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CHOLESTEROL METABOLISM BY *CHEIRANTHUS CHEIRI* LEAF AND TISSUE CULTURE HOMOGENATES

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SUMMARY

Cheiranthus cheiri leaf and tissue culture homogenates readily metabolize [4-^{14}C] cholesterol to 4-cholesten-3-one. The cytoplasmic fraction has the greatest metabolic activity, although other fractions are active as well. Homogenates from *Nerium oleander* leaves and *Dioscorea deltoidea* tissue cultures are devoid of metabolic activity under identical conditions. *Digitalis* leaf homogenates are only about $\frac{1}{10}$th as active as the *Cheiranthus* systems. The first step in cholesterol side chain cleavage by plants may be the formation of 4-cholesten-3-one.

INTRODUCTION

CURRENT theory assumes that the biosynthesis of steroids in plants and animals proceeds via similar routes. That cholesterol does exist in plants and can function as a precursor of a variety of plant steroids has been shown in an ever increasing number of cases. The involvement of cholesterol in biosynthesis of cardenolides, sapogenins, steroidal alkaloids, steroidal hormones and other steroids in plants has been reviewed [1–3]. Although it is known that cholesterol can be metabolized to a variety of other steroids in plants, in many cases the intervening steps in the metabolic processes have not been elucidated. We have begun to examine the most active metabolic routes of cholesterol in a variety of steroid producing plants.

EXPERIMENTAL

Plants. Cheiranthus cheiri, Digitalis purpurea, and *Nerium oleander* were grown in the greenhouse. *C. cheiri* and *D. purpurea* were 4–6 months old. The *N. oleander* was over 10 yr old. Tissue suspension cultures of embyroid *C. cheiri* [4, 5] and undifferentiated *Discorea deltoidea* [6] have both been maintained for approx. 4 yr and were grown as previously described.

Homogenates. Thirty per cent homogenates of leaves or tissue cultures were prepared in a buffer medium containing 0·25 M sucrose, 0·05 M Tris chloride pH 7·4. 1·0 mg/ml bovine serum albumin (Sigma Chemical Co.), 0·005 M $MgCl_2$, 0·045 M mercaptoethanol, and 0·003 M L-cysteine HCl. Homogenates were prepared using a Sorvall Omni-Mixer Homogenizer operated for two 20-sec intervals at maximum speed. The vessel was maintained in an ice-bath during the homogenization. The homogenates were filtered through a layer of washed muslin under vacuum and the resulting filtrates used as the enzyme source.

Incubations. [4-^{14}C] cholesterol was obtained from New England Nuclear and has a specific activity of 57 mCi/mmol. It exhibited approx. 98% radiochemical purity upon t.l.c. The homogenate or the subcellular fraction from one gram of plant leaves or tissue culture was incubated with 0·10 μCi[4-^{14}C] cholesterol in a

5·0 ml total volume of homogenization buffer to which was also added 1·5 mg $NADP^+$, 7·0 mg glucose-6-phosphate, and 2·5 units glucose-6-phosphate dehydrogenase (Sigma Chemical Co.). Incubations were conducted at 30°C on a water bath shaker. aerating with 95% O_2–5% CO_2. The [4-^{14}C]cholesterol was added to the incubation mixture in 0·10 ml 70% ethanol. The results of incubations ranging from 0·25 to 6·0 h are reported.

Following incubation, each reaction mixture was extracted with 40 ml ethyl acetate–glacial acetic acid (100:1, v/v) for 1 h on a shaker at room temperature and the resulting emulsion was broken by centrifugation. The organic phase was removed by pipette, dried over anhydrous sodium sulfate and evaporated to dryness. An extraction efficiency of 86 ± 7% was routinely obtained.

Thin layer chromatography. Silica gel H (Brinkman) plates were routinely developed 3 successive times in the same chamber containing diisopropyl ether–petroleum ether–acetic acid (70:30:1 by vol.). Plates were air dried 15 min between developments. Initial studies indicated that cholesterol was converted to a single metabolite with a mobility identical to 4-cholesten-3-one. Co-chromatography in benzene–ethyl acetate (4:1, v/v) and methylene dichloride-methanol (97:3, v/v) confirmed these results, as can be seen in Table 2.

