PROPERTIES OF FLAGELLAR "RIGOR WAVES" FORMED BY ABRUPT REMOVAL OF ADENOSINE TRIPHOSPHATE FROM ACTIVELY SWIMMING SEA URCHIN SPERM

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ABSTRACT

Sea urchin sperm were demembranated and reactivated with a solution containing 0.04% Triton X-100 and 0.03 mM ATP. The ATP concentration was then lowered abruptly by diluting the sperm suspension 50-fold into reactivating solution containing no ATP. The flagella of the sperm in the diluted suspension were not motile, but they were bent into a variety of stationary rigor wave forms closely resembling the wave forms occurring at different stages of the flagellar bending cycle during normal movement. The form of these rigor waves was unchanged upon storage for several hours in the presence of dithiothreitol and EDTA. Addition of 1 μM ATP induced slow relaxation of the waves, with most of the sperm becoming partially straightened over a period of about 30 min; somewhat higher concentrations gave a more rapid and complete relaxation. Concentrations of ATP above 10 μM induced resumption of normal beating movements. Addition of ITP, GTP, or GDP (up to 1 mM) produced no relaxation of the rigor waves. Digestion with trypsin to an extent sufficient to disrupt the radial spokes and the nexin links caused no change in the rigor wave forms, suggesting that these wave forms could be maintained by the dynein cross-bridges between the outer doublet tubules of the flagellar axoneme. Study of the effects of viscous shear on the rigor wave axonemes has shown that they are resistant to distortion by bending, although they can be twisted relatively easily.

Considerable experimental evidence now exists to support a sliding-tubule model of flagellar motility, in which the normal propagated bending waves are the result of localized sliding movements between adjacent doublet tubules of the flagellar axoneme (11, 21, 23, 24). Recent work has strongly indicated that these sliding movements are generated by a mechanism involving the interaction of ATP with the arms, composed of dynein ATPase, that extend between the doublet tubules (8, 23, 24). In particular, it has been shown that partial removal of the dynein arms causes a proportionate decrease in the speed of sliding between tubules (10). The detailed mechanism by which the dynein arms generate sliding is not yet known, but it has been postulated that this mechanism involves the cyclic detachment and reattachment of the arms to successive binding sites along the adjacent doublet tubule in a mechanochemical cycle associated with the binding and hydrolysis of ATP (11, 23). Such a mechanism implies that during normal movement the dynein arms form transient cross-bridges between adjacent doublet tubules.

In order to investigate the properties of these

postulated cross-bridges, we have now studied the effect on actively beating axonemes of abruptly lowering the ATP concentration to a level below the minimum required for motility. This sudden withdrawal of ATP causes the motile axonemes to set into stationary waves whose forms resemble those present at different phases of the normal beating cycle. By analogy to muscle, we propose that these stationary waves produced by abrupt deprivation of ATP be termed "rigor waves."

In this paper, we describe a method for obtaining preparations of sea urchin sperm with their axonemes in rigor waves, and give the results of various chemical and mechanical experiments on their properties. Our results suggest that the form of the rigor waves is maintained largely by the dynein arms, which in the absence of ATP, form fixed cross-bridges between adjacent doublet tubules of the axoneme. A preliminary account of this work has been published previously (9). The cross-bridges responsible for maintaining rigor waves in sea urchin sperm may well be related to those proposed by Lindemann et al. to account for the effect of ATP on flagellar stiffness in bull sperm (16).

MATERIALS AND METHODS

Sperm from the sea urchin *Colobocentrotus atratus* were collected and stored as described previously (8). Before use, they were diluted with 1–2 vol of sea water to give a suspension with a concentration of 15–25 mg protein/ml and maintained at 0°C.

Preparations of rigor wave sperm were made by demembranating the live sperm with Triton X-100 in a solution containing 0.03 mM ATP. In this solution the demembranated sperm were reactivated by the ATP, with their flagella beating at a frequency of two to four beats per second. While the sperm were actively moving in this way, the ATP concentration was lowered abruptly by diluting the suspension with a relatively large volume of solution containing no ATP. The sperm in the resultant diluted suspension were immotile with their flagella bent into stationary rigor waves. In order to obtain uniform preparations of rigor wave sperm it was found critical that the procedure used to dilute the sperm into ATP-free solution be carefully controlled to give as rapid mixing as possible without subjecting the sperm to excessive viscous shear. Since the undiluted suspension contained a high density of sperm and a low concentration of ATP, the ATP concentration varied significantly with time and it was necessary to standardize the time elasping before dilution. With the sperm densities given below, we estimate that the initial ATP concentration of 0.03 mM had decreased to 0.015–0.02 mM by the time dilution occurred 45–60 s after the start of the incubation. The sperm suspensions were too dense to observe in detail by light microscopy, but the sperm could be seen to swim slowly until they had exhausted the ATP supplied, which happened after about 90 s of incubation.

In a typical preparation, 25 μl of a sperm suspension in sea water were added to 0.3 ml of demembranating solution (0.15 M KCl, 2 mM $MgSO_4$, 0.5 mM EDTA, 1 mM dithiothreitol [DTT], 0.04% Triton X-100, wt/vol, 0.03 mM ATP, and 10 mM Tris-HCl buffer, pH 8.1) at room temperature (23°C). After gentle mixing for 15 s, 100 μl of the demembranated sperm suspension were withdrawn into a capillary pipette (approx. 1.3 mm diameter and 70 mm long), and immediately added at a rate of about 4 μl/s to 5 ml of suspension solution (0.15 M KCl, 2 mM $MgSO_4$, 0.5 mM EDTA, 1 mM DTT, and 10 mM Tris-HCl buffer, pH 8.1) which were being stirred in a 10-ml beaker with a small magnetic bar turning at about 60 rpm. The resultant stock suspension of rigor wave sperm was used for experiments as desired. If cooled to 0°C, the sperm could be stored for up to 2 h without loss of their potential for motility upon addition of ATP.

The rigor wave sperm were studied by light microscopy by diluting a sample of the stock suspension into more suspension solution in a Petri dish. For photographic work, it was found most convenient to let the sperm settle to the bottom of the dish for a few hours, or overnight at 4°C, in order to obtain a larger proportion of the sperm within the focal plane of the microscope. Sticking of the sperm to the glass surface was minimized by precoating the dish with egg white (8). The bend angles of the axonemal waveforms were measured as described previously (8).

Light microscopy of the rigor wave sperm was usually performed with a 10× 0.22 NA objective lens and a 0.6/0.75 NA dark-field condenser. This combination of lenses with an XBO 150 lamp required about a 0.5-s exposure on Kodak 2475 film (4,000 ASA) at a magnification of 40. For occasional work at higher resolution we used a 40× 0.75 NA water-immersion objective with a 0.8/0.95 NA dark-field condenser.

For study of the effect of trypsin digestion on the rigor waves, a preliminary assay was made at the start of each experiment by digesting a small sample of the sperm with trypsin in the presence of ATP in order to determine the time necessary to destroy motility and to cause the doublet tubules of the axoneme to slide apart. At the trypsin concentration used (0.2 μg/ml), this time was 6–9 min. A portion of the sperm preparation was then digested with the same concentration of trypsin in the absence of ATP for twice the predetermined sensitization time, after which the digestion was stopped by adding a 10-fold excess of soybean trypsin inhibitor (2 μg/ml). Another portion of the sperm preparation was digested with five times the trypsin concentration also for twice the sensitization time. Samples of the digested sperm

were examined immediately by light microscopy, and were then allowed to settle for photography. Other samples of the digested sperm were tested with 0.1 mM ATP in order to verify that the axonemes disintegrated with sliding of the doublet tubules (23).

