# ADENOSINE 3',5'-CYCLIC MONOPHOSPHATE

# IN *CHLAMYDOMONAS REINHARDTII*

## Influence on Flagellar Function and Regeneration

### ROBERT W. RUBIN and PHILIP FILNER

From the MSU/AEC Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48823. Dr. Rubin's present address is the Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, Colorado 80302.

### ABSTRACT

Adenosine 3',5'-cyclic monophosphate (cAMP) influences both flagellar function and flagellar regeneration in *Chlamydomonas reinhardtii*. The methylxanthine, aminophylline, which can cause a tenfold increase in cAMP level in *C. reinhardtii*, inhibits flagellar movement and flagellar regeneration by wild-type cells, without inhibiting cell multiplication. Caffeine, a closely related inhibitor, also inhibits flagellar movement and regeneration, but it inhibits cell multiplication too. Regeneration by a mutant lacking the central pair of flagellar microtubules was found to be more sensitive than wild type to inhibition by caffeine and to be subject to synergistic inhibition by aminophylline plus dibutyryl cAMP. Regeneration by three out of seven mutants with different flagellar abnormalities was more sensitive than wild type to these inhibitors. We interpret these results to mean that cAMP affects a component of the flagellum directly or indirectly, and that the responsiveness of that component to cAMP is enhanced by mutations which affect the integrity of the flagellum. The component in question could be microtubule protein.

## INTRODUCTION

Evidence for a functional link between adenosine 3',5'-cyclic monophosphate (cAMP) and microtubules can be found in work on pancreas islets cells, melanocytes, neoplastic mammalian cells in culture, nerve cells, and blood platelets (see 1 and 2 for references). The cAMP-microtubule relationship may be rather direct, since brain microtubule protein can be phosphorylated in vitro by cAMP-stimulated protein kinase (3). The phosphorylated protein interacts with a soluble factor from brain, while the unphosphorylated protein does not (4). Treatment with cAMP *in situ* may affect the colchicine-binding property of microtubule protein (2). Eipper (5) recently reported that brain microtubule protein of rat is phosphorylated in vivo. It remains to be seen if that phosphorylation is stimulated by cAMP in vivo.

The similarities of microtubule protein from sources as diverse as mammalian brain and algal flagella (6) suggested to us that if cAMP affected microtubules in animals, it could be expected to do so in green plants as well. The flagella of the green alga, *Chlamydomonas reinhardtii*, have been studied in some detail (6–10). Therefore, we chose to look for a cAMP-microtubule relationship in this system.

Amrhein and Filner[1] have shown that cAMP and a cyclic nucleotide phosphodiesterase which can be inhibited by theophylline occur in *C. reinhardtii*. When cells are incubated in aminophylline (theophylline₂ethylenediamine) for 1 h, their cAMP level increases more than tenfold.

If cAMP affects microtubule function or formation, then treatment with agents which mimic cAMP or increase the endogenous cAMP level may be expected to influence flagellar movement or regeneration, respectively. Therefore, we have investigated the effects of the methylxanthines, aminophylline and caffeine, and the lipophilic cAMP derivative, 6N, O²′-dibutyryl cAMP (11), on flagellar movement and flagellar regeneration in *C. reinhardtii*.

## MATERIALS AND METHODS

### Cultures

*C. reinhardtii* was grown on a Tris-acetate-phosphate (TAP) medium as described elsewhere[1]. The usual procedure for obtaining cells was to dilute an aliquot of a stationary phase culture (about $1.2 \times 10^6$ cells/ml) tenfold into fresh TAP medium and allow the cells to grow for 2 days. Satisfactory results were also obtained with a fivefold dilution and overnight growth.

### Titers and Viable Counts

Motile cells were either stunned by 1–2 min agitation on a Vortex mixer, fixed with 2.5% glutaraldehyde, or treated with 5 mM caffeine to render them immotile. Mutants with severely impaired motility could be titered without these treatments. Titers were determined on a counting slide with a sample depth of 0.1 mm. Viable counts were made by plating about $10^2$ cells on each of four Petri dishes containing 2% agar TAP medium. Colonies were counted after 1 wk.

### Chemicals

Aminophylline, caffeine, and 6N, O²′-dibutyryl cAMP were purchased from Sigma Chemical Corp. (St. Louis, Mo.).

