FLAGELLAR ELONGATION AND SHORTENING IN *CHLAMYDOMONAS*

The Use of Cycloheximide and Colchicine to Study the Synthesis and Assembly of Flagellar Proteins

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ABSTRACT

Flagella can be removed from the biflagellate *Chlamydomonas* and the cells begin to regenerate flagella almost immediately by deceleratory kinetics. Under usual conditions of deflagellation, more than 98% of all flagella are removed. Under less drastic conditions, cells can be selected in which one flagellum is removed and the other left intact. When only one of the two flagella is amputated, the intact flagellum shortens by linear kinetics while the amputated one regenerates. The two flagella attain an equal intermediate length and then approach their initial length at the same rate. A concentration of cycloheximide which inhibits protein synthesis permits less than one-third of each flagellum to form when both flagella are amputated. When only one is amputated in cycloheximide, shortening proceeds normally and the degree of elongation in the amputated flagellum is greater than if both were amputated in the presence of cycloheximide. The shortening process is therefore independent of protein synthesis, and the protein from the shortening flagellum probably enters the pool of precursors available for flagellar formation. Partial regeneration of flagella occurs in concentrations of cycloheximide inhibitory to protein synthesis suggesting that some flagellar precursors are present. Cycloheximide and flagellar pulse-labeling studies indicate that precursor is used during the first part of elongation, is resynthesized at mid-elongation, and approaches its original level as the flagella reach their initial length. Colchicine completely blocks regeneration without affecting protein synthesis, and extended exposure of deflagellated cells to colchicine increases the amount of flagellar growth upon transfer to cycloheximide. When colchicine is applied to cells with only one flagellum removed, shortening continues normally but regeneration is blocked. Therefore, colchicine can be used to separate the processes of shortening and elongation. Radioautographic studies of the growth zone of *Chlamydomonas* flagella corroborate previous findings that assembly is occurring at the distal end (tip growth) of the organelle.

INTRODUCTION

An earlier report (25) described flagellar regeneration in the protozoan flagellates *Euglena*, *Astasia*, and *Ochromonas* and documented the usefulness of these systems in the study of organelle development. It was shown that flagellar regeneration was partially dependent on new protein synthesis, and that the flagellum was probably elongating by the assembly of precursors at the tip of the organelle.

This report will describe some aspects of flagellar regeneration in the biflagellate *Chlamydomonas*. The organism is well suited to this type of investigation, for a number of reasons: genetic (see reviews, references 13 and 28) and ultrastructural studies (8, 11, and see reference 22 for review) are extensive; it can be grown synchronously in large quantities (1, 12); it can be deflagellated in a simple and reproducible manner (5, 24). The techniques of flagellar isolation and fractionation, radioautography, and incorporation of labeled amino acids can all be applied to the organism without difficulty and, equally important, mutants useful for the study of flagella are available (14, 15, 18, 36).

In the present report,[1] the kinetics of flagellar regeneration in single cells and populations of *Chlamydomonas* are described under conditions in which either one or both of the flagella are amputated. The requirements for new protein synthesis during flagella formation are investigated with pulse-labeling and by the use of cycloheximide, and flagellar assembly is studied by using colchicine. Light microscope radioautography and electron microscopy are used to observe the flagellar growth zone.

MATERIALS AND METHODS

Cultures

Experiments were performed with three strains of *Chlamydomonas reinhardi:* 21 gr (wild-type), pf 16 (nonmotile, normal-length flagella), and Arg 1 (arginine-requiring mutant). Cultures were grown as follows: 250-ml Erlenmeyer flasks containing about 75 ml of Medium I of Sager and Granick (27) were incubated at 25°C and bubbled with air. Arginine (0.1 mg/ml) was added to the medium used for the arginine-requiring mutant. A cycle of 14 hr light and 10 hr dark was used to induce synchronous growth (1, 12). The organisms were used at a density of 1–4 × 10^6 cells/ml, between the 2nd and 4th hr of the light cycle.

Flagellar Amputation

Flagella were removed from populations of cells by mild shearing forces in a homogenizer. 15 ml of culture were placed in the fluted glass receptacle of a Virtis homogenizer.[2] Treatment of the sample at a speed of 14,000 rpm (setting 50 on our machine) for 60 sec removed at least 98% of the flagella without otherwise injuring the cells. Treatment at 9,000 rpm (our setting 30) for 30 sec produced partial deflagellation, resulting in cells of the following types: (*a*) both flagella removed; (*b*) both flagella remaining; (*c*) flagella partially off, and (*d*) one flagellum removed and the other intact. As much as 15–20% of the cell population were of the last type, and observations on this class of cells will be reported below. In all cases, regeneration times were measured from the beginning of amputation treatment.

Measurement of Regeneration

POPULATIONS: Regenerating cultures were kept under constant conditions of illumination, temperature (25°C), and aeration. Samples of the regenerating cultures were taken at regular intervals and fixed by the addition of an equal volume of 2.5% glutaraldehyde in 0.01 M potassium phosphate buffer (pH 7.0). Fixed cells were stored in the cold until the end of the experiment, when the flagella were measured under a phase microscope with an ocular micrometer. Flagellar lengths refer to the distance of the flagellar tip from just below the outer limit of the cell wall (See Figs. 22 and 23). The flagella of at least 50 cells were measured for each sample.

SINGLE CELLS: Since pf 16 cells are nonmotile, regeneration in single cells could easily be studied under the microscope. A thin ring of Vaseline petroleum jelly was made on a microscope slide, a small drop of cell suspension was applied within the ring, and a cover slip was gently pressed over it. A single cell or group of cells was selected for observation and then photographed at intervals as frequent as once per minute. Microscope illumination was filtered through a green interference filter and turned off between observations so as to prevent excessive heating of the slide. The major elements of the photographic system were a Zeiss 40 X apo-oil phase-contrast objective, a 60 watt-second Zeiss electronic flash, and high contrast copy film developed in Dektol.[3] The photographic negatives were projected at a standard enlargement, tracings were made of the projections, and the tracings were measured. Data from cells which were obviously tipped or out of focus were excluded. Regeneration data on the wild-type strain were obtained by the use of a rotocompressor.[4] to hold the cells immobile.

Amino Acid Incorporation

In vivo amino acid incorporation experiments were carried out in 10-ml Erlenmeyer flasks. 1 ml of cells at a density of 2–4 × 10^6 cells/ml was incubated

[1] Preliminary notes describing parts of the work reported here appeared in the Abstracts of papers presented at the American Society for Cell Biology (*J. Cell Biol.* 1967. **35**:113A.) and (*J. Cell Biol.* 1968. **38**: 97A.).

[2] The Virtis Co., Gardiner, N. Y.

[3] Eastman Kodak, Rochester, N. Y.

[4] The Biological Institute, Philadelphia, Pa.

at 25°C in light while being stirred with a magnetic stirrer. Arginine-^{14}C[5] was added to the incubating culture, and the mixture was sampled at regular intervals. Samples for scintillation counting were prepared by using a modification of the procedure of Mans and Novelli (17). 50-μl samples were withdrawn with disposable capillary pipettes, placed on Whatman 3MM 2.3 cm filter discs, and dropped immediately into cold 10% TCA.[6] The accumulated filters were kept in cold TCA until the end of the experiment and then were treated in bulk as follows: 2 × 15-min wash, cold 10% TCA; 2 × 10-min wash, hot (90°C) 5% TCA; 2 × 5-min wash, hot 80% ethanol; 2 × 5-min wash 1:1 ethanol-ether; 2 × rinse, absolute ether. The treated filters were dried and placed in vials for scintillation counting.

Radioautography

Cell samples (0.05–0.1 ml) were withdrawn from the incubation mixture and fixed for 30 min in the cold with an equal volume of 2.5% glutaraldehyde in 0.01 M potassium phosphate buffer (pH 7.0). Samples were then sedimented at 150 *g* for 3 min, washed twice with 10 ml of distilled water, and spread with a glass rod into a thin film on ethanol-cleaned slides. The slides were allowed to air dry and then were treated as follows: 2 × 15-min wash, cold 5% TCA; 30-min wash, hot (90°C) TCA; overnight wash, 70% ethanol. Slides were dehydrated in 70, 95, and 100% ethanols, dried, and dipped in Kodak NTB-2 emulsion.[3] After development, silver grains over the flagella were counted with a Zeiss Neofluar oil-phase objective (100 ×) with water as a temporary mounting medium. Zero-time controls, fixed immediately after the addition of isotope, showed only a background distribution of silver grains over the flagella.

Electron Microscopy

Cells were fixed for 5 min at room temperature by the addition of 1 ml of 12% glutaraldehyde in 0.02 M potassium phosphate buffer, pH 7.5, to 4 ml of cells suspended in culture medium. The organisms were then concentrated by gentle centrifugation in a clinical centrifuge and postfixed for 1–1.5 hr in the cold in 2.5% glutaraldehyde in 0.005 M potassium phosphate buffer, pH 7.5, and 0.5% osmium tetroxide. Dehydration was carried out using 10-min changes of 50, 70, and 95% cold ethanol, the sample being allowed to warm to room temperature in 95% ethanol. After two to three changes of absolute ethanol over a period of 1 hr, the samples were infiltrated and embedded in Epon or Araldite according to standard procedures (16). Sections were cut with a Porter-Blum Sorvall MT-2 microtome and were mounted on uncoated 400-mesh grids. The sections were stained with uranyl acetate and lead citrate (21) and observed with a Philips 200.

