

MICROTUBULE BIOGENESIS AND CELL SHAPE IN *OCHROMONAS*

III. Effects of the Herbicidal Mitotic Inhibitor

Isopropyl *N*-Phenylcarbamate on Shape and Flagellum Regeneration

DAVID L. BROWN and G. BENJAMIN BOUCK

From the Department of Biology, University of Ottawa, Ottawa, Canada K1N 6N5 and the
Department of Biological Sciences, University of Illinois, Chicago, Illinois 60680

ABSTRACT

The role of microtubules and microtubule nucleating sites in the unicell, *Ochromonas* has been examined through the use of two mitotic inhibitors, isopropyl *N*-phenylcarbamate (IPC) and isopropyl *N*-3-chlorophenyl carbamate (CIPC). Although IPC and CIPC have little or no effect on intact microtubules, the *assembly* of three separate sets of microtubules in *Ochromonas* has been found to be differentially affected by IPC and CIPC. The assembly of flagellar microtubules after mechanical deflagellation is partially inhibited; the reassembly of rhizoplast microtubules after pressure depolymerization is totally inhibited (however, macrotubules may form at the sites of microtubule initiation or elsewhere); and, the reassembly of the beak set of microtubules after pressure depolymerization may be unaffected although similar concentrations of IPC and CIPC completely inhibit microtubule regeneration on the rhizoplast. These effects on microtubule assembly, either inhibitory or macrotubule inducing, are fully reversible. The kinetics of inhibition and reversal are found to be generally similar for both flagellar and cell shape regeneration. Incorporation data suggest that neither IPC nor CIPC has significant effects on protein synthesis in short term experiments. Conversely, inhibiting protein synthesis with cycloheximide has little effect on microtubule regeneration when IPC or CIPC is removed. Although the exact target for IPC and CIPC action remains uncertain, the available evidence suggests that the microtubule protein pool or the microtubule nucleating sites are specifically and reversibly affected. Comparative experiments using the mitotic inhibitor colchicine indicate some similarities and differences in its mode of action with respect to that of IPC and CIPC on assembly and disassembly of microtubules in these cells.

In the first two papers of this series (Bouck and Brown, 1973; Brown and Bouck, 1973) evidence was presented that the characteristic asymmetric shape of *Ochromonas* is mediated by two appar-

ently independent sets of cytoplasmic microtubules which (*a*) differ from each other in sensitivity to the microtubule depolymerizing agents colchicine and hydrostatic pressure, (*b*) are

initiated both at structurally distinct nucleating sites and at different times during shape regeneration after depolymerization, and (c) are each related to the development and maintenance of specific portions of the asymmetric cell form. Since alterations in shape of living cells (reflecting alterations in the cytoplasmic microtubule system) can be monitored by light microscopy and can be quantitated in whole populations, *Ochromonas* has proved to be useful as a model system to investigate the source and possible control of nucleating site in microtubule initiation and polymerization. Hepler and Jackson (1969) proposed that the differential effects of the mitotic inhibitor isopropyl *N*-phenylcarbamate (IPC) and the microtubule depolymerizing agent colchicine on the spindle apparatus in endosperm cells may be due to an indirect action of IPC on microtubules by binding to the structurally amorphous spindle organizing material (i.e., nucleating site). This suggested that examining the response of *Ochromonas* to carbamate herbicides could provide a mechanism for confirming and clarifying the microtubule-nucleating site relationships in this system.

Ochromonas offers an additional advantage in investigating the action of IPC and CIPC (isopropyl *N*-3-chlorophenyl carbamate) in that the effects of these agents on both the flagellar and cytoplasmic microtubule systems can be readily and separately assayed. During shape regeneration after hydrostatic pressure or colchicine treatment, the two cytoplasmic nucleating sites (the kinetobeak fiber and rhizoplast, c.f. Bouck and Brown, 1973 for terminology and description of sites) apparently act to initiate microtubule growth, and one of these sites, the rhizoplast, also provides the point for spindle microtubule attachment during mitosis (Slankis and Gibbs, 1972; Bouck and Brown, 1973). Because of the highly regular disposition of oanelles and of well-defined nucleating sites any alteration in microtubule distribution caused by the experimental treatment is readily apparent. Similarly, methods are available to deflagellate cells, and flagellar regeneration occurs reasonably synchronously in populations of *Ochromonas* (Dubnau, 1961; Rosenbaum and Child, 1967). Therefore, the effects of microtubule-active reagents (e.g., colchicine) and protein synthesis inhibitors on regeneration can be quantitated and their effects on incorporation of labeled amino acids can be assayed in both cytoplasmic and flagellar systems.

Our results indicate that IPC and CIPC have similar effects in inhibiting both shape and flagellar regeneration but do not provide unequivocal evidence for the site of action of these reagents. In nonregenerating cells, IPC and CIPC have no apparent effect on flagellar microtubules and only a slight effect on cytoplasmic microtubules and cell shape. However, electron microscopy of cells made spherical by exposure to simultaneous application of IPC (or CIPC) and hydrostatic pressure reveals a transformation of cytoplasmic microtubules into larger diameter macro-tubules. Significantly, macro-tubules are frequently associated with microtubule nucleating sites. In cells recovering from an IPC-pressure treatment, macro-tubules disappear and micro-tubules reappear at the same nucleating sites concomitant with the reappearance of normal shape. Experiments using cycloheximide demonstrate that this regeneration occurs independent of measurable levels of new protein synthesis. Comparisons made with the action of the microtubule depolymerizing agent colchicine suggest that IPC may be especially useful in studies of microtubule assembly in *Ochromonas* and possibly in other organisms, particularly with organisms exhibiting high tolerance levels or insensitivity to colchicine.

MATERIALS AND METHODS

Cultures

Ochromonas danica was grown in defined medium as previously described (Bouck and Brown, 1973). Cells were harvested in the log phase of growth at a density of $2-5 \times 10^6$ cells/ml.

Flagellar Amputation and Regeneration

Cells were harvested by low speed centrifugation, concentrated to a 2-ml volume, and mechanically deflagellated in a fluted glass tube essentially as described earlier (Rosenbaum and Child, 1967). Regeneration of flagella in control media or treatment solutions was assayed by fixing samples in Lugol's iodine (6 g KI, 4 g I₂/100 ml water) at intervals during regeneration and by measuring the flagella of at least 50 cells/sample with an ocular micrometer.

Disassembly of Cytoplasmic Microtubules and Shape Regeneration

The pressure apparatus used to produce microtubule disassembly and concomitant loss of normal

cell shape has been previously described (Brown and Bouck, 1973). Cells were subjected to 6,000–8,500 lb/in² for 30 min in control media or treatment solutions; the pressure was released, and recovery was monitored in samples resuspended in control media or the appropriate treatment solution. During shape regeneration after an experimental treatment, 1-ml samples were fixed by addition of 1 ml of phosphate-buffered 4% glutaraldehyde, and at least 100 cells/sample were scored as possessing: the normal elongate form including the posterior tail (Fig. 10 *d*); intermediate shape, no tail but with some anterior beak asymmetry (Fig. 10 *b, c*); or completely spherical shape (Fig. 10 *a*).

Chemical Treatments

Reagents used to modify shape and flagellar regeneration were dissolved in fresh, sterile culture media just before use. Colchicine and cycloheximide (Sigma Chemical Co., St. Louis, Mo.) were dissolved directly in media and cells resuspended in these solutions were incubated under constant light and temperature. Purified IPC and CIPC (generously provided by PPG Inc., Chemical Div., Pittsburgh, Pa.), which have low solubility in water, were dissolved in 1 ml of ethanol and diluted to 100 ml with fresh media. Control media used in regeneration studies involving IPC or CIPC also contained 1% ethanol.

Microscopy

For observation of living cells during treatment or regeneration periods, a Zeiss 1.30 NA planapochromat bright-field objective and Nomarski optics were utilized. Photographs of swimming cells were obtained with a Zeiss photomicroscope equipped with a Zeiss 30 W.S. electronic flash and Kodak Pan-x 35-mm film developed in Diafine (Acufine, Inc., Chicago, Ill.) Measurements of flagellar regeneration and counts of shape regeneration were made directly using Nomarski optics at a magnification of $\times 1,600$.

For electron microscopy, 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 postfixation in cold 1% osmium tetroxide in the same buffer. Cells were dehydrated in a graded acetone series and embedded in Spurr's (1969) epoxy mixture. Sections cut with a DuPont diamond knife were collected on carbon-stabilized Formvar-coated grids, stained in uranyl acetate and lead citrate (Reynolds, 1963) and examined in an AEI-6B or a Hitachi HU 11E electron microscope.

Amino Acid Incorporation

The in vivo incorporation of amino acids into trichloroacetic acid-(TCA) precipitable protein was determined using the filter disk method (Mans and Novelli, 1961). 5-ml samples of cells from log phase cultures were incubated at constant light and temperature in the presence of uniformly labeled (¹⁴C) amino acid mixture (New England Nuclear, Boston, Mass.) at a specific activity of 5 μ Ci/ml. Details of methods used to increase uptake of labeled amino acids and to compensate for increase in cell number in control samples have been described previously (Brown and Bouck, 1973).

