The Plastids of *Polytrichum* II. The sporogenous cells¹

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Introduction

Weier (1931a) studied sporogenesis in the moss, *Polytrichum commune*, and reported that the staining reaction of a plastid in a sporogenous cell is characteristic of a cell's stage of development. According to his account, at the origin of the archesporium each of the several plastids in an archesporial cell contains a darkly staining reticulum. During the multiplication of the archesporial cells, a single, flattened, circular plastid persists in each cell. Weier described this plastid as having a darkly staining rim (plastonema) and a lightly staining interior (plastosome).

After the final mitotic division, the sporogenous cells separate from each other. Each sporocyte becomes tetrahedrally lobed, foreshadowing the configuration of the spore tetrad. Prior to meiosis, the single plastid in the sporocyte divides into four daughter plastids that appear to be granular. The daughter plastids migrate, one into each lobe of the sporocyte, and pass through a phase in which elongate "granules" form in the plastids. Subsequent to meiosis, the single plastid in each spore again contains a darkly staining reticulum. Weier reported that a typical chloroplast is found in the young spore, and that this plastid divides to form two plastids as the spore ages, in preparation for shedding. The present study is a continuation of an earlier effort (Paolillo 1964b) to combine light and electron microscopy in characterizing the morphology of the plastids in the sporogenous cells of *Polytrichum*.

Material and methods

Capsules of various ages were collected from *Polytrichum ohioense*, and *P. juniperinum*. Four classes of materials were examined. Capsules of the first class, referred to as *field collections*, were fixed 4-24 hours after their removal from the field, with care being taken to prevent harm by drying during the interim period. These capsules are assumed to be representative of the stage of development that would be found in similar capsules in the

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field at the time of fixation. The second category, the *greenhouse plants*, were cultivated in late winter and early spring for two weeks or longer in a greenhouse. This group of capsules was much accelerated in development compared to plants of similar age in the field at the time of fixation. A third group of capsules includes all those greenhouse plants that were subjected to experimental regimes of darkness and/or low temperature for 12–96 hours before fixation. The fourth series of capsules was obtained by culturing young sporophytes, one to two inches long, without their calyptras on Knudson's agar with 1% dextrose, after the sporophytes were surface sterilized with 5% Chlorox solution.

Materials for light microscopy were either fresh, or fixed and embedded in wax, depending on the observations that were required. Sections of materials killed with Regaud's fixative were stained with hematoxylin. Those from specimens treated with glacial acetic acid-alcohol (1:3, in 95% ethanol) were stained with acridine orange for fluorescence of nucleic acids (Paolillo 1964a). Native fluorescence of chlorophyll was observed using fresh materials. A Leitz Ortholux microscope and Osram HBO 200 lamp were used for fluorescence microscopy with a blue excitation filter and a yellow barrier filter.

Materials for electron microscopy were fixed in aqueous 2.5% KMnO_4 for 20–30 minutes or for 1–1/2 hours in buffered glutaraldehyde with 1 hour post-fixation in OsO_4 (Ledbetter and Porter 1963). Spore sacs were removed from the capsules in buffer or distilled water and cut into two parts. One portion was placed directly into the fixative and cut into smaller pieces. The other part of the same spore sac was used to prepare hand sections for observing native fluorescence of chlorophyll, for evaluating the stage of development of the sporogenous cells, or for fixation for light microscopy. For late stages of development, spores were removed from the capsules by cutting off the capsule lids and squeezing the capsules against a glass slide with a dissecting needle. The spores were retrieved from the fixatives and dehydrating fluids by low speed centrifugation. Materials for electron microscopy were embedded in a 3:7 mixture of methacrylates (Paolillo 1962) and in Mollenhauer's (1964) mix #1 of epon and araldite. Thin sections were viewed on RCA EMU 3 and 4 microscopes. Sections of the glutaraldehyde-fixed materials were post-stained with lead citrate (Reynold 1963).

Observations

Electron microscopy. The observations made on field and greenhouse plants will be combined with those from sporophytes grown in culture because the observations on these materials agree as far as the developmental history of the sporogenous cells is concerned. Likewise, observations on the two species will be taken together for this description, which will emphasize only features of plastid development throughout sporogenesis.

