Photocontrol of Anthocyanin Synthesis in Milo Seedlings [1]

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Prolonged irradiation at moderately high intensities is a requirement for anthocyanin synthesis in many plants. Action spectra for anthocyanin formation, however, are different for nearly every species examined (1, 5, 6, 7, 9). In general, a maximum is present between 400 and 500 mμ (blue) or one between 400 and 500 mμ and another between 600 and 800 mμ (blue & red). In some species a second controlling photoreaction which requires brief irradiances at relatively low intensities is present (1, 6). The latter exhibits all the characteristics of phytochrome (4) with a promotive action on anthocyanin production by the red region of the spectrum (600–700 mμ) that is reversed by far red (700–800 mμ).

A somewhat more responsive and experimentally convenient plant than the species previously used was desired to facilitate work on anthocyanin and examination of the photoreactions involved. Sorghum seedlings attracted attention because of marked reddening of the plants at an early stage of growth. Preliminary experiments showed that seedlings of many varieties of sorghum produced a great deal of anthocyanin. The availability of large lots of seeds of Wheatland milo (*Sorghum vulgare* Pers.) prompted its selection for further studies, some results of which are described herein.

Materials & Methods

Seeds of Wheatland milo were germinated in darkness on 8-mesh stainless-steel screens immersed in aerated tap water. Temperatures of 25 to 26 C during germination and subsequent shoot growth produced seedlings of adequate size in 3½ to 4 days. The temperature during the treatments and during the post-treatment incubation period was maintained at 20 C. When intact seedlings were placed in petri dishes the root tended to lift the shoot away from the substrate. Excising the roots resulted in no measurable effect on the amount of anthocyanin produced in the internode so long as the seed remained attached to the shoot. Therefore, the standard procedure was to remove the roots prior to treatment. The intact shoots with seeds attached were placed on water-soaked filter paper in petri dishes or plastic boxes. These shoots were exposed to various light regimes, then harvested immediately or after a period of incubation in complete darkness. When a dark-incubation period was used, the seedlings were generally harvested 24 hours after the beginning of the light treatments. In this way the total time allowed for anthocyanin synthesis was kept constant irrespective of the duration of the light period. Experimental evidence indicated that with light periods up to 9 hours, dark-incubation periods of 24 hours produced only about 15 % more anthocyanin than was formed with dark-incubation periods of 15 hours. At the conclusion of the experiment the seed and the coleoptile were discarded and the first internode was cut into small segments. Usually, five first internodes were extracted in 5 milliliters of 1 % HCl methanol and held 24 hours at 5 C. Anthocyanin content was determined from absorbancy values (1 = 1 centimeter) at 525 mμ in the acid solutions.

Studies of the first photoreaction were usually made with radiant energy from cool-white fluorescent lamps that gave a maximum unfiltered illumination of 2,400 ft-c. The radiant flux density, 470 mμ, was about 50 microwatts per square centimeter. Action spectra for both the first and second photoreactions were determined on a carbon-arc spectrograph (7). Filters of red, dark blue, or a combination of red and dark-blue cellophanes were used in certain experiments. The transmissions of these cellophane filters are given by Downs et al. (3).

Results

Preliminary experiments were performed to determine the optimum conditions for anthocyanin synthesis in milo seedlings. The temperature during the dark incubation period exerted a definite control over the amount of anthocyanin produced. Maximum production occurred in response to a temperature of 20 C during dark incubation period. The temperature during the light period also influenced anthocyanin production (table I), but since 20 C temperatures during the light period enabled the seedlings to make a substantial amount of anthocyanin, no detailed studies were made.

Wheatland milo seedlings produced anthocyanin in the roots, in the internodes, and in the coleoptiles during a 24-hour period of light. Removal of the root and the coleoptile, or coleoptile alone, had no influence on the amount of anthocyanin found in the

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Table I

Amount of Anthocyanin Formed by Wheatland Milo Seedlings at Various Temperatures during a 3-Hour Period*

Temperature		Anthocyanin
Light	Dark	
C	C	A × 10^{-2}
5	20	4
20	15	40
20	20	43
20	25	40
20	30	24
20	35	10

* At an illuminance of 2,400 ft-c from fluorescent lamps and during the succeeding 24-hour dark incubation period.

internode. Removal of the seed from the base of the internode drastically curtailed anthocyanin production, and addition of various sugars to the substrate only partially substituted for the absence of the seed.

