LOCAL ACTIVATION AND INACTIVATION EXPERIMENTS ON FLAGELLA

Thesis by

Stuart Frederick Goldstein

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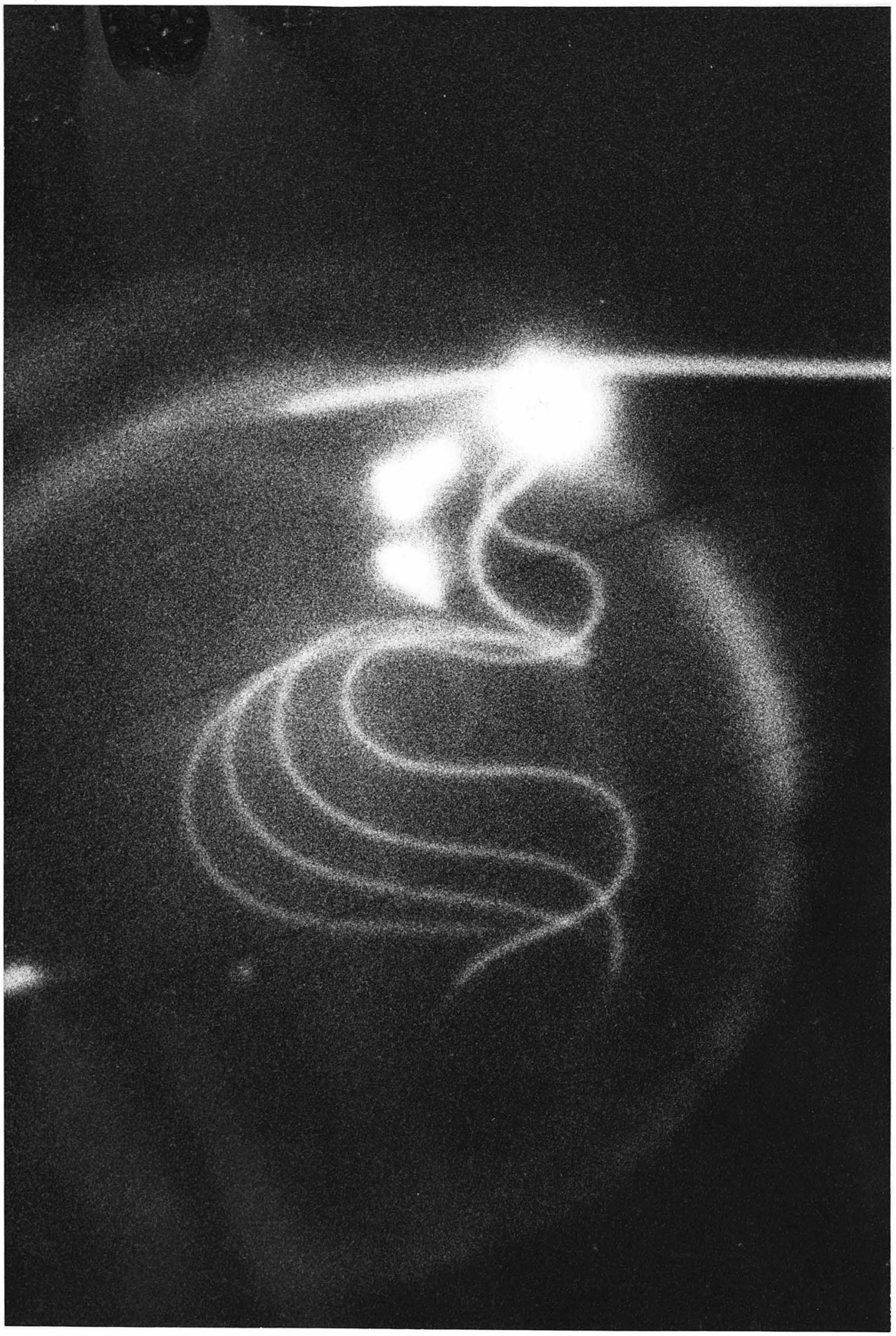
"The tails of the animalcula, so far from assisting locomotion, impede it, and produce an unstable oscillatory movement. They are, in fact, long filaments of the viscid seminal substance which is trailed after the moving globule."

Needham, 1749, from Cole (27)



Frontispiece. A dark-field, multiple-exposure photograph of an irradiated spermatozoon of a starfish, Pisaster brevispinus. The tail was beating 15.5 times/second before irradiation. The brightest exposure was taken about 0.5 milliseconds before irradiation; the other exposures were taken at a rate of 50 exposures/second after irradiation. The irradiated point has attached to the glass, and appears in the same position on all exposures. Beating has stopped between that point and the head, and one of the bends behind that point can be seen progressing to the tip of the tail after irradiation. The large light circle in the background is due to slight scattering of some of the unfocussed laser beam by a dielectric mirror in the optical system.

ACKNOWLEDGMENTS

When I arrived at Caltech, I was given a copy of the Biology Division policies and procedures manual, which stated that the basis of all the rules "is the principle that anything that facilitates instruction and research is desirable and anything that impedes is undesirable." I first read this in cynical disbelief; I now read it in grateful disbelief. But it's true; and for that principle, and for the friendliness and competence of that principle's administration, I thank the Biology Division of the California Institute of Technology (such a cold name for such a warm group!).

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ABSTRACT

The mechanisms of flagellar movement were investigated by studying the ability of various regions of a flagellum to initiate bends, and to propagate bends independently of activities in other regions. Two experimental approaches were used: the establishment of an artificial gradient of ATP along a flagellum, and the inactivation of a small region of a flagellum by localized irradiation. Flagella of the spermatozoa of sea urchins and a few other marine invertebrates were used in this study. Glycerinated flagella were activated by ATP gradients established by means of diffusion from the tips of micropipettes. These gradients could be made broad enough to produce a gradual decrease in ATP concentration along the entire flagellum, or narrow enough to supply ATP to only part of the flagellum. Localized supply of ATP to regions of the flagellum other than the basal end produced no beating. Beating properties along the flagellum appeared quite sensitive to ATP concentration at the basal end, but rather insensitive to ATP concentration at other points, and centering a gradient about points other than the basal end did not cause beating to start at those points.

Small regions of flagella were irradiated at preselected phases of beating by means of a pulsed ruby laser microbeam. Multiple-exposure dark field photographs of the spermatozoa were taken immediately before and after irradiation. The region of a flagellum between the head and the irradiated point continued beating for at least a few beats if that region was at least a quarter of the length of the tail, and stopped

immediately if it was shorter. Bends which were already established beyond the irradiated point continued to the tip, but showed a variety of changes in their properties. No new bends were formed in this region. Irradiation within a bent region caused that region to straighten immediately.

These experiments indicate that the basal end of the flagellum is necessary for bend initiation, and largely responsible for the determination of wave properties. Although a portion of a flagellum can independently propagate established bends, the bend properties at any point are influenced by activities along the rest of the flagellum.

The relevance of these observations to current models of flagellar beating is discussed.

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CHAPTER I

General Introduction to Problems of Flagellar Motility

Early Observations on Cilia and Flagella

In November of 1677, in a letter to the Royal Society of London (27), Antony van Leeuwenhoek announced the discovery of "living animalcules" in human semen. They had been found and shown to him by a medical student, Johan Ham. Leeuwenhoek observed that:

> Their bodies were rounded, but blunt in front and running to a point behind, and furnished with a long tail . . . . The animalcules moved forward with a snake-like motion of the tail, as eels do when swimming in water.

Leeuwenhoek had observed ciliated protozoa (31), and he later discovered flagellated ones (32).

Leeuwenhoek's observations on spermatozoa were the subject of much controversy. During the following century his descriptions were amply verified by many investigators, although as late as 1833 Treviranus (93) ascribed the apparent motion of sperm tails to Brownian movement. Many of the early studies on spermatozoa were both stimulated and hampered by contemporary theories of reproduction. The preformation doctrine dominated thought on the subject until about the middle of the eighteenth century, so that even workers who admitted seeing spermatozoa often considered the tail to be a miniature backbone or umbilicus.

By 1835, cilia were known to be widely distributed among animals. Cloquet (26) in 1827, noticed that small patches removed from mussel gills resembled spermatozoa. This was perhaps the first suggestion of the basic similarity of cilia and flagella. Some theories on the mechanism

of ciliary beating began to appear about this time. Some workers have assumed, even quite recently (83,56), that cilia are moved by muscles, or muscle-like fibers, attached to their bases. Grant (42) suggested that cilia might beat by the flow of water in and out of a tubular structure. Sharpey (84), however, noted that cilia bend along much of their length, and suggested that contractile material extends all along the cilium. This suggestion attempted to answer two distinct questions: (1) Is the passage of bending waves along the flagellum passive or active? (2) If the bending waves are active, are they produced by a contractile apparatus, or by some other mechanism? Each of these questions has evoked much further discussion (86).

Interest in cilia and flagella increased toward the end of the nineteenth century, and observations became more refined as the quality of microscopes improved. Rootlet fibers were seen running from the bases of cilia and flagella in a number of cells, and were postulated to cause beating. This role for them — or for any extra-ciliary structures — was ruled out by several investigators. Peter (74) found that cilia of protozoa can continue to beat after the cell is crushed into small fragments, showing that an intact cellular structure is unnecessary. Verworn (98) observed beating in cilia isolated with only a bit of their basal cytoplasm, and Engelmann (33) observed beating in frog spermatozoa severed between their heads and mid-pieces. They concluded that very little of the cell body is needed for beating. The role of rootlet fibrils is still unknown, although they may help anchor the cilium. They are absent in spermatozoa. Engelmann found no motility in spermatozoa

severed between their mid-pieces and tails, and several workers tried unsuccessfully to completely remove cilia from cells without destroying their ability to beat. They concluded that the basal apparatus is necessary for movement, but their results may now be interpreted as being due to interruption of the normal supply of chemical energy substrates to the active parts of the cilium. There were, however, some workers who thought that cilia could beat without any extra-basal material. Erhard (34) claimed he could destroy basal granules by heating without affecting the motility of cilia.

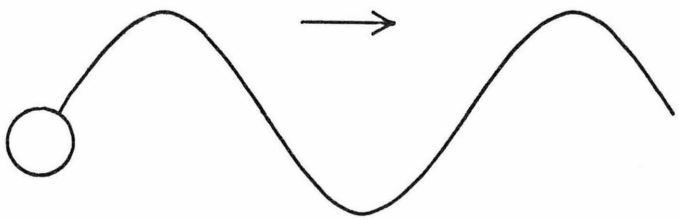
By 1900, workers noticed that cilia and flagella seem to develop from basal bodies. Henneguy (37) and von Lenhossek (58) noticed the similarity of basal granules and centrioles; von Lenhossek suggested that basal bodies and centrioles are identical.

Even though the width of flagella (about 0.2μ) is small compared to the resolving power of light microscopes, some workers managed to observe internal fibrils after disrupting the flagellar membranes. The work of Ballowitz (6) and others demonstrated that fibrils are widespread in cilia and flagella, and these workers suggested that the fibrils they observed were contractile.

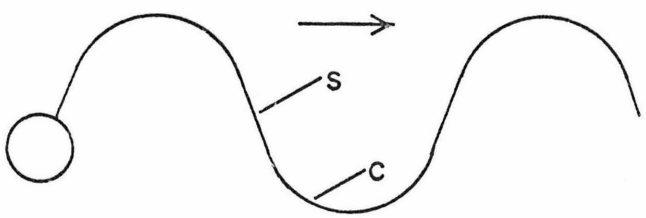
Contemporary Observations and Calculations on the Movement of Cilia and Flagella

The present period of study began largely with the work of James Gray, in the early 1920's. Earlier observations have been refined and enlarged, and new approaches have been developed.

Detailed descriptions of various types of waveforms have been made, using cilia and flagella from a number of types of cells, since the introduction of the stroboscope (64) and flash photomicrography (44) for viewing and recording their movements. Gray's (45) description of the movement of sea urchin spermatozoa has formed the basis for much of the discussion about the waveforms and possible mechanisms of beating. He described the beating as planar, with a waveform which could be fitted by a sinusoidal curve, as shown in Figure 1a. He emphasized the fact that the amplitude of the waves did not diminish as they passed along the tail. Gray (43) had pointed out earlier that if a passive flagellum were moved only at its base the wave amplitude should decrease towards its tip as the wave energy was dissipated in propelling the cell forward, and that the demonstration of a constant wave amplitude along the tail would provide strong evidence that flagella beat actively along their entire length. Machin (59) has developed this argument quantitatively, and has shown that a passive flagellum driven at its base could not produce the waveforms recorded by Gray. Machin proposed that the energy could be supplied by contractile elements, which were triggered to contract as a wave reached them. In a detailed analysis



a. Sinusoidal waveform



b. Waveform composed of circular regions connected by straight regions

Figure 1. Flagellar waveforms. Arrows indicate direction of propagation of waves. C = circular region; S = straight region.

of this possibility (60), he showed that this model would develop spontaneous standing waves if the elements were identical along its length, and that distally propagating waves could be established by the presence of a dominating region at the basal end. Brokaw and Wright (20) obtained photographs of the posterior flagellum of a dinoflagellate protozoan, Ceratium, with high enough resolution to determine that its waveform is planar, but not sinusoidal. The bent regions appeared to be circular, with a constant radius of curvature; these arcs appeared to be connected by straight regions, as shown in Figure 1b. This waveform has since been found in some invertebrate spermatozoa (14). This waveform can be generated by switching regions along the flagellum among only three states: a straight state and two bent states. Brokaw (16) has proposed that the transition from straight to bent state at any point might be induced by bending just proximal to that point. This localized propagation does not require the long-range viscoelastic interactions implicit in Machin's (60) model, and can more easily explain the more asymmetrical waveforms of cilia (16). Other types of waveforms have also been described. The plane of bending of a bull spermatozoon (46,77) changes as the wave progresses along the tail, giving it a partially helical character, and causing the cell to rotate as well as move forward. In addition, the amplitude of the waveform increases as the wave passes distally. Numerous other three-dimensional waves have been observed, and some flagella (such as those of the protozoa Peranema and Monas) exhibit quite bizarre waveforms, for which no simple models have been proposed. Successful photography of these three-dimensional bending

patterns has not been accomplished; detailed knowledge of flagellar bending patterns is only available for a very few cases, in which the movements are planar and have been successfully photographed.

In 1955, Hoffman-Berling (49) obtained beating by supplying adenosine triphosphate (ATP) to flagella which had been suspended in an aqueous solution of glycerin. Although the flagella bent and straightened rhythmically, waves did not propagate along their length. Brokaw (11), in 1961, obtained wave propagation in glycerinated flagella of Polytoma uvella by reactivating them with ATP. Unlike glycerin-extracted muscles, flagella reactivate after only a few minutes in glycerin; their motility diminishes markedly within hours, even when stored at -20° C. The glycerin apparently damages the membrane enough to allow ATP diffusion inward, but leaves the axoneme intact.

A number of workers have carried out calculations concerning the energetics and hydrodynamics of cilia and flagella. Bidder (8) noted that the Reynolds number for flagellar movement, which is a measure of the ratio of inertial to viscous forces operating on them during movement, is very small. Gray and Hancock (47) developed equations relating the swimming velocity of a flagellum with sinusoidal waves to the frequency, amplitude and wavelength of its waveform, and obtained good agreement with measurements on living sea-urchin spermatozoa. Brokaw (14) developed an equation relating swimming velocity and wave parameters of a flagellum whose waveform consists of circular arcs and straight lines, and obtained results differing only slightly from those of Gray and Hancock. The speed of the organism, then, does not seem very sen-

sitive to the detailed shape of the waveform. Brokaw also calculated the bending moments due to external viscous forces and concluded that the flagellum must be very stiff in the straight and bent regions to maintain its waveform as it moves against the viscous resistance of the water, but this stiffness must be appreciably reduced at the junctions between these regions, where bending and unbending occur. These hydrodynamic analyses also make it possible to estimate the rate at which a flagellum does work against external viscous resistance as it moves. Measurements of ATPase activity have been made on cilia and flagella from a number of organisms, after treating them to remove permeability barriers to ATP (9). They break down ATP at high enough rates to satisfy the calculated energy requirements, assuming a reasonable efficiency of conversion.

Variations of wave parameters with changes in temperature (52), viscosity (15) and hydrostatic pressure (55) have been studied. At present, it is difficult to interpret these changes in terms of a mechanism of flagellar beating.

Structure and Composition of Cilia and Flagella

Since Grigg and Hodge (48) introduced the electron microscope as a tool for the study of flagellar morphology in 1949, electron micrographs of cilia and flagella of many organisms have been published. The familiar pattern of nine outer and two inner fibrils first published by Manton and Clarke (63) has proven to be quite widespread. More subtle

features have since been demonstrated. Each of the outer fibers is composed of two subfibrils, of approximately equal size, with a pair of arms projecting from one of the subfibrils. These fibrils appear to belong to a general class of cellular structures known as microtubules. A set of radial spokes and secondary fibrils are sometimes seen between the central and outer fibrils, but these structures may vary or be absent in electron micrographs of different cells. The central fibrils are often surrounded by a sheath, and an extra central fibril is sometimes observed.

The set of nine plus two fibrils, together with the matrix and auxiliary structures, is referred to as the "axoneme," and is illustrated in Figure 2. Details vary among different cilia and flagella. Afzelius (2) has reported a motile spermatozoon which lacks the central fibrils. The mitochondria of spermatozoa are arranged around the basal portion of the axoneme, in a region of the cell called the "mid-piece." This region is quite short in invertebrate spermatozoa, but may extend for some distance in vertebrate spermatozoa. In other cells, the mitochondria are generally found only within the cell body, with some mitochondria near the flagella or cilia. Mammalian spermatozoa contain a set of large bodies distal to the mitochondria. These bodies run parallel to the axonemal fibrils, between the axoneme and the flagellar membrane, and often taper distally. Their function is unknown. The tails of these spermatozoa are often surrounded by a heavy sheath.

Although Miescher (66) found lipid and protein in plasmolysed salmon sperm flagella, and Marza (65) found proteins in the flagella of

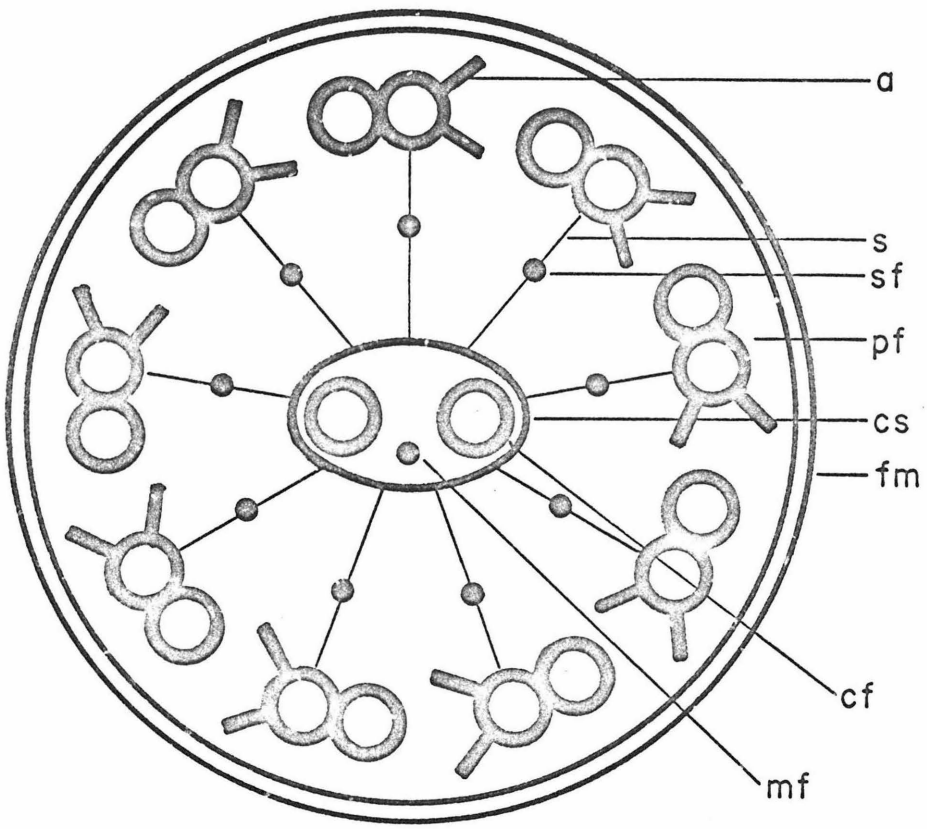


Figure 2. Diagram of cross-section of a flagellum viewed from basal end. Various structures, including secondary fibrils, spokes, central sheath and midfibrils are absent in some flagella. a = arm; cf = central fibril; cs = central sheath; fm = flagellar membrane; mf = midfibril; pf = peripheral fibril; s = spoke; sf = secondary fibril.

Modified from Gibbons and Grimstone (39).

various spermatozoa by histochemical techniques, detailed study of flagellar chemistry began with the work of Zittle and O'Dell (102), in 1941. They fractionated bull spermatozoa, and analysed the head, mid-piece and tail regions. 23% of the tail fraction was lipid, presumed due to the sheath. 13.6% of the remaining material was nitrogen. The lipid fraction of flagella has since been studied by a number of workers (9). Tibbs (92) examined flagella from several types of cells, and found flagella of Polytoma to contain 0.6% ribonucleic phosphorus, but found none in tails of fish spermatozoa. Infrared absorption indicated that the protein existed substantially in the α-helix form, and paper chromatography of Polytoma flagella revealed little hydroxyproline, ruling out collagenous protein as a major constituent. Culbertson (29) did find some hydroxyproline in cilia of Tetrahymena pyriformis, but considered the total amino acid pattern to be unlike that of collagen. He found a small amount of nucleic acid (0.4%), as have other investigators (25,100).

Muscle-like proteins have been found in flagella. Burnasheva (21) extracted "spermosin" from bull sperm homogenates, using standard procedures for the extraction of myosin from muscle. It has high ATPase activity and combines with actin to form "actospermosin", which shows a reversible configurational change on the addition of ATP, virtually identical to that of actomyosin. Cytochemical studies by Nelson and Plowman (71) on rat spermatozoa suggest that a myosin-like component is localized in the large bodies surrounding the axoneme. Plowman and Nelson (75) obtained "flactin" from starfish sperm flagella with standard

procedures for the extraction of actin from muscle. It polymerizes in 0.1 M KCl, contains bound nucleotide, and combines with rabbit myosin. Pautard (73) extracted an actomyosin-like gel from fish sperm flagella, which was observed to undergo rhythmic oscillation on addition of ATP. At present, it is unclear whether these observations on muscle-like proteins reflect phenomena actually involved in flagellar beating.

