FLAGELLAR REGENERATION IN PROTOZOAN FLAGELLATES

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ABSTRACT

The flagella of populations of three protozoan species (*Ochromonas*, *Euglena*, and *Astasia*) were amputated and allowed to regenerate. The kinetics of regeneration in all species were characterized by a lag phase during which there was no apparent flagellar elongation; this phase was followed by elongation at a rate which constantly decelerated as the original length was regained. Inhibition by cycloheximide applied at the time of flagellar amputation showed that flagellar regeneration was dependent upon de novo protein synthesis. This was supported by evidence showing that a greater amount of leucine was incorporated into the proteins of regenerating than nonregenerating flagella. The degree of inhibition of flagellar elongation observed with cycloheximide depended on how soon after flagellar amputation it was applied: when applied to cells immediately following amputation, elongation was almost completely inhibited, but its application at various times thereafter permitted considerable elongation to occur prior to complete inhibition of flagellar elongation. Hence, a sufficient number of precursors were synthesized and accumulated prior to addition of cycloheximide so that their assembly (elongation) could occur for a time under conditions in which protein synthesis had been inhibited. Evidence that the site of this assembly may be at the tip of the elongating flagellum was obtained from radioautographic studies in which the flagella of *Ochromonas* were permitted to regenerate part way in the absence of labeled leucine and to complete their regeneration in the presence of the isotope. Possible mechanisms which may be operating to control flagellar regeneration are discussed in light of these and other observations.

INTRODUCTION

By studying the development of flagella we may uncover mechanisms and control processes which are applicable to the development of cellular organelles in general. Cells which are regenerating flagella provide systems of developing organelles which have several advantages, the major advantage being the ease with which flagella may be amputated from large numbers of cells and the apparent synchrony of regeneration which follows (5, 9, 25).

The chemistry and ultrastructure of flagella and cilia are becoming better known (for reviews, see references 11, 13, 22, 23, 29, 32). Most of the information concerning development of flagella has come from electron microscopic studies of spermatogenesis in various vertebrates (2, 10, 20, 33) and invertebrates (12, 21, 24, 33) in addition to a few reports both on cilia formation in vertebrate tissues (34, 35), and on flagellar morphogenesis in slime molds (31) and amebo-flagellates

(8, 29, 30). Most of these studies have been concerned with the more complex problem of the origin of the flagellar basal body (kinetosome, centriole) rather than with the mechanisms of elongation of the shaft of the organelle. Nevertheless, they have served to emphasize our ignorance of the nature of the processes controlling the synthesis of ciliary and flagellar proteins and other components, as well as the subsequent assembly of these components into the precise and characteristic structure of the motile organelle. We are interested in the cellular localization of the sites(s) of synthesis and assembly, the mechanisms operating to control the size of the organelle, and the possible role of the basal body in the process of regeneration.

Flagellar regeneration in *Chlamydomonas* has been studied by Lewin (18) and by Hagen-Seyfferth (15), who induced regeneration by detaching the flagella with high temperature, low or high pH or ethanol. Grebecki and Kuznicki (14) were able to strip the cilia from *Paramecium* with chloral hydrate and observe ciliary regeneration, and Child has designed a system for studying cilia regeneration in the ciliated protozoan *Tetrahymena pyriformis* (6). Dubnau (9), using the chrysomonad protozoan *Ochromonas*, detached the flagella from populations of cells by mechanical agitation and then analyzed the kinetics of regeneration and the effect of inhibitors of energy metabolism, amino acid, purine, and pyrimidine analogues on the regeneration kinetics.

We have extended Dubnau's studies and have developed systems of regenerating flagella in the flagellated protozoans *Euglena*, *Astasia*, and *Ochromonas* (25, 26). These systems have several advantages. First, large numbers of cells can be grown axenically in defined medium under controlled conditions. Second, the peninsular location of the flagellum facilitates its amputation by relatively gentle chemical or mechanical procedures which do not disrupt the organisms. The kinetics of the subsequent flagellar regeneration can then be determined by direct measurement of flagellar lengths. The location of the flagellum also facilitates the design of radioautographic and cytochemical studies, as well as other experimental manipulations (38), which can be carried out without interference from the general cytoplasmic milieu. Third, methods are available for isolating flagella and parts thereof (13) at various times during the regeneration in quantities sufficient for biochemical or isotopic analysis.

MATERIALS AND METHODS

Culture of Organisms

All of the flagellates used in this investigation were maintained axenically at room temperature (23–24°C) in 16 × 125 mm screw-cap tubes containing 5 ml of medium. These cultures were used to inoculate larger volumes of medium in Roux or Erlenmeyer flasks.

(*a*) *Astasia longa* was grown in Cramer-Myers' medium (7) at pH 6.8 with 0.02 M sodium acetate as a carbon source. The cultures were transferred every 5–6 days, and the entire contents of a 5–6 day, 5 ml culture were used to inoculate 150 ml of medium in a Roux flask.

(*b*) *Euglena gracilis* (Z) was grown in constant light or dark in the same medium used for *Astasia*, and the cultures were transferred every 4–5 days. ½ ml of a 4 day culture was used to inoculate 100 ml of medium in a Roux flask. When nondividing cultures of *Euglena* were required, 100 ml of a 3 day, Roux flask culture were harvested and washed three times by gentle centrifugation in the resting medium described by Stern et al. (36) at pH 6.8, and then suspended in 300 ml of this medium and shaken gently on a reciprocal shaker. All the operations were carried out sterilely. After 3 days in this medium in constant light there was very little division, and the cells remained viable for at least 20 days.

(*c*) *Ochromonas danica* was grown in constant light in Aaronson and Baker's medium (1). The cultures were transferred every 4 days, and 0.5 ml of a 4 day culture was used to inoculate 100 ml of medium in a 500 ml Erlenmeyer flask. When nondividing cultures of *Ochromonas* were required, 100 ml of a 2 day, Erlenmeyer flask culture were harvested and washed three times under sterile conditions with the defined medium lacking biotin, and the cells suspended in 200 ml of this medium in a 1000 ml Erlenmeyer flask. After 3 days in constant light there was very little cell division and the cells were still viable.

Flagellar Amputation Procedures

Euglena and Astasia: (*a*) 25 ml of a 4 day, Roux flask culture were concentrated to 5 ml by gentle centrifugation in a clinical centrifuge, and this volume was deflagellated in a 20 × 150 mm fluted test tube[1] by agitation with a vortex mixer[2] running at top speed for 45 sec.

(*b*) 75 ml of the culture were concentrated to

[1] Available from Kontes Glass Co., Vineland, N. J. Specification sketch No. F-1024-J.

[2] Variable Speed Super Mixer, Lab-Line Instruments, Inc., Melrose Park, Ill.

15 ml and the flagella amputated in the 50 ml fluted receptacle of the Virtis[3] homogenizer, running at 7–8000 rpm for 15–20 sec. Larger volumes could be deflagellated equally well in the larger receptacles available with this homogenizer.

(c) The pH of 100 ml of culture in Cramer-Myers' medium (7) at pH 6.8 was quickly lowered with 1 N acetic acid to pH 4.7 for 2–2.5 min and then raised to pH 6.8 with 1 N KOH. The culture was magnetically stirred throughout the procedure.

Ochromonas: The flagella of 3-day, Erlenmeyer flask cultures were detached with the vortex mixer and fluted tube as described above for *Euglena* and *Astasia* except that the agitation time was extended to 60 sec.

There was considerable flexibility in the amputation procedures described above, and they could easily be varied to accommodate larger or smaller volumes of cells, and the concentration of cells could be increased without affecting the efficiency of the procedures. Following flagellar amputation, the cells were either transferred directly to 125 ml Erlenmeyer flasks or were gently concentrated (clinical centrifuge) and resuspended in defined medium prior to transferral. All the regeneration experiments were carried out at 26.5°C in constant light in a gently shaking water bath.

Measurement of Flagellar Lengths

The kinetics of flagellar regeneration were routinely obtained by fixation of samples of cells in Lugol's iodine (6 g KI, 4 g I_2/100 ml water) at intervals following the amputation procedure and by measurement of flagellar lengths with an ocular micrometer. The mean flagellar length was determined for each time from a minimum of 100 measurements. Initial flagellar lengths were obtained from cells fixed prior to the amputation.

There were always some cells in both the regenerating and nonregenerating control samples which did not appear to have flagella, and these were included as zeroes in the determination of mean lengths. Several physiological and/or experimental factors may have contributed to this apparent lack of flagella by some cells. Many flagellates normally lose their flagella during cytokinesis (38), new ones being grown by the daughter cells. This problem was circumvented in certain experiments by use of nondividing organisms in resting media (see Materials and Methods). There was also a slight tendency for organisms with long flagella (*Euglena* and *Astasia*) to lose their flagella when Lugol's-fixed samples were placed on slides for length determinations, or for the flagella to be lying underneath the organisms.

[3] Virtis Co. Inc., Gardiner, N. Y.

