

# A Lithium-sensitive Regulator of Sperm Flagellar Oscillation Is Activated by cAMP-dependent Phosphorylation

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**Abstract.** Specific effects of both in vivo activation and in vitro activation by cAMP-dependent phosphorylation on bending wave parameters of demembrated, reactivated, tunicate (*Ciona intestinalis*) and sea urchin (*Lytechinus pictus*) sperm flagella can be reversed by exposure to protein phosphatase. The effects of protein phosphatase incubation can be imitated by inclusion of

LiCl in the reactivation solutions. The primary effect of cAMP-dependent phosphorylation appears to be activation of a regulatory mechanism controlling flagellar oscillation, rather than activation of the active sliding mechanism. Lithium appears to act on the same regulatory mechanism.

CURRENT models for flagellar oscillation involve switching between groups of outer microtubular doublets that produce active sliding in different directions (Brokaw, 1972; Satir, 1985). These models appear to be inadequate to fully determine the parameters of flagellar oscillation (Brokaw, 1985b). Other evidence suggests, for example, that there is a regulatory mechanism that directly controls the frequency of flagellar oscillation, in addition to effects on frequency caused by changes in sliding velocity that result from changes in the intensity of the active sliding process (Okuno and Brokaw, 1979). Identification of these control mechanisms involved in causing, and determining the parameters of, flagellar oscillation and bend propagation is one of the major requirements for further advance in our understanding of how flagella and cilia work. Progress in understanding these control mechanisms may come from understanding the mechanisms by which flagellar oscillation is activated by cAMP-dependent phosphorylation (Brokaw, 1982).

Motility of spermatozoa from trout and from the tunicate, *Ciona intestinalis*, has been shown to be totally dependent on an activation step that can be imitated in vitro by incubation of demembrated spermatozoa with cAMP (Morisawa and Okuno, 1982; Brokaw, 1982, 1985a). cAMP-dependent phosphorylation of flagellar proteins has been shown to occur under these conditions (e.g., Opresko and Brokaw, 1983). On the other hand, activation of motility of spermatozoa of the sea urchin *Strongylocentrotus purpuratus* has been suggested to be a direct result of an elevation of intracellular pH by Na<sup>+</sup>/H<sup>+</sup> exchange, which may regulate dynein-driven active sliding without involvement of cAMP-dependent phosphorylation (Christen et al., 1982; Lee et al., 1983; Johnson et al., 1983). Spermatozoa from another sea urchin, *Lytechinus pictus*, appear to have properties intermediate between these two extremes. *Lytechinus* sper-

matozoa, after demembration and suspension in reactivating solutions containing 1 mM MgATP<sup>2-</sup>, are only partially active, with 50–90% of the sperm population remaining quiescent (Brokaw, 1985a). These sperm also require incubation with cAMP for full activity. Changes in percentage motility accompanying phosphorylation and dephosphorylation have also been described for spermatozoa of starfish and sea urchins found in Japan (Ishiguro et al., 1982; Takahashi et al., 1985; Murofushi et al., 1986).

The present paper presents detailed information about the changes in parameters of flagellar motility of demembrated and reactivated spermatozoa of *Ciona* and *Lytechinus* accompanying incubation with cAMP, the catalytic subunit of the cAMP-dependent protein kinase, or a protein phosphatase preparation from rabbit skeletal muscle. No direct measurements of phosphorylation or dephosphorylation of flagellar proteins under the conditions used for these incubations are reported here. Such measurements have been detailed in other publications (Opresko and Brokaw, 1983; Murofushi et al., 1982, 1986), and phosphorylation and dephosphorylation are therefore assumed to be the significant biochemical events occurring in the experiments reported here. The significant substrates for phosphorylation have not been identified in these species, but work with dog spermatozoa has identified a supernatant phosphoprotein, axokinin, that may be a principal regulator of the state of motility of these sperm flagella (Tash and Means, 1982; Tash et al., 1984).

Lithium has previously been described as a reversible inhibitor of a system regulating the motility of demembrated, reactivated sea urchin spermatozoa (Gibbons and Gibbons, 1984). Our further characterization of the effects of lithium indicates that this reagent is acting on the same regulatory system that is modulated by cAMP-dependent phosphorylation, thus making lithium a useful reagent for examining the effects of modification of this system.

## Materials and Methods

Spermatozoa were collected in as concentrated a form as possible, and stored on ice for up to 6 h during a series of experiments. No significant changes in results were observed during these short storage times. Demembration with Triton X-100 and reactivation with solutions containing ATP used procedures similar to those reported previously (Brokaw, 1985a, 1986). Demembration solutions for *Ciona* spermatozoa contained 0.25 M KCl, 1 mM MgSO<sub>4</sub>, 1 mM dithiothreitol (DTT), 0.4 mM EGTA, 0.04% Triton X-100, and 20 mM Tris-HCl buffer, pH 8.0. Demembration solutions for *Lytechinus* spermatozoa contained 0.25 M KCl, 2 mM MgSO<sub>4</sub>, 1 mM DTT, 1 mM EGTA, 0.04% Triton X-100, and 10 mM Tris-HCl buffer, pH 8.2. All reactivation solutions contained 0.25 M K acetate, 20 mM Tris-HCl buffer, 1 mM DTT, and 0.5% polyethylene glycol (PEG), and were buffered with EGTA and/or EDTA to maintain a low Ca<sup>++</sup> concentration (usually 10<sup>-8</sup> M). The *Ciona* reactivation solutions contained 2 mM EDTA and 25 μM EGTA, a free Mg<sup>++</sup> concentration of 0.5 mM, and pH 8.0. The *Lytechinus* reactivation solutions contained 1 mM EGTA, a free Mg<sup>++</sup> concentration of 2.5 mM, and pH 8.2. ATP and additional MgSO<sub>4</sub> were added to the reactivation solutions to give the desired MgATP<sup>-</sup> concentration. For some experiments, 0.1% methyl cellulose was included in the reactivation solutions to increase the viscosity. This modification counteracts the increase in wavelength that occurs at lower beat frequencies, and improves the analysis of the bending patterns to obtain parameters. All experiments and measurements were carried out at 18°C.

Measurements were made on spermatozoa swimming at the upper surface of an open drop of reactivation solution on a microscope slide, using either stroboscopic illumination to determine flagellar beat frequencies, or multiple-flash photography on moving film (Brokaw, 1986). Photographs were analyzed with computer-assisted methods (Brokaw, 1984) to obtain bending wave parameters for individual spermatozoa. Data points presented in this paper are typically based on samples of 20 spermatozoa; in a few cases data were missing and the samples contained only 18 or 19 spermatozoa. Beat frequency measurements by stroboscopic illumination typically were based on samples of 20 or 30 spermatozoa.

