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TRANSLOCATION OF CHOLESTEROL FROM LEAVES TO RIPENING FRUITS OF *SOLANUM KHASIANUM*

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Key Word Index—*Solanum khasianum*; Solanaceae; sterol translocation; sterol metabolism; cholesterol; cholesterol esters; solasodine.

Abstract—After foliar application of [4-^{14}C]cholesterol to a *Solanum khasianum* shrub during a 6-week period, cholesterol was recovered not only from untreated leaves, but also from fruits at three different stages of maturity. In addition to free [4-^{14}C]cholesterol, small amounts of [4-^{14}C]cholesteryl esters but no [4-C^{14}]cholesteryl glycosides were found in the fruits, treated, and untreated leaves. Thus, cholesteryl glycosides are probably not involved in the translocation of cholesterol. The implications of cholesterol translocation in the kinetics of solasodine production are discussed.

INTRODUCTION

The transport of cholesterol and other sterols has been demonstrated in both tracheophytes [1, 2] and mammals [3]. While they are transported in the form of a sterol–lipoprotein complex in mammals [3], the mechanism of translocation in plants is not well understood. Some authors believe that steryl glycosides [4] or hydrosoluble sterols [5] may act as the transportable form of plant sterols, while others have suggested a sterol–protein carrier [1]. Atallah *et al.* [1] found differences between the translocation of sitosterol and cholesterol in sunflower and geranium. For instance, labeled sitosterol but not labeled cholesterol was translocated in mature sunflower plants. In the course of our work on the biosynthesis of solasodine in *Solanum khasianum*, we have observed that cholesterol is translocated from one plant organ to another, and that cholesteryl glycosides are not apparently involved in this process. What is significant for the biosynthesis of solasodine is that the fruits, which are the richest source of the alkaloid, import cholesterol from the leaves.

RESULTS

Evidence for the identity of free cholesterol and cholesteryl esters in untreated leaves and fruits

When leaves treated with [4-^{14}C]cholesterol, untreated leaves, and fruits at three different stages of maturity (immature green, green–yellow, and fully mature yellow fruits) were extracted with hexane, the highest total radioactivity was found in the treated leaves, and the lowest radioactivity in the immature green and green–yellow fruits (Table 1). Untreated leaves and mature yellow fruits showed total radioactivity of equal order of magnitude. Aliquots of each of the hexane extracts were chromatographed on Si gel plates with Solvent System I (see Experimental) and then scanned. Only three radioactive zones were observed, viz. at R_f 0, 0.30, and 0.96, corresponding to unidentified material, cholesterol, and cholesteryl esters, respectively. The radioactive zones were eluted from the plate and assayed in a scintillation counter. The per cent distribution of radioactivity among the five plant parts is shown in Table 1.

The identity of cholesterol in the fruits was established by subjecting the eluate from the zone at R_f 0.30 to reversed-phase HPLC, collecting fractions corresponding to sterols which eluted between 20 and 45 min, and then subjecting the eluates to MS. Among the sterols thus isolated (see Experimental), cholesterol was a minor component and its identity was confirmed by a comparison with the fragmentation pattern of an authentic sample [6]. Cholesterol was shown to be labeled by diluting the eluate (*ca* 24 min) from reversed-phase HPLC with carrier cholesterol and rechromatographing it until the specific activity was constant. The presence of cholesterol in the ester fraction of the fruits was demonstrated by eluting the zone at R_f 0.96 and saponifying with 10% methanolic KOH under reflux. When the nonsaponifiable material from the preceding hydrolysis was again subjected to TLC, over 85% of the applied radioactivity was found at R_f 0.30 and the rest at R_f 0.

A different method was employed to show that labeled cholesterol was present in untreated leaves. An aliquot of the hexane extract from untreated leaves was saponified and the nonsaponifiable material was subjected to TLC. The zone at R_f 0.30 was eluted and carrier cholesterol was added. After recrystallization from MeOH, the specific activity was 204 ± 8 cpm/μmol. This was followed by recrystallizations from MeOH–CH_2Cl_2 (1:1) (209 ± 10 cpm/μmol) and acetone (204 ± 8 cpm/μmol).

