J. Cell Sci. 38, 105–123 (1979)
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INHIBITION OF MOVEMENT OF TRITON-DEMEMBRANATED SEA-URCHIN SPERM FLAGELLA BY Mg^{2+}, ATP^{4-}, ADP AND P_i

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SUMMARY

Three distinct patterns of inhibition of $MgATP^{2-}$-activated flagellar motility have been found by measuring the motility of Triton-demembranated sea-urchin spermatozoa beating with their heads attached to a glass surface.

Inhibition of beat frequency by the reaction products, ADP and P_i, is competitive with the normal substrate, $MgATP^{2-}$, and the inhibitory effects are similar to a reduction in $MgATP^{2-}$ concentration.

Inhibition of beat frequency by ATP^{4-} is competitive with $MgATP^{2-}$, but is accompanied by an inhibition of bending, as measured by the angle between the straight regions on either side of a bend, which is not seen when $MgATP^{2-}$ concentration is reduced.

Inhibition of beat frequency by Mg^{2+} is not competitive with $MgATP^{2-}$, and is accompanied by an increase in bend angle, so that there is no change in the rate of sliding between flagellar tubules.

These differences suggest unexpected complexity of dynein ATPase action in flagella.

The beat frequencies of both swimming and attached spermatozoa show a linear double reciprocal dependence on $MgATP^{2-}$ concentration, with identical slopes. The calculated sliding velocities between tubules also give linear relationships, but the slopes are different, suggesting that beat frequency may be the more fundamental dependent variable in this system.

INTRODUCTION

A strong dependence of beat frequency on ATP concentration is one of the most dramatic, and easily measured, properties of the ATP-reactivated oscillation of demembranated flagella (Hoffmann-Berling, 1955; Kinoshita, 1958). Simple saturation, or Michaelis–Menten, kinetics have been demonstrated for this relationship (Brokaw, 1967, 1975*a*), suggesting a direct control of frequency by $MgATP^{2-}$ concentration. However, current ideas about the generation of flagellar oscillation by an active sliding process similar to the actin-myosin interaction in muscle suggest that sliding velocity, which is proportional to the product of the frequency and amplitude of oscillation, should be a primary dependent variable.

We began these experiments on flagella with the aims of trying to determine whether frequency or sliding velocity was the more fundamental dependent variable determined by the ATP concentration, and whether the effects of other components of the ATP dephosphorylation system – Mg^{2+}, ATP^{4-}, ADP, and P_i – were similar to the effects observed with the actin-myosin system. We have been only partially

successful, but our experiments have provided some more detailed characterization of the dynein ATPase system which generates flagellar movement, which may add to the useful information available for deducing mechanisms of bending, oscillation, and bend propagation by flagella.

MATERIALS AND METHODS

Spermatozoa were obtained from the sea urchin, *Lytechinus pictus*, as described previously (Brokaw, 1975*a*). In some experiments, involving low concentrations of magnesium in the reactivation solutions, the spermatozoa were washed twice with cold 0·5 M NaCl. The demembranation and reactivation of the spermatozoa were carried out according to the method of Gibbons & Gibbons (1972) with a little modification. The high-cation extraction solution (HES) for demembranating the spermatozoa was the same as that used previously (Brokaw, 1975*a*) which contained 0·15 M KCl, 2 mM dithiothreitol (DTT), 2 mM Tris buffer, 2 mM $MgCl_2$, 5 mM $CaCl_2$, 0·5 mM EDTA and 0·04 % (vol/vol) Triton X-100, adjusted to pH 8·2. When the reactivation was carried out in low Mg^{2+} reactivation solution, the demembranated spermatozoa were prepared with a low-cation extraction solution (LES), which had no $MgCl_2$ or EDTA, and only 0·1 mM $CaCl_2$. We found that the requirement for $CaCl_2$ in the extraction solution in order to obtain symmetrical bending waves of reactivated flagella (Brokaw, Josslin & Bobrow, 1974) was greatly reduced when $MgCl_2$ was omitted from the extraction solution. The base of the reactivation solution (RBASE) always contained 20 mM Tris buffer, 2 % (wt/vol) polyethyleneglycol, 2 mM DTT, and KCl at 0·20 M or a lesser amount as required to maintain constant ionic strength when other components were added. Other components included ATP, $MgCl_2$, EGTA, and for some experiments, ADP and KH_2PO_4. $Na_2ATP.\ 3H_2O$ was obtained from Boehringer-Mannheim Corp. and assumed to have a formula weight of 605·2; $Na_2ADP.\ 2H_2O$ was obtained from P-L Biochemicals and assumed to have a formula weight of 507·2. We used $MgCl_2$ instead of $MgSO_4$ used in the previous experiments (Gibbons & Gibbons, 1972; Brokaw, 1975*a*) to avoid the inhibitory effect of SO_4^{2-} ion.

The spermatozoa were demembranated and reactivated using the extraction and reactivation solutions as follows. A 5-μl portion of sperm suspension was mixed with 0·2 ml of extraction solution and gently shaken for 30 s at room temperature. Then 2 μl of extracted sperm suspension were put into 0·5 ml of reactivation solution. A drop of reactivated sperm suspension was placed in a well slide (Brokaw, 1977), covered with a coverslip and examined with dark-field illumination using a ×40 oil-immersion objective.

All of the experiments were carried out at a room temperature of 18 ± 1 °C and the microscope stage was kept at 18 ± 0·5 °C by circulating water.

Most of these measurements were made on reactivated spermatozoa which were firmly attached to the glass slide surface by their heads, with the flagellum beating freely in the solution with a beat plane approximately parallel to the slide surface. These are referred to as attached spermatozoa. Swimming spermatozoa were observed to determine the differences between swimming and attached spermatozoa. They were swimming under the coverslip very close to it and the bending planes were usually parallel to the glass surface.

For experiments involving only beat frequency measurements, the frequencies were counted stroboscopically on a sample of at least 15 spermatozoa under each condition studied. For experiments in which bend angle and wavelength were also measured, multiple-flash photographs were taken with the flash frequency adjusted to 4 times the flagellar beat frequency, as described previously (Brokaw, 1977). For these experiments, the smallest sample size was 7 spermatozoa under each condition. During a series of experiments with a particular sperm sample, there was usually a gradual change in the beat frequency measured under standard conditions, and the measurements were adjusted to compensate for this change. Each experiment was repeated 2–4 times, and the results presented represent the mean values for the mean and standard deviation obtained in each experiment. The method of least squares was used to fit straight lines to the data points.

Measurements on the films were made using a microfilm viewer, as described previously (Brokaw, 1977). Bend angles were measured between the straight regions on either side of a

bend, assuming that these straight regions formed tangents to the circular arcs of adjacent bends. Bends near the middle of the flagellum were measured independently for principal and reverse bends (Gibbons & Gibbons, 1972), and then averaged. Wavelengths were measured by overlaying the image with a flexible polyethylene tubing. Average sliding velocities were approximated as twice the product of beat frequency and bend angle for individual spermatozoa.

