FLAGELLAR ELONGATION AND SHORTENING
IN CHLAMYDOMONAS

IV. Effects of Flagellar Detachment, Regeneration, and Resorption on the Induction of Flagellar Protein Synthesis

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ABSTRACT

Synthesis of new proteins is required to regenerate full length Chlamydomonas flagella after deflagellation. Using gametes, which have a low basal level of protein synthesis, it has been possible to label and detect the synthesis of many flagellar proteins in whole cells. The deflagellation-induced synthesis of the tubulins, dyneins, the flagellar membrane protein, and at least 20 other proteins which co-migrate with proteins in isolated axonemes, can be detected in gamete cytoplasm, and the times of initiation and termination of synthesis for each of the proteins can be studied.

The nature of the signal that stimulates the cell to initiate flagellar protein synthesis is unknown. Flagellar regeneration and accompanying pool depletion are not necessary for either the onset or termination of flagellar protein synthesis, because colchicine, which blocks flagellar regeneration, does not change the pattern of proteins synthesized in the cytoplasm after deflagellation or the timing of their synthesis. Moreover, flagellar protein synthesis is stimulated after cells are chemically induced to resorb their flagella, indicating that the act of deflagellation itself is not necessary to stimulate synthesis.

Methods were defined for inducing the cells to resorb their flagella by removing Ca²⁺ from the medium and raising the concentration of K⁺ or Na⁺. The resorption was reversible and the flagellar components that were resorbed could be re-utilized to assemble flagella in the absence of protein synthesis. This new technique is used in this report to study the control of synthesis and assembly of flagella.

KEY WORDS flagella · Chlamydomonas · flagellar resorption · control of protein synthesis · regeneration · calcium

Systems of ciliary and flagellar regeneration provide us with the means to investigate the control of synthesis of specific proteins and their assembly into a cell organelle (9, 31, 36, 41). It has been shown by use of inhibitors that the regeneration of Chlamydomonas flagella (36) or Tetrahymena cilia (34) is dependent on new protein synthesis. If these organisms are cultured under "stepped-down" or starvation conditions (producing gametes in Chlamydomonas), the appear-
The synthesis of the flagellar or ciliary proteins in the cytoplasm is reflected by the ability of polyosomes isolated from regenerating cells to synthesize tubulin in vitro, whereas tubulin synthesis cannot be detected on polyosomes from control cells before deflagellation or deciliation (13, 22, 45). In addition, Guttmann (12) and Guttmann and Gorovsky (13) have shown that synthesis of ciliary proteins in *Tetrahymena* is accompanied by the synthesis of new RNA, which can be labeled after deciliation. Thus, by detaching an organelle at the cell surface, a chain of events which appears to include transcription of messenger RNA, translation of the message in the cytoplasm, and assembly of the proteins into the end product organelle—the cilium or flagellum—is initiated. Most of the compartments involved in this series of events can be isolated. Methods are available for the isolation of cilia and flagella, and the organelles have been fractionated and extensively characterized (25, 29, 33, 39, 40, 47). Recently, the major structural protein of the organelle, flagellar tubulin, has been assembled into microtubules in vitro (2, 7, 20). Polyosomes active in protein synthesis have been obtained (13, 22, 45), and the cytoplasmic compartment can be isolated and analyzed for the appearance of many of the flagellar proteins after translation but before assembly into the organelle (23). Work is just beginning on the nuclear or transcriptional compartment in these regenerating systems.

Although the formation of cilia and flagella has been studied in several different protistan and multicellular systems (6, 35, 41, 42), *Chlamydomonas* has one major advantage over other systems currently in use: the genetics of *Chlamydomonas* have been well defined. Since the pioneering work of Lewin, a large number of conditional and nonconditional mutants with defects in flagellar development and motility have been isolated (15, 18, 24, 31, 43). The ease of isolation of conditional mutants for flagellar development will provide direct access to mechanisms regulating the transcriptional, translational, and assembly events that lead to the development of the flagellum.

One of the questions of general importance which can be asked in these regenerating systems is: What signal travels from the cell surface upon flagellar detachment and initiates the chain of events leading to the synthesis of new flagellar proteins, which, in a cell such as *Chlamydomonas*, can be seen within 5 min after deflagellation? Several possibilities are obvious and testable: (a) Flagellar regeneration and the resulting decrease in the "flagellar precursor pool" in the cytoplasm could initiate new flagellar protein synthesis. (b) The act of flagellar detachment itself, in which there is initial membrane damage followed by re-sealing, could provide such a signal. (c) The cell could recognize the presence or absence of its flagella and initiate flagellar protein synthesis after deflagellation in response to the absence of flagella.

To study these possibilities, it was necessary to develop methods to monitor the synthesis of flagellar proteins in the cytoplasm at intervals after deflagellation. In addition, methods to induce the resorption of flagella into the cytoplasm were developed, and could be used to artificially increase the pool of flagellar proteins and also to produce flagella-less cells without deflagellation. The following report describes the development of these methods, and presents results which suggest that the cell can recognize the absence of its flagella and respond by initiating synthesis of flagellar proteins.

**MATERIALS AND METHODS**

**Cell Culture and Gametogenesis**

Wild-type *Chlamydomonas reinhardtii*, strain 21gr, were used in all experiments. Cells were grown in either medium 1 (referred to as "M" here) of Sager and Granick (37) or M with reduced sulfur levels for labeling experiments with 35S (see below). Cultures in either of these media were grown at 25°C in 250-ml Erlenmeyer flasks on a light-dark cycle of 14 h light/10 h dark; the cells were bubbled with air at all times. The cells for regeneration and resorption studies (Results) were used as soon as the cells reached a density of 2-4 x 10⁶/ml. Gametes were prepared by washing cells into nitrogen-free medium (M-N) (19), then placing the culture in continuous light overnight. The resulting gametes were reduced in size relative to vegetative cells, with ~5% of the protein content of the parent cells, but the flagella were as long as, or longer than, the flagella of vegetative cells, in agreement with the observations of Randall (30).