Aliquots of incubation extracts were co-chromatographed with the reference standards cholesterol and 4-cholesten-3-one (Ikapharm, Ramat-Gan, Israel) on plates divided into 2 cm wide columns. The reference standards were located by exposing the developed t.l.c. plates to iodine vapors. The iodine was allowed to evaporate from the plates overnight, and the areas corresponding to the standards as well as the remainder of each column were transferred with a razor blade to scintillation counting vials. Omnifluor (New England Nuclear) or toluene counting solution was added to each vial and ^{14}C was counted in a Beckman LS-100 liquid scintillation counter equipped with external standard. Samples containing 1000 c.p.m. or more above background were normally counted for 20 min while lesser amounts of radioactivity were counted for correspondingly longer periods of time. A background count of 10–15 c.p.m. was routinely obtained for ^{14}C.

Co-crystallization of 4-cholesten-3-one. The metabolite resulting from the incubation of [4-^{14}C] cholesterol with *C. cheiri* tissue culture homogenates from several experiments was isolated by preparative t.l.c. on thick silica gel H plates, developing with CH_2Cl_2–CH_3OH (97:3, v/v). The metabolite was extracted from the silica gel with $CHCl_3$ using a Soxhlet apparatus. To approx. $2{\cdot}4 \times 10^5$ dpm was added 100 mg non-radioactive 4-cholesten-3-one. The 4-cholesten-3-one was recrystallized successively from 85% ethanol. 90% methanol. acetone–methanol and 85% ethanol. The results are presented in Table 3.

Aliquots from each crystallization were counted for ^{14}C and the results are expressed as the average d.p.m./mg with the standard deviation.

Tissue fractionation. Leaf homogenates were prepared as described above. The homogenates were centrifuged at $600 \times g$ for 10 min in a Sorvall RC2-B refrigerated centrifuge. The pellet, called the nuclear fraction, was washed once by resuspending and recentrifuging in the homogenation buffer. The $600 \times g$ supernatant fraction was centrifuged at $10{,}000 \times g$ for 10 min. The pellet, called the mitochondrial fraction, was washed once. The $10{,}000 \times g$ supernatant fraction was centrifuged at approx. $100{,}000 \times g$ for one hr in a Spinco model L ultracentrifuge equipped with a Titanium 50 rotor. The pellet, referred to as the microsomal fraction, was washed once. The supernatant or cytoplasmic fraction was used as

such. These fractions are very similar to those used by others in the study of plant steroid [7, 8] and alkaloid [9] metabolism.

RESULTS

The results of the metabolic studies of cholesterol with various plant homogenates are given in Table 1. Initial results indicated that leaf and tissue culture homogenates of the cardenolide producing *C. cheiri* could transform 70–75% of the cholesterol into a single major less polar metabolite in two hrs. Heating the homogenate from *C. cheiri* leaves for 10 min in a boiling water bath completely destroyed this metabolic activity. Homogenates from sapogenin producing *D. deltoidea* undifferentiated tissue cultures failed to demonstrate metabolic activity under these conditions. Homogenates from *N. oleander* failed to yield this metabolite, but did convert 10% of the cholesterol into a second as yet unidentified product. Leaf homogenates of the cardenolide producing plant *D. purpurea* converted only about 1% of the incubated cholesterol to the metabolite produced by the *C. cheiri* systems.

