Plant Physiol. (1982) 70, 506–510
0032-0889/82/70/0506/05/$00.50/0

Induction of Flavonoid Synthesizing Enzymes by Light in Etiolated Pea (*Pisum sativum* cv. Midfreezer) Seedlings

Received for publication March 15, 1982 and in revised form April 29, 1982

GEZA HRAZDINA AND GRACE F. PARSONS
Department of Food Science and Technology, Cornell University, Geneva, New York 14456

ABSTRACT

Etiolated pea (*Pisum sativum* cv. Midfreezer) seedlings respond to illumination with white light by changes in the activity of phenylpropanoid and flavonoid synthesizing enzymes. Unlike in cell cultures, changes in enzyme activity in pea seedlings are not concerted. Phenylalanine ammonia-lyase (EC 4.3.1.5) activity peaked approximately 18 hours after onset of illumination. The phenylacetate path did not interfere with the measurement of phenylalanine ammonia-lyase activity. Activity of cinnamic acid 4-hydroxylase (EC 1.14.13.11) showed an early peak after 8 hours illumination, declined thereafter sharply, then gradually increased during the remainder of the experiment. Activities of chalcone synthase and UDP glucose:flavonol 3-*O*-glucosyltransferase (EC 2.4.1.91) increased steadily and reached a plateau after approximately 70 hours illumination time. Activity of 4-hydroxycinnamate:coenzyme A ligase (EC 6.2.1.12) remained relatively unchanged, whereas that of chalcone isomerase (EC 5.5.1.6) declined steadily during the course of the experiment. The relative *in vitro* enzyme activities suggest that the rate-limiting step for the phenylpropanoid path is the cinnamic acid 4-hydroxylase, that of the flavonoid pathway is the chalcone synthase. Integration of enzyme activity curves, however, show that only the curve deriving from phenylanine ammonia-lyase activity matches closely the production of the flavonol glycosides.

Detailed investigations on the effect of light on dark-grown parsley cell suspension cultures resulted in recognition of coordinated induction in phenylpropanoid- and flavonoid-metabolizing enzyme activities (6). This coordinated induction of enzyme activity is due to *de novo* synthesis of the subunits and assembly of the enzymes (20) and not to activation of preformed inactive components.

There are indications that in intact plants the change in enzyme activities upon illumination are not so straight forward (3, 15). Etiolated pea seedlings respond to exposure of light with complex pattern of transitory changes in the concentration of their flavonoid components (22). These flavonoids are the 3-triglucosides and 3-*p*-coumaryl triglucosides of kaempferol and quercetin, and the 3-sophoroside 5-glucosides and 3-sambubioside 5-glucosides of cyanidin and delphinidin (4, 25). Correlation between changes in PAL[1] activity and flavonoid glycoside production suggested the involvement of two different systems in flavonoid synthesis: a low-magnitude, phytochrome-mediated response, and a long-term response of greater magnitude dependent on continuous illumination (21).

In this paper we report the changes in flavonoid glycoside production and in the activity of phenylpropanoid and flavonoid synthesizing enzymes in etiolated pea (*Pisum sativum* cv. Midfreezer) seedlings upon illumination.

[1] Abbreviation: PAL, phenylalanine ammonia-lyase.

MATERIALS AND METHODS

All experiments were performed at least in triplicate.

Plant Material. Pea (*Pisum sativum* cv. Midfreezer) seeds were germinated and grown in the dark in flats containing sterilized sand at 20°C day and 13°C night temperatures in a growth chamber. Eight d after planting, the seedlings were illuminated continuously with Cool White fluorescent lights (200 μE/m·s) and samples taken in intervals up to 110 h.

Chemicals. CoASH, glucose 6-P, glucose 6-P dehydrogenase, $NADP^+$, ATP, naringenin, and *p*-coumaric acid were obtained from Sigma Chemicals, [3-^{14}C]cinnamic acid (57 mCi/mmol) from Amersham Radiochemicals, *S*-adenosyl methionine [^{14}C]methyl, uridine diphosphoglucose [UL-^{14}C]glucose (223 mCi/mmol) from International Chemical and Nuclear Corp., [U-^{14}C]phenylalanine (420 mCi/mmol) and [2-^{14}C]malonyl CoA (45.6 mCi/mmol) from New England Nuclear, and 4-hydroxycinnamyl-CoA was synthesized as reported previously (11). Naringenin chalcone was synthesized according to Moustafa and Wong (17); quercetin 3-glucoside was synthesized as previously described (2).