**Deflagellation and Flagellar Length Measurements**

The cells were deflagellated by mechanical shearing in the fluted glass chamber of a VirTis homogenizer (VirTis...
Labeling Experiments

Sulfur-starved gametes labeled with $^{35}$S were used to study flagellar protein synthesis in whole cells. The vegetative cells used to prepare these gametes were grown to a density of $2-4 \times 10^9$ cells/ml in M, with the amount of MgSO$_4$ in the medium reduced to 1% of the original concentration. The Mg$^{++}$ supplied in the medium by the MgSO$_4$ was replaced by MgCl$_2$. The low-sulfur gamete medium used was M-N, again with the MgSO$_4$ reduced to 1% of the original concentration and the Mg$^{++}$ replaced by MgCl$_2$. $^{35}$S as H$_2$^{35}SO$_4$ obtained from New England Nuclear (Boston, Mass.) (sp act 43 Ci/mg) was used as the label for all experiments, from a 10 mCi/ml stock solution in sterile distilled water.

For experiments to label the proteins synthesized in cells before and after deflagellation, sulfur-starved gametes were used at a density of $1-2 \times 10^7$ cells/ml, 18 h after the parent vegetative cells were placed in nitrogen-free medium. One 5-ml sample of cells (1-2 $\times 10^7$/ml) was labeled in the absence of deflagellation (nondflagellated control) and other 5-ml samples (same cell density) were labeled for successive 30-min pulses during regeneration, i.e., 0-30 min, 32-62 min, and so on for 320 min after deflagellation. 0.5 mCi of label (50 $\mu$l of the 10 mCi/ml stock solution) was added to the sample at the beginning of each pulse period. The cells were stirred and illuminated with fluorescent light at all times. At the end of the 30-min labeling period, the 5-ml samples of cells (with their flagella, if any, still in place) were pelleted at 350 g for 2 min (IEC model HN centrifuge, 1,500 rpm, Damon/IEC Div., Needham Heights, Mass.). The supernate was discarded and the pellet resuspended in 800 $\mu$l of sodium dodecyl sulfate/dithiothreitol (SDS/DTT) sample buffer (see below) and boiled for 2 min. The sample was then centrifuged at 3,000 g for 10 min (IEC PR-6, rotor no. 253, 3,200 rpm) to pellet the "ghosts" of the cell walls, which were discarded. The supernate was then stored at -20°C before electrophoresis. To insure that the cells were completely disrupted and the proteins prepared for electrophoresis, it was important to keep the density of cells boiled in the sample buffer below $1-2 \times 10^7$ cells/ml of final sample buffer.

The $^{35}$S incorporation into protein was measured during each pulse period. Duplicate 25-$\mu$l samples were spotted onto filter disks (Whatmann 3MM) 25 min after the addition of label, and the disks were immediately placed in 10% TCA at 0°C. The disks were processed to determine label incorporation into TCA-precipitable protein by a modification of the method of Mans and Novelli (26) as described previously (36).

Another set of experiments — to study flagellar protein synthesis after deflagellation in the absence of regeneration — followed the procedure just described, except that 3 mg/ml of colchicine, which completely blocks flagellar regeneration without inhibiting protein synthesis (reference 34 and Figs. 7 and 10), was included in all samples.

Experiments to label newly synthesized proteins in whole cells before, during, and after flagellar resorption also used the above procedure for labeling, except that sulfur-starved vegetative cells were used instead of gametes. It was found (see Results) that the resorption media inhibited $^{35}$S incorporation into protein in both gametes and vegetative cells, but the inhibition was greater in gametes. Therefore, experiments to label newly synthesized proteins before, during, and after resorption (Fig. 14) were carried out using vegetative cells grown in low-sulfur medium (above) to a density of $2-4 \times 10^9$/ml.

Gel Electrophoresis and Autoradiography

SDS-urea slab gel electrophoresis followed by autoradiography was used to identify proteins labeled during $^{35}$S pulses. To compare the proteins synthesized in cells before and after deflagellation, identical quantities of protein from each pulse period were loaded onto SDS-urea slab gels, which were then run, dried, and autoradiographed. It was found that the simplest and most accurate way to load all channels in a gel with equal amounts of protein was to load each channel with protein from the same number of cells. 10$^7$ cells (25 $\mu$g of protein) was the optimal load for producing good gel patterns. A sample of $^{35}$S-labeled, isolated axonemes was run on all gels as a standard to mark the position of bands corresponding to flagellar proteins. These axonemes were isolated from gametes prepared in low-sulfur M-N medium (above). 4 liters of vegetative cells in M medium (2-4 $\times 10^8$ cells/ml) were transferred to 1.5 liters of low-sulfur M-N medium. 5 mCi of $^{35}$S (0.5 mCi of the 10 mCi/ml $^{35}$SO$_4^-$ stock solution) was added and gametogenesis allowed to occur in the continuing presence of $^{35}$S. The flagella were then isolated by the sucrose-pH procedure previously described (47). Axonemes were prepared by treating these flagella with 0.04% Nonidet NP-40 (Shell Chemicals, London) in M-N medium at 0°C and immediately collecting the axonemes (36). Axonemes prepared in this manner were contaminated with a small amount of the flagellar membrane, as seen in the gel shown in Fig. 8; this trace contamination served as a flagellar membrane protein marker.

The gel system employed the following modifications of the gel system of Laemmli (21). (a) The separation gel was made with a 3- to 8-M gradient of urea and a 4-16% gradient of acrylamide (4). (b) The buffer system...
was the Tris-glycine buffer of Laemmli, except that SDS was omitted from the stacking and separation gels and was present only in the electrode buffer. The sample buffer contained 0.6 M DTT instead of 2-mercaptoethanol as the reducing agent. The gel slabs (21 cm × 30 cm × 0.15 cm) were run at 20 mA/gel for 12 h. Gels were stained with Coomassie Blue, dried on a Hoefer (model SE 540) gel drier (Hoefer Scientific Instruments, San Francisco, Calif.), and exposed for autoradiography on Kodak X-Omat XR-5 X-ray film.