Effects of dephosphorylation were studied using a crude protein phosphatase preparation from rabbit skeletal muscle. Spermatozoa after demembration and activation were diluted 1:4 with ATP-free reactivation solution containing phosphatase and chicken egg-white trypsin inhibitor (#T1886; Sigma Chemical Co., St. Louis, MO) at a concentration of 10 μg/ml. Phosphatase action was terminated by a 1:100 dilution into reactivation solution. To distinguish effects of dephosphorylation by the phosphatase from other effects that might be caused by proteases or other components of this crude preparation, control experiments examined the effects of 8 mM Na pyrophosphate present during the incubation with protein phosphatase (cf. Fig. 4). This concentration of pyrophosphate probably gives only partial inhibition of the protein phosphatase (Takahashi et al., 1985). The protein phosphatase was prepared by following the procedure of Silberman et al. (1984) through ethanol precipitation and the first DEAE-Sephadex chromatography step. The high salt peak from this column was concentrated by ultrafiltration, mixed with an equal volume of glycerol, and stored at -20°C; its activity did not decrease measurably over 2 mo. A typical preparation, used for most of these experiments, had a specific activity, measured by casein dephosphorylation, of 24 nM/min per mg protein (24 U/mg). Protein phosphatase activity was measured at 22°C, using casein (#C6780; Sigma Chemical Co.; dialyzed against assay buffer before use) as a substrate, at a concentration of 10 mg/ml, in an assay solution similar to that used during incubations of sea urchin spermatozoa with the phosphatase preparation (reactivation solution lacking added MgATP<sup>-</sup>, and with PEG omitted). Phosphate release was measured with the malachite green method of Carter and Karl (1982).

## Results

### In Vitro Activation of *Ciona* Spermatozoa

When concentrated *Ciona* spermatozoa are demembrated and suspended directly in reactivation solution, without prior in vivo activation, they are nonmotile. Motility requires incubation under conditions in which cAMP-dependent phosphorylation can occur. This in vitro activation was carried out by mixing 100 μl of demembrated sperm suspension with 150 μl of activation solution contain-

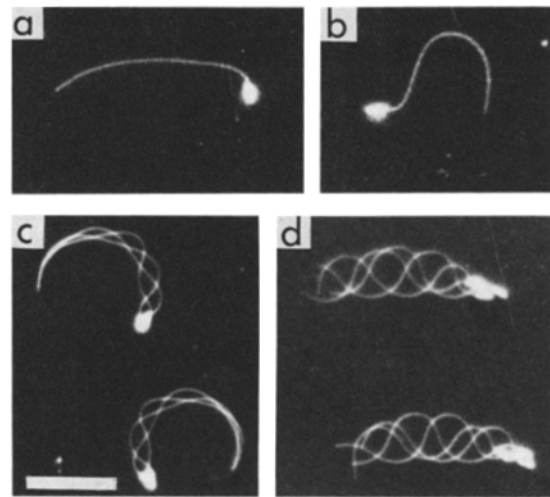


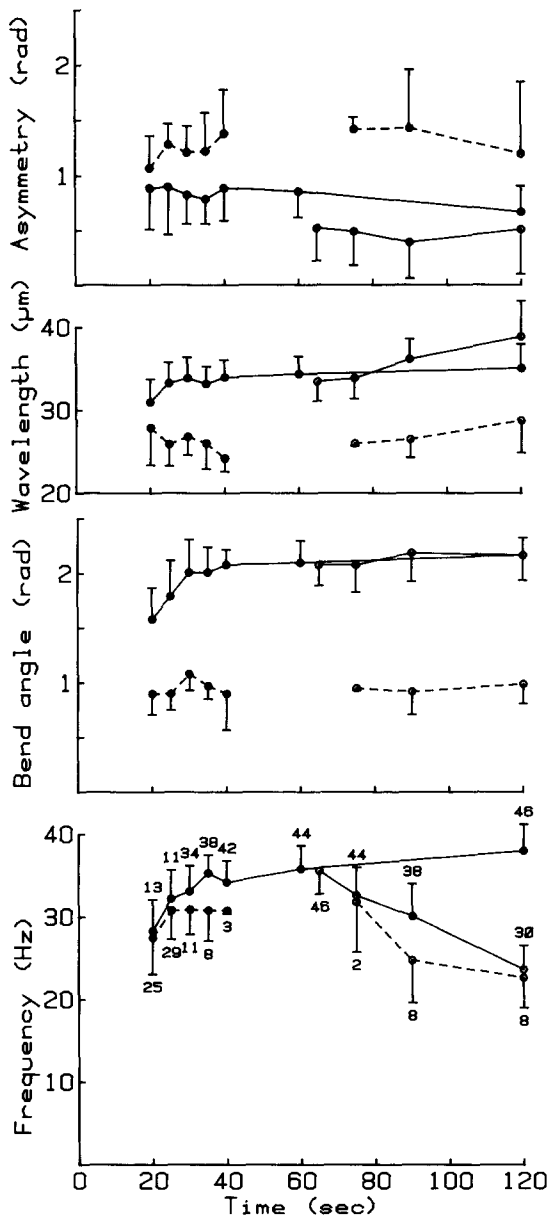
Figure 1. Multiple exposure photomicrographs of demembrated *Ciona* spermatozoa taken with three or four exposures on stationary 35-mm film, with flashes at 100 Hz, showing the four types of bending patterns obtained with progressively increasing amounts of cAMP-dependent phosphorylation (see text). *a* and *b* were taken with a 16× objective, of spermatozoa floating at the upper surface of an open drop. *c* and *d* were taken with a 40× objective, using a cover glass on the sperm preparation. Bar, 20 μm.

ing 1 mM MgSO<sub>4</sub>, 1 mM DTT, 0.2 mM EGTA, 1 mM ATP, 10 μM cAMP, and 20 mM Tris-HCl buffer, pH 8.0, so that the KCl concentration was reduced to 0.1 M. Phosphorylation was terminated when the suspension was diluted (usually 1:100 or more) into reactivation solution containing 0.25 M K acetate. The combined effects of dilution and increased salt concentration inhibit further phosphorylation (Opresko and Brokaw, 1983). Depending on the duration of the incubation with cAMP and ATP, four distinct bending patterns can be obtained when the spermatozoa are subsequently diluted into standard reactivation solution with 1 mM MgATP<sup>-</sup>.

