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A Method for the Synchronous Induction of Large Numbers of Telotrochs in *Vorticella convallaria* by Monocalcium Phosphate at Low pH

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ABSTRACT. We have devised a two step method for the synchronous induction of telotrochs in the peritrich ciliate, *Vorticella convallaria*. The method is easy, reliable, and allows us to study the earliest events of telotroch formation at the ultrastructural, biochemical, and molecular levels. The steps involved are: (1) excising the cell body from the stalk in a large population (7.4×10^4 cells) of EDTA-treated, attached cells by the application of monocalcium phosphate monohydrate solution at pH 3.2, (2) rinsing and suspending the isolated cell bodies in inorganic medium. Within 90 min, 80% of the population forms telotrochs. Analysis of factors that are important for maximum stalk excision and transformation shows that the population must not be older than 2 d and the most effective concentration of monocalcium phosphate is 4.8 mM for a 20 min exposure. The most effective monocalcium phosphate is in the monohydrated form. A pH value of 3.2, produced by the addition of hydrochloric acid in the presence or absence of calcium is not sufficient to initiate stalk excision and telotroch formation. This observation leads us to conclude that stalk excision is dependent on monocalcium phosphate or its hydrolysis products.

Supplementary key words. Sessile peritrich, stalk excision, telotroch formation.

POPULATIONS of the peritrich ciliate *Vorticella convallaria* exhibit two major morphological types. The primary type is the sessile trophont termed the stalked zooid and the secondary form called a telotroch is the motile, dispersive form. In nature, when environmental conditions deteriorate sufficiently, the trophont transforms into a telotroch, detaches from the stalk, and swims away leaving the stalk behind. When the telotroch finds a new suitable location, it re-attaches, regrows its stalk, and begins feeding. Recently, the morphology of these transitions, both at the light microscopical [7, 8] and ultrastructural levels [10, 11], has been described for *Vorticella convallaria*. Briefly, when forming a telotroch the trophont stops feeding and begins to elongate; its inverted bell-shape becomes cylindrical. The oral cilia are partially resorbed and the cell produces an aboral ciliary wreath, which serves as a motor. Following a series of convulsive contractions and twists, the newly formed telotroch lifts off from the stalk and swims away [11]. The detachment of the cell from the stalk always occurs at the same position, the stalk/scopular junction. The stalk/scopular junction of the trophont is semipermanent: at its proximal end, the stalk fits into a socket made up of a ring of scopular cilia (inside) and the scopular lip (outside). Small fibrils extend from the sheath to the scopular lip and cilia and may serve to anchor the stalk to the cell body. Rupture of these fibrils has been hypothesized to facilitate stalk excision [11].

There have been several attempts to initiate mass telotroch formation from cultured peritrichs. Most of these methods involve producing some injury to the stalk. For instance, telotroch formation has been induced by scraping attached cells with a rubber policeman or briefly exposing the attached cells to air [1, 2, 6, 8]. At best about 30% of the attached cells transform and are found in a wide range of different ontogenic stages. Another method involves changing the culture medium to induce cell division [3, 8]. In this case, one of the division products is a telotroch [8]. The problems with this scheme are that the telotrochs are contained in a large volume of medium, telotroch development occurs non-synchronously, and most of the early events of transformation have been completed by the time division concludes.

A more rigorous analysis of the trophont to telotroch transition at the ultrastructural, biochemical, and/or molecular levels requires both large numbers of cells in the earliest stages of transformation and their synchronous development throughout ontogeny. This challenge led us to investigate a variety of chemical methods of telotroch induction and to look for similar systems of morphogenesis in other cell types. One of these turned out to be flagellar excision in *Chlamydomonas* [4, 5], which has been hypothesized to involve calcium.

The purpose of this study is to describe a method for mass, synchronous telotroch formation that allows us to investigate early events governing trophont to telotroch formation. Our procedure subjects upwards of 7.0×10^4 trophonts to monocalcium phosphate (MCP) at low pH, initiating better than 80% stalk excision. Following rinsing and suspension of cells at a more basic pH, more than 80% of the detached cells transform into telotrochs.

