FINE STRUCTURAL CHANGES IN PROPLASTIDS DURING PHOTODESTRUCTION OF PIGMENTS

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ABSTRACT

Etiolated bean leaves supplied δ-amino-levulinic acid in the dark synthesize large amounts of protochlorophyllide which is not converted to chlorophyllide upon illumination of the leaves. The fine structure of the proplastids is not affected by the treatment. When leaves containing "inactive" protochlorophyllide are exposed to light of 700 ft-c for 3 hours, they lose practically all their green pigments. During this period large stacks of closed membrane structures are built up in the region of the prolamellar body. These lamellar structures remain even when no or only traces of pigment are left in the leaves. In untreated control leaves the pigment content remained constant during similar illumination and the structural changes in the plastids consisted of a rearrangement of the vesicles from the prolamellar bodies into strands dispersed through the stroma; lamellae and grana formation occurred later.

INTRODUCTION

Leaves on etiolated, dark-grown bean seedlings contain Mg-vinyl pheoporphyrin (protochlorophyllide), with an *in vivo* absorption peak at 650 mμ ($Pchl_{650}$). Upon illumination the pigment is rapidly transformed to chlorophyllide and subsequently to chlorophyll (14, 19). Later on, new pigment is formed (14, 17) and the plastids, the fine structure of which begins to change immediately after exposure to light, assume their mature form and structure (4).

δ-Amino-levulinic acid (δ-AL) is an early precursor of porphyrins and has been used frequently in investigations into porphyrin synthesis in animals and plants (1, 5). Etiolated leaves of barley and beans supplied δ-AL in the dark accumulate large amounts of protochlorophyllide, as well as smaller amounts of earlier chlorophyll precursors. However, protochlorophyllide formed from exogenous δ-AL has an absorption peak at 630 to 633 mμ *in vivo* ($Pchl_{631}$) and it is not converted to chlorophyllide in light (5). The conversion of the endogenous $Pchl_{650}$ to chlorophyllide and the subsequent chlorophyll accumulation seems to be unaffected or even somewhat enhanced (13) in weak light by the presence of $Pchl_{631}$ (5). Leaves containing $Pchl_{631}$ as a consequence of treatment with δ-AL die when exposed to strong light. The deterioration of the leaves is accompanied (and/or preceded) by rapid destruction of the chlorophylls. Thus, while normal etiolated plants become green in light, leaves fed δ-AL during a preceding period in the dark lose their pigment upon illumination.

It has been often assumed that grana formation is coupled to chlorophyll accumulation, and that loss of pigment leads to a destruction of the lamellar system. In this work we investigated the fine structure of δ-AL treated leaves containing large amounts of $Pchl_{631}$ and the structural changes

which occurred in the plastids when these leaves were exposed to strong light. The results showed that, during the period of decrease in chlorophyll content, stacks of elongated, closed membrane structures were formed in the plastids, which remained even when no or only traces of pigment were left.

MATERIAL AND METHODS

Red kidney bean seedlings (*Phaseolus vulgaris*) were grown in Vermiculite in a dark room at a constant temperature of 26°C. After 14 to 16 days the first pair of leaves was detached for use. Usually six leaves were placed on filter paper above a thin layer of cotton in petri dishes containing 5 ml of 0.01 M δ-aminolevulinic acid or tap water. The Petri dishes remained in the dark room or were exposed to white fluorescent light. The material was handled under a weak green safelight. Pigment content of single leaves was measured *in vivo* in a Cary spectrophotometer by the method of Shibata (11). For electron microscopy, sections of the leaves were fixed in 2 per cent $KMnO_4$ for ¾ hour in the dark at room temperature. During the first 5 to 10 minutes the sections were maintained at reduced pressure. So that changes in both pigment content and structure could be checked in the same leaf, frequently sections were cut from the same leaf after various periods of light exposure and spectrophotometric measurements made. The tissue sections were dehydrated in graded alcohols, embedded in Epon, and cut with a diamond knife on a Porter-Blum ultramicrotome. The sections were examined in an RCA C3 electron microscope.

