Regeneration of Cilia in Starved *Tetrahymena thermophila* Involves Induced Synthesis of Ciliary Proteins but not Synthesis of Membrane Lipids

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The synthesis of ciliary-membrane phospholipids and ciliary proteins was studied after deciliation in starving *Tetrahymena thermophila* cells. Deciliated cells regenerated the new ciliary membrane without any induced phospholipid synthesis. The constant cell volume found during the regrowth of the cilia suggests that renewal of ciliary membranes takes place by insertion of intracellular membrane material into the cell surface. In contrast with the absence of induced phospholipid synthesis during ciliary regeneration, the synthesis of ciliary proteins was found to be induced. This enhanced synthetic activity was made possible by an increased rate of intracellular protein degradation in regenerating cells. It was found that the extent of the induced synthesis strongly depends upon the growth conditions of the cells before starvation. Furthermore, it was shown that the degree of induced protein synthesis is greater for higher-molecular-weight ciliary proteins than for lower-molecular-weight species.

The regeneration of cilia and flagella in both unicellular and multicellular organisms has emerged in recent years as a useful model system for the study of the biogenesis of non-autonomous cell organelles. The problem has been studied most extensively in *Chlamydomonas*, where both biochemical (Rosenbaum *et al.*, 1969; Weeks & Collis, 1976; Weeks *et al.*, 1977; Lefebvre *et al.*, 1978) and mutational (Luck *et al.*, 1977; Shiota *et al.*, 1979) analyses have been carried out. Some of the same advantages for the study of these problems are afforded by the ciliated protozoan, *Tetrahymena*. *Tetrahymena* is a microbial eucaryote with about 800 cilia per cell, it grows well in culture, deciliation methods applicable to *Tetrahymena* have been published (Rosenbaum & Carlson, 1969; Thompson *et al.*, 1974), and a great deal of background information is available on its morphology, physiology, and biochemistry (Hill, 1972; Elliott, 1973). This system is especially well suited for the study of macromolecular biosynthesis and the related assembly processes involved in the regeneration of cilia.

Tubulin is the most prominent structural protein in both cilia and flagella, and the synthesis of this molecule has been intensively studied in several systems, including *Tetrahymena* (Nelsen, 1975; Williams, 1975; Guttman & Gorovsky, 1979), sea urchin embryos (Stephens, 1977; Merlino *et al.*,

1978), and *Chlamydomonas* (Weeks & Collis, 1976; Weeks *et al.*, 1977). These reports focus on the regulation of biosynthesis of a single protein (tubulin), but the recent developments in the understanding of the biochemistry and biophysics of the cell membranes of *Tetrahymena* (Thompson & Nozawa, 1977; Allen, 1978), together with the fact that 50% of the cell-surface membrane covers the cilia (Satir & Satir, 1974), prompt an effort to extend the study of ciliary regeneration to include aspects of the renewal of the ciliary membrane. We have therefore initiated a detailed study of the renewal of both ciliary structural proteins and the ciliary membrane. In this paper we report on the induced synthesis of ciliary proteins, but find no induction of phospholipid synthesis associated with ciliary regeneration in *Tetrahymena thermophila*. Moreover, it was found that a large proportion of the protein used in the regeneration of cilia comes from pre-existing pools within the cell. The importance of control mechanisms responsible for the mobilization of stored phospholipid and protein reservoirs for our understanding of organelle biogenesis in general is discussed.

Experimental

Growth conditions and volume determinations

Tetrahymena thermophila was grown axenically at 22°C in 200 ml of an enriched 2% (w/v) proteose

Abbreviation used: SDS, sodium dodecyl sulphate.

peptone medium (Thompson, 1967) in a 500 ml Erlenmeyer flask incubated on a reciprocal shaker until the culture reached the mid-to-late-logarithmic phase of growth. Cells to be studied under starvation conditions were harvested by gentle centrifugation (300 g for 5 min) and after two washes in an inorganic medium (Hamburger & Zeuthen, 1957) they were suspended in inorganic medium at the same cell density as before the wash. Cell number was measured with a Coulter counter model B. Cells used for volume determinations were fixed for 1–2 h in 2% glutaraldehyde in inorganic medium, and a cell-volume distribution was obtained using a Coulter Channelyzer attached to a model 2B Coulter counter.

Deciliation

Cells for deciliation, either in proteose peptone or in inorganic medium, were concentrated to 1×10^7 – 7×10^7 cells in 3 ml of their own medium and deciliated using the calcium-shock method of Rosenbaum & Carlson (1969). The cilia were harvested immediately after the deciliation by centrifugation at 25000 g for 15 min, and the deciliated cells were allowed to regenerate new cilia for 3–4 h in either proteose peptone or inorganic medium. At this time, phase contrast microscopy showed that the full complement of the new cilia had emerged, and the cells were exposed to a second round of deciliation. The cilia were collected as described above.

