

THE REGENERATION OF CILIA IN PARTIALLY DECILIATED *TETRAHYMENA*

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ABSTRACT

Partial deciliation of *Tetrahymena* resulted in cells losing 75% of their cilia, with the balance being paralyzed. The paralyzed cilia are resorbed in the first 20 min after partial deciliation, and regeneration of cilia begins before resorption is completed. Inhibition of protein synthesis with cycloheximide does not inhibit ciliary resorption or regeneration, whereas vinblastine sulfate inhibits regeneration but not resorption. Inhibition of regeneration occurs in completely deciliated cells when they are treated with cycloheximide or vinblastine sulfate. It is concluded that the resorbing cilia contribute materials which allow regeneration to occur in the absence of protein synthesis.

The volume of cilia regenerated in the presence of cycloheximide in partially deciliated cells is greater than the ciliary volume which is resorbed. This suggests the *Tetrahymena* cells have a pool of ciliary precursors. This pool does not contribute materials for regeneration in completely deciliated cells which are treated with cycloheximide. It is concluded that resorbing cilia in partially deciliated cells contribute materials which potentiate assembly of cilia from the pool of precursors.

Cilia and flagella provide many desirable advantages as model systems which can be exploited to develop our understanding of the cellular mechanisms which control organellogenesis. Numerous studies have been made on the ultrastructural and biochemical nature of cilia and flagella, proving invaluable to furthering our understanding of their formation (for reviews see, 4, 6, 7, 8, 12, 13). The process of ciliary regeneration in *Tetrahymena* has been described by others (13), and it has been shown that ciliary regeneration requires protein synthesis and the assembly of microtubule protein(s). Other studies of flagellate systems have yielded similar results. In the biflagellated organism *Chlamydomonas*, however, it has also been shown that regeneration of flagella can occur in the absence of protein synthesis. Resorbed flagellar proteins are reutilized, and flagella are assembled

from a cytoplasmic pool of flagellar proteins (3, 10). The striking similarity between ciliary and flagellar ultrastructures may imply that an equal similarity exists in the mechanisms controlling their organellogenesis.

This report deals with an attempt to further explore ciliogenesis in *Tetrahymena*. The results obtained demonstrate that *Tetrahymena*, like *Chlamydomonas*, can regenerate cilia in the absence of protein synthesis if deciliation is incomplete.

MATERIALS AND METHODS

Materials

Tetrahymena pyriformis, strain GL-C, was obtained from a stock maintained by Dr. Norman Williams at the University of Iowa.

Solutions of 5 mM CaCl₂ and 5 mM EDTA in 50 mM

sodium acetate buffer, pH 6.0, were used for the deciliation of *Tetrahymena*. The cells recovered from deciliation in 0.1 M potassium phosphate buffer, pH 7.0.

The reagents used for scanning electron microscopy of *Tetrahymena* were 2% osmium tetroxide for fixation, an ethyl alcohol series for dehydration, and an ethyl alcohol-amyacetate series for critical point drying (performed in a simplified apparatus developed by Ruffolo (11)).

A tritiated amino acid mixture, no. 3130-08, was obtained from Schwarz/Mann, Div., Becton, Dickinson and Co. Orangeburg, N. Y., cycloheximide from the Sigma Chemical Co., St. Louis, Mo., and vinblastine sulfate from the Eli Lilly and Co., Indianapolis, Ind.

Culture Methods

T. pyriformis (GL-C) were grown on bactotryptone medium (5) at 28°C. Except where otherwise noted, all experiments were performed on cultures growing logarithmically at densities between 1×10^4 and 5×10^4 cells/ml.

Methods of Ciliary Amputation

Complete removal of somatic cilia was carried out at room temperature by the same technique used by Rosenbaum and Carlson in their study of ciliary regeneration in *T. pyriformis*, strain W (2, 13). Partial deciliation was accomplished in the same manner except that the shearing force applied as the final step in the total deciliation technique was omitted. Rather than being subjected to shearing forces, the cells were simply suspended in the recovery buffer. Under these circumstances, approximately 70–75% of the cilia were shed and the remaining 25–30% were paralyzed.

