

# ATP-INDUCED SLIDING OF MICROTUBULES IN BULL SPERM FLAGELLA

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## INTRODUCTION

ATP-induced sliding of doublet microtubules has been recently demonstrated with axonemes of sea urchin sperm flagella which had been briefly digested with trypsin (13). The specific chemical conditions in which such sliding occurs are the same as those required for the reactivation of beating in demembrated sea urchin sperm cells (4). The conclusion from these observations, that the bending of sea urchin sperm flagella is based on a mechanism which produces local sliding between adjacent doublet microtubules, supports theoretical studies of flagellar motility which find that a "sliding filament" model is better than a "contracting filament" model in explaining some of the characteristics of the flagellar bending waves (2, 10).

The application of these results from sea urchin sperm flagella to explain the mechanism of mammalian sperm motility is complicated by the presence of large additional structures found in the flagella of mammalian sperm. Mammalian sperm flagella have in common with sea urchin sperm flagella a basic axoneme composed of a cylinder of nine parallel doublet microtubules surrounding a central pair of single microtubules (3, 1). These 11 microtubules are held together by various accessory structures including pairs of arms which contain the ATPase protein *dynein* which seem to serve as mechanically active bridges between adjacent doublet tubules (5).

In mammalian sperm the basic axoneme is supplemented by nine coarse fibers which lie parallel and adjacent to the nine doublet microtubules forming a second cylindrical layer in the flagellar structure (3, 1). The coarse fibers are much larger than the doublet tubules near the head of the sperm cell and taper gradually until they disappear near the tip of the flagellum. The microtubules and coarse fibers are enclosed by a third cylindrical layer consisting of a mitochondrial sheath near the sperm head and a fibrous sheath along the rest of the length of the flagellum. In order to produce motion in the more bulky mammalian sperm, it is generally assumed that the

coarse fibers participate actively in the motile process although an inactive elastic role has also been suggested (7). An inactive elastic role has also been proposed for the fibrous sheath (9).

In order to determine whether the doublet microtubules of mammalian sperm flagella are capable of motility similar to the doublets of sea urchin sperm flagella the effects of ATP on trypsin-digested bull sperm were examined.

## MATERIALS AND METHODS

Ejaculated bull semen was cooled and stored at 0°C until used. The motility of the sperm was checked by suspending samples of the semen in 3.4% sodium citrate at room temperature. The percent of fully active sperm declined slowly over a period of 3-5 days of storage but nearly all sperm retained some activity in the form of twitching or slow flexing.

Small quantities of the sperm were demembrated by suspending semen (about 1:10) in a solution containing 0.1 M KCl, 5 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 1 mM dithiothreitol, 10 mM Tris-phosphate pH 7, 0.1% Triton X-100 at 0°C for 30-60 min. The Triton-washed sperm were diluted 1:40 into a second solution containing 0.15 M KCl, 4 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 1 mM dithiothreitol, 2% Carbowax, 0.3 mM ATP, 10 mM Tris-phosphate pH 7.0. Trypsin was added (1.25 µg/ml) and a drop of the suspension was placed on a microscope slide, under a cover glass sealed with silicone grease.

Samples were fixed for electron microscopy by mixing the suspensions with equal volumes of 6% glutaraldehyde in 0.2 M phosphate buffer, pH 7.0. Some fixed samples were then pelleted, embedded in Epon-Araldite, and examined in thin section (12). For negatively stained specimens the fixed cells were either allowed to settle out of the fixing solution onto a Formvar film-covered grid, rinsed with distilled water, and stained with uranyl acetate; or they were pelleted by centrifuging at 200g and gently resuspended in distilled water. Drops of the distilled water suspensions were allowed to dry on film-covered grids and then negatively stained with uranyl acetate.

## RESULTS

Observation of the samples by dark-field light microscopy was carried out for periods of 3-4 h

during which no major damage to the sperm cell occurred. Under the conditions of these experiments the Triton-washed sperm were found to be completely nonmotile in the presence of ATP until a few minutes after the addition of the trypsin when the flagella gradually began twitching or flexing. The reason for this effect is unknown but may be due to the disruption of some barrier between the ATP in solution and part of the motile system. This twitching could be observed in some cases even after several hours of digestion.

After about 30 min of digestion, microtubules began to be observed sliding out of the tips of the flagella (Fig. 1). The rate of sliding varied so that the process occurred over a time span of from a couple of seconds to several minutes. Occasionally, microtubules slid out of twitching flagella but more often they slid out of nonmoving flagella. A few of the microtubule groups were found to have slid upward, escaping at the point of attachment of the flagellum to the sperm head. No examples were found where any microtubules came through the side of a flagellum.