Lipid analysis

Rates of phospholipid synthesis and cellular phosphate uptake were measured by pulse labelling deciliated and control cells with from 35 to 150 μ Ci of carrier-free [32 P]P_i for a 30 min or 2 h period. The lipids were extracted from whole cells and cilia by the method of Bligh & Dyer (1959), and were washed twice (Folch *et al.*, 1957) to remove any remaining trace of water-soluble phosphate label. The phosphorus content was measured by the method of Bartlett (1959), as modified by Marinetti (1962).

Protein analysis

Cells that were regenerating cilia were analysed for induced protein synthesis by a 14 C/ 3 H double-labelling experiment. Cells from the mid-logarithmic phase of growth were washed and suspended in inorganic medium to give a final cell density of 4×10^5 cells/ml. A portion (50 ml) of the culture was labelled with 250 μ Ci of DL-[1- 14 C]leucine (sp.act. 53 Ci/mol) for 4 h. Then the culture was washed and 'chased' with unlabelled 1 mM-leucine for 1 h. The culture was split in halves, one of which was deciliated; the other half was used as the control. Control and deciliated cells were each incubated for 3 h in inorganic medium containing 500 μ Ci of

L-[4,5- 3 H]leucine (sp. act. 59 Ci/mmol). At this time both cultures were deciliated, and the cilia were solubilized in hot SDS.

The samples were analysed by SDS/polyacrylamide gel electrophoresis by the procedure of Laemmli (1970). The proteins were separated in 7.5% tube gels 6.5 cm long, and the gels were run at 3 mA/tube. The gels were stained for 3 h in 0.25% (w/v) Coomassie Brilliant Blue solution in methanol/water/acetic acid (5:5:1, by vol.), destained in the same solution without dye, and stored in 7.5% (w/v) acetic acid. Absorbance profiles were obtained by scanning the destained gels at 550 nm in a Beckman spectrophotometer.

The radioactivity contained in the separated proteins in the gels was determined after cutting the gels into successive 1 mm slices with a Joyce Loeb gel slicer. Each slice was put into a scintillation vial and solubilized by shaking overnight with 100 μ l of water and 500 μ l of Protosol solubilizer. Scintillation fluid [10 ml of Packard Permafluor/toluene (1:24, v/v)] was added and the radioactivity was counted in a Beckman LS 335 liquid scintillation counter.

A semi-quantitative method was also used to detect the induced synthesis of both ciliary and non-ciliary proteins. Cells growing in the standard medium was harvested in logarithmic phase, washed and suspended in 50 ml of inorganic medium at a density of 2.5×10^5 cells/ml. After 5 h, the culture was divided into two samples. One sample was deciliated and allowed to regenerate cilia for 4 h in the presence of [14 C]leucine (Amersham) at a concentration of 5.24 μ Ci/ml. The control sample was kept in the presence of [14 C]leucine without deciliation. After 4 h, the samples were deciliated, the cilia and the deciliated cell bodies from both samples were solubilized, and the proteins were separated by SDS/polyacrylamide gel electrophoresis in a 7.5% slab gel. The gel was stained with Coomassie Blue, dried and incubated for 4 days next to Kodak SB-5 X-ray film before development.

Effect of growth conditions on the degree of induced protein synthesis

The proportions of newly synthesized tubulin and dynein in regenerated cilia were determined after growing the cells under various conditions. Cells were grown to stationary phase (10^6 cells/ml) in the standard rich medium, to logarithmic phase (3×10^5 cells/ml) in this medium, and to logarithmic phase (10^5 cells/ml) in a relatively poor medium, 1% (w/v) proteose peptone. The cells were washed twice and suspended in 50 ml of inorganic medium at 5×10^5 cells/ml and starved for 5 h in the presence of 1 mCi of [3 H]leucine (Amersham). Each culture was then washed twice in inorganic medium and divided into two samples. One sample was deciliated and allowed to regenerate cilia (4 h) in the presence of

unlabelled 1 mM-DL-leucine; the other sample was kept in unlabelled 1 mM-leucine for 4 h without deciliation. Both samples were then deciliated, the cilia were solubilized in 250 μ l of SDS solubilizing solution (Laemmli, 1970), and the proteins were separated in 7.5% polyacrylamide gels. The gels were stained in Fast Green (Gorovsky *et al.*, 1970) and scanned using an Isco model UA-5 recording densitometer. The gels were then sliced and counted for radioactivity using a Beckman Model LS-200B liquid scintillation counter. Specific activities of tubulin and dynein in the regenerated and control cilia were determined and are reported as c.p.m./mg of paper wt. under the peaks in the densitometric scans. The deciliation procedure was monitored using scanning electron microscopy.