MATERIALS AND METHODS

Stock cultures of *Vorticella convallaria* were grown on Cerophyl medium according to Vacchiano et al. [8]. For telotroch transformation experiments, cells were grown on Cerophyl medium for 2 d in 2,800-ml Fernbach flasks. This medium was then removed and replaced with fresh Cerophyl and the cultures were agitated at 100 rpm for 24 h on a New Brunswick model G25 incubator/shaker (New Brunswick Scientific, New Brunswick, NJ). This treatment caused some of the cells to detach from the substrate and others to initiate division to form telotrochs [8]. The detached cells and telotrochs were used to inoculate several sterile Petri dishes. The cells were allowed to re-attach and regrow their stalks for 24 hr. These re-attached, stalked cells (1-d-old), were used as starting material for most of the experiments described in this study.

For experiments, the growth medium from the 1-d-old cells was removed and the cells were rinsed twice in 1× rinse buffer (RB) (0.187 mM EDTA, 3.9 mM NaCl, 0.41 mM TRIS-HCl, 0.5 mM maleic acid, pH adjusted to 6.8) and suspended for 5 min in RB. The RB was removed and the test solution was then added to the attached cells. The Petri dish containing the cells was agitated on a shaker (Warner Chilott Laboratory model 21516-1, Richmond, CA) at 70 rpm for 20 min. In the second step of this two-step procedure, the detached cells were immediately rinsed several times by filtration in calcium-free inorganic medium (CFI) (0.24 mM KCl, 0.24 mM $MgSO_4$) or in inorganic medium (IM) (0.048 mM $CaH_4(PO_4)_2$, 0.24 mM KCl, 0.24 mM $MgSO_4$) [7] and then concentrated by filtration to about 25 ml.

The filtration apparatus was fabricated by modifying a Tup-

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Abbreviations: CFI, calcium-free inorganic medium; IM, inorganic medium; MCP, monocalcium phosphate; RB, rinse buffer.

Table 1. The effect of different concentrations of MCP-H_2O on stalk excision and telotroch formation.

Treatment	% stalk excision[a] ± SEM	% telotroch formation[b] ± SEM	% dead cells ± SEM
CFI n = 27	0.27 ± 0.35	NR[c]	0
0.048 mM[d] MCP-H_2O n = 3	0	NR[c]	0
0.96 mM MCP-H_2O n = 3	28 ± 2	NR[c]	0
4.8 mM MCP-H_2O n = 27	84.72 ± 3.45	88.82 ± 2.66	1.5 ± 0.5
9.6 mM[e] MCP-H_2O n = 3	82 ± 0	20.5 ± 0.5	14.5 ± 10.5

[a] Exposure time of 20 min.
[b] Telotroch formation at 90 min post-treatment.
[c] No Results: the levels of stalk excision were too low to accurately assess telotroch formation.
[d] The concentration found in inorganic medium.
[e] Concentrations of MCP-H_2O above 9.6 mM precipitated.

Table 2. The effect of the length of time of exposure to 4.8mM MCP-H_2O on stalk excision and telotroch formation (n = 4).

Time (min)	% stalk excision ± SEM	% telotroch formation[a] ± SEM	% dead cells ± SEM
IM	3.5 ± 1.4	NR[b]	0
5	0	NR[b]	0
10	4	NR[b]	0
15	23 ± 11	NR[b]	0
20	84.72 ± 3.45	88.82 ± 2.66	1.5 ± 0.5
25	87 ± 3	ND[c]	94 ± 2

[a] Telotroch formation at 90 min post-treatment.
[b] No Results: the levels of stalk excision were too low to accurately assess telotroch formation.
[c] No Data: The cells died.

perware™ type plastic storage container (15 × 15 × 8 cm). The bottom of the container and the center of the lid were cut out and discarded. A Nytex filter (3-5/2 μm, 100% polyamide nylon) (Tetko Inc., Briarcliff Manor, NY) was positioned under the lid with its edges protruding beyond the lid rim. The filter was held in place by the edges of the container and a pouch formed at the center when the lid and the bottom snapped together. The filtering apparatus was then inverted and placed on a large tray. Because of the large surface area the liquid passed quickly through the filter into the tray and the isolated cell bodies were easily concentrated to 25 ml. The 25 ml of cells were pipetted into a Petri dish; transformation to the telotroch form was monitored using a Leitz Ortholux microscope.

