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Chloroplast Biogenesis

XX. ACCUMULATION OF PORPHYRIN AND PHORBIN PIGMENTS IN CUCUMBER COTYLEDONS DURING PHOTOPERIODIC GREENING[1]

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ABSTRACT

A study of greening in cucumber (*Cucumis sativus* L.) cotyledons grown under a light (14-hour) dark (10-hour) photoperiodic regime was undertaken. The pools of protoporphyrin IX, Mg-protoporphyrin IX monoester, protochlorophyllide, and protochlorophyllide ester were determined spectrofluorometrically. Chlorophyll *a* and *b* were monitored spectrophotometrically. Pigments were extracted during the 3rd hour of each light period and at the end of each subsequent dark period during the first seven growth cycles. Protoporphyrin IX did not accumulate during greening. Mg-protoporphyrin IX monoester and longer wavelength metalloporphyrins accumulated during the light cycles and disappeared in the dark. Their disappearance was accompanied by the accumulation of protochlorophyll. Higher levels of protochlorophyll were observed in the dark than in the light, and the greatest accumulation occurred during the third and fourth dark cycles. Protochlorophyllide was present in 3- to 10-fold excess over protochlorophyllide ester; it was detectable during the period of net chlorophyll accumulation as well as afterward. In contrast, protochlorophyllide ester was observable only during the first four photoperiodic cycles, suggesting that it was a metabolic intermediate only during the early stages of chlorophyll accumulation. Between the third and fourth growth cycles, a rapid increase in area and fresh weight per cotyledon began. This was accompanied by a 250-fold increase in the level of chlorophyll *a* + *b* during the three subsequent growth cycles. No lag period in the accumulation of chlorophyll *b* was observed, and at all stages of greening, the chlorophyll *a*/*b* ratio was approximately 3.

Although the sequence of structural changes that occurs during plastid development suggests that prothylakoid membranes are used in some way in the formation of photosynthetically active thylakoids, many of the biochemical details of this process remain to be elucidated. Conventional studies have been confined mainly to the greening of etiolated plant tissues, grown in continuous darkness for several days or weeks, then irradiated either with continuous light (7, 19) or with brief light flashes interspaced with dark periods (1). It has been tacitly assumed that the sequence of biochemical events taking place during these treatments is identical to that occurring during greening under field conditions.

As part of an effort to investigate the biochemistry of Chl formation and photosynthetic membrane biogenesis as they occur in nature, we have undertaken a systematic investigation of the greening process in plants grown under a light-dark photoperiodic regime. In this study, the pools of biosynthetic intermediates between protoporphyrin IX and Pchl were measured in parallel with the accumulation of Chl, during both the light and dark cycles, on successive days of the greening process. In this context, Pchl refers to the mixture of Pchlide and Pchlide ester encountered in plant tissues.

A preliminary communication was presented at the annual meeting of the American Society of Plant Physiologists in May, 1976 (5).

MATERIALS AND METHODS

Plant Material and Growth Conditions. Cucumber seed (*Cucumis sativus* L. cv. Beit Alpha MR) was purchased from the Niagara Chemical Division, FMC Corporation, Modesto, Calif.

Two liters of vermiculite (Terra Lite) were placed in a Pyrex tray (22 × 34 cm) and uniformly moistened with 1.2 liters of Hoagland nutrient solution. Seeds were evenly distributed over the moist vermiculite and covered with a thin layer of vermiculite (1 cm) which was moistened with a small amount of distilled H_2O. Finally, the planted tray was covered with an inverted Pyrex tray. Covered trays were placed in a growth chamber at 28 C under a 14-hr light 10-hr dark photoperiodic regime, starting at the beginning of the first light cycle. Illumination was generated by 14 banks of cool white fluorescent lights and six 60 w tungsten bulbs. The light intensity as measured with a calibrated Isco spectroradiometer model SR and a Weston light meter model 756 was 3,538 μw cm^{-2} (900 ft-c). On the 3rd day after sowing, the cover trays were removed and the seedlings were watered daily thereafter with Hoagland solution.

Harvesting. When cotyledons were harvested during a dark cycle, all manipulations were performed under a dim green safelight. When they were harvested during a light cycle, manipulations were performed under subdued laboratory lighting (about 10 ft-c).

Measurement of Cotyledonary Areas. The outlines of excised cotyledons were traced on paper, and the enclosed areas were determined by planimetry. At least one dozen cotyledons were used for each area determination.

