THE EFFECTS OF ISOPROPYL N-PHENYL CARBAMATE

ON THE GREEN ALGA *OEDOGONIUM CARDIACUM*

I. Cell Division

RONALD A. COSS and JEREMY D. PICKETT-HEAPS

From the Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, Colorado 80302

ABSTRACT

Cell division in vegetative filaments of the green alga *Oedogonium cardiacum* is presented as an experimental system. We report on how we have used this system to study the effects of isopropyl N-phenylcarbamate (IPC) on the mitotic apparatus and on the phycoplast, a planar array of cytokinetic microtubules. Polymerization of microtubules was prevented when filaments, synchronized by a light/dark regime and chilled (2°C) while in metaphase or just before phycoplast formation, were exposed to 5.5×10^{-4} M IPC and then returned to room temperature. Spindles reformed or phycoplasts formed when these filaments were transferred to growth medium free of IPC. However, the orientation of both microtubular systems was disturbed: the mitotic apparatus often contained three poles, frequently forming three daughter nuclei upon karyokinesis; the phycoplast was often stellate rather than planar, and it sometimes was displaced to the side of both daughter nuclei, resulting in a binucleate and an anucleate cell upon cytokinesis. Our results suggest that IPC (*a*) prevents the assembly of microtubules, (*b*) increases the number of functional polar bodies, and (*c*) affects the orientation of microtubules in *O. cardiacum*. High voltage (1,000 kV) electron microscopy of 0.5-μm thick sections allowed us to visualize the polar structures, which were not discernible in thin sections.

Isopropyl N-phenylcarbamate (IPC) and Isopropyl N-3-chlorophenyl carbamate (CIPC) are herbicides known to decrease rates of growth in plants by rapidly disrupting mitosis and cytokinesis (1–4). Initially, these carbamates were assumed to act like colchicine, and later it was shown that ICP reversibly inhibits the regeneration of cilia in *Stentor*, as indeed does colchicine (5). If flagella are amputated, they will regenerate rapidly in *Ochromonas*, but, again, IPC prevents this regeneration. If cells of *Ochromonas* are treated with periods of high pressure, microtubules disappear and later reassemble when the pressure is removed; IPC rapidly and completely prevents reassembly of microtubules in such experiments (6, 7). However, in the presence of IPC, macrotubules appeared on the rhizoplast (where microtubules arise in normal cells), apparently within a few minutes after release of the pressure. Treatment with colchicine never induced macrotubules. These experiments with *Ochromonas* have since been repeated using the volvocalean alga *Polytomella*, and apparently similar results were obtained (D. Brown, personal communication).

IPC also affects the orientation of spindle microtubules in dividing endosperm cells of the African blood lily, *Haemanthus katerinae* (8). Treatment with this drug appears to alter mitosis by inducing formation of multipolar spindles, while not affecting the appearance of the microtubules. These results suggest that IPC breaks up polar centers organizing the spindle apparatus, resulting in more than two functional poles. However, upon a careful examination of the foci of these multiple poles, no structure or organelle was found which could be implicated in the orientation of the microtubules directed towards these foci.

We have repeated and extended the experiments on *Haemanthus* but using the filamentous green alga *Oedogonium cardiacum*. The effect of IPC on the spindle and also on the phycoplast, a system of cytokinetic microtubules all oriented in the plane of cell division (9–11), was investigated. [The phycoplast is peculiar to certain green algae alone, and contrasts with the phragmoplast, the system of microtubules characteristic of cytokinesis in higher plants and a few green algae (11).] Normal and experimentally altered cells have been examined in the living state by Nomarski optics, and after fixation by conventional electron microscopy and high-voltage electron microscopy. We have, in particular, searched for macrotubules induced to form during and after treatment with IPC.

MATERIALS AND METHODS

Culture

The haploid male strain of *Oedogonium cardiacum* used in this investigation was obtained from the Indiana Culture Collection of Algae (catalogue number LB 39).

Growth Conditions

The alga was grown vegetatively in the defined medium of Hill and Machlis (12) (referred to as "H/M" medium) in 65-mm × 15-mm plastic petri dishes at 19°C under a light/dark regime of 15 h light/9 h darkness. Nuclei in vegetative filaments grown under these conditions enter mitosis approximately 4 h after the onset of darkness.

Light Microscopy

Mitosis in living cells was followed by Nomarski differential interference optics. When filaments were to be examined continuously for more than 5 min, the edge of the cover slip was sealed with Valap (13), allowing only for a small pore through which additional H/M medium was administered when necessary.

Electron Microscopy

Material to be prepared for electron microscopy was initially fixed in 2.5% glutaraldehyde in H/M medium, pH 6.9, at 22°C for 2 h. The filaments were then washed four times, 30 min each, in H/M medium, and post-fixed in 1% OsO_4 (made up in H/M medium) for 1 h at room temperature. The filaments were slowly dehydrated in acetone over the next 3 days. Dehydration was carried out on ice. The material was then slowly infiltrated with Spurr's (14) resin, flattened between two microscope slides and polymerized for 12 h at 70°C. Preselected cells were cut out, mounted on epoxy stubs and sectioned on a Reichert OM-U2 ultramicrotome (C. Reichert, Sold By American Optical Corp., Buffalo, N. Y.) with a diamond knife. Sections were picked up on Formvar-coated (0.3% Formvar) slot grids, stained for 10 min in 2% aqueous uranyl acetate (UAc) and poststained for 5 min in lead citrate at room temperature. Sections were examined in a Philips 200 electron microscope operating at 60 kV.

Filaments prepared for high-voltage electron microscopy were stained initially after fixation and before dehydration for 12 h in a 2% solution of aqueous UAc at room temperature. They were then slowly dehydrated in acetone, slowly infiltrated with Spurr's resin, flat embedded, and polymerized in the oven. Selected cells were cut out, mounted on epoxy stubs, and sectioned with glass knives with a clearance angle of 50°. 0.5-μm thick sections were picked up on Formvar-coated slot grids (three coats of 0.4% Formvar) and stained. Sections from material stained before dehydration were poststained with 2% aqueous UAc for 20–30 min and with lead citrate for 10–15 minutes at room temperature (Figs. 20, 21, 23, 25, 26). Later sections from unstained blocks were found to give similar or better results when stained with 2% UAc in absolute methanol for 20 min and then with lead citrate (Figs. 14–17) than material pretreated before dehydration with aqueous UAc. Currently, all sectioned material is stained with methanolic UAc. After staining, the grids were coated on both sides with a layer of carbon and the sections then examined in a JEM 1000 electron microscope operating at 1,000 kV.

Drug and Cold Treatments

Vegetative filaments were transferred to plastic petri dishes containing varied concentrations of IPC (obtained from Sigma Chemical Co., St. Louis, Mo.) to determine the concentration range that reversibly alters mitosis and cytokinesis. It was found that 2.2×10^{-4} M (40 ppm)–7.8 $\times 10^{-4}$ M (150 ppm) IPC was suitable for experiments. Concentrations greater than 7.8×10^{-4} M IPC were lethal to some cells.

