CONTROL OF FLAGELLAR MOTION IN CHLAMYDOMONAS AND EUGLENA BY MECHANICAL MICROINJECTION OF Mg$^{2+}$ AND Ca$^{2+}$ AND BY ELECTRIC CURRENT INJECTION

KATHLEEN M. NICHOLS AND ROBERT RIKMENSPOEL
Department of Biological Sciences, State University of New York at Albany, Albany, New York 12222, U.S.A.

SUMMARY

Upon impalement with a microelectrode in a Ca$^{2+}$-free medium containing 5 mM ATP, a Chlamydomonas cell lost its flagellar activity within 45 s, and the injection of either positive or negative direct current did not stimulate the flagella to beat after that time. When 3 mM Mg$^{2+}$ was added to the external medium, the impaled cell exhibited a flagellar frequency of 22 ± 7 Hz. With 5 mM ATP and 3 mM Mg$^{2+}$ in a Ca$^{2+}$-free medium, negative direct electric current inhibited flagellar frequency and positive direct electric current enhanced flagellar activity. The flagella recovered to approximately their characteristic frequency (20 ± 6 Hz) upon the cessation of current.

Euglena and Chlamydomonas cells were mechanically microinjected with Ca$^{2+}$ or Mg$^{2+}$ ions contained in 1 M KCl-filled microelectrodes. In both cells, the injection of Ca$^{2+}$ resulted in a decrease in flagellar frequency dependent on the amount of Ca$^{2+}$ injected. The frequency decreased to zero Hz upon the injection of 16 x 10$^{-14}$ l in Euglena and 3.5 x 10$^{-14}$ l in Chlamydomonas. The microinjection of 10 x 10$^{-14}$ l of 0.2 M Mg$^{2+}$ into Euglena cells resulted in an approximately 2-fold increase in flagellar frequency. Chlamydomonas flagella, which stop beating upon impalement in a Mg$^{2+}$-free medium, began to beat when the cell was injected with Mg$^{2+}$ ions. The flagella exhibited an average frequency of 16 ± 3 Hz when injected with 5 x 10$^{-14}$ l of 0.2 M Mg$^{2+}$. The data indicate that an increase in internal Mg$^{2+}$ stimulates flagellar frequency. The microinjection of Ca$^{2+}$ inhibits flagellar motility.

INTRODUCTION

Recent experiments in this laboratory have indicated that Mg$^{2+}$ plays a role in the control of flagellar motion in Euglena. A Euglena cell in a Ca$^{2+}$-free medium containing ATP and EGTA, impaled with a microelectrode through which electric current was passed, exhibited flagellar motion that was a function of the amount of current applied. If the medium contained gramicidin also (an antibiotic which affects membrane integrity; Henderson, McGivan & Chappell, 1969; Bamberg & Lauger, 1973), the flagellum remained stopped after 2 current injections, each for a period of 10-30 s. In the presence of Mg$^{2+}$, however, pre-injection flagellar frequencies were restored upon the cessation of current (Nichols & Rikmenspoel, 1977).

To reaffirm the participation of Mg$^{2+}$ in the control of motility, electrical control experiments analogous to those performed on Euglena were carried out on Chlamydomonas cells. The results indicate that the flagellar motility in Chlamydomonas is dependent on Mg$^{2+}$.

To investigate further the effect of Mg$^{2+}$ on flagellar motility, Euglena (which has a
volume of approximately $4 \times 10^{-12} \text{l.}$) and *Chlamydomonas* ($\approx 5 \times 10^{-13} \text{l.}$) were mechanically microinjected with solutions containing $\text{Mg}^{2+}$. The effects of the injection of $\text{Ca}^{2+}$ were also investigated. Microinjection experiments have been reported in the literature, but not on cells of the size of *Euglena* or *Chlamydomonas*. *Limulus* photoreceptors, aequorin droplets, *Aplysia* neurons, *Paramecium*, and sea-urchin eggs with volumes ranging from $5 \times 10^{-6}$ to $5 \times 10^{-11} \text{l.}$, have been microinjected with volumes which were not less than $1 \times 10^{-12} \text{l.}$ (Lisman & Brown, 1975; Kusano, Miledi & Stinnakre, 1975; Stinnakre & Tauc, 1973; Saiki & Hiramoto, 1975; Hamaguchi, 1975). In this paper a set up is described which permits microinjection of volumes as small as $10^{-14} \text{l.}$ The results of our experiments show that the microinjection of $\text{Mg}^{2+}$ activates flagellar motion in *Chlamydomonas* and accelerates flagellar frequency in *Euglena*. The injection of $\text{Ca}^{2+}$, however, decreases flagellar frequency. The data presented here have been reported in abstract form (Nichols & Rikmenspoel, 1976).

