MACROMOLECULAR PHYSIOLOGY
OF PLASTIDS

IX. DEVELOPMENT OF PLASTID MEMBRANES DURING
GREENING OF DARK-GROWN BARLEY SEEDLINGS

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SUMMARY

The development of plastid membranes was studied in relation to chlorophyll accumulation
in dark-grown barley seedlings of various ages after transfer to light. Quantitative electron
microscopy showed that the prolamellar body membranes are reorganized into primary
lamellar layers which contain sufficient membranes to support grana formation during 24 h
of greening. Structural reorganization of the plastid membranes is completed rapidly in young
seedlings, but is slow in older seedlings. Chlorophyll accumulates rapidly in young leaves after
a short lag. In older leaves there is a longer lag phase before the onset of chlorophyll synthesis,
and the final rate of synthesis is lower.

Shortly after transferring to light, the crystalline prolamellar bodies in the etioplasts are
transformed and then dispersed into lamellar layers with numerous perforations and pro-
tuberances. Before the phase of rapid chlorophyll synthesis, many small-diameter 2-disk grana
are formed. When chlorophyll begins to accumulate, the perforations are rapidly eliminated
from the lamellar layers and a maximum number of 2-disk grana are formed. As greening
proceeds additional disks are added to these original 2-disk grana.

During the phase of rapid chlorophyll synthesis, pairing of the lamellae is positively corre-
lated with the accumulation of chlorophyll. During greening less chlorophyll appears to be
incorporated into the paired regions of the lamellae in young leaves as compared to old leaves.
The results on the structural aspects of plastid development are discussed in relation to the
formation of photosynthetic capacity.

INTRODUCTION

The formation of prolamellar bodies during the development of etioplasts in dark-
grown seedlings of angiosperms has been studied in some detail (von Wettstein, 1958;
Mühlethaler & Frey-Wyssling, 1959; von Wettstein & Kahn, 1960; Gunning & Jagoe,
cf. also Kirk & Tilney-Bassett, 1967). The accumulation of protochlorophyll(ide) in
dark-grown leaves and the capacity for its resynthesis depends on the seedling age
The formation of prolamellar bodies is dependent on the synthesis of protochlorophyll(ide)
(Boynton & Henningsen, 1967; von Wettstein, Henningsen, Boynton, Kannangara &

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Light energy over a prolonged period is required for the formation of grana and further accumulation of chlorophyll (Eriksen, Kahn, Walles & von Wettstein, 1961). Continuous illumination of dark-grown seedlings with moderate to high intensity of white light results in typical chloroplasts with a system of lamellae (i.e. thylakoids) differentiated into grana comprised of stacked or paired regions of the lamellae (i.e. disks), and unpaired stroma lamellae connecting two or several grana (cf. Kirk & Tilney-Bassett, 1967). Crystalline prolamellar bodies are formed in addition to grana and stroma lamellae in plastids of dark-grown seedlings greening in low intensity of white light (Mühlethaler & Frey-Wyssling, 1959; Eilam & Klein, 1962; Wrischer, 1966; Henningsen & Boynton, 1970) or in red light (Boardman, Anderson, Kahn, Thorne & Treffry, 1971). Irradiation of dark-grown bean leaves with 200–300 light flashes separated by dark periods of 15 min (Sironval, Michel, Bronchart & Englert-Dujardin, 1969), or prolonged irradiation with far-red light (De Greef, Butler & Roth, 1971) results in long profiles of largely unpaired lamellae arranged in loose stacks.

The initial photoconversion of protochlorophyllide to chlorophyllide is often followed by a lag period before additional chlorophyll is accumulated (Virgin, 1955; Wolff & Price, 1957; Virgin, 1960). The duration of the lag depends on the age of the dark-grown seedlings (Sisler & Klein, 1963; Akoyunoglou & Argyroudi-Akoyunoglou, 1969), the rate of chlorophyll synthesis on temperature and light intensity (Smith & Koski, 1948; Virgin, 1955). Shortly after the onset of illumination a small amount of chlorophyll b is detectable (Shlyk, Rudoi & Vezitskii, 1970; Thorne & Boardman, 1971; Henningsen & Boardman, 1973).

During greening of dark-grown mutant cells of _Chlamydomonas_ (Ohad, Siekevitz & Palade, 1967; Goldberg & Ohad, 1970) and _Chlorella_ (Bryan, Zadylak & Ehret, 1967), the formation of plastid lamellae parallels the increase in chlorophyll. The development of photosynthetic activity of the cells correlates with the chlorophyll content, although pairing of the lamellae to form grana was delayed. Studies on the changes in the content of plastid specific proteins and lipids suggest that functional photosynthetic membranes in green algae are assembled from single components in a multi-step process (Petrocellis, Siekevitz & Palade, 1970; Eytan & Ohad, 1970, 1972; Hoober, 1970; Bar-Nun, Wallach & Ohad, 1972).
During greening of dark-grown angiosperm seedlings the photosynthetic capacity develops through a sequence of steps. Photosystem I activity can be detected earlier than photosystem II, which precedes or coincides with the appearance of Hill activity (Gyldenholm & Whatley, 1968; Boardman et al. 1970; Thorne & Boardman, 1971; Oelze-Karow & Butler, 1971; Henningsen & Boardman, 1973). It appears that the photosynthetic units are assembled at an early stage of greening, whereas accumulation of further chlorophyll molecules at later stages of greening serve to increase the size of the units (Henningsen & Boardman, 1973). It appears that during the development of chloroplasts in higher plants, the lamellae are completed by stepwise addition of single components.

