ADENOSINE TRIPHOSPHATE-INDUCED MOTILITY AND SLIDING OF FILAMENTS IN MAMMALIAN SPERM EXTRACTED WITH TRITON X-100

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ABSTRACT

Bull sperm that had been extracted with 0.2% Triton X-100 could be reactivated with ATP, and their movement closely resembled the motion of intact live sperm. Their motility required the presence of ATP, magnesium, and a medium of suitable salt concentration and pH. When Triton-extracted bull sperm were digested briefly with trypsin at pH 9.0, they appeared to retain most of their normal structure, but subsequent exposure of the digested sperm to ATP caused a disintegration of the flagellar structure. Direct observation of this disintegration by light microscopy, using dark-field illumination, combined with an electron microscope study of preparations of the disintegrated sperm, demonstrated the presence of an active sliding mechanism of filament interaction in bull spermatozoa. Human sperm subjected to the same procedures showed similar patterns of reactivation and of disintegration.

It has been known for some time that addition of exogenous ATP can reactivate motility in sperm that have been subjected to procedures which increase the permeability of the plasma membrane. Various types of invertebrate sperm have been shown to respond with regularly propagated flagellar waves and forward progression (5, 19, 20). Recently it has been demonstrated that the nonionic detergent Triton X-100 is effective in completely removing the plasma membrane of the sperm of the sea urchins *Colobocentrotus atratus* and *Tripneustes gratilla*, and, further, that the demembranated sperm are capable of apparently normal motility in the presence of ATP and Mg^{++} (11, 16). In addition, Summers and Gibbons (43) have shown that if these demembranated sperm are first digested briefly with trypsin, the subsequent addition of ATP results in an immediate disintegration of the axonemal structure. Observation of this disintegration using dark-field light microscopy led to the conclusion that ATP generated active sliding movements between the doublet tubules of the axoneme.

In spite of these successes in reactivating the sperm of invertebrates, there has been some difficulty in achieving comparable results with mammalian sperm, which have a more complex structural organization. Several workers have reported that glycerinated mammalian sperm reactivated with ATP show only limited activity, characterized as a twitching or uncoordinated movement (3, 19, 29). Recently Lindemann and Rikmenspoel were able to restore slow coordinated motility to segments of bull sperm flagella (23, 24), and they determined that mechanical bending of the flagellum and the presence of ADP helped to stabilize the maintenance of coordination. Similar slow coordinated waves have also been reported re-

cently by Morton (28) in mammalian sperm reactivated with low concentrations of ATP. Direct measurement of the stiffness of bull sperm flagella, after cells had been impaled with a microprobe, demonstrated that the presence of ATP causes a substantial decrease in the stiffness of the flagellum (25); this result would be expected if the longitudinal fibers in the flagella are bridged to each other by ATP-sensitive links. The presence of such cross-bridging has been described in sea urchin sperm flagella (12, 13, 18, 44). This indirect evidence has suggested that a sliding filament process similar to that described for sea urchin sperm (43) may also be present in mammalian sperm.

Recent studies have attempted to adapt the Triton-extraction procedure developed by Gibbons and Gibbons (11) and the trypsin-digestion procedure of Summers and Gibbons (43) to mammalian sperm, and preliminary reports of such work have appeared (22, 28, 42). We now present a more detailed account of the application of these techniques to bull sperm, and some preliminary work on human sperm.

MATERIALS AND METHODS

Sperm Preparation

Bull sperm were routinely obtained as live, frozen semen from Curtis Breeders through a local distributor (Robert Toledo, Twin Pine Dairies, Waianae, Hawaii). When large volumes of sperm were required (as when pellets for electron microscope examination were desired), epididymal bull sperm were used. For this purpose, sperm were flushed from the caudae epididymides of freshly slaughtered bulls with the medium in which they were to be stored at 0°C (see below). For the experiments with human sperm, semen was collected from volunteer donors and treated for storage at 0°C as described below. Sperm of the sea urchin *C. atratus* were collected and stored as described by Gibbons and Gibbons (11).

Most motility experiments were performed with washed bull sperm from a frozen straw of semen. Since the number of sperm per straw was approximately constant, the concentration of sperm in the extracting solution remained roughly constant. When human sperm or epididymal bull sperm were used, the sperm concentration in the washed suspension was adjusted visually to give about the same concentration as was obtained from the frozen bull sperm. Concentrations of sperm protein were assayed by the Lowry method (26).

Reactivation of Motility

To prepare bull or human sperm for reactivation experiments, they were first suspended in a storage medium which consisted of 0.2 M sucrose, 0.07 M K_2SO_4, 5 mM $MgSO_4$, 3-5 μM $CaCl_2$, and 2mM phosphate buffer, pH 7.4 (24). In the case of frozen bull semen, the samples were thawed rapidly, diluted 10-fold with storage medium, and washed twice by centrifugation and resuspension in fresh storage medium. The sperm were then stored at 0°C. The same procedure was used for human sperm in order to remove the seminal fluid. The condition of the sperm in the stock suspensions was always inspected immediately before use by examining the motility of a small sample diluted into storage medium at 25°C. The percentage of sperm showing normal motility was usually about 90% in fresh samples of epididymal bull sperm, 30-50% in frozen bull sperm, and 50% in fresh human sperm.

Reactivated sperm of optimal quality were obtained using the following procedure. 50 μl of washed sperm suspension were added to 0.5 ml of extracting solution containing 0.2% Triton X-100, 0.2 M sucrose, 0.07 M potassium glutamate, 1 mM dithiothreitol (DTT), 0.03 M Tris-HCl buffer, pH 7.9 at room temperature. After about 30 s in extracting solution, a 50-μl sample of the extracted sperm was transferred to an optical slide Petri dish containing 2 ml of reactivation medium (0.13 M sucrose, 0.045 M potassium glutamate, 5 mM $MgSO_4$, 0.6 mM DTT, 0.02 M Tris-HCl buffer, pH 7.9). After the sperm had been mixed with reactivating solution, ATP was added from a 0.1 M stock solution to give a final concentration of 5 mM. On occasion, this procedure was varied in order to test the conditions necessary for motility.

Disintegration

Two procedures have been used to treat the sperm with trypsin for disintegration experiments. In the first procedure, the sperm were suspended in a Triton-extraction solution containing 1% Triton X-100, 0.2 M sucrose, 0.035 M K_2SO_4, 5 mM $MgSO_4$, 1 mM DTT, and 2 mM phosphate buffer, pH 9.0-9.5, at a concentration of 0.5-1.0 mg protein/ml. Trypsin was added to give a trypsin to sperm protein ratio of 1:20, and the suspension was incubated for 5 min at room temperature. The pH of the suspension was then adjusted to 7.5-7.9 by addition of a suitable volume of 0.1 M phosphate buffer pH 6.0, and stored for up to 2 h at 0°C. To observe the process of disintegration by light microscopy, a few drops of the sperm suspension were placed in a slide chamber consisting of a cover glass supported on a microscope slide by a layer of silicone grease along two edges. A droplet of suspension medium containing 0.2 mM ATP, or 0.2 mM ATP mixed with 4 mM ADP, was then placed at one edge of the cover glass while the sperm were under observation. The medium containing ATP slowly moved across the chamber, and the resulting disintegrations could be observed and photographed at the boundary of this medium.

