1983

A light and electron microscopic study of the development of antheridia in Onoclea sensibilis L

Jane Louise Kotenko
Iowa State University

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Iowa State University

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A light and electron microscopic study of the development of antheridia in *Onoclea sensibilis* L.

by

Jane Louise Kotenko

Volume 1 of 2

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Major: Botany

Approved:

Signature was redacted for privacy.

In Charge of Major Work

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Iowa State University
Ames, Iowa

1983
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This dissertation is dedicated to the memory of Gregory Bateson, anthropologist/psychologist/cyberneticist, son of William Bateson, geneticist. Bateson spent his whole life searching for the "pattern which connects" all living creatures with each other.

My interests, either directly or indirectly, have involved studying the continuum or cycling of life. A fundamental question concerns how an organism can recreate itself by producing two very specialized cells, the sperm and the egg.

This present study was conducted to investigate part of a fern life cycle; in particular, the formation of male gametangia and the eventual formation of sperms. I have experienced similar feelings to those expressed by Lewis Thomas (1974), Chancellor of Sloan-Kettering Cancer Institute, as he wrote about human embryology. "For the real amazement, if you want to be amazed, is the process. You start out as a single cell derived from the coupling of a sperm and an egg, this divides into two, then four, then eight, and so on, and at a certain stage there emerges a single cell which will have as all its progeny, the human brain. The mere existence of that cell should be one of the great astonishments of the earth. People ought to be walking around all day, all through their waking hours, calling to each other in endless wonderment, talking of nothing except that cell."

To be realistic, my amazement of living systems is often tempered with everyday frustrations and pressures, spawning self-doubts about the importance of science. Lewis Thomas (1974) has been able to place
into perspective for me the phenomena of science and scientists. "The most mysterious aspect of... science is the way it is done. Not the routine, not just the fitting together of things that no one had guessed at fitting, not the making of connections; these are merely the workaday details, the methods of operating. They are interesting, but not as fascinating as the central mystery, which is that we do it at all, and that we do it under such compulsion.... It sometimes looks like a lonely activity, but it is as much the opposite of lonely as human behavior can be. There is nothing so social, so communal, so inter­dependent.... It is instinctive behavior in my view, and I do not understand how it works.... While it is going on, it looks and feels like aggression: get at it, uncover it, bring it out, grab it, it's mine! It is like a primitive running hunt, but there is nothing at the end to be injured. More probably the end is a sigh. But then, if the air is right and the science is going well, the sigh is immediately interrupted, there is a yawping new question, and the wild, tumbling activity begins once more, out of control all over again."

I believe that being a scientist is instinctual, for I have experienced total dissolution of frustrations during the discovery of a new developmental stage. When I wonder whether my work is significant or not, I think of the answer given by Gandhi. "Whatever you do will be insignificant, but it is very important that you do it."
INTRODUCTION

This study was designed to investigate, using light and electron microscopy, the morphological and anatomical changes which occur during the initiation and subsequent development of sperm-producing structures known as antheridia. All sexually reproducing homosporous plants form antheridia during the gametophytic stage of their life cycle, but antheridium morphology and mode of development vary greatly among different taxonomic groups.

_Onoclea sensibilis_ L., an advanced, leptosporangiate fern was selected as the experimental organism in this study due to the simplicity of its antheridia, composed of only three sterile, jacket cells surrounding the spermatogenous tissue. Each antheridium arises from an asymmetric cell division in one of the vegetative cells of a morphologically simple gametophyte plant. The fast-growing gametophytes are less than 1 mm in length and width at sexual maturity and are composed of only one layer of chlorenchymatous vegetative cells.

_Onoclea sensibilis_ L. gametophytes respond strongly to an antheridium-inducing substance known as Antheridiogen Pt (A\text{pt}). The A\text{pt} is produced by _Pteridium aquilinum_ (L.) Kuhn gametophytes and has the ability to induce antheridia in _Onoclea_ gametophytes within a few days, whereas, _Onoclea_ gametophytes grown in standard culture conditions do not form antheridia in the time period covered by experiments in this study.

The simple morphology of _Onoclea_ gametophytes readily allows for measurements of vegetative cell sizes, shapes, and numbers and for observations on the location of new antheridium initials and the number of cells within maturing antheridia. Using these characteristics as
parameters, the effects of axenic culture conditions and A treatment times and concentrations on the induction period of antheridium formation and the number and location of antheridia can be monitored.

The simplicity, controllability, and predictability of gametophyte vegetative and reproductive growth forms provide an excellent system in which to study cellular differentiation and antheridium development. Gametophytes can be selectively harvested and processed for examination of various stages in antheridium development using light and electron microscopic techniques.

Although fern gametophytes are morphologically simple, the stability of their morphology under specified conditions and their ability to form differentiated organs and cell types, such as rhizoids and trichomes, attest to an internal physiological complexity. Representatives of all five hormone groups found in higher plants are also present in fern gametophytes and the ultrastructure of gametophyte vegetative cells is comparable to chlorenchyma in higher plants. Thus, fern gametophytes, as experimental organisms, have the advantage of providing a simple system in which to study complex developmental pathways, comparable in many ways to higher plants.

No previous studies have investigated the ultrastructural changes which accompany the initiation of antheridia in ferns and only three previous reports have mentioned changes which occurred at the light microscope level. Nevertheless, a generalized description of the process of fern antheridium initiation arose in the early literature and has been accepted unquestioningly in the recent literature.

Alternatively, there is much controversy in the literature in-
volving developmental aspects of the first four cell divisions in the
initials with respect to the orientation of the spindle apparatus, the
orientation of the subsequent cell wall, and the manner in which the
phragmoplast and cell wall proceed to form. Three different theories
have been proposed to explain the development of the sterile jacket
cells during the first four cell divisions.

Due to the availability of older stages in antheridium development,
this study is expanded to include observations of events during
spermatogenesis. Ultrastructural changes will be observed both in
spermatogenous cells during the division phase and in spermatids developing
into spermatozoids during the differentiation phase. Spermatogenic
events in three other homosporous fern species have been investigated
in detail, but some key developmental events have not yet been described.

In essence, this study is concerned with all aspects of fern
spermatogenesis, which in reality, begins with the formation of the
antheridium initial. Specifically, this study serves six main pur-
poses: 1) to add comparative information on the effects of sterilization
and time and concentration of A sub pt application on gametophyte growth and
antheridium formation; 2) to add correlative information on antheridium
location sites with respect to internal gradients of fern gametophytes;
3) to provide clarifying information on antheridium morphology and de-
velopment in advanced, leptosporangiate ferns; 4) to provide comparative
information on developmental pathways which involve asymmetric cell
divisions and differentiation; 5) to provide new and comparative in-
formation on fern spermatogenesis; and 6) to provide a structural
foundation for future studies investigating physiological and physical factors involved in antheridium initiation.
LITERATURE REVIEW

Taxonomic Classification

The onocleoid ferns, composed of three genera, *Onoclea*, *Onocleopsis*, and *Matteuccia*, have been variously classified over the years. The most recent study on the systematics of the group was done by Lloyd (1971) who placed the group in the family Aspidiaceae as a subfamily, Onocleoideae. Lloyd used the phylogenetic scheme proposed by Wagner (1969). In the most recent scheme by Wagner (1974a), the Aspidiaceae has been combined into the subfamily Dryopteridoideae within the family Aspleniaceae. For the comparative purposes of this study, the scheme proposed by Wagner (1974a, b) will be used and is diagrammatically shown in Figure 1. *Onoclea sensibilis* is thus classified as a member of the most advanced family of ferns.

Gametophyte Morphology

The most recent, comprehensive treatise on comparative morphology of fern gametophytes was compiled by Nayar and Kaur (1971). They recognized eight different patterns of gametophyte (commonly known as a prothallus) development with five ultimate shapes, including tuberous, filamentous, cordate, strap-like, and ribbon-like. The most common shape is the cordate- or heart-shape, which is exemplified by the Aspleniaceae and which occurs in many other families.

Development of a prothallus begins with the germination of a haploid spore. The chlorophyllous spores of *Onoclea* germinate rapidly in moist environments, as do all chlorophyllous spores, in about 1-3
Figure 1. A phylogenetic scheme of the homosporous ferns, adapted from Wagner (1974a, b)
days (Dyer, 1979; Lloyd, 1971). According to Nayar and Kaur (1971), prothallial development in *Onoclea* is of the Aspidium-type. The spore cell divides transversely four times to produce a filament of four prothallial cells and a basal rhizoid. The apical cell then divides obliquely to begin the formation of a two-dimensional plate of prothallial cells. Eventually, when there are about 20 cells in the plate, one of the marginal, anterior cells functions as an apical initial which forms a "notched" meristem composed of a U-shaped, marginal row of cells. Due to cell division and elongation in this region, a cordate prothallus forms. This description is consistent with the description given by Lloyd (1971). However, according to Miller and Miller (1964), *Onoclea* spores germinated and typically formed a filament of two cells. These two cells immediately divided longitudinally to form a two-dimensional thallus of four cells. The upper two cells continued to divide to form a plate of prothallial cells. They did not follow later development, but it is presumably similar to the above description.

A cordate prothallus has four basic regions, consisting of the notch meristem, two wing regions, a median midrib or central region, and a basal region (Nayar and Kaur, 1971). The basal cells generally form rhizoids, which are long, thin, clear cells implicated in anchorage and absorption (Cran, 1979; Nayar and Kaur, 1971). The cells in the central and wing regions normally do not divide unless they are induced to form gametangia or trichomes (Crotty, 1967; Lloyd, 1971). In mature gametophytes of *Onoclea*, antheridia are formed both in the central region and on the inner two-thirds of the wings (Lloyd, 1971). Marginal
cells of the wings of *Onoclea* commonly form trichomes, small, unicellular, papillate cells, and eventually cells in the central region will form these trichomes which often secrete waxy substances (Lloyd, 1971; Nayar and Kaur, 1971). The sole function of the notch meristem regions is to produce new vegetative cells (Crotty, 1967).

In cordate gametophytes, the cells of the meristem are small and isodiametric, the cells in the central and wing regions undergo the greatest elongation, while the cells at the base are quite large and often isodiametric (Nāf et al., 1975; Reuter, 1953). Cordate gametophytes do maintain a plasticity of shape and growth that can be released under variable culture conditions involving light, temperature, pH, nutrients, chemicals and hormones, and biotic factors such as spore density (Dyer, 1979; Miller, 1968). For example, gametophytes grown in red light will often grow indefinitely as filaments, whereupon transfer to blue light causes them to grow two-dimensionally (Miller, 1968).

There is some evidence that fungal contamination affects gametophyte development, either enhancing growth or, in some cases, inhibiting growth (Dyer, 1979). In order to keep experimental gametophytes free of other organisms, the spores are often treated with chemical sterilants, the most commonly used sterilant being sodium hypochlorite (Miller, 1968). Although this sterilant is widely used, there are few reports on the effects it has on gametophyte growth or reproductive structures.

Steeves, Sussex, and Partanen (1955) found that the plasticity of gametophytes increased in sterilized cultures, with many of the gametophytes forming filamentous proliferations or coral-like aggrega-
tions. These abnormal growth forms reportedly maintained their ability to produce antheridia. Soon after, Marengo (1956) reported that sodium hypochlorite removed the perispore layer from *Onoclea* spores. Only members of the Aspleniaceae have this extra layer of material, known as a perispore, covering the exine of the spore. In a study by Schraudolf (1962), it was reported that spore sterilization did not affect the development of antheridia in *Anemia*.

Crotty (1967) mentioned, without explanation, that Clorox, whose active ingredient is sodium hypochlorite, inhibits cell wall growth. In a later study, Howland and Boyd (1974) found that up to 50% suppression of *Pteridium* spore germination occurred with a brief treatment of dilute sodium hypochlorite. Most recently, Dyer (1979) reported that spore sterilization with 2-5% sodium hypochlorite for 2-5 min may cause a reduction in percent germination and a loss of synchrony during germination.

In studies using *Onoclea sensibilis* gametophytes as the experimental organism, the effects of sterilization were not mentioned, but growth rates were presented (Lloyd, 1971; Miller and Miller, 1964; Naf, 1956). Naf (1956) reported that in spore-sterilized cultures exposed to 200 ft-c of continuous light at 23°C, seven-day-old gametophytes had an average of 9.4 cells. Lloyd (1971) reported that in unsterilized cultures exposed to 210-290 ft-c of continuous light, six-day-old gametophytes had an average of 3 cells. Miller and Miller (1964) found that cultures exposed to 16 hours of 200 ft-c of light per day at 25°C in which only the sporangia had been sterilized, six-day-old gametophytes had an average of 27 cells.
It is evident from these studies that gametophyte growth is affected by different culture conditions, but due to the number of variables among the studies, including the growth media used, it is not clear how sterilization affected growth.

Cellular Anatomy of Fern Gametophytes

The ultrastructure of *Onoclea* gametophytes has not been previously studied, except for some aspects of sporogenesis (Bassel et al., 1981; Marengo, 1977, 1979; Marengo and Badalamente, 1978). Ultrastructural studies on other species of fern gametophytes have been limited to the early filamentous stage (Cran, 1979). But, with complementary studies at the light microscope (LM) level, many features of gametophyte cell anatomy have become clear.

The small, meristematic cells are densely cytoplasmic, staining strongly with pyronin Y, a stain specific for RNA (Crotty, 1967). As the cells derived from the meristem begin to expand and become part of the other regions in the gametophyte, the cytoplasm becomes increasingly vacuolated (Cran, 1979; Crotty, 1967). These highly vacuolate cells, with their thin layer of peripheral cytoplasm, do not stain very much with pyronin Y (Crotty, 1967).

All of the cells of a gametophyte, excluding the rhizoids, contain numerous chloroplasts. An increasing gradient of chloroplast size was reported for 7-celled filaments of *Dryopteris pseudo-mas* growing in blue light. The average dimensions of the organelles in the apical cells were 6 μm long x 4 μm wide compared to 10 μm x 6 μm in the basal cells. This
gradient was also noted in *Pteris vittata* (Ootaki and Furuya, 1969).

No gradient of chloroplast size was observed in filaments grown in red light (Bergfeld, 1970). The chloroplasts in the apical cells grown in red light were on average larger than the chloroplasts in apical cells grown in blue light (Bergfeld, 1970; Stetler and DeMaggio, 1972). Proplastids have not been observed in vegetative cells of gametophytes, although they do occur in spores of some species and in gamete cells (Cran, 1979). In most studies, chloroplasts are described as having moderate to extensive grana stacks with interconnecting membranes and large starch grains (Cran, 1979; Stetler and DeMaggio, 1972). Wada and O'Brien (1975) described two morphological types of chloroplasts in the tip cells of the filaments of *Adiantum capillus-veneris*. One type was cigar-shaped and appeared to be "anchored" by one end close to the cell membrane. The other type was smaller and more rounded and apparently pleomorphic. Both types had starch grains.

Smith and Smith (1969) also reported two different types of chloroplasts in *Todea barbara* (Osmundaceae). One type had well-developed grana and intervening membranes with little starch accumulation. The other type consisted of amyloplasts, containing abundant starch deposits and stacks of membranes that were unconnected. Even with little starch present, the latter type had few internal membranes. Both types apparently occurred together in the cells of the gametophytes. Amyloplasts have also been reported to occur in *Blechnum* (Aspleniaceae) gametophytes (Beisvåg, 1970).

Reversibility of chloroplast size has been reported by Ootaki and Furuya (1969) who found that larger chloroplasts in white light revert
to smaller sizes within 48 hr after interstitial cells were stimulated
to divide by physically isolating them.

All of the prothallial cells in gametophytes contain the usual
components found in cells of higher plants, including ribosomes,
mitochondria, Golgi bodies, rough endoplasmic reticulum, vacuoles, oil
droplets, and microbodies (Cran, 1979). Faivre-Baron (1977) found
abundant microbodies in early cells of Gymnogramme (Adiantaceae) fila­
ments, but very few in older cells. In 4-5-celled protonema of
Dryopteris pseudo-mas, microbodies were found in all of the cells, but
were most common in the apical cells (Cran, 1979). Dense, amorphous
inclusions have been observed in microbodies of fern gametophytes, but
no crystalline structures (Cran, 1979).

Microtubules are present, and according to Cran (1979), they are
more frequent at the anterior end of protenemal cells, around the
nuclear envelope, or running parallel in a band interior to the
plasmalemma of cells about ready to divide. The only report of a
band of microtubules prior to divisions comes from Wada et al. (1980),
who reported the occurrence of cortical, circumferentially aligned
microtubules (CCAM) during pre-prophase in tip cells of Adiantum capillus-
veneris filaments grown in red light. The CCAM disappeared during
metaphase. As described, CCAM appear to be synonymous with the pre-
prophase bands of microtubules (PPBM) described from higher plants
(Busby and Gunning, 1980; Gunning et al., 1978; Palevitz and Hepler,
1974; Pickett-Heaps and Northcote, 1966). The PPBM occurred along
the region of the cell wall where the new cell wall fused.

The CCAM in the tip cells of A. capillus-veneris occur 10-15 µm
from the tip of the cell, in the region that the new cell wall formed. No microtubules occurred in the first 5 \( \mu m \) of the cell. In the region posterior to 15 \( \mu m \), many microtubules occurred which were aligned parallel to the long axis of the cell (Wada et al., 1980).

Stetler and DeMaggio (1972) investigated the presence and orientation of microtubules (MT) in the tip cells of *Dryopteris filix-mas* grown in both red and blue light. They also did not find MT in the first 5 \( \mu m \) of the cells, but in the 10-15 \( \mu m \) region, they found randomly oriented MT in both red and blue light. They found a difference in MT orientation in red and blue light that occurred in the 15-30 \( \mu m \) region. In red light, the MT were aligned parallel to the long axis of the cell, but in blue light, the MT were randomly oriented. These observations by Stetler and DeMaggio (1972) lend support to the hypothesis proposed by Miller and Stephani (1971) that MT orientation may play a role in the phototropic response exhibited by gametophytes transferred from red light to blue light mentioned earlier.

All of the cells in the gametophytes are connected by plasmodesmata (Cran, 1979). Variously shaped nuclei in the cells have been reported, including spherical, lobed, and more commonly, ellipsoidal (Cran, 1979). The different shapes were not correlated with any particular growth states. Nucleoli and irregular aggregation of chromatin were reported as consistent features in the nuclei of the gametophytes (Cran, 1979).

Ootaki (1965) noted that in cordate gametophytes of *Pteris vittata* (Adiantaceae), there was an increasing apico-basal gradient of nuclear volume. Takei and Tanaka (1974) examined the DNA content of the nuclei in all the cells of *Microlepia marginata* (Cyatheoideae) gametophytes and
found that they all contained 1C complement of DNA. Alternatively, Partanen (1965) found that endopolyploidy occurred in cells of *Osmunda* gametophytes.

In a histological study of *Pteris vitatta* gametophytes, Crotty (1967) found that in divisions of prothallial cells which form two similar daughter cells, the nucleus became suspended in the center of the vacuolate cell by cytoplasmic strands. In dividing nuclei, the organelles tended to aggregate around the nucleus, after which they became dispersed in the peripheral cytoplasm of the daughter cells (Gran, 1979). In the regions where cell divisions occur, the nondividing cells had a thick layer of material surrounding the nucleus and also a layer adhering to the cell walls; both layers stained for carbohydrates and proteins (Crotty, 1967). The material occurred only along the walls in expanding cells and not in the mature cells, suggesting that it was related to cell wall growth.

There is indication that some cells of gametophytes form an extracellular cuticular layer. In *Onoclea*, Lloyd (1971) found that marginal cells, along with cells in the wings, secreted a fatty substance that stained with Sudan IV. Lloyd (1971) also noted a waxy deposit on the surface of antheridium that stained with Sudan IV. Supporting evidence of cuticular material comes from a study by Wada and Staehelin (1981) who, using freeze-fracture electron microscopy, found a multilayered, lipid-like coat covering the first filamental cell of *Adiantum capillus-veneris*. They also discovered rosettes of 8-9 nm-wide particles on the P-face of the plasma membrane coinciding with the region of maximal cell-wall growth and expansion.
The ultrastructure of vegetative cells that have been induced to form antheridia has not been investigated. Changes that have been observed at the LM level in induced vegetative cells will be reviewed in the later section on Antheridium Morphology.

Antheridiogens and Antheridium Formation

According to Naf (1979), most strains of *Onoclea* fail to form antheridia spontaneously at any stage in development in cultures grown in continuous light. But Klekowski and Lloyd (1968) found that a small percentage of gametophytes formed antheridia after 45 days in culture under continuous light. Voeller and Weinberg (1969) claimed that gametophytes of *Onoclea* not grown under continuous light will soon form antheridia, but they did not specify how soon. Naf et al. (1974) found that gametophytes grown in the light for 7 days and then transferred to near-darkness formed antheridia in about 2-3 wk.

Over 30 years ago, a discovery was made of a substance that could induce antheridia in *Onoclea* gametophytes within days of spore germination. Döpp (1950) discovered that medium harvested from maturing cultures of *Pteridium aquilinum* hastened the onset of antheridium formation in young prothalli of the same species by several days and in the prothalli of *Dryopteris filix-mas* by several weeks. The active substance in the extract was named Antheridiogen Pt (A\textsubscript{pt}). Naf (1956) discovered that *Onoclea* gametophytes responded strongly to A\textsubscript{pt} at a dilution of 1:30,000 of the crude extract. Pringle (1961) isolated A\textsubscript{pt} from the crude extract and found that it was active at a concentration
of 1 part per 10 billion or $10^{-7}$ mg/ml with *Onoclea*.

Since the discovery of $A_{pt}$ by Dopp (1950), antheridiogens have been discovered from ten other species of ferns, including *Onoclea* and at the present time, 28 different species of ferns in advanced families have shown the ability to respond to $A_{pt}$ (Naf, 1979). Although *Onoclea* produces its own antheridiogen, $A_{on}$, the gametophytes respond very weakly to it, producing only a few antheridia 17 days after treatment (Naf, 1979). But Naf (1969) found that if $A_{on}$ was boiled at pH 2.0, the activity dramatically increased.

Only the antheridiogen from *Anemia phyllitidis* has been chemically characterized, revealing a molecular formula of $C_{19}H_{22}O_6$ (Endo et al., 1972) that has the configuration of a derived gibberellin (GA) (Nakanishi et al., 1971). Not surprisingly, GA was shown to possess the ability to induce antheridia in some species of gametophytes (Emigh and Farrar, 1977; Schraudolf, 1962, 1966a; Voeller, 1964a). GA was found to be effective at concentrations between $5 \times 10^{-5}$ and $5 \times 10^{-7}$ g/ml. Although GA did not hasten the onset of antheridia in *Onoclea* gametophytes, Schraudolf (1966b) found that treatment with GA increased the percentage of antheridium-bearing gametophytes.

The time that $A_{pt}$ was applied to maturing gametophytes of *Onoclea* was found to influence the length of the induction period. When $A_{pt}$ was applied at 1/10 or 1/50 full strength on the day of sowing, antheridia did not form until 7 days later when there were about 11 vegetative cells per gametophyte (Naf, 1958). When the same concentrations of $A_{pt}$ were applied six and nine days after sowing, antheridia formed 2-1/2 to 3 days later (Naf, 1959).
A similar correlation between time of treatment and length of induction periods was observed for *Anemia* (Näf, 1959). Schraudolf (1966a) concluded that the induction of antheridia was possible only after a certain state of development of the prothalli had been reached. Näf et al. (1975) hypothesized that a physiological state antagonistic to antheridium formation became operative if the cultures were treated on the day of sowing.

Eventually *Onoclea* gametophytes became totally insensitive to \( A_{pt} \) (Näf, 1958). Näf (1958) claimed that the gametophytes lost sensitivity to \( A_{pt} \) within a period of 2 days, shortly after they attained a notch meristem, which was about 14 days after sowing. Voeller and Weinberg (1969) also found a decrease in sensitivity to \( A_{pt} \) in *Onoclea* gametophytes, but the decrease occurred over a period of a few weeks.

Döpp (1959) and Näf (1961) have provided indirect evidence that a substance inhibitory to antheridium formation is produced in the notch meristem. Döpp (1959) obtained an extract from macerated tissue of *Pteridium* gametophytes that inhibited the action of \( A_{pt} \). Näf (1961) found that insensitive gametophytes of *Onoclea* regained the ability to form antheridia if the notch meristems were excised. The inhibitory substance has not been isolated. Auxins and abscisic acid have been implicated as inhibitors to antheridium formation (Döpp, 1962; Hickok, 1983). Further information on the physiology of antheridiogens can be found in reviews by Miller (1968), Näf et al. (1975), Näf (1979), and Voeller (1964b).

Along with inducing antheridia, \( A_{pt} \) and GA were found to inhibit vegetative cell divisions (Näf, 1956; Schraudolf, 1966a). Although
inhibition of vegetative growth by nonspecific effects such as low light
intensities and lack of nutrients has been correlated with antheridium
formation (Döpp, 1927; Prantl, 1881, Sossountzov and Delaporte, 1949),
Döpp (1950) and Näf (1956) provided evidence which showed that A\textsubscript{pt}
was a specific organ-inducing substance. Döpp (1950) found a concentra­
tion of A\textsubscript{an} that induced antheridia without reducing the number of
vegetative cells compared to the control gametophytes. Näf (1956)
found that the total number of cells in A\textsubscript{pt}-treated gametophytes was
always higher than in untreated gametophytes. Näf (1956) concluded that
the energy normally channeled into vegetative growth was somehow diverted
into antheridium growth.

Location of Antheridium Initials

Information is completely lacking on the location of antheridia in
young prothalli of Onoclea. However, complementary information does
exist on other cordate, antheridiate gametophytes. In Anemia,
Schraudolf (1966a) found that the first antheridia were always formed
on lateral, marginal cells. Hofmeister (1862) generalized that in many
young prothalli, antheridia were formed on marginal cells and Atkinson
(1894) and Nayar (1965) found a relationship between crowded conditions
and marginal antheridia.

Näf (1959) found that when older gametophytes of Anemia were
treated with A\textsubscript{an}, only nonmarginal cells behind the marginal, lateral
meristem formed antheridia. Lygodium gametophytes, which have apical
meristems, first formed antheridia in the basal region, and then ex-
clusively in the apical, central region (Näf, 1960). In both *Anemia* and *Lygodium*, there was a tempo-spatial loss of cellular ability to form antheridia.

Momose (1958a) summarized information on the antheridial areas in mature, cordate prothalli of ferns from 20 previous years of his work on gametophyte development. He claimed that there were three basic types of antheridium location patterns, including axial, laminal, and marginal. In the axial type, the antheridia are formed on the central cells between the notch meristem and the basal cells. This distribution supposedly occurred in advanced families of ferns. The laminal type pattern was characterized by antheridia forming on the surface of the wings and was common in the primitive families of ferns. The marginal type prothalli produce antheridia on the margins of the wings. *Ceratopteris* was the only example given in this category.

Factors involved in controlling the location of antheridia are not known. The interaction of hormones may be involved. Abscisic acid (Cheng and Schraudolf, 1974), auxin in the form of IAA (Hotta, 1959), ethylene (Miller et al., 1970), GA₃ (Schraudolf, 1966c), and ribosylated zeatin (Schraudolf and Fischer, 1979), along with antheridiogens, all have been isolated from fern gametophytes. Very little is known about their sites of synthesis or their modes of translocation. Only IAA has been shown to move both acropetally and basipetally (Albaum, 1938; Faivre-Baron, 1981; Reynolds, 1979). There is some evidence which shows that antheridiogens are probably produced in the notch meristem. For a review of the effects of hormones on gametophyte development, see Howland and Edwards (1979), Miller (1968), and Smith (1979).
It has been well-documented that polar gradients do exist in gametophytes with respect to cell age and position. Obvious gradients related to age occur in terms of cell divisions, elongation, orientations, and differentiation (Igura, 1955; Miller, 1968; Reuter, 1953). Other apico-basal gradients related to age include cell permeability, osmotic potential of the cells, cell sensitivity to UV radiation, nuclear volume, the ability of the cells to regenerate new prothalli, and the ability of cells to respond to auxin (Miller, 1968; Miller and Miller, 1964; Smith, 1979).

Gradients related to cell position also have been shown to exist through the use of various stains (Reuter, 1953). When Dryopteris gametophytes were stained with toluidine blue at pH 6.0, only the cells in the meristem region stained. At pH 7.0, both the meristem cells and the marginal region of the wings stained. At pH 8.0, all the regions excluding the central region, stained. This pattern of staining with toluidine blue is similar to the pattern involving the loss of ability of cells to form antheridia.

From numerous observations of many species of fern gametophytes, it is apparent that only a fraction of the cells in a responding region actually form antheridia (Döpp, 1962; Momose, 1958b; Naf et al., 1975; Stokey, 1951). There are no developmental studies on fern gametophytes which contribute to an understanding of this differential cell response within similar regions. A possibility exists that some of the cells may act as "target cells." In a somewhat similar system involving moss protonemal gametophytes, target cells have been identified which accumulate cytokinins, in contrast to adjacent cells (Brandes and
Kende, 1968). Only the cells that accumulated cytokinin proceeded to form buds.

According to Naf et al. (1975), antheridia in Onoclea have a preferred cellular location, which was at the anterior end of elongate, vegetative cells. Antheridia produced by the large, isodiametric, basal cells did not have a preferred cellular site of formation. Nayar and Kaur (1971) claimed that leptosporangiate-type of antheridia develop toward the middle of the peripheral wall of the mother cell. The only other reference to cellular location of antheridia was reported by Naf et al. (1975) who claimed that Dennstaedtia antheridia have no preferred cellular site of formation.

Antheridium Morphology

The life cycle of seedless plants did not receive much attention until the mid-nineteenth century, by which time most aspects of the life cycle of angiosperms were understood (Harvey-Gibson, 1919). In 1842, Bischoff was the first to notice wart-like structures on fern gametophytes, structures which would eventually be identified as antheridia by Nägeli in 1844 (Hofmeister, 1862). Although Nägeli thought that the antheridium was the male reproductive structure, he did not understand its role in reproduction. He was unaware of fern archegonia and further, his description of antheridium morphology could even apply to archegonium morphology. He stated that antheridia consisted of two to five tiers of cells, each with four cortical cells lying in a circle around a space filled with opaque granules, known as a canal (Hofmeister,
By 1862, Hofmeister had determined the true nature of antheridia and presented his own description of their morphology (Harvey-Gibson, 1919; Hofmeister, 1862).

Although it is well-known today that antheridia in advanced ferns have three, sterile jacket cells, the earliest workers claimed that the antheridium had numerous cells and probably developed similar to the multicellular moss antheridia (Hofmeister, 1862; Strasburger, 1868). Atkinson (1894), Campbell (1886), and Kny (1895) provided the earliest descriptions of antheridia with three jacket cells. Their descriptions and some aspects of the description given by Strasburger (1868) collectively have come to be known as the classical theory of antheridium development in advanced ferns (Verma and Khullar, 1966). The classical theory, which appeared in early textbooks by Bower (1923), Eames (1936), and Smith (1938) claimed that an antheridium arises as a papillate projection which is then cut off from an epidermal cell by a periclinal division (Figure 2A, B). The initial then enlarges and divides with the formation of a funnel-shaped wall, which in most cases reaches the basal wall of the initial cell (Figure 2C). In some species, the first wall in the initial may be less concave or even flat (transverse). If the wall is formed transversely, it remains so throughout the development of the antheridium. The 2-celled antheridium then consists of a cup-shaped or barrel-shaped basal cell and an upper cell.

The next division occurs in the upper cell resulting in the formation of a hemispherical wall parallel to the outer wall of the upper cell (Figure 2D). The 3-celled antheridium consists of a basal cell, an upper jacket cell, and a central spermatogenous cell.
Figure 2. Diagramatic representation of antheridium formation in advanced, leptosporangiate ferns. A-E. Formation according to the classical theory. F-K. Formation according to Davie's theory.
The upper jacket cell then is divided by a funnel-shaped wall forming a cap cell and a doughnut-shaped ring cell (Figure 2E). This completes the formation of the jacket cells of an antheridium. The spermatogenous cells then continue to divide and eventually the cells differentiate into spermatozoids.

An explanation for how the uniquely shaped walls are deposited was not given in most cases. Kny (1869) purportedly claimed that the funnel-shaped wall which forms the cap cell, forms simultaneously in all regions (Davie, 1951).

Davie (1951), unable to comprehend how such walls could form, re-investigated the problem of antheridium development using *Pityrogramma calomelanos* (Adiantaceae). The workers who proposed the classical theory had used *Pteris serrulata* (Adiantaceae), *Onoclea sensibilis*, *Matteuccia struthiopteris*, and *Dryopteris filix-mas* (Aspleniaceae).

According to Davie (1951), the antheridium begins development as postulated in the classical theory, as a hemispherical protuberance cut off from the underlying prothallial cell (Figure 2A, B), but the similarity ceases there. Davie claimed that the first cell wall in the initial invariably forms transversely (Figure 2F) and either remains flat throughout development or secondarily becomes concave or funnel-shaped due to internal pressures (Figure 2G).

The next cell wall is also originally transverse, and secondarily assumes a hemispherical orientation due to the expansion of the central cell (Figure 2H, I).

The third cell wall which forms the cap cell is also transverse, like the previous two cell walls (Figure 2J). Due to the continuing
expansion of the central cell, the two cell walls come into contact (Figure 2K). The final morphology of the antheridium jacket cells, according to Davie's theory, is similar to the morphology described by the classical concept, although the hypothesized development is completely different.

Davie (1951) made his observations from living material mounted in chloral hydrate and presented his evidence in the form of drawings. Davie's theory of antheridium development was readily accepted and appeared in many textbooks after 1951 including Smith (1955) and Sporne (1962). Foster and Gifford (1974) presented both Davie's theory and the classical theory without commenting on the validity of either theory.

Numerous descriptive studies on gametophyte morphology appeared in the next decade due mainly to Momose, Nayar, and Stokey (Nayar and Kaur, 1971), but usually only diagrams of mature antheridia were included. Kachroo (1955), Kachroo and Nayar (1953) and Stokey and Atkinson (1957) were some of the few workers who discussed antheridium development after Davie (1951). In all three papers, the descriptions of antheridium development for members of the Adiantaceae and Aspleniaceae were consistent with Davie's theory. In all cases, only drawings were presented as evidence for the way antheridia developed.

Stone (1958, 1961, 1962) conducted the next comprehensive studies on antheridium development using the genera Blechnum, Doodia (Aspleniaceae), and Polyplegium (Hymenophyllaceae; Cyatheaceae). She found that in all cases antheridium development followed the tenets of the classical theory. In Blechnum and Doodia, the first cell wall
in the initial remained flat forming a barrel-shaped basal cell, while in *Polypheleium*, the first cell wall in the initial was originally deposed in the shape of a funnel (Stone, 1961, 1962). Stone presented photographs showing that the orientation of the spindle equator and the subsequent funnel-shaped walls were the same, indicating that the funnel-shape was primarily and not secondarily derived.

Stone (1961) also determined that all sections of the funnel-shaped wall forming the cap cell are not formed simultaneously, as suggested by Kny (1869). She observed the phragmoplast initially forming between the telophase nuclei in the upper jacket cell prior to cap cell formation.

Schraudolf (1963) provided evidence that the cap cell in antheridia of *Anemia* is formed by an initial, funnel-shaped wall, as proposed by the classical theory.

Even with supporting evidence from Stone (1958, 1961, 1962) and Schraudolf (1963), the classical theory of antheridium development was not readily accepted. In the next review of gametophyte morphology of homosporous ferns, Atkinson and Stokey (1964) supported Davie's theory on how the basal cell of an antheridium is formed. They claimed that if a basal cell has an upper, funnel-shaped wall, that shape must be derived secondarily due to internal pressures. Atkinson and Stokey (1964) did not address the question of how the other antheridial jacket cells are formed.

Verma and Khullar (1966) worked with genera in the Adiantaceae to help clarify the process of cell wall formation in antheridia. They concluded that the first wall in the initial is formed transversely,
and in some species is secondarily displaced downwards to take the shape of a funnel-shaped wall, which is consistent with Davie's theory. They claimed that the second wall is formed in the shape of a hemisphere, as proposed in the classical theory, and they felt that the third wall which formed the cap cell, is initially formed in a transverse or slightly concave position, but is not initially in contact with the lower wall of the upper jacket cell from which it is formed. Their interpretation of the formation of the third wall is consistent with Davie's theory.

Verma and Khullar (1966) included both drawings and photographs as evidence for their conclusions. The antheridial cells shown in the photographs were in a nondividing state.

Verma and Khullar (1966) attributed a theory of antheridium jacket cell formation to Stone, which is contrary to the interpretations presented by Stone, herself (1958, 1961, 1962). Stone's theory, according to Verma and Khullar (1966), claimed that the first cell division in the initial is transverse, the second division is hemispherical, and the third division is funnel-shaped. Verma and Khullar (1966) mentioned the work by Stone (1962) with *Polyphlebium* in which the first wall is funnel-shaped, but they chose not to emphasize that work. Thus, by 1966, there were four proposed theories on how jacket cells form in fern antheridia. These proposals are summarized in Table 1.

Stone (1969) examined eight more species in the Blechnaceae and found that antheridium development in *Woodwardia, Sadleria, and Brainea* was similar to development in *Blechnum* and *Doodia* (Stone, 1962). Stone (1969) was critical of the interpretations of Verma and Khullar (1966) regarding the original orientation of the third wall which forms the
Table 1. Orientation of the first three cell walls in antheridium initials according to four proposed theories of antheridium development in advanced, leptosporangiate ferns

<table>
<thead>
<tr>
<th>Theory and author(s)</th>
<th>Orientation of the first three walls in the antheridium initial</th>
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<tr>
<td></td>
<td>First wall</td>
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<tr>
<td>Classical</td>
<td></td>
</tr>
<tr>
<td>Atkinson (1894)</td>
<td>Flat,</td>
</tr>
<tr>
<td>Campbell (1886)</td>
<td>concave,</td>
</tr>
<tr>
<td>Kny (1869)</td>
<td>or funnel-shaped</td>
</tr>
<tr>
<td>Strasburger (1868)</td>
<td></td>
</tr>
<tr>
<td>Stone (1962)</td>
<td></td>
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<tr>
<td>Davie's Theory</td>
<td></td>
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<tr>
<td>Davie (1951)</td>
<td>Transverse</td>
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<tr>
<td></td>
<td>May be</td>
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<td></td>
<td>secondarily concave or funnel-shaped</td>
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<td>Verma and Khullar's Theory</td>
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<td>Stone's Theory according to Verma and Khullar</td>
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<td>Verma and Khullar (1966)</td>
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<td>Nayar and Kaur (1971)</td>
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cap cell. Stone (1969) claimed that it is impossible to determine the manner in which a wall is laid down from a study of the cells after cytokinesis has been completed for some time.

Schraudolf (1968) presented persuasive evidence that the first cell wall in *Polypodium* antheridia is initially funnel-shaped. He included LM photographs which show metaphase chromosomes oriented at a $40^\circ$ angle to the base of the initial, the same angle at which the subsequent wall forms. Schraudolf concluded that the wall begins to form on one side of the initial and then proceeds circularly until the advancing edges meet. Schraudolf (1968) also contended that the second wall is hemispherical and the third wall is funnel-shaped, in agreement with the classical theory.


However, Sporne (1975), in the latest edition of his textbook, included both Davie's theory and the observations by Stone (1962). Sporne (1975) supported the interpretations of Stone (1962), that for at least three genera in the Blechnaceae and Hymenophylloideae, the classical theory of antheridium development is correct.

In a study on gametophytes in the Schizaeaceae, Bierhorst (1975) provided supporting evidence for the classical theory of antheridium development. He determined that the cap cell in *Actinostachys* antheridia is formed by a funnel-shaped wall and that the wall begins as a
phragmoplast between the telophase nuclei and proceeds to encircle the nucleus of the future cap cell.

Schraudolf and Richter (1978) are the only workers to present ultrastructural data on fern antheridia. In Polypodium and Platycerium (Polypodiaceae), they found plasmodesmata transversing the wall where the funnel-shaped wall is in contact with the basal wall of the antheridium. If, as Davie (1951) contended, the funnel-shaped wall is secondarily formed, plasmodesmata would not traverse the wall in the area of contact.

Plasmodesmata also traversed the contact wall between the cap cell and the central, spermatogenous cell, indicating that the cap cell also is formed initially by a funnel-shaped wall (Schraudolf and Richter, 1978).

The last published information on antheridium morphology was provided by Leung and Näf (1979), using Onoclea sensibilis. Their observations were made from whole mounts of gametophytes, fixed and stained with pyronin Y and methyl green. According to Leung and Näf, the antheridium initials of Onoclea arise as hemispherical outgrowths. The first cell wall formed in the initial is funnel-shaped, according to the classical theory. They felt, however, that the wall forms in a vertical manner, from the base to the rim. They suggested that as the funnel-shaped wall forms, it increases in diameter and height until the circular, upper rim intersects the mid-region of the outer, curved wall of the initial. This contradicts the interpretation by Schraudolf (1968), who claimed that the funnel-shaped wall forms laterally beginning at one side of the initial and circling around until the edges meet.
Leung and Naf (1979) then concluded that the second wall is formed transversely, according to Davie's theory. They claimed that the wall first forms from a transversely oriented phragmoplast in the upper region of the upper cell, then moves down to complete cell wall formation, and finally is pushed back up to the original position of the phragmoplast by internal pressures. Speculations on the genesis of the second wall are lacking in previous studies.

Leung and Naf (1979) were unable to follow the formation of the cap cell wall, but did observe that the equator of the spindle apparatus was oriented perpendicularly to the upper and lower walls of the upper jacket cell. This position was previously noted by Stone (1961), Schraudolf (1968), and Bierhorst (1975), who all claimed that the subsequent funnel-shaped wall begins as a phragmoplast between the telophase nuclei and proceeds to form in a lateral, circular fashion around the nucleus of the future cap cell. Leung and Naf (1979), contended that the wall probably forms simultaneously in all areas, as suggested by Kny (1869).

Most of the studies on antheridium development have dealt mainly with the orientation of the cell walls. Only a few of the studies mentioned the concomitant cytoplasmic changes which occur during development. Stone (1958, 1961) noticed a dense accumulation of cytoplasm in the region of the forming initial in Polyphlebium and Blechnum. Verma and Khullar (1966) indicated in their drawings that the initial protuberance was densely cytoplasmic. Bierhorst (1975) reported an accumulation of cytoplasm in the bulge that formed the initials in Actinostachys. Similarly, in Onoclea, Leung and Naf (1979) found that
cytoplasm rich in RNA accumulates ahead of the mother nucleus which forms the initial.

In the expanded initials, Stone (1961, 1962) found a polar distribution of cytoplasm resulting in a large vacuolate region in the basal portion of the cell. Leung and Nâf (1979) reported the presence of a "bouquet" of RNA anterior to the nucleus in the initials of Onoclea. Stone (1961), Schraudolf (1968), and Leung and Nâf (1979) all noted that the nucleus in the initial appears much larger than the nucleus in the vegetative mother cell.

The only other reports of cytoplasmic changes during development pertain to the upper cell in the two-celled antheridium. Stone (1961, 1962) reported a polar distribution of cytoplasm in the upper cell, with a vacuolate region occurring in the anterior portion. Leung and Nâf (1979) found that a "bouquet" of RNA reappears and is located in the basal portion of the upper cell. Atkinson (1894) and Verma and Khullar (1966) both presented drawings which indicate that a dense region of cytoplasm occurs in the basal region of the upper cell.

Spermatogenesis

Spermatogenesis can be divided arbitrarily into two phases, the divisional phase and the differentiation phase. During the divisional phase, the central spermatogenous cell undergoes a number of mitoses to form a specified number of spermatid cells. During the differentiation phase, the spermatid cells are transformed into spermatozoids.

Very little information is available on cellular changes during
the divisional phase. Reportedly, antheridia of advanced, leptosporangiate ferns produce either 16 or 32 spermatids (Bell and Duckett, 1976; Bower, 1923; Eames, 1936; Foster and Gifford, 1974; Nayar and Kaur, 1971). Anemia was reported to produce 8, 16, or 32 spermatids (Voeller and Weinberg, 1967). The minimum number of spermatids produced per antheridium was reported for 5 species of Drynaria (Polypodiaceae) with 4-8 spermatids (Nayar, 1965).

Bierhorst (1975) claimed that the spermatogenous cells divide synchronously. According to Atkinson (1894), Campbell (1886), and Stone (1961), the first three sets of mitoses follow a consistent pattern, with the first division oriented longitudinally, the second set of mitoses oriented longitudinally and perpendicularly to the first division, and the third set of mitoses oriented transversely to all the previous divisions resulting in 8 spermatogenous cells. Bower (1923) and Campbell (1913) claimed that the second set of mitoses is oriented transversely, while the third set is oriented longitudinally and perpendicularly to the first longitudinal division.

Most ultrastructural studies involving spermatogenesis have pertained only to the differentiation phase (Bell, 1979b; Duckett, 1975), but Duckett (1975) also noted some changes during the divisional phase. The few changes mentioned included a change in the appearance of the mitochondria, the dedifferentiation of the chloroplasts into proplastids, and a change in Golgi body activity correlated with the cell cycle.

The transformation of spermatids into spermatozoids involves many
profound changes within the cell. The angular, meristematic spermatids round up, much of their cytoplasm degenerates, the nuclear chromatin condenses, the nucleus elongates and coils into 2-3 gyres, and numerous flagella form. The ultrastructure of these events has been well-documented in the homosporous ferns, _Pteridium_, _Dryopteris_, and _Ceratopteris_ (Bell, 1974, 1979b; Bell and Duckett, 1976; Duckett, 1975; Duckett et al., 1979; Schedlbauer et al., 1973) and in one heterosporous fern, _Marsilea_ (Hepler, 1976; Mizukami and Gall, 1966; Myles, 1979; Myles and Bell, 1975; Myles and Hepler, 1977).

