Critical Reviews in Biochemistry and Molecular Biology, 34(2):59–69 (1999)

Tracing Steroid Synthesis in Plants

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CONTENTS

I. INTRODUCTION

Radioactive tracers have played an important role in elucidating the biochemical pathway of plant steroids. Tavormina, Gibbs and Huff[1] made the first successful incorporation of [2-^{14}C]-mevalonic acid (MVA) into sterols with a rat liver homogenate. The discovery of MVA as a key intermediate and its efficient incorporation into cholesterol opened a new phase in the field of biochemical research concerning the conversion of acetate into isoprenoids.[2,3] Although after almost 40 years some details still remain to be clarified, the broad outline of the sterol biosynthetic pathway leading to plant sterols and their derivatives is now understood.[4] Squalene was proven to occupy a key position in sterol biogenesis[5] and its cyclization pattern turned out to be the branching point in the synthesis in various types of steroids: the tetracyclic phytosterols, the cardenolides and steroid alkaloids, the ecdysteroids, the pentacyclic triterpenoids, triterpene acids, the triterpene saponins, the whitanolides, etc. The basic building block of all these compounds is derived from mevalonic acid.

In tracer studies confusing and sometimes conflicting results may arise, which sometimes are not even recognized. Although most of these can be understood as arising from complex interconversions of added precursors or major alterations in an ongoing metabolism, it is important to appreciate the limitations of the radiochemical feeding techniques that can be used in plant physiology. Banthorpe et al.[6] have described a set of working rules representing an ideal experimental design in monoterpene biosynthesis. Most of this experimental design can be applied in tracing steroid synthesis and is outlined in this chapter.

II. TYPE OF PRECURSOR

A. SPECIFIC PRECURSORS

Acetate and mevalonate are the most celebrated labeled precursors in steroid labeling experiments. Both substrates are water soluble and easily taken up by excised plant tissues and rapidly metabolized. Mevalonic acid is a rather specific and unique intermediate in isoprenoid synthesis and ^{14}C-labeled mevalonic acid gives a rather high yield in ^{14}C-steroids. The percentage incorporation of ^{14}C-acetate in steroids is in general considerably lower than obtained with ^{14}C-mevalonate, which may be due to any of several reasons. The preceding conversion of acetate into mevalonate can be a limiting factor: the

thiokinase converting acetate into acetyl CoA may be a rate-limiting step. The enzyme 3-hydroxy-3-methylglutaryl CoA (HMG CoA) reductase has a key role in the regulation of cholesterol biosynthesis.[7–9] Excessive dilution of ^{14}C-acetate with endogenous acetate and the utilization of acetyl CoA by competing metabolic pathways will lower the ^{14}C incorporation into steroids.

Specifically, ^{14}C- and ^{3}H-labeled mevalonic acid, now commercially available, have played a major role in elucidating the biochemical pathway in sterol synthesis and [2-^{14}C]-MVA has been used by numerous investigators to trace an ongoing steroid synthesis. Cycloartenol turned out to be the starting intermediate for phytosterol synthesis in which the methyl groups at C-4 are subsequently lost.[10,11] These desmethyl sterols in higher plants generally have a methylene, ethylidene, methyl or an ethyl group at C-24 in the side chain. These C-24 alkyl groups are formed by a trans methylation reaction from methionine via *S*-adenosylmethionine.[12,13]

Labeled sterols have been used to investigate sterol interconversions and to trace, e.g., cardenolide and withanolide synthesis. Most of these are not available commercially and are to be synthesized by the user. The chemical synthesis of a radiolabeled sterol is to be preferred as its chemical purity is to be assured. Tritium-labeled sterols have been used extensively because their synthesis is a little easier and less costly than the ^{14}C-labeled counterpart. Procedures to prepare radiolabeled sterols have been described by Goad.[14]

Cholesterol, a major sterol in animal cells, but occurring in trace amounts in plants, is the starting compound for cardenolide synthesis. For the typical conversions leading to the digitoxigenin moiety in digitoxine, specifically labeled sterols and pregnanes have been employed successfully. A major problem in these incorporation experiments is the lipophilic properties of these precursors. Special applications are required in the administration of these labeled substrates. Aberhart et al.[15] removed the leaf wax from the upper surface of *Digitalis lanata* (Scrophulariaceae) leaves with ethylacetate and [8-^{3}H,4-^{14}C]-cholesterol (dissolved in hexane) was applied to the upper surface with a glass rod. After evaporation of the hexane, the leaves were slightly sprayed with a 1% silicone oil in hexane to restore a waxy layer. Although some of the leaves showed brown spots as a result of the toxicity of the hexane, most of the leaves grew normally and were harvested after a 37 day incorporation period. The conversion of [8-^{3}H,4-^{14}C]-cholesterol into digitoxigenin and digoxigenin indicated the absence of several postulated intermediates in the elaboration of these cardenolides. Leaves from *Acnistus breviflorus* (Solanaceae) synthesized with anolides from [24-^{14}C]-24-methylenecholesterol absorbed by the petiole from a concentrated Tween-20 suspension.[16]

Incorporation experiments with specific precursors provide the details of the biochemical pathway only. Possible impacts of the detergents employed are ignored, and the physiological aspects of steroid synthesis are not captured. Mevalonate is easily taken up and although it is available in high specific activities the introduction of this labeled precursor into plant tissue may lead to a distortion of the flux of precursors through the pathway. Exogenously supplied MVA enters the biosynthetic pathway after a primary regulatory point (HMG CoA reductase[7]) and this may lead to an abnormal accumulation of an intermediate at a secondary control point. Many investigations with ^{14}C-MVA mention the accumulation of ^{14}C-squalene.[17,18] This phenomenon may also be used to identify intermediates, but the incorporation values of ^{14}C-MVA do not necessarily give a true picture of the normal physiological process. The main effect of excess MVA supplied to cell suspension cultures of celery (*Apium graveolens*, Umbelliferae) was an increase in the synthesis of steryl esters. In MVA treated cells, the esterified 4,4-dimethylsterols were more highly labeled than 4-mono- and 4-desmethylsterols, suggesting a restriction in the flow of carbon downstream from cycloartenol and 24-methylenecycloartenol, which indicates the presence of a post-mevalonic acid control point in the sterol biosynthesis pathway.[19] A very low incorporation of ^{14}C in the fatty acid moiety of the steryl esters indicated a limited breakdown of excess MVA into acetate by a mevalonate shunt as depicted by Nes and Bach.[20]