The morphological changes which accompanied disintegration were examined by preparing samples of the

preparations of disintegrated sperm for examination by electron microscopy. In order to distinguish the effects of the different steps in the treatment procedure, a series of control preparations were also examined in which sperm had been (*a*) not treated; (*b*) extracted with Triton at pH 7.5; (*c*) extracted with Triton at pH 9-9.5; (*d*) extracted with Triton at pH 9-9.5 with trypsin present. The preparations were all chilled to 0°C after treatment, and centrifuged at 12,000 *g* to obtain pellets for electron microscopy.

For some experiments, a different procedure was used to treat the sperm with trypsin. This second procedure involved extracting the sperm with 0.2% Triton at pH 7.5-8.0, and then diluting the sperm into a reactivating solution that contained 5 mM $MgSO_4$, 0.2 mM ATP, 4 mM ADP, and trypsin (trypsin/sperm protein ratio about 1:20). Except for the presence of trypsin in the reactivation solution, these conditions were essentially the same as those used in motility experiments. Preparations treated in this way could be observed directly by light microscopy. The sperm initially showed normal motility with a beat frequency of 2-3 Hz, but began disintegrating after the digestion had progressed sufficiently. Samples of the fully disintegrated preparations were centrifuged and fixed for examination by electron microscopy.

Light Microscopy

Light microscopy was performed with 10× and 25× objectives, using dark-field illumination (15). The motility of reactivated sperm was filmed with a 16-mm camera at 12 and 40 frames/s. Frequencies and velocities of moving cells were determined from these films, and from direct microscope observation with stroboscopic illumination. The disintegration of trypsin-treated sperm was recorded by photographs on 35-mm film.

Electron Microscopy

Samples to be examined by electron microscopy were centrifuged into pellets, and then fixed by addition of a solution containing 0.2 M sucrose, 0.035 M K_2SO_4, 5 mM $MgSO_4$, 2% glutaraldehyde, and 10 mM phosphate buffer, pH 7.5. After this initial fixation, the pellets were washed, postfixed with 1% OsO_4, dehydrated in acetone, and embedded in epoxy resin. Thin sections were cut, and then stained with uranyl acetate and lead citrate. Electron micrographs were taken with a Philips EM 300 at 80 kV.

Chemicals

ATP was obtained from Boehringer Mannheim Corp. (New York). Tris, L-glutamic acid, Triton X-100, and DTT were obtained from Sigma Chemical Co. (St. Louis, Mo.). The ADP was obtained from P-L Biochemicals, Inc. (Milwaukee, Wis.). Potassium glutamate was prepared by titration of L-glutamic acid with potassium hydroxide. The Tris was recrystallized from 1 mM EDTA, and then from 80% methanol before use.

RESULTS

When Triton-extracted bull sperm were observed by light microscopy with dark-field illumination, it was apparent that the intensity of the light scattered by the proximal portion of the flagellum and by the acrosomal region of the head was markedly reduced. Electron microscope examination of the extracted sperm indicated that the plasma membrane which normally surrounds the cell had been removed. The usual Triton extraction procedure at pH 7.8 also removed most of the mitochondrial complex that normally surrounds the proximal portion of the flagellum, but left a remnant structure, apparently consisting of the empty outer mitochondrial membrane (Fig. 2 *a*, *b*). The residual structure could be removed by a modified treatment described under the subheading of Disintegration.

Motility

Triton-extracted bull sperm can be reactivated with ATP and Mg^{++}, and a wide variety of conditions permit the reinstatement of some degree of movement. However, optimal motility was obtained by using the Triton-extraction and reactivation media specified in Materials and Methods, and the movement of such reactivated sperm very closely resembled that of live sperm. A series of films were taken of the reactivated sperm.

The general characteristics of these reactivated preparations have been compared with those of corresponding live preparations (Table I). The average beat frequency for the reactivated sperm was 10 Hz, almost identical with that of the live sperm. The forward velocities for reactivated cells ranged from 13 to 100 μm/s, with an average velocity and standard deviation of 50 ± 22 μm/s. This is higher than the average velocity of 39 ± 19 μm/s measured for the live sperm preparation, but the difference is probably not statistically significant. The percentages of motility were 61% and 71% in the live and reactivated preparations, respectively.

Both the reactivated and the live sperm rotated about their long axis as they progressed upward (35, 37). The reactivated preparations usually remained motile for 15-60 min.

Thus, the reactivated sperm in our preparations at least equaled and possibly surpassed the live sperm in their average forward velocity, rotation

TABLE I

Motility of Live and Reactivated Bull Sperm

	Beat frequency*	Velocity‡	Rotation‡		Motility§
			% rotating	Rotation frequency	% motile
	Hz	*μm/s*	%	*Hz*	%
Live	11 ± 4	39 ± 19	80	2.7 ± 2.3	61
Reactivated	10 ± 3	50 ± 22	90	4.2 ± 1.5	71

All reactivated preparations were studied in standard medium, containing 4–6 mM ATP and 5 mM Mg^{2+} (see text). All measurements reported were taken from 16-mm motion picture film. Beat frequency measurements were confirmed by direct stroboscopic measurements, which gave a range of frequency for the reactivated bull sperm of 8–14 Hz.

* Beat frequency measurements were made on nonrotating sperm swimming close to a glass surface in order to avoid interfering effects of rotation (35). Each value represents average of 36–45 sperm.

‡ Measurements are made on freely swimming sperm not close to a glass surface. Each value represents average of 10–13 sperm.

§ Each value represents average of about 120 sperm.

frequency, and percentage of motile cells. The presence of normal frequencies and forward progression has not previously been observed in extracted mammalian sperm.

The waveforms of live and reactivated sperm appear generally similar (Fig. 1), although the amplitude of the reactivated flagella appears somewhat greater, particularly in the proximal region of the flagellum. This difference in amplitude may be a result of the decreased stiffness resulting from the removal of the mitochondria from the proximal region of the flagellum. A more detailed analysis of the swimming motion must await high-speed film data.

The necessity for each of the ingredients in the reactivation solution was examined separately. Sucrose could be omitted without affecting the ability to obtain reactivation, but it prevented osmotic shock upon placing the sperm in the extraction solution, yielding sperm which had undistorted flagella after Triton extraction, and it seemed to improve the general appearance of the waveform of the reactivated sperm. The Tris-HCl buffer could be omitted but the need for constant pH adjustment then became a detriment to smooth execution of experiments.

The reactivating solution used for preliminary experiments was formulated to be isotonic with live bull sperm. However, we found that a considerable improvement in the motility could be obtained by diluting this medium with 1 part distilled water to 2 parts isotonic reactivating solution. This dilution constituted the only significant difference between the reactivating and extraction solutions (the latter being left at isotonic concentration to prevent osmotic shock).

The pH requirement for motility was rather stringent, with optimal reactivation being obtained only between pH 7.6 and 8.1. Outside this range there was a more or less abrupt decrease in the percentage of motile cells. Outside the optimal range of pH, cells that were not actively swimming either twitched or did not move at all, and they appeared to be very limp with their flagella swaying slowly with any currents in the solution. In a typical reactivated preparation, 30-80% of the sperm were fully active at the optimal pH, a figure which compared well with that of the live sperm in the same preparation before treatment (Table I).

