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Regulation of 5-Aminolevulinic Acid Synthesis in Developing Chloroplasts[1]

I. Effect of Light/Dark Treatments *in Vivo* and *in Organello*

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ABSTRACT

Intact chloroplasts isolated from greening cucumber (*Cucumis sativus* L. var Beit Alpha) cotyledons regenerated protochlorophyllide (Pchlide) in the dark with added cofactors from either exogenous glutamate or endogenous substrates. No other intermediates of the chlorophyll biosynthetic pathway accumulated. When inhibitors of 5-aminolevulinic acid (ALA) dehydratase were added, the Pchlide that failed to form was replaced by an excessive amount of ALA. When greening seedlings were returned to the dark, ALA-synthesizing activity in the isolated chloroplasts decreased dramatically and recovered if the dark-treated seedlings were again exposed to continuous white light prior to chloroplast isolation. Both the decline and the recovery of ALA-synthesizing activity were complete in approximately 50 minutes. Changes in chloroplast structure during *in vivo* light to dark and dark to light transitions (as evidenced by electron microscopy) were much slower. Exposing isolated chloroplasts from dark-treated seedlings to short white flashes before incubation transformed nearly all the endogenous Pchlide, but hardly stimulated ALA synthesis, suggesting that Pchlide does not act as a feedback inhibitor on ALA synthesis. Chloroplasts isolated from dark-treated tissue did not form Pchlide from glutamate when incubated in the dark with added cofactors; moreover, the endogenous Pchlide did not turn over *in organello*. However, these chloroplasts did synthesize Pchlide from added ALA at the normal rate and synthesized ALA from glutamate at a reduced, but still significant, rate. Mg chelation was not affected by *in vivo* dark treatment.

The original literature on the effect of light upon Chl biosynthesis has been reviewed by Virgin (27), Virgin and Egneus (28), and Kasemir (20). Here it suffices to mention briefly those aspects of this large field that have a direct bearing on the present study.

It is generally accepted that the coarse control of Chl biosynthesis occurs at the level of ALA[2] formation (2, 4, 11, 12, 18, 23). Phytochrome-regulated ALA synthesis is well documented in the initial stages of greening, when dark grown seedlings are exposed to light (20, 27, 28). In the later stages, however, light is thought to exert its effect on ALA formation not through phytochrome, but through a feedback inhibition by Pchlide (7, 10, 20, 25, 27).

When greening plant tissues are returned to the dark, Pchlide accumulates. If such tissues are also treated with an inhibitor of ALA dehydratase (the enzyme that catalyzes the next step beyond ALA in the protoporphyrin synthesis pathway), Pchlide synthesis is blocked while ALA accumulates. In many cases this ALA accumulation exceeds the amount of ALA equivalents accumulated in the controls (7, 10, 25). This phenomenon, termed 'ALA overproduction,' has been likewise explained in terms of a feed-back inhibition of ALA synthesis by Pchlide.

From what precedes, it can be seen that one of the central questions in the regulation of the Chl biosynthetic pathway remains that of the mechanism whereby light modulates the level of ALA in chloroplasts, especially during the later stages of chloroplast development. In this connection, it should be noted that recent studies with soluble ALA-synthesizing systems have failed to reveal any obvious chromophores in the conversion of Glu to ALA (19). The present article and the studies reported in our companion paper (17) deal with this and closely related questions.

In this study we have taken advantage of an intact cucumber chloroplast preparation that is competent to carry out both ALA and Pchlide synthesis *in vitro* (14, 15, 30) to examine the effect of light exposure given *in vivo* on the capacity of isolated chloroplasts to synthesize ALA; and, using the same cell-free preparation, we have delineated the relationship between Pchlide level and ALA synthesis. A preliminary account of this work has been presented elsewhere (16).

MATERIALS AND METHODS

Materials

Cucumber seed (*Cucumis sativus* L. var Beit Alpha) was a generous gift of Harris Moran Seeds, Salinas, CA 93901.

The following were purchased from Sigma: ATP, NADPH, glucose-6-phosphate, Glu, LVA, SAM, *p*-dimethylaminobenzaldehyde, cysteine, EDTA, Hepes, Tes, sorbitol, and Coomassie brilliant blue G.

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[2] Abbreviations: ALA, 5-aminolevulinic acid; [H], reducing power consisting of 4 mM NADPH and 4 mM glucose-6-phosphate (15); LVA, levulinic acid; Mg-Proto, magnesium protoporphyrin IX; Mg-Proto Me, magnesium protoporphyrin IX monomethyl ester; Proto, protoporphyrin IX; SAM, *S*-adenosyl-L-methionine; fr wt, fresh weight.

