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26-HYDROXYCHOLESTEROL AND CHOLEST-4-EN-3-ONE, THE FIRST METABOLITES OF CHOLESTEROL IN POTATO PLANTS

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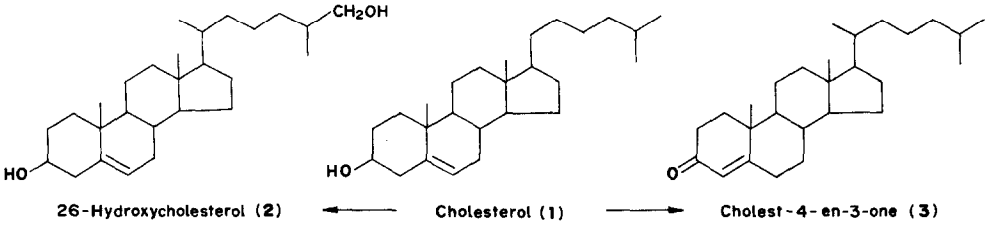
Key Word Index—*Solanum tuberosum*; Solanaceae; cholesterol metabolism; 26-hydroxycholesterol; cholest-4-en-3-one; sterols.

Abstract—Etiolated potato sprouts convert administered cholesterol-4-^{14}C to radioactive 26-hydroxycholesterol and cholest-4-en-3-one. These two steroids must be the first products of cholesterol metabolism in potato plants.

INTRODUCTION

SINCE the discovery of cholesterol (**1**) in potato plants 10 yr ago,[1] it has been shown to function as a precursor for the biosynthesis of many other steroids in plants,[2] including the steroidal sapogenins and alkaloids.[3] The identification of all these products of cholesterol metabolism has raised a host of questions about the biochemical mechanisms and pathways whereby they are produced.[4]



Some time ago, we have established that 26-hydroxycholesterol (**2**) is converted to diosgenin by *Dioscorea floribunda* plants.[5] However, evidence for the conversion of cholesterol to 26-hydroxycholesterol has been lacking so far. The presence of 26-hydroxycholesterol in the human aorta has recently been established,[6] but to our knowledge this compound has not been detected in plants heretofore. The conversion of cholesterol to cholest-4-en-3-one (**3**) was first observed in animals[7] and later in isolated potato leaves.[8] Tschesche *et al.*[9] have shown that cholestenone acts as a precursor of sapogenins in *Digitalis lanata*

[1] JOHNSON, D. F., BENNETT, R. D. and HEFTMANN, E. (1963) *Science* **140**, 198.
[2] HEFTMANN, E. (1971) *Lipids* **6**, 128.
[3] HEFTMANN, E. (1967) *Lloydia* **30**, 209.
[4] HEFTMANN E. *Lipids*, in press.
[5] BENNETT, R. D., HEFTMANN E. and JOLY, R. A. (1970) *Phytochemistry* **9**, 349.
[6] SMITH, L. L. and PANDYA, N. L. (1973) *Atherosclerosis* **17**, 21.
[7] HEFTMANN, E. (1970) *Steroid Biochemistry*, Academic Press, New York.
[8] JOHNSON, D. F., WATERS, J. A. and BENNETT, R. D. (1964) *Arch. Biochem. Biophys.* **108**, 282.
[9] TSCHESCHE, R., HULPKE, H. and FRITZ, R. (1968) *Phytochemistry* **7**, 2021.

plants, but direct evidence for the conversion of cholesterol to cholestenone in intact plants is necessary to establish its role as a biosynthetic intermediate.

In the present paper we present direct evidence for the conversion of cholesterol-4-^{14}C to both labeled 26-hydroxycholesterol and cholestenone in growing potato plants. These two oxidations constitute the first transformations of cholesterol in the biosynthetic pathways leading to the steroidal sapogenins and alkaloids.

RESULTS AND DISCUSSION

Cholesterol-4-^{14}C in a 96% ethanol solution containing silicone oil and tocopherol[10] was administered to a Russet potato plant growing in the dark for 1 month. The plant was then homogenized and boiled in 1 N HCl for 3 hr. The hydrolyzate was extracted with dichloromethane, and aliquots of the extract were fractionated by TLC.

