

TABLE IV
ANTAGONISM OF ENDOGENOUS INHIBITOR BY STIMULATOR
(TEST SPORES WERE DEPLETED OF INHIBITOR BY
FLOATING ON WATER PRIOR TO USE)

TEST SOLUTION	% GERMINATION
Water	80
Water + stimulator	97
Inhibitor *	10
Inhibitor + stimulator	97

* Inhibitor solution prepared by floating 200 mg spores on 20 ml distilled water at 16° C overnight.

even at its optimal concentration, was nearly as effective as the endogenous stimulator.

Prolonged exposure of spores to stimulator solutions at appropriate temperatures, induced, in addition to increased germination, the formation of structures resembling appressoria, infection hyphae, infection pegs, and substomatal vesicles. Definitive evidence that these responses were brought about by the same substance that stimulates germination is not yet available. Dilution curves for germination and for vesicle formation are shown in figure 4. The curves exhibit a measure of similarity but also appear to differ appreciably.

MECHANISM OF STIMULATION: Although the detailed mechanism is unknown, it is evident that the stimulator operates by overcoming the influence of an endogenous inhibitor. Thus, the self-inhibition exhibited by concentrated masses of spores on water or agar was abolished by addition of stimulator extract. Furthermore, the stimulator was shown to overcome

the influence of endogenous inhibitor solution on inhibitor-depleted spores (table IV).

SUMMARY

Extracts which accelerate markedly the germination of rust uredospores were prepared from resting spores. The active material is relatively volatile and is soluble in water and various organic solvents. Although it has not yet been isolated or identified, evidence from inactivation tests with various chemical reagents suggests that there may be two active substances, one possessing a carbonyl group and the other a hydroxyl group.

The stimulator promotes germination by overcoming the self-inhibition due to an endogenous germination inhibitor. Under suitable environmental conditions crude preparations of the germination stimulator also induce differentiation of the germ tubes into structures resembling appressoria, infection hyphae, infection pegs, and substomatal vesicles.

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PHOTOCONTROL OF ANTHOCYANIN FORMATION IN TURNIP AND RED CABBAGE SEEDLINGS¹

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Syntheses of the prominent red and blue anthocyanin pigments of many plant tissues either require radiation or are enhanced by it. The older work was discussed by Arthur (1) and Blank (3). Arthur (2) suggested that red color formation in apple skin after irradiation was greatest in the regions of 2900 to 3100 Å and 3600 to 4500 Å. More recently, Eddy and Mapson (8), Slabecka-Szweykowska (19, 20), Eberhardt (7), and Withrow, Klein, Price, and Elstad (21) studied anthocyanin synthesis in several plant tissues as influenced by radiation. Both Slabecka-Szweykowska and Withrow and his associates noted synthesis at wavelengths longer than 6000 Å.

The effectiveness of radiation for inducing anthocyanin formation in red cabbage seedlings, *Brassica*

oleracea var. Red Acre, and turnip seedlings, *Brassica rapa* var. Purple Top White Globe, is reported here.

MATERIALS AND METHODS

Seeds of red cabbage and turnips were germinated in darkness on three sheets of Whatman No. 2 filter paper moistened with water at 25° C in Petri dishes. The moistening solution for the turnip seed was an aqueous solution containing 32 ppm of chloramphenicol, which was observed to suppress chlorophyll formation and increase anthocyanin production in a manner similar to that reported for terramycin (14).

Seedlings were irradiated with a spectrograph source or with a fluorescent or incandescent source of radiation after 3 days of germination time, when the cotyledons had spread out after shedding the seed coats. They were then returned to darkness for 16

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to 24 hours. This period was found to be adequate for anthocyanin synthesis induced by the radiation. Four or more replicates were used in the general irradiation experiments and duplicate lots for the spectrographic ones. Each type of experiment was repeated many times.

Analyses of the anthocyanin content of the seedlings were made at the conclusions of the dark periods. Counted numbers of seedlings were extracted in 0.01 molal HCl in aqueous 25% 1-propanol. The seedlings were extracted overnight at 5° C and finally in a boiling water bath for 1 minute. Analyses were based on the differences in absorptions at 6200 Å before and after addition of Na₂CO₃ to make the solutions alkaline. This procedure permitted correction for scattering of light due to a small amount of suspended material. Adjustments were made in the volume of the extracting solution and in the number of seedlings used to give an optical density of 0.1 to 1.0 where possible. The optical density values were transformed to concentrations by using the molecular extinction coefficient of 2.45×10^4 at 5350 Å, for cyanidin (18). A factor was used to convert the optical density values determined at 6200 Å in the presence of Na₂CO₃ to those at 5350 Å for cyanidin in acid solution. This procedure does not distort the relative scale and gives an approximate estimate of the absolute values, which depend on the conditions chosen for growing the seedlings.

