DEVELOPMENTAL CHANGES OCCURRING IN ISOLATED INTACT ETIOPLASTS

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SUMMARY

When dark-grown plants are illuminated, the characteristic morphology of the etioplast changes. The tubules of the paracrystalline prolamellar body disperse as the membranes reorganize and 'grow out' as 'perforated' thylakoids. Similar changes are expected during the greening of the isolated etioplasts of *Avena*. Under certain preparative conditions the morphological changes vary from those previously reported for intact tissue.

Following low illumination at a low temperature the process of outgrowth is slowed down, resulting in the outgrowth of regularly spaced tubules from the prolamellar body into the stroma. These tubules appear to develop slowly into normal thylakoids by a process of tubule fusion to form perforated thylakoids. A diagram is included to show how this is believed to take place. The outgrowing tubules have an outer diameter of 25-28 nm with a lumen of 7.0-7.6 nm diameter.

Two types of protein body are observed, the 'stromacentre' which is a regular feature of *Avena* plastids and a 'crystallite' which is only observed under certain conditions. The 'stromacentre' is a fibrillar spherulite with fibrils (diameter 9 nm) in bundles up to 200 nm long which in turn are aggregated to form the characteristic spherulite of the stromacentre. The crystallite is composed of fibrils 7.5-8.5 nm in diameter lying parallel to each other with a regular spacing of 12.5 nm. The crystallite appears to develop when the etioplast is damaged and has started to vesiculate.

Particles which are larger than either plastid or cytoplasmic ribosomes have also been observed. These vary in diameter from 18 to 24.5 nm and are arranged in rows adjacent to the thylakoids.

INTRODUCTION

During proplastid development in dark-grown plants, the inner layer of the envelope invaginates to provide the primary internal membranes (Wettstein & Kahn, 1960; Menke, 1964). The vesicles formed migrate into the stroma and fuse together to form the network of tubules composing the paracrystalline prolamellar body (Wellburn, 1968), the structure of which has been described by Gunning (1965b), Gunning & Jagoe (1967) and Wehrmeyer (1965a, b, c). In this form the plastid is called an etioplast (Kirk & Tilney-Bassett, 1967).

Following illumination, the paracrystallinity of the prolamellar body is disrupted and the tubule membranes reorganize to push out into the stroma as flat, membranous sheets, the so-called 'perforated' thylakoids, the perforations being relics of the pores of the prolamellar body (Gunning & Jagoe, 1967). Sustained illumination causes the thylakoids to 'overlap' into bi-thylakoids and eventually grana are formed (Wehrmeyer & Röbbelen, 1965).

Ultrastructural studies have shown that the non-membranous stroma includes ribosome-like particles (Jacobson, Swift & Bogorad, 1963) which may be aggregated...
into polysomes (Brown & Gunning, 1967; Bartels & Weier, 1967; Falk, 1969), DNA fibril areas (Ris & Plaut, 1962; Bisalputra & Bisalputra, 1967; Kislev, Swift & Bogorad, 1967) and enzyme molecules (see Kirk & Tilney-Bassett, 1967). One enzyme, carboxydismutase, is thought to occur in sufficient quantities in *Avena* to become visible as the fibrillar 'stromacentre' (Gunning, Steer & Cochrane, 1968; Steer, Gunning, Graham & Carr, 1968). Protein crystals have also been shown in the young chloroplasts of *Equisetum limosum* (Manton, 1967).

Plastids are thought to be autonomous with respect to some aspects of metabolism and in the isolated condition retain some biosynthetic capabilities similar to those found *in vivo* (see Kirk & Tilney-Bassett, 1967). They can incorporate amino acids into proteins (Spencer & Wildman, 1964; Chen & Wildman, 1967; Margulies & Parenti, 1968; Goffeau, 1969) and synthesize RNA (Kirk, 1964) and DNA (Spencer & Whitfield, 1967; Herrmann, 1969). Scott & Smillie (1967) have shown that one function of the chloroplast DNA is to code for the RNA of chloroplast ribosomes. It is also thought that proplastids can reproduce independently of the nucleus (see Bell, 1970). However, current evidence indicates that plastid differentiation is semi-autonomous involving the participation of both nuclear and plastid DNA (Berger, 1967; Surzycki, Goodenough, Levine & Armstrong, 1970).

During the development of a method for the isolation of intact etioplasts with 'normal' greening characteristics, it was noted that certain conditions produced some unexpected morphological variations. These variations are reported and discussed below. The preparative procedure was altered until these variations no longer appeared and the resulting method is reported in Wellburn & Wellburn (1971).

**MATERIALS AND METHODS**

**Plant materials**

Seedlings of *Avena sativa* (var. Mostyn) were grown in a moist peat/vermiculite mixture in total darkness at 22 °C for 13 days. Harvesting of the laminae and all subsequent procedures were carried out as quickly as possible, either in total darkness or under a dim green safelight (Wilkins, 1965).