**EXPERIMENTAL METHODS**

**Organisms and materials**

*Euglena gracilis* and *Chlamydomonas reinhardtii* were obtained from the American Type Tissue Culture Co., Rockville, Maryland. Both cell types were grown under constant illumination at 21 °C in a modified Cramer Meyers Medium (Cramer & Meyers, 1951) containing 7.3 mM $\text{KH}_2\text{PO}_4$, 7.5 mM $(\text{NH}_4)_2\text{PO}_4$, 1.7 mM $\text{MgSO}_4$, 0.2 mM $\text{CaCl}_2$ and trace metals.

Experiments were conducted in 1 ml of deprived medium (DM) containing 7.3 mM $\text{KH}_2\text{PO}_4$ and 7.5 mM $(\text{NH}_4)_2\text{PO}_4$ in glass-double-distilled H$_2$O, adjusted to pH 7.0, to which < 0.05 ml of *Euglena* or *Chlamydomonas* culture was added. In this medium the normal motility of the cells was maintained for up to 2 h.

$\text{ATP}$, disodium salt, was obtained from Boehringer Mannheim Co., New York, New York. Solutions of 0.1 M $\text{ATP}$ at pH 7.0 were frozen in aliquots of 1 ml. For experiments, additions of ATP were made from an aliquot thawed just prior to use. EGTA, ethylene glycol-bis-(β amino ethyl ether) N,N' tetra-acetic acid, at pH 7.0, and gramicidin, which were used as described before (Nichols & Rikmenspoel, 1976), were obtained from Sigma Chemical Co., St Louis, Missouri.

Additions were made to the DM with a Hamilton syringe, Hamilton Co., Reno, Nevada. The DM and all additions were adjusted to pH 7.0 prior to use. The experiments were performed at room temperature (21 ± 1 °C).

**Apparatus**

Cells were viewed during experimentation in a Zeiss Universal Microscope with a 40 × Zeiss water-immersion objective. The techniques for impaling and positioning the cells as well as the details concerning the methods for preparing microelectrodes and suction pipettes have been described previously (Nichols & Rikmenspoel, 1977).

Electrical current was injected into an impaled *Euglena* or *Chlamydomonas* cell through the microelectrode from a direct current source with an internal resistance of 100 MΩ.

The apparatus for electrical control experiments was redesigned to enable the microinjection of substances into the cells as well as to retain the ability to inject electrical current (see Fig. 1). The microelectrode, mounted in a piezoelectric driver, was connected with polyethylene tubing to a glass chamber which, in turn, was connected with tubing to a 1-μl Hamilton syringe. The Hamilton syringe was securely mounted on an aluminium block. Also fixed on the aluminium block was a micrometer wheel which, when turned, advanced the plunger of the Hamilton syringe and delivered an amount of liquid from the syringe. The syringe, all plastic tubing and the glass connector were filled with Versilube-F-50 silicon oil (Silicon Products Dept., General Electric Co., Waterford, N.Y.) to prevent evaporation of the electrolyte in the micro-
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electrode and to prevent air bubbles in the tubing which could affect the amount of solution microinjected. The oil provided electrical insulation for the wire connecting the microelectrode to the current source. Also, the ions in the electrolyte are not miscible in the oil. Turning the micrometer wheel resulted in an amount of oil being delivered from the syringe into the tubing-connector-tubing system, and a volume of solution was delivered into an impaled cell from the microelectrode.

![Diagram of microinjection apparatus](image)

**Fig. 1. Diagram illustrating the apparatus for the microinjection of ions into Euglena and Chlamydomonas.** The Hamilton syringe, all plastic tubing, and the glass connector were filled with silicon oil. Shading represents areas filled with oil.

An Ag-AgCl wire connected to the direct current source was permanently fixed into the glass connector and through the plastic tubing to enable contact between the electrolyte in the microelectrode and the direct current source. In this way, cells could be both electrically stimulated and mechanically microinjected with the same experimental apparatus.

**Cinemicrography**

Experimental preparations were illuminated under dark field, as described before (Nichols & Rikmenspoel, 1977). A 1 kW Xenon arc light (Hanovia Co., Newark, New Jersey) with a wavelength band restricted to 420-700 nm with 3-mm GG420 and 4-mm KG 3 glass filters (Schott Gen., Mainz, W. Germany), provided illumination for microscopy and filming.

All data were recorded on 16-mm Plus X film at 200 frames/s using a Milliken DBM5C camera (Teledyne Corp., Arcadia, Calif.).