### Motility Inhibition Experiments

Wild-type cells were diluted in TAP medium to $2 \times 10^6$ cells/ml and shaken under fluorescent light

[1] Amrhein, N., and P. Filner. 1972. Adenosine 3′,5′-cyclic monophosphate in *Chlamydomonas reinhardtii*. Isolation and characterization. Submitted for publication.

for 1 h. The chemical to be tested was dissolved in TAP medium at twice the desired final concentration, then mixed with an equal volume of cell suspension. The titer of immotile cells and the total titer were then determined. In the case of aminophylline, the cells were incubated for at least 10 min in the presence of the compound before the titer of immotile cells was determined.

### Regeneration Experiments

5 min before shearing, 0.75 ml of culture, usually about $5 \times 10^6$ cells/ml, were mixed with 0.75 ml TAP medium containing twice the desired final concentration of compound. A 0.2 ml aliquot was taken immediately and fixed with 2.5% glutaraldehyde. The remainder was placed in the stainless steel microchamber of a Virtis homogenizer (The Virtis Co., Inc., Gardiner, N. Y.) (8) and sheared at a setting of 80 for 1 min, or 1.5 min in later experiments. Immediately after shearing, another 0.2 ml aliquot was taken and fixed. The remainder was incubated at ambient temperature (22°C) on a reciprocal shaker under fluorescent light. Aliquots were taken and fixed periodically thereafter. Fixed samples were stable and could be stored until a convenient time was found for measuring flagellar lengths.

### Flagellar Length Measurements

A phase-contrast microscope equipped with an eyepiece micrometer was used at 1,250 × magnification. The scale divisions corresponded to 0.5 μm and measurements were made to the nearest micron. The slide was systematically scanned until 20 flagella had been measured. These values were then averaged $(\overline{\mu m})$. Only one flagellum was measured on a given cell, and severely curled flagella were not measured. The fractions of cells with flagella after shearing and regeneration in the absence of inhibitor $(f_o)$ and in the presence of inhibitor $(f_i)$ were determined by examining 50 cells in each sample. A corrected average flagellar length $(\overline{\mu m}_c)$ was then calculated which included in the average those flagella of "zero length" which resulted from the presence of the inhibitor:

$$\overline{\mu m}_c = \overline{\mu m}\left(\frac{f_i}{f_o}\right) + 0\left(\frac{f_o - f_i}{f_o}\right) = \overline{\mu m}\left(\frac{f_i}{f_o}\right).$$

### Mutants

Spontaneous mutants exhibiting impaired motility were selected by the procedure of Warr et al. (7), starting from a clone of (+) wild type. Among the first 30 mutant clones examined, seven with distinct and stable phenotypes were encountered, each phenotype including a flagellar abnormality.

## Electron Microscopy

The cells were fixed in 2.5% glutaraldehyde, post-fixed in 2% osmium tetroxide, and rapidly dehydrated in ethanol. After propylene oxide infiltration, they were embedded in Epon and sectioned. The sections were stained with lead citrate and uranyl acetate, then photographed with a Philips 300 electron microscope.

## RESULTS

### Flagellar Movement

Caffeine and aminophylline completely inhibit swimming of *C. reinhardtii* (+) wild type at 2 mM (Fig. 1). Cells which have ceased swimming exhibit little or no flagellar movement. Caffeine inhibits very quickly, since all cells stop swimming within the few seconds required to mix a culture aliquot with caffeine solution and examine a drop under the microscope. Cells exposed to aminophylline do not stop swimming until about 10 min after introduction of the compound. Inhibited cells remained stationary for at least 45 min. Dibutyryl cAMP, up to 5 mM, did not inhibit swimming.

### Cell Multiplication

At 3.3 mM, a concentration which inhibits swimming, aminophylline does not affect the
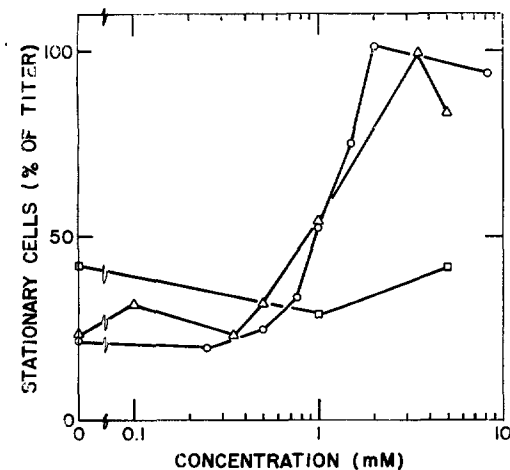


