Plant Physiol. (1982) 69, 1392–1400
0032-0889/82/69/1392/09/$00.50/0

Development of Photochemical Activity in Relation to Pigment and Membrane Protein Accumulation in Chloroplasts of Barley and Its Virescens Mutant[1]

Received for publication August 24, 1981 and in revised form December 31, 1981

DAVID J. KYLE[2] AND SAUL ZALIK
Department of Plant Science, University of Alberta, Edmonton, Alberta T6G2E3 Canada

ABSTRACT

The development of photochemical activity in relation to pigment and membrane protein accumulation in chloroplasts of greening wild-type barley (*Hordeum vulgare* L. cv. Gateway) and its virescens mutant were studied. The rate of chlorophyll accumulation per plastid was faster in the wild-type than in the mutant seedlings upon illumination after 6 days of etiolation, but was not different after 8 days. Although the protein content per plastid did not vary during greening, there was a change in the sodium dodecyl sulfate-polyacrylamide gel polypeptide profiles. High molecular weight proteins of 96,000 and 66,000 decreased whereas those at 34,000, 27,000 and 22,000 increased in relative quantity as a function of greening. The fully greened mutant seedlings were not deficient in the light-harvesting chlorophyll protein complex (LHC) or the reaction centers of photosystem I and photosystem II. Photosystem I-associated photochemical activities appeared within the first hour of plastid development and photosystem II associated activities and O_2 evolution within the next 6 hours. In all cases, the developmental rates per unit protein were slower in the mutant following 6 days of etiolation, but no differences between the two genotypes could be seen after 8 days due to a decrease in the developmental rate of the wild-type chloroplasts. An increase in photosynthetic unit size associated with plastid morphogenesis was faster in the wild-type seedlings after 6 days, but again the difference was negligible after 8 days. It was concluded that no single measured photochemical parameter is affected by this mutation, but rather, all aspects of chloroplast development are affected similarly by an overall reduction in the rate of chloroplast morphogenesis. This mutant, therefore, undergoes the normal pattern of proplastid to chloroplast development, but at a markedly reduced rate.

Under natural conditions, proplastids in undifferentiated tissue will develop into chloroplasts as the tissue matures (16). The study of greening under natural light conditions, however, is confounded by the problem that the proplastid is rapidly transformed into a chloroplast when the tissue is no longer meristematic, and therefore, plastid preparations are usually heterogeneous with respect to the developmental state of the chloroplasts. In order to synchronize the development of all the chloroplasts, plants can be grown in the dark to a certain stage and then placed in the light. This synchronization leads to more homogeneous preparations with respect to plastid development, and allows the study of the appearance of various parameters associated with greening (3, 16). Although this procedure works well with algae (26, 28), the major difficulty when angiosperm seedlings are used is that the proplastid develops up to a 'resting stage' the etioplast (16). This artifical stage is never seen under natural conditions of plastid development.

Another effective method of studying chloroplast morphogenesis involves the use of specific mutations which delay or halt development at a particular point in the sequence of events. One such mutant, the virescens mutant of Gateway barley is characterized by a nonlethal developmental mutation which exhibits a lag in Chl biosynthesis that is affected by light and temperature (19). Several mutant lines of corn, soybean, and cotton have also been described which exhibit a temperature-induced virescens (1). In order to circumvent the problems associated with the initial lag in Chl accumulation in the virescens mutant of barley, we chose to keep the seedlings etiolated for 6 to 8 d before studying the development of photosynthetic function during the etioplast to chloroplast conversion. In this manuscript, we present a characterization of this mutant with respect to the appearance of photochemical activities, and the development of thylakoid membrane proteins.

MATERIALS AND METHODS

Plant Material. Wild-type barley (*Hordeum vulgare* L. cv. Gateway) (N) and its virescens mutant (M) used in this study were described previously (22).

Seeds of the two lines were planted in trays of vermiculite and watered with a full-strength Hoagland solution. For etiolation/greening studies, the plants were kept in the dark at 22°C for 6 or 8 d and then illuminated at 8000 lux for various periods of time. Plants were also grown under continuous light for 6 or 8 d.

Chloroplast Isolation. Chloroplasts were isolated by a modified method of Leese and Leech (17). Fifty g of leaf laminae were homogenized for 15 s with 250 ml of 67 mM phosphate buffer (KH_2PO_4/Na_2HPO_4; pH 7.6) containing 0.5 M sucrose, 1 mM $MgCl_2$, and 0.2% BSA. The homogenate was filtered through eight layers of cheesecloth and two layers of nylon mesh (50 μm pore size), and centrifuged at 4000*g* for 2 min. The crude pellet was washed twice with resuspending buffer consisting of 50 mM Hepes (pH 7.6); 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, and 1 mM $MnCl_2$. Any remaining intact chloroplasts were broken by osmotic shock and pipetting during the second wash.

Electrophoresis. Electrophoresis of membrane proteins was carried out using the discontinuous buffer system originally described by Laemmli (15). Chloroplast membrane pellets were resuspended in a sample buffer consisting of 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromo-

[1] Supported by a Natural Sciences and Engineering Research Council of Canada grant to S. Z. D. J. K. is recipient of a Natural Sciences and Engineering Research Council Post Graduate Scholarship.

[2] Present address: MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824.

phenol blue, with an SDS to Chl ratio of 20:1. The samples were then immersed in boiling water for 5 min and centrifuged for 1 min in a Beckman microfuge. Electrophoresis was carried out at 10 mamp/slab for 5 to 6 h in the dark at 5°C. For the separation of Chl proteins, the chloroplast membranes were extracted in the dark at 6°C for 30 min in the sample buffer at an SDS to Chl ratio of 10:1 prior to electrophoresis.

Photoreactions. Water to FeCN[3] measurements were carried out at 20°C using a Hansatech O_2 electrode as originally described by Delieu and Walker (6). The sample cell (1.0 ml) contained the chloroplast preparation (5–20 μg of Chl), 1.5 mM FeCN, and 10 mM DL-glyceraldehyde, 0.33 M sorbitol, 1 mM $MgCl_2$, and 10 mM phosphate buffer (Na_2HPO_4/KH_2PO_4; pH 7.4 at 20°C). The sample was illuminated with a 500 w projector lamp fitted with a Corning 2-62 red glass filter (intensity = 950 kerg/cm^2·s). After 30 s of illumination, 20 μl of 0.5 M NH_4Cl were added to the cell to obtain a rate for the uncoupled reaction.

H_2O to DPIP was assayed spectrophotometrically by the photoreduction of DPIP. The actinic light (1.1×10^3 kerg/cm^2·s) was filtered through a Corning 2-62 red glass filter. A 3.0 ml quartz cuvette contained the chloroplast preparation (1–2 μg Chl/ml), 10 mM phosphate buffer (pH 7.4), 0.33 M sorbitol, 1 mM $MgCl_2$, and 1.5 mM DPIP. PSII activities were calculated using a mM extinction coefficient of 17.4 for DPIP. All values were corrected for the DCMU-insensitive reduction of DPIP by repeating the experiment with the inclusion of 5 μM DCMU.

The photoreduction of DPIP with DPC as electron donor (DPC→ DPIP) was carried out essentially as described for the H_2O→ DPIP measurements with the inclusion of 0.5 mM DPC in the reaction medium.

PSI was assayed polarographically as TMPD→ MV by a method similar to that of Henningsen and Boardman (9). The reaction mixture contained 4 mM TMPD, 2 mM MV, 60 mM sodium ascorbate, 1 mM DCMU, and chloroplast membranes (1–2 μg Chl/ml in 10 mM phosphate buffer [pH 7.4], 0.33 M sorbitol, and 1 mM $MgCl_2$).

