MICROTUBULE BIOGENESIS AND CELL SHAPE IN *OCHROMONAS*

II. The Role of Nucleating Sites in Shape Development

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ABSTRACT

The proposal made in the preceding paper that the species-specific shape of *Ochromonas* is mediated by cytoplasmic microtubules which are related to two nucleating sites has been experimentally verified. Exposure of cells to colchicine or hydrostatic pressure causes microtubule disassembly and a correlative loss of cell shape in a posterior to anterior direction. Upon removal of colchicine or release of pressure, cell shape regenerates and microtubules reappear, first in association with the kineto-beak site concomitant with regeneration of the anterior asymmetry, and later at the rhizoplast site concomitant with formation of the posterior tail. It is concluded that two separate sets of cytoplasmic tubules function in formation and maintenance of specific portions of the total cell shape. On the basis of the following observations, we further suggest that the beak and rhizoplast sites could exert control over the position and timing of the appearance, the orientation, and the pattern of microtubule distribution in *Ochromonas*. (a) the two sites are accurately positioned in the cell relative to other cell organelles; (b) in regenerating cells microtubules reform first at these sites and appear to elongate to the cell posterior; (c) microtubules initially reappear in the orientation characteristic of the fully differentiated cell; (d) the two sets of tubules are polymerized at different times, in the same sequence, during reassembly or resynthesis of the microtubular system. Experiments using cycloheximide, after a treatment with colchicine, have demonstrated that *Ochromonas* cannot reassume its normal shape without new protein synthesis. This suggests that microtubule protein once exposed to colchicine cannot be reassembled into microtubules. Pressure-treated cells, on the other hand, reassemble tubules and regenerate the normal shape in the presence or absence of cycloheximide. The use of these two agents in analyzing nucleating site function and the independent processes of synthesis and assembly of microtubules is discussed.

The preceding paper (Bouck and Brown, 1973) examines the fine structure of vegetative and dividing cells of *Ochromonas* and describes, (a) a close correlation between the distribution of cytoplasmic microtubules and the asymmetric cell shape, and (b) the association of these microtubules with two distinct sites in the cell anterior. These observations implied a cytoskeletal role for microtubules and further suggested that control of microtubule distribution, and hence control of cell shape, may be effected by restricting microtubule assembly to specific nucleating sites (terminology of Tilney and Goddard, 1970).

The present study was undertaken to determine if the observed microtubule distribution is obligatory to development and/or maintenance of cell shape, as well as to clarify whether the presumptive nucleating sites do in fact function as sites of initial microtubule assembly. It was found that microtubule depolymerizing agents (colchicine

or hydrostatic pressure) produce a loss of cell shape concomitant with tubule disassembly. In cells regenerating the normal shape microtubules reassemble at and appear to elongate from two nucleating sites. Since changes in cell shape could be observed by light microscopy and occurred in approximate synchrony in populations of *Ochromonas*, the investigation was extended to examine the kinetics of shape loss and regeneration and the dependence on new protein synthesis in regenerating cells. By inhibiting protein synthesis with cycloheximide it is demonstrated that colchicine may bind irreversibly to microtubule monomers, suggesting that colchicine may be less suitable for studying microtubule assembly than pressure or isopropyl *N*-phenylcarbamate (IPC), in this organism (Brown and Bouck, 1971).

MATERIALS AND METHODS

Cultures

Ochromonas danica was grown on defined medium as described previously (Bouck and Brown, 1973, adjoining paper). Under those conditions, increase in cell number is logarithmic to 7-10 $\times$ 10^6 cells/ml 4 days after inoculation. For the following experiments cells were harvested in the log phase of a growth at a density of 2-5 $\times$ 10^6 cells/ml.

Chemical Treatments

Reagents used to induce shape changes or to modify shape regeneration were dissolved in fresh, sterile culture medium just before use. Cells were harvested by low speed centrifugation, resuspended in the treatment solution (colchicine or cycloheximide, Sigma Chemical Co., St. Louis, Mo.), and incubated under constant light and temperature (22°C). To investigate recovery after a period of treatment, cells were again centrifuged and gently resuspended in fresh control medium.

Hydrostatic Pressure

The apparatus for pressure treatment consisted of a hydraulic press and French pressure cell (American Instrument Co., Inc., Silver Spring, Md.) connected in series by high pressure tubing to a pressure gauge shut-off valve, and a pressure-fixation chamber similar to that described by Landau and Thibodeau (1962). Cells were subjected to 4000-8000 psi for 10, 20, or 30 min and were then either fixed under pressure or the pressure was released and recovery was monitored. To determine the effects of different reagents on recovery, samples were suspended in the appropriate treatment solution just before pressure

application and were not changed during the recovery period.

Microscopy

Samples of living cells were assayed at appropriate intervals during exposure to colchicine or cycloheximide, or during recovery from experimental treatment. Photographs of swimming cells were obtained with a Zeiss Photomicroscope equipped with Nomarski optics, a Zeiss 30 W·s electronic flash and ADOX KB 14 film developed in Diafine (Acufine, Inc., Chicago, Ill.). At the same time intervals samples were also fixed for quantitative counts of shape change (see Results) and for electron microscopy. For cell counts 1 ml samples were fixed by addition of 1 ml of buffered 4% glutaraldehyde. For electron microscopy all fixations were carried out at room temperature for 1.5 h in 3% glutaraldehyde (0.1 M sodium phosphate buffer, pH 6.9) followed by a 2-12 h postfixation in cold 1% osmium tetroxide in the same buffer. Techniques for subsequent handling of cells, sectioning, and electron microscopy are described in the preceding paper.

Amino Acid Incorporation

The incorporation of amino acids into trichloroacetic acid (TCA) precipitable protein was determined using the filter disc method (Mans and Novelli, 1961). 5-ml samples of cells were incubated at constant light and temperature in 25-ml Erlenmeyer flasks. Radioactive amino acids [^{3}H]leucine, [^{14}C]protein hydrolysate; Schwarz Bio Research Inc., Orangeburg, N.Y., or uniformly labeled [^{14}C]amino acid mixture; New England Nuclear Corp., Boston, Mass.) were added to the samples to give a final specific activity of 5 μCi/ml. At appropriate intervals 100-μl samples were taken with sterile capillary pipettes, spotted on 2.3 cm filter discs, and stored in cold 10% TCA. At the end of an experiment the filter discs were processed for liquid scintillation counting after the extraction schedule of Rosenbaum et al., (1969).

To increase uptake of the labeled amino acids, cells were harvested by low speed centrifugation, washed, and resuspended (all under aseptic conditions) in medium lacking amino acids at least 3 h before addition of label. Periodic visual examination of samples was made to ensure absence of bacterial contamination.

The cultures used in these studies were in log phase but nonsynchronous growth. Therefore, even over short terms cell divisions occurred in control samples but were not observed in samples exposed to certain reagents (e.g. the antimitotic agent colchicine). To compensate for this difference in cell number during an experiment, cell counts were made at regular

intervals and incorporation is expressed on a per cell basis. The validity of this assay was originally tested on nondividing, biotin-starved cultures obtained as described by Dubnau (1961). Since no significant differences in regeneration or incorporation kinetics were noted in these nondividing cells all subsequent studies were carried out on log phase cultures growing in normal biotin-supplemented media.

RESULTS

In untreated 4-day cultures of *Ochromonas* between 70 and 90% of the cells exhibit the characteristic asymmetric form and posterior tail. Treatment of such cells with colchicine or hydrostatic pressure results in a dramatic modification of cell shape within minutes. The cells lose their elongate form in each case and with continued treatment may become spherical. The percentage of cells sphering in response to either of these agents is dependent on the magnitude and the duration of exposure. These fully reversible changes in cell form can be observed quantitatively in the entire cell population so that the kinetics of shape change can be determined. Samples taken at intervals during a treatment or recovery period were examined and at least 100 cells per sample were scored as pos-
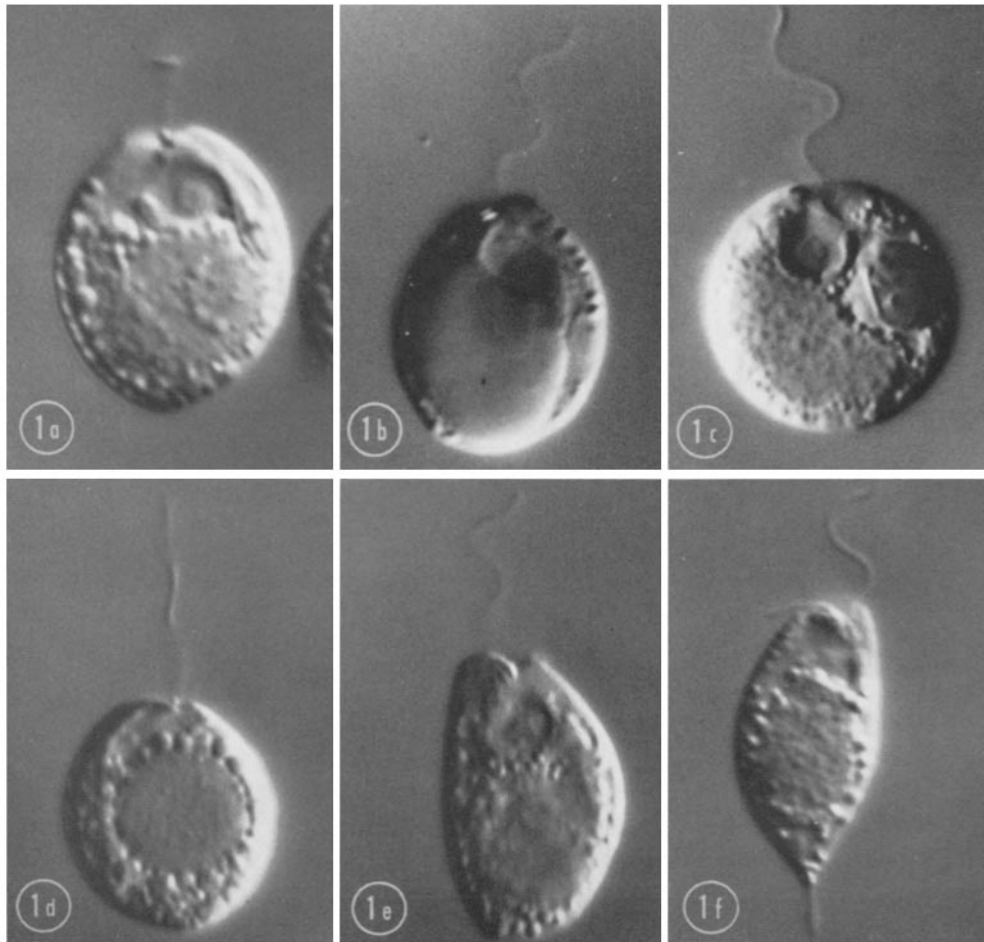
FIGURES 1 a–f Flash photomicrographs of swimming *Ochromonas* showing loss of shape in 10 mg/ml of colchicine (1 a–1 c), and subsequent shape regeneration when colchicine is removed (1 d–1 f). Fig. 1 a, 15-min exposure to colchicine; the elongate tail has disappeared. Fig. 1 b, 2-h exposure; note rounding of the cell posterior. Fig. 1 c, 4–5-h exposure; cells are completely spherical. Fig. 1 d, 3-h recovery; early indication of anterior asymmetry. Fig. 1 e, 6-h recovery; anterior shape regenerated and cell has elongated. Fig. 1 f, 10-h recovery; normal cell shape fully regenerated. × 2500.

