites.

The results of the incubation of 4-^{14}C-progesterone with leaf homogenates for 2 hr at 30° is given in Table 3. Again leaf homogenates of *C. cheiri* were most active, with approximately 19% 5α-pregnan-3,20-dione being present after 2 hr. A small amount of ^{14}C-5α-pregnan-3β-ol-20-one was also formed. *S. kombé* demonstrated greater metabolic activity

TABLE 2. CO-CRYSTALLIZED PREGNENOLONE-7-^{3}H METABOLITES FROM *Cheiranthus cheiri* LEAF HOMOGENATES

Metabolite	Solvent	CPM/MG
Progesterone	Methanol	3575 ± 85
	Methylene dichloride–methanol–water	3461 ± 62
	Methylene dichloride–light petroleum	3512 ± 50
	Methylene dichloride–light petroleum	3523 ± 24
5α-Pregnan-3,20-dione	Ethanol (95%)	685 ± 23
	Methylene dichloride–methanol–water	628 ± 10
	Methylene dichloride–light petroleum	630 ± 10
	Methylene dichloride–light petroleum	625 ± 11

The radioactive metabolites of ^{3}H-pregnenolone following incubation with *C. cheiri* homogenates for 2 hr were isolated by preparative TLC on silica gel H plates developed with CH_2Cl_2–CH_3OH (97:3) (developed 2×). The metabolites were recovered from the silica gel by Soxhlet extraction with CH_2Cl_2, and co-crystallized with the corresponding non-radioactive reference materials.

than either *D. purpurea* or *Corchorus olitorius*, although with all three tissues the amounts of ^{14}C-5α-pregnan-3,20-dione (III) and ^{14}C-5α-pregnan-3β-ol-20-one (IV) formed were not extensive.

TABLE 3. METABOLISM OF 4-C^{14}-PROGESTERONE BY PLANT LEAF HOMOGENATES

	% Recovered radioactivity as	
Tissue source	5α-Pregnan-3,20-dione	5α-Pregnan-3β-ol-20-one
Control	0·27 ± 0·15	0·30 ± 0·20
C. cheiri	19·46 ± 3·16	1·36 ± 0·47
D. purpurea	1·79 ± 0·54	2·39 ± 0·88
C. olitorius	0·67 ± 0·26	2·05 ± 1·02
S. kombé	6·93 ± 2·02	6·13 ± 1·73

Homogenates from 1 g of 4–6-month-old leaves were incubated with 0·10 μCi 4-^{14}C-progesterone for 2 hr at 30° in the presence of an NADPH generating system. The reaction mixtures were extracted and assayed as described in the Experimental. Each value represents the average of 3–4 determinations with the standard deviations.

The two metabolites resulting from 4-^{14}C-progesterone were isolated by preparative TLC and recrystallized to constant specific activity with the corresponding non-radioactive

chemical. The solvents used and the results are given in Table 4. The identity of these two metabolites was therefore confirmed.

TABLE 4. CO-CRYSTALLIZED PROGESTERONE-4-^{14}C METABOLITES FROM *Cheiranthus cheiri* LEAF HOMOGENATES

Metabolite	Solvent	Specific activity (CPM/MG)
5α-Pregnan-3,20-dione	Ethanol (95%)	1185 ± 10
	Methylene dichloride–methanol–water	1225 ± 15
	Methylene dichloride–light petroleum	1215 ± 25
	Methylene dichloride–light petroleum	1180 ± 40
5α-Pregnan-3β-ol-20-one	Hexane–acetone	552 ± 11
	Methanol–water	522 ± 16
	Ether–acetone–water	527 ± 18
	Ethyl acetate	528 ± 8

5α-Pregnan-3-20-dione and 5α-pregnan-3β-ol-20-one were isolated by preparative TLC following the incubation of *C. cheiri* leaf homogenates with 4-C^{14}-progesterone. The metabolites were isolated by preparative TLC on silica gel H plates developed with CH_2Cl_2–CH_3OH (97:3) (developed 2×) recovered from the silica gel by Soxhlet extraction with CH_2Cl_2, and recrystallized with the corresponding non-radioactive reference material.

DISCUSSION

Most investigators of steroid metabolism in plants have relied on the application of the steroid to be studied onto the leaves of intact plants, and allowing 2–9 weeks before extraction.[1–6,8–11] We have shown that significant metabolism of pregnenolone and progesterone can occur in as little as 2 hr under suitable conditions employing leaf homogenates.