Mann (61) identified ATP in bull spermatozoa in 1945, and Burnasheva (22) later found them to contain guanosine triphosphate (GTP) and guanosine diphosphate (GDP) along with adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP). Felix, et. al (35) found ATPase in salmon sperm flagella. ATP and ATPase activity have since been found in many flagella. Gibbons (38) separated isolated cilia from Tetrahymena pyriformis into fractions of axonemes, outer fibrils and plasma membrane. The axoneme accounted for about half of the total protein, the soluble matrix and membrane for about a quarter each. About 70% of the Mg^{++}-dependent ATPase activity was associated with the axoneme; the remainder, which was activated by Ca^{++} and Mg^{++}, was associated with the membranes. The ATPase activity of the axoneme was correlated with the presence of the arms on the outer fibrils. Mitochondria in the mid-piece of spermatozoa appear to contain the normal complement of respiratory enzymes and carry out oxidative phosphorylation in the presence of oxygen (40). Sea urchin spermatozoa are normally functional in sea water, which supplies dissolved oxygen but little or no substrate for glycolysis; they exhibit little or no glycolytic activity, and do not beat in the presence of respiratory inhibitors (78).

Spermatozoa of animals with external fertilization generally exhibit little or no glycolytic activity (62). On the other hand, spermatozoa of animals with internal fertilization (such as mammals), which are normally supplied with sugars, are capable of glycolysing a number of hexoses (62,76). Terner (90) found turnover in the lipid fraction of bull spermatozoa, and suggested that energy is used for active synthesis of lipids as well as for motility.

Although ATP appears to be the primary energy source for motility, the implication of intermediate phosphagens (72) and the discovery of bound GTP along the outer fibrils (89) suggest that much remains to be learned about the chemical aspects of flagellar energetics.

Acetylcholinesterase activity has been found in some flagella (69), and nerve-like transmission of a bending signal has been suggested. The effects of a number of chemicals - even LSD (41) - have been studied but the results are difficult to interpret, and these substances may be acting indirectly, through other parts of the cell. Sleigh (86), Bishop (9), Holwill (51), and Nelson (70) have extensively reviewed recent work.

Current Speculations About the Mechanism of Bending

It is generally agreed that flagella are self-contained organelles which utilize energy obtained from glycolysis or respiration to bend actively along their length. Beyond this, little is known about the

mechanism of beating. Two very general models are currently popular.

The more popular model achieves bending by means of contractile elements arranged along the flagellum. These elements may contract so as to amplify the undulations of an otherwise passively vibrating elastic flagellum (59,60) or a bending wave may be propagated along the flagellum as contraction at one site triggers contraction of the next site (16). This model works equally well, of course, if the elements on the convex side expand instead of those on the concave side contracting. These contractile elements have usually been presumed to be located in the outer fibrils of the axoneme. Satir (81), however, has obtained electron micrographs of the tips of straight and bent cilia, in which the relative lengths of the outer fibrils can be compared. While the nine outer fibrils end in the same plane in straight cilia, the fibrils on the concave side of bent cilia extend farther at the tip than those on the convex side, by an amount consistent with the supposition that the length of the outer fibrils remains unchanged during bending. Horridge (53), using photographs of the macrocilia of a ctenophore, has reached a similar conclusion. These observations suggest that, if bending is due to contraction, the contractile elements are not the outer axonemal fibers.

The second model achieves bending by means of the sliding of incompressible and inextensible filaments. The outer axonemal fibrils in Satir's photographs could be interpreted as having caused the cilium to bend as they slid past one another, instead of having slid passively as a result of bending (81). Both models are considered in more detail in Chapter 5.

Summary of Remaining Problems

While a number of features of flagellar motility are known, much remains to be elucidated before a model can be constructed at the molecular and electron micrograph levels of structure. Almost nothing is known about the mechanism of energy transfer along the flagellum, the chain of transfer of the high-energy phosphates, or the coupling of the energy reactions and the reactions concerned with beating.

Although bending waves are normally initiated at the basal end of a flagellum (except for a few flagella, in which bends can be initiated at either end), it is not known whether other regions along the flagellum are capable of initiating waves. In addition, the mechanism of wave transmission is not understood.

It has been suggested that flagellar waves are merely an amplification of the undulations of a passive, elastic flagellum (59) but the waveforms seen in ciliary beating are difficult to produce with this model. The stiffness of an actively beating flagellum has not been measured, and might well change during the beat cycle. The mechanism of transmission may be an integral part of bend formation, or it may be a separate process.

Much remains to be discovered about the nature of the bends themselves. Their possible contractile nature has already been mentioned. Only further comparative studies will tell whether flagellar bending is inherently a process of switching between discrete states or a continuous bending process. The question of how unbending, and the various

wave characteristics, are controlled — which may, indeed, turn out to be a large set of questions — remains mostly unanswered.

CHAPTER 2

Introduction to the Study of the Localization of Function along Flagella

Previous Work

Few observations have been reported concerning the nature and effects of interactions along the flagellum or the localization of functions to given regions. Holwill (50) reported seeing flagella of a trypanosome flagellate, Strigomonas oncopelti, which were prevented from moving at one point by an obstruction. Flagella of this organism are normally capable of passing waves in either direction; distally directed waves were observed proximal to the obstruction, and proximally directed waves distal to it. Nelson (67,70) observed rat spermatozoa beating distally while appearing quiescent and relatively rigid proximally. Baker (5) observed that in tails of spermatozoa of a urodele, Crypotobranchus, impeded by other spermatozoa or debris, cessation of beating near the proximal end did not prevent beating of more distal regions. In fact, any part of the flagellum could be motile while other parts were immotile. The tails of urodele spermatozoa, however, are not simple flagella. They are large organelles, called "undulating membranes," and contain structures in addition to the axoneme.

Terni (91) found that pricking the undulating membrane of a spermatozoan of a urodele, Geotriton fuscus, with a fine needle caused beating to stop at the point touched, and to reverse direction on both sides of that point. He claimed that, if he cut a tail into two or more pieces, each piece was capable of beating. He irradiated small parts of a tail, using an ultraviolet beam of 8 μ diameter (the tail was

about 700 μ long). The irradiated region stopped immediately, but beating remained normal on both sides of that region. The complexity of urodele sperm tails makes these results difficult to interpret.

The study of Engelmann (33) on frog spermatozoa severed behind the neck has been mentioned in the section on Early Observations in Chapter 1.

Kaneda (54) impaired the beating of frog spermatozoa by either pressing a point on their tails to the coverslip with a glass microplate or drawing the spermatozoa into short lengths of capillary tubing. He found that the parts of the tail on the two sides of the microplate could beat at different frequencies. However, if less than 20% of the tail was proximal to the obstruction, beating usually stopped in the proximal region while the remainder of the tail continued to beat. Restricting the amplitude of part of the tail with capillary tubing caused a decrease in frequency. The frequency was decreased equally in and out of the capillary, but he did not state whether the amplitude was also decreased outside of the capillary. Walker (99) irradiated short regions of flagella of a trypanosome, Trypanosoma, with a focused beam of visible light (4300-4650 Å) while the organism was in a medium containing acriflavine, which rendered it sensitive to this light. Beating ceased in the irradiated region, but continued on both sides of it. He did not give the directions of wave propagation or compare the beating on the two sides of the injury. Since trypanosomes are usually capable of initiating waves at either end of the flagellum, the results of these experiments are difficult to assess.

Gray (45) reported observing a sea urchin spermatozoon attached to a slide by a point in the central part of its flagellum. The region of the tail proximal to the point of restraint beat, while the remainder of the tail did not. As the spermatozoon moved slowly forward, points on the tail beat as they became proximal to the constraint, suggesting that beating cannot pass a point of restraint. This not only disagrees with the observations mentioned above, but is contrary to the behavior of several sea urchin spermatozoa which I have observed, which continued to beat distally to regions that had become attached to the slide.

The previous observations on constrained or damaged spermatozoa are somewhat confusing. Beating appears to be possible on both sides of an immobilized region, but it is usually unclear whether beating was initiated independently in each region or some sort of bending signal passed through the immobilized area. This distinction is only clear on unusual types of flagella (50,91). If waves are initiated only at the basal end, propagation through a region apparently does not depend on undulation of the flagellum in that part and some regions of a flagellum can beat while others are quiescent. As yet, the ability of any region to initiate and sustain beating independently of other regions remains largely unexplored.

Potentialities of Microbeam Irradiation

Ultraviolet microbeams have been used since 1912 (94) for destruction at subcellular levels. Monochromatic sources may be used, and both

reflecting and quartz optics have been developed for focusing the collimated beams, so that spot diameters of 1μ or less can be achieved. Alpha particle, proton, electron and X-ray beams have also been used to a small extent, but are generally less convenient to control than ultraviolet beams. The major drawback to using these beams for irradiation of flagella is the relatively long exposures needed to produce significant damage. This disadvantage has been overcome with pulsed ruby laser microbeams. These lasers produce highly monochromatic visible light (usually 6943 Å) from a small (about 3 mm diameter), well-collimated source, and are normally focused by the microscope that is used for viewing the specimen to be irradiated. The laser is aimed into one of the oculars, and the beam emerges from the objective reduced and concentrated by a factor equal to the magnification of the microscope. The pulse duration is a fraction of a millisecond, providing very quick destruction. Q-switched lasers, in which the pulse duration has been decreased to a fraction of a microsecond, provide even faster, more powerful tools. The use of a pulsed ruby laser microbeam for partial destruction of cells (human leukocytes) was first described in 1962 by Bessis, Gires, Mayer and Nomarski (7). They realized that these cells are transparent to visible light, and stained them with Janus green B to absorb the red beam. When this stain is used, the mitochondria are selectively damaged (3). The main effect from absorption of visible laser irradiation appears to be simple thermal destruction of the irradiated region, but second-order effects might arise from the intense electrical field of the beam, which could cause

compressive (electrostrictive) effects (81a), and from ionization and rapid vaporization, which could give rise to shock waves (30,36).

The pulsed ruby laser provides a means of rapidly damaging a small region of an actively bending flagellum at a preselected point, and at a predetermined phase of its beat cycle at that point. It has been used for that purpose in the present study. Photomicrographs have been taken immediately before and after irradiation, which show the changes in the movement of the flagellum resulting from the damage. The spermatozoa of a number of invertebrates have been used. These flagella have a relatively simple ultrastructure, and the components of their tails are probably common to most cilia and flagella. They have planar waveforms, so that photographs were obtained in which the entire tail is in focus.

Localized Application of ATP to Selected Regions of the Tail

No mechanism is known for the active transfer of ATP from mitochondria to the various parts of the flagellum, and adequate ATP to supply the energy needed for beating can probably be supplied by simple diffusion from the basal region (17). However, an appreciable diffusion gradient would result, the distal region of the flagellum having a lower concentration of ATP than the proximal end. If any of the wave parameters at a point are sensitive to the ATP concentration at that point, variations in waveform along the flagellum should be noticeable. Since bending waves normally appear constant all the way to the tip (45,14),

this suggests that either beating is insensitive to local ATP concentration or there is a mechanism for supplying a constant ATP concentration to the entire flagellum.

When glycerinated spermatozoa are diluted into solutions containing ATP, their beat frequency and wave velocity increase with increasing ATP concentration over very wide ranges (12), while other parameters appear to be relatively insensitive to ATP concentration, suggesting that at least some part of the flagellum is sensitive to ATP concentration. Glycerinated spermatozoa offer a system on which the effect of ATP gradients along a flagellum may be studied. They lack an internal ATP supply and their membranes seem permeable to ATP supplied from an external source. In the experiments described in the section on ATP Diffusion Experiments in Chapter 3, external ATP gradients were developed along glycerinated flagella by means of diffusion of ATP solutions from the tips of micropipettes. The gradient could be centered around any region of a flagellum, to test the possibility that waves might be initiated at, and their parameters therefore determined by, the region with the greatest ATP concentration. Very steep gradients were used to test the effects of supplying ATP to a limited region of a flagellum, and the ability of any region to beat independently of the rest of the organelle.

CHAPTER 3

Materials and Methods

Solutions Used

The following solutions were used in this study.

1. Prepared sea water: 10^{-3} M ethylenediaminetetraacetate (EDTA) (96), 2% (w/v) polyvinyl pyrrolidinone (PVP) in filtered sea water, adjusted to pH = 8.1 with 0.5 M tris (hydroxymethyl) aminomethane (tris).

2. Tris-thioglycolate buffer (TTG): 0.2 M thioglycolic acid adjusted to pH = 7.8 with tris. This stock solution was diluted down to 0.02 M for use in solutions (3) and (4).

3. Basic glycerol salt solution: 0.01 M $MgCl_2$, 0.25 M KCl, 0.02 M TTG, 2% PVP, adjusted to pH = 7.6 with tris.

4. Basic ATP salt solution: 0.004 M $MgCl_2$, 0.25 M KCl, 0.02 M TTG, 2% PVP, adjusted to pH = 7.6 with 0.5 M tris.

5. Glycerol solution: 55% glycerol, 0.01 M $MgCl_2$, 0.25 M KCl, 0.02 M TTG, 2% PVP adjusted to pH = 7.6 with 0.5 M tris.

ATP Diffusion Experiments

Collection of Spermatozoa

Experiments involving the diffusion of ATP from the tips of micropipettes were performed on spermatozoa of two species of sea urchins,

Strongylocentrotus purpuratus and Lytechinus pictus, collected in or near Newport Bay, California. The animals were induced to spawn by the injection of 1-2 ml of 0.5 M KCl into their perivisceral cavity (95,97) and about 1 ml of the spermatozoa was collected from the dorsal surface with a Pasteur pipette.

Glycerination of Spermatozoa for ATP Diffusion Experiments

Spermatozoa were glycerinated by a method similar to that of Brokaw (18). The concentrated spermatozoa were diluted with an approximately equal volume of prepared sea water. This procedure helped to prevent clumping of the spermatozoa when they were introduced into the glycerol solution. This sperm suspension in sea water was then introduced with a Pasteur pipette into about 20 volumes of basic glycerol solution. The tip of the Pasteur pipette had usually been drawn to a diameter of a few tenths of a millimeter so that a fine stream of spermatozoa could be injected into the glycerol solution to facilitate the rapid suspension of the spermatozoa in the glycerol solution. These preparations were stored between -10°C and -20°C, and used within a few hours.

Apparatus for ATP Diffusion Experiments

Pipettes were pulled on an automatic pipette puller from Kimax thin-wall capillary tubing with an outside diameter of 1.2 to 1.5 millimeters. The tubing had been cut into lengths of 3 inches, fire-polished, washed with hot 3 N HCl and rinsed with boiling filtered distilled water. Two pipettes, with tip diameters of less than 1 μ, were produced from each piece. A pipette was broken to a final tip

diameter of about 1 μ by touching its tip lightly to the frosted end of a frosted glass slide, and was filled through the shank with a solution containing basic ATP salt solution and varying amounts of ATP. This solution was filtered through a Millipore filter of pore size 0.45 μ. The ATP concentrations ranged from 10^{-4} M to 10^{-2} M; most observations were made using 5 x 10^{-4}M ATP. A short length of polyethylene tubing, partially filled with this solution, was placed over the tip to act as a reservoir until the pipette was ready for use. The end of the shank was cleaned with acetone and sealed with melted paraffin.

The pipette was manipulated by means of a Brinkmann Instruments RP micromanipulator. It was placed in the micromanipulator and allowed to stand for about 15 minutes before use, to assure equilibration of temperature and of flow of the solution in the pipette. All observations were made with an ambient temperature of 18°C.

Observations were made with a Zeiss GFL microscope, using dark-field optics and continuous illumination. It was convenient to view the edge of the slide through the microscope while the pipette was being introduced under the coverslip; however, the mechanical stage of the microscope could not be moved back far enough to place the front edge of standard one-inch wide slides in the viewing field, so one-half inch slides were used. These were prepared by scoring 3" x 1" slides lengthwise with a diamond pencil and breaking them. 22mm x 22mm #1½ cover glasses were similarly divided into 3 equal pieces, and mounted above the slides. Vaseline was initially used to mount the cover glasses, but was replaced with a mixture of 3 parts vaseline to 1 part paraffin

(by weight), which allowed prepared slides to be stored for long periods. Earlier observations were made with cover glasses mounted 50μ above the slides; later ones were made with cover glasses mounted about 1 mm above the slides, supported by pieces of cut slides.

Manipulation of Apparatus and Spermatozoa

A drop of concentrated glycerinated spermatozoa was diluted into about 1 ml of basic ATP salt solution, and enough of this suspension was placed on a slide to completely fill the space under the cover glass. The reservoir was removed from the tip of the pipette and the pipette was maneuvered under the cover glass and into the center of the viewing field. The mechanical stage was used to position spermatozoa; the micromanipulator was used to withdraw and reintroduce the pipette from a region of the field, as a means of regulating the ATP supply to that region. The apparatus is diagramed in Figure 3.

Laser Irradiation Experiments

Collection of Spermatozoa

Most of the experiments involving microbeam irradiation were performed on the spermatozoa of a sea urchin, Strongylocentrotus purpuratus, collected as described above. Spermatozoa for these experiments were also obtained from a starfish, Pisaster brevispinus, allowed to spawn spontaneously; from a tunicate, Ciona intestinalis, by dissection of the sperm duct; and from a keyhole limpet, Megathura crenulata, by dissection of the gonad.

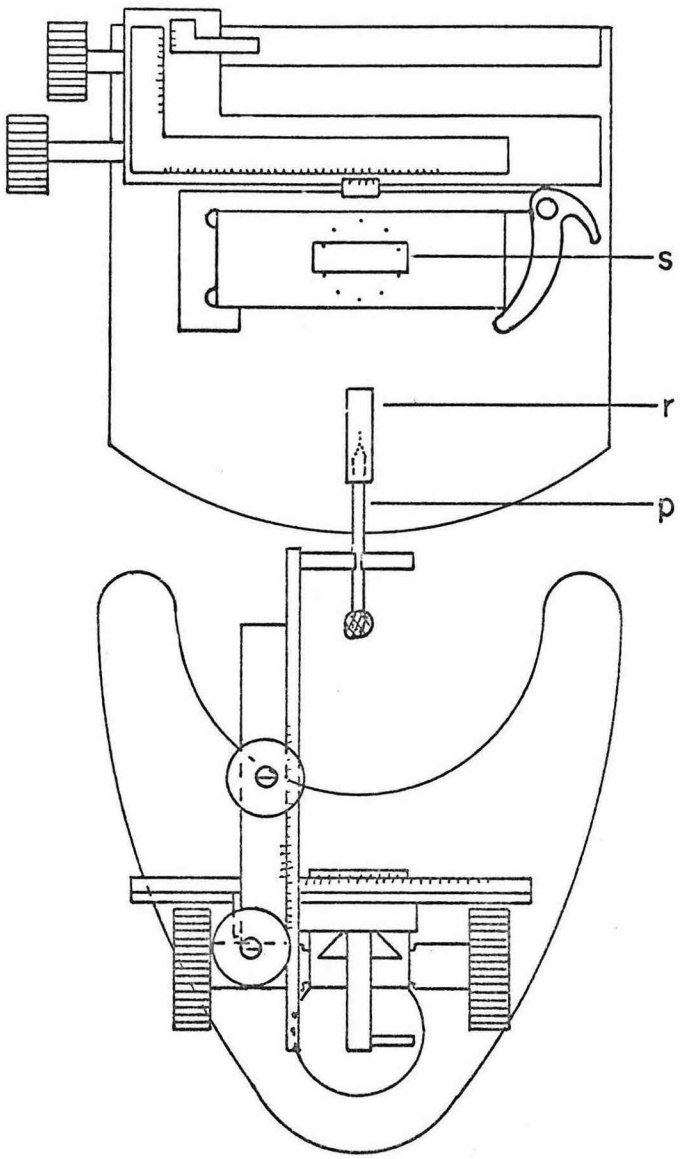


Figure 3. Apparatus used in ATP diffusion studies. Reservoir was removed immediately before pipette was introduced into sperm suspension. p = pipette; r = reservoir; s = sperm suspension.

Absorption of Beam Energy

Cells are usually transparent to visible light and the need for a dye to increase absorption of light at 6943 Å was anticipated. Although the diameter of the focused laser beam was 1 to 2 μ in diameter, flagella are only about 0.2 μ in diameter, and only a fraction of the energy in the beam intersected a flagellum. In addition, the small diameters of flagella give them a large surface-to-volume ratio, allowing them to dissipate energy rapidly and further increasing the difficulty of heating them. These difficulties were circumvented by dissolving a dye in the medium, so that the entire volume of water intersected by the beam absorbed light. Any dye that may have adsorbed to the spermatozoa should have further increased heating of the tail. Various dyes were tested for ability to absorb light in the region of 6943 Å , using a Bausch and Lomb colorimeter. An optical density (O.D.) of about 1.5-2 was sought for reasons discussed in Appendix 1. In practice, concentrations were adjusted to give convenient readings, and the concentrations necessary for O.D. = 1.5 were calculated from the Beers-Lambert law. Methylene blue, brilliant cresyl blue, Janus green B, and Food, Drug and Cosmetic (FD&C) blue #1* were examined and tested for toxicity to sea urchin spermatozoa in prepared sea water.

A large fraction of methylene blue precipitated out when it was added to sea water, because of the high salt concentration. Dissolving 0.1%(w/v) methylene blue in sea water and filtering yielded an approxi-

*Colour Index number 42090 (28). Obtained in 89% pure form from H. Kohnstamm & Co., Chicago, N.Y., Huntington Park. Also available as brilliant blue FCF.