Extraction and Determination of Chl. Three g of cotyledons were sliced into sections and homogenized at 0 to 4 C for 2 min in 20 ml of acetone-0.1 N NH_4OH (9:1, v/v) (15) in a Sorvall Omni-Mixer. After centrifugation, the pellet was washed once with 3 ml of 80% acetone (v/v) and recentrifuged. The resulting supernatant was combined with the original extract. The amount of Chl in the 80% acetone extract was determined spectrophotometrically according to Anderson and Boardman (2).

Although most of the Chl was extracted in acetone-0.1 N NH_4OH, a small amount remained bound to the washed pellet. The amount of bound Chl was determined spectrofluorometrically as described earlier (13), but with the following modifications. The acetone-precipitated lipoprotein pellet was thoroughly suspended in 15 ml of 0.5 M sucrose, 0.2 M tris-HCl

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(pH 7.7) with the aid of a TenBroeck homogenizer. The corrected fluorescence emission spectrum of the bound Chl was recorded at an excitation wavelength of 440 nm, and exhibited an emission band between 640 and 700 nm, with an emission maximum at about 676 nm (13). The fluorescence integral between 640 and 700 nm (*i.e.* the area between 640 and 700 nm) was determined by planimetry and was converted to nmol of Chl *a* + *b* by reference to a standard curve. The standard curve was calibrated in nmol of Chl *a* + *b* (3:1)/ml of pellet suspension. Within the concentration range of 0.05 to 1.6 nmol of Chl *a* + *b*/ml of pellet suspension, the fluorescence integral between 640 and 700 nm was proportional to the Chl *a* + *b* concentration.

Extraction and Determination of Protoporphyrin IX, Mg-Protoporphyrin IX Monoester, and Pchl. After adjusting the Chl extract to 75% acetone with H_2O, Pchlide ester was transferred to hexane and determined spectrofluorometrically as described earlier (15). Protoporphyrin IX and Pchlide remained in the hexane-extracted acetone fraction, and were determined spectrofluorometrically as reported elsewhere (15). Mg-protoporphyrin IX monoester and the longer wavelength metalloporphyrins (14) (*i.e.* the MPE-equivalent)[3] were determined according to Smith and Rebeiz (18).

Spectrophotometry. Spectrophotometric measurements were performed with an Aminco dual wavelength spectrophotometer, model DW-2, operated in the split beam mode.

Spectrofluorometry. Corrected fluorescence emission spectra of pigment extracts were recorded at room temperature on a Perkin-Elmer spectrofluorometer, model MPF-3, equipped with a corrected spectra accessory (14, 15).

Chromatography. In order to determine whether the hexane extract (containing lipophilic pigments including Pchlide ester and Chl) was contaminated with Pchlide, this fraction was concentrated under N_2 gas. The pigments were segregated by chromatography on thin layers of Silica Gel H, developed in benzene-ethyl acetate-ethanol (8:2:2, v/v/v) (12).

RESULTS

Cotyledon Growth during Photoperiodic Greening. The cucumber cotyledons exhibited a significant increase in fresh weight and expanded considerably during the first seven growth cycles (Fig. 1). The radicle pierced the seed coat within 24 hr after sowing. During the second growth cycle, the radicle elongated, further splitting the seed coat. Between the third and fourth growth cycles, the seeds began to emerge from beneath the vermiculite, and extensive splitting of the seed coats occurred, thus exposing the cotyledonary surfaces. At this stage, a rapid growth of the cotyledons began which, by the seventh cycle, resulted in an 18-fold increase in fresh weight and surface area. Concomitantly, a considerable amount of Chl was formed as the cotyledons acquired a deep green color.

Accumulation of Chl during Photoperiodic Greening. Beside the increase in fresh weight and surface area, the most obvious change induced by photoperiodic growth was the accumulation of Chl which followed sigmoid kinetics. Since dry seeds were devoid of detectable tetrapyrroles, any Chl that accumulated was synthesized *de novo*. During the emergence of the radicle and cotyledons from the seed coat, and until the cotyledons had become fully exposed (by the fourth growth cycle), Chl *a* + *b* biosynthesis proceeded slowly. The first traces of Chl *a* and *b* were usually detected during the second light cycle (Fig. 2). Before this time, these pigments could not be detected either by spectrophotometry or by the sensitive spectrofluorometric technique of Boardman and Thorne (4). A rapid linear accumulation of these pigments began during the third growth cycle that paralleled the emergence and subsequent physical development of the cotyledons (Fig. 1). By the seventh growth cycle, a 250-fold increase in the level of Chl *a* + *b* was observed. At all stages of greening, the Chl *a*/*b* ratio was approximately 3.