RESULTS

Cycloheximide Inhibition Studies

It has been known for some time that vegetatively growing *Chlamydomonas* contain a pool of unassembled protein available for flagellar regeneration (36). This was demonstrated by deflagellating cells in the presence of a concentration of cycloheximide sufficient to block protein synthesis and showing that the cells were able to regenerate only 5.5 μm of flagella, about one-half of the length attained when protein synthesis was permitted (Fig. 1, curves A and B). This result indicated that the flagellar protein pool was sufficient to assemble half-length flagella and that flagellar protein synthesis was required to complete regeneration. For the results which follow in this section, the term “flagellar protein pool” is defined as the total complement of proteins necessary to assemble flagella, where the size of this pool was determined by measuring the length of flagella which could be assembled in the absence of protein synthesis (presence of cycloheximide).

The size of the flagellar protein pool in the cell at various times after deflagellation was used as a measure of the kinetics of flagellar protein synthesis during regeneration. At the time of deflagellation, \( t_0 \), all of the flagellar protein in the cell (5.5 μm) was in the unassembled pool (Fig. 1). As new flagellar protein was synthesized after deflagellation, it became part of either the unassembled pool or the growing flagella. Therefore, the total flagellar protein in the cell at any time after deflagellation was the sum of protein assembled in the flagella plus the protein unassembled in the pool. All of the flagellar protein in the cell in excess of the 5.5-μm pool which was initially present at \( t_0 \) must have been newly synthesized after deflagellation. In other words:

\[
\text{[new synthesis between } t_0 \text{ and } t] = \text{[total assembled and unassembled protein at } t] - \text{[the pool of unassembled protein at } t_0 \text{ (5.5 μm)}].
\]

An example of the method used to determine the amount of newly synthesized flagellar protein at times after deflagellation is shown in Fig. 2. A culture of cells was deflagellated and allowed to regrow flagella. Cycloheximide was added to a sample 40 min after deflagellation (Fig. 2a), when the flagella had regenerated to 7 μm (assembled flagellar protein). The cells were immediately re-deflagellated and allowed to regenerate once again as far as possible in cycloheximide. They regenerated to a total of 2 μm, which represents the size of the unassembled flagellar protein pool present in the cell at 40 min after deflagellation. The total flagellar protein present at this time equals the 7 μm present in the flagella plus the 2 μm unassembled in the pool for a total of 9 μm. Therefore, the flagellar protein newly synthesized during the first 40 min of regeneration equals the 9 μm of total flagellar protein minus the 5.5-μm pool which was present in the cell at \( t_0 \) for a net synthesis of 3.5 μm.

Similarly, when cycloheximide was added to cells at 120 min after deflagellation (Fig. 2b), the flagella had already regenerated to nearly full length, 10 μm. When these cells were re-deflagellated, they regenerated 6 μm of flagella in cycloheximide (curve B). Therefore, the total flagellar protein in cells at 120 min after deflagellation was
synthesis of flagellar protein began within 10 min after deflagellation and was almost complete by 90 min. The rate of net flagellar protein synthesis could be determined by taking the first derivative of the curve in Fig. 4 (dashed line, Fig. 5). The rate increased rapidly after synthesis began, reached a maximum of 0.275 μm/min by 30 min, and declined to <0.1 μm/min by 60 min after deflagellation. A similar rate of synthesis was

equal to the 10 μm (assembled) plus the 6 μm (unassembled) for a total of 16 μm, and the newly synthesized flagellar protein was equal to this 16 μm minus 5.5 μm for a net synthesis of 10.5 μm.

By use of the procedure described above, new flagellar protein synthesis was measured at various times after deflagellation (Fig. 3). Curve A in Fig. 3 shows the length of the flagella at each time of cycloheximide addition, and curve B shows the amount of regeneration that occurred after re-deflagellation of these same cells in cycloheximide. At each time after the initial deflagellation, the total flagellar protein in the cells was the sum of the points on curve A (assembled flagellar protein) plus the points on curve B (unassembled flagellar protein in the pool). The amount of newly synthesized flagellar protein at each point, therefore, was equal to this sum minus the original amount in the unassembled protein pool at t₀ (5.5 μm) and is shown in Fig. 4. Fig. 4 shows that the

FIGURE 2 Re-deflagellation of regenerating vegetative cells in cycloheximide to determine the size of the unassembled flagellar protein pool at different times during regeneration. Open circles, normal flagellar regeneration kinetics. (a) At 40 min after the initial deflagellation (dashed line), cycloheximide was added to a sample of regenerating cells; they were then re-deflagellated and allowed to regrow flagella in cycloheximide (triangles). (b) Cycloheximide addition and re-deflagellation at 120 min after deflagellation.

FIGURE 3 The size of the unassembled flagellar protein pool in cells at various times during regeneration. Curve A, normal regeneration kinetics. Curve B, the amount of regeneration which occurred after cycloheximide addition and re-deflagellation (see Fig. 2) of cells at each time shown.

FIGURE 4 The amount of new flagellar protein synthesis after deflagellation. Points were determined from the data in Fig. 3 as described in the text.
directly determined by measuring incorporation of 

3H into regenerating flagella (36).

**Measurement of Synthesis of Flagellar Proteins by 35S Labeling**

The measurements of flagellar protein synthesis described above are indirect, in that they can only measure the cytoplasmic pool size of those flagellar proteins which are limiting for regeneration. To examine the synthesis of the individual flagellar proteins directly, therefore, methods to label newly synthesized proteins in whole cytoplasm after deflagellation had to be developed (Materials and Methods). Gametes were chosen for these labeling experiments because they are nondividing starved cells and have a reduced basal level of protein synthesis relative to dividing vegetative cells (19). Moreover, by the use of gametes cultured in low-sulfur medium and pulse-labeled with 35S, it was possible to study the synthesis of flagellar proteins in whole cells at intervals after deflagellation, with a minimum of interference from nonflagellar proteins. A similar method was used by Guttmann and Gorovsky to study synthesis of ciliary proteins in the cytoplasm of starved *Tetrahymena* (13).