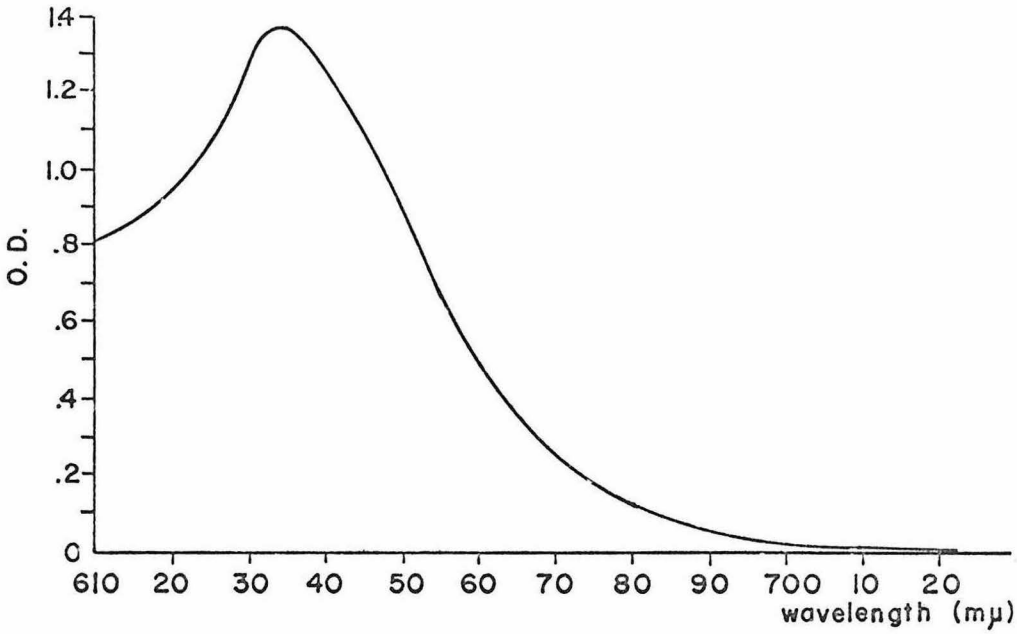
mately correct absorption value, but when sea urchin spermatozoa were added to a filtered solution containing 0.1% methylene blue, they showed appreciably decreased activity.

Brilliant cresyl blue and Janus green B also partially precipitated out when added to sea water. 0.5% (w/v) of brilliant cresyl blue, and 0.1% (w/v) of Janus green B, absorbed enough light after filtration, but when sea urchin spermatozoa were added to a filtered solution of either dye, the proximal portion of the tails beat normally, while the waves died out quickly toward their tip.

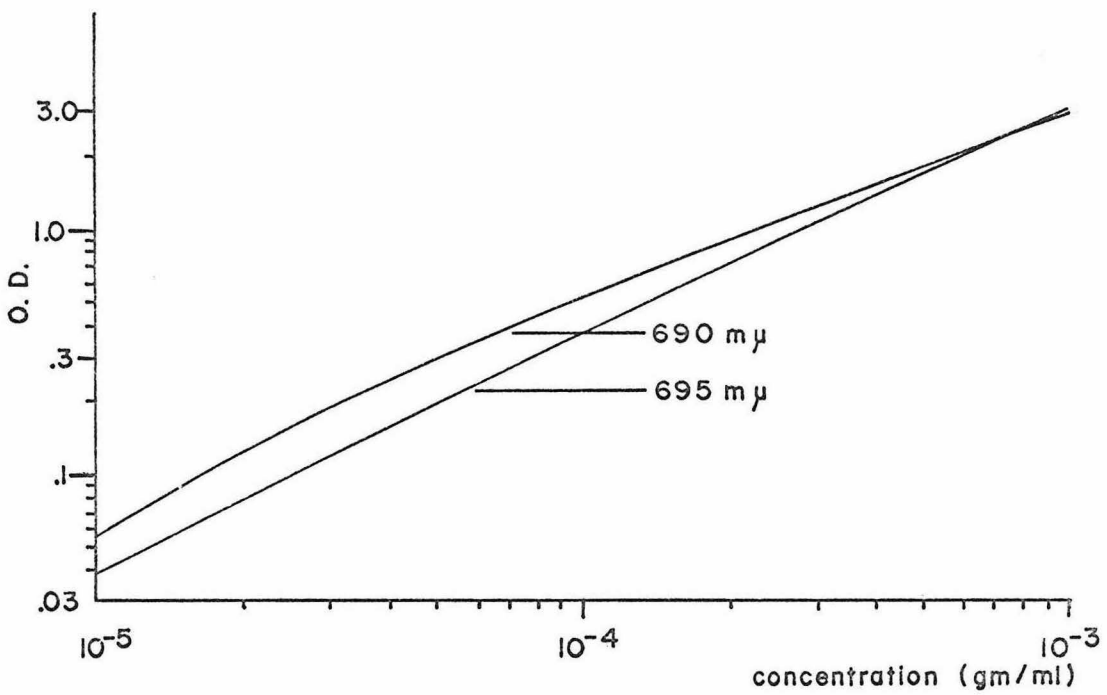
FD&C blue #1 did not precipitate out in sea water. The absorption spectrum of a 10^{-5} gm/ml solution, as measured on a Bausch & Lomb colorimeter, is shown in Figure 4a. The optical density at 695 mμ = 0.02. A concentration $C = \frac{2}{0.02} \cdot 10^{-5} = 10^{-3}$ gm/ml is needed for O.D. = 2. Measurements of O.D. vs. log C for this dye in the region of 6943 Å is shown in Figure 4b, and the Beers-Lambert law can be seen to hold fairly well for it. This dye, at a concentration of 10^{-3} gm/ml, caused no noticeable effects on intact spermatozoa, and caused fairly slight reduction in motility when added to suspensions of glycerinated spermatozoa in ATP solutions. Irradiation of spermatozoa in FD&C blue #1 at a concentration of 10^{-3} gm/ml caused the desired damage to the flagellum and the dye was used at this concentration in all the microbeam experiments.

Attachment of Spermatozoa to Glass

Spermatozoa were far easier to hit if their heads adhered to the slide or cover glass, holding them in position. Spermatozoa of Strongylocentrotus rarely attach this way, and several techniques for increasing



a. Absorption spectrum



b. Absorption vs. log concentration

Figure 4. Absorption properties of FD&C blue #1, in distilled water.

the number adhering were tried.

Silicone-coating slides and cover glasses with Siliclad(a water soluble silicone concentrate of Clay-Adams, Inc., N.Y.) did not noticeably affect the number of spermatozoa attaching to the glass.

Finely ground pieces of cover glass were added to the sea water in an attempt to increase the surface area available for attachment. Spermatozoa did not adhere to these particles, nor did they attach to droplets of paraffin vapor which were condensed onto the slides for the same purpose.

Fertilizin was prepared from sea urchin eggs (97) and added to the sperm suspension. About ½ cc of Strongylocentrotus purpuratus eggs was washed 3 times in 10 ml of ice cold sea water and added to 2 ml of sea water at room temperature, which had been adjusted to pH = 2.8 with HCl. After 10 minutes, the eggs were spun down, the supernatant was added to 4 parts of sea water and the pH was adjusted to 8.1 with 0.5 M tris. This fertilizin solution was diluted with about 4 parts of sperm solution. Fertilizin increased the number of spermatozoa attaching to the glass, but spermatozoa tended to attach by the tip of their tails as well as by their heads, and often attached only by their tails.

Strongylocentrotus purpuratus eggs were added to a dilute sperm solution, so that there were a few eggs on a slide. The eggs burst when the cover glass was placed on the slide, and a few spermatozoa could usually be found in the vicinity of each egg which were attached only by their heads and beating normally.

Bovine serum albumin (BSA) and PVP were added, both with and without eggs, but neither seemed to improve attachment.

Preparation of Live Spermatozoa

A very small drop of spermatozoa was diluted just before use with about 1 ml of prepared sea water. Strongylocentrotus purpuratus eggs were added to the sperm suspension in all experiments performed on Strongylocentrotus purpuratus spermatozoa. Spermatozoa of the other animals used (as well as glycerinated spermatozoa) attached only by their head often enough so that no special methods were used.

A small drop of this suspension was placed on a slide, and a cover glass was lowered over the drop. The drop was small enough to form a very thin film, and all the spermatozoa appeared to be swimming in the same plane. Such a thin film maximized the chance of spermatozoa attaching to the glass and minimized the chance of tails drifting out of focus after irradiation. The slides and cover glasses had been carefully washed, because the dark-field optics were very sensitive to light scattered from debris.

Glycerination of Spermatozoa for Microbeam Experiments

Glycerinated spermatozoa did not beat well in the thin films used for the microbeam experiments. In addition, the dye impaired their motility somewhat, so that glycerinated spermatozoa prepared by the method described for ATP diffusion experiments did not beat well enough for use in the microbeam experiments, and a modified preparative procedure was therefore used. Concentrated semen was diluted with an

approximately equal volume of prepared sea water. This sperm suspension was then introduced with a Pasteur pipette into about 5 volumes of glycerol solution. The tip of the pipette had been drawn to a diameter of a few tenths of a millimeter. This preparation was stored at -10°C for 12 hours, and was then sedimented in a Beckman Spinco centrifuge at 10,000 rpm in a 40A rotor for 10 minutes at -5°C. Most of the spermatozoa were spun down, and the supernatant medium, containing a very dilute suspension of spermatozoa, was pipetted off and stored at -10°C until used.

Preparation of Glycerinated Spermatozoa

A few drops of the glycerinated sperm suspension were mixed with a small amount of a solution which contained basic ATP salt solution, 0.3% (w/v) BSA, 3×10^{-4} M ATP and 10^{-3} gm/ml FD&C blue #1 and had been adjusted to pH = 8.1 with 0.5 M tris, and a small drop of this suspension was placed on a slide and covered in the manner described in the section on preparation of live sperm. The initial concentration of spermatozoa in the glycerol solution, and the final concentration of the glycerol solution during observation, were both increased by this procedure.

Apparatus for Microbeam Experiments

A general description of the equipment and its use is given in this section. A detailed description of the components is given in Appendix 2. A block diagram of the system described in this section is shown in Figure 5. A simplified schematic diagram is shown in Figure 6.

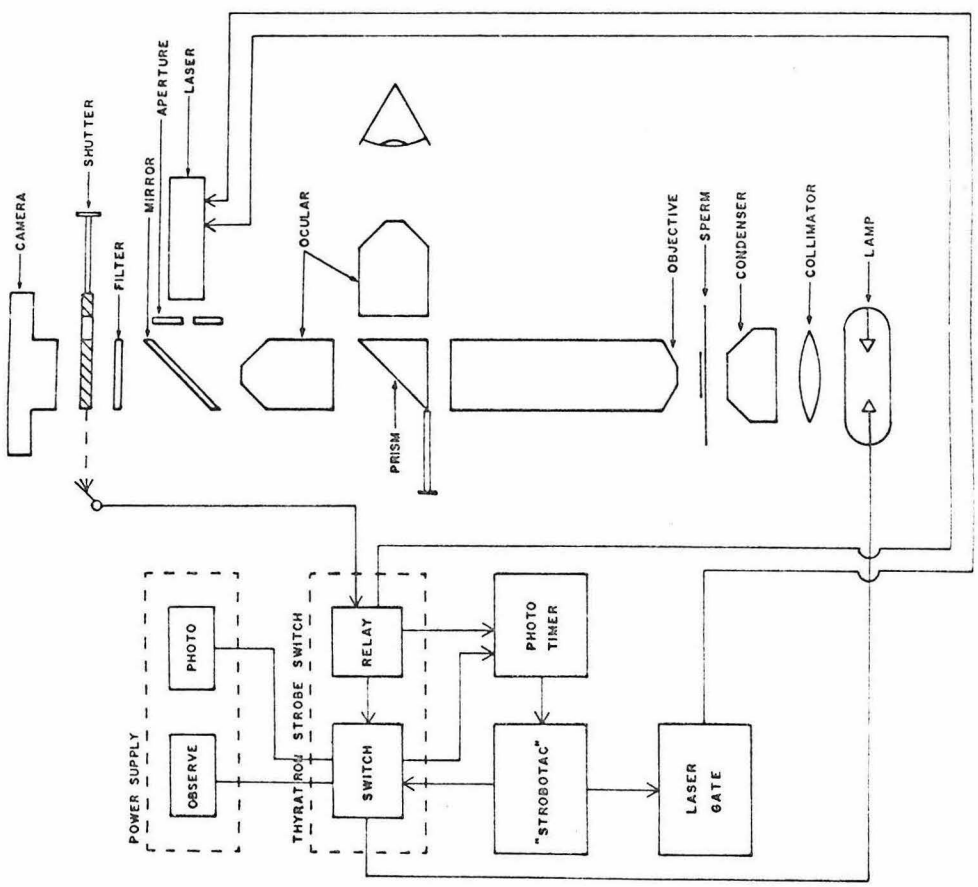


Figure 5. Laser microbeam apparatus

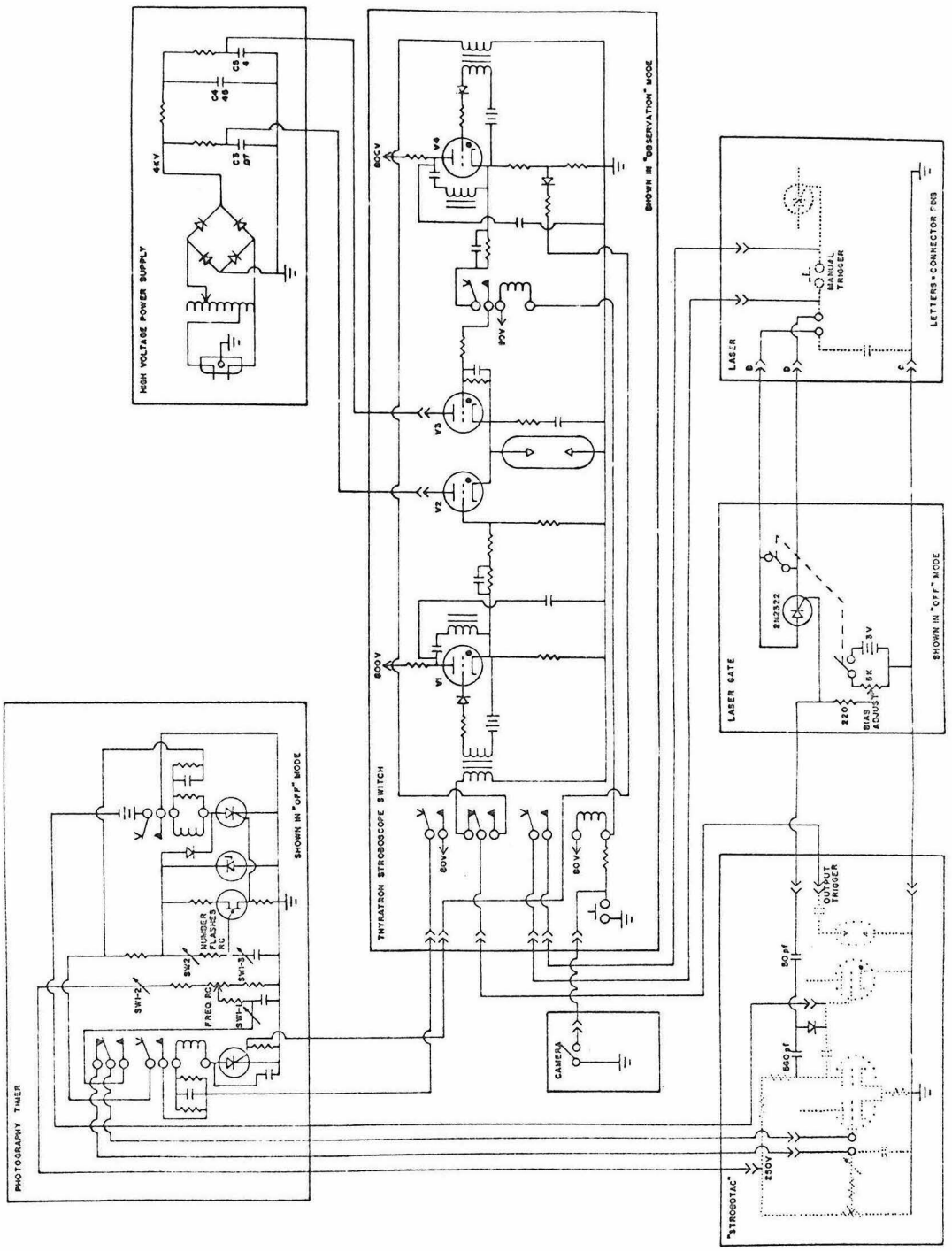


Figure 6. Laser microbeam apparatus. Simplified schematic diagram.

A Zeiss GFL microscope, with a trinocular head and a 40X variable aperture (n.a. 0.6-1.0), oil immersion objective, was used with dark-field stroboscopic illumination. The microscope was mounted on a heavy Zeiss frame, which also supported a 35mm camera and associated reflex accessory above the vertical (monocular) tube of the microscope. The camera was a Robot Vollautomat star II, and was mounted on a Zeiss reflex adapter with a magnification of 0.5X. Photographs were taken on Kodak Tri-X or Iford Mark V films, at a magnification of 200X. They were developed in Acufine for 30 minutes at 18-20°C, and stopped, fixed and washed in a conventional manner. The photographs are negative prints, on DuPont Varigam paper at contrast grade 4, made from intermediate positive transparencies on Kodak Contrast Process Ortho film to increase contrast.

A TRG model 513 biolaser system was used. The laser head and housing were mounted directly on the vertical tube of the microscope. Although the housing was designed for use with a Leitz Ortholux microscope, it was easily mounted on the Zeiss microscope by means of an adapter made for this purpose. The unit was light enough to be supported by the microscope without aid. The laser head was cooled with filtered air. Laser emission was reflected down the monocular tube by a dielectric mirror between the monocular eyepiece (American Optical, 10X, wide angle) and the camera. A filter, which absorbed the red light of the laser, but passed the bluish light of the xenon flash lamp, was interposed between the mirror and the camera to diminish this image and the image of the focused laser spot. The size of the beam was

reduced by an aperture between the mirror and laser head. It was further reduced by the microscope optics, and emerged from the objective with a diameter of about 2 μ, as determined by the manufacturer's specifications and the microscope optics.

The laser was aligned in the following manner. A thin layer of a 4.4×10^{-2} gm/ml solution of light green SF yellowish stain* was allowed to dry on a slide (79), and a cover glass was cemented over it with Canada balsam. This slide was used as a target. A single pulse produced a hole in the dye film. The position of this hole was aligned to coincide with crosshairs in one of the binocular (viewing) eyepieces by adjustment of controls on the mirror.

Stroboscopic illumination was used for observation and photography. A General Electric FT-230 short gap xenon flash lamp was used as a light source. This lamp discharges without external triggering when a potential of greater than 2,500-3,000 volts is applied. It was ignited by connecting across a capacitor which had been charged to 4,000 volts. This capacitor served as the flash capacitor to store the energy for the discharge. A 0.07 μ f capacitor (C3 in Figure 6) was used for the low-intensity flashes for observation; a 4μf capacitor (C5 in Figure 6) was used for the high-intensity flashes needed for photography. A 45 μ f capacitor (C4 in Figure 6) was placed across the voltage supply for the larger flash capacitor, to store energy for multiple-exposure photographs. The intensity of these exposures diminished as

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* Colour Index number 42095. It is also available as FD&C green #2, and might be useful as a biological stain for laser adsorption.

this storage capacitor discharged, and the first image was easily distinguished from subsequent ones.

A type 5949 high-power hydrogen thyratron was used as a switch between the flash lamp and each flash capacitor. It was triggered by a C1K xenon thyratron, which was driven by pulses from the "output trigger" connector of a General Radio type 1531-A "Strobotac" stroboscope. Two such pairs of thyratrons were used: one to connect the flash lamp to the small flash capacitor and one to connect it to the large flash capacitor. Output pulses from the "Strobotac" were directed to the appropriate thyratron by the electronic-flash contacts of the camera, and high-intensity flashes were produced only when the shutter was opened.

During observation of spermatozoa, the "Strobotac" flashed at the frequency indicated on its control dial, which was usually adjusted to be equal to the beat frequency of the spermatozoon being observed. During photography, the "Strobotac" could be operated at the same frequency or another, predetermined, frequency. This frequency was determined by the "photography timer". This timer also controlled the number of exposures on each photograph, by clamping the thyratron grid of the "Strobotac" to -9 volts a preselected time after the first high-intensity flash. From 1 to 5 exposures were available, at flash rates of 5, 10, 25, 50 and 100 flashes per second. The control dial of the "Strobotac" was calibrated against 60 Hz line current. An oscilloscope was calibrated against the "Strobotac" dial at each of the above frequencies, and in turn was used to calibrate the timer to an accuracy of within a few per cent.

Figure 7 shows some spermatozoa which were not damaged by the laser. They were all beating at about 25 Hz, and the timer was set to 4 flashes at a rate of 50 flashes/second. The flash rate can be seen to be about twice the frequency of the tails, and the first exposure is noticeably more intense than the subsequent ones.

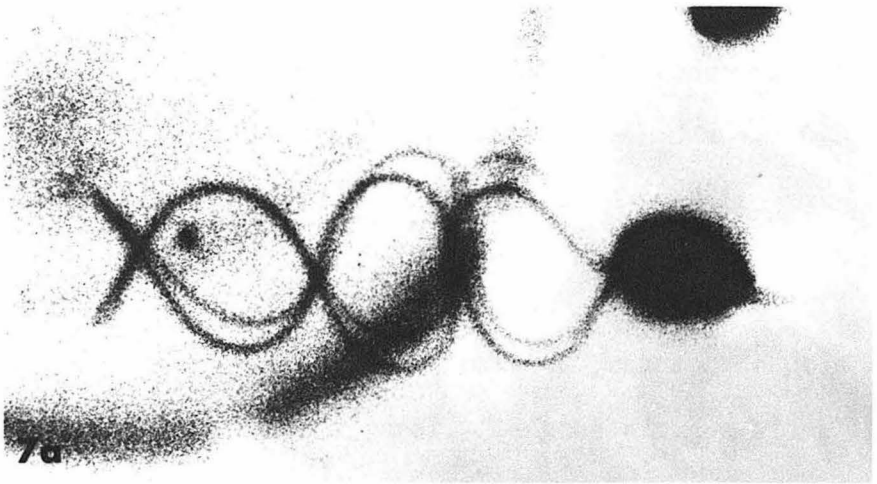
The TRG model 513 biolaser system is normally triggered by a manual switch. A silicon controlled rectifier (SCR) was placed in series with this manual switch, and a pair of contacts on the relay activated by the camera was placed in parallel with it. The SCR was triggered to conduct by pulses from the multivibrator of the "Strobotac". When the camera shutter was opened, the laser was triggered to fire on the next flash. The laser was thus synchronized with the stroboscope. When the stroboscope flashed at the same frequency as a beating flagellum, the laser was synchronized with that flagellum.