Plastid Counts, Chl, and Protein Determinations. Platid concentrations were calculated from counts made under a microscope using a hemocytometer. Chl was extracted by grinding leaf tissue in a TenBroeck homogenizer or suspending an aliquot of a chloroplast preparation in 80% acetone and calculations were made using the coefficients of MacKinney (18). Protein content was determined by the method of Bradford (4).

RESULTS

Chl Changes Associated with Greening. The changes in the Chl *a/b* ratio and total Chl/g fresh weight in both M and N preparations as a function of greening are shown in Figure 1a. There was a 2-h lag in the drop of the Chl *a/b* ratio in the M compared to the N after 6 d of etiolation. It took a longer illumination time for the Chl *a/b* ratios to drop in both N and M chloroplasts after 8 d of etiolation (Fig. 1b). There was a considerable SE for the Chl *a/b* ratios in the early stages of greening due to an extremely low Chl content in the isolated chloroplast membranes. The Chl *a/b* ratio of mutant seedlings grown under continuous illumination for 6 to 8 d was significantly lower than that of the wild-type. The changes in the Chl content of seedings on a fresh weight basis indicated that the Chl content of the N was always greater than that of the M. This difference was reduced, however, after 8 d of dark pretreatment.

The protein and Chl per plastid are shown in Figure 2. On a

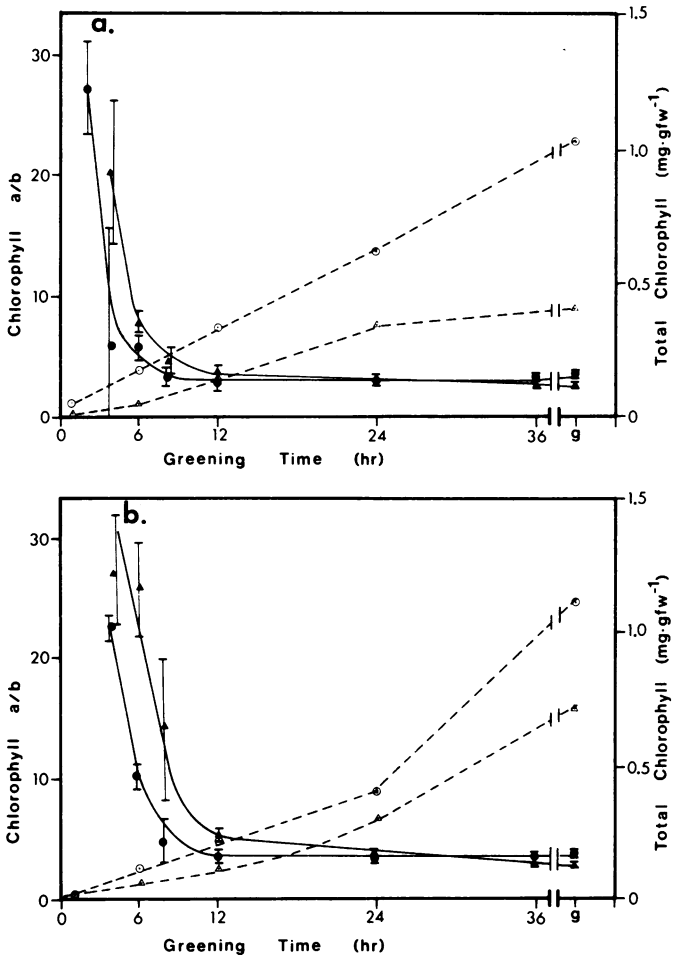

FIG. 1. Chl accumulation during greening in etiolated normal and virescens mutant seedlings. Chl content in normal (○) and mutant (△); Chl *a/b* in normal (●) and mutant (▲); g, values for seedlings grown under continuous illumination. Seedlings were etiolated for 6 d (a) and 8 d (b). Bars = ±2 SE.

qualitative basis, curves for Chl accumulation on a fresh weight basis (Fig. 1) and on a plastid basis (Fig. 2) were quite similar. Mutant seedlings greened for 24 h, following 6 d of etiolation, had a Chl content of 51% of the N on a plastid basis and 58% on a fresh weight basis. After 8 d of etiolation followed by 24 h of greening, these values had increased to 72% and 71%, respectively. The total membrane protein levels per plastid did not appear to change extensively throughout greening. After 6 d of etiolation, the protein content of the M etioplasts was 75% that of the N (Fig. 2), and after 8 d this value had risen to 88% (data not shown). However, the M appeared to have a higher protein content than the N measured on a plastid basis after the 6- or 8-d etiolated seedlings were illuminated for 36 h (Fig. 2).

Membrane Proteins. SDS-polyacrylamide gel electrophoresis of the Chl-proteins from the N and M seedlings grown for 6 and 8 d under continuous illumination resulted in the gel patterns shown in Figure 3a. A sharp green band was seen at an apparent mol wt of 96 kd, corresponding to the P_{700} Chl *a* protein complex; a broader diffuse band was seen at 28 to 30 kd, corresponding to the LHC; and the third band, at the gel front, was free pigment. These bands migrated to the same positions in both the M and N preparations. When the membrane preparations were heated to 100°C in SDS before electrophoresis, no Chl-protein bands were visible in the unstained gels other than the free pigment band at the gel front. Heating the samples resulted in a sharpening of the LHC band at 29 kd and protein bands at 96, 55, and 48 kd

[3] Abbreviations: FeCN, ferricyanide; DPIP, 2,6-dichlorophenol indophenol; DPC, 1,5-diphenyl carbazide; TMPD, *N,N,N'N'*-tetramethyl-*p*-phenylene diamine; MV, methyl viologen; LHC, light-harvesting chlorophyll protein complex.

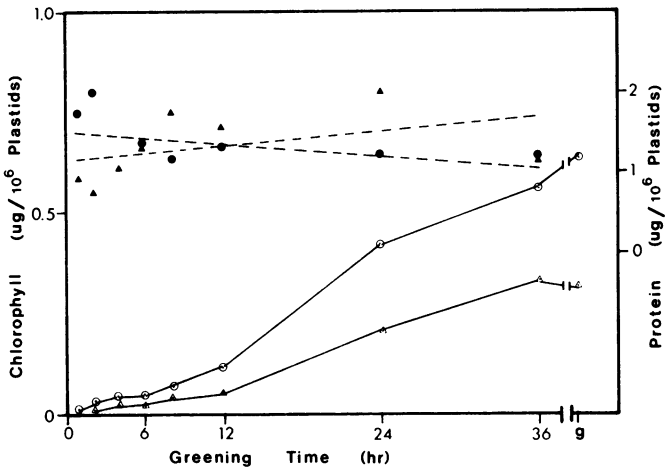

FIG. 2. Accumulation of Chl and protein on a plastid basis in 6-d etiolated normal and virescens mutant seedlings. Chl content of normal (○) and mutant (△); protein content of normal (●) and mutant (▲); g, values for seedlings grown under continuous illumination for 6 d.

the appearance of any new bands (Fig. 3b). Thus, the proteins from the disappearing bands either ran to a position(s) which superimposed a band(s) already present in the samples or failed to enter the gel.

The polypeptide profiles changed markedly as a function of greening as seen for the 6-d etiolated N-plants (Fig. 4a). A general trend can be seen in the disappearance of the high (96 and 66 kd) and very low (>20 kd) mol wt proteins. A diffuse band at 38 kd, seen in the early stages of development, split into a doublet at 38 and 40 kd in the later stages. A 34 kd band showed significant increases in the later stages of greening, although it was present in low amounts even after 2 h in the light. The most obvious change was the development of a doublet around 29 kd which, in green tissue, was perhaps the major protein band. Other bands between 22 and 27 kd also intensified during greening; however, most of these were also faintly visible after 2 h.

