ON THE POSSIBLE ROLE OF SEROTONIN IN THE REGULATION OF REGENERATION OF CILIA

NURI RODRÍGUEZ and FERNANDO L. RENAUD

From the Biology Department, University of Puerto Rico, Río Piedras, Puerto Rico 00931. Dr. Rodríguez's present address is the Department of Microbiology, Medical Sciences Campus, University of Puerto Rico, San Juan, Puerto Rico 00936.

ABSTRACT

A study was made of the interrelationship of serotonin, cAMP, and calcium ions in the regulation of regeneration of cilia by *Tetrahymena pyriformis*. All these compounds stimulated the regeneration, whereas a blocker of serotonin synthesis, *p*-chlorophenylalanine, and a calcium chelator, EGTA, inhibited the process. This inhibition could be overcome by the addition of any of the stimulatory compounds. cAMP was also found to be inhibitory at high concentrations. The intracellular concentration of this nucleotide was found to increase during the regeneration, and this increase occurred precociously in the presence of serotonin. It was concluded that serotonin may regulate ciliary regeneration by a mechanism involving cAMP and calcium ions, but that the causal relationships among these compounds still need to be established.

Serotonin is a neurotransmitter in invertebrate nervous systems and a putative neurotransmitter in vertebrates (7). However, its biological role is not necessarily restricted to the transmission of chemical signals by nerve cells, because it has also been reported in a wide variety of non-neural systems. For example, it has been found in unfertilized vertebrate and invertebrate eggs (3–5, 9), in protozoa (16), and in blood platelets (22). However, the information we have about its mechanism of action is very limited. In most systems studied so far, it seems to involve both cyclic nucleotides and divalent cations. For example, the stimulation of fly salivary gland secretion by serotonin has been studied in detail and is known to involve cAMP and calcium ions (23). An increase in the level of cAMP has also been found during ciliary movement (10), but in the case of blood platelet activation (1), serotonin causes an increase in cGMP but no changes in cAMP. Calcium ions have also been implicated in some of these systems; for example, it is known that a calcium ionophore will activate both blood platelets and salivary gland secretion (18, 24). On the other hand, the role of serotonin in unfertilized eggs and protozoan cells is even more obscure. These systems are particularly intriguing because, being unicellular, the target of the neurohormone would be the secretory cell itself. Buznikov et al. speculated that serotonin may act as a regulator of cell division in the sea urchin egg, as they found that anti-serotonin agents caused delays in the first division (4). In addition, Gustafson and Toneby (14) found that *p*-chlorophenylalanine (PCPA), a specific inhibitor of serotonin synthesis, caused developmental abnormalities in sea urchin embryos. The effects of this drug could be overcome by the addition of 5-hydroxytryptophane (5-HTP), the immediate precursor of serotonin.

In *Tetrahymena pyriformis*, serotonin has been reported to be present in stationary-phase cultures (2), but its specific biological role in this organism

J. CELL BIOLOGY © The Rockefeller University Press · 0021-9525/80/05/0242/06 $1.00
Volume 85 May 1980 242–247

is unknown. There is some evidence that it may activate phagocytosis in this ciliate (8). In addition, it has been reported that this neurohormone may indeed activate adenylate cyclase in *Tetrahymena* (32). In this paper, we present evidence that serotonin may act as a regulator of ciliary regeneration in this protozoan. A preliminary account of this work has been presented previously (28).

