those seen on membranes of freeze-etched species of known mycoplasmas (*8*).

We do not yet know how widespread helical filaments are among organisms associated with other yellows diseases. Careful examination of published work, however, reveals a possible hint of helical filaments in thin-section electron micrographs of citrus with stubborn disease (*9*).

The findings so far obtained indicate that the helical filaments described here are associated with CS disease development in plants in a manner highly consistent with expectations for the CS agent itself. Preliminary data also suggest that tetracycline antibiotics interfere with development of CS symptoms and with formation of the helical filaments in inoculated plants. Moreover, the helical filaments coincide in internal ultrastructural detail and in distribution in CS-infected tissues with the mycoplasma-like bodies previously described by others (*10*) as the probable CS agent. We recognize that our findings are indirect and that alternative explanations for our data cannot be discarded, but the agreement of results from the several lines of investigation lead us to suggest that the helical filaments described here may derive from the CS agent itself.

ROBERT E. DAVIS
JOSEPH F. WORLEY
ROBERT F. WHITCOMB
TAKASHI ISHIJIMA
RUSSELL L. STEERE

Plant Science Research Division, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705

References and Notes

1. Y. Doi, M. Teranaka, K. Yora, H. Asuyama, *Ann. Phytopathol. Soc. Japan* **33**, 259 (1967).
2. T. Ishiie, Y. Doi, K. Yora, H. Asuyama, *ibid.*, p. 267.
3. R. F. Whitcomb and R. E. Davis, *Annu. Rev. Entomol.* **15**, 405 (1970).
4. R. E. Davis and R. F. Whitcomb, *Annu. Rev. Phytopathol.* **9**, 119 (1971).
5. R. E. Davis, J. F. Worley, R. F. Whitcomb, T. Ishijima, R. L. Steere, *Phytopathology*, in press.
6. H. P. Chu and R. W. Horne, *Ann. N.Y. Acad. Sci.* **143**, 190 (1967); D. R. Anderson and M. F. Barile, *J. Bacteriol.* **90**, 180 (1965).
7. R. L. Steere, *Cryobiology* **6**, 137 (1969).
8. T. W. Tillack, R. Carter, S. Razin, *Biochim. Biophys. Acta* **219**, 123 (1970).
9. D. LaFleche and J. M. Bové, *Fruits* **25**, 455 (1970).
10. T. Chen and R. R. Granados, *Science* **167**, 1633 (1970); R. R. Granados, *Contrib. Boyce Thompson Inst.* **24**, 173 (1969); K. Maramorosch, E. Shikata, R. R. Granados, *Trans. N.Y. Acad. Sci.* **30**, 841 (1968).
11. We thank Lynn Loetterle, Joel Rosen, and Michael Coan for their technical assistance, and Dr. Anthony F. Demsey for preparation of freeze-etched specimens.

7 February 1972

5 MAY 1972

Reactivated Triton-Extracted Models of Paramecium: Modification of Ciliary Movement by Calcium Ions

Abstract. *Triton-extracted models of* Paramecium *were reactivated to swim in solutions of adenosine triphosphate and magnesium ions. The cilia beat in the normal direction (toward the rear) when the calcium ion concentration was less than 10^{-6}M, and they beat in the "reversed" direction (toward the front) when calcium ion concentration was raised above 10^{-6}M. These results support the proposal that ciliary reversal, hence backward swimming, of live paramecia is mediated by an increased cytoplasmic calcium concentration around the ciliary system by calcium-dependent membrane responses to external stimuli.*

The locomotor behavior of *Paramecium* is primarily dependent on ciliary "reversal"—the change in direction of the ciliary power stroke—which causes a reversal of locomotion in response to external stimuli (*1*). *Paramecium* produces a graded, regenerative depolarization in response to an applied outward current or a mechanical or chemical stimulation of the anterior membrane. This is always followed closely in time by ciliary reversal (*2*). The regenerative response is mediated by an influx of Ca^{2+} caused by a temporary increase in the membrane conductance to Ca^{2+} (*3*). Ciliary reversal is also closely associated with the liberation of Ca^{2+} bound to the cell membrane (*4*). Therefore, it has been proposed that an increase of cytoplasmic concentration of Ca^{2+} around the cilia might be responsible for ciliary reversal (*4*, *5*).