**Motility analysis**

The films were projected to a final magnification of 1000 x in a Vanguard Motion Analyzer. On successive frames the outline of the flagellum and a portion of the cell were traced on transparent paper. The flagellar frequency was obtained from the period between repeating flagellar shapes as described by Nichols & Rikmenspoel (1977).

**Calibration of the microinjection volume**

The usual methods for measuring small amounts of ions, such as radioactive tracers or atomic absorption measurements were not feasible for calibrating the microinjection deliveries due to the extremely small volumes in question, approximately $10^{-14}$. During the actual experiments described below in the Results section, the precise volumes of solution injected were not known. It was noted, however, that frequently a ‘vacuole’ became visible in the Euglena or Chlamydomonas, immediately after the microinjection had been performed. The term ‘vacuole’ is used here to describe an area in the cell from which organelles have been
displaced by the flow of fluid from the injection pipette. The cinemicrographs of the mechanical microinjection on *Euglena* and *Chlamydomonas* were examined for the appearance of a vacuole after injection of various volumes. Fig. 2 shows an enlargement of 2 film frames of a *Chlamydomonas*, before and after injection, on which the formation of a vacuole is apparent. It appeared that estimating the volume of these vacuoles, as illustrated in Fig. 2, was the only way of obtaining a (albeit a posteriori) calibration of the microinjection method.

**Fig. 2.** A, *Chlamydomonas* cell impaled on a microelectrode before microinjection with 1 M KCl and 0.2 M MgSO₄. B, the same cell after microinjection of approximately $2 \times 10^{-14}$ l. of solution. Note the appearance of a vacuole as a result of microinjection. Approximately 30 s have elapsed between the cinemicrographs of the cell before and after microinjection. Due to the hard photographic printing technique used to make the vacuole stand out clearly, the flagellum is not visible.

It was difficult to discern the exact boundaries of the vacuole caused by microinjection, due to the change in the position of granules, chloroplasts, and other inclusions in the cytoplasm during a single film sequence of an impaled cell before and after injection. Only vacuoles which appeared in the cell at the tip of the implanted electrode and directly following a microinjection event were considered vacuoles due to microinjection. The additional observation that upon injection of current, a vacuole of the kind described above does not appear in the cell, supports the conclusion that the vacuole represents a microinjected volume. Cells into which current has been injected exhibit a slight swelling, approximately 5–20 % of the original volume, of the entire cell boundary, and undergo a rearrangement of cellular material but do not show the repeated appearance of a vacuole at the implanted electrode tip. The outline of the vacuole in the cell was, in most cases, a circle, and the volume was calculated as a sphere. In some cases, the vacuole appeared as an ellipse. The volumes for these vacuoles were taken as cylinders. The vacuole was measured within 30 s after injection to avoid errors due to the dispersal and subsequent disappearance of the vacuole within 30–45 s after micro-injection.

**Fig. 3** shows the results obtained for the measurement of the volume of the microinjection vacuole as a function of the advance of the 1-µl Hamilton syringe in inches for both *Euglena* and *Chlamydomonas*. As the volume delivered from the Hamilton syringe increases, the volume actually injected into the cells increases approximately linearly. It is possible that the calibration curve for *Chlamydomonas* differs from that of *Euglena* due to the smaller volume of the *Chlamydomonas* cell, the increased leakage which results on impalement, and the subsequent failure to retain some of the microinjected volume.

It can be seen from Fig. 3 that the reproducibility of the microinjection set-up was quite good. The measured vacuole volumes did not correspond to the amount of oil delivered from the Hamilton syringe, however. One 0.0012-in. (0.003-cm) advance of the Hamilton syringe (one turn of the micrometer) should result in a delivery of approximately $6 \times 10^{-18}$ l. The calibrated volumes are of the order of $10^{-19}$ l. The entire microinjection apparatus is not tight at the con-
nexus of the plastic tubing to the syringe needle, the glass connector and the microelectrode. The major part of the silicon oil delivered by the Hamilton syringe presumably leaks out at these junctions.

The rather flexible polyethylene tubing used to connect the various parts of the microinjection apparatus probably expands slightly when a volume of oil is delivered from the Hamilton syringe. The resulting hydrostatic pressure causes an outflow of oil through the various connections and a (very small) outflow from the micropipette. Since the hydrostatic pressure is bled rapidly through the various leaks it is not probable that the outflow from the micropipette continues after the initial pulse.

The estimates of the microinjected volumes made from Fig. 3 could be interpreted as lower limits. We believe, however, that the volumes microinjected into the cells in our experiments were correctly estimated with the aid of Fig. 3 to within a factor of 2 or 3.