The amount of anthocyanin formed by seedlings of Wheatland milo increased with increasing illumination. When the illumination was continuous and exceeded 1,000 ft-c, about 6 hours were required for the appearance of anthocyanin. At lower levels of continuous illumination about 12 hours were required. In each case the rate of accumulation was linear once the anthocyanin appeared, but the rates of accumulation at high and low illuminations were not the same (fig 1). Figure 2 shows that milo seedlings illuminated for different periods and allowed to incubate in the dark, accumulate anthocyanin at an immediate constant rate provided the illumination exceeds 1,200 ft-c. Another experiment indicated that after 16 hours of illumination the rate decreased and after about 30 hours an apparent saturation was approached. During the first 16 hours about 20 units of anthocyanin were formed per hour: 10 units per hour were formed from the 16th to the 32nd hour, and only 3 units per hour were formed between the 32nd and the 48th hours. However, when the illumination was decreased below 1,200 ft-c, a lag period or pre-induction period occurred (fig 2).

The reciprocity law is not obeyed in the first photoreaction for anthocyanin formation in Wheatland milo seedlings since time appears to operate independently of intensity. For example, plotting response against illumination indicates that exposure to light for 2 to 16 hours caused an apparent saturation at 1,200 ft-c because increasing the illumination from

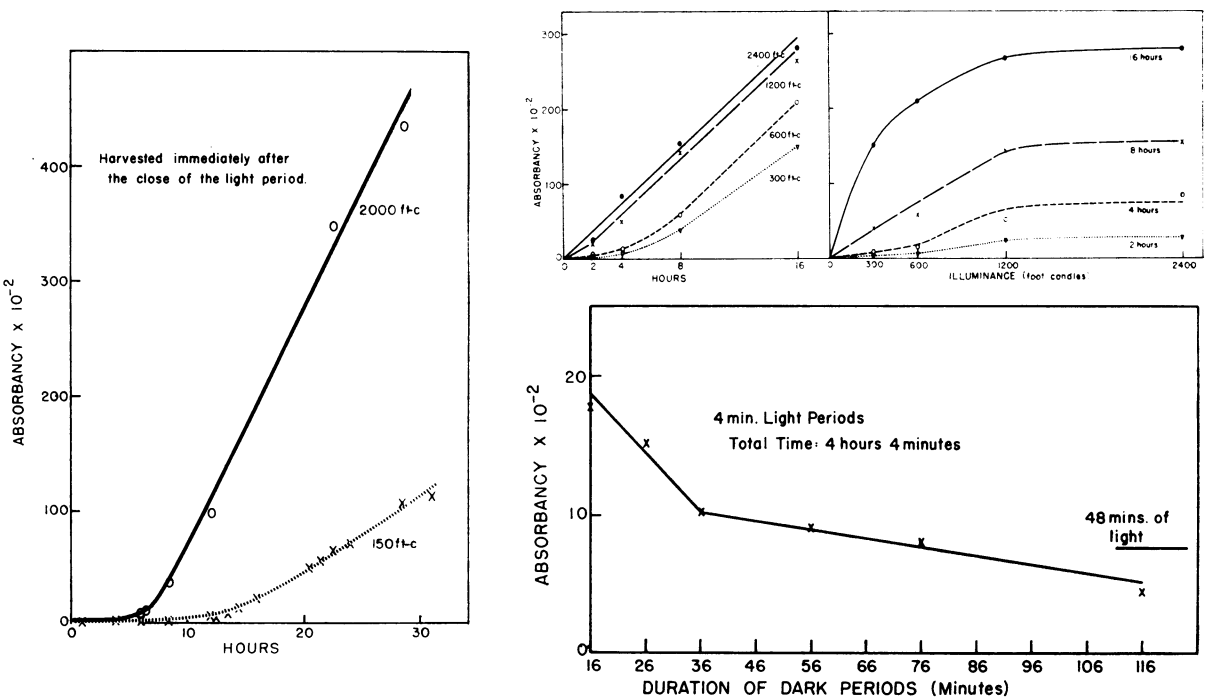
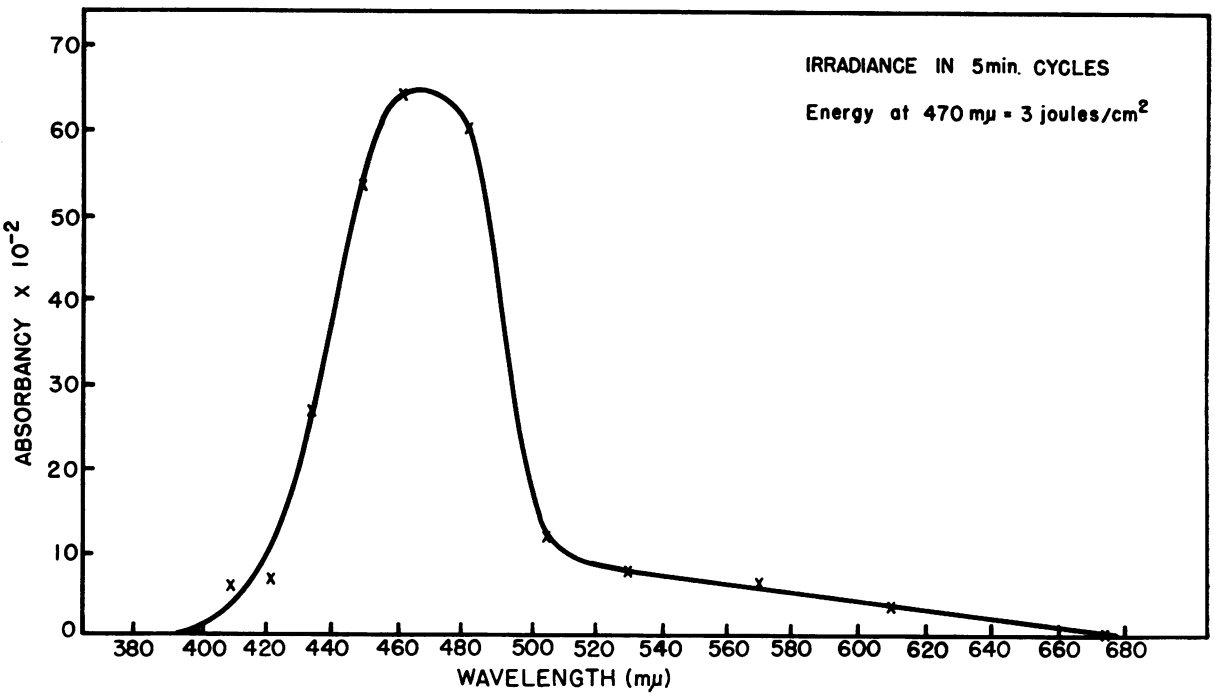