Because the differentiation phase of spermatogenesis was not an integral part of this study, events during this phase are not detailed in the literature review. The literature pertinent to the differences found in _Onoclea_ spermatogenesis are incorporated into the Discussion.
MATERIALS AND METHODS

Culture Conditions

Spore collection

Fertile fronds of *Pteridium aquilinum* (L.) Kuhn were collected along a trail in Backbone State Park near the Primitive Camping Upper Area, Delaware Co., Iowa, on June 29, 1980. The fronds were placed in large manila envelopes to dry. The envelopes were pressed manually to dislodge the spores from the sporangia and were collected in a glass vial and stored in a refrigerator at 4°C.

Fertile fronds of *Onoclea sensibilis* L. were collected from a small, dense stand of plants growing along the east side of County Road S70 about 7 miles north of Highway 30, Story Co., Iowa, on November 14, 1980. The fronds were soaked in a dilute solution of Tween 80 (2 drops/100 ml) for 2 min and rinsed twice in double distilled water (d.H₂O). They were then sterilized in 10% Clorox for 3 min, rinsed twice in sterilized d.H₂O and once in sterilized tap water. The fronds were placed in glassine envelopes to dry. The amount of pressure necessary to separate the spores from the sporangia resulted in a mixture of spores and fine pieces of sporangium walls and fronds. That mixture was sieved through a double layer of 0.37 mm wire mesh onto a piece of weighing paper. To remove the very fine pieces of nonspore tissue still present, the weighing paper was agitated and tilted. It was found that the spores were more strongly attracted to the paper than the nonspore material. The spores could then be collected and stored at 4°C.
**Spore sowing and transferring**

All spores were sown on culture medium containing 8.0 gm of Difco Bacto Agar per liter of nutrient medium composed of Bold's macronutrients (Bold, 1967), Nitsch's micronutrients (Nitsch, 1951), and a supplement of ferric chloride (1 drop of 1% FeCl₃/liter; Appendix C). The medium was autoclaved for 15 min and poured into sterile, disposable, plastic petri plates to solidify.

If the spores were to be sown steriley, 0.015 gm of *Pteridium* or *Onoclea* spores were placed in a 15 ml tapered centrifuge tube with 4 ml of the previously described dilute Tween 80 solution and capped with a piece of aluminum foil. After shaking for 1 min, the suspension was centrifuged for 1 min, until the spores formed a pellet at the bottom of the tube. The supernatant was pipetted off and the spores were rinsed twice with d.H₂O, using the centrifugation procedure. The second d.H₂O rinse was replaced with tap water for 24 hr at room temperature to allow fungal spores to germinate. The spores were then spun down into a pellet and the tap water removed, being replaced by a 2% filtered solution of calcium hypochlorite. The centrifuge tube was shaken vigorously for 30 sec and then centrifuged. The sterilant was pipetted off and replaced by sterile d.H₂O. The spores were in the sterilant for less than 2 min. Following two rinsings in sterile d.H₂O, the spores were immersed in 10 ml of Bold and Nitsch nutrient medium.

Multispore cultures of *Pteridium* gametophytes were obtained by pipetting 1 ml aliquots of the spore suspension onto the culture medium contained in 100 x 15 mm petri plates. This method of sowing is termed liquid-sowing and for this study, liquid-sowing also implies a 1 day
incubation period in tap water. This procedure and all other procedures requiring sterile techniques were performed within a Plexiglas, sterile, transfer hood.

Multispore cultures of Onoclea gametophytes were obtained by pipetting 0.5 ml aliquots of spore suspension onto agar medium contained in 60 x 20 mm petri plates.

All Pteridium cultures were sown sterilely. For comparison purposes, Onoclea spores were sown both sterilely and unsterilely. Two unsterile sowing methods were used. One method followed the sterilization procedure described above with the omission of the calcium hypochlorite step (liquid-sowing). The other method involved sealing dry spores in glassine envelopes. Small holes were made in the envelope with a straight pin and the spores were tapped onto the agar medium (dry-sowing).

In some preliminary experiments, Onoclea gametophytes or germinating spores were transferred from multispore cultures onto agar in either 60 x 20 mm petri plates, where each plate received nine gametophytes spaced 1 cm apart, or onto agar in 100 x 15 mm petri plates, where each plate received 24 gametophytes spaced 1 cm apart. These resultant cultures are referred to as "transferred multispore cultures." The germinating spores consisted of a small rhizoid protruding from the spore wall. The gametophytes that were transferred were composed of three to six prothallial cells and one basal rhizoid. Only one stage was present per plate. Transfers were made with the aid of a sterilized dissecting needle and a Bausch and Lomb dissecting microscope fitted with a 20X objective lens.
Both the multispore and transferred multispore cultures were placed in continuous fluorescent light at an intensity of 350 ft. c. To prevent the agar from drying out too rapidly, the plates were placed in clear plastic vegetable crispers.

**Antheridiogen (A\textsubscript{pt}) collection**

Eighteen multispore cultures of *Pteridium*, sown on September 24, 1980, were collected on November 15, 1980 and frozen overnight. After thawing, the resultant liquid fraction was collected, Millipore-filtered using HA, 0.45 \textmu m filters, and autoclaved for 15 min at 15 psi. This sterile liquid extract contained A\textsubscript{pt} and in this undiluted form, it is referred to as 100% A\textsubscript{pt}. Dilutions were made by adding portions of the extract to various quantities of sterile nutrient medium.

**A\textsubscript{pt} treatments**

To obtain background information on the biology of both treated and untreated *Onoclea* gametophytes in culture, the following preliminary experiments were performed.

Transferred multispore cultures of young gametophytes were treated with 2 ml of either 100%, 10%, 1%, 0.1%, or 0.01% A\textsubscript{pt} 3 days after spore transfer to determine a general optimal range of A\textsubscript{pt} concentrations.

Transferred multispore cultures of either germinating spores or young gametophytes were treated with 1 ml of 100% or 50% A\textsubscript{pt} to compare the effects of treatment on two different developmental stages of gametophytes. In order to determine the best means of application, the A\textsubscript{pt} was applied to the media of the transferred cultures either 2 days before transfer and allowed to soak into the agar or it was applied to
the cultures on the day of transfer.

The following preliminary experimental conditions were designed to determine the optimal concentration of the antheridiogen, the optimal time of application, and the effects of spore sterilization on untransferred spore cultures. For each experimental condition, plates of untreated gametophytes were used as controls.

1) Sterilized multispore cultures were treated with 1 ml of either 100%, 50%, 25%, 10%, or 1% A<sub>pt</sub> 4 days after sowing.

2) Sterilized multispore cultures were treated with 1 ml of 25% A<sub>pt</sub> on the day of sowing or 6 days after sowing.

3) Unsterilized multispore cultures were treated with 1 ml of 25% A<sub>pt</sub> on the day of sowing.

After observing the growth and reproductive responses of the cultured gametophytes in the preliminary experiments, six different culture and treatment conditions were selected to analyze comparative growth data in detail. The six different conditions include the following:

1) Sterilized multispore cultures treated with 1 ml of 25% A<sub>pt</sub> on the day of sowing,

2) Sterilized multispore cultures treated with 1 ml of 25% A<sub>pt</sub> 4 days after sowing,

3) Sterilized multispore cultures untreated,

4) Unsterilized multispore cultures, sown according to the procedure used for sterilized spores minus the step involving the sterilant, untreated,

5) Unsterilized multispore cultures, sown by tapping spores from glassine envelopes onto dry agar, treated with 1 ml of 25%
on the day of sowing, and

6) Unsterilized multispore cultures, sown as described for condition 5, untreated.

For the sake of clarity, a diagram of the derivation of the six culture conditions is shown in Figure 3.

Gametophytes were collected from these cultures on certain days and cleared. The gametophyte clearing technique is described in Appendix D. The number of gametophytes collected and the days on which they were collected are summarized in Table D.1.

Gametophytes fixed for light microscopy (LM), transmission electron microscopy (TEM), or scanning electron microscopy (SEM) were all collected from sterilized multispore cultures that were treated with 1 ml of 25% A<sub>pt</sub> 4 days after sowing (condition 2).

Light Microscopy (LM)

Cleared gametophytes

Semipermanent slides were made of cleared gametophytes, collected from the six different culture conditions, by the following procedure. The gametophytes were placed into a drop of 1% aqueous acid fuchsin on a slide and cover-slipped. A few drops of Hoyer's clearing solution were added to one edge of the cover slip and drawn slowly through the stain with a tissue wick placed at the opposite edge of the cover slip (Appendix D).

Within the next few days, photographs were taken of the cleared gametophytes on a Leitz Wetzlar Ortholux research microscope equipped
Onoclea spores

Sterilized

Unsterilized

Liq. Sow.

Dry Sow.

Tr. day 0

Tr. day 4

Untr.

Tr. day 0

Tr. day 4

Untr.

Code:  
Liq. Sow. — Liquid sowing of spores with 0.5 ml of nutrient medium  
Dry Sow. — Dry sowing of spores by tapping them from envelopes onto agar  
Tr. — Treated with 1 ml of 25% A₆₇₇₉  
Untr. — Untreated  
Tr. day 0 — Treated same day as sowing  
Tr. day 4 — Treated 4 days after sowing

Figure 3. Derivation of the six culture and treatment conditions used for gametophyte clearings
with phase-contrast optics. Kodak Plus-x ASA 125 and Pan-x ASA 32 film were used in the attached Leitz Orthomat automatic camera. A living gametophyte, prior to being cleared, is shown in Figure 4. A cleared and stained gametophyte is shown in Figure 5.

A sketch was made of each gametophyte before it was photographed, and antheridium locations and the number of nuclei present in each antheridium were noted. It was necessary to focus through the antheridia to determine the number of nuclei present, which could not, in all cases, be determined from the photographs. The information from each sketch was transferred to the corresponding photograph. The photographs were traced onto transparent acetate sheets with a water-resistant, felt-tipped pen to clarify the position of the prothallial cell walls, the location of antheridia, and the number of cells present in each antheridium. A tracing of the cleared gametophyte shown in Figure 5 is shown in Figure 6. From these drawings, the following growth data were collected: the total number of prothallial cells per gametophyte, the total number of antheridia per gametophyte, the number of cells within each antheridium, the relative location of each antheridium (Figure 7), and the area of each gametophyte. Methods of data collection, analyses, and presentation are discussed in Appendix D.

**Cytochemistry**

In order to locate the nuclear DNA and the cytoplasmic RNA within prothallial and antheridial cells of gametophytes, a combination stain of methyl green and pyronin Y (modified from Long and Taylor, 1956) was used according to the following procedure outlined in detail in Ap-
Figure 4. Living gametophyte showing four antheridia (arrows) located below notch meristem. X 190

Figure 5. Gametophyte cleared and stained with acid fuchsin. Dark arrows indicate a 2-celled and a 4-celled antheridium. Clear arrow indicates a trichome. X 200

Figure 6. Line drawing of cleared gametophyte shown in Figure 5. The thick-lined circles indicate antheridium locations and numbers within circles represent number of cells present in antheridium. A measuring axis is superimposed on drawing to illustrate method of determining relative locations of antheridia. Y-axis is aligned with central, longitudinal axis of gametophyte and coordinate point X ≈ 0, Y ≈ 0 corresponds to center point of gametophyte

Figure 7. A standardized, unit axis on which relative coordinate points are plotted. Relative coordinate point for 2-celled antheridium shown in Figure 6 is X ≈ -1/2, Y ≈ +1/2. Actual coordinate of that antheridium is X ≈ -18, Y ≈ +20
Appendix E. Nine-day-old treated and untreated gametophytes were fixed in a 3:1 absolute ethanol-glacial acetic acid mixture for 2 hr at room temperature. They were hydrated through an ethanol dilution series. The gametophytes were stained for 45 min in methyl green/pyronin Y at room temperature. They were rinsed 4X in distilled water, 15 min each, and placed on a slide with a drop of water and a cover-slip.

Photographs were taken using the microscopic setup previously described. Kodak Ektachrome ASA 50 tungsten film was used with an 82A filter.

Azure B (modified from Flax and Himes, 1952; Jacqmard et al., 1972) was also used to observe the nucleic acid components in the cells of the gametophytes. The procedure (Appendix E) entailed fixing the treated and untreated gametophytes in 3:1 ethanol-glacial acetic acid and hydrating to water through an ethanol dilution series. The gametophytes were then placed in a solution of azure B for 2 hr at 50°C. After four rinses in water, 15 min each, the gametophytes were dehydrated up to absolute tertiary butyl alcohol (TBA) through a TBA series. After three changes in 100% TBA, 7 min each, they were put through a series of TBA:xylene until the gametophytes were in pure xylene. They were slowly infiltrated with the xylene-soluble, mounting medium Picoxyte, then placed on slides with pure Picoxyte and cover-slipped to form permanent slides.

Resin embedded material

One to two weeks after treatment, gametophytes were collected and fixed with either 3% glutaraldehyde in a phosphate buffer (0.05 M,
pH 7.0; Glauert, 1975), or with a combination of 3% glutaraldehyde and 0.5% paraformaldehyde in the same buffer (Hall, 1978).

In both cases, they were fixed for 2 hr at room temperature, rinsed in buffer 4X, 20 min each, and placed into buffered 1% osmium tetroxide for 45 min. The gametophytes were then rinsed 4X in buffer, 5 min each, and in d. H₂O twice, 5 min each (Appendix E).

Due to the small size of the prothalli, they were placed into porous, styrene baskets (Flo-Thru Specimen capsules, #9-00-09-50, American Optical Corp.) before dehydration. Dehydration to absolute ethanol consisted of a series of changes in 10% increases of ETOH beginning with 10% ETOH. After three changes in 100% ETOH, the gametophytes were tediously removed from the baskets and placed into a solution of 100% ETOH and gradually infiltrated with propylene oxide. After three changes in pure propylene oxide, they were slowly infiltrated and then embedded with Spurr's epoxy resin (Spurr, 1969).

Sections were cut at 1 to 2 μm with a glass or diamond knife on a Reichert OM-U2 ultramicrotome. To obtain a serial order of both stained and unstained sections, pairs of slides were used for mounting sections. The slides were divided into regions: regions 1, 2, and 3 occur from left to right on the upper half of the slide; regions 4, 5, and 6 occur from left to right on the bottom half of the slide.

For every six sections that were cut, three were placed in a drop of sterile distilled water in each of two corresponding regions on the pair of slides. When all six regions were filled (18 sections per slide), the slide pairs were placed on a heating plate to dry. One slide from each pair was stained with toluidine blue 0 (Appendix E).
Both the stained and unstained slides of each pair were mounted using Permount mounting medium. Unstained sections were obtained to be viewed with Nomarski interference contrast optics.

Photographs were taken on either a Leitz Wetzlar Ortholux microscope equipped with bright field optics or on a 1966 model Zeiss photomicroscope equipped with Nomarski interference contrast optics.

Transmission Electron Microscopy (TEM)

The resin embedded material prepared for LM was also used for TEM. Gray and silver sections were cut using glass and diamond knives on a Reichert OM-U2 ultramicrotome. The sections were chloroformed and collected on 300 mesh copper grids. They were stained for 30 min in methanolic uranyl acetate (Stempak and Ward, 1964), followed by 1 hr of staining in lead citrate (Reynolds, 1963).

Observations were made on an Hitachi HU-11C TEM. Images were recorded on 3-1/4 x 4 in. Dupont Cronar Ortho S Litho sheet film.

Scanning Electron Microscopy (SEM)

Except for some three-week-old gametophytes observed in the living state with SEM, the gametophytes were processed for SEM in the following way. One- to four-week-old protahalli were fixed with 3% glutaraldehyde in a phosphate buffer (0.05 M, pH 7.0) for 2 hr at room temperature and post-fixed with 1% osmium tetroxide, in the same phosphate buffer. After dehydration in an ethanol dilution series, the gametophytes were slowly infiltrated with either Freon TF(113) or amyl acetate. They were
critical point dried with liquid carbon dioxide and mounted on brass discs with silver paint. The preparations were coated with about 15 nm of gold-palladium in a Polaron E5100 sputter coating unit.

The live specimens were prepared for observation by simply removing them from culture, quickly placing them onto a piece of magnetic tape that was silver-painted to a brass disc, and immediately viewing and photographing them with a JEOL JSM-35 SEM at an accelerating voltage of 5 to 8 kV. The fixed specimens were viewed at an accelerating voltage of 20 kV.

Images were recorded on 3-1/4 x 4 in. Polaroid type 665 positive/negative film. Kodak Kodabromide paper (F series), or Kodak Polycontrast paper was used for positive prints made during this study.
RESULTS

Preliminary Experiments

Preliminary experiments revealed that the following five variable factors affect the vegetative and reproductive responses of *Onoclea* gametophytes grown in culture: 1) concentration of \(A_{pt}\), 2) method of applying \(A_{pt}\), 3) time of \(A_{pt}\) application, 4) the act of transferring gametophytes, and 5) sterilization of spores. Quantitative data were not collected from the preliminary experiments. The information reported in this section comes from casual observations. These qualitative data, reported in the following subsections, were used to determine relevant, external factors which influence gametophyte growth and reproduction. These factors were then incorporated into the design of later experiments to quantify their effects on gametophyte growth and reproduction. The quantified information is reported in the Gametophyte Growth Data section and the Antheridium Developmental Data section.

**Concentration of \(A_{pt}\)**

High concentrations of \(A_{pt}\), when applied to the transferred multisporo cultures, are more effective at inducing antheridia than lower concentrations. When the cultures are treated with 100%, 50%, 10%, 1%, 0.1%, or 0.01% \(A_{pt}\), only the 100% and 50% \(A_{pt}\) elicit a strong response in terms of the number of antheridia produced per gametophyte during the first week of treatment. The 10% \(A_{pt}\) induce far fewer antheridia per gametophyte during the same time period, while the responses to concentrations lower than 10% are negligible.

When nontransferred multisporo cultures are treated with 100%,
50%, 25%, 10%, and 1% \text{A}_{\text{pt}}$, the three highest concentrations all induce numerous antheridia per gametophyte during the first week after treatment. The lower concentrations induce far fewer antheridia per gametophyte during the same time period.

In order to conserve the amount of \text{A}_{\text{pt}} to insure that the same source was used in the entire study, the lowest concentration of \text{A}_{\text{pt}} that elicits the strongest response (25% \text{A}_{\text{pt}}) was selected to be used in the later experiments.

**Method of applying \text{A}_{\text{pt}}**

It is found that the method of applying \text{A}_{\text{pt}} affects the reproductive responses of the gametophytes in the transferred multispore cultures. If the \text{A}_{\text{pt}} is allowed to soak into the agar before the young gametophytes are transferred to it, as opposed to being applied to the surface of the agar after the gametophytes are transferred, fewer antheridia are initiated per gametophyte during the first week after treatment. For the later experiments, the method of applying the \text{A}_{\text{pt}} onto the surface of gametophytes already growing on agar is used.

**Time of \text{A}_{\text{pt}} application**

The first attempt to determine the best time to apply \text{A}_{\text{pt}} was made with transferred multispore cultures. Cultures of germinating spores and cultures of young gametophytes (3-5 prothallial cells, 1 rhizoid per gametophyte) were treated with 100% \text{A}_{\text{pt}} the day they were transferred. Germinating spores do not grow beyond the 2-celled stage. However, the young gametophytes have between 20 to 40 cells 4 days
after treatment with three to four antheridia per gametophyte. It is not known what prevents the germinating spores from growing. The nontreated, germinating spores that are transferred grow normally during the first few weeks.

To obtain more information on an optimal time to apply $A_{pt}$, nontreated, sterile, multispore cultures were treated with 25% $A_{pt}$ at different times in their development. The antheridiogen was applied to the cultures either on the day they were sown, 4 days after sowing, or 6 days after sowing. Most of the spores treated on the day of sowing or the prothalli treated 4 days after sowing, grew normally and produced 2-10 antheridia per gametophyte during the first week after treatment. However, very few gametophytes treated 6 days after sowing produce antheridia. The gametophytes that are induced have very few antheridium initials during the first week after treatment. For the later experiments, cultures are treated with 25% $A_{pt}$ either on the day of sowing or 4 days after sowing.

**Transferring gametophytes**

Gametophytes in transferred, multispore cultures grow more slowly than gametophytes in nontransferred, multispore cultures. Also, after 2-3 wk of growth in the transferred cultures, many of the gametophytes die. Few, if any, gametophytes die in the nontransferred cultures.

The primary reason for transferring the gametophytes is to obtain a group of morphologically similar gametophytes which will have the same growth rates and responses to $A_{pt}$. However, it was found that, although the gametophytes are similar on the day of transfer, they proceed to
grow at different rates and respond differently to $A_{pt}$ with respect to the number of initials produced and the location of the initials on the thalli. Lacking any advantages, transferred multispore cultures are not used in the later experiments.

**Spore sterilization**

The first observable effect of calcium hypochlorite applied for 2 min is the removal of the perispore layer of the spore. The intact perispore of an unsterilized spore is shown in Figures 8 and 9, with a typical jagged appearance, due to a reticulate pattern of deposition. Unsterilized spores are dark brown in appearance. After sterilization, the spores turn bright green revealing the internal cytoplasmic components at the LM level. SEM micrographs of sterilized spores reveal the disappearance of much of the reticulate material of the perispore (Figures 10 and 11). The exine layer below the perispore is shown in Figures 12 and 13 after the perispore was physically cracked off of a nonsterilized spore.

If the spores are sterilized for less than 2 min, there is no observable change in the morphology of the gametophytes, although the gametophytes appear to grow much more slowly than gametophytes in unsterilized cultures. If the spores are sterilized for more than 2 min, morphological abnormalities do occur in which the typical cordate shape of the thallus becomes highly branched. The branched gametophytes, however, are still capable of initiating antheridia in response to $A_{pt}$. In the later experiments, both sterilized and unsterilized spores are sown to produce multispore cultures, in order to quantify the ef-
Figures 8-9. Unsterilized spore of *Q. sensibilis* showing jagged, peaked appearance of outer, perispore layer

8. X 1,118

9. X 8,250

Figures 10-11. Sterilized spores of *Q. sensibilis* revealing disappearance of much of perispore layer

10. X 690

11. X 10,200

Figures 12-13. Unsterilized spore of *Q. sensibilis* showing exine layer below perispore layer which has been physically cracked off

12. X 1,070

13. X 10,320
fects of spore sterilization.

In the preliminary experiments, the unsterilized spore cultures were sown by tapping the spores onto dry agar from glassine envelopes (dry-sown). The sterilized spores were sown suspended in a liquid solution after one day of incubation in tap water (liquid-sown). For a more direct comparison between sterilized and unsterilized spore cultures in later experiments, unsterilized spores were also sown suspended in liquid after one day of incubation in tap water. However, this liquid-sowing of unsterilized spores applied only to the cultures that were not treated with $A_{pt}$. The unsterilized spore cultures that were $A_{pt}$-treated were dry-sown and immediately treated with 1 ml of 25% $A_{pt}$. Although this procedure superficially transforms the dry-sown cultures into liquid-sown cultures, they are recognized as being different from the other liquid-sown cultures due to the fact that they lack a one-day incubation in tap water. The effects of an incubation period versus no incubation period were investigated by comparing data from dry-sown, unsterilized spore cultures and liquid-sown, unsterilized spore cultures.

Gametophyte Growth Data

Quantitative data are obtained from cleared gametophytes collected daily to investigate the effects of the following variable factors on gametophyte growth: 1) dry-sowing (no incubation period) versus liquid-sowing (one-day incubation period), 2) spore sterilization versus no sterilization, 3) $A_{pt}$ treatment (day 0) versus no treatment of unsterilized spore cultures, 4) $A_{pt}$ treatment (day 0) versus no treatment
of sterilized spore cultures, and 5) treatment (day 4) versus no

treatment of sterilized spore cultures. The effects of the variable

factors were studied in depth in order to determine an optimal culture

and treatment regime for growing gametophytes to be processed and sec-

tioned for viewing with LM, TEM, and SEM.

The results presented in this section are expressed in terms of

the average number of vegetative cells produced per gametophyte, the

average total number of cells (vegetative and antheridial) produced per

gametophyte, and the average area formed per gametophyte. These data

are obtained directly from drawings of cleared gametophytes collected
daily from the six culture conditions.

Mean values and confidence intervals (0.01 significance level)
determined for these expressions of growth rates are summarized in Ap-

pendix F, Tables F.1-F.6. The t-test for unpaired observations and

equal variances is used as a test for significant differences between

means at the significance level of either 0.05 or 0.01. If the t-test

is used, the significance level is given. In some cases, linear regres-

sion analysis is used. The results are presented in the form of regres-

sion equations and $r^2$ values.

Effects of no incubation versus one-day incubation

To compare the effects of dry-sowing (no incubation period)

versus liquid-sowing (one-day incubation period), gametophytes were col-

lected from untreated, unsterilized spore cultures that were either
dry-sown or liquid-sown (culture conditions 4 and 6). The method of
sowing does not affect the average number of vegetative cells produced per gametophyte until after 6 days in culture (Figure 14). After 5 days of growth, the gametophytes grown in liquid-sown cultures have an average of 18.9 cells compared to 18.5 cells per gametophyte grown in dry-sown cultures. From 6-11 days in culture, the average number of cells per gametophyte grown in liquid-sown cultures is significantly higher than in dry-sown cultures ($P < 0.01$). After 11 days in culture, the average number of cells per gametophyte from liquid-sown cultures is 231.4 cells compared to 186.0 cells per gametophyte from dry-sown cultures.

There is no significant difference, however, in the average area per gametophyte from dry-sown versus liquid-sown cultures during the first 11 days in culture (Figure 15). After 5 days in culture, the average area per gametophyte grown in liquid-sown cultures is $0.021 \text{ sq mm (mm}^2\text{)}$ and the average area per gametophyte grown in dry-sown cultures is $0.019 \text{ mm}^2$. After 11 days in culture, the averages are $0.214$ and $0.215 \text{ mm}^2$, respectively.

The relationship between the number of cells per gametophyte and the area per gametophyte is analyzed using linear regression analysis. For gametophytes grown in liquid-sown cultures, the equation for the regression line is $Y = 918X + 2030$ with an $r^2$ value of 0.9779. For gametophytes grown in dry-sown cultures, the equation for the regression line is $Y = 1168X - 5084$ with an $r^2$ value of 0.9771. In each culture condition, the area of the gametophyte is highly dependent on the number of cells produced per gametophyte.

Although the effects of an incubation period are minimal, the
Figures 14-17. Vegetative gametophyte growth data

14. Average number of vegetative cells per gametophyte in liquid-sown versus dry-sown cultures, unsterilized and untreated (culture conditions 4 and 6)

15. Average area per gametophyte in liquid-sown versus dry-sown cultures, unsterilized and untreated (culture conditions 4 and 6)

16. Average number of vegetative cells per gametophyte in sterilized versus unsterilized, untreated cultures (culture conditions 3 and 4)

17. Average area per gametophyte in sterilized versus unsterilized, untreated cultures (culture conditions 3 and 4)
tendency for incubated cultures to produce gametophytes with slightly more cells per unit area is considered when analyzing data from A^-treated, sterile and unsterile spore cultures that are either liquid-sown or dry-sown, respectively (culture conditions 1 and 5).

**Effects of spore sterilization**

To determine the effects of spore sterilization on gametophyte growth rates, data are obtained from untreated, liquid-sown gametophytes grown from either sterilized spores or unsterilized spores (culture conditions 3 and 4). Spore sterilization strongly affects the average number of vegetative cells produced per gametophyte during the first eleven days in culture (Figure 16). The gametophytes grown from sterilized spores have significantly fewer cells ($P < 0.01$) than gametophytes grown from unsterilized spores. Four days after sowing, the gametophytes from sterilized spore cultures have an average of only 4.5 cells compared to 10.6 cells per gametophyte grown in unsterilized spore cultures. Seven days later, the averages are 91.1 and 231.4 cells, respectively. The gametophytes from unsterilized spore cultures have an average of more than 2.5 times as many cells as gametophytes from sterilized spore cultures after 11 days of growth.

The average area per gametophyte during the same time period is graphed in Figure 17. The average area per gametophyte grown in sterilized spore cultures is significantly smaller ($P < 0.01$) than the average area per gametophyte grown in unsterilized spore cultures. After 4 days in culture, the average area of gametophytes grown in sterilized spore
cultures is 0.007 mm\(^2\) as compared to 0.013 mm\(^2\) per gametophyte grown in unsterilized spore cultures. Seven days later, the averages are 0.021 and 0.100 mm\(^2\), respectively.

The relationship between the number of vegetative cells per gametophyte and the area per gametophyte is analyzed using linear regression analysis. For gametophytes grown in sterilized cultures, the equation for the regression line is \(Y = 1078X + 197\) with an \(r^2\) value of 0.952. For gametophytes grown in unsterilized cultures, the equation of the regression line is \(Y = 918X + 2030\) with an \(r^2\) value of 0.978. In each culture condition, the area of the gametophyte is highly dependent on the number of cells produced per gametophyte.

In view of the dependency of gametophyte area on cell number and the significant difference between each of the corresponding variables due to sterilization versus unsterilization, the inhibition of gametophyte growth in sterilized cultures is attributed to an inhibition of cell division.

**Effects of Apt treatment (day 0) versus no treatment of unsterilized spore cultures**

Within the first 4 days of growth, there is no significant difference in the average number of vegetative cells per treated or untreated gametophytes (culture conditions 4 and 5; Figure 18). From 5-11 days in culture, there are significantly fewer vegetative cells per treated gametophyte compared to untreated gametophyte (\(P < 0.01\)). After 5 days in culture, treated gametophytes have an average of 13.94 vegetative cells compared to 18.90 vegetative cells per untreated gametophyte. After 11 days in culture, the averages are 101.50 and 231.40 cells, respectively.
Figures 18-21. Gametophyte growth data

18. Average number of vegetative and total cells per gametophyte in \( A_{pt} \)-treated versus untreated, unsterilized cultures (culture conditions 4 and 5). \( A_{pt} \) treatment occurred on day 0

19. Comparison of average areas per gametophyte in treated and untreated, sterilized and unsterilized cultures (culture conditions 1-5)

20. Average number of vegetative and total cells per gametophyte in treated versus untreated, sterilized cultures (culture conditions 1 and 3). \( A_{pt} \) treatment occurred on day 0

21. Average number of vegetative and total cells per gametophyte in treated versus untreated, sterilized cultures treated four days after sowing (culture conditions 2 and 3)
Treating unsterilized spore cultures on day 0 strongly inhibits the number of vegetative cells per gametophyte compared to unsterilized spore cultures from 5-11 days in culture (Figure 18).

Spore sterilization and A treatment on day 0 appear to have similar inhibitory effects on the number of vegetative cells produced per gametophyte. The number of vegetative cells per gametophyte from treated, unsterilized cultures is not different than from untreated, sterilized cultures (compare Figures 16, 18).

The average areas per treated and untreated gametophytes grown in unsterilized cultures are not significantly different until 7 days after sowing (Figure 19). From 7-11 days after sowing, the average area of treated gametophytes is significantly smaller (P < 0.01).

By day 11, the average area per gametophyte in untreated, unsterilized cultures is 0.214 mm² compared to 0.134 mm² for treated, unsterilized cultures (Figure 19).

The average area per treated, unsterilized gametophytes is slightly greater than the average area per untreated, sterilized gametophytes during most of the time between 4-11 days in culture (P < 0.01; Figure 19). Although spore sterilization and A treatment on day 0 appear to have similar inhibitory effects on the number of vegetative cells produced per gametophyte, it appears that spore sterilization inhibits the area formed per gametophyte slightly more than treatment of A on day 0.

To determine the relationship between the area and the number of vegetative cells per gametophyte from unsterilized cultures treated on day 0, the data are analyzed using linear regression analysis. The equation for the regression line is \( Y = 1270.81X + 1045.64 \) with an
$r^2$ value of 0.9624. The area per gametophyte, thus, is highly dependent on the number of vegetative cells produced per gametophyte, as it was with untreated gametophytes in sterile or unsterile cultures.

Due to the dependency of the area on cell numbers and to the significant difference between these corresponding variables in treated and untreated cultures, the inhibition of gametophyte growth in treated cultures is attributed to an inhibition of the rate of cell division.

The number of total cells per treated gametophyte becomes significantly higher than the number of vegetative cells per treated gametophyte 5 days after sowing and treatment ($P < 0.01$; Figure 18). The number of total cells per treated gametophyte does not exceed the number of vegetative cells per untreated gametophyte until after 8 days in culture. By 11 days in culture, there is an average of 378.30 vegetative and antheridial cells per treated gametophyte compared to 231.40 vegetative cells per untreated gametophyte. Although treatment with $A_{pt}$ significantly inhibits the cell divisions of vegetative cells compared to untreated gametophytes, the number of total cell divisions in treated gametophytes is equal to or greater than the number of total cell divisions in untreated gametophytes.

**Effects of $A_{pt}$ treatment (day 0) versus no treatment of sterilized spore cultures**

Figure 20 shows the effects of treatment day 0 versus no treatment of sterilized spore cultures (culture conditions 1 and 3). During the first 5 days in culture, there are slightly more vegetative cells per treated gametophyte than untreated gametophyte ($P < 0.01$). The average number of vegetative cells per treated gametophyte 4 days after sowing was
9.70 compared to 4.50 per untreated gametophyte. From 6-11 days in culture, there is no significant difference in the average number of vegetative cells per treated or untreated, sterilized gametophytes. The number of vegetative cells per treated, sterilized gametophytes is also very similar to treated, unsterilized gametophytes (Figures 18, 20). Thus, although sterilization and A treatment both strongly inhibit the number of vegetative cells produced per gametophyte, their inhibitory effects are not in any degree additive or synergistic (Figures 18, 20).

The average area per treated gametophyte is similar to the area per untreated gametophyte until after 11 days in sterile culture (Figure 19). On day 11, the average area per untreated gametophyte (0.100 mm$^2$) is larger than the average area per treated gametophyte (0.079 mm$^2$; $P < 0.01$). Just as the inhibitory effects of spore sterilization and A treatment are not in any degree additive or synergistic with respect to the number of vegetative cells produced per gametophyte, they are also not additive or synergistic with respect to the area formed per gametophyte during the first 10 days of culture (Figure 19).

As in treated, unsterilized cultures, the area per gametophyte is highly dependent on the number of vegetative cells produced per gametophyte in treated, sterilized cultures. The regression equation is $Y = 854.11X + 4171.67$ with an $r^2$ value of 0.962.

The number of total cells per treated gametophyte becomes significantly higher than the number of vegetative cells per treated gametophyte 6 days after sowing and treatment ($P < 0.01$; Figure 20). This is similar to the increase in total cells versus vegetative cells per treated gametophyte observed after 5 days in unsterilized spore cultures (Figure 18).
The number of total cells per treated gametophyte exceeds the number of vegetative cells per untreated gametophyte 4 days from sowing and treatment (Figure 20), which is about 6 days earlier than in unsterilized cultures (Figure 18). By 9 days after sowing, treated gametophytes have an average of 161.70 total cells and 56.8 vegetative cells compared to 53.7 vegetative cells per untreated gametophytes (Figure 20). The number of total cells per treated gametophyte from sterilized spore cultures is always equal to or greater than the number of vegetative cells per untreated gametophyte, as is the case with treated and untreated gametophytes from unsterilized spore cultures.

There is not a large difference in the number of total cells per treated gametophyte between sterilized and unsterilized cultures (Figures 18, 20). On day 5 and day 7, there are slightly more cells per treated gametophytes from unsterilized cultures compared to sterilized cultures. On day 5, the averages are 18.30 and 13.95 cells, respectively, and on day 7, the averages are 44.40 and 37.50 cells, respectively. These differences are significant at the 0.01 significance level. When the average number of total cells per gametophyte from sterilized cultures on day 9 (161.70) is compared to the averages from unsterilized cultures on day 8 (113.90) and day 10 (223.60), it is found that these three mean values occur along a common line. This indicates that after day 7, the number of total cells per gametophyte from either sterilized or unsterilized spore cultures is not different for corresponding days.

Although spore sterilization and A_{pt} treatment both inhibit vegetative cell divisions, after 1 wk of growth in treated cultures,
the number of total cell divisions per either sterilized or unsterilized gametophyte is similar to vegetative cell divisions in uninhibited gametophytes from untreated, unsterilized cultures (Figures 18, 20).

**Effects of Apt treatment (day 4) versus no treatment on sterilized spore cultures**

Figure 21 shows the effects of treating sterilized spore cultures 4 d after sowing versus not treating sterilized spore cultures (culture conditions 2 and 3). There is no difference in the number of vegetative cells per gametophyte grown in either treated or untreated cultures until 7 days after sowing. From 7-11 days, there are significantly fewer vegetative cells per gametophyte in treated cultures (P < 0.01). By day 11, there is an average of 69.00 vegetative cells per gametophyte from treated cultures compared to 91.10 vegetative cells for untreated cultures. This observation differs from the observation made from sterilized spore cultures treated on day 0, which shows that there is no significant difference in the number of vegetative cells per treated gametophyte compared to untreated gametophyte (Figure 20). Whereas treatment of sterilized spore cultures on day 0 does not cause an additional inhibition to the production of vegetative cells per gametophyte already inhibited by spore sterilization, the treatment of sterilized spore cultures on day 4 does appear to cause an additional inhibition of vegetative cell numbers (Figure 21). The average area per gametophyte treated day 4, though, is not different from the average area per untreated sterilized gametophyte within the first 11 days of culture (Figure 19). Thus, it appears that there is slightly more cell enlargement in gametophytes treated day 4 as opposed to untreated,
sterilized gametophytes.

Linear regression analysis is used to determine the relationship between the area per gametophyte treated on day 4 and the number of vegetative cells produced per gametophyte. The equation of the regression line is $Y = 1271.79X + 935.36$ with an $r^2$ value of 0.9546. As in all previous cases, the area per gametophyte treated day 4 is highly dependent on the number of vegetative cells produced per gametophyte.

Figure 21 also shows that the number of total cells per gametophyte treated on day 4 becomes significantly higher than the number of vegetative cells per treated gametophyte 7 days after sowing, or 3 days after treatment ($P < 0.01$). This is in comparison to the increase of total cells over vegetative cells per gametophyte by 5 and 6 days after sowing and treatment day 0 in unsterilized and sterilized cultures, respectively.

The initial rate of total cell division is higher during the first 7 days after treatment day 4 compared to treatment day 0. After 7 days from the day of treatment, gametophytes from sterilized spore cultures treated on day 4 have an average of 192.15 total cells and 69.00 vegetative cells, whereas the gametophytes from sterilized cultures treated on day 0 have an average of only 37.50 total cells and 25.00 vegetative cells. The gametophytes from unsterilized cultures treated on day 0 have an average of 44.40 total cells and 25.40 vegetative cells.

Figure 21 shows that the number of total cells per gametophyte treated on day 4 exceeds the number of vegetative cells per untreated gametophytes 9 days after sowing, or 5 days after treatment ($P < 0.01$). This is in comparison to the increase occurring 4 and about 10 days after
sowing and treatment day 0 in sterilized and unsterilized cultures, respectively (Figures 18, 20). The average number of total cells per gametophyte treated day 4 is equal to or greater than the number of vegetative cells per untreated gametophyte, as is the case with sterilized and unsterilized cultures treated day 0.

After 11 days of growth, it appears that the number of total cells per gametophyte treated day 4 will equal or exceed the number of vegetative cells per gametophyte in untreated, unsterilized spore cultures (Figures 18, 21). This observation, along with similar observations for the other culture conditions, suggests that although spore sterilization and Aₚt treatment both inhibit vegetative cell divisions, the number of total cell divisions in treated gametophytes eventually equals or exceeds the number of vegetative cell divisions in uninhibited gametophytes from untreated, unsterilized cultures.

Summary of the gametophyte growth data

Due to the extensive amount of quantitative data presented on the effects of various factors on gametophyte growth, the pertinent results are summarized as follows:

Effect of dry-sowing versus liquid-sowing Gametophytes grown in liquid-sown, unsterilized spore cultures (one-day incubation) appear to have more vegetative cells per unit area after 7 days in culture compared to gametophytes grown in dry-sown, unsterilized spore cultures (no incubation). This information was originally obtained to facilitate interpreting the data from later experiments involving treated, sterilized spore cultures (liquid-sown) compared to treated, unsterilized
spore cultures (dry-sown).

But in these latter, comparative cases, the number of vegetative cells per gametophyte in dry-sown cultures was either equal to or higher than for gametophytes in liquid-sown cultures, thus rendering the preliminary information unapplicable.

**Inhibitors of vegetative growth** Spore sterilization strongly inhibits the growth of untreated gametophytes. This inhibition is attributed to an inhibition of vegetative cell division.

A treatment day 0 inhibits the number of vegetative cells per gametophyte in unsterilized spore cultures to the same degree that spore sterilization inhibits vegetative cell divisions of untreated gametophytes. Treatment does not inhibit the area formed per gametophyte from unsterilized spore cultures as much as spore sterilization inhibits the area formed per untreated gametophyte, although the effects are similar.

The inhibitory effects of spore sterilization and treatment are not additive with respect to the growth of gametophytes in sterilized spore cultures treated day 0. Treatment on day 4 causes a slight, additional decrease in the number of vegetative cells per gametophyte in sterilized spore cultures.

Gametophytes from sterilized and unsterilized spore cultures treated day 0 had similar numbers of vegetative cells per gametophyte. From 5-7 days in culture, the number of total cells per gametophyte was higher in unsterilized cultures.

**Enhancement of reproductive growth** The number of total cells (vegetative and antheridial) per treated gametophyte equals or exceeds the number of vegetative cells per untreated gametophyte.
The number of total cells per gametophyte treated day 0 exceeds the number of vegetative cells per treated gametophyte 5 and 6 days after treatment in unsterilized and sterilized cultures, respectively. The number of total cells per gametophyte treated day 4 exceeds the number of vegetative cells per treated gametophyte 3 days after treatment.

Antheridium Developmental Data

Quantitative data are obtained from Aₐ₉ -treated gametophytes to investigate the effects of spore sterilization and the time of treatment on the following characteristics of antheridium development: 1) the length of the induction period of antheridium initials, 2) the rate of antheridium formation, 3) the length of the maturation period of antheridia, and 4) the location of antheridium initials on the gametophytes. The data are obtained from drawings of cleared gametophytes collected daily from culture conditions 1, 2, and 5.

The information gained serves two purposes: one, as a contribution to the basic knowledge of reproductive biology of Onoclea gametophytes, and two, as a determinant, in conjunction with the data on gametophyte growth rates, in selecting an optimal culture and treatment regime for growing gametophytes to be processed and sectioned for LM, TEM, and SEM.

Mean values and confidence intervals at the 0.01 significance level were determined for some of the data. The t-test for unpaired observations and equal variance is used as a test for significant differences between the means at a significance level of either 0.05 or 0.01. If the t-test is used, the significance level is given. In some cases,
correlation analysis is used to measure the relationship between two effects due to an external controlling factor. When correlation analysis is used, the correlation coefficient, $r$, is given.

**The induction period and the rate of antheridium formation**

The induction period is defined as the length of time between the day of $A_{pt}$ application and the day on which there is an average of one antheridium initial per gametophyte. The induction period for gametophytes in unsterilized cultures treated day 0 is about 4-1/4 days. For gametophytes in sterilized cultures treated day 0 and day 4, the induction period is about 5-1/2 days and 2-1/2 days, respectively (Figure 22).

Spore sterilization and time of $A_{pt}$ treatment both affect the length of the induction period.

The induction period is about 1 day longer in sterilized cultures treated day 0 compared to unsterilized cultures. Because the number of vegetative cells per gametophyte in these two culture conditions is similar (Figures 18, 20), the inhibition due to sterilization is independent of vegetative growth.

The rate of antheridium formation is greater in cultures treated day 4 compared to day 0 during the first week after treatment (Figure 22). The seventh day after treatment, gametophytes treated day 4 have an average of eight antheridia compared to less than three for cultures treated day 0. The rate of vegetative cell divisions is also greater in cultures treated day 4 during the first week after treatment (Figures 18, 20, 21).

After 7 days from treatment, the rate of antheridium formation in
Figure 22. The average number of antheridia per gametophyte following treatment with $A_{pt}$ in three culture conditions: culture condition 1 (○), culture condition 2 (●), and culture condition 5 (△).
Ster., treated day 0
- Ster., treated day 4
- Unster., treated day 0

Days after treatment

Aver. no. of antheridia / gametophyte

1 2 3 4 5 6 7 8 9 10 11 12
cultures treated day 0 increases to equal the rate found in cultures treated day 4 (Figure 22). The rate of vegetative cell divisions also begins to increase after the first week of treatment in cultures treated day 0 (Figures 18, 20).

This concomitant increase in vegetative cells with the number of antheridia indicates that a possible correlation exists between the two variables. The average number of cells present when the first initial forms varies among the three treated culture conditions (Figure 23). Gametophytes with one antheridium in unsterilized cultures have a lower average of cells (11.6) than gametophytes in sterilized cultures treated day 0 (17.1; P < 0.01).

Although it appears that there is an initial threshold of numbers of vegetative cells that a treated gametophyte needs to produce before forming antheridia, there apparently are other requirements which need to be met before antheridium initiation can occur. Spore sterilization initially interferes with the ability of the gametophytes to meet those requirements.