When a suitable spermatozoon was selected for injury, the frequency of illumination was adjusted to be equal to that of the tail, so that the tail was in the same position each time the lamp fired. The mechanical stage was then positioned so that the desired point on the tail appeared under the crosshair. The microscope prism was set to the "monocular" position and the camera shutter was opened. On the next flash (high-intensity), the timer and the laser were activated. Although the xenon flash lamp of the laser probably flashed within a few microseconds of the illuminating flash, there is typically a delay of about 0.5 milliseconds from the start of the xenon flash until stimulated emission begins and the laser fires (57). This delay

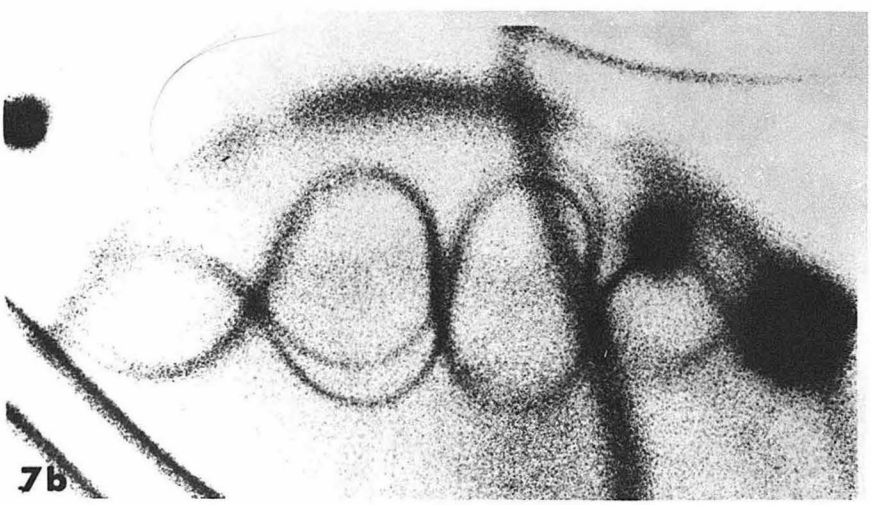
Figure 7. Undamaged spermatozoa, beating at approximately half the exposure frequency.

a. S. purpuratus spermatozoon. Frequency before irradiation = 26.2 Hz.

b. C. intestinalis spermatozoon. Frequency before irradiation = 25.2 Hz.



7a



7b

10 μ

allowed one exposure before injury, but did not allow the spermatozoon to move significantly far from the position at which the laser was aimed. The period of beating was typically about 40 milliseconds, and the amplitude was about 5 μ , so a point on the tail moved about 0.3μ between the xenon flash and the firing of the laser.

Measurements of Parameters from Photographs

All measurements were taken on positive prints made at a magnification of 4,000 X on grade 5 paper. Lengths along a flagellum were determined by measuring lengths of polyethylene tubing laid along it. Radii of curvature of circular regions were measured by overlaying circles drawn on transparent plastic; radii of curvature of bent regions with varying radii were measured by overlaying Archimedean spirals drawn on transparent plastic.

CHAPTER 4

Observations

Experiments With ATP Gradients

When a pipette containing ATP was introduced into a field of spermatozoa, as described in the section on ATP diffusion experiments in Chapter 3, beating began within a few seconds and occurred within a radius of 50-100 μ from the tip of the pipette. Beating always began at the basal end of a flagellum and proceeded distally, even when the tip of the pipette was placed near the distal end, thus presumably reversing any normal head-to-tail gradient.

Most observations were made on spermatozoa which remained fixed in the field of view because their heads became attached to the slide or cover glass. As a pipette was brought nearer to a flagellum, the beat frequency increased continuously, while the amplitude and wavelength appeared to remain constant. When the pipette was brought to within a few μ of the basal end, the frequency increased markedly and the amplitude fell to almost a quiver, as if a bend started before the previous one had developed fully. The critical frequency was not measured stroboscopically, but was probably about 20 or 30 Hz. The frequency decreased and the amplitude increased to their previous values as the pipette was withdrawn, and beating stopped completely when the pipette was removed from the field. These results were reversible: the pipette could be moved up to and away from a spermatozoan several times, with the same results occurring each time.

No gradient of activity along the tail was noticed, except that the most distal portion (approximately the last quarter) often showed

no activity. Lack of activity in the distal portion is also found, however, in glycerinated spermatozoa in uniform ATP solutions.

When the pipette was removed, bends almost always propagated to the tip before beating stopped; a spermatozoon almost never stopped beating with a bend having progressed only part way along its flagellum. Sometimes, however, a bend could be stopped part way along by placing the pipette tip far enough from the flagellum so bends progressed very slowly, and quickly withdrawing the pipette. When the pipette was returned, the bend continued.

With a separation of 50 μ between the slide and cover glass, the region in the vicinity of the pipette tip quickly became saturated with ATP, and the ATP concentration was less localized than if the pipette had been diffusing into an unlimited volume (see Appendix 3). To reduce this effect and produce sharper gradients, the experiments were repeated with the cover glasses mounted 1 mm above the slides. Using these slides, the tip of a pipette filled with 10^{-3} M ATP could be brought to within 15 μ of the basal end of a flagellum without producing beating. As the pipette was moved nearer a spermatozoon, bending waves began at the basal end of the tail and proceeded distally. When the pipette was placed near the distal end of the tail, the tail remained inactive until the pipette was moved near the basal end. Once beating began, no changes in velocity or amplitude were observed as the bend propagated along the tail, except in the tip region, as noted previously. Beating could not be initiated in other regions by placing the pipette tip near them.

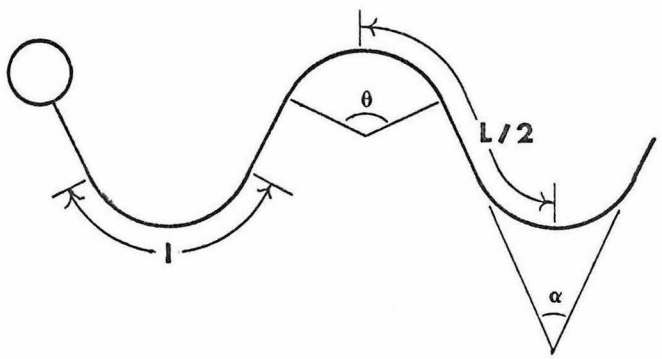
Laser Irradiation Experiments

General Considerations

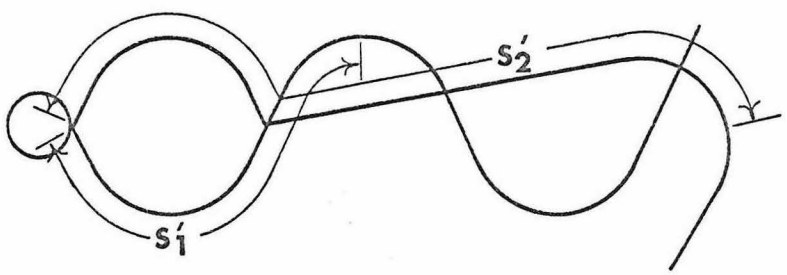
The results of irradiation were recorded on multiple-exposure photographs, as described in Chapter 3. All photographs shown were taken at a flash rate of 50/second and have a final magnification of 3,000 X, except where otherwise noted. Damaged regions generally appear as sharp breaks, with visible damage limited to a region of 1 μ or less in length.

The beat frequency (f) of a spermatozoon before lasing was determined from the "Strobotac", which had been adjusted to flash at the same frequency. The wavelength (L) of a spermatozoon before lasing always refers to the wavelength as measured along the flagellum, and was determined by direct measurement on a photograph. Measurement of the position of a bent region was made to the center point of that region. The normal velocity (V_s) at which bends moved along the flagellum, was calculated from these observed values of f and L. The distance (S') that a wave travelled between two successive exposures after irradiation of the flagellum was determined from direct measurements on a photograph. The time (t') between exposures was known, and the velocity V_s' ($V_s' = S'/t'$) was calculated. These measurements are illustrated in Figure 8.

An irradiated flagellum often attached to the slide or cover glass at the damaged point, which appears in the same position on the last few images of a photograph.



a. Spermatozoon before lasing.



b. Spermatozoon after lasing. $S' = S'_2 - S'_1$ = distance travelled between exposures.

Figure 8. Values Measured From Photographs

That portion of a flagellum between the head and the irradiated point is referred to as "proximal"; that portion of the tail beyond the point of damage is referred to as "distal." Observations on the effects of irradiation on the proximal and distal regions are described separately.

Although the microbeam experiments were usually performed on spermatozoa of Strongylocentrotus purpuratus, spermatozoa of some other invertebrates were also irradiated and photographed, as mentioned previously and noted in the photographs. The effects were similar on all types of spermatozoa, and conclusions drawn from these experiments apply to all spermatozoa observed.

Behavior of the Proximal Portion of Live Spermatozoa After Laser Microbeam Irradiation

The irradiated region of live spermatozoa almost invariably adhered to the slide or cover glass. Such spermatozoa, in which the proximal portion of the flagellum stopped immediately after irradiation, are shown in Figure 9. Spermatozoa which adhered to the glass and continued to beat proximally are shown in Figure 11. The frequency of the spermatozoon shown in Figure 11a was 26 Hz before irradiation, slightly faster than half the frequency at which the exposures were taken (50 flashes/second). If the proximal beating had continued unchanged after injury, the phase of the waveform in images 3 and 4 would be slightly more advanced than that of images 1 and 2, respectively. However, the reverse is true, indicating that beating slowed down after irradiation.

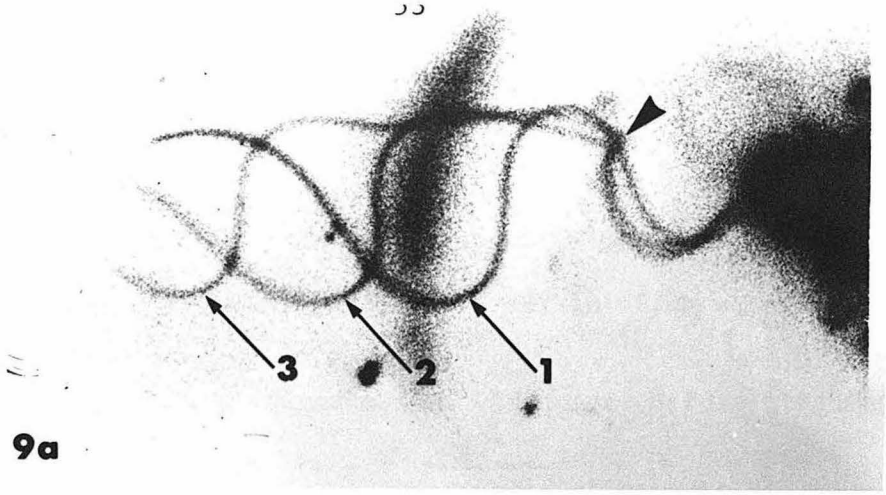
Figure 9. Spermatozoa in which the proximal portion (arrows) did not beat after irradiation. Arrowhead indicates point irradiated. Numbers indicate order in which exposures were taken.

a. S. purpuratus spermatozoon. Frequency before irradiation = 18.3 Hz.

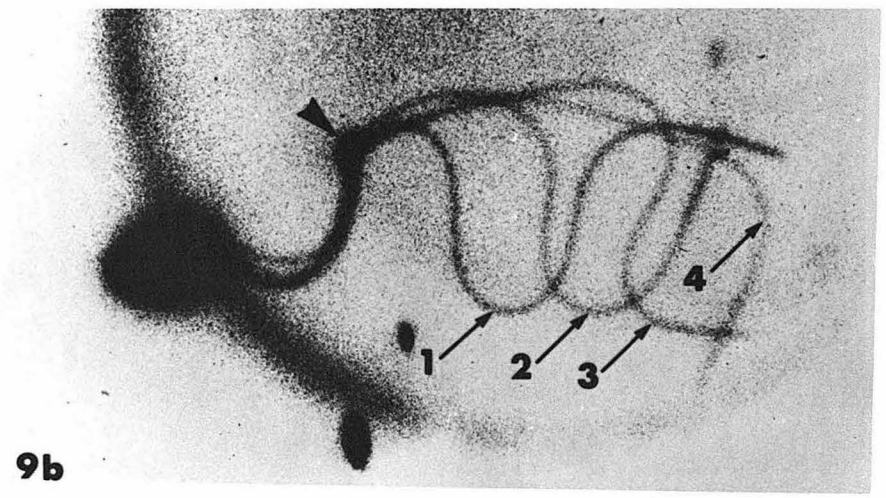
b. S. purpuratus spermatozoon. Frequency before irradiation = 20.0 Hz.

Figure 10. Spermatozoon in which the irradiated point (arrowhead) did not attach to the glass. First two exposures were taken before irradiation. Numbers indicate order in which exposures were taken.

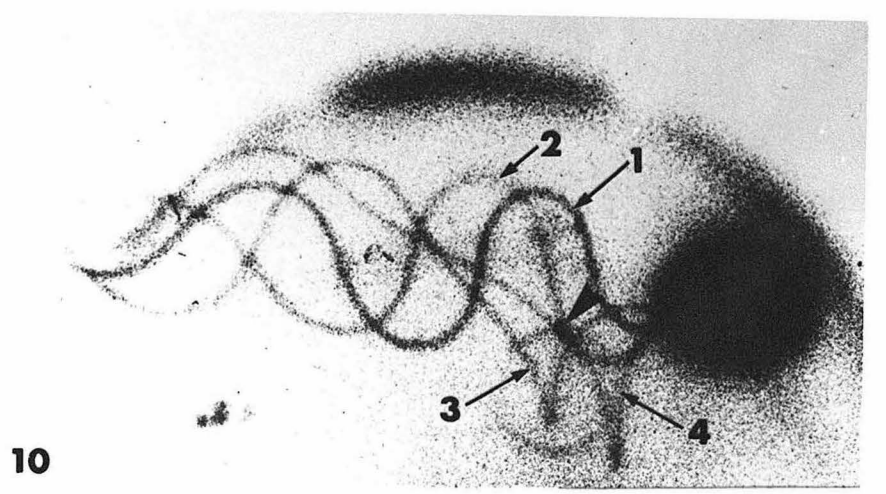
S. purpuratus spermatozoon. Frequency before irradiation = 20.2 Hz.



9a



9b



10

10 μ

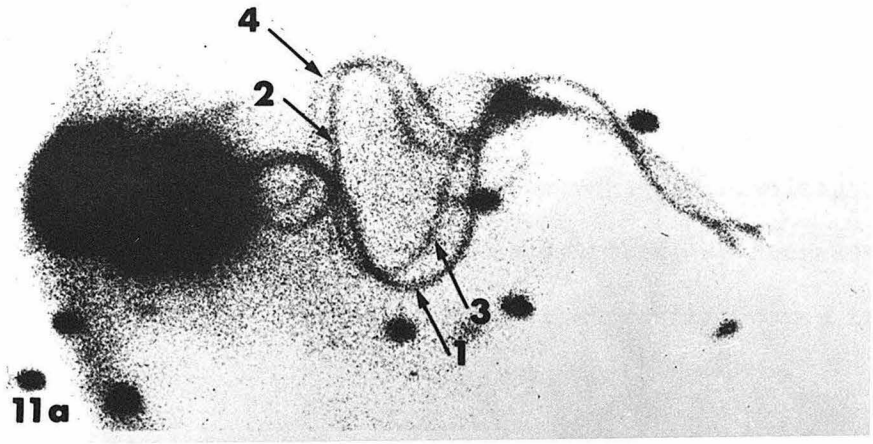
Figure 11. Spermatozoa in which the proximal portion continued to beat after irradiation. Numbers indicate order in which exposures were taken.

a. S. purpuratus spermatozoon. Frequency before irradiation = 26.0 Hz.

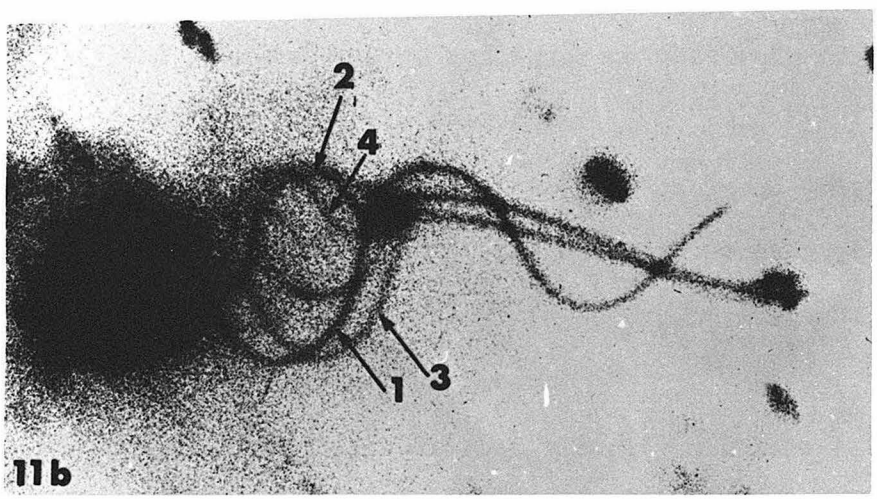
b. S. purpuratus spermatozoon. Frequency before irradiation = 29.3 Hz.

Figure 12. Spermatozoon in which the radius of curvature of a proximal bent region (arrows) is smaller in exposure 3 than exposure 2. Numbers indicate order in which exposures were taken.

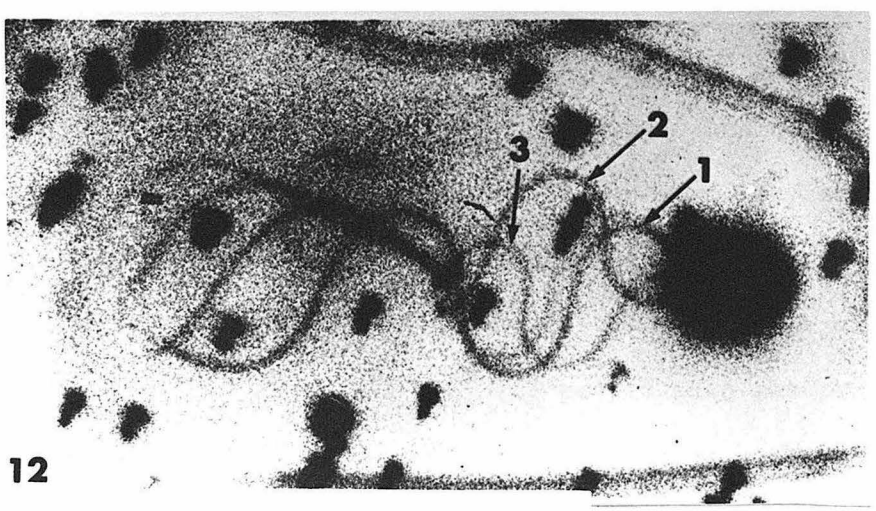
S. purpuratus spermatozoon. Frequency before irradiation = 20.0 Hz.



11a



11b



12

10 μ

In normal steady state beating, the relationship between the frequency of bend initiation and the velocity of bend propagation is well defined, and the two are easily distinguished. However, the wavelength is not constant near the head even in intact spermatozoa, so decreases in these parameters could not be distinguished in the proximal waves in these photographs.

The continuation of beating in a proximal region after irradiation was related to the length of that region. This relationship is shown in Figure 13. The proximal region continued to beat only if it was at least 25-30% of the length of the entire tail.

Figure 10 shows a spermatozoon in which the injured point did not adhere to the glass. Some bending of the proximal portion occured after irradiation, although no beating was occurring when the spermatozoon was observed a second or two later.

Spermatozoa were generally observed visually within a second or two after injury. Beating had often stopped in the proximal region, and when it did continue its frequency was usually much lower than before irradiation. The amplitude sometimes appeared normal, but in other cases it was greatly reduced. The beating often slowed gradually to a stop within a few seconds, although the proximal regions of some spermatozoa beat spasmodically for several seconds before stopping.

When the injured point was attached to the glass, the radius of curvature of a bend decreased as the wave passed down the proximal region and the bend was forced into a smaller radius between the attached point and a new bend coming along behind. This can be seen in exposures 2 and 3 of Figure 12.

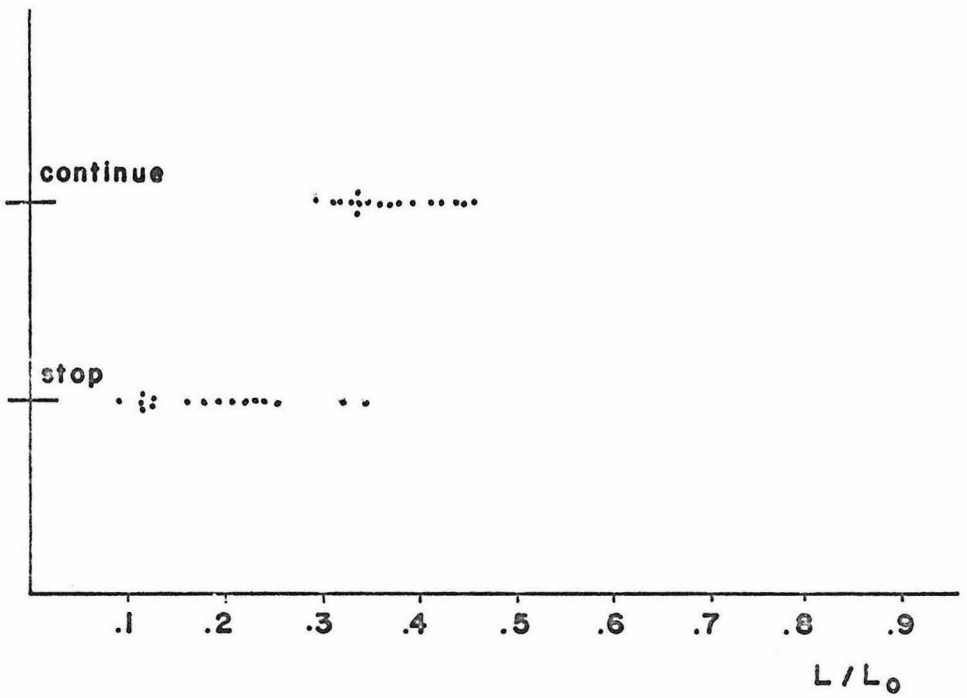


Figure 13. Continuation of beating vs. length. L = length of proximal region; Lo = length of entire tail.

Behavior of the Proximal Portion of Glycerinated Spermatozoa After Laser Microbeam Irradiation

Glycerinated spermatozoa beat rather slowly and asymmetrically under the conditions necessary for irradiation. Stroboscopic determination of the beat frequency of such spermatozoa is very difficult. The "Strobotac" could not be synchronized with these spermatozoa, and exact measurements of their beat parameters were not made. The proximal regions of irradiated glycerinated spermatozoa often appeared to beat quite normally after damage, without obvious changes in amplitude, frequency or wavelength. In contrast to irradiated live spermatozoa, the glycerinated spermatozoa often continued beating proximally for several minutes.