Experimental lots of seedlings were irradiated with a bank of white fluorescent lamps giving a maximum illumination of 1,000 ft-c and an irradiance for anthocyanin synthesis at 7000 Å of about 0.6×10^{-3} watts/cm² or with a two-prism spectrograph with a carbon arc source as previously described (15). The irradiance with the two-prism spectrograph was 0.17×10^{-3} watts/cm² in the region of 6100 Å, 0.24×10^{-3} watts/cm² at 7000 Å, and 0.29×10^{-3} watts/cm² at 8000 Å. The effective slit width at 7000 Å was about 200 Å. For irradiation of seedlings in the region of 4000 to 5400 Å, the spectrograph was used as a single-prism instrument, and the focal length was reduced to give a dispersion of 5 Å/mm at 5000 Å and 2 Å/mm at 4000 Å. The energies in part of this region were doubled or trebled by reduction in width of the spectrum obtained by reflection from front-surface mirrors. Combinations of neutral filters were used to give an irradiance of 0.50×10^{-3} watts/cm² with a slit width of 200 Å at 5000 Å, constant within 10% from 4000 to 5400 Å. The absorption of the glass prisms in the spectrograph, which causes the energy to fall rapidly at wavelengths shorter than 3950 Å, limited the region studied.

RESULTS

COURSE OF ANTHOCYANIN FORMATION: The amount of anthocyanin formed is linearly dependent upon the irradiance with the fluorescent source after 15 minutes for red cabbage seedlings and 120 minutes for turnip seedlings (fig 1). In darkness there is an endogenous synthesis of 26×10^{-10} moles anthocya-

TABLE I
ANTHOCYANIN FORMATION IN TURNIP SEEDLINGS AS A
FUNCTION OF INTENSITY AT CONSTANT RADIANT
ENERGY FROM A FLUORESCENT SOURCE

LIGHT INTENSITY	EXPOSURE	ANTHOCYANIN PER SEEDLING
<i>ft-c</i>	<i>hrs</i>	10^{-10} moles
1000	1	0.72
500	2	1.6
250	4	2.3
125	8	3.5

nin/seedling for red cabbage. The small amount, 1.2×10^{-10} moles anthocyanin/seedling, indicated for the response in darkness of turnip seedlings, was not identified as anthocyanin. It should be considered as an arbitrary base.

Anthocyanin synthesis in turnip seedlings depends upon the intensity at constant energy (table I). Reciprocity fails in part at least because of the long induction period.

LOW ENERGY RESPONSE: Possible control of anthocyanin synthesis by the photomorphogenic system (4) was examined because of its response in the spectral region involved in anthocyanin synthesis. Red cabbage seedlings were exposed in some experiments for 5 minutes to radiation in the wavelength regions

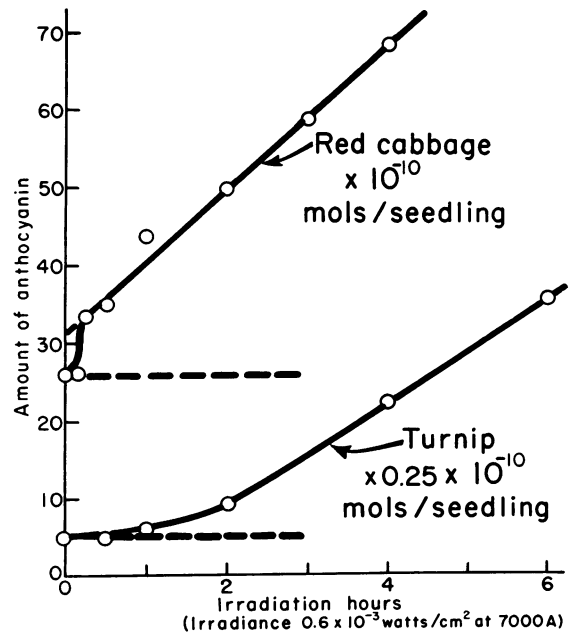


FIG. 1. Variation in anthocyanin synthesis in red cabbage and turnip seedlings with time of irradiation at a constant irradiance equivalent to photochemical effectiveness to 0.6×10^{-3} watts/cm² at 7000 Å. The seedlings were extracted for analysis 24 hrs after the beginning of irradiation. Dash lines indicate synthesis in unirradiated seedlings.