Pattern C, illustrated in Fig. 1 *a*, is a quiescent configuration similar to the "cane configuration" described for the quiescent state of sea urchin spermatozoa by Gibbons (1980). It is obtained if demembrated spermatozoa are examined without exposure to phosphorylating conditions.

Pattern Q, illustrated in Fig. 1 *b*, is a quiescent configuration that is also seen when live *Ciona* spermatozoa undergo intermittent quiescence (Omoto and Brokaw, 1983). In contrast to Pattern C, the flagellum is bent in both directions, with a sharp bend near the basal end and a long bend in the opposite direction occupying the distal portion of the flagellum. Observation of spermatozoa undergoing intermittent quiescence clearly shows that the long distal bend has the same sense as the principal bends of asymmetrically beating flagella. A brief exposure to phosphorylating conditions causes demembrated spermatozoa to change from pattern C to pattern Q.

Pattern D, illustrated in Fig. 1 *c*, is an active bending pattern. Oscillatory bend formation occurs in the basal part of the flagellum, but the bends are damped, or attenuated, when they start to propagate, so that there is little or no bending in the distal part of the flagellum. However, the sperm head oscillates through an angle of 1 radian or more; this suggests



**Figure 2.** Results from an experiment showing the effect of the time of incubation with cAMP on the parameters of movement of *Ciona* sperm flagella (solid symbols). Each point includes data from two samples; the mean, standard deviation, and number of spermatozoa in the combined samples are shown for each time point. Values of parameters for flagella showing P bending patterns were averaged separately and are indicated by the points connected by solid lines; values for flagella showing D bending patterns are connected by dashed lines. A total of 16 spermatozoa were omitted from these samples because they had intermediate bending patterns that were not readily classifiable as either D or P. This figure also shows (open symbols) results obtained after exposure of demembrated spermatozoa to a protein phosphatase preparation after 60 s of incubation with cAMP. These results are plotted vs. the time of exposure to 30 U/ml of protein phosphatase; actual exposure times ranged from 30 to 120 s, and concentrations ranged from 4 to 15 U/ml.

that sliding is occurring between microtubules in the distal part of the flagellum. Demembrated spermatozoa change from pattern Q to pattern D after further exposure to phosphorylating conditions.

Pattern P, illustrated in Fig. 1 *d*, is an active bending pattern in which bends initiated at the basal end of the flagellum propagate along the full length of the flagellum with little or no attenuation. It is considered to be the "normal" form of flagellar motility for these spermatozoa. Demembrated spermatozoa change from pattern D to pattern P after extended exposure to phosphorylating conditions. At intermediate times, a sperm population containing a mixture of D and P bending patterns can be obtained; in such populations individual spermatozoa can sometimes be observed to switch between these two patterns with little or no change in beat frequency. With some sperm samples, the quality of the P bending waves of reactivated spermatozoa also appears to increase with time of phosphorylation, as discussed below.

### *Time Course of In Vitro Activation of Ciona Spermatozoa*

Fig. 2 summarizes measurements of bending wave parameters for spermatozoa as a function of the time of incubation with cAMP and ATP. These results show that after the transition from quiescence to motility there is a further gradual increase in beat frequency which is not just a transition from slower D bending patterns to faster P bending patterns; instead there is a clear increase in the beat frequencies of the P bending patterns. This is accompanied by a small increase in bend angles of spermatozoa with P bending patterns, resulting at least in part from a decreasing number of spermatozoa with intermediate values of bend angle. Increases in wavelength are small. A decrease in asymmetry with cAMP incubation time is suggested, but is small compared with the standard deviations. However, a decreased asymmetry after long incubation times (120 s or longer) is consistently seen in experiments of this type.

Fig. 3 shows histograms of the values of beat frequency and bend angle obtained at the 25- and 30-s time points of the experiment in Fig. 2. The distribution of bend angles is bimodal, with major peaks corresponding to the typical bend angles of D and P bending patterns shown in Fig. 2. In addition, there is a significant number of spermatozoa with bending patterns which have intermediate bend angles, some of which are not readily classifiable as D or P bending patterns. In contrast to the bend angle distribution, the distribution of beat frequencies has no bimodal character.

Better resolution of the early stages of activation was obtained in experiments where the rate of activation was reduced by using a reduced cAMP concentration (0.2  $\mu$ M) during the incubation. No significant oscillation was seen until sufficient phosphorylation occurred to sustain a frequency of  $\sim$ 25 Hz (data not shown). D bending patterns were obtained after 50–120 s of incubation; P bending patterns appeared only after 90 s of incubation.

### *In Vivo Activation of Ciona Spermatozoa*

*Ciona* spermatozoa can be activated in vivo by exposure to conditions causing an increase in internal pH. Dilution of the concentrated spermatozoa 1:25 with a solution containing 90% 0.5 M NaCl, 10% sea water, and 80 mM histidine, at pH 8.4, for 20 s transforms the spermatozoa to an activated state that persists after demembration, so that no incubation with cAMP is required before reactivation of motility. These activated spermatozoa can be diluted 1:10 with de-

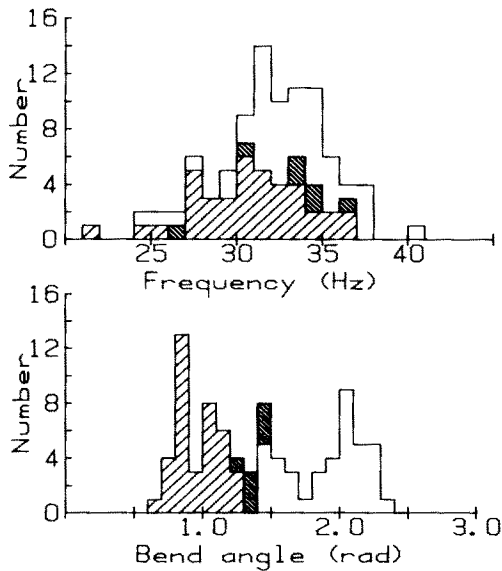


Figure 3. Histograms showing the distributions of frequency and bend angle for *Ciona* spermatozoa in the 25- and 30-s cAMP incubation samples from the experiment reported in Fig. 2. Spermatozoa with P bending patterns are shown without cross-hatching. Spermatozoa with D bending patterns are shown with light cross-hatching. Spermatozoa with bending patterns that could not be readily classified as either P or D are shown with heavy cross-hatching.

membration solution, incubated for 20–30 s, and then diluted into reactivation solution where they are 100% motile and almost all display the P bending pattern. Bending wave parameters are similar to those of spermatozoa activated in vitro, except that the asymmetry of the bending pattern is slightly less for in vivo-activated spermatozoa than for in vitro-activated spermatozoa.

