CENTRIOLE REPLICATION

II. Sperm Formation in the Fern, *Marsilea*, and the Cycad, *Zamia*

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ABSTRACT

Sperm formation was studied in the fern, *Marsilea*, and the cycad, *Zamia*, with particular emphasis on the centrioles. In *Marsilea*, the mature sperm possesses over 100 flagella, the basal bodies of which have the typical cylindrical structure of centrioles. Earlier observations by light microscopy suggested that these centrioles arise by fragmentation of a body known as the blepharoplast. In the youngest spermatids the blepharoplast is a hollow sphere approximately 0.8 μ in diameter. Its wall consists of closely packed immature centrioles, or procentrioles. The procentrioles are short cylinders which progressively lengthen during differentiation of the spermatid. At the same time they migrate to the surface of the cell, where each of them puts out a flagellum. A blepharoplast is found at each pole of the spindle during the last antheridial mitosis, and two blepharoplasts are found in the cytoplasm before this mitosis. Blepharoplasts are also found in the preceding cell generation, but their ultimate origin is obscure. Before the last mitosis the blepharoplasts are solid, consisting of a cluster of radially arranged tubules which bear some structural similarity to centrioles. In *Zamia*, similar stages are found during sperm formation, although here the number of flagella on each sperm is close to 20,000 and the blepharoplast measures about 10 μ in diameter. These observations are discussed in relation to theories of centriole replication.

INTRODUCTION

Since the earliest studies of Van Beneden and Boveri (1, 2) the centriole has often been regarded as "a permanent and autonomous cell-organ that arises only by the division of a preexisting body of the same kind" (3). The details of the supposed division, however, were never clear from classical investigations. Indeed, the best evidence has long suggested that centriole replication by binary fission is unlikely and that a kind of budding process might be involved (4, 5). It has also been known for many years that centrioles can arise in cells which seem to lack preexisting centrioles. Chief among these examples are the cells which give rise to the flagellated sperms in various plants (algae, mosses, liverworts, ferns, cycads, and *Ginkgo*). Such "de novo" origin of centrioles is of considerable theoretical interest and quite early attracted the attention of cytologists (6–12). In particular, Sharp (11, 12) provided a carefully detailed account of the events in *Marsilea* and *Equisetum*. These cases and others demand a cautious appraisal of the theory of centriole

autonomy and suggest that centriole formation may be partially under the control of other parts of the cell.

With the increased resolution afforded by electron microscopy, it is now clear that the centriole has a complex morphology and that its replication probably involves several distinct steps. Bernhard and de Harven (13) were the first to show that immature centrioles are similar to but smaller than the fully developed centrioles with which they are associated. Such immature centrioles, or procentrioles, have since been seen in several different forms (14–17), and it is probable that they represent a common stage in centriole development. However, the macromolecular events leading to procentriole formation are completely obscure, and it is by no means proven that the procentriole is physically derived from a mature centriole.

The present study of sperm formation in the fern *Marsilea* and the cycad *Zamia* was begun in order to extend ultrastructural information to the classical cases of "de novo" centriole origin. The formation of multiflagellated sperms in these plants has turned out to parallel closely the development of multiflagellated sperms in the snail, *Viviparus* (14). The electron microscope has also revealed an unsuspected complexity in the structure known traditionally as the blepharoplast.

MATERIALS AND METHODS

Marsilea

Sporocarps of *Marsilea* are readily obtained from commercial sources; our material was purchased from the Carolina Biological Supply, Elon College, North Carolina.

The sporocarps can be stored indefinitely in the dry condition. To obtain developing gametophytes, one places a sporocarp in water after first nicking the heavy wall with a razor blade. The sporocarp imbibes water and extrudes a gelatinous, fingerlike projection several centimeters in length. Clusters of micro- and megaspores are attached to this projection. The complete development of the male gametophyte takes place within the microspore wall; mature sperms emerge after 10 to 12 hr and immediately swim to the female gametophytes, which have been developing simultaneously.

After many failures to obtain adequately preserved material, we discovered that fixation in itself poses no serious problems, but that the usual embedding plastics will not penetrate the intact microspore wall. The following simple procedure was finally adopted. Gametophytes were fixed for 2 hr in 1% OsO_4 buffered to pH 7.4 with veronal-acetate. After a brief rinsing in buffer, groups of gametophytes were placed on a microscope slide beneath a coverslip. They were subjected to light finger pressure accompanied by a rotary motion. The appropriate amount of grinding leaves the cells intact but cracks the microspore wall. There are, of course, some gametophytes which are badly damaged and still others which remain unaffected. Those with cracked walls become well infiltrated with plastic, while the remainder shrivel to an almost unrecognizable mass during subsequent steps. Groups of gametophytes were next embedded in 2% agar pellets, dehydrated through an acetone series, and embedded in Vestopol W. Individual gametophytes in the correct stage and orientation were found by searching 1-μ sections stained by the Giemsa technique (Fig. 2). The block was trimmed to include only the desired gametophyte before thin sectioning was carried out. Sections were picked up on Formvar-coated grids and stained by floating on the surface of 1% uranyl acetate. Micrographs were made at initial magnifications of 2,000 to 16,000 with an RCA EMU-3 microscope.

Zamia

Sperm formation in the cycads is a long process taking place within the pollen tube during the several months between pollination and fertilization. A time scale for development in the genus *Zamia*, found in Florida, is given in the monograph of Webber (10). We were particularly interested in the mature body cell, its division into two spermatids, and the immediately ensuing sperm formation. Female cones were collected during the months of May, June, and July on the Gainesville campus of the University of Florida. We are indebted to Dr. William Johnson and Dr. Frank Nordlie who kindly shipped us material in the summer of 1963. Individual seeds were removed from the cone and the nucellus was exposed by dissection. The positions of the pollen tubes are marked by brownish lines on the apex of the nucellus, but we had no success in dissecting the delicate pollen tubes from the living material. Therefore, the tip of the nucellus was excised and placed intact into the fixative. A double fixation technique was used, beginning with 1% OsO_4 in a 10% sucrose solution buffered to pH 7.4 with veronal acetate. After 3 hr, the material was rinsed in buffer and placed in 2% formaldehyde in 10% sucrose buffered to pH 7.4 with phosphate. After fixation for an additional 2 hr, followed by washing, the individual pollen tubes were removed, embedded in agar, dehydrated in an acetone series, and embedded in Vestopal W. The percentage of well fixed pollen tubes was very low. The more advanced stages came through better than the earlier ones, but in no case was the preservation entirely satisfactory.

