EXTRACTION MODEL OF THE LONGITUDINAL
FLAGELLUM OF CERATIUM TRIPOS
(DINOFLAGELLIDA): REACTIVATION OF
FLAGELLAR RETRACTION

TADASHI MARUYAMA*
Department of Biology, Tokyo Metropolitan University, Fukasawa, Setagaya-ku,
Tokyo 158, Japan

SUMMARY
The mechanism of retraction of the longitudinal flagellum of Ceratium tripos was studied by
making extracted models of the flagellum. Non-detergent models extracted in low ionic strength
medium containing 1 M-glucose, 10 mM-EDTA, and 50 mM-Tris-HCl buffer (pH 8.0), retracted
when Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, Ba\textsuperscript{2+}, Sr\textsuperscript{2+}, Mn\textsuperscript{2+} or Cd\textsuperscript{2+} was applied locally with a glass capillary. A
demembranated model of the flagellum was made with an extraction medium containing 0.8-1.0 M-
glucose, 20 mM-Tris-acetate (pH 7.8), 2 mM-EGTA, 5-7 mM-MgSO\textsubscript{4}, 0.1 M-potassium glutamate
and 0.1% Triton X-100. The model required a concentration of Mg\textsuperscript{2+} of a few mmol/l for successful
reactivation of both retraction and undulation, and about 0.1 M-potassium glutamate (or sodium
glutamate) for reactivation of undulation. Neither type of motion of the models could be reactivated
above 35 °C. Ca\textsuperscript{2+} induced the retraction at pCa 5.5 or less. In addition to Ca\textsuperscript{2+}, Mn\textsuperscript{2+}, Ba\textsuperscript{2+}, Sr\textsuperscript{2+}
and Cd\textsuperscript{2+} also induced retraction but Mg\textsuperscript{2+}, La\textsuperscript{3+} or Tb\textsuperscript{3+} did not. Although ATP was required for
undulation, it was not required for retraction. Co-incubation with hexokinase to remove
contaminating ATP did not suppress the retraction. The potent ATPase inhibitor, orthovanadate,
inhibited undulation at 10 \mu M but did not inhibit retraction even at 2 mM. SH blockers, N-
ethylmaleimide and dithio-bis-nitrobenzoic acid strongly suppressed undulation but had no effect
on retraction. Calmodulin inhibitors, trifluoperazine and chlorpromazine, also had no effect
on retraction. These data indicate that undulation is generated by a 9+2 microtubular axoneme using
energy released by hydrolysis of ATP and that retraction can be induced by Ca\textsuperscript{2+} without a
requirement for ATP.

INTRODUCTION
Two dissimilar types of motion of the longitudinal flagellum of Ceratium tripos have been shown to be
generated by two different motility mechanisms (Maruyama, 1981, 1982). An axoneme composed of 9+2
microtubules was thought to generate undulatory flagellar motion, whilst the R-fibre was believed to contract to fold the
flagellum.

As is the case for many forms of eucaryotic motility, the retraction appears to be
regulated by Ca\textsuperscript{2+} (Maruyama, 1982). Transmembrane Ca\textsuperscript{2+} influx into the flagellum

* Present address: Toa Nenryo R & D Laboratories, Division of Life Science, Tsuruga-oka, Ohi-
machi, Iruma-gun, Saitama 354, Japan.

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possibly triggers contraction of the R-fibre (Maruyama, 1985). However, it is not clear whether Ca^{2+} interacts directly with the R-fibre or what kind of energy source is used for contraction. In order to answer these questions, we made extracted models of the longitudinal flagellum. The demembranated model was reactivated to undulate in the presence of Mg^{2+} and ATP, while Ca^{2+} induced contraction of the R-fibre in the absence of ATP. The dynein inhibitor, orthovanadate, and SH blockers, N-ethylmaleimide and dithio-bis-nitrobenzoic acid, strongly suppressed undulation but did not affect retraction. The data suggest that contraction of the R-fibre is independent of ATP but is dependent solely on a divalent cation, normally Ca^{2+}.

