Flagellar Microtubule Dynamics in *Chlamydomonas*: Cytochalasin D Induces Periods of Microtubule Shortening and Elongation; and Colchicine Induces Disassembly of the Distal, but Not Proximal, Half of the Flagellum

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Abstract. To study the mechanisms responsible for the regulation of flagellar length, we examined the effects of colchicine and Cytochalasin D (CD) on the growth and maintenance of *Chlamydomonas* flagella on motile wild type cells as well as on *pf* 18 cells, whose flagella lack the central microtubules and are immobile. CD had no effect on the regeneration of flagella after deflagellation but it induced fully assembled flagella to shorten at an average rate of 0.03 μm-min^{1}. Cells remained fully motile in CD and even stubby flagella continued to move, indicating that flagellar shortening did not selectively disrupt machinery necessary for motility. To observe the effects of the drug on individual cells, *pf* 18 cells were treated with CD and flagella on cells were monitored by direct observation over a 5-hour period. Flagella on control *pf* 18 cells maintained their initial lengths throughout the experiment but flagella on CD-treated cells exhibited periods of elongation, shortening, and regrowth suggestive of the dynamic behavior of cytoplasmic microtubules observed in vitro and in vitro. Cells behaved individually, with no two cells exhibiting the same flagellar behavior at any given time although both flagella on any single cell behaved identically. The rate of drug-induced flagellar shortening and elongation in *pf* 18 cells varied from 0.08 to 0.17 μm-min^{-1}, with each event occurring over 10–60-min periods. Addition of colchicine to wild type and *pf* 18 cells induced flagella to shorten at an average rate of 0.06 μm-min^{-1} until the flagella reached an average of 73% of their initial length, after which they exhibited no further shortening or elongation. Cells treated with colchicine and CD exhibited nearly complete flagellar resorption, with little variation in flagellar length among cells. The effects of these drugs were reversible and flagella grew to normal stable lengths after drug removal. Taken together, these results show that the distal half to one-third of the *Chlamydomonas* flagellum is relatively unstable in the presence of colchicine but that the proximal half to two-thirds of the flagellum is stable to this drug. In contrast to colchicine, CD can induce nearly complete flagellar microtubule disassembly as well as flagellar assembly. Flagellar microtubules must, therefore, be inherently unstable, and flagellar length is stabilized by factors that are sensitive, either directly or indirectly, to the effects of CD.

WITHIN the past several years it has become evident that microtubules are highly dynamic structures that can rapidly assemble and disassemble in vitro and in vitro by the addition or loss of tubulin from the microtubule plus end (see Avila, 1990; Cassimeris and Salmon; 1991; Hotani and Miyamoto, 1990). Mechanisms that regulate tubulin addition or loss from a microtubule are poorly understood, although the presence of GTP-tubulin at the plus end has been proposed to stabilize, or cap, individual microtubules (reviewed in Avila, 1990). Furthermore, the presence of various microtubule-associated proteins, capping structures, or posttranslational modifications of tubulin itself may alter the assembly and disassembly properties of the microtubules. Besides the dynamics exhibited by individual microtubules, cells can completely disassemble and reorganize whole arrays of microtubules, as occurs during the transition from interphase to mitosis, and are able to specify the assembly and disassembly of individual groups of microtubules, as occurs in the complex *Actinospherium* axopodia. Although it is certain that cells can switch individual microtubules from assembly to disassembly states, we have little understanding of the nature of that switch.

Isolated ciliary and flagellar microtubules are very stable structures, but they can be relatively dynamic in vivo. In some cells, they are regularly disassembled during the cell

cycle (see Bloodgood, 1974). In *Chlamydomonas* (Rosenbaum et al., 1969) and *Tetrahymena* (Rannestad, 1974), they can be induced to disassemble upon amputation of one or more flagella or cilia, by alterations in the tonicity of the culture media, or by addition of low concentrations of amiprophosmethyl, caffeine, isobutyl methylxanthine, and halothane (see Lefebvre and Rosenbaum, 1986). Flagellar microtubule assembly is highly regulated at the level of individual microtubules (see Dentler, 1981, 1987, 1990) and does not appear to be dependent on a pool of assembly-competent protein within the cell body (see Lefebvre and Rosenbaum, 1986; Jarvik and Chojnacki, 1985). Based on the availability of a variety of *Chlamydomonas* mutants with abnormally long or short flagella, it is apparent that flagellar length is under genetic control (Jarvik and Chojnacki, 1985).

We decided to ask if flagella are dynamic structures and, if so, could we shift the dynamics to make the flagella less stable, or more dynamic. Since the plus ends of each of the A tubules and both central tubules are capped by microtubule capping structures in virtually all cilia and flagella (Dentler and Rosenbaum, 1977; Dentler, 1980, 1990), we have focused on the role of these caps on microtubule assembly. Our identification of an antigen in common with *Tetrahymena* ciliary tips and mammalian kinetochores, which binds to the assembly sites of microtubules in the mitotic apparatus (Miller et al., 1990; Miller and Dentler, 1992), and our observation that microinjection of the antibody to this antigen blocks chromosome movement in mammalian cells (Miller, J. M., D. Wise, and W. J Dentler, manuscript submitted for publication), suggests that studies of the capping structures may shed light on the assembly of cytoplasmic as well as flagellar microtubules.

Recently, Boisvieux-Ulrich et al. (1990) reported that cytochalasin D (CD)[1] treatment of quail oviduct cells undergoing ciliogenesis resulted in the inhibition of ciliary microtubule assembly and the failure of the cilia to form capping structures. Basal bodies attached to cytoplasmic vacuolar membranes nucleated ciliary microtubule assembly, but microtubules were unable to grow longer than 1–2 μm and capping structures were not formed. This is consistent with our previous study showing that capping structures are formed during the first 1–2 μm of ciliary growth in ciliated amphibian cells and that the remainder of ciliary assembly occurs in the presence of fully formed microtubule caps (Portman et al., 1987). This was also the first study to correlate the absence of capping structure assembly with the inability of a cilium to assemble microtubules.

