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Controls on Chlorophyll Synthesis in Barley[1]

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ABSTRACT

In 7- to 10-day-old leaves of etiolated barley (*Hordeum vulgare*), all of the enzymes that convert δ-aminolevulinic acid to chlorophyll are nonlimiting during the first 6 to 12 hours of illumination, even in the presence of inhibitors of protein synthesis. The limiting activity for chlorophyll synthesis appears to be a protein (or proteins) related to the synthesis of δ-aminolevulinic acid, presumably δ-aminolevulinic acid synthetase. Protein synthesis in both the cytosol and plastids may be required to produce nonlimiting amounts of δ-aminolevulinic acid. The half-life of a limiting protein controlling the synthesis of δ-aminolevulinic acid appears to be about 1½ hours, when determined with inhibitors of protein synthesis. Acceleration of chlorophyll synthesis by light is not inhibited by inhibitors of nucleic acid synthesis, but is inhibited by inhibitors of protein synthesis. A model for control of chlorophyll synthesis is proposed, based on a light-induced activation at the translational level of the synthesis of proteins forming δ-aminolevulinic acid, as well as the short half-life of these proteins. Evidence is presented confirming the idea that the holochrome on which protochlorophyllide is photoreduced to chlorophyllide functions enzymatically.

Chlorophylls are the end products of the multienzyme, branched, biosynthetic pathway that also produces hemes and bile pigments (11).

Several lines of evidence indicate that this pathway is regulated at the first step, *i.e.*, the synthesis of δ-aminolevulinic acid (ALA)[3]. Virgin (33) demonstrated that the rates of chlorophyll synthesis and protochlorophyllide regeneration during the lag phase are limited by some earlier step in the pathway. Granick (8) found that etiolated seedlings produce large amounts of protochlorophyllide and other chlorophyll precursors when fed ALA; he suggested that the enzymes that convert ALA to protochlorophyllide are present at nonlimiting activities in dark-green plants and that the chlorophyll biosynthetic pathway was regulated by controlling the rate of ALA synthesis. Kirk and Allen (15), Schiff and Epstein (26), and others have suggested that the rate of ALA synthesis is controlled by a negative feedback in which protochlorophyllide or the protochlorophyllide holochrome inhibits the activity of ALA-synthetase. Gassman and Bogorad (5, 6) have proposed the hypothesis that light regulates chlorophyll synthesis in young bean leaves but that synthesis of RNA was required as a precursor step in this process.

In this paper we shall present further evidence to support the hypotheses that light stimulates the synthesis of an enzyme which forms δ-aminolevulinic acid, that this is the rate-limiting enzyme in chlorophyll synthesis, and that, at least in barley, RNA synthesis may not be required for early chlorophyll synthesis.

METHODS AND MATERIALS

Barley (*Hordeum vulgare* var. Wong; W. A. Burpee Co., Philadelphia, Pa.) seedlings were grown in vermiculite beds in the dark in closed cabinets. The seedlings were watered with tap water every second day. The temperature in the growth room was regulated at 25 ± 2 C. The growth rate of the seedlings is shown in Figure 1. The period of most rapid growth was over after 7 days. The first leaf was almost fully formed at this time. A second, smaller leaf appeared after 10 days. Unless noted otherwise, seedlings, 7 to 10 days old, were used.

All handling of the seedlings was performed under dim, green safelight (less than 1 ft-c with a filter combination that passed only 540–560 nm of light). The dark-grown seedling "tops" were harvested by cutting them off just above the bed; the lowest inch (approximately the coleoptile region) was discarded in seedlings older than 7 days. Approximately 10 g of seedling tops, prepared as above, were placed in 15-ml beakers containing 6 ml of solution, with the bottom 3 cm of the tops immersed in the solution usually for 2 hr in the dark before illumination. The uptake of solutions was facilitated by gently circulating air around the tops with a small fan (40 cubic feet per min). The solutions in the beakers were replenished to ensure maintenance of turgor of the seedlings throughout the experiment. That a 2-hr dark preincubation permitted adequate uptake of solution by the leaves was shown by the following observations. (*a*) ALA-fed leaves synthesized enough PCHLD in the dark preincubation period to form more CHL than controls in a subsequent 2-hr period of low intensity illumination; (*b*) CHX decreased the rate of CHL formation within 1½ hr after it was fed to the leaves.

All illuminations were performed with 7 ft-c of tungsten light unless otherwise noted. The effect of the slight variation in light intensity over the samples was minimized by frequently shifting the beakers about the illuminated field during the course of the experiment.

At the end of the incubation and illumination period, tops were blotted to remove excess moisture; then they were weighed, and 10 g fresh weight were immediately extracted into 80 ml of alkaline acetone (9 volumes of acetone to 1 volume of 0.1 M K_2CO_3) at an initial temperature of −20 C with the aid of a Waring

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[2] The work reported is part of a thesis of K. Nadler to satisfy requirements of the doctorate degree at the Rockefeller University. More extensive references than those cited and tables of data not included in the manuscript may be obtained from the thesis. The present address of K. Nadler is: Botany Department, Michigan State University, East Lansing, Michigan 48823.

[3] Abbreviations: ALA (=δAL) = δ-aminolevulinic acid; Chlorophyllide = CHL minus phytol; CHL = chlorophyll; PCHLD = Protochlorophyllide = Mg vinyl pheoporphyrin.