MATERIALS AND METHODS

Organism

_Ceratium tripos_ was grown as described previously (Maruyama, 1981). Actively swimming vegetative cells were used for experiments after one or more months of incubation.

Reagents

Orthovanadate was purchased from Wako Pure Chemicals Co. (Tokyo). Its final concentration was determined by absorbance at 265 nm using the molar extinction coefficient, ε_{265} = 2925 M^{-1}cm^{-1} (Goodno, 1979). Ethyleneglycol-bis(aminomethyl ether)-N,N'-tetraacetic acid (EGTA) and SH reagents, N-ethylmaleimide (MalNEt) and dithio-bis-nitrobenzoic acid (DTNB), were purchased from Sigma Co. (U.S.A.). Other chemicals were purchased from Wako Pure Chemicals Co. (Tokyo). Potassium glutamate was made by neutralizing glutamic acid to pH 7.5 with KOH.

**Extracted model type 1**

Cells harvested using a hand-operated centrifuge were washed twice with the extraction medium 1, which contained 10 mM-EDTA, 1 M-glucose, 60 mM-Tris (pH 8.0). A 300 μl sample of the cell suspension in extraction medium 1 was placed in a chamber, which was a depression in a glass slide. Test media were applied to the model locally through a glass capillary with a tip of approx. 10 μm, using an inverted microscope with bright contrast phase optics at ×100. The standard test medium contained 1 M-glucose, 60 mM-Tris HCl (pH 8.0) and 1 mM-CaCl₂. In order to test for the effects of the other cations, CaCl₂ was replaced by them. In the case of Mg^{2+}, Mg^{2+} buffer was made up with EGTA as will be described below.

**Extracted model type 2**

Cells harvested using a hand centrifuge were washed with 10 ml of the extraction medium 2, which contained 0.8-1.0 M-glucose, 2 mM-EGTA, 5-7 mM-MgSO₄, 0.1 M-potassium glutamate, 0.1 % (v/v) Triton X-100, 20 mM-Tris and 1 mM-dithiothreitol (DTT). The pH was adjusted to 7.8 with acetic acid. Cells were then washed with a bathing medium of the same composition except that Triton X-100 was omitted. Some flagella were lost during these procedures. The models were placed in the observation chamber. Because the models tended to adhere to the glass, the chamber was treated with approx. 1% egg albumin solution to prevent attachment before the experiment (Gibbons & Gibbons, 1972). The models were then reactivated by applying the reactivation medium through a glass capillary with a tip of approx. 10 μm. Usually 40-100 models were reactivated during 4-20 min after extraction. Reactivity was measured as the percentage of models that were reactivated. The reactivation medium for undulation contained 0.4 mM-Na₂ATP in addition to the components in the bathing medium. The reactivation medium for retraction contained 1-2 mM-calcium acetate, 5 mM-MgSO₄, 0.1 M-potassium glutamate, 0.8-1 M-glucose. Other ions were substituted for Ca^{2+} in some reactivation tests. The effects of the concentration of
Reactivation of flagellar retraction

Each component of the media were studied by changing one of the components in all three (extraction, bathing and reactivation) media.

In order to determine the effect of temperature on the reactivity of the models, they were incubated in the bathing medium at various temperatures from that of ice-water (0°C) to 40°C for 3 min after extraction.

ATP in the supernatant of the bathing solution was determined by using a luciferine–luciferase kit, ATP Bioluminescence CLC (Boehringer-Manheim, West Germany) with an Aminco Chem-Glow photometer.

All the experiments were carried out at room temperature (20–28°C).