In experiments involving a combination of cold and IPC treatments, the following schedule was used: filaments with nuclei in metaphase or just after karyokinesis were transferred to H/M medium at 2°C for 20 min and then transferred to a solution of 5.5×10^{-4} M (100 ppm) IPC in H/M medium at 2°C for 15 min. The cold, IPC-

containing cultures were then warmed to room temperature (22°C) and left for 15 min. The filaments were then transferred through three changes of fresh H/M medium at 22°C and prepared for electron microscopy 15-60 min later.

RESULTS

For 3–10 days after inoculation of new medium, greater than 80% of the nuclei in filaments of *O. cardiacum* enter mitosis about 4 h after the onset of darkness. The mitotic nucleus is characterized by large chromosomes, distinct spindle fibers (bundles of microtubules), and an intact nuclear envelope. After karyokinesis, the widely separated daughter nuclei flatten against each other and a phycoplast forms between them. The synchrony that can be induced in these filaments and the good images obtained with Nomarski optics provide an excellent system for studying the effects of various treatments on mitosis and phycoplast formation in living cells. The sequence of mitosis and cytokinesis in live cells of *O. cardiacum* is shown in Figs. 1–12. We now report on how we have used this system in experimental studies.

The poles of the mitotic apparatus in living cells of *O. cardiacum* are seen as pointed extensions of the nuclear envelope containing spindle fibers (Figs. 7, 18). Conventional electron microscopy of these poles shows spindle microtubules converging

in the extensions, but no organizing center can be identified at the area of convergence (Fig. 13) (9, 10). However, in 0.5-μm thick sections viewed at 1,000 kV, an electron-dense mass distinguishable from the nucleoplasm is visible in each polar extension (Fig. 14), and bands of spindle microtubules, the most prominent being the kinetochore microtubules (Fig. 15), converge on these foci. For brevity and because we believe that there is circumstantial evidence that the electron-dense bodies function in organizing the spindle microtubules, we will refer hereafter to the polar structures as polar "microtubule organizing centers" or MTOCs.

When filaments containing prophase nuclei were placed into a solution of 2.2 × 10^{-4} M IPC, some nuclei were prevented from further progression through mitosis, some nuclei continued through mitosis normally, and some nuclei underwent an abnormal mitosis, giving rise to more than two daughter nuclei. When nuclei that had just finished dividing were exposed to 2.2 × 10^{-4} M IPC, variable results were again encountered: some phycoplasts did not form, normal-appearing phycoplasts arose, and some abnormal, maloriented phycoplasts developed. Higher concentrations of IPC blocked formation of the metaphase spindle or formation of the phycoplast. To increase the number of abnormal mitotic apparatuses and

FIGURES 1–12 Light micrographs (Nomarski optics) showing various stages of cell division in living cells of *O. cardiacum*. Figs. 1–10, × 750; Figs. 11 and 12, × 650.

FIGURE 1 Preprophase, arrowheads indicate the division ring.

FIGURE 2 Prophase, chromosomes are condensing and the division ring is larger (arrowheads).

FIGURE 3 Prometaphase, chromosomes are fully condensed.

FIGURE 4 Metaphase, cordlike chromosomal fibers run from the kinetochores to the poles of the closed spindle.

FIGURE 5 Early anaphase, chromatids just separating.

FIGURE 6 Midanaphase.

FIGURE 7 Late anaphase, note the polar extensions of the nuclear envelope (arrowheads).

FIGURE 8 Telophase, showing collapse of the interzonal spindle.

FIGURE 9 Phycoplast, extending from between closely appressed daughter nuclei, has reached the cell periphery (arrowheads).

FIGURE 10 Separation of the daughter nuclei after the phycoplast has fully formed (arrowheads), before wall rupture.

