MOTILITY OF TRITON-DEMEMBRANATED SEA URCHIN SPERM FLAGELLA DURING DIGESTION BY TRYPSIN

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ABSTRACT

The survival curves for a population of reactivated spermatozoa exposed to digestion by trypsin indicate that a large number of trypsin-sensitive targets must be digested before the flagellum disintegrates.

Changes in flagellar movement during trypsin digestion can be very small, especially when the spermatozoa are reactivated at 0.25 M KCl. They are not the changes which would be expected if elastic resistance of the trypsin-sensitive structures responsible for maintaining the integrity of the axoneme is a significant determinant of flagellar bend amplitude.

By carrying out trypsin digestion under a variety of conditions, at least six distinct effects of trypsin digestion on parameters of flagellar movement have been detected. These include a gradual increase in the rate of sliding between tubules, gradual and abrupt changes in beat frequency accompanied by reciprocal decreases in bend angle, changes in the symmetry and planarity of bending, and selective interference with mechanisms for bend initiation and bend propagation.

KEY WORDS flagella · motility · trypsin · biological oscillation · microtubule

Study of the effects of trypsin on flagellar axonemes was initiated by Summers and Gibbons (25, 26). They found that the addition of ATP to axonemes which had been briefly digested with trypsin caused the axonemes to disintegrate, under conditions where the addition of ATP to undigested axonemes caused reactivation of oscillatory bending and bending wave propagation. Direct observations of the disintegration process by darkfield light microscopy revealed an ATP-driven extrusion of tubules (25), which has been shown by more recent electron microscope studies on ciliary axonemes to involve translation of an A-tubule and its dynein arms towards the base of the neighboring B-tubule with which its dynein arms form cross bridges (20). These observations provide very firm experimental support for the sliding microtubule model of flagellar bending which was based on earlier indirect evidence, particularly on evidence that bending occurs without changes in length of the axonemal microtubules (1, 21, 22, 29).

The observations of Summers and Gibbons indicate that some protein components responsible for maintaining the integrity of the axoneme are more sensitive to trypsin digestion than the components responsible for the active sliding process itself. Electron microscope observations on briefly digested axonemes showed damage to the interdoublet or "nexin" (28) linkages and to the radial spoke–central sheath interconnections (26). Since these structures have been suggested to play a role in converting active sliding into oscillatory bending and in regulating the parameters of bending (4, 29), we thought that additional information about the function of these structures might be obtained by observations on motile

flagella during the course of trypsin digestion. Initial observations showed that there was little change in waveform during digestion, and then an abrupt cessation of motion. More detailed observations reveal a variety of subtle changes in movement during the course of trypsin digestion and provide a new source of information about the roles of flagellar proteins in controlling movement.

MATERIALS AND METHODS

These experiments were carried out with spermatozoa from the sea urchin *Lytechinus pictus*, using the Triton-demembranation procedure of Gibbons and Gibbons (16), as previously modified (7, 12). Spermatozoa were diluted with 1–2 vol of 0.5 M NaCl to obtain a stock sperm suspension which gave an optical density reading of 0.19 to 0.21 when diluted 1:500 with 0.5 M NaCl (14). Protein measurements, using the Lowry assay with bovine serum albumin as standard, indicated that an optical density reading of 0.20 corresponded to a sperm protein concentration of 42 mg ml^{-1} in the stock sperm suspension. This sperm suspension was stored on ice; all subsequent work was done at 16°C.

Spermatozoa were demembranated as needed by mixing 10 μl of the stock sperm suspension with 1.0 ml of Triton extraction solution (0.15 M KCl, 2 mM $MgSO_4$, 2 mM dithiothreitol (DTT), 2 mM Tris buffer, 0.5 mM EDTA, 5 mM $CaCl_2$, and 0.04% (vol/vol) Triton X-100, at pH 8.2). After 30 s, an appropriate volume, usually 10 μl, of a stock solution of trypsin (Sigma Chemical Co., St. Louis, Mo., T 8003) in distilled water was added to 1.0 ml of reactivation solution (0.15 M KCl, 2 mM $MgSO_4$, 2 mM DTT, 20 mM Tris buffer, 2.0 mM ethylene glycol-bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetate [EGTA], 0.5 mM $CaCl_2$, 2% (wt/vol) polyethylene glycol, and 0.2 mM ATP, at pH 8.2). Stock trypsin solutions were usually 0.08 mg ml^{-1} for measurements of disintegration times and 0.04 mg ml^{-1} for observations of motility during digestion, which gave sperm protein:trypsin ratios of 5:1 and 10:1, respectively, but were varied as required. After an additional 30 s, 10 μl of extracted sperm suspension was added to the 1.0 ml of reactivation solution containing trypsin, and timing of trypsin digestion was begun.

For observations and photographic recording of trypsin digestion of individual spermatozoa, a drop of reactivated sperm suspension was transferred to a well slide (11) and covered with a cover glass. Measurements of beat frequency were obtained from records of the flash frequency used for stroboscopic observation and photography. Multiple-flash photographs used for measurements of bend angles and wavelengths and records of the flash frequency and time of each photograph were obtained using techniques described previously (11).

Average disintegration times for a sperm sample were measured using a slightly different procedure. A drop of reactivated sperm suspension containing trypsin was placed on a microscope slide and observed without a cover glass, using a ×16 objective. Three spermatozoa which were beating normally with their heads attached to the microscope slide were selected, and observed continuously until they disintegrated. The disintegration time for each spermatozoon was recorded. However, if any of these three spermatozoa were lost prematurely by detachment or by the flagellum sticking to the slide before disintegration, data from that preparation were discarded. The procedure was repeated until eight successful preparations were measured, to obtain disintegration times for a sample of 24 spermatozoa under similar conditions. In experiments which compared different conditions, such as two different KCl concentrations in the reactivation solutions, the two different conditions were alternated for successive preparations, until 24 spermatozoa were measured under each condition.

RESULTS

When demembranated spermatozoa are reactivated in standard reactivation solution containing 0.4 μg ml^{-1} trypsin, they swim for about 3 min with no obvious change in the characteristics of their movement except for some decrease in the radius of their swimming circles. Then, for a period of 10–20 s, their movement usually becomes erratic and difficult to observe or photograph. If they are swimming next to a glass surface, they almost always impinge on the surface and stick to it by their heads during this period. Their movements then stop abruptly, and the axoneme disintegrates within 1–2 s. Tubule sliding can usually be visualized during disintegration, but it is modified by the trypsin-resistant connections between tubules which remain at the basal end of the axoneme (25). The observed distintegration time for a particular sperm flagellum will be signified by t_D.

Measurements of Disintegration Time

While observing reactivated spermatozoa which were being digested by trypsin, it became obvious that the values of t_D for a population were fairly narrowly distributed. Results of an experiment in which 24 values of t_D were obtained for a sperm sample, using the procedure described in Materials and Methods, are illustrated by the curve for normal length spermatozoa in Fig. 1. When the results of this experiment are replotted on probability coordinate paper, they are distributed around a straight line, indicating that the values of t_D are normally distributed. The intercept and slope of this straight line provide estimates of the

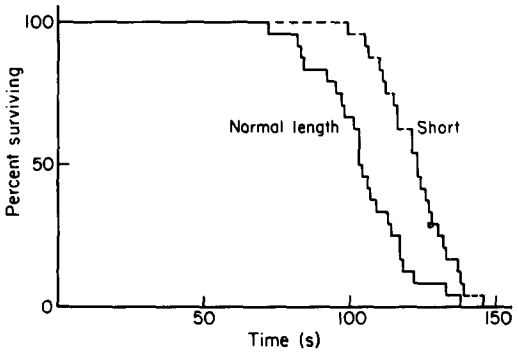

FIGURE 1 Survival curves comparing the disintegration times for normal, unbroken spermatozoa, and broken spermatozoa retaining only about a 5-μm length of flagellum, selected from a homogenized sperm preparation. The trypsin concentration in the reactivation solution was 0.8 μg ml^{-1}.

mean disintegration time, signified by $\bar{t}_D$, and the standard deviation of t_D. For this sample, $\bar{t}_D$ = 103 s and the standard deviation = 14 s. If the distribution of t_D is entirely determined by the statistical fluctuations inherent in the random digestion of n targets, the relative standard deviation should be $1/\sqrt{n}$, so for this sample, the estimate of n would be 51. From 18 experiments of this type at the same trypsin concentration for which reasonably normal distributions of t_D were obtained, the mean value of the estimate of n was 49, and the estimated standard deviation for the values of n was 24.

