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THE EFFECTS OF LIGHT AND KINETIN ON AMARANTHIN SYNTHESIS IN RELATION TO PHYTOCHROME*

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Abstract—The effects of anaerobic conditions and of various inhibitors on amaranthin synthesis, stimulated by short or continuous irradiation with white light in the eventual presence of kinetin were investigated. The results suggest that both light and kinetin act on the pigment synthesis at two distinct levels, activation of genes and control of the availability of energy-rich compounds. Phytochrome seems to be involved only in the responses depending on short irradiations, while under continuous illumination the photosynthetic system is the photoreceptor for the pigment synthesis.

INTRODUCTION

PREVIOUS investigations on amaranthin synthesis induced by short irradiations in kinetin-treated seedlings of *Amaranthus tricolor* have shown that the effect of light is controlled by phytochrome with the involvement of the synthesis of enzymes.[1]

This paper deals mainly with the effects of various inhibitors on pigment synthesis stimulated by short or continuous illumination, in the presence or absence of kinetin, and their relationship with the action of phytochrome.

RESULTS

Amaranthin synthesis in *Amaranthus tricolor* seedlings was stimulated by one of these treatments; (a) short irradiations with white light ranging from 15 min to 6 hr, (b) kinetin in darkness, (c) kinetin and short irradiations, (d) continuous illumination, and (e) kinetin under continuous illumination. Salicylaldoxime (SAL), 3-(3,4-dichlorophenyl)-1,1-dimethyl-urea (DCMU), 2,4-dinitrophenol (DNP) and puromycin were used as inhibitors. The first compound, a copper chelating agent, at a concentration 2×10^{-2} M completely inhibits the electron transport chain while at a lower concentration (10^{-3} M) it affects only cyclic photophosphorylation and oxidative phosphorylation.[2,3] DCMU at 10^{-5} M inhibits non-cyclic photophosphorylation.[4] DNP is a classic uncoupler of oxidative phosphorylation. Puromycin is a known inhibitor of protein synthesis.

The Effects of Anaerobiosis, SAL, DCMU, DNP and Puromycin on the Amaranthin Synthesis Induced by Short Irradiations

The effects of anaerobiosis and various inhibitors on amaranthin synthesis induced by short irradiations are presented in Table 1. Seedlings irradiated for 6 hr under nitrogen and

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[2] A. TREBST, *Z. Naturforsch.* **18b**, 817 (1963).
[3] W. URBACH and H. GIMMLER, *Progress in Photosynthesis Research* **3**, 1274 (1969).
[4] W. URBACH and W. SIMONIS, *Biochem. Biophys. Res. Commun.* **17**, 39 (1964).

TABLE 1. EFFECT OF ANAEROBIOSIS, SAL, DNP, DCMU AND PUROMYCIN ON LIGHT-INDUCED AMARANTHIN SYNTHESIS

Treatment	Amaranthin/seedling (10^{-2} nmoles)*
Dark control (water)	6·8 ± 0·1
Light control (6 hr light then 18 hr darkness)	54·4 ± 1·2
6 hr light (N_2) then 18 hr darkness (air)	7·3 ± 0·1
6 hr light (air) then 18 hr darkness (N_2)	33·7 ± 0·8
6 hr light (air) then 10 hr darkness (N_2) and then 8 hr darkness (air)	33·7 ± 0·8
6 hr light then 18 hr dark (air)	
10^{-3} M SAL added at the 9th hr after darkening	54·4 ± 1·2
Puromycin (100 μg/ml) added at the 9th hr after darkening	54·4 ± 1·2
10^{-3} M SAL added at the moment of darkening	41·3 ± 1·0
Puromycin (100 μg/ml) added on darkening	44·1 ± 1·0
2×10^{-2} M SAL added on illumination	0
10^{-3} M SAL added on illumination	21·2 ± 0·6
10^{-4} M DNP added on illumination	19·0 ± 0·6
10^{-5} M DCMU added on illumination	54·0 ± 1·1

* 90% confidence level.

then kept in darkness under air do not produce more pigment than dark controls. When seedlings irradiated in air are kept in darkness under nitrogen the amount of amaranthin is approximately 40% less than that produced by control seedlings under aerobic conditions and is not modified by restoring the aerobic conditions after 10 hr or later. The application of SAL or puromycin at the moment of darkening causes a 20% decrease of the amaranthin accumulation, and this is indicative of dark processes related to oxidative phosphorylation and protein synthesis. When applied at the onset of illumination SAL has a stronger inhibitory effect, similar to that of DNP.

