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LEUCOANTHOCYANIN FORMATION IN BUCKWHEAT SEEDLING HYPOCOTYLS*

JAMES R. TROYER

Department of Botany and Bacteriology,
North Carolina State of the University of North Carolina at Raleigh, U.S.A.

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Abstract—The leucoanthocyanin content of hypocotyls of buckwheat seedlings cultured in continuous darkness increases until the seventh day of growth and then decreases. The maximum content occurs in seedlings which are also at a peak in their ability to form anthocyanin following exposure to light. However, the synthesis of anthocyanin is accompanied by no change in leucoanthocyanin content, and 11-day-old hypocotyl materials of different anthocyanin-forming abilities do not show corresponding differences in leucoanthocyanin content. Also, augmentation of anthocyanin formation by supplying sucrose does not result in a change in the leucoanthocyanin level. These observations do not permit a clear conclusion to be drawn concerning the relation of the leucoanthocyanin to anthocyanin synthesis in this plant.

INTRODUCTION

THE metabolic relation of leucoanthocyanins and anthocyanins in the living plant is still uncertain.[1,2] The complex chemistry of the former substances[3] has made study of them difficult. As a result, most of the evidence bearing on the problem of whether some leucoanthocyanins may be precursors of anthocyanins *in vivo* is circumstantial.

In connexion with certain observations of anthocyanin synthesis in buckwheat seedlings,[4] two particular problems involving the leucoanthocyanin of this material[5] have been investigated. The rate of formation of leucoanthocyanins during growth in darkness has been examined and compared with the anthocyanin-forming capability of the seedlings, and variation in the leucoanthocyanin content of 11-day seedlings has been followed during their synthesis of anthocyanins. These studies have shown a coincidence of peak leucoanthocyanin content with maximum anthocyanin-synthetic capacity, and that leucoanthocyanin levels do not vary as anthocyanin is formed under certain conditions.

RESULTS

Leucoanthocyanin Content of Developing Hypocotyls

Leucoanthocyanin is present in the hypocotyls of dark-grown buckwheat seedlings as early as 3 days and as late as 11 days after the onset of germination (Fig. 1). Moreover, the amount of leucoanthocyanin varies with the stage of development of the seedling, increasing during the period from 3 to 7 days and then decreasing. Since growth of the hypocotyl as

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[1] L. BOGORAD, *Ann. Rev. Plant Physiol.* **9**, 417 (1958).
[2] T. SWAIN and E. C. BATE-SMITH, *Comparative Biochemistry* **3**, 755, Academic Press, New York (1962).
[3] M. M. BOKADIA, *J. Indian Chem. Soc.* **38**, 616 (1961).
[4] J. R. TROYER, unpublished.
[5] J. R. TROYER, *J. Elisha Mitchell Sci. Soc.* **77**, 137 (1961).

measured by wet weight continues throughout the period in question, the formation of leucoanthocyanin clearly does not keep pace with growth; in fact, the leucoanthocyanin content expressed on a wet-weight basis decreases steadily throughout the growth period.

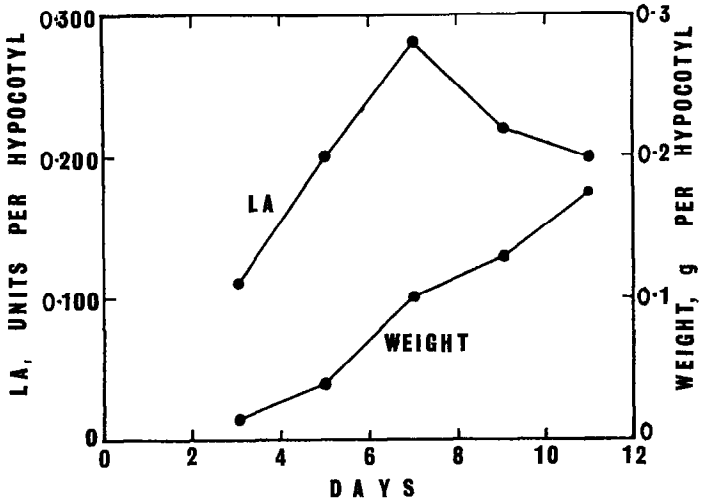

FIG. 1. LEUCOANTHOCYANIN CONTENT (LA) AND WET WEIGHT OF HYPOCOTYLS OF BUCKWHEAT SEEDLINGS OF DIFFERENT AGES CULTURED IN CONTINUOUS DARKNESS.
Each point represents the mean of four replicates.