Direct light microscopic observations of rigor wave sperm being subjected to mechanical stress were made as follows. A rectangular open-ended chamber 0.20 mm high, 7 mm wide, and 20 mm long was prepared by glueing strips of microscope cover glass along the edges of a microscope slide and then covering with an intact cover glass. The chamber was filled by flowing in a suspension of rigor wave sperm from one end, and left for a few minutes to permit some of the sperm to become stuck to the microscope slide. The remaining unstuck sperm were then removed by drawing through fresh suspension solution. Partially attached sperm having a portion of their axoneme stuck to the cover glass and the other portion free in the fluid were selected for observation. Fluid was then made to flow through the chamber by sucking from a reservoir of suspension solution at one end with strips of dry filter paper applied to the other end. The rate of flow was controlled by varying the number of thicknesses of filter paper in the strip. With practice, a reasonably constant and reproducible rate of flow could be maintained for 5–10 s with this procedure. The effect of the fluid flow on the wave forms of the partially attached sperm was studied from photographs made before, during, and after the flow. In some cases the direction of flow was then reversed by interchanging the reservoir and filter paper.

The approximate velocity of the fluid in the vicinity of the sperm was obtained by calculating the velocity gradient at the glass surface. For a chamber of height $2a_h$ and width w, the velocity gradient at the surface is:

$$\left(\frac{dv}{da}\right)_{a = a_h} = \frac{3Q}{2wa_h^2}$$

where Q is the total fluid flow through the chamber per second. Since the fluid layer at the glass surface is stationary, the velocity v_d at a small distance d from the surface is given by:

$$v_d = \frac{3Qd}{2wa_h^2}. \qquad (1)$$

A second type of flow experiment enabled us to subject rigor wave sperm to greater degrees of viscous shear, but did not permit simultaneous observation by light microscopy. The sperm preparations were forced through a narrow Teflon tube (0.34 mm diameter, 30 cm long) with a syringe pump at rates up to 0.25 ml/s. The stream exiting from the tube was decelerated by being directed obliquely against the side of a small beaker. Since the shear experienced by a particular sperm depends on its radial position in the capillary tube, each preparation was passed three times through the tube before observation in order to obtain an averaging of the shear. The form of the rigor waves in the resultant sheared sperm, as well as the degree of motility regained upon addition of 1 mM ATP, were compared with those of unsheared sperm from the original suspension.

Assuming laminar flow, the velocity v of the fluid at radius r in a capillary tube of radius r_o and length l is given by $v = (\Delta P/4\eta l)(r_o^2 - r^2)$ where η is the viscosity and ΔP the pressure difference between the ends of the tube. If Q is the total volume of fluid passing per second, this expression converts to $v = (2Q/\pi r_o^4)(r_o^2 - r^2)$. The average velocity gradient experienced by the sperm is given by:

$$\left(\frac{dv}{dr}\right)_{av} = \frac{32Q}{15\pi r_o^3} \qquad (2)$$

Under our conditions of maximal flow, Reynolds number $\rho\, r_0 v_{av}/\eta$ has a value of 1,150, which is approximately half the lower critical value for laminar flow (4). In these experiments, therefore, it may be expected that the flow in the tube was mostly laminar. However, it is possible that some turbulence occurred as the fluid was being decelerated after its exit from the tube.

Sources of chemicals were the same as reported previously (8). Solutions of trypsin (Worthington Biochemical Corp., Freehold, N. J.), and soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, Mo.) were freshly prepared on the day of use, and kept at 0°C.

RESULTS

Preparation and Form of Rigor Waves

When the ATP concentration of a suspension of motile reactivated sperm is lowered abruptly by dilution, the sperm become immotile with their flagella remaining bent in stationary rigor wave forms. By using suitable conditions for the reactivation and subsequent dilution, we have been able to obtain uniform preparations of sperm with rigor wave forms that closely resemble the wave forms occurring at different phases of the flagellar bending cycle during normal movement.

A typical preparation of rigor wave sperm is illustrated in Fig. 1. The wave form of each individual sperm is completely stationary and shows no changes with time. When the sperm are allowed to settle to the bottom of a Petri dish, the majority lie flat within the plane of focus, showing that the rigor waves are planar in form, like those of beating flagella. In order to make a more detailed comparison of the rigor and beating wave forms, it is convenient to assemble a selection of micrographs showing sperm whose rigor waves represent the various phases of the bending cycle

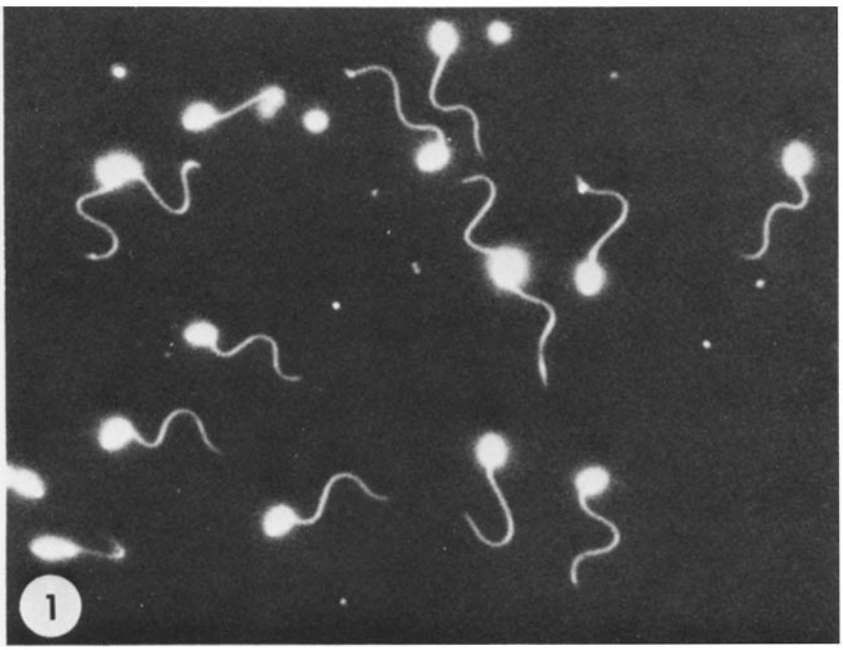

FIGURE 1 Dark-field micrograph of a standard preparation of rigor wave sperm. The majority of sperm have settled so that the plane of axonemal bending lies parallel to the focal plane. A few sperm (e.g., bottom left corner) are lying at an angle to the focal plane. × 450.

(Fig. 2). Comparison of these rigor wave forms with the beating wave forms of live and reactivated sperm of the same species (see Figs. 3 and 4 of reference 8) makes clear their close resemblance. All stages of the beating cycle appear to be represented among the rigor wave forms. The distinct characteristics of the bends on each side of the axoneme are maintained, with the bend on one side (principal bend) having a somewhat larger angle than that on the other side (reverse bend). In addition to their difference in angle, the principal and reverse bends are also distinguished by the fact that the former usually appears to die away just before it reaches the distal tip of the axoneme while the latter propagates smoothly over the tip with little change in curvature (8). In these qualitative details the rigor wave forms closely resemble the beating wave forms. Our present experimental technique has not permitted us to determine whether the rigor wave forms are quantitatively the same as the beating wave forms. The bend angles and curvatures of the rigor wave forms appear generally similar to those of the beating wave forms of live and reactivated sperm. There appears to be a slightly greater average bend angle in the rigor wave sperm, but it is not certain this difference is significant because the waves were not formed under equivalent ATP concentrations. In addition, there are variations in bend angle and curvature among individual sperm of the same preparation, and in order to be valid quantitatively, the comparison would have to be made with single individual sperm.

