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METABOLISM OF CHOLESTEROL-4-^{14}C IN *NICOTIANA* PLANTS

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Abstract—Cholesterol-4-^{14}C was supplied to intact *Nicotiana tabacum* L. Maryland Robinson plants through the stem by wick feeding. After 24 or 56 hr, translocation and metabolism of cholesterol were found to take place. A higher recovery of supplied compound was observed in 24 hr than in 56 hr. Interconversion among cholesterol, campesterol and stigmasterol appeared to take place in *Nicotiana* plants.

INTRODUCTION

AMONG the most widely distributed C_{27}-C_{29} sterols in plants, cholesterol (Δ^5-cholesten-3β-ol), campesterol (24α-methyl-Δ^5-cholesten-3β-ol), stigmasterol (24α-ethyl-$\Delta^{5,22}$-cholestadien-3β-ol), and β-sitosterol (24α-ethyl-Δ^5-cholesten-3β-ol) are the major 3β-hydroxysterols occurring in tobacco (*Nicotiana tabacum* L.). These 3β-hydroxysterols occur as free alcohols, esters, or glucosides.[1,2] The 3β-hydroxysterols were synthesized *in vivo* from the common source of acetate or mevalonic acid[3–5] in tobacco as in other plants.[6–10] In plants, cholesterol is a precursor of different types of steroids.[11] This paper reports the metabolism of cholesterol-4-^{14}C supplied to green tobacco plants.

RESULTS AND DISCUSSION

The levels of total 3β-hydroxysterol content in the leaf, stem, and root of the experimental plants, 24 or 56 hr after administration of cholesterol, are shown in Tables 1 and 2, respectively. Despite the slight variation among tissue weights because of unavoidable plant size differences, the patterns of 3β-hydroxysterol distribution in all experimental plants are the same. Generally, leaf tissue contained the highest concentration as well as total amount of sterols, root tissue is the next, while stem tissue contained the lowest concentration and least amount of sterol.

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Table 1. Phytosterol content in experimental plants 24 hr after supplying with cholesterol

	Leaf	Stem	Root	Total
Plant A. Supplied with 2 mg *Cholesterol*				
Tissue weight (g)	22 4	4·7	6·5	33·6
Total sterol (mg)	38·30	3·24	7·15	48·69
Plant B. Supplied with 2 mg *Cholesterol Containing* 100 μc *of Cholesterol*-4-^{14}C				
Tissue weight (g)	23·3	4·5	7·5	35·3
Total sterol (mg)	38·40	3·28	7·80	49·48
Specific activity (counts/min/mg)	$1·88 \times 10^4$	$2·62 \times 10^6$	$1·83 \times 10^3$	
Total activity (counts/mın)	$7·22 \times 10^5$	$8·59 \times 10^6$	$1·43 \times 10^4$	$9·32 \times 10^6$

Table 2. Phytosterol content in experimental plants 56 hr after supplying with cholesterol

	Leaf	Stem	Root	Total
Plant C. Supplied with 2 mg *Cholesterol*				
Tissue weight (g)	27·2	3·9	8·2	39·3
Total sterol (mg)	43·80	2·73	8·69	55·22
Plant D. Supplied with 2 mg *Cholesterol Containing* 90 μc *of Cholesterol*-4-^{14}C				
Total weight (g)	29·4	4·3	8·7	42·4
Total sterol (mg)	49·39	3·40	10·09	62·88
Specific activity (counts/min/mg)	$2·92 \times 10^3$	$1·36 \times 10^6$	$3·26 \times 10^3$	
Total activity (counts/min)	$1·44 \times 10^5$	$4·62 \times 10^6$	$3·29 \times 10^4$	$4·79 \times 10^6$

The specific activity of total sterol fraction remained highest in the stem tissue, where the feeding of cholesterol-4-^{14}C took place. In fact the stem retained the majority of the recovered material which was gradually translocated to other parts of plants as time progressed. After 24 hr of cholesterol-4-^{14}C administration, 4·2% of the ^{14}C activity was recovered in the sterol fraction, of which 92% was in the stem, 7·7% in the leaf, and 0·3% in the root. After 56 hr, only 2·4% of the ^{14}C was recovered in the sterol fraction, of which 96·3% was in the stem, 3% in the leaf and 0·7% in the root. These data appear to indicate the translocation and metabolism of 3β-hydroxysterol in plant tissue.