TABLE II

ANTHOCYANIN FORMATION IN TURNIP* AND RED CABBAGE SEEDLINGS IRRADIATED WITH AN ENERGY OF ABOUT 0.1 JOULE/CM² OF RED (5800 TO 6900 Å) AND/OR FAR-RED (6900 TO 8000 Å)

TYPE OF IRRADIATION	ANTHOCYANIN CONTENT PER SEEDLING	
	RED CABBAGE	TURNIP
None	25	2.97
Red	38	2.94
Far-red	26	2.97
Far-red, red	37	...
Red, far-red	29	...

* The turnip seedlings were irradiated after induction of anthocyanin synthesis by exposure for 4 hrs to a fluorescent source.

greater than 6900 Å (far-red), which transforms the photomorphogenic pigment to the red-absorbing form. In other experiments the photomorphogenic pigment was transformed in 5 minutes to the far-red-absorbing form by exposure to radiation between 5800 to 6900 Å (red). The irradiances in both cases were about 0.30×10^{-3} watts/cm². An increase in anthocyanin synthesis as compared with synthesis in darkness was found only after exposure to red radiation (table II). This effect of red radiation of low energy on anthocyanin synthesis by the red cabbage seedlings is reversed by far-red radiation.

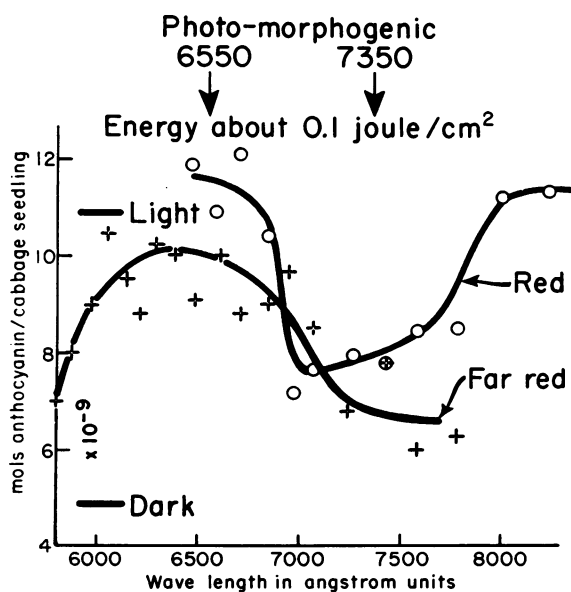


FIG. 2. Action spectra for low-energy anthocyanin synthesis by red cabbage seedlings. The curves marked red and far-red, respectively, were for lots given radiation of these types for 5 min potentiating anthocyanin synthesis as indicated by lines for light and dark immediately prior to 5-min exposures in the spectrum.

Anthocyanin synthesis in the turnip seedlings was unresponsive to the state of the photomorphogenic pigment. Turnip seedlings were irradiated for 4 hours with the fluorescent source to induce some anthocyanin formation. They were then irradiated for short times with red or far-red radiation, both of which were without effect on the synthesis (table II).

The action spectra for the low-energy anthocyanin synthesis in red cabbage seedlings were measured in the region of 5800 to 8300 Å (fig 2). Seedlings were first exposed to about 0.1 joule/cm² of either red or far-red radiation as indicated for the respective curves on figure 2. They were then placed in the spectrum for 5 minutes (radiant energy at 7000 Å of about 0.1 joule/cm²), after which they were returned to darkness for 24 hours before extraction of the anthocyanin. Anthocyanin formation in the seedlings given the prior exposure to far-red radiation is enhanced by red radiation; such enhancement accounts for the initial increase shown for red cabbage in figure 1. The synthesis in seedlings given the prior exposure to red radiation is suppressed by far-red radiation (fig 2).