BSA and 4,6-dioxoheptanoic acid were obtained from Calbiochem; $MgCl_2 \cdot 6H_2O$ was from Mallinckrodt. Acetone, hexanes, diethyl ether (for anesthesia), and O_sO_4 were from Fisher Scientific. Ethyl acetoacetate was from Aldrich, Percoll was from Pharmacia, and SDS (Duponol) was from DuPont. Cation Exchange Resin (AG 50W-X8, 100-200 mesh, hydrogen form) was purchased from Bio-Rad Laboratories. Proto was from Porphyrin Products, Logan, UT 84321, and glutaraldehyde was obtained from Polysciences, Inc., Warrington, PA 18976. Methylene blue was purchased from Matheson, Coleman, and Bell.

Methods

Plant Material and Chloroplast Isolation

Cucumber seeds were germinated in complete darkness at room temperature for 6 d. After 20 h illumination with white fluorescent light (60–70 $\mu E \cdot m^{-2} \cdot s^{-1}$ PAR, at 28 to 30°C), the cotyledons were harvested, and developing chloroplasts were isolated (9). Intactness of the isolated chloroplasts was approximately 90% (14). The seedlings that received the 20 h illumination are referred to as 'control seedlings' or 'control tissue.' If the control seedlings were returned to the dark at 28 to 30°C, they are referred to as 'dark-treated seedlings.' Chloroplasts were isolated from dark-treated seedlings under dim green safe light. If the dark-treated seedlings were again exposed to light, they are referred to as 'dark-light-treated seedlings.' Conditions for the second light treatment were the same as for the light exposure of control seedlings.

Electron Microscopy

Cotyledon pieces approximately 1 mm^3 were fixed for 2 h using 4% glutaraldehyde in 0.15 M phosphate (pH 7.2). The material was washed three times in the same buffer and treated for 1 h with 1% O_sO_4 followed by three 15 min washes with 0.15 M phosphate buffer. Dehydration followed in an ethanol concentration series (10, 30, 50, 70, 95, 100, 100, 100% ethanol) with the tissue being left to equilibrate for 30 min in each solvent. The fixation and dehydration were carried out at room temperature.

The tissue was then embedded for 2 d in 'Spurr's plastic' (24), sectioned with an LKB IV microtome, and viewed with a JEOL 100 S electron microscope.

Light Treatments of Isolated Chloroplasts

For experiments presented in Figures 1 and 2, the chloroplast preparation (7–8 mL) contained in a 50-mL beaker sitting on ice was illuminated for 5 min with white fluorescent light (about 70 $\mu E \cdot m^{-2} \cdot S^{-1}$ PAR) prior to incubation in order to remove the Pchlide initially present in the isolated chloroplasts (6). The light treatment of chloroplasts isolated from dark treated tissue was done with short white flashes from a photographic flash light (Zykkor, model 375D). Flashes were each 1/50 s in duration with dark intervals between them of about 15 s. The chloroplast preparation, in 3 to 5 mL of incubation buffer containing 4 mM [H], was in a Petri dish (5.5 cm in diameter) sitting in a water bath at 28 to 30°C. All the flash manipulations were carried out in the dark room under dim green safelight.

Incubation Conditions

Routine incubations of isolated chloroplasts were carried out in 25-mL Erlenmeyer flasks in 1 mL total volume containing 500 μmol sorbitol, 10 μmol Hepes, 20 μmol Tes, 1 μmol EDTA, 1 μmol $MgCl_2$, 2 mg BSA, and chloroplasts equivalent to 3 to 5 mg of plastid protein as determined by the Bradford method (3). Incubations were at 28 to 30°C (pH 7.7), either in dark or in the light (approximately 110 $\mu E \cdot m^{-2} \cdot s^{-1}$ PAR) (14, 15). Dark incubations lasted for 45 or 60 min, whereas incubations for ALA synthesis under photosynthetic conditions were for 20 min. Flasks were shaken at 60 to 65 double strokes per min. The reactions were initiated by the addition of chloroplasts, and terminated by freezing and/or by the addition of citric acid to pH 2.8 and SDS to 3.33% (29). Modifications of the general protocol are given in figure and table legends.

ALA Assay

ALA was extracted, purified, and determined spectrophotometrically (29).