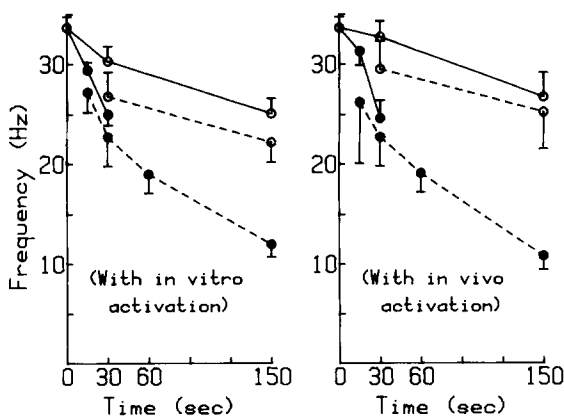


Figure 4. Effects of the time of incubation with protein phosphatase (30 U/ml) on the frequency of oscillation of reactivated *Ciona* sperm flagella following either in vivo activation or in vitro activation by incubation with cAMP for 75 s. This incubation time was selected to give a frequency comparable to the frequency obtained after in vivo activation. Solid circles indicate the results obtained after incubation with protein phosphatase for the time shown. Open circles indicate the results obtained when 8 mM sodium pyrophosphate was included in the phosphatase incubation mixture. Solid lines connect measurements on P bending patterns; dashed lines connect measurements on D bending patterns.

### Deactivation of *Ciona* Spermatozoa

Fig. 2 also shows results of exposing in vitro-activated spermatozoa to a crude protein phosphatase preparation from skeletal muscle, after 60 s of incubation with cAMP. Fig. 4 compares the results of exposing in vivo- and in vitro-activated spermatozoa to another crude protein phosphatase preparation. The initial effect of this exposure, indicated by the results in Figs. 2 and 4, is a decrease in beat frequency of P bending patterns. This appears to be a reversal of the beat frequency increase seen in the later stages of in vitro activation (Fig. 2). A later effect of the incubation with protein phosphatase is an increase in numbers of spermatozoa with D bending patterns, without any decrease in the bend angle of P bending patterns. This is accompanied by further decrease in beat frequency, which eventually results in lower beat frequencies than can be recorded during activation. After more extended phosphatase exposure, the distinction between the P and D patterns begins to become obscured by the presence of an erratic type of bending, which is not seen at any time during activation. Little or no decrease in bend angle is seen after phosphatase treatment of spermatozoa activated in vitro. However, a decrease in bend angle is seen after phosphatase treatment of spermatozoa activated in vivo, in experiments such as the one summarized in Fig. 4, and is accompanied by an increase in asymmetry (not shown) rather than the decrease seen after in vitro activation (Fig. 2).

Both types of activated demembrated sperm preparations can be almost fully returned to type Q or C quiescent patterns by extended incubation with the phosphatase preparation (5 min or longer). However, this is not proven to be entirely the result of dephosphorylation, since considerable deterioration of the motility also occurs in the pyrophosphate-inhibited phosphatase incubations after these extended incubation times. Rephosphorylation with the catalytic subunit of the cAMP-dependent protein kinase also failed to completely restore the motility of spermatozoa after extended exposure to the protein phosphatase preparation (not shown). Another method that can be used to identify effects of dephosphorylation is the use of thiophosphorylation with ATP $\gamma$ S instead of ATP during the incubation with cAMP, to produce a phosphatase-resistant state of activation. Although ATP $\gamma$ S can activate *Lytechinus* spermatozoa, *Ciona* spermatozoa incubated with cAMP and 2 mM ATP $\gamma$ S for 120 s displayed only C bending patterns when diluted into reactivation solution, indicating that not even a small amount of activation by thiophosphorylation occurred.

### Effects of Lithium or Vanadate on *Ciona* Sperm Flagellar Motility

Fully activated spermatozoa generating P bending waves can be switched to D bending by addition of LiCl to the reactivation solution. At intermediate LiCl concentrations, where a clearly bimodal mixture of P and D bending patterns is obtained, individual spermatozoa can be observed to switch back and forth between these two discrete bending patterns. Fig. 5 illustrates typical examples of P and D bending patterns obtained in reactivation solution containing 2.5 mM LiCl after in vitro incubation with cAMP and ATP for 120 s. Higher concentrations of LiCl will induce quiescence. However, Q patterns are seen in only a small fraction of the sperm population, and the transition to quiescence does not appear

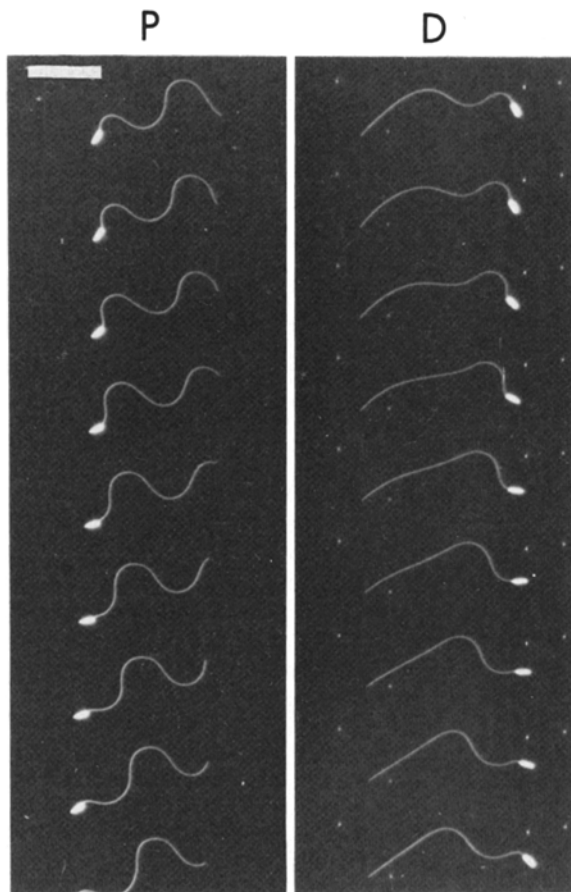


Figure 5. Typical P and D bending patterns of *Ciona* spermatozoa in reactivation solution containing 2.5 mM LiCl. Photographed on moving film with flashes at 300 Hz. Bar, 20  $\mu$ m.

to be sufficiently synchronous to obtain predominantly Q populations, as can be seen during initial phosphorylation.