Behavior of the Distal Portion of Live Spermatozoa After Laser Microbeam Irradiation

Bent regions distal to the injured point at the time of irradiation usually continued propagating to the tip of the tail, but the radius of curvature, total angle and velocity of the bent regions were all affected by the damage. No new bends ever developed in the distal region after injury. Propagating distal bent regions can be seen in most of the photographs, and are particularly clear in Figure 9. The effects of radiation on the above parameters are described separately, and the relationships between these effects are discussed.

Radius of Curvature of Distal Bent Regions

The radius of curvature of distal bent regions never decreased, and in approximately one out of four of the injured spermatozoa it

remained constant or nearly constant (within 10% of its original length) after irradiation, as shown in Figure 14. In approximately one out of ten of the injured spermatozoa, the radius quickly increased to a new value at which it remained,as shown in Figure 15. It increased by approximately 25% in Figure 15a and approximately 50% in Figure 15b; these values are typical. In the remaining cases, the radius usually continued increasing as it proceeded along the tail, as shown in Figure 16. The radius was clearly not constant within a given bent region, but increased distally. However, within a bent region, the radius of curvature at a given point on the flagellum remained constant in two successive exposures, even though it was in a more proximal part of that region on the later exposure; that is, the radius of curvature at a point on the flagellum remained constant while that point was within a curved region. The only exceptions to this were relatively rare spermatozoa in which distal waves appeared to have stopped propagating and begun straightening, as shown in Figure 17.

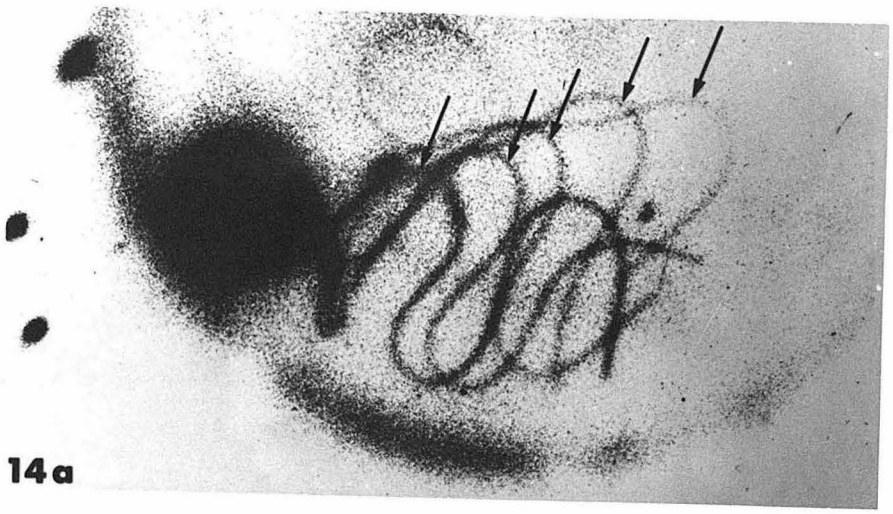
When the radius of a bent region remained constant, it did so even as that region approached the tip of the tail and shortened, as shown in Figure 18. This is also true of intact spermatozoa (14), as shown in Figure 19.

There were often two distal bent regions. When the radius of the more proximal bent region increased, that of the more distal one sometimes increased, as shown in Figures 16a and 21a. It was also common, however, for the radius of the more proximal bent region to increase while that of the more distal one remained constant, as shown in Figure 20.

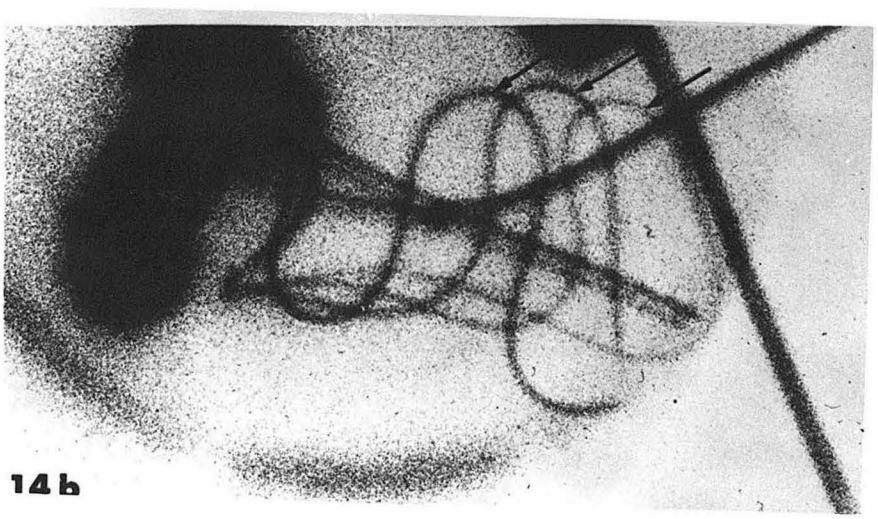
Figure 14. Spermatozoa in which the radius of curvature of a distal bent region (arrows) has remained constant.

a. S. purpuratus spermatozoon. Frequency before irradiation = 17.5 Hz.

b. P. brevispinus spermatozoon. Frequency before irradiation = 19.2 Hz.



14a



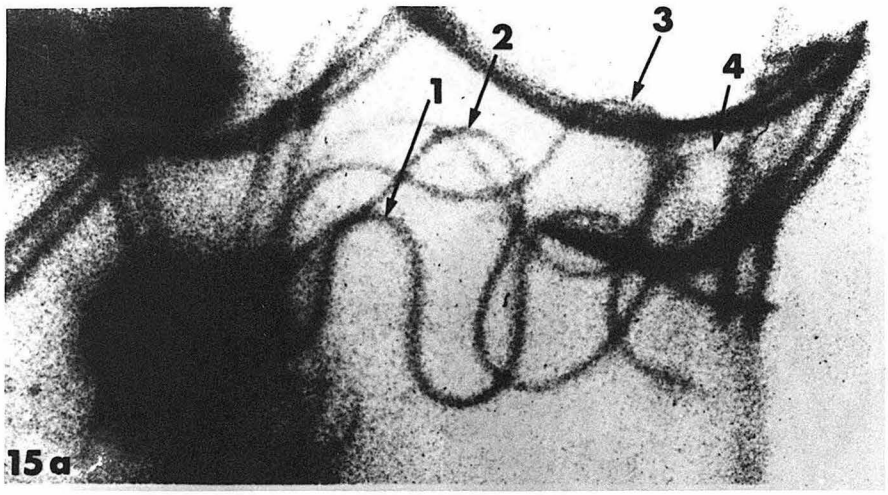
14b

10 μ

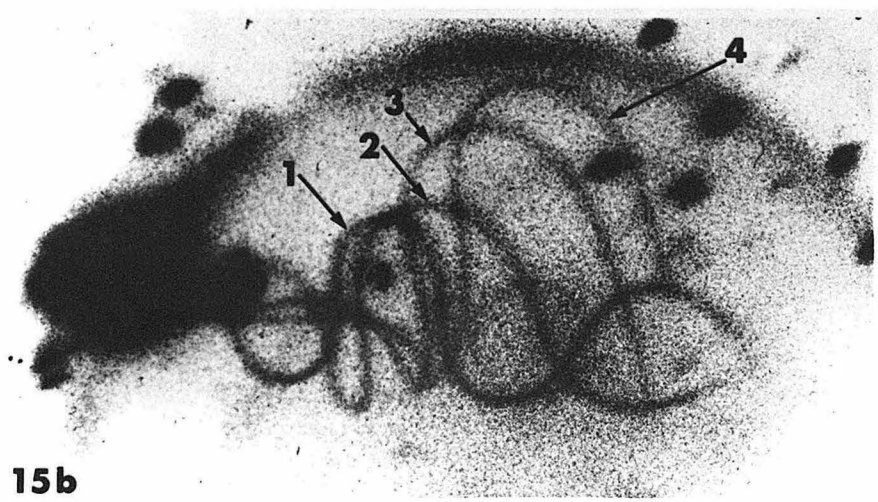
Figure 15. Spermatozoa in which the radius of curvature of a distal bent region (arrows) quickly increased to a new constant value. Numbers indicate order in which exposures were taken.

a. S. purpuratus spermatozoon. Frequency before irradiation = 28.2 Hz.

b. S. purpuratus spermatozoon. Frequency before irradiation = 25.2 Hz.



15a



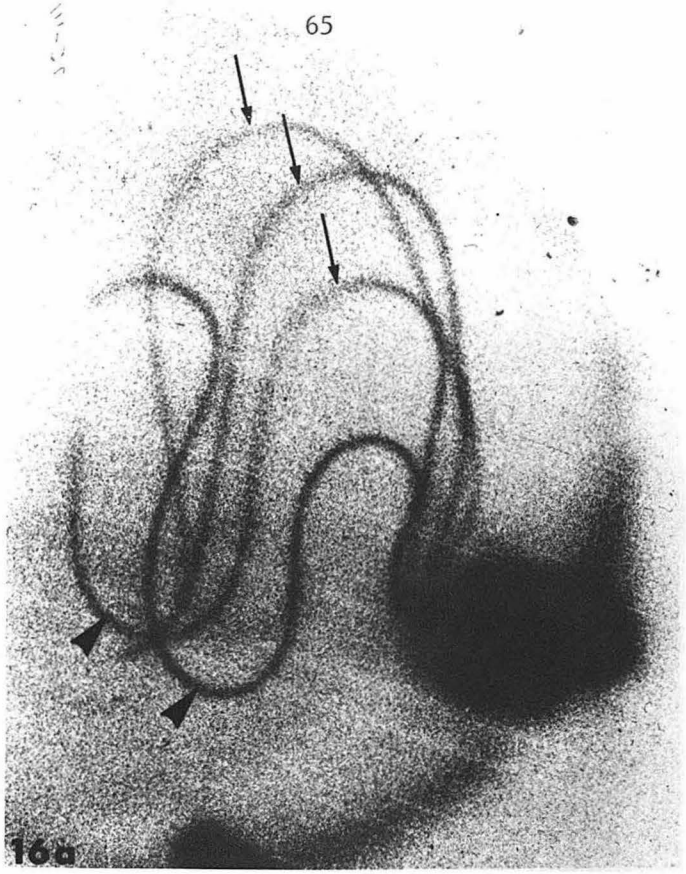
15b

10 μ

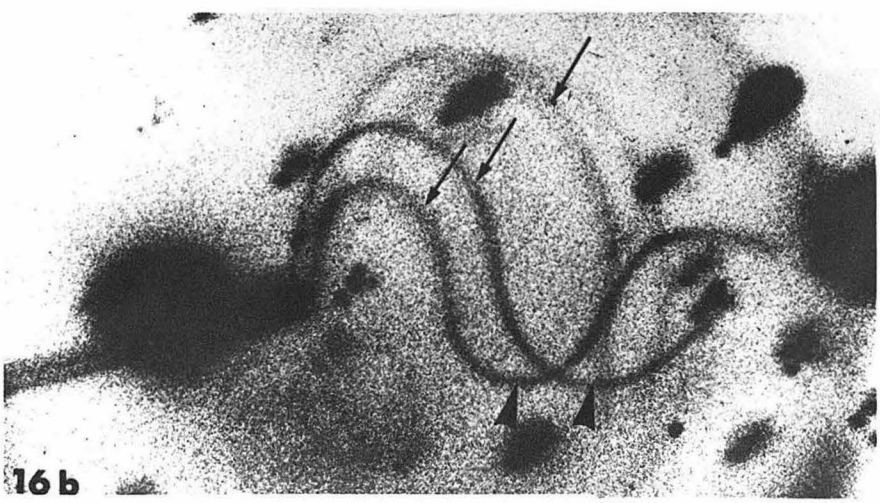
Figure 16. Spermatozoa in which the radius of curvature of both the more proximal (arrows) and the more distal (arrowheads) of two distal bent regions have increased. The flagella were irradiated within a few μ of their basal end.

a. P. brevispinus spermatozoon. Frequency before irradiation = 16.7 Hz

b. S. purpuratus spermatozoon. Frequency before irradiation = 17.2 Hz



16a



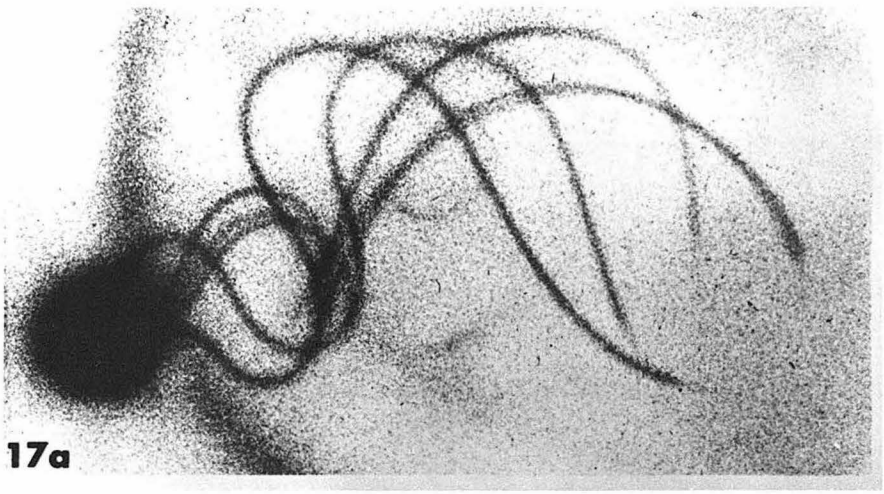
16b

10 μ

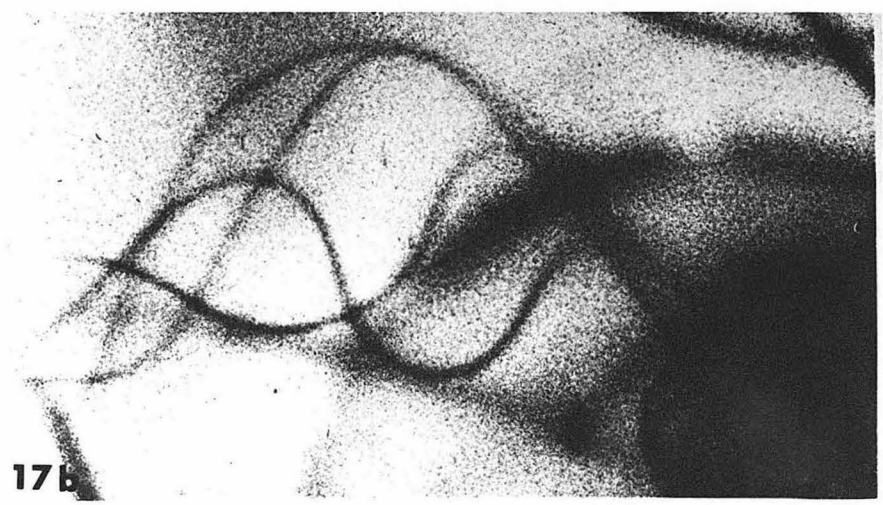
Figure 17. Spermatozoa in which a distal bent region appears to have stopped propagating and started to straighten.

a. P. brevispinus spermatozoon. Frequency before irradiation = 16.7 Hz

b. M. crenulata spermatozoon. Frequency before irradiation = 20.3 Hz



17a



17b

10 μ

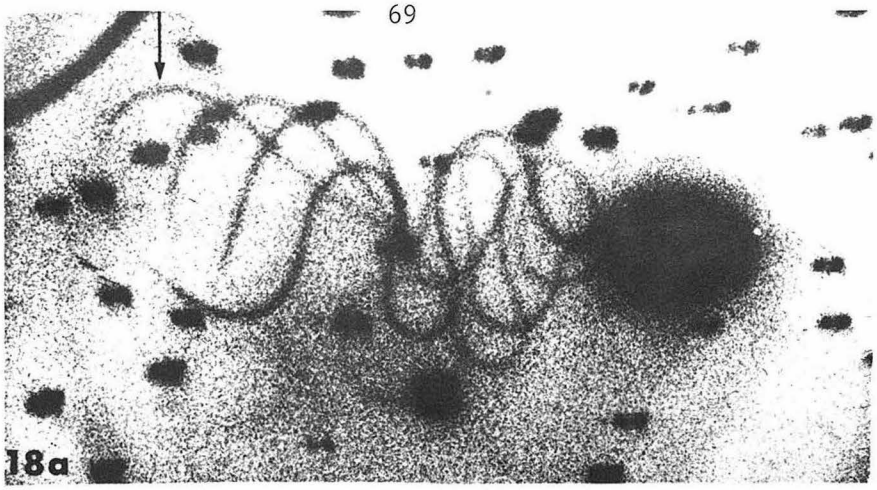
Figure 18. Spermatozoa in which distal bent regions (arrows) can be seen approaching the tip of the flagellum.

a. S. purpuratus spermatozoon. Frequency before irradiation = 21.8 Hz

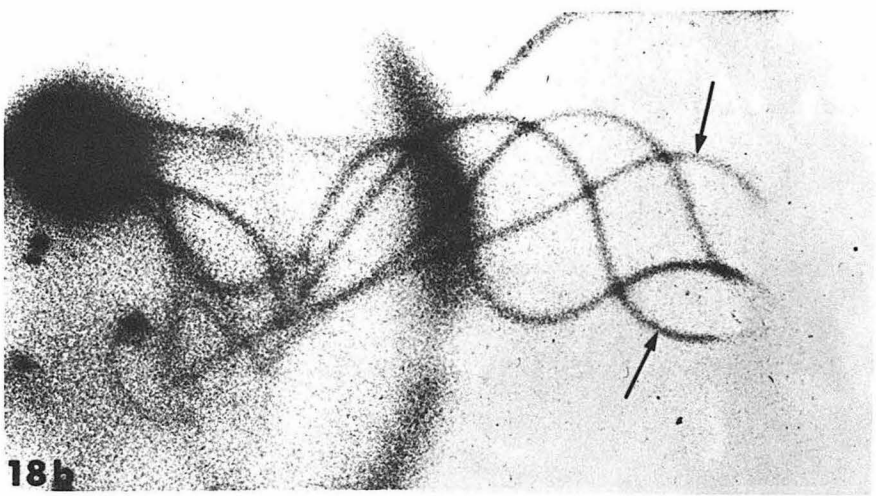
b. S. purpuratus spermatozoon. Frequency before irradiation = 20.5 Hz

Figure 19. Intact spermatozoon in which bent regions (arrows) can be seen approaching the tip of the flagellum.

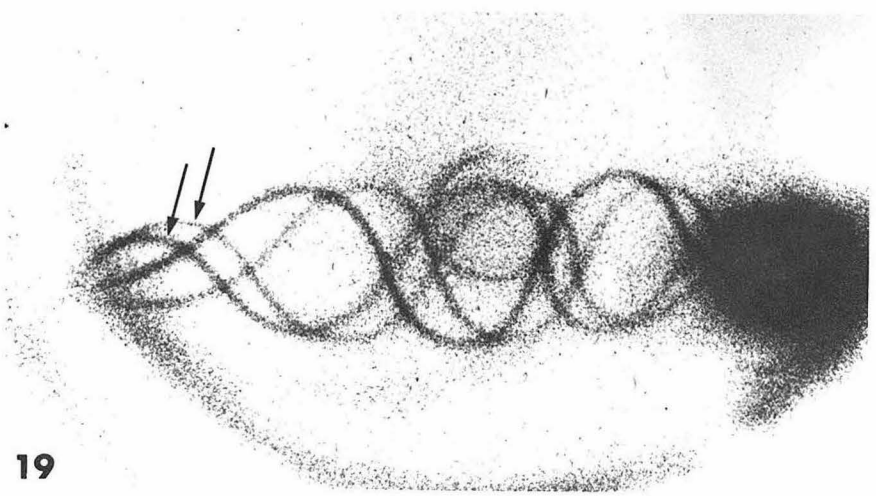
S. purpuratus spermatozoon. Frequency before irradiation = 22.7 Hz



18a



18b



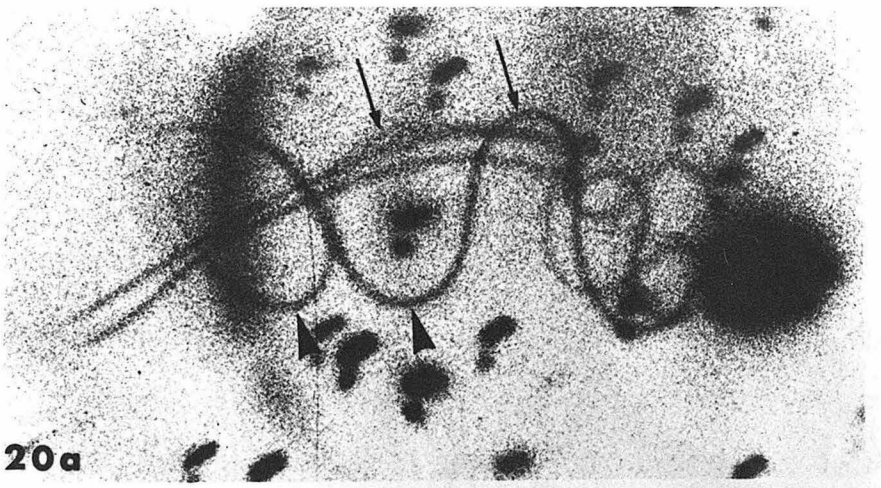
19

10 μ

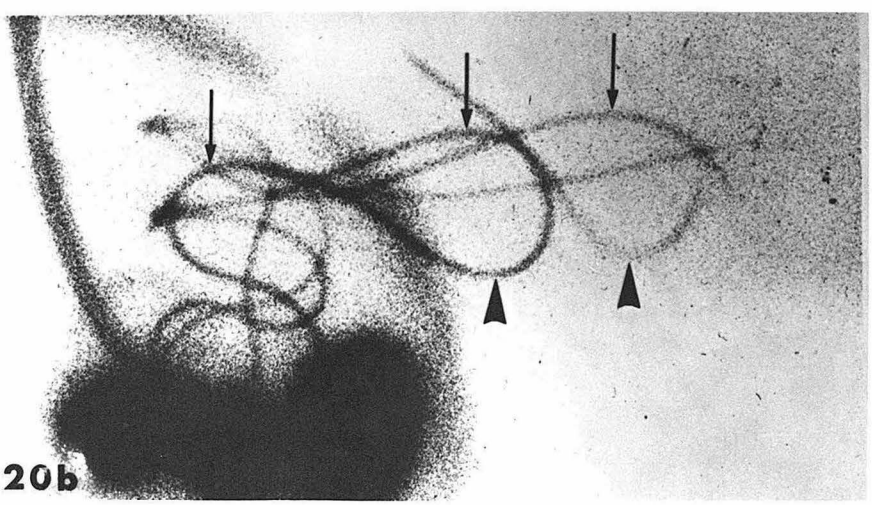
Figure 20. Spermatozoa in which the radius of the more proximal of two distal bent regions (arrows) increased, while that of the more distal bent regions remained constant.