RESULTS

Impalement and electrical control of Chlamydomonas

It was necessary to hold a Chlamydomonas cell in position by suction through a micropipette so that the cell could be impaled with a microelectrode. After the cell was impaled, the suction in the micropipette was released and the pipette was withdrawn from the cell leaving the Chlamydomonas firmly impaled on the microelectrode. Cells held on a suction pipette, but not impaled, had an average flagellar frequency of 28 ± 5 Hz.

In deprived medium (DM) with no additions, the Chlamydomonas lost flagellar activity within 1 s after impalement of the cell with a microelectrode. When 3–5 mM ATP had been added to the DM flagellar activity continued for 30–45 s after impalement of the cell. With 5 mM ATP and 3 mM MgSO₄ added to the external medium a flagellar frequency of 22 ± 7 Hz was maintained for many minutes after impalement. These observations indicate that ATP diffuses out of the cells much faster than Mg²⁺.
Fig. 4 shows enlargements of 4 film frames on which an impaled *Chlamydomonas* with a motile flagellum is visible. In almost all of our experiments only one of the two flagella of *Chlamydomonas* was visible on the films made. It proved not possible to have these cells impaled and also in an orientation in which both flagella were visible. All data related below refer therefore to one flagellum on each *Chlamydomonas*.

At an external Mg\(^{2+}\) concentration of less than 1 mM or more than 4 mM, and with 1–5 mM ATP in the preparation, the flagellar activity of the impaled cell was much reduced. Less than 3 mM ATP in the experimental preparations did not sustain motility for more than one minute after impalement. All current injection experiments on *Chlamydomonas* reported here were performed in DM with 3 mM Mg\(^{2+}\) and 5 mM ATP added. The addition of ATP and Mg\(^{2+}\) to the external medium of *Chlamydomonas* was necessary due to the failure of the membrane to seal around the implanted electrode. Similarly, impaled bull spermatozoa require Mg\(^{2+}\) and ATP in the external medium to maintain flagellar motility (Lindemann & Rikmenspoel, 1971). Impaled *Euglena* cells in DM, however, require only ATP and not Mg\(^{2+}\) in the external medium to maintain motility (Nichols & Rikmenspoel, 1977).

The injection of negative or positive direct current of 0–3 μA or more through the microelectrode into an impaled *Chlamydomonas* cell caused flagellar motion to stop. When, after approximately 30 s, the current injection was discontinued, the flagellar motility resumed in less than 1 s as estimated by visual observation. The wave shape of the flagellum after a 30-s exposure to current (an injection cycle) was essentially unchanged compared to that prior to impalement as shown in Fig. 5A, B. The frequency recovered to 20 ± 6 Hz.

Two to four cycles of current injection could be performed on a *Chlamydomonas* cell, after which the flagellum failed to recover preinjection frequency. The addition of 1 mM EGTA, a strong Ca\(^{2+}\) chelator which binds only slightly to Mg\(^{2+}\), to the DM containing 5 mM ATP and 3 mM Mg\(^{2+}\) allowed the flagellum to recover from 5 to 8 cycles of current injection.

In the presence of 5 mM ATP, 3 mM Mg\(^{2+}\) and 1 mM EGTA the flagellar frequency varies smoothly with the amount of direct current injected. Fig. 6 presents the ratio of flagellar frequency during and before the second cycle of current injection as a function of current. Flagellar frequency declines as increasing amounts of negative current are injected into the cell, whereas positive currents of up to 0.15 μA cause an increase in
flagellar frequency. More than $+0.15 \mu A$ of injected current results in a sharp decrease in motility, which reaches zero at $+0.3 \mu A$.

The currents applied to the cells in the experiments illustrated in Fig. 6 are large for a cell 7-10 $\mu m$ in diameter. However, in a previous report (see fig. 9 of Nichols & Rikmenspoel, 1977), it was calculated from resistance measurements on impaled Euglena that the greater part of the applied current flows through a leakage path adjacent to the impaling microelectrode and only a very small amount of current flows through the cell. This same assumption has been made for the experiments in Chlamydomonas.

These results closely parallel those obtained in Euglena where it was shown that electrically controlled flagellar frequency was dependent on Mg$^{2+}$ and that negative direct current applied through microelectrodes decreased flagellar frequency and that positive direct currents increased the frequency of a Euglena flagellum (Nichols & Rikmenspoel, 1977). The mechanism which mediates the electrical control of flagellar motion is therefore probably the same in Chlamydomonas and in Euglena.

