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EFFECT OF IMPOSED HEAD VIBRATION ON THE STABILITY AND WAVEFORM OF FLAGELLAR BEATING IN SEA URCHIN SPERMATOZOA

BY CHIKAKO SHINGYOJI[1], IAN R. GIBBONS[2], AKIRA MURAKAMI[1] AND KEIICHI TAKAHASHI[1]

[1]*Zoological Institute, Faculty of Science, University of Tokyo, Hongo, Tokyo 113, Japan,* and [2]*Pacific Biomedical Research Center, University of Hawaii, Honolulu, HI 96822, USA*

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Summary

The heads of live spermatozoa of the sea urchin *Hemicentrotus pulcherrimus* were held by suction in the tip of a micropipette mounted on a piezoelectric device and vibrated either laterally or axially with respect to the head axis. Within certain ranges of frequency and amplitude, lateral vibration of the pipette brought about a stable rhythmic beating of the flagella in the plane of vibration, with the beat frequency synchronized to the frequency of vibration [Gibbons *et al.* (1987), *Nature* **325**, 351–352]. The sperm flagella, with an average natural beat frequency of 48 Hz, showed stable beating synchronized to the pipette vibration over a range of 35–90 Hz when the amplitude of vibration was about 20 μm or greater. Vibration frequencies below this range caused instability of the beat plane, often associated with irregularities in beat frequency. Frequencies above about 90 Hz caused irregular asymmetrical flagellar beating with a marked decrease in amplitude of the propagated bends and a skewing of the flagellar axis towards one side; the flagella often stopped in a cane shape. In flagella that were beating stably under imposed vibration, the wavelength was reduced at higher frequencies and increased at lower frequencies. When the beat frequency was equal to or lower than the natural beat frequency, the apparent time-averaged sliding velocity of axonemal microtubules, obtained as twice the product of frequency and bend angle, decreased with beat frequency in both the proximal and distal regions of the flagella. However, at vibration frequencies above the natural beat frequency, the sliding velocity increased with frequency only in the proximal region of the flagellum and remained essentially unchanged in more distal regions. This apparent limit to the velocity of sliding in the distal region may represent an inherent limit in the intrinsic velocity of active sliding, while the faster sliding observed in the proximal region may be a result of passive sliding or elastic distortion of the microtubules induced by the additional energy supplied by the vibrating pipette.

Axial vibration with frequencies either close to or twice the natural beat

Key words: flagella, stability of waveform, beat frequency, sliding velocity, *Hemicentrotus pulcherrimus*.

frequency induced cyclic changes in the waveform, compressing and expanding the bends in the proximal region, but did not affect bends in the distal region or alter the beat frequency.

Introduction

Flagellar movement is characterized by oscillation. Although the mechanism of flagellar oscillation is still little understood, it appears to operate not only at the base of the flagellum, where the new bends are cyclically initiated, but also along the length of the flagellum, where the bends are propagated without a decrease in the bend angle. It is possible that the mechanism of flagellar oscillation is closely associated with the mechanisms controlling microtubule sliding in the axoneme. A useful approach to understanding the oscillatory mechanism would therefore be to analyze the patterns of bending and microtubule sliding in the beating flagella under conditions that change their beat frequency.

Several techniques are available for indirectly lowering the beat frequency of sperm flagella by changing either the viscosity or the chemical composition of the medium (Brokaw, 1966, 1975; Ogawa *et al.* 1977; Okuno and Brokaw, 1979; Asai and Brokaw, 1980; Cosson *et al.* 1983). However, the effects of changing the beat frequency by direct mechanical means are simpler to interpret because of the absence of chemical side effects and because the natural frequency can be both increased and decreased from its natural value. Okuno and Hiramoto (1976) found that the flagellar beat frequency of starfish spermatozoa changed when their movement was mechanically constrained by microneedles (see also Kaneda, 1965). They reported that a flagellum could be synchronized to the vibration of a microneedle placed close to its proximal region, enabling modulation of the flagellar beat frequency by ±8 Hz. Sleigh and Jarman (1973) found that both the angular velocity and the rate of bend propagation in the effective stroke in ctenophore cilia were increased significantly when the frequency at which the cilia were driven externally was increased. However, no systematic study has been performed on the relationship between such changes in beat frequency and the other parameters of flagellar beating.

We have recently developed a new experimental procedure by which the flagellar beat frequency can be either increased or decreased more extensively by imposing a sinusoidal vibration on the head of a sea urchin spermatozoon held in the tip of a vibrating micropipette. In this paper, we report the effects of vibration at various amplitudes and frequencies applied to the heads of live spermatozoa in a direction lateral to their longitudinal axis. Stable flagellar beating could be obtained over a wide range of vibration frequencies, ranging from about 25 % below to 100 % above the natural beat frequency. A brief description of the effect of vibration along the sperm axis is also included.

Materials and methods

Sperm of the sea urchin *Hemicentrotus pulcherrimus* were diluted with Ca^{2+}-free artificial sea water containing 465 mmol l^{-1} NaCl, 10 mmol l^{-1} KCl,

25 mmol l^{-1} $MgSO_4$, 0.2 mmol l^{-1} EDTA and 10 mmol l^{-1} Tris–HCl (pH 8.2). All experiments were carried out at room temperature (24–25.5 °C).

The flagellar movement was observed with an inverted microscope (Nikon Diaphot TMD) with phase-contrast optics (Nikon BM X40). A Strobex System 236 power supply and a model 271B xenon flash lamp (Chadwick–Helmuth) were used as the light source. The pulses for triggering the stroboscopic flashes were provided by an electronic stimulator (Nihon Kohden MSE-3R) and the flash frequency was monitored with a frequency counter (Iwatsu universal counter SC-7201). The movements of the spermatozoa and the micropipette were recorded with a Sony video camera (AVC 1150D) fitted with a Nikon zooming camera adaptor and a Sony U-matic video cassette recorder (VO-5850).