Saponification of the steryl ester fraction from untreated leaves and from mature fruits both yielded

Table 1. Radioactivity in hexane extracts of *S. khasianum* leaves and fruits

	R_f	% Distribution in TLC* 0	0.30	0.96	Total cpm $\times 10^{-4}$
Treated leaves		60.2	30.0	9.8	374
Untreated leaves		69.7	17.8	14.5	1.69
Immature green fruits		64.0	16.0	20.0	0.45
Green–yellow fruits		33.0	35.0	32.0	0.29
Mature yellow fruits		14.3	49.7	36.0	5.66

*C_6H_6–Et_2O (9:1)/Si gel G.

nonsaponifiable material which gave two radioactive zones in TLC: one having an R_f 0.30 (cholesterol) and representing 85% of the applied radioactivity and one having an R_f 0 (unidentified metabolite) and representing 15% of the applied radioactivity. The sterol composition of the esters in fruits and leaves was similar to the free sterol composition (see Experimental).

Evidence for the absence of radioactive cholesteryl glycosides from the leaves

When the hexane extract of the leaves treated with [^{14}C]cholesterol was subjected to TLC with Solvent System I, a radioactive zone was observed at the origin, which corresponds to the location of steryl glycosides (Table 2). It was eluted from the plate, and a portion was rechromatographed with Solvent System II (see Experimental). Another portion was subjected to reversed-phase TLC with Solvent System III (see Experimental). Substantial radioactivity was found near the steryl glucoside standard in the latter but not in the former TLC system. The radioactive material was recovered and subjected to reversed-phase HPLC. No significant radioactivity corresponding to steryl glucoside was eluted from the column. Instead, most of the radioactivity (*ca* 80%) was eluted earlier (*ca* 6–8 min) than steryl glucoside (cf Table 2). Further evidence for the absence of cholesteryl glycosides from the hexane extract was obtained when the material remaining at the origin of the thin-layer chromatogram after development with Solvent System I was treated with either cellulase or 2 N HCl. Neither of these treatments generated free cholesterol.

Steryl glycosides may not be easily extractable from plant tissues with hexane. Therefore, the leaves were re-extracted with EtOH. An aliquot of this extract was fractionated by development in Solvent System I followed by Solvent System II. The resulting radioactive zones were similar to those in the hexane extract, and none of them corresponded to steryl glycosides. A wide band, including all radioactive material close to the steryl glucoside standard, was scraped off the plate and subjected to reversed-phase HPLC. As in the hexane extract, the radioactivity was not associated with steryl glucoside, but was eluted earlier from the column than the steryl glucoside standard.

When the hexane extracts of the leaves and fruits were examined for the presence of radioactive acylated steryl glycosides, no radioactive zone corresponding to the reference standard was found in any of the three TLC systems, except in the leaf extract where three radioactive zones were located near the standard. However, these radioactive materials, after saponification and cellulase hydrolysis, did not migrate from their original positions.

Table 2. Chromatographic behavior of steroids in TLC and HPLC

Steroids	1*	2*	3*	4*
Cholesterol	0.30	0.75	0.29	0.24
24-Methylcholesterol	0.30	0.75	0.29	0.28
24-Ethyl-5,22-cholestadienol	0.30	0.75	0.29	0.28
24-Ethylcholesterol	0.30	0.75	0.29	0.33
Steryl glucosides	0	0.35	0.47	0.11
Acylated steryl glucosides	0	0.42	0.16	—
Steryl esters	0.96	0.99	0	—
Solasodine	0	0.53	0.59	0.8
Solamargine	0	0	0.83	—

*1. R_f in TLC, C_6H_6–Et_2O (9:1)/Si gel G.

2. R_f in TLC, $CHCl_3$–MeOH–H_2O–HOAc (90:8:1:1)/Si gel G.

3. R_f in reversed-phase TLC, MeOH–C_6H_{14} (19:1)/Whatman $KC_{18}F$.

4. HPLC elution time, in min, Zorbax BP–ODS, 96% aq. MeOH.

DISCUSSION

The work presented here is part of a study concerned with the effect of water stress on solasodine production in *S. khasianum*. We have observed that increasing water stress induces an increase in the amount of solasodine in the mature berries of this plant (Yaniv, Weissenberg and Palevitch, unpublished observations).