FIGURE 1  Inhibition by methylxanthines of swimming of *C. reinhardtii* (+) wild type. Caffeine (o); aminophylline (△); dibutyryl cAMP (□). The data for methylxanthines are from one experiment and those for dibutyryl cAMP are from a different experiment.

rate of cell multiplication, while caffeine slows but does not stop cell multiplication (Table I). Approximately a 100-fold increase in cell titer occurred over 89 h, indicating a doubling time of about 13 h compared to the normal 8 h. Cells which grow up in the presence of 3.3 mM caffeine exist in clumps and lack flagella, while in a normal culture the great majority of the cells are single cells with flagella (rarely as low as 85%, usually more than 95%). Dibutyryl cAMP at 1 mM did not inhibit cell multiplication, nor did aminophylline plus dibutyryl cAMP (Table I).

### Flagellar Regeneration

Flagella can be removed from more than 95% of the cells by shearing for 1 min, without a decrease in viable count (Fig. 2). In fact, the viable count approximately doubled during the first 15 sec in this experiment, presumably as a result of dispersion of clusters of vegetatively dividing cells. Since shearing does not kill cells, while removing the flagella from virtually all cells, our observations on regeneration have been made on typical cells, not some extraordinary subpopulation which happened to survive the shear treatment.

Usually more than 95% of the cells had flagella before shearing (Table II). Between 85 and 100% of the cells regenerated flagella, in most experiments with wild-type cells regenerating in the absence of inhibitor. Regeneration was relatively synchronous: no cells regenerated extraordinarily quickly, i.e., nearly completely within 15 or 30 min, and no cells regenerated extraordinarily slowly, i.e., produced only an extremely short stub after 60 min.

The initial flagellar length and the regenerated flagellar length at 60 min are reasonably reproducible: the averages of three experiments conducted with cells from different cultures on different days were 10.7 ± SD 0.5 µm before shearing and 8.4 ± SD 0.5 µm after 60 min of regeneration. These figures are comparable to the published data for single cells (8, 9).

Cells without flagella were not included in calculations of average flagellar length before shearing or after regeneration in the absence of inhibitor. However, the frequency of cells without flagella increased markedly when regeneration occurred in the presence of an effective inhibitor (Table II). Therefore, we have treated those cells which do not regenerate flagella due to the presence of inhibitor as having flagella of zero

TABLE I

Growth of C. reinhardtii in the Presence of Aminophylline, Caffeine and
Dibutyryl cAMP

| | Medium | Time | Titer $(10^{-6})$ | |
| | | | Cells/ml | Clumps/ml |
| --- | --- | --- | --- | --- |
| | | h | | |
| Exp. I | TAP | 0 | 0.004 | — |
| | TAP | 89 | 10.3 | — |
| | TAP, 3.3 mM amino-phylline | 89 | 13.8 | — |
| | TAP, 3.3 mM caffeine | 89 | >0.44* | 0.11 |
| Exp. II | TAP | 0 | 0.35 | |
| | TAP | 48 | 15 | |
| | TAP, 1 mM dibutyryl cAMP | 48 | 16 | |
| | TAP, 1 mM dibutyryl cAMP, 3.3 mM aminophylline | 48 | 13 | |

Stationary phase cells which had grown up in TAP medium were subcultured into the indicated media at zero time. The normal doubling time of these cells in TAP is 8-9 h, and stationary phase occurs at 10-15 $\times$ $10^6$ cells/ml.

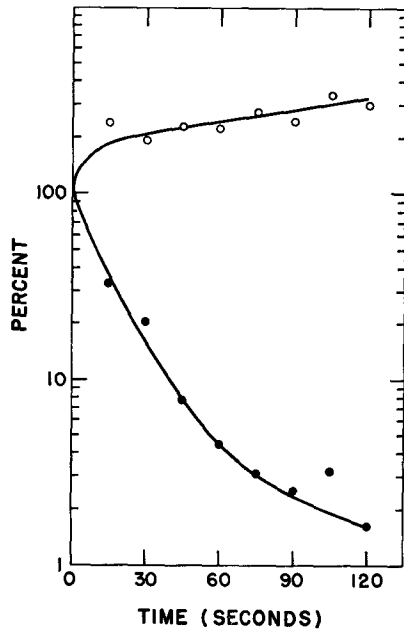* The number of cells per clump could not be accurately counted, but was at least four.