Buffers. The buffers used are as follows: A, 0.2 M K_2HPO_4/ KH_2PO_4 (pH 8.0); B, 0.2 M K_2HPO_4/ KH_2PO_4 (pH 7.5); C, 0.1 M Tris-HCl (pH 7.6). All buffers contained 4 mM 2-mercaptoethanol.

Determination of the Flavonol Glycoside Content. Approximately 1 g seedlings was ground in a chilled mortar with 100 mg granular silica in 3.0 ml methanol. The homogenate was centrifuged for 2 min at 11,500*g* and the supernatant filtered through a 100-μm mesh nylon screen. The filtrate was used for the determination of the flavonol glycoside content. The reaction mixture consisted of 10 μl methanolic plant extract, 50 μl 5% $AlCl_3$ in methanol, and 940 μl methanol. Absorbance was measured at 421 nm and the flavonol glycoside content calculated using $\epsilon = 29{,}200$ for both kaempferol and quercetin glycosides. ϵ was determined with pure samples of quercetin and kaempferol 3-glucosides.

Preparation of Plant Extracts. Approximately 1 g seedlings was ground in a chilled mortar with 300 mg PVP and 150 mg granular silica in 4.0 ml of the appropriate buffer. The homogenate was centrifuged at 11,500*g* for 2 min, the supernatant treated with approx. 200 mg of a 2:1 mixture of Dowex 1x2 (equilibrated with the appropriate buffer) and Amberlite XAD-4 for 0.5 min, centrifuged for 1 min at 11,500*g*, and filtered through a 100-μm mesh nylon screen. The extract obtained was used for the determination of enzyme activities.

Determination of Enzyme Activities. PAL activity was determined by a radiotracer method modified from Amrhein and Zenk (1). The incubation mixture contained 100 μl plant extract and 5 μl [U-^{14}C]phenylalanine (0.2 nmol, 10^5 dpm) and was incubated 20 min. The reaction was stopped by adding 20 μl concentrated acetic acid, extracted for 5 min with 200 μl ethyl acetate, centrifuged 1 min at 11,500*g* and 100 μl of the ethyl acetate extract was

used directly in 5 ml toluene cocktail (2.5 g PPO/L) for radioactivity determination by liquid scintillation spectrometry.

Determination of Phenylacetate-Path Activity. Seedlings (1.87 g) were homogenized with 400 mg PVP and 200 mg granular silica in 4.0 ml buffer A and treated as above. Four 100-μl aliquots were incubated with 5 μl [U-^{14}C]phenylalanine (0.2 nmol, 10^5 dpm) for 60 min at 30°C, the reaction stopped by addition of 20 μl concentrated acetic acid and the reaction mixture extracted with 2 × 250 μl ethyl acetate. The extracts were combined, evaporated to 50 μl under N_2, and chromatographed in two-dimension on 100 μm cellulose thin layer plates (Eastman) using the solvent systems described in the work of Stafford and Lewis (24) with authentic phenylalanine, cinnamic acid, and phenylacetic acid as references. The chromatograms were evaluated by x-ray radiography (14- and 21-d exposure).

Cinnamic acid 4-hydroxylase activity was determined by a modified method of Hahlbrock *et al.* (5). The reaction mixture contained 250 nmol $NADP^+$, 50 nmol glucose 6-P, 1.0 unit glucose 6-P dehydrogenase, 30 nmol [3-^{14}C]cinnamic acid, and 200 μl plant extract in a total volume of 505 μl. The reaction mixture was incubated 40 min, 500 μg *p*-coumaric acid was added in 50 μl concentrated acetic acid, and the reaction product extracted into 300 μl ethyl acetate as above. One hundred μl aliquots of the extract were chromatographed on 4 cm wide Whatman 3 mm chromatography paper strips for 2.5 h in the upper phase of benzene:acetic acid:water (2:2:1) (18), the *p*-coumaric acid area cut out, and radioactivity determined by liquid scintillation spectrometry in 17 ml toluene cocktail.