The 3β-hydroxysterols were separated employing reverse-phase TLC into three distinct bands. Cholesterol had the greatest mobility ($R_f = 0·67$), campesterol and stigmasterol had the same mobility ($R_f = 0·65$), while β-sitosterol has the lowest mobility ($R_f = 0·62$). Radioautograms of the sterol fraction from stem, leaf, and root obtained from plants 24 hr after cholesterol-4-^{14}C administration showed that most of the ^{14}C label was in the cholesterol band. Radioautogram bands of other sterols were also detected, but their R_f values were too close to permit good separation into individual sterols. In the samples obtained from plants 56 hr after administration, the ^{14}C radioautogram was only detected in the cholesterol band from sterol fraction, where the stem ^{14}C material was originally supplied. The ^{14}C activities in sterol fractions from leaf and root tissues were probably below the sensitivity for radioautography in the allowed time of exposure.

It is of interest to note that over 90% of the ^{14}C activity was not recovered in the 3β-hydroxysterol fraction. The labeling in the terpenoid skeleton usually is considered rather stable; the apparent loss of the labeled steroid may remain in other 3β-hydroxysterols or related compounds not included in this study.[12,13] Some of the sterols from tobacco, such as ergosterol, are known to be metabolically active and water soluble.[14] It is also likely that in plant tissue such as *Nicotiana tabacum*, the terpenoid compounds may take part in the general metabolic pool. The reduction of ^{14}C recovery with metabolic time appeared to suggest such a possibility. It has been suggested that cholesterol may be transformed to Δ^4-cholesten-3-one and cholestan-3β-ol in *Solanum tuberosum*.[15] Enzymatic aromatization of the β-ring of $\Delta^{5,7}$ sterols was also reported.[16]

The quantity of cholesterol, campesterol, stigmasterol, and β-sitosterol in leaf, stem, and root tissues of the experimental plants supplied with cholesterol-4-^{14}C is shown in Table 3, as determined by GLC. The relative distribution of these sterols in experimental plants follows the general pattern of a normal tobacco plant, indicating that the administration of foreign cholesterol did not disturb the endogenous sterol balance. In the leaf and stem tissues, stigmasterol usually occurs in the highest amount, followed in decreasing order by β-sitosterol, campesterol, and cholesterol, while in the root system, campesterol generally is higher than β-sitosterol.

TABLE 3. DISTRIBUTION OF 3β-PHYTOSTEROLS IN TISSUES FROM PLANTS SUPPLIED WITH CHOLESTEROL-4-^{14}C

	Cholesterol mg	Campesterol mg	Stigmasterol mg	β-Sitosterol mg	Total
Plant B 24 hr *After Cholesterol-4-^{14}C administration*					
Leaf	4·72	5·95	18·74	8·99	38·40
Stem	0·41	0·51	1·22	1·14	3·88
Root	0·27	2·25	3·67	1·61	7·80
Plant D 56 hr *After Cholesterol-4-^{14}C administration*					
Leaf	5·63	7·61	25·78	10·37	49·39
Stem	0·45	0·52	1·31	1·12	3·40
Root	0·35	2·69	4·95	2·10	10·09

In examining the ^{14}C activity in various 3β-hydroxysterols, only the sterol fraction from stem tissue was used because of its high specific activity. Eluates of the four sterols were measured from GLC and the results are shown in Table 4. In test plants that had metabolized either 24 hr or 56 hr, cholesterol maintained the highest total activity as well as specific activity, indicating its relative stability during the observation period. Campesterol has the next highest specific activity, followed by stigmasterol. The total ^{14}C activity of campesterol is, however, lower than that of stigmasterol. These results appear to suggest a closer metabolic relationship between cholesterol and campesterol than between cholesterol and stigmasterol. No ^{14}C activity of β-sitosterol was detectable by methods employed in this

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Table 4. ^{14}C activity in 3β-phytosterols recovered from stem tissue of plants supplied with cholesterol-4-^{14}C