RESULTS

Effects of Mg^{2+}

Results of measurement of the beat frequencies of attached spermatozoa demembranated with HES in reactivation solutions containing a fixed ATP concentration (0·1 or 0·2 mM) and varying concentrations of $MgCl_2$ are shown in Fig. 1. As previously noted (Gibbons & Gibbons, 1972), there is an optimum $MgCl_2$ concentration. At low $MgCl_2$ concentrations, increasing $MgCl_2$ causes an increase in the concentration of $MgATP^{2-}$, which is considered to be the effective substrate for the motility of

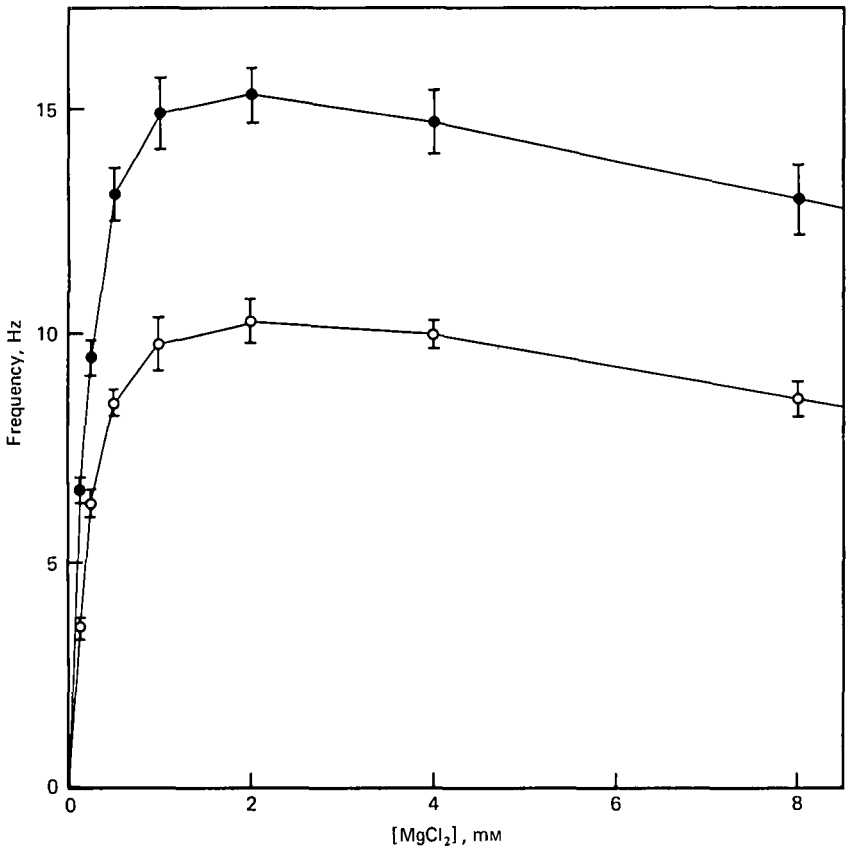


Fig. 1. Effect of $MgCl_2$ concentration on flagellar beat frequency of attached spermatozoa. Results obtained with 0·1 mM ATP in the reactivation solution are indicated by open circles (○); results obtained with 0·2 mM ATP are indicated by solid circles (●). Extraction solution: HES. Reactivation solution: RBASE plus 1·8 mM EGTA. Data were obtained from 2 experiments with different sperm samples.

these flagella (Gibbons & Gibbons, 1972; Brokaw, 1975*a*). The reactivation solution in these experiments contained 1·8 mM EGTA, so that free Mg^{2+} ion concentration increases almost linearly with $MgCl_2$ concentration in the range above 2 mM $MgCl_2$, with very little increase in $MgATP^{2-}$ concentration. These experiments showing a frequency decrease with increasing $MgCl_2$ concentration above 2 mM therefore provide a direct indication of an inhibitory effect of Mg^{2+} on flagellar beat frequency.

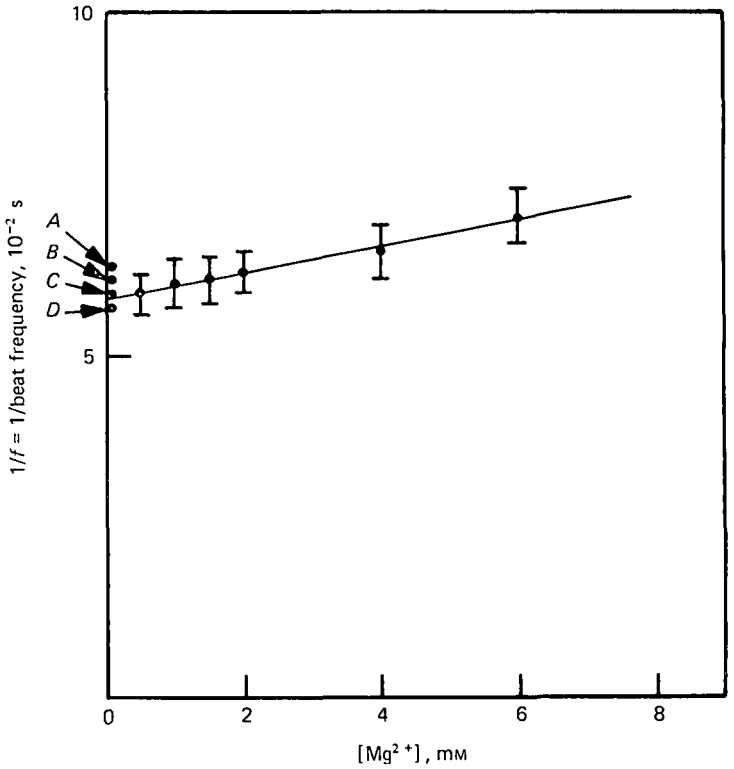


Fig. 2. Effect of Mg^{2+} concentration on beat frequency of attached spermatozoa extracted with LES and reactivated with RBASE plus 0·1 mM EGTA. $MgCl_2$ and ATP concentrations were calculated to maintain a constant $MgATP^{2-}$ concentration of 0·2 mM. At the lowest Mg^{2+} concentration, the points labelled *A*, *B*, *C*, and *D* indicate, respectively, the beat frequencies obtained using MgATP association constants of 1·4, 1·2, 1·0, and $0{\cdot}8 \times 10^4$ M^{-1}, to calculate the $MgCl_2$ and ATP concentrations. Data from 3 experiments.

When the $MgATP^{2-}$ concentration is maintained constant, by calculations using a $MgATP^{2-}$ association constant of $1{\cdot}0 \times 10^4$ M^{-1} (Sillen & Martell, 1964), the relationship between beat frequency and Mg^{2+} concentration is shown in Fig. 2. For these experiments, the spermatozoa were demembranated with LES and reactivated with RBASE containing 0·1 mM EGTA so that the Mg^{2+} concentration is more directly dependent on the added $MgCl_2$. ATP and $MgCl_2$ were added to maintain a $MgATP^{2-}$ concentration of 0·2 mM. The inhibitory effect of Mg^{2+} can be represented by a linear relationship between the reciprocal of beat frequency ($1/f$) and Mg^{2+}

concentration. At higher Mg^{2+} concentrations, the bending waves tended to become asymmetrical and erratic, and the flagella easily attached to the slide surface. It was difficult to find normally beating flagella at 10 mM Mg^{2+} or above.

At Mg^{2+} concentrations of 1 mM or greater, most of the added ATP is in the form of $MgATP^{2-}$, and calculations of the $MgATP^{2-}$ concentration for the experiment shown in Fig. 2 are relatively insensitive to the values used for the $MgATP^{2-}$ and MgEGTA association constants. At Mg^{2+} concentrations below 1 mM, the value of the $MgATP^{2-}$ association constant becomes a major factor, while in these experiments, using only 0·1 mM EGTA, the MgEGTA association constant is relatively uninfluential. The 4 points at the left of Fig. 2 represent the mean beat frequencies measured in reactivation solutions containing 0·4 mM total ATP (and approximately 0·2 mM Mg^{2+}) using appropriate amounts of $MgCl_2$ to give 0·20 mM $MgATP^{2-}$, calculated with $MgATP^{2-}$ association constants of 1·4, 1·2, 1·0 and $0{\cdot}8 \times 10^4\ M^{-1}$. Since a continuation of a linear relationship between $1/f$ and Mg^{2+} concentration is obtained by using the accepted value of $1{\cdot}0 \times 10^4\ M^{-1}$, this value appears to be satisfactory for the conditions of these experiments.