Figure 2 Kinetics of removal of flagella of C. reinhardtii (+) wild type by shearing. Flagellated cells (●); viable count (○).

length in calculating the length of the average flagellum regenerated in the presence of inhibitors (see Methods and Table II).

Aminophylline and caffeine inhibit regeneration of flagella by wild-type cells (Fig. 3), with 50% inhibition at about 3.3 mM and complete in-hibition at about 6 mM. Dibutyryl cAMP alone had little effect on regeneration (Fig. 3, Table II).

## Mutants

The seven mutants with impaired motility have been designated fm 8, 9, 10, 12, 15, 24, and 30. Fm 8 and 9 have half-length flagella. Those of fm 8 function, since the cells swim slowly, while those of fm 9 are paralyzed. Fm 10 and 12 have normal length flagella which beat actively, but fm 10 cells spin in place and often have super-numerary flagella (3 to 5), while fm 12 cells have flagella which detach very easily. Fm 15 and 24 have normal length flagella, but semiparalyzed and paralyzed, respectively. Those of fm 30 are slightly shorter than normal and are semipara-lyzed. When flagellar cross sections were examined in the electron microscope, it was found that flagella of fm 24 lacked the central pair of micro-tubules, flagella of fm 30 had 0, 1, or 2 central pair microtubules (Fig. 4) and the other mutants had flagella which appeared normal in cross sections. Mutants without the central pair of microtubules are well known in Chlamydomonas (7).

## Inhibition of Regeneration in Flagellar Mutants

The absence of the central pair of microtubules in fm 24 led us to concentrate our efforts on a

TABLE II

*Interaction of Dibutyryl cAMP and Aminophylline in Inhibition of Flagellar Regeneration*

| Treatment | Regeneration medium | Time | Fraction with flagella $(f_o, f_i)$ | | $f_i/f_o$ | | Flagellar length | | Corrected flagellar length | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | W.T. | Fm 24 | W.T. | Fm 24 | W.T. | Fm 24 | W.T. | Fm 24 |
| | | *min* | | | | | $\overline{\mu m}$* | | $\overline{\mu m}_c$‡ | |
| Unsheared | — | −5 | 0.95 | 0.97 | | | 9.1 | 9.5 | | |
| Sheared | TAP | 0 | 0.00 | 0.00 | | | 0.0 | 0.0 | | |
| Sheared | TAP | +60 | 0.89 | 0.96 | | | 7.6 | 7.5 | | |
| Sheared | TAP, 1 mM dibutyryl cAMP | +60 | 0.75 | 0.89 | 0.84 | 0.93 | 6.1 | 7.6 | 5.1 | 7.1 |
| Sheared | TAP, 3.3 mM aminophylline | +60 | 0.83 | 0.83 | 0.93 | 0.86 | 4.2 | 4.4 | 3.9 | 3.8 |
| Sheared | TAP, 1 mM dibutyryl cAMP, 3.3 mM aminophylline | +60 | 0.52 | 0.32 | 0.58 | 0.33 | 4.9 | 1.7 | 2.8 | 0.6 |

* $\overline{\mu m}$ is the average of 20 flagella, in microns.

‡ $\overline{\mu m}_c = \overline{\mu m}\left(\dfrac{f_i}{f_o}\right) + \left(\dfrac{f_o - f_i}{f_o}\right) = \overline{\mu m}\left(\dfrac{f_i}{f_o}\right).$
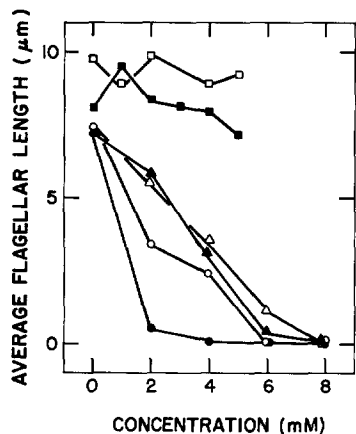


