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METABOLISM OF 4-CHOLESTEN-3-ONE TO 5α-CHOLESTAN-3-ONE BY LEAF HOMOGENATES

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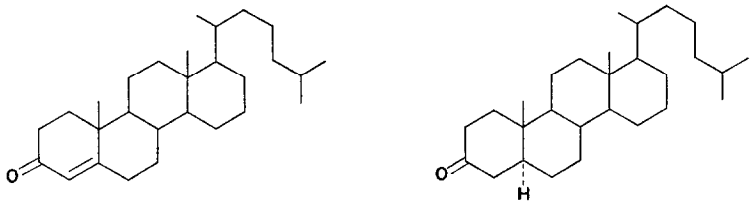
Key Word Index—*Digitalis purpurea*; *Cheiranthus cheiri*; *Strophanthus kombe*'; 4-cholesten-3-one; 5α-cholestan-3-one.

Abstract—Young leaf homogenates of *Digitalis purpurea*, *Cheiranthus cheiri* and *Strophanthus kombe*' were incubated at 30° for 2 hr with 4-cholesten-3-one-[4-^{14}C] in a buffered medium containing an NADPH generating system. A single identical metabolite was observed with each tissue. The metabolite was identified by co-chromatography and co-crystallization to constant specific activity as 5α-cholestan-3-one. Leaf homogenates of *D. purpurea* exhibited the greatest metabolic activity. Addition of non-radioactive progesterone effectively competed with the radioactive substrate, markedly reducing the yield of 5α-cholestan-3-one.

INTRODUCTION

ALTHOUGH cholesterol has been isolated from a relatively small number of plants, it is incorporated into a variety of plant steroids.[1–4] However, few of the intervening steps in the active metabolic routes of cholesterol in plants have been examined.

We have previously shown that leaf and tissue culture homogenates of *Cheiranthus* and *Digitalis* can readily metabolize ^{14}C-cholesterol to 4-cholesten-3-one, within 2 hr.[5] Similar results have been obtained using sitosterol as the substrate.[6] The occurrence of 4-cholesten-3-one, and its formation from cholesterol in the red alga *Meristotheca papulosa* following a 40-hr incubation, has also recently been demonstrated.[7] Johnson *et al.*[8] have isolated 4-cholesten-3-one as the major transformation product after incubating *Solanum tuberosum*



(I) 4-Cholesten-3-one (II) 5α-Cholestan-3-one

SCHEME 1.

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leaves with ^{14}C-cholesterol for 5 days, and have shown that 4-cholesten-3-one can be further metabolized to cholestan-3β-ol and possibly cholestan-3α-ol.

We have examined the subsequent metabolism of 4-cholesten-3-one (I) by leaf homogenates of several steroid-producing plants, employing a buffered medium and 2-hr incubations, demonstrating 5α-cholestan-3-one (II) as the major product.

RESULTS

Homogenates from young leaves of *Digitalis purpurea*, *Cheiranthus cheiri* and *Strophanthus kombe*' were incubated with 4-cholesten-3-one-[4-^{14}C] in a buffered medium containing an exogenously added NADPH generating system. Following incubation, the reaction mixtures were extracted and examined by TLC. After a 2-hr incubation, a single major metabolite was observed with each tissue. Co-chromatography of the metabolite with a variety of standards in three TLC systems, revealed that for all three tissues, the metabolite had R_fs identical to 5α-cholestan-3-one.

TABLE 1. 5α-CHOLESTAN-3-ONE FROM 4-CHOLESTEN-3-ONE-[4-^{14}C]

Leaf homogenate source	Estracted radioactivity as 5α-cholestan-3-one (%)	Leaf homogenate source	Extracted radioactivity as 5α-cholestan-3-one (%)
Control	0·38 ± 0·24	*Cheiranthus cheiri*	8·51 ± 1·49
Digitalis purpurea	16·03 ± 2·71	*Strophanthus kombe*'	9·92 ± 0·42
Heated *D. purpurea*	0·57 ± 0·11		

Homogenates from 1 g of the above plant leaves were incubated with 0·10 μCi 4-cholesten-3-one-[4-^{14}C] in a buffered system containing an NADPH-generating system. The results of 2 hr incubations at 30° are reported. The average of 4–6 determinations with the standard deviation is given. The results are expressed as the percentage of total extractable ^{14}C present as 5α-cholestan-3-one. The heated *D. purpurea* leaf homogenate was boiled for 10 min in a water bath.