sessing: (a) the normal elongate form including tail (Fig. 1 f); (b) intermediate shape, no tail (Figs. 1 a, 1 b, 1 d, and 1 e); or (c) completely spherical shape (Fig. 1 c). Electron microscopy of cells fixed at the same time intervals permitted correlations between changes in cell shape and the distribution of cytoplasmic microtubules.

Light Microscope Observations

EFFECTS OF COLCHICINE ON CELL SHAPE

LOSS OF SHAPE IN COLCHICINE: Cells suspended in colchicine solutions of appropriate concentration lose their characteristic form and ultimately become spherical. Change in shape begins almost immediately after suspension in colchicine and follows a regular pattern. The posterior tail rapidly shortens and then disappears, resulting in a cell which is still elongate and in which the morphology of the beak and platform area is unchanged (Fig. 1 a). This is followed by a gradual rounding of the cell which is first apparent in the posterior region and then progresses toward the cell anterior (Fig. 1 b). The anterior asymmetry is the most resistant to the action of colchicine but ultimately a uniform population of spherical cells is obtained (Fig. 1 c). The spherical cells are still fully motile, show the normal forward movement, but no longer rotate as they swim.

The degree of shape loss is dependent on the concentration and the duration of exposure to colchicine. Fig. 2 shows the effects on cell shape of a 5-hr exposure to increasing colchicine concentrations. Little or no effect is observed below 2 mg/ml even after prolonged treatment (up to 24 h). Concentrations of 20 mg/ml are still fully
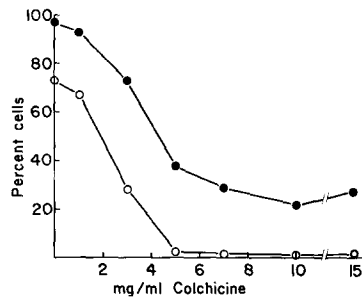
FIGURE 2 Shape loss in increasing concentrations of colchicine. Open circles, percentage of cells with tails (see Fig. 1 f); closed circles, percentage of cells with tails plus those with intermediate shape (see Figs. 1 a, 1 b, 1 d, and 1 e) after 5-h exposure to colchicine.
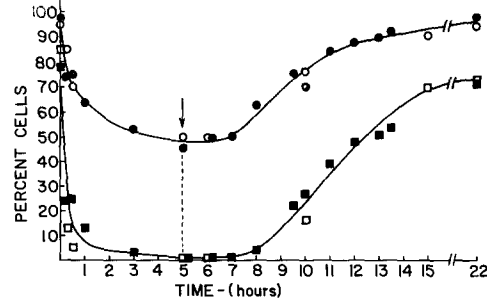
FIGURE 3 Kinetics of shape loss and regeneration in two populations of *Ochromonas* (open and closed symbols) exposed to 10 mg/ml colchicine. Colchicine washed out and cells resuspended in fresh media at 5 h (arrow). Squares, percentage of cells with tails; circles, percentage with intermediate shape.

reversible. The lowest concentration of colchicine which consistently produced uniform populations of rounded cells is 10 mg/ml (2.5 × 10⁻² M).

Fig. 3 shows the kinetics of shape loss and regeneration in two separate populations exposed to 10 mg/ml of colchicine for 5 h. As already indicated by the pattern of shape loss (Figs. 1 a–1 c) the tail appears to be most sensitive to colchicine since within a 15–30 min exposure most tails have disappeared. The gradual rounding of cells proceeds slowly throughout the period of treatment. By 5 h no tails are observed and between 50 and 75% of the cells are completely spherical (Fig. 1 c). A portion of the cells (25–50%) retain a small anterior ridge that is not affected by extended colchicine treatment.

REGENERATION OF SHAPE IN COLCHICINE-TREATED CELLS: The effects on cell shape of a 5-h exposure to colchicine are slowly but completely reversible (Fig. 3). When the cells are resuspended in colchicine-free medium there is a 2–3-h lag during which no change in cell shape is detected. Then in the succeeding 6–8-h period the population regenerates the anterior asymmetry, followed by elongation of the tail. 10 h after treatment the population appears identical to a control culture never exposed to colchicine.

Light microscope observation of cells during the recovery period indicate that shape regeneration proceeds in a polar fashion but in the reverse sequence to shape loss. Regenerating cells first reform the beak and platform (Fig. 1 d), elongate (Fig. 1 e), and finally form the tail (Fig. 1 f). In no case where cells observed to reform the tail before regeneration of the anterior region. The

swimming cell resumes rotation about its long axis as soon as the beak and platform asymmetry has regenerated.

EFFECT OF HYDROSTATIC PRESSURE ON CELL SHAPE

Hydrostatic pressure has been reported to disassemble microtubules and produce shape changes in several cell types (Tilney et al., 1966; Tilney and Gibbins, 1969; Kennedy and Zimmerman, 1970) and the effects of this agent on a variety of cell functions have been extensively studied (see Zimmerman, 1970 for review). As an alternative to the use of chemical agents we have tested the effects of pressure on the pattern, kinetics, and fine structure of shape loss and regeneration in *Ochromonas*.

LOSS OF SHAPE WITH PRESSURE: No observation of living cells was made during exposure to pressure. However, observation of cells fixed under pressure after 10, 20, or 30 min of exposure shows a pattern of shape loss identical to that in colchicine-treated cells. Fig. 4 summarizes the changes in shape at different pressures. 7000–8000 psi for 30 min produces completely spherical cells, while lower pressure (5000–7000 psi) produces a population with normal anterior asymmetry but no tails. At any pressure that yields spherical cells (e.g. 8000 psi) the cells first lose the tail, progressively round from the posterior to anterior, and lastly lose the beak and platform. At pressures near 8000 psi a small percentage of cells also lose their flagella. Extending the pressure above 8000 psi results in deflagellation of most cells, and above 12,000 psi it results in complete cytolysis.

REGENERATION OF SHAPE: The pattern of shape regeneration in pressure-treated cells confirms the observations on regeneration of colchicine-treated cells. Regenerating cells first reform the beak and platform, then elongate, and finally reform the tail.

Although the pattern of regeneration appears identical in colchicine- and pressure-treated cells, the kinetics of recovery are very different. Regeneration of shape begins almost immediately upon release of pressure and is complete within 2 h (Fig. 19). The duration of the recovery period is dependent on the magnitude of the pressure applied (at least to 8000 psi), but is reproducible at any one pressure. At pressures above 8000 psi the
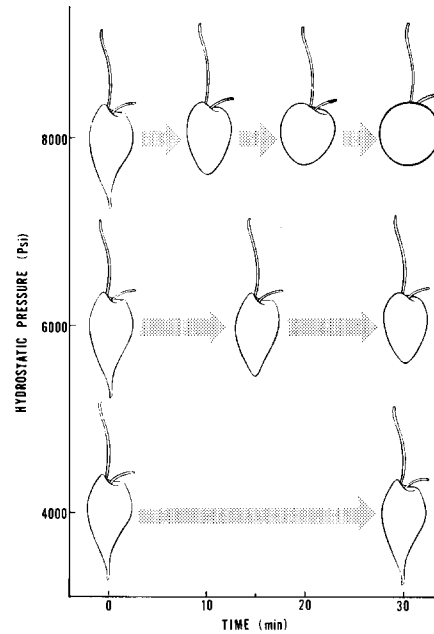

FIGURE 4 Diagrammatic representation of shape loss during a 30-min exposure to 4000, 6000, or 8000 psi hydrostatic pressure.

synchrony of regeneration is lost, apparently resulting from the presence of two populations of regenerating cells in the sample; spherical cells with flagella and spherical cells lacking flagella. Cells deflagellated by the pressure treatment regenerate flagella and cell shape simultaneously, perhaps in competition. This deflagellated population regenerates cell shape more slowly than spherical cells with flagella.