The experiment shown in Fig. 1 was carried out to examine the effect of flagellar length on disintegration time. The stock sperm suspension was briefly homogenized by mixing for 5 s with a vortex mixer at full speed, and the spermatozoa which were selected for measurement were ones which either appeared to be completely undamaged, with flagella of normal length, or were ones which had only a short portion of the flagellum, about 5 μm in length, attached to the head and continuing to beat. In each of four experiments of this type in which reasonably normal distributions of t_D were obtained for both short and normal length flagella, the results were similar to those shown in Fig. 1, with a longer $\bar{t}_D$ for the short flagella than for the normal length flagella, and a smaller standard deviation for the short flagella. The values of $\bar{t}_D$ for the short flagella averaged 13.5% greater than for the normal length flagella, with a standard deviation of 4.5%. The estimates of n had a mean and standard deviation of 39 ± 12 for the normal length flagella, and 118 ± 74 for the short flagella.

Another series of experiments was carried out to examine the effect of the KCl concentration in the reactivation solutions on $\bar{t}_D$. In each experiment, two KCl concentrations were compared, using the same trypsin concentration. For KCl concentrations of 0.1 to 0.3 M, one member of the pair was always 0.15 M KCl as reference. For lower KCl concentrations, a pair of KCl concentrations such as 0.06 and 0.10 M was examined, and the mean value of $\bar{t}_D$ for 0.10 M KCl obtained from experiments in which 0.10 and 0.15 M KCl were paired, was used to calculate the value of $\bar{t}_D$ for 0.06 M KCl, etc. The trypsin concentration used for each experiment was adjusted so that the value of $\bar{t}_D$ for the lower KCl concentration was in the range of 40-90 s, but the calculated values of $\bar{t}_D$ all correspond to a trypsin concentration of 0.8 μg ml^{-1}. As shown in Fig. 2, $\bar{t}_D$ decreases as the KCl concentration is decreased. However, there is no significant change in the estimate of n: in this series of experiments the estimate of n was 60 ± 28 for 16 measurements at 0.15 M KCl, 67 ± 29 for 12 measurements at 0.2-0.3 M KCl, and 59 ± 32 for 19 measurements at 0.04-0.10 M KCl.

Data from these experiments also provided a comparison of the values of $\bar{t}_D$ obtained at two different trypsin concentrations, at 0.15 M KCl. With 0.8 μg ml^{-1} trypsin, 18 values of $\bar{t}_D$ gave a mean and standard deviation of 96.1 ± 12 s, and with 1.6 μg ml^{-1} trypsin, 12 values of $\bar{t}_D$ gave a mean and standard deviation of 49.3 ± 3.3 s. As expected, the rate of trypsin digestion is proportional to the trypsin concentration.

We also measured the overall rate of digestion

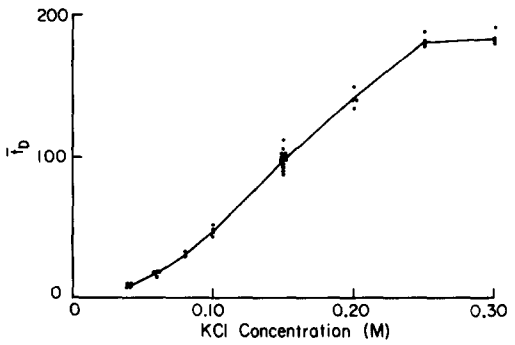

FIGURE 2 Effect of KCl concentration in the reactivation solution on the mean time, $\bar{t}_D$, for disintegration of the flagellar axoneme by trypsin.

of flagellar proteins by trypsin, using isolated axonemes and pH-stat measurements of the H^+ ions released during protein hydrolysis (26). These measurements required a much higher protein:trypsin ratio (2,500:1), which reduced the effect of KCl concentration on $\bar{t}_D$. Under conditions where $\bar{t}_D$ at 0.05 M KCl was approximately half of the value of $\bar{t}_D$ obtained at 0.15 M KCl, the overall rate of protein hydrolysis was twice as great at 0.05 M KCl as at 0.15 M KCl. The large effect of KCl concentration on $\bar{t}_D$ in our other experiments (Fig. 2) may therefore be simply a result of the effect of KCl concentration on the activity of trypsin, rather than a more interesting effect such as a change in the number of targets required to be digested by trypsin before disintegration, or a specific change induced by KCl in the trypsin sensitivity of structures responsible for maintaining the structure of the axoneme.

Movement Parameters during Trypsin Digestion

Detailed measurements of two spermatozoa during trypsin digestion under standard reactivating conditions are shown in Fig. 3, along with measurements on two control spermatozoa. Representative photographs from these two trypsin digestion series are shown in Figs. 4 and 5. The bend angle measurements show that the difference between the angles of the principle and reverse bends (16) increases during the latter part of the trypsin digestion period, especially in the case of swimming spermatozoa, where the increased curvature of the swimming path is easily detected by visual observations. The measurements also show a gradual increase in beat frequency throughout the period of trypsin digestion. Near the end of the trypsin digestion period, the frequency often

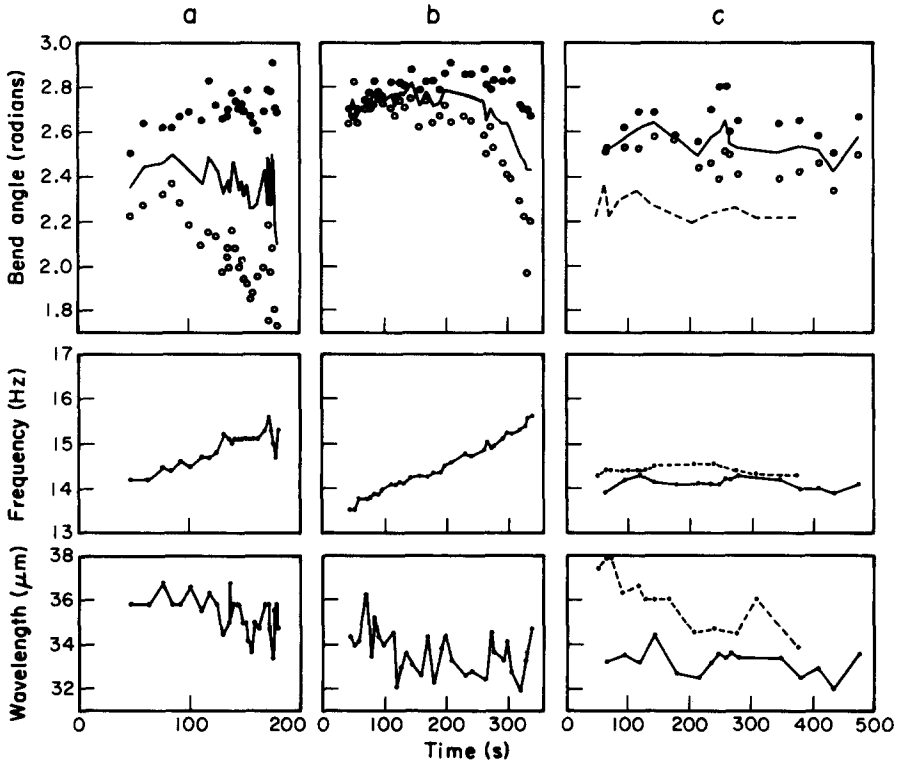

FIGURE 3 Measurements of bend angle, beat frequency, and wavelength (measured along the flagellum) for two spermatozoa during exposure to trypsin, in (*a*) and (*b*), and two control spermatozoa, in (*c*). The spermatozoon in (*a*) was swimming, and t_D was 200 s. The spermatozoon in (*b*) was attached to the slide by its head, and t_D was 360 s. In (*c*), the solid lines identify measurements for an attached spermatozoon and the dashed lines identify measurements for a swimming spermatozoon. Bend angles were measured separately for the principle and reverse bends, and are indicated by solid and open circles, respectively, and the lines indicate the mean bend angle.