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**Control experiments.** Euglena in DM, with 1 mM EGTA, 10 mM ATP, and 1 $\mu$m gramicidin added to the external medium, were impaled with a microelectrode. In control experiments Euglena cells were mechanically microinjected through the impaling electrode with a solution of 1 M KCl. Fig. 7A shows the effect on flagellar frequency of the microinjection of 1 M KCl into impaled Euglena. Up to $1.3 \times 10^{-13}$ l. of 1 M KCl injected did not affect beating frequency; $2.3 \times 10^{-13}$ l. of injected 1 M KCl caused a 50% reduction in flagellar frequency.

Impaled Chlamydomonas cells in DM with 1 mM EGTA, 5 mM ATP and 3 mM
MgSO$_4$ were microinjected with 1 mM KCl, the control solution. Fig. 7B shows that the flagellar frequency remained unchanged after $3 \times 10^{-14}$ l. of 1 mM KCl were microinjected. Increasing amounts of injected KCl caused the flagellar activity to diminish and reach a frequency of zero when $1.1 \times 10^{-13}$ l. were microinjected.

The injected volume does therefore not affect the flagellar frequency up to $1.3 \times 10^{-13}$ l. in *Euglena* and up to $3 \times 10^{-14}$ l. in *Chlamydomonas*. The injected amount of electrolyte has no effect below $1.3 \times 10^{-13}$ mol in *Euglena* and $3 \times 10^{-14}$ mol in *Chlamydomonas*.

![Flagellar frequency during the second current injection of *Chlamydomonas*](image)

**Fig. 6.** Flagellar frequency during the second current injection of *Chlamydomonas* in DM with 1 mM EGTA, 5 mM ATP and 3 mM Mg$^{2+}$ added, as a function of injected current. The frequencies are normalized to those before the second current injection, to reduce the scatter. The line in this figure, and those in Figs. 7-9, were drawn by eye to indicate the manner in which we interpret the data points.

**Microinjection of Mg$^{2+}$.** *Euglena* cells were suspended in DM with 1 mM EGTA, 10 mM ATP and 1 $\mu$M gramicidin, but no Mg$^{2+}$, added, and impaled. All impaled *Euglena* were routinely given a 30-s injection of 0.5-$\mu$A negative current. Approximately 25% of the cells did not show flagellar activity after termination of the electrical current. Approximately one half of these immotile cells started flagellar motility after injection with $1 \times 10^{-13}$ l. of 1 mM MgSO$_4$ + 1 mM KCl. The frequency of the flagellar beat (averaged over 5 cells) was $12 \pm 6$ Hz. This restored motility was from visual observation maintained from 2–5 min, after which it decreased noticeably.

Approximately 75% of the *Euglena* exhibited flagellar activity after impalement and one cycle of negative current injection. The flagellar frequency of these cells was...
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10 ± 4 Hz which was about half of the value immediately after impalement (16 ± 4 Hz). The reduced frequency was due presumably to the removal of Mg²⁺ from the cell (Nichols & Rikmenspoel, 1976). It should be noted that the application of negative current does not result in an exit of (positive) Mg²⁺ ions from the microelectrode.

![Graph](image1)

Fig. 7. Flagellar frequency normalized to that before the injection as a function of the volume of 1.0 M KCl microinjected. A, Euglena; B, Chlamydomonas.

![Graph](image2)

Fig. 8. Flagellar frequency of A, Euglena, and B, Chlamydomonas as a function of the volume of 1.0 M KCl and 0.2 M MgSO₄ microinjected into the cell in DM containing 1 mM EGTA. For Euglena, 1 μM gramicidin and 10 mM ATP were added to the external medium. For Chlamydomonas, 5 mM ATP was added to the external medium. In this figure and in Fig. 9, ⋄ represent single cells given only one microinjected volume, resulting from a single advance of the syringe plunger; ○ represent single cells given more than one volume; × represents an open circle given the initial injected volume.

Two procedures were used to investigate the effect on microinjection of Mg²⁺ into the Euglena that exhibited the reduced activity described above. In the first procedure the injected volume was given in one motion of the micrometer. In the alternative
procedure a volume was microinjected and after approximately 20 s the cell was given an additional volume. Some cells were given up to two more additional injected volumes. By trial and error it was determined that microelectrodes filled with 0.2 M MgSO$_4$ and 1 M KCl were the most satisfactory for the microinjection experiments.