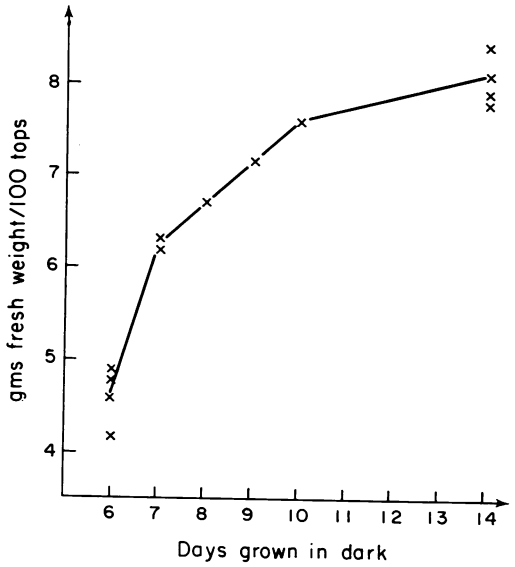

FIG. 1. The growth of Wong barley seedlings in the dark. Growth is plotted on the ordinate as grams fresh weight per 100 seedling tops. Tops are prepared as described in Materials and Methods section. The first leaf completed its most rapid growth before the 7th day; seedlings germinated and grown in the dark for 8 to 10 days were used in the following experiments.

Blendor run at high speed for 1 min; another 20 ml were used for rinsing the blender and residue. After the extract had remained at 4 C for several hours and had been filtered, the extract was no longer turbid. The absorbances of the clear filtrates were determined, with a Cary 14 spectrophotometer with 1- or 5-cm-long cells. Pigment concentrations for CHL were calculated according to the equations of MacKinney (19) for 1-cm length cells: [Chl] in μg/ml = 11.9 A_{663}. During early stages of greening the amount of CHL *b* was negligible to that of CHL *a*. Variations in chlorophyll concentrations on duplicates were within 10%.

RESULTS

Nutrients Do Not Overcome the Lag Phase in Chlorophyll Synthesis. Greening in etiolated seedling tops exposed to continuous light is shown in Figure 2. There was a rapid formation of chlorophyll(ide) in the first few minutes of illumination due to the photoreductive conversion of most of the protochlorophyllide present in etiolated seedlings to chlorophyllide. This burst of chlorophyllide production was followed by a period of several hours in continuous light, during which the rate of CHL synthesis was very low, *i.e.*, the "lag phase." After the lag phase, *i.e.*, in the "linear phase," CHL was more rapidly formed. During the first 24 hr of illumination, an 8-day-old leaf developed about 10% of the CHL (80 μg/g fresh wt) compared to the leaf of a seedling which had been illuminated for 16 hr each day.

The length of the lag phase and the ability of the seedlings to synthesize CHL once past the lag phase were strikingly age-dependent as is noted by Akoyunoglou and Siegelman (1). Etiolated barley seedlings older than 7 days had an increasingly longer lag phase and a decreasing ability to form CHL once past the lag phase. For example, a 9-day-old seedling had a 2- or 3-hr lag as compared to a 1-hr lag in 7-day-old seedlings; similarly, the rate of CHL production in the linear phase decreased with the age of the plant. The decreased CHL synthetic ability as the primary leaf matures probably reflects the general loss of plastid protein in etiolated leaves older than 7 days (25). The occurrence of a lag phase may be interpreted as indicating that some component(s) essential for CHL synthesis and (or) chloroplast development is limiting during this period. The end of the lag phase would then correspond to the rapid production of this limiting component(s).

A number of investigators have sought to identify the component(s) that limits CHL synthesis in the lag phase. In etiolated bean seedlings, feeding sugars to the leaves prevented an increase in the lag phase (34) and facilitated development of chloroplast lamellae (4). In greening wheat leaves, Tolbert and Gailey (32) observed that a 5-hr induction period for photosynthetic CO_2 fixation could be shortened but not eliminated by feeding ribose or glucose to the leaves. In our experiments with barley leaves,

