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Effects of Microtubule-Inhibitors on Nuclear Migration and Rhizoid Differentiation in Germinating Fern Spores (*Onoclea sensibilis*)

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Summary

Germinating spores of the sensitive fern, *Onoclea sensibilis* L., undergo premitotic nuclear migration before a highly asymmetric cell division partitions each spore into a large protonemal cell and a small rhizoid initial. Nuclear movement and subsequent rhizoid formation were inhibited by the microtubule (MT) inhibitors, colchicine, isopropyl-N-3-chlorophenyl carbamate (CIPC) and griseofulvin. Colchicine prevented polar nuclear movement and cell division so that spores developed into enlarged, uninucleate single cells. CIPC and griseofulvin prevented nuclear migration, but not cell division, so that spores divided into daughter cells of approximately equal size. In colchicine-treated spores, MT were not observed at any time during germination. CIPC prevented MT formation at a time coincident with nuclear movement in the control and caused a disorientation of the spindle MT. Both colchicine and CIPC appeared to act at a time prior to the onset of normal nuclear movement. The effects of colchicine were reversible but those of CIPC were not. Cytochalasin b had no effect upon nuclear movement or rhizoid differentiation. These results suggests that MT mediate nuclear movement and that a highly asymmetric cell division is essential for rhizoid differentiation.

Keywords: Cell polarity; Differentiation; Microtubules; Nuclear migration; *Onoclea sensibilis;* Spore germination.

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1. Introduction

There are many examples in which asymmetric cell division precedes cellular differentiation: generative cells in germinating pollen (SANGER and JACKSON 1971), stomates and root hairs (AVERS 1963, CUTTER and HUNG 1972, PALEVITZ and HEPLER 1974, STEBBINS and SHAH 1960), and rhizoids in germinating spores of *Equisetum* and most ferns (CROTTY 1967, DYER and CRAN 1976, NAYER and KAUR 1971, v. WETTSTEIN 1965). In each of these examples nuclear migration is necessary to establish the highly asymmetric division of a mother cell into daughter cells of unequal size and widely divergent morphogenic fates. The smaller daughter cell differentiates or gives rise to cells that differentiate into a distinct cell type. Despite the widespread occurrence of asymmetric division and its importance in cellular differentiation, very little is known about the mechanisms of nuclear movement in plants (see BRITZ 1979).

In spores of the sensitive fern, *Onoclea sensibilis* L., nuclear migration is one of the earliest visible events of germination and occurs prior to an unequal division that forms a rhizoid and protonemal initial. Spore germination is synchronous, and nuclear movement occurs simultaneously in almost all spores during a short interval. The spores are oblong and monolete; they have a linear raphe (seam) along their flattened proximal side (BASSEL *et al.* 1981). The nucleus remains in the center of each spore until 16 hours after initiation of germination when it migrates to the raphe, and then along the raphe to one end. The nucleus moves with an apparent velocity of 0.26 μm minute^{-1} and undergoes changes in shape as it migrates (VOGELMANN and MILLER 1980). During migration an aggregation of mitochondria has been observed at the leading edge of each nucleus and microtubules appear in the perinuclear region (BASSEL *et al.* 1981). Cytoplasmic streaming has not been observed in germinating spores and the nucleus appears to displace passively the chloroplasts and other organelles as it moves through the cytoplasm. After completion of nuclear movement the spore divides into two cells of unequal size and widely divergent morphogenic fates. The larger daughter cell divides and develops into a filamentous protonema, whereas the smaller lens-shaped cell immediately differentiates into a rhizoid.

Previous studies have shown that nuclear migration and the completion of a highly asymmetric cell division appear to be necessary for rhizoid differentiation (MILLER and GREANY 1976, VOGELMANN and MILLER 1981). The presence of low concentrations of methanol prevents nuclear migration to the end of the spore, such that the initial cell division is symmetric, and the daughter cells are approximately equal in size. As a consequence, both daughter cells develop into protonemata but neither differentiates into a rhizoid.

In view of the apparent obligate role of polar nuclear movement in rhizoid formation, the mechanism of nuclear migration was studied further by treating

spores with inhibitors of cytoskeletal components. The effects of each inhibitor were examined at different stages of germination and at critical times spores were processed for electron microscopy. The effects of the inhibitors upon the polarity of development were observed both in spores exposed continuously and in those removed from inhibitors at different stages of germination.

2. Materials and Methods

2.1. Spore Isolation and Culture

Fertile fronds of *Onoclea sensibilis* L. were collected in the fall of 1977 in Jericho, Vermont, and stored in plastic bags at 4 °C. Spores were isolated using methods of STOCKWELL and MILLER (1974). The outer spore coat, which interferes with the observation of nuclear migration and cell division, was removed from the spores with commercial sodium hypochlorite bleach (VOGELMANN and MILLER 1981). The spores were then dried and stored in petri dishes at 4 °C. To initiate germination, spores were sown on the surface of 3.0 ml of Knop's mineral medium in 25 × 100 mm culture tubes under continuous cool-white fluorescent light at 20.3 W m^{-2} and 26 ± 1 °C.

2.2. Inhibitors

Colchicine (Tridom Chemical Inc., Hauppauge, N.Y., U.S.A.) was dissolved in Knop's medium. The antimitotic compound, isopropyl-N-3-chlorophenyl-carbamate (CIPC, Sigma Chemical Co., St. Louis, Mo, U.S.A.), and the fungal metabolite, griseofulvin (Aldrich Chemical Co., Milwaukee, Wis, U.S.A.), have limited solubility in aqueous solution and were first dissolved in acetone. Cytochalasin b (Sigma) was dissolved in dimethylsulfoxide (DMSO). Dilutions were made by dissolving aliquots of the inhibitor solutions in Knop's medium, the maximum amount of solvent not exceeding 1% in the final solution. Controls had similar amounts of solvent but were without inhibitor. Control experiments showed that these amounts of solvents did not change the extent or pattern of spore germination, compared with spores germinated on plain Knop's solution. The effects of inhibitor concentration were examined by germinating spores in the continuous presence of the inhibitor for 60 hours. Preliminary experiments showed that this period was sufficient to allow full expression of the effects of the inhibitors on nuclear migration and rhizoid differentiation.

2.3. Data Collection

At the end of an experiment, gametophytes were mounted on a microscope slide in a drop of acetocarmine-chloral hydrate solution (EDWARDS and MILLER 1972) which stained the nuclei and lysed the chloroplasts so that the position of the cell walls was easily observed. The preparation was sealed with a coverslip ringed with heavy mineral oil, the slides were examined with a microscope, and a random sample of 200 gametophytes was scored for the presence of rhizoids. None of the inhibitors reduced spore viability or changed the percent germination which reached about 95% under all control and experimental conditions. The basic datum was the percentage of the sample with rhizoids. The absence of rhizoids was used as an index of the effectiveness of the inhibitors in preventing nuclear migration and asymmetric cell division; those spores which did not form rhizoids had suffered a blockage of nuclear movement. This was established by experiments in which a detailed time course for nuclear movement and cell division was determined in germinating spores treated with either CIPC or colchicine.

2.4. Electron Microscopy

Spores were prepared for electron microscopy by the method described by BASSEL *et al.* (1981). Spores grown on Knop's medium containing 50 μM CIPC or 8 mM colchicine

for 18 or 27 hours were fixed for 12 or 24 hours in 2.5% glutaraldehyde in PM buffer which contains 100 mM PIPES, 1 mM GTP, 1 mM $MgSO_4$, 2 mM EGTA, pH 6.9 (LUFTIG *et al.* 1977). Cells were postfixed in 1% OsO_4. Spores that were 18 hours old were treated with 2.75% NaOCl in 0.1 M mannitol for 1.5 minutes to rupture the spore coat (intine). Cells were dehydrated in ethanol, and embedded in Spurr's low viscosity epoxy resin (SPURR 1969). Sections were stained with uranyl acetate and lead citrate and examined with a Siemens Elmiskop IA or 102.