The results of incubations of leaf homogenates of *D. purpurea*, *C. cheiri* and *S. kombe*' with 4-cholesten-3-one are given in Table 1. *D. purpurea* exhibited the greatest activity and was able to convert about 16% of the substrate to 5α-cholestan-3-one in 2 hr. *S. kombe*' homogenates yielded about 10% of the extractable radioactivity as the product, while over 8% of the extractable radioactivity following incubations with *C. cheiri* leaf homogenates was present as 5α-cholestan-3-one. Heating the *D. purpurea* leaf homogenate for 10 min in a boiling water bath completely destroyed the metabolic activity.

TABLE 2. CO-CRYSTALLIZATION OF 5α-CHOLESTAN-3-ONE TO CONSTANT SPECIFIC ACTIVITY

Solvent	Cpm/mg	Solvent	Cpm/mg
95% EtOH	866 ± 15	Ether–methanol–60% EtOH	917 ± 11
Acetone–Methanol	855 ± 43	95% EtOH	906 ± 30

The radioactive metabolite of 4-cholesten-3-one following incubation with leaf homogenates of *D. purpurea* was isolated by preparative TLC. The metabolite was removed from the silica gel by Soxhlet extraction with $CHCl_3$, and co-crystallized to constant specific activity with nonradioactive 5α-cholestan-3-one.

Final verification that the metabolite was 5α-cholestan-3-one was obtained by co-crystallization of the metabolite to constant specific activity with non-radioactive 5α-cholestan-3-one (Table 2). The metabolite was isolated by preparative TLC of extracts from *D. purpurea* incubations.

Specificity of the Δ^4-reductase enzyme system was investigated by incubating leaf homogenates of *D. purpurea* with 4-cholesten-3-one-[4-^{14}C] in the presence of either 1·0 mg nonradioactive 4-cholesten-3-one or 1·0 mg nonradioactive progesterone (4-pregnen-3,20-dione). The addition of the nonradioactive 4-cholesten-3-one produced an effective dilution of the radioisotopically labeled form by a factor of 1770. As can be seen in Table 3, the per cent of extractable radioactivity recovered as 5α-cholestan-3-one after 2-hr incubation was reduced from 20% to *ca.* 2% by the presence of nonradioactive 4-cholesten-3-one in the reaction mixture. The addition of 1·0 mg progesterone to each incubation flask resulted in a complete elimination of homogenate-catalyzed formation of 5α-cholestan-3-one. The results indicate that the Δ^4-reductase enzyme system is lacking in specificity, since progesterone is capable of competing with and eliminating the metabolism of the 4-cholesten-3-one-[4-^{14}C].

TABLE 3. METABOLISM OF 4-CHOLESTEN-3-ONE-[4-^{14}C] BY *Digitalis purpurea*

Leaf homogenate	Other additives	Extracted radioactivity as 5α-cholestan-3-one (%)
Control	—	0·46 ± 0·21
D. purpurea	—	20·10 ± 3·24
D. purpurea	1·0 mg 4-cholesten-3-one	2·35 ± 0·25
D. purpurea	1·0 mg progesterone	0·36 ± 0·14

Homogenates from 1 g of *D. purpurea* leaves were incubated with 0·10 μCi 4-cholesten-3-one-[4^{14}C] in a buffered system containing an NADPH generating system. To each of 3 flasks was added either 1·0 mg nonradioactive 4-cholesten-3-one or progesterone. Each value represents the average of 3 determinations with the standard deviation. The results are expressed as percentage of the total extractable ^{14}C as 5α-cholestan-3-one.

DISCUSSION

The metabolism of 4-cholesten-3-one to 5α-cholestan-3-one can be readily demonstrated by employing 2-hr incubations with leaf homogenates from cardenolide- and spirostanol-producing plants. No subsequent metabolism of 5α-cholestan-3-one was observed under our incubation conditions. Leaf homogenates of the plants employed in this study have previously been shown to be capable of metabolizing progesterone (4-pregnen-3,20-dione) to 5α-pregnan-3,20-dione and subsequently to 5α-pregnan-3β-ol-20-one.[9] In the present study, the addition of 1·0 mg of progesterone per flask, producing a theoretic molar dilution of the radioactive substrate of *ca.* 1770, completely eliminated the formation of 5α-cholestan-3-one, demonstrating the lack of specificity of the enzyme; progesterone may be more readily bound by the enzyme.