B. NONSPECIFIC PRECURSORS

Steroids do occur on various sites in a plant tissue and some cell organelles like chloroplasts[21] have their own steroid synthesis. Laticifers of many latex bearing plant species are well known for their synthesis and accumulation of triterpenes, e.g., *Euphorbia* with triterpenols,[22,23] and various species of *Asclepias* producing cardenolides. The laticifers of many *Hoya* species (Asclepiadaceae) contain suspensions of solid terpenoid particles with 0.01 to 2 µm diameter.[24,25] Within the genus *Hoya* these submicroscopic particles consist mainly of triterpenyl cinnamates[26] and their abundance makes these compounds the major steroids in the aerial tissues of these species. The nonarticulated laticifers are to be considered as tubular cells in which the thin wall lining cytoplasm produces triterpene esters, which in turn are secreted

as solid particles into the large central vacuole. Most of the latex is lost upon incision, but excised stem parts of this plant restore the laticifer content and after a few days newly synthesized latex and its lipids exude upon repeated incision.

When the excised stem parts of *Hoya australis* were supplied with ^{14}C-labeled acetate or mevalonate none of the label did proceed to the triterpenyl cinnamates.[27] Although both substrates are soluble in water and easily taken up and translocated through excised tissue they did not enter the cytoplasm of the laticifers. Exogenously supplied sugars, however, did reach the laticifers and were subsequently used in the massive triterpenoid synthesis of these specialized cells. Although sucrose and glucose are not specific precursors in steroid synthesis, they are excellent substrates to reach the hot spots of terpenoid synthesis in *Hoya* and *Euphorbia*, the nonarticulated laticifers. And both the triterpenoid and the cinnamoyl moiety of the esters gain label from the ^{14}C sugars supplied. No accumulation of ^{14}C was measured in squalene.

Incorporation experiments with [1-^{14}C]-glucose and [6-^{14}C]-glucose produced equal amounts of ^{14}C-triterpenylcinnamates. This indicates that glycolysis inside the laticifer is the major route in glucose catabolism providing the substrates for the MVA pathway and the required ATP and reduction equivalents. When [3,4-^{14}C]-glucose was administered, only traces of ^{14}C proceeded to the typical latex triterpenoids. These results were interpreted as indicative that in these cells the oxidative pentose phosphate cycle is not involved in the elaboration of triterpenoids from glucose.[28,29]

III. AMOUNT OF SUBSTRATE

Intermediates of the mevalonate pathway should be fed at the highest possible specific radioactivities and at the lowest feasible concentrations. Typical intermediates of this pathway (like MVA, isopentenyl diphosphate) are normally not translocated *in vivo* from cell to cell and the compounds supplied must perturb the system. Since the concentrations of intermediates are well balanced in a living tissue an extra supply of one of the intermediates may evoke unnatural patterns of metabolism during the period after feeding and until any excess of additive has been degraded and eliminated by salvage mechanisms. Intercellular translocation of sugars, amino acids, and organic acids occurs in plants and these compounds may be supplied in greater amounts. In most cases they are stored in the vacuoles after uptake and released and used in metabolism later. These substrates are no specific precursors in steroid synthesis, but specialized cells, like laticifers in various plant species, synthesize relatively large amounts of triterpenoids or cardenolides and these heterotrophic cells depend on carbohydrates or organic acids from neighboring cells.

A. SUGARS

In *Hoya* and *Euphorbia* sucrose and glucose appeared to be efficient precursors in latex triterpenoid synthesis.[27,30–33,44] As milligram amounts of sugars were taken up by excised stem parts of *Hoya* and substantial amounts of ^{14}C were traced in the triterpenyl cinnamates the production of esterified triterpenes from exogenously supplied sucrose can be calculated. Results presented in Figure 1 show that an increasing supply of sucrose up to about 30 µmol was mainly used in the synthesis of esterified triterpenes (laticifers) while production of sterols (which are absent in the laticifers) was saturated at about 10 µmol supply.

B. AMINO ACIDS

Radiolabel from amino acids was not incorporated into the triterpenoids of laticifers, but substantial amounts of ^{14}C proceeded to the sterols in *Euphorbia*.[34,35] The branched amino acids isoleucine, leucine, and valine and the hydroxy amino acid threonine appeared to be efficient precursors in triterpene ketones (occurring in the wax layer) after uptake by the cotyledons of intact *Euphorbia lathyris* seedlings. In this species about 10% of the sterols and wax triterpenoids were produced from amino acids.[36–38]

C. ORGANIC ACIDS

Several organic acids have been used in incorporation experiments and malonate appeared to be one of the most effective precursors in sterol, triterpene, and cardenolide synthesis.[39–42] Malonic acid is a common constituent of mature leaves of certain species of Leguminosae and was found to accumulate in the stem of *Asclepias curassavica*.[42] This organic acid is rapidly absorbed and distributed over excised tissues. Excised 6 cm stem parts of *Hoya diversifolia* absorbed 3 µmol of [2-^{14}C]-malonate in about 24 h and a time course experiment showed the labeling of four classes of terpenoids (Figures 2A, 2B). After 24 h the (free) triterpenols had reached their maximum ^{14}C level, while the sterols continued to gain ^{14}C