In order to obtain a better understanding of the relationship between the structural and biochemical aspects of plastid development during greening, we have examined the effects of seedling age and light intensity on the time courses of chlorophyll accumulation, of reorganization of the prolamellar bodies into lamellae, and of the formation of grana. The dark-grown seedlings of various ages provided leaf tissues with different capacities for chlorophyll synthesis and grana formation. This facilitated the distinction of steps in the development of plastid structures in relation to chlorophyll accumulation.

MATERIALS AND METHODS

Plant material. Seedlings of barley (Hordeum vulgare L., cultivar Svalöfs Bonus) were grown in darkness at 23 ± 2 °C and 70–80 % relative humidity. The seeds were planted in Vermiculite and the developing seedlings watered with tap water. The seedlings were transferred to light on the 5th, 7th, 9th and 11th day after planting. The illumination was either with 3200 or 20 lux of white light from a bank of fluorescent tubes (Philips, 1 daylight + 2 warm white), at an air temperature of 25 ± 2 °C and a relative humidity of 60–70 %. For each treatment the same 10 seedlings were sampled for electron microscopy and analysed for pigment content by in vivo spectrophotometry. The apical 10-mm segments of the primary leaves from 7-, 9- and 11-day-old seedlings were discarded, and the adjacent 15-mm segments used for the investigation. From 5-day-old seedlings the coleoptiles were removed, the apical 5-mm segments of the primary leaves discarded, and the adjacent 15-mm segments studied.

Electron microscopy. A short piece from each of the 10 leaf segments was fixed in 4·2 % glutaraldehyde in 0·07 M phosphate buffer, pH 7·2. The leaf tissue was dissected in dim light, but kept dark during the 2-h fixation at 0 °C. All subsequent treatments were carried out in normal room light. After 4 changes of phosphate buffer (0·07 M, pH 7·2) at 4 °C overnight, a 2-h postfixation with 2 % OsO₄ in 0·07 M phosphate buffer, pH 7·2, was carried out at room temperature. Dehydration in a graded ethanol series preceded embedding in epoxy resin (Spurr, 1969). Thin sections were cut with a diamond knife on a Cambridge ultramicrotome. The sections were mounted, unsupported, on grids and contrasted with uranyl acetate and lead citrate. Micrographs of non-serial sections were obtained with a Siemens Elmiskop I or a Zeiss EM 9 A electron microscope. Quantitative measurements of various parameters from the plastid sections were carried out on photoenlargements (× 3) of micrographs taken at ×10000. Measurements of areas were carried out with a planimeter (Haff, No. 315) and the length of distinct membrane profiles was determined with a precision map distance reader (Minerva). From every sample 2 or more leaf pieces were used to obtain measurements from 30 to 40 plastid sections.

Pigment determinations. In vivo absorption spectra were obtained with a Zeiss RPQ-20 A recording spectrophotometer equipped with an integrating sphere attachment modified to allow correction for unspecific absorption. The correction was carried out at 750 nm. The 10 leaf pieces were arranged parallel in a single layer between the glass windows of a metal frame which could be attached to the integrating sphere.
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The chlorophyll content in greening seedlings of the different age groups was determined in extracts of leaf pieces from two or more separately grown batches of seedlings, which otherwise received the same treatments as the leaves used for electron microscopy. One-gramme of leaf pieces from the 10–25 mm band or in the case of 5-day-old seedlings from the 5–20 mm band was extracted with 80% acetone. Total chlorophyll \((a + b)\) was determined by the method of Bruinsma (1961). For each sample of leaves used for chlorophyll extraction, the in vivo absorption spectrum was recorded from two or three sets of 10 leaf segments.

The chlorophyll concentration in the leaf segments used for electron microscopy was estimated from the in vivo absorbance.

RESULTS

Characteristics of the plant material. During development of barley seedlings in darkness the fresh weight of the shoots and the length of the primary leaves increased until the 9th day after planting, whereafter no significant increase in these two parameters was observable (Henningsen & Boynton, 1969). The elongation of the primary leaves was slowed down or ceased when the seedlings were transferred to light prior to the 9th day. The growth characteristics of the seedlings used for the electron-microscopic analysis of plastid development are shown in Table 1. The primary leaf of dark-grown barley seedlings was tightly rolled up to the 7th day, and then the leaf started to unroll. Unrolling of the leaf took place after transfer to light. For the 9- and 11-day-old leaves, unrolling was completed after about 6 h in the light (3200 lx), but took from 8 to 16 h in leaves 7 and 5 days of age, respectively. With 7-day-old seedlings illuminated at 20 lx the leaves were not completely unrolled until after 12 h in the light.

