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Ultrastructural Investigations on Spore Germination in the Fern Woodwardia Virginica (L.) Smith

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ULTRASTRUCTURAL INVESTIGATIONS ON SPORE GERMINATION IN THE FERN *WOODWARDIA VIRGINICA* (L.) SMITH

A Thesis

Presented to

the Faculty of the Department of Biology

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In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

James Rodney McCurry

May 1982

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ULTRASTRUCTURAL INVESTIGATIONS ON SPORE GERMINATION IN THE FERN WOODWARDIA VIRGINICA (L.) SMITH

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Particular thanks go to Drs. Kenneth Nicely and Gary Dillard for their faith in my ability and guidance when needed. Mr. Guy Briggs and Ms. Bobbie Dowden are especially thanked for their professional assistance with part of the photography.

Last but not least, I would like to thank my wife and son for their patience and support during the long hours of research needed to complete this project.

This thesis is dedicated to the memory of Dr. Ernest O. Beal, an outstanding scientist, teacher and friend, who originally proposed the idea for this study.

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James Rodney McCurry May 1982 58 pages

Directed by: E. J. Hoffman, K. A. Nicely and G. E. Dillard

Department of Biology Western Kentucky University

This investigation represents the first electron microscopic study of spore germination in the fern Woodwardia virginica (L.) Smith. Samples of fern spores were induced into synchronous division by a dark, red light and white light sequence and examined for the early events of germination. During early germination the nucleus, which was located in a central position, assumed a pointed trailing end and a broadened amoeboid proximal face while migrating to a proximal position in the spore. At this time the nucleus became spherical, and chromosomes began to condense even before the disruption of the nuclear envelope. Chloroplastid and mitochondrial structure give clues to the phylogenetic position of Woodwardia. It was determined that chloroplast structure in the protonema resembled that for other organisms intermediate between the bryophytes and angiosperms. The mitochondria contained both tubular and flattened cristae, again indicating an intermediate position for the ferns. Scanning electron microscopy was used to study the spore and spore germination through the second mitotic division of the basal cell.

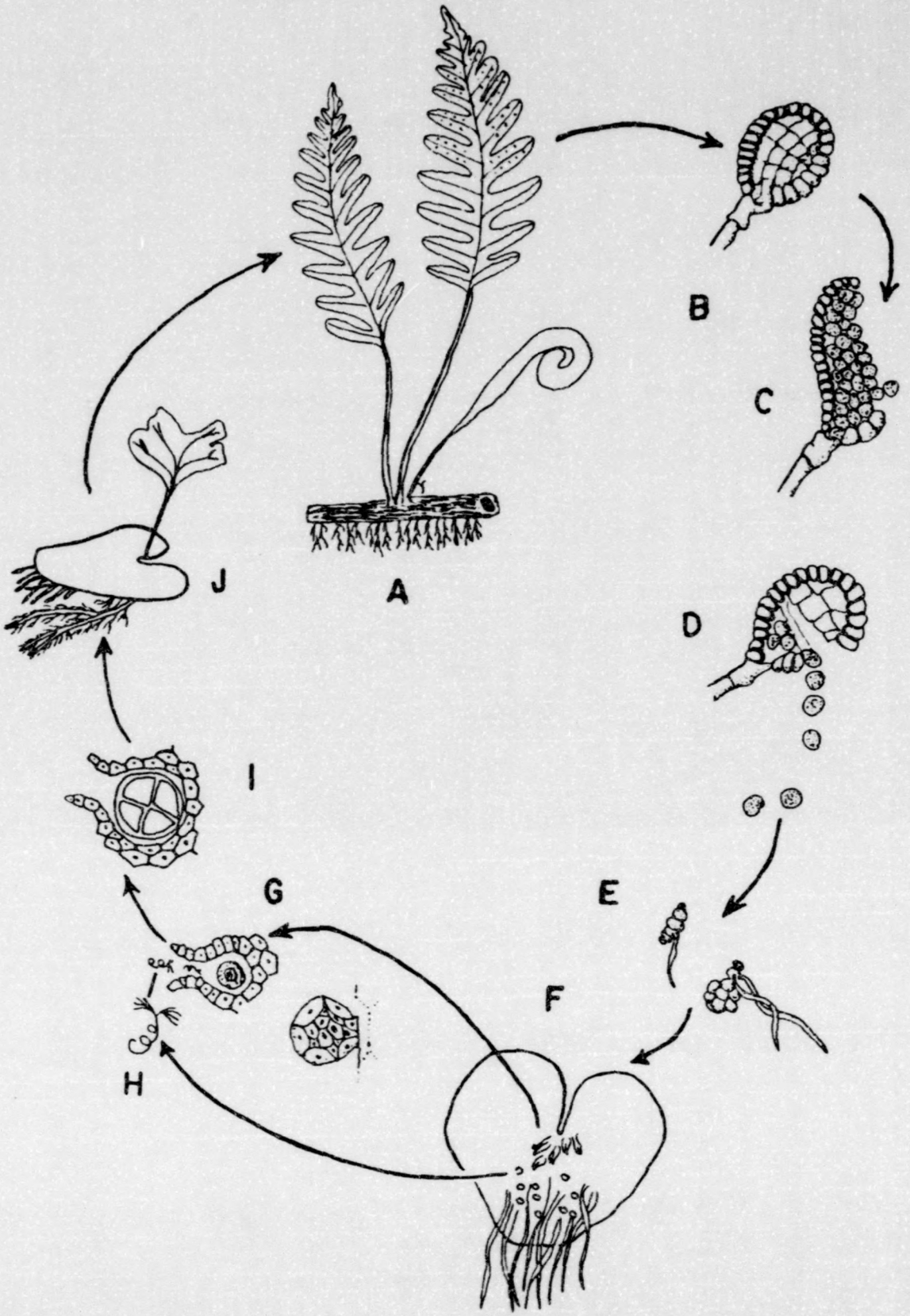
INTRODUCTION

The ferns are of considerable importance in the study of plant phylogeny and cell biology. Their position between primitive non-vascular plants and the most advanced vascular plants, the angiosperms, makes them especially useful tools for investigation. As the "least complex" organisms exhibiting higher plant characteristics, they can be used to study many processes without the complications normally associated with advanced systems.

Ferns exhibit a life cycle that is similar in many ways to that of higher plants. A haploid (N) gametophyte generation, which produces the gametes, alternates with a diploid (2N) sporophyte generation which produces the spores (Fig. 1). This thesis deals exclusively with the spore and its initial germination. As a result of sporogenesis, four "D"-shaped spores are formed by meiosis of the spore mother cell within the sporangium.

Of particular interest are the events leading to and including the unequal first division of the spore nucleus. Certain properties of the growth of spores, particularly those relating to cell division, are reminiscent of the algae and bryophytes. This emphasizes the taxonomic position of the pteridophytes as being between the algae and angiosperms in the evolutionary scheme. Certain members of the pteridophytes have been examined by electron microscopy with regard to

Figure 1. Diagram of a typical fern life cycle. Note particularly the stages of spore formation, release and early germination.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particular aspects of their growth and morphology, e.g., organelle production in Pteridium (Bell and Muhlethaler 1962; Bell 1964), plastids in Psilotum triquetrum (Sun 1961) and in Isoetes howellii (Paollilo 1962) and spore germination in Matteuccia struthiopteris (Gantt and Arnott 1965; Marengo 1977), Blechnum spicant (Beisvag 1970), Dryopteris borrei (Cran 1970), Equisetum (Gullvag 1968, 1971), Polypodium vulgare (Fraser and Smith 1974) and Onoclea sensibilis (Miller and Bassel 1980; Bassel et al. 1981). In addition, one ultrastructural report has been presented on the effects of microtubule inhibitors on germinating fern spores (Vogelmann et al. 1981). The rhizoids of Dryopteris borrei were extensively examined by Dyer and Cran (1976). With the exception of the papers of Fraser and Smith (1974) and Miller (1980, 1981), all reports utilized potassium permanganate as a fixative. This fixative is a strong oxidizing agent which destroys much of the cytoplasmic and nuclear detail leaving only membranes clearly visible. All of the above reports except Miller (1980, 1981) utilized protonemata of three or more cells. Only occasional micrographs of dormant and germinating spores have appeared in the literature. No extensive studies have been performed because of the extreme difficulty of fixation and embedding as well as the difficulty in preparing successful thin sections. An examination of dormant and germinating spores of ferns could add significantly to the understanding of the phylogenetic position of ferns.

Only within the last 25 years has the value of fern spores and protonemata as tools for experimental investigation of cellular events been recognized (Mohr 1956; Miller 1968; Dyer 1979). Their usefulness lies in the fact that they have a simple cellular structure, are easy to obtain in relative large quantities, and are viable for years in refrigerator storage. Also, culture of large numbers of spores approaches the ease of bacterial cells, and their position among the plant phyla makes them important. In addition, fern spores consisting solely of single cells give a homogeneous collection of cells free from dissimilar surrounding tissues that might interfere with the results of experimental procedures. The uniformity of a population of spores confers several advantages, since, when conditions are changed, the majority of the population is affected in a similar manner. Thus, by examining small samples one can be reasonably sure that they are typical of the population as a whole. This characteristic is especially desirable for electron microscope investigations because longer periods of time and effort are necessary to examine a single cell.

If one examines any textbook dealing with plant structure very little information can be found concerning the ultrastructure of pteridophyte cells. On the other hand, a wealth of information is available about virtually every cell type found in angiosperms. There have also been numerous investigations on the ultrastructure of algal and fungal cells and to a lesser extent the bryophytes, but the pteridophytes have

not received the same attention. Most pteridophyte studies have concentrated on sporophyte tissues and cells, but there is no cohesive body of information at the ultrastructural level regarding the structure of the various cell types and organelles found in early division stages of fern spores. Extensive studies on fern spores and gametophytes have been performed at the light microscopic level. Much is known about the light, moisture and temperature requirements for germination. Physiological events during germination have also been studied.

The species studied in this investigation was Woodwardia virginica (L.) Smith. It was used because it was readily obtainable in large quantities, spores can be stored for a long period of time without appreciable decrease in viability and previous light microscopic studies had shown a relatively rapid germination time.

MATERIALS AND METHODS

Collection of Spores

Fronds bearing ripe sporangia were collected along the shores of Lake Mattamuskeet, North Carolina, in June 1980 and 1981 by Carolina Biological Supply Company. Spores were obtained by laying the fronds, sporangial side downward, on sheets of smooth white paper and allowing them to dry in a warm draft-free room for 24 hr; those which had been shed were transferred to shell vials closed with cotton plugs. All collected spores were stored in a refrigerator at 4 C, except during shipping, until needed. Storage under these conditions maintained at least a 50% germination rate for the two years required in this investigation.

Cell Culture

The medium selected was a modified Knop's solution. It consisted of $MgSO_4 \cdot 7H_2O$ (0.51 g), KNO_3 (0.12 g), $Ca(NO_3)_2 \cdot H_2O$ (1.44 g), $FeCl_2 \cdot 6H_2O$ (0.17 g), KH_2PO_4 (0.25 g), and distilled H_2O (1000 ml). If a solid medium was required, 1.0-1.5% agar was added to the solution before autoclaving. Eight ml of liquid medium was added to 18 x 100 mm loose fitting screw cap glass culture tubes. All media were sterilized by autoclaving for 15 min at 121 C. Cotton swabs were used to inoculate the spores by tapping the swab dipped into the spores onto the edge of the culture tube and allowing the spores to

fall onto the surface of the culture medium. For agar culture the solid agar was melted at 100 C and, after cooling slightly, was poured into a 35 mm plastic petri dish to a depth of 3-5 mm. When the agar solidifed, spores were tapped onto the surface as previously described. A spore density giving a slightly speckled surface appearance was found to be desirable.

After sowing, the spores were placed in the dark for 12 to 48 hr at room temperature to allow imbibition of water. This procedure was followed to synchronize spore germination as much as possible. At the completion of the dark period, the spores were placed in red light (GEF1578-Red) at 150 ft-c for 6 to 18 hr to initiate germination. Nuclear division was initiated by placing the red light activated cultures under 150 ft-c of illumination from a Grolux (Sylvania F15T8-Gro) light. All cultures were maintained at 21-25 C in growth chambers.

A one-step staining method developed by Edwards and Miller (1972) was used for determining spore germination. Nuclear and cytoplasmic stains generally fail to penetrate the spore within less than 24 hr, and division of the nucleus cannot be detected within less than 24 hr. Acetocarmine-choral hydrate stain was prepared by adding 16 g choral hydrate to 10 ml of acetocarmine. Acetocarmine was prepared by the conventional method of adding excess carmine dye to a solution of 50% distilled water and 50% glacial acetic acid, boiling, adding dilute ferric sulfate and filtering

to remove undissolved particles. The acetocarmine-choral hydrate solution acted as a clearing agent as well as a stain. Stained spores were examined under 100X magnification with a Zeiss phase contrast microscope.

Transmission Electron Microscopy

Many fixation and embedding techniques were tested on agar and liquid grown cultures. These included 6% glutaraldehyde + 2% OsO_4 post fix, simultaneous glutaraldehyde-OsO_4 and 1.5% $KMnO_4$. Phosphate, cacodylate, PIPES and a microtubule polymerizing buffer were used. All buffers were adjusted to a pH of 7.2. Dehydration was accomplished with a graded series of acetone or ethanol. Embedding plastics were either Epon 812 or Spurr Low Viscosity Plastic. Both vacuum and room temperature infiltration were utilized.

The final procedure selected used techniques from those reported by Hepler (1980) and Bassel *et al*. (1981). Spores cultured for 0-99 hr were fixed in a 15 ml centrifuge tube with a solution of 2.5% glutaraldehyde in 0.05 M cacodylate buffer containing 5 mM $CaCl_2$, pH 7.2, for 12 to 24 hr at 23 C. Three ml of 10% TWEEN 80 (Sigma Chemical Company) was added to cause the spores to sink. After centrifuging and undergoing three washes in buffer containing $CaCl_2$, the spores were treated with 1 ml of 2.75% NaOCl in 0.3 M mannitol for 45 to 90 sec. An additional 10 ml of buffer was added to further dilute the NaOCl, and the sample was centrifuged at 1200 rpm for 5 min. The pellet of cells remained in the

bottom of the tube while the supernate was slowly poured off. After each additional treatment centrifugation was necessary. The spores were washed with 0.3 M mannitol followed by buffer. Post fixation consisted of adding a solution of 1% OsO_4 containing 0.8% potassium ferricyanide, $K_3Fe(CN)_6$ and 0.05 M cacodylate buffer, pH 7.2. The spore pellet was broken by shaking, and the fixative was allowed to remain for 2 hr. A brief buffer wash removed excess osmium fixative, and a second treatment with 2.75% NaOCl was performed as above. After rinsing, a second OsO_4-potassium ferricyanide fixation was utilized for an additional 30 min. Following a buffer rinse, the spores were stained for 2 hr in 2% aqueous uranyl acetate.

After staining, the fixed spores were mixed with one drop of 2% agar to facilitate handling the sample. The agar sample was cut into 1 mm cubes and placed in 1 dram shell vials for additional processing.

A 15 min rinse in 2-methoxyethanol was used before dehydrating with a standard graded series of ethanols from 20 to 100%. Propylene oxide was used for two 15 min rinses as an intermediate solvent. Epon 812 (Shell Chemical Company) was used for embedding in gradually increasing amounts by adding one part Epon to three parts propylene oxide for 2 hr to overnight, followed by two parts Epon to two parts propylene oxide for 8 hours to overnight, three parts Epon to one part propylene oxide overnight and finally 100% Epon for 12 hr. Specimens were transferred to fresh Epon in flat embedding

molds. After infiltration, the samples were placed in a 100 C oven for 4 hr or a 70 C oven for 4 days to polymerize the Epon.

Blocks were sectioned on a Reichert OmU2 ultramicrotome with a diamond knife. Near serial sections of silver or grey interference colors were picked up on 300 mesh copper grids and examined with a Zeiss EM 9S2 transmission electron microscope.

Scanning Electron Microscopy

Appropriately germinated spores and protonema were fixed overnight with 3% glutaraldehyde in cacodylate buffer, pH 7.2. A standard ethanol dehydration sequence was used to remove all water from the specimen. The dehydrated samples were placed in containers made from modified BEEM capsules and nylon or brass mesh for critical point drying with a Samdri-790 Critical Point Dryer. After critical point drying the samples were mounted on silver coated stubs, sputter coated with gold and examined with an Hitachi S-500 or an ISI Super IIIA scanning electron microscope.

RESULTS

Culture, Fixation and Embedding

The medium for the culture of Woodwardia virginica was not critical. Very little difference in germination rate was noted except that when distilled water was used a slightly reduced rate of germination resulted. Agar or liquid media gave similar final germination rates. Cultures sown on agar were 24-28 hr slower in initial germination. The medium finally selected was utilized because it was readily available.

