

CALCIUM-INDUCED QUIESCENCE IN REACTIVATED SEA URCHIN SPERM

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ABSTRACT

Sperm flagella of the sea urchin *Tripneustes gratilla* beat with asymmetrical bending waves after demembration with Triton X-100 in the presence of EGTA and reactivation at pH 8.1 with 1 mM ATP in the presence of 2 mM MgSO₄. Addition of 0.1–0.2 mM free Ca²⁺ to these reactivated sperm induces 70–95% of them to become quiescent. This quiescence can be reversed by reduction of the free Ca²⁺ concentration with EGTA, or by dilution to reduce the MgATP²⁻ concentration below 0.3 mM. The quiescent waveform is characterized by a sharp principal bend of ~5.6 rad in the proximal region of the flagellum, a slight reverse bend in the midregion that averages ~0.3 rad, and a principal bend of ~1.1 rad in the tip. The quiescent sperm are highly fragile mechanically, and disruption, including microtubule sliding, occurs spontaneously at a slow rate upon standing or immediately upon gentle agitation. Mild digestion by trypsin causes a gradual appearance of normal, symmetrical flagellar beating. Addition of increasing concentrations of vanadate to quiescent sperm causes a graded decrease in the proximal bend angle, with 50 μM vanadate reducing it to ~2.6 rad. In the presence of 0.1 mM free Ca²⁺ and 10 μM vanadate, a characteristic, crescented stationary bend is induced in the demembrated sperm, without intermediate oscillatory beating, by the addition of either 0.1 or 1 mM ATP. In the absence of vanadate, these two concentrations of ATP produce asymmetric beating and quiescence, respectively. The results support the hypothesis that quiescence in live sperm is induced by an elevated concentration of intracellular Ca²⁺. In addition, they demonstrate that bending can occur in flagella in which oscillatory beating is inhibited and emphasize the close relationship between asymmetric beating and quiescence.

KEY WORDS dynein arms · regulation of microtubule sliding · vanadate · quiescent waveform · asymmetric beating

In this work we have sought to investigate directly the hypothesis of the preceding paper (11) that the quiescent phase of the intermittent swimming observed in sperm of *Tripneustes gratilla* is caused by a transient increase in the intracellular free Ca²⁺

concentration. Following the studies of others on the involvement of Ca²⁺ in ciliary arrest (35, 36), and in regulating the direction of ciliary and flagellar beating (24, 25, 29), we have looked to see if Ca²⁺ can induce a quiescent state in demembrated sea urchin sperm reactivated with MgATP²⁻.

Since the work of Brokaw et al. (4), the importance of Ca²⁺ in regulating the form of flagellar

bending waves in sea urchin sperm has been recognized. These workers showed that in reactivated sperm of the Californian sea urchin, *Strongylocentrotus purpuratus*, the degree of asymmetry of the bending waves can be assayed by the turning rate of the swim path, and that it depends on the Ca^{2+} concentration in both the demembrating and reactivation solutions. When *Strongylocentrotus* sperm had been demembrated with Triton X-100 in the presence of 0.5 mM EDTA and no added Ca^{2+} , their flagellar beat was asymmetric at a Ca^{2+} concentration as low as 1 nM in the reactivation solution and became increasingly asymmetrical in response to increasing free Ca^{2+} . At a Ca^{2+} concentration of 1 μM , the asymmetry was so extreme that the turning rate of most of the sperm was too great to be measured by the technique used. On the other hand, when the demembrating solution had contained 5 mM Ca^{2+} , although the response to increasing Ca^{2+} in the reactivating solution was qualitatively similar, a relatively much higher concentration of Ca^{2+} was required to produce a given degree of asymmetry, and in reactivating solution containing $<0.1 \mu\text{M}$ Ca^{2+} , the flagellar beat was so nearly symmetrical that the sperm swam in paths with little or no curvature.

In the present work, we have confirmed that sperm of a Hawaiian species of sea urchin, *T. gratilla*, behave similarly to those of *Strongylocentrotus*. For ease of description, we propose to call sperm that have been demembrated in the presence of EGTA with no added Ca^{2+} "potentially asymmetric sperm," because when these sperm are subsequently reactivated with 1 mM MgATP^{2-} their flagellar waveforms are markedly asymmetric, even in reactivating solution that contains EGTA and no added Ca^{2+} .¹ Likewise we shall use the term potentially symmetric sperm for preparations demembrated in the presence of 5 mM Ca^{2+} , because the waveforms of these sperm are nearly symmetric when they are reactivated with

¹ We use the term "potentially asymmetric" for these sperm because the asymmetry of their flagellar waveform is expressed only when they are reactivated with a high concentration of ATP. In unpublished experiments, we have found that the flagellar asymmetry of such sperm decreases markedly when they are reactivated in low concentrations of ATP; and, for example, at 12 μM ATP, which is just above the minimum concentration needed for motility, the flagella beat almost symmetrically, with nearly equal angles in the principal and reverse bends.

1 mM MgATP^{2-} in the presence of EGTA and no added Ca^{2+} .

Sperm of a second Hawaiian species of sea urchin, *Colobocentrotus atratus*, show a substantially diminished susceptibility to Ca^{2+} under most conditions, although their susceptibility is increased if the demembrated sperm are depleted of dynein by brief extraction with 0.5 M KCl (13).

In this report, we describe conditions under which potentially asymmetric *Tripneustes* sperm reactivated with MgATP^{2-} become quiescent in response to added Ca^{2+} , and exhibit a quiescent waveform that resembles that of quiescent live sperm (11). The quiescent reactivated sperm resume swimming when the free Ca^{2+} concentration is reduced by addition of EGTA. We have investigated the detailed conditions that are required to produce quiescence and have examined some of its properties, including the effect of vanadate on the waveform. The results corroborate the hypothesis of the preceding paper that quiescence is induced by an elevated concentration of intracellular Ca^{2+} , although it is possible that there may be some differences between the regulatory pathways involved in live and demembrated cells. We also present evidence that the mechanisms responsible for quiescence and for asymmetric beating are closely related, and that quiescence in reactivated sperm is an active state in which certain of the dynein arms remain potentiated while others are inactive.

MATERIALS AND METHODS

T. gratilla sperm were collected in seawater containing 0.2 mM EDTA, and stored as a stock suspension (3–10 mg protein/ml) for up to 4 h at room temperature as described in the preceding paper (11).

In our standard procedure for producing quiescence in reactivated sperm, the sperm were demembrated by transferring 25 μl of the stock suspension of sperm to 0.3 ml of Triton-EGTA extraction solution (10 mM Tris-HCl, pH 8.1, 2 mM EGTA, 1 mM dithiothreitol, and 0.05% (wt/vol) Triton X-100) at room temperature (22°–23.5°C). This extraction solution differs from the one in our preceding papers (12, 15) in that it contains no KCl or MgSO_4 , because we have found that this change greatly improves the quality and longevity of reactivated sperm of *Tripneustes*, and in that it contains 2 mM EGTA rather than 0.5 mM EDTA, because this is required to produce potentially asymmetric sperm. After 20–30 s extraction, 50 μl of extraction solution containing sperm were transferred to reactivating solution (10 mM Tris-HCl, pH 8.1, 0.15 M KCl, 2 mM MgSO_4 , 0.1 mM EGTA, 1 mM dithiothreitol, 2% [wt/vol] polyethylene glycol [20,000 mol wt], and 1 mM ATP), the swimming motion and percentage of motile sperm were checked under the microscope, and then 0.2–0.3 mM Ca^{2+} (from a stock solution of 0.1 M CaCl_2 in water) was added. This protocol will be referred to as the standard procedure. In some experiments, we varied the order of

the addition of sperm, Ca^{2+} , and ATP, or we varied the concentration of MgSO_4 , Ca^{2+} , or ATP, as described in Results. For the sake of brevity, we shall use the term "free Ca^{2+} " to denote the total Ca^{2+} concentration minus the EGTA concentration (which is 0.1 mM in most cases). Some of this Ca^{2+} will in fact be bound to ATP and to proteins, but we believe that this amount is not significant to the discussion except where noted. Magnesium concentrations are expressed as total added MgSO_4 . Light microscopy and photography were performed at room temperature as described in the preceding paper (11), except that with the water immersion lens, it was found best to omit polyethylene glycol from the reactivating solution.