Proteins extracted from plastids of green M seedlings etiolated for 6 d are shown in Figure 4b. This pattern was virtually identical to that of the N plastids with the time frame delayed slightly. The same patterns of appearance and disappearance of protein bands were seen in the 8-d SDS gels as in the 6-d gels, but the time frame did not appear to be affected by the mutation (data not shown). Although little change was seen in the time frame of the developmental sequence of the M patterns whether etiolated for 6 or 8 d, the 8-d N patterns lagged considerably behind the 6-d N patterns to a developmental rate that was almost indistinguishable from the M.

PSII Measurements. Electron transport through both PSI and PSII was measured as O_2 evolution upon illumination of thylakoid membrane preparations using FeCN as the electron acceptor. No O_2 evolving activity on a Chl basis could be detected within the first 4 h of greening for either N or M membranes following 6 d of etiolation (Fig. 5a). The rate of development of the O_2 evolving capacity after this lag, however, was faster in the N than in the M. Although consistently high activities were observed in N plastid preparations, both N and M samples had achieved activities of nearly 100% that of the light-grown controls within 24 h or greening. Since the level of Chl was significantly reduced in the M, the data was replotted on a unit protein basis (Fig. 5b). The development of the M activities was not complete at 24 h on a protein basis, and preparations from N chloroplasts were the first to reach a stable activity level. An increase in activity on a protein basis with no change on a Chl basis can be explained by either a drop in plastid protein between 24 and 36 h, or an increase in Chl associated with the increase in activity. Because there is little or no change in the protein content/plastid (Fig. 2), the increase in rate of electron transport between 24 and 36 h of greening in the M plastids must be directly related to an increase in Chl content.

PSII activity was also measured spectrophotometrically by the photoreduction of DPIP with water as electron donor. The rate of photoreduction of DPIP was very high in the presence of DCMU during the first few h of greening. Therefore, all measured activities were corrected for DCMU-insensitive DPIP photoreduction. Photochemical activity on a Chl basis showed a rapid rise to a maximum at 3 to 4 h of greening of both N and M thylakoids after 6 d of etiolation and a decline to a stable level at the 12-h stage (Fig. 6a). The activities peaked after 6 and 9 h for N and M plastids, respectively, after 8 d of etiolation, and stable activities were not obtained until at least 24 h of greening (data not shown). In light-grown seedlings, these PSII activities were higher for the N than the M after 6 d, but almost equal after 8 d of continuous illumination.

A continuous increase in PSII activity is seen over the 36 h of period, when the data are plotted as activity per unit protein (Fig. 6b). The rate of increase in activity of the M-chloroplasts is unchanged by the duration of the dark etiolation, but that of the N is considerably reduced after 8 d in the dark (Fig. 6c). In all cases, just as for O_2 evolution, the PSII activity per unit protein continues to rise long after stable rates are reached on a Chl basis.

The additional electron transport stimulated by DPC over that with H_2O as the donor is shown in Figure 7. The activities were corrected for DCMU-insensitive photoreduction to negate the effects of any DPC donation to PSI. In the first few h of greening, DPC is as potent donor to PSII. This stimulation declines sharply over the first 4 h in seedlings etiolated for 6 days as the $H_2O \rightarrow$ DPIP activity becomes functional. This decay component is extended to 8 and 12 h for the N and M, respectively, in plants greened after 8 d of etiolation (Fig. 7b). The rate of decrease over the 12 to 36-h greening period, however, is the same for both N and M after both 6 and 8 d of etiolation.

PSI Measurements. The development of photochemical activity associated with PSI (TMPD→ MV) is shown in Figure 8. Significant activities were obtained in all cases within the 1st h of greening. Maximum rates of PSI activity, on a Chl basis, were seen after 8 h of greening in both N and M plants etiolated for 6 d, whereas, after 8 d of etiolation, maximum activities were reached after only 4 h of greening (data not shown). PSI activity of N thylakoid membranes was high during the initial stages of greening but attained a stable rate within 24 h which was lower than that of the M. After 8 d of etiolation, although the maximum activities were achieved earlier in plastid development, the photochemical activities had not reached stable levels even after 36 h of development (data not shown). The initial activities were artificially high due to the low Chl content of the plastids. When plotted on a protein basis (Fig. 8b), the rates of PSI development were consistently higher in the N regardless of the duration of etiolation. PSI activities always began to level off after about 24 h of greening except in the case of the 6 d etiolated M. On a unit protein basis, very little activity was apparent in the 1st of greening. However, significant PSI activity was seen after 2 h. Thus, PSI appeared earlier and reached stable levels sooner during the greening process than PSII.

Photosynthetic Units. The development of the photosynthetic unit as defined by light saturation kinetics of PSII (DPC→ DPIP) is shown for 6-d etiolated N and M seedlings in Figure 9. These data have been normalized so that the activity at the maximum light intensity is unity. The light intensity required for saturation of PSII activity decreased as green progressed. The half saturation intensities decreased from 210 to 69 kerg/cm^2·s during the first 24 h of greening in the N (Fig. 9a, insert), while it decreased from 470 to 66 kerg/cm^2·s over the same period in M chloroplasts (Fig. 9b, insert). The photosynthetic unit size increased more rapidly in