Flagellar Isolation Procedure

The flagella could be isolated at different times during the regeneration period by use of a modification of Watson and Hopkins' (39) procedure for the isolation of *Tetrahymena* cilia. The method for isolating *Euglena* flagella is outlined here, although the procedure will also work with the other flagellates used in this study with minor modifications in centrifugal forces.

The *Euglena* were concentrated by low-speed centrifugation, washed once with 0.025 M sodium acetate (pH 7.0) at room temperature, and suspended in 5 ml of this solution in a 50 ml conical centrifuge tube. The cell concentration was not critical although we usually used 1.0–3.0 × 10^7 cells/ml. The suspension was then cooled to 4°C in an ice bath, and 25 ml of the solution used by Watson and Hopkins (39) (0.025 M sodium acetate, 12% ethanol v/v, 0.12% EDTA-2 Na w/v, pH 7.0) containing 7% sucrose w/v and 1 ml of 1 M $CaCl_2$ was added with vigorous stirring. After several minutes on ice with occasional stirring, or until microscopic observation revealed that most of the flagella were detached, the suspension was centrifuged at 500 *g* (IEC PR-2 centrifuge, swinging bucket head No. 269, International Equipment Co., Needham Heights, Mass.) for 10 min at 4°C to sediment most of the cell bodies. The upper ¾ of the supernatant, the portion containing most of the flagella, was removed and centrifuged at 3000 *g* in the SS-34 head of the Sorvall centrifuge for 10 min at 4°C to sediment the flagella. When shorter and lighter flagella from organisms in the early stages of regeneration were isolated, this centrifugal speed was increased to 7500 *g*. The flagellar pellet, which still contained some cell bodies, was resuspended in 2 ml of 7% sucrose by gentle swirling, was layered over 2 ml of 14% sucrose in a 12 ml conical centrifuge tube, and was centrifuged as described above at 500 *g* for 10 min. The supernatant and interphase, which contained most of the flagella, were relayered over 14% sucrose and centrifuged again at 500 *g*. The purified flagella could then be concentrated by centrifugation at 3000 *g* (7500 *g* for shorter flagella) in the Sorvall centrifuge. By this method, large quantities of pure flagella, which showed a minimum of breakage of swelling, could be isolated from organisms at different stages in the regeneration (see Fig. 1). The method could be scaled up or down as necessary. Flagellar counts obtained with a Petroff-Hauser counting chamber indicated that the isolation procedure was 50–60% efficient.

Radioautography

Samples of organisms were taken from the incubation flasks which contained 20 μc/ml L-leucine-^{3}H (5 c/mM New England Nuclear Corp., Boston, Mass.), washed three times with fresh medium by gentle

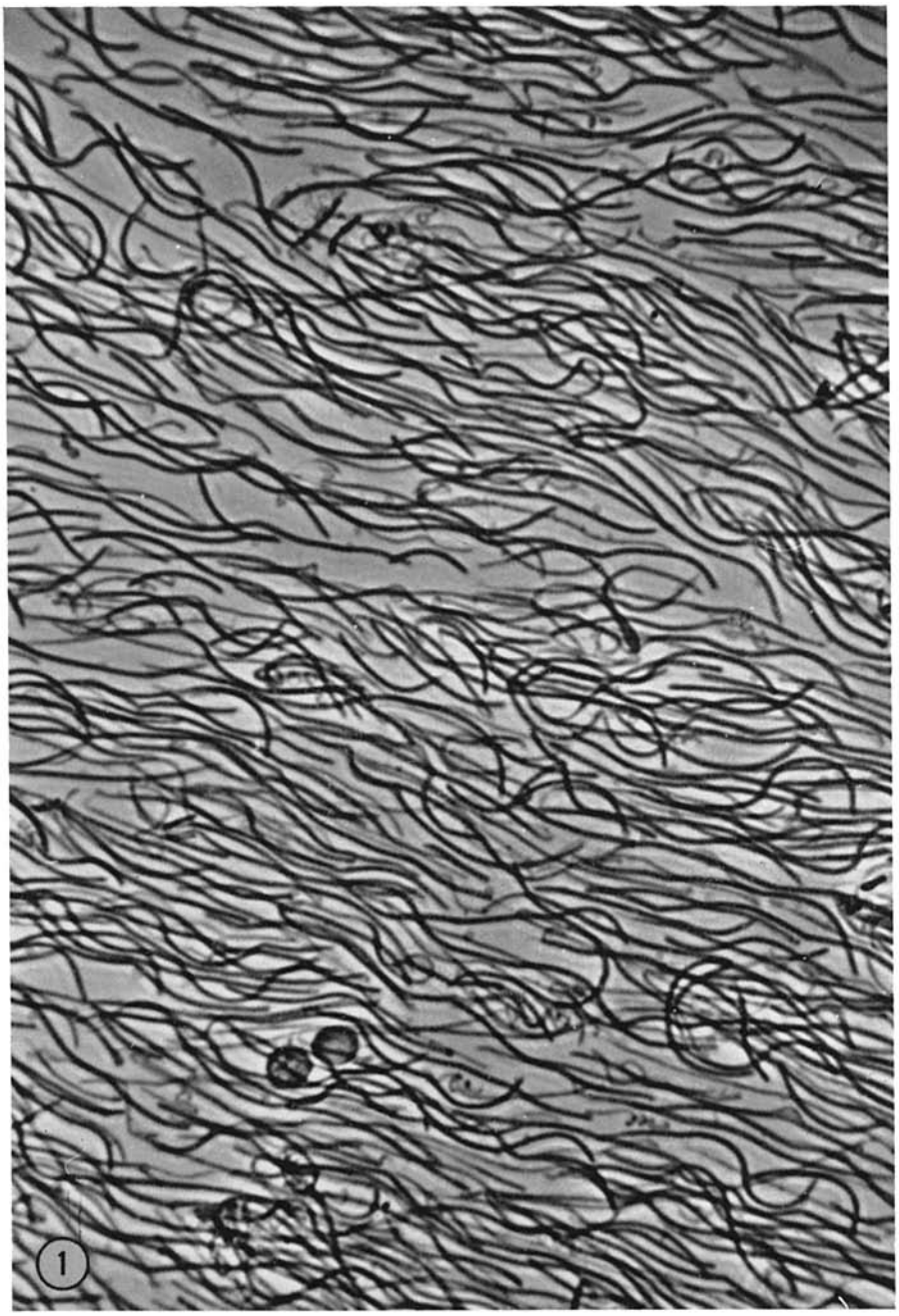

FIGURE 1 Isolated flagella (unfixed) of *Euglena gracilis* (Z). The round bodies in the lower left of the phase photomicrograph are "swollen" flagella, i.e. flagella whose axonemes have curled up within the flagellar membranes. × 1100.