Electron Microscope Observations

In the preceding paper (Bouck and Brown, 1973) we showed that cytoplasmic microtubule distribution is related to intracellular sites which appear to serve as focal points for two main groups of microtubules; the beak and the rhizoplast microtubules. The location of these sites and of the cellular organelles in *Ochromonas* is constant and remains apparently unchanged by the experimental conditions used in this study. Therefore, sections of a particular orientation through a specific part of the cell can be recognized and selected from control or treated samples and examined for any alteration in microtubule number or distribution.

MICROTUBULE INTEGRITY IN COLCHICINE

MICROTUBULE DISASSEMBLY: Exposure of cells to 10 mg/ml of colchicine results in complete disassembly of cytoplasmic microtubules over a 5–6-h period. Electron microscope examination of cells fixed at intervals during this period reveals that depolymerization appears to proceed from the posterior toward the cell anterior, and that the two groups of microtubules differ in their sensitivity to the drug.

The first microtubules to be affected are those inserting on the rhizoplast. Within 30 min of exposure to colchicine this group disappears completely from most cells (Fig. 5) concomitant with the disappearance of the posterior tail (Figs. 1 a, 3). The loss of these microtubules appears to occur through depolymerization to subunits, since we see no evidence for the accumulation of fibrillar material (protofilaments), retraction of the bundle of microtubules comprising the tail, or displacement of the rhizoplast tubules to another part of the cell. The beak tubules are similarly but more slowly affected and are still present after 30 min (Figs. 5 and 6). However, comparison of similar sections of control and treated cells does show some decrease in total number of beak microtubules. Samples taken between 30 min and 5 h show a further progressive loss of microtubules. After 2 h tubules are still observed near the beak but are only rarely seen in the cell posterior. By 5–6 h of exposure the cells are rounded and microtubules are completely absent (Fig. 7). The rhizoplast and other kineto-fibers appear unaltered by the treatment but there are no associated microtubules (Fig. 8).

The differential sensitivity of the rhizoplast microtubules is also evident in cells exposed to lower concentrations of colchicine for the same time period (Fig. 2). For example, in a sample exposed to 3 mg/ml of colchicine for 5 h most cells possess an intermediate form but lack tails. Electron microscope observation shows a loss of rhizoplast microtubules from most cells with little or no change in the beak tubules. At the light and electron microscope levels this population appears identical to a sample exposed to 10 mg/ml of colchicine for 30 min. In concurrence with the observations of others (eg. Behnke and Forer, 1967; Tilney and Gibbins, 1968), we could detect no effect of colchicine on flagellar microtubules at any of the concentrations used. In addition we could find no apparent change in structure and

distribution of organelles or kinetosome fiber attributable to the colchicine treatment.

MICROTUBULES IN REGENERATING CELLS: Within 3 h after removal of colchicine (after exposure to 10 mg/ml) microtubules were again evident in the anterior of most cells near the beak (Fig. 9), but not associated with the rhizoplast (Fig. 10). This reappearance of microtubules occurs concurrently with early regeneration of the beak asymmetry (Figs. 1 d and 3). By 5 h many more tubules have reformed and they frequently extend to the cell posterior. The rhizoplast microtubules are the last to reform and their reappearance coincides with regeneration of the tail. By this time (7–10 h of recovery) regeneration of cell shape is complete and the cytoplasmic microtubular system is apparently reformed (Figs. 11 and 12)

MICROTUBULE INTEGRITY UNDER PRESSURE

MICROTUBULE DISASSEMBLY: Hydrostatic pressure in our system does not produce a complete depolymerization of all microtubules, even in spherical cells which have been subjected to 8000 psi for 30 min (Fig. 13). Rather, this level of pressure appears to selectively depolymerize the rhizoplast microtubules and either depolymerize or disrupt the normal distribution of the other cytoplasmic microtubules. The selective depolymerization confirms certain observations of colchicine-treated cells. Again, the rhizoplast tubules appear the most sensitive and the loss of these tubules is correlated with loss of the elongate cell tail. At 6000 psi, apart from the absence of rhizoplast microtubules, the fine structure of the cell appears unchanged. However, at higher pressures additional effects are observed. Cytoplasmic microtubules are present in much reduced numbers and appear randomly distributed in contrast to the peripheral localization in untreated cells. Frequently, extra kinetosomes are found near the cell anterior and occasionally these are continuous with microtubules extending through the cytoplasm (Fig. 14). These may represent "intracellular flagella" since the typical 9 + 2 axoneme cross-section is also occasionally seen in the cytoplasm of these cells.