Effect of Ca^{2+} and ionophore A 23187 on protein degradation

The effect of Ca^{2+} and calcium ionophore on intracellular protein degradation was analysed in starving cells. After two washes, a logarithmic-phase culture was suspended in 150 ml of inorganic medium containing 50 μ Ci of L-[4,5- ^3H]leucine (sp. act. 48 Ci/mmol) and incubated for 5 h at 28°C. The cells were washed twice, suspended in inorganic medium, and the culture was divided into four parts. One part was deciliated using the normal procedure, one was exposed to 5.0 μM - Ca^{2+} , and another was exposed to 5 μM - Ca^{2+} + 5.5 mM-ionophore A 23187 for 10 min, which is the maximum concentration that could be used without any spontaneous deciliation or excessive mucus production. The remaining part was kept as a control. The degradation of proteins was measured over an 18 h period in the presence of unlabelled 5 mM-leucine to prevent any re-incorporation of trichloroacetic-acid-soluble material. At each time point at which protein degradation was measured, a portion (400 μ l) was removed in triplicate and 100 μ l of bovine serum albumin solution (7 mg/ml) was added to ensure complete precipitation of all the labelled proteins; the cells were then broken in 4% trichloroacetic acid. The acid-insoluble material was removed by centrifugation. From each supernatant a 400 μ l aliquot was removed for scintillation counting. The total cellular activity was determined in two ways: either a portion (400 μ l) of the culture was removed for liquid scintillation counting, or the trichloroacetic-acid precipitate was resolubilized in a mixture of 0.1 M-NaOH and 0.4% sodium dodecyl cholate. In the latter case, the total activity was found by adding the c.p.m. from the supernatant to those of the redissolved precipitate. There was good agreement between the data obtained from both methods.

Chemicals

All chemicals were reagent grade and used without any further purification. The Ca^{2+} -iono-

phore, A 23187, was a gift from the Lilly Research Laboratories to Professor U. V. Lassen, August Krogh Institute, University of Copenhagen.

Results

Ciliary regeneration in inorganic medium

Completely deciliated *Tetrahymena* regenerated cilia at essentially the same rate in proteose peptone or inorganic medium, as judged by phase-contrast microscopy of the swimming ability of the deciliated cells (Fig. 1). Furthermore, when the starving cells were exposed to three consecutive rounds of deciliation spaced 3 h apart, we found that this extensive deciliation did not significantly affect the viability and the regeneration kinetics of the cells. The ability of the cells to overcome this massive loss of ciliary material shows that the system is favourable for detailed studies of the biogenesis of both ciliary membranes and ciliary axonemal structures.

Phospholipid synthesis

The rate of phospholipid synthesis in starving cells was assessed by pulse labelling the cells for 30 min intervals with [^{32}P]P_i. It was found that the rate of phospholipid synthesis in cells, that had been starved for 5 h, was less than 1% of the rate observed in exponentially growing cells (Table 1).

Approximately 50% of the cell surface area is lost during the deciliation (Satir & Satir, 1974). Since phospholipid synthesis in starved non-deciliated cells after 5 h in the inorganic medium is almost abolished, it seemed unlikely that this rate of

Fig. 1. *Ciliary regeneration in Tetrahymena*
Recovery of cellular motility of deciliated *Tetrahymena* was measured. O, Exponentially growing cells suspended in rich medium after deciliation; Δ, cells starved for 5 h prior to deciliation, after which the cells were incubated in inorganic medium.

Table 1. *Phospholipid synthesis during ciliary regeneration*

The incorporation of ^{32}P into whole cell phospholipids was measured in exponentially growing, starved, and starved deciliated cells. Two 20ml aliquots from each of the cultures were labelled with carrier-free $^{32}\text{P}[\text{P}_i]$ for 30 min, after which the incorporation of label was stopped by pouring the cells into chloroform/methanol (6:1, v/v). The lipids were extracted by using the method of Bligh & Dyer (1959), washed twice according to Folch *et al.* (1957), and the lipid specific activity determined as c.p.m./ μg lipid phosphorus.

