Changes in the Etiolated Tobacco Leaf During Greening. I. Structure of the Chloroplast[1]

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Introduction

Avery (1) in 1933 provided the first definitive histological and cellular study of the growth of tobacco leaves. He carefully documented the pattern of enlargement from the time of emergence of the leaf from the bud until it was fully expanded. Very little cell division occurs after the leaves are one-fifth to one-sixth their final length. Cellular enlargement continues, of course, and results from water intake following a multitude of changes within the cells. Initiation of some of these changes follows light absorption and energy transformation. Protochlorophyll is rapidly transformed by light (4) and certain types of protein synthesis is accelerated (10). Phytochrome and chlorophyll as primary energy receptors (8, 10) are, in part, responsible for channeling the energy that is used in the formation of cellular components which further expansion and cellular differentiation processes.

Developing cell components and organelles in *Nicotiana tabacum* L. cv. 18a will be treated in this series of reports. Structural changes during chloroplast development have been described for species such as *Euglena* (3, 17), *Agapanthus* (6, 19), *Aspidistra* (8, 13), barley (22) and *Chlorophytum* (19) but not tobacco.

Three excellent general summaries of present knowledge of chloroplast structure and morphogenesis have recently appeared (5, 7, 14). Changes during plastid development owing to age, leaf excision and cultural conditions have been taken into account by us and are described in this and subsequent reports.

Materials

Etiolated tobacco leaf tissue was obtained from a single variety of aromatic tobacco, *Nicotiana tabacum* L. cv 18a, originally supplied by Professor F. A. Wolf. Seed were sown in ten-inch clay pots on sterile, sandy loam and covered with a thin layer of soil and "Vermiculite." Germination and early growth were under a glass cover. After about five weeks, plants were transplanted into six-inch clay pots and grown in sandy loam under greenhouse conditions. During the subsequent four weeks of growth, plants were fertilized weekly with one tablespoon of each of the following salts $Ca(NO_3)_2$, $MgSO_4$, and KH_2PO_4 dissolved in one gallon of tap water. Growth appeared normal in every way. When the number of nodes per plant ranged from 19 to 25, the top seven nodes were removed (unless flower buds had developed, in which case 11 nodes were discarded) and the next lower axillary bud was allowed to develop in a light-tight container. These containers were 9.0 x 16.3 cm cylindrical cardboard cartons previously painted with flat-black paint inside and aluminum paint outside and dipped in hot paraffin for waterproofing. Each chamber was provided with ventilation by means of a small section of black rubber tubing, curled to prevent light from entering. The opening in the bottom of the box was sealed around the stem with modeling clay plasticised with castor oil. Support was provided by two small stakes. Bud development continued in the dark box at the expense of photosynthate from the exposed leaves.

After 14 to 18 days of growth, leaves produced at the apex in the light tight chamber ranged in width from 1 to 3 cm and in length from 2 to 7 cm on different plants. On most shoots, six or eight leaves developed in the dark. If darkened shoots were allowed to grow for as long as three weeks, some of the older leaves usually showed necrosis which began on the tips and edges. In a few crops, however, initiation of growth in the dark was retarded owing to unknown causes and shoots with normal numbers of healthy leaves were not produced until 20-24 days had elapsed.

Dark-grown shoots were routinely harvested in the dark between 10 and 12 pm and placed immediately in White's inorganic nutrient (23) supplemented with glucose, glycine

and succinic acid. (Bases for this treatment were established and are presented in a subsequent report in this series.) Individual leaves were excised after eight hours, placed on the rim of Petri dishes with petioles in fresh solution, and exposed to light. Unless otherwise indicated, the light intensity was 100 fc.

Methods

Etiolated leaves having been 18 days in development were excised, separated according to age or position on the stem, and exposed to light intensities of 60, 100, and 150 fc. After 2, 4, 12, 24, and 36 hours of exposure, sections of approximately 3 x 5 mm size, or less, were taken from the distal half of the leaf and placed in Altmann's fixative (6) until the edges were blackened (an indication of complete fixation). Tissues were washed overnight in water, dehydrated, infiltrated, embedded in Fisher Tissuemat, and sectioned at 10 u. Sections were carried through a xylol, xylol-butyl alcohol, and an ethyl alcohol series to water, then placed in 20-30% hydrogen peroxide until bleached. Peroxide was removed with 30% ethyl alcohol (2-3 hours) and two changes of water. Staining (15) was with acid fuchsin in aniline water (20 g in 100 ml) for 30 minutes at 60° C and in picric acid in alcohol. This staining technique followed the recommendation of Grave (6), who investigated the use of some 17 different fixatives and five staining techniques for young plastids. Altmann's fixative and 2% osmic acid were considered the only ones giving good fixation, but the former caused least blackening of the tissues. The most acceptable stain for the proplastids was found to be acid fuchsin following fixation with Altmann's. As plastids developed, however, the stroma as well as the granules took up some of the stain and definition of granules was reduced.

