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A FUNCTION OF SITOSTEROL

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Abstract—Sitosterol-3-^{14}C was converted by a *Digitalis lanata* plant to progesterone, digitoxigenin, gitoxigenin, and digoxigenin. The results indicate that sitosterol functions as a starting material for the biosynthesis of other steroids in plants.

INTRODUCTION

THE PREDOMINANT sterols of higher plants are C_{29} compounds, e.g. sitosterol (24α-ethylcholesterol) (I), accompanied by small amounts of C_{28} sterols and the C_{27} sterol cholesterol. The biosynthesis of sitosterol has been studied rather thoroughly,[1] but nothing is known about the metabolism of this sterol in plants, except for reactions involving its hydroxyl group, such as glycoside and ester formation. In fact, the literature contains no convincing evidence that sitosterol is metabolized at all by growing plants. Cholesterol, on the other hand, is now known to be a precursor of several different types of plant steroids, which suggests that, as in animals,[2] this sterol may be a key biosynthetic intermediate in plants. However, both sitosterol and cholesterol could, hypothetically, act as precursors of the C_{21} steroids (and compounds derived from the latter) by removal of all the carbon atoms following C-21. We have now tested this hypothesis by administering labelled sitosterol to *Digitalis lanata*, a plant which contains three cardenolides known to be biosynthesized from cholesterol via progesterone.[1] A preliminary account of the conversion of sitosterol to progesterone has appeared elsewhere.[3]

RESULTS

A *Digitalis lanata* plant was treated with sitosterol-3-^{14}C and then extracted. The extract was subjected to mild acid hydrolysis, and the hydrolyzate was fractionated by column chromatography on alumina. Fractions 1 and 2 were shown by TLC to contain mostly sterols, but some radioactivity also appeared to be associated with progesterone (II). These two components were separated on a second alumina column, and the progesterone-containing fraction was purified by preparative TLC and diluted with carrier progesterone. This material was crystallized from two solvents and then reduced to Δ^4-pregnene-3β,20β-diol, which was

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[1] E. HEFTMANN, *Lloydia* **31**, 293 (1968).
[2] E. HEFTMANN and E. MOSETTIG, *Biochemistry of Steroids*, Reinhold, New York (1960).
[3] R. D. BENNETT, E. HEFTMANN and B. J. WINTER, *Naturwissenschaften*, in press.

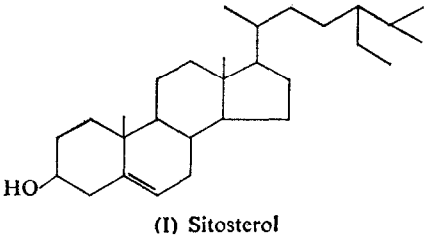
isolated by preparative TLC and further crystallized. The specific activity remained constant during these operations (Table 1, A).

TABLE 1. CRYSTALLIZATION OF RADIOACTIVE STEROIDS*

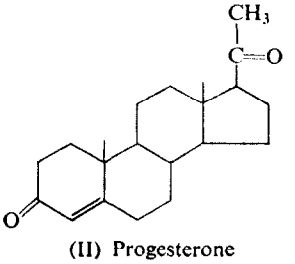
Compound	Solvent used for crystallization	Counts/min/μmole†
A. Progesterone		34·1 ± 1·7
	Hexane–acetone	33·6 ± 1·7
	MeOH–H_2O	33·8 ± 1·7
Δ^4-Pregnene-3β,20β-diol	Hexane–acetone	34·4 ± 1·7
B. Gitoxigenin diacetate		103 ± 5
	Hexane–acetone	109 ± 5
	Benzene	100 ± 5

* Portions of 0·2 mg or less were plated from solution on ringed planchets over an area of 12·7 cm^2 and counted in duplicate on a Beckman Widebeta II instrument. Counter efficiency was 34 per cent and background was 2 counts/min.

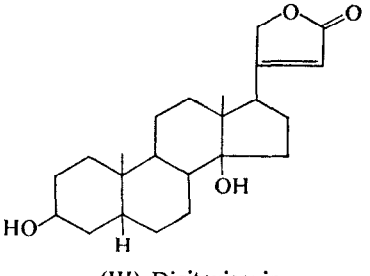
† 90 per cent confidence level.