Certain differences between vegetative cells and gametes in the rate of flagellar regeneration and the size of the unassembled flagellar protein pool deserve mention. Gametes regenerated flagella more slowly than vegetative cells (Fig. 6a and b), and maintained a much smaller flagellar protein pool, as shown by the observation that only 2 μm of flagella regenerated after gametes were deflagellated in cycloheximide (triangles, Fig. 6b), whereas vegetative cells deflagellated in cycloheximide regenerated 5.5 μm of flagella. In addition, sulfur-starved gametes regenerated their flagella even more slowly than normal gametes, and maintained only 1 μm of unassembled flagellar precursor in the cytoplasm instead of 2 μm (Fig. 6c).

To determine which proteins in the cytoplasm of gametes became labeled in response to flagellar amputation, cultures of *Chlamydomonas* were labeled for 30-min periods before and during flagellar regeneration; whole cell protein was then subjected to slab gel autoradiography (Materials and Methods). Incorporation of 35S into protein remained constant during each 30-min pulse period before and after deflagellation (Fig. 7); there-
INCORPORATION OF $^{35}$S INTO TCA-PRECIPITABLE PROTEIN DURING PULSE-LABELING PERIODS BEFORE AND AFTER DEFLAGELLATION OF SULFUR-STARVED GAMETES. TCA filter disks (Materials and Methods) were taken at 25 min after the addition of $^{35}$S for each labeling period. The x's show the total incorporation of $^{35}$S into TCA-precipitable protein in samples of pulse-labeled *Chlamydomonas* gametes used subsequently for the comparative gel autoradiography shown in Fig. 9b. The total label incorporation into TCA-precipitable protein was relatively constant for each 30-min pulse in this experiment and showed no dramatic increase after deflagellation. "ndf," Labeling of nondeflagellated cells; "0-30, 32-62, 64-94," etc., labeling of cells during each of the pulse periods after deflagellation.

Therefore, the incorporation of label into any individual protein band on the autoradiograph (Fig. 9b) could be used as a measure of the rate of synthesis of that protein during a pulse period.

As shown in Fig. 9b, deflagellation stimulated the labeling of a number of proteins above pre-deflagellation levels. Many of these proteins comigrated with proteins in the axonemal standard (Fig. 9b channel "axo"). For purposes of discussion, these bands are called "co-migrating bands," to indicate their tentative identification as flagellar proteins. All of the co-migrating bands stimulated by deflagellation were labeled during the first 30 min of regeneration. Some of the changes in the synthesis of individual proteins that occurred in response to deflagellation are described below. Labeled bands other than the identified flagellar proteins were given arbitrary numbers for identification.

IDENTIFIABLE FLAGELLAR PROTEINS: A few flagellar proteins have been identified with some certainty (10, 14, 47): the tubulins ($\alpha + \beta$), dyneins ("dyn"), and the membrane protein ("m") (Fig. 8). Bands in the total cell protein comigrating with each of these identifiable flagellar proteins became labeled in response to deflagellation. Each of these proteins was labeled within the first 30-min pulse period after deflagellation, and labeling at the same or higher level continued through the next two 30-min pulse periods. Labeling began to decrease after 96 min but continued above pre-deflagellation levels for at least 280 min. This continued synthesis reflected the slow gamete regeneration kinetics in Fig. 6c, which showed that flagellar growth in these cells continued for 4 h after deflagellation. However, even after regeneration was complete, synthesis of tubulins, dyneins, and the membrane protein continued above pre-deflagellation levels.

OTHER FLAGELLAR PROTEINS: In addition to the flagellar tubulins, dyneins, and membrane protein, a number of other bands that comigrated with proteins in the axonemal standard became labeled after deflagellation. It is not known which of the axonemal structures these proteins represent. Label incorporation into bands

![Figure 8](image-url)
2 and 5, for example, followed the same kinetics seen for the tubulins, dyneins, and membrane protein, with label appearing within 30 min of deflagellation, continuing at a high level for 90 min, then slowly decreasing over several hours. Not all of the co-migrating bands labeled with these kinetics. For example, the majority of the labeling of bands 8 and 10 was confined to the first 30 min after deflagellation. Band 4 was labeled with different kinetics than all of the above bands; incorporation of label into band 4 was stimulated by deflagellation, and remained at the same high level for 4 h. In summary, labeling of all of the bands co-migrating with flagellar proteins was stimulated within 30 min after deflagellation, but the increased synthesis terminated at different times for the different proteins.

**INCREASED SYNTHESIS AFTER DEFLAGELLATION OF PROTEINS NOT CO-MIGRATING WITH FLAGELLAR PROTEINS:** Deflagellation stimulated the synthesis of at least three bands that did not co-migrate with flagellar proteins. The appearance of label in two of these bands, 7 and 9 (Fig. 9b), was delayed until 60–90 min after deflagellation, as opposed to the increased labeling seen within 30 min for the flagellar proteins discussed above. A third non-co-migrating band, number 1, was synthesized with the same kinetics as the co-migrating identifiable flagellar proteins. Two features of the band 1 protein are of interest: it is the most heavily labeled of all of the proteins in gametes after deflagellation, and its apparent molecular weight is greater than that of the largest major flagellar protein, the membrane protein.

**DECREASED OR UNCHANGED LABELING OF CO-MIGRATING AND NON-CO-MIGRATING PROTEINS AFTER DEFLAGELLATION:** The amount of label in many of the bands in whole gamete protein was the same before and after deflagellation, indicating that the increased label observed in the bands described above was in fact due to new synthesis and not to some nonspecific increase in sulfur incorporation after deflagellation (see also Fig. 7). For example, the labeling of band 11 was constant before and after deflagellation. The position of this band indicates that it is probably the large subunit of ribulose-diphosphate carboxylase, which has a mol wt of 55,000 (11). The SDS-urea gradient gel system used was necessary to resolve this protein from tubulin, as standard Laemmli (21) gels usually produced a superposition of band 11 and α tubulin. The incorporation of label into several bands—3, 6, 12, and 13, for example—decreased from predeflagellation levels between 30 and 120 min postdeflagellation, then gradually returned to predeflagellation levels.