The concentration of LiCl required to induce D bending increases with the time of incubation under phosphorylating conditions (not shown). Control experiments were carried out showing that the addition of up to 10 mM LiCl during incubation with cAMP for *in vitro* activation did not produce any noticeable effects on the subsequent reactivated motility (data not shown).

The results shown in Fig. 6 are typical of experiments comparing the inhibitory effects obtained with reactivation solutions containing either lithium or vanadate on spermatozoa after *in vitro* activation. Vanadate also causes the appearance of D bending patterns, but only after much greater inhibition of frequency. This frequency inhibition is accompanied by a large decrease in bend angle for the P bending patterns, which is not seen with inhibition by lithium or after protein phosphatase incubation. The combined effect of these changes is that with vanadate inhibition the transition to D bending patterns occurs after a large ( $\sim 60\%$ ) decrease in sliding velocity (proportional to the product of frequency and bend angle) for the P bending patterns. This decrease in sliding velocity presumably results from the inhibitory effect of vanadate on the mechanism that generates active sliding in these flagella (Gibbons et al., 1978). Vanadate has negligible effect on the wavelength or

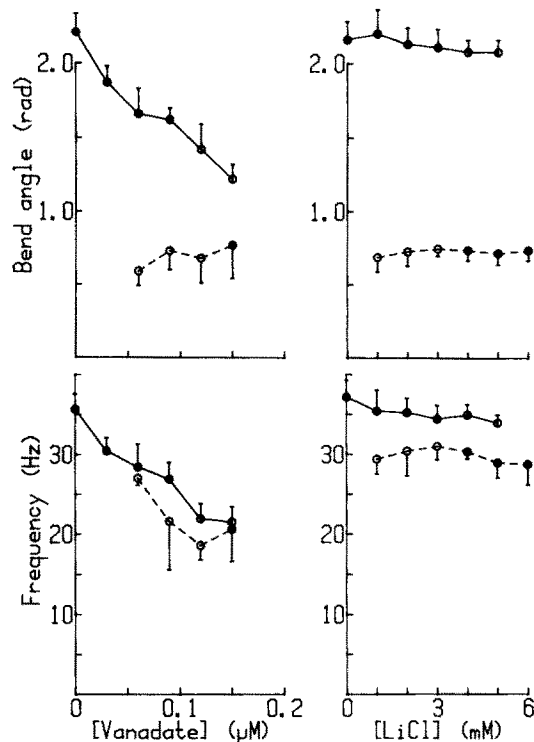
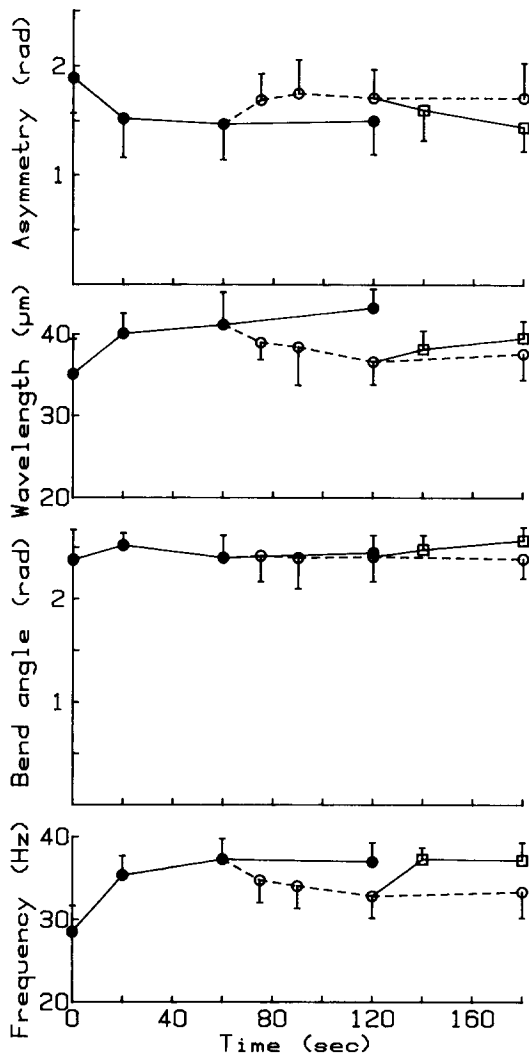


Figure 6. Effects of vanadate or LiCl on frequency and bend angle of *Ciona* sperm flagella. Points connected by solid lines are for flagella with P bending patterns; points connected by dashed lines are for flagella with D bending patterns. Solid circles represent the predominant type of bending pattern in the samples; with 0.12  $\mu$ M vanadate there was an approximately equal mix of P and D bending patterns. Between 21 and 30 spermatozoa were analyzed at each concentration of inhibitor. At 0.06  $\mu$ M vanadate, D bending patterns were very rare and the data shown are for a sample of only three spermatozoa; at 0.15  $\mu$ M vanadate, P bending patterns were very rare and the data shown are for a sample of only two spermatozoa. Similarly, D bending patterns were rare at 1 mM LiCl ( $n = 3$ ) and P bending patterns were rare at 5 mM LiCl ( $n = 5$ ).

asymmetry of these bending patterns. In contrast to the effects of vanadate, the transition to D bending patterns caused by lithium occurs with only a 10% decrease in sliding velocity for the P bending patterns. In addition, vanadate-inhibited flagella generating D bending patterns can be obtained with frequencies as low as 12 Hz, whereas with lithium inhibition all of the sperm become quiescent before such low frequencies are obtained. Under conditions where all of the intact spermatozoa in the sample have become quiescent, for example with 12 mM LiCl for the sample shown in Fig. 6, short spermatozoa with only 5–15  $\mu$ m of flagellum, resulting from breakage of the flagellum, are still beating with high beat frequencies ( $\sim 40$  Hz compared to 44 Hz for short flagella in the absence of lithium). In contrast to this, at, for example, 0.3  $\mu$ M vanadate, only a few spermatozoa are motile, but most of these have normal length flagella and beat frequencies of  $\sim 10$  Hz, and short flagella appear to be just as thoroughly inhibited by vanadate as full length flagella.