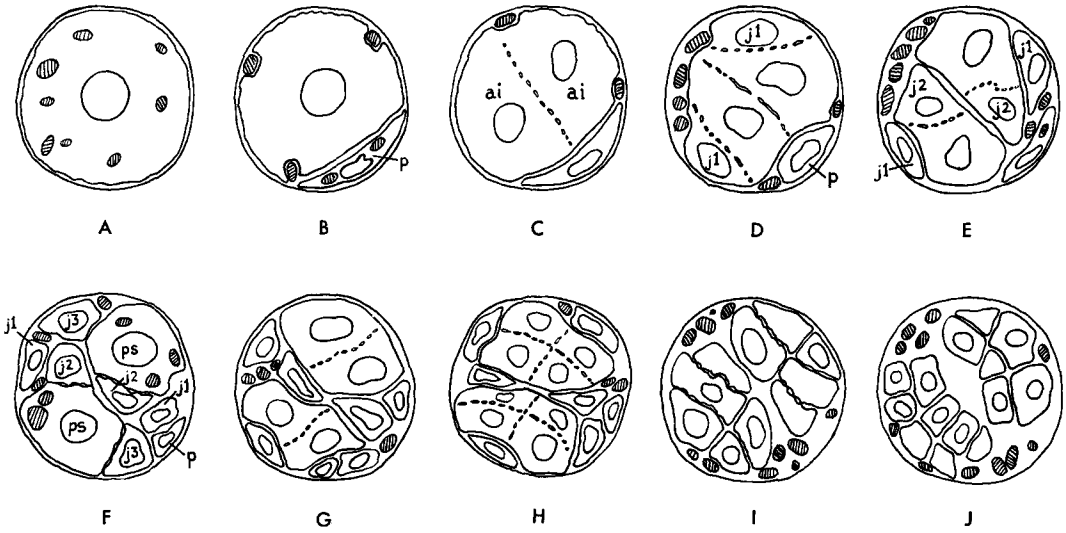


FIGURE 1 Scheme of microgametophyte development, in *Marsilea*, to the spermatid stage. The entire process depicted requires about 6 hr after the spores are placed in water. *A*, undivided microspore. *B*, The first division produces a prothallial cell (*p*) and an apical cell. *C*, Division of the apical cell yields two antheridial initials (*ai*). *D*, *E*, and *F*, Each antheridial initial cell divides three times to produce three jacket cells (*j*1, *j*2, and *j*3) and one primary spermatogenous cell (*ps*). *G*, *H*, *I*, and *J*, Each primary spermatogenous cell gives rise, through four successive mitoses, to 16 spermatids. Spermiogenesis follows the stage shown in *J* to give a total of 32 flagellated sperms still enclosed in the microspore wall.

OBSERVATIONS

DEVELOPMENT OF THE MICROGAMETOPHYTE: The entire gametophyte generation of *Marsilea* takes place within the microspore wall (Fig. 1). Development begins shortly after the microspores are exposed to water. By the end of 5 or 6 hr, 32 spermatids have been formed, which then undergo metamorphosis into multiflagellated sperms. By 10 to 12 hr, the spore wall cracks and free swimming sperms are observed in the water.

The first division of the microgametophyte generation is highly unequal, cutting off a small prothallial cell from the larger apical cell (Fig. 1 B). In the second division the apical cell gives rise to two antheridial initials (Fig. 1 C). In our material the plane of this division appears to be more or less perpendicular to that of the first, rather than parallel as in Sharp's Fig. 5 (12). Cytokinesis is incomplete and abundant cytoplasmic connections are observed between the two antheridial initials. The first jacket cell is cut off obliquely from each antheridial initial by the next division (Fig. 1 D). The jacket cells are sterile. The second and third jacket cells are formed by the succeeding two divisions (Figs. 1 E and F). Now there are seven sterile cells (one prothallial cell and six jacket cells) and two primary spermatogenous cells within the spore wall. Since there is one spermatogenous cell in each antheridium, this stage will be referred to as the one-cell stage. By this time, cytokinesis is complete and all cells appear to be independent from each other. The primary spermatogenous cells next undergo four divisions to produce sixteen spermatids in each antheridium. Through the two- and four-cell stages abundant cytoplasmic connections are observed between the spermatogenous cells, and all sterile cells can be identified (Figs. 1 G and H). The eight-cell stage is distinct in that the cytoplasmic connections disappear, and the sterile cells disintegrate (Fig. 1 I). Some asynchrony has been noted between the two antheridia, but it is almost always possible to identify the stage being studied, even in electron microscope sections. The transformation of the spermatid into the mature sperm involves complex changes in the nucleus and in the cytoplasmic components, details of which can be found in the accounts of Shaw (8), Belajeff (9), and Sharp (12).

The cytological events of particular interest here concern the formation of the so called blepharo-

plasts[1] and their transformation into the basal bodies of the mature sperm. Sometime during the four-cell stage two small spheres appear in the cytoplasm of each spermatogenous cell. The exact fate of these blepharoplasts is not clear, but a similar pair of spheres is seen in the cytoplasm of each spermatogenous cell in the eight-cell stage. These migrate to the poles of the spindle during the last division, thus playing the role of centrioles. The blepharoplasts at this stage are approximately 0.8 μ in diameter and are hollow, as clearly drawn by the earlier workers. After the last division, the blepharoplast breaks up into a cluster of centrioles, which line up near the nucleus. As the nucleus elongates and takes on its characteristic spiral structure (Figs. 3 to 5), the centrioles migrate to the surface where they become the basal bodies for the flagella of the mature sperm (Fig. 7).

THE BLEPHAROPLAST: With one exception noted later, we have found the first evidence of a blepharoplast when there are four cells in each antheridium. At this time we have observed two small blepharoplasts, approximately 0.3 μ in diameter, side by side in each spermatogenous cell. According to Belajeff (9) and Sharp (12), these blepharoplasts disintegrate during the third spermatogenous division and a pair of new blepharoplasts are formed at the poles. We have very little evidence concerning this point since we have studied only one gametophyte actually undergoing this division; in this we found blepharoplasts in the cytoplasm.

During the eight-cell stage, the blepharoplasts are conspicuous cytoplasmic components. Each spermatogenous cell contains two blepharoplasts, a fact which we have confirmed by serial sections through all the cells in a single gametophyte. These apparently lie near each other during early interphase, but later migrate to opposite sides of the nucleus (Fig. 6). At this stage, each blepharoplast is approximately 0.6 μ in diameter and consists of a closely packed aggregate of radially arranged tubules (Figs. 8 to 14). Sections cut tangential to the blepharoplast surface show a number of circular outlines, which are transverse sections of the tubules. Cuts near the center of the blepharoplast show longitudinal sections of the tubules. Most of the tubules are straight, although a slight waviness in outline is common. Some of the tubules run from the periphery to the center of the blepharoplast, while others may extend from one side to the other. However, it is structurally impossible for more than a small fraction of such

[1] The term *blepharoplast* ($\beta\lambda\epsilon\phi\alpha\rho\iota\varsigma$, eyelash or cilium; $\pi\lambda\alpha\sigma\tau\circ\varsigma$, formed) was introduced by Webber (10) to describe prominent cytoplasmic spheres found in the pollen tubes of *Zamia*, which are involved in the formation of the flagella. Although the term is sometimes used synonymously with basal body (e.g., Wilson, 3), the blepharoplast of plant cells is not one basal body but the precursor to many. In keeping with the majority of botanical writers (e.g., 18) we have found it useful to retain the original meaning.