Pigment Extraction and Assay

Nonphytylated porphyrins and Mg porphyrins were extracted into diethyl ether and assayed spectrofluorimetrically as described earlier (5). Chl was assayed spectrophotometrically according to Arnon (1).

Mg Chelatase

Mg chelatase in isolated chloroplasts was assayed according to Fuesler *et al.* (9).

Error Range of Replicate Chloroplast Isolations

Replicate experiments using chloroplasts isolated from four different batches of identically treated tissue agreed within ± 10% at the 95% confidence level.

RESULTS

Initially, dark-grown cucumber cotyledons contained approximately 1.4 μg of Pchlide $\cdot$ g fr wt^{-1}. After 20 h of exposure to continuous white light, they contained 463 μg of Chl $\cdot$ g fr wt^{-1}, which was increasing linearly at the rate of 23 μg Chl $\cdot$ g fr $wt^{-1} \cdot h^{-1}$, but retained approximately 0.1 to 0.2 μg of Pchlide $\cdot$ g fr wt^{-1}. If the seedlings after 24 h of continuous illumination were placed in the greenhouse and sampled at regular intervals, it was observed that the Chl content leveled off at approximately 1.02 mg $\cdot$ g fr wt^{-1} (data not shown). This level was reached after 24 h in the greenhouse and remained constant for at least another 48 h. Therefore, our studies were carried out with a tissue that had already accumulated approximately 45% of its final Chl content on a fresh weight basis. It would not have been possible to measure small increments of Pchlide in a tissue with this much Chl, were it

not for our assay (5), which separates Pchlide from the phytylated pigments and determines Pchlide spectrofluorimetrically under conditions minimizing Chlide interference.

Electron microscopy revealed that the chloroplasts in our 20 h greened, 'control,' tissue had numerous fairly well-developed grana, but still contained many prolamellar bodies (data not shown). In contrast, chloroplasts in the fully mature tissue no longer exhibited prolamellar bodies, had many starch grains, and the grana, on the average, appeared to be somewhat larger. The simultaneous occurrence of grana and prolamellar bodies was demonstrated in various tissues under continuous illumination of low intensity (28).

Comparison of ALA and Pchlide accumulation in chloroplasts isolated from control tissue and assayed in the dark as a function of time revealed that ALA was produced at a faster rate and reached a higher level than Pchlide (Fig. 1). When the same two assays were compared as a function of increasing LVA concentration (Fig. 2), it was seen that LVA stimulated ALA accumulation more than it inhibited Pchlide accumulation. No other intermediates in the Chl biosynthetic pathway were detected spectrophotometrically or fluorimetrically. Similar results were obtained if LVA was replaced by 4,6-dioxoheptanoic acid (data not shown). Figures 1 and 2 agree in describing what is known as 'ALA-overproduction,' a phenomenon which has been noted in studies with intact seedlings, but which has not been reported *in organello*. ALA overproduction has been explained in terms of a direct or indirect feedback mechanism by which accumulated Pchlide

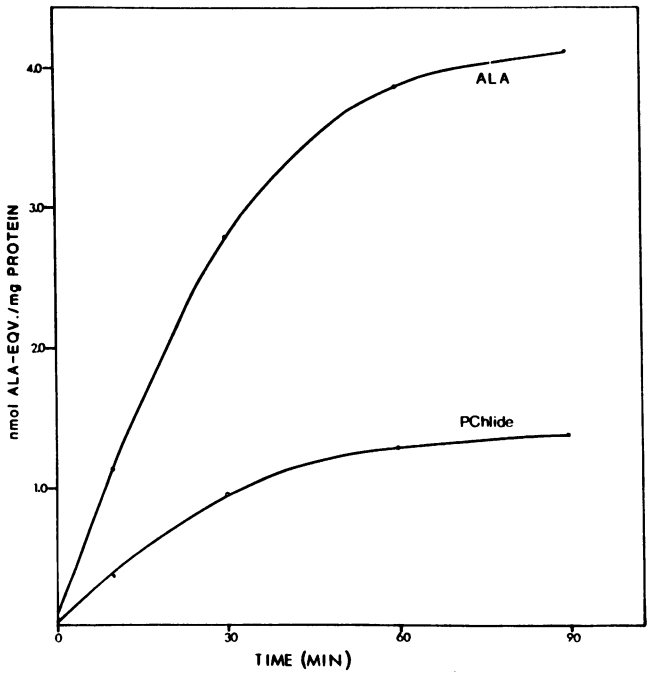

Figure 1. Synthesis of ALA and Pchlide in chloroplasts isolated from control seedlings. Chloroplasts isolated from control seedlings were incubated in the dark with 6 mM Glu, 3 mM ATP, 4 mM [H], 1 mM SAM, and other additions indicated in "Materials and Methods." Ten mM LVA was added only to the incubations for ALA accumulation (top curve). The values for Pchlide were converted into ALA-equivalents by multiplying by 8.

