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CONTROL OF CILIARY ACTIVITIES BY ADENOSINETRIPHOSPHATE AND DIVALENT CATIONS IN TRITON-EXTRACTED MODELS OF *PARAMECIUM CAUDATUM*

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INTRODUCTION

The locomotor behaviour of *Paramecium* and many other protozoans depends on the movements performed by cilia distributed over the cell surface (Jennings, 1906). Recent evidence indicates that the movements of the cilia are regulated by electric events in the surface membrane. A depolarization of the membrane, which occurs in response to an injection of outward current (Naitoh, 1958; Naitoh & Eckert, 1968; Eckert & Naitoh, 1970; Machemer & Eckert, 1973), or to mechanical stimulation of the front end of the specimen (Naitoh & Eckert, 1969*a*, *b*) or spontaneously (Kinosita, 1954; Naitoh, 1966), is always followed by a transient reversal in the direction of the effective power stroke of cilia (i.e. ciliary reversal). Reversal of the ciliary beat causes the animal to swim backward. On the other hand, a hyperpolarization induced by an injection of inward current (Naitoh, 1958; Naitoh & Eckert, 1968) or by mechanical stimulation of the rear end of the animal (Naitoh & Eckert, 1969*a*, *b*; 1973) is followed by an inhibition of ciliary reversal and an increase in the beat frequency of the cilia in the normal direction. This causes the animal to swim forward with increased velocity.

Depolarization of the surface membrane by injected current or mechanical stimulation of the cell anterior induces a voltage-dependent increase in calcium conductance, which permits Ca^{2+} to flow inward down its electrochemical gradient. This results in a regenerative depolarizing action potential (Naitoh, Eckert & Friedman, 1972; Eckert, Naitoh & Friedman, 1972).

Ciliary reversal also occurs when the external cationic conditions are changed so as to liberate bound calcium from the membrane (Jahn, 1962; Naitoh, 1968). The liberated calcium is believed to activate a reversal mechanism in the cilia (Naitoh, 1969).

In order to understand how modifications of ciliary movement are coupled to electric and/or ion exchange events of the surface membrane, it is important to know the effects on the ciliary apparatus of the ions involved in the membrane events. We therefore examined the effects of various cations on the ATP-reactivated cilia of detergent-extracted models of *Paramecium*. In such models the ciliary membrane is functionally

disrupted so that the externally applied cations have direct access to the ciliary apparatus.

Our preliminary report (Naitoh & Kaneko, 1972) showed that external application of calcium ions led to a reversal of the orientation of effective power stroke in ATP-Mg^{2+}-reactivated cilia. Thus, calcium ions cause the models to swim backward as do live specimens upon receiving a depolarizing stimulus.

MATERIALS AND METHODS

Extraction of Paramecia

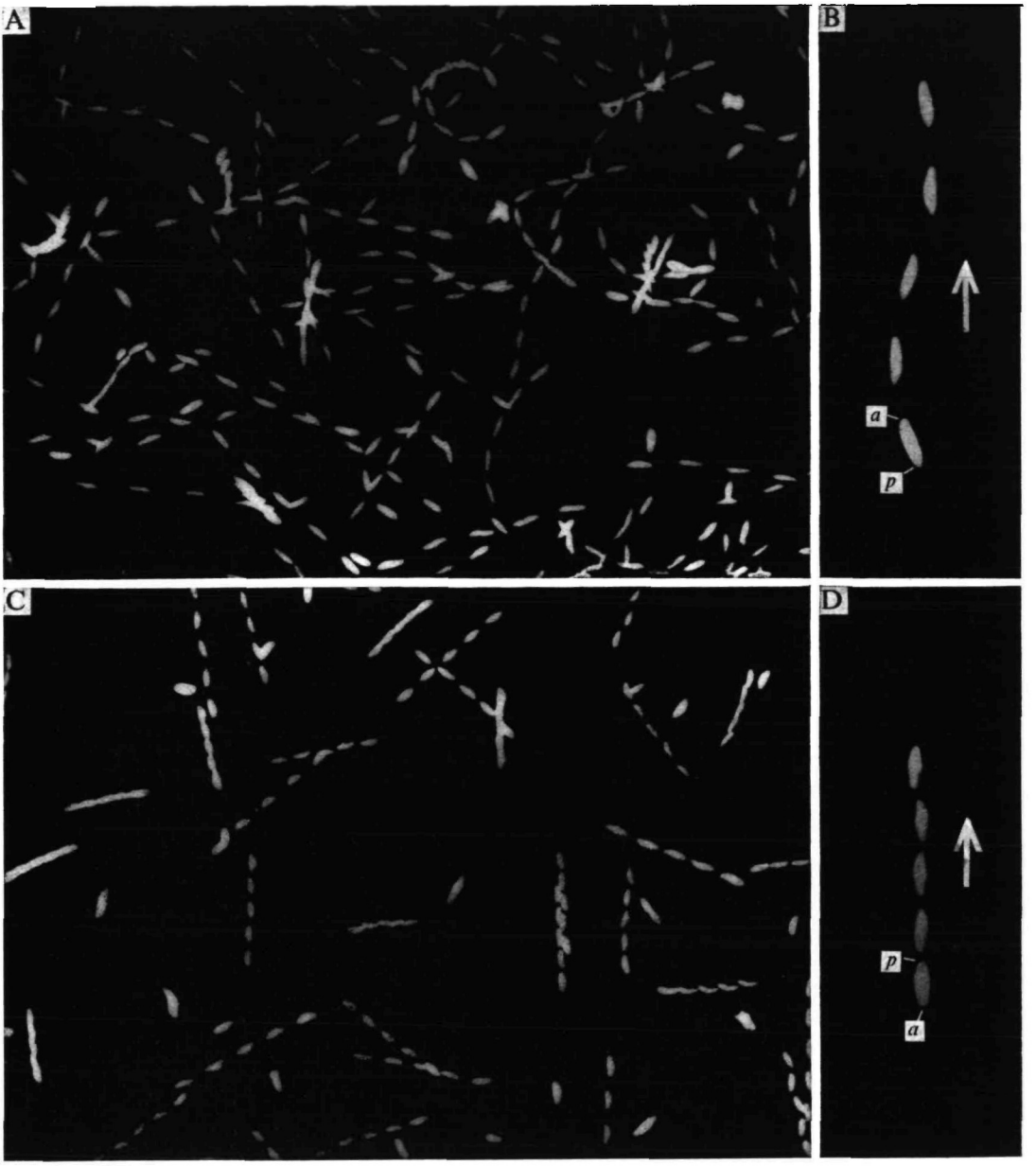
Specimens of *P. caudatum* (mating type I of syngen 1) reared in a hay infusion were washed thoroughly with 2 mM-$CaCl_2$ solution buffered to pH 7·2 by 1 mM Tris-HCl buffer. A concentrated suspension of the specimens was cooled in an ice bath and centrifuged gently to make a loose pellet. The pellet was then resuspended in a cold (0–1 °C) extraction medium which consisted of 0·01% (by volume) octylphenoxy polyethoxyethanol (Triton X-100 obtained from Wako Pure Chemicals Co., Tokyo), 20 mM-KCl, 10 mM ethylen-diaminetetraacetic acid (EDTA: neutralized by KOH) and 10 mM Tris (hydroxymethyl) aminomethane (Tris)-maleate buffer (pH was adjusted to 7·0 with NaOH). The suspension was then stored in an ice bath (0–1 °C) for 35–45 min. The extracted specimens thus obtained were washed gently three times with a cold (0–1 °C) washing medium which consisted of 50 mM-KCl, 2 mM EDTA, and 10 mM tris-maleate buffer (pH 7·0), and kept in this medium for 15 min to remove the Triton. They were then washed again with a cold (0–1 °C), buffered (pH 7·0 by 10 mM Tris-maleate) 50 mM-KCl solution to remove the EDTA. The washed models were equilibrated in the KCl solution for at least 15 min at 0–1 °C before experimentation.