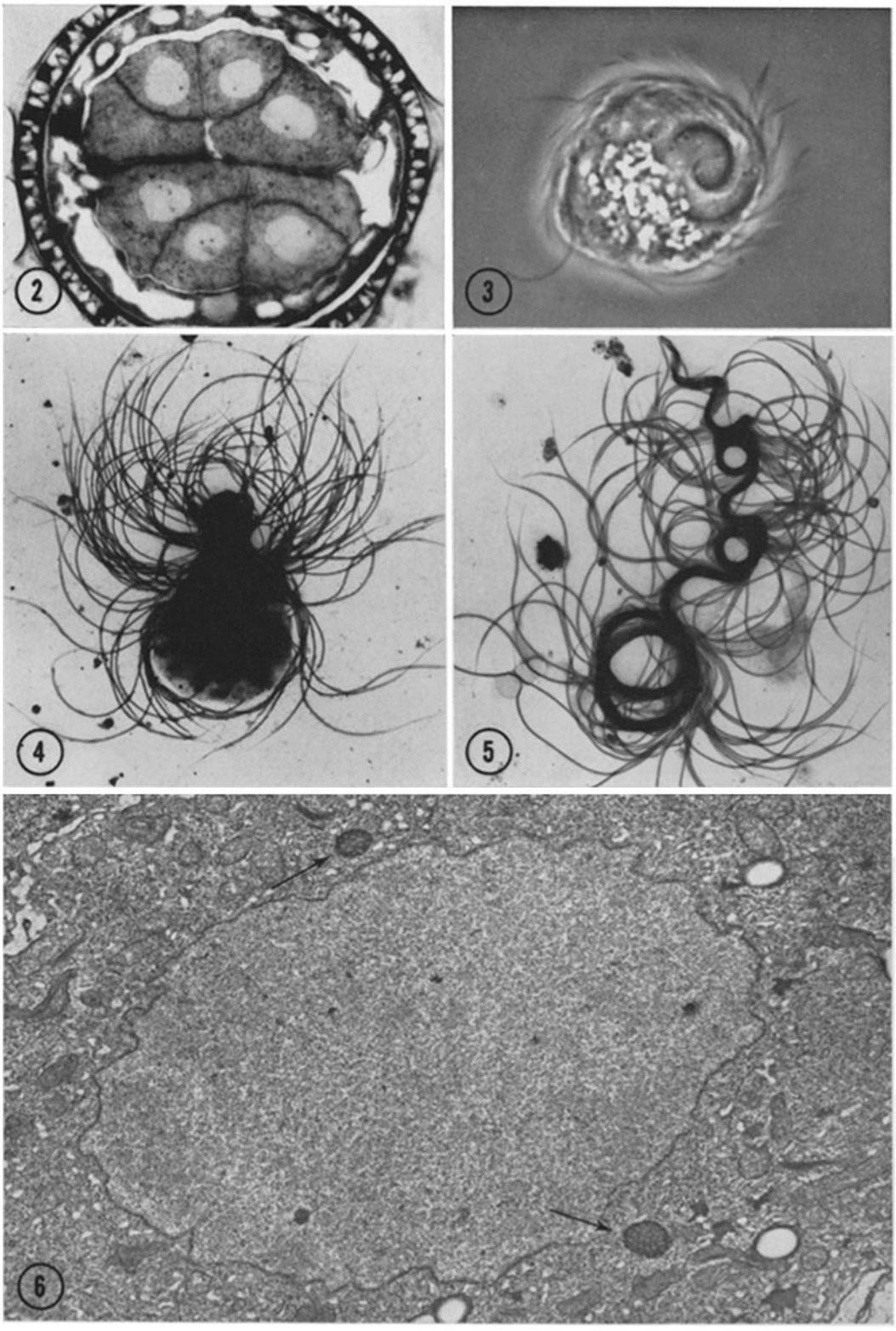
FIGURE 2 Section through the microgametophyte at the stage of eight cells per antheridium. The section includes parts of four cells in each antheridium (above and below the horizontal cleavage line). OsO_4-fixed section, 1 μ thick, stained with Giemsa. × 970.

FIGURE 3 Mature flagellated sperm after release from the microspore wall. The flagella originate from basal bodies aligned along the thin, spiralized nucleus. Fixed for a few seconds in OsO_4 vapor; phase contrast. × 1300.

FIGURE 4 A mature sperm similar to that in Fig. 3. Fixed for a few seconds in OsO_4 vapor, and dried on a Formvar film. Preparations of this sort were used for estimating the number of flagella. Electron micrograph. × 2500.

FIGURE 5 A sperm similar to the preceding except that the nucleus has detached from the cytoplasmic mass and is partially uncoiled. The same change is seen when the sperm penetrates the jelly surrounding the megagametophyte. × 2500.

FIGURE 6 Section through a spermatogenous cell at the eight-cell stage, showing the two blepharoplasts on opposite sides of the nucleus (arrows). Compare with Fig. 2, which illustrates the same stage by light microscopy. × 12,500.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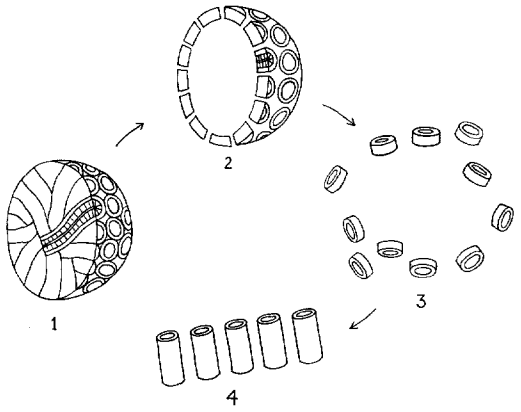


FIGURE 7 Scheme of stages in the transformation of solid blepharoplast into the basal bodies of the flagella. *1*, Solid blepharoplast as found before the last mitosis. *2*, Hollow blepharoplast consisting of radially arranged procentrioles, found during and immediately after the last mitosis. *3*, Stage of breakup of the blepharoplast in the early spermatid. *4*, Alignment of the procentrioles along the nuclear envelope is followed by elongation to form definitive centrioles. Each centriole then puts out a flagellum.

tightly packed tubules to extend to the center of a sphere, and consequently most of the tubules must be shorter than the radius of the blepharoplast. Although these tubules are not structurally identical to centrioles, they are clearly related. The tubules are about 0.09 to 0.11 μ in diameter, somewhat less than the diameter of typical mature centrioles of *Marsilea* and other organisms. Their walls are indistinctly divided into subunits. Although one cannot see the nine triplet fibers found in centrioles, there appear to be about nine subunits. However, the resolution so far obtained in our sections and the close packing of the tubules make a definite enumeration impossible. Transverse sections of the tubules display an indistinct "hub and spokes" arrangement, similar to that seen in the basal region of several kinds of centrioles (14, 15, 19, 20). The "hub" is a hollow fiber which extends the length of the tubule (Fig. 12). The "spokes" are projections from the hub to the periphery (Fig. 10).

