A Selective Effect of Ni$^{2+}$ on Wave Initiation in Bull Sperm Flagella

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**ABSTRACT**

Bull sperm that are extracted with 0.1% Triton X-100 and restored to motility with Mg$^{2+}$-ATP lose coordination and stop swimming in the presence of 0.5 mM NiSO$_4$. Although spontaneous coordination of flagellar waves is lost after exposure to Ni$^{2+}$, other functions of the flagellum remain intact. The capacity for wave propagation along the flagellum is maintained together with the capacity for microtubular sliding. Wave motility can be restored to Ni$^{2+}$-inhibited sperm by inducing a permanent bend onto the flagellum by micromanipulation. In the absence of such intervention, the loss of wave coordination is complete and irreversible. Ni$^{2+}$-inhibited demembranated cells that are kept active by maintaining a bend in the flagellum exhibit a normal beat frequency. Both intact and demembranated sperm can retain spontaneous wave production at considerably slower rates of motion than Ni$^{2+}$-inhibited cells. Short segments from the distal tip of the flagellum contain only the 9 + 2 microtubular axoneme. These short segments are able to propagate imposed bends even in the presence of Ni$^{2+}$. In addition to wave propagation Ni$^{2+}$-treated sperm can be shown to exhibit a normal sliding tubule phenomenon by direct assay. Although Ni$^{2+}$-treated cells have a functional sliding tubule mechanism, and consequently the axoneme can propagate bends, it appears that these retained functions are not sufficient to cause spontaneous bend initiation. Our findings show that bend initiation is inhibited by Ni$^{2+}$, and therefore is an independent process separate from the sliding tubule mechanism responsible for wave propagation.

Mammalian sperm can be restored to motility with exogenous ATP after the plasma membrane has been damaged or removed. The earliest attempts to induce swimming in membrane-disrupted mammalian sperm relied on glycerol treatment. The motility obtained was described as a twitching motion devoid of normal flagellar wave formation (1, 11). A later microdissection study of bull sperm reported a similar uncoordinated motion in the presence of ATP, and found that although the sperm could not initiate motility on their own they could be started using a microprobe. The microprobe was used to force the flagellum into a constantly bent configuration at which point the flagellum started beating. When the flagellum was released, motility once again stopped (15). More recently, swimming motion was demonstrated in a preparation of demembranated mammalian sperm (13). The same study also determined that a regression of the motility to an uncoordinated twitching could be easily affected by changes in pH or by changes in the concentration of ATP relative to Mg$^{2+}$.

On the basis of these earlier findings it is clear that sperm can remain uncoordinated even in the presence of ample ATP. No explanation has been afforded that accounts for the behavior of sperm in this alternate state of motility. In the twitching state, sperm will not initiate wave motility, but they will propagate bends that are forced onto the flagella. At the time this information was first reported it was tacitly assumed that the twitching reflected a less than optimal functioning of the motile apparatus because of poor conditions for ATPase activity and cross-bridge formation in the axoneme. Now, Ni$^{2+}$ inhibition of motility suggests that the coordination of motility into waves can be blocked even when other conditions are optimal for swimming.

Certain heavy metal ions (Ni$^{2+}$, Cu$^{2+}$) block swimming in demembranated bull sperm (13). Preliminary experiments found that sperm were still responsive to manipulation after treatment with Ni$^{2+}$ and would propagate bending waves that were forced into the flagella (17).

In this study, the techniques of single cell manipulation and membrane extraction are employed separately and in combination to explore the effects of Ni$^{2+}$ on motility in bull sperm. We have specifically tested the effects of Ni$^{2+}$ on microtubule
sliding, axonemal wave propagation, and sustained motile activity. The implications of the new information for the mechanism of motility in mammalian sperm are discussed.

MATERIALS AND METHODS

Sperm Collection and Storage

In the initial stages of the study, sperm were generously supplied by the Eastern Artificial Insemination Cooperative (Ithaca, N.Y.). The semen was diluted to five times its volume with citrate-egg yolk diluent (19) after collection. The diluted sperm were shipped to the laboratory on ice and arrived within 24 h of collection. After arrival, sperm were stored at 0°-5°C until used for experiments.