Reactivation of the models

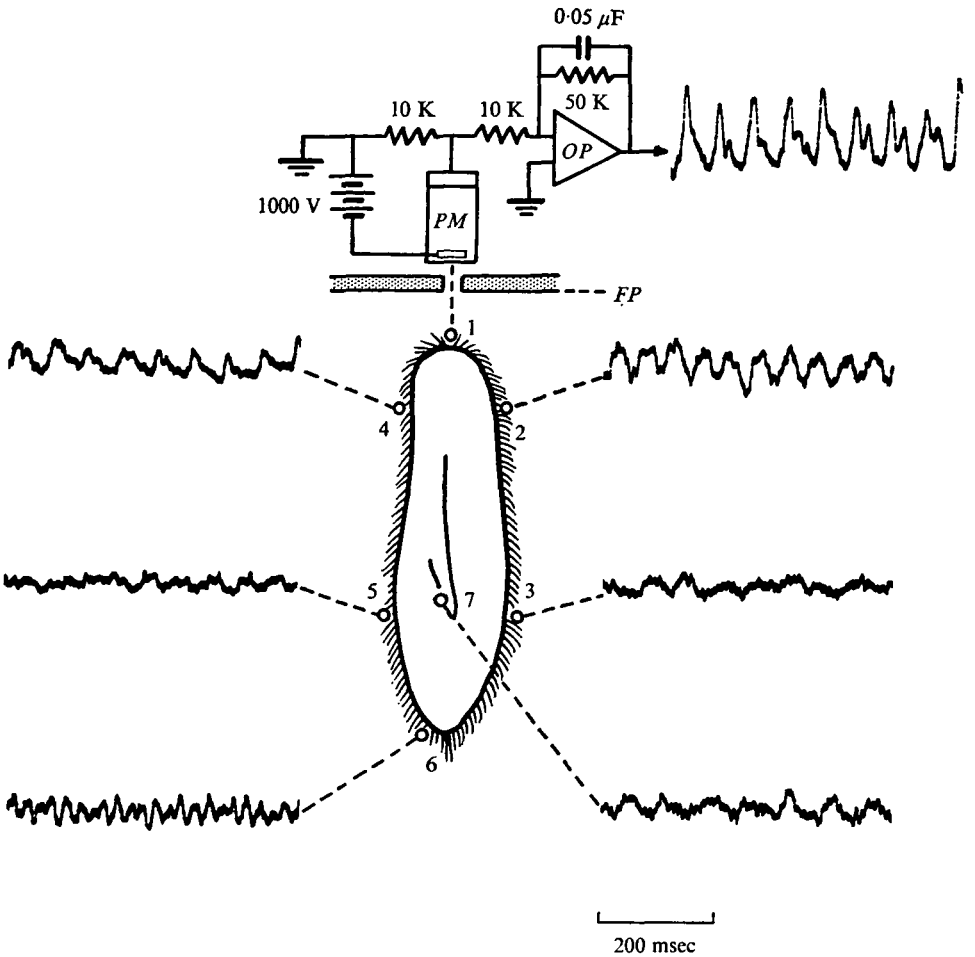
About 500 cell models with a minute amount of KCl solution (about 10^{-4} ml) were pipetted into a large amount of a reactivation medium (about 1 ml) in a depression slide and stirred gently by a blunt glass needle at a room temperature of 19–21 °C. The reactivation media consisted of 50 mM-KCl containing test substances (pH was adjusted to 7·0 by 10 mM Tris-maleate buffer). After an appropriate exposure in the reactivation solution the models were observed and photographed.

Swimming velocity and direction

About 200 reactivated models were introduced into a thin (70 μm) space between a glass slide and a coverslip. This spacing was sufficient for the models to swim freely in parallel with the glass plates but too shallow for locomotion in the vertical direction (median size of the models was 250 μm in length and 50 μm in width). The models were then photographed in this space with five consecutive xenon flashes (1 flash/sec) with dark-field illumination. The first image of the sequence was brighter than the others because the first flash was more intense, and so the direction of swimming is readily evident (Text-fig. 1). Swimming velocity was calculated from the distance between each photographic image of a swimming model. Slowly swimming models produced an overlapping bead-like image. A bright single image indicates that the model did not swim at all.



Text-fig. 1. Sequential exposure of swimming models of *Paramecium* made with five successive xenon flashes, 1 sec apart, in a thin (70 μm) layer of reactivation medium. Since the first flash was the strongest, the first image in each sequence is brightest. (A) Forward-swimming models in a medium of 4 mM ATP, 4 mM-$MgCl_2$ and 3 mM EGTA (free Ca^{2+} below 10^{-8} M) (about $\times$ 12). (B) A forward-swimming model of higher magnification (about $\times$ 28). (C) Backward-swimming models in 4 mM ATP, 4 mM-$MgCl_2$ and 5×10^{-5} M-$CaCl_2$ (about $\times$ 12). (D) Backward-swimming model at higher magnification (about $\times$ 28). (*a*, *p*) Anterior and posterior ends of the models respectively.



Text-fig. 2. Photometric monitoring of the beating frequency of reactivated cilia of *Paramecium* model. The photometer sampled small areas of the image (small circles with numbers beside or on a model show the locations). This portion of the image was projected on to a photomultiplier (*PM*) through a small hole (0·5 mm in diameter) in a screen (*FP*). Changes in light intensity due to metachronal waves were amplified, displayed on an oscilloscope and photographed. *OP*, Operational amplifier. Frequency of the electrical signal from location 6 was doubled due to lashing-type beat (without metachronal waves) of the cilia at that location.

Beating frequency of the reactivated cilia

The frequency of metachronal waves passing a point on the model was monitored photometrically. Several models in a reactivation medium were pipetted on to a glass slide and compressed gently by a coverslip to prevent free swimming. The image of metachronal waves (× 200), was focused on a screen through a phase-contrast objective (Nikon bright contrast; × 40), and a small part of the image went on to a photomultiplier (Toshiba MS–9S, anode voltage, 1000 V) through a small hole (0·5 mm in diameter) in the screen. Cyclic changes in the light intensity due to passage of metachronal waves were displayed by this means on a cathode-ray tube and photographed (Text-fig. 2).

Electrical characteristics

Long (250 msec) electric current pulses in both inward and outward directions were introduced through a glass microcapillary electrode (less than 1 μm in tip diameter; filled with 0·1 M-KCl; 100–200 MΩ; see Naitoh & Eckert, 1972) into a live non-extracted specimen in a dilute saline solution. Resulting intracellular potential changes were monitored through a recording electrode of the same type and displayed on a cathode-ray tube. The external solution was then replaced with cold (1–2 °C) extraction medium. Electrical characteristics, such as resting membrane potential and responses to the current pulses, were monitored during the extraction.