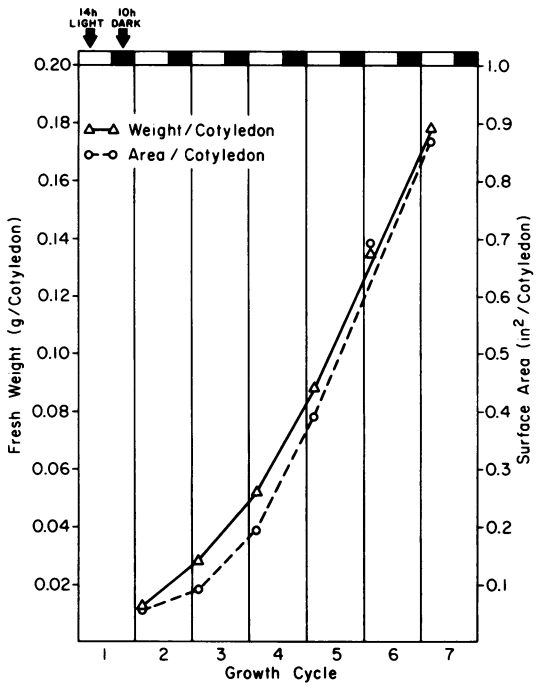

FIG. 1. Increase in fresh weight (△) and surface area (○) of cucumber cotyledons during photoperiodic growth. Measurements were made during the 3rd hr of each light cycle.

Accumulation of Pchl during Photoperiodic Greening. The levels of Pchlide and Pchlide ester, the respective immediate precursors of Chlide *a* and Chl *a*, were also monitored during the light and dark cycles of the greening process. Neither of these pigments could be detected in dry seeds. Their synthesis and accumulation was first observed during the first dark cycle (Fig. 3).

Pchlide constituted the bulk of the Pchl pool (Fig. 3A); after the second growth cycle, it was detectable at all times during the light and dark phases of greening. Although its rate of accumulation was maximal during the third and fourth dark cycles, significant amounts (approximately 50 pmol/cotyledon) were unambiguously detected during the subsequent dark cycles, and even in 3-week-old cotyledons (22nd growth cycle).

In contrast, Pchlide ester was detectable only during the first four growth cycles, and its concentration was only about $^1/_{10}$ that of Pchlide (Fig. 3B). Chromatography of the Pchlide ester fraction on thin layers of silica gel revealed no contamination by Pchlide.

The smaller amounts of Pchlide ester measured during the light phases as compared to the dark phases of photoperiodic greening suggested that it was photoconverted to Chl in the light. After the fourth dark cycle, it was difficult to assess whether traces of Pchlide ester were still present or not, due to the relatively large amounts of Chl in the tissue which interfered with the spectrofluorometric assay.

Light-dependent Accumulation of Mg-Protoporphyrin IX Monoester + Longer Wavelength Metalloporphyrins during Photoperiodic Greening. It was reported previously that during greening under continuous illumination in distilled H_2O, etiolated cucumber cotyledons synthesized and accumulated Mg-protoporphyrin IX monoester + longer wavelength metalloporphyrins (14). The longer wavelength metalloporphyrins were

[3] Abbreviation: MPE-equivalent: Mg-protoporphyrin IX monoester + longer wavelength metalloporphyrins.