The protochlorophyllide content of the primary leaves reached its maximum on the 7th day after planting and then declined rapidly (Henningsen & Boynton, 1969). The distribution of protochlorophyllide and chlorophyll over the length of the primary leaf was analysed for 7-day-old seedlings (Fig. 1). The apical 45 mm of the leaf contained about 80% of the protochlorophyllide present in 7-day-old dark-grown leaves. After illumination for 4 h the highest amount of chlorophyll was accumulated in the 15–45 mm leaf segment. After 24 h illumination the chlorophyll content was maximal in the 15–60 mm leaf segment and appeared to be evenly distributed in this region of the leaf. Thus, the apical leaf segment of the 7-day-old seedlings greens more slowly than the proximal 15–60 mm leaf segment, indicating that the cells in the apical

<table>
<thead>
<tr>
<th>Seedling age, days</th>
<th>Fresh weight of shoot, mg</th>
<th>Length of primary leaf, mm</th>
<th>Length of secondary leaf, mm</th>
<th>Length of coleoptile, mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>89 ± 3</td>
<td>88 ± 2</td>
<td>21 ± 3</td>
<td>59 ± 1</td>
</tr>
<tr>
<td>7</td>
<td>116 ± 5</td>
<td>152 ± 1</td>
<td>67 ± 6</td>
<td>60 ± 2</td>
</tr>
<tr>
<td>9</td>
<td>170 ± 5</td>
<td>235 ± 5</td>
<td>193 ± 7</td>
<td>64 ± 1</td>
</tr>
<tr>
<td>11</td>
<td>181 ± 8</td>
<td>247 ± 6</td>
<td>233 ± 8</td>
<td>67 ± 1</td>
</tr>
</tbody>
</table>
leaf segment are physiologically the oldest. From visual inspection of the seedlings during greening, the tips of the leaves of 9- and 11-day-old seedlings were also greening more slowly than the rest of the leaf, whereas the leaves of 5-6-day-old seedlings appeared to green uniformly over the whole length of the leaf.

Fig. 1. Distribution of protochlorophyllide and chlorophyll in the primary leaves of 7-day-old barley seedlings. The in vivo absorbance at the red absorption maximum is used as a measure of the pigments in consecutive 15-mm segments of the leaves. 

A, in darkness; B, 4 h, 3200 lx; C, 24 h, 3200 lx.

To allow comparison with our previous studies of plastid development in dark-grown seedlings (Henningsen & Boynton, 1969, 1970), we chose to analyse the 10–25 mm leaf segment of 7-, 9- and 11-day-old seedlings and the 5–20 mm leaf segment of 5-day-old seedlings.

Chlorophyll formation. Photoconversion of protochlorophyllide in dark-grown barley seedlings was completed after less than 5 min illumination with white light at either 20 or 3200 lx. The spectral shift of the in vivo absorption maximum from 684 to 672 nm was completed in less than 10 min in 5- and 7-day-old leaves, but took 20–40 min with leaves of age 9 and 11 days. The shift of the maximum from 672 to 678 nm also depended on seedling age. In 5- and 7-day-old seedlings this red shift started after 2 h in the light and was completed at 4–6 h. With 9–11-day-old seedlings, a 16–24 h illumination was required before the absorption maximum reached 678 nm. The red shift to 678 nm also depended on the light intensity. In 7-day-old seedlings the absorption maximum reached 678 nm after 4–6 h at 3200 lx, but 8–16 h were required at 20 lx. In summary, a considerable amount of chlorophyll (150–200 µg per gram fresh weight) was present in the plastids, before the in vivo absorption at 678 nm dominated.

The accumulation of chlorophyll in the primary leaves of 5- and 7-day-old seedlings had an initial lag of 30 min. Chlorophyll accumulated slowly during 30 min to 2 h in the light (Fig. 2), whereupon the rapid phase of chlorophyll synthesis began. In
7-day-old seedlings the rate of chlorophyll accumulation was reduced after 12 h whereas in the 5-day-old seedlings the high rate of chlorophyll synthesis continued throughout the 24-h period studied. As is also apparent from Fig. 2, the accumulation of chlorophyll in 7-day-old seedlings kept at 20 lx is slower than in those given 3200 lx. With seedlings of age 9 and 11 days, the lags were 1 and 4–6 h, respectively. Chlorophyll accumulated thereafter at lower rates than in the young seedlings. Increasing age of the dark-grown seedlings results in a longer lag phase and a decrease in the rate of chlorophyll synthesis.

![Diagram](image)  
**Fig. 2.** Accumulation of chlorophyll (a + b, μg/g fresh weight) upon illumination of dark-grown barley seedlings of various ages. △, 5 days, 3200 lx; ●, 7 days, 3200 lx; ○, 7 days, 20 lx; ▲, 9 days, 3200 lx; ■, 11 days, 3200 lx.