Assay for Recovery of Motility

Cells that are either completely or partially deciliated by these methods are nonmotile. Recovery from either of these conditions is indicated when the cells begin to swim. The degree of recovery can be gauged by counting the numbers of cells in a culture sample which are and are not swimming and by calculating percent motility. Two methods were routinely used to determine the percent of motile cells. The first technique, used by Rosenbaum and Carlson (9), involved placing culture samples in capillary tubes. These, in turn, were submerged in mineral oil for observation under a dissecting microscope. In the second method, cells were observed directly in their culture flasks on an inverted microscope. In both methods motile vs. nonmotile cells were scored and the percent motility was calculated. The two methods were found to produce concordant results.

Radioactive Assay of Normal and Inhibited Protein Synthesis

For each experiment a mixture of (2 μ Ci/ml) tritiated amino acids (1 mCi/ml) was added to three cultures of

either deciliated or logarithmically growing, normal *T. pyriformis*. 30 min later, cycloheximide (1 or 5 μ g/ml) was added to these cultures to inhibit protein synthesis. At intervals of 5, 30, 60, and 90, and 120 min, a 1-ml aliquot of each culture was withdrawn and assayed for incorporation of tritiated amino acids into total cell protein. Each aliquot was run into 1 ml of 10% trichloroacetic acid, swirled, and heated in a water bath at 90°C for 30 min. The precipitated proteins were washed four times with distilled water, dried, and solubilized with 0.5 N NaOH at 50°C. The solubilized samples were diluted to 0.1 N NaOH with distilled water. An 0.2-ml aliquot of each sample was mixed with 15 ml of scintillation fluid consisting of 33% Triton X-100, 67% toluene, and 0.27% PPO (vol/vol/wt). The samples were then counted in a Beckman LS-133 scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

Determination of Ciliary Frequency, Relative Lengths, and Volumes

Scanning electron micrographs of *Tetrahymena* were photographically enlarged to magnify the cells to $\times 4,000$. The relative length and frequency data for cilia were obtained by measuring the lengths of all somatic cilia on at least six cells (always more than 500 cilia) with a plastic rule. The mean frequency per cell of each ciliary length class was determined by dividing the number in each class by the number of cells from which data were obtained. The sum of the lengths per cell was used as a relative measure of ciliary volume. Cilia are cylindrical and the cross-sectional areas are uniform. Ciliary volume, therefore, is directly proportional to length. All the somatic cilia were measured on cells on which tangling of cilia was minimal.

Although deviations in the angular orientation of cilia produced consistent underestimation of total ciliary length, randomness of orientation (assumed) results in consistent error in all determinations. Deviations in orientation can, therefore, be ignored.

The number of somatic cilia on untreated log cells is less than previously reported for *Tetrahymena*. The reason for this difference is unknown. It is possible that the loss of a consistent proportion of cilia occurs as a result of some step in preparation of specimens for scanning electron microscopy. It is assumed that the same proportional loss of cilia also occurs in the preparation of partially deciliated cells for microscopy.

Inhibition of Ciliary Regeneration by Vinblastine Sulfate

Cultures of partially deciliated *T. pyriformis* in "recovery buffer" were treated by adding 0.5, 1, 2.5, 10, 15, 20, or 25 μ l of a 0.2% solution of vinblastine sulfate in "recovery buffer." 2 h later the cultures were examined and scored for motility, and samples were prepared for scanning electron microscopy.

Scanning Electron Microscopy

Aliquots of culture were fixed for 2 min by adding an equal volume of 2% osmium tetroxide and were then washed with water by centrifugation. The fixed cells were dehydrated in an ethyl-alcohol series and the alcohol was subsequently replaced with 100% amylacetate. The fixed cells were then critical point dried in the simplified apparatus developed by Ruffolo (11), mounted on stubs, and coated with a mixture of gold and palladium. The scanning electron microscopy was done on a Cambridge Stereoscan.

RESULTS

Recovery of Motility in Partially Deciliated *Tetrahymena*

Partially deciliated *Tetrahymena* recovered motility with the same kinetic pattern as cells that had been completely deciliated (Fig. 1). In each case a 30-min lag occurred before any signs of motility could be seen. Approximately 30–35 min after the onset of recovery, 90–95% of the cells in the culture were fully motile. The number of cells motile in an untreated log culture never exceeded 95%. Therefore, according to the motility criterion, both partially and completely deciliated cells were fully recovered approximately 1 h after treatment.