Light Microscopy

Synchrony in division stages was attempted by using dark, red light and white light sequences. Both 24 and 48 hr of dark imbibition worked equally well. The minimum red light exposure necessary to initiate germination was found to be 6 hr. Periods of time up to 18 hours were also satisfactory; however, if this time limit were exceeded, an occasional mitotic division would have occurred. The final procedures selected are summarized in Figures 2 and 3. The maximum germination rate obtained was 50%.

The acetocarmine-choral hydrate stain aided in identifying germinating spores. Ungerminated spores retained a green color in the cytoplasm; germinating spores exhibited a light pink cytoplasm. A spore was considered to have germinated when nuclei from the basal cell and rhizoidal cell could be

Figure 2. Diagram of the timing of events during early development of gametophytes of *Woodwardia virginica*.

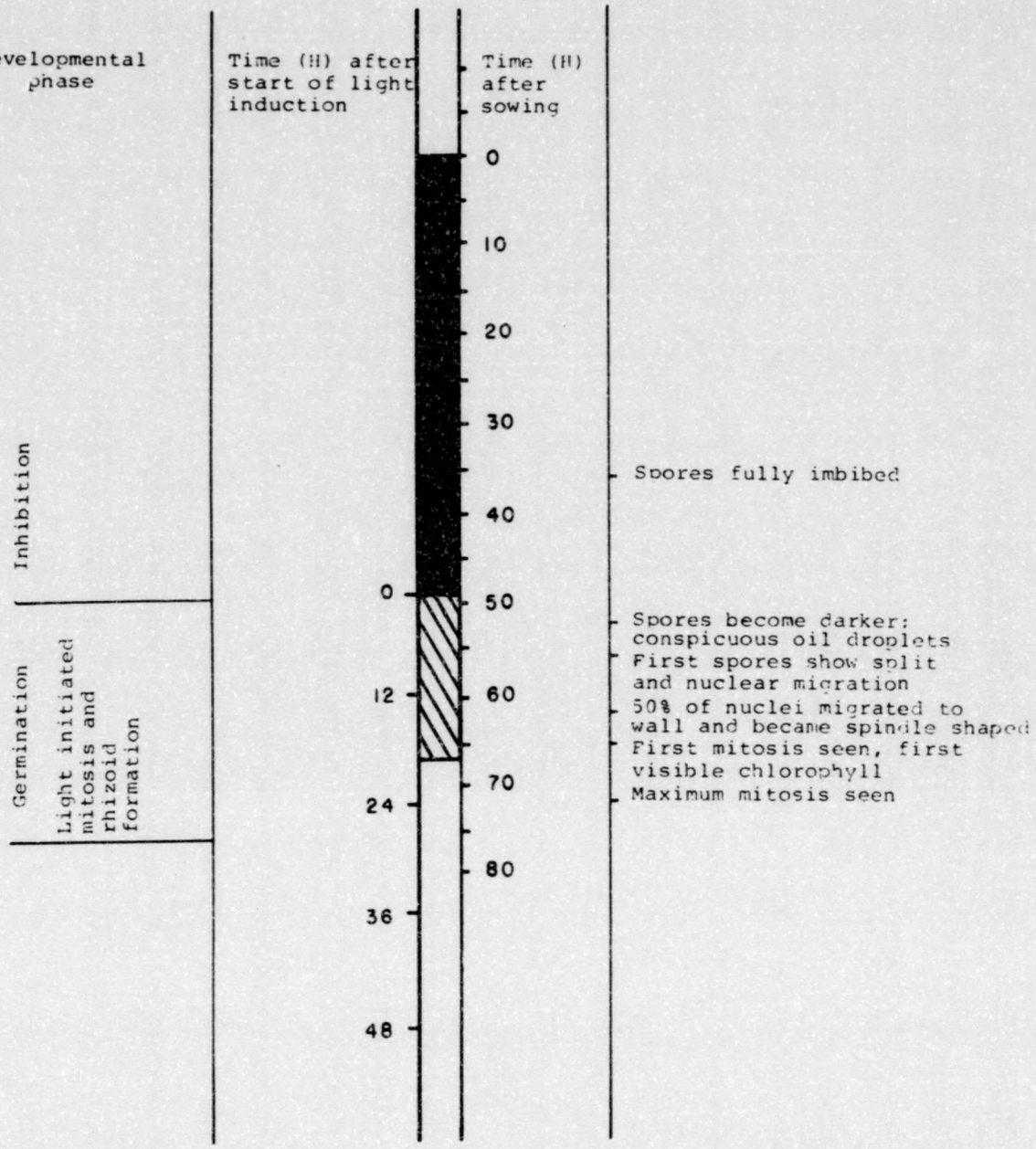
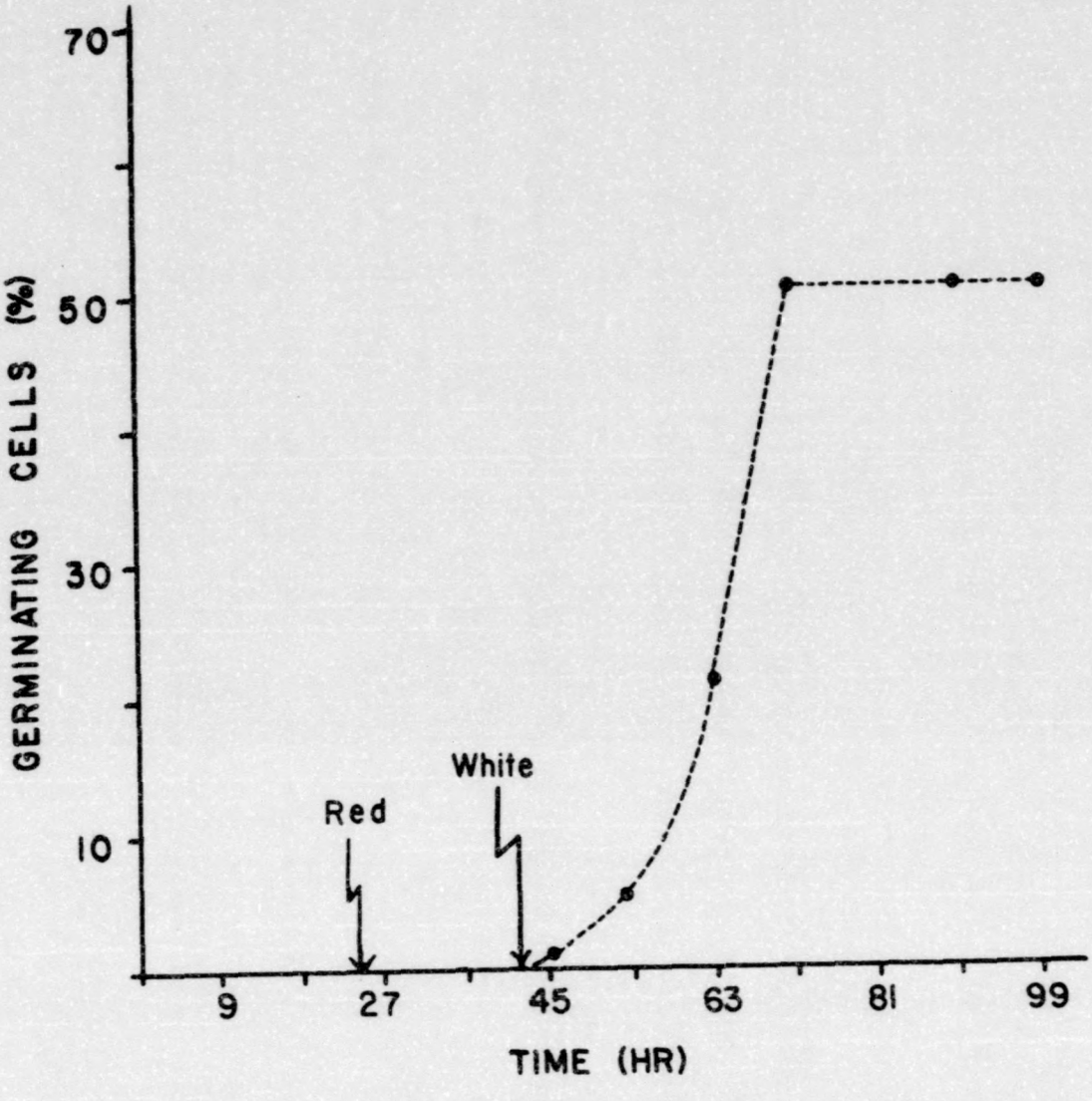

Developmental phase
Time (H) after start of light induction
Time (H) after sowing
Inhibition
Germination
Light initiated mitosis and rhizoid formation
0
10
20
30
40
50
60
70
80
0
12
24
36
48
Spores fully imbibed
Spores become darker; conspicuous oil droplets
First spores show split and nuclear migration
50% of nuclei migrated to wall and became spindle shaped
First mitosis seen, first visible chlorophyll
Maximum mitosis seen

Figure 3. Graph of timing for first mitotic division in Woodwardia virginica. Note that most initial nuclear division occurred between 70 and 72 hours.

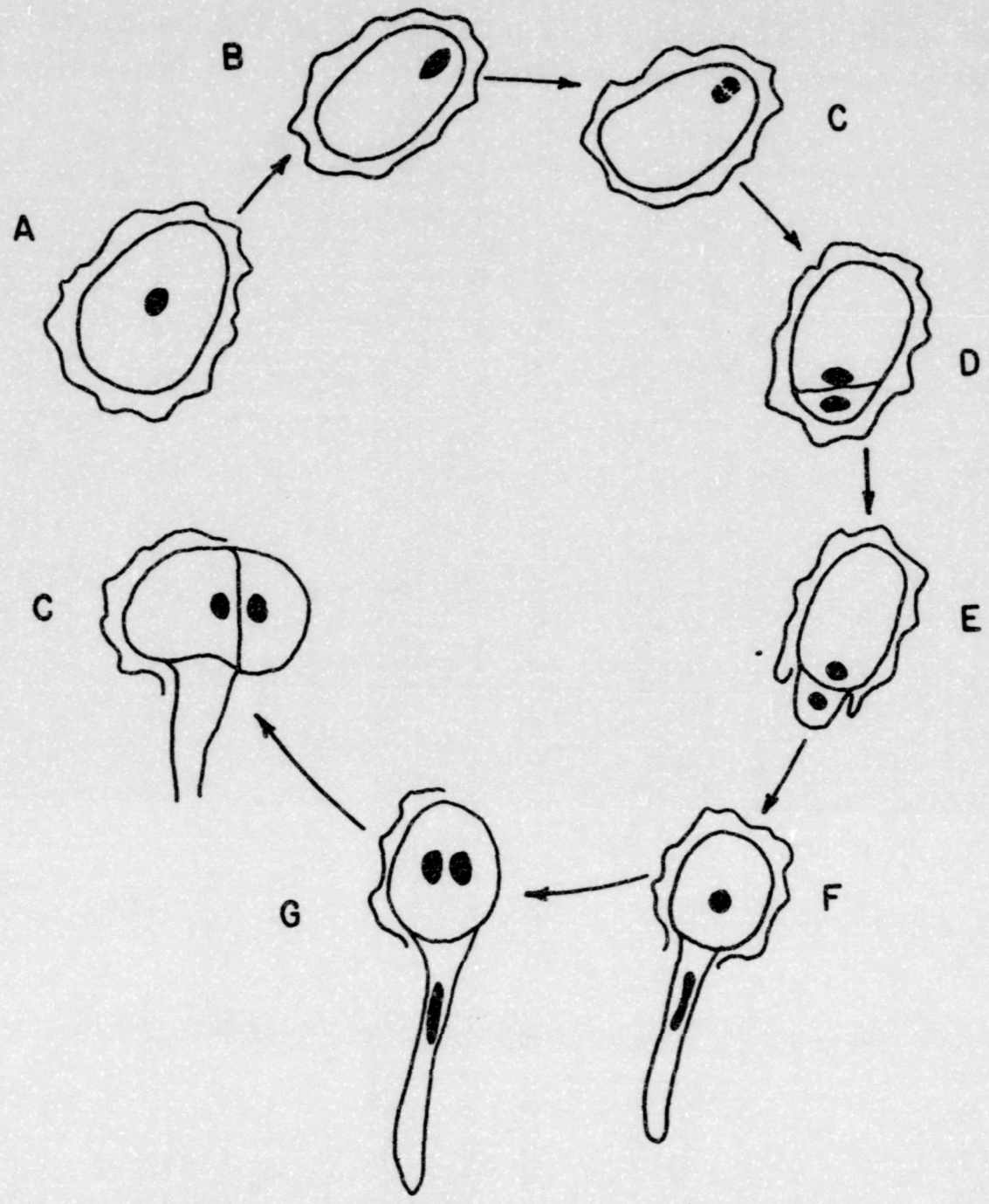

70
50
30
10
GERMINATING CELLS (%)
Red
White
9
27
45
63
81
99
TIME (HR)

observed as two deeply staining pink spots. The events leading to and including the first two nuclear divisions can be readily observed (Fig. 4). The nuclei were not normally seen until 48 hr of culture because rupture of the spore wall was necessary for penetration of the stain. Earlier stages of nuclear movement could be seen by treating the spore with a solution of NaOCl until the spore coat ruptured. The maximum mitotic rate was found to occur between 70 and 72 hr. By this time practically every viable cell had undergone the first nuclear division.

When potassium permanganate was used as a fixative, the spore cytoplasm appeared different from that fixed with glutaraldehyde or osmium tetroxide. The large lipid droplets noted in the ungerminated spore appeared as holes with a dark, irregular border. Cytoplasmic ground substance appeared light, and no ribosomes or chromatin could be seen with this strong oxidizing fixative. Endoplasmic reticulum and chloroplasts could be located. The standard fixative for plant tissue utilizing 6% glutaraldehyde for 4 hr plus a 2 hr post-fixation in 2% OsO_4 was also tried. Figure 5 shows an example of the results of this procedure. Lipid droplets were partially preserved and occasionally appeared to be bounded by a membrane. The quantity of these droplets appeared less than with the final fixation method chosen. The exine and its associated sculptured perine were readily observed. Cytoplasmic detail was not well preserved as the only organelles easily identified were the chloroplasts with included starch grains.

Figure 4. Diagram of early gametophyte development of Woodwardia virginica.

A. Non-germinated spore with a centrally located nucleus.
B. Nuclear migration to proximal edge. Before mitosis the nucleus becomes spindle shaped.
C. First mitotic division. The location of the nucleus results in a division which unequally divides the cell.
D. After mitosis, cytokinesis divides the cytoplasm into a small rhizoidal initial and large basal cell. A new cell wall is laid down between the two newly formed nuclei.
E. Growth of the rhizoidal initial causes rupture of the spore coat. At this time the two nuclei are easily stained.
F. The rhizoidal initial elongates. The nucleus migrates down the rhizoid from its original position near the basal cell. The basal cell nucleus returns to a more central position.
G. The basal cell nucleus undergoes a second division.
H. A cell wall develops between the two nuclei resulting in the formation of a basal cell and a protonemal cell. The protonemal cell would continue to divide to form the thread-like protonema.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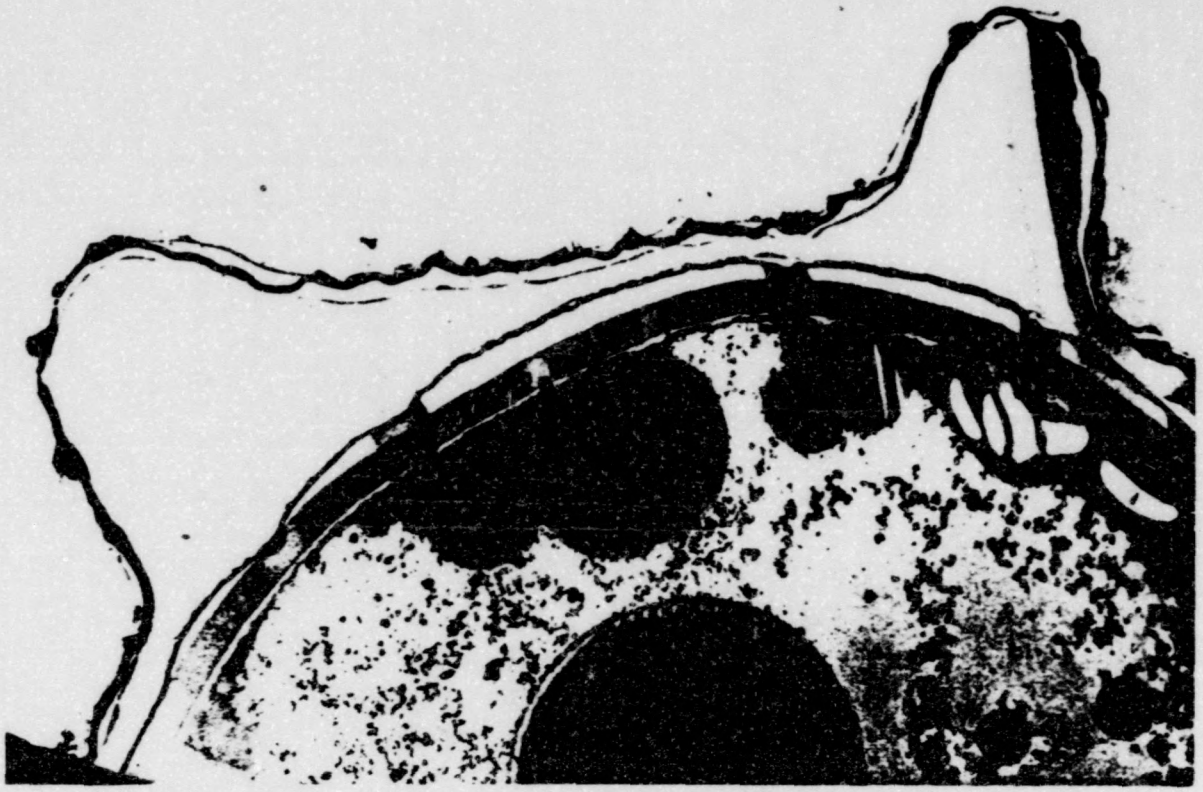
D
E
F
G
C

Figure 5. Fixation with 6% glutaraldehyde post-fixed with 2% OsO_4. Note especially the poor preservation of cytoplasmic contents. The lipid bodies (L) appear to be bounded by a membrane. Starch grains are apparent in the chloroplasts (C) as white refractile bodies. Chatter is seen as thick and thin bands in this thin section.