To determine if drugs that disrupt actin filament assembly may have a role in the formation or maintenance of capping structures, we examined the affect of CD on flagellar regeneration in *Chlamydomonas*. *Chlamydomonas* was chosen because their flagella are easily observed and because they can be deflagellated and will regenerate new flagella within 2 h. CD was chosen because it is the Cytochalasin most specifically targeted to actin filament formation (Cooper, 1987). The results reported here show that CD does not affect flagellar assembly during regeneration but that it does significantly alter the ability of a *Chlamydomonas* cell to regulate or maintain the length of its flagella. Direct observation of CD-treated cells shows that the flagella oscillate in length, as opposed to control cells, which maintain constant length flagella, and colchicine-treated cells, which show nearly uniform shortening of flagella. CD has no apparent affect on the structure of the flagellum or basal bodies nor does it affect flagellar motility even as the flagella shorten. Additionally, these results show that colchicine induces flagellar shortening over a 60–120-min period, but that shortening then ceases and the flagellum achieves a new stable length. These results suggest that the distal portion of a flagellum can undergo a turnover of flagellar tubulin and, presumably, microtubule-associated proteins, but that the proximal 50–70% of the flagellum either does not undergo turnover or that the rate of turnover is considerably slower than that of the distal portion of the flagellum.

1. *Abbreviations used in this paper*: CD, Cytochalasin D; WGA, wheat germ agglutinin.

Materials and Methods

Chlamydomonas cells were cultured sterilely and synchronously in "R medium" (Harris, 1989) with constant aeration in 250-ml flasks on a 12-h light/dark cycle. For each experiment, cells were chosen ~2–3 h after the lights were turned on and cultures were checked to insure that cells had equal length flagella. Cultures with dividing cells were discarded. *Chlamydomonas* gametes were made by washing wild-type cells in "M-N" medium (Harris, 1989) and incubating cells in fresh "M-N" medium in constant light overnight. Both wild-type (cc-124) and paralyzed flagellar mutants (*pf* 18) were obtained from the *Chlamydomonas* Genetics Center (see Harris, 1989). All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Experimental cells were mixed with drugs and incubated in six-well plastic tissue culture plates (Falcon # 3008). For drug additions, stocks of CD (10 mg/ml in DMSO), colchicine (200 mg/ml in culture medium), and cycloheximide (1 mg/ml in culture medium) were added in 1–5-μl aliquots to 1-ml aliquots of cells in the wells. The plates were then covered with their transparent tops, placed on a sheet of white paper to reflect light, and were agitated on a Lab Line Jr. Orbital Shaker (Lab Line Instruments, Melrose Park, IL) at 100 RPM under continuous illumination from a desk lamp fitted with two standard fluorescent bulbs. Living cells were observed by suspending a coverslip on pillars of Vaseline placed on a glass slide. For continuous observation of *pf* 18 cells, the coverslips were sealed with VALAP (1:1:1 vaseline/lanolin/paraffin). Cells were observed with a Zeiss Photomicroscope equipped with a 40× phase lens and 2.0× optovar and were illuminated with a 12 W halogen lamp filtered with a green interference filter, a UV-reflecting heat filter, and two glass heat filters. Cells were either recorded with a video camera and tape recorder or cells were observed directly with the microscope. Photomicrographs were taken using Kodak TMAX 400 film from a TV monitor using a Nikon FE camera or using the internal camera on a Zeiss Photomicroscope II.

For most experiments, cells were fixed in Lugol's iodine (6% KI, 4% iodine in water) and assayed within two days after fixation. Lugol's iodine was chosen because the flagella were straight and were easy to see and measure in phase microscopy. To prevent formation of a precipitate with Lugol's, the stock solution was diluted 1:3 with distilled water. For fixation, 20-μm aliquots of cells were removed from the culture using 200-μl pipette tips, whose ends were cut off to minimize the possibility of flagellar shearing, and were mixed with an equal volume of diluted Lugol's solution. In many experiments, separate aliquots of cells were removed and fixed with 1-2% (final concentration) glutaraldehyde to insure that flagellar loss or length changes were not induced by the fixation procedure. Because the glutaraldehyde-fixed flagella were curved, they were not, generally, measured other than to insure that the flagella fixed by each procedure produced identical results.

Flagellar lengths were either measured from video tapes of living cells by placing a ruler on the TV monitor or by using an ocular micrometer and a phase microscope. For fixed cells, an average of 50 different cells (50 flagella) were measured for each data point. To avoid scoring cells whose flagella were broken during fixation or preparation of slides for microscopy, cells were only measured if they contained flagella or short flagellar stubs. This procedure may skew the data towards longer average length flagella but avoided skewing the data in the opposite direction due to shearing of

flagella during preparation for microscopy. Flagellar lengths were then analyzed and graphed using Canvas and Cricket Graph programs on a Macintosh IIcx or IIci computer. Statistical analysis was made using Statpack and the Macintosh II cx.

For EM, cells were fixed by dilution into 2% glutaraldehyde in culture medium. Subsequent fixation and embedding followed the method used by Hoops and Witman (1983). To preserve actin filaments, the phalloidin method described by Katoh et al. (1991) was also used.

Results

Initial experiments were designed to determine if CD blocked the assembly of *Chlamydomonas* flagella or microtubule capping structures, as it did in quail oviduct (Boisvieux-Ulrich et al., 1990). Flagella were amputated from wild type cells by pH shock and were allowed to regenerate in the presence of 100 or 200 μM CD, concentrations of CD lower than that used to inhibit actin filament assembly in *Chlamydomonas* gametes (Detmers, et al., 1983). As shown in Fig. 1, CD did not significantly affect flagellar regeneration. The average rate of growth in drug-treated cells was slightly slower than that in control cells (0.181 μm-min^{-1} in controls, 0.175 μm-min^{-1} in 100 μM CD, 0.14 μm-min^{-1} in 200 μM CD), but the distributions of flagellar length in drug-treated cells (Fig. 2) suggested that the slower average growth rate was due to the presence of a few cells with very short flagella. In these experiments, CD was added immediately after flagellar amputation but identical results were obtained when the drug was added 10 min before deflagellation as well as during regeneration. In both cases, the cells were observed to swim normally. In preliminary observations of the flagellar microtubules using negative staining and EM (see Dentler, 1980), we found no obvious differences in the structures of the microtubule caps or any other flagellar structures between control and CD-treated cells. In contrast to the initial growth of flagella, flagella on drug-treated cells gradually began to shorten after reaching 85–90% of the flagellar lengths on control cells (Fig. 1). Although the average length of flagella on drug-treated cells gradually decreases (Fig. 1), a more accurate picture of the cells can be determined by examining the range of flagellar lengths at 74 and 440 minutes after deflagellation, as shown in Fig. 2. In contrast to the control cells, the length of flagella on drug-treated cells is quite variable and cells with very short and very long flagella

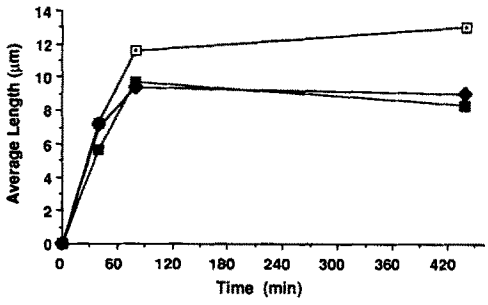

Figure 1. Flagellar regeneration after amputation. Flagella on wild type cells were amputated with pH shock and samples were fixed during regeneration in the presence of 100 and 200 μM CD or without drugs. The average flagellar length at each time point was calculated from 50 flagella. Cells were incubated with 100 μM CD (*diamonds*) or 200 μM CD (*closed squares*) and were compared with cell incubated without drugs (*open squares*).