Experiments were carried out to determine the relationship of protein synthesis to recovery of motility in both partially deciliated and completely deciliated cells. Fig. 2 shows the effect of various concentrations of cycloheximide on synthesis of total cell protein. It was found that 5 $\mu\text{g}/\text{ml}$ of cycloheximide was sufficient to eliminate protein

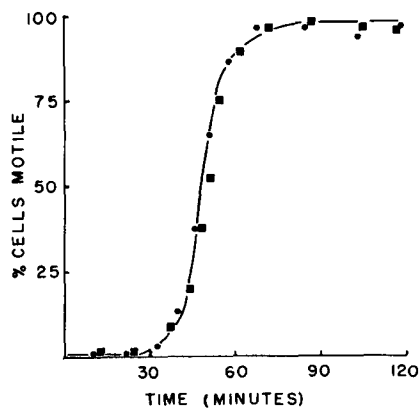


FIGURE 1 The kinetics of motility recovery in completely deciliated (■—■) and partially deciliated (●—●) *T. pyriformis* (GL-C). No difference in the kinetic pattern of motility recovery was observed.

synthesis in deciliated cells. The effect of inhibition of protein synthesis on the recovery of motility was determined for both partially and completely deciliated *Tetrahymena*. Fig. 3 shows a comparison between the recoveries of partially deciliated cells and of completely deciliated cells both treated with an excess of cycloheximide. As previously shown by Rosenbaum and Carlson (9), completely deciliated cells were unable to recover motility in the presence of cycloheximide.

On the other hand, partially deciliated cells were

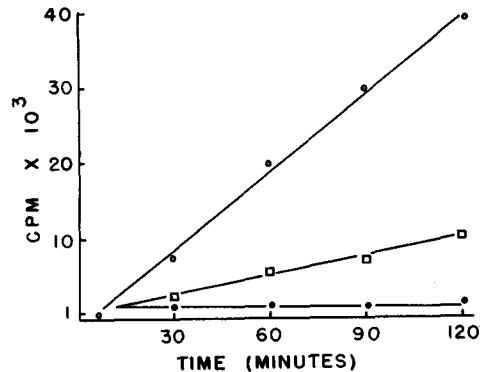


FIGURE 2 The effect of cycloheximide on the incorporation of tritiated amino acids into total cell protein. Cells were grown in bactotryptone media and labeled with tritiated amino acids for 30 min. The culture was then divided into three portions: no cycloheximide was added to the first portion (○—○). The second and third portions were treated with 1 $\mu\text{g}/\text{ml}$ (□—□) and 5 $\mu\text{g}/\text{ml}$ (●—●) cycloheximide, respectively.

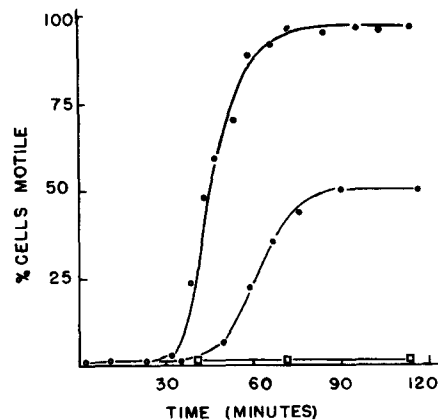


FIGURE 3 The effect of cycloheximide on the recovery of motility in partially deciliated (●—●) and completely deciliated (□—□) *Tetrahymena*. Cycloheximide-treated, partially deciliated cells recovered motility more slowly than partially deciliated untreated cells (○—○).

able to recover a significant level of motility. The onset of recovery of motility was delayed by approximately 10 min in partially deciliated cells treated with 20 $\mu\text{g}/\text{ml}$ of cycloheximide relative to the onset in untreated, partially deciliated cells. Not only was recovery of motility delayed, but the maximum number of cells motile at any observation never exceeded 50%. A significant level of recovery of motility can therefore occur in the presence of cycloheximide in partially deciliated *Tetrahymena*.