FIGURES 11 and 12 Wall rupture and cell elongation. In both micrographs the position of the phycoplasts is indicated by arrowheads; the edge of the ruptured parental wall is indicated by the paired arrows.
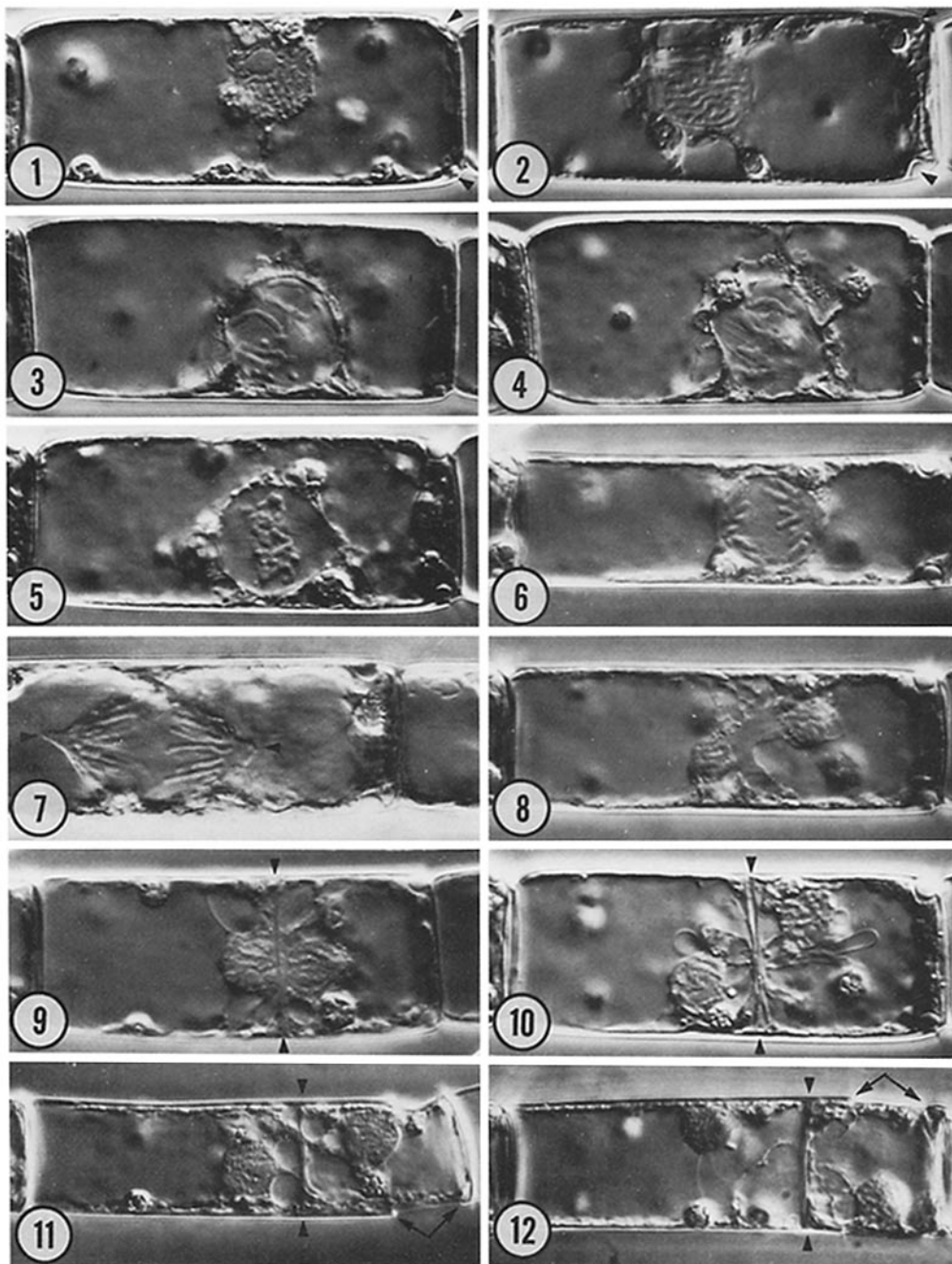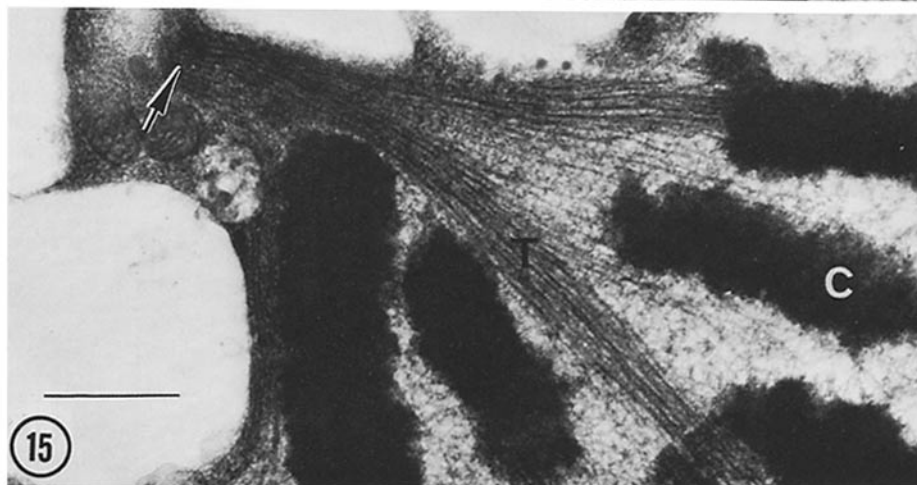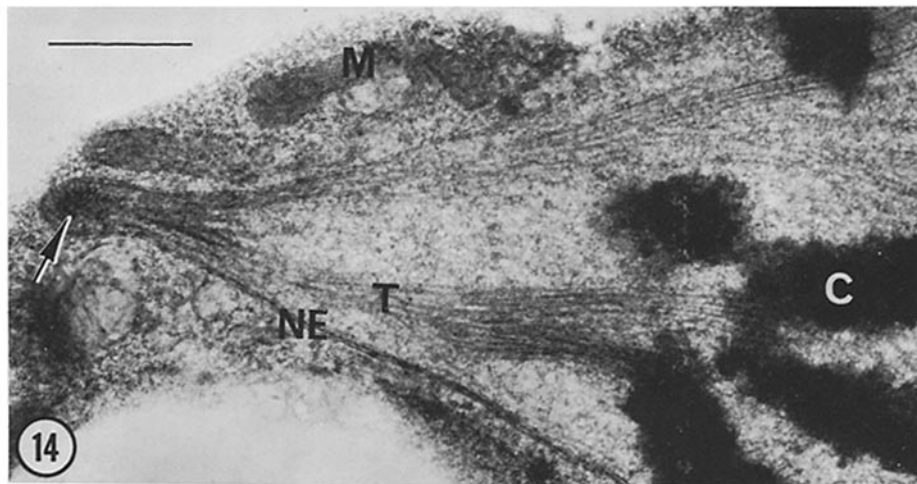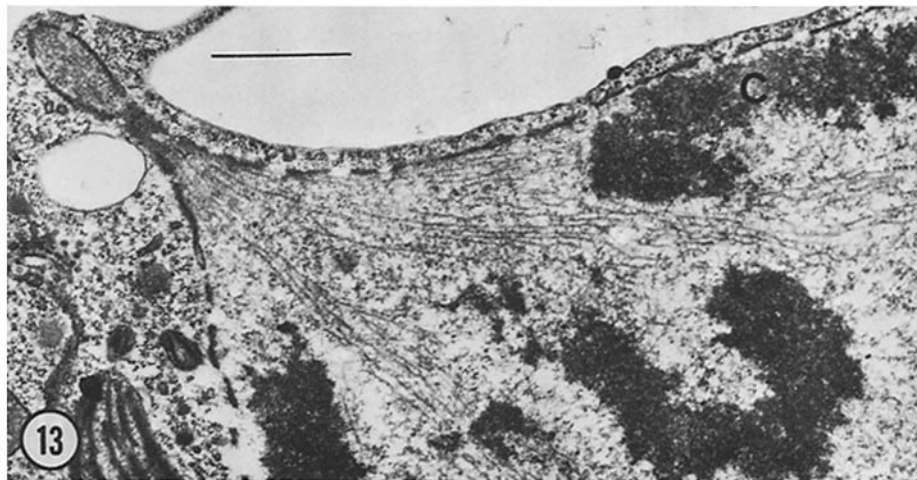
FIGURE 13   Conventional electron micrograph of a polar extension (C, chromosome). × 18,000.

FIGURES 14 and 15   High-voltage electron micrographs (HVEMs) of 0.5-μm thick sections through polar extensions. Spindle microtubules (T) embed into the polar MTOCs (arrowheads) (C, chromosome; NE, nuclear envelope; M, mitochondrion). Fig. 14, × 19,000; Fig. 15, × 18,000.

phycoplasts, and to make the results more reproducible, we devised the procedure outlined in the Materials and Methods section. This treatment: (a) disassembles the spindle or blocks formation of the phycoplast; (b) exposes the cell to the drug; and (c) then allows the spindle to reform or the phycoplast to form (in the warm) in normal culture medium minus the drug.

Treatment of filaments with cold alone for 20 min depolymerizes most spindle microtubules. A few short remnants of kinetochore microtubules remain, but the chromosomes are scattered in the nucleus (Fig. 16). When such cold-treated filaments were warmed up to room temperature, the spindle reformed and mitosis progressed in a normal manner (Fig. 17).

When cold-treated filaments were transferred to cold IPC and the solution was warmed to room temperature, the spindle apparatus did not reform. High-voltage electron micrographs of such nuclei give a picture similar to Fig. 16. However, when these cold/IPC-treated filaments were transferred to fresh medium (i.e. without IPC) at room temperature, the spindles reformed, but now they were abnormal. The spindle was rotated, often up to 90°, and the spindle fibers of the metaphase plate usually appeared to converge on more than two poles (compare Figs. 18 and 19). Three to four distinct poles defined by the kinetochore microtubules converging upon dense foci (MTOCs) were found in thick sections through such metaphase plates (Fig. 20). These MTOCs were no longer located within extensions of the nuclear envelope, as they are in normal mitotic nuclei.

The reorientation of the spindle was maintained as the chromosomes started to segregate (Fig. 21) to the poles, either two or three in number (Fig. 22). A distinct, electron-dense MTOC, into which the spindle microtubules embed, was also located at each of the poles in such anaphase nuclei when examined in the high-voltage electron microscope. Fig. 23 shows one of the polar MTOCs. Further anaphase elongation resulted in the nuclear envelope being stretched into the shape of a "Y" (Fig. 24), with chromosomes located at each apex of the "Y" (Fig. 25). Eventually the nuclei divided, each usually giving rise to three daughter nuclei (Fig. 26).