Cells were counted at all stages of telotroch induction to determine the efficiency of the processes. The number of living cells attached to the bottom of the Petri dish was determined by direct cell counts using a Bausch and Lomb stereo zoom microscope. Given the possibility that the cells may not be uniformly dispersed throughout the Petri dish, we examined eight randomly placed, nonadjacent, 0.25 cm^2 sample areas. The number of cells was determined for each of these areas and from these numbers we were able to extrapolate the total number of cells present in the Petri dish.

The number of non-attached cells, either zooids or telotrochs, was determined by removing a 1-ml aliquot, fixing the cells by addition of a few drops of Bouin's fluid, and counting them directly using a Segdwick-Rafter counting chamber.

Photographs were taken with an Orthomat automatic camera attached to a Leitz Ortholux microscope equipped with phase-contrast optics.

RESULTS

In preliminary experiments, we tested several calcium-containing compounds for their effect at initiating transformation to the telotroch form. These included calcium chloride, calcium sulfate, and monocalcium phosphate (MCP). Of these compounds only MCP at pH 3.2 induced significant transformation. Unless otherwise noted, MCP utilized in these experiments was obtained from Fisher Scientific, Pittsburgh, PA. Addition of 4.8

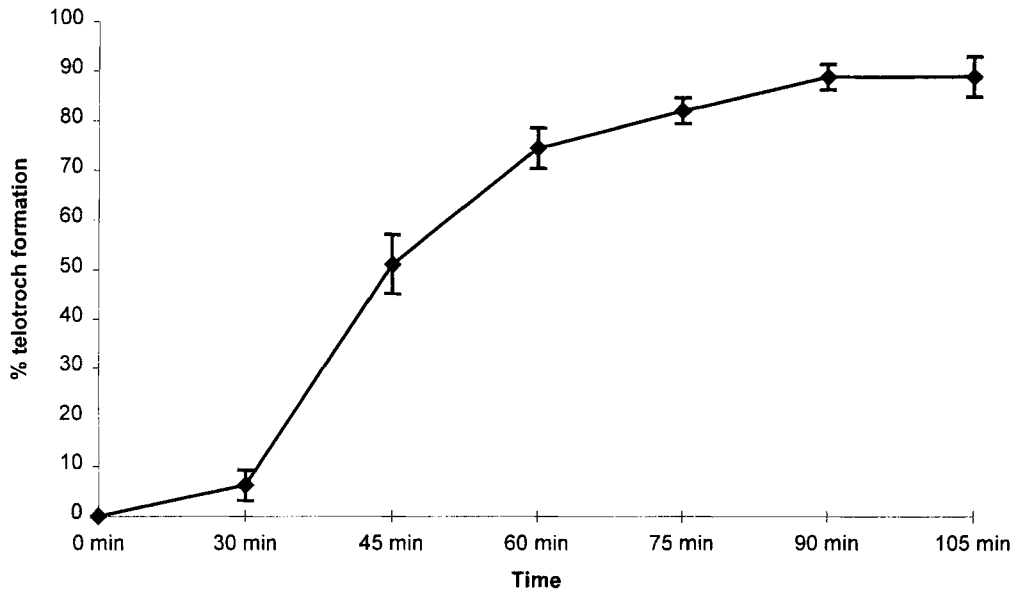

Fig. 1. Time course of telotroch formation in *Vorticella convallaria* induced by the two-step method using monocalcium phosphate (MCP). By 90 min over 90% of the excised cell bodies have transformed into the telotroch form (n = 27). Bars represent 1 standard error.