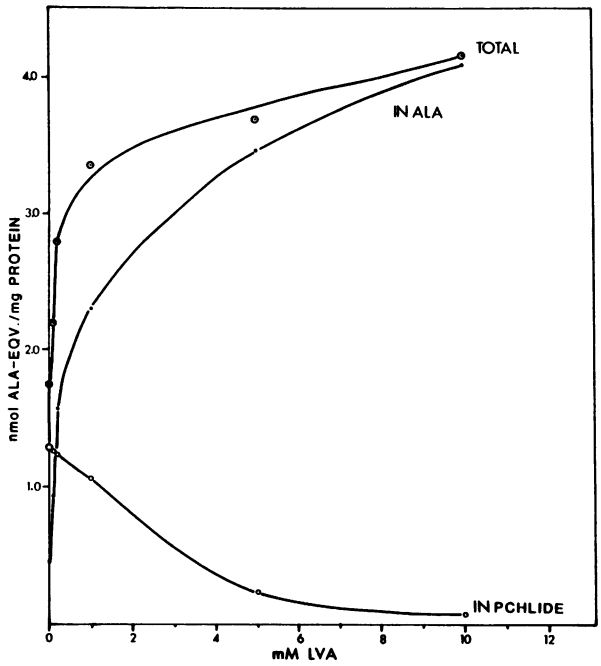

Figure 2. Distribution of ALA-equivalents between Pchlide and ALA at various concentrations of LVA. Chloroplasts isolated from control seedlings were incubated in the dark for 1 h with various concentrations of LVA. Other incubation conditions were the same as in Figure 1.

inhibits its own formation at the level of ALA synthesis (7, 10, 18, 25).

To test that hypothesis, we placed cucumber seedlings back into the dark prior to chloroplast isolation. Pchlide accumulation in intact cotyledons was complete in about 100 min, when the tissue contained about 1.7 nmol Pchlide·g fr wt^{-1}. Chloroplasts isolated from such dark-treated tissue contained 150 to 200 pmol Pchlide·mg protein^{-1}, which could be largely eliminated by exposure to light. To remove the endogenous Pchlide in chloroplasts isolated from dark-treated tissue, photographic flashes were used rather than continuous illumination in order to minimize damage to the chloroplasts, and in order to avoid drastic changes in internal ATP and NADPH concentrations due to photosynthesis. In Figure 3, 20 flashes at 30°C in the presence of 4 mM [H] removed 93% of the endogenous Pchlide but did not appreciably stimulate ALA synthesis.

Chloroplasts isolated from dark-treated tissue had decreased capability for synthesizing ALA from Glu compared to the chloroplasts from control tissue. This is clearly seen in Figure 4 ('dark' curve). The decline in ALA-synthesizing ability was completed in about 50 min. When dark-treated tissue was exposed again to white light, the ability to synthesize ALA *in organello* was largely restored, although frequently not quite to the initial level. The recovery of ALA-synthesizing ability was completed in about 50 min (Fig. 4, 'light' curve). Each of the curves in Figure 4 has four time points, which represent four separate chloroplast isolations. Other experiments (data not shown) used different selections of time points but agreed with the findings shown here, strengthening our confidence in these curves.