Figure 6. Simultaneous glutaraldehyde and osmium fixation. This procedure preserves the cell contents better than the previous example. Lamellae and starch grains within the chloroplast (C) are retained. Lipid bodies (L) and mitochondria (M) can be observed in the cytoplasm. In this germinated cell dense staining granular material is located around the organelles and between lipid droplets.



Simultaneous glutaraldehyde-OsO_4 fixation was also ineffective. Results of this procedure were superior to the previous examples (Fig. 6) but were not considered satisfactory for the intended study. The final selected procedure gave superior results to all others examined.

Spurr embedding plastic (Spurr 1969) did not cure properly and the resulting soft blocks were difficult to section without chatter and folding (Fig. 7). Epon 812 proved to be a successful plastic. Only occasional chatter or folding was noted. Both plastics preserved cytoplasmic contents equally well.

Electron Microscopy

The Spore and Spore Coat--The spores of Woodwardia virginica were 15 x 23 μm in size. The spore coat was highly structured, rising in irregular folds and surface projections. Figure 8 shows a scanning electron micrograph of a spore rupturing to release the developing rhizoid initial. A simple ridge marked the point where the coat split during germination. The spore coat consisted of several layers which comprised a substantial proportion of the dry mass of the spore. The outer surface of the exine exhibited a complex characteristic pattern of ridges and furrows and was surrounded by a thin membranous exosporium or perine (Fig. 9). Layering of the exine was recognizable by differences in electron density, there being three distinct zones (Fig. 10). The cell wall, which was considered to be the spore intine,

Figure 7. Use of Spurr embedding plastic. The fixation procedure was as in Figure 6. Note the folding of the plastic (f), chatter (c) and distortion of cytoplasmic contents. The arrow shows the location of a knife scratch.

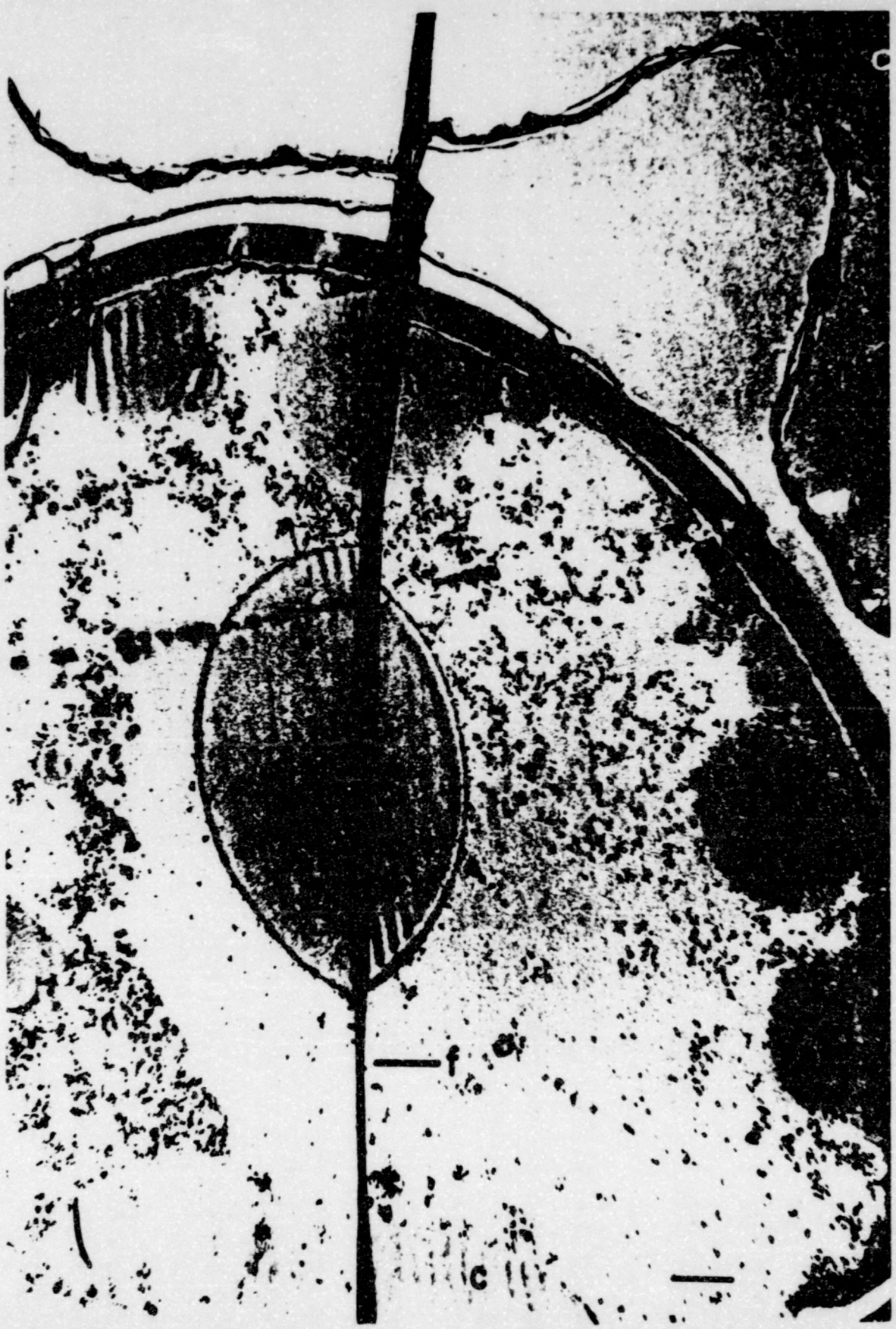

f
c

Figure 8. Scanning electron micrograph of a spore of Woodwardia virginica. The spore has germinated as evidenced by the split along the raphe (R). Note also the sculpturing of the spore surface which can be used as a taxonomic tool to identify individual fern species.

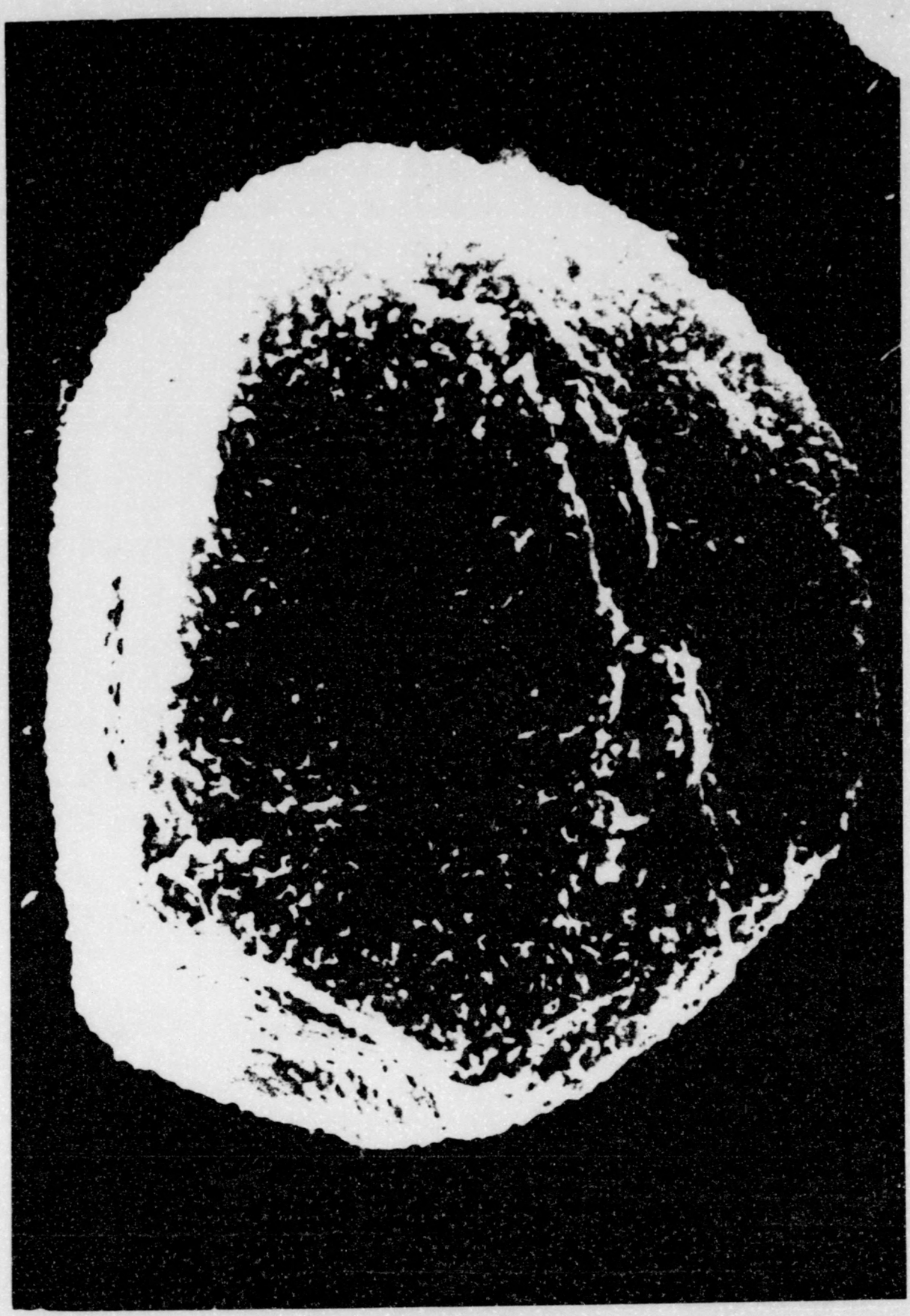


Figure 9. Transmission electron micrograph of spore showing perine (P) with projections on the outer surface. In this germinated spore large vacuoles are present as a result of utilization of stored lipid droplets.

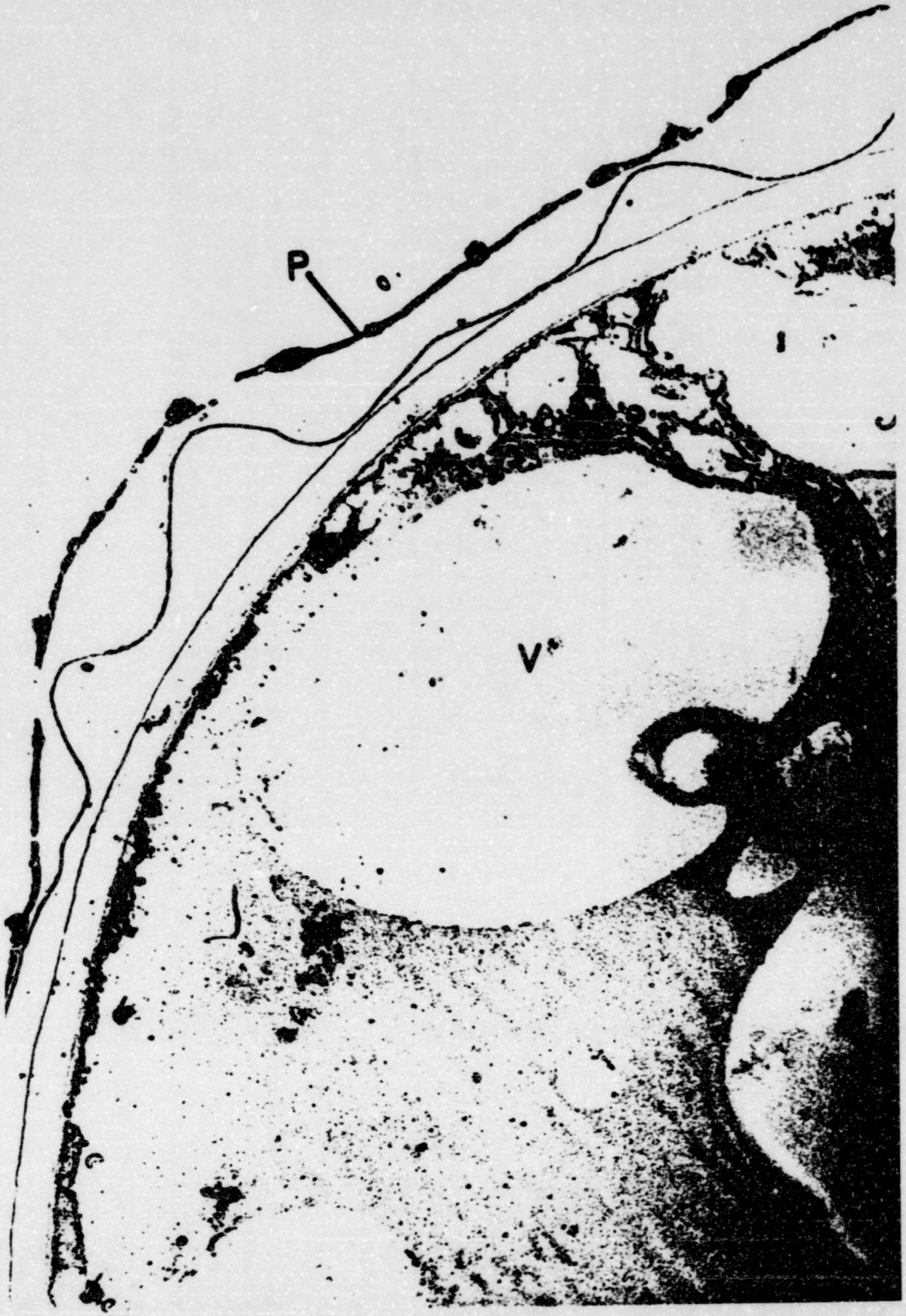

P
V

Figure 10. Electron micrograph showing layering of the exine. Three distinct layers can be identified: (1) An electron dense outer layer 90 nm in thickness. This layer has broken away in places (arrow). (2) A layer of uniformly fibrous material that is believed to cause major difficulty with fixation and embedding. (3) An interior dense layer that appears similar to layer 1. The new cell wall of the developing spore would form immediately interior to this layer.

could be convoluted with invaginations into the cell (Fig. 11). Dense staining material is frequently found between the intine and the new cell wall formed before germination (Figs. 11, 12).

The Imbibed Spore--The major component of the cytoplasm of the non-germinated spore was the mass of lipid droplets (Fig. 13). These range in size from 1 to 6 μm. The center of the non-germinating spore appeared to be composed of a single lipid mass 20 μm in size. It could not be determined if a true membrane existed around the periphery of each drop. There appeared to be some polarity in the spore as the larger lipid bodies (other than the central one) were located predominantly on one end, and the smaller lipid structures and organelles were at the opposite end. No abundant protein granules such as those found in Matteuccia struthiopteris (Gantt and Arnott, 1965) were observed. A small number of Golgi bodies, mitochondria, microbodies and proplastids could be seen among smaller peripheral lipid bodies. A small number of the proplastids had developed membranous structures, and starch granules could be seen. Free ribosomes as well as rough endoplasmic reticulum were also present. The nucleus appeared rounded and centrally located with an intact nuclear membrane delimiting the outer boundary. A prominent nucleolus or nucleoli could be seen. Chromatin at this stage was very diffuse, and a limited number of dense chromatin masses were scattered throughout the nucleoplasm.

Figure 11. Invagination of the cell wall (spore intine) into the cytoplasm. Dense staining granular material (DM) frequently fills the exterior of the invaginated area. An extension of the cell wall (arrow) appears to divide the region into two areas.