In accordance with its specific inhibitory action on photophosphorylation, DCMU has no effect on pigment synthesis stimulated by a light treatment too short to induce photosynthesis.

The Effect of Anaerobiosis, SAL, DNP and DCMU on the Amaranthin Synthesis Induced by Kinetin and Short Irradiations

In kinetin-treated seedlings, both irradiated (15 min) or not, anaerobiosis completely blocks the amaranthin synthesis which, on reverting to aerobic conditions, takes place at a normal rate (Fig. 1). Thus, it appears that oxygen deprivation does not affect irreversibly the ability of the seedlings to synthesize amaranthin. If anaerobic conditions are established 9 hr after the application of kinetin, i.e. when the activating action of the hormone is achieved,[1] the enhancing effect of the irradiation is nullified. SAL at 2×10^{-2} M totally blocks pigment synthesis, which is strongly depressed by the same inhibitor at a concentration 10^{-3} M and by 10^{-4} M DNP. DCMU has no effect (Table 2). These results indicate that the stimulating effect of kinetin on amaranthin synthesis must be ascribed to an enhancement of oxidative phosphorylation.

The Effect of Anaerobiosis, SAL, DNP and DCMU on the Amaranthin Synthesis Stimulated by Continuous Illumination

From the data reported in Table 3 it appears that amaranthin synthesis stimulated by continuous illumination with white light is completely suppressed under nitrogen (in which

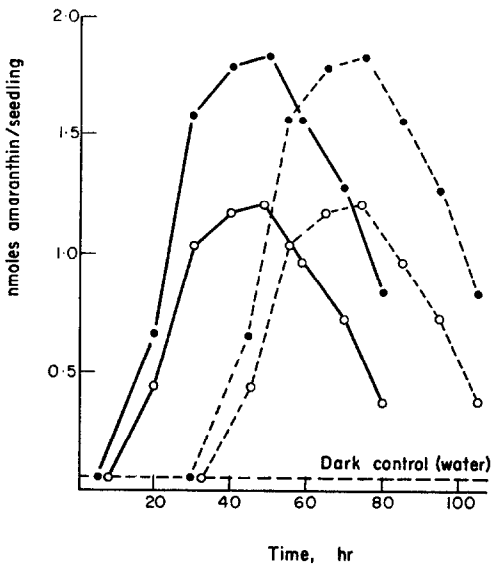

FIG. 1

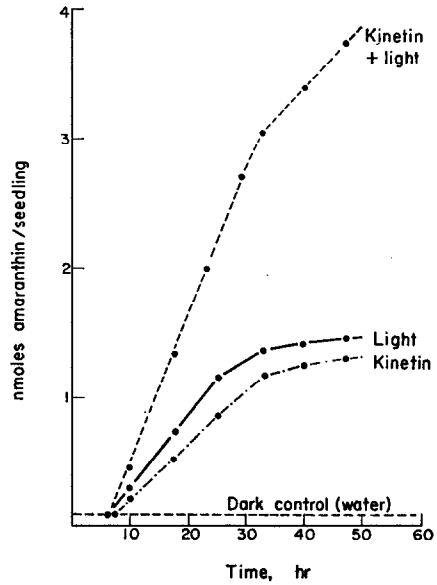

FIG. 2

FIG. 1. TIME COURSE OF AMARANTHIN SYNTHESIS IN KINETIN-TREATED SEEDLINGS.
——○——, in darkness; ——●——, 15 min white light at the onset of the experiment; - - -○- - -, in darkness under nitrogen until the 24th hr; - - -●- - -, 15 min white light at the onset of the experiment and under nitrogen until the 24th hr.

FIG. 2. TIME COURSE OF AMARANTHIN SYNTHESIS.
—·—·—·—·, in the presence of kinetin; ————, under continuous illumination with white light; ....., in the presence of kinetin and under continuous illumination with white light.