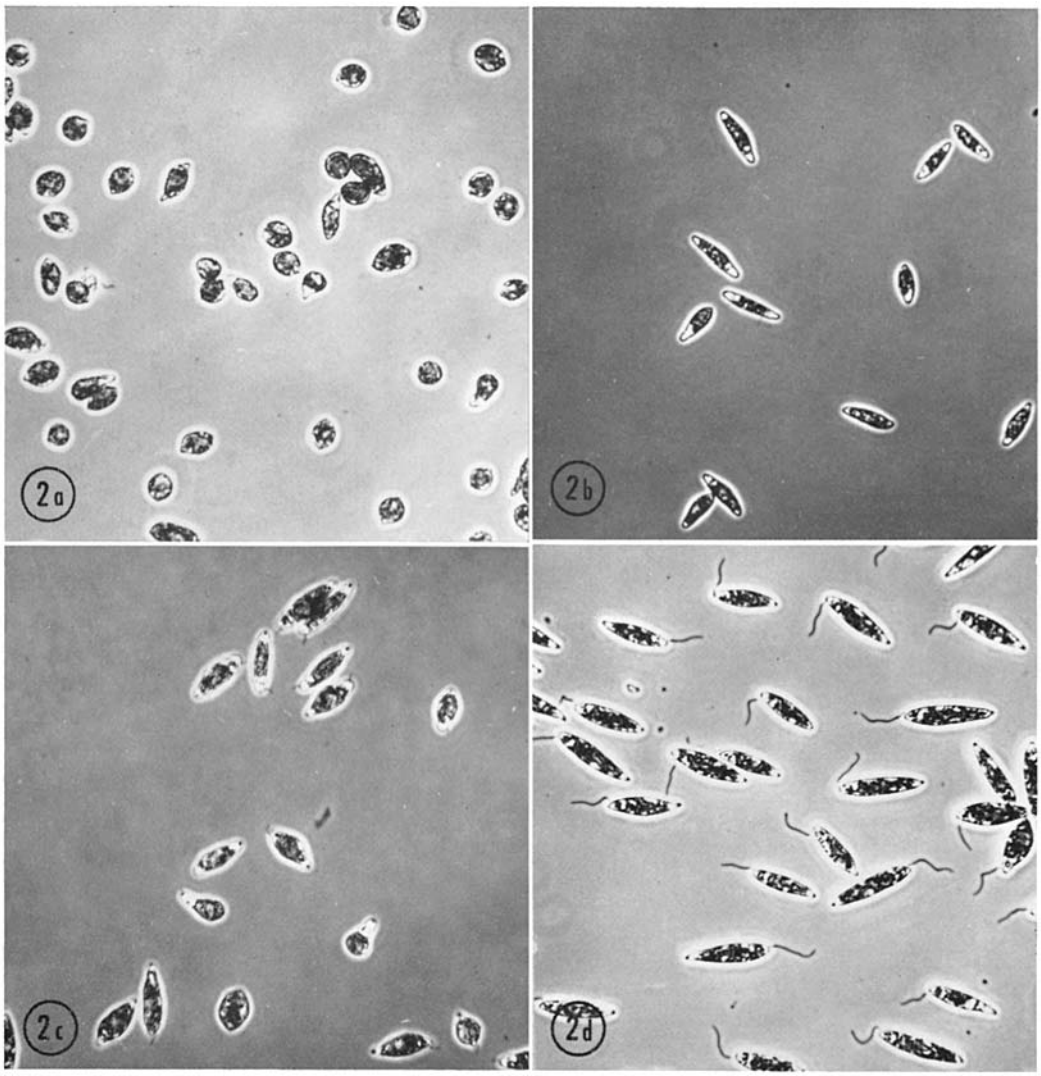

FIGURE 2 Flagellar regeneration in a culture of *Euglena gracilis* (Z). Fig. 2 *a* Rounded up *Euglena* immediately following flagellar amputation; Fig. 2 *b* The typical elongate form of *Euglena* in mid-lag phase; Fig. 2 *c* Appearance of the flagellar stubs following the lag phase; Fig. 2 *d* Flagella in mid-elongation phase. × 200.

centrifugation (clinical centrifuge) and resuspension, suspended in a minimal amount of distilled water and fixed with Champy's fluid for 2–3 hr. They were then washed three times with distilled water by centrifugation and resuspension and were suspended in a minimal amount of distilled water; a few drops of the suspension were placed on a subbed slide (3). After the organisms had settled, the slide was tipped to remove excess fluid and to spread the organisms and was immediately placed in 70% ethanol for 15 min. The slides were removed, allowed to dry, and passed through three 20-min changes of 5% trichloroacetic acid. They were then washed thoroughly with 70% ethanol and allowed to remain in the ethanol for 24 hr (with several changes) in the cold. This was followed by alcohol dehydration and air drying. Radioautography was carried out with NTB-2 liquid emulsion (Eastman Kodak Company, Rochester, N. Y.) according to the procedures described by Kopriwa and LeBlond (17). After development, the

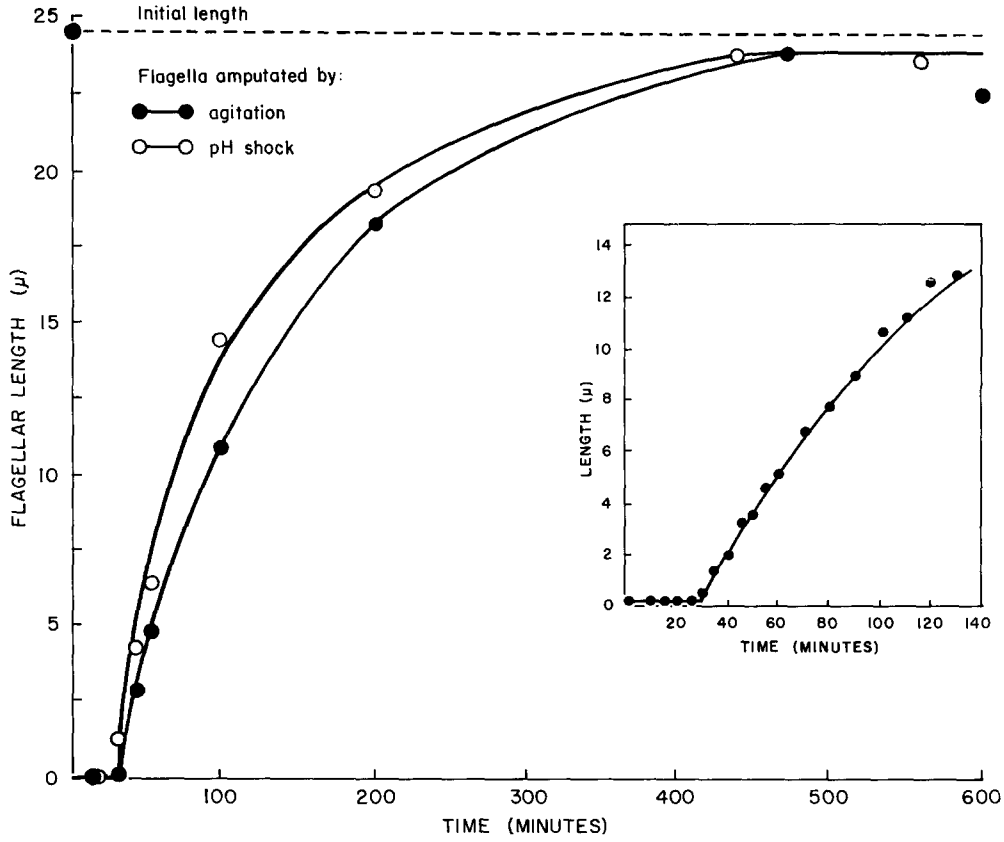

FIGURE 3 The kinetics of flagellar regeneration in cultures of *Astasia longa*. Open circles, flagella amputated by pH shock; closed circles, flagella amputated by mechanical agitation. The inset shows the early kinetics of regeneration, each point representing the mean of 500 measurements.

silver grains over the flagella were counted with a Zeiss Neofluar oil-phase objective (100 times) with water as a temporary mounting medium. The osmium tetroxide-containing fixative did not interfere with the radioautographic procedure, and was found to be the most satisfactory fixative for preserving the flagella and for maintaining their attachment to the organisms throughout the procedure. Zero-time controls, fixed immediately after the addition of leucine-^{3}H, showed few or no silver grains above background in the flagella.

In Vivo Amino Acid Incorporation

The in vivo incorporation of L-leucine-^{14}C (New England Nuclear Corp., 222 mc/mM) into trichloroacetic acid-insoluble proteins of whole *Euglena* and isolated *Euglena* flagella was assayed by the procedure of Mans and Novelli (19). Aliquots of labeled organisms or flagella were spread on 1 × 3 inch strips of Whatman No. 2 filter paper, were dried with a heat lamp or blower, and then were washed in bulk using 10 ml of fluid per strip, with three changes of 5% trichloroacetic acid, two changes of hot 80% ethanol, two changes of 3:1 hot methanol-chloroform, and one change of ether, and were dried. The radioactivity on the paper was then assayed by liquid scintillation counting.

RESULTS

Flagellar Regeneration Systems (General)

The amputation procedures resulted in the detachment of 95–99% of the flagella from populations of flagellates without disrupting the organisms. Electron microscopy[4] revealed that amputation did not remove the basal bodies, and that the flagella of *Ochromonas* had been broken off just

[4] We wish to thank Miss Susan Langreth, Whitman Laboratory, University of Chicago, for the electron microscopy.

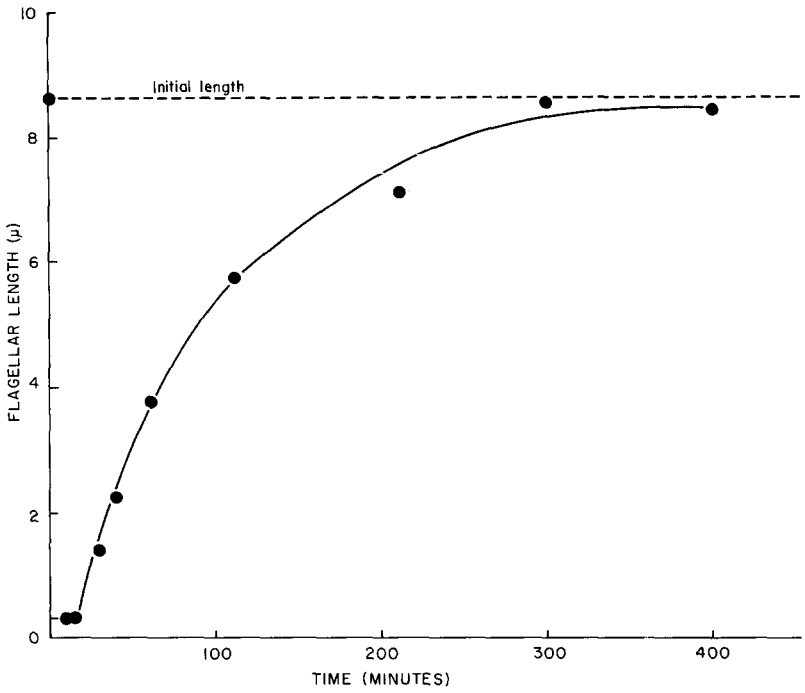

FIGURE 4 The kinetics of flagellar regeneration in a culture of *Ochromonas danica*. Flagella amputated by agitation in a fluted tube with a vortex mixer.

above the cell walls, while those of *Euglena* and *Astasia* were severed at levels where the flagella emerged from the reservoirs. Immediately following amputation *Euglena* and *Astasia* tended to round up and remain motionless, but they soon began crawling. In about 15 min they again assumed their typical elongate shape, and by 30 min the new flagellar stubs could be observed. The stubs were motile as soon as they appeared. This sequence of events is shown for *Euglena* in Fig. 2 *a–d*.