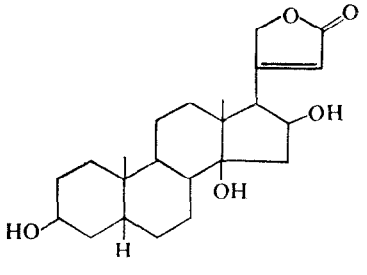
(I) Sitosterol



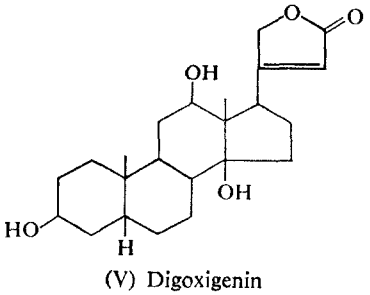
(II) Progesterone



(III) Digitoxigenin



(IV) Gitoxigenin



(V) Digoxigenin

Fraction 3 from the first alumina column contained digitoxigenin (III), and fractions 5 and 6 contained a mixture of gitoxigenin (IV) and digoxigenin (V). TLC indicated that each of these cardenolides was radioactive. The three cardenolides were then isolated and purified by column chromatography and preparative TLC until each was radiochemically pure by TLC in two different systems. After acetylation, each of the cardenolide acetates was also shown to be radiochemically pure by TLC. Finally, the gitoxigenin diacetate was diluted with carrier material and crystallized twice without change in the specific activity (Table 1, B).

Table 2 shows the total radioactivity of each of the steroid metabolites of sitosterol-3-^{14}C. The estimates are based upon fractions that are radiochemically homogeneous by TLC, after suitable corrections for aliquots removed for testing during earlier purification steps.

TABLE 2. RADIOACTIVITY OF INDIVIDUAL STEROIDS

Compound	Counts/min	Per cent of original radioactivity
Progesterone	2,500	0·043
Digitoxigenin	12,800	0·22
Gitoxigenin	6,300	0·11
Digoxigenin	5,000	0·086

DISCUSSION

The results indicate that sitosterol may act as a precursor of other steroids in plants. Although the conversion rates were relatively low (Table 2), they compare favorably with those previously reported for cholesterol in *Digitalis*. Under similar conditions, other workers observed an incorporation of cholesterol-4-^{14}C into the cardenolides of about 0·2 per cent.[4] The conversion of cholesterol to progesterone has not been demonstrated directly in *Digitalis*, but in separate experiments the yield of pregnenolone from cholesterol-4-^{14}C was about 0·01 per cent,[5] and the incorporation of pregnenolone-4-^{14}C into progesterone was 0·4 per cent.[6] Thus, sitosterol appears to be at least as efficient a precursor of progesterone and the cardenolides as cholesterol.

EXPERIMENTAL

TLC techniques were as described previously.[7] Aliquots of radioactive samples were counted on planchets at infinite thinness under a gas-flow detector (see Table 1, legend, for details). Sitosterol-3-^{14}C (5·67 μc/μmole) was obtained from Central Laboratorium TNO, Delft, Netherlands, and was purified by TLC. Acetylation reactions were carried out by a vapor phase method.[8,9]

Sitosterol-3-^{14}C was administered in doses of $5{\cdot}82 \times 10^5$ counts/min to the leaves of a potted *Digitalis lanata* plant, 2·5 months old, by the technique previously described.[10] A total of ten such treatments were given, twice weekly. One week after the last treatment, the plant was harvested, frozen in liquid N_2, and lyophilized. The dried material (12 g) was homogenized in a blender with 100 ml of H_2O, and the homogenate was diluted with 100 ml of EtOH, refluxed for 5 min, and filtered. The filter cake was then extracted by refluxing for 5 min with successive 100-ml portions of 50, 60, 70, 80, 90 and 100 per cent EtOH. All of these filtrates were

[4] J. A. F. WICKRAMASINGHE, P. C. HIRSCH, S. M. MUNAVALLI and E. CASPI, *Biochemistry* **7**, 3248 (1968).
[5] E. CASPI, D. O. LEWIS, D. M. PIATAK, K. V. THIMANN and A. WINTER, *Experentia* **22**, 506 (1966).
[6] H. H. SAUER, R. D. BENNETT and E. HEFTMANN, *Phytochem.* **6**, 1521 (1967).
[7] R. D. BENNETT and E. HEFTMANN, *Phytochem.* **5**, 747 (1966).
[8] J. K. NORYMBERSKI and A. RIONDEL, *Experentia* **23**, 318 (1967).
[9] H. H. SAUER, R. D. BENNETT and E. HEFTMANN, *Phytochem.* **8**, 69 (1969).
[10] R. D. BENNETT and E. HEFTMANN, *Phytochem.* **4**, 475 (1965).

