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Changes in Chlorophyll *a* and *b* Content in Dark-Incubated Cotyledons Excised from Illuminated Seedlings

THE EFFECT OF CALCIUM

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ABSTRACT

Cucumber seedlings were illuminated for various time periods, cotyledons excised, placed in the dark, and changes in chlorophyll *a* and *b* content monitored. During the dark periods, chlorophyll *b* content decreased while chlorophyll *a* did not. When the illumination time was lengthened, the percentage of chlorophyll *b* decomposition from initial levels decreased. Ca^{2+} at 50 millimolar prevented the decrease in chlorophyll *b* and caused a decrease in chlorophyll *a*. The effect of Ca^{2+} decreased with increased illumination time. Cycloheximide and chloramphenicol inhibited chlorophyll *b* decrease, but did not induce chlorophyll *a* decrease.

When Ca^{2+} was applied to cotyledons, excised from 4 hour illuminated seedlings and preincubated with water in the dark until chlorophyll *b* content had decreased to a low level, chlorophyll *b* began to accumulate in the dark. Cycloheximide and chloramphenicol had no such effect. Ca^{2+} also induced an accumulation of chlorophyll *b* in cotyledons excised from seedlings treated with periodic light (2 minutes light followed by 98 minutes dark) during dark incubation. Our results indicate that there is unstable chlorophyll present in the early phase of greening, and that Ca^{2+} induces chlorophyll *b* synthesis from chlorophyll *a* in the dark.

When etiolated tissues are exposed to light, Chl is synthesized after a few hours of lag period (13) and then becomes functional in the photochemical reactions of photosynthesis (16), perhaps by being incorporated into photosynthetic membranes as greening proceeds. But during the greening phase, some Chl is unstable and rapidly decomposes in the dark (4, 17). Kupke (4) reported that Chl *b* in the early phase of greening disappeared soon after the plant tissue was returned to the dark. But Chl *b* in the late phase of greening is stable in the dark (4, 17). We found that Chl was not stabilized in the early phase of greening in cucumber cotyledons treated with Ca^{2+} (14). These facts suggest that in the early phase of greening, there exists some easily decomposed Chl population which may not be situated in the photosynthetic membranes. Shlyk *et. al.* (11) reported that Chl *b* was formed from "young" molecules of Chl *a* in the biosynthetic center, and that CP[1] hindered the maturation of Chl *a*, resulting in its conversion to Chl *b*. Little is known about the induction and regulation of Chl *b* synthesis. Using Ca^{2+}, which breaks down unstable Chl, we attempted to estimate Chl stability in greening tissue. We report the changes in Chl stability that occur during greening, and the Ca^{2+}-induction of Chl *b* formation.

[1] Abbreviations: CP, chloroamphenicol; CH, cycloheximide.

MATERIALS AND METHODS

Cucumber seeds (*Cucumis sativus* L. cv. Aonagajibai) were soaked in distilled H_2O for 3 h and then germinated on moist vermiculite at 28 C in the dark. Four days after germination, the seedlings were illuminated continuously for various time periods with white light (Mitsubishi FLR 80H W/A) at an intensity of 10,000 lux (19.4 w m^{-2} at 380 to 710 nm) at 28 C. In experiments using periodic illumination, seedlings were illuminated for 2 min followed by 98 min of darkness. This cycle was repeated from one to nine times. At the end of illumination, cotyledons were excised without their hypocotyl hook. Twenty cotyledons were wrapped in aluminum foil and weighed. Then the cotyledons were taken out of the foil in the dark, and placed with their abaxial surface up on 5.5-cm filter paper (Toyo No. 2) wetted with 1.6 ml of test solution in a 6-cm Petri dish. The dishes were kept in the dark at 28 C. The solutions used were distilled H_2O, 50 mM $CaCl_2$, 10 μg/ml CH,[1] or 100 μg/ml CP. At the end of incubation in the dark, cotyledons of each sample were blotted with filter paper, wrapped in aluminum foil, and stored at −20 C until use. All manipulations were performed under dim green safe light.