Concentrations of adenine nucleotides in the models

Changes in the amounts of adenosinetriphosphate (ATP), adenosinediphosphate (ADP) and adenosinemonophosphate (AMP) in the specimens were enzymically determined during the course of extraction. About 0·2 ml of loosely packed models were homogenized and extracted in 4% perchloric acid for 30 min and then centrifuged in the cold. The neutralized supernatant was subjected to the enzymic determinations of adenine nucleotides by the method described by Bergmeyer (1963).

RESULTS

Changes in the ciliary activities during the extraction

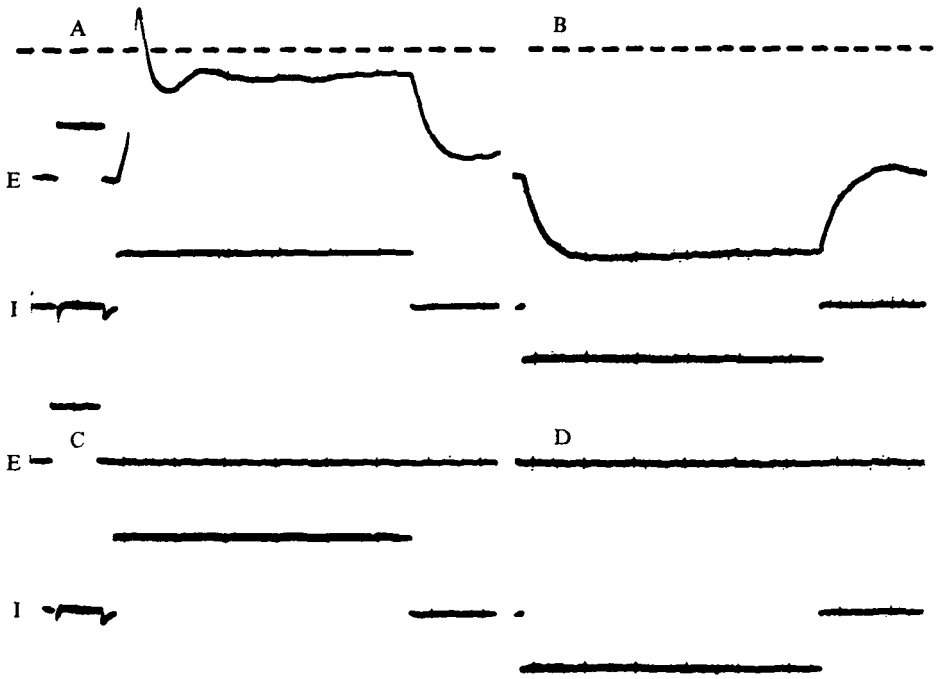
When specimens of *Paramecium* were immersed into the extraction medium they swam forward without showing any avoiding reactions, either spontaneously or upon collision with an obstacle, which are characteristic of non-extracted live specimens in their culture medium or in dilute saline solutions. The swimming velocity gradually decreased, and the specimens stopped swimming after 20 min due to a slowing of ciliary activity. Complete cessation of ciliary beating occurred after 30–40 min.

Changes in the electrical characteristics of the membrane during the extraction

Non-extracted live specimens in a saline solution (4 mM-KCl + 1 mM-$CaCl_2$, buffered to 7·2 by 1 mM Tris-HCl) showed graded regenerative calcium spikes (Naitoh *et al.* 1972) followed by damped potential oscillations in response to long (250 msec) depolarizing current pulses (10^{-9} A) (Text-fig. 3 A). The surface membrane behaved as an ohmic resistance (about $1{\cdot}5 \times 10^7\Omega$) in parallel with a capacitance (about 0·001 μF) to a hyperpolarizing pulse (Text-fig. 3 B). A delayed anomalous rectification was observed in response to stronger hyperpolarization.

Upon replacement of the saline with the extraction medium a sudden depolarization and a transient (several seconds) sign reversal of the membrane potential (about +20 mV) occurred, then the potential gradually approached the reference (zero) level. The membrane thereafter never showed any kind of electrical activity.

An injection of a square pulse did not induce a measurable potential shift across the membrane (Text-fig. 3 C, D), indicating a great increase in the membrane conductance.



Text-fig. 3. Electrical responses of *Paramecium* to long (250 msec) square pulses before (A, B) and after (C, D) the treatment with Triton X-100. Upper trace (E), membrane potential. Lower trace (I), current intensity. Calibration pulse on the upper trace; 10 mV, 10^{-9} A and 50 msec. (A, B) Live specimen in a solution with 4 mM-K^+ and 1 mM-Ca^{2+}. Dotted line shows reference level. (C, D) The same specimen after 5 min treatment with Triton X-100. There is no resting potential and no measurable electrotonic potential shift in response to injected current.

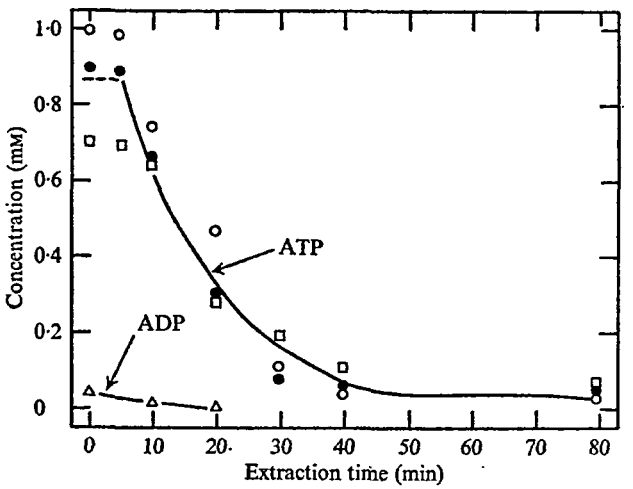
Changes in the intracellular concentrations of adenine nucleotides during the extraction

The concentration of ATP was 0·87 mM in non-extracted live specimens (a mean of three measurements on different groups of the specimens; approximate volume of specimens was $5{\cdot}6 \times 10^{-7}$ ml; see Fortner, 1925). As shown in Text-fig. 4, the ATP concentration decreased gradually with extraction time and became less than one-fifth the original value in 30 min. It continued to decrease during washing and storage of the models in the KCl solution.

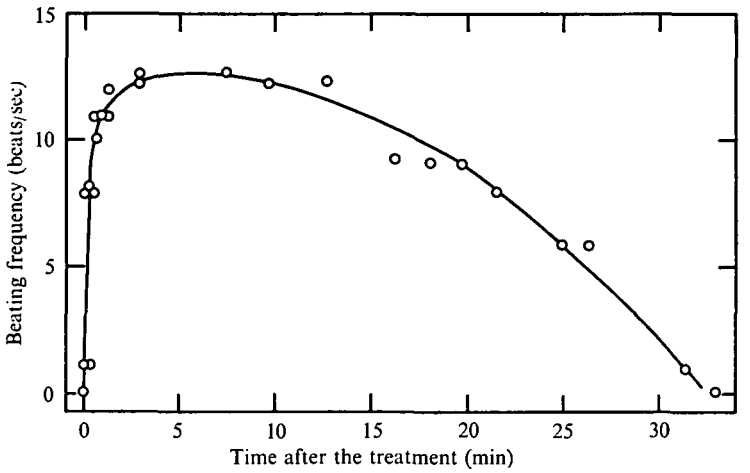
The amounts of ADP and AMP were very small (less than 0·05 mM) and became undetectable after 20 min of extraction.