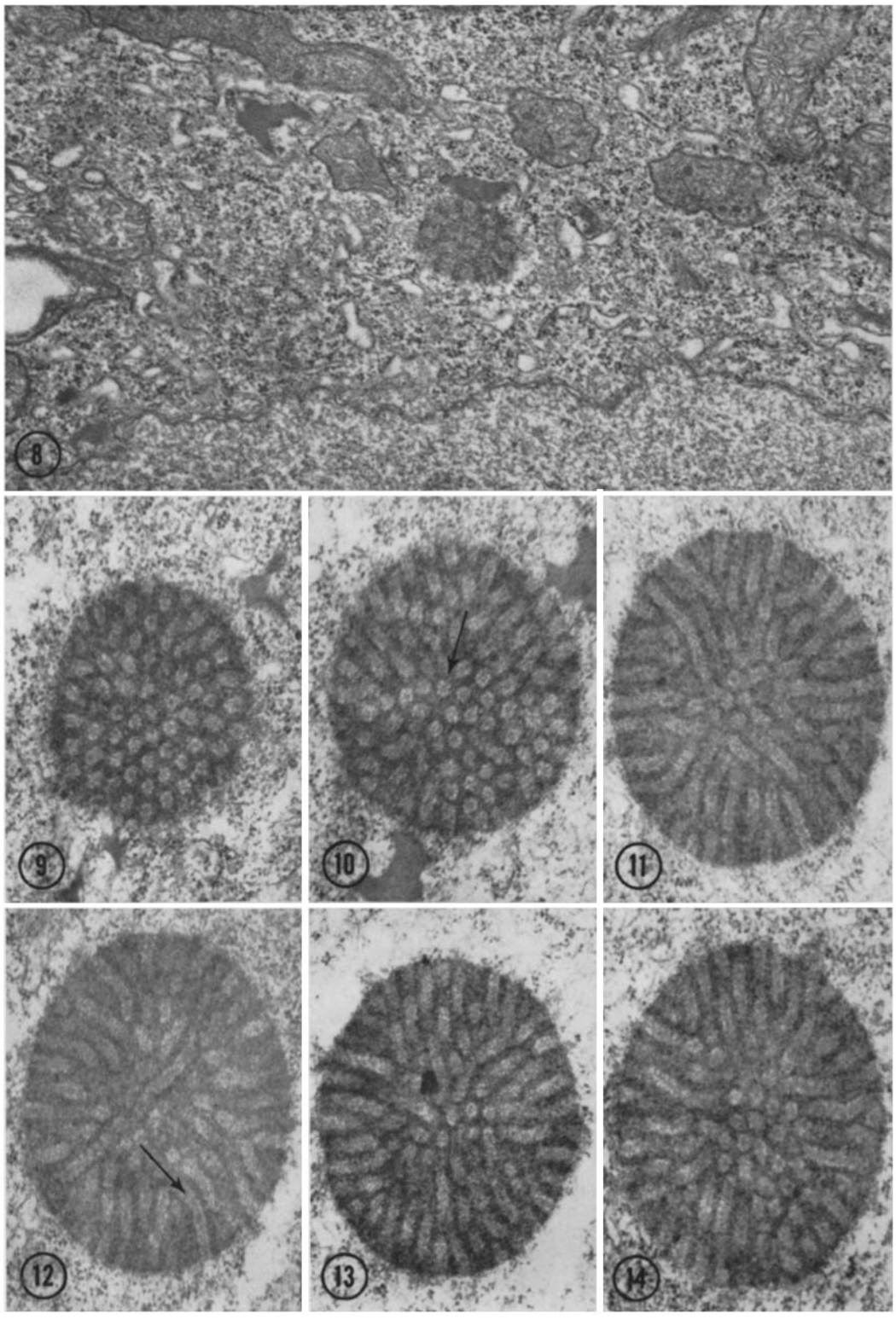
Since the tubules are packed very closely, and each extends perpendicularly to the surface, it is relatively easy to calculate the total number of tubules at the surface. For a blepharoplast of radius $R = 0.30$ μ consisting of tubules of radius $R_1 = 0.05$ μ, this number is about 130 $(4\pi R^2 (0.91)/\pi R_1^2$. The factor 0.91 is the fractional area occupied by close packed circles on a plane).

We have found a single exception to the outline given above. In a cell which was either an antheridial initial or at the two-cell stage, we found four blepharoplasts. The largest of these was about 1 μ in diameter and is illustrated in Figs. 9 to 14. We found no other cells with blepharoplasts at such an early stage.

THE LAST MITOSIS AND SPERMIOGENESIS: Sometime before metaphase of the final spermatogenous mitosis, a striking change takes place in the structure of the blepharoplast. In some manner, it is converted from a more or less solid sphere of tightly packed tubules into a hollow sphere whose surface is composed of annular or short cylindrical structures similar in cross-section to the earlier tubules (Figs. 7, 15 to 23). These cylinders are now quite clearly related to centrioles; they appear to be identical to the structures described as *procentrioles* in the snail, *Viviparus* (14). They are 0.10 to 0.11 μ in diameter but no more than 0.07 to 0.08 μ in length (Fig. 16). Their walls are divided into nine subunits whose arrangement is not so orderly as in mature centrioles. Each displays a conspicuous hub and spoke when cut transversely. These features are reasonably clear from direct inspection of the micrographs (Figs. 18, 19), but can be accentuated (Figs. 21 to 23) by use of the rotation technique described by

FIGURE 8 A blepharoplast situated near the nuclear envelope during interphase of the eight-cell stage. × 37,000.