The amount of chlorophyll in the leaf segments used for the electron-microscopic analysis of the plastid membranes was judged by measuring the *in vivo* absorbance (Fig. 3). The chlorophyll accumulation curves obtained revealed similar trends to those obtained when the chlorophyll was extracted (Fig. 2). Exceptions noted were that the 9- and 11-day-old seedlings had longer lag periods and were greening more slowly, suggesting that the material used for the actual electron microscopy were physiologically older than the average 9- and 11-day-old seedlings. Thus, the material used for the ultrastructural analysis accentuates the differences between the 4 age groups, an advantage for the present investigation.

**Plastid size and shape.** The etioplasts in the primary leaf of barley seedlings developing in darkness increase in volume up to the 7th day, and then the volume decreases again (Henningsen & Boynton, 1969; and Table 2). The average area of the plastid sections in the primary leaves was determined during greening and the significant changes found have been presented in Table 2. Changes in the area of plastid sections represent changes in volume as well as in shape of the plastids. Thus, 5- and 7-day-old
Fig. 3. Changes in the in vivo absorbance at the chlorophyll absorption maximum upon illumination (3200 lx) of dark-grown barley seedlings of various ages. Results for 7-day-old seedlings illuminated with 20 lx are also shown (○). The data were taken on the leaf segments which were used for electron-microscopical analysis. The average of the measurements from 14 separate plantings of 7-day-old seedlings illuminated for 4 and 24 h, respectively, are indicated with X. △, 5 days old; ●, 7 days; ▲, 9 days; □, 11 days.

Table 2. Changes in the size of plastids in primary leaves of barley seedlings grown in darkness and then illuminated with white light

<table>
<thead>
<tr>
<th>Age of seedlings, days</th>
<th>Condition</th>
<th>Average area of plastid sections, μm² ± s.e.</th>
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<tbody>
<tr>
<td>5</td>
<td>darkness</td>
<td>4.2 ± 0.2</td>
</tr>
<tr>
<td>0.5–12 h</td>
<td>3200 lx</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td>16–24 h</td>
<td>3200 lx</td>
<td>8.2 ± 0.3</td>
</tr>
<tr>
<td>7</td>
<td>darkness</td>
<td>6.5 ± 0.3</td>
</tr>
<tr>
<td>0.5–24 h</td>
<td>3200 lx</td>
<td>5.8 ± 0.3</td>
</tr>
<tr>
<td>0.5 h</td>
<td>20 lx</td>
<td>6.8 ± 0.8</td>
</tr>
<tr>
<td>1–24 h</td>
<td>20 lx</td>
<td>7.9 ± 0.3</td>
</tr>
<tr>
<td>9</td>
<td>darkness</td>
<td>4.5 ± 0.2</td>
</tr>
<tr>
<td>0.5 h</td>
<td>3200 lx</td>
<td>4.4 ± 0.4</td>
</tr>
<tr>
<td>1–24 h</td>
<td>3200 lx</td>
<td>6.7 ± 0.4</td>
</tr>
<tr>
<td>11</td>
<td>darkness</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td>0.5–24 h</td>
<td>3200 lx</td>
<td>4.5 ± 0.5</td>
</tr>
</tbody>
</table>
etioloplasts were elongated ellipsoids, whereas 11-day-old etioplasts are almost spherical (cf. Fig. 13). During greening of 5-day-old seedlings the plastid volume remained constant up to 12 h in the light, and then doubled between 12 and 16 h. No changes in plastid size were observed during greening of 7-day-old leaves at 3200 lx; however, at 20 lx a significant increase of plastid size was observed. In 9-day-old leaves illuminated at 3200 lx, the average plastid section area increased between 30 min and 1 h, and then it remained constant. The increase appeared to represent a change from a spherical to an ellipsoidal shape (cf. Fig. 14). This change in shape was also observed for plastids of the 11-day-old leaves (cf. Figs. 13 and 18).

Reorganization of prolamellar body membranes. In leaves of all ages, structural transformation of the prolamellar bodies (cf. Fig. 13) was completed in less than 30 min after transfer to light. With respect to the older leaves (9 and 11 days), tube transformation was completed faster in continuous illumination at 3200 lx, than in darkness after a brief illumination at a high light intensity (Henningsen & Boynton, 1969). Dispersal of the prolamellar bodies was measured by the number of plastid sections containing a prolamellar body, and by the area of the prolamellar bodies per area plastid section (Fig. 4). In 7-day-old leaves illuminated for 30 min at 3200 lx (cf. Fig. 12), the number of plastid sections with a prolamellar body was reduced to a third of the dark value. In leaves of age 5, 9 (Fig. 14) and 11 days (Fig. 13), a significant decrease in the number of prolamellar bodies was not detected until after 1 h of
illumination. The size of the prolamellar bodies remaining at 1 h was drastically reduced. In leaves illuminated at 3200 lx the remaining prolamellar bodies were slowly dispersed, particularly in the 11-day-old leaves where prolamellar bodies were detected for up to 12 h in the light. In general, the dispersal of the prolamellar bodies was slower in leaves continuously illuminated at 3200 lx than in darkness following a brief illumination at high light intensity. In the 7-day-old leaves with a maximum content of protochlorophyllide, dispersal of the prolamellar bodies is faster than in leaves of the other age groups. The shift of the chlorophyllide absorption maximum in vivo from 684 to 672 nm precedes or accompanies the dispersal of the major portions of the prolamellar bodies.