**Extraction of etioplasts**

A new column procedure described in Wellburn & Wellburn (1971) was used to isolate intact etioplasts. The isolated etioplasts were illuminated by Philips 'warm-white' fluorescent tubes providing 150 and 5000 lx respectively either on the Sephadex column at 4 °C or after fraction collection at 20 °C.

**Electron microscopy**

The etioplast suspension was fixed for 2 h at 0 °C in an equal volume of 5 % (v/v) glutaraldehyde in 0.025 M phosphate buffer, pH 7.3, containing 9 % sucrose. The suspension was pelleted at 750 g for 3 min. The pellet was washed 3 times using successive buffer solutions (as above) containing 9 %, 8 % and 7 % sucrose, respectively. Post-fixation was carried out for 2 h in 2 % osmium tetroxide in buffer (as above) containing 5 % sucrose, and dehydration was carried out using a graded ethanol series with decreasing amounts of sucrose and no sucrose after 50 % ethanol. The pellet was embedded in Epon and sectioned on a Reichert OMU2 ultramicrotome. The sections were double stained in uranyl acetate and lead citrate (Reynolds, 1963) and examined in a GEC-AEI EM801 electron microscope.
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OBSERVATIONS

Tubules

Prior to illumination the internal membranes of the etioplast are arranged as tubules of the paracrystalline prolamellar body (Fig. 11) and following illumination under 5000 lx at 20 °C the crystallinity is observed to be disrupted. The tubule membrane is redistributed and extended outwards from the prolamellar body into the stroma as flat sheets of perforated thylakoids (Fig. 10, and Wellburn & Wellburn, 1971; also Gunning & Jagoe, 1967, and Wellburn, 1968, for intact tissue).

Under 150 lx at 4 °C, morphological variations are apparent. After 5 min of illumination (Fig. 3) the tubules of the prolamellar body become disrupted and appear to re-unite as long tubules with no interconnexions. Further illumination results in emergence of the tubules from the prolamellar body into the stroma, but instead of the expected immediate conversion into perforated thylakoids the tubules persist (Figs. 2, 4). They have an outer diameter of 25–28 nm with a central lumen of diameter 7·0–7·8 nm and are similar in dimensions to the tubules of the prolamellar body. They can be extensive; for example the longest tubule in Fig. 6 measures 2·3 μm. In longitudinal section they are seen to emerge from the dispersing prolamellar body in regular rows. In transverse section the tubules are seen to be alternate (Fig. 4). The spacing between the individual tubules corresponds to the tubule spacing in the paracrystalline prolamellar body.

With continued illumination the tubules become less distinct and are not observed in plastids illuminated for 3 h. Such plastids are structurally similar to plastids illuminated for 2·5 h with 5000 lx at 20 °C. There appears (Fig. 4) to be a longitudinal fusion from the tubule tips towards the prolamellar body followed by a recombination of the membrane laterally across the rows of tubules resulting in the formation of membrane sheets, the so-called perforated thylakoids. This process occurs basipetally so, as the proximal ends of the tubules are reorganizing into perforated thylakoids, the distal ends are being added to as the prolamellar body continues to disperse. This process continues slowly so that after 2 h of illumination only a small number of tubules persist close to the still dispersing prolamellar body with most of the membrane in the form of perforated thylakoids or developing grana (Fig. 7).

Rough thylakoids

Particulate arrangements are observed in the serial sections in Figs. 8 and 9. In transverse section they appear as a single layer of heavily stained particles adjacent to the thylakoids. In the glancing surface view of a thylakoid the particles (gp) are seen to be arrayed in rows. The diameter of these particles (18–24·5 nm) varies according to the plane of section and they are larger than the ribosome-like particles in the stroma and those in the stroma component of the prolamellar body (Figs. 8, 9 and 11), which range from 13·5 to 15·5 nm in diameter. It is possible that because of the positioning of the particles relative to the thylakoid, the size difference and the altered staining characteristics, these regular particles may be polysomes actively synthesizing thylakoid protein. Previous evidence by the author (Brown & Gunning, 1967)
and also by Bartels & Weier (1967) and Falk (1969), indicates that membrane-aligned and free polysomes exist in *Avena*, *Triticum* and *Phaseolus*. These polysomes may function in the synthesis of both enzymic and structural protein and in this case (Figs. 8, 9) probably additional membrane protein immediately prior to overlapping.

*Protein bodies*

Two types of protein body have been observed. Examples of a fibrillar spherulite or 'stromacentre' (Gunning, 1965a) are shown in Figs. 2, 6, 7, 10 and 12. The spherulite fibrils may be observed to be up to 200 nm long with a cross-sectional diameter of 8-9 nm (Fig. 7) and they tend to form bundles which aggregate as a spherulite. Thus in any one plane of section the fibrils can be viewed transversely, longitudinally and obliquely.