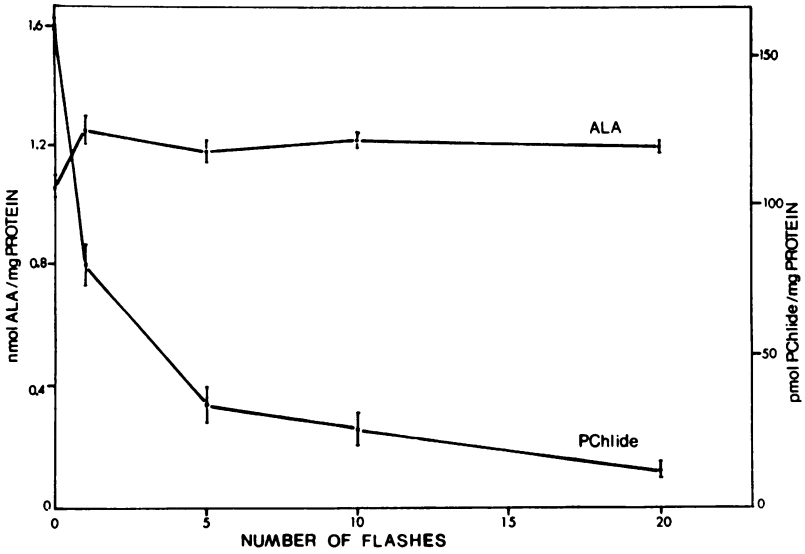

Figure 3. Effect of light flashes *in organello* on Pchlide reduction and ALA synthesis. Greening seedlings were returned to darkness for 100 min, chloroplasts were then isolated under dim green safelight. Four mM [H] was added to the chloroplast suspension. Aliquots of the suspension were given various numbers of photographic flashes at 30°C as described in "Materials and methods," and their remaining Pchlide determined. These chloroplast suspension samples were further incubated in the dark for 45 min to determine their ALA-synthesizing activity. Ten mM LVA, 6 mM Glu, 3 mM ATP, 4 mM [H], and other additions indicated in "Materials and Methods" were included in all samples. Each point represents the mean and range of two measurements.

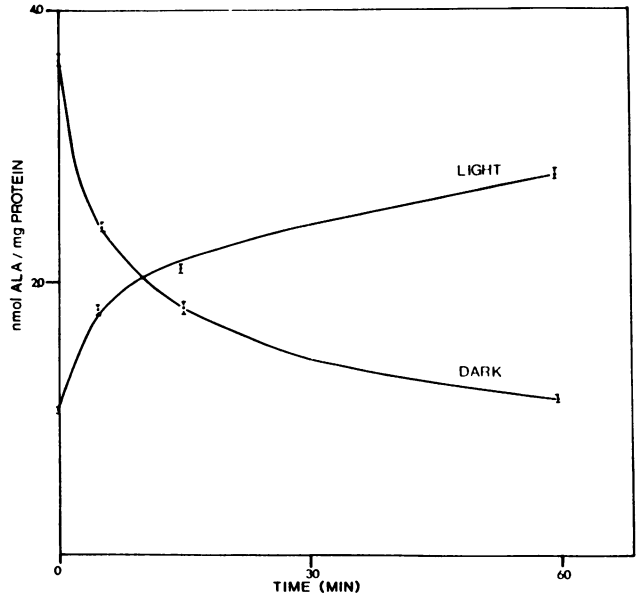

Figure 4. Decline and recovery of ALA-synthesizing capacity upon transfer of cucumber seedlings from light to dark and from dark back to light. Greening seedlings were returned to darkness, cotyledons were harvested, and chloroplasts isolated at indicated intervals under dim green safelight (dark curve). For the light curve, seedlings dark treated for 120 min were again exposed to continuous light, cotyledons were harvested and chloroplasts isolated at indicated intervals under dim white fluorescent light (<1 $\mu E \cdot m^{-2} \cdot s^{-1}$). In both experiments, the isolated chloroplasts were further incubated in the dark for 45 min to determine the ALA-synthesizing activity. Same additions as in Figure 3 were included in all samples. Each point represents the mean and range of duplicate *in organello* assay measurements.

The chloroplasts isolated from dark-treated tissue, in contrast to chloroplasts from control tissue, were not able to synthesize any additional Pchlide from Glu *in organello* (Fig. 5). Moreover, during the *in vitro* incubation, there was no change in the amount of Pchlide either in the absence (Fig. 5A) or in the presence (Fig. 5B) of LVA, indicating that the endogenous Pchlide did not turn over *in vitro*. On the other hand, in these chloroplasts, ALA synthesis from Glu still proceeded at a significant rate, albeit considerably lower than the rate obtained with control chloroplasts (approximately one-third of the control rate; compare Fig. 5 with Fig. 1 or the low and high points of the 'dark' curve in Fig. 4).