FIGURES 9 to 14 Six sections through the same blepharoplast, proceeding from near the surface to slightly beyond the center (section numbers 1, 3, 8, 10, 12, and 13). The hub and spoke arrangement is suggested in several places where the tubules are cut transversely (arrow, Fig. 10). That the hub is a hollow fiber is shown well at the arrow in Fig. 12. This blepharoplast was exceptional in being found in a cell younger than the four-cell stage. × 45,000.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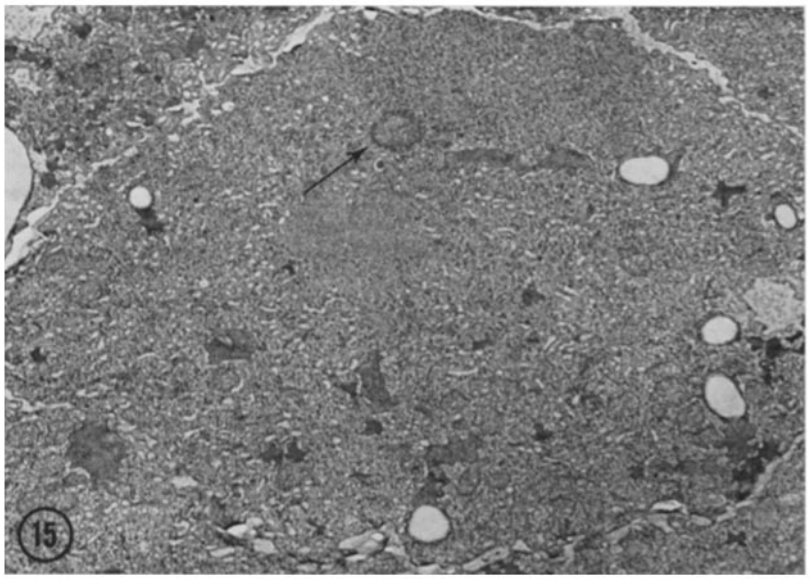
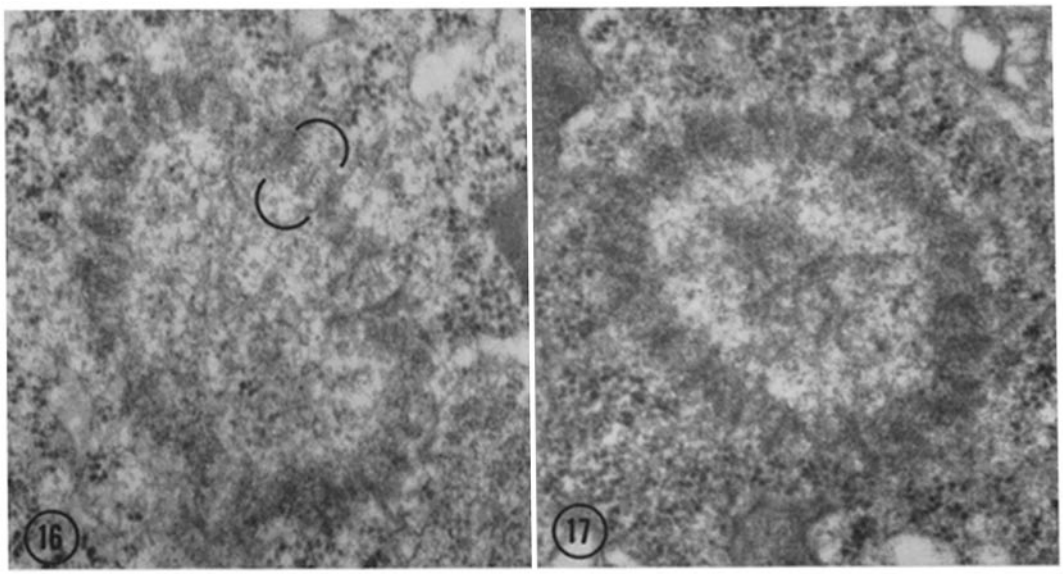


FIGURE 15 Section through a spermatid just after completion of the last mitosis. The hollow blepharoplast (arrow) lies at the spindle pole opposite the newly formed cell plate. × 8500.

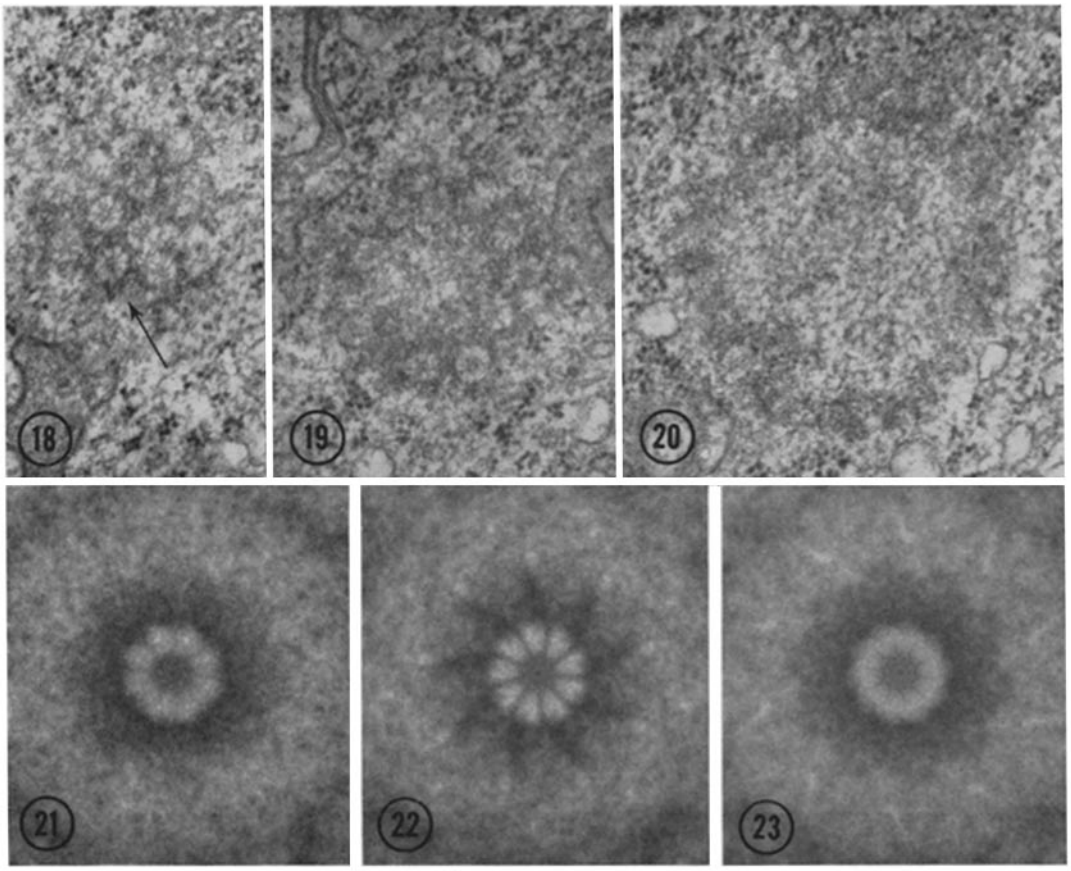


FIGURES 16 and 17 Median sections through two different blepharoplasts at late telophase or early interphase of the last mitosis. Each blepharoplast consists of somewhat over 100 procentrioles which form the wall of a hollow sphere. The axis of each procentriole is radial to the sphere. That the hub of the hub-and-spoke arrangement is a tubule running the length of the procentriole is shown well in the procentriole bracketed in Fig. 16. × 65,000.

Markham, Frey, and Hills (21). As in the case of the solid blepharoplast, the number of procentrioles constituting the hollow sphere can be estimated by dividing the surface area of the blepharoplast by the area of a single procentriole. Values for five clusters ranged from 110 to 150. The estimate is quite sensitive to the assumed average radius of a cluster and the radius of the procentri-

oles, since these values are squared. It seems probable that the number of procentrioles is equal to the number of tubules in the earlier solid blepharoplast. One can imagine the transformation of the solid into the hollow blepharoplast as a simple "digestion" of the interior of the sphere. The actual intermediate stages, however, have not been seen, nor is the significance of this change understood.