**Is Flagellar Regeneration Necessary for the Initiation of Flagellar Protein Synthesis?**

The means by which removal of flagella stimulates flagellar protein synthesis is unknown. One possibility to be considered is whether deflagellation itself is sufficient to induce flagellar protein synthesis or whether the flagellar regeneration and accompanying pool depletion which follow deflagellation are also required. If regeneration is necessary, then the cell probably initiates the series of synthetic events seen in Fig. 9 in response to removal of flagellar precursor protein from the pool during regeneration.

This possibility was tested using the antimicrotubule drug, colchicine, which blocks flagellar regeneration after deflagellation without inhibiting cell protein synthesis (36). Cells were labeled for 30-min pulse periods before and after deflagellation, as described above, but in this experiment a concentration of colchicine (3 mg/ml) that completely blocked flagellar assembly was included in the medium at all times. This concentration did not inhibit 35S incorporation into protein during the course of the experiment (Fig. 10).

The pattern of protein synthesis changes induced by deflagellation in colchicine (Fig. 11) was nearly identical to the changes in synthesis induced by deflagellation when regeneration was allowed to occur (Fig. 9b). For example, identifiable flagellar proteins—the tubulins, dyneins, and the membrane protein—were all labeled within the first 30 min after deflagellation in colchicine. Label in these proteins then decreased from 96 to 280 min. In fact, Fig. 9b and 11 (synthesis in the presence or absence of regeneration) differ in only one major band which is seen only in colchicine-treated cells: band 1a, which migrated slightly more slowly than band 1 and did not co-migrate with any flagellar protein. It is clear from these results that the stimulation of flagellar protein synthesis does not require flagellar regeneration and accompanying pool depletion. Therefore, the cell must respond either to the act of deflagellation itself (e.g., because of membrane damage) or to the absence of flagella caused by deflagellation.

**LEFEBVRE ET AL. Control of Flagellar Synthesis**
FIGURE 9  Labeling of newly synthesized protein in gametes before and after deflagellation. Equal amounts of whole cell protein (cell bodies and flagella) from sulfur-starved gametes, pulsed for equal lengths of time (30 min) with $^{35}$S, either before deflagellation (channel "ndf"), or for successive pulse periods after deflagellation (channels "0-30, 32-62," etc.) were loaded on gels. (a) Coomassie Blue-stained. The gel was stained with Coomassie Blue, then dried and photographed. The staining pattern confirms that equal amounts of protein from each labeling period were loaded on the gel.
FIGURE 9 (b) Autoradiograph of the same gel, exposed 19 h. (The amount of $^{35}$S incorporated into protein during the first 25 min of each labeling period is shown in Fig. 7.) Channel “axo” is the labeled axonemal standard.
To distinguish between these two possibilities, methods were developed to induce resorption of flagella, thereby producing flagella-less cells whose flagella had never been detached and whose membranes were therefore not damaged.

Induction of Flagellar Resorption in Chlamydomonas

Chlamydomonas cells spontaneously resorb their flagella before cell division or after amputation of one of the pair of flagella (3, 5, 19). Such resorption requires <45 min, and the flagella are motile during the entire process.

Several chemical treatments were found to artificially induce the resorption of Chlamydomonas flagella. When cells in M medium were treated with 5 mM GTP or ATP, 10 mM pyrophosphate (PPi), or 25 mM citrate, the flagella became paralyzed and began to shorten. The kinetics of flagellar resorption induced by 20 mM PPi were approximately linear, with the flagella shortening at a rate of 0.1 μm/min (Fig. 12). Gamete flagella resorbed at a rate similar to that for vegetative cells for the first 90 min of PPi treatment, then the rate of resorption slowed, such that, even after 360 min of PPi treatment, gamete flagella were not completely resorbed (Fig. 12). All of the agents that caused resorption also completely blocked flagellar regeneration in gametes or vegetative cells after deflagellation. Transmission electron microscopy of thin-sectioned cells showed that intact flagella were not pulled into the cell during resorption. The localization of flagella and their basal bodies at the cell surface did not change during resorption.2

It was noted initially (23) that all four of the agents found to cause resorption—ATP, GTP, Citrate, and PPi—are good chelators of divalent cations. Furthermore, because Na+ or K+ salts of these compounds were used, the monovalent cation concentration of the medium was raised to over 30 mM by their addition, as opposed to the 3 mM normally present in M medium. By varying the concentrations of Na+, K+, Ca++, and Mg++ in the medium, we were able to develop a simple medium which induced resorption of flagella (Table 1). Flagellar resorption could be induced by removing Ca++ from the medium and raising the Na+ or K+ concentration to 25 mM; reversal of resorption occurred when the Ca++ concentration was restored to 0.3 mM, even in the continued presence of the monovalent cation.

Treatment with either the calcium-free high salt medium or PPi had no long-term toxic effect on cells, and the cells divided for many generations in either medium. However, when grown in these media, the cells at no point in the cell cycle had flagella and they did not separate after cell division. Clumps containing over 100 individual cells were seen after 6 days in resorption medium, in agreement with a similar phenomenon noted by Iwasa and Murakami (17).