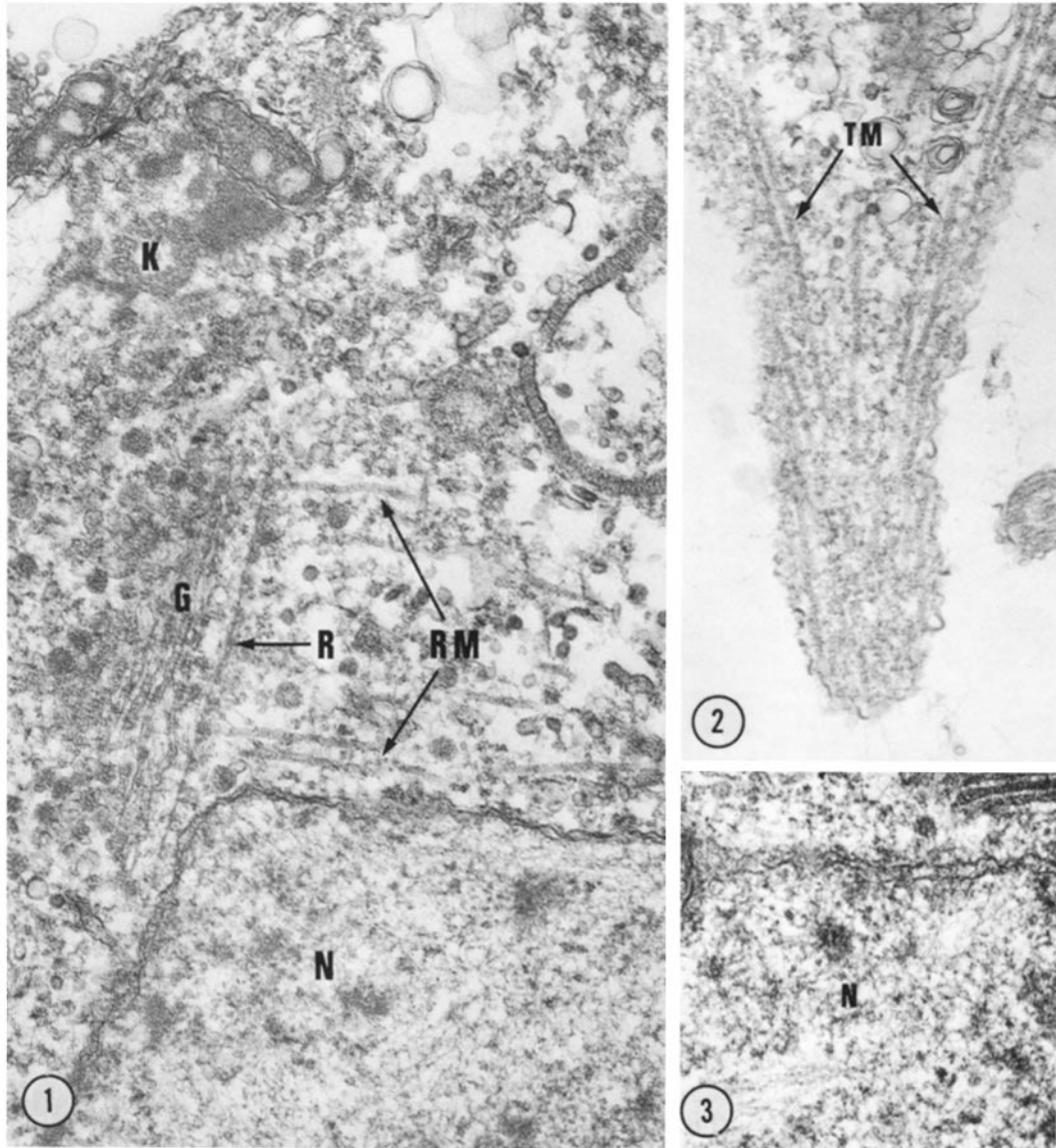
RESULTS

Effects of IPC and CIPC on Nonregenerating Cells

Initial studies on the effects of IPC and CIPC were carried out on nonregenerating cells. In control cultures of *Ochromonas*, between 70 and 90% of the cells exhibit the characteristic asymmetric form and posterior tail (Fig. 8 *a*). Exposure to high concentrations of IPC (up to 1.5×10^{-3} M) or CIPC (2×10^{-4} M) for 3–5 h had no observable effects on cell motility but did produce partial shape loss in some cells. In a variable percentage of cells, the posterior tail shortened or disappeared although in no case were completely spherical cells produced. Exposure for short periods (30 min) resulted in only a slight shortening of the tail in some cells.

A detailed description of the normal microtubule distribution in *Ochromonas* has been published (Bouck and Brown, 1973). Cytoplasmic microtubules are associated with intracellular sites (nucleating sites), which were interpreted to initiate the assembly of two main sets of microtubules, the beak and rhizoplast microtubules, in cells regenerating the normal shape. The basic features of this association are shown in Figs. 1 and 4 taken of cells exposed to IPC alone, and Figs. 22 and 23 taken of cells regenerating shape after an IPC-pressure treatment. Figs. 4 and 23 show the attachment point (the kinetobeak site) of the set of microtubules related to the anterior beak asymmetry of the cell. The other principal site of microtubule attachment, the rhizoplast (Figs. 1, 22), initiates microtubules which extend from the lower surface of the rhizoplast towards the cell posterior where they are believed to terminate in the tail (Figs. 2, 24).

The normal microtubule complement (Figs.



FIGURES 1-3 Cells exposed to 10^{-3} M IPC for 30 min. Fig. 1, Longitudinal section through cell anterior showing the rhizoplast (*R*) with the Golgi complex (*G*) on its upper surface and rhizoplast microtubules (*RM*) attached at its lower surface. *K*, kinetosome; *N*, nucleus. $\times 46,000$. Fig. 2, Longitudinal section through the cell posterior with microtubules (*TM*) converging into the cell tail. $\times 43,000$. Fig. 3, Portion of nucleus (*N*) showing fibrillar nuclear material in IPC-treated cell. $\times 50,000$.

1, 2, 4) is observed in cells exposed to 10^{-3} M IPC for 30 min, a time equivalent to that used in subsequent pressure-IPC experiments. In cells exposed to IPC for longer periods (3-5 h), there is no apparent effect on any cell organelles other than an increase in the amount of fibrillar material seen in the nucleus (Fig. 3). The flagellar

microtubules (Fig. 6) and most cytoplasmic microtubules (Fig. 5) appear unaltered by this treatment but a few larger diameter macro-tubules are observed coexistent with microtubules in some cells. Prolonged exposure to IPC (up to 17 h) does result in increasing numbers of macro-tubules, although large numbers of microtubules

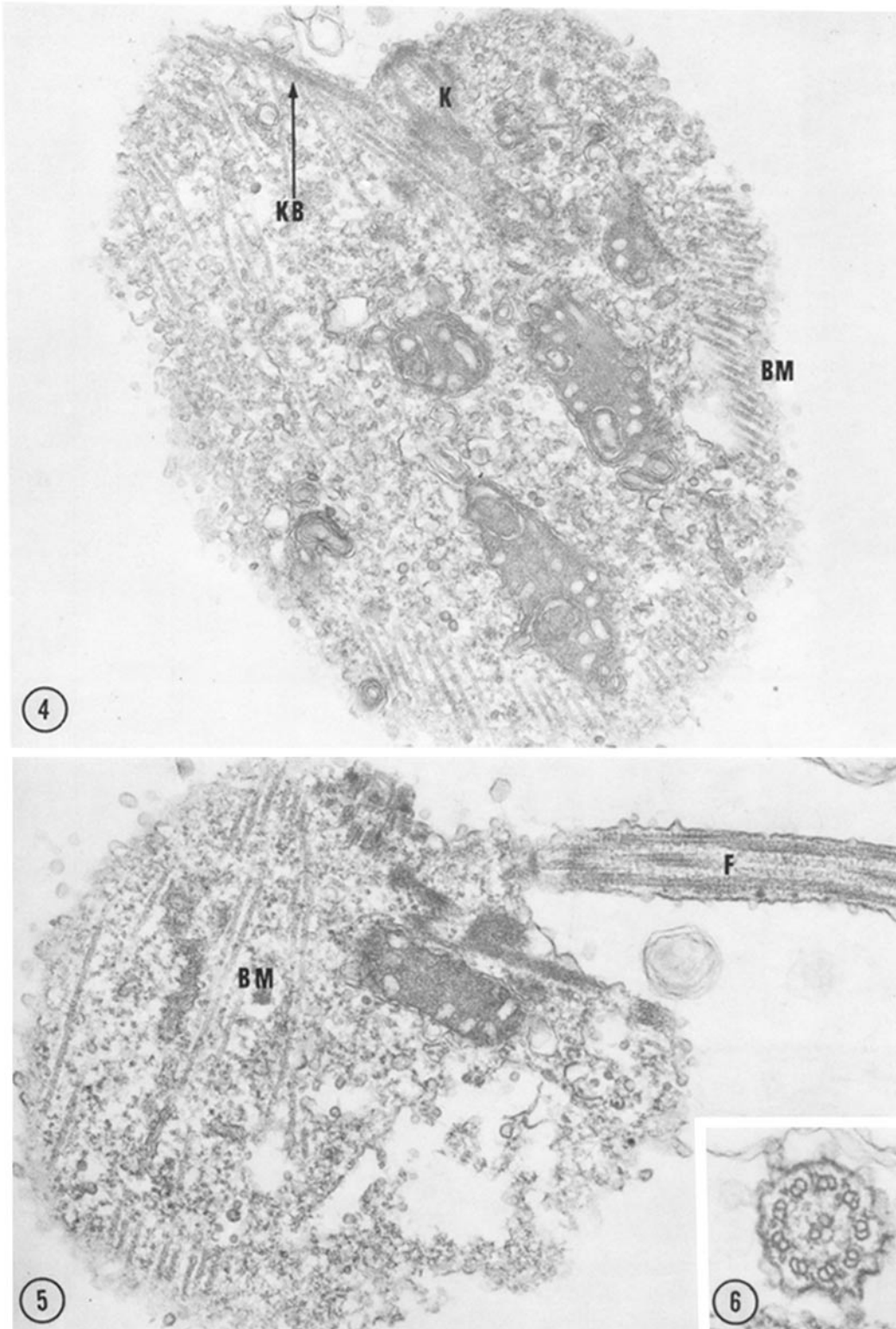


FIGURE 4 Section cut tangential to the beak illustrating the kineto-beak complex (*KB*) and numerous beak microtubules (*BM*) just beneath the cell surface. *K*, kinetosome. Cell exposed to 10^{-3} M IPC, 30 min. $\times 32,000$.

FIGURES 5 and 6 Cells exposed to 10^{-3} M IPC for 3 h. Note the presence of normal beak microtubules (*BM*, Fig. 5) and flagellar microtubules (*F*, Fig. 5 and Fig. 6). Fig. 5, $\times 59,000$; Fig. 6, $\times 83,000$.

are still observed. No macrotubules or other obvious effects are observed in nonregenerating cells exposed to 2×10^{-4} M CIPC.