Fig. 1. Accumulation of anthocyanin measured immediately after various periods of irradiation at two levels of illumination from fluorescent lamps.

Fig. 2. (*left*). Amount of anthocyanin formed in response to various durations of light from fluorescent lamps at several illumination levels. Measurement was made after a 24-hour dark incubation period. (*right*) Amount of anthocyanin formed in response to various illumination levels after several durations of exposure to light from fluorescent lamps. Measurements were made after a 24-hour dark incubation period.

Fig. 3. Amount of anthocyanin formed when various durations of darkness intervene between several brief exposures to an illuminance of 2,400 ft-c from fluorescent lamps. Total time 4 hours and 4 minutes.



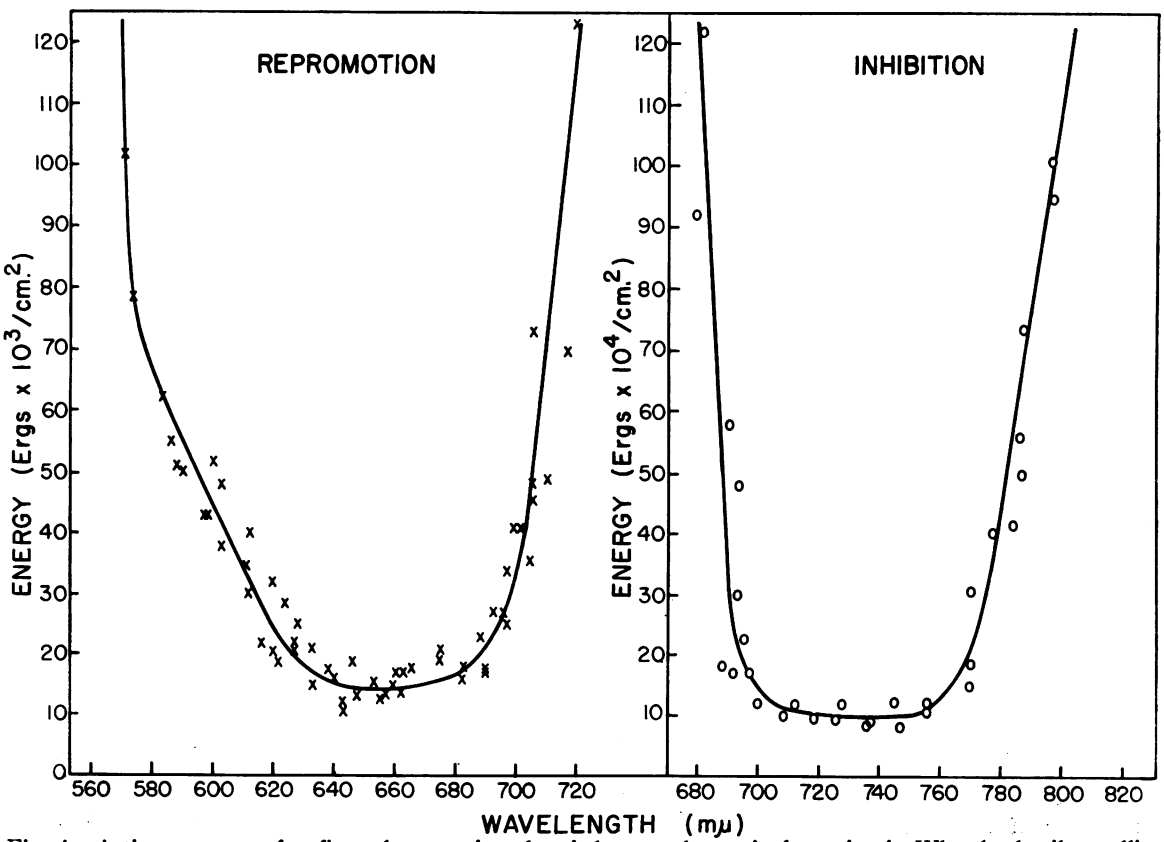


Fig. 4. Action spectrum for first photoreaction that induces anthocyanin formation in Wheatland milo seedlings.
Fig. 5. Action spectra for far-red inhibition and red repromotion of anthocyanin induced by first photoreaction.

1,200 to 2,400 ft-c failed to induce much of an increase in anthocyanin content (fig 2). Doubling the duration of exposure at 1,200 ft-c, however, doubled the amount of anthocyanin produced, and plotting response against time shows no saturation effect (fig 2). Moreover, 48 minutes of light given in cycles of

Table II

Relative Accumulation of Anthocyanin per Unit of Light*

Light/cycle min	Relative accumulation/minute of light A × 10^{-2}
2	11.0
4	8.0
6	6.7
8	5.4
10	4.0
12	3.8
14	3.4
16	3.1
18	3.0
20	3.2

* (1 min) in each 20-minute cycle at an illuminance of 2,400 ft-c from fluorescent lamps. Total time was 4 hours.

4 minutes light and 16 minutes dark over a period of 4 hours produced twice as much anthocyanin as did 48 minutes given in a single exposure (fig 3). Decreasing the number of 4-minute light periods during the 4-hour period by increasing the dark period of each cycle reduced the amount of anthocyanin formed. Finally, when the total amount of light was reduced to 16 minutes in four cycles of 4 minutes light and 76 minutes dark, about the same amount of anthocyanin was formed as with a single exposure of 48 minutes (fig 3). A single 16-minute exposure produced very little anthocyanin. Cycles of 2 minutes of light, 18 minutes of dark over a 4-hour period were very efficient for promoting anthocyanin formation. The efficiency per minute of light decreased as the period of light in each cycle increased (table II). Finally, the accumulation of anthocyanin per minute of light reached a constant value when the light periods were 16 minutes or more in each 20-minute cycle.

Action Spectrum for First Photochemical Reaction. An action spectrum for anthocyanin formation was determined by using a cyclic-exposure technique. Because energy from the spectrograph was not adequate to induce an immediate linear response, seedlings were given a 4-hour pre-induction period of white fluorescent light at an illumination of 100 ft-c to overcome the lag period of anthocyanin formation. This pre-induction period was followed by 6 hours of cyclic irradiation on the spectrograph. The spectrographic irradiation consisted of 5 minutes in the spectrum alternating with 5 minutes of dark. At the close of the spectrograph-irradiation period all seedlings were given a 5-minute exposure to red radiant energy from a filtered source to shift phytochrome (4) to the far-red-absorbing form, which is most favorable for anthocyanin synthesis. The action spectrum for this first photoreaction for anthocyanin formation showed a pronounced maximum at about 470 mμ (fig 4). The first photoreaction was not active at the longer wavelengths.