Experiment	Time in starvation medium (h)	$10^{-7} \times$ Number of cells harvested	$^{32}\text{P}[\text{P}_i]$ added (μCi)	$10^{-4} \times$ Radioactivity in lipid (c.p.m.)	Lipid P (μg)	Specific activity (c.p.m./ μg of lipid P)
Logarithmic cells	—	0.96	35	340	66.0	5200
Non-growing control	5.5	1.0	35	0.248	56.0	44.3
Non-growing deciliated	5.5	0.92	35	0.157	41.0	38.3
Non-growing control	6.5	1.8	150	1.62	116.5	139.0
Non-growing deciliated	6.5	1.6	150	1.24	112.5	110.2

phospholipid biosynthesis would be able to support the renewal of the new ciliary membrane. We therefore tested the possibility that deciliation may trigger an induced synthesis of phospholipid of sufficient magnitude to support the extensive membrane renewal associated with ciliary regeneration. Several experiments were carried out in which cells in the process of regenerating cilia were incubated with ^{32}P for up to 2 h. The complete absence of ^{32}P incorporation into the regenerating ciliary membranes in every case suggested that these membranes are formed from previously existing membrane material within the cell rather than by the assembly of newly synthesized phospholipids.

Since our labelling periods did not exceed 2 h, failure to detect any lipid labelling in the ciliary membranes could be accounted for by a time lag between time of synthesis and assembly of the ciliary membrane, as described by Nozawa & Thompson (1972). We therefore turned to an analysis of phospholipid synthesis in whole cells. Deciliated and non-deciliated starved cells were pulse labelled with ^{32}P for 30 min either immediately after deciliation, or at the terminal stage of ciliary elongation (1–1.5 h after deciliation). The results in Table 1 clearly show that no induced phospholipid synthesis takes place at the time of active ciliary renewal. On the contrary, a 15–20% decrease in ^{32}P incorporation into phospholipids was detected, thus favouring the idea that deciliation and reciliation do not give rise to any immediate phospholipid biosynthesis. The regeneration of new ciliary membranes must therefore occur at the expense of previously formed membranes.

Volume changes during ciliary regeneration

In view of the fact that regenerated ciliary membranes are formed in the absence of phospholipid synthesis, the possibility arises that the cells might compensate for the extensive loss of surface membrane by reducing the cellular volume. Accordingly, the cellular volume was measured in glu-

taraldehyde-fixed cells throughout the initial starvation period and during ciliary regeneration in inorganic medium. As expected, control non-deciliated cells showed a significant reduction in volume during the first few hours of starvation (Table 2). Following this, the cell size was then stable over the next 24 h. Light-microscopic examination of the 3 h starved culture revealed that up to 40% of the cells were in division; this effect is likely to be responsible for the initial decrease in mean cell size. No further cell divisions were seen after 5 h in starvation medium.

Cells that were deciliated after 5 h in starvation medium showed an initial cell volume only slightly smaller than that of the non-deciliated control cells. In addition, no significant change in cell volume occurred in these cells during the period of ciliary regeneration (3 h). These data do not support the hypothesis that the bulk of the membrane associated with regenerated cilia originates as a result of a redistribution of the pre-existing cell surface remaining after deciliation; this would require a decrease in cell volume which was not seen. Rather, it appears that the membrane associated with regenerated cilia is in fact new membrane, formed largely from precursors within the cell. No explanation can be offered for the significantly smaller size of the regenerated cells after 24 h in starvation medium (Table 2).

Protein synthesis

The synthesis of ciliary proteins in starved and in starved deciliated cells was analysed by determining the amount of induced synthesis during ciliary regeneration. A cell culture in the initial starvation period was labelled with ^{14}C leucine for 5 h, 'chased' with excess unlabelled leucine, then halved; one half was deciliated and the other half was kept as a control. Control cells and cells regenerating cilia were then labelled with ^3H leucine for 3 h in order to determine the rates of protein turnover in accord-

Table 2. *Cell volume changes during ciliary regeneration*

Cell volume measurements of deciliated and non-deciliated *Tetrahymena* in proteose peptone or in inorganic medium were carried out. The results are means $\pm$ s.d. for the number of different experiments indicated. The cells were fixed and sized as described in the text. The average cell spherical volume was calculated from the frequencies of cell volumes obtained in 100 channels. Each channel volume was found by calibration with latex beads having known diameters.

	Time in starvation medium (h)	Cell volume (μm^3)	Cell surface (μm^2)	Number of experiments	Surface area (% of control)	r (μm)
Log phase cells		9176 $\pm$ 540	2121 $\pm$ 82	6		13.00
Control non-growing	5	4742 $\pm$ 294	1365 $\pm$ 57	4	100	10.42
Deciliated (15 min) non-growing	5	4038 $\pm$ 185	1226 $\pm$ 37	4	90	9.88
Control non-growing	8	5322 $\pm$ 230	1475 $\pm$ 43	4	100	10.83
Deciliated (3 h) non-growing	8	4021 $\pm$ 151	1223 $\pm$ 31	4	83	9.87
Control non-growing	24	5005	1416	2	100	10.62
Deciliated (21 h) non-growing	24	3101	1029	2	73	9.05

Fig. 2. *Double-labelling of ciliary proteins in control cells and in cells regenerating cilia*