[5] Radioactive arginine was obtained from Schwarz BioResearch, Orangeburg, N. Y., and from New England Nuclear, Boston, Mass.

[6] Abbreviations used in this report: TCA, trichloroacetic acid.

RESULTS

The Kinetics of Flagellar Regeneration in Populations of Chlamydomonas

In light-dark synchronized cultures of *Chlamydomonas*, the cell size and flagellar length prior to amputation are very homogeneous and, following amputation, more than 98% of the cells regenerate flagella. The kinetics of normal flagellar regeneration in a population of cells can be determined by the use of the methods described in the preceding section. Regeneration begins almost immediately following amputation, with little or no lag occurring during the initial period. The flagella proceed to elongate at a decelerating rate (initial rate of 0.4 μ/min), almost regaining their initial length after 70–90 min (Fig. 1). Flagellar growth then continues at a slow rate (about 0.15–0.2 μ/hr) similar to that of normal elongation during interphase (Fig. 2). Regeneration curves are subject to some error because of variation within the populations studied. However, kinetic data obtained from several different experiments indicate the synchrony of regeneration to be quite reproducible (see Fig. 1).

The Kinetics of Flagellar Regeneration in Single Cells

A series of photographs of a single cell (paralyzed strain) can be used to obtain a regeneration curve corresponding to the population curve (Fig. 3). The kinetics of regeneration are found to be essentially identical to those determined for a population. Experimental errors arise from mechanical and optical factors, although the uniformity of regeneration and the correlation between population and single cell kinetics indicate the method to be accurate.

Regeneration Kinetics Following Amputation of One Flagellum

Flagellar regeneration was also studied in paralyzed cells in which one flagellum had been amputated and the other left intact (long-zero cells). These cells present a complex situation, the intact

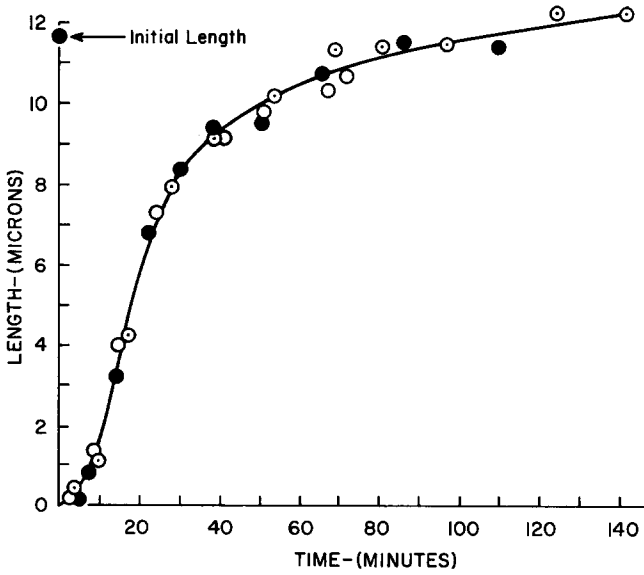

FIGURE 1 The kinetics of flagellar regeneration in a population of *Chlamydomonas reinhardi* (21 gr). Open circles, closed circles, and circles with dots represent three different experiments and show the reproducibility of regeneration kinetics in populations.

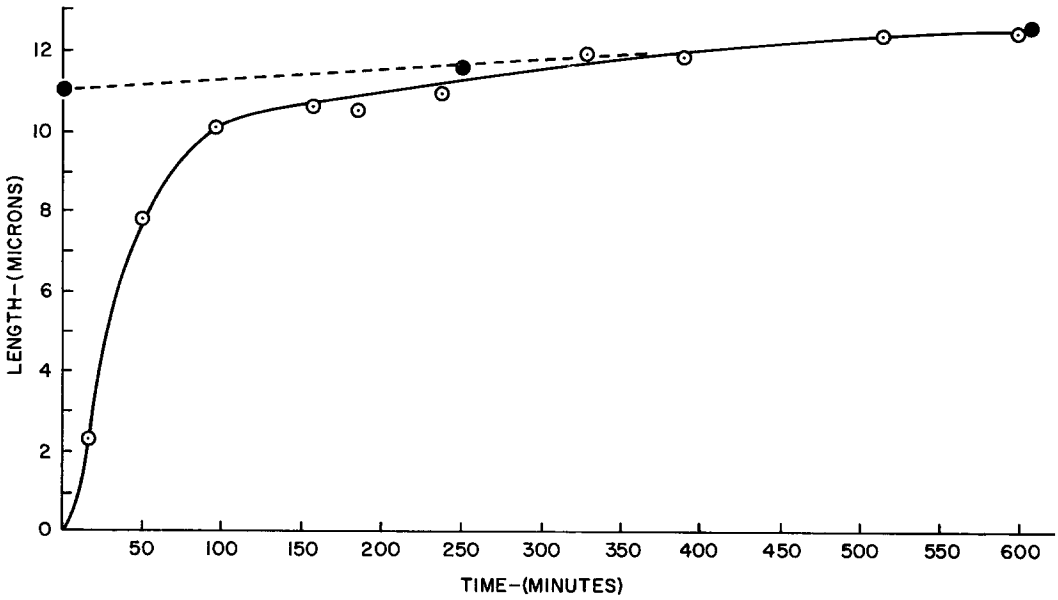

FIGURE 2 The kinetics of flagellar regeneration in a population of *Chlamydomonas* (21 gr) showing the slow elongation phase over an 8-hr period (100–600 min). Closed circles and dashed line indicate the increase in flagellar length in a population of unamputated interphase cells over the same time period.

flagellum becoming shorter while the amputated flagellum is simultaneously elongating. The kinetics of regeneration in all observed long-zero cells fall into two distinct classes. In one class (Fig. 4), the long (intact) flagellum partially shortens by linear kinetics and is maintained at some intermediate length. Concomitantly, the amputated flagellum grows out by normal ki-

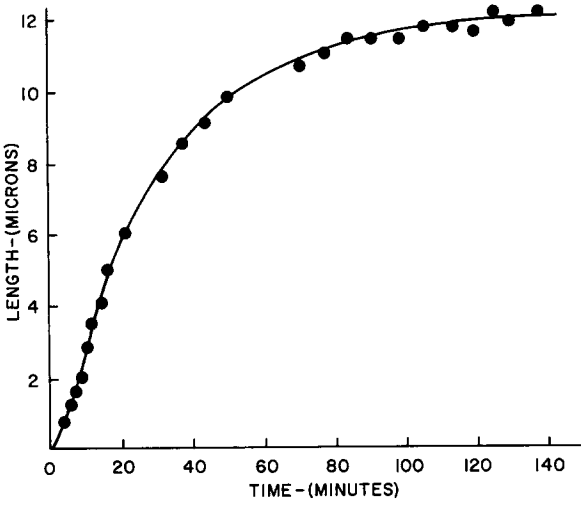

FIGURE 3 The kinetics of flagellar regeneration in a single *Chlamydomonas*. Compare with Fig. 1.

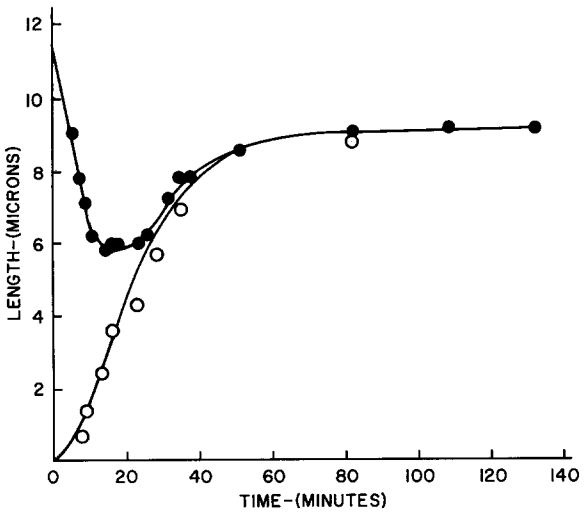

FIGURE 4 Amputation of one of the two flagella of a single *Chlamydomonas* (pf 16) showing partial shortening of the intact flagellum. Open circles, amputated flagellum; closed circles, intact flagellum.

netics. When both flagella have reached the same intermediate length, they grow out simultaneously to a final length somewhat less than the original length of the unamputated flagellum or the control length of the population. In the second class (Fig. 5), the intact flagellum shortens to zero or nearly zero length and immediately begins to elongate again. The amputated flagellum grows out to an intermediate length which is maintained until the other flagellum has completed its shortening and has grown out again. When both flagella are at the same intermediate length, they continue to elongate together, again reaching a length somewhat less than normal. The common elements of these two patterns of long-zero kinetics are: (*a*) shortening of the intact flagellum by linear kinetics at a rate of about 0.4 μ/min, (*b*) regrowth of both the intact flagellum and amputated flagellum by normal kinetics, and (*c*) a period when the growth of one flagellum or the other lags. The factor that distinguishes the two classes of long-zero kinetics seems to be whether or not the shortening flagellum attains a length less than that of the regenerating, amputated flagellum. If it does, the regenerating flagellum lags (Fig. 5); if it does not, the shortening flagellum lags (Fig. 4). In either case, the net effect is that both flagella attain the same intermediate length before elongating simultaneously. Selected frames from a long-zero sequence of the second

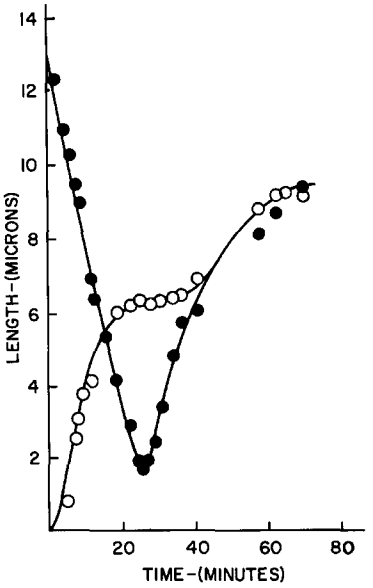

FIGURE 5 Amputation of one of the two flagella of a single *Chlamydomonas* (pf) showing almost complete shortening of the intact flagellum and the lag in the regeneration of the amputated flagellum. Open circles, amputated flagellum; closed circles, intact flagellum.

class (almost complete shortening of the intact flagellum) are shown in Fig. 6. Although all the studies mentioned of long-zero cells were done with the paralyzed strain, it can be shown that a similar situation occurs in the wild type. Since photography of the motile wild-type cells was difficult with the usual Vaseline-ringed slide, these cells were immobilized with a roto-compressor (see Methods). One of the two flagella could be removed by gently compressing the cells to a pressure slightly beyond that necessary for immobilization. Fig. 7 shows two single flagellum amputations carried out on the same cell, successively removing opposite flagella. The kinetics follow those described in the first class mentioned above.