Action Spectra for 2nd Photochemical Reaction. After the first photoreaction runs for several hours anthocyanin formation in milo seedlings is controllable by the phytochrome-photoreceptor system. The anthocyanin potentiated by as much as 8 hours of high-intensity light (fig 6) was appreciably inhibited by a subsequent 5-minute exposure to far red. Like all typical phytochrome responses, the action of far red is reversed by a subsequent exposure to red radiant energy. Moreover, the response is repeatedly reversible (table III). The action spectra for the far-red inhibition of anthocyanin formation and repromotion by red (fig 5) are also typical of phytochrome. No fine structure appeared in either action spectrum; this finding is consistent with other studies made in this laboratory (2, 6, 7).

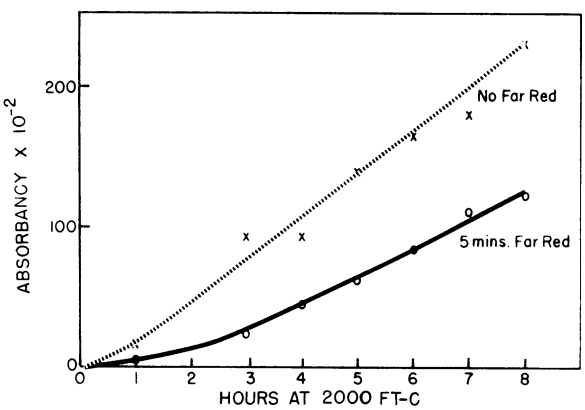


Fig. 6. Far-red inhibition of anthocyanin formation after various durations of high-intensity light (2,000 ft-c) from fluorescent lamps.

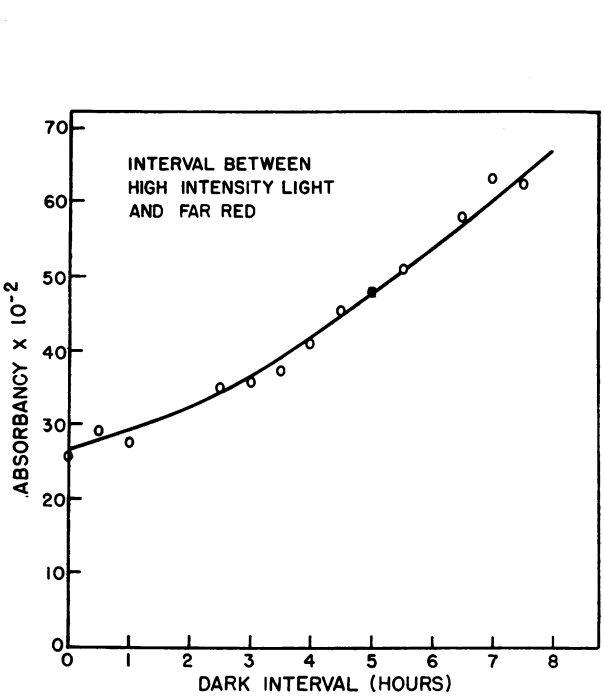
Table III

Reversibility of Anthocyanin Formation by Far-Red & Red Radiant Energy*

Exposures**		Anthocyanin A × 10^{-2}
Far red number	Red number	
0	0	106
1	0	48
1	1	106
27	26	45
27	27	109
38	37	48
38	38	97
42	41	49
42	42	103

* After 3-hour exposure to an illuminance of 2,000 ft-c from fluorescent lamps.