Authentic 26-hydroxycholesterol and cholest-4-en-3-one were added to the appropriate fractions of radioactive plant sterols. The diluted sterols were repeatedly chromatographed until they were radiochemically pure. 26-hydroxycholesterol-4-^{14}C was then converted to the diacetate and cholest-4-^{14}C-en-3-one to the 2,4-dinitrophenylhydrazone. The derivatives were again purified by repeated TLC until the specific radioactivity was constant within the analytical error. Table 1 shows the specific activities of all compounds after chromatography in the solvent systems indicated.

TABLE 1. PURIFICATION BY CARRIER DILUTION AND TLC*

Compound	Developing solvent	cpm/μM†
26-Hydroxycholesterol	CH_2Cl_2–MeOH (9 : 1)	468 ± 24
	CH_2Cl_2–MeOH (23 : 2)	428 ± 20
	cyclohexane-EtOAc (1 : 1)	522 ± 26
	CH_2Cl_2–MeOH (24 : 1)	555 ± 28
26-Hydroxycholesterol diacetate	*n*-hexane-EtOAc (17 : 3)	88 ± 5
	n-hexane–Me_2CO (19 : 1)	75 ± 4
	CH_2Cl_2–Me_2CO (9 : 1)	80 ± 4
Cholestenone	*n*-hexane-EtOAc‡ (17 : 3)	1157 ± 58
	CH_2Cl_2–Me_2CO (9 : 1)	1023 ± 52
	n-hexane-EtOAc (17 : 3)	944 ± 48
	CH_2Cl_2–Me_2CO (23 : 2)	889 ± 44
Cholestenone dinitrophenylhydrazone	CH_2Cl_2–MeOH (24 : 1)	167 ± 8
	CH_2Cl_2-EtOAc (23 : 2)	219 ± 10
	CH_2Cl_2	230 ± 11

* Samples were counted in a toluene solution of PPO and POPOP, using a Packard Tricarb Model 3003 scintillation counter with a counting efficiency of 76% and a background of 11 cpm.
† 90% Confidence level.
‡ Developed twice.

The purification of radioactive steroids by TLC in different solvent systems constitutes an improvement over the crystallization with different solvents we have previously used. As Wortmann and Touchstone[11] have pointed out, TLC is not only more efficient and rapid than crystallization, but it also produces better yields and higher purity of substances.

[10] HEFTMANN, E. and SCHWIMMER, S. (1972) *Phytochemistry* **11**, 2783.
[11] WORTMANN, W. and TOUCHSTONE, J. C. (1973) In: *Quantitative Thin Layer Chromatography* (TOUCHSTONE, J. C., ed.), p. 23. Wiley-Interscience, New York.

Scanning of the radiochromatograms has revealed a large number of cholesterol metabolites in potato plants grown in the light and dark. Work on their identification in connection with the biosynthesis of glycoalkaloids is in progress.

EXPERIMENTAL

Methods. Unless specifically stated, the analytical methods were the same as previously described.[10]

Administration of cholesterol-4-^{14}C. A piece of tissue containing an actively growing sprout and weighing 4·4 g was cut from a Russet Burbank potato tuber. It was kept in a small beaker with some H_2O in a laboratory cupboard for a month. During the first 2 weeks 0·5 ml of a soln containing $4{\cdot}66 \times 10^7$ cpm cholesterol-4-^{14}C and *ca* 0·5 μl of each, silicone oil D.C. 200 and DL-α-tocopherol in 96% EtOH was administered to the growing tips of the sprout at the rate of 0·1 ml every third day. In spite of some evidence of tissue damage, the etiolated plant grew well and reached a weight of 9 g at the time of harvest.