Table I compares chloroplasts isolated from three types of tissue (control, dark-treated, and dark-light-treated) with respect to their abilities to synthesize Pchlide in the absence of added substrate, with 6 mM Glu, or with 20 μM ALA (this concentration was chosen because, in control chloroplasts, 20 μM ALA was shown to be as effective as 6 mM Glu, the substrate normally used for *in organello* Pchlide synthesis). As seen also in Figure 5, chloroplasts from dark-treated tissue were unable to make Pchlide from either added Glu or endogenous substrate, but were fully as capable to make Pchlide from ALA as chloroplasts isolated from the other two tissues (Table I). On the other hand, chloroplasts isolated from control and dark-light-treated tissue were able to convert endogenous substrate and added Glu to Pchlide, to about equal extent.

In all the experiments mentioned thus far, ALA synthesis was measured *in organello*, in the dark, with exogenous cofactors. It is possible also to follow ALA synthesis in isolated chloroplasts in the light without addition of ATP or [H] (15). Chloroplasts isolated from the same three tissues (control, dark-treated, and dark-light treated) were compared with respect to their abilities to synthesize ALA from added Glu and endogenous substrates under photosynthetic conditions. The chloroplasts isolated from dark-treated tissue were obviously less active in the synthesis of ALA than the chloroplasts isolated from either control or dark-light-treated tissue (Table II).

The possible influence of *in vivo* light and dark treatments on Mg chelation in isolated chloroplasts was also investigated. Formation of Mg-Proto (Me) from added Proto proceeded at the same rate in chloroplasts isolated from dark-treated tissue as in chloroplasts from control tissue (Table III). Exposure of chloroplasts isolated from dark-treated tissue to 20 flashes

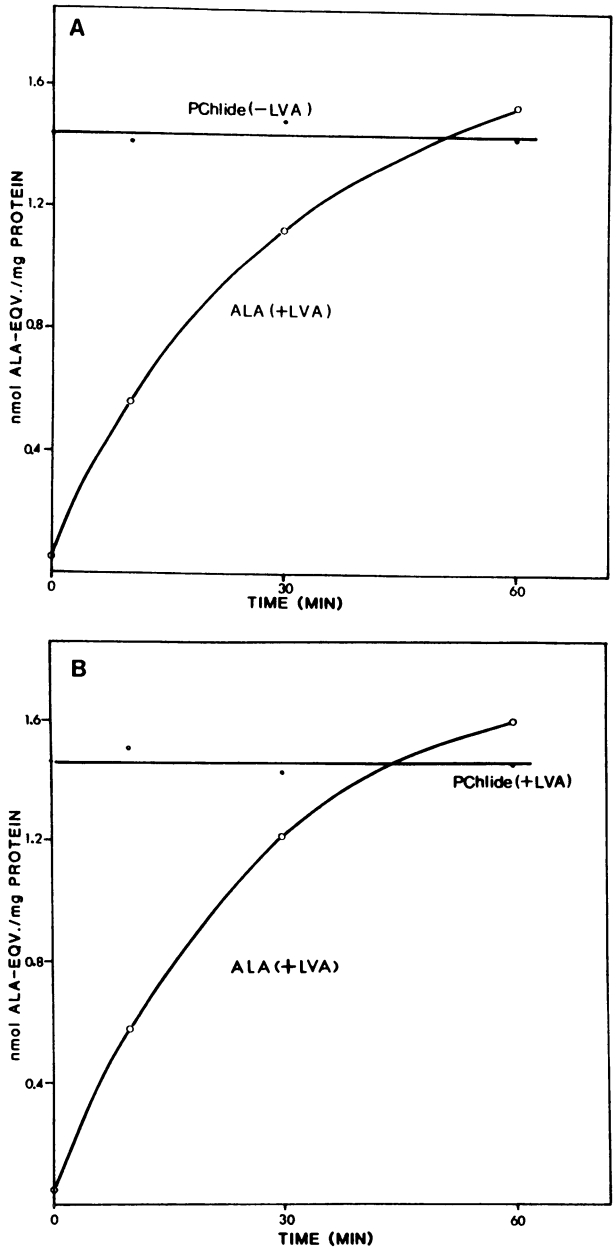

Figure 5. ALA and Pchlide synthesis in chloroplasts isolated from dark treated seedlings. A, Greening seedlings were returned to darkness for 2 h. Chloroplasts were then isolated under dim green safelight and incubated further in the dark with 6 mM Glu, 3 mM ATP, 4 mM [H], 1 mM SAM, and other additions indicated in "Materials and Methods." Ten mM LVA was included only in the incubations for ALA accumulation. B, Same as in A, except that 10 mM LVA was included in all incubations.

removed 87% of the endogenous Pchlide but caused no enhancement of Mg chelation (data not shown).