TABLE 2. EFFECT OF ANAEROBIOSIS, SAL, DNP AND DCMU ON AMARANTHIN SYNTHESIS INDUCED BY KINETIN OR BY KINETIN AND SHORT IRRADIATION

Treatment	Amaranthin/seedling (10^{-2} nmoles)*
Dark control (water)	6·8 ± 0·1
24 hr kinetin	
dark control	68·7 ± 0·7
under N_2	0
plus 2×10^{-2} M SAL	0
plus 10^{-3} M SAL	19·9 ± 0·4
plus 10^{-4} M DNP	20·6 ± 0·5
plus 10^{-5} M DCMU	68·0 ± 0·7
10^{-3} M SAL added at the 9th hr	34·3 ± 1·5
under N_2 from the 9th hr	29·7 ± 1·5
plus 15 min white light on adding kinetin	104·6 ± 4·2
15 min light under N_2	0
15 min light 2×10^{-2} M SAL added with kinetin	0
15 min light 10^{-3} M SAL added with kinetin	40·8 ± 1·6
15 min light 10^{-4} M DNP added with kinetin	31·4 ± 1·3
15 min light 10-5 M DCMU added with kinetin	103·5 ± 4·0
9 hr kinetin	
then 15 hr water	49·7 ± 2·0
then 15 hr 10^{-3} M SAL	46·7 ± 1·9
plus 15 min white light, then 15 hr water	68·6 ± 2·2
plus 15 min white light, then 15 hr water under N_2	48·6 ± 1·9
plus 15 min white light, then 15 hr 10^{-3} M SAL	52·7 ± 2·1

* 90% confidence level.

case chlorophyll does not form). The pigment synthesis is inhibited 50% if anaerobic conditions are applied 7 hr after commencing illumination, and approximately to the same extent by the addition of 10^{-3} M SAL or 10^{-4} M DNP at the onset of the experiment.

TABLE 3. EFFECT OF ANAEROBIOSIS, SAL, DNP AND DCMU ON AMARANTHIN SYNTHESIS UNDER CONTINUOUS ILLUMINATION

Treatment	Amaranthin/seedling (10^{-2} nmoles)*
Dark control (water)	6·8 ± 0·1
Light control (24 hr white light)	93·5 ± 4·6
Light (24 hr)	
under N_2	0
under N_2 at the 7th hr	46·7 ± 1·8
10^{-5} M DCMU applied on illumination	73·0 ± 3·0
10^{-5} M DCMU applied at the 7th hr	72·9 ± 2·9
10^{-5} M DCMU applied and under N_2 at the 7th hr	26·2 ± 1·1
10^{-4} M DNP applied on illumination	44·8 ± 1·8
2×10^{-2} M SAL applied on illumination	0
10^{-3} M SAL applied on illumination	48·6 ± 1·9
SAL as above; under N_2 at the 7th hr	24·3 ± 1·0
SAL as above; 10^{-5} M DCMU applied at the 7th hr	20·6 ± 1·2
SAL as above; 10^{-5} M DCMU applied and under N_2 at the 7th hr	0

* 90% confidence level.

DCMU at 10^{-5} M inhibits 22% either when applied at the onset of the irradiation or 6 hr later, i.e. at the beginning of the photosynthetic activity. This effect must be considered as specific on photophosphorylation, since DCMU lacks any action on amaranthin formation when photosynthesis is not operating (Tables 1 and 2). The inhibition is enhanced when SAL and DCMU are given simultaneously or in combination with anaerobiosis. In the presence of both inhibitors and under nitrogen the pigment synthesis is completely blocked.

These results show that amaranthin synthesis induced by continuous illumination is dependent not only on oxidative phosphorylation but also on cyclic and non-cyclic photophosphorylation.

The Effect of Anaerobiosis, SAL, DNP and DCMU on the Amaranthin Synthesis Induced by Kinetin and Continuous Illumination

The rate of formation of amaranthin under continuous illumination and in the presenc of kinetin was found to be much greater than that observed when light and kinetin were given individually (Fig. 2). Table 4 provides data for the effects of oxygen deprivation and inhibitors of phosphorylation on the synthesis of amaranthin in kinetin-treated seedlings under continuous illumination with white light. It is apparent that pigment formation is strongly depressed when anaerobic conditions are established 6 hr after the beginning of the irradiation, and this is probably due to the block of the hormone effect, since the amount of pigment under continuous illumination and in a nitrogen atmosphere is similar either in the presence or absence of kinetin. The inhibitory effects of SAL, DNP and DCMU are quite similar to those observed in seedlings continuously irradiated in the absence of the hormone.