From the results given in Tables 1 and 3, the metabolic sequence appears to be a pregnenolone → progesterone → 5α-pregnan-3,20-dione → 5α-pregnan-3β-ol-20-one. One or both of the latter two products have been reported following the incubation of progesterone in intact plants,[4] with plant tissue cultures,[7,12,13] and with plant microsomes.[14] The conversion of progesterone to 5α-pregnan-3,20-dione appears to be the rate limiting step in this sequence, most notably with homogenates of *D. purpurea*, *C. olitorius* and *S. kombé*, where the amount of 5α-pregnan-3β-ol-20-one exceeds the amount of 5α-pregnan-3,20-dione.

Cardenolides are 5β derivatives, and it is surprising that a number of investigators have observed 5α derivatives following plant tracer feeding studies with progesterone and pregnenolone, with little or no appearance of 5β derivatives. Either the cardenolide biosynthetic pathway is not a major steroid metabolic route in these plants, or the 5α to 5β inversion occurs at some later step in the sequence. However, progesterone → 5β-pregnan-3,20-dione would be a logical point at which this could occur. It is possible that if very small amounts of 5β derivatives were formed in our *in vitro* metabolic system, they may have gone undetected. If 5α-pregnan-3β-ol-20-one is not involved in cardenolide biosynthesis, questions relating to its biochemical significance and metabolic fate are yet to be answered.

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EXPERIMENTAL

Homogenates. Leaf homogenates (30%) of 4–6-month-old greenhouse grown *C. cheiri*, *D. pupurea*, *S. kombé*, and *C. olitorius* were prepared as previously described, in a buffer medium containing 0·25 M sucrose, 0·05 M Tris chloride pH 7·4, 0·05 M $MgCl_2$, 0·045 M mercaptaethanol, 0·003 M cysteine HCl, and 1 mg/ml bovine serum albumin fraction V (Sigma Chemical Co.).[15–17]

Incubations. Pregnenolone-7-^{3}H (New England Nuclear) with a specific activity of 17 Ci/μmol showed greater than 97% radiochemical purity upon TLC. Progesterone-4-^{14}C (Nuclear Chicago) had a specific activity of 36·1 μCi/μmol and was more than 99% radiochemically pure. The homogenate from 1 g of leaves was incubated either with 0·10 μCi progesterone-4-^{14}C or 1·0 μCi pregnenolone-7-^{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. Both substrates were added to the reaction flasks in 0·10 ml 70% EtOH. All incubation flasks were maintained at 30° on a water bath shaker, aerating with 95% O_2–5% CO_2. Each reaction mixture was extracted with EtOAc–HOAc (100:1) as previously described after a 2-hr incubation.[15–17] The organic phase of each was dried over anhydrous Na_2SO_4, and evaporated to dryness under vacuum. The extraction efficiency for pregnenolone-7-^{3}H and its metabolites was routinely 70–90%, and for progesterone-4-^{14}C and its metabolites was 85–95%.

Identification of metabolites. Aliquots of the extracts containing approximately 10 000 cpm were co-chromatographed with reference standards on silica gel H (Brinkman) plates which were divided into 2 cm wide columns. The solvent systems used were CH_2Cl_2–CH_3OH (97:3) (developed 2×), $CHCl_3$–acetone (9:1), and CH_2Cl_2–CH_3OH–H_2O (188:12:1). Reference standards were located with iodine vapor. After evaporation of the iodine from the plates, the areas which corresponded to the reference standards were transferred to counting vials with the aid of a razor blade. The silica gel from the remainder of each 2 cm wide column were similarly transferred to counting vials, and Omnifluor (New England Nuclear) or toluene counting solution was added to each vial. All vials were counted for 20 min in a Beckman LS-100 liquid scintillation counter equipped with an external standard. Backgrounds of 10–15 cpm and 30–35 cpm were routinely obtained for ^{14}C and ^{3}H, respectively. The two metabolites of pregnenolone (I) that co-chromatographed with progesterone (II) and 5α-pregnan-3,20-dione (III) were isolated by preparative TLC using thick (0·5 mm) silica gel H plates and the solvent system CH_2Cl_2–CH_3OH (97:3). The metabolites were removed from the silica gel by Soxhlet extraction with CH_2Cl_2 for 18 hr. The two metabolites of progesterone that co-chromatographed with 5α-pregnan-3,20-dione (III) and 5α-pregnan-3β-ol-20-one (IV) were isolated by preparative TLC as described above for pregnenolone metabolites. Extracts from a number of *C. cheiri* incubation reactions were pooled and subjected to TLC to isolate the above metabolites. The metabolites corresponding to ^{3}H-progesterone, ^{3}H-5α-pregnan-3-20-dione, ^{14}C-5α-pregnan-3,20-dione and ^{14}C-5α-pregnan-3β-ol-20-one were recrystallized to constant specific activity following the addition of 50–100 mg of the corresponding authentic non-radioactive chemical. The solvents used for the recrystallization, and the results are given in Tables 2 and 4. Each value represents the specific activity in cpm/mg of triplicate samples following each recrystallization.

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