In the usual preparations of rigor wave sperm, about 1–5% of the sperm showed abnormal bending patterns, such as a single sharp bend, a wave of diminished bend angle, a twisted nonplanar wave form, or a nearly straight axoneme (Fig. 3). These presumably represent sperm that were not swimming well at the time they were diluted out of the ATP, either because they had been damaged previously or because they happened to be subjected to a greater than average viscous shear during dilution. If the conditions of preparation departed from optimal, then the fraction of sperm with abnormal wave forms increased. It will perhaps be useful if we summarize here the factors we have found helpful in obtaining uniform preparations of undistorted rigor waves. First, the sperm should be swimming well with normal wave forms in the demembranating solution before the ATP concentration is lowered. Second, the dilution into ATP-free solution should be accomplished as uniformly and rapidly as possible without exposing

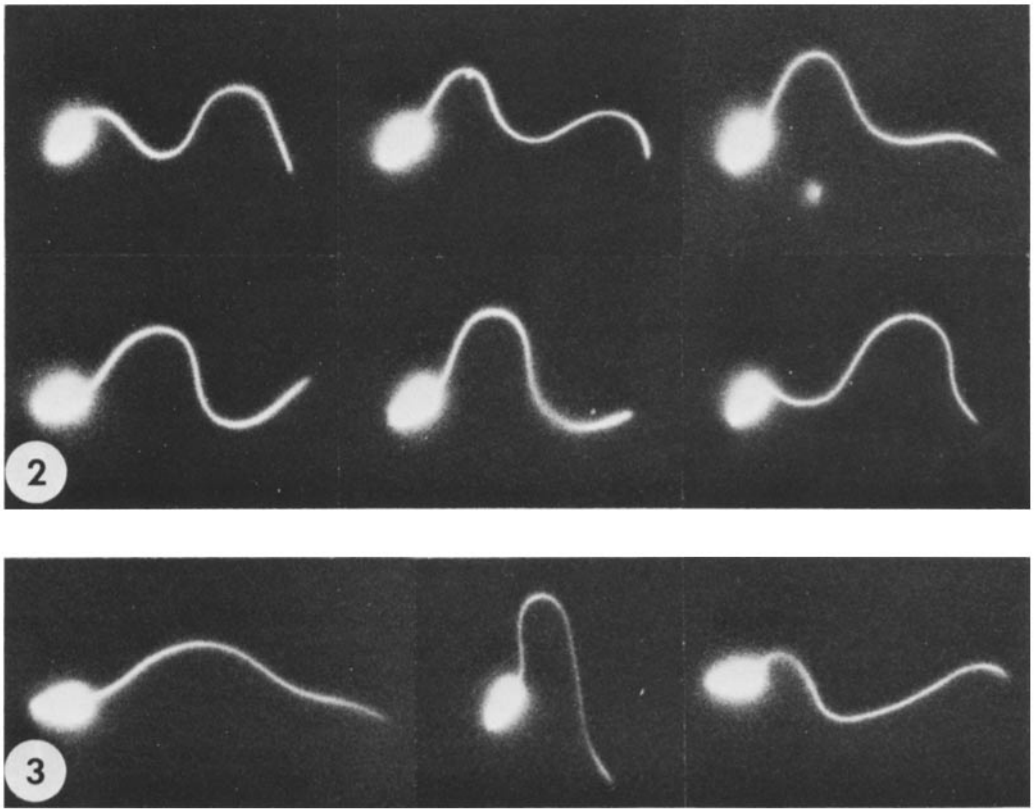

FIGURE 2 Dark-field micrographs of different individual rigor wave sperm showing typical wave forms. The micrographs have been arranged into a series depicting the progression of the waves as they would appear in a normal bending cycle. × 1,050.

FIGURE 3 Some abnormal wave forms typical of those found in low percentage in standard preparations of rigor wave sperm. × 1,050.

the sperm to excessive viscous shear. The wave forms of the beating axonemes are sensitive to viscoelastic stress (11), and excessive shear during dilution causes a flattening of the resultant rigor wave forms. Third, the ATP concentration in the demembranating solution should not be substantially greater than is necessary to give complete reactivation of all the sperm. If the ATP concentration is too high, the resultant rigor wave forms do not resemble the wave forms of the beating flagella, but are considerably flattened, or even straight, in the majority of the sperm.

Preparations of rigor wave sperm can be stored in suspension solution containing DTT and EDTA for several hours at room temperature, or for up to 4 days at 4°C, without noticeable change in their wave forms. After prolonged storage, the attachment between the sperm head and axoneme becomes greatly weakened, so that the axonemes are detached by gentle agitation, still maintaining their rigor wave forms. This weakening of the head-axoneme attachment only occurs when the sperm are stored in the presence of a reducing agent such as DTT, suggesting that disulfide bonds play a major role in maintaining the normal strength of the head-axoneme attachment, as has been reported previously in mammalian sperm (3).

The presence of DTT and EDTA is important in maintaining the form of the rigor waves during storage. If these protective compounds are omitted from the storage solution, the rigor waves undergo a characteristic change in form within a few hours, with the distal portions of the waves twisting relative to the proximal portions, so that the waves lose their planarity and assume a three-dimensional form. Few sperm, if any, undergo this

change in wave form so long as DTT and EDTA are present in the suspension solution.

The significance of the twisting of the rigor wave forms upon aging in the absence of EDTA and DTT has not been determined. A similar change in wave form can be produced more rapidly by treating rigor wave sperm with glutaraldehyde or with osmium tetroxide. It is possible that this twisting of the rigor wave forms may be related to the tendency of isolated doublet tubules to coil into helical forms under similar conditions (23, 27).

Reactivation of Motility

If 1 mM ATP is added to a fresh preparation of rigor wave sperm at room temperature, almost all the sperm become motile, and their movement appears essentially the same as that of sperm that have not previously been set into rigor. However, the capacity for motility decreases fairly rapidly with storage, so that after being kept overnight in the presence of DTT and EDTA at 0°C, the sperm retain normal rigor wave forms but very few of them become motile upon addition of ATP.

Relaxation of Rigor Waves

Addition of low concentrations of ATP to a suspension of rigor wave sperm in suspension solution at room temperature induced relaxation of the waves, causing the axonemes to straighten slowly; the speed and extent of this relaxation were dependent on the concentration of ATP. Addition of 1 μM ATP caused partial straightening over a period of about 30 min (Fig. 4 A, C), with the bend angles decreasing on the average to about half of their initial values; no further change in wave form occurred on more prolonged standing. In 3 μM ATP, the waves became partially relaxed within a few minutes, but relaxation was not complete and most sperm retained a gentle residual curvature (Fig. 4 D). In 6 μM ATP, the waves relaxed within 10–15 s, and the axonemes became nearly straight. Addition of 10 μM ATP was sufficient to straighten the sperm nearly completely within less than 10 s. Concentrations of ATP above 10 μM induced reactivation of the sperm axonemes with resumption of the normal beating movement.

The addition of 1 mM ADP produced no immediate change in the form of the rigor waves, but they slowly relaxed so that by 1 min most appeared to have about half straightened. We interpret this slow relaxation with a high concentration of ADP as resulting either from the conversion of ADP to ATP by the adenylate kinase present in the sperm (7), or from contamination of the ADP used by traces of ATP, and we conclude that ADP probably has no direct relaxing effect on the rigor waves.

Other nucleotides, including ITP, GTP, or GDP, produced no relaxation of the rigor waves, even when they were used at a concentration of 1 mM (Fig. 4 B). The presence of those nucleotides did not prevent relaxation occurring when ATP was subsequently added to the preparation.