Effect of Cycloheximide on Flagellar Regeneration

Cycloheximide can be used successfully to inhibit protein synthesis in *Chlamydomonas*. Fig. 8 indicates that cycloheximide at a concentration greater than 1 μg/ml rapidly and completely inhibits amino acid incorporation into the TCA-insoluble protein of whole cells. This concentration of cycloheximide is equally effective on all strains of *Chlamydomonas* used in this study.

EFFECT OF CYCLOHEXIMIDE ON REGENERATION IN PF 16 WHEN BOTH FLAGELLA ARE AMPUTATED: Flagellar regeneration was examined in paralyzed cells under conditions of cycloheximide inhibition (in the absence of new protein synthesis), and was found to proceed to a limited extent. Fig. 9 shows regeneration in a population of the mutant pf 16 with and without cycloheximide. This experiment and others on single cells indicate that each of the flagella elongates to about 1–2 μ. In the wild-type and arginine-requiring mutants there is considerably more regeneration in the presence of cycloheximide, the flagella regenerating to about one-half their normal length. Studies investigating the effect of cycloheximide on wild-type cells are presented in detail below.

EFFECT OF CYCLOHEXIMIDE ON REGENERATION IN PF 16 WHEN ONE FLAGELLUM IS AMPUTATED: The above results indicate that the paralyzed mutant is able to form a total of about 2–4 μ of flagellum (1–2 μ/flagellum) when both flagella are amputated in the presence of cycloheximide. However, long-zero cells in which only one flagellum is amputated have a flagellar shortening and elongation pattern which is virtually identical whether cycloheximide is present or not. Figs. 10 and 11 show the two classes of long-zero kinetics found in the presence of 10 μg/ml cycloheximide; these results should be compared with those in Figs. 4 and 5. The results from these experiments indicate that in the absence of new protein synthesis, more flagellum is formed when one flagellum is amputated than when both are amputated.

EFFECT OF CYCLOHEXIMIDE ON FLAGELLAR REGENERATION IN WILD-TYPE CELLS: When both flagella of the wild-type strain are amputated in the presence of cycloheximide, the flagella elongate to about one-half normal length and maintain this length (Fig. 12). The regeneration kinetics in the presence of cycloheximide are similar to the kinetics of the controls until just prior to the inhibition of elongation. These results indicate that the cells have a limiting amount of some protein, possibly flagellar precursor, which is necessary for regeneration.

The following experiment was performed to determine if the limiting protein suggested in the experiment above was actually being used for flagella formation and, if so, at what stage of regeneration. The flagella of a population of cells

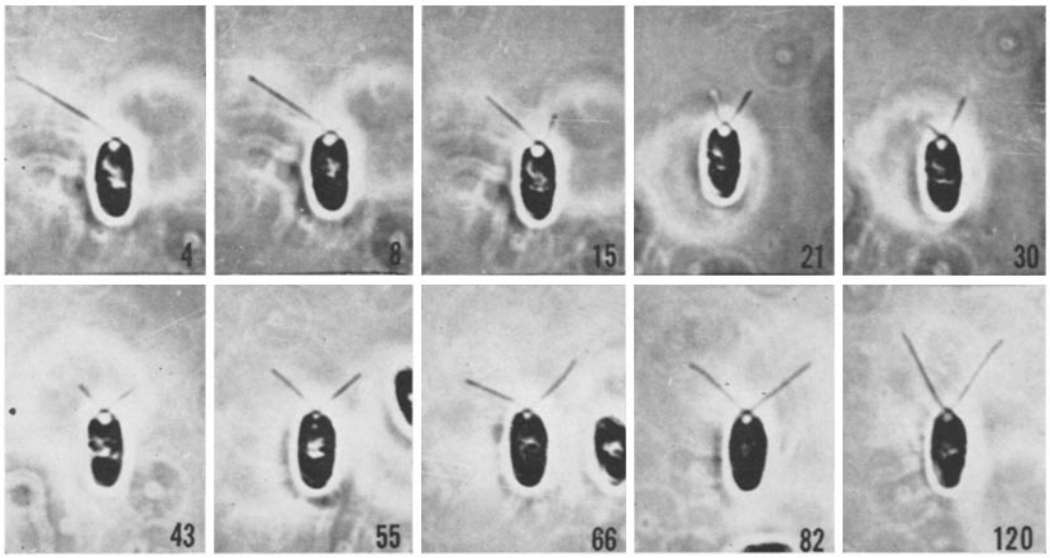

FIGURE 6 Photomicrographs chosen from the experiment described in Fig. 5 to illustrate the simultaneous shortening and elongation processes in a single cell. The time (minutes) after amputation of the right flagellum is shown in the lower right corner of each micrograph. × ca. 1100.

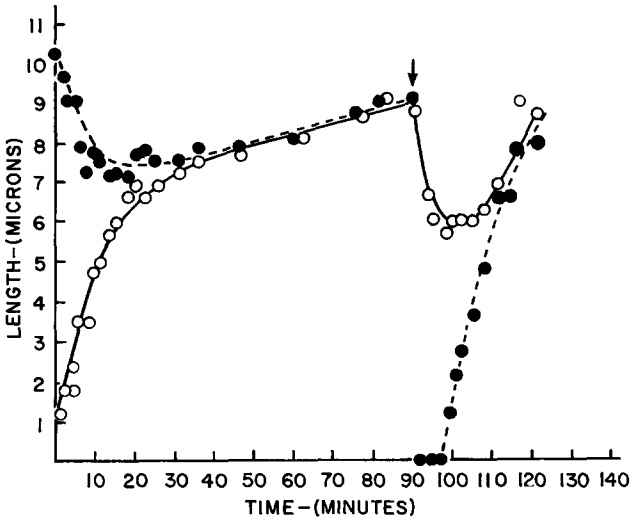

FIGURE 7 Two single flagellum amputations carried out on a wild-type *Chlamydomonas* (21 gr), successively removing opposite flagella. Open circles, amputated flagellum in the first regeneration, intact flagellum in the second regeneration; closed circles, intact flagellum in the first regeneration, amputated flagellum in the second regeneration. The arrow indicates the time of the second amputation.

were amputated, and the cells were allowed to regenerate flagella to various lengths. Re-amputation was then carried out in the presence of cycloheximide, and the amount of elongation was assessed. Fig. 13 shows that the flagella regenerate to about one-half normal length when cycloheximide is added at the time of amputation (control). When the inhibitor is added to cells which have already regenerated flagella to about ¾ length and are then re-amputated, regeneration is much less. If the cells are allowed to regenerate flagella of almost normal length and are re-amputated, the amount of regeneration in cycloheximide is almost similar to that obtained in the controls.

The Rate of Incorporation of Arginine-^3H into Protein of Regenerating Flagella

The previous result indicated that there might be some use of flagellar precursor proteins during

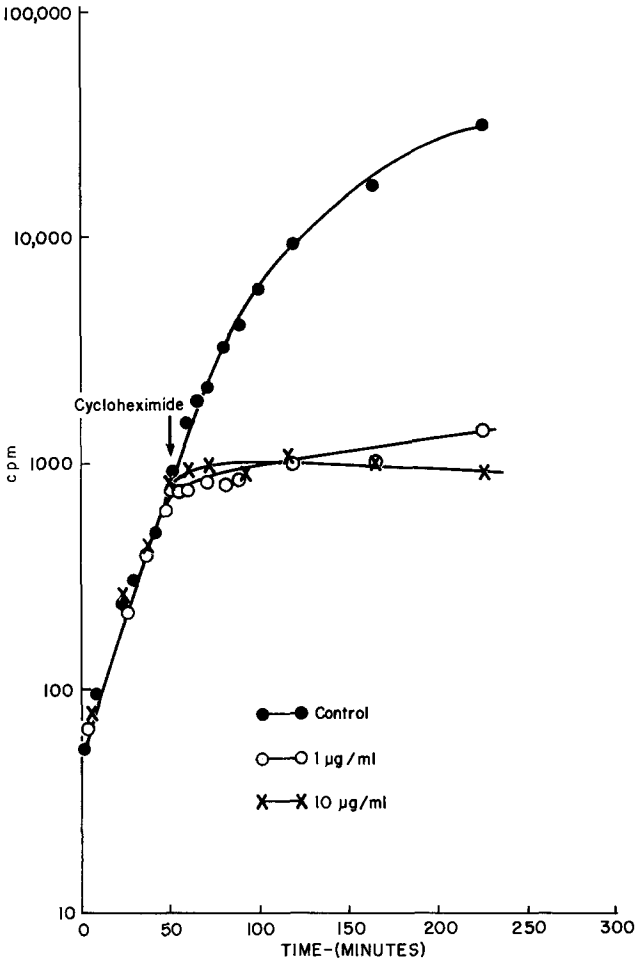

FIGURE 8 Effect of cycloheximide on arginine-^{14}C incorporation into TCA-insoluble protein of *Chlamydomonas* (pf 16). Arginine-^{14}C (2.5 µc/ml, 172 mc/mmole) added at time zero and cycloheximide added at 50 min. Closed circles, no cycloheximide: open circles, 1.0 µg/ml; crosses, 10.0 µg/ml.