 Calcium and magnesium buffers

Ca\(^{2+}\) buffer for the type 1 models (Ca buffer 1) consisted of 10 mM-EDTA or EGTA with various amounts of CaCl\(_2\). Mg\(^{2+}\) buffer consisted of 10 mM-EDTA or EGTA with various amounts of MgCl\(_2\). The apparent stabilization constants of EDTA for Ca\(^{2+}\) and Mg\(^{2+}\) are 8·32 and 6·42, respectively, at pH 8·0, and of EGTA, 8·63 and 2·99 (Amos, Routledge, Weis-Fogh & Yew, 1976). Ca\(^{2+}\) buffer for the type 2 models (Ca buffer 2), which contained 7 mM-Mg\(^{2+}\), consisted of 5 mM-EGTA or EDTA with various amounts of CaCl\(_2\). The apparent stabilization constants of EDTA for Ca\(^{2+}\) and Mg\(^{2+}\) are 8·12 and 6·22, respectively, at pH 7·8, and of EGTA, 8·25 and 2·68 (Amos et al. 1976).

Scanning electron microscopy

For scanning electron microscopy, the models were fixed with a fixative containing 5% glutaraldehyde, 1 M-glucose, 0·1 M-sodium phosphate buffer (pH 7·4) for 30 min. The contracted flagellar models were fixed after washing with the reactivation medium. The fixed samples were dehydrated through a graded series of ethanol, acetone and isoamylacetate. They were then dried in a critical-point drier (Hitachi, HCP-2). After coating with gold using an ion coater (Eiko Engineering Co. Japan), they were observed in a JEOL JSM 25 scanning electron microscope.

RESULTS

Extracted model type 1

The longitudinal flagella that were extracted with medium 1 were relaxed and quiescent, though some continued to undulate for a few minutes. The models retracted when 1 mM-Ca\(^{2+}\) was applied. Calcium ions sometimes stimulated retraction as well as undulation; such models began to undulate and then the stimulated portion started to fold while the distal portion was still undulating. The threshold for retraction determined by using calcium buffer 1 was at a pCa of approximately 5·0. Magnesium ions induced undulation only if applied within 5 or 10 min after extraction but gradually began to induce retraction after 10 min. In order to remove contaminating Ca\(^{2+}\) from the Mg\(^{2+}\) test medium, and to determine the threshold for the retraction, magnesium buffer was used. The threshold was approximately 4·0 in pMg. It is noteworthy that Mg\(^{2+}\) could not induce retraction of the type 1 model flagella when extracted with media containing 2 mM-EDTA instead of 10 mM.

The effects of other cations were studied with test media containing various cations instead of Ca\(^{2+}\) or Mg\(^{2+}\). Divalent cations, Mn\(^{2+}\), Sr\(^{2+}\), Cd\(^{2+}\) and Ba\(^{2+}\) triggered retraction at 0·1 mM, but Na\(^{+}\), K\(^{+}\), Co\(^{2+}\), Ni\(^{2+}\), Zn\(^{2+}\), La\(^{3+}\) or Tb\(^{3+}\) did not at 1 or 10 mM (Table 1). Because no chelator that has a higher stabilization constant to
Ca$^{2+}$ than to other ions was used, the possibility of Ca$^{2+}$ contamination remained. However, those cations that triggered retraction did so at 0.1 mM in the presence of 10 mM-EDTA in the surrounding medium; under these conditions contamination by Ca$^{2+}$ is not likely to be present at a concentration high enough to trigger retraction. Longitudinal flagella immersed in extraction medium 1 were not stable and gradually disintegrated into thin threads, which could no longer be reactivated to undulate or retract. Because model 1 was not extracted with detergent, the flagellar membrane may have remained intact.

**Extracted model type 2**

Fig. 1 shows the effect of potassium glutamate concentration in the extraction, bathing and reactivation media on the undulation of the model. The undulation in K$^+$-free medium was weak and the percentage of reactivated models was less than 50%. The undulation became stronger and the percentage of reactivation increased at higher concentrations of potassium glutamate, but the percentage decreased again at over 0.2 M.