The divalent cation requirements for relaxation were examined by centrifuging preparations of rigor wave sperm, and resuspending them in suspension solution from which the magnesium had been omitted. When the sperm were suspended in this divalent cation-free solution, the addition of 1 mM ATP induced no relaxation of the rigor waves. When the suspension solution contained 2 mM Ca^{2+}, the waves could be relaxed by ATP, but much higher concentrations were required than in the presence of Mg^{2+}. A concentration as high as 0.1 mM ATP gave only a slow partial relaxation, and 1 mM was required for a rapid and complete effect.

As mentioned above, the rigor wave sperm could be stored overnight at 0°C without change in their wave form. However, these aged sperm had lost their ability to be relaxed, and addition of ATP (1 μM to 1 mM) to the suspension had no effect on the rigor wave forms.

These results show that the nucleotide and divalent cation requirements for relaxation closely parallel those for motility (8), with $MgATP^{2-}$ being required for full effect, and $CaATP^{2-}$ being a rather inefficient substitute. Aging of the sperm overnight destroyed their ability for relaxation as well as their potential for regaining motility.

We have studied briefly a variety of other conditions for a possible relaxing effect on the rigor waves. Changing the pH of the suspension solution between pH 5.5 and 9.7 had no effect on the rigor waves, either in the presence or the absence of magnesium. At pH 10.1 the axonemes straightened within a few minutes, but this pH is close to that at which the whole axoneme becomes solubilized (12). The addition of 1 M urea to standard suspension solution at pH 8.1 had no visible effect on the rigor waves. Increasing the KCl concentration in suspension solution to 0.5 M caused a slow relaxation, with the waves becoming partially straightened over a period of about 30 min. Further experiments will be required to

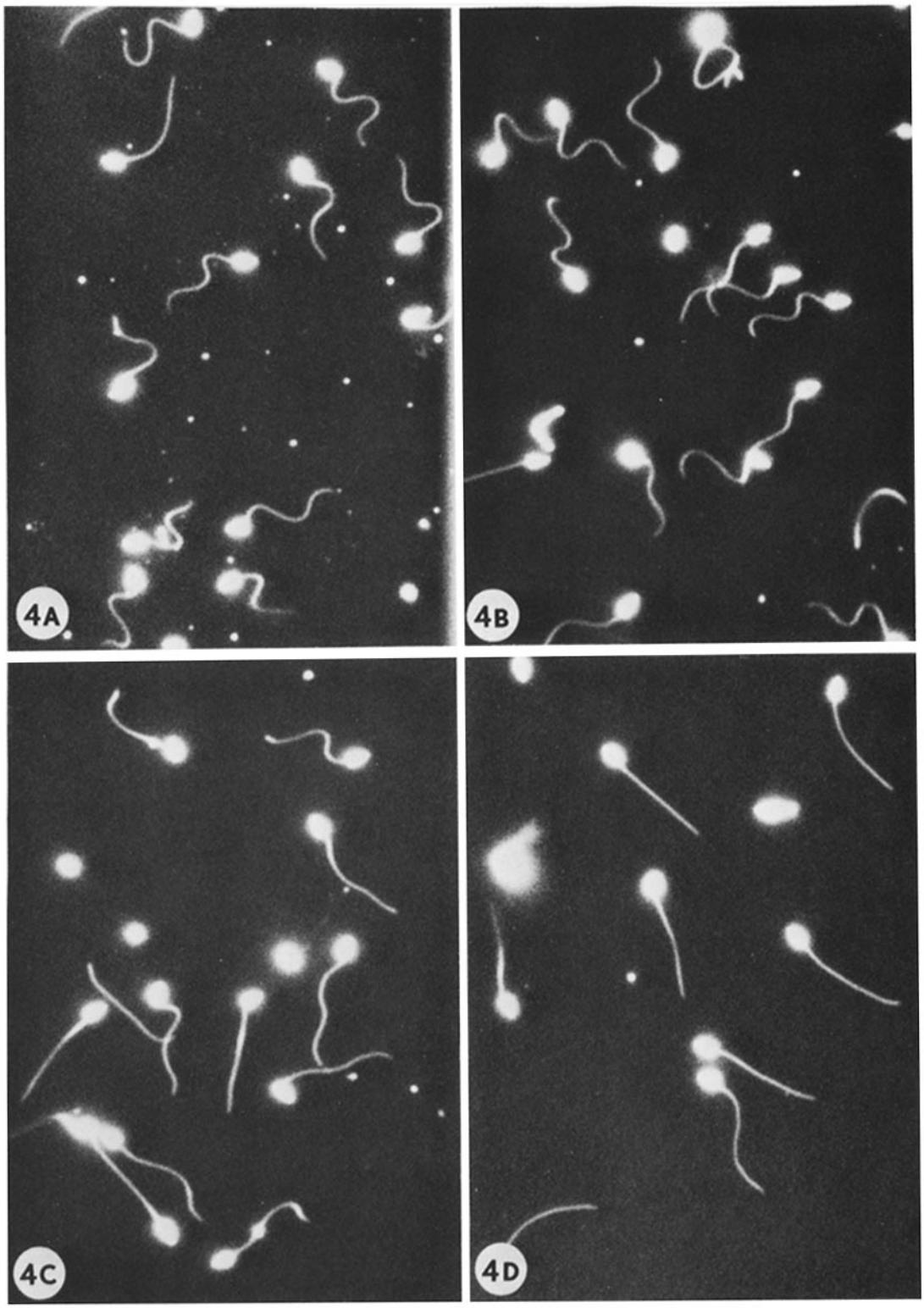

FIGURE 4 The effects of ITP and ATP in relaxing rigor wave sperm. × 450. Fig. 4 A, initial preparation. Fig. 4 B, a sample from the same preparation 30 min after the addition of 1 mM ITP. Fig. 4 C, Another sample of the same preparation 30 min after the addition of 1 μM ATP. Fig. 4 D, another sample 30 min after the addition of 3μM ATP.

determine whether this relaxing effect of 0.5 M KCl is due to the action of this solvent in partially solubilizing the dynein arms (10).

Effect of Trypsin Digestion on Rigor Waves

Previous work has shown that the various structural components of the axoneme are destroyed at different rates by digestion with trypsin (24). Therefore, we have examined the effects of trypsin digestion in order to obtain information about which structural components of the axoneme are required to maintain the form of the rigor waves. The extent of trypsin digestion has previously been expressed by the decrease in turbidity of a suspension of isolated axonemes. However, the presence of the sperm heads in preparations of rigor wave sperm makes it impossible to use the turbidimetric assay, and so we first determined the digestion time required to sensitize the axonemes to disintegration with ATP, and then examined the effects on the rigor wave sperm of digesting for two times and for 10 times this sensitization time.

Digestion of rigor wave sperm with trypsin for twice the sensitization time produced no apparent change in the form of the rigor waves, which maintained their initial planarity and bend angles (Fig. 5). Even when the sperm were digested for 10 times the sensitization time, there was little immediate change in the rigor wave forms; however, in this case, the wave forms degenerated upon storage overnight, with some axonemes splitting, some becoming straight, and some showing coiling of the wave forms.

Preliminary Experiments on the Mechanical Properties of Rigor Waves

We have not yet been able to make quantitative estimates of the rigidity and strength of rigor wave axonemes, but we have performed two types of experiment which provide qualitative information about these properties.