Although no long-term toxic effects were observed under resorption conditions, there were changes in cell metabolism associated with these treatments. In either gametes or vegetative cells, 35S incorporation into protein was inhibited by up to 75% during resorption (Fig. 13). The cells gradually recovered their ability to incorporate 35S into protein in the continued presence of resorption medium until incorporation had returned to pre-resorption levels, or higher, after about 6 h. This inhibitory effect was highly variable from culture to culture. In some experiments, no inhibition of 35S uptake was seen during resorption and yet resorption occurred as well as during those experiments in which 35S incorporation was inhibited. Incorporation of [3H]acetate into protein showed a similar, variable inhibition when cells were treated with PPi. Inhibition of protein synthesis itself was not sufficient to induce resorption, 2

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2 N. Pierpont and P. Lefebvre. Unpublished observations.
FIGURE 11. Labeling of newly synthesized proteins in gametes before and after deflagellation in colchicine. A sample of sulfur-starved gametes in colchicine was labeled for a 30-min period before deflagellation, and other samples were labeled for successive 30-min periods after deflagellation in the presence of colchicine, which completely blocked flagellar regeneration. Equal amounts of protein from each sample were loaded on a gel along with the labeled axonemal standard; this figure is the autoradiograph of that gel. Channel “ndf,” cells labeled without deflagellation; channels “0-30, 32-62,” etc., cells labeled for successive 30-min periods after deflagellation; channel “axo,” labeled axonemal standard.
FIGURE 12 The rate of flagellar resorption for vegetative cells and gametes. Resorption was induced by treating cells with 20 mM NaPPI in M medium (vegetative cells) or M-N medium (gametes).

**TABLE I**

**Alterations in M or M-N Medium Which Induce Resorption**

| Concentration in the medium | Resorption |
|-----------------------------|------------|
| K⁺ Na⁺ Ca⁺⁺ Mg⁺⁺             |            |
| mM                          |            |
| 0.7 2.5 0.36 1.2*           | -          |
| 0.7 2.5 0.36 0              | -          |
| 0 2.5 0.36 0               | -          |
| 0.7 0 0.36 0               | -          |
| 0.7 25 0.36 0             | -          |
| 25 2.5 0.36 0            | -          |
| 0.7 2.5 0 1.2            | -          |
| 0.7 25 0 1.2             | +          |
| 25 2.5 0 1.2            | +          |

Cells were washed from M (or M-N for gametes) into media with the concentrations of ions shown. Resorption was scored with a phase microscope equipped with an ocular micrometer.

* Concentrations normally present in M or M-N media.

because neither cycloheximide nor chloral hydrate, both of which are potent inhibitors of *Chlamydomonas* protein synthesis (27, 36), induced flagellar resorption or paralysis, even after 8 h of treatment. In addition, during prolonged treatment with PPi no regrowth of flagella occurred, even after ³⁵S uptake had returned to normal levels.

**Induction of Flagellar Protein Synthesis after Resorption**

Experimentally induced resorption of flagella can be used to ask the question raised earlier—Is the act of deflagellation needed to stimulate flagellar protein synthesis, or can cells synthesize...
flagellar proteins simply in response to the absence of flagella? Using resorption, cells can be produced that have no flagella but have never been deflagellated. These cells should show labeling of the flagellar proteins in the cytoplasm if flagellar protein synthesis is stimulated by the absence of flagella (i.e., by resorption). Unfortunately, labeling of gametic cells during and after resorption was complicated by the variable inhibition of sulfur incorporation into protein associated with resorption in these cells. However, it was possible to study the cytoplasmic synthesis of flagellar proteins in vegetative cells during and after resorption, because inhibition of \( \text{S} \) incorporation was not so great in vegetative cells as in gametes, and recovery from inhibition was rapid after resorption was reversed.

The cell proteins labeled when vegetative cells were pulsed with \( \text{S} \) before, during, and after resorption are shown in Fig. 14. Fewer identifiable flagellar bands could be detected in vegetative cells than in gametes because of the high background of synthesis of nonflagellar proteins. However, by comparing channel “B4” (cells labeled before resorption) with channel “R150–180” (cells labeled from 150 to 180 min in resorption medium) in Fig. 14, it can be seen that little if any labeling of flagellar proteins occurred in cells kept in resorption medium, even after flagella were completely resorbed. Resorption in this experiment was complete within 120 min of treatment.

However, when the cells were washed out of resorption medium after resorption was complete, or if calcium was added to the medium to reverse resorption, a number of bands co-migrating with flagellar proteins became labeled. The flagellar membrane protein, dyneins, and \( \beta \) tubulin (\( \alpha \) tubulin was obscured by RUDP carboxylase) were all labeled, as was a band co-migrating with band 4 in the axonemal standard. Measurement of flagellar length of several hundred cells at each of several times during resorption showed that the cells had not spontaneously lost their flagella while shortening, and therefore the turn-on of flagellar protein synthesis seen after calcium addition was not an artifact caused by flagellar breakage and loss during shortening. Instead, the cell synthesized flagellar proteins after resorption in response to the absence of flagella; the act of deflagellation and possible accompanying membrane damage were not necessary to stimulate flagellar protein synthesis. Although calcium was necessary to stimulate flagellar protein synthesis after resorption, regrowth of flagella, which began immediately after calcium was added, was not necessary, because the same stimulation of synthesis occurred if colchicine was present to prevent regeneration after calcium addition (results not shown).

To compare the resorption-induced stimulation of flagellar protein synthesis with deflagellation-induced synthesis, a culture of cells was treated with PPI to induce flagellar resorption, washed out of PPI, and allowed to regenerate 3 \( \mu \)m of flagella. These cells were then mechanically deflagellated and labeled for three 30-min periods during regeneration (channels “DF 0–30, 32–62, 64–94”). Synthesis of the major flagellar proteins after deflagellation was similar to the synthesis of flagellar proteins induced by adding \( \text{Ca}^{++} \) to reverse resorption (channels “+ \text{Ca}^{++} 0–30, 32–62, 64–94”). It should be noted, however, that the synthesis of \( \beta \) tubulin under these conditions appeared to terminate sooner if synthesis was induced by resorption than if synthesis was induced by resorption followed by deflagellation.

This experiment shows, therefore, that the synthesis of flagellar proteins can be stimulated in the cytoplasm without mechanically detaching the flagella, by inducing the resorption of flagella and then reversing resorption by the addition of calcium. This observation is consistent with the theory that cells turn on flagellar protein synthesis after deflagellation in response to the absence of flagella rather than in response to the act of deflagellation itself. This experiment does not, however, rule out the possibility that flagellar protein synthesis can be stimulated by two different mechanisms—one responding to the absence of flagella and the other responding to flagellar amputation.

