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Control of Chlorophyll Production in Rapidly Greening Bean Leaves[1]

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Summary. The possible involvement of nucleic acid and protein synthesis in light-regulated chlorophyll formation by rapidly greening leaves has been studied.

Removing leaves from illumination during the phase of rapid greening results in a reduction in the rate of pigment synthesis; cessation occurs within 2 to 4 hours. Etiolated leaves which exhibit a lag in pigment synthesis when first placed in the light do not show another lag after a 4 hour interruption of illumination during the phase of rapid greening.

Actinomycin D, chloramphenicol, and puromycin inhibit chlorophyll synthesis when applied before or during the phase of rapid greening. Application of δ-aminolevulinic acid partially relieves the inhibition by chloramphenicol.

It is suggested that light regulates chlorophyll synthesis by controlling the availability of δ-aminolevulinic acid, possibly by mediating the formation of an enzyme of δ-aminolevulinate synthesis. This process may result from gene activation or derepression; the involvement of RNA synthesis of some sort is suggested by the inhibitory effect of actinomycin D on chlorophyll production by rapidly greening leaves.

The greening of etiolated leaves under constant illumination can be conveniently considered to occur in 3 stages: I) the photoconversion of accumulated protochlorophyllide a to chlorophyllide a (18, 20, 36, 41). II) A lag phase during which little or no additional pigment accumulates (36). III) A period of rapid chlorophyll synthesis which continues until pigment content approaches that of the normal green leaf (36).

Stage I, according to Smith and Benitez (34), is a nonenzymatic photochemical reaction involving the reduction of protochlorophyllide a to chlorophyllide a, possibly with concomitant oxidation of the holochrome protein. The reaction is essentially completed during a few minutes of illumination at medium light intensities; at high intensities, the reduction is accomplished in seconds or fractions of seconds. The action spectrum corresponds closely to the absorption spectrum of protochlorophyllide holochrome (19). The chlorophyllide a so formed is converted enzymatically to chlorophyll a, without the need for light, within one-half hour after this transformation (41).

Stage II has been studied extensively by Price and Klein (31), Virgin (37, 38, 39), and others. Its duration, when it occurs, depends upon many factors and varies with the species and age of the plant. It is also influenced by temperature and certain chemicals. The red-far red photomorphogenic system (13) has been shown to exert a control over the lag phase in beans (31) and in wheat (39). Sisler and Klein (32) found that preincubation of leaves with ALA (δ-aminolevulinic acid) eliminated Stage II when the leaves were placed in low intensity red light.

Of the 3 stages, Stage III is generally of longest duration. Depending upon environmental conditions and plant material, it may continue for as long as 24 to 48 hours (36). The action spectrum for chlorophyll production during this stage has not been determined, but it is very likely that 1 component corresponds to the absorption spectrum of protochlorophyllide a (9, 38).

Structural changes in the chloroplast have been shown to accompany, and be correlated with, these stages of pigmentation (17, 40).

One of the events which occurs in etiolated leaves upon illumination is an acceleration of protein synthesis (8, 29). Studies with chloramphenicol, an inhibitor of protein synthesis (7), have shown that when the antibiotic is administered prior to illumination, the greening of etiolated leaves is partially inhibited (26) and protein synthesis is suppressed in chloroplasts in vivo (27) and in vitro (35). It has been proposed that light

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induces the synthesis of enzymes necessary for chlorophyll production. Various photosynthetic enzymes have already been demonstrated to be photoinduced (25, 33).

Nucleic acid synthesis as a prerequisite for chlorophyll synthesis is indicated from studies with actinomycin D on higher plants (3, 4) and this antibiotic as well as 5-fluorouracil on *Euglena* (24, 30, 33). Further data are included here.

The present studies were undertaken to determine: if and how Stage III differs from earlier stages with regard to the nature of the photocontrolling mechanisms; for example, does a lag phase similar to Stage II follow an interruption of illumination during Stage III? Is continuous nucleic acid and protein synthesis necessary for pigment formation during Stage III? A preliminary report of some of this work has appeared (10).

Materials and Methods

Plant Materials. Plants of *Phaseolus vulgaris* var. Red Kidney were employed throughout these studies. Seeds were allowed to imbibe distilled water at 20° from 4 to 6 hours and were then sown in vermiculite held in polyethylene trays. The plants were grown in darkness at about 25° for 7 to 9 days.

All manipulations of the plants prior to illumination were performed under a dim green safelight. Primary leaves were excised, and leaf-halves placed on 2 layers of filter paper in Petri dishes containing 0.2 M sucrose with or without inhibitors.