RESULTS

The Effect of δ-AL Treatment on Chlorophyll Formation

When 14-day-old etiolated bean leaves were treated with δ-AL in the dark, inactive protochlorophyllide ($Pchl_{631}$) accumulated rather rapidly in their plastids. After 3 to 4 hours the amounts of active and inactive protochlorophyllide were equal, as indicated by the height of the absorption bands at 631 mμ and 650 mμ. (Protochlorophyllide which is not converted to chlorophyllide upon illumination of the leaf is termed "inactive" in this report; "active" protochlorophyllide is that which is converted under these circumstances.) After 24 hours there was 6 to 10 times more inactive than active protochlorophyllide present. This is in agreement with the observations of Granick (5) and Sisler and Klein (13). No significant changes in amount of convertible protochlorophyllide could be detected during this period. The δ-AL-treated leaves were much greener than the water-treated controls, which were yellow, and remained turgid and healthy looking. Frequently the greening was not uniform, and patches, having the same yellow color as the controls, remained.

Leaves which had been treated for 16 hours or longer with δ-AL in the dark lost essentially all their pigments after 3 to 4 hours' exposure to 700 ft-c of white light (Figs. 1 and 2). However, after only a 10 second exposure to the same light source the 633-mμ absorption peak in δ-AL-treated leaves remained unchanged, while conversion of the endogenous active protochlorophyllide ($Pchl_{650}$) to chlorophyllide occurred to the same extent as in the controls. The protochlorophyllide-chlorophyllide transformation did, however, differ in one respect: in the water-treated control leaves spectral shifts described by Shibata (11) could be observed upon illumination: $Pchl_{650}$ changed immediately to Chl_{682} and then, gradually during the next 30 to 45 minutes, back to Chl_{673}. In the δ-AL-treated leaves, on the other hand, $Pchl_{650}$ changed upon illumination directly to $Chl_{673-675}$; no further spectral changes occurred.

During 3-hour exposures to light the green areas of the leaves faded gradually and turned greyish-white. The yellow patches remained somewhat longer and faded only later. In control leaves the chlorophyll content remained essentially the same during a 3 to 4 hour period and greening began somewhat afterwards. After 24 hours in the light the chlorophyll content in the control leaves generally increased 20- to 40-fold.

Structural Changes in the Plastids During Photodestruction of Pigments

Structural Changes in Control Leaves

The structural changes found in plastids of 14-day-old etiolated leaves exposed to 700 ft-c of white light are in agreement with the ones described by Eriksson *et al.* (4) and Virgin *et al.* (18). In the etiolated leaves prior to illumination an extensive prolamellar body accompanied by a few single strands of vesicles was seen in the rounded or ameboid proplastids. At this stage the units in the prolamellar body appeared to be fused into a network of regularly arranged tubular units, giving a crystalline appearance. After 10 seconds' exposure to 700 ft-c of white light this regular appearance of