2.5. Time of Sensitivity to CIPC and Colchicine

To measure the time when germinating spores became sensitive to CIPC or colchicine, they were germinated on medium containing the inhibitor and were then transferred at 4 hours intervals to medium without inhibitor. They were allowed to grow until 60 hours, and were then stained and examined for the presence of rhizoids. Sensitivity during germination was measured by the time of transfer which resulted in the suppression of rhizoids in 50% fo the gametophyte population. To measure the time when they became insensitive to inhibitor, spores were germinated on Knop's medium and were transferred at 4 hours intervals to medium with inhibitor. The amount of inhibitor carried over was minimized by transferring the spores to 10 ml of medium and then again 1 hour later. Insensitivity was measured by the transfer time in which rhizoids were subsequently formed in 50% of the gametophyte population.

Nuclear migration was not prevented when colchicine or CIPC was administered to spores that had passed a certain stage in germination. In an attempt to estimate whether penetration rate of the inhibitor influenced the apparent time of insensitivity, other experiments were done in which spores were transferred and then immediately chilled to 4 °C for 12 hours in darkness. Chilling was expected to arrest germination and hold spores in an inactive state while allowing more time for the inhibitor to enter or exit from the cells. After the incubation period, the cultures were returned to the growth chamber. Samples of gametophytes were stained 2 d later and examined for rhizoids. Control spores were germinated for 12 or 24 hours and then chilled for 12 hours. After warming they were examined at intervals to assess the effects of the cold treatment upon the time of onset and rate of completion of nuclear migration and cell division.

3. Results

3.1. Effects of Inhibitors on Rhizoid Formation

Under continuous treatment with different concentrations of colchicine (Fig. 1 *a*), three different gametophyte morphologies were produced in different proportions: 1. two-celled gametophytes in which one cell was a rhizoid, 2. two-celled gametophytes in which there was no rhizoid, and 3. single cells. The percentage of gametophytes with rhizoids decreased with increasing colchicine concentration. At 2.5 mM, there were about equal numbers of gametophytes with rhizoids, bicells without rhizoids, and single cells. At 8 mM colchicine, 7% were bicellular and without rhizoids, 88% were unicellular, and only 5% of the population underwent an asymmetric cell division and formed rhizoids.

Detailed observation of the course of germination showed that gametophytes with rhizoids developed from spores in which the nucleus underwent migration to one end of the spore, an asymmetric division occurred, and the smaller

of the two daughter cells differentiated into a rhizoid. Bicellular gametophytes without a rhizoid developed from spores in which nuclear migration was inhibited but cell division was not. In these spores the initial cell division was symmetric, and both daughter cells gave rise to protonemata. In some spores, both nuclear migration and cell division were inhibited—these developed into larger single uninucleate cells.

The developmental effects of continuous treatment with different concentrations of CIPC (Fig. 1 *b*) were similar to those of colchicine. Some spores germinated normally, some divided symmetrically without prior nuclear

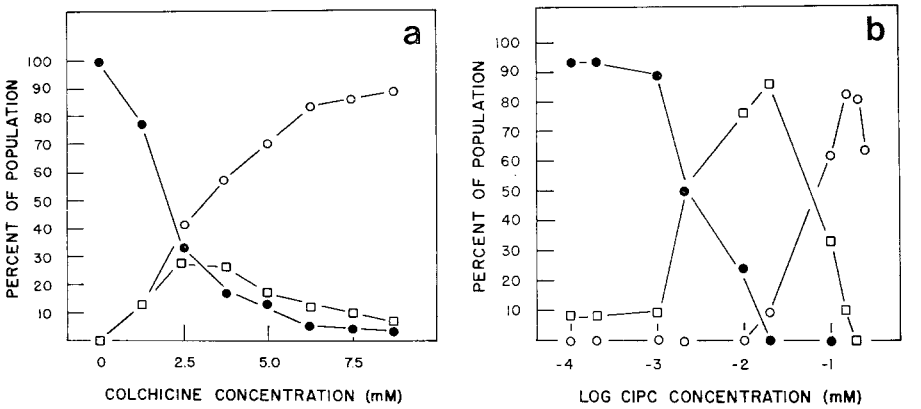

Fig. 1. Inhibition of rhizoid formation by colchicine (*a*) and CIPC (*b*). Spores were germinated in the continuous presence of colchicine or CIPC and examined 60 hours into germination; percentage of gametophytes with rhizoids (●), two-celled gametophytes without rhizoids (□), single uninucleate cells (○)

movement, and some formed single cells with one nucleus. The percentage of gametophytes with rhizoids decreased with increasing CIPC concentration. At 50 μM, rhizoid formation was prevented and about 90% of the spores developed into bicellular gametophytes (Fig. 3 *c*). Although the cells were able to complete an initial division, CIPC completely inhibited further cell division. The nuclei in many cells were observed to undergo some stages of the mitotic cycle but metaphase figures appeared aberrant and chromosomes did not separate. Higher concentrations of CIPC inhibited almost all cell division.

Griseofulvin inhibited nuclear movement and rhizoid formation in a manner similar to CIPC. In spores germinated on Knop's medium which was saturated with griseofulvin (ca. 0.5 mM), only 15% of the gametophytes formed rhizoids (data not shown). In the remainder of the population, the initial cell division was symmetric and both daughter cells had protonemal characteristics.

Cytochalasin b at 10, 20, and 100 μg · ml^{-1} did not prevent nuclear migration,

the occurrence of asymmetric cell division, or rhizoid formation in any fraction of the spore population. Rhizoid elongation, however, was strongly inhibited at all of the above concentrations (data not shown).

3.2. Effect of Inhibitors upon Nuclear Migration and Cell Division

Colchicine (8 mM) inhibited both nuclear migration and cell division (Fig. 2 *a*) so that each spore developed into a highly vacuolate cell (Figs. 3 *a* and *b*). Division did not occur as long as colchicine was present. Rate of germination appeared unaffected by the inhibitor as measured by the time when acetocarmine penetrated the spores (Fig. 4). This red stain enters the cells at the

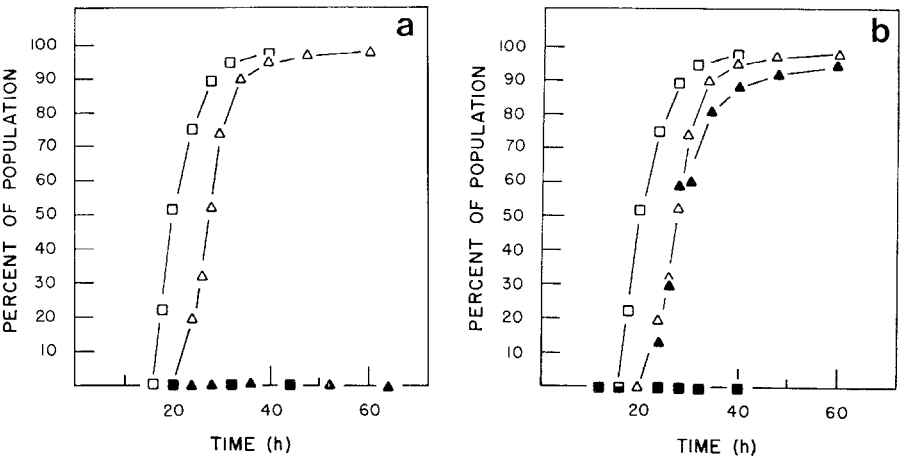

Fig. 2. The effects of 8 mM colchicine (*a*) and 50 μM CIPC (*b*) upon nuclear migration and cell division in germinating spores. Solid symbols show data from inhibitor treatments, open symbols show data from the control; completion of nuclear migration (□, ■), completion of cell division (△, ▲)

time the spore coat ruptures, usually about the time of normal cell division (FISHER and MILLER 1978). Spores are not stained at earlier times because the intine is impermeable to carmine. Since neither nuclear migration or cell division occurred in this concentration of colchicine, stain uptake was the only available index of germination rate.