The kinetics of flagellar elongation obtained in populations of logarithmically growing *Astasia*, *Ochromonas*, and *Euglena* are illustrated in Figs. 3–5. In each case following amputation there was a short, reproducible lag phase followed by a phase of elongation. The elongation began at a maximal rate which decelerated as the initial length was regained. The lag phase and early kinetics of elongation in *Astasia* are shown more clearly in the inset of Fig. 3 where each point represents the mean of 500 measurements. The duration of the lags, the initial rates of elongation, and the time required to regain the initial length for all three species are given in Table I.

The degree of simultaneity with which amputated cells go from the lag phase to the phase of elongation may be seen in Fig. 6. In this experiment, more than 95% of the cells regenerated flagella.

The kinetics of regeneration were not significantly altered when the flagella of *Astasia* were amputated by the pH shock or mechanical procedures (Fig. 3). In *Euglena*, flagellar regeneration occurred equally well when the cells were grown either in the light or dark on Cramer-Myers' medium with acetate as the carbon source and regenerated in either the light or dark. In addition, all the flagellates used in this investigation could regenerate their flagella in distilled water with kinetics similar to those obtained in defined media. This is illustrated for *Euglena* in Fig. 5.

Reregeneration of Flagella

Once the flagella had been detached and regeneration had begun, the flagella could be reamputated and would regenerate again by the same kinetics as in the first regeneration. An example of such a reregeneration in *Ochromonas* is shown in Fig. 7.

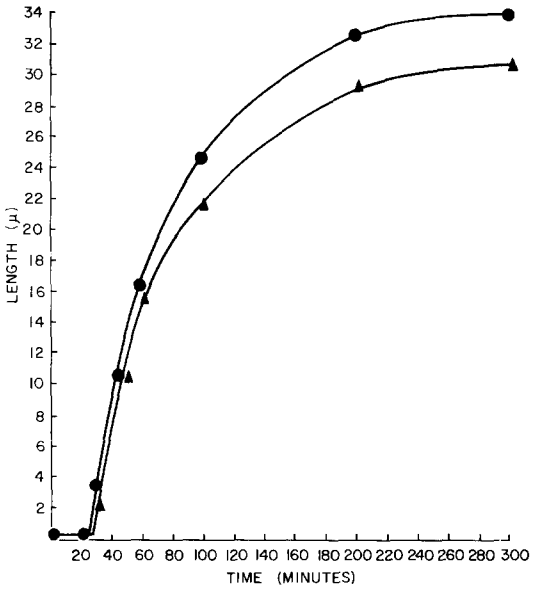

FIGURE 5 The kinetics of flagellar regeneration in cultures of *Euglena gracilis* (Z). Flagella amputated with the Virtis homogenizer. Deltas, regeneration in defined medium; circles, regeneration in distilled water.

TABLE I

Parameters of Flagellar Regeneration Kinetics in Cultures of Three Species of Flagellated Protozoans

Organism	Length of Lag	Initial rate of elongation	Initial length of flagella	Time required to regain initial length
	min	*μ/min*	*μ*	*min*
Astasia longa	25–30	0.14	25–30	400–450
Ochromonas danica	20–25	0.08	8–10	300–350
Euglena gracilis	25–30	0.26	28–33	250–300

Amino Acid Incorporation into Regenerating and Nonregenerating Flagella

The resolution obtainable by light microscope radioautography was sufficient to permit the quantitation of L-leucine-^{3}H incorporation into proteins of the flagella of *Ochromonas* and *Euglena*. In addition, some of the incorporation studies were carried out by assaying leucine-^{14}C in the proteins of isolated flagella. *Astasia* was impermeable to leucine under the conditions used. A typical radioautograph of a *Euglena* from a culture in which leucine-^{3}H was present during regeneration is shown in Fig. 8. We have focused on the flagellum, so the silver grains over the cell body do not appear in the radioautograph, although the cell bodies become labeled prior to the appearance of label in the flagella.

To determine if free amino acids were utilized for flagellar protein synthesis during regeneration, the incorporation of L-leucine-^{3}H into the proteins of regenerating flagella of nondividing *Ochromonas* and *Euglena* was assessed radioautographically

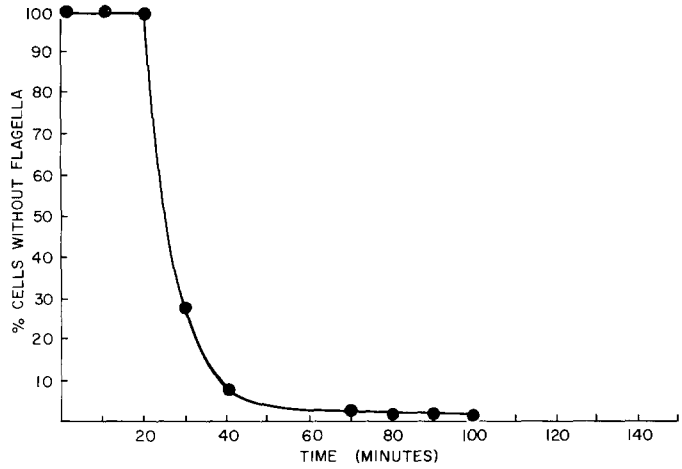

FIGURE 6 The per cent of *Astasia* without flagella at various times following flagellar amputation. Each point represents the mean of 500 organisms counted.

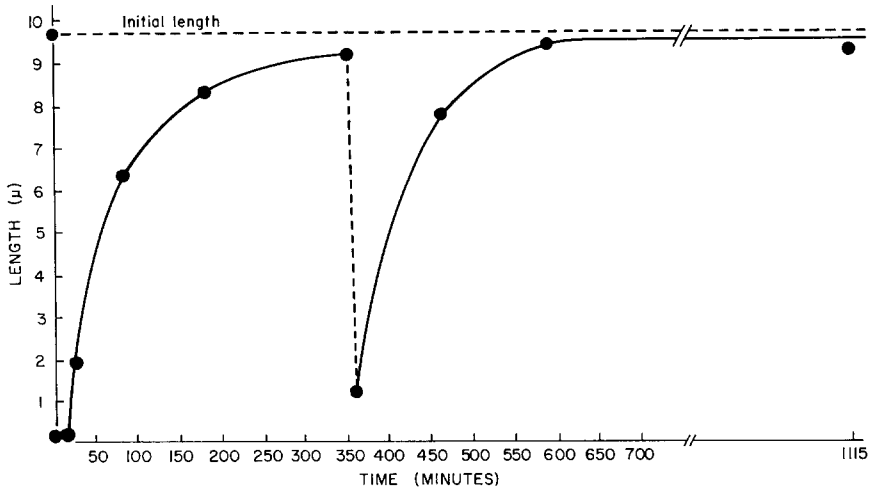

FIGURE 7 Reregeneration of the flagella of *Ochromonas*. The flagella were reamputated at the completion of the first regeneration. Although this figure does not show a lag prior to the reregeneration, this does occur (9, 37).[5]

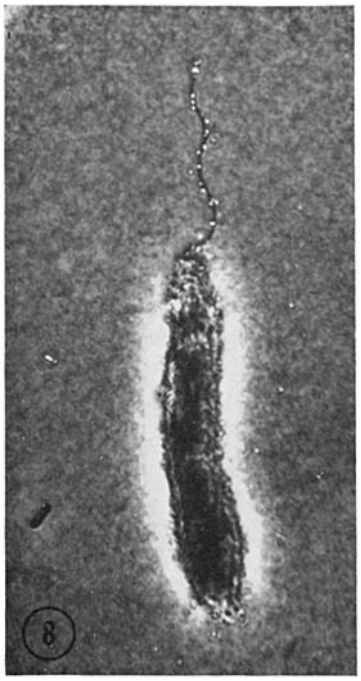

FIGURE 8 Radioautograph of a *Euglena* from a culture in which L-leucine-^{3}H (20 μc/ml) was present during flagellar regeneration. × 900.

and compared to the incorporation into the flagellar proteins of nonregenerating, full-grown flagella of organisms from the same culture. Previous work had shown that amino acid incorporation into the total trichloroacetic acid-insoluble proteins of whole cells was the same in regenerating and control cells, i.e. the amputation procedure did not change the over-all pattern of amino acid incorporation into total cell protein. The results (Fig. 9 a, b) indicated that the regenerating flagella of *Ochromonas* and *Euglena* incorporated 75 and 50% more leucine into their respective flagellar proteins than the nonregenerating flagella of these organisms by the end of the regeneration period. The kinetics of incorporation of label into the regenerating flagella were not the same as the kinetics of elongation since it could be calculated that the grains/unit flagellar length increased with time. The incorporation kinetics were doubtlessly influenced by the time taken for the amino acid to penetrate the organisms, the size of the free amino acid pool and the possible turnover of flagellar proteins.