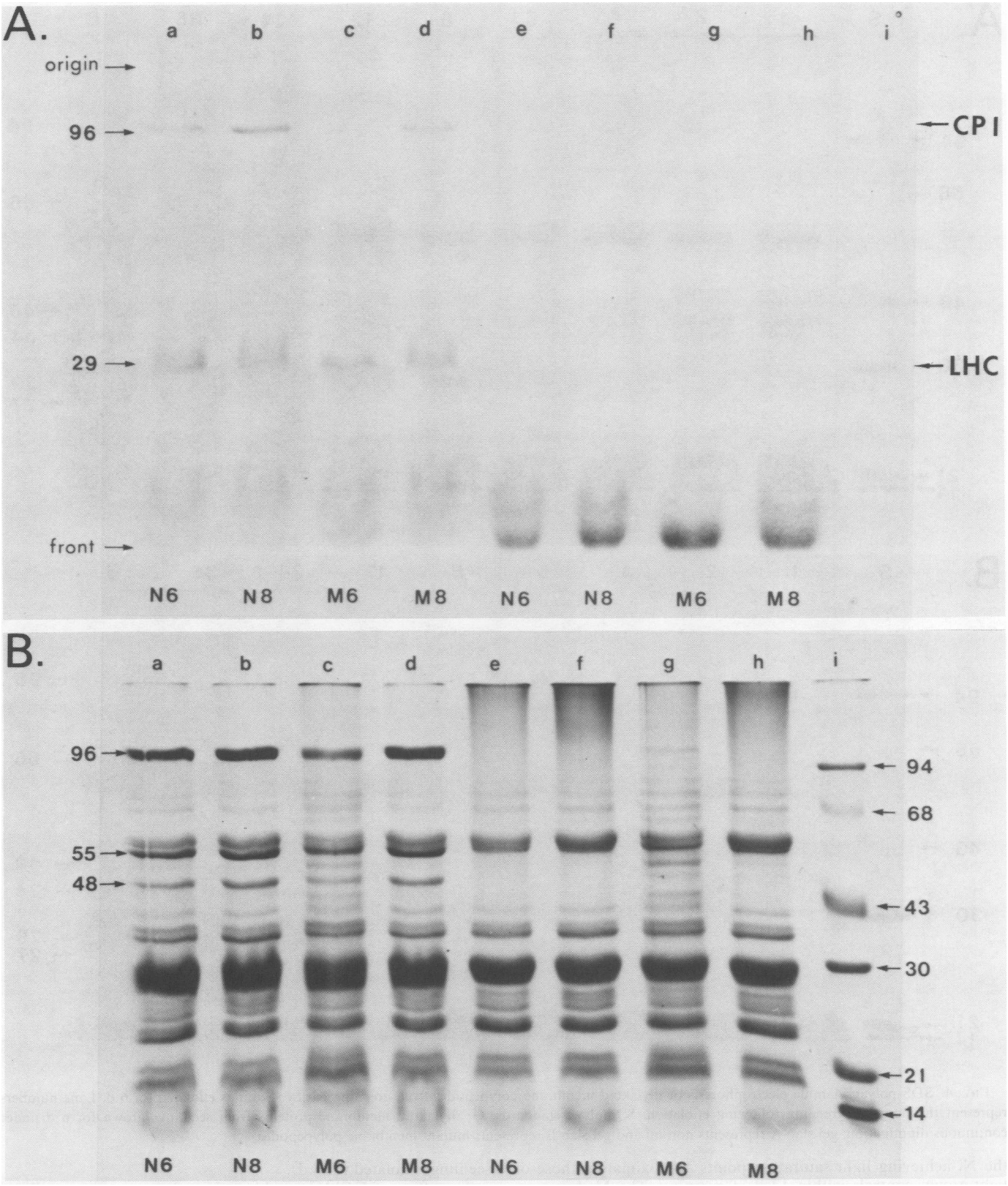

FIG. 3. SDS-polyacrylamide gel electrophoresis of Chl-proteins extracted from thylakoids of normal and mutant barley grown for 6 or 8 d in continuous light. A, Unstained gel indicating the green bands; B, gel stained with Coomassie blue. Lanes a to d, thylakoid membranes treated with SDS for 30 min at 5°C; lanes e to h, membranes treated with SDS for 5 min at 100°C; lane i, protein mol wt standards (Bio-Rad lysozyme, 14.3 kd; soybean tripsin inhibitor, 21 kd; carbonic anhydrase, 30 kd; ovalbumin, 43 kd; phosphorylase b, 94 kd). The lanes are also marked to indicate the 6-d normal (N6), 8-d normal (N8), 6-d mutant (M6), and 8-d mutant (M8).

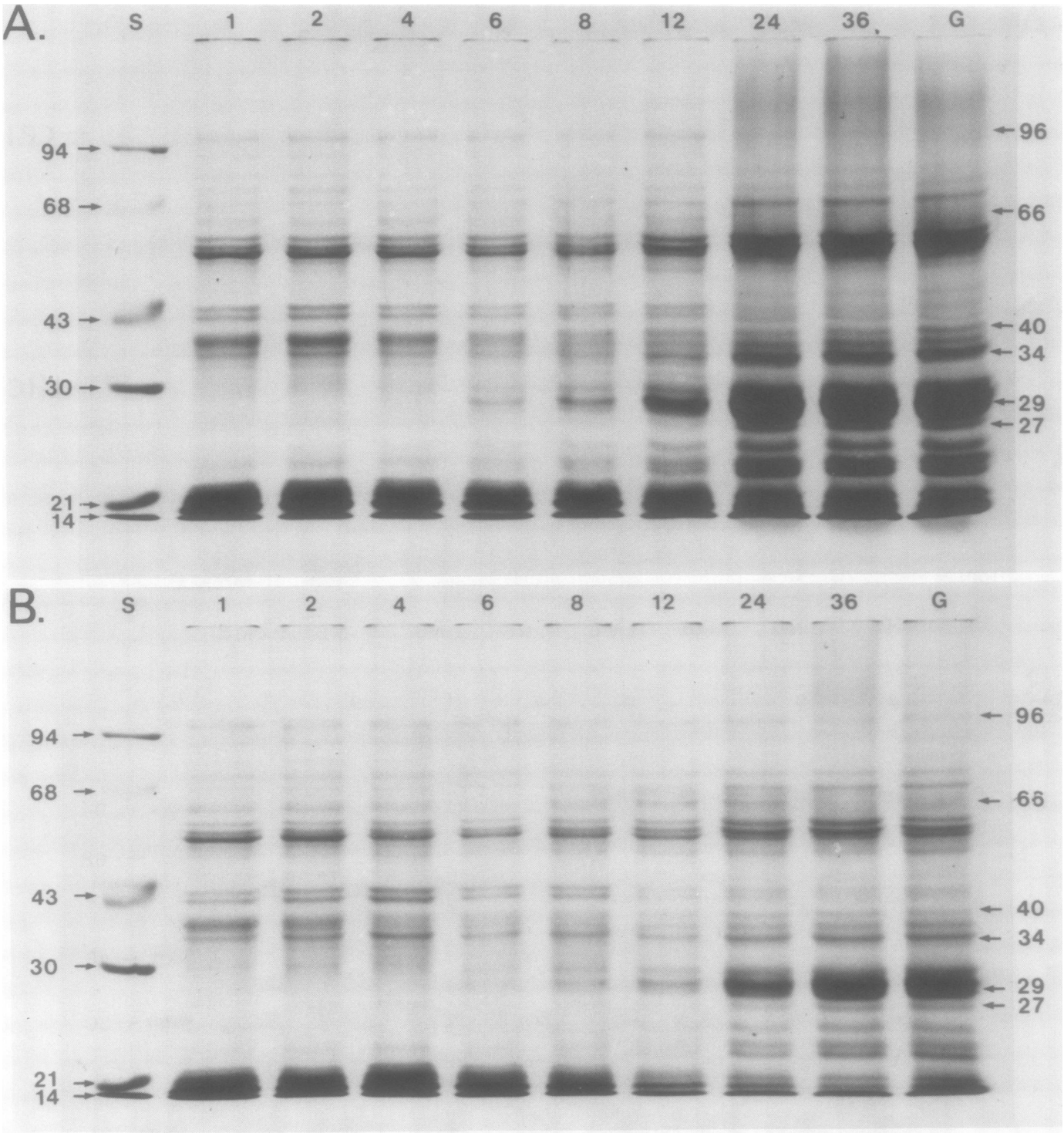

FIG. 4. SDS-polyacrylamide electrophoresis of thylakoid membrane polypeptides from greening barley seedlings etiolated for 6 d. Lane numbers represent the hours of greening following etiolation; S, mol wt standards; G, thylakoid membrane proteins from seedlings grown for 6 d under continuous illumination; gel slab A represents normal and gel slab B represents mutant membrane polypeptides.

the N, achieving light saturation points approximating those of light-grown controls within 12 h of greening. The M, however, took about 24 h to attain the light-grown control photosynthetic unit sizes. The half-saturation intensities decreased from 180 to 71 kerg/cm^2·s over the first 24 h of greening in the chloroplasts isolated from N-plants etiolated for 8 d (data not shown). This rate of decrease was closely paralleled by the M (190 to 62 kerg/cm^2·s) over the same time period. Thus, little difference in the course of development could be seen between the N and M seedlings etiolated for 8 d.