#### *In Vitro* Activation of *Lytechinus* Spermatozoa

Demembrated *Lytechinus* spermatozoa diluted directly



**Figure 7.** Results from a typical experiment demonstrating effects of incubation under phosphorylating or dephosphorylating conditions on the parameters of movement of *Lytechinus* sperm flagella measured in reactivation solutions containing 1 mM MgATP<sup>-</sup>. The filled circles, connected by solid lines, show results of incubation with cAMP under standard conditions, for the time indicated on the abscissa. Open circles connected by dashed lines show the results of incubation with a protein phosphatase preparation after 60 s of incubation with cAMP. For these incubations, the cAMP incubation mixture was diluted 1:4 with reactivation solution lacking MgATP<sup>-</sup> and containing protein phosphatase. This dilution, in combination with the increased salt concentration, should inhibit further phosphorylation during the phosphatase incubation. The squares show the results of incubation with cAMP and protein kinase catalytic subunit after 60 s of incubation with cAMP followed by 60 s of incubation with protein phosphatase. For these incubations, the protein phosphatase incubation mixture was diluted 1:4 with an appropriate mixture of demembration buffer and activation solution to obtain conditions similar to those for the initial cAMP incubations; the concentration of protein kinase catalytic subunit was ~500 U/ml. This dilution is not sufficient to eliminate continued phosphatase activity that may compete with phosphorylation during the final incubation. Most data points show means and standard deviations for samples of 20 spermatozoa; in a few cases, data from two such samples were combined.

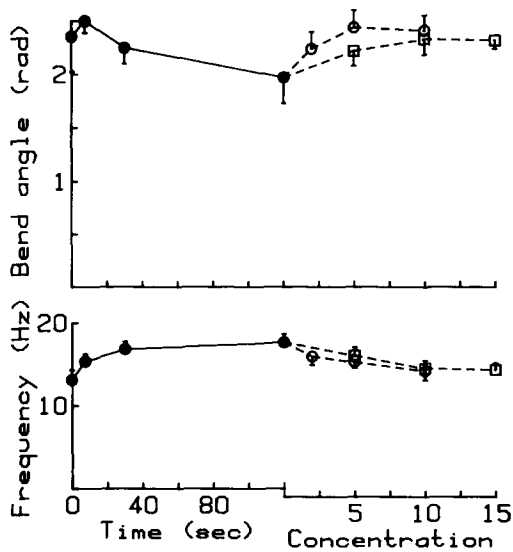
into reactivation solution containing 1 mM MgATP<sup>-</sup> typically show 50–90% quiescence, in a C pattern. Most of the remaining, motile spermatozoa show extremely asymmetric flagellar bending patterns, with highly attenuated bending waves.

In vitro activation of motility was carried out as in the experiments with *Ciona* spermatozoa, except that 100 μl of demembrated sperm suspension was diluted with 300 μl of activation solution containing 0.05 M KCl, to obtain a final KCl concentration of 0.10 M (Brokaw, 1985a). After only a few seconds of incubation, almost all of the spermatozoa become motile; it has not been practicable to quantitate the percentage motility as a function of time under these activation conditions. However, there are more gradual changes in parameters of flagellar bending which can be quantitated, as shown in Fig. 7 (reactivation solutions containing 1 mM MgATP<sup>-</sup>) and Fig. 8 (0.1 mM MgATP<sup>-</sup>). Although relatively few points are shown for the effect of cAMP incubation time on movement parameters for these experiments which were designed to include observations of the reversal of the effects of phosphorylation, the results are typical of many other experiments in which more extensive data on the effects of cAMP incubation time were collected (not shown). The magnitude of the changes in parameters with duration of incubation is variable, because there is large variation between different sperm samples in the state of the spermatozoa before cAMP incubation. The changes in parameters with increasing duration of incubation with cAMP include increases in beat frequency and wavelength and a decrease in asymmetry. However, the asymmetry remains high, even after extended incubation, unless the spermatozoa are incubated with high Ca<sup>++</sup> and Triton X-100 to extract calmodulin (Brokaw and Nagayama, 1985). When assayed in reactivation solutions containing 1 mM MgATP<sup>-</sup> (Fig. 7), some samples show an increase in bend angle with duration of cAMP incubation. However, when assayed in reactivation solutions containing 0.1 mM MgATP<sup>-</sup> (Fig. 8), a decrease in bend angle with duration of cAMP incubation is consistently observed. With 0.1 mM MgATP<sup>-</sup> (Fig. 8), usually all of the spermatozoa are motile even without incubation with cAMP.

Full in vitro activation is prevented by omission of cAMP from the activation solution or by increasing the KCl concentration to 0.25 M (data not shown), as previously reported for *Ciona* spermatozoa (Opresko and Brokaw, 1983). Activation is normal with 0.1 mM ATP, but significantly slower with 0.02 mM ATP in the incubation medium (not shown). Activation can be obtained with 2 mM ATPγS substituted for ATP (not shown). These characteristics are all consistent with the inference that in vitro activation by incubation with cAMP is associated with phosphorylation of flagellar proteins by the cAMP-dependent protein kinase.

#### Deactivation of *Lytechinus* Spermatozoa

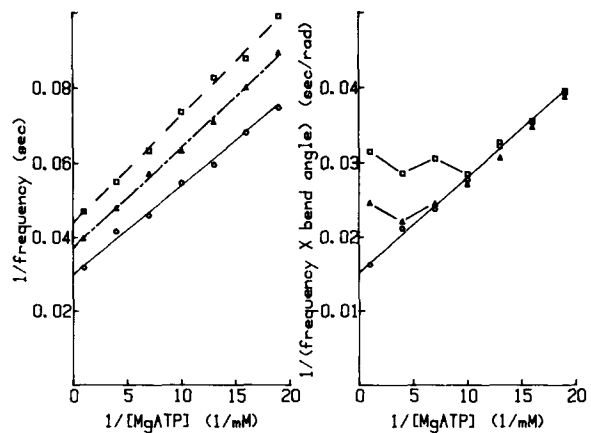
The effects of incubation with cAMP can be reversed by incubation with a protein phosphatase preparation, and then restored by incubation with the catalytic subunit of the cAMP-dependent protein kinase. An example of this type of experiment is shown in Fig. 7. Incubation with the phosphatase preparation partially restored the lower beat frequencies and wavelengths and higher asymmetries that were seen in spermatozoa that were reactivated after incubation without



**Figure 8.** Effects of time of incubation with cAMP and the effects of dephosphorylation and LiCl on the parameters of movement of *Lytechinus* sperm flagella reactivated with 0.1 mM MgATP<sup>-</sup>. The solid circles show the effects of the time of incubation with cAMP. The open circles show the effect of incubation with a protein phosphatase preparation for 30 s after 120 s of incubation with cAMP, before dilution into reactivation solution; the concentration scale indicates the  $\mu$ l of protein phosphatase preparation added to 100  $\mu$ l of sperm suspension. The casein phosphatase activity of this phosphatase preparation was 0.6 U/ $\mu$ l. The squares show the effects of LiCl after 120 s of incubation with cAMP; the concentration scale indicates the LiCl concentration in the reactivation solution, in mM.

cAMP. The effects of the relatively mild phosphatase treatment used here appeared to level off after  $\sim$ 60 s of incubation, and did not reduce the beat frequency to levels below that observed for nonactivated spermatozoa. In some of our phosphatase-treated preparations, quiescent spermatozoa appeared after the longest incubations used. Takahashi et al. (1985) reported complete quiescence after longer incubations with a purified protein kinase preparation with comparable levels of activity.