On the other hand, retraction of the models could be reactivated in the absence of K$^+$ (Fig. 1). The percentage reactivation decreased in reactivation media containing 0.2 M or more of potassium glutamate and lost contractility at 0.25 M-potassium glutamate. The model lost the ability to retract in the presence of 0.1 M-NaCl, KCl, KNO$_3$, KI, KSCN, NH$_4$Cl, 0.05 M-K$_2$SO$_4$ or 0.1 M-sodium isothionate (sodium hydroxyethanesulphonate) but the retractability was well preserved in 0.1 M-

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**Fig. 1.** Effect of K-glutamate concentration in the extraction, bathing and reactivation media on the reactivity of the type 2 extraction model. Ordinate, percentage of models that were reactivated; abscissa, concentration of potassium glutamate (M). (○) Undulation reactivated by reactivation medium containing 0.4 mM-ATP; (●) retraction reactivated by reactivation medium containing 1 mM-Ca$^{2+}$.
potassium or sodium acetate, potassium or sodium glutamate. All anions tested, except acetate and glutamate, inhibited retraction of these models, but K⁺ can be replaced with Na⁺ without affecting the reactivity. However, glutamate was better than acetate because the model lost retractability in 0·15 M-potassium acetate but retained it in 0·15 M-potassium glutamate (Fig. 1). Therefore, 0·1 M-potassium glutamate was used in the extraction, bathing and reactivation media in the following experiments.

Fig. 2 shows the effect of MgSO₄ concentration in the extraction, bathing and reactivation media on the undulation and retraction of the model. The models could not be reactivated to undulate in the absence of Mg²⁺ but were reactivated well between 1 and 30 mM; the percentage reactivation decreased at concentrations over 30 mM. Models extracted and bathed in Mg²⁺-free medium (in the presence of EDTA) lost some of their retractability. Higher concentrations than 10 mM-MgSO₄ had a deleterious effect on retractability and the model could not be reactivated at 20 mM or more. In the following experiments, the extraction medium contained 5 or 7 mM-MgSO₄.

The reactivation of undulation of the models was not affected from 0 to 30 °C, but was severely suppressed at over 35 °C (Fig. 3). The model retained the ability to retract from 0 to 30 °C but lost it above 35 °C (Fig. 3). This indicates that both the contractile mechanism and the undulatory mechanism are heat-labile.

Almost 100% of the models were reactivated to undulate at pH between 7·0 and 8·5 (Fig. 4). The percentage of reactivation, however, decreased at lower pH values. The model could not be reactivated to undulate at pH 6·0. On the other hand, retraction
was reactivated well at lower pH values, between 6.0 and 8.0, but models lost retractability at pH values above 8.0. Therefore, the undulatory mechanism is inactive at pH values lower than 7.0 while the retraction mechanism is susceptible to pH values higher than 8.0. The model was not relaxed completely at pH 6.0 or below; under these conditions the flagellum remained half-retracted. It is not clear whether

Fig. 3. Effect of temperature on the reactivity of type 2 models. Duration of treatment at all temperatures was 3 min. Ordinate, % reactivation; abscissa, temperature. (○) Undulation; (●) retraction.

Fig. 4. Effect of pH on the reactivity of type 2 models. Ordinate, % reactivation; abscissa, pH of the extraction, bathing and reactivation media. (○, △) Undulation; (●, ▲) retraction; (○, ●) the buffer was 20 mM-Tris-acetate; (△, ▲) 20 mM-Tris and 10 mM-2-(N-morpholino)-ethanesulphonic acid (MES), the pH was adjusted with acetate.
H+ induces R-fibre contraction or not. Because the apparent Ca²⁺ binding constant of EGTA is smaller at lower pH, the free Ca²⁺ concentration could be higher.

**Energy sources for undulation and retraction**

Because the undulation of the longitudinal flagellum is probably generated by the 9+2 axoneme (Maruyama, 1982), undulation is likely to depend on ATP as an energy source. However, the energy source for retraction is less obvious. Fig. 5 shows the relation between ATP concentration in the reactivation medium and the percentage of models showing undulation. Undulation could not be reactivated by reactivation media containing less than 20 µM-ATP, though 20 µM-ATP relaxed curved flagella which were probably in rigor (Gibbons & Gibbons, 1974). Undulation could be reactivated by reactivation media containing more than 50 µM-ATP. The model undulated for a few minutes and then ceased undulation. Once a model had ceased to undulate it could not be reactivated. Application of concentrations of ATP of over 1 mM seems to suppress undulation. Although a high concentration of ATP may have some inhibitory effects on undulation, it is evident that ATP is necessary for the undulation of the longitudinal flagellum.

