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*Dedicated to Prof. K. Mothes on the occasion of his seventieth birthday*

# RELATIONSHIP BETWEEN ORGAN DEVELOPMENT AND ACTIVITY OF ENZYMES INVOLVED IN FLAVONE GLYCOSIDE BIOSYNTHESIS IN YOUNG PARSLEY PLANTS

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**Abstract**—The activities of five enzymes directly related to the biosynthesis of apiin, (7-*O*[β-D-apiofuranosyl(1→2)β-D-glucosyl]-5,7,4′-trihydroxyflavone, and to the corresponding glycoside of chrysoeriol (3′-methoxyapigenin) as well as the accumulation of these flavone glycosides were studied during the growth of young parsley plants (*Petroselinum hortense*). The enzymes examined were phenylalanine ammonia-lyase (E.C. 4.3.1.5), chalcone-flavanone isomerase, UDP-apiose synthetase, and a glucosyl- and an apiosyltransferase. On a fresh weight basis, the glycoside content as well as the activities of all of the five enzymes were found to be relatively high in very young cotyledons or leaves and decreased to a rather low level within a few days. The striking similarity among the five curves obtained when enzyme activities were plotted versus growth time suggests that the isolation of other enzymes involved in this biochemical pathway is likely to be most successful in the initial stages of cotyledon or leaf development.

## INTRODUCTION

THE TWO main flavone glycosides of parsley (*Petroselinum hortense* Hoffm.) are apiin (7-*O*-[β-D-apiofuranosyl(1→2)-β-D-glucosyl]-5,7,4′-trihydroxyflavone) and the corresponding glycoside of chrysoeriol (3′-methoxyapiin).[1]

Earlier work from this laboratory[1] has shown that 5,7,4′-trihydroxyflavanone (or the corresponding chalcone)[2] but not 3,5,7,4′-tetrahydroxyflavanone is a good precursor for apigenin and chrysoeriol in this plant.

Up until now the following five enzymes involved in the flavone glycoside pathway[3] have been demonstrated to be present in parsley: Phenylalanine ammonia-lyase (E.C. 4.3.1.5) (PAL)[4a,b], chalcone-flavanone isomerase,[5a,b] UDP-apiose synthetase,[6] and a glucosyl[7] and an apiosyl[8] transferase. The steps which are catalysed by these enzymes are shown in Fig. 1.

In connection with our efforts to isolate new enzymes of flavonoid biosynthesis it seemed important to us to study the relationship between organ development and the

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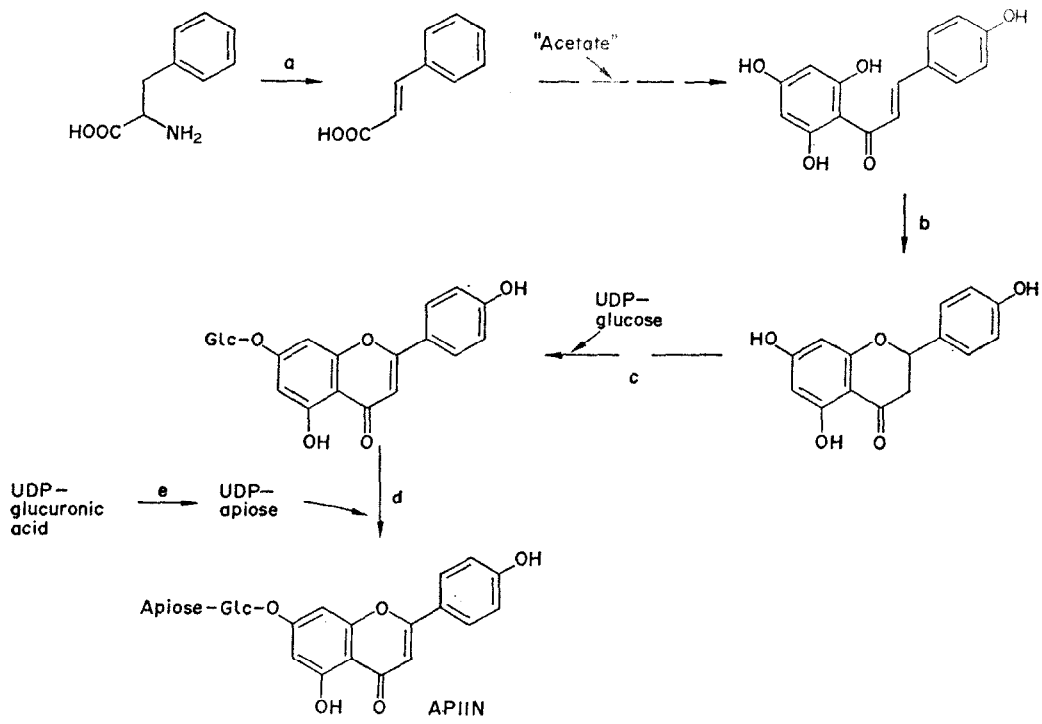