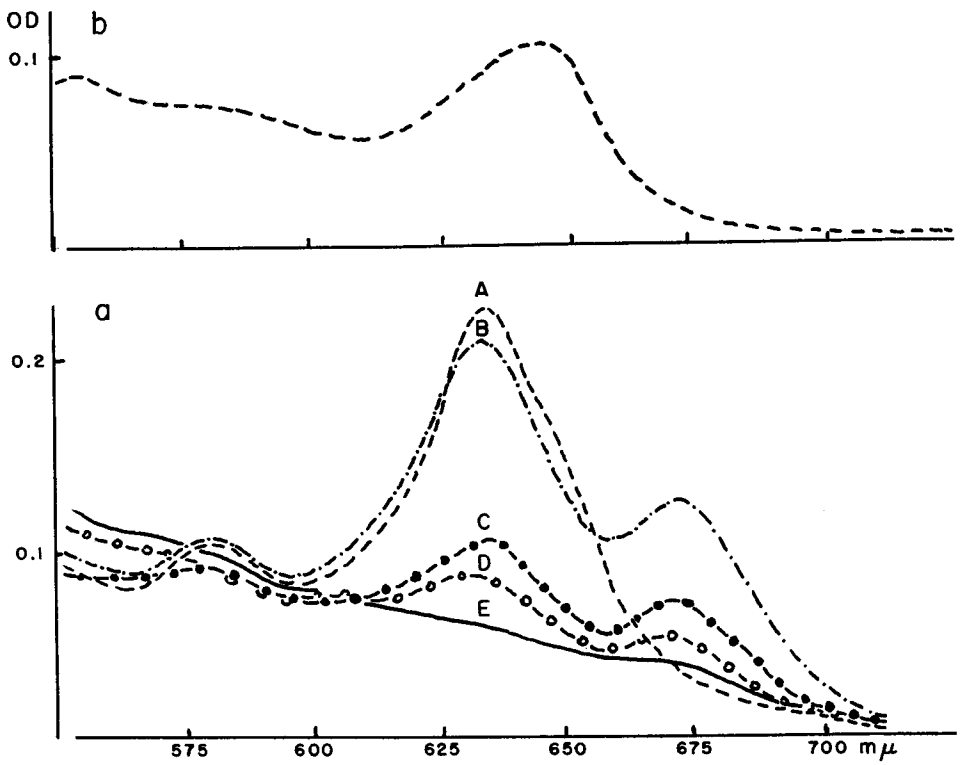


FIGURE 1 Fig. 1 *a, in vivo* absorption curves obtained from an etiolated bean leaf treated for 18 hours with δ-amino-levulinic acid in darkness and then exposed to light of 700 ft-c. Curve *A*, before exposure to light. Curve *B*, after 0.2 minutes of illumination; *C*, after 60 minutes; *D*, after 120 minutes; and *E*, after 180 minutes. Fig. 1 *b, in vivo* absorption curve from an untreated etiolated control leaf.

the prolamellar body was lost and disconnected vesicles appeared. After 3 to 4 hours of continuous illumination the prolamellar body was diminished in size and many of the vesicles were arranged in single strands, frequently in the form of loops protruding from the prolamellar body into the stroma. The prolamellar body may have disappeared completely and the strands of vesicles frequently formed concentric ring structures. These structures were spaced throughout the stroma and seemed not to be restricted to the area originally occupied by the prolamellar bodies. During this period no evidence was found of additional vesicle formation from the plastid membranes, and stacking of vesicles (grana formation) was rather exceptional and occurred very infrequently. After 24 hours of exposure to light the plastids contained a number of grana and interconnecting lamellar structures.

STRUCTURAL CHANGES IN THE δ-AL-TREATED LEAVES

Before exposure to light, the fine structure of the proplastids in the leaves supplied with δ-AL for 16 to 20 hours was the same as in proplastids of the controls (Fig. 3). These plastids contained, in addition to their "endogenous" $Pchl_{650}$, an up to 10 times larger amount of inactive $Pchl_{631}$. Under the fluorescence microscope an increased fluorescence could be observed restricted to certain areas in the proplastids which in the phase microscope appeared darker than the stroma and which are probably identical with the prolamellar bodies (9). Granick (5) found also slight fluorescence in the stroma; this could not be observed in our material.

Also, after a 10 second exposure to light no effect of the δ-AL treatment on plastid structure could be found and the changes observed in sections from treated and control leaves were identical. Both

showed the transformation of the regularly fused, crystal-like prolamellar body into an irregularly arranged mass of single units. However, after 1 hour of exposure to light the plastids from the δ-AL-treated leaves were radically different. Closed membrane structures, arranged parallel to one another and packed into stacks of frequently more than ten units with even spaces between them (Fig. 4), took the place of the prolamellar

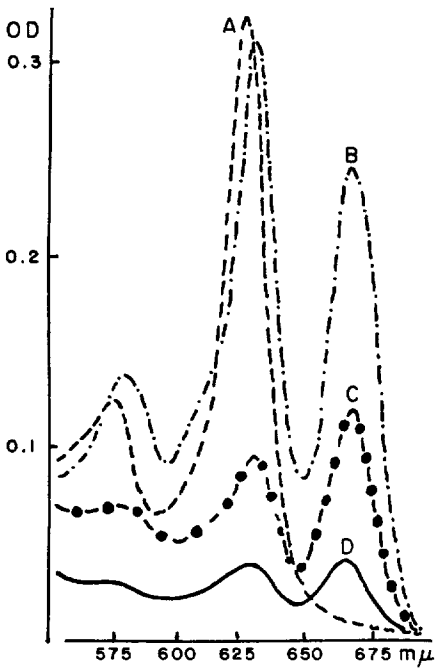


FIGURE 2 Absorption curves of acetone extracts of 1tiolated bean leaves treated as explained in Figure e *a*. Curve *A*, before exposure to light; *B*, after 0.2 minutes of illumination; *C*, after 90 minutes; and *D*, after 180 minutes.

