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# BIOSYNTHESIS OF STEROLS AND TRITERPENES IN *PELARGONIUM HORTORUM*

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**Key Word Index**—*Pelargonium hortorum*; geranium; *Geraniaceae*; sterol biosynthesis; sitosterol; stigmasterol; α- and β-amyrin; tetracyclic triterpenes.

**Abstract**—Although all parts of the geranium plant (*Pelargonium hortorum*) are capable of synthesizing sterols and triterpenes and their esters *in vitro* from mevalonic acid-[2-$^{14}$C], the aerial portions are more active than other tissues. All plant parts were shown to incorporate mevalonic acid-[2-$^{14}$C] into isoprenoids for at least 3 days. The leaves and petioles had the greatest incorporation on a wet weight basis. Chopped preparations showed comparable incorporations of mevalonate whereby rootlets incorporated about one half as much as most parts; the flower petals incorporated five times the average amount. In leaves the principal sterol synthesized was sitosterol. Metabolic studies with isolated leaves indicated a fairly rapid conversion of free tetracyclic triterpenes to 4-desmethylsterols, while β-amyrin was synthesized at a different rate than α-amyrin. Esterified tetracyclic triterpenes exhibited only a slight amount of conversion to 4-desmethylsterols.

## INTRODUCTION

There has been no report in which the capacity of all morphologically distinct plant parts to synthesize sterols has been measured at the same time. Since the question of whether or not all plant parts can synthesize terpenoids may have some bearing on their translocation [1], this problem has been investigated using the common geranium (*Pelargonium hortorum*). The principal sterol and triterpene constituents of the plant are sitosterol and β-amyrin respectively [2]; sitosterol glycoside is also present in trace amounts [1].

## RESULTS

*Incorporation of mevalonic acid-*[2-$^{14}$C] *into isoprenoids by isolated whole plant parts.* A study was first made of the ability of individual isolated plant parts to incorporate mevalonic acid-[2-$^{14}$C] into isoprenoids as a function of time. The experiments (Fig. 1), demonstrated that all parts of the geranium plant could incorporate mevalonate into neutral isoprenoid lipid. The aerial parts, the leaves and petioles demonstrated the greatest uptake of label and were continuing to do so at an almost linear rate even after 4 days incubation. The flower petals initially incorporated mevalonate quite rapidly but then leveled off. With the rootlets the uptake of labeled mevalonate initially was rather slow, but increased appreciably in the last days of incubation. The main stem and root incorporated the least mevalonic acid-[2-$^{14}$C]. Their radioactive isoprenoid lipid content did, however, increase gradually over the 4-day course of the experiment.

*Incorporation of mevalonic acid-*[2-$^{14}$C] *into crude sterol–triterpene and ester fractions of Geranium.* The results for the neutral fractions, isoprenoid hydrocarbon, esterified sterols and triterpenes and the respective free compounds formed as the result of incubation of mevalonic acid-[2-$^{14}$C] with intact plant parts are shown in Table 1. The lower portions of the plant again had the least capacity to synthesize free and esterified sterols and triterpenes. Only the roots contained any labeled isoprenoid hydrocarbon.

Chopped tissues from the various major parts

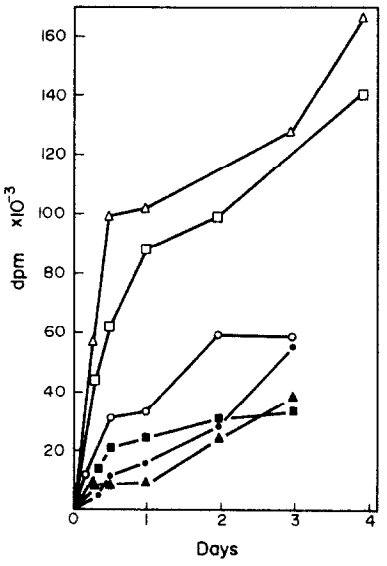


Fig. 1. Incorporation of mevalonic acid-[2-$^{14}$C] into isolated whole plant parts. △ Leaves, □ petioles, ○ flower petals, ▲ main stem, ■ roots, ● rootlets.