The relative concentrations of Mg^{2+} and ATP appear to be highly important in obtaining coordianted motility in bull sperm. We first noticed that if ATP was placed at a given concentration in the medium and the Mg^{2+} concentration was then increased by subsequent additions, there was a concentration at which the sperm would begin showing coordinated movement, continued addition of Mg^{2+} defined a range of activity, and then finally at an upper threshold of Mg^{2+} concentration the coordinated activity ceased (Table II). For an ATP concentration of 4 mM, the range of Mg^{2+} concentration for coordinated activity was 3–5 mM. Above and below this range, the sperm underwent uncoordinated twitching to a greater or lesser extent depending on the degree of removal of the Mg^{2+} concentration from the optimal range. A

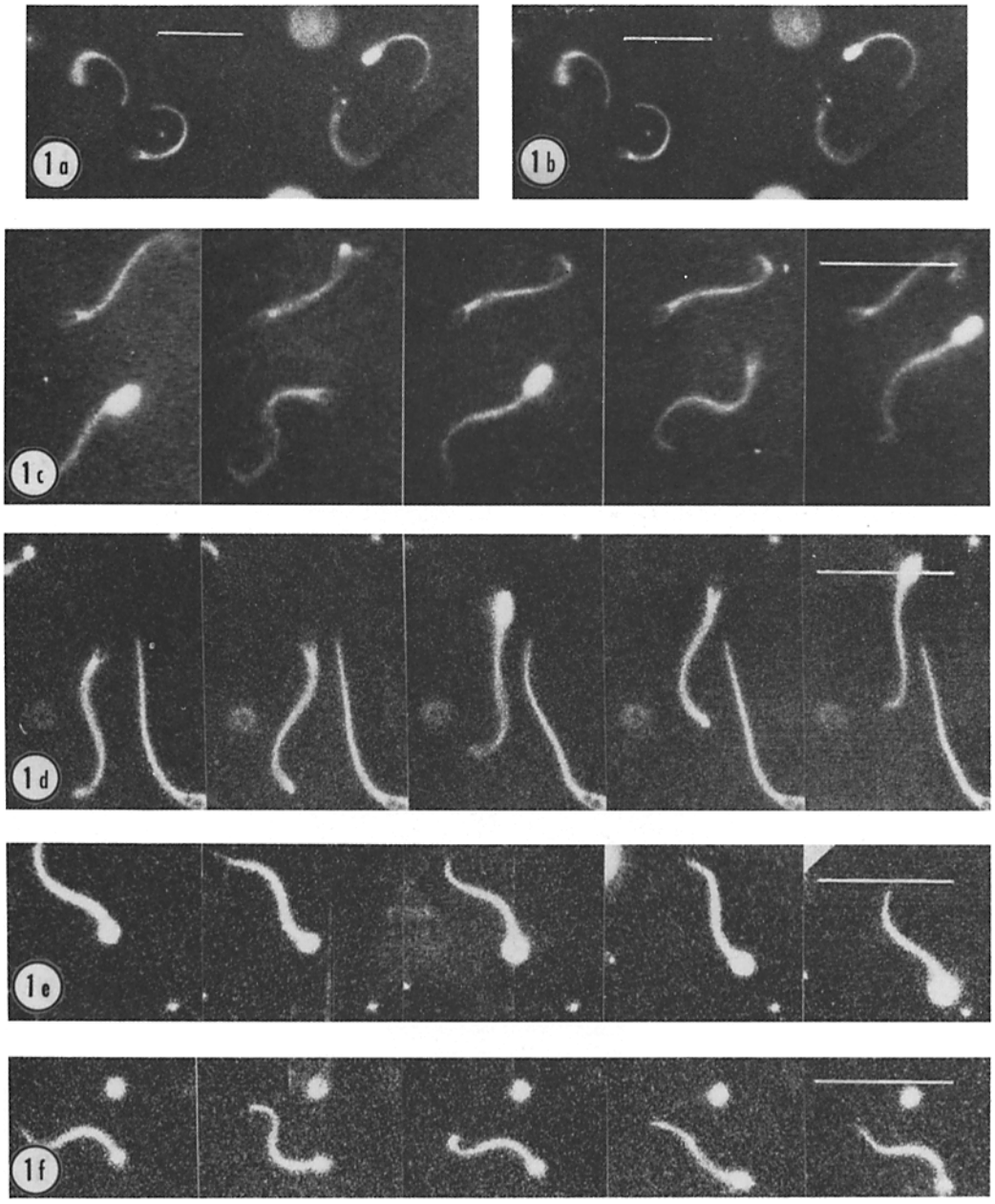


FIGURE 1 Several sequences of frames taken from 16-mm motion picture film exhibiting the motility of live and reactivated sperm. (*a*) Two frames showing a preparation of bull sperm that had been demembranated with Triton at pH 7.8, and then placed in standard reactivating solution containing 4 mM Mg^{2+} and 1 mM ATP at pH 7.9; (*b*) The same preparation of sperm as in Fig. 1 *a* after increasing the ATP concentration to 5 mM; (*c*) Live bull sperm; (*d*) Live human sperm; (*e*) Demembranated human sperm in standard reactivating solution containing 1 mM Mg^{2+}, 1 mM ATP. The forward progress and the rotation of the free swimming reactivated sperm appear comparable to those of the live sperm. All sperm shown in these micrographs were swimming freely in the medium. The bars indicate 50 μm.

TABLE II

The Response of Triton-Treated Bull Sperm at Various Concentrations of Mg^{++}, ATP, and ADP

ATP	Mg^{++}	ADP	Reactivation*
mM	*mM*	*mM*	
0.08–12.0	=[ATP] ± 25%	—	+
0.4–4.0	=[ATP] ± 25%	≥[ATP]	+
≤1.0	[ATP] ≤ Mg <1.5	—	+
≤1.0	≥2.0	—	–
≤1.0	≥2.0	>[Mg^{++}] – 1	+
1.0–12.0	>2[ATP] or <0.5[ATP]	—	–‡
0.4–4.0	0	—	–

* Experiments were only recorded as negative when a portion of the same preparation could be reactivated under standard reactivation conditions of 4 mM ATP and 5 mM Mg^{++}. The presence of coordinated flagellar beating and forward progression of the sperm constituted the criterion of success. No coordinated motility was obtained with Mg^{++} or ATP concentrations below 0.05 mM. All trials were performed using standard reactivating solution at pH 7.9. The top line of Table II represents the results of 14 experiments, and the other lines each represent the results of three to six experiments.

‡ One experiment at 4 mM Mg^{++} and 10 mM ATP gave a reactivation. This represents one experiment out of six experiments observed in this range, or 17% reactivation.

similar effect could be demonstrated by fixing the Mg^{2+} concentration and varying the concentration of ATP. Therefore, unlike the situation for the motility reported for sea urchin sperm (11), it was not possible with bull sperm to set the Mg^{2+} concentration at a given value and obtain reactivation over a wide range of ATP concentration.