the first half of elongation. Pulse-labeling experiments were carried out to determine the actual *rate* of incorporation into the proteins of elongating flagella. Arginine-requiring cells were shown to have regeneration kinetics similar to those of the wild-type cells and to incorporate labeled arginine into their protein at a rate which proceeded linearly after about 2 min in the presence of the isotope. The cells were deflagellated, pulsed with arginine-^{3}H for six 10-min intervals during the course of regeneration, and radioautographed. The rate of incorporation (grains/µ/min) was determined by assessing the number of silver grains over the flagella for each time interval. Cells with nonregenerating flagella (interphase cells) were also found to incorporate some amino acid into their flagellar proteins.[7] This turnover incorporation was corrected for by pulsing cells which had nonregenerating flagella for 10 min and then subtracting this incorporation from that obtained for each 10-min pulse in the regenerating cells. The results in Fig. 14 show that the greatest rate of incorporation occurs at the time when the flagella are at about one-half length. The rate then decreases as the flagella attain their normal length. Thus, the highest rate of incorporation into flagellar protein occurs at about the time when, the cycloheximide studies indicate, the cells have the lowest amount of flagellar precursors.

[7] Cells with nonregenerating flagella will incorporate amino acids into their flagellar proteins. The protein turnover in *Chlamydomonas* flagellar axonemes, membranes, and matrix components will be the subject of another report (Rosenbaum, J., K. Carlson, and G. Witman. In preparation).

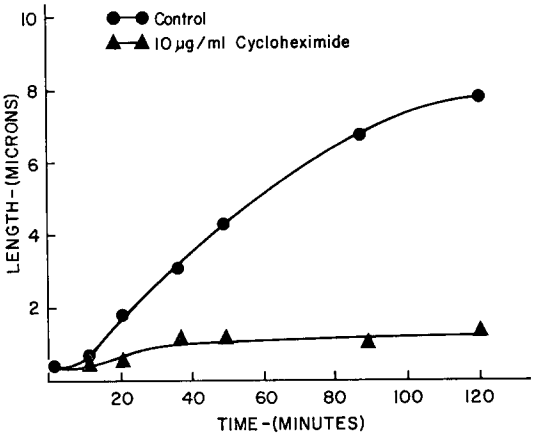

FIGURE 9 Effect of cycloheximide on flagellar regeneration in a population of *Chlamydomonas* (pf 16). Closed circles, control; closed triangles, 10.0 μg/ml cycloheximide.

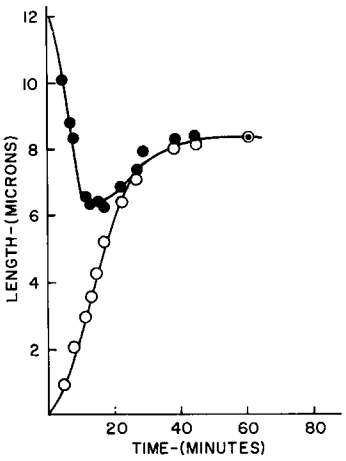

FIGURE 10 Effect of cycloheximide (10.0 μg/ml) on flagellar regeneration in a single *Chlamydomonas* (pf 16) after removal of one flagellum and when partial shortening of the intact flagellum occurs. Open circles, amputated flagellum; closed circles, intact flagellum.

The Effect of Colchicine on Flagellar Regeneration in Chlamydomonas

Colchicine is a well known inhibitor of the formation of microtubular systems (see references 9, 10 for reviews). Its mode of action has been investigated, and it has been shown to bind to the subunits of microtubules isolated from various organelles, including the mitotic apparatus and flagella (3, 4, 29, 35). It was therefore used in studying flagellar regeneration in *Chlamydomonas*.

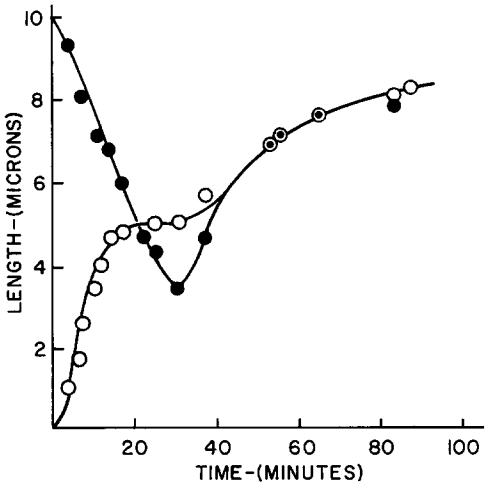

FIGURE 11 Effect of cycloheximide (10.0 μg/ml) on flagellar regeneration in a single *Chlamydomonas* (pf 16) after removal of one flagellum and when the intact flagellum shortens past the regenerating flagellum. Open circles, amputated flagellum; closed circles, intact flagellum.

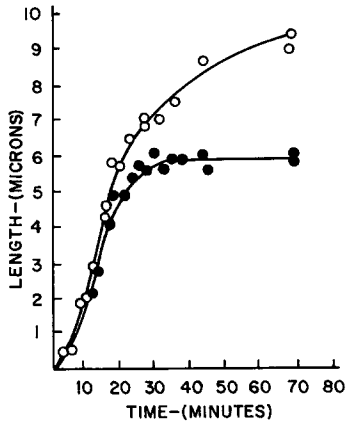

FIGURE 12 Effect of cycloheximide (10.0 μg/ml) on flagellar regeneration in a population of wild-type *Chlamydomonas* (21 gr). Open circles, control; closed circles, cycloheximide added at time of amputation.

EFFECT OF COLCHICINE ON FLAGELLAR REGENERATION WHEN BOTH FLAGELLA ARE AMPUTATED: When used at the proper concentration, colchicine was found to prevent flagellar elongation in *Chlamydomonas*.[8] Fig. 15 shows

[8] The potency of colchicine as an inhibitor of flagellar regeneration will vary somewhat with different lots of colchicine. Dose response curves run on each new batch of colchicine show that 1.0–1.5 mg/ml will usually completely inhibit flagellar regeneration.

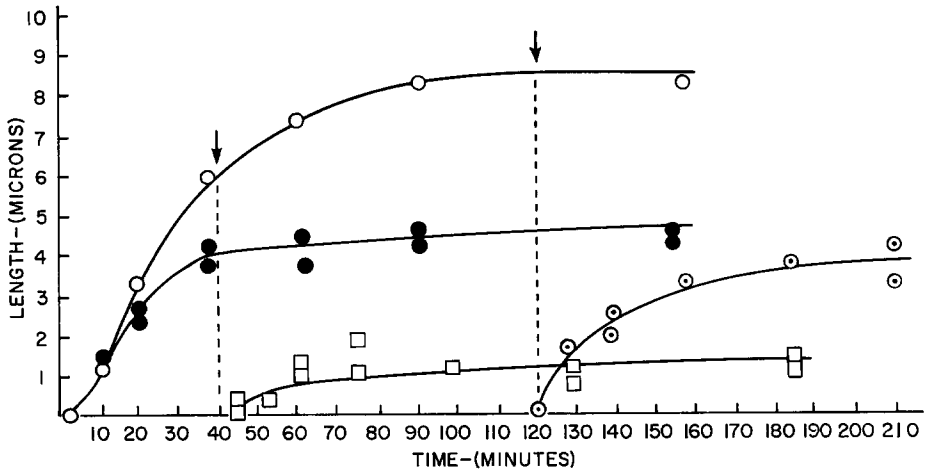

FIGURE 13 Effect of cycloheximide (30.0 μg/ml) on flagellar regeneration in *Chlamydomonas* (21 gr) after prior regeneration to various flagellar lengths. Open circles, no cycloheximide; closed circles, addition of cycloheximide at time of amputation; squares, addition of cycloheximide following 40 min of regeneration (arrow) and re-amputation; circles with dots, addition of cycloheximide after 120 min of regeneration (arrow) and re-amputation.

the effect of varying concentrations of colchicine on flagellar regeneration. At 0.1 mg/ml, normal regeneration occurs; at 0.4 mg/ml, there is partial regeneration; and at 1.0 mg/ml, flagellar regeneration is completely inhibited as long as colchicine is present. These results are shown more clearly in Fig. 16 in which the complete kinetics of regeneration in the presence of varying amounts of colchicine are described. It can be seen that under conditions of partial inhibition (0.4 mg/ml) the rate of elongation is decreased from the very beginning of regeneration. This is in contrast to the partial inhibition observed with cycloheximide (Fig. 12) where regeneration kinetics are normal until the time of inhibition at half-length.

