THE DEVELOPMENT AND STRUCTURE OF PYRENIOIDS IN BULBOCHAETE HILOENSIS

B. RETALLACK AND R. D. BUTLER
Department of Botany, University of Manchester, Manchester 13, England

SUMMARY

Chloroplasts in zoospore mother cells, zoospores, and sporelings of Bulbochaete are shown to possess spherical granular areas which are interpreted as incipient pyrenoids, which arise de novo within the stroma. Very young pyrenoids are often seen adjacent to pale, fibril-containing areas of the chloroplast. Later, the incipient pyrenoids are often associated with a single thylakoid and become invested with starch grains and cytoplasmic channels as they develop into forms morphologically indistinguishable from the mature pyrenoids of the vegetative cell. In young oogonia, degenerating pyrenoids are seen to possess much starch. Infrequently, the matrix of a degenerating pyrenoid is invaded by the stroma, its dimensions decreasing to those of an incipient pyrenoid. The appearance of young pyrenoid-like structures within lateral outgrowths of the chloroplasts of young oogonia and sporelings may represent a further method of pyrenoid formation.

INTRODUCTION

The pyrenoids of algal cells may arise by a number of means. In some instances, mature pyrenoids may divide within a cell, giving rise to 2 or more daughters (Manton, 1966), whilst in certain brown algae new pyrenoids arise as lateral outgrowths of the chloroplast in the region of old pyrenoids (Evans, 1966). A de novo formation of pyrenoids is known to occur in Tetracystis excentrica (Arnott & Brown, 1966) and Oedogonium cardiacum (Hoffman, 1968a, b). In the latter species, Hoffman has shown that the zoospores may possess several incipient pyrenoids which lack the associated starch grains and membrane-bounded channels which are typical of the mature pyrenoids.

This paper deals with pyrenoid development and structure in Bulbochaete hiloensis, of the family Oedogoniaceae, as revealed through an electron-microscope investigation of zoospore and gamete development. Evidence for a de novo formation of pyrenoids in a manner similar to that in Oe. cardiacum is given, and a number of additional distinctive features not previously reported are described.

MATERIALS AND METHODS

Cultures, obtained from the Culture Collection of Algae at Indiana University, were maintained in a biphasic, soil-water medium (without additional CaCO₃), at 20 °C under a 16:8 h light-dark cycle illuminated by fluorescent warm white tubes at about 4300 lx (400 ft candles). Zoospore formation was induced by subjecting vegetative filaments to the appropriate
temperature and illumination changes as previously described (Trerice-Retallack & von Maltzahn, 1968).

For electron microscopy, filaments or zoospore suspensions were fixed for 2–3 h at 0–4 °C in 6% glutaraldehyde in phosphate buffer at pH 7.5 and post-fixed in buffered 2% OsO₄. Material was dehydrated through an ethanol series and embedded via propylene oxide in either Epon 812 (Luft, 1961) or a mixture of Araldite and Epon 812 (Mollenhauer, 1964). Sections were cut with glass knives, mounted on uncoated grids, stained with 2% aqueous uranyl acetate for 5 min followed by either 5 min in lead citrate prepared according to Reynolds (1963) or 20 s in lead citrate prepared according to Venable & Coggeshall (1965) and examined with an AEI EM6B electron microscope.

**OBSERVATIONS**

Uninduced, vegetative material contains several pyrenoids per cell, one of which is usually closely associated with the nucleus. The mature pyrenoids are roughly spherical, with an electron-dense matrix surrounded by starch grains. The matrix is penetrated by channels whose contents resemble the general cytoplasm. These cytoplasmic channels are bounded by an envelope continuous with that of the chloroplast (Fig. 1). The pyrenoid matrix usually has a granular appearance but it may occasionally assume a lattice-like character in which the basic component consists of planes of parallel lines with a 6–7 nm centre-to-centre spacing. In some areas, layers of such arrays cross each other at an angle of approximately 70° (Fig. 2).