Most of our other experiments used reactivation solutions containing 1·8 mM EGTA, and a calculation of Mg binding by EGTA was necessary to obtain an accurate Mg^{2+} concentration. Using association constants for EGTA given by Sillen & Martell (1964), the apparent association constant for MgEGTA at pH 8·2 is $2{\cdot}0 \times 10^3\ M^{-1}$. (In this case, MgEGTA represents $MgEGTA^{2-}$ and $MgHEGTA^{-}$, and free EGTA includes $EGTA^{4-}$, $HEGTA^{3-}$ and H_2EGTA^{2-}.) However, preliminary experiments of the type shown in Fig. 2 at fixed EGTA concentrations indicated the value of $1{\cdot}6 \times 10^3\ M^{-1}$ was more appropriate for the conditions of our experiments. The suitability of this value was also examined in the experiment shown in Fig. 3, where spermatozoa were demembranated with LES and reactivated with RBASE plus 0·4 mM ATP. The EGTA concentration was varied from 0·1 to 10 mM, with $MgCl_2$ added to maintain 0·10 mM Mg^{2+} and 0·20 mM $MgATP^{2-}$. When the amount of $MgCl_2$ added was calculated using a MgEGTA association constant of $1{\cdot}6 \times 10^3\ M^{-1}$, the beat frequency was independent of EGTA concentration, as shown in Fig. 3. Interpretation of this result assumes that none of the EGTA species themselves has a direct effect on flagellar beat frequency.

The effect of Mg^{2+} on flagellar beat frequency was examined at various fixed concentrations of $MgATP^{2-}$. Fig. 4A shows that the degree of inhibition by Mg^{2+}, defined by the slope of $1/f$ vs. Mg^{2+} concentration, was independent of $MgATP^{2-}$ concentration. The intercept of each of these straight lines on the ordinate gives an 'ideal' frequency free from effects of Mg^{2+} and also free from effects of ATP^{4-}. The line (*1*) in Fig. 4B was derived from these ideal values, and the line (*2*) represents the results obtained with 5·0 mM Mg^{2+}. The results in Fig. 4 indicate that Mg^{2+} is an uncompetitive inhibitor of beat frequency, with an apparent K_I of about 21 mM under the conditions of this experiment.

The inhibition of beat frequency by Mg^{2+} is accompanied by an increase in bend angle. Figs. 7–10 (pp. 114–117) show the results obtained from experiments in which attached spermatozoa were photographed to obtain measurements of wave parameters

in addition to beat frequency. Spermatozoa were demembranated with LES and reactivated with reactivation solutions containing RBASE and 1·8 mM EGTA, with the $MgATP^{2-}$ concentration maintained at 0·20 mM. Beat frequencies, shown in Fig. 7A, decreased with increasing Mg^{2+} concentration, as already described. As

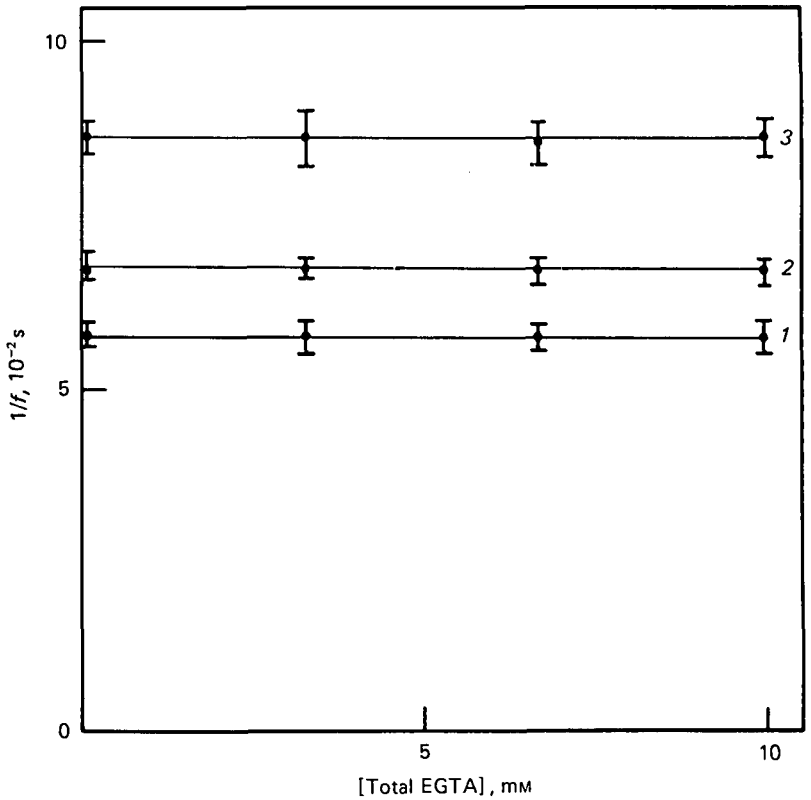


Fig. 3. Verification of Mg-association constants, using RBASE plus 0·4 mM ATP. At each EGTA concentration, the calculated amount of $MgCl_2$ was added to give 0·1 mM Mg^{2+} and 0·2 mM $MgATP^{2-}$. Line *1* shows beat frequencies obtained at each EGTA concentration, using a MgEGTA association constant of $1{\cdot}6 \times 10^3$ M^{-1}. Line *2* shows beat frequencies obtained when the reactivation solution also contained 30 mM P_i, using an association constant for $MgHPO_4$ of 80 M^{-1}. Line *3* shows beat frequencies obtained when the reactivation solution contained 1 mM ADP, using a $MgADP^{-1}$ association constant of $1{\cdot}0 \times 10^3$ M^{-1}. Extraction solution: HES. Combined data from 3 experiments.

shown in Fig. 8A, the bend angle increased linearly with Mg^{2+} concentration from 0·1 up to 6·0 mM, but showed no further increase at 8·0 mM Mg^{2+}. An approximate measure of the average rate of sliding between flagellar microtubules during the propagation of a bend is twice the product of frequency and bend angle. Since this is also a rate measurement, its reciprocal was plotted as a function of Mg^{2+} concentration in Fig. 9A. In the range of 0·1–6 mM Mg^{2+}, there is no change in sliding velocity with Mg^{2+} concentration. The results in Fig. 10A show a very small increase in wavelength of the flagellar bending waves with Mg^{2+} concentration.

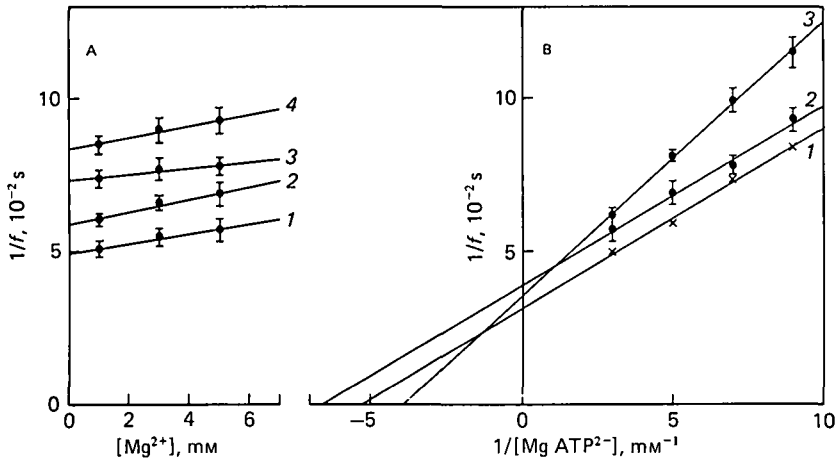


Fig. 4. Inhibition of beat frequency of attached spermatozoa by Mg^{2+} and by ATP^{4-}. Extraction solution: LES. Reactivation solution: RBASE plus 1·8 mM EGTA. Data from 4 experiments. A, reciprocal plots of frequency vs. Mg^{2+} concentration. Lines labelled *1*, *2*, *3* and *4* correspond to $MgATP^{2-}$ concentrations of 1/3, 1/5, 1/7, and 1/9 mM, respectively. B, double reciprocal plots of frequency vs. $MgATP^{-2}$ concentration. Line *1* is a plot of frequencies extrapolated to o Mg^{2+} concentration, from A. Line *2* is a plot of the frequencies obtained at 5 mM Mg^{2+}, in A. Line *3* shows results obtained in the presence of 5 mM ATP^{4-}.