HIGH ENERGY ACTION SPECTRA: The formation of anthocyanin in response to high radiant energy was measured. Dark-grown cabbage seedlings were placed along the spectrum in the region of 5500 to 9000 Å and irradiated for 4 hours. They were then irradiated with the red source for 5 minutes to convert the photomorphogenic pigment to the far-red-absorbing form (4) and then returned to darkness for 24 hours prior to extraction of anthocyanin (fig 3). The energies indicated in figure 3 were for the region of 6900 to 7100 Å. The indicated amounts of anthocyanin formed (fig 3) were those found in a single ex-

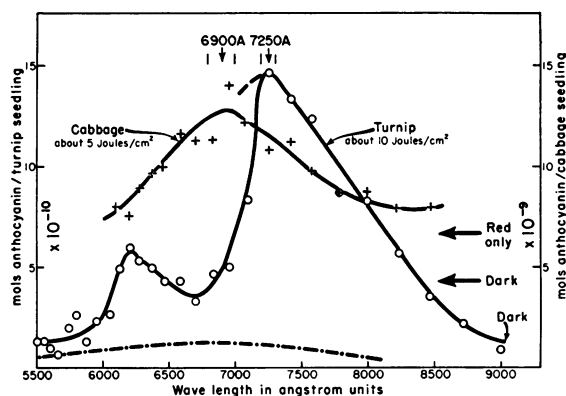


FIG. 3. Action spectra in the region 5500 to 9000 Å for anthocyanin synthesis in red cabbage and turnip seedlings. The dash line is the expected direction of energy absorption for turnip seedlings in the absence of energy transfer. The dot-dash-curve shows the relative absorption of oxidized butyryl coenzyme A dehydrogenase after Mahler (12). The heavy arrows indicate anthocyanin synthesis for darkness and a short exposure to red light. The synthesis of anthocyanin in the dark by the turnip is indicated.

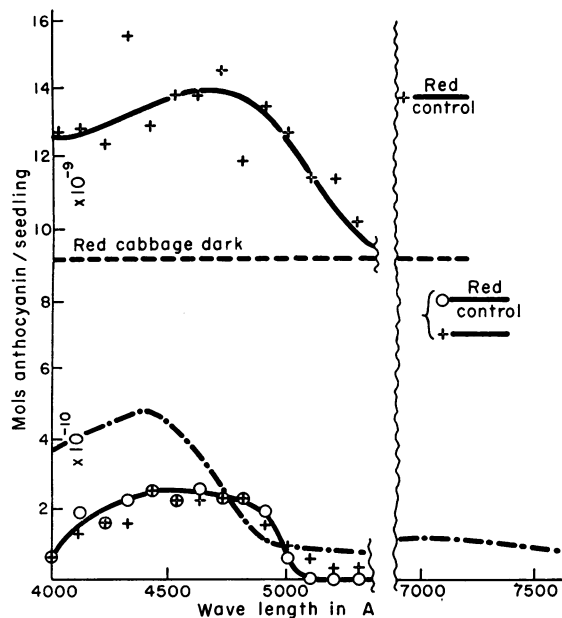


FIG. 4. Action spectra in the region of 4000 to 5330 Å for anthocyanin synthesis in red cabbage (upper) and turnip seedlings (lower) for radiant energies of about 5 and 8 joules/cm², respectively. The values in the region of 7000 Å are for control stations at the same energies with a broad slit width (400 Å). The dot-dash-curve shows the relative absorption of oxidized butyryl Co A dehydrogenase after Mahler (12).

periment. Similar results were obtained in three other experiments.

Turnip seedlings grown in darkness were first irradiated for 2 hours under the fluorescent source at 15° C to overcome the induction period shown in figure 1. They were then placed along the spectrum and exposed for 8 hours, after which they were returned to darkness for 24 hours prior to extraction of anthocyanin. Results obtained in the region of 5500 to 9000 Å are shown in figure 3. Irradiation for 2 hours at 5° C with the fluorescent source was not effective in overcoming the induction period in contrast to the effectiveness at 15° C.

The action spectra for anthocyanin synthesis by red cabbage and turnip seedlings in the region of 4000 to 5330 Å are shown in figure 4. A reference point, for synthesis in the red part of the spectrum, was run simultaneously with the same energy as for the blue part of the spectrum, but at a low resolving power with a spectral width of about 400 Å centered at 6900 Å for the red cabbage seedlings and at 7200 Å for the turnip seedlings. Duplicate samples were taken at each wavelength position and the action spectra were measured in two independent experiments for the two kinds of seedlings.

DISCUSSION

PHOTOEFFECTS ON ANTHOCYANIN SYNTHESIS: The details of the photocontrol of anthocyanin syn-

thesis are apt to be confusing because several photoreceptive pigments are probably involved. The action maxima of these pigments is in the region of 6500 to 7400 Å. The photoreversible receptor for the photoperiodic and photomorphogenic responses of plants has an effect on anthocyanin synthesis in red cabbage seedlings but not in turnip seedlings. The two forms of this photoreversible-pigment system are indicated as red- and far-red absorbing (4). This pigment system in the far-red form probably exerts its effect on anthocyanin synthesis indirectly by influencing the metabolic status of the seedlings.