of the plant, when incubated with mevalonate, were also capable of biosynthesizing isoprenoid lipid (Table 2). Unlike the previous experiments, the flower petals exhibited the greatest biosynthetic ability on a fresh wt basis. Chopped roots were, comparatively, also more active than their intact counterparts. Appreciable labeled isoprenoid hydrocarbon was present in all parts, but proportionately, represented a much greater percentage of the total neutral isoprenoid radioactivity of the roots and rootlets. The distribution of radioactivity in the free sterol fraction among the major sterol classes was fairly similar for all plant parts with the exception of the flower petals, which were relatively higher in labeled 4,4-dimethylsterols.

*Incorporation of mevalonic acid-*[2-$^{14}$C] *into sterol–triterpene and the corresponding ester fractions of Geranium leaves vs time.* The data in Fig. 1 indicates that leaves are the tissues with the greatest capacity for synthesizing sterol and triterpenes. Also our previous work [1] indicated

Table 1. Incorporation of mevalonic acid-[2-$^{14}$C] into isoprenoids by isolated parts of *Pelargonium hortorum*

| Plant part | Total neutral lipid (dpm) | Isoprenoid hydrocarbon (dpm) | Steryl esters (dpm) | Free sterol (dpm) |
|---|---|---|---|---|
| Flower petals | 64700 | 0 | 8960 | 55700 |
| Leaves | 119000 | 0 | 15800 | 103000 |
| Petioles | 106000 | 0 | 9680 | 96300 |
| Main stem | 36500 | 0 | 5880 | 30600 |
| Roots | 30600 | 11200 | 1800 | 17600 |

Each incubation contained 2 g of intact geranium tissue and 2 $\mu$Ci of mevalonic acid-[2$^{14}$C] in a total volume of 5 ml. The incubations were for 2 days.

Table 2. Incorporation of mevalonic acid-[2-$^{14}$C] into isoprenoids by chopped tissues of *Pelargonium hortorum*

| Plant parts | Total neutral lipid (dpm) | Isoprenoid hydrocarbon (dpm) | Steryl esters (dpm) | Free sterol (dpm) | Percent distribution of labeled free sterols | | |
|---|---|---|---|---|---|---|---|
| | | | | | 4,4-Dimethyl-sterols | 4α-Methyl-sterols | 4-Desmethyl-sterols |
| Flower petals | 252000 | 22900 | 22000 | 207000 | 81·5 | 9·9 | 9·4 |
| Leaves | 50100 | 646 | 2440 | 47000 | 69·9 | 8·4 | 21·6 |
| Petioles | 45800 | 2080 | 5420 | 38300 | 59·1 | 11·2 | 29·6 |
| Main stem | 41900 | 4030 | 7800 | 30100 | 58·2 | 11·1 | 30·6 |
| Roots | 46800 | 18800 | 8190 | 19800 | 54·6 | 11·4 | 34·2 |
| Rootlets | 23400 | 7050 | 4550 | 11800 | 42·7 | 21·0 | 36·3 |

Each incubation contained 2 g of chopped tissue and 5 $\mu$Ci of mevalonic acid-[2-$^{14}$C] in a total volume of 3 ml. Incubations were carried out for 2 days.

that leaves are the primary source for sterols and triterpenes to be translocated to other parts of the plant. Two geranium leaves each were presented with mevalonic acid-[2-$^{14}$C] and subsequently processed after varying time periods to obtain a neutral fraction. Chromatography of the neutral fraction yielded the hydrocarbons, ester and free sterol–triterpene fractions. The hydrocarbon fraction exhibited the rapid turnover shown by squalene in other tissues [3, 4] and was not investigated further. Fig. 2 shows the incorporation of radioactivity into the crude ester and free sterol–triterpene fractions respectively. Both exhibited a rather pronounced rate of turnover between 1 and 48 hr, with the esterified sterol and triterpenes decreasing in total $^{14}$C-incorporation, after an initial increase, and the unesterified sterol and triterpenes undergoing a gradual increase after an initial decrease.

