Cytological Studies in the Protococcales.

I. Zoospore Formation in *Characium Sieboldii*, A. Br.

BY

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With Plate XI and two Figures in the Text.

INTRODUCTION.

THE first account of reproduction in *Characium* is that of Braun (1) in which the process is described for several species. *Characium acuminatum* (A. Br.), De Toni, he says, has a cell structure of a pale green cytoplasm and a single pyrenoid which is located in the middle of the cell. Reproduction takes place by the gradual disappearance of the pyrenoid and a cytoplasmic division into irregular polygons. These pieces then separate, become rounded up, and elongate to form 80-150 zoospores, which are liberated by a lateral rupture of the mother-cell wall and remain in motion 2-3 hours, then affix themselves to the substratum, elongate, and become pointed. In *C. Sieboldii*, A. Br., division may take place before the cell is fully grown, the cell contents dividing transversely. There may also be a median longitudinal division of the cytoplasmic contents. Each division of the cytoplasm is preceded by a division of the pyrenoid so that every cytoplasmic fragment contains 1-4 pyrenoids. The cytoplasmic parts which have been formed by these repeated divisions are the zoospores; they vary somewhat in size and shape. The smallest number formed is 8, most frequently 16, but there may be 30-60. Microzoospores are also formed.

West (11) states that there is a repeated division of the cell contents, transverse divisions occurring before the longitudinal ones. In a short time each portion loses its angular character, becomes rounded up, and forms a biciliate zoospore. In some species the pyrenoid disappears during the formation of the zoospores and reappears upon their germination.

Lambert (5) has described the reproduction of two forms that are parasitic on Crustaceans. The chromatophore of *Characium gracilipes*, Lamb., is single, parietal, and situated in the convex side of the cell, while the pyrenoid is always a small hyaline body separated from the chlorophyll-bearing portion of the cell. The elongation of the pyrenoid is the first evidence of reproduction. It assumes a dumb-bell shape and finally separates

into two parts, each of which rounds up like the original pyrenoid. This pyrenoid division is followed by a division of the chromatophore into two parts. Repeated transverse divisions in the protoplasm produce a single row of 32 cells within the old mother-cell wall. This is followed by the longitudinal division of 30 of these cells, the two terminal cells again dividing transversely, thus forming 64 cells in all. Longitudinal division may occur, however, after 4, 8, or 16 cells have been formed by transverse division, but there is always a longitudinal cleavage of the cells at the end of the series of transverse cleavages. Lambert finds in *C. cylindricum*, Lamb., that there are two chromatophores containing oil drops, but no pyrenoids, and that the nucleus is located between the chromatophores. By repeated transverse divisions the contents of the cell form 8, 16, or 32 parts, and then there is a series of longitudinal divisions. Another type of spore-formation in which the contents of the mother-cell form 100–2,000 small zoospores is also found in this species.

MATERIAL AND METHODS.

The material for this investigation was collected from a small sluggish stream on the campus of the University of Wisconsin, where the alga was found growing abundantly on blades of grass lying in the water. The shape and dimensions of the cells agree very well with *Characium Sieboldii*, A. Br. (1). The blades of grass were cut into pieces about a half-centimetre long and then dropped directly into the fixing fluid. Several different fluids were tried, but Eisen's weak solution, prepared according to the formula of Timberlake (8), proved the best. After embedding in paraffin, the material was cut on a microtome into sections from 9 to 12 microns thick. Heidenhain's iron-alum-haematoxylin gave fairly good results, but Flemming's safranin-gentian violet-orange G combination proved much better. The best preparations, however, were obtained by substituting light green FS for the orange G in the Flemming's triple stain.

THE STRUCTURE OF THE CELL.

The chlorophyll in the species studied is not located in a definite chromatophore, but apparently evenly distributed throughout the cell. The cytoplasm is not visibly alveolate in structure at any stage of development, but has always a uniform granular structure.