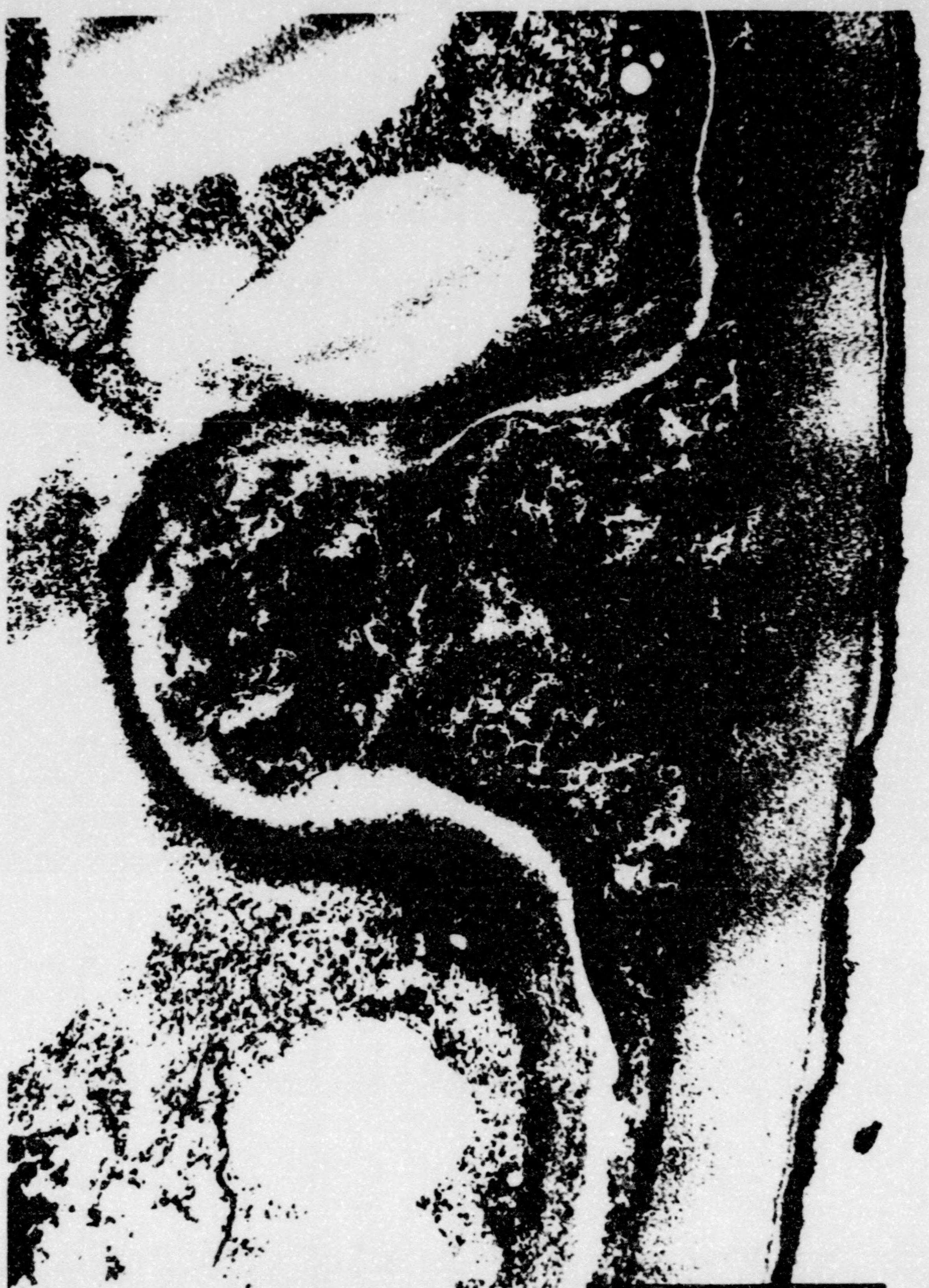


Figure 12. Dense staining material (DM) between exine and intine. A granular dense material is often located near the region where the exine fragments during germination.

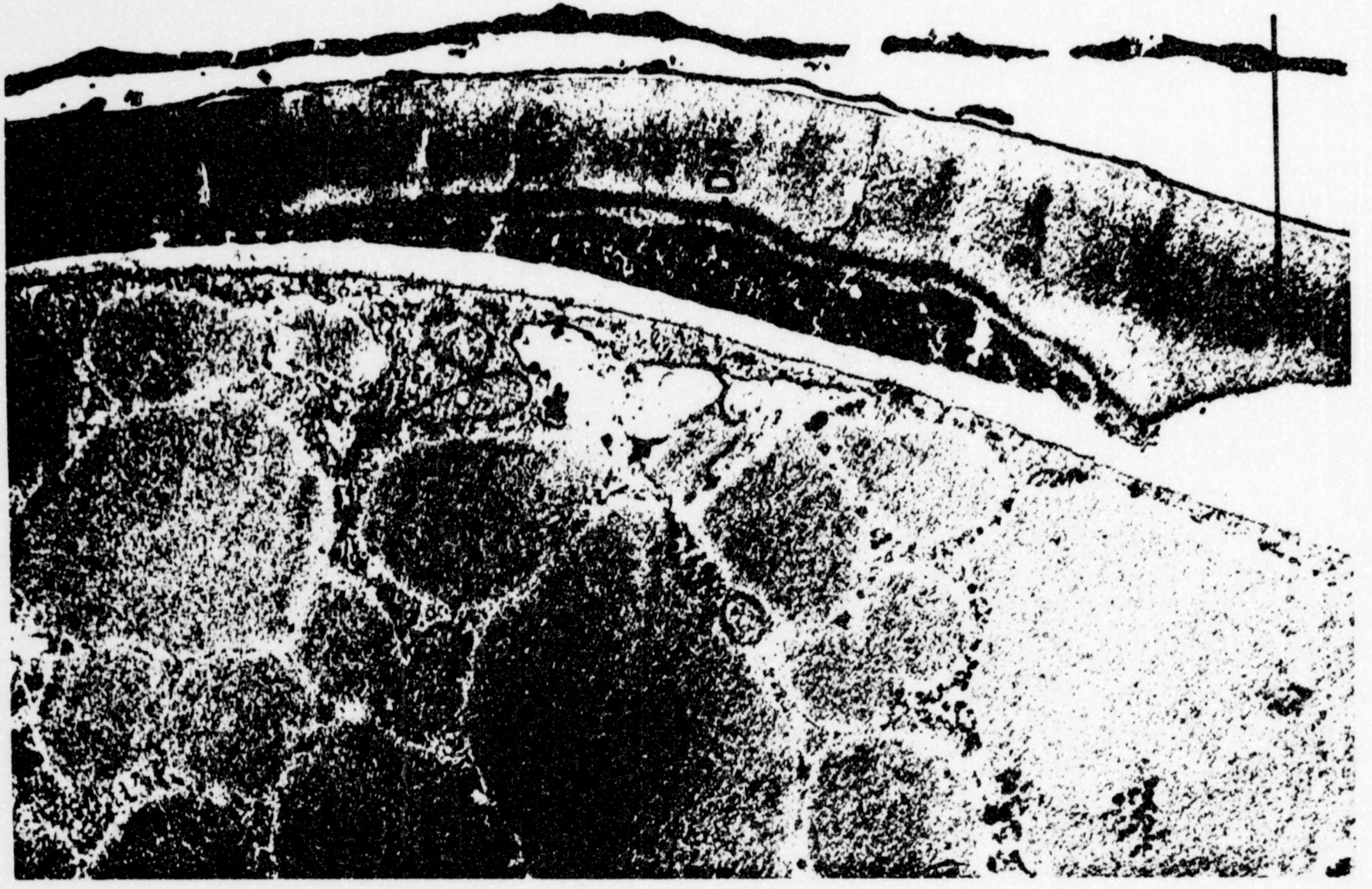
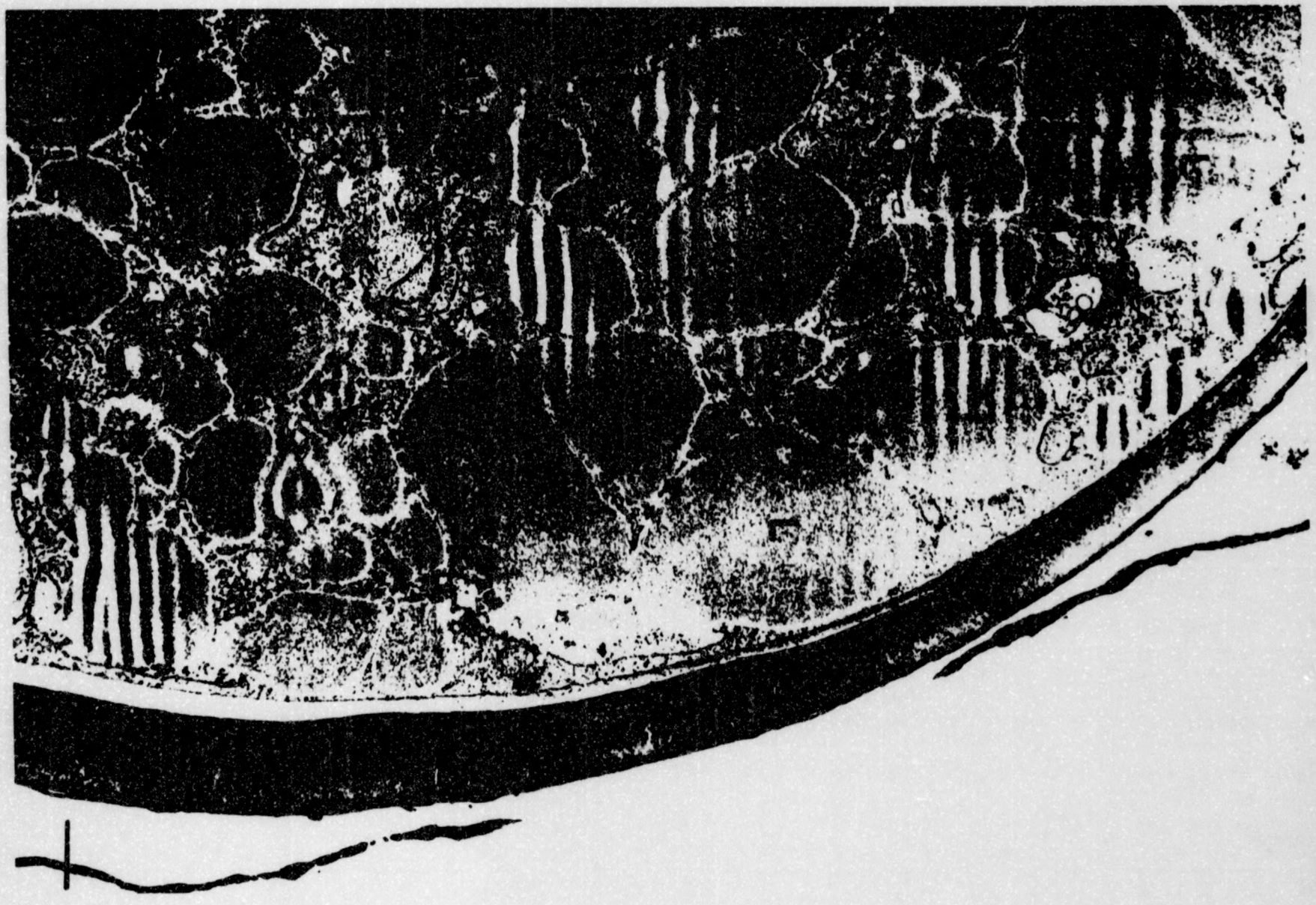


Figure 13. Lipid droplets (LD). The entire peripheral area of cytoplasm is filled with lipids. These vary in size from 1 to 6 μm. Larger droplets are usually located near the center and the smaller lipid bodies are found near the periphery.



The Germinating Spore--During early germination, fat bodies were the prominent feature (Fig. 14). With glutaraldehyde-osmium fixation they were spherical, had uniform contents and varied greatly in size, 1 to 6 μm. It was still not clear if they were bound by a membrane. Usually, no bounding layer could be detected, but on occasion when lipid droplets were closely adpressed, a dark line was visible at their periphery. When this was the case, it generally did not extend completely around the globule. When $KMnO_4$ was used as a fixative, lipids showed an irregular electron opaque margin with a transparent core.

Organelles identified as microbodies were bounded by a single membrane (Fig. 15). They were usually spherical but occasionally were elongate or dumbbell-shaped in profile. A granular matrix with varying density was seen. The microbodies were never frequent, but they were more common in actively dividing spores. One or more dense inclusions were occasionally seen. The association of microbodies with elements of rough endoplasmic reticulum was not a constant feature.

No peculiarities were noted with regard to the Golgi apparatus which would distinguish it from other plant cells (Figs. 14, 15). The Golgi bodies generally consisted of a stack of about six cisternae from the ends of which vesicles were produced. Two types of Golgi vesicles were seen, one type had a clear matrix and the other type had a denser one.

Figure 14. The germinating spore. The region of the nucleus contains a normal complement of cell organelles. Lipid droplets (L) decrease in size as the cell develops. Proplastids (Pp) contain starch granules (SG). Vacuoles (V) are present in areas where the lipid droplets have been utilized. Golgi (G) are frequently associated with the lipid droplets and the nuclear membrane. Both electron dense and electron transparent Golgi vesicles can be found originating from the same Golgi. Mitochondria (M) are numerous in the area surrounding the nucleus.

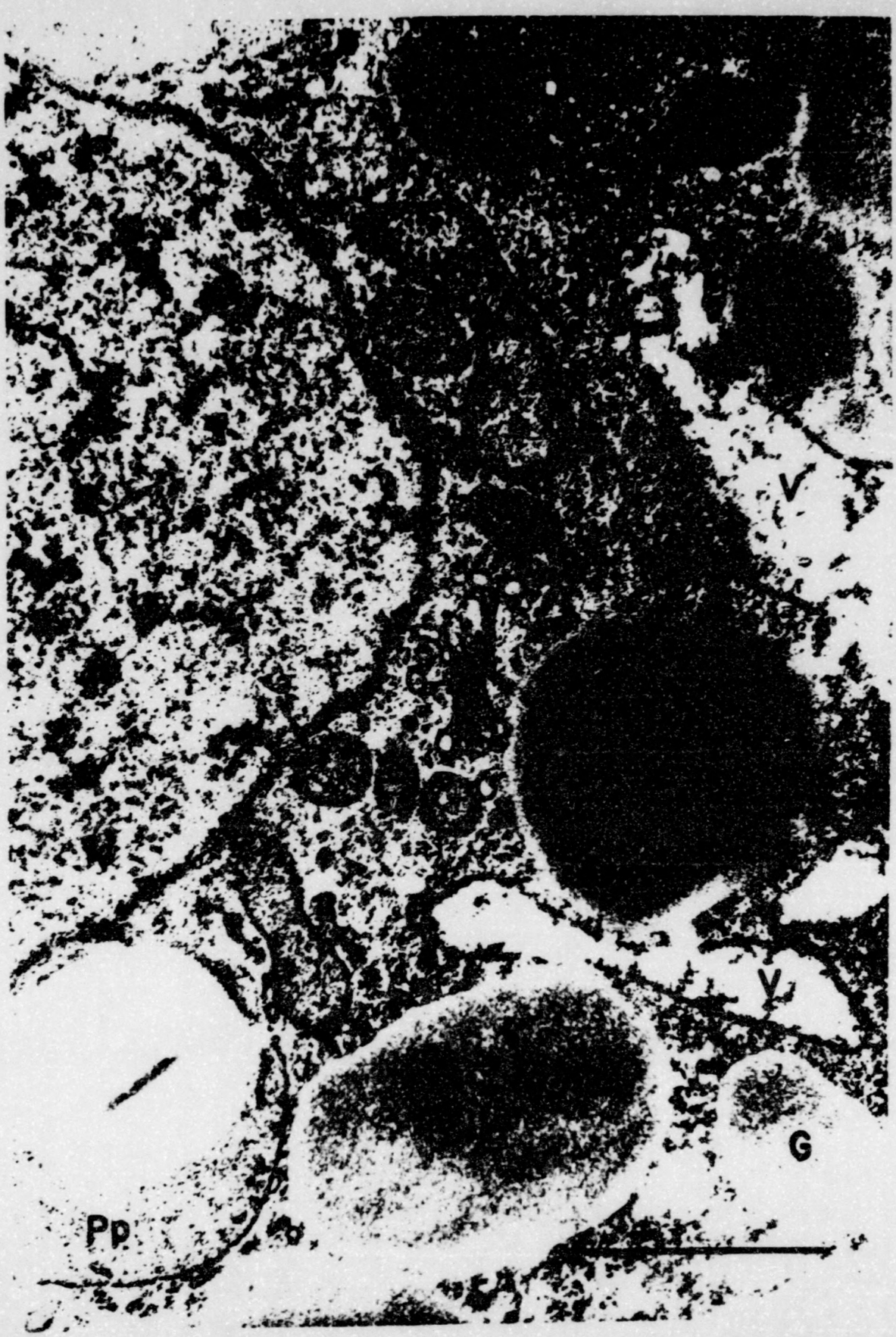
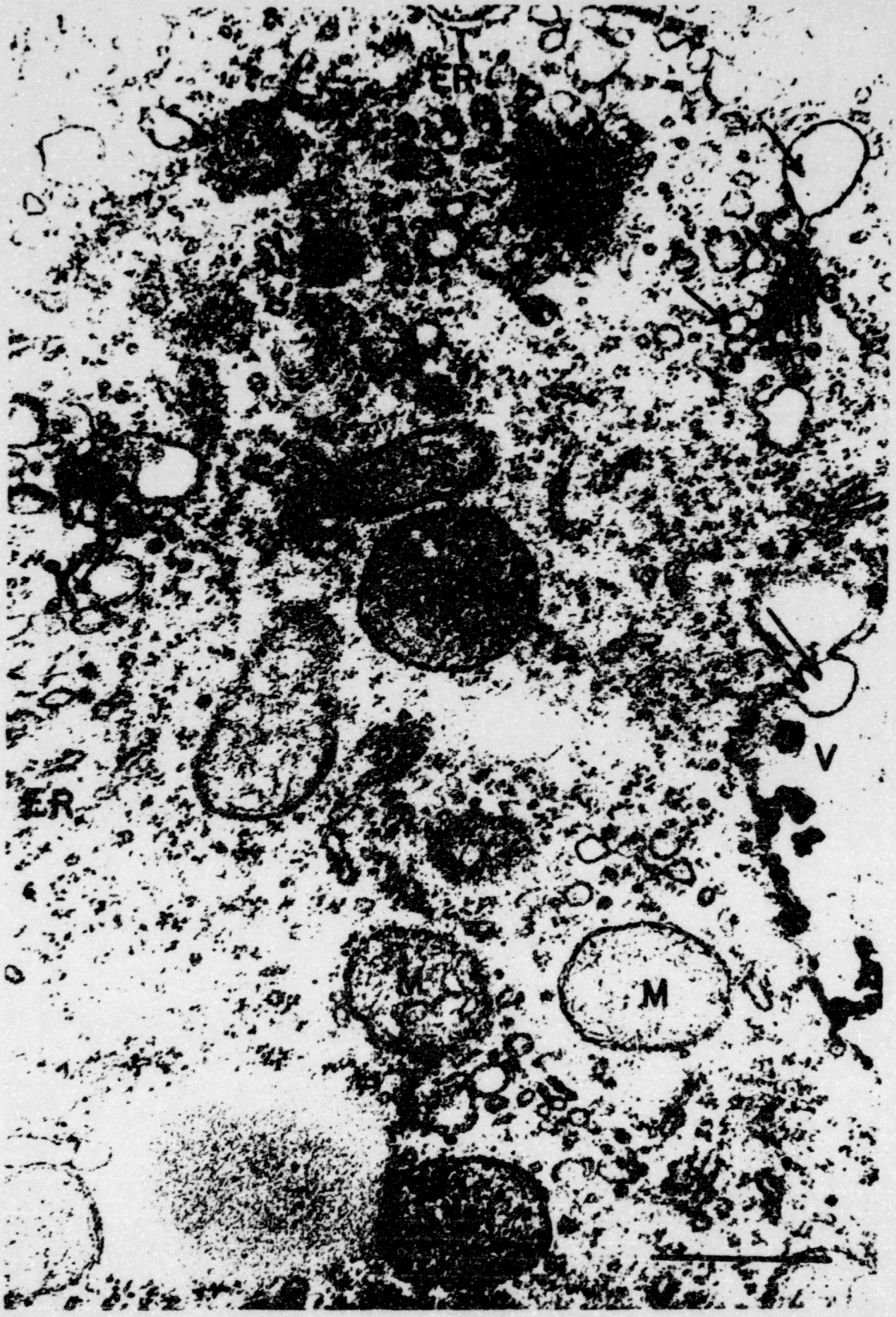