Induced material shows numerous stromal areas central to the chloroplast which have an electron-dense and granular appearance like that of the pyrenoid matrix. From serial sections, it is evident that these areas are not connected to any other structure and they appear to arise *de novo*. That these structures are indeed incipient pyrenoids is supported by the fact that an obvious series of intermediate forms culminating in the mature pyrenoid may be observed, particularly in the later stages of zoospore development (Figs. 3, 5–11). The incipient pyrenoids which coexist with but are independent of mature pyrenoids (Fig. 3) may first be observed approximately 12 h after the beginning of the induction treatment. At this stage, the young pyrenoids are spherical, approximately 25–40 nm in diameter and consist of granular aggregations, each with rather greater electron density than the surrounding stroma. Frequently, these young pyrenoids are located near a relatively electron-transparent area of the stroma which is itself traversed by fibrils measuring 2–3 nm in thickness (Figs. 4, 5). These fibrillar areas have not been found in association with older pyrenoids. Also evident are larger subspherical granular aggregates, up to 80 nm in diameter, which are interpreted as older pyrenoids.

In the early stages of development, the developing pyrenoid is, in some instances, partially bordered by one or a pair of membranes which are continuous with, but divergent from, the regular thylakoid system of the chloroplast (Fig. 5). In a few sections, microtubules are also observed in the vicinity of an incipient pyrenoid but this is not a regular feature. In some sections, an association of the incipient pyrenoid with starch is evident. This may be seen in both the younger and older forms (Figs. 6, 7, 9–11). Invariably, these starch grains are found intercalated between the divergent thylakoid and the pyrenoid matrix itself. At this stage, or just before, the matrix
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contains profiles of various shapes (Fig. 6) which are bounded by a double-membrane envelope continuous with that of the chloroplast, and a direct connexion between these profiles and the cytoplasmic ground plasm may be demonstrated (Fig. 8). It becomes apparent that the pyrenoid matrix is gradually being penetrated by a system of cytoplasmic channels.

In progressively older pyrenoids, the accumulation of matrix-associated starch and the degree of cytoplasmic channelling become more pronounced (Figs. 9–11), culminating in a mature pyrenoid which may be associated with the nucleus and is indistinguishable from that present in the vegetative cell, as already described. At this stage, it may be determined from serial sections that the matrix bordering the cytoplasmic channels can exhibit an electron density considerably greater than the more deep-seated areas (Figs. 10, 11).

Observations of sexual material suggests that during oogenesis the mature pyrenoid undergoes some form of degeneration. In a young oogonium, the pyrenoid matrix becomes markedly more dominated by starch (Figs. 12, 13), the matrix finally recessing to a state morphologically reminiscent of that in the young pyrenoid. Simultaneously, the matrix also becomes invested with a material similar in electron density to the chloroplast stroma (Figs. 13, 14). These processes ultimately result in an almost complete obliteration of the pyrenoid matrix (Fig. 14). In conjunction with these degenerative sequences, young oogonia are also seen to develop stromal areas which have an electron density and granularity similar to that of incipient pyrenoids but which are enclosed by a lateral outgrowth of the chloroplast envelope (Fig. 14). Examination of serial sections confirms that these structures are separate from the mature pyrenoid, and are also evident in sporelings, where they may be associated with small starch grains (Fig. 15).

DISCUSSION

An electron-microscope study of zoosporogenesis and gametogenesis in B. hiloensis has shown that pyrenoids may arise de novo in the chloroplast stroma and that these incipient pyrenoids may then develop into mature pyrenoids by the association of starch grains and the development of cytoplasmic channels within the matrix. The situation is analogous, in some respects, to that in Oe. cardiacum, the zoospores of which are also endowed with numerous, incipient pyrenoids similar in structure to those in B. hiloensis (Hoffman, 1968a). In Oedogonium, however, unlike Bulbochaete, the incipient pyrenoids of the zoospore at no stage include cytoplasmic channels and Hoffman suggests that they probably develop during the germling stage. It would appear that in Bulbochaete, as in Oedogonium, the comparatively large numbers of pyrenoids in various stages of development in both zoospores and sporelings provide the newly formed germlings with a ready source of pyrenoids to be distributed amongst the daughter cells.