Recovery of motility in partially deciliated cells could be due either to the replacement of paralyzed cilia with functional cilia or the repair of paralyzed cilia. Replacement requires assembly of microtubules, whereas repair need not. Partially deciliated cells were, therefore, subjected to treatment with vinblastine which has little effect on protein synthesis, but is known to inhibit assembly of microtubules (1). Fig. 4 shows the results of this experiment. Partially deciliated cells treated with as little as 10 $\mu\text{g}/\text{ml}$ vinblastine are unable to recover motility during 2 h. This suggests that ciliary regeneration and, in particular, microtubule assembly are involved in recovery of motility in partially deciliated cells, as they are for fully deciliated cells. Thus, the results in Fig. 3 suggest that ciliary regeneration can occur without protein synthesis in partially deciliated cells, but not in fully deciliated cells.

Ciliary Regeneration in Partially Deciliated *Tetrahymena*

Analysis of scanning electron micrographs (SEM) (Figs. 5 and 6) demonstrates that partially deciliated *Tetrahymena* resorb paralyzed cilia and generate new cilia during recovery of motility. This

occurs even in the presence of an excess of cycloheximide. Fig. 5 is a series of scanning electron micrographs showing *Tetrahymena* in various stages of recovery after partial deciliation. Figs. 5 *a* and 5 *b*, respectively, show cells before and immediately after partial deciliation. 72% of the normal number of cilia were lost by cells during partial deciliation (Table I). Fig. 5 *c* depicts a partially deciliated cell after 20 min of recovery in the presence of cycloheximide. At this stage (10 min before the first signs of motility), many more cilia were present than immediately after partial deciliation. Regenerated cilia in cells 2 h after partial deciliation in the presence and absence of an excess of cycloheximide are shown in Fig. 5 *d* and 5 *f*, respectively, and little difference is noted between them.

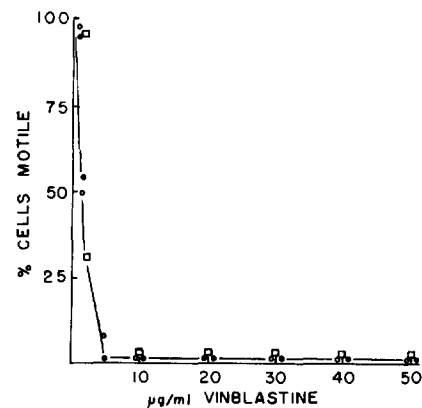
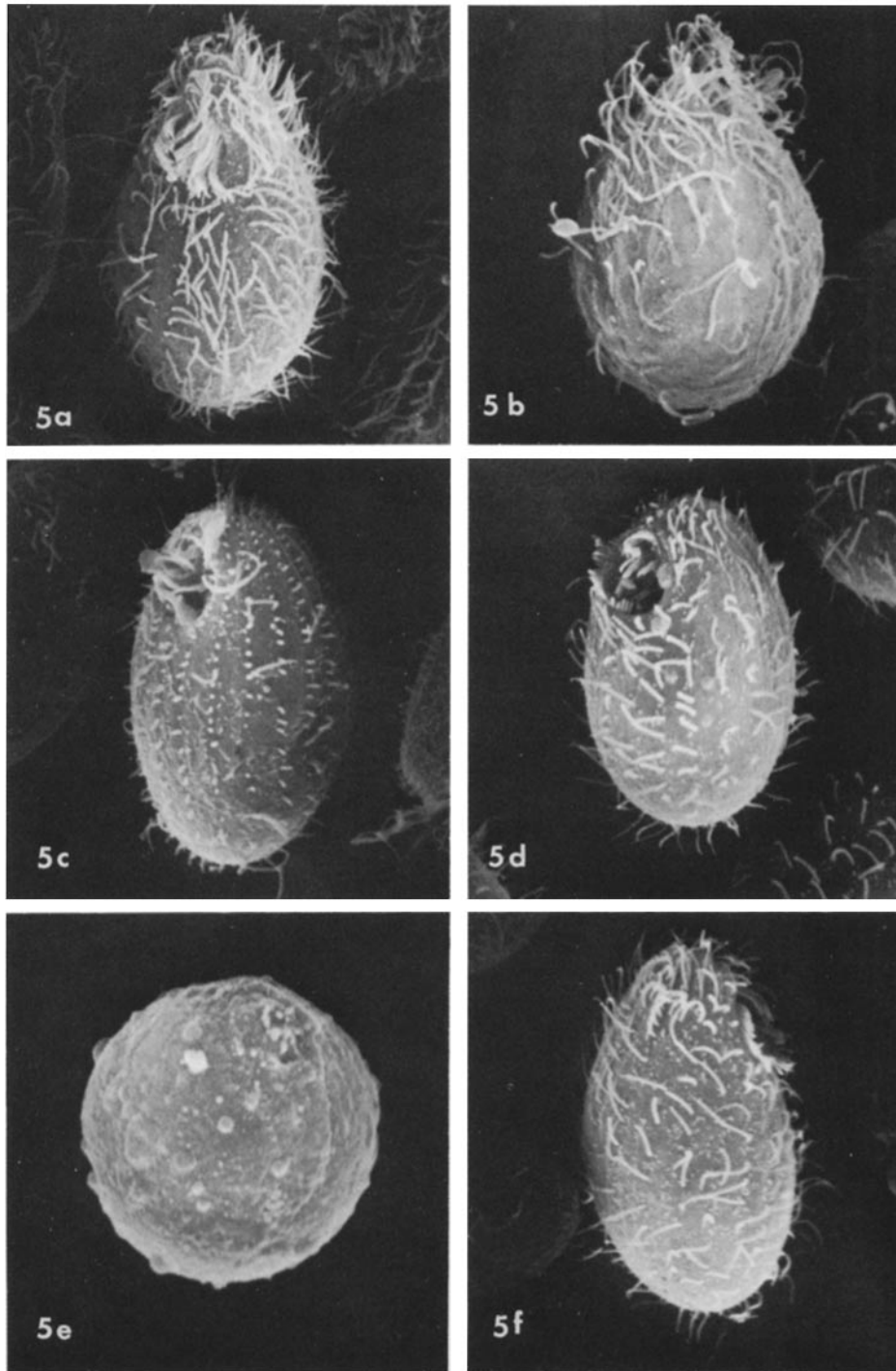