Although cell counts were not made in this study, IPC appeared to inhibit cell division. Light microscopy of populations exposed to IPC for periods up to 17 h (normal generation time about 9 h) revealed very few dividing cells, and complete cytokinesis was not observed. Dividing cells were only rarely seen by electron microscopy,

suggesting that most cells are blocked at some stage before mitosis. One of the few dividing cells observed, which appears to be in late prophase, is shown in Fig. 7. Portions of the two mitotic rhizoplasts, each with a Golgi complex on the outer surface, are evident. However, the complement of spindle microtubules which normally extends between the two rhizoplasts (Bouck and Brown, 1973) is absent and is replaced by large numbers of macro-

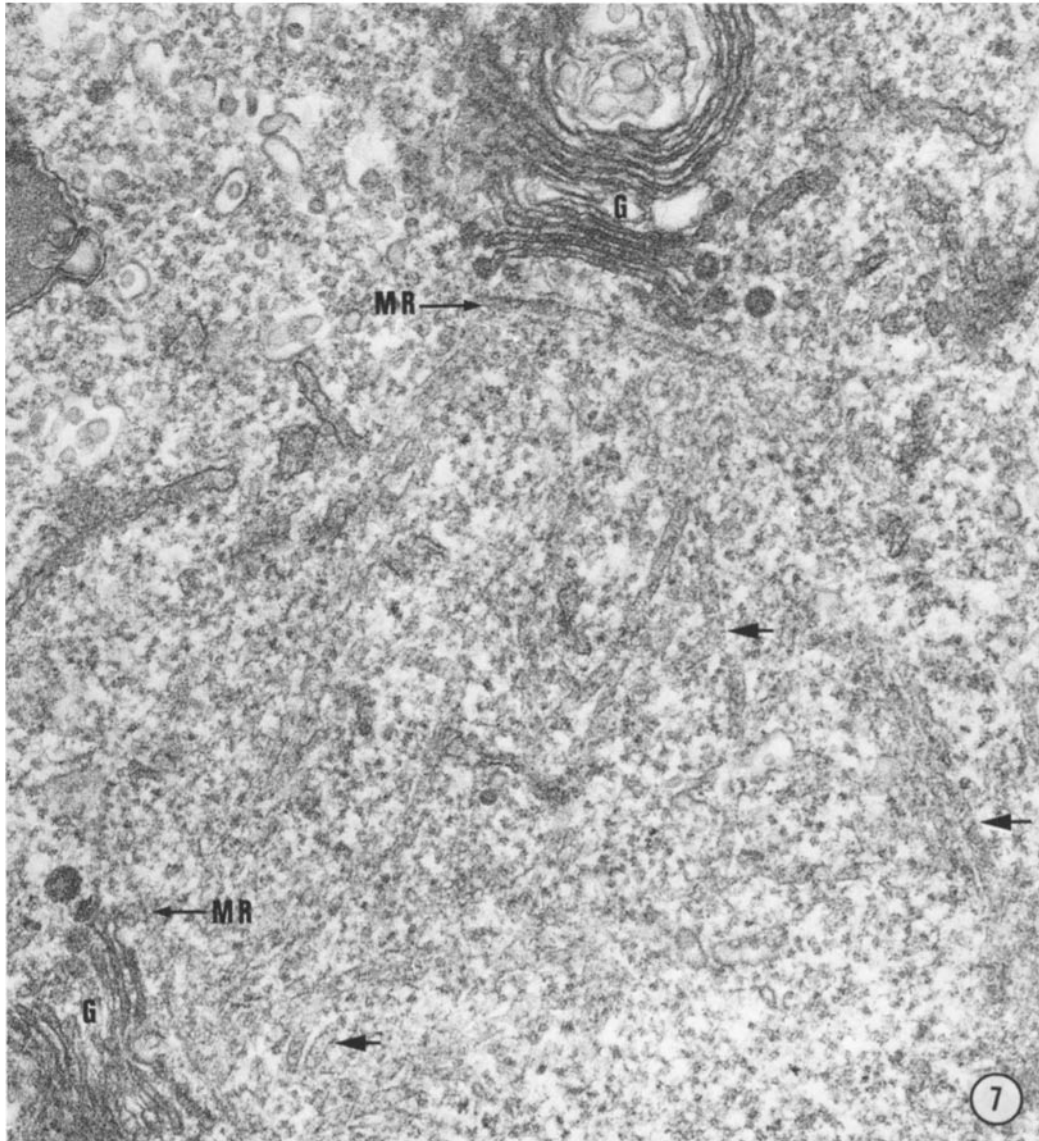


FIGURE 7 Cell in 10^{-3} M IPC for 3 h blocked in prophase. Spindle microtubules are replaced by macro-tubules (arrows) which appear to converge on the mitotic rhizoplast (MR). G, Golgi complex. $\times 53,000$.

In the following experiments, the effects of IPC on regenerating microtubule systems will be described in detail. CIPC, within a very narrow concentration range, proved to be effective at lower concentrations than IPC and some comparative data are included.

Effect of IPC on Shape Regeneration of Pressure-Treated Cells

The absence of substantial action of IPC on cytoplasmic microtubules or cell shape in non-regenerating cells, even at concentrations at least 10- to 50-fold higher than those effective in other organisms, suggested that IPC may affect only developing microtubule systems. We have shown previously (Brown and Bouck, 1973) that relatively high hydrostatic pressure (8,000 lb/in², 30 min) will reversibly alter the normal cell shape, producing a population of uniformly spherical cells. However, 8,000 lb/in² in our system did not depolymerize all cytoplasmic microtubules as revealed by electron microscopy. Higher pressure deflagellated most cells.

IPC (10⁻³ M) added during or immediately after exposure to 8,000 lb/in² for 30 min completely blocks the reassembly of cytoplasmic microtubules and shape regeneration as long as it is present. IPC has no apparent effect on microtubules that were not depolymerized by the prior exposure to pressure. Hydrostatic pressure is interpreted to act on other microtubule systems by shifting a dynamic equilibrium to the depolymerized state (Inoué and Sato, 1967). Reagents which bind to microtubule protein and prevent repolymerization (e.g., colchicine), therefore, exhibit a synergistic action with pressure (Marsland, 1966). These observations suggested that IPC, regardless of its specific site of action, by blocking microtubule assembly would also act synergistically with hydrostatic pressure.

Combined Effects of IPC and Hydrostatic Pressure

LOSS OF SHAPE IN IPC PRESSURE

Preliminary observations were made on cells exposed to varying pressures (4,000–8,500 lb/in²) for 30 min in the presence of a high concentration of IPC (10⁻³ M), shown above to block shape regeneration of pressure-treated cells. 4,000–6,000 lb/in² for 30 min in 10⁻³ M IPC produces a

population of cells with the normal interior asymmetry but no tails. At higher pressures (6,500–8,500 lb/in²) the anterior asymmetry is lost as well, resulting in a uniform population of spherical cells (Fig. 8 *b*). At the higher pressures (above 6,500 lb/in²) an increasing percentage of cells is deflagellated, and these cells regenerate shape more slowly than spherical cells

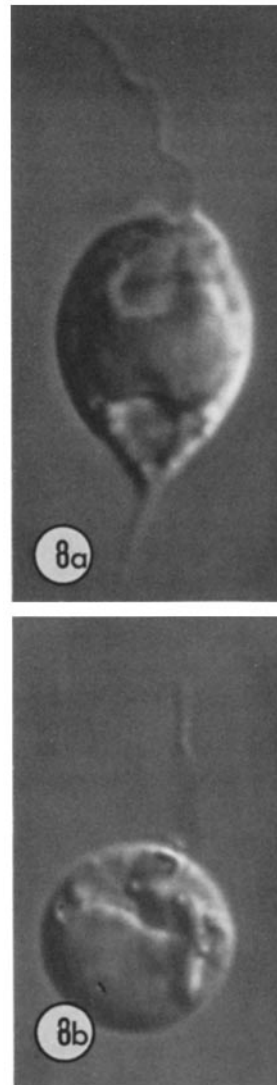


FIGURE 8 *a-b* Flash photographs showing return to normal cell shape of *Ochromonas* 2.5 h after pressure release (8,500 lb/in², 30 min) (*a*) and spherical cells (*b*) after treatment with IPC pressure (8,500 lb/in², 30 min in 10⁻³ M IPC followed by 2.5 h of IPC at 10⁻³ M).

with flagella. To minimize this problem, cells pressurized at 6,500 lb/in² in IPC were used in some regeneration studies even though microtubule depolymerization was not complete (see below).

The effective concentration range for IPC was determined by pressurizing cells at 6,500 lb/in² for 30 min in varying concentrations of IPC and by counting the percentage of cells regenerating the complete normal shape after 80-min recovery in the same IPC concentration (Fig. 9). At 10⁻⁴ M IPC, normal regeneration occurs, at 10⁻³ M, regeneration is completely blocked as long as IPC is present, and at intermediate concentrations partial shape regeneration occurs. The lowest concentration of IPC which consistently blocked shape regeneration (10⁻³ M) was used in subsequent experiments.

REGENERATION OF SHAPE IN IPC-PRESSURE TREATED CELLS

The effects on cell shape of a 30-min exposure to 10⁻³ M IPC in conjunction with pressure (up to 8,500 lb/in²) are completely reversible. The pattern of shape regeneration is identical to that observed in cells recovering from exposure to colchicine or pressure alone (Brown and Bouck, 1973). Regenerating cells first reform the anterior beak asymmetry, then elongate, and lastly reform the posterior tail (Fig. 10 a-d). Shape regeneration occurs rapidly and in close synchrony within the population when the pres-

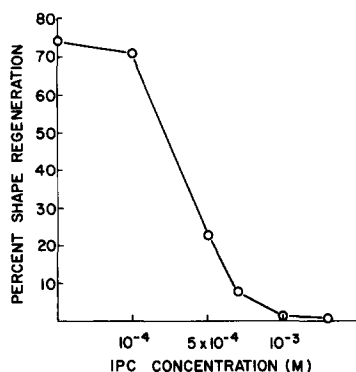


FIGURE 9 Effect of pressuring cells (6,500 lb/in², 30 min) in increasing concentrations of IPC on subsequent shape regeneration in IPC. Cells pressurized and allowed to recover in the same IPC concentration plotted against percentage of cells regenerating complete cell shape 80 min after pressure release.

sure is released and when cells are placed in fresh medium without IPC. After 90-min recovery, the population appears identical to a control culture never exposed to IPC and pressure (Fig. 10 d). Including 1% ethanol in the regeneration medium which had no detectable effect on the kinetics or pattern of shape regeneration.

Effect of Cycloheximide on Shape Regeneration

The rapidity of shape regeneration when IPC pressure-treated cells are returned to control media suggested that regeneration involved a reassembly of cytoplasmic microtubules, as was shown in cells recovering from exposure to pressure alone (Brown and Bouck, 1973). Fig. 11 shows that cycloheximide, at a concentration which effectively inhibits incorporation of amino acids into TCA-precipitable protein (Fig. 12), has little effect on the kinetics or total percentage of shape regeneration in IPC pressure-treated cells. The slightly lower total percent regeneration after 45-min recovery in cycloheximide is probably due to the presence of some deflagellated cells in the population. Deflagellated cells regenerate only about one-fourth of the flagellum in cycloheximide, and these cells regenerate shape more slowly than deflagellated cells recovering in control medium.

IPC (10⁻³ M) has no detectable effect on the incorporation of labeled amino acids into TCA-precipitable protein (Fig. 12). The inhibition of shape regeneration and flagellum regeneration by IPC, therefore, is not due to a general inhibition of protein synthesis.