Chl was extracted with methanol and determined by the hydroxylamine method (7). Cotyledons were homogenized with absolute methanol in the dark and the homogenate was centrifuged at 20,000*g* for 15 min. The pellet was resuspended with methanol and centrifuged again. One-twentieth the volume of the combined supernatants of 5.76 M hydroxylamine solution was added to the supernatant, and the mixture was incubated at room temperature for 10 min, then centrifuged at 10,000*g* for 10 min. The resultant supernatant *A* at 666 nm was measured against a blank without hydroxylamine. Chl was expressed as μg/g fresh weight.

RESULTS

In water controls during dark incubation after a short time of illumination (4 h), a large part of the Chl *b* disappeared but Chl *a* did not. In Ca^{2+}-treated cotyledons, Chl *a* decreased and Chl *b* did not (Table I). As the illumination period was prolonged (up to 12 h), the decrease of Chl *b* in water controls and that of Chl *a* in Ca^{2+}-treated samples became smaller. No decrease in Chl content was observed in cotyledons in the dark when illumination periods of 24 h were used.

Figure 1 shows the time course of Chl decomposition in the dark after illumination for 4 or 9 h. When 4-h illuminated cotyledons were treated with water, almost all Chl *b* disappeared during the first 24 h of dark incubation, but Chl *a* remained unchanged. In Ca^{2+}-treated samples, Chl *b* decreased slightly during the first 6 h and then remained at a constant level which was higher than that of the water control. Chl *a* in Ca^{2+}-treated samples markedly decreased during the first 24 h, then remained

Table I. *Effect of Ca^{2+} on the Decomposition of Chl in the Dark in Cotyledons from Seedlings Illuminated for Different Periods*

Four-day-old etiolated seedlings were illuminated for different periods. After that, cotyledons were excised and incubated with water or 50 mM Ca^{2+} in the dark for 24 h. Each value represents the mean of four samples with SD.

Illumination	Dark Incubation	Chl *a*	Chl *b*
h		*μg/g fresh wt*	
4	Initial	57.6 ± 2.4	15.7 ± 0.5
	Water	66.8 ± 4.1	3.2 ± 0.7
	$CaCl_2$	51.4 ± 2.8	10.8 ± 1.0
6	Initial	146 ± 7	46 ± 1
	Water	172 ± 8	18 ± 2
	$CaCl_2$	137 ± 6	30 ± 2
12	Initial	423 ± 8	106 ± 2
	Water	453 ± 16	83 ± 2
	$CaCl_2$	396 ± 33	93 ± 6
24	Initial	722 ± 30	166 ± 7
	Water	740 ± 45	168 ± 7
	$CaCl_2$	721 ± 48	171 ± 12

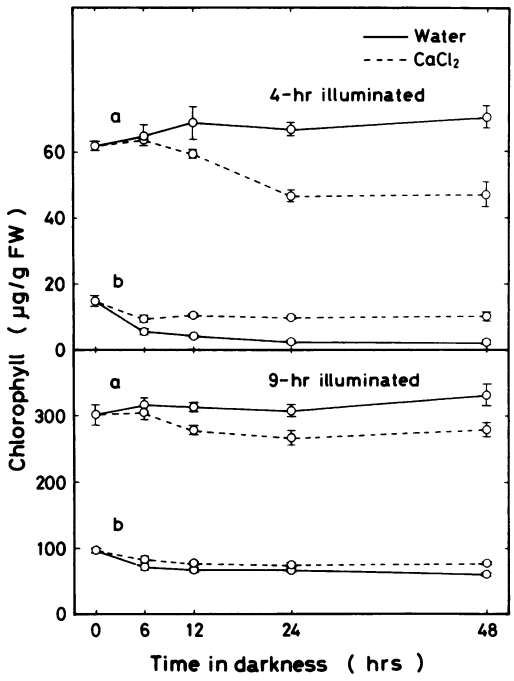

FIG. 1. Effect of Ca^{2+} on Chl decomposition in the dark in cotyledons from seedlings illuminated for 4 or 9 h. Four-day-old seedlings were illuminated for 4 h (upper panel) or 9 h (lower panel) and cotyledons were excised. The cotyledons were incubated with water (O——O) or 50 mM Ca^{2+} (O---O) in the dark. Each plot represents the mean of four samples. Vertical bars indicate the standard error of the mean. Upper curves: Chl *a*; lower curves: Chl *b*.