On the other hand, models could be caused to retract with Ca²⁺ without adding ATP. In order to remove possible contamination by ATP released from the cell body, hexokinase was added to the bathing solution. This enzyme hydrolyses ATP in the presence of glucose as a substrate. Retraction was unaffected when models were incubated with up to 0.4 IU/ml hexokinase for periods of 4–30 min, indicating that the retraction occurred without ATP. Moreover, the concentration of ATP in the bathing solution was determined by the luciferase–luciferin method after removal of

![Fig. 5. Effect of ATP concentration in the reactivation medium on the undulation of type 2 models. Ordinate, % reactivation; abscissa, Na₂ATP concentration (µM) in the reactivation medium. The reactivation medium contained 60 mM-Tris-acetate (pH 7.8), for triangles, and 20 mM, for circles.](image-url)
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the cells. The medium contained only trace amounts (less than 1 nM) of ATP. Therefore, retraction seemed to occur in the absence of ATP, though there remained the possibility that sufficient ATP to provide energy for several contractions might bind firmly to the R-fibre.

Threshold of calcium ion concentration for retraction

In order to determine the threshold of Ca\(^{2+}\) for reactivation of retraction, calcium buffer 2 was applied to the model through a glass capillary. Fig. 6 shows the relationship between free calcium concentration (pCa) and the retraction of the model. The R-fibre did not contract at pCa values greater than 5.5 but contracted at pCa 5.5 or below. Because the surrounding medium contained 2 mM-EGTA, the actual free Ca\(^{2+}\) concentration in the model might be less than that in the application medium and because the axoneme may be in rigor in the absence of ATP, the real threshold may be a larger pCa value. However, the threshold was roughly the same in the presence of 200 \(\mu\)M-ATP in the application medium.

Effect of other cations

The extracted type 2 model was insensitive to Mg\(^{2+}\), though it induced the retraction of the type 1 model. Table 1 shows the effects of various cations on the retraction of these models. Among those tested, some divalent cations (Ca\(^{2+}\), Ba\(^{2+}\), Sr\(^{2+}\), Cd\(^{2+}\), Mn\(^{2+}\)) triggered retraction, while monovalent cations (K\(^{+}\) or Na\(^{+}\)), some divalent (Ni\(^{2+}\), Co\(^{2+}\), Fe\(^{2+}\), Cu\(^{2+}\), Zn\(^{2+}\)) or some trivalent (La\(^{3+}\) or Tb\(^{3+}\)) did not. While Ca\(^{2+}\) triggered retraction in 95% \((n = 96)\) of the models even at 100 \(\mu\)M without

![Fig. 6. Threshold of Ca\(^{2+}\) concentration for retraction of type 2 models. Ordinate, % reactivation; abscissa, free Ca\(^{2+}\) concentration expressed as pCa. (●) In the absence of ATP; (○) in the presence of 200 \(\mu\)M-ATP in the reactivation medium.](image)
Reactivation of flagellar retraction

using \( \text{Ca}^{2+} \) buffer, \( \text{Ba}^{2+}, \text{Sr}^{2+}, \text{Mn}^{2+} \) and \( \text{Cd}^{2+} \) induce it in 18% (\( n = 72 \)), 49% (\( n = 43 \)), 80% (\( n = 45 \)) and 57% (\( n = 53 \)), respectively. The R-fibre probably has less sensitivity for these cations. Other cations did not trigger the retraction even at 1 or 10 mM. No chelating agent, such as EGTA, was used to remove contaminating calcium in the test media of these cations, except \( \text{Mg}^{2+} \). A possibility of calcium contamination in the media, therefore, could not be ruled out.