Cold treatment of filaments whose nuclei had just finished dividing blocked the formation of the phycoplast (Fig. 27). When these filaments were warmed up, microtubules polymerized, the phycoplast appeared (Fig. 28), and cytokinesis followed.

Phycoplasts did not form when cold-treated filaments were transferred to a solution of cold IPC and then warmed up in the presence of IPC. When such filaments were transferred to fresh H/M medium, phycoplasts did form. However, though the phycoplast was composed of microtubules and vesicles, their orientation was not necessarily in the normal, transverse plane. These phycoplasts were often stellate, containing strands of cytoplasm radiating out into the vacuole (Figs. 29-31). Sometimes the phycoplast did not arise between the daughter nuclei, but to one side of both (Figs. 31, 32). Such a phycoplast was still functional, the product of cytokinesis later being a binucleate and an anucleate cell (Fig. 33). Unfortunately, we have not been able to identify the MTOC(s) responsible for the nucleation and orientation of the microtubules of the phycoplast as distinct structures in either thick or thin sections.

We carefully examined filaments for the occurrence of macrotubules during and after treatment with IPC. At no time were macrotubules found associated with either the mitotic apparatus or the phycoplast.

DISCUSSION

O. cardiacum provides an unusual experimental system for the study of mitosis and cytokinesis. Several light microscopists have described cell division in the Oedogoniales [for references see Fritsch (15) and Smith (16)]; while mitosis is classical in most respects, cytokinesis is unusual, and cell elongation, involving expansion of a preformed ring of wall material, is unique. Recent ultrastructural studies of cell division in Oedogonium (9, 10, 17, 18) have verified that the nuclear envelope does not break down during mitosis. Further, these studies revealed that bundles of microtubules run from complex kinetochores into the polar extensions of the nuclear envelope, and that the cytoplasmic septum or phycoplast which forms between daughter nuclei is composed of transversely oriented microtubules interspersed with vesicles. It is now clear that O. cardiacum is excellent material for studying mitosis in live cells. The chromosomes are large and the spindle fibers are very distinct. In the absence of polar centrioles, the intact nuclear envelope and the spindle fibers define the position of the poles with great precision (Fig. 18). [Vegetative and mitotic cells are devoid of centrioles which are formed de novo in large numbers during zoosporogenesis and spermiogenesis (19, 20).] The
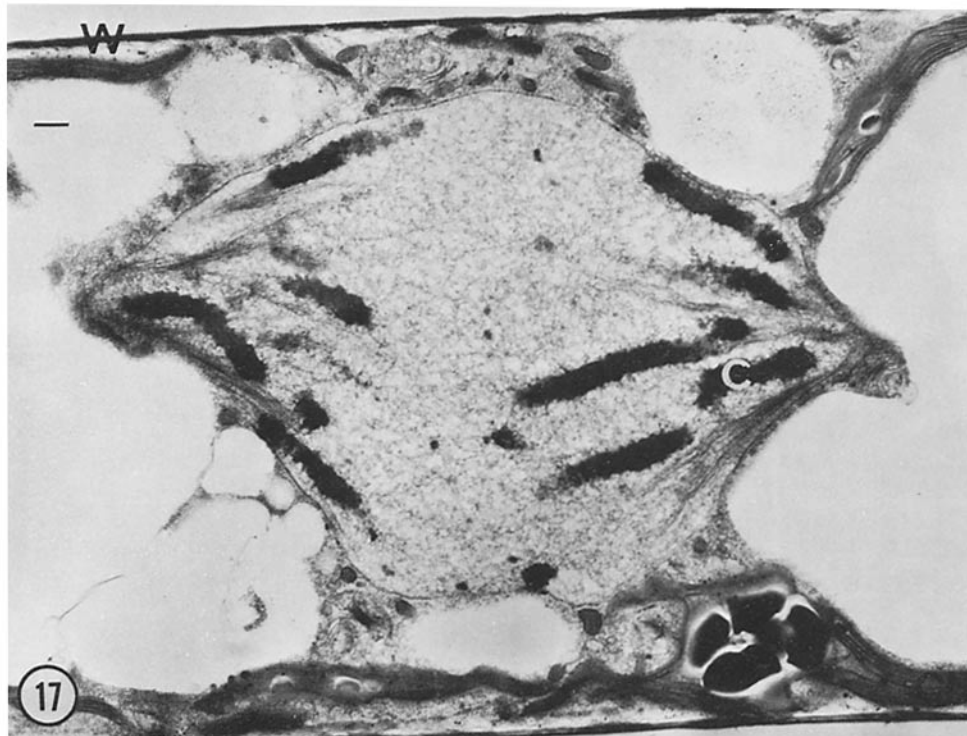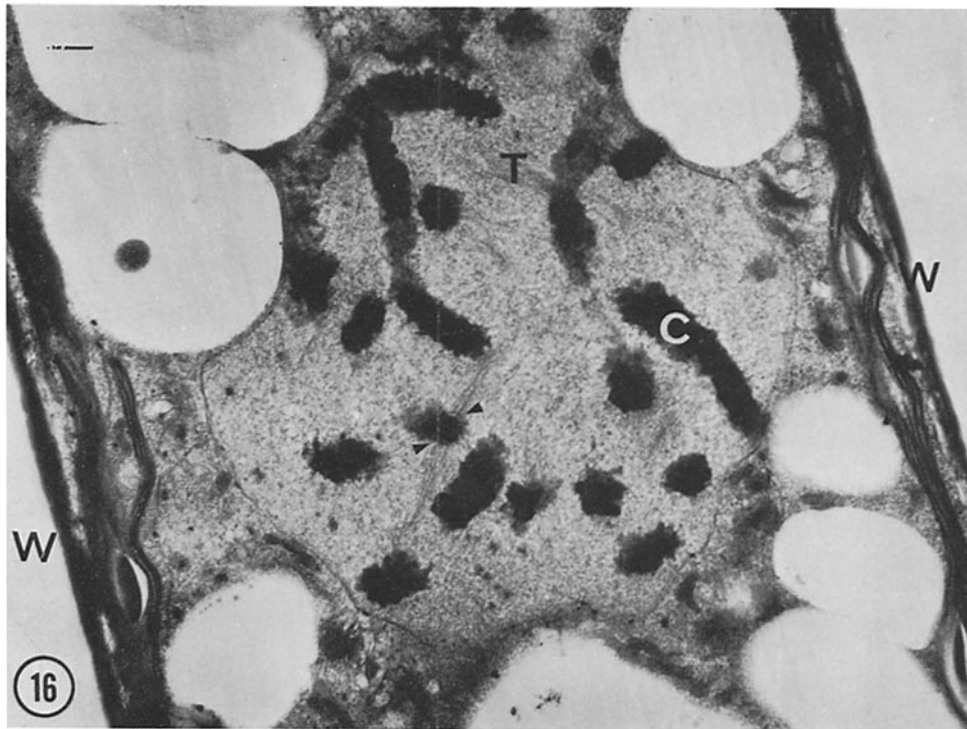
FIGURE 16 HVEM of a metaphase nucleus that had been chilled at 2°C for 20 min. Only a few spindle microtubules (T) remain, specifically those associated with the kinetochores, and the chromosomes (C) are randomly oriented within the nucleus. The paired kinetochores are indicated by the arrowheads (W, cell wall). × 6,000.