The average number of cells per gametophyte with one antheridium from sterilized cultures treated day 4 is 20.2 which is significantly higher than for unsterilized and sterilized cultures treated day 0 (P < 0.01, P < 0.05, respectively). Gametophytes treated on day 4 have an average of five vegetative cells (Figure 21). Even though treatment on day 4 reduces the induction period by about 3 days in sterilized cultures, 2 days are still necessary for the induction of antheridial initials. This observation supports the earlier supposition that the number of vegetative cells per gametophyte is not the only determining
Figure 23. Average number of vegetative cells per gametophyte with respect to number of antheridia present per gametophyte in sterilized and unsterilized cultures treated on day 0 and day 4.

Figure 24. Average area per gametophyte with respect to number of antheridia present per gametophyte in sterilized and unsterilized cultures treated on day 0 and day 4.
factor in the initiation of the first antheridium per gametophyte.

The average number of vegetative cells per gametophyte with two antheridia present is the same for gametophytes from sterilized and unsterilized cultures treated on day 0 (Figure 23). The second antheridium is not initiated on gametophytes in unsterilized cultures until an average of eight additional vegetative cells is produced compared to only three additional vegetative cells for gametophytes from sterilized cultures treated day 0. Four additional vegetative cells are formed per gametophyte from cultures treated day 4 before the second antheridium is produced. The average number of vegetative cells present per gametophyte with two antheridia from cultures treated day 4 is slightly higher than the averages for gametophytes from cultures treated day 0 (P < 0.05). But there is no difference in the average number of vegetative cells per treated gametophyte that possess three antheridia regardless of the culture condition in which they grow. This same relationship is true for gametophytes with four to six antheridia and for gametophytes with seven to ten antheridia. Once gametophytes begin forming antheridia, it appears that there is a high correlation between the number of vegetative cells per treated gametophyte and the number of antheridia produced per gametophyte among all three culture conditions.

To support this supposition, correlation coefficients (r values) are determined with respect to the number of vegetative cells per gametophyte and the number of antheridia present per gametophyte for each of the treated culture conditions. The r value for gametophytes from unsterilized cultures treated day 0 is 0.861. The r value for gametophytes from sterilized cultures treated day 0 is 0.881 and for
gametophytes from cultures treated day 4, \( r = 0.914 \). These high \( r \) values indicate that for each culture condition, there is a high correlation between the number of vegetative cells and the number of antheridia present per gametophyte. This is not indicative of a cause and effect relationship between vegetative cell numbers and antheridium initiations, but it does imply that a certain number of vegetative cells is necessary, in conjunction with other factors, to support or stimulate the formation of an antheridium.

The relationship of the number of antheridia to the area per gametophyte is similar in many respects to the relationship between the number of antheridia and the number of vegetative cells per gametophyte (Figure 24). This is not surprising, since the area per gametophyte previously was shown to be highly dependent on the number of vegetative cells produced per gametophyte for each of the three treated culture conditions.

The area per gametophyte with one antheridium from sterilized cultures treated day 0 is larger than for gametophytes from unsterilized spore cultures treated day 0 (\( P < 0.01 \)). Both of these average areas are less than the average area per gametophyte treated day 4 (\( P < 0.01 \)). The relationship among the averages of area is the same as the relationship among the averages of vegetative cells per gametophytes with one antheridium. The averages of the area per gametophyte with two, three, four to six, or seven to ten antheridia from unsterilized cultures treated day 0 and sterilized spore cultures treated day 4 are the same. But the averages of the area per gametophyte from sterilized cultures treated day 0 with corresponding numbers of antheridia tend to be smaller in each case. This observation is somewhat different
from the observation that the averages of the number of vegetative cells per gametophyte from all three culture conditions are the same by the time the gametophytes have formed the third antheridium initial.

Correlation analysis was used to determine the relationship between the number of antheridia present per gametophyte and the area per gametophyte. The $r$ value for gametophytes from unsterilized cultures treated day 0 is 0.879. For gametophytes from sterilized cultures treated day 0, $r = 0.907$ and for gametophytes from sterilized cultures treated day 4, $r = 0.914$. These high $r$ values indicate that there is a high correlation between the area per gametophyte and the number of antheridia formed per gametophyte just as there is a high correlation between the number of vegetative cells per gametophyte and the number of antheridia formed per gametophyte. The area, the number of vegetative cells, and the number of antheridia per gametophyte all appear to be interrelated.

The length of the maturation period of antheridia

It is not possible to directly observe how long it takes for an antheridium initial to develop into a sperm-releasing antheridium due to the fact that the number of cells present per antheridium in living gametophytes cannot be determined. An indirect method was employed to determine the approximate time intervals between different developmental stages. For each day that gametophytes were collected from treated cultures (conditions 1, 2, and 5), a constant number was selected for observation for each condition and the number of antheridia present and the number of cells in each antheridium were recorded. It is found that there are ten observable stages of antheridium develop-
ment with respect to the number of cells present. An antheridium can have 1, 2, 3, 4, 5, 7, 11, 19, 35, or 67 cells including the jacket cells of the antheridium. The 35- and 67-celled stages can have 32 or 64 spermatids or 32 or 64 spermatozoids (32-sp/64-sp) bringing the total number of observable stages up to twelve. Although there are observable stages during spermatid differentiation in terms of the number of coils present, only the spermatid stage and the mature spermatozoid stage were recorded.

Data on the number and stages of antheridia present per gametophyte from unsterilized cultures treated day 0 are graphed in Figure 25. Data were taken from 33 gametophytes each day they were collected. The first antheridium initials are found on gametophytes collected the fourth day after treatment. The most advanced antheridium stage present is the 3-celled stage, although most of the antheridia have 1 or 2 cells. Five days after treatment, 5-celled antheridia are present. By the seventh day, there are 19-celled stages present and by the eighth day, many 35-celled stages are observed. A few 67-celled antheridia are also seen by the eighth day. By the eleventh day, numerous 32-sp- and 64-sp-celled stages are present. It takes an average of 8-9 days from the time of treatment for an antheridium to form and produce spermatids and about three more days for the spermatids to differentiate into spermatozoids.

The time interval between the day the first initials are formed and the day the first spermatid-containing antheridia are observed is about 4 days, indicating that there is an average of two rounds of mitotic divisions every 24 hr. Table 2 is a summary of the profiles of gametophytes
Figure 25. Number and stages of antheridia present per 33 gametophytes collected daily from un-sterilized cultures treated on day 0
Table 2. Profile of gametophytes collected per day from unsterilized spore cultures treated day 0

<table>
<thead>
<tr>
<th>Day collected (from treatment)</th>
<th>Aver. # veg. cells</th>
<th>Aver. area (sq mm)</th>
<th>Aver. # antheridia</th>
<th>Stages present</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>9.9</td>
<td>0.014</td>
<td>0.60</td>
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<td>5</td>
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<td>1.91</td>
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<td>0.032</td>
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<tr>
<td>8</td>
<td>45.3</td>
<td>0.059</td>
<td>5.32</td>
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</tr>
<tr>
<td>10</td>
<td>67.8</td>
<td>0.083</td>
<td>7.33</td>
<td>1, 2, 3, 4, 5, 7, 11, 19, 35, 67</td>
</tr>
<tr>
<td>11</td>
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<td>0.133</td>
<td>10.80</td>
<td>1, 2, 3, 4, 5, 7, 11, 19, 35, 67, 32-sp, 64-sp</td>
</tr>
</tbody>
</table>

collected on specific days in terms of their average number of vegetative cells, average area, average number of antheridia, and stages of antheridia present.

Figure 26 shows the number and stages of antheridia present per 30 gametophytes observed per day from sterilized spore cultures treated day 0. Five days after treatment, 1- and 2-celled stages are present. By the ninth day, many 35-celled stages are present along with a few 67-celled stages. The 35-celled stage is probably present by the eighth day, but gametophytes were not collected on that day. By the eleventh day, some of the antheridia have 32 mature spermatozoids. It takes an average of 8 days from the time of treatment for an antheridium to form and produce spermatids and about three more days for the spermatids
Figure 26. Number and stages of antheridia present per 30 gametophytes collected daily from sterilized cultures treated on day 0.
Number of antheridia / 30 gametophytes

Days after treatment
4 5 6 7 9 11

Stage:
to differentiate into spermatozoids. The time interval between the day the first initials are formed and the day the first spermatid containing antheridia are observed is between 3-4 days, indicating that an average of about two mitotic divisions occurs every 24 hr. Table 3 is a summary of the profiles of gametophytes collected per day with respect to the average number of cells, the average area, the average number of antheridia and the stages of antheridia present per gametophyte from sterilized spore cultures treated day 0. Spore sterilization does not appear to affect the length of the maturation period of antheridia or the length of time necessary for the differentiation of spermatids into spermatozoids.

Table 3. Profile of gametophytes collected per day from sterilized spore cultures treated day 0

<table>
<thead>
<tr>
<th>Day collected (from treatment)</th>
<th>Aver. # veg. cells</th>
<th>Aver. area (sq mm)</th>
<th>Aver. # antheridia</th>
<th>Stages present</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>9.7</td>
<td>0.010</td>
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<tr>
<td>9</td>
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<td>5.97</td>
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</tr>
<tr>
<td>11</td>
<td>89.3</td>
<td>0.079</td>
<td></td>
<td>1, 2, 3, 4, 5, 7, 11, 19, 35, 67, 32-sp, 64-sp</td>
</tr>
</tbody>
</table>
Figure 27 shows the number and stages of antheridia present per 26 gametophytes collected per day from sterilized spore cultures treated day 4. Two days after treatment, 1- and 2-celled stages are present and by the sixth day, antheridia containing 32 spermatids are observed. By the seventh day, there are antheridia present with 64 spermatids. It takes an average of three more days for both the 32 and the 64 spermatids to differentiate into 32 and 64 spermatozoids, respectively. It takes an average of 9-10 days from the time of treatment for an antheridium to form and produce mature spermatozoids. This about 2 days less than for gametophytes from cultures treated day 0.

The time interval between the day the first initials are formed and the day the first spermatids are observed is about 4 days, indicating that an average of about two mitotic divisions occurs every 24 hr. Table 4 is a summary of the profiles of gametophytes collected per day with respect to the average number of cells, the average area, the average number of antheridia, and the stages of antheridia present per gametophyte from sterilized spore cultures treated day 4. The length of the maturation period, including the time intervals for cell divisions and cell differentiation, is not affected due to the time of A treatment.

**Location of antheridium initials**

The locations of initials (1- and 2-celled stages) on gametophytes were observed daily for both pragmatic and theoretical reasons. Pragmatically, the information enhances the ability to predict which regions or cells will most likely produce antheridia before the
Figure 27. Number and stages of antheridia present per 26 gametophytes collected daily from sterilized cultures treated on day 4.
Days after treatment

Number of antheridia / 26 gametophytes

Stage: 123457 1111

Days after treatment: 2 3 4 5 6 7 8 9 10
Table 4. Profile of gametophytes collected per day from sterilized spore cultures treated day 4

<table>
<thead>
<tr>
<th>Day collected (from treatment)</th>
<th>Aver. # veg. cells</th>
<th>Aver. area (sq mm)</th>
<th>Aver. # antheridia</th>
<th>Stages present</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>16.5</td>
<td>0.020</td>
<td>0.22</td>
<td>1, 2</td>
</tr>
<tr>
<td>3</td>
<td>22.2</td>
<td>0.028</td>
<td>1.79</td>
<td>1, 2, 3, 5</td>
</tr>
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<td>29.7</td>
<td>0.040</td>
<td>2.77</td>
<td>1, 2, 3, 4, 5, 7</td>
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<tr>
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<td>0.052</td>
<td>4.14</td>
<td>1, 2, 3, 4, 5, 7, 11, 19</td>
</tr>
<tr>
<td>6</td>
<td>49.4</td>
<td>0.064</td>
<td>6.03</td>
<td>1, 2, 3, 4, 5, 7, 11, 19</td>
</tr>
<tr>
<td>7</td>
<td>69.0</td>
<td>0.088</td>
<td>8.27</td>
<td>1, 2, 3, 4, 5, 7, 11, 19, 35, 67</td>
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<tr>
<td>8</td>
<td>85.2</td>
<td>0.110</td>
<td>9.88</td>
<td>1, 2, 3, 4, 5, 7, 11, 19, 35, 67</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td>32-sp</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td>67-sp</td>
</tr>
</tbody>
</table>

Initials are visibly evident. These regions can then be sectioned to obtain data on pre- and post-inductional cellular changes in induced vegetative cells while avoiding the cells likely to produce trichomes or rhizoids. Theoretically, information on location of antheridium initials will contribute to the prior knowledge of the internal physiology of fern gametophytes.

To obtain data on locations of initials, information is derived from the drawings of cleared gametophytes. Each initial is assigned a relative X and Y coordinate point with respect to the center of the gametophyte, represented by the intersection of the X and
Y axes, and the margins of the gametophyte, represented by the coordinate extremes. The initials present on n number of gametophytes observed each day are plotted on a two-dimensional coordinate system for each culture condition. The value for n (the number of gametophytes observed) is given below each graph.

Figure 28 shows the location of antheridium initials on gametophytes from unsterilized cultures treated day 0. The majority (86%) of the initials formed on gametophytes the fourth day after treatment are produced by peripheral cells, all of which are located in the upper half of the gametophytes. Drawings of nine of the gametophytes collected on the fourth day are shown in Figure 29, showing the location and stage of the antheridium initials.

On the fifth day after treatment, 76% of the initials are located on peripheral cells, but again most of the induced cells are located in the upper half of the gametophytes. By the tenth day, only 24% of the initials are induced on peripheral cells, but with the majority (83%) of all the induced cells occurring on the upper half of the gametophytes. Initials are never formed on anterior, marginal cells of the gametophytes, regions which correspond to the notch meristem and outer wings of the gametophytes. With time, the preferred locations of antheridium initials shift from upper peripheral cells to upper central cells below the notch meristem region. A summary of the data on the location of initials on gametophytes from unsterilized spore cultures treated day 0 is given in Table 5.

Figure 30 shows the relative locations of antheridium initials on gametophytes collected daily from sterilized cultures treated day 0.
Figures 28 A-E. Relative location of antheridium initials (1- and 2-celled) on gametophytes collected from unsterilized cultures treated on day 0, plotted on standardized, unit axes

A. Relative location of initials from 49 gametophytes collected 4 days after treatment

B. Relative location of initials from 33 gametophytes collected 5 days after treatment

C. Relative location of initials from 47 gametophytes collected 7 days after treatment

D. Relative location of initials from 44 gametophytes collected 8 days after treatment

E. Relative location of initials from 36 gametophytes collected 10 days after treatment
A day 4, n = 49
B day 5, n = 33
C day 7, n = 47
D day 8, n = 44
E day 10, n = 36
Figure 29. Drawings of nine representative gametophytes collected 4 days after treatment in unsterilized cultures treated on day 4 showing location and number of cells in first-formed initials. X 215
Table 5. Data on the location of antheridium (anth.) initials in gametophytes from unsterilized spore cultures treated day 0

<table>
<thead>
<tr>
<th>Days after treatment</th>
<th># of gametophytes observed</th>
<th># of 1- and 2-celled anth.</th>
<th>% anth. on peripheral cells</th>
<th>% anth. on upper half of gametophyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>49</td>
<td>29</td>
<td>86</td>
<td>100</td>
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<td>5</td>
<td>33</td>
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<td>7</td>
<td>47</td>
<td>22</td>
<td>46</td>
<td>86</td>
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<td>8</td>
<td>44</td>
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<td>98</td>
</tr>
<tr>
<td>10</td>
<td>36</td>
<td>42</td>
<td>24</td>
<td>83</td>
</tr>
</tbody>
</table>

The distribution pattern observed in gametophytes grown in unsterilized cultures is also observed for gametophytes grown in sterilized cultures. On the fourth day after treatment, the one initial observed occurs on a peripheral cell midway between the apex and base of the gametophyte. By the fifth day, many more initials are present and they are also all located on peripheral cells, 90% of which are found in the upper half of the gametophytes. Drawings of nine of the gametophytes collected on the fifth day are shown in Figure 31, revealing the location and stage of the antheridium initials.

By the ninth day, only 26% of the initials are formed on peripheral cells, but almost all of the induced cells are located in the upper half of the gametophytes. The initials are never formed on anterior, marginal cells of the gametophytes, occupied by the notch meristem and the outer wing regions, as was the case for gametophytes from unsterilized cultures. A summary of the data on the location of
Figures 30 A-E. Relative location of antheridium initials (1- and 2-celled) on gametophytes collected from sterilized cultures treated on day 0, plotted on standardized, unit axes

A. Relative location of initials on 32 gametophytes collected 4 days after treatment

B. Relative location of initials on 40 gametophytes collected 5 days after treatment

C. Relative location of initials on 33 gametophytes collected 6 days after treatment

D. Relative location of initials on 30 gametophytes collected 7 days after treatment

E. Relative location of initials on 33 gametophytes collected 9 days after treatment
day 4, n=32
C day 6, n=33
D day 7, n=30
E day 9, n=33
Figure 31. Drawings of nine representative gametophytes collected 5 days after treatment from sterilized cultures treated on day 0 showing location and number of cells in first-formed initials. X 255
antheridium initials on gametophytes from sterilized cultures treated
day 0 is given in Table 6. These data are consistent with the previous
observations that over time the initial locations change from peripheral
cells in the upper half of the gametophytes to central cells in the
upper half of the gametophytes below the notch meristem. Spore steriliza-
tion does not appear to affect the location of antheridium initiations
during the first week after treatment.

Table 6. Data on the location of antheridium initials in gametophytes
from sterilized spore cultures treated day 0

<table>
<thead>
<tr>
<th>Days after treatment</th>
<th># of gametophytes observed</th>
<th># of 1- and 2-celled anth.</th>
<th>% anth. on peripheral cells</th>
<th>% anth. on upper half of gametophyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>32</td>
<td>1</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>40</td>
<td>17</td>
<td>100</td>
<td>89</td>
</tr>
<tr>
<td>6</td>
<td>33</td>
<td>22</td>
<td>68</td>
<td>77</td>
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<td>7</td>
<td>30</td>
<td>19</td>
<td>74</td>
<td>89</td>
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<tr>
<td>9</td>
<td>33</td>
<td>53</td>
<td>26</td>
<td>93</td>
</tr>
</tbody>
</table>

Figure 32 shows the relative locations of antheridium initials on
gametophytes collected daily from sterilized cultures treated day 4.
The second day after treatment, when the first initials are observed,
all of the initials occur on peripheral cells in a mid-region between
the apex and base of the gametophytes. The third day after treatment,
70% of the initials are formed on peripheral cells, but these cells
are located both in the upper and lower halves of the gametophytes.
This observation differs from the observation on initial locations in
Figures 32 A-G. Relative location of antheridium initials (1- and 2-celled) on gametophytes collected from sterilized cultures treated on day 4, plotted on standardized, unit axes.

A. Relative location of initials from 45 gametophytes collected 2 days after treatment

B. Relative location of initials from 49 gametophytes collected 3 days after treatment

C. Relative location of initials from 44 gametophytes collected 4 days after treatment

D. Relative location of initials from 28 gametophytes collected 5 days after treatment

E. Relative location of initials from 31 gametophytes collected 6 days after treatment

F. Relative location of initials from 26 gametophytes collected 7 days after treatment

G. Relative location of initials from 31 gametophytes collected 8 days after treatment
gametophytes treated day 0, where it is shown that the induced peripheral cells are located mainly in the upper half of the gametophytes. Drawings of nine of the gametophytes collected the third day after treatment from cultures treated day 4 are shown in Figure 33 revealing the location and stage of the initials. The difference in the locations of induced peripheral cells is attributed to the fact that the gametophytes treated day 4 versus day 0 have more vegetative cells per gametophyte when the first initials are forming. Most of those additional vegetative cells are located in the notch meristem and wing regions where antheridia do not form.

The eighth day after treatment, 90% of the initials are formed on central cells, with the majority occurring in the upper half of the gametophytes. Initials are not formed on anterior marginal cells of the gametophytes, as was the case in the other culture conditions. A summary of the data on the location of initials in gametophytes from sterilized cultures treated day 4 is given in Table 7. The basic trends reported for gametophytes from cultures treated day 0 are also observed for gametophytes from cultures treated day 4. The preferred location for antheridium initiations shifts in time from lateral, peripheral cells to central cells below the notch meristem.

The only differentiating cell types that antheridia can be confused with are the early stages in the formation of trichomes and rhizoids. But due to the fact that trichomes and rhizoids occur in predictable regions of the thalli, these regions were conveniently avoided while studying antheridium development. Rhizoids are always produced by cells in the basal region of the gametophytes. Trichomes, which form
Figure 33. Drawings of nine representative gametophytes collected 3 days after treatment from sterilized cultures treated on day 4 showing location and number of cells present in first-formed initials. X 255
Table 7. Data on the location of antheridium initials on gametophytes from sterilized spore cultures treated day 4

<table>
<thead>
<tr>
<th>Days after treatment</th>
<th># of gametophytes observed</th>
<th># of 1- and 2-celled anth.</th>
<th>% anth. on peripheral cells</th>
<th>% anth. on upper half of gametophyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>45</td>
<td>9</td>
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<td>28</td>
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<tr>
<td>8</td>
<td>32</td>
<td>39</td>
<td>10</td>
<td>97</td>
</tr>
</tbody>
</table>

as early as the fourth day after sowing, are formed first on anterior, marginal cells of the gametophytes. With time, more trichomes are formed on lateral, peripheral cells, but usually not on cells in the basal regions. Only after the eleventh day in culture are trichomes observed forming on upper, central cells, thus allowing sufficient time for investigating trichome-free regions of antheridium initial formations.

Summary of antheridium developmental data

Induction period

The induction period for gametophytes from un-sterilized cultures versus sterilized cultures treated day 0 is about 4-1/4 days compared to 5-1/2 days, respectively. Spore sterilization lengthens the induction period by about 1 day.

The induction period for gametophytes from sterilized cultures
treated day 4 is about 2-1/2 days. Treatment on day 4 versus day 0 in sterilized cultures shortens the induction period by about 3 days.

The average number of vegetative cells per gametophyte with one antheridium from unsterilized cultures treated day 0 is 11.6 cells. The average for sterilized cultures treated day 0 is 17.1 cells and the average for sterilized cultures treated day 4 is 20.2 cells. Although it appears necessary for gametophytes to surpass a threshold of numbers of vegetative cells before forming their first initial, it is apparent that other factors are also involved in determining the length of the induction period and the formation of antheridium initials.

Rate of antheridium initiations Once the initials begin to form in sterilized and unsterilized cultures treated day 0, the rate of formation is similar.

Both the rate of antheridium formation and the rate of vegetative cell divisions are higher in cultures treated day 4 compared to day 0 during the first 7 days after treatment.

Correlation analysis shows that for each culture condition, the number of vegetative cells per gametophyte is highly correlated with the number of antheridia present. After the first two initials are formed, the gametophytes produce the same average number of additional vegetative cells before the next initial is formed, regardless of the culture condition.

Correlation analysis also shows that for each culture condition, the area per gametophyte is highly correlated to the number of antheridia present per gametophyte. This is not surprising, due to
the fact that the area per gametophyte was shown to be highly dependent on the number of vegetative cells produced per gametophyte.

**Length of maturation period** The 12 observable stages in antheridium development include the 1-, 2-, 3-, 4-, 5-, 7-, 11-, 19-, 35-, and 67-celled stages and the 32 and 64 spermatozoid stages (32-sp and 64-sp).

In cultures treated day 0, gametophytes form antheridia with 32 and 64 spermatids on the eighth and ninth days after treatment, respectively. In cultures treated day 4, gametophytes form antheridia with 32 or 64 spermatids on the sixth and seventh days after treatment, respectively.

For each culture condition, there is about 4 days between the day the first initials are observed and the day that the first spermatid-containing antheridia are observed, indicating that an average of two mitotic divisions occur every 24 hr in order to complete the cell-forming phase of antheridium development.

Three days are necessary for spermatids to differentiate into spermatozoids, regardless of the culture condition in which the gametophytes grow.

**Location of antheridium initials** The first formed initials occur mainly on lateral, peripheral cells of the gametophytes. On gametophytes treated day 0, the induced peripheral cells are located mainly in the upper half of the gametophytes. On gametophytes treated day 4, the induced peripheral cells are located both in the upper and lower halves of the gametophytes.

No antheridia are ever formed on anterior, marginal cells of the gametophytes, areas occupied by the forming notch meristem and the
wings of the gametophytes.

The preferred location of antheridium initials shifts from peripheral cells to central cells in the upper half of the gametophytes below the notch meristem during the 5 days following the first day of initiations.

Rhizoids and trichomes occurred in consistently predictable regions and thus were conveniently avoided while studying antheridium development.

Selection of the optimal culture and treatment regime

The information on gametophyte growth and antheridium development presented above was used in determining an optimal culture and treatment regime for growing gametophytes to be sectioned and/or viewed with LM, TEM, or SEM. From the beginning, it was deemed desirable to grow gametophytes in axenic conditions to assure definition of the system, but not at the expense of disturbing the normal growth patterns of the gametophytes. Although spore sterilization is found to inhibit the vegetative growth rate of gametophytes, this inhibition is not evident after sterilized and unsterilized spore cultures are treated with $A_{pt}$. Spore sterilization also lengthens the induction period by one day, but the duration of the division and differentiation phases of antheridium development is not affected, nor are the locations of the initials affected compared to unsterilized cultures. The one extra day in the induction period for gametophytes from sterilized spore cultures is considered acceptable in return for the maintenance of uncontaminated gametophyte cultures. Thus, spore sterilization was adopted as part of
the optimal culturing procedures.

The optimal day on which to treat sterilized cultures is shown to be the fourth day after sowing. Treatment day 4 results in the highest rate of antheridium formation and the shortest induction period. Ideally, during a shorter induction period, the cellular changes are more concentrated and more easily detected.

Thus, for growing gametophytes to be processed for LM, TEM, and SEM, the culture and treatment regime consisted of treating sterilized cultures with 1 ml of 25% A<sub>pt</sub> the fourth day after sowing. The gametophytes were harvested between 6-12 days after treatment.

Vegetative Cell Development and Structure

The development of A<sub>pt</sub>-treated and untreated Onoclea gametophytes follows a modified "Aspidium-type" prothallial developmental pattern as described by Nayar and Kaur (1971). The first division of the bilateral spore is an unequal division in a plane perpendicular to the longitudinal axis of the spore, forming a small rhizoidal cell and a much larger prothallial cell (Figures 34A, B). The prothallial cell then divides one or two times in a plane parallel to the first division, resulting in a filament of two or three cells with an elongating basal rhizoid (Figures 34C, D). These filament-forming, transverse divisions occur within two to three days after sowing. The cells of the filament then divide longitudinally with respect to the long axis of the filament to form a plate of four or six cells (Figures 34E, F). The cells of the plate continue to divide both transversely and longitudinally and
Figures 34 A-J. Prothallium developmental pattern of *O. sensibilis* illustrated with drawings of gametophytes collected from sterilized cultures treated on day 4.

A-F. Gametophytes collected 4 days after sowing. X 255

G-H. Gametophytes collected 6 days after sowing (2 days after treatment). Arrows indicate apical cell. X 255

I. Gametophyte collected 8 days after sowing (4 days after treatment). X 215

J. Gametophyte collected 11 days after sowing (7 days after treatment). X 215
expand until a plate with about 12-15 cells is formed. A wedge-shaped, apical initial cell is then formed in one of the central, anterior, marginal cells by an oblique cell division (Figures 34G, H). The two-dimensional prothalli with apical initials are usually present by the sixth day after sowing. These prothalli usually have two or three basal rhizoids, a trichome initial on one of the off-center, anterior, marginal cells, and occasionally, an antheridial initial cell. By the eighth day, many of the gametophytes have produced small, notched meristems (Figure 34I) which become well-developed by the eleventh day (Figure 34J).

As the prothalli mature, they develop distinct morphological, and presumably, physiological regions which include a meristem region, two wing regions, a central region, and a basal region (Figure 35; this figure and all subsequent figures occur in Appendix B). These regions are notably different from each other with respect to cell shape and size and to the type of differentiated cells and/or organs produced within each region.

The cells in the meristem region of three-week-old gametophytes are quite small, averaging about 10-15 μm X 10-15 μm (Figure 36). Figure 37 shows a fixed gametophyte stained with azure B, a stain specific for nucleic acids. The cells in the meristem region are densely cytoplasmic in comparison to the cells in the other regions. The primary function of the meristem cells is the production of new vegetative cells.

The cells in the wing regions are larger than the meristematic cells, but much smaller than the cells in the central and basal regions (Figures 35, 36). They average about 25 μm X 40 μm. The cells in the
outer wing regions have been observed producing only trichomes. Cells in the inner wing region are capable of forming antheridia.

The cells in the central region have more diverse shapes and sizes than the other regions. The cells in the upper, central part of the central region are the smallest and most isodiametric (Figures 35, 38). The smallest cells are about 30 \( \mu m \) X 30 \( \mu m \). The cells increase in size from the upper central part of the region to the lower central part of the region, where they reach a size of about 70 \( \mu m \) X 120 \( \mu m \). The longitudinal axis of a cell in the central part of the central region is parallel to the longitudinal axis of the gametophyte. The cells in the peripheral zones of the central region are much longer than wide, with various measurements including 35 \( \mu m \) X 160 \( \mu m \), 40 \( \mu m \) X 120 \( \mu m \), and 60 \( \mu m \) X 150 \( \mu m \). The longitudinal axes of the cells in the lateral parts of the central region diverge from the longitudinal axis of the gametophyte by an angle of about 45 degrees (Figures 35, 38). The cells in the central region mainly produce antheridia, although after the eleventh day in culture, some of the central cells form trichomes.

The cells in the basal region are quite large and usually isodiametric (Figure 38). Some of the basal cells measure 140 \( \mu m \) X 150 \( \mu m \). The basal cells mainly produce rhizoids, but they also can form antheridia. Although it would be of interest to study the cytological differences among the four regions, this study was limited to observations of vegetative cells categorized broadly as either meristematic or nonmeristematic cells (i.e., apical region versus nonapical region).
Meristematic cells

Transverse and longitudinal sections were made through the meristematic regions of the gametophytes (Figure 38) to study their internal structure. Figure 39 shows a longitudinal section through a meristematic cell and Figure 40 shows a transverse section through the meristem region. The centrally located nucleus is surrounded by cytoplasm and numerous small- and medium-sized vacuoles. The cytoplasm contains numerous mitochondria, chloroplasts, Golgi bodies, rough endoplasmic reticulum (RER), microbodies, and variously oriented microtubules (Figures 41-43). The chloroplasts have few starch grains and lack an extensive lamellar system. The anticlinal walls of the meristem cells are thin (0.13 μm) and are traversed by numerous plasmodesmata. In comparison, the periclinal walls (exterior walls) are relatively thick (0.35 μm). Oblique, longitudinal sections through meristem cells reveal that there are numerous plasmodesmata traversing the posterior, transverse wall (Figures 44-49). In cross-section, the plasmodesmata consist of an outer circular membrane, which is a continuation of the plasmalemma of the adjacent cell, and an inner core of electron dense material separated from the membrane by a uniform space. The average diameter of the plasmodesmata is about 30 nm (Figure 49).

Numerous microtubules are oriented in the same direction parallel to the posterior, transverse wall (Figures 46-48). Golgi bodies near the cell wall produce large vesicles containing some fibrous material (Figure 47). Similar appearing fibrous material occurs between the plasmalemma and the cell wall (Figure 47). Occasionally, meristematic
cells contain unidentified, osmiophilic bodies measuring as large as 0.45 \( \mu \text{m} \) (Figures 46, 49).

**Nonmeristematic cells**

The cells formed by the meristem which are destined to become vegetative cells in the other three regions, undergo a number of cellular changes in the process. The first noticeable change is an increase in cell volume. As the cells begin to expand, the numerous small vacuoles coalesce to form larger vacuoles (Figures 50, 51). The larger vacuoles continue to combine until the central nucleus is surrounded by four or five very large vacuoles separated by thin cytoplasmic strands (Figures 52, 53). As the large vacuoles form, the major portion of the cytoplasm becomes localized near the periphery of the cell (Figure 54).

The transverse and radial walls of the expanding cells become thicker (0.20-0.25 \( \mu \text{m} \)) compared to similar walls of the meristematic cells, while the periclinal walls remain about the same thickness. Fewer plasmodesmata are present traversing the inner walls (Figure 54).

The nucleus also moves over to the periphery of the maturing vegetative cell, while still maintaining contact with cytoplasm along opposite and adjacent walls of the cell via thin cytoplasmic strands (Figures 45, 55-58). The cytoplasmic strands, which are composed of free ribosomes and an occasional small vacuole, progressively become fewer in number. Numerous microtubules have been observed in the cytoplasm surrounding some of the peripheral nuclei. In Figures 46-48, numerous microtubules are shown aligned parallel to the cell wall next to the peripheral nucleus with many more microtubules oriented tangentially
to the nuclear membrane envelope.

In the much expanded, vacuolate cells of the central and basal regions of the gametophytes, the cytoplasm is distributed in a very thin layer around the periphery of the cells and thin cytoplasmic strands are no longer apparent (Figures 59-61). Figure 62 shows a transverse section through a region of mature vegetative cells revealing nuclei in various positions along the cell walls. The nuclei have elongated and flattened (Figures 62-64). Nucleoli are present and occasionally a lobed nucleus is observed (Figure 65).

The organelles which occur in the cytoplasm of the meristematic cells are also present in the fully expanded vegetative cells. The many chloroplasts found in the large, vacuolate cells are located, almost exclusively, in the cytoplasm along the periclinal walls (Figures 55-57, 59-63). Most of the chloroplasts have a well-developed lamellar system with many grana, along with plastoglobuli and small starch grains (Figures 64, 66).

Interspersed among the chloroplasts are microbodies and mitochondria (Figures 67, 68). Some of the mitochondria have accumulated densely staining, amorphous material (Figures 69, 70). Mitochondria also occur in the cytoplasm along the inner walls of the cells (Figure 57).

Also present in the ribosome-filled cytoplasm are small vacuoles, RER, and Golgi bodies. Golgi bodies near the cell walls form vesicles containing fibrous material, similar in appearance to the material found between the plasmalemma and the cell wall (Figure 71). Occasionally present are single-membrane bound organelles containing many tubules
and/or vesicles (Figures 72, 73). They are similar in appearance to paramural bodies as described by Marchant and Robards (1968), although their membranes are not continuous with the plasmalemma in these micrographs. Very few microtubules are observed in highly vacuolate cells, although they are occasionally seen near a cell wall (Figures 66, 73).

Cellular Changes during the Division Phase of Antheridium Initiation and Development

Initiation of 1-celled stage

The first-formed antheridium initials typically occur on marginal cells along the sides of the gametophyte, as reported earlier in the section on "Antheridium Developmental Data," and during the first week of initiations, the preferred sites of initiation shift to cells in the upper, central region of the gametophytes. Regardless of where the induced vegetative cells are located in the gametophyte, the antheridium initials predominantly form at the anterior end of the vegetative cells (Figures 35, 37, 38, 74, 75) with the exception of the cells in the basal region of the gametophytes. These larger, isodiametric cells do not appear to have a preferred cellular region for antheridium initiation, with antheridia occurring both in the anterior and central regions of the cells (Figure 38).

Marginal cells have three possible cell wall regions from which an initial can form; dorsal or ventral periclinal wall regions, and curved, radial wall regions. The initial is usually formed in the anterior, radial cell wall region (Figures 37, 75). Nonmarginal cells have only
two possible anterior cell wall areas from which an initial can arise, those being the dorsal and ventral periclinal walls. Antheridia are observed on either, but not both, of these walls in any one cell. In general, a vegetative cell produces only one antheridium, but if a second antheridium is formed on a vegetative cell, it usually occurs immediately posterior to the first-formed antheridium (Figure 35).

Prior to the formation of an initial, the nucleus of the induced vegetative cell moves into the anterior region of the cell, either in the transverse-ventral, transverse-dorsal, or transverse-radial corner. When a nucleus is observed in one of these corners, indicating a high potential for forming an antheridium, the nucleus is often accompanied by an increase in cytoplasmic material. Gametophytes stained for DNA (methyl green) and RNA (pyronin Y) are shown in Figures 76-87. Nuclei in anterior, radial-transverse corners of marginal cells are surrounded by an accumulation of pink-stained cytoplasm, presumably composed of a large amount of RNA (Figures 76-78, 81, 86). A longitudinal section through a vegetative cell in the central region of a gametophyte shows a nucleus in the transverse-dorsal corner of the cell surrounded by an increased amount of cytoplasmic material (Figure 88). A micrograph of a similar region reveals the presence of an interphase nucleus, as evidenced by the highly dispersed chromatin (Figure 89). Between the nucleus and cell walls are chloroplasts in association with RER, mitochondria, and many vesicle-forming Golgi bodies. An adjacent section shows that the cytoplasm next to that nucleus is dense with ribosomes and mitochondria (Figure 90). A few microtubules are present within the cytoplasm, oriented in various directions. These cellular conditions
are presumed to precede antheridium initiation.

Nuclear division occurs before an antheridium initial cell is formed. A nucleus in mitotic metaphase is shown in the radial-transverse corner of a vegetative cell presumably initiating an antheridium initial (Figure 77). A nucleus in mitotic telophase is shown in a similar corner of another vegetative cell (Figure 78). The set of chromosomes nearest the radial wall is surrounded by a large amount of RNA-stained cytoplasm. The other set of chromosomes occurs near the inner periphery of the accumulated cytoplasm near the large central vacuole. Similar observations are made in transversely sectioned material, showing a telophase nucleus in one of the vacuolate cells of the central region embedded in accumulated cytoplasm (Figure 91). The set of chromosomes nearest the outer wall is surrounded by a large amount of cytoplasm, while the other set of chromosomes occurs near the inner edge of the accumulated cytoplasm next to the central vacuole. A thin strand of cytoplasm traverses the central vacuole between the innermost set of chromosomes and the opposite cell wall. The phragmoplast is forming between the two sets of chromosomes at about a 45° angle to the outer cell wall. The phragmoplast develops into the basal cell wall of an antheridium initial, resulting in the formation of a small, wedge-shaped initial at the anterior end of the vegetative cell (Figure 92).

One-celled stage

The basal wall of the initial always forms at an oblique angle (about 45°) to the outer wall of the vegetative cell, intercepting the anterior, transverse wall about 10-15 μm from the outer edge of
the cell (Figure 92). The majority of the accumulated cytoplasm is partitioned into the initial cell. The remaining cytoplasm, surrounding the daughter nucleus in the vegetative cell, contains numerous small vacuoles, some mitochondria, RER, and Golgi bodies (Figure 93). Thin strands of cytoplasm, mainly composed of ribosomes, have been observed connecting the nucleus in the vegetative daughter cell to cytoplasm along the opposite wall (Figures 91, 94).

A surface view of a newly formed antheridium initial is shown in Figure 79. The nucleus in the initial appears larger than the nucleus in the vegetative mother cell. RNA-stained cytoplasm can be seen surrounding the initial nucleus. A side view of a newly formed initial reveals a centrally located nucleus surrounded by dense cytoplasm stained for RNA (Figure 80). The nuclei in vegetative daughter cells migrate to various regions in the cell after the initial is formed. In Figure 80, the vegetative cell nucleus is near the center of one of the outer periclinal walls, either dorsal or ventral. The thin strands of cytoplasm shown in Figures 91 and 94 may play some role in the mobility of the nucleus within the vegetative cell.

The base of the initial is about 30 μm long and the height of the initial through the center point is about 10 μm (Figures 95-97). The width of an initial varies between 25-30 μm (Figure 98). This variation may be due to the size of the vegetative cell which produces the initial. The upper, curved wall of the initial is about 0.30-0.35 μm thick, while the anterior, transverse wall is about 0.20 μm thick and the basal wall is only about 0.10 μm thick (Figures 95, 97). Plasmodesmata occur in both the anterior, transverse and basal walls of the initial.
Within the initial, the nucleus is centrally located and surrounded by dense cytoplasm (Figures 95, 96). Chloroplasts occur in all areas of the initial except the area between the nucleus and the basal wall. Some of the chloroplasts appear to be dividing and most of them contain starch grains (Figures 95, 97). The longitudinal axis of the chloroplasts near the upper wall of the initial are aligned parallel to the wall and parallel to the longitudinal axis of the initial. The chloroplasts located on the sides of the nucleus are oriented in various directions (Figures 95, 97). Small- to medium-sized vacuoles occur in the same areas of the initial as the chloroplasts.

Other organelles which occur in the dense cytoplasm of antheridium initials include polysomes, often found near the nuclear pores of the nuclear envelope (Figure 99). The nuclear pores have an average diameter of about 70 μm. Microtubules occur throughout the initial with many of them located next to the cell walls (Figures 99-101). Numerous mitochondria are present and occasionally a mitochondrion with an inclusion of densely staining amorphous material is observed (Figure 102). A few microbodies are always present within the initial (Figures 102, 103) along with Golgi bodies forming numerous vesicles containing fibrous material (Figure 102). Although RER occurs throughout the initial, it is often quite prevalent in the region between the nucleus and the basal cell wall (Figure 104). Paramural bodies are also present (Figure 105).

The antheridium initial then enters an expansion phase of growth. Due to the expansion of the upper, curved wall, the initial increases both in height and diameter, measured through the center of the initial (Figures 106, 107). An initial, shown in transverse section in Figure 107,
has expanded to a height of about 20 μm. The small vacuoles that were in the lower half of the initial have coalesced to form a few large, basal vacuoles (Figures 106, 107). The cytoplasm is displaced into the upper half of the initial where the organelles appear to be distributed in a polar gradient. Most of the chloroplasts are located near the periphery of the upper wall of the initial. The nucleus is located in the central, lower part of the cell with the rest of the organelles occurring mainly in a region between the nucleus and the chloroplasts.

A near-median, longitudinal section through an expanding initial reveals one large, basal vacuole, with the major portion of the cytoplasm confined to the upper region of the cell (Figure 108). Microtubules are present along the cell wall and throughout the cytoplasm (Figures 109-111). Occasionally, lobed microbodies are observed (Figure 111). Golgi bodies are actively forming vesicles containing a fibrous material near the upper wall (Figure 109). The wall appears to be uniform in thickness (0.30 μm) in all areas except for a small region near the junction of the wall with the anterior, transverse wall, where the upper wall is somewhat thicker (0.45 μm). The basal and transverse walls of the initials are still relatively thin (0.15 and 0.20 μm, respectively).

The expanding phase of antheridium initials was also observed in gametophytes stained for nucleic acids. Initials, in which the large, basal vacuole is beginning to form, are shown in Figure 81. An initial, after the large vacuole has formed, is shown in Figure 82. The nucleus in each of these initials is centrally located near the base of the
initial and the RNA-stained cytoplasm is localized in the upper, central region of the initial.

As the initial continues to expand in height and diameter, the basal vacuoles become larger and the nucleus begins to elongate perpendicular to the base of the initial (Figure 112). Serial sections through an initial during this phase of expansion reveal the relative distribution of the cytoplasm, nucleus, and vacuoles (Figures 113-115). The nucleus remains in contact with all of the walls of the initial through thin, cytoplasmic strands. There are a few chloroplasts along the lower walls, but most of the organelles are located in the upper, central part of the cell.

By the time the initial expands to about 28-30 μm in height and 38-40 μm in diameter, the cell is ready to divide. Serial sections through an initial during the late anaphase stage of mitosis are shown in Figures 116-119. In a near-median section, the upper area is dense with cytoplasm with peripherally located chloroplasts (Figure 116). A median section reveals the first set of chromosomes along with a portion of the forming phragmoplast near the base of the initial (Figure 117). In a section anterior to the median, the first set of chromosomes is still evident and the dense region of cytoplasm in the upper part of the initial contains more chloroplasts (Figure 118). The portion of the phragmoplast present in this section is at a greater distance from the base of the initial compared to the location of the phragmoplast in the previous section (Figure 117). In a more anterior section, a portion of the second set of chromosomes is evident near the base of the initial beneath the phragmoplast (Figure 119). The
The highest point of the phragmoplast is about 12 μm from the base of the initial, which is about two-fifths the height of the initial. Knowing the approximate height of the phragmoplast and the approximate distance from the center of the basal wall to an area along the upper, curved wall 12 μm high, the angle of the forming phragmoplast to the base of the initial can be determined to be about 40° using the inverse sine function. This proposed configuration is supported by data from a nucleic acid-stained initial during a similar stage of mitosis (Figure 83). The two sets of chromosomes are positioned such that a plane equidistant between them occurs at an angle of about 40-45° to the base of the initial.

Prior to the telophase stage of mitosis, the metaphase chromosomes are also observed to be located in a plane angled at about 40-45° from the base of the initial (Figure 120). The metaphase chromosomes occur in the central part of the anterior half of the initial, which is where the phragmoplast initially forms. An unusual structure occurs along the cell wall near the uppermost chromosome. It is composed of closely packed vesicles embedded in densely staining amorphous material (Figure 121). A similar structure occurs at about the same height along the curved, upper wall directly opposite the first structure (Figure 122).