Fig. 8A shows that the microinjection of 0.2 M Mg$^{2+}$ increased the flagellar frequency in *Euglena* up to an injected volume of $10 \times 10^{-14}$ l. Greater amounts caused the frequency to be decreased after the injection. The effects of the microinjection persisted in *Euglena* for a period of 2 to 5 min, after which the cells gradually returned to the preinjection level of activity, as judged by visual observation. The cells which had ceased flagellar motion upon a total injection of $20 \times 10^{-14}$ l. did not recover activity, however.

It can be seen in Fig. 8A that the effects of multiple doses of microinjection are additive to produce the same effect as the total doses given at once.

For microinjection experiments *Chlamydomonas* were suspended in DM to which 5 mM ATP and 1 mM EGTA had been added. Upon impalement the *Chlamydomonas* flagella ceased beating approximately 45 s after the microelectrode penetrated the cell.

Almost all *Chlamydomonas* flagella could be restarted by microinjection of 0.2 M MgSO$_4$ + 1 M KCl. The flagellar motion induced by the microinjection of Mg$^{2+}$ appeared to be of the normal type as illustrated in Fig. 5C. For quantitation of the effect, the microinjection was performed with the 2 alternative procedures described above for *Euglena*. Fig. 8B shows the flagellar frequency obtained in *Chlamydomonas* as a function of the microinjected volume of 0.2 M Mg$^{2+}$. It can be seen in Fig. 8 that a frequency of approximately 18 Hz was observed after the injection of 1 to $2 \times 10^{-14}$ l. This value of the frequency is close to that of impaled *Chlamydomonas*, in the presence of 3 mM external Mg$^{2+}$, described above. The flagellar activity initiated by the microinjection of Mg$^{2+}$ was maintained for approximately 1 min (as visually observed), after which it gradually declined.

It can be seen in Fig. 8A, B that for *Chlamydomonas* as well as for *Euglena* the effects of multiple doses of injected Mg$^{2+}$ were additive, and approximately equal to those of a single injected dose. This indicates that the dose delivered from the micropipette represents a distinct event, and that effects due to a continuous flowing of solution from the micropipette after the pulsed delivery are probably small.

**Microinjection of Ca$^{2+}$.** *Euglena* in DM with 10 mM ATP, 1 mM EGTA and 1 $\mu$m gramicidin added, when impaled with a microelectrode filled with 1 M KCl and 0.2 M CaCl$_2$, ejected their flagella in less than 1 s upon impalement in over 50% of the cells observed. The cells that retained their flagella upon impalement either ejected their flagella or ceased flagellar motility with $< 1 \times 10^{-14}$ l. of 0.2 M Ca$^{2+}$ microinjected. If the Ca$^{2+}$ concentration in the microelectrodes was reduced to 0.02 M and all other conditions were unchanged, a *Euglena* retained its flagellum upon impalement of the cell. Cells were given an injection of negative direct current to decrease the flagellar frequency to $10 \pm 4$ Hz upon recovery. An impaled *Euglena* was then given an injection of 0.02 M CaCl$_2$ and 1 M KCl. Data were obtained from cells that were given one microinjection of a specific volume or from cells that were given more than one injected volume to produce an additive effect.

Fig. 9A depicts the dependence of the flagellar frequency (normalized to that prior
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to injection) on the amount of 0.02 M Ca\(^2+\) solution injected. It can be seen in Fig. 9A that an injected volume of \(> 1.3 \times 10^{-13}\) l. leads to flagellar arrest. The decreased flagellar frequency due to the injection of \(< 1.3 \times 10^{-13}\) l. of 0.02 M Ca\(^2+\), as shown in Fig. 9A lasted for 1 to 2 min. After that period the *Euglena* flagella increased in activity and in several minutes the preinjection activity, as judged from visual observation, was restored. Cells injected with \(1.3 \times 10^{-13}\) l. or more did not recover flagellar motility.

![Graph A](image1)

**Fig. 9.** Flagellar frequency of *A, Euglena*, and *B, Chlamydomonas* as a function of the volume of 1 mM KCl and 0.02 M CaCl\(_2\) microinjected into the cell in DM. One micromolar gramicidin and 10 mM ATP were added to the external medium of *Euglena*; 5 mM ATP and 3 mM MgSO\(_4\) were added to the external medium of *Chlamydomonas*.

*Chlamydomonas*, in DM with 1 mM EGTA, 5 mM ATP and 3 mM MgSO\(_4\), were impaled with a microelectrode. The addition of Mg\(^2+\) to the external medium was necessary to maintain flagellar activity after impalement. Microinjection with Ca\(^2+\) was done in *Chlamydomonas* as described above for *Euglena*.