Examples of sliding involving both individual microtubules and groups of microtubules were identified on the basis of the brightness of the sliding groups as seen in the dark field. In some cases groups of microtubules were seen which had split open along part of their length, and had forced loops of microtubules out of the main body of the group, indicating that relative sliding was occurring between microtubules in the extruded group (Fig. 1). The extruded microtubules were found in three general patterns. Larger groups and microtubules which became attached to the glass surface as they slid out of the flagellum lay in relatively straight patterns. Individual microtubules and small groups which did not become attached to the microscope slide were usually found coiled into uniform helicies. Similar coiling of microtubules has been observed previously (6, 13). In a few cases the microtubules apparently coiled back on themselves as they were extruded, and by re-binding upon themselves formed microtubule rings (Fig. 1). The rings observed were about  $6 \times 10^{-3}$ – $10^{-2}$  mm in diameter. They usually were found floating free in suspension. In some cases the ends of the microtubules extended out of the rings. In other cases no loose ends were seen. The rings were usually inactive but in one case a ring was observed to disintegrate into a tangled helix of individual microtubules. The nature of these microtubular rings is being further investigated.

The number of sperm cells showing extruded groups of microtubules increased over a period of about 1 h until about 20% of the sperm had extruded microtubules. The fact that more flagella did not show sliding of microtubules is probably due in part to the combination of two effects of the trypsin digestion, one of which tends to free the microtubules from their restraining structural attachments within the flagellum, and the other which tends to destroy the structures which participate in the production of the mechanical sliding forces (14). Attachment of the tips of the microtubules and the flagella to the microscope slide also probably prevents sliding in some instances.

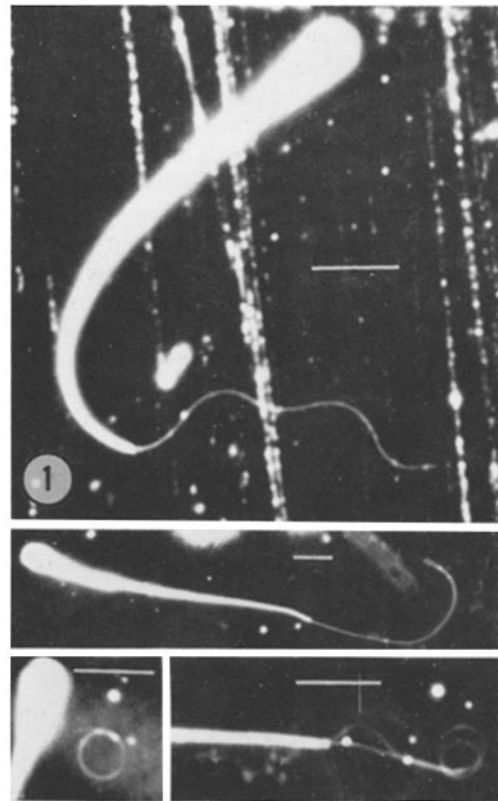
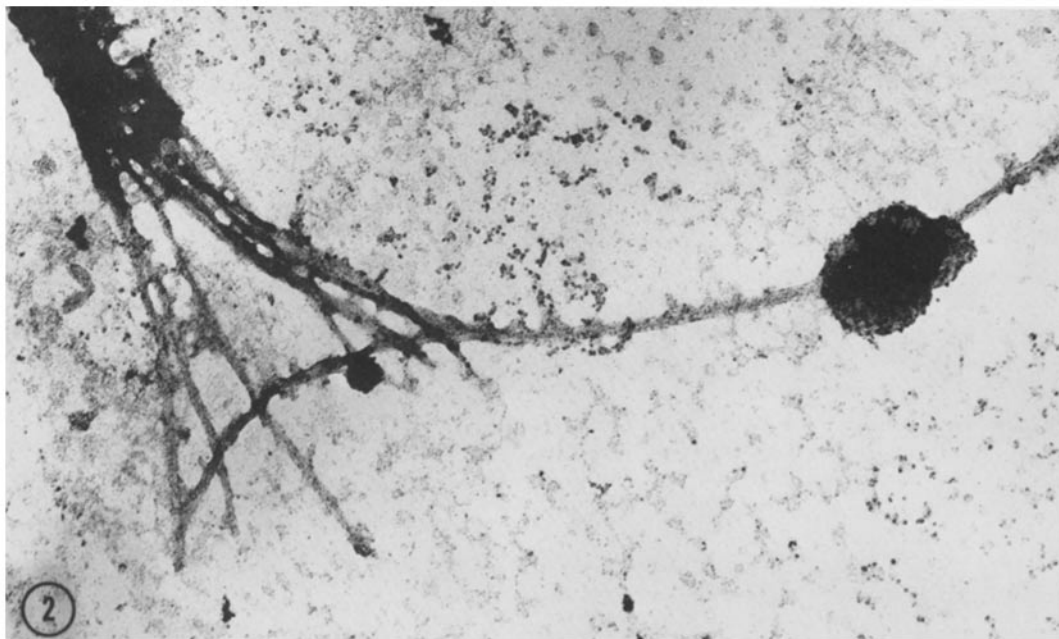
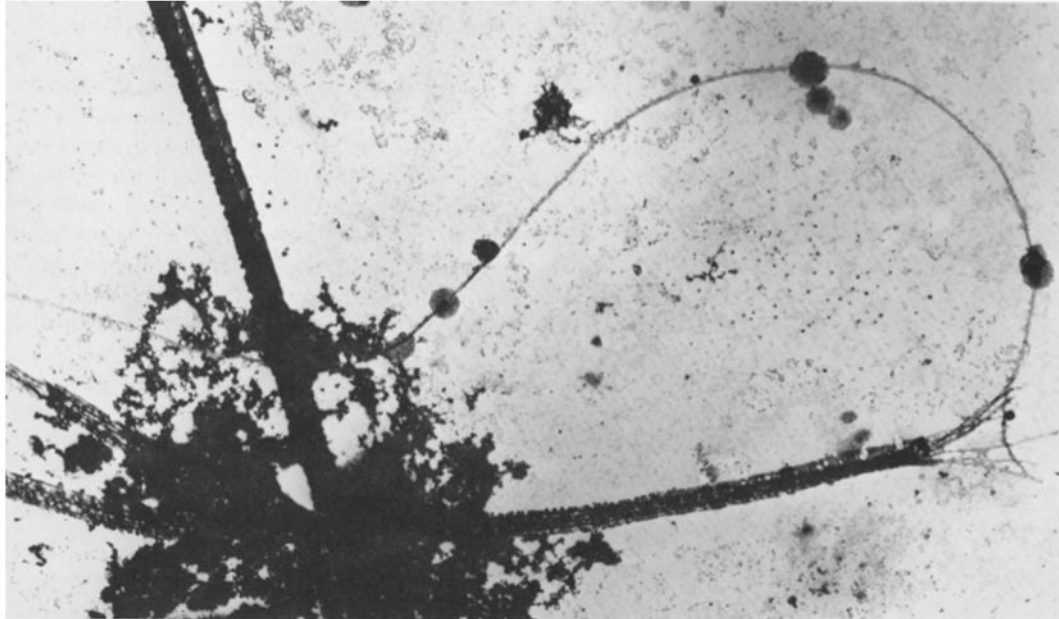