The suction pipettes used to vibrate the sperm heads were prepared from glass capillary micropipettes made by using a microelectrode puller (Narishige PN-3). The tip of each micropipette was broken off to obtain an enlarged opening 1–2 μm in diameter. Heat polishing of the pipette tip was then done on a microforge to obtain a smooth surface as well as to reduce the inner diameter to 0.8 ± 0.1 μm. The micropipette was connected to flexible polyethylene tubing which led to a 1 ml hypodermic syringe. The plunger of the syringe was moved by a micrometer caliper. The tubing and the syringe were filled with liquid paraffin and the micropipette was filled with Ca^{2+}-free artificial sea water. The micropipette was attached to a piezoelectric driver (see below) mounted on a hydraulic micromanipulator (Narishige MO-103 combined with MN-1), which was fixed to the body of the microscope so that the micropipette could be positioned independently of the movement of the microscope stage.

To hold a spermatozoon with the micropipette, a drop of sperm suspension was placed on a coverglass on the microscope stage. The tip of the micropipette was brought close to the head and, by manually controlling the micrometer caliper, the anterior one-third to one-quarter of the head was gently sucked into the micropipette. The distance between the tip of the micropipette and the surface of the coverglass (10–21 μm) was measured during each experiment by reading the scale on the focusing knob of the microscope. The best optical condition for recording the flagellar waveform was obtained when this distance was small, largely because of a hydrodynamic interaction with the glass surface that caused the flagella to beat in a plane parallel to the surface and therefore in the plane of focus of the microscope. Although this interaction with the surface may affect flagellar beat parameters other than the beat plane (Brennen and Winet, 1977), no quantitative analysis of this effect was made in the present study.

The suction pipette was oscillated by means of a piezoelectric driver capable of vibrating along any axis at frequencies up to 150 Hz, with the direction being determined by the distribution of sinusoidal voltage applied to three sets of piezo elements in a Π-shaped configuration (Corey and Hudspeth, 1980; Gibbons *et al.* 1987). A waveform generator (Trio-Kenwood sweep/function generator FG-271) was used to supply the voltage, with an Iwatsu counter (SC-7201) to monitor the frequency. All vibration amplitudes are given as peak-to-peak values.

The term lateral vibration will be used in this paper to designate vibration in a plane perpendicular to the longitudinal axis of the sperm head and parallel to the plane of the observation slide. Vibration along the head axis will be termed axial vibration.

To analyze the flagellar waveform, tracings of the spermatozoa and the micropipette tip were made by hand from the screen of a video monitor (Panasonic WV-5470) onto sheets of transparent film. The final magnification on the CRT display was ×28 000, corresponding to 2.5 horizontal video lines per micrometre. During a vibration experiment, the flash frequency was kept 1–3 Hz below or above the vibration frequency. Since the flagella generally synchronized their beat frequency to the vibration imposed on their head (see Results), this enabled us to observe, by a stroboscopic effect, the movement of the flagella at a greatly reduced apparent speed and, at the same time, to record the various phases of the beat cycle. We have analyzed 7–40 images of all sperm flagella under each set of conditions.

The true beat frequency was calculated as the algebraic sum of the flash frequency and the apparent beat frequency on the monitor screen. At the beginning and end of each experiment, the natural beat frequency of the spermatozoon held by the non-vibrating pipette was determined similarly.

The wavelength of the flagellar bending waves was determined from plots of local angular orientation of the flagellum (shear angles) as the length measured along the flagellum between two adjacent bends of opposite curvature. If the flagellum had fewer than two complete bends (as in Fig. 1D), the wavelength was approximated as twice the length of a single bend.

We chose to represent the tubule sliding velocity at a given point on the flagellum by the product 2×(beat frequency)×(averaged angle of principal and reverse bends centred at the point in question) (Brokaw, 1971; Gibbons, 1982; Takahashi *et al.* 1982). This product is proportional to the time-averaged speed of sliding between each pair of microtubule doublets around the axoneme, with the constant of proportionality differing for the various microtubule pairs.

In both vibrated and unvibrated spermatozoa, the flagellar waveforms were asymmetrical, with the angles of bends on one side (principal bends) being larger than those on the other side (reverse bends) (Gibbons and Gibbons, 1972). However, under most conditions, the difference between the angles of principal and reverse bends was substantially less than in free-swimming spermatozoa, presumably as a result of the sperm head being held by the pipette (Gibbons, 1982).

Results

Effect of lateral vibration on the frequency and stability of beating

When a spermatozoon was caught by a micropipette, the flagellum usually continued to beat regularly with an apparently normal waveform (Fig. 1A). The mean natural frequency, i.e. the flagellar beat frequency of the spermatozoa held by a stationary micropipette, was 48.4 Hz (range 38–56 Hz, N=30).

