

Structure and Function of Developing Barley Plastids¹

Received for publication June 20, 1973 and in revised form March 20, 1974

DIANE ROBERTSON² AND WATSON McMILLAN LAETSCH

Department of Botany, University of California, Berkeley, California 94720

ABSTRACT

Five different regions of the first foliage leaf of etiolated barley seedlings were studied with respect to leaf growth, plastid growth and replication, differentiation of etioplasts, and conversion of etioplasts into chloroplasts upon illumination. Ultrastructural changes of the plastids were correlated with chlorophyll synthesis and development of photosynthetic activity as measured by ¹⁴CO₂ incorporation and O₂ evolution. The first foliage leaf has greater linear growth over a longer period of time in the dark than in the light. Only the bottom two regions (4 and 5) are still growing in the 5-day etiolated leaf. Region 4 grows by cell elongation, and region 5 grows by both cell division and elongation. Plastids in all five regions of the leaf are capable of enlarging when exposed to light. This is true both for the intact plant and for excised sections. Plastid replication occurs predominantly in the younger regions of the leaf (regions 3, 4, and 5). The amount of chlorophyll synthesized by different regions in the intact plant is significantly higher (3-40 times) than that made by excised sections. Ultrastructural changes occurring in each region when excised sections are illuminated were classified into five stages involving increased membrane synthesis and appression into grana, and these changes were correlated with the first appearance of photosynthetic activity. The earliest detectable photosynthetic activity occurs in region 1 after 2 hours of illumination when chloroplasts show only a few overlaps in the thylakoids. Plastids in younger regions of the leaf require up to 24 hours of light to form grana and develop photosynthetic activity. Plastids in each region of the leaf are in different stages of development when photosynthesis is initiated, indicating that development of photosynthetic activity is not strictly correlated with a certain stage of plastid development. Membrane appression is not indicative of photosynthetic activity since overlaps are formed in the dark, but it was always present when photosynthetic activity was detectable. Likewise, there does not appear to be any strict correlation between the presence of chlorophyll and membrane appression. These results show that the particular structural and functional correlations that can be made depend to a large degree on age of the tissue.

number of investigators have attempted to correlate structural changes of etioplasts when exposed to light with development of photosynthetic activity. Rhodes and Yemm (39) measured CO₂ exchange in dark-grown barley plants when exposed to light, and they followed ultrastructural changes during this same period, but they stated only that grana formation was promoted by light and coincided with Chl formation and increase in photosynthetic activity. Wieckowski (49), using very young bean seedlings, found that O₂ evolution was first detectable after about 2 hr of illumination, and this corresponded with the appearance of appressed lamellae in the plastids. DeGreef *et al.* (11), however, followed O₂ evolution and plastid ultrastructural changes in bean leaves illuminated with far red light. They concluded that grana formation was not necessary for development of photosynthetic activity or accumulation of Chl. Light-driven redox changes of Cyt *f* in greening bean leaves were measured by Hiller and Boardman (27), but they found no ultrastructural features correlated with the onset of redox changes.

Attempts have also been made to correlate ultrastructural changes in plastids in greening leaves with partial photosynthetic reactions in plastids isolated from the leaves (1, 21). Photosystem II activity was generally correlated with the formation of grana. Oelze-Karow and Butler (36), however, pointed out that this may reflect the greater stability of the grana against photosystem II inactivation. Sane *et al.* (40) used a French press to disrupt spinach chloroplasts and separated two fractions, one containing only photosystem I activity and the other with both photosystem I and II activity. Thin sectioning and freeze-fracturing of the two fractions revealed that the former fraction originated almost entirely from the stroma lamellae and the latter came from the grana regions. These results indicate a strong correlation between photosystem II activity and membrane appression.

Work with various mutants has confused the picture still further. Homann and Schmid (29), using tobacco mutants, concluded that a fully active fraction containing photosystem I activity can be associated with unappressed thylakoids, but the complete photosynthetic electron transport system including the fraction containing photosystem I and II activity required a close packing of at least two thylakoids. On the other hand, a *Chlamydomonas* mutant studied by Goodenough *et al.* (18) has no membrane stacking in its chloroplasts, yet it showed substantial CO₂ fixation, photophosphorylation, and photosynthetic electron transport. In addition, a barley mutant lacking Chl *b* (5, 17, 26) exhibited high photosystem I and II activity in spite of a reduction of appressed lamellae.

It is still unclear which membrane structure configuration can be correlated with photosynthetic activity. Conflicting claims as to the necessity of thylakoid appression for various photosynthetic activities make it difficult to obtain a clear picture of this important developmental process. The differences reported might be due merely to species differences or to different tissue age.

Seedlings of angiosperms grown in the dark do not synthesize Chl but their proplastids do differentiate into etioplasts. A

¹ Supported by a National Science Foundation Predoctoral Fellowship to Diane Robertson and by National Science Foundation Grant GB-12964.

² Present address: Ohio Agricultural Research and Development Center, Wooster, O. 44691.

In an attempt to clarify this situation, a detailed study of leaf growth, plastid growth and replication, etioplast differentiation, Chl synthesis, and photosynthetic activity in five different regions of the first foliage leaf of barley was undertaken. Owing to the activity of the leaf's basal meristem, these five regions represent tissue of five different ages in a single leaf. With the information from this study, we should be able to compare the development of a variety of plastid types and ages in a single leaf and from that draw conclusions concerning the correlation of structure with function.

MATERIALS AND METHODS

Seeds of barley, *Hordeum vulgare* L., var. Atlas 54 were sterilized in a 1% sodium hypochlorite solution for approximately 5 min, rinsed several times in tap water, and spread on the bottom of a covered plastic box in a small amount of water. They were germinated in the dark at 25 C for 24 hr. Seeds with an emergent coleorhiza were then placed on moist paper toweling in large covered glass jars. The seeds were returned to the dark for approximately 5 more days and kept moist with tap water. All handling of seeds and seedlings was conducted under illumination provided by a green safelight containing a 15-w incandescent bulb and a green Kodak filter (No. 54) with a transmission band of 510 to 570 nm.

After 5 to 6 days of growth in the dark, seedlings with first foliage leaves 80 to 100 mm long were collected. Seeds, roots, and coleoptiles were removed, and each leaf was measured and divided into four equal portions. A 6-mm section was excised from the middle of each portion, and these were designated regions 1, 2, 3, and 4 (Fig. 1). The remaining portion below region 4 was also used and designated region 5. Any pieces of the second foliage leaf which were present inside tissue from regions 4 and 5 were removed. For greening experiments, tissue from each region was floated on distilled water in covered Petri dishes under white fluorescent bulbs (F72PG17-D). For experiments with intact plants, dark-grown seedlings 80 to 100 mm long were placed on moist paper toweling in covered jars and illuminated under the same light conditions.

Leaf growth was measured by marking each region with India ink with a needle or Rapidograph pen. The coleoptiles were removed from intact dark-grown plants, and each region was marked at 1-mm intervals. The plants were returned to the dark, and the regions were measured again after 24 hr.

The cell separation technique used for chloroplast counts was modified from Dale and Heyes (10). Leaf tissue was fixed in 6% aqueous glutaraldehyde for at least 1 hr and stored in glutaraldehyde in the refrigerator until needed. Leaf pieces were then rinsed briefly in tap water and placed in a solution of 5% chromic acid made up in 1 N HCl and heated to 60 C for 1/2 to 1 hr (younger tissues required longer heating). Each piece was then placed on a slide in a drop of water and teased apart with needles. Tissue from the older regions of the leaf (regions 1, 2, and 3) would separate easily, but tissue from regions 4 and 5 only partially separated even with prolonged heating. The number and size of chloroplasts in individual cells (Fig. 1) were determined with a 100 \times phase contrast oil immersion lens on a Zeiss Photomicroscope. Counts were made on dark-grown tissue and on tissue which had been in the light for 5, 12, 24, and 48 hr and represent the average number of plastids per mesophyll cell. Plastid sizes represent their longest dimension.

Pigments for Chl determinations were extracted by grinding leaf tissue in 80% acetone with a mechanically driven ground glass homogenizing tube. Chlorophylls *a* and *b* were determined by the method of Arnon (2). The detectable limit of

Chl for the methods and equipment used was about 2 $\mu\text{g/g}$ fresh weight or 0.02 $\mu\text{g/ml}$.

Tissue for electron microscopy was fixed in 2% glutaraldehyde in phosphate buffer (0.05 M, pH 6.8) containing 20 $\mu\text{g/ml}$ CaCl_2 for 2 hr at room temperature. The tissue was both pressure and vacuum infiltrated while in glutaraldehyde. Three rinses in buffer preceded postfixation in 2% OsO_4 in phosphate buffer at 0 C for 2 hr. Tissue was dehydrated in a graded acetone series and propylene oxide, stained in uranyl nitrate added to the 70% acetone dehydration step, and embedded in Luft Epon (32). Sections were cut with a diamond knife on a Reichert OmU2 ultramicrotome, stained with lead citrate (38), and examined with a Siemens Elmskop I microscope.