The rigidity has been examined by observing the effects of fluid flow past axonemes that are partially attached to a microscope slide forming the bottom surface of a trough. Under these conditions, the fluid velocity is given by Eq. 1 (see Materials and Methods), and varies directly proportional to the distance from the surface of the slide. With our procedure we were able to attain velocity gradients of up to 1,200/s, corresponding to a velocity of 240 μm/s at a distance of 0.2 μm from the slide. Partially attached axonemes that lay parallel and close to the surface were selected for observation. The effect of fluid flow on these axonemes depended on their orientation relative to the direction of flow. When the unattached portion of the axoneme lay downstream from the attached portion, the wave form was not changed greatly by the flow, but some sperm showed a slight flattening of the waves corresponding to a decrease of about 0.4 rad in bend angle (Figs. 6 A–C). A more obvious change in wave form frequently occurred when the unattached portion of the axoneme lay upstream from the attached portion, in which case the flow would often cause the unattached portion to twist completely over and come to rest in a more downstream position (Figs. 6 A–C). This movement appeared to result from a twisting through 180° of a limited region of axoneme located very close to the point where the axoneme became attached to the slide. The twisting could occur in either a straight (Figs. 6 A, C) or a bent region (Fig. 6 B) of the axoneme, and in the latter it was possible to see that the twisting was confined to a limited region of the axoneme, not more than 2–3 μm long. The portion of the axoneme distal to the twisted region appeared to have been rotated through 180° without substantial change in its wave form, the bend angles remaining constant or changing by less than 0.4 rad (see legend to Fig. 6). In the majority of sperm, the axonemes became partially twisted in a velocity gradient of about 100/s, and the twisting had gone to completion (180° twist) in a gradient of 400/s; further increases in the velocity gradient up to 1,200/s had no further effect on the wave form. The changes in bend angle and the twisting were fully reversible, and when the flow was terminated the axonemes returned to their original wave form, apparently as a result of their internal elasticity.

The strength of the rigor wave axonemes has been studied by forcing the preparations through a capillary tube at various rates. This procedure subjects the sperm to shear from the radial velocity gradient in the tube, with the average velocity gradient being given by Eq. 2 (see Materials and Methods). After a preparation of sperm had been sheared in this way, one portion of it was examined by light microscopy to see whether the shear had caused permanent distortion of the rigor wave forms, and another portion was placed in a solution containing 1 mM ATP to determine whether the sperm were still capable of motility.

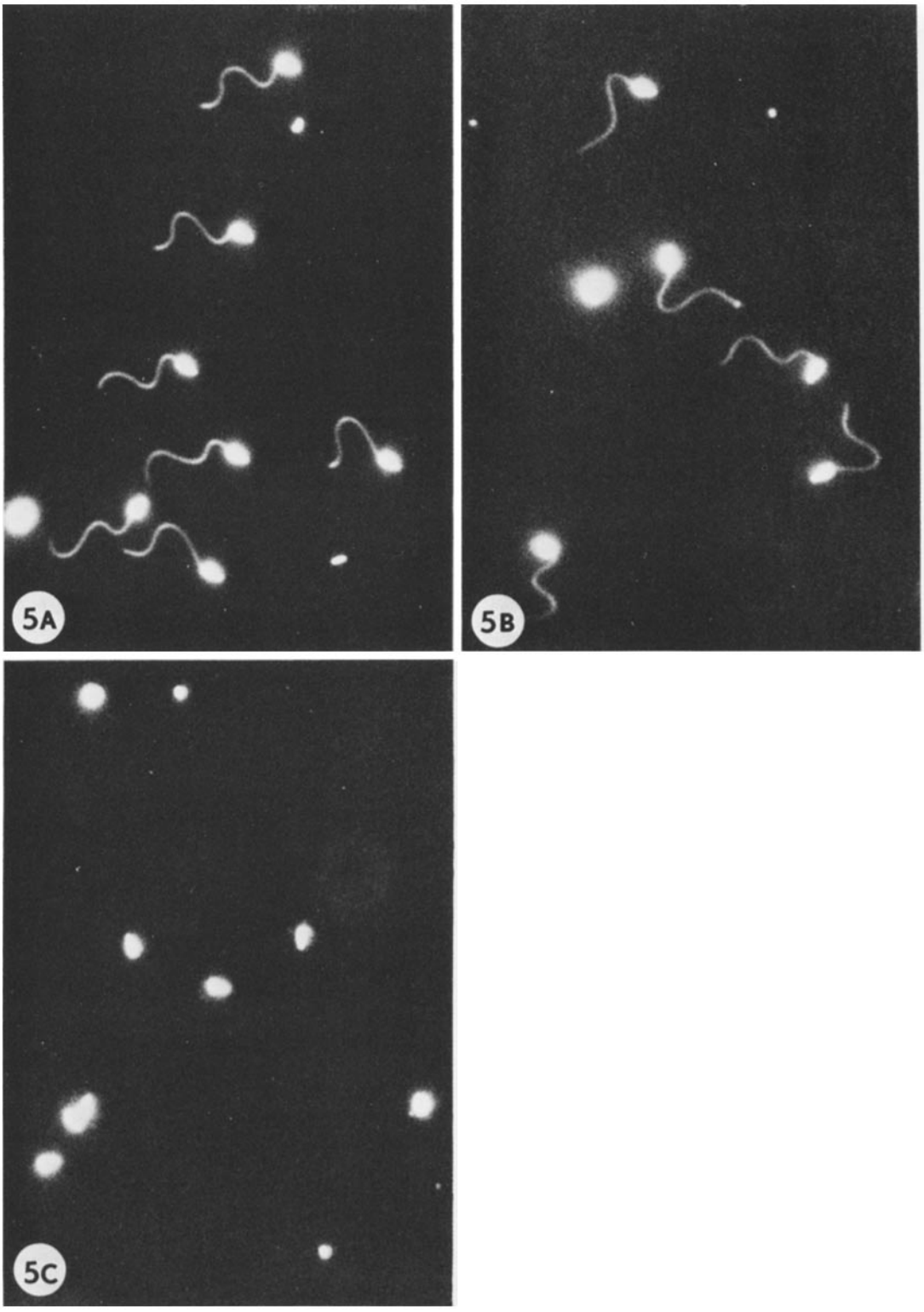

FIGURE 5 Dark-field micrographs showing the effect of trypsin digestion on a preparation of rigor wave sperm. × 450. Fig. 5 A, initial preparation. Fig. 5 B, same preparation after digestion with trypsin for twice the sensitization time. Fig. 5 C, same preparation as in Fig. 5 B after addition of 1 mM ATP. The axonemes have disintegrated by sliding apart into individual tubules, which are not visible under these photographic conditions.