Tschesche and Fritz have previously shown that 5α-cholestan-3-one-[4-^{14}C] can be incorporated into tomatidine by leaves of *Solanum lycopersicum* (tomato).[10] Twenty-day

[9] STOHS, S. J. and EL-OLEMY, M. M. (1972) *Phytochemistry* **11**, 2409.
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incubations were employed. Tschesche *et al.* have also found that 24-days after the administration of 5α-cholestan-3β-ol-[4-^{14}C] and 5α-cholestan-3-one-[4-^{14}C] to *Digitalis lanata* plants, radioactive tigogenin and gitogenin could be isolated.[11] 5α-Cholestan-3β-ol-[4-^{14}C] was converted to 5α-cholestan-3-one, while 5α-cholestan-3-one-[4-^{14}C] was also metabolized to 5α-cholestan-3β-ol. These authors believe that the formation of 5α-cholestan-3β-ol is a side reaction in the formation of spirostanols as tigogenin and gitogenin from 5α-cholestan-3-one.[11] Tschesche *et al.* had previously demonstrated that 4-cholesten-3-one is an obligatory intermediate in the incorporation of cholesterol into spirostanols.[12] We have previously observed that cholesterol is readily converted to 4-cholesten-3-one by leaf homogenates in 2 hr.[5] These results, in conjunction with our present observations that 4-cholesten-3-one is metabolized by leaf homogenates to 5α-cholestan-3-one, support the thesis that the above sequence constitutes a major metabolic route of steroids, particularly in spirostanol producing plants.

EXPERIMENTAL

Homogenates. Plants of *Cheiranthus cheiri, Digitalis purpurea* and *Strophanthus kombe*' were greenhouse-grown for 6–8 months. *Ca.* 30% homogenates were prepared as previously described, in a buffer solution containing 0·25 M sucrose, 0·05 M Tris chloride (pH 7·4), 0·045 M mercaptoethanol, 0·05 M $MgCl_2$, 0·003 M cysteine HCl, and 1 mg/ml bovine serum albumin fraction V.[4,5,13]

Incubations. 4-Cholesten-3-one-[4-^{14}C] (Amersham-Searle) with a sp. act. of 55·7 μCi/μmol was shown to have a radiochemical purity of greater than 98% upon TLC. Homogenate equivalent to 1 g leaves was incubated with 0·10 μCi 4-cholesten-3-one-[4-^{14}C] in 5·0 ml homogenization buffer which additionally contained 1·5 mg $NADP^+$, 7·0 mg glucose-6-phosphate and 2·5 units glucose-6-phosphate dehydrogenase. Substrate was added to all reaction flasks in 0·10 ml 70% EtOH. Control flasks contained the homogenization buffer without homogenate to give a final 5·0 ml vol. In experiments where nonradioactive 4-cholesten-3-one or progesterone were added to incubation flasks, 1·0 mg of either was added in a total vol. of 0·20 ml 70% EtOH. The EtOH was also added to all control flasks with and without leaf homogenate. The flasks were incubated at 30° on a water bath shaker for 2 hr, aerating with 95% O_2–5% CO_2. The reaction in each flask was stopped by the addition of 0·50 ml HOAc, followed by the addition of 50 ml EtOAc and extracted as previously described.[4,5,13] The organic phase of each extraction was dried over anhyd. NA_2SO_4 and evaporated to dryness under vacuum. The extraction efficiency for 4-cholesten-3-one-[4-^{14}C] and its metabolite was 75–95%.

Metabolite identification. Ca. 5000–10000 cpm from each of the extracts was co-chromatographed with reference standards on silica gel H (Brinkman) plates which were divided into 2-cm wide columns. The solvent systems employed were diisopropyl ether–petrol–HOAc (70:30:1), $CHCl_3$ (developed 4 times), and $CHCl_3$–EtOH (9:1). I_2 vapor was used to locate reference standards. After allowing the iodine to evaporate from the plates, the areas corresponding to the reference standards were transferred to scintillation counting vials with the aid of a razor blade. The remainder of each 2-cm wide silica column was similarly transferred to counting vials, and Omnifluor (New England Nuclear) or toluene counting solution was added to each vial. All sample vials were counted in a Beckman LS-100 liquid scintillation counter equipped with an external standard. A background of 10–15 cpm was routinely obtained for ^{14}C. A single metabolite was observed which corresponded chromatographically with 5α-cholestan-3-one (II). This metabolite was isolated by preparative TLC using thick (0·5 mm) silica gel H plates and the solvent system diisopropyl ether–petrol–HOAc (70:30:1). The metabolite was subsequently removed from the silica gel by Soxhlet extraction with $CHCl_3$ for 18–20 hr. The metabolite was co-crystallized to constant specific activity following the addition of 100 mg of the authentic nonradioactive 5α-cholestan-3-one to *ca.* 90000 cpm of the metabolite. The solvents employed for the recrystallization and the results obtained are given in Table 2. Each value represents the specific activity in cpm/mg of triplicate samples following each recrystallization.

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