Electron Microscopy of IPC (CIPC)-Pressure Effects

MICROTUBULE ALTERATIONS IN IPC PRESSURE

DIFFERENTIAL SENSITIVITY OF CYTOPLASMIC MICROTUBULES: Exposure of *Ochromonas* to 6,000 lb/in² for 30 min in the presence of 10⁻³ M IPC produces a uniform population of motile cells possessing the normal anterior beak asymmetry but lacking tails. The beak set of microtubules is still present in these cells, but the rhizoplast microtubules have disappeared and short segments of a larger diameter macrotubule are observed associated with the rhizoplast (Fig. 13). If these cells are left in IPC after the IPC-pressure treatment there is no shape regeneration, the

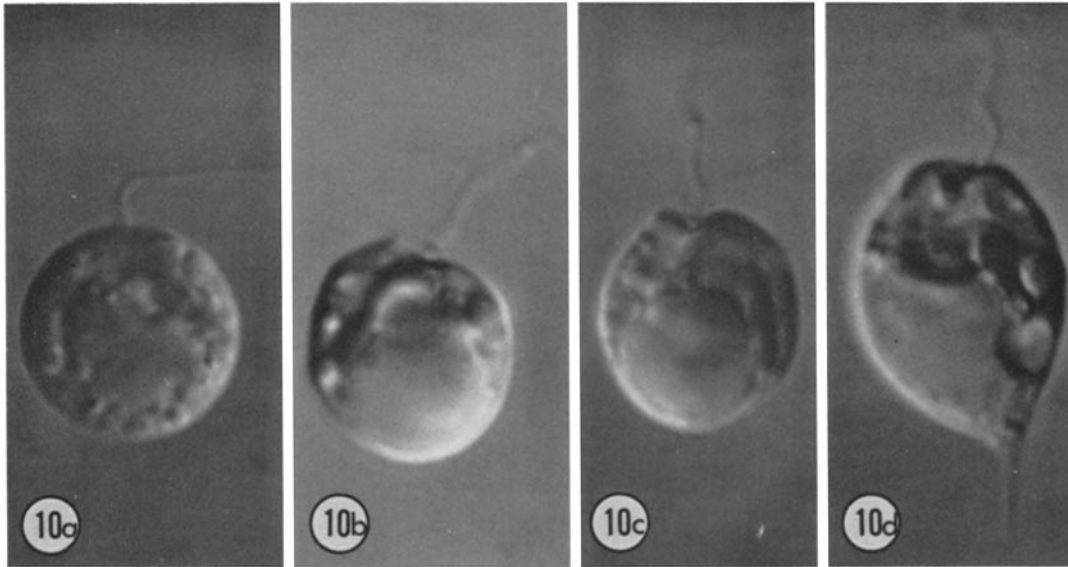


FIGURE 10 *a-d* Flash photographs of swimming *Ochromonas* illustrating the sequence of shape regeneration when IPC is removed after pressure-IPC treatment. (a) 0 time recovery, spherical cell; (b) 25-min recovery, regeneration of anterior asymmetry; (c) 50-min recovery, cell somewhat elongated; (d) 95-min recovery, normal cell fully regenerated. $\times 1,300$.

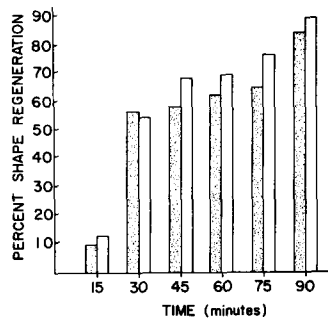


FIGURE 11 Effect of cycloheximide on shape regeneration of pressure IPC-treated *Ochromonas*. Cells pressurized (6,500 lb/in², in 10⁻³ M IPC) and regeneration of the complete cell shape assayed in either control media (open bars) or 20 µg/ml cycloheximide (stippled bars).

beak microtubules appear unaltered, and macro-tubules remain associated with the rhizoplast as long as IPC is present. At slightly higher pressures (6,500–7,000 lb/in², 30 min) the beak microtubules are also affected. Cells appear spherical, fewer beak microtubules are present, and more macro-tubules are found in the cytoplasm in addition to those associated with the rhizoplast. At these pressures, few cells are deflagellated and when the cells are placed in

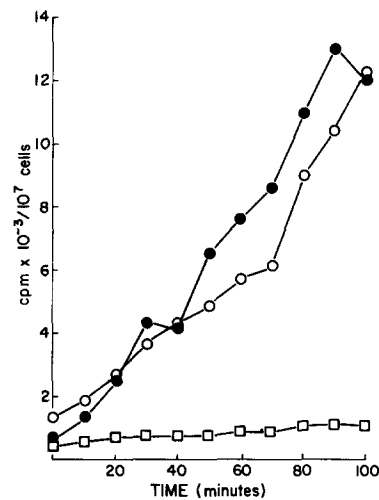


FIGURE 12 Effect of cycloheximide and IPC on incorporation of labeled (¹⁴C) amino acids into TCA-precipitable protein. Open circles, control; closed circles, 10⁻³ M IPC; squares, 20 µg/ml cycloheximide.

fresh medium without IPC, the normal shape is regenerated within 90 min (Fig. 11).

The differential sensitivity of beak and rhizoplast microtubules is also evident in cells exposed to CIPC and hydrostatic pressure. Although CIPC appears to affect the rhizoplast micro-

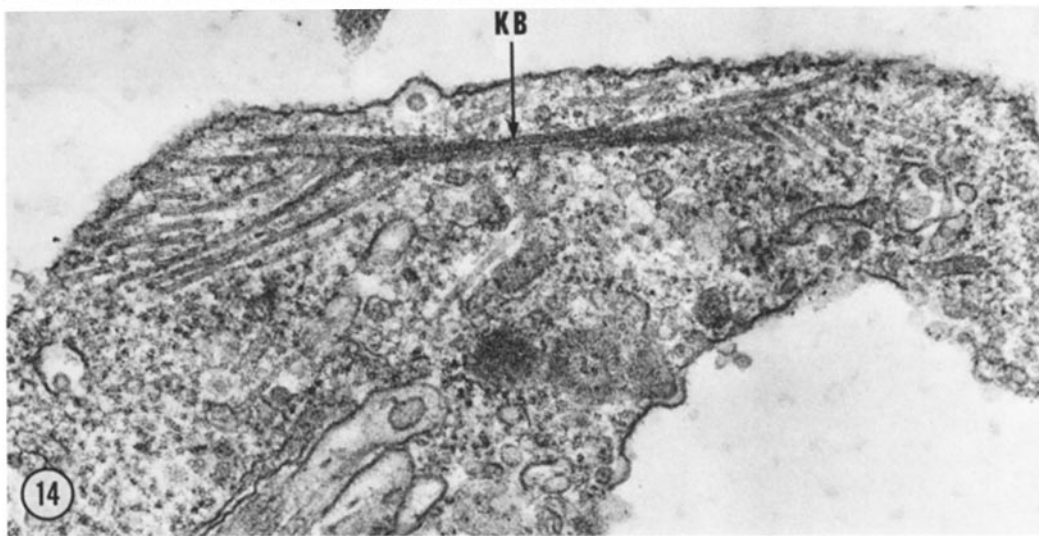


FIGURE 13 Longitudinal section through anterior of a cell exposed to 10^{-3} M IPC, 6,000 lb/in² for 30 min and fixed immediately after pressure release. Note that microtubules of the beak (*Mi*) are present but only macrotubules (*Ma*) insert on the rhizoplast (*R*). *K*, kinetosome; *G*, Golgi complex; *N*, nucleus. $\times 54,000$.

FIGURE 14 View of the regenerated kineto-beak complex (*KB*) in a cell exposed to 8,500 lb/in², 30 min in 2×10^{-4} M CIPC after 150-min recovery in the same CIPC concentration. $\times 44,000$.

tubules in lower concentrations than IPC, the concentration range is very critical and only the rhizoplast system of microtubules is affected. Cells were exposed to 8,500 lb/in² for 30 min (a pressure shown to depolymerize nearly all microtubules, Brown and Bouck, 1973) in the presence of varying concentrations of CIPC and were permitted to recover for 150 min in the same CIPC concentration. Under these conditions, 3×10^{-4} M CIPC produced cells with bizarre shapes or resulted in cell lysis. Both the beak and rhizoplast microtubules reassemble in cells recovering in 10^{-4} M CIPC. At intermediate concentrations (2×10^{-4} M) microtubules return to the beak complex (Fig. 14) but macrotubules only are found on the rhizoplast. At the light and electron microscope levels, this population appears identical to a sample exposed to lower pressure, 6,000 lb/in² for 30 min, and 10^{-3} M IPC (i.e., are without tails).

MACROTUBULES IN IPC PRESSURE: In contrast to the effects of CIPC and higher pressures, exposure of cells to 8,000–8,500 lb/in² for 30 min in 10^{-3} M IPC results in a uniform population of spherical cells which exhibit no shape regeneration as long as IPC is present. Cytoplasmic microtubules are completely absent from these cells and are replaced by larger macrotubules 350–400 Å in diameter. The tubule wall is 60–70 Å thick and each tubule usually has a central electron-opaque granule (Fig. 18), which appears as a thin line down the center of the macrotubule in longitudinal section (Fig. 17). Macrotubules occur in several arrangements in the cytoplasm and nucleus (Figs. 15–18) but never show the peripheral localization of microtubules in untreated cells. Macrotubules not associated with the rhizoplast occur either as short randomly scattered segments (Fig. 15) or form bundles of long (3–4 μm), parallel, closely associated tubules in the cytoplasm (Fig. 17) and nucleus (Fig. 16).

MACROTUBULE-NUCLEATING SITE RELATIONSHIP: The most striking feature of IPC and CIPC pressure-treated cells is the close association of macrotubules with one of the microtubule nucleating sites, the rhizoplast. In most of the cells examined, the rhizoplast does not appear to be altered in structure or disposition in the cell by this treatment. As in control cells, the rhizoplast extends from the kinetosome region to the nucleus (Fig. 20), the upper surface is associated with the Golgi complex (Figs. 13, 20, 21), and the electron-

opaque material, which forms crossbandings on the lower rhizoplast surface, appears unaltered. However, the normal complement of rhizoplast microtubules is completely absent and is replaced by macrotubules which clearly terminate or originate directly on the lower rhizoplast surface (Fig. 21). At the higher pressures used (8,000–8,500 lb/in²), fewer rhizoplasts were encountered and, in a few cells, fragments of the rhizoplast which have lost their kinetosome attachment were observed. This may be the result of the pressure-induced retraction of flagellar axonemes into the cytoplasm (Fig. 19), and it occurred in some cells at pressures above 8,000 lb/in² in the presence or absence of IPC.

