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Time Course of Steroid Biosynthesis and Metabolism in Haplopappus heterophyllus

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Summary. A single dose of mevalonic acid-2-^{14}C was administered simultaneously to 6 *Haplopappus heterophyllus* plants. They were harvested at intervals ranging from 3 days to 6 months. Four groups of biosynthetically related sterols were found to be radioactive in each plant, and the changes in radioactivity with time were studied. The most striking finding was a radioactive phenolic material present only in the 6-month plant, which had flowered.

Change in concentrations of steroids during growth and development of plants have frequently been studied (11), but only for major constituents which can be measured easily. The approach used in the present work involved labeling the steroids of *Haplopappus heterophyllus* seedlings by administration of a single dose of mevalonic acid-2-^{14}C as a precursor and studying changes in the distribution of radioactivity as the plants matured and progressed to the flowering state.

Materials and Methods

Haplopappus heterophyllus (A. Gray) Blake plants were grown from seeds in a greenhouse. About 2 months after germination, in January 1966, 6 plants were selected for uniformity (height 6–10 cm). The leaves of the plants were dipped into a 0.1 % aqueous solution of Tween 20 and allowed to dry. Mevalonic acid-2-^{14}C dibenzylethylenediamine salt (7.56 mc/mmole) (New England Nuclear Corporation)[2] in water, containing 2 % ethanol and 0.1 % Tween 20, was then applied to the leaves, in a dose of 25 μc per plant. After the leaves were dry, an equal volume of solution, 2 % in ethanol and 0.1 % in Tween 20 in water was applied. Mevalonic acid applied by this method is almost quantitatively absorbed into the leaves (7).

The plants were worked up individually 3 days, 7 days, 14 days, 1 month, 2 months, and 6 months after application of mevalonic acid. The shoot system of each plant was harvested, frozen in liquid nitrogen, and lyophilized. The dry material was homogenized in a tissue grinder with 25 ml of methanol, and the homogenate was filtered. The filter cake was extracted in a Soxhlet apparatus with 80 ml of benzene-methanol (3:1) for 6 hours. This extract and the filtrate previously obtained were combined and evaporated to dryness. The residue was refluxed with 50 ml of 5 % potassium hydroxide in methanol for 2 hours under nitrogen. The solvent was evaporated in vacuum, and the residue was then refluxed with a mixture of 25 ml of benzene, 55 ml of water, and 20 ml of concentrated hydrochloric acid for 3 hours. The benzene layer was separated, and the aqueous layer was extracted with 2 25-ml portions of dichloromethane. The organic layers were washed with 15 ml of water, filtered, combined, and evaporated. The residue was taken up in 25 ml of benzene and extracted with 2 15-ml portions of 2 % sodium bicarbonate solution and 15 ml of water. The extracts were passed through 10 ml of benzene and discarded. The original benzene layer was then extracted with 2 15-ml portions of 0.5 N sodium hydroxide and 15 ml of water. The extracts were passed through the same 10 ml of benzene as above, filtered, combined, and immediately acidified with concentrated hydrochloric acid. The mixture was then extracted with 2 25-ml portions of dichloromethane, and the extracts were passed through 10 ml of water, filtered, combined, and evaporated (Phenolic Fraction).

The original benzene layer from above was combined with that used for washing the aqueous extracts, filtered, and evaporated. The residue was

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[2] Reference to a company or product name does not imply approval or recommendation of the product by the United States Department of Agriculture to the exclusion of others that may be suitable.

taken up in 2.5 ml of ethanol, and 2.5 ml of 2 N sodium hydroxide was added to hydrolyze any mevalonolactone remaining from the mevalonic acid. After 10 minutes, 25 ml of benzene was added, and the mixture was extracted with 2 15-ml portions of water. The extracts were passed through 10 ml of benzene and discarded. The benzene layers were combined, filtered, and evaporated (Neutral Fraction).

Because the last plant of the series was much larger than the others, the volumes used in its workup were increased by a factor of 4.

Methods for thin-layer chromatography (TLC), counting of radioactive samples, and evaporation of solutions were as described previously (5).

Results

The 3 plants harvested during the first 14 days had not increased significantly in size. The growth rate was slow for the first 2 months and rapid thereafter. The 6-month plant was 118 cm high and had flowered.

The radioactivity of the neutral fractions declined continuously during the course of the experiment, while that of the phenolic fractions fluctuated but increased to a maximum in the 6-month plant. In all of the plants, however, the neutral fraction was much more radioactive than the phenolic fraction.