MICROTUBULES IN REGENERATING CELLS: The pattern of reappearance or reorganization of microtubules in pressure-treated cells is essentially the same as observed in colchicine-treated cells,
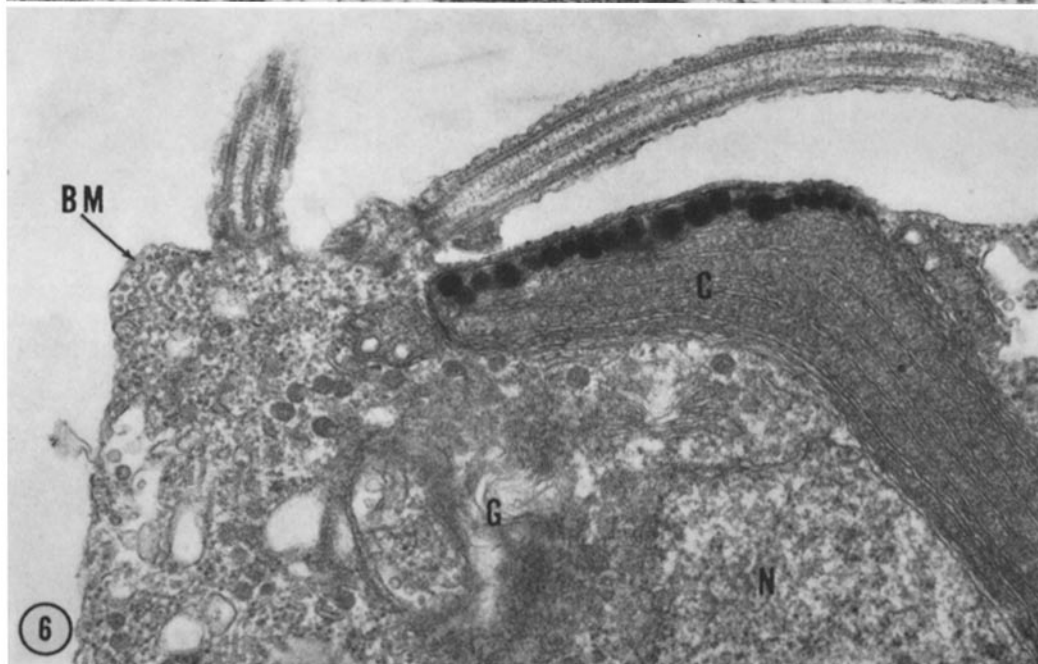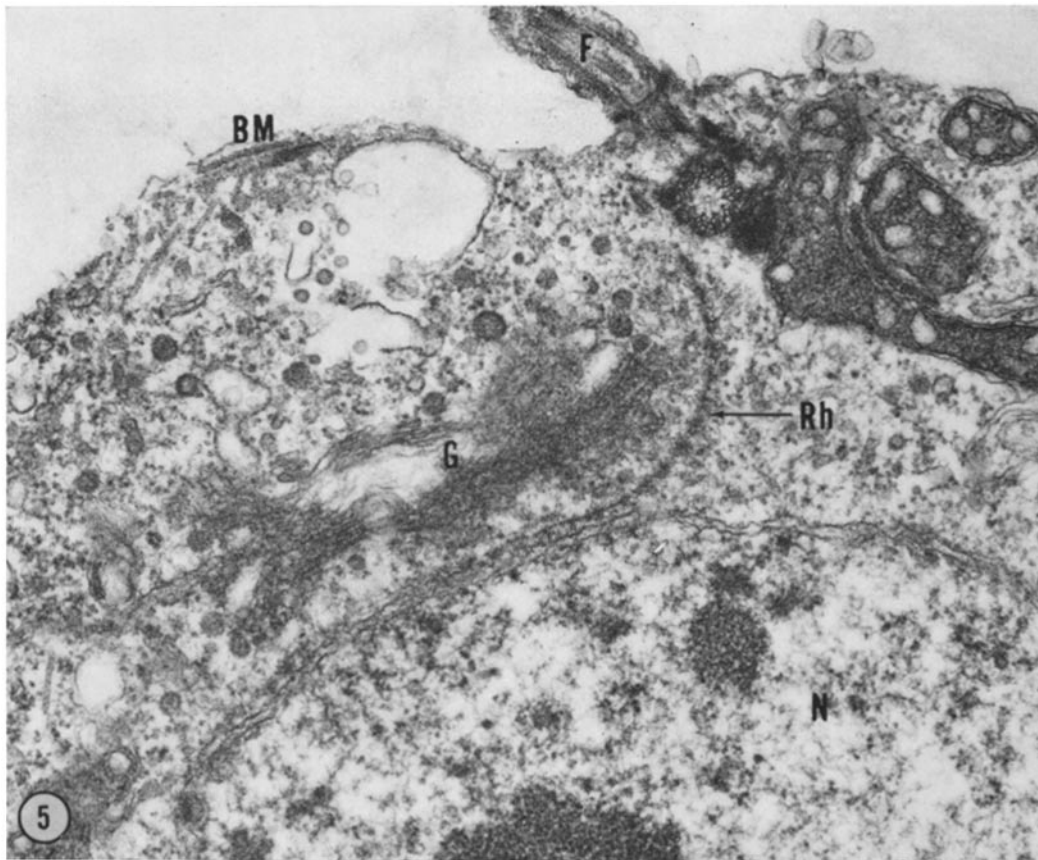
FIGURES 5 and 6   Longitudinal sections through the anterior of cells exposed to 10 mg/ml of colchicine for 30 min. In Fig. 5 note the absence of microtubules on the rhizoplast (*Rh*) with some beak tubules still evident at this time. *N*, nucleus; *G*, Golgi complex; *F*, flagellum; *BM*, beak microtubules. × 37,000. Fig. 6 is a section at approximately right angles to Fig. 5. Both the long flagellum (left) and the short flagellum (right) arching over the eyespot region of the chloroplast (*C*) are apparent. Beak microtubules (*BM*) are seen in cross-section and oblique views near the base of the long flagellum. *N*, nucleus; *G*, Golgi complex. × 35,000.
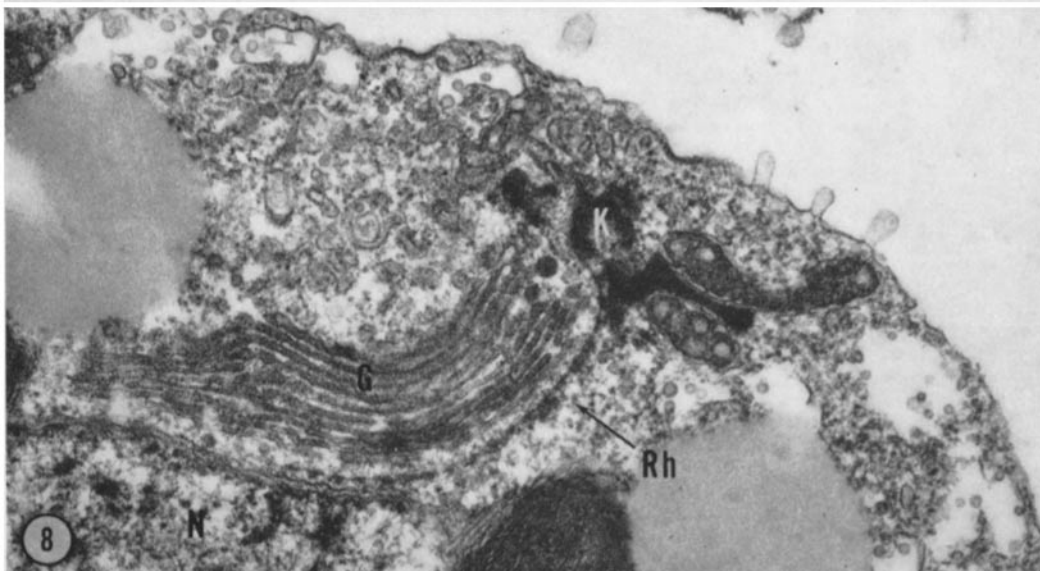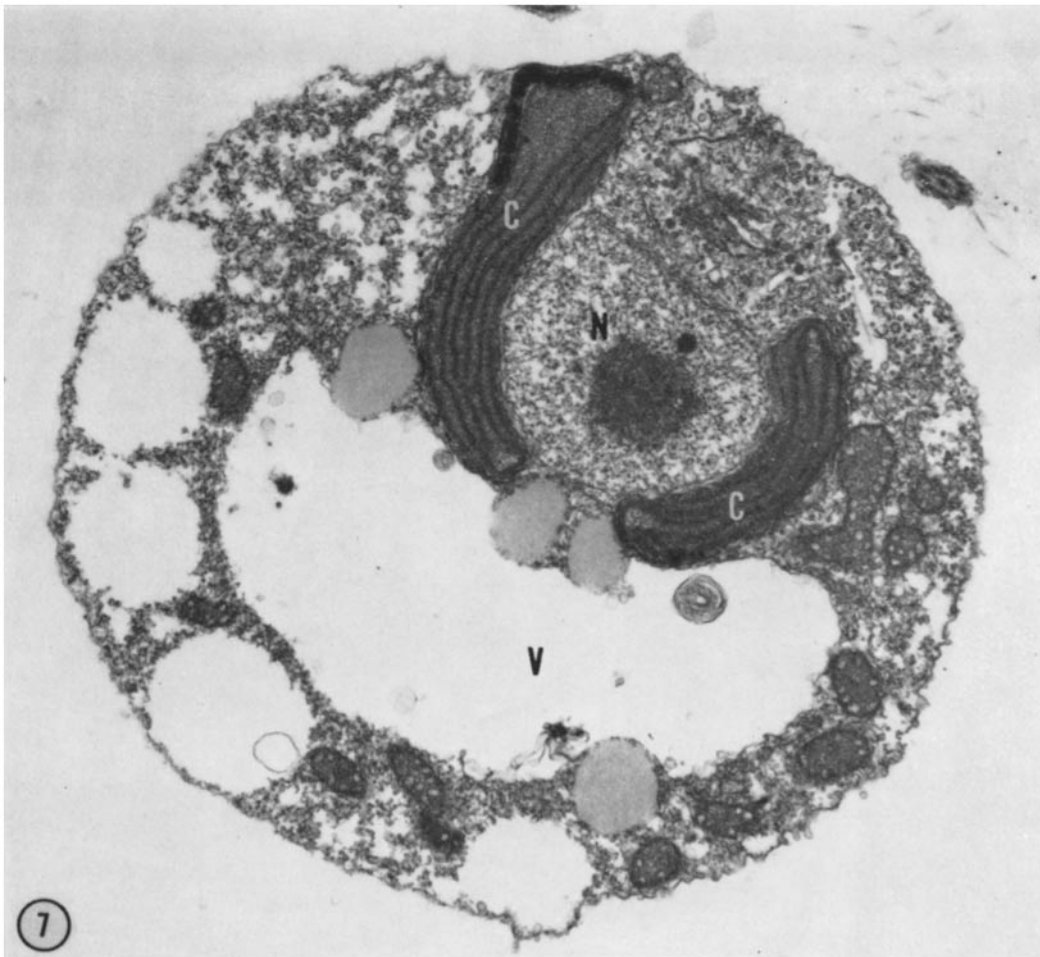
FIGURES 7 and 8   Cells exposed to 10 mg/ml of colchicine for 5 h. Fig. 7, Longitudinal section through a spherical cell. No cytoplasmic microtubules present. N, nucleus; C, chloroplast; V, vacuole. × 18,000. Fig. 8, Longitudinal section showing rhizoplast extending from the kinetosome (K) to the surface of the nucleus (N). No microtubules are seen. Rh, rhizoplast; G, Golgi complex. × 32,000.
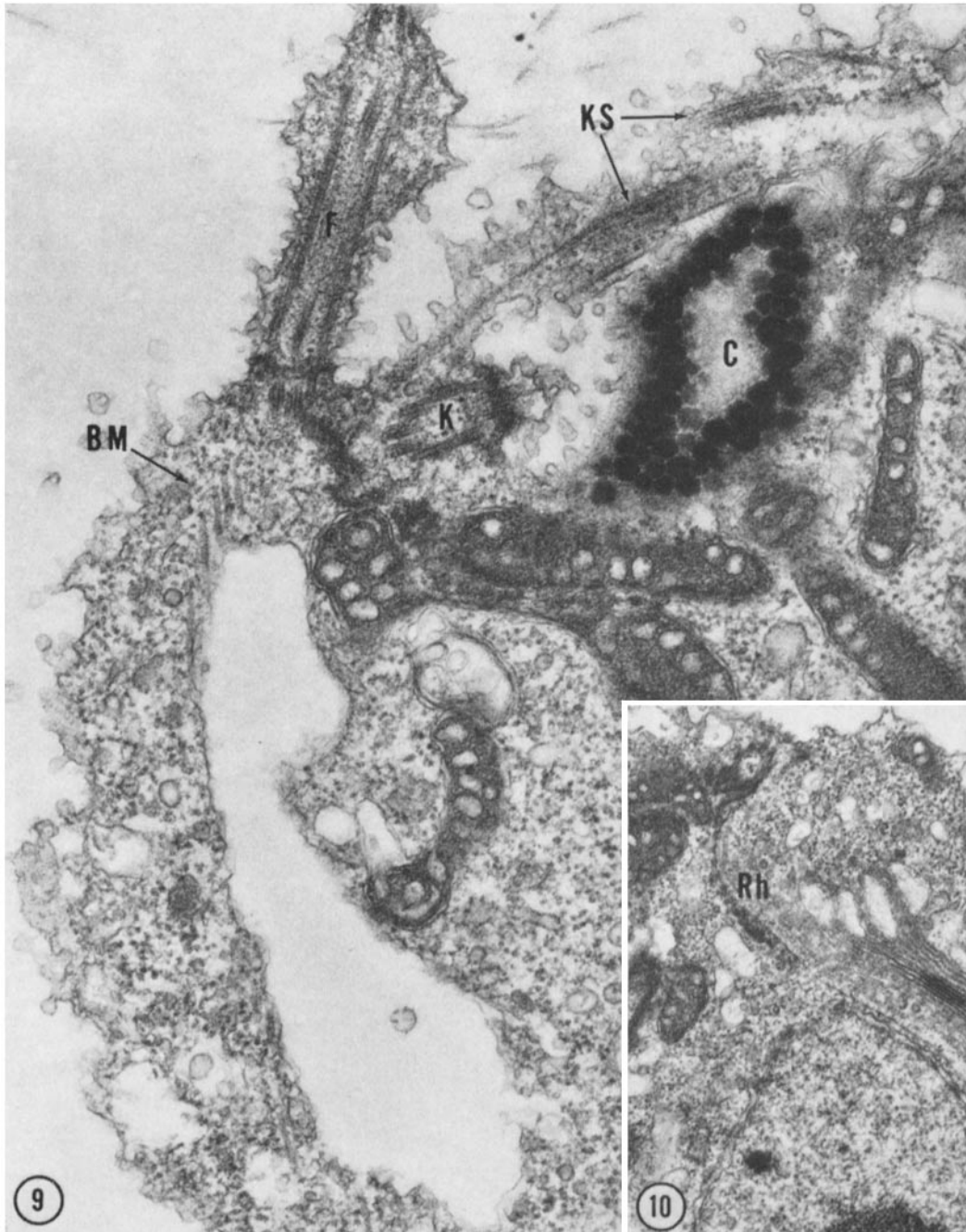
FIGURE 9 Oblique section through the cell anterior after 3-h recovery, after 5 h in 10 mg/ml of colchicine. A few beak microtubules (*BM*) are evident near the long flagellum (*F*). *K*, kinetosome of the short flagellum; *C*, eyespot extension of the chloroplast; *KS*, kineto-stigmatic fibers. × 41,000.