Densitometry scans of proteins from (b) original cilia and (d) regenerated cilia are shown. The upper parts of the figure show the incorporation of [^{14}C]leucine during 5 h of incubation in inorganic medium followed by 3 h of incubation in the same medium containing [^3H]leucine in (a) control cilia and (c) regenerated cilia. The radioactivity patterns are from the scanned gels shown in the lower parts. For experimental detail see the text.

ance with the method of Arias *et al.* (1969). At the end of the 3 h [^3H]leucine labelling period both control and regenerated cilia were harvested, solubilized in hot SDS solution, and the proteins were separated by SDS/polyacrylamide gel electrophoresis. The gels were stained with Fast Green, scanned with a recording densitometer, then sliced, and the amounts of ^{14}C and ^3H were determined by liquid scintillation counting.

The absorbance profiles of both control (Fig. 2b) and regenerated (Fig. 2d) cilia show the major proteins, tubulin and dynein (arrows). The proteins present in the regenerated cilia correspond well with the control ciliary proteins, except for minor differences in the amounts of low-molecular-weight proteins. However, one protein with an apparent molecular weight of about 70000 is much more predominant in the regenerated cilia.

While the [^{14}C]leucine incorporation into ciliary proteins in control (Fig. 2a) and regenerated (Fig. 2c) ciliary proteins does not differ significantly, the incorporation of [^3H]leucine into the regenerated cilia is almost an order of magnitude higher than in control cilia. Since the labelling of ciliary proteins with [^3H]leucine reflects the rate of ciliary protein synthesis after the removal of the original cilia, the large increase in labelling with ^3H must be due to an enhanced synthesis of ciliary proteins caused by the deciliation. Significantly, all proteins in the regenerated cilia were synthesized at an enhanced rate. According to Arias *et al.* (1969), the ratio $^3\text{H}/^{14}\text{C}$ reflects the rate of turnover of proteins. This ratio has been calculated for control (Fig. 3, lower curve) and for regenerated (Fig. 3, upper curve) cilia. Only minor variations in the rate of turnover exist among the original ciliary proteins, but the regenerated ciliary proteins show a wider variation of turnover rates. Furthermore, the negative relationship between the $^3\text{H}/^{14}\text{C}$ ratio and the molecular weight of the proteins from regenerated cilia indicates that higher-molecular-weight proteins exhibit a greater degree of induced protein synthesis than do lower-molecular-weight species. This correlation mimics the relative rate of membrane-protein degradation in liver cells, where higher-molecular-weight proteins show the fastest turnover rate (Dehlinger & Schimke, 1971).

Although the double-labelling experiment shows the relative amounts of induced synthesis for all proteins found in regenerated cilia, it does not indicate what proportion of the total this may represent for any protein contained in cilia. Accordingly, we attempted to determine this in a separate experiment. The cells were first pulsed with [^3H]leucine (20 $\mu\text{Ci}/\text{ml}$) for 5 h in starvation medium. The culture was then divided, one half was deciliated, and both halves were incubated for 4 h in the presence of unlabelled 1 mM-leucine, during which time cilia regenerated in the deciliated sample. At the

end of this interval, both samples were deciliated, and the cilia were solubilized in hot SDS solution. The specific activities of the major ciliary proteins, tubulin and dynein, from both regenerated and non-regenerated cilia were determined as described in the Experimental section. The results are presented in Table 3. The experiment was carried out using cells grown under three different conditions, as indicated.

The percentage difference in specific activity between the proteins in control and regenerated cilia is a measure of the proportion of the total amount of each protein in regenerated cilia that is the result of induced synthesis. For cells grown to stationary phase in rich medium, it can be seen (Table 3) that only 15% of the tubulin found in regenerated cilia was the result of induced synthesis, whereas about 32% of the dynein was induced. For cells grown to logarithmic phase in rich medium, induced tubulin was 27% and induced dynein 62%, and for cells grown in poor medium induced tubulin was 66% of the total. The first point to be made is that the amount of induced synthesis for dynein was always greater than for tubulin (where both were measured), thus confirming the relationship between the amount of induced synthesis and the molecular weight, first seen in the double-labelling experiment.

In addition, it can be seen that the amount of a given protein in regenerated cilia that is the result of

Fig. 3. Turnover rates of proteins from original cilia and regenerated cilia from starving *Tetrahymena*

The $^3\text{H}/^{14}\text{C}$ ratios of ciliary proteins were calculated on the basis of the radioactivity profiles of original and regenerated cilia (Figs. 2a and c, respectively).

induced synthesis varies according to the way the cells are grown. For example, the proportion of the total amount of tubulin in regenerated cilia resulting from induced synthesis varied from 15% in the rich medium to 66% in the poor medium. Cells grown in rich medium apparently rely much more heavily upon previously synthesized pools of ciliary precursors than do cells grown in poor medium. This in turn suggests that ciliary precursor pool sizes in *Tetrahymena* may vary widely, depending upon how the cells are grown.