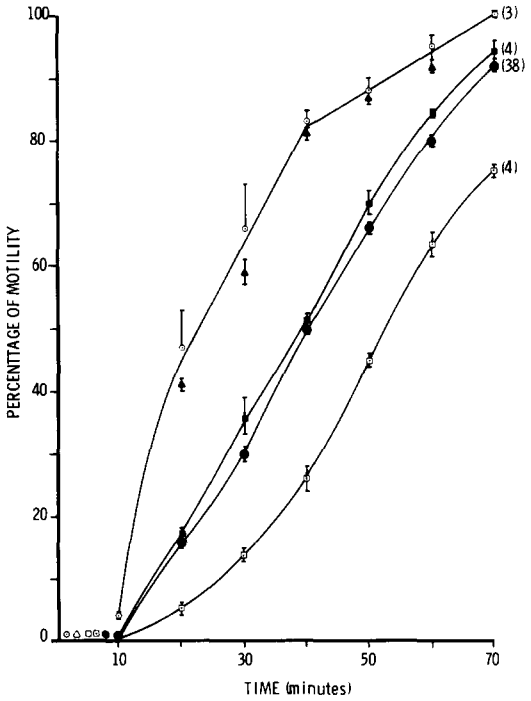
MATERIALS AND METHODS

Tetrahymena pyriformis W strain was cultured in 2% proteose-peptone (Difco Laboratories, Detroit, Mich.), 0.1% dextrose in 1-liter wide-bottom flasks with surface aeration. Log phase cultures were deciliated by a technique similar to that of Rosenbaum and Carlson (30). The cells were first washed with a buffered salt-sucrose solution (0.2 M sucrose, 0.18% NaCl, 10 mM imidazole-HCl, pH 6.8) and then resuspended with 10 vol of deciliating solution at 5°C (50 mM sodium acetate, pH 5.0, 0.1% EDTA, and 0.2 M sucrose). This was followed by the addition of 0.01 vol of 1 M $CaCl_2$ and vigorous agitation with a test tube mixer for 15–20 s. The deciliated cells were then quickly spun down with a hand centrifuge and resuspended in 10 vol of regeneration medium (0.2 M sucrose, 10 mM PIPES buffer, pH 6.8). 10-ml aliquots of the cell suspension were then transferred to 50-ml flasks in a constant-temperature shaker bath at 25°C. Ciliary regeneration was assayed by a modification of the procedure of Rosenbaum and Carlson (30). Samples were taken at 10-min intervals and examined microscopically (× 400; Zeiss phase microscope with Neofluar objectives) under a vaseline-ringed coverslip, and the percentage of motile cells was scored. At least 100 cells were observed in each sample. Several compounds were added to the regeneration medium, singly or in various combinations, to test their effect on the regeneration process: serotonin creatine sulfate (5-HT), PCPA, 5-HTP, cAMP and its dibutyryl derivative (But_2 cAMP), EGTA, $CaCl_2$, and the calcium ionophore A23187. All the compounds tested were obtained from Sigma Chemical Co. (St. Louis, Mo.), except the ionophore that was a gift of Eli Lilly and Co. (Indianapolis, Ind.).

The concentration of cAMP was determined by the receptor protein displacement method of Gilman and Murad (13). 5-ml aliquots of regenerating cells are taken at different time intervals and centrifuged for 2 min at 0°–5°C. The cell sediment was then resuspended in 5% TCA, the precipitated protein was removed by centrifugation, and the TCA was extracted from the supernate with water-saturated ether. The samples were then lyophilized and stored at −20°C until just before the assay. The protein concentration was determined by the method of Lowry et al. (17), using bovine serum albumin as a standard.

RESULTS

When the deciliated *Tetrahymena* cells are resuspended in regeneration medium there is a lag period of 10 min before any motility is observed (Fig. 1). The regeneration then proceeds with exponential kinetics and is essentially complete in 80 min. In the presence of 5-HT the duration of the lag period is not affected, but the period of the exponential regeneration is accelerated (Fig. 1). Similar results were observed with concentrations



FIGURES 1–5 The bars at each point indicate the standard error of the mean. The numbers in parentheses in each graph indicate the number of times each experiment was performed.