FIG. 1. SOME OF THE ENZYMATIC REACTIONS INVOLVED IN THE BIOSYNTHETIC FORMATION OF APIIN. a = Phenylalanine ammonia-lyase, b = chalcone flavanone isomerase, c = UDP-glucose:apigenin-7-*O*-glucosyltransferase, d = UDP-apiose:apigenin-glucoside apiosyltransferase, e = UDP-apiose synthetase.

activities of the five enzymes mentioned above. In the present paper evidence is presented that drastic changes occur in the activity of these enzymes in cotyledons and leaves during the growth of young parsley plants. The changes are related to the synthesis of the flavone glycosides.

## RESULTS

All plant material was harvested at the same time of day to ensure comparable growth conditions. No significant changes in enzyme activity during the day were observed. Incubations were carried out immediately after preparation of a crude extract from the tissue. Figure 2 shows the relationship between growth time and fresh weight of cotyledons and leaves. In both cases there are only minor deviations from the sigmoidal curves. Furthermore, the amount of protein extracted with buffer decreased only insignificantly with increasing age of the tissue. Hence the shape of the curves obtained when enzyme activities per fresh weight were plotted vs. growth time would be the same if enzyme activities were calculated on the basis of the amount of protein isolated. In the following figures all enzyme activities are based on the fresh weight of the tissue.

### *Flavone Glycoside Content*

Changes of the flavone glycoside content during growth of parsley cotyledons and leaves are shown in Fig. 3. In both organs, the glycoside content is very high during the initial

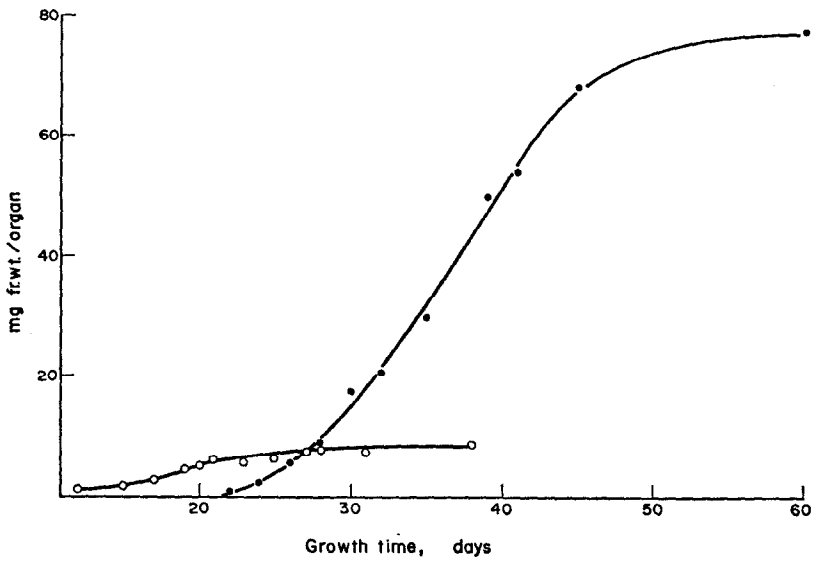


FIG. 2. FRESH WEIGHT OF DEVELOPING PARSLEY COTYLEDONS (○——○) AND LEAVES (●——●).