FIGURE 4 The effect of vinblastine on motility recovery in partially deciliated *T. pyriformis* (GL-C). At the time of partial deciliation, vinblastine, in varying concentrations, was added to samples of cells recovering in phosphate buffer. Recovery of motility was determined at 120 min post-deciliation.

FIGURE 5 Partial deciliation in *T. pyriformis* demonstrated with the scanning electron microscope. Fig. 5 *a*: a ciliated cell before partial deciliation. Fig. 5 *b*: a partially deciliated cell immediately after treatment. 75% of the somatic cilia were amputated; the remaining 25% were paralyzed. Fig. 5 *c*: a cell which was recovering in phosphate buffer containing 20 $\mu\text{g}/\text{ml}$ cycloheximide and fixed 20 min after partial deciliation. The cells in this sample were not motile, and the micrograph shows that essentially all cilia are short. Fig. 5 *d*: a cell fixed 2 h after partial deciliation. The cells were recovering in phosphate buffer containing 20 $\mu\text{g}/\text{ml}$ cycloheximide. The cells were motile in this sample, and the micrograph shows that many relatively long cilia are present. Fig. 5 *e*: a cell 2 h after partial deciliation, which immediately after partial deciliation, was placed in phosphate buffer containing 20 $\mu\text{g}/\text{ml}$ vinblastine. The remaining cilia were resorbed and no regeneration occurred. Fig. 5 *f*: a partially deciliated cell untreated with drugs at 2 h postdeciliation. After 2 h 90% of the normal ciliary volume is present. For calculation of ciliary volume, see Table II and text.



The histograms in Fig. 6 show the relative lengths and numbers of cilia per cell for cells in the presence of cycloheximide over a period of 120 min after partial deciliation. Ciliary lengths shifted progressively toward the shorter classes through the first 20 min. This shows that the old cilia were resorbed. From 30 min through 120 min, ciliary lengths shifted progressively to longer classes. At 12 min the distribution of lengths was marginally bimodal. Table I shows, by relative measure, changes in the absolute numbers of cilia per cell over the course of the experiment. The numbers of cilia decreased from 0 min to 6 min as the frequencies of ciliary lengths (Fig. 6) shifted toward the shorter classes. The earliest indication of increase in the number of cilia per cell occurred at 12 min. By 20 min, however, the numbers of cilia again decreased, probably a result of ongoing resorption. This second decrease was followed by a steady increase in numbers of cilia throughout the remainder of the experiment.