FIGURE 1 The effect of serotonin on ciliary regeneration. Note that cells regenerating in the presence of 1 mM serotonin (○) or 10^{-5} M serotonin (▲) regenerate faster than control cells (●), whereas the regeneration is delayed by 1 mM PCPA (□). In the presence of both 1 mM serotonin and 1 mM PCPA, the regeneration kinetics are very similar to those of control cells (■).

of 5-HT as low 10^{-5} M, and in the presence of 5-HTP, the immediate precursor of serotonin. On the other hand, the regeneration is inhibited by PCPA (Fig. 1), a compound that is reported to block 5-HT synthesis by inhibiting the hydroxylation of tryptophan. However, in the presence of both PCPA and 5-HT, the regeneration proceeds with control kinetics (Fig. 1). Similar results were obtained when 5-HTP was substituted for 5-HT in the presence of PCPA.

The effect of cAMP or But_2 cAMP on the regeneration is concentration dependent. A high concentration of But_2 cAMP slows down the regeneration, whereas an intermediate concentration (5 mM) accelerates it (Fig. 2). However, a But_2 cAMP concentration lower than 1 mM has no effect on the regeneration kinetics. Similar results

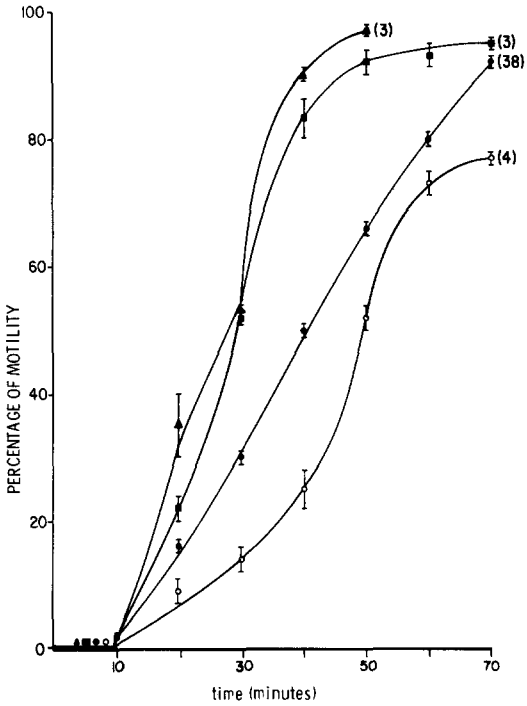


FIGURE 2 The effect of But_2 cAMP on ciliary regeneration. 5 mM But_2 cAMP stimulates the regeneration rate (▲) whereas 10 mM (○) inhibits it. The stimulatory effect of 5 mM But_2 cAMP is seen even in the presence of 1 mM PCPA (■). Control cells (●).

are obtained when cAMP is substituted for But_2 cAMP. The stimulatory effect of But_2 cAMP is observed even in the presence of PCPA (Fig. 2), suggesting that this effect is independent of serotonin synthesis.

Calcium ions seem to play an important role in the regulation of regeneration, and the addition of calcium ions to the regeneration medium also shows concentration-dependent effects: a high calcium concentration (10 mM) results in a marked slowdown of the regeneration process, whereas a concentration of 1 mM accelerates it (Fig. 3). The stimulatory effect is obtained with even much lower concentrations, down to 10^{-6} M. Addition of EGTA, a calcium-chelating agent, to the regeneration medium will inhibit the process (Fig. 4). However, in the presence of both EGTA and 5-HT the cells regenerate with control kinetics (Fig. 4). The results obtained with the calcium ionophore A23187 resemble those observed after the direct addition of calcium ions. Micromolar concentrations of the ionophore inhibit the regeneration, whereas lower concentrations ($1.0–0.5 \times 10^{-9}$ M) stimulate it (Fig. 5). In addition, it should be observed that calcium ions will counteract the inhibitory effect of PCPA (Fig. 4).

The intracellular concentration of cAMP during the course of the regeneration is illustrated in Fig. 6. Notice that the cyclic nucleotide level remains constant up to 10 min after the deciliation and then starts to increase, reaching a maximum after 20 min. It then starts to decrease and reaches pre-regeneration levels 40 min after the deciliation. If 5-HT is present in the regeneration medium, the maximal level of cAMP is reached immediately and, after 20 min, starts to decrease, reaching the same level as the control cells (Fig. 6).