FIGURE 17 HVEM of a nucleus treated as in Fig. 16, but after recovery by transfer to room temperature. The anaphase nucleus is normal in all respects (C, chromosome; W, cell wall; Ch, chloroplast). × 5,000.
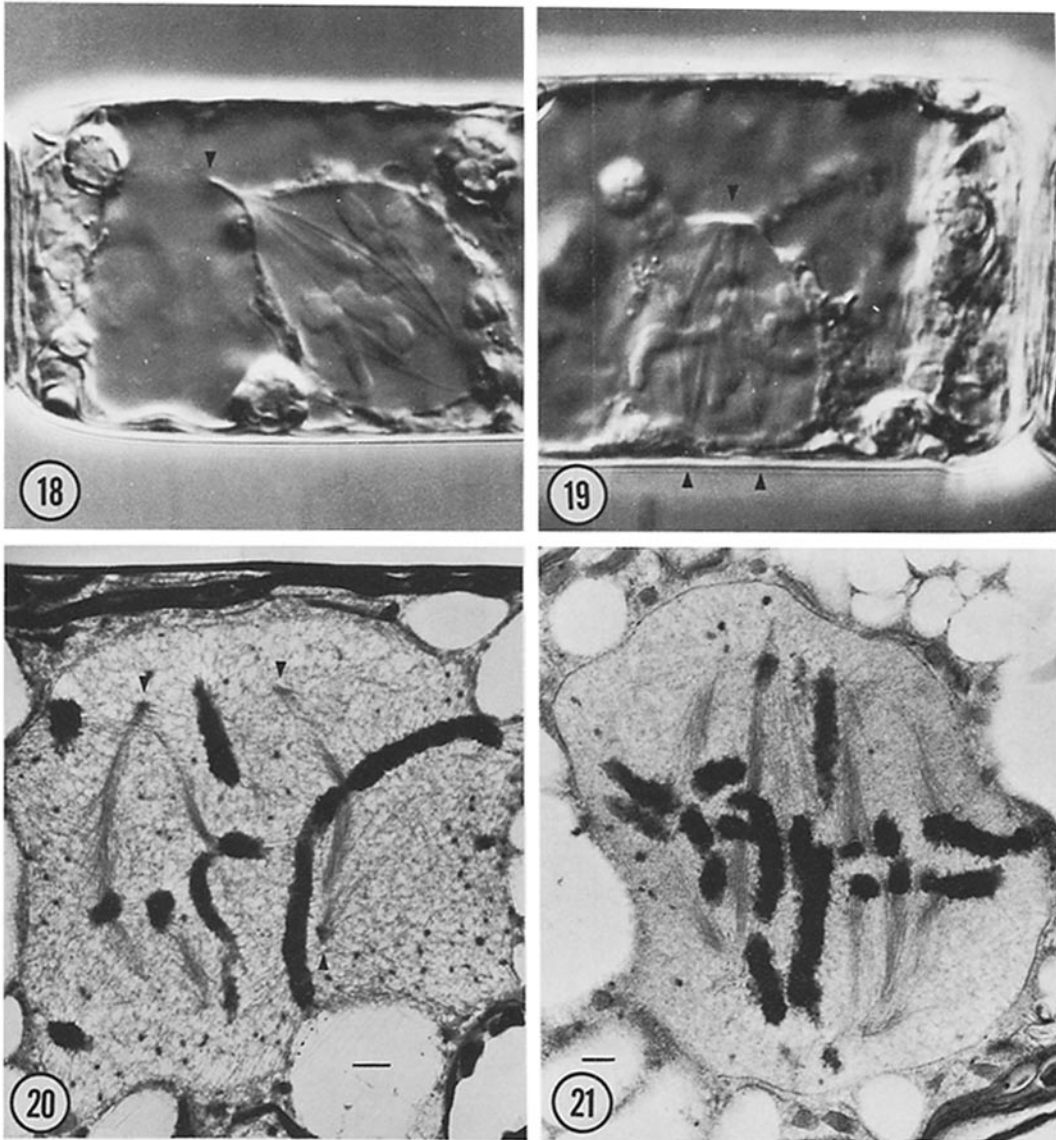
FIGURE 18   Normal metaphase spindle in a live cell of *O. cardiacum*. Note the polar extension (arrowhead). × 1,600.

FIGURE 19   This cell has been chilled (2°C) at metaphase and then treated with IPC. Later the cell was brought to room temperature and the IPC washed out. The spindle has reformed, but at right angles to the cell axis, and it is now tripolar (arrowheads). × 1,400.

FIGURE 20   HVEM of a cell similar to that in Fig. 19. Three polar bodies are seen (arrowheads). × 5,000.

FIGURE 21   HVEM of early anaphase in a cell whose metaphase plate was similar to that in Fig. 19. Though the sister chromatids have separated, the abnormal axis of the spindle is preserved. × 4,500.