Two-celled stage

The phragmoplast forms a funnel-shaped cell wall which divides the initial cell into a basal cell and an inverted cone-shaped upper cell (Figures 123-127). It is not known how the cell wall forms within the initial, but it is assumed that the wall forms in either a uni- or
bidirectional manner, pivoting around a central area of the basal wall. If the wall forms symmetrically, it spans a distance between the center of the basal wall to an area around the upper, curved wall at a height of about one-half the total height of the antheridium. The ratio of the height of the antheridium through the center point to the diameter of the base of the antheridium is about 3:4. The funnel-shaped cell wall is oriented at about a 40° angle to the base of the presumptive antheridium (Figures 123, 125). An idealized diagram of a symmetrical, two-celled antheridium drawn to these specifications is shown in Figure 128. To determine the volume of the structure occupied by each cell, the structure is divided into measurable geometric shapes as shown in Figure 129. The basic components are an upper half-sphere, BDF, and a lower cylinder, ABFG. The height and radius of the half-sphere each measure 2 units. The height of the cylinder measures 1 unit and the radius of the cylinder measures 2 units, resulting in a height to diameter ration of 3:4 for the entire structure. To determine the relative volume of the upper and basal cells, the total volume of the antheridium is determined first by adding the volume of the half-sphere to the volume of the cylinder. The summation of these volumes equals 29.3 cubic units (C.U.). The volume of the basal cell is determined by subtracting the volume occupied by the cone, CEO, from the volume occupied by the approximate cylinder, ACEG. The volume occupied by the basal cell is 14.7 C.U. The volume of the upper cell is simply the total volume of the antheridium minus the volume of the basal cell. The volume of the upper cell is 14.6 C.U. It appears that the upper and basal cells of symmetrical, two-celled antheridia have equal volumes even though they
have strikingly different shapes.

The basal cells in some two-celled antheridia are not symmetrical (Figure 127). The angle between the funnel-shaped wall and the basal wall varies in different regions of the antheridium. The length of the outer wall of the basal cell in the region where the angle is smallest is about one-third of the height of the antheridium. The length of the outer wall of the basal cell in the region where the angle is greatest is between two-thirds and three-quarters the height of the antheridium. An idealized diagram of an asymmetric two-celled antheridium is shown in Figure 130. The outer basal walls are one-third and three-quarters of the height of the antheridium. The asymmetric antheridium is drawn to the same height as the symmetric antheridium shown in Figure 128, which is 3 units. When a diagram is drawn to these specifications, the angle between the funnel-shaped wall and the basal wall varies from about 21° to 60°. To determine the relative volumes of the upper cell and basal cell, measurable, geometric figures are drawn within the structure (Figure 131). The half-sphere, BDF, corresponds exactly in size and shape to the half-sphere, BDF, in the symmetric antheridium in Figure 129. The cylinder, ABFG, is superimposed upon the asymmetric antheridium such that it corresponds exactly in size, shape, and location as the cylinder, ABFG, in the symmetric drawing in Figure 129.

Under these conditions, the volume of the upper cell in the asymmetric antheridium equals the volume of the upper cell in the symmetric antheridium. Both upper cells, circumscribed by CDEO have a volume of 14.6 C.U.
To determine the volume occupied by the asymmetric basal cell, SCOET, it is transformed into a symmetric cell by the subtraction and addition of specific portions. A wedge-shaped portion formed by the 180° rotation of the area circumscribed by OGT is subtracted from the asymmetric basal cell. A wedge-shaped portion of equal volume and shape is added to the other side of the basal cell indicated by the area ASO. The resultant symmetric basal cell, ACOEG, has the same shape and volume as the basal cell, ACOEG, in Figure 129, which is 14.7 C.U. Even if the funnel-shaped wall is asymmetrical, the volumes of the upper and basal cells appear to remain equal. It is proposed that the position of the funnel-shaped cell wall is determined by the shape of the initial cell such that the two daughter cells will occupy equal volumes.

The large, basal vacuoles that were in the initial cell (Figures 112-116) are partitioned into the funnel-shaped basal cell (Figures 123-127). The major portion of the cytoplasm is located in the upper cell. The nucleus in the upper cell is centrally located and appears to be much larger and less intensely stained than the nucleus in the basal cell (Figures 123, 125, 126). The nucleus in the basal cell occurs in the angle formed by the funnel-shaped wall and the basal wall (Figures 123, 126). Nucleoli are present in the nuclei of both cells. Figure 84 shows a 2-celled antheridium at a similar stage stained for nucleic acids. The centrally located nucleus in the upper cell is embedded in RNA-stained cytoplasm, while the basal cell is highly vacuolate.

Within the upper cell, the chloroplasts are confined mainly to a region between the nucleus and the outer, curved wall as shown in serial sections (Figures 132, 133). The chloroplasts are oriented in
various directions and most of them contain starch grains. Small vacuoles occur in the same regions as the chloroplasts. In the area between the nucleus and the lower half of the funnel-shaped wall, only ribosomes, mitochondria, RER, and some microtubules are found (Figures 132, 134). Microtubules are abundant around the nuclear envelope (Figure 135) and are also found along the cell walls (Figure 136). Microbodies and Golgi bodies are found within the cytoplasm of the upper cell (Figures 132, 133, 137) and occasionally osmiophilic bodies are observed (Figure 138).

The same types of organelles found in the upper cell are also found in the basal cell, but appear to be fewer in number and occur in a thin layer of cytoplasm along the cell wall. There is more heterochromatin present in the nucleus of the basal cell than the nucleus of the upper cell (Figures 132, 133).

The outer wall of the 2-celled antheridium is thinnest near the apex (0.25-0.30 μm) and gradually becomes thicker near the base of the structure (0.35-0.40 μm). The funnel-shaped wall is only about 0.10 μm thick, while the basal wall of the initial is about 0.20 μm thick. The latter two walls have very few plasmodesmata and in the region where they fuse, the combined wall can become quite thick (0.90 μm; Figure 139).

The basal cell will undergo no further divisions. Before the upper cell divides, many changes will occur within the cytoplasm. Sections through a 2-celled antheridium in which the upper cell nucleus is in prophase of mitosis are shown in Figures 140-142. The upper cell nucleus is much larger than the basal cell nucleus and has moved from a central location to an upper, central location (Figure 141). Many
nuclear pores are evident in the nuclear envelope (Figure 142) and average about 70 nm in diameter. Large vacuoles are now present in the region between the nucleus and the sides of the upper cell. Concomitant with the formation of the vacuoles is a redistribution of the chloroplasts which now occur in the cytoplasm immediately surrounding the nucleus (Figures 140-142). The longitudinal axes of the chloroplasts which are located between the nucleus and the top of the cell appear to be oriented parallel to the base of the antheridium. This same orientation holds true for the chloroplasts below the nucleus. The longitudinal axes of the chloroplasts located between the nucleus and the large vacuoles appear to be oriented perpendicular to the base of the antheridium. The largest chloroplasts measure about 5 μm in length.

A major portion of the cytoplasm is localized within the basal region of the upper cell (Figures 140-142). Figure 143 shows a median section through this accumulated cytoplasm between the nucleus and the base of the cell. In the lower portion of this area, the cytoplasm is composed mainly of mitochondria and ribosomes, with an occasional microtubule, small vacuole, and some smooth endoplasmic reticulum (SER; Figure 144). In the portion of the cytoplasm nearer the nucleus, the composition changes to mostly SER and ribosomes with some small vacuoles and microtubules present (Figure 144). An off-median section through this region is shown in Figure 145. The numerous profiles of mitochondria are interspersed with an abundance of SER. In three dimensions, the basal region of the upper cell is visualized as being lined with mitochondria and filled with ribosomes. A gradient of SER increases from bottom to top.
The cytoplasm surrounding the nucleus, which contains most of the chloroplasts, also contains some mitochondria, an abundance of SER, RER, and vesicle-forming Golgi bodies, an occasional microbody and numerous microtubules (Figures 140, 142, 146, 147). Very few microtubules are observed near the walls of the upper cell.

This redistribution stage in antheridium development was also observed in nucleic acid-stained gametophytes as shown in Figure 85. The enlarged nucleus of the upper cell is located in the upper, central region of the cell with the majority of the RNA-stained cytoplasm occurring within the basal portion of the cell.

The size of the antheridium has changed little during the redistribution of cytoplasm leading up to the next cell division. The height of the antheridium still averages about 28-30 \( \mu \text{m} \) and the basal diameter averages about 38-40 \( \mu \text{m} \).

The nucleus in the upper cell next enters into mitosis as shown in Figure 148. The centromeres of the metaphase chromosomes are aligned in a plane parallel to the base of the 2-celled antheridium about 10 \( \mu \text{m} \) from the apex of the upper cell. Telophase of this division is shown in a nucleic acid-stained antheridium shown in Figure 86. One set of daughter chromosomes occurs in the upper one-third of the cell and the other set occurs within the upper portion of the accumulated cytoplasm in the basal portion of the cell. A plane equi-distant from the two sets of chromosomes occurs parallel to the base of the antheridium and is presumed to be the orientation of the first formed portion of the succeeding phragmoplast. The actual formation of the phragmoplast was not observed, but sections through 3-celled antheridia all reveal
the presence of a curved cell wall dividing the newly formed daughter cells (Figures 149-151).

Three-celled stage

The 3-celled antheridium is comprised of a basal cell, a central cell, and a cup-shaped upper cell. The curved wall between the upper and central cells is oriented parallel to the outer curved wall of the upper cell. The distance between these two parallel curved walls is about one-fifth the total height of the antheridium, or about 6 μm. The inner, curved wall intercepts the funnel-shaped wall about 6 μm from the outer edge. An idealized diagram of a 3-celled antheridium is shown in Figure 152. To determine the relative volumes occupied by the upper and central cells, the figure is divided into measurable, geometric shapes in Figure 129.

To determine the volume of the central cell, the volume of the region circumscribed by points HKN (Figure 129) is first calculated by adding the volume of the half-sphere (IKM) to the volume of the cylinder (HIMN). The volume of HKN is 12.0 C.U. Next, the volume of the area circumscribed by the points HJOLN is calculated by subtracting the volume of the cone (JLO) from the volume of the approximate cylinder (HJLN). The volume of HJOLN is 5.0 C.U. Now, the relative volume of the central cell can be determined by subtracting the volume of the area, HJOLN, from the volume of HKN. The volume of the central cell is 7.0 C.U. As calculated earlier, the entire antheridium occupies a volume of 29.3 C.U., which means that the central cell occupies about 25% of the total volume.
The volume of the upper cell is determined by subtracting the volume of the central cell, JKLO, from the volume of the previous upper cell of the 2-celled antheridium which is circumscribed by the points CDEO. The relative volume of the upper cell is 7.6 C.U. which is about 25% of the total volume of the antheridium. It appears that the volumes of the upper and central cells of a symmetrical 3-celled antheridium are, on average, equal to each other, even though the cells have strikingly different shapes.

**Central cell**  The major portion of the cytoplasm in a 3-celled antheridium is partitioned into the central cell (Figures 78, 87, 149-151). Numerous small vacuoles occur in the cytoplasm surrounding the nucleus. The cytoplasm is densely packed with ribosomes and many mitochondria are present (Figure 153). Occasionally, a mitochondrion is observed containing an inclusion of densely staining, amorphous material (Figures 153, 154). Some of the mitochondria appear to be cup-shaped (Figures 155, 156) while others are dumbbell-shaped (Figure 153), indicating a metabolically active cell. Microbodies also occur within the cytoplasm (Figure 153, 157) along with vesicle-forming Golgi bodies (Figure 157). The vesicles are either smooth-walled and large (0.10 \( \mu \text{m} \)) or have coated walls and are smaller (0.03 \( \mu \text{m} \)). The large, smooth-walled vesicles often contain fibrous material (Figure 157). ER occurs throughout the cytoplasm along with many chloroplasts, some of which appear to be dividing (Figures 153, 155, 158). An average length of a chloroplast profile is about 3 \( \mu \text{m} \) in the central cell compared to 5 \( \mu \text{m} \) in the vegetative cells.

Microtubules are often found near the walls of the central cell
Microtubules also occur in other areas of the cytoplasm, oriented in various directions (Figure 157).

Occasionally, an invagination of the nuclear envelope (Figure 156) and more shallow indentations (Figure 162) are observed. The indentation in an envelope shown in Figure 162 occurs in the nuclear region facing the base of the antheridium. Numerous microtubules occur in the cytoplasm between the indentation and the base of the antheridium (Figure 163) and appear to be randomly oriented. A smaller indentation occurs in the nuclear envelope 180° from the larger indentation and faces the top of the antheridium (Figure 164). Microtubules are also observed near this indentation. The purpose of these indentations or invaginations is not known. However, their presence may be correlated to the next division, which in the developing antheridium, will occur in the central cell.

Most of the 3-celled antheridia have a height of about 28 μm and a basal diameter of 38 μm. One C.U. would equal 857 μm^3. The estimated volume of the central cells is about 6000 cubic μm (μm^3), using the method previously described for determining cell volumes.

**Upper cell** The upper cell of the 3-celled antheridium retains most of the large vacuoles that were present in the upper cell of the 2-celled antheridium (Figures 148-151, 153). They occur in the cytoplasm surrounding the centrally located nucleus. The types of organelles present in the central cell also are present in the upper cell. SER and RER are quite abundant (Figures 165-167), along with Golgi bodies producing both large, smooth-walled, vesicles containing fibrous material and smaller, coated vesicles (Figure 165). The vesicles
have about the same dimensions as the vesicles found in the central cell. Chloroplasts appear throughout the cytoplasm, some with constrictions (Figure 166). The average length of a chloroplast profile is about 3 μm. Microtubules occur along the cell walls of the upper cell (Figure 161) and deeper within the cytoplasm (Figure 165).

The upper cell is part of the developing sterile jacket of an antheridium and will undergo one more mitotic division.

**Basal cell** The basal cell of a 3-celled antheridium remains the same in appearance as the basal cell in a 2-celled antheridium. The volume remains unchanged and the basal wall is still about 0.20 μm thick. The thin walls of the central cell are about 0.10 μm thick and the outer curved wall averages about 0.30 μm in thickness. Plasmodesmata occur in all of the interior walls (Figure 153).

**Division of the central cell** The central cell is the first spermatogenous cell of the antheridium and is the next cell to divide in the developmental sequence. During telophase of the central cell division, the phragmoplast typically forms in a plane perpendicular to the base of the antheridium (Figure 168). Near-median sections through one set of telophase chromosomes are shown in Figures 169-172. The dense cytoplasm in the dividing cell contains many polysomes (Figures 170, 171). Many microtubules occur in the vicinity of the condensed chromosomes (Figure 171) along with double membrane segments closely associated with portions of the chromosomes (Figure 172). Presumably, the double membranes are the newly forming nuclear envelope.
Four-celled stage

Eventually, the phragmoplast develops into a cell wall resulting in two spermatogenous cells of equal volume and shape. Occasionally, the cell wall will form at a slightly oblique angle to the base of the antheridium (Figures 77, 173, 174). The 4-celled antheridia have about the same dimensions as the 3-celled antheridia, with a height of about 30 μm through the vertical center and a basal wall diameter of about 40 μm (Figures 173, 174). The cell walls are about as thick as the corresponding walls in the 3-celled antheridia.

Spermatogenous cells The ultrastructure of the two spermatogenous cells is similar to the ultrastructure of the one previous spermatogenous cell, except that the chloroplasts appear to be smaller than the chloroplasts in the jacket cells (upper and basal cells; Figure 173). Also, some of the chloroplasts are less differentiated in the spermatogenous cells than in the jacket cells (Figure 175), lacking an extensive lamellar system. Microtubules are present along all of the cell walls, including the newly formed cell wall (Figures 176, 177) and the outer walls of the two cells (Figures 175, 176). Microtubules also occur deeper in the cytoplasm (Figures 175, 177).

Upper cell The upper cell of a 4-celled antheridium is similar in appearance to the upper cell of a 3-celled antheridium (Figures 153, 173). The centrally located nucleus is surrounded by cytoplasm containing mitochondria, chloroplasts, RER, and an occasional microbody (Figures 173, 178, 179). Golgi bodies have produced many vesicles in the cytoplasm near the nucleus (Figure 178). Large vacuoles occupy a large portion of the peripheral cell volume (Figures 173, 174).
Microtubules are present along the cell walls and deeper within the cytoplasm (Figures 175, 178, 179). The next cell division in the developmental sequence will occur in the upper cell.

**Basal cell** The ultrastructural appearance of the basal cell has not changed from that described for the 3-celled antheridia.

**Division of the upper cell** Figures 180-185 show serial sections through a 4-celled antheridium in which the upper cell is undergoing cytokinesis following nuclear division. The sections are cut at an angle oblique to the base of the antheridium. To conveniently refer to various areas in the upper cell, the outer curved wall of the cell is geometrically divided into 180 degrees. Zero degrees (0°) is arbitrarily assigned to the junction of the funnel-shaped wall of the basal cell and the outer, curved wall of the antheridium on the left-hand side. At 180° from that junction is the junction of the funnel-shaped wall with the outer curved wall on the right-hand side of the antheridium (Figure 180). In Figure 180, the phragmoplast is observed at about 55°. There is a substantial amount of cytoplasm surrounding the phragmoplast in this region. In the area between 110° and 130°, there are many cytoplasmic strands traversing the cell. In a more median section, a portion of one daughter nucleus occurs at about 85° (Figures 181, 187). The phragmoplast can be seen in two regions, one at 50° and one at about 120°. In the region near 120°, there is a large amount of cytoplasm surrounding the phragmoplast (Figures 181, 187, 188). The phragmoplast is oriented in a plane perpendicular to both the outer and inner curved walls of the upper cell and consists of numerous small and larger, smooth-walled vesicles. Within the
larger vesicles, cell wall material is beginning to accumulate. Small, coated vesicles are also present in the phragmoplast. The coalescing vesicles are surrounded with a densely staining, amorphous material, particularly in the lower portion of the phragmoplast. At the lowest end of the phragmoplast, near the inner wall, there is a globular group of small vesicles embedded in the densely staining material. This structure is reminiscent of the previously described structure which occurs in the dividing initial cell (Figures 121, 122). It appears that the upper portion of the phragmoplast is more developed than the lower portion, evidenced by a greater amount of vesicle fusion and a lack of densely staining material near the vesicles in the upper portion.

The surrounding cytoplasm contains many polysomes, SER and RER, some mitochondria and very active Golgi bodies (Figures 188, 189). Microtubules are abundant and appear to be oriented perpendicular to the phragmoplast. The portion of the phragmoplast near the 50° region is surrounded by much less cytoplasm (Figures 181, 187, 190), particularly to the left of the phragmoplast. This portion of the phragmoplast appears to be in a later stage of development than the portion which occurs at 120°, exhibiting more vesicular fusion and lacking any densely staining material near the fused vesicles. Microtubules occur mainly near the lower portion of the forming wall, oriented perpendicular to the new wall. The cytoplasm to the right of the forming wall contains active Golgi bodies, mitochondria, RER, chloroplasts and microbodies (Figures 187, 190).

In a median section through the dividing upper cell, the forming cell wall is still visible both at about 50° and 120°, but the amount
of associated cytoplasm has decreased in both areas (Figure 182). A large profile of the daughter nucleus occurs in the median section at about 90°.

Moving through the center of the antheridium to an off-median section (Figure 183), a smaller profile of the nucleus is still present at about 90° while the other daughter nucleus is now apparent at about 60°. It is not clear from this micrograph where the forming cell wall is located, but it is presumed to be around 70° and 115°.

In a more peripheral section, the first-described nucleus is no longer in view, while the second nucleus has a larger profile near 60° (Figure 184). The presence and position of the forming cell wall are not evident in this micrograph, but it is highly likely that some of the cellular contents located between 80° and 100° are part of a para-dermal section of the new cell wall.

An even more peripheral section through the upper cell (Figures 185, 191) reveals a much larger profile of the second, peripheral nucleus centered at 70° with no evidence of the forming cell wall. A diagramatic interpretation of the location of the two nuclei, the phragmoplast, and new cell wall regions looking down through the outer curved wall of the upper cell is shown in Figure 186. The centrally located nucleus is completely encircled by the cylindrical phragmoplast/cell wall formation. The other nucleus occurs outside the newly forming cell wall in a peripheral region of the upper cell. It appears that the youngest stage of the phragmoplast occurs on the side opposite the peripheral nucleus, and that a more differentiated stage of wall formation exists as the wall curves around the central nucleus approaching.
the vicinity of the peripheral nucleus. If phragmoplast formation occurs in a continuous manner, either uni- or bidirectionally, the most advanced stage of wall formation would occur between the two nuclei. The only other interpretation would invoke an assumption that phragmoplast formation occurs in a discontinuous manner, which seems improbable in the absence of any supporting evidence.

**Five-celled stage**

After cytokinesis is completed, the centrally located, disc-shaped cell is known as the cap cell. The remaining volume of the upper cell is partitioned into a doughnut-shaped cell known as the ring cell (Figures 192, 193). The formation of the sterile jacket of an *Onoclea* antheridium is now completed, consisting of a basal cell, a ring cell, and a cap cell. The three jacket cells enclose two central, spermatogenous cells at this stage in development (Figure 81).

Prior to, or during the formation of the cap cell, the antheridium increases in height from about 30 μm to about 40 μm, while maintaining a basal diameter of about 40 μm (Figure 185). The height to diameter ratio is now about 1:1 compared to the earlier ratio of 3:4. Because of this increase in size during the cap cell formation, an estimate of the initial volume of a cap cell may vary, but an approximate estimation is made according to the following observations. The distance between the upper and lower walls of the cap cell and ring cell is still about one-fifth of the total height of the antheridium (Figure 191). The funnel-shaped wall of the basal cell intersects the outer wall of the antheridium at a point equi-distant from the apex and base.
of the antheridium. The phragmoplast and subsequent cell wall of the new cap cell occur in an area about 30-40° from the vertical axis of the antheridium (Figures 180-185, 192). An idealized diagram of a symmetrical, 5-celled antheridium drawn to these specifications is shown in Figure 193. To determine the relative volumes of each cell, the antheridium is divided into measurable geometric shapes as shown in Figure 194. The basic components are an upper half-sphere, BDF, and a lower cylinder, ABFG. The height and radius of the half-sphere each measure 2 units. The height and radius of the cylinder each also measure 2 units, resulting in a height to diameter ratio of 1:1 for the entire antheridium. The cell wall of the cap cell, indicated by the lines CJ and EL, are extrapolated back to the vertical axis line, DO, such that they form an angle of 70°. To determine the volume of the cap cell, circumscribed by the points CDELKJ, the volume of the half-sphere, IKM, is first subtracted from the volume of the larger half-sphere, BDF, to obtain the volume of the hollow half-sphere, BDFMKI. The volume of the hollow half-sphere is 13.1 C.U. The volume of the cap cell occupies 70° of the total 180° of the hollow, half-sphere, which is 39% of the total volume. Thus, the volume of the cap cell is 39% of 13.1 C.U., or 5.1 C.U.

The volume of the ring cell is determined by first calculating the combined volume of the cap cell and ring cell, circumscribed by the points BDFNKH. This is accomplished by first determining the volume of the half-sphere/cone combination, BDFO, and from this volume, subtracting the volume of the half-sphere/cylinder/cone combination, HKNP. The volume of the combined cap and ring cell equals 16.1 C.U. The volume
of the ring cell equals the volume of the cells combined minus the volume of the cap cell, or 11.0 C.U. It appears that the cap cell occupies about one-third of the previous upper cell volume, while the ring cell accounts for the other two-thirds. This estimate of the initial cap cell volume may be conservative. If the cap cell were to form prior to the increase in antheridium height, that is, when the antheridium has a height to diameter ratio of 3:4, the estimated cap cell volume would be greater than for antheridia with a height to diameter ratio of 1:1, with all other specifications being equal. Figure 195 shows an idealized drawing of a 5-celled antheridium which has a height to diameter ratio of 3:4, with the cap cell wall located at 60° and 120°. When the wall of the cap cell is extrapolated back to the vertical axis, an angle of 60° is formed (Figure 196). Using the same methods for determining relative volumes as previously described, the relative volume of the cap cell, UVXW, is calculated to occupy 50% of the volume of the previous, upper cell, and thus is equal in volume to the ring cell.

Care must be taken when estimating the initial volume of the cap cell since the cap cell is forming during a period of rapid growth of the antheridium. Cell volume estimates may range anywhere from 25-50% of the total upper cell volume, depending on when estimates are made.

**Cap cell** A major portion of the ribosome-dense cytoplasm in the upper cell is partitioned into the cap cell (Figures 187, 197), containing chloroplasts, mitochondria, SER and RER, microbodies, vesicle-forming Golgi bodies, and small- and medium-sized vacuoles.
Microtubules occur near the cell walls and throughout the cytoplasm (Figure 198). Many plasmodesmata traverse the wall separating the cap cell from the ring cell. The upper wall is about 30 μm thick, while the lower wall is about 10 μm thick.

**Ring cell** The ring cell retains most of the large vacuoles that were present in the upper cell (Figures 191, 192, 199). The cytoplasm contains the same kinds of organelles present in the cap cell. Many Golgi bodies are present, producing numerous vesicles (Figure 200). The outer wall is about 30 μm thick, while the other walls of the ring cell are about 10 μm thick.

**Basal cell** The ultrastructure of the basal cell has changed little since the cell was first formed, although the cell continues to enlarge. The basal cell is still mainly composed of one large vacuole with a thin-layer of cytoplasm along the cell walls containing chloroplasts, mitochondria, vesicle-forming Golgi bodies, ER, microbodies, and microtubules (Figures 201, 202). There are few plasmodesmata in the walls of the basal cell. The funnel-shaped wall is about 10 μm thick, the basal wall is about 20 μm thick, and the outer wall is about 30 μm thick.

**Spermatogenous cells** These meristematic appearing cells are densely packed with ribosomes (Figure 81) and contain all of the types of organelles found within the jacket cells (Figures 201-204). Many mitochondria are present, some of which are dumbbell-shaped (Figure 201), while others have a lobed appearance (Figure 203). Some of the chloroplasts are constricted and appear to be dividing (Figures 201, 204) and numerous Golgi bodies are present, actively forming vesicles.
Figure 204). Microtubules are found throughout the cytoplasm, both along the cell walls and deeper within the cytoplasm (Figures 203, 204). These two cells appear to be metabolically active and indeed, will soon divide mitotically to form a 7-celled antheridium containing four spermatogenous cells. The jacket cells will no longer divide, but will continue to expand in size as the spermatogenous tissue grows.

It is estimated that the actual volume of each spermatogenous cell is about $2500 \mu m^3$, if the 5-celled antheridium has a height to diameter ratio of $30 \mu m \times 40 \mu m$. After the 5-celled antheridium reaches the dimensions of $40 \mu m \times 40 \mu m$, the volume of each spermatogenous cell is estimated to be $4500 \mu m^3$.

Seven-celled stage

The 7-celled antheridium is formed when the two spermatogenous cells in the 5-celled antheridium divide to form four spermatogenous cells. If the previous division of the initial spermatogenous cell was in a plane perpendicular to the base of the antheridium, the succeeding division is also in a plane perpendicular to the base of the antheridium but at a $90^\circ$ angle to the first division. Sections through 7-celled antheridia are shown in Figures 205-209. Figures 205-208 show serial sections through an antheridium. In Figure 205, the first nucleus is evident in the cell on the right. In Figure 206, that nucleus is barely evident, while a second nucleus is apparent in the cell on the left. In Figure 207, the second nucleus is no longer present, but a third nucleus is now apparent in the second cell on the right. In Figure 208, the third nucleus is no longer visible, while a fourth
nucleus is now apparent in the second cell on the left. The 7-celled antheridia have a height of about 40 μm. The diameter, measured both at the base and through the center of the antheridia is about 40 μm.

To estimate the volume occupied by the four spermatogenous cells, various measurements were taken from median sections of the antheridium and transformed into an idealized drawing (Figure 210). Figure 210B shows a drawing of the spermatogenous region of the antheridium shown in Figure 207. The line AD represents the distance across the region measured at the height of the junction of the funnel-shaped wall with the inner wall of the ring cell. Line BC represents the diameter of the bottom wall of the cap cell. Line GF is the distance between the lines BC and AD and line FE is the distance from line AD to the base of the antheridium. When lines AB and CD are extrapolated upward to point H, measurable geometric figures emerge from which the volume of the spermatogenous region can be calculated.

To determine the volume occupied by the spermatogenous cells, represented in two dimensions by ABCDE, the volume represented by ABCD is first determined. To do this, the volume of the cone BHC is subtracted from the volume of the cone AHD. The volume of the inverted cone ADE is then added to the volume of ABCD. The volume occupied by the four spermatogenous cells is estimated to be 10,500 μm³. The volumes of spermatogenous regions in two other 7-celled antheridia are estimated to be 9,500 and 10,000 μm³. The average of 10,000 μm³ is about twice the volume of the first spermatogenous cell, that, as reported earlier, has an average volume of about 5,000 μm³. Thus, each of the four spermatogenous cells, with average volumes of about
2,500 \mu m^3, are about one-half the size of the first spermatogenous cell. The described method of estimating volumes of spermatogenous cells is applied to all succeeding stages in antheridium development.

**Spermatogenous cells** Aside from their reduced size, all four spermatogenous cells have the same meristematic appearance as the previous two spermatogenous cells in 5-celled antheridia (Figures 209, 211). Many of the mitochondria appear to be either dumbbell-shaped or cup-shaped. Infrequently, a mitochondrion accumulates a large amount of densely staining amorphous material (Figure 212). Many of the chloroplasts are smaller than the chloroplasts in the jacket cell and also appear to be less differentiated. Microtubules are quite abundant and occur near the cell walls, near the nuclear envelope, and deeper within the cytoplasm (Figures 213, 214, 215). Occasionally, an osmiophilic body is observed within the cytoplasm (Figure 215).

**Cap and ring cells** The ultrastructure of the cap and ring cells is very similar to the ultrastructure of the corresponding cells in the 5-celled antheridia (Figures 209, 211). They are highly vacuolate with the same types of organelles occurring in the peripheral cytoplasm. The shapes of these two cells differ somewhat from the previous stage. The inner walls of both cells are now flat, as opposed to their previous concave appearance in the 5-celled antheridium (Figures 197, 201, 209). The inner walls of the cap and ring cells are still about 0.10 \mu m thick, while the outer wall is about 0.30 \mu m thick.

**Basal cell** The ultrastructure of the basal cell has changed little since its inception. The basal wall is still about 0.20 \mu m thick, while the funnel-shaped wall is only about 0.10 \mu m thick. The
outer wall varies in thickness. Near the base of the cell, the wall is about 0.50 \( \mu m \) thick, while near the top of the basal cell, it is about 0.30 \( \mu m \) thick (Figure 211).

**Eleven-celled stage**

The 11-celled stage is formed when the four spermatogenous cells in the 7-celled antheridia divide synchronously to form eight spermatogenous cells. Serial sections cut in a plane perpendicular to the base of an 11-celled antheridium are shown in Figures 216-219. The divisions are in a plane parallel to the base of the antheridium and at a 90° angle to all the previous divisions, which were perpendicular to the base of the antheridium. If the previous divisions were not in a perpendicular plane to the base of the antheridium, the most recent divisions, forming eight cells, are still oriented in a plane at an angle of 90° to the first two synchronized divisions (Figures 220, 221).

The 11-celled antheridia have a height and central diameter of about 45 \( \mu m \). To estimate the volume occupied by eight spermatogenous cells, measurements are taken from perpendicular, median sections, such as the one shown in Figure 217 and transformed into an idealized drawing shown in Figure 210C. The volume is estimated to be about 16,000 \( \mu m^3 \). Estimated volumes of two other 11-celled antheridia are 11,500 and 14,000 \( \mu m^3 \). Thus, the volume of each spermatogenous cell ranges from 1,400 to 2,000 \( \mu m^3 \). The eight spermatogenous cells each have a volume between four-fifths and one-half of each of the previous four spermatogenous cells, which have volumes of about 2,500 \( \mu m^3 \).
Spermatogenous cells

The spermatogenous cells are meristematic in appearance and densely packed with ribosomes (Figures 222, 223). Figure 222 shows a section through the antheridium perpendicular to the base, while Figure 223 shows a section through an antheridium parallel to the base. The mitochondrial profiles range in appearance from circular to oblong to dumbbell-shaped and cup-shaped. As in previous stages, an occasional mitochondrion contains some densely staining amorphous material (Figure 224). Most of the chloroplasts are smaller than the chloroplasts in the jacket cells. The average longitudinal length of the chloroplasts in the spermatogenous cells is about 1.5 \( \mu \text{m} \) compared to an average length of about 4.0 \( \mu \text{m} \) for chloroplasts in the jacket cells. The spermatogenous cell chloroplasts are less differentiated than those in the jacket cells, with respect to the amount and organization of lamellae and the lack of starch grains. Some chloroplasts are highly lobed and appear to be dividing (Figure 223). Golgi bodies occur throughout the cytoplasm producing both smooth and coated vesicles (Figures 222, 223, 225). In some 11-celled antheridia, the spermatogenous cells contain more osmiophilic bodies than observed in previous stages (Figures 226, 227). SER and RER occur throughout the cytoplasm and occasionally a microbody is observed (Figure 226, 227).

Microtubules are abundant near the cell walls, oriented in various directions (Figures 226, 227). They occur singly or in small groups oriented parallel to each other (Figures 228, 229). A tangential section through a nuclear envelope reveals the presence of many microtubules oriented in different directions around the nucleus (Figure 230). Polysomes are evident in the cytoplasm (Figure 230). Invagina-
tions of the nuclear envelope are occasionally observed (Figures 222, 225).

The cell walls between the spermatogenous cells are very thin, measuring only about 0.10 μm. Many plasmodesmata traverse the connecting walls (Figures 222, 223).

Cap cell The ultrastructure of the cap cell has changed little since the 7-celled stage. Large vacuoles occupy the major portion of the cell (Figures 222, 231). The peripheral cytoplasm contains mitochondria, usually with circular or oblong profiles, and chloroplasts with extensive lamellar systems and many, large starch grains. Vesicle-forming Golgi bodies are present, along with ER (Figures 222, 231) and microbodies (Figure 232).

The thickness of the outer wall of the cap cell varies between 0.30 and 0.40 μm. The inner walls are about 0.10 μm thick and contain a few plasmodesmata.

Ring cell The ultrastructure of the ring cell, as with the cap cell, has changed little in appearance since the previous stage. The ring cell contains the same kinds of organelles found within the cap cell, and like the cap cell is composed mainly of large vacuoles. In Figure 233, microtubules are shown near the outer wall of the ring cell, aligned parallel to each other and to the wall.

The outer wall varies in thickness between 0.30 and 0.40 μm, while the wall between the ring cell and the spermatogenous cells is only about 0.10 μm thick. The wall between the ring cell and the basal cell is about 0.15 μm thick. A few plasmodesmata occur in all the walls except for the outer wall. The wall between the ring cell and the
spermatogenous cells tends to be more convex than in the 7-celled antheridia (Figures 222, 231).

**Basal cell** Aside from an increase in volume, the ultrastructure of the basal cell remains unchanged with respect to previous stages. The cell contains one very large vacuole with a thin layer of cytoplasm near the cell walls (Figures 222, 234). The cytoplasm contains the same types of organelles present in the cap and ring cells.

The funnel-shaped wall is about 0.15 μm thick, the outer wall is between 0.30 and 0.40 μm thick, and the basal wall is about 0.20 μm thick. All of the walls have a few plasmodesmata except for the outer wall.

**Nineteen-celled stage**

Nineteen-celled antheridia are formed after the eight spermatogenous cells of 11-celled antheridia undergo synchronized mitotic divisions to form 16 spermatogenous cells. Figures 235 and 236 show perpendicular and oblique sections through 19-celled antheridia, respectively. The orientation of this set of divisions was not followed. Nineteen-celled antheridia have increased to a height and central diameter of about 50 μm. To estimate the volume occupied by the 16 spermatogenous cells, measurements are taken from the antheridium shown in Figure 235 and transformed into the drawing shown in Figure 210D. The volume of the spermatogenous tissue is estimated to be about 26,000 μm³. The volume occupied by each cell is about 1,600 μm³. Due to a lack of measurable 19-celled antheridia, an average volume and range are not available. The volume per each of the 16 spermatogenous cells varies between
four-fifths less to one-tenth greater than the volume per spermatogenous
cell in some 11-celled antheridia. The fact that some groups of 16
spermatogenous cells have a greater volume per cell than some groups
of eight spermatogenous cells may be due to natural variation between
antheridia or may be due to the fact that some antheridia will produce
32 spermatozoids while others will produce 64 spermatozoids. Spermato-
genous cell volumes in early stages of antheridium development may be
indicative of the terminal number of spermatozoids.

Spermatogenous cells    The ultrastructure in the spermatogenous
cells of 19-celled antheridia appears to be very similar to the ultra-
structure of spermatogenous cells in 11-celled antheridia. The cyto-
plasm, densely packed with ribosomes, contains many mitochondria, ranging
in shape from circular to cup-shaped (Figures 237-239). As in previous
stages, an occasional mitochondrion contains an inclusion of some densely
staining, amorphous material (Figures 237, 238). The chloroplasts,
with an average length of about 1.5 μm, lack an extensive lamellar net-
work and contain few starch grains. Vesicle-forming Golgi bodies, small
vacuoles, SER and RER occur throughout the cytoplasm (Figures 237,
239-241). In some regions, the SER appears to be continuous with the
outer membrane of the nuclear envelope (Figure 240). Osmiophilic bodies,
with a diameter of about 0.35 μm, are quite prevalent in the spermatogenous
cells of some antheridia (Figures 237, 238).

Numerous microtubules occur throughout the cells. They occur near
the nuclear envelope, oriented in various directions (Figure 240), or in
groups parallel to each other (Figure 241). Many microtubules also are
found near the cell walls (Figure 238).
The walls between the spermatogenous cells are still quite thin (0.10 \mu m), and contain many plasmodesmata (Figures 237-239).

**Cap cell** The highly vacuolate cap cell contains large chloroplasts with many large starch grains (Figure 242). The chloroplasts have an average length of about 5 \mu m. The other types of organelles described in cap cells of earlier stages are still present in the peripheral cytoplasm. The outer wall of the cap cell is relatively thick. There are regions that are as thick as 0.60 \mu m (Figure 242). The side and lower walls of the cap cell are between 0.10 and 0.20 \mu m thick, and contain a few plasmodesmata.

**Ring cell** The highly vacuolate ring cell also contains large chloroplasts that have accumulated many large starch grains (Figure 243). The peripheral cytoplasm contains the same types of organelles previously described for ring cells in earlier stages. The outer wall of the ring cell is quite thick (0.40 \mu m), but not as thick as the outer wall of the cap cell (0.60 \mu m). The internal walls are about 0.10 to 0.20 \mu m thick, and contain a few plasmodesmata.

**Basal cell** The highly vacuolate basal cell has changed little in ultrastructural appearance since the cell was formed (Figures 235, 239, 244). The funnel-shaped wall is about 0.15 to 0.20 \mu m thick and the basal wall is about 0.25 \mu m thick. Both walls have a few plasmodesmata.

**Thirty-five-celled stage**

The 35-celled antheridium is formed when the 16 spermatogenous cells divide synchronously to form 32 spermatogenous cells. The size
of the 35-celled antheridia varies. Larger antheridia are shown in Figures 245 and 246 with a height and central diameter of about 55 μm. Measurements taken from Figure 245 are transformed into the drawing shown in Figure 210E. The volume occupied by the 32 spermatogenous cells is estimated to be about 36,000 μm³. The volume of the spermatogenous cells in the antheridium shown in Figure 246 is estimated to be about 33,000 μm³. The volume per cell is between 1,100 and 1,000 μm³. Each spermatogenous cell volume is about 35% less than the cell volume of each of the previous 16 spermatogenous cells. Not all 35-celled antheridia are this large though.

Figure 247 shows an antheridium that has terminated the division phase with 32 spermatogenous cells. The 32 cells are in an early stage of spermatid differentiation, the details of which will be presented in the section on "Cellular Changes during the Differentiation Phase of Spermatids." The antheridium in Figure 247 has a height and central diameter of only 50 μm. The volume of the spermatogenous cells is estimated to be about 24,000 μm³, with each cell occupying about 750 μm³. Each of these 32 cells is about 50% less in volume than each of the previous 16 cells. The possibility exists, although conclusive data are lacking, that the smaller groups of 32 spermatogenous cells may enter directly into spermatid differentiation, while the larger groups of 32 spermatogenous cells will divide one more time before differentiating into spermatozoids.

Due to the limiting amount of observations of this stage in
development, it cannot be ascertained that the spermatogenous cells shown in Figures 245 and 246, which are larger than those in Figure 247, will divide one more time. If they are going to divide one more time, the cells are known as spermatid mother cells. If they enter directly into the differentiation phase, the cells are known as spermatids, in which case the previous 16 cells are known as spermatid mother cells.

Spermatogenous cells in larger, 35-celled antheridia The cytoplasm of these cells, densely packed with ribosomes, contains small, dedifferentiated chloroplasts, with some vesiculate, internal membranes (Figures 248-250). Numerous mitochondria are present and range in shape from circular to cup-shaped. As in previous stages, an occasional mitochondrion is observed with an inclusion of amorphous material (Figure 251). Golgi bodies are abundant and are actively producing vesicles (Figure 249). Many of the larger, smooth-walled vesicles contain some fibrous material. ER and small vacuoles occur throughout the cytoplasm (Figures 248-250), along with some osmiophilic bodies (Figure 250).

A new structure, the blepharoplast, has appeared within the cytoplasm (Figures 248, 252). The blepharoplast is composed of a circular mass of densely staining material (presumably spherical in shape), penetrated by numerous lightly stained channels (Figures 253-255). Figure 255 reveals a sharply bent channel that extends through the blepharoplast. A dark staining tubule runs lengthwise through the center of each channel, with thin filaments radiating out from the tubule to the sides of the channel (Figures 253-255).

Numerous microtubules occur in the cytoplasm near the blepharoplast
oriented perpendicular to the tangent of the blepharoplast (Figures 253-255). The ends of some of the microtubules appear to terminate in the outer region of the densely stained material (Figures 253-255). Microtubules also occur near the cell walls and the nuclear envelope, either singly or in small groups oriented parallel to each other (Figures 249, 250).

Early stages in blepharoplast formation are observed in spermatogenous cells of larger, 35-celled antheridia. An aggregation of moderately stained, flocculent material, about 0.5 µm in diameter, appears in the periphery of the cell (Figure 256). Two plaques of densely staining material arise about 40-50 nm apart from each other in the flocculent material (Figures 257, 258). Each plaque is triple-layered, with two dense, plate-like layers separated by a light layer. The distal layer of each plaque is thicker than the proximal layer. The plaques are about 200 nm in length.

The distal layers begin to accumulate dense material as the flocculent material decreases in amount (Figure 259). Light-staining channels appear in these growing distal layers (Figure 259), each which begins to resemble a blepharoplast. Microtubules are found in the area of these developing blepharoplasts (Figure 260).

Further stages in the development of blepharoplasts were not observed in Onoclea. If their development follows the development described for Marsilea, a heterosporous fern, the two growing distal layers will become spherical blepharoplasts that will separate at prophase of the spermatid mother cell division. After moving to opposite poles of the
spindle, cytokinesis insures that each spermatid will have one blepharoplast.

In previous studies of spermatogenesis in homosporous ferns, blepharoplasts always have been observed in both spermatid mother cells and spermatids. From these studies, it has been determined that at some point in time, the blepharoplast in spermatid mother cells divides to form two blepharoplasts. Although only one blepharoplast has been observed in spermatogenous cells of larger 35-celled antheridia, the presence of more than one blepharoplast is not ruled out.

As previously mentioned, about one-half of Onoclea antheridia produce 32 sperm. Therefore, about one-half of the 19-celled Onoclea antheridia contain 16 spermatogenous cells that function as spermatid mother cells. The fact that no blepharoplasts were observed in spermatogenous cells of 19-celled antheridia is attributed to the small sample size (2) of the 19-celled stage.

The cell walls between the 32 spermatogenous cells are thicker than in previous stages, with a thickness of about 0.20 µm (Figures 248, 252). Numerous plasmodesmata still connect the cells.

If the 32 spermatogenous cells are functionally spermatid mother cells, they will divide one more time to form 64 spermatids.

**Sixty-seven-celled stage**

Median sections through 67-celled antheridia, cut perpendicular to the base, are shown in Figures 261 and 262. These antheridia have a height and central diameter of about 60 µm. Measurements taken from the antheridium shown in Figure 261 are transformed into the drawing
shown in Figure 210F. The volume of the spermatogenous cells is estimated to be about 46,000 $\mu m^3$. The volume of the spermatogenous cells shown in Figure 262 is estimated to be about 51,000 $\mu m^3$. The volume per spermatid varies between 720 and 790 $\mu m^3$, which is about the same volume estimated for each of the 32 spermatids within smaller, 35-celled antheridia (750 $\mu m^3$).

The volumes per spermatogenous cell for all the stages in the division phase of antheridium development are shown graphed in Figure 263 on a logarithmic scale. The average volume of the initial spermatogenous cell is about 5,000 $\mu m^3$. The final cell products of the division phase of development, the spermatids, have a volume of about 750 $\mu m^3$, whether they are in groups of 32 or 64. Their volume is about 15% of the volume of the initial spermatogenous cell.

The volume per spermatogenous cell in the middle stages of development lie either approximately on or well-below the line drawn between the initial volume and the volume of each of the 64 spermatids. It is proposed that a second line can be drawn parallel to the first line, representing the developmental pathway terminating with 32 spermatozoids. It is hypothesized that the initial spermatogenous cell in this pathway will have a volume of about 4,000 $\mu m^3$ and that the size of the initial spermatogenous cell is highly correlated to the final number of spermatozoids produced per antheridium. From the graph, it appears that for every doubling of cells, the volume per cell decreases by about three-tenths. A decrease in cytoplasmic volume below a threshold level may be correlated with a switch from the division phase of development to a differentiation phase of development.
Cellular Changes during the Differentiation of Spermatids into Spermatozoids

Early spermatids

Blepharoplasts and centrioles The blepharoplast in each spermatid enlarges as it is transformed into numerous procentrioles (Figures 264-266). Sets of doublet microtubules forming the procentrioles can be seen in Figure 266. Eventually, nine sets of triplet microtubules will form the mature centriole. The procentrioles have a length of about 0.30 μm and an outer diameter of about 0.15 μm. The inner diameter is about 0.11 μm. A central tubule is present in the procentrioles with thin filaments radiating outward along the entire length of the tubule, spanning the lumen between the central tubule and the sets of microtubules. The internal structure of the procentrioles is reminiscent of the internal structure of the channels in newly formed blepharoplasts.