In the later stages of the study, sperm were obtained through the cooperation of NOBA Inc. (Tiffin, Ohio). Raw semen was diluted to 25 x 10^6 cells/ml with a Tris base extender containing 2% egg yolk (9). Diluted sperm were shipped on ice and arrived at the lab in 24-36 h. After arrival, sperm were stored at 0°-5°C until used for experiments.

Preparation of Sperm

Sperm were removed from the original diluent (extender) and suspended in a citrate-buffered medium immediately before use. The citrate-buffered medium consisted of 0.07 M sodium citrate, 5 mM MgSO₄, 2 mM fructose, and 1 mM CaCl₂ (pH 7.4). Transfer of the sperm was accomplished by diluting 2 ml of sperm stock suspension with 4 ml of citrate, then centrifuging at low speed (1,700 rpm) for 5 min. The supernate was discarded and the pellet resuspended in 6 ml of citrate buffer after that a second centrifugation was performed. The final pellet was suspended in 2 ml of citrate buffer.

Impalement Studies

The initial investigation of the effect of Ni²⁺ was performed by rupturing the plasma membrane of the sperm with a drawn glass microprobe. For this part of the study, one drop of citrate sperm suspension was placed into 2 ml of working medium that contained 0.2 M sucrose, 0.07 M K₂SO₄, 0.5 mM MgSO₄, 3-5 µM CaCl₂, 4 mM ADP, and 2 mM sodium phosphate buffer, pH 7.4 (16). This dilute suspension was then placed into two wells as described earlier by Lindemann and Rikmenspoel (14), thereby providing a control and test sample from the same initial mixture. Probing of the individual sperm was done with a drawn glass microneedle pulled with a Chowdhury micropipette puller (Scientific International, Midwest City, Okla.). The microneedle was mounted in a Piezoelectric driver (22) that was in turn carried on a Brinkmann CP-VI micromanipulator (Brinkmann Instruments, Westbury, N.Y.).

Entry of the probe into the sperm head was assisted by means of rapidly advancing the probe with the Piezoelectric driver. In the medium described above, motility continues without interruption after impalement (16). For the purposes of this study, it was often necessary to repeat the impalement process until distinct damage of the sperm head could be seen, thereby insuring that the plasma membrane had indeed been ruptured. NiSO₄ (0.01 M) was added to the test samples to a concentration of 0.3 mM, and cells were impaled to observe the effect of Ni²⁺.

During the tests with Ni²⁺, the probe was repositioned after impalement and used to bend the flagellum. The results were observed and filmed through a Zeiss universal microscope (Carl Zeiss, Inc., New York) which employed a x 40 water immersion objective. Cinematographic records were taken on 16-mm film at 20 frames per second (fps). All experiments were run at ambient temperature (~23°C).

Demembranation Studies

The effects of Ni²⁺ on demembranated bull sperm were determined using Triton X-100 extracted model. 50 µl of sperm in citrate buffer was added to 3 ml of working medium containing 0.024 M potassium glutamate, 0.132 M sucrose, 0.02 M Tris-HCl, 1 mM dihydrothreitol (DTT), 0.1% Triton X-100 (wt/vt), and 1 mM MgSO₄ (pH 7.9) (12). Sperm were observed microscopically to verify the loss of motility, a consequence of membrane removal, and then activated by addition of 30 µl of 0.1 M ATP solution. After motility was verified, 0.01 M NiSO₄ solution was added to a final concentration of 0.5 mM in the sperm suspensions. Samples without nickel were routinely prepared as controls. Five experiments were also done without DTT in the medium by including 4 mM ADP in the working medium. With the inclusion of ADP, sperm will swim without DTT, but at a slower rate of motility (13, 16). These experiments were necessary as DTT precipitates with Ni²⁺ at pH 7-9, and the possibility that the precipitation of DTT was solely responsible for the loss of sperm motility had to be explored.