For illumination, leaves were placed in a control room maintained at 20° under light intensities of 400 to 700 ft-c as measured by a Weston illumination meter. General Electric cool white Power Groove fluorescent tubes provided the illumination. When leaves were supplied 10 mM ALA hydrochloride (Calbiochem), illumination, when desired, was provided by a slide projector equipped with a 500 watt tungsten lamp with a 660 mμ interference filter interposed between the source and the leaves. Energy flux was kept at 3 μw/cm² and monitored with an ISCO spectroradiometer. Leaves supplied ALA form protochlorophyllide and become photosensitive; upon strong illumination with white light, the protochlorophyllide and chlorophyll are destroyed (16, 32). Consequently, in the present experiments, illumination was with light of low intensity and of wavelength beyond that absorbed by the bulk of the protochlorophyllide formed from exogenous ALA.

Inhibitors. Chloramphenicol (Parke, Davis, and Co.), puromycin dihydrochloride (Nutritional Biochemical Co.) neutralized with KOH prior to use, actinomycin D (kindly provided by Merck), 5-fluorouracil (Hoffman-LaRoche), 5-bromouracil and 2-thiouracil (Calbiochem), and DL-ethionine and *p*-fluorophenylalanine (Sigma Chemical Co.) were diluted to the indicated concentrations in 0.2 M sucrose.

Pigment Determination. To terminate each experiment, the leaves were cooled, protected from light, weighed, and frozen prior to extraction with 80% acetone. Leaves which had been maintained in darkness during an experiment were illuminated for 3 minutes at 0° at the conclusion of the dark period to photoconvert newly accumulated protochlorophyllide to chlorophyllide prior to freezing of the tissue. The total chlorophyll content was determined spectrophotometrically by the method of Arnon (2). Protochlorophyllide was determined using the extinction coefficients of Anderson and Boardman (1). Values reported here are averages of 3 replicates per experiment, reflecting at least 2 separate experiments. No detectable increase in fresh weight occurred in the material during the course of these studies and the results are valid when expressed as pigment content per leaf.

Results

The Participation of Light in Stage III. The effect of light on chlorophyll synthesis during Stage III was investigated in leaves of 7-day-old dark-grown plants which had been illuminated 4 to 6 hours.

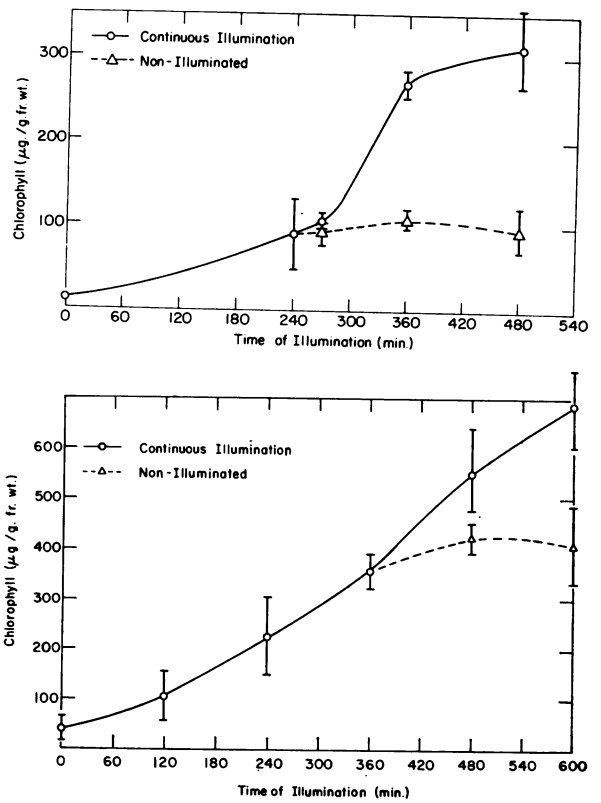

FIG. 1. The effect of light on chlorophyll synthesis during Stage III. a) (top) Light removed after 4 hours. b) (bottom) Light removed after 6 hours.

The lag phase in such leaves is completed within 3 hours after the initiation of illumination. After 4 hours in the light, the leaves were visibly green. Under continuous illumination Stage III begins at about the third hour of illumination in this material (fig 1a, b). Leaves returned to darkness after 4 or 6 hours of illumination continue pigment synthesis [producing protochlorophyllide (38)], but at a greatly diminished rate as compared to continuously irradiated controls (fig 1a, b). After approximately 2 to 4 hours in the dark, protochlorophyllide synthesis ceases.

When rapidly greening leaves are allowed to remain in the dark until protochlorophyllide synthesis stops, i.e. about 4 hours, and are then re-illuminated, pigment accumulation begins immediately, i.e. the leaves resume protochlorophyllide production without an intervening lag (fig 2).