Fig. 9B shows the decrease of the flagellar frequency in *Chlamydomonas* as a function of the injected volume of 0.02 M Ca\(^2+\) solution. The effects shown in Fig. 9B persisted for approximately 30-45 s in *Chlamydomonas*, after which the flagella returned to the (visually judged) preinjection level of activity.

It should be noted that in *Chlamydomonas*, as well as in *Euglena*, the flagella that had been completely stopped by the microinjection of Ca\(^2+\), did not resume beating. This occurred in *Chlamydomonas* with the injection of \(> 3 \times 10^{-14}\) l.

It can be seen in Fig. 9 that the effects of the microinjection of Ca\(^2+\) are quite similar in *Euglena* and *Chlamydomonas*. The volume of Ca\(^2+\) solution necessary to reduce the flagellar frequency to one half is approximately \(4 \times 10^{-14}\) l. in *Euglena* and \(8 \times 10^{-15}\) l. in *Chlamydomonas*. The ratio of these injected volumes is similar to that of the cellular volumes of the two organisms which are \(\approx 4000 \mu\text{m}^3\) and \(\approx 500 \mu\text{m}^3\), respectively. It can also be observed in Fig. 9 that the multiple injections of Ca\(^2+\) appear to elicit the same effect as the total dose given at once, but the scattering of the experimental points seems too large to allow us to conclude with certainty that the effects are additive.
DISCUSSION

The results of the microinjection experiments with Mg^{2+} show that a variation of the internal content of Mg^{2+} can control the flagellar frequency in *Euglena* and *Chlamydomonas*. An increased internal Mg^{2+} level increases the frequency up to a maximum, beyond which the frequency falls off. From a comparison of Figs. 7 and 8 it can be seen that the drop in frequency occurs where the volume and the amount of electrolyte injected do not have an appreciable effect.

The dependency of the flagellar frequency in both *Euglena* and *Chlamydomonas* on the amount of Mg^{2+} microinjected resembles quite closely the dependency of that frequency on injected direct electric current, as reported earlier (Nichols & Rikmenspoel, 1977) and in this paper. This strongly supports the notion that the electrical control of the flagellar frequency is affected through a regulation of the internal Mg^{2+} concentration in these cells. It should also be noted that the medium in which the experiments with electrical control were conducted does not contain an ion other than Mg^{2+} which could mediate the control.

A comparison can be made between the amount of Mg^{2+} necessary to mediate electrical control of a cell and the amount of Mg^{2+} required to be microinjected into a cell to cause a flagellar response. The volume of a *Euglena* cell used in our experiments is approximately 4000 μm^3 or 4 x 10^{-12} l. We have found previously (Nichols & Rikmenspoel, 1977) that the concentration change of Mg^{2+} which is induced in a *Euglena* by current injection is of the order of 1 mM. This means that the electrical control is affected through the changing of the Mg^{2+} content of the *Euglena* cells by amounts of the order of 4 x 10^{-16} mol. The microinjection of Mg^{2+} has its maximal stimulation action with the delivery of 5-10 x 10^{-14} l of 0.2 M Mg^{2+}, as shown in Fig. 8A. The amount of Mg^{2+} microinjected is then 10-20 x 10^{-15} mol.

The volume of a *Chlamydomonas* cell of 10 μm diameter is approximately 500 μm^3 or 5 x 10^{-13} l. An impaled *Chlamydomonas* cell through which negative current has been passed, requires the presence of 3 mM Mg^{2+} in the external medium to recover flagellar beating when current is discontinued. The amount of Mg^{2+} through which the electrical control is mediated in *Chlamydomonas* is thus in the order of 1.5 x 10^{-15} mol. Optimal flagellar frequencies were obtained in *Chlamydomonas* by the microinjection of 1 x 10^{-14} l of 0.2 M Mg^{2+} equivalent to 2 x 10^{-15} mol Mg^{2+}. These results indicate that the amounts of Mg^{2+} delivered by microinjection into a cell to stimulate (Euglena) or to initiate (Chlamydomonas) flagellar motion are comparable to the amounts of Mg^{2+} which mediate the electrical control of flagellar activity in both of these cells.

The application of negative direct current to impaled cells should cause positively charged ions, particularly Mg^{2+}, to move by electrostatic attraction into the cell. Negative current caused a decrease in flagellar frequency. However, the microinjection of Mg^{2+} into cells caused an increase in flagellar beating. This suggests that the effect of negative current is the opposite of that of the microinjection of Mg^{2+} and that Mg^{2+} is lost from the cell in response to the application of direct negative current. It should
be noted that if this interpretation is correct, Mg\(^{2+}\) is transported against an electrical and a chemical gradient in response to the application of direct negative current (Nichols & Rikmenspoel, 1977).