Lateral vibration of the pipette with frequencies different from the natural

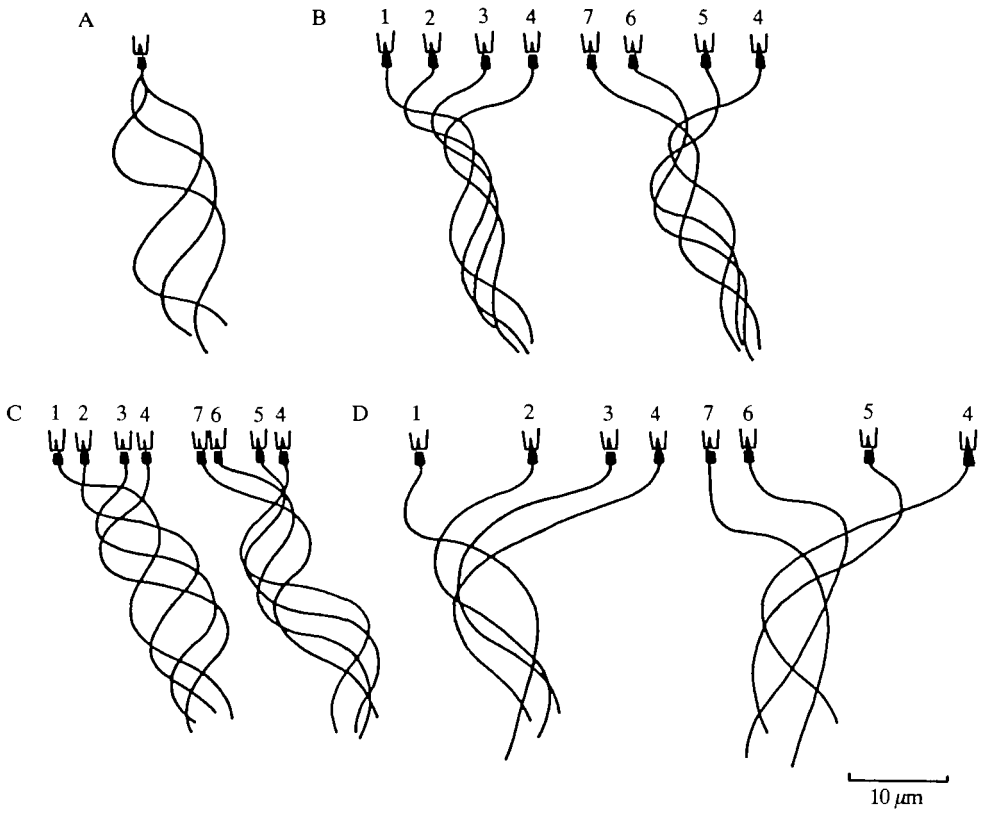

Fig. 1. Typical tracings of live spermatozoon during lateral vibration. The spermatozoon was held by a suction pipette in Ca^{2+}-free artificial sea water. (A) Natural beating at 48.0 Hz without vibration. (B) Vibration at 70.9 Hz. Numbers by sperm heads indicate the order of successive images representing one vibration cycle. For clarity, the tracings for each half cycle (1–4 and 4–8) are drawn separately. (C) Vibration at 50.6 Hz. (D) Vibration at 30.5 Hz.

frequency caused the beating of the flagellum to become synchronized with the movement of the pipette, while the flagellum continued stable beating as long as the frequency and the amplitude of vibration were within certain limits. The pipette vibration also caused the plane of flagellar beat to coincide with the plane of pipette vibration (Gibbons *et al.* 1987). Fig. 1B–D shows typical tracings of a sperm flagellum beating stably at various vibration frequencies. Since the beat frequency was always equal to vibration frequency during stable beating, there was a one-to-one correspondence between the position of the pipette in its vibration cycle and the phase of the flagellar waveform.

The effect of lateral vibration was studied in 20 spermatozoa with vibration frequencies of 15.0–101.5 Hz and vibration amplitudes of 0.7–33.0 μm. Fig. 2 summarizes the effect of vibration frequency and amplitude on the stability of flagellar beating. The broken lines in Fig. 2 indicate the boundaries of the region in

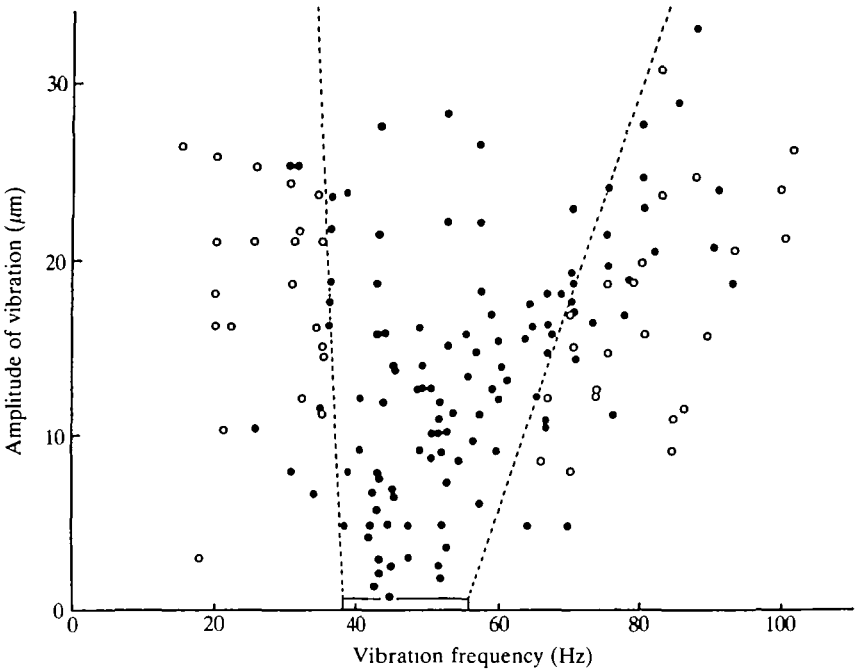

Fig. 2. Range of stable flagellar beating observed in a typical population of spermatozoa at different vibration frequencies and amplitudes. Filled circles, stable beating synchronized to pipette vibration. Open circles, unstable beating. Dashed lines show approximate boundaries of the region of stable beating. The rectangle on the abscissa indicates the range of beat frequencies in this population of spermatozoa held in a stationary micropipette.

which stable beating was invariably induced. The unstable beating is described in the next section. The range of vibration frequency capable of maintaining stable beating increased with the amplitude. When the vibration amplitude was 20 μm or greater, stable beating was obtained at frequencies between about 35 and 90 Hz. The effect of increasing the amplitude was most evident at higher frequencies. At lower frequencies, increasing the amplitude did not significantly reduce the critical frequency beyond which the beat became unstable.

When the vibration amplitude was less than about 2 μm, the flagellar beat did not become synchronized to the pipette vibration even when the vibration frequency was near the natural beat frequency, and the flagellum kept beating more or less stably at its natural frequency.