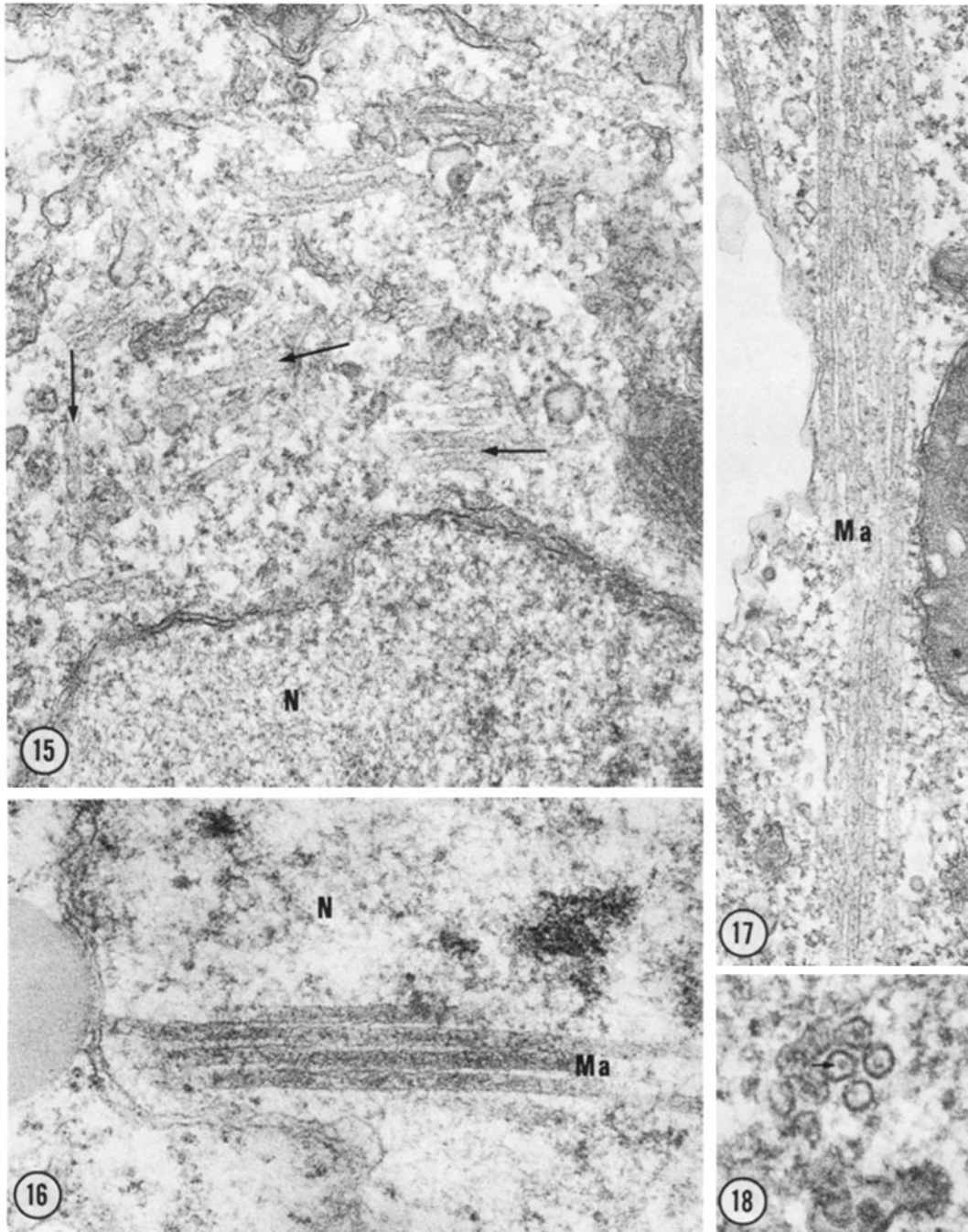
MICROTUBULES IN REGENERATING CELLS

Cells were fixed at intervals during shape regeneration in control medium after an exposure to pressure (8,000 lb/in², 30 min) and 10^{-3} M IPC. Within 10 min after return to control medium, microtubules of the beak complex appeared completely reassembled (Fig. 23) concurrent with regeneration of the beak asymmetry. Between 10- and 30-min recovery, microtubules reappear in association with the lower rhizoplast surface (Fig. 22) coincident with regeneration of the tail (Fig. 24), and by 60–80 min the cells have the normal microtubule complement and cell shape. During the recovery period, macrotubules disappear from the cytoplasm as microtubules reassemble. In the first 10 min of recovery, macrotubules and microtubules appeared coexistent in the cytoplasm; after 10 min, cytoplasmic macrotubules were rare and by 30-min recovery were never observed. Nuclear macrotubules persist longer, were frequently seen after 30-min recovery, but disappeared by 80 min. Cells recovering in 20 μg/ml cycloheximide (after exposure to IPC pressure) showed an identical pattern and time-course of macrotubule loss and microtubule reassembly.

Effects of IPC and CIPC on Flagellar Regeneration

CONTROL EXPERIMENTS—STANDARD CURVES

In different regeneration experiments, the percentage of cells deflagellated was reproducible and the total percentage of cells regenerating flagella in control media was comparable. How-



FIGURES 15-18 Macrotubules in IPC or CIPC pressure-treated cells. Fig. 15, Randomly oriented macro-tubules (arrows) near the nucleus (*N*). $\times 41,000$. Fig. 16, Aligned macrotubules (*Ma*) inside the nucleus (*N*). $\times 60,000$. Fig. 17, Longitudinal section through a group of cytoplasmic macrotubules (*Ma*). $\times 53,000$. Fig. 18, Cross section through a group of macrotubules. Arrow indicates central granule $\times 115,000$.

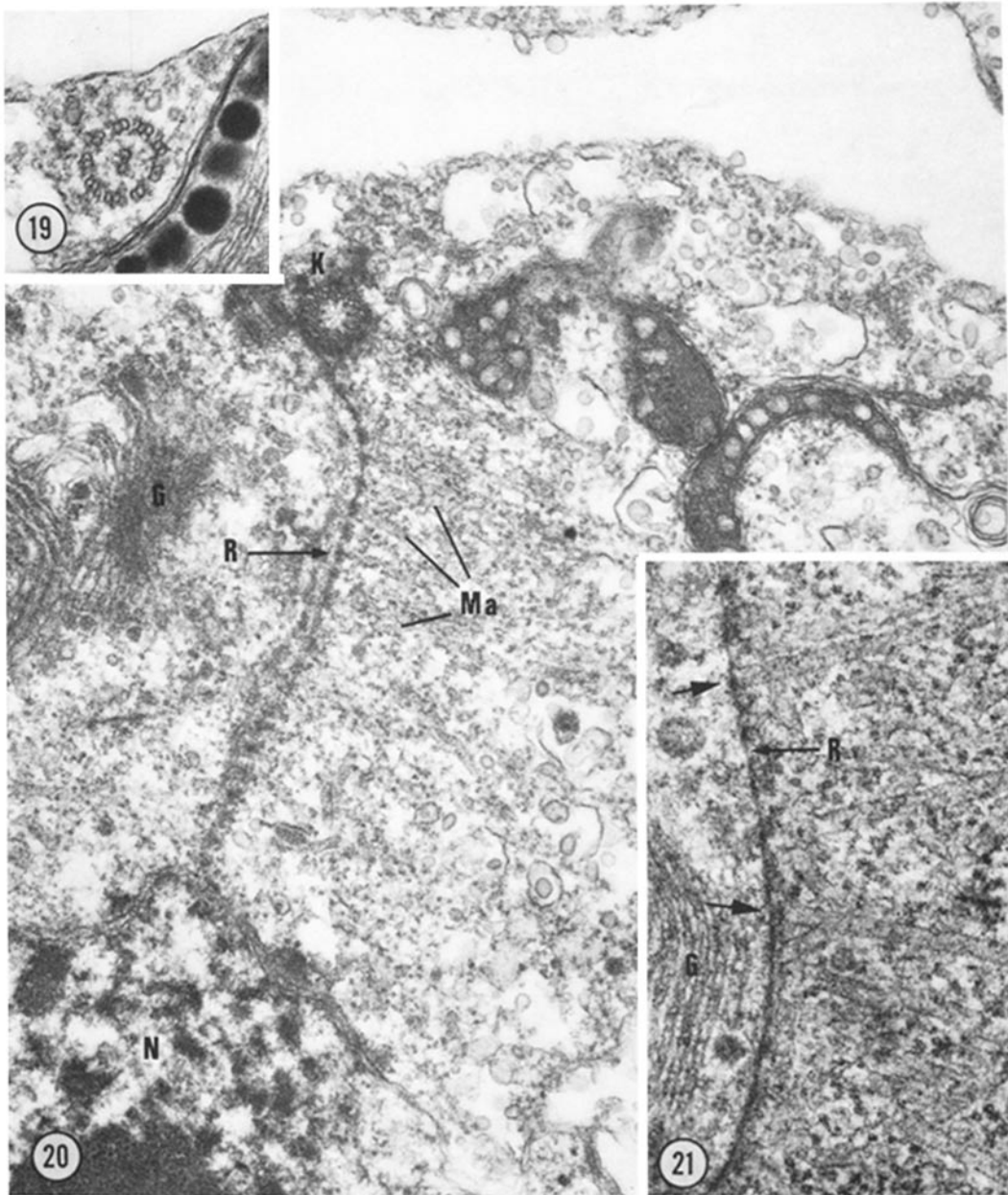
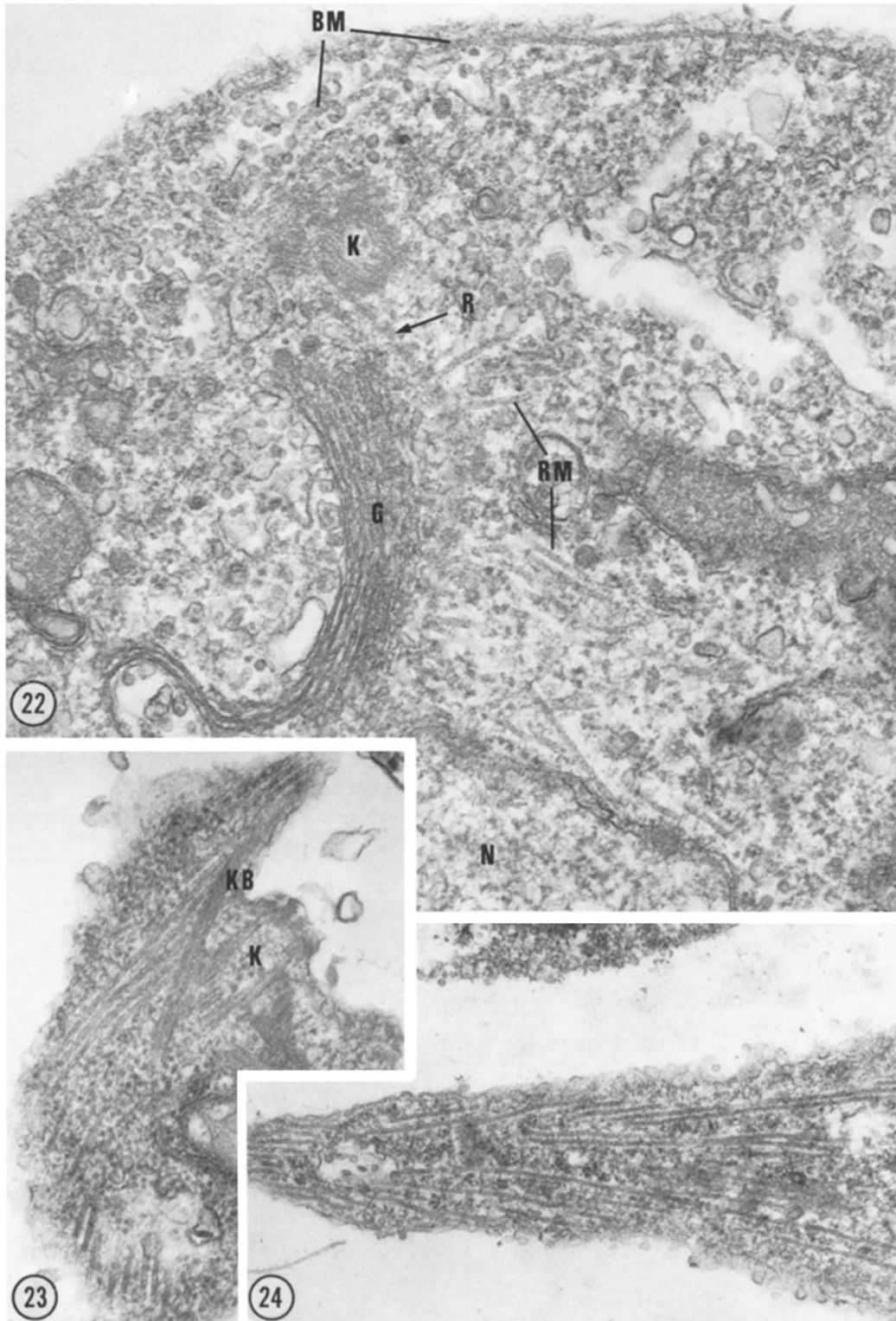


FIGURE 19 Section from a cell exposed to 8,000 lb/in² for 30 min to 10⁻³ M IPC showing a flagellar axoneme in cross section within the cytoplasm. $\times 51,000$.