Hydroxycinnamate:CoA ligase activity was determined in buffer C by the hydroxamic acid assay as described in the work of Knobloch and Hahlbrock (14).

Chalcone synthase activity was determined as in the work of Hrazdina *et al.* (9, 10).

Malonyl-CoA hydrolase activity was determined similarly as the activity of chalcone synthase by omitting *p*-coumaryl-CoA from the reaction. Chalcone isomerase activity was determined in buffer B according to Hahlbrock *et al.* (7). The reaction mixture contained 36 nmol naringenin chalcone in 10 μl ethylene glycolmonomethylether, 5 μmol KCN, and 10 μl tissue extract in 1.0 ml buffer B. Chemical isomerization of the chalcone was determined by substituting buffer for the tissue extract. UDPglucose:flavonoid 3-*O*-glucosyltransferase activity was determined as described in the work of Hrazdina *et al.* (12) with quercetin as substrate. UDPglucose hydrolyase activity was determined as above by omitting the flavonoid co-substrate. Protein was determined according to Schaffner and Weissman (19).

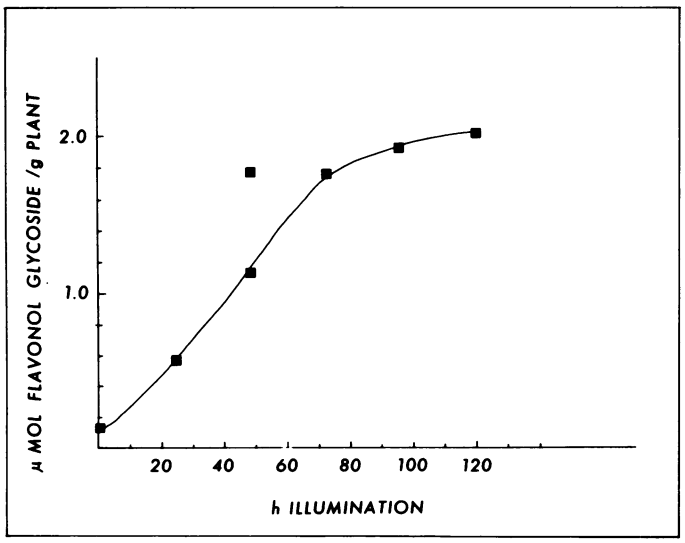

FIG. 1. Production of quercetin and kaempferol glycosides upon illumination in etiolated pea (*Pisum sativum* cv. Midfreezer) seedlings. Flavonol glycoside content of dark-grown seedlings remained at a low level, corresponding to 0 h illumination value.