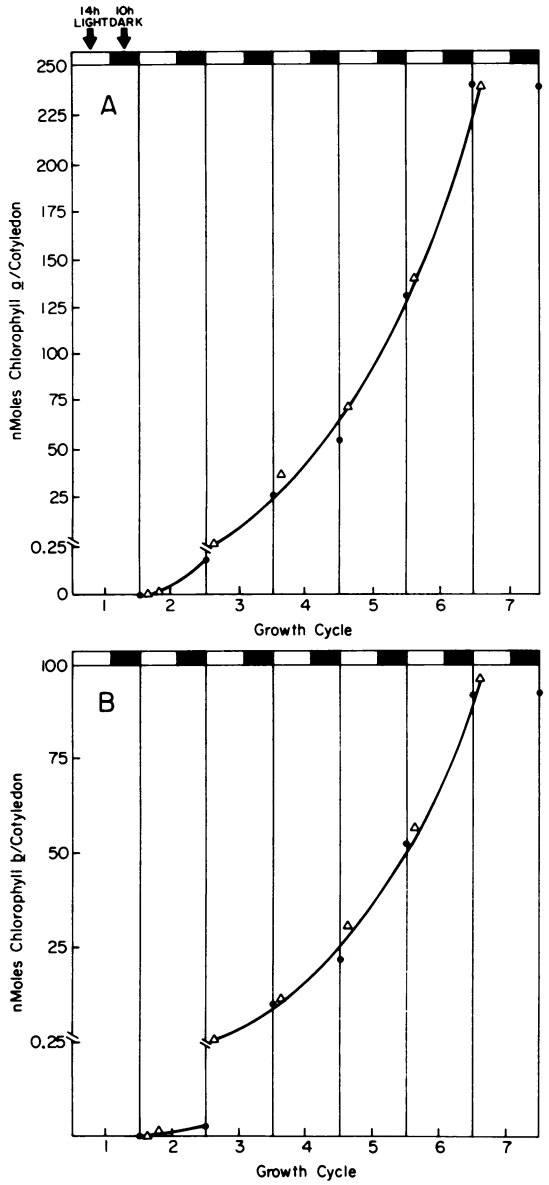

FIG. 2. Accumulation of Chl *a* (A) and Chl *b* (B) in photoperiodically grown cucumber cotyledons. Seeds were planted at the beginning of the first light cycle. Determinations were made during the 3rd hr of each light cycle (△) and at the end of each dark cycle (●), unless otherwise indicated. In all experiments, the Chl *a*/*b* ratio was approximately 3.

postulated to represent the metabolic intermediates between Mg-protoporphyrin IX monoester and divinyl Pchlide (14). It was therefore of interest to investigate the behavior of these putative intermediates of the Pchl biosynthetic pathway during photoperiodic greening.

The MPE-equivalent accumulated only during the light cycles of photoperiodic greening, and disappeared during the subsequent dark cycles (Fig. 4). This was compatible with their presumed metabolic role.

Accumulation of Protoporphyrin IX during Photoperiodic Greening. Protoporphyrin IX did not accumulate either during the light or dark cycles of photoperiodic greening.

DISCUSSION

During the past 3 decades, the greening process in higher plants has been most conveniently studied in seedlings grown in darkness for several days or weeks and then subsequently illuminated. The interference of Chl with the spectrophotometric determination of the metabolic pools between protoporphyrin IX and Pchl precluded a detailed quantitative study of the greening process in tissues already containing significant amounts of Chl. The present investigation was made possible by the availability of newly developed spectrofluorometric assays for the quantitative determination of protoporphyrin IX, Mg-protoporphyrin IX monoester, longer wavelength metalloporphyrins, Pchlide, and Pchlide ester in the presence of large amounts of Chl (14, 15).

One of the characteristics of etiolated tissues greening under continuous light is the observation of a time lag in the biosynthesis and accumulation of Chl *b* in comparison to that of Chl *a*. The extent of this lag phase seems to depend on the plant species and on the age of the etiolated tissue (6, 11, 19). For example, Thorne and Boardman (20), using a sensitive spectro-