DISCUSSION

The experimental findings described in this paper focus on two major points: (a) the hypothesis that ALA synthesis is regulated by a Pchlide feedback inhibition, and (b) the effect of light and dark treatments *in vivo* upon the ALA-synthesizing capacity of isolated chloroplasts.

Our findings are inconsistent with any view of the regulation of Pchlide synthesis that is based on feedback inhibition by Pchlide on ALA formation. Chloroplasts isolated from dark-treated tissue contained high levels of Pchlide (Figs. 3 and 5; Table I) and had much lower ALA synthesizing capacity than chloroplasts isolated from control tissue (Fig. 4; Table II). However, this decline was not reversed by an *in vitro* light treatment of the isolated chloroplasts which removed 93% of the endogenous Pchlide (Fig. 3). Therefore, ALA synthesizing activity in the chloroplasts isolated from dark-treated tissue was low regardless of whether the initial high level of endogenous Pchlide was retained (Figs. 3 and 5) or removed (Fig. 3), indicating that endogenous Pchlide did not inhibit ALA synthesis by a reversible feedback mechanism. In chloroplasts isolated from control tissue, the initial rate of ALA synthesis was much higher than the initial rate of Pchlide synthesis (Fig. 1); whereas, if ALA synthesis were regulated solely by a Pchlide feedback mechanism, the initial rate of ALA synthesis and the initial rate of Pchlide synthesis (expressed in terms of ALA equivalents) should be identical, because in both cases the content of the alleged feedback inhibitor, Pchlide, is the same, that is, essentially nil.

ALA overproduction, previously shown in intact tissue (10, 25) has now been demonstrated *in organello*, not only with chloroplasts that were isolated from control tissue and had little endogenous Pchlide at the beginning of the incubation (Figs. 1 and 2), but also with chloroplasts that were isolated from dark-treated tissue and contained a high level of endogenous Pchlide throughout the incubation (Fig. 5). As a matter of fact, in the latter case, ALA overproduction was more striking because in these chloroplasts Pchlide synthesis was virtually absent, while ALA synthesis was still appreciable. These results suggest that ALA synthesis is independent of endogenous Pchlide level. Endogenous Pchlide accumulated during the dark treatment did not affect Mg chelation (Table III), ruling out one of the variants of the feedback hypothesis (4) involving two feedback loops, a first loop, from Pchlide to Mg chelatase, followed by Proto buildup and inhibition of ALA synthesis by protoheme.

Endogenous Pchlide accumulated *in vivo* in the dark did not turn over *in vitro* (Fig. 5B). On the other hand, the turnover of ALA in chloroplasts isolated from either control or dark-treated tissue was not determined. Indeed, it would be technically very difficult to do so, or to establish the destination of ALA in chloroplasts that are not making Pchlide. It appears probable that conversion of ALA to metabolites of the iron-porphyrin branch is an important factor in the overall fate of ALA in the dark (4).

The ability of isolated chloroplasts to form Pchlide from Glu, which decreased to virtually nil during *in vivo* dark treatment (Fig. 5; Table I), was restored by reillumination of the dark-treated tissue (Table I). However, the ability of isolated chloroplasts to convert added ALA to Pchlide was not affected by either dark or light treatments (Table I). Taken together, these findings suggest that *in vivo* dark and light treatments affect the Pchlide pathway at the synthesis of ALA. This interpretation is in line with the conventional wisdom

Table I. *Effect of Dark and Light Treatments in Vivo on Pchlide Synthesis from Various Substrates in Isolated Chloroplasts*

Chloroplasts were isolated from control tissue, 100 min dark-treated-tissue and 100 min dark-100 min light-treated tissue, as described in "Materials and Methods." All three chloroplast preparations were incubated in the dark for 45 min in the presence of the substrate indicated plus 3 mM ATP, 4 mM [H], 1 mM SAM, and other additions indicated in "Materials and Methods." The columns headed by the symbols Δ indicate the change from the zero-time controls.

Substrate	Type of Tissue					
	Control	Δ	Dark treated	Δ	Dark-light treated	Δ
	pmol Pchlide·mg protein^{-1}					
Zero time	38		175		42	
No substrate	87	49	178	3	95	53
6 mM Glu	165	127	177	2	173	131
20 μM ALA	167	129	310	135	176	134

Table II. *Effect of Dark and Light Treatments in Vivo on Light-Supported ALA Synthesis in Isolated Chloroplasts*

Chloroplasts were isolated as described in Table I and incubated under continuous white fluorescent light for 20 min in the presence of 10 mM LVA. Each entry gives the mean and range of duplicate *in organello* assay measurements.