Anthocyanin Formation and Leucoanthocyanin Content

The synthesis of anthocyanin can be induced in excised buckwheat hypocotyls by a brief light period followed by a longer dark period.[4] As the results in Table 1 indicate, when synthesis of considerable anthocyanin occurs in hypocotyls taken from dark-grown seedlings 6 days old, there is no significant change in the level of leucoanthocyanin. In seedlings 11 days old the apical and basal regions of the hypocotyl possess different anthocyanin-forming capabilities.[6] From the data of Table 1 it is clear, however, that for a given region of the hypocotyl there is no significant change in the leucoanthocyanin content accompanying anthocyanin formation in response to light. Moreover, the apical region, in which the major amount of anthocyanin appears, contains less leucoanthocyanin than the basal region.

The synthesis of anthocyanin in basal segments of 11-day-old hypocotyls can be enhanced by floating them on sucrose solutions during the dark period following exposure to light.[4] This effect is illustrated by the results in Table 1. However, while supplying sucrose increases the amount of anthocyanin formed, there is no accompanying change in the level of leucoanthocyanin.

DISCUSSION

The commonly used procedure of leucoanthocyanin analysis by heating with HCl is subject to errors of at least two kinds. The yield of anthocyanidin from leucoanthocyanin is probably quite low.[7,8] Also, other substances present in the crude extracts used here may

[6] W. K. H. KARSTENS, *Rec. trav. bot. neerl.* **36**, 85 (1939).
[7] E. C. BATE-SMITH and T. SWAIN, *Chem. and Ind.*, 377 (1953).
[8] F. E. KING and W. BOTTOMLEY, *J. Chem. Soc.*, 1399 (1954).

yield red products with strong light absorption at 535 mμ.[5,8–10] Since these defects of the method act in opposite directions, it is difficult to guess to what extent the results obtained reflect true leucoanthocyanin quantities. It is assumed here that the data presented are at least relative estimates of leucoanthocyanin.

TABLE 1. THE INFLUENCE OF LIGHT ON ANTHOCYANIN AND LEUCOANTHOCYANIN IN HYPOCOTYL MATERIAL FROM DARK-GROWN BUCKWHEAT SEEDLINGS.*

Age of seedling	Experimental treatment	Portion of hypocotyl	Relative units per hypocotyl	
			Anthocyanin	Leuco-anthocyanin
6 Days	6 hr dark, 25° 72 hr dark, 10°	Entire	0·000	0·237
	6 hr light, 25° 72 hr dark, 10°	Entire	0·380	0·224
11 Days	Initial Content	Apical Segment	0·000	0·107
		Basal Segment	0·000	0·159
	6 hr dark, 25°	Apical Segment	0·000	0·099
	72 hr dark, 10°	Basal Segment	0·000	0·142
	6 hr light, 25°	Apical Segment	0·132	0·107
	72 hr dark, 10°	Basal Segment	0·044	0·147
11 Days	6 hr light, 25° 72 hr dark, 10°	Basal Segment		
	on H_2O		0·033	0·111
	on 0·015 M sucrose		0·049	0·113
	on 0·060 M sucrose		0·065	0·106

* Each value is the mean of five replicates.

Leucoanthocyanin Content of Developing Hypocotyls

The occurrence of a peak leucoanthocyanin content in hypocotyls of 7-day-old buckwheat seedlings, with a decline thereafter, does not correspond to the situations found in dark-grown seedlings of *Brassica*[11] and *Impatiens*.[12] In these two cases the amounts of leucoanthocyanin in the hypocotyls increased steadily until the ends of the experimental periods (10 and 13 days, respectively). In *Impatiens* this increase continued even after the growth of the hypocotyl ceased, whereas in buckwheat the decline occurs at a time when the wet weight of the hypocotyl is still increasing. It is interesting to note that the maximum leucoanthocyanin content occurs at the same seedling age (7 days) at which the anthocyanin-forming ability of the dark-grown buckwheat hypocotyl is at a maximum.[4] This coincidence might suggest a biogenetic relation between the leucoanthocyanin and the anthocyanin, especially since the former is converted on heating to cyanidin, the aglycone of the latter.[5] On the other hand, it might merely reflect a particular metabolic status of the seedlings at this point in their development.

Anthocyanin Formation and Leucoanthocyanin Content

The results of the studies of the leucoanthocyanin content of hypocotyls in which anthocyanin synthesis occurs do not clearly indicate whether there is or is not a biogenetic relation

[9] H. RAUDNITZ, *Science* **128**, 782 (1958).
[10] W. D. MCFARLANE, *J. Inst. Brewing* **67**, 502 (1961).
[11] F. EBERHARDT, *Planta* **43**, 253 (1954).
[12] R. E. ALSTON, *Amer. J. Bot.* **45**, 289 (1958).

between the two types of substances. Circumstantial evidences discouraging to the hypothesis of such a relation have been reported for various plant materials by several investigators [13–17]. However, the tracer studies of Bopp and Matthis [18] provide strong support for the idea that in *Impatiens* seedlings the anthocyanin originates by conversion of the leucoanthocyanin. In buckwheat, the lack of changes in leucoanthocyanin levels when sizeable amounts of anthocyanin are formed might result if there is no direct metabolic connexion between the two. On the other hand, the same result would be observed if anthocyanin were formed from leucoanthocyanin, but a steady level of the latter were maintained by simultaneous synthesis. In this matter the observations presented permit no clear conclusion.