It is clear from these studies that chlorophyll synthesis during Stage III can be controlled by light.

Inhibition of Greening in Stage III by Various Agents. A) *Inhibition of Protein Synthesis.* The administration of chloramphenicol (CAM) to leaves inhibits further chlorophyll production in illuminated leaves at various stages in the greening process (fig 3). Exposure of isolated leaf halves to 5 mM CAM before Stage I inhibited greening during a subsequent 9-hour period of illumination. If CAM was applied at the beginning of Stage III, chlorophyll accumulation during the next 2 to 4 hours of illumination was reduced. Even when leaves were exposed to CAM after 7 hours of irradiation, well into Stage III, significant inhibition was detected during the following 2-hour period.

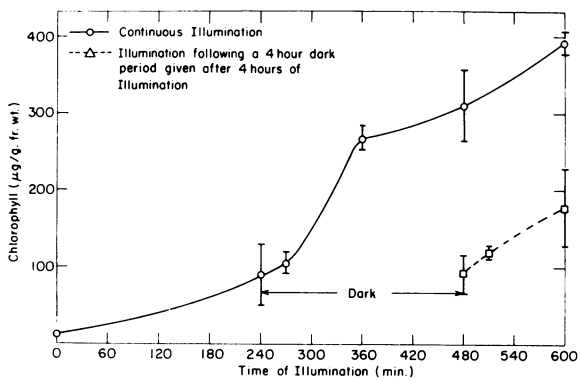

FIG. 2. The course of chlorophyll formation upon resumption of illumination during Stage III.

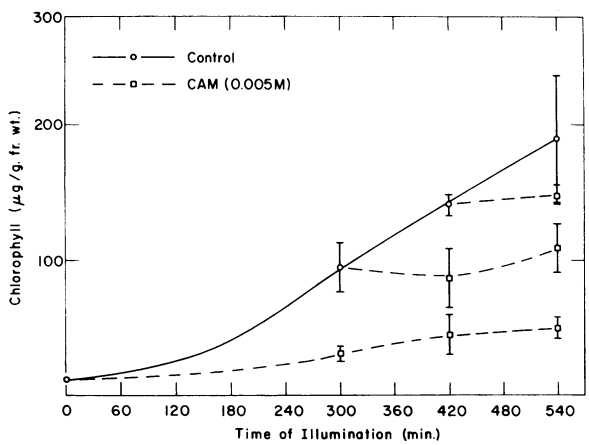

FIG. 3. The effect of chloramphenicol on chlorophyll synthesis during Stages II and III.

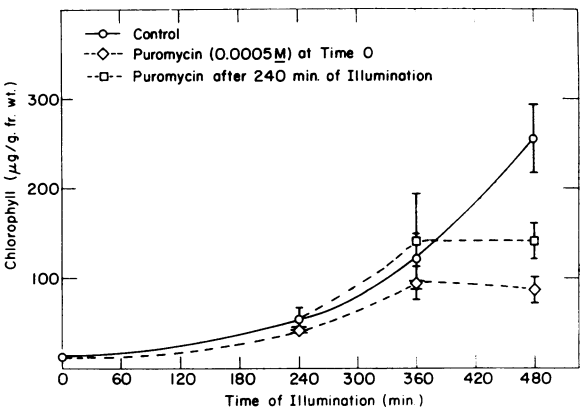

FIG. 4. The effect of puromycin on chlorophyll synthesis during Stages II and III.

Puromycin, which potently inhibits protein synthesis in a variety of organisms (35, 42), produced the same type of effect (fig 4) but up to 3 hours elapsed between the initial exposure of leaves to puromycin and detectable inhibition of greening. This lag may result from the use of lower concentrations of puromycin (0.5 mM) than of CAM (5 mM) and/or differences in the mode of action of the 2 compounds. However, since inhibition with puromycin was manifested initially as green patches on the chlorotic leaf while CAM exerted a uniform effect, it seems likely that puromycin is taken up with more difficulty and is distributed less evenly than CAM. DL-Ethionine (1 mM) and p-fluorophenylalanine (1 mM) inhibited chlorophyll syn-

Table I. *The Effect of Some Inhibitors of Protein and RNA Synthesis on Chlorophyll Synthesis by Etiolated Leaves*

Leaves were preincubated 12 hours in dark with each inhibitor at a concentration of 1 mM; chlorophyll content was determined after 8 hours of illumination.