Unstable beating caused by lateral vibration

When the flagella were beating stably during lateral vibration, the plane of beating coincided with that of pipette vibration and did not depend on the original beat plane. However, when the vibration frequency was too low to maintain stable beating (below about 35 Hz), the beat plane often changed in an unpredictable

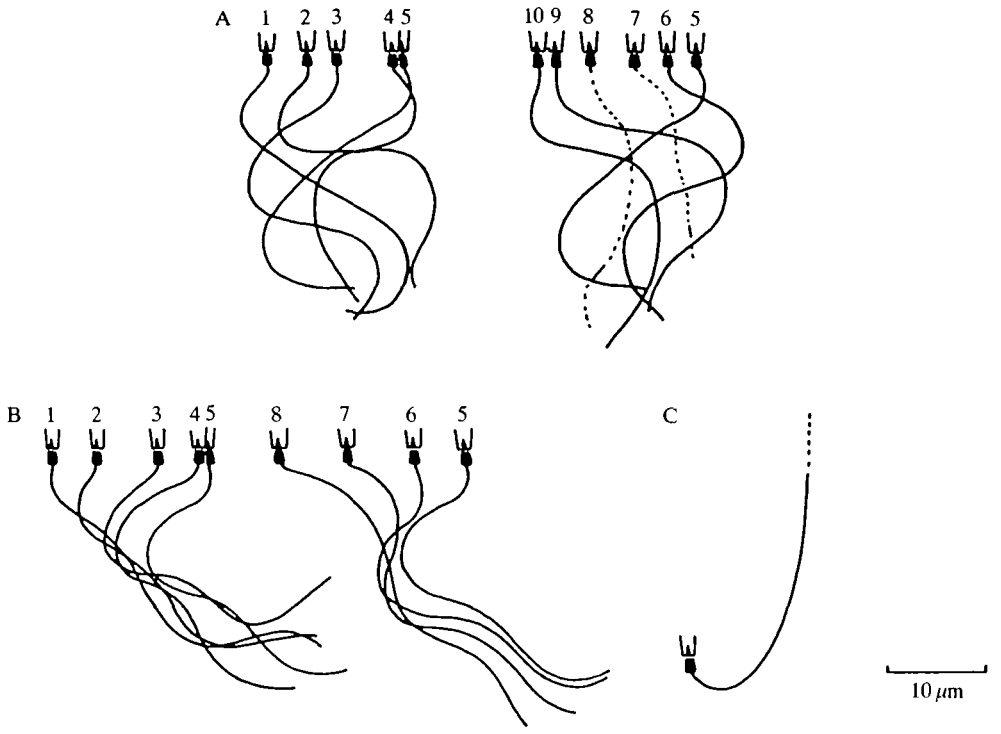

Fig. 3. Tracings of spermatozoa showing unstable beating at low and high vibration frequencies. (A) Frequency, 34.2 Hz; amplitude, 16.1 μm. (B) Frequency, 99.7 Hz; amplitude, 20.7 μm. (C) Flagellar arrest observed just after vibration at 93.1 Hz; this flagellum resumed beating when the vibration frequency was lowered to 50 Hz.

manner. Bend propagation appeared not to be disturbed by the change of the beat plane (Fig. 3A), although the beat frequency often became irregular.

In contrast, when the vibration frequency was greater than about 80 Hz, no such changes of beat plane occurred, but the waveform showed irregular, sometimes jerky, changes. The unstable beating in this range was characterized by a prominent decrease in the maximum angle attained by reverse bends and a decrease in amplitude of all bends during their propagation (Fig. 3B). At frequencies above about 100 Hz, no bends propagated into the distal region of the flagellum. During such unstable beating, the flagellum sometimes became arrested in a cane shape, with a sharp bend at its base (Fig. 3C). After transient periods of arrest, the flagellum could resume normal beating when the vibration frequency was lowered. However, after prolonged arrest, beating never resumed, presumably because of mechanical damage.

During unstable beating at high frequency, the axis of the flagellar beat became conspicuously skewed relative to the axis of the sperm head. This occurred within the plane of pipette vibration, and the direction of skew was the same as that in which the flagella became bent in the arrest response.

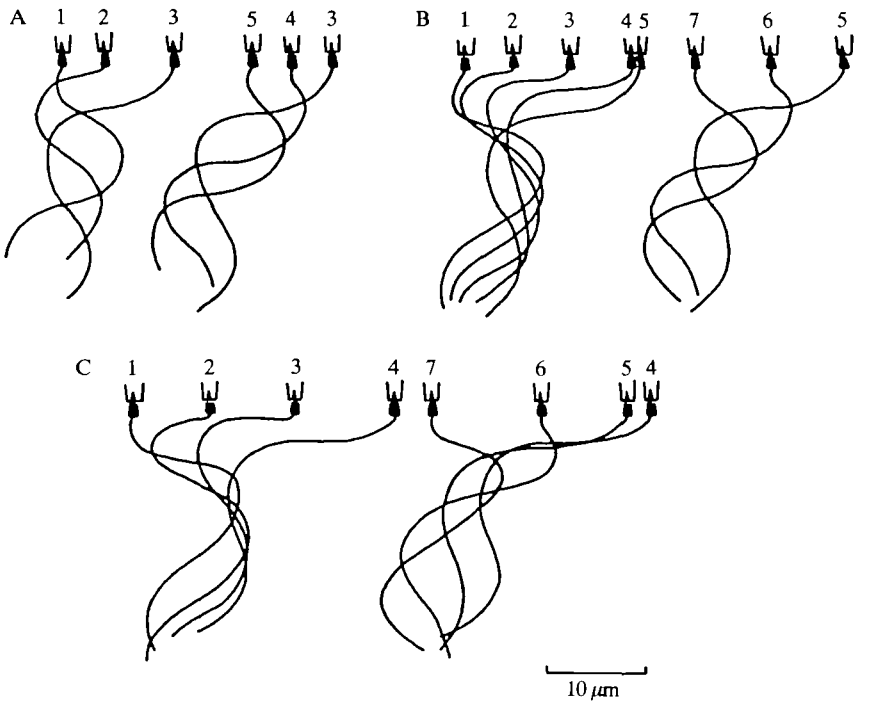

Fig. 4. Tracings showing the effect on the waveform of changing the vibration amplitude at constant frequency. Note that the shape of the bends in the proximal region changes with the amplitude, while that of bends in the distal region appears to be unaffected. Vibration frequency 47 Hz. (A) Amplitude, 11.8 μm; (B) amplitude, 18.2 μm; (C) amplitude, 24.4 μm.