FIGURE 3  Inhibition of regeneration of flagella by methylxanthines. Open symbols, wild type; filled symbols, fm 24; circles, caffeine; triangles, aminophylline; squares, dibutyryl cAMP. The data for methylxanthines are from one experiment and those for dibutyryl cAMP are from a second experiment. The cells regenerated for 60 min.

comparison of this mutant with wild type. The kinetics of regeneration were the same in wild type and fm 24 (Fig. 5). Aminophylline inhibits regeneration by wild type and by fm 24 to about the same degree (Table II, Fig. 3). However, regeneration by fm 24 is exceptionally sensitive to caffeine (Fig. 3, Table III) and the combination of 1 mM dibutyryl cAMP plus 3.3 mM amino-

phylline (Tables II and III, Fig. 5). In contrast, the same combination inhibits regeneration by wild type about as much as aminophylline alone. The uniqueness of the response of fm 24 lies in the synergistic interaction between aminophylline and dibutyryl cAMP.

The greater sensitivity of fm 24 to caffeine or aminophylline plus dibutyryl cAMP can be demonstrated with cells from cultures in early, middle, or late exponential phase, but both wild-type and fm 24 cells lose their capacity to regenerate flagella as they pass into stationary phase (Table III). Neither butyric acid, cAMP, nor adenosine 2′,3′-cyclic monophosphate could replace dibutyryl cAMP in the inhibition of regeneration by fm 24.

The combination of aminophylline plus dibutyryl cAMP also inhibits strongly flagellar regeneration by fm 8 and 15, while regeneration by fm 9, 10, 12, and 30 can be inhibited to about the same degree as wild type (Table IV). In another experiment, however, 3.3 mM caffeine completely inhibited regeneration by fm 30, while regeneration by fm 9, 10, and 12 was little affected.

## DISCUSSION

The hypothesis that cAMP mediates the regulation of microtubule-dependent processes led us to predict that flagellar function or formation should be affected by treatments which alter the level of endogenous cAMP. The prediction has
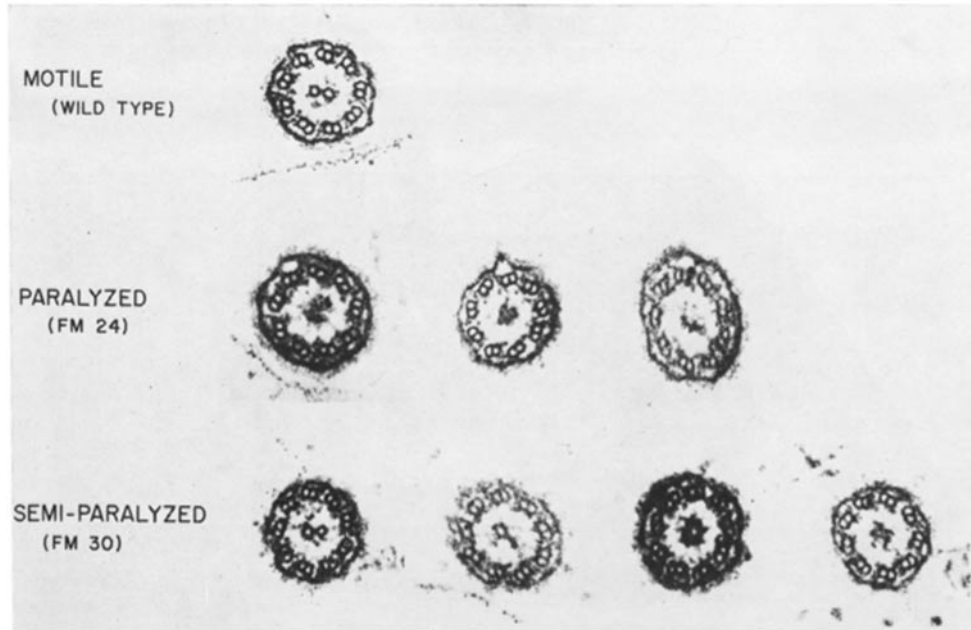
FIGURE 4   Structural abnormalities in flagella of motility mutants.
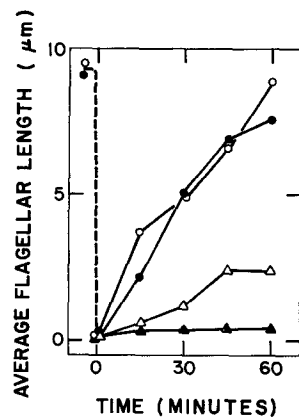