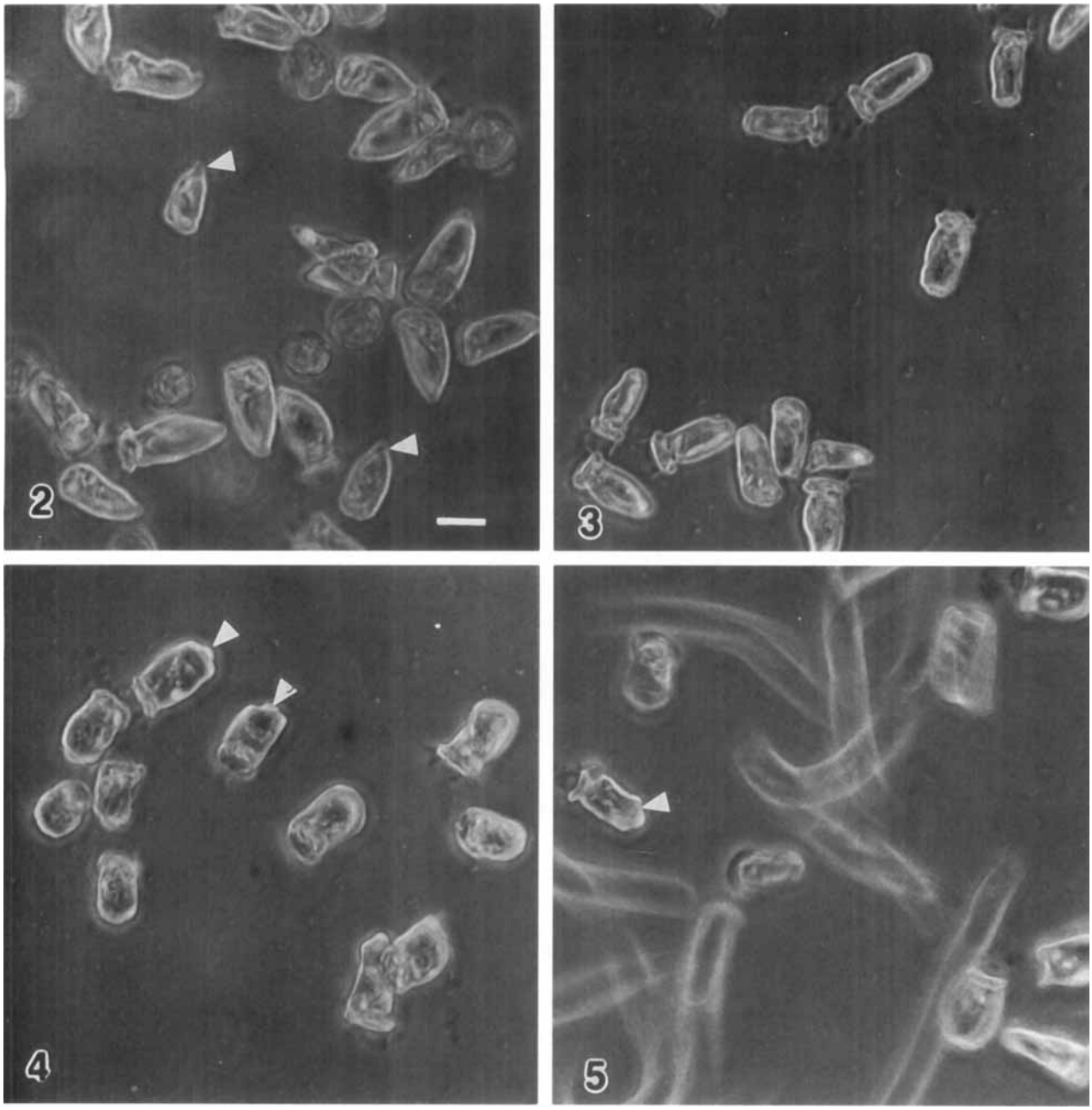

Fig. 2–5. Photomicrographs of populations of isolated cell bodies of *Vorticella convallaria* following stalk excision. Bar = 25 μm. **2**. A population of cells 30 min following stalk excision. Note inverted bell-shape of the cells with the broader portion containing the oral apparatus. The narrow, tapered end (arrowhead) is the scopular region that was attached to the stalk. **3**. A mixed population of cells about 50 min following excision. Note that cells are becoming broader at the aboral region. **4**. Transforming cells about 70 min after excision. The aboral region is characterized by formation of a cupola-like structure (arrowhead) housing the scopular cilia [13]. **5**. Telotrochs approximately 85 min following excision. Many cells are swimming (snake-like images) while a few have yet to complete formation of the ciliary wreath (arrowhead).

mM MCP (pH 3.2) (the pH value remained stable for over 1 mo) for 20 min initiates maximal stalk excision, while lower concentrations or shorter times of exposure significantly reduced levels of excision. Concentrations of MCP higher than 4.8 mM or exposure times longer than 20 min greatly increased cell mortality (Tables 1 and 2, respectively). Therefore, in order to prevent cell death, a second step was developed to rapidly remove the cells from the low pH environment. This two-step method resulted in 88% transformation to the telotroch (Tables 1 and 2). Maximal transformation, as measured by telotroch mobility, occurred between 75 and 90 min after completion of the second step in the procedure (Fig. 1). Following stalk excision, the cell bodies were immobile and retained their inverted bell-shape for about 30 min (Fig. 2). By 55 min some of the cells began to elongate (Fig. 3), and by 70 min, most of the cells possessed the aboral ciliary wreath (Fig. 4). Around 85 min most of the cells were swimming around in the Petri dish (Fig. 5).

Next, we examined the effects of several other factors on stalk excision and telotroch formation using the standard two-