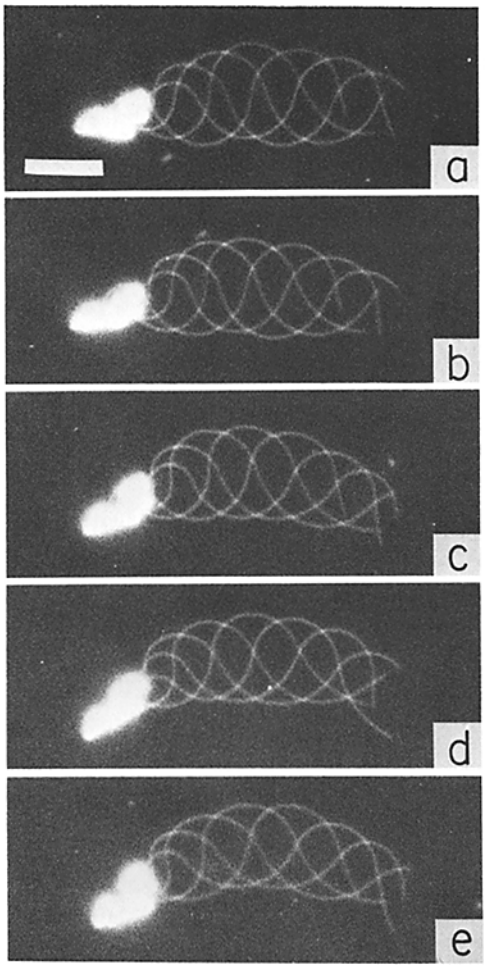

FIGURE 4 Photographs of a swimming spermatozoon during trypsin digestion in standard reactivation solution. Data for this spermatozoon are given in Fig. 3*a*; t_D = 200 s. The times and flash frequencies for each photograph are: (*a*) 0.32 t_D, 57.0 Hz; (*b*) 0.46 t_D, 58.0 Hz; (*c*) 0.71 t_D, 60.4 Hz; (*d*) 0.81 t_D, 60.4 Hz; and (*e*) 0.90 t_D, 60.8 Hz. The scale bar in this figure and in Figs. 5, 9, 11, 13, and 14 represents a length of 10 μm.

becomes erratic, but this is not fully shown by the measurements in Fig. 3, since the method for measuring beat frequency, although very precise when the frequency is steady, does not respond rapidly to changes in frequency.

Changes in mean bend angle and wavelength are more difficult to detect since there are relatively large fluctuations in both the experimental and control data. Some of the fluctuation in the bend angle and wavelength measurements may be artifact resulting from having to make measurements at different positions along the flagellum, since the flash frequency and the beat frequency are not phase-locked. The effects of these fluctuations can be minimized by averaging results from several experiments.

Fig. 6 shows combined data from measurements of movement parameters during trypsin digestion of 14 spermatozoa beating with their heads attached to the glass surface of the microscope slide. The measurements for each spermatozoon were plotted as in Fig. 3*b*, and values of frequency and bend angle at times of 0.2, 0.3, . . . 0.9 $\bar{t}_D$

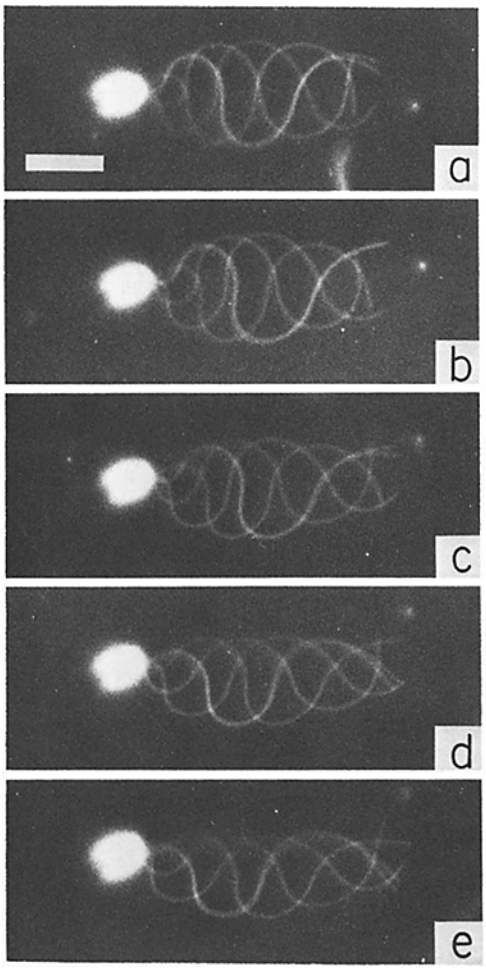

FIGURE 5 Photographs of an attached spermatozoon during trypsin digestion in standard reactivation solution. Data for this spermatozoon are given in Fig. 3*b*; t_D = 360 s. The times and flash frequencies for each photograph are: (*a*) 0.21 t_D, 55.1 Hz; (*b*) 0.47 t_D, 57.1 Hz; (*c*) 0.77 t_D, 59.8 Hz; (*d*) 0.91 t_D, 61.6 Hz; and (*e*) 0.94 t_D, 62.5 Hz.

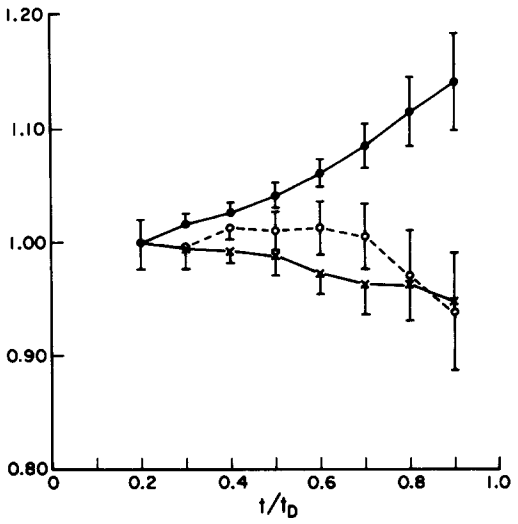

FIGURE 6 Mean values and standard deviations for measurements of beat frequency (solid points and line), mean bend angle (open points and dashed line), and wavelength (x's and solid lines) on a sample of 14 attached spermatozoa exposed to trypsin in reactivation solutions containing 0.15 M KCl. Trypsin concentrations ranged from 0.2 to 0.4 μg ml^{-1}, and the mean value of t_D was 319 s. The results are shown relative to the value measured at 0.2 t_D, where the mean beat frequency was 13.9 Hz, the mean bend angle was 2.66 radians, and the mean wavelength was 34.8 μm. Standard deviations for the wavelength measurements are shown only for 0.4, 0.5, 0.6, and 0.7 t_D; similar values were obtained for the other points.

were read from the graphs, using linear interpolation between points. The average values of the five points for 0.2–0.6 t_D were used to normalize the data from each spermatozoon to the mean value for the sample of 14 spermatozoa. The mean and standard deviation were then computed at each value of t_D. The results are plotted in Fig. 6 relative to the value at 0.2 t_D, to facilitate comparison of the changes in frequency and bend angle. The combined beat frequency measurements for this sample, and for another sample analyzed in the same manner (Fig. 8; data at 0.15 M KCl), show a smooth increase in beat frequency of about 20%/t_D. The bend angle remains almost constant from 0.2 to 0.7 t_D, and then decreases. The wavelength declines gradually throughout the period of trypsin digestion.

The changes in movement parameters which occur during trypsin digestion can be modified by changing the ionic strength of the reactivation solution, but little difference was noted between the results obtained with reactivation solutions containing 0.10 or 0.15 M KCl. With 0.25 M KCl, the movement parameters remain more constant during trypsin digestion. Examples where there is very little change in beat frequency during the course of trypsin digestion, as in Fig. 7, are frequently obtained with 0.25 M KCl, and the average increase in beat frequency for a larger sample of spermatozoa is only about half of that obtained at 0.15 M KCl (Fig. 8). Swimming spermatozoa digested at 0.25 M KCl also show a change in symmetry which is illustrated by the bend angle measurements in Fig. 7. When first examined, the spermatozoon in Fig. 7*a* was swimming in a clockwise circle, when viewed towards the surface at which it was swimming. The predominant sense of the swimming circles, for live spermatozoa, or reactivated spermatozoa at 0.15 M KCl, is counterclockwise. The radius of the swimming circles gradually enlarged during the course of trypsin digestion until the spermatozoon was swimming with a straight path, and then after a period of straight swimming it began to swim in counterclockwise circles of gradually decreasing radius. Comparison of Fig. 7 with Fig. 3*a* suggests that the time-course of changes in the angle of the principle and reverse bends is the same at 0.15 and 0.25 M KCl, but that at 0.25 M KCl the angle of the principle bend is initially reduced to a value less than the reverse bend. This description involves an assumption about the nomenclature of principle and reverse bends; that the principle bend, which normally has a larger bend angle (16), is determined by a fixed asymmetry in the axonemal bend generating mechanisms. Further work will be required to establish the validity and usefulness of this assumption. The changes in relative bend angles, shown in Fig. 7, which accompany the change in direction of circling on the slide surface, must be accompanied by an additional change in the direction of roll of the spermatozoon around a longitudinal axis. Stable clockwise circling at the slide surface requires the spermatozoon to roll around its longitudinal axis in the direction which will produce a right-handed helix if swimming freely at a distance from surfaces (18). To remain swimming stably at the slide surface when the bend angle symmetry changes, either the direction of roll must change, or the spermatozoon must flip over so that it is again swimming in a clockwise circle, which did not occur in cases such as Fig. 7*a*.