That flagellar protein turnover may be occurring was indicated by the incorporation of leucine-^{3}H into the full-grown (control) flagella of nondividing *Ochromonas* and *Euglena*. In attempts to determine the stability of the flagellar proteins of *Euglena*, nondividing organisms were permitted to regenerate their flagella in the presence of labeled leucine (^{3}H or ^{14}C), which was subsequently removed by washing the organisms twice in resting medium containing a 10,000 fold excess of unlabeled leucine (chase medium). The organisms were then suspended in the chase medium for 18 hr. Samples were taken prior to and after the

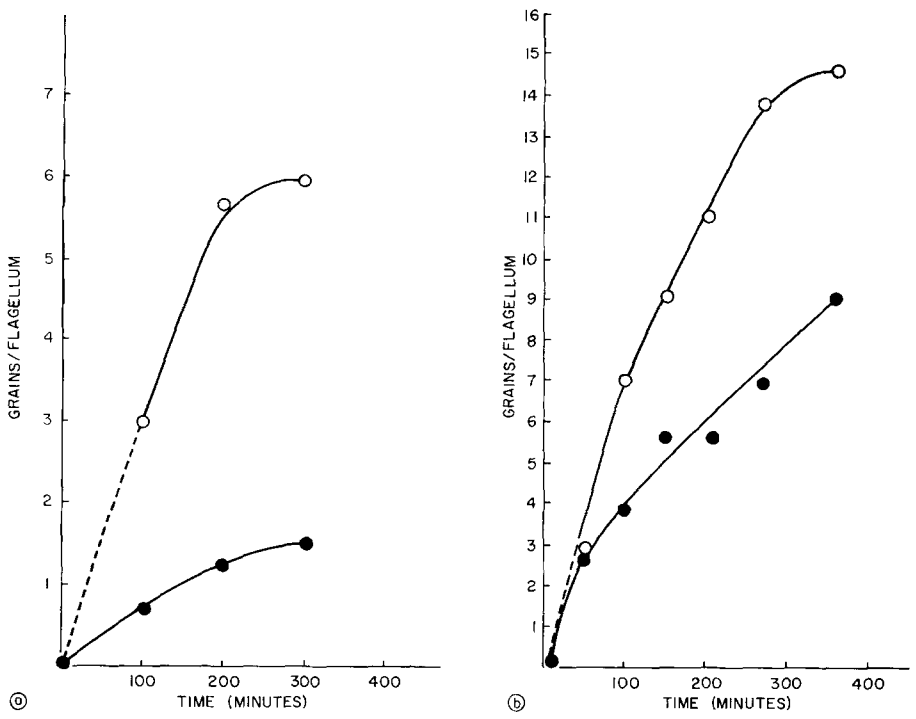

FIGURE 9 Incorporation of L-leucine-^{3}H (20 μc/ml) into regenerating and nonregenerating flagella of nondividing *Ochromonas* (Fig 9 *a*) and *Euglena* (Fig. 9 *b*). The isotope was added at the time of flagellar amputation and samples taken for radioautography at the indicated times. The dashed lines indicate that there are no zero time points for the regenerating flagella since the organisms have no flagella at this time. Open circles, regenerating flagella; closed circles, nonregenerating flagella.

18 hr chase and assayed for flagellar radioactivity (in trichloroacetic acid-insoluble protein) either by radioautography or by scintillation counting of isolated flagella. Both assay procedures showed that 36–37% of the radioactivity incorporated into the regenerating flagella was removed by the 18 hr chase in unlabeled leucine (Table II).

Effect of Cycloheximide on Amino Acid Incorporation and Flagellar Regeneration

Further evidence for the role of protein synthesis in flagellar elongation was obtained by use of the protein synthesis inhibitor cycloheximide (40) with the *Euglena* flagella-regenerating system. When used at a concentration of 10–20 μg/ml, cycloheximide completely inhibited the incorporation of L-leucine-^{14}C into the trichloroacetic acid-insoluble proteins of nondividing *Euglena* immediately following its addition to the culture (Fig. 10). Addition of the inhibitor to nondividing cells within 5 min following flagellar amputation resulted in greater than 90% inhibition of flagellar elongation (Fig. 11). On the other hand, if the inhibitor was added at 40 min following amputation, the flagella continued to grow about 10 μ before their growth was finally inhibited (Fig. 11). This latter result is shown in greater detail in Fig. 12, where cycloheximide was added at 6, 30, 45, and 83 min postamputation. In every case where the inhibitor was added at times other than immediately following amputation, considerable flagellar growth occurred before elongation ceased.

It should also be noted that in all of these inhibitor experiments cycloheximide did not have a lethal effect on *Euglena*, and once flagellar regeneration had been inhibited, resuspension of the organisms in fresh medium permitted flagellar growth to occur again albeit at a slightly slower rate than normal (Fig. 13). Moreover, if nondividing *Euglena* with full-grown flagella were placed in concentrations of cycloheximide up to 50 μg/ml, they maintained their flagellar lengths

TABLE II

Loss of Radioactivity from Labeled Flagella of Nondividing Euglena after Resuspension in Medium with Unlabeled Leucine

L-leucine-^{3}H (20 μc/ml) or -^{14}C (1 μc/ml) was added to cultures at the time of amputation. After 360 min, the organisms were washed and resuspended in resting medium containing 0.01 M unlabeled L-leucine for 18 hr.

Procedure	Before chase	After chase	Lost
			%
Autoradiography (gr/μ)	0.49	0.32	36
Isolated flagella (dpm/10^{7} flagella)	12,800	8084	37

and were still actively moving even after several hours in the inhibitor.

The Flagellar Growth Zone

The *Ochromonas* and *Euglena* flagella-regenerating systems were used in conjunction with light microscope radioautography to determine if the regenerating flagella had a growth zone. After flagellar amputation, the experimental organisms were allowed to regenerate their flagella to about one-half their initial length in the absence of leucine-^{3}H followed by the addition of leucine-^{3}H (20 μc/ml) to the culture for the remainder of the regeneration period. Control cultures were placed in medium containing leucine-^{3}H prior to and at the time of flagellar amputation and permitted to complete their regeneration in the presence of the isotope. Samples were taken for radioautography from all three cultures at the time of isotope addition (zero time controls) and at intervals thereafter until the end of the regeneration period. The distribution of silver grains over the proximal and distal halves of the flagella of at least 100 organisms/sample were determined after a 7 day exposure. The results of such an experiment with nondividing *Ochromonas* are presented in Table III as the per cent of the total flagellar silver grains which appeared over the proximal and distal halves of the flagella. These results show that there were no striking differences in the labeling pattern along the length of the flagella when leucine-^{3}H was added either prior to or at the time of flagellar amputation (Table III, lines 1 and 2; Fig. 14 *a*). On the other hand, when leucine-^{3}H was added

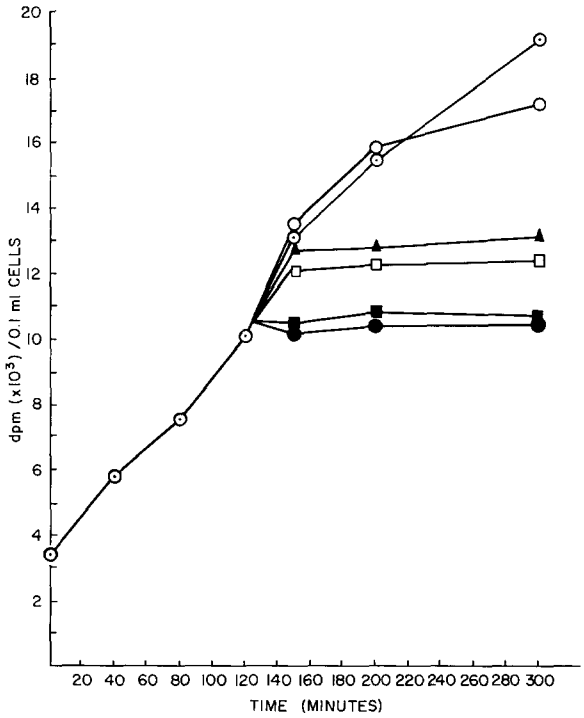

FIGURE 10 Effect of cycloheximide concentration on the incorporation of L-leucine-^{14}C (1 μc/ml) into trichloroacetic acid-insoluble proteins of nondividing *Euglena*. Cycloheximide was added 125 min after the addition of the isotope. Open circles with dots, 0 μg/ml; open circles, 0.01 μg/ml; deltas, 0.1 μg/ml; open squares, 1 μg/ml; closed squares, 10 μg/ml; closed circles, 20 μg/ml.