Ciliary volumes of cells undergoing regeneration in the presence and absence of cycloheximide are compared in Table II. Immediately after partial deciliation, the ciliary volume is 24% of the normal ciliary volume. By 2 h, cells regenerating in the presence of cycloheximide have a ciliary volume 38% of the normal, and untreated cells a ciliary volume of 92% of the normal. Partially deciliated cells treated with cycloheximide regenerate more cilia to short lengths, rather than fewer cilia to normal lengths. This is indicated by the observation that partially deciliated cells treated with cycloheximide regenerate 84% of the normal number of cilia (Table I) and only 38% of the normal ciliary volume (Table II).

After treatment of partially deciliated cells with vinblastine, resorption of cilia occurred but regeneration was prevented. By 2 h the cells were devoid of cilia (Fig. 5 e). At 4 h cilia were still absent, but when vinblastine was removed from the culture a recovery of motility began 30 min later and the normal pattern for recovery of motility followed.

DISCUSSION

Tetrahymena can regenerate cilia in the presence of cycloheximide when old cilia are available for resorption. This result is in contrast to that reported for completely deciliated cells where cycloheximide prevents ciliary regeneration (9). The coupling of ciliary resorption with ciliary regeneration when cycloheximide is present suggests that

resorbing cilia may provide materials which are reused by the cell in the development of replacement cilia.

In *Tetrahymena* that are partially deciliated and treated with cycloheximide the volume of the somatic ciliature immediately after partial deciliation is 24% of the normal, and the volume which is regenerated is 38% of normal (Table II). This difference (representing a 58% increase in ciliary volume over the starting volume) raises questions concerning the source of the ciliary components from which excess ciliary volume is formed. There are several possible explanations for these results. First, the excess ciliary volume may be developed from ciliary subunits provided by the resorption of that portion of the oral ciliature which remains after partial deciliation. Rosenbaum and Carlson have observed that the oral ciliature which remains after total deciliation is sloughed rather than resorbed (9). Partially deciliated *Tetrahymena* in this present study behaved in the same manner. Second, the excess ciliary volume may be illusory due to the differential fragility of the cilia at various times after partial deciliation, i.e., early culture samples of partially deciliated cells may be more sensitive to the manipulations required for preparation for the scanning electron microscope than later samples, resulting in proportionally more cilia being lost from the early samples. However, to minimize this problem, samples were removed from regenerating cultures by gentle pouring to limit shearing forces which might strip off cilia. Once cells are fixed, the differential fragility may not be a significant problem since the process of fixation is likely to strengthen the weakened linkage of cilia to the cell by the cross-linking of proteins. Nevertheless, the problem of differential fragility has not been entirely eliminated. Third, the excess ciliary volume may represent a contribution of components from a pool of ciliary precursors within the cytoplasm of the cell. The possibility that the excess ciliary volume is derived from a pool of ciliary precursors is of importance as a hypothesis, because it raises questions concerning the nature of precursor pools of proteins in relation to the control of ciliary regeneration in *Tetrahymena*.

Rosenbaum (9, 10) in several studies has assumed that the amount of regeneration which exceeds the amount of resorption without the synthesis of proteins is a measure of the size of the precursor pool. The data for partially and com-