It was subsequently found that the presence of ADP as well as ATP in the reactivating solution appeared to facilitate coordination. When 3-10 mM Mg^{2+} were present in the medium, 0.1–2.0 mM ATP did not produce coordinated motion, but coordination could be regained by adding ADP to the preparation. The concentration of ADP necessary was approximately such as to make the summed concentrations of ATP and ADP equal to the Mg^{2+} concentration (Table II), suggesting that the coordinating action of ADP was a result of its binding excess free Mg^{2+} ions as $Mg\text{-}ADP^{-}$. The effect of ADP is complex, however, for its addition to sperm which were fully reactivated with ATP caused a decrease in their beat frequency, without affecting their ability to coordinate.

The duration of motility varied from several minutes to an hour in different reactivated preparations. The deterioration could be retarded by including freshly prepared 1 mM DTT in the solutions. The preparations were still more stable if 5-10 mM ethyleneglycol-bis-(β-aminoethyl ether) *N,N'*-tetraacetic acid (EGTA) was included in the reactivating solution. As well as demonstrating the increased longevity of reactivated sperm, these experiments employing EGTA confirm earlier reports (11, 24) that free Ca^{2+} is not required for motility or coordination. Although EGTA was sometimes included in the medium to stabilize the preparation, we found that it was not necessary to use it routinely. Changing the predominant anion in the reactivating solution from glutamate to sulfate or chloride did not affect the ability to reactivate cells, but did produce differences in longevity. Glutamate was chosen as the standard anion because it yielded the best longevity. These results suggest that the extracted sperm flagella are extremely sensitive to traces of contaminating substances, presumably heavy metals, which may be present in the reactivating solutions.

In a small number of experiments, we have attempted to reactivate Triton-extracted human sperm by the same procedure used to reactivate bull sperm. The motility of sperm reactivated with solutions containing 1 mM ATP and 1 mM Mg^{2+}, and 2 mM ATP and 2 mM Mg^{2+}, was studied by cinematography. The reactivated human sperm showed normal wave propagation and forward swimming (Fig. 1). Measurements of forward velocity averaged 15 μm/s for reactivated cells as compared to 17 μm/s for a preparation of live sperm at the same temperature. However, the average flagellar beat frequency of the reactivated

cells (3.8 Hz) was less than that of the live sperm (7.5 Hz), and the percentage of motile sperm in the reactivated preparation was only 30% as compared to 50% in the same preparation before treatment. Although further experiments will be necessary to optimize the reactivating conditions specifically for human sperm, it is clear that a fair degree of coordinated motility can be obtained with the procedures developed for bull sperm.

Disintegration

Bull sperm are more resistant than sea urchin sperm to complete denuding of the flagellar structure by Triton X-100. Although the plasma membrane of bull sperm is removed completely by extraction with Triton at pH 7.5-8.0, the fibrous sheath surrounding the principal piece of the flagellum is not removed at all, and the helical mitochondrial complex surrounding the proximal portion of the flagellum is removed only partially. The inner mitochondrial membrane, cristae, and mitochondrial matrix appear almost completely removed by the extraction with Triton, but the outer membranes of the mitochondria are resistant under these conditions and they remain as apparently empty membranous sacs still in the form of a helical sheath around the proximal portion of the flagellum (Fig. 2 *a*, *b*). This remnant of the mitochondrial sheath can be completely removed by treating the sperm for several minutes with extraction solution at pH 9.0–9.5 (Fig. 2 *c*), instead of at the usual pH 7.5–8.0. This removal of the residual mitochondrial sheath can be detected by dark-field light microscopy as a further decrease in light scattering of this region of the flagellum. The reports of extensive disulfide bridging in sperm mitochondrial membranes (2) suggest that the solubilization of the outer membrane at pH 9.5 may be due to the quicker reduction of disulfide bonds at the higher pH, although we have not verified this possibility.

Brief digestion of the Triton-extracted sperm with trypsin has little apparent effect on the flagellar structure (Fig. 2 *d*), but it sensitizes the flagella so that subsequent exposure to ATP causes them to disintegrate. The manner in which the flagella disintegrate depends markedly on whether or not the residual mitochondrial sheath on the proximal portion of the flagellum is still present. When this mitochondrial sheath has been completely removed by extraction at pH 9.0–9.5, the disintegration occurs by extrusion of elements from the denuded proximal region of the flagellum (Fig. 3 *a*). When the mitochondrial sheath is still present, it prevents this disruption of the proximal region, and disintegration occurs by an extrusion of elements from the distal tip of the flagellum (Fig. 3 *b*). These two patterns of disintegration will be referred to as proximal and distal disintegration, respectively.

We have found that the most uniform preparations for proximal disintegration were obtained by treating the live sperm with extracting solution containing Triton, DTT, and trypsin at pH 9.0–9.5, as described in Materials and Methods. After the pH of the suspension had been brought down to 7.5–8.0, the sperm were placed in a trough slide, and solution containing ATP was added at one edge. When the diffusing front of ATP reached a sperm, there was a short period of quivering motion of the flagellum, followed by the commencement of disintegration with the development of a sharp bend in the denuded proximal region of the flagellum, a splitting of this region into two strands, and a gradual buckling out of one of the two strands (Fig. 3 *a*, *d*). In most cases, these strands then split further into a number of fibers which slid out successively from the denuded region of the flagellum. This pattern of sliding made it appear that each successive fiber to emerge was riding "piggyback" on the next emerging fiber (Fig. 3 *d*). As the distal tip of each fiber came free from the fibrous sheath covering the distal portion of the flagellum, it curved gently back toward the sperm head. In sperm where the process of disintegration had gone to completion, it could frequently be seen that eight of the fibers had emerged and spread themselves out in this manner, while a last and ninth fiber was left behind within the fibrous sheath (Fig. 3 *e*). From the location and tapered shape of the spread fibers, it seemed probable that they corresponded to the nine coarse auxiliary fibers of the flagellum. This was confirmed by electron microscopy, which showed that only one to eight coarse fibers and axonemal doublets remained in the flagella after disintegration (Fig. 2 *e*), while large numbers of coarse fibers and doublet tubules were free in the medium. There appears to be a structural association between each coarse fiber and its neighboring doublet tubule in the flagellum, for large numbers of the paired coarse fiber-doublet tubule combinations are present lying free in the medium. As reported previously by Fawcett (10), this structural association of coarse fibers and doublet tubules appears to extend along most of the distal portion of each coarse