Characteristics of the bending waves during stable beating

Bend angle

As long as stable beating was maintained, changing the amplitude of vibration at a constant frequency had only a limited effect on the flagellar waveform. Fig. 4 shows tracings of a spermatozoon that was vibrated with amplitudes ranging from 11.8 to 24.4 μm. Although the changes in amplitude affected the waveform in the proximal region, possibly due to passive bending of the axoneme under the hydrodynamic force, the angles of bends propagated beyond the proximal quarter of the flagellar length did not change significantly with the vibration amplitude (Fig. 5).

Changing the vibration frequency, in contrast, had a marked effect on the angles of propagated bends, particularly in the high frequency range (Fig. 6). The angles of fully developed bends initiated at a frequency of less than about 55 Hz remained nearly constant as these bends propagated through the region 12–30 μm from the base, and then gradually decreased as the bends propagated further towards the tip. This decrease in angle resembled that observed during normal beating without vibration, but was greater in extent. As the frequency was increased above about

55 Hz, the decrease in angle of the propagating bends began to occur closer and closer to the flagellar base.

Fig. 7 compares the effect of frequency on the maximal bend angle observed in the proximal and middle regions of the flagellum with that on the bend angle in the middle region 25–30 μm from the base. The maximal bend, that is the fully developed proximal bend, occurred at a distance of 5–17 μm from the flagellar

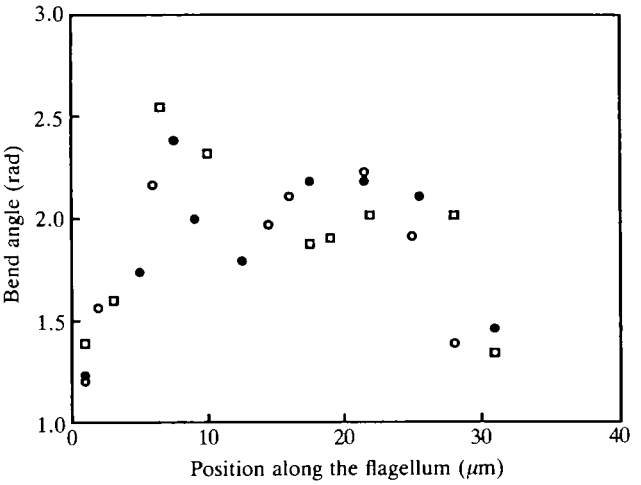

Fig. 5. Changes in angle of principal bends, showing that the vibration amplitude has little effect on the bend angle in the middle region of the flagellum. Data were obtained from the same tracings as those in Fig. 4. Vibration frequency was 47 Hz. Open circles, amplitude 11.8 μm; filled circles, amplitude 18.2 μm; open squares, amplitude 24.4 μm.

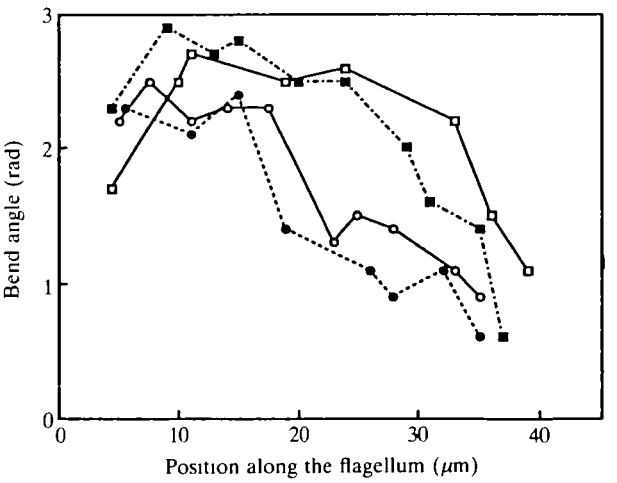

Fig. 6. Effects of vibration frequency on the angles of principal bends centred at different positions along the flagellum. Open squares, 36.1 Hz; filled squares, 48.6 Hz; open circles, 78.7 Hz; filled circles, 93.1 Hz. Vibration amplitudes were 16–18.6 μm. Data represent results from a single sperm flagellum.

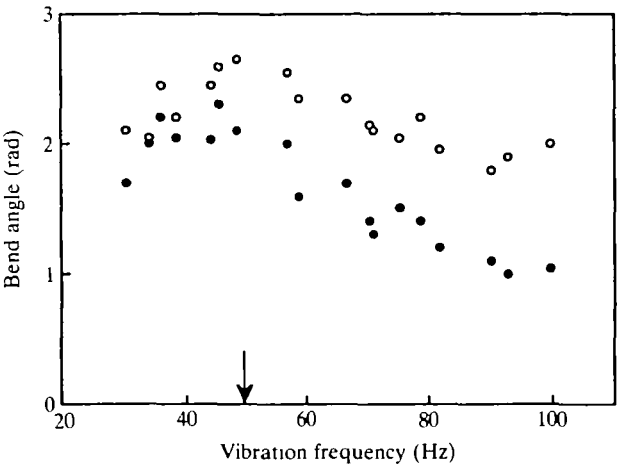

Fig. 7. Relationship between bend angle and the vibration frequency. Angle shown represents the average of principal and reverse bend angles. Open circles, maximal bend angles (which occurred between 5 and 17 μm from the flagellar base). Filled circles, bend angles in the middle region (25–30 μm from the base). Vibration amplitude was 14.6–24.3 μm. Data were collected from three spermatozoa. An arrow indicates the natural beat frequency (48 Hz) of the spermatozoa.

base. Above about 50 Hz, the maximal bend angle in the proximal region appeared to decrease only slightly with increasing frequency, whereas the bend angle in the middle region decreased sharply. Below about 45 Hz, the bend angle decreased with the frequency in both the proximal and middle regions of the flagellum.