Table 3. The effects of culture age on MCP-H_2O-induced stalk excision and telotroch formation (n = 4).

Age of culture	% stalk excision[a] ± SEM	% telotroch formation[b] ± SEM
CFI/IM (neg. control)[c]	0	NR[d]
1 day	88.33 ± 5.17	94.67 ± 1.45
2 day	72.33 ± 6.7	75 ± 10
3 day	37.67 ± 12	71 ± 6.66
4 day	26.67 ± 11.46	39.33 ± 9.2

[a] Time of exposure is 20 min.
[b] Telotroch formation at 90 min post-treatment.
[c] IM and CFI yield statistically similar results and were used interchangeably as negative control.
[d] No Results: the levels of stalk excision were too low to accurately assess telotroch formation.

step method described above. As the cells age the numbers of MCP-induced stalk excisions and telotroch formations decreased significantly: young cells, 1-d-old following inoculation into the Petri dish, exhibited 88% stalk excision and 95% telotroch transformation, while 4-d-old cells exhibited 27% excision and 39% transformation (Table 3).

Due to the fact that there are several methods for manufacturing MCP [12] we compared the effects of MCP obtained from three vendors in the USA, Fisher Scientific, Pittsburgh, PA; J. T. Baker, Phillipsburg, NJ; and Sigma Chemical Co., St. Louis, MO (Table 4). The MCP obtained from Fisher and Baker was monohydrated, while the Sigma product was anhydrous. Monocalcium phosphate obtained from Fisher and Baker initiated comparatively high levels of stalk excision at pH 3.2 (75% and 63%, respectively), but at its initial pH of 4.5 the Baker product initiated low levels of stalk excision (10%). This may be explained in part by assuming that at pH 4.5 the solubility of the Baker MCP decreases. The Sigma MCP at pH 3.2 initiated significantly lower levels of stalk excision (22%), explained, in part, by solubility problems with this anhydrous product. Levels of transformation to the telotroch form initiated by the Fisher, Baker, and Sigma products were relatively high (81%, 57%, and 95% respectively).