CaCl_2 was either reagent grade from J. T. Baker Chemical Co., Phillipsburg, N. J., or "ultrapure" from Merck Chemical Div., Merck & Co., Inc., Rahway, N. J., and the two gave identical results. ATP was obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind., and EGTA was from Sigma Chemical Co., St. Louis, Mo. Sodium metavanadate from Fisher Scientific Co., Pittsburgh, Penn., was recrystallized as described earlier (19).

RESULTS

Quiescence in Reactivated Sperm

Before the addition of Ca^{2+} in the final step of our standard procedure, the level of free Ca^{2+} in the reactivating solution is very low, since the total Ca^{2+} carried over from the seawater is about 10 μM and is far less than the EGTA concentration. The flagella of the potentially asymmetric sperm added to this solution beat in a highly asymmetric manner that causes the sperm to swim in small circles that correspond to a turning rate of ~ 0.6 rad/beat, in general agreement with the observations of Brokaw et al. (4). We have found that addition of 0.1 mM free Ca^{2+} to such preparations causes 70–95% of the sperm to stop swimming (Fig. 1) with their flagella uniformly bent into a quiescent waveform that resembles the quiescent waveform seen in live sperm (Fig. 2 of reference 11), although the angles of the proximal and tip bends are larger. In most preparations the percentage of quiescent sperm reaches its final value within the time required for mixing the added Ca^{2+} , although we occasionally found that the percentage would increase by 10–20% in the minute or two after addition of Ca^{2+} or that the percentage could be increased by increasing the temperature of observation to 25°C. Among the sperm that remain motile, the flagella of some beat irregularly with waves of very low amplitude in the proximal region only and barely produce any forward progression, while others beat more vigorously with waves propagating about two-thirds of the length of the flagellum, causing the sperm to swim at the bottom of the dish in circles of even

smaller radius than before the addition of Ca^{2+} .

It was of interest to determine whether non-beating flagella would bend into the same quiescent waveform when the ATP was added last to reactivating solution already containing nonmotile demembrated sperm and 0.1 mM free Ca^{2+} , and to see if the result was dependent upon whether the flagella of the nonmotile sperm were straight, or bent in stationary rigor waves before the addition of ATP. When the suspension of sperm that have been in the demembrating solution for 20–30 s according to the usual procedure is diluted into reactivating solution containing no ATP or Ca^{2+} , the flagella of most of the sperm become set into rigor waves because the level of sperm-derived endogenous ATP in the extracting solution at this time is at an appropriate low level for preservation of rigor waves upon dilution (14). If, on the other hand, the sperm remain in the extraction solution long enough for this endogenous ATP to be hydrolyzed (~ 4 min), then the flagella are more nearly straight after dilution (Fig. 2a). The addition of 0.1 mM free Ca^{2+} to either of these two forms of potentially asymmetric sperm in the absence of ATP causes no apparent change in the shape of their flagella. However, upon subsequent addition of 1 mM ATP the flagella of both forms bend into a quiescent form (Fig. 2b) which is indistinguishable from that of sperm prepared by the standard procedure. This result suggests that it is not necessary for the flagella to be beating to bend into the quiescent waveform, although the possibility that a brief period of flagellar beating occurred during the mixing of the sperm with ATP is not completely excluded.

Effect of Ca^{2+} Concentration

Experiments in which different concentrations of Ca^{2+} were used to induce quiescence have shown that free Ca^{2+} in the range 0.1–0.2 mM seems to be the most effective and typically produces quiescence in 70–95% of the sperm in our standard procedure. However, quiescence in up to 50% of the sperm has been obtained with a free Ca^{2+} concentration of ~ 0.05 mM. With supraoptimal levels of free Ca^{2+} , such as 1 or 2 mM, only 5–20% of the sperm become quiescent, while the rest swim vigorously and uniformly in circles of small radius at the bottom of the dish, with approximately the same asymmetry as before the addition of Ca^{2+} . The percentage of quiescent sperm is even lower if 2 mM free Ca^{2+} is added to the dish before addition of the ATP.