The neutral fractions were examined by TLC scans for differences in radioactivity of the following 4 classes of sterols: 4,4-dimethyl-, 4-methyl-, Δ^5-, and Δ^7-sterols. Lanosterol (4,4,14α-trimethyl-$\Delta^{8,24}$-cholestadien-3β-ol), lophenol (4α-methyl-Δ^7-cholesten-3β-ol), β-sitosterol (24α-ethyl-Δ^5-cholesten-3β-ol), and Δ^7-cholesten-3β-ol were cochromatographed as representatives of these 4 classes. Figure 1 shows a scan of the neutral fraction from the 7-day plant. Under these chromatographic conditions no subfractionation occurs within each class.

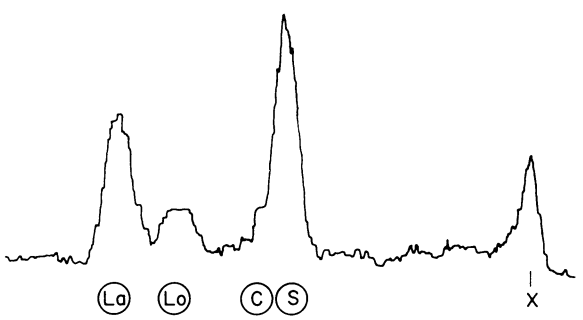

FIG. 1. Radiochromatogram of the neutral fraction of a *H. heterophyllus* plant 7 days after administration of mevalonic acid-2-^{14}C. Letters indicate positions of standards: La, lanosterol; Lo, lophenol; C, Δ^7-cholestenol; S, β-sitosterol; X, origin. A 5 × 20-cm Silica Gel plate was developed continuously with benzene-methanol (199:1) for 2.5 hours and scanned at 1.5 inches/hour, with a time constant of 100 seconds and a slit width of 4 mm.

Since 5α-sterols do not separate from Δ^7-sterols in the system used, it was necessary to investigate further the material corresponding to Δ^7-cholestenol. This was accomplished by acetylation followed by continuous development TLC, which separates Δ^7- from 5α-sterol acetates. Only the former group was present in *H. heterophyllus*, and it gave the expected positive color reactions in the Tortelli-Jaffe (14) and selenium dioxide (9) tests. TLC on Anasil B (1, 3) separated the Δ^7-sterol acetate fraction into 2 components, corresponding in mobilities to Δ^7-stigmasten-3β-ol acetate and $\Delta^{7,22}$-stigmastadien-3β-ol (α-spinasterol) acetate. By the same method β-sitosterol, cholesterol, and stigmasterol had previously been identified as the constituents of the Δ^5-sterol fraction of this species (4). In all of the plants the 2 Δ^7-sterols were present in much higher concentrations than the 3 Δ^5-sterols.

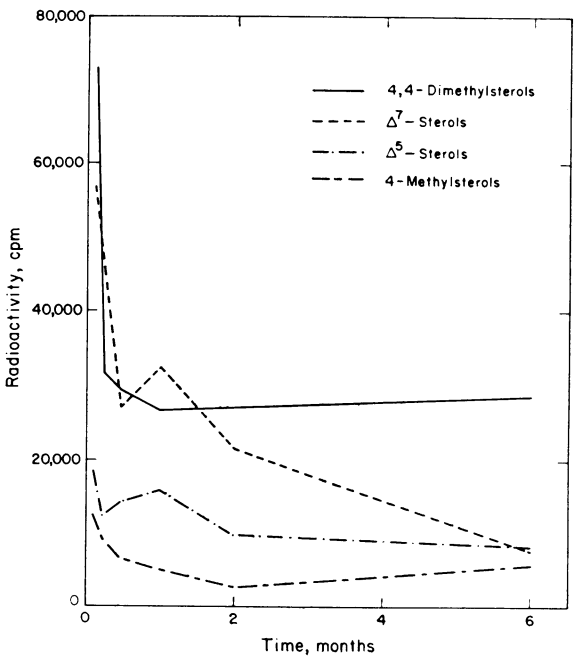

FIG. 2. Radioactivity of sterols in neutral fractions of *H. heterophyllus* plants at various time intervals after administration of mevalonic acid-2-^{14}C.

To obtain quantitative data on the changes in radioactivity observed by TLC scans, the zones corresponding to the 4 standards were removed from the plates, eluted, and aliquots of the eluates counted. Figure 2 presents these results graphically.