The measurement of plastid sizes and size changes was accomplished by using a microscope slide projector. With a stage micrometer the distance between the object and projected image was set so that one micron on the stage represented one millimeter on the screen. Since tobacco chloroplasts are somewhat elongated and plano-convex in shape, measurements of both length and thickness were taken. Dimensions of palisade and mesophyll cell plastids were taken separately from the first and fifth leaves (termed A and E respectively) developed on a shoot. Measurements were mainly of plastids that were flat against the cell

wall. This procedure was necessary in getting thickness measurements, and, too, length measurements were most easily obtained this way. However, some difficulty was encountered in making measurements on small-sized very thin particles.

When studies were made on living plastids, identification was achieved with Rhodamine B, a vital stain used by many workers (see Strugger 20) in chloroplast studies. According to Morthland et al (12), Rhodamine B becomes bound to nucleic acid molecules, either by attachment to free phosphoric acid groups or displacement of proteins. Without the use of this stain, however, proplastids could be distinguished from starch grains by their lack of refractive power and/or their somewhat granular appearance. An iodine-potassium iodide solution made up in 0.5 M sucrose was also used for identification of starch but this stain is not particularly sensitive for amylopectin.

Results

Size Changes in Plastids. Changes in plastids of different age, in different tissues, and under different intensities of light are recorded in **Table 1**.

From the data, it appears that plastids in older leaves increased in size sooner than those from younger

leaves. After only two hours of light their average thickness was measurably greater. These plastids also showed a length, or diameter, growth increment before one was seen in plastids of young leaves. After twenty-four hours of exposure to light, it was found the mode size was six microns in diameter, even though the average diameter under these conditions was no greater than that of young-leaf plastids. (These figures were rounded off to the nearest whole number.) No correlation of the growth differences were found between old and young leaves so far as protein, carotene, or chlorophyll formation were concerned. Data for this will be provided in a later report.

In order to more clearly determine the response of plastids to differences in light intensity, plastids from old and young leaves were measured. Effects attributable to age appeared at only 2- and 24-hour periods. Each figure in **Table 2** represents an average of measurements made on 48 plastids. Apparently, the higher the light intensity used the faster the plastids enlarged. Expansion in size was especially marked shortly after exposure to light.

One hundred fc caused the quickest response and resulted in plastids which were relatively "long" and thin; these plastids then increased

Table 1—Effect of leaf age and light intensity on plastid size and development[a]

Exposure Time (hr.)	2				4				12				24				36			
Tissue[b]	P		M		P		M		P		M		P		M		P		M	
Dimension[c]	L	T	L	T	L	T	L	T	L	T	L	T	L	T	L	T	L	T	L	T
Old Leaf																				
Light (fc)																				
60	3	2	4	2	4	2	4	2	5	2	6	2	5	2	6	2	5	2	5	2
100	4	1	4	1	4	2	5	2	4	2	5	2	6	2	5	2	6	2	6	2
150	4	2	4	2	5	2	5	2	5	2	5	2	5	2	6	2	5	2	6	2
Avg.	4	2	4	2	4	2	5	2	5	2	5	2	5	2	6	2	5	2	6	2
Young Leaf																				
Light (fc)																				
60	3	2	3	2	4	2	5	2	5	1	6	1	5	2	5	2	5	2	6	2
100	5	1	5	1	5	2	5	1	5	1	5	2	6	2	6	2	6	2	6	2
150	4	1	4	1	4	2	4	2	5	2	5	2	5	2	5	2	5	3	6	3
Avg.	4	1	4	1	4	2	5	2	5	2	5	2	5	2	6	2	5	2	6	2

[a] Size measurements are given in microns and expressed as the nearest whole number.
[b] P = palisade tissue; M = mesophyll.
[c] L = length; T = thickness.

Table 2—Effect of light duration and intensity on plastid size expressed in microns

Exposure Time in hours	2		4		12		24		36	
Dimensions of Measurement[a]	L	T	L	T	L	T	L	T	L	T
60 fc	3	2	4	2	6	2	5	2	5	2
100 fc	5	1	5	2	5	2	6	2	6	2
150 fc	4	2	5	2	5	2	5	2	6	3

[a] L = length; T = thickness.

in thickness to equal results with other treatments and after 24 hours they showed an additional increment in length.

Plastids grew in length slowest at 60 fc and even after 36 hours were not as long as those allowed to develop at higher intensities. Initially, however, they showed a thickness equal to or greater than those of other treatments.

Response by growth in length to an intensity of 150 fc was intermediate between the other two treatments. After 36 hours, however, these plastids were found to be thicker than others, though previously they were comparable.