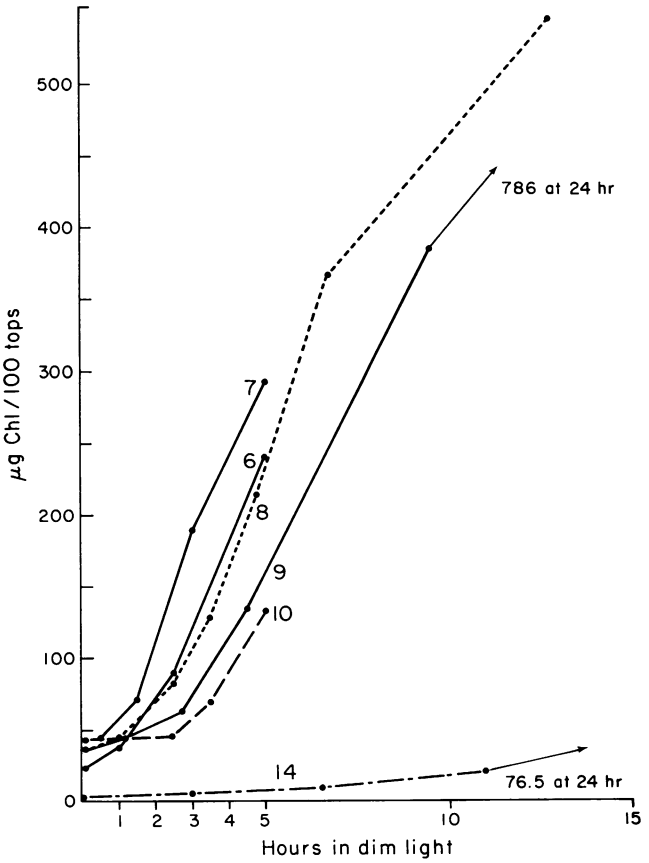

FIG. 2. The time course of chlorophyll formation in 7 ft-c of tungsten light in leaves of increasing age. Chlorophyll concentration is expressed in μg/100 tops. The age in days of the etiolated tops is adjacent to each greening curve. Seedling tops with the bottom 3 cm in H_2O were illuminated with 7 ft-c of tungsten light as described in Materials and Methods section.

Table I. *Effect of Succinate and Glycine on Chlorophyll Formation in Etiolated 10-Day-Old Seedling Tops*

Substrate of ALA	Chlorophyll Formed during Illumination			
	5 min	2 hr	6½ hr	20 hr
	μg chlorophyll/g fresh wt			
Water	6.1 ± 0.6[1]	10.0 ± 2.0	38.0 ± 0	84
Na_2 succinate (0.01 M)	5.8 ± 0.0	9.1 ± 1.4	47.0 ± 6	87
Glycine (0.01 M)	5.5 ± 0.6	14.0 ± 1.4	39.0 ± 4	80
Glycine + succinate	6.2 ± 0.6	12.0 ± 1.4	36.0 ± 1	84

[1] Standard deviation from two samples.

neither substrates for ALA formation (Table I), sugars, amino acids, potassium gibberellate (40 mg/liter), kinetin (saturated aqueous solution), nor vitamins including pyridoxal, a precursor to the coenzyme for ALA synthetase, were found to shorten the lag phase in CHL synthesis. Indeed, 40 mg/liter of indoleacetate inhibited CHL formation by 40%. We conclude from these data that the lag phase of CHL synthesis is not due to a general nutritional deficiency.

ALA Overcomes the Lag Phase in Chlorophyll Synthesis. Feeding ALA to the leaves was found to stimulate CHL synthesis during the lag phase; 1 mg of ALA per ml caused CHL to double in 2¼ hr of low intensity illumination (Fig. 3). Similar results were obtained by Sisler and Klein (27). From this experiment and others (*vide infra*), it appeared that the rate of formation of ALA was the rate-limiting step in CHL synthesis in the lag phase.

The rate of ALA formation might be limited by the activity of the enzyme that formed ALA or by the availability of the substrates used by the enzyme. As yet, the enzyme that forms ALA in higher plants has not been identified in plastids. In *Rhodopseudomonas spheroides*, in mitochondria of animal cells, and presumably in mitochondria of plant cells, the enzyme ALA-synthetase is present. This enzyme uses succinyl-CoA and glycine as substrates and uses pyridoxal phosphate as coenzyme. When succinate and glycine were fed to the leaves in the lag phase, there was no enhancement of CHL synthesis (Table I).

Could succinyl-CoA be a limiting substrate? Succinyl thiokinase, which forms succinyl-CoA, has been reported to be present in wheat chloroplasts (21), in Jerusalem artichoke mitochondria (23), and in etiolated bean leaf homogenates (31). If succinyl-CoA, produced in the mitochondrion is used there for ALA formation, then calculations based on respiration data from the lag phase of greening *Chlamydomonas* (22) and bean leaves (34) indicate that the succinyl-CoA that can be formed from α-ketoglutarate in the citric acid cycle is at least 100 to 1000 times that required for ALA formation. If succinyl-CoA is formed by succinyl thiokinase, then the activity of this enzyme in *Rhodopseudomonas spheroides* (17, 18) and in wheat chloroplasts is clearly in excess and is not limiting ALA production. These calculations suggest, but do not prove, that succinyl-CoA probably is not a limiting substrate. In *Anabaena variabilis*, Pearce *et al.* (24) have found that this alga lacks both succinyl thiokinase and α-ketoglutarate dehydrogenase; succinyl-CoA is probably formed by a CoA-transferase from acetoacetyl-CoA to succinate.

Still another route to ALA synthesis was suggested by Gassman *et al.* (7). They showed that γ, δ-dioxovaleric acid (K*m*, 2.6 × 10^{-3} M) could become aminated to form ALA by extracts of *Chlorella vulgaris* with the use of one of several amino acids as a source of the NH_2 group. As yet, the presence of this ketoaldehyde has not been demonstrated in plants.

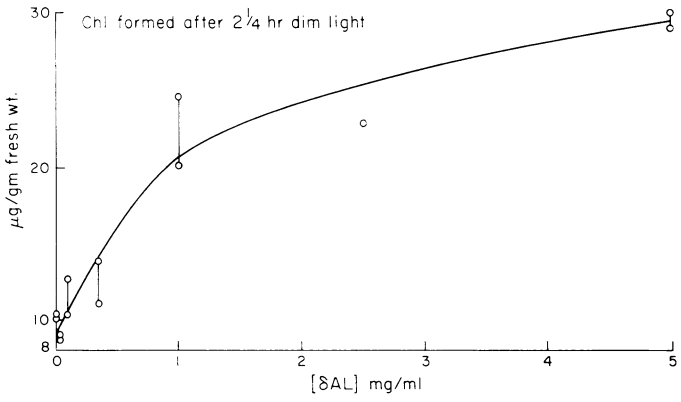

FIG. 3. The effect of δ-aminolevulinic acid (ALA = δAL) on the amount of chlorophyll formed after 2¼ hr of light (approximately the end of the lag phase). Chlorophyll is expressed in μg/g of fresh weight of tops. Nine-day-old leaves were preincubated in the dark for 2½ hr in increasing concentrations of ALA and were then illuminated for 2¼ hr in 7 ft-c of tungsten light.