Crystallites are shown in Figs. 10–12. Unlike the stromacentre they are not a regular feature of isolated *Avena* plastids but are observed only in isolated etioplasts which are considered to be damaged. The component fibrils are regularly oriented, so that, in any one plane of section, all the fibrils in each crystallite have the same orientation. Consequently, the crystallite appears sharp when the fibrils are viewed transversely and longitudinally but amorphous when viewed obliquely. The fibrils have a diameter of 7.5–8.5 nm and are spaced approximately 12.5 nm apart. The crystallites, like the 'stromacentre', are not membrane bound and tend to occur adjacent to the plastid envelope, presumably because of space limitation due to the presence of the prolamellar body (Figs. 10, 11). When fully developed they may be cubic in form and puncture the etioplast envelope (Fig. 10). They are not restricted to areas where there is no 'stromacentre' for both structural types can occasionally be seen adjacent to each other (Fig. 12).

Crystallites are always observed in tissue having vesicles immediately below the plastid envelope. This vesiculation is produced by the isolation technique. It is not observed in plastids isolated using the column method described in Wellburn & Wellburn (1971) but is found in those plastids isolated by methods using solutions of high osmolarity, for example, zonal centrifugation or the methods of Boardman & Wildman (1962) or Jacobson (1968). In the micrographs accompanying the description Figs. 1. A diagrammatic representation of granum development. A, The paracrystalline prolamellar body (derived from Wehrmeyer, 1965a, b, c). The component tubules have numerous interconnexions. B, Following illumination at 4 °C, the interconnexions break and long intact tubules are formed. C, As the prolamellar body tubules continue to disperse they are pushed out into the stroma as regularly spaced rows corresponding to their original positions in the prolamellar body. D, With continued illumination the process of membrane fusion commences and progresses basipetally until the tubule membranes have reorganized as perforated thylakoids. The perforations arise from faults in the fusion process. More extensive faults are also possible. E (i)–(iv), Transverse profiles to illustrate the possible transition from tubules to perforated thylakoids. F, Membrane growth commences, the perforations and faults providing the weak points for movement and overlapping for the formation of bi-thylakoids. G, Continued growth causes further overlapping resulting in granum stacks shown in H.
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Fig. 1. For legend see facing page.
of the method described by the last-named author, vesicle formation similar to that associated with the crystallites is clearly to be seen. Vesicle formation similar to that observed in these isolated etioplasts is not found in situ and must be due to organelle damage during isolation.

DISCUSSION

Stroma tubules are formed when isolated etioplasts are illuminated with a light of relatively low intensity (150 lx) at a low temperature (4 °C) but are not observed when plastids are illuminated with 5000 lx at 20 °C (Wellburn & Wellburn, 1971). Similar tubules have been observed by Appelqvist, Stumpf & von Wettstein (1968) in isolated barley chloroplasts and by Henningsen & Boynton (1970) in the plastids of the primary leaves of dark-grown barley which had been illuminated for 8 h under 20 lx. No explanation was offered by these authors. Current evidence (Treffry, 1970) indicates that, following illumination at 0 °C, the photoconversion of protochlorophyllide to chlorophyllide is not accompanied by the expected morphological changes of prolamellar body disruption and thylakoid outgrowth. Treffry (1970) suggests that this is due to the inhibition of the phytylation of the chlorophyllide by the low temperature. It is our view that at 4 °C the process of outgrowth has been considerably slowed down. Disruption of the tubules of the prolamellar body takes place and subsequent membrane changes between disruption and thylakoid formation can be observed in detail. A diagrammatic interpretation of these results is shown in Fig. 1. The paracrystallinity of the prolamellar body is disrupted (Fig. 1 A) but, because the membranes cannot reorganize quickly enough at the low temperature, rows of tubules appear round the periphery of the prolamellar body (Fig. 1 B) and appear to be pushed out from it as the prolamellar body disperses, the dispersing tubules contributing to the outgrowing tubules. This process is shown clearly in Fig. 10. Many of the membranes retain their ability to reorganize into flat sheets by fusion of the adjacent tubule walls (Fig. 1 D). This process appears to occur basipetally (Fig. 1 E (i) to (iv)). It is thought that perfect lateral membrane fusion does not always take place; thus small perforations or longitudinal faults may be formed in the thylakoids (Fig. 1 E (iv)) giving rise to possible sites for overlapping during bi-thylakoid and subsequent granum formation (Fig. 1 F-H).

In the case of development at higher temperatures and higher light intensities the tubules are still formed within the disrupting prolamellar body but the process of tubule fusion occurs at the surface of the prolamellar body and is not extended into the stroma.