**Re-utilization of Resorbed Flagellar Proteins**

Flagellar resorption was completely reversed when the cells were transferred into fresh medium, and the resorbed flagellar material was reused to assemble flagella without new protein synthesis, as shown by the following experiment: Gametes were treated with PPI for 1, 2, and 3 h, to induce flagellar resorption to lengths of 8, 5, and 3 \( \mu \)m, respectively, and then the cells were washed out of PPI into M medium containing cycloheximide (Fig. 15). The cells in each sample regrew flagella to nearly full length. Almost all of the regrowth that occurred after cells were washed...
FIGURE 15 Re-usability of flagellar protein in gametes in the absence of protein synthesis. Resorption was induced by treatment of gametes with 20 mM P Pi (open circles). At three times during resorption—60 min (triangles), 120 min (squares), and 180 min (x's)—a sample of cells was washed out of P Pi into M medium containing 10 μg/ml of cycloheximide, and allowed to regrow flagella as far as possible in the absence of protein synthesis. In a parallel experiment (filled circles), 10 μg/ml of cycloheximide was included in the medium during resorption; then the cells were washed out of P Pi after 2 h to reverse resorption in the continuing presence of cycloheximide.

out of P Pi and into cycloheximide-containing medium represented the re-use of resorbed material, because gamete cytoplasm contains a very small pool of unassembled flagellar protein (Fig. 6b) that could have contributed little (a maximum of 2 μm) to flagellar regrowth. Clearly, flagellar material was resorbed into the cell and was re-used in the absence of new protein synthesis. Furthermore, regrowth of flagella after resorption was not dependent on flagellar proteins which were newly synthesized during resorption, because the same amount of regrowth occurred if the entire resorption and regrowth was done in the presence of cycloheximide (Fig. 15, filled circles).

Because P Pi-induced resorption increased the pool size of assembly-competent flagellar protein in the cytoplasm, this technique was used to determine whether the rate of flagellar regeneration is sensitive to flagellar precursor pool size. Gametes were treated with P Pi for 180 min to induce resorption, then washed out of P Pi and allowed to regenerate flagella (triangles, Fig. 16). The kinetics of regrowth were then compared to the regeneration rate of flagella after deflagellation (x's, Fig. 16). The initial rate of flagellar growth after 3 h of resorption was 0.4 μm/min and full length flagella were produced after 45 min. By comparison, the initial rate of flagellar regeneration after deflagellation was 0.12 μm/min, and regeneration of full length flagella was not complete until 180 min. To exclude the possibility that damage to the cell was responsible for the slower flagellar growth after deflagellation, gametes were treated with P Pi for 180 min to induce resorption to a length of 3 μm, washed into fresh M-N medium, and the short flagella remaining on the cells at this time were amputated (circles, Fig. 16). After this deflagellation, the

FIGURE 14 Labeling of proteins synthesized in vegetative cells before, during, and after resorption. Sulfur-starved vegetative cells were induced to resorb their flagella by raising the NaCl concentration in calcium-free medium to 25 mM. Cells in calcium-free medium were labeled for 30-min periods before resorption was initiated (channel "B4"), and another culture was labeled from 150 to 180 min during resorption (channel "R150-180"). After 180 min in resorption medium, by which time the flagella had been completely resorbed, CaCl₂ was added to a concentration of 0.3 mM to reverse resorption. Samples of cells were then labeled for the first three 30-min periods after calcium addition and initiation of flagellar regrowth (channels "+Ca 0-30, 32-62, 64-94"). A parallel culture of cells was also induced to resorb flagella for 180 min, then CaCl₂ was added to 0.3 mM to reverse resorption and initiate flagellar regrowth. These cells, however, were allowed to regrow flagella for 10 min, then they were deflagellated. Samples of these deflagellated cells were labeled for the first three 30-min pulse periods after deflagellation (channels "Df 0-30, 32-62, 64-94"). Equal amounts of protein from each pulse period were run on gels (Materials and Methods), which were then dried and autoradiographed. For this figure, channels "B4" and "R150-180" were exposed for 180 h, compared to 45 h for the other channels to compensate for the increase in sulfur incorporation into protein associated with the reversal of resorption (Fig. 13).
FIGURE 16 Acceleration of the rate of flagellar regeneration by prior flagellar resorption. Triangles, Rate of flagellar growth of gametes after cells were induced to resorb flagella to a length of 3 μm in 20 mM PPI (3 h) and then washed out of PPI into fresh calcium-containing medium. Circles, the rate of flagellar growth after resorption followed by deflagellation. Flagella were resorbed to 3 μm, then the cells were washed into fresh, calcium-containing medium. The remaining 3-μm long flagella were then amputated and the cells allowed to regrow new flagella.

cells regenerated flagella as rapidly as cells that were treated with PPI and washed directly into fresh medium without deflagellation, showing that the act of deflagellation did not slow the rapid kinetics of flagellar growth after resorption. Therefore, the resorption of flagellar material into gamete cytoplasm before deflagellation accelerated the subsequent flagellar regeneration. This result indicated that the rate of flagellar elongation could be modified by the size of the flagellar protein pool in the cytoplasm.

DISCUSSION

Initiation of Flagellar Protein Synthesis after Deflagellation

It has been known for some time that flagellar regeneration in *Chlamydomonas* (36) as well as ciliary regeneration in *Tetrahymena* (34) requires new protein synthesis. In this report, it is shown that this synthesis is independent of flagellar regeneration and the concomitant decrease in flagellar protein pool size. As shown by Figs. 9b and 11, the synthesis of more than 20 proteins, many of them co-migrating with flagellar proteins, is stimulated by deflagellation. This stimulation is almost identical whether or not regeneration is allowed to occur. This result extends the observation of Rosenbaum, Moulder and Ringo (36), based on inhibitor studies (confirmed by Weeks et al. for tubulin synthesis [46]), that new flagellar protein synthesis is stimulated by deflagellation in the absence of regeneration.