Photosynthetic activity was measured with a Clark polarographic electrode (Yellow Springs Instrument Co., model 5331) and a YSI model 53 biological oxygen monitor combined with a Texas Instruments Servo-riter II recorder. A 5-ml sample chamber with a water jacket was attached to a Haake circulating water bath that held the temperature at 25 C. The chamber was illuminated with a Lite-Scope (Iota Cam Corp.) filtered through two infrared filters (Corning No. 1-69, and a Zeiss thermoprotective reflectance filter [Calflex] 46 78 32) giving a final intensity of approximately 1000 ft-c. It was not determined whether this intensity was saturating for all the tissues. The chamber was filled with distilled water and bubbled with 5% CO_2 in air for 1 min before adding the tissue sample. Eleven to 42 pieces of leaf tissue (0.036–0.192 g fresh wt; 0–0.081 mg Chl) were cut into 1-mm strips and inserted into the chamber. A constant rate of O_2 uptake in the dark due to respiration was established before and after illumination, and it served as a basal rate from which O_2 evolution was calculated. Rates were linear during the measurements, which were approximately 10 min long for each dark and light period (Fig. 2). The entire series of experiments was carried out twice, and each point represents the average of these two measurements. The limit of detectability for this method was approximately 1.1 $\mu\text{moles O}_2/\text{g fresh wt/hr}$.

Incorporation of $^{14}\text{CO}_2$ by leaf tissue was measured using the apparatus shown in Figure 3. Three to 12 leaf pieces (0.01–0.05 g fresh wt; 0–0.008 mg Chl) were held upright on the pin in the center of the chamber and allowed to equilibrate for 5 min in the light with ambient air moving slowly through the chamber. A damp piece of cotton was wrapped around the base of the pin to prevent drying of the tissue. Illumination of approximately 2500 ft-c was provided by a Tiyoda microscope illuminator with infrared filter (Corning No. 1-69). After equilibration, the apparatus was closed, and 20 μl of $\text{NaH}^{14}\text{CO}_3$ (53.5 mc/mole) were injected through the serum cap into 1 N HCl in the side arm liberating 0.085 μmole of $^{14}\text{CO}_2$ (4.54 μc in an 18-ml volume). The leaf tissue was exposed to $^{14}\text{CO}_2$ for 5 min, and the reaction was stopped by rapidly removing the tissue from the chamber and plunging it into liquid nitrogen. The samples were kept frozen in liquid nitrogen until they were combusted in a Packard Tri-Carb sample oxidizer, model 305. The combusted samples were counted with a Packard Tri-Carb liquid scintillation spectrometer, model 3375, with an external standard. The scintillation solution consisted of 15 gm Omnifluor, 1000 ml of toluene, and small amounts of methanol and ethanolamine. Calculations showed that the highest incorporations used only about 20% of the CO_2 in the chamber.

Calculations of the per cent of appressed membrane relative to total membrane present at different stages of development (Table III) were based on measurements from electron micrographs. The amount of appressed and total membrane was measured with a map mileage tracer.

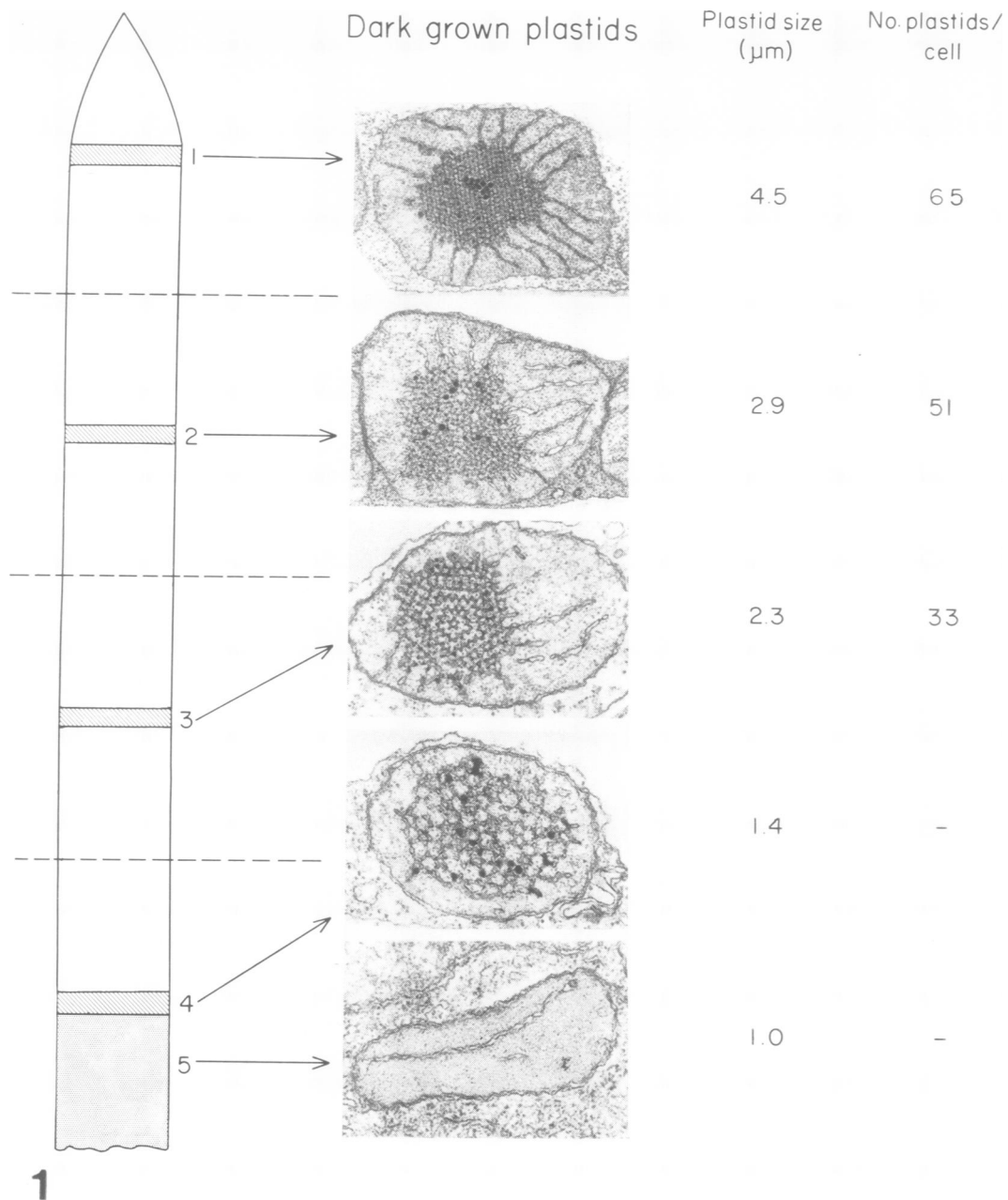


FIG. 1. Diagram showing regions of the first foliage leaf of barley used for experimentation, micrographs of dark-grown plastids found in each region, average size of plastids (μm), and average number of plastids per cell in each region. Regions: 1: $\times 15,810$; 2: $\times 18,144$; 3: $\times 25,315$; 4: $\times 34,710$; 5: $\times 36,706$.

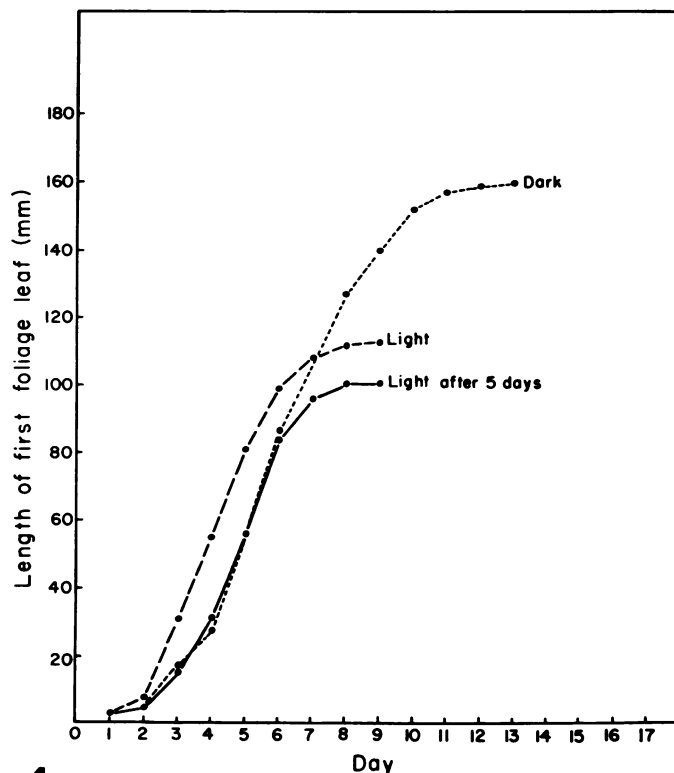
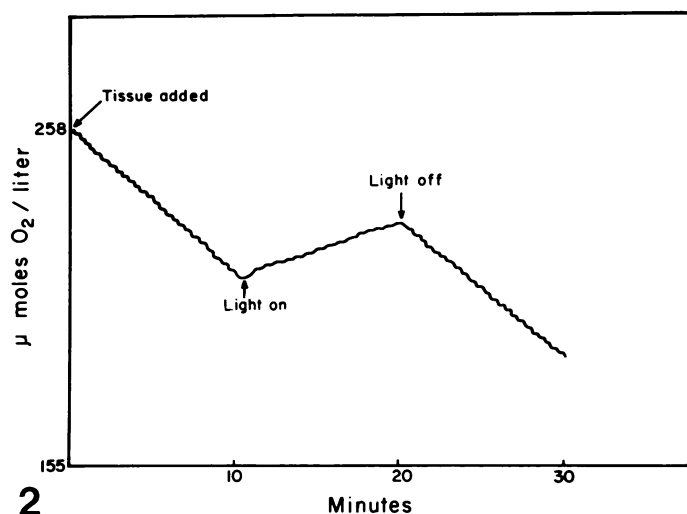
RESULTS

Leaf Growth. The growth curves for the first foliage leaf of barley show marked differences in light and dark (Fig. 4). Both curves are S-shaped, but light-grown plants grow more rapidly during the first 4 days. The first leaf of light-grown plants does not continue to grow after about 9 days, though most growth occurs during the first 7 days. The first leaf of dark-grown plants continues to grow for about 13 days with most of its growth occurring in the first 11 days.