Nuclear migration was almost totally inhibited by continuous treatment with 50 μM CIPC (Fig. 2 *b*). Cell division was largely unaffected and the time course of division was similar in treated and control spores. Almost all spores divided into daughter cells of equal size, none of which differentiated into a rhizoid (Fig. 3 *c*).

3.3. Ultrastructure of Spores Treated with Colchicine and CIPC

During normal nuclear movement, microtubules and mitochondria are associated with the nucleus, particularly the leading face (Fig. 5 *a*). In CIPC- or

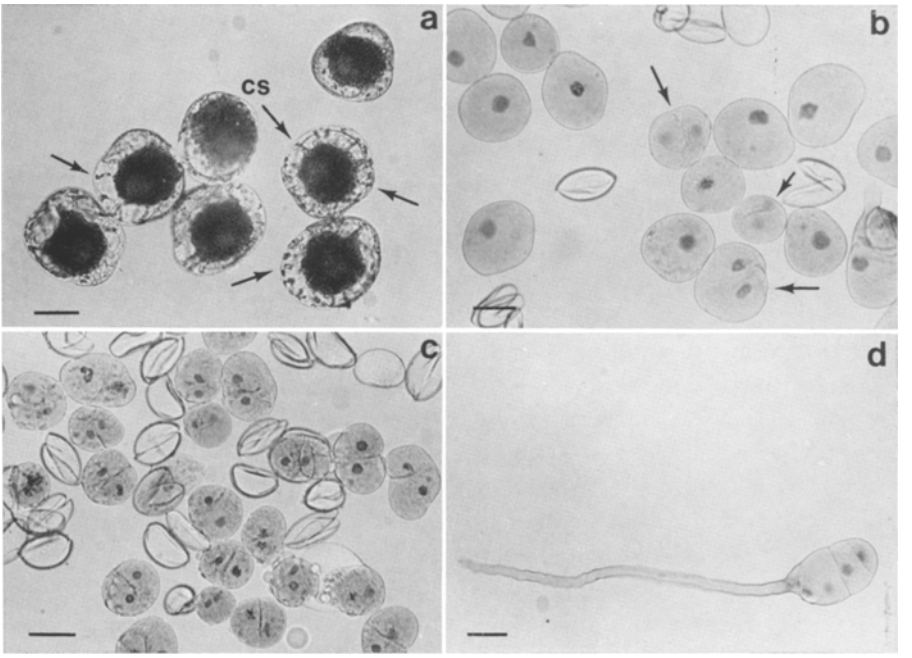

Fig. 3. The effect of colchicine and CIPC upon early gametophyte development. Spores treated with 8 mM colchicine developed into enlarged cells (*a*) in which the nucleus is positioned centrally and is surrounded by many chloroplasts. Note the many cytoplasmic strands (*CS*) and the absence of rhizoids. Staining with acetocarmine-chloral hydrate shows nuclear position more clearly (*b*). Some spores underwent a single symmetric division (arrows) and formed daughter cells of equal size but neither cell differentiated into a rhizoid. Spores treated with 50 μM CIPC underwent a single symmetric division and did not form rhizoids (*c*). The cells are surrounded by the clear spore coats that become detached during staining. The control shows the length of a normal rhizoid (*d*). All gametophytes are 60 hours old. Marker bar = 50 μM

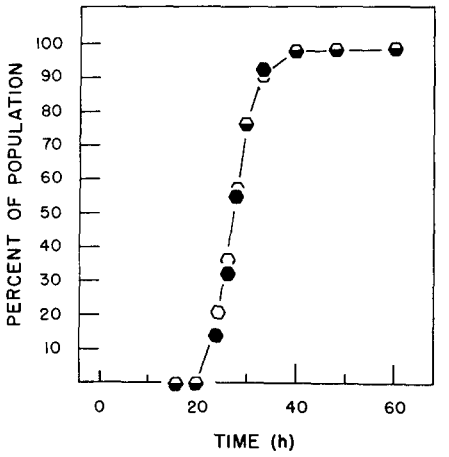

Fig. 4. Time of penetration of acetocarmine stain in spores treated with 8 mM colchicine. Stain penetration marks the timer the spore coat ruptures; spores treated with colchicine (solid symbols), control (open symbols)

colchicine-treated spores, the nucleus does not migrate from its central position (Fig. 6 *a*), and microtubules are not found near the nucleus at any time prior to breakdown of the nuclear membrane during mitosis (Figs. 5 *b* and *c*). In colchicine-treated spores MT were not seen at any time in development, but in CIPC-treated spores, MT appeared to attach and extend from the chromosomes after the nuclear membrane disappeared (Figs. 7 *a* and *b*). In contrast to a normal orderly bipolar arrangement of the spindle, in CIPC-treated spores the chromosomes fibers appeared to run in many directions (Figs. 7 *a* and *b*).

During normal spore germination, large aggregates of mitochondria develop at the prospective spindle poles (BASSEL *et al.* 1981). In the presence of colchicine, some spores exhibited a very large mitochondrial accumulation at the side of the nucleus adjacent to the proximal face of the spore (Fig. 8 *a*). This may be an exaggerated form of the mitochondrial accumulation that normally occurs at the leading edge of each migrating nucleus, or it may be related to the aggregate that forms at the prospective spindle poles seen in mitotic prophase. This type of mitochondrial accumulation was not observed in CIPC-treated spores; instead, small groups of mitochondria appear randomly distributed at the periphery of the "spindle" (Fig. 6 *b*). Some colchicine-treated spores have small "spindle pole" aggregates of mitochondria, but in the absence of MT, the chromosomes show no specific alignment relative to them (Fig. 8 *b*).

Another striking ultrastructural observation was the aberrant cell plate and cross-wall that formed in CIPC-treated spores, an effect which was also found after treatment of spores with methanol or chloroform (unpublished results). During cytokinesis in normal spores, a single layer of vesicles appears in the center of the telophase spindle (Fig. 9 *a*). The vesicles soon fuse to form a narrow cell plate. In CIPC-treated spores a large multilayered assembly of vesicles surrounded by mitochondria concentrates between the two daughter nuclei (Fig. 9 *b*). The vesicles eventually fuse to form a thick, "foamy", branching wall.

3.4. Time of Sensitivity to CIPC and Colchicine During Germination

The time of onset and termination of sensitivity to colchicine and CIPC during germination was measured by transfer experiments in which spores

Fig. 5. Nuclear region of control and treated spores. *a* Control spore with nucleus migrating to one end of the spore. Many microtubules (arrows) are seen adjacent to the nucleus and the associated mitochondria. Black arrows point to MT in contact with mitochondria in this section. *b* Nuclear region of a cell treated with 50 μM CIPC for 18 hours. The nucleus remains in the center of the cell, and no MT can be seen around the nucleus. *c* Portion of the nuclear region of a spore treated with 8 mM colchicine for 27 hours. The nucleus, which is in prophase, remains in the center of the spore, and no MT can be seen. (*a* and *c* are at the same magnification). Abbreviations used in electron micrographs: *C* chloroplast, *cp* cell plate, *L* lipid droplet, *M* mitochondrion, *N* nucleus, *np* nuclear pore, *P* protein granule. Marker bar = 1 μm unless indicated otherwise