V
V
G
Pp

Figure 15. Microbody. Microbodies (MB) bounded by a densely staining membrane are frequently seen located near the nucleus in the germinating spore. Note the Golgi (G) with their dense and electron transparent vesicles (arrows). A Golgi vesicle (double arrow) appears to be fusing with a large vacuole (V). Both dense and fibrillar material can be seen in the cavity of the vacuole. Numerous ribosomes arranged in spirals, aggregates or free are present. Most of the endoplasmic reticulum (ER) has ribosomes on its external surface. Mitochondria (M) are also numerous at this stage of development.


ER
ER
V
M

The endoplasmic reticulum was usually seen in short lengths and was almost always covered with ribosomes.

Mitochondria appeared to be typical of those found in higher plants. They were oval to round with dimensions approximately 1 to 1.5 μm (Fig. 15, 16). The cristae appeared platelike or rounded. Mitochondria were more numerous in the region of the nucleus but were distributed more sparsely throughout the cytoplasm.

A readily observed effect of light initiation was the rapid increase in starch build-up in the developing chloroplasts (Fig. 16). Each chloroplast contained one to several starch grains. Starch grains were generally elliptical and up to 2 μm in length. Their presence contributed to the "loose" appearance of the chloroplast. These grains frequently had a less dense outer layer and a more dense mottled matrix. As germination progressed the number of membranes within the chloroplast increased. By the time mitosis occurred the chloroplasts were well developed (Fig. 17). The plastids were generally elliptical, measuring 1.5 x 3 μm. The internal organization appeared typical of higher plants--i.e., a well ordered arrangement of grana connected by intergranal lamellae running in the long axis of the plastid. The morphology differed, however, in that the thylakoidal arrangement tended to be rather loose, somewhat resembling that in Isoetes (Paollilo, 1962). The grana were variable in number, ranging from two to eight or more thylakoids. Thylakoids

Figure 16. Mitochondria. Oval to round shaped mitochondria (M) with plate-like or rounded cristae are located around the nucleus. The chloroplasts (C) have further differentiated and contain grana lamellae. The size of the starch granule (SG) within the chloroplasts has increased. Both light and dark Golgi vesicles (arrows) appear associated with lipid droplets (L).

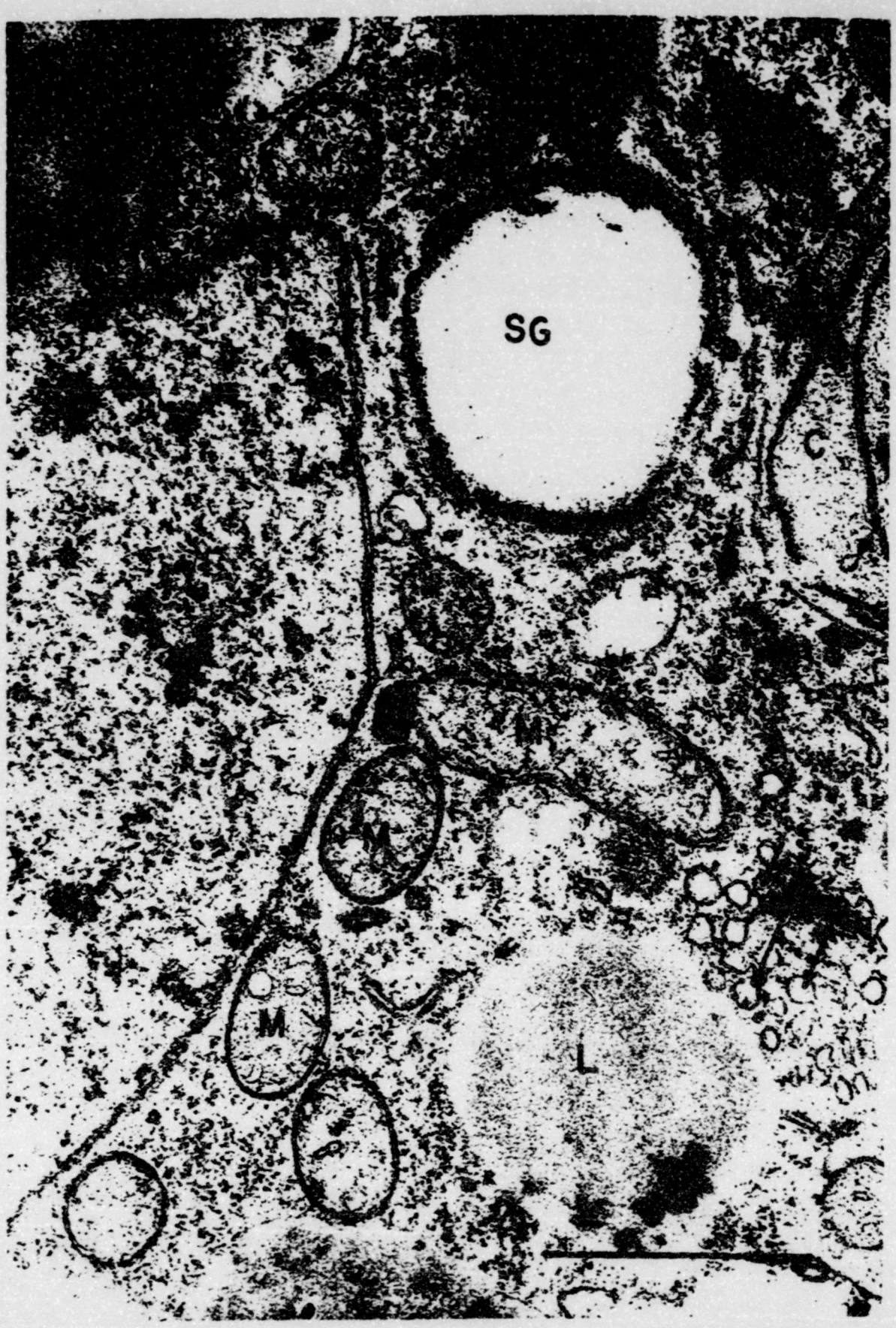

SG
C
M
M
L

Figure 17. Mature chloroplast. By the time mitosis occurs the chloroplasts (C) are well formed and most contain starch granules (SG). Chloroplasts frequently aggregate in groups of three or more near the periphery of the cell. Mitochondria (M) and Golgi (G) are less frequently observed. The lipid bodies (L) appear less dense. A fine granular deposit (possibly artifact) is located in the center of many of the larger lipids.


C
SG
L
L
SG

in Woodwardia are lamellar, not tubular as seen in some pteridophyes. Few interconnecting lamellar membranes were seen.

Ribosomes were found in locations normal for plant cells. They were either bound to the endoplasmic reticulum or were more commonly found free in the cytoplasm (Fig. 15). The most common configuration was that of small groups of three to ten. Larger groupings of ribosomes occasionally occurred as spirals. The magnification of micrographs used in this report made it difficult to detect ribosomes in mitochondria or chloroplasts. Ribosomes were also found associated with the outer membrane of the nuclear envelope.

Even at an early age of development small vacuoles were present in the cytoplasm between the lipid bodies largely in the peripheral region (Figs. 15, 18, 19). These increased in size and coalesced, eventually surrounding the central cytoplasmic aggregation. The interior surface of the bounding membrane contained dense aggregations.

The nucleus of the germinating spore appeared spherical, lobed or ellipsoidal depending on the stage of germination. A spherical (Fig. 20) or lobed appearance (Fig. 21) was more commonly associated with a nucleus that had not begun to migrate or with one that had completed movement to the proximal end of the cell. Cytoplasmic contents frequently deformed the nuclear surface (Fig. 22). Lipids and chloroplasts usually were observed in close association with these deformities.

Figure 18. Vacuoles. Even at an early age, numerous vacuoles (V) are present. These vacuoles increase in number and size with maturity. Golgi vesicles (arrows) appear to contribute to this increase in size. The dense staining material on the interior of the vacuolar membrane is of unknown origin.

V
V

Figure 19. Vacuole fusion. A zone of fusion (arrow) appears when two of the larger vacuoles join together. This process would continue with growth as lipids are utilized until the major component of the basal cell would be vacuole.


R

Figure 20. Spherical germinating nucleus. The nucleus (N) of a germinating cell migrates to a proximal position before dividing. In this fern condensation of chromatin (Ch) appears to occur before the nuclear membrane breaks down. The nucleolus (Nu) can be divided into two regions, a granular pars granulosa (Pg) and a fibrillar pars amorphora (Pa). The two dense areas in the middle are probably chromatin granules. The nuclear membrane is intact at this stage of germination.

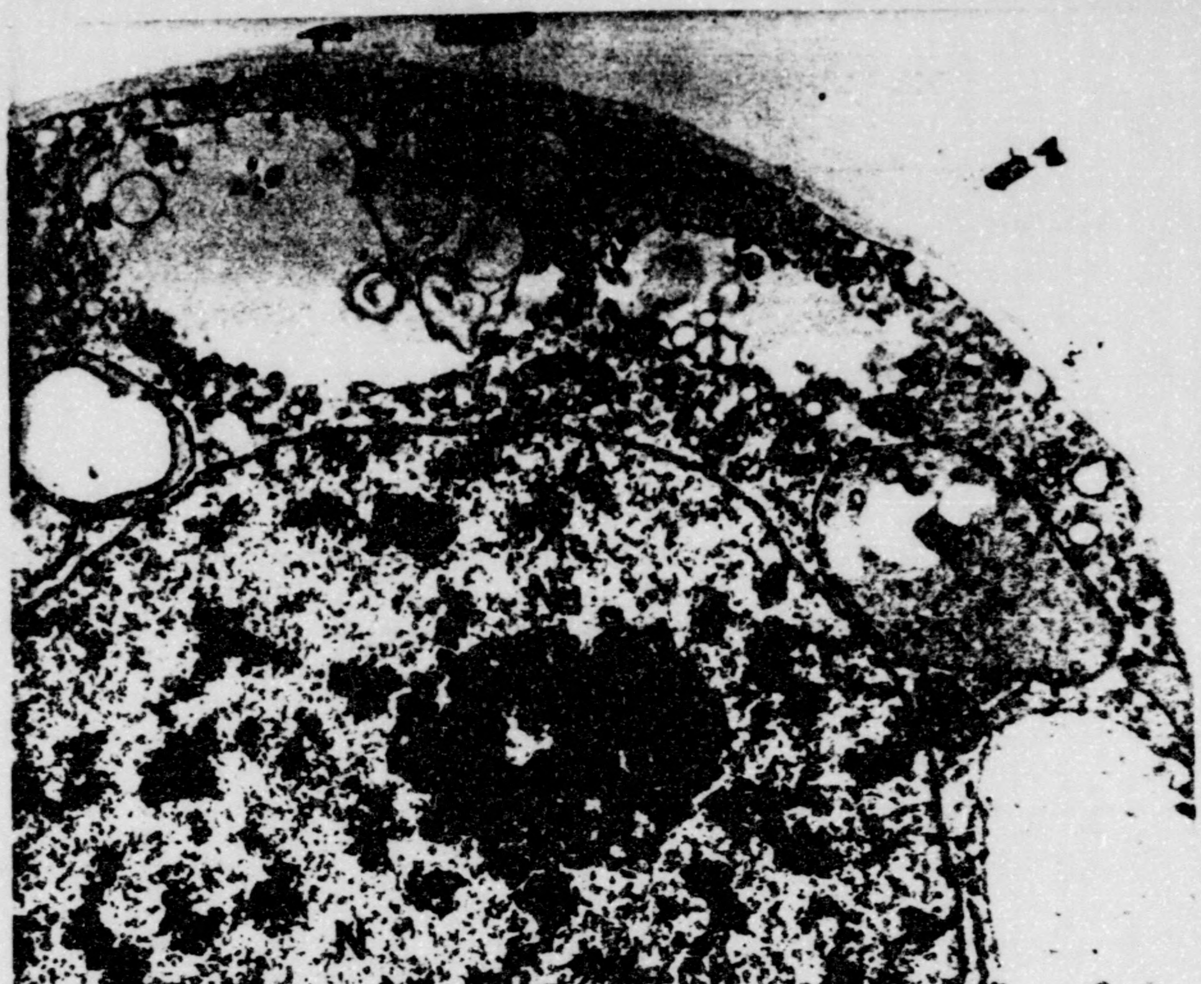
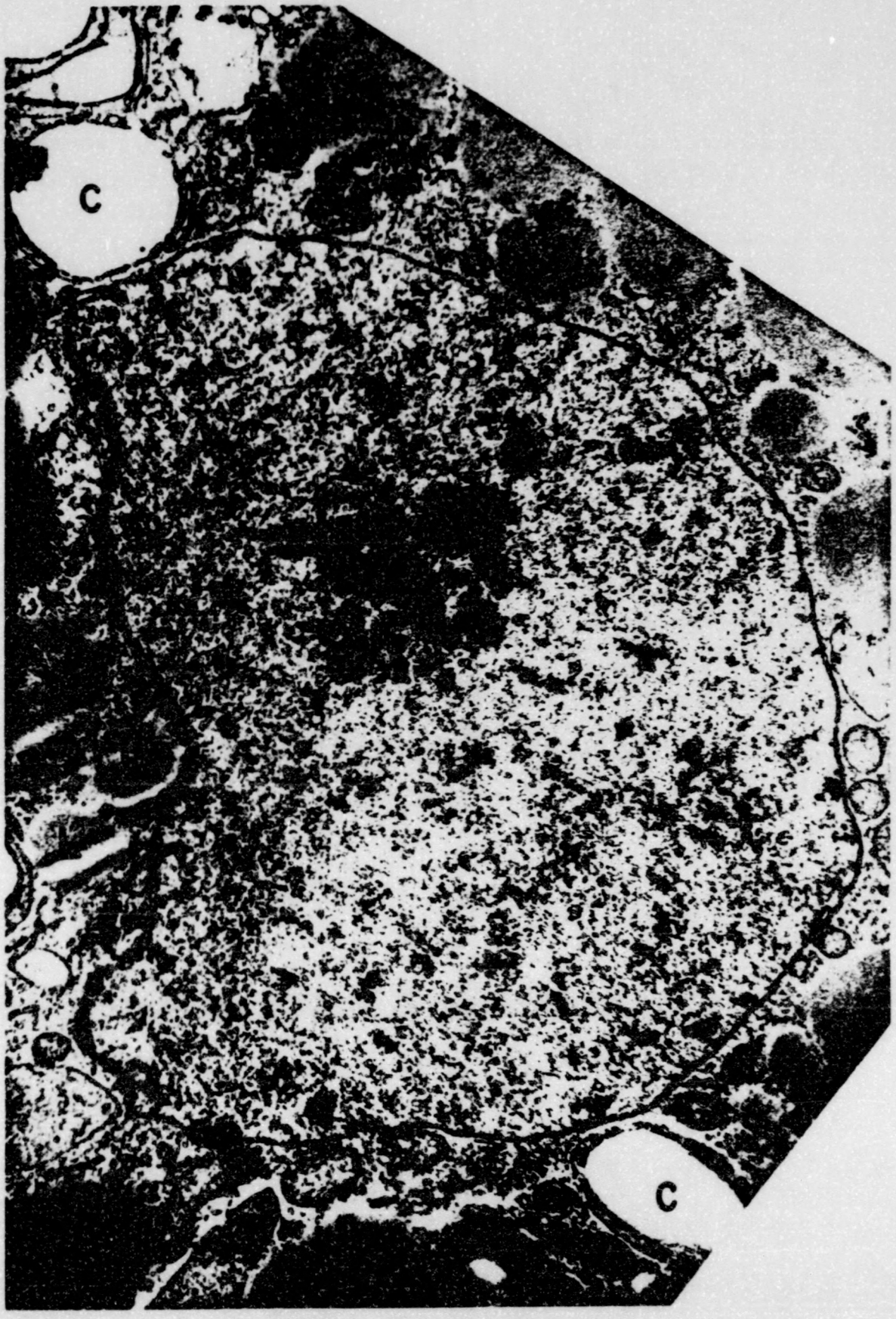