Illuminating plants midway through their growth in the dark (Fig. 4) has no effect on the shape of the curve, but final length of the first leaf is less than in either dark or light. This is due to the combined effect of the slower growth rate in the dark and the later inhibitory effect of light on total leaf growth.

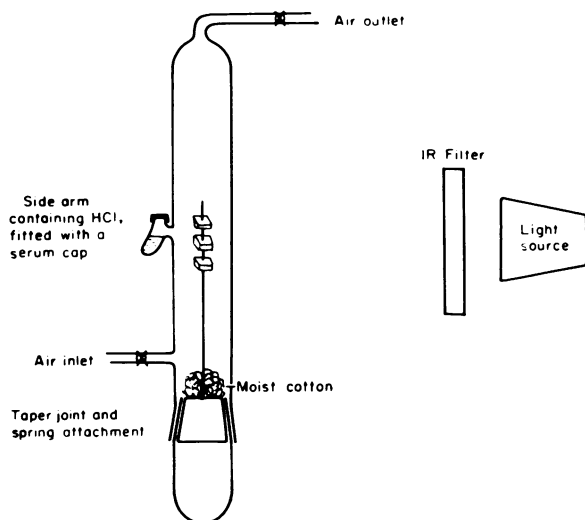
Marking experiments were conducted on dark-grown plants in order to determine which of the five leaf regions was still growing after 5 days. Only regions 4 and 5 grew. Region 4 elongated about 4 mm, and region 5 grew so much that the marks were always obliterated. Examination of longitudinal sections of tissue from these two regions revealed mitotic figures almost exclusively in region 5. Thus, growth in region 5 was due to both cell elongation and cell division.

Plastid Growth and Replication. Growth and replication of plastids in the first foliage leaf of barley were determined by comparing the size and number of plastids per cell in the five different regions of the dark-grown leaf and at intervals during 48 hr of illumination. This comparison was made with both excised sections and intact plants. The figures for excised sections and intact plants are given in Tables I and II.



2

4



3

FIG. 2. Oxygen evolution of tissue from region 3 which has been illuminated 12 hr with 500 ft-c white light.

FIG. 3. Diagram of apparatus used for ¹⁴CO₂ incorporation studies.

FIG. 4. Growth curves of the first foliage leaf of barley in continuous light (500 ft-c), in darkness, or placed in light after 5 days growth in darkness.

Where no figures are given, the plastids were too small to be seen. Attempts to count them using thick stained leaf sections also proved unsuccessful. Electron micrographs show that the plastids are indeed very small and few in number in these regions, but such profiles cannot be used for determining plastid numbers. Estimates of plastid size can be obtained by measuring plastid profiles in electron micrographs, but these are not very accurate compared with light microscope measurements. From these, however, we have obtained a value of about 1.4 μm for the plastids of region 4 and 1.0 μm for those of region 5. The measurements from electron micrographs were made on excised tissue only.

A comparison of the values, from base to tip, of the dark-grown leaf (Tables I and II) shows that the dark-grown plastids are capable of enlarging and dividing as each successive region matures.

In the excised sections, all five regions showed a significant increase in plastid size during the 48 hr of illumination (Table I). For the *t* test, dark values were compared to each light value in regions 1, 2, and 3. In regions 4 and 5, the 12-hr number was compared to the 24- and 48-hr values, since dark values were not available. Since plastids enlarged enough to be

Table I. Plastid Size

Region ¹	Intact Plants					Excised Sections				
	Hours in light									
	0	5	12	24	48	0	5	12	24	48
	μm									
1	4.5	5.1	5.4	6.2	6.5	4.5	5.5	5.0	5.0	5.1
2	2.9	4.8	5.3	5.2	6.2	2.9	3.7	4.3	4.2	5.3
3	2.3	3.2	4.1	4.8	6.7	2.3	1.7	3.4	4.0	4.4
4	—	2.3	3.8	4.0	5.9	—	—	1.8	2.1	2.9
5	—	2.1	3.8	3.3	5.4	—	—	1.6	2.1	3.2

¹ Within a given region all values differ significantly from either the 0-hr value (regions 1, 2, and 3) or the 5- or 12-hr values (regions 4 and 5) at a level of significance of 0.005 in a *t*-test.

seen with the light microscope after 12 hr, it seems likely that there was a significant increase in plastid size during the first 12 hr.

The average number of plastids per cell (Table II) showed

Table II. *Plastid Number*

Region ¹	Intact Plants					Excised Sections				
	Hours in light									
	0	5	12	24	48	0	5	12	24	48
	<i>no./cell</i>									
1	65	64	75	76	65 NS	65	53	67	64	64 NS
2	51	61 ²	60 ²	69	65 S	51	47	52	60	54 NS
3	33	55	68	62	65 S	33	39	46	52	58 NS
4	—	32	50	63	72 S	—	—	36	28	41 NS
5	—	30	55	56	71 S	—	—	23	28	44 NS

¹ Within a given region all values are either significantly different (S) or not significantly different (NS) from the 0-hr values (regions 1, 2, and 3) or the 5- or 12-hr values (regions 4 and 5) at a level of significance of 0.005 in a *t* test.

² Values not significantly different in *t* test.

no significant increase during 48 hr of illumination in regions 1 and 2 but did increase significantly in region 3. There is no significant increase in regions 4 and 5 if the 12-hr value is compared to that for 24 and 48 hr.

In intact plants, all five regions showed significant increases

in plastid size in the light (Table I). All regions except region 1 showed a significant increase in plastid number per cell during the same period (Table II). In regions 4 and 5, the 5-hr light values were used as the basis for comparison.

Etioplast Ultrastructure. Figure 1 shows a model of the first foliage leaf of barley with specific regions used in this investigation indicated by numbers 1 through 5. Electron micrographs illustrate the state of development of dark-grown plastids found in each region. Figure 1 also gives average plastid size and average number of plastids per cell.

Each region, starting from the base of the leaf, represents successively older tissue, and so a comparison of plastids from one region to the next represents a developmental sequence and provides a basis for comparison of ultrastructural changes occurring in light.

Plastids in region 5 are typical proplastids and contain almost no membranes. These plastids are very small and few in number. Those in region 4 are also very small but do contain more membrane, most of which is arranged in a small prolamellar body. Not all plastid profiles in this region contain prolamellar bodies. The plastids in regions 3, 2, and 1 are larger and more numerous and have well developed prolamellar bodies. There are usually a number of single thylakoids extending into the stroma from the periphery of the prolamellar bodies. Plastids in regions 1 and 2 are often characterized by small overlaps of the primary thylakoid layers (Fig. 5).

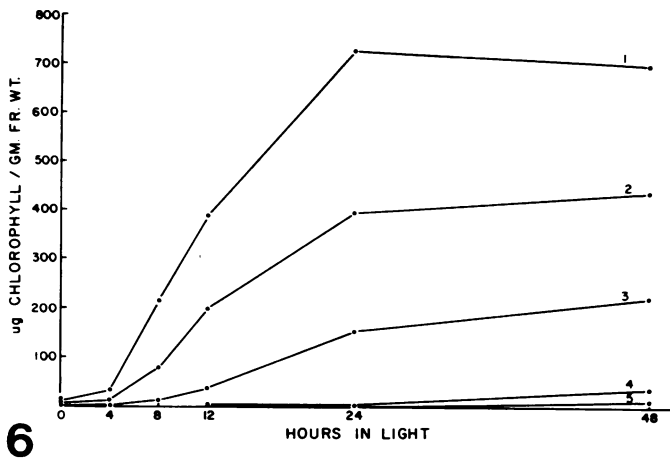


FIG. 5. Plastid from region 1 of the dark-grown barley leaf. Arrow indicates short regions of grana-like pairing of the single thylakoids. \times 68,250.

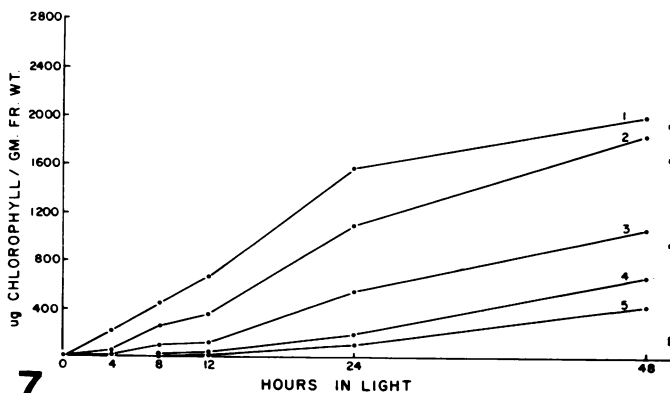
Morphological development of proplastids into mature, highly differentiated etioplasts involves an increase in both size and synthesis of a large amount of membrane, most of which is stored in the prolamellar body. The prolamellar body forms a reserve of membrane material that can be rapidly rearranged into primary thylakoid layers when the plastids are exposed to light.