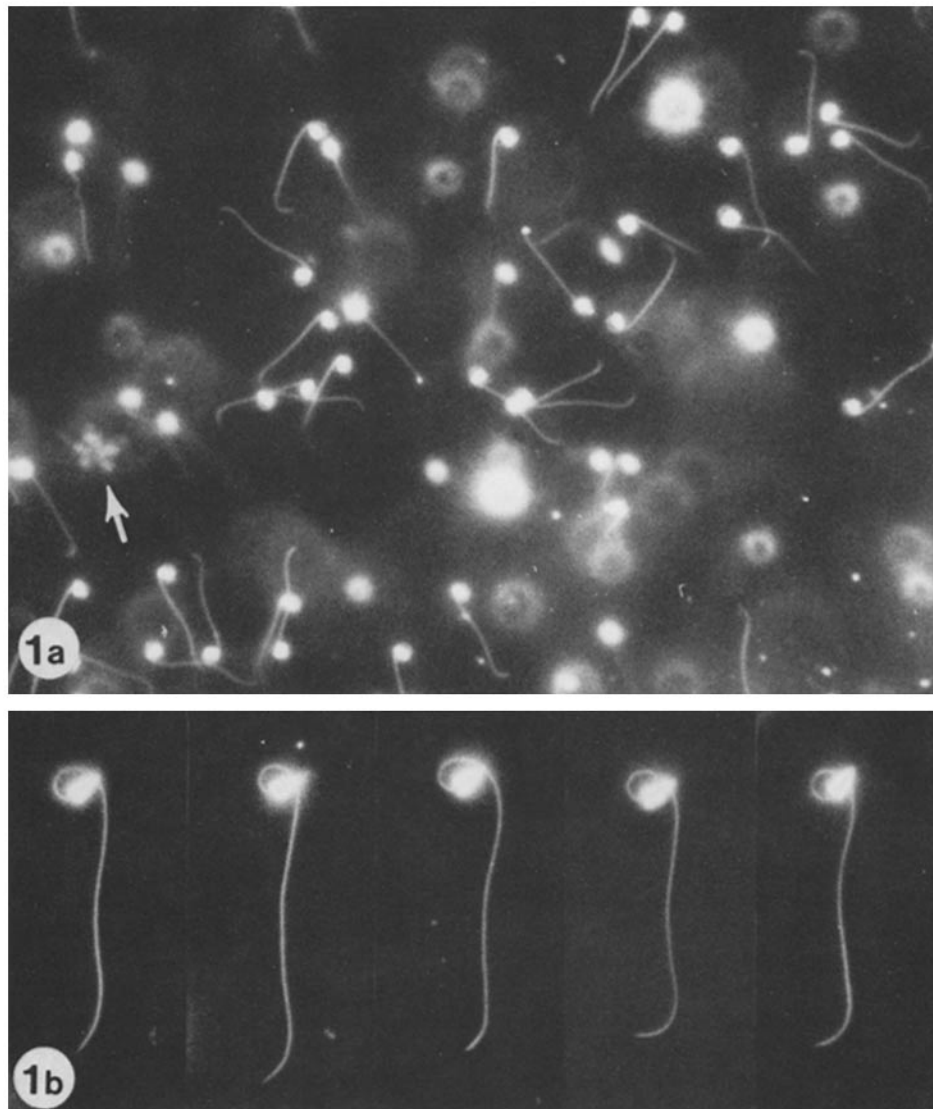


FIGURE 1 Dark-field micrographs of reactivated sperm of *Tripneustes* that have become quiescent after addition of 0.1 mM free Ca^{2+} according to the standard procedure. (A) Micrograph made at low magnification. The asymmetric movement of one sperm may be seen as a star-shaped image at left center (arrow). The resolution of the axoneme close to the head in these sperm is poorer than that of quiescent live sperm (Figs. 1 and 4 of reference 11), because of the greater angle of the proximal bend and because demembrated flagella scatter less light than do intact ones. Since the sperm are floating in free fluid away from the bottom surface, only a few are lying in a plane perpendicular to the viewer. Exposure, $\frac{1}{2}$ s \times 380. (B) Micrographs taken at high magnification showing the flagellar waveforms of individual quiescent reactivated sperm. Exposure: flash. \times 930.

Effect of Changes in Composition of Reactivating Solution

We have studied the effect of ATP concentration on the induction of quiescence by Ca^{2+} in our

standard procedure and have found that a minimum concentration of ~ 0.7 mM ATP (in the presence of 2 mM MgSO_4 and 0.1 mM free Ca^{2+}) is required to obtain the maximum percentage of quiescence. Raising the ATP concentration to 5

mM with either 5 or 7 mM MgSO_4 appears to produce the same results as 1 mM ATP, and in particular the percentage of quiescent sperm does not increase beyond the usual value of 70–95%. Lowering the ATP concentration produces a substantial effect, however, and in 0.5 and 0.3 mM ATP, only ~50 and 10%, respectively, of the sperm become quiescent.

Changes in the MgSO_4 concentration (in the presence of 1 mM ATP and 0.1 mM free Ca^{2+}) have shown that ~0.6 mM MgSO_4 is needed to produce maximum quiescence. At MgSO_4 concentrations below this level, an increasing percentage of the sperm remain motile after the addition of 0.1 mM free Ca^{2+} , until almost all the sperm

remain motile at MgSO_4 concentrations of 0.3 mM and less. Taken together these results indicate that a MgATP^{2-} concentration of at least 0.6 mM is necessary to obtain a maximum percentage of quiescence. However, in apparent contradiction to the result obtained with ionophore-treated live sperm (11), addition of 10 mM MgSO_4 to a standard preparation of quiescent reactivated sperm did not induce any resumption of beating.

Preliminary results on the effect of pH indicate that quiescence can be produced when the reactivating solution is at any pH within the range 7.7–8.5 and the standard procedure is followed in other respects. It is notable that the angle of the proximal bend decreases substantially, and the reverse bend

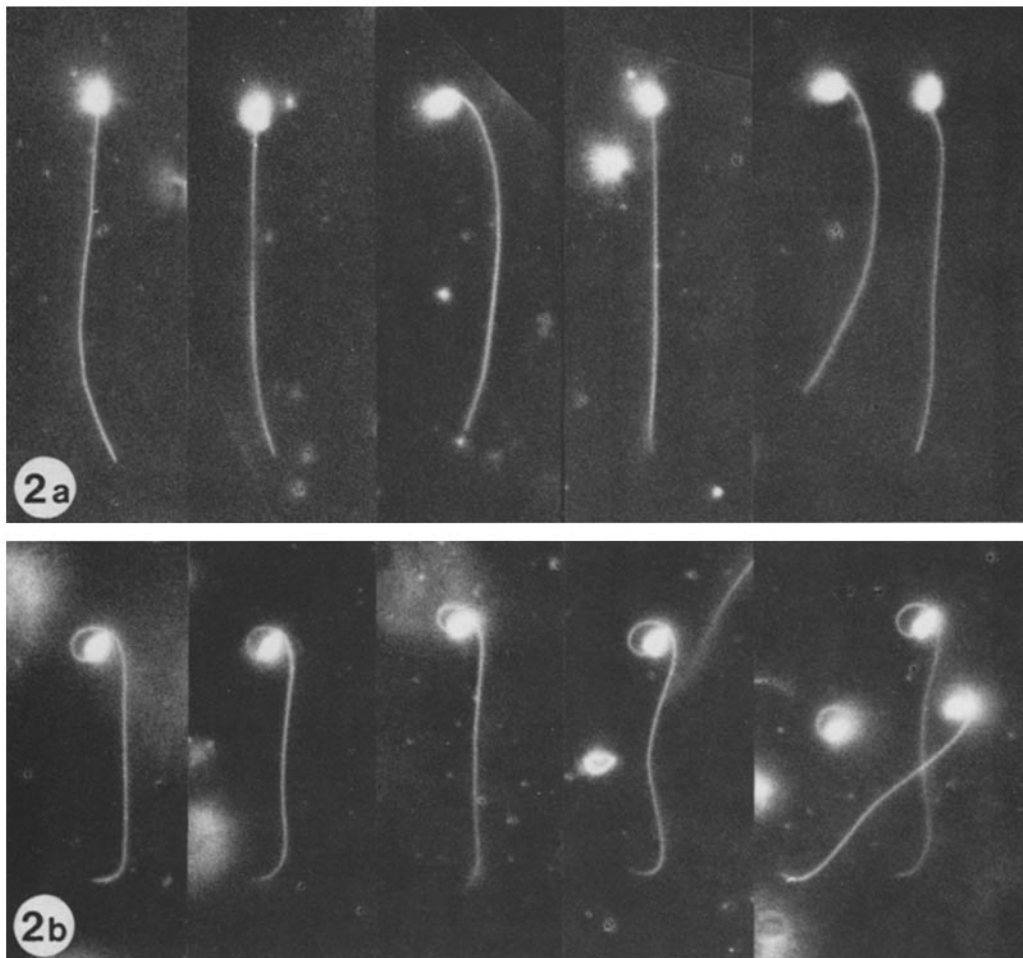


FIGURE 2 Dark-field micrographs showing individual, potentially asymmetric sperm suspended in reactivating solution containing 0.1 mM free Ca^{2+} . (A) Without ATP; (B) same preparation after addition of 1 mM ATP. Exposure: flash. $\times 930$.

in the midregion of the flagellum is lost, as the pH is reduced through the narrow range 8.0–7.7, so that the quiescent waveform of the reactivated sperm at the lower pH resembles more closely that of the quiescent live sperm. A more complete study of the properties of the reactivated quiescent sperm at lower pH is in progress.