Control experiments, in which ^{14}C-cholesterol was left for the same length of time in blanched potato tissue indicated that the observed reactions were not oxidative artifacts.[8] Further details of the method of application of ^{14}C-cholesterol have been given earlier.[10]

Isolation of labeled steroids. The plant was homogenized in H_2O for 5 min, and then H_2O and HCl added to make 300 ml of 1 N soln. After 3 hr of refluxing, the hydrolyzate was made alkaline with NaOH soln and then extracted with three 200-ml portions of CH_2Cl_2. The extract was dried over Na_2SO_4 and evaporated to 100 ml. From an aliquot the recovery of radioactivity was determined as $3{\cdot}87 \times 10^7$ cpm. A 5-ml aliquot of the steroid extract was chromatographed on a Silica Gel G layer, 50 × 200 × 1 mm, with CH_2Cl_2–Me_2CO (23 : 2) and scanned. Most of the radioactivity was localized in the middle of the TLC plate and was recovered with a vacuum zone collector by continuous extraction with 300 ml Me_2CO in a Soxhlet for 4 hr. Faster- and slower-moving radioactive mateial was similarly recovered by continuous extraction with 20 ml Me_2CO for 4 hr. The main fraction was concentrated and applied to a Silica Gel G layer, 50 × 200 × 0·25 mm. Chromatography with CH_2Cl_2–Me_2CO (9 : 1) again gave 3 radioactive zones. The middle zone was recovered by extraction with 20 ml Me_2CO, as before, and then rechromatographed on a 0·25-mm Silica Gel G layer with CH_2Cl_2–Me_2CO (23 : 2). The slowest moving zone now had the highest radioactivity and was rechromatographed with CH_2Cl_2–Me_2CO (9 : 1), as before. At this stage, the radiochromatogram showed a peak in the region of 26-hydroxycholesterol, a sample of which had been applied next to the starting line and detected by treatment of the pilot chromatogram with 50% H_2SO_4.

The faster-moving radioactive material in the first thin-layer chromatogram was applied to a Silica Gel G layer, 50 × 200 × 0·25 mm, and developed twice with *n*-hexane–EtOAc (17 : 3). The middle zone from this chromatogram was rechromatographed with CH_2Cl_2–Me_2CO (23 : 2), as described above. The zone containing most of the radioactivity was now located near the solvent front. When rechromatographed by double development with *n*-hexane–EtOAc (17 : 3), a radioactive band in the middle of the plate coincided with authentic cholestenone, which had been chromatographed alongside the extract.

Identification of 26-hydroxycholesterol-4-^{14}C. To the radioactive zone coinciding with 26-hydroxycholesterol and containing 13,124 cpm, 11·3 mg authentic 26-hydroxycholesterol was added. It was purified to constant sp. act. by TLC on Silica Gel G layers, 50 × 200 × 1 mm, with the solvent systems showns in Table 1. To the final product another 12·5 mg of carrier sterol was added, prior to acetylation with 10 ml acetic anhydride and 5 ml anhyd. pyridine. The acetylation mixture was diluted with Et_2O and washed, in succession, with 0·1 N HCl, H_2O, 0·05 N $NaHCO_3$, and H_2O. The diacetate of 26-hydroxycholesterol was purified to constant specific activity by TLC on Silica Gel G layers, 50 × 200 × 1 mm, with the solvent systems shown in Table 1.

Identification of cholest-4-^{14}C-en-3-one. To the radioactive zone coinciding with cholestenone and containing 32,138 cpm, 10·7 mg authentic cholestenone was added. It was purified to constant sp. act. by TLC on Silica Gel G layers, 50 × 200 × 1 mm, with the solvent systems shown in Table 1. To the final product another 9·2 mg of carrier sterol was added, prior to the preparation of the 2,4-dinitrophenylhydrazone.[12] To the dry sample 10 ml 0·2% dinitrophenylhydrazine in EtOAc was added. After evaporation to dryness in a flask, the residue was dissolved in 100 ml 0·03% trichloroacetic acid in C_6H_6. The flask was kept tightly stoppered at 40° for 40 min, then evaporated under N_2 at 40°. The product was purified to constant specific activity by TLC on Silica Gel GF layers, 200 × 200 × 1 mm, with the solvent systems shown in Table 1.

[12] KNAPSTEIN, P. and TOUCHSTONE, J. C. (1968) *J. Chromatogr.* **37**, 83.