Since cholesterol is the precursor of steroidal alkaloids [7], its biosynthesis, translocation, and metabolism are presumably somehow involved in this effect. Water stress could stimulate cholesterol production, its translocation to the sites of alkaloid synthesis, and its transformation into the alkaloids. The results of our present study show that cholesterol originally present in the leaves can be translocated to the fruits. If solasodine synthesis were restricted to the fruits, the imported cholesterol would contribute to solasodine production. The absence of labeled

cholesteryl glycosides indicates that glycosidation of cholesterol does not precede side-chain hydroxylation [8] in the formation of glycoalkaoids. Further, our results indicate that the metabolic conversion of cholesterol to a glycoside is not a prerequisite for translocation. The absence of cholesteryl glycosides from *S. khasianum* is not without precedent. The fruits of *S. xanthocarpum* contain sitosterol, stigmasterol, campesterol, and cholesterol, but only sitosteryl and stigmasteryl glucosides were found along with glycoalkaloids [9]. While the leaves of *S. andigena* seedlings contain glucosides of sitosterol, stigmasterol, and cholesterol, the leaves of mature plants do not contain cholesteryl glucoside [10]. After the application of labeled cholesterol and sitosterol to geranium plants, only sitosterol was found to be in the form of a glucoside [1].

EXPERIMENTAL

A single *S. khasianum* shrub, grown at the Experiment Station of the Volcani Center, was treated with 12.5 μCi of [4-^{14}C]cholesterol (6.17 mCi/mmol). [4-^{14}C]Cholesterol (50 μCi) was dissolved in 25 ml 96% EtOH, containing 1 μl/ml silicone oil (Dow-Corning 200) and 1 μl/ml DL-tocopherol [11]. Treatment was started when the plant was 4 months old. It consisted of applying *ca* 0.4 ml of the above soln with a brush to the upper surface of three young, fully expanded leaves. Treatment was given to different leaves 3× a week over a 6-week period. The plants were harvested 2 weeks after the last treatment. All treated and untreated leaves and mature, yellow-green, and immature berries were collected separately, dried in an oven at 50°, ground, and sent to the Western Regional Research Laboratory.

Powdered leaves (150 g) and fruits (1.0 g) were extracted in a Soxhlet apparatus overnight with *n*-hexane or EtOH. The hexane extracts were concentrated under vacuum and aliquots were counted in a scintillation counter or used for chromatography.

Plant extracts were saponified by refluxing with 20 ml 10% methanolic KOH for 30 min. The mixture was diluted with 50 ml H_2O and extracted with Et_2O (3 × 50 ml). The Et_2O extract was dried over Na_2SO_4. Hydrolysis was carried out by adding 2 mg cellulase (Calbiochem) and 5 ml 0.2 M Pi buffer, pH 4.6, to the residue from TLC fractionation. After 48 hr incubation in the dark at 35 ± 1°, the mixture was diluted with 10 ml H_2O and extracted with Et_2O (3 × 15 ml). The Et_2O extract was dried over Na_2SO_4. For acid hydrolysis, the plant material was refluxed with 15 ml 2 N HCl for 1 hr, 0.5 N NaOH was then added to the mixture until it was basic to litmus, and it was extracted with Et_2O as above.

Precoated Si gel G TLC plates (Analtech) were developed in Solvent Systems I, C_6H_6–Et_2O (9:1); or II, $CHCl_3$–MeOH–H_2O–HOAc (90:8:1:1). Reversed-phase TLC plates (Whatman $KC_{18}F$) were developed with Solvent System III, MeOH–C_6H_{14} (19:1).

HPLC used a 250 × 4.6-mm i.d. stainless-steel chromatography tube packed with Zorbax BP-ODS (7–8 μm; Du Pont). For elution, 96% aq. MeOH was used at a flow rate of 2 ml/min. The UV detector was set at 202 nm.

GLC was performed at 235° on 1% XE-60.

Reference steroids were in part those isolated previously and those obtained from Supelco (Belfonte, PA). The free and esterified sterol composition of the leaves and fruits, as determined by GLC and MS [6], were essentially identical. In the leaves, we found cholesterol:campesterol:stigmasterol:sitosterol:unknown, in proportions 10:8:17:65:trace, and in the fruits in proportions 5.5:4:9.5:81.5:trace.

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