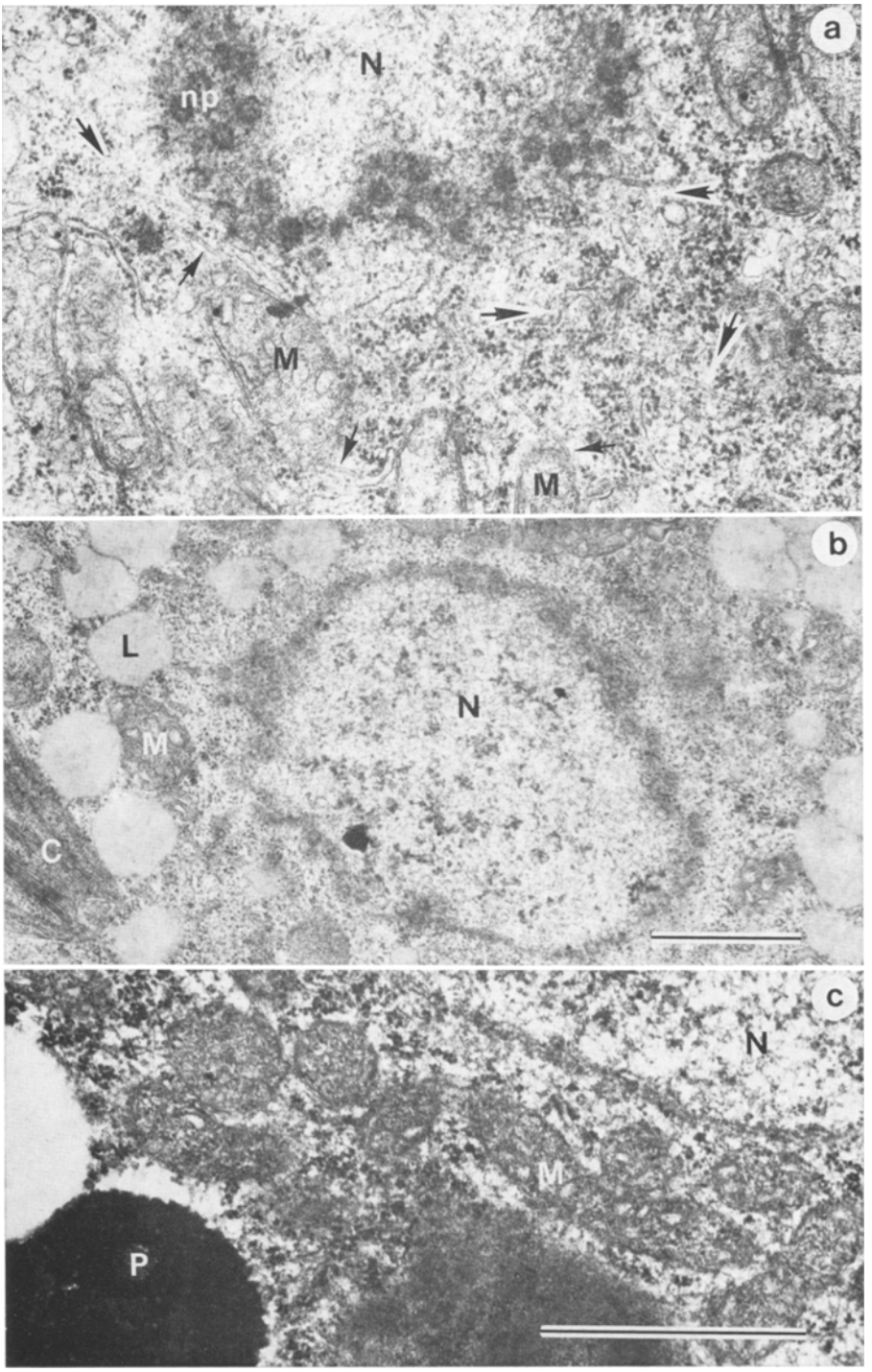

Fig. 5

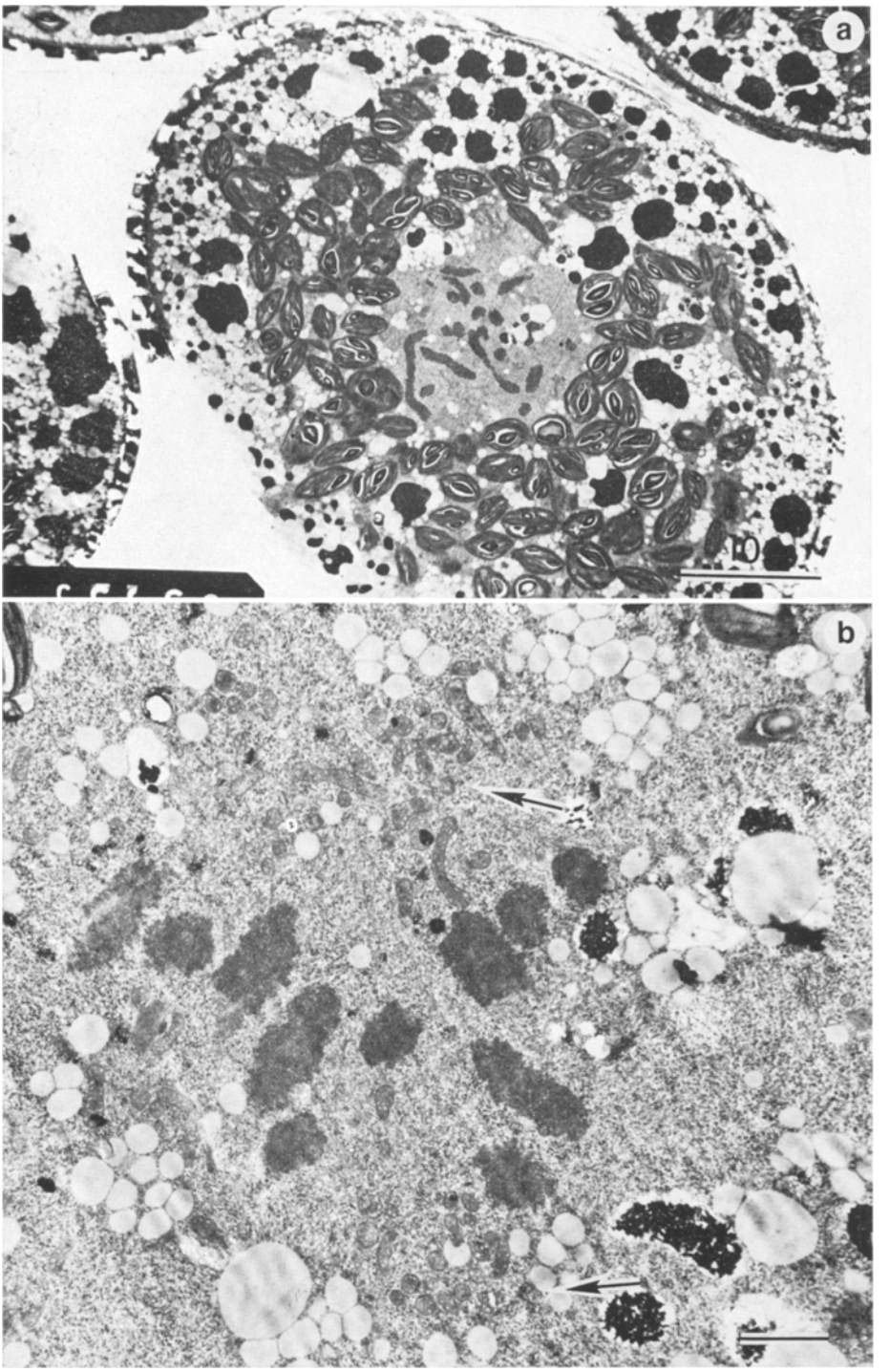

Fig. 6. *a* Spore treated with 50 µM CIPC for 27 hours. The condensed chromosomes are centrally located. *b* Spore treated with CIPC for 27 hours. Arrows indicate aggregates of mitochondria. The upper aggregate is one pole of the spindle in this anaphase cell. The mitochondrial aggregate at the pole is much smaller than that found in normal mitosis of untreated spores