*Turnover of esterified and free sterol–triterpene fractions of Geranium leaves.* Saponification of the crude ester fractions followed by preparative TLC, separated the complex mixture into 4-desmethylsterols, 4α-methylsterols and 4,4-dimethylsterols. Preparative TLC of the crude unesterified sterol–triterpene fractions was also performed and the results are presented in Fig. 3. The 4-desmethyl- and the 4α-methylsterols from both the free and esterified fractions exhibited a steady increase in $^{14}$C-incorporation. Although the esterified 4,4-dimethylsterols appeared to undergo some turnover during the experimental period, the unesterified 4,4-dimethylsterols exhibited a pronounced turnover, presumably to 4α-methyl- and 4-desmethylsterols.

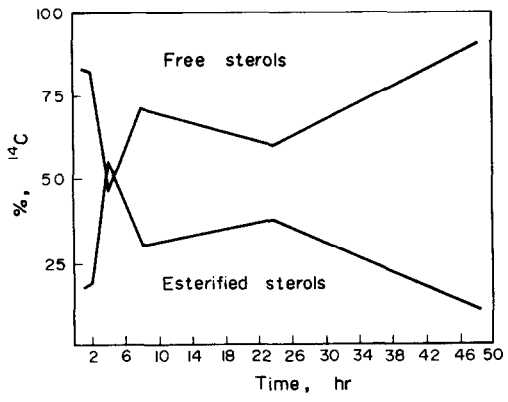


Fig. 2. Rate of incorporation of mevalonic acid-[2-$^{14}$C] into esterified and unesterified sterol and triterpene fractions from isolated geranium leaves.

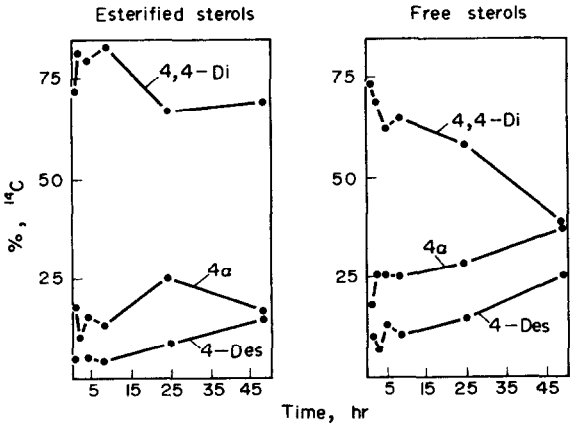


Fig. 3. Distribution of radioactivity in the esterified and unesterified sterols and triterpenes from geranium leaves incubated with mevalonic acid-[2-$^{14}$C].

Each of the unesterified sterol fractions was subjected to GLC-RC to determine the identity of the labeled compounds. The 4-desmethylsterols consisted primarily of labeled sitosterol (80%) and campesterol (20%); no radioactive peak was indicated for stigmasterol. The 4α-methylsterols yielded radioactive peaks whose retention times were identical to lophenol (4%), obtusifoliol (27%), and cycloeucalenol (69%).

Acetylation of the 4,4-dimethylsterol fraction followed by argentation chromatography and GLC–RC showed that cycloartenyl acetate, 24-methylene cycloartanyl acetate, α-amyrin acetate and β-amyrin acetate were the labeled constituents. The distributions of the radioactive material within the 4,4-dimethylsterol fraction (Fig. 4)

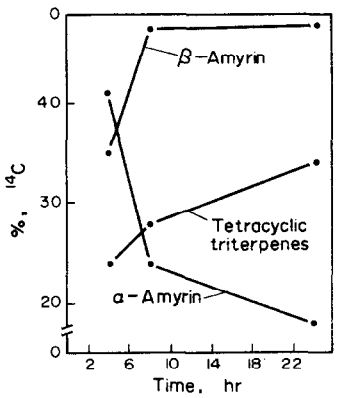


Fig. 4. Distribution of radioactivity in unesterified triterpenes of geranium leaves incubated with mevalonic acid-[2-$^{14}$C].

suggest that there are different rates of formation of α- and β-amyrin.