There is no uniformity in either the shape or location of the pyrenoid. In certain cells the pyrenoid is spherical (Pl. XI, Figs. 3 and 6), in others variously lobed (Figs. 1, 2, and 9). Although the pyrenoid of the algae is generally reported as being spherical, Timberlake (10) has found that in cells of *Hydrodictyon* that are forming starch rapidly the pyrenoids are apt to be angular, while I have found the same thing in *Scenedesmus obliquus* (Turp.), Ktz., and *S. quadricauda* (Turp.), de Breb. (7). The pyrenoid of *Characium*

always has lobed projections and never angular ones as in *Hydrodictyon* and *Scenedesmus*. At times the lobes are deeply constricted and the pyrenoid dumb-bell shaped, or there may be several lateral projections, of various sizes, that are almost completely cut off from the main body. These irregular pyrenoids are the rule rather than the exception, a few variations in shape being shown in Text-fig. 1. Usually there is one large pyrenoid near the centre of the cell, but not infrequently other pyrenoids are found scattered about in the cytoplasm. These vary in size from those that are scarcely visible to those that are 10–12 microns in diameter. The smallest ones are generally spherical and some distance from the large irregular ones, a fact which suggests their origin *de novo* rather than a formation by a division of existing pyrenoids. In the development of the cell there is a single pyrenoid at first, but later on others may appear. If there is a division of the pyrenoid it is not into two equal parts. While I am not willing absolutely to deny that new pyrenoids in *Characium Sieboldii* are

TEXT-FIG. 1. Outline drawings of pyrenoids showing their irregular contour. ($\times 2,000$.)

formed by the division of an already existing pyrenoid, I am of the opinion that in a great majority of cases the pyrenoids are formed *de novo* within the cell.

Wille (12) states that the existence of starch in *Characium* is questionable. Fig. 12 shows the ring of starch plates usually found around a pyrenoid, but these differ from the starch plates in most other algae in that they are very much thinner. Owing to the comparatively large size of the pyrenoid, many starch plates are seen when it is viewed in cross-section. In certain preparations these starch plates are found just inside the plasma membrane (Fig. 12). Similar starch plates in *Hydrodictyon* have been called 'stroma' starch by Klebs (4), but Timberlake (10) has shown that these 'stroma' starch plates in *Hydrodictyon* are of pyrenoidal origin. In *Hydrodictyon* the 'stroma' starch plates are found close to the pyrenoid starch plates, but in *Characium* they are always just inside the plasma membrane. The reason for this may possibly be that the plates are so light that surface tension causes them to migrate to the periphery in much the

same manner as Czapek (2) believes that the lipoids in the cell tend to migrate towards the surface film. An indication that surface tension governs the arrangement of these small plates is seen in the transverse cleavage of the cytoplasm, when after the new plasma membrane has been formed the starch plates are located along the middle of the cell, where there were none before. If surface tension is the factor governing the distribution of these starch plates it is easily seen that with the formation of the daughter-cells there is a change in the surface tension and a readjustment of the starch plates so that they are equally distributed over the surface of the new daughter-cells. On the other hand, if these plates were dragged in mechanically by the inward growth of the cleavage furrows from the plasma membrane, there would be an unequal distribution of the starch plates and a tendency for them to be more numerous on the edges of the newly formed plasma membrane near the mother-cell wall. Since, however, the starch plates are evenly distributed along the newly formed plasma membranes the assumption that surface tension is the controlling factor in their distribution does not appear unreasonable.

The pyrenoids appear absolutely homogeneous in structure, with no light and dark areas as Timberlake (10) has shown for *Hydrodictyon* and Lutman (6) for *Closterium*. I have been able to demonstrate this irregularity in structure for *Scenedesmus* (7) and *Pediastrum*, so I believe that the failure to find the light and dark areas is not a matter of faulty technique.

Mature cells of *Characium Sieboldii* are multinucleate. The number of nuclei varies, but is always (so far as I have observed it) a multiple of two, usually 32 or 64. The nuclei are distributed throughout the cytoplasm and are approximately equidistant from each other, varying in size from $\frac{3}{4}$ to $1\frac{1}{2}\mu$ in diameter. Usually the only structures noticeable in the nucleus are the single nucleolus and a sharply defined nuclear membrane (Fig. 1), but in some cases a few chromatin granules can be distinguished (Fig. 13). These granules are comparatively large, and occasionally are connected with one another by strands. The number of granules is approximately the same as the number of chromosomes, so that it is possible that the nucleus shown in Fig. 13 is not a resting nucleus, but is in the spireme stage.