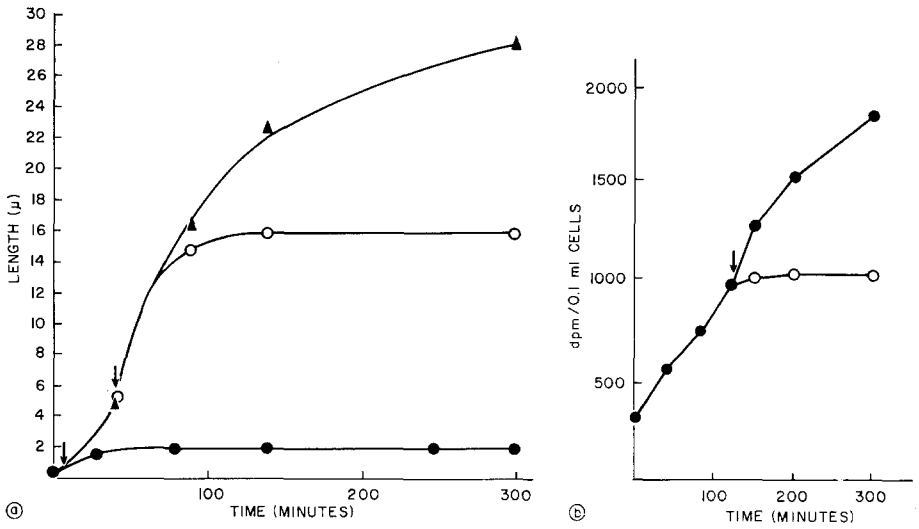

FIGURE 11. Effect of cycloheximide (20 μg/ml) on flagellar regeneration in nondividing *Euglena*. Fig 11 *a* Cycloheximide added immediately following amputation, closed circles; at 40 min postamputation, open circles; control, deltas. Fig 11 *b* Simultaneous leucine-^{14}C incorporation experiment showing inhibition of incorporation by 20 μg/ml cycloheximide added 125 min following addition of the isotope. Closed circles, 0 μg/ml cycloheximide; open circles, 20 μg/ml cycloheximide.

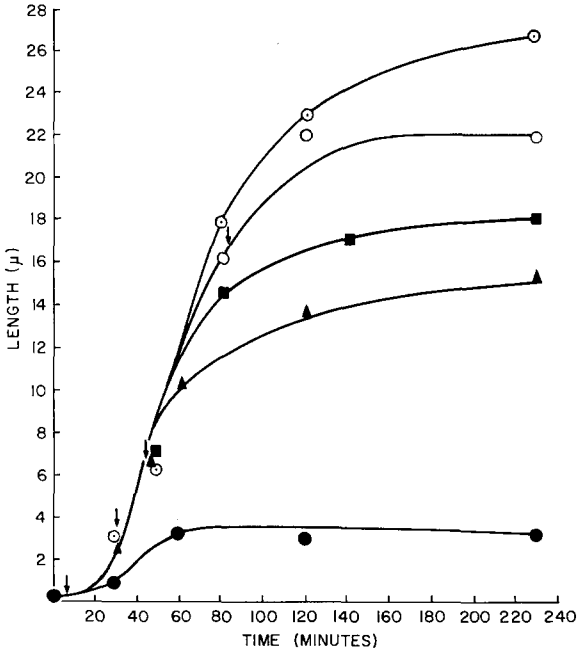

FIGURE 12 Effect of time of addition of cycloheximide (20 μg/ml) on flagellar regeneration in nondividing *Euglena*. Cycloheximide added at 6 min (closed circles), 30 min (deltas), 45 min (squares) and 83 min (open circles) postamputation; open circles with dots, control.

after the flagella had already grown out part way, i.e. 60 min postamputation, and when the cells were radioautographed near the end of the regeneration, there was a predominance of labeling (about 80% of the total label) in the distal portions of the flagella (Table III, line 3; Fig. 14 *b*). The photomicrographs in Fig. 14 *a* and *b*, which show radioautographs of individual cells selected from slides made from the experimental and control cultures, represent the extreme situations for uni-

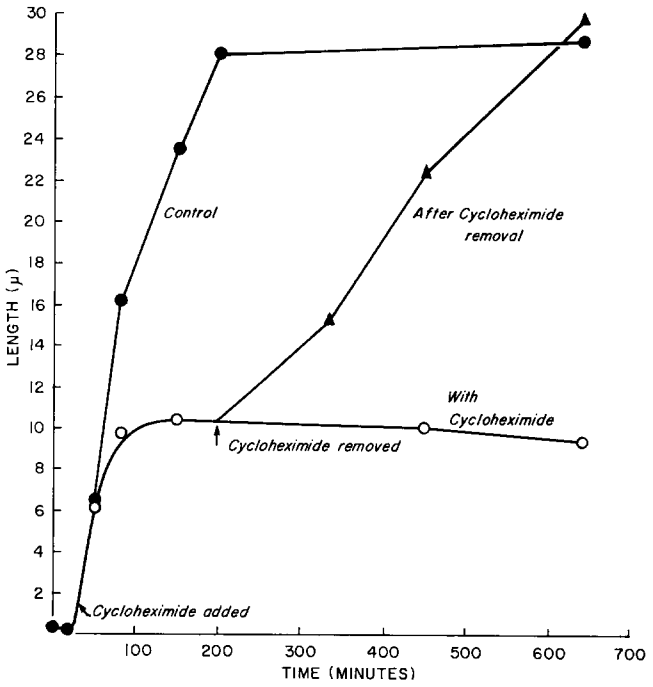

FIGURE 13 Reversibility of cycloheximide (30 μg/ml) inhibition of flagellar regeneration in *Euglena*. Cycloheximide added at 35 min postamputation. Closed circles, control; open circles, continued presence of inhibitor; deltas, resuspension in fresh medium at 200 min postamputation.

TABLE III

Growth Zone of Regenerating Flagella of Ochromonas danica

See text for procedure and discussion

Time of leucine-^{3}H addition	Ratio of proximal and distal grains to total grains: Proximal/ total	Distal/ total	No. of flagella counted
45 min prior to amputation	0.45	0.55	116
At the time of amputation	0.42	0.58	114
60 min after amputation	0.21	0.79	139

form labeling along the flagellum (leucine-^{3}H added at time of, or prior to amputation) and for distal labeling (leucine-^{3}H added at 60 min postamputation), respectively. They are included only to further illustrate the general conclusion (arrived at by counting silver grains over at least 100 flagella from each sample) that *Ochromonas* flagella have a tip growth zone.

No pronounced pattern of distal labeling could be observed when similar experiments were carried out with the *Euglena* flagella-regenerating system. Since full-grown, nonregenerating *Euglena* flagella became quite heavily labeled (see Results, Fig. 9 *b*) it was possible that any labeling pattern was obscured.

DISCUSSION

The Kinetics of Flagellar Regeneration

Flagellar elongation was preceeded by a lag phase during which there was no apparent growth, followed by growth at a maximal rate which constantly decelerated until the initial length was reached. This same pattern of regeneration kinetics had previously been described by Dubnau (9) in studies of flagellar regeneration in populations of *Ochromonas*. The duration of the lag and the regeneration rate were characteristic of each species, and there was no relationship between the length of the lag and the subsequent rate of regeneration or final length to which the flagella grew (see Table I). Each of the phases of the regeneration is discussed in detail below.

THE LAG PHASE

The lag appeared regardless of the method of flagellar amputation or whether the observations were made on single cells (5, 38)[5] or populations (see inset of Fig. 3). The specific nature of the lag

[5] Rosenbaum, J. L. Unpublished results.

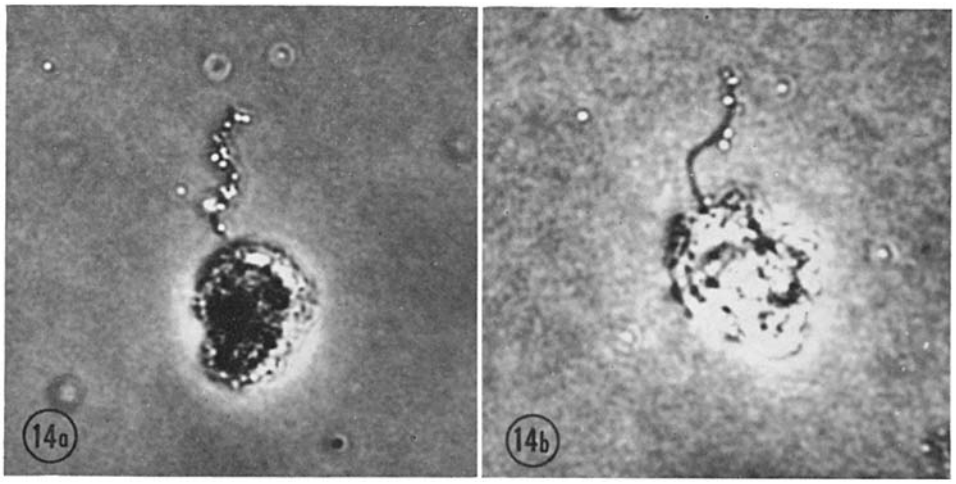

FIGURE 14 Growth zone of regenerating *Ochromonas* flagella. Fig 14 *a* L-leucine-^{3}H (20 μc/ml) added at time of flagellar amputation; Fig 14 *b* L-leucine-^{3}H added at 60 min postamputation. × 2000. See text for procedure and discussion.

is unknown, but it would not seem to be related to a possible trauma to the cell caused by the amputation procedure since individual cells whose flagella have been gently amputated by micromanipulation still have a lag (38). In addition, Dubnau (9) has shown that the lag in *Ochromonas* flagellar regeneration could not be changed by increasing the severity of the amputation procedure. Neither is it reasonable that the appearance of the lag was an artifact caused by withdrawal of the basal body into the cytoplasm at the time of amputation, such that the flagellum would actually be growing but would appear not to be doing so. Tamm (38) has shown in *Peranema* that the level of amputation had little effect on the length of the lag phase, but that the initial rate of elongation was a function of stump length. Hence, if the lag period was an artifact caused by the withdrawal of the basal body into the cytoplasm during the initial phase of elongation, the rate of the basal body's withdrawal from and subsequent movement toward the cell surface would have to be precisely regulated in relation to the level of amputation. This possible complication may be dismissed at present since electron microscope investigation of sections of regenerating *Astasia* did not give evidence for any movement of the basal body away from the cell surface.