Incubation with the protein phosphatase preparation, or addition of LiCl to the reactivation solution, both cause a reduction in beat frequency and an increase in bend angle in reactivation solutions containing 0.1 mM MgATP, reversing the effects of cAMP-dependent phosphorylation, as shown in Fig. 8. A major change in sensitivity to LiCl is also associated with phosphorylation. Demembrated spermatozoa which are observed without incubation with cAMP and are normally nearly 100% motile at 0.1 mM MgATP<sup>-</sup> become entirely quiescent in reactivation solutions containing 1–5 mM LiCl (depending on the sperm sample). On the other hand, after 120 s of incubation with cAMP, they are still mostly motile in the presence of 20 mM LiCl, with only a few spermatozoa in the quiescent state. This change in sensitivity to LiCl does not appear to be paralleled by a change in sensitivity to the protein phosphatase; very little quiescence was induced by incubating unphosphorylated spermatozoa with 5  $\mu$ l of phosphatase for 30 s—a treatment that is equivalent to  $\sim$ 10 mM LiCl for phosphorylated spermatozoa (Fig. 8).



**Figure 9.** Double reciprocal plots showing the effect of phosphorylation and dephosphorylation of demembrated *Lytechinus* spermatozoa on the relationship between motility parameters and substrate concentration. The left panel shows frequencies; the right panel shows the product of frequency and bend angle, which is proportional to sliding velocity. Squares (top line) show data for spermatozoa that were not incubated with cAMP. Circles (bottom line) show data for spermatozoa that were incubated with cAMP for 120 s. Triangles (middle line) show data for spermatozoa that were incubated with a protein phosphatase preparation for 120 s after 120 s of incubation with cAMP. The lines were drawn using parameters for  $f_{\max}$  and  $K_m$  obtained by nonlinear least squares fitting of the data by a standard relationship of the form  $f = f_{\max}/(1 + [\text{MgATP}^-]/K_m)$ . The values of  $K_m$  for beat frequency were, from top to bottom, 0.065, 0.073, and 0.081 mM. The value of  $K_m$  for sliding velocity, obtained from only the data following 120 s of incubation with cAMP, was 0.085 mM.

Several other inhibitors—vanadate, sodium pyrophosphate, ADP—were tested under the same conditions; none of these produced the same combination of decreased frequency and increased bend angle obtained with LiCl (data not shown).

#### Reactivation of *Lytechinus* Spermatozoa as a Function of MgATP<sup>-</sup> Concentration

The difference between the results of cAMP incubation seen in Fig. 7 at 1 mM MgATP<sup>-</sup> and Fig. 8 at 0.1 mM MgATP<sup>-</sup> can be understood more clearly by looking at the results of experiments comparing spermatozoa before and after activation, in reactivation solutions covering a range of MgATP concentrations, as shown in Fig. 9. Spermatozoa incubated with cAMP for 120 s show a simple linear relationship on a double reciprocal plot of frequency  $\times$  bend angle (which is proportional to sliding velocity) and MgATP<sup>-</sup> concentration. Spermatozoa that were not incubated with cAMP, or that were exposed to protein phosphatase after incubation with cAMP, have similar sliding velocities at low MgATP<sup>-</sup> concentrations, but at high MgATP<sup>-</sup> concentrations the results diverge from a linear relationship, because the bend angles decrease as the sperm start to become quiescent. In contrast to this, the relationship between frequency and substrate concentration gives a linear double reciprocal plot, regardless of the state of phosphorylation, with a major effect of phosphorylation on the maximum frequency and little effect on  $K_m$ .



## Discussion

### The Requirement for Phosphorylation

These new observations add to an already strong body of evidence indicating that phosphorylation of an important component (or components) of the flagellar machinery is required for activation of normal motility of sperm flagella. For *Ciona* spermatozoa, evidence that activation is dependent upon addition of cAMP or the catalytic subunit of cAMP-dependent protein kinase to the incubation mixture, and is inhibited by protein kinase inhibitor, was presented by Opresko and Brokaw (1983), along with evidence that many sperm proteins were phosphorylated under these conditions. In particular the new observations on deactivation of motility by treatment with a protein phosphatase preparation, combined with earlier observations of Takahashi et al. (1985), provide evidence against the possibility that phosphorylation activates a protease that is responsible for converting the flagellum to an active state. This is an important concern, since it has been shown previously that quiescent *Lytechinus* spermatozoa can be activated, at least partially, with trypsin (Brokaw, 1985a). Evidence that the same phosphorylation that can activate motility in vitro is also required for in vivo activation of *Ciona* spermatozoa has been provided by observations comparing protein phosphorylation patterns after in vivo or in vitro activation (Opresko and Brokaw, 1983) and by observations on inhibition of in vivo activation by the protein kinase inhibitor, H8 (Brokaw, 1987). Additional evidence is provided by experiments of the form shown in Fig. 4, illustrating that both in vivo and in vitro activation produce sperm flagella that are similarly sensitive to deactivation by exposure to protein phosphatase.

### Effects of Phosphorylation on Parameters of Flagellar Motility

Most previous studies of activation of sperm flagellar motility by cAMP-dependent phosphorylation have simply monitored the percentage of motile spermatozoa as an indicator of activation. A more detailed examination of the parameters of sperm flagellar motility, as carried out here, provides additional information.

Activation includes a variety of changes, including progressive, quantitative changes in parameters such as beat frequency (also seen during cAMP-activation of mammalian spermatozoa by Lindemann [1978], etc.) as well as sometimes abrupt qualitative transitions in bending pattern. These transitions include transitions from quiescence to oscillation (as in the activation of *Lytechinus* sperm flagella and the Q to D transition of *Ciona* sperm flagella) and transitions from nonpropagated to propagated bending (the D to P transition of *Ciona* sperm flagella). Therefore, activation clearly does not involve a single "on-off" switch that is regulated by phosphorylation in a "one-hit" manner. The events occurring during phosphorylation might be most simply explained by a model in which multiple copies of a single protein substrate are phosphorylated. Abrupt transitions, such as the transition from quiescence to oscillation, occur when a sufficient fraction of the substrate protein has been phosphorylated. Quantitative parameters, such as beat frequency, vary continuously as a function of phosphorylation of the substrate protein.