Since cycloheximide will partially inhibit flagellar formation by inhibiting protein synthesis, it was necessary to determine whether colchicine had a similar effect. In Fig. 17, it can be seen that a concentration of 1 mg/ml colchicine has little or no effect on arginine-^{14}C incorporation into total *Chlamydomonas* TCA-precipitable protein. Therefore, the inhibition by colchicine of flagellar regeneration in *Chlamydomonas* occurs by a mechanism other than inhibition of protein synthesis.

The results also indicate that colchicine acts rapidly to inhibit flagellar regeneration. When colchicine is added to cells which have partially regenerated their flagella, inhibition of further elongation occurs almost immediately (Fig. 18). Once this inhibition has occurred, the flagellar length is maintained, and little or no shortening has been observed in the continued presence of the drug.[9]

EFFECT OF COLCHICINE ON REGENERATION WHEN ONE FLAGELLUM IS AMPUTATED: Regeneration studies made on long-zero cells in the presence of 1.0 mg/ml colchicine indicate the shortening process in the intact flagellum to continue normally; however, no regeneration occurs in the amputated flagellum. Fig. 19 shows the effect of colchicine on the two classes of long-zero regeneration. The intact flagellum is found to shorten either completely or partially as in the controls (Figs. 4 and 5) and, in the case of partial shortening, to maintain this intermediate length in colchicine.

THE REVERSIBILITY OF COLCHICINE INHI-

[9] This is in contrast to the effect of colchicine on the mitotic apparatus and other cytoplasmic microtubules in which the equilibrium between microtubules and microtubule subunits can be shifted towards the subunits by treatment with colchicine (see references 9, 10, 35). The differential effects of colchicine and other antimitotic agents, high and low temperature, and enzymatic digestion on cytoplasmic and ciliary microtubules have recently been treated in reports by L. G. Tilney and J. R. Gibbins (*Protoplasma*. 1968. **65**:167.) and O. Behnke and A. Forer (*J. Cell Sci.* 1967 **2**:169.).

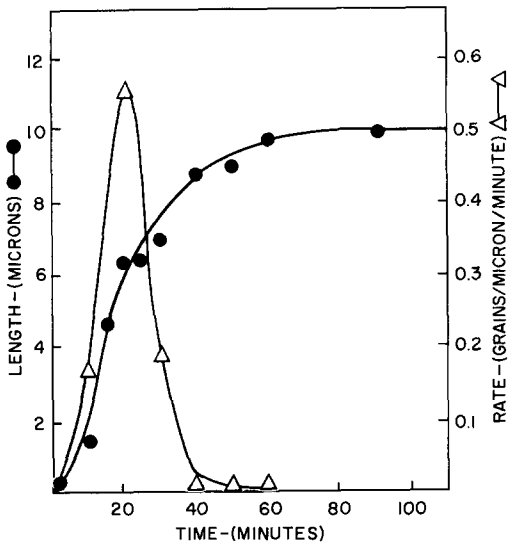

FIGURE 14 Rate of arginine-^{3}H incorporation into regenerating *Chlamydomonas* (Arg 1) flagella. Closed circles, flagellar regeneration kinetics from the same population used for the rate study; triangles, rate of incorporation of arginine-^{3}H (110 μc/ml, 382 mc/mmole) into flagellar protein. See text for procedure.

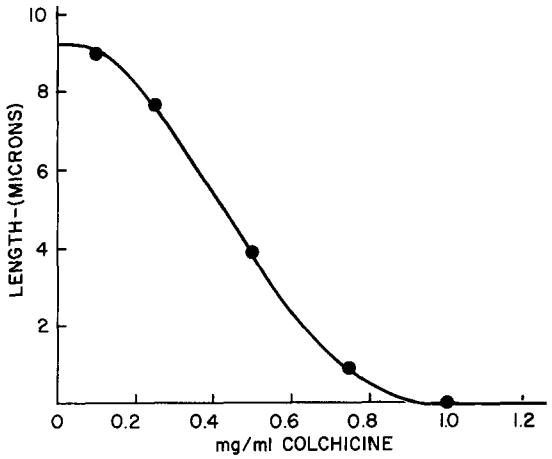

FIGURE 15 Effect of varying concentrations of colchicine on flagellar regeneration in *Chlamydomonas* (21 gr). Colchicine added at time of amputation and amount of elongation assessed after 80 min.

BITION: The inhibition of flagellar regeneration by colchicine can be reversed by placing the organisms in fresh medium (Fig. 20). A lag always occurs in regeneration after the removal of colchicine; other experiments have shown that this lag becomes longer as the period of colchicine inhibition is increased. The kinetics of recovery after colchicine inhibition are somewhat slower than the kinetics of normal regeneration, although the flagella eventually reach the same length as the controls.

Effect of Sequential Addition of Colchicine and Cycloheximide on Flagellar Regeneration in Wild-Type Cells

It was shown that colchicine completely blocks flagellar regeneration without affecting protein synthesis, while cycloheximide inhibits protein synthesis and permits about one-half of the flagellum to form. It was of interest to determine whether cells amputated in the presence of colchicine could continue to produce flagellar precursors. If this were the case, removal of colchicine and simultaneous addition of cycloheximide should cause the flagella to recover and elongate farther than if cycloheximide were added immediately following amputation. Fig. 21 shows that this situation does occur. One group of cells was placed in colchicine and deflagellated immediately. In a portion of the cells, colchicine was washed out in the presence of cycloheximide within 1 min of deflagellation; these cells were then replaced in cycloheximide-containing medium. The flagella elongated to a length of about 4 μ. Another portion of the deflagellated cells was left in colchicine for 47 min. The colchicine was then washed out in the presence of cycloheximide, and the cells were replaced in cycloheximide. In this situation, the flagella elongated to about 6 μ. It is apparent that the amputated cells which were left in colchicine for 47 min accumulated additional precursors which could be utilized for flagellar elongation.

The Growth Zone of Chlamydomonas Flagella

Earlier radioautographic studies (25) on the flagellate *Ochromonas* suggested that elongation occurred by the assembly of precursors at the distal end of the regenerating flagellum ("tip growth zone"). The arginine-requiring mutant of *Chlamydomonas* presents favorable material for the verification of this result since the cells incorporate arginine into their proteins very rapidly. It is therefore possible to pulse-label regenerating cells for short periods of time and still obtain sufficient isotope in the flagellar protein for radioautographic analysis. In addition, the results

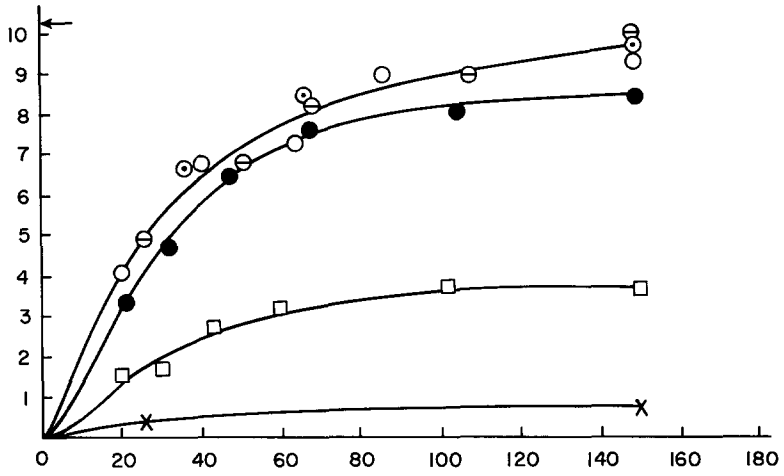

FIGURE 16 Kinetics of flagellar regeneration in varying concentrations of colchicine. Colchicine added at time of amputation. Open circles, control; circles with bars, 0.08 mg/ml; circles with dots, 0.15 mg/ml; closed circles, 0.4 mg/ml; squares, 0.7 mg/ml; crosses, 1.0 mg/ml. With this batch of colchicine, slightly more than 1.0 mg/ml (1.5 mg/ml) was needed for complete inhibition of regeneration (see footnote 8).

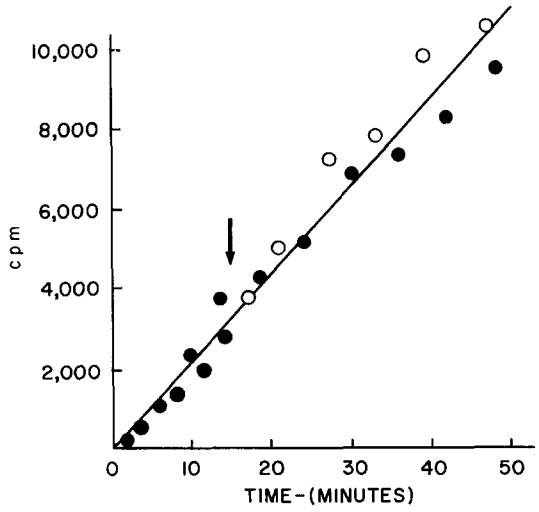

FIGURE 17 Effect of colchicine on arginine incorporation into TCA-precipitable protein of *Chlamydomonas* (Arg 1). Arginine-^{14}C (2.5 μc/ml, 172 mc/mmole) added at zero time and colchicine (1.0 mg/ml) at 15 min (arrow). Closed circles, control; open circles, colchicine.

from studies on the rate of incorporation of arginine into elongating flagella (Fig. 14) indicated at about what time during regeneration the greatest rate of arginine incorporation into the flagella occurred.