Table 1. Metabolism of [4-^{14}C] cholesterol by plant leaf and tissue culture homogenates

Tissue source	Per cent recovered radioactivity in 4-cholesten-3-one (%)
Control	$0{\cdot}33 \pm 0{\cdot}03$
Cheiranthus cheiri leaf, heated	$0{\cdot}39 \pm 0{\cdot}05$
Cheiranthus cheiri leaf	$69{\cdot}9 \pm 11{\cdot}8$
Cheiranthus cheiri tissue culture	$75{\cdot}8 \pm 6{\cdot}2$
Nerium oleander leaf	$0{\cdot}48 \pm 0{\cdot}23$
Digitalis purpurea leaf	$1{\cdot}35 \pm 0{\cdot}45$
Dioscorea deltoidea tissue culture	$0{\cdot}38 \pm 0{\cdot}06$

Homogenates from one gram of leaves or tissue culture were incubated with 0·10 μCi/[4-^{14}C] cholesterol for 2 h at 30°C in the presence of an NADPH generating system. The reaction mixtures were extracted and assayed as described under *Experimental*. Each value represents the average of 3–6 determinations with the standard deviations.

Further investigations demonstrated that the metabolite from the *C. cheiri* systems had a t.l.c. mobility identical to 4-cholesten-3-one, as can be seen in Table 2. In each system approx. 82% of the radioactivity following a four hour incubation was associated with the 4-cholesten-3-one reference compound.

Final verification that the metabolite was indeed 4-cholesten-3-one was obtained by co-crystallization of the metabolite with non-radioactive 4-cholesten-3-one to constant specific activity (Table 3).

The rate at which 4-cholesten-3-one was formed from cholesterol by homogenates of embryoid *C. cheiri* tissue cultures under our experimental conditions was examined (Table 4). Maximum conversion of cholesterol to 4-cholesten-3-one occurs at approx. 6 h with over 80% transformation. However, the greatest metabolic activity per unit of time occurs within 15 min and subsequently

Table 2. Co-chromatography of [4-^{14}C] cholesterol metabolite with 4-cholesten-3-one

Solvent system	R_f Values cholesterol	R_f Values 4-cholesten-3-one	Per cent radioactivity recovered with 4-cholesten-3-one (%)
Diisopropyl ether–petroleum ether–acetic acid (70 : 30 : 1, by vol.) (Develop 3×)	0·58	0·68	82·0 ± 2·0
Benzene–ethyl acetate (4 : 1, v/v)	0·32	0·50	82·2 ± 0·4
Methylene dichloride–methanol (97 : 3, v/v)	0·38	0·57	81·6 ± 1·6

[4-^{14}C] cholesterol was incubated with *C. cheiri* tissue culture homogenates for 4 h. Aliquots (approx. 7000 c.p.m.) of a pooled extract were co-chromatographed with cholesterol and 4-cholesten-3-one on silica gel H plates employing the above solvents. The standards were located with I_2 vapors and the areas corresponding to the standards as well as the remainder of each column were transferred to counting vials and the ^{14}C counted.

Table 3. Recrystallization of [4-^{14}C]4-cholesten-3-one to constant specific activity

Recrystallization	Solvent	Specific activity (d.p.m./mg)
1	85% ethanol	2240 ± 20
2	90% methanol	2210 ± 30
3	Acetone–methanol	2305 ± 85
4	85% ethanol	2270 ± 50

The radioactive metabolite of cholesterol following incubation with homogenates from *C. cheiri* was isolated by preparative t.l.c. on thick silica gel H plates, developing with CH_2CL_2–CH_3OH (97 : 3, v/v). The metabolite was removed from the silica gel by Soxhlet extraction using $CHCl_3$, and co-crystallized with non-radioactive 4-cholesten-3-one.

decreases. If for example the transformation per unit time at 15 min is taken as 1·00, the rate at one h is 0·45 and at 2 h is only 0·28.

The ability of various subcellular fractions of *C. cheiri* tissue cultures to convert cholesterol to 4-cholesten-3-one was examined (Table 5). In 2 h the total homogenate yielded approx. 72% 4-cholesten-3-one while an equivalent amount of the 100,000 × *g* supernatant (cytoplasmic) fraction metabolized almost 80% of the [4-^{14}C] cholesterol to 4-cholesten-3-one. Equivalent amounts of nuclei, mitochondria, and microsomes all converted approx. 50% or more of the substrate to 4-cholesten-3-one in 2 h. It is readily apparent that the sum of the metabolic capabilities of the individual fractions far exceeds the transforming capacity of the initial homogenate.