The data for the spermatozoon in Fig. 7*b* also

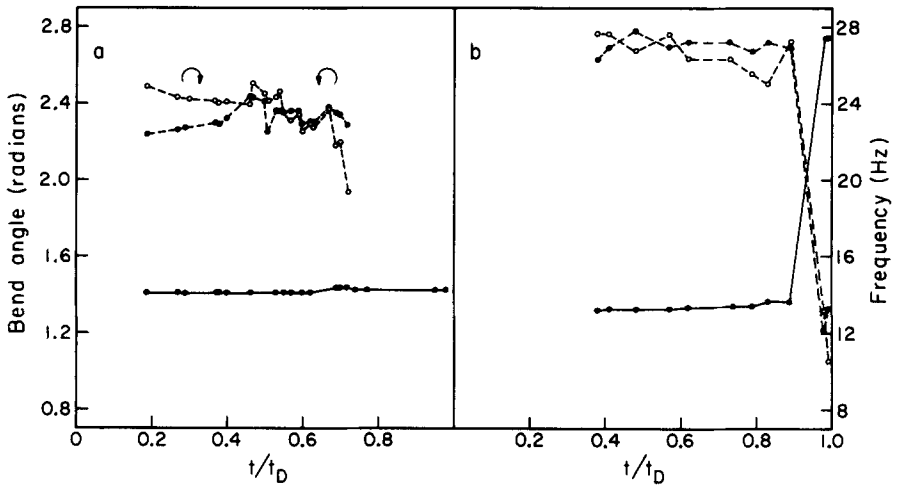

FIGURE 7 Bend angle and beat frequency measurements for two spermatozoa exposed to trypsin in reactivation solutions containing 0.25 M KCl. A swimming spermatozoon, with t_D = 278 s, is shown in (*a*), and an attached spermatozoon, with t_D = 218 s, is shown in (*b*). Principal and reverse bends are distinguished by closed and open circles, as in Fig. 3, and are connected by dashed lines; beat frequency points are connected by solid lines. The spermatozoon in (*a*) attached to the slide at 0.74 t_D and no accurate bend angle measurements were obtained after attachment. Its circling direction at the slide surface is indicated by the curved arrows.

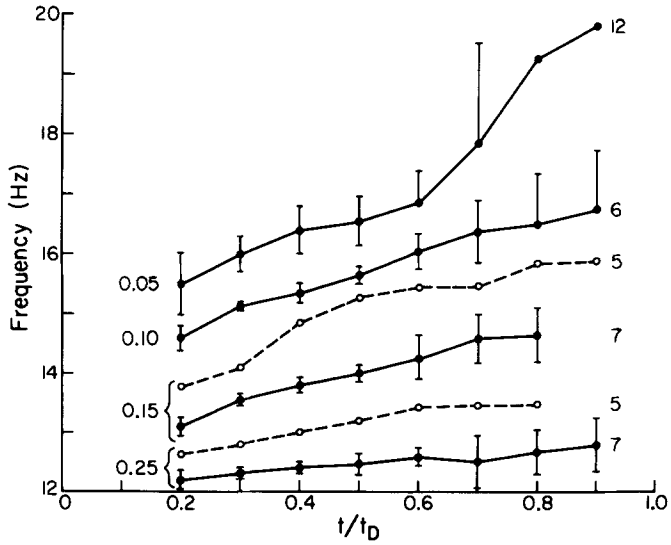

FIGURE 8 Measurements of beat frequency for spermatozoa exposed to trypsin at various KCl concentrations, identified by the numbers at the left ends of the curves. The number of spermatozoa in each sample is indicated at the right end of each curve. Swimming spermatozoa are identified by open circles and dashed lines; attached spermatozoa are identified by solid points and lines. Standard deviations are shown for the attached spermatozoa; the values for swimming spermatozoa were similar. The standard deviations for the curve labeled 0.05 M KCl were 3.0 Hz at 0.8 t_D and 2.2 Hz at 0.9 t_D and could not be shown on the graph without confusion. The curve labeled 0.05 M KCl actually combines data from four spermatozoa observed at each of three KCl concentrations: 0.04, 0.05, and 0.06 M.

show an additional phenomenon which is frequently observed with either 0.10, 0.15, or 0.25 M KCl in the reactivation solutions, and which will be referred to as "frequency doubling". During the last few seconds before t_D, there is an abrupt change from the normal movement mode to another mode which has exactly twice the beat frequency and approximately half the bend angle. In many cases, the precision of frequency doubling was made evident by an abrupt change from exactly four flashes per beat to exactly two flashes per beat. The bend angles in the higher frequency mode cannot be measured accurately enough to tell whether there is an exact division of bend angle by 2, which would maintain a constant rate of sliding between flagellar tubules. Examples of frequency doubling are shown by the photographs in Fig. 9. The duration of frequency doubling is variable. In the example shown in Figs. 7*b* and 9*a–c*, it occurred for about 5 s before t_D at 218 s. Longer durations have been occasionally observed, but it is often more brief, and may last for only a few beats just before t_D. In the example shown in Fig. 9*d* and *e*, the last photograph in the normal mode was obtained while the spermatozoon was swimming; it then attached and switched to the frequency-doubled mode. On a few occasions, frequency doubling was observed while a spermatozoon continued to swim, but usually frequency doubling is preceded by a period of erratic movement which results in attachment.

At lower KCl concentrations, in the range of 0.04–0.06 M, there is much more variation between individual spermatozoa, and the changes in beat frequency and bend angle during the latter portion of the trypsin digestion period are often enhanced. Combined data, shown in Fig. 8, show that the beat frequency increase for t less than 0.6 t_D is similar to the rate at 0.15 M KCl, and that the rate increases after 0.6 t_D. The two examples shown in Fig. 10 illustrate behavior that is typically seen at these lower KCl concentrations. Just before t_D, the movement may be very similar to the movement seen after frequency doubling at higher KCl concentrations. However, this mode is not reached by an abrupt doubling, but by a more gradual change in frequency, especially in the case shown in Fig. 10*b* and the photographs in Fig. 11. In the example in Fig. 10*a*, there was a period of alternation between two modes of movement, but in this case the two modes were closer together, and the transitions were more gradual, although usually too fast to follow with frequency measurements. The frequency was frequently too unstable to permit measurement by adjustment of the flash frequency; this was the case between 0.72 and 0.95 t_D in Fig. 10*a*.

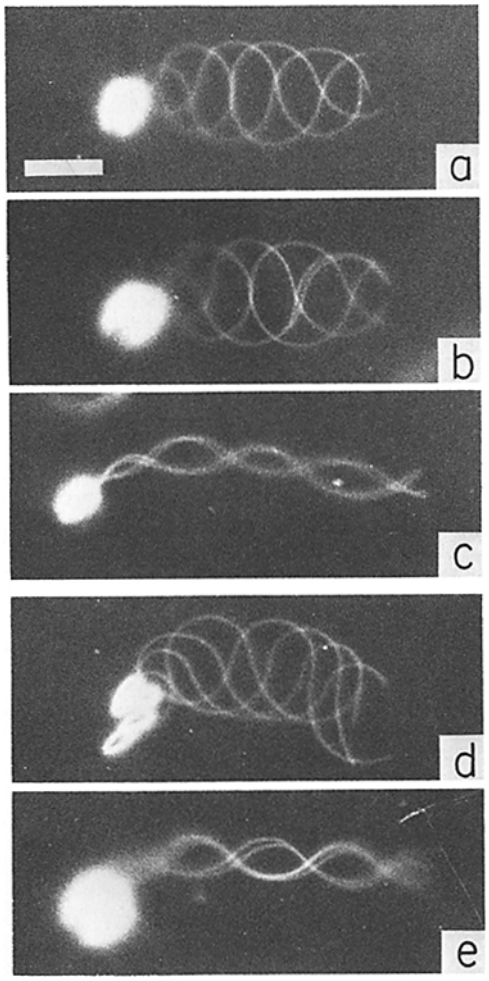

FIGURE 9 Photographs showing frequency doubling during trypsin digestion of reactivated spermatozoa. The spermatozoon shown in (*a*)-(*c*) was reactivated at 0.25 M KCl, and t_D = 218 s; data for this spermatozoon are shown in Fig. 7*b*. The spermatozoon shown in (*d*) and (*e*) was reactivated at 0.10 M KCl, and t_D = 117 s. The times and flash frequencies for each photograph are: (*a*) 0.38 t_D, 52.3 Hz; (*b*) 0.83 t_D, 54.3 Hz; (*c*) 0.98 t_D, 54.7 Hz; (*d*) 0.73 t_D, 68.5 Hz; and (*e*) 0.87 t_D, 73.7 Hz.