FIGURE 5   Kinetics of flagellar regeneration. Open symbols, wild type; filled symbols, fm 24; circles, regeneration in TAP; triangles, regeneration in 1 mM dibutyryl cAMP plus 3.3 mM aminophylline.

proved to be correct for the flagella of *C. reinhardtii*. We have shown that treatment of cells with aminophylline under conditions which result in more than a tenfold increase in endogenous cAMP causes inhibition of both flagellar movement and flagellar regeneration. These inhibitory effects are relatively specific since the same concentration of aminophylline does not inhibit cell

multiplication. If, for instance, aminophylline inhibited flagellar regeneration by inhibiting general protein synthesis, one would expect that cell multiplication would also have been inhibited. Caffeine, another methylxanthine which is well known as an inhibitor of mammalian cyclic nucleotide phosphodiesterase (11), also inhibits flagellar function and regeneration, although not with the same degree of specificity as aminophylline, since caffeine slows cell multiplication.

It is conceivable that, in addition to increasing endogenous cAMP, aminophylline and caffeine have other effects on *Chlamydomonas* and that one of these other effects may account for the inhibition of flagellar regeneration. However, we have also found that mutations which affect flagellar structure have a high probability of rendering the flagellar regeneration mechanism very sensitive to synergistic inhibition by aminophylline plus dibutyryl cAMP, as well as caffeine alone. Since the only well-established common denominator of aminophylline and dibutyryl cAMP is that they both increase endogenous cAMP activity (11), the synergism clearly points to cAMP as the factor responsible for inhibition of flagellar regeneration.

The available data do not rule out the possi-

TABLE III

*Inhibition of Flagellar Regeneration as a Function of Cell Titer*

| Cell line | Titer, cells $(10^{-6})$/ml | Unsheared length | Regenerated length, 60 min ($\overline{\mu m}_c$) | | |
|---|---|---|---|---|---|
| | | | Control | 1 mM dibutyryl cAMP, 3.3 mM aminophylline | 3.3 mM caffeine |
| | | $\overline{\mu m}$ | | | |
| Wild type | 0.45 | 10.1 | 9.2 | 4.2 | 5.7 |
| | 1.13 | 9.7 | 9.7 | 8.0 | 8.9 |
| | 2.72 | 8.4 | 9.4 | 5.7 | 6.9 |
| | 5.65 | 10.3 | 7.1 | 4.6 | 8.6 |
| | 6.40 | 11.8 | 2.5 | 0 | 0 |
| Fm 24 | 0.17 | 10.3 | 6.3 | 4.9 | 0 |
| | 0.90 | 7.8 | 8.2 | 1.5 | 0 |
| | 3.20 | 10 | 5.5 | 0 | 0.7 |
| | 5.56 | 11.7 | 4.9 | 1.6 | 2.1 |
| | 12.9 | 10.3 | 5.2 | 0 | 0 |
| | 13.0 | 9.1 | 0 | 0 | 0 |

Stationary phase cultures of wild-type and fm 24 cells were diluted to $10^4$ cells/ml in TAP. Aliquots were removed twice a day for titer determination and shear regeneration experiments. Both cultures multiplied exponentially with a doubling time of 8.5–9 h. The stationary phase titer of wild type was unusually low in this experiment. The same pattern of results has been obtained with wild-type cells that attained a stationary phase titer above $10^7$.

TABLE IV

*Inhibition of Flagellar Regeneration in Mutants Exhibiting Impaired Motility*

| Cell line | Unsheared length | Regenerated length, 60 min ($\overline{\mu m}_c$) | | Regeneration, % control |
|---|---|---|---|---|
| | | Control | 1 mM dibutyryl cAMP, 3.3 mM aminophylline | |
| | $\overline{\mu m}$ | | | |
| Wild type (+) | 11.6 | 8.9 | 6.9 | 78 |
| Fm 8 | 4.4 | 3.7 | 0.0 | 0 |
| Fm 9 | 4.6 | 4.3 | 2.7 | 63 |
| Fm 10 | 10.1 | 8.0 | 5.7 | 71 |
| Fm 12 | 11.4 | 8.6 | 7.2 | 84 |
| Fm 15 | 8.3 | 7.8 | 1.0 | 13 |
| Fm 24 | 9.4 | 7.0 | 2.8 | 40 |
| Fm 30 | 7.6 | 5.1 | 4.2 | 82 |