Aggregates within the stroma of plastids are not uncommon. They have been reported by Buvat (1959), Lemoine (1966), Gunning (1965a) and Gunning et al. (1968) in normally grown plants; by Thompson, Dugger & Palmer (1965, 1966) in peroxyacetyl nitrate- and ozone-treated plants; by Cronshaw, Hoefert & Esau (1966), Purcifull, Edwardson & Christie (1966) and Esau & Cronshaw (1967) in virus-infected plants and by Gunning et al. (1968) in artificially wilted plants. *Avena* is considered unusual in having an aggregate as a permanent feature. It is
thought that this 'stromacentre' consists of Fraction I protein (Gunning et al. 1968) which in turn is believed to be a form of carboxydismutase (Steer et al. 1968). The spherulite present in the isolated etioplasts is assumed to be chemically similar to that in intact tissue because of the similarity in ultrastructure. The ultrastructural differences between the crystallite and the spherulite indicate that the crystallite protein may not be Fraction I. Alternatively Fraction I may be more than one protein and the crystallite an aggregate of just one of them. The results indicate that crystallite formation and vesiculation are associated events. Vesiculation close to the plastid envelope may interfere with a control mechanism ensuring the optimum production of this protein. High osmolarity in the isolation medium enhances vesiculation and use of such media should be avoided if levels of particular proteins are to be determined accurately.

It is unlikely that the crystallite consists of lamellar protein because crystallites of similar structure were seen both in illuminated and non-illuminated plastids. Observations of the plastids in Avena leaves treated with actinomycin D and chloramphenicol (Wellburn, 1968) indicate that growth of thylakoids (by addition of new membrane to form bi-thylakoids) does not take place until at least 60 min after illumination. It is unlikely that the membrane structural protein would be formed in any quantity in the non-illuminated etioplasts before illumination initiates the photoconversion of phytochrome which in turn starts the sequence of developmental changes. The ability to synthesize certain proteins is indicated by the regular polysomes adjacent to the thylakoids in Figs. 8, 9. It seems likely, because of their position, that these are ready to engage in active synthesis of membrane proteins for thylakoid expansion. The DNA-dependent RNA polymerase present in the plastid (Scott & Smillie, 1967) may transcribe from the plastid DNA the appropriate messenger RNA for this protein.

Current evidence suggests that the harmony between the genome and plastome is essential for the formation of normal grana, and it is therefore unlikely that fully formed and active grana would develop in a homogeneous etioplast suspension. From the work showing that chloramphenicol affects mature granum formation (Wellburn, 1968) and the work of Schötz (1970) on mutants of Oenothera, the stages from initial overlapping to mature granum stack formation appear to be dependent upon the interrelated control of genome and plastome.

We have observed granum stacks of up to 5 thylakoids in etioplasts illuminated at 20 °C with 5000 lx and hope in the future to carry out longer periods of illumination and investigate this important interrelationship.

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REFERENCES


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Fig. 2. Part of an isolated etioplast after 30 min of illumination with 150 lx at 4 °C. d, DNA fibrils; e, the intact envelope; p, dispersing prolamellar body; pt, perforated thylakoid; s, stromacentre; tu, tubules in T.S.; tu/l, tubules in L.S. x 50000.

Fig. 3. Part of an isolated etioplast showing the intact 'unconnected' prolamellar body tubules (arrowed) after 5 min of illumination with 150 lx at 4 °C. x 45000.
Fig. 4. An isolated etioplast (30 min illumination with 150 lx at 4 °C), showing the intact envelope (arrowed), alternate rows of tubules in T.S. (tu) and L.S. (tu/l) which are developing into perforated thylakoids (pt). × 41 000.

Fig. 5. Part of an isolated etioplast (illumination as Fig. 4) showing a tubule which may have altered its direction of 'growth'. × 52 000.

Fig. 6. An isolated etioplast (45 min illumination as Fig. 4), sectioned to show only tubules in L. S. (tu/l), 'stromacentre' and DNA fibrils (d). × 28 000.
Fig. 7. Part of an isolated etioplast (2 h illumination with 1500 lx at 4 °C) showing bi-thylakoids (arrowed), perforated thylakoids 'stromacentre' (s) and few tubules. $\times 55000$.

Figs. 8, 9. Serial sections showing 'polysomes' aligned adjacent to the thylakoids in transverse ($tp$) and glancing surface ($gp$) section. $\times 37000$. 
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Fig. 10. An isolated etioplast (2 h illumination with 5000 lx at 20 °C) showing both protein bodies, the 'stromacentre' spherulite (s) and the crystallite (c), which appears to perforate the envelope. \( \times 35,000 \).

Fig. 11. An isolated etioplast showing many crystallites which have developed adjacent to the envelope. \( \times 32,000 \).

Fig. 12. A higher-magnification view of an isolated etioplast showing adjacent 'stromacentre' (s) and crystallite (c). \( \times 71,000 \).
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