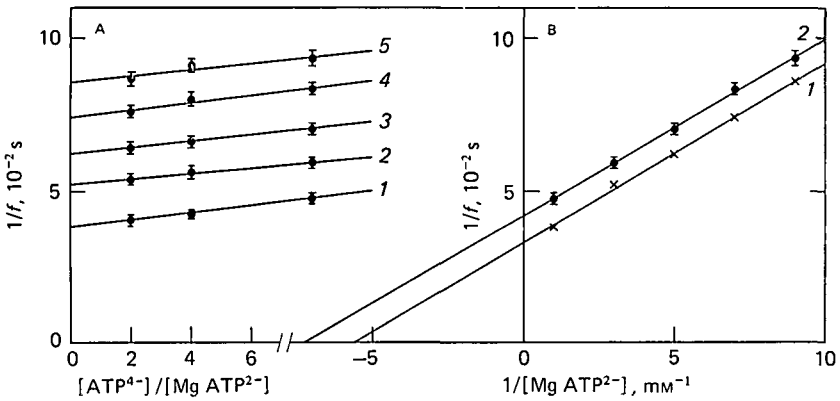


Fig. 5. Inhibition of beat frequency of attached spermatozoa by ATP^{4-}. Conditions as in Fig. 4. A, reciprocal plots of frequency vs. ratio of ATP^{4-} : $MgATP^{2-}$. Lines labelled *1–5* correspond to $MgATP^{2-}$ concentrations of 1, 1/3, 1/5, 1/7 and 1/9 mM respectively. B, double reciprocal plots of frequency vs. $MgATP^{2-}$ concentration at constant ATP^{4-} : $MgATP^{2-}$ ratios. Line *1* is a plot of frequencies extrapolated to o ATP^{4-} concentration from A. Line *2* is a plot of frequencies obtained at an ATP^{4-} : $MgATP^{2-}$ ratio of 8.

Effects of ATP^{4-}

ATP^{4-} has inhibitory effects on both beat frequency and bend angle, as shown by Figs. 7B and 8B. These 2 effects together give a relatively large inhibition of sliding velocity, as shown by Fig. 9B. There is a small increase in wavelength with ATP^{4-} concentration, as shown by Fig. 10B. When the ATP^{4-} concentration was greater than 10 mM, the bending waves were irregular and more asymmetrical, and showed a noticeable decrease in amplitude as the bends propagated along the length of the flagellum.

At a constant ATP^{4-} concentration of 5 mM, the variation in beat frequency with $MgATP^{2-}$ concentration is approximately that expected if ATP^{4-} is acting as a competitive inhibitor (Fig. 4B, curve 3). A more extensive test is shown by the results in Fig. 5. In these experiments, the $MgATP^{2-}$ concentration was varied, at several fixed values of the ratio of ATP^{4-} and $MgATP^{2-}$ concentrations. The nearly parallel lines obtained in these experiments indicate that ATP^{4-} is acting as a competitive inhibitor, with a K_I of approximately 6 mM.

Since extraction of dynein from cilia by solutions containing high ATP concentrations (20 mM) has been reported by Raff & Blum (1969), the reversibility of the inhibitory effect of ATP^{4-} on beat frequency was examined. Spermatozoa were exposed to reactivation solution containing 8 mM ATP^{4-}, and then diluted to an ATP^{4-} concentration of 0·2 mM for measurement of beat frequencies. With this preparation, the beat frequency obtained at a $MgATP^{2-}$ concentration of 0·2 mM was $16{\cdot}4 \pm 1{\cdot}4$ Hz without exposure to 8 mM ATP^{4-}, and $16{\cdot}2 \pm 1{\cdot}3$, or $16{\cdot}0 \pm 1{\cdot}4$ Hz after exposure to 8 mM ATP^{4-} for 5 or 7 min, respectively. In the presence of 8 mM ATP^{4-}, the measured beat frequency was $9{\cdot}3 \pm 0{\cdot}8$ Hz. We conclude that there are no irreversible effects sufficient to interfere with our interpretation of the inhibitory effects of ATP^{4-}.

Effects of ADP and P_i

ADP and inorganic phosphate, P_i, which is primarily in the form of HPO_4^{2-} at pH 8·2, are the products of the dephosphorylation of ATP which supplies energy for flagellar movement. Before examining their effects on flagellar movement, we carried out tests of the Mg association constants, using the procedure shown in Fig. 3. The experiment described previously for evaluating the MgEGTA association constant was repeated in the presence of 1 mM ADP and an amount of additional $MgCl_2$ (0·091 mM) calculated to be bound to ADP in the presence of 0·10 mM Mg^{2+} if the association constant is $1{\cdot}0 \times 10^3$ M^{-1} (Smith & Alberty, 1956). If this calculation is incorrect, the presence of ADP will significantly change the $MgATP^{2-}$ concentration at the lowest EGTA concentration, where the total Mg concentration is low. Since our results showed the frequency to remain independent of EGTA concentration in the presence of 1 mM ADP, this association constant appears to be satisfactory for the conditions of these experiments. Similarly, in the presence of 30 mM P_i, a $MgHPO_4$ association constant of 80 M^{-1} appeared to be satisfactory, which is close to the value of 76 M^{-1} given by Smith & Alberty (1956).

The effects of ADP and P_i on the relationship between $1/f$ and $MgATP^{2-}$ concentration are shown in Fig. 6, using HES-demembranated spermatozoa and reactivation solutions containing RBASE plus 1·8 mM EGTA, 0·5 mM $CaCl_2$ and 1·0 mM Mg^{2+}. The line for ADP represents reactivation solutions containing 1·0 mM total ADP (0·5 mM $MgADP^-$ and 0·5 mM ADP^{3-}); the line for P_i represents reactivation

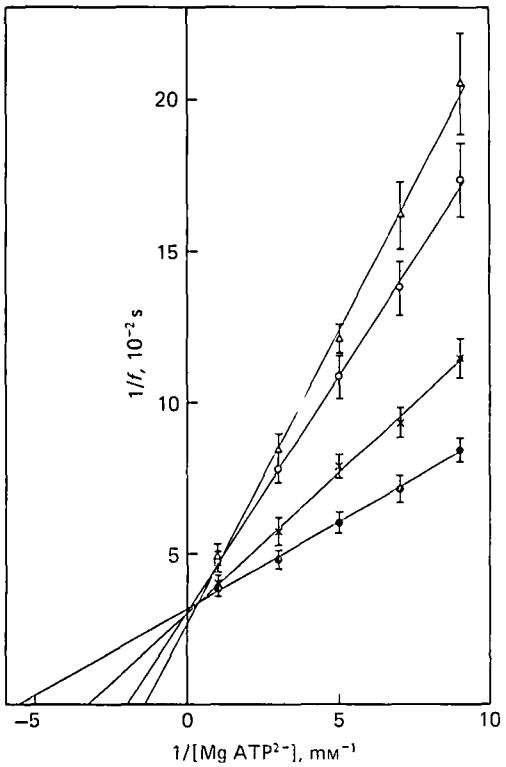
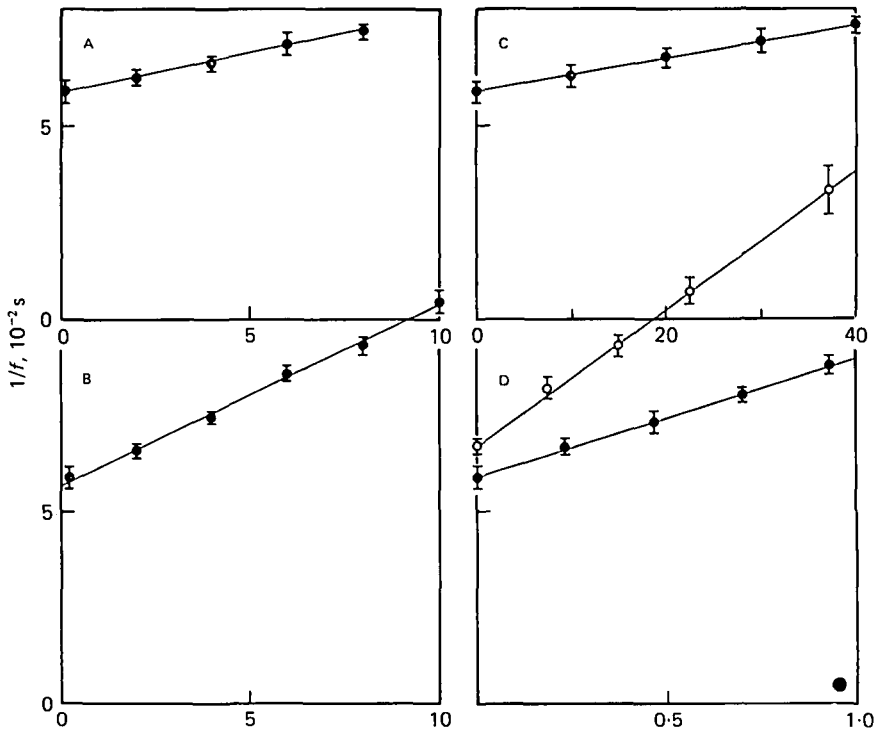