FIGURE 1 Upper micrographs: sperm cells which have extruded groups of microtubules. Lower right: an extruded group of microtubules which is split open near the end of the flagellum due to relative sliding of microtubules within the group. The microtubules are coiled at the end of the group. Lower left: a microtubule ring (near the head of a sperm cell). The scale markers are  $10^{-2}$  mm long.  $\times 1,000$  except center micrograph  $\times 600$ .



**FIGURE 2** Negatively stained bull sperm flagella, from distilled water suspension, showing one or two tightly joined microtubules extruded from a flagellum. The lower micrograph shows details of the microtubules at the tip of the flagellum. The fibrous sheath is seen as a network surrounding the flagella which terminates just before the tip. Upper micrograph,  $\times 9,000$ ; Lower micrograph,  $\times 32,000$ .

In control experiments it was found that microtubules were not extruded if either ATP or trypsin were omitted from the solution.

In no case did it seem that the digestion in the

presence of ATP caused any of the coarse fibers to slide or otherwise separate from the flagella. Free coarse fibers would have been recognizable due to their tapered form. The separation of the coarse

fibers from the flagella should also cause a decrease in the brilliance of the flagella in the dark field but no such change was detected during the experiments. Examination in the electron microscope of fixed, sectioned specimens showed that the tryptic digestion had little effect on the fibrous sheath. The presence of this sheath throughout the experiments may have prevented the demonstration of possible motile activity by the coarse fibers. The electron micrographs also served to verify the absence of a membrane on the Triton-treated flagella.

Negatively stained sperm cells which had been digested in the presence of ATP were examined in the electron microscope. Fewer cells were found which had long segments of microtubules extending out of their flagella than were seen in the preparations under the light microscope (Fig. 2). It is probable that the longer extensions of microtubules were broken or pulled out of the flagella during the handling of the samples. Short ( $2-10 \times 10^{-3}$  mm) segments of extruded microtubules involving from one to four doublet microtubules were quite common.

#### DISCUSSION

These experiments provide evidence that the doublet microtubules of bull sperm flagella possess an ATP-activated motile system which is similar to that of the simpler sea urchin sperm flagella and suggest that the nine doublet microtubules perform the same motile function in both systems. It is not possible, on the basis of these experiments, to determine whether the single microtubules were ever extruded from the flagella. They may be present in some groups of extruded doublet microtubules. However, experiments performed with simpler flagella suggest that the single tubules play an inactive role in motility (13, 14).

Although these experiments give no indication of the function of the nine coarse fibers of the mammalian sperm flagella, an active model consistent with the results of these experiments can be proposed in which the microtubular axoneme of the bull sperm performs a primary motile function which parallels that proposed for the

axoneme of sea urchin sperm flagella. If the active production of force by the coarse fibers were controlled by the beating of the microtubular axoneme through some form of localized mechanical coupling, then the nature of the flagellar beat would tend to correspond to the sliding filament model regardless of whether the coarse fibers functioned by a contractile or bending or sliding mechanism. A model for such mechanical coupling of motile structures is found in the synchronized beating of closely opposed individual flagella and cilia (11).

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