Properties of the Quiescent Sperm

In quiescent reactivated sperm produced by the standard procedure (Fig. 1), as in live sperm, the flagellum curves around sharply in the region near the head, forming a large angle proximal bend measuring ~ 5.6 rad (range 5.4–5.9 rad; number of sperm measured, $n = 12$). By analogy to the similar waveform in quiescent live sperm (11), this bend will be considered to be in the principal bend direction. The middle portion of the flagellum usually exhibits a slight reverse bend with an average angle of 0.3 rad, although in some sperm this bend is absent while in others it is quite pronounced (0–0.5 rad, $n = 12$) (Figs. 1*b* and 2*b*). In the tip region of the flagellum, there is a second principal bend with an average angle of 1.1 rad, (0.5–1.6 rad; $n = 12$). The total change in angular direction between the axis of the head and the tip of the flagellum (ϕ_{tip}) is thus extremely high, being as much as 7.2 rad in some cases (Fig. 1*b*), and averaging 6.4 rad.

The average angle of the proximal bend in these quiescent demembrated sperm is substantially greater than the value of 3.5 rad found in quiescent live sperm (11), and it causes the tip of the sperm head to cross back over the flagellum. The resultant nonplanarity of the quiescent waveform is apparent in the lack of perfect focus in the head region of the images in Fig. 1*b*. This twisting can be seen directly in flagella of sperm that are floating with their plane of bending parallel to the optical axis, and preliminary observations suggest that right-handed and left-handed twisted forms occur in about equal numbers. The average angle of the tip bend, 1.1 rad, in quiescent demembrated sperm is also larger than the average value of 0.4 rad found in quiescent live sperm (11).

A striking characteristic of quiescent reactivated sperm is their mechanical fragility, which is very different from the mechanical stability of rigor wave sperm (14). Gentle agitation of quiescent reactivated sperm, such as that caused by drawing them up into and delivering them from a Pasteur pipette, produces a variety of changes including a

decrease in angle of the proximal bend, fraying of microtubules from the tip end of the axoneme, and kinks and other lesions in the axonemal structure. When allowed to stand for a short time at room temperature, even without agitation, most preparations of quiescent sperm show a gradual distortion of the initial quiescent waveform, and after 20–30 min a majority of the sperm flagella exhibit drastic shape changes that often include sliding and looping out of some microtubules from the main axonemal bundle (Fig. 3). Sperm flagella in which some tubules have slid out from the axonemal bundle usually show a diminution in proximal bend angle, presumably because the sliding of some tubules partially alleviates the shear stress in the quiescent axoneme. In a few instances, these separated bundles of tubules have been observed to propagate repeated bending waves at a frequency of ~ 2 Hz, but we have not yet been able to characterize their motion in detail. Similar bending waves have been described in split flagella of *Chlamydomonas* by Nakamura and Kamiya (30).

We have investigated the reversibility of the quiescent state by adding 2 mM EGTA subsequent to the addition of Ca^{2+} . The degree of reversal obtained has varied from one preparation to another, and we think it likely that the considerable

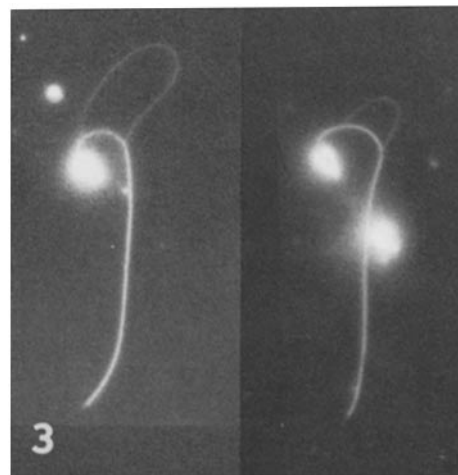


FIGURE 3 Dark-field micrographs showing flagellar disruption that occurs in a preparation of quiescent reactivated sperm on brief standing. Two examples show looping out and sliding of a small bundle of microtubules, with concomitant decrease in proximal bend angle. Exposure: flash. $\times 930$.

shear strain in the quiescent waveform causes fairly rapid mechanical tearing of the axonemal structure in a way that interferes with reversal of quiescence. In some instances, when EGTA was added immediately after the preparation had been checked for quiescence, ~80% of the quiescent sperm resumed swimming asymmetrically in the manner seen before the addition of Ca^{2+} . However, if the addition of EGTA was delayed for 4–5 min, then little reversal was usually obtained. If 2 mM EGTA was added to a dish containing reactivating solution, 0.2 mM free Ca^{2+} , and 1 mM ATP before the addition of potentially asymmetric sperm, the sperm swam in the manner seen in the standard procedure before the addition of Ca^{2+} , indicating that the failure to obtain complete reversal upon addition of EGTA after quiescence has been induced is not due to the affinity of the flagellar receptor protein for Ca^{2+} being too high for the Ca^{2+} to be removed by the EGTA.

Quiescence could also be reversed by lowering the ATP concentration to 0.2 mM or less by diluting the suspension of quiescent sperm into additional reactivating solution containing 0.1 mM free Ca^{2+} , but no ATP. With a given preparation of quiescent sperm, the degree of reversal obtained by lowering the ATP concentration was usually greater than that obtained by lowering the Ca^{2+} concentration, and in particular the former appeared to be less dependent on the age of the quiescent sperm. In seven trials performed at times ranging up to 5 min after the induction of quiescence, between 80 and 100% of the quiescent sperm became motile when the ATP concentration was lowered to 0.2 mM.

Treatment of freshly prepared quiescent sperm with trypsin (0.1 $\mu\text{g}/\text{ml}$ reactivating solution) causes gradual resumption of flagellar beating. This beating is at first of very low amplitude, and is largely confined to the proximal region of the flagellum. Over a period of a few minutes of incubation with trypsin, the flagella beat more and more actively, and the large angle of the proximal bend diminishes together with much of the wave asymmetry, until after ~10 min, close to 100% of the sperm swim in circles of large radius similar to those of potentially symmetric sperm reactivated at very low Ca^{2+} concentration. More prolonged exposure to trypsin eventually causes cessation of movement and axonemal disintegration. The exact manner in which the quiescent flagella resume beating and in which the large proximal bend is

dissipated during the initial stages of digestion should be amenable to analysis by microcinematography.

Effect of Vanadate on Quiescence

In view of the recently discovered usefulness of vanadate as an inhibitor of ciliary and flagellar motility (7, 19, 26, 32), we have examined its effect on quiescent reactivated sperm, adding it either before or after the addition of ATP. To investigate whether the quiescent waveform can form in flagella in which oscillatory beating is completely inhibited with vanadate, we added 5 μM vanadate and 0.1 mM free Ca^{2+} to preparations of potentially asymmetric sperm with nearly straight flagella. In the absence of ATP, these additions caused no change in the waveform. Upon subsequent addition of 1 mM ATP, the flagella bent uniformly into the waveform shown in Fig. 4a, with a sharp bend at the proximal end of the flagellum as it leaves the head, followed by a gradual, fairly uniform bend of curvature $\sim 0.022 \mu\text{m}^{-1}$ over the rest of the flagellum. Since there is no straight portion in the midregion of the flagellum and consequently no well defined proximal bend, we used the angle between the axis of the head and the flagellar tip, ϕ_{tip} , as a measure of the degree of bending. In 5 μM vanadate, the value of ϕ_{tip} averages 3.1 rad (3.0–3.3 rad; $n = 16$). With a higher concentration of vanadate, 50 μM , subsequent addition of 1 mM ATP produces the gently curving waveform shown in Fig. 4b. In this case, ϕ_{tip} averages 1.1 rad (0.8–1.4 rad; $n = 11$) and the sperm appear to form a fairly smooth arc from the tip of the head to the tip of the flagellum. The curvature of this arc is about the same as that of the flagella in 5 μM vanadate (Fig. 4a), and the principal difference between the waveforms at the two concentrations of vanadate is that the flagella of the sperm in 50 μM vanadate completely lack the sharp bend between the axis of the head and the basal region of the flagellum. When the concentration of vanadate is raised as high as 1 mM, then subsequent addition of 1 mM ATP induces no significant bending of the flagellum (Fig. 4c and d). Although most of these experiments were performed with sperm that initially had straight flagella, trial experiments with 5 μM vanadate suggested that the same results were obtained starting with sperm that initially had their flagella bent into rigor waves (14).