Reactivation of ciliary movement

A few seconds after the models were transferred into a reactivation medium, which contained ATP, Mg^{2+} and EGTA (see later section), the cilia began to beat, first slowly and soon quickly, exhibiting antiplectoidal (Machemer, 1972) metachronal waves (Pl. 1 B). Metachronal waves could be seen even in cilia which beat very slowly (less than 1 beat/sec) at the beginning of their reactivation. Whereas some models, especially those extracted for long periods or stored for a long time before reactivation, showed lashing-type beatings with the frequency higher than that in the good re-



Text-fig. 4. Changes in the cellular concentrations of ATP and ADP during the extraction with Triton X-100. Measurements of ATP concentration were made in three different groups of *Paramecium* from different cultures. ADP concentrations were determined for one group of specimens corresponding to black circles in the ATP concentration curve.



Text-fig. 5. Change in beating frequency of cilia after the treatment of the models by a reactivation medium with 4 mM ATP, 4 mM-$MgCl_2$ and 3 mM EGTA.

activated models but without metachronal co-ordination. The effective stroke was directed toward the rear and somewhat to the right, causing the models to swim forward as the live specimens do under non-stimulated conditions (Text-fig. 1A, B).

The beating frequency of the cilia reached its maximum about 2 min after the transfer, then decreased slowly. The cilia finally stopped beating in 40–60 min (Text-fig. 5). Frequent renewals of the reactivation medium did not prolong the time of the ciliary reactivation. All the measurements of beating frequency and swimming velocity, therefore, were made two minutes after the transfer.

In an optimum reactivation medium (4 mM ATP, 4 mM-$MgCl_2$, 3 mM EGTA)

Table 1. *Beating frequency of cilia at seven different locations on* Paramecium *model*

Location	Beating frequency (beats/sec)
1. Most anterior	12·3 ± 0·6
2. Anterior right	11·7 ± 0·6
3. Posterior right	11·6 ± 0·6
4. Anterior left	11·3 ± 0·6
5. Posterior left	11·7 ± 0·6
6. Posterior	11·3 ± 0·5
7. Pharyngeal	11·4 ± 0·7

Location number corresponds to that in Text-fig. 2. The 'right' side is at the observer's right hand when the side of the specimen bearing the oral grove (i.e. ventral side) is kept down and the anterior end points away from the observer. Each beating frequency is a mean from twelve models.

more than 95% of the models were reactivated and swam immediately after 15 min storage in the cold KCl solution. However, only 50% of the models were reactivated after 3 h of storage in the KCl solution.

The beating frequency, as well as the swimming velocity, in a reactivation medium differed among specimens obtained from different cultures. Therefore, a series of experiments was carried out on fresh models (within 1 h after the extraction) made at the same time from one culture.

Sometimes, especially in a medium with low ATP and/or Mg^{2+} concentrations, only cilia on a limited area of the model were reactivated. This caused a curved or circular swimming of the model (Text-fig. 1A). However, there was no tendency for cilia over a definite region to exhibit greater sensitivity to ATP and Mg^{2+} than the rest. The local reactivation occurred indiscriminately on any portion of the model in a diluted ATP-Mg^{2+} medium.

The beating frequency was found to be almost identical in all the cilia over the surface of one model when they were well reactivated in an optimum medium (Table 1), although local differences in beating frequency are normally observed in live specimens.

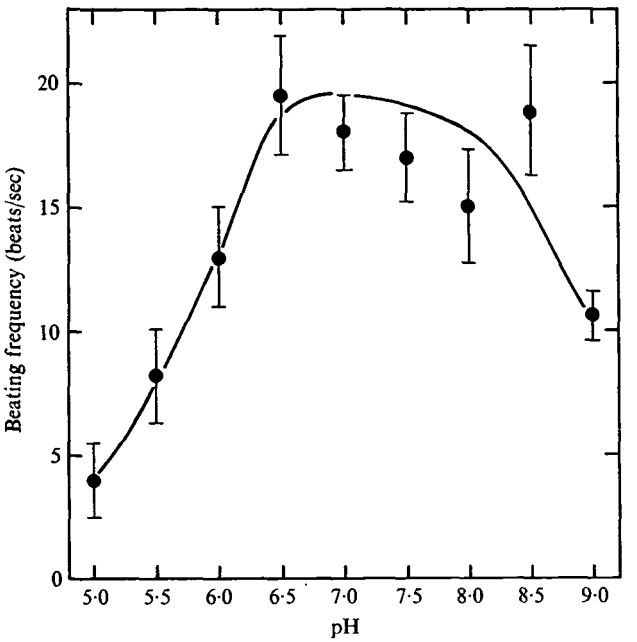
Optimum pH for the ATP-Mg^{2+} reactivation

The beating frequency and swimming velocity of the models were determined in nine ATP-Mg^{2+} media (4 mM ATP, 4 mM-$MgCl_2$, 3 mM EGTA) with the pH controlled by 10 mM Tris-maleate-NaOH buffer between pH 5 and 9.

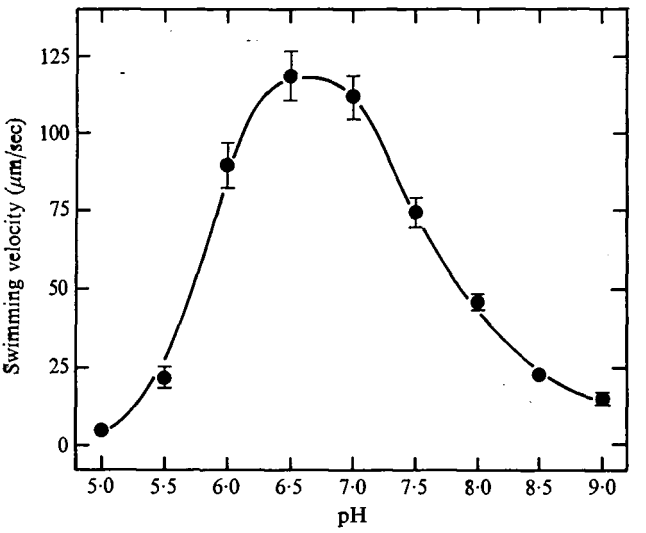
As shown in Text-fig. 6 (beating frequency) and Text-fig. 7 (swimming velocity), maximum values were obtained in a pH range from 6·5 to 7·0. For that reason all the following experiments were carried out at pH 7·0.

