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THE INCREASE IN PHENYLALANINE AMMONIA-LYASE ACTIVITY IN STRAWBERRY LEAF DISKS AND ITS CORRELATION WITH FLAVONOID SYNTHESIS

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Abstract—Phenylalanine ammonia-lyase (PAL) activity increased rapidly on exposure of leaf disks to light. The activity increase was necessary to permit the high rates of flavonoid synthesis in leaf disks and was correlated with the rates of synthesis of flavonoids. Although light stimulated the increase in PAL activity, the increase was not altered by short exposure to Red or Far-red light as was flavonoid synthesis.

INTRODUCTION

THE control mechanisms for flavonoid synthesis in plants are of considerable interest and have been sought from several different directions.[1,2] It is reasonable to assume that any control mechanism must ultimately be associated with genetic phenomena but equally reasonable to expect that the mode of expression is enzymatic. The intact wild strawberry plant as grown under greenhouse conditions produces only trace amounts of flavonoids[3] whereas once leaf disks are prepared, flavonoids rapidly accumulate.[4,5] We have been investigating the rapid change which permits this accumulation in leaf disks, although the more intriguing problem would be the more gradual changes which permit flavonoid synthesis in the intact plant during autumn coloration. Phenylalanine ammonia-lyase (PAL) is an enzyme of wide distribution[6] which was discovered by Koukol and Conn.[7] In many plants it is the principal link between primary and a large sector of secondary plant metabolism. PAL activity has been shown to increase in potato tuber disks and is correlated with the synthesis of chlorogenic acid.[8]

RESULTS AND DISCUSSION

Leaves from strawberry plants grown in the greenhouse normally had a low activity of phenylalanine ammonia-lyase. The average of 50 samples during 1966 was 1·20 μmoles cinnamic acid/gram fresh weight/hr. The PAL activity of greenhouse derived leaves varied considerably throughout the year due to a number of unresolved factors. When leaf disks (1·0 cm dia.), cut from the lamina of these leaves were placed in light the PAL activity increased (Fig. 1). The rate of increase of PAL activity in leaf disks was influenced by the

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[2] D. HESS, *Planta* **61**, 73 (1964).
[3] L. L. CREASY, E. C. MAXIE and V. L. SINGLETON, *Proc. Am. Soc. Hort. Sci.* **85**, 325 (1964).
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[5] L. L. CREASY and T. SWAIN, *Phytochem.* **5**, 501 (1966).
[6] M. R. YOUNG, G. H. N. TOWERS and A. C. NEISH, *Can. J. Botany* **44**, 341 (1966).
[7] J. KOUKOL and E. E. CONN, *J. Biol. Chem.* **236**, 2692 (1961).
[8] M. ZUCKER, *Plant Physiol.* **40**, 779 (1965).

composition of the solutions on which they were floated. The largest observed stimulations in PAL activity resulted from sucrose (Fig. 2). If sucrose was present at the same concentrations as other metabolites, the stimulation was similar for several substances (Table 1).

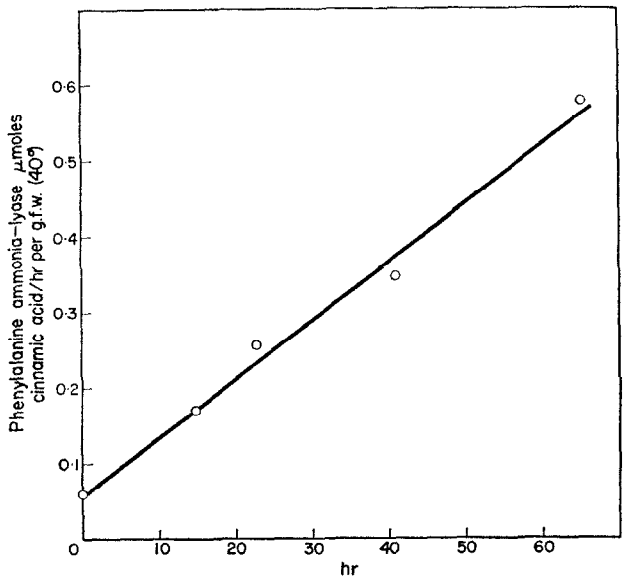

FIG. 1. INCREASE IN PHENYLALANINE AMMONIA-LYASE ACTIVITY OF STRAWBERRY LEAF DISKS FLOATED ON DISTILLED WATER AT 25° WITH 1·6 m W/cm² CONSTANT LIGHT.