Chlorophyll Synthesis in Relation to Cell Age. The time course for Chl synthesis in five regions of etiolated barley leaves has been studied in both excised sections and intact plants. Data are given in μg total Chl/g fresh wt in Figures 5 and 7. The Chl reported at zero time represents the amount of protochlorophyllide converted to Chl after 5 min of illumination. Since each region of the leaf represents tissue of a different age, it can be seen from the graphs that age greatly influences the rate of Chl synthesis. Successively older regions synthesize Chl at a faster rate and reach higher concentrations in 48 hr. The amount made by intact plants in 48 hr (Fig. 7) is significantly higher than that made by excised sections (Fig. 6). For example, it is over three times as much for region 1 and over 40 times as much for region 5.

Excised sections do not continue to synthesize Chl as rapidly as intact plants during the 24- to 48-hr period. In fact, region 1 shows a slight decrease in Chl content after 48 hr. Examination of electron micrographs of these plastids reveals that they are becoming filled with starch which may be inhibiting further Chl synthesis by causing a reduction in the amount of plastid membrane present. Chlorophyll levels attained by intact



6



7

FIG. 6. Time course of Chl synthesis in excised sections from the first foliage leaf of etiolated barley.

FIG. 7. Time course of Chl synthesis in five regions of the intact first foliage leaf of etiolated barley. The points in squares give the Chl content of green controls.

Table III. *Appressed Membrane Present in Plastids Illuminated for Various Lengths of Time*

Region	Hours in Light					
	2	3	5	12	24	48
	%	%	%	%	%	%
1	4	29	59	74	59	68
2	2	11	28	58	72	67
3	0	09	06	51	74	71
4	0	0	04	04	67	66
5	0	0	0	0	62	82

plants after 48 hr in the light are slightly higher than those of green controls that have been growing continuously in light (Fig. 7). This shows that etiolated barley plants still retain their full Chl-synthesizing abilities.

It is interesting to compare the times at which Chl is first detected in each of the five regions. Regions 1, 2, and 3, if intact plants, have Chl immediately after illumination because they are the only ones that contain protochlorophyllide in the etiolated plant which is transformed to Chl by a brief light exposure. Regions 4 and 5 have no detectable Chl until they have been in the light 8 hr. Chlorophyll first appears in the excised sections at 12 hr in region 4 but not until 24 hr in region 5.

Ultrastructural Changes in Relation to Cell Age. Dark-grown plastids in each region of the leaf are different, and so a developmental study was undertaken to determine if they would all develop in the same manner and at the same rate when exposed to light. Tissue excised from each region of the leaf was placed in 500 ft-c white light for varying periods of time. Samples of each region were prepared for electron microscopy after 2, 3, 5, 12, 24, and 48 hr in the light.

After comparing developmental stages of each region during 48 hr of illumination, it appears that morphological changes which plastids undergo consist largely of increases in amount of membrane, and perhaps more importantly, the amount of appressed thylakoids. These developmental changes were quantified by determining the percentage of appressed thylakoid membrane relative to total membrane present for each region and stage of development. These figures are given in Table III. Similarities in figures for certain regions and times, as well as similarities in the morphological appearance of the plastids, led to simplification of the developmental changes into five stages. It should be kept in mind, however, that this simplification is only a convenience for discussing a much more complicated process.

The five stages that were established are shown diagrammatically in Figure 8 and are illustrated with representative electron micrographs in Figures 9 through 13. The latter are of plastid profiles taken from tissue of different ages. Stage A (Fig. 9) has a few single thylakoids in the stroma. Stage B (Fig. 10) has small overlaps corresponding to approximately 6% appression (the actual figures ranged from 2 to 11%). Stage C (Fig. 11) has a few small grana with about 28% appression (28–29%); stage D (Fig. 12) has larger and more numerous grana corresponding to 56% appression (51–62%). And stage E (Fig. 13), the fully differentiated chloroplast with large grana stacks, has approximately 71% appression (66–82%).

Figure 14 shows the relationship between the stage of development of each region and its length of time in the light. The important points to note are the different times at which plastids in each region reach full development or stage E. This happens in region 1 after only 12 hr of illumination. Plastids in regions 2, 3, and 4 take 24 hr, and those in region 5 need 48

Developmental stages of plastids in light

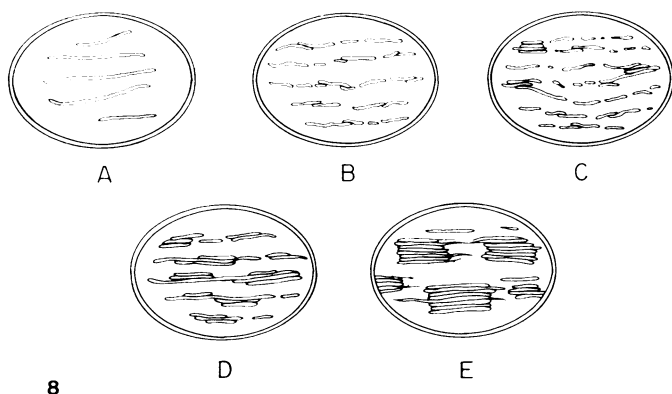


FIG. 8. Diagram of the five stages of plastid development in light.

hr. Plastids in some regions appear to skip certain stages (*i.e.*, region 3 seems to skip stage C). This is very likely due to the rather long time between samples which prevented us from catching the plastids in that particular stage of development.

We can conclude from this graph that the plastids in all the regions of the leaf go through similar stages of development but do not develop at the same rate. Younger regions require longer periods of illumination than older ones to form fully developed chloroplasts.

DEVELOPMENT OF PHOTOSYNTHETIC ACTIVITY IN
RELATION TO CELL AGE

Oxygen Evolution. Photosynthetic activity was measured in excised tissue at six different times during 48 hr of illumination. Figure 15 shows the time course for the development of O_2 -evolving capacity by tissue from each of the five regions of the barley leaf. Note the different times at which each region first exhibits measurable photosynthetic activity. Successively younger regions require longer periods of illumination in order to initiate photosynthesis. Tissue from region 1 evolves O_2 after only 2 hr of light, region 2 after 3 hr, and region 3 after 5 hr of light. Tissue in the youngest regions, 4 and 5, do not begin to evolve O_2 until they have had 24 to 48 hr of illumination, respectively.

Successively older regions have a higher total photosynthetic capacity at all the times measured. The 48-hr values approach those of the green controls (given in boxes) and show that etiolated tissue can acquire a photosynthetic capacity equivalent to that of plants grown in continuous light.

Carbon Dioxide Incorporation. Photosynthetic activity was also measured by the incorporation of $^{14}CO_2$. Table IV gives the μ moles of CO_2 incorporated by tissue from each region that had been illuminated for various periods of time. The times at which photosynthetic CO_2 incorporation was significantly greater than dark fixation for each region were the same as those found when measuring O_2 evolution. Tissue from region 1 showed incorporation after only 2 hr in light, from region 2 after 3 hr, and from region 3 after 5 hr. Region 4 tissue began incorporating CO_2 after 24 hr of light and that from region 5 after 48 hr of illumination. The μ moles of CO_2 incorporated by tissue of the regions was somewhat less than the μ moles of O_2 evolved/g fresh wt. This would be partly offset since the $^{14}CO_2$ incorporation method does not take into account the dark respiratory loss of CO_2 that would occur during the incorpora-

tion. In addition, high levels of CO_2 (5%) were used for the O_2 evolution measurements, and any concentration of CO_2 over 0.1% produces a great inhibition of photorespiration. Cereal crops are known to have photorespiratory rates of 3 to 5 times dark respiration (50). The CO_2 incorporation values would be subject to CO_2 loss due to photorespiration that would not occur in the O_2 measurements because of the high CO_2 levels used.

DISCUSSION

Growth of barley plastids in the dark has been studied by both Smith (41) and Henningsen and Boynton (23). Smith found that the number of plastids per cell in the apical 1 cm of successively older leaves increased during early growth and then reached a plateau. His final numbers were similar to those of region 1: about 65 plastids per cell. Henningsen and Boynton showed that plastid size increased through 7 days of growth in the dark and then declined thereafter. They were actually following the senescence of barley plastids since they started with well developed etioplasts in their 5-day-old tissue and followed their decline through 11 days. Other degenerative processes also became evident during this period.

Fasse-Franzisket (16) found that plastids in etiolated leaves of *Agapanthus umbellatus* multiplied very slowly in the dark but underwent rapid division when exposed to light. Boasson and Laetsch (6) also found light-induced division of tobacco etioplasts. This is in contrast to Glydenholm's (20) findings that there was no increase in chloroplast numbers in etiolated bean leaves during greening. However, Holowinsky *et al.* (28) and Mego and Jagendorf (33) found that dark-grown bean plastids did increase in size when illuminated.

Our results show that plastids in all regions of the leaf are capable of enlarging when exposed to light, whether in the intact plant or excised sections, but plastid replication occurs predominantly in the younger regions of the leaf (regions 4 and 5) where some cell division and elongation are taking place.

The intact plant seems to offer a better environment for plastid growth than does the excised tissue. This is indicated by the larger plastid sizes and generally higher plastid numbers for cells of the intact plant compared to the excised material. This is not surprising in view of the fact that each region of the intact plant has the benefit of materials made by the rest of the plant as it greens, whereas the excised sections have only the resources and developmental potential of their own cells on which to rely.