To verify the hypothesis, we examined the effects of Ca^{2+}, and of Mg^{2+} and adenosine triphosphate (ATP), on the cilia of *Paramecium* specimens that had been extracted with detergent; in these models, the ciliary apparatus is expected to remain functional but the cell membrane is disrupted. When the model was in a mixture of ATP and Mg^{2+}, ciliary beating was in the normal direction (toward the rear), which caused the model to swim forward. When Ca^{2+} was added, the ciliary power stroke was reoriented to the front which caused the model to swim backward.

Specimens of *Paramecium caudatum* (mating type I, syngen 1) reared in hay infusion were washed in a solution of 2 m*M* $CaCl_2$ and 1 m*M* tris(hydroxymethyl)aminomethane (tris) hydrochloride (*p*H 7.2) and were then centrifuged to a loose pellet. The pellet was suspended in extraction medium (0° to 1°C), which contained 0.01 percent (by volume) Triton X-100 (*6*), 20 m*M* KCl, 10 m*M* of the tripotassium salt of ethylenediaminetetraacetic acid (EDTA) (Wako Pure Chemical Co., Tokyo), and 10 m*M* tris-maleate buffer adjusted to *p*H 7.0 with NaOH. After 30 minutes of extraction (*7*, *8*) the specimens were washed to remove Triton and EDTA with a solution (0° to 1°C) of 50 m*M* KCl and 10 m*M* tris-maleate buffer adjusted to *p*H 7.0 with NaOH. Specimens were kept in this solution for at least 30 minutes before being tested.

About 10^{-4} ml of the KCl solution, containing 100 to 500 models, was pipetted into about 1 ml of test solution, which contained test substances and 50 m*M* KCl, and was 19° to 21°C. A few seconds after the models were transferred into medium with ATP and

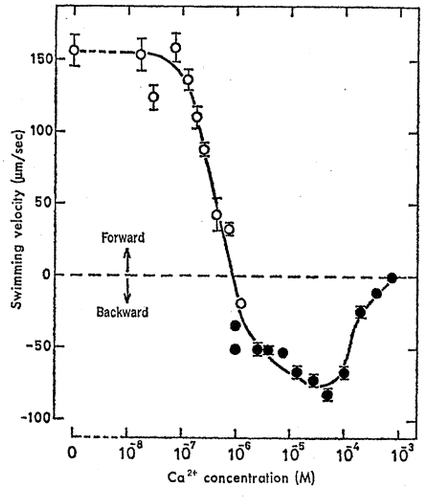

Fig. 1. Effect of Ca^{2+} concentration on both swimming velocity and direction of models of *Paramecium* reactivated by ATP and Mg^{2+}. The graph gives means and standard errors for measurements on 100 to 500 specimens. Concentrations of Ca^{2+} were adjusted by Ca^{2+} buffers in the range between 10^{-8} and 10^{-6}*M* (open circles) and by simple addition of $CaCl_2$ in the range above 10^{-6}*M* (solid circles). Although each Ca^{2+} concentration greater than 10^{-6}*M* is the sum of Ca^{2+} contaminants (*12*) plus Ca^{2+} added to the media, other factors (for instance, formation of complexes between Ca^{2+} and ATP) undoubtedly affect the free Ca^{2+} concentration. Therefore, the concentrations shown are approximate.

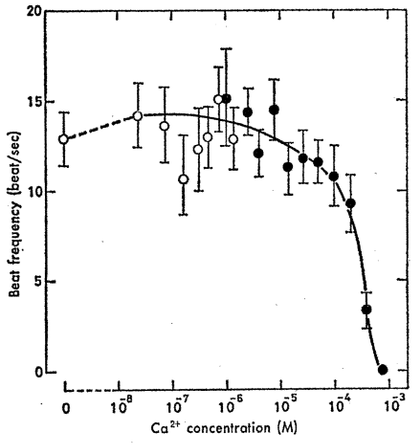

Fig. 2. Effect of Ca^{2+} concentration on beat frequency of cilia in models of *Paramecium* reactivated by ATP and Mg^{2+}. Means and standard errors for measurements on 50 to 100 specimens are given. Open circles and solid circles have same meanings as in Fig. 1.