*Incorporation of $^{14}CO_2$ into a Geranium plant through a leaf.* One leaf of an intact potted geranium plant was exposed to $^{14}CO_2$ for 3 weeks. The EtOH extracts of the plant parts were taken to dryness and then the residue dissolved in $C_6H_6$–EtOH (1:1). This left much insoluble material behind and resulted in a relative enrichment of lipid in the soluble fraction. The radioactive content of the soluble material (Table 3) indicated a great deal of radioactivity in the exposed leaf, its petiole and the branch to which it was attached. The same was true of leaves on the same branch and leaves on lower branches. Significant radioactivity was also present in the roots and rootlets. In contrast, the leaves on higher branches were rather poorly labeled. No radioactivity was found in a flower that was present on a higher branch. When several of the more radioactive extracts were examined by alumina column chromatography, from 4 to 34% of the total radioactivity was eluted in the isoprenoid fractions.

Further examination of the steryl ester and free sterol fractions from the pooled leaves and petioles indicated that 85% of the steryl ester fraction could be recovered as nonsaponifiable sterol and that *ca* 61% of the free sterol fraction was actually steroidal material. Because of large amounts of endogenous sterol most of the fractions could not be assayed by GLC–RC. However, it was found that 46·9% of the radioactivity of the esterified 4,4-dimethylsterols was β-amyrin and 53·1% was associated with the unresolved α-amyrin-24-methylene cycloartanol peak. The free 4,4-dimethylsterols gave only one radioactive peak corresponding to the unresolved α-amyrin and 24-methylene cycloartanol.

## DISCUSSION

The data presented here show that all parts of the geranium plant are capable of synthesizing sterol and triterpenes and their respective esters but this synthetic capacity is less in the underground portion of the plant. The question then arises, if all portions of a plant can synthesize sterols and triterpenes, what is the necessity for translocation? One possible answer is that since plant sterol biosynthesis is essentially one requiring light [5], perhaps translocation is designed to augment the concentration of these substances in those portions of the plant receiving diminished (stem) or no (root and rootlets) light.

Active turnover of some established triterpenoid precursors to sitosterol and the pentacyclic triterpenes α- and β-amyrin was demonstrated by isolated geranium leaves. Presumably this turnover occurs in other isolated geranium parts, although at different rates. The observation that α-amyrin is biosynthesized before β-amyrin in

Table 3. Radioactivity in the ethanol extracts of *Polargonium hortorum* tissues after exposure of one leaf of an intact plant to $^{14}CO_2$

| Plant part | Radioactivity in ethanol extract (dpm × $10^{-3}$) | Hydrocarbon (dpm × $10^{-3}$) | Steryl esters (dpm × $10^{-3}$) | Free sterol (dpm × $10^{-3}$) |
|---|---|---|---|---|
| Exposed leaf, L-1 | 8940 | 258 | 1150 | 1630 |
| Petiole of L-1 | 4070 | 9·77 | 320 | 261 |
| One leaf above L-1 on same branch as L-1 | 9430 | 4·9 | 94·4 | 296 |
| Other two leaves above L-1 on same branch | 4770 | — | — | — |
| Leaves on higher branches | 23·5 | — | — | — |
| Leaves on lower branches | 256 | — | — | — |
| All petioles other than L-1 petiole | 1070 | — | — | — |
| Branch bearing L-1 | 2360 | — | — | — |
| Other branches and main stem | 553 | — | — | — |
| Roots | 94 | — | — | — |
| Rootlets | 235 | — | — | — |
| Flower, on a higher branch | 0 | — | — | — |
| Pooled leaves and petioles* | — | 71·0 | 71·0 | 1050 |

* Ethanol extract partitioned between ethyl ether and 5% NaOH before application to column. Total dpm of organic phase, 3·62 × $10^6$.

these isolated preparations should be further investigated since it may shed some light on the squalene–pentacyclic triterpene cyclization process [7,8].

## EXPERIMENTAL

*Plants.* Geranium plants (*Pelargonium hortorum*) were purchased locally.