The earliest stage observed thus far is from a capsule that had begun to

inflate, with the formation of the typical air chambers. At this stage, the sporogenous layer has been set off (Fig. 1) and is beginning to increase in cell number. Neither the cells of the sporogenous layer nor the surrounding cells are vacuolated at this time, but the cytoplasm of the sporogenous cells is visibly denser than that of the adjacent vegetative cells (Fig. 1). The cells of the sporogenous layer are interconnected with plasmodesmata, but plasmadesmata were not found between the sporogenous cells and vegetative cells.

At this early stage in the development of the spore sac, the differentiation of a tapetum with a photosynthetic layer and a putatively secretory layer (Paolillo 1964b) has not yet been accomplished. The cells that will become the tapetum contain typical chloroplasts (Fig. 1), like those found in the leaves of the gametophore (Paolillo and Reighard 1967). In the capsule of Fig. 1 the sporogenous cell plastids had already begun to flatten. But like the plastids of the vegetative cells, each of the plastids in a young sporogenous cell contains a grana-fretwork system. The plastids of the sporogenous cells are differentiated from the vegetative plastids by the lesser complexity of their lamellar systems and by the diversity of the grana found in them (Figs. 1-3). The diameters of the grana and the numbers of compartments are highly variable (Figs. 2 and 3) compared to the more or less uniform character of the grana found in the vegetative plastids. The stacking of the compartments is often skewed, so that the margins of the grana are tilted in sectional views (Fig. 2).

As the sporogenous cells multiply, the number of plastids per interphase cell is maintained at one. The plastid becomes a flattened, circular structure, and it is often found cupped around the interphase nucleus (Fig. 4). The lamellar component of each plastid at this stage is much simplified from its earlier condition. The plastid profile is sparsely populated with extensive lamellae, and many grana contain only 2-3 compartments of small diameter. In the materials at hand, the plastid morphology remains essentially constant throughout the multiplication of the sporogenous cells, in spite of the continued division of the plastid with each nuclear and cell division. The plastids of the tapetal layers maintain their vegetative condition and continue to provide a marked contrast to the plastids in the sporogenous cells.

When the sporocyte stage is reached, the plastids become polymorphic. Lobing and division occur (Fig. 5) and some specimens show the wide spacing (100 Å) of parallel stacked lamellae (Fig. 6) already described for the plastids of sporocytes in *Polytrichum* (Paolillo 1964b). Other specimens retain small grana of 2-3 closely stacked compartments into the sporocyte stage.

The daughter plastids formed in the sporocyte begin to round up, and each spore in a tetrad contains a highly simplified, nearly spherical plastid (Fig. 7) when the exine is well formed. No grana were found in these plastids. The plastids contained instead a number of dispersed lamellae and what appear to be small vacuoles. While the spore is completing the

Figs. 1-4.

formation of its inner wall layer and accumulating its reserve food (lipid droplet), the plastid becomes polymorphic and starts to increase its internal complexity. The lamellar components in the plastid increase, but the first manifestation of this is the formation of a loosely organized, non-crystalline prolamellar body (PLB) (Fig. 8). Serial sections reveal that two or more PLBs may be present in a single plastid. The lamellar system continues to gain in complexity as the PLBs disperse and small grana appear (Figs. 9 and 10). By the time the PLBs are entirely gone, typical grana are present (Fig. 11), and the plastid is no longer polymorphic. Instead it has the shape of an oblate or prolate ellipsoid, as is typical of chloroplasts in the gametophyte and sporophyte. The plastid may divide before it becomes a typical chloroplast of afterwards. In either case, the two chloroplasts that result may again divide before the spore is shed (Reighard 1967).

Starch is present in the chloroplasts of the aging spore (Fig. 11) in relative abundance. No starch was detected at earlier stages, except for occasional minute grains in some sporocytes (Fig. 5). The translucent areas in Figs. 7 and 10 appear to be vacuoles rather than starch grains, but the staining reaction of these areas can vary from plastid to plastid, and their contents are unidentified.

Fluorescence microscopy. Plastids in the young sporogenous tissues (like those in Figs. 1-4) fluoresce a brilliant red in blue light. During the later stages in multiplication of the sporogenous cells, fluorescence in their plastids diminishes, whereas fluorescence in the vegetative plastids does not change. Fluorescence is dim in sporocyte plastids (like those of Figs. 5 and 6) and it is virtually undetectable in the newly formed spores. The yellow-fluorescing spore wall (exine) is elaborated before any restoration of fluorescence begins in the plastid. The plastids of Figs. 7-9 were obtained from samples that showed little or no chlorophyll fluorescence. A plastid that has begun to restore its internal complexity with the formation of grana (Figs. 10-11), fluoresces brilliantly in blue light. All daughter plastids in the spore maintain this high level of fluorescence (see also Reighard 1967).