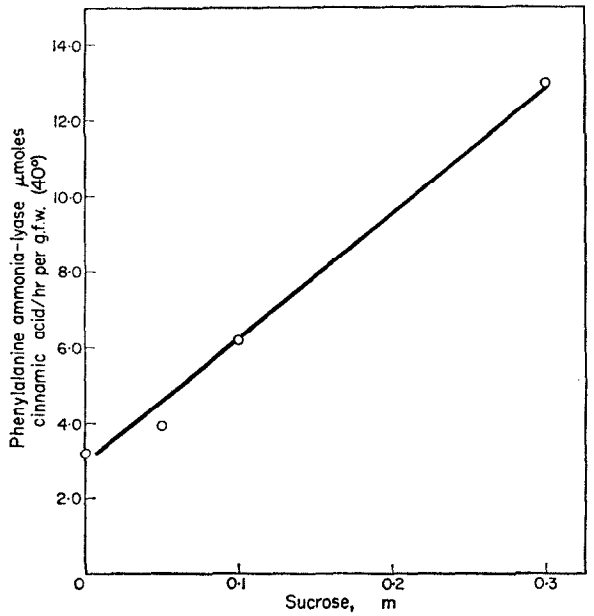

FIG. 2. EFFECT OF SUCROSE CONCENTRATION ON THE PHENYLALANINE DEAMINASE ACTIVITY OF STRAWBERRY LEAF DISKS AFTER 48 hr OF CONSTANT LIGHT (1·6 m W/cm²) AT 25°.

Phenylalanine, although stimulating the increase in PAL activity did not act as an inducer of this enzyme.[8]

Phenylalanine ammonia-lyase is on the biosynthetic path to a large number of secondary plant constituents including cinnamic acids, flavonoids and lignin and its activity should be at least sufficient to account for the production of all substances which are formed from its product. Since strawberry leaf phenylalanine ammonia-lyase is a stable enzyme and can be isolated and assayed easily, we assumed that the rate of activity in acetone powders was similar to the potential rate in intact leaves. In an experiment with 5 replicate groups of disks, the std deviations of the means of PAL, anthocyanin and flavolan content were $\pm 5{\cdot}7$ per cent, $\pm 7{\cdot}8$ per cent and $\pm 7{\cdot}5$ per cent respectively. The low PAL activity in greenhouse grown plants is quite sufficient to account for the production of lignin during leaf maturation

TABLE 1. RELATIVE ACTIVITY OF PHENYLALANINE AMMONIA-LYASE IN LEAF DISKS AFTER VARYING LENGTHS OF TIME UNDER 1·6 m W/cm² LIGHT COMPARED TO THE ACTIVITY IN LEAF DISKS FLOATING ON WATER

Treatment		PAL activity, as % of H_2O control
0·0067 M	Sucrose	122
0·05 M	Sucrose	262
0·1 M	Sucrose	375
0·3 M	Sucrose	692
0·0067 M	Phenylalanine	80
0·01 M	Phenylalanine	160
0·0067 M	Cinnamic acid	63
0·0067 M	*p*-Coumaric acid	88
0·01 M	*p*-Coumaric acid	212
0·0067 M	Caffeic acid	135
0·01 M	Shikimic acid	122
0·01 M	ATP	280
10^{-5} M	DCMU	32

and the low rate of production of cinnamic acids and flavonoids characteristic of such plants[5] but is not sufficient to permit the rates of accumulation of flavonoids and cinnamic acids in leaf disks floating on solutions in the light.[5] Figure 1 showed that there was an increase in the activity of PAL in leaf disks under these conditions and Table 2 shows that the enhanced activity is sufficient to support the accumulation rates of cinnamic acids, flavonoids and lignin. Table 2 gives the measured or estimated rates of accumulation of major products, ultimately derived through PAL, known in this tissue. The rates of accumulation of (+)catechin, strawberry leucoanthocyanin (biflavan) (SLA),[9] and anthocyanin were directly measured while the rate of accumulation of flavolans (condensed leucoanthocyanins) was estimated from the production of cyanidin during hydrolysis in BuOH/HCl (95:5) corrected by a percent reaction of synthetic polymer.[9] The rate of accumulation of cinnamic acids was estimated from the measured accumulation of chlorogenic acid and from the relative rates

[9] L. L. CREASY and T. SWAIN, *Nature* **208**, 151 (1965).