FIGURE 20 Longitudinal section through a spherical cell after 30-min exposure to 8,000 lb/in² in 10⁻³ M IPC. No cytoplasmic microtubules are present. Macrotubules (*Ma*) are associated with the rhizoplast (*R*). Note the regular appearance of electron-opaque material on the lower rhizoplast surface. *K*, kinetosome; *G*, Golgi complex; *N*, nucleus. $\times 36,000$.

FIGURE 21 High magnification of a portion of the rhizoplast (*R*) in a pressure CIPC-treated cell (8,500 lb/in², 30 min, 2×10^{-4} M). Arrows indicate points of macrotubule termination on the rhizoplast. *G*, Golgi complex. $\times 44,000$.



FIGURES 22-24 Reassembled microtubules in cells regenerating shape after a 30-min exposure to 8,000 lb/in² in 10⁻³ M IPC. Fig. 23, Regenerated kineto-beak complex (*KB*) after 10-min recovery. *K*, kinetosome. $\times 55,000$. Fig. 22, Longitudinal section through cell after 20-min recovery. Beak microtubules (*BM*) and rhizoplast microtubules (*RM*) reassembled at the lower rhizoplast (*R*) surface are evident. *K*, kinetosome; *G*, Golgi complex; *N*, nucleus. $\times 49,000$. Fig. 24, Section through the tail of fully recovered cell showing microtubules converging in the tail. $\times 37,000$.

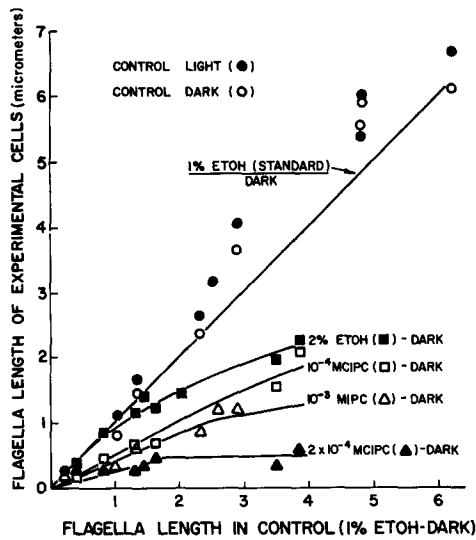


FIGURE 25 Flagellar regeneration plotted using a standard curve obtained from regeneration in 1% ETOH. See text for figure explanation.

ever, the rate of flagellar elongation in control media did vary with the age of the culture used and the experimental conditions during regeneration. To compensate for this variability among different experiments, a standard curve was constructed based on a 1% ethanol control. The data from each experiment were then plotted against the 1% ethanol control for that particular experiment (Fig. 25).

The choice of regeneration in 1% ethanol in the dark as the standard was a result of two considerations: (a) ethanol was used as the solvent for IPC and CIPC at a final concentration of 1%, and (b) studies of flagellar regeneration in another photosynthetic flagellate, *Euglena* (unpublished data), showed a reversal of IPC inhibition in the light but not in the dark. 1% ethanol is the lowest concentration in which IPC and CIPC, at the experimental concentrations used, will remain in solution. There is a slight inhibitory effect of 1% ethanol on the rate of flagellar elongation relative to regeneration in the light or dark without ethanol (Fig. 25). 2% ethanol is clearly unacceptable.

Both IPC and CIPC used at concentrations shown above to completely block shape regeneration cause a partial inhibition of flagellar regeneration (Fig. 25). Again, CIPC proved to be an effective inhibitor at lower concentrations than IPC. Cells regenerating in 2×10^{-4} M CIPC

form only about 0.5–1 μm of flagellum in 150 min, whereas, in 10^{-3} M IPC, 1.5–2 μm of flagellum regenerates in the same time period.

FLAGELLAR REGENERATION IN IPC

The characteristics of flagellar regeneration in *Ochromonas* described in detail by Dubnau (1961) and Rosenbaum and Child (1967) have been verified in this study. After mechanical amputation, there is a 20–25-min lag period, after which flagella elongate, following deceleratory kinetics, and regain about 90% of their initial length by 180 min. Fig. 26 shows the effect of varying concentrations of IPC on flagellar regeneration. Above 10^{-4} M, there is increasing inhibition, with about 1.5 μm of flagellum regenerating after 180 min in 10^{-3} M IPC. Higher concentrations of IPC do result in greater inhibition (closed circles, Fig. 26) but the effects are only slowly reversible when the cells are returned to control media. In any inhibitory concentration of IPC or CIPC, the rate of flagellar elongation is decreased from the start of regeneration (Fig. 27). Addition of the inhibitor 30 min before amputation resulted in only a slightly greater inhibitory effect.

COMPONENTS OF IPC INHIBITION

Further analysis of IPC and CIPC effects reveals that the suppressed flagellar regeneration in populations of *Ochromonas* is a result of both a decline in the absolute growth rate of flagella and a decline in the percentage of cells regenerating flagella. These two components of inhibition, which together result in the regeneration kinetics in Fig. 27, have been examined separately. Fig.

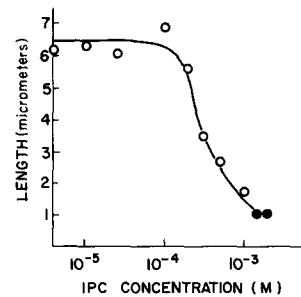


FIGURE 26 Effect of increasing IPC concentration on flagellar regeneration. IPC added immediately after amputation and flagellum length measured after 180 min. Inhibition is fully reversible to 10^{-3} M IPC, open circles.

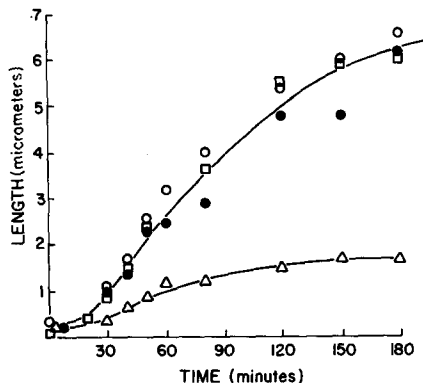


FIGURE 27 Effect of IPC on flagellar regeneration. Triangles, 10^{-3} M IPC; closed circles, 1% ETOH control in the dark; open circles, control minus ETOH in the light; squares, control minus ETOH in the dark.

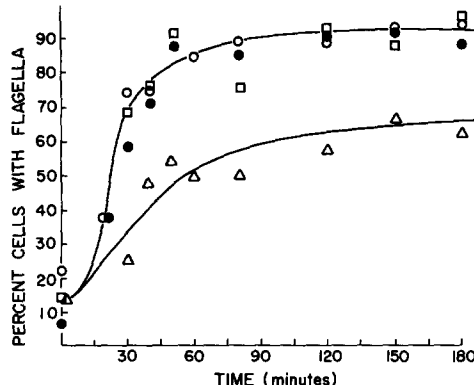


FIGURE 28 Effect of IPC on percentage of cells regenerating flagella. Symbols as for Fig. 27.

28 shows the percentage of cells with flagella at various times after flagellar amputation, and Fig. 29 shows the average length of the regenerated flagella at the same times. 90% of the cells have reformed short flagella (average length $2.5 \mu\text{m}$) after 50-min recovery in control media, and these elongate in close synchrony to about $6.5 \mu\text{m}$ by 180 min. By contrast, in populations regenerating in 10^{-3} M IPC, only about 60% of the cells reform flagella (Fig. 28) and these flagella elongate very slowly after 60 min to an average length of $2.7 \mu\text{m}$ by 180 min (Fig. 29). In 2×10^{-4} M CIPC, both the percentage of cells reforming flagella and the rate of flagellar elongation are lower than in IPC.

REVERSIBILITY OF INHIBITION

The inhibitory effects of IPC and CIPC on flagellar regeneration are completely reversible when cells are returned to fresh medium. Fig. 30 shows the length of flagella regenerating after a 3-h recovery in control media after a 3-h exposure to varying concentrations of IPC. A comparable length of flagellum is reformed ($7\text{--}8 \mu\text{m}$) after inhibition, up to a maximum of 10^{-3} M IPC. After exposure to concentrations greater than 10^{-3} M, the rate of elongation during recovery is slower but flagella eventually reach the control length.

Comparative Data: Effects of Colchicine and Cycloheximide

The results in the preceding sections clearly implicate microtubules as the target for IPC and

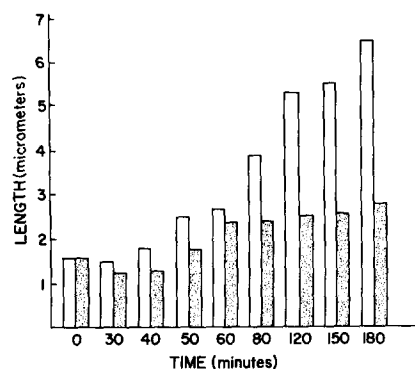


FIGURE 29 Average flagellar length on those cells regenerating flagella in control media (1% ETOH, dark), open bars, or 10^{-3} M IPC, stippled bars.

CIPC effects. The regeneration of flagella after mechanical amputation and shape regeneration of pressure-treated cells are dependent on microtubule assembly, and in both cases IPC has inhibitory effects, although to a different degree, over the same concentration range (Fig. 31). In the absence of direct evidence for the specific mode of action of IPC, comparative studies were carried out using the known inhibitor of microtubule assembly, colchicine, and the protein synthesis inhibitor, cycloheximide. The results of these studies are summarized in Fig. 33 and Tables I and II.