	Chlorophyll	Inhibition
	μg/g fr wt	%
Control	653 ± 38	. . .
5-Fluorouracil	680 ± 55	0
5-Bromouracil	572 ± 45	10
2-Thiouracil	380 ± 26	41
p-Fluorophenylalanine	353 ± 53	46
DL-Ethionine	135 ± 14	78

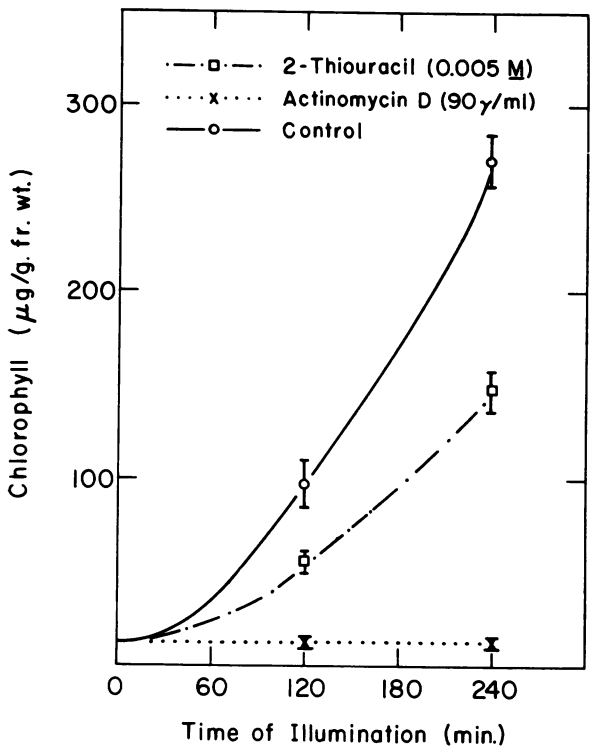

FIG. 5. The effect of 2-thiouracil and Actinomycin D on chlorophyll synthesis. Etiolated leaves preincubated for 16 hours in darkness with antimetabolite.

thesis by 78 and 46 % respectively when administered 12 hours before an 8 hour illumination period (table I).

B) *Inhibition of RNA Synthesis.* The inhibitory effects of 2-thiouracil (5 mM) and actinomycin D (90 μg/ml) on the course of chlorophyll accumulation during the first 4 hours of illumination of etiolated bean leaves are shown in figure 5. Leaves were exposed to the antimetabolites for 16 hours prior to exposure to light. Treatment with actinomycin completely blocked chlorophyll accumulation; incubation with 2-thiouracil reduced chlorophyll production to about 45 % of that in the controls.

Table II. *The Effect of Actinomycin D (90 μg/ml) on Chlorophyll Synthesis During the Rapid Phase of Greening*

Hrs of illumination	Chlorophyll	Inhibition
	μg/g fr wt	%
6	488 ± 21	. . .
6 + Act. D after 4 hrs	555 ± 67	0
8	550 ± 116	. . .
8 + Act. D after 4 hrs	661 ± 27	0
10	755 ± 42	. . .
10 + Act. D after 4 hrs	500 ± 76	33.8

Chlorophyll accumulation during 8 hours of illumination was sharply curtailed by treatment of etiolated leaves with 2-thiouracil (1 mM); 5-fluorouracil and 5-bromouracil had essentially no effect (table I).

In other experiments the effect on chlorophyll accumulation of the administration of 2-thiouracil and actinomycin D after greening Stage II was examined. Actinomycin D prevented pigment synthesis when it was applied after Stage II, i.e., after 4 hours of light, inhibition beginning only after an additional 4 hours of illumination (table II); it is likely that the delay reflects slow penetration and/or the lifetime of an RNA species required for chlorophyll production. On the other hand, if leaves were illuminated for 2 hours, to pass Stage II, and then incubated with these inhibitors for 16 hours in darkness, the response to subsequent illumination was similar to that displayed by leaves treated with inhibitors prior to any illumination (e.g. as in fig 5).

These data indicate that DNA-dependent RNA production is required for continuous chlorophyll synthesis during Stages II and III.

Granick (12) found that etiolated leaves supplied with ALA formed large amounts of protochlorophyllide, thus demonstrating that production of this pigment in darkness is limited by the availability of ALA and therefore presumably by the lack of activity of some enzyme involved in ALA production. (This extra pigment which absorbs at 632 mμ in vivo may be protochlorophyllide not associated with holochrome protein; it is not phototransformed to chlorophyllide but can act as a photosensitizing pigment.)