The wax embedded materials that were sectioned and stained with acridine orange showed a brilliant metachromatic (orange) fluorescence in the cytoplasm of archesporial cells and sporocytes. These observations indicate the presence

Figs. 1-4. 1, single layer of sporogenous cells, Spg, among vegetative cells, Veg, early during the inflation of the capsule. Simplification and flattening of the sporogenous plastids, SP, has begun. Compare with vegetative plastids, VP. Both sporogenous and vegetative cells lack large vacuoles. Nuclei, N, are prominent. $\times 6,500$. 2, plastid from the sporogenous layer represented in Fig. 1. The grana are rather small and the fretwork is sparse. Stacking in the larger grana is noticeably skewed, at *Sk*. $\times 25,000$. 3, like Fig. 2, but with both large and small grana. The fretwork is sparse and irregular. $\times 25,000$. 4, later stage in multiplication of the sporogenous cells. The plastid is flattened, nearly circular in the third dimension, and is sparsely populated with expansive lamellae and minute grana of 2-3 compartments. The plastid is here seen cupped around the nucleus, N. The cytoplasm is rich in ribosomes. $\times 25,000$. Figs. 1-3, KMnO_4 fixation, Fig. 4, glutaraldehyde fixation.

Figs. 5-9. 5, lobed, possibly dividing, plastid in a sporocyte. The cytoplasm is rich in ribosomes. The profiles marked M are mitochondria. Starch, St, cytoplasmic lipid droplets, L. $\times 25,000$. 6, sporocyte plastid showing stacked lamellae that are not as closely juxtaposed as they would be in normal grana. Mitochondria, M, and cytoplasmic lipids, L, are abundant. Golgi body, GB. $\times 30,000$. 7, simplified, spherical plastid within a spore that has elaborated its ornamented exine, E. The plastid contains dispersed lamellae and some small vesicles. The numerous white areas seem to be vacuoles rather than starch grains.

of a high concentration of RNA in the cytoplasm and harmonize with the observed density of ribosomes visualized in cells of these stages that were examined with the electron microscope (Figs. 4 and 5).

Other light microscopy. Observations were made on Regaud-fixed hematoxylin-stained cells to complement the electron-microscopic observations. The intention was to uncover the ultrastructural basis for the images observed by light microscopists. The parallel between the reported light microscopic images and the electron-microscopical findings has already been drawn, in part (Paolillo 1964b). It can be added here that the simplification of the interior of the plastid accounts for the disappearance of the darkly staining reticulum, which must be the light microscopic visualization of the grana-fretwork system. This observation harmonizes with the writings of both Weier (1931a) and Eyme (1954). Furthermore, the elaboration of a new grana-fretwork system in the plastid of the aging spore harmonizes with the ideas of Weier (1931a) and Eyme (1954), regarding the return of typical chloroplast structure before the spores are shed.

On the question of a plastonema-plastosome in the plastids of the multiplying archesporial cells, I have been unable to obtain any substantiation for Weier's (1931a) description. All plastids observed at interphase during this stage of development were flattened, circular and cup-shaped structures that stained uniformly. This staining reaction is easily explained by reference to the ultrastructure of the plastids in the multiplying archesporial cells, which have extensive lamellae but no conspicuous grana (Fig. 4). When the light microscope is adjusted to the proper level of focus on a hematoxylin-stained plastid that is appropriately curved and positioned, the false impression of a plastonema-plastosome configuration is obtained. One might then argue, as did Eyme (1954), that Weier's (1931a) report is inaccurate in describing the plastonema-plastosome configuration. The "false plastonema" was observed by me in light-microscopic preparations of an archesporium where the plastids were shown to have sub-microscopic structure like that illustrated in Fig. 4.

The resemblances between my light microscopic preparations and Weier's (1931a) figures are so convincing that I find it necessary to favor Eyme's (1954) explanation of the structure of the plastid during the multiplication of the archesporial cells. In doing so I am faced with an enigma, because Weier (1931b) also described the plastonema-plastosome configuration for plastids of multiplying spermatogenous cells of *Polytrichum*. Eyme (1954) rejected this description, but investigations of these plastids reveal a sound ultrastructural basis for the reported staining image. The plastids are biconcave (Fig. 12), as they are in the meristem of *Isoetes* (Paolillo 1962)

×20,000. 8, portion of polymorphic plastid in aging spore. A non-crystalline prolamellar body, PLB, has formed. Lamellae extend from the PLB. Vesicles and tubules, VT, occur elsewhere in the plastid. ×30,000. 9, dispersing prolamellar body, PLB, and associated lamellae, some of which are paired, Pr, in the beginnings of grana formation. ×47,000.