Effect of ATP concentration

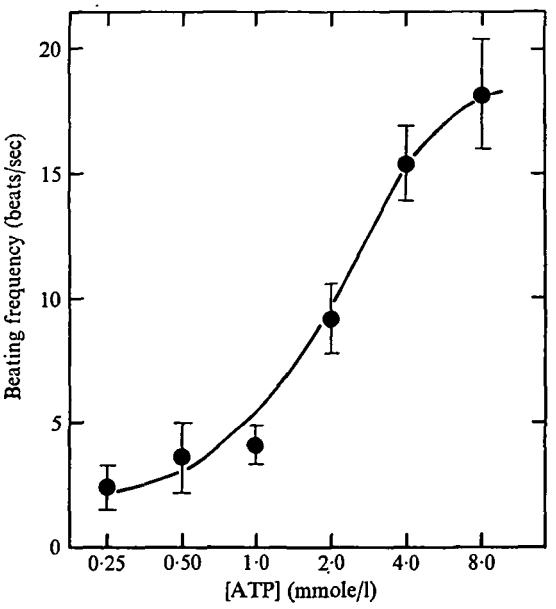
Beating frequency and swimming velocity were determined in a series of reactivation media with different ATP concentrations (0·25–8·0 mM), while other ionic compositions were kept constant (4 mM-$MgCl_2$, 3 mM EGTA). Increase of frequency was sigmoidal when plotted against a logarithmic scale of ATP concentration between 0·25 and 8·0 mM (Text-fig. 8). The response was most sensitive in the concentration



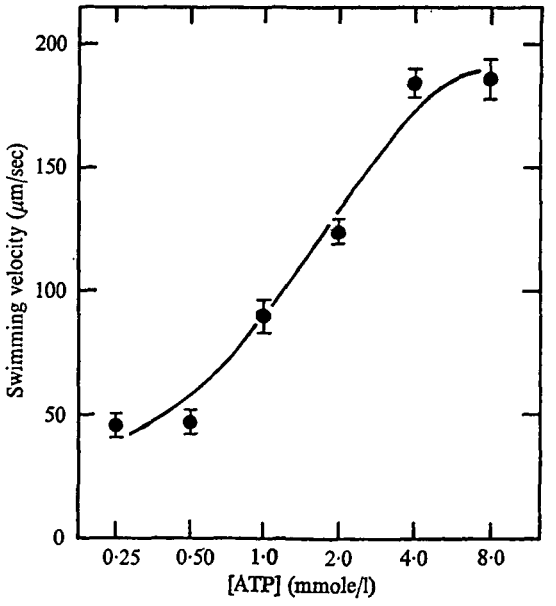
Text-fig. 6. Beating frequency of cilia as a function of pH in the reactivation medium (4 mM ATP, 4 mM-$MgCl_2$ and 3 mM EGTA).



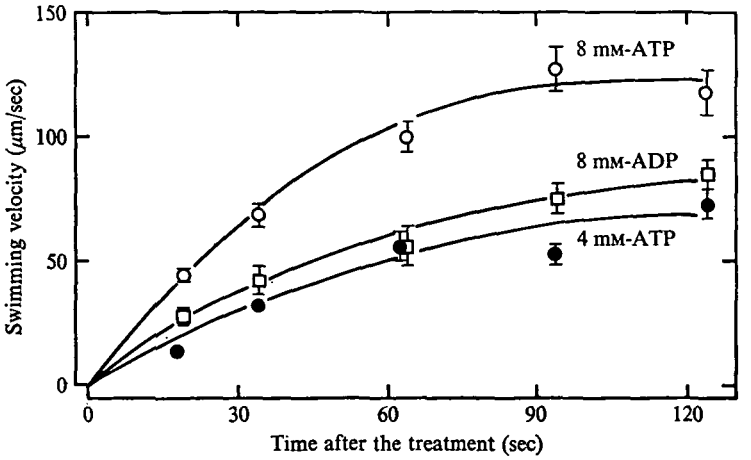
Text-fig. 7. Swimming velocity of the models as a function of the external pH. The reactivation medium contains 4 mM ATP, 4 mM-$MgCl_2$ and 3 mM EGTA.



Text-fig. 8. Beating frequency of cilia of the models as a function of ATP concentration in the reactivation medium. Concentrations of Mg^{2+} (4 mM), H^+ (pH 7·0) and EGTA (3 mM) were kept constant throughout.



Text-fig. 9. Swimming velocity of the models as a function of ATP concentration in the reactivation medium. Concentrations of other ions were kept constant throughout (4 mM-Mg^{2+}, 3 mM EGTA and pH 7·0).



Text-fig. 10. Time course of the change in swimming velocity of the models after treatment with reactivation media containing ATP or ADP. Concentrations of Mg^{2+} (4 mM), H^+ (pH 7·0) and EGTA (3 mM) were kept constant throughout.

range of 1–4 mM. With further increase in ATP concentration the frequency tended toward a plateau value. Swimming velocity showed the same pattern of change in response to increased ATP concentration (Text-fig. 9).

Effect of nucleotide phosphates other than ATP

Guanosine triphosphate (GTP), uridine triphosphate (UTP), cytocine triphosphate (CTP), ADP and AMP were tested to determine whether these can reactivate the models in the presence of Mg^{2+}.

Only ADP was found to induce ciliary beating. The time course of the reactivation by 8 mM ADP was similar to that by 4 mM ATP as shown in Text-fig. 10, but the maximum swimming velocity in the ADP solution was significantly smaller than that in an ATP solution of the same concentration (8 mM).

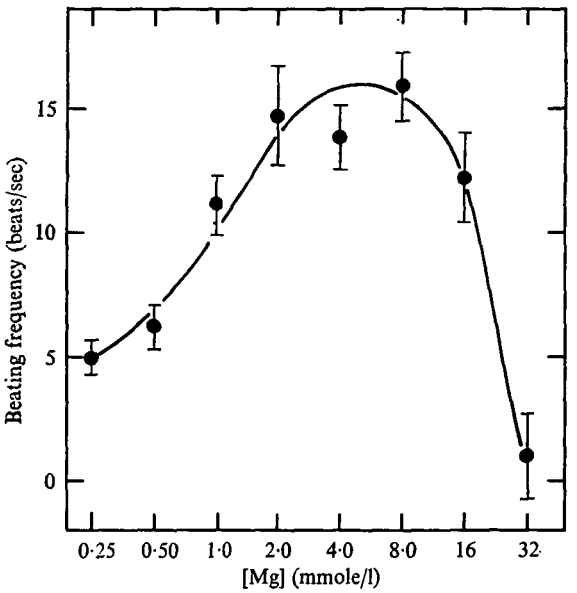
Effect of divalent cations

Magnesium. Beating frequency and swimming velocity were determined in a series of media with Mg^{2+} concentrations ranging from 0·25 to 32 mM at a constant ATP concentration (4 mM).

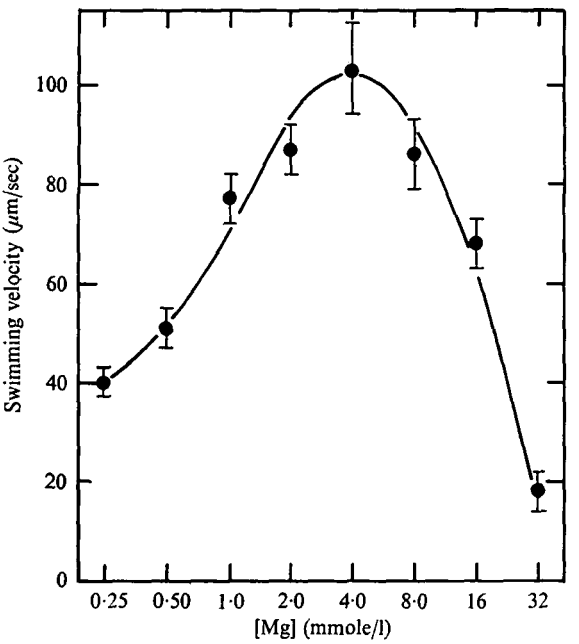
Both beating frequency (Text-fig. 11) and swimming velocity (Text-fig. 12) increased with increasing Mg^{2+} concentration, reached a maximum at 4–8 mM, then decreased with further increase in the concentration.