From these results it can be deduced that amaranthin synthesis stimulated by kinetin

TABLE 4. EFFECT OF ANAEROBIOSIS, SAL, DNP AND DCMU ON AMARANTHIN SYNTHESIS INDUCED BY KINETIN UNDER CONTINUOUS ILLUMINATION

Treatment	Amaranthin/seedling (10^{-2} nmoles)*
Dark control (water)	6·8 ± 0·1
Dark control (24 hr kinetin)	68·7 ± 2·7
Light control (24 hr kinetin under continuous illumination)	173·6 ± 6·9
Kinetin and light treatment as above	
under N_2 at the 7th hr	41·7 ± 2·5
10^{-5} M DCMU applied on illumination	143·1 ± 5·8
10^{-5} M DCMU applied at the 7th hr	142·3 ± 5·7
10^{-5} M DCMU applied and under N_2 at the 7th hr	24·3 ± 1·0
2×10^{-2} M SAL applied on illumination	0
10^{-3} M SAL applied on illumination	95·5 ± 3·8
10^{-4} M DNP applied on illumination	86·8 ± 3·4
Kinetin, light and SAL treatment as above	
under N_2 at the 7th hr	33·2 ± 0·7
10^{-5} M DCMU applied at the 7th hr	59·4 ± 2·4
10^{-5} M DCMU applied and under N_2 at the 7th hr	0

*90% confidence level.

under continuous illumination is dependent on photophosphorylation and oxidative phosphorylation, the latter being greatly enhanced by the hormone.

DISCUSSION

Although amaranthin biosynthesis is not yet clear, it is known that tyrosine acts as a precursor of the pigment.[5] However, administration of the amino acid to *A. tricolor* seedlings is effective in increasing the pigment accumulation only when light or kinetin are concurrently given. In darkness and in the absence of the hormone the pigment increase is negligible.[6] Since recent results using actinomycin D have shown that light and kinetin affect amaranthin synthesis through the stimulation of DNA-directed RNA synthesis,[7,8] it can be inferred that in etiolated seedlings in the absence of the hormone the lack of pigment formation is due to a gene repression and not to a limited availability of substrate. However, little is known for certain regarding the mechanism by which light and kinetin act in de-repressing genes. It is apparent from the results described in the preceding paper that the action of light on DNA-directed RNA synthesis is mediated by an unknown photoreceptor while the action of photoactivated phytochrome seems to be confined to the control of translation. This result conflicts with Mohr's hypothesis on the mode of action of phytochrome[9] according to which this photoreceptor, through an increase in the concentration of ascorbic acid, causes the dissociation of certain histones from DNA and this would result in a de-repression of the corresponding genes.

With regard to the action mechanism of kinetin, Matthysse[10] has shown that in pea buds the hormone forms a complex with an active protein which has been supposed to facilitate

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[7] M. PIATTELLI, M. GIUDICI DE NICOLA and V. CASTROGIOVANNI, *Phytochem.* **9**, 785 (1970).
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[9] H. MOHR, *Photochem. Photobiol.* **5**, 469 (1966).
[10] A. G. MATTHYSSE and M. ABRAMS, *Biochim. Biophys. Acta* **199**, 511 (1970).

the polymerase binding on DNA and the initiation of RNA synthesis, but no evidence is available at present to decide whether this mechanism holds true in the case of *A. tricolor* also.