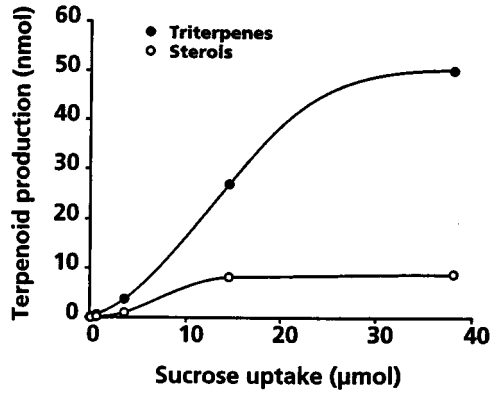

Figure 1 Triterpene (●) and sterol (○) production from various amounts of exogenously supplied labeled sucrose by 8 cm stem parts from the fourth internode from *Hoya diversifolia*. Incorporation time, 72 h.

in a 100 h period. Esterified sterols gained little activity and the curve of the ^{14}C esterified triterpenols almost paralleled the uptake curve: a rapid synthesis during ^{14}C uptake and slow, but steady, increase after 12 h (Figure 2B). The triterpene cinnamates did not gain ^{14}C and these findings suggested that exogenous malonate does not participate in the synthesis of latex triterpenes. After 90 h of incorporation all the occurring steroids totalled 43.500 dpm ^{14}C. This figure represents 1.17 % incorporation, from which a yield of 2.1 nmol sterol can be calculated. In *Hoya* the production of sterols from exogenous malonate is one order of magnitude lower than produced from sucrose (Figure 1).

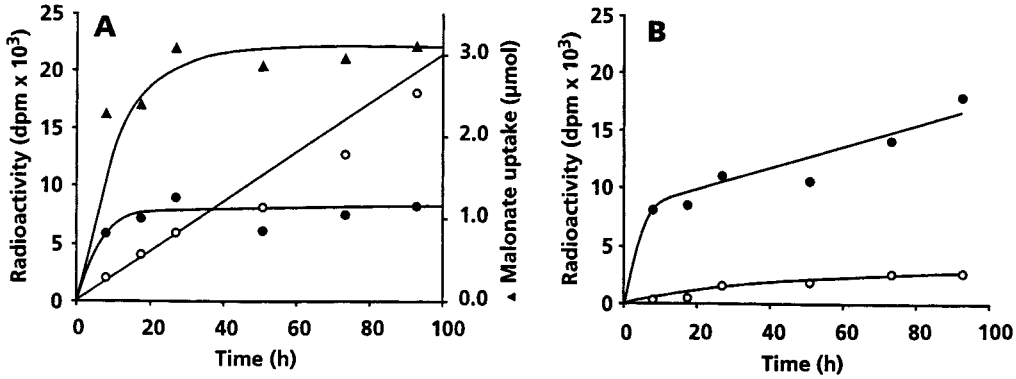

Figure 2 (A) Time course incorporation of [2-^{14}C]-malonate into free triterpenols (●) and sterols (○) by 6 cm stem parts excised from the fourth internode from *Hoya diversifolia*. 1.667 µCi [2-^{14}C]-malonate, spec. act. 0.516 mCi/mmol. (B) Time course incorporation of [2-^{14}C]-malonate into esterified triterpenes (●) and esterified sterols (○) by 6 cm stem parts excised from the fourth internode from *Hoya diversifolia*. 1.667 µCi [2-^{14}C]-malonate, spec. act 0.516 mCi/mmol.

Malonate levels in the stem of *Asclepias curassavica* rose to 114 µmol/g dry weight during plant development. Excised 10 cm stems showed a rapid metabolism of this organic acid, which turned out to be an efficient precursor in 5ß-cardenolides. A supply of 3.5 µmol of malonate per stem was well within the physiological limits of this plant and an appropriate amount of ^{13}C-cardenolides could be synthesized for nuclear magnetic resonance (NMR) analysis. However, the obtained ^{13}C enrichment pattern in the major cardenolide uscharidin did not match exactly the expected labeling pattern from the MVA pathway.[40,42]

The metabolic fate of the nonspecific precursors not only depends on enzyme activity but primarily on the way they are distributed over the tissue. The epidermal cells of *Euphorbia lathyris* seedlings do produce triterpene ketones in their wax layer and several amino acids apparently do reach these specialized cells.[36,37] Labeled acetate is hardly involved in triterpene ketone synthesis and it may be assumed that exogenous acetate does not reach the epidermal cells.

Taking the quantitative aspects into account, the nonspecific precursors may be more advantageous than acetate or MVA. The fatty endosperm of *Euphorbia lambii* seedlings converts triacylglycerols into acetyl-CoA which in turn is converted into sucrose. The sucrose is taken up by the cotyledon and a part of it is used in steroid synthesis via acetyl-CoA and MVA. As far as the steroid synthesis is concerned, an uptake of acetate by the cotyledons would avoid the metabolic detour over sucrose. Although ^{14}C-acetate is easily taken up by the cotyledons, the uptake capacity is a limiting step. The uptake capacity for sucrose is higher and that does more than compensate the nonspecific character as a substrate in steroid synthesis.[43] A similar phenomenon may be the case in triacylglycerol and sterol synthesis in developing *Cuphea lutea* seeds (Lythraceae). The level of acetate incorporation in the *in vitro* experiments was two orders of magnitude less than C_2 utilization required to sustain endogenous lipid synthesis.[18] The high level of squalene synthesis from labeled MVA was unexpected and the authors proposed two pools of squalene to explain the incorporation levels. Developing seeds are supplied *in vivo* with sucrose, which is converted into triacylglycerols and *in vitro* incorporation experiments with ^{14}C-sucrose might have equalled the lipid production as well as a concurrent sterol synthesis.