** Three minutes of far red; 1 minute of red.



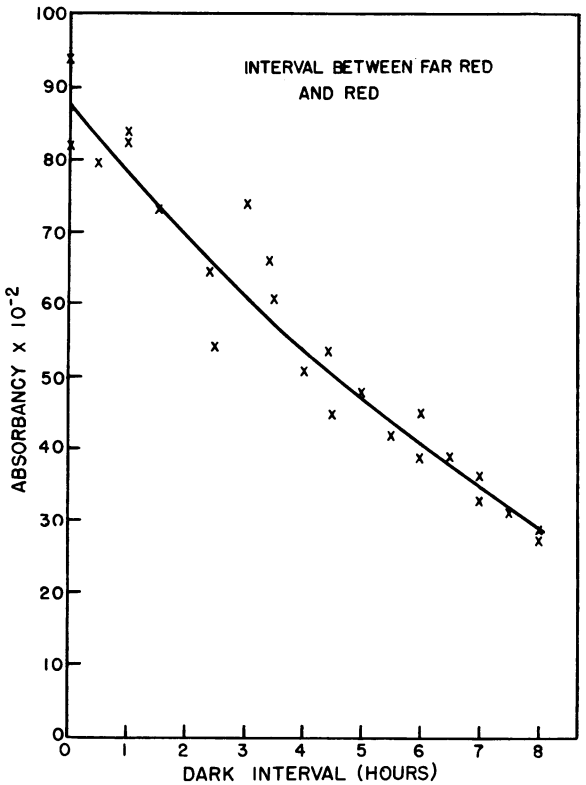


Fig. 7. Loss of far-red inhibition of anthocyanin formation when successively longer dark periods intervene between first photoreaction and the irradiation with far red.

Fig. 8. Loss of repromotion of anthocyanin formation by red when successively longer dark periods intervene between far-red and red irradiations.

The inhibiting effect of far red decreased during the dark incubation period that followed the first high-energy light exposure. When 4 hours of darkness intervened between the close of the first light period and the far-red irradiation, about 50 % of the inhibiting effect of the far red was lost (fig 7). A similar effect was obtained when dark periods of various durations intervened between the inhibitory far-red and the repromoting red irradiations. Again, about 50 % of the response was lost after 4 hours (fig 8).

Anthocyanin Formation in Roots. Intact or excised roots of milo in a well-aerated medium are able to synthesize anthocyanin. Excised roots were placed in petri dishes on filter paper moistened with a 3 % sucrose solution. Anthocyanin formation in the excised roots first required the operation of an apparently high-energy photoreaction; then, as in the shoots, the amount of anthocyanin formed depended upon the form of phytochrome present. A far-red irradiation inhibited and a subsequent exposure to red repromoted anthocyanin formation in the excised roots in the same way as in the shoots.

Discussion

Two photoreceptors control the formation of anthocyanin in Wheatland milo seedlings. The first photoreceptor requires more energy than the second one and exhibits a maximum response at 470 mμ. The 470 mμ photoreceptor can be light saturated for a particular period of illumination but additional anthocyanin can be produced at that level of illumination by increasing the exposure time. The time dependence of anthocyanin formation is illustrated by experiments with cyclic lighting where energy distributed in small pulses (short cycles) for several hours was much more effective than the same energy given as a single exposure. The time dependence of anthocyanin synthesis indicates that some substrate is being made available at a rate too slow to provide for maximum efficiency of the irradiation. The limiting material apparently originates in the seed and then moves to the site of the light action. However, a certain amount of the substrate is already available in the shoot when the seed is excised, since a substantial amount of anthocyanin is produced without the seed.

The duration of the induction period for anthocyanin synthesis in Wheatland milo seedlings depends on the intensity of the light used to excite the 470 mμ photoreaction. The induction photoreaction may produce substrates for reactions preceding or involved in the steady-state photoreaction (6). The induction period in Wheatland milo seedlings can be

lessened and finally removed by higher light intensities indicating that the substrates must be produced about as rapidly as required for the subsequent reactions. No action spectra were determined for the induction period, but the photoreceptor is probably the same as for the steady-state photoreaction (7).

The first photoreaction during the linear phase of anthocyanin production showed a single action maximum at 470 mμ. The 470 mμ action maximum is possibly suggestive of the absorption of a flavin. Action spectra for anthocyanin synthesis in some plant tissue show action maxima at wavelengths greater than 600 mμ. Apple skins (7, 8) produce maximum anthocyanin at about 650 mμ, red cabbage (6) and white mustard seedlings (5) at about 700 mμ, and turnip seedlings exhibit a maximum response at about 725 mμ (6).