DISCUSSION

The regeneration of cilia and flagella has been the subject of previous studies by several workers in various systems such as *Tetrahymena* (15, 26, 35), *Chlamydomonas* (31, 33), and sea urchin blastulae (34). However, very little is known about the regulation of these processes. The present work sug-

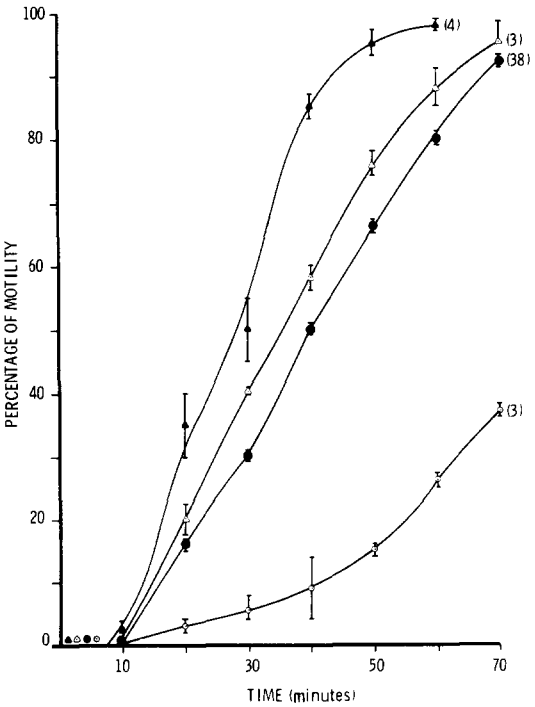


FIGURE 3 The effect of calcium ions on ciliary regeneration. A calcium concentration of 10 mM will inhibit regeneration (○) whereas a concentration of 1 mM (▲) or 10^{-6} M (△) will stimulate it. Control cells (●).

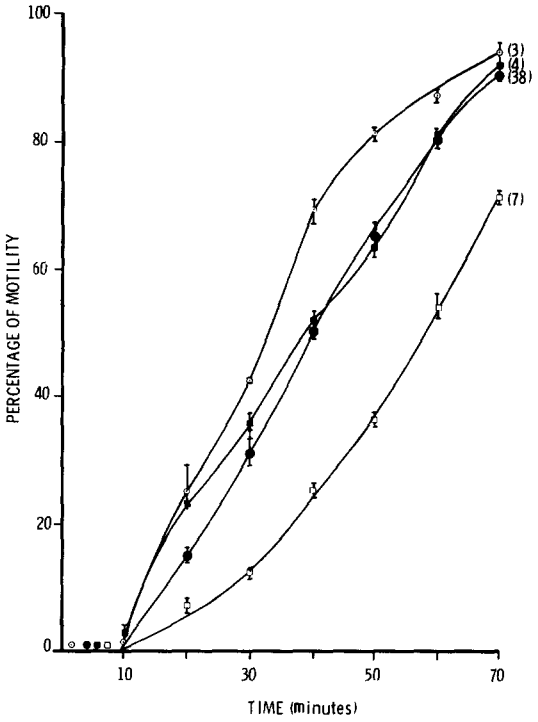


FIGURE 4 1 mM calcium will overcome the inhibitory effect of 1 mM PCPA (○). A calcium-chelating agent, 1 mM EGTA, will inhibit regeneration (□), but in the presence of both 1 mM EGTA and 1 mM serotonin (■) the cells regenerate with control kinetics (●).