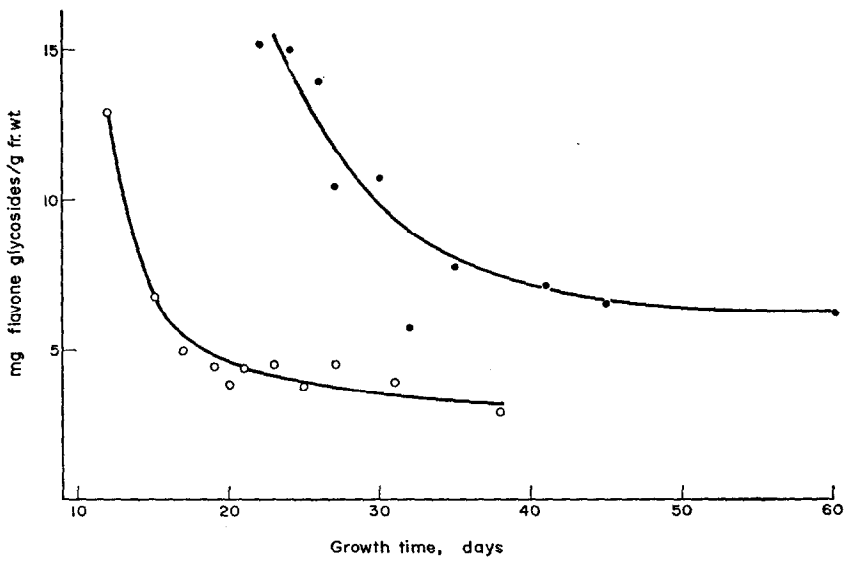


FIG. 3. FLAVONE GLYCOSIDE CONTENT OF DEVELOPING PARSLEY COTYLEDONS (○——○) AND LEAVES (●——●).

stages of development and decreases to about one third of this amount within a few days. The term "flavone glycosides" comprises apiin, apigenin-7-glucoside, chrysoeriol-7-apiosyl-glucoside, and possibly small amounts of apigenin itself.

*Enzyme Activities*

The activities of the enzymes mentioned above in relation to growth time of young parsley plants are shown in Fig. 4.