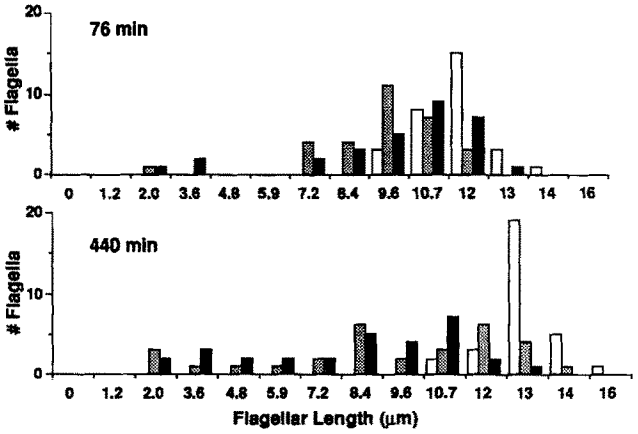

Figure 2. Distribution of flagellar lengths of wild type cells sampled at 76 and 440 min during regeneration (see Fig. 1). (*Open bars*) Control; (*gray bars*) 100 μM CD; (*filled bars*) 200 μM CD.

are present, although the two flagella on every individual cell were equal in length. These results show that CD does not affect the ability of a cell to assemble functional flagella, but that it does affect the ability of a cell to maintain or regulate flagellar length.

Since the major effect of CD appeared to be on the regulation of length, and not growth per se, wild type cells with fully grown flagella were treated with CD and were fixed and measured. As shown in Fig. 3, CD induced flagella to shorten to nearly 60% of the control flagellar lengths. Cells washed out of the drug recovered normal length flagella within 2 h. As in all experiments, unfixed cells were routinely checked throughout the experiment and there were no differences in morphology or swimming behavior between the control and drug-treated cells. The effects of increasing concentrations of CD on flagellar shortening are tabulated in Fig. 4. Increasing concentrations of CD lead to more rapid flagellar shortening, but cells recover normal length flagellar lengths after drug removal. Cells were also treated with 1 and 2% DMSO (used to dissolve CD), but flagella on these cells were identical to those on control cells, in which flagellar lengths remained stable throughout the 5-h experimental period (data not shown). The same concentrations of CD also induced flagellar shortening in *Chlamydomonas* gametes made from wild-type cells (data not shown).

These results indicated that CD disrupts the normal regulation of flagellar length in wild-type *Chlamydomonas* cells. Based on the variations of flagellar lengths observed in the presence of the drug, (see Fig. 2), we suspected that flagella on individual cells in CD may grow or shorten. To examine the shortening phenomenon alone, we tested the effects of

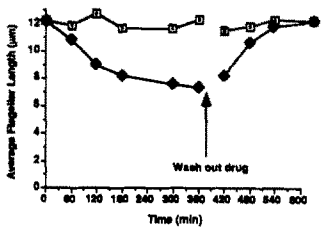

Figure 3. Effect of cytochalasin D on flagellar shortening. Comparison of the average flagellar lengths on wild type cells with fully grown flagella that were incubated in the absence (*open squares*) or presence (*diamonds*) of 200 μM CD.

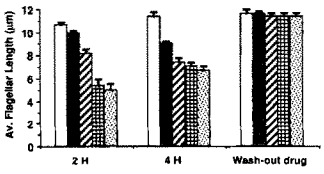

Figure 4. Flagellar resorption and cytochalasin D concentration. Average lengths of flagella on wild type cells incubated in increasing concentrations of CD as compared with cells incubated without drug. (*Open bars*) Control cells; (*gray bars*) 50 μM CD; (*diagonally hatched bars*) 100 μM CD; (*cross-hatched bars*) 200 μM CD; (*spotted bars*) 400 μM CD. After four hours, cells were washed into fresh drug-free culture medium and cells were fixed two hours after removal of CD. Error bars represent the standard error of the mean.

colchicine on CD-treated cells, with the rationale that colchicine would bind the tubulin dimers that dissociate from disassembling flagella and would shift the equilibrium toward the disassembly state. As shown in Fig. 5, 2 mg/ml colchicine alone induced flagellar shortening. Over a four hour period in colchicine, flagella shortened to an average of 80% of their initial length (and of the length of flagella on control cells), which is close to the amount of shortening observed in CD-treated cells. In contrast to CD-treated cells, in which there was a broad distribution of flagellar lengths, colchicine-treated cells exhibited little variation in flagellar length (data not shown), which suggested that the two drugs affected the flagellar length regulation in different ways. As with CD, colchicine-treated cells regained normal flagellar lengths when cells were washed out of the drug and incubated in fresh medium.

When colchicine was added with increasing concentrations of CD, the flagella shortened and, within 4 h, most flagella appeared as ~1–2-μm stubs that barely extended past the cell wall (Figs. 5, 6, and 10). These results confirm that colchicine or CD alone can cause flagellar shortening but that the combination of the two drugs leads to nearly complete flagellar disassembly. This occurred in two steps, with an initial rapid shortening over 60–120 min followed by a gradual shortening over the duration of the experiment (Fig. 5). The distribution of flagellar lengths in CD-treated cells was considerably broader than that found in the controls or in the colchicine-treated cells, in which the distribution of flagellar lengths was tightly grouped about the mean length (Fig. 6). Cells treated with colchicine and CD exhibited a broad range of flagellar lengths during the first 2 h of drug treatment (Fig. 6, 120 min) but flagella on most cells were <1 μm long after 360 min. A few wild type cells did retain long flagella even in the presence of both drugs (Fig. 6, 360 min). Virtually all cells recovered normal length flagella after removal of the drugs and the distributions of flagellar lengths on control cells and cells washed out of the drugs were virtually identical. Flagellar motility was not altered by the drugs, and even the short stubs of flagella in CD and colchicine-treated cells continued to move.