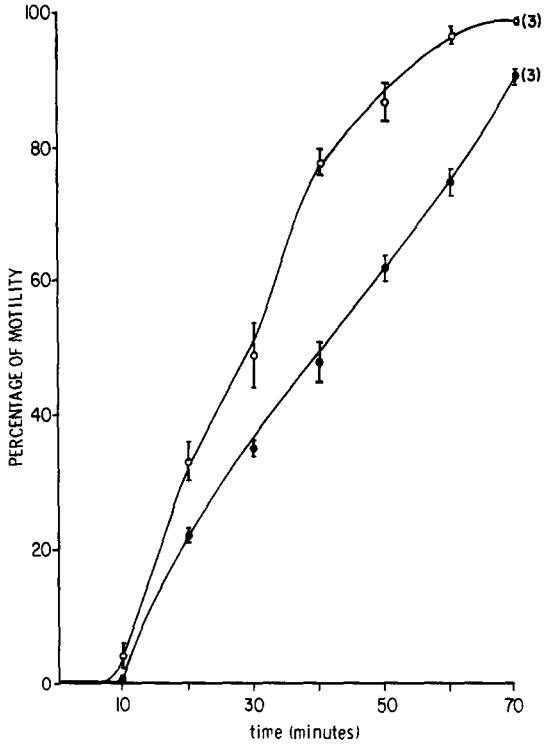


FIGURE 5 The effect of the ionophore A23187 on ciliary regeneration. Control cells (●); 0.5 nM ionophore (○).

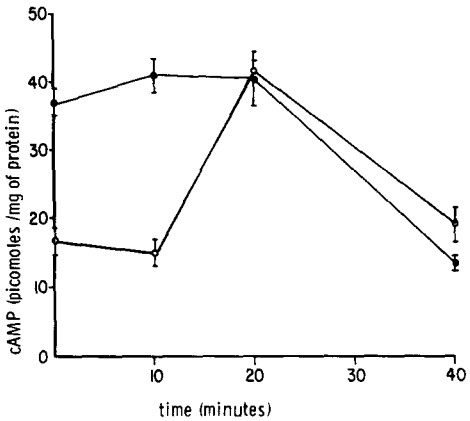


FIGURE 6 The effect of serotonin on the intracellular concentration of cAMP during ciliary regeneration. Samples were prepared and assayed as described in Materials and Methods. Each time point represents the mean of at least five experiments; the bars indicate the standard error of the mean. It should be noted that the abscissa represents the time when the samples were taken after deciliation. Control cells (○); cells regenerating in the presence of 1 mM serotonin (●) from zero time.

gests that serotonin may play an important role in this regulation in *Tetrahymena* because: (*a*) exogenous serotonin accelerates the regeneration kinetics (Fig. 1), and this effect is noticed even with micromolar concentrations of the neurohormone; (*b*) blocking serotonin synthesis with a specific inhibitor (PCPA) will also cause a delay in the regeneration kinetics (Fig. 1); (*c*) the delay caused by PCPA can be counteracted by the addition of serotonin or of 5-HTP, the immediate precursor of serotonin (Fig. 1); (*d*) serotonin will counteract the inhibitory effect of other agents such as EGTA (Fig. 4). That the effect of the neurotransmitter may be at the post-translational level is suggested by the fact that neither serotonin nor PCPA has any effect on total RNA or protein synthesis during the regeneration process (29). However, an effect of serotonin at the transcriptional and translational level cannot be excluded at the moment. Guttman and Gorovsky (15) have demonstrated that both RNA and protein syntheses are impor-

tant in ciliary regeneration in starved *Tetrahymena* cells.

It is of interest to note that it had been previously reported that melatonin, a molecule derived from serotonin, can inhibit ciliary regeneration in *Tetrahymena* (6). Perhaps the inhibition is caused by a competition between melatonin and serotonin for the same receptor sites on the membrane. Other workers have also reported that treatment with the monoamine oxidase inhibitor pargyline caused ciliogenesis in normally nonciliated rat brain cells (19). The authors speculated that the drug caused increased intracellular levels of serotonin, which would be in agreement with our hypothesis.