FIGURE 10 Longitudinal section through the rhizoplast (*Rh*) after 3-h recovery. No microtubules evident. × 22,000.

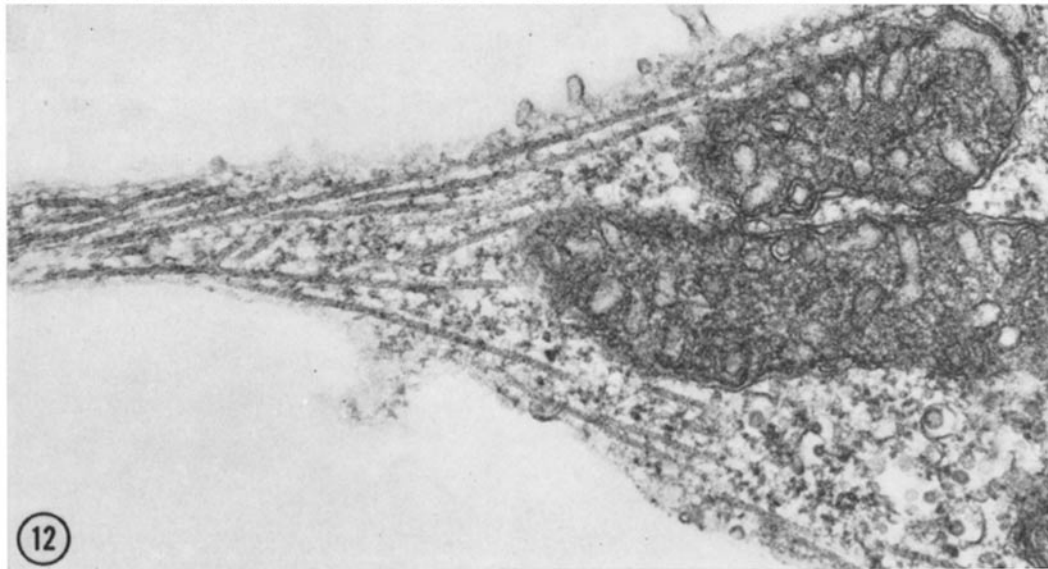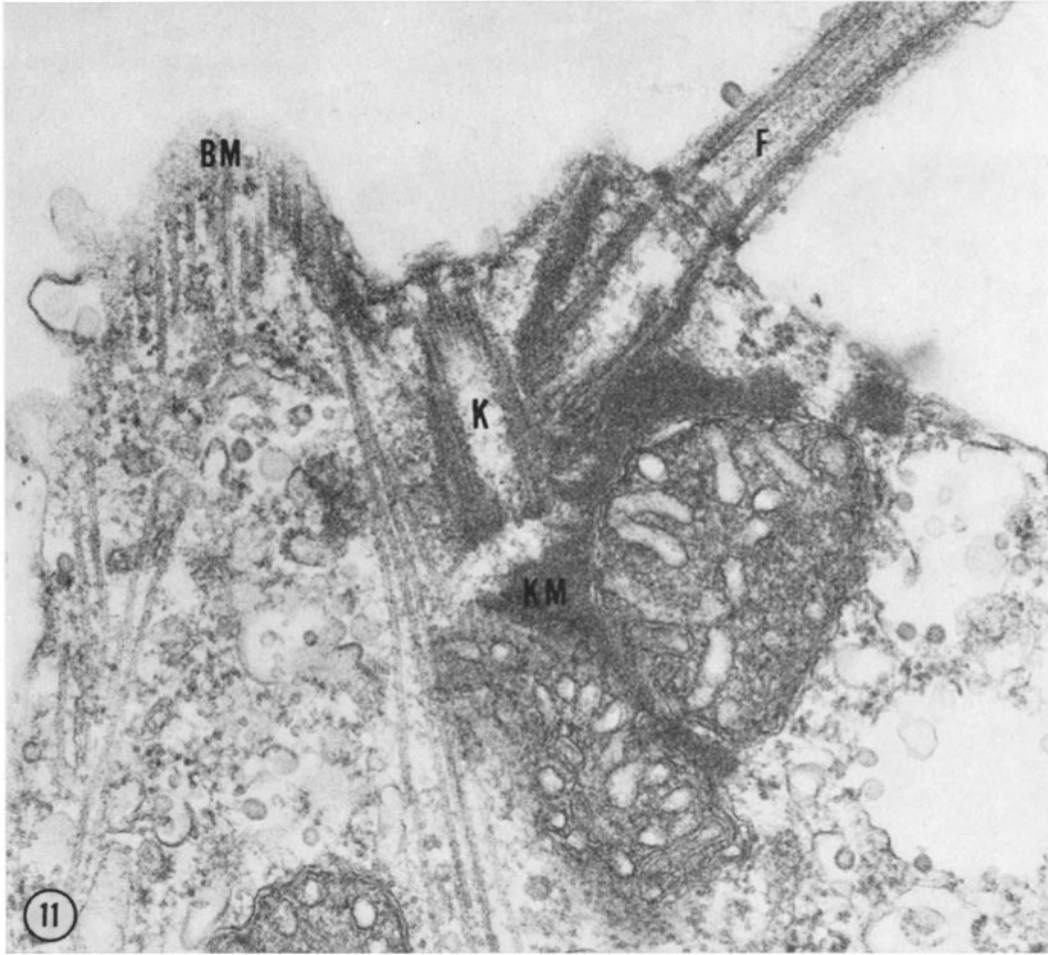368   THE JOURNAL OF CELL BIOLOGY · VOLUME 56, 1973

FIGURE 11 Glancing section through the beak of fully recovered cell (after 5-h in 10 mg/ml of colchicine), showing beak microtubules (*BM*) extending just beneath the plasma membrane. *F*, short flagellum; *K*, kinetosome of long flagellum; *KM*, kinetomitochondrial fiber. × 53,000.