The normal reactivation conditions include a high Ca^{2+} ion concentration in the Triton-extraction solution, and a very low Ca^{2+} ion concentration in the reactivation solution, in order to produce nearly symmetrical movement (12). If the 5 mM $CaCl_2$ normally included in the Triton-extrac-

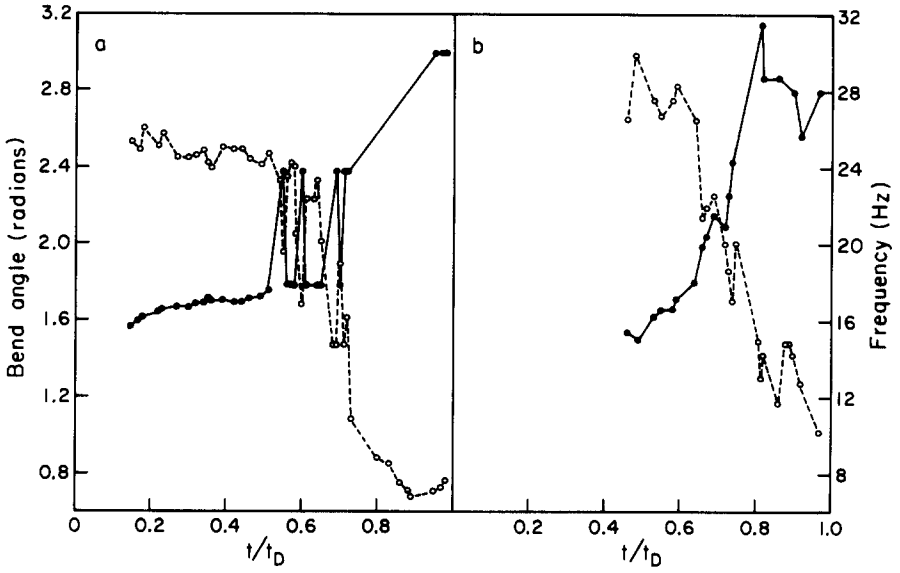

FIGURE 10 Bend angle and beat frequency measurements for two attached spermatozoa exposed to trypsin in reactivation solution containing 0.05 M KCl in (*a*) (t_D = 461 s) and 0.04 M KCl in (*b*) (t_D = 297 s). Beat frequency is indicated by solid points and lines, and mean bend angle is indicated by open points and dashed lines.

tion solution is omitted, reactivated spermatozoa swim in circles of small radius, even when the reactivation solution has a very low Ca^{2+} ion concentration. During trypsin digestion under these conditions, the spermatozoa swim in small circles, in the normal (counterclockwise) sense, when first examined. The radius of these swimming circles gradually increases from initial values of 50 μm or less, to ~200 μm. At this stage, many of the spermatozoa either leave the surface of the slide, or flip over and swim in clockwise circles. This indicates that at this stage, trypsin digestion causes a change in the direction of roll of the spermatozoa around their longitudinal axis, from a left-handed to a right-handed screw sense. Subsequently, the swimming path continues to straighten and then reverse its circling direction, as described for spermatozoa reactivated in 0.25 M KCl. Bend angle measurements on a spermatozoon showing this behavior are shown in Fig. 12*a*. Similar results were obtained when 0.1 mM $CaCl_2$ was included in the Triton-extraction solution. When 0.5 mM $CaCl_2$ was included in the Triton solution, the results were similar to those obtained with normal Triton-extraction solution containing 5 mM $CaCl_2$.

After Triton-extraction with 5 mM $CaCl_2$, reactivation in a solution containing increased Ca^{2+} ion causes asymmetrical swimming (12). With the $CaCl_2$ concentration in the standard reactivation solution increased to 2.2 mM, the spermatozoa swim erratically, with circular paths of small (~10 μm) radius; both directions of circling are seen. During trypsin digestion under these conditions, the swimming paths become gradually straighter, and are nearly straight at t_D. Reversals of circling direction, either with or without leaving the surfaces and turning over, are seen, but there is much more individual variation so that a consistent pattern of changes in roll was not demonstrated. Bend angle measurements on one spermatozoon under these conditions are shown in Fig. 12*b*. In this case, there was probably no change in the sense of roll, so that the clockwise circling after reversal of the relative magnitudes of the principle and reverse bends at 0.54 t_D was unstable, and the spermatozoon flipped over and reverted to counterclockwise circling between 0.63 and 0.64 t_D. The movement of the flagellum of this spermatozoon was more erratic, and the bend angle measurements record several episodes of more asymmetrical beating in addition to the gradual trends during trypsin digestion. The spermatozoon in Fig. 12*b* showed a large asymmetry in bending in the distal portion of the flagellum, as shown by the photographs of another spermatozoon under these conditions, in Fig. 13. At the beginning of observation it was swimming in

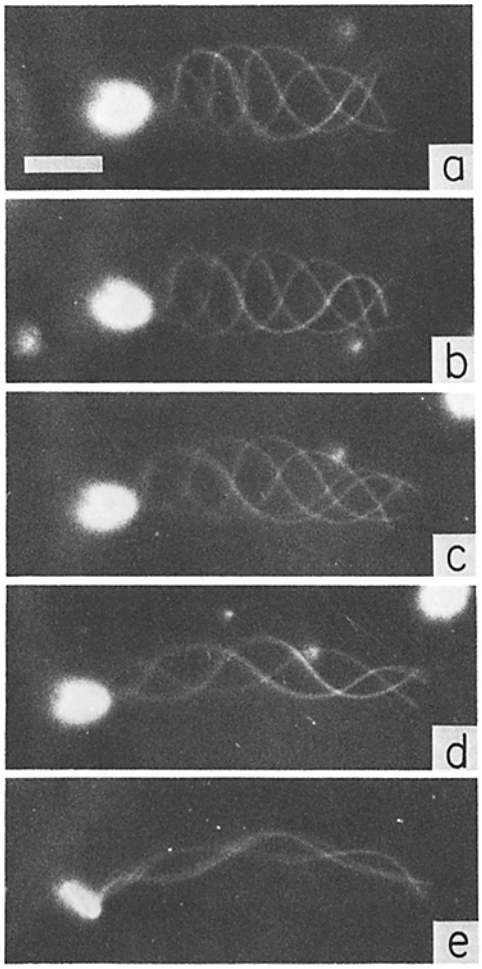

FIGURE 11 Photographs of an attached spermatozoon during trypsin digestion in reactivation solution containing 0.05 M KCl. Data for this spermatozoon are given in Fig. 10*b*; t_D = 461 s. The times and flash frequencies for each photograph are: (*a*) 0.30 t_D, 66.6 Hz; (*b*) 0.51 t_D, 70.1 Hz; (*c*) 0.65 t_D, 71.2 Hz; (*d*) 0.71 t_D, 71.2 Hz; and (*e*) 0.98 t_D, 59.4 Hz.

smaller circles than the spermatozoon in Fig. 12*a*, even though this is not reflected by the bend angle measurements made near the middle of the flagellum.

During the latter stages of trypsin digestion in reactivation solutions containing 2.2 mM $CaCl_2$, when most of the spermatozoa have moderately straight swimming paths, some of the spermatozoa show an additional effect of trypsin digestion which is different from the effects observed under other conditions. This is illustrated by the photographs in Fig. 13. There is an abrupt transition from a reasonably normal wave form to a wave form which shows bends initiating near the base of the flagellum and rapidly decreasing in amplitude as they propagate along the flagellum. Most spermatozoa showing this low amplitude mode have smaller amplitudes than the spermatozoon shown in Fig. 13, which was selected for presentation here because the characteristics of the wave form can be seen more clearly when the amplitude is larger. In most cases, the transition between these two modes of beating occurs without any change in beat frequency.