Crystalline prolamellar bodies were reformed in the plastids of 7-day-old leaves illuminated at 20 lx (Figs. 4, 20). Under this condition the dispersal of the prolamellar bodies appeared inhibited in comparison to leaves illuminated at 3200 lx. The reformation of crystalline prolamellar bodies and the reaccumulation of protochlorophyllide in leaves of barley seedlings illuminated at 20 lx have been described in detail elsewhere (Henningsen & Boynton, 1970). Small crystalline prolamellar bodies were occasionally observed in 5-day-old leaves illuminated for 6–12 h with 3200 lx. It is conceivable that portions of the tightly rolled leaves received a lower intensity of light and this might lead to accumulation of some protochlorophyllide during the rapid phase of chlorophyll synthesis.

Dispersal of the prolamellar bodies resulted in the formation of primary lamellar layers. This involved a restructuring of membraneous tubules into membrane sheets.
The newly formed lamellar layers had numerous perforations and protuberances (Figs. 12, 14). The total amount of lamellae (thylakoids) present at various stages of greening was estimated by determining the length of distinct lamellar profiles per plastid section (Fig. 5). The total length of the lamellae increased simultaneously with the decrease in size of the prolamellar bodies. Following the initial increase in length of the lamellar profiles due to dispersal of the prolamellar bodies, a small decrease was observed. This decrease is presumably related to the elimination of perforations from the primary lamellar layers, and would imply that the perforations are eliminated by contraction of the membrane material. The total amount of lamellae was also calculated as profile length of lamellae per plastid section area (Fig. 5). In 5-day-old leaves the total length of lamellar profiles per plastid section gradually increased up to 12 h in the light, and then remained about constant over the next 12-h period. The membranes participating in the differentiation of the lamellar system in the etioplasts during greening of 7-, 9- and 11-day-old leaves (during the first 24 h) were largely derived from the prolamellar body membranes. This was also true for the membranes participating in the formation of grana. Additional membranes were synthesized only at later stages of greening. In the etioplasts of 5-day-old leaves the lamellar system was formed from reorganized prolamellar body membranes as well as additional membranes synthesized during greening.

The perforations in the lamellar layers of the etioplasts and those formed by dispersal were eliminated during greening. The number of perforations in profiles of the lamellar layers were quantitated in 7-day-old leaves illuminated with 3200 or 20 lx (Fig. 6). The number of perforations reached a maximum after 2 h in the light. During the following 6 h, the number decreased to a low value that remains constant. Leaves illuminated 24 h at 20 lx contained both grana, prolamellar bodies and lamellar layers with few perforations. Transfer of the seedlings from low (20 lx) into high light (3200 lx) resulted in the dispersal of the prolamellar bodies which accumulated in dim light, and resulted in an increase in the number of perforations observed (Fig. 6). In the 5-day-old leaves the elimination of the perforations from the lamellar layers was faster than in 7-day-old leaves illuminated at 3200 lx. Most of the perforations had already been eliminated after 4 h in the light. In 9-day-old leaves the perforations were eliminated at about the same time as in 7-day-old leaves, but in the 11-day-old leaves many perforations remained in the lamellar layers for up to 12 h.

**The occurrence of fine tubules.** Fine tubules (cf. Figs. 12, 14) were found in the stroma of plastids in dark-grown barley seedlings (Henningsen & Boynton, 1969). The fine tubules occurred in bundles of 10–42 parallel tubules, and reached a maximum length of 2 μm. The fine tubules could be found in the plastids at any stage of greening. Fine tubules occurred with particular frequency in plastids of 5- and 7-day-old leaves illuminated with 3200 lx for 30 min to 2 h, as well as in 7-day-old leaves illuminated 2–4 h at 20 lx. Fine tubules were also frequent in 5-day-old leaves illuminated for 6 h at 3200 lx and in 7-day-old leaves after 8 h at 20 lx.

**Grana formation.** A granum was considered to consist of two or more closely paired, appressed lamellae (cf. Figs. 15, 17). The formation of grana was followed by determining the number of grana per plastid section area (Fig. 7), the number of disks per
granalum (Figs. 8, 9) and the total length of paired lamellar profiles per plastid section area (Fig. 10).

In etioplasts of all ages, between 0-2 and 1 cross-sectioned granum was found per \( \mu m^2 \) plastid section area (Fig. 7). It remains to be investigated whether these grana are homologous to those developing during greening. After 24 h of greening, seedlings of all ages contained about 3 sectioned grana per \( \mu m^2 \) plastid section area. In 5-day-old leaves the number of grana increased rapidly, reaching a maximum of 4-5 between 6 and 12 h of illumination, and then declined thereafter to the level reached by all plastids at 24 h.