On the other hand, the evidence which has been accumulated suggests that the lag represents a physiological event or series of events, possibly synthetic in nature, which must occur prior to the onset of flagellar elongation. Dubnau (9) observed that *Ochromonas*, which has a lag of 20–25 min, when placed at 1°C following flagellar amputation, had a complete lag when returned to room temperature. Cells which were permitted to undergo their lag at room temperature and were then placed at 1°C for varying periods of time had to undergo a second lag when the temperature was raised. The duration of this second lag was dependent on the length of the cold treatment; a 10 min treatment usually was sufficient to cause the reappearance of a complete second lag. Dubnau concluded from these results that (*a*) the processes determining the length of the lag were temperature sensitive, i.e. could not occur at 1°C, and were possibly enzymatic in nature, (*b*) that elongation could not occur at 1°C even after a normal lag, and (*c*) that cold treatment could abolish the effect of the lag and that this deterioration was probably nonenzymatic in nature. In addition, Dubnau showed that the lag occurred only after the amputation procedure; once flagellar regeneration had actually started, cold treatment could not induce a second lag, but reamputation of partially grown flagella always caused the reappearance of the lag.

Our results showed that the addition of cycloheximide, at a concentration sufficient to inhibit amino acid incorporation into protein, immediately following flagellar amputation resulted in almost complete inhibition of flagellar elongation,

whereas addition of the inhibitor after the lag permitted considerable elongation to occur before growth finally stopped. Thus, protein synthesis was required during the lag in order for the subsequent elongation to occur but, after having undergone the lag, some elongation could take place in the absence of protein synthesis. These results might be interpreted in light of Dubnau's observation that the effect of the lag could be abolished by cold treatment by the following hypothesis: during the lag flagellar precursor proteins (monomers and/or aggregates of monomers) may be synthesized and accumulated until a threshold amount is reached, whereupon assembly and visible elongation could take place. Prior to the lag there would not be enough preformed precursors in the cell to initiate elongation, as inferred from the fact that cycloheximide applied at the time of amputation almost completely inhibited elongation. Once assembly (and elongation) had begun, a secondary irreversible stabilization process would immediately occur; thus, cold treatment could not cause a breakdown of the structural elements of the flagellum once they had been assembled and stabilized. We tested the effect of cold treatment on regenerating flagella and, although it inhibited further elongation, there was no shortening. However, we know from Dubnau's (9) work that cold treatment applied at the end of the lag caused a reappearance of the lag. This might have resulted from the reversible breakdown of monomer aggregates in the cold after they had been accumulated during the first lag and before they were assembled and stabilized. This speculative series of events has some analogies to the effect of cold treatment on the breakdown of the mitotic apparatus whose tubules have structural similarities to the flagellar tubules (28). The mitotic apparatus seems to be in constant equilibrium with its precursors in the cytoplasm (16) and probably is not in a stabilized state, i.e. cold treatment can cause its disappearance and warming permits its reformation. Thus, the labile state of the mitotic tubules might be analogous to the state of the flagellar precursors during the lag phase, when cold temperature can cause the deterioration of the lag.

The above hypothesis is still highly speculative since we have no information concerning the nature of the precursors or how they assemble. In addition, we have made the assumption that the cycloheximide inhibition of regeneration was the result of a direct effect on the synthesis of flagellar proteins.

The Elongation Phase

Flagellar elongation appeared to begin abruptly following the lag, and there did not seem to be a gradual transition between the two phases. This was shown quite clearly in *Astasia longa* (inset of Fig. 3). For a more detailed analysis of these elongation kinetics, one is referred to Tamm's report on flagellar regeneration in *Peranema* (38) and Dubnau's analysis of *Ochromonas* regeneration kinetics (9). It should be noted, however, that all of the aforementioned studies were carried out on log-phase cells and, although the general pattern of kinetics seems to remain the same in stationary phase cells, preliminary results[5] indicate that lag phases are longer and regeneration rates slower in the stationary phase. Doubtlessly, the nutritional environment and past history of the organisms are factors to be considered in analyzing flagellar regeneration kinetics. It is also interesting in this respect that log-phase *Euglena* and *Astasia* could regenerate their flagella at the same or increased rates in distilled water in comparison to defined medium (Fig. 5). The reasons for such variations will require further investigation.

The question of whether the flagellum reaches a maximum length or continues to elongate has also been investigated by Tamm (38) in studies on single *Peranema*. That the latter situation occurred was indicated from his accurate measurements of flagellar lengths after amputation of the flagella of young, postdivision cells. Following the typical rapid and deceleratory elongation, he observed that the flagella continued to elongate slowly with a decreased rate constant, but still by deceleratory kinetics. This slow growth continued for 15–18 hr throughout interphase until cytokinesis, at which time the flagella shortened and a new flagellum grew out for each daughter cell. Even so, his mathematical model did predict that there was a theoretical maximum flagellar length "equal to the constant asymptote" of the model, and that growth would not continue indefinitely if division, and shortening, could be inhibited. Although our own results and those of Dubnau (9) showed a leveling-off of growth at the regained initial length, the method of measurement may not have been sufficiently accurate to show a subsequent slow increase in length. Whether this slow elongation is a general phenomenon of flagellar growth will require similar single cell investigations on other flagellates.

Protein Synthesis Requirements for Flagellar Regeneration

THE EFFECT OF CYCLOHEXIMIDE ON FLAGELLAR REGENERATION IN EUGLENA: Cycloheximide, which has been shown to inhibit protein synthesis at the translational level (40), proved to be an excellent inhibitor of amino acid incorporation into *Euglena* proteins. Unlike other inhibitors which were tried, e.g. puromycin, cycloheximide blocked the incorporation of amino acids into proteins almost immediately following its addition to the culture. Moreover, its inhibitory effect on flagellar regeneration could be reversed by resuspending the cells in fresh medium.

We have already discussed results with this inhibitor which indicated that protein synthesis was required during the lag in order for elongation to occur (Discussion). In addition, cycloheximide added at any time after the lag phase, although permitting some elongation, prevented the flagella from reaching their initial length. If one assumes that cycloheximide was acting directly on the synthesis of flagellar proteins, then these results would suggest that (*a*) there were few flagellar precursor proteins available for flagellar growth prior to amputation, (*b*) flagellar protein synthesis occurred at a rapid rate during the lag so that addition of the inhibitor after the lag permitted some flagellar elongation in the absence of protein synthesis, and (*c*) flagellar protein synthesis was also occurring throughout the actual elongation phase.

The fact that the flagella could re-regenerate two or three times by kinetics similar to the first regeneration (Fig. 7, and refs. 9, 37) also supports the suggestion that de novo protein synthesis was required for flagellar growth, unless the organisms had a sufficient reserve of flagellar precursor proteins which could be mobilized to form several flagella. This seems unlikely in view of the inhibitor evidence.

Although the inhibitor results support Dubnau's (9) conclusion that at least some de novo protein synthesis was necessary for flagellar growth, he observed that the addition of inhibitors of protein synthesis (as well as amino acid, purine and pyrimidine analogues) to *Ochromonas* at the time of amputation only depressed the initial rate of elongation, complete inhibition occurring only after the flagella had grown out about half way. This suggested to him that the cells had enough precursor proteins (prior to amputation) to form about half of the flagellum and that de novo protein synthesis was required only for the completion of growth. However, this lag in inhibition of elongation could be attributed to a slow penetration of the inhibitor. Thus, in repeating some of Dubnau's experiments, we were able to show that addition of inhibitors (*e.g.* 8-azaguanine) to *Ochromonas* prior to amputation, for a period of time equivalent to the lag in inhibitory effect observed when the inhibitor was added at the time of amputation, resulted in almost complete inhibition of elongation,[5] suggesting that Dubnau's observations were the result of a permeability phenomenon.