Fig. 5, glutaraldehyde fixation, Figs. 6-9, KMnO₄ fixation.

where a "true plastonema" is visualized with the light microscope. It seems awkward to speculate at this time that Weier was correct in one interpretation (1931b) but incorrect in the other (1931a). The materials I have studied may

Figs. 10-12. 10, a plastid that was increasing in complexity with small grana, G, vesicles and tubules, VT. The plastid was still somewhat polymorphic. $\times 25,000$. 11, typical chloroplast of maturing spore. Grana, G, starch, St, $\times 25,000$. 12, profile of a biconcave, circular plastid from spermatogenous cell of a young antheridium. The rim of the plastid, Pn, could provide the densely stained "plastonema" of the light microscopic image. The thin central region of the plastid would account for the lightly stained "plastosome". Mitochondrion, M. $\times 20,000$. Figs. 10-11, KMnO_4 fixation, Fig. 12, glutaraldehyde fixation.

not adequately represent the range of morphology of the sporogenous plastids, and the question of the plastonema-plastosome configuration cannot be regarded as completely closed.

One further point can be made at this juncture. The electron-microscopic images of sporocytes support Eyme's (1954) contention that the "granularity" of the plastids in young sporocytes is due to the clustering of cytoplasmic particles around the plastids. Figures 5 and 6 show mitochondria, golgi bodies, and cytoplasmic lipid droplets in the vicinity of the plastids (also, see Figs. 6 and 7 in Paolillo 1964b). The fact remains, however, that the elongate granules described by Weier (1931a) may correspond to stacked lamellae, as in Fig. 6 (Paolillo 1964b).

The experimental plants. Sporophytes containing sporocytes or spores were left attached to their gametophores, and subjected to 12, 24, 48, 72, or 96 hours of darkness at room temperature, 4°C, or -5° dry or embedded in ice. These regimes did not produce any remarkable departure from the images obtained with control plants, except that the wet-frozen plants showed extensive vesiculation of the protoplast after 24 hours of treatment. Because the samples used were thawed for fixation, it is impossible to separate the effect of thawing from the effect of freezing.

Discussion

The changes in plastid morphology that occur during sporogenesis in *Polytrichum* being with a progressive simplification of the grana-fretwork system. This simplification is not without parallel in other phases of the life history. Changes during spermatogenesis leave the plastid of each sperm cell a terminally simplified organelle (Paolillo *et al.* 1968a, 1968b). But the simplification of the plastid during sporogenesis is not terminal. The initial reduction in complexity is followed by a morphological steady state until meiosis. Then a greater simplification of the plastid occurs, and the spore starts to build its wall layers and to accumulate its food reserve. Through this period, the sporogenous cells are suspended in a spore sac that is lined with a layer of putatively secretory tissue, backed by a layer of photosynthetic tissue (Paolillo 1964b).

The decline in chlorophyll fluorescence from archesporial plastid to spore plastid correlates with the simplification of structure. It also supports the notion that the plastid is being shut down, photosynthetically. New plastid materials are probably synthesized as the total number of plastids increases in the sporogenous tissue. Apparently, this synthesis is not devoted to maintaining the internal complexity of the plastid, nor its photosynthetic capacity. The situation invites the speculation that the plastid has returned to a condition conducive to the controlled rate of division that is correlated with nuclear and cell division. But simplification of plastids is not a necessary feature of sporogenesis in the bryophytes. It is already known that sporogenous

cells in *Riccardia*, a liverwort, contain typical chloroplasts throughout their development (Horner *et al.* 1966).