Chlorophyll levels attained by the intact plants were very similar to those found by Obendorf and Huffaker (35) for 7-day-old barley. They too found that the age of the dark-grown barley seedlings had a significant effect on the amount of Chl that various leaf sections accumulated after 24 hr of illumination. Sections from 5-day-old plants synthesized over three times as much Chl as those from 10-day-old plants.

Chlorophyll synthesis is much slower in those regions of the leaf in which active cell and plastid division and enlargement are taking place (regions 4 and 5). In fact, it does not even begin until the intact plants have received 8 hr of light. This indicates that as long as all the resources of the cells and plastids are diverted to replication and growth, very little will be available for synthesizing Chl. There is very rapid Chl synthesis in regions 1 and 2 where no cell division and almost no plastid division is taking place. Region 3 tissue lies somewhere between these extremes in having a moderate amount of Chl synthesis, no cell division, but some chloroplast replication.

Much of the early work on bean and barley plastid ultra-

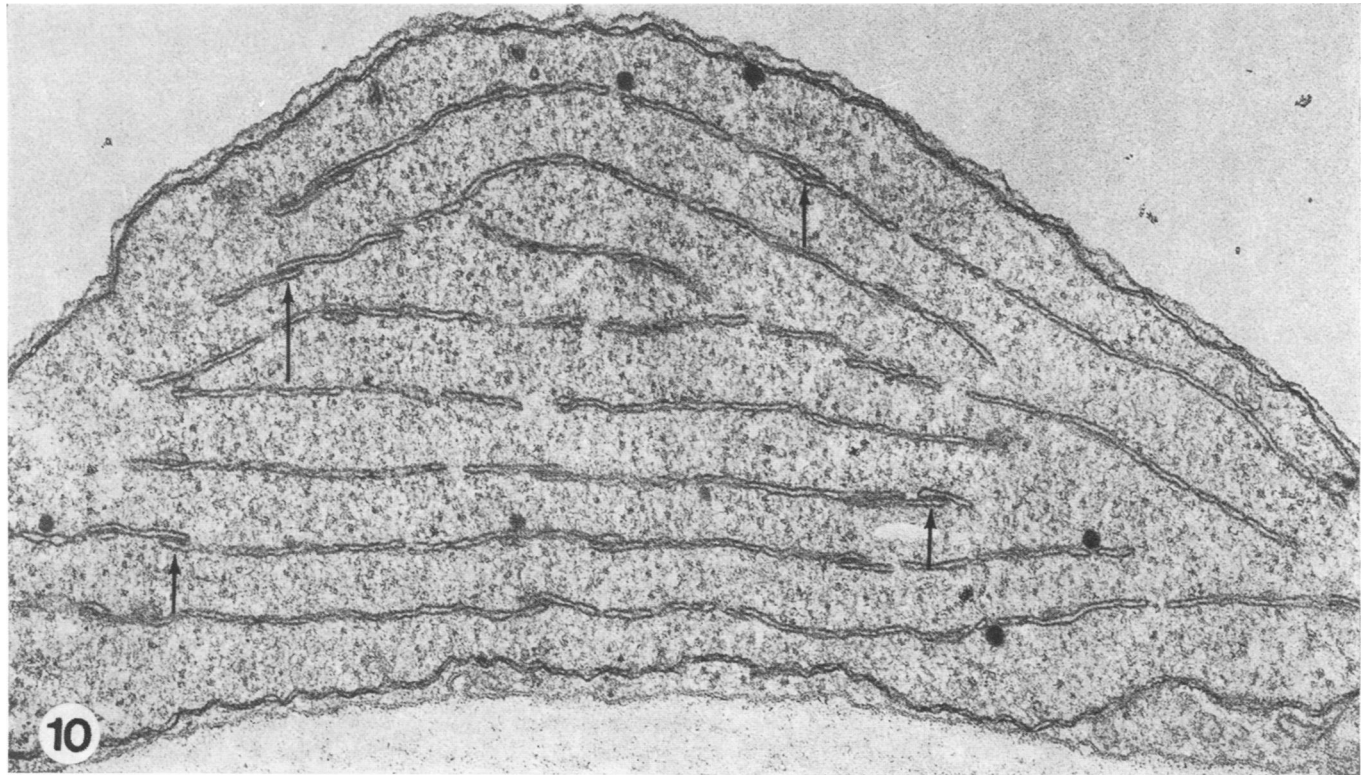
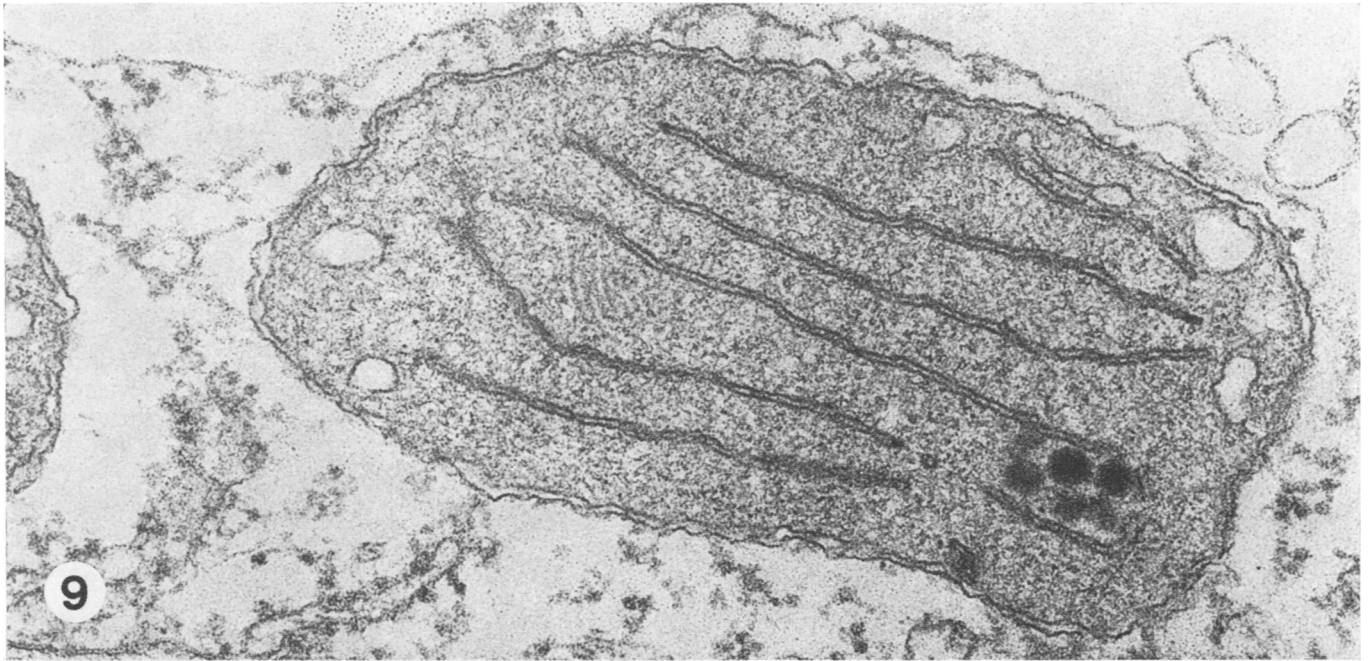


FIG. 9. A plastid in stage A of development with no appressed thylakoids. $\times 94,300$.

FIG. 10. A plastid in stage B of development with very short regions of thylakoid appression indicated by arrows. $\times 60,000$.

structure (15, 45, 47, 48) involved the use of KMnO_4 as the fixative for electron microscopy, and it produced artifacts that prevented sound interpretations of developmental events. In addition, most studies have used tissue containing only mature, highly differentiated etioplasts (11, 14, 19, 23, 24, 30). Rhodes and Yemm (39) looked at a few young stages of dark-grown barley plastids, but they did not follow their development in light.

Weier and Brown (46) presented a thorough description of the formation of prolamellar bodies in etiolated bean plastids from a system of porous lamellae which appear to arise from the inner membrane of the plastid envelope. They also pointed out the presence of numerous small overlaps in the peripheral lamellae which resemble small, two-compartmented grana. Henningsen and Boynton (23) also mentioned these in barley, and they were described in the present study. They seem to be