At the end of the last mitosis, each of the two antheridia contains 16 spermatids. These transform without further division into mature multiflagellated sperms. This transformation involves far-reaching alterations in nearly every cell component and has been the subject of much study in *Marsilea* and in other ferns and their allies. For purposes of this study, only the changes in the cluster of procentrioles will be described. Each spermatid contains one group of procentrioles which played the role of centrosome or centriole at the last mitosis. Shortly after reconstitution of the nucleus the hollow sphere begins to disintegrate into a loosely organized cluster of procentrioles (Fig. 24). Concomitantly, each procentriole starts to elongate and in traverse section shows a more regular arrangement of wall elements. Soon each procentriole has reached the condition of a typical animal or plant centriole, namely a cylinder



FIGURES 18 to 20 Three sections through the same blepharoplast. In Fig. 18, the procentrioles are cut almost transversely; that is, the section is tangential to the surface of the blepharoplast. Note the clear hub-and-spoke arrangement in the center of several procentrioles. Compare Fig. 7. × 63,000.

FIGURES 21 to 23 Rotation micrographs of the procentriole indicated by an arrow in Fig. 18. In each case, n successive prints were made after a rotation of $360°/n$; n = 8, 9, and 10 for Figs. 21, 22, and 23, respectively. Note the generally good reinforcement of both the wall elements and the spokes in Fig. 22. × 190,000.

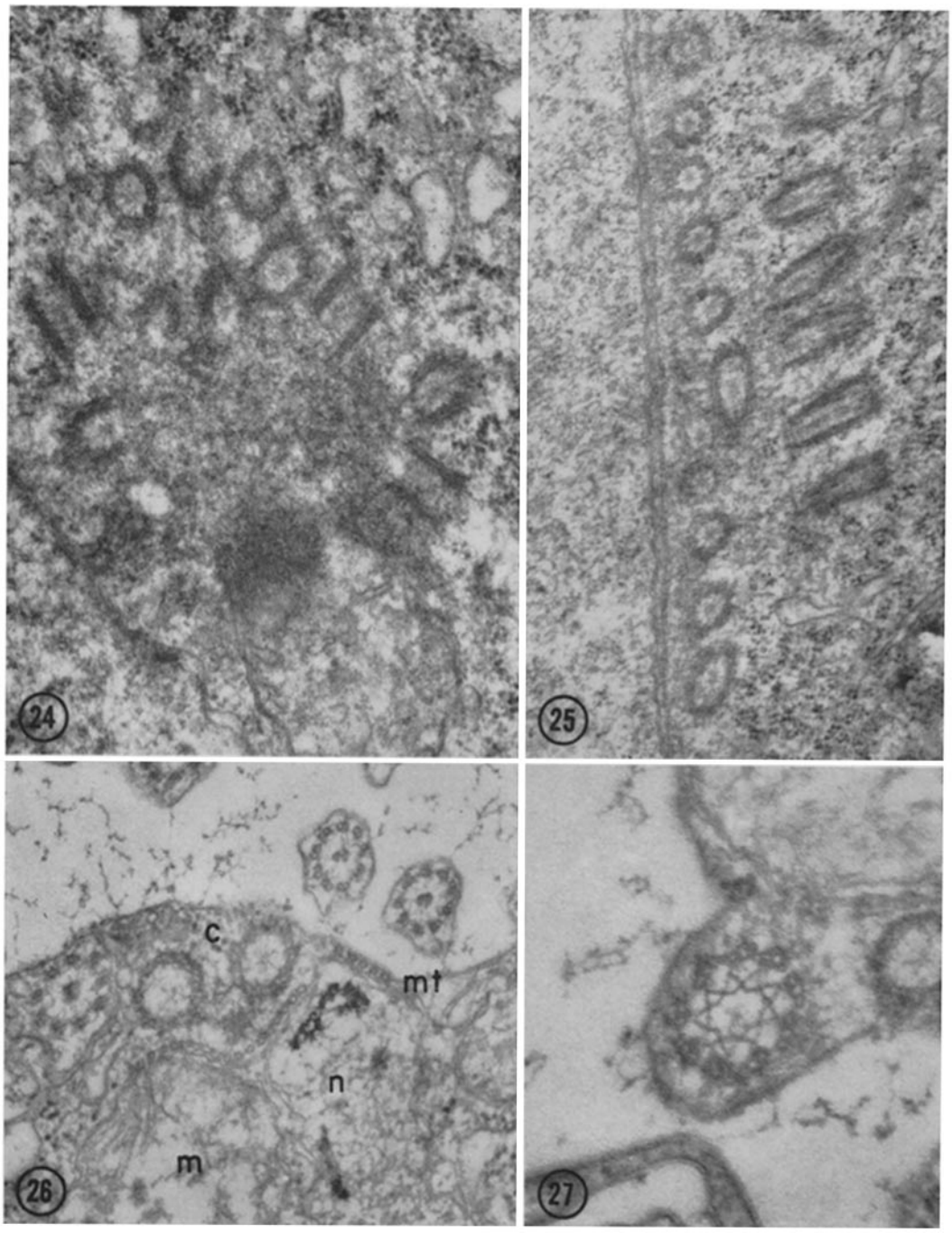


FIGURE 24 Breakup of the hollow blepharoplast shortly after the end of the last mitosis. As the individual procentrioles begin to elongate, their wall elements become more distinct. × 62,000.

FIGURE 25 Centrioles lined up along the nuclear envelope in two perpendicular rows. The nucleus has begun to elongate but has not yet spiralized. There are many more centrioles in each cell than shown here. × 48,000.

FIGURE 26 Section through the edge of one gyre of the spiralized sperm. The nucleus (*n*) is now a thin ribbon in which the chromatin appears as densely stained clumps. The mitochondria have fused into a spiral structure (*m*) which runs along the nuclear border. Microtubules (*mt*) and two centrioles (*c*) are also present. × 70,000.

FIGURE 27 Transverse section through a centriole (basal body) near the origin of the flagellum. The star arrangement in the center has been described in other plant materials. × 140,000.