The cells used in this experiment were from overnight cultures which had been started from slants. The titers varied between 1.3 and 9.5 × $10^6$ cells/ml.

bility that cAMP affects some entity remote from the flagella, which in turn acts upon the flagella, perhaps again through intermediates. The influence of cAMP on flagella, therefore,

may be rather indirect. However, the heightened sensitivity to cAMP of the regeneration process in mutants with structurally abnormal flagella indicates that, regardless of whether cAMP acts directly or indirectly, some link in the chain of components by which cAMP inhibits regeneration is strongly dependent upon the integrity of the flagellum. The simplest explanation is that the component in question is in the flagellum itself.

Is that component microtubule protein? It is impossible to say at this time. A direct relationship, i.e., the phosphorylation of microtubule protein by a cAMP-dependent protein kinase (see Introduction), seems unlikely: in feeding experiments with $^{32}PO_4^{3-}$, at a specific activity more than sufficient for detection of one phosphorus atom per molecule of microtubule protein, no detectable radioactivity was found incorporated into microtubule protein that had been extracted from isolated flagella, reduced and alkylated, and subjected to electrophoresis on sodium dodecyl sulfate-containing acrylamide gels (N. Amrhein, R. W. Rubin, and P. Filner, unpublished observations). It seems, therefore, that microtubule protein in the *Chlamydomonas* flagellum, i.e., polymerized microtubule protein, does not contain covalently bound phosphate. One remaining possibility is that depolymerized

microtubule protein may be phosphorylated, in which case cAMP could inhibit regeneration by stimulating phosphorylation and maintenance of microtuble protein in the depolymerized state.

## REFERENCES

1. RASMUSSEN, H. 1970. Cell communication, calcium ion and cyclic adenosine monophosphate. *Science (Wash. D. C.).* 170:404.
2. GILLESPIE, E. 1971. Colchicine binding in tissue slices. Decrease by calcium and biphasic effect of adenosine-3′,5′-monophosphate. *J. Cell Biol.* 50:544.
3. GOODMAN, D. B. P., H. RASMUSSEN, F. DiBELLA, and C. E. GUTHROW. 1970. Cyclic adenosine 3′:5′ monophosphate stimulated phosphorylation of isolated neurotubule subunits. *Proc. Natl. Acad. Sci. U. S. A.* 67:652.
4. MURRAY, A. W., and M. FROSCIO. 1971. Cyclic adenosine 3′:5′ monophosphate and microtubule function: specific interactions of the phosphorylated protein subunits with a soluble brain component. *Biochem. Biophys. Res. Commun.* 44:1089.
5. EIPPER, B. A. 1972. Rat brain microtubule protein: purification and determination of covalently bound phosphate and carbohydrate. *Proc. Natl. Acad. Sci. U. S. A.* 69:2283.
6. OLMSTED, J. B., G. B. WITMAN, K. CARLSON, and J. L. ROSENBAUM. 1971. Comparison of the microtubule proteins of neuroblastoma cells, brain and *Chlamydomonas* flagella. *Proc. Natl. Acad. Sci. U. S. A.* 68:2273.
7. WARR, J. R., A. McVITTIE, SIR J. RANDALL, and J. M. HOPKINS. 1966. Genetic control of the flagellar structure in *Chlamydomonas reinhardii. Genet. Res.* 7:335.
8. ROSENBAUM, J. L., J. E. MOULDER, and D. L. RINGO. 1969. Flagellar elongation and shortening in *Chlamydomonas.* The use of cycloheximide and colchicine to study the synthesis and assembly of flagellar proteins. *J. Cell Biol.* 41:600.
9. COYNE, B., and J. L. ROSENBAUM. 1970. Flagellar elongation and shortening in *Chlamydomonas.* II. Reutilization of flagellar proteins. *J. Cell Biol.* 47:777.
10. JACOBS, M., and A. McVITTIE. 1970. Identification of the flagellar proteins of *Chlamydomonas reinhardii. Exp. Cell Res.* 63:53.
11. ROBINSON, G. A., R. W. BUTCHER, and E. W. SUTHERLAND. 1971. Cyclic AMP. Academic Press Inc., New York.