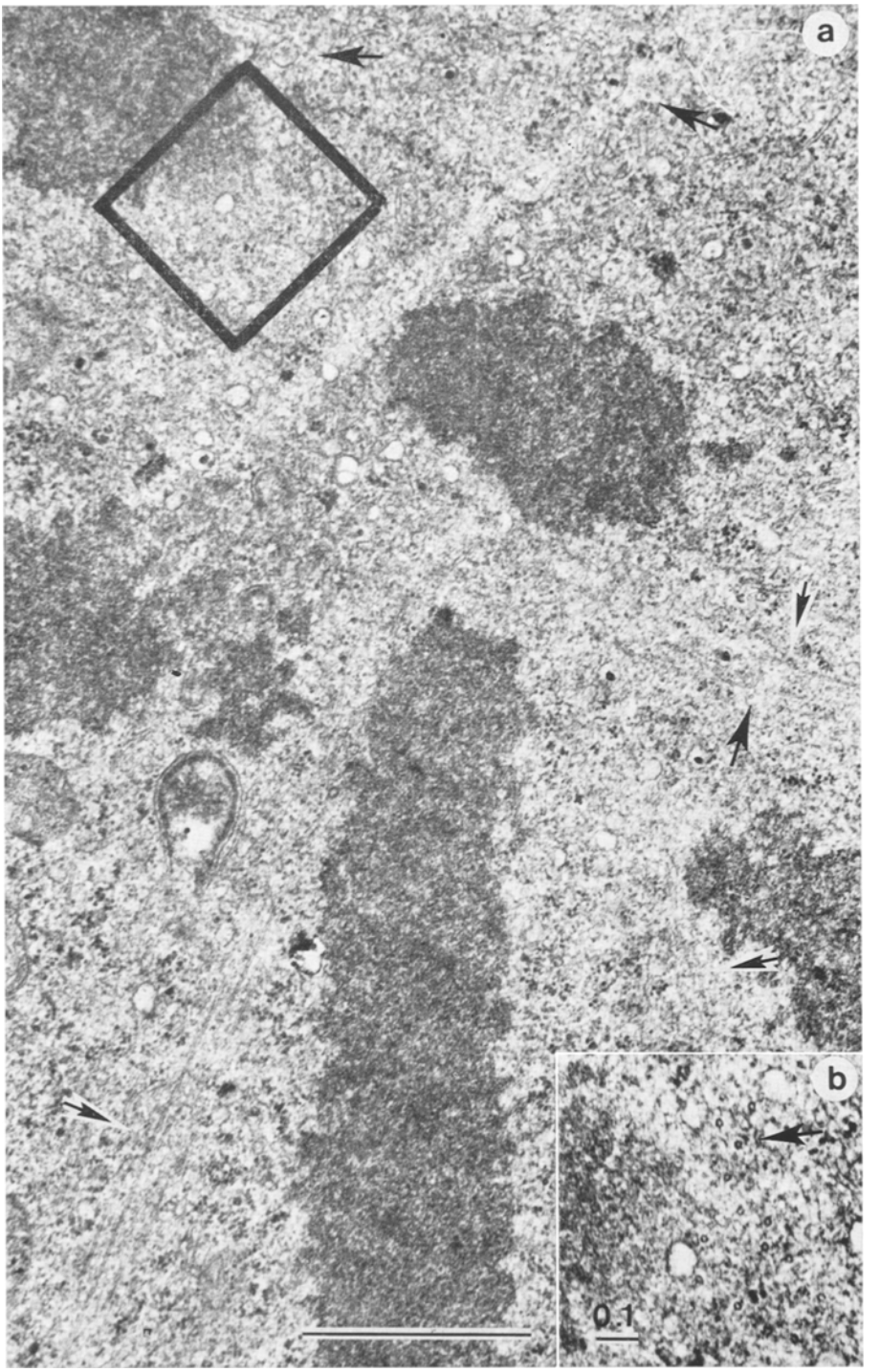

Fig. 7. Spindle region of a spore treated with CIPC for 27 hours. Instead of the orderly arrangement of MT seen in a normal spindle, MT in this cell run in many different directions as indicated by the arrows. Black on white arrows point to some of the MT in longitudinal section, black arrows to cross-sectioned MT. The boxed region is enlarged (*b*) so that the cross-sectioned MT can be seen more clearly

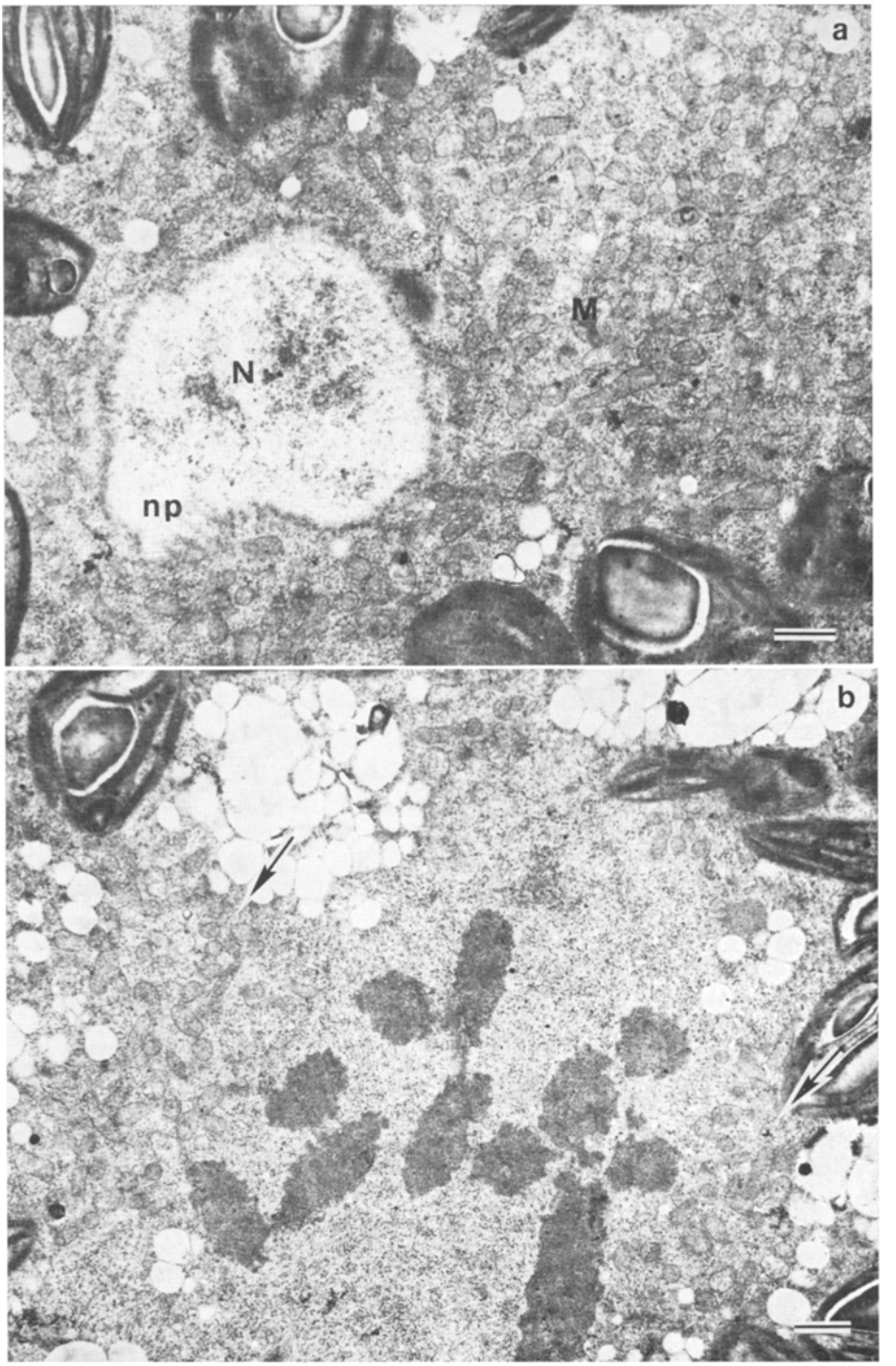

Fig. 8. *a* Spore treated with 8 mM colchicine for 27 hours. Mitochondria have accumulated on the side of the central nucleus nearest to the proximal face of the spore. This single mitochondrial aggregate is similar to, but much larger than the spindle pole mitochondrial aggregate seen in untreated spores at prophase. No MT are present. *b* Colchicine-treated spore after nuclear membrane breakdown. Arrows point to mitochondrial aggregates, but MT are not seen, and chromosomes are not aligned perpendicular to these presumptive "spindle pole" mitochondrial aggregates