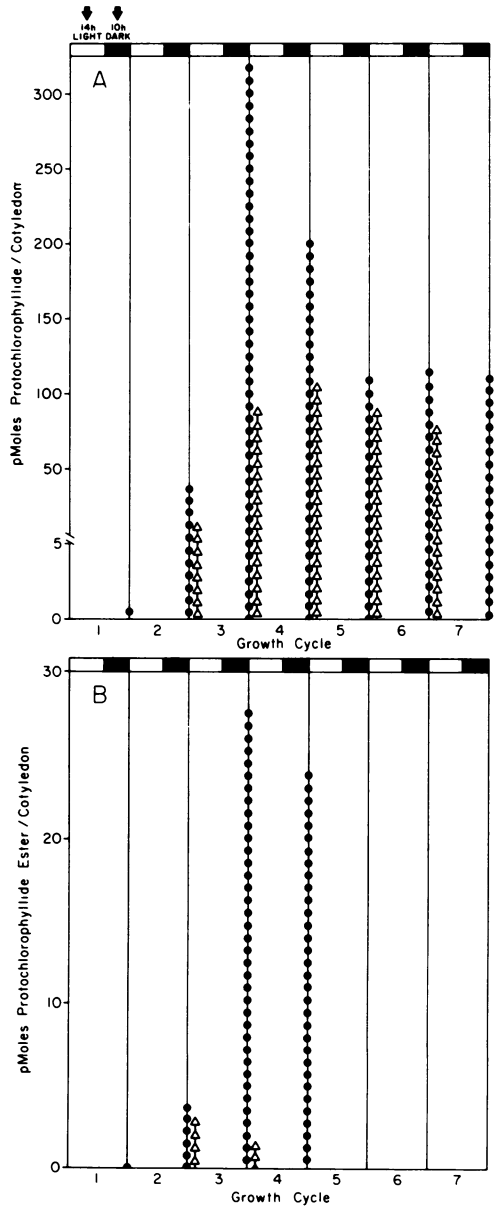

FIG. 3. Levels of Pchlide (A) and Pchlide ester (B) in greening cotyledons during the 3rd hr of each light cycle (△) and at the end of each dark cycle (●). In some experiments, traces of Pchlide were observed in extracts prepared during the second light cycle.

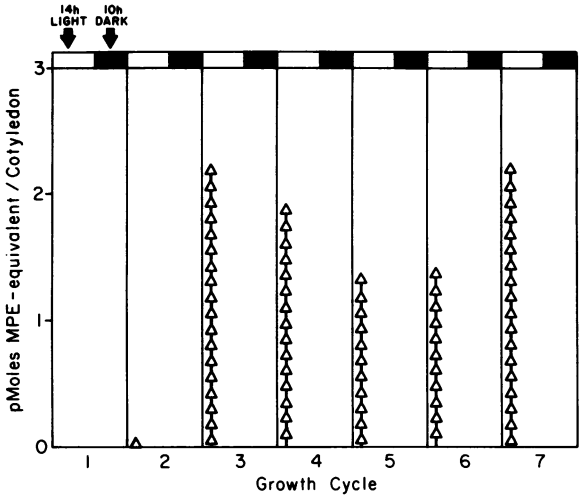

FIG. 4. Light-dependent accumulation of Mg-protoporphyrin IX monoester + longer wavelength metalloporphyrins (MPE-equivalent) during photoperiodic greening. Determinations were made during the 3rd hr of each light cycle (△) and at the end of each dark cycle (●). The MPE-equivalent was detected only during the light cycles.

fluorometric assay, reported a lag phase of 10 min in the formation of Chl *b* in etiolated pea seedlings; afterward, the ratio of Chl *a* to *b* decreased from 300 at 10 min to 15 after 1 hr. In contrast, no lag phase in the biosynthesis of Chl *b* was observed during photoperiodic greening. Chl *a* and *b* appeared to be formed simultaneously as soon as Chl formation became detectable (Fig. 2).

Protoporphyrin IX did not accumulate during photoperiodic greening. On the other hand, Mg-protoporphyrin IX monoester and longer wavelength metalloporphyrins accumulated only during the light phases of the growth cycles. They are present continuously during active Chl accumulation in the light, and gradually disappear in the dark (Smith and Rebeiz, unpublished data). The conversion of Mg-protoporphyrin IX monoester into Pchlide was recently reported by Mattheis and Rebeiz (10), thus demonstrating unambiguously the metabolic role of this tetrapyrrole. The longer wavelength metalloporphyrins were postulated to represent the intermediates between Mg-protoporphyrin IX monoester and divinyl Pchlide (14–16, 18). The MPE-equivalent was thus formed in excess during active greening, and was presumably converted to Pchl in the dark.

Pchlide was always present in higher amounts than Pchlide ester, and was more ubiquitous during the life of the cotyledons (Fig. 3). Furthermore, the levels of Pchlide were always higher in the dark than in the light. These observations are compatible with the acknowledged photoconvertibility of Pchlide to Chlide *a* in the light, and the role of Pchlide as the major precursor of Chl in higher plants (3, 6, 17).

In contrast, Pchlide ester was detectable only during the first four growth cycles (Fig. 3B). Like Pchlide, it was present at higher levels during the dark phases of photoperiodic greening than during the light phases (Fig. 3B). Since the phototransformation of Pchlide ester has been recently demonstrated in several laboratories (8, 9), it is concluded that this pigment contributes to Chl accumulation only during the early stages of photoperiodic greening.

The Pchl holochromes which appear to participate in Chl biosynthesis during photoperiodic greening will be described in forthcoming papers.

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