A population of arginine-requiring cells was deflagellated and divided into two parts. To one part, arginine-^{3}H was added as soon after amputation as possible (about 2–3 min); to the second part, the isotope was added after the flagella had regenerated for 15 min (flagellar length, about 4 μ). Samples from both cultures were prepared for radioautography at 5-min intervals until regeneration was complete. The results in Table I are from radioautographs assessed after the first 10 min in isotope. It can be seen that arginine-^{3}H added at the time of amputation results in a tendency for a distal localization of silver grains. This distal localization of silver grains is amplified when the isotope is added at 15 min postamputation and the cells are radioautographed 10 min later. In the latter case, about 70% of the total label in the flagellar protein is localized over the distal half of the flagellum.

Electron Microscopy of Flagellogenesis in Chlamydomonas

Thin sections of *Chlamydomonas* were prepared for electron microscopy at various times after flagellar amputation, in an effort to substantiate the tip growth zone results described above. Some representative electron micrographs are shown in Figs. 22 and 23. The results indicate that: (*a*) the flagella are detached just above the transitional region of the flagellar shaft and below the outer limit of the cell wall; (*b*) the flagellar membrane heals over almost immediately after ampu-

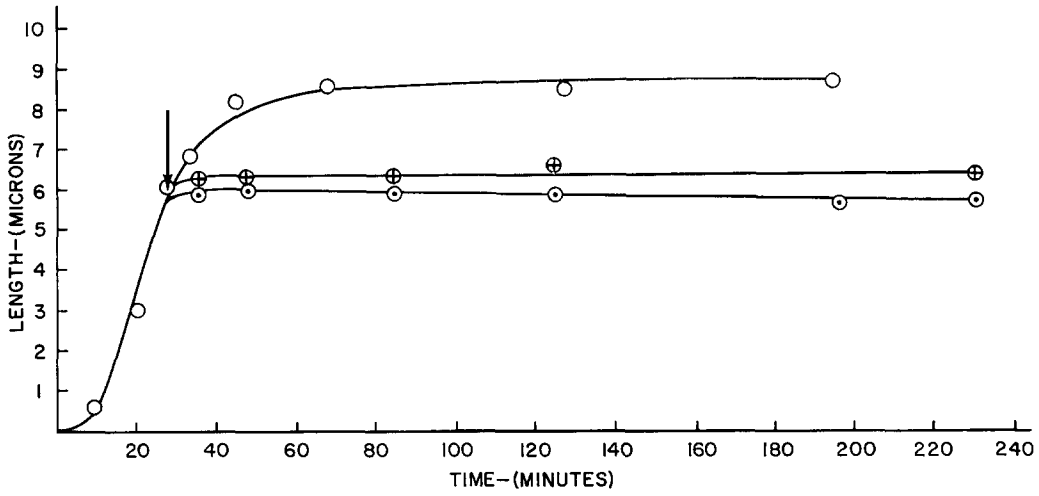

FIGURE 18 Rapidity of colchicine inhibition of flagellar elongation in *Chlamydomonas* (21 gr). Circles with crosses, 2.5 mg/ml colchicine; circles with dots, 10.0 mg/ml colchicine. The arrow indicates the time of colchicine addition. Following inhibition of elongation, there is no observable shortening during a 4-hr time period in the continued presence of an excess of colchicine.

tation; and (*c*) the flagellar membrane may be distended at the tip of the regenerating flagellum. Many of our electron micrographs showing longitudinal sections of the flagella in early stages of regeneration indicate the localization of some disorganized material in the tip and shaft (particularly between the axoneme and flagellar membrane) of the organelle (Fig. 22). Micrographs taken at later stages of regeneration indicate that the tubules apparently extend to the tip of the elongating flagellum (Fig. 23).

DISCUSSION

An earlier investigation of flagellar regeneration in the flagellates *Euglena*, *Astasia*, and *Ochromonas* (25) made it apparent that amino acids were incorporated into the proteins of these organisms relatively slowly. Because of this, pulse-labeling of the flagellar proteins was difficult to carry out during the brief regeneration periods. The motility of the flagellates also hindered studies of regeneration kinetics in single cells. So as to avoid these experimental problems, the biflagellate *Chlamydomonas* was used for further studies of flagellar regeneration. The problems concerned with pulse-labeling of the flagellar proteins were overcome by use of the arginine-requiring mutant (arginine-^{14}C incorporation into TCA-precipitable proteins is rapid and linear after about 2 min),

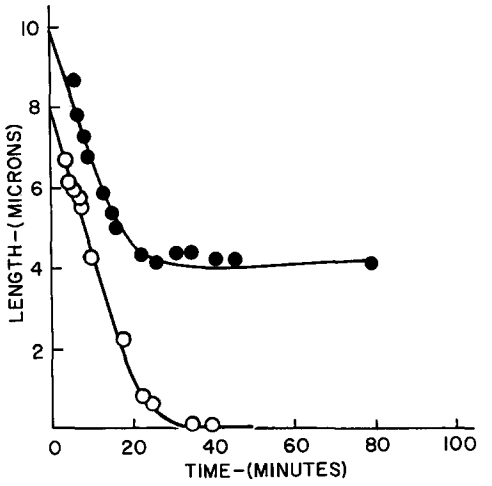

FIGURE 19 Effect of colchicine (1.0 mg/ml) on flagellar regeneration in single *Chlamydomonas* (pf 16) following amputation of one flagellum. Two different experiments showing partial and complete shortening are plotted on the same graph. Closed circles, partial shortening of the intact flagellum; open circles, complete shortening of the intact flagellum. No regeneration occurs in the amputated flagella.

and single cell studies were facilitated by the availability of various paralyzed mutants. Also, the biflagellate nature of the organism permitted studies on regeneration after removal of only one of the two flagella. *Chlamydomonas* has the addi-

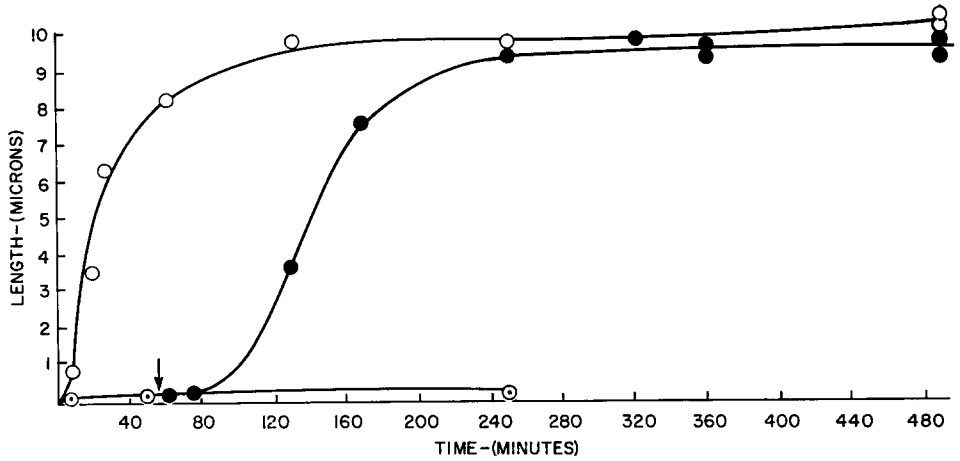

FIGURE 20 Reversibility of colchicine inhibition of flagellar regeneration in *Chlamydomonas* (21 gr). Open circles, no colchicine; circles with dots, cells in continued presence of colchicine (1.5 mg/ml) from time of amputation; closed circles, sample of colchicine-blocked cells placed in fresh medium at 50 min (arrow).

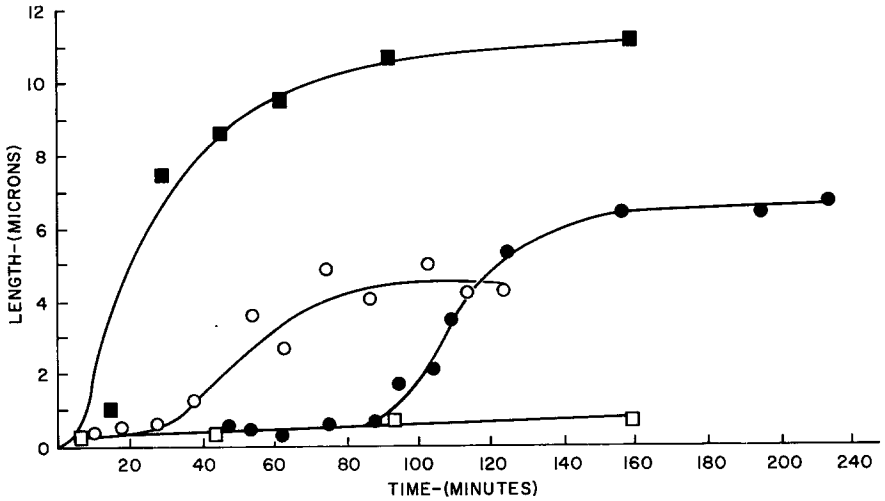

FIGURE 21 Effect of sequential additions of colchicine and cycloheximide on flagellar regeneration in *Chlamydomonas* (21 gr). Closed squares, no inhibitors; open squares, deflagellated cells in colchicine (1.5 mg/ml) for duration of experiment; open circles, colchicine (1.5 mg/ml) added just before amputation, washed out in the presence of cycloheximide within 1 min after amputation, and cells placed in 30 μg/ml cycloheximide; closed circles, colchicine added just before amputation, washed out in the presence of cycloheximide after 47 min, and the cells placed in cycloheximide.

tional advantage of being easily grown in large quantities as synchronously dividing cultures (1, 12); this enabled the isolation of large amounts of flagella which could then be fractionated into component parts.[7] In addition, literature on the ultrastructure of the organism and its flagella is extensive (see references 22, 23).