Both double- and pulse-labelling experiments reveal a molecular-weight-dependent enhancement of protein synthesis following the deciliation. The deciliation procedure itself (Rosenbaum & Carlson, 1969) involves the administration of high concentrations of Ca^{2+} , which has been shown to accelerate the degradation of Z-discs in muscle fibres (Dayton *et al.*, 1976). Our results might therefore be explained by a Ca^{2+} -activated proteolysis of the more susceptible higher-molecular-weight proteins, due to the elevated calcium concentration during the

deciliation, leaving behind cells depleted of pools of some ciliary proteins. In order to simulate the elevated Ca^{2+} concentration causing deciliation this ion was administered together with the Ca^{2+} ionophore A 23187 in concentrations low enough to avoid deciliation. Starved cells were labelled with [^3H]leucine, 'chased' with unlabelled leucine and either deciliated or exposed to Ca^{2+} with or without the ionophore. The rate of protein degradation was measured as the production of trichloroacetic acid-soluble radioactivity in the samples indicated in Table 4. The deciliated cells clearly showed an enhanced cellular protein degradation. Neither of the samples exposed to Ca^{2+} nor the combination of ionophore and the ion showed any difference in the rate of protein degradation. These results lead to the conclusion that it is the removal of ciliary material, not secondary factors, that causes both the increased breakdown of existing proteins and the induced synthesis of ciliary proteins.

Changes in the pattern of protein metabolism accompanying ciliary regeneration were also in-

Table 3. *Proportions of newly synthesized proteins in regenerated cilia*

For experimental details see the text. Specific activities are reported as c.p.m./mg of paper wt. under peaks in densitometric tracings of the gels. The amount of ^3H was 10^4 – 10^5 c.p.m. in all peaks. The values reported are based on a minimum of three gels in each case and are rounded off to the nearest whole number. The values are means $\pm$ s.d. Within each pair of measurements, the value in regenerated cilia is given as a percentage of the control value (= 100%).

Growth conditions	Protein	Specific activities	
		(c.p.m./mg)	(%)
Enriched medium; stationary phase	Tubulin	Control	156 $\pm$ 7
		Regenerated	133 $\pm$ 2
	Dynein	Control	278 $\pm$ 30
		Regenerated	188 $\pm$ 8
Enriched medium; logarithmic phase	Tubulin	Control	486 $\pm$ 48
		Regenerated	354 $\pm$ 38
	Dynein	Control	1781 $\pm$ 344
		Regenerated	675 $\pm$ 81
Proteose-peptone; logarithmic phase	Tubulin	Control	362 $\pm$ 22
		Regenerated	122 $\pm$ 11

Table 4. *Protein degradation in deciliated cells and in cells exposed to ionophore A 23187 and to Ca^{2+}*

Exponentially growing cells were washed and suspended in inorganic medium containing $0.33\mu\text{Ci}$ of L-[^3H]leucine/ml for 5 h. Intracellular protein degradation was tested in the presence or absence of Ca^{2+} ($5\mu\text{M}$) and ionophore (5.54 nM) as indicated. Aliquots were removed in triplicate from the cultures and analysed for trichloroacetic-acid-soluble radioactivity as described in the text.

	Time	Acid-soluble radioactivity (% of total)				
		15 min	30 min	1 h	3 h	18 h
Deciliated cells		4.7	7.6	10.6	22.1	40.0
Control non-deciliated cells		5.0	6.4	7.8	11.8	36.6
+ Ionophore		4.9	6.3	7.4	12.0	38.7
+ Ca^{2+}		4.8	6.2	7.8	11.8	36.1
+ Ionophore + Ca^{2+}		4.9	7.9	7.5	12.6	38.7

investigated using an autoradiographic method. Cells regenerating cilia and non-deciliated control cells were pulsed for 4 h with [^{14}C]leucine (5.24 $\mu\text{Ci/ml}$). Both cilia and deciliated cell bodies of control and regenerating samples were solubilized and the proteins were separated by SDS/polyacrylamide-gel electrophoresis. The gels were stained with Coomassie Blue, dried, and incubated for 4 days next to X-ray film before developing. The results are

Fig. 4. Changes in the pattern of protein synthesis accompanying ciliary regeneration in starving *Tetrahymena*