Sperm treated in the Triton X-100 extraction medium were used for the microdissection studies. Microdissection was performed using drawn glass microprobes, observations and films were made through a Reichert zetopan microscope (American Optical Corp., Scientific Instrument Div., Buffalo, N.Y.) equipped with a Zeiss x 40 water immersion objective (Carl Zeiss, Inc.). Data were recorded on 16-mm motion picture film at 24 fps. Experiments were run at ambient temperature.

Microtubular Sliding

The effects of Ni²⁺ on microtubular sliding were tested using the same extraction medium as given in “Demembranation Studies.” Sperm were prepared for these experiments by freezing the sperm without adding ATP and storing them frozen for 48 h at ~20°C in the Triton X-100 containing medium. After the frozen storage period the sample was thawed and divided into two equal aliquots. Nickel sulfate was added to one aliquot to a final concentration of 0.5 mM. After a 5-min incubation, ATP was added to both samples to a final concentration of 1 mM. Sperm were microscopically observed before and after the addition of ATP, and 35-mm photographs were taken of the sperm using Kodak 2475 recording film to record low intensity details. In five of these experiments the sperm were thawed and centrifuged for 3 min at 1,700 rpm and then resuspended in fresh media without DTT before the addition of Ni²⁺. In this way the precipitate of Ni²⁺-DTT could be avoided so as to allow better photographic clarity. All experiments were run at ambient temperature.

RESULTS

Bull sperm that were demembranated, impaled, or dissected lost all spontaneous wave motility in the presence of Ni²⁺. A concentration of 0.5 mM Ni²⁺ was sufficient to stop endogenous wave generation regardless of the means employed to damage or remove the membrane. Motility in Ni²⁺-inhibited modeled sperm was not restored after a 30-fold dilution into new media. This experiment was repeated three times and no recovery of motility was observed. Intact sperm from the same washed stock suspension assumed vigorous motility when added to the diluted sample in the same dish with the Ni²⁺-inhibited sperm. Apparently, the sperm are irreversibly inhibited by the original exposure to Ni²⁺. After exposure to Ni²⁺, the sperm flagella usually retain a slight, often difficult to detect, twitching motion that is not organized into regular waves.

Addition of Ni²⁺ to solutions containing 1 mM DTT resulted in the formation of a precipitate. The concentration of Ni²⁺ needed to stop motility was 0.5 mM in the DTT-containing media and was lower (0.3 mM) in media prepared without DTT, indicating that DTT provides some protection for the sperm.

Demembranated cells, impaled cells, and dissected distal segments of the flagellum were initiated into a pattern of coordinated motility by manipulation with a microprobe. To establish coordinated motion on an Ni²⁺-treated flagellum, two points along the flagellum were fixed in position such that the flagellum was at all times in a bent configuration. Fig. 1 is a tracing from 16-mm motion picture film that shows the motion of a sperm manipulated to initiate beating. This cell was in 0.3 mM Ni²⁺, in a medium not containing DTT (as described for the impalement studies). The impaled cell was positioned so as to bend the flagellum. Although the beat frequency of the sperm was low, the bends appear to propagate towards the tip of the flagellum in a regular pattern with a propagation velocity of ~40 µm/s. The manipulated cells remained active for as long as the mechanical positioning was maintained, in one instance for 30 min without loss of motility.

The beat frequencies of Ni²⁺-treated sperm were recorded to provide a measure of the level of flagellar activity that could be compared with that of untreated sperm. Table I contains a summary of the beat frequencies of sperm that were impaled and restarted in an ADP-containing medium with 0.3 mM

Sperm

Ni²⁺ inhibition of Bull Sperm
Ni²⁺. Sperm treated in this way were very slow. Data for control samples prepared in the same way but without Ni²⁺ are included in Table I. These cells also exhibit low beat frequencies. The ranges of data for control and Ni²⁺-treated cells clearly overlap, indicating that the untreated cells, that do not require manipulation for motility, can initiate bending waves at similarly low frequencies. The difference in activity between the control and Ni²⁺-treated impaled cells may be because of an effect of Ni²⁺ on adenylate kinase activity. The adenylate kinase-mediated conversion of ADP to ATP is a complicating factor when ADP is the exclusive substrate for motility.