Wavelength

Varying the frequency caused a marked change in the wavelength of the flagellar waves (Fig. 8). Lateral vibration at frequencies higher than the natural beat frequency reduced the natural wavelength by up to 30 %, whereas vibration at lower frequencies increased it by as much as 50 %. At very low frequencies the wavelength became so large that only a single complete bend was present on the flagellum during much of the beat cycle; the curvature of such single bends was notably non-uniform (Fig. 1D).

Sliding velocity

Fig. 9 shows the relationship between frequency and apparent sliding velocity, calculated from the data shown in Fig. 7. In the proximal region of the flagellum, the sliding velocity increased with the frequency (open circles). In the middle region of the flagellum, in contrast, the sliding velocity increased with frequency only up to about 45 Hz, but then levelled off with no further increase at higher frequencies (filled circles).

Fig. 10 shows sliding velocities calculated for several points along the flagellar

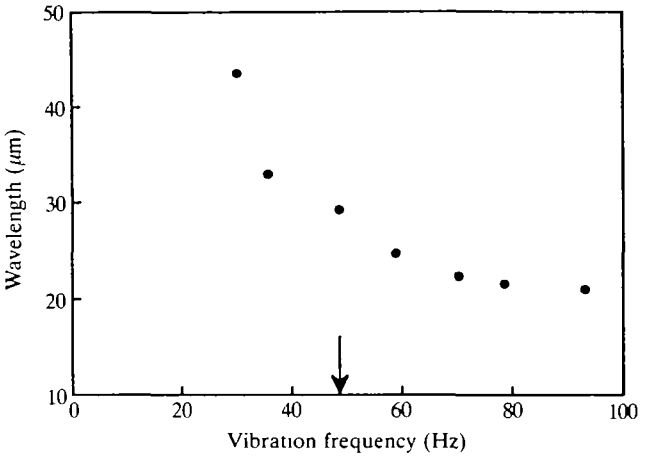

Fig. 8. Change in wavelength with vibration frequency. Each point represents an average wavelength from 7–10 images at different phases of the beat cycle for a single spermatozoon. The wavelengths were measured from shear angle curves as described in Materials and methods. The arrow indicates the natural beat frequency (48 Hz).

length for various frequencies applied to a single spermatozoon. During vibration at 45.3 Hz, a nearly constant sliding velocity was maintained as the bend propagated along the flagellum (line *b*). As the frequency increased, the sliding velocity in the proximal region of the flagellum increased, whereas the sliding velocity in the distal region beyond about 20 μm from the base of the flagellum remained almost the same as at 45.3 Hz (lines *c*, *d*, *e*, *f*). Thus, the higher the frequency, the more sharply the sliding velocity decreased towards the tip. When

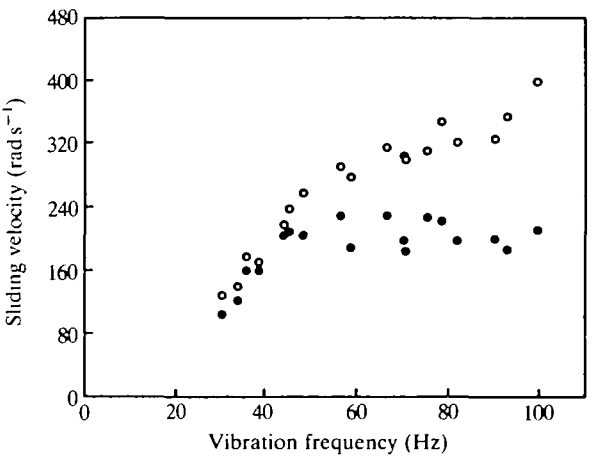

Fig. 9. Changes in apparent sliding velocity with vibration frequency. Open circles, sliding velocity in the proximal region (obtained from the maximal bend angle). Filled circles, sliding velocity in the middle region (25–30 μm from the base).

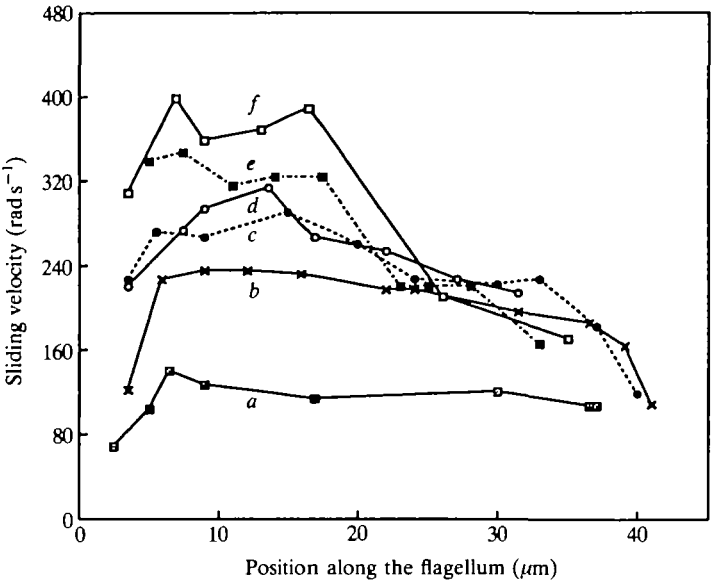

Fig. 10. Changes in apparent sliding velocity as a function of position along the flagellum. *a*, frequency, 34.2 Hz; amplitude, 16.1 μm; *b*, frequency, 45.3 Hz; amplitude, 14.6 μm; *c*, frequency, 56.7 Hz; amplitude, 14.6 μm; *d*, frequency, 66.7 Hz; amplitude, 14.6 μm; *e*, frequency, 78.7 Hz; amplitude, 18.6 μm; *f*, frequency, 99.7 Hz; amplitude, 23.9 μm.

the frequency was lowered to 34.2 Hz, the sliding velocity was significantly reduced not only in the proximal region but along the entire flagellum (line *a*). However, the difference between the sliding velocities in the proximal and distal regions of the flagella diminished at these low frequencies.