Mg^{2+}, all the cilia on the models began to beat, first slowly and irregularly, soon quickly and regularly; this caused the models to swim forward.

The beat frequency of the cilia and the swimming velocity of the models were measured in different test solutions. Beat frequencies were determined by photometrically monitoring the frequency of metachronal waves passed at the most anterior part of the model. Swimming velocity was calculated from the distance between photographic images of swimming models, which were taken with five consecutive xenon flashes (1 sec^{-1}) in a 70-μm layer of a test solution. The first image of the sequence was brighter than the others because the first flash was about twice as intense; thus swimming direction could be determined (see cover).

Full reactivation of cilia was obtained in a mixture of 4 m*M* ATP, 4 m*M* $MgCl_2$, 3 m*M* ethyleneglycol-bis(aminoethylether)tetraacetic acid (EGTA), 50 m*M* KCl, and 10 m*M* tris-maleate (adjusted to *p*H 7.0 with NaOH). In this medium, beat frequency was 12.3 $\pm$ 0.6 sec^{-1} (mean and standard error for 50 models). The frequency value approaches that for the unextracted live specimen (10 to 30 sec^{-1}) (*9*). In this solution the swimming velocity was 162 $\pm$ 12 μm/sec (mean and standard error for 240 models); this velocity is less than that of the normal forward-swimming specimen. In general, ciliary beating was toward the rear and caused the model to swim forward (Fig. 1). Data for ciliary reactivation will be given (*8*).

Reactivation of ciliary beating in the presence of EGTA indicates that Ca^{2+} is not essential for ciliary beating in *Paramecium*. That Mg^{2+} and ATP are required for reactivation is consistent with reactivation of ciliary activity in other extracted models (*10*).

The effect of Ca^{2+} concentration on cilia reactivated by ATP and Mg^{2+} was then examined. The Ca^{2+} concentrations between 10^{-8} and $10^{-6}M$ were obtained by changing the ratio of $CaCl_2$ to EGTA (3 m*M*) in the medium containing ATP and Mg^{2+} (*11*), and concentrations greater than $10^{-6}M$ were obtained by addition of an adequate amount of $CaCl_2$ to a solution containing ATP and Mg^{2+} but free of EGTA (*12*).

We observed that, whereas in Ca^{2+} concentrations less than $10^{-7}M$ ciliary beating on the model was toward the rear and swimming motion was forward, the ciliary power stroke gradually shifted toward the front as the Ca^{2+} concentration was increased. This caused decreased forward velocity of swimming at Ca^{2+} concentrations of 10^{-7} to $10^{-6}M$, and finally a reversal of swimming direction occurred at concentrations greater than $10^{-6}M$ (Fig. 1). Maximum velocity of backward swimming was observed in $5 \times 10^{-5}M$ Ca^{2+}. Beat frequency of cilia reactivated by ATP and Mg^{2+} was not much affected by increasing Ca^{2+} concentration to $10^{-4}M$, although some perturbations appear in the relation between frequency and concentration (Fig. 2). Ciliary beating of the model, however, became slower as Ca^{2+} concentration was increased above $10^{-4}M$, and beating stopped in $10^{-3}M$ Ca^{2+} (*13*). This resulted in decreased velocity of backward swimming and finally in its cessation (Fig. 1).

Treatment of the model with a mixture of Ca^{2+} ($5 \times 10^{-5}M$) and ATP (4 m*M*) without Mg^{2+} induced no ciliary beat, but instead produced a reorientation of the nonbeating cilia toward the front. This position of cilia corresponds to backward swimming (*14*).