Control cells and cells regenerating cilia were pulse-labelled with [^{14}C]leucine (5.24 $\mu\text{Ci/ml}$). After 4 h, cilia were removed, samples were solubilized, and the proteins were separated by SDS/polyacrylamide gel electrophoresis. The dried gels were subjected to autoradiography, then developed after 4 days. Equal amounts of regenerated (a and c) and control (b and d) cilia were compared, and equal amounts of the deciliated cell bodies from regenerating (e) and control (f and g) cells were compared. The results indicate an induced synthesis of ciliary proteins accompanied by a decrease in the rate of synthesis of cell-body proteins. The one exception (labelled X) apparently represents the induced synthesis of a non-ciliary protein.

presented in Fig. 4. Whereas Coomassie Blue staining of lanes (a)–(d) was equal, the autoradiogram shows that all proteins in regenerating cilia (a and c) are much more radioactive than in the control (b and d), indicating the induced synthesis of ciliary proteins. In general, the opposite effect was seen in the deciliated cell bodies. Whereas Coomassie Blue staining of lanes (e)–(g) was equal, the autoradiogram shows that non-ciliary cellular proteins in the regenerating cells (lane e) are less heavily labelled than in the control cells (f and g). The one exception (labelled X) apparently represents the induced synthesis of a non-ciliary protein.

Discussion

It was first shown by Nozawa & Thompson (1972) that synthesis of phospholipids in starving *Tetrahymena* is negligible. The present study shows that this condition persists during the regeneration of large amounts of ciliary surface membrane. A possible explanation for this is that the newly regenerated cilia are covered with old surface membrane which migrates over the cilia during the regeneration process. This hypothesis predicts that a significant decrease in cell volume should accompany the regeneration of cilia. However, our measurements show that no such decrease in cell volume occurs. This leads to the alternative conclusion that ciliary regeneration is supported by the formation of new surface membrane from membrane precursors that are stored within the cell.

A possible internal source of surface membrane in *Tetrahymena* has been suggested by Satir *et al.* (1976). Working with *T. pyriformis*, these authors have presented ultrastructural evidence that suggests that the membrane surrounding the cortex organelles of exocytosis, called mucocysts, may integrate into the surface at the time of their discharge; in this way, it may contribute to the formation of surface membrane. Moreover, these authors find that mucocyst discharge accompanies the dibucaine-induced deciliation of *T. pyriformis*. Unfortunately, the mucocysts are unstable organelles that rapidly disintegrate when outside the cell or in cell-free preparations. Our attempts to isolate the organelle for biochemical analysis have been unsuccessful, and no information about mucocyst membrane composition is available. Further experimental work on mucocyst behaviour may allow a judgement of the role of these membranes for surface-membrane assembly during ciliary regeneration.

The double-labelling experiment with radioactive amino acids (Figs. 2 and 3) has shown that ciliary proteins, unlike phospholipids, are synthesized at accelerated rates during the regeneration of cilia in *Tetrahymena thermophila*. This was confirmed by the autoradiographic experiment (Fig. 4). The

induced synthesis of proteins has also been shown in studies of the regeneration of cilia in both sea urchin embryos (Stephens, 1977) and *T. pyriformis* (Guttman & Gorovsky, 1979), and in the regeneration of flagella in *Chlamydomonas* (Weeks & Collis, 1976; Lefebvre *et al.*, 1978) and *Polytomella* (Brown & Rogers, 1978). However, the methods employed in the present study have additionally revealed a novel relationship between synthesis and size: a direct relationship was found between protein molecular weight and the extent of induced synthesis during ciliary regeneration. The suspicion arose that this relationship might be an artifact induced by the Ca^{2+} shock used in the deciliation procedure. It is known that large proteins are degraded by proteases at faster rates than smaller ones, and it has been shown recently that intracellular proteases can be activated by high levels of Ca^{2+} in some systems (Dayton *et al.*, 1976). Accordingly, we compared the degradation rates of intracellular proteins in deciliated cells with undeciliated cells, some of which were treated with high levels of Ca^{2+} in the presence and absence of ionophore A 23187. The results showed that there is an elevated level of protein degradation in deciliated cells that is not mimicked by artificially elevated intracellular Ca^{2+} levels in non-deciliated cells. We are led to the conclusion that it is the removal of cilia *per se* that leads to a molecular-weight-dependent induced synthesis of ciliary proteins. Other direct cellular responses to deciliation apparently include an increase in the degradation rate (Table 4), and a decrease in the synthesis rate (Fig. 4), for general cellular (non-ciliary) proteins.

Although deciliation induced the synthesis of ciliary proteins as described above, it was found that much of the protein actually used in the regeneration of cilia comes from cytoplasmic pools which pre-exist within the cell. Our experiments have shown that a variable amount of newly synthesized tubulin and dynein, and presumably all other proteins as well, are found in regenerated cilia; the exact amount depends on how the cells are grown prior to starvation (Table 3). Under all conditions tested, however, the induced synthesis of dynein was greater than the induced synthesis of tubulin. This confirms the relation between the extent of induced synthesis of ciliary proteins and their molecular weight first seen in the double-labelling experiment.