Table 1 confirms that the variation shown in Fig. 10 is typical by summarizing the data obtained at two positions along the length of several spermatozoa. At frequencies higher than the natural beat frequency the sliding velocity increased only in the proximal region of the flagella, whereas the sliding velocity determined 25–30 μm from the flagellar base was not significantly changed from that observed near the natural beat frequency. In contrast, vibration at frequencies lower than the natural beat frequency reduced the sliding velocity in both the proximal and distal regions of the flagellum.

Axial vibration

In some preliminary experiments, we applied axial vibration to spermatozoa at frequencies that were either close to their natural beat frequency or at double this value. At both these vibration frequencies, the flagella maintained regular cyclic beating at their own natural frequency. Fig. 11 shows tracings of a single sperm flagellum during axial vibration at twice its natural beat frequency. Although the bends in the proximal region of the flagellum underwent a cycle of substantial

Table 1. *Sliding velocities in proximal and middle regions of flagella at various frequencies of lateral vibration**

Range of frequency (Hz)	Apparent sliding velocity ($rad\,s^{-1}$)		N
	Proximal region bend†	Middle region bend‡	
30.5–38.6	154±18	135±24	4
44.2–48.6	230±18	205±7	5
56.7–66.7	293±15	214±18	3
70.5–99.7	332±31	202±14	8

* The amplitude of pipette vibration was between 14.6 and 24.3 μm.
† Maximal bend angle observed in the region 10–15 μm from the flagellar base.
‡ Bend angles observed in the region 25–30 μm from the flagellar base.

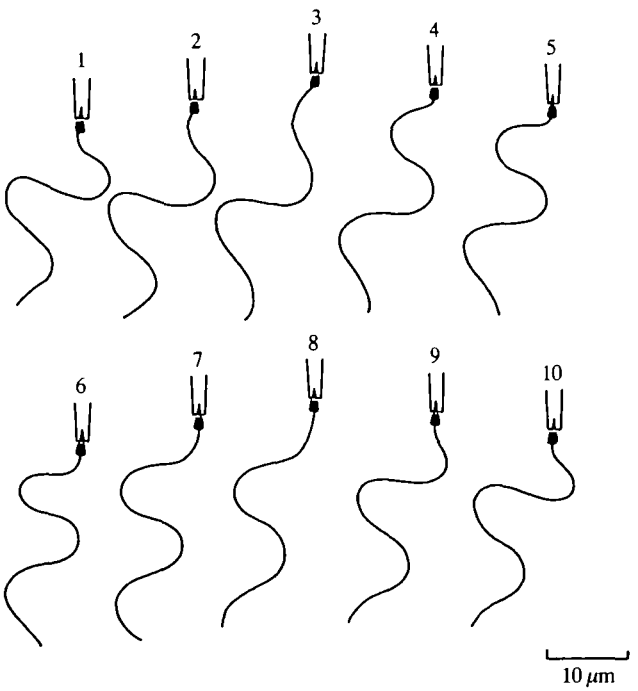

Fig. 11. Tracings of a sperm flagellum during axial vibration at twice its natural beat frequency. Beat frequency, 35.7 Hz; vibration frequency, 71.4 Hz; vibration amplitude, 6.1 μm.

compression and extension during each half beat cycle as a result of this vibration along the axis, the waveforms in the distal region appeared essentially unaffected. The results with an axial vibration frequency equal to the natural beat frequency (data not shown) resembled those at twice the natural beat frequency (Fig. 11), except for the occurrence of only a single cycle of compression and extension per

beat cycle of the bends in the proximal region. All sperm flagella studied (N=6) showed essentially the same response to vibration at the natural beat frequency and at twice that frequency.

To one of the sperm studied, we applied a broader range of vibration frequencies (40, 60, 70 and 80 Hz, compared to the natural beat frequency of 50 Hz). The response of the flagellum appeared basically similar to that described above, but the frequency of bend formation did not synchronize to the frequency of pipette vibration. This lack of synchronization made the effects on the waveform difficult to study in detail. In the middle and distal region of the flagellum, bends appeared stable and propagated with approximately the natural beat frequency. At low frequencies, such as 35 or 30 Hz, the beat became unstable and the bends appeared and propagated in different planes.

Although axial vibration appeared to have a relatively low degree of coupling to the mechanism governing flagellar beat frequency, it did have sufficient effect to lock the phase of beating to the phase of vibration when the vibration frequency was close to or twice the natural beat frequency (Fig. 11, and data not shown).

Discussion

General effects of imposed head vibration

In this paper, we demonstrate that a head vibration imposed perpendicular to the longitudinal axis of the spermatozoon can change most parameters of the flagellar movement, including beat frequency, bend angle and wavelength. A remarkable effect of this lateral vibration is that it can induce stable, regular beating over a very broad (nearly threefold) range of frequency. Together with the previously reported results on the rotation of the flagellar beat plane induced by changing the plane of imposed vibration (Gibbons *et al.* 1987), our results show that such vibration applied to the sperm head stabilizes the flagellar beating (1) by synchronizing the beat to the imposed movement so that the beat frequency equals the vibration frequency over the approximate range 35–90 Hz, and (2) by rotating the plane of the flagellar beat so that it coincides with the plane of pipette vibration. The observed synchronizing effects indicate plasticity and mechano-sensitivity of both the mechanism initiating new bends at the flagellar base and that determining the beat plane.

The changes in bend angle caused by lateral vibration of the head were smaller than the changes in beat frequency, while axial vibration of the head changed the bend angle in the proximal region greatly, with no significant effect on the beat frequency. These results support the view that the mechanism regulating the beat frequency is independent of the mechanism controlling the bend amplitude (Gibbons, 1974; Brokaw, 1977).