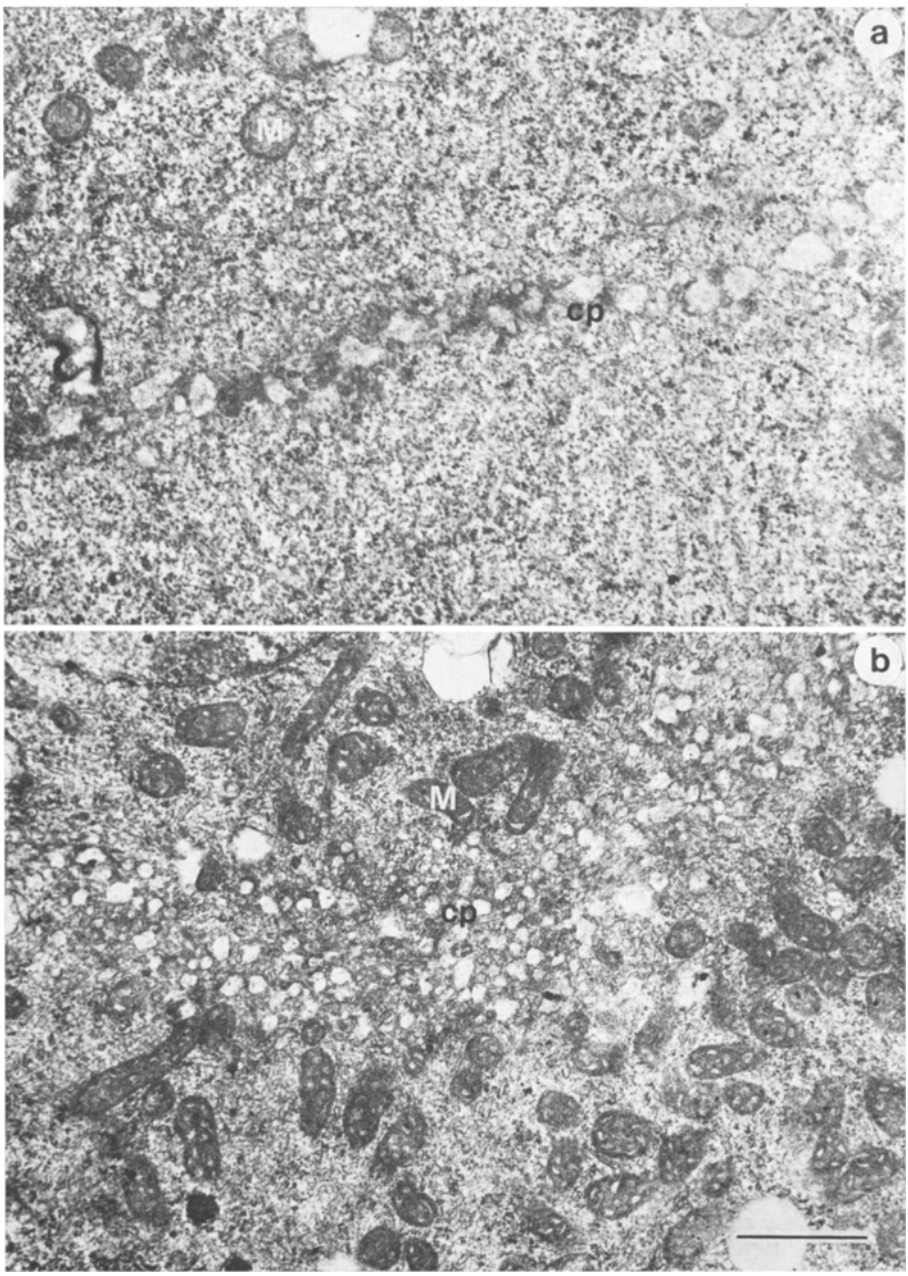

Fig. 9. Cell plate formation. *a* Untreated spore at telophase. A single layer of vesicles forms at the center of the spindle and fuses to form a narrow cell plate. *b* Spore treated with CIPC. The cell plate region contains large aggregates of vesicles which eventually fuse and form a thick, "foamy" branching cell wall. Many mitochondria lie adjacent to the cell plate. (*a* and *b* are at the same magnification)

were either placed onto or taken off media with inhibitor at different times during germination. Some experiments were done in which spores were held in darkness at 4 °C for 12 hours to allow more time for the entry or exit of an inhibitor. Chilling delayed the onset of nuclear migration and mitosis, but only for the duration of incubation in the cold (Fig. 10). Both nuclear migration and cell division resumed after warming and there did not appear to be any permanent effects of this treatment upon spore germination.

The onset of sensitivity to colchicine is shown by the relationship between the time spores were transferred from colchicine to medium free of colchicine,

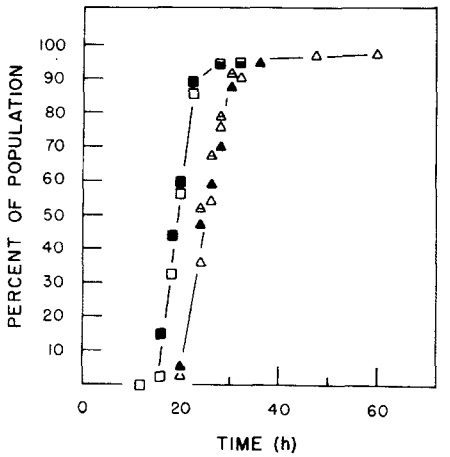

Fig. 10. The effect of incubation in the cold upon nuclear migration and cell division. Spores were germinated for 12 hours (solid symbols) and 24 hours (▲) and chilled to 4 °C for 12 hours. They were then warmed and the time of completion of nuclear migration (□ ■) and cell division (△ ▲ ▲) compared with the control (open symbols). Data from the chilled spores are shifted 12 hours towards the time of initiation of germination to compensate for the period of chilling

and the proportion of gametophytes which failed to develop rhizoids (Fig. 11 *a*). Onset of sensitivity to colchicine occurred several hours prior to the time of normal nuclear movement. Treatment for only the initial 16 hours of germination prevented subsequent rhizoid formation in 50% of the gametophyte population, even though at 16 hours no nuclear movement had occurred. A paired experiment in which transfer was followed by cold treatment gave very similar results.

Sensitivity to CIPC (Fig. 11 *c*) appeared to develop at times earlier than for colchicine. Treatment for only the initial 9 hours of germination prevented nuclear movement and rhizoid formation in 50% of the population, and a 12-hour treatment almost completely prevented rhizoid formation. The time