The effect of DCMU on chloroplasts from greening seedlings etiolated for 6 d is shown in Figure 10. These data have been normalized so that the activity in the absence of DCMU is equal to unity and that in the presence of 10 mM DCMU is equal to zero. Lower concentrations of DCMU were required for 50% inhibition of activity in less developed chloroplasts. These values increased from 50 to 100 nM for the N (Fig. 10a) and from 8 to 150 nM for the M plastids (Fig. 10b) during the first 24 h of

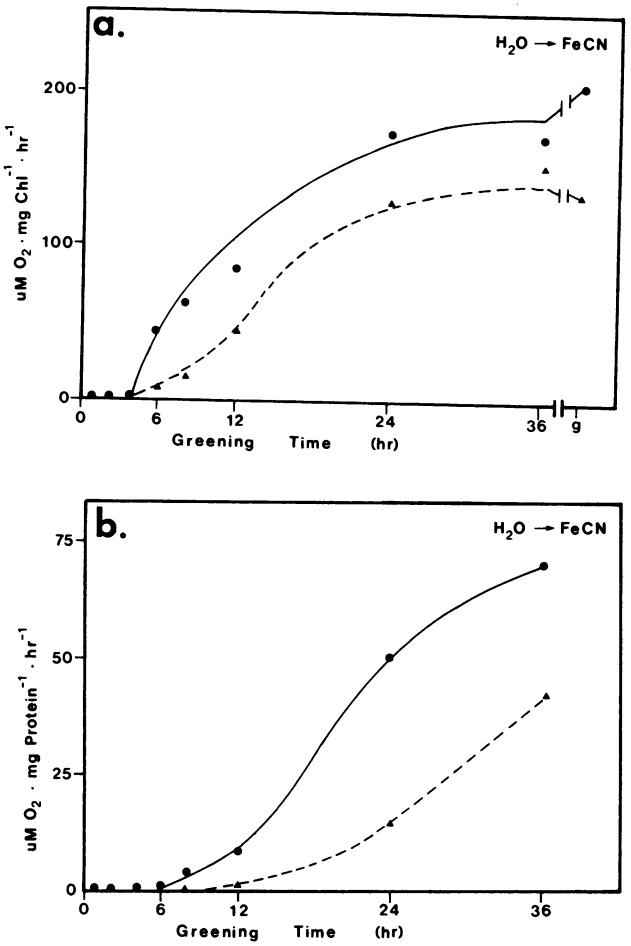

FIG. 5. Development of PSII activity ($H_2O \rightarrow$ FeCN) in chloroplasts from normal and virescens mutant seedlings etiolated for 6 d. Activities of normal (●) and mutant (▲) plastids are plotted on a Chl basis (a) and a plastid protein basis (b); g, values for seedlings grown for 6 d under continuous illumination.

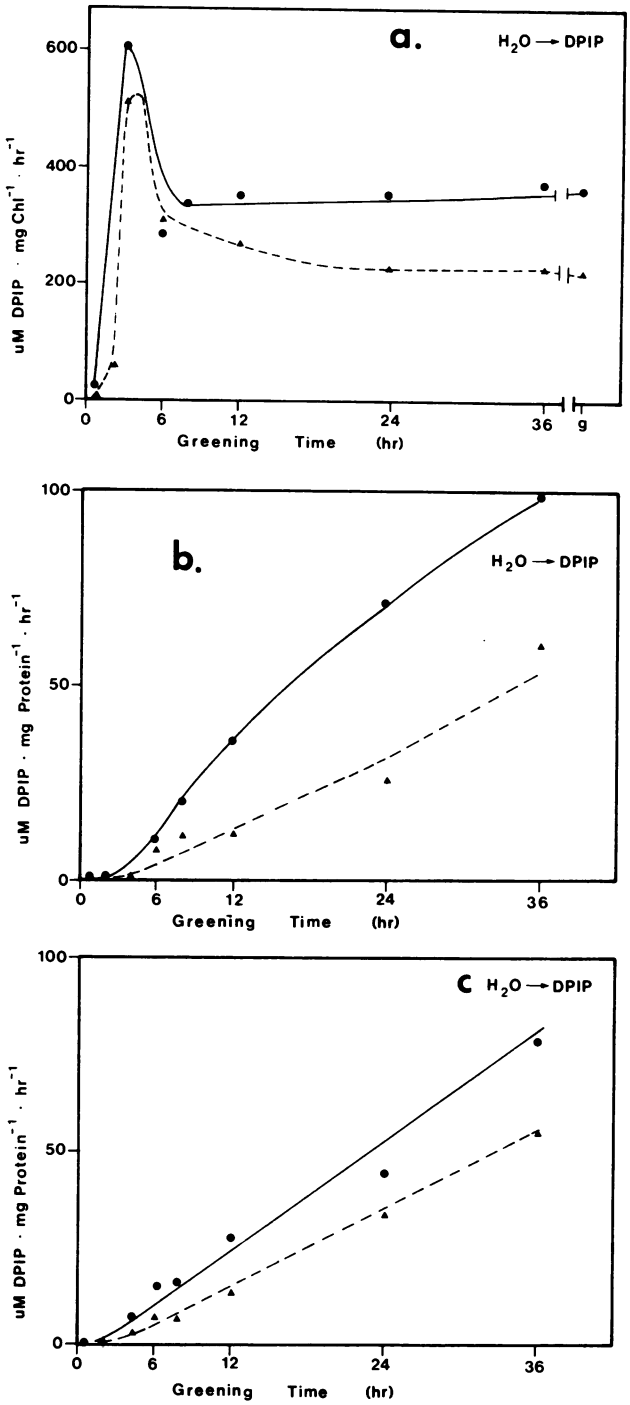

FIG. 6. Development of PSII activity ($H_2O \rightarrow$ DPIP) in chloroplasts from normal and virescens mutant seedlings. Activities of normal (●) and mutant (▲) plastids are plotted on a Chl basis for 6-d etiolated seedlings (a), and on a palstid protein basis for 6-d (b) and 8-d (c) etiolated seedlings; g, values for seedlings grown for 6 d under continuous illumination.

greening. The slope of the Hill plots approximate unity for all but the 2-h N sample, in which case it was 0.59. The results from seedlings etiolated for 8 d and allowed to green were similar to those for 6-d etiolated seedlings in that the DCMU I_{50} increased as greening progressed (data not shown). These values increased from 14 to 200 nM over the first 24 h of greening in the N, and from 40 to 200 nM in the M (2-h M values could not be determined). The Hill coefficients for all curves approximated unity except for the 2-h N sample which was 0.63. The consistently low Hill coefficients in very young samples may suggest a substantial amount of DCMU insensitive photoreduction of DPIP.

DISCUSSION

Normal and mutant seedlings were etiolated for 6 or 8 d prior to greening in order to align the tissues developmentally. In seedlings etiolated for 6 d, however, the subsequent greening rate was still slower in M compared to N seedlings (Fig. 1). After 8 d in the dark, followed by 24 h in the light, a smaller difference was seen in the Chl content of N and M leaves. Since the Chl content on a plastid basis was not different at this stage, the decreased Chl content in the M-leaves probably represents fewer chloroplasts per unit fresh weight of leaf.

The rapid decline of the Chl *a/b* ratio is a common indicator of chloroplast development (20, 24, 27). Stable *a/b* ratios have been previously reported within 4 to 8 h of greening in barley seedlings (8, 9). This greening rate however, was dependent on the environmental conditions, especially temperature and light intensity (19). We observed that the time required for the Chl *a/b* ratio to stabilize was longer after prolonged etiolation (Fig. 1). This decrease in the rate of chloroplast membrane development was further demonstrated in the reduced rates of Chl synthesis by N seedlings after 8 d of etiolation compared with 6 d. The virescens mutant was much less affected by the longer duration in the dark.