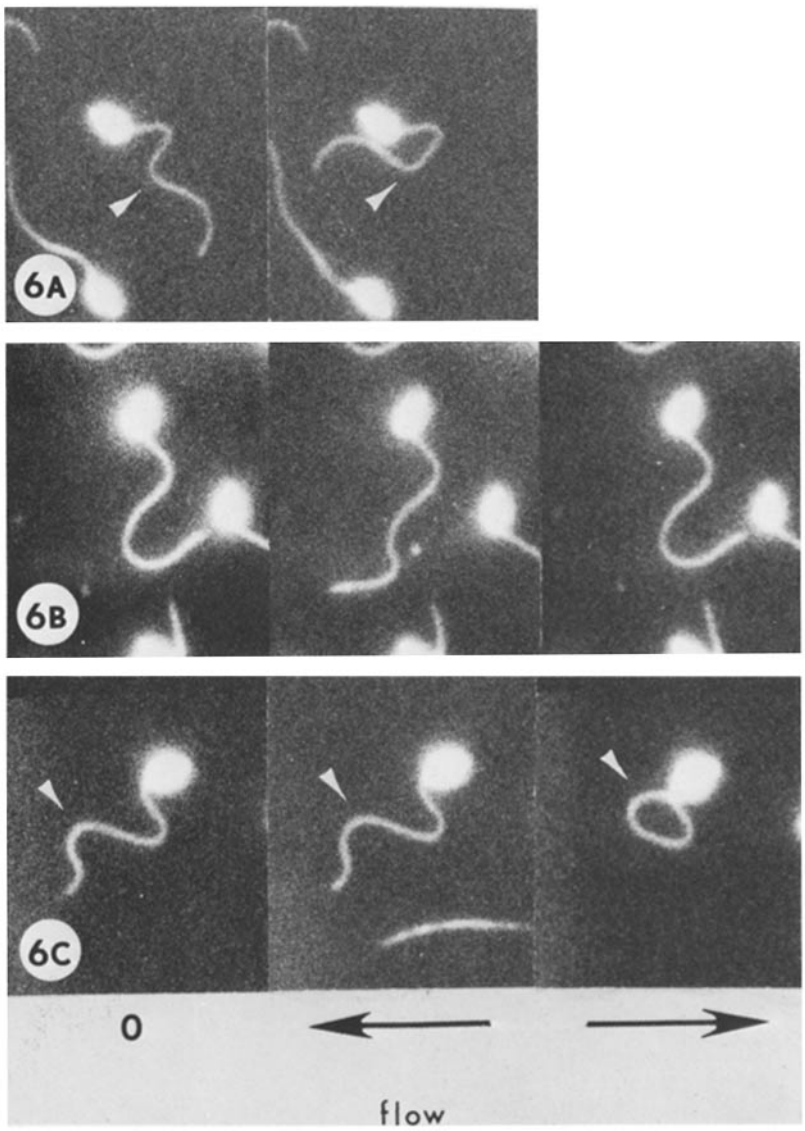

FIGURE 6 The effects of fluid flow on rigor wave sperm that are partially attached to the bottom surface of a trough. × 800. The left-hand column shows the sperm with no fluid flow. The center column shows the same sperm with fluid flow directed from right to left (velocity gradient approximately 400/s). The right-hand column shows two of the same sperm with the fluid flow directed from the left to right. The angles of the bends indicated by arrows are: Fig. 6 A, 1.74, 1.74 rad; Fig. 6 C, 2.35, 2.09, 2.75 rad. In the original photograph of the right-hand image of Fig. 6 C, the axoneme is visible passing close by the sperm head, but this detail has been largely lost in reproduction.

Fig. 7 and Table I summarize the effects of exposing the sperm to various velocity gradients in the range 0.7–3.5 × 10^4/s. At the highest velocity gradient, almost all of the axonemes showed considerably straightened wave forms with some of them being completely straight (Fig. 7 D); however, this velocity gradient also caused breakage of more than half the sperm, resulting from a transverse fracture of the axoneme, most often in the region close to the sperm head. At the lowest velocity gradient used, the wave forms of most axonemes appeared unaffected by the shear, and

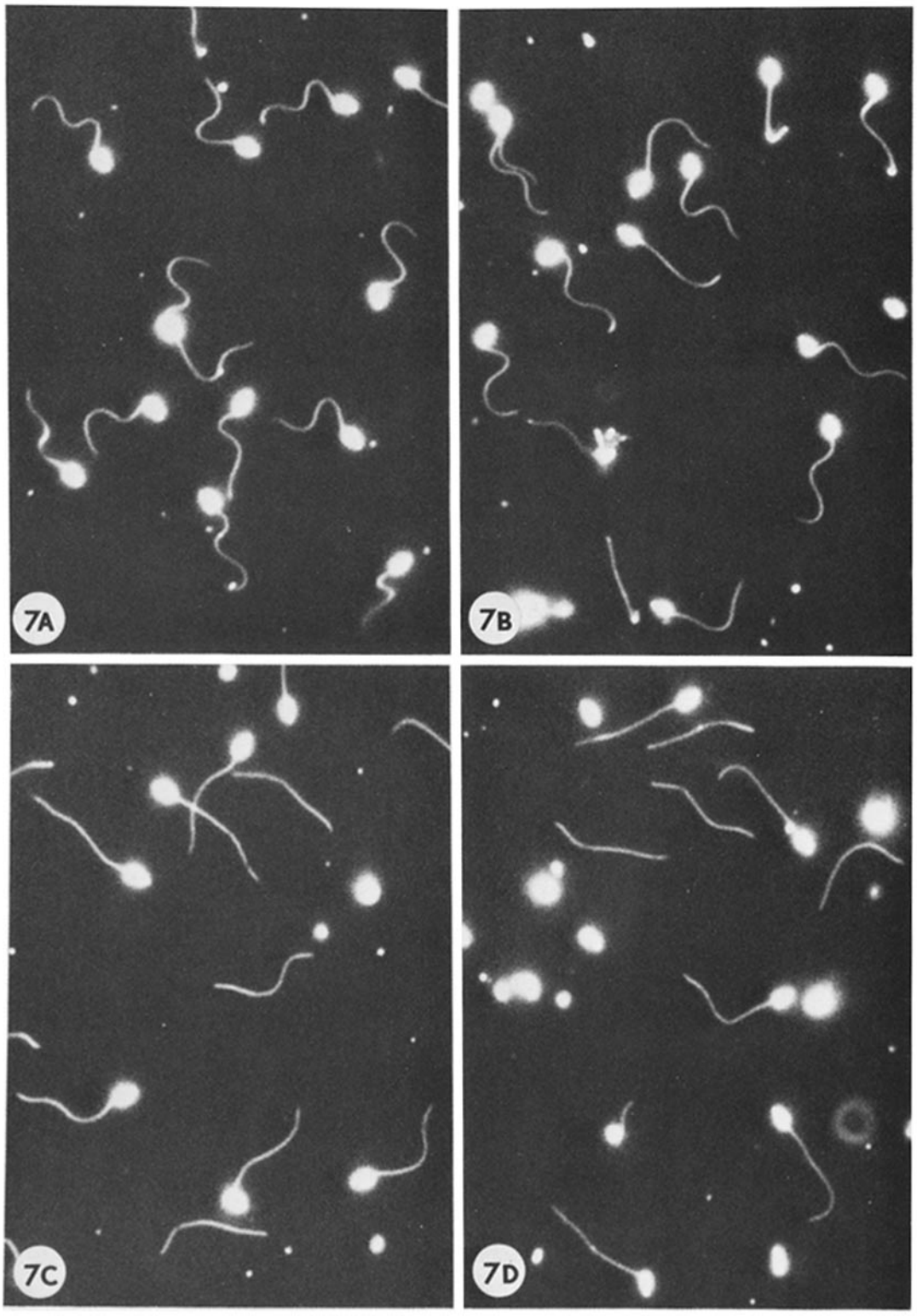

FIGURE 7 The effect of viscous shear on a preparation of rigor wave sperm. × 450. Separate photographs show the sperm after being subjected to a velocity gradient of Fig. 7 A, zero (control); Fig. 7B, 0.7×10^4/s; Fig. 7 C, 2.0×10^4/s; Fig. 7 D, 3.5×10^4/s.

TABLE I

Effects of Viscous Shear on Rigor Wave Forms

Average velocity gradient	Broken axonemes	Waveforms of non-broken axonemes	Motility of non-broken axonemes in ATP
s^{-1}	%		%
0	0	Normal	100
0.7×10^4	17	85% normal 15% straightened*	100
2.0×10^4	35	17% normal 83% straightened*	70
3.5×10^4	60	100% straightened*	80

* Straightened refers to wave forms that were either partially or completely straightened. The average degree of straightening appeared to increase with increasing viscous shear, but we did not attempt to quantitate it.

only about 20% of the sperm were broken. These results indicate that the rigor wave forms are permanently distorted only when the magnitude of the velocity gradient approaches that necessary to cause transverse fracturing of the axoneme.