Effect of ATPase inhibitors on the motility of the type 2 models

Orthovanadate is a potent inhibitor of dynein ATPase (Gibbons & Gibbons, 1978; Gibbons et al. 1978; Kobayashi, Martensen, Nath & Flavin, 1978). Fig. 7 shows the effects of orthovanadate on the undulation and retraction of the model. A low concentration of orthovanadate (\( \mu \text{M} \)) in the bathing and reactivation media inhibited undulation of the models after 10–20 min of incubation. The inhibitory effect of orthovanadate was abolished when it was reduced by 2.5 mM catechol before the experiment (Fig. 7). Reduction of vanadate by catechol was reported to abolish its inhibitory effect on the beating of sperm flagella (Gibbons et al. 1978). On the other hand, the retraction induced by \( \text{Ca}^{2+} \) was not inhibited by orthovanadate even at 1 or 3 mM.

Flagellar undulation and ciliary beating are reported to be suppressed by SH reagents (Blum & Hayes, 1974; Cosson, Tang & Gibbons, 1983). The sulphphydryl reagent MalNEt suppressed undulation of the type 2 model at 10 \( \mu \text{M} \) or more (Fig. 8). Retraction, however, was not affected even at values above 1 mM (Fig. 8). DTNB, which is also a sulphphydryl reagent, inhibited the undulation at 100 \( \mu \text{M} \) but did not affect retraction at 1 mM (data not shown).

### Table 1. Effect of cations on the retraction of the models

<table>
<thead>
<tr>
<th>Cations</th>
<th>Concentration (M)</th>
<th>Retraction of models</th>
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<td>Type 1</td>
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<td>( K^+ )</td>
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<tr>
<td>( Na^+ )</td>
<td>( 1 \times 10^{-1} )</td>
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<td>( Mg^{2+} )</td>
<td>( 5 \times 10^{-2} )</td>
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<tr>
<td>( Ca^{2+} )</td>
<td>( 1 \times 10^{-4} )</td>
<td>+</td>
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<tr>
<td>( Sr^{2+} )</td>
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<tr>
<td>( Ba^{2+} )</td>
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<td>( Cd^{2+} )</td>
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<td>( Tb^{3+} )</td>
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* Type 2 model is not sensitive to \( \text{Mg}^{2+} \) at 10 mM.
Fig. 7. Effect of orthovanadate on the reactivity of type 2 models. Ordinate, % reactivation; abscissa, concentration of orthovanadate in the bathing medium. (○) Undulation between 10 and 20 min; (△) undulation in the medium containing orthovanadate that had been reduced with 2.5 mM-catechol before the experiment. (●) Retraction between 10 and 20 min. Note that the retraction was not affected at all by orthovanadate.

Fig. 8. Effect of MalNEt on the reactivity of type 2 models. Ordinate, % reactivation; abscissa, MalNEt concentration in the bathing medium. (○, △) Undulation after 10–20 min incubation; (●) retraction after 10–20 min incubation; (○, ●) no DTT in the media; (▲) 1 mM-DTT.
Effect of calmodulin inhibitors on retraction

Since calcium-induced contraction of the R-fibre, it was interesting to know whether the Ca\(^{2+}\)-dependent regulator protein, calmodulin, is involved in the process of contraction. Trifluoperazine and chlorpromazine are known to inhibit calmodulin in the presence of Ca\(^{2+}\) (Levin & Weiss, 1978). A concentration of 50 \(\mu\text{M}\)-trifluoperazine or 200 \(\mu\text{M}\)-chlorpromazine in the bathing medium did not affect retraction within 25 min of incubation, suggesting that calmodulin plays no part in the mechanism of contraction.