Table II. *Suppression of Chlorophyll Formation in Greening Barley Seedling Tops by Inhibitors of Protein Synthesis*

Chlorophyll formed is expressed as a percentage of the pigment of uninhibited tops. The tops were preincubated with the inhibitors in the dark for 2 hr before illumination. Several experiments are tabulated. Tops in 0 to 10 μg of acetoxycycloheximide/ml were illuminated for 36 hr before chlorophyll extraction; tops in higher concentrations of acetoxycycloheximide were extracted after 15 hr of light; tops in cycloheximide were extracted after 12 hr of light, while those in chloramphenicol were extracted after 36 hr of light.

Inhibitor (μg/ml)	% Control
Acetoxycycloheximide	
0	100
0.01	135 ± 33
0.1	138 ± 33
1.0	62 ± 15
10	43 ± 10
0	100
50	24 ± 0.3
0	100
100	5 ± 1
Cycloheximide	
0	100
1	119
10	93
100	18 ± 9
Chloramphenicol succinate salt	
0	100
1	73
10	54
100	36 ± 4
500	39 ± 2

By considering the above information it may be surmised that metabolic precursors for the rapid synthesis of ALA will probably not be limiting in the young cells rich with substrates. The localization and identification of the enzyme(s) that forms ALA for CHL synthesis, whether in mitochondria alone or also in plastids, are not known. There is some evidence that all of the enzymes that convert ALA to CHL may be localized in the plastids (9, 11).

Chlorophyll Synthesis is Inhibited by a Variety of Protein Synthesis Inhibitors. If an enzyme limiting the synthesis of ALA or a substrate of ALA is produced during greening, then CHL synthesis should be blocked by inhibitors of protein synthesis. Almost complete inhibition of CHL production was obtained with cycloheximide and acetoxycycloheximide, whereas severe inhibition was obtained with chloramphenicol (Table II). For example, 100 μg/ml of cycloheximide or acetoxycycloheximide inhibited CHL production 82 and 95%, respectively; chloramphenicol (100 μg/ml) inhibited incompletely, to the extent of 64%, and 500 μg/ml did not increase this level of inhibition. However, in bean, inhibition with chloramphenicol was 100% (5, 6). Cycloheximide and acetoxycycloheximide are believed to block protein synthesis at cytoplasmic (80S) ribosomes, whereas chloramphenicol is considered to block at chloroplast and mitochondrial (70S) ribosomes (3, 20, 28).

Chlorophyll Synthesis Continues in the Presence of δ-Aminolevulinic Acid and Inhibitors of Protein Synthesis. As reported above, CHL synthesis can be blocked by inhibitors of protein synthesis. If the synthesis of only the enzyme that forms ALA or a substrate of ALA was limiting CHL formation and all other enzymes of the CHL biosynthetic chain were in excess, then it should be possible to replace this enzyme(s) with ALA, even in the presence of protein synthesis inhibitors. The results of these experiments are shown in Figures 4 and 5 and Table III. These data show that CHL formation was severely depressed by inhibitors of protein synthesis, but that when ALA was simultaneously administered, CHL was as rapidly produced in ALA-treated leaves as in leaves without inhibitors during the first 6 to 12 hr of greening. Even after 25 hr of illumination, leaves fed cycloheximide plus ALA formed 78% of the CHL of control leaves (Fig. 4); similarly, those fed acetoxycycloheximide plus ALA formed 87% of the CHL of controls after 11 hr of light (Fig. 5), while leaves fed chloramphenicol plus ALA synthesized 74% of the CHL of control leaves during 21 hr of illumination (Table III). Clearly, in the presence of ALA, protein synthesis in the early stages of greening was not essential for CHL formation. If it is assumed that at the levels of inhibitors used, protein synthesis was completely blocked, then several conclusions may be drawn from the above results. (*a*) The synthesis of none of the enzymes converting ALA to CHL is necessary for normal CHL production during the first 6 to 12 hr of greening. (*b*) Since protein synthesis is not required for CHL formation when ALA is provided, the synthesis of a protein(s) connected with ALA formation controls greening. This protein may be the enzyme that forms ALA.