Triton X-100-demembranated sperm that are given ATP as the exclusive substrate do not show a similar reduction in average beat frequency with the inclusion of 0.5 mM Ni²⁺. Table I includes the data for demembranated sperm that were Ni²⁺ inhibited. These sperm were naturally sustained in motion by two points of flagellar attachment, a condition that was fairly common in preparations of Triton X-100-extracted sperm allowed to stand for 5–10 min. Before Ni²⁺ treatment, some active sperm stick to the microscope slide by their heads and subsequently also become attached to the slide by a distal part of their flagella. As a result, these sperm generally continued their motion after Ni²⁺ administration, regardless of the fact that all the surrounding sperm with free flagella stopped immediately. Sperm constrained in this manner provided a means to obtain an average frequency for a large population of Ni²⁺-inhibited sperm, and also to draw a direct comparison with freely moving demembranated control sperm under similar conditions. The beat frequency of Triton-extracted models does not appear to decrease as a result of Ni²⁺ treatment in the case of those sperm that were attached to the microscope slide at two points. Table I also includes the average beat frequency taken for spontaneously beating cells with free (unrestrained) flagella in the same reactivation media but containing 6 mM ADP in addition to 1 mM ATP. These sperm do not require any mechanical constraint to sustain their motion and yet maintain an average beat frequency well below that of the Ni²⁺-treated samples.

Our results strongly suggest that Ni²⁺-treated sperm are not less energetic, but rather are incapable of initiating bending waves. It is clear that untreated sperm can continue to generate bending waves at lower beating frequencies than are observed for Ni²⁺-inhibited cells; therefore, it is not possible that Ni²⁺ exerts its effects by slowing the sperm beyond the limits needed for autonomous motility.

The microprobing technique was also used to remove a part of the flagellum from a Triton-extracted sperm in the presence of 0.5 mM Ni²⁺. The free segment could then be manipulated and tested for its ability to propagate bending waves. In five experiments a total of 15 flagellar segments were dissected and reactivated by microprobing. The shortest of these segments was ~15 µm in length and was sliced from the distal end of the flagellum. This segment is one of those included in Fig. 2. The small distal segments were observed to beat with regular frequencies, one to three cycles per second, and to continue motion for as long as a bend was maintained. It was possible on one occasion to stop and restart the same segment three times in succession. The only apparent limitation to the minimum length of flagellum that could be reactivated was the authors' facility at the manipulator. Fig. 2 includes three sequences, each showing a slight variation of the reactivation procedure. It can be seen that reactivation of a short distal segment is possible with the sperm still intact (Fig. 2A), or with the distal portion of the flagellum cut free (Fig. 2B). And reactivation can also be maintained in the absence of the probe if the piece can be kept bent by sticking it to the slide at both ends (as in Fig. 2C).

It is known that the coarse fibers in bull sperm taper (3) and

![Figure 1](image_url)

**Figure 1** The motion of mechanically reactivated bull sperm after inhibition with Ni²⁺. This figure was composed from tracings of 16-mm motion picture film and shows the position of an Ni²⁺-inhibited bull sperm after a manipulation and the resulting motility. The sperm was impaled in medium containing 0.3 mM Ni²⁺, 4 mM ADP, and 0.5 mM Mg²⁺. The series of superimposed tracings shows the motion of sperm over a one-cycle interval. The progression of the induced bend towards the distal tip of the flagellum is followed with arrows. Note the development of a reverse bend as the original bend nears the tip of the flagellum. The filming rate was 20 fps. The time of each traced frame measured from the first position is given at the base of the arrows in seconds. Magnification is indicated by a 20-µm bar. The experiment was performed at 23°C.

| TABLE I  |
|----------|
| Effect of Ni²⁺ on Beat Frequencies in Bull Sperm |
|          | No. Frequency | Average frequency |
|          | range (Hz)    | ± SD (Hz)         |
| Treatment | sperm         |                   |
| Control Cells | 15 | 2.9–1.3 | 2.0 ± 0.6 |
| 0.3 mM Ni²⁺ manipulated† | 15 | 1.8–0.7 | 1.0 ± 0.3 |

| Treatment | No. exp | No. sperm | Average frequency ± SD (Hz) |
|-----------|---------|-----------|-----------------------------|
| Triton X-100 demembranated cells§ | 20 | 240 | 3.4 ± 0.9 |
| Control samples 0.5 mM Ni²⁺‡ | 4 | 57 | 3.4 ± 1.1 |
| 6.0 mM ADP | 4 | 44 | 2.1 ± 0.6 |