*Incubations.* All incubations were conducted at room temp. under normal laboratory conditions. Mevalonic acid-[2-$^{14}$C] as the dibenzylethylenediamine salt (sp. act. 5·8 mCi/mmol, New England Nuclear Corporation, Boston, Massachusetts) was used as labeled precursor. Chopped tissues refers to plant organs cut with scissors into pieces approximately 0·5 × 0·5 cm, or 0·5 cm in length in the case of rootlets. Chopped tissues were placed in a covered petri dish, and $H_2O$ was added to maintain the viability of the plant tissue. Except for the time study of various intact plant parts, the mevalonic acid-[2-$^{14}$C] incubations were terminated after 2 days. Complete absorption of precursor soln usually occurred within 30 min to 1 hr, after which more distilled $H_2O$ was added in sufficient amount to keep the parts viable during the entire experimental period.

*Uptake of* $^{14}CO_2$. One leaf, while still attached to a potted geranium plant, was placed into an airtight Plexiglas box. The leaf was then exposed to 1 mCi of $^{14}CO_2$ derived from $Ba^{14}CO_2$ (sp. act. 21·9 mCi/mmol, Nuclear Chicago, Chicago, Ill.) for a period of 3 weeks. The plant was watered and maintained normally. The plant appeared generally healthy throughout the course of the exposure. After 3 weeks the plant was sectioned appropriately and immediately extracted with hot EtOH [1].

*Preparation of neutral fractions and hydrolysis of ester fractions.* A neutral fraction of each incubation was prepared by partitioning an EtOH extract of the wet plant tissue between $Et_2O$ and 10% NaOH. The $Et_2O$ extract was subjected to column chromatography and $^{14}$C-determination. Ester fractions were hydrolyzed by refluxing for 2 hr in EtOH–$C_6H_6$–$H_2O$ (8:1:1) containing 15% KOH, followed by extraction with $Et_2O$.

*Chromatography.* Samples in 40 ml petrol (bp 30–60°) were separated on a column of Merck acid-washed alumina (60 g, 1 cm dia). The first 40 ml was collected as fraction 1. Nine fractions of petrol (20 ml) were collected followed by 10 $C_6H_6$ fractions (20 ml) and one EtOH fraction (200 ml). Hydrocarbons, including squalene, were recovered in fractions 3 to 7, sterol esters in fractions 12–14, and free triterpenes and 4-desmethyl sterols in the EtOH fraction. Preparative-TLC [6] was employed to separate 4-desmethylsterols ($R_f$ 0·44), 4α-methylsterols ($R_f$ 0·54), and 4,4-dimethylsterols ($R_f$ 0·64). Visualization was by spraying with 5% anisaldehyde in 95% EtOH–$H_2SO_4$, (19:1). Bands corresponding to standard sterols and triterpenes were scraped from the plates, and eluted with EtOAc–MeOH (1:1). Sterols were acetylated ($Ac_2O$–$C_5H_5N$, 2:1) at room temp. overnight and the acetates subjected to preparative-TLC on 12% $AgNO_3$–Si gel G developed with petrol–$C_6H_6$ (1:1).

*Determination of radioactivity.* Radioactivity was determined as previously described [3]. GLC–RC was performed as described before [1]. The analysis was made on a 3% OV-17 column at 270°.

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## REFERENCES

1. Atallah, A. M., Aexel, R. T., Ramsey, R. B. and Nicholas, H. J. *Phytochemistry*. In Press.
2. Aexel, R. T., Ramsey, R. B. and Nicholas, H. J. (1972) *Phytochemistry* **11**, 2353.
3. Kelley, M. T., Aexel, R. T., Herndon, B. L. and Nicholas, H. J. (1969) *J. Lipid Res.* **10**, 166–174.
4. Capstack, E., Rosin, N., Blondin, G. A. and Nes, W. R. (1965) *J. Biol. Chem.* **240**, 3258–3263.
5. Knapp, F. F. and Nicholas, H. J. (1971) *Phytochemistry* **10**, 85–95.
6. Rahman, R., Sharpless, K. B., Spencer, T. A. and Clayton, R. B. (1970) *J. Biol. Chem.* **245**, 2667–2671.
7. Nes, W. R. and Cannon, J. W. (1968) *Phytochemistry* **7**, 1321.
8. Eschenmoser, A., Ruzicka, L., Jeger, O. and Arigoni, D. (1955) *Helv. Chim. Acta* **38**, 1890.