FIGURE 12 Longitudinal section through the cell posterior of a fully recovered cell showing microtubules converging into the cell tail. × 44,000.
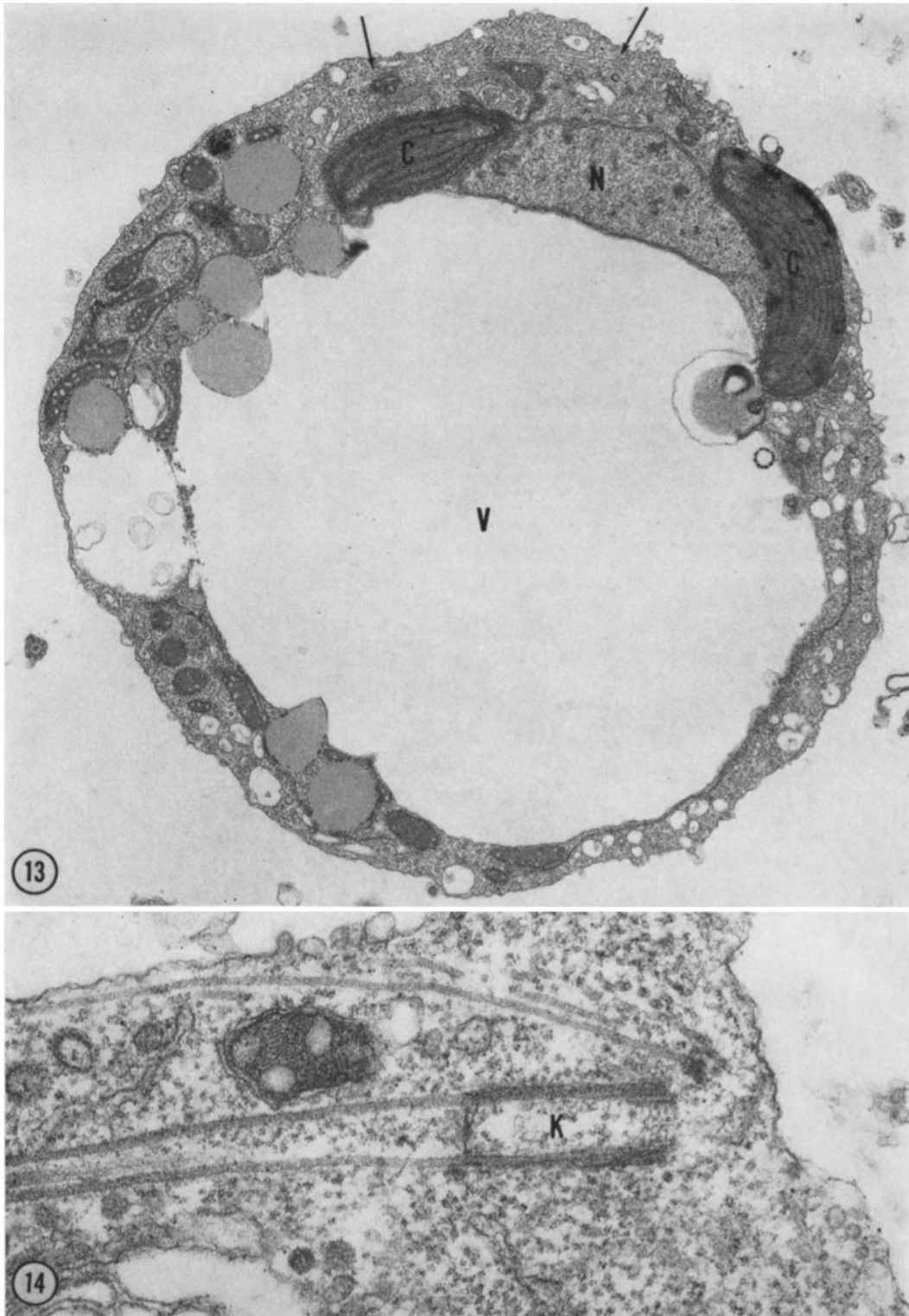
FIGURE 13   Longitudinal section through a spherical cell fixed after 30 min at 8000 psi. Arrows indicate presence of a few microtubules. N, nucleus; C, chloroplast; V, vacuole. × 13,000.

FIGURE 14   Section from a cell exposed to 8000 psi for 30 min showing what appears to be a kinetosome (K) with microtubules extending from one end into the cytoplasm. × 54,000.
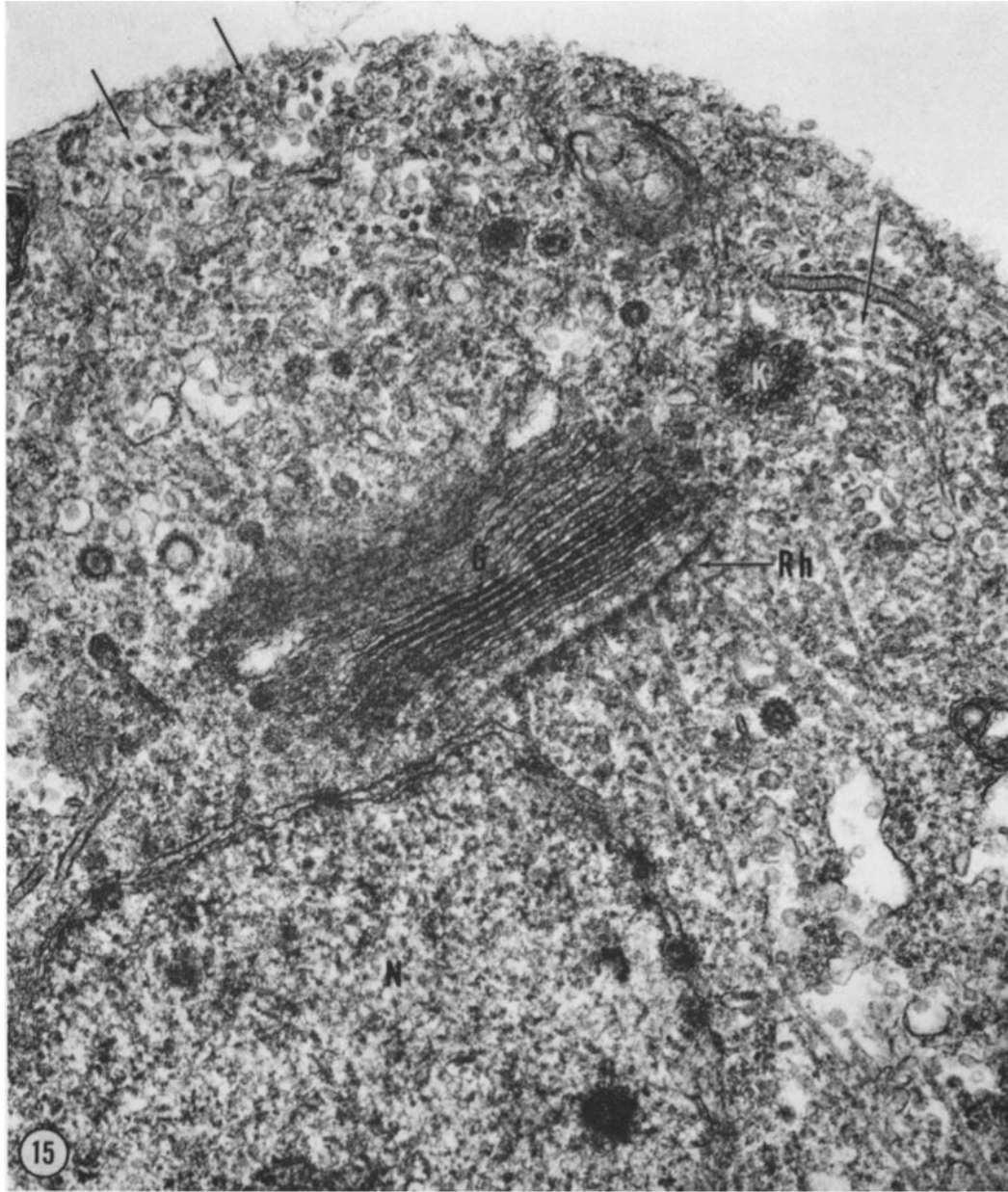
FIGURE 15 Oblique section through the anterior of a cell fixed after 15-min recovery after a 30-min exposure to 8000 psi. Microtubules of the kineto-beak complex appear to be completely reformed (arrows) and the first microtubules to reassemble at the rhizoplast (*Rh*) are also evident. *K*, kinetosome; *N*, nucleus; *G*, Golgi complex. × 47,000.

although the period of regeneration is much shorter. Microtubules reappear in association with the beak complex within 10 min of pressure release, concurrent with development of the an-

terior asymmetry. Between 15 and 30 min of recovery microtubules reappear on the rhizoplast (Fig. 15) and by 60–80 min most cells appear completely normal.

Reassembly or Resynthesis of Microtubules during Regeneration

The results in the preceding section indicated that control of microtubule distribution in regenerating cells may be related to sites which function in initial polymerization of microtubules. Colchicine and pressure are generally believed to cause a reversible depolymerization of microtubules, with colchicine acting specifically by binding to microtubule subunit protein. This suggests that regeneration in *Ochromonas* could involve a reuse of the same microtubule protein disassembled by pressure or colchicine. However, since the rates of shape regeneration after the two treatments were very different (Figs. 3 and 19), it was of interest to determine whether regeneration was independent of new protein synthesis in both cases.

Fig. 16 shows that cycloheximide at a concentration of 20 µg/ml rapidly inhibits incorporation of amino acids into TCA-precipitable protein. Colchicine, at 10 mg/ml also causes some inhibition immediately upon addition. However, if the colchicine is washed out after a 3-h exposure and the cells are regenerating shape, there appears to be no long-lasting effect of colchicine on incorporation (Fig. 17). The effects of 20 µg/ml of cycloheximide on incorporation are also fully

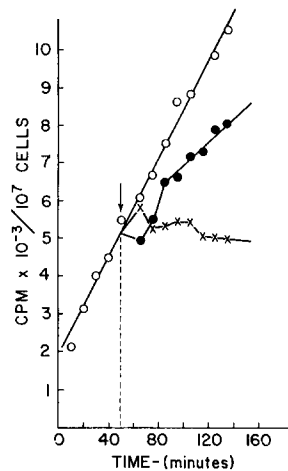

Figure 16   Effect of colchicine and cycloheximide on [3H]leucine incorporation into TCA-precipitable protein. [3H]Leucine added at time zero and colchicine (10 mg/ml) or cycloheximide (20 µg/ml) added at 50 min (arrow). Open circles, control; closed circles, colchicine; crosses, cycloheximide.

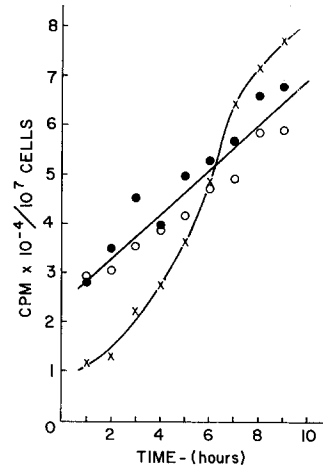

Figure 17   Reversibility of colchicine and cycloheximide inhibition of [14C]amino acid incorporation into TCA-precipitable protein. Three samples preincubated for 3 h in either control medium (open circles), 10 mg/ml of colchicine (closed circles), or 20 µg/ml of cycloheximide (crosses). Cells then resuspended in control medium and [14C]amino acid mixture was added at zero time.

reversible over a period of several hours. It is important to point out here that the concentration of cycloheximide used has no apparent effect on cell shape or microtubule integrity in nonregenerating cells. After a 5-h exposure to 20 µg/ml of cycloheximide, cell shape and motility are normal, and extending the exposure to 24 h produces only a shortening of the tail.