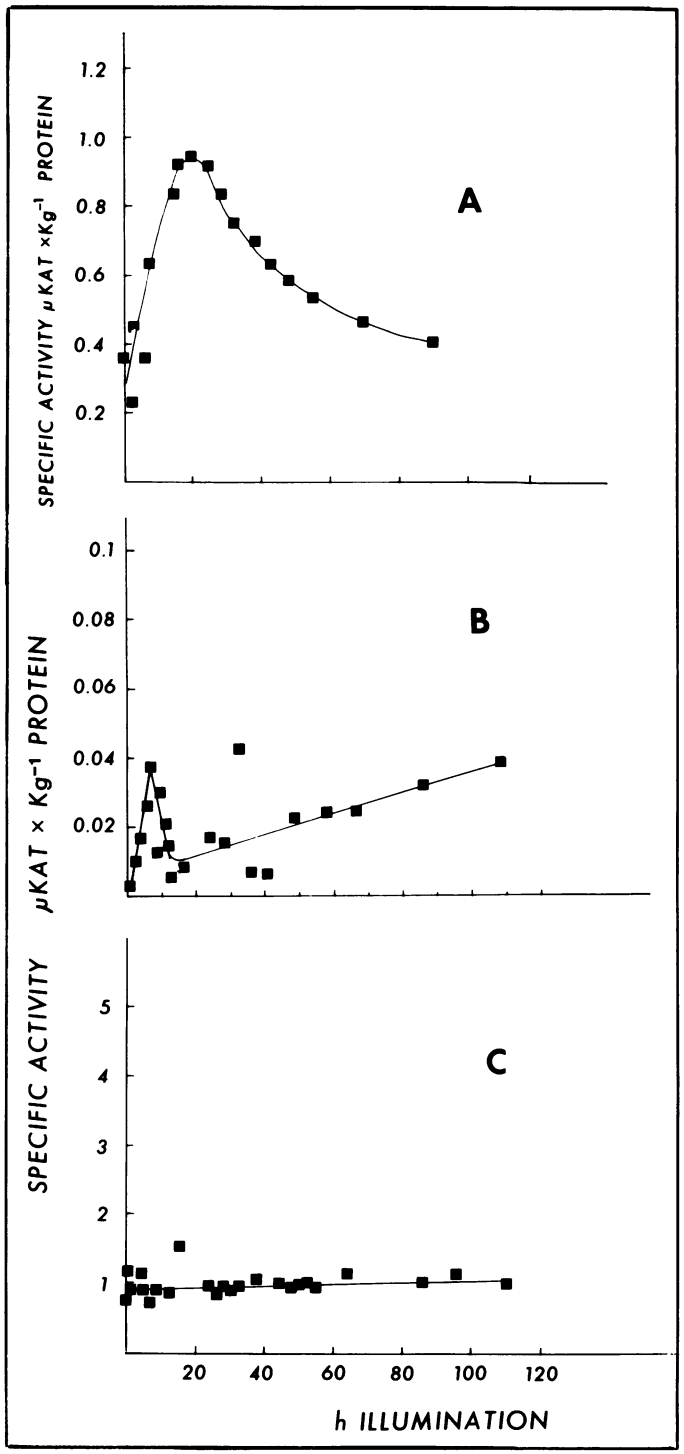

FIG. 2. Changes in activity of general phenylpropanoid pathway enzymes in etiolated pea seedlings upon illumination. A, PAL; B, cinnamic acid 4-hydroxylase; C, hydroxycinnamate:CoA-ligase. Dark values of enzyme activity showed no significant change and corresponded to that measured at 0 h illumination time.

RESULTS

Accumulation of Flavonol Glycosides upon Illumination. The etiolated pea seedlings contained minute, but measurable,