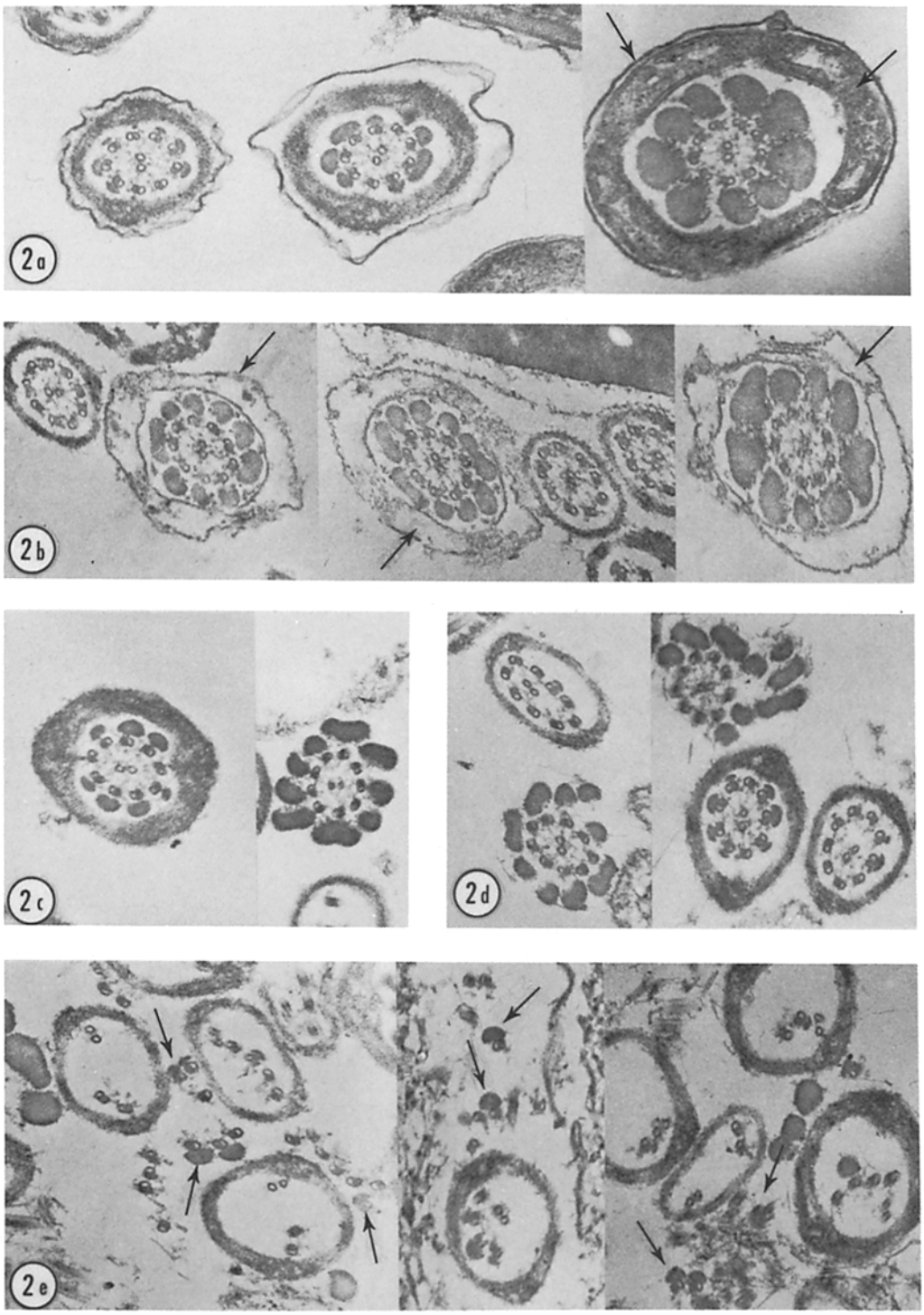


FIGURE 2 Electron micrographs showing cross sections of bull sperm flagella at successive stages in the treatment used to induce proximal disintegration. (*a*) Intact sperm (untreated). Note the presence of the plasma membrane, and the cristae and dense matrix in the mitochondria (third section from left) indicated by arrows; (*b*) Sperm extracted with 1% Triton at pH 7.5. The plasma membranes are absent, and the mitochondria show only a remnant "ghost" structure (arrows); (*c*) Sperm extracted with 1% Triton at pH 9.0. The mitochondrial remnants are no longer present; (*d*) Sperm extracted with 1% Triton at pH 9.0, and then digested with trypsin for 5 min (trypsin sperm protein was approximately 1:20); (*e*) Sperm extracted with 1% Triton at pH 9.0, trypsin treated (as in Fig. 2 *d*), and then ATP added just before fixation. Note the presence of many doublet tubule-auxiliary fiber combinations (arrows), although free doublet tubules and auxiliary fibers are present as well. × 55,000.

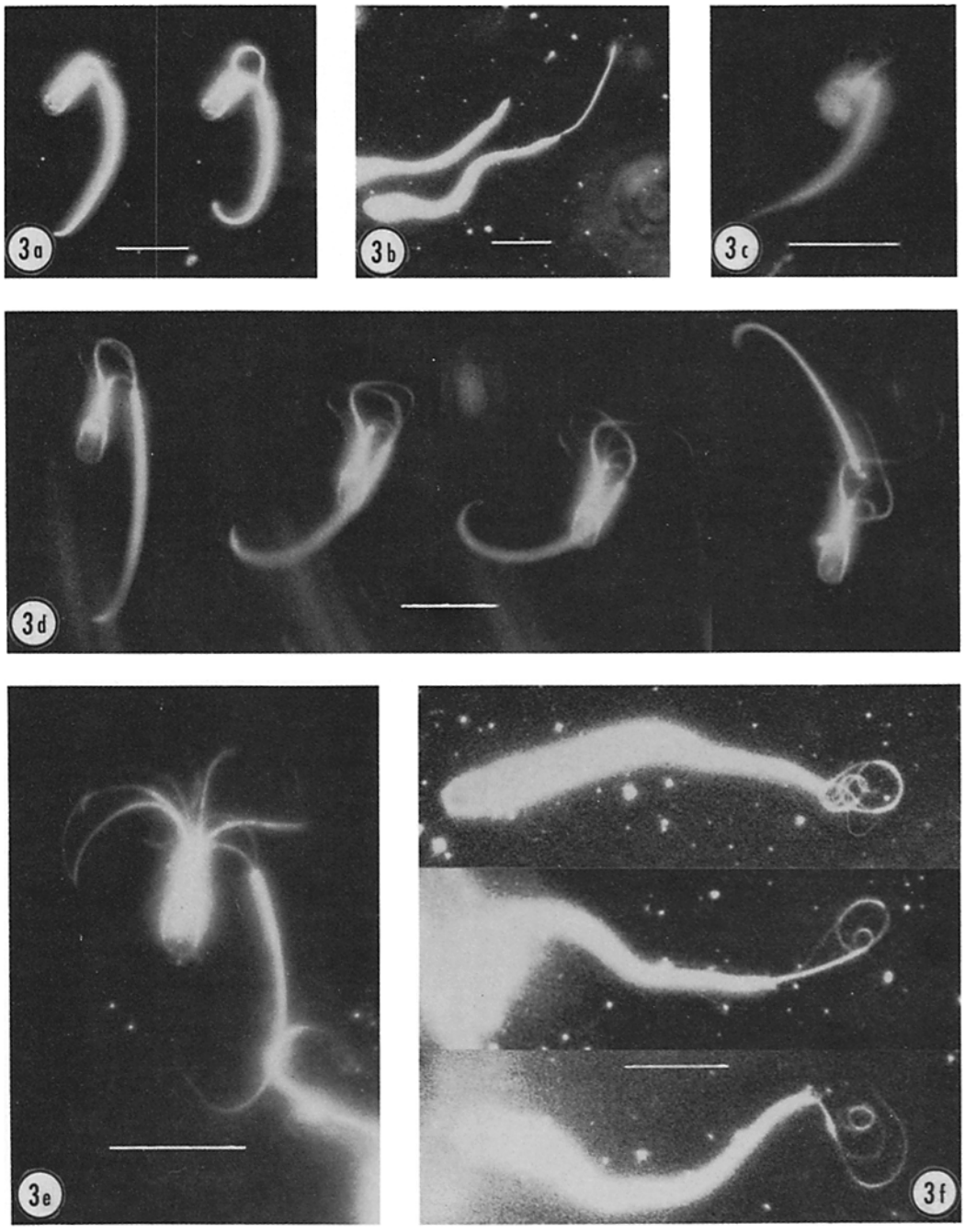
fiber, but it is apparently not present in the proximal portion, in the region of the original mitochondrial sheath. The strength of the fiber-tubule association is evidently great enough to withstand the mechanical stress inherent in disintegration, but we have no evidence concerning its possible role in normal motility.