Lastly, we examined the effects of low pH on stalk excision and telotroch formation initiated by the addition of HCl in the presence or absence of calcium (Table 5). For these experiments a pH value of 3.2 was achieved by buffering HCl with either $Ca(OH)_2$ or NaOH. Cells exposed to 24 mM HCl at pH 3.2 showed very low levels of stalk excision (5.5%) when compared to that induced by MCP treatment (85%). Furthermore, these low, HCl-induced stalk excision values were independent of whether or not exogenous calcium was present (5.5% without calcium, 4% with calcium). Because stalk separation was so poor, it was impossible to accurately assess transformation levels initiated by HCl (Table 5).

DISCUSSION

We have devised a two-step method for inducing the formation of large numbers of telotrochs from a population of attached *Vorticella*. We can induce more than 7.0×10^4 cells to transform synchronously into telotrochs within 90 min. The technique is reliable, easy, and apparently does little damage to cells compared to the more invasive techniques of scraping or exposure to air [1, 2, 6, 7, 8].

Injury to the stalk has been postulated to induce telotroch formation [1, 2, 6, 7, 8]. Typically, trophonts with such an injury remain attached during most of the transformation process,

Table 4. Comparison of the effects of MCP from Fisher, Baker, and Sigma on stalk excision and telotroch formation (n = 4).

4.8 mM MCP	% stalk excision[a] ± SEM	% telotroch formation[b] ± SEM
Fisher monohydrate. Lot #795374 pH 3.2	75 ± 4	81 ± 7
Baker monohydrate. Lot #J49711 Buffered to pH 3.2 with 1 drop H_3PO_4/ 500 mL	63 ± 9	57 ± 20
Baker monohydrate Lot #J49711. pH 4.5	10 ± 2.6	NR[c]
Sigma anhydrous Lot #94H0253. pH 3.2	22.5 ± 0.5	95 ± 0.5
IM[d] (negative control)	0	NR[c]

[a] Time of exposure is 20 min.
[b] Telotroch formation at 90 min post-treatment.
[c] No Results: the levels of stalk excision were too low to accurately assess telotroch formation.
[d] IM contains 0.048 mM MCP-H_2O (Fisher).

jettisoning the stalk at the very end of the process. In spite of the fact that stalk severing induced by MCP differs from other induction methods, the sequence of transformation events leading to telotroch formation is similar, whether or not the cell body remains attached to the stalk. These events involve a change in shape from the inverted bell-shape to the elongate, torpedo-shape of the telotroch, partial resorption of the oral cilia, and concomitant with these changes, formation of the aboral ciliary wreath which will power the cell during its motile stage. Investigators using this method of telotroch induction should be able to enhance our knowledge of the molecular, biochemical, and morphological aspects of telotroch formation, particularly, the earliest stages of telotroch development.

Table 5. The effect of HCl pH 3.2 on stalk excision and telotroch formation (n = 4).

Compound	% stalk excision ± SEM	% telotroch formation[a] ± SEM
CFI/IM[b,c]	1.9 ± 0.9	NR[d]
4.8mM MCP-H_2O pH 3.2	85 ± 3	88 ± 3
24mM HCl buffered to pH 3.2 with NaOH 15 min exposure	5.5 ± 5.5	NR[d]
24mM HCl pH 3.2 with $Ca(OH)_2$ 15 min exposure.	4 ± 3.5	NR[d]
24mM HCl buffered to pH 3.2 with $Ca(OH)_2$ 20 min exposure.	0	NR[d]

[a] Telotroch formation at 90 min post-treatment.
[b] IM contains 0.048 mM MCP-H_2O, CFI is calcium free.
[c] IM and CFI yield statistically similar results and were used interchangeably as negative control.
[d] No Results: the levels of stalk excision were too low to accurately assess telotroch formation.