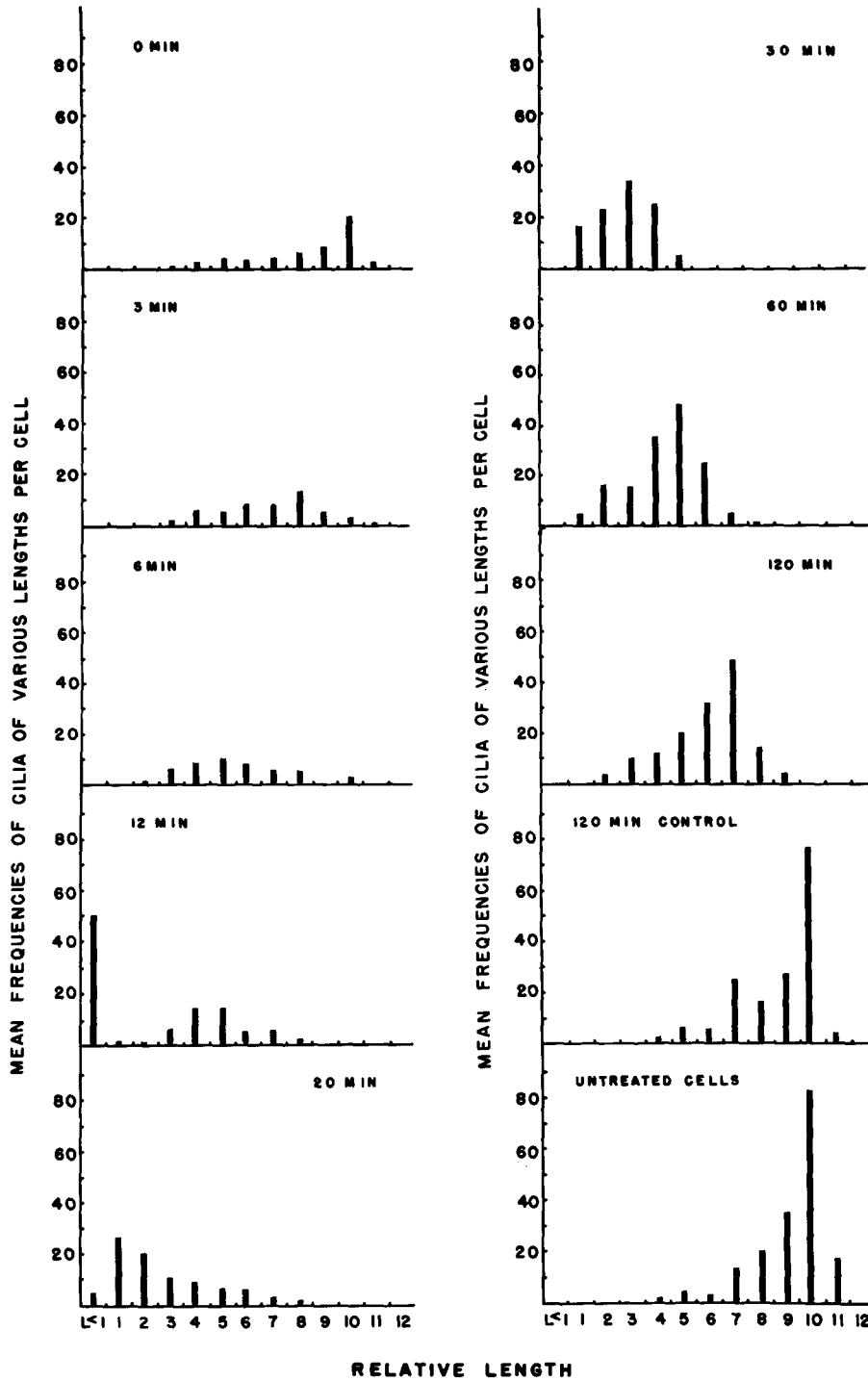


FIGURE 6 A series of histograms visualizing the frequency of cilia and their relative lengths per cell at various times after partial deciliation. Histograms labeled "0 min" through "120 min" show changes in the number and length of cilia observed in cells recovering in 20 $\mu\text{g}/\text{ml}$ cycloheximide. The length and frequency of cilia formed by 2 h, in partially deciliated cells untreated with cycloheximide, are shown in the histogram labeled "120 min control." The frequency distribution of relative ciliary length for logarithmically growing cells is shown in the histogram labeled "untreated cells."

pletely deciliated *Tetrahymena* taken together are in conflict with this assumption, since it appears unlikely that a pool would exist in the former case and not the latter.

The correlation of utilization of the pool of ciliary precursors with the resorption of a portion of the old ciliature indicates that the old ciliature may provide some factor(s) which functions to potentiate assembly of cilia from the pool of precursors. The term potentiation is suggested by the observation that the volume of cilia regenerated by partially deciliated cells treated with cycloheximide is greater than that which was

resorbed. This implies that the ratio of potentiating factor(s) to other ciliary components is less in the regenerated cilia than in the cilia which were resorbed. The nature of the potentiating factor(s) suggested by this study is unknown. The factor(s) may exist, as such, in mature cilia. It is also unknown if the potentiating factor(s) has any role in the function of mature cilia.