The shape of relaxed and contracted type 2 models

Fig. 9A shows a scanning electron micrograph of a relaxed model 2 longitudinal flagellum. The flagellar membrane is evidently lost. The R-fibre, which was reported to be connected with the packing material at regular intervals (Maruyama, 1982), runs...
along the axonemal complex. The contracted R-fibre seems to be a little thicker than the relaxed one (Fig. 9B), and the fully contracted R-fibre seems to take a helical course as described previously (Maruyama, 1982). Fig. 9 also shows that the axonemal complex consists of two fibres connected together side-by-side. One is presumably the microtubular axoneme and the other is probably the packing material that is connected to the R-fibre. Although the tip of the R-fibre has not been seen for certain, it seems to terminate at the connection with the axonemal complex (Fig. 9B).

The process of retraction of the model

Fig. 10 depicts a schematic representation of the reactivation of undulation and retraction of the model of the longitudinal flagellum. In the presence of Mg$^{2+}$ and ATP, but in the absence of Ca$^{2+}$, the model undulates (Fig. 10E), while in the presence of Ca$^{2+}$ the model contracts. When the model was preserved well and the connection between the axonemal complex and the R-fibre was firm enough, the axonemal complex was folded to a configuration similar to that of the retracted living flagellum (Fig. 10B). After removal of Ca$^{2+}$, the model relaxed but the folding of the axonemal complex did not fully relax; it took a slightly zigzag form (Fig. 10B). The contracting R-fibre sometimes detached from the axonemal complex during retraction (Fig. 10C). After sequestration of Ca$^{2+}$ the model could not resume its original straight configuration but took the shape of a relaxed bow; namely, a slightly curved axonemal complex and a straight R-fibre. The contracting R-fibre sometimes peeled

![Diagram](image-url)
off from the axonemal complex; when this occurred the detached free end of the contracting R-fibre rotated counterclockwise, and the R-fibre became thicker as the contraction proceeded. When Ca²⁺ was sequestered by the surrounding EGTA, the contracted R-fibre relaxed. It elongated and became thin and flexible (Fig. 10D). These observations indicate that the R-fibre actively generates tension when it contracts.

**DISCUSSION**

The present data on the demembranated model of the longitudinal flagellum show differences between the mechanisms for undulation and retraction; (1) retractability was lost in the presence of 20 mM-MgSO₄ but the reactivation of undulation was not affected; (b) concentrations above 0.2 M-potassium glutamate inhibited retractile motility but not undulation; (3) undulation was not reactivated at pH values below 7.0, while retraction was reactivated even at pH 6.0; (4) ATP was needed for undulation but not for retraction; (5) sulphhydryl reagents, MalNEt and DTNB strongly inhibited undulation but not retraction; (6) orthovanadate severely suppressed undulation but not retraction; (7) Ca²⁺ induced contraction of the R-fibre. This evidence, together with the previous observations that the longitudinal flagellum contains a 9+2 microtubular axoneme and that the arrangement of protofilaments of the R-fibre changes during contraction (Maruyama, 1982), is consistent with our previous view that undulation is generated by the 9+2 microtubular axoneme and the R-fibre contracts to fold the flagellum (Maruyama, 1981, 1982).

It is now widely accepted that calcium ions play a regulatory role in various motility mechanisms (Ebashi, 1980). The contraction of the R-fibre has been shown also to be regulated by Ca²⁺ at pCa 5.5 or below (Fig. 6). However, both type 1 and type 2 models were reactivated to retract by several alkaline earth ions, Sr²⁺ and Ba²⁺, and by some other divalent cations, Cd²⁺ and Mn²⁺ (Table 1). Although these are competent to induce contraction, Ca²⁺ is likely to function in vivo because it has the strongest activity and because sea water contains 10 mM-Ca²⁺ but only low concentrations of the other ions.

It has been reported that Ca²⁺ functions to stop flagellar or ciliary motion in various organisms (Tsuchiya, 1976). However, it is not clear what kind of effect it has on the axoneme of the longitudinal flagellum in *Ceratium*. It possibly stops undulation, otherwise the two motions would antagonize each other during contraction.