at a constant level. In 9-h illuminated cotyledons, the decrease in Chl *b* in water controls and Chl *a* in Ca^{2+}-treated samples was less pronounced compared to the decrease in 4-h illuminated cotyledons. In 9-h illuminated cotyledons as well, the levels of Chl *a* and *b* remained constant after the initial 24 h in the dark.

After various periods of dark incubation of 4-h illuminated cotyledons with water, they were treated with Ca^{2+} for a subsequent 24 h in the dark, and the changes in Chl content were measured (Fig. 2). The Ca^{2+}-induced effect of Chl *a* decrease became smaller as the dark preincubation with water was prolonged. In the first 12 h of the dark preincubation with water Chl *b* decreased to a low level which did not subsequently change.

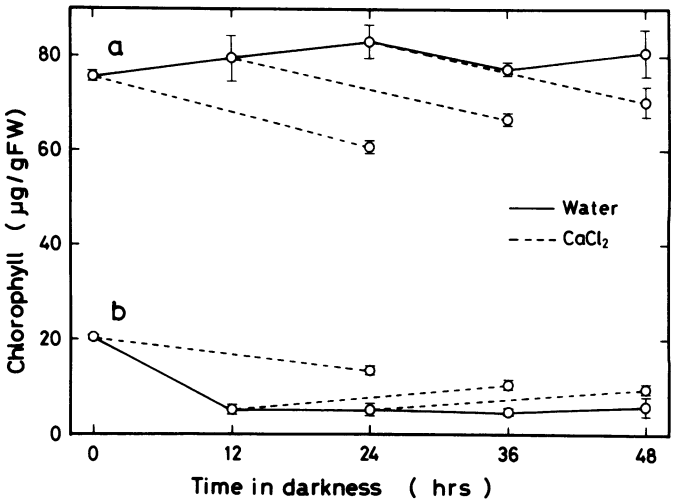

FIG. 2. Effects of Ca^{2+} on the amounts of Chl *a* and *b* after various periods of preincubation with water in the dark. Four-day-old seedlings were illuminated for 4 h and excised cotyledons were preincubated with water in the dark (O——O). After 0, 12, or 24 h of preincubation with water, cotyledons were incubated with 50 mM Ca^{2+} (O---O) for another 24 h in the dark. Each plot represents the mean of four samples. Vertical bars indicate the standard error of the mean. Upper curves: Chl *a*; lower curves: Chl *b*.

Table II. *Effects of Ca^{2+} and Inhibitors of Protein Synthesis on the Decomposition of Chl in the Dark in Cotyledons from Seedlings Illuminated for 4 h*

Four-day-old seedlings were illuminated for 4 h. After that, cotyledons were excised and incubated with Ca^{2+}, CH or CP in the dark for 24 h. Each value represents the mean of four samples with SD.

Dark Incubation	Chl *a*	Chl *b*
	μg/g fresh wt	
Initial	63.4 ± 1.4	17.0 ± 1.8
Water	68.9 ± 1.7	4.5 ± 0.7
50 mM $CaCl_2$	51.4 ± 3.3	13.7 ± 0.7
10 μg/ml CH	62.2 ± 5.3	10.9 ± 0.8
100 μg/ml CP	65.7 ± 0.6	11.6 ± 0.8

However, when cotyledons were incubated with Ca^{2+} after the Chl *b* level had decreased, Chl *b* began to increase in the dark. The longer the period of preincubation with water, the smaller the amount of Chl *b* accumulated during the Ca^{2+} incubation.