When the reactivation of the sheared preparations was tested with 1 mM ATP, 70–100% of the unbroken sperm became motile, while all the broken axonemes remained immotile (Table I). In the preparations subjected to the highest shear, where all the rigor wave forms appeared partially or completely straightened, the movement of the reactivated sperm was noticeably altered from that of control sperm that had not been subjected to shear. The movement of the sheared sperm was characterized by a marked tendency for the axonemal bending waves to die away before they had propagated the full length of the axoneme, by a decrease of about 20% in average beat frequency, and by the presence of occasional irregular twitching movements which interrupted the regular beating of the axoneme. In the preparation subjected to the lowest shear, where most of the rigor wave forms remained undistorted, the sperm reactivated normally with their motility apparently similar to that of the control sperm. These results show that forcible straightening of the rigor wave forms by shear usually damages the motile apparatus of the axoneme, but does not necessarily completely prevent its functioning. Shear forces that are insufficiently great to distort the rigor wave forms do not damage the motile apparatus. The critical level of velocity gradient required to distort the rigor wave forms was about 2×10^4/s, corresponding to a shearing force per unit area of approximately 2×10^2 g/cm s^2 for a medium of this velocity.

DISCUSSION

It is now generally accepted that the propagated bending waves typical of flagellar beating are the result of localized sliding movements between the doublet tubules of the axoneme (6, 11, 21, 23, 24). The geometrical relationships in the axoneme are such that, if the tubules are inextensible and there is no twisting of the axonemal structure, then the amount of sliding displacement between tubules in a bent region of axoneme is proportional to the angle of the bend. Under these conditions, a particular pattern of sliding displacement between tubules determines a unique pattern of axonemal bending. During normal axonemal beating, the pattern of sliding displacement between tubules will be continually changing in a manner corresponding to the formation and propagation of bending waves.

In this paper, we have shown that abrupt removal of ATP from beating axonemes causes the axonemes to set into stationary rigor waves. This preservation of rigor wave forms implies that the removal of ATP results in the formation of fixed intra-axonemal cross-links which prevent any further sliding displacement between tubules, so that the axoneme is forced to remain in whatever pattern of bending it happens to be in at the time of cross-link formation.

In order to obtain information about the nature of the axonemal cross-links responsible for maintaining the form of the rigor waves, we have examined the effects of controlled digestion by trypsin. Previous electron microscope studies correlating the extent of trypsin digestion with its effect on axonemal structure have shown that the digestion time required to sensitize the axonemes to disintegration by ATP corresponds to approximately the time necessary for the digestion to disrupt the majority of the nexin links between adjacent outer doublet tubules, and of the radial links between each doublet tubule and the central sheath (24). After twice the sensitization time, corresponding to about 85% of the initial turbidity for isolated axonemes, the nexin links and radial spokes appear completely disrupted, while about

80% of the dynein arms and almost all the central tubules and outer doublet tubules remain apparently intact. After 10 times the sensitization time, corresponding to about 60% of the initial turbidity for isolated axonemes, the central tubules are largely destroyed, although about half of the outer doublet tubules still remain; at this stage the axonemes begin to lose their integrity with the bundles of nine doublet tubules breaking up into smaller groups (24).

Our results in this paper have demonstrated that digestion of rigor wave sperm for twice the sensitization time has no apparent effect on the form of rigor waves, and that even digestion for 10 times the sensitization time has little immediate effect. Since the only axonemal components which appear to have a comparable resistance to trypsin are the dynein arms and the outer doublet tubules, it seems probable that in the absence of ATP the dynein arms form fixed cross-bridges between adjacent doublet tubules, and that it is the presence of these fixed dynein cross-bridges preventing sliding movement between tubules which is principally responsible for the maintenance of the rigor wave forms. The lack of sensitivity to trypsin digestion indicates that the presence of the radial spokes and of the nexin links is not required to maintain the rigor wave forms, but does not exclude the possibility that these components may constitute additional cross-links stabilizing the wave forms in undigested axonemes.

In most earlier electron micrographs of flagella, there appeared to be a gap of 5–10 nm between the dynein arms on one doublet tubule and the B component of the adjacent doublet so that the arms did not form a complete cross-bridge between the two doublets (2, 13). However, recent work has suggested that the apparent absence of cross-bridges may be a result of the difficulty of preserving them during fixation for electron microscopy (10). By using appropriate conditions of specimen preparation, we have been able to demonstrate the presence of dynein arms forming numerous cross-bridges between the doublet tubules in electron micrographs of rigor wave axonemes (Gibbons and Gibbons, results in preparation).

A variety of recent evidence has indicated that the dynein arms play a major functional role in generating the active sliding movements between tubules during normal flagellar beating, with the arms forming transient cross-bridges between tubules, and the energy for sliding being provided by hydrolysis of ATP (8, 10, 11, 23, 24). The detailed mechanism of force generation remains unknown, but each mechanochemical cycle of a dynein arm presumably involves its detachment and reattachment to successive binding sites on the adjacent doublet tubule in some manner associated with its binding and hydrolysis of a molecule of ATP. The simplest hypothesis for explaining rigor waves would be to assume that at the final stage of each cycle involving binding and hydrolysis of an ATP molecule the dynein arm remains in a resting state attached to the adjacent tubule until such time as it binds another ATP molecule and commences a new cycle of detachment and attachment. On this basis, all the dynein arms that happened to be attached at the moment ATP was effectively removed from the solution would remain attached and constitute fixed cross-bridges, while the arms that happened to be detached at that moment would form cross-bridges later when they had completed their ongoing enzymic cycle. However, such a hypothesis may well be a considerable oversimplification, for it takes no account of the coordinating mechanisms that must be assumed to regulate the sliding movements of the tubules to produce propagated bending waves. It should be noted that we do not, at present, have a satisfactory way to determine what percentage of the total number of dynein arms is involved in forming the fixed cross-bridges that stabilize the rigor wave forms. The preservation of the wave forms requires only that there be no sliding between the tubules, and this could, in principle, be accomplished by just a small percentage of the dynein arms forming fixed cross-bridges. However, the considerable resistance of the rigor wave axonemes to forcible straightening by viscous shear suggests that there is actually a fairly high degree of cross-bridging between the tubules. This finding is supported by our preliminary electron microscope observations, which have shown a moderately high percentage of the dynein arms forming cross-bridges, although the difficulties with specimen preparation have so far prevented this procedure being used for quantitative assay of cross-bridges (Gibbons and Gibbons, results in preparation).

The relaxation of rigor waves provides a convenient visual indication of conditions that detach the cross-bridges between tubules so that elasticity can cause straightening of the axoneme. Our results have shown that the rigor waves are slowly and partially relaxed by low concentrations of ATP. The slowness with which straightening occurs in 1–3 μM ATP indicates that relaxation under these

conditions is not a result of a complete detachment of the dynein cross-bridges, with the tubules then sliding freely at a speed determined by the passive elastic and viscous forces on them. The slowness of the straightening suggests, rather, that relaxation is a result of a controlled sliding of the tubules involving the same mechanochemical cycle of dynein arm detachment and reattachment coupled to hydrolysis of ATP that is responsible for generating the much more rapid sliding between tubules that occurs at higher concentrations of ATP. During relaxation in low concentrations of ATP, the sliding between tubules would thus occur at a slow speed determined by the turnover rate of the mechanochemical dynein arm cycle at the particular ATP concentration involved. Under these conditions, the active moment generated by the dynein arms would presumably be too small to oppose the intrinsic elastic forces tending to straighten the axoneme so that sliding would be forced to occur in the energetically "downhill" direction favored by the elastic forces. This general hypothesis is supported by the fact that the divalent cation and nucleotide specificity for inducing relaxation closely parallels that for inducing motility. $MgATP^{2-}$ is the most effective for producing either relaxation or motility, while $CaATP^{2-}$ is very much less effective and needs to be present at a concentration about 10^5-fold greater than $MgATP^{2-}$ to produce an equivalent effect (8). The other nucleotides tested, $MgITP^{2-}$, $MgGTP^{2-}$, and $MgGDP^{-}$ appear to be completely incapable of inducing either relaxation or motility, in spite of the fact that inhibition studies have shown that they are able to compete with $MgATP^{2-}$ for binding to the functional enzymic sites (8).