Anthocyanin production in apple skin and turnip seedlings was apparently not influenced by the condition of phytochrome at the close of the primary high-energy photoreaction. However, an exhaustive investigation was not conducted. Moreover, the phytochrome action may have been so entangled with the long wavelength absorption of the first photoreaction that separation could not be made. Phytochrome was clearly demonstrated to control anthocyanin synthesis in red cabbage seedlings (6), but because of the 700 mμ action maximum, a clean separation of the two photoreactions was not possible. The action of phytochrome in controlling anthocyanin formation in Wheatland milo seedlings is clearly separate from the first photoreaction. The high-energy photoreceptor does not have appreciable action beyond 600 mμ in this tissue. Red or far red given alone or an exposure to one followed by an exposure to the other with no previous high-intensity light, has no measurable effect on anthocyanin production. After the process is started by an exposure to high-intensity light, however, far red will inhibit and red after far red will repromote anthocyanin formation. Phytochrome control of anthocyanin synthesis in Wheatland milo seedlings exhibits most of the characteristics found in the control of other plant responses (2), such as similarity of action spectra, reversibility, and low-energy requirements.

Inhibition of anthocyanin formation by far-red irradiation, which converts phytochrome to the inactive red-absorbing form, is never complete in Wheatland milo seedlings. Moreover, if 4 hours elapse before phytochrome is changed from the far-red to the red-absorbing form by an exposure to far-red radiation, 50 % of the response escapes control. The time course of loss of inhibition by far red is in part a measure of the rate of the processes leading to the formation of anthocyanin.

The presence of the active far-red-absorbing form of phytochrome is a necessary condition for producing anthocyanin in Wheatland milo. The processes leading to anthocyanin formation continue at the same rate if phytochrome is changed from the red- to the far-red-absorbing form by an exposure to red radiant energy within a few minutes after an exposure to far red. However, if 4 hours of darkness intervene between the far-red and red irradiations, only 50 % of the maximum repromotion is obtained. Presumably, a component in the chain of biosynthetic events leading to anthocyanin accumulates behind the block imposed by the absence of the far-red-absorbing form of phytochrome and is slowly diverted to some other reaction sequence when the block remains in effect for an appreciable time.

Summary

Anthocyanin synthesis in seedlings of Wheatland milo is controlled by two photoreactions. The first photoreaction requires more energy than the second and exhibits a maximum response at about 470 mμ. Alternate light and dark periods induce formation of more anthocyanin than continuous light of the same total energy indicating that the first photoreaction may be substrate limited.

After the first photoreaction runs for several hours, anthocyanin formation is further controlled by a second photoreaction. An exposure to far-red-radiant energy following the first photoreaction inhibits anthocyanin formation. The inhibitory effect of the far red is reversed by a subsequent exposure to red. Action spectra for the second photoreaction are typical of those obtained from other phytochrome controlled responses.

Literature Cited

1. Arnold, A. W. 1962. Factors affecting anthocyanin formation & morphogenesis in the hypocotyl of *Impatiens balsamina*. Ph.D. thesis, Univ. Rhode Island.
2. Borthwick, H. A. & S. B. Hendricks. 1960. Photoperiodism in plants. Science 132: 1223–1228.
3. Downs, R. J., H. A. Borthwick, & A. A. Piringer. 1958. Comparison of incandescent & fluorescent lamps for lengthening photoperiods. Proc. Am. Soc. Hort. Sci. 71: 568–578.
4. Hendricks, S. B. 1960. Rates of change of phytochrome as an essential factor determining photoperiodism in plants. Cold Spring Harbor Symp. Quan. Biol. 25: 245–248.
5. Mohr, H. 1957. Der Einfluss monochromatischer Strahlung auf das Längenwachstum des hypocotyls und auf die anthocyanbildung bei Keimlingen von *Sinapsis alba*. Planta 49: 389–405.
6. Siegelman, H. W. & S. B. Hendricks. 1957. Photocontrol of anthocyanin formation in turnip & red cabbage seedlings. Plant Physiol. 32: 393–398.
7. Siegelman, H. W. & S. B. Hendricks. 1958. Photocontrol of anthocyanin synthesis in apple skin. Plant Physiol. 33: 185–190.
8. Siegelman, H. W. & S. B. Hendricks. 1958. Photocontrol of alcohol, aldehyde, & anthocyanin production in apple skin. Plant Physiol. 33: 409–413.
9. Withrow, R. B., W. H. Klein, L. Price, & V. Elstad. 1953. Influence of visible & near infrared radiant energy on organ development & pigment synthesis in bean & corn. Plant Physiol. 28: 1–14.