It appears that for a 24-hour exposure period, 100 fc is the optimum light intensity for plastids development. But it should be noted that at 150 fc maximum thickness for any treatment was observed after 36 hours of exposure to light. Further data would be desirable for confirmation.

Figure 1A shows the structure of the plastids as it appeared after four hours' exposure of the leaf to light. The granules which were heavily stained with acid fuchsin have been termed *Granascheibchen* by Strugger (20). Mitochondrial-like particles stain similarly. Another component appeared to be either starch grains or vacuoles. Still another component was a distinct group of granules of very small size that stained deeply with acid fuchsin. They were detectable in very few plastids and in these there were only a few of the small, stained particles. Again, there seem to be two possibilities as to identity. One is that this represents the primary granum described for several species by Mühlethaler (13) and also by Leyon (9). The other possibility is that these are small fat particles since they faintly resemble a component identified in a like manner by Mühlethaler. Since they disappeared during the next two hours of development and did not reappear,

it is not likely that they were either fat bodies or the primary granum of Mühlethaler (13). Their identity was not further investigated.

A delimiting membrane surrounding the plastid appears to be a major difference between the four-hour stage of development (**Figure 1A**) and earlier stages. After only two hours of exposure to light, plastids could barely be distinguished from surrounding cytoplasm owing to the indefinite character of the membrane and apparently complete homogeneity of the granular cytoplasm. The several states of chloroplast membranes have been discussed by Park (14).

Between the fourth and twelfth hour of exposure there was an increase in apparent thickness of the limiting membrane, a decrease in the number of granules (seemingly through coalescence), and an increase in the size of starch grains as well as a slight increase in size of the plastids themselves. During the next 12 hours, the number of granules was reduced to one or two in most cases and these were greatly enlarged. These changes correspond with those described by Strugger (20) for development of the primary granum, but seems at variance with those of Mühlethaler (13). Apparent increases in plastid membrane thickness were due to a centrifugal arrangement of the granules. After 36 hours, these changes had proceeded still further and plastids were clearly in the *Amyloplastische Proplastiden* stage (13, 20). Grave (6), using light microscopy and *Agapanthus umbellatus* discerned these changes in granular structure and illustrated them very clearly. It may be interjected here that Sorokin (16) has observed organelles of similar appearance in epidermal cells from the pod of *Phaseolus vulgaris*. In her preparations the organelles were stained with Janus Green B and were, therefore, identified as mitochondria.
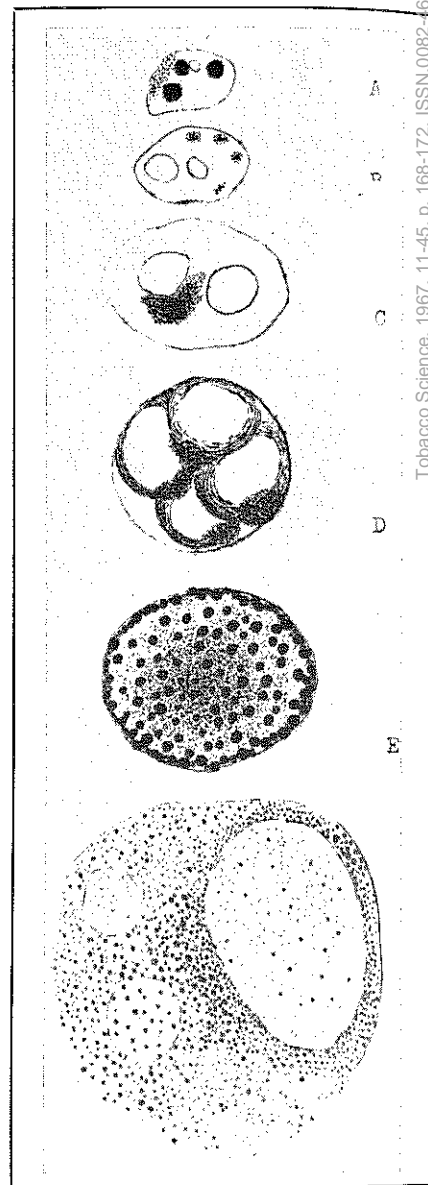
Efforts were made to determine

Figure 1. The developing plastid as seen following fixation with Altmann's fixative and staining with acid fuchsin. Key: A, after exposure to 100 fc for four hours; B, after twelve hours exposure; C, after twenty-four hours exposure; D, after thirty-six hours; E, plastid in a young leaf (same age as A-D) which developed on the plant under greenhouse conditions; F, plastid from a mature leaf of the middle of the plant.

how plastids developing in etiolated excised leaves compared with those developing in leaves of fully illuminated plants. Leaf sections from shoots grown in the light were fixed in formalin-propionic acid and stained with Conant's quadruple stain. A typical chloroplast from this tissue is seen in **Figure 1E**. Grana were prominent. No starch was present but plastid diameter was approximately the same as found in the experimental material. These findings are in good agreement with those of Mühlethaler (13), who reported that following the *Amyloplastische Proplastiden* stage, a starch-free plastid developed

in which grana were formed.