We have also examined the time-course of trypsin digestion in reactivation solution containing 0.04 M $KHCO_3$, at pH 8.4. In the absence of trypsin, this $KHCO_3$ concentration lowers the bend angle immediately by about 10%, and then after about 5 min causes a gradual decrease in bend angle, accompanied in its later stages by some decrease in frequency. Movement normally stops abruptly, rather than decreasing gradually to 0 bend angle (11). In the presence of trypsin,

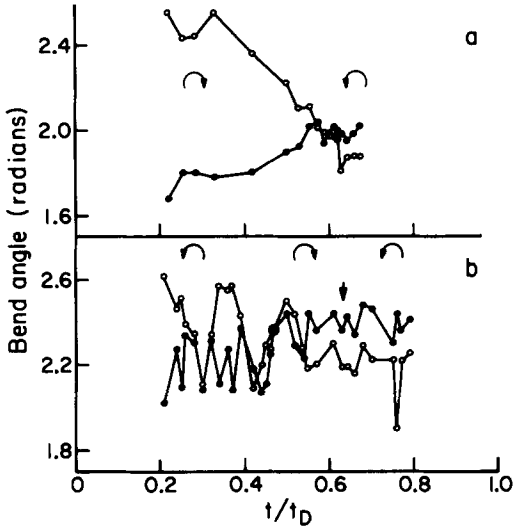

FIGURE 12 Bend angle measurements for spermatozoa exposed to trypsin. The spermatozoon in (*a*) was demembranated in a Triton solution without $CaCl_2$, and reactivated under standard conditions; t_D = 200 s. The spermatozoon in (*b*) was demembranated under standard conditions, and reactivated in reactivation solution with the $CaCl_2$ concentration increased to 2.2 mM; t_D = 401 s. Bend directions are identified by open and solid circles, as in Figs. 3 and 6, and the circling direction at the slide surface is indicated by the curved arrows. The vertical arrow in (*b*) indicates the time at which this spermatozoon flipped over and reversed its circling direction.

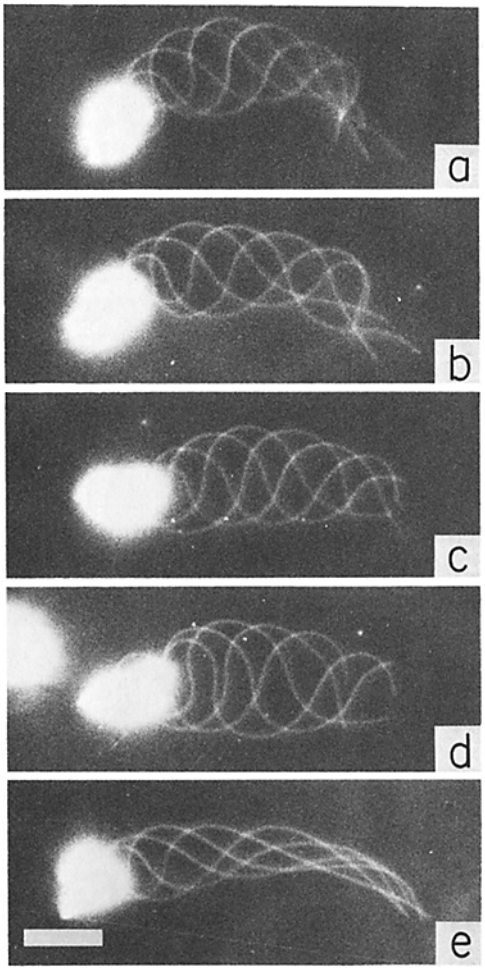

FIGURE 13 Photographs of a swimming spermatozoon during trypsin digestion in reactivation solution containing 2.2 mM $CaCl_2$; t_D = 368 s. The times and flash frequencies for each photograph are: (*a*) 0.24 s, 52.8 Hz; (*b*) 0.36 t_D, 53.3 Hz; (*c*) 0.64 t_D, 49.4 Hz; (*d*) 0.78 t_D, 43.5 Hz; and (*e*) 0.94 t_D, 42.9 Hz.

these changes occur much more rapidly. The sequence of photographs in Fig. 14 shows a typical example. A gradual decrease in bend angle is evident after 0.5 t_D, and then, at about 0.67 t_D there is an abrupt cessation of normal bending, followed by a long period of very small amplitude oscillation (Fig. 14*d*) and sporadic propagation of bends of larger amplitude, in one direction (Fig. 14*e*). During the last third of the trypsin digestion period, almost all of the sperm flagella have lost normal motility and show only very low amplitude oscillation. This is in striking contrast to the situation after 3 min of exposure to either tryspin or $KHCO_3$ alone, where most of the spermatozoa are still beating with nearly normal bending patterns. After making these observations, reexamination of flagella reactivated in the presence of 0.04 M $KHCO_3$ suggests that after about 10 min, when most normal movement has ceased, some of the flagella appear to be making very low amplitude oscillations, but they are seldom as large as those in Fig. 14*d*, and this type of oscillation is generally easier to see during trypsin digestion than with $KHCO_3$ alone. Frequency doubling was never observed under these conditions.

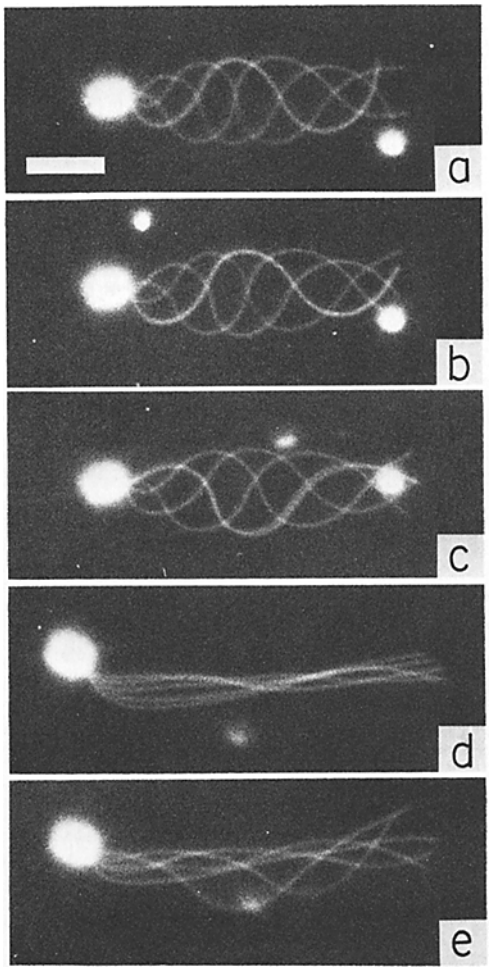

FIGURE 14 Photographs of an attached spermatozoon during trypsin digestion in reactivation solution containing 0.04 M $KHCO_3$, at pH 8.4; t_D = 218 s. The times and flash frequencies for each photograph are: (*a*) 0.41 t_D, 54.0 Hz; (*b*) 0.54 t_D, 54.9 Hz; (*c*) 0.62 t_D, 57.0 Hz; (*d*) 0.68 t_D, 56.7 Hz; and (*e*) 0.78 t_D, 51.5 Hz.

DISCUSSION

Effects of Trypsin on Structures which Maintain Axonemal Structure

During the period of exposure to trypsin before flagellar disintegration, the ATP-reactivated movement of demembranated sea urchin spermatozoa can continue with only small changes in movement parameters, especially when the reactivation solution contains 0.25 M KCl. If each flagellum has only one trypsin-sensitive target, so that there is no significant trypsin damage until this target is hit, causing disintegration, then no changes in movement parameters would be expected before disintegration. However, our observations show that the distribution of t_D is inconsistent with a one-target hypothesis, and that under standard conditions the number of targets appears to be of order 50 or greater. If the distribution of t_D were a reliable measure of the number of targets, we would expect that in flagella of ~10% of the normal length, the number of targets would be ~10% of normal, and the standard deviation of t_D would be greatly increased. Since we did not observe such an increase, the measured values of t_D appear to vary for other reasons which do not have the same length-dependence as the number of targets. The standard deviation of t_D therefore does not provide an accurate measure of the number of targets, although it still provides a lower limit. Extrapolation from the results with short flagella to flagella of normal length indicates that the number of targets in intact flagella may be >1,000. Since the nexin linkages between tubules have a longitudinal spacing of 86 nm (30) or 96 nm (17), an intact flagellum will contain ~4,000 of these nexin linkages between axonemal tubules. Our estimate for the lower limit for the number of trypsin-sensitive targets is not inconsistent with the hypothesis that the nexin linkages are the trypsin-sensitive targets which are responsible for maintaining the structural integrity of the axoneme.