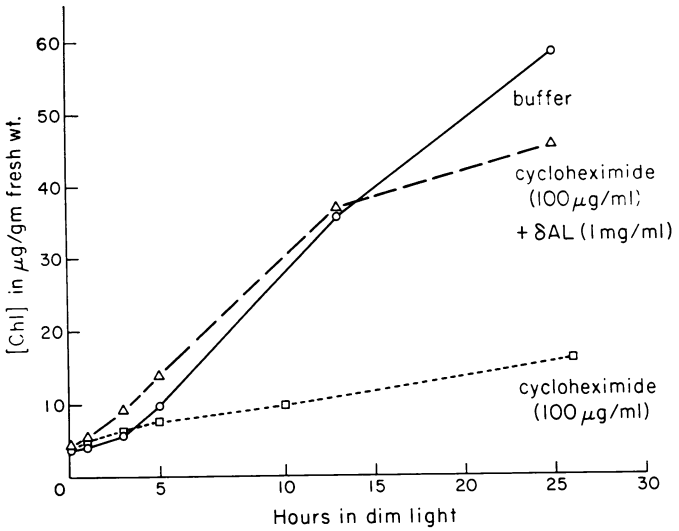

FIG. 4. Chlorophyll formation in seedling tops in the presence of 100 μg of cycloheximide per ml ± 1 mg of ALA per ml and 0.01 M potassium phosphate buffer, pH 7.1. The leaves were preincubated for 2 hr in the dark, then illuminated with 7 ft-c of tungsten light, and extracted at successive times.

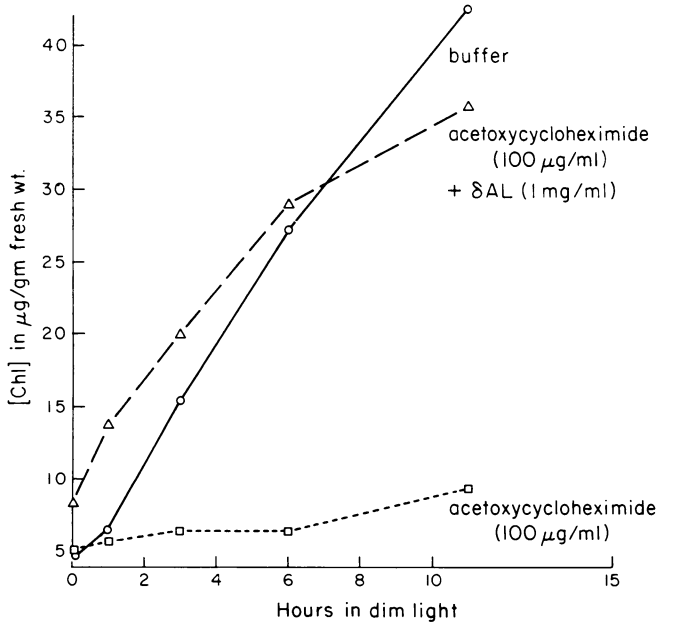

FIG. 5. Chlorophyll formation in seedling tops preincubated in the presence of 100 μg of acetoxycycloheximide per ml ± 1 mg of ALA per ml and 0.01 M potassium phosphate buffer, pH 7.1 for 3½ hr in the dark, then illuminated with 7 ft-c of tungsten light, and extracted at successive times.

Table III. *Chlorophyll Formation with Time in 9-Day-Old Seedling Tops Preincubated for 2½ Hours in the Presence of 0.1 mg of Chloramphenicol per ml ± 1 mg of ALA per ml and then Illuminated with 7 ft-c of Tungsten Light*

Time in Light	Water Controls	Chloramphenicol (0.1 mg/ml)	Chloramphenicol (0.1 mg) + ALA (1 mg) per ml
hr	*μg chlorophyll/g fresh wt*		
0.1	4.1 ± 0.0	4.8 ± 0.0	6.1 ± 1.0
1.5	4.9 ± 0.5	5.3 ± 0.2	14.0 ± 3.0
3.5	9.5 ± 1.2	9.3	26.0 ± 1.0
21.0	76.0		56.0

Table IV. *Chlorophyll Formation at the End of the Lag Phase in the Presence of Cycloheximide*

The 9-day-old seedling tops were preilluminated for 2½ hr, and then the tops were incubated with cycloheximide (0.1 mg/ml) under continued illumination. All illumination was with 7 ft-c of tungsten light.

Time in Light	Water Controls	After 2½ hr of Dim Light 100 μg/ml of Cycloheximide was Fed
hr	*μg chlorophyll/g fresh wt*	
0.1	5.6	
2.5	11.0 ± 0	13.0 ± 0
4.0	28.0 ± 1	19.0 ± 1
6.0	40.0 ± 0	22.0 ± 1

Estimate of the Half-life of the Limiting Enzyme in ALA Synthesis. If we assume that the enzyme that forms ALA controls CHL synthesis, then its activity should be proportional to the rate of CHL production. The rate of CHL production is low during the lag phase and is high and constant during the linear phase. When cycloheximide was given at the end of the lag phase (*i.e.*, when the rate of the synthesis of ALA had become high), a subsequent decrease in the rate of CHL formation was observed (Table IV and Fig. 6). The half-life of the enzyme that forms ALA was estimated to be less than 1½ hr on the basis of the following data. Seedlings fed cycloheximide at the end of the lag phase produced only 6 μg of additional CHL per g fresh weight in 1½ hr, whereas uninhibited controls produced 16 μg of additional CHL per g fresh weight. The rate of CHL synthesis was decreased approximately 60% within 1½ hr (Table IV). A similarly short half-life was calculated from data in Figure 6.