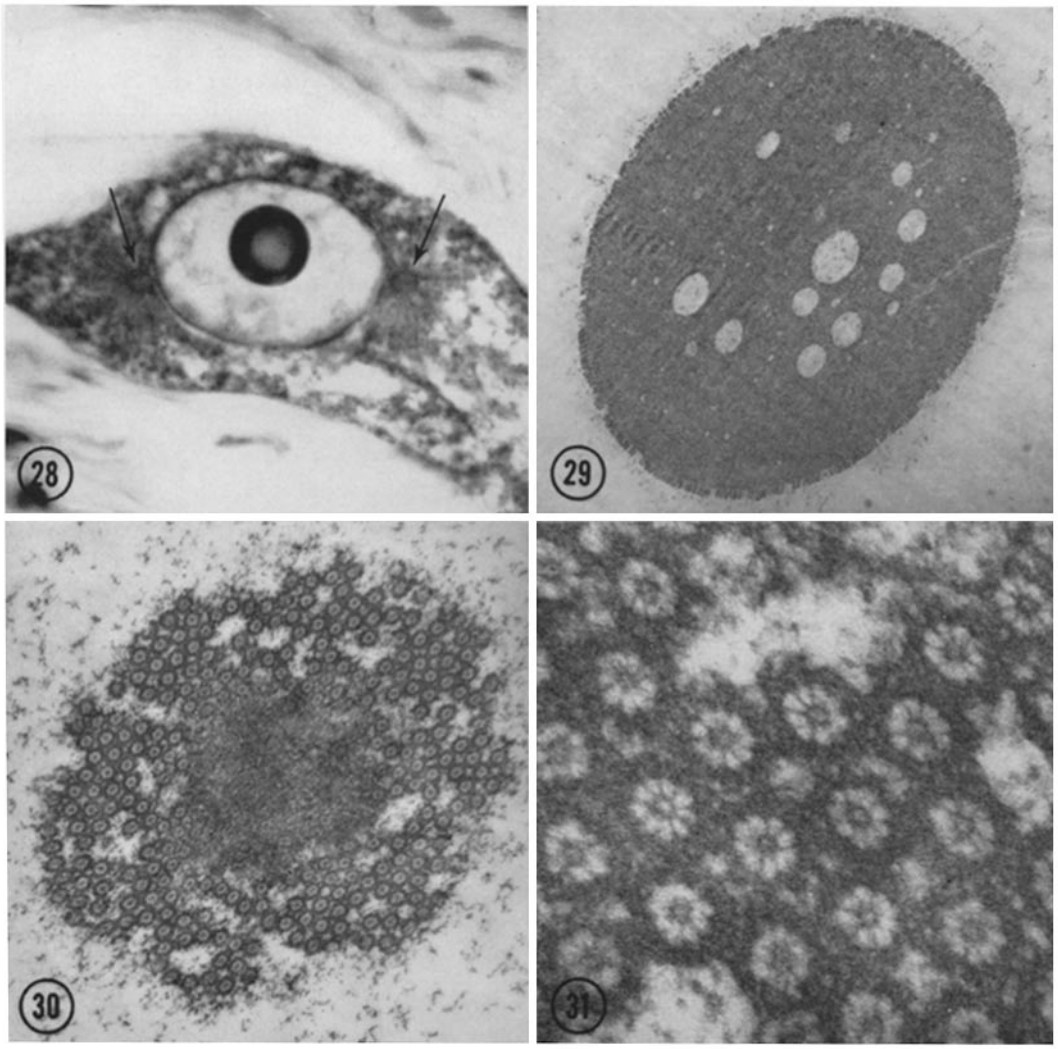


FIGURE 28 Section through the body cell of the pollen tube of *Zamia*. The very pale nucleus is characterized by a prominent nucleolus. In the cytoplasm are two blepharoplasts (arrows) from which astral rays radiate. This is a relatively young stage in which the blepharoplast diameter is about 4 μ. × 830.

FIGURE 29 Low magnification electron micrograph of a section through the blepharoplast of *Zamia*. The surface layer of procentrioles is barely discernible at this magnification. × 7100.

FIGURE 30 Section cut tangentially to the surface of the blepharoplast, showing numerous closely packed procentrioles. × 21,000.

FIGURE 31 Individual procentrioles from Fig. 30 at greater enlargement. The central hub and nine radiating spokes are clearly shown in several cases. The exact arrangement of the wall elements is difficult to determine. × 120,000.

whose walls consist of nine triplet fibers. The central hub and spoke is retained throughout these early transformations. Eventually, the centrioles line up along the nuclear envelope, as in Fig. 25. The nucleus elongates, spiralizes, and comes to lie just under the surface in the anterior part of the cell. Between the nucleus and the cell surface is a row of centrioles, along with an

elaborate mitochondrial derivative, and a series of microtubules (Fig. 26). Flagella grow out from the centrioles, which thereby become basal bodies. The centrioles show a tapering in diameter from the distal toward the proximal end. At a certain level near the cell surface an elaborate network of fibers is developed, resembling a star (Fig. 27). An identical arrangement has been described in the basal bodies of several other plant species (20, 22).

Of particular interest is a comparison between the number of procentrioles in the anaphase cluster and the number of basal bodies and flagella in the mature sperm. As already mentioned, the number of procentrioles is between 110 and 150. Counts of flagella have been made on mature sperms mounted intact on electron microscope grids (Figs. 4 and 5). Values from about 85 to over 120 have been obtained. Since some flagella are usually wrapped closely around the main body of the sperm, and hence cannot be counted, the higher values are probably closest to the true number. It thus seems reasonable to suppose that there is no centriole replication after the last mitosis, and that the procentrioles of the hollow cluster merely transform into an equivalent number of mature centrioles. In keeping with this argument is the observation that the maturing centrioles in a given cell are of uniform size, i.e. once the procentrioles have begun to separate from each other and to elongate, no new small procentrioles can be recognized.

THE BLEPHAROPLAST OF ZAMIA: The most spectacular development of blepharoplasts occurs during the development of the microgametophyte (pollen tube) in the cycads. The body cell of the pollen tube develops two enormous blepharoplasts, which are said to reach a diameter of 27 μ in *Ceratozamia* (23). These blepharoplasts, as in *Marsilea*, occupy the spindle poles during the single division of the body cell. The two spermatids resulting from this division develop into globular sperms each bearing as many as 25,000 flagella (24). We have studied the structure of the blepharoplast in *Zamia* by light and electron microscopy. Unfortunately, we missed the stages of transformation of the blepharoplast into a cluster of centrioles, but earlier accounts, based on light microscopic observations, make it quite clear that the sequence of events is similar to that in *Marsilea*. We have also examined the mature sperms by electron microscopy.

In body cells of *Zamia*, the blepharoplasts are up to 10 μ in diameter and are surrounded by conspicuous cytoplasmic asters (Figs. 28, 29). They are unstained by the Feulgen reaction for DNA, and are also negative after the more sensitive azure-A Feulgen technique (25). The body cell nucleus shows Feulgen-positive strands as expected. The azure-B stain for RNA (26) shows a general cytoplasmic basophilia which contrasts sharply with the unstained or very lightly stained blepharoplast. It is difficult to say with certainty that the blepharoplasts are completely unstained, because of the confusion of underlying and overlying cytoplasm. In sections thin enough to include only the blepharoplast, they appear completely negative.

Sections through a full-sized blepharoplast, shortly before the division of the body cell, show that the bulk of the organelle consists of a rather structureless matrix of moderate electron density (Fig. 29). The entire surface is covered by a single layer of procentrioles (Figs. 30, 31) whose number is readily calculated to be about 20,000. These procentrioles display a prominent hub-and-spoke arrangement in the center, and their over-all ninefold symmetry is quite evident. Formally, the blepharoplast of *Zamia* is comparable to the cluster of procentrioles at the poles of the last division in *Marsilea*. Both consist of a single layer of procentrioles on the surface of a sphere. In *Marsilea*, the sphere is imaginary in the sense that the interior appears much like the "ground" cytoplasm; in *Zamia*, the sphere consists of an ill defined dense material.

We have examined blepharoplasts from several immature body cells. In general, the preservation of our material has been very poor, but it does appear that some sort of tubular network exists in these blepharoplasts comparable to the tubules seen before the last division in *Marsilea*. This point needs further work, however.