If the flagellar axoneme is structurally uniform along its length, we would expect that, as we observed, $\bar{t}_D$ for short flagella would not be very different than for long flagella. The small increase in $\bar{t}_D$ observed for short flagella may indicate that the trypsin-resistant tubule connections at the base of a flagellum (25) have a stronger influence on maintaining the integrity of a short flagellum than on a flagellum of normal length.

Models for flagellar movement have been studied (4, 13) in which the amplitude of the bending waves is regulated by a balance between active shear moment and the moment produced by elastic shear and bending resistances. Structures responsible for maintaining the integrity of the axoneme, such as the nexin linkages, would be expected to introduce an elastic shear resistance. If this elastic shear resistance is important in regulating the amplitude of bending, then the gradual breakage of these structures during trypsin digestion would be expected to cause a gradual increase in the amplitude of bending, which might be accompanied by a decrease in beat frequency, as a secondary effect. Since this type of change is not shown by the results, the observations on movement during trypsin digestion provide evidence against the hypothesis that the shear elasticity of the structures which maintain axonemal structure is a major factor in regulating the amplitude of flagellar bending waves. However, this evidence is not conclusive; since there are many small changes in movement parameters during trypsin digestion, a small increase in amplitude resulting from a decrease in shear elasticity might be obscured by other effects. If the digestion of a small fraction of the structures which maintain axonemal structure is sufficient to initiate a cooperative disintegration of the axoneme, the amplitude increase might be very small.

Changes in Movement Parameters during Trypsin Digestion

Our experiments reveal six distinct, and possibly independent, effects of trypsin digestion on parameters of movement of reactivated spermatozoa. In addition, there are small effects on wavelength, which may be secondary effects resulting from changes in other parameters of movement, and have not been examined in detail.

INCREASE IN SHEAR RATE: The gradual increase in frequency, with no decrease in bend angle, which occurs during the first two-thirds of the trypsin digestion period, represents a gradual increase in the rate of sliding between flagellar tubules. The shear rate increase is in the range of 10–20%/t_D, depending on the KCl concentration. If there is no decrease in wavelength, L, this beat frequency increase will correspond to an increase in the shear moment resulting from external viscous resistances. However, the shear moment is proportional to L^3 (5), so that the gradual decrease in wavelength shown in Fig. 3 would be

sufficient to eliminate the need for an increase in active shear moment.

This effect could result from a direct effect of trypsin on a protein which determines active shear rate or beat frequency under conditions where bend angle is maintained constant, or from an effect on a wavelength regulating mechanism under conditions where frequency is determined by a balance of active and viscous moments, and active moment and shear rate are reciprocally related as expected for a cross-bridge mechanism for generating active sliding.

RECIPROCAL INCREASE IN BEAT FREQUENCY AND DECREASE IN BEND ANGLE: This is usually seen only after 0.6 t_D, and may then be superimposed on the first effect. Very large changes may occur after 0.8 t_D. At low KCl concentrations (0.04–0.06 M) the changes may be gradual. With 0.10–0.25 M KCl, they frequently involve an abrupt doubling of frequency.

This effect might represent a continuation of the first effect beyond the point where a constant bend angle can be maintained, or it might represent a different effect on either a frequency-regulating mechanism or a bend angle-regulating mechanism under conditions where the shear rate remains constant. This effect suggests that the shear rate is an important independent variable, as predicted by some models for flagellar oscillation (8, 10); previous attempts to obtain evidence for this were unsuccessful (7).

Frequency doubling may be a highly significant observation. It is not an expected property of a model in which frequency and bend angle are entirely determined by a balance of active moment and moments resulting from elastic and viscous resistances, such as the models studied by computer simulation (4, 13). It might indicate that either frequency or bend angle is regulated by mechanisms which operate with relatively large steps. This does not appear to be a likely characteristic of the frequency regulating mechanism, since it is known from other work that the beat frequency can be smoothly varied over a large range by changes in ATP concentration (7). Changes in bend angle produced by CO_2 can also be gradual, but over a much more limited range. When this range is exceeded, there is typically an abrupt cessation of bending, rather than a transition to half the bend angle (11).

In other oscillatory systems, frequency doubling is often interpreted as evidence for a population of coupled oscillators, which can either all oscillate with the same phase, or can oscillate with half the population out of phase with the other half, to produce twice the beat frequency (19). This might occur if the individual dynein cross bridges which generate active sliding in flagella have an intrinsic, or at least an optimum, oscillation frequency. Observations of proportionality between ATP turnover and beat frequency of flagella (3, 15) are consistent with this idea. Indications of an optimum cross-bridge oscillation frequency have also been found for insect fibrillar muscle (22, 27).

Frequency doubling was previously observed in intact spermatozoa from a tunicate, *Ciona*, during exposure to thiourea, which causes an inhibition of beat frequency with no change in bend angle (2). The two frequency modes obtained under those conditions are very similar to the two modes seen during trypsin digestion (Fig. 9). Further insight might be gained by attempts to obtain frequency doubling under conditions which produce different movement patterns. There is no obvious similarity between the action of thiourea and trypsin which can assist interpretation of their induction of frequency doubling.

CHANGES IN THE SYMMETRY OF BENDING: Changes in the symmetry of bending during trypsin digestion often occur at the same time as a gradual decrease in mean bend angle, with the result that the bends in the principle direction may remain nearly constant, while a large decrease occurs in the bends in the reverse direction. It is therefore difficult to determine whether the change in symmetry is an independent effect, or whether both the change in symmetry and the change in mean bend angle result from trypsin sensitivity of the reverse bends. In the latter case, the difference between the results obtained with spermatozoa demembranated with or without $CaCl_2$ (Figs. 3*a* and 12*a*) might represent a change in the level of the principal bend angle as a result of the $CaCl_2$ effect, while in both cases the reverse bend angle decreases during trypsin digestion.

The large changes in bend angle found with low KCl concentrations and during frequency doubling clearly cannot be explained by changes in only the reverse bends.

CHANGES IN ROLL SENSE: Roll around the longitudinal axis during swimming probably results from a small twist in the bending plane along the length of the flagellum. The mechanisms which cause bending to occur in one plane are

not sufficiently well understood (6) to provide any basis for attempting to interpret the changes caused by trypsin digestion.

FAILURE OF BEND PROPAGATION: The abrupt change observed in the later stages of trypsin digestion in reactivation solutions containing 2.2 mM $CaCl_2$, illustrated by Fig. 13, might be interpreted as a transition to a state which shows normal bend initiation and defective bend propagation. A rather similar transition, in the opposite direction, was described previously for live sea urchin spermatozoa at increased viscosity (2). Under conditions where the viscosity appears to be too high for normal bend propagation, so that bends which initiate near the base of the flagellum are rapidly damped out, the addition of thiourea can restore normal bend propagation. As the thiourea diffuses in through the sperm membrane, there is a gradual reduction in beat frequency, and then an abrupt transition to normal bend propagation which occurs with little change in beat frequency. In both of these cases, there is an abrupt change in wave form with no change in frequency, even though there must be large changes in the rate of sliding between flagellar tubules and in the viscous moments experienced by the active sliding process. These observations, and the observations of frequency doubling, strongly suggest that the frequency of flagellar oscillation is not simply determined by a balance between active moment and viscous resistances, as in some simple models for flagellar oscillation (4, 10); but it is still necessary to explain the changes in frequency which can be produced by changing the viscosity of the medium.

A separation of bend initiation and bend propagation has also been demonstrated by Shingyoji, Murakami, and Takahashi (24), who found that an ATP concentration sufficient to allow propagation of mechanically initiated bends was not sufficient for spontaneous bend initiation.

INCREASED SENSITIVITY TO REDUCTION OF BEND ANGLE BY CO_2: The reduction of bend angle by CO_2 is not associated with an increase in beat frequency, in contrast to the trypsin-induced decreases in bend angle discussed in the second and third of these subsections. There may therefore be little connection between the earlier reduction in bend angle which occurs during trypsin digestion in the presence of $KHCO_3$ and the changes in bend angle which occur during trypsin digestion in the absence of $KHCO_3$.