Magnesium ions induced retraction of the type 1 models but not of the type 2 models. At present this cannot be explained, but the evidence that it took about 10 min for type 1 models to acquire Mg²⁺ sensitivity in medium 1 and that models did not gain Mg²⁺ sensitivity when they were extracted in 2 mM-EDTA suggests the possibility that Mg²⁺ bind firmly to the R-fibre in situ. Once bound Mg²⁺ is removed, the R-fibre would become sensitive to Mg²⁺ for contraction but would also become labile. The R-fibre would be stabilized somehow in the flagellar matrix of the type 1
model, whose flagellar membrane remains more or less intact. However, type 2 models could not be reactivated to contract even when the Mg$^{2+}$ is chelated by EDTA. This Mg$^{2+}$-induced contraction could be explained in another way by the presence of a Ca$^{2+}$ reservoir in the flagellum, though no membranous structure similar to the sarcoplasmic reticulum in skeletal muscles (Ebashi & Endo, 1968), or the tubules in the spasmoneme of Vorticellidae (Amos, 1972), has been found in the longitudinal flagellum (Maruyama, 1982). If this is true, Ca$^{2+}$ would have to be released somehow from the reservoir.

Calcium ions induced retraction in a medium that contained only trace amounts of ATP with 1 mM-orthovanadate, or mM amounts of sulphydryl reagents such as MalNEt and DTNB. Weis-Fogh & Amos (1972) showed that energy for contraction of the spasmoneme in Vorticella could be supplied by Ca$^{2+}$-binding to the spasmoneme. Energy for contraction of the R-fibre may also be provided by Ca$^{2+}$-binding to the R-fibre. These data also indicate that sulphhydryl groups do not play a role in contraction of the R-fibre.

The calcium-dependent regulatory protein calmodulin has been found in Tetrahymena cilia (Ohnishi, Suzuki & Watanabe, 1982) and Chlamydomonas flagella (Gitelman & Witman, 1980). Although Ca$^{2+}$ plays a key role in triggering or generating retraction of the longitudinal flagellum, calmodulin does not seem to play a role in retraction, because trifluoperazine or chlorpromazine did not affect retraction.

The R-fibre resembles the spasmoneme of the contractile stalk in Vorticellids because: (1) neither structure requires ATP for contraction; (2) SH reagents do not affect the contractility of either structure; (3) alkaline earth ions Ca$^{2+}$, Ba$^{2+}$ or Sr$^{2+}$ induce contraction but Mg$^{2+}$ does not (Hawkes & Rahat, 1976; Hoffmann-Berling, 1958). There are some differences: (1) La$^{3+}$ and Tb$^{3+}$ induce contraction of the spasmoneme (Routledge & Amos, 1977) but not of the R-fibre; (2) reorganization of the protofilaments in the R-fibre changes parallel arrays, with striations, in the relaxed state to a rather random swirling arrangement around the longitudinal axis, without striations, in the contracted state (Maruyama, 1982). Although reorganization of the protofilaments was reported in the myoneme of Stentor (Huang & Mazia, 1975), the striation pattern is unique to the R-fibre. The contracted spasmoneme was reported to be isotropic but became birefringent when relaxed (Weis-Fogh & Amos, 1972). However, no fine-structural change was reported in the filaments of the spasmoneme (Routledge & Amos, 1977). It is reported that the spasmoneme contains calcium-binding proteins called spasmin (Amos, Routledge & Yew, 1975; Amos et al. 1976) or spastin (Yamada & Asai, 1982). It would be interesting to know whether a similar protein occurs in the R-fibre.

The R-fibre twists during contraction (Fig. 9). The retracted flagellum is twisted into a right-handed helix (Maruyama, 1982), but the R-fibre pursues a left-handed helix. The protofilaments of the contracted R-fibre seemed to swirl around its longitudinal axis. Therefore, the parallel arrays of protofilaments in the relaxed R-fibre are possibly reorganized to take a swirling arrangement during the contraction to generate a force not only to fold the axoneme but also to twist it into a right-handed helix.
Further investigation of the reorganization of the protofilaments of the R-fibre during contraction will be necessary to understand the mechanism underlying the contraction.

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