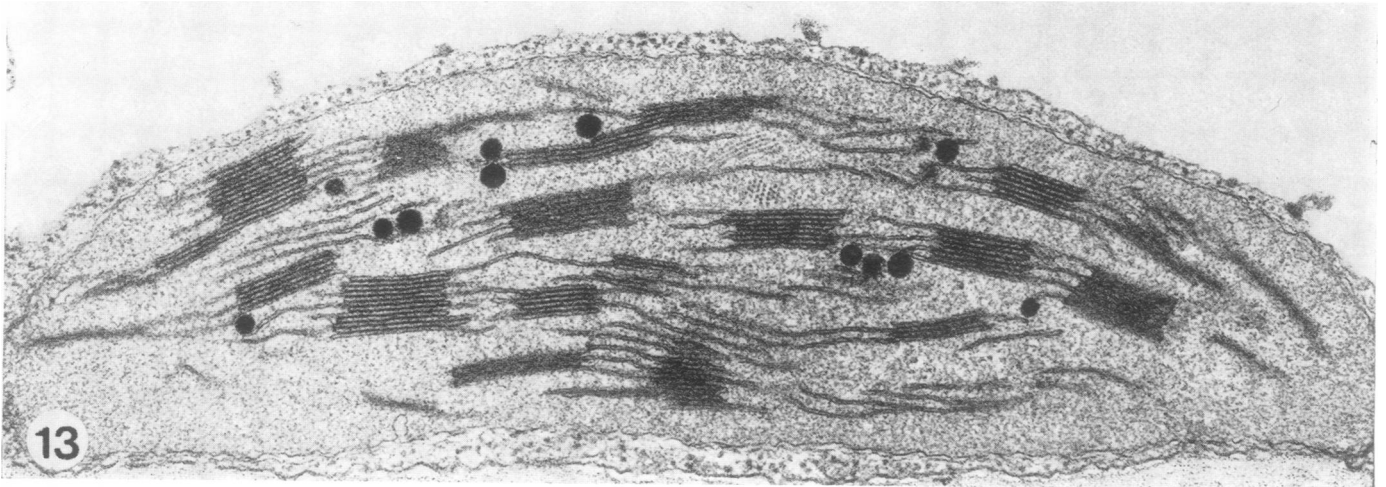
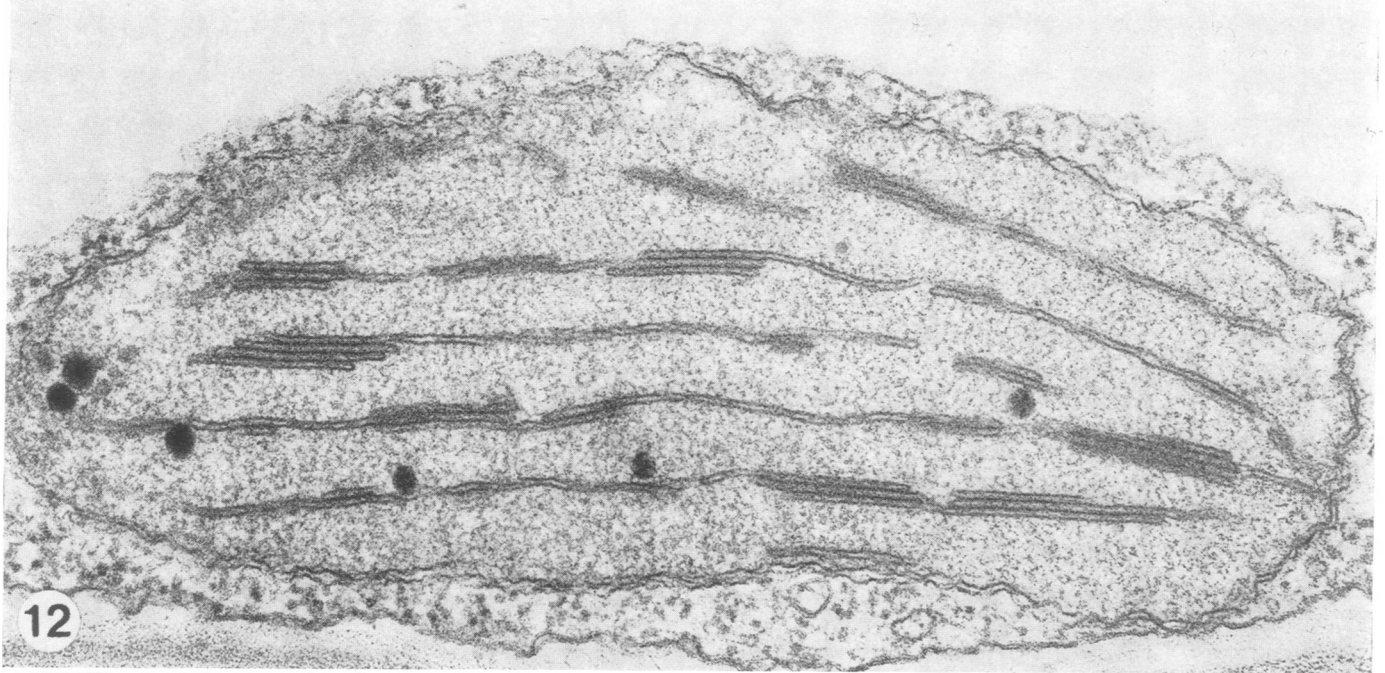
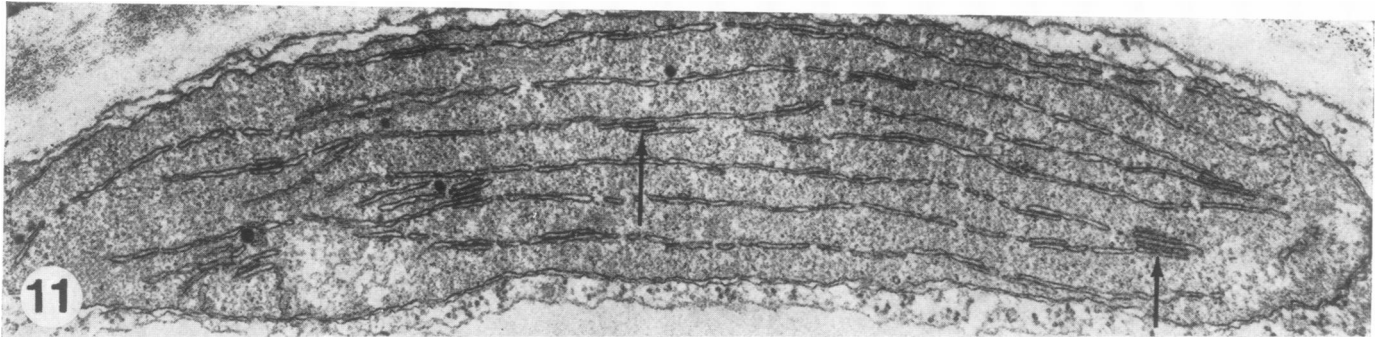


FIG. 11. A plastid in stage C of development with a few small grana indicated by arrows. $\times 35,000$.

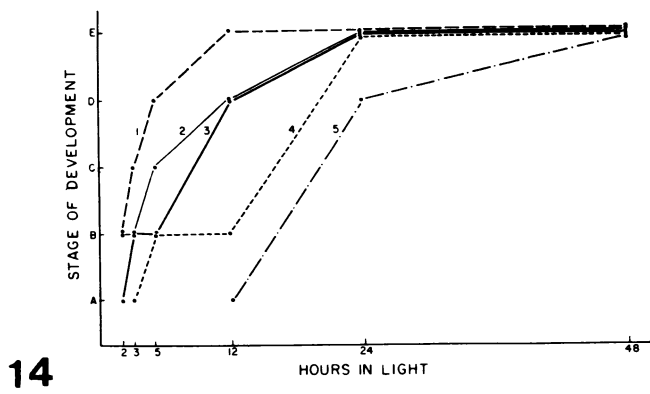
FIG. 12. A plastid in stage D of development with larger and more numerous grana. $\times 75,750$.

FIG. 13. A plastid in stage E of development with large grana stacks. $\times 43,500$.

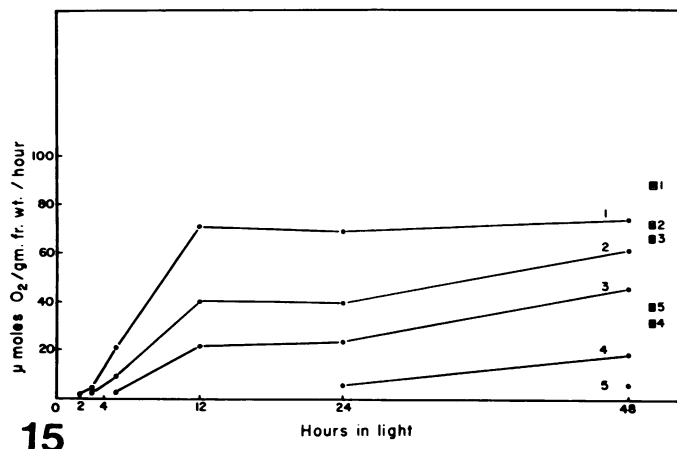
characteristic of older, more fully developed etioplasts containing a large amount of membrane, since they were not seen in plastids in younger regions of the leaf.

There have been a number of studies on the development of photosynthetic activity in greening tissue, each using different species and methods (3, 4, 9, 12, 22, 27, 34, 36, 39, 42, 44).

The earliest appearance of photosynthetic activity in barley was measured by Smith (42) and Henningsen and Boardman (22) after only 30 min of illumination. However, Nadler *et al.* (34) found no O_2 evolution in barley until after the lag phase of Chl synthesis, which was about 5 hr. Their tissue was greened under very low intensity light (7 ft-c) which would



14



15

FIG. 14. Time course of plastid development during 48 hr illumination of the five regions of an etiolated barley leaf.

FIG. 15. Time course for the development of O₂-evolving ability by tissue excised from five regions of the dark-grown barley leaf and illuminated for various lengths of time. Values in boxes are for green controls.

Table IV. Incorporation of CO₂ by Barley Leaf Sections

Region	Hours in Light						
	0	2	3	5	12	24	48
	μ moles CO ₂ /g fresh wt·hr						
1	0.10	0.30	1.07	3.91	8.87	14.34	11.16
2	0.09	0.10	0.56	3.33	6.95	23.16	23.41
3	0.13	0.12	0.32	2.32	3.62	17.45	18.22
4	0.17	0.13	0.14	0.08	0.17	1.37	2.87
5	0.14	0.11	0.14	0.06	0.08	0.09	0.38

not stimulate very rapid greening of the tissue and might account for the delay in development of photosynthetic activity. More recently Egneus *et al.* (13) detected O₂ evolution in intact barley leaf tissue after about 1.5 hr of greening, a value very close to that reported here for the oldest region.