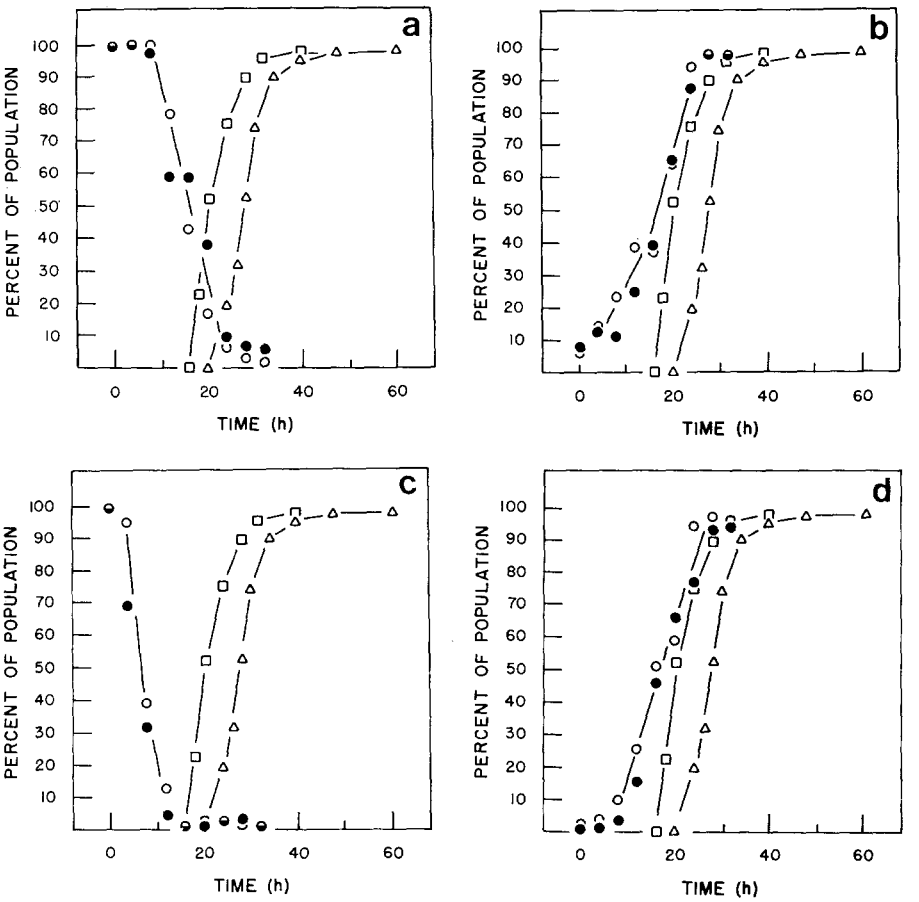

Fig. 11. Time of onset and termination of sensitivity to colchicine and CIPC during germination. For determination of the time of onset of sensitivity, spores were germinated for different lengths of time on 8 mM colchicine or 50 μM CIPC and were then transferred to medium without inhibitor. The time of development of sensitivity was determined from the percentage of spores that germinated into gametophytes with rhizoids (○ ●). Those gametophytes without rhizoids developed from spores in which nuclear migration was inhibited. Percent rhizoid formation is plotted as a function of time the spores were transferred and allows comparisons between the time of sensitivity to colchicine or CIPC and the completion of nuclear migration (□) and cell division (△) in the controls; rhizoids formed from spores transferred at room temperature (○), and from spores transferred and immediately chilled to 4 °C for 12 hours (●). The time of termination of sensitivity to colchicine and CIPC was determined similarly except that spores were germinated for different times and then transferred to medium with inhibitor. *a* Onset of sensitivity to colchicine. *b* Termination of sensitivity to colchicine. *c* Onset of sensitivity to CIPC. *d* Termination of sensitivity to CIPC

of action of CIPC was unaltered by cold treatments. In view of other irreversible side effects, the apparent sensitivity to CIPC during early stages of germination must be interpreted with caution.

The onset of insensitivity to colchicine was determined by transferring spores to colchicine at different times after initiation of germination. Insensitivity developed prior to nuclear movement, and the 50% response time occurred at 17 hours (Fig. 11 *b*). Colchicine (8 mM) either inhibited both nuclear movement and cell division or neither. Spores that were exposed to colchicine after they had completed nuclear movement, but several hours prior to the onset

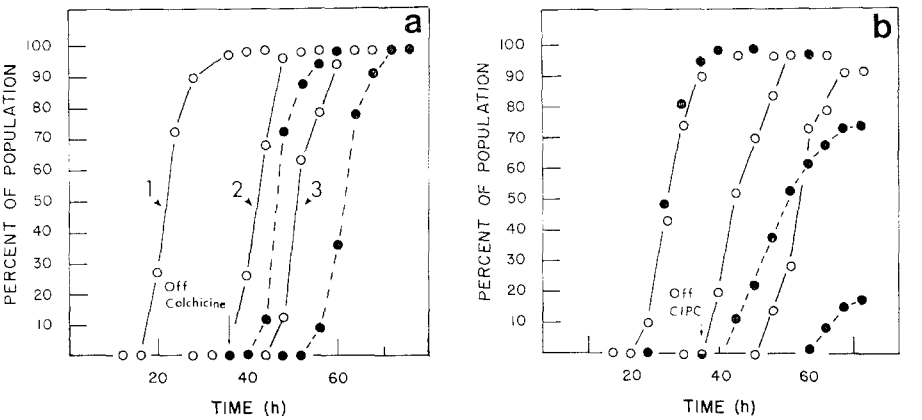

Fig. 12. Resumption of cell division after removal from colchicine or CIPC. Control (○), cells removed from inhibitor (●). *a* Resumption of cell division after 36 hours colchicine-treatment. *b* Resumption of cell division after 36 hours CIPC-treatment

of cell division, were able to divide and form rhizoids. Those exposed at earlier times developed into uninucleate enlarged single-cells. Data shown from experiments with chilling are similar.

Onset of insensitivity to CIPC (Fig. 11 *d*) with a 50% response time at 17 hours is similar to that of colchicine. At the concentration of CIPC used in the transfer experiments, the initial cell division was either symmetric or asymmetric, and the relative frequency of each type of division was determined by the time of transfer to CIPC. No further cell division occurred, so that at the end of the experiment all gametophytes were two-celled whereas the control had 4–6 cells.

3.5. Gametophyte Development after Removal from Colchicine and CIPC

Experiments were done to examine the degree to which polar development was reestablished after the cells were removed from the MT inhibitors. Spores were treated with colchicine or CIPC for 36 hours and then were transferred

to medium without inhibitor. Following removal from colchicine (Fig. 12 *a*) each single cell divided into two protonemal cells of equal size. The onset of this round of division occurred 8 hours after removal from colchicine. The nucleus in one of the daughter cells then migrated to one side and an asymmetric division formed a rhizoid initial (Fig. 13 *a*). Subsequent rhizoid elongation occurred perpendicular to the main axis of the gametophyte whereas primary rhizoids in untreated gametophytes elongated parallel to the axis. However, after several days additional growth and the formation of other numerous secondary rhizoids, there was no visible difference between the control and gametophytes initially treated with colchicine.

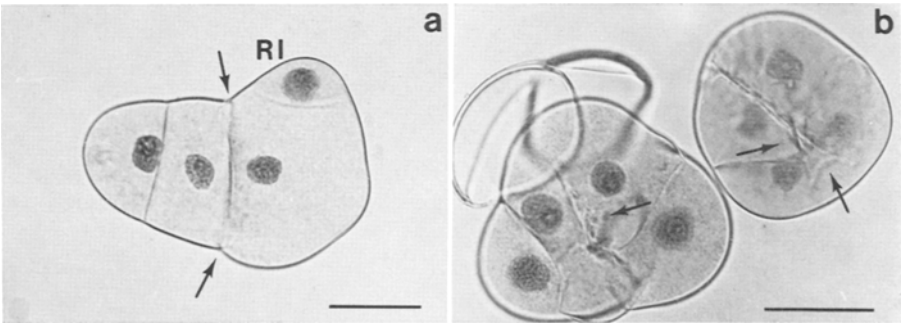

Fig. 13. Gametophyte development after removal from colchicine or CIPC. Spores were treated as in Fig. 12. Marker bar = 50 μm. *a* Gametophyte that developed following colchicine-treatment. This gametophyte was unicellular at the time it was removed from colchicine but during the recovery period it underwent a symmetric division (wall indicated by arrows) and then two other divisions. The first asymmetric division followed nuclear movement and formed a rhizoid initial (*RI*) whereas the other division occurred in the protonemal cell. *b* Gametophytes that developed following CIPC-treatment. In contrast to recovery from colchicine, cell division in these cells was disoriented so that normal protonemata did not form. Rhizoids sometimes differentiated from different parts of these cellular aggregates. Note the abnormal branching cross-walls (arrows) in both gametophytes

CIPC appeared to have lasting effects upon cell division (Fig. 12 *b*). In the presence of CIPC, an initial symmetric division occurred at a time coincident with the initial asymmetric division in the control. After transfer to medium without CIPC the onset and rate of completion of subsequent rounds of division was inhibited. Further division did not occur at all in 20% of the gametophytes. Normal filamentous protonemata were not formed; instead, the plane of division was disoriented so that clusters of cells formed (Fig. 13 *b*). Sometimes these gametophytes formed rhizoids but this did not occur at specific intervals, nor with any regularity. These effects were observed after spores were treated with CIPC for only a few hours and persisted despite repeated washing in medium without CIPC.