During greening of the 5- and 7-day-old leaves the average number of disks per

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**Fig. 6**

Number of perforations in profiles of the lamellae in plastids of 7-day-old dark-grown barley seedlings illuminated from 0 to 24 h with 3200 or 20 lx (\( \bullet \) and \( \bigcirc \), respectively). The 20-lx specimen was transferred to 3200 lx at arrow.

**Fig. 7**

Number of grana per plastid section area during illumination of dark-grown barley seedlings of various ages. \( \triangle \), 5 days, 3200 lx; \( \bullet \), 7 days, 3200 lx; \( \bigcirc \), 7 days, 20 lx; \( \blacktriangle \), 9 days, 3200 lx; \( \blacksquare \), 11 days, 3200 lx.
granum remained close to 2 during the first 4 h (Figs. 8, 15). Few grana containing 3–6 disks were observed during the first 4 h (Fig. 9) of greening, although successively more grana with 3 or more disks appeared thereafter. Figs. 8 and 9 demonstrate that the more rapidly greening 5-day-old leaves contained grana with more disks earlier than in the 7-day-old leaves. The increase in the number of disks per granum occurred in a similar manner in 7-day-old leaves greening at 20 lx and at 3200 lx. In the 9- and 11-day-old leaves grana containing 2 disks remained dominating during the 24 h studied (Figs. 8, 9, 18). The analysis reveals that the differentiation of the lamellar system in etioplasts occurred in the following way: first, a maximum number of 2-disk grana were produced, and then more disks were added to these original grana as greening proceeded. Eventually two or more grana may be incorporated into a single granum consisting of a very large number of disks which in some cases traverses the entire chloroplast (e.g. Weier, Stocking & Shumway, 1967).

Loose stacks of several lamellar layers are common in plastid sections from 9- and 11-day-old leaves in darkness as well as after prolonged exposure to light (Figs. 13,
Pairing between 2 of the lamellar layers in a loose stack is observed (Fig. 16). This type of membrane arrangement was rather frequent during the first 4–8 h in the light and might be similar to the regions of grana-like pairing between the lamellar layers found in unilluminated leaves.

Finally, we attempted to analyse whether the total profile length of paired regions in the grana of a plastid was correlated with its chlorophyll content during the various stages of greening. The increase in profile length of the paired regions of lamellae per area of the plastid sections during illumination of the seedlings is shown in Fig. 10. The chlorophyll content of the analogous leaf segments is given for comparison. It can be seen that there was a reasonably parallel increase in the 2 parameters during the greening of 7-day-old seedlings at 3200 lx. The detailed correlation between the two, however, breaks down. While the chlorophyll content increased smoothly after 2 h, the length of the paired regions rose in a distinct step between 4 and 6 h and only after 12 h did it begin to rise smoothly. In the 7-day-old leaves illuminated with 20 lx, the lag in chlorophyll synthesis was paralleled by a lag in the formation of paired regions. Again, a distinct step in the formation of the paired regions occurred between 4 and 6 h, and again between 16 and 24 h. In the 9- and 11-day-old seedlings...
(cf. Fig. 3) there was a slow greening paralleled by a slow increase in the length of the paired profiles. The best correlation between increase in length of paired lamellar profiles and chlorophyll was obtained in the 5-day-old seedlings (Fig. 10). During the first 1–2 h of illumination the paired regions per plastid section area increased significantly, although this initial increase was not accompanied by a comparable increase in the chlorophyll content of the leaves. It can be concluded that the length of paired lamellae per \( \mu \text{m}^2 \) plastid section area is the parameter that gives the best correlation with the amount of chlorophyll in the developing plastids. On the other hand, it is equally clear that the paired grana membranes can incorporate different amounts of chlorophyll per unit of membrane area depending on the stage of greening.
and the age of the seedlings. In the initial phase of greening, particularly in young leaves, pairing of the lamellae precedes chlorophyll accumulation. At any stage of greening a small amount of chlorophyll can possibly be present in unpaired regions of the lamellae.

The general relation between the length of paired lamellar profiles and the chlorophyll content of the leaves during greening for each seedling age and light condition is given in Fig. 11. The length of paired regions of the lamellae is positively correlated with the chlorophyll content of the leaf segments. The curves of the relation between paired regions of the lamellae and the chlorophyll content have different slopes for the different seedling ages (Fig. 11). Assuming that the increase in profile length is proportional to an increase in area of the paired regions of lamellae, the results suggest that less chlorophyll is incorporated per area of paired lamellae in young leaves than in older leaves. In 5-day-old seedlings 100 μg chlorophyll per g fresh weight correspond to 1 μm profile of paired lamellae per μm² of plastid section. In 9-day-old seedlings this figure is about 500 μg chlorophyll per g fresh weight. Thus, about 5 times as much chlorophyll is associated with the paired membranes in the greening etioplasts of 9-day-old seedlings as in those of 5-day-old seedlings. At later stages in the greening of young seedlings, increasing amounts of chlorophyll are incorporated into the already formed membranes of the grana (cf. 5-day-old seedlings, Fig. 11). The 7-day-old seedlings illuminated with 3200 lx display a slope intermediate between the 5- and 9-day-old seedlings. Probably both in young and old seedlings the chlorophyll content per membrane area in the grana eventually will reach the same value.
DISCUSSION