It is not yet certain whether the halt in relaxation before straightening is complete represents the attainment of an equilibrium between the active and passive forces in the axoneme, or whether it is simply the result of the denaturation of the dynein ATPase with time, but it may be noted that the concentration of ATP required to achieve straightening (approx. 10 μM) is close to that required to restart beating movements of the axoneme (8). Although these and other details are not yet clear, the general hypothesis that relaxation in low concentrations of ATP involves the mechanochemical cycle of the dynein arms appears to provide a reasonable basis for understanding the speed and specificity of relaxation. The present evidence supporting the hypothesis is mostly indirect, but it may be possible to examine the hypothesis more directly by measuring the rate of ATP hydrolysis during relaxation.

The behavior of partially attached axonemes when subjected to the stress of fluid flow demonstrates some general mechanical properties of the rigor state. The rigor axonemes have considerable resistance to bending, for the moment exerted by the maximum fluid flow obtainable in this type of experiment (approx. 150 μm/s in the vicinity of an axoneme close to the glass surface) produced only a small change of 0.4 rad or less in the bend angles of the rigor waves. This rather small change in bend angle indicates that elastic stretching and compression of the tubules probably does not play a major role in accomodating the shearing forces between the tubules during normal flagellar movement. However, the fact that a moderate applied bending moment does produce a measurable change in angle suggests that the elasticity of the tubules may represent a factor that cannot be completely neglected.

Although the rigor axonemes are resistant to bending, they can be twisted relatively easily. The same applied bending moments that cause only a small change in bend angle are sufficient to twist the axoneme through 180° (3.1 rad). It is evident that the cross-links within the axonemal structure that are responsible for maintaining the form of the rigor waves do not prevent the axoneme from twisting reversibly through 180° within a region only 2–3 μm long. Experiments with a scale model of an axoneme (unpublished observations) have indicated that the observed twisting requires only a slewing of the outer tubules through an angle of 0.10–0.16 rad relative to the central longitudinal axis of the axoneme, and does not require a significant amount of relative sliding motion between adjacent tubules. This slewing of the tubules could well be accommodated by a corresponding change in the angle of the cross-bridges between tubules, without the need for stretching or breakage.

The velocity gradient of the fluid flow makes it difficult to estimate the effective magnitude of the bending moment produced on the unattached portion of a partially attached rigor axoneme. Nevertheless, the fact that twisting through 180° can be produced by velocity gradients of about 400/s, corresponding to an average fluid velocity of about 40 μm/s in the vicinity of an axoneme lying close to the glass surface, provides an indication of the sensitivity of the axoneme to torsion. During the ordinary movement of a freely

swimming sperm, the velocity of flagellar wave propagation relative to the surrounding fluid is often as high as 600 μm/s (5), so that the magnitude of the active forces generated within the axoneme to oppose the viscous resistance of the medium would appear to be of at least sufficient magnitude to cause significant twisting of the axonemal structure. This poses the question of the torsional stability of the axoneme, and suggests that if axonemal tubules do not twist during normal flagellar movement it is principally because of a balance of torsional moments and not because the axonemal structure has a high resistance to torsion. The alternative possibility to be considered is that the axonemal structure does not remain untwisted, but undergoes a dynamic twisting in localized regions associated with the formation and propagation of bending waves. Electron microscopy has provided evidence that twisting of the axonemal structure occurs in certain cilia which have a three-dimensional form of beat (1, 20, 25), but there is as yet no evidence of axonemal twisting during normal movement of sea urchin sperm flagella, which have a planar wave form.

The effects of subjecting preparations to high degrees of viscous shear in a capillary tube have provided confirming evidence of the high resistance to bending in rigor axonemes. As discussed above, the rigor wave forms appear to be maintained largely by the dynein arms, which form cross-bridges preventing relative sliding motion between the outer doublet tubules of the axoneme. The straightening of rigor wave forms by high degrees of shear could, in principle, be the result of either the stretching or the tearing of the cross-bridges between tubules, or of the stretching and compression of the tubules themselves. Consider what each of these possibilities would involve for the straightening of a typical bend with an angle of 2 rad and a radius of curvature of 5 μm. The average amount of sliding between tubules in such a bend amounts to about 90 nm (23), so that straightening by stretching of cross-bridges would require a 600% increase in the length of the dynein arms, whose initial length is only about 15 nm; this seems improbable. The second possibility is that straightening is a result of tearing of the cross-bridges so that sliding movement can occur between tubules. This possibility cannot be excluded, but the high degree of mechanical cooperativity between the cross-bridges, the partial straightening of waves observed at intermediate degrees of shear, and the fact that straightening does not necessarily destroy the potential for motility, all suggest that tearing of cross-bridges is not the major factor underlying straightening. The third, and perhaps most likely, possibility is that straightening involves stretching and compression of the tubules themselves. In this case, a small change in relative length, amounting to about 5% between the tubules on the inside and outside of the bend, would be sufficient to permit complete straightening of the above typical bend. The irreversible nature of the straightening we observed would indicate that these changes in length are largely inelastic, such as might be expected to result from dislocations and changes in the packing arrangement of the molecular subunits in the tubules. This hypothesis has the advantage of providing a ready explanation for the fact that irreversible straightening of the rigor wave forms occurs at approximately the same level of viscous shear as that required to cause complete transverse fracturing of the axonemes, and it can also account for partial straightening of the wave forms.

The presence of rigor waves in flagella constitutes an obvious parallel to the rigor state of muscle, in which the removal of ATP causes formation of fixed cross-bridges between the myosin filaments and the actin filaments so that the muscle becomes set at its existing length. In muscle, these cross-bridges are formed by the globular head portions of the myosin molecules which contain the sites for ATP hydrolysis as well as those for binding to actin. A wide variety of evidence has established that during normal muscular contraction, the myosin filaments slide relative to the actin filaments as a result of a mechanochemical cycle of the myosin cross-bridges (14). Study of the kinetics of ATP hydrolysis by actomyosin has suggested a model of cross-bridge activity in which the binding of a molecule of ATP initiates the mechanochemical cycle by causing the cross-bridge to detach from the actin filament (17). To this extent, the mechanism generating sliding in muscle appears to resemble that generating sliding between tubules in flagella. However, it must not be forgotten that myosin differs substantially from dynein in both its physical-chemical and its enzymatic properties, just as actin differs substantially from tubulin (15, 19, 22, 26). Moreover, attempts to demonstrate that proteins of the actomyosin system can cross-interact with those of the tubulin-dynein system have so far met with largely negative results (18). Thus, although the occurrence of a rigor state in flagella and in muscle

constitutes an interesting point of similarity, the two systems are clearly different in many other major respects.

Further study of the mechanical and chemical properties of rigor waves in flagellar axonemes may provide a useful approach to obtaining more detailed information about both the elastic properties of the axonemal structures and the mechanochemical activity of dynein.

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