Normal Kinetics of Flagellar Regeneration

The homogeneity of cell size and flagellar length in synchronized cells, the fact that more than 98% of the cells regenerate flagella after amputation, and the accuracy of the flagellar measurement methods made possible the reproducible determi-

nation of regeneration kinetics in single cells and populations.

The pattern of flagellar regeneration kinetics in *Chlamydomonas* is similar to that described previously for other flagellates (7, 25, 34). The major difference is the apparent lack of a lag phase prior to flagellar elongation. Otherwise, the cells show the typical rapid and deceleratory elongation kinetics by which the majority of the flagellum is formed in 70–90 min at an initial rate of about 0.4 μ/min at 25°C. Similar regeneration kinetics in *Chlamydomonas* were first described by Child (5), and more recently by Randall et al. (19). Following the period when the majority of elongation has occurred (0–90 min), there is a phase of very slow elongation over a period of several hours; this has been observed in both single cells and

TABLE I

Growth Zone of Chlamydomonas (Arg 1) Flagella

See text for procedure and discussion.

Time of isotope* addition	Ratio of proximal and distal to total grains/flagellum‡	
	Proximal/Total	Distal/Total
At time of amputation	0.43	0.57
15 min after amputation	0.28	0.72

* Arginine-^{3}H, 110 μc/ml, 382 mc/mmole.

‡ At least 50 flagella counted.

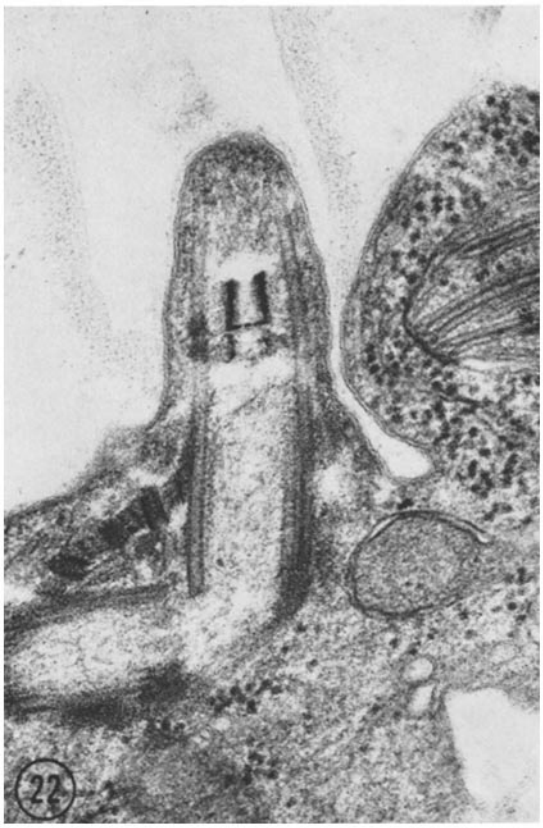

FIGURE 22 A longitudinal section of a flagellar bud 1.5 min after flagellar amputation. See Discussion. × 70,000.

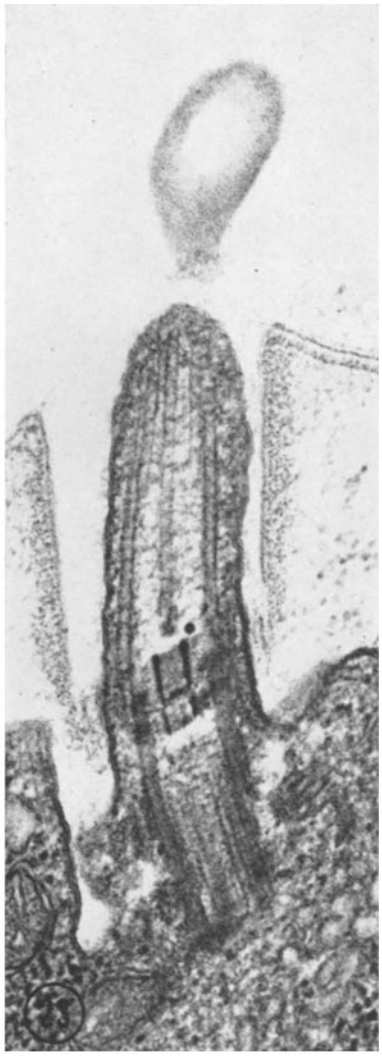

FIGURE 23 A longitudinal section of a flagellum 4.0 min following flagellar amputation. See Discussion. × 70,000.

populations. The slow elongation phase of the kinetics has also been shown by Tamm (34), who accurately measured the kinetics of flagellum formation in single *Peranema* at known stages of the division cycle. He demonstrated that the slow elongation phase continued throughout interphase until the time of cytokinesis. The significance of this slow growth phase is not known and will require further work for elucidation.

Effect of Cycloheximide on Flagellar Regeneration

On addition to the culture, cycloheximide will immediately inhibit more than 98% of the incorporation of amino acids into the TCA-precipitable protein of *Chlamydomonas*. When it is added to wild-type cells at the time of amputation, one-half of each flagellum forms by kinetics similar to those in control cells. This result suggests that the cells have sufficient flagellar precursor to form part of the flagellum even in the absence of protein synthesis. However, it is possible that cycloheximide is inhibiting the formation of some *nonflagellar* protein which is necessary for flagellar elongation, and it is the inhibition of the synthesis of this protein which permits only partial flagellar regeneration. The results of the experiment shown in Fig. 13 argue against the latter possibility. They indicate that the regenerating flagella are actually utilizing the limiting protein as they elongate; this strongly suggests that the protein is part of the flagellar structure rather than an accessory protein required for elongation. In the remainder of this discussion, the protein will tentatively be called flagellar precursor.

The amount of precursor is found to be highest at the time of amputation; it then drops as the flagella near mid-length, and almost regains its original level as the flagella approach their normal length. These data indicate that the cell during the middle of elongation would be required to synthesize more precursor to (*a*) permit the completion of elongation and (*b*) explain the fact that cells which have almost completely regenerated their flagella again have sufficient precursor to permit one-half flagellar regeneration in cycloheximide. Studies performed using pulse labeling with arginine-^{3}H tend to support this possibility (Fig. 14). These results show the greatest *rate* of arginine incorporation into the protein of flagella to occur at about the time the flagella reach mid-length. This could mean either (*a*) that the greatest rate of flagellar protein synthesis is occurring at the time the flagella reach mid-length, or (*b*) that the specific activity of the flagellar precursor is highest at mid-length because at this time any preexisting unlabeled precursor is at a low level (see Fig. 13). These possibilities are being tested by actually measuring the amount of flagellar tubular precursors at different times during regeneration.

The cycloheximide results also indicate that inhibitor applied at the time of amputation permits varying amounts of elongation in different strains of *Chlamydomonas*. Wild-type cells will regenerate flagella to about one-half length in cycloheximide while the paralyzed mutant (pf 16) will regenerate less than one-third of the flagellum under similar conditions. This result suggests that the different strains may have varying amounts of flagellar precursor proteins.

Regeneration Kinetics Following Amputation of One Flagellum

In most flagellates, the flagella shorten and disappear prior to cytokinesis; this phenomenon has been thoroughly reviewed and documented by Tamm (34). In this system, however, flagellar shortening can be made to occur prematurely by amputating only one of the two flagella. The cause of this phenomenon is not clear, although the results enumerated below have been accumulated. (*1*) Shortening occurs abruptly by linear kinetics at a rate of about 0.4 μ/min at 25°C and is complete in about 30 min. The simultaneous elongation of the amputated flagella occurs by decelerator kinetics at an initial rate almost similar to the rate of shortening. Tamm (34) described a linear pattern of flagellar shortening in precytokinesis *Peranema*, although the rate of shortening in this organism was about 1.2 μ/min. Whether the kinetics of amputation-induced shortening and of the normal precytokinesis shortening in *Chlamydomonas* are similar is not yet known, although Randall et al. (19) report that precytokinesis shortening in *Chlamydomonas* takes about 30 min. Johnson and Porter, in their ultrastructural study of division in *Chlamydomonas* (11), observed that flagella which are shortening prior to cytokinesis show disorganized, particulate material at their tips, suggesting that the flagellum is degenerating by disaggregation of tubules at the distal end. In the present work, phase and dark-

field microscope observations indicate that the intact flagellum (or axoneme) is not being pulled into the cell and that flagellar material is not being cast off into the medium. An ultrastructural study will be necessary to determine if there is tip degeneration during amputation-induced shortening. (*2*) Flagellar shortening can occur in the presence of cycloheximide. This result indicates that the initiation, rate, and completion of shortening do not depend on new protein synthesis. (*3*) The simultaneous shortening and elongation processes in opposite flagella seem to be related since the pattern of regeneration in the amputated flagellum is always dependent on the degree of shortening of the intact flagellum. (*4*) The cycloheximide inhibition results, along with those in (*3*) above, suggest that precursors from the shortening flagellum may be used for the formation of the elongating flagellum. Thus, when both flagella of the paralyzed mutant are amputated in the presence of cycloheximide, there is a total flagellar elongation of about 2–4 μ (1–2 μ/flagellum). Conversely, when only one flagellum is amputated in cycloheximide, the pattern of regeneration is similar to that in the long-zero controls in which there can be a total flagellum formation of 14–18 μ. So as to determine if cycloheximide-inhibited long-zero cells have sufficient precursor to support all of the elongation observed in the absence of protein synthesis, the amount of flagellar material "resorbed" in long-zero cells was added to the amount of precursor pool in the cell (as determined by the amount of elongation when *both* flagella are amputated in cycloheximide). This quantity is found to be relatively close to the total elongation of the intact and amputated flagella in cycloheximide-inhibited long-zero cells. This suggests (but does not prove) that protein from the shortening flagellum can enter a common pool of precursors available for flagellar formation and that this pool is sufficient to support all of the elongation in cycloheximide-inhibited long-zero cells.