There are several obvious and distinctive differences between the effects of colchicine, cycloheximide, and IPC (or CIPC). (a) Exposure to colchicine alone (10 mg/ml) results in disassembly of all cytoplasmic microtubules concurrent with loss of cell shape. (b) Concentrations of colchicine which cause shape loss (above 7 mg/ml) also

completely inhibit flagellar regeneration (Fig. 32). (c) The effects of colchicine on cell shape and flagellar regeneration are only slowly reversible (7–10 h) when cells are permitted to recover in control medium. (d) Cycloheximide completely inhibits shape regeneration and the reappearance of microtubules in cells made spherical by prior exposure to colchicine (Fig. 33). (e) Comparable flagellar regeneration occurs (1.5–2 μm of flagellum formed) in 20 $\mu\text{g}/\text{ml}$ cycloheximide, which inhibits amino acid incorporation, and in 10^{-3} M IPC, which has no detectable effect on incorporation (Fig. 12). However, in contrast to the action of IPC or CIPC, cycloheximide does not inhibit micro-

tubule reassembly and shape regeneration in cells recovering from exposure to hydrostatic pressure (Fig. 33). (f) In the absence of IPC, no macrotubules were observed in cells exposed to pressure, colchicine, cycloheximide, or any combination of these agents.

DISCUSSION

There is increasing evidence that microtubule assembly in vivo (for reviews see Porter, 1966; Pickett-Heaps, 1969; Tilney, 1971) and in vitro (Borisy et al., 1972; Weisenberg et al., 1972) is

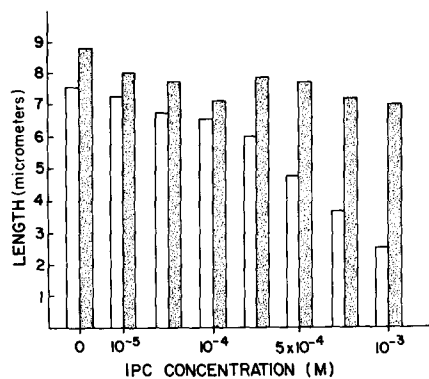


FIGURE 30 Reversibility of IPC inhibition of flagellar regeneration. Open bars, flagellar length after 180 min in varying concentrations of IPC. Stippled bars, flagellar lengths after 180-min recovery in control media after the period of IPC inhibition.

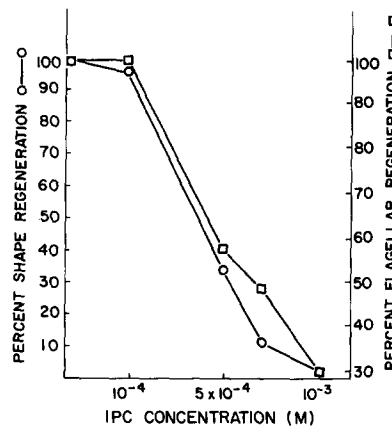


FIGURE 31 Comparison between shape regeneration and flagellar regeneration in increasing IPC concentration. Both graphed as percent of the control for that experiment. Actual flagellar length in control was 7.6 μm after 180-min recovery; actual percentage of cells regenerating complete shape after 80 min following exposure to 6,500 lb/in^2 for 30 min was 70%.

TABLE I
Effect of Various Reagents on Cell Shape, Microtubules, and Amino Acid Incorporation

Treatment	Cell shape	Effect assayed	
		Cytoplasmic microtubules	Amino acid incorporation
Colchicine	Spherical	Disassembly	Slight inhibition
IPC (10^{-3}M)	Tails shortened or absent	Most intact, few macrotubules	No effect
CIPC ($2 \times 10^{-4}\text{M}$)	Tails shortened	No effect	—
Cycloheximide (20 $\mu\text{g}/\text{ml}$)	No effect	No effect	>95% inhibition
IPC pressure (8,000 lb/in^2 , 30 min)	Spherical	Macrotubules	—
CIPC pressure (8,500 lb/in^2 , 30 min)	Spherical	Macrotubules	—

See text for discussion.

TABLE II
Effects of Various Reagents on Shape and Flagellar Regeneration

Recovery medium	Shape regeneration-microtubule reassembly			Flagellar regeneration
	after colchicine	after IPC pressure	after CIPC pressure	
Control	+	+	+	Complete re-generation
	(7-10 h)	(60-80 min)	(60-80 min)	
		Macrotubules → microtubules	Macrotubules → microtubules	
Cycloheximide (20 μg/ml)	-	+	+	Partial regeneration (1.5-2 μm)
		(60-80 min)	(60-80 min)	
		Macrotubules → microtubules	Macrotubules → microtubules	
IPC (10 ⁻³ M)	-	-	NT	Partial regeneration (1.5-2 μm)
		(Macrotubules)		
CIPC (2 × 10 ⁻⁴ M)	-	NT	+ -	Partial regeneration (0.5-1 μm)
			Macrotubules and micro-tubules	
Colchicine	-	-	NT	Complete inhibition
		Macrotubules disassemble		

See text for discussion.

Complete shape regeneration (+); no shape regeneration (-); not tested (NT).

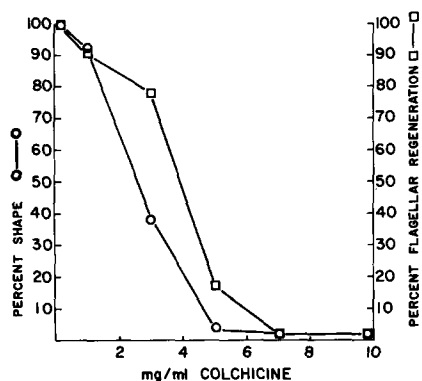


FIGURE 32 Comparison of the effects of increasing colchicine concentration on cell shape and flagellar regeneration. Both graphed as percent of the control for that experiment. Cell shape assessed after 5-h exposure to colchicine or control media; flagellar length measured after 180-min recovery in colchicine or control media.

mediated by some kind of nucleating site. The extent to which nucleating sites, by providing the initial template for microtubule assembly, may regulate expression of a specific cell form is strikingly evident in *Ochromonas*. Our earlier investigations (Bouck and Brown, 1973; Brown

and Bouck, 1973) indicated the presence of two independent nucleating sites, the beak complex and the rhizoplast, which appeared to initiate assembly of cytoplasmic microtubules. These sites were interpreted to function in regulating the position and timing of the appearance, the orientation, and the total pattern of microtubule distribution in the cell, thereby controlling the development of cell form. The complexity of regulation implied in this interpretation suggested that experimental analysis of these microtubule-nucleating site relationships could provide clues to the precise mechanisms involved in shape development in *Ochromonas* as well as in other organisms. The major experimental problems in the earlier studies, the extensive deflagellation at pressures required to depolymerize all microtubules (above 8,500 lb/in²) and the apparent irreversible alteration of microtubule protein exposed to colchicine, were overcome by the use of the antimetabolic herbicides IPC and CIPC in conjunction with relatively low hydrostatic pressures.

IPC and CIPC appear to act synergistically with pressure. Used separately, 10⁻³ M IPC or 2 × 10⁻⁴ M CIPC causes only slight loss of

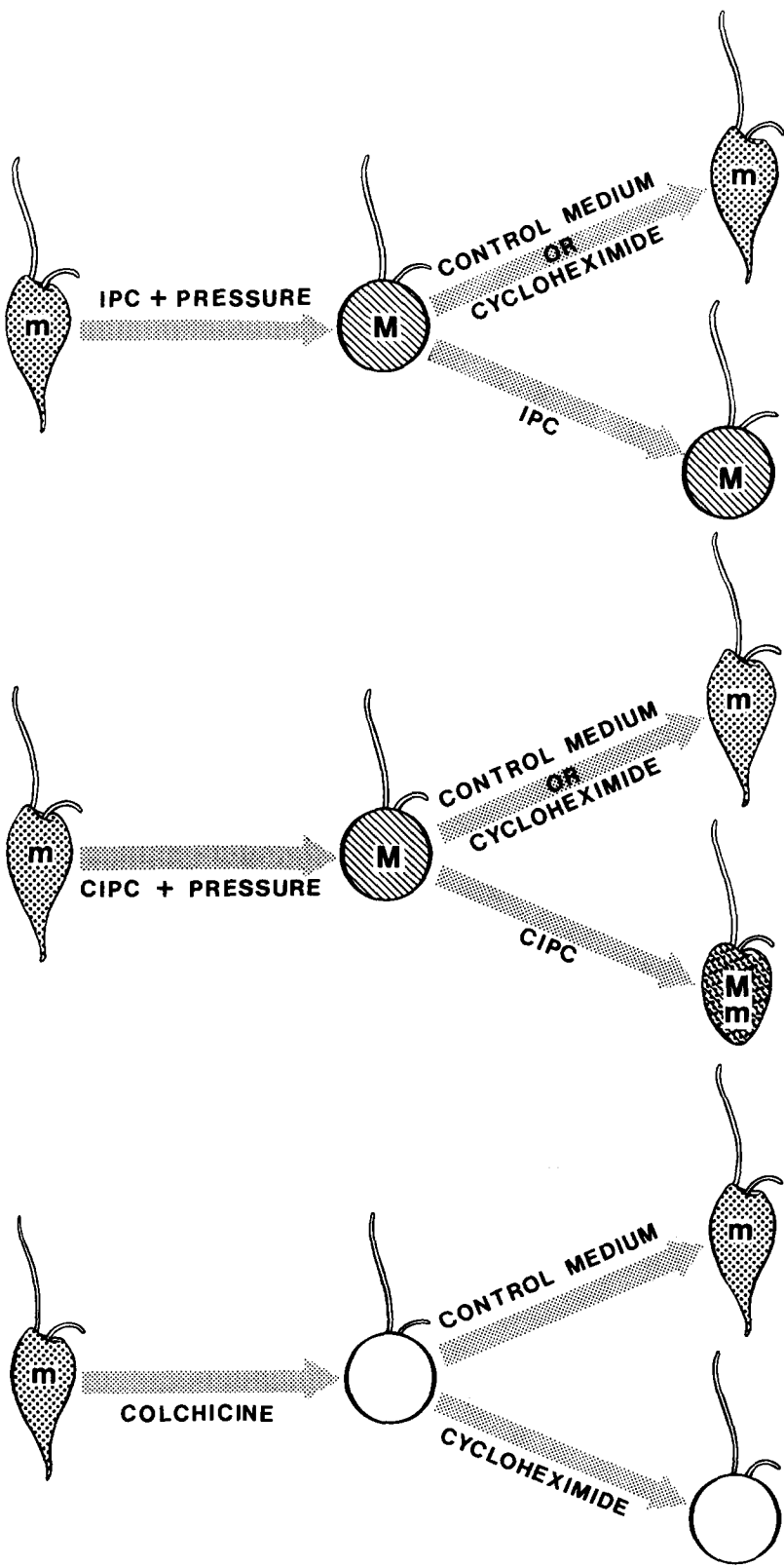


FIGURE 33 Summary diagram comparing the effects of different treatments on cell shape and microtubule integrity. Stippled (*m*), microtubules; hatched (*M*), macro-tubules; open, no microtubules or macro-tubules.

shape, and 8,000 lb/in² hydrostatic pressure produces incomplete microtubule disassembly. However, in cells exposed to IPC and pressure in combination, all cytoplasmic microtubules are either depolymerized or transformed into larger diameter macrotubules which no longer function in shape maintenance. In addition, when cells are resuspended in IPC immediately after a pressure treatment, microtubule reassembly is inhibited but there is no apparent effect on microtubules that were not previously depolymerized. This is most evident in cells exposed to lower pressures (6,000 lb/in², 30 min) in 10⁻⁸ M IPC. The rhizoplast set of microtubules is completely transformed to macrotubules with no apparent effect on the beak set (Fig. 13), and regeneration is blocked as long as IPC is present. CIPC has similar effects on the rhizoplast tubules but does not inhibit regeneration of the beak microtubules.