bodies which had almost entirely disappeared. The space taken up by these structures was approximately the same as that occupied previously by the vesicular aggregates, and there was only a small tendency for units to protrude into the surrounding stroma. Even after 3 hours of light exposure, when no or only traces of pigments were left, the stacks of units remained unaltered (Figs. 5 to 7). The appearance of plastids containing these localized stacked membrane structures was confined to areas of the leaf which were green before the process of fading began. As mentioned above, occasionally yellow spots remained in the leaves even after 1 to 3 hours of light exposure. Sections through those areas contained plastids with structures typical for control leaves after 1 to 3 hours of illumination and showed remnants of vesicular centers together with loops or ring structures (Fig. 8). It can be assumed that during illumination pigment destruction as well as the described structural changes are confined to cells which have taken up δ-AL.

DISCUSSION

The main sequence of events in chloroplast development, in both light and darkness, has been described by a number of workers. More detailed investigations into the structural changes following exposure to light of etiolated tissue have been made in von Wettstein's laboratory (4, 18). Work by Klein *et al.* (8) and the data from our controls in the work described here confirm the findings of this group. At least three different phases in plastid development could be found after light exposure:

1. A rapid disruption of the orderly arranged system of interconnected units in the prolamellar body into single elongated vesicles. These changes occurred simultaneously with the protochlorophyllide-chlorophyllide conversion.
2. A dispersal of vesicles originating from the vesicular center throughout the stroma. The vesicles are usually arranged in strands, forming loops and, later on, ring structures. The formation of these structures under the conditions of the experiment described here coincided approximately with the lag period in chlorophyll accumulation.
3. Grana formation, which occurs after the dispersal of the vesicles through the stroma. This process is temperature-dependent (6) and begins at the same time as the rapid accumulation of chlorophyll.

Proplastids in etiolated bean leaves incubated with δ-AL for 16 to 20 hours in darkness are identical in fine structure with those in untreated leaves. The δ-AL-treated leaves contain 4 to 10 times more protochlorophyllide than the controls. The protochlorophyllide derived from exogenous δ-AL is of the 631-mμ absorbing type ($Pchl_{631}$) which is not converted to chlorophyllide upon illumination of the leaf; the two properties of the "new" protochlorophyllide suggest that the physical state of $Pchl_{631}$ differs from that of $Pchl_{650}$. $Pchl_{631}$ is either complexed with a different specific