Only three green bands were seen immediately after electrophoresis corresponding to the P_{700} Chl *a* protein complex at 96 kd, the

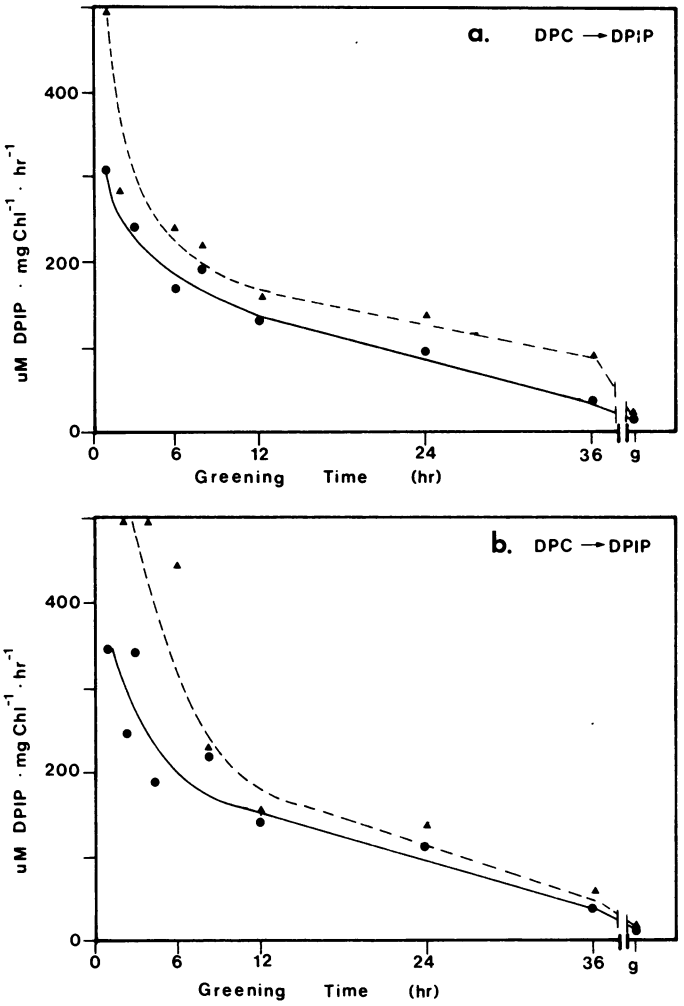

FIG. 7. Photoreduction of DPIP-stimulated by DPC as a function of greening in chloroplasts from normal and virescens mutant seedlings etiolated for 6 (a) and 8 (b) d. (●), normal; (▲), mutant; g, values for seelings grown under continuous illumination.

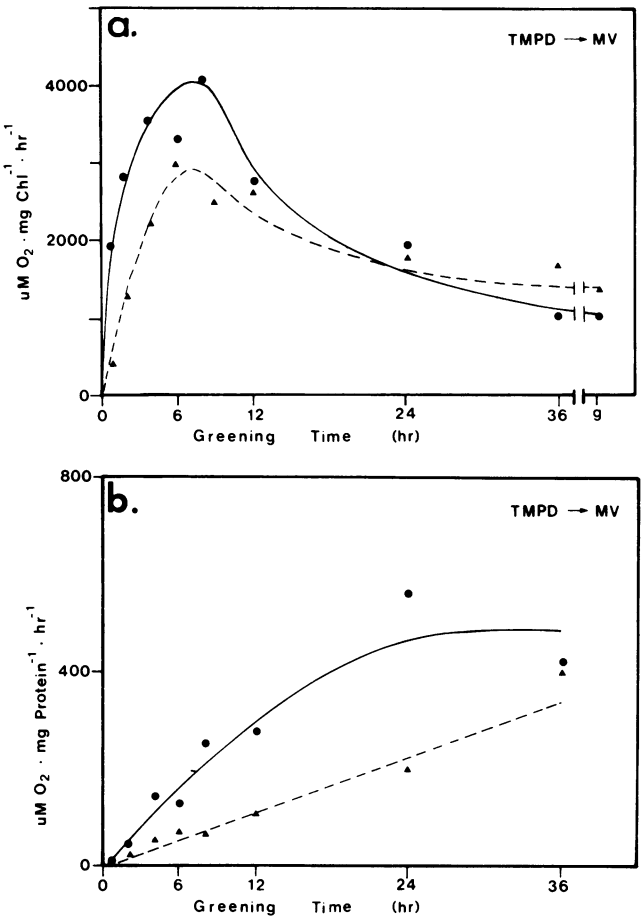

FIG. 8. Development of PSI activity (TMPD→ MV) in chloroplasts from normal and virescens mutant seedlings etiolated for 6 d. Activities of normal (●) and mutant (▲) plastids are plotted on a Chl basis (a) and a plastid protein basis (b); g, values for seedlings grown for 6 d under continuous illumination.

LHC at 29 kd, and a zone-free pigment at the gel front (Fig. 3). A loss of protein bands at 95, 53, and 47 kd were reported by Wessels and Borchert (25) upon boiling spinach chloroplast membranes in SDS for 2 to 5 min, or delipidation in acetone before SDS-electrophoresis. They further suggested that the 47 kd band is the PSII reaction center (see also Ref. 5), and the 53 kd band is a dimer of the LHC. Although no Chl was detected in these areas immediately after electrophoresis, we observed Coomassie staining bands at 55 and 48 kd to be lost upon boiling in SDS. Estimates of the mol wt for the LHC polypeptides vary from 21 to 30 kd (23). Before boiling, the LHC band was broad and ranged from 25 to 30 kd. After boiling in SDS, however, the bands sharpened and a dimer could be distinguished around 29 kd in all samples. Thus, on the basis of identification of these Chl bands by previous workers (5, 23, 25), it appears that the LHC, PSII reaction center, and the P_{700} Chl *a* protein were all present in the N and M seedlings after 6 and 8 d.

The polypeptide profiles in Figure 4 indicate that no significant thylakoid membrane protein present in the N was absent from the M chloroplasts. Jhamb and Zalik (12) also found no difference in gel patterns at the 8-d stage, but some minor differences were noted at the 6-d stage which were due to the different developmental stages of the N and M when the seedlings were grown under high intensity continuous illumination.

Prolamellar bodies are thought to contain a very high amount of Pchl holochrome (14, 21). Ultrastructural studies have indicated that the M is apparently deficient in prolamellar bodies (13). The M also has a lower initial protein content per plastid than the N (Fig. 2). Since the protein content per plastid changed very little during chloroplast development, we feel that the breakdown of certain membrane proteins must have occurred concurrently with the insertion of newly synthesized proteins such as LHC. Several high mol wt proteins do indeed disappear as a function of greening (Fig. 4). The breakdown of these proteins might be responsible for the appearance of the low mol wt bands seen during the first few h of greening. The developmentally related appearance and disappearance of chloroplast membrane proteins in barley was also recently studied by Hoyer-Hansen and Simpson (11). These authors noted that 15 polypeptides decreased in staining intensity during greening and also found a general pattern of loss of high and low mol wt proteins as a function of greening. A loss of high mol wt proteins during greening has also been reported in Euglena (28).

Chl *b* is an integral component of the LHC, and the appearance of the 29 kd band coincided with the stabilization of the Chl *a/b* ratio (Figs. 1 and 4). As in the case of Chl accumulation, changes in the Chl *a/b* ratio and changes in the polypeptide profiles indicated a developmental time lag in the M which was not corrected by prolonged etiolation. Rather, the M approached N parameters because of a decrease in the developmental rate of the N as a result of prolonged etiolation.