When the sperm flagella are made to bend into

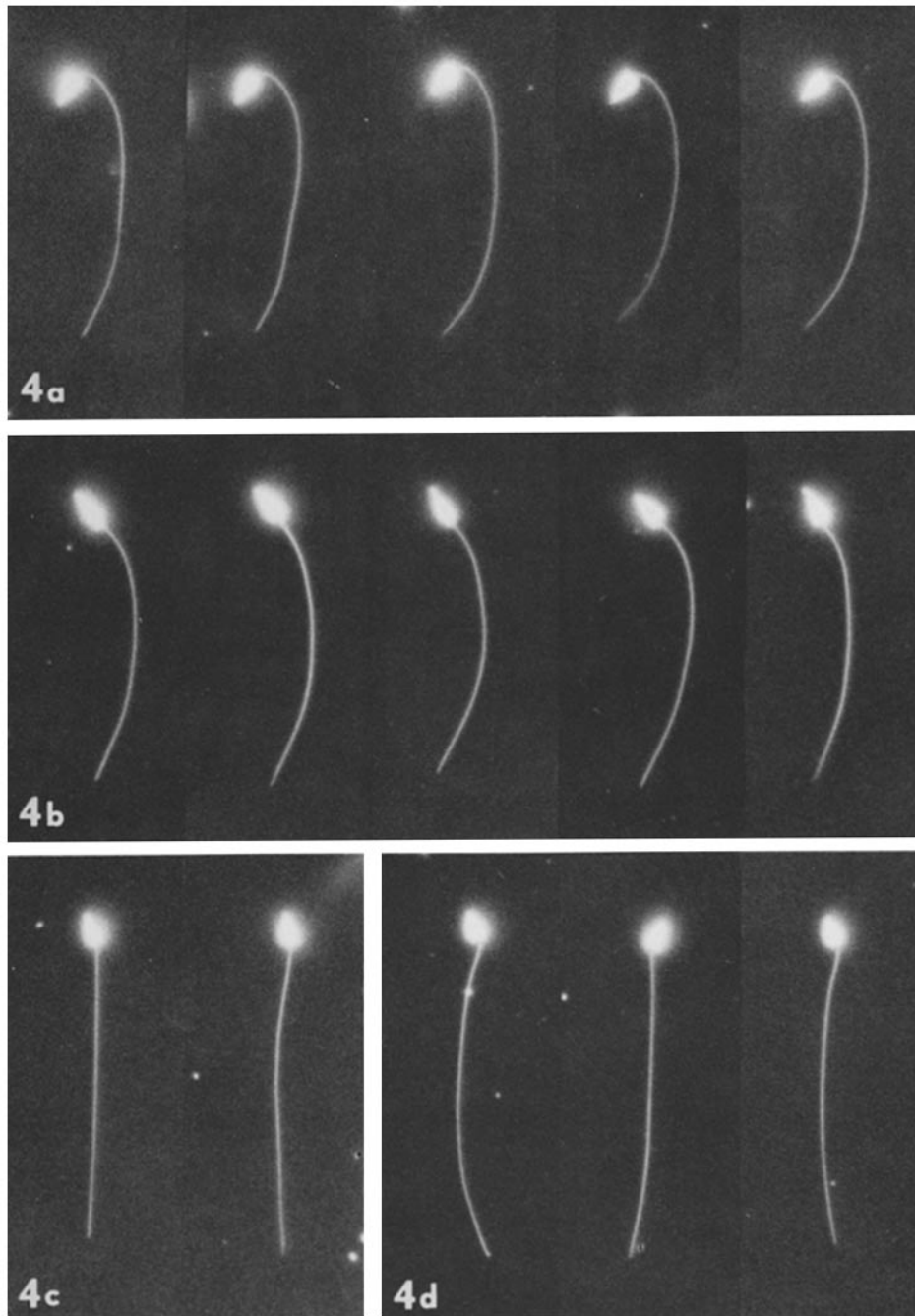


FIGURE 4 Dark-field micrographs showing the flagellar bending induced when 1 mM ATP is added to potentially asymmetric sperm in which oscillatory beating is inhibited by vanadate. Sperm were suspended in reactivating solution containing 0.1 mM free Ca^{2+} and vanadate before the addition of ATP. The sperm are stationary. (A) 5 μM vanadate, followed by ATP. $\times 930$. (B) 50 μM vanadate, followed by ATP. $\times 930$. (C) 1 mM vanadate, without ATP. $\times 880$. (D) 1 mM vanadate, followed by ATP. $\times 880$. Exposure: flash.

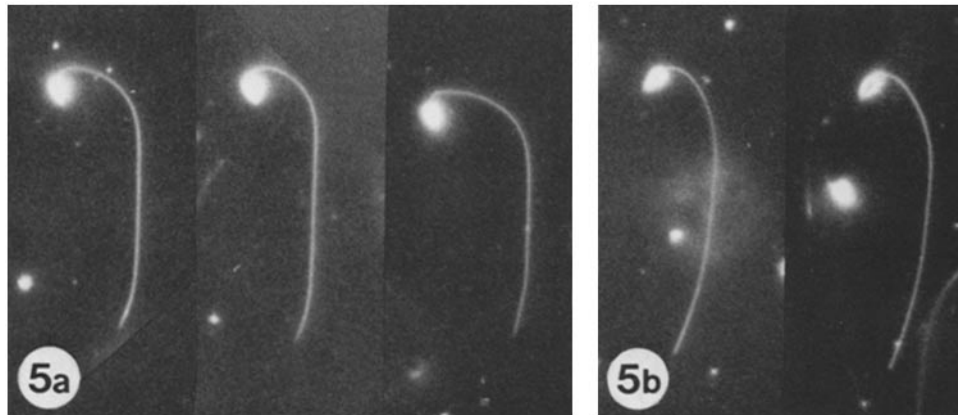


FIGURE 5 Dark-field micrographs showing the effect of adding vanadate to preparations of sperm that have already been made to bend into the quiescent waveform by the addition of 0.1 mM free Ca^{2+} and 1 mM ATP. (A) 10 μM vanadate added ~ 2 min after induction of quiescence. (B) Same, but with 50 μM vanadate added. Exposure: flash. $\times 830$.