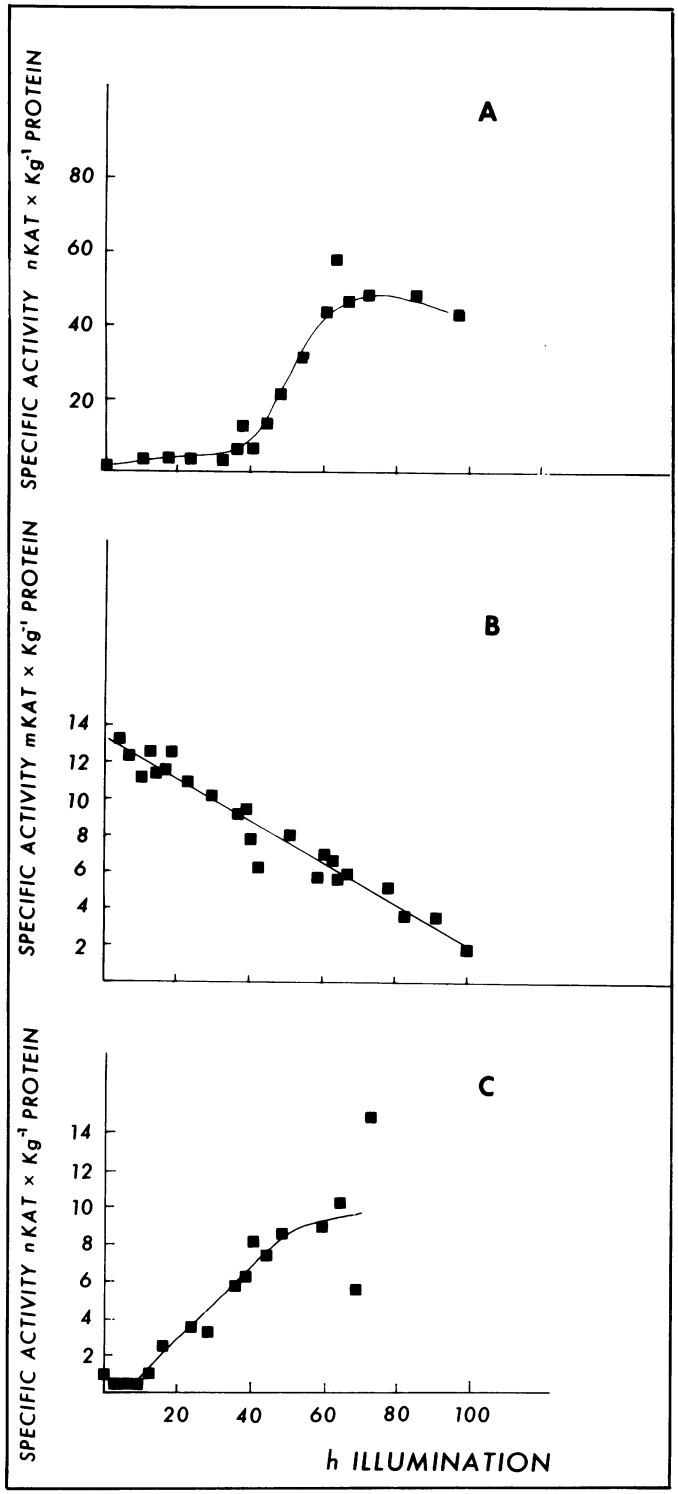

FIG. 3. Changes in activity of specific flavonoid path enzymes in etiolated pea seedlings upon illumination. A, chalcone synthase; B, chalcone isomerase; C, UDPglucose:flavonoid 3-*O*-glucosyltransferase. Enzyme activities in seedlings remaining in dark showed no significant change from 0 h illumination values.

amounts of flavonol glycosides (0.17 μmol/g tissue). The flavonol glycoside content increased steadily upon illumination and reached a plateau after approx. 100 to 120 h illumination time (3 μmol/g tissue, Fig. 1). There was no significant change in the flavonol glycoside content during the same course of time in seedlings remaining in the dark.

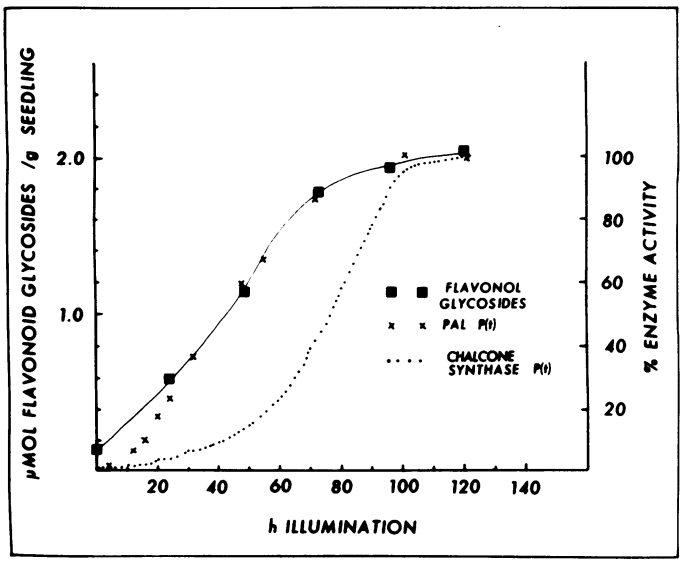

FIG. 4. Production of flavonol glycosides and integration of the activities of PAL and chalcone synthase using the equation $P(t) = {}_0\int^t E(\tau)\, d\tau$ in etiolated pea seedlings upon illumination.