Figure 21. Lobed germinating nucleus. Serial sectioning frequently showed that the nucleus was lobed instead of spherical. Spherical or lobed nuclei have the same general ultrastructural features.

Figure 22. Nuclear deformation. Cytoplasmic contents frequently deform the nuclear surface. The most frequent structures causing deformation are lipids (L) and chloroplasts (C). An occasional association between these structures and the nuclear envelope can be detected.


C
C

The nucleus was surrounded by a typical double membrane. The outer surface of the envelope was occasionally connected with an element of endoplasmic reticulum. Nuclear pores were common and appeared to be closed by a single membrane. The interphase nucleus was highly granular. Scattered irregular aggregations of chromatin were a consistent feature (Fig. 23). One or more nucleoli were present.

When located midway between the center and the periphery of the cell, the nuclei showed a spindle shape, with a narrow inward end and a broadened face in the direction of migration (Fig. 24). Once migration was completed the nucleus again became spherical before division occurred. Shortly after migration was completed, the chromatin began to condense into dark staining aggregates (Fig. 20). The nucleoli could be recognized as being composed of two distince regions, a fibrillar and granular zone corresponding to the pars amorphora and pars granulosa first noted in animal cells (Marinozzi 1964).

The appearance of the basal cell nucleus shortly after germination is shown in Figure 25. A distinctive feature of this nucleus was an extremely large nucleolus. Pars amorphora and pars granulosa areas could easily be detected. The chromatin appeared very diffuse and scattered at this time.

Rhizoid--Shortly after nuclear migration to the proximal end of the spore, mitosis occurred producing two cells of unequal size. The larger basal cell later divides to form the green prothallial filament. The smaller rhizoidal initial

Figure 23. The interphase nucleus. A consistent feature of this nucleus is the scattered aggregation of chromatin. Most of the cytoplasmic organelles are located around the periphery of this nucleus. Proplastids (P) are commonly present.

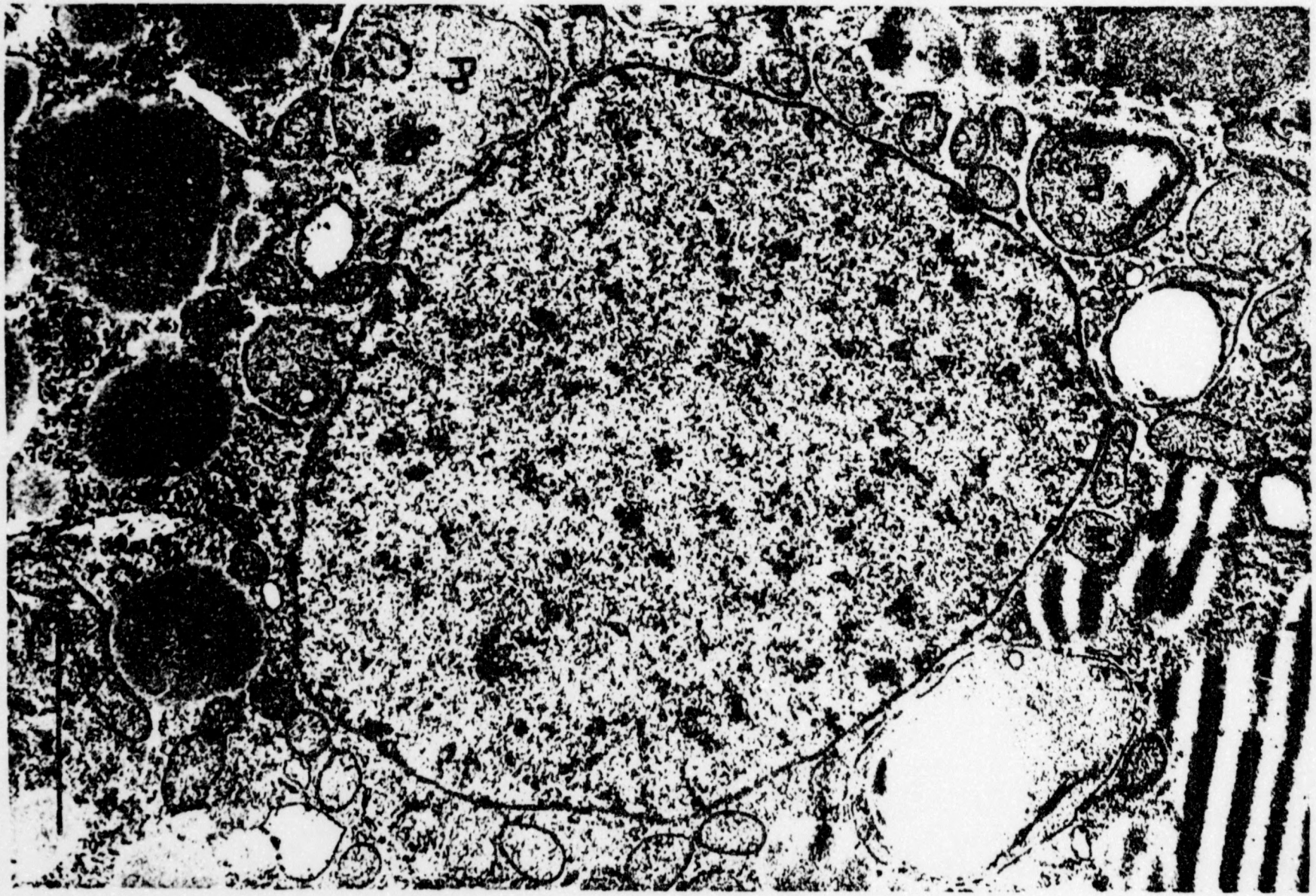

P
P

Figure 24. Migrating nucleus. During migration from a more central position to the proximal end of the cell the nucleus assumes an amoeboid shape with a broadened proximal and a tapered distal end. Once migration is complete the nucleus will again assume a spherical shape. Most of the cytoplasmic organelles follow along this path of migration.

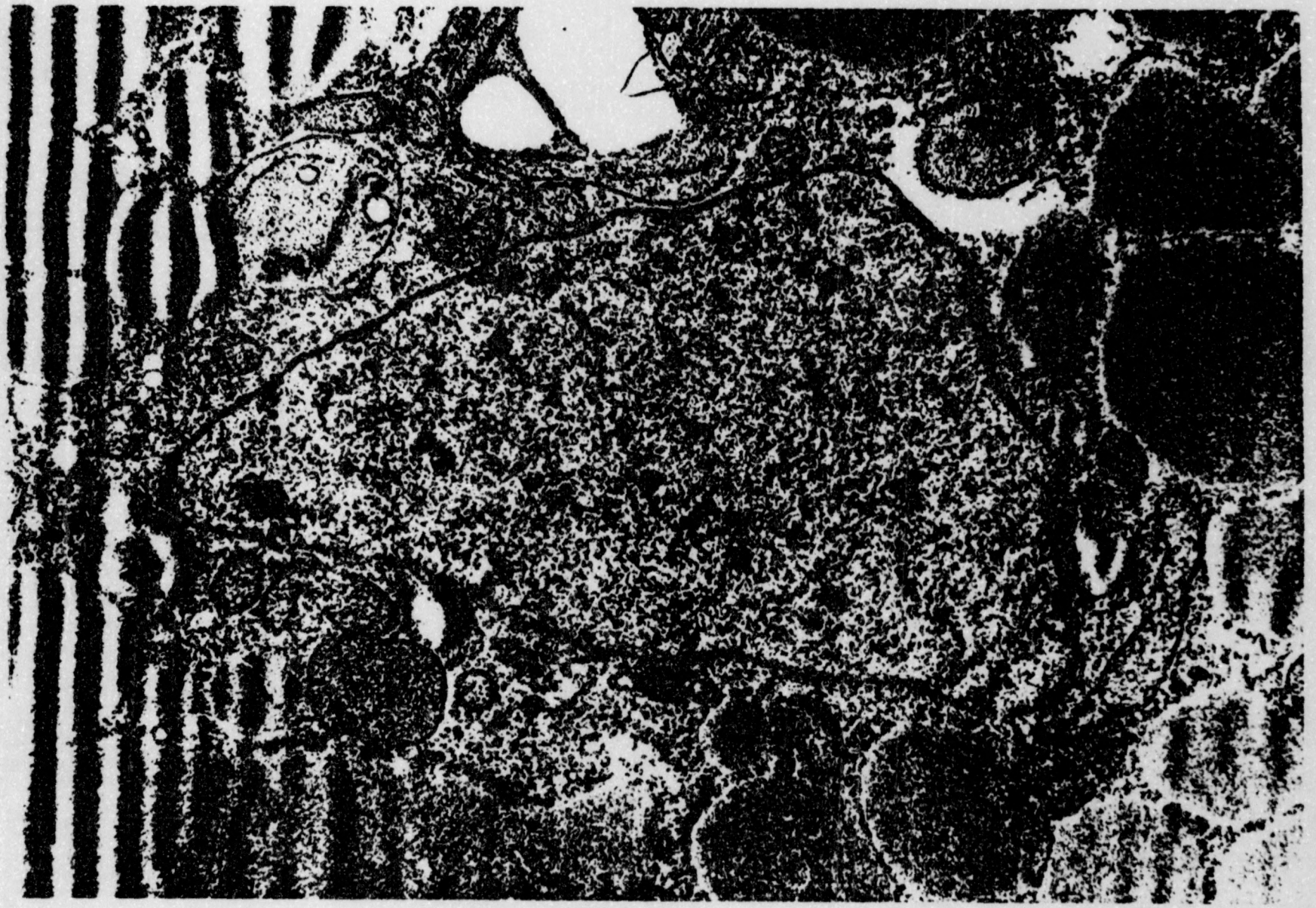
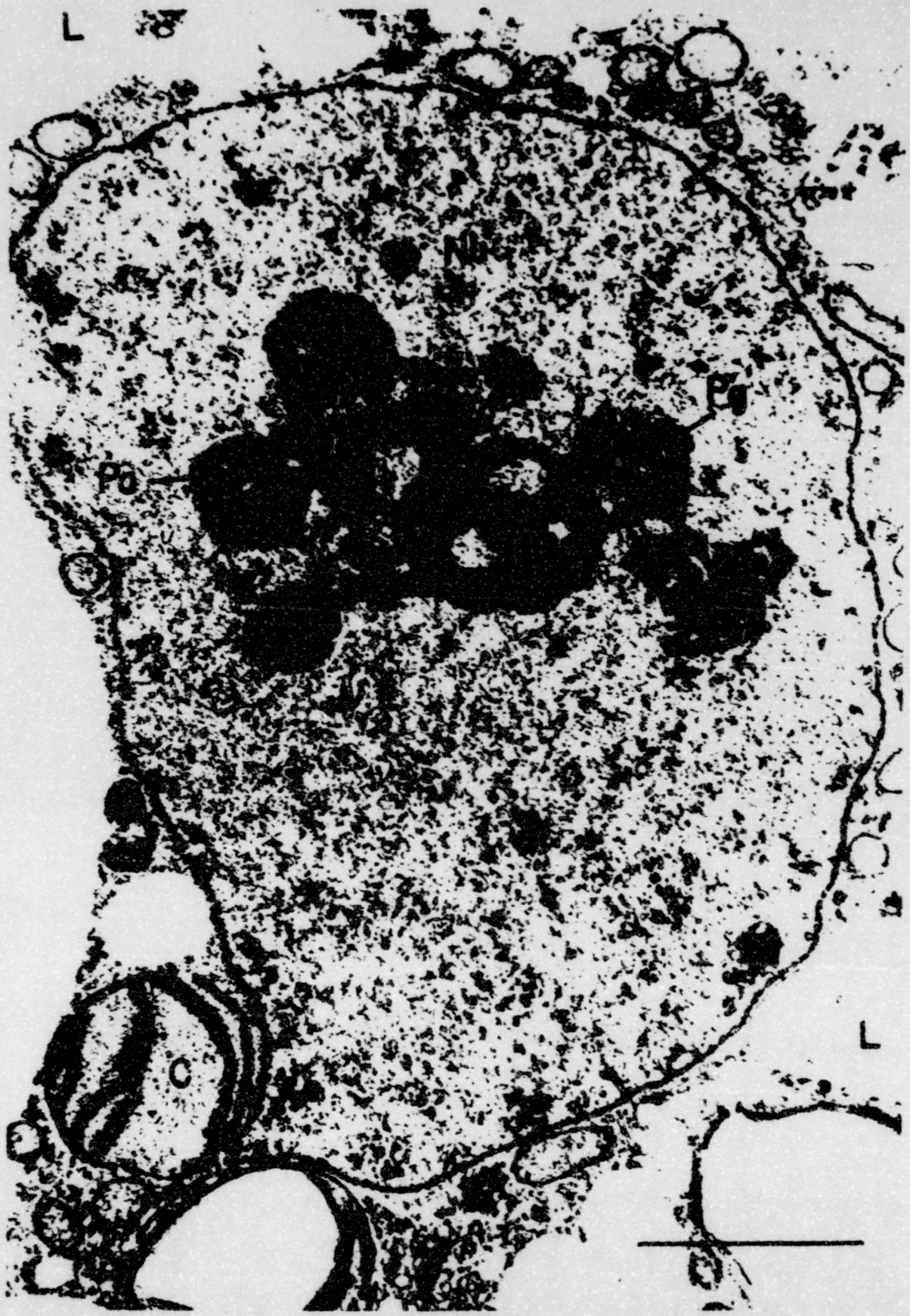


Figure 25. Basal cell nucleus after germination. The nucleolus (Nu) distinctly shows two areas, pars amorpha (Pa) and pars granulosa (pg). Chromatin masses are less distinct in this recently divided cell. Chloroplasts (C) are well formed and many of the former lipid droplets (L) appear as open spaces.


L
Pd
Pd
C
L

increases in length, ruptures the spore coat along the raphe and becomes the rhizoid. SEM micrographs of the exterior appearance of the rhizoidal cell are shown in Figures 26 and 27. The basal cell remained enclosed by the spore coat during germination.