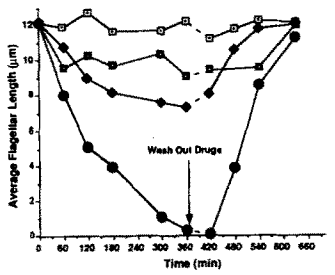

Figure 5. Flagellar shortening and recovery in cytochalasin D and colchicine. Average flagellar lengths on wild type cells with fully grown flagella that were treated with no drug (*open squares*), 2 mg/ml colchicine (*filled squares*), 200 μM CD (*white dotted squares*), and 2 mg/ml colchicine + 200 μM CD (*filled circles*). Cells that were washed into fresh drug-free medium after 360 min of drug treatment recovered normal flagellar lengths.

In addition to CD and colchicine, cells were treated with 10 μg/ml cycloheximide to determine if the affect of CD may be due to the inhibition of protein synthesis (Ornelles et al., 1986; Zambetti et al., 1991). Cycloheximide had no significant affect on flagellar length and cells treated with 10 μg/ml cycloheximide, cycloheximide plus CD, cycloheximide plus colchicine, showed no effects other than those observed with colchicine or CD alone. Flagellar length regulation is not, therefore, dependent on protein synthesis during the 4–5-h experimental period.

Taken together, these results suggested that CD abolished or altered mechanism(s) that maintained flagellar stability in the wild-type cells. Based on the broad distribution of flagellar lengths in drug-treated, but not control cells, we wondered if flagella on individual cells underwent events of shortening and, possibly, elongation independently of other cells, but, since the wild type cells used in these experiments were fully motile throughout the period of drug treatment, we could not observe the effects of the drug(s) on individual cells. To examine the behavior of individual cells, we examined *pf* 18, a *Chlamydomonas* mutant with paralyzed flagella that we could observe in situ for several hours. Moreover, since *pf* 18 lacks the two central pair microtubules, the effects of CD and other drugs could be compared between wild type cells, with the 9 + 2 complement of microtubules, and *pf* 18, which only contains the nine doublet microtubules.

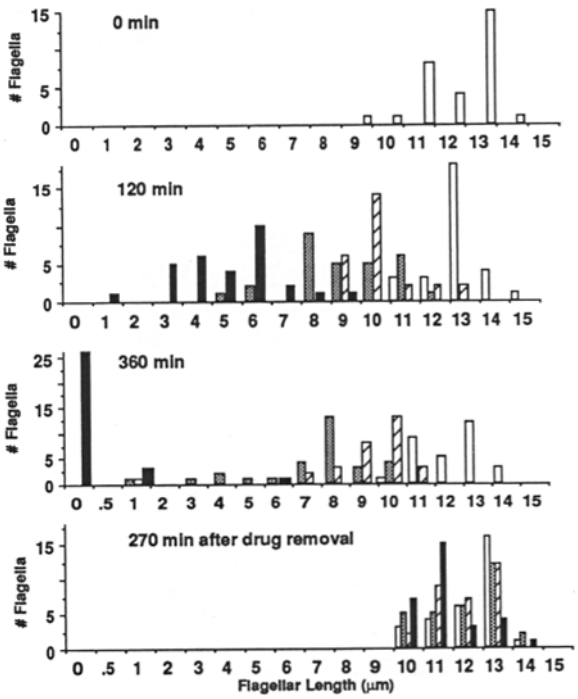

Figure 6. Distribution of flagellar lengths on cells treated with no drugs (*open bars*), 100 μM CD (*gray bars*), 2 mg/ml colchicine (*hatched bars*), and 100 μM CD + 2 mg/ml colchicine (*filled bars*). Cells were fixed and measured at 120 and 360 min after addition of the drugs as well as 270 min after drug removal.

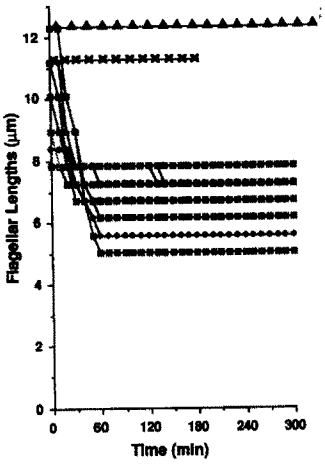

Figure 7. Lengths of flagella on 11 individual *pf 18* cells incubated in 2 mg/ml colchicine monitored for 300 min. Initial flagellar lengths varied from <7 μm to >12 μm on individual cells. Flagella on each of the cells shortened within the first 60 min after colchicine addition and remained at the shortened length throughout the remainder of the experiment. Flagellar lengths on two representative control cells (*triangles* and *crosses*) are also plotted but these flagella, typical of all control cells, did not change in length throughout the 3–5-h observation period.

Initial observations were made of control *pf* 18 cells to determine if flagellar length changes occurred during the period of observation. Seventeen cells were examined over a 5-h period and data for 2 representative cells are presented in Fig. 7. The initial lengths of flagella on individual *pf* 18 cells ranged from 10.5 to 15.5 μm and showed no significant length changes during the 3–5-h observation period.

Upon addition of colchicine, individual *pf* 18 cells shortened their flagella to 86–71% of their initial length within the first hour of drug treatment and then maintained the shortened flagella for the next 4 h (Fig. 7). The maximum amount of colchicine-induced shortening ever observed occurred in one experiment in which six cells shortened their flagella to 50% of their initial length during the first hour and then maintained the shortened length throughout the following four hours of observation. Virtually every cell in a given experiment behaved identically, which is consistent with the narrow distribution of flagellar lengths on colchicine-treated wild type cells (Fig. 6). The rate of initial shortening ranged from 0.02 to 0.08 μm-min^{-1}, with an average rate of 0.06 μm-min^{-1} (Table I). These data revealed that the distal half of the flagellum is relatively unstable and can be induced to disassemble by the addition of colchicine whereas the proximal half of the flagellum is relatively stable to colchicine.

Since the flagella on control cells maintained constant lengths and those on colchicine-treated cells maintained a constant length after the initial shortening period, the effects of CD on individual *pf* 18 cells were examined. Examples of two cells incubated in the presence of 200 μM CD and observed over a 5-h period are shown in Fig. 8. Both of the cells photographed were on the same slide, and, until they drifted apart, were in the same field of view. Each cell maintained two equal length flagella and their normal cell shape throughout the observation period. However, the flagella on each cell varied in length over the observation period. The cell shown in the top panel shortened its flagella during the first hour, then flagella elongated over the next 3 h and, finally, during the last hour the flagella gradually shortened. The cell shown in the bottom panel maintained normal flagellar length for the first hour but the flagella shortened during the next 3 h before growing to nearly normal length during the last hour. Since these cells were in the same field of view, these data emphasize the autonomous behavior of individual cells in the presence of DC.