IV. CALCULATION OF STEROID PRODUCTION FROM LABELED SUBSTRATES

Once the biochemical pathway of the conversion of a substrate into a sterol is known in detail and the labeling pattern of the end product has been established or predicted, the contribution of this exogenously supplied substrate to sterol production can be calculated. An example of a conversion of [U-^{14}C]-sucrose into triterpenols in *E. lathyris* has been worked out in Table 1. Sucrose is catabolized to acetyl-CoA, which is subsequently used in the MVA pathway. So the biosynthesis of 1 mol of triterpenol requires 4.5 mol of sucrose.[28,29,32] From 54 carbon atoms from [U-^{14}C]-sucrose, only 30 carbon atoms are incorporated in the triterpenol skeleton and 24 are lost as $^{14}CO_2$. The 113,750 dpm ^{14}C encountered in the triterpenols are to be corrected for the calculated losses of $^{14}CO_2$ and the total investment in triterpenol synthesis becomes 204,750 dpm. This radioactivity was supplied by [U-^{14}C]-sucrose and equals 73.78 nmol sucrose. As 4.5 mol of sucrose are required to produce 1 mol of triterpenol, 73.78/4.5 = 16.4 nmol of triterpenol are produced from exogenously supplied sucrose. This figure is to be considered as a minimum value. Endogenous sucrose is also involved, but to what extent is rather difficult to predict without data on the distribution of the labeled substrate over the tissue incorporated, as outlined in Section VII.

Table 1 Incorporation of ^{14}C from [U-^{14}C]-sucrose into Triterpenols of *Euphorbia lathyris*

Uptake	5,328,000 dpm
Specific activity	1.25 mCi/mmol
^{14}C-triterpenols	113,750 dpm
^{14}C loss in synthesis	91,000 dpm
Total ^{14}C investment	204,750 dpm
^{14}C-sucrose investment	73.78 nmol
^{14}C-triterpenols produced	16.40 nmol
% incorporation	3.843 %

V. SIZE OF THE TISSUE

Tissues are composed of different cell types and differences in steroid composition and capacity in steroid production are to be anticipated. The size of the tissue was shown to act upon the incorporation pattern in *Hoya diversifolia*. The nonarticulated laticifers in this climbing tropical plant occur as long vessels running near the phloem and in the cortex. Supplied sucrose is mainly used in triterpenyl cinnamate synthesis in the laticifers as far as steroid synthesis is concerned. The incorporation of ^{14}C into triterpenyl cinnamates depended on the length of the stem part used. Results presented in Figure 3 show a linear relationship between the length of the stem part used and the amount of ^{14}C-sterols produced. A similar linear relationship was observed for the triterpenyl cinnamates, but these typical latex constituents were not produced by excised stem parts shorter than 0.9 cm. Bathing 2 mm thick stem slices in a ^{14}C-sucrose solution resulted in a considerable uptake of the sugar and although some ^{14}C-triterpenols and sterols are detectable after a 24 h incubation period, the triterpenyl cinnamates were not radioactive.

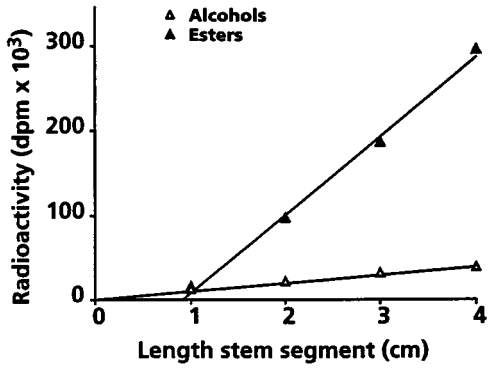

Figure 3 Incorporation of ^{14}C from [U-^{14}C]-sucrose into esterified triterpenes, (free) triterpenols + sterols (alcohols) by stem parts excised at different lengths from the fifth internode of *Hoya diversifolia*. 10 µCi [U-^{14}C]-sucrose, 1.97 µmol, 40 h incorporation time.

VI. SUBSTRATE UPTAKE BY INTACT PLANTS

Carbon dioxide is the only radioactive precursor that can be fed to all green plants under physiological conditions. A variety of water-soluble ^{14}C-labeled substrates can be fed to various growing seedlings. Seedlings from many endospermous dicots obtain their assimilates and nutrients from a living endosperm. Species with an easily removable endosperm are appropriate plants with which to trace radioactivity from water-soluble ^{14}C-labeled substrates under physiological conditions.[44]

The fatty endosperm of *Euphorbia lathyris* is a living tissue which converts triacylglycerols into sucrose and hydrolyzes storage proteins into amino acids. Seeds of *Euphorbia lathyris* germinate 4 days after imbibition and from that day the cotyledons of the growing seedling take up the sucrose and the amino acids produced in the endosperm. When grown in the dark at 25°C the endosperm was depleted in about 12 days. Per day an uptake rate of 9.5 µmol of sucrose per seedling was measured in the period 6–12 days after imbibition. At 9 days after imbibition the endosperm was easily removed (within 20 seconds) and immediately replaced by a ^{14}C-labeled solution. Although growth was not fully maintained, the cotyledons continue to absorb substantial amounts of sugars and amino acids. Upon removal of the endosperm a daily production of 11 µg triterpenols per seedling was maintained for two subsequent days. From the third day after endosperm removal a 50% reduction in triterpenol production was observed.

When the liberated cotyledons of a 9-day-old seedling were supplied with a 100–150 µl solution containing 1 µmol [U-^{14}C]-sucrose, most of the ^{14}C-labeled substrate was actively taken up and translocated over the seedling (Figure 4A). This amount of sucrose is about 10% of the daily uptake and was found to be the minimum value to promote ^{14}C transport to hypocotyl and root. After 24 h the seedling was cut into 9 pieces (as depicted in Figure 4B) and in each part levels of sucrose and glucose were assayed and the ^{14}C content in the sterols, triterpenols, and triterpene ketones measured. Results presented in Figure 4B show that about 42% of the radioactivity was retained by the cotyledons, 32% was recovered from the apical part of the hypocotyl, and only 2.2 % was traced in the roots (losses in respiration were not measured).

Highest levels in ^{14}C-triterpenols were encountered in the cotyledons. The apical part of the hypocotyl contained the most ^{14}C-triterpene ketones and had the highest ^{14}C-sterol production, but ^{14}C-sterols were also abundant in the root tip.