Fig. 6. Inhibition of flagellar beat frequency by ADP and P_i. Extraction solution: HES. Reactivation solution: RBASE plus 1·8 mM EGTA, 0·5 mM $CaCl_2$ with Mg^{2+} maintained at 1·0 mM. Results obtained without ADP or P_i are indicated by (●); with 30 mM P_i by (×); with 1 mM ADP by (○); and with 30 mM P_i plus 1 mM ADP by (△). Data from 3 experiments.

solutions containing 30 mM free phosphate (29 mM HPO_4^{2-} and 1 mM $H_2PO_4^-$) and 2·5 mM $MgHPO_4$. Both reaction products have inhibitory effects, but the P_i effect is very weak. In the presence of both ADP and P_i, the inhibitory effects appear to be simply additive.

Fig. 7C shows the effect of P_i concentration on beat frequency with 0·2 mM $MgATP^{2-}$ and 0·10 mM Mg^{2+}. The linear plots in Figs. 6 and 7C suggest that P_i is a competitive inhibitor of flagellar beat frequency, but no distinction between the effects of $MgHPO_4$, HPO_4^{2-} or $H_2PO_4^-$ is possible since HPO_4^{2-} was the predominant species in both of these experiments. The apparent dissociation constant, K_i, is about 60 mM for P_i. Effects of P_i on bend angle and wavelength, shown in Figs. 8C and 10C, are extremely small and may not be significant.

Fig. 7D shows the effect of ADP concentration on beat frequency with 0·20 mM $MgATP^{2-}$, at 2 different Mg^{2+} concentrations, 4 and 0·1 mM. With 1 mM ADP, the $MgADP^{-}$ concentrations should be 0·80 and 0·09 mM at these 2 Mg^{2+} concentrations, respectively. Since the inhibitory effect observed at the low Mg^{2+} concentration is more than 0·09/0·80 times that observed at the high Mg^{2+} concentration, ADP^{3-}



Figs. 7–10. Data from photographs of attached spermatozoa, showing the effects of inhibitors at a constant $MgATP^{2-}$ concentration of 0·20 mM. Extraction solution: LES. Reactivation solution: RBASE plus 1·8 mM EGTA. Combined data from 4 experiments. In each figure, the effect of Mg^{2+} is shown in A, the effect of ATP^{4-} is shown in B, the effect of P_i is shown in C, and the effect of ADP is shown in D, with concentrations, in mM ,as indicated for each figure. In C, the concentration of Mg^{2+} was maintained at 0·1 mM. In D, the concentration of Mg^{2+} was maintained at 0·1 mM for the experiments shown by (●), and 9 % of the added ADP should be associated to give $MgADP^{-}$; for the experiments shown by (○), the Mg^{2+} concentration was maintained at 4 mM and 80 % of the added ADP should be associated to give $MgADP^{-}$.

Fig. 7. Beat frequency effects.

must be having an inhibitory effect. The results in Figs. 6 and 7D indicate that both $MgADP^{-}$ and ADP^{3-} are competitive inhibitors of flagellar beat frequency, with K_I values of about 0·4 and 1·3 mM, respectively, under the conditions of these experiments. At the low Mg^{2+} concentration, where ADP^{3-} is responsible for most of the inhibitory effect on beat frequency, there is no significant effect of ADP on bend angle and wavelength, as shown in Figs. 8D and 10D. At the high Mg^{2+} concen-

trations, where $MgADP^-$ is responsible for most of the inhibitory effect on beat frequency, there is a definite increase in wavelength with ADP concentration, and a very slight increase in bend angle.

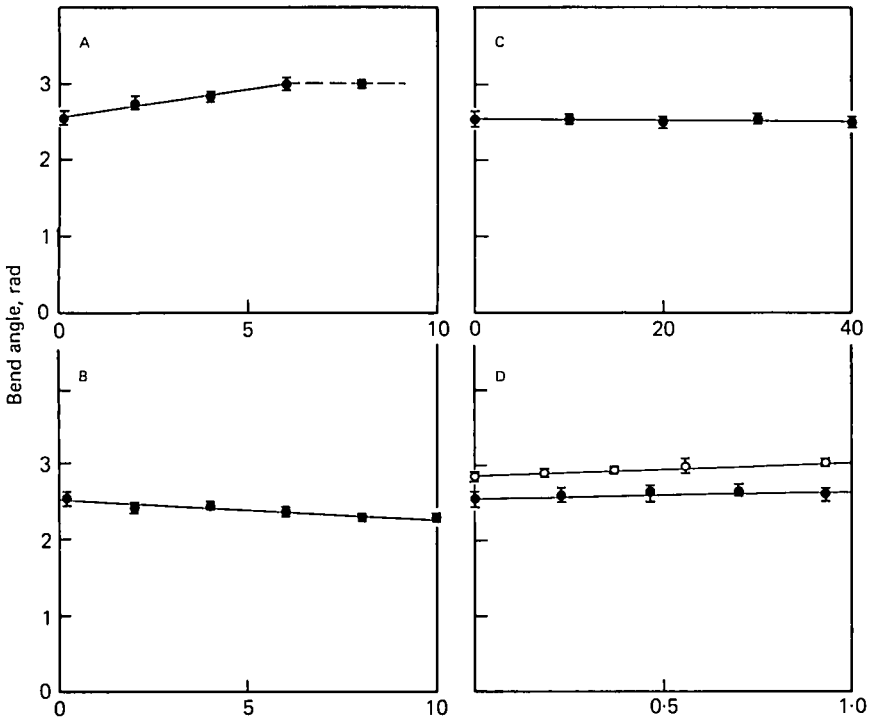


Fig. 8. Bend angle effects.

In experiments with ADP, ATP generated by the adenylate kinase activity of flagella (Brokaw, 1961; Brokaw & Gibbons, 1973) might introduce errors into interpretation of the effects of ADP. However, under the conditions of our experiments, no movement was observed in reactivation solution containing 1 mM ADP and no ATP, so inhibition of adenylate kinase activity with diadenosine pentaphosphate (Lienhard & Secemski, 1973) was considered to be unnecessary.