To quantify the effects of CD on *pf* 18 cells, the flagellar lengths for 16 randomly chosen cells observed over a 5-h period were measured and graphed (Fig. 9). These data were collected from four separate experiments, since we were only able to follow four to five cells in any single experiment. Examples of two typical cells incubated in 200 μM CD for 5 h are shown. Virtually every cell initially shortened its flagella in response to CD and, with only a few exceptions in *pf* 18 (and wild type cells), both flagella on a single cell were the same length at any given time. After the initial shortening period, three major patterns of flagellar behavior were observed, and these different patterns are shown in Fig. 9. Flagella on some cells (Fig. 9 *A*) shortened 2–3 μm during the first 30 min and then oscillated ± a few microns over the 5-h observation period. Other cells (Fig. 9 *B*) shortened their flagella for the first hour and then started a period of flagellar regrowth, after which flagella oscillated in length for the remainder of the observation period. Still other cells (Fig. 9 *C*) shortened their flagella to <1 μm, and remained at that length for 1–2 h before growing again. Some flagella continued to grow throughout the observation period, others exhibited periods of growth and stability and others oscillated in length, with periods of growth and disassembly. Most flagella did not grow longer than their lengths at the time of drug addition, although flagella on a few cells have been observed to grow 1–2 μm longer than their initial lengths. The rates of flagellar assembly and disassembly by *pf* 18 cells were measured during each period of growth and shortening (Table II). The average rate of disassembly was nearly 0.1 μm-min^{-1} while that of flagellar assembly was averaged nearly 0.14 μm-min^{-1}. The average assembly rate is nearly identical to that observed in wild-type cells whose flagella were amputated and regenerated in the presence of CD (see above). These data show that CD destabilizes the normal regulation of flagellar length in *pf* 18 cells and that individual flagella behave autonomously.

Table I. Average Rate of Colchicine-induced Flagellar Shortening in Chlamydomonas Wild Type and PF-18 Cells

Experiment	Initial length	Final length	Maximum shortening rate	Final length	Shortening
	μm	μm	μm/min	%	%
PF 18					
1 (n = 19)	9.5 ± 1.5	7.3 ± 1.0	0.036 ± 0.017	77.33 ± 6.6	22.5 ± 3.1
2 (n = 6)	10.6 ± 1.4	5.5 ± 0.59	0.083 ± 0.02	49 ± 2	50 ± 2
Wild type					
1 (n = 30)	12.2 ± 0.2	9.7 ± 0.2	0.074	80	20

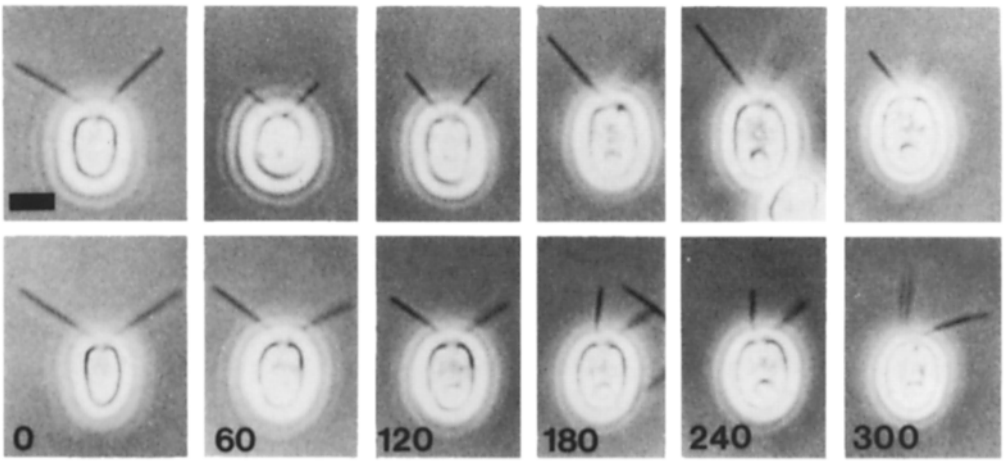

Figure 8. Two *pf 18* cells photographed at the indicated times in 200 μM CD. Both of these cells were on the same slide throughout the observation period. The period of flagellar assembly and disassembly on the two cells occurs independently of one another but both flagella on a single cell behave nearly identically. Bar, 5 μm.

Cells were fixed and processed for EM to examine any structural changes that may be induced by CD. No differences were seen when cells were fixed by conventional methods (Hoops and Witman, 1983) or by using saponin-phalloidin to preserve actin filaments (Katoh et al., 1991) nor were differences observed when control and CD-treated cells were compared. When colchicine was added with CD, cells accumulated increased numbers of small vesicles (Fig. 10 *E*) compared to wild type or CD-treated cells, but otherwise appeared to be identical to control cells. Filamentous structures between the tips of shortened flagella and the membrane were frequently observed in cells treated with CD plus colchicine (Fig. 10, *B* and *C*), although the nature of this material is not known. The lumen of each of the basal bodies was filled with filaments that ran parallel to the long axis of the microtubules in control and in drug-treated cells (Fig. 10). There were no apparent differences between the quantity or arrangement of filamentous structures in control cells and in those treated with CD and/or colchicine.

Figure 9 Flagellar lengths on *PF* 18 cells in Cytochalasin D

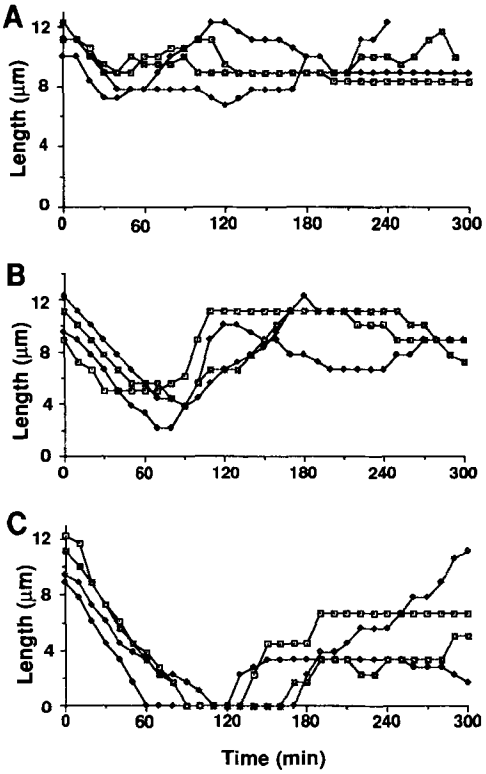

Figure 9. Lengths of flagella on individual *pf 18* cells in the presence of 200 μM CD in four separate experiments. The data are grouped to show the three general types of flagellar behavior observed (see text).