a. S. purpuratus spermatozoon. Frequency before irradiation = 25.7 Hz

b. P. brevispinus spermatozoon. Frequency before irradiation = 18.5 Hz



20a



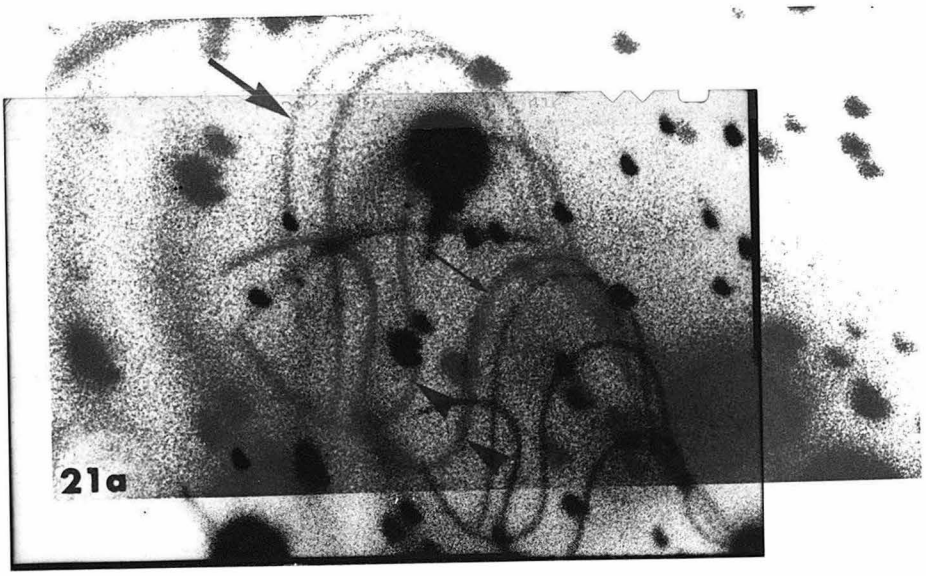
20b

10 μ

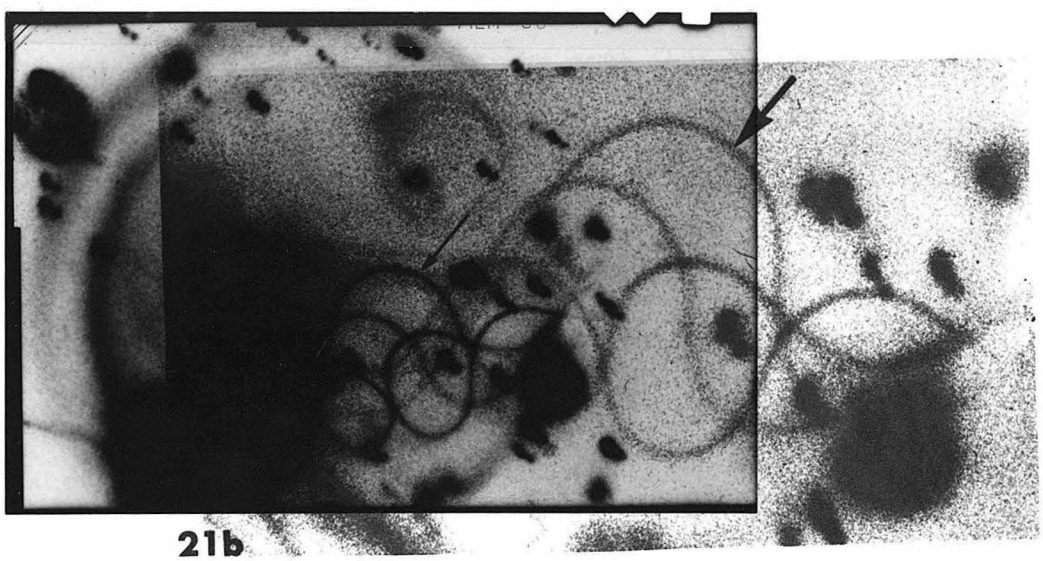
Figure 21. Spermatozoa in which the shape of a distal bent region was preserved as it expanded. The photograph of the spermatozoa in the overlying transparencies has been reduced so that exposure 4 of the bent region in the transparency coincides with exposure 1 of that region in the print.

a. Transparency reduced to 73% of print size. The distance travelled by this region is significantly less between exposures 3 and 4 than between exposures 1 and 2. The radius of the more distal of the two distal bent regions (arrowhead) has also increased. S. purpuratus spermatozoon. Frequency before irradiation = 21.3 Hz.

b. Transparency reduced to 53% of print size. S. purpuratus spermatozoon. Frequency before irradiation = 20.2 Hz.



21a



21b

10 μ

This was not due to a signal having reached the more distal region later than the more proximal one, since the small radius persisted for 2 or 3 exposures after injury.

Length of Distal Bent Regions

The length of a bent region never decreased, and never increased when the radius of curvature remained constant; however, it commonly increased with increasing radius of curvature. Although exact length measurement of such regions is difficult, the shape of the bent region was retained in some spermatozoa as the radius increased, so that a photograph can be reduced until the reduced image of a later exposure coincides with the full-sized image of an earlier exposure. In Figure 21a, the overlying transparency has been reduced to 73% of the print size; in Figure 21b, it has been reduced to 53% of the print size. Length often increased by 25% or more, but no definite relationship was found between increases in length and radius of curvature.

Angle of Distal Bent Regions

Bent regions are bordered by straight regions, and the angle between these straight regions (α) was measured, as shown in Figure 22. The angle remained constant, of course, in spermatozoa such as those shown in Figure 21, in which the shapes of bends were retained; but in spermatozoa such as those shown in Figure 22, this angle increased after injury, although the length of the bent region remained rather constant. Figure 22c shows a spermatozoon irradiated just behind the head, in which a bent region appears to have straightened completely.

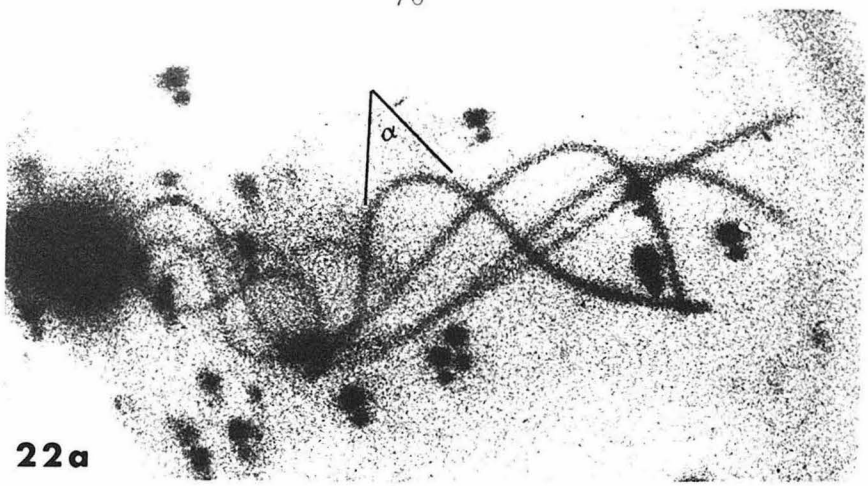
Figure 22. Spermatozoa in which the total angle (α) of a distal bent region has increased as the region propagated.

a. S. purpuratus spermatozoon. Frequency before irradiation = 24.8 Hz

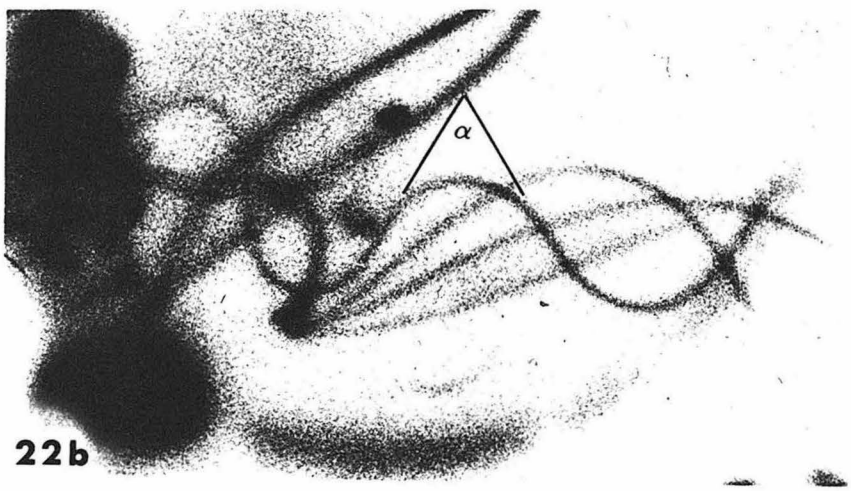
b. S. purpuratus spermatozoon. Frequency before irradiation = 24.0 Hz

c. A bent region (arrows) appears to have straightened completely with no propagation. C. intestinalis spermatozoon. Frequency before irradiation approximately 12 Hz.

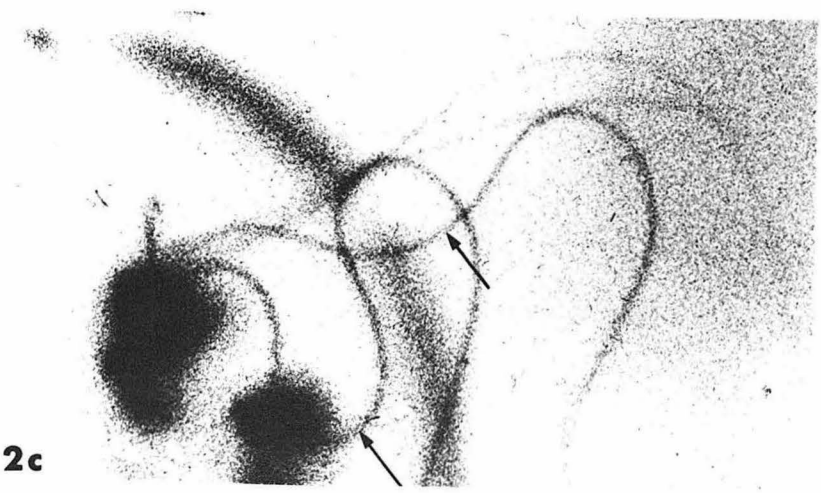
Exposures taken at 10/second.



22a



22b



22c

10 μ

Velocity of Distal Bent Regions

When a flagellum was irradiated, the velocity of distal bent regions almost invariably decreased. In approximately one out of ten of the injured spermatozoa, this velocity quickly decreased to a new value, which varied from less than 30% of normal to over 90%, but no definite relationship was found between velocity and position irradiated. Because the normal velocity of bend propagation is constant, the distinction could not usually be made between the velocity having decreased to a constant velocity, which depended only on conditions at the irradiated point, or having decreased to a constant fraction of the velocity at each point along the flagellum. This distinction can be made, however, in the spermatozoon shown in Figure 23, where the wave velocity increased towards the tip before irradiation. Exposures 1 and 2 were taken before irradiation; exposures 3 and 4 were taken after. The normal positions of bent regions as a function of time, as measured in exposures 1 and 2, are shown by the solid line in Figure 25. If the velocity (V_s') after irradiation was a constant fraction (k) of the normal velocity (V_s) at each point, the time (t') needed to propagate a given distance (S) would have been

$$t' = \int_0^{S'} \frac{dS'}{V_s'} = \frac{1}{k} \int_0^{S'} \frac{dS'}{V_s} = t/k, \text{ where } t = \text{the time}$$

needed to propagate the distance S before irradiation, as indicated by the dashed line. The position of the distal region in exposure 4 is consistent with the velocity having decreased to a constant fraction of the normal velocity at each point along the flagellum.

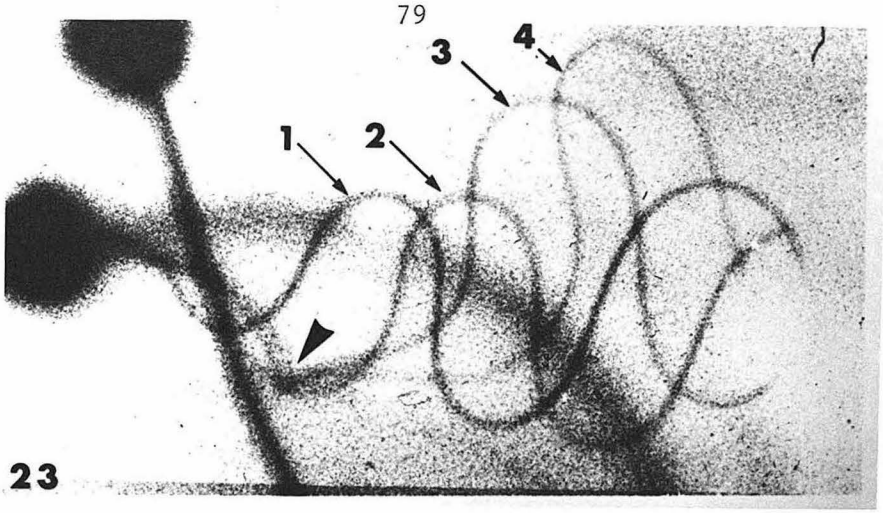
Figure 23. Spermatozoon in which the velocity of bent regions increased distally before irradiation. Numbers indicate order in which exposures were taken. Exposures 1 and 2 were taken before irradiation. Arrowhead indicates point irradiated. Arrows indicate distal bent region followed.

P. brevispinus. Frequency before irradiation = 20.3 Hz

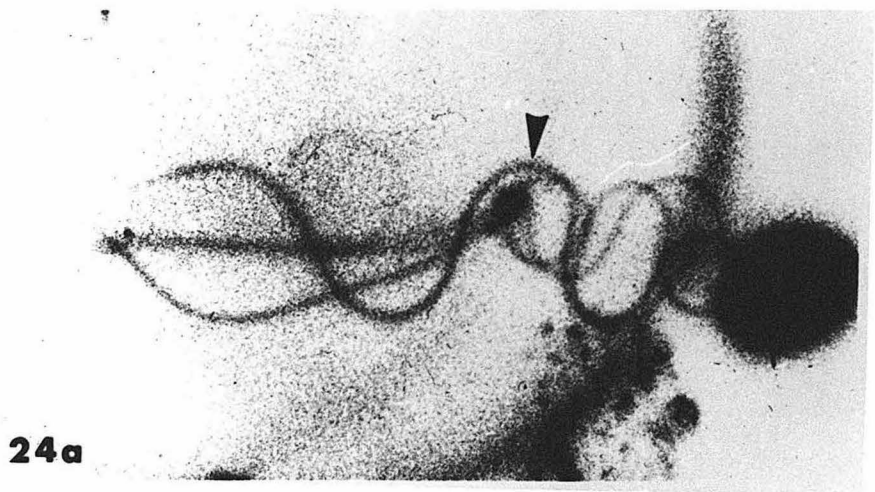
Figure 24. Spermatozoa irradiated near the center of a bent region. Arrowhead indicates point irradiated.

a. S. purpuratus spermatozoon. Frequency before irradiation = 23.2 Hz

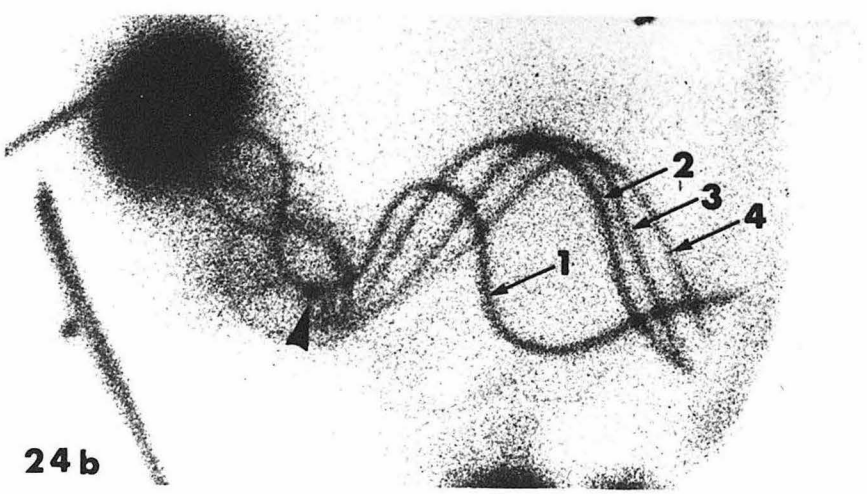
b. A bubble appears to have formed at the point irradiated. Numbers indicate order in which exposures were taken. The distal bent region (arrows) has travelled significantly less between exposures 3 and 4 than between exposures 1 and 2. S. purpuratus spermatozoon. Frequency before irradiation = 26.8 Hz.



23



24a



24b

10 μ

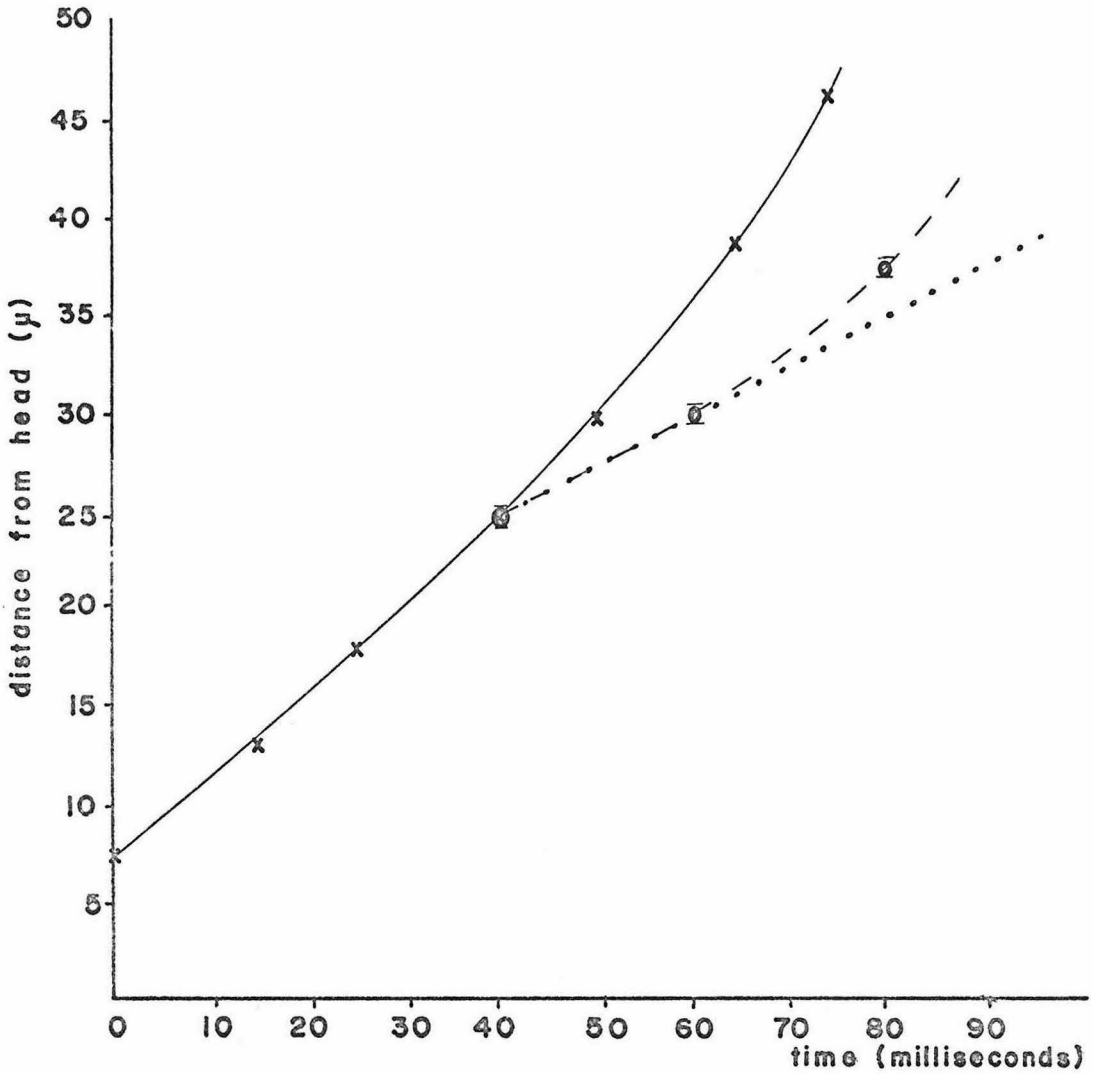


Figure 25. Propagation of bends in spermatozoon shown in Figure 23. Solid line = position of bent regions before irradiation; dashed line = position of a bent region after irradiation; dotted line = linear extrapolation of position of that bent region in exposures 2 and 3.

A second class of spermatozoa was observed, in which the velocity of distal bends did not assume a constant value, but continually decreased as the wave proceeded along the tail, as shown in Figure 21a and 24b. In Figure 21a the average velocity has decreased from 42% of normal between exposures 1 and 2 to 17% between exposures 3 and 4; in Figure 24b it has decreased from 41% of normal between exposures 1 and 2 to 17% between exposures 3 and 4.

Relationships of Effects

The effects of irradiation have been described for radii of curvature and velocities of distal bent regions, and changes in total length and angle subtended from those bent regions whose radii of curvature increased have been described. Those regions which exhibited the greatest increase in radius usually showed the greatest decrease in velocity. Beyond this, no well-defined relationships were observed, and changes in velocity were not necessarily related in any flagellum. The angle and length of a region are, of course, directly related for a given radius. They remained constant with constant radius, and neither ever decreased as the radius increased. Beyond this, no definite relationship was observed between them and either the radius or velocity.

A bent region has been considered to be immediately distal to the irradiated point if the point was anywhere from the proximal end of that region to the distal end of the following bent region. No relationship was observed between the locus of damage within those limits and the effects produced.