the standard quiescent waveform by addition of 0.1 mM Ca^{2+} and 1 mM ATP before the addition of vanadate, the final waveforms obtained depend on both the concentration of vanadate and the length of time the flagella spend in the quiescent waveform before the vanadate is added. When 5, 10, or 50 μM vanadate is added immediately after addition of the ATP, the waveforms obtained appear essentially the same as those described above for vanadate added before the ATP (Fig. 4a and b). However, if the addition of vanadate is delayed so that the sperm spend longer than ~ 1 min in the fully bent quiescent form, then the waveforms obtained upon addition of vanadate differ in certain characteristic and reproducible properties. In particular, the curvature is distributed less uniformly over the length of the flagellum, and the value of ϕ_{tip} is greater. With 10 μM vanadate (Fig. 5a), the curvature is localized into the proximal 10 μm of the flagellum so that the midregion is nearly straight, and the value of ϕ_{tip} averages 3.6 rad (3.3–3.9 rad; $n = 15$). With the same concentration of vanadate added pre-ATP, the waveform is essentially like that in Fig. 4a, and the average value of ϕ_{tip} is 3.2 rad. When 50 μM vanadate is added >1 min post-ATP (Fig. 5b), the whole flagellum is curved, but the degree of curvature increases toward the base with a fairly sharp bend in the proximal 2 μm ; the value of ϕ_{tip} averages 2.6 rad (2.1–3.1 rad; $n = 15$). Comparison with the waveform obtained with 50 μM vanadate added pre-ATP (Fig. 4b), suggests that most of the greater bend angle of the former results from the

greater curvature in the basal region of the flagellum. The basis of the altered waveform obtained when vanadate is added $\sim >1$ min post-ATP is not yet clear, but it does not appear to be the result of progressive random structural damage to the flagellum while it is bent into the highly stressed rigor waveform, because the final waveform obtained is not altered when the time of vanadate addition is changed within the range 1–5 min post-ATP.

The value of the proximal bend angle in quiescent flagella is highly sensitive to low concentrations of vanadate, and addition of as little as 1 μM vanadate post-ATP causes a decrease in ϕ_{tip} from its usual value of 5.6 rad to ~ 4 rad. The reverse bend in the midregion of quiescent flagella disappears completely at a vanadate concentration of 5 μM .

Addition of 2.5 mM catechol (19) reverses the effects of vanadate concentrations up to 50 μM , and over a period of ~ 1 min the waveform reverts to the typical quiescent form (Fig. 1b).

In standard preparations of quiescent sperm to which 10 μM vanadate or more has been added, the waveform is stable with time, and the flagella appear to be resistant to mechanical damage when the sperm are subjected to gentle agitation with a Pasteur pipet. No looping out or fraying of microtubule bundles is seen, and if the inhibition by vanadate is reversed by adding 2.5 mM catechol after 15 min, the sperm resume the standard quiescent waveform, with visible damage in only $\sim 10\%$ of the sperm as compared to 90% in otherwise

equivalent sperm aged in the absence of vanadate. When the quiescent sperm are aged in the presence of 5 μM or less vanadate added pre-ATP, their waveform is often unstable, and over a period of 15–30 min it changes slowly from that shown in Fig. 4a to one that resembles that in Fig. 5a.

To obtain information about the relationship between asymmetric beating and quiescence, we examined the effect of two concentrations of ATP, 0.1 mM and 1 mM, on the bending of straight flagella of potentially asymmetric sperm in which oscillatory beating had been inhibited by addition of 10 μM vanadate before the addition of ATP. The rationale for this experiment is that were it not for the presence of vanadate the flagella would be beating asymmetrically in 0.1 mM ATP and would be quiescent in 1 mM ATP. The results showed that, in the presence of 0.1 mM Ca^{2+} and 10 μM vanadate, addition of either concentration of ATP caused the flagella to assume crescented waveforms closely resembling those in Fig. 4a, with an average ϕ_{tip} of 2.6 rad in 0.1 mM ATP and 3.0 rad in 1 mM ATP.

Lack of Quiescence in Symmetrically Beating Sperm

As yet we have been unable to induce a high percentage of quiescence in potentially symmetric sperm reactivated with ATP. The highest levels of quiescence were obtained by adding 0.1–0.3 mM free Ca^{2+} to the reactivated sperm first so as to induce relatively asymmetric beating (4), and then adding an additional 0.5 mM Ca^{2+} . This procedure usually caused ~5–10% of the sperm to become quiescent with the typical waveform, although in occasional preparations the percentage was as high as 50%. In all cases, the rest of the sperm in the preparations continued to swim with asymmetric waveforms.

We have also attempted to induce quiescence in reactivated sperm of *Colobocentrotus* using the standard procedure developed for *Tripneustes*, but the percentage of quiescence obtained was usually only 5–10%. In quiescent reactivated sperm of *Colobocentrotus*, the angle of the proximal bend was ~3 rad, substantially less than that in reactivated sperm of *Tripneustes*, although similar to that in quiescent live sperm of *Tripneustes* (11).

DISCUSSION

The present results demonstrate that a uniform quiescent state can be induced by addition of Ca^{2+}

to demembrated *Tripneustes* sperm, and that many of the properties of this state resemble those of quiescence in live sperm. The waveform of quiescent reactivated sperm is similar to that of quiescent live sperm, although the angle of the proximal bend is substantially greater. With appropriate care, quiescence in reactivated sperm can be reversed by reduction in free Ca^{2+} concentration through addition of excess EGTA, or by a reduction in ATP concentration through dilution. In both reactivated sperm and ionophore-treated live sperm, a free Ca^{2+} concentration in the range 0.05–0.1 mM is sufficient to induce quiescence in most of the sperm (11).

Our observations indicate that Ca^{2+} -induced quiescence represents an active state of the flagellum in which many of the dynein cross-bridges remain potentiated to produce sliding between the doublet tubules of the axoneme. This conclusion is supported primarily by the mechanical fragility of quiescent flagella and by their requirement for the continued presence of a high concentration of MgATP^{2-} , both of which are completely contrary to the properties of flagella in a rigor state (14).

On the basis of our results with both reactivated and live sperm (11), we think it most likely that the waveform of quiescent flagella is largely the result of an asymmetrical distribution of activity among the dynein arms, in which the arms responsible for generating reverse bends on the flagellum are inhibited, presumably in a relaxed state, by the presence of Ca^{2+} , while those responsible for generating principal bends remain potentiated. This conclusion is supported most directly by the fact that aging or gentle mechanical agitation of quiescent reactivated flagella causes small bundles of microtubules to slide out and form a loop on the axoneme, with a concomitant decrease in the angle of the proximal bend, and by the fact that small groups of tubules that have separated from quiescent axonemes are capable of propagating repeated bending waves. Evidence that tubule sliding of the former type is powered by the dynein arms on adjacent doublet tubules by a mechanochemical cross-bridge cycle driven by dephosphorylation of MgATP^{2-} (13, 34, 37) has been obtained previously by study of isolated flagellar axonemes briefly digested with trypsin. Additional, less direct support is provided by two other properties of quiescent flagella, namely, the graded decrease in proximal bend angle with increasing concentrations of vanadate, which is in qualitative accordance with the known sensitivity of the dy-

nein cross-bridge to inhibition by vanadate (19, 32), and the requirement for continued presence of a high concentration of MgATP^{2-} . The latter, in particular, appears to exclude the possibility of a purely Ca^{2+} -driven mechanism like that underlying the contraction of the spasmoneme in the ciliate *Vorticella* (1), since such a mechanism would require only a low level of MgATP^{2-} sufficient to maintain the dynein arms in a detached state. Bending as a result of active sliding between the two central tubules appears to be precluded, because this would produce bending within the plane of the central tubules, perpendicular to the normal plane of beat, and so would presumably lead to three-dimensional waves during stopping and starting transients; such nonplanar beating has not been observed (20, 22). Thus, at the present time, the most likely basis of the quiescent waveform appears to be a Ca^{2+} -induced asymmetry in the distribution of dynein cross-bridge activity on the nine doublet tubules of the axoneme. This hypothesis receives some independent support from the preliminary report of three distinct classes of dynein (from *Paramecium* cilia) that differ in their response to regulatory concentrations of Ca^{2+} (10).