Comparison of effects of $MgATP^{2-}$ on swimming and attached spermatozoa

A linear double-reciprocal relationship between beat frequency and $MgATP^{2-}$ concentration was reported previously for freely swimming *Lytechinus* spermatozoa under conditions similar to the present experiments (Brokaw, 1975*a*). Attachment of the sperm head to the microscope slide changes the frequency and waveform of the movement (Brokaw, 1965, 1975*a*). When freely swimming, demembranated spermatozoa propagate bending waves which appear to retain a constant circular curvature in the bent regions. Upon attachment to a slide surface, or other restraint, the frequency decreases, and the waveform appears compressed, with a larger bend angle

and a tendency for the wavelength to increase and the bend angle to decrease in the distal region, as the bends propagate along the length of the flagellum.

A more detailed comparison was made using spermatozoa demembranated with HES and reactivated in reactivation solution containing RBASE, 1·8 mM EGTA, 0·5 mM $CaCl_2$, and 1·0 mM Mg^{2+}, with varying $MgATP^{2-}$ concentration. Results of measurements of beat frequencies are shown in Fig. 11. The double-reciprocal plots for swimming and for attached spermatozoa form 2 straight lines which are nearly parallel, with the swimming spermatozoa having a consistently higher beat frequency.

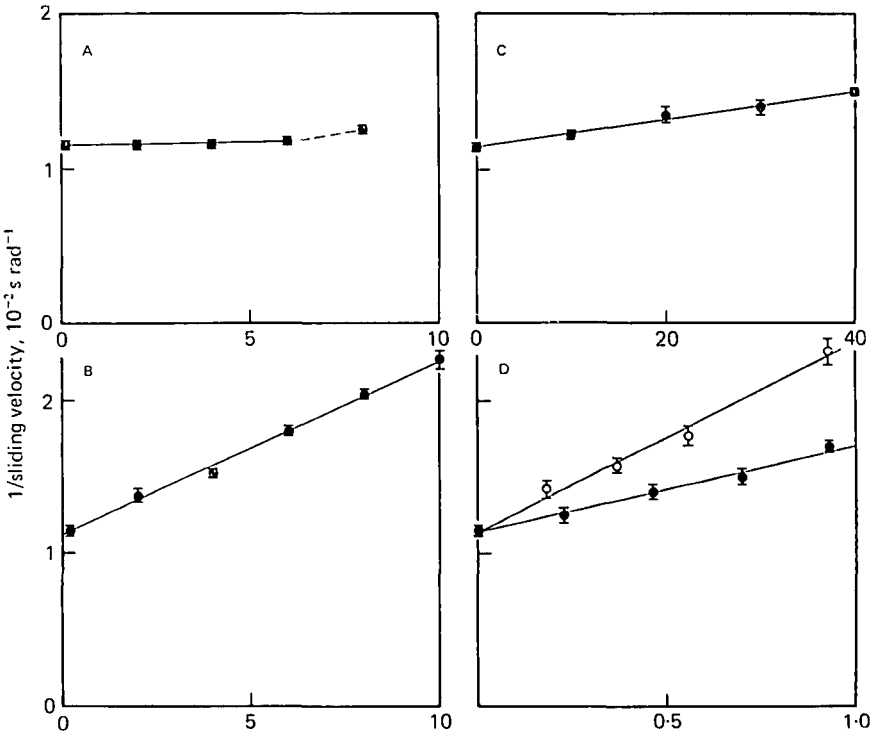


Fig. 9. Sliding velocity effects.

Bend angle and wavelength measurements are shown in Fig. 12. The attached spermatozoa show only a slight change in bend angle in the range of 0·1–1·0 mM $MgATP^{2-}$, but the bend angle of swimming spermatozoa decreases from about 2·3 to about 2·0 radians over this range. The decrease in beat frequency caused by attachment is less than the increase in bend angle caused by attachment, so that the sliding velocities estimated for attached spermatozoa are greater than for swimming spermatozoa. As shown in Fig. 13, the double reciprocal plots of sliding velocity and $MgATP^{2-}$ concentration for swimming and attached spermatozoa are not parallel, because of the different effects of $MgATP^{2-}$ concentration on bend angle.

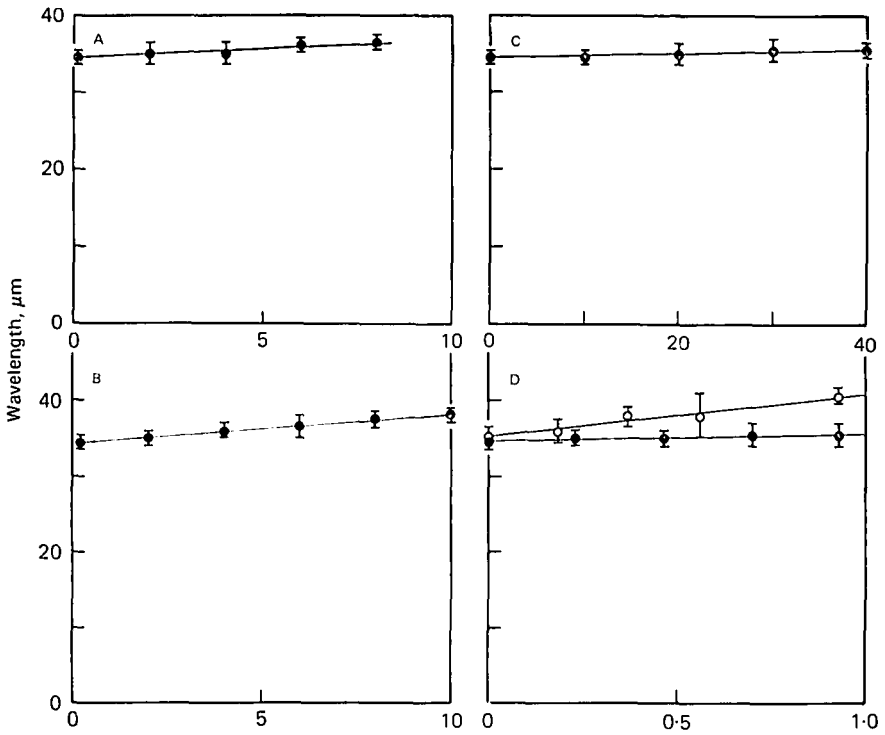


Fig. 10. Wavelength effects.

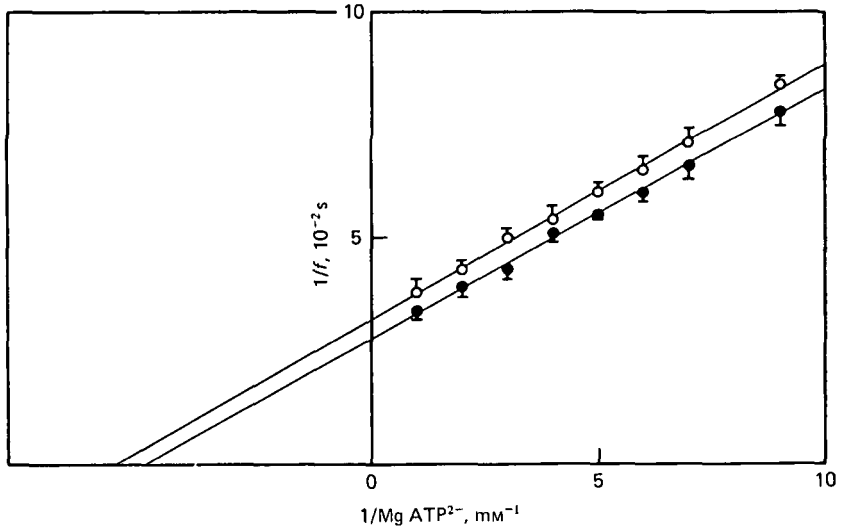


Fig. 11. Effect of $MgATP^{2-}$ concentration on beat frequency of swimming (●) and attached spermatozoa (○). Extraction solution: HES. Reactivation solution: RBASE plus 1·8 mM EGTA, 0·5 mM $CaCl_2$, with Mg^{2+} maintained at 1·0 mM. Data from 4 experiments.