Most of the endogenous sugars appeared to be concentrated in the lower part of the hypocotyl. The cotyledons and the apical part of the hypocotyl contained only trace amounts of glucose and sucrose. If the endogenous sugar amounts are taken into consideration to calculate the absolute amounts of steroids produced from ^{14}C-sucrose the cotyledons provide a marginal contribution to the triterpenol production in the seedling. Most of the triterpenols are synthesized in the basal part of the hypocotyl and this part of the seedling produces most of the latex triterpenols (Figure 4C). The root appeared to produce 40% of the sterols in the seedling. From 10.9 µmol of endogenous sucrose equivalents 0.7 µg sterols, 11.3 µg of triterpenols and 2 µg triterpene ketones were synthesized. The calculated triterpenol production matches nicely the daily triterpenol production (11 µg d^{-1}), as judged by GLC.

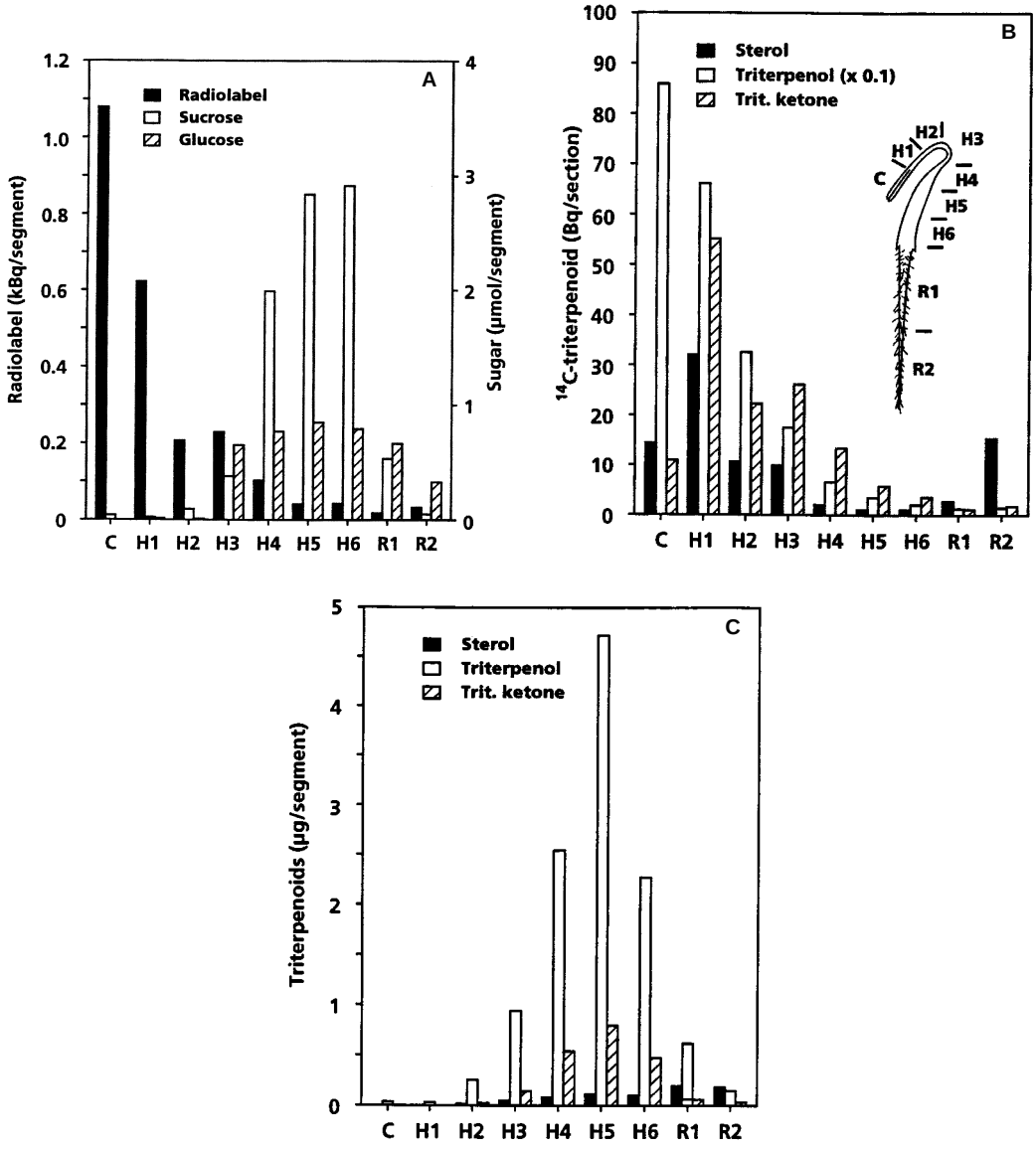

Figure 4 (A) Distribution of ^{14}C, sucrose and glucose over 100 seedlings (*E. lathyris*, 9-day-old) after incubation of the cotyledons of an intact seedling in 100 µl 10 mmol [U-^{14}C]-sucrose (spec. act. 0.0865 mCi/mmol) for 24 h. Sugar levels and ^{14}C content were measured in the cotyledons (C), the hypocotyl (sectioned in six 0.7 cm parts, H1–H6) and the root (cut in two 3 cm parts, R1–R2). Glucose level in C, H1, and H2 was 3, 7, and 4 nmol seedling^{-},[1] respectively. (B) Incorporation of ^{14}C from [U-^{14}C]-sucrose into sterols, latex triterpenes and wax triterpene ketones in various parts of 100 intact *E. lathyris* seedlings. Uptake: 97 µmol [U- ^{14}C]-sucrose, 0.0865 mCi/mmol, incorporation time 24 h. The drawing represents a 9-day-old-seedling with the sectioning pattern. (C) The amount of sterols, triterpenols and triterpene ketones produced from endogenous sugars in a 24 h incorporation period. The triterpenoid production in the cotyledons (C), the hypocotyl (sectioned in six parts, H1–H6) and the root (R1, R2) is calculated from the data presented in (A) and (B). (From Koops, A. J. and Groeneveld, H. W., *J. Plant Physiol.*, 138, 142, 1991. With permission.)