Fig. 18 summarizes studies on the effects of cycloheximide on shape regeneration of colchicine and pressure-treated cells. Cells made spherical by exposure to colchicine are unable to regenerate shape or reassemble microtubules as long as cycloheximide is present. If the cycloheximide is washed out and cells are resuspended in control media the kinetics of regeneration are similar to the kinetics of cells exposed only to colchicine (Fig. 3). These results suggest that microtubule protein once exposed to colchicine cannot be reutilized in regenerating cells. Pressure-treated cells, on the other hand, reassemble microtubules and regenerate the normal cell shape in the absence of new protein synthesis (Fig. 18). After an exposure to pressure, the kinetics of shape regeneration are identical in control or cycloheximide-containing media (Fig. 19). Furthermore, the reassembly of microtubules appears to be
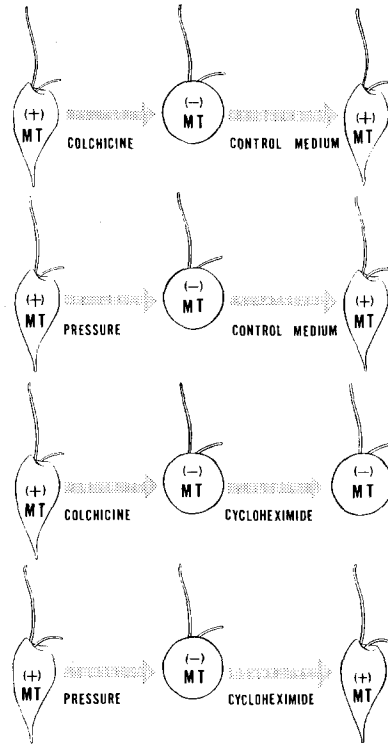
FIGURE 18 Summary diagram showing the effects of cycloheximide on shape regeneration and microtubule reassembly in colchicine- and pressure-treated cells.
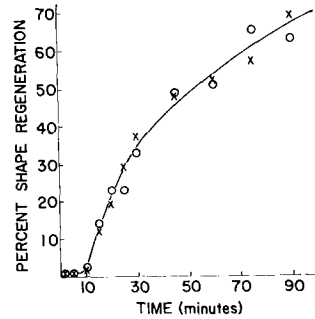

FIGURE 19 Effect of cycloheximide on kinetics of shape regeneration in pressure-treated *Ochromonas*. Cells pressurized (6500 psi, 30 min) and regeneration of the complete cell shape assayed in either control medium (open circles) or 20 μg/ml of cycloheximide (crosses).

initiated at the same sites in the beak and the rhizoplast and the same pattern of shape regeneration is observed (Figs. 1 d–1 f) in the presence or absence of cycloheximide.

DISCUSSION

In the accompanying paper (Bouck and Brown, 1973) the interpretation presented was that two independent sets of cytoplasmic microtubules, related to specific sites and portions of the cell asymmetry, together contribute to the characteristic form of *Ochromonas*. Since in vegetative and dividing cells there is no indication of a cell wall, pellicle, or other structure which might perform a cytoskeletal function it was suggested that cytoplasmic microtubules played a dual role in formation and maintenance of cell shape in this organism. A cytoskeletal function for microtubules has been proposed and confirmed experimentally in a variety of cell types including several protozoans (Tilney et al., 1966; Kennedy and Zimmerman, 1970; Tucker, 1970), ectodermal cells of

Arbacia blastulae (Tilney and Gibbins, 1969), disk-shaped blood cells (Behnke, 1970), nerve axons (Yamada et al., 1971), and cultured fibroblast-like cells (Goldman, 1971). However, most of these studies have not distinguished between the separate roles of microtubules in development or maintenance of shape, and as Tilney and Gibbins (1969) have pointed out, in several organisms microtubules do little to maintain shape once it is established. In contrast, microtubules in nerve cell axons appear responsible for maintenance of axonal shape but elongation is more directly dependent on a network of cytochalasin-sensitive neurofilaments in the growth cone (Yamada et al., 1970; 1971).

The results presented in this paper clearly demonstrate that microtubules play an obligatory role in both developing and maintaining shape in *Ochromonas*. The normal asymmetric form appears to be directly dependent on the continued presence of cytoplasmic microtubules. Treatment of cells with the microtubule depolymerizing agents colchicine or hydrostatic pressure causes tubule disassembly and a concomitant gradual loss of cell shape, eventually producing a uniform population of spherical cells. Cell shape is not regenerated unless microtubules are permitted to reassemble by releasing the pressure or washing out the colchicine. In regenerating cells the reassembly of microtubules correlates precisely with formation of the characteristic cell shape. In addition, because of the close synchrony of shape loss and regeneration in populations of *Ochromonas* it has been possible to relate the development of functionally distinct sets of microtubules to sites which appear to initiate and orient microtubule assembly.

The Independent Roles of Beak and Rhizoplast Microtubules

In fully differentiated cells of *Ochromonas* the cytoplasmic microtubules appear to form two separate sets associated with the beak complex or the rhizoplast. The differential sensitivity of these microtubules to colchicine and pressure demonstrates the independence of the two sets and relates each set to a particular portion of the cell shape.

At relatively high concentrations of colchicine (10 mg/ml) or high hydrostatic pressure (8000 psi) both sets of microtubules are affected, but at different rates. Cells fixed at intervals during shape loss show first a disassembly of rhizoplast tubules correlating with disappearance of the posterior tail, followed by loss of the beak tubules and the beak asymmetry. Lower concentrations of colchicine (3–5 mg/ml) or lower pressure (6000 psi) selectively disassemble the rhizoplast microtubules with little or no effect on the beak set. Cells treated this way exhibit the normal anterior shape but tails are completely absent.

Exposure of cells to two other antimitotic agents produces similar effects. The *Vinca* alkaloid, vinblastine sulfate, has been shown to depolymerize existing microtubules and form microtubular crystals in vivo (Bensch and Malawista, 1969) and in vitro (Bensch, et al., 1969). A 1-h exposure of *Ochromonas* to high, but still fully reversible, concentrations of vinblastine (100–200 µg/ml) results in disassembly of rhizoplast microtubules with formation of crystals and a concurrent loss of the cell tail (unpublished results). The beak asymmetry is not altered by this treatment, or by longer exposures (at least to 5 h) to vinblastine. The mitotic inhibitor IPC, in conjunction with low hydrostatic pressure (6000 psi), selectively converts the rhizoplast microtubules into short segments of a larger diameter "macrotubule" with a concomitant loss of the tail (Brown and Bouck, 1971). Higher pressures (7000–8000 psi) convert the beak set as well, producing spherical cells.

These observations implicate the more labile rhizoplast set of cytoplasmic microtubules in maintenance of the posterior tail extension. The beak microtubules, therefore, appear responsible for maintaining the complex anterior asymmetry with the exception of the platform ridges. The kineto-stigmatic fibers associated with these ridges are resistant to prolonged colchicine treatment and are assumed to be nonmicrotubular in nature. The flagellar microtubules were not affected by any of the agents employed. We were unable to confirm the report of Kennedy and Zimmerman (1970) that the central ciliary tubules in *Tetrahymena* were partially degraded at 10,000 psi since this pressure deflagellated most *Ochromonas* cells, and at lower pressures no degradation was observed.

The differential effects of several chemical and physical agents on the stability of cytoplasmic and flagellar (or ciliary) microtubules have been well documented (Behnke and Forer, 1967; Tilney and Gibbins, 1968; Rosenbaum et al., 1969). The suggestion that these differences in stability indicate different tubulin compositions (Behnke and Forer, 1967) has been supported by biochemical (Stephens, 1970) and serological studies (Fulton et al., 1971) and recently Olmsted et al. (1971) have clearly demonstrated the presence of distinctive tubulins in cytoplasmic (neurotubules) and flagellar microtubules. It is possible that the differences in stability of flagellar and cytoplasmic microtubules in *Ochromonas*, and the differential sensitivity of the beak and rhizoplast sets of cytoplasmic tubules, might also reflect unique tubulin compositions.

Alternatively, the beak and rhizoplast sets may represent two equilibrium systems competing for a common pool of microtubule protein. The implication here is that the difference in equilibrium state of the two systems, and hence their differential sensitivity, is not due to dissimilar tubulins but may be a characteristic of the sites which initiate polymerization (see below). The observations reported here on development of cytoplasmic microtubules during cell division or regeneration of the normal cell shape after treatment with pressure or colchicine are consistent with this interpretation. In regenerating cells, those microtubules which were the least sensitive to depolymerizing agents are the first to reappear if the agent is removed. Thus, initially the more stable beak microtubules reassemble and the anterior asymmetry of the cell is established, and only later the tail is reformed and microtubules are again observed on the rhizoplast. The same sequence of tubule assembly and correlative shape development is seen in dividing *Ochromonas*. The formation of a new beak for each of the daughter cells is

evident early in mitosis while the tail is the last part of the cell shape to differentiate (see Bouck and Brown, 1973, adjoining paper). The microtubules that are most susceptible to depolymerizing agents (i.e. the rhizoplast set), therefore, are those which were last polymerized during development.