Control of plastid division is altered just prior to meiosis to give four rather than two plastids before the final nuclear division, but other conditions must change at this time as well. For example, the plastid of the newly formed spore appears more like a senescent plastid rather than a proplastid, because it resembles the terminally simplified plastids of the *Polytrichum* sperm (Paolillo *et al.* 1968b), rather than the archesporial plastids or plastids of the young spermatogenous cell. When it is again "turned on", the plastid of the spore does not rejuvenate to become like the plastids of the multiplying archesporial cells. Instead, it forms a prolamellar body, like an etiolated plastid, and then grana. Division occurs in a plastid of increasing complexity, or in a typical chloroplast. The chloroplasts divide in the germinating spore and the protonema (Reighard 1967), where there are again numerous plastids per cell. The fact that the chloroplasts of the spore contain starch may indicate that the spores begin vegetative activity before they are shed (see also Valanne 1966).

During its simplification and subsequent return to the vegetative state, the plastid assumes certain forms that are associated with abnormal development in the plastids of higher plants. Specifically, the 100 Å spacing of stacked lamellae prior to meiosis resembles the condition of xantha mutants in barley, and the prolamellar body found after meiosis resembles what is found in etiolated plastids (von Wettstein 1960, 1961). Shortly after meiosis, the plastids of the spores resemble the plastids of albino tissues. The point must be made here that the occurrence of the "abnormalities" is a routine part of the developmental cycle in the *Polytrichum* plastid. In this regard, there is a strong resemblance to what is found in certain virescent mutants of corn (Paolillo and Reighard 1968) where abnormal plastids are converted to normal ones as the seedling ages. A better grasp of these developmental phenomena in higher plants may shed some light on what is taking place as the life history of the moss unrolls.

The continuity of the plastids through spore formation in *Polytrichum* is relatively certain. There is no evidence for degeneration of plastids. Nor is there evidence for blebbing of the nucleus to form organelles, as may be suggested for higher plants (Heslop-Harrison 1964). On the other hand it must be admitted that when the plastid is polymorphic, some profiles that are indistinguishable from mitochondria could be sectional views of the lobes of the plastid. The question of whether such lobes can be freed to form independent organelles must remain open, but it seems more likely that there is a simple clustering of organelles in the sporocytes.

The materials utilized in this study were collected in three successive growing seasons and cultured under a variety of conditions, and all of the observations made relate to a general pattern of development. With regard

to the materials of *P. commune* that were studied earlier (Paolillo 1964b), the occurrence of stacked, widely spaced lamellae in the sporocyte plastids and of prolamellar bodies (plastid centers) in the spore plastids is confirmed. But the present materials showed no stacked, widely spaced lamellae in the spore plastids, whereas this feature was found in the earlier investigation (Paolillo 1964b). This difference could be related as much to individual physiological variability as it is to species differences. A scatter method of collection, with heavy reliance on field and greenhouse plants is relatively ineffective in deciding this type of problem because of the great number of events in the developmental sequence. It is unlikely that such sampling can provide the required collections for a more precise study, especially if a time scale is to be imposed on the developmental sequence, and if the control mechanisms are to be elucidated. For example, the failure of the experimental regimes to alter plastid structure is probably due to inappropriate timing of the start and duration of the experiments with relation to the stage of development of the capsule. In this regard, the successful attempts to mature sporophytes on agar with simple culture techniques offers great promise. Such cultured sporophytes can be collected at ideal intervals, hopefully determined by external morphology. There is also the opportunity for more precise and accurate control of the physical and chemical environment of the sporophyte than can be obtained with the gametophore attached.

It must be noted that the sporophytes that grew to maturity on agar showed differential green banding at the capsular end as well as red coloration of the seta. However, the calyptras were still firmly attached and no inflation of the capsules had taken place. Future efforts will include an attempt to culture younger sporophytes, and the spore sac and sporocytes apart from the intact capsule.

Summary

As the cells of the archesporium multiply, the plastids flatten and become internally simplified, and no longer contain conspicuous grana. The plastids in the sporocyte round up with the onset of meiosis. The single plastid in each spore is highly simplified and nearly spherical. After the exine of the spore is elaborated, the plastid becomes polymorphic, forms one or more prolamellar bodies and begins to rebuild the grana-fretwork system. Typical chloroplasts are developed before the spores are shed.

During the early phases of development of the archesporium, its plastids fluoresce a brilliant red in blue light. This chlorophyll fluorescence dims by the time sporocytes are developed, and is virtually undetectable in the newly formed spores. No increase in chlorophyll fluorescence is noted until after the elaboration of the yellow-fluorescing exine. These observations indicate a shut-down in vegetative plastid activities during sporogenesis, and a return to the vegetative state before the spores are shed. The continuity of the plastids

through sporogenesis is virtually certain.

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