Changes in enzyme activities upon illumination. PAL activity showed an immediate response to illumination. Its activity increased almost linearly up to 16 h illumination time, reaching a sharp peak at approx. 18 h, and declined from there on during the course of the experiment (Fig. 2A). There was no conversion of phenylalanine to phenylacetate in the assay mixture, therefore the data presented in Figure 2A represent the true PAL activity.

Cinnamic acid 4-hydroxylase showed a sharp, linear increase in the first 6 to 8 h of illumination time, declined at 14 h illumination to nearly that of its original dark level. From here on, the activity increased linearly at a much lower rate (Fig. 2B). The cinnamic acid 4-hydroxylase activity remained at the original dark level in plants not exposed to light.

4-Hydroxycinnamate:CoA ligase activity was investigated with 4-hydroxycinnamic, caffeic, and ferulic acids. Activity with all three substrates remained essentially the same throughout the course of the experiment and did not differ from that in plants kept in the dark (Fig. 2C). The activity of the enzyme(s) with caffeic acid was slightly higher but parallel to that with 4-hydroxycinnamic or ferulic acids.

Chalcone synthase activity was found to be 2 orders of magnitude lower than that of the preceding enzyme, the hydroxycinnamate:CoA ligase. The activity of this enzyme started to increase after 40 h illumination, reaching a maximum after 70 to 80 h (Fig. 3A). Dark control plants showed no significant change during the same period.

Malonyl CoA hydrolase activity followed the course of the chalcone synthase during the illumination period. Since malonyl-CoA was not only utilized for the enzymic synthesis of the chalcone, but was concurrently hydrolyzed by the malonyl-CoA hydrolase, the direct measurement of chalcone synthase activity (8) from ethyl acetate extracts in toluene cocktail could not be carried out without chromatographic separation of the reaction products.

Chalcone isomerase activity, present originally in the dark-grown seedlings at 13 mkat/kg protein, decreased sharply and constantly during the experiment to 2 mkat/kg protein (Fig. 3B). The lowest level of activity, however, was still 3 orders of magnitude higher than the highest activity of all other enzymes in the phenylpropanoid and flavonoid pathway.

UDPglucose:flavonol 3-*O*-glucosyltransferase showed approx. a 10-h lag period before any changes in its activity were noticeable. From there on the activity increased steadily to approx. 60 to 70

h illumination, when it approached a plateau (Fig. 3C).

UDPglucose hydrolase activity increased during the illumination period similarly to that of the glucosyltransferase, preventing a direct measurement of glucosyltransferase activity from the ethyl acetate extracts in toluene cocktail.

Correlation between Enzyme Activities and Flavonol Glycoside Production. Integration of the assumed rate limiting enzyme activities (*e.g.* PAL, chalcone synthase) of the general phenylpropanoid and specific flavonol glycoside path using the equation $P(t) =_0 \int^t E(\tau)\, d\tau$ showed that only the curve obtained by integrating PAL activities matched in shape and time sequence the production of flavonol glycosides (Fig. 4). Integration of the activities of chalcone synthase produced a curve of similar sigmoid shape, its time sequence was, however, off by approximately 30 to 40 h. None of the curves produced by integrating the enzyme activities matched quantitatively that of the production of the flavonol glycosides.

DISCUSSION

The quantitative determination of flavonol glycosides in solutions is based on absorbance measurements at the λ_{max} of their visible spectrum (320–380 nm). Because of interference by other, nonflavonoid compounds in this spectral region, these compounds could not be quantitated directly in plant extracts. The visible absorption maximum of flavonol glycosides in alcoholic solutions is known to undergo a bathochromic shift in the presence of $AlCl_3$ (13). This property of the compounds has been used in the past as one of the criteria in their structural identification. This spectral shift also permits the direct quantitative determination of the total flavonol glycoside content in pea seedlings, and measurement in their change during development.