Both light and electron microscopy indicated that disintegration did not go to completion in all the sperm in a preparation but often stopped before all of the fiber-tubule combinations had emerged from the fibrous sheath. In preparations made from frozen sperm, around 90% of the trypsin-treated flagella usually disintegrated to some extent, in spite of the fact that only 30–50% of the sperm were motile in the same preparations before treatment. The fraction of sperm showing complete disintegration, with extrusion of seven to eight fiber-tubule combinations, varied with the conditions, but was sometimes as high as 50% when disintegration was made to occur slowly by perfusing with a mixture of 0.2 mM ATP and 4 mM ADP. When trypsin-treated bull sperm were placed in a medium containing 0.2 mM ATP and 4 mM ADP, the first fiber became free of the sheath within about 5 s, the process of disintegration was complete after about 15 s, and after that time no further motion of the fibers was observed. Thus, the speed of sliding of the fibers would appear to have been of the order of 6 μm/s under these conditions.

The best preparations showing distal disintegration of flagella were obtained by treating the sperm by the standard procedure for reactivating motility, except that the reactivating solution contained 0.2 mM ATP and 4 mM ADP, and then adding trypsin to the suspension. The sperm remained normally motile with a beat frequency of 2–3 Hz for several minutes after the addition of trypsin, and then the coordinated flagellar beating degenerated into a feeble uncoordinated twitching movement. At about the same time, structural disintegration of the flagella began by means of an extrusion of a very fine strand of material from the distal end of each flagellum. This strand appeared to be extruded at a slow uniform speed of around 1 μm/s, and frequently attained a final length nearly equal to that of the original flagellum. We did not observe extrusion to lengths significantly greater than that of the original flagella. During the later stages of extrusion the strand often appeared to separate into two to three extremely fine filaments, which then usually adopted a coiled configuration (Fig. 3*f*). Both the original extruded strand and the subfilaments which it split into usually appeared to have a uniform light-scattering intensity along their length. This pattern of distal disintegration appears generally similar to that described recently by Summers who digested bull sperm with trypsin under slightly different conditions (42).

The extreme fineness and uniform light intensity of the extruded strand suggested that it was composed of a small number of tubules from the flagellar axoneme, and that the subfilaments which the original strand split into consisted of the individual tubules. This has been confirmed by electron micrographs of preparations that have undergone distal disintegration (Fig. 4). Flagellar cross sections show that the central pair of tubules and from one to three of the outer doublet tubules are usually missing, while all nine of the coarse auxiliary fibers are still present. The doublet tubules remaining in the flagellum often adjusted their positions to close up the gap left by missing doublets, and thus formed a smaller complete cylinder containing six, seven, or eight doublets. The extruded central and outer doublet tubules were frequently visible, close to the residual flagellar structure. The pair of central tubules from an axoneme usually remained together as a unit, but most often they did not appear to have any doublet tubules associated with them. In a few flagellar cross sections (Fig. 4), some of the coarse auxiliary fibers were missing as well as the doublet axonemal tubules, giving an appearance like that seen in the preparations that had undergone proximal disintegration. This is consistent with the observed condition of these preparations before fixation, where a small percentage of proximal disintegrations were seen under the light microscope.

As already mentioned, the most uniform preparations of distal disintegration were obtained by adding trypsin to preparations of normally reactivated sperm already containing ATP. Under these conditions, the majority of sperm that were initially motile disintegrated in the manner discribed above. It appeared characteristic that only sperm that were initially motile, at least to some degree, would disintegrate in this way, and that nonmotile sperm did not disintegrate. Disintegration of the distal type could also be obtained by treating the Triton-extracted sperm with trypsin at pH 7.5–8.0 in the absence of ATP, and then subsequently infiltrating with ATP. However, the results obtained with this procedure were less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satisfactory and usually only a small percentage of the sperm disintegrated. The difference in results obtained by these two methods, together with the lack of disintegration by nonmotile sperm, suggests that the beating and twitching movements of sperm before and during disintegration may play some role in producing the extrusion of tubules during distal disintegration. However, the difference between the two types of procedure could also be explained on the basis that distal disintegration occurs only within a critical narrow range of trypsin digestion which is difficult to attain by pretreatment with trypsin.

We have incidentally noted that disintegrations of the proximal type would occur in preparations of Triton-extracted sperm incubated for an extended period at pH 8.5–9.0 in the presence of ATP, but in the absence of trypsin. It is reasonable to suppose that this disintegration is a result of digestion by the endogenous proteolytic activity released from the sperm acrosome by the extraction with Triton (32, 45).

The flagella of human sperm treated by the pH 9.0–9.5 Triton-trypsin procedure and subsequently exposed to ATP showed essentially the same pattern of proximal disintegration (Fig. 3 *c*) as bull sperm.

Direct Comparison of Mammalian Sperm and Sea Urchin Sperm

We performed a small number of experiments to make a direct comparison between the properties of reactivated bull sperm and reactivated sea urchin sperm. Triton-extracted sea urchin sperm showed good motility in the reactivation medium developed for bull sperm, but they did not lose coordination when the ATP and Mg^{2+} concentrations were changed so as to uncoordinate the bull sperm. Moreover, when bull sperm were treated by the previously described technique for reactivating sea urchin sperm (11), they did not show coordinated motility.

DISCUSSION

The requirements for the maintenance of active, coordinated motion in Triton-extracted bull sperm are remarkably similar to those of sea urchin sperm. However, there is one important difference in that the presence of significant concentrations of Mg^{2+} or of ATP^{4-} in the uncomplexed form tends to be antagonistic to the coordination process in bull sperm. This finding may explain why some of the procedures used in earlier attempts to reactivate mammalian sperm resulted in only uncoordinated twitching motion (3, 19, 29). This finding and the specificity of the coordination process for a limited range of pH probably explain the earlier results reported for impaled bull sperm in which the flagella were flaccid with only slight twitching activity in high concentrations of ATP at pH 7.4 (24, 25). The reason why free Mg^{2+} and ATP^{4-} are antagonistic to coordination is not yet clear. It is possible, although seemingly unlikely, that the presence of free Mg^{2+} or of ATP^{4-} interferes with the interaction of $Mg\text{-}ATP^{2-}$ at the sites of ATPase activity. Alternatively, the amounts of free Mg^{2+} and ATP^{4-} may need to be minimized for some aspect of coordination not directly involved in ATP hydrolysis, since the flagella do