Irradiation of Bent Regions

Irradiating the proximal end of a bent region did not prevent propagation of that region. The propagation of such regions is shown in Figure 27. When a flagellum was irradiated near the center of a bent region, that region is absent in subsequent exposures, as shown in Figure 24; shortened bent regions were never maintained. Figure 26 shows a spermatozoon which appears to have been incompletely damaged in a bent region. The bend appears to have passed through the irradiated point, and to have regained its original length.

Irradiation of Basal Regions

Figures 14a, 16, 17b and 21 show spermatozoa which were irradiated within a few μ of their heads. The effects appear identical to those in spermatozoa irradiated more distally. Figures 15a and 28 show similarly irradiated flagella which exhibited a second effect: the region behind the head curved. This could be due to the force of waves passing back along the tail, if the tail became unusually flexible in that region. These flagella were straight when observed a second or two later. No new bent regions ever developed after injury to the basal end of the flagellum.

Distal Portion of Glycerinated Spermatozoa

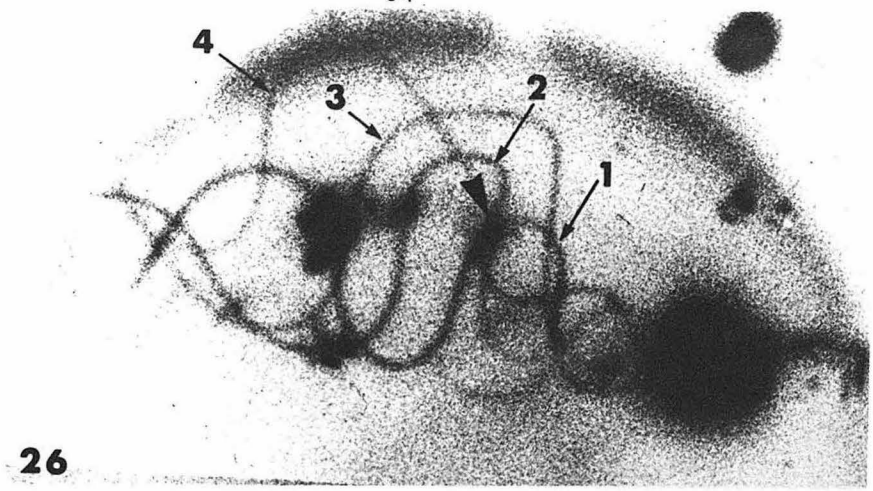
Exact determination of the beat parameters of glycerinated spermatozoa before irradiation were not made, for reasons discussed earlier in this chapter. Glycerinated spermatozoa were not as bright as live spermatozoa under dark-field illumination, and were very difficult to photograph. A glycerinated spermatozoon which was irradiated near the head

Figure 26. Spermatozoon which appears to have been incompletely damaged by irradiation in a bent region (arrows). Arrowhead indicates point irradiated. Numbers indicate order in which exposures were taken. S. purpuratus spermatozoon. Frequency before irradiation = 25.0 Hz.

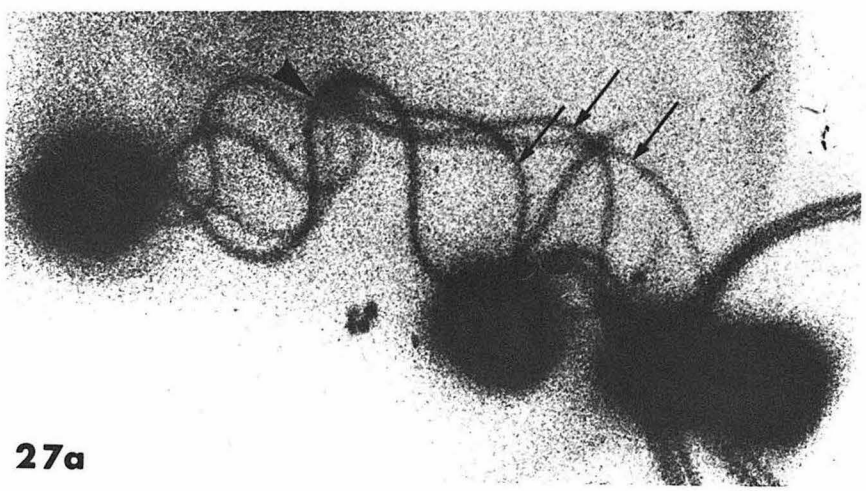
Figure 27. Spermatozoa irradiated at the proximal end of a bent region (arrows). Arrowhead indicates point irradiated.

a. S. purpuratus spermatozoon. Frequency before irradiation = 20.2 Hz

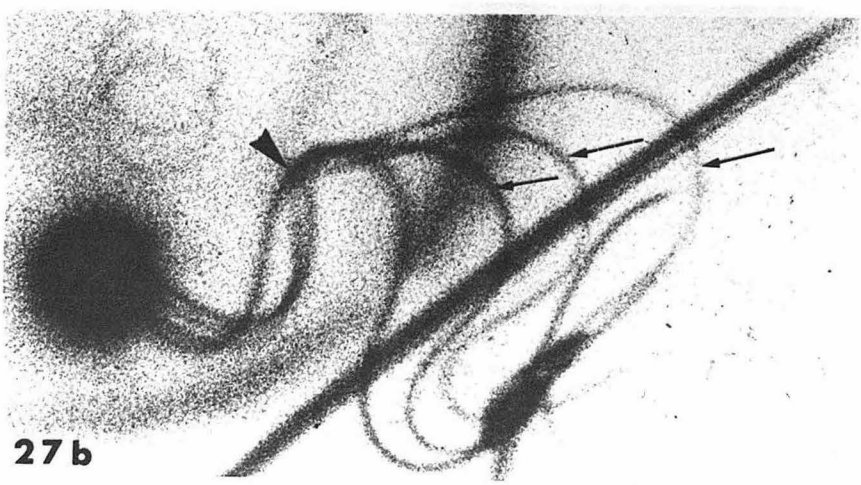
b. P. brevispinus spermatozoon. Frequency before irradiation approximately 15 Hz



26



27a



27b

10 μ

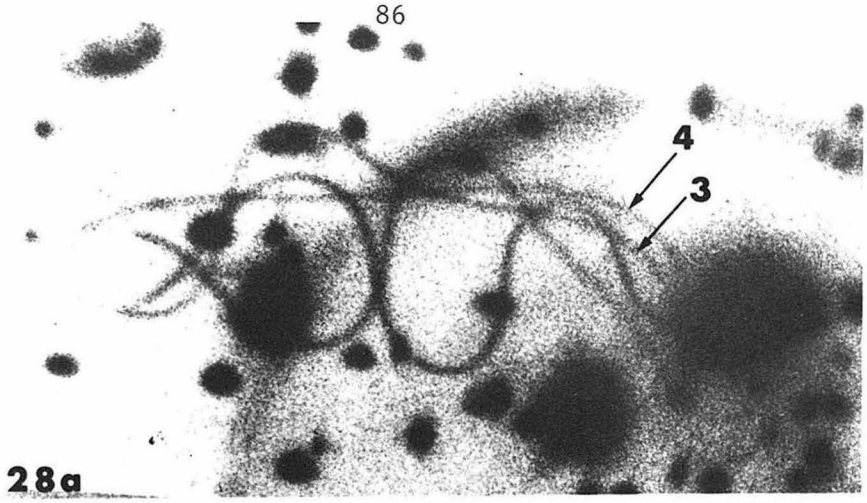
Figure 28. Spermatozoa irradiated within a few μ of the basal end of their flagellum, showing a curvature (arrows) behind their head. Numbers indicate order in which exposures were taken.

a. S. purpuratus spermatozoon. Frequency before irradiation = 24.8 Hz

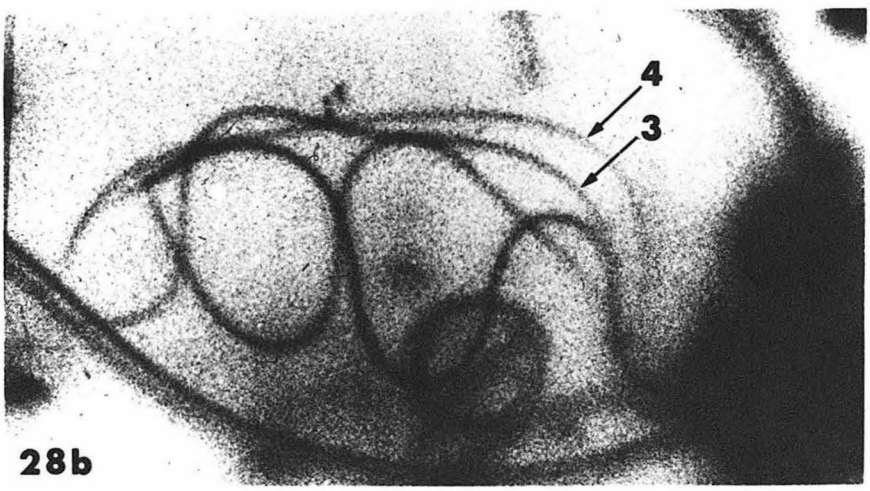
b. S. purpuratus spermatozoon. Frequency before irradiation = 26.0 Hz

Figure 29. Glycerinated spermatozoon, irradiated near the head.

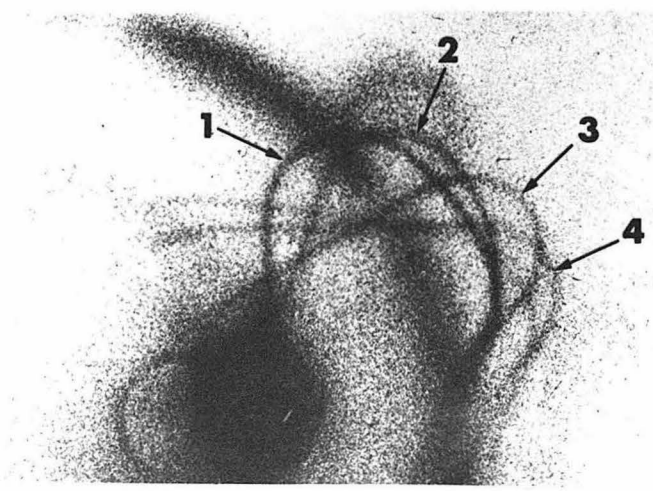
S. purpuratus spermatozoon. Frequency before irradiation approximately 10 Hz. Exposures taken at 25/second.



28a



28b



29

10 μ

is shown in Figure 29. The distal bend propagated to the tip of the tail. No bends ever developed distal to the point of irradiation, even when the proximal section continued to beat actively.

CHAPTER 5

Discussion

Role of the Basal End of the Flagellum in Initiating Bends and Determining Wave Parameters

Experiments with ATP Gradients

Glycerinated spermatozoa receive energy from the ATP in the external medium, which presumably enters all along the flagellum. Even in intact spermatozoa, the tails have been reported to be permeable to some ions (88). When external ATP gradients were established along a flagellum, bending waves were only initiated at the basal end, even when the ATP source was placed near other regions. These regions did not beat when the ATP supply was localized to them. ATP gradients established by the micropipettes caused no obvious variations in wave parameters along the flagellum, even though these parameters could be changed by changing the ATP concentration at the basal end of the flagellum. The determination of wave parameters by ATP concentration appears to be localized to the basal region.

Laser Microbeam Experiments

When a point on a flagellum was irradiated, bent regions distal to the point of damage continued to the tip of the flagellum, but new bent regions never formed distal to the irradiated point, even though the proximal region often continued beating.

Although the mechanism of energy transfer from the mitochondria to points all along the flagellum is not known, it could possibly be diffusion of an energy-rich compound such as ATP (17). However, the cessation of beating does not seem to have been simply due to interruption

of an ATP gradient along the tail, since, for a gradient to produce enough ATP to reliably supply energy for every beat, there must be an excess supply of ATP, so that at least a few beats should have occurred distally after irradiation. This was never seen to occur. In addition, glycerinated spermatozoa, in which ATP presumably entered all along the flagellum from the external medium, never initiated distal bends after irradiation.

This inability of distal regions to beat independently indicates that the basal region of a flagellum is specialized for the initiation of bends. This agrees well with the observation that mechanically damaged glycerinated spermatozoa often beat proximal to the damaged point, while the distal region is inactive, but independently beating proximal and distal portions of glycerinated spermatozoa are never observed. In fact, breakage of tails by mechanical shearing has been used to inhibit the motility of distal tail fractions (19).

Distal regions failed to initiate bends even when a tail was irradiated within 1 or 2 μ of its basal end, indicating that initiation is localized very close to the base of the flagellum. Cilia and flagella have been removed from cells by several investigators (11) and can be made to beat on the addition of ATP to the medium, even though they have been separated from their basal bodies. The region of the flagellum within a few μ of the basal end therefore appears to be the site of bend initiation.

Formation and Propagation of Bends

Current Models

The mechanical models for flagellar bending which are currently popular fall into two classes. According to the more prevalent of these, bending is caused by contractile elements arranged serially along the flagellum. In the second of these, bending is achieved by the active sliding of incompressible and inextensible filaments, which extend the length of the flagellum. In both models, the outer axonemal fibrils are generally identified as the active elements.

Local Contraction Model

Several investigators (45,59,85) have suggested that bending is achieved by the contraction of elements on one side of a bent region. A simple version of this model is shown in Figure 30. Some longitudinal component of the flagellum must be incompressible enough for bending to result rather than contraction. Connections between the elements on the two sides keep bending localized to the region of the contracting elements. Development of circular bent regions consists of a transition of the elements on the concave side of that region from a relaxed to a contracted state, perhaps accompanied by a passive extension of the elements on the convex side. Contraction of one element may trigger the contraction of the next element. This model could work equally well, of course, with elements which actively extend rather than contract.

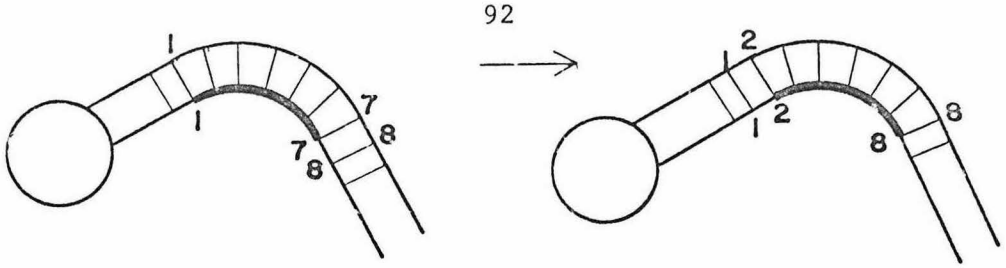
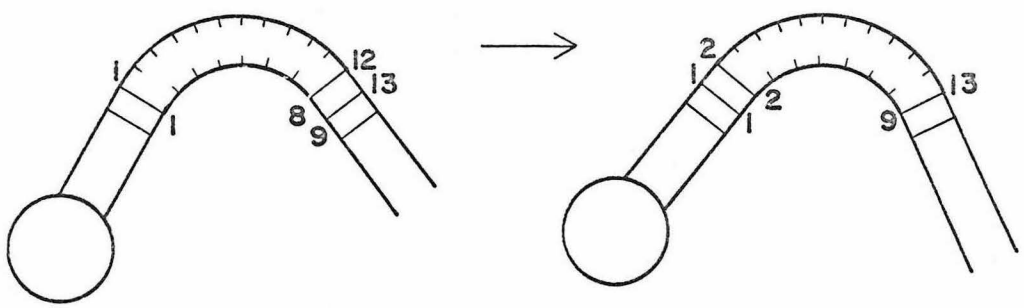
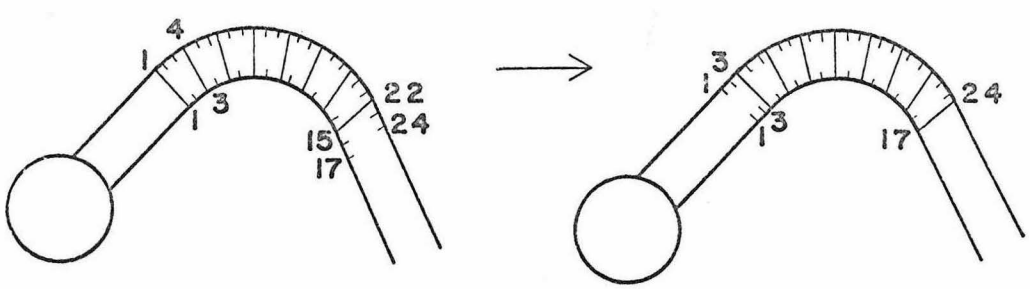


Figure 30. Local contraction model.



a. Links in straight regions.



b. Links in bent regions

Figure 31. Sliding filament model

Sliding Filament Model

Bending may also be produced if fibrils are caused to slide with respect to one another, without contracting (80). Two simple versions of this model are shown in Figure 31. Bending is kept localized to a small region by cross-links, as in the local contraction model, and the filaments are assumed to be flexible, but incompressible and inextensible. The cross-links can be in the straight regions, as shown in Figure 31a, or in the bent regions, as shown in Figure 31b, or both. A bent region is propagated in the former model by making connections at its proximal end and breaking them at its distal end; in the latter model the region is propagated by making connections at its distal end and breaking them at its proximal end, and connections in the bent region continually shift as the region propagates.

The sliding filament model imposes more severe constraints on bending than the local contraction model, and should react differently to microbeam irradiation.

Experimental Implication on Models of Beating

In the microbeam irradiation experiments, the proximal region stopped beating when the flagellum attached to the glass at the irradiated point if that region was less than 25 to 30 % of the length of the entire tail. The length of a normal bent region of a sea urchin spermatozoon is approximately 25% of the length of the entire tail (14). The unattached spermatozoon shown in Figure 10 has continued to bend after irradiation, and in glycerinated spermatozoa, which showed less

tendency to adhere to the glass than live spermatozoa, proximal beating often continued for several minutes. In mechanically broken spermatozoa, very small pieces of tail remaining attached to the heads are regularly observed beating. The inability of short proximal sections to beat may therefore be due to mechanical constraint caused by their attachment to the glass. These results agree well with the observations of Kaneda (54) that frog spermatozoa usually stopped beating proximally when a point on the tail was pressed to the slide so that less than 20% of the flagellum was proximal to the constraint. This behavior would be expected of a sliding filament model, if the filaments were prevented from sliding past one another at the constrained point.

Irradiation affected the radius of curvature, length, total angle and velocity of distal bent regions. Bending and unbending at a point are therefore affected by activities in other regions of the flagellum, indicating that bend propagation is not a completely localized phenomenon. The outer axonemal fibrils do not appear to have slid past one another at their basal ends in electron micrographs, so that the development of new bends in a sliding filament model necessitates movement of the active filaments along the entire flagellum. Interruption of this movement in the distal portion by irradiation might easily result in immediate changes in the beating in that region.

On the other hand, some of the changes in the parameters of distal bent regions are difficult to explain in terms of a sliding filament model. Figure 32 illustrates the parameters of a bent region. An increment of length of the inner filament is $dS_i(\theta) = r(\theta)d\theta$. Similarly,

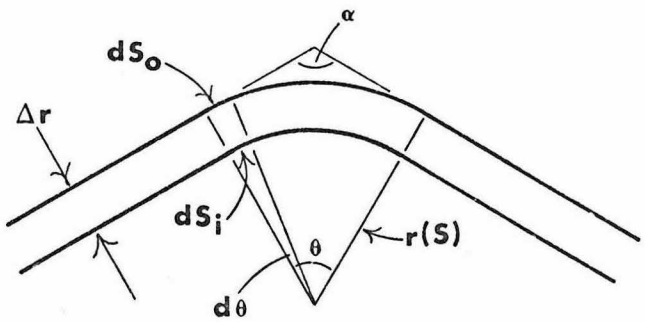


Figure 32. Parameters of a bent region.

$dS_o = (r(\theta) + \Delta r)d\theta$. The incremental difference in the length of the inner and outer filaments is $dS(\theta) = dS_o - dS_i = \Delta r d\theta$. The total difference in the length of the filaments within the bent regions is $S = \int_\theta ds(\theta) = \int_\theta \Delta r d\theta = \theta \Delta r = (180 - \alpha) \Delta r$. So $\alpha = 180 - S/\Delta r$, independent of the shape of that region or the mechanism of bending; the total angle (α) of a bent region is determined only by the difference of length of the inner and outer fibers in that region. The total angle of distal bent regions often increased in irradiated spermatozoa, without preventing them from propagating. An increase in α only requires elements to contract less than normally in a contractile model, but requires filaments to slide past one another in a sliding filament model. A sliding filament model has the difficult task of maintaining and propagating a bent region by cross-links between the filaments while those filaments are sliding and breaking the cross-links within or on at least one end of that region. A similar difficulty has been mentioned by Gibbons and Grimstone (39), who point out that flagellar bending would require continual sliding all along the flagellum due to the formation of new bent regions at the basal end.

Laser irradiation never produced "partially damaged" bent regions; damage within a bent region caused that region to quickly straighten. Two types of partial damage might have been expected: 1) irradiation within a bent region might have led to propagation of a shortened bent region; 2) bending might have continued to propagate, without being followed by unbending, causing the flagellum to curl up. Either of these results might be expected from a local contraction model for bend propa-

gation in which bending and unbending are relatively independent events. However, bent regions consistently appear to be maintained as units.

Summary of Conclusions

Bending waves appear to be initiated only in the basal region of a flagellum, and the wave parameters are determined primarily by conditions in this region.

The type of change occurring in the proximal portion and the rapidity of change occurring along the distal portion upon irradiation suggest constraints imposed by incompressible and inextensible sliding filaments, while the types of changes occurring within distal bent regions suggest that the actual forces accomplishing bending involve some form of contraction.

Bending at any point is affected by activities at other points, but propagation of bent regions does not require that the entire flagellum remain intact. However, it does require that the entire bent region remain intact.