Discussion

Numerous studies of ciliary and flagellar growth have focused on the assembly process, with the goal of identifying proteins or other factors that may be present in low quantities that could limit the extent of microtubule growth in these organelles (see Lefebvre and Rosenbaum, 1986). An alternate approach to studying mechanisms responsible for the regulation of flagellar microtubule assembly is to examine the effects of treatments that alter the ability of the flagella to maintain their full length. *Chlamydomonas* flagella shorten during the cell cycle or when one of the two flagella is lost from the cell (Rosenbaum et al., 1969; Coyne and Rosenbaum, 1970), and shorten in response to changes in ionic strength (Solter and Gibor, 1978), pyrophosphate (Handa A. K., J. Cherniack, and P. Filner. 1977. *J. Cell Biol.* 75: 285a; Dentler and Rosenbaum, 1977), halothane (Tesler, 1977), and other treatments (for review see Lefebvre and

Table II. Rates and Duration of Assembly and Disassembly of Cytochalasin D-treated Flagella

Average rate of flagellar disassembly	0.097 ± 0.007 μm/min
Average rate of flagellar assembly	0.136 ± 0.015 μm/min
Duration of flagellar disassembly periods	46 ± 6 min
Duration of flagellar assembly periods	31 ± 4 min
Duration of periods of no length change	37 ± 4 min

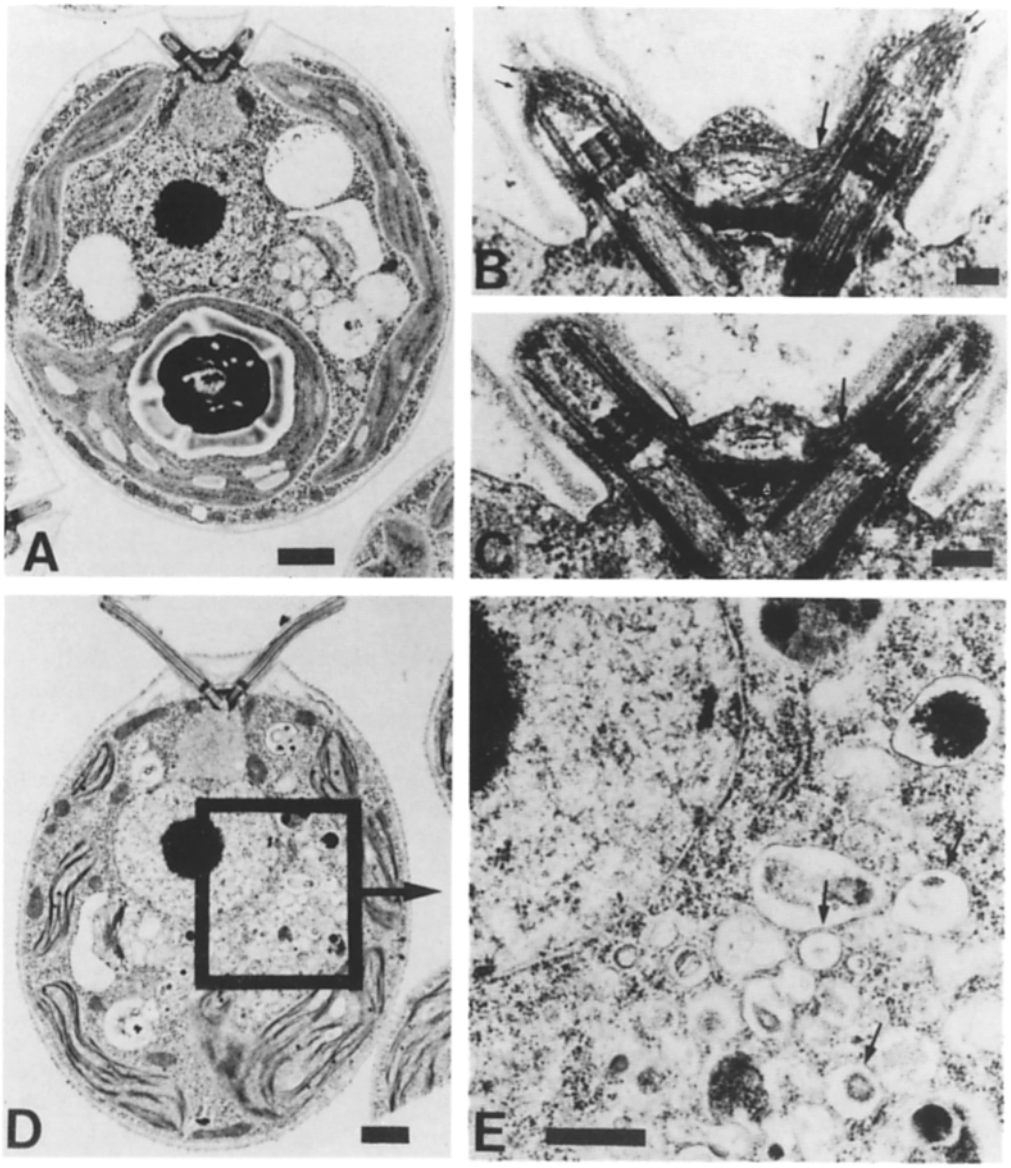

Figure 10. Electron micrographs of cells treated with CD and colchicine. (*A*) Cell treated for 4 h with 200 μM CD but no colchicine. Flagella shortened to the transition zone but the overall cell structure is indistinguishable from controls. (*B* and *C*) Flagella on cells treated with 200 μM CD and 2 mg/ml colchicine for 4 h. Flagella have been nearly completely disassembled and numerous filamentous structures (*arrows*) appear near the ciliary tips (*B*) and along the sides of the basal bodies (*B* and *C*). These filamentous structures are not generally seen in control cells. Filamentous structures running parallel to the long axis of the basal body fill the basal body lumen in control and in drug-treated cells. (*D* and *E*) Cell treated with CD and colchicine showing the accumulation of membrane vesicles. The portion of the cell outlined by the box in *D* is magnified in *E* to illustrate these vesicles. Arrows point to vesicular membranes. Similar vesicles appear in control cells and those treated with CD alone (see *A*) but cells treated with CD and colchicine show increased numbers of the vesicles. Bars: (*A*) 1 μm; (*B* and *C*) 200 nm; (*D*) 1 μm; (*E*) 0.5 μm.