The disruption caused by mild mechanical agitation indicates that the flagella of quiescent reactivated sperm are very highly stressed, and are at the point of tearing themselves apart. If we accept for the present that axonemal twisting does not occur to any major extent in quiescent reactivated flagella and that the effective diameter of the axoneme is 165 nm (17, 39), then the average proximal bend angle of 5.6 rad and the tip angle of 1.1 rad correspond to total sliding displacements between each pair of adjacent doublet tubules of ~280 nm in the midregion of the flagellum and 360 nm at the tip (33). This displacement would represent at least a tenfold extension of the nexin links that are thought to be the principal structures limiting the amount of sliding between tubules and maintaining the integrity of the beating axoneme, for the nexin links appear to have a rest length of ~18–30 nm in straight axonemes (16). While so great an extensibility is quite exceptional for a protein structure, there is independent electron microscope evidence that the nexin links are highly elastic and capable of being stretched to ~300 nm (8, 31, 38). Our data showing the mechanical fragility of quiescent reactivated flagella suggest that this great an extension is close to the elastic limit of the nexin links. The random frac-

ture of a small number of nexin links in any highly stressed region of the axoneme might be expected to place an additional shear stress on neighboring intact links so that these would become more liable to fracture, causing the disrupted region to spread cooperatively along much of the length of the axoneme. The amount of distortion of waveform seen in quiescent flagella that have been aged for 15–30 min suggests that such extended disrupted regions are present in most axonemes by this time. The fact that quiescent sperm aged in the presence of 10 μM vanadate (which causes an ~50% decrease in proximal bend angle, and presumably also a roughly proportional decrease in the sliding displacement between tubules) do not show such structural disintegration when the vanadate inhibition is reversed with catechol after aging, argues against the alternative possibility that degradation of axonemal structures by a Ca^{2+} -activated sperm protease is a major factor in the observed disruption. It seems likely that the tearing that occurs as a consequence of the excessive strain constitutes the limiting factor in determining the degree to which quiescence can be reversed by subsequent reduction in free Ca^{2+} concentration with EGTA. The fact that a somewhat greater reversal can usually be obtained by lowering the ATP concentration than by lowering the free Ca^{2+} may be the result of reactivation at low ATP concentrations being less demanding on axonemal integrity and optimal reactivation conditions than reactivation at high ATP concentration (3).

The existence of a small reverse bend in the midregion of quiescent demembrated flagella seems unlikely to be due to residual activity of the dynein cross-bridges that usually produce reverse bends in beating flagella, for it is seldom seen in quiescent live sperm. A more likely possibility, perhaps, is that it helps accommodate the enormous shear strain that results from the large proximal bend angle in quiescent reactivated sperm. If this shear strain produces a significant amount of axonemal twisting around the long axis (17, 23), then the reverse bend in the flagellar midregion might be a reaction that partially alleviates the shear strain in this region. It is worth noting that the reverse bend is completely lost upon addition of low concentrations of vanadate (e.g., 5 μM) that cause only a relatively small reduction in the angle of the proximal bend. The overlap of sperm head and axoneme in the proximal region of quiescent reactivated sperm indicates that there has to be some nonplanarity of the bend in this region, but

our observations do not indicate that the quiescent reactivated flagellum shows any major departure from a planar waveform.

Our results indicating that a MgATP^{2-} concentration of 0.6 mM or greater is required to initiate and maintain quiescence in the majority of sperm could be interpreted in two ways. One possibility is that a high rate of cycling of the dynein arms that generate principal bends is required to make the flagellum bend sufficiently to become quiescent. At a MgATP^{2-} concentration of 0.3 mM, only ~10% of the sperm become quiescent, although the flagellar beat frequency (25 Hz) is 69% of that in 1 mM MgATP^{2-} (36 Hz) (19), which would appear to indicate that a near maximum rate of cross-bridge cycling is required for quiescence in most sperm under these conditions. The second possibility is that the Ca^{2+} -induced inhibition of the dynein arms that generate reverse bends may require a high MgATP^{2-} concentration to be fully effective; this would also provide an explanation for the asymmetry of the flagellar bending waves decreasing in a graded manner as the MgATP^{2-} concentration is lowered from 0.6 to 12 μM . It may be noted that these two possibilities are not mutually exclusive.

The graded decrease in proximal bend angle with vanadate concentrations of 1–50 μM indicates that there is sufficient residual cross-bridge activity to maintain a substantial principal bend angle at concentrations of vanadate up to ten times those required to stop completely oscillatory flagellar beating under similar conditions (19, 26). This is in general agreement with the observations of Cande and Wolniak (7) and of Sale and Gibbons (32), although it is not possible to make a quantitative comparison, because, unlike the other cases studied, the quiescent flagellum is a static system doing no external work. Our results indicate that in experiments where complete inhibition of dynein cross bridge activity is desired, the concentration of vanadate should be $>50 \mu\text{M}$, and possibly as high as 1 mM.

The mechanisms involved in quiescence appear to be closely related to those in asymmetrical beating, for the conditions that favor obtaining a high percentage of quiescence are the same as those that produce a high degree of asymmetry, and vice versa. Drawing on the work of Brokaw and collaborators on asymmetry (2, 4), which we have corroborated here with the species *Tripneustes*, it is clear that (a) both asymmetry and quiescence can be regulated by changing the con-

centration of free Ca^{2+} in the reactivating solution, (b) both are favored by the absence of Ca^{2+} during demembration, so that the demembrated sperm are in the potentially asymmetric state, (c) for full effect, both require the presence of a relatively high concentration of MgATP^{2-} (~1 mM) in the reactivating medium, (d) both are eradicated by mild digestion with trypsin (3, 6), and (e) both occur only to a limited extent in sperm of the species *Colobocentrotus*. The narrow ranges of Ca^{2+} and MgATP^{2-} concentration that appear to be required to obtain a high percentage of quiescence may explain why it has not been observed previously in asymmetrically swimming reactivated sea urchin sperm (2, 4).

Although our results bear only indirectly on the mechanisms involved in asymmetrical beating, they tend to support the view that the latter is the result of an asymmetrical activity of dynein arms that produces a pattern of beating in which the apparent "rest position" of the flagellum is curved in the principal bend direction (2, 20). The strongest evidence for this view derives from comparison of the effects of adding either 0.1 or 1 mM ATP to initially straight, potentially asymmetric sperm in a medium containing 2 mM MgSO_4 , 0.1 mM free Ca^{2+} , and 10 μM vanadate. Under such conditions, if the vanadate were not present, the addition of 0.1 mM ATP would cause asymmetric beating, while the 1 mM ATP would induce the standard quiescent waveform. However, in the presence of 10 μM vanadate, oscillatory beating is prevented and the flagella assume a similar, gently bent form in both cases. The similarity of these waveforms emphasizes the close relationship of asymmetric beating and quiescence.