Discussion

Plastids appear to respond somewhat differently to light depending upon the leaf tissue in which they are located. From the data one may conclude that growth in diameter of plastids in spongy mesophyll cells precedes that in palisade cells. In five out of six leaves examined, that had been exposed to light for 36 hours, the plastids were equal or greater in length or diameter in spongy mesophyll cells than in palisade cells. This faster growth might be attributed to larger size of the spongy cells and less physical compaction of the contents. If this were the case, it might be expected that chloroplasts in palisade cells would show the greatest growth in thickness because there the plastids abut one another as they are pushed against the cell wall during vacuolar expansion. It would seem that as growth ensues plastids next to the vacuolar membrane should thicken most quickly. The data, however, do not bear this out; hence, the observed plastid growth differences cannot be attributed to plastid pressure against adjacent plastids or the absence of turgor pressure from the vacuoles.

Growth of the plastids was neither uniform in rate nor direction. The data show that during the second two hours of exposure the increase in "length" of mesophyll plastids was one micron. Twenty hours of exposure was required for the second such increment.

Internally, plastid development shows changes which appear to be progressive for several constituents. Starch grains in the plastid are one of these. Such granules have been found in the amoeboid stage of the protoplastid by many workers. Mühlethaler (13) showed starch formation to be among the first changes detectable in the developing proplastid. At the early stage in development represented here, Grave (6) and Strugger (20) have reported evidence that supports the view starch grains appear before vacuoles. The occurrence of vacuoles in slightly older proplastids has been reported by Strugger (20). Bald (2) found them in considerably older plastids and termed them stromatic gaps. They are shown in electron micrographs by Mercer et al (11) and described as intralamella vacuoles containing chloroplasm. Under continuous illumination, the structures observed here continued to increase in size but their number seemed constant.

The developmental changes in the tobacco chloroplast follow reasonably well those described by Grave (6) and Strugger (20) for other species. However, the new information obtained with tobacco emphasizes two points. One is that there is a polygranular stage that seemingly leads to the formation of the primary granum and the second is that the grana develop after the formation of the primary granum. Concerning the first of these points, it was the view of Grave (6) and Strugger (18, 19) that a monogranular proplastid (observed in only a few slides made of tobacco) developed through a starch-forming stage and then a greening stage. Following this, grana were formed through an ordering of the granules of the primary granum which, itself, had developed from *Granascheibschens*.

Strugger (21) using fluorescence microscopy, demonstrated the presence of chlorophyll in the primary granum, and it has become a practice to identify the plastid containing a primary granum as a greening proplastid. As will be shown subsequently in this series of reports, detectable amounts of chlorophyll are formed in tobacco after exposure of leaves to light for two hours; whereas the primary granum is not conspicuous until after 24 hours' exposure. However, it is during the period of formation of the primary granum that chlorophyll concentration rises most rapidly; i.e., between 12 and 24 hours. The question may logically be raised, therefore, as to whether or not the early-formed chlorophyll is as effective or efficient in photosynthesis as that produced in the greening plastid stage.

Plastids developing in excised etiolated leaves and continuous illumination may not be expected to represent in every respect the plastid developing under natural conditions. An indication of the differences may be obtained from a comparison in **Figure 1** of **D** and **E**. There was little size difference between these. But plastids developing under natural light conditions were in a true polygranular stage when their counterparts in detached etiolated leaves were still in the early primary granum stage. Thus it appears that under normal greenhouse conditions plastids showed essentially an effect of exposure to light of a longer duration than those studied in excised etiolated leaves and exposed for 36 hours.

Development typical for the upper middle region of a plant grown under normal greenhouse conditions is shown in **Figure 1F**. Even though

collections were made early in the morning after several cloudy days, the starch grains were very prominent and large.

Summary

Developing plastids in excised etiolated leaves respond more rapidly to a 100 fc light intensity than to 60 fc. Under 100 fc, they measurably increased in thickness and diameter during four hours of exposure. Another 20 hours of light was required for measurable increases in diameter. There was some indication that plastids in older leaves reacted more rapidly than those in younger ones. Alternatively, they could have been larger at zero time in the experiment.

Internally, a steady increase in size of starch grains (and/or vacuoles) was observed. Small grana (*Granascheibchens*) developed early and appeared to coalesce to form the primary granum. There is good evidence that plastids develop more slowly in excised etiolated than in attached leaves, although the studies were not comparable in all respects.

Plastid development in variety 18a of aromatic tobacco followed, in general, the pattern described for other species. The size obtained by starch grains (and/or vacuoles) under experimental conditions seemed unique but paralleled that in plastids developed under greenhouse conditions.

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