Calcium. Addition of Ca^{2+} (more than 10^{-5} M; without either Mg^{2+} or ATP) always induced longitudinal shortening of the model (i.e. contraction; see Weis-Fogh & Amos, 1972) (Pl. 1 C) and discharge of trichocysts; then the cilia came off the body and finally the models disintegrated.

When ATP was applied in association with Ca^{2+}, non-beating cilia, which pointed toward the rear in the KCl solution (Pl. 1 A), swung forward so as to point toward front (Pl. 1 D). Ciliary beating usually was not observed in the Mg^{2+}-free ATP-Ca^{2+}



Text-fig. 11. Beating frequency of cilia as a function of Mg^{2+} concentration in the reactivation medium. Concentrations of ATP (4 mM), H^+ (pH 7·0) and EGTA (3 mM) were kept constant throughout.



Text-fig. 12. Swimming velocity of the models as a function of Mg^{2+} concentration in the reactivation medium. Ionic compositions other than Mg^{2+} were kept constant throughout (4 mM ATP, 3 mM EGTA, pH 7·0).

medium. However, cilia sometimes beat for a short period (10–20 sec) after they became detached from the body of the model.

The most striking effect of Ca^{2+} on the ciliary orientation occurred when Ca^{2+} was applied to the reactivated, forward-swimming models in an ATP-Mg^{2+} medium. The orientation of the effective power stroke was reversed when the calcium concentration exceeded 10^{-6} M, causing the models to swim backward like backward-swimming live specimens in response to an appropriate stimulus (Text-figs. 1C, D; Pl. 1E; see also fig. 1 in Naitoh & Kaneko, 1972).

An increase in Ca^{2+} concentration up to 5×10^{-4} M did not affect the beating frequency of ATP-Mg^{2+}-reactivated cilia. Ciliary beating, however, slowed with further increase in Ca^{2+} concentration and stopped at 10^{-3} M (see fig. 2 in Naitoh & Kaneko, 1972).

Symplectoidal metachronal waves were observed in the Ca^{2+}-induced backward-swimming models (Pl. 1E).

Barium and strontium. Effects of Ba^{2+} and Sr^{2+} on the models were essentially the same with those of Ca^{2+}. They induced backward swimming in the ATP-Mg^{2+}-reactivated models. Velocity of the backward swimming was somewhat slower than that produced by Ca^{2+} ($66{\cdot}8 \pm 6{\cdot}8\%$ with Sr^{2+}, mean of 222 models; $89{\cdot}2 \pm 6{\cdot}5\%$ with Ba^{2+}, mean of 325 models; $100 \pm 5{\cdot}7\%$ with Ca^{2+} and $35{\cdot}2 \pm 2{\cdot}0$ μm/sec, mean of 421 models).

Manganese, cobalt and iron. The models were reactivated in a medium with ATP (4 mM) and Mn^{2+} (4 mM) with the swimming velocity of less than 45% of that in ATP-Mg^{2+} of the same concentration.

Co^{2+} (4 mM) was also effective in reactivating cilia of the models in the presence of ATP (4 mM), but it was less effective than Mn^{2+}, so that most of the reactivated models did not swim freely.

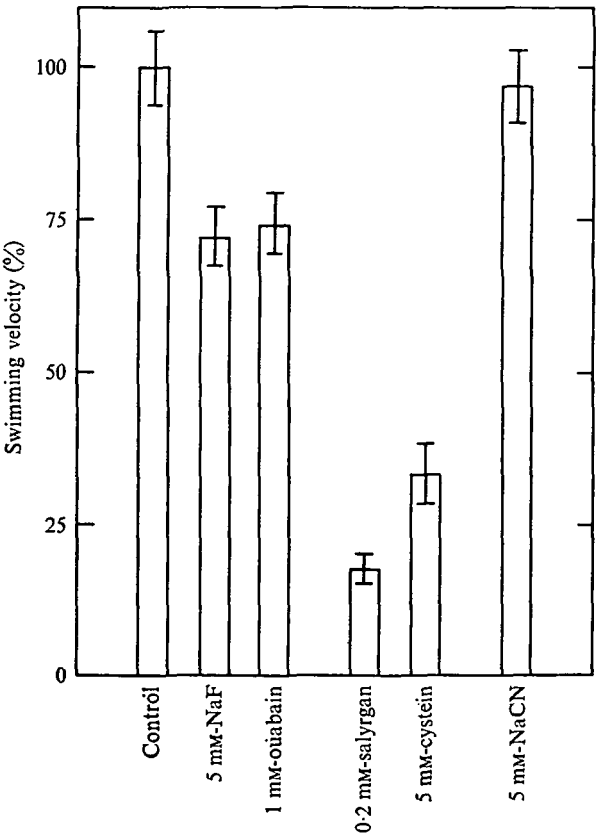
In a medium with ATP (4 mM) and Fe^{2+} (4 mM) only delayed (1–2 min after the treatment) weak reactivation of ciliary beating was observed. Beating frequency was less than 2 beats/sec.

Nickel. When a small amount of Ni^{2+} ions (0·5 mM) was applied to the ATP-Mg^{2+} reactivated models, cilia ceased to beat within 1 min. The pointing direction of the stopped cilia was toward the rear. Addition of Ca^{2+} (more than 10^{-6} M) in the Ni^{2+} containing ATP-Mg^{2+} medium caused the stopped cilia to point toward the front. Ni^{2+}, therefore, inhibits only Mg^{2+}-dependent beating but not Ca^{2+}-dependent change in the orientation of cilia.

Effects of some inhibitors

Effects of several metabolic inhibitors, such as NaF (5 mM), ouabain (1 mM), salyrgan (0·2 mM), and NaCN (5 mM), on the ATP-Mg^{2+}-reactivated ciliary beating were examined.

As shown in Text-fig. 13, salyrgan, which is a potent SH-blocking reagent, strongly depressed ciliary activity. Washing the salyrgan-inhibited models with 5 mM cystein caused a small recovery of the ciliary activity. Fluoride and ouabain reduced ciliary activity a little, but the models continued to swim for as long a time as the models in normal reactivation medium. Cyanide showed no effect.



Text-fig. 13. Effects of several inhibitors on the ATP-Mg^{2+}-reactivation of cilia. Degree of ciliary reactivation is expressed in percent of swimming velocity in a standard reactivation medium (4 mM ATP, 4 mM-$MgCl_2$ and 3 mM EGTA).

DISCUSSION

Criterion for the present model

Electron microscopy showed that the membrane remained at least partially visible around each cilium and covering the cell surface of the present models (Y. Naitoh, 1972 unpublished). However, loss of the electrical potential across the membrane and large increase in the electrical conductivity (Text-fig. 3) indicate that the Triton-extraction rendered the membrane very permeable to ions. The large decrease in cellular adenine nucleotides during the extraction (Text-fig. 4) indicates that the extracted membrane is also permeable to these substances. Therefore, externally applied inorganic ions and ATP (or other nucleotides phosphates) have direct access to the cell interior and can influence the function of the ciliary apparatus without much restriction by the surface membrane.