TABLE 2. PHENYLALANINE AMMONIA-LYASE ACTIVITY AND ACCUMULATION RATES OF DERIVED PRODUCTS IN STRAWBERRY LEAF DISKS AFTER 65 hr OF LIGHT ON H_2O AT 25° SEE TEXT AND EXPERIMENTAL SECTION FOR METHODS OF DETERMINING RATES OF ACCUMULATION

Enzyme activity = 2·8 μmoles cinnamic acid/hr/gram fresh weight at 25°.

Product	μmoles/hr/g.f.w.	
	Product	Utilization of cinnamic
Anthocyanin	0·02	0·02
SLA	0·19	0·38
(+) Catechin	0·75	0·75
Cinnamic acids	0·42	0·42
Flavolans	—	0·83
Lignin	—	0·004
Total		2·40

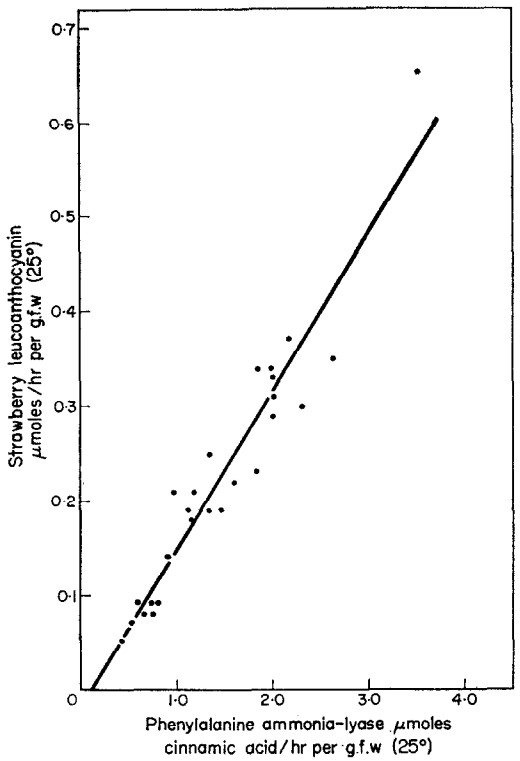

FIG. 3. CORRELATION OF PHENYLALANINE AMMONIA-LYASE (PAL) ACTIVITY WITH THE RATE OF STRAWBERRY LEUCOANTHOCYANIN (SLA) ACCUMULATION IN LEAF DISKS. DATA DRAWN FROM 5 EXPERIMENTS. (REGRESSION EQUATION, SLA = −0·02 + 0·167 PAL, r = 0·923).

of accumulation of the other cinnamic acids to chlorogenic acid in this tissue.[10] The rate of lignin synthesis was presumably low as corroborated by low rates of ^{14}C incorporation from phenylalanine-^{14}C into non-extractable residue of leaf disks.[10] Table 2 reveals that the activity of PAL is only just sufficient to permit the rates of synthesis of recognized products. This realization permits the interpretation of Fig. 3 which correlates the activity of PAL with the rate of accumulation of strawberry leucoanthocyanin. This correlation should preferably be applied to the total accumulation of products of PAL rather than to any single product. Similar correlations were found for other rapidly accumulated products but not for chlorogenic acid which is apparently not an end product in this tissue.

The rate of accumulation of PAL derived products represents a considerable commitment of the synthetic activity of strawberry leaf disks. This commitment, of 2·4 μmoles C_9 units/hr/gf.w., is about the same as the amount of carbon utilized in respiration in strawberry leaf disks.

The rate of increase of PAL activity in leaf disks is altered by changes in light quality. Leaf disks placed under different filtered light sources show subsequent differences in their PAL activity. Table 3 gives the PAL activity after 7 days constant exposure to 0·55 mW/cm^2

TABLE 3. EFFECT OF LIGHT QUALITY ON THE INCREASE IN PHENYLALANINE AMMONIA-LYASE IN STRAWBERRY LEAF DISKS. DISKS WERE FLOATED ON 0·1 M SUCROSE IN 0·55 m W/cm^2 LIGHT FOR 7 DAYS AT 25°

Light source	PAL-Activity μmoles/hr/g.f.w.	Light source	PAL-Activity μmoles/hr/g.f.w.
Dark	2·4	Green	5·3
White	5·3	Red	3·9
Blue	6·7	Far-red	2·3

light from cellulose acetate filters. An action spectrum for the increase of PAL activity in jerusalem artichoke has been published[11] which shows considerable similarity to the general response in Table 3. Although the enzyme activity is correlated with flavonoid synthesis in these experiments, we feel that the excessively long treatment time required with the low light energies prohibits interpretation of these results.