Our study of flagellar waveforms during stopping and starting transients suggests that a flagellum becomes quiescent when the level of asymmetry in its bending waves attains a certain critical level at which no new reverse bends can be initiated (20). This hypothesis is supported by the fact that the degree of asymmetry is the parameter of the flagellar waves that usually shows the greatest range of variation among the different individual sperm in reactivated preparations (2, 12), and that this broad scatter in asymmetry appears roughly to correspond to the broad range of free Ca^{2+} concentrations that are required to make the different sperm in a preparation become quiescent. However, while the view that a certain critical level of asymmetry must be attained before quiescence occurs may well be valid for the sperm in

a given reactivated preparation, it is clearly an oversimplification for it provides no explanation for the different levels of asymmetry (as indicated by ϕ_{tip}) in the quiescent waveforms of reactivated sperm and of live and ionophore-treated sperm. For example, ϕ_{tip} in quiescent reactivated sperm averages 6.4 rad, substantially greater than its value of 3.6 rad in quiescent live and ionophore-treated sperm, which is in turn greater than the angle of 2.4 rad in quiescent live sperm with short flagella (11, 20). This variation indicates that while a high degree of asymmetry may well be a necessary prerequisite for quiescence, it is not in itself sufficient, and that there are additional factors that influence the critical level of asymmetry at which the transition to quiescence occurs. Our preliminary experiments mentioned above, together with the results of Goldstein (21), suggest that one of these additional factors may be pH.

The degree of asymmetry in reactivated sperm depends partly on the concentration of Ca^{2+} in the reactivating medium, but is also influenced to a major extent by whether the sperm were demembrated under potentially symmetric or potentially asymmetric conditions, i.e., in the presence or absence of Ca^{2+} (4). The chemical nature of the difference produced by the presence or absence of Ca^{2+} in the demembrating solution has not yet been identified, but the fact that we do not see the extreme forms of Ca^{2+} -induced asymmetry of reactivated sperm (2, 18, 20) in ionophore-treated sperm, or in live sperm swimming in seawater with elevated K^+ (11), suggests that live sperm are usually in a state resembling potentially symmetric reactivated sperm.

A Ca^{2+} concentration of between 0.1 and 1 μM has been reported to produce arrest in demembrated lateral cilia in gill filaments of *Elliptio*, whereas a 100-fold higher concentration is needed to arrest the latero-frontal and frontal cilia (36). In demembrated cells of several other organisms, two distinct modes of ciliary or flagellar behavior, corresponding approximately to Ca^{2+} concentrations below and above 1 μM , have been described (24, 25, 29). To induce a maximum percentage of quiescence in preparations of reactivated sea urchin sperm, it appears that a Ca^{2+} concentration of 50–100 μM is required, which suggests that the affinity of the Ca^{2+} -binding sites in the sperm flagella is more like that of the latero-frontal and frontal gill cilia than that of the lateral cilia. The fact that supraoptimal concentrations of free Ca^{2+} (1–2 mM) induce motility rather than

quiescence in both demembrated and ionophore-treated sperm of *Tripneustes* may be a consequence of the formation of $CaATP^{2-}$, for this will reduce the concentration of $MgATP^{2-}$, and also compete with the remaining $MgATP^{2-}$ for binding sites on the axoneme (12). Since quiescence requires a relatively high concentration of $MgATP^{2-}$ for its initiation and maintenance, such dual competition might be expected to have a very noticeable effect.

At present, nothing is known about the properties of the protein(s) involved in Ca^{2+} -dependent regulation of ciliary and flagellar beating. The Ca^{2+} -dependent regulator protein of rat testis (calmodulin) has been characterized and shown to have four equivalent binding sites for Ca^{2+} , each with a dissociation constant of 2.4 μM (9). Such a high affinity is compatible with a regulation of the direction of ciliary beating by μM levels of Ca^{2+} , as it seems to be in several organisms. However, in sea urchin sperm flagella, a free Ca^{2+} concentration of $\sim 100 \mu M$ has been reported to be required for maximum asymmetry (2, 4), and we have found about the same concentration necessary to obtain a maximum percentage of quiescence. These findings together suggest that at least some of the Ca^{2+} binding sites in sea urchin sperm flagella have lower affinities of the order of 100 μM under the conditions used for flagellar reactivation. It is possible that the properties of the flagella Ca^{2+} -binding sites are modified by demembration with Triton X-100 in the presence of 5 mM Ca^{2+} . Holwill and McGregor (24) reported that preparations of *Crithidia* demembrated with Nonidet P-42 could not be made to propagate flagellar waves proximally in a sustained fashion as do the live cells, whereas glycerol-treated cells behave like the live cells and propagate waves in either direction depending upon the intracellular free- Ca^{2+} concentration, and interpreted this to indicate that either the presence of the membrane is essential for proximally-directed wave propagation, or the detergent treatment itself alters the Ca^{2+} -dependent regulatory mechanism.

The Ca^{2+} -induced quiescence which we have described here, and the related phenomenon of arrest in cilia, are not the only known procedures for obtaining a reversible cessation of flagellar beating. The previous work of Brokaw and Simonick (5) described a reversible inhibition of motility in reactivated sea urchin sperm by CO_2 and indicated that the inhibition affects one of the

regulatory mechanisms underlying flagellar movement rather than the active sliding process itself. A reversible inhibition of motility by lowered pH has been described by Goldstein (21). In this case the sperm are usually nearly straight in the quiescent state, and the results demonstrated that the principal and reverse bends possess different sensitivities to pH. A third form of reversible cessation of motility occurs upon deprivation of ATP (14, 21). The possible relationships between the mechanisms underlying these various induced quiescent states remain to be determined.

The results in this paper demonstrate clearly that demembrated flagella in which the dynein cross-bridges are potentiated by the presence of $MgATP^{2-}$, but in which oscillatory bending is inhibited are capable of bending, straightening, and rebending under appropriate conditions. Clear instances of such bending and straightening are provided by the ATP-induced bending of straight flagella of potentially asymmetric sperm in which oscillatory beating is inhibited by $10 \mu M$ vanadate added before the ATP, by the decrease of $\sim 50\%$ in proximal bend angle upon adding $50 \mu M$ vanadate to sperm flagella previously made quiescent by addition of Ca^{2+} and ATP, and by the recovery of the standard quiescent waveform upon reversal of vanadate inhibition with catechol (19). A change in the angular orientation of non-beating cilia in *Paramecium*, upon addition of Ca^{2+} , ATP, and Zn^{2+} , has been reported by Naitoh (28), and a response of non-beating cilia of *Mytilus* to mechanical stimulation has been described by Motokawa and Takahashi (27).

It has not so far been possible to determine the localization of the dynein cross-bridge activity responsible for generating bends during normal flagellar beating. However, the bending of the whole length of a non-beating flagellum into a nearly circular arc, such as Fig. 4b, is reminiscent of a bimetallic strip, and it suggests that under these conditions the shear stress produced by a uniform cross-bridge activity distributed along the whole length of the flagellum is opposed by a similarly uniform resistance to sliding and bending. The accretion of curvature in the proximal regions of flagella with a somewhat higher level of cross-bridge activity (Fig. 4a), suggests in turn that an increase in shear strain beyond a certain level results in transfer of much of that strain to the vicinity of the centriolar block to sliding at the proximal end of the flagellum. At the present time, the relationship of these patterns of bending in the

presence of vanadate to those that occur during normal oscillatory beating is not clear, but it seems possible that the general approach of investigating the bending and straightening of demembrated flagella under non-oscillatory conditions may be a useful way to obtain information about the factors regulating the activity of dynein cross-bridges at different positions on the flagellum, as well as about the properties of the structural components that resist active sliding between tubules and convert it into bending.

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