Several factors are important for high telotroch yields induced by MCP. The age of the cells is important both for stalk excision and transformation. A greater percentage of the younger, stalked trophonts can be induced to jettison their stalks than can older, attached cells. While other explanations are conceivable, it is possible that as cells age, there are changes in the structural integrity of the stalk/scopular junction. One possible candidate is a group of small fibrils that connect the stalk (sheath) to the scopular lip and to the scopular cilia: Wibel et al. [11] have correlated stalk severing with the loss of these fibrils. Thus, it is possible that over time more and more fibrils are added to anchor the stalk to the cell body, making the semipermanent junction progressively more refractory to the MCP treatment.

The specific events that initiate MCP-induced stalk excision remain unclear at this time. Our results indicate that low pH in the presence of calcium is in and of itself insufficient to initiate stalk excision. Evidence to support this conclusion comes from the observation that HCl at pH 3.2, both with and without added calcium, failed to excise significant numbers of stalks while MCP at pH 3.2 initiated significant excision. So one conclusion is that initiation of stalk excision is specifically dependent upon MCP or its hydrolysis products. In addition to ionic calcium, these products include dicalcium phosphate (insoluble), ionized forms of phosphoric acid, and the fully protonated form of phosphoric acid [9].

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LITERATURE CITED

1. Nakijima, Y., Hashimoto, K. & Asai, H. 1986. Behavior and function of scopular organelles during differentiation of a sessile *Carchesium polypinum* into a free swimming telotroch. *Cytobios.*, **47**:179–185.

2. Rahat, M., Friedlander, H. & Pimstein, R. 1975. Colchicine inhibition of stalk elongation in *Carchesium* sp.: effect of Ca^{2+} and Mg^{2+}. *J. Cell Sci.*, **19**:183–193.

3. Rose, C. R. & Finley, H. E. 1976. A method for mass cultivation of sessile peritrich protozoa. *Trans. Am. Microsc. Soc.*, **95**:541–544.

4. Sanders, M. A. & Salisbury, J. L. 1989. Centrin-mediated microtubule severing during flagellar excision in *Chlamydomonas reinhardtii*. *J. Cell Biol.*, **108**:1751–1760.

5. Sanders, M. A. & Salisbury, J. L. 1994. Centrin plays an essential role in microtubule severing during flagellar excision in *Chlamydomonas reinhardtii*. *J. Cell Biol.*, **124**:795–805.

6. Succhard, S. J. & Goode, D. 1982. Microtubule-dependent transport of secretary granules during stalk secretion in a peritrich ciliate. *Cell Motility*, **2**:47–71.

7. Vacchiano, E. J., Kut, J., Wyatt, M. L. & Buhse Jr., H. E. 1991. A novel method for mass culturing *Vorticella*. *J. Protozool.*, **38**:608–613.

8. Vacchiano, E. J., Dreisbach, A., Locascio, D., Castaneda, L., Vivian, T. & Buhse Jr., H. E. 1992. Morphogenic transitions and cytoskeletal elements of the stalked zooid and telotroch stages in the peritrich ciliate *Vorticella convallaria*. *J. Protozool.*, **39**:101–106.

9. Varma, S., Misra, N. P. & Singh, J. P. 1979. Hydrolysis of monocalcium phosphate monohydrate in water. *Fertiliser News*, **24**:12–16.

10. Wibel, R., Vacchiano, E. J. & Buhse Jr., H. E. 1993. Ultrastructural study of the cortex and membrane skeleton of *Vorticella convallaria* (Ciliophora: Peritricha). *Trans. Am. Microsc. Soc.*, **112**:107–120.

11. Wibel, R., Vacchiano, E. J., Maciejewski, J. J., Buhse Jr., H. E. & Clamp, J. 1997. The fine structure of the stalk-scopula region of *Vorticella convallaria*. *J. Euk. Microbiol.*, **44**:457–466.

12. Windholz, M., Budavari, S., Blumetti, R. F. & Otterbein, E. S. 1983. The Merck Index, an Encyclopedia of Chemicals, Drugs and Biologicals, 10th ed., Merck & Co., Rahway, New Jersey. Pg. 233.

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