Reactivation of the ciliary beating

In the present models the reactivation of ciliary beating by ATP required Mg^{2+} or Mn^{2+} ions. The other divalent cations tested (Ca^{2+}, Co^{2+}, Fe^{2+} and Ni^{2+}) were far

less effective than Mg^{2+} and Mn^{2+}. The swimming velocity in Mg^{2+} was double that in Mn^{2+}. A high degree of specificity of Mg^{2+} for ciliary reactivation has been reported in the extracted models of other ciliary systems (spermatozoa: Bishop, 1962; Gibbons & Gibbons, 1972. Protozoan flagella: Brokaw, 1961, 1963. Protozoan cilia: Seravin, 1961; Gibbons, 1965. Ciliated epithelium: Child & Tamm, 1963; Satir & Child, 1963; Eckert & Murakami, 1972. For detailed references, see Arronet, 1971).

Recently Summers & Gibbons (1971) demonstrated that Mn^{2+} is as effective as Mg^{2+} in producing disintegration of isolated trypsin-digested axonemes from sea-urchin spermatozoa in the presence of ATP, caused by active sliding between the peripheral tubules of the axonemes. The sliding is thought to be the underlying mechanism of ciliary bending in intact cilia. Reactivation of ciliary beating by ATP-Mg^{2+} in the presence of EGTA indicates that Ca^{2+} ions are not primarily necessary for the cyclic bending mechanism in the cilia (Gibbons, 1965).

High ATP-specificity for the ciliary reactivation found in the present models has also been reported in other ciliary models (Brokaw, 1961; Gibbons, 1965; Gibbons & Gibbons, 1972). The fact that ADP is effective for ciliary reactivation suggests that an adenylate kinase system is present in the models (Brokaw, 1961; Gibbons, 1965; Winicur, 1967; Naitoh, 1969). Some investigators (Gibbons, 1965; Winicur, 1967) reported a delay in reactivation of ciliary beating by ADP. However, in the present models the time course of reactivation by ADP (8 mM) was essentially similar to that by ATP (4 mM) (Text-fig. 10).

Optimum pH for the ATP-Mg^{2+} reactivation was 6·5–7·0 (Text-figs. 6, 7); the value is similar to that in isolated glycerinated cilia of *Tetrahymena* (Gibbons, 1965), and somewhat lower than that in Triton-extracted sperm models (Gibbons & Gibbons, 1972).

Similarity between beating frequency and swimming velocity curves as a function of concentration of H^+, ATP or Mg^{2+} (cf. Text-figs. 6 and 7, 8 and 9, 11 and 12) indicates that the swimming velocity of the reactivated models is primarily dependent on the beating frequency of their cilia.

The beating frequency in an optimum reactivation medium (4 mM ATP, 4 mM-$MgCl_2$ and 3 mM EGTA) was $12{\cdot}3 \pm 0{\cdot}6$ beats/sec (mean and standard errors for 50 models), approaching the value for the unextracted live specimen (Sleigh, 1962; Kinosita, Dryl & Naitoh, 1964*b*). The mean swimming velocity (240 models) was 162 ± 12 μm/sec; this was less than half that of the normal live specimen (Kinosita *et al.* 1964*b*). The lower swimming velocity might be due to somewhat diminished co-ordination in the reactivated cilia.

Metachronal co-ordination

The reactivated cilia usually showed metachronal co-ordination. This finding indicates that metachronal co-ordination is basically independent of the membrane function. It depends most probably on mechanical interactions between the cilia (Sleigh, 1962; Kinosita & Murakami, 1967). The idea that metachronal waves are propagated by impulses passing along the cell membrane (Grebecki, 1965) is no longer credible (see Eckert & Naitoh, 1970). But this should not be confused with the fact that bioelectric events in the membrane regulate the frequency and the orientation of

ciliary beating (Kinosita, 1954; Kinosita *et al.* 1964*a*, *b*, 1965), thereby secondarily affecting the metachronal wave pattern (see the next section).

Orientation of effective stroke

In Ca^{2+} concentrations below 10^{-7} M the direction of effective power stroke was toward the rear of the model, and the swimming motion was forward. The 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 reversal of the swimming direction occurred at concentrations above 10^{-6} M. This corresponds to the backward swimming of non-extracted live animals in response to an appropriate stimulus (Pl. 1 E; see also fig. 1 in Naitoh & Kaneko, 1972).

Treatment of the models with a mixture of Ca^{2+} and ATP (without Mg^{2+}) induced no ciliary beating, but a reorientation of the non-beating cilia toward the front. This position of the immobile cilia corresponds to backward swimming with motile cilia (Pl. 1 D).

Naitoh (1969) found that cilia of *Paramecium* lost their ability to beat in response to ATP and Mg^{2+} during extraction by glycerol (10–15 days at -15 °C); however, they still reversed their pointing direction in response to ATP and Ca^{2+} similarly to the present models. Sr^{2+} and Ba^{2+} also induced reversed orientation of nonbeating cilia.

These findings strongly support the proposal (Naitoh, 1966) that the ciliary system of *Paramecium* has at least two kinds of motile component; one component concerned with the cyclic bending of the cilium and the other regulating the orientation of the effective power stroke in beating cilia, or the pointing direction in non-beating cilia. Both use ATP as the energy source. The former requires Mg^{2+} and the latter Ca^{2+} as co-factors for the ATP-energized reactivation.

Bioelectric control of ciliary movement

Since the end of the last century it has been known that ciliary reversal of *Paramecium* and other ciliated protozoans occurs on the cell surface facing the cathode when the specimen is placed in an electric field (Verworn, 1889; Jennings, 1906). More recently ciliary reversal was found to occur in close association with a membrane depolarization (Kinosita, 1954; Kinosita *et al.* 1964*a*, 1965; Naitoh, 1966; Eckert & Naitoh, 1970). It was also noted that external Ca^{2+} is indispensable for ciliary reversal (Bancroft, 1906; Kamada, 1940; Kinosita, 1954; Okajima, 1954). Recent electrophysiological examination of the *Paramecium* membrane showed that a regenerative depolarization is mediated by an influx of Ca^{2+} in response to an increase in the calcium conductance of the membrane (Naitoh *et al.* 1972). Thus, the idea was put forward that an increase in cytoplasmic calcium concentration within the cilium due to this calcium influx is responsible for the manifestation of ciliary reversal (Eckert, 1972; Eckert & Naitoh, 1972). The present finding that the extracted models of *Paramecium* show ciliary reversal in the presence of Ca^{2+} (above 10^{-6} M) strongly supports the proposal (see also 'calcium hypothesis' in Naitoh, 1968, 1969). Eckert (1972) calculated the approximate increment in free Ca^{2+} concentration within a cilium corresponding to a 1 mV depolarization, which approximates to a threshold membrane depolarization for the least ciliary reversal in live specimens (Machemer & Eckert, 1972). The value was about 10^{-6} M, which is comparable to an effective concentration for the induction

of ciliary reversal in the present models. Furthermore, a behavioural mutant of *Paramecium aurelia*, which never shows ciliary reversal in response to stimuli, fails to exhibit the increased Ca^{2+} conductance in response to a stimulus (Kung & Eckert, 1972). Triton-extracted models of the same mutant do, however, exhibit normal ciliary reversal in response to applied calcium (Kung & Naitoh, 1973). These lines of evidence strongly indicate Ca^{2+} – mediated membrane regulation of ciliary orientation in *Paramecium*.