We have missed the stages in which the blepharoplast is converted into a cluster of centrioles. There is every reason to suppose that the process parallels the comparable stages in *Marsilea*. The earlier workers, in particular Ikeno (7), Webber (10), and Chamberlain (23), describe a breakdown of the blepharoplast into a cluster of granules with subsequent alignment at the cell surface. Moreover, the mature sperm of *Zamia* possesses many thousands of flagella, as would be predicted if the procentrioles of the blepharoplast are converted directly into basal bodies. The basal bodies of the

Zamia sperm are unusually long, about 4 μ in our material, and are differentiated into several distinct regions.

DISCUSSION

In the following discussion we will use the term *procentriole* as follows. 1. The procentriole is a short cylinder or toroid approximately 90 to 110 mμ in diameter and 70 to 80 mμ long. It is, therefore, narrower and shorter than a typical mature centriole, average values for which would be over 150 mμ in diameter and over 300 mμ in length. 2. It has a less orderly arrangement of the nine wall components than a typical centriole. 3. From the limited observations available (*Viviparus*, *Marsilea*, *Zamia*, *Allomyces*), it seems probable that it regularly contains a central hub and spoke arrangement (Figs. 22 and 31). In this respect, it resembles only the proximal or basal end of a mature centriole (14, 19, 20). 4. It is probable that in the development of the mature centriole the procentriole becomes the proximal end (cf. discussion in 14).

In the present study, we have shown that the centrioles which give rise to the flagella of the *Marsilea* sperm are derived from a cluster of procentrioles present at each pole of the last antheridial mitosis. Although accurate counts are not possible, we estimate that the number of procentrioles equals the number of future flagella. It seems probable, therefore, that no replication occurs after the last antheridial mitosis. The formation of the mature centrioles seems to be by extension of preexisting procentrioles. This mode of growth rules out a simple scheme of centriole self-replication whereby a single mature centriole gives rise to two, which, in turn, give four, eight, sixteen, etc. Indeed, in this case there are no typical centrioles at all until the full complement has matured.

Our observations on *Zamia*, although fragmentary, suggest that essentially the same process occurs here. The major difference is the very large number of centrioles and flagella, and the related enormous size of the blepharoplast. The classical literature on plant spermiogenesis contains many references to blepharoplasts in those forms with multiflagellated sperms (ferns, cycads, *Equisetum*, and *Ginkgo*, among others). Where details are complete, as in *Equisetum* (11), the sequence of events is very similar to that in *Marsilea*. As a working hypothesis, therefore, we suggest that the blepharoplast in all cases consists of a cluster of procentrioles which mature after the last mitosis of the spermatogenous cells.

Centriole formation in *Marsilea* closely resembles that in the multiflagellate sperms of the snail, *Viviparus* (14). In *Viviparus*, two groups of procentrioles appear in the primary spermatocytes and mature into typical centrioles during the first meiotic division. The procentriole clusters in the spermatocyte are similar to the hollow blepharoplast of *Marsilea*, the major difference being that the two mature centrioles from the preceding mitosis are found in the centers of the clusters.

From the association of the procentrioles with a mature centriole in *Viviparus*, as indeed from the association of a procentriole with a mature centriole during mitotic divisions in general, one is tempted to regard the procentriole as a daughter physically derived from the mature centriole. On this view, the important step in centriole self-replication would be the splitting or budding off of the daughter procentriole. The events in *Marsilea*, however, show that a *mature* centriole is not necessary at all during the formation of new centrioles, and suggest the possibility that procentriole formation may be under control of other parts of the cell. One could argue that, in *Marsilea*, the procentriole itself is a persistent self-replicating body, and that it matures into a typical centriole only at the time of sperm formation. If this were true, the apparent *de novo* origin of centrioles in this plant and elsewhere would be illusory. That such a situation may exist is shown by the excellent study of Renaud and Swift (15) on the origin of motile gametes in the mold, *Allomyces*. These authors describe small centrioles, similar to what are called procentrioles here, in close association with the nuclei of the hyphal tip. At the time of gamete formation, the small centriole elongates to three times its original length and becomes the basal body of a flagellum. In a recent study, Robinow and Marak (27) have discovered a centriolelike body in yeast. The centriolar plaque, as it is called, is about 1500 A in width but only a few hundred A long. Two of them are found closely associated with pores in the nuclear envelope at opposite ends of the intranuclear spindle. These findings in *Allomyces* and yeast force a reexamination of all cases of apparent absence of centrioles. Unfortunately, it is very difficult to recognize short centrioles even when they are next to the nuclear envelope or to a

mature centriole. Free in the cytoplasm they would be exceedingly difficult to identify.

The cytological evidence now available is adequate to rule out certain simple modes of centriole replication (e.g., binary fission), but many schemes, ranging from complete nuclear dependence to complete organelle autonomy are, in fact, compatible with the morphological data. As an example, we could assume that the macromolecular events leading to the formation of the procentriole take place at the gene and ribosome level and that the mature centriole serves merely as a locus of "self-assembly" of the procentriole. Such a scheme would have interesting consequences in the case of permanently ciliated cells like many of the Protozoa. One could imagine that the centriole proteins are formed in a typical fashion, under ultimate nuclear control, and migrate to the cortex in which they are organized next to the ciliary basal bodies (centrioles). The distribution of the new centrioles on the surface of the organisms would be dictated by the already existing distribution, and in this way the pattern could be "self-replicating" without the individual units themselves being so. Hypotheses of this type have been discussed by Sonneborn (28); they differ fundamentally from the strict self-replication of ciliary basal bodies postulated by Lwoff (29).

In the present study, we failed to obtain evidence for DNA in the blepharoplast using the Feulgen reaction. Similar results for blepharoplasts in other fern species were reported by Yuasa (30). An independent study of the blepharoplast of *Zamia* by Turner (31) likewise failed to disclose Feulgen-positive material. An apparent contradiction is the report of Lee (32) that the blepharoplast in *Ginkgo* stains slightly pink after the Feulgen reaction. His results are difficult to interpret, however, since his wording implies that fresh (unfixed?) material was treated with the Feulgen reagent.

A negative Feulgen reaction merely sets an upper limit to the concentration of DNA possibly present. Before its fine structure was investigated, it seemed that the blepharoplast of *Zamia* should be an ideal place to test for the presence of DNA in centrioles. However, the fact that only the surface is covered with centriole material greatly reduces the significance of the negative Feulgen reaction. DNA has been reported in basal bodies of the ciliate *Tetrahymena* on the basis of chemical studies of isolated material (33–35). More recently, Randall and Disbrey (36) describe acridine orange staining of basal bodies which they believe is due to the presence of trace amounts of DNA; they also obtain thymidine incorporation into the basal bodies. It is clear that the presence of DNA in centrioles, if substantiated as a general phenomenon, will add weight to theories of centriole autonomy. Attention in the near future will undoubtedly focus on the existence and nature of centriolar DNA.

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