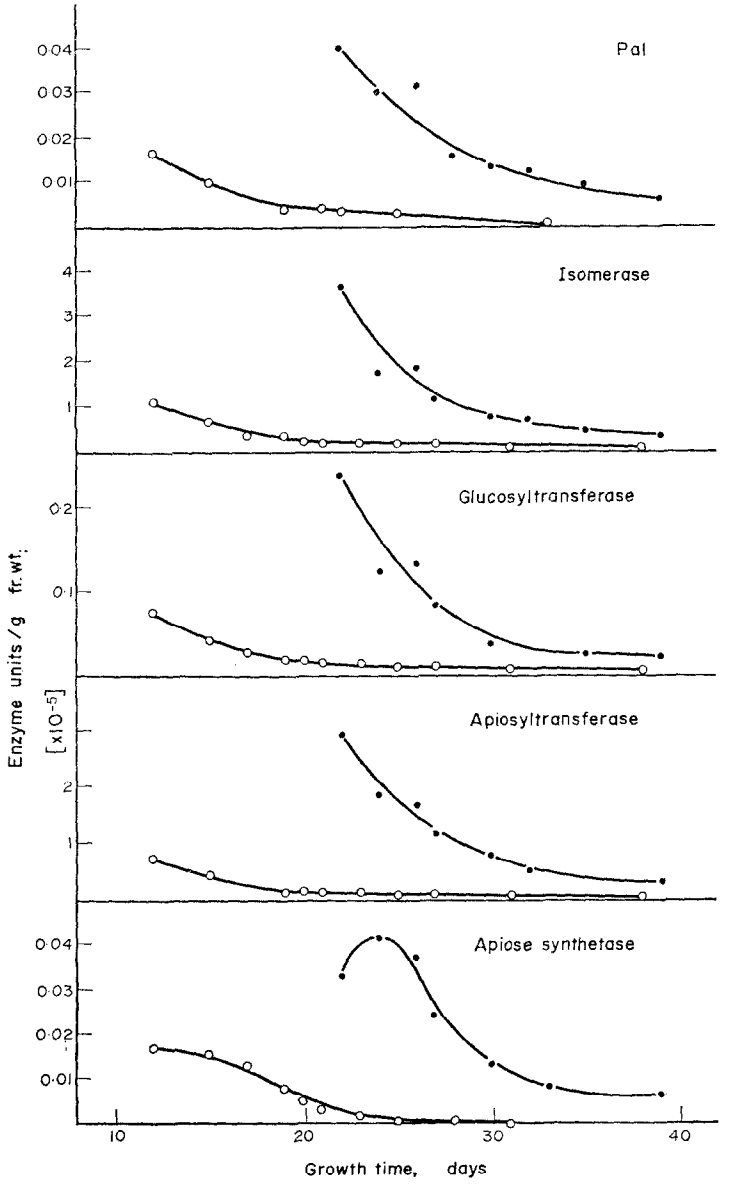


FIG. 4. ENZYME ACTIVITIES IN DEVELOPING PARSLEY COTYLEDONS (○——○) AND LEAVES (●——●).

Although the absolute amounts of isolated enzyme activity differ greatly under our assay conditions the shape of all of the curves in Fig. 4 is almost identical. Furthermore, the relative activities of all of the five enzymes are roughly the same in cotyledons and leaves.

## DISCUSSION

With the exception of the investigations of those enzymes involved in the formation of phenylalanine and tyrosine from non-aromatic precursors,[9-12] all studies concerned with the time course of the biosynthesis of flavonoid compounds at an enzymatic level have been confined to PAL, the first enzyme of the pathway shown in Fig. 1. Most of these studies were concerned with the effects of light or wounding on PAL activity in various plants.[13-18] Maier and Hasegawa[19] recently studied the changes of PAL activity in developing grape fruit under normal growth conditions (without wounding or abnormal light treatment) and reported a high degree of correlation with the rate of naringenin glycoside accumulation.

All of the five enzymes investigated in our work, with the exception of UDP-apiose synthetase, exhibited the highest specific activities when extracted from the youngest samples of cotyledon or leaf tissue (Fig. 4). A sharp and progressive decline in the specific activities was observed in extracts made from progressively older organs. Comparison of the specific activities (Fig. 4) with the concentrations of flavone glycosides in the respective organs at the various stages of development (Fig. 3) reveals a substantial correlation. This perhaps assumes added significance when we consider that specific activity is an expression of that portion of total protein whose function is devoted to a particular catalytic activity, and is thus an expression of concentration.

UDP-apiose synthetase, the only enzyme that deviated from the general pattern presented in Fig. 4, exhibited a maximum in its specific activity in leaves 2 days after the first sampling. Thereafter, its specific activity underwent a decline which paralleled that of the other four enzymes. It is tempting to speculate that the specific activities of the other four enzymes undergo a similar increase to maximum values at some stage prior to our first harvest and therefore, very early in the development of the leaves. In this regard, it should be pointed out that in these experiments, the cotyledons were first examined when they had reached the surface of the soil and became fully exposed to light (12 days after sowing). In a separate experiment with seedlings grown on filter paper, little activity was found in 6-day-old etiolated cotyledons, and no activity could be detected in the seed. These enzymes therefore seem to reach maximum specific activities in the cotyledons before day 12 after sowing. These observations are in good agreement with the results obtained for PAL activity in developing mung bean seedlings by Minamikawa *et al.*[12] These authors report that PAL activity drops in mung bean cotyledons to a rather low level after an initial increase, whereas in the root–shoot axis the specific activity decreases steadily from high

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initial values in a manner very similar to the results presented in Fig. 4. In general, however, a comparison of results concerning PAL activity in different plants or different organs of the same plant and its relationship to tissue development is only possible if comparable stages of growth and cell differentiation are considered.

The general shape of the curves presented in Fig. 4 is identical in cotyledons as well as in leaves for all of these enzymes, except UDP-apiose synthetase, regardless of their relative activities. This suggests that, at least under our growth conditions and during the period of organ development investigated, the enzymes of flavonoid biosynthesis are regulated interdependently. However, it is not clear from these results whether PAL and/or one or more other enzymes are the initial point of regulation of this biosynthetic pathway.