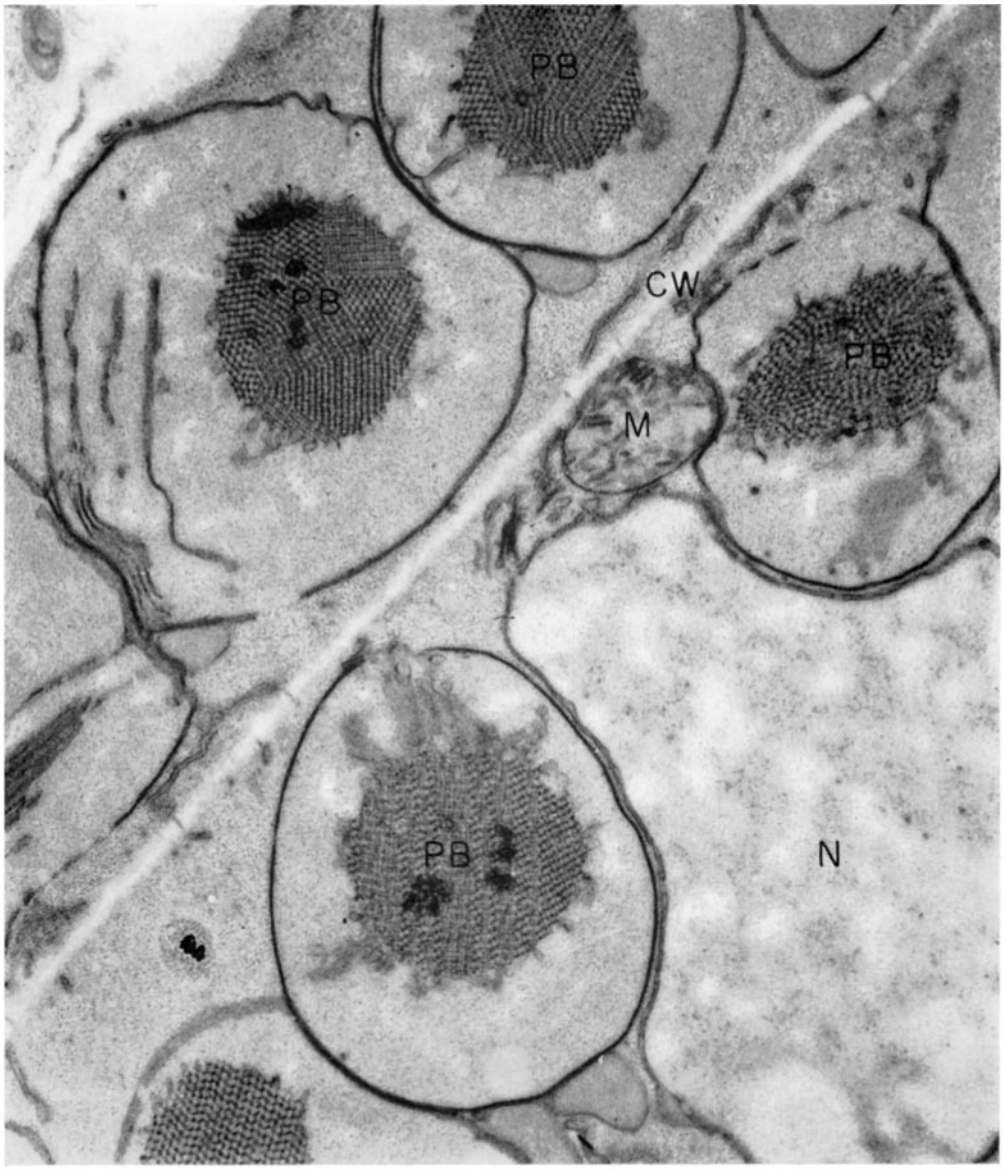


FIGURE 3 Section through proplastids of a leaf treated with 0.01 M δ-amino-levulinic acid for 18 hrs. in darkness. The treatment resulted in a large increase in inactive protochlorophyllide ($Pchl_{631}$). The fine structure of the proplastids appears normal. PB, prolamellar body; N, nucleus; M, mitochondrion; CW, cell wall. × 14,000.

protein or is non-specifically adsorbed. The fluorescence data make it plausible that it is located in close vicinity of the active $Pchl_{650}$, probably within the prolamellar body. Because of the lack of structural differences between plastids of δ-AL-treated and control leaves, it can be assumed that an increase in pigment content is in itself insufficient for structural development. For this to occur, other changes are required, presumably affecting the structural proteins in the plastids. So far, it has been possible to induce this change in higher plants only photochemically.