FIGURE 3 Dark-field micrographs of trypsin-treated demembranated sperm undergoing disintegration as ATP is perfused through the preparation: (*a*) Two micrographs sequential in time showing the onset of disintegration in bull sperm which have been extracted with Triton at pH 9, and then treated briefly with trypsin. The middle piece of the sperm is denuded of mitochondrial remnants (see Fig. 2). The pattern of splitting into two strands, one of which buckles out from the middle piece region, is typical of the initial stage of proximal disintegration; (*b*) A bull sperm which has been extracted with Triton at pH 7.9 (same conditions as for reactivation) and digested with trypsin. The onset of disintegration is marked by the emergence of a strand from the distal end of the flagellum. Note the uniform intensity of the extruded strand; (*c*) A human sperm treated as in Fig. 3 *a*. Tapered fibers are visible extending from the middle piece region; (*d*) A sequence of micrographs showing the emergence of tapered fibers from the midpiece region of a single bull sperm. Note the sequential order of emergence of the fibers, most marked in the second and third frames; (*e*) The final stage of the proximal disintegration process in a typical bull sperm. Nine tapered fibers are visible on the original micrograph; (*f*) Several sperm undergoing distal disintegration after extraction with Triton at pH 7.9, and subsequent digestion with trypsin. The strands that have emerged from the distal end of the flagella have separated into several subfilaments, which have tended to curl up. The strands do not possess a gradual taper, but undergo incremental changes in intensity with their progressive separation into individual subfilaments. The bars indicate 25 μm.

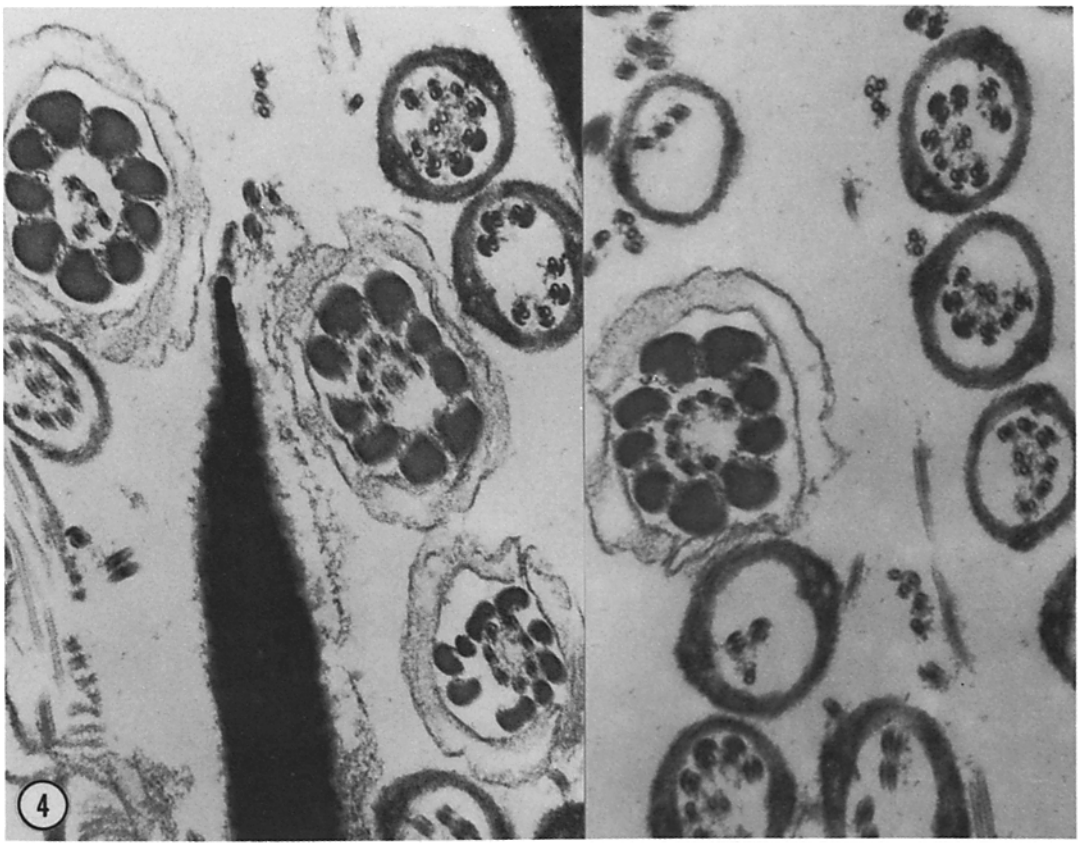

FIGURE 4 Electron micrographs of a preparation of bull sperm in which the flagella had undergone distal disintegration. Fresh epididymal bull sperm were treated with Triton-extraction solution containing 0.2% Triton X-100, 0.2 M sucrose, 0.6 M KCl, 1 mM DTT, and 30 mM Tris buffer, pH 7.9, at room temperature. After 2 min, the suspension was diluted with 3 vol of standard reactivation solution containing 1:20 trypsin/protein, and 0.2 mM ATP and 2 mM ADP. After 15 min, when the majority of sperm flagella had disintegrated, the suspension was centrifuged, and the pellet fixed for electron microscopy. × 55,000.

exhibit some uncoordinated movement in the presence of free Mg^{2+} or ATP^{4-}.

We have observed, as did Lindemann and Rikmenspoel (24), that when the ATP/Mg^{2+} ratio was below that necessary for coordination, occasional sperm which happened to be attached to the slide in such a way that their flagellum was bent would retain coordination. Such observations suggest that when the ATP and Mg^{2+} concentrations are unbalanced, the forces generated by the active elements fall below a critical threshold necessary to induce the bending required for coordination, and that bending the flagellum artificially supplies the mechanical end point necessary to initiate coordination. A possibly similar effect was observed by Brokaw and Gibbons (8) in partially demembranated flagella of sea urchin sperm in which the rapid beating of a distal membrane-covered region was intermittent, and was turned on and off in phase with the much slower bending cycle in the proximal region of naked axoneme. Such observations suggest that some mechanical feature of the bending participates in the regulation of sliding required for coordination.

In bull sperm, the regulation of free Mg^{2+} and ATP in intact cells would occur as a natural outcome of the constant total concentration of (ATP + ADP + AMP). Measurements of the ATP concentration in live bull sperm have given values of 5–10 mM (4, 21), and this is the same concentration range that yields the most lifelike motility in our extracted preparations. There thus seems to be no need to invoke complex interpretations involving compartmentalization of ATP to

reduce its concentration within the flagellum during normal movement (28).

Our results have shown that ADP appears to have a double effect on motility: it facilitates coordination by binding excess Mg^{2+}, and it decreases the beat frequency possibly as a result of $Mg\text{-}ADP^{-}$ competing with $Mg\text{-}ATP^{2-}$ for binding at the active sites of force production. In the intact cell, the concentrations of Mg^{2+} and ATP will presumably not vary greatly under normal physiological conditions. However, the ATP concentration can be altered experimentally by changing the metabolic conditions, or by inhibiting oxidative phosphorylation with KCN, and the beat frequency then decreases gradually while coordination is maintained (39, 41). The fact that the decrease in intracellular ATP concentration will be accompanied by an increase in the total concentrations of ADP and AMP provides an adequate explanation for the slower motion with maintenance of coordination under these conditions of reduced metabolism.