The fact that cilia of the model beat in the reversed direction when Ca^{2+} concentration is increased gives support to proposals that ciliary reversal, and hence backward swimming in live specimens, is induced by an increase in cytoplasmic Ca^{2+} concentration mediated by Ca^{2+}-dependent membrane phenomena (*4*, *5*). The present results also support the proposal (*14*) that ciliary activity of *Paramecium* is controlled by at least two functionally separable motile systems, one of which produces cyclical bending while the other determines the orientation of the ciliary movement. Both use ATP as energy source, the former requiring Mg^{2+} as cofactor for activation and the latter, Ca^{2+} (*14*, *15*).

Hyperpolarization and depolarization of the surface membrane in live paramecia both result in an increase in ciliary beating frequency (*9*). That Mg^{2+} is required for ciliary beating suggests that movement and distribution of Mg^{2+} that are regulated by the cell membrane may be the basis of bioelectric control of frequency. At present this possibility remains unresolved.

YUTAKA NAITOH*
HIROKI KANEKO†
Zoological Institute, Faculty of Science, University of Tokyo, Tokyo, Japan

References and Notes

1. H. S. Jennings, *Behavior of the Lower Organisms* (Columbia Univ. Press, New York, 1906), pp. 41–109.
2. Y. Naitoh and R. Eckert, *Z. Vergl. Physiol.* **61**, 427 (1968); *Science* **164**, 963 (196[illegible] R. Eckert and Y. Naitoh, *J. Gen. Physiol.* **55**, 467 (1970).
3. Y. Naitoh, R. Eckert, K. Friedman, *J. Exp. Biol.*, in press.
4. Y. Naitoh and I. Yasumasu, *J. Gen. Physiol.* **50**, 1303 (1966); Y. Naitoh, *ibid.* **51**, 85 (1968).
5. R. Eckert, *Science* **176**, 473 (1972).
6. Octylphenoxy polyethoxyethanol was obtained from Wako Pure Chemical Co., Tokyo, Japan.
7. A 30-minute extraction in this medium resulted in a loss of membrane potential and a 100-fold drop in the input resistance. Intracellular adenine nucleotide concentrations, determined enzymatically, were reduced to less than one-tenth those of the unextracted specimen. These lines of evidence indicate that externally applied ions and ATP can pass through the extracted membrane to act directly on the contractile elements of cilia (*8*).
8. Y. Naitoh, in preparation.
9. H. Kinosita, S. Dryl, Y. Naitoh, *J. Fac. Sci. Univ. Tokyo Sect. 4* **10**, 291 (1964).
10. N. J. Arronet, *The Muscle and Cell Contractile (Motile) Models* (Academy of Sciences of U.S.S.R., Leningrad, 1971).
11. H. Portzehl, P. C. Caldwell, J. C. Rüegg, *Biochim. Biophys. Acta* **79**, 581 (1964).
12. The EGTA-free mixture of ATP and Mg^{2+} contains about $10^{-6}M$ Ca^{2+} contaminant from the KCl reagent, so that the models swim backward in the medium without an extra addition of Ca^{2+}.
13. Adenosine triphosphate and Mg^{2+} have little effect on ciliary movement and swimming behavior of live speciments. Increased extracellular Ca^{2+} concentration does not produce ciliary reversal in unextracted specimens, but does influence electrical properties of the membrane (*2*, *4*).
14. Y. Naitoh, *J. Gen. Physiol.* **53**, 517 (1969).
15. L. G. Worley, *J. Cell. Comp. Physiol.* **5**, 53 (1934); Y. Naitoh, *Science* **154**, 660 (1966); L. Kuznicki and E. Mikotajczyk, *Progr. Protozool.* (1969), p. 172.
16. This report is dedicated to H. Kinosita for his 60th birthday. We thank R. Eckert for help in preparing the manuscript.

* Present address: Department of Zoology, University of California, Los Angeles 90024.
† Present address: Department of Physiology, School of Medicine, Kitazato University, Kanagawa, Japan.

6 December 1971; revised 17 February 1972