There have been several studies of the development of photosynthetic activity using isolated plastids rather than intact tissue. Photoreduction of NADP first appeared after 2 hr of continuous illumination in barley (37) but not before 15 to 20 hr of illumination in bean (1, 21). Egneus *et al.* (13) found that a cooperation between photosystems I and II was first detected after 3 hr of greening. One should keep in mind that times at which photosynthesis becomes detectable with isolated plastids

may be later than they would be with intact tissue because of damage to plastids during isolation (7, 22).

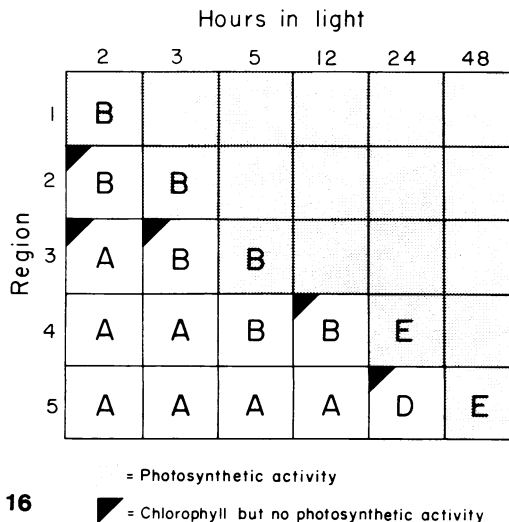
Many differences reported by workers using intact tissue are probably due to species differences or to use of different experimental methods. Conditions under which plants are grown and their subsequent handling could also be contributing factors. Briggs (8) found some time ago that differences in age of tissue affect the rapidity with which it develops the ability to liberate O₂ and incorporate CO₂. Etiolated bean leaves from 13-day-old seedlings developed much more O₂-evolving power during a given exposure to light than did leaves from 8-day-old seedlings. Our study has shown that this is also true for barley tissue, and it emphasizes the usefulness of barley in providing well characterized tissue of different ages from a single leaf.

These results on photosynthetic activity, combined with the data on plastid ultrastructure and Chl synthesis, enable us to draw some conclusions regarding the correlation of these distinct yet highly interrelated processes.

SUMMARY

Relationships between plastid ultrastructure, photosynthetic activity, and Chl content have been compared in Figure 16 as a function of leaf cell age and time of illumination. The letters in boxes represent stages of plastid development at those particular times (see Fig. 8). Shaded areas represent photosynthetic activity measured as O₂ evolution and ¹⁴CO₂ incorporation. The darkened corners represent the presence of Chl when no photosynthetic activity was measurable.

There are several interesting features to be noted in this chart. All of the regions are not in the same stage of development when photosynthesis is first detectable. Regions 1, 2, and 3 have only a few small overlaps corresponding to stage B while regions 4 and 5 have well developed chloroplasts with large grana corresponding to stage E. This indicates that the development of photosynthetic activity is not strictly correlated with a certain stage of plastid development; if it were, we would expect all regions to exhibit photosynthetic activity at the same stage of plastid development. Some other factor must be limiting photosynthesis. In addition, some regions may have both overlaps or even grana (*i.e.*, region 5, 24 hr) and Chl but no measurable photosynthetic activity.



16

FIG. 16. Chart relating plastid development, Chl content, and photosynthetic activity in five regions of the barley leaf during 48 hr illumination.

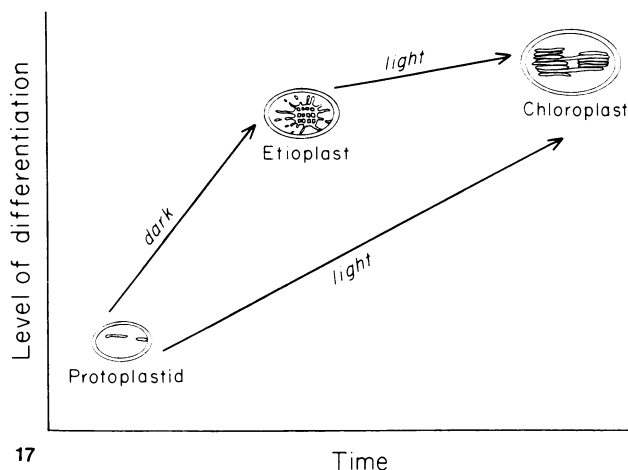


FIG. 17. Diagram of plastid development in barley.

It can be concluded that membrane appression in barley chloroplasts, either as a few small overlaps or large grana, always accompanies photosynthetic activity, but thylakoid appression and Chl can be present when photosynthetic activity is not measurable. The occurrence of small regions of appression even in some dark-grown plastids makes it impossible to say that a strict correlation exists between appressed membrane and photosynthetic activity. It seems likely from these data that photosynthesis does require regions of membrane appression, as several authors have suggested (29, 49), but these appressions can exist in the absence of photosynthesis and are not necessarily indicative of it.

Likewise, there does not appear to be any strict correlation between the presence of Chl and membrane appression. It has already been shown that overlaps can occur in dark-grown plastids that contain only protochlorophyllide, and Chl can exist without membrane appression, as is found in region 3. In the dark, plastids of region 3 have no overlaps, but they do contain a small amount of protochlorophyllide which is transformed to Chl upon illumination. After 2 hr in the light, more Chl has been synthesized, but overlaps still do not appear.

Chlorophyll usually appears before photosynthetic activity in barley, and it is, of course, always present when photosynthesis is occurring. Other workers have studied the relationship between Chl content and photosynthesis to determine if the amount of Chl present was the limiting factor in the development of photosynthetic activity. Smith *et al.* (43) measured Hill reaction activity with barley plastids isolated from greening tissue. They concluded that Hill activity was closely related to and developed concurrently with Chl content when etiolated leaves were greened. Two years later, however, Smith (42) concluded from further work on barley that Chl content and O_2 -evolving power are not closely correlated. He found that dark-grown leaves that had been illuminated and returned to the dark built up an agent by metabolic processes which enabled them to liberate O_2 when illuminated again. He felt that the agent could not be entirely identified with Chl because it increased about 2-fold during the second illumination, but the O_2 -liberating capacity increased about 200-fold. He went on to say that Chl could be present in living organisms without affording the ability for photosynthesis. This was found to be the case in the present study.

Dowdell and Dodge (12) also found no direct relationship between chlorophyll content and photosynthetic activity in pea leaves, while Herron and Mauzerall (25) reported that photosynthetic O_2 evolution considerably precedes the rise in Chl

content during greening of a mutant *Chlorella*. By manipulating the light conditions during greening, they produced cells with $1/10$ as much Chl as fully greened cells but with full photosynthetic activity. And Nadler *et al.* (34), by using various protein inhibitors, concluded that the development of photosystem II activity was limited by the synthesis of proteins in both the cytoplasm and the plastids, and not by Chl synthesis. These findings emphasize the fact that many other factors in addition to pigment concentration and plastid ultrastructure influence the development of photosynthetic activity.

Based on the data gathered from the comparative study of plastid development in each region of the barley leaf, two distinct pathways of development from a proplastid into a mature functional chloroplast can be described. These are illustrated in Figure 17. If a small undifferentiated proplastid is allowed to develop for a time in the dark, it will increase in size and synthesize a large amount of membrane which forms the prolamellar body. If allowed to develop long enough in the dark, single thylakoids may be produced from the periphery of the prolamellar body and a few small overlaps may develop on them. This developmental sequence can be seen when plastids from region 5 through region 1 of the dark-grown barley leaf are compared. The resulting fully developed etioplast then requires a relatively brief period of illumination to be transformed into a mature, functional chloroplast. The transformation of the plastids in region 1 illustrates this process very well. If the undifferentiated proplastid is exposed to light, it requires a much longer period of illumination to develop into a mature chloroplast. This is, presumably, because it has not already synthesized the membranes, pigments, and enzymes that are present in the mature etioplast. The plastids in region 5 undergo this type of development. This is the "normal" developmental pathway producing chloroplasts in light-grown plants. Similar developmental pathways have been proposed by von Wettstein (47) for barley and by Klein and Schiff (31) for bean. It is being emphasized again in this paper, because of the all too common impression that there is a causal developmental relationship between etioplasts and chloroplasts.

All of these data point out that physiological age of the cell and its plastids determines the rate of chloroplast development in the light. In addition, the particular structural and functional correlations that can be made depend to a large degree on the age of the tissue. Unless this fact is taken into account in studies attempting to correlate structure with function, it will be impossible to make generalizations regarding the specific structures associated with photosynthesis.