The results in this report also show that initiation of flagellar protein synthesis occurs after flagellar resorption and, therefore, that amputation and accompanying membrane damage are not required for stimulation of flagellar protein synthesis. The fact that flagellar protein synthesis is stimulated after resorption suggests that the cell recognizes the absence or shortening of the flagella and responds by inducing the synthesis of flagellar proteins. The nature of the signal that the cell uses to recognize that its flagella are lost or shortened is unknown. Such a signal could involve flagellar components that shuttle between the flagella and the cell. Candidates for these components are the flagellar proteins that can be seen by labeling experiments to turn over rapidly in gametic and vegetative flagella. Alternatively, changes in the amount of some small molecule whose concentration is modulated by flagellar components, e.g., an ion pumped by a flagellar membrane ATPase or the product of some flagellar enzyme (8, 44), could communicate the state of the flagella to the cell. The fact that flagellar protein synthesis did not begin after resorption until calcium was added to the cells (Fig. 14) is consistent with a role for calcium, at least as a co-factor, in the signaling mechanism.

There is evidence in other systems that stimulation of synthesis of ciliary proteins after deciliation requires both transcription and translation. Guttmann (12) and Guttmann and Gorovsky (13) showed that ciliary regeneration was blocked by both actinomycin D and cycloheximide and that labeled, newly synthesized RNA could be detected in polysomes isolated from regenerating, starved cells. Weeks and Collis (45) have also shown, using an in vitro translation system, that

2 P. Lefebvre, J. Rosenbaum, and J. Jarvik. Unpublished observations.
deflagellation caused the appearance of tubulin mRNA in Chlamydomonas polysomes.

Termination of Flagellar Protein Synthesis

The experiments reported here also provide new information about the termination of flagellar protein synthesis. As shown in Figs. 9b and 11, even though all of the presumed flagellar proteins were stimulated within the first 30 min after deflagellation, the synthesis terminated at different times for the different proteins. Synthesis of some proteins, for example the tubulins, did not return to resting levels for 4 h after deflagellation, while other proteins, for example band 10 in Fig. 9b, were synthesized for <90 min after deflagellation and, in fact, the vast majority of label in this band was incorporated in the first 30 min. Thus, flagellar protein synthesis is not coordinate: individual proteins are synthesized with a wide variety of kinetics.

The termination of flagellar protein synthesis is clearly not linked to flagellar regeneration. The synthesis of the proteins labeled after deflagellation was almost identical whether or not colchicine was present to prevent flagellar regeneration. This observation, which applies to the identifiable flagellar proteins as well as to the other co-migrating bands, shows that termination of synthesis of at least these proteins is not regulated by any feedback from the regenerating flagella. In addition, the flagellar proteins that accumulate in the cell when regeneration is blocked by colchicine do not themselves inhibit flagellar protein synthesis, since synthesis terminates at the same time in the presence or absence of regeneration.

One might expect that cells that have been induced to synthesize flagellar proteins in the absence of regeneration (presence of colchicine) could subsequently use these proteins to assemble almost full-length flagella in the absence of new protein synthesis. However, results reported earlier (36) indicated that only a small amount of these proteins that appear in the cytoplasm during colchicine blockage of assembly could be used for flagellar regeneration in the presence of cycloheximide. That is, if cells were deflagellated, left in colchicine for up to 2 h, then washed out of colchicine into cycloheximide, only 2 μm of flagella was assembled in excess of the 5.5-μm pool present in the cell at the time of deflagellation. Several explanations for this observation are possible: (a) All of the necessary flagellar proteins are synthesized after deflagellation in colchicine, but some post-translational processing step that requires regeneration must occur before the newly made proteins can assemble. (b) All of the necessary flagellar proteins are synthesized after deflagellation in colchicine, but one or more proteins needed for assembly are degraded rapidly if regeneration is prevented. (c) Some minor protein that has not been detected on our one-dimensional gel system is not synthesized after deflagellation in colchicine, and the absence of this protein(s) limits regeneration. This possibility is being tested by using two-dimensional gel electrophoresis (28) to increase the number of flagellar proteins detectable in the cytoplasm after deflagellation in the presence and absence of colchicine. Another approach to the problem will be the use of mutants such as cs89 (18) which is able to synthesize assembly-competent flagellar protein sufficient to assemble at least two full-length flagella after the flagella are amputated, and the cells are placed in colchicine and washed 2 h later into cycloheximide. This mutant grows flagella up to three times normal length under several conditions, and therefore may also be defective in the normal control of flagellar protein synthesis.

Use of Induced Flagellar Resorption

The induced resorption of assembled flagella reported here will be useful for investigating the regulation of flagellar stability and for manipulating the size of the flagellar protein pool in cells. Flagella are much more stable than other microtubule-containing organelles. The isolated flagellum is insensitive to cold, colchicine, and pressure—treatments that depolymerize cytoplasmic and mitotic spindle microtubules (1, 16, 32, 38). The cell itself, however, breaks down its own flagellar microtubules while resorbing its flagella, either before cell division or in response to the amputation of one of the pair of flagella; the literature pertaining to the natural resorption of flagella by many organisms has recently been reviewed (3). The mechanisms that control the conditional stability of flagella are unknown, and it is hoped that the ability to induce the reversible resorption of flagella as described in this paper will provide some insight into the control of flagellar stability.

Resorption can also be used to increase the pool of unassembled flagellar proteins in the cell and thereby accelerate morphogenesis; an exam-
ple of the use of this technique is shown in this report in experiments in which the flagellar regen-
eration rate of gametes can be increased by increas-
ing the size of the flagellar protein pool by flagellar resorption.

Finally, because PPI and the other resorption treatments prevent flagellar regeneration, they can be used as a substitute for colchicine in blocking flagellar assembly in vivo. Colchicine has toxic effects on cells after prolonged exposure, but cells will divide for days in the presence of any of the resorption agents, indicating that they produce little toxic effect on cells.

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