The effect of Ca^{2+} on Chl content in the dark was compared with the effects of the protein synthesis inhibitors CH and CP (Table II and III). Both inhibitors prevented the decrease in Chl *b*, though to a lesser extent than Ca^{2+} (Table II). Neither caused a decrease in Chl *a* levels. After preincubation in water for 24 h in the dark, cotyledons were transferred to medium containing CH, CP, or Ca^{2+} (Table III). Ca^{2+} caused an increase in Chl *b* and a concomitant decrease in Chl *a* (Fig. 2), but CH and CP had no effect whatsoever.

It has been reported that Chl *b* is not synthesized under intermittent illumination (9). In our experiments with cucumber cotyledons, very little Chl *b* was accumulated after 9 cycles of periodic light (Fig. 3). Chl *a* synthesized under periodic light was unstable compared with that synthesized under continuous illumination. Decomposition of Chl *a* occurred to some extent in the former (Table IV), but never in the latter (Table I, Fig. 1). When cotyledons were incubated with Ca^{2+} after treatment with periodic illumination, Chl *b* was synthesized in parallel with a substantial decrease in Chl *a* content.

Table III. *Induction of Chl b Formation by Ca^{2+} in Cotyledons Preincubated with Water in the Dark*

Four-day-old seedlings were illuminated for 4 h. Excised cotyledons were incubated with water for 24 h and then with Ca^{2+}, CH, and CP, for another 24 h in the dark. Each value represents the mean of four samples with SD.

Incubation[a]	Chl *a*	Chl *b*
	μg/g fresh wt	
Before preincubation	56.4 ± 4.1	14.8 ± 1.5
After preincubation	55.8 ± 2.8	2.0 ± 0.5
After incubation with:		
Water	56.2 ± 4.9	1.1 ± 1.0
50 mM $CaCl_2$	44.7 ± 4.5	6.0 ± 1.1
10 μg/ml CH	53.1 ± 3.6	1.2 ± 0.2
100 μg/ml CP	51.1 ± 4.4	1.4 ± 0.4

[a] Preincubation, water, 24 h dark; incubation, test solution, 24 h dark.

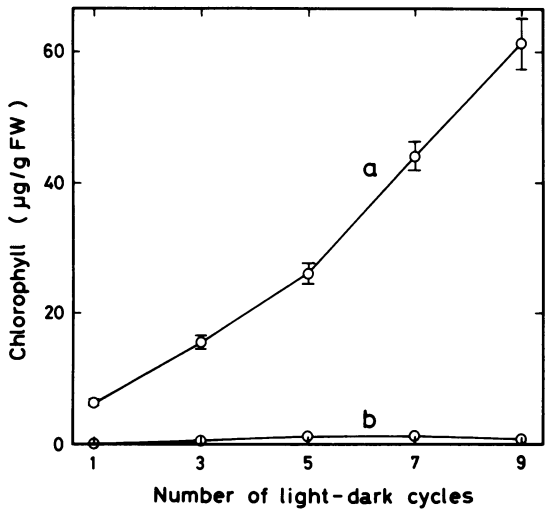

FIG. 3. Accumulation of Chl *a* and *b* in cotyledons exposed to cycles of periodic illumination. Four-day-old seedlings were illuminated for 2 min followed by 98 min of darkness and the cycle repeated. Cotyledons were sampled at the end of the 2 min illumination. Each plot represents the mean of four samples. Vertical bars indicate the standard error of the mean. Upper curve: Chl *a*; lower curve: Chl *b*.

Table IV. *Induction of Chl b Formation by Ca^{2+} in the Dark in Cotyledons from Seedlings Treated with Periodic Illumination*

Four-day-old seedlings were treated with 4 or 8 cycles of 2 min light and 98 min dark. At the end of 2 min illumination of the last cycle, cotyledons were excised. The cotyledons were incubated with water or 50 mM Ca^{2+} in the dark for 24 h. Each value represents the mean of four samples with SD.