The induction of enzyme activities in the general phenylpropanoid and flavonoid biosynthesis in intact pea seedlings differs from those observed in tissue culture with other plants. Although in tissue cultures the induction of activities is concomitant for all enzymes (6), in illuminated pea seedlings only PAL showed activity changes similar to the group I enzymes of the parsley tissue culture. Changes in the activity of cinnamic acid 4-hydroxylase suggests the involvement of two different mechanisms as described by Smith and Attridge (21). The one is a short-term effect, showing maximum enzyme activity at approximately 8 h after onset of illumination and declining to nearly the original dark level after 14 h. This short-term effect, reminiscent of phytochrome-mediated responses (21, 22), is followed by a steady increase in activity likely to be derived from the high intensity response of the seedlings to illumination (16, 22). Parallel increase in activity of PAL, cinnamic acid 4-hydroxylase, and the hydroxycinnamate:CoA ligases reported for pea seedlings (28), could not be observed in this cultivar under the conditions of the experiments. Activity of the hydroxycinnamate:CoA ligase was high in etiolated pea seedlings similarly to that found by Wallis and Rhodes (27).

In the past, changes in enzyme activities upon illumination in the flavonoid biosynthetic pathway were reported chiefly in tissue cultures. Tissue cultures provide a greatly simplified model system for the investigation of biochemical events. However, because of the simplicity of the tissue culture system, where in culture media totipotent, undifferentiated cells of approximately similar age respond in unison to the light stimulus, events cannot be related to the differentiated system in intact plants relying on active transport of nutrients and metabolites, and on cell-to-cell communication capability.

Calculation of flavonol glycoside production by integrating the changes in activities of PAL, chalcone synthase, and the glucosyl transferase resulted in curves of shape similar to that of the measured flavonol glycoside production. However, only that obtained with PAL matched the flavonol gycoside production curve in shape and time sequence, suggesting a rate-controlling function of this enzyme.

There was great discrepancy between the measured amount of flavonol glycoside production (t = 55 h, 1.8 μmol) and that calculated by integration of enzyme activities (t = 55 h, 4.1 nmol). Recent evidence for the existence of phenylpropanoid and flavonoid pathway enzymes as multienzyme complexes associated with the ER in the living cell (23, 26) suggest that the intermediary products are channeled in the pathway. In channeling, the intermediary pathway metabolites would be utilized at a higher rate than exogenously introduced substrates for the individual enzymic reaction steps. Such channeling would explain the higher rate of production of flavonoid glycosides *in vivo* than that measured by integration of the individual enzyme activities *in vitro*.