VII. INCORPORATION PATTERN AND PLANT SIZE

The various classes of steroids occurring in plant species are mostly composed of various components. The sterols are composed of 3 to 4 major compounds, the latex triterpenoids in *Euphorbia lathyris* were shown to contain 5 major triterpenols, 3 major 5ß-cardenolides were detected in *Asclepias curassavica* and in *Digitalis lanata* 7 major cardenolides in a mixture of at least 30 have been reported.[45] Such a complex composition may be an advantage in labeling experiments. If all the compounds occurring are labeled with more or less specific activity, the entire steroid synthesis in the tissue is covered. A variable specific activity may be due to any of several reasons. It may mark an ongoing change in sterol composition. The substrate is not equally distributed over the tissue, not all the sites of steroids are reached by the substrate (e.g., the laticifers in *Hoya*), or the tissue gradually loses its viability with a concurrent change in metabolism.

To measure the specific activity an adequate separation of the components is required. Radio-gas chromatography is a powerful tool to establish the ^{14}C distribution in sterol and triterpenol mixtures, but the small amounts per injection require very high incorporation values and high specific activities. A successful analysis of sterol and triterpenol synthesis with this method has been performed with *Euphorbia* seedlings by Groeneveld and Roelvink.[31]

High performance liquid chromatography (HPLC) is a more convenient technique to measure the specific radioactivity of various components in a mixture of steroids.[46] Sample size is bigger and the ^{14}C profile can easily be measured by liquid scintillation counting of the individual drops of the eluent.[42,46]

Results of a ^{14}C incorporation from [2-^{14}C]-malonate into the various cardenolides of *Digitalis lanata* have been reported by Groeneveld et al.[42] This time course experiment with excised leaves showed a gradual change in the labeling of the various cardenolides, suggesting glucoevatromonoside to be a key intermediate in cardenolide synthesis in this species. As excised leaves gradually lose their viability they cannot be used for prolonged incorporation experiments. A change in their metabolism may occur within 24 h upon excision as Chibnall noticed in 1924.[47]

An irreversible loss in viability does not occur when whole plants are supplied with tracers. The feeding of tracers can be performed with a wick or a cut petiole, but the uptake is rather slow and distribution over the plant is difficult to predict. The administration of substrates through a cut root system can be a good alternative. Uptake and distribution are mainly controlled by transpiration. A promising ^{14}C incorporation profile into the 5α-cardenolides of *Digitalis purpurea* was obtained with [2-^{14}C]-malonate uptake via cut roots. The plants were grown on a water culture with full nutrient supply. Roots were cut at 1 cm below the rosette and uptake of 0.5 ml labeled substrate occurred within 3 h. The plants were transferred to aerated tap water for 1 day and then grown again on a full nutrient solution. Three days after ^{14}C-malonate uptake the cardenolides were extracted and separated by HPLC. Results presented in Figure 5 show that the ^{14}C profile nicely matches the cardenolide profile. All the occurring cardenolides had gained ^{14}C according to their natural abundance. The rosette had ceased growth in this period, but some regrowth of the root system had already started at the end of the 3-day incorporation period.

Growing plants perform a continuous synthesis of sterols and in many plant species the sterol composition changes during growth and development.[48,49] To trace the synthesis of sterols and steroid derivatives nonspecific labeled substrates (like sugars, amino acids, or organic acids) may have the advantage of easy distribution and accumulation in the tissue. In most cases plant parts have to be cut to enable a rapid uptake of labeled substrates and growth will cease for a certain period with a concurrent reduction in sterol synthesis. A recovery of growth by the tissue incorporated will presumably restore the synthesis of sterols and steroid derivatives. This recovery of growth may be essential in tracing sterol synthesis. The technique used to supply partly derooted plants with appropriate water-soluble ^{14}C tracers may be a valuable tool to obtain quantitative data about sterol synthesis in the aerial part of plants and provide the data to understand the mechanism of a changing sterol composition.

(The procedures and techniques discussed in this chapter have been described in detail by Koops[36–38] and Groeneveld et al.[29–35,40–44])

ACKNOWLEDGMENT

I thank Marjolein Kortbeek, who made the drawings, and Renger Jansen van 't Land and Fred Siesling (Botanical Gardens, University of Utrecht) for their continuous supply of healthy plants.