The evidence presented here for the occurrence of active sliding induced by ATP in bull sperm flagella is in general agreement with previous results obtained with flagella of sea urchin sperm (43). However, the greater structural complexity of bull sperm flagella naturally causes some differences in the detailed patterns of sliding. The general features of the two patterns which we have termed proximal and distal disintegration are undoubtably determined by the presence or absence of a residual mitochondrial sheath on the proximal portion of the flagellum. Both patterns of disintegration can be explained on the basis that sliding is a result of interaction between the tubules of the flagellar axoneme. When the mitochondrial remnant has been removed from the sperm so that disintegration of the proximal type can occur, the coarse auxiliary fibers would tend to be carried along passively by their attachments to the sliding doublet tubules, and to loop out from the denuded proximal portion, since the attachment of the fibers to the sperm head would prevent sliding at that end. The pattern of proximal disintegration by looping out is very similar to that observed in sea urchin sperm flagella when the presence of the centriole prevents sliding of the tubules at the proximal end (43). When the mitochondrial remnant is still present, it prevents the looping out of the fibers in the proximal region and forces the disintegration to occur by extrusion of tubules from the distal end of the flagellum. Judging from the similarity between the resulting photographs (Fig. 3 *f*) and those presented by Summers (42), it is very likely that this mode of disintegration is the same as that demonstrated by Summers for bull sperm. While it is true that there are several differences in the medium and procedure employed in the two studies, the essential ingredients and conditions necessary for distal disintegration are maintained in both. This distal extrusion presumably occurs after the trypsin digestion has proceeded far enough to free the doublet tubules from their normal attachment to the coarse fibers, for the presence of tapered fibrous sheath and their joint attachment to the sperm head would prevent the coarse fibers from sliding distally in this way. The two patterns of disintegration we have observed can thus both be reasonably explained on the basis of active sliding between adjacent doublet tubules. However, the possibility that the central tubules and/or the coarse fibers also participate actively in the disintegration cannot be excluded.

Although the basic features of proximal and distal disintegration appear to be determined by the presence or absence of a mitochondrial remnant, the two types of disintegration differ also in certain respects of detail that are not so readily explained. These differences concern the relationship of disintegration to motility, the participation of central tubules, and the final length after disintegration. The distal disintegration of bull sperm appears to be closely dependent upon motility, for it occurs only in sperm that were initially motile. Proximal disintegration in bull sperm, as in sea urchin sperm, seems less dependent upon motility, for often 90–100% of the flagella in a digested preparation will disintegrate in spite of the fact that only 20–50% of the original undigested flagella were capable of motility with ATP. The central tubules are almost always extruded during distal disintegration, even when only one of the doublet tubules is lost, whereas in proximal disintegration the central tubules usually remain within the fibrous flagellar sheath even after six to eight of the doublet tubule-coarse fiber combinations have been extruded from the flagella. A further difference concerns the final length after disintegration. The length of the strand extruded distally from bull sperm has not been observed to exceed that of the original flagellum, whereas in sea urchin sperm axonemes the final length is often as much as five to seven times the original length (16, 43). The expulsion of the central tubules and the fact that the final length does not exceed twice

the original suggest that the force for distal disintegration might be derived from interaction between the outer and the central tubules rather than between adjacent outer tubules. However, the significance of this rather speculative possibility, like the question of a possible active role of the coarse auxiliary fibers in proximal disintegration, will have to await further study.

The close similarity between the axonemal structure of bull sperm flagella and that of sea urchin flagella suggests that a similar mechanism is responsible for producing the sliding movements between the doublet tubules. In sea urchin sperm a variety of evidence indicates that the sliding movements between the doublet tubules are generated by the projections ("arms") which form transient cross-bridges between adjacent pairs of doublet tubules and are composed of the ATPase protein dynein (13, 18, 43, 44). Since arms of similar appearance are also present on the doublet tubules in the axoneme of bull sperm flagella (13), it seems likely that they are responsible for generating the sliding movements of these tubules in bull sperm, although direct evidence of this is lacking so far.

In view of our finding that ATP induces active sliding between the doublet tubules in trypsin-treated bull sperm flagella, it appears likely that similar sliding movements contribute the basis of the normal propagated bending waves in intact bull sperm flagella. In the intact flagella, these sliding movements would presumably be coordinated and restrained by the axonemal structure, leading to the occurrence of limited sliding which is repetitively initiated giving rise to the motion of the flagellum. Theoretical analyses have shown that such repetitive sliding, suitably coordinated along the axoneme, can produce bending waves in a model system similar to those observed in actual flagella and cilia (6, 27, 40).

In comparing the mechanism of movement in mammalian sperm flagella with that in the simpler sperm of invertebrates, the major question concerns the role of the coarse fibers. Two hypotheses appear possible at present. The active forces producing motility may be generated by the dynein arms on the axonemal doublet tubules, while the coarse fibers play a passive role in increasing the stiffness and mechanical strength of the sperm flagellum. Alternatively, it is possible that the auxiliary fibers play an active role, and function to amplify the forces generated by the axonemal doublets. Our study of the patterns of disintegration observed after trypsin treatment has not as yet permitted us to determine whether or not the coarse fibers play an active role in this sliding. Comparative study of structure and movement in a variety of mammalian sperm (33) has shown that the apparent stiffness of the sperm is higher in the species having the thicker coarse fibers. This observation strongly suggests that the coarse fibers contribute a major proportion of the flagellar stiffness, but it does not exclude their possessing an active role in addition. If we accept this evidence that the coarse fibers are responsible for a major portion of the flagellar stiffness, we must interpret measurements of the stiffness as reflecting the condition of these structures. Direct measurements of the stiffness of bull sperm flagella have demonstrated a 15-fold change in stiffness as a function of ATP concentration (25). This change in stiffness is consistent with the coarse fibers having an active role mediated by ATP, although it could also be explained by the presence of ATP-sensitive cross-bridges between the doublet tubules since each doublet tubule has a coarse fiber attached to it along much of its length.

Isolated bull sperm flagella possess ATPase activity, and much of this activity can be solubilized by extraction with 0.6 M KCl (9). However, the physical-chemical characteristics of the solubilized ATPase have not yet been determined in sufficient detail to assess its homogeneity and its degree of similarity to dynein, the ATPase protein isolated from cilia and from invertebrate sperm flagella (14, 17). Several cytochemical studies have reported the presence of ATPase activity associated with the coarse fibers of mammalian sperm flagella (1, 30, 31). However, these findings have not so far been confirmed by biochemical studies on the isolated coarse fibers which have reported them to be composed largely of a keratin-like protein without ATPase activity, and possessing numerous free SH groups that might interfere with cytochemical ATPase determinations (1, 34).

If the coarse fibers of mammalian sperm play an active role in motility, then it would be expected that the active bending moment, power output, and metabolic rate of mammalian sperm would be significantly greater than those of typical invertebrate sperm with a simple "9 + 2" flagellum. The available evidence taken at face value would indicate that this is the case (7, 36, 38, 39, 41). However, the data for invertebrate sperm were mostly obtained at 16°C (7), and those for bull sperm at 37°C (38, 39). Therefore, it is not clear whether a straight comparison is valid, since the

energetics of sperm motility are clearly affected by temperature. Further studies of mechanochemical energy conversion in different types of spermatozoa over a range of temperatures may make it possible to obtain more definite information as to whether or not the coarse auxiliary fibers play an active role in the motility of mammalian sperm.

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