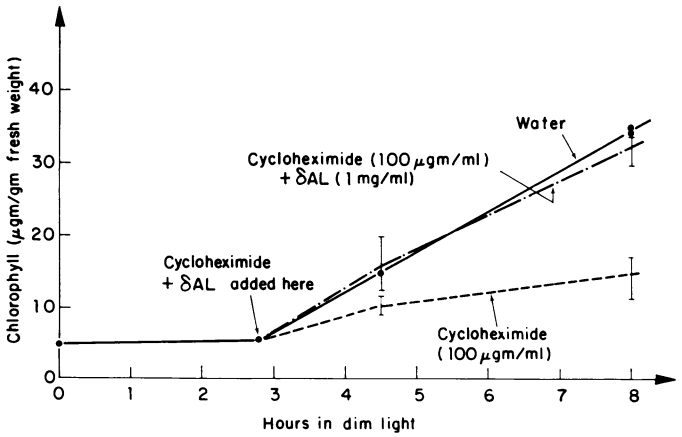

FIG. 6. Chlorophyll formation in seedling tops placed in 100 μg of cycloheximide per ml ± 1 mg of ALA per ml after 2¾ hr of 7 ft-c of tungsten light (approximately at the end of the lag phase). All solutions contained 0.01 M potassium phosphate buffer, pH 7.1. Error bars represent standard deviations of two measurements.

Table V. *Effect of Base Analogues and Mitomycin-C on Chlorophyll Formation (Expressed as a Percentage of Uninhibited Controls)*

The seedlings were preincubated in the dark and were allowed to green up as described in the footnotes.

Inhibitor (mg/ml)		% Control
0		100
0.6	5-Thiouracil[1]	93
0		100 ± 11[2]
0.5	5-Bromouracil[1]	85 ± 15
0.5	8-Azaguanine[1]	94 ± 19
0.5	2,6-Diaminopurine[1]	84 ± 4
0		100 ± 10
0.1	5-Fluorouracil[3]	104 ± 10
0.1	5-Fluorouracil[4]	112
0		100 ± 21
0.050	Mitomycin-C[5]	95

[1] 4 hr of dark preincubation and 16½ hr of light.
[2] Standard deviation of two samples.
[3] 1½ hr of dark preincubation and 20½ hr of light.
[4] 12 hr of dark preincubation and 9½ hr of light.
[5] 12 hr of dark preincubation and 36 hr of light.

Because of the short half-life of the enzyme(s) that limits ALA synthesis, it is evident that continual *de novo* synthesis of this enzyme is required for greening to proceed.

RNA Synthesis and Greening. With inhibitors of RNA synthesis, one may determine whether RNA is limiting CHL synthesis. We used actinomycin D, Mitomycin-C, and a number of base analogues. No inhibitory effect by 5-fluorouracil (100 μg/ml) could be obtained even when the leaves were preincubated in the dark with the inhibitor for 12 hr before illumination; similar negative results were obtained with saturated aqueous solutions of 5-thiouracil, 5-bromouracil, 8-azaguanine, and 2,6-diaminopurine (Table V). Some (38%) inhibition of CHL formation was obtained with a concentrated solution of actinomycin D (100 μg/ml), but only when tops were preincubated in the dark for 24 hr before illumination with 7 ft-c of red light (to avoid photodestruction of the actinomycin D); however, the long dark

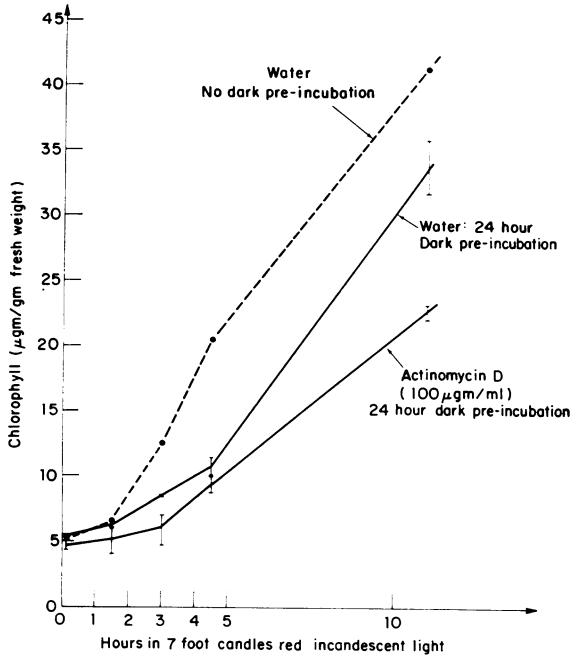

FIG. 7. The effect of actinomycin D on chlorophyll formation in 9-day-old seedling tops. The leaves were treated with actinomycin D (100 μg/ml) for 24 hr in the dark prior to illumination. Some controls imbibed water for 24 hr in the dark, and others were freshly excised. To avoid photodestruction of the inhibitor, the leaves were illuminated with 7 ft-c of red tungsten light obtained by interposing two layers of red cellophane between the tops and a 60-w lamp adjusted to the proper distance. Error bars represent standard deviation of two samples.

incubation itself had an inhibitory effect on CHL formation (Fig. 7). From these data, it appears that the inhibitors of RNA synthesis do not affect appreciably the synthesis of CHL. One may therefore infer that rRNA, tRNA, and at least the mRNA that codes for the limiting enzyme of ALA synthesis are stable and nonlimiting in etiolated barley leaves. On the other hand, this negative result may be explained by the not too likely assumption of the impermeability of the cells to all of these RNA inhibitors even when supplied at relatively high concentrations.