Type of Tissue	Added Substrate	Activity
		nmol ALA·mg protein^{-1}
Control	None	1.20 ± 0.03
	6 mM Glu	2.31 ± 0.05
Dark treated	None	0.41 ± 0.01
	6 mM Glu	0.66 ± 0.02
Dark-light treated	None	1.09 ± 0.03
	6 mM Glu	2.06 ± 0.03

Table III. *Effect of in Vivo Dark Treatment on Mg Chelation in Isolated Chloroplasts*

Chloroplasts isolated from control and dark-treated tissues were incubated in the dark with 8 mM ATP and indicated amounts of exogenous Proto. Ten mM LVA was added to prevent Proto formation from endogenous ALA; 20 μM methylene blue was added to prevent cyclization of Mg-Proto Me (6). After 45 min, Pchlide and Mg-Proto (Me) were determined. Before incubation, the Pchlide content was 29 pmol·mg protein^{-1} in chloroplasts from control tissue and 183 pmol·mg protein^{-1} in chloroplasts from dark-treated tissue. Mg-Proto and Mg-Proto Me are not distinguished in this assay.

Proto	Type of Tissue			
	Control		Dark-treated	
	Pchlide	Mg-Proto (Me)	Pchlide	Mg-Proto (Me)
μM	*pmol·mg protein^{-1}*			
0	29	8	178	2
2	25	195	179	153
10	26	596	183	659

(2, 11, 12, 18, 23), which places the regulation of tetrapyrroles at the level of ALA. Moreover, the fact that dark treatment *in vivo* did not affect Mg chelation (Table III) is entirely consistent with the observation that conversion of added ALA to Pchlide in isolated chloroplasts was not altered by the *in vivo* dark treatment prior to chloroplast isolation (Table I).

The fluctuations in ALA-synthesizing ability when seedlings are dark treated or reilluminated can be followed using two different *in organello* assays (15): by incubating chloroplasts either in the dark with added ATP and [H], or by incubating them in the light, relying on the photosynthetic production of endogenous ATP and NADPH. The effect of *in vivo* light to dark and dark to light transitions on the ALA-synthesizing capacity was qualitatively identical regardless of which assay was used (*cf.* Fig. 4 to Table II), indicating that these fluctuations are not a matter of the uptake of exogenous cofactors across the chloroplast envelope, but of inherent enzymic capacity to convert Glu to ALA. The recovery of the ALA synthetic capacity when dark-treated seedlings are exposed to light (Fig. 4, Table II) was examined further in the companion paper (17) and turned out to be a typical phytochrome-regulated process.

When control seedlings are placed back into the dark, the chloroplasts undergo certain structural changes. With our material, however, these changes were slower than the physiological changes pertaining to the biosynthesis of ALA and Pchlide (Figs. 4 and 5; Tables I and II). In fact, in cucumber cotyledons, changes in chloroplast ultrastructure did not begin to be noticeable until 2 h in the dark (data not shown), when Pchlide accumulation *in vivo* had already reached a maximum value, whereas, changes in the ability of isolated chloroplasts to synthesize ALA were complete in about 50 min (Fig. 4).

It would be rash at the present time to formulate a hypothesis on the mechanism whereby light regulates the synthesis of ALA and Pchlide in cucumber chloroplasts. That task must wait for additional experimental information. Moreover, our findings are not directly comparable to those obtained by other workers (7, 8, 10, 18, 21, 25, 26) using other biological materials and different experimental techniques. For example, as we have seen, cucumber cotyledons respond particularly fast to changes in illumination (Fig. 4). On the other hand, the ALA synthesizing capacity of barley plastid stroma changed very slowly when greening seedlings were transferred from light to dark (18). This difference between cucumber cotyledons and grass leaves in the rapidity of the light responses was noticed also by Stumpf and coworkers (13, 22)

working on fatty acid desaturation in cucumber and maize. In cucumber, Murphy and Stumpf (22) observed fast light responses which were inhibited by cycloheximide, whereas in maize the same process was largely light independent (13). Therefore, the possibility must be considered that this difference in the light regulation of ALA synthesis between cucumber and barley reflects a broader difference in the way in which chloroplast development is controlled by light in different angiosperm tissues.

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