Studies on the development of photochemical activities on a

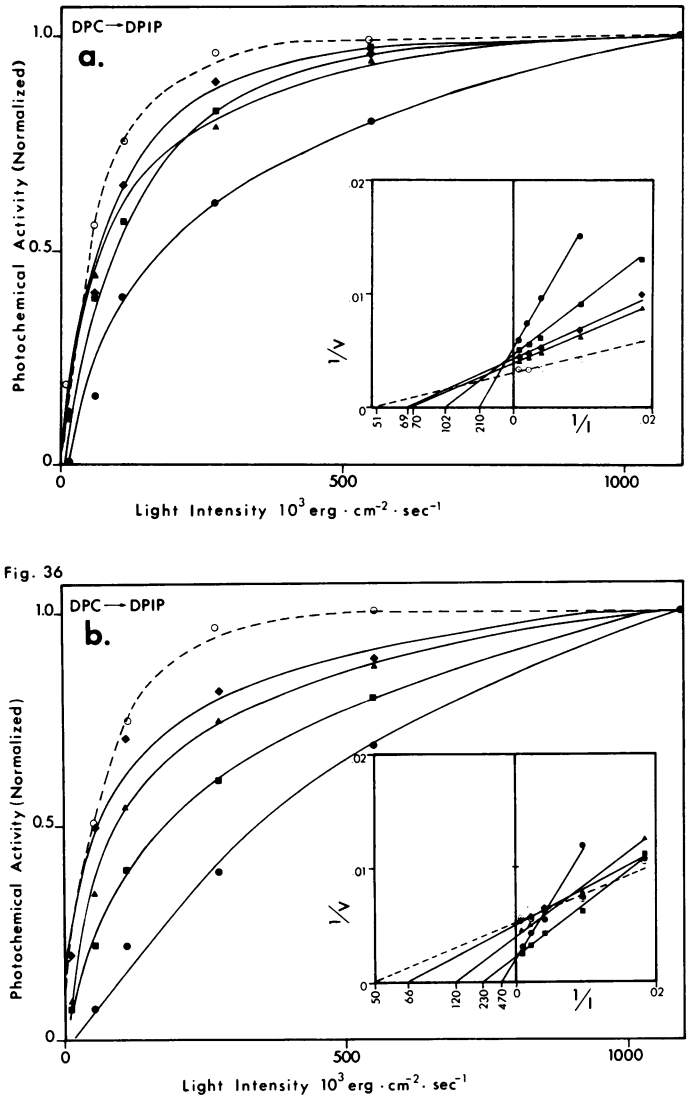

Fig. 36

FIG. 9. Light saturation curves for developing normal (a) and virescens mutant (b) chloroplasts from seedlings etiolated for 6 d. The photoreductive activities of PSII (DPC→ DPIP) have been normalized to a value of 1.0 at the maximum light intensity. Actinic light intensities were varied with neutral density filters (Balzers). Data are shown for chloroplasts greened for 2 h (●), 6 h (■) 12 h (▲), and 24 h (◆) as well as for chloroplasts from seedlings grown for 6 d under continuous illumination (○). The double reciprocal plots of the data before normalization are shown in the inserts.

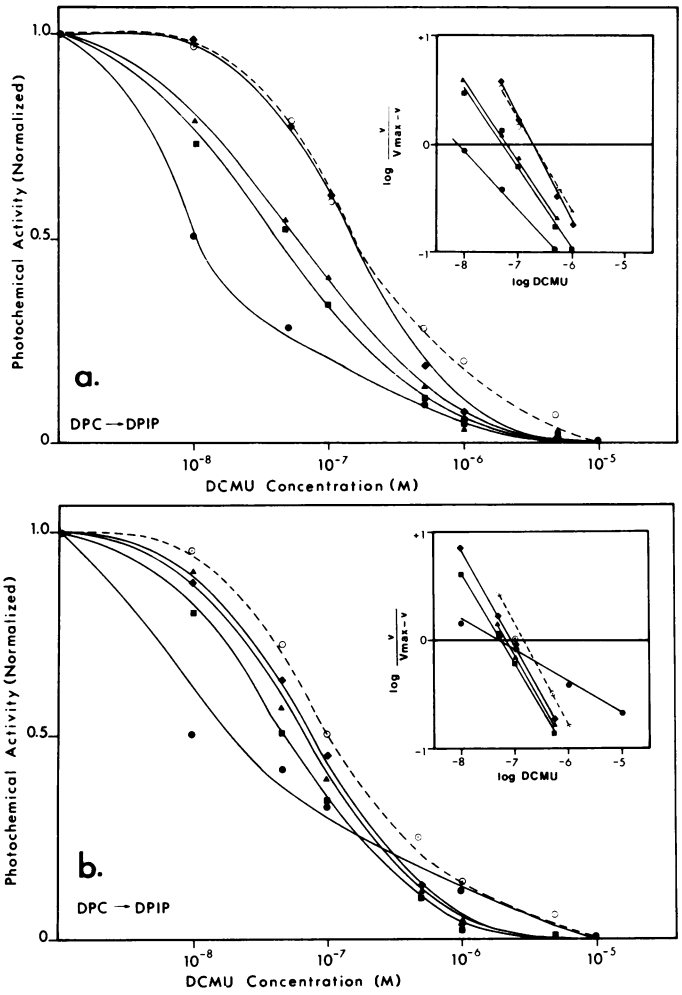

FIG. 10. DCMU inhibition curves for the developing normal (a) and virescens mutant (b) chloroplasts from seedlings etiolated for 6 d. The PSII activities (DPC→ DPIP) have been normalized to a value of 1.0 in the absence of DCMU and to 0 in 10 mM DCMU. Data are shown for chloroplasts greened for 2 h (●), 6 h (■), 12 h (▲), and 24 h (◆) as well as for chloroplasts from seedlings grown for 6 d under continuous illumination (○). The Hill replots of the data before normalization are shown in the inserts.

Chl basis during greening are obviously confounded by changing levels of Chl. Since the protein content of the plastids during development was more stable than the CHl content, it was considered to be a more useful base for the expression of development of photochemical activities. The activity expressed on a unit Chl, however, was useful in detecting the onset of photochemical activity. In addition, a decline in activity after a sharp rise may be related to changes in the photosynthetic unit size (numbers of Chls/reaction center).

The development of PSII activity was measured by the H_2O→ FeCN (O_2 evolution) and H_2O→ DPIP (DPIP reduction) reaction sequences. Under our conditions, O_2 evolution was nonexistent on a protein basis during the first 6 h of greening in N plastids (Fig. 5b). The plastid Chl content at this time was less than 10% of the fully greened tissue (Fig. 2). Activity in the M was slower to develop than in the N and did not appear to be affected by the extended dark period.

On a protein basis, DPIP photoreduction was measurable within 4 h of greening (Fig. 6b). As a result of this early onset, the activity per unit Chl showed a characteristic peak and decline to stable levels as Chl accumulated (Fig. 6a). The earlier onset of the H_2O→ DPIP activity, compared to H_2O→ FeCN, may indicate that some compound other than water in the plastid or buffer was responsible for electron donation in these early stages of chloroplast development, or may simply reflect the differential accessibility of DPIP and FeCN to the membranes. On a unit protein basis, the M was unaffected by the prolonged period in the dark, whereas the development rate in the N seedlings was reduced.

DPC is an artificial donor to PSII, and high rates of DPIP photoreduction were seen with DPC as electron donor within the 1st h of greening (Fig. 7). Thus, even though the H_2O-splitting capacity had not yet developed, the PSII traps were present and functional. Initial activities were high on a Chl basis because of the small amount of Chl in the system.