No. of Cycles	Dark Incubation	Chl *a*	Chl *b*
		μg/g fresh wt	
4	Initial	22.0 ± 1.1	0
	Water	19.7 ± 2.5	0
	$CaCl_2$	11.5 ± 1.2	1.9 ± 0.5
8	Initial	49.1 ± 2.6	0.6 ± 0.3
	Water	43.1 ± 1.1	0
	$CaCl_2$	36.8 ± 0.5	4.7 ± 0.3

DISCUSSION

It has been reported that Chl molecules bind to specific proteins (15) that are situated at definite sites in the photosynthetic membranes (3) after which they become functional in the photochemical reactions of photosynthesis. Tissues in the earliest phase of greening lack photosynthetic activity that develops as greening proceeds. Although the site of Chl synthesis and how it is incorporated into the photosynthetic membrane are poorly understood, the newly synthesized Chl molecules are probably "free" or not bound to a specific site. Such Chl may be subjected to decomposition or conversion to Chl *b*. There is some evidence for the existence of labile Chl (4, 11, 14). Shlyk *et al.* (9, 10) proposed the existence of "young" Chl *a* that easily converts to Chl *b* in tissues at an early stage of greening. They claimed that "young" Chl *a* was located in or near the biosynthetic center. Kupke (4) showed that Chl *b* in tissues at an early phase of greening soon decomposed in the dark but became stable as greening proceeded. We showed in a previous paper (14) that Ca^{2+} broke down newly synthesized Chl in the dark as well as in the light. Taking advantage of this fact, we used Ca^{2+} in the present study to examine the stability of Chl during greening.

When the cotyledons of illuminated seedlings were placed in the dark, Chl *b* decreased in water controls and Chl *a* decreased in Ca^{2+}-treated samples, eventually stabilizing at constant levels. As the illumination period was prolonged, the changes in Chl *a* and *b* content diminished. These results indicate that some portion of the Chl population is unstable but becomes stable as greening proceeds. Chl *a* stabilization also takes place in the dark, since a long preincubation with water made Chl *a* less susceptible to the effect of Ca^{2+}. It has not yet been determined whether this unstable Chl population is the same as Shlyk *et al.*'s "young" Chl *a*.

Many authors have reported the conversion of Chl *a* to Chl *b* (10) but very little information is known about the site and regulation of Chl *b* synthesis. A substantial increase in Chl *b* takes place after the end of the lag period of Chl formation under continuous illumination (2). Preillumination which eliminates the lag period also stimulates Chl *b* accumulation during continuous illumination (5). Chl *b* synthesis is dependent on both the wavelength of the light used (6) and the intensity (8). Chl *b* is synthesized from Chl *a* in the dark after a pulse of light (12), and preillumination with a pulse of light enhances Chl *b* synthesis after a second pulse of light (5), while treatment with CP mimicks the effect of the first pulse of light (11).

All above studies concerning Chl *b* synthesis in the dark were those with etiolated tissues exposed to light for a short time. We found that Ca^{2+} induces Chl *b* synthesis in the dark in cotyledons of seedlings which had been illuminated for long periods of time, and were actively synthesizing Chl *a* and *b*. Since Ca^{2+} inhibits the rapid decrease in Chl *b* content during dark incubation, Ca^{2+} may inhibit Chl *b* decomposition, or enhance Chl *b* synthesis from Chl *a* (since new Chl is not synthesized in the dark). In tissues that were either preincubated for long periods in water so that Chl *b* content was low, or in cotyledons illuminated with intermittent illumination, Ca^{2+} causes an increase in Chl *b* and a concomitant decrease in Chl *a*. These results suggest that Chl *b* is formed from Chl *a* in the presence of Ca^{2+}.

The protein synthesis inhibitors CH and CP inhibit Chl *b* decomposition in the dark, but do not induce Chl *b* formation and Chl *a* decomposition or transformation. They may affect protease or chlorophyllase activity.

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