Owing to the small size of the nuclei it is impossible to make out clearly the details of karyokinesis. Nuclear division in the Chlorophyceae frequently takes place at night, but I have found no regular time for the occurrence of karyokinesis in *Characium*, and division figures are as apt to be found in material fixed during the daytime as in that fixed at night. I have not been able to determine the early stages in the formation of the spindle, but when the equatorial plate stage is reached the spindle is sharply defined. Karyokinesis takes place simultaneously in all the nuclei of a cell, this being contrary to the condition found by Timberlake (9) in the coenocytic *Hydrodictyon*, where, although all the nuclei in a cell are dividing at the

same time, those at one end are not at the same stage of division as those at the other. The mass of chromosomes in the equatorial plate is always clearly recognizable, but the spindle fibres are not seen so easily. The number of chromosomes is about 10 or 12. The individual fibres of the spindle form a cone-shaped structure that ends in a small body (Figs. 15 and 16). In some division figures the small body at either end of the spindle can be seen even though the spindle itself is indistinguishable. This small body at the spindle pole was not observed excepting during the metaphases. The nature of this body is doubtful since there are no indications of polar radiations. In the telophase figures found, the spindle could not be seen, but the two groups of chromosomes were easily recognizable, forming deeply staining masses. The details of the reconstruction of the daughter-nuclei are also lacking, but there are frequently paired nuclei in the cell, indicating cases where nuclear division has just been completed.

FORMATION OF ZOOSPORES.

There is no definite stage in the development of the alga at which zoospore development takes place. Instances were not found, however, of the formation of zoospores in cells containing less than eight nuclei. The process of zoospore formation is that which has been called 'progressive cleavage' by Harper (3). The first indication may be a single transverse cleavage of the cytoplasm, or several transverse cleavages may take place at the same time. When there is a single cleavage plane formed it is not located in the middle of the cell but at either end (Fig. 2). The process of cleavage is a furrowing in, the furrow starting at the plasma membrane and gradually growing towards the centre. Cross-sections show furrows starting towards one another from opposite sides of the cell, but the furrows probably appear at first as a ring-shaped furrow in the plasma membrane and extending entirely around the cell. In some cases the protoplasmic masses formed by the cleavage furrows contain approximately the same number of nuclei (Fig. 3), while in others the number of nuclei is decidedly unequal (Fig. 2). The position of the pyrenoids remains unchanged during the formation of the first cleavage furrows, so that whether or not a protoplasmic segment will contain a pyrenoid when it is cut off is merely a matter of chance. Fragments may also be formed in which there are two pyrenoids (Fig. 3). The number of transverse cleavage planes formed varies, but the greater the number of nuclei in the cell the greater the number of transverse planes. After several transverse cleavage planes have been formed the cleavage furrows appear in the long axis of the cell. These do not extend from one end of the mother-cell wall to the other, but are formed between two transverse cleavage planes (Figs. 3 and 4), the new furrows appearing first in the plasma membranes of the central protoplasmic masses. Furrows generally begin at opposite sides and

grow towards the centre. These furrows are broadly V-shaped and not narrow as Harper has shown them for *Synchytrium* (3). That this broadening at the outer end is the normal condition, and not due to plasmolysis, is shown by the fact that the protoplasts are not separated from one another by spaces of any considerable width along the transverse cleavage planes. At other times the secondary furrows are not formed at right angles to the primary cleavage planes but are formed at varying angles (Fig. 4). With the completion of the formation of these furrows the protoplasm is divided into a number of irregular multinucleate masses with irregular outlines. At this time also there is considerable variation in the number of nuclei in each protoplasmic mass. The fact that there is no division of the pyrenoid accompanying the cytoplasmic cleavage is much more marked after the longitudinal cleavage furrows have been formed. The pyrenoids remain unchanged during the process and are still homogeneous in structure with the ring of starch plates showing quite plainly around each (Fig. 5). The further steps in the process consist in the formation of uninucleate masses of protoplasm. The cleavage furrows that cut out these uninucleate protoplasts may be formed parallel to the long axis of the mother-cell, although they are usually formed at varying angles to the already existing cleavage planes. When the process of cleavage has been completed the whole interior of the mother-cell wall is filled with angular uninucleate masses of protoplasm (Fig. 5). These daughter-cells contain no pyrenoids, the pyrenoids of the original mother-cell remaining between the uninucleate cells in certain cases, while in others no pyrenoids can be found. The fate of the pyrenoids of the mother-cell was not determined, but their disappearance certainly occurs after the process of cleavage is well advanced.