Effect of Light on a Limiting Step in Chlorophyll Synthesis. Light absorbed by the pigment, phytochrome, has been implicated in a process that triggers ALA synthesis in etiolated barley seedlings. A comprehensive review of this subject is given by Kirk and Tilney-Bassett (16). The data presented in Figure 8 suggest that an illumination of short duration enhanced the synthesis of an unstable protein(s) which overcame the limiting step in CHL synthesis. A 5-min exposure to 35 ft-c of tungsten light, followed by a 5-hr dark period increased the rate of CHL formation in the initial hours of continuous illumination. Administration of cycloheximide (100 μg/ml) 2 hr before the 5-min preillumination completely blocked this stimulation. A progressively greater transitory stimulation of CHL production was observed when the cycloheximide was given during or after the 5-min preillumination flash; CHL formation eventually ceased in all seedlings fed cycloheximide. Because light stimulated the CHL synthesis and the stimulation was transient, and because an inhibitor of protein synthesis blocked this stimulation, it is inferred that light induced the formation of an unstable enzyme(s) which increased the rate of ALA formation.

DISCUSSION

The results, by a number of investigators of studies on CHL synthesis in dark-grown seedlings and the effects of inhibitors of protein and nucleic acid synthesis, in general, are in agreement

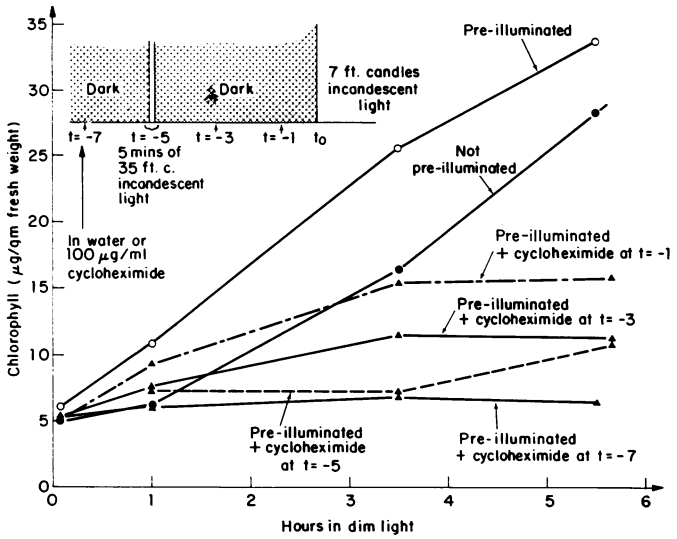

FIG. 8. The antagonistic effects of preillumination and preincubation with cycloheximide on the rate of chlorophyll formation during the lag phase. Preilluminated tops were those irradiated with 35 ft-c of tungsten light for 5 min at t = −5 hr. Tops were then transferred to cycloheximide solutions at various times before or after preillumination (*i.e.*, at t = −7, −5, −3, and −1 hr as diagrammed in the inset). Cross-hatched areas represent dark periods.

qualitatively; however, quantitatively these results diverge considerably. One reason may be that the amounts of storage material, such as proteins, mRNA, and ribosomes deposited in the proplastids where CHL synthesis is to occur, depend on the species, environmental conditions, and age of the seedlings. In an attempt to define the limiting conditions for CHL synthesis, we chose barley, because in barley the proplastids enlarge considerably as compared, for example, to bean proplastids. It has been estimated that by 7 to 10 days the amount of stored protein in the prolamellar bodies is sufficient, to become reorganized on brief illumination into about 10 onion-like layers of primary thylakoids (13); these layers could stabilize an amount of CHL equivalent to 5 to 10% of the CHL of a mature leaf. (It is perhaps significant that an etiolated leaf top, fed cycloheximide and ALA, can only produce approximately 10% of the CHL of a seedling grown in 16 hr of light each day.)

Without light, differentiation of the plastid ceases, and CHL biosynthesis is arrested at the protochlorophyllide stage even in the presence of abundant materials for synthesis. In the bean plant the protochlorophyllide available for CHL synthesis was shown to be equivalent to that on the holochrome and also about 15% extra (12).

In the 7- to 10-day-old barley leaves, our data indicate that all of the enzymes converting ALA to CHL are present at activities which are nonlimiting during the first 6 to 12 hr in the light, even when protein synthesis had been inhibited. This result has been demonstrated by the following experiments. (*a*) In the absence of light, the feeding of ALA to the etiolated barley leaves led to the formation of excessive amounts of protochlorophyllide in the proplastids. Therefore the enzymes from ALA to protochlorophyllide were not limiting even during the lag phase in the dark. (*b*) Exposure of ALA-fed etiolated leaves to light of low intensity (to avoid photobleaching) resulted in the formation of relatively large amounts of CHL during the first 6 to 12 hr compared to leaves not fed ALA. Therefore the holochrome protein that photoreduces protochlorophyllide to chlorophyllide was not limiting. (*c*) The feeding of various nutrients, including possible substrates for ALA synthesis, were probably not limiting because they did not enhance CHL synthesis.