In view of the fact that the pyrenoid matrix has a high protein content (Smith, 1955), the period of growth of the incipient pyrenoid marks a period of intense synthesis and/or accumulation of protein within the chloroplast. It is of interest, there-
fore, that young incipient pyrenoids are found to be in association with areas of low electron density, traversed by 2–3 nm fibrils. Similar areas have been observed in the chloroplasts of *Egregia menziesii* (Bisalputra & Bisalputra, 1967a) and *Laurencia spectabilis* (Bisalputra & Bisalputra, 1967b) and have been found to contain DNA. In other species, DNA has also been located adjacent to pyrenoids (Ris & Plaut, 1962; Steffenson & Sheridan, 1965). One may speculate, therefore, whether these areas of low electron density in *B. hiloensis* also contain DNA fibrils, and if their association with incipient pyrenoids represents the functional association of chloroplast DNA with *de novo* formation of pyrenoids, as previously suggested by other workers (Brown, 1967; Hoffman, 1968a).

The early association of the diverging thylakoid and the pyrenoid matrix has not been observed in other species. In *Bulbochaete*, however, this represents a regular feature, even though it may seemingly be absent in a few instances due to the plane of sectioning. Its role, like that of the areas of low electron density, may be functional. It may well act, in conjunction with the matrix, as a source of substrate and/or enzymes resulting in the observed later association with a starch grain. It is also possible, of course, that this thylakoid may, in addition, have some purely structural significance, perhaps physically influencing the position of the pyrenoid.

The formation of the cytoplasmic channels is a further point of interest. This exceptional feature has already been identified in mature pyrenoids of *Platymonas* (Manton & Parke, 1965) and *Oe. cardiacum* (Hoffman, 1968a). In no instance, however, has their actual development been observed. Hoffman, from his work on *Oedogonium*, suggests that, as the pyrenoid expands centrifugally and unequally, there is a passive invasion of the matrix by the cytoplasm, thus forming the cytoplasmic channels. This interpretation may explain the sequence of events we have observed in *B. hiloensis*, although the early expansion of the pyrenoid appears to be remarkably regular (Figs. 6, 8). Furthermore, in our material it should be noted that the young pyrenoids are usually situated in the inner regions of the stroma and are rarely found adjacent to the chloroplast envelope, whatever the plane of sectioning. Consequently, the alternative idea of an active invagination of the chloroplast envelope to form these cytoplasmic channels seems equally attractive and some of our micrographs (Fig. 8) may certainly be interpreted along these lines.

The function of these cytoplasmic channels has yet to be determined. In *Chrysochromulina*, it is held that metabolic products from the chloroplast enter the cytoplasm via the pyrenoid surface (Manton, 1966). If this is so in *Bulbochaete*, and *Oedogonium*, the additional surface area of the channels would bestow obvious advantages. It is interesting that in *Bulbochaete*, the matrix bordering the channels may show a considerably greater electron density than the more deep-seated regions. These denser areas may perhaps correspond to areas with a high level of metabolite accumulation and/or synthesis and it may therefore be that the adjacent cytoplasmic channels are indeed functionally operative in metabolite transfer.

The significance of the lattice-like substructure apparent in some pyrenoids is uncertain. This appearance, and the angle of intersection, are reminiscent of the situation in crystal-containing bodies (Thornton & Thimann, 1964; Cronshaw,
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1964) and microbodies (Frederick, Newcomb, Vigil & Wergin, 1968) in higher plants. In the diatom, *Achnanthes brevipes* (Holdsworth, 1968), a crystalline matrix of similar dimensions to those of *Bulbochaete* has been observed. This feature has to date not been reported in any other non-diatomaceous algae and it is too early to say whether this will prove to be a regular feature of algal pyrenoids.