considerable degree of synchronization of cell division obtainable in *O. cardiacum* and the ease with which this alga may be cultured are added attractions for such studies. *O. cardiacum* provides a good experimental system for studying the formation and orientation of cytoplasmic (i.e. extranuclear) microtubules, specifically those of the phycoplast, in live cells for many of the same
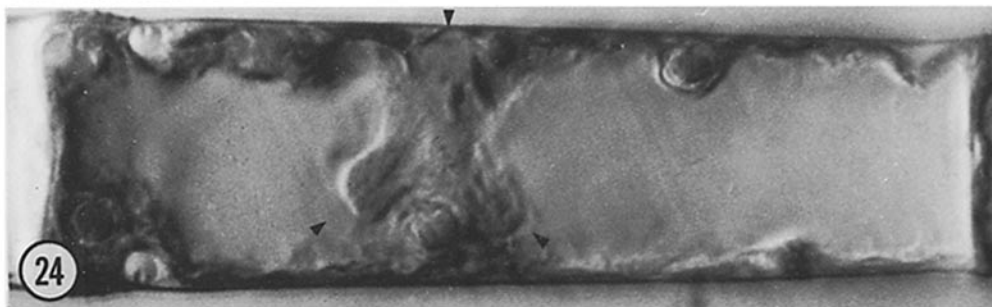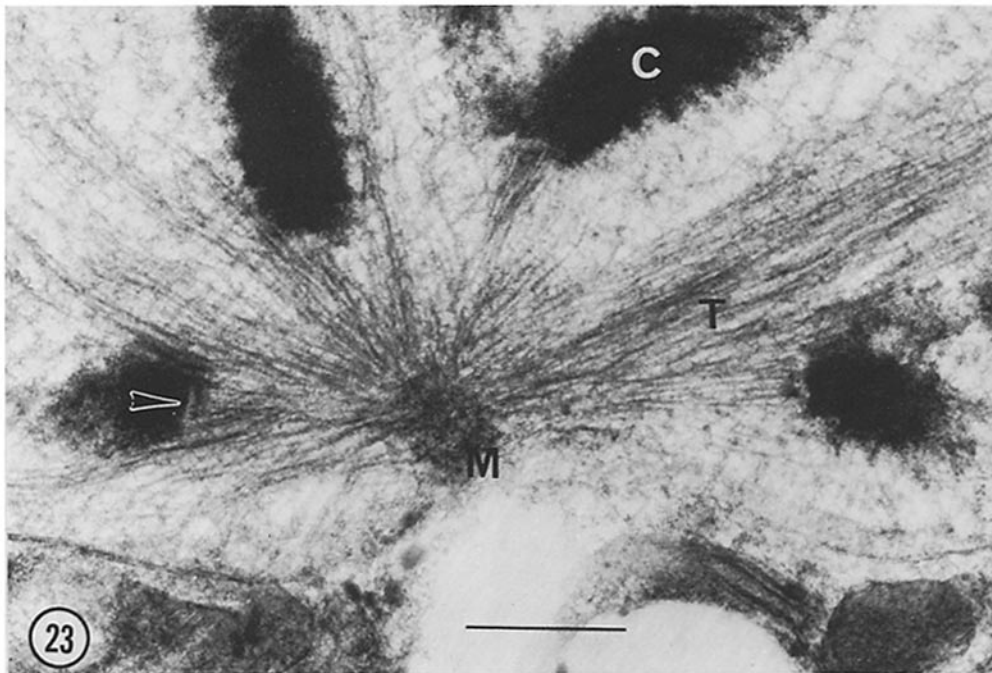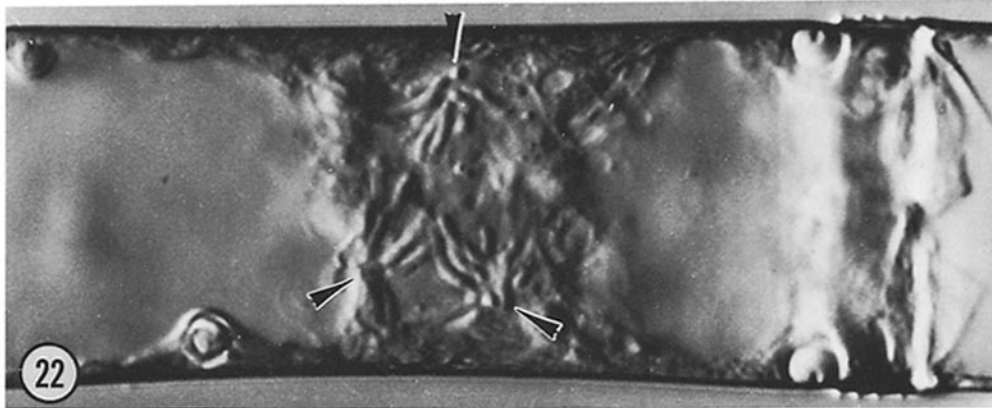
FIGURE 22 Tripolar anaphase (arrowheads) in a cell treated as in Fig. 19, but after a longer period of recovery. × 1,700.

FIGURE 23 HVEM of one of the poles of a tripolar nucleus similar to that in Figure 22. Numerous spindle microtubules (*T*) embed into the polar MTOC (*M*) (*C*, chromosome; arrowhead, kinetochore). × 21,000.

FIGURE 24 Late anaphase in a cell after recovery from treatment as in Fig. 19. The nucleus is stretched into the shape of a "Y" (arrowheads). × 1,500.
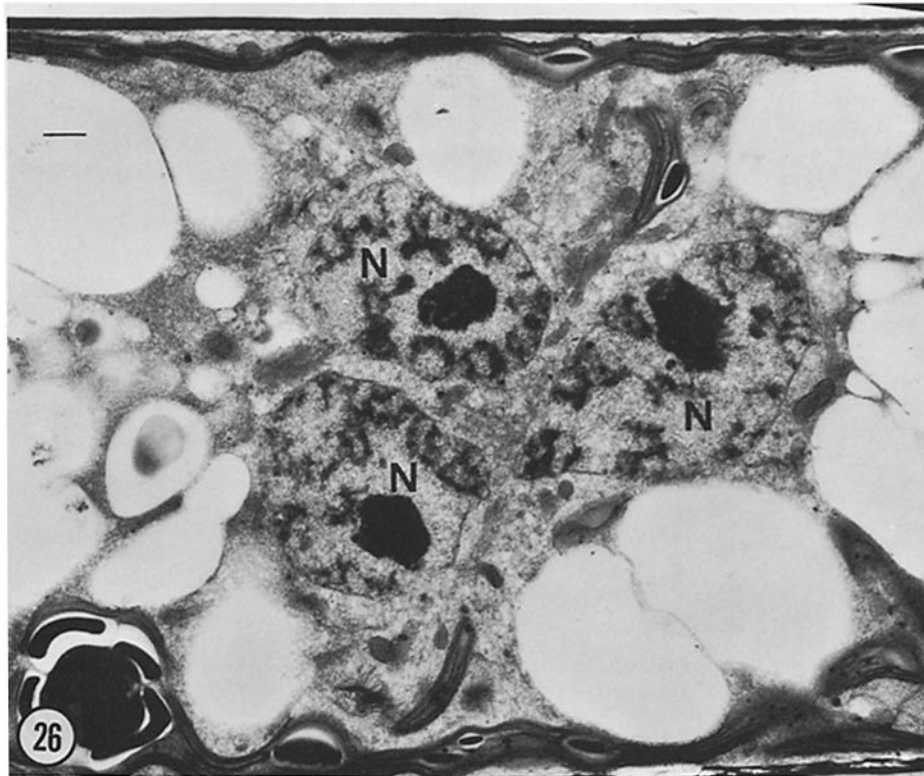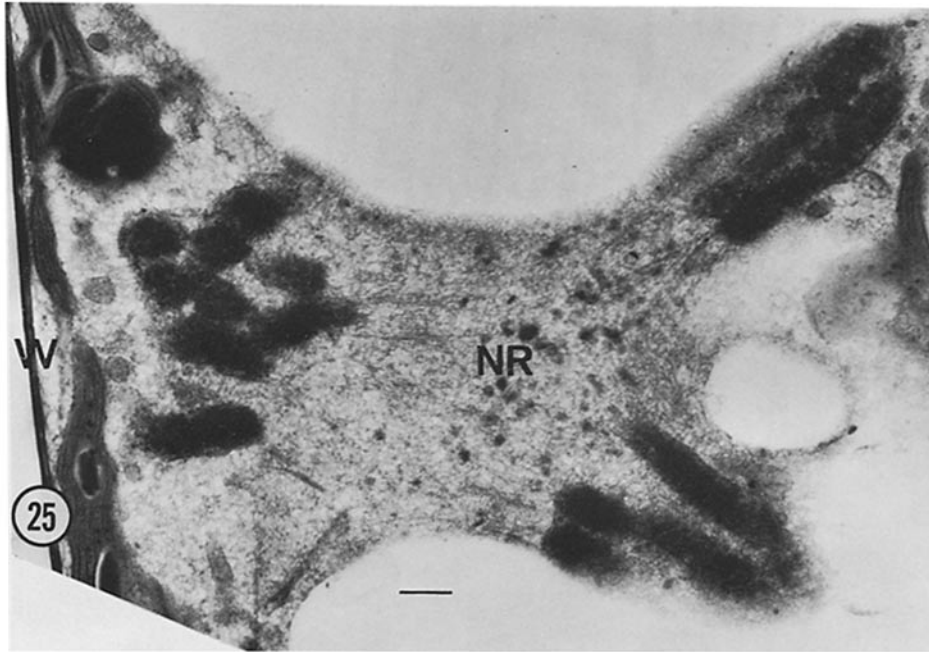
FIGURE 25   HVEM of a cell similar to that in Fig. 24, showing chromosomes at each apex of the "Y"-shaped nucleus (NR, nucleolar remnants; W, cell wall). × 7,000.