The concentrations of the sterols in these plants were too low to permit quantitative evaluation, but some changes could be observed by TLC. The concentration of 4,4-dimethylsterols remained ap-

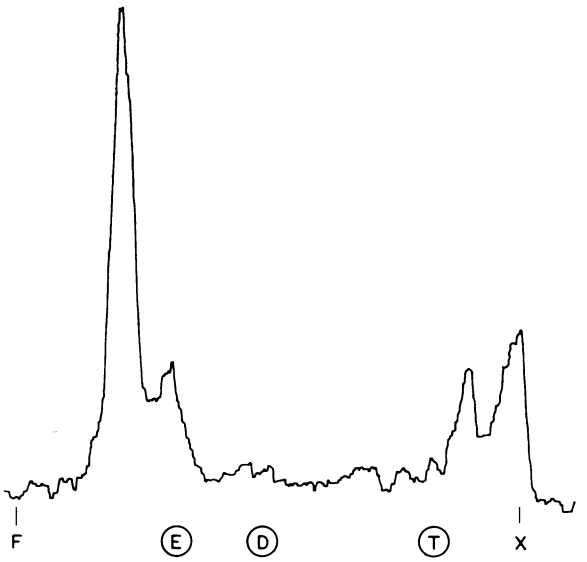

FIG. 3. Radiochromatogram of the phenolic fraction of a *H. heterophyllus* plant 6 months after administration of mevalonic acid-2-^{14}C. F, solvent front; E, estrone; D, estradiol; T, estriol; X, origin. A 5 × 20-cm Silica Gel G plate was developed with dichloromethane-methanol (23:2) and scanned at 0.75 inch/hour, with a time constant of 300 seconds and a slit width of 4 mm.

proximately constant during the first 2 months, but was greatly increased in the 6-month plant. In each plant 4-methylsterols were present in amounts below the limit of detection by TLC. Δ^7-Sterols increased gradually in concentration from the second week to the second month and then more rapidly in the next 4 months. The amount of Δ^5-sterols remained at a low level throughout, although a slight increase was observed in the 6-month plant.

Figure 3 shows a radiochromatogram of the phenolic fraction from the 6-month plant. In the corresponding chromatographs of the phenolic fractions from the 3-, 7-, and 14-day plants, the level of radioactivity was low and there were no well-defined peaks. The highly radioactive material near the front appeared in the 1-, 2-, and 6-month plants. This substance is volatile and was not further investigated, since it cannot be a steroid. The 2 peaks close to the origin in figure 3 were also present in the 1- and 2-month plants. The peak corresponding in mobility to estrone, however, was observed only in the 6-month plant. In other solvent systems its mobility differed from that of estrone.

Discussion

In animals the biosynthesis of sterols from mevalonic acid via the sequence squalene → 4,4-dimethylsterols → 4-methylsterols → Δ^7-sterols → Δ^5-sterols is fairly well established (8). In higher plants, however, direct evidence regarding this biosynthetic pathway is lacking. Only the conversion of squalene to the Δ^5-sterol, β-sitosterol, has been demonstrated experimentally (2). However, sterols of each of the intermediate groups have been biosynthesized from mevalonic acid and acetate (10, and references therein), and the time course of sterol biosynthesis from sodium acetate-1-^{14}C in tobacco tissue cultures (6) was consistent with the above pathway.

Mevalonic acid and squalene are apparently rapidly metabolized by *H. heterophyllus,* since each of the 4 groups of sterols attained its maximum radioactivity in the 3-day plant, and little or no radioactive squalene was present.

The changes in radioactivity thereafter (fig 2) are, to some degree, indicative of the expected biosynthetic relationships, but it also appears that other complicating factors must have been involved. For instance, both acidic and basic hydrolysis was used in the workup, so that only free sterols would have been isolated from any sterol esters or glycosides, which are common constituents of plants. Such sterol derivatives may be metabolized at quite different rates than the free sterols.

The radioactive phenolic material which was found only in the flowering plant did not correspond chromatographically to any of the common steroidal estrogens. The possibility that it is nevertheless a steroid cannot be dismissed, although an analogous radioactive material was not observed in the phenolic fractions of flowering *H. heterophyllus* plants treated with cholesterol-4-^{14}C or pregnenolone-4-^{14}C (4), both estrogen precursors in animals. These plants contained several other radioactive phenolic compounds, none of which could be identified with known estrogens.

We have meanwhile also observed a different radioactive phenolic compound in the fruits of pomegranate plants treated with pregnenolone-4-^{14}C. Furthermore, we have detected phenolic compounds, showing colors and fluorescences on TLC plates sprayed with 50 % sulfuric acid (12) much like those of the steroidal estrogens, in extracts of flower buds of Trembling Aspen (*Populus tremuloides*) and fruits of *Yucca aloifolia*. None of them, however, correspond in chromatographic mobilities to known steroidal estrogens. Some of the latter have previously been isolated from plants (13, and references therein).

No evidence was found for the presence of radioactive 5α-androstane-3β, 16α, 17α-triol, which Zalkow et al. (15) isolated from *H. heterophyllus.* The closely related species *H. tenuisectus* has likewise failed to produce this steroid.

Acknowledgment

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