Rosenbaum, 1986). These results suggest that the flagellum is inherently unstable, at least in *Chlamydomonas* and that factors that regulate length in the cell may act by stabilizing the microtubules against disassembly.

The results reported here show that colchicine, a drug that binds tubulin dimers and prevents their assembly into microtubules, and Cytochalasin D, a drug that binds to actin and disrupts actin filaments, induces the disassembly of *Chlamydomonas* flagella. Although each of these drugs appears to cause different effects on the disassembly process, it is likely that they act on some central regulatory system, since they induce both of the two flagella on an individual cell to resorb (or, with CD, grow) at the same size rate and extent. They also have the same effects on wild type cells, whose flagella contain the normal "9+2" complement of microtubules, and *pf*-18 cells, whose flagella contain the nine doublet microtubules but lack the central microtubules, so the targets of these drugs must be related to all the flagellar microtubules and not only the doublets or central microtubules.

Colchicine Effects on Flagellar Disassembly

This is the first report to show that colchicine induces flagellar disassembly. The colchicine-induced disassembly of wild type cells was not as dramatic as that of the *pf* 18 cells but this is probably more a function of the methods employed to measure flagella than the behavior of the flagella themselves. The flagella on a population of cells before drug addition varied 1–2 μm, so small changes of flagellar length caused by colchicine or CD could be overlooked by averaging a large population of cells. In contrast to wild type cells, *pf* 18 cells were immotile so individual *pf* 18 cells were observed throughout the experimental period. Each change in the length of individual flagella was, therefore, directly observed and recorded.

The flagellar shortening induced by colchicine probably reflects a certain amount of flagellar protein turnover that occurs at the tips of the fully grown flagellar microtubules. If the tubulin slowly exchanges with the soluble pool of tubulin

in the flagellum, the addition of colchicine would be expected to bind to the free tubulin and shift the equilibrium toward microtubule disassembly, which are the results we observed in this study. The amount of tubulin flux in flagellar microtubules is probably very small, since a study of *Chlamydomonas* cells with fully grown flagella that were pulse labeled for 10 min revealed a small amount of newly labeled tubulin in the detergent-solubilized "membrane + matrix" fraction but no newly labeled tubulin that was incorporated in the detergent-insoluble axoneme fraction (Remillard and Witman, 1982). Based on our data, the rate of colchicine-induced flagellar disassembly averaged 0.02 μm/min, and the maximum rate of disassembly we ever observed was 0.08 μm/min. If this is a reflection of the rate of tubulin flux in the flagellum, then the maximum amount of tubulin incorporation during a 10-min labeling period would be equal to the tubulin in 0.2–0.8 μm of flagellar microtubules, ~1.7%, and no greater than 6.7%, of the total axonemal tubulin. The amount of tubulin flux in the fully grown flagellum during a 10-min pulse is probably much less than 2–7% of the total tubulin, since the lengths of flagellar microtubules are normally constant, within the limits of our measuring abilities. It is unlikely that exchange would occur uniformly throughout the 0.2–0.8 μm of the flagellar microtubules during the labeling period because this would require nearly complete disassembly and reassembly of the linear microtubule polymer. The lack of label found in the fully grown axonemes is probably due to the low levels of tubulin exchange being too little to be detected. Alternatively, since the ends of the flagellar microtubules are very fragile, and prone to breakage during isolation or extraction (Dentler and Rosenbaum, 1977), and labeling tubulin that was incorporated into the distal 0.2–0.8 μm of the flagellar microtubules may have been lost into the "membrane + matrix" fraction, an observation that is consistent with the small amount of labeled tubulin reported by Remillard and Witman (1981).

Since colchicine did not induce the disassembly of more than one-half of the flagella and since, once shortened, flagella maintained a constant length in the presence of colchicine, the proximal half of the flagellar microtubules must be more stable than the distal half. This is the first report of differential stability of the proximal and distal halves of a flagellum, but others have reported structural differences between the proximal and distal halves of the flagellum. Hoops and Witman (1983) described beak-shaped projections in three of the B-tubules that were found in the proximal but not distal half of the flagellum, and Piperno and Ramanis (1991) reported that the proximal half of a flagellum contains a set of inner dynein arms that are not found in the distal half. These inner arm components were also more tightly bound to the doublets than were other inner arms and the outer arms, which may partially explain our results showing that the proximal half of the flagellum is more stable to colchicine than the distal half. Whether the beaks or inner arm component directly or indirectly alter the stability of the flagellar microtubules in the presence of colchicine remains to be determined.

Cytochalasin D Effects on Flagellar Disassembly and Assembly

The mechanism of CD action that causes flagellar disassembly is more puzzling than that induced by colchicine. Our initial hypothesis, based on the results reported by Boisvieux-Ulrich et al., (1990), that the capping structures would not be assembled, does not appear to be correct, since caps were formed on flagella that grew in the presence of CD and caps were present on fully grown flagella whose microtubules oscillated in length in CD. CD may have effects on the functions of capping structures, but it is certain that CD does not prevent the caps from forming, so we have no data regarding cap function or the effects of CD on the caps.

We considered the possibility that the high concentrations of CD used in these experiments may have general metabolic effects on the cells that could alter their ability to regulate flagellar length. While the concentrations of CD and colchicine used in these experiments are high, relative to concentrations used in cultured mammalian cells, they are the same or lower than the concentrations used by others to study microtubule (Rosenbaum et al., 1969) and actin assembly (Detmers et al., 1983) in *Chlamydomonas* cells. This may reflect the relative insensitivity of tubulin and actin to the drugs or may be due to the inability of these drugs to penetrate these free-living protozoans, It is unlikely that CD had any major effects on metabolic processes, because (wild type) cells continued to swim rapidly or, when flagella were extremely short, the flagella continued to wiggle, despite their being incubated for up to 6 h in CD, colchicine, or colchicine plus CD. This is quite different from other agents, including changes in ionic strength, sodium pyrophosphate, and IBMX, that induce flagellar shortening but also paralyze the flagella during the shortening period. Since the tonicity of the medium affects flagellar length (Solter and Gibor, 1978; Telser, 1977), we monitored the activity of the contractile vacuole using time lapse video microscopy but found that the contractile vacuole size and rate of contraction showed no noticeable differences when control cells were compared with drug-treated cells. Since the tonicity of the medium would not be affected by the concentrations of drugs used here and since the cells showed no obvious alterations of the contractile vacuole or flagellar motility, we feel that it is unlikely that the drugs effect the cells simply by altering their ability to regulate internal ionic strength.