In our system we are not measuring regeneration directly, but indirectly using motility as a regeneration index. Nevertheless, we feel that motility is a true index of regeneration, as has been stated by other authors (15, 30, 35); because cell motility is dependent on the existence of cilia, it should be directly proportional to the number of regenerating cells. In addition, observations on fixed cells suggest that there is a direct correlation between the number of ciliated cells and the motility of the population. Unfortunately, newly formed cilia detach very easily after fixation and make statistical studies difficult. However, if the stimulatory or inhibitory compounds are added to nondeciliated cells in regeneration medium, they have no noticeable effect on cell motility (29). If serotonin or cAMP had a stimulatory effect on motility, or if PCPA or EGTA had an inhibitory effect, these same effects should be noticed even on undeciliated cells, but this is not observed. Therefore, it cannot be argued that the observed effects are caused by inhibition or stimulation of motility, but by a direct effect on the regeneration rate.

In addition, it should be emphasized that a stimulatory or inhibitory effect on motility, and not on regeneration rate, would not be shown by our data. A direct effect on motility would cause already motile cells to move either more slowly or more rapidly, depending on whether the compound is inhibitory or stimulatory. However, in our assay we are not discriminating between slower or faster movements, but between moving cells, regardless of the speed, and immobile cells. In our observations with phase microscopy, we have never detected cilia in immobile cells.

Serotonin has been reported to act in various systems by mechanisms involving cyclic nucleotides and calcium ions. In our system, the evidence suggests that serotonin may stimulate ciliary regeneration by a cAMP-mediated mechanism, as exogenous serotonin will cause a precocious rise in the intracellular concentration of cAMP in regenerating cells (Fig. 6). Thus, there is a direct correlation between the concentration of cAMP and the regeneration rate. In addition, dibutyryl cAMP can counter the inhibitory effect of PCPA (Fig. 2), suggesting that in the presence of exogenous nucleotide, serotonin synthesis is not required for regeneration.

Our results show that the effect of cAMP on ciliary regeneration is dose dependent; relatively high concentrations of the nucleotide are inhibitory, but lower concentrations are stimulatory (Fig. 2). cAMP has been reported to have similar dose-dependent effects in other systems involving microtubule assembly (11, 12). However, the basis of the stimulation or inhibition remain so far unknown. Other workers have reported previously that cAMP inhibited ciliary or flagellar regeneration (33, 35). Wolfe (35) found that both 5 and 10 mM but_2 cAMP inhibited ciliary regeneration, 5 mM only slightly. However, his assay system was different from ours, as he made only one motility measurement, 90 min after deciliation. Therefore any differences in regeneration kinetics that occur early in the process would not be detected by his procedure. Rubin and Filner (33) also reported that 1 mM But_2 cAMP inhibited flagellar regeneration in *Chlamydomonas* in the presence of aminophylline. 5 mM But_2 cAMP by itself had no effect on the regeneration rate. However, as in the previously discussed work, only one time point was examined, 60 min after deflagellation, and any early stimulation by the nucleotide of the regeneration rate would not be detected.

Our data so far indicate that calcium ions play an important role in the regulation of regeneration, as this process can be stimulated by moderate and low concentrations of these ions (Fig. 3) and by a calcium ionophore (Fig. 5). A similar dose-dependent effect by calcium ions has been reported in other systems (11, 21). It has also been reported that calcium ions are essential for flagellar regeneration in *Chlamydomonas* (25). However, it is known that calcium ions will inhibit microtubule assembly "in vitro" (20). This suggests that the stimulatory effect of calcium on the regeneration is probably not caused by a direct stimulation of microtubule assembly, but by some other unknown mechanism.

Serotonin seems to be involved in the calcium metabolism of regenerating cells, as calcium ions

can counteract the inhibitory effect of PCPA (Fig. 4), and serotonin can counteract the inhibitory effect of the calcium-chelating agent EGTA (Fig. 4). These results suggest that regenerating cells may utilize calcium ions either from the external medium or from internal stores. When the external calcium is sequestered by EGTA, the regeneration is delayed because one of the calcium sources is blocked. However, in the presence of serotonin, regeneration proceeds normally because serotonin may help to mobilize calcium ions from intracellular stores. The latter process cannot take place when serotonin synthesis is blocked, and the regenerating cells then depend mostly on external calcium. That is why regeneration may proceed normally in the presence of PCPA if the calcium concentration of the external medium is increased. A somewhat similar hypothesis has been proposed to explain the action of serotonin on the fly salivary gland (27).