In response to light an increase in size and a change in shape of the plastids were observed. The increase in plastid volume in greening barley leaves occurred earlier than reported for plastids isolated from greening bean leaves (Mego & Jagendorf, 1961). In darkness following a brief illumination the plastid volume increases rapidly, and then returns to the same volume as before illumination (Henningsen & Boynton, 1969). It is conceivable that the changes in size and conformation of the plastids reflect changes in the metabolic activity of the plastids.

If the rate of chlorophyll accumulation is used as a measure, then the 5-day-old dark-grown barley seedlings used in this study concerning the structural aspects of chloroplast development are comparable to the 6-day-old dark-grown seedlings used for earlier studies about the development of the photochemical activities (Henningsen & Boardman, 1973). Furthermore, they are comparable to the seedlings used for analysis of esterification of chlorophyllide (K. W. Henningsen & S. W. Thorne, in preparation) and for changes in the properties of chlorophyll(ide) holochromes at the initial stage of greening (K. W. Henningsen, S. W. Thorne & N. K. Boardman, in preparation).

The spectral shift from 682–684 to 672 nm and esterification of newly formed chlorophyllide have similar time courses and both processes are temperature dependent in a similar manner (Sironval et al. 1965; K. W. Henningsen & S. W. Thorne, in preparation). Also the dispersal of the prolamellar bodies into primary lamellar layers has been correlated with the spectral shift of chlorophyllide from 682–684 to 672 nm (Henningsen & Boynton, 1969; Henningsen, 1970). In leaves of mutants in barley where the spectral shift from 682 to 672 nm of newly formed chlorophyllide is lacking, esterification and subsequent processes are also blocked (K. W. Henningsen & S. W. Thorne, in preparation). Thus, the 682–684 to 672 nm spectral shift, the esterification of chlorophyllide and the dispersal of the prolamellar body appear interconnected.

Ultracentrifugation (Bogorad, Laber & Gassman, 1968), gel chromatography (Henningsen & Kahn, 1971; K. W. Henningsen, S. W. Thorne & N. K. Boardman, in preparation) and fluorescence spectroscopy (Thorne, 1971), give results consistent with the translocation of the newly formed chlorophyll a protein complex in the plastid membranes. The time course for the increase in fluorescence efficiency of newly formed chlorophyll a in barley leaves (K. W. Henningsen & S. W. Thorne, in preparation), correlates well with the appearance of the first photochemical activities (Henningsen & Boardman, 1973). Appreciable photosystem I activity is detected with plastids from barley leaves illuminated for 15 min. Chlorophyll holochrome extracted with saponin from such leaves has photosystem I activity and appears to contain associated cytochromes (K. W. Henningsen, S. W. Thorne & N. K. Boardman, in preparation). Since cytochromes are not associated with protochlorophyllide holochrome extracted with saponin from dark-grown leaves (Henningsen & Kahn, 1971), the association between chlorophyll holochrome and cytochromes appears to be formed during the
light period. Thus, it appears that the newly formed chlorophyll-protein complex is translocated from the original site in the prolamellar body membranes and incorporated into reaction centre sites in the primary lamellar layers where it becomes associated with components of the electron-transport system.

Photosynthetic oxygen evolution from leaves can be detected as soon as 30 min after the onset of illumination. On a chlorophyll basis, the rate of oxygen evolution from both leaves and isolated plastids reaches a maximum after 1.5–2 h of greening (Henningsen & Boardman, 1973). In young dark-grown barley seedlings, photosynthetic CO₂ fixation could also be detected after 3 h illumination (Rhodes & Yemm, 1966). These results when considered in light of the observations reported above show that both photosystem I and II are functional at a stage when the lamellae are either single or paired into small diameter 2-disk grana. The early appearance of 2-disk grana and capacity for photosynthetic oxygen evolution in greening barley leaves agree with the studies on lamellar structure and photosynthetic reactions in mutants of *Nicotiana tabacum* by Homann & Schmid (1967). They concluded that photosystem I activity can occur with single lamellae, but oxygen evolution (photosystem II) requires pairing between at least 2 lamellae. Oxygen evolution and photophosphorylation were detected in dark-grown bean leaves irradiated with far-red light (De Greef et al. 1971). Under this condition most of the lamellae are unpaired; however regions of pairing between 2 or 3 lamellae are evident.