LITERATURE CITED

- ANDERSON, J. M. AND N. K. BOARDMAN. 1964. Studies on the greening of dark-grown bean plants. II. Development of photochemical activity. *Aust. J. Biol. Sci.* 17: 93-101.
- ARNON, D. I. 1949. Copper enzymes in isolated chloroplasts: polyphenol-oxidase in *Beta vulgaris*. *Plant Physiol.* 24: 1-15.
- BIGGINS, J. AND R. B. PARK. 1966. Carbon dioxide assimilation by etiolated *Hordeum vulgare* seedlings during the onset of photosynthesis. *Plant Physiol.* 41: 115-118.
- BLAACW-JANSEN, G., J. KOMEN, AND J. B. THOMAS. 1950. On the relation between the formation of assimilatory pigments and the rate of photosynthesis in etiolated oat seedlings. *Biochim. Biophys. Acta* 5: 179-185.
- BOARDMAN, N. K. AND H. R. HIGHKIN. 1966. Studies on a barley mutant lacking chlorophyll *b*. I. Photochemical activity of isolated chloroplasts. *Biochim. Biophys. Acta* 126: 189-199.
- BOASSON, R. AND W. M. LAETSCH. 1969. Chloroplast replication and growth in tobacco. *Science* 166: 749-751.
- BRADBEER, J., A. O. GYLDENHOLM, M. E. WALLIS, AND F. R. WHATLEY. 1969. Studies on the biochemistry of chloroplast development. In: H. Metzner, ed., *Progress in Photosynthesis Research*, Vol. 1. International Union of Biological Sciences, Tübingen, pp. 270-279.
- BRIGGS, G. E. 1920. Experimental researches on vegetable assimilation and

- respiration. XIII. The development of photosynthetic activity during germination. Proc. Roy. Soc. B 91: 249-268.
9. BUTLER, W. 1965. Development of photosynthetic systems 1 and 2 in a greening leaf. Biochim. Biophys. Acta 102: 1-8.
 10. DALE, J. E. AND J. K. HEYES. 1970. A virescens mutant of *Phaseolus vulgaris*: growth, pigment and plastid characteristics. New Phytol. 69: 733-742.
 11. DEGREEF, J., W. L. BUTLER, AND T. F. ROTH. 1971. Greening of etiolated bean leaves in far red light. Plant Physiol. 47: 457-464.
 12. DOWDELL, R. J. AND A. D. DODGE. 1970. The photosynthetic capacity of pea leaves with a controlled chlorophyll formation. Planta 94: 282-290.
 13. EGNÉUS, H., S. RAFTEL, AND G. SELLDÉN. 1972. The appearance and development of photosynthetic activity in etiolated barley leaves and isolated etio-chloroplasts. Physiol. Plant. 27: 48-55.
 14. ENGELBRECHT, A. H. P. AND T. E. WEIER. 1967. Chloroplast development in germinating safflower (*Carthamus tinctorium*) cotyledon. Amer. J. Bot. 54: 844-856.
 15. ERIKSSON, G., A. KAHN, B. WALLE, AND D. VON WETTSTEIN. 1961. Zur makromolekularen Physiologie der Chloroplasten. III. Ber. Deut. Bot. Ges. 74: 221-232.
 16. FRASSE-GRANZISKET, U. 1956. Die Teilung der Proplastiden und Chloroplasten bei *Agapanthus umbellatus* L'Herit. Protoplasma 45: 194-227.
 17. GOODCHILD, D. J., H. R. HIGHKIN, AND N. K. BOARDMAN. 1966. The fine structure of chloroplasts in a barley mutant lacking chlorophyll *b*. Exp. Cell Res. 43: 684-688.
 18. GOODENOUGH, J., J. ARMSTRONG, AND R. LEVINE. 1969. Photosynthetic properties of ac-31, a mutant strain of *Chlamydomonas reinhardi* devoid of chloroplast membrane stacking. Plant Physiol. 44: 1001-1012.
 19. GUNNING, B. E. S. AND M. P. JAGOE. 1965. The prolamellar body. In: T. W. Goodwin, ed., Symposium on Biochemistry of Chloroplasts, Vol. II. Academic Press, London, pp. 655-676.
 20. GYLDEHOLM, A. O. 1968. Macromolecular physiology of plastids. V. On the nucleic acid metabolism during chloroplast development. Hereditas 59: 142-168.
 21. GYLDEHOLM, A. O. AND F. R. WHATLEY. 1968. The onset of photophosphorylation in chloroplasts isolated from developing bean leaves. New Phytol. 67: 461-468.
 22. HENNINGSEN, K. W. AND N. K. BOARDMAN. 1973. Development of photochemical activity and the appearance of the high potential form of cytochrome *b*-599 in greening barley seedlings. Plant Physiol. 51: 1117-1126.
 23. HENNINGSEN, K. W. AND J. E. BOYNTON. 1969. Macromolecular physiology of plastids. VII. The effect of a brief illumination on plastids of dark-grown barley leaves. J. Cell Sci. 5: 757-793.
 24. HENNINGSEN, K. W. AND J. E. BOYNTON. 1970. Macromolecular physiology of plastids. VIII. Pigment and membrane formation in plastids of barley greening under low light intensity. J. Cell Biol. 44: 290-304.
 25. HERRON, H. A. AND D. MAZERALL. 1972. The development of photosynthesis in a greening mutant of *Chlorella* and an analysis of the light saturation curve. Plant Physiol. 50: 141-148.
 26. HIGHKIN, H. R. AND A. W. FRENKEL. 1962. Studies of growth and metabolism of a barley mutant lacking chlorophyll *b*. Plant Physiol. 37: 814-820.
 27. HILLER, R. G. AND N. K. BOARDMAN. 1971. Light driven redox changes of cytochrome *f* and the development of photosystems I and II during greening of bean leaves. Biochim. Biophys. Acta 253: 449-458.
 28. HOLOWINSKY, A. W., P. B. MOORE, AND J. B. TORREY. 1965. Regulatory aspects of chloroplast growth in leaves of *Xanthium pennsylvanicum* and etiolated red kidney bean seedling leaves. Protoplasma 60: 94-110.
 29. HOMANN, P. AND G. SCHMID. 1967. Photosynthetic reactions of chloroplasts with unusual structures. Plant Physiol. 42: 1619-1632.
 30. KIRK, J. T. O. AND R. A. E. TILNEY-BASSETT. 1967. The Plastids. W. H. Freeman and Co., San Francisco.
 31. KLEIN, S. AND J. SCHIFF. 1972. The correlated appearance of prolamellar bodies, protochlorophyllide species, and the Shibata shift during development of bean etioplasts in the dark. Plant Physiol. 49: 619-626.
 32. LUFT, J. 1961. Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. 9: 409-414.
 33. MEGO, J. L. AND A. T. JAGENDORF. 1961. Effect of light on growth of Black Valentine bean plastids. Biochim. Biophys. Acta 53: 235-254.
 34. NADLER, K., H. HERRON, AND S. GRANICK. 1972. Development of chlorophyll and Hill activity. Plant Physiol. 49: 388-392.
 35. OBENDORF, R. L. AND R. C. HUFFAKER. 1970. Influence of age and illumination on distribution of several Calvin cycle enzymes in greening barley leaves. Plant Physiol. 45: 579-582.
 36. OELZE-KAROW, H. AND W. L. BUTLER. 1971. The development of photophosphorylation and photosynthesis in greening bean leaves. Plant Physiol. 48: 621-625.
 37. PHUNG NHU HUNG, S., A. HOARAU, AND A. MOYSE. 1970. Etude de l'Evolution en Chloroplastes des Plastes étiolés d'Orge. II. Photophosphorylation et Photoreduction du NADP, Formation de Ferredoxine, an Eclaircissement continue et par l'Action d'Eclairs. Z. Pflanzenphysiol. 62: 245-258.
 38. REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17: 208-212.
 39. RHODES, M. J. C. AND E. W. YEMM. 1966. The development of chloroplasts and photosynthetic activities in young barley leaves. New Phytol. 65: 331-342.
 40. SANE, P. V., D. J. GOODCHILD, AND R. B. PARK. 1970. Characterization of chloroplast photosystems 1 and 2 separated by a nondetergent method. Biochim. Biophys. Acta 216: 162-178.
 41. SMITH, H. 1970. Changes in plastid ribosomal-RNA and enzymes during the growth of barley leaves in darkness. Phytochemistry 9: 965-975.
 42. SMITH, J. H. C. 1954. The development of chlorophyll and oxygen-evolving power in etiolated barley leaves when illuminated. Plant Physiol. 29: 143-148.
 43. SMITH, J. H. C., C. S. FRENCH, AND V. M. KOSKI. 1952. The Hill reaction: development of chloroplast activity during greening of etiolated barley leaves. Plant Physiol. 27: 212-213.
 44. TOLBERT, N. E. AND F. B. GALEY. 1955. Carbon dioxide fixation by etiolated plants after exposure to white light. Plant Physiol. 30: 491-499.
 45. VIRGIN, H. I., A. KAHN, AND D. VON WETTSTEIN. 1963. The physiology of chlorophyll formation in relation to structural changes in chloroplasts. Photochem. Photobiol. 2: 83-91.
 46. WEIER, T. E. AND D. L. BROWN. 1970. Formation of the prolamellar body in 8-day, dark-grown seedlings. Amer. J. Bot. 57: 267-275.
 47. WETTSTEIN, D. VON. 1959. The formation of plastid structures. Photochemical apparatus, its structure and function. Brookhaven Symp. Biol. 11: 138-159.
 48. WETTSTEIN, D. VON AND A. KAHN. 1960. Macromolecular physiology of plastids. Eur. Regional Conf. Electron-microscopy (Delft) 2: 1051-1054.
 49. WEICKOWSKI, S. 1969. Studies on the activity and ultrastructure of the photosynthetic apparatus in the earliest stages of primary bean leaves development. Acta Soc. Bot. Pol. 38: 103-114.
 50. ZELITCH, I. 1971. Photosynthesis, Photorespiration and Plant Productivity. Academic Press, New York.