* All sperm in a medium that contained 4 mM ADP and 0.5 mM Mg²⁺.
† Sperm were impaled and after cessation of motility the flagella were bent with a microprobe to reintiate motility.
‡ All experiments utilized Triton-extracted sperm supplied with 1 mM ATP and 1 mM Mg²⁺.
§ Only cells that were fortuitously attached to the microscope slide by the head and tip of the flagellum exhibited any wave motion in this condition.
that they are not present in electron micrographs of cross sections of the distal end of the flagella. The length of the distal portion of the flagellum that does not contain coarse fibers was estimated by examining a collection of electron micrographs. The electron micrographs were taken from thin sections of tightly packed, pelleted sperm, and therefore can be assumed to show a sampling of sperm cross sections distributed randomly along the flagellum. It was found that of 178 cross sections of the principal piece, 112 showed some coarse fibers present, and 66 did not. On the basis of these figures, we estimate that the longest coarse fibers terminate at 2/3 the length of the principal piece. The principal piece in bull sperm is ~50 μm in length; therefore the last 17 μm of the principal piece should be completely devoid of coarse fibers.

The three short segments that are displayed in Fig. 2 were induced to propagate bending waves and were active for many complete cycles of bending in the presence of Ni2+. The segments are of the appropriate length to test the hypothesis that the microtubular axoneme without coarse fibers is capable of propagating waves after Ni2+ treatment. On the basis of our findings, it appears that the activity observed after Ni2+ inhibition can be entirely accounted for by the microtubular axoneme, because the presence of coarse fibers is not necessary.

The effect of Ni2+ on the ATP-dependent sliding of microtubules was tested. Lindemann and Gibbons demonstrated that an ATP-dependent sliding of microtubules could be shown directly in bull sperm flagella (13). We found that it was possible to prepare samples of spermatozoa in a suitable manner to test the sliding interaction by freezing Triton-extracted sperm for 48 h at -20°C. This was done in the same medium used to produce demembranated sperm and reliably yielded sperm that would slide apart when ATP was added. Fig. 3A shows the condition of sperm after the freezing procedure. Note that the brightness of the middle piece is less than that of the principal piece, indicating the removal of the mitochondrial sheath. When ATP was added to the mixture the sperm were

![Figure 2](image-url)

**Figure 2** Reactivation of motility in small distal segments of flagella after Ni2+ treatment. Three series of photographs from motion picture film are displayed showing the motility that results when a short distal piece of a bull sperm flagellum is bent with a microprobe. In each series, alternate frames are shown in order to follow the motility through one cycle or more. The sperm were subjected to 0.5 mM Ni2+ for at least 5 min before manipulation and had lost all spontaneous motility. In series A the distal segment that is reactivated remains attached to the sperm. It can be seen that only the distal portion of the flagellum that is bent by the probe initiates movement. In series B a very short distal piece of the flagellum (~15 μm) is completely free of the parent cell and is manipulated into motility by bending it with the probe. Series C gives the best view of wave shape and wave propagation on an isolated short segment stuck to the slide at both ends. Note that the remainder of the sperm cell in each series is not spontaneously motile. Series A and B include a frame at the end of the sequence that shows the same short sections released from the probe and once again immotile. These released segments are useful to judge the length of the manipulated segments. A micrometer is provided in the lower right of the figure, ruled at 20-μm intervals. All three sperm displayed were extracted with 0.1% Triton X-100 in a medium containing 4 mM ADP, 1 mM ATP, and 1 mM Mg2+. The experiments were performed at 23°C and filmed at 24 fps. Phase-contrast microscopy using a × 40 Zeiss water immersion objective was employed for both manipulation and filming. Elapsed time is given in milliseconds above the columns of photographs.
observed during the extrusion of the internal fibers. The fibers appeared to buckle out from the middle piece region and slide out of the principal piece one after another or several at a time. Fig. 3 (B and C) shows the resulting condition of the sperm after sliding.