In contrast to the higher plants, the ac-31 mutant in *Chlamydomonas* has a functional photosystem II, but all the lamellae are unpaired (Goodenough, Armstrong & Levine, 1969). During greening of dark-grown cells of the mutant y-1 in *Chlamydomonas*, the developing lamellae are unpaired at the time when oxygen evolution is first detected (Ohad et al. 1967).

While the photosynthetic membranes in green algae appear to be homogenous (Eytan & Ohad, 1972), the lamellar system in chloroplasts of higher plants is differentiated into grana and stroma regions with distinctly different functions and composition (Sane, Goodchild & Park, 1970; Allen, Good, Trosper & Park, 1972). This distinction between the lamellar system in algae and higher plants could explain why pairing can be lacking in chloroplasts of algae, while some paired regions between at least two lamellae occur in chloroplasts of higher plants under conditions where photosystem II is functional.

During the early stages of greening high light intensity is required to saturate the photosystems (Kirk & Goodchild, 1972; Bar-Nun et al. 1972; Henningsen & Boardman, 1973). During this period the chlorophyll content is low and the plastid membranes consist of unpaired lamellae and small-diameter 2-disk grana. At later stages of greening when grana with 3 or more disks are formed and more chlorophyll accumulates the photochemical reactions are saturated at a lower light intensity. It appears that the accumulation of chlorophyll in the light harvesting system and also the appearance of the high potential form of cytochrome b-559 parallels the increase in paired regions with 3 or more lamellae.

The development of chloroplasts in 7-, 9- and 11-day-old barley seedlings follows the same sequence as described for the 5- to 6-day-old seedlings. Chloroplast develop-
ment is slower in the older seedlings and it is evident that during greening for up to 24 h, the lamellar membranes are all derived from membrane material present in the etioplast. However, the newly dispersed lamellar layers may function as a framework for the insertion of further membrane components required for the formation of grana.

This work was supported by grants from the National Institute of Health, U.S. Public Health Service (GM 10819), the Carlsberg Foundation, and the Danish Natural Science Research Council to Professor D. von Wettstein. The authors thank Professor Diter von Wettstein, Dr A. Kahn and Dr Niels Nielsen for valuable discussions. The technical assistance of Miss K. B. Pauli and Mr P. Eriksen is gratefully acknowledged.

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(Received 29 August 1973)
Fig. 12. Plastid section from the primary leaf of a 5-day-old dark-grown barley seedling illuminated for 30 min with 3200 lx of white light. Remnants of a transformed prolamellar body (pb) connected to primary lamellar layers with numerous perforations (arrows) and protuberances (tr). Groups of fine tubules (f) in the stroma. × 37000.

Fig. 13. Plastid section from the primary leaf of an 11-day-old dark-grown barley seedling illuminated for 30 min with 3200 lx of white light. The plastid membranes consist of a transformed prolamellar body and a stack of long parallel lamellar layers largely without perforations. × 37000.
Fig. 14. Section through a plastid in the primary leaf of a 9-day-old dark-grown barley seedling illuminated for 1 h with 3200 lx of white light. Primary lamellar layers are radiating from the periphery of a transformed prolamellar body. The lamellar sheets have numerous perforations (p) and protuberances (arrows and insets). A bundle of fine tubules (f) is present in the stroma. × 61,000. Insets: Protuberances from the primary lamellar layers, in cross-section (at left) and in surface view (at right). × 108,000; scale line represents 0.2 μm.
Fig. 15. Plastid section from the primary leaf of a 5-day-old dark-grown barley seedling illuminated for 4 h with 3200 lx of white light. Most of the perforations have been eliminated from the lamellar layers and grana (g) are forming. Bundles of fine tubules in the stroma are seen in longitudinal and cross-section (arrows). $\times 51000$.

Fig. 16. Plastid section from the primary leaf of a 9-day-old dark-grown barley seedling illuminated for 4 h with 3200 lx of white light. Many perforations are still present in the lamellar layers. Regions of the lamellar layers are arranged in loose stacks and some of the lamellae participate in grana-like pairing. $\times 30000$. 
Fig. 17. Plastid section from the primary leaf of a 5-day-old barley seedling illuminated for 16 h with 3200 lx of white light. The lamellar system is well differentiated into grana consisting of several paired disks and inter-grana lamellae which connect adjacent grana. $\times 33000$.

Fig. 18. Plastid section from the primary leaf of an 11-day-old dark-grown barley seedling illuminated for 24 h with 3200 lx of white light. Several lamellar layers are arranged in a loose stack. Some short regions of the lamellar layers are paired (arrows). $\times 41000$. 
Figs. 19, 20. Plastid sections from the primary leaves of 7-day-old dark-grown barley seedlings illuminated for 8 h with white light at 3200 lx (Fig. 19) and at 20 lx (Fig. 20). Many grana, with several disks in each, are formed in the plastids under both conditions. The plastid from a leaf illuminated at 20 lx (Fig. 20) contains in addition crystalline prolamellar bodies. The small prolamellar bodies are connected to several adjacent lamellar layers. Fig. 19, ×48000; Fig. 20, ×41000.