Effect of Colchicine on Flagellar Regeneration

Stubblefield and Brinkley were able to show that the elongation of ciliary buds in cultured fibroblasts is inhibited by Colcemid (33). More recently, it has been shown that the elongation of cilia in cilia-regenerating systems in *Tetrahymena* is also inhibited by Colcemid and colchicine (26). In addition, the latter report demonstrated that the inhibitory concentration of colchicine had no effect on either RNA or protein synthesis. The present report shows that colchicine will completely (and reversibly) inhibit flagellar regeneration in *Chlamydomonas*. As in *Tetrahymena*, the colchicine has no effect on cell protein synthesis. Colchicine acts rapidly to inhibit regeneration; when it is added after the flagella are partially regenerated, inhibition can be observed to occur almost immediately. Once flagellar elongation is inhibited, there is no apparent decrease in flagellar length in the presence of colchicine over a period of several hours; this result suggests the relative stability of the flagellar tubules.[7, 9]

While cycloheximide permits partial flagellar elongation in the absence of protein synthesis, colchicine completely inhibits elongation during normal protein synthesis. This result would be expected if colchicine is acting by binding to the flagellar microtubular precursors available for partial regeneration under conditions of cycloheximide inhibition. This suggestion is supported by work from Taylor's laboratory (3, 4, 29, 35) showing that the mechanism of action of colchicine in vivo is probably due to its great binding affinity for the subunits of microtubules. In order to provide more definitive evidence that colchicine acts as an inhibitor of regeneration by binding microtubular precursors, studies are currently in progress utilizing colchicine-^{3}H binding for assaying the amount of tubular subunits present in the cells at various times during regeneration.

EFFECT OF COLCHICINE ON LONG-ZERO CELLS: When colchicine is applied to cells in which only one flagellum is amputated, elongation is completely inhibited while the shortening process continues normally. As in control long-zero cells, the flagella shorten either totally or partially, and in the latter case they maintain the length to which they shorten. Therefore, the initiation and rate of shortening are not influenced by the absence of simultaneous elongation, and colchicine can be used to effectively separate the elongation and shortening processes under conditions of normal protein synthesis.

EFFECT OF COLCHICINE AND CYCLOHEXIMIDE ON FLAGELLAR REGENERATION: Colchicine has been found to reversibly inhibit flagellar regeneration without affecting protein synthesis in *Chlamydomonas*. Therefore, experi-

ments were performed to determine whether colchicine-blocked amputated cells would continue to synthesize flagellar precursors to be used for elongation in the absence of protein synthesis. When amputated cells were blocked with colchicine for 1 min and 47 min and then washed free of colchicine and placed in cycloheximide, elongation was greater in the cells which had been blocked for the longer time. These results suggest that cells in which regeneration is inhibited by colchicine continue to make flagellar precursors and that deflagellation specifically stimulates flagellar protein synthesis.[10] Investigations are currently in progress to determine if there is an increase in colchicine-binding protein in colchicine-blocked deflagellated cells. Conceivably, conditions could be established in colchicine-blocked deflagellated cells which permit the formation of a complete flagellum in the presence of cycloheximide.

Growth Zone of Chlamydomonas Flagella

The results of earlier studies on flagellar regeneration (25) indicated that *Ochromonas* flagella were elongating by the addition of precursors to the distal end of the organelle (tip growth). Similar results were obtained in the present study by use of the arginine-requiring mutant of *Chlamydomonas*. Cells with partially elongated flagella were given a short pulse of arginine-^{3}H, and radioautography showed that a greater percentage of the total flagellar silver grains was located on the distal portions of the flagella than in control cells in which the isotope was present from the beginning of elongation. A discussion of the various problems encountered with this interpretation can be found in the original report (25). As in the *Ochromonas* growth zone work, the present study still does not define the part of the flagellum elongating at the tip, *i.e.* membrane or axoneme. This problem is currently being investigated by (*a*) studying the membrane growth zone using labeled lipid precursors and fluorescent antibodies made for isolated *Chlamydomonas* flagellar membranes,[11] and (*b*) carrying out similar growth zone experiments on flagella whose membranes have been removed prior to radioautography.

Electron Microscopy of Elongating Chlamydomonas Flagella

The studies on the flagellar growth zone suggested that flagellar axonemes may be elongating by the assembly of precursors at the distal end of the shaft (tip growth). So as to illuminate this point further, electron microscopic observations on thin sections of *Chlamydomonas* at various intervals following flagellar amputation have been made. Many of the sections of flagella observed early in the regeneration show disorganized particulate material in the tips of the flagella. These observations are somewhat similar to those reported by others who have done ultrastructural studies of flagello- and ciliogenesis (2, 6, 20, 30–32) and support the hypothesis of axonemal tip assembly. However, observations on *Chlamydomonas* flagella at later stages in regeneration show that the tubules may extend to the tip of the flagellum. Thus, the electron microscopic results at this time are still somewhat equivocal with respect to the growth zone of elongating flagella. The results do indicate that the flagellar membrane is reformed immediately following mechanical amputation, that the transitional region of the flagellum (see reference 22) remains intact during amputation, that the flagella are detached just above the transitional region of the shaft, and that the flagellar membrane may be "ballooned out" at the tip. The cytoplasmic particles labeled "axonemal precursors" by Steinman in his report on ciliogenesis in salamander trachea (32) have not been observed. However, since these results indicate that flagellar precursors may be accumulated during colchicine inhibition of flagellar regeneration, the colchicine-blocked system may aid in the observations of such particles if they are indeed axonemal precursors. The ultrastructural investigations of normal and colchicine-blocked regeneration in the *Chlamydomonas* flagella-regenerating and *Tetrahymena* cilia-regenerating systems (26) are therefore still in progress.

Control Processes in Flagellar Elongation

It is premature to hypothesize about control mechanisms for flagellar growth although the

[10] Other results indicate that amino acid incorporation into total cell protein of regenerating cells is identical with that into total cell protein of nonregenerating cells, while the specific activity of flagellar axonemes from regenerating cells is at least ten times that of nonregenerating flagellar axonemes. This result also indicates that amputation is initiating the synthesis of flagellar proteins. See footnote 7.

[11] Witman, G. Unpublished results.

results in this report do allow for a description of some of the events underlying the process of flagellar elongation. Precursor which is available for flagellar formation prior to amputation is apparently depleted during the first part of growth. Enough precursor is then synthesized to permit completion of elongation and to bring the precursor pool back to the level found in the cell prior to amputation (Fig. 13). When additional precursor is accumulated in amputated cells during a colchicine block, the subsequent *rate* of regeneration in cycloheximide does not increase even though there is *more* elongation than if the cells were not previously blocked with colchicine (Fig. 21). Taken together, these results support the hypothesis that, under normal conditions of flagellar growth, neither the rate nor the amount of regeneration is limited by the precursor supply and that the assembly process is probably rate limiting. It is not known why the rate of flagellar elongation slows after 70–90 min of regeneration when the results show an apparently sufficient precursor pool in the cell. These results may be explained by the tip growth zone. It could be hypothesized that the movement of precursor to the tip assembly site is either diffusion-limited or that some barrier is formed during the end of regeneration which impedes the movement of precursor to the growing tip; in either case, it would take a longer time to accumulate the amount of precursor necessary for assembly. The slow elongation which does occur after the majority of regeneration is completed might result from the slow accumulation of precursor at the assembly site.

The studies presented in this report have helped elucidate the processes of flagellar elongation and shortening in *Chlamydomonas*. However, many other aspects of this system need to be examined before questions concerning the initiation, rate, and termination of flagellar growth can be answered. A few of these are: the quantitative determination of the precursors of flagellar components in the cell at various times during regeneration; the rates of net synthesis and turnover of the different parts of the flagellum; the mechanisms of assembly of flagellar microtubules and membranes; the biochemical processes underlying the simultaneous elongation and shortening of the two flagella; the genetic control of flagellar synthesis and assembly. The various advantages of the *Chlamydomonas* flagella-regenerating system provide a means of attacking these problems.

We should like to thank Mrs. B. Rudolph for technical assistance, Dr. J. G. Gall for use of the electron microscope, and Joanna Olmsted for assistance in editing the manuscript. This work is supported by United States Public Health Service grant GM-14642 to J. L. Rosenbaum, USPHS postdoctoral fellowship HD-35631 to D. Ringo, and a National Science Foundation predoctoral fellowship to J. Moulder.

Received for publication 1 November 1968, and in revised form 26 December 1968.

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