Cellular features The early spermatids are still angular in shape and are separated by a uniformly thick cell wall of about 0.20 μm (Figure 264). Many plasmodesmata traverse the adjoining cell walls. The spermatid nucleus is spherical to oblong with an abundance of heterochromatin (Figure 264).

Cytoplasmic isolation of spermatids

Initial production of cell wall material The spermatids become spherical in contrast to the previous angular-shaped cells (Figure 267). Concomitant with their changing shape, there is a large accumulation of fibrous cell wall material between the plasmalemma and certain regions of the previously formed cell wall (Figures 267, 268). Although a
few cytoplasmic connections occur where the cell wall remains thinnest, the spermatids are essentially cytoplasmically isolated.

**Internal cellular features** Golgi bodies are present forming numerous vesicles containing fibrous material similar to the material accumulating outside the plasmalemma (Figures 267, 268). Also present within the cytoplasm are polysomes, RER, and dedifferentiated chloroplasts (Figure 268). Some of the plastids have a few starch grains and occasionally some osmiophilic globuli. The few thylakoids present tend to be somewhat dilated (Figure 268).

**Blepharoplasts and multilayered structures (MLS)** The procentrioles have now developed into centrioles which become distributed in a row along the length of a microtubular platform (Figure 269). The centrioles are embedded in densely staining, amorphous material from the blepharoplast. The microtubular platform consists of closely associated microtubules aligned parallel to each other in the same plane. These microtubules are part of a larger body known as the multilayered structure (MLS). To complete the MLS, a layer of lamellar plates is formed subtending the microtubule platform (Figure 270). The MLS becomes closely associated with a mitochondrion (MA), with contact occurring between the lamellar plates and the mitochondrion (Figure 270). The appearance of the MLS varies, depending on the plane of section. When a section is cut perpendicular to the long axis of the microtubules, the lamellar plates appear as a dense plaque (Figure 271). If the section is cut at an angle oblique to the long axis of the microtubules, the lamellar plates are readily apparent (Figure 272). The long axis of the microtubule platform is oriented at an angle of about 40° to the long axis.
of the lamellar plates (Figure 273).

The mitochondrion and lamellar plates elongate in the same direction (Figure 274). During this elongation process, microtubules continue to be added to the platform at a 40° angle to the lamellar plates. At some point in time, elongation ceases and the number of microtubules becomes constant. A diagrammatic reconstruction of the MLS at this point in time is shown in Figure 275 as modified from Duckett (1975). The lamellar plates are collectively known as the lamellar strip (LS) and the microtubules are collectively known as the microtubule band (MB).

Either during or after elongation, the entire assemblage of centrioles, MLS and MA becomes associated with the nucleus in a very specific way. The mitochondrion moves into a groove formed by an indentation of the nuclear envelope (Figures 271, 272). The microtubules, which extend beyond the lamellar strip, become closely appressed to the nuclear envelope. In the view shown in Figure 271, there are 85 microtubules evident in the MB. The microtubules will continue to elongate in the direction that the nucleus will elongate and eventually coil. A diagram of the initial relationship between the MLS and the spherical nucleus is shown in Figure 276 as modified from Duckett (1975).

The centrioles, now functioning as basal bodies, are distributed in a row along the MB (Figures 274, 277). Densely staining, amorphous material occurs between each of the basal bodies and also between the basal bodies and the MB (Figure 277). The long axes of the basal bodies are oriented at angles oblique to the long axis of the MB (Figure 278).

Flagella are being formed from the basal bodies. The transition zone of the flagellum proper is characterized by the appearance of a
nine-pointed, star-shaped, internal substructure (Figure 277). The appearance of the internal substructure varies along the length of the forming flagellum (Figures 272, 277). Nine sets of triplet microtubules are present in the basal bodies and the adjacent transition zone.

Non-MB microtubules are also present within the cytoplasm of the spermatids (Figure 279). These more randomly-oriented microtubules appear to be inserted in the dense, amorphous material presumably derived from the blepharoplast.

Additional production of cell wall material As the MB elongates to the point of encircling at least half of the spherical nucleus, a new layer of fibrous material is deposited outside of the plasmalemma (Figures 280, 281). This material is more dense than the fibrous material deposited previously when the spermatids first became more rounded in shape. This new layer of extracellular material is about 0.10 \( \mu \text{m} \) thick. Plasmodesmata are rare at this point in development.

Golgi bodies are still actively producing vesicles containing fibrous material (Figure 281). Polysomes are apparent along with both SER and RER (Figures 281, 283). The plastids, with their dense stromas and few, dilated thylakoids, are present near the nuclear envelope (Figure 283). When starch grains or osmiophilic plastoglobuli are not present, it is difficult to distinguish a plastid from a mitochondrion.

Separation of spermatids from their cell walls A decrease in the volume of the spermatid creates a large amount of space between the plasmalemma and the cell wall (Figure 283). Certain regions of the cytoplasm are highly vesiculate (Figure 283). There are also membrane-bound, vacuolate organelles present containing cytoplasmic remnants (Figure 283).
These organelles may be lysosomal in function.

At this point in development, the spermatogenous tissue appears to be exerting some type of pressure on the jacket cells of the antheridium (Figure 284). The inner walls of the cap and ring cells appear to be indented to accommodate the spermatids.

As the spermatids decrease in volume, internal cellular forces result in the separation of the anterior portion of the MLS and MA from the nucleus which is now assuming a crescent shape (Figure 283). It is not clear from this study whether the separation is due to an anterior elongation of the MLS and MA or if the MB is extending anteriorly and simply displacing the MLS and MA both anteriorly and laterally. Duckett (1975) has shown in Pteridium that the MB extends anteriorly somehow and displaces the MLS and MA. A diagrammatic representation at this stage in development of the relationship among the MLS, MA, MB, and the nucleus is shown in Figure 285 as modified from Duckett (1975).

Flagella are beginning to appear in the newly formed extracellular space (Figure 286). The flagella have a typical 9 + 2 arrangement of microtubules. Osmiophilic material is still evident between the basal bodies and the MB.

**Mid-spermatids**

The mid-spermatid stage is characterized by the continued elongation and coiling of the nucleus until about 1-1/2 gyres are formed and the coiling of the MLS and MA until about 3/4 of a gyre are formed (Figures 287, 288). Condensed chromatin now lines the inside of the nuclear envelope which is in contact with the MB.
An osmiophilic crest (OC) is now visible along the outer anterior edge of the MB (Figures 288, 289), separated from the MB by a distance of about 50 nm. The OC is about 60 nm thick and about 0.25 μm wide. A structure, not previously described in fern spermatids, occurs between the anterior plasmalemma and the MA, oriented at a right angle to the MLS (Figures 288, 289). The structure appears to consist of three layers of osmiophilic material, with the two layers nearest the plasmalemma connected by uniformly spaced osmiophilic regions. In other studies of ferns, there is usually one layer of osmiophilic material in this position known as the osmiophilic strip (OS).

A diagramatic representation of the relationship between the MLS and the nucleus is shown in Figure 290 as modified from Duckett (1975). Previous studies of ferns have shown that the nucleus coils sinistrally as viewed from the anterior end. This is also true of Onoclea spermatids.

Basal bodies are found in the cytoplasm posterior to the OC along the MB (Figures 288, 289). Numerous flagella are present within the extracellular space.

The volume of the mid-spermatids is less than the volume of the early spermatids, evidenced by the increase in extracellular space. A significant amount of autolysis is occurring in the centrally located cytoplasm (Figures 288, 289, 291). Flattened stacks of Golgi cisternae are present along with numerous large vesicles (Figure 289). There appears to be additional fibrous cell wall material coating the previous wall material added during the early spermatid stage (Figure 287).

The plastids present in the mid-spermatids no longer have a dense stroma and their bounding double membranes are becoming less dis-
tistinguishable (Figures 288, 291). Larger starch grains are present. Some of the mitochondria have developed internal spaces filled with thin filaments, presumably DNA (Figure 291). Other mitochondria have large inclusions of densely staining amorphous material.

The spermatogenous tissue still appears to be exerting some type of pressure on the jacket cells of the antheridium. The inner walls of the ring and cap cells are indented where there is contact with the walls of the spermatids (Figure 287). The outer wall of the ring cell is relatively thin (0.25 μm) while the outer wall of the cap cell is quite thick (0.70 μm; Figure 287).

**Mature spermatozoids**

The mature spermatozoid is characterized by the completion of nuclear elongation and coiling and MLS coiling. Previous studies of homosporous ferns have shown that the nucleus forms 2-1/2 gyres and the MLS forms an additional 1-1/4 gyres. From the longitudinal sections of spermatozoids shown in Figures 292 and 293, it appears that in *Onoclea*, there are between 4 and 5 total gyres. Observations necessary to determine the number of nuclear and MLS gyres are lacking.

The nuclear chromatin of mature spermatozoids is in a totally condensed state (Figures 294, 295). The diameter of the nucleus has diminished to the point that the MB has folded over and remains appressed to the nuclear envelope over more than three-quarters of the nuclear circumference (Figure 295). A diagrammatic representation of the relationship of the MLS and OC to the coiled nucleus is shown in Figure 296 as modified from Duckett (1975). The relationship of the MB to the
LS and the nucleus is shown in Figure 297, modified from Duckett (1975), with the spermatozoid uncoiled. A longitudinal section through a spermatozoid would reveal not only a cross-section of an anterior nuclear gyre but also the MB which connects that region of the nucleus with the coiled LS (Figure 295). Basal bodies occur along the outside of the MB only in the anterior gyres (Figure 295). The two basal bodies shown in Figure 295 are embedded in a reticulum of osmiophilic material which appears to extend as a plug into them. This association has been noted in previous studies.

The mid-spermatids have continued to decrease in volume until at the spermatozoid stage, there is little cytoplasm left within the anterior gyres (Figure 298, 299). Within the peripheral cytoplasm near the MB, there are numerous, individual mitochondria (Figures 295, 299). These mitochondria, along with the elongate mitochondrion associated with the MLS, have very swollen cristae.

Cytoplasm is more abundant within the more posterior gyres (Figures 295, 298, 300). This centrally located cytoplasm is quite vesiculate, and contains a few mitochondria and plastids with large starch grains. The plastid membranes are not clearly distinguishable in all regions.

The mature spermatozoids have an accessory band of microtubules between the OC and the plasmalemma, containing 25-35 microtubules (Figure 298). This band of microtubules has been noted in other homosporous ferns during the late spermatid stage (Duckett, 1975).

The spermatozoids have a diameter of about 7 μm at their widest point (Figures 292, 293). The length of the cells ranges from about 7 to 9 μm. The spermatozoids shown in Figures 292 and 293 are from 67-
celled antheridia. However, spermatozoids from 35-celled antheridia have about the same dimensions (Figure 294). This sized cell can be accommodated easily within the neck canal of Onoclea archegonia. The diameter of the neck canal is about 15 μm and the length of the neck canal is about 70 μm (Figure 301).

Spermatozoid release

A group of mature antheridia is shown in Figure 302. When the antheridia are immersed in water, the disc-shaped cap cell becomes detached from the ring cell, allowing the release of the spermatozoids. Figures 303-305 show different views of cap cell detachment. It appears that the previously adjoining walls of the ring cell and cap cell remain intact after detachment. Either the internal pressures due to water imbibition are great enough to mechanically break the cell walls apart, or a combination of increased pressure with the dissolution of the middle lamella region results in the removal of the cap cell.

Spermatozoids are evident within the antheridia shown in Figures 303-305 and are shown being released from an antheridium in Figure 298. They are each enclosed within a layer of cell wall material that was deposited during differentiation (Figures 294, 298). The first layer of cell wall material deposited during the early spermatid stage apparently dissolves during spermatozoid release (Figure 298). This observation may indicate a difference in chemical composition of the various wall layers deposited during differentiation.

The two differently shaped spermatozoids shown in Figures 306 and 307 reveal the fibrous nature of their encapsulating walls. Observa-
vations of living spermatozoids during this study have shown that this wall material is disposed of within 1 min after release, thus allowing free movement of the flagella. The flagellar movements cause the spermatozoids to move in an anterior direction along a helical path.

A recapitulation of the entire developmental sequence, beginning with the formation of an antheridium initial and terminating with the formation of 32 spermatozoids, is diagrammatically illustrated in Figure 308.
DISCUSSION AND CONCLUSIONS

Gametophytes of *Onoclea sensibilis* were induced to form antheridia in order to study many different aspects of developmental biology. Five general developmental areas of interest emerged during this study. Correspondingly, the discussion is divided into five sections.

The first section pertains to the growth rate of gametophytes as affected by spore sterilization and A_pr treatment. The second section concerns the induction period and location of antheridium initials on the gametophyte in terms of the physiological gradients found within the thallus. The third section deals with the cellular location of the initials in terms of cellular and organismal gradients and external physical forces. The fourth section is reserved for a discussion on the comparative morphology of fern antheridia. The fifth section pertains to spermatogenesis in *Onoclea* compared to spermatogenesis in other pteridophytes.

Growth of Gametophytes

In the interest of developing an optimal culture condition for growing antheridiate gametophytes that was also aseptic, it was necessary to determine the effects of spore sterilization on the vegetative growth rate of gametophytes using a dilute solution of calcium hypochlorite.

Compared to the control cultures, the gametophytes in the sterilized cultures grew significantly more slowly due to an inhibition of cell division. This observation may be related to the claim that Clorox
inhibits cell growth (Crotty, 1967). However, it should be mentioned that fungal contamination was often present in the control cultures and some fungi are known to stimulate the growth of gametophytes (Bell, 1958; Hutchinson, 1967, Parès, 1958). Pirozynski and Malloch (1975) have proposed that endotrophic fungi are conspicuous in gametophytes or sporophytes or both in all groups of extant ferns and that the association is obligatory. Even if fungi are enhancing growth in the control cultures, it seems highly likely that the sterilant is affecting a real inhibition in the sterilized cultures. When the exposure to the sterilant was extended past 2 min, severe abnormalities resulted in the gametophytes. The gametophytes became highly branched, suggesting that calcium hypochlorite somehow disrupted the polarity of the apical cell. Steeves, Sussex, and Partanen (1955) also found abnormal gametophyte growth forms in Pteridium, including filamentous proliferations and coral-like aggregates, after treatment with sodium hypochlorite.

During this study, the oxidative ability of calcium hypochlorite was evidenced by the partial degradation of the perispore during treatment. Vogelmann and Miller (1980) have utilized this sterilant to purposefully remove the perispore of Onoclea in order to clearly view the migrating nucleus during germination. They incorrectly stated that sodium hypochlorite removes the exine layer. How the sterilant may affect prothalli other than by degrading the perispore is not known.

The Aₚ was found to inhibit the growth rate to the same extent as the sterilant did. This inhibitory effect was first noticed by Döpp (1950). Earlier observations had shown that antheridiate prothalli of many species of ferns were generally much smaller than archegoniate
gametophytes and that conditions interfering with growth such as low light intensity, crowding, and poor mineral supply favor the production of antheridiate prothalli (Döpp, 1927; Prantl, 1881; Sossountzov and Delaporte, 1949). Döpp (1950) determined that the effects of \( A_{pt} \) on antheridium formation were due to a specific effect and not to a nonspecific inhibition of growth. Naf (1956) reconfirmed this finding. Observations from this study are in agreement with the observations by both Döpp (1950) and Naf (1956). The total number of cells in treated gametophytes quickly exceeded the number of cells in untreated gametophytes, although the number of vegetative cells in treated gametophytes was significantly less than the number of vegetative cells in untreated gametophytes after antheridia began to form. These observations suggested that the energy normally channeled into vegetative growth is somehow partially diverted into antheridial growth (Naf, 1956; Schraudolf, 1962, 1966a).

Although the effects of calcium hypochlorite and \( A_{pt} \) were not additive with respect to vegetative growth rate in this study, there appeared to be a slight additive effect with respect to the onset of antheridium formation. Gametophytes in unsterilized cultures began forming antheridia one day earlier than gametophytes in sterilized cultures treated day 0. This inhibition was not due to a nonspecific decrease in growth rate because the gametophytes in each culture condition had equal numbers of cells on corresponding days. Schraudolf (1962) reported that the \( GA_3 \)-induced antheridium formation in Anemia is independent of sterilization versus nonsterilization of spores with sodium hypochlorite.
Seven days after treatment, the rate of antheridium formation in *Onoclea* was comparable regardless of the time of $A_{pt}$ treatment and sterilization conditions. If calcium hypochlorite, in the presence of $A_{pt}$, does mediate a specific inhibition, it appears to involve the prolongation of the induction period and not the actual formation of antheridia.

However, the concentration of $A_{pt}$ was found to influence the rate of antheridium formation. When concentrations of less than 25% (1:3 dilution) were applied, the number of antheridia produced per gametophyte and the number of gametophytes that produced antheridia markedly decreased. Näf (1956) also found a reduced response to lower concentrations of $A_{pt}$, but he was able to detect a response to dilutions of 1:31,250 and occasionally 1:156,250. The reason for the difference of $A_{pt}$ activity in this study compared to the earlier study by Näf (1956) may be due to many factors. The number of gametophytes producing $A_{pt}$, the pH of the medium, photoperiod, temperature, light intensity, or type of nutrient medium have been shown to affect the titre of $A_{pt}$ (Voeller, 1964b). For instance, Voeller (1964b) found a 100X higher titre of $A_{pt}$ at pH 4.5 compared to pH 7.0.

**Induction and Location of Initials in Relation to Gametophyte Physiology**

**Induction period**

The time of $A_{pt}$ application influenced the length of the induction period of antheridium formation in *Onoclea*. Gametophytes treated day 0 had an induction period of about 5-1/2 days compared to 2-1/2 days when treated
on day 4. Näf (1956) found a 7-day lag period between Ap application on
day 0 and the first evidence of antheridium formation. When Näf (1959)
treated six- and nine-day-old gametophytes, the induction period was
only 2-1/2-3 days. This lead Näf (1959) to speculate that the events
leading to the appearance of antheridium initials either proceed more
slowly in very young prothalli or that very young prothalli lack the
competence to respond to the active factor.

During this study, if Ap was applied on the sixth day after sowing,
the response was very weak in terms of the number of antheridia formed per
gametophyte and the number of gametophytes producing antheridia. Näf
(1958) previously showed that Onoclea gametophytes enter a stage in which
they are insensitive to Ap. This stage occurred shortly after the pro-
thalli attained heart shapes, which was about 12 days after sowing. Näf
(1958) claimed that this state of insensitivity is reached within 2 days.
Voeller and Weinberg (1969) repeated the experiments of Näf (1958) and
found that the decrease in sensitivity occurs over a period of 2 wk.

The gametophytes in this study became insensitive to Ap about 5 days
earlier than the gametophytes cultured by Näf (1958). This difference is
attributed to a faster growth rate of gametophytes in this study. The
seventh day after sowing, the gametophytes had about 20 cells and were
forming notch meristems. During the study by Näf (1958), the gameto-
phytes had an average of 9.4 cells on the seventh day after sowing and
did not form notch meristems until about 12 days after sowing.

The loss of sensitivity of gametophytes to Ap has been attributed
to an inhibitory substance produced by the notch meristem (Döpp, 1959).
This substance has not been isolated. Further information on the nature
of the inhibitory state and substances of gametophytes is reviewed by Miller (1968), Naf et al. (1975), and Naf (1979).

**Location of antheridia**

When $A_{pt}$ was applied during the first 4 days of growth, the initials were always located on the lateral marginal cells. The later initials were located on cells in the upper, central zone. If the gametophytes were treated after 6 days, the few initials that formed were located on cells in the upper, central zone or on the interior of the wings. There appeared to be a tempero-spatial loss of sensitivity to $A_{pt}$ which proceeds in a general acropetal manner. As mentioned earlier, Naf (1959) claimed that all the cells of *Onoclea* become insensitive to $A_{pt}$ simultaneously within 2 days. Voeller and Weinberg (1969) repeated Naf's experiments and found that there was a gradual decrease in sensitivity. Neither study included information on the location of the initials.

A similar pattern of tempero-spatial loss of sensitivity to $GA_3$ occurred in *Anemia phyllitidis* (Naf, 1959; Schraudolf, 1966a). When moderate amounts of $GA_3$ were used, the initials were always formed on lateral, marginal cells (Schraudolf, 1966a). When Naf (1959) treated older gametophytes, the responding cells were limited to an area immediately behind the marginal, lateral meristem. In this species, the loss of sensitivity occurred first in the anterior part of the prothallus followed by a loss of sensitivity in the posterior region. The difference in the loss of sensitivity patterns between *Onoclea* and *A. phyllitidis* may be due to the difference in location of their notch meristems. The meristem in *Onoclea* is apical, while the initial meristem...
in *A. phyllichtidis* is lateral. *Lygodium japonicum*, a species in the same family as *Anemia*, has an apical meristem and exhibits the same loss of sensitivity pattern as *Onoclea* (Näf, 1960).

More studies are necessary to determine if the first initials are typically found on lateral, marginal cells of autotrophic, cordate prothalli. There are a few indications from the literature that marginal initials on young prothalli may be common. Atkinson (1894), Hofmeister (1862), and Nayar (1965) made references to a correlation between young prothalli and marginal antheridia. Nayar and Atkinson both noticed a relationship between crowded conditions and marginal initials, whereas in uncrowded conditions, the initials were superficial.

There are more than enough studies on the location of antheridia on older cordate gametophytes to support the generalization that the preferred location of later initials occurs on nonmarginal cells (Atkinson, 1894; Hofmeister, 1862; Momose, 1958a; Nayar and Kaur, 1971; Stokey, 1951). So far, there appears to be only one exception to this generalization. *Ceratopteris* commonly has marginal antheridia on both young and old prothalli (Momose, 1958a).

It is possible that the internal chemical and physical gradients of *Onoclea* gametophytes are correlated to the tempero-spatial pattern of antheridium formation. During this study, the cells of the very young prothalli appeared insensitive to $A_{pt}$. The gametophytes quickly entered a phase sensitive to $A_{pt}$ and then very quickly lost their sensitivity. The loss of sensitivity correlates highly with the onset of the notch meristem. Näf (1958) also observed this response pattern with *Onoclea*. Both Näf (1958) and Döpp (1959) attributed the loss in sensitivity to
an inhibitory substance produced by the notch meristem. Experiments by Czaja (1921), Parès (1958), and Nüf (1961) support this conclusion.

The cause of a longer induction period for gametophytes treated day 0 as opposed to day 4 is not known. Previously mentioned, Nüf (1959) speculated that events leading to the appearance of antheridium initials either proceed more slowly in very young prothalli or alternatively, that very young prothalli lack competence to respond to the active factor. It seems reasonable to assume that germinating spores are preprogrammed to proceed through specific pathways necessary for the germination event to occur and while in these pathways, the cells are unable to sense the presence of the A<sub>pt</sub> molecules. Through feedback mechanisms, they then eventually enter a pathway receptive to A<sub>pt</sub>, perhaps due to the formation of specific receptor molecules. On the other hand, and equally likely, an inhibitory substance may be present in the spores and young cells of the prothalli. The time necessary for the dilution or inactivation of this substance would be reflected in the longer induction period. Abscisic acid (ABA), a known inhibitory substance, has been isolated from spores and young prothalli of <i>A. phyllitidis</i> (Bürcky, 1977; Cheng and Schraudolf, 1974) and recently, ABA was shown to inhibit antheridium formation in <i>Ceratopteris</i> (Hickok, 1983). All of the other major plant hormone groups are represented in fern gametophytes (Hotta, 1959; Miller et al., 1970; Schraudolf, 1966c; Schraudolf and Fischer, 1979), some perhaps also inhibitory.

In order to explain the tempero-spatial loss of sensitivity pattern in <i>Onoclea</i>, one must assume that different physiological regions exist in the gametophytes independent of an apico-basal age gradient.
Perhaps an appropriate ratio of different hormones conducive to antheridium formation is present in the central cells for only a short period of time. As the hormones continue to be synthesized and translocated, an inductive ratio may no longer exist in any regions of the gametophyte.

It is possible that \( A_{pt} \) may selectively enter the thallus through the rhizoids in the basal region and move acropetally through the cells, while the inhibitory substance produced in the notch meristem is moving basipetally. The rhizoid has been shown to differentially absorb vital dyes which then gradually move into the prothallial cells and through the thallus in an acropetal manner (Reuter, 1953; Smith, 1972). Labeling gametophytes with radioactive \( A_{pt} \) or tagging \( A_{pt} \) with an immunofluorescent dye would help to clarify the mode of translocation of the antheridiogen.

On the other hand, hormonal gradients may not be significant in these small prothalli, which are generally much less than 1 mm in length. Physiological differences between regions of the prothallus independent of hormone concentrations may need to be invoked. There is some evidence in the literature which suggests that gradients do exist with respect to cell position (Reuter, 1953). The cells in the center of young gametophytes of Dryopteris stain differently in response to toluidine blue and acid fuchsin than do all other regions. Although an explanation for these differential responses was not given, the observation is indicative of a physiological difference in the centrally located cells. Cells in this region are also the last to remain responsive to \( A_{pt} \).

Lloyd (1971) also presented some evidence that the central cells
are physiologically different from the other regions. Using *Onoclea*, he found that cells in the margin, as well as those in the wings of archegoniate gametophytes, secrete a fatty substance which reacts with sudan IV. He did not report whether antheridiate gametophytes exhibited the same phenomenon.

Although different physiological regions apparently exist in gametophytes, much more work is needed to characterize the differences and relate them to antheridium formation.

Also puzzling is the observation that only a few cells within an apparently uniform region form antheridia. In *Onoclea*, a high correlation exists between the number of vegetative cells present and the number of antheridia produced. For about every six cells formed per day, one antheridium was induced. In older gametophytes of many other species, it also has been shown that only a fraction of the cells in the central region form antheridia (Momose, 1958b; Nayar and Kaur, 1971; Stokey, 1951).

This situation could be due to one of a number of factors. Perhaps a certain threshold amount of A_\text{pt} must accumulate in a cell in order to induce an initial and certain cells may be better adapted to sequestering A_\text{pt}. And, perhaps the critical amount can be obtained only by depleting the surrounding four or five cells. Näf (1962) claimed that A_\text{pt} must be continuously present to facilitate antheridium formation and maturation. If the supply of A_\text{pt} was prematurely discontinued, "green antheridia" developed, in which the large jacket cells are filled with chloroplasts. No sperm were ever observed in "green antheridia." These atypical antheridia were also observed during the transition to the
Insensitive phase in *Onoclea* (NMF, 1962). No "green antheridia" were observed during this study. This observation that $A_{\text{pt}}$ must be continuously present to induce antheridia is consistent with a theory based on differential accumulation.

Alternatively, instead of certain cells actively accumulating $A_{\text{pt}}$, certain cells may be target cells which differentially react to $A_{\text{pt}}$. Labeling experiments will be necessary in order to choose between these two possibilities. Target cells are known to exist in gametophytes of mosses (Brandes and Kende, 1968). Only cells which form buds accumulate cytokinins and the cytokinin must remain within the cells until the buds are completely formed or they will develop into chloronema. Specific proteins, which are found only in caulonemal cells, have been found to bind kinetin 10X more strongly than other isolated proteins (Erichsen et al., 1977).

If target cells do exist in fern gametophytes, a question immediately arises concerning the underlying causal factor(s). The importance of inorganic ions as external effectors of morphogenetic events has already been well-established (Jaffe, 1980; Maclean and Hilder, 1977; Weisenseel and Kircherer, 1981). In all of the plants that have been investigated electrophysiologically, an ionic current always preceded a pattern formation and local growth (Weisenseel and Kircherer, 1981). It may be possible that ionic currents and/or electrical currents exist within the developing prothallus such that only certain cells occur in sites susceptible to antheridium induction. The effects of the currents may eventually be overridden by other controlling factors, such as hormones,
which at some point in time affect an inhibitory state over the entire prothallus.

Any reasonable model which attempts to interpret antheridium formation of necessity will have to include information on hormones present, their sites of synthesis, modes of translocation, synergistic and antagonistic interactions, and integrate that information with underlying electrical fields. Intimately involved with cellular currents are changes in membrane potentials and often changes in cell wall components (Jaffe, 1980; Quatrano, 1978). The possible importance of ionic currents in antheridium initiation will be discussed further in the next section on the cellular location of initials.

Formation of Initials from Asymmetric Cell Divisions

In this study, antheridium initials were formed on highly vacuolate cells in the basal, central, and interior wing regions. There was no apparent ultrastructural difference among these cells. The only ultrastructural gradient occurred between the apical meristem region and all of the other regions. The most noticeable differences involved an increase in the amount of vacuolation and in the size and differentiation of the chloroplasts, from the apex to the base of the thallus. Changes in less noticeable structures, such as microtubules, were not systematically quantified, but there appeared to be many more microtubules present in the meristematic cells. Their presence may be correlated with the high mitotic index in that region.

Cells in the meristem region were densely cytoplasmic, and contained
few vacuoles. Many medium-sized, rounded chloroplasts, with a limited amount of grana and connecting lamellae were present. Cells in the other regions had one very large central vacuole and contained many large, well-differentiated chloroplasts along their outer walls. The ultrastructure of the cells in *Onoclea* gametophytes appeared very similar to that of other cordate gametophyte species previously studied (Cran, 1979). Cran (1979) mentioned that there is an increasing gradient in chloroplast size from apex to base without mention of differences in their state of differentiation.

Näf et al. (1975) proposed that one of the first changes in the induction of an initial is a decrease in chloroplast size in the vegetative mother cell. Such a change is plausible due to the fact that chloroplasts in interstitial cells revert to smaller sizes after the cell is stimulated to prepare for division by surgical isolation (Ootaki and Furuya, 1969). This change occurs only after 48 hr. Application of Apt also indirectly or directly releases a block to interstitial mitoses, but a change in chloroplast size was not noticed in induced cells in this study.

During this study, the first observable cellular change in induced cells of *Onoclea* was the migration of the nucleus to an anterior corner of the cell. Except for the basal cells, antheridia were always formed at the anterior end of the vegetative cells. This observation is consistent with the observations of Näf (1962) for *Onoclea*. *Onoclea* appears to be somewhat unique in terms of having a preferred anterior location for antheridium formation. The only other specific report in the literature on cellular location of initials is for *Dennstaedtia*,
which was reported to have no preferred location for initials (Nayar et al., 1975). However, from published drawings, it appears that Osmunda (Atkinson and Stokey, 1964), Ceratopteris (Nayar and Kaur, 1971), and some members of the Grammitidaceae (Stone, 1960) may have anteriorly formed antheridia. Nayar and Kaur (1971) stated that, in general, antheridia in advanced, leptosporangiate ferns develop toward the middle of the mother cell.

The next noticeable cellular change in antheridium induction is a large accumulation of RNA-staining cytoplasm surrounding the nucleus in the anterior corner. A dense accumulation of cytoplasm in the region of antheridium formation has also been observed in Actinostachys (Bierhorst, 1975), Polyphlebium (Stone, 1958), and Blechnum (Stone, 1961). In Onoclea, the accumulated cytoplasm is composed of a dense ribosome matrix containing a large number of mitochondria, some chloroplasts, Golgi bodies and a few microbodies. Very few microtubules are observed either along the cell walls or within the cytoplasm. It has previously been observed that growth in cells of gametophytes occurs in the region where RNA is most dense (Nakazawa and Tanno, 1965; Ootaki, 1963).

The period of time necessary for these changes to occur and the sequential order of the changes are not known. Leung and Näf (1979) claimed that a "bouquet" of RNA appears prior to prophase of the migrated nucleus in Onoclea. However, I viewed this report by Leung and Näf (1979) with reservations. The reasons for these reservations will be discussed in the next section where most of their interpretations of later events are shown to be erroneous. Thus, it is not known whether
1) the RNA-stained material accumulates in the anterior corner before nuclear migration, or 2) whether the migrating nucleus is accompanied by increasing numbers of organelles and RNA molecules, or 3) whether the cytoplasm accumulates after the nucleus migrates.

In rhizoid formation in germination of fern spores, which also entails nuclear migration followed by an asymmetric cell division, an aggregation of RNA-staining cytoplasm accumulates around the migrating nucleus (Bassel et al., 1981; Cohen and Crotty, 1979; Gantt and Arnott, 1965). The RNA is found to first accumulate in the nucleus before appearing in the cytoplasm (Cohen and Crotty, 1979). They suggested that the nucleus is active in directing differentiation and is not passively caught in differential cytoplasm. More observations on events prior to the asymmetric cell division, along with labeling experiments with RNA precursors, will be necessary to determine if antheridium formation is analogous in any way to rhizoid formation.

It is known that intense RNA synthesis and accumulation is associated with the prophase stage of mitosis in most organisms during their normal cell cycle (Mitchison, 1971). Perhaps, the bouquet of RNA observed by Leung and Nøf (1979) is only a reflection of a typical mitotic division and not an accumulation of RNA specific to antheridium formation.

To determine if there are specific antheridium-inducing RNA molecules present, Schraudolf (1967) treated *A. phyllitidis* gametophytes, grown in continuous light with both GA$_3$ and RNA-synthesis inhibitors and concluded that there was no inhibition of the induction process, although further development was affected. But Voeller and Weinberg
(1969) repeated Schraudolf's experiments with gametophytes growing in the dark and concluded that a new species of RNA is a limiting step in antheridial induction. When Iqbal and Schraudolf (1977) looked for specific proteins associated with antheridium induction, they found a quantitative difference in certain protein components but they were unable to find any qualitative differences. However, they did not rule out the possibility that special proteins may exist, but in quantities too small to detect with their methods. The identification of specific antheridium-inducing RNA or proteins awaits further experimentation.

Because nuclear movement appears to play an integral role in antheridium induction in *Onoclea*, either as a determinant of cell polarity or as a result of factors governing cell polarity, understanding the basis for the movement will be important in understanding the induction process. Microtubules have been implicated in nuclear migration in some developmental systems (Hepler and Palevitz, 1974; Schmiedel and Schnepf, 1979; Wada and O'Brien, 1975; Zeiger, 1971). If germinating fern spores are treated with antimicrotubule drugs, nuclear migration is inhibited and rhizoids fail to form (Vogelmann et al., 1981). There are other systems, such as guard mother cell formation in onions, in which the premitotic nuclear migration prior to an asymmetric cell division is unaffected by antimicrotubule drugs (Hepler and Palevitz, 1974). Due to the few observations of microtubules in the vacuolate cells of *Onoclea*, the importance of microtubules in nuclear migration cannot be assessed.

Microfilaments also have been shown to affect nuclear movements (Britz, 1979; Hepler and Palevitz, 1974). But cytochalasin-b, a pur-
ported inhibitor of microfilaments, did not affect nuclear movement in germinating fern spores or the formation of the rhizoids (Vogelmann et al., 1981). Although microfilaments are reported to occur in all eucaryotic cells (Hepler and Palevitz, 1974), they are often difficult to visualize in fixed cells and were not observed in the cells of *Onoclea*. The role of microfilaments and microtubules in antheridium induction awaits further investigation.

The membranes within the cells, including the organellar membranes, the tonoplast, and the plasmalemma, probably play a major role in establishing cellular polarity by facilitating intracellular movements (Poo and Robinson, 1977; Schmiedel and Schnepf, 1980; Weisenseel and Kicherer, 1981). As the nucleus moves in *Onoclea* cells, the nuclear membrane is in very close proximity to the tonoplast and the chloroplast membranes at all times. The chloroplasts line the plasmalemma along the outer wall, often establishing contact between their membranes. Schmiedel and Schnepf (1980) presented evidence which suggests that movement of nuclei in *Funaria* caulonema is inhibited due to a change in the nuclear envelope, which is brought about by different stages in the cell cycle.

When Miller and Bassel (1980) treated germinating spores of *Onoclea* with membrane active compounds such as methanol, ethanol, or chloroform, the nuclear movement associated with the periphery of the spore was inhibited. The initial movement of the nucleus from the center of the spore to the side of the spore was unaffected. Even though the nucleus proceeded to divide near the side wall, rhizoid formation did not occur. Two vegetative cells were formed instead. These observa-
tions suggested that there may be a special region of the membrane that acts as a target site for nuclear movement.

Perhaps in *Onoclea* gametophytes, $A_{pt}$ affects the membranes in the anterior regions of certain cells, which in turn somehow triggers the nucleus to begin its migration into that region. $A_{pt}$ may mediate its affect by establishing ionic or electrical currents through the cells of the gametophyte as mentioned in the previous section. Electrical fields have been shown to redistribute receptors in cell membranes (Poo and Robinson, 1977). Weisenseel and Kicherer (1981) hypothesized that localized receptors in cell membranes initially lead to unstable, asymmetric distribution of molecules including membrane pumps or channels. An asymmetry of ionic conductivity occurs which then leads to a transcellular ionic current.

In studies investigating the electrical currents in cells, evidence is presented which shows that there is one point on a cell in which the current enters, while it exits over a large area of the remaining surface. The point of influx is also the area that differentiates into a new organ or is an area of localized growth (Nuccitelli and Jaffe, 1974; Weisenseel et al., 1975; Weisenseel et al., 1981).

Certain ions have been linked to the formation of currents in cells, in particular, $H^+$, $K^+$, and $Ca^{2+}$. There is generally a buildup of ions in the region of influx which may then affect the activation or inactivation of enzymes or hormones.

In caulonema of *Funaria* treated with cytokinin, a large increase in $Ca^{2+}$ occurred in the anterior region of the cell where the bud will form (Saunders and Hepler, 1981). Bud formation in this system involved
a premitotic nuclear migration followed by an asymmetric cell division. And, as mentioned in the previous section, Brandes and Kende (1968) found that the presence of cytokinin was necessary during the entire developmental sequence.

It is tempting to hypothesize that one of the roles of \( A_{pt} \) in the formation of antheridia involves initiating an electrical field within the gametophytes which in turn affects the membranes in the apical regions of cells located in specific areas of the field. Within these cells, ions would accumulate and, in turn, trigger nuclear migration and eventually, differential gene activity and nuclear division.

The need for the continued presence of \( A_{pt} \) in the cell during induction may indicate a more specific role for \( A_{pt} \) in terms of insuring that the new cell will become an antheridium as opposed to a rhizoid or a trichome. The presence of \( A_{pt} \) in combination with the new ionic environment may be important in directing the differential gene activity necessary for antheridium initiation.

A loss of sensitivity to \( A_{pt} \) may entail the loss of the necessary membrane components that need to be localized in the anterior of the cell, or as mentioned in the previous section, an inhibitory substance may simply bind or deactivate \( A_{pt} \) by some unknown mechanism. Although this hypothesis is purely speculative, many aspects of it can be readily tested.

The actual partitioning of the accumulated cytoplasm in the anterior region by an oblique cell wall may be essential for insuring the continued development of the antheridium. Miller and Bassel (1980) presented evidence from germinating fern spores, which showed that a
migrating nucleus must remain near a specific region of the spore wall for a specified amount of time in order for rhizoid formation to be initiated. If the nucleus moved away from that area prematurely, rhizoid formation did not occur.

In this study, the new cell wall of *Onoclea* antheridia is oriented at an angle oblique to the outer wall of the vegetative cell and intersects the anterior, transverse wall about 10 μm in from the upper edge. Orientation of cell walls in some other plant cells has been shown to be related to preprophase bands of microtubules (Busby and Gunning, 1980; Gunning et al., 1978; Palevitz and Hepler, 1974). Although preprophase bands of microtubules were not observed in the vegetative cells of *Onoclea*, not enough sections were obtained at the appropriate stage to discount the possibility of their presence. Preprophase bands of microtubules are known to occur in protonemal cells of another fern species, *Adiantum capillus-veneris* (Wada et al., 1980).

On the other hand, the orientation of the new, oblique wall simply may be under the control of the physical forces present within the cell. Recently, Lintilac (1974a, b), Lintilac and Jensen (1974), and Miller (1980) have revived the theories of Thompson (1917) which promoted physical forces as primary causal mechanisms in growth and form of organisms. They suggested that in a cell exposed to anisotropic stress, either compression or tension, a shear-free plane exists perpendicular to the direction of the largest principal stress exerted on the cell.

Thompson (1917; page 356) showed a drawing of an elongate cell with a small, wedge-shaped cell cut off at the anterior end, which
bears a striking resemblance to the actual situation in antheridium-forming cells of *Onoclea*. Thompson claimed that in this example, the chemical concentration on which polarity depends in unsymmetrical, with one of its poles being deflected to one side where the small cell is being formed. The polar axis becomes a curved axis. If the principal stress follows the curved axis, the new wall would form transverse to the axis, but would then lie obliquely to the apparent axis of the cell.

The problem with showing a relationship between cell wall orientation and shear-free planes is the difficulty in determining the direction of the largest principal stress component. Miller (1980) was able to ingeniously provide some evidence that the orientation of the new cell wall in elongate versus short tip cells of fern gametophytes is related to differences in physical stresses. He first plasmolyzed the cells and then watched the redistribution of the cytoplasm as the cells were allowed to deplasmolyze. The pattern of redistribution was very specific to the cell type and correlated highly with the position of the new cell wall.

Antheridium induction in fern gametophytes should prove to be a very useful system for investigating many different aspects of morphogenesis which involve nuclear migrations and asymmetric cell divisions.

**Comparative Antheridium Morphology**

Each cell division in the formation of antheridial jacket cells is unique and will be discussed separately. Comparisons to antheridia of
other species at corresponding stages in development will be made when possible. Comparisons will be limited to other leptosporangiate ferns which produce antheridia with five or less jacket cells. The classification system used for this discussion is adopted from Wagner (1974a, b) which is shown diagrammatically in Figure 1.

Formation of initials

This study showed that Onoclea forms antheridia by an asymmetrical cell division at the anterior end of vegetative cells, contrary to previous reports in the literature. Campbell (1886) claimed that antheridia in Onoclea arose as papillae, although his accompanying drawing depicts a wedge-shaped cell at the anterior end of a vegetative cell. More recently, Davie (1951) concluded that the initial cell of Onoclea antheridia began its development as a hemispherical protuberance, which was soon cut off from the underlying prothallial cell. Leung and Naf (1979) concluded after a three-year study of Onoclea antheridia, that the initial arises as a hemispherical outgrowth on a nonspecialized vegetative cell, after which an unequal cell division results in a small one-celled initial. Leung and Naf (1979) used whole mounts of gametophytes for their entire study which may explain their misinterpretation.

It is difficult to determine from the literature whether a wedge-shaped initial is typical or atypical in antheridium formation in fern gametophytes. In early textbooks on ferns, a generalization was made that antheridium initials of leptosporangiate ferns are produced by a protuberance or papillae followed by the formation of a cell wall
(Atkinson, 1894; Bower, 1923; Campbell, 1913). In the most recent review of the morphology of homosporous fern gametophytes, Nayar and Kaur (1971) stated that leptosporangiate-type antheridia develop as a superficial, thick, papilla-like initial cell formed towards the middle of the peripheral wall of the mother cell. They noted an exception in Ceratopteris, which formed wedge-shaped initials at the anterior ends of mother cells. However, they claimed that eusporangiate-type of antheridia originate as wedge-shaped initials formed towards one end of a superficial prothallial cell. Atkinson and Stokey (1964) also found that the more primitive ferns which produce antheridia with numerous jacket cells, form wedge-shaped initials. If these generalizations are true, that would mean, in terms of antheridium initiation, that Onoclea is more similar to the primitive ferns than to members in its own advanced family. This conclusion is difficult to accept.

I have found only three reports in the literature that state or infer from drawings that leptosporangiate ferns have wedge-shaped initials (Campbell, 1886; Nayar and Kaur, 1971; Stone, 1961). Aside from the drawings of Onoclea initials by Campbell (1886) and the reference to Ceratopteris by Nayar and Kaur (1971), Stone (1961) found that Grammitis, in the Polypodiaceae, has wedge-shaped initials.

Besides the general references to the leptosporangiate ferns mentioned above, there are specific references to members of leptosporangiate families which concluded that initials are formed either as protuberances or as papillae: Adiantum (Atkinson, 1894; Kachroo and Nayar, 1953; Verma and Khullar, 1966); Athyrium, Cyathea, Dicksonia, Diellia, Dryopteris, Onoclea, Pityrogramma, Polypodium, and Woodwardia (Davie, 1951); and
Pteris and Onychium (Verma and Khullar, 1966). These genera occur in all the leptosporangiate families except the Schizaeaceae and the Osmundaceae. These studies either presented no evidence or only drawings as evidence that initials arise as protuberances or papillae. This information, along with the fact that Davie (1951) characterized Onoclea initials as papillae are reasons to view these reports with serious reservations. A definite need exists to critically reexamine the formation of antheridia in all of these genera.

However, it should not be inferred that the protuberance-type of initials do not exist. Unquestionable evidence shows that Actinostachys (Schizaeaceae; Bierhorst, 1975), Polyphyllum (Cyatheaceae; Stone, 1958), and Blechnum (Aspleniaceae; Stone, 1961) form a protuberance-type initial.

According to Bierhorst (1975), in Actinostachys, "the mother cell of the antheridial initial bulges at the surface. The degree of bulging determines whether or not the antheridium will be stalked.... The nucleus of the mother cell assumes a position within the bulge. The accumulated cytoplasm comes to be present in the antheridium initial."