Thus, the results obtained in this work suggest strongly that serotonin, cAMP, and calcium ions are part of the regulative "loop" of ciliary regeneration, although the causal relationships among these parameters are not yet clear.

This work was supported by National Institutes of Health grant RR-8102.

Received for publication 26 June 1979, and in revised form 27 December 1979.

REFERENCES

1. AGARWAL, K. C., and K. C. STEINER. 1976. Effect of serotonin on cyclic nucleotides of human platelets. *Biochem. Biophys. Res. Commun.* **69:** 962–969.
2. BRIZZI, G., and J. J. BLUM. 1970. Effect of growth conditions on serotonin content of *Tetrahymena pyriformis*. *J. Protozool.* **17:**553–555.
3. BUZNIKOV, G. A., I. V. CHUDAKOVA, and N. D. ZVEDZINA. 1964. The role of neurohumours in early embryogenesis. I. Serotonin content of developing embryos of sea urchin and loach. *J. Embryol. Exp. Morphol.* **12:**563–573.
4. BUZNIKOV, G. A., A. N. KOST, N. F. KUCHEROVA, A. L., MUNDZHOYAN, N. N. SUVOROV, and L. V. BERDYSHEVA. 1970. The role of neurohumours in early embryogenesis. III. Pharmacological analysis of the role of neurohumours in cleavage division. *J. Embryol. Exp. Morphol.* **23:** 549–569.
5. BUZNIKOV, G. A., A. V. SAKHAROVA, B. N. MANUKHIN, and L. N. MARKOVA. 1972. The role of neurohumours in early embryogenesis. IV. Fluorometric and histochemical study of serotonin in cleaving eggs and larvae of sea urchins. *J. Embryol. Exp. Morphol.* **27:**339–351.
6. CASTRO, W., N. COLÓN, and F. RENAUD. 1973. The effect of melatonin on cilia regeneration and cell division. *J. Cell Biol.* **59**(2, Pt. 2): 47*a* (Abstr.).
7. COOPER, J. R., F. E. BLOOM, and R. H. ROTH. 1974. The Biochemical Basis of Neuropharmacology. Oxford University Press, New York.
8. CSABA, G., S. U. NAGY, AND T. LANTOS. 1976. Are biogenic amines acting on *Tetrahymena* through a cyclic AMP mechanism? *Acta Biol. Med. Ger.* **35:**259–261.
9. EMANUELSON, H. 1974. Localization of serotonin in cleavage embryos of *Ophryotrocha labronica*. La Graca and Bacci. *Wilhelm Roux's Arch. Dev. Biol.* **175:**253–271.
10. GENTLEMAN, S., and T. E. MANSOUR. 1977. Control of Ca^{+2} efflux and cyclic AMP by 5-hydroxytryptamine and dopamine in abalone gill. *Life Sci.* **20:**687–694.
11. GILLESPIE, E. 1971. Colchicine binding in tissue slices. Decrease by Ca^{++} and biphasic effect of adenosine 3,'5' monophosphate. *J. Cell Biol.* **50:**544–549.
12. GILLESPIE, E. 1975. Microtubules, cyclic AMP, calcium and secretion. *Ann. N. Y. Acad. Sci.* **253:**771–779.
13. GILMAN, A. G., and F. MURAD. 1974. Assay for cyclic nucleotides by receptor protein binding displacement. *Methods Enzymol.* **38:**49–61.
14. GUSTAFSON, T., and M. TONEBY. 1970. On the role of serotonin and acetylcholine in sea urchin morphogenesis. *Exp. Cell Res.* **62:**102–117.
15. GUTTMAN, S. D., and M. A. GOROVSKY. 1979. Cilia regeneration in starved *Tetrahymena*: An inducible system for studying gene expression and organelle biogenesis. *Cell.* **17:**307–317.