	Cholesterol*	Campesterol	Stigmasterol	β-Sitosterol	Total
Plant B 24 hr *After Cholesterol-4-^{14}C administration*					
Total activity (counts/min)	$5{\cdot}84 \times 10^6$	$1{\cdot}20 \times 10^6$	$1{\cdot}55 \times 10^6$	—	$8{\cdot}59 \times 10^6$
Cal. Specific activity (counts/min/mg)	$1\,42 \times 10^7$	$2{\cdot}35 \times 10^6$	$1{\cdot}27 \times 10^6$	—†	
Plant D 56 hr *After Cholesterol-4-^{14}C administration*					
Total activity (counts/min)	$2{\cdot}77 \times 10^6$	$8{\cdot}78 \times 10^5$	$9{\cdot}70 \times 10^5$	—	$4\,62 \times 10^6$
Cal. Specific activity (counts/min/mg)	$6{\cdot}16 \times 10^6$	$1{\cdot}69 \times 10^6$	$7\,40 \times 10^5$	—	

* Specific activity of originally supplied cholesterol-4-^{14}C is 42·2 mC/mM or $2{\cdot}43 \times 10^8$ counts/min.
† Not detected.

study. Earlier reports indicated that the conversion between β-sitosterol and stigmasterol was not observed in 5 days,[7] but was demonstrated in 6 weeks.[17] Another report[18] suggested that no significant conversion of cholesterol to β-sitosterol or stigmasterol occurred. This may partially explain the absence of ^{14}C activity in our β-sitosterol fraction, especially since the longest metabolic period in the present study was only 56 hr. It can be concluded, however, based on data presented here, that conversion from cholesterol to campesterol and stigmasterol does take place in *Nicotiana* plants.

EXPERIMENTAL

Plant Material and Cholesterol Administration

Plants of *Nicotiana tabacum* L. var. Maryland Robinson Medium Broadleaf were grown in nutrient solution. They were placed under controlled laboratory conditions for 4 weeks of active growth before experimentation. 1 week before the test, the plants were decapitated, a normal practice to encourage leaf development. 2 plants of identical size were used for each set of studies. Within each set, one plant was supplied with 2 mg of unlabelled cholesterol by wick feeding through the stem, and the other with the same amount of cholesterol containing 90–100 μc of cholesterol-4-^{14}C (spec act. 42·2 mc/mM). For feeding, cholesterol was dissolved in a minimum amount of bis (2-methoxyethyl) ether. After the solution was completely absorbed, the container was washed with a few drops of EtOH, and then with 2 ml $H_2O \times 3$. One set of plants was harvested 24 hr after completion of feeding; another set was harvested 32 hr later.

Total 3β-Hydroxysterol Extraction

Isolation and determination of total sterols was carried out as described previously.[19] Briefly, it follows the semi-micro gravimetric method of Stedman and Rusaniwskyj.[20]

Radioactivity Assay

Extracted radioactive 3β-hydroxysterols were dissolved in 15 ml of toluene with (per l.) 2,5-diphenyl-oxazole (0 25 mg PPO) and 1,4-bis-[2-(5-phenyloxazolyl)] benzene (5 g-POPOP). The solutions were assayed in a Packard* Tricarb liquid scintillation spectrometer. Quench corrections were made by means of the automatic external standard.

* Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

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TLC

Solutions of extracted free 3β-hydroxysterols were spotted on paraffin-impregnated Kieselguhr* G chromatoplates[21] with wedge-tip divisions.[22] The chromatograms were developed with the reversed-phase system,[21] paraffin–acetone H_2O (4:1). The sterols appeared as bands and were further resolved by a continuous flow of solvent. The plates were sprayed with 3% phosphomolybdic acid in EtOH and heated at 100° for 5 min. R_f values of unknown sterols were obtained by comparing with those of authentic compounds. Radioautograms were made by placing Koolac* AA X-ray film (Eastman Kodak Company, Rochester, N.Y.) in contact with TLC plates from 1 to 4 weeks.

GLC

Sterols were released from sterol-digitonin complex by heating with pyridine and then extracting with Et_2O. The free sterols were converted into acetate derivatives with pyridine and acetic anhydride (1:1). A mixture of steryl acetates were dissolved in tetrahydrofuran, and analyzed with a F and M* 400 series gas chromatograph (flame ionization detector). The instrument was equipped with a 1·2 × 6 mm glass column containing 3·8% UCW98, on Gas Chrom 80-100S* (Applied Science Laboratory, Inc.). The column was at 240°, the detector at 250° and flash heater at 300°. The carrier gas was He at 60 ml/min. Cholestane was used as an internal standard. Identification of steryl acetates was made by comparison of their retention times with those of authentic compounds.

Individual sterols were collected in a glass capillary tube as they eluted from the GLC column.

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