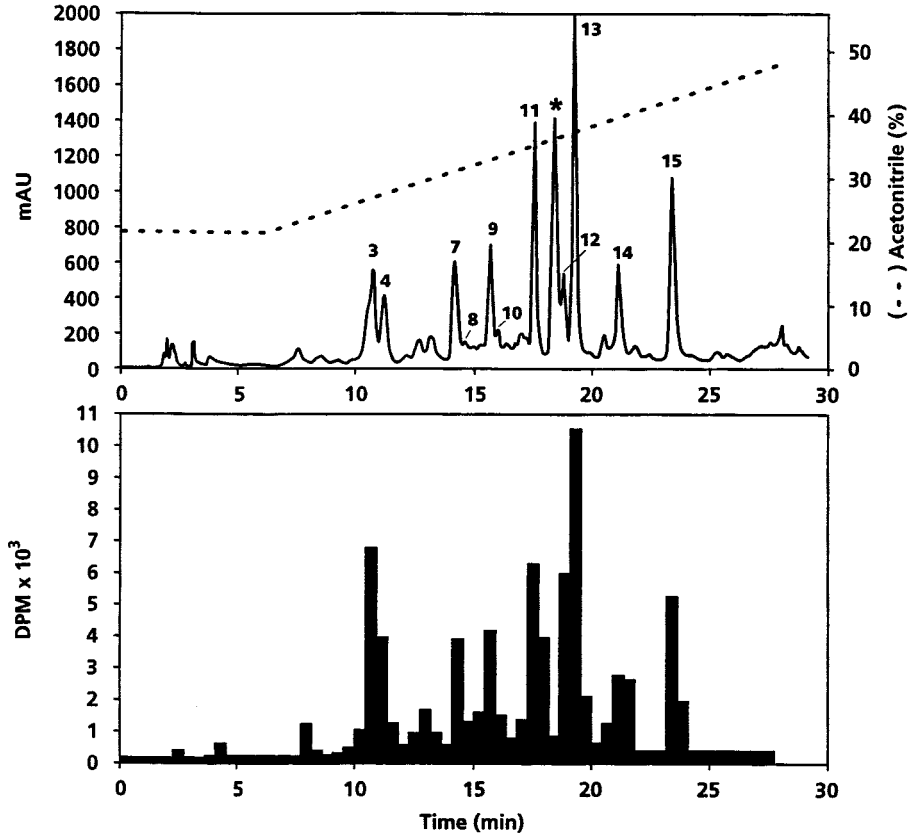

Figure 5 Incorporation profile [2-^{14}C]-malonate into the cardenolides of *Digitalis purpurea*. 5 µCi [2-^{14}C]-malonate, 5 µmol, incorporation period: 72 h, derooted plant 3.9 g fresh weight. ^{14}C-cardenolides: 393,360 dpm. The numbered peaks have a UV spectrum typical for cardenolides. 11: purpureaglycoside B, 12: gitoxin, 13: purpureaglycoside A, 14: gitaloxin, 15: digitoxin, * :digitoxigenin, int. standard. Column: Chromspher C_{18} reversed phase, 20 × 0.3 cm, 5 µm particle size (Chrompack, Middelburg, The Netherlands), acetonitrile-water gradient, flow 0.4 ml min^{-1}. Detection 220 nm.

REFERENCES

1. **Tavormina, P. A., Gibbs, M. H., and Huff, J. H.,** The utilization of ß-hydroxy-ß-methyl-δ-valerolactone in cholesterol biosynthesis, *J. Am. Chem. Soc.*, 78, 4498, 1956.
2. **Wright, L. D., Creson, E. L., Skeggs, H. R., MacRae, G. D. E., Hoffman, C. H., Wolf, D. E., and Folkers, K.,** Isolation of a new acetate-replacing factor, *J. Am. Chem. Soc.*, 78, 5273, 1956.
3. **Bloch, K.,** The biological synthesis of cholesterol, *Vitam. Horm.*, 15, 119, 1957.
4. **Benveniste, P.,** Sterol biosynthesis, *Annu. Rev. Plant Physiol.*, 37, 275, 1986.
5. **Cornforth, J. W., Cornforth, R. H., Donninger, C., Popják, G., Shimizu, Y., Ichii, S., Forchielli, E., and Caspi, E.,** The migration and elimination of hydrogen during biosynthesis of cholesterol from squalene, *J. Am. Chem. Soc.*, 87, 3224, 1965.
6. **Banthorpe, D. V., Charlwood, B. V., and Francis, M. J. O.,** The biosynthesis of monoterpenes, **Chem. Rev.,** 72, 115, 1972.
7. **Bach, T. J.,** Synthesis and metabolism of mevalonic acid in plants, **Plant Physiol. Biochem.,** 25, 163, 1987.
8. **Bach, T. J., Boronat, A., Caelles, C., Ferre, A., Weber, T., and Wettstein, A.,** Aspects related to mevalonate biosynthesis in plants, *Lipids*, 26, 637, 1991.
9. **Sabine, J. R. (Ed.),** *Monographs on Enzyme Biology: HMG-CoA Reductase*, CRC Press, Boca Raton, 1983.
10. **Benveniste, P., Hewlins, M. J. E., and Fritig, B.,** La biosynthèse des stérols dans les tissus de tabac cultivés in vitro. Cinétique de formation des sterols et de leurs precurseurs, *Eur. J. Biochem.*, 9, 526, 1969.
11. **Gibbons, G. F., Goad, L. J., Goodwin, T. W., and Nes, W. R.,** Concerning the role of lanosterol and cycloartenol in steroid biosynthesis, *J. Biol. Chem.*, 246, 3967, 1971.