Microtubule Nucleating Sites

The experiments described here indicate that the association of cytoplasmic microtubules with kinetosome-related sites is a result of initial tubule assembly at these sites during shape differentiation. The loss of cell shape in response to colchicine, pressure, or other agents acting to depolymerize microtubules always proceeds from the posterior toward the anterior of the cell (Figs. 1 a–1 c). The last indications of cell shape to disappear are those most closely related to the presumptive nucleating sites, and the last microtubules to depolymerize are short segments associated with these sites. In regenerating cells the first indication of shape regeneration is in the cell anterior (Fig. 1 d) and microtubules appear in the same area oriented such that one end of the microtubule approaches or contacts the beak site. At a later stage of regeneration these microtubules have lengthened to the cell posterior, and a second set of tubules appears to assemble at and extend from the rhizoplast (Fig. 15).

Particularly in developing systems, cytoplasmic microtubules have been generally observed to be associated at one end with sites composed of an amorphous, electron-opaque material. This association has prompted conjecture that these sites nucleate initial microtubule assembly and presumably could function in regulating the position and timing of appearance, the orientation, and the pattern of microtubule distribution in the cell (Porter, 1966; Tilney and Gibbons, 1968; Pickett-Heaps, 1969). However, in the experimental studies carried out thus far there is some question as to whether all of these presumed functions are necessarily related to nucleation. In Arbacia ectoderm cells microtubules are assembled at and extend in all directions from satellites in the cell anterior. The subsequent orientation of these tubules is assumed to be controlled by other, as yet unidentified, factors (Tilney and Goddard, 1970). Tucker (1970) has suggested that the ordered patterns of microtubules established during cytopharyngeal development in Nassula are defined at the points of tubule initiation (i.e. the

nucleating sites). Cross-connections or bridges between microtubules are formed secondarily and function to stabilize the preexisting microtubule patterns. A conflicting interpretation of the role of microtubule bridges has been presented by Tilney (1971). The centroplast of Raphidiophrys nucleates the assembly of several groups of microtubules which are ultimately ordered into a characteristic pattern in the axonemes. The orientation of tubules into the axonemal pattern occurs at some distance from the centroplast, apparently as a result of bridge formation between adjacent microtubules (Tilney, 1971).

Unlike the situation in Arbacia and Raphidiophrys, in Ochromonas the microtubules of the beak complex initially reassemble in the characteristic bidirectional orientation and those inserting on the rhizoplast are assembled only at the lower rhizoplast surface. The simplest interpretation, in concurrence with the suggestion of Tucker (1970), is that the nucleating sites in this organism serve as templates which specify the points of individual microtubule initiation. A close examination of the rhizoplast indicates how such a template could be constructed. The amorphous, electron-opaque material which is generally presumed to function in tubule initiation forms crossbandings on the rhizoplast. In longitudinal section the crossbandings are evident as dense aggregates only on the lower rhizoplast surface (Figs. 9 and 10 in Bouck and Brown, 1973, adjoining paper), and the microtubules terminating on this surface appear to attach at the dense regions. The crossbanding material does not appear to be altered by agents which depolymerize all of the rhizoplast tubules (Figs. 5 and 8). In regenerating cells microtubules reassemble at the lower rhizoplast surface, apparently in the same arrangement observed in untreated cells. These observations suggest that the material forming the nucleation site is prepatterned in such a way as to provide a template which defines the pattern of microtubules at the point of initiation. The accurate positioning of the two nucleating sites in Ochromonas relative to the kinetosome and other kineto-fibers, therefore, probably governs the direction of microtubule growth and the total distribution of microtubules in the cell.

In addition to controlling the spacial distribution, nucleating sites have also been suggested to function in regulating the time of appearance of microtubules in developing systems. For

example, during formation and differentiation of primary mesenchyme cells in *Arbacia*, microtubules disappear and reform relative to different sites in what appears to be a programmed sequence of "activation and repression of nucleating sites" (Gibbins et al., 1969). In differentiating *Ochromonas* the beak set of microtubules appears to be completely formed before tubule assembly is initiated at the rhizoplast. This could indicate that the two sets of microtubules (or the two nucleating sites) are synthesized at different times during differentiation of shape. However, observations on the regeneration of pressure-treated cells in the presence of cycloheximide argue against this possibility. In pressurized cells both sets of microtubules are depolymerized, presumably to monomers, while the nucleating sites appear unaltered. When the pressure is released, microtubules are reassembled in the absence of new protein synthesis in the normal sequence and pattern, first at the beak site and later at the rhizoplast. If we assume the initial step in microtubule assembly involves a binding of monomers at the nucleating site these results suggest that the beak site has a greater affinity for microtubule protein than the rhizoplast site. The beak set of microtubules, therefore, polymerizes first even though sufficient microtubule protein is present in the disassembled state to completely reform both sets of tubules. Extending this argument further, one would predict that the beak set of tubules would be more stable than the rhizoplast set in the presence of depolymerizing agents. This is, in fact, observed (see preceding section, Discussion). Whether the temporal control of microtubule assembly indicates an activation of nucleating sites at specific times (Gibbins et al., 1969) or reflects differences in the nucleating sites themselves cannot be determined from the evidence now available but must await isolation and biochemical characterization of the nucleating material.

Effect of Cycloheximide on Shape Regeneration

Cycloheximide, at a concentration which rapidly inhibits incorporation of amino acids into TCA-precipitable protein (20 μg/ml, Fig. 16), completely blocks microtubule assembly and shape regeneration in colchicine-treated cells. This suggests that microtubule protein once exposed to colchicine cannot be reassembled into new microtubules. The relatively slow shape regenera-

tion when cells are resuspended in control media after a colchicine treatment (Figs. 3, 9–12) probably indicates the time required for resynthesis of the microtubular system. It does not appear that the previous colchicine treatment affects this resynthesis, since the partial inhibition of amino acid incorporation is completely reversed when the colchicine is washed out (Fig. 17). These results suggest that, in *Ochromonas*, colchicine either binds irreversibly or inactivates microtubule protein so that it cannot be reutilized in regenerating cells. In vitro studies of microtubule protein from chick embryo brain have shown that subunit protein bound to colchicine undergoes an inactivation process, releases the colchicine, and thereafter cannot bind to colchicine (Wilson, 1970). It was suggested that inactivation resulted in a conformation change of the subunit protein so that the colchicine binding sites were no longer accessible. It is possible that a similar inactivation process in *Ochromonas* may alter the ability of the subunit protein to repolymerize into microtubules.

Although colchicine has been reported to have many nonspecific effects (see for example, Mueller et al., 1971), the primary mechanism of action is by binding to microtubule subunit protein (Borisy and Taylor, 1967; and many others). This binding apparently prevents reassembly of the subunit protein and leads to depolymerization of existing microtubules. In contrast to the situation in *Ochromonas*, the binding of colchicine to spindle microtubule protein in *Pectinaria* (Inoué and Sato, 1967) and flagellar microtubule protein in *Chlamydomonas* (Rosenbaum et al., 1969) is completely reversible. In both cases, microtubule protein kept in the depolymerized state by colchicine is reassembled in the absence of new protein synthesis when the colchicine is removed. Conceivably, differences in tubulin composition between, for example, flagellar and cytoplasmic microtubules (Olmsted et al., 1971) could account for the difference in reversibility of colchicine disassembly.

However, this does not appear to be true for *Ochromonas*. If cells are deflagellated and allowed to regenerate in the presence of cycloheximide about one fourth of the flagellum is reformed (Rosenbaum and Child, 1967; unpublished results). This suggests that a pool of flagellar microtubule protein is present and available for polymerization. Colchicine, at the same concentrations which depolymerize cytoplasmic micro-

tubules (Fig. 2), completely inhibits flagellar regeneration when added at the time of amputation. If the colchicine is then washed out, and the cells are resuspended in cycloheximide containing media, no regeneration is observed. It appears, therefore, that subunit protein from the flagellar and cytoplasmic microtubules of *Ochromonas* is irreversibly altered by exposure to colchicine.

The kinetics of shape regeneration of pressure-treated *Ochromonas*, on the other hand, is identical in the presence or absence of new protein synthesis (Fig. 19). The reappearance of microtubules in these cells probably involves a reassembly of the same subunit protein within minutes of pressure release. Unfortunately, pressures necessary to depolymerize all cytoplasmic microtubules also deflagellate many cells, resulting in asynchronous shape regeneration. In more recent studies we have circumvented this problem by combining exposure to lower pressure in the presence of the anti-mitotic herbicide, IPC (Brown and Bouck, 1971). In cells exposed to IPC and pressure simultaneously, all microtubules are depolymerized or converted to larger tubules without deflagellation. The kinetics of shape regeneration and the pattern of microtubule reassembly in the pressure-IPC cells is identical in control or cycloheximide containing media. In addition, IPC rapidly and reversibly inhibits shape regeneration in pressure- or colchicine-treated cells, and flagellar regeneration in deflagellated cells.

The results presented here and in the preceding paper have shown that it is possible to detail the entire complement of microtubules in *Ochromonas*. The use of microtubule depolymerizing agents has demonstrated an obligatory function for cytoplasmic microtubules in establishing and maintaining the species-specific cell shape. Observation of regenerating cells has further suggested that temporal and spacial control of microtubule distribution is related to nucleating sites which initiate tubule assembly. The apparent dependence on resynthesis of microtubules in colchicine-treated cells in contrast to microtubule reassembly after exposure to pressure-IPC suggest that *Ochromonas* may be particularly suitable as a model system to investigate synthesis and assembly of cytoplasmic microtubules.

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