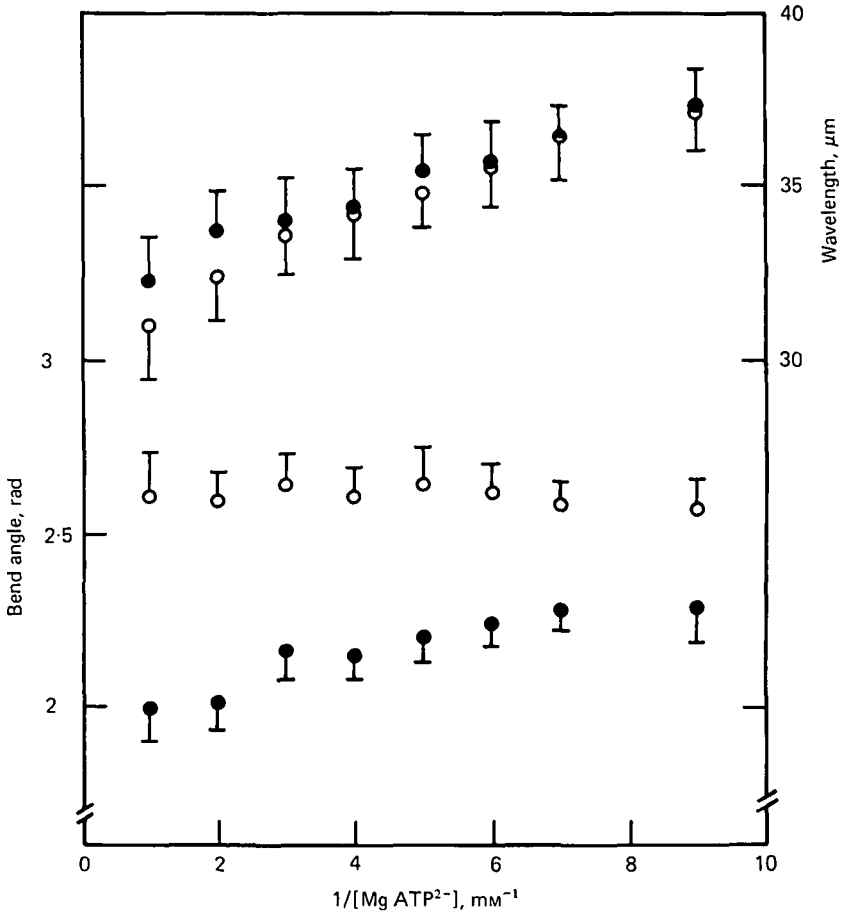


Fig. 12. Data from the same experiments as in Fig. 11, showing the effects of $MgATP^{2-}$ concentration on the bend angle and wavelength of swimming (●) and attached spermatozoa (○).

DISCUSSION

Effects on frequency and sliding velocity

The results of a previous study of the effects of viscosity and ATP concentration on the movement of freely-swiming, reactivated spermatozoa of *Lytechinus* were summarized by

$$1/f = c_1\eta^{\gamma} + c_2/C, \tag{1}$$

where f is the beat frequency, η is the viscosity, C is the ATP (or $MgATP^{2-}$) concentration, and c_1, c_2 and γ are constants (Brokaw, 1975*a*). For analysis of the present results, which were all obtained at the same viscosity, it is convenient to use

$$1/f = c_0 + c_2/C. \tag{2}$$

We have now shown that this expression also describes the relationship between beat frequency and $MgATP^{2-}$ concentration for attached spermatozoa, with the same

value of c_2 for both swimming and attached spermatozoa, but different values for c_0 (Fig. 11).

In earlier kinetic studies such as those of Holwill (1969), it was assumed that flagellar beat frequency is directly related to a rate constant involved in the turnover of ATP, leading to a direct relationship between frequency and $MgATP^{2-}$ concentration. However, the recent accumulation of evidence for a sliding-microtubule mechanism for the generation of flagellar motility has led to speculative comparisons

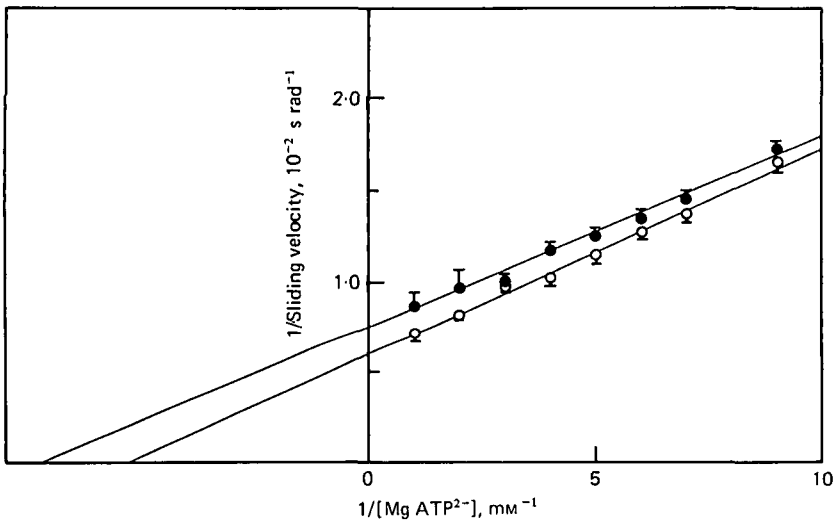


Fig. 13. Data from the experiments of Figs. 11 and 12, showing the effect of $MgATP^{2-}$ concentration on sliding velocity for swimming (●) and attached spermatozoa (○).

(Brokaw, 1975*b*) with the cross-bridge mechanisms proposed for generation of force and movement between actin and myosin filaments in striated muscle. The existence of stable 'rigor' states (in which the flagellar microtubules are immobilized by stable cross-bridges) in flagella (Gibbons & Gibbons, 1974) as well as in muscle in the absence of ATP, suggests that $MgATP^{2-}$ may be required for a cross-bridge detachment step in the cyclic operation of cross-bridges. This $MgATP^{2-}$-dependent detachment step may then be the rate-limiting step controlling the rate of sliding between flagellar tubules.

These ideas suggest that the sliding velocity, rather than the beat frequency, should be directly related to $MgATP^{2-}$ concentration. A detailed examination of the shear oscillation of cross-bridge models containing a $MgATP^{2-}$-dependent rate function (Brokaw & Rintala, 1977) demonstrated that results consistent with equation (1) could be obtained only if the elastic resistance was adjusted to maintain a constant amplitude, so that frequency was proportional to sliding velocity.

In principle, if flagellar bend angle changes with $MgATP^{2-}$ concentration it is unlikely that both beat frequency and sliding velocity will obey an equation such as equation (1). We had hoped to make this distinction in the present experiments, but

the amount of change in bend angle with $MgATP^{2-}$ concentration (Fig. 12) is insufficient, so that essentially linear double-reciprocal plots are obtained when either frequency or sliding velocity are plotted against $MgATP^{2-}$ concentration (Figs. 11, 13). If there were sufficiently strong grounds to argue that the different mechanical and hydrodynamic situations represented by attached spermatozoa and by swimming spermatozoa should, like changes in viscosity, only influence c_0, and not c_2, these results would support a more direct relationship between $MgATP^{2-}$ concentration and beat frequency than between $MgATP^{2-}$ concentration and sliding velocity. We do not find this argument compelling.