Hyperpolarization of the membrane inhibits ciliary reversal (i.e. it normalizes the direction of the power stroke) and increases the beating frequency (Naitoh, 1958; Kinosita *et al.* 1964*b*; Eckert & Naitoh, 1970). Present results clearly indicate that the beating frequency is a direct function of both Mg^{2+} and ATP concentrations (Text-figs. 8, 11). It might therefore be conjectured that changes in the Mg^{2+} concentration associated with bioelectric events in the membrane are responsible for the mechanism underlying the control of the beating frequency.

An increase in the beating frequency is also observed in association with ciliary reversal by membrane depolarization (Kinosita *et al.* 1965; Machemer & Eckert, 1973). Eckert & Murakami (1972) found an increase in beating frequency by ionophoretic application of Ca^{2+} to the ciliated cell of amphibian oviduct epithelium. They suggested an activation of ATP-yielding enzyme systems by Ca^{2+} as a cause of the frequency increase. However, this is not likely in the case of *Paramecium* because an increase in the frequency following a depolarization (and also following a hyperpolarization) occurs with very short latency (5–10 msec). Perhaps an antagonism between Mg^{2+} and Ca^{2+} in the ciliary motile system and/or calcium (or magnesium) sequestering sites (possibly in the membrane; see Naitoh, 1968) might be involved in the mechanism.

Cilia on the anterior half of a live specimen respond to membrane depolarization with shorter latency and stronger shifts to the anterior than do cilia on the posterior half (Okajima, 1953; Eckert & Naitoh, 1970). The similar localized differences of the sensitivity and the degree in changing the ciliary orientation in response to applied Ca^{2+} are seen in the models (Naitoh, 1969). This suggests that the differences are due to factors intrinsic in the ciliary apparatus. On the other hand, the present finding that reactivity of the extracted cilia to ATP and/or Mg^{2+} is the same almost all over the model (Table 1) supports the possibility that the localized difference in beating frequency found in a live animal results from localized differences in the regulatory function of the surface membrane. Some examples of localized differentiation in membrane function are well demonstrated in *Opalina* and *Paramecium*, namely, specialized mechanoreceptive (Naitoh & Eckert, 1969*a*) and chemosensitive (Naitoh, 1961) areas.

SUMMARY

1. Cilia of *Paramecium candatum* extracted with Triton X-100 were reactivated in the presence of ATP and Mg^{2+}.

2. The beating frequency of the reactivated cilia is a function of both the ATP and Mg^{2+} concentrations.

3. The reactivated cell models swam forward when the Ca^{2+} concentrations in the ATP-Mg^{2+} medium was kept below 10^{-7} M. They swam backward when the Ca^{2+}

concentration was above 10^{-6} M. This was due to a reversed orientation of the effective power stroke of the reactivated cilia.

4. In the absence of Mg^{2+} the cilia failed to beat, even though ATP was present. If Ca^{2+} was then added the cilia assumed a new orientation, pointing toward the anterior without beating.

5. Ni^{2+} inhibited ciliary beating in the reactivated models, but has no influence on changes in the orientation of the cilia produced by ATP and Ca^{2+}. This suggests that one ATP-activated system is responsible for beating, while another governs the direction of the effective stroke.

6. Mn^{2+} is half as effective as Mg^{2+} for inducing ciliary beating in the extracted models in the presence of ATP.

7. Salyrgan strongly inhibits Mg^{2+}-ATP activated ciliary activity of the model.

8. Bioelectric control of ciliary activity by the cell membrane of live animals is discussed.

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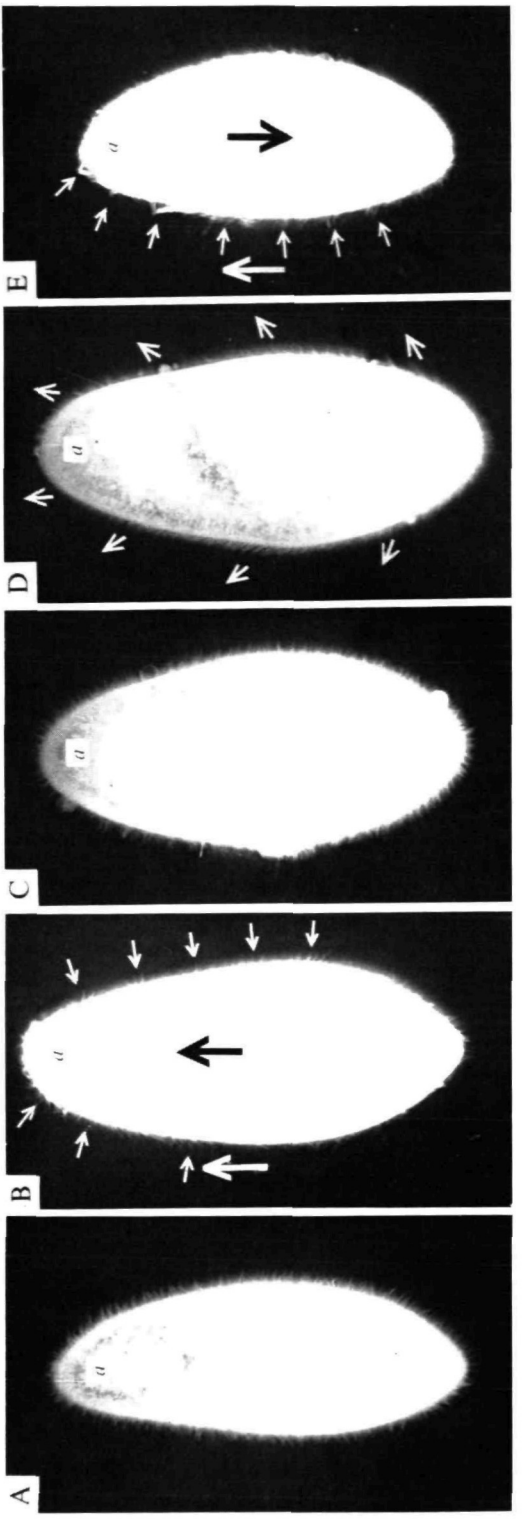
EXPLANATION OF PLATE I

Photographs of the models in various solutions taken by single xenon flashes through a bright-contrast phase objective (Nikon X20) (Magnification about ×230). (A) 50 mM-KCl solution. Cilia did not beat. Pointing direction of the cilia was toward posterior or perpendicular to the surface. (B) A forward-swimming model in the KCl solution with 4 mM ATP, 4 mM-$MgCl_2$ and 3 mM EGTA. Black arrow indicates the swimming direction. Small white arrows indicate metachronal waves, which are propagated in the direction of large white arrow (antiplectoidal). (C) 50 mM-KCl solution with 0·05 mM-$CaCl_2$. Cilia do not beat and point perpendicular to the surface. (D) 50 mM-KCl solution with 0·05 mM-$CaCl_2$ and 4 mM ATP. Cilia do not beat, but had reoriented once to point toward the front. Small white arrows show approximate pointing direction of cilia. (E) A backward-swimming model in the KCl solution with 4 mM ATP, 4 mM-$MgCl_2$ and 0·05 mM-$CaCl_2$. Black arrow shows the swimming direction. Small white arrows indicate the metachronal waves which were conducted in the direction of the large white arrow (symplectoidal). (*a*) Anterior end of the models.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