Anaerobic conditions, which must be assumed to inhibit the action of P_{730} without interfering with the purely photochemical activation of phytochrome,[11] SAL and DNP inhibit amaranthin synthesis stimulated by short irradiations (Table 1). This demonstrates the implication of oxidative phosphorylation in the regulation of pigment production, and since it has been shown in the previous paper that phytochrome acts on amaranthin formation through the stimulation of protein synthesis, it appears likely that this photoreceptor controls translation through its effects on the availability of energy-rich compounds. The fact that oxygen deprivation, SAL, DNP or puromycin at the moment of darkening reduce the pigment accumulation is evidence for dark processes depending on oxidative phosphorylation and protein synthesis. These processes are probably under the control of phytochrome, since the inhibitors have no effect if they are applied 9 hr after the moment of darkening, when photoactivated phytochrome is presumably decayed completely. Thus, the effect of phytochrome would be non-specific and this could explain the variety of responses controlled by this photoreceptor (germination, RNA and protein synthesis, flavonoid synthesis, hypocotyl elongation, etc.). In fact, in the literature results can be found on photocontrolled processes other than synthesis of plant pigments which can be explained according to this hypothesis of the mode of action of phytochrome. For instance, in the case of lettuce seed germination[11] the observed inhibiting effect of anaerobiosis applied after the activation of phytochrome can find a ready explanation assuming that P_{730} controls the oxidative phosphorylation.

Amaranthin synthesis can take place also in darkness in the presence of kinetin, whose action has been ascribed to gene activation on the basis of the inhibitory effect of actinomycin D. Furthermore, from the fact that the antibiotic had no more effect when it was administered 9 hr after the moment of kinetin application, it has been deduced that during this period the gene activation stimulated by the hormone is completed.[8] From the data reported in the present paper (Table 2) it appears that the effect of kinetin is counteracted by anaerobiosis, SAL or DNP and therefore involves an action on oxidative phosphorylation. However, this action is probably not dependent on its effect on genes since seedlings removed from kinetin at the end of gene activation synthesize less amaranthin when compared with controls kept continuously in the hormone and are insensitive to anaerobic conditions or SAL, while both these two factors exert a remarkable inhibition if at the time of their application kinetin has not been removed.

When in the presence of kinetin phytochrome is photoactivated, an increase in pigment synthesis is observed (Table 2) which can be ascribed to the stimulation of the enzyme synthesis caused by a further increase of the level of oxidative phosphorylation. In fact, if kinetin is removed after 9 hr and then seedlings are irradiated, an enhancement of the pigment synthesis is observed which is totally blocked by anaerobiosis or SAL.

The inhibiting effects of oxygen deprivation, SAL, DNP and DCMU, both alone and in combination, on amaranthin synthesis stimulated by continuous illumination (Table 3) clearly show that in these conditions not only oxidative phosphorylation is involved, but also the cyclic and non-cyclic photophosphorylations. Therefore, the photoreceptor for betacyanin synthesis under continuous irradiation with white light is the photosynthetic system. This is in agreement with some findings reported for HER anthocyanin synthesis.[12]

[11] H. IKUMA and K. V. THIMANN, *Plant Physiol.* **39**, 756 (1964).

[12] R. J. DOWNS, H. W. SIEGELMAN, W. L. BUTLER and S. B. HENDRICKS, *Nature, Lond.* **205**, 909 (1965); M. J. SCHNEIDER and W. R. STIMSON, *Plant Physiol.* **46** (suppl.), 25 (1970).

Under continuous illumination and in the presence of kinetin, *A. tricolor* seedlings synthesize a very large amount of amaranthin, and from the effects of inhibitors it can be deduced that this is due to the stimulation of oxidative phosphorylation operated by the hormone and the simultaneous induction by light of photophosphorylation.

All the above results point to the conclusion that both light and kinetin act on amaranthin synthesis at two different levels, activation of genes and control of the availability of energy-rich compounds. The effect of light on the genic system appears to be mediated by other photoreceptor(s) than phytochrome, while that on the availability of the energy-rich compounds is mediated by phytochrome during the early hours of irradiation and successively by the photosynthetic system.

EXPERIMENTAL

Plant material, experimental treatments and amaranthin estimation. Seedlings of *Amaranthus tricolor* were grown in the conditions previously reported[1] and used when 2-days-old. The sources of white and far-red light were as described earlier.[13] Administration of kinetin was carried out as described previously.[8] When inhibitors were used they were applied to the paper on which the seedlings were growing. In the experiments with DCMU, seedlings from which the radicle had been removed were used. The quantitative determination of amaranthin was carried out as previously reported.[13]

[13] M. Piattelli, M. Giudici de Nicola and V. Castrogiovanni, *Phytochem.* **8**, 731 (1969).

Key Word Index—Amaranthus tricolor; Amaranthaceae; betalains; amaranthin synthesis; light; kinetin; phytochrome.