The influence of growth conditions upon the amount of induced protein synthesis shown here may explain an apparent disagreement between two previous reports. Working with *T. pyriformis*, Nelsen (1975) and Guttman & Gorovsky (1975, 1979) independently discovered that *Tetrahymena* can regenerate cilia in starvation medium, and both attempted to determine whether induced tubulin synthesis could be detected under these conditions.

Nelsen (1975) reported little or no induced tubulin synthesis and Guttman & Gorovsky (1975, 1979) reported that 30–45% of the tubulin in regenerated cilia was synthesized *de novo* during regeneration. The present results with *T. thermophila* suggest that the difference may be due to the way the cells were grown.

These results also serve to emphasize the importance of precursor pools of protein in the regeneration of cilia in *Tetrahymena*. Under no condition tested was all of the ciliary protein used in regenerating cilia synthesized *de novo*, and under some conditions most of the protein must have been supplied from pre-existing reservoirs within the cell. For example, only 15% of the tubulin in regenerated cilia was due to induced synthesis in cells grown to stationary phase in the rich medium. This heavy use of precursor pools suggests the possibility that the induced synthesis of proteins may be only indirectly related to the biogenesis of new cilia, perhaps being primarily intended to replenish pools of ciliary precursors rather than to drive the assembly process directly. This conclusion is consistent with findings by Guttman & Gorovsky (1979), who have shown that the synthesis of tubulin does not begin until after ciliary regeneration is well under way in *T. pyriformis*, and that it continues after the cilia are completely regenerated. If the induced synthesis of proteins is a secondary response to deciliation, and in addition there is no induced synthesis of phospholipids at all, as shown here, the central problem relating to the biogenesis of cilia becomes one of understanding the mechanisms responsible for the mobilization of stored reservoirs during ciliary outgrowth. The regeneration of cilia in *Tetrahymena* is a favourable system for the study of this important cellular and developmental problem.

Though large pools of ciliary precursors are involved in the regeneration of cilia in *Tetrahymena*, it is nevertheless true that ciliary regeneration is dependent upon synthesis of protein *de novo* (Rosenbaum & Carlson, 1969; Rannestad, 1974). This suggests the possibility that the formation of cilia may be regulated by the temporally controlled synthesis of some limiting protein or proteins necessary for the assembly of other components that pre-exist within the cell. A prediction of this hypothesis is that the limiting protein(s) might be recognized by its high specific activity in experiments in which cilia are regenerated in the presence of labelled amino acids. Stephens has identified such components in sea-urchin cilia (Stephens, 1977). In a double-labelling experiment of the type we have used, the postulated limiting component would show the highest $^3\text{H}/^{14}\text{C}$ ratio, i.e. it would have the smallest pool and show the largest amount of induced synthesis. The finding that the $^3\text{H}/^{14}\text{C}$ ratio varies directly with molecular weight

(Fig. 3) suggests that some high-molecular-weight component may be limiting for the assembly of ciliary components in *Tetrahymena*. The direct relationship between molecular weight and the amount of induced synthesis further suggests that there may be a hierarchical arrangement in which each protein is limiting for all others of lower molecular weight.

There is independent evidence that the limiting proteins are structural components of cilia. Working with *T. pyriformis*, Rannestad (1974) showed that the requirement for protein synthesis *de novo* can be spared if cilia are broken but not detached from the cell. Under these circumstances, the cells resorb the broken cilia and replace them with newly regenerated ones. It is this regeneration coupled to resorption that can occur in the absence of protein synthesis. This suggests that the old cilia may thus constitute a 'pool' of ciliary precursors, complete with 'limiting' or control proteins ordinarily requiring synthesis *de novo*.

It has been suggested that certain proteins that are not structural components of cilia may be important in controlling ciliary regeneration in *Tetrahymena*. Guttman & Gorovsky (1979) found that deciliation in *T. pyriformis* leads to the induced synthesis of a cytosol protein of mol.wt. 80000 that is not selectively localized in cilia. They suggest this might be an important control element in the formation of cilia. In the present study, we have found a protein of mol.wt. 35000, also apparently not present in cilia, that similarly shows induced synthesis during ciliary regeneration in *T. thermophila*. However, it remains to be seen whether or not either of these proteins is actually involved in ciliary regeneration. The fact that cilia can regenerate in the absence of protein synthesis, provided the resorption of old cilia is permitted, suggests that all elements controlling assembly are structural components of cilia. This in turn suggests that catalytic, non-ciliary proteins are probably not involved in the control of ciliary development in *Tetrahymena*. Further work will be required to determine the significance of the induced non-ciliary proteins.

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