The action spectra for anthocyanin synthesis influenced by the photoreversible system (fig 2) can be separated from the other photoreactions because essential saturation is attained at low energies. Saturation for synthesis is approached at an energy of about 0.1 joule/cm² at the action maximum, while about 50 times this energy is required for an equivalent effect by the photoresponsive system next considered. The low-energy action spectra, which must be measured both for seedlings with the photoreversible pigment initially in the red-absorbing form and the far-red-absorbing form, are similar to those found for other photomorphogenic and photoperiodic responses (4).

Attention is now turned to the action spectrum (fig 3) for anthocyanin synthesis by the red cabbage seedlings in the region of linear dependence of synthesis on radiant energy (fig 1). A symmetrical curve is obtained in the region of 6000 to 8000 Å as might be expected for a single photoreceptive pigment (fig 3). The action maximum is near 6900 Å, which could have been confounded with that of the red-absorbing form of the low-energy-requiring photoreversible system (6500 Å).

The nature of the induction period for anthocyanin synthesis by the turnip seedlings is now considered. A steady state is established (fig 1) after about 2 hours with an irradiance of 0.6×10^{-3} watts/cm² in the region of 7000 Å. This induction requires radiant energy and thus involves a photoreceptive pigment. An action spectrum was not obtained for the induction period. The induction photoreaction very likely gives rise to products required as substrates for reactions preceding the steady-state photoreaction. Dark reactions are also present in the sequence of reactions between the induction and the steady-state photoreactions as indicated by the marked effect of reducing the temperature during the induction period.

Anthocyanin synthesis in turnip seedlings is most simply approached by considering the photoreactions involved in the steady-state condition (the linear portion of the lower curve, fig 1). The action spectrum in the region between 5500 to 9000 Å is shown in figure 3. A pronounced maximum is observed near 7250 Å, with a very sharp decrease in action towards shorter wavelengths. The action of a single photoreceptive pigment with a maximum near 7250 Å might have followed the direction of the dashed line

shown in figure 3 or perhaps one with a maximum effectiveness at a wavelength shorter than 7250 Å. There is evidently a "bite" being taken out of the action of the single rate-limiting photoreceptor. This "bite" is a reflection of the action spectrum of the red cabbage seedlings. This indicates that energy is probably transferred in the turnip seedlings from the receptor with the indicated maximum at 7250 Å to one with a maximum near 6900 Å, which alone is operative in the red cabbage seedlings. The energy transfer is apparently most effective from 6200 to 7200 Å. Between 6000 to 6200 Å a small action maximum is observed.

The probable energy transfer between two photoreceptors in the turnip seedlings after steady-state conditions are established tells much about the nature of these photoreceptors. First, it is unusual to find the receptor with the absorption maximum at the longer wavelength (7250 Å) transferring energy to the one with the maximum at a shorter wavelength (6900 Å). Actually, all that is required in such a transfer is for the absorption from the 7250 Å acceptor to correspond in energy to a transition from a lower energy level that is partially filled for the 6900 Å receptor. The rapid decrease with energy in the distribution for the filling of the energy levels of the ground state of the 6900 Å receptor can cause the rapid rise of the action in the turnip seedlings between 6900 to 7250 Å.

The second feature of the energy transfer in the turnip seedlings is that the photoreceptor (7250 Å) has a long half-life as indicated by the fact that it holds its excitation until the energy transfer can take place. According to Pringsheim's (17) rule on fluorescence, this indicates that the photoreceptor (7250 Å) has a conjugate double-bond system involved in the effective transition as is also probably the case in the other photoreceptor (6900 Å).

A third feature of the energy transfer in the turnip seedlings is that the two photoreceptors must be contiguous, since the energy transfer is surely not second order in kinetics.

Both photoreceptors probably contain copper, which is a reasonable expectation from the region of action. This expectation is also in harmony with the considerable inhibiting effects obtained by the use of copper chelating compounds by Edmondson and Thimann (9) on anthocyanin formation in *Spirodela oligorrhiza*. It is further indicated by reduced anthocyanin synthesis in red cabbage plants grown on copper-deficient muck found by J. C. Brown, of Plant Industry Station, Beltsville, Maryland (unpublished work).