Observations of the effects of protein phosphatase treatment suggest that the model involving a single protein substrate, described in the preceding paragraph, may also be too simple. In several ways, the effects of protein phosphatase treatment are not the exact reversal of the effects of incubation with cAMP. (a) Protein phosphatase treatment of *Ciona* spermatozoa reduces beat frequencies to levels well below the minimum frequency at which oscillation begins during cAMP incubation (Figs. 2 and 4). (b) The increase in bend angle of P bending patterns of *Ciona* spermatozoa seen between 20 and 30 s after the start of cAMP incubation is not reversed by treatment with protein phosphatase (Fig. 2).

These observations, and the minor differences seen between the effects of in vivo and in vitro activation on *Ciona* sperm flagellar motility, suggest that there may be several different substrate proteins for the cAMP-dependent phosphorylation that occurs during activation of *Ciona* sperm flagellar motility. However, the primary effect of phosphorylation might be on a single regulatory protein substrate.

### Lithium Acts on the Same Regulatory System as Phosphorylation

Lithium has been identified previously as a reversible inhibitor of flagellar oscillation in ATP-reactivated, demembrated sea urchin sperm flagella (Gibbons and Gibbons, 1984). It was also reported in that work that the inhibitory effects of lithium could be counteracted by addition of cAMP, but this observation was difficult to interpret at that time because of lack of information about the effects of cAMP-dependent phosphorylation on sea urchin sperm motility. The new observations reported here show similarities between the effects of addition of lithium to reactivation solutions and the effects of dephosphorylation by incubation with protein phosphatase. The most important effects observed are the transformation from P bending patterns to D bending patterns in *Ciona* sperm flagella (Figs. 5 and 6), and the combination of reduced beat frequency and increased bend angle in *Lytechinus* sperm flagella assayed at 0.1 mM MgATP<sup>-</sup> (Fig. 8). Additional evidence that cAMP-dependent phosphorylation decreases the sensitivity of these spermatozoa to inhibition by lithium, supporting the original observations by Gibbons and Gibbons (1984), has also been found. These observations all suggest that lithium is acting on the same regulatory system that is modulated by cAMP-dependent phosphorylation.

The possibility that lithium is simply activating an endogenous protein phosphatase is eliminated by the observations that the effects of lithium can be reversed by dilution (Gibbons and Gibbons, 1984) and that lithium has no effect when added to the cAMP incubation medium, where any endogenous enzymes will be present in far higher concentration than in the reactivation solutions.

There are minor differences between the effects of lithium and protein phosphatase that suggest that the regulatory system is complex, and that the points of action of lithium and phosphorylation on the regulatory system may not be identical. Nevertheless, the correspondence between the effects is close enough to suggest that LiCl is a useful reagent, more readily available than protein phosphatase, for investigating the behavior of this regulatory system.



## The Regulatory System

An obvious extension of the simple model for regulation by phosphorylation of a single protein substrate would be to assume that the functioning of the active sliding system responsible for the motility of these flagella is directly proportional to the fraction of the substrate that is phosphorylated. According to this model, the generation of P bending waves in *Ciona* spermatozoa would require that the active sliding system be activated to 80–90% of its maximum level, in order to explain the transitions between P and D bending waves that occur during phosphorylation and dephosphorylation and with lithium inhibition (Figs. 2, 4, and 6). However, the experiments with vanadate (Fig. 6), which is known to be an inhibitor of the active sliding system (Gibbons et al., 1978), clearly show that vanadate inhibition of the active sliding system to 80% of its maximum level is not sufficient to cause a transition from P to D bending patterns. This model also predicts that phosphorylation and dephosphorylation would always produce changes in mean bend angle and frequency of flagellar oscillation that correspond to changes in sliding velocity. The measurements on *Lytechinus* sperm flagella at low MgATP<sup>2-</sup> concentration (Figs. 8 and 9) clearly show that this is not the case. Together, these observations provide strong evidence against the idea that the effects of phosphorylation and dephosphorylation are just the direct result of regulating the activity of the active sliding system. This conclusion is supported by the observations of Gibbons and Gibbons (1984), who reported that lithium did not inhibit the sliding disintegration of trypsin-treated axonemes and did not inhibit the activity of dynein ATPase. On the other hand, some direct effects on the active sliding system may be indicated by the increases in both frequency and bend angle of P bending waves during the incubation of *Ciona* spermatozoa with cAMP (Fig. 2) and the decrease in the product of frequency and bend angle of P bending waves of *Ciona* spermatozoa as a result of inclusion of lithium in the reactivation solutions (Fig. 6).

An alternative version of this model is suggested by the observation that phosphorylation causes a decrease in flagellar bend angle in *Lytechinus* sperm flagella assayed at low MgATP<sup>2-</sup> concentration. This suggests that phosphorylation may be decreasing a threshold for switching the direction of active sliding. With a lower threshold, less bending or sliding would be required before switching, giving a lower amplitude and higher frequency for the same sliding velocity. With a higher threshold, spermatozoa might enter a quiescent state if they were unable to generate sufficient sliding or bending to reach the threshold required for switching the direction of active sliding. This kind of model can explain many of the observations reported here, but it is still difficult to explain why such a large inhibition of the active sliding system by vanadate (Fig. 6) is required before the system falls below the threshold required for the generation of P bending waves.

Both of the above versions of the model assume that the regulatory system under discussion is an integral part of the mechanism for flagellar oscillation. On the other hand, observations of the effects of trypsin on the motility of demembrated sea urchin sperm flagella suggest that the regulatory system can be destroyed by trypsin, leaving the basic oscillatory mechanism intact. These observations include the ob-

servation that lithium-inhibited spermatozoa can be activated with trypsin (Gibbons and Gibbons, 1984) and that quiescent spermatozoa that have not been incubated with cAMP can be activated with trypsin (Brokaw, 1985a).

Perhaps the most novel aspect of the results reported here is the differential sensitivity of oscillation and bend propagation to phosphorylation and to lithium, as evidenced by the distinction between D and P bending wave patterns of *Ciona* spermatozoa. Further work is required to demonstrate that this is truly a difference between the processes of oscillation and bend propagation, as opposed to a differential sensitivity of basal and distal regions of the flagellum. Detailed analysis of the D bending patterns of *Ciona* spermatozoa of various lengths is needed, to identify the changes in active sliding that occur during the transitions between D and P bending patterns. These studies may provide important information about the still largely unknown regulatory mechanisms responsible for flagellar oscillation and bend propagation.

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