12. **Castle, M., Blondin, G., and Nes, W. R.,** Evidence for the origin of the ethyl group of ß-sitosterol, *J. Am. Chem. Soc.*, 85, 3306, 1963.
13. **Goad, L. J., Lenton, J. R., Knapp, F. F., and Goodwin, T. W.,** Phytosterol side chain biosynthesis, *Lipids*, 9, 582, 1974.
14. **Goad, L. J.,** Biosynthesis and interconversion of sterols in plants and marine invertebrates, *Methods Enzymol.*, 111, 311, 1985.
15. **Aberhart, D. J., Lloyd-Jones, J. G., and Caspi, E.,** Biosynthesis of cardenolides in *Digitalis lanata*, *Phytochemistry*, 12, 1065, 1973.
16. **Monteagudo, E. S., Burton, G., and Gros, E. G.,** Biosynthesis of whitanolides in *Acnistus breviflorus*. Incorporation of labeled 24-methylenecholesterol, *Phytochemistry*, 35, 927, 1994.
17. **Rees, H. H., Mercer, E. I., and Goodwin, T. W.,** The stereospecific biosynthesis of plant sterols and α- and ß-amyrin, *Biochem. J.*, 99, 726, 1966.
18. **Singh, S. S., Nee, Th. Y., and Pollars, M. R.,** Acetate and mevalonate labeling studies with developing *Cuphea lutea* seeds, *Lipids*, 21, 143, 1986.
19. **Wilkinson, S. C., Powls, R., and Goad, L. J.,** The effects of excess exogenous mevalonic acid on sterol and steryl ester synthesis in celery (*Apium graveolens*) cell suspension cultures, *Phytochemistry*, 37, 1031, 1994.
20. **Nes, W. D. and Bach, T. J.,** Evidence for a mevalonate shunt in a tracheophyte, *Proc. R. Soc. Lond. Ser. B*, 225, 425, 1985.
21. **Buggy, M. J., Britton, G., and Goodwin, T. W.,** Terpenoid biosynthesis by chloroplasts isolated in organic solvents, *Phytochemistry*, 13, 125, 1974.
22. **Groeneveld, H. W.,** Biosynthesis of latex triterpenes in *Euphorbia*; evidence for a dual synthesis, *Acta Bot. Neerl.*, 25, 459, 1976.
23. **Piazza, G. J. and Holzwarth, J. A.,** Enhancement of terpenoid biosynthesis from mevalonate in a fraction of the latex from *Euphorbia lathyris*, *Plant Physiol.*, 89, 681, 1989.
24. **Groeneveld, H. W.,** Some morphological and chemical characteristics of the purified terpenoid particles in the latex of *Hoya australis*, *Acta. Bot. Neerl.*, 25, 1, 1976.
25. **Groeneveld, H. W.,** A comparative investigation of the particle fractions of *Hoya*, *Dischidia* and *Euphorbia* latices. *Acta Bot. Neerl.*, 25, 167, 1976.
26. **Warnaar, F.,** Aromatic and fatty acids of triterpene esters and rubber content of *Hoya* latices and their taxonomic significance, *Phytochemistry*, 23, 1049, 1984.
27. **Groeneveld, H. W. and Koning, J.,** Biosynthesis of phytosterols and latex triterpenes in *Hoya australis* and *Hoya carnosa*, *Acta Bot. Neerl.*, 25, 227, 1976.
28. **Groeneveld, H. W.,** The biochemical pathway in triterpene synthesis in the laticifers of *Hoya* and *Euphorbia*, *Bull. Soc. Bot. Fr.*, 129, Actualitées Botanique, 1, 71, 1982.
29. **Groeneveld, H. W., Peters, E. G. M., Kroese, G. W., and Papenhuyzen, C.,** Lipid synthesis and ultrastructure in the laticifers of etiolated *Euphorbia lathyris* seedlings, *Ann. Bot.*, 51, 307, 1983.
30. **Groeneveld, H. W., Hageman, J., and Vellenga, A. Th. N.,** The involvement of sucrose, glucose and other metabolites in the synthesis of triterpenes and DOPA in the laticifers of *E. lathyris*, *Phytochemistry*, 21, 1589, 1982.
31. **Groeneveld, H. W. and Roelvink, P. W.,** Lipid synthesis in the laticifers of *E. lathyris* seedlings, *Planta*, 154, 347, 1982.
32. **Groeneveld, H. W. and Roelvink, P. W.,** Quantitative aspects of latex lipid synthesis in *E. lathyris* seedlings, *Planta*, 156, 264, 1982.
33. **Groeneveld, H. W. and van der Burg, B.,** Quantitative aspects of triterpene synthesis in the laticifers of *Hoya diversifolia*, *Plant Sci. Lett.*, 33, 81, 1984.
34. **Groeneveld, H. W. and Elings, J. C.,** The involvement of amino acids in latex lipid synthesis in *Euphorbia lathyris* seedlings, *Phytochemistry*, 23, 275, 1984.
35. **Groeneveld, H. W. and Mahlberg, P. G.,** Serine in sterol synthesis in *E. lathyris* seedling, *Phytochemistry*, 25, 1111, 1986.
36. **Koops, A. J. and Groeneveld, H. W.,** The quantitative contribution of sucrose and threonine to triterpenoid production in the etiolated seedling of *E. lathyris*, *J. Plant Physiol.*, 138, 162, 1991.
37. **Koops, A. J., Italiaander, E., and Groeneveld, H. W.,** Triterpenoid biosynthesis in the seedling of *E. lathyris* from sucrose and amino acids, *Plant Sci.*, 74, 193, 1991.
38. **Koops, A. J., Baas, W. J., and Groeneveld, H. W.,** The composition of phytosterols, latex triterpenols and wax triterpenoids in the seedling of *E. lathyris*, *Plant Sci.*, 74, 185, 1991.
39. **Higgins, M. J. P. and Kekwick, R. G. O.,** An investigation into the role of malonyl-coenzyme A in isoprenoid synthesis, *Biochem. J.*, 134, 295, 1973.
40. **Groeneveld, H. W., van den Berg, B., Elings, J. C., and Seykens, D.,** Cardenolide biosynthesis from malonate in *Asclepias curassavica*, *Phytochemistry*, 29, 3479, 1990.
41. **Groeneveld, H. W., van Tegelen, L. P., and Versluis, K.,** Cardenolide and neutral lipid biosynthesis from malonate in *Digitalis lanata*, *Planta Med.*, 58, 229, 1992.
42. **Groeneveld, H. W., van Berkel, Y. E. M., Binnekamp, A., and Seykens, D.,** Some quantitative aspects of cardenolide synthesis from malonate in *Asclepias curassavica*, *Phytochemistry*, 37, 1605, 1994.

43. **Groeneveld, H. W., Elings, J. C., Jorge Blanco, M. S., and Bramwell, D.,** Quantitative aspects of triacylglycerol metabolism and sterol synthesis from ^{14}C-acetate in etiolated seedlings of *Euphorbia lambii*, *Physiol. Plant.*, 75, 227, 1989.
44. **Koops, A. J. and Groeneveld, H. W.,** Triterpenoid synthesis in the etiolated seedling of *E. lathyris*. Developmental changes and the regulation of local triterpenoid production, *J. Plant Physiol.*, 138, 142, 1991.
45. **Wichtl, M. and Freier, R.,** Veränderungen der Zusammensetzung des Glykosidgemisches in den Blättern von *Digitalis lanata* Ehrh. im Verlaufe der ersten Vegetationsperiode, *Dtsch. Apo. Ztg.*, 118, 798, 1978.
46. **Heupel, R. C. and Nes, W. D.,** Evidence for differences in sterol biosynthesis and derivatization in sorghum, *J. Nat. Prod.*, 47, 292, 1984.
47. **Chibnall, A. C.,** Investigations on the nitrogenous metabolism of the higher plants. The role of asparagine in the metabolism of the mature plant, *Biochem. J.*, 18, 395, 1924.
48. **Heupel, R. C., Sauvaire, Y., Le, P. H., Parish, E. J., and Nes, W. D.,** Sterol composition and biosynthesis in Sorghum: importance to developmental regulation, *Lipids*, 21, 69, 1986.
49. **Fenner, G. P., Patterson, G. W., and Lusby, W. R.,** Developmental regulation of sterol biosynthesis in *Cucurbita maxima* L., *Lipids*, 24, 271, 1989.