APPENDIX 1

Dye Concentration Needed for Irradiation of Spermatozoa

The laser beam intercepted a volume of dye solution approximately 2 μ deep and 2 μ in diameter. This volume would absorb approximately 3×10^{-9} joules in heating from 18°C (at which the slide was maintained) to 100°C, if the water were heated instantaneously so that no conduction losses occurred. The rate of cooling of an instantaneously heated sphere is discussed by Carslaw and Jaeger (23), who present a graphical solution. One or 2 microseconds is needed for the center of a sphere of the above volume to cool halfway down to its initial temperature; the dye solution must therefore absorb at least 3×10^{-9} joules/microsecond in order to reach a temperature of around 100°C. The energy of the laser pulse is approximately 15 millijoules, and is produced over a period of about 300 microseconds, so the average power is approximately 5×10^{-5} joules/microsecond; the dye solution must absorb about 6×10^{-5} of the beam in a path length of 2 u, so that the theoretically required optical density (O.D.) is 0.13. Because of the very approximate nature of the calculation, I desired an extra order of magnitude as a safety factor, so an O.D. = 2 was sought in evaluating dye solutions. Since this value was not always sufficient to produce noticeable damage, lower concentrations were not tested, and satisfactory results were obtained by using this dye concentration in all the microbeam experiments.

APPENDIX 2

Description of Apparatus for Microbeam Experiments

A detailed description of the equipment used in the laser microbeam experiments is given in the Appendix. A simplified schematic diagram of the complete system is shown in Figure 6.

Laser gate

A type 2N2322 SCR is in series with the manual trigger switch of the laser power supply, as shown in Figure 6. The SCR is triggered by a pulse from the multivibrator of a General Radio "Strobotac." The gate of the SCR is biased just below threshold by the "bias adjust" potentiometer.

General Description of Stroboscope

The illumination system for flash photography and stroboscopic illumination designed by Brokaw (13) utilized two General Electric FT-230 short gap xenon flash lamps. Each flash lamp was connected to its own flash capacitor, and was triggered to discharge by a high voltage pulse from the "Strobotac" through a pulse transformer. The flash lamps were mounted on a moveable platform beneath the microscope, and only the flash lamp in proper position for illumination of the microscope stage was triggered. The flash capacitors were operated at potentials of 2500 volts or less; at this voltage the flash lamps did not fire until triggered.

Switching that system from observational to photographic mode required about a second to turn off the "strobotac," slide the flash lamps into position, open the camera shutter and turn the "strobotac" on again.

During this time the spermatozoon could drift slightly out of position, and turning the "Strobotac" off changed its phase with respect to the flagellum. For the present study, a new system was designed, using a single flash lamp. The photographic capacitors were switched in simultaneously with the laser and timing circuitry while the strobotac was running.

High Voltage d.c. Power Supply

The stroboscope power supply, shown in Figure 33, is used to supply the high voltage for the flash lamp, and delivers a maximum potential of 4,000 volts. This voltage is high enough to cause the flash lamps to conduct when the potential is applied, and to allow the hydrogen thyratrons to conduct when they are triggered. The voltage is adjusted with variable autotransformer T2. Flash capacitors C3-1 and C3-2 supply energy for observation; either one or both of them can be switched in. Flash capacitor C5 supplies energy for photography, and capacitor C4 stores energy for recharging C5 during multiple exposure photographs. Meter M1 indicates the voltage across C2, which is a very close approximation to the peak voltage across the bridge. Meter M2 indicates the voltage across C4. When the power supply is turned off, the contacts on relay K1 close, discharging the capacitors through R2.

Thyratron Stroboscope Switch

The thyratron stroboscope switch consists of two almost identical units, one (V1 and V2) used to connect flashlamp FL1 to the smaller flash capacitor for observation, and the other (V3 and V4) used to

Figure 33 High Voltage Power Supply

Symbol	Description
C1	capacitor, .01 uf
C2	" , .5 "
C3-1	" , .02 "
C3-2	" , .05 "
C4	" , 45 "
C5	" , 4 "
D1	diode stack, RCA CR104
D2	" " " "
D3	" " " "
D4	" " " "
F1	fuse, 3AG, 5A SLO-BLO
K1	relay, 6.3 VAC
M1	meter, 0-6000 VDC
M2	" " "
PL1	pilot lamp, 6v
R1	resistor, 20M, 2W
R2	" 25K, 50W
R3	" 10M, ½W
R4	" " "
R5	" 100K, 100W
R6	" " "
R7	" 1K, 100W
SW1	switch, SPST, power
SW2	" , SPST
SW3	" , SPST
T1	filament transformer, 6.3V
T2	variable autotransformer
T3	transformer, TRIAD P217AL

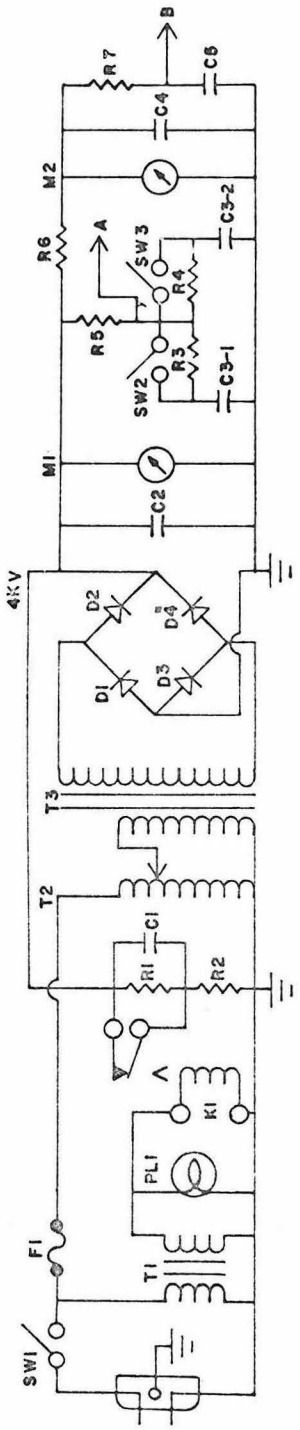


Figure 33. High Voltage Power Supply

connect it to the larger flash capacitor for photography, as shown in Figure 34. In each unit a type 5949 high-power hydrogen thyratron (V2 and V3) acts as a switch between the flash lamp and the appropriate capacitor. The circuit is designed to be actuated by pulses from the "trigger output" connector of the "Strobotac." Type C1K xenon thyrotrons (V1 and V4) amplify these pulses enough to trigger the hydrogen thyratrons.

The circuit normally operates in the observational (low intensity) mode, with relays K2 and K3 de-energized, as shown in Figure 34. In this mode, pulse transformer T10 receives a negative pulse from the "Strobotac." The polarity of the pulse is reversed by the transformer and used to trigger V1 into conduction. V1 in turn triggers V2 into conduction, and is turned off by the back-voltage of inductor L1. When V2 conducts, almost 4,000 volts is placed across flash lamp FL1, causing it to fire. It discharges the smaller flash capacitor (C3 of Figure 33), and V2 and FL1 turn off. Continuous operation is possible at up to 75 flashes/second. For photographs, relays K2 and K3 can be energized by either pressing the "test" button or closing the "electronic flash" contacts of the camera. The incoming pulse from the "Strobotac" then goes into pulse transformer T11, and V4, V3 and FL1 then function as described above for V1, V2 and FL1, respectively, discharging the larger flash capacitor (C5), which is then rapidly recharged by the energy stored in C4. It has been used for photographs at up to 100 flashes/second. V4 is prevented from firing while the circuit is in the observational mode by resistors R13-R17, capacitors C15-C19 and relay K3.

Figure 34 Thyratron Stroboscope Switch

Symbol	Description
C1,C2	Capacitor, 9,000, 150 V
C3, C4	" , 22, 20V
C5	" , 500, 25V
C6,C7	" , 22, 20V
C8	" , 250, 25V
C9, C10	" , 24, 450V
C11	" , .001
C12	" , .2
C13-C20	" , .01
C21-C23	" , .1
D1-D6	diode, IN538
D7,D8	diode stack, 4-IN3549
D9-D11	diode, IN459
F1	fuse, 3AG, 5A
FL1	flash lamp, GE, FT230
H1,H2	hydrogen reservoir of V2,V3
J1,J3	plug, 3-terminal phone
J2,J4	" , 2-terminal phone
K1	relay, 115 VAC
K2	" , 4PST
K3	" , reed, Magnecraft W102 VX-13
L1,L2	choke, swinging, UTC,H170
M1	meter, 150 VAC
PL1	pilot light, neon
R1,R2	resistor, .5, 20W
R3	" , 10K, 2W
R4	resistor, 1250, 20W adjust.
R5	" , 50K, 20W
R6	" , 330, 2W
R7,R22	" , 22K, 1W
R8,R20	" , 25K, 15W
R9, R18	" , 220K, 1W
R10	" , 150, 4W
R11	" , 220K, 2W
R12	" , 10, 25W
R13-R17	" , 56, ½W
R19	" , 220, ½W
R21	" , 220, 1W
R23-R25	" , 100, 2W
SW1	switch, SPST
SW2	" , micro
SW3	" , DPDT
SW4	" , pushbutton
T1	transformer, auto
T2,T3	" , filament, Chicago-Standard, P-6454
T4-T7	transformer, filament, Thordarson, 21F76
T8	transformer, filament, TRIAD F45X
T9	transformer, filament, power, TRIAD R-22B
T10,T11	transformer, pulse, Pacific Coil 105PC
V1,V4	thyratron, Xenon, C1K
V2,V3	thyratron, hydrogen, 5949

Notes:
1) The cathodes of V2 and V3 are internally connected to the electrical centers of their heaters.
2) To turn on, switch SW1, SW2 and SW3 from positions shown and turn T1 to zero. Then slowly increase T1 until M1 reads 100-115 V.
3) All capacitances given in microfarads. All resistances given in ohms.

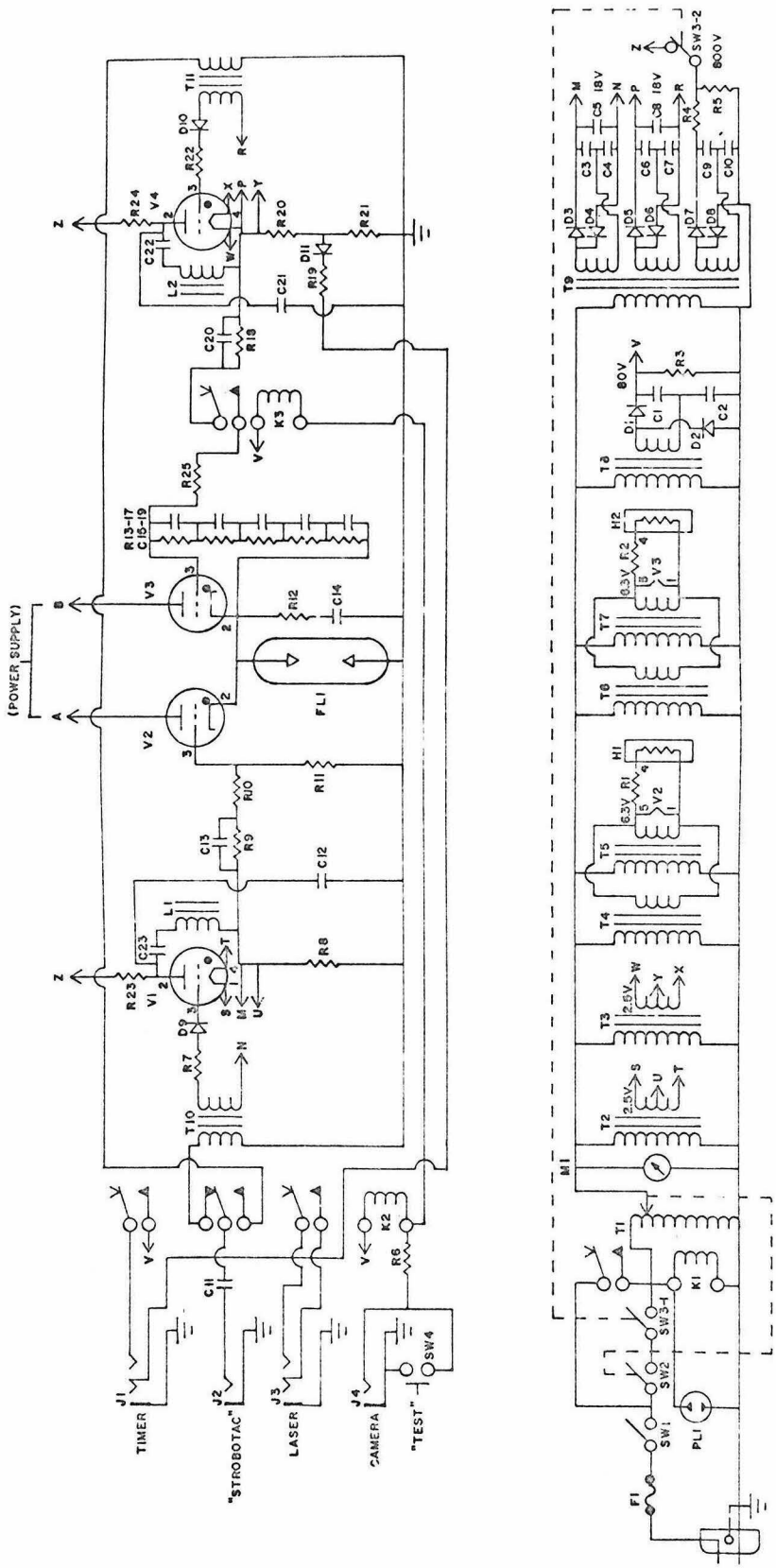


Figure 34. Thyratron Stroboscope Switch

Relay K2 closes the manual trigger contacts of the laser and turns on the power to the photography timer, which is then activated by the next pulse through R19. The closing time of relay K2 is about 1 millisecond longer than that of K3, and the "strobotac" occasionally fires after K3 has closed but before K2 has closed, resulting in 2 exposures before irradiation. When the "test" button is released or the camera contacts are opened, the circuit reverts to the observational mode.

Photography Timer

The photography timer has two parts, as shown in Figure 35: a variable R-C timing network to determine the frequency of the "Strobotac" during photography, and a unijunction-SCR time delay circuit (82) to control the number of exposures on the photographs.

Closure of relay K1 causes the substitution of a particular RC network, determined by the positions of switches SW-1 and SW-2, for the RC circuit which normally controls the multivibrator frequency of the "Strobotac." Frequencies of 5, 10, 25, 50 or 100 flashes/second can be chosen, or the "Strobotac" can be allowed to continue at its normal frequency. The frequencies may be adjusted by trimpots R8-R12. The power for the circuit is turned on by a relay (K2 of Figure 34), as described in the last section, and the first pulse from the thyratron switch triggers SCR Q1 into conduction, energizing relay K1. This connects the frequency-controlling RC network and activates the time delay circuit. There is an extra 3 or 4 milliseconds between the first and second exposures, due to the closing time of relay K1.

Figure 35 Photography Timer

Symbol	Description
B1	battery, 9V, Eveready, 216
C1,C13	capacitor, 100
C2	" , .015
C3,C4	" , .15
C5,C6	" , .033
C7	" , .005
C8	" , .47
C9	" , .22
C10	" , .094
C11	" , .047
C12	" , .022
D1	diode, IN538
K1,K2	relay, DPDT, LEACH 9227-3969
P1	plug, 7-pin miniature
P2	plug, 3-terminal phone
Q1,Q4	SCR, C20B
Q2	transistor, unijunction 2N494
Q3	diode, Zener, 18V, 400mW
R1,R31	resistor, 180,2W
R2	" , 150
R3	" , 30K, 5%
R4	resistor, 5.1K, 5%
R5	" , 33K "
R6	" , 3.3K "
R7	" , 39K "
R8-R12	trimpot, 20K
R13	resistor, 24K, 5%
R14	" , 51K, "
R15	" , 22K "
R16	" , 43K "
R17	" , 15K "
R18-R22	" , 5.1M "
R23	" , 100K, 1%
R24	" , 590K, "
R25	" , 910K, "
R26	" , 1.2M, "
R27	" , 1.5M, "
R28	" , 100
R29	" , 33
R30,R32	" , 3.3K, 1W
SW1	switch, rotary, 3P6T
SW2	switch, rotary, SP5T

Notes: 1) Switches viewed from rear.

2) All capacitances given in microfarads. All resistances given in ohms, and ½watt unless otherwise specified.

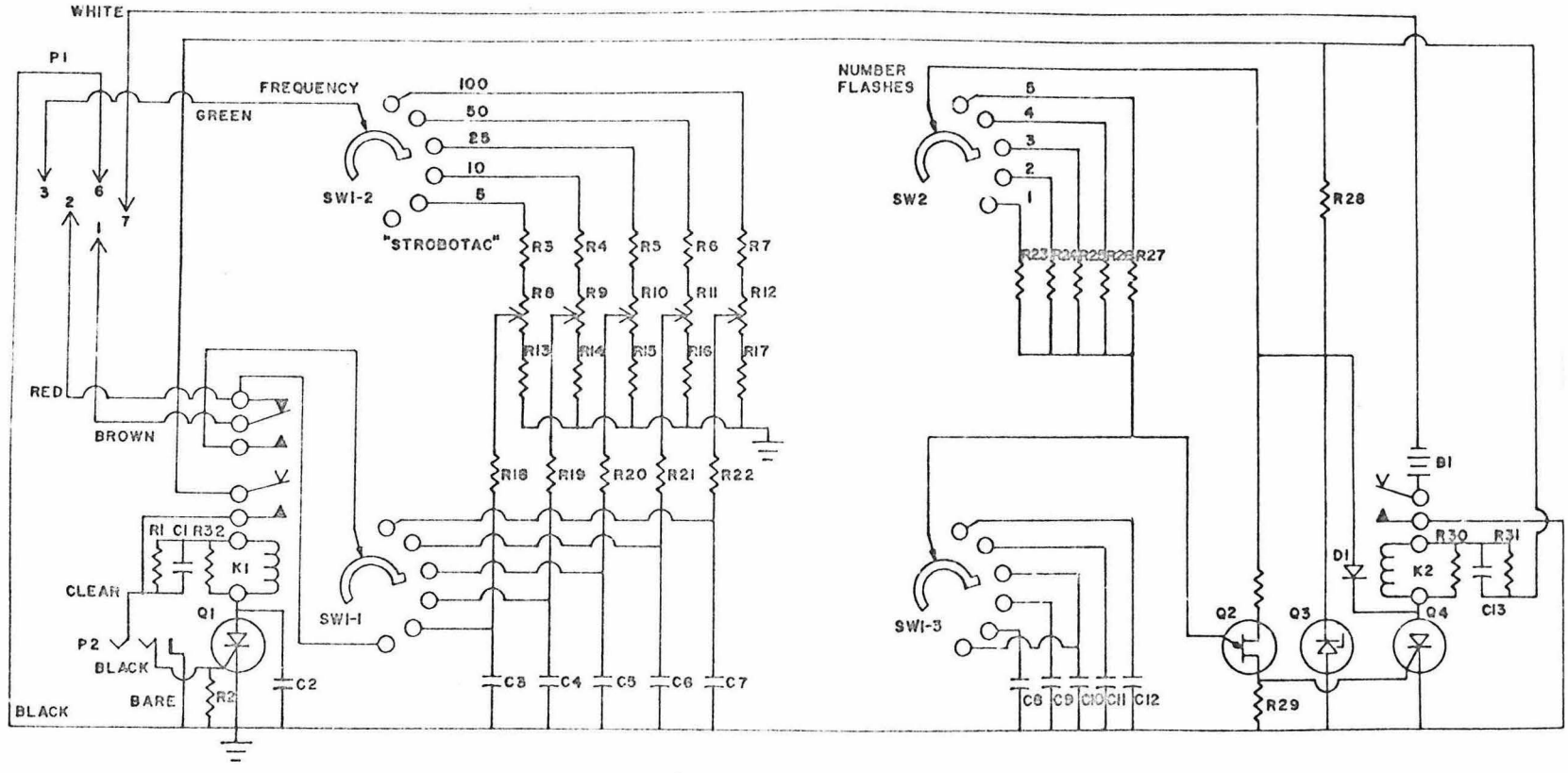


Figure 35. Photography Timer

Photographs are difficult to interpret if they contain more than about 4 exposures, so a second part of the timer circuit was used to turn off the "Strobotac" after a selected number of flashes. After a time delay determined by SW1-3 and SW2, unijunction transistor Q2 triggers SCR Q4 into conduction, energizing relay K2, which causes the grid of the thyratron of the "Strobotac" to be biased to -9 volts, preventing further firing. Delays allowing from 1 to 5 flashed may be selected at any of the predetermined frequencies. If SW1 is set to allow the "Strobotac" to continue at its normal frequency, time delays assume a frequency of 25 flashes/second. Diode D1 conducts, lowering the voltage across Zener diode Q3 to almost zero, preventing the delay capacitor from charging before the next photograph. When the thyratron stroboscope switch reverts to the observational mode, power to the timer is turned off and the timer is ready for the next photograph.

APPENDIX 3

Diffusion from Pipettes

If ATP were diffusing from the tip of a pipette into an unbounded volume, the pipette tip could be approximated by a continuous spherical or point source. A solution for the problem of a spherical source of radius (a) with a constant concentration (C) at its surface may be obtained from Carslaw and Jaeger (24). The concentration (c) at any distance (r) from its center at any time (t) is:

$$c = \frac{aC}{r} \operatorname{erfc} \frac{r-a}{2\sqrt{(Dt)}} \quad , \; r \geq a,$$

where D = diffusion constant of ATP, approximately 4×10^{-6} Cm^2/second (10), and $\operatorname{erfc}(x) = 1 - \frac{2}{\pi} \int_0^x e^{-Z^2} dZ$. The gradient resulting from this source approaches a steady-state solution of $c = \frac{aC}{r}$, so the concentration of ATP at a distance of 50 μ from a pipette tip of 1 u diameter should be 1% of the concentration at the tip, and a large gradient should be produced along a sperm tail. However, in the initial diffusion experiments the pipette was placed between a slide and cover glass separated by 50 u; the tip was within a few u of the cover glass. The time-dependent part of the concentration equation at a distance of 50 μ, is: $\operatorname{erfc} \left(\frac{1.2}{\sqrt{t}}\right) = 20\%$ of its steady-state value at t = 2 seconds; the solution in this region of the tip becomes saturated within a few seconds, decreasing the slope of the concentration gradient in that region. Separating the slide and cover glass by 1 mm lengthens the time scale by a factor of 400, allowing a gradient of the form $c = \frac{1}{r} \operatorname{erfc} \frac{r}{\sqrt{t}}$ to be maintained for several minutes. The reflection due to the cover glass was within several μ of the pipette tip, so this refection caused little effect on the shape of the gradient at distances (r) greater than several μ.

In practice, the concentration gradient about a pipette tip was tested by moving it towards the basal end of a spermatozoon. A pipette could be brought to within approximately 15 μ of a spermatozoon before beating began, with a large increase in beat frequency as it was moved nearer, for a few minutes after the pipette was first placed in the sperm suspension.

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