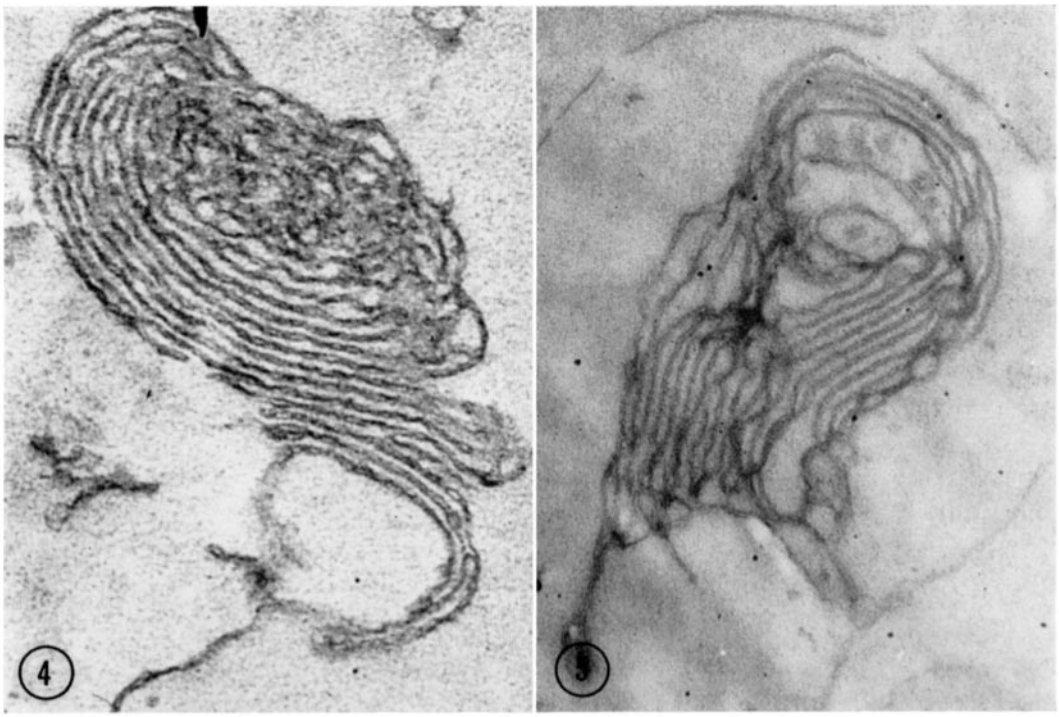


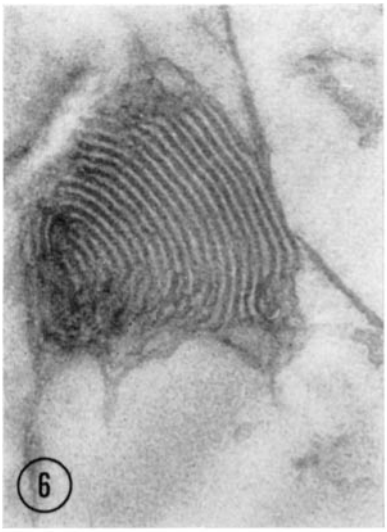
FIGURE 4 Section through the former prolamellar body of a plastid in a leaf treated with 0.01 M δ-AL for 18 hours in darkness and then exposed for 1 hour to white light of 700 ft-c. Pigment content was reduced to about one-half due to photodestruction. × 54,000.

FIGURE 5 Section through a plastid of the same leaf after 2 hours of illumination. × 48,000.

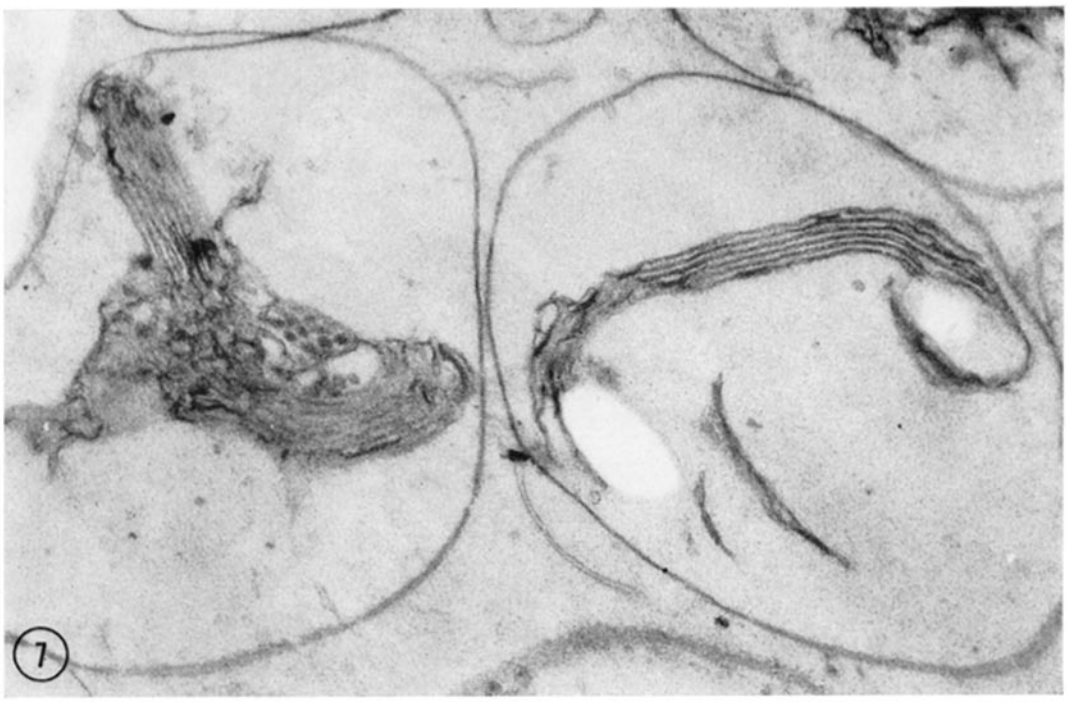
When δ-AL-treated leaves are exposed to light, the endogenous $PChl_{650}$ is converted to Chl_{675} instead of to Chl_{682} as in untreated leaves. A similar "shift of the shift" was found in illuminated glycerin extracts of holochrome (15), although in this case the active Pchl had a peak at 635 mμ. Isolated proplastid suspensions from non-treated etiolated leaves behave similarly (7). The appearance of Chl_{675} in the δ-AL-treated leaves after illumination may be due to physical changes in the arrangement of the holochrome, but it is also possible that it may result from an overlapping of the absorbtion-spectra for the $Pchl_{631}$ and the newly formed chlorophyll. In any case, the appearance of the peak at 675 mμ instead of 682 mμ does not interfere with the structural transformation usually found during pigment conversion.