DISCUSSION

We have shown that selected cardenolide producing plants can produce significant metabolism of cholesterol in a relatively short incubation time. Johnson

Table 4. Rate of 4-cholesten-3-one formation from cholesterol by *C. cheiri* tissue culture homogenates

Incubation time (h)	Per cent recovered radioactivity as 4-cholesten-3-one (%) Control	Experimental
0·25	—	33·5 ± 2·3
0·50	—	43·9 ± 4·3
0·75	—	51·1 ± 5·1
1·00	0·3 ± 0·1	60·9 ± 3·6
2·00	—	75·5 ± 3·2
6·00	2·1 ± 0·5	83·3 ± 2·6

Flasks (250 ml) containing the homogenate from 6 g *C. cheiri* tissue culture and 0·60 μCi [4-^{14}C] cholesterol with the NADPH generating system were incubated at 30°C and aerated with 95% O_2–5% CO_2. Aliquots were removed at the indicated times, extracted and assayed as described under *Experimental*. Controls contained no homogenate. Each value represents the average of four values with the standard deviation.

Table 5. Formation of 4-cholesten-3-one from cholesterol by subcellular fractions of *C. Cheiri* tissue suspension cultures

Fraction	Per cent recovered radioactivity as 4-cholesten-3-one
Control	0·8 ± 0·3
Homogenate (total)	72·5 ± 2·0
Nuclei	55·2 ± 0·6
Mitochondria	47·7 ± 4·2
Microsomes	55·6 ± 0·4
Supernatant (cytoplasm)	79·7 ± 0·5

The homogenate or subcellular fraction derived from one gram of tissue was incubated for 2 h with 0·10 μCi [4-^{14}C] cholesterol. The extraction and assay is as described under *Experimental*. Each value represents the average of four determinations with the standard deviation.

et al.[10] have shown that 4-cholesten-3-one is an intermediate in the conversion of cholesterol to 3β-cholestanol by potato leaves. However, five day incubation periods were used. Tschesche *et al.*[11] have shown that in the biosynthesis of spirostanols such as gitogenin and tigogenin, cholesterol must pass through 4-cholesten-3-one. Incubations of up to 3 weeks were employed. However, 4-cholesten-3-one does not function as an intermediate in the biosynthesis of ecdysterone from cholesterol in *Podocarpus elata*[12]. It is evident that 4-cholesten-3-one functions as an intermediate in the metabolism of cholesterol to certain steroids in plant systems.

Why *N. oleander*, *D. purpurea*, and *D. deltoidea* fail to yield 4-cholesten-3-one is not known. We have previously shown that suspension tissue cultures of *D. deltoidea* can extensively metabolize cholesterol within five days[13]. Either the plants simply do not produce high levels of these enzymes or our incubation conditions and/or additives are not suitable.

The fact that the sum of the metabolic capabilities of the individual intracellular fractions was approx. 3·3 times the transforming activity of the total homogenate is surprising. Several possible explanations exist. During the subcellular fractionation and washing process, inhibitors such as phenolic compounds may have been removed. Endogenous and competing steroids may have been removed during the fractionation and washing. Also, since the actual molar amount of radioactive cholesterol was small, some of it may have been bound nonspecifically to lipoproteins, and therefore not be readily available to attack by the metabolizing enzymes.

In plants the synthesis of cardenolides such as digitoxigenin is believed to involve the pathway cholesterol → 20α-hydroxycholesterol → 20, 22(R) dihydroxycholesterol → pregnenolone → progesterone → digitoxigenin[1–3]. An identical pathway exists from cholesterol to progesterone in mammalian tissues [14]. However, in mammalian systems it has recently been demonstrated that 4-cholesten-3-one[15] and 20α-hydroxy-4-cholesten-3-one[16] can be converted to progesterone. The precise role of these two compounds in cholesterol side chain cleavage in both mammalian and plant systems is not known. Since 4-cholesten-3-one is the initial metabolic product of cholesterol under our experimental conditions by such cardenolide producing plants as *Cheiranthus* and *Digitalis*, this may represent the first step in the synthesis of cardenolides from cholesterol.

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