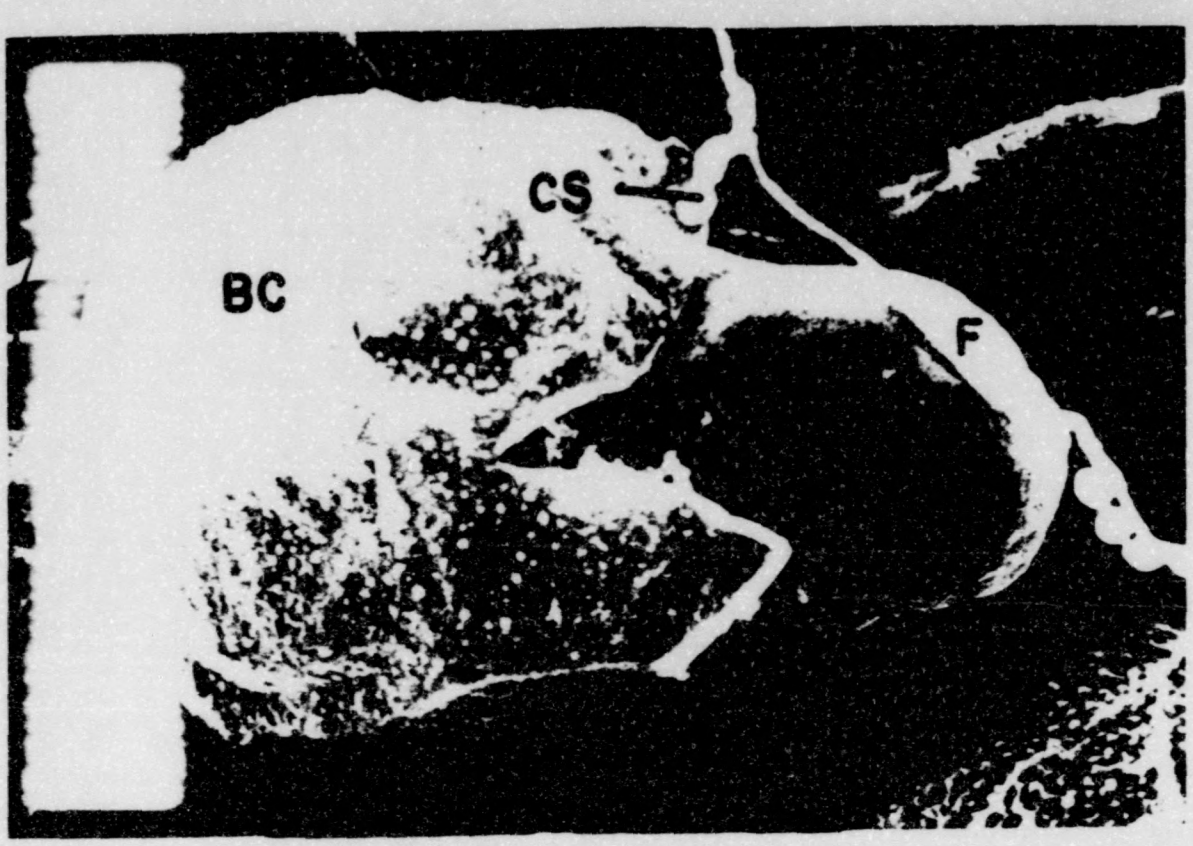
The nucleus of the rhizoidal initial was dome shaped with flattening along the newly formed cell wall separating the rhizoid from the basal cell (Fig. 28). Dense chromatin masses could be seen in the nucleoplasm but no nucleoli were observed. There did not appear to be any preferential distribution of cell organelles. The cell wall cut across a section of the basal cell resulting in the indiscriminate distribution of organelles into the two cells. Numerous small vacuoles could be seen in the rhizoid cytoplasm. Plastids and other organelles were present during this stage.

After rupturing the spore coat, the rhizoid initial underwent rapid growth to become the rhizoid. In longitudinal section the fusion of many smaller vacuoles could be observed (Fig. 29). These vacuoles contained deposits of electron dense material on their inner surface. The chloroplasts, if present, began to degenerate and became functionless. Most of the cell organelles appeared to have migrated toward the tip. Fat bodies, if present initially, quickly disappeared. Endoplasmic reticulum was often found parallel to the cell wall.

In cross section, the presence of large central vacuoles was noted (Fig. 30). The electron dense areas in the vacuoles

Figure 26. SEM of germinating spore. After unequal division two cells are formed, the basal cell (BC) and the rhizoidal initial (RI). Growth of the rhizoidal initial causes rupturing along the raphe. A fungal contaminant (F) with conidiospores (CS) can be seen along the surface of these cells. Note the sculpturing of the spore surface.

Figure 27. SEM of rhizoidal initial. The rhizoidal initial rapidly elongates to become a rhizoid. The basal cell (BC) nucleus has now migrated to a more central position. The basal cell may remain enclosed in the spore coat during much of germination.


CS
BC
F

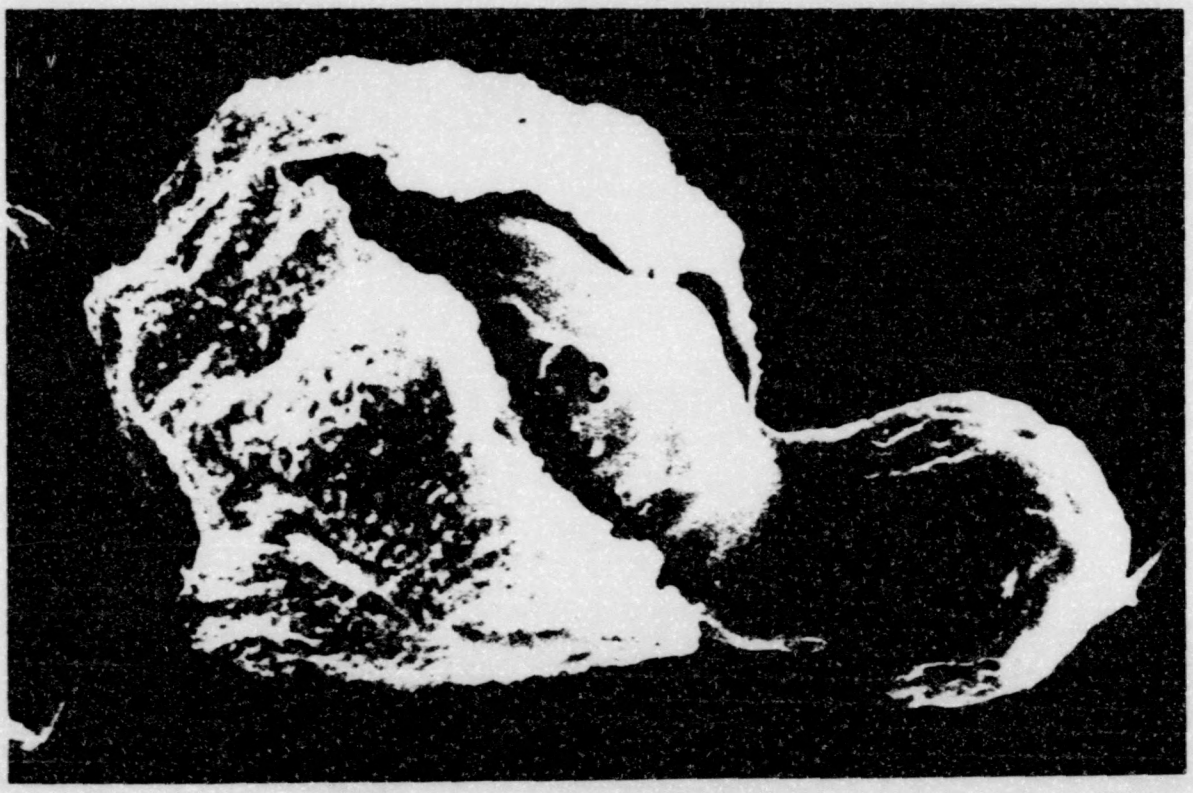


Figure 28. Rhizoidal initial. After the first mitosis in the spore a new cell wall is laid down dividing the cytoplasm into two cells, a basal cell (BC) and a rhizoidal initial (RI). The contrast in the basal cell is much more evident. The dome shaped nucleus of the rhizoidal initial remains adjacent to the newly formed cell wall (CW). All normal cytoplasmic organelles can be found in this new cell. Chloroplasts (C) will shortly degenerate and become functionless. Most organelles are located in the apical region of the cell. Small vacuoles (V) which will later fuse are seen. Most of the lipid in the basal cell has been utilized leaving large open spaces, many with a granular center.

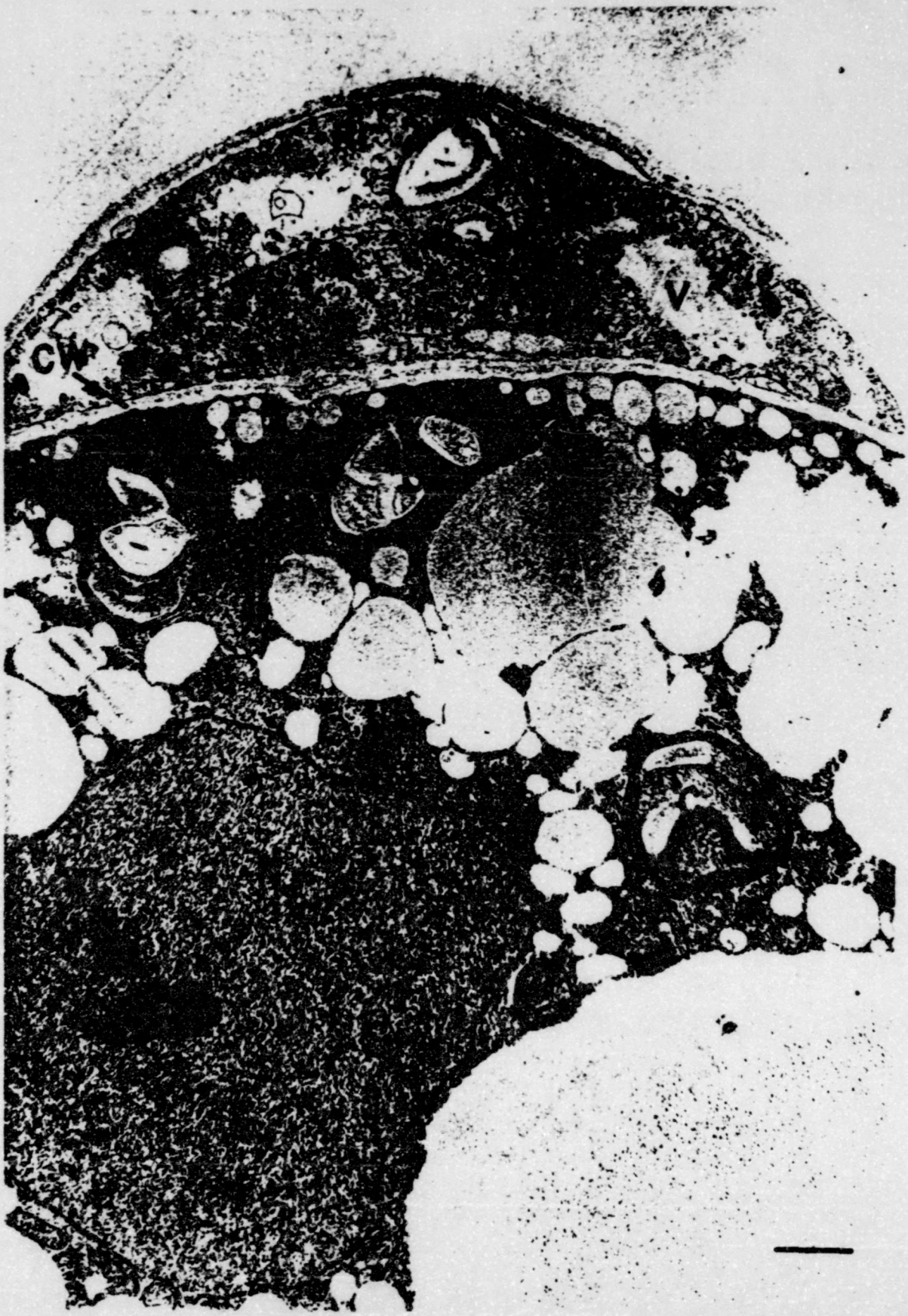

CW
V

Figure 29. Rhizoid. After rupturing the spore coat the rhizoidal initial elongates to become the rhizoid (R). Fusion of many of the smaller vacuoles has occurred to make a large, branched vacuole (V) the most prominent feature. Chloroplasts (C) will continue to degenerate until their identity will be lost. Most other organelles will migrate to the apical tip. No lipid bodies remain in the rhizoid at maturity. Well developed chloroplasts are retained in the basal cell (BC).

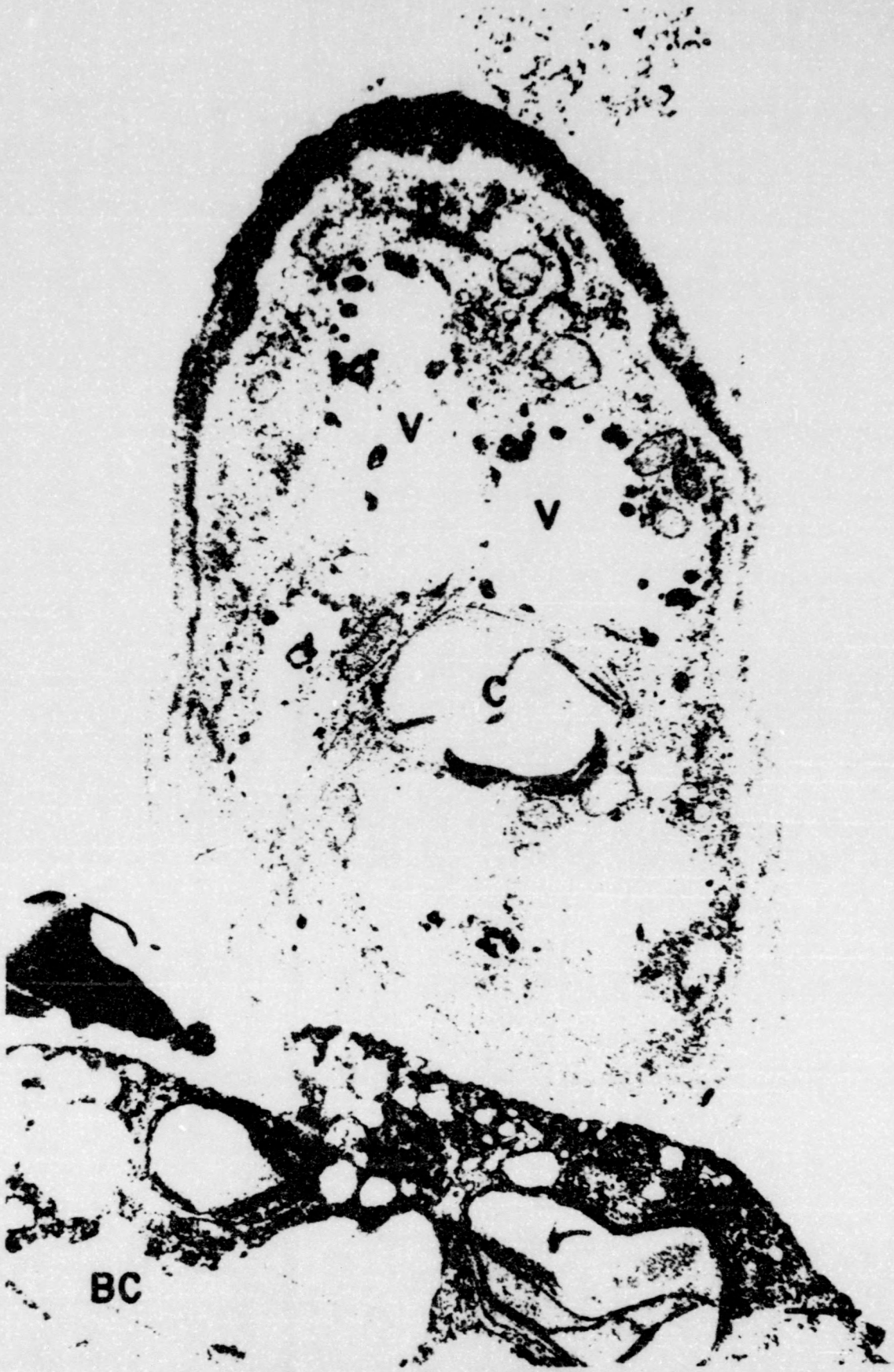
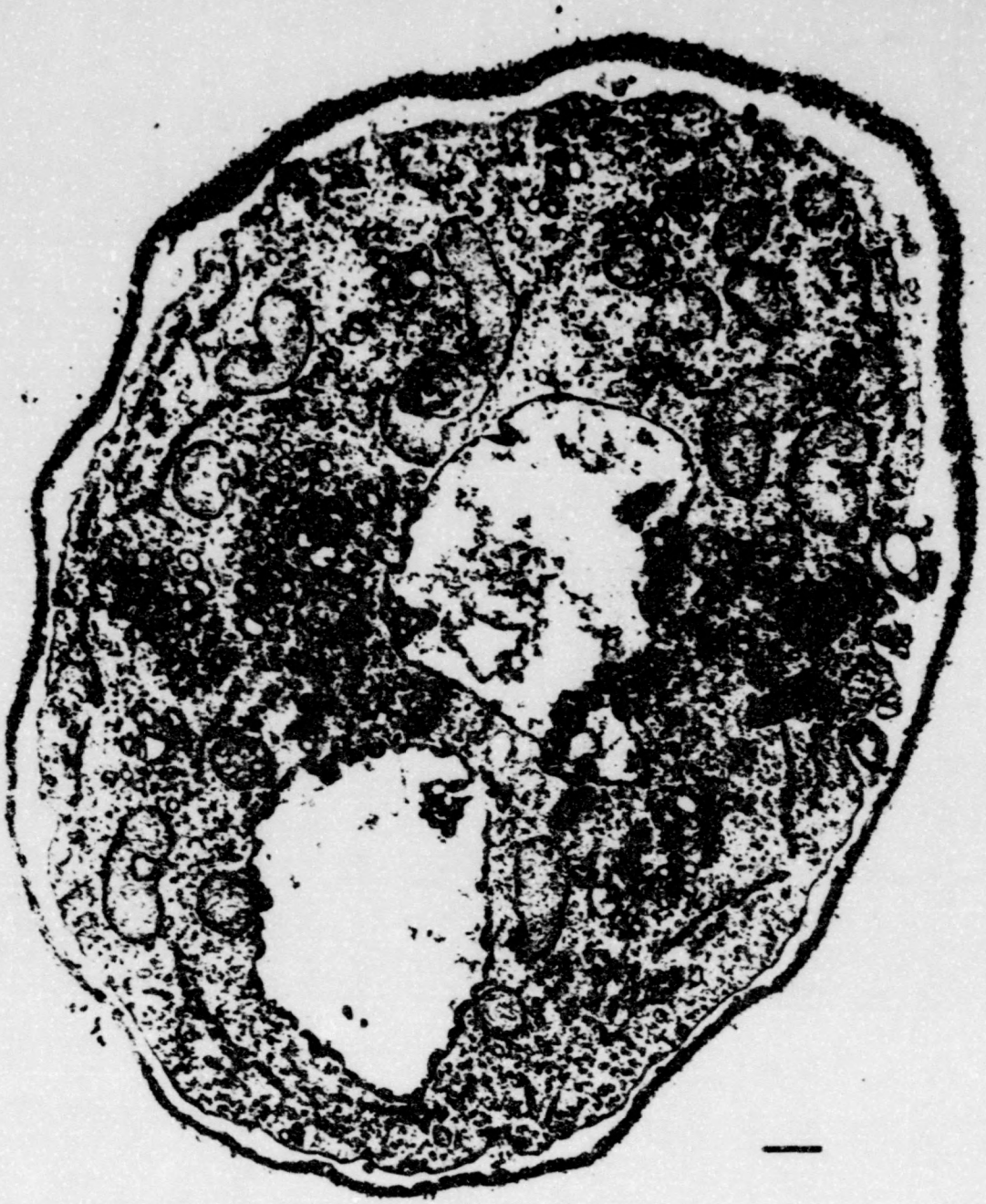
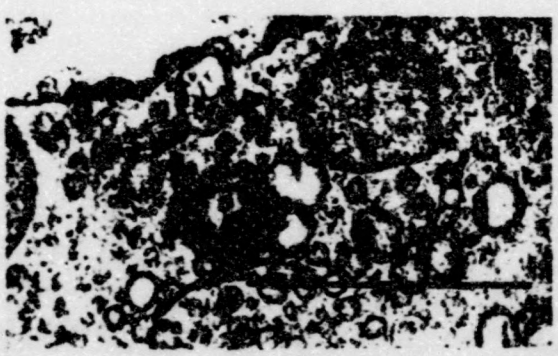