EXPERIMENTAL

Plant Materials

Seedlings of Japanese buckwheat (*Fagopyrum sagittatum* Gilib.) were cultured and handled by methods described in detail elsewhere.[4] The seeds germinated and the seedlings grew on wet filter paper in continuous darkness at 20° for periods ranging from 3 to 11 days. At appropriate times either entire hypocotyls or hypocotyl segments were taken for further treatment and analysis. Hypocotyl segments 25 mm long were used in some experiments involving seedlings 11 days old. With material of this age, apical segments from the region just below the hook and basal segments from the region just above the roots were taken for comparison.

The formation of anthocyanin was induced by exposing the plant material to light at room temperature (about 25°) for a period of 6 hr. The light source used consisted of daylight fluorescent lamps which gave a maximum illumination of 11,000 to 13,000 meter-candles at the surface of the plant material. After the light treatment the hypocotyls or hypocotyl segments were placed in darkness at 10° for an additional period of 72 hr. During this dark period the hypocotyls were kept on wet filter paper or, in one experiment, on aqueous sucrose solutions.

Anthocyanin and Leucoanthocyanin Analysis

In the study of variation of leucoanthocyanin content with seedling age, leucoanthocyanin analyses were made by a quantitative modification of the procedure of Bate-Smith.[19] For each sample 50 hypocotyls were ground in a mortar with 25 ml of aqueous 2 N HCl. The mixture was heated to the boiling point and held there for 5 min. The residue of plant material was then filtered off and treated in the same manner with three additional 25-ml portions of 2 N HCl. The combined aqueous filtrates were next shaken in a separatory funnel with two successive 15-ml portions of iso-amyl alcohol. The separated amyl alcohol layers were combined, three drops of methanol were added, and the volume was brought up to 50 ml with additional iso-amyl alcohol. The absorbance at 535 mμ of the iso-amyl alcohol solution was then measured in a Bausch and Lomb Spectronic 20 colorimeter. The anthocyanidin so measured was assumed to reflect the leucoanthocyanin originally present. It should be noted,

[13] N. W. SIMMONDS, *Nature*, **173**, 402 (1954).
[14] R. E. ALSTON and C. W. HAGEN, *Nature*, **175**, 990 (1955).
[15] S. KRUGMAN, *Forest Sci.* **2**, 273 (1956).
[16] M. BOPP, *Z. Bot.* **47**, 197 (1959).
[17] M. NEYLAND, Y. L. NG and K. V. THIMANN, *Plant Physiol.* **38**, 447 (1963).
[18] M. BOPP and B. MATTHIS, *Z. Naturforsch.* **17b**, 811 (1962).
[19] E. C. BATE-SMITH, *Biochem. J.* **58**, 122 (1954).

however, that the plant residue previously filtered off was red with a colouring material not extracted by the aqueous acid. In accordance with previous practice,[4] the absorbance reading was multiplied by the volume of the solution to give a measure in relative units of the total quantity of pigment in the sample.

For experiments in which hypocotyls contained anthocyanin a different procedure was employed. A sample of 100 hypocotyls or segments was extracted at 10° with methanol–conc. HCl (99:1, v/v). The extract was filtered and brought to a final volume of 100 ml. The relative anthocyanin content was then determined by an absorbance reading at 535 mμ. The entire extract and the original hypocotyl material were then combined and 50 ml of aqueous 2 N HCl was added. The mixture was heated at the boiling point for 15 min, during which time most of the methanol escaped. The solution was filtered, and the plant residue heated with three successive 10-ml portions of aqueous 2 N HCl. After the filtrates were combined, cooled, and made up to 100 ml, the absorbance of the resulting solution was measured at 535 mμ. This reading was taken as a measure of total anthocyanidin from both leucoanthocyanin and anthocyanin sources.

In order to correct for a change in absorbance occasioned by the change of solvent or by hydrolysis or destruction of the anthocyanin originally present, three samples of the buckwheat anthocyanin purified by repeated paper chromatography in several solvents were treated in accordance with the above procedure. This resulted in a decrease in absorbance to a value 0·90 times the original value. Subtraction of 0·90 times the absorbance of the original extract from the absorbance of the solution after boiling with acid gave a value which was taken as a measure of the anthocyanidin produced from leucoanthocyanin, and consequently as a quantitative estimate of the latter.