FIGURE 26   The product of division of a nucleus similar to that in Fig. 25 is a cell with three nuclei (N). × 5,500.
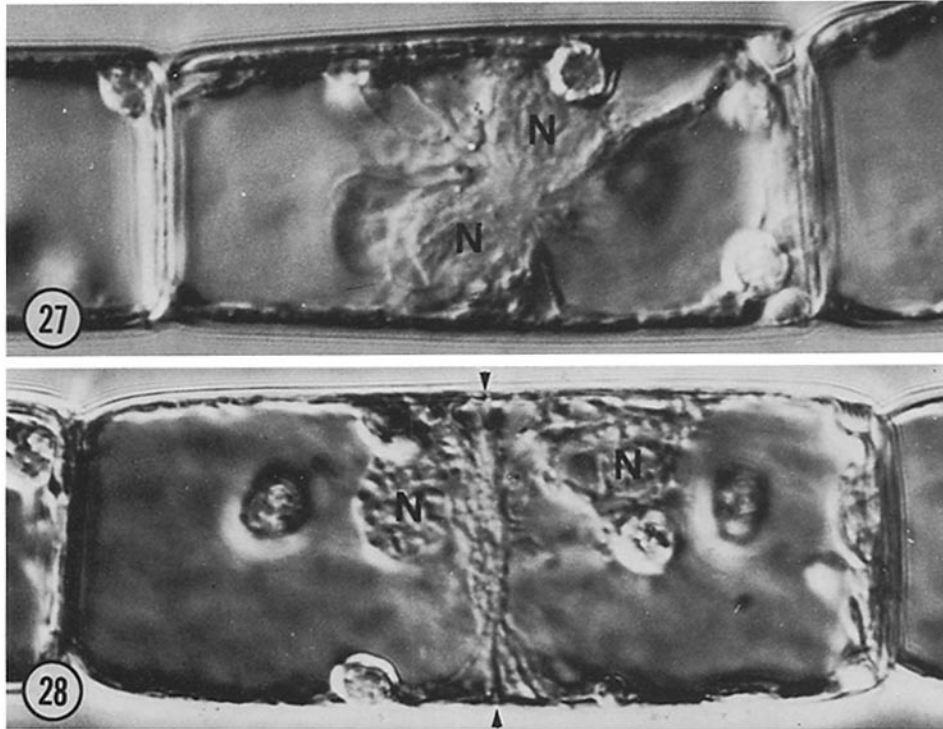
FIGURE 27 The phycoplast is prevented from forming in this cell by chilling it (2°C) just after nuclear division (*N*, nucleus). × 1,500.

FIGURE 28 A cell as in Fig. 27, but after recovery by transfer to room temperature. The phycoplast has formed between the daughter nuclei (*N*) and extends to the cell periphery (arrowheads). × 1,500.

reasons. Finally, live cells can be successfully prepared for electron microscopy, so the ultrastructure of dividing cells can be compared with results obtained with light optics.

A technique most valuable to this research has been high-voltage electron microscopy of thick sections. Although the nuclear envelope is ex-tended at the poles of mitotic nuclei, no recognizable structure has been identifiable in thin sections of such poles (Fig. 13) (9, 10). The presence of polar structures in spermatogenous nuclei undergoing division (21) suggests that they are probably also present in vegetative filaments, but cannot be distinguished from the nucleoplasm in thin sec-

FIGURES 29–33 Cells of *O. cardiacum* that were chilled (2°C) just after nuclear division, exposed to IPC, returned to room temperature and allowed to recover in IPC-free medium.

FIGURE 29 A cell whose newly formed phycoplast is stellate rather than planar. × 1,200.

FIGURE 30 A cell showing another stellate phycoplast (*N*, nucleus). × 1,200.

FIGURE 31 The abnormal phycoplast in this cell did not form between the daughter nuclei; the division ring has just ruptured (paired arrows). × 1,100.

FIGURE 32 A cell with a fully formed phycoplast (arrowheads) dividing the cell into binucleate and anucleate portions; the wall has just ruptured at the division ring (paired arrows). × 1,200.