These small uninucleate cells now round up and become zoospores. Fig. 6 shows a condition in advance of that found in Fig. 5, the young cells having lost their angular shape to a certain extent, but the rounding-up process is not fully completed. This figure also shows that a pyrenoid reappears in the young cells before the rounding up has become completed. At first these new pyrenoids are very minute, red-staining bodies, when Flemming's triple stain is used, showing practically nothing of the hyaline area with which they are surrounded when the zoospore is ready for liberation. When the process of rounding up has been completed, the zoospores thus formed are ovoid or pyriform (Fig. 7), each having a pyrenoid at one end and a nucleus at the other. These two bodies are not always on the line of the long axis of the young zoospore as Fig. 17 shows. The relation of the cilia to the zoospore could not be determined from the preparations.

THE GROWTH OF THE YOUNG CELL.

Since stages in the liberation of the young zoospores were not observed the manner of their coming to rest could not be determined. From our

knowledge of other algae it would seem probable that they always come to rest with the cilia downward, and yet from the examination of the relationship between the nucleus and pyrenoid in uninucleate stages there is no definite relationship between the pyrenoid, nucleus, and base of the cell. Text-fig. 2 shows that the pyrenoid may be between the base of the cell and the nucleus, or the position of the two may be reversed, while at other times both pyrenoid and nucleus are side by side in the middle of the cell.

After the zoospore becomes attached the young cell grows considerably before becoming binucleate (Figs. 8 and 9), but after the cells have reached a certain size each is found to contain two or more nuclei, the position of which, in the binucleate cells, is quite variable, that shown in Fig. 9 being the most common. With the further growth of the cell there is a simultaneous mitotic division of the nuclei into four (Fig. 10), eight (Fig. 11)

TEXT-FIG. 2. Uninucleate cells showing that the relative position of nucleus and pyrenoid is not constant. ($\times 2,000$.)

sixteen, and so on until the maximum number of nuclei is reached. Division stages in the two- and four-nucleate cells were not found in great abundance, but in all cases found the nuclei were dividing simultaneously in each cell. In the youngest cells the pyrenoids are generally spherical, but in older cells various irregularities, that have been described above, are found. Irregularly shaped pyrenoids may be found as early as the binucleate stage, but they are not found in the majority of cells until the eight-nucleate stage has been reached. The fact that these pyrenoids are regular in the earliest stages may be considered a proof that irregularities found in the older cells are not artifacts due to the treatment during fixation and embedding.

SUMMARY.

Mature cells of *Characium Sieboldii* contain 32–64 nuclei and one or more irregularly shaped pyrenoids.

The process of zoospore formation is one of progressive cleavage, the first cleavage planes being transverse and the later ones longitudinal. The cleavage continues until angular uninucleate protoplasts are formed. The pyrenoid does not divide, but disappears during the process.

The angular protoplasts become zoospores by rounding up, forming pyrenoids and cilia.

After coming to rest the zoospore develops without cell division into the new plant, but the nuclei increase in number by simultaneous division as the cell enlarges.

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DESCRIPTION OF FIGURES ON PLATE XI.

Illustrating Mr. G. Morgan Smith's paper on *Zoospore Formation in Characium Sieboldii*.

All the figures were drawn with the aid of the Abbé camera lucida, the drawings being made at the level of the base of the microscope. Figs. 1 and 7 were drawn with the Leitz oil-immersion objective $\frac{1}{8}$ and ocular 4 (magnification about 2,000 $\times$); while in Figs. 2-6 and 8-12 the ocular 3 was used (magnification about 1,650 $\times$). Figs. 13-17 were drawn with the Zeiss 2 mm. objective, 1.40 N.A., and compens. oc. 18 (magnification about 3,800 $\times$).

- Fig. 1. Mature cell showing position of pyrenoids and nuclei.
- Fig. 2. Cell with simultaneous division of nuclei and the beginning of cleavage.
- Fig. 3. A stage at the beginning of longitudinal cleavage.
- Fig. 4. Showing the cleavage into several multinucleate masses.
- Fig. 5. After the cleavage into uninucleate masses.
- Fig. 6. Uninucleate masses partially rounded up to form zoospores.
- Fig. 7. Young zoospores nearly ready for liberation.
- Figs. 8-11. Stages in the development of the young colony.
- Fig. 12. A cell showing 'stroma' starch.
- Figs. 13-16. Stages in nuclear division.
- Fig. 17. Nearly mature zoospore.

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Smith, Gilbert Morgan. 1916. "Cytological studies in the Protococcales. I. Zoospore formation in *Characium sieboldii*, A. Br." *Annals of botany* 30, 459–466. <https://doi.org/10.1093/oxfordjournals.aob.a089615>.

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