Frozen sperm were thawed and treated with 0.5 mM Ni$^{2+}$ 5 min before the administration of ATP. The process of sliding was not altered by the presence of Ni$^{2+}$. Fig. 3 shows a direct comparison of several sperm, slid both with (Fig. 3 C) and without (Fig. 3 B) Ni$^{2+}$. The number of sperm showing extruded microfilaments is not significantly reduced in the Ni$^{2+}$-treated samples. Table II presents the results of seven individual experiments to allow direct comparison of the sliding activity of the same sperm samples with and without 0.5 mM Ni$^{2+}$. The day-to-day variation in the sperm stock was generally a greater factor than the effect of Ni$^{2+}$. Overall (as presented in the bottom row of Table II), a slight decrease in responding cells may be present after Ni treatment. The difference in response in treated and untreated cells is probably not significant, for even with a reasonably large sample size it is not possible to assign a 0.05 level of confidence to the result using a one-tailed t-test analysis of the data. It is apparent that Ni$^{2+}$ does not cause serious impairment of microtubular sliding in bull sperm flagella.

![Figure 3](image-url)

Figure 3. Microtubular sliding in Ni$^{2+}$-inhibited bull sperm. (A) Triton X-100-treated bull sperm after 48 h of frozen storage at -20°C. Note the diminished brightness of the middle piece region (arrows), as compared to the principal piece, and the uneven appearance of that region. Sperm pictured were in Triton X-100 extraction media without ATP (see Materials and Methods). (B) Sperm prepared as in A but with the addition of 1 mM ATP. The figure shows the internal fibers of the sperm as they appear after extrusion from the sperm. (C) Sperm prepared as in A but with 5 min of incubation in 0.5 mM Ni$^{2+}$ and then subsequent addition of 1 mM ATP. Note that the extrusion of fibers is apparently the same as with no Ni$^{2+}$ present (in B). The sperm were viewed using dark-field light microscopy, and the prints were made from 35-mm still photographs taken with Kodak 2475 recording film to record fine detail at low light intensity. All experiments were done at ambient temperature (23°C). The horizontal bar indicates 20 μm.
A one-tailed test does not justify the assignment of a 0.05 level of confidence to the deviation of Ni$^{2+}$ tests from control samples.

### DISCUSSION

Bull sperm treated with Ni$^{2+}$ exhibit a pattern of responses consistent with normal microtubular function. The sperm can exhibit microtubular sliding after exposure to Ni$^{2+}$, and can propagate bending waves which testifies to the efficacy of the sliding mechanism in these sperm. The rapidity of motion that can be obtained from Ni$^{2+}$-treated sperm controverts any explanation for their spontaneous motility that is based on a decreased capacity to utilize energy. Yet, despite the apparently normal responses of the microtubules, Ni$^{2+}$-inhibited sperm cannot produce autonomously generated waves without imposed manipulation or restraint. When sperm are manipulated to induce motility the positioning that is effective always involves the induction of a bend between two fixed portions of the flagellum. The active response of the flagellum that follows involves a propagation of the formed bend distally along the flagellum and the formation of a new recurved (opposite) bend in the region proximal to the induced bend. This sequence of activity is not much different from that observed in normal sperm, with two notable exceptions. The inhibited sperm will not generate an initial bend without assistance and will not continue to form new bends if released from the restraining conditions.

It appears that the axoneme is the component of the sperm that retains function after Ni$^{2+}$ inhibition and that the propagation of formed waves is undoubtedly a role of the axoneme in bull sperm. The forces generated by the axoneme during the propagation of a formed wave would indeed tend to cause the formation of a recurve wave solely because of the confined condition of the flagellum during these observations. Theoretical models to explain the propagation of bending in cilia (20) and flagella (2, 10, 21) on the basis of a sliding tubule mechanism have produced remarkable facsimiles of the observed movement of flagella and cilia during wave propagation. From these studies and our current results it is altogether likely that microtubule sliding is a necessary and sufficient force-generating mechanism to explain the propagation of a formed wave on a flagellum or a cilium. It is also likely that a second mechanism within the axoneme or associated structures may play a crucial role in permitting or causing the development of the initial bend that is then propagated by the sliding tubule mechanism.