The inhibitory effect observed in the presence of $KHCO_3$ has been shown to be the result of action of CO_2 on some mechanism responsible for control of flagellar bend angle, possibly a mechanism for selective inhibition of dynein cross-bridge activity (11). This interpretation implies that CO_2-inhibited flagella still have functional cross bridges, but that they are uniformly activated on both sides of the flagellum so that little or no net bending moment can be generated. The effect of $KHCO_3$ during trypsin digestion provides additional support for this interpretation, because very low amplitude oscillation and sporadic attempts to generate normal amplitude bends can be seen to continue after the abrupt cessation of normal oscillatory bending. These effects are more obvious when $KHCO_3$ exposure is combined with trypsin digestion, possibly because trypsin digestion may weaken flagellar structure enough to make the axoneme more sensitive to fluctuations in active shear moment. In contrast to the situation shown in Fig. 13, the combination of $KHCO_3$ and trypsin digestion appears to produce a defect in bend initiation under conditions where bend propagation is not inhibited (Fig. 14*e*).

These results also show that the failure of $KHCO_3$ to inhibit active tubule extrusion by trypsinized axonemes (14) is not likely to be the result of a trypsin-induced loss of sensitivity to $KHCO_3$ inhibition.

Conclusion

The large number of effects of trypsin digestion observed in these experiments indicates the presence in flagella of a larger number of mechanisms controlling movement than is incorporated into the current computer models which are capable of generating oscillatory bending and propagated bending waves, such as those of Brokaw (4) and Brokaw and Rintala (13). A similar conclusion might be reached on the basis of the failure of the current computer models to duplicate important details of flagellar behavior (6, 9) and on the basis of the known complexity of the protein components and the ultrastructure of the axoneme. The trypsin digestion experiments are valuable because they reveal possible dimensions for change in parameters of movement, but the rather unspecific action of trypsin makes it difficult to use this approach to relate flagellar structure and function. Some progress in this direction may be possible by exploiting some of the differences in effects of trypsin digestion under different condi-

tions. It will be necessary to determine whether these differences indicate that trypsin digestion alters the sensitivity of the flagellar mechanisms to conditions such as KCl, Ca^{2+}, and CO_2, or whether these conditions modify the action of trypsin on various flagellar proteins.

At this stage, little correlation of structure and function is possible. As already noted, the changes in motility during trypsin digestion are not those which would be expected to result from a gradual reduction in the shear elasticity of structures such as nexin linkages which might be involved in maintaining axonemal structure. Warner and Satir (29) have presented evidence which suggests that the radial spokes may undergo cyclic attachment and detachment from the central sheath during part of the ciliary bending cycle. Such a process might be equivalent in effect to an internal viscous shear resistance, which would retard sliding between tubules. In this case the gradual disruption of the radial spokes or the structures with which they interact, leading to the ultrastructural damage detected by Summers and Gibbons (26), might decrease their action as an internal viscous shear resistance, and cause an increase in shear rate similar to that observed during the early part of the trypsin digestion period. At this stage, it is not clear that the suggestion that the radial spoke interactions are functionally equivalent to a viscous shear resistance is of any value in understanding normal flagellar function.

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REFERENCES

1. Brokaw, C. J. 1965. Non-sinusoidal bending waves of sperm flagella. *J. Exp. Biol.* **43:**155–169.
2. Brokaw, C. J. 1966. Effects of increased viscosity on the movements of some invertebrate spermatozoa. *J. Exp. Biol.* **45:**113–139.
3. Brokaw, C. J. 1967. Adenosine triphosphate usage by flagella. *Science (Wash. D. C.).* **156:**76–78.
4. Brokaw, C. J. 1972. Computer simulation of flagellar movement. I. Demonstration of stable bend propagation and bend initiation by the sliding filament model. *Biophys. J.* **12:**564–586.
5. Brokaw, C. J. 1972. Viscous resistances in flagella: Analysis of small amplitude motion. *J. Mechanochem. Cell Motility.* **1:**151–155.
6. Brokaw, C. J. 1972. Flagellar movement: A sliding filament model. *Science (Wash. D. C.).* **178:**455–462.
7. Brokaw, C. J. 1975. Effects of viscosity and ATP concentration on the movement of reactivated sea-urchin sperm flagella. *J. Exp. Biol.* **62:**701–719.
8. Brokaw, C. J. 1975. Molecular mechanism for oscillation in flagella and muscle. *Proc. Natl. Acad. Sci. U. S. A.* **72:**3102–3106.
9. Brokaw, C. J. 1975. Cross-bridge behavior in a sliding filament model for flagella. *In* "Molecules and Cell Movement". S. Inoue and R. E. Stephens, editors. Raven Press, New York. 165–178.
10. Brokaw, C. J. 1976. Computer simulation of flagellar movement. IV. Properties of an oscillatory two-state cross-bridge model. *Biophys. J.* **16:**1029–1041.
11. Brokaw, C. J. 1977. CO_2-inhibition of the amplitude of bending of Triton-demembranated sea-urchin sperm flagella. *J. Exp. Biol.* In press.
12. Brokaw, C. J., R. Josslin, and L. Bobrow. 1974. Calcium ion regulation of flagellar beat symmetry in reactivated sea urchin spermatozoa. *Biochem. Biophys. Res. Commun.* **58:**795–800.
13. Brokaw, C. J., and D. Rintala. 1975. Computer simulation of flagellar movement. V. Models incorporating cross-bridge kinetics. *J. Mechanochem. Cell Motility.* **3:**77–86.
14. Brokaw, C. J., and T. F. Simonick. 1976. CO_2 regulation of the amplitude of flagellar bending. *In* Cell Motility. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 933–940.
15. Brokaw, C. J., and T. F. Simonick. 1977. Mechanochemical coupling in flagella. V. Effects of viscosity on movement and ATP-dephosphorylation of triton-demembranated sea-urchin spermatozoa. *J. Cell Sci.* **23:**227–241.
16. Gibbons, B. H., and I. R. Gibbons. 1972. Flagellar movement and adenosine triphosphatase activity in sea urchin sperm extracted with Triton X-100. *J. Cell Biol.* **54:**75–97.
17. Gibbons, I. R. 1977. Structure and function of flagellar microtubules. *In* International Cell Biology 1976–1977. B. Brinkley and K. R. Porter, editors. Rockefeller University Press, New York. 348–357.
18. Gray, J. 1955. The movement of sea-urchin spermatozoa. *J. Exp. Biol.* **32:**775–801.
19. Pavlidis, T. 1973. Biological oscillators: their mathematical analysis. Academic Press, Inc., New York. 160.
20. Sale, W. S., and P. Satir. 1977. The direction of active sliding of microtubules in *Tetrahymena* cilia. *Proc. Natl. Acad. Sci. U. S. A.* **74:**2045–2049.
21. Satir, P. 1965. Studies on cilia. II. Examination of the distal region of the ciliary shaft and the role of

the filaments in motility. *J. Cell Biol.* **26:**805–834.
22. SATIR, P. 1968. Studies on cilia. III. Further studies of the cilium tip and a "sliding filament" model of ciliary motility. *J. Cell Biol.* **39:**77–94.
23. SCHÄDLER, M., G. J. STEIGER, and J. C. RÜEGG. 1971. Mechanical activation and isometric oscillation in insect fibrillar muscle. *Pfluegers Archiv Gesamte Physiol. Menschen Tiere.* **330:**217–229.
24. SHINGYOJI, C., A. MURAKAMI, and K. TAKAHASHI. 1977. Local reactivation of triton-extracted flagella by iontophoretic application of ATP. *Nature (Lond.).* **265:**269–270.
25. SUMMERS, K. E., and I. R. GIBBONS. 1971. Adenosine triphosphate-induced sliding of tubules in trypsin-treated flagella of sea-urchin sperm. *Proc. Natl. Acad. Sci. U. S. A.* **12:**3092–3096.
26. SUMMERS, K. E., and I. R. GIBBONS. 1973. Effects of trypsin digestion on flagellar structures and their relationship to motility. *J. Cell Biol.* **58:**618–629.
27. STEIGER, G. J., and J. C. RÜEGG. 1969. Energetics and efficiency in the isolated contractile machinery of an insect fibrillar muscle at various frequencies of oscillation. *Pfluegers Archiv Gesamte Physiol. Menschen Tiere.* **307:**1–21.
28. STEPHENS, R. E. 1970. Isolation of nexin—the linkage protein responsible for the maintenance of the nine-fold configuration of flagellar axonemes. *Biol. Bull.* **139:**438.
29. WARNER, F. D. and P. SATIR. 1974. The structural basis of ciliary bend formation. Radial spoke positional changes accompanying microtubule sliding. *J. Cell Biol.* **63:**35–63.
30. WARNER, F. D. 1976. Ciliary inter-microtubule bridges. *J. Cell Sci.* **20:**101–114.