Significant PSI activities were observed within the first 2 h of greening in both N and M seedlings. On a Chl basis, maximum rates of the TMPD→ MV reactions were seen at 8 h for both N

and M after 6 d of etiolation (Fig. 8a). After 8 d of etiolation, these maximum rates were achieved within 4 h of greening. This earlier peak in activities is most likely due to an unaltered rate of PSI development superimposed on a reduced rate of Chl synthesis after 8 d of etiolation (Fig. 1).

A decrease in the light intensity required for saturation of the DPIP photoreduction is indicative of an increase in photosynthetic unit size with chloroplast development (2). Changes similar to those in Figure 9 have been reported in photosynthetic algae for O_2 evolution (10, 27) as well as DPIP photoreduction (7). Half-saturation intensities of 300 kerg/cm^2·s have been reported for the $H_2O\rightarrow$ DPIP reactions in seedlings of barley greened for 2 h, and 50 kerg/cm^2·s after 24 h of greening (9). After 6 d of etiolation, the light intensity required for saturation of the developing M plastids decreased more slowly than in the N (Fig. 8) indicating a reduced rate of greening in the M. After 8 d however, these rates were almost the same for the N and M.

The number of Chl per DCMU inhibition site (reaction center) decreased with greening in both N and M seedlings (Fig. 10). After 6 d of etiolation and 2 h of greening, there was 1 DCMU inhibition site per 3,000 (N) and 18,000 (M) Chl. We believe that these values may, however, reflect a change in sensitivity to DCMU rather than a decrease in photosynthetic unit size during greening. A change in DCMU sensitivity as a function of greening has been reported previously in barley (9). Substantial amounts of DCMU-insensitive PSII activity were seen in all the photochemical measurements performed on very young chloroplasts. The change in DCMU sensitivity was also suggested by the decrease in the Hill slopes to values less than 1.0 in the early stages of greening. The I_{50} concentrations in greening seedlings after 6 d of etiolation were usually lower and approached the light-grown control values more slowly in the M than in the N chloroplasts. After 8 d of etiolation, the approach to the light-grown values occurred at about the same rates for the N and M due to a marked reduction in the rate of development of the N.

In all the parameters studied in this manuscript, we have reached the basic conclusion that the developmental rate of the virescens mutant is unaffected by the duration of etiolation up to 8 d, whereas the wild type barley developmental rate is substantially reduced upon prolonged etiolation. This has the effect of equalizing the greening rates of the normal and virescens mutant seedlings by the 8-d etiolation period.

Acknowledgment The authors wish to thank Mr. B. Zytaruk for his help in the preparation of the figures.

LITERATURE CITED

1. ALBERTE RS, JD HESKETH, G HOFSTRA, JP THORNBER, AW NALOR, RL BERNARD, C BRIM, J ENDRIZZI, RJ KOHEL 1974 Composition and activity of the photosynthetic apparatus in temperature-sensitive mutants of higher plants. Proc Natl Acad Sci USA 71: 2414–2418
2. BAKER NR, K HARDWICK 1974 A model for the development of photosynthetic units. Proc Int Congr Photosynth Res 3: 1897–1906
3. BRADBEER JW 1976 Chloroplast development in greening leaves. *In* N Sunderland, ed. Perspectives in Experimental Botany, Vol 2. Pergamon Press, New York, pp 131–143
4. BRADFORD MM 1976 A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254
5. CHUA N-H, P BENNOUN 1975 Thylakoid membrane polypeptides of *Chlamydomonas reinhardtii* wild-type and mutant strains deficient in photosystem II reaction center. Proc Natl Acad Sci USA 72: 2175–2179
6. DELIEU T, DA WALKER 1972 An improved method for the measurement of photosynthetic oxygen evolution by isolated chloroplasts. New Phytol 71: 201–225
7. CAHEN D, S MALKIN, S SHOCHAT, I OHAD 1976 Development of photosystem II complex during greening of *Chlamydomonas reinhardtii* y-1. Plant Physiol 58: 257–267
8. EGNEUS H, S SELLDEN, L ANDERSON 1977 Appearance and development of P_{700} oxidation and photosystem I activity in etio-chloroplasts prepared from greening barley leaves. Planta 133: 47–52
9. HENNINGSEN KW, NK BOARDMAN 1973 Development of photochemical activity and appearance of the low potential form of cytochrome b_{559} in greening barley seedlings. Plant Physiol 51: 1117–1126
10. HERRON HA, D MAUZERALL 1972 The development of photosynthesis in a greening mutant of *Chlorella* and an analysis of the light saturation curve. Plant Physiol 50: 141–148
11. HOYER-HANSEN G, DJ SIMPSON 1977 Changes in the polypeptide composition of internal membranes of barley plastids during greening. Carlsberg Res Commun 42: 379–389
12. JHAMB S.M S ZALIK 1973 Soluble and lamellar proteins in seedlings of barley and its virescens mutant in relation to chloroplast development. Can J Bot 51: 2147–2154
13. JHAMB S, S ZALIK 1975 Plastid development in a virescens mutant of barley and chloroplast microtubules. Can J Bot 53: 201462025
14. KAHN A 1968 Developmental physiology of bean leaf plastids II. Negative contrast electronmicroscopy of tubular membranes in prolamellar bodies. Plant Physiol 43: 1769–1780
15. LAEMMLI UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond) 227: 680–685
16. LEECH RM 1976 Plastid development in isolated etiochloroplasts and isolated proplastids. *In* N Sunderland, ed. Perspectives in Experimental Botany, Vol 2. Pergamon Press, New York, pp 145–162
17. LEESE BM, RM LEECH 1976 Sequential changes in the lipids of developing proplastids isolated from green maize leaves. Plant Physiol 57: 789–794
18. MACKINNEY G 1941 Absorption of light by chlorophyll solutions. J Biol Chem 133: 315–322
19. MILLER RA, S ZALIK 1965 Effect of light quality, light intensity and temperature on pigment accumulation in barley seedlings. Plant Physiol 40: 569–574
20. PLESNICAR M, DS BENDALL 1973 The photochemical activities and electron carriers of developing barley leaves. Biochem J 136: 803–812
21. RASCIO N, M ORSENIGO, D ARBOTT 1976 Prolamellar body transformation with increasing cell age in the maize leaf. Protoplasma 90: 253–263
22. THOMSON L, S ZALIK 1981 Acyl lipids, pigments, and gramine in developing leaves of barley and its virescens mutant. Plant Physiol 67: 646–654
23. THORNBERG JP, JP MARKWELL, S REINMAN 1979 Plant chlorophyll-protein complexes: recent advances. Photochem Photobiol 29: 1205–1216
24. WELLBURN AR, R HAMPP 1979 Appearance of photochemical function in prothylakoids during plastid development. Biochim Biophys Acta 547: 380–397
25. WESSELS JSC, MT BORCHERT 1978 Polypeptide profiles of chlorophyll-protein complexes and thylakoid membranes of spinach chloroplasts. Biochim Biophys Acta 502: 78–93
26. WILD A 1978 Studies on chloroplast development in *Chlorella fusca*. *In* G Akoyunoglou, JH Argyroudi-Akoyunoglou, eds, Chloroplast Development. Elsevier, Amsterdam, pp 5336538
27. WILD A, U TROSTMANN, I KLETZMANN, KH FALDNER 1978 Development of the photosynthetic apparatus during light-dependant greening of a mutant of *Chlorella fusca*. Planta 140: 45656
28. WOLFF B, R SCHANTZ 1978 Thylakoid membrane proteins during chloroplast development in *Euglena gracilis Z*. *In* G Akoyunoglou, JH Argyroudi-Akoyunoglou, eds, Chloroplast Development. Elsevier, Amsterdam, pp 245–250