Both $Pchl_{631}$ and Chl_{675} disappear rapidly when δ-AL-treated leaves are continually illuminated with 700 ft-c of white light. It is not known what the breakdown products of photochemically destroyed chlorophylls are. Neither *in vivo* (Fig. 1) nor in acetone (Fig. 2) or ether extracts could we find spectrophotoscopic evidence for any breakdown products after 1 to 3 hours of illumination. It can be assumed that the pigments are rapidly oxidized and/or broken down to forms which do not absorb visible light.

The most conspicuous structural change in the plastids during the 1 to 3 hours of photodestruction of the pigments is the formation of stacked lamellar structures in the region of the prolamellar body instead of the dispersal of the vesicles through the stroma. The structures resemble stacks of thylacoids (10) rather than myelin figures (16). We assume that they are formed by a fusion of the existing vesicles. Lamellar formation, although quite frequent in etiolated corn and barley, is rather unusual in plastids of dark-grown bean leaves and does not usually occur in the light



FIGURES 6 and 7 Section through plastids of the same leaf as in Fig. 4 after 3 hours of illumination. Only traces of pigment are left. Fig. 6, × 48,000; Fig. 7, × 40,000.



before the end of the lag period in chlorophyll accumulation. However, the appearance of these structures in the δ-AL-treated leaves, while the pigment content diminishes, indicates that their formation and arrangement into stacks may not necessarily be correlated with an increase in chlorophyll content. The stacks of these lamellae differ from the grana found at later stages of plastid development in untreated bean leaves. They resemble more the "magnograna" found by Döbel (2) in tomato leaves bleached after treatment with chloramphenicol or streptomycin. A similar formation of large stacks of lamellae without increase in pigment content has been reported

by Eilam and Klein (3) for leaves floated on a sucrose solution in the dark after exposure to low light. Siegesmund *et al.* (12) have reported the occurrence of concentric lamellae in plastids of streptomycin-bleached cells of *Euglena.* It is not surprising that under all these conditions the lamellar stacks differ from the normally occuring grana (or, as in the case of *Euglena*, from the lamellae may persist, at least temporarily, even when there are no or only traces of chlorophyll left.

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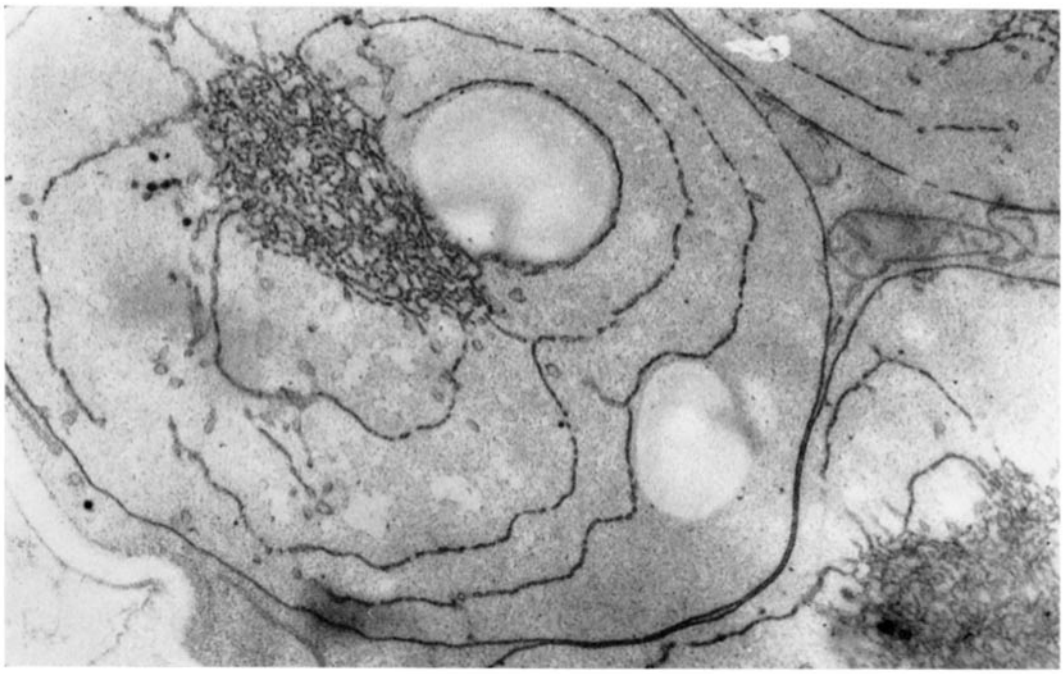