16. JANAKIDEVI, K., V. C. DEWEY, and G. W. KIDDER. 1966. Serotonin in protozoa. *Arch. Biochem. Biophys.* **113:**758–759.
17. LOWRY, O. H., N. K. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–276.
18. MASSINI, P., and E. F. LUSCHER. 1974. Some effects of ionophores for divalent cations on blood platelets. *Biochim. Biophys. Acta.* **732:**109–121.
19. MILHAUD, M., and G. D. PAPPAS. 1968. Cilia formation in the adult cat brain after pargyline treatment. *J. Cell Biol.* **37:**599–609.
20. OLMSTED, V., and G. G. BORISY. 1973. Characterization of microtubule assembly in porcine brain extracts by viscometry. *Biochemistry.* **12:** 4282–4289.
21. PERCHELLET, J. P., and R. K. SHARMA. 1979. Mediatory role of calcium and guanosine 3':5 monophosphate and adrenocorticotropin-induced steroidogenesis by adrenal cells. *Science (Wash. D. C.).* **203:**1259–1261.
22. PLETSHER, A., and M. DAPRADA. 1975. The organelles storing 5-hydroxytryptamine in blood platelets. *Ciba Found. Symp.* **35:**261–279.
23. PRINCE, W. T., J. BERRIDGE, and H. RASMUSSEN. 1972. Role of calcium and adenosine 3':5'-cyclic monophosphate in controlling fly salivary gland secretion. *Proc. Natl. Acad. Sci. U. S. A.* **69:**553–557.
24. PRINCE, W. T., H. RASMUSSEN, and M. BERRIDGE. 1973. The role of calcium in fly salivary gland secretion analyzed with the ionophore A 23187. *Biochim. Biophys. Acta.* **329:**98–107.
25. QUADER, H., J. CHERNIAK, and P. FILNER. 1977. Chemically induced shortening and regeneration in *Chlamydomonas reinhardii*. *Exp. Cell. Res.* **113:**295–301.
26. RANNESTAD, J. 1974. The regeneration of cilia in partially deciliated *Tetrahymena*. *J. Cell Biol.* **63:**1009–1017.
27. RASMUSSEN, H., and B. P. GOODMAN. 1977. Relationships between calcium and cyclic nucleotides in cell activation. *Physiol. Rev.* **57:**421–509.
28. RODRÍGUEZ, N., and F. L. RENAUD. 1976. On the possible role of serotonin in the regulation of cilia regeneration. *J. Cell Biol.* **70**(2, Pt. 2):94*a* (Abstr.).
29. RODRÍGUEZ, N. 1978. Effect of serotonin, cyclic nucleotides and calcium ions on ciliary regeneration in *Tetrahymena pyriformis*. Ph. D. Thesis. University of Pureto Rico, School of Medicine, Río Piedras, P. R.
30. ROSENBAUM, J., and K. CARLSON. 1969. Cilia regeneration in *Tetrahymena* and its inhibition by colchicine. *J. Cell Biol.* **40:**415–425.
31. 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:**600–619.
32. ROSENSWEIG, Z., and S. H. KINDLER. 1972. Epinephrine and serotonin activation of adenyl cyclase from *Tetrahymena pyriformis*. *FEBS.* (*Fed. Eur. Biochem. Soc.*) *Lett.* **25:**221–223.
33. RUBIN, R., and P. FILNER. 1973. Adenosine 3':5-cyclic monophosphate in *Chlamydomonas reinhardtii*. Influence on flagellar function and regeneration. *J. Cell Biol.* **56:**628–635.
34. STEPHENS, R. E. 1972. Studies on the development of the sea urchin *Strongylocentrotus droebachiensis*. III. Embryonic synthesis of ciliary proteins. *Biol. Bull.* **142:**489–504.
35. WOLFE, J. 1973. Cell division, ciliary regeneration, and cyclic AMP in a unicellular system. *J. Cell Physiol.* **82:**39–48.