The mediation of phytochrome in the synthesis of flavonoids in strawberry leaf disks is confounded by the action of sugar on the response.[5] It is necessary to balance the desire for rapid synthesis of flavonoids resulting from sugar feeding with the resulting nullification of phytochrome response at higher sugar levels. The experiment reported in Table 4 demonstrates that the flavonoid shows no Far-red inhibition of its subsequent dark synthesis as the sucrose concentration is increased, but there are no changes in the activity of PAL associated with short exposure to Red or Far-red light even in water. The lack of phytochrome control of PAL activity changes suggests that phytochrome exerts its influence in controlling the availability or reactivity of acetate units[12] ultimately destined for flavonoid synthesis, rather than in controlling the metabolism of C_9 units.

[10] L. L. CREASY, in preparation.
[11] C. NITSCH and J. P. NITSCH, *C. R. Acad. Sci. (Paris)* **262**, 1102 (1966).
[12] M. HACKER and H. STOHR, *Planta* **68**, 215 (1966).

TABLE 4. THE INFLUENCE OF FINAL BRIEF EXPOSURES OF LEAF DISKS TO RED OR FAR-RED LIGHT ON THEIR SUBSEQUENT DARK INCREASE OF STRAWBERRY LEUCOANTHOCYANIN (SLA) AND PHENYLALANINE AMMONIA-LYASE (PAL) ACTIVITY AT DIFFERENT SUGAR CONCENTRATIONS

Treatment	Dark synthesis Far-red as % of Red	
	PAL	SLA
Water	103	22
0·05 M Sucrose	90	88
0·3 M Sucrose	103	96

EXPERIMENTAL

Plant Material

Plants of *Fragaria vesca* var. "Alpine" were grown in the greenhouse at Ithaca, New York. Leaf disks (1 cm in dia.) were cut from the lamina of freshly cut leaves and floated with the lower epidermis upward on solutions in petri dishes.

PAL-Assay

Eighty leaf disks/sample were frozen and stored at −25°. Fifty ml of −25° acetone was added and the tissue immediately homogenized at 40,000 rev/min for 60 sec (Virtis-45 Homogenizer) and poured into a cold büchner funnel. The acetone was filtered off and the residue washed twice with cold acetone. The residue was air dried on the büchner for a few minutes and then under vacuum for several hours when it was transferred to a vial and stored dry at −25°. The powders were stable for several months. The PAL activity was measured by suspending the powder (at 10 mg/6 ml) in pH 8·8, 0·1 M Sodium Borate buffer for 15 min, centrifuging at 5000 g for 20 min and transferring 2 ml of the clear supernatant to each of 2 spectrophotometer cells, one containing 1 ml H_2O (Reference) and the other 1 ml of 0·06 M L-phenylalanine (Sample). The cells are placed in a temperature controlled cell-holder (at 40°) in a spectrophotometer and the rate of change of absorbance at 280 mμ was measured for at least an hour. An extinction coefficient of 16,500 was determined for cinnamic acid at 280 mμ in pH 8·8 Borate.

Preparation and Analysis of Extracts

Ten blotted leaf disks were extracted 4 times with hot methanol and the extracted residue was saved for direct estimation of non-extractable flavolans. Flavonoid analyses were done as previously reported.[5] Chlorogenic acid was determined according to the method of Zucker and Ahrens.[13]

Light Sources

White, blue, green and red light was provided by fluorescent tubes with appropriate filters made from cellulose acetate. Far-red light was supplied with a tungsten lamp and a filter made from 5 cm water and red and green cellulose acetate. Light sources were equalized by measuring the total radiant energy with a radiometer and adjusting the distances.

[13] M. ZUCKER and J. F. AHRENS, *Plant Physiol.* **33**, 246 (1958).