Observation of correlative shape changes after IPC (CIPC) pressure and during the recovery period confirms our earlier proposal of independent roles for beak and rhizoplast microtubules (Brown and Bouck, 1973). At low pressures (6,000 lb/in²) in IPC, the more labile rhizoplast set of microtubules is selectively converted into macrotubules with a concomitant loss of the cell tail. Higher pressures (7,000–8,500 lb/in²) convert the beak set as well, producing spherical cells. Furthermore, CIPC in appropriate concentrations affects only the rhizoplast microtubules. During shape regeneration, the more stable beak microtubules initially reassemble in the characteristic bidirectional orientation (Figs. 14, 23) as the anterior asymmetry of the cell is established, followed by microtubule reassembly on the lower rhizoplast surface concurrent with formation of the tail. The kinetics, sequence, and pattern of shape regeneration are identical in the presence or absence of new protein synthesis. These results are consistent with the suggestion that the differential sensitivity of the beak and rhizoplast microtubules and the different time of appearance of the two sets of cytoplasmic tubules during shape regeneration are characteristics of the nucleating sites (for a more complete discussion of this point see Brown and Bouck, 1973).

IPC has been shown to alter or inhibit the normal development of microtubule systems in a variety of organisms. Exposure to low concentrations of IPC (5.5×10^{-5} M) disorients spindle

microtubules in dividing *Haemanthus* endosperm cells and results in formation of a multipolar spindle apparatus. Hepler and Jackson (1969) proposed that IPC is indirectly affecting microtubules in this system by binding to the spindle organizing material (i.e., nucleating site). A similar interpretation is suggested for the effect of IPC in inhibiting cell division in *Marsilea* microspores (Hepler, 1972), and in altering the distribution of reassembling spindle and phyco-plast microtubules in *Oedogonium* (Coss and Pickett-Heaps, 1973). IPC has also been shown to affect the development of microtubule systems other than the spindle apparatus, including: (a) reversible inhibition of cilia regeneration in *Stentor coeruleus* (Banerjee and Margulis, 1969); (b) inhibition of the postmitotic nuclear migration in *Micrasterias* (Kiermayer and Hepler, 1970); (c) inhibition of the cytoplasmic microtubule-dependent elongation of developing generative cells in *Haemanthus* (Sanger and Jackson, 1971), apparently through a loss of existing microtubules; and, (d) delay of flagellum morphogenesis in the slime mold *Didymium nigripes* (Kerr, 1972).

Although microtubules, or microtubule associated material, are implicated as the target of IPC action in all of these studies, the wide variation in concentration of IPC used, the different assay methods, and the variable responses in these widely diverse organisms make it difficult to propose a uniform mode of action for IPC. In addition, earlier reports that the 3-chlorophenyl derivative of IPC, CIPC, inhibits protein synthesis in several plant systems (Mann et al., 1965; 1967) and the hypothesis that IPC and CIPC may inhibit messenger RNA transcription and hence, new protein synthesis (Mann et al., 1967; Keitt, 1967) suggested that some of the responses listed above (e.g., inhibition of cilia regeneration and flagellum morphogenesis) may be due to inhibition of protein synthesis, including the synthesis of microtubule protein.

Our results show that in *Ochromonas*, IPC and CIPC alter normal microtubule assembly without affecting amino acid incorporation into TCA-precipitable protein (Fig. 12). IPC and CIPC, unlike colchicine, have little or no effect on intact microtubules but inhibit the development of spindle, cytoplasmic, and flagellar microtubule systems. The effects of IPC on spindle and cytoplasmic microtubules clearly suggest a similar mode of action. In both systems microtubules are replaced by (nonfunctional) macrotubules, which

may be associated with microtubule-nucleating sites.

We cannot state unequivocally that IPC affects only the assembly of spindle microtubules. Since no intact spindle microtubules were observed in cells exposed to IPC alone (10^{-3} M, 30 min), it is possible that spindle microtubules present when IPC was added are also converted into macro-tubules. Coss and Pickett-Heaps (1973) have shown that the reassembly of cold depolymerized spindle (and phycoplast) microtubules in *Oedogonium* is inhibited by 5.6×10^{-4} M IPC. However, no macro-tubules are observed, and when the IPC is washed out a multipolar spindle is formed with three or four distinct microtubule organizing centers (i.e., nucleating sites).

The effects of these herbicides on flagellar regeneration are more difficult to interpret. Flagellar regeneration is at least partially inhibited over the same range of IPC concentrations which affect reassembly of cytoplasmic microtubules (Fig. 31). However, at no concentration was complete inhibition of regeneration observed. These results superficially resemble the effects of cycloheximide on flagellar regeneration in *Ochromonas* (Table II) and other flagellates (Rosenbaum and Child, 1967; Rosenbaum et al., 1969) where partial regeneration in the absence of new protein synthesis is interpreted to show the presence of a pool of flagellar precursors, including microtubule protein. More than 90% of the cells regenerate 1.5–2.0 μm of the flagellum in 20 $\mu\text{g}/\text{ml}$ cycloheximide (unpublished results), suggesting that most of the population has a similar size precursor pool. The observation that a comparable average flagellar length (1.5–2.0 μm) is regenerated in 10^{-3} M IPC might then indicate that the precursor pool is insensitive or inaccessible to IPC. However, 40% of the cells do not regenerate any flagellum in IPC (Fig. 28) and the percentage of nonregenerating cells is even higher in cells recovering in CIPC. The difference in response within a population may result from the use of nonsynchronous cultures in these experiments, and studies in progress using synchronous populations of *Euglena* should resolve this problem.

The transformation of microtubules into larger diameter macro-tubules was an unexpected result and has not been reported in other studies using IPC. However, similar transformation has been produced in other organisms by a variety of chemical and physical agents, including: low

temperature (Tilney and Porter, 1967), digitonin (Hanzely and Olah, 1970), the anesthetic halothane (Allison et al., 1972; Hinkley and Samson, 1972), vinblastine sulfate (Tyson and Bulger, 1972), and the enzyme hyaluronidase (Burton and Fernandez, 1973). (See Tyson and Bulger [1973] for a more complete review of the occurrence of macro-tubules.) Macro-tubules are believed to represent a different conformational state of microtubules since microtubules disappear as macro-tubules are formed and vice versa (Tilney and Porter, 1967; Hinkley and Samson, 1972), and macro-tubules are colchicine sensitive (Hinkley and Samson, 1972; also confirmed in this study). Tilney and Porter (1967) discussed two alternative explanations for this transformation at low temperatures: (a) a direct shortening of microtubules by sliding of adjacent protofilaments, and (b) breakdown of microtubules into subunits and reassembly as macro-tubules. Both of these may occur in *Ochromonas*. Exposure to high hydrostatic pressure (8,500 lb/in²) alone depolymerizes most microtubules, presumably to subunits, and if IPC is then added, macro-tubules are formed. In contrast, when cells are exposed to IPC in conjunction with pressure, only macro-tubules are seen in cells fixed immediately after pressure release. It is possible that macro-tubules are reassembled from pressure-dissociated subunits while still under pressure (8,500 lb/in²) but a direct transformation seems equally likely. In cells recovering from pressure IPC, macro-tubules and microtubules were occasionally observed coexistent and inserting side by side on the same rhizoplast, suggesting an *in situ* transformation. However, as in all other studies of microtubule-macro-tubule interconversions, no structural intermediates were observed in *Ochromonas*.

On the basis of the experimental evidence (Balasubramanian and Wetlaufer, 1966) that halothane can induce conformational changes in globular proteins, Hinkley and Samson (1972) suggested that macro-tubules in anesthetic-treated axons are reassembled from halothane-modified microtubular subunit strands. Also, Tyson and Bulger (1973) have hypothesized that the formation of macro-tubules in cells exposed to vinblastine may result from a vinblastine-induced conformation change or the stabilization of a particular conformation of microtubule protein. It is tempting to speculate that IPC (and CIPC) may have a similar direct molecular action

on microtubule protein in *Ochromonas*. Moreland and Hill (1959) proposed that CIPC inhibition of photolysis in isolated chloroplasts might be due to H bonding of the carbamate to peptide linkages, which could alter secondary and tertiary protein structure. Extending this argument, we could hypothesize that IPC binds to free microtubule subunits, producing a conformation change that only permits reassembly into macro-tubules. The effect of hydrostatic pressure would be to make the IPC "sites" more accessible in intact microtubules. Alternatively, the IPC-induced change in microtubule structure may reflect an alteration in microtubule organizing material as suggested by Hepler and Jackson (1969). Some of the macro-tubules formed in *Ochromonas* are directly associated with micro-tubule nucleating sites (Figs. 13, 20, 21), and it may be that the organizing material for the nuclear and the randomly oriented cytoplasmic macro-tubules is simply not easily recognized. The differential sensitivity of the spindle, rhizoplast, and beak, and the flagellar microtubules would then indicate subtle differences in the nucleating sites for the different microtubule systems.

Clearly, from the data now available, we cannot distinguish between these alternatives or other possible modes of IPC action. This problem is currently being investigated by: (a) the isolation and characterization of IPC-stabilized macro-tubules from *Ochromonas*, (b) binding studies using labeled CIPC and microtubule protein from CIPC sensitive cells, and (c) testing the effects of IPC and CIPC on in vitro microtubule polymerization.

Regardless of the specific site of IPC and CIPC action, the results presented here have shown that these carbamate herbicides can be used to rapidly and reversibly inhibit microtubule assembly without affecting other cell structures or total protein synthesis in short term experiments. The apparent specificity of this effect has permitted analysis of the separate roles of the beak and rhizoplast sets of cytoplasmic microtubules and their nucleating site relationships.

The authors thank Dr. L. Simone for his skillful illustration of Fig. 33.

Supported by grant A6353, National Research Council of Canada, to D. L. Brown; and grants GM 11100 and GM 19637 from the United States Public Health Service, to G. B. Bouck.

Received for publication 4 September 1973, and in revised form 14 January 1974.

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