In *Rhodopseudomonas spheroides* bacteriochlorophyll production and the activity of ALA synthetase, the enzyme which has been shown to catalyze the formation of ALA from glycine and succinate in this bacterium, respond similarly to various factors: inhibitors of RNA synthesis, inhibitors of protein synthesis, light intensity, and oxygen tension (5, 14, 21, 22, 23). Consequently it appears that these environmental factors and inhibitory compounds act on pigment synthesis by affecting this enzyme. Bacterial ALA synthetase appears to be extremely labile (6, 14). Similarly, it seems possible that alterations in chlorophyll production brought about by removing light from greening leaves may reflect reduced availability of ALA, especially since no porphyrin precursors appear to accumulate under these circumstances. An effect of darkness, and of CAM in light, might be to arrest the production of an enzyme of ALA synthesis without simultaneously altering its rate of decay.

Support for the contention that some enzyme(s) required for ALA synthesis falls in activity (decays?) in leaves treated with CAM or puromycin during Stage II is provided by the observation that the inhibition of chlorophyll synthesis during the

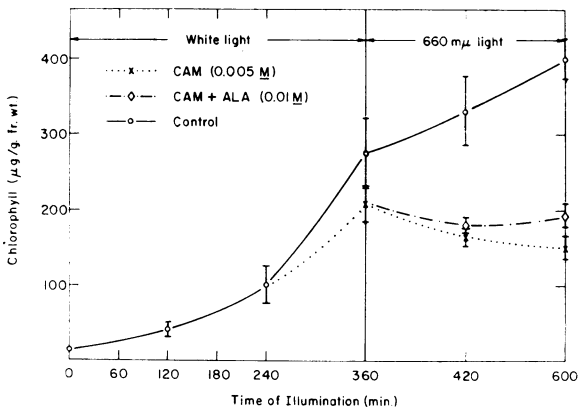

FIG. 6. The enhancement of chlorophyll synthesis by ALA in dim red light in the presence of CAM.

phase of rapid greening can be overcome, in part, by administration of 10 mM ALA to leaves maintained in low intensity red light (660 mμ) (fig 6). The absorption spectra of leaves manipulated in this manner are in no way irregular; this indicates that normal sites are available for locating the chlorophyll newly formed from exogenous ALA in CAM or puromycin-treated leaves. Either more sites than chlorophyll molecules exist at this point in plastid development or production of lamellar proteins is not completely arrested by the administration of these inhibitors to rapidly greening leaves.

The failure to restore chlorophyll production to the normal level by supplying ALA to illuminated CAM-treated leaves has a counterpart in the effect of CAM on protochlorophyllide production by etiolated leaves supplied ALA in darkness; in the latter case pigment production is less than in controls (table III). This suggests that some enzyme(s) of porphyrin biogenesis, besides those concerned with ALA formation, may need to be constantly replaced and that this regeneration is halted or slowed in chloramphenicol-treated leaves.

Discussion

Studies with inhibitors of nucleic acid and protein synthesis indicate the need for nucleic acid and protein synthesis throughout the greening process. This is consistent with the possible existence of a short-lived messenger RNA whose synthesis is governed by light and whose function is to provide information for the synthesis of a labile protein required for ALA synthesis.

Kirk and Allen (15) have proposed that the synthesis of protochlorophyllide holochrome may be the basis of the control of greening, an alternative to the above suggestion. They believe, from the studies of the effect of cycloheximide on greening of *Euglena*, that protein synthesis is required for the production of holochrome to bind with the pigment. However, the observation here that the inhibition of chlorophyll production by CAM or puromycin during Stage III can be partially overcome by the administration of ALA to weakly illuminated leaves appears to bear on this as well as other questions. Furthermore, Gassman and Bogorad (11) find that ALA can relieve the CAM inhibition of protochlorophyllide resynthesis in etiolated bean leaves. If all protochlorophyllide molecules are photoconverted only when associated with the specific holochrome protein, the latter must be present in inhibitor-treated ALA-fed leaves. In any event, spectrally normal chlorophyll can be produced from exogenous ALA in inhibitor-treated leaves.

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Table III. *The Effect of Chloramphenicol (CAM) on the Incorporation of δ-Aminolevulinic Acid (ALA) into "Inactive" Protochlorophyllide in Darkness*

	Protochlorophyllide†		Inhibition	
	μg/g fr wt		%	
	6 hrs	24 hrs	6 hrs	24 hrs
10 mM ALA at T_0	163 ± 8.3	570 ± 20	. . .	. . .
10 mM ALA + 5 mM CAM at T_0	119 ± 11.8	347 ± 14.5	26.9	39.2
10 mM ALA at T_0 + 5 mM CAM after 6 hrs	. . .	330 ± 3.8	. . .	42.2

† Pigment extractions and determinations were made after 3 minutes of illumination using the extinction coefficients of Anderson and Boardman (11).

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