In considering possible types of photoreceptors having the required characteristics, attention was directed to the recently described acyl dehydrogenases (13). These enzymes are flavoproteins and in the case of butyryl coenzyme A dehydrogenase, the green enzyme from liver, Mahler (12) has given evidence for associated copper. The butyryl coenzyme A dehydrogenase has an absorption maximum in the region

of 6800 to 6850 Å (12) for the oxidized form, which is in agreement with that for the red cabbage photoreceptor (6900 Å). Recently, Steyn-Parve and Beinert (16) have raised questions about the necessity of copper for the functioning of the enzyme. They suggest that absorption in the red region of the spectrum is present for the copper-free enzyme.

Irrespective of the exact nature of the acyl dehydrogenases other than as flavoproteins, their consideration suggests that the photoreceptive pigments for anthocyanin might be effective in the blue portion of the spectrum, where the flavin moiety absorbs. Attention was accordingly given to the nature of the action spectra in the region of 4000 to 5000 Å, which had not earlier been measured because of the experimental difficulties in obtaining the requisite irradiances. To attain this end the spectrograph was modified to a single-prism instrument of shorter focal length and otherwise changed as previously mentioned. The action spectra were then measured (fig 4). The results are in agreement with action arising from a flavin absorption and are evidence for the flavoprotein nature of the photoreceptors.

The action (amount of anthocyanin formed) per incident erg or per incident quantum is greater in the region of the red maxima than between 4000 to 5000 Å, particularly by the turnip seedlings, while the absorption coefficient of butyryl coenzyme A dehydrogenase enzyme is about a third as great. This indicates that the quantum efficiency is greater for absorption in the red than in the blue part of the spectrum. In the red part of the spectrum the action could be associated with the copper atom. Mahler (12) showed by poisoning with cyanide that the copper atoms are probably required for single-electron transport.

The photoreactions are possibly concerned with electron or hydrogen transfer in the formation of the aromatic A- or B-ring of the anthocyanin molecules or on precursors of these rings such as acyl radicals. (See Geissman and Hinreiner (11) for the most recent discussion of anthocyanin biosynthesis.) Since the probable photoreceptor is the oxidized form of the flavoprotein, the photoactions are effective in its return to the reduced form.

The effects of light on anthocyanin synthesis by cress seedlings noted by Eddy and Mapson (8) were probably due to the influence of the reversible pigment system that produces photomorphogenic and photoperiodic responses as indicated by the effectiveness of the low radiant energies used. Effectiveness of radiation in the region of 7250 Å for inducing anthocyanin formation in bean seedlings was noted by Withrow, Klein, Price, and Elstad (21). They commented that this synthesis paralleled the photomorphogenic response although they did not consider their experiments complete in this respect. The observed enhancement of anthocyanin synthesis at 7250 Å in the bean seedlings was more likely due to a photoreceptor similar to the one found in turnip seedlings with a maximum at 7250 Å. The irradiance

(1500 microwatts/cm²—7250 Å treatment) in the experiments on anthocyanin formation in the bean seedlings was sufficient for promoting synthesis by such a photoreceptor. Also, anthocyanin production was energy-dependent for high energies.

QUENCHING OF PHOTOSYNTHESIS: The probable energy transfer between photoreceptors involved in anthocyanin synthesis suggests an explanation for the rapid decrease in photosynthesis in *Chlorella* in the region of 6750 to 7000 Å as observed by Emerson and Lewis (10) and verified by others. The speculation here is that a flavoprotein with an absorption maximum near 6900 Å in the plastid quenches energy transfer in the chlorophyll-carotinoid coupled system of the plastid. This flavoprotein could be one associated with electron and hydrogen transfer resulting in oxygen evolution and transferring of hydrogen from water (6). The implication should not be made that the system for anthocyanin synthesis is associated with the plastid.

SUMMARY

Action spectra were measured for anthocyanin synthesis in 3-day-old seedlings of Red Acre red cabbage, *Brassica oleracea*, and Purple Top White Globe turnip, *Brassica rapa*. A single photoreceptive pigment is present in the red cabbage seedlings leading to action maxima at 6900 Å and near 4500 Å. The receptor is possibly a flavoprotein similar to the butyryl coenzyme A dehydrogenase enzyme of liver mitochondria, and it possibly contains copper. Action maxima for anthocyanin synthesis in turnip seedlings were found near 7250 Å, 6200 Å, and 4500 Å. The action spectrum in the region of 6200 to 7200 Å suggests that energy of activation is transferred between two photoreceptors. A speculation is advanced that a similar quenching of photosynthesis takes place in the plastid in the region of 6750 to 7000 Å, accounting for the decreased action in this region as observed by others.

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