V
V
C
BC

Figure 30. Rhizoid. A cross section near the apex reveals numerous cell organelles. Mitochondria (M), Golgi (G), endoplasmic reticulum (ER), microbody (Mb), and ribosomes can all be found. Conspicuously absent are the chloroplasts and lipid droplets. Note the increase in dense staining material on the inner vacuole membrane (V). Structures that appear to be coated vesicles (CV) are located along the plasmalemma of the rhizoid.

Figure 31. Golgi vesicles. Two types of Golgi vesicles can easily be recognized in the rhizoid. In type I (arrows) an electron transparent core is surrounded by a deeply staining membrane. An electron dense core is occasionally present. These vesicles appear to fuse with the central vacuole (double arrow). They range in size from 60 to 300 nm. Type II Golgi Vesicles (circles) appear to have the same density as the Golgi cisternae. No function was determined for either type vesicle in this experiment.

Figure 32. Paramural bodies and cell wall. The rhizoid cell wall consists of two layers--an outer dense layer (DL) and an inner electron transparent layer (ET). Paramural (PM) bodies are located exterior to the plasmalemma.



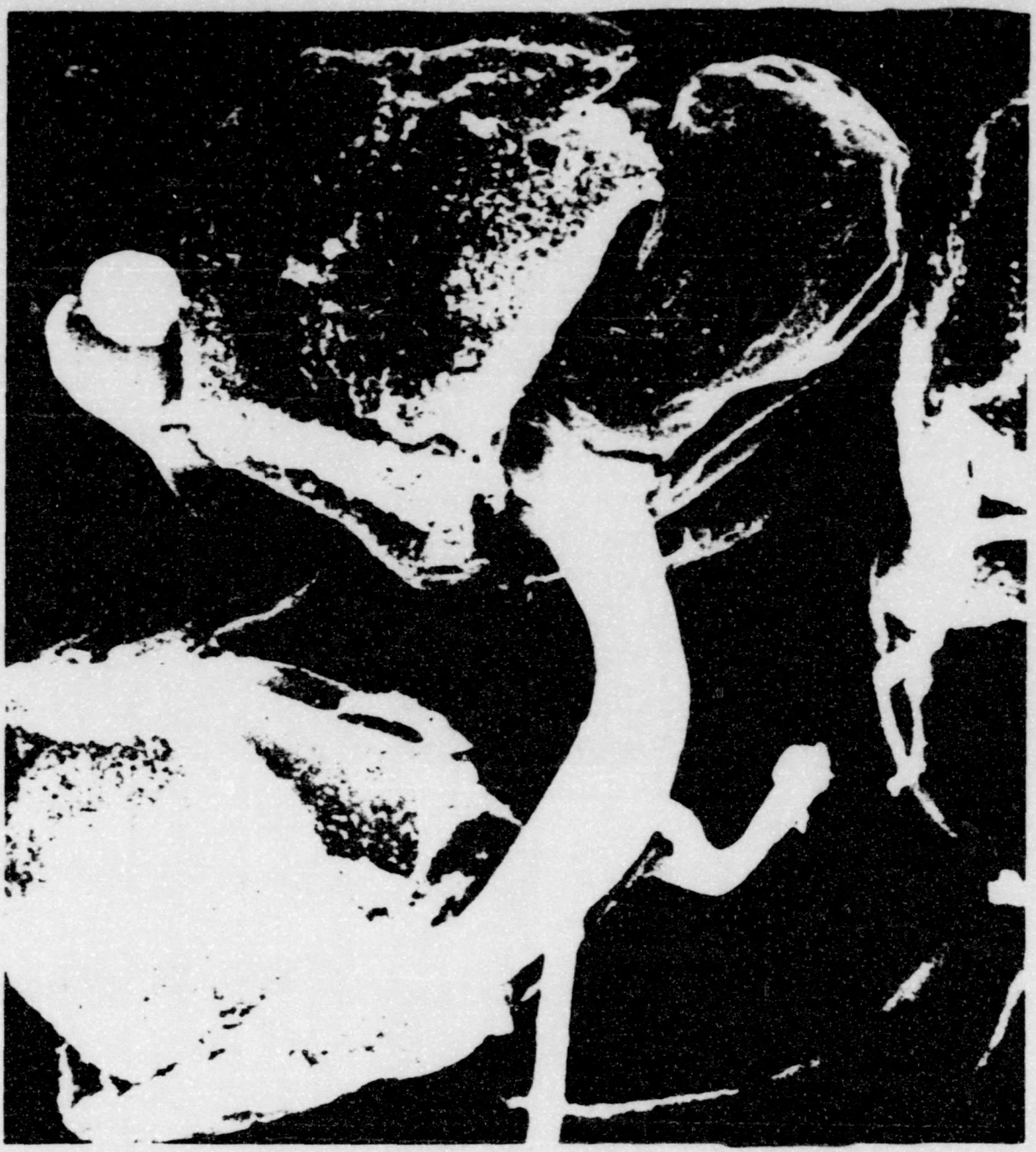


appeared to contain vesicles. Mitochondria were abundant, and occasional microbodies were seen. With the possible exception of the large vacuole, the most conspicuous feature was the appearance of large numbers of Golgi and associated vesicles. At least two types of vesicles were seen. One type had a densely staining outer surface and an electron transparent interior which often contained internal densities (Fig. 31). The second type had a density similar to the Golgi cisternae and had a lighter staining outer boundary (Fig. 31). Both types of vesicles appeared to be derived from the Golgi bodies.

The rhizoid cell wall consisted of two layers, an outer dense layer which appeared to have parallel arranged fibers and an inner layer of electron transparent material. Paramural bodies (Fig. 32) (Marchant and Robards 1968) could be seen exterior to the plasmalemma. Structures that appeared to be coated vesicles were also present along the surface of the plasmalemma (Fig. 30).

A scanning electron micrograph demonstrates the next stage of development where a second division of the basal cell results in formation of the first protonemal cell (Fig. 33). No further division of the basal cell occurred. Continued division of the apical protonemal cell would have produced the long green filament characteristic of a fern protonema.

Figure 33. Three-celled protonema. After the initial germination the basal cell nucleus will return to a more central location and divide to produce a new basal cell and protonemal cell. Further division will occur only at the apical protonemal cell.



DISCUSSION

The scanning and transmission electron microscopes provide excellent tools for the study of cellular development, and fern spores and protonemata are favorable material to utilize in these investigations. Their usefulness lies in their simple cellular structure, generally consisting of a 2 to 6 cell filament. This feature permits individual examination of each cell, and its entire developmental history can be followed. In addition changes in one cell can be readily correlated with the development of adjacent cells.

Such a system has advantages over the root tip cell system usually chosen. In that multicellular system difficulty is often encountered in following particular cells, and it is often difficult to obtain a homogenous collection of cells. Dissimilar surrounding tissue may interfere with experimental results.

Culture

The culture medium proved to be no problem as almost any medium would work, even distilled water. Agar or liquid media were equally successful, but slower growth on agar allowed more rapid build-up of fungi, which sometimes interfered with the growth of the spores. The exact concentration of spores was not critical, even in liquid culture where clumping of inoculated spores frequently occurred. Further

experimentation with media, light and temperature and staining regimes might enable one to determine more accurately the mitotic stages of division at the light microscope level. If this could be accomplished the next logical step would be to apply this knowledge of germination to samples prepared for the electron microscope.

Fixation, Dehydration and Embedding

Sampling for electron microscopy presented considerable difficulty. The hard spore coat made penetration of fixatives and embedding plastic almost impossible before rupture of the coat. The procedure by Bassel *et al*. (1981) using NaOCl to weaken the exine facilitates fixation and embedding. Also, spores and protonema under three cells contain a large quantity of lipids which kept the samples floating on the surface of fixation chemicals and dehydrating agents. This problem was solved by adding the wetting agent, Tween 80, to lower the surface tension of the culture medium enough for the spores to sink.

Ultrastructure

It would be interesting to examine the first or second asymmetric divisions giving rise to the rhizoid and protonemal cells. The major problems in studying mitosis at the ultrastructural level in fern spores were precise identification of mitotic times and the small sample size. Synchrony of development, even with a dark, red, white light procedure

was poor. Only 5% of the cells could be induced into division at any one time. The chances of finding one of these steps in a sample prepared for electron microscopy were slim. Under living conditions the first mitotic division could not be detected even at the light microscope level because of the thick spore coat. Even the second or later divisions could not be observed in living tissue because a dense ring of chloroplasts encircled the nucleus. The NaOCl technique of Bassel et al. (1981) enabled easier detection of early division stages that occur within the spore. The major problem with this method was that the acetocarmine-choral hydrate procedure required a staining time of 2.5 hr before dividing nuclei could be seen. For electron microscopy to be successful, simultaneous samples would have to be processed for electron microscopy. The light microscope could be used to improve the chances of success. Complete mitosis apparently took place within one hour after starting. Division stages were observed previous to mitosis and immediately following the first division.

It was clear from the description of organelles that their general ultrastructural characteristics were very similar to those thought to be characteristic of higher plants. In a general way, many of the organelles could be disregarded as indicators of the evolutionary position of a group of plants. For example, the basic structure of Golgi bodies and nuclei was similar throughout the plant kingdom. Many variations do exist, but these were not thought to be

characteristic of any particular group. Many times these differences could be associated with a particular function or environment. The plastid and, to a lesser extent, the mitochondria are considered to be reliable organelles indicative of evolutionary status (Cran 1970). The progression from a simple lamellate structure in many green algae to that of a complex system of grana interconnected by simple thylakoids of angiosperms was noted. With the mitochondria a similar change in structure exists, ranging from tubular cristae in more primitive forms to flattened cristae in more advanced groups.

As was noted in this study, the thylakoids in the gametophyte chloroplasts of Woodwardia virginica were arranged in a loose system of grana, the distinction between which was often not great. Sporophyte tissue was not examined; but based on the work of Cran (1970) on Dryopteris, it would not be surprising to find that the chloroplasts in the sporophyte more closely resemble those of the angiosperms.

Since Woodwardia is a leptosporangiate fern, one of the more highly evolved members of the pteridophytes, it might be expected that its position within the group would be revealed by its fine structure. A lower member of the group, such as Isoetes, has a much less defined structure (Paolillo 1962). The thylakoids intermesh to form small grana, but there was little similarity to the ordered structure in the angiosperms. Sufficient work has not been completed on the ultrastructure of the plastids of the pteridophytes to

determine if there is a gradation in the differentiation of the thylakoids from such plants as Isoetes to Woodwardia. With the limited investigations performed, it has been shown that the chloroplasts of the lower members are more similar to those of the algae and bryophytes than to those of the higher plants (Cran 1970).

No comparisons of the above type have been made with the mitochondria. It would be expected that Isoetes would have more tubular cristae. Cran (1970) reported the cristae in Dryopteris to be flat and plate-like. In this investigation of Woodwardia both tubular and plate-like elements could be found within the same mitochondrion. Thus, from the viewpoint of their chloroplasts and mitochondria, it appears that Woodwardia was indeed an intermediate genus, resembling the lower plants in some characteristics and the higher plants in other features. While this comparison was incomplete, it does provide a basis for continued studies.

During the course of this work the presence of organelle aggregation around the nucleus was noted. Such a distribution is called systrophy and has been seen in higher plants as a transitory light response, but it appears as a stable condition associated with division in algae and bryophytes (Strasburger 1965). Since many of the lower plants have few chloroplasts, it is necessary that chloroplast division be closely related to nuclear division. In unicellular algae with only one chloroplast, if the plastid divided without reference to the nucleus, many inviable cells would result. In such

organisms division of the nucleus and chloroplast take place at about the same time, and the daughter chloroplasts migrate to the region of the spindle poles. Thus the resultant cells are guaranteed a chloroplast. In Chlamydomonas the chloroplast occupies a considerable volume of the cell in comparison to other organelles and thus tends to envelope the nucleus. In Isoetes (Paolillo 1962), the apical meristem contains cells with a single chloroplast closely applied to the nucleus. At prophase the chloroplast divides and daughters move to opposite poles of the cell. When cells are examined at increasing distances from the meristem, an increasing number of chloroplasts are found ranging from two proximal to the meristem to sixty-four distally. As the number of chloroplasts per cell increases, their positional relationship to the nucleus decreases. Thus, the nucleus may decrease its control over chloroplast division.

The location of chloroplasts in the basal cell of Woodwardia may be a stage in the progression outlined above, in that a close spatial arrangement between plastids and nucleus was observed prior to cell division, but several were involved. In the future it would be interesting to make direct counts to determine the level of nuclear control over distribution of chloroplasts. The fact that the rhizoid receives a random number of plastids may indicate less control than for Isoetes. In the angiosperms, since chloroplast division can occur at any time during the cell cycle, there appears to be even less nuclear control over distribution.

This study showed the ulstrastructure of a spore prior to the second mitotic division. Only one report (Bassel et al. 1981) has shown micrographs of this stage of development. The presence of proplastids in a fern spore is reported for the first time in the present study. Previous techniques made this observation impossible, although their presence has been postulated (Fraser and Smith 1974). A second observation, previously unreported, was that the chromosomes apparently condense before the nuclear membrane disrupts during mitosis. Since later stages were not found, it is impossible to speculate on the significance of this discovery. Further research is planned to clarify this important event.

A third new observation is the change in shape of the nucleus during migration to the proximal face of the spore. The broadened "amoeboid" proximal face and a spindle shaped trailing end was noted. Again, significance of this event cannot be determined at this time.

This investigation has contributed observations on mitosis and the evolutionary position of the ferns. A useful continuation would be to not only examine organelle ultrastructure, but also to examine organelle distribution and mitosis in a wide variety of ferns from "primitive" to "advanced" groups. It is hoped that the writer will be able to continue with this project.

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