The limiting activity for CHL synthesis appears to be due to more than one protein which is directly or indirectly related to the synthesis of ALA. This was shown by the following experiments. (*a*) Illumination in the presence of protein synthesis inhibitors blocked CHL synthesis; nevertheless, when exogenous ALA was also present, CHL synthesis was not impaired during the first 6 to 12 hr. Therefore CHL synthesis was limited by the synthesis of some protein or proteins that formed ALA. All of the other enzymes from ALA to CHL were nonlimiting, including the holochrome photoenzyme which converted protochlorophyllide to chlorophyllide. Because little holochrome protein would be produced in the presence of protein synthesis inhibitors, the existing holochrome must have been used many times for chlorophyllide formation, indicating that the holochrome can function as a photoenzyme; this confirms the data of Bogorad *et al.* (3). (*b*) Inhibitors of RNA synthesis did not prevent appreciably the synthesis of CHL in the light during the first 6 to 12 hr. Therefore none of the mRNA, tRNA, or rRNA required for ALA formation appeared to be in limiting concentrations. (*c*) Not only did cycloheximide inhibit ALA formation, but chloramphenicol did also to a lesser degree. Because chloramphenicol inhibition is considered to occur on plastid 70S ribosomes (whereas cycloheximide inhibition is believed to occur on cytoplasmic 80S ribosomes), the synthesis of ALA may require the synthesis of at least one protein or enzyme in the cytoplasm, as well as the synthesis of at least one other protein in the plastid. (*d*) Exposure of the leaves to cycloheximide or chloramphenicol for more than 12 hr resulted in a cessation of CHL formation, even from ALA, probably because proteins converting ALA to CHL slowly turned over and became limiting.

Some properties of the protein or proteins bringing about ALA synthesis were shown by the following experiments. (*a*) When cycloheximide was added in the linear phase of CHL synthesis in the light, CHL synthesis diminished at a rate indicative of a 1½-hr half-life of the limiting step. This is presumably the half-life of the system that synthesizes ALA. This relatively short half-life is compatible with the finding of Bogorad (2), that when leaves which have been in the linear phase of CHL synthesis are returned to the dark, they lose the ability to form CHL within 2 hr. The rapid decrease in CHL-synthesizing ability may be explained by the short half-life of one or another of the enzymes involved in the system that synthesizes ALA. It is interesting to note that a short half-life of about 1 hr also has been reported for the ALA synthetase of liver mitochondria; this is based on experiments in which the rats were fed cycloheximide (14). We suggest that CHL synthesis, like heme formation in animal cells (10), is regulated by the synthesis and rapid turnover of ALA synthetase

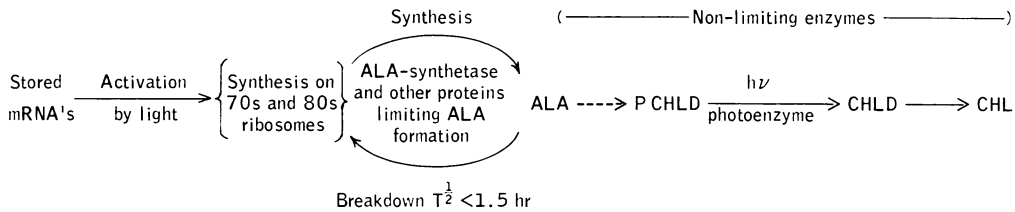

FIG. 9. A tentative working model for the control of chlorophpyll biosynthesis in barley.

and other proteins associated with and necessary for ALA synthetase activity. (*b*) Illumination of the leaves with red light for 5 to 10 min caused an increase in the rate of CHL synthesis (29), perhaps mediated by a phytochrome system (16). The more rapid CHL synthesis was prevented by inhibitors of protein synthesis, but not by inhibitors of nucleic acid synthesis. Bogorad (2) has shown that a number of enzymes rapidly appear shortly after illumination of leaves in the lag phase. Our results suggest that brief illumination leads to an enhanced synthesis, not merely activation, of at least one protein that limits ALA synthesis, and for which a mRNA may already be present. The control by light of earliest ALA synthesis may thus be at the translational, not transcriptional, level. The concept of inactive, stored mRNA's (30) activated by light into bringing about the synthesis in the lag phase of a number of enzymes, including the one for ALA synthesis, may be a useful concept to consider in this connection.

Our data do not rule out a separate mechanism of feedback inhibition control of the synthesis of δ-aminolevulinic acid by some product of the biosynthetic chain, such as that of heme or of protochlorophyllide-holochrome (26).

In analogy with what is known about ALA synthesis in animal cells, it may be speculated that in barley, the enzyme for ALA synthesis may be coded by a nuclear gene, synthesized in the cytoplasm, and then transferred to the mitochondria and plastids. Cycloheximide may inhibit the synthesis of this enzyme in the cytoplasm, and chloramphenicol may inhibit the synthesis of some protein(s) in the plastid required for transport or localization of the enzyme in the plastid.

A possible mechanism for the control of CHL synthesis is represented in Figure 9. The control on the rate of ALA synthesis and therefore the control on the rate of CHL synthesis may depend, in part, on the rate of the synthesis of a limiting protein that forms ALA, presumably ALA synthetase and the rate of its breakdown. This is in agreement with proposals by Gassman and Bogorad (5, 6). We propose, furthermore, that illumination, perhaps via phytochrome, may activate stored mRNA to make the requisite proteins for ALA synthesis. The synthesis of the proteins requires the participation of 80S ribosomes of the cytoplasm and 70S ribosomes of the plastids. The breakdown of the ALA synthetase system proceeds with a half-life of 1½ hr. Once ALA is made, it is converted to CHL by the other enzymes of the biosynthetic chain, these enzymes being nonlimiting and stable for at least 6 to 12 hr. In this model, CHL synthesis is controlled by the steady-state activity of ALA synthetase, an enzyme with a rapid turnover rate.

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