Stone (1958) claimed that, for Polyphyllum, "the antheridium arises from a filament cell as an outgrowth which produces a spherical head either before or after it is cut off from the parent cell. In Blechnum, Stone (1961) reported that "the antheridium arises from a prothallial cell as a protuberance, which forms above the nucleus which is enlarged and surrounded by denser cytoplasmic material than the rest of the cell.... Mitosis occurs and a wall forms in a plane parallel with the surface of the prothallus if it is a superficial antheridium or perpendicular to
the surface if it is a marginal antheridium."

Due to the lack of data from many genera and the probable erroneous data from a number of other genera, no phylogenetic or developmental assessments can be made at the present time in terms of the initiation event in antheridium formation.

Cytology of the initials

The initials of *Onoclea* are densely cytoplasmic with a centrally located nucleus. The nucleus appeared to occupy a larger volume than the nucleus in the mother cell. This observation was also made by Leung and Naf (1979) for *Onoclea*, by Stone (1961) for *Blechnum* and *Doodia*, and by Schraudolf (1968) for *Polypodium*. Nuclear enlargement and chromatin dispersal have been shown to occur in nuclei transplanted into different cytoplasmic environments, indicating an exposure of the genes to the new regulatory conditions (Gurdon and Woodland, 1970). But nuclear swelling also has been associated with DNA synthesis and mitosis (Gurdon and Woodland, 1970). The significance of the larger nucleus in the initial is not known.

In *Onoclea*, microtubules were evident in the initials, particularly along the outer, curved wall. Their presence may be important in directing the distribution of new layers of microfibrils into the cell wall as the initial rapidly expands both in height and width before the next cell division. Golgi bodies forming vesicles filled with fibrillar material were also present. These vesicles may be transporting the new cell wall material or precursors of cell wall material to the plasmalemma. If the initial is papillate, as in some species, the expansion phase occurs before the
first division.

During the expansion of the initial in *Onoclea*, most of the cytoplasm became localized in the anterior region of the initial. The lower region became highly vacuolate. This polar arrangement of cytoplasm was also noted by Leung and Naf (1979) for *Onoclea* and Stone (1961, 1962) who observed cytoplasmic polarity in the initials of *Blechnum* and *Doodia*. Atkinson (1894) and Stokey and Atkinson (1957) included drawings which indicated that a cytoplasmic polarity exists in the initials of *Adiantum* and *Elaphoglossum*, respectively.

**Formation of the basal cell**

In *Onoclea*, the metaphase chromosomes and the phragmoplast of the first division in the initial were lined up at a 41° angle to the base of the initial. The subsequent cell wall was oriented at the same angle, intersecting the basal wall near the center. Only rarely was this newly formed wall transverse (parallel) to the base of the initial. Leung and Naf (1979) claimed that in *Onoclea*, the phragmoplast was oriented parallel to the base of the initial, but that the subsequent cell wall was funnel-shaped, with the base of the funnel in contact with the center of the basal wall. They also rarely found a transversely oriented new wall. They did not present photographic evidence for the presence of transverse phragmoplasts and I feel that their interpretation of the orientation is incorrect.

The occurrence of funnel-shaped walls in basal cells is consistent with the classical theory of antheridium development proposed by Strasburger (1869), Campbell (1886, 1913), and Atkinson (1894). Although the
classical theory is generally interpreted to mean that the upper wall of the basal cell is always funnel-shaped, many of the authors accredited with the theory actually claimed that the orientation of the first wall formed in the initial varies from funnel-shaped to more-or-less transverse (Atkinson, 1894; Campbell, 1886, 1913). The observations made of the first cell wall in *Onoclea* initials also agrees with observations of Stone (1962) for *Polypodium*.

Although observations from *Onoclea* are consistent with the classical theory, they directly contradict Davie's theory, which states that the first cell wall of the initial is always oriented transversely to the base of the initial and either remains in that position or is pushed downward by the expanding upper cells. Davie (1951) based his claim on observations of numerous antheridia, many of which had transverse walls and many others which had walls varying in their degrees of concavity. He presented no conclusive evidence showing that these differently shaped walls are ontogenetically related.

Evidence from species other than *Onoclea* also contradicts Davie's theory. Stone (1962) showed in *Polypodium* that the mitotic axis of the first division is oriented at approximately 45° to the basal wall and that the subsequent cell wall is funnel-shaped. Schraudolf (1968) presented similar data for *Polypodium polycarpon*. Schraudolf and Richter (1978) presented further evidence in *Polypodium crassifolium* and *Platycerium* that the orientation of the funnel-shaped wall is not a secondary trait due to internal pressures. He showed the presence of plasmodesmata traversing the basal wall in the region where the angled, funnel-shaped wall intersects the basal wall. Plasmodesmata would not traverse the wall if part
of the wall had secondarily come into contact with the other part of the wall. This observation was also made using *Onoclea* during this study.

Although there is sufficient data to show that at least four different genera of ferns have antheridia with primary funnel-shaped walls in the basal cells, there are some species of ferns that typically do produce disc- or barrel-shaped basal cells due to a transversely oriented new cell wall (*Vittaria*, Farrar, Botany Department, Iowa State University, pers. comm.; *Diplazium*, Kawasaki, 1957; *Blechnum*, Stone, 1961). There are other species that apparently produce both kinds of basal cells with equal frequency (*Dryopteris*, Momose, 1939; *Camptosorus*, Momose, 1941a; *Cystopteris*, Momose, 1941b; *Pentarhizidium*, Momose, 1958b; *Asplenium*, Momose, 1959). Although the basal cell in antheridia of members of the Schizaeaceae was always a thin, disc-shaped cell (Atkinson, 1960; Bierhorst, 1975; Schraudolf, 1963), its development is not analogous to basal cell formation in the other advanced, leptosporangiate families. In the Schizaeaceae, the basal cell is not formed until after another division occurs in the initial, and then the basal cell is derived from the vegetative cell supporting the initial.

Although it is easy to envision how a transverse wall is formed, it is much more difficult to imagine how a funnel-shaped wall is deposited. In fact, very little is known about the process or the mechanisms controlling the formation of the funnel-shaped wall.

In *Onoclea*, the phragmoplast was first formed in the region between the daughter nuclei and spanned the distance from the center of the base of the initial to a lateral midpoint along the curved cell wall of the initial. The completion of cytokinesis was not observed. It seems
reasonable to assume that the phragmoplast proceeds to form in a pivotal manner, most likely bidirectionally, until the advancing edges meet and fuse. The occurrence of small structures composed of densely packed vesicles embedded in dense staining matrix material near the wall of the initial at the upper limit of the phragmoplast may be important in directing the formation of the wall. Similar structures were observed by Gunning et al. (1978) in cells in the root apices of Azolla. They felt that they may be microtubule organizing centers (MTOC) and are probably related to preprophase bands of microtubules. After preprophase bands of microtubules (PPBM) disappear during prophase, it has been speculated that the site of PPBM is still morphogenetically active and may have the ability to orient new cell walls (Palevitz and Hepler, 1974). Perhaps the vesiculate structures observed in Onoclea initials are MTOC or a reflection of a morphogenetically-active site along the wall, which serves as a "track" for the developing phragmoplast and subsequent cell wall.

Kny (1869) and Leung and Näf (1979) felt that the funnel-shaped wall is formed simultaneously in all parts of the initial. Leung and Näf (1979) claimed to have visualized an RNA-stained circular ribbon that occurs just above the base of the initial after the nucleus divides. They felt that this ribbon rises up in the initial and increases in diameter until the ribbon is in contact with the curved wall of the initial about midway between the apex and base of the initial. They felt that the cell wall is deposited behind the ribbon as it rises and that the upper nucleus rotates around the ribbon, supplying it with additional RNA. As mentioned earlier, Leung and Näf (1979) only used
whole mounts of gametophytes in their observations, a situation in which artifacts would be difficult to avoid. I consider their interpretation to be erroneous.

In this study, cytokinesis resulted in a densely cytoplasmic upper cell with a large, centrally located nucleus and a highly vacuolate basal cell, with a smaller nucleus. The nucleus in the upper cell was probably enlarged due to the fact that it was in a state of synthesis prior to the next mitosis. The nucleus in the lower cell is arrested in its cycle and normally will undergo no further divisions.

The upper and basal cells were approximately equal in volume in *Onoclea*, and from drawings and photographs of other antheridia with funnel-shaped basal cells, it appears that both cells in other species also are about equal in volume. No previous studies have investigated the geometry of the antheridia. In the literature, it is taken for granted that the basal cell is much smaller than the upper cell in a typical 2-celled antheridium (Näf, 1962).

The significance of the relationship between equal cell volumes and funnel-shaped walls is not known. If the laws of physical forces apply to the orientation of cell walls (Lintilac, 1974a, b; Miller, 1980; Thompson, 1917), then it may be assumed that in an antheridium with a height to diameter ratio of 3:4, which is positioned at an oblique angle to the lower vegetative cell, the plane of shear-free stress occurs at an angle of about 41° to the base of the initial. Thompson (1917; page 409) briefly discussed the orientation of cell walls in antheridia. He made an analogy between the funnel-shaped wall and the position that a soap bubble would take in a cylindrical dish or beaker.
The angles would be similar. Supposedly, the film of soap would lie in the shear-free plane. He further suggested that if the initial were a perfect hemisphere, the new cell wall would form in a transverse plane.

Perhaps the orientation of the first cell wall in an initial depends on the shape of the initial when mitosis is stimulated. Miller's work with tip cells of fern protonemata supports this supposition (Miller, 1980). In antheridia with very elongate basal cells, the upper wall of the basal cell is almost invariably transverse. Examples include *Blechnum* (Stone, 1961, 1962), *Vittaria* (Stokey, 1951), and some species of *Asplenium* (Momose, 1959). The shape of the initial in *Asplenium* when mitosis occurs is not known, but the initials in *Vittaria* and *Blechnum* during division are much longer than wide, as opposed to the initials of *Onoclea* (Farrar, Botany Department, Iowa State University, pers. comm.; Stone, 1961). More investigations involving the shape of initial cells during mitosis will be necessary before any assumptions can be made.

The factors controlling the orientation of new cell walls are probably numerous and interactive. Schraudolf (1967, 1977) found that RNA and protein synthesis inhibitors greatly affected the cell pattern in antheridia. The compound, 5-bromodeoxyuridine (BudR) caused antheridia to resemble short vegetative filaments with transverse walls. BudR causes AG/TC transitions during DNA replication or transcription. It is not known how these mutations mediate cell wall orientation.

This observation may not be incompatible with the theory of physical forces influencing cell wall orientation. The mutations may influence the expansion of the cell such that when mitosis is triggered, the new
cell wall is positioned in response to the physical forces on the shape of the cell at that point in time.

**Formation of the second wall in the initials**

Many cytoplasmic changes occurred in the upper cell of the 2-celled *Onoclea* antheridium before the next mitosis. The upper portion of the cell became vacuolate, while the RNA-stained cytoplasm accumulated in the basal portion of the cell. The chloroplasts, originally in the upper portion of the cell, became distributed tangentially around the nucleus. Microtubules occurred both near the nuclear envelope and along the cell walls, but PPMB were not observed. General observations of this redistribution of cytoplasm have been previously made (Atkinson, 1894; Leung and Näf, 1979; Stone, 1961, 1962).

During metaphase of this division, the chromosomes lined up in the upper one-third of the cell, parallel to the base of the initial. The new cell wall was oriented parallel to the outer, curved wall of the initial and intersected the funnel-shaped wall about 6 µm in from the edge, thus forming a central, spermatogenous cell and an upper, jacket cell. Observations of this curved, periclinal wall are consistent with the classical theory of antheridium development, but contradict Davie's theory. Davie (1951) stated that the second cell wall is transverse, just like the first wall and that it becomes curved secondarily, due to expansion of the internal cell pushing the wall upward. No photographic evidence was presented to support Davie's claim.

Most previous observations of the second wall formation are consistent with the classical theory of development (Atkinson, 1894;
Bower, 1923; Campbell, 1913; Nayar and Kaur, 1971; Schraudolf and Richter, 1978; Stone, 1961, 1962; Verma and Khullar, 1966). It appears that there is less flexibility in the orientation of the second wall as compared to that of the first wall.

However, there were still some workers who supported Davie's theory (Atkinson and Stokey, 1964; Kachroo, 1955; Smith, 1955; Sporne, 1962; Voeller, 1964a, b). There is no convincing evidence from any of these studies.

In the schizaeaceous ferns, the wall which forms a central spermatogenous cell and an upper ring cell tends to be oriented transversely (Bierhorst, 1975; Schraudolf, 1963). But this wall is actually the first wall formed in the initials of the schizaeaceous ferns and not the second wall as in other families, making significant comparisons difficult.

The complete formation of the phragmoplast and subsequent curved periclinal wall in Onoclea were not observed, nor have these stages been followed in previous studies. Most likely, a disc-shaped phragmoplast which forms between the two nuclei in the upper part of the cell continues to form along its entire circumference in an outward and downward direction, following the curvature of the outer wall until it reaches and fuses with an area on the funnel-shaped wall. Ultrastructural information is lacking for this stage and it is not known whether PPMB or vesiculate structures are involved in the cell wall formation.

Leung and Naf (1979) offered an alternative interpretation which is difficult to accept. Although they agreed that the initial portion of the phragmoplast is located in the upper portion of the cell oriented
parallel to the base of the initial, they claimed that this portion physically moves down to the level of the upper rim of the funnel-shaped wall. The cell wall is then formed at this level and acquires its curved, periclinal shape secondarily in response to increasing pressures in the central cell or cells. There is no convincing evidence to support this explanation either from their study or this study.

The volumes of the central spermatogenous cell and the upper jacket cell were approximately equal in Onoclea and from previously published drawings and photographs, this relationship may hold for other genera as well. The significance of the relationship between the cell volumes and the position of the new cell wall is not known. If a shear-free plane formed by external physical forces is determining the position of the wall, the plane must be curved and the axes of principal stresses must be perpendicular to all points on the surface of the outer curved wall.

The majority of the dense cytoplasm was partitioned into the central spermatogenous cell. There were no special cytoplasmic structures in this meristematic cell to disclose its spermatogenic nature. Occasionally, a mitochondrion is observed in the spermatogenous cell which has an inclusion of densely staining amorphous material. Such mitochondria were also seen in vegetative cells and in spermatogenous cells at later stages. The nature and function of these inclusions are not known, although there is some reason to suspect that the material may be calcium phosphate precipitate. Mitochondria are known to actively accumulate Ca$^{2+}$ in the presence of permeant anions, in particular, phosphate (Lehninger, 1970). Masses of calcium phosphate have ranged up
to 0.3 μm in diameter. It has been suggested that Ca$^{2+}$ uptake may be correlated to a cell's ability to regulate its ionic environment. The nature and function of these inclusions await further investigation.

In *Onoclea*, the next division was always observed in the spermatogenous cell which is consistent with observations by Campbell (1886) for *Onoclea*. Leung and Nöf (1979) indicated that the next division is always in the upper jacket cell in *Onoclea*. Stone (1961) found with *Blechnum* that the first division of the spermatogenous cell usually takes place before the upper jacket cell divides, but occasionally it divided either simultaneously with or rarely after the upper jacket cell division. Bierhorst (1975) made similar observations with *Actinostachys*. Schraudolf (1963) stated that in *Anemia* the central cell divides before the upper jacket cell, but in *Polypodium*, his drawings indicated that the upper cell divides first (Schraudolf, 1968). Davie (1951) claimed that in *Pityrogramma*, the upper jacket cell divides first and that this sequence of divisions is typical of many other species, including *Onoclea*. In general, Nayar and Kaur (1971) claimed that the upper jacket cell divides first. In summary, it appears that the order of divisions involving the central cell and the upper jacket cell are not under strict controls.

**Formation of the cap cell**

The side wall of the cap cell in *Onoclea* was in the shape of a modified cylinder, such that each end of the cylinder is perpendicular at the intersection with the upper and lower walls of the jacket cell. Stages prior to cytokinesis were not observed in this study, but Leung and Nöf
(1979) observed that the equator of the spindle apparatus was oriented perpendicular to the upper and lower walls of the upper jacket cell. This is the orientation that is predicted from this study due to the shape and position of the forming cell wall which was observed. Although Leung and Näf (1979) were unable to follow the development past the spindle stage, they speculated that the wall probably forms simultaneously in all regions after division just as Kny (1869) suggested. Evidence from this study showed that all areas of the wall do not form simultaneously. The phragmoplast was at different stages in formation in different regions of the cylinder and the most mature stage of the cell wall appeared to exist between the two nuclei. It is hypothesized that an initial disc-shaped phragmoplast forms between the nuclei during telophase and that it continues to grow around its circumference until it fuses with the upper and lower wall of the ring cell. Then, unknown forces direct the continued formation at a specific curvature which will result in the growing edges meeting at a location opposite the two nuclei where they will fuse.

Bierhorst (1975) supported this hypothesis for cap cell formation. In Actinostachys, he found that the entire cap cell wall is not formed simultaneously, and that the wall forms first between the two nuclei. Stone (1961, 1962) made similar observations with Blechnum and Polyplebeium. The earliest phragmoplast was observed between the two nuclei and after the cell wall began to form between the nuclei, Stone (1961, 1962) could distinguish a halo of, presumably, phragmoplast material on the edges of the circular plate. Schraudolf (1963, 1968) presented drawings which show that the equator of the spindle is oriented
perpendicular to the upper and lower walls of the jacket cell and that
the subsequent, cylindrical cap cell wall is oriented in the same
plane. The genera investigated were *Polypodium* and *Anemia*. Thus, it
appears that along with *Onoclea*, *Actinostachys*, *Anemia*, *Blechnum*,
*Polyphelebium*, and *Polypodium* all form cap cells according to the
classical theory, which claims that the jacket cell divides anticlinically
by means of a funnel cell.

The examples given above contradict Davie's theory, which states
that the cell wall of the cap cell forms transversely and when the upper
wall of the central cell grows or is pushed upward, the two cell walls
come into contact giving the impression of a disc-shaped cap cell and
a ring cell (Davie, 1951). Davie felt that cap cells of all advanced
ferns are formed by a transverse wall. This obviously has been proven
false in this study, and even Davie (1951) included drawings of
*Pityrogramma* which show the equator of the mitotic spindle to be oriented
perpendicular to the upper and lower walls of the jacket cell. This
indicates that the cell wall will not be transverse.

Verma and Khullar (1966) studied six species in the Adiantaceae
and concluded that the cap cell is formed by a transverse wall. They
used whole mounts of gametophytes and included photographs of *Adiantum*
and *Pteris* antheridia in nondividing stages. The information in the
photographs is inconclusive at best and could be easily misinterpreted.

Stone (1969) was critical of the interpretation of Verma and Khullar
(1966) with respect to the formation of the cap cell. Stone (1969)
claimed that it is impossible to determine the manner in which a wall is
laid down from a study of cells after cytokinesis has been completed
for some time.

Unfortunately, Nayar and Kaur (1971) in their treatise on gametophyte morphology endorsed most of Davie's concepts involving, in particular, the first cell wall forming the basal cell, and the formation of the cap cell, even though there simply is no solid evidence to support most of Davie's ideas. Although the first cell division in the initial may be transverse in some species, the second two divisions are most likely never transverse. To prove this conclusively, many more species will need to be examined and most of the species already investigated will need to be reexamined.

The volume of the cap cell, in relation to the jacket cell from which it was derived, depends on the dimensions of the antheridium during the division. If the antheridium still has a 3:4 height to diameter ratio, the volume of the cap cell is one-half the volume of the original upper jacket cell. If this is the typical condition, then every division in the initial involved in the formation of jacket cells is an equal division in terms of volume, even though the cell shapes are strikingly different. The significance of this fact remains to be determined.

The factors which control the formation and orientation of the cap cell are not known. Ultrastructural information is lacking for the early stages in division. It is difficult to imagine PPMB oriented in a circular fashion near both the upper and lower walls. If the orientation depends totally on external physical forces, the axes of principal stress would have to be aligned parallel to the base of the initial and radiate outward in all directions from the center of the cell, or off-
center, depending on the final position of the cap cell. Although this may be the case, it would be very difficult to prove.

All three jacket cells, the basal cell, the doughnut-shaped ring cell, and the disc-shaped cap cell, are highly vacuolate and generally have few plasmodesmata connections between themselves and the spermatogenous cells. All three cells contain, in varying ratios, the same types of organelles found in the vegetative cells. Golgi bodies are particularly abundant and microtubules often occur near the cell walls. The presence of these organelles is probably related to the fact that each of the cells will be expanding to accommodate the growing spermatogenous tissue.

Spermatogenesis

In *Onoclea*, the central spermatogenous cell underwent five or six synchronized mitotic divisions resulting in either 32 or 64 spermatids. The first division was longitudinal, the second divisions were longitudinal and perpendicular to the first division, and the third set of divisions was transverse to the previous two sets of divisions resulting in 8 spermatogenous cells. These observations are consistent with previous reports by Atkinson (1894), Campbell (1886), and Stone (1961). A few authors have reported the second divisions to be transverse and the third divisions to be longitudinal (Bower, 1923; Campbell, 1913). Division patterns after the first three sets are difficult to follow and were not investigated. There are no previous reports in the literature on division patterns after the first three synchronized sets.
The divisional phase of spermatogenesis takes about 4 days with one set of mitoses every 16-20 hr. Iqbal and Schraudolf (1977) inferred that spermatogenous divisions in *Anemia* take about 3 days. The species they used typically forms only 16 spermatids (Voeller and Weinberg, 1976). The only other report concerning the length of the divisional phase involves the heterosporous fern, *Marsilea*, which forms 32 spermatids in 3-1/2 hr (Hepler, 1976). Differences in the reproductive strategy of *Marsilea* as opposed to homosporous ferns probably accounts for the notably reduced divisional phase of spermatogenesis. In *Marsilea*, a semi-aquatic fern, expedient fertilization must occur before a transient water supply evaporates or before the sperms are displaced from the eggs due to water currents or wave action.

In *Onoclea*, the time necessary for differentiation of the spermatids into spermatozoids was about 3 days. The only other reference to the length of differentiation in homosporous ferns comes from work by Schedlbauer et al. (1973) in which Schedlbauer indicated that for cultures of *Ceratopteris* growing in red light, differentiation is of the order of 48 hr. Bell (1979b) speculated that for homosporous ferns, the period of differentiation is probably less than 1 day. As expected, *Marsilea* has a very reduced period of differentiation, which is less than 8 hr (Myles and Hepler, 1977). With so little information on the length of differentiation in other ferns, it is not known whether 3 days for *Onoclea* is typical of other homosporous ferns.

Teleologically, the reduced period of differentiation in *Marsilea* is understandable, but the controlling factors which contribute to an
expedient differentiation phase are not known. Perhaps *Marsilea* spermatids have preformed mRNA, proteins, or enzymes for which only activation is necessary for differentiation, whereas *Onoclea* spermatids may need to undergo extensive RNA or protein synthesis. But there are indications that little protein synthesis occurs in spermatids of *Ceratopteris* (Schedlbauer et al., 1973). They cautioned that an impermeable wall surrounding the spermatids may have inhibited the uptake of labeled amino acids and, thus, the actual state of protein synthesis in the cells may not have been revealed. Alternatively, Duckett (1975) suggested that cysteine, the labeled amino acid Schedlbauer et al. (1973) used, would not be expected to occur in very large quantities in tubulin, the predominant protein in spermatid cells. Factors involved in controlling the length of differentiation await further investigation.

During this study, antheridia with either 32 or 64 spermatids were found with equal frequency on gametophytes. The factors which control the final number of spermatids per antheridium are not known. In *Onoclea*, it appeared that when spermatogenous cells reach a specific volume, their mitotic cycle is repressed and the cells are induced to differentiate into spermatozoids. I hypothesize that the volume of the initial spermatogenous cell is correlated with the final number of spermatids. More divisions may be accommodated with a larger, initial spermatogenous cell before the resultant cells reach the presumed threshold volume. Many more measurements will be necessary to statistically show that a correlation exists between the volume of the initial spermatogenous cell and the final number of sperms. It will be essential to investigate other species of ferns which commonly produce
a different number of sperms per antheridium.

However, there is a paucity of information on the number of spermatids per antheridium in other species of ferns. Eames (1936) and Bower (1923) indicated that, in general, homosporous, leptosporangiate fern antheridia contain 32 sperms. Foster and Gifford (1974) and Nayar and Kaur (1971) claimed that 16 or 32 sperms per antheridium are common for higher ferns. Voeller and Weinberg (1967) found that Anemia gametophytes treated with GA$_3$ or A$_n$ formed antheridia with 16 spermatids. In un-supplemented cultures, they found many antheridia with 16 sperms, some with 32 sperms, and occasionally an antheridium with 8 sperms. Generally, only one size of antheridium occurred per gametophyte. Bell and Duckett (1976) claimed that Pteridium forms 32 sperms per antheridium.

The lowest number of sperms encountered per antheridium was reported for Drynaria (Polypodiaceae) which has 4-8 sperms (Nayar, 1965). Accounts of antheridia with more than 32 sperms in the most advanced ferns (Aspleniaceae) were not encountered in the literature. Eames (1936; pp. 280-281) reported that in the Schizaeaceae, there are typically more than 32 sperms per antheridium and in the Cyatheaceae, there are commonly antheridia with 64 sperms and possibly some with 256 sperms. From this study, it is felt that antheridia with 64 sperms is more common in the Aspleniaceae than previously reported.

The size, shape and number of jacket cells in an antheridium also may be highly correlated with the final number of sperms. Their ability to expand with the increasing number of spermatogenous cells may play a key role in determining the final number of sperms. It would be informative to examine the more primitive groups of ferns.
with antheridia composed of numerous jacket cells to determine the volume of the initial spermatogenous cell compared to final number of sperms.

Not all workers assume that spermatogenous cells divide synchronously, as expressed by the geometric progression $2^n$, where $n$ equals the number of synchronized divisions (Nayar, 1965). Only in this study and in a study by Bierhorst (1975) have synchronized divisions been reported. But it appears from the number of sperms reported for other species, that, in general, spermatogenous cells divide synchronously. Studies on other systems which involve synchronized, dividing cells surrounded by a nondividing layer of cells, such as sporangia, may provide relevant information on the factors controlling the final number of differentiated cells.

The means through which cell volume might influence cell differentiation is not known. Perhaps the nuclear:cytoplasmic ratio is important in the process, due to either a decreased or increased concentration of a substance in the reduced cytoplasm which then either activates or de-represses genetic information. Alternatively, the increased proximity of the nuclear membrane with the plasmalemma may play a role in inhibiting mitosis and directing differentiation.

**Ultrastructure of spermatogenous cells**

In *Onoclea*, the meristematic, spermatogenous cells changed little in their ultrastructural appearance from the inception of the spermatogenous initial cell to the spermatid mother cell stage. The cytoplasm, dense with ribosomes, contained numerous pleomorphic mitochondria with
dense stromas and vesiculate cristae. Duckett (1975) observed pleomorphic mitochondria in early spermatogenous cells of *Pteridium* and *Dryopteris*, but described them as having transparent matrices with short, vesiculate cristae. He noted that the stroma of the mitochondria became denser in the spermatids. The reason for this discrepancy in mitochondrial appearance is probably due to fixation artifacts in Duckett's material. In poorly fixed spermatogenous tissue in *Onoclea*, the mitochondria were the first organelles to lose their internal structure.

The dedifferentiation of chloroplasts during spermatogenesis, as observed in *Onoclea*, has also been observed in other ferns and mosses (Carothers, 1975; Duckett, 1975; Hepler, 1976; Paolillo and Cukierski, 1976). Golgi bodies were present in all of the cells of *Onoclea* antheridia, producing large vesicles containing fibrous material, similar in appearance to material in the cell walls. Active Golgi bodies are implicated in the formation of the numerous, new cell walls formed during the divisional phase. Duckett (1975) found Golgi body behavior to be strictly related to the cell cycle. He found Golgi bodies with small, marginal vesicles during interphase and large vesicles during telophase with their contents becoming incorporated into new cell plates.

There were always numerous microtubules present in the spermatogenous cells of *Onoclea*. Although definite preprophase bands of microtubules have not been identified, small bands of parallel microtubules occurred near the cell wall in certain regions of the cell. Many microtubules were also found oriented tangentially around the nuclei. Observa-
tions on microtubule numbers or distribution in spermatogenous cells of other species are lacking.

RER and SER occurred throughout the cells in Onoclea during the division phase, as reported for other ferns by Duckett (1975). Mitochondria with inclusions of densely staining amorphous material were occasionally observed in the spermatogenous cells of Onoclea. These inclusions have not previously been reported for any other plant species. As mentioned earlier, the chemical nature and function of the inclusions are not known.

Also observed in the older spermatogenous cells of Onoclea were some osmiophilic bodies. No previous mention has been made of such bodies in spermatogenous cells, but Hepler (1976) included micrographs of Marsilea spermatids containing osmiophilic bodies resembling those seen in Onoclea. Two large osmiophilic bodies arose de novo with the blepharoplast in Ginkgo (Gifford and Larson, 1980) but their function is not known and no speculation was offered.

**Ultrastructural changes during spermatid differentiation**

Many profound changes occurred during differentiation which resulted in nuclear coiling, chromatin condensation, flagella formation, and the loss of a great deal of cytoplasm. The formation of a de novo organelle, the blepharoplast, appeared to be intimately related to all of these changes. Events during differentiation in Onoclea are very similar to events previously observed in other homosporous ferns (Bell, 1974, 1979b; Bell and Duckett, 1976; Duckett, 1975), Equisetum (Duckett, 1975), and Marsilea (Hepler, 1976; Mizukami and Gall, 1966; Myles, 1979; Myles
and Bell, 1975; Myles and Hepler, 1977). Only differences in observations on *Onoclea* compared to these earlier studies will be stressed in this discussion.

The blepharoplast of *Onoclea* is very similar in appearance to the blepharoplasts of the other species. *Onoclea* blepharoplasts have a diameter of about 0.95 μm which falls in the range given for the other species of 0.50-1.0 μm, and the hub and spoke substructure of the channels is also very similar among all of the species. The number of channels in *Onoclea* blepharoplasts appears to equal the number in homosporous ferns (Bell, 1974, 1979b; Bell and Duckett, 1976), but the number is much less than in *Marsilea* (Mizukami and Gall, 1966). Although Bell (1974) and Bell and Duckett (1976) reported that the channels in homosporous ferns are about 50 nm in diameter, the channels in *Onoclea* are closer to 90 nm in diameter. The channels in *Marsilea* blepharoplasts measure about 90-100 nm according to Mizukami and Gall (1966) or 70 nm according to Hepler (1976). Although the blepharoplasts of *Onoclea* and *Marsilea* have about the same diameter, the channels in *Marsilea* blepharoplasts are packed much closer together in the densely staining matrix. Mizukami and Gall (1966) and Hepler (1976) claimed there were about 100-150 channels in *Marsilea* blepharoplasts. The number of channels in *Marsilea* blepharoplasts corresponds to the number of flagella formed by the spermatid; thus, Mizukami and Gall (1966) and Hepler (1976) felt that there was a one to one relationship between channels and subsequent procentrioles. If this relationship holds for homosporous ferns, such as *Pteridium*, which has about 40 flagella (Duckett, 1975; Elmore and Adams, 1976), *Pteridium* would also have about
40 channels in its blepharoplast.

Bell and Duckett (1976) were not convinced that a direct relationship exists between the number of blepharoplast channels and the number of subsequent procentrioles. In Ceratopteris, the number of channels appeared to be half the number of basal bodies (Bell and Duckett, 1976). Many more observations and measurements will be necessary before the relationship between channel numbers and procentriole or flagellar numbers is elucidated. If a one-to-one relationship does hold for homosporous ferns, judging from the similarity in appearance between Onoclea and Pteridium blepharoplasts, Onoclea probably has about 40 channels per blepharoplast and 40 subsequent flagella.

Early stages in blepharoplast formation have not been observed in homosporous ferns prior to this study. In Onoclea, the early stages are similar to Marsilea blepharoplast formation (Hepler, 1976). In Onoclea, two triple-layered plaques formed within a spherical mass of flocculent material. Material condensed on the distal layers of each plaque in which channels began to form. Further stages were not observed in Onoclea, but they are probably similar to events that occur in Marsilea, in which the two distal layers become spherical blepharoplasts. They separate from each other during prophase with each daughter spermatid retaining one blepharoplast. In Onoclea, the early stages were observed in antheridia containing 32 spermatogenous cells. For Equisetum, Pteridium, and Dryopteris, Duckett (1975) claimed that two blepharoplasts occur in the spermatid mother cells and that each spermatid retains one blepharoplast. Bierhorst (1975) also observed these sequential events in Actinostachys. The sequence is somewhat dif-
ferent in Marsilea, however (Hepler, 1976). The blepharoplast first arises at telophase of the second to last division and degenerates during late prophase of the next to last division. It reforms at telophase of the next to last division into two blepharoplasts, as explained above, which separate during prophase of the last division, resulting in spermatids containing one blepharoplast each.

There is not enough evidence from this study or previous studies of homosporous ferns to conclusively say that blepharoplasts arise de novo only once in spermatogenesis. Onoclea is not an ideal system in which to determine this matter, due to the fact that antheridia form both 32 and 64 spermatozoids. Early stages of blepharoplast formation in 19-celled antheridia would be ambiguous in terms of not knowing whether the 16 spermatogenous cells would terminate in 32 or 64 spermatids.

Hepler (1976) and Pickett-Heaps (1969) felt that the blepharoplast acts as a MTOC, but Bell (1979b) disagreed. Although the chemistry of the blepharoplast is not known, the dense matrix does not appear to be a pool of tubulin monomers, due to the lack of affinity of the material to radioactive colchicine (Bell and Duckett, 1976). Schedlbauer et al. (1973) found the incorporation of radioactive cysteine to be more prominent at the periphery of the blepharoplast than anywhere else. They speculated that if S-S bonds are involved in the polymerization of the flagellar proteins, cysteine might be expected to figure in the syntheses immediately preceding assembly. Supposedly, microtubules contain a very low concentration of cysteine (Olmsted et al., 1970), but microtubule-associated proteins (MAP) may contain cysteine. MAP
are not required for the assembly of tubulin dimers into microtubules, but they facilitate the process (Dustin, 1980). Perhaps the dense matrix of blepharoplasts is composed of MAP.

The blepharoplast eventually forms procentrioles and an MLS. In Onoclea, this happened in the spermatids after their cell walls began to thicken. This is also the case in other homosporous ferns (Bell, 1974). But in Marsilea, the MLS and procentrioles form at the end of telophase of the last division (Myles and Hepler, 1977).

In previously studied homosporous ferns, the lamellar plates of the MLS have been found to form simultaneously with the microtubular band of the MLS, whereas in Equisetum, the microtubular band forms first (Duckett, 1975). The MLS of Onoclea is formed in the same manner as other homosporous ferns. The growth of the MLS, its attachment to a mitochondrion, the distribution of the basal bodies, and the attachment of the entire complex to the nucleus in Onoclea all appear to conform to previous descriptions of other homosporous ferns.

According to Duckett (1975), an osmiophilic crest (OC) is present from the inception of the MLS and occurs along the anterior edge of the MLS. In Onoclea, the osmiophilic crest was not apparent until the mid-spermatid stage.

A structure occurs in Onoclea that has not been previously described in other ferns. The structure consisted of three layers of densely staining material between the mitochondrion of the MLS and the anterior plasmalemma. The outer two layers were connected by uniformly spaced plugs of densely staining material. In Pteridium, one layer of osmiophilic material occurred in the corresponding area known as an
Duckett (1975) found that one of the differences between *Equisetum* spermatozoids and homosporous fern spermatozoids is the presence of an accessory band of microtubules separating the OC and the plasmalemma in ferns, but not in *Equisetum*. There are 25 microtubules in the band in *Pteridium* and 35 in *Dryopteris*. *Onoclea* has an accessory band of microtubules which has between 25 and 35 microtubules.

The mature spermatozoids of *Onoclea* appear to have 4 to 5 gyres. This is consistent with observations made in *Pteridium* (Duckett, 1975). In *Pteridium*, the nucleus occupies 2-1/2 to 3-1/2 gyres, while the MLS occupies 1-3/4 gyres. It appears that *Onoclea* has a similar ratio of nuclear to MLS gyres. In *Equisetum*, the MLS occupies 2-1/2 gyres and the nucleus occupies only 1-1/4 gyres, which is definitely different than *Onoclea*. *Marsilea*, on the other hand, has between 8 and 10 gyres, with the MLS occupying only 3/4 of the anterior gyre (Myles and Hepler, 1977).

The forces involved in the nuclear coiling and chromatin condensation are not known. Myles and Hepler (1977) presented an in-depth discussion on this topic. In summary, they felt that the microtubular ribbon acts as a guide for nuclear elongation and that a force-generating system such as actin or actin-like proteins may be situated near the nuclear envelope. Bell (1979b) generally agreed with the hypothesis presented by Myles and Hepler (1977) and Duckett et al. (1979) provided further evidence for the role of microtubules as a "former" for nuclear coiling in a mutant species of *Ceratopteris*. 
Cell wall formation during spermatid differentiation

Throughout the division phase of spermatogenesis in *Onoclea*, the cell walls separating the spermatogenous cells were only about 0.10 μm thick. The walls began to uniformly thicken to about 0.20 μm during the formation of the blepharoplast in the spermatid mother cells. As the blepharoplast differentiated into procentrioles and an MLS, a large amount of fibrous material was differentially deposited between the plasmalemma and the original cell wall, synchronous with the rounding-up of the spermatids. A few cytoplasmic connections, much larger in diameter than plasmodesmata, occurred between the spermatids. A second uniformly thin layer of fibrillar-granular material was deposited between the plasmalemma and the previous deposition after the MLS had encircled about one-half of the spherical nucleus. Golgi bodies were actively producing vesicles filled with fibrillar material during these two stages of cell wall deposition. No previous studies have described these two phases of cell wall deposition. Analysis of the chemical nature of these two layers was not done during the course of this study, but it has previously been shown that callose, hemicellulose, and lipids are all present in the thickened walls between the spermatids of *Ceratopteris* (Cave and Bell, 1973). During the early stage in differentiation, they found that the thickened walls stained homogeneously for the three different compounds, but later in differentiation, only a thin layer stained for hemicellulose and lipids near the spermatozoid, and no portion of the wall stained for callose. Gorska-Brylass (1968) found that callose accumulates around spermatids in *Adiantum cuneatum*, but that it disappears when the nucleus begins to coil.
Cave and Bell (1973) assumed that only one layer of material was deposited around fern spermatids and, thus, concluded that the deeper staining of a thin layer later in differentiation was due to loss of water in that layer which dilutes the interstitial layer. But the fact that there are probably two chemically different layers deposited during differentiation, as found in this study, may account for this differential staining pattern. The first layer may hydrate to a certain extent due to the hemicellulosic component, but probably not due to the loss of water in the later, thin layer.

If cell wall deposition in *Onoclea* during differentiation is similar to *Ceratopteris*, the first large deposition of material is probably composed of hemicellulose, lipids, and callose, while the second, thinner layer is probably composed of hemicellulose and lipids. Future studies will be necessary to confirm these assumptions.

Callose occurs quite often in the plant kingdom, including around liverwort spores (Górska-Brylass, 1969), pteridophyte sperms, eggs, and megasporocytes (Górska-Brylass, 1968; Bell, 1979a), *Selaginella* micro- and megaspores (Górska-Brylass, 1968; Horner and Beltz, 1970), and micro- and megaspores of higher plants (see Horner and Beltz, 1970). Callose is known for its sealing properties and often occurs during periods of a life cycle when the plant is making a transition from the gametophyte generation to the sporophyte generation or vice versa. Bell (1979a) speculated that the preparation of a cell, within the tissue of its parent, for a different phase of growth may be impossible if the cell continues to suffer the ingress of informational molecules.

The function of hemicellulose in the layer of deposition may be
related to the dispersal of the sperms. Hemicellulose is hydrophilic and the uptake of water is apparently necessary for sperm release. Late in differentiation, the spermatogenous tissue is observed bulging into the jacket cells in Onoclea. Bierhorst (1975) also made this observation with Actinostachys. This bulging is probably due to the pressure caused by the absorption of water in the large layer of hemicellulosic material. A counter-pressure builds up in the jacket cells due to their high osmotic potential (Igura, 1959, 1960). Presumably, the interaction between these two pressures plays an important role in sperm release.

Hartman (1931) found that the cap cells in Onoclea antheridia are removed during dehiscence as opposed to being ruptured. SEM micrographs from this present study of Onoclea showing antheridia without cap cells support the earlier observations of Hartman (1931).

The cap cell appears to have a number of special characteristics compared to the other jacket cells. This study showed that the outer wall of the cap cell becomes extremely thick during spermatid differentiation, sometimes reaching a thickness of about 0.70 μm. This thick wall may serve as support against the increasing pressure of the spermatogenous cells. Lloyd (1971) observed an accumulation of waxy material on the surface of Onoclea antheridia, especially near the margin of the cap cell, and Hartman (1931) found that the cap cell of Onoclea antheridia stained much more heavily for sugars than the other jacket cells. Perhaps all of these features function in strengthening the cap cell, which may be the most susceptible cell to internal pressures.

However, these special characteristics of cap cells in Onoclea may indicate that sperm release involves a process not totally dependent on
osmotic processes. Hausmann and Paolillo (1977) found that the tip cells of antheridia in *Polytrichum* produce very thick walls late in spermatid differentiation, and that these walls consist of a gel that swells (Paolillo, 1981). There is also a cuticle around the antheridia of *Polytrichum*, just as there appears to be a cuticle around the antheridia of *Onoclea* (Lloyd, 1971; Paolillo, 1981), which led Paolillo (1981) to conclude that the consequential slow exchange of water could not account for the opening of the antheridia in *Polytrichum*. As supporting evidence, Paolillo (1981) found that *Polytrichum* antheridia could open rapidly in hypertonic solutions, which could not be the case if opening depended on the uptake of water. From observations that mechanical disturbances set off antheridium openings in *Polytrichum*, Paolillo (1981) hypothesized that a disturbance, such as the action of the first raindrop, sets off a number of reactions within the antheridium cells. These reactions result in the movement of water from surrounding antheridial cells into the tip cells and finally, into walls of the tip cells, which then swell and cause the tip cells to burst.

Perhaps antheridia of *Onoclea* are also in a metastable condition at maturity. They presumably would have a large amount of potential energy stored due to water uptake during growth, in the jacket cells, in the thick, hemicellulosic walls around the spermatids and possibly in the space created between the maturing spermatids and the cell walls. Paolillo and Cukierski (1976) found that as the protoplasts of the maturing spermatids of *Polytrichum* withdrew from the cell wall, the resultant space appeared to be filled with an aqueous solution.

When the cap cells of *Onoclea* antheridia were developmentally
prepared to receive a signal, only after the spermatozoids were mature, the antheridium would be ready to respond, with an ensuing swelling of the cap cell wall causing the cap cell to burst from the connecting ring cell. The remaining stored potential energy in the jacket cells and sperm mass would be sufficient to force the sperms out of the antheridium cavity. This mechanical signal process, as opposed to a purely osmotic process, may better insure the opening of the antheridium only after the sperms are ready for fertilization. There are probably numerous modifications of these two suggested processes throughout the more primitive plant groups. As an example, Paolillo (1981) found that in Funaria, sperms are expelled entirely by the swelling of the sperm mass. Many more observations of fern antheridia will be necessary to determine conclusively the method of sperm release.

The presence of lipids in the walls immediately surrounding the spermatozoids in Ceratopteris (Cave and Bell, 1973) may also be present around Onoclea spermatozoids and may play an accessory role in sperm dispersion. Paolillo and Cukierski (1976) found that lipids are present in the walls surrounding some species of moss sperms as they are released from antheridia. The lipids cause a dispersion at the air-water interface of sperms that are still in their vesicles. In some mosses, the sperms coat water droplets due to the action of their lipid coats, which are then splashed to the female plants, reducing the amount of swimming necessary for fertilization. The role of lipids in fern spermatozoid dispersion awaits further investigation.
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ACKNOWLEDGMENTS

I first would like to thank the Graduate College of Iowa State University for their financial assistance, making this study possible.

I would like to thank Dr. Donald Farrar, co-chairman, for his support of and interest in my academic adventures and especially for his friendship and respect. I would like to thank Dr. Harry T. Horner, Jr., co-chairman, for his interest in my goals, enthusiasm for this project, and for ensuring financial support for this study. I would also like to thank Dr. Clifford LaMotte, Dr. Robert Chapman, and Dr. Alan Atherly for serving as members on my committee.

Special thanks go to Bruce Wagner, supervisor of Bessey Hall EM facility, for his constant good humor and EM expertise.

I would like to express my gratitude to my Texas colleague, Joan E. Nester, for her interest in my work, her insights, and most of all for her friendship.

I especially would like to thank Michael J. Krogmeier for numerous, helpful discussions on data interpretation and presentation, for his computer expertise, and for his deep understanding and moral support.

To my parents, Joseph and Mabel, I extend my deepest appreciation for the opportunities that they initially provided for me and for their continuing support of my efforts.
A light and electron microscopic study of the development of antheridia in *Onoclea sensibilis* L.

by

Jane Louise Kotenko

Volume 2 of 2
A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of
DOCTOR OF PHILOSOPHY
Major: Botany

Approved:
Signatures have been redacted for privacy.