FIGURE 33 The result of division of the cell shown in Fig. 32 is a binucleate cell (double arrowhead) next to an anucleate cell (single arrowhead). × 600.
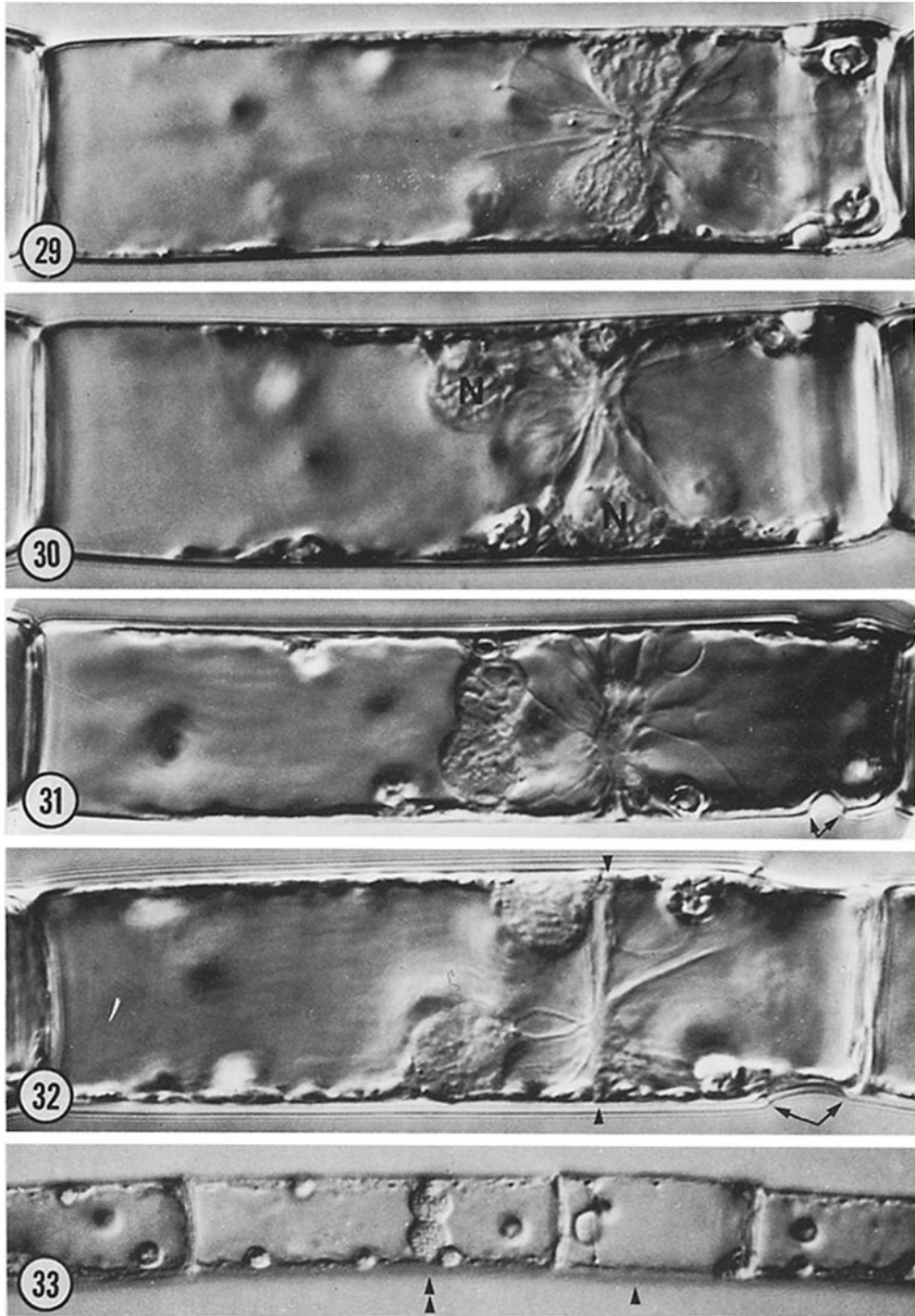
tions. Polar material, denser than the rest of the nucleoplasm, was found at the tip of each of the polar evaginations in 0.5-μm thick sections viewed at 1,000 kV (Fig. 14), and these structures are further defined by the numerous spindle microtubules that converge on them. The polar MTOCs become even clearer as distinct entities in some experimentally treated cells (Fig. 23).

IPC affected at least three phenomena in mitotic cells of *O. cardiacum*. First, in agreement with earlier reports (5–8), this drug prevented the assembly of microtubules and/or did not depolymerize existing microtubules. Secondly, the orientation of forming and reforming spindles was profoundly altered (Figs. 19–23). Thirdly, treatment of filaments with IPC increased the number of polar MTOCs. The second and third effects were observed after the drug was washed out. These findings support the suggestion made by Hepler and Jackson (8) that IPC disrupts mitosis by leading to the formation of multipolar spindles. Both Doxey (2) and Hepler and Jackson (8) documented the segregation of chromosomes to more than two sites in cells treated with IPC. However, they were unable to find any structure at the foci of spindle microtubules. In this paper we have shown through the use of high-voltage electron microscopy of thick sections that a morphologically distinct MTOC is found at the foci of chromosome segregation in both normal mitotic spindles (Figs. 14, 15) and in the experimentally induced multipolar spindles (Fig. 23). We further show that treatment with IPC increases the number of functional polar MTOCs; three daughter nuclei were often formed in experimentally treated cells and this number corresponded with the number of polar MTOCs observed in many cells at metaphase.

We can offer no explanation for the reorientation of the spindle in experimentally treated cells (Figs. 19, 20, 21); the lack of polar extensions seems to be associated with the kinetochore fibers being shorter than normal (compare Figs. 18 and 19). Neither phenomenon is induced by cold alone, because mitosis progressed in a normal manner in cold-treated filaments that were warmed up to room temperature (Fig. 17). Nor are they a result of the combined use of cold and IPC, since filaments initially treated at room temperature with IPC demonstrated similar abnormalities, although less frequently. These two phenomena appear to follow from treatment with IPC alone.

IPC affected both the polymerization and orientation of cytoplasmic (phycoplast) as well as nuclear (spindle) microtubules in *O. cardiacum*. Cytoplasmic microtubules were prevented from polymerizing in filaments exposed to IPC at room temperature, but shortly after these filaments were transferred to fresh H/M medium, the phycoplast formed. When cold/IPC-treated filaments were transferred to fresh medium (22°C), phycoplasts were also formed. The orientation of the phycoplast was changed, however, often being stellate rather than planar (Figs. 29, 30, 31). Treatment with cold alone was not responsible for the altered disposition of the phycoplast, since filaments about to form phycoplasts when blocked from doing so with cold (Fig. 27) can later form normal appearing and functional phycoplasts upon warming (Fig. 28). Also the phycoplast may not even form between IPC-treated daughter nuclei (Figs. 31, 32). However, such a phycoplast was still functional; an anucleate and a binucleate pair of daughter cells were often formed by the IPC treatment (Fig. 33).

In this investigation, we confirm that IPC disturbs the orientation of the microtubules arising from the polar MTOCs and the phycoplast MTOC(s). Since treatment with IPC results in altered orientation of the phycoplast microtubules after the drug has been washed out, we suggest that it causes this effect by acting on the MTOC(s), rather than on the subunits of the microtubules. Perhaps this drug changes the number or structure of the MTOC(s) in the phycoplast as it does to polar MTOCs, resulting in a change in the orientation of the microtubules after the drug is washed out; but until the MTOC(s) of the phycoplast and changes in them induced by IPC can be clearly visualized, this must remain speculation.

IPC prevented polymerization of microtubules in *O. cardiacum*. Thus, it probably also reversibly affects the subunits of the microtubules as well as having a longer term effect on the MTOCs. Vermani and Bouck (manuscript in preparation) have recently obtained results that also suggest that IPC interacts with the components of microtubules. They found that if *Ochromonas* was continuously treated with vinblastine and subjected to brief pressure treatment (sufficient to depolymerize microtubules), no macrotubules formed upon release of the pressure. However, macrotubules did appear on the rhizoplast after pressure treatment in the continuing presence of both IPC and

vinblastine. They interpret their results as showing that ... "the IPC/macrotubular tubulin or proto-filaments may well be in the same form as the tubulin assumes after prolonged incubation with vinblastine alone (*i.e.* paracrystal, presumably of closed-packed macrotubules)" (G. B. Bouck, personal communication). At no time in our research have we encountered macrotubules after treatment with IPC or with a combination of cold and IPC.

In our experiments and those on *Ochromonas* (6, 7) and *Polytomella* (D. Brown, personal communication), the concentrations of IPC used to alter cell division and/or microtubular structure are 10 or more times higher than the concentrations used to block normal spindle alignment in *Haemanthus* endosperm cells (8) and in dividing microspores of *Marsilea* (P. Hepler, personal communication). We agree with Hepler's suggestion that the higher concentrations of IPC necessary to alter microtubular systems in these algae may be due to lack of uptake of IPC, and may also reflect a more highly organized or tightly structured spindle pole in *Oedogonium* than is found in *Haemanthus*.

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