The potentials induced by the negative direct electric current have not been measured directly in *Euglena* or *Chlamydomonas*. Such a measurement would require a second impaling electrode. In our experimental set-up the 3 elements necessary for a measurement of induced potentials (one holding pipette, one current electrode and one voltage electrode) could not be accommodated. In *Euglena* we have reported previously on the basis of resistance measurements (Nichols & Rikmenspoel, 1977) that the potentials induced by the electrical current injection are probably in the physiological range. In a series of experiments on bull spermatozoa, the potentials induced by currents comparable to those used in *Euglena* and *Chlamydomonas* have been measured directly (O'Day, Rikmenspoel & Lindemann, 1976). In impaled bull spermatozoa the potentials induced by the negative direct electric current, in experimental conditions very similar to those for *Euglena* and *Chlamydomonas*, were found to be of the order of \(-10\) to \(-50\) mV (O'Day *et al.* 1976). These results indicate that in our experiments on electrical control, indeed, physiological potentials were induced.

Upon impalement in a deprived medium in the absence of ATP and Mg\(^{2+}\), flagellar motility stopped within 10–20 s in *Euglena* and within less than 1 s in *Chlamydomonas*. This indicates that the diffusion times for ATP out of the cells, through the opening around the impaling electrode are of the order of 10–20 s for *Euglena* and 1 s for *Chlamydomonas*. The microinjection effect of Mg\(^{2+}\) persists in *Euglena* for approximately 2–5 min, and in *Chlamydomonas* for 1–2 min. The characteristic diffusion times of the Mg\(^{2+}\)-ATP complex should be quite similar to those of ATP; the times for Mg\(^{2+}\) ions should be faster than those of ATP. The observation that the microinjection effects of Mg\(^{2+}\) last for periods which are one to two orders of magnitude longer than the characteristic diffusion times indicates that the Mg\(^{2+}\) which actually affects the flagellar motion is bound at a site in the cell. The notion that Mg\(^{2+}\) is bound at a site in the cell is supported by other observations. Impaled *Euglena* cells retain flagellar activity in DM with ATP and EGTA, but no external Mg\(^{2+}\), added (Nichols & Rikmenspoel, 1977). *Chlamydomonas* cells continue to exhibit flagellar activity for approximately 45 s after impalement in DM with ATP and EGTA present, but in the absence of external Mg\(^{2+}\). The existence in flagella of a Mg\(^{2+}\) site of action, different from the binding in a complex with ATP, has been proposed previously by Gibbons & Fronk (1972) from the observed non-linear kinetics of the dynein-tubulin ATPase activity as a function of Mg\(^{2+}\) concentration. Our experiments do support the view that such a second site of action for Mg\(^{2+}\) exists. The data do not indicate, however, whether that site is in the cell body or in the flagellum.

Ca\(^{2+}\) has an inhibitory action on the flagellar activity in *Euglena* and *Chlamydomonas*, as shown by the microinjection experiments. This action might well be analogous to the ciliary arrest brought about in mussel gill preparations by Ca\(^{2+}\) reported by several investigators recently (Satir, 1975; Murakami & Takahashi, 1975; Motokawa, Murakami & Takahashi, 1975). The effects of Ca\(^{2+}\) in causing a ciliary reversal in *Paramecium* (Naitoh & Eckert, 1974) and in causing a reversal in flagellar wave
propagation in *Crithidia* (Holwill & McGregor, 1975) and flagellar reversal in *Chlamydomonas* (Hyams & Borisy, 1975; Schmidt & Eckert, 1976) are probably by a different mechanism.

The microinjection experiments with Ca$^{2+}$ were done in the presence of 1 mM EGTA, a strong Ca$^{2+}$ chelator. That effects of Ca$^{2+}$ were observed, and that the effects were maintained over a period of approximately one order of magnitude longer than the diffusion times, shows that the Ca$^{2+}$ ions causing the effects were strongly bound to a site in the cells.

The Ca$^{2+}$ ion is not an inhibitor of the ‘contractile’ tubulin-dynein system in flagella as reported by Gibbons & Gibbons (1972). Partial activation of the dynein ATPase activity is possible with Ca$^{2+}$ instead of Mg$^{2+}$ (Gibbons & Gibbons, 1972; Gibbons & Fronk, 1972). These observations indicate that the probable site of the inhibitory action of Ca$^{2+}$ reported here is not the dynein ATPase of the flagella.

Our present experiments do not indicate whether Mg$^{2+}$ and Ca$^{2+}$ act on the same site or not. This information can probably be obtained only by localizing binding sites for the 2 different ions.

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REFERENCES


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