LITERATURE CITED

1. AMRHEIN N, MH ZENK 1971 Untersuchungen zur Rolle der Phenylalanin Ammonium-Lyase (PAL) bei der Regulation der Flavonoidsynthese in Buchweizen (*Fagopyrum esculentum* Moench). Z Pflanzenphysiol 63: 145–168
2. CHEN LJ, G HRAZDINA 1981 Structural aspects of anthocyanin-flavonoid complex formation and its role in plant color. Phytochemistry 20: 297–303
3. DIXON RS, DS BENDALL 1978 Changes in the levels of enzymes of phenylpropanoid and flavonoid synthesis during phaseollin production in cell suspension cultures of *Phaseolus vulgaris*. Physiol Plant Pathol 13: 295–306
4. FURUYA M, AW GALSTON 1965 Flavonoid complexes in *Pisum sativum*. Nature and distribution of the major components. Phytochemistry 4: 285–296
5. HAHLBROCK K, J EBEL, R ORTMANN, A SUTTER, E WELLMANN, H GRISEBACH 1971 Regulation of enzyme activities related to the biosynthesis of flavone glycosides in cell suspension cultures of parsley (*Petroselinum hortense*). Biochim Biophys Acta 244: 7–15
6. HAHLBROCK K, KH KNOBLOCH, F KREUZALER, JRM POTTS, E WELLMANN 1976 Coordinated induction and subsequent activity change of two groups of metabolically interrelated enzymes. Light induced synthesis of flavonoid glycosides in cell suspension cultures of *Petroselinum hortense*. Eur J Biochem 61: 199–206
7. HAHLBROCK K, E WONG, L SCHILL, H GRISEBACH 1970 Comparison of chalcone-flavanone isomerase heteroenzymes and isoenzymes. Phytochemistry 9: 945–958
8. HELLER W, K HAHLBROCK 1980 Highly purified 'flavanone synthase' from parsley catalyses the formation of naringenin chalcone. Arch Biochem Biophys 200: 617–619
9. HRAZDINA G, R ALSCHER-HERMANN, VM KISH 1980 Subcellular localization of flavonoid synthesizing enzymes in *Pisum*, *Phaseolus*, *Brassica* and *Spinacia* cultivars. Phytochemistry 19: 1355–1359
10. HRAZDINA G, LL CREASY 1979 Light induced changes in anthocyanin concentration, activity of phenylalanine ammonia-lyase and flavanone synthase and some of their properties in *Brassica oleracea*. Phytochemistry 18: 581–584
11. HRAZDINA G, F KREUZALER, K HAHLBROCK, H GRISEBACH 1976 Substrate specificity of flavanone synthase from cell suspension cultures of parsley and structure of release products *in vitro*. Arch Biochem Biophys 175: 392–399
12. HRAZDINA G, GJ WAGNER, HW SIEGELMAN 1978 Subcellular localization of enzymes of anthocyanin biosynthesis in protoplasts. Phytochemistry 17: 53–56
13. JURD L, TA GEISSMANN 1956 Absorption spectra of metal complexes of flavonoid compounds. J Org Chem 21: 1395–1401
14. KNOBLOCH KH, K HAHLBROCK 1975 Isoenzymes of p-coumarate:CoA ligase from cell suspension cultures of *Glycine max*. Eur J Biochem 52: 311–320
15. MCCLURE JW, GG GROSS 1975 Diverse photoinduction characteristics of hydroxycinnamate:Coenzyme A ligase in dicotyledonous seedlings. Z Pflanzenphysiol 76: 51–55
16. MOHR H 1972 Lectures on Photomorphogenesis. Springer-Verlag, New York
17. MOUSTAFA E, E WONG 1967 Purification and properties of chalcone-flavanone isomerase from soybean flour. Phytochemistry 6: 625–632
18. POTTS JRM, R WEKLYCH, EE CONN 1974 The 4-hydroxylation of cinnamic acid by sorghum microsomes and the requirement for cytochrome P-450. J Biol Chem 246: 5019–5026
19. SCHAFFNER W, C WEISSMANN 1973 A rapid, sensitive and specific method for the determination of protein in dilute solutions. Anal Biochem 56: 502–514
20. SCHROEDER J, F KREUZALER, E SCHAEFER, K HAHLBROCK 1979 Concomitant induction of phenylalanine ammonia lyase and flavanone synthase mRNAs in irradiated plant cells. J Biol Chem 254: 57–65
21. SMITH H, TH ATTRIDGE 1970 Increased phenylalanine ammonia lyase activity due to light treatment and its significance for the mode of action of phytochrome. Phytochemistry 9: 487–495
22. SMITH H, DB HARPER 1970 The effects of short and long term irradiation on the flavonoid complement of the terminal buds of *Pisum sativum* var. Alaska. Phytochemistry 9: 477–485
23. STAFFORD HA 1981 Compartmentation in natural product biosynthesis by multienzyme complexes. *In* E E Conn, ed, The Biochemistry of Plants, Vol 7. Academic Press, New York, pp 118–138
24. STAFFORD HA, LL LEWIS 1977 Interference by a phenylacetate pathway in

isotopic assays for phenylalanine ammonia-lyase in leaf extracts. Plant Physiol 60: 830–834
25. STATHAM CM, RK CROWDEN, JB HARBORNE 1972 Biochemical genetics of pigmentation in *Pisum sativum*. Phytochemistry 11: 1083–1088
26. WAGNER GJ 1982 Compartmentation in plant cells: the role of the vacuole. *In* LL Creasy and G Hrazdina, eds, Cellular and Subcellular Specialization in Plant Metabolism. Plenum, New York, pp 1–45
27. WALLIS PJ, MJC RHODES 1977 Multienzyme form of hydroxycinnamate:CoA ligase in etiolated pea seedlings. Phytochemistry 16: 1891–1894
28. WILKINSON M, VS BUTT 1978 Enzyme changes accompanying liquification and flavonoid synthesis in illuminated pea shoots. FEBS Proc Meet 12: Abstr. no. 0307, pp 147–154