Ciliary components from resorbing cilia, including potentiating factor(s), may be reassembled into the structure of regenerated cilia in the presence of cycloheximide. However, the components of resorbed cilia need not be reincorporated into regenerated structures to explain regeneration in the presence of cycloheximide. All that the cell needs is a pool of ciliary precursors and some source of potentiating factor(s). There is information available suggesting that organelle components in *Tetrahymena* may be reused in the regeneration of replacement structures, but the evidence is not strong enough to preclude other hypotheses such as utilization of pooled organelle components never before assembled into organellar structure. Williams and Nelson, in their study of the replacement of oral structures in *Tetrahymena*, provide evidence which can be explained by either the pool hypothesis or the reutilization hypothesis (14). They have studied synchronous oral replacement under experimental conditions where net protein synthesis was prevented; the only label available for protein synthesis was from turnover of cell proteins and reincorporation of labeled turnover amino acids was minimized by an amino acid chase. They found that under these conditions new

TABLE I
Number of Cilia Per Cell During the Course of Ciliary Regeneration in Partially Deciliated *Tetrahymena* Treated with Cycloheximide*

Time†	Number of cilia per cell	Percent of normal
<i>min</i>		%
0	49 ± 5.9	28
3	48 ± 6.3	27
6	46.8 ± 6.48	26
12	122.3 ± 11.8§	70
20	87.5 ± 8.7§	50
60	147.5 ± 25.9	84
120	148.3 ± 23.5	84
120, no cycloheximide	168.5 ± 22.6	96
Log cells	176.9 ± 26.38	100

* $\mu\text{g/ml}$ cycloheximide.

† The lapse of time after partial deciliation.

§ The difference is significant at the 0.05 level but not at the 0.025 level using the t-distribution.

TABLE II
Relative Ciliary Volumes at Various Times during the Course of Ciliary Regeneration in Partially Deciliated *Tetrahymena*

Time*	Relative ciliary volumes‡	Percent of normal relative ciliary volume
<i>min</i>	<i>units/cell</i>	%
0	390.77 ± 49.17	24
120, 20 $\mu\text{g/ml}$	616.8 ± 87.48	38
120, cycloheximide	1470.38 ± 173.52	92
Normal log cells	1606.3 ± 210.20	100

* The lapse of time after partial deciliation.

‡ Total ciliary volume for each sample is calculated from the data presented in Fig. 6 by multiplying the frequency in each length class by the length, and summing all length classes. For each sample all the somatic cilia were measured on no less than six cells and at least 500 cilia were measured. Ciliary length is directly proportional to volume; cilia are cylindrical and uniform in diameter.

oral structures formed during synchronous oral replacement were only slightly less heavily labeled than those they replaced. These authors indicate that reutilization of oral apparatus components is a possibility in oral replacement, but suggest that there may be other sources for oral apparatus subunits as well. In both the replacement study by Williams and Nelson (14) and this ciliary regeneration study, it is difficult to determine whether or not there is any reutilization of organelle components from resorbed structures in the development of regenerated structures. A decision regarding this question may be possible if some direct means is developed for measuring the actual quantity of organelle components in regenerated structures which have been derived from resorbed organelles.

The process of ciliary regeneration and resorption can occur simultaneously in *Tetrahymena*. This conclusion is supported by the partial bimodality shown in the histogram plot in Fig. 6 of the relative ciliary lengths at 12 min. Data on the numbers of cilia present at various times throughout regeneration in the presence of cycloheximide which are presented in Table I further support this conclusion by showing, first, an increase, and then, a decrease in the number of cilia between 6 and 20 min. These data suggest that a resorbing population and a regenerating population of cilia are present between these two points of time. Further evidence supporting this conclusion is the observation that both partially and completely deciliated cells begin recovery of motility at the same time. If initiation of regeneration in partially deciliated cells required the completion of resorption, then regeneration would be delayed for a period at least as long as the period required for resorption. This was not observed.

It is likely that resorption is completed for most individual cilia before regeneration is initiated at the same site. This is suggested by the decrease in the number of cilia between 12 and 20 min and is probably due to the resorbing population, which existed at 12 min, having been resorbed, or nearly so, by 20 min. Two distinct and separate mechanisms may control regeneration and resorption of cilia. It is common in biochemical systems for synthesis and degradation to occur as separate processes rather than one being simply the reverse of each other. Similarly, assembly and disassembly may not be the reverse of each other.

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