Iowa State University
Ames, Iowa
1983
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Figure 35. Three-week old gametophyte showing four morphological regions. Antheridia are present on cells in central region, inner wing region, and basal region. Trichomes occur on margins of wings and on cells in central region, indicated by smaller, dark arrow. Rhizoids only occur in basal region, with one rhizoid labelled with a clear arrow. Two antheridia have arisen from one vegetative cell (larger, dark arrow), with younger antheridium located posterior to older antheridium. X 98

Figure 36. A U-shaped, notch meristem and upper wing regions of a three-week old gametophyte. X 120

Figure 37. A gametophyte during second week of growth, fixed and stained for RNA with azure B. Meristematic region stains intensely. Dark arrows indicate presence of antheridia at varying stages of development. Clear arrows indicate presumptive sites of antheridium initiation. All antheridia arise at anterior end of vegetative cells. Marginal cells give rise to antheridia on their radial, anterior wall, compared to nonmarginal cells which give rise to antheridia on either their dorsal or ventral anterior wall. X 160

Figure 38. A three-week old gametophyte showing orientation of transverse and longitudinal sections. Some antheridia formed on cells in basal region are located in a more central position (arrow) than antheridia formed from cells in central and wing regions. X 170

Figure 39. Longitudinal section through meristematic region. Nucleus is centrally located surrounded by chloroplasts (arrows) and coalescing vacuoles. Cell posterior to meristematic cell has a peripherally located nucleus and a large central vacuole. Chloroplasts occur along the dorsal and ventral walls (arrow). X 825

Figure 40. Transverse section through a meristematic region (central three cells) flanked on both sides by cells in upper wing regions. Dark arrow indicates a chloroplast and clear arrow indicates a nucleolus. X 845
Figures 41-43. A meristematic cell of a young, antheridiate gametophyte

41. A centrally located nucleus is shown with a large nucleolus. The surrounding cytoplasm contains numerous chloroplasts lacking an extensive lamellar system, many small vacuoles, mitochondria, and Golgi bodies (dark arrow). Plasmodesmata traverse the inner, thin walls (clear arrow). X 12,090

42-43. Higher magnifications of two regions shown in Figure 41. Present within the meristematic cell are microbodies, microtubules (small, clear arrow), ER (larger, clear arrow), and Golgi bodies (dark arrow)

42. X 18,650

43. X 18,300
Figures 44-45. Longitudinal sections through meristematic cells. Outer, curved, periclinal wall (dark arrows) is much thicker (0.35 \( \mu m \)) than interior walls (0.13 \( \mu m \)) such as posterior wall shown in Figure 45 (clear arrow)

44. X 6,730

45. X 6,660
Figures 46-49. Higher magnifications of posterior wall of meristematic cell shown in Figure 44 (clear arrow)

46. Near the posterior wall, which is traversed by many plasmodesmata (arrow), are numerous microtubules oriented parallel to each other. In cell on left, posterior to meristematic cell on right, peripheral nucleus is surrounded with microtubules. X 13,440

47. Microtubules are indicated by dark arrows. Golgi bodies are present forming vesicles containing fibrous material (clear arrow on left). Invaginations of the plasmalemma also contain fibrous material (clear arrow on right). X 48,720

48. Microtubules nearest cell wall are oriented parallel to each other and wall. Next to those microtubules are other microtubules oriented parallel to each other but at a right angle to first-described microtubules. X 44,550

49. An osmiophilic body is shown in meristematic cell. Plasmodesmata (arrow) are composed of an outer, circular membrane and an inner core of electron-dense material. X 45,190
Figures 50-51. Meristematic cells differentiating into vacuolate, vegetative cells

50. Small vacuoles are coalescing into larger vacuoles. Many dedifferentiated chloroplasts occur around centrally located nucleus. Numerous plasmodesmata traverse interior, thin walls. X 6,250

51. Two meristematic cells with centrally located nuclei are shown next to a nonmeristematic cell on left with a peripherally located nucleus and a large central vacuole. X 900
Figures 52-54. Transformation of meristematic cells into vacuolate, vegetative cells

52. Longitudinal section through a meristematic cell in which four to five large vacuoles have formed. Cytoplasm is assuming a peripheral location, transiently connected to nuclear region with thin cytoplasmic strands (arrows). X 835

53. Transverse section through meristematic region. X 575

54. Peripheral cytoplasm in a newly formed, vacuolate, vegetative cell revealing chloroplasts lacking extensive lamellar systems and containing little starch. Internal cell walls are thicker (0.20-0.25 μm) than in meristematic cells (clear arrows). Periclinal wall (dark arrow) is still about 0.35 μm thick. X 12,260
Figures 55-58. Vacuolate, vegetative cells showing recently migrated nuclei near cell wall

55-56. Peripheral nuclei still maintain contact with cytoplasm along opposite and adjacent walls through cytoplasmic strands (dark arrows). Clear arrows indicate thin layer of cytoplasm now localized near cell walls

55. X 865
56. X 770

57-58. Cytoplasmic strands are composed mainly of free ribosomes and an occasional small vacuole

57. X 7,000
58. X 7,800
Figure 59. A longitudinal section through near center of a young, antheridiate gametophyte. Cells in central and basal regions have one large central vacuole. Chloroplasts (dark arrows) in peripheral cytoplasm are located mainly along periclinal walls (dorsal and ventral). X 435

Figure 60. A paradermal section through a young, antheridiate gametophyte. No chloroplasts are present along internal walls (clear arrows), but do occur along outer walls (dark arrows). X 305
Figure 61. A highly vacuolate, mature vegetative cell in a young, antheridiate gametophyte. Chloroplasts are much more differentiated than in younger, meristematic cells. X 6,225
Figure 62. Transverse section through mature, vegetative cells revealing different cellular positions of the nuclei (dark arrows). Clear arrow indicates a chloroplast. X 1,315

Figure 63. Highly vacuolate, vegetative cell. Peripheral nucleus is often flat and ellipsoidal and is separated from plasma-lemma by well-differentiated chloroplasts. X 7,570
Figures 64-66. Portions of mature, vegetative cells

64. Chloroplasts with extensive lamellar systems, plastoglobuli, and some starch grains. Mitochondria and small vacuoles occur with chloroplast and separate ellipsoidal nucleus from plasmalemma. X 11,655

65. A lobed nucleus with an osmiophilic body near nuclear envelope. X 17,035

66. Microtubules occur near cell walls of this vegetative cell. X 25,765
Figures 67-73. Cytoplasmic contents in mature, vegetative cells of young, antheridiate gametophytes

67-68. Microbodies. X 22,670

69-70. Mitochondria with densely staining amorphous inclusions (dark arrows)

   69. X 12,640

   70. X 16,880

71. Golgi body forming vesicles. X 29,025

72-73. Structures containing vesicles and tubules, resembling paramural bodies, although connections with plasmalemma are not evident in these sections

   72. X 31,120

   73. X 26,890
Figure 74. Antheridia on anterior, dorsal surface of vegetative cells in central region of a young gametophyte. Dark arrow indicates a presumptive antheridium initial. An antheridium and a trichome occur on same vegetative cell in lower right-hand corner. X 765

Figure 75. An antheridium developing from anterior, radial corner of a marginal, vegetative cell. X 1,630
Figures 76-87. Fixed gametophytes stained with methyl green for DNA and pyronin Y for RNA. DNA stains greenish-blue and RNA stains dark pink to purple.

76. Nucleus embedded in an accumulation of RNA-stained cytoplasm in anterior, radial region of a marginal, vegetative cell, a situation preceding antheridium initiation. X 600

77. Clear arrow indicates an anterior, radial corner of a marginal, vegetative cell in which metaphase chromosomes are embedded in RNA-stained cytoplasm, a situation immediately preceding formation of an antheridium initial. Dark arrow shows a 4-celled antheridium with a basal cell, an upper cell, and two spermatogenous cells. Wall dividing spermatogenous cells is oblique to base of antheridium. X 370

78. Dark arrow points toward anterior, radial corner of a marginal, vegetative cell in which telophase chromosomes are embedded in an accumulation of RNA-stained cytoplasm. More peripheral set of chromosomes will become enclosed within antheridium initial after cytokinesis. Inner set of chromosomes will be retained within vacuolate, vegetative cell. Clear arrow shows a 3-celled antheridium with a basal cell, an upper cell, and a central, spermatogenous cell, which contains most of RNA-stained cytoplasm. There is an 11-celled antheridium in lower, central part of photograph. Basal cell has a transverse, upper wall as opposed to typical, funnel-shaped, upper wall. X 370

79. Arrow points toward a newly formed antheridium initial in anterior, dorsal region of a vegetative cell. Enlarged nucleus in initial is surrounded by RNA-stained cytoplasm. Nucleus which remains in vegetative, mother cell is located just posterior to initial. X 370

80. Arrow indicates an antheridium initial at anterior, radial corner of a vegetative cell. Initial is filled with RNA-stained cytoplasm. X 370

81. Dark arrows indicate antheridium initials at anterior, radial corners of marginal, vegetative cells. Nuclei are centrally located and large vacuoles are beginning to form at base of initials. Cytoplasm is becoming concentrated in upper, central region of initials. Clear arrow shows a nucleus embedded in RNA-stained cytoplasm in anterior, radial corner of another marginal, vegetative cell, potentially a site of antheridium initiation. A 5-celled antheridium is
present in lower, left-hand corner, with a basal cell, a ring cell, a cap cell, and two spermatogenous cells. Cell wall dividing spermatogenous cells is perpendicular to base of antheridium. X 370

82. Arrow indicates an expanded initial with large, basal vacuoles and a nucleus in a central, lower position. Cytoplasm has accumulated in upper central region. X 600

83. Initial indicated by arrow has entered into mitotic telophase. A line drawn equidistant between two sets of chromosomes would be oriented at about 40-45° to base of initial. RNA-stained cytoplasm occurs mostly in upper, left-hand side of initial. X 370

84. A 2-celled antheridium, with a vacuolate basal cell and a densely cytoplasmic upper cell. X 600

85. A 2-celled antheridium in which cytoplasm in upper cell has accumulated in basal region of cell. Nucleus in upper cell is enlarged and less densely stained compared to nucleus in basal cell. Upper cell nucleus is now located in upper, central region of cell. X 370

86. Dark arrow shows a 2-celled antheridium with upper cell in mitotic telophase. Phragmoplast will form in a plane parallel to base of antheridium. Cytoplasm in upper cell is localized in basal region. Clear arrows show nuclei which have migrated to anterior, radial corner of marginal, vegetative cells and are embedded within RNA-stained cytoplasm. X 370

87. Dark arrow shows a 3-celled antheridium with a vacuolate basal and upper cell and a densely cytoplasmic central, spermatogenous cell. Clear arrow indicates a 2-celled antheridium with a vacuolate basal cell and a densely cytoplasmic upper cell. X 370
Figure 88. Arrow indicates nucleus in outer, anterior corner of vacuolate, vegetative cell embedded in cytoplasm containing many chloroplasts. X 880

Figures 89-90. Sections through an outer, anterior corner of a vegetative cell into which a nucleus has migrated. Surrounding nucleus are a large number of mitochondria. Large dark arrows indicate outer, periclinal wall. Smaller, dark arrow indicates presence of some microtubules.

89. X 10,570

90. X 8,545
Figure 91. A nucleus near periclinal wall of a vegetative cell during telophase of mitosis. Clear arrow indicates innermost set of chromosomes. White arrow indicates outermost set of chromosomes. Phragmoplast forming in region indicated by white star will intersect the outer wall at an oblique angle. A cytoplasmic strand, faintly shown in this photograph, traverses large vacuole from innermost set of chromosomes to opposite wall of vegetative cell (dark arrow). X 780

Figures 92-93. Antheridium initials at anterior end of vegetative cells

92. A small, wedge-shaped antheridium in which basal wall intersects anterior wall of vegetative cell about 10 μm from edge (dark arrow) and periclinal wall of vegetative cell near a central region. Nucleus in vegetative mother cell is located just below initial surrounded by small vacuoles. X 700

93. An antheridium initial densely cytoplasmic with many small vacuoles in lower, anterior region. Nucleus in vegetative, mother cell is surrounded by many small vacuoles. X 8,000
Figure 94. Montage of a vegetative, mother cell after formation of an initial towards top of micrograph. Nucleus in mother cell is connected to cytoplasm along opposite wall by a thin, cytoplasmic strand composed mainly of free ribosomes. X 17,740
Figures 95-98. Internal and external views of antheridium initials

95-96. TEM and LM of same antheridium initial showing centrally located nucleus within dense cytoplasm containing numerous, well-differentiated chloroplasts, mitochondria, small vacuoles, and a few microbodies. Dark arrows indicate periclinal wall and clear arrow shows anterior wall of initial. Chloroplasts are present in all areas of initial except for between nucleus and basal wall. Base of initial is about 30 μm long and height of initial is about 10 μm through center point.

95. X 5,410

96. X 555

97. A nonmedian section through an initial. A few plasmodesmata occur in anterior wall of initial (clear arrow). Dark arrow refers to periclinal wall of initial. Very few plasmodesmata occur in basal wall. X 5,455

98. A surface view of two antheridium initials at anterior ends of nonmarginal, vegetative cells. Width of initials vary between 25 and 30 μm. X 535
Figures 99-105. Cytoplasmic components of antheridium initials

99-101. Polysomes (largest, dark arrow) are present near nuclear pores in nuclear envelope (clear arrow). Microtubules (smaller, dark arrows) are present near cell walls and deeper within cytoplasm of initials.

99. X 19,650

100. X 20,920

101. X 29,535

102. A mitochondrion with an amorphous inclusion and Golgi bodies forming vesicles in initial. X 17,030

103. Microbodies in initial. X 11,980

104. RER between nucleus and base of initial. X 10,420

105. Paramural bodies in initial. X 24,400
Figures 106-107. Expanding antheridium initials revealing a polar distribution of cytoplasmic organelles. Chloroplasts are located mainly in upper region along curved, periclinal wall (small, dark arrow). Nucleus is in a central, lower region. Basal region of expanding initial is composed mainly of large vacuoles, with major portion of cytoplasm occurring above nucleus containing many mitochondria and small vacuoles. Larger, dark arrow indicates periclinal wall of initial and clear arrow refers to basal wall of initial.

106. X 7,810

107. X 800
Figures 108-111. An expanding antheridium initial

108. One large basal vacuole has formed with major portion of cytoplasm localized in upper region of initial. Curved, periclinal wall is thickest (0.45 μm) near junction with anterior wall of initial (clear arrow). Wall near apex is about 0.30 μm thick. X 5,965

109. A higher magnification of area indicated by smaller, dark arrow in Figure 108. Vesicle-forming Golgi bodies are present with fibrous material occurring within vesicles (dark arrow on the left). Dark arrow on right shows some microtubules near cell wall. X 17,900

110. A higher magnification of larger, dark arrow in Figure 108. Microtubules are present near both outer cell wall (small, dark arrow) and within cytoplasm (larger, dark arrow). X 28,675

111. A higher magnification of area indicated by smaller, clear arrow in Figure 108 showing presence of a lobed microbody and some microtubules (dark arrow). X 26,605
Figures 112-115. Antheridium initials near end of expansion phase

112. An expanded initial at anterior end of a vegetative cell (arrow). Nucleus has elongated somewhat in a vertical direction. Base of initial is vacuolate. X 525

113-115. Serial sections through an expanded initial. Most of cytoplasm is localized in upper, central region. Nuclear region remains in contact with all sides of initial through thin cytoplasmic strands (arrows). Nucleolus is quite large. Basal region is highly vacuolate. X 680

Figures 116-119. Serial sections through an antheridium initial during mitotic telophase

116. Near-median section revealing localized cytoplasm in upper, central region with large basal vacuoles. Height of initial is about 28-30 μm and basal diameter is about 38-40 μm. X 835

117. A more anterior section showing first observed set of chromosomes (clear arrow). A portion of phragmoplast is apparent near base of initial (dark arrow). X 835

118. A more anterior section showing another portion of phragmoplast at a much higher level above base of initial (dark arrow). Set of chromosomes observed in Figure 117 are still evident above phragmoplast (clear arrow). X 835

119. A more anterior section revealing a portion of second set of chromosomes below phragmoplast (clear arrow). Highest point of phragmoplast is about two-fifths height of antheridium (dark arrow). First-observed set of chromosomes are barely evident above phragmoplast (clear arrow). X 835
Figures 120-122. Antheridium initial during mitotic metaphase

120. A montage of an initial during metaphase with base of initial out of view and parallel to right-hand edge of micrograph. Chromosomes are located in central portion of anterior half of initial with centromeres aligned in a plane oriented about 40-45° to base of initial. Cytoplasm is localized in upper, central portion of initial. A structure, composed of closely packed vesicles in dense-staining material, occurs near cell wall and uppermost chromosome (dark arrow). X 10,780

121. Higher magnification of structure indicated by arrow in Figure 120. X 37,680

122. A similar structure to that shown in Figure 121 occurs at about same height along curved, periclinal wall directly opposite first structure. X 34,905
Figures 123-127. Sections through 2-celled antheridia composed of a basal cell and an upper cell

123-124. Serial sections through a 2-celled antheridium. Cell wall dividing initial into a basal cell and an upper cell is funnel-shaped and fuses both with periclinal wall and basal wall (dark arrows). Wall is angled at about 40° to base of antheridium. Basal cell is highly vacuolate with a densely staining nucleus near center of cell. Upper cell acquired most of cytoplasm and has a centrally located nucleus that is larger and less densely stained. Nucleus in vegetative mother cell has migrated to a central position along outer, periclinal wall. A cytoplasmic strand connects nuclear region with cytoplasm along base of 2-celled antheridium

123. X 800
124. X 840

Figures 125-126. Serial sections through another 2-celled antheridium. There is clearly a polar distribution of cellular organelles in upper cell. Chloroplasts and small vacuoles occur along curved, periclinal wall and are excluded from basal region of upper cell. Intersection points of funnel-shaped wall are indicated by arrows in Figure 125. Nucleus in upper cell is larger and less densely stained than in the basal cell. Dark arrows in Figure 126 indicate two nuclei

125. X 820
126. X 860

Figure 127. An asymmetric, 2-celled antheridium. Intersection points of funnel-shaped wall are indicated by arrows. X 580

Figure 128. An idealized diagram of a symmetrical, 2-celled antheridium with a height to diameter ratio of 3:4. The angle, θ, is 41°. Upper rim of funnel-shaped cell intersects curved, periclinal wall at about one-half height of antheridium
Figure 129. Measurable, geometric shapes superimposed on idealized diagram shown in Figure 128 in order to determine volume occupied by cells in 2- and 3-celled antheridia. In a 2-celled antheridium, upper cell is circumscribed by points CDEO and basal cell by ACOEG. As described in text, volume of two cells are calculated to be equal. In a 3-celled antheridium, upper cell is circumscribed by points CDELKJ, basal cell by ACOEG, and central, spermatogenous cell by points JKLO. As described in text, volumes of upper cell and central cell are calculated to be equal and both, together, equal volume of basal cell.

Figure 130. An idealized diagram of an asymmetric, 2-celled antheridium. Length of outer wall of basal cell in region where angle formed by funnel-shaped wall is smallest is about one-third height of antheridium. Length of outer wall in region where angle is greatest is about three-quarters height of antheridium. Height to diameter ratio of antheridium is 3:4. With these specifications taken from antheridium shown in Figure 127, angle between funnel-shaped wall and basal wall varies from 21 to 60°.

Figure 131. Measurable, geometric shapes superimposed on drawing shown in Figure 130. As described in text, volume of upper cell, circumscribed by points CDEO, was calculated to be equal to volume of basal cell, circumscribed by points SCOET.
Figures 132-133. Serial sections through a 2-celled antheridium. Organelles are polarly distributed in upper cell. Chloroplasts and small vacuoles occur between the centrally located nucleus and curved, periclinal wall. Many mitochondria occur in ribosome-rich cytoplasm between nucleus and funnel-shaped wall. Nucleus in upper cell has dispersed chromatin, while nucleus in basal cell has more condensed chromatin. Dark arrow indicates funnel-shaped wall and clear arrow indicates basal wall of antheridium.

132. X 5,040

133. X 5,510
Figures 134-139. Cytoplasmic components in cells of 2-celled antheridia

134. Region in upper cell between nucleus and funnel-shaped wall is composed mainly of ribosomes, mitochondria, RER, and microtubules. X 26,950

135. Microtubules (arrows) surround nucleus in upper cell. Part of nuclear envelope with nuclear pores is evident in center of micrograph. X 19,935

136. Microtubules (arrows) are found along periclinal cell wall in upper cell. X 33,945

137. Golgi bodies and microbodies are found in upper cell. X 18,960

138. An occasional osmiophilic body is observed in upper cell. X 18,000

139. Region where funnel-shaped cell contacts the basal wall can become quick thick (0.90 μm). X 10,620
Figures 140-141. TEM and LM of a 2-celled antheridium during mitotic prophase of upper cell. Cytoplasm of upper cell has become redistributed with nucleus assuming an upper, central location (clear arrow). Large vacuoles have formed between nucleus and sides of upper cell and chloroplasts are oriented tangentially around nucleus. Major portion of cytoplasm is located in basal region of upper cell. A large number of mitochondria are found near funnel-shaped wall. Dark arrow indicates nucleus in basal cell

140. X 8,160

141. X 595
Figure 142. A peripheral section through 2-celled antheridium shown in Figures 140 and 141. Many nuclear pores are evident in prophase nucleus (arrow) which measure about 70 nm in diameter. X 11,225
Figures 143-145. Cytoplasmic components in cytoplasm which has accumulated in base of upper cell of a 2-celled antheridium

143. Low magnification of basal region in a near-median view showing numerous mitochondria aligned along funnel-shaped wall. X 6,090

144. Higher magnification of right-hand region shown in Figure 143. Near funnel-shaped wall, cytoplasm is composed largely of ribosomes, mitochondria, an occasional, small vacuole, microtubules (arrow), and some SER. Near nucleus, composition consists mainly of ribosomes, SER, and some vacuoles and microtubules. X 23,425

145. A more peripheral section of upper cell shown in Figure 143. SER is quite abundant and is interspersed with mitochondria near funnel-shaped wall. X 35,020
Figures 146-147. Cytoplasm surrounding nucleus in upper cell of a 2-celled antheridium containing SER, mitochondria, Golgi bodies, and microtubules

146. X 28,585

147. X 24,735
Figure 148. Mitosis in upper cell of a 2-celled antheridium. The centromeres of chromosomes are aligned in a plane parallel to base of antheridium about 10 μm from apex of upper cell (arrow). X 800

Figures 149-151. Three-celled antheridia composed of a basal cell, an upper cell, and a central spermatogenous cell. New cell wall dividing upper cell of 2-celled antheridium into new upper cell and spermatogenous cell is curved and parallel to outer, curved periclinal wall, intersecting funnel-shaped wall about 6 μm in from edge (dark arrows). Nuclei in three cells are indicated either by clear arrows or dark stars. Major portion of the cytoplasm in a 3-celled antheridium is contained within spermatogenous cell where nucleus is largest and less densely stained. Height to diameter ratio of 3-celled antheridia is about 3:4, with an average height of about 30 μm and diameter of about 40 μm

149. X 770
150. X 910
151. X 770
Figure 152. An idealized diagram of a 3-celled antheridium with a height to diameter ratio of 3:4 and distance from upper to lower wall of upper cell measuring one-fifth total height of antheridium. Funnel-shaped wall intersects outer, periclinal wall at about one-half height of antheridium with the angle measuring about 41°. As mentioned in the legend of Figure 129, upper cell and spermatogenous cell each occupy about 25% of total volume, while basal cell occupies about 50% of the volume.
UPPER CELL

SPERMATOCYTOGENOUS CELL

BASAL CELL

41°
Figure 153. Montage of a 3-celled antheridium. Central spermatogenous cell is densely cytoplasmic. Clear arrow indicates one of many dumbbell-shaped mitochondria present and dark arrow indicates a mitochondrion with a densely staining, amorphous inclusion. Average length of chloroplasts in spermatogenous cell of 3-celled antheridia is about 3 \( \mu \text{m} \) compared to 5 \( \mu \text{m} \) in vegetative cells. X 6,740
Figures 154-158. Cytoplasmic components in spermatogenous cell of 3-celled antheridia

154. Higher magnification of mitochondrion shown in Figure 153. X 17,815

155-156. Smaller, dark arrows point toward cup-shaped mitochondria. Larger, dark arrow points toward an invagination in nuclear envelope

155. X 9,850

156. X 11,950

157. Golgi bodies are present along with coated vesicles (clear arrow) and smooth vesicles (dark arrow). A few microbodies are present along with some microtubules. X 25,655

158. A chloroplast with two constrictions, indicating a dividing plastid. X 6,640
Figures 159-161. Location of microtubules in cells of a 3-celled antheridium

159. Microtubules occur parallel in groups or singly along upper, curved wall of spermatogenous cell, indicated by dark arrows. X 19,130

160. Microtubules also occur along funnel-shaped wall in spermatogenous cell, indicated by dark arrows. Note a plasmodesm traversing wall region where funnel-shaped wall contacts basal wall of antheridium. X 17,765

161. Microtubules are shown occurring along walls in spermatogenous cell in upper, right-hand corner of micrograph and along all walls in upper cell, located in left portion of micrograph. X 19,065
Figures 162-163. Low and high magnifications of basal region of a spermatogenous cell in a 3-celled antheridium. Nucleus has an indentation in region facing attenuated base of cell (large, dark arrow). Between indentation and base of cell, cytoplasm consists of abundant ribosomes, mitochondria, Golgi bodies, smooth and coated vesicles, and many microtubules oriented in various directions. This cell would have soon undergone mitosis

162. X 8,335
163. X 38,180

Figure 164. A small indentation (dark arrow) occurs in nuclear envelope 180° from larger indentation shown in Figure 162. A microtubule is shown in vicinity of indentation. X 32,075
Figures 165-167. Cytoplasmic components in upper cell of 3-celled antheridia

165. Upper, dark arrow is located to right of a microtubule and lower, dark arrow is pointing toward a microtubule. Types of organelles present in spermatogenous cell are also present in upper cell. X 22,575

166. Arrow shows a chloroplast with a constriction. X 7,265

167. X 11,145
Figures 168-172. Sections through 3-celled antheridia in which spermatogenous cell is in telophase of mitosis

168. A median, longitudinal section showing phragmoplast (arrow) forming in a plane perpendicular to base of antheridium. X 725

169-170. A nonmedian section through a 3-celled antheridium showing one set of telophase chromosomes.

169. X 910
170. X 8,775

171-172 Higher magnification of chromosomes in central, spermatogenous cell. Dark arrows indicate poly­somes. Small, clear arrows indicate microtubules. Large, clear arrow shows a double membrane sur­rounding chromosome, presumably reassembling into a nuclear envelope

171. X 23,520
172. X 20,155
Figures 173-174. TEM and LM of sections through a 4-celled antheridium. In this antheridium, new cell wall separating two spermatogenous cells (larger, dark arrow) is oriented at an angle oblique to base of antheridium. Thus, in some planes of section, such as one shown in Figure 174, one spermatogenous cell nucleus may be positioned above other spermatogenous cell nucleus. Two, lower arrows in Figure 174 point toward nuclei in spermatogenous cells. Upper arrow indicates nucleus in upper cell. Chromatin in spermatogenous cell nucleus is more dispersed than in upper and basal cells. Nucleus in basal cell still has a large nucleolus. Some of chloroplasts are beginning to dedifferentiate in spermatogenous cells (smaller, dark arrow in Figure 173). Four-celled antheridia have a height of about 30 μm and a diameter of about 40 μm.

173. X 6,330

174. X 715
Figures 175-177. Higher magnification of cytoplasmic components in spermatogenous cells of 4-celled antheridium shown in Figure 173

175. Dedifferentiated chloroplast noted in Figure 173. Lamellar system is much reduced and no starch is present. Microtubules are oriented in various directions near both sides of the cell wall separating spermatogenous cells from upper cell (dark arrows). Microtubules also occur deeper within cytoplasm of spermatogenous cell (clear arrow). X 27,485

176. Microtubules occur near new wall which formed two spermatogenous cells (dark arrow) and along funnel-shaped wall (clear arrow). X 26,265

177. Microtubules near new wall in spermatogenous tissue (dark arrow) and deeper within spermatogenous cell cytoplasm (clear arrow). X 37,580
Figures 178-179. Cytoplasmic components in upper cell of a 4-celled antheridium. Arrows indicate microtubules within cytoplasm and near nuclear envelope. Much of cell is composed of large vacuoles, as was case in 3-celled antheridia

178. X 25,835

179. X 21,165
Figures 180-185. Serial sections through a 4-celled antheridium in which upper cell is undergoing cytokinesis following division of nucleus in upper cell to form cap cell and ring cell

180. Upper cell is divided into 180 degrees to facilitate referring to various regions within cell. Zero degrees (0°) is arbitrarily assigned to junction of funnel-shaped wall and outer, curved periclinal wall on left-hand side of antheridium. In this periclinal section, a portion of the phragmoplast occurs at about 55° (arrow). X 835

181. In this more median section, phragmoplast occurs at both 50° and 120° (dark arrows). There is a large amount of cytoplasm surrounding phragmoplast at 120°. Phragmoplast is oriented at right angles to both upper and lower cell walls of upper cell. First observed nucleus (clear arrow) will be partitioned into cap cell by cylindrical phragmoplast. X 785

182. A near-median section showing developing cell wall still at 50° and 120°. There is much less cytoplasm surrounding phragmoplast/cell wall. A larger profile of cap cell nucleus is evident between two arrows. X 785

183. Ring cell nucleus is evident in this section (dark arrow), just to left of still apparent cap cell nucleus (clear arrow). Forming wall is presumed to be about 70° and 115°. X 785

184. Cap cell nucleus is no longer visible, while profile of ring cell nucleus is much larger (dark arrow). A portion of forming cell wall is presumably present just to left of ring cell nucleus. X 695

185. A large profile of ring cell nucleus is present in upper cell at 70° with no evidence of forming cell wall. Height of 4-celled antheridium is about 40 µm and the diameter is still about 40 µm. X 800

186. A diagrammatic representation of serial sections shown in Figures 180-185 looking down through apex of upper cell showing relative location of nuclei and cell wall/phragmoplast
Figures 187-188. TEM of section shown in Figure 181

187. Phragmoplast can be identified in both sides of cap cell nucleus. 6,335

188. A higher magnification of portion of phragmoplast occurring on right side of nucleus in Figure 187. Coalescing vesicles are accumulating fibrous cell wall material (larger, clear arrows). More vesicle fusion has occurred in upper portion of upper cell (left side of micrograph). Dense material, present around the vesicles, is most abundant around smaller vesicles. A globular group of small vesicles embedded in densely staining material occurs at lower end of phragmoplast (larger, dark arrow). Smaller, dark arrows indicate coated vesicles, both near phragmoplast and in cytoplasm away from phragmoplast. Smaller, clear arrows show presence of polysomes. Many microtubules are still present and oriented perpendicular to phragmoplast. Bracketed region refers to an area of intense Golgi body activity. X 26,795
Figure 189. A section serial to section shown in Figure 188 in region indicated by bracket. Area in bracket reveals large Golgi body responsible for vesiculate region observed in Figure 188. X 24,975

Figure 190. Higher magnification of phragmoplast shown to left of cap cell nucleus in Figure 187. More vesicle fusion and a lack of dense material around vesicles distinguish this portion of phragmoplast as being more advanced than region shown in Figure 188. Microtubules are more abundant near lower portion of phragmoplast (near bottom of micrograph). X 27,925
Figure 191. TEM of section shown in Figure 185. Chloroplasts in two spermatogenous cells are progressively becoming smaller and less differentiated than chloroplasts in jacket cells and vegetative cells. Microbodies are evident in all three cell types shown in this micrograph. X 6,925

Figure 192. A 5-celled antheridium consisting of a vacuolate basal cell, a vacuolate, doughnut-shaped ring cell, a moderately vacuolate, disc-shaped cap cell, and two, densely cytoplasmic, spermatogenous cells. Arrows indicate location of new cell wall forming cap cell and ring cell. Height to diameter ratio is now about 1:1, both being about 40 μm. X 525
Figure 193. Idealized diagram of a 5-celled antheridium drawn according to measurements taken from the sections shown in Figures 180-185 and 192. Height to diameter ratio is 1:1, distance between upper and lower walls of cap cell and ring cell is about one-fifth height of antheridium, and funnel-shaped wall intersects outer, periclinal wall at a height of about one-half height of antheridium. Wall of cap cell is drawn such that a line extrapolated back to vertical axis of antheridium forms an angle of 35°.

Figure 194. Measurable geometric shapes superimposed on drawing shown in Figure 193. As described in text, volume of basal cell, ABOFG, was calculated to be 40% of total antheridium volume. Volume of cap cell, CJLE, is about 12% of total volume and ring cell, BCJI-FELM, is about 26% of total volume. Two spermatogenous cells, together, OHKN, occupy 22% of total volume.

Figure 195. An idealized diagram of a 5-celled antheridium with a height to diameter ratio of 3:4. Wall of cap cell is drawn at 60° and 120°, perpendicular to upper and lower wall of upper cell. Distance between upper and lower walls of cap cell and ring cell is one-fifth height of antheridium. Funnel-shaped wall intersects outer, periclinal wall at a height of about one-half height of antheridium.

Figure 196. Measurable geometric shapes are superimposed on drawing shown in Figure 195. When wall of cap cell is extrapolated back to vertical axis, an angle of 30° is formed. As described in text, volume of cap cell, UWXV, and ring cell, CUWJ-VELX, each equal about 12.5% of total volume and each spermatogenous cell, JKO, KOL, has a volume equal to about 12.5% of total volume. Basal cell, ACOEG, occupies about 50% of total volume.
Figures 197-200. Cytoplasmic components of cap cell and ring cell in a 5-celled antheridium

197-198. Outer wall of cap cell (largest, dark arrow) is about 0.3 \( \mu \text{m} \) thick, while inner wall (clear arrow) is about 0.1 \( \mu \text{m} \) thick. Microtubules occur near cell wall and deeper within cytoplasm (smaller, dark arrows). Wall adjoining ring cell and cap cell, located in lower portion of Figure 198, has a number of plasmodesmata and is about 0.1 \( \mu \text{m} \) thick

197. X 13,360
198. X 17,700

199-200. Ring cell is more vacuolate at this stage than cap cell. Outer wall (large, dark arrow) is about 0.3 \( \mu \text{m} \) thick, while inner wall is about 0.1 \( \mu \text{m} \) thick (clear arrow). Numerous Golgi bodies are present forming many vesicles filled with fibrous material

199. X 8,500
200. X 24,080
Figures 201-202. Cytoplasmic components in cells of a 5-celled antheridium

201. Some chloroplasts in spermatogenous cells are constricted (smaller, clear arrows). Some mitochondria are dumbbell-shaped (dark arrow). Numerous, small vacuoles occur in spermatogenous cells. Inner walls of cap cell and ring cell (large, clear arrow) are still concave. X 6,300

202. A section showing basal portion of a spermatogenous cell and upper portion of basal cell. Few plasmodesmata occur in connecting funnel-shaped cell. Some microtubules (dark arrows) occur near wall which is about 0.15 μm thick. X 22,600
Figures 203-204. Cytoplasmic composition of spermatogenous cells in a 5-celled antheridium. Some mitochondria are lobed. Some chloroplasts are constricted (clear arrow). Microtubules (dark arrows) occur along cell walls and deeper within cytoplasm.

203. X 19,120

204. X 21,900
Figures 205-208. Serial sections through a 7-celled antheridium which is composed of a vacuolate basal cell, a vacuolate ring cell, a vacuolate cap cell, and four densely cytoplasmic, spermatogenous cells

205. First spermatogenous cell nucleus is evident in cell on right (dark arrow). Clear arrow indicates ring cell nucleus. X 560

206. Second spermatogenous cell nucleus is evident in cell on left (left, dark arrow). A small portion of first-observed nucleus in right-hand cell is still evident (right, dark arrow). Cap cell nucleus is indicated by clear arrow. X 560

207. Third-observed spermatogenous cell nucleus is shown in right-hand cell (dark arrow). Cap cell and basal cell nuclei are indicated by clear arrows. X 560

208. Fourth-observed spermatogenous cell nucleus is evident in left-hand cell (dark arrow). This 7-celled antheridium has a height and diameter of 40 μm each. Plane of cell division forming four spermatogenous cells from two spermatogenous cells is longitudinal, but perpendicular to first spermatogenous cell division plane. X 560

Figure 209. TEM of a 7-celled antheridium. Densely cytoplasmic spermatogenous cells have many oddly shaped mitochondria (dark arrows). Many chloroplasts are less differentiated than chloroplasts in jacket cells (smaller, clear arrows). Some chloroplasts have constrictions. Inner walls of cap cell and ring cell are now flat to slightly convex. Basal wall of antheridium, near lower right-hand corner of micrograph, is about 0.20 μm thick; outer, periclinal wall, in upper right-hand corner is about 0.30 μm thick; while most of internal walls are about 0.10-0.15 μm thick. X 5,335
Figures 210A-F. Outlines of median, longitudinal section through spermatogenous tissue in various stages of antheridium development drawn to scale shown in center of page. The numbers in parentheses indicate the number of spermatogenous cells

210A. Outline of one spermatogenous cell in a 3-celled antheridium. Volume of cell, determined by method described in text is about 6000 μm$^3$

210B. Solid lines represent outline of median section through four spermatogenous cells in a 7-celled antheridium shown in Figure 207. Dashed lines are drawn in to provide a means for determining volume which would be generated by rotating area, GCDE, 360° around vertical axis, GE. Volume of spermatogenous tissue was determined to be 10,000 μm$^3$ by method described in the text. Average volume per each of four spermatogenous cells is 2500 μm$^3$. Same method of determining volumes was applied to Figures 210C-210F

210C. Outline of median section of eight spermatogenous cells within an 11-celled antheridium shown in Figure 217. Volume is 16,000 μm$^3$, with an average volume of 2000 μm$^3$ for each of eight spermatogenous cells

210D. Outline of median section of 16 spermatogenous cells within a 19-celled antheridium shown in Figure 235. Volume of spermatogenous tissue is estimated to be 26,000 μm$^3$ with an average spermatogenous cell volume of 1600 μm$^3$

210E. Outline of median section of 32 spermatogenous cells within a 35-celled antheridium shown in Figure 245. Volume of spermatogenous tissue is about 36,000 μm$^3$ with an average spermatogenous cell volume of about 1100 μm$^3$

210F. Outline of median section of 64 spermatogenous cells within 67-celled antheridium shown in Figure 261. Volume of tissue is estimated to be 46,000 μm$^3$, with an average volume per spermatogenous cell of 720 μm$^3$
Figures 211-212. Section through a 7-celled antheridium

211. Densely cytoplasmic spermatogenous cells contain many oddly shaped mitochondria. Chloroplasts are much smaller than chloroplasts in jacket and vegetative cells. Outer wall of basal cell is thicker near base (0.5 μm; larger, dark arrow) than near top (0.3 μm). X 7,250

212. Higher magnification of a region in lower part of spermatogenous cytoplasm shown in Figure 211. A mitochondrion has accumulated some densely staining, amorphous material in central stroma. X 21,860
Figures 213-215. Cytoplasmic composition of spermatogenous cells in 7-celled antheridia. Dark arrows indicate microtubules which occur along cell walls, deeper within cytoplasm, and near nuclear envelope. An osmiophilic body is occasionally observed. Walls between spermatogenous cells are thin (0.1 μm) and have many plasmodesmata.

213. X 13,940
214. X 24,225
215. X 31,097
Figures 216-221. Longitudinal sections through 11-celled antheridia composed of three jacket cells and eight spermatogenous cells

216-219. Serial sections through an 11-celled antheridium. First four observed spermatogenous cells are present in Figure 216. Second four spermatogenous cells are apparent in Figure 218. Plane of cell divisions resulting in eight spermatogenous cells is parallel to base of antheridium (transverse) and perpendicular to two previous sets of cell divisions. Dark arrow on left in Figure 216 indicates transverse plane. Other arrow indicates one of previous longitudinal, division planes. X 465

220-221. Sections through two 11-celled antheridia in which first two sets of cell divisions were oriented obliquely to base of antheridia. In each case, plane of cell division forming eight spermatogenous cells is still oriented perpendicular to previous two sets of divisions. Arrows indicate different planes of division. Height and central diameter of 11-celled antheridia are about 45 μm each

220. X 555
221. X 565
Figure 222. A longitudinal section through an 11-celled antheridium. Although cell walls of spermatogenous cells are at right angles to each other, they are not confluent at center. Dedifferentiating chloroplasts have an average length of about 1.5 μm compared to 4.0 μm in jacket cells. Dark arrow indicates an invagination in nuclear envelope. Inner wall of ring cell (clear arrow) is convex in appearance. Thin walls (0.1 μm) between spermatogenous cells have numerous plasmodesmata. Few plasmodesmata traverse walls between spermatogenous cells and jacket cells. X 9,165
Figure 223. A transverse section through an 11-celled antheridium. Clear arrows indicate dedifferentiating chloroplasts, some of which are highly lobed. Cell walls shown in micrograph are products of first two sets of longitudinal divisions which result in four spermatogenous cells. These walls also are not confluent in center. X 11,070

Figure 224. Dark arrow indicates a mitochondrion with a dense inclusion observed in spermatogenous cell of an 11-celled antheridium. X 11,015
Figures 225-227. Cytoplasmic components in cells in 11-celled antheridia

225. A higher magnification of area indicated by arrow in Figure 222 showing more clearly invagination in nuclear envelope (large, dark arrow). Golgi body next to invagination has formed many vesicles containing fibrous material (clear arrow). Some coated vesicles occur near cell wall (smaller, dark arrow). X 28,560

226-227. Section through periphery of spermatogenous tissue in an 11-celled antheridium. Many elongated and oddly shaped mitochondria are present. Dark arrows show osmiophilic bodies. Many microtubules occur near cell wall oriented in various directions

226. X 4,030

227. X 29,895
Figures 228-230. Cytoplasmic composition of spermatogenous cells in 11-celled antheridia

228-229. Microtubules are abundant, occurring either singly or in groups oriented parallel to each other (dark arrows). Plasmodesmata are present in walls between spermatogenous cells but are few in number in walls between spermatogenous cells and jacket cells. Invaginations in plasmalemma occur containing fibrous material. X 31,110

230. A section through a spermatogenous cell cut tangentially through nuclear envelope. A portion of nuclear envelope is evident in center of micrograph, showing some nuclear pores. Microtubules occur around nuclear envelope (dark arrows). Polysomes are present in cytoplasm (clear arrows). X 12,305
Figures 231-234. Cytoplasmic composition of jacket cells in 11-celled antheridia

231. Both cap cell and ring cell are highly vacuolate. Mitochondria are usually circular to oblong and chloroplasts are well-differentiated and contain numerous starch grains. Thickness of outer wall varies between 0.3 and 0.4 μm. Vesicle-forming Golgi bodies are present. X 9,455

232. Microbodies occur in jacket cells. X 17,785

233. Microtubules occur along outer wall of ring cell (dark arrows). X 40,745

234. A section through basal cell. Outer wall (larger, dark arrow) is 0.3-0.4 μm thick. Basal wall (smaller, dark arrow) is about 0.2 μm thick, while funnel-shaped wall (clear arrow) is about 0.15 μm thick. Chloroplasts have very large starch grains. X 4,295
Figures 235-238. Sections through 19-celled antheridia, which are composed of three jacket cells, and 16 spermatogenous cells.