AMINO ACID INCORPORATION INTO EUGLENA AND OCHROMONAS FLAGELLA: Additional evidence for the de novo synthesis of flagellar proteins came from a comparison of the amino acid incorporation into the proteins of regenerating and nonregenerating flagella of *Euglena* and *Ochromonas*. In most of these studies division-associated changes in the flagella were circumvented by use of nondividing cells. The results showed that there was always more incorporation into the proteins of regenerating than nonregenerating flagella, suggesting that de novo flagellar protein synthesis from amino acids was occurring during elongation. It should be noted, however, that (*a*) the studies only measured the *accumulation* of amino acid into protein and told us nothing about rates of synthesis at different stages of regeneration, and (*b*) we do not know which of the various flagellar components were being made de novo. Information in respect to (*a*) could be obtained by pulsing cells with a labeled amino acid at different times during the regeneration and isolating the flagella for counting, and in respect to (*b*) by assaying the radioactivity in fractionated flagella.

Amino acid incorporation was always observed in nonregenerating, full-grown flagella of nondividing cells; the amount was dependent on the organisms used. Nonregenerating *Euglena* flagella incorporated almost 50% as much amino acid as did the regenerating flagella, while nonregenerating *Ochromonas* flagella incorporated 25% as much as the regenerating flagella. This incorporation into full-grown flagella may have resulted from (*a*) turnover of one or more of the flagella proteins, or (*b*) a nonspecific migration of labeled cellular proteins into the shaft of the flagellum. Assuming that the labeling was the result of turnover, we attempted to determine the turnover characteristics of flagellar proteins by labeling regenerating flagella of nondividing *Euglena* and then chasing with the unlabeled amino acid at the end of the

regeneration period. That approximately 36–37% of the label was lost after an 18 hr chase suggests that flagellar proteins were slowly turning over. However, these results must be interpreted with caution because the flagellum consists of several proteins, some of which may be more stable than others. The stability of the various parts of the flagellum could be determined by fractionation of flagella isolated at intervals during the chase period. Experiments such as the latter would be crucial for differentiating between turnover of flagella structural proteins and the possibility that the labeling of nonregenerating flagella resulted from the nonspecific migration of labeled cytoplasmic proteins back and forth between the cytoplasm and the flagellum. There is no ultrastructural evidence of a barrier which might prevent a movement of cytoplasmic proteins into the shaft, at least between the axoneme and the flagellar membrane.

The Growth Zone of Regenerating Flagella

When nondividing *Ochromonas* were permitted to regenerate their flagella part way without leucine-^{3}H and the isotope was added only for the latter part of the regeneration, radioautography showed a predominance of silver grains over the distal portions of the flagella. Control organisms which were placed in leucine-^{3}H either prior to or from the time of amputation until regeneration was completed showed a relatively uniform distribution of silver grains along the flagellum. These results suggested that *Ochromonas* flagella had a tip growth zone. When similar experiments were carried out with *Euglena*, there was only a slight tendency toward distal labeling. This might be explained by the observation that there was considerable amino acid incorporation into nonregenerating *Euglena* flagella (50% of that incorporated into regenerating flagella) (see Discussion) and such (turnover?) incorporation might be expected to obscure any specific labeling pattern appearing during regeneration. On the other hand, nonregenerating *Ochromonas* flagella incorporated only 25% as much amino acid as the regenerating flagella and the chances of observing a specific labeling pattern during regeneration would be greater. There was always some proximal labeling in *Ochromonas* flagella when leucine-^{3}H was added after the flagella had already grown out part way (Table III, line 3) and this may have resulted from turnover of flagellar proteins, nonspecific migration of labeled cytoplasmic proteins, or from movement of flagellar precursors up to the tip growth zone.

We have no way of knowing from these growth zone studies whether the flagellar membranes, axonemes, or different parts of the axonemes all have a tip growth zone. Indeed, the 20–25% proximal labeling observed in *Ochromonas* flagella might have been due, in part, to a proximal growth zone in one of the flagellar components. This could be determined by similar growth zone studies where parts of the flagella, e.g. membranes, were removed by Gibbons' (13) procedures prior to radioautography. There is, however, cytological and ultrastructural evidence suggesting that both membranes and axonemes of the flagella have tip growth zones. Tamm (37) was able to amputate a *Peranema* flagellum to one-half its length, leaving a piece of the flagellar sheath hanging from the stub. As the flagellum started to grow, he recorded (photographically) the changes in distance between the cell body and the piece of dangling sheath which served as a marker of the cut end. That the distance did not change as the flagellum continued to grow suggests that at least the sheath had a tip growth zone. In addition, there have been several electron microscopic investigations of ciliary and flagellar growth in various vertebrates and invertebrates (8, 24, 29–31, 34, 35) which also provide some evidence for a tip growth zone. In some of these studies, vesicles and electron-opaque granules of various sizes have been observed in the growing flagellum, and Sotelo and Trujillo-Cenoz (34), in studies of developing cilia of the chick neural epithelium, found these vesicles located in the tip of the growing cilium. Likewise, Schuster (30) observed particles and vesicles, called proaxonemal particles, strung out along the length of the shaft and occassionally grouped at the tip of developing flagella of the slime mold *Didymium*. However, in investigations of developing flagella in *Allomyces* (24), *Naegleria* (8), and cilia of *Tetrahymena*[5] the axonemal fibers were sometimes observed to extend right to the tip of the growing cilium or flagellum with no evidence of possible "morphogenetic material" at the tip. Clearly, the ultrastructural changes occurring during flagellar elongation will require further investigation. However, the radioautographic results presented here, along with Tamm's cytological study of the *Peranema* flagellar growth zone (30) and the avail-

able ultrastructural evidence, argue strongly in favor of a tip growth zone.

The results described above, which suggest that the flagella grow by addition of precursors to the tip, raise questions concerning the site of synthesis of flagellar precursor proteins. One might postulate that the flagellar proteins are synthesized either (*a*) on cytoplasmic ribosomes which are found in abundance adjacent to the basal bodies, and subsequently transferred up the shaft to the tip assembly site, or (*b*) that the growing flagellum has a self-contained protein synthetic system. At the present time there is not sufficient evidence to discount either possibility. Although a self-contained flagellar protein synthetic system may seem improbable on the basis of the available biochemical evidence showing that cilia have little RNA (4, 39) and activating enzyme activity (27) and, ultrastructurally, an absence of ribosomes, these investigations have all been carried out on full-grown cilia and flagella.

Control of Flagellar Growth

From the data presented in this report, along with Dubnau's results on *Ochromonas* flagellar regeneration (9), Tamm's study of flagellar growth in single *Peranema* (37, 38), and Child's observations on cilia regeneration in *Tetrahymena* (6), some general comments can be made on mechanisms which may be operating to control the maximum flagellar length and the deceleratory nature of the elongation kinetics.

First, as Dubnau originally demonstrated, the maximum flagellar length cannot be explained by any simple limiting substance type of hypothesis in the strictest sense whereby amputation would cause the mobilization of just enough raw material to form one flagellum. He showed that after reamputation of flagella which had grown out half way, the flagella still reregenerated to their normal length, rather than one and one-half times their normal length as would be expected if each amputation caused the cell to produce just enough precursors to form one flagellum. On this basis and from an analysis of the kinetics of regeneration, he postulated that some type of feedback mechanism was operating whereby the cell would know the length of the flagellum at any time during its growth. The postulation of such a feedback control was strengthened by some of our observations on flagellar regeneration in the biflagellate *Chlamydomonas* (5, 23).[5] It was shown that when the two flagella were unequally amputated, both stumps would elongate at decelerating rates, the longer stump decelerating more rapidly than the shorter, so that both reached the initial length at the same time. These results have been corroborated by Tamm's observations that *Peranema* flagella amputated to different stump lengths regenerated at decreasing initial rates the longer the stump, and that the flagella always grew out to their normal length regardless of the level of amputation (38).

The control of the growth rate and maximum length could be operating at the level of the protein synthetic process or could somehow be localized within the structure of the organelle itself. We know that flagellar and ciliary growth are dependent upon protein synthesis from the inhibitor and incorporation results presented in this report and from Child's observations on the inhibition of ciliary growth in *Tetrahymena* by cycloheximide and puromycin (6). However, we do not know whether the *rate* of flagellar protein synthesis is decreasing during elongation and could, therefore, account for the decelerating elongation kinetics. On the other hand, indirect evidence suggests that the control may not be operating at this level alone. Thus, it was observed that, if one of the two flagella of *Chlamydomonas* was cut off, only the amputated flagellum elongated.[5] The unamputated flagellum could not utilize the proteins being formed for the elongation of the amputated flagellum. It would seem reasonable to postulate on this basis that, even though flagellar elongation is dependent upon protein synthesis, the control of rate and maximum length ultimately lies within the structure of the organelle itself.

Many different hypotheses could be presented on how such an organelle-regulated control might be exerted. However, at this time, any model of a flagellar growth control mechanism is necessarily premature until information can be acquired on the chemical nature of flagellar precursor proteins, the cellular site and rate of their synthesis, the mechanism by which their assembly takes place, and further amplification of the ultrastructural changes occurring during flagellar elongation. The flagella-regenerating systems described in this report offer promising approaches to these problems.

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preliminary observations on the ultrastructural changes occurring during flagellar regeneration.

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