The oogonial pyrenoid is particularly interesting. It is known that oogonia show an increase in storage products. That this should happen to the exclusion of the matrix may expand support for the role of the pyrenoid matrix in carbohydrate synthesis and/or polymerization (Bouck, 1965). The increase and accumulation of pyrenoidal starch may thus represent a functional decrease of the pyrenoid matrix. Indeed, the matrix has been restricted to the dimensions of an incipient pyrenoid.

The existence of the incipient pyrenoid-like structures within peripheral outgrowths of the chloroplast of the young oogonia arouses speculation, for their association with starch in sporelings suggests that they may be functional. Similar bulging pyrenoids occur in some euglenoids (Leedale, 1967), in some brown algae, where new pyrenoids develop in lateral buds from the base of the original form (Evans, 1966), and in *Chrysochromulina*, where the lateral pyrenoids undergo division (Manton, 1966). In *Bulbochaete*, however, these lateral outgrowths are not associated with mature pyrenoids and appear to arise de novo. It is tempting to envisage that this is another possible pathway for pyrenoid development.

One of us (B. R.) wishes to acknowledge receipt of a Commonwealth Scholarship.

REFERENCES


BROWN, R. M. (1967). The pyrenoid; its structure, distribution, and function. *J. Phycol. 3* (Suppl.), 5-7 (Abstr.).


(Received 24 April 1969)

**ABBREVIATIONS ON PLATES**

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<tr>
<td>ci</td>
<td>cytoplasmic invasion</td>
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<tr>
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<td>lo</td>
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Fig. 1. Mature pyrenoid from uninduced material, showing a granular matrix, starch and cytoplasmic invasions. Inset shows double-membraned envelope of cytoplasmic invasion.

Fig. 2. Portion of a mature pyrenoid from uninduced material showing a crystalline matrix. Note layers of parallel lines crossing each other at an angle of 70° (arrowed).

Fig. 3. Mature pyrenoid near nucleus in a zoospore. Note incipient pyrenoid nearby.
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Fig. 4. Pale fibril-containing area in the central stroma of a zoospore chloroplast.

Fig. 5. A zoospore incipient pyrenoid bounded by diverging thylakoid and bordered by one of the pale fibril-containing areas.

Fig. 6. Developing pyrenoid in a zoospore. Note associated starch grain bounded by a thylakoid, and a cytoplasmic invagination.

Fig. 7. Developing pyrenoid in a sporeling, showing associated starch grains and profiles of cytoplasmic invaginations.
Fig. 8. Developing pyrenoid in a sporeling, showing profiles of cytoplasmic invaginations. Note the finger-like invasion of the pyrenoid matrix by the channel filled with ground plasm and bounded by a double envelope continuous with that of the chloroplast.

Fig. 9. Developing pyrenoid in a zoospore showing cytoplasmic channels and starch grains.

Fig. 10. A zoospore pyrenoid at a later stage than seen in Fig. 9. Note dense areas within the matrix.

Fig. 11. Almost mature pyrenoid in a zoospore, showing starch, cytoplasmic channels and dense areas of the matrix.
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Figures 3, 9, 10, 11 show various structures labeled with 'ci', 'dp', 'n', 'm', and 's'. The images depict microscopic views, possibly of algal cells, with annotations indicating different parts of the cells.
Fig. 12. Pyrenoid of a young oogonium showing marked starch accumulation together with cytoplasmic and stromal invaginations of the matrix.

Fig. 13. Pyrenoid in oogonium at later stage than that shown in Fig. 12. Note increase in stroma invagination and morphological decrease of pyrenoid matrix.

Fig. 14. Degenerating pyrenoid in an old oogonium. Note the lateral outgrowth of the chloroplast envelope which encloses an area of electron granularity and density similar to that of incipient pyrenoids.

Fig. 15. Sporeling chloroplast showing lateral outgrowth possessing matrix with an associated starch grain.
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