combined, concentrated in vacuum to 100 ml, and extracted with three 100-ml portions of $CHCl_3$–EtOH (2:1). The extracts were washed with 20 ml of H_2O, combined, and evaporated. The residue was refluxed with 100 ml of 0·1 N H_2SO_4 in 50% MeOH for 30 min. The MeOH was removed in vacuum, and the aqueous residue was extracted with three 100 ml portions of $CHCl_3$. The extracts were washed with 20 ml of 10 per cent $KHCO_3$ and 20 ml of H_2O, combined, and evaporated, to yield 377 mg ($3·52 \times 10^6$ counts/min).

This material was chromatographed on an 8-g column of Grade III Woelm alumina. Fractions of 100 ml each were collected with the following eluents: 1, benzene; 2, 10 per cent ether in benzene; 3, ether; 4, 1 per cent; 5, 5 per cent; and 6, 20 per cent methanol in ether. The fractions were monitored by TLC with CH_2Cl_2–MeOH (23:2).

Fractions 1 and 2 (190 mg, $3·09 \times 10^6$ counts/min), along with 100 μg of progesterone added as carrier, were chromatographed on an 8-g column of Grade III alumina. Fractions of 30 ml each were collected with the following eluents: 1–2, hexane; 3–5, 5 per cent; 6–8, 10 per cent; 9–10, 25 per cent; 11–13, 50 per cent benzene in hexane; 14–15, benzene; 16–17, 5 per cent; and 18–19, 10 per cent ether in benzene. TLC showed that fractions 14–16 (37 mg, $4·1 \times 10^4$ counts/min) contained progesterone. This material was freed of some sitosterol and more polar radioactive impurities by preparative TLC with cyclohexane–EtOAc (1:1) and then with CH_2Cl_2–MeOH (49:1). The isolated progesterone ($2·2 \times 10^3$ counts/min), which was radiochromatographically homogeneous by TLC in two systems, cyclohexane–EtOAc (1:1) and CH_2Cl_2–acetone (24:1), was diluted with 12 mg of carrier material and crystallized as shown in Table 1. The material from the second crystallization was reduced with $NaBH_4$ to Δ^4-pregnene-3β,20β-diol,[6] and the latter was isolated by preparative TLC with CH_2Cl_2–MeOH (47:3) and again crystallized.

Fraction 3 from the first alumina column (14 mg, $3·39 \times 10^4$ counts/min), which contained digitoxigenin, was rechromatographed on a 4 g column of Grade III alumina. Fractions of 10 ml each were collected with the following eluents: 1–2, 10 per cent; 3–4, 25 per cent; 5–6, 50 per cent ether in benzene; 7–8, ether; 9, 1 per cent; 10–11, 5 per cent; and 12–13, 10 per cent MeOH in ether. TLC of fraction 10 (7·8 mg, $1·24 \times 10^4$ counts/min) with CH_2Cl_2–MeOH (49:1) and with EtOAc–cyclohexane (9:1) showed a single radioactive peak corresponding to digitoxigenin. After acetylation, the radioactivity was shown to be associated with digitoxigenin acetate by TLC with CH_2Cl_2–MeOH (19:1).

Fractions 5 and 6 from the first alumina column (20 mg, $5·53 \times 10^4$ counts/min) contained a mixture of gitoxigenin and digoxigenin, which was separated by preparative TLC with CH_2Cl_2–MeOH (9:1). TLC of the gitoxigenin with EtOAc showed a major peak corresponding to the latter, with some minor impurities which were removed by preparative TLC in the same system. The isolated gitoxigenin (3·4 mg, $5·8 \times 10^3$ counts/min) was radiochemically homogeneous by TLC with CH_2Cl_2–MeOH (9:1) and, after acetylation, by TLC with CH_2Cl_2–MeOH (47:3). The gitoxigenin diacetate was then diluted with 18 mg of carrier material and crystallized (Table 1).

The digoxigenin fraction from above was purified by preparative TLC with EtOAc to give chromatographically homogeneous digoxigenin (3·2 mg, $4·5 \times 10^3$ counts/min), as shown by TLC with EtOAc, CH_2Cl_2–MeOH (9:1), and, after acetylation, CH_2Cl_2–MeOH (19:1).