Another aspect of the relationship between changes in enzyme activity and product accumulation can be studied much better if these are based on the individual organ as the reference. In Fig. 5, changes in PAL activity and accumulation of flavone glycosides per

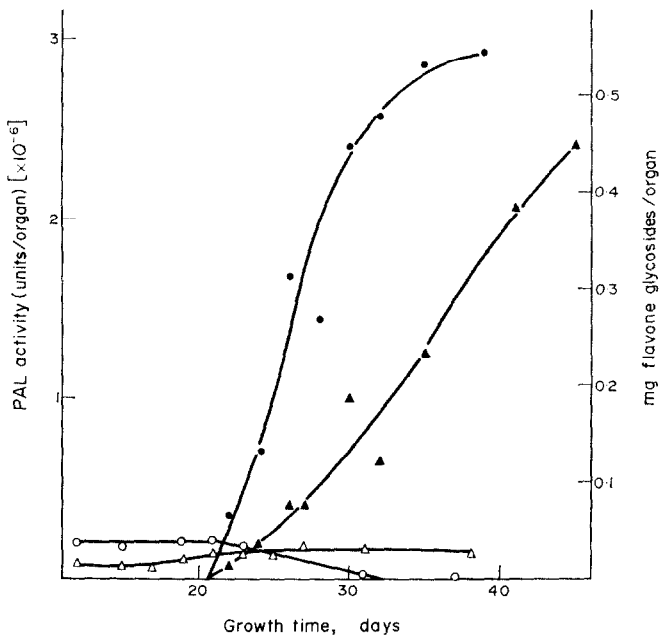


FIG. 5. PAL ACTIVITY AND FLAVONE GLYCOSIDE CONTENT OF COTYLEDONS AND LEAVES OF PARSLEY PLANTS.

Cotyledons: (○———○) PAL; (△———△) flavone glycosides. Leaves: (●———●) PAL; (▲———▲) flavone glycosides.

organ are plotted vs. growth time. All of the other enzymes examined give curves of identical shape and can be substituted for PAL. When the first leaf is formed, PAL activity in the cotyledons decreases from a nearly constant level to very low values. At the same time, a dramatic increase in total enzyme activity is observed at the initial stages of leaf development, followed after a short delay by an increase in the accumulation of flavone glycosides.

As a result of our investigations, chances for a successful isolation of other enzymes involved in this pathway seem to be highest in young leaves about 22 days after sowing of the parsley seed.

## EXPERIMENTAL

### *Plant Material*

Seeds of parsley (*Petroselinum hortense* Hoffm.) were purchased from a commercial source. Plants were grown under carefully controlled conditions in a growth chamber; illumination, 10,000 lx (Xenon arcs, Osram XQO, 3000 W), temperature 18°, or in the darkness, 16°. The relative humidity was kept at 75–80%. All plant material was harvested 3 hr after onset of illumination and immediately extracted.

### *Isolation and Quantitative Determination of Apigenin Glycosides*

Cotyledons or leaves (150–200 mg) were extracted with EtOH for 9 hr in a soxhlet apparatus. The solution was evaporated to dryness, and the flavone glycosides were dissolved in 10 ml of hot 50% EtOH. Undissolved material was removed by centrifugation. The amount of flavone glycosides were determined by measuring the absorptivity at 340 nm. The extinction coefficients for the flavone glycosides concerned are very similar at this wavelength.[20] The value determined for apiin was used for the mixture of glycosides (log $\epsilon = 4{\cdot}26$). No significant amounts of other compounds absorbing at this wavelength could be detected.

### *Enzyme Preparation*

With the sole exception of PAL, all the enzymes were extracted from the tissue by the following procedure. The plant material was ground at 0–4° with one half of its weight of quartz sand and 3 vol. (cotyledons) or 6 vol. (leaves) of 0·1 M Tris–HCl buffer, pH 7·5, containing EDTA (10 mM) and mercaptoethanol (20 mM). The homogenate was centrifuged for 10 min at 30,000 *g*, and aliquots of the supernatant solution were assayed for enzyme activities.