FIGURE 8 Section through a plastid in a part of the leaf that was not affected by the treatment after 3 18 hours of illumination. Note the aggregation of vesicles in the prolamellar body and their normal dispersal through the stroma. × 40,000.

normal arrangement of the lamellae). The normal development of grana is probably regulated by a number of factors and feed-back systems the equilibrium between which has probably been upset by the various treatments. The point is that lamellae and lamellar stacks can be formed in plastids during a period of a decrease in chlorophyll content, as shown here, as well as in plastids with low chlorophyll content.

It has been frequently assumed that destruction of chlorophyll goes hand in hand with a disappearance of the lamellar system in plastids. The present work (see Figs. 6 and 7) shows that

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BIBLIOGRAPHY

1. BOGORAD, L., *in* Biogenesis of Natural Compounds, (P. Bernfeld, editor), London, Pergamon Press Limited, 1963, 183.
2. DÖBEL, P., *Biol. Zentr.*, 1963, **82,** 277.
3. EILAM, Y., and KLEIN, S., *J. Cell Biol.*, 1962, **14,** 169.
4. ERIKSSON, G., KAHN, A., WALLES, B., and VON WETTSTEIN, D., *Ber. Bot. Ges.*, 1961, **74,** 221.
5. GRANICK, S., *Proc. Internat. Congr. Biochem., 5th, Moscow*, 1961, **5,** 176.
6. KLEIN, S., *J. Biophysic. and Biochem. Cytol.*, 1960, **8,** 529.
7. KLEIN, S., and BOGORAD, L., data unpublished.
8. KLEIN, S., BRYAN, G., and BOGORAD, L., *J. Cell Biol.*, 964, **22,** 433.
9. KLEIN, S., and POLJAKOFF-MAYBER, A., *J. Biophysic. and Biochem. Cytol.*, 1961, **11,** 433.
10. KREUTZ, W., and MENKE, W., *Z. Naturforsch.*, 1962, **17 b,** 675.
11. SHIBATA, K., *J. Biochem.*, (*Tokyo*), 1957, **44,** 147.
12. SIEGESMUND, K. A., ROSEN, W. G., and GAWLIK, S. R., *Am. J. Bot.*, 1962, **49,** 137.
13. SISLER, E. C., and KLEIN, W. H., *Physiol. Plantarum*, 1963, **16,** 315.
14. SMITH, J. H. C., *in* Comparative Biochemistry of Photoreactive Systems, (M. B. Allen, editor), New York, Academic Press, Inc., 1960, 257.
15. SMITH, J. H. C., and BENITEZ, A., *Carnegie Inst. Wash. Year Book*, 1963, **52,** 149.
16. STOECKENIUS, W., *in* The Interpretation of Ultrastructure, (R. J. C. Harris, editor), New York, Academic Press, Inc., 1962, 349.
17. VIRGIN, H. I., *Physiol. Plantarum*, 1958, **11,** 347.
18. VIRGIN, H. I., KAHN, A., and VON WETTSTEIN, D., *Photochem. and Photobiol.*, 1963, **2,** 83.
19. WOLFF, J. B., and PRICE, L., *Arch. Biochem. and Biophysics*, 1957, **72,** 293.