Inorganic phosphate, P_i, and ADP (probably both ADP^{3-} and $MgADP^-$) act as simple competitive inhibitors of flagellar beat frequency and sliding velocity. Their effects on bend angle are similar to the effects of reduction in $MgATP^{2-}$ concentration: either no effect or a slight increase in bend angle. Their effects on beat frequency can therefore be incorporated into equations (1) or (2) by replacing c_2 with $c_2(1+K_1[ADP]+K_2[P_i])$. Kinetic analysis of actomyosin ATP dephosphorylation indicates that P_i is released first, and that under normal conditions ADP release effectively involves an exchange of ADP for ATP at the active site (Bagshaw & Trentham, 1974; Koretz & Taylor, 1975). If the actomyosin scheme is applicable to flagella, and non-competitive inhibition by P_i is undetectable, the expected form for c_2 in equations (1) or (2) is $c_2(1+K_1(1+K_3[P_i])[ADP])$. There are 2 possible explanations for this discrepancy: product release by the flagellar ATPase may be random rather than sequential as in actomyosin; or K_3 may be small, and K_2 may result from a competitive binding of P_i at the $MgATP^{2-}$ binding site independently of P_i release (Hsu, Cleland & Anderson, 1966). Our results do not distinguish between these 2 possibilities. Evidence for the second type of P_i effect has been found for the myosin subfragment 1 ATPase (Bagshaw & Trentham, 1974).

A multiplicative interaction between the inhibitory effects of P_i and ADP would also be expected if these inhibitory effects result from a limitation on the free energy from ATP hydrolysis; our results provide no indication of such a limitation.

A much larger inhibition (about 80% inhibition by 5 mM P_i) of the axonemal ATPase activity of *Tetrahymena* cilia was reported by Otokawa (1973). However, in Otokawa's experiments, no additional $MgCl_2$ was added to compensate for the Mg binding by phosphate, and the experiments were carried out at pH 7·5, where most of the phosphate will be in the $H_2PO_4^-$ form.

ATP^{4-} also appears to act as a competitive inhibitor of beat frequency or sliding velocity, according to the results shown in Figs. 4, 7B, and 9B. Its effect on bend angle is significantly different from the effect of a reduction in $MgATP^{2-}$ concentration, and in this respect it does not appear to be acting as a simple inhibitor competitive with $MgATP^{2-}$. However, in these experiments, when ATP^{4-} concentration is increased at constant $MgATP^{2-}$ concentration, the Mg^{2+} concentration decreases to values below the lowest value for which data are given in Figs. 7A, 8A, etc. Since increased Mg^{2+} concentration was found to increase the bend angle (Fig. 8A), the decrease in bend angle obtained at increased ATP^{4-} concentrations might be a result of the decrease in Mg^{2+} concentration. For this to be the case, however, at Mg^{2+}

concentrations below 0·1 mM the relationship between bend angle and Mg^{2+} concentration must deviate sharply from the linear relationship drawn for Mg^{2+} concentrations greater than 0·1 mM. It would be expected that this continued decrease in bend angle at very low Mg^{2+} concentration would also be accompanied by an increase in beat frequency sufficient to maintain a constant sliding velocity, and the effect of Mg^{2+} on beat frequency would also be non-linear. This would appear as an uncompetitive enhancement of beat frequency at very low Mg^{2+} concentration, and would lead to an underestimation of the inhibitory effect of ATP^{4-} on beat frequency by experiments such as those summarized by Figs. 4 and 5. Since this explanation requires a non-linear effect of Mg^{2+} concentration on bend angle and beat frequency with apparently linear relationships if either high or low Mg^{2+} concentrations are considered separately, we favour the simpler explanation that ATP^{4-} has a direct effect on bend angle.

Hayashi (1974) has found an inhibitory effect of ATP^{4-} on the ATPase activity of sea-urchin axonemes and dynein. However, the relationship between ATPase activity and ATP^{4-} concentration obtained by Hayashi is very different from the linear relationships between $1/f$, sliding velocity, and ATP^{4-} concentration seen in Figs. 7B and 9B. Hayashi concluded that ATP^{4-} was acting as a modifier of the ATPase, and changing its affinity for $MgATP^{2-}$, rather than as a competitive inhibitor. We cannot draw this conclusion from our data. ATP^{4-} appears to bind at the active site, to competitively reduce $MgATP^{2-}$ binding. However, bound ATP^{4-} has an additional effect resulting in a decrease in bend angle, which is not shown by bound ADP or P_i.

The effect of Mg^{2+} is clearly different from the effects of P_i, ADP, and ATP^{4-}, since the frequency inhibition is not competitive with $MgATP^{2-}$ and there is no inhibition of sliding velocity. If $MgATP^{2-}$, and its competitive inhibitors, have a direct effect on control of sliding velocity, our observation of a lack of inhibition of sliding velocity by Mg^{2+} is consistent with the finding of Hayashi (1974), using an experiment similar to that shown in Fig. 1, that there is no inhibition of sea-urchin axonemal ATPase activity by Mg^{2+}. The only other situation which is known to produce reciprocal changes in beat frequency and bend angle is found when demembranated spermatozoa are exposed to trypsin digestion (Brokaw & Simonick, 1977). For a brief period before disintegration, digested flagella show abrupt increases in beat frequency, which are always associated with decreases in bend angle. In these cases, the changes are large enough so that it can be seen that the decrease in bend angle is slightly greater, so that there appears to be a small decrease in sliding velocity associated with these changes (Brokaw, unpublished observations).

The finding that inhibition by Mg^{2+} is uncompetitive indicates that the conclusions of previous studies of the effect of $MgATP^{2-}$ concentration on flagellar movement, which have usually been carried out with a constant excess Mg^{2+} concentration, as in the experiments shown in Fig. 11, do not need to be modified to obtain an accurate value for c_2 in equations (1) and (2). The converse type of experiment, in which Mg^{2+} concentration is varied in the presence of excess ATP, will give a different value for c_2 unless the ATP^{4-} : $MgATP^{2-}$ ratio is maintained constant, as in Fig. 5B. With this procedure, the values of c_2 obtained from Figs. 4B and 5B are essentially identical.

Evidence has recently been presented showing that, in the absence of ATP, Mg^{2+} induces the formation of stable cross-bridges between flagellar tubules (Warner, 1978). We might therefore suggest that in reactivated flagella, Mg^{2+} increases the rate of formation of force-generating cross-bridges, and therefore increases the steady-state cross-bridge population and the force that it generates. If a balance between active forces and elastic resistances is important in determining the bend angle, this effect of Mg^{2+} might favour an increase in bend angle. Cross-bridge detachment kinetics would not be altered by Mg^{2+}, but would be influenced by the concentration of $MgATP^{2-}$ and its competitors.

The uncompetitive inhibition of beat frequency by Mg^{2+} also differs from the uncompetitive inhibition by vanadate, which is accompanied by a decrease in amplitude and an inhibition of dynein ATPase activity (Gibbons *et al.* 1978).

Effects on wavelength

Most of the decreases in beat frequency caused by the inhibitors we have examined here are accompanied by small increases in wavelength. This result, and previous observations of wavelength decreases when the viscosity of the medium is increased (Brokaw, 1966, 1975*a*) are consistent with the hypothesis that the wavelength of actively propagated bending waves is determined by mechanical interactions between flagellar elasticity and the viscosity of the surrounding fluid, just as with passively propagated waves on an inert filament (Rikmenspoel, 1965, 1978). All our experiments were carried out at a constant viscosity. If we assume that none of the inhibitors alters the elasticity of the flagella, the simplest form of this hypothesis would predict that the wavelength should change in proportion to $f^{-0.25}$ when the frequency, f, is changed. We have examined the relationship between wavelength and frequency in each of our experiments. Good agreement with this prediction is found for attached spermatozoa when the frequency is changed by changing the concentrations of $MgATP^{2-}$ (Figs. 11, 12) or Mg^{2+} (Figs. 7A, 10A). With the other inhibitors, the changes in wavelength of attached spermatozoa are less than predicted. The changes of wavelength of swimming spermatozoa are also significantly less than predicted (Figs. 11, 12; Brokaw, 1975*a*). No explanation is at hand for these differences, and the differences found in other work (Brokaw & Josslin, 1973; Rikmenspoel, 1978).

We thank Tom Simonick for extensive assistance with these experiments, and the United States Public Health Service for support by grant No. GM-18711 awarded by the National Institute of General Medical Sciences and by Biomedical Research Support Grant No. RR 07003 awarded by the Division of Research Resources.

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(*Received 8 January* 1979)