235. A median, longitudinal section through a 19-celled antheridium showing vacuolate nature of jacket cells and densely cytoplasmic spermatogenous cells. Antheridium has a height and diameter of about 50 μm each. X 405

236. An oblique section through a 19-celled antheridium. Chloroplasts are evident in jacket cells and vegetative cells, but not in spermatogenous cells at this magnification. X 420

237. A TEM of the section shown in Figure 236 revealing the presence of many small plastids containing little starch. Many small vacuoles are also present. X 6,900

238. A higher magnification of lower, left-hand region of spermatogenous tissue shown in Figure 237. A mitochondrion, with a large amount of dense material in stroma is clearly shown along with an osmiophilic body. Microtubules are shown near cell wall (arrows). X 40,250
Figures 239-241. Cytoplasmic composition of spermatogenous cells in 19-celled antheridia

239. A spermatogenous cell revealing an almost circular nucleus and some cup-shaped and dumbbell-shaped mitochondria. X 6,775

240-241. Microtubules are abundant in spermatogenous cells, both near nucleus and deeper within cytoplasm, sometimes occurring in parallel arrays. SER is present and in some instances, SER appears to be continuous with outer membrane of nuclear envelope (dark arrows). Golgi bodies are forming vesicles containing fibrous material

240. X 24,905

241. X 26,825
Figures 242-244. Cytoplasmic composition in jacket cells of a 19-celled antheridium

242. Cap cell has very large chloroplasts with many large starch grains in contrast to plastids in spermatogenous tissue (dark arrow). A major portion of cap cell is comprised of large vacuoles. Outer wall, at top of micrograph, is quite thick (0.6 μm). Inner wall is convex (larger, dark arrow) and is quite thin (0.1 μm). The side wall (clear arrow) is somewhat thicker (0.2 μm). X 7,930

243. Highly vacuolate ring cell also has large chloroplasts with many starch grains. Outer wall (dark arrow) is about 0.4 μm thick, while inner wall is about 0.15 μm thick. X 6,750

244. Section through a portion of basal cell. Basal wall (clear arrow) is about 0.25 μm thick, while funnel-shaped wall near upper, right-hand corner of micrograph is only about 0.15 μm thick. X 8,500
Figures 245-248. Sections through 35-celled antheridia, which are composed of three jacket cells and 32 spermatogenous cells

245-246. Median, longitudinal sections through 35-celled antheridia. Spermatogenous cells are angular in shape. Average height and central diameter are each about 55 \( \mu \text{m} \)

245. X 480
246. X 246

247. Median, longitudinal section through a 35-celled antheridium in which spermatogenous cells (spermatids) have begun to round up and differentiate into spermatozoids. Height and central diameter are each only about 50 \( \mu \text{m} \). X 480

248. TEM of spermatogenous cells in a 35-celled antheridium. Walls between cells have thickened to about 0.2 \( \mu \text{m} \). Chloroplasts have dilated lamellae. A blepharoplast has arisen de novo in each spermatogenous cell consisting of a spherical mass of densely staining material penetrated by numerous lightly stained channels. Nuclear envelope is slightly indented in area near blepharoplast. X 9,880
Figures 249-251. Cytoplasmic components in spermatogenous cells of 35-celled antheridia

249-250. Microtubules are abundant within cells (dark arrows). Many, active Golgi bodies are present. An occasional osmiophilic body is observed

249. X 16,895

250. X 21,860

251. A mitochondrion with a densely staining amorphous inclusion in central stroma. X 27,100
Figure 252. A spermatogenous cell in a 35-celled antheridium showing angular nature of cell and location of blepharoplast with respect to other cell components. Nucleus is slightly indented in area near blepharoplast. X 17,160
Figures 253-255. Higher magnification of blepharooplasts and their immediate, cytoplasmic environment. A tubule occurs in center of each lightly stained channel within blepharooplast (dark arrows) with thin filaments radiating outward from tubule along its entire length. Microtubules are abundant near blepharooplast and are oriented perpendicular to tangent of blepharooplast, appearing to terminate at periphery of structure. Microtubules are also present near nuclear envelope. Some channels in blepharooplast are bent and extend through entire structure (arrow in Figure 255)

253. X 80,135
254. X 48,585
255. X 26,170
Figures 256-260. Early stages in formation of a blepharoplast observed in spermatogenous cells of a 35-celled antheridium

256. A circular area of densely staining, flocculent material, measuring 0.5 μm in diameter, arises in spermatogenous cell near cell wall (arrow). Blepharoplast presumably arises de novo within this structure. X 16,830

257. Low magnification of spermatogenous cells showing location of a developing blepharoplast (arrow) in cell in upper, left-hand corner. Spermatogenous cells are angular in shape with thin adjoining walls (0.2 μm). Nuclei are spherical in shape. X 4,380

258-260. Serial sections through the area indicated by the arrow in Figure 257

258. Two, trilayered plaques are present surrounded by a small amount of flocculent material. Two plaques are separated by about 40-50 nm. Each plaque consists of two, dense, plate-like layers separated by a light layer. Each plaque is about 200 nm in length. Thicker, distal layer of each plaque is indicated by a clear arrow. X 39,550

259. Adjacent section to section shown in Figure 258. Distal layers have accumulated dense material in this region and channels have begun to form within material (clear arrows). Central tubule in upper channel is evident. Each distal layer presumably gives rise to one blepharoplast. X 53,505

260. Microtubules occur in area near developing blepharoplast which is no longer in view. Plastids and mitochondria both have dilated lamellae. X 27,340

Figures 261-262. Median, longitudinal sections through 67-celled antheridia, composed of three jacket cells and 64 spermatids. Antheridia have a height and central diameter of 60 μm each

261. X 440

262. X 395
Figure 263. A graph of the average volume per spermatogenous cell with respect to number of spermatogenous cells present within antheridium. Dotted line represents a threshold line in terms of volume. Spermatogenous cells which reach this volume leave mitotic cycle and enter their differentiation phase.
No. of spermatogenous cells/ antheridium

Volume/spermatogenous cell (cubic μm x 100)
Figures 264-266. Early spermatid differentiation

264. Section through two spermatids in which blepharoplast is forming procentrioles (dark arrows). Spermatids are still angular in shape and an abundance of heterochromatin occurs within nuclei. X 12,815

265. A higher magnification of area indicated by lower, dark arrow in Figure 264 showing developing procentrioles. Procentrioles have a length of about 0.3 μm and an outer diameter of about 0.15 μm. A central tubule (dark arrow) runs length of procentriole, with thin filaments radiating outward along its length. X 36,520

266. Sets of doublet microtubules (arrow) are shown arranged in a cylinder around central tubule. X 46,550
Figures 267-268. Stage during which spermatids round up and additional layers of cell wall material are deposited.

267. Cytoplasmic connections occur between spermatids in areas where wall remains thin (small, dark arrow). Centriole-MLS complex is forming within each spermatid (larger, dark arrows). X 10,530

268. Plastids in round spermatids have dense matrices, some starch grains, and osmiophilic globuli. Few lamellae present in plastids tend to be dilated (clear arrow). Long-chain polysomes are present (dark arrow) along with numerous vesicles. X 26,350
Figure 269. Higher magnification of developing centriole-MLS complex in spermatid in upper, right-hand corner of Figure 267. Centrioles are spreading out in a row within a large accumulation of osmiophilic material located above a microtubular platform (MB). X 45,820

Figure 270. Centrioles, not yet aligned in same plane, are shown above a portion of developing microtubule band. Subtending microtubule band are lamellar plates which have become associated mitochondrion (MA). There is an inclusion within MA. Numerous vesicles occur within cytoplasm containing fibrous material. X 39,535
Figures 271-272. Association of MLS complex with nucleus in early spermatid stage. MA and lamellar plates elongate in same direction and fit into a groove formed by nucleus. Microtubules in MB extend beyond MA and LP and become closely appressed to nuclear envelope. Centrioles, which are beginning to function as basal bodies, are aligned in a row along MB. Eighty-five microtubules are evident in MB in Figure 271. Golgi bodies have formed vesicles containing fibrous material.

271. X 34,540

272. X 34,320
Figure 273. A section showing orientation of microtubules in MB to lamellar plates of LS. They are oriented at about 40° to each other. Non-MB microtubules occur within cytoplasm near MLS complex in this early spermatid cell. X 34,420

Figure 274. An early spermatid showing an elongating MA and MLS. Basal bodies are shown arranged in a row above MB. Nuclear to cytoplasmic ratio is very high. Plastids, mitochondria, and active Golgi bodies occur within cytoplasm. Cytoplasmic connections (arrow) are still present traversing thin regions of adjoining cells. X 18,760
Figure 275. A diagrammatic representation of elongating MA, elongating lamellar plates, known as lamellar strip, and microtubules in MB as adopted from Duckett (1975).

Figure 276. A diagrammatic representation of initial association of complex shown in Figure 275 with nucleus in early spermatid stage as adopted from Duckett (1975).

Figure 277. A section of an early spermatid showing different regions in basal bodies aligned in a row above MB. Dark arrow indicates a section through a transition zone in a basal body from which flagella will arise. Transition zone typically has a nine-pointed, star-shaped, internal structure. In this region, some microtubules occur as triplets, while others are now reduced to doublets. Proximal region of a basal body is shown in structure labelled BB, consisting of a 'hub and spoke' internal structure. Nine sets of imbricated, triplet microtubules surround hub and spokes. Some microtubules occur singly near basal bodies (clear arrow). Basal bodies are embedded in osmiophilic material. X 83,000.

Figure 278. A section through an early spermatid showing orientation of basal bodies to axis of MB. Basal bodies are oriented at oblique angles to longitudinal axis of MB. X 13,270.

Figure 279. Non-MB microtubules are present within cytoplasm of early spermatid cells. Many appear to terminate in osmiophilic material in which basal bodies are embedded. X 23,555.
Figures 280-282. Sections of early spermatid cells in which MB extends at least halfway around spherical nucleus

280. Dark arrow indicates MLS within nuclear groove. Clear arrow indicates a portion of MB which has extended around half of nuclear circumference. A second, additional layer of cell wall material has been deposited outside plasmalemma. X 6,265

281. A peripheral section through an early spermatid showing second layer of wall material. Golgi bodies are actively forming vesicles containing fibrous material similar to material in new cell wall layer (dark arrows). Polysomes are also present within cytoplasm (clear arrows). X 17,070

282. Plastids in early spermatids have a very dense matrix and contain some starch grains. Few lamellae present are dilated in appearance. X 21,080
Figure 283. As spermatids continue to differentiate, a space develops between proplast and multilayered cell wall due to a loss of spermatid cytoplasm. MLS and MA have become displaced from nucleus which has assumed a crescent shape. Cytoplasm is highly vesiculate. Membrane-bound, vacuolate organelles are present containing unidentifiable particles (dark arrow) and are perhaps lysosomal in nature. X 15,425

Figure 284. Section through a 67-celled antheridium containing spermatids in which nucleus has assumed a crescent shape and a space has developed between spermatids and their cell walls. Indentations occur within inner jacket cell walls where spermatids appear to be exerting an outward pressure. X 390

Figure 285. A diagrammatic representation of spermatid stage shown in Figure 283 revealing 3-dimensional relationship of LS, MB, and MA to nucleus as it begins to coil (adopted from Duckett, 1975)

Figure 286. Flagella have begun to form at this stage and have extended into space created by loss in spermatid volume. Flagella are composed of typical 9+2 arrangement of microtubules, with nine doublet microtubules surrounding two central microtubules. X 25,210
Figure 287. The mid-spermatid stage in which nucleus has coiled into about 1-1/2 gyres and MLS/MK have coiled into about 3/4 gyres. Multilayered cell wall is shown surrounding each mid-spermatid (dark arrows). More than three layers appear to be present now. Numerous flagella are present in space surrounding mid-spermatids. Clear arrow is pointed toward one indentation in inner wall of ring cell. Outer wall of ring cell is only about 0.25 μm thick, while outer wall of cap cell is about 0.7 μm thick. X 6,750
Figure 288. A higher magnification of one mid-spermatid shown in Figure 287. Chromatin is condensing along nuclear envelope which is in contact with MB. An osmiophilic crest is now apparent along anterior edge of MLS. OC is about 60 nm thick and 0.25 μm wide. Autolysis is occurring in centrally located cytoplasm (white arrow). Cytoplasm is quite vesiculate in some regions. Matrix of plastids is much less dense than during early spermatid stage. A structure occurs between anterior plasmalemma and MA consisting of three layers of osmiophilic material, with two layers nearest plasmalemma connected by uniformly spaced dense regions. X 19,855
Figure 289. A section through anterior region of a mid-spermatid. Trilayered structure described in Figure 288 is more clearly shown in this micrograph (clear arrow). MLS and OC are evident on both sides of spermatid. Autolysis is occurring in central cytoplasm at anterior end of spermatid. X 42,390

Figure 290. A diagrammatic representation of mid-spermatid stage showing relationship of lamellar strip, microtubule band, associated mitochondrion, osmiophilic crest and partially coiled nucleus (adopted from Duckett, 1975)

Figure 291. Centrally located cytoplasm in a mid-spermatid. Autolysis is occurring in organelle indicated by large, dark arrow. Vacuole-like organelle containing many, dense granules indicated by clear arrow may be a lysosome. Mitochondria occur both with internal spaces filled with thin filaments (smaller, dark arrow) and densely staining, amorphous material. X 47,000
Figures 292-293. Sections through 67-celled antheridia releasing mature spermatozoids. Remnants of cap cell are indicated by clear arrow. Dark arrows point toward longitudinal sections through spermatozoids. There are about 4-5 total gyres. Width of spermatozoids at their widest point is about 7 $\mu$m. Lengths range from about 7 to 9 $\mu$m.

292. X 840

293. X 630

Figure 294. Section through a 35-celled antheridium showing mature spermatozoids within their multilayered, cell wall compartments. Chromatin in coiled nuclei is totally condensed. Length and width of these spermatozoids is about same as ones shown in Figures 292 and 293. X 4,900
Figure 295. Section through a posterior portion of a mature spermatozoid. Microtubule band encircles more than three-quarters of nuclear circumference. Double membrane of plastids is indistinguishable in some areas. Large starch grains are present in plastids. Basal bodies are embedded in osmiophilic material with a plug of osmiophilic material entering base of basal bodies (clear arrows). X 24,390

Figure 296. A diagrammatic representation of a mature spermatozoid showing 3-dimensional relationship of coiled MLS to coiled nucleus (adopted from Duckett, 1975)

Figure 297. A diagrammatic representation of relationship of microtubule band to lamellar strip and nucleus when mature spermatozoid is uncoiled (adopted from Duckett, 1975)
Figure 298. A section through an antheridium during spermatozoid release. Direction of sperm departure is indicated by dark arrow. Sperms are ensheathed by cell wall material deposited during differentiation. First addition of cell wall material during early spermatid stage appears to be dissipating. Second and later layers of cell wall material are denser and form a thin layer around sperms. Most cytoplasm in spermatozoid is present within posterior gyres of nucleus. Large starch grains are present in cytoplasm. Basal bodies only occur in anterior gyres. An accessory band of microtubules now exists between osmiophilic crest and plasmalemma (clear arrow). Number of microtubules present in accessory band is between 25-35 (inset)

298. X 7,375

Inset. X 40,835
Figure 299. An anterior, transverse section through a spermatozoid showing lack of centrally located cytoplasm. Mitochondria in peripheral cytoplasm have very swollen cristae. X 16,780

Figure 300. An oblique section through a spermatozoid showing presence of cytoplasm in posterior region. A lysosome-like organelle is present in posterior cytoplasm (dark arrow). Double membranes of plastids are difficult to discern. X 25,500

Figure 301. A median, longitudinal section through an archegonium of *O. sensibilis*. Diameter of neck canal averages about 15 μm and length of neck canal is about 70 μm. X 350
Figures 302-305. External views of mature antheridium

302. Unfixed, uncoated gametophyte showing many mature antheridia. X 290

303-305. Fixed and coated antheridia in which cap cell is either absent or partially removed. Spermatozoids are evident within antheridia. Side wall of ring cell remains intact after cap cell removal (dark arrows). Side wall of cap cell also appears to be intact during removal

303. X 1,100
304. X 1,560
305. X 1,425

Figures 306-307. Higher magnification of two spermatozoids being released from antheridia. They are ensheathed in fibrous wall material, presumably second and later additions to wall during differentiation

306. X 8,115
307. X 9,400
Figures 308A-Q. A diagrammatic representation of the entire developmental sequence of antheridia in *O. sensibilis*

308A. Nuclear migration to an outer, anterior corner of vegetative mother cell with a concomitant increase in RNA-stained material in that region

308B. Nuclear division

308C. Partitioning of accumulated cytoplasm into a small, wedge-shaped antheridium initial cell

308D. Expansion of antheridium initial to about 28-30 μm in height and 38-40 μm in diameter. Cytoplasm accumulates in upper, central region of initial

308E. First nuclear division in initial. Phragmoplast is oriented at a 40-45° angle to base of initial

308F. Formation of funnel-shaped cell wall after nuclear division resulting in a 2-celled antheridium composed of a basal cell and an upper cell. Nucleus is enlarged and less densely stained in upper cell

308G. Cytoplasm accumulates in basal portion of upper cell

308H. Nuclear division in upper cell. Phragmoplast is oriented parallel to base of antheridium

308I. Formation of a curved, periclinal wall parallel to outer periclinal wall of antheridium resulting in formation of a 3-celled antheridium. Three-celled antheridium consists of a vacuolate basal cell, an upper, cup-shaped cap cell, and a densely cytoplasmic, central, spermatogenous cell

308J. Nuclear division in spermatogenous cell. Phragmoplast is oriented in a plane perpendicular to base of antheridium

308K. Formation of longitudinally oriented cell wall in spermatogenous tissue followed closely by nuclear division in upper cell. Phragmoplast is oriented perpendicular to outer and inner walls of upper cell. Dividing nucleus is positioned slightly off-center in upper cell
308L. Formation of a cylindrical cell wall dividing upper cell into a disc-shaped cap cell and a doughnut-shaped ring cell. Five-celled antheridium consists of a basal cell, a ring cell, a cap cell, and two spermatogenous cells.

308M. Two spermatogenous cells divide in a longitudinal plane, perpendicular to first division plane in spermatogenous cell resulting in a 7-celled antheridium. Seven-celled antheridium consists of three jacket cells and four spermatogenous cells.

308N. Four spermatogenous cells divide in a transverse plane, perpendicular to first two sets of cell division in spermatogenous tissue resulting in eight spermatogenous cells. Eleven-celled antheridium consists of three jacket cells and eight spermatogenous cells.

308O. Eight spermatogenous cells undergo mitotic division resulting in 16 spermatogenous cells enclosed in three jacket cells forming a 19-celled antheridium. In this sequence, these 16 spermatogenous cells function as spermatid mother cells.

308P. Sixteen spermatid mother cells divide to form 32 spermatids enclosed in three jacket cells forming a 35-celled antheridium.

308Q. Thirty-two spermatids differentiate into 32 spermatozoids. Cap cell is forcibly removed from antheridium and spermatozoids are released into an aqueous environment ensheathed in a layer of cell wall material. Cell wall is soon disposed of, allowing flagellated spermatozoids to swim toward an archegonium.
APPENDIX C: CULTURE MEDIUM PREPARATION

The culture medium used was a combination of Bold's macronutrients (Bold, 1967), Nitsch's micronutrients (Nitsch, 1951), and a supplement of ferric chloride. Stock solutions were prepared for each macronutrient component (Table C.1) and one stock solution was prepared containing all of the micronutrient components (Table C.2).

Ten ml of each macronutrient stock solution and 1 ml of the micronutrient stock solution were added per liter of d.H₂O. One drop of a 1% solution of ferric chloride (FeCl₃) was also added per liter of medium. The pH of the medium is 6.2.

After thoroughly mixing the solution, 8.0 gm of Difco Bacto Agar were added per liter of medium and the mixture was autoclaved for 15 min at 15 lbs pressure. The medium was then poured into 100 x 15 or 60 x 20 mm sterile disposable plastic petri plates and allowed to cool.

Table C.1. Stock solutions of Bold's macronutrient components

<table>
<thead>
<tr>
<th>Component</th>
<th>Formula of salt used</th>
<th>Concentration in stock (gm/400 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium nitrate</td>
<td>NaNO₃</td>
<td>10</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>CaCl₂ · 2H₂O</td>
<td>1</td>
</tr>
<tr>
<td>Potassium phosphate</td>
<td>K₂HPO₄</td>
<td>3</td>
</tr>
<tr>
<td>(dibasic)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potassium phosphate</td>
<td>KH₂PO₄</td>
<td>7</td>
</tr>
<tr>
<td>(monobasic)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Magnesium sulfate</td>
<td>MgSO₄ · 7H₂O</td>
<td>3</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>NaCl</td>
<td>1</td>
</tr>
</tbody>
</table>
Table C.2. Stock solution of Nitsch's micronutrients

<table>
<thead>
<tr>
<th>Component</th>
<th>Formula</th>
<th>Concentration (gm/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfuric acid</td>
<td>H$_2$SO$_4$</td>
<td>0.50</td>
</tr>
<tr>
<td>Manganese sulfate</td>
<td>MnSO$_4$$\cdot$4H$_2$O</td>
<td>3.00</td>
</tr>
<tr>
<td>Zinc sulfate</td>
<td>ZnSO$_4$</td>
<td>0.50</td>
</tr>
<tr>
<td>Boric acid</td>
<td>H$_3$BO$_3$</td>
<td>0.50</td>
</tr>
<tr>
<td>Copper sulfate</td>
<td>CuSO$_4$$\cdot$5H$_2$O</td>
<td>0.025</td>
</tr>
<tr>
<td>Sodium molybdate</td>
<td>Na$_2$MoO$_4$$\cdot$2H$_2$O</td>
<td>0.025</td>
</tr>
</tbody>
</table>
APPENDIX D: GAMETOPHYTE CLEARING TECHNIQUE AND METHODS OF DATA COLLECTION

Clearing Technique and Slide Preparation

Semipermanent slides were made of cleared gametophytes collected from six different culture and treatment conditions.

Slides of cleared gametophytes were made by first placing the gametophytes into a drop of 1% aqueous acid fuchsin and cover-slipping them. A few drops of Hoyer's clearing solution, composed of 50 ml distilled water, 30 gm gum arabic (USP, flake), 200 gm chloral hydrate, and 20 ml of glycerin, were added to one edge of the cover-slip and drawn slowly through the stain with a tissue wick placed at the opposite edge of the cover-slip (Anderson, 1954).

Gametophyte Collection

The days on which gametophytes were collected to be cleared and the number of gametophytes observed from each collection are summarized in Table D.1 for each of the six different culture conditions.

Data Collection and Analysis

Data were collected from cleared gametophytes on the total number of vegetative cells per gametophyte, total number of antheridia per gametophyte, the number of cells in each antheridium, the relative location of each antheridium, and the area of each gametophyte. Data on these various aspects of growth were collected, analyzed and presented
### Table D.1. Collection days and number of observed gametophytes

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>Days after sowing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>1) Sterilized, Tr., day 0, Liq. Sow.</td>
<td>30</td>
</tr>
<tr>
<td>2) Sterilized, Tr., day 4, Liq. Sow.</td>
<td>45</td>
</tr>
<tr>
<td>3) Sterilized, Untr., Liq. Sow.</td>
<td>30</td>
</tr>
<tr>
<td>4) Unsterilized, Untr., Liq. Sow.</td>
<td>30</td>
</tr>
<tr>
<td>5) Unsterilized, Tr., day 0, Dry Sow.</td>
<td>49</td>
</tr>
<tr>
<td>6) Unsterilized, Untr., Dry Sow.</td>
<td>30</td>
</tr>
</tbody>
</table>

in a variety of ways. Except for determining the area of each gametophyte, all of the other data were obtained directly from the traced drawings made from the photographs of each cleared gametophyte.

The area of the gametophytes were determined using an Apple II microcomputer, an Apple II software program for determining areas, and an Apple II Graphics Art Tablet. Because the gametophyte drawings have enlargement factors of either 215X or 255X, a length of 1 mm on the Art Tablet was converted by the computer to either 4.65 μm or 3.92 μm, respectively. The actual areas of the gametophytes were then calculated and presented in square μm (μm²).

Mean values and confidence intervals (C.I.) at a significance level of 0.01 were calculated for the number of vegetative cells per gametophyte.
phyte, the number of antheridia per gametophyte, the total number of cells per gametophyte, and the area per gametophyte for each day of collection. A t-test was used to determine the significant differences between mean values, with the significance level reported in each case.

Linear regression was used to determine the causal relationship between the number of vegetative cells per gametophyte and the area per gametophyte. The regression equation and $r^2$ value is reported in each case.

The number of vegetative cells per gametophyte and the area per gametophyte were both bar-graphed with respect to the number of antheridia present per gametophyte. Correlation analysis was also used to determine the relationship between the number of antheridia present per gametophyte and either the number of vegetative cells or the area per gametophyte. In each case, the r value is reported.

Data on the number of antheridia per gametophyte and the number of cells in each antheridium were combined and presented in bar graphs. For each day of collection, an equal number of gametophytes, chosen randomly, were observed and the number of each stage of antheridium development was recorded and graphed to determine the length of maturation of an average antheridium.

Distributions of antheridium initials (1- and 2-celled) over time were presented using a standardized two-dimensional coordinate axis. The top and bottom of the Y-axis correspond to the apex and base of each gametophyte. The ends of the X-axis correspond to the sides of the gametophyte. The location of the initials on gametophytes from each collection day were assigned relative coordinate points and plotted.
on a standardized unit axis.

A measuring axis is superimposed on the drawing of the gametophyte shown in Figure 6, such that the Y-axis lies on the central, longitudinal axis of the gametophyte and the point X = 0, Y = 0 corresponds to the center point of the gametophyte. The relative coordinate point of each initial is determined in reference to the quadrant in which the initial occurs.

The actual coordinate point of the two-celled initial shown in Figure 6 is X = -18, Y = +20, while the relative coordinate point is X = -1/2, Y = +1/2. This relative point is then plotted on a standardized unit axis shown in Figure 7. The points for initials on all of the gametophytes collected on the same day are plotted on the same unit axis to determine their relative distribution.
APPENDIX E: FORMULAE AND SCHEDULES

I. Cytochemical staining schedules for light microscopy (LM)

A. Methyl green/pyronin Y for DNA and RNA (modified from Long and Taylor, 1956)

1. Fix gametophytes in 3:1 100% ETOH:glacial acetic acid for 2 hr at room temperature. Hydrate through the following series of solutions
2. 70% ETOH, 5 min
3. 50% ETOH, 5 min
4. 20% ETOH, 5 min
5. d.H2O, 3X, 5 min each
6. Stain gametophytes with methyl green/pyronin Y in an acetate buffer, pH 4.4, for 45 min
7. Rinse 4X in d.H2O, 15 min each
8. Place gametophytes on slide with drop of water and coverslip them
9. Photograph them immediately

B. Azure B for DNA and RNA (modified from Flax and Himes, 1952; Jacqmard et al., 1972)

1. Fix gametophytes in 3:1 100% ETOH:glacial acetic acid for 2 hr at room temperature. Hydrate through the following series of solutions
2. 70% ETOH, 5 min
3. 50% ETOH, 5 min
4. 20% ETOH, 5 min
5. d.H2O, 3X, 5 min each
6. Stain the gametophytes in Azure B in a McIlvaine buffer, pH 4.0, at 50°C for 2 hr
7. Rinse 4X in d.H2O, 15 min each and dehydrate through the following series of solutions
8. 20% ETOH, 7 min
9. 30% ETOH, 7 min
10. 40% ETOH, 7 min
11. 50% ETOH, 7 min
12. 50% ETOH, 10% tertiary butyl alcohol (TBA), 7 min
13. 50% ETOH, 20% TBA, 7 min
14. 50% ETOH, 35% TBA, 7 min
15. 50% ETOH, 50% TBA, 7 min
16. 25% ETOH, 75% TBA, 7 min
17. 100% TBA, 3X, 20 min each
18. 100% TBA, 2 hr
19. 3:1 TBA:xylene
20. 1:1 TBA:xylene
21. 1:3 TBA:xylene
22. Pure xylene, 3X, 7 min each
II. Specimen preparation of resin embedded material for LM and TEM

A. Fixation and dehydration (Glauert, 1975; Hall, 1978)

1. Fix gametophytes in either 3% glutaraldehyde or 3% glutaraldehyde and 0.5% paraformaldehyde in a 0.05 M phosphate buffer at pH 7.0 for 2 hr at room temperature
2. Rinse in buffer 4X, 20 min each
3. Post-fix in 1% OsO_4 in same phosphate buffer for 45 min at room temperature
4. Buffer rinse 4X, 5 min each
5. Rinse in d.H_2O 2X, 5 min each
6. Place into Flo-thru specimen capsules (#9-00-09-50 from American Optical Corp.) with 10% ETOH, 7 min
7. 20% ETOH, 7 min
8. 30% ETOH, 7 min
9. 40% ETOH, 7 min
10. 50% ETOH, 7 min
11. 60% ETOH, 7 min
12. 70% ETOH, 7 min
13. 80% ETOH, 7 min
14. 90% ETOH, 7 min
15. 100% ETOH, 3X, 7 min each
16. Remove from capsules and place into glass vials with 100% ETOH, 7 min

B. Infiltration and embedment (Spurr, 1969)

1. 2:1 ETOH:propylene oxide
2. 1:2 ETOH:propylene oxide
3. Pure propylene oxide, 3X, 7 min each
4. 3:1 propylene oxide:resin on a rotating mixer, 1 hr
5. 1:1 propylene oxide:resin on a rotating mixer, 2 hr
6. 1:3 propylene oxide:resin on a rotating mixer, 3 hr
7. Pure resin, overnight
8. Drain gametophytes on paper towel and place into pure resin in molds, Beem flat embedding molds or aluminum weighing trays
9. Cure for 48 hr at 65°C
C. Sectioning, mounting, and general staining for LM

1. Trim resin block with razor
2. Cut sections 1-2 \( \mu \text{m} \) thick with a glass or diamond knife on a Reichert OM-U2 ultramicrotome
3. Collect sections with an orange stick applicator and place on slides in a drop of d.H\textsubscript{2}O
4. Place slides on heating plate (70-80°C) to expand and adhere the sections to the slide
5. Form a puddle on the slides, while on the heating plate, with toluidine blue 0 (1% toluidine blue 0 in a 1% solution of sodium borate) until the edge of puddle begins to dry (Jensen, 1962)
6. Rinse slide with a stream of d.H\textsubscript{2}O
7. Dry on heating plate
8. Place cover slip with permount on slide
9. Place slide on warming tray (50-55°C) with lead weights for 4 days

D. Sectioning, mounting and staining for TEM

1. Determine regions of the gametophyte to be thin-sectioned by examining the previously cut 1-2 \( \mu \text{m} \) sections
2. Cut thin sections having grey to silver interference colors (60-85 nm) using glass or diamond knives using a Reichert OM-U2 ultramicrotome
3. Expand sections with chloroform fumes and arrange for collection with an eyelash attached to orange stick applicator
4. Collect and dry sections on 300 mesh copper grid
5. Stain grids in 20% uranyl acetate in 100% methanol (Stempak and Ward, 1964) for 30 min and rinse in the following series of solutions
6. 100% methanol, 50 dips
7. 100% methanol, 50 dips
8. 100% methanol, 50 dips
9. 50% methanol, 50 dips
10. 50% methanol, 50 dips
11. d.H\textsubscript{2}O, 50 dips
12. d.H\textsubscript{2}O, 50 dips
13. Stain grids with lead citrate (Reynolds, 1963) for 1 hr by placing grids, section side down, on the surface of a drop of lead citrate on a piece of wax in a petri plate containing pellets of NaOH
14. Rinse in six changes of d.H\textsubscript{2}O, 50 dips each
15. Air dry and place in grid holders
III. Specimen preparation for SEM

A. Fixation and dehydration

1. Fix gametophytes with 3% glutaraldehyde in a 0.05 M phosphate buffer at pH 7.0 for 2 hr at room temperature
2. Rinse in buffer, 4X, 15 min each
3. Post-fix in 1% OsO₄ in the same phosphate buffer for 45 min at room temperature
4. Rinse in buffer, 4X, 5 min each
5. Rinse in d.H₂O, 2X, 5 min each
6. Place in Flo-thru specimen capsules and dehydrate through the following series of solutions
7. 10% ETOH, 7 min
8. 20% ETOH, 7 min
9. 30% ETOH, 7 min
10. 40% ETOH, 7 min
11. 50% ETOH, 7 min
12. 60% ETOH, 7 min
13. 70% ETOH, 7 min
14. 80% ETOH, 7 min
15. 90% ETOH, 7 min
16. 100% ETOH, 3X, 7 min each
17. 3:1 ETOH:freon TF 113 or amyl acetate, 15 min
18. 1:1 ETOH:freon TF 113 or amyl acetate, 15 min
19. 1:3 ETOH:freon TF 113 or amyl acetate, 15 min
20. Pure freon or amyl acetate, 15 min
21. Pure freon or amyl acetate, 2X, 30 min each
22. Pure freon or amyl acetate, overnight at 4°C
23. Place capsules into critical point apparatus
24. Flush system with CO₂ 7-8X
25. Close system and bring through critical point (310°C, 1072 psi)
26. Mount dried specimens on brass discs with silver paint
27. Coat specimens with gold-paladium for 3 min in a Polaron E5100 Sputter coating unit
28. Store specimens in a desiccator

IV. Formulae of stains for LM, stains for TEM, fixatives, and embedding medium

A. Stains for LM

1. Methyl green/pyronin Y in an acetate buffer (0.1 M, pH 4.4; Modified, Long and Taylor, 1956)

   a. Make acetate buffer

   1). 3.0 ml glacial acetic acid/500 ml d.H₂O
   2). 6.75 gm sodium acetate/500 ml d.H₂O
   3). 310 ml (1) + 190 ml (2)
b. Add 2.5 gm of methyl green to 500 ml of the acetate buffer
c. Extract mixture several times with chloroform to remove the methyl violet
d. Add 1 gm of pyronin Y to the extracted mixture

2. Azure B in McIlvaine buffer at pH 4.0 (Flax and Himes, 1952; Jacqmard et al., 1972)
   a. Make McIlvaine buffer
      1). 24.6 ml 0.1 M citric acid
      2). 15.4 ml of 0.2 M disodium phosphate
   b. Add 0.25 mg of azure B/1 ml of McIlvaine buffer

B. Stains for TEM
   1. 20% uranyl acetate in 100% methanol (Stempak and Ward, 1964)
      a. Add 10 gm of hydrate uranyl acetate (UO₂(CH₃COO)₂.2H₂O) to 50 ml of absolute acetone-free, methanol and mix with a magnetic stirrer
      b. Filter the solution into a glass flask and cork securely
   2. Lead citrate (Reynolds, 1963)
      a. Boil d.H₂O
      b. Add 30 ml of boiled d.H₂O to 1.33 gm of lead nitrate (Pb(NO₃)₂) and 1.76 gm of sodium citrate Na₃(C₆H₅O₇)·2H₂O
      c. Stir mixture with a magnetic stirrer for 1 min
      d. Stir intermittently for 30 min
      e. Add 8 ml of freshly made NaOH and mix gently
      f. Dilute to 50 ml with boiled d.H₂O
      g. Place solution in a glass tube with a screw-on cap with a thin layer of mineral oil over the stain

C. Fixatives
   1. 3% glutaraldehyde in phosphate buffer (0.05 M, pH 7.0)
      a. Make phosphate buffer (0.1 M, pH 7.0)
         1). 13.609 gm KH₂PO₄/liter d.H₂O
         2). 14.190 gm Na₂HPO₄/liter d.H₂O
         3). 170 ml (1) + 330 ml (2)
b. Make phosphate buffer (0.05 M, pH 7.0)
   1). 250 ml 0.1 M phosphate buffer
   2). 250 ml d.H2O

c. Add the following components together
   1). 47 ml 0.05 M phosphate buffer
   2). 3 ml 50% glutaraldehyde

2. 3% glutaraldehyde and 0.5% paraformaldehyde in a 0.05 M phosphate buffer at pH 7.0 (Hall, 1978)

a. Make 10% paraformaldehyde
   1). 10 gm paraformaldehyde/100 ml d.H2O
   2). Heat to 60-70°C while stirring
   3). Add 2-3 drops of 1 N NaOH until solution becomes clear

b. Add the following components together
   1). 2 ml 10% paraformaldehyde
   2). 3 ml d.H2O
   3). 20 ml 0.1 M phosphate buffer
   4). 15 ml 8% glutaraldehyde

D. Embedding medium

1. Spurr's epoxy resin (Spurr, 1969)
   a. Add the following four components together gravimetrically to obtain a firm to hard resin
      1). 5.0 gm of D.E.R. 736
      2). 0.3 gm of DMAE
      3). 10.0 gm of VCD
      4). 25.0 of NSA
   b. Stir with automatic stirrer for 1-2 hr
APPENDIX F:

MEAN VALUES AND CONFIDENCE INTERVALS
Table F.1. Culture condition 1 (sterilized, treated day 0, liquid-sown)

<table>
<thead>
<tr>
<th>Days after sowing</th>
<th># veg. cells/ gametophyte X ± C.I.*</th>
<th># total cells/ gametophyte X ± C.I.</th>
<th># antheridia/ gametophyte X ± C.I.</th>
<th>Area/gametophyte (µm²) X ± C.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>4.87 ± 1.05</td>
<td>-</td>
<td>-</td>
<td>6124 ± 911</td>
</tr>
<tr>
<td>4</td>
<td>9.70 ± 1.10</td>
<td>9.90 ± 1.27</td>
<td>.06 ± .16</td>
<td>10023 ± 1169</td>
</tr>
<tr>
<td>5</td>
<td>13.00 ± 1.30</td>
<td>13.95 ± 1.79</td>
<td>.45 ± .30</td>
<td>14471 ± 1739</td>
</tr>
<tr>
<td>6</td>
<td>17.60 ± 1.96</td>
<td>22.80 ± 3.39</td>
<td>1.61 ± .50</td>
<td>20701 ± 2299</td>
</tr>
<tr>
<td>7</td>
<td>25.00 ± 2.92</td>
<td>37.50 ± 6.26</td>
<td>2.03 ± .44</td>
<td>27595 ± 2852</td>
</tr>
<tr>
<td>9</td>
<td>56.76 ± 8.46</td>
<td>161.70 ± 37.00</td>
<td>5.97 ± 1.04</td>
<td>56198 ± 7821</td>
</tr>
<tr>
<td>11</td>
<td>89.30 ± 14.30</td>
<td>223.00 ± 37.00</td>
<td>-</td>
<td>78818 ± 11605</td>
</tr>
</tbody>
</table>

*Confidence intervals are reported at the significance level of 0.01 for Tables G.1-G.9.

Table F.2. Culture condition 2 (sterilized, treated day 4, liquid-sown)

<table>
<thead>
<tr>
<th>Days after sowing</th>
<th># veg. cells/ gametophyte X ± C.I.</th>
<th># total cells/ gametophyte X ± C.I.</th>
<th># antheridia/ gametophyte X ± C.I.</th>
<th>Area/gametophyte (µm²) X ± C.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>4.90 ± .89</td>
<td>4.90 ± .89</td>
<td>0 ± 0</td>
<td>7016 ± 1101</td>
</tr>
<tr>
<td>6</td>
<td>16.50 ± 1.24</td>
<td>16.80 ± 1.24</td>
<td>1.22 ± .16</td>
<td>19814 ± 1552</td>
</tr>
<tr>
<td>7</td>
<td>22.15 ± 1.47</td>
<td>25.73 ± 1.69</td>
<td>1.79 ± .29</td>
<td>28379 ± 1817</td>
</tr>
<tr>
<td>8</td>
<td>29.66 ± 2.92</td>
<td>39.25 ± 3.43</td>
<td>2.77 ± .35</td>
<td>40485 ± 4511</td>
</tr>
<tr>
<td>9</td>
<td>38.86 ± 4.29</td>
<td>64.57 ± 8.51</td>
<td>4.14 ± .64</td>
<td>51737 ± 6185</td>
</tr>
<tr>
<td>10</td>
<td>49.40 ± 5.28</td>
<td>106.26 ± 19.42</td>
<td>6.03 ± .92</td>
<td>64313 ± 8157</td>
</tr>
<tr>
<td>11</td>
<td>69.00 ± 9.75</td>
<td>192.15 ± 25.60</td>
<td>8.27 ± 1.17</td>
<td>88599 ± 11938</td>
</tr>
<tr>
<td>12</td>
<td>85.20 ± 11.85</td>
<td>258.50 ± 44.60</td>
<td>9.88 ± 1.39</td>
<td>109896 ± 15461</td>
</tr>
</tbody>
</table>
Table F.3. Culture condition 3 (sterilized, untreated, liquid-sown)

<table>
<thead>
<tr>
<th>Days after sowing</th>
<th># veg. cells/</th>
<th># total cells/</th>
<th># antheridia/</th>
<th>Area/gametophyte (μm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X ± C.I.</td>
<td>X ± C.I.</td>
<td>X ± C.I.</td>
<td>X ± C.I.</td>
</tr>
<tr>
<td>3</td>
<td>4.50 1.71</td>
<td>0 0</td>
<td>7016 1101</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>17.00 1.71</td>
<td>0 0</td>
<td>18250 1850</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>26.20 3.19</td>
<td>0 0</td>
<td>27308 3421</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>36.50 3.75</td>
<td>0 0</td>
<td>35119 3239</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>53.70 6.50</td>
<td>0 0</td>
<td>55877 6697</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>66.20 10.00</td>
<td>0 0</td>
<td>76591 12794</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>91.10 9.37</td>
<td>0 0</td>
<td>99716 10219</td>
<td></td>
</tr>
</tbody>
</table>

Numbers are the same as vegetative cells per gametophyte since no antheridia were formed.

Table F.4. Culture condition 4 (unsterilized, untreated, liquid-sown)

<table>
<thead>
<tr>
<th>Days after sowing</th>
<th># veg. cells/</th>
<th># total cells/</th>
<th># antheridia/</th>
<th>Area/gametophyte (μm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X ± C.I.</td>
<td>X ± C.I.</td>
<td>X ± C.I.</td>
<td>X ± C.I.</td>
</tr>
<tr>
<td>3</td>
<td>7.13 1.74</td>
<td>0 0</td>
<td>7812 1113</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>10.60 2.45</td>
<td>0 0</td>
<td>13101 2197</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>18.90 2.07</td>
<td>0 0</td>
<td>21268 2213</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>23.80 4.35</td>
<td>0 0</td>
<td>23467 3597</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>45.40 8.00</td>
<td>0 0</td>
<td>40553 6985</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>137.10 16.45</td>
<td>0 0</td>
<td>128594 14871</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>231.40 25.00</td>
<td>0 0</td>
<td>214520 28378</td>
<td></td>
</tr>
</tbody>
</table>

Numbers are the same as vegetative cells per gametophyte since no antheridia were formed.
Table F.5. Culture condition 5 (unsterilized, treated day 0, dry-sown)

<table>
<thead>
<tr>
<th>Days after sowing</th>
<th># veg. cells/ gametophyte</th>
<th># total cells/ gametophyte</th>
<th># antheridia/ gametophyte</th>
<th>Area/gametophyte (μm^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X  ± C.I.</td>
<td>X  ± C.I.</td>
<td>X  ± C.I.</td>
<td>X  ± C.I.</td>
</tr>
<tr>
<td>4</td>
<td>9.90 ± 1.02</td>
<td>10.80 ± 1.15</td>
<td>0.60 ± 0.29</td>
<td>13645 ± 1340</td>
</tr>
<tr>
<td>5</td>
<td>13.94 ± 1.40</td>
<td>18.30 ± 2.00</td>
<td>1.91 ± 0.28</td>
<td>20788 ± 2563</td>
</tr>
<tr>
<td>7</td>
<td>25.40 ± 2.82</td>
<td>44.40 ± 6.03</td>
<td>2.68 ± 0.38</td>
<td>31761 ± 3272</td>
</tr>
<tr>
<td>8</td>
<td>45.30 ± 3.68</td>
<td>113.90 ± 17.50</td>
<td>5.32 ± 0.78</td>
<td>58857 ± 5246</td>
</tr>
<tr>
<td>10</td>
<td>67.75 ± 10.70</td>
<td>223.60 ± 40.60</td>
<td>7.33 ± 1.42</td>
<td>83546 ± 11808</td>
</tr>
<tr>
<td>11</td>
<td>101.50 ± 12.80</td>
<td>378.30 ± 46.00</td>
<td>10.80 ± 1.31</td>
<td>133519 ± 18699</td>
</tr>
</tbody>
</table>

Table F.6. Culture condition 6 (unsterilized, untreated, dry-sown)

<table>
<thead>
<tr>
<th>Days after sowing</th>
<th># veg. cells/ gametophyte</th>
<th># total cells/ gametophyte</th>
<th># antheridia/ gametophyte</th>
<th>Area/gametophyte (μm^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X  ± C.I.</td>
<td>X  ± C.I.</td>
<td>X  ± C.I.</td>
<td>X  ± C.I.</td>
</tr>
<tr>
<td>5</td>
<td>18.40 ± 2.07</td>
<td>-</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>35.50 ± 3.72</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>67.20 ± 8.13</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>120.20 ± 18.00</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>186.00 ± 26.40</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\)Numbers are the same as vegetative cells per gametophyte since no antheridia were formed.
Table F.7. Number of vegetative cells and area per gametophyte with n number of antheridia from culture condition 1, 3-9 days after sowing

<table>
<thead>
<tr>
<th># antheridia/gametophyte</th>
<th># veg. cells/gametophyte</th>
<th>Area/gametophyte (um²)</th>
<th># gametophytes sampled</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>X</td>
<td>C.I.</td>
<td>X</td>
</tr>
<tr>
<td>1</td>
<td>17.1</td>
<td>2.3</td>
<td>19504</td>
</tr>
<tr>
<td>2</td>
<td>20.4</td>
<td>2.2</td>
<td>22892</td>
</tr>
<tr>
<td>3</td>
<td>30.3</td>
<td>7.3</td>
<td>32422</td>
</tr>
<tr>
<td>4-6</td>
<td>49.8</td>
<td>12.3</td>
<td>48684</td>
</tr>
<tr>
<td>7-10</td>
<td>65.4</td>
<td>14.3</td>
<td>65936</td>
</tr>
</tbody>
</table>

Table F.8. Number of vegetative cells and area per gametophyte with n number of antheridia from culture condition 2, 4-10 days after sowing

<table>
<thead>
<tr>
<th># antheridia/gametophyte</th>
<th># veg. cells/gametophyte</th>
<th>Area/gametophyte (um²)</th>
<th># gametophytes sampled</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>X</td>
<td>C.I.</td>
<td>X</td>
</tr>
<tr>
<td>1</td>
<td>20.2</td>
<td>3.3</td>
<td>25967</td>
</tr>
<tr>
<td>2</td>
<td>24.5</td>
<td>2.7</td>
<td>31951</td>
</tr>
<tr>
<td>3</td>
<td>30.1</td>
<td>3.5</td>
<td>39745</td>
</tr>
<tr>
<td>4-6</td>
<td>41.2</td>
<td>3.1</td>
<td>54885</td>
</tr>
<tr>
<td>7-10</td>
<td>58.3</td>
<td>8.1</td>
<td>78060</td>
</tr>
</tbody>
</table>
Table F.9. Number of vegetative cells and area per gametophyte with n number of antheridia from culture condition 5, 4-10 days after sowing

<table>
<thead>
<tr>
<th># antheridia/gametophyte</th>
<th># veg. cells/gametophyte</th>
<th>Area/gametophyte (μm²)</th>
<th># gametophytes sampled</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X</td>
<td>C.I.</td>
<td>X</td>
</tr>
<tr>
<td>1</td>
<td>11.6</td>
<td>1.5</td>
<td>16537</td>
</tr>
<tr>
<td>2</td>
<td>20.0</td>
<td>4.7</td>
<td>27002</td>
</tr>
<tr>
<td>3</td>
<td>29.0</td>
<td>5.6</td>
<td>37325</td>
</tr>
<tr>
<td>4-6</td>
<td>44.9</td>
<td>6.9</td>
<td>55222</td>
</tr>
<tr>
<td>7-10</td>
<td>64.9</td>
<td>11.2</td>
<td>83358</td>
</tr>
</tbody>
</table>