Evidence to support the existence of a separate mechanism for the initiation of bending can be found in recent studies of flagellar motility. Goldstein reported a two-step onset of motility after induced quiescence in sea urchin sperm (7, 8). First, a bend would form at the basal end of the flagellum; after a short delay, this initial bend would begin to propagate along the flagellum. Gibbons has shown that under certain conditions live sea urchin sperm will exhibit the initial bend without consequently propagating the bend along the flagellum in the usual fashion (4). This gives rise to the candy cane shape observed in some quiescent sperm. Further investigation of this curious behavior has shown that the same pattern of quiescence can be induced in demembranated sea urchin sperm models by controlled administration of calcium (6). Therefore, bend initiation can occur without consequent propagation, and the two functions can also be controlled experimentally (at least to a limited extent).

The most convincing evidence that a separate structure is responsible for the process of bend initiation is presented in the work of Witman et al. (25). They showed that a mutant strain of *Chlamydomonas* that is immotile also lacks the network of spokes that normally interconnect the doublet tubules and the central pair of tubules. In addition, they succeeded in demonstrating that the doublet tubules of the same mutant strain were still capable of exhibiting a normal sliding tubule mechanism. There is other good evidence to support the contention that the spoke assembly plays an active role in the generation of motility. Warner and Satir (24) have reported observing transitions in the positioning of the radial spokes that correlated with the bending of the flagella. Therefore, it is conceivable that the spoke assembly could serve as the structural basis for a mechanism of bend initiation in simple flagella.

It is reasonable to suggest from our data that the behavior of Ni$^{2+}$-inhibited sperm reflects the capabilities of an axoneme with normal dynein-tubulin function. It is tempting to imply that the coarse fibers are selectively inhibited by Ni$^{2+}$ and that they could be responsible for wave initiation in mammalian sperm. Although this may be the case in mammalian sperm, the argument does not seem so attractive when it is taken in the light of at least two reports that mention a toxic effect of Ni$^{2+}$ on invertebrate cilia and flagella (5, 18). The invertebrate flagellum has only the axoneme and no coarse fibers. Therefore, if the mechanism of action of Ni$^{2+}$ is the same in these simpler flagella, it would suggest that the target is within the axoneme but not at the dynein-tubulin interaction.

Recently, Sale and Gibbons (23) have demonstrated a direct inhibition of dynein-tubulin cross-bridge formation with vanadate. After vanadate treatment, both motility and microtubular sliding are blocked in sea urchin sperm flagella. This is in sharp contrast to the results we have obtained with Ni$^{2+}$. The work done with vanadate provides a demonstration of the effects produced by cross-bridge inhibition and makes it apparent that Ni$^{2+}$ is not inhibiting motility by the same action. In the light of the work reported for sliding and wave initiation in the simpler nonmammalian flagella, it is appropriate to suggest that the most likely site of action of Ni$^{2+}$ is the network of radial spokes.

The balance of ATP and Mg$^{2+}$ in the suspension media can produce a pattern of behavior in bull sperm almost identical to
that produced by Ni\(^{2+}\) (13). Unlike the effects of Ni\(^{2+}\), this effect is reversible. Both Ni\(^{2+}\) and the ratio of ATP to Mg\(^{2+}\) appear to interplay in some way on a mechanism controlling wave initiation. Our current results make it appear unlikely that microtubular sliding within the axoneme is the target of Ni\(^{2+}\). Experiments that are in progress to study the effect of Ni\(^{2+}\) on sperm ATPase and to locate the site of action of Ni\(^{2+}\) may shed further light on the mechanism responsible for wave initiation.

At present it can be concluded that wave initiation in bull sperm is not a necessary consequence of an intact microtubular sliding mechanism. Our work supports the hypothesis that the processes of wave propagation and wave initiation have a fundamentally different basis in origin.

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