Finally, since actin often is associated with membranes in eukaryotic cells, we considered the possibility that changes in actin filament assembly may alter the ability of the flagellum to recruit membrane and that this may alter the ability of the flagellum to regulate growth. We consider this to be unlikely for two reasons. First, the rate of flagellar regeneration, which requires the recruitment of flagellar membrane as well as axonemal components, was virtually identical in control and CD-treated cells, so flagella can recruit membrane in the presence of CD. Second, as flagellar lengths oscillated in the presence of CD, the rates of growth (0.01–0.25 $\mu m \cdot min^{-1}$) were similar to those during flagellar regeneration (0.15 $\mu m \cdot min^{-1}$), so even long periods of CD exposure failed to alter the ability of the membrane to add to or be removed from the flagellum.

We also considered the possibility that actin may be associated with the flagellar membrane and may have some effects on the movement of flagellar proteins up or down the flagellum. To examine this possibility, we added polystyrene beads to control and CD-treated *pf* 18 cells and looked for any changes in the attachment or movement of beads along

the flagellum (Bloodgood, 1990). In two separate experiments (data not shown), we observed no difference in the frequency of attachment or rates of movement of beads on *pf* 18 flagella in the presence or absence of CD, even though flagellar length changes occurred in the CD-treated cells. In addition to having no (obvious) effect on flagellar motility, CD also has no apparent effect on the energy-dependent movement of particles along the flagellar surface.

Since CD inhibits or alters the synthesis of specific proteins in mammalian cells (Ornelles et al., 1986; Zambetti, et al., 1991), we tested the effect of cycloheximide with and without CD on *Chlamydomonas* cells (data not shown). We found that the addition of cycloheximide at concentrations that inhibited *Chlamydomonas* protein synthesis did not effect flagellar length nor did it alter the effects of CD or colchicine, and conclude that the effect of CD is probably not on protein synthesis in *Chlamydomonas*, although we cannot completely exclude this possibility because we did not measure the amounts of protein synthesis in the drug-treated cells. Taken together, these results indicate that the effect of CD is not on general metabolic processes, on the assembly of the microtubule capping structures, or on protein synthesis.

Is There any Flagellar Actin?

The best known effect of Cytochalasins and, in particular, CD, is to alter the assembly of cytoplasmic actin filaments (Cooper, 1987), so it is logical to ask if there are any actin filaments in the flagellum. Filamentous actin has been found in proximal portion of epithelial cell cilia (Chailley et al., 1986) and it forms a large aggregate near the distal ends of the doublet microtubules in the photoreceptor cilia of *rds* mutant mice (Chaitin, 1991), so the presence of actin filaments in cilia is not without precedent. During these studies we searched for filamentous actin using both light and electron microscopy. Using NBD-Phallacidin and Bodipy-Phalacidin, which are sensitive fluorescent dyes that bind to and to stabilize filamentous actin, we were able to observe a weak fluorescence along the length of the flagellum, but this fluorescence was too weak to convincingly record using a silicon-intensified video camera, and we found no concentration of fluorescent staining in any region of the flagellum, so we conclude that the amount of filamentous actin in the flagellum, if any is present, is extremely low. We have found filamentous material at the ends of flagellar microtubules (see Fig. 10*b*) and have not seen this material on cells that were not treated with the drugs. However, the quantity of this material is extremely small, it was only seen on very short flagella, and we have not identified its composition at this time, so we have no evidence that it is (or is not) filamentous actin. Based on our current data, we cannot conclude that filamentous actin is present in the *Chlamydomonas* flagellum.

An actin-like protein does appear to be associated with one or more of the inner arms of *Chlamydomonas* flagella (Piperno, 1988; Piperno and Luck, 1979), but whether it is filamentous or monomeric actin is not known. Although the function of the flagellar actin is not known, the assembly of one or two of the inner arms with which it is associated appears to be related to flagellar growth and axonemal stability. Piperno and Ramanis (1991) recently reported that *pf* mutants that lack inner arms 2 and 3 fail to grow longer than 6 μm, approximately half of the normal flagellar length, and that these inner arms are added to the proximal portion of the flagellum only after the flagellum has grown longer than 6 μm. Thus, the addition of these arms to the proximal portion of the flagellum permits the flagellum to continue its growth and may confer some stability to the axoneme. Both our colchicine and CD experiments are consistent with differences in stability between the proximal and distal portions of the flagellum. Colchicine induced disassembly of the distal third or half of the flagellum in wild type and *pf* 18 cells, but the proximal portion was never disassembled in colchicine. In the presence of CD, most (but not all) cells had flagella that oscillated in length from 12 to 4 μm, which is also consistent with the proximal portion of the flagellum being more stable than the distal half. However, the relationship between the drug-induced disassembly of full length flagella, the presence of unique inner arm dyneins in the proximal third to half of the flagellum, and the function of inner arm–associated actin remains to be determined.

Are Flagellar Microtubule Dynamics Similar to Those of Cytoplasmic Microtubules?

Although the site of CD action is unknown, it is certain that it alters the controls that normally maintain flagellar length. The oscillations in flagellar length observed in the CD-treated *pf* 18 cells are similar to cytoplasmic microtubule dynamics observed in vivo and in vitro (Avila, 1990; Cassimeris and Salmon, 1991; Hotani and Miyamoto, 1990). However, this flagellar behavior differs from the "dynamic instability" of cytoplasmic microtubules in that each of the microtubules in the "9 + 2" arrays within each of the two flagella on an individual cell behave identically. The dynamics observed here, therefore, are more like the transitions between the disassembly of one microtubule array and the assembly of another microtubule array that occurs in cells transiting from interphase to mitosis or in dividing cells transiting from prophase or prometaphase to anaphase. In each of these examples, an individual cell can switch between microtubule assembly and disassembly in response to yet unknown signals.

The assembly and disassembly rates as well as the duration of these processes are considerably slower in flagella than those reported for cytoplasmic microtubules (Cassimeris and Salmon, 1991; Hotani and Miyamoto, 1990), but this is not surprising, since microtubules compose less than half of the total flagellar protein and since flagellar assembly and disassembly require the coordinated changes in the membrane, the two central and nine doublet microtubules, and all the proteins necessary to generate flagellar motility. At present, it would appear that the molecular target for CD is associated with an unknown cytoplasmic target that is responsible for length regulation, with one of the capping structures that are bound to the sites of flagellar microtubule assembly (Dentler, 1980), or with one of the inner dynein arms that are necessary for flagellar growth and may be associated with an actin-like protein (Piperno and Ramanis, 1991). CD is the first treatment shown to destabilize both the assembly and disassembly of flagellar microtubules and studies of its effects may help us to understand the mechanisms necessary for normal flagellar growth control.

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