PAL was similarly extracted by this procedure with 0·1 M borate buffer, pH 8·8. In order to remove phenolic material that interferes with the spectrophotometric assay, 500 mg of charcoal were added per g fr. wt.[21] This method proved to be much more efficient than the preparation of an acetone powder for PAL determination.

One unit of activity is defined as the amount of enzyme which catalyses the formation of 1 μmole of product in 1 min at 30°.

In all cases, the rate of product formation was linear with time and amount of protein added during the incubation period. The presence of activators or inhibitors could not be demonstrated when active and inactive preparations were mixed.

### *Enzyme Assays*

PAL was assayed by the spectrophotometric method described by Zucker.[13] The incubation mixture contained 100 μmoles of borate (pH 8·8), 10 μmoles of L-phenylalanine, and 0·1–1 mg of protein in a volume of 1 ml. After preincubation for 15 min the increase in absorbance at 290 nm was followed for 60 min.

A spectrophotometric assay for chalcone-flavanone isomerase from parsley has been described elsewhere.[5b] The reaction mixture contained 50 μmoles of Tris–HCl (pH 7·6), 4 μmoles of 2′,4,4′,6′-tetrahydroxychalcone,[5a] dissolved in 10 μl of ethylene glycol monomethyl ether (EGME), and 10–100 μg of protein in a total volume of 1 ml. The disappearance of chalcone was measured by recording the absorbance at 366 nm.

UDP-glucose:apigenin 7-*O*-glucosyltransferase was assayed with $^{14}$C-labelled apigenin[7] as substrate. A mixture containing 43 μmoles of Tris–HCl (pH 7·5) containing EDTA and mercaptoethanol as described above, 0·01 μmole of [2-$^{14}$C]-apigenin (2 mc/m-mole) dissolved in 10 μl of EGME, 0·05 μmoles of UDP-glucose, and 1–20 μg of protein in a volume of 230 μl was incubated at 30° for 17 min. After addition of 0·2 μmole of unlabelled apigenin-7-*O*-glucoside the reaction was stopped by applying the mixture to a silica gel TLC plate which was immediately dried in a stream of air. The chromatogram was developed in toluene–ethyl formate–formic acid (10:8:3, by vol.).The radioactive zones corresponding to the glucoside and the aglycone were scraped from the plate and the radioactivity determined by scintillation counting. The counting efficiency for both zones was independent of the amounts applied to the plate.

The transfer of UDP-apiose to 7-*O*-(β-D-glucosyl)-apigenin was measured by incubating a solution of $4{\cdot}6 \times 10^{-6}$ μmoles of UDP-[U-$^{14}$C]-apiose[6] (3300 dis/min 50 μl) with 0·1 μmole of 7-*O*-(β-D-glucosyl)-apigenin, dissolved in 20 μl of EGME, 34 μmoles of Tris–HCl, pH 7·5, 3·4 μmoles of EDTA, 3·4 μmoles of mercaptoethanol, and 4–250 μg of protein in a volume of 170 μl for 12 min. 400 μg of apiin were then added and the solution was applied to paper (Schleicher-Schuell 2043b). After chromatography in 15% acetic acid, the zone of apiin was detected under u.v. light, cut out of the paper, and the radioactivity was measured in a scintillation counter.

The assay mixture for UDP-apiose formation consisted of 2·5 μmoles of Tris–HCl, pH 7·5, $2 \times 10^{-4}$ μmoles of UDP-[U-$^{14}$C]-glucuronic acid (30,000 dis/min), 0·1 μmole NAD, 0·5 μmole of mercaptoethanol

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0·25 μmole EDTA, and 2–25 μg of protein in a volume of 50 μl. After incubation for 5 min, the reaction was stopped by addition of 10 μl of glacial acetic acid and heating the mixture for 20 min on a boiling water bath in order to hydrolyze the sugar nucleotides. Apiose was identified by paper chromatography in ethyl-acetate–pyridine–water (8:2:1, by vol.).

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