4. Discussion

Polar nuclear migration was prevented when spores were treated at critical times during germination with the MT inhibitors colchicine, CIPC or griseofulvin. When the nucleus remained in the center of each spore rhizoid differentiation did not occur. Colchicine (8 mM) inhibited both nuclear movement and cell division, and spores developed into uninucleate enlarged cells that were highly vacuolate. CIPC (50 μM) selectively inhibited nuclear movement but not the initial cell division and each spore was partitioned into two cells of equal size.

Colchicine binds directly to tubulin, can prevent the assembly of MT, and can lead to their disappearance from the cytoplasm (DUSTIN 1978). CIPC does not appear to bind directly to tubulin (BARTELS and HILTON 1973), nor destroy MT, although it can prevent polymerization (BROWN and BOUCK 1974). Its mechanism of action is unknown but is probably similar to that of isopropyl-N-phenylcarbamate which disorients MT and may act on MT organizing centers (MTOC) or intermicrotubule bridges (COSS *et al.* 1975). Similar to CIPC, griseofulvin does not appear to bind directly to tubulin but may inhibit MT formation by acting upon MT associated proteins (ROOBOL *et al.* 1977).

In a study of normal germinating O. *sensibilis* spores (BASSEL *et al.* 1981), MT were observed around the nuclear envelope and associated mitochondria of migrating nuclei. The absence of MT from the nuclear region in CIPC- or colchicine-treated spores combined with the lack of nuclear movement indicates that MT probably play an essential role in movement of the nucleus from the center to the end of germinating spores. MT also appear to play an essential role in transport of secondary nuclei in *Acetabularia* (KOOP and KIERMAYER 1980) and the movement of nuclei into newly formed side branches of *Funaria* caulonema (SCHMIEDEL and SCHNEPF 1979). There is also a close relationship between nuclear position and the distribution of cortical MT in dividing *Adiantum* protonemata (WADA *et al.* 1980). Microfilaments have been implicated in wound-mediated nuclear movement in *Tradescantia* (SCHNEPF and VON TRAITTEUR 1973) but there is no evidence for their involvement during nuclear migration in O. *sensibilis* spores. Cytoplasmic streaming is absent during spore germination and cytochalasin b had no effect. Although cytochalasin b strongly inhibited rhizoid elongation, it is possible that the inhibitor did not penetrate prior to rupture of the spore coat.

As observed with electron microscopy, colchicine had different effects than CIPC in germinating O. *sensibilis* spores. Colchicine (8 mM) appeared to completely prevent MT assembly for nuclear movement and cell division. CIPC (50 μM), however, appeared to inactivate the MTOC's normally involved in nuclear movement and bipolar spindle formation without affecting the MTOC function of the chromosome kinetochore. An aggregation of mitochondria at what would be the leading edge of normal migrating nuclei was

sometimes seen in spores treated with colchicine but not CIPC. Somehow CIPC appeared to interfere with its formation.

In *O. sensibilis,* nuclear movement was more sensitive than cell division to the MT inhibitors. At suboptimal concentrations of colchicine, up to 30% of the spores were able to complete cell division but not nuclear movement. This differential sensitivity was even more pronounced in CIPC treatments in which it was possible to eliminate nuclear movement in almost all spores without preventing cell division. Differential resistance to MT-inhibitors by different "classes" of MT have been described in a number of studies (BROWN and BOUCK 1974, HARDHAM and GUNNING 1979, 1980, PICKETT-HEAPS 1967).

Sensitivity to colchicine (Fig. 11 *a*) developed prior to nuclear movement and several hours after nuclear DNA replication (FISHER and MILLER 1978). The time of sensitivity nearly coincides with the time of development of insensitivity (Fig. 11 *b*) which suggest that colchicine acts at a critical stage of short duration, presumably the time when MT form.

The early development of sensitivity to CIPC (Fig. 11 *c*) is difficult to interpret. Contrary to other studies in which the effects of CIPC were readily reversible (BARTELS and HILTON 1973, KOOP and KIERMAYER 1980) short treatments of *O. sensibilis* spores with CIPC had lasting effects upon later cell division. BROWN and BOUCK (1974) noted similar inhibitory effects of IPC upon cell division in *Ochromonas* although there was no information about the point in the division cycle at which IPC acted. The cellular target of CIPC (MTOC) is different than that of colchicine (MT), and this could explain the difference between the time of development of sensitivity to the two inhibitors. On the other hand, it is possible that CIPC did not wash out of the cells so that there was an apparent development of sensitivity at a time earlier than the execution point. Further experimentation is needed to resolve this ambiguity.

Both colchicine and CIPC lost their effectiveness when applied after a critical time in germination (16–17 hours) but prior to nuclear movement. While both inhibitors can prevent MT polymerization, CIPC does not appear to affect intact MT, and there are many examples in which intact MT are colchicine-resistant. The execution point at which colchicine acts suggests that MT may be formed for both nuclear movement and cell division at similar times. It seems unlikely that these effects can be explained by a change in permeability to the inhibitors. Major changes in spore permeability have been observed when the spore coat ruptures, about the time of cell division, but not before (MILLER 1980).

The morphogenic effects of the MT inhibitors support conclusions made from earlier studies in which membrane-active compounds, such as methanol, also inhibited nuclear migration and polar rhizoid formation (MILLER and GREANY 1976, VOGELMANN and MILLER 1981). In the presence of methanol, the

nucleus in each spore sometimes moved to the raphe but did not migrate to the end of the spore. Cell division was symmetric and each daughter cell developed into a protonema and neither into a rhizoid. The 50% response time for the development of insensitivity to methanol occurred 20 hours after initiation of germination—several hours after the time of action of the MT inhibitors. Methanol did not inhibit rhizoid differentiation after the completion of a normal asymmetric cell division.

There is a growing amount of evidence that indicates that the molecular basis of cellular polarity exists in the peripheral cytoplasm which includes the plasmalemma and the underlying cytoskeleton (QUATRANO 1978, WEISENSEEL 1979). Although both methanol and the MT inhibitors prevented asymmetric division and rhizoid formation, the sites of action are probably different. The target of the MT inhibitors appears to be the MT associated with nuclear movement whereas methanol may act by altering the association between membranes and MT, or the polarity directing processes of the plasmalemma itself.

Germinating *O. sensibilis* spores behave differently than *Fucus* zygotes (MILLER and BASSEL 1980) which have been proposed as a model system for the study of development of polarity in higher plants (QUATRANO 1978). *Fucus* zygotes undergo an initial symmetric division prior to rhizoid formation which is inhibited by cytochalasin b but not colchicine (NELSON and JAFFE 1973, QUATRANO 1973). The opposite occurs in *O. sensibilis* and although there may be similarities in the molecular basis of cellular polarity, the early events that lead to cellular differentiation are clearly different.

Rhizoids are formed following nuclear movement and asymmetric division many times during the growth and development of mature *O. sensibilis* prothalli. The function of asymmetric division may be to contain the nucleus in a special cytoplasmic region. Agents that disrupt membranes or MT destroy polar development and prevent rhizoid formation. Because cells are able to recover from colchicine treatment, it is possible to study further the destruction of polar development apart from the events associated with the reestablishment of polarity after cells are removed from inhibitors. This should allow further clarification of the relationship between membranes, cytoskeletal components, and polar plant cell development.

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