Unstable beating

When the vibration frequency was less than about 35 Hz, the flagellar beat became unstable and the beat plane often changed in an unpredictable manner

(Fig. 3A). In many cases the instability of the beat plane was associated with irregularity in the beat frequency. This may indicate that the mechanism governing bend initiation is associated with the mechanism determining the beat plane. Since increasing the vibration amplitude at these low frequencies did little to improve the stability of the beat, it seems unlikely that the velocity of pipette movement, or the force exerted by the external medium, is primarily responsible for the lower threshold for stabilization. Rather, the vibration seems to provide mechanical forces that modulate the timing of the intrinsic pacemaker that governs beat frequency by regulating the initiation of new bends at the flagellar base. There is a striking discrepancy between the percentages of frequency increase and decrease within which stable beating can be maintained. Increases are possible up to nearly 100 % above the natural beat frequency, whereas decreases are limited to about 25 %. This difference suggests that the upper and lower limits may be the result of different constraints on the beating. One possibility is that the upper limit is due to a limitation on the rate of energy supply while the lower limit is due to some unknown conflict between the intrinsic and the external rhythms.

Instability of a different nature occurred at high frequencies. When the vibration frequency was greater than a critical value that depended on the amplitude, a conspicuous change occurred in the asymmetry of the flagellar waveform. Both principal and reverse bends continued to be formed at the base of the flagellum, but the angles of reverse bends decreased very rapidly as they propagated along the flagellum (Fig. 3B). The stability of the beat plane was, however, not affected. In these asymmetrically beating flagella, the axis of flagellar beating was conspicuously skewed relative to the axis of the sperm head, and the beating often became arrested with the flagellum bent over sharply to one side. This position of arrest resembles that seen in Ca^{2+}-induced quiescence (Gibbons, 1980), suggesting that the two may represent different aspects of a common mechanism. One possibility is that the high-frequency vibration induces damage to a membrane that permits release of Ca^{2+} from an internal store. It should be noted that many spermatozoa were irreversibly damaged after a prolonged arrest response during high-frequency vibration.

Changes in wavelength

The present study has shown that the flagellar wavelength can be changed reversibly over a wide range, such that the flagellar length may comprise from only about one half wave to as much as two complete waves. Wavelength changes of somewhat smaller magnitude have been reported previously to be dependent upon changes in viscosity or in MgATP concentration (Brokaw, 1966, 1975; Okuno and Brokaw, 1979). The fact that the extensive changes observed here are possible indicates that the phase relationships of oscillatory sliding are not determined intrinsically by the length of the flagellum or by its structural characteristics. The observed differences in wavelength could be interpreted either in terms of the velocity of bend propagation in the middle and distal regions of the flagellum, calculated as the product of wavelength and beat frequency, or in terms of the rate

of viscous energy dissipation in these regions of the flagellum (Brokaw, 1966, 1975).

Calculations based upon the data in Fig. 8 indicate that the bend propagation velocity in the middle region of the flagellum, which is approximately 1400 $\mu m\,s^{-1}$ in sperm flagella beating at their natural frequency of 48 Hz, changes linearly with, but not in direct proportion to, the imposed frequency. Thus, when the imposed frequency is increased by about 230 %, from its lower limit of 30 Hz to its upper limit of 100 Hz, the bend propagation velocity increased by only about 60 %, from 1200 $\mu m\,s^{-1}$ to 1940 $\mu m\,s^{-1}$. This suggests that the changes in wavelength cannot be interpreted as being due to maintenance of a constant propagation velocity.

Sliding velocity

Our data indicate that the effects of head vibration on the apparent velocity of tubule sliding are quite different in the proximal and distal regions of the sperm flagellum. In the proximal region, the sliding velocity can increase by as much as 80 % from its natural value of $220 \pm 20\,rad\,s^{-1}$ to about $400\,rad\,s^{-1}$ as the beat frequency is increased from 45 to 100 Hz (Fig. 9), whereas the sliding velocity in the distal region of the flagellum remains essentially constant at its natural value over the same frequency range (Figs 9,10). This distinction between these two regions does not occur when the beat frequency is decreased, for in this case the apparent sliding velocity decreases with beat frequency to an equal extent in both the proximal and distal regions of the flagellum (Fig. 9; Table 1).

It is possible that the increase in apparent sliding velocity in the proximal region of the flagellum at high imposed beat frequencies may be due to passive sliding between the microtubules or to elastic distortion of the microtubules or some other component of the axonemal structure. This hypothesis is favoured by the decrease in angle of bends as they propagate along the flagellum at these frequencies, as well as by the observation that sliding velocities in the distal region do not appear to be able to exceed their natural value of about $220\,rad\,s^{-1}$.

However, we cannot exclude the possibility that the high apparent sliding velocities are due to active sliding that is able to occur at an enhanced velocity as a result of much of the normal viscous loading on this region of the flagellum being borne by the external forces on the head and the bulk fluid motion induced by the oscillating pipette. The highest value of the time-averaged velocity of sliding, about $400\,rad\,s^{-1}$, in the proximal region of a sperm flagellum being vibrated at high frequency (Fig. 10), corresponds to an average sliding velocity of approximately 22 $\mu m\,s^{-1}$ between doublets 3 and 4 (Takahashi *et al.* 1982), whereas sliding velocities at vibration frequencies close to the natural beat frequency, about $240\,rad\,s^{-1}$, correspond to 13 $\mu m\,s^{-1}$. Comparable values of 10–14 $\mu m\,s^{-1}$ for the average sliding velocities have been reported in trypsin-treated axonemes undergoing sliding disintegration in the presence of $1\,mmol\,l^{-1}$ ATP (Gibbons, 1974; Yano and Miki-Noumura, 1980; Takahashi *et al.* 1982).

The present study has shown that the flagellar beat frequency can be modulated by imposed head vibration over a wide range without loss of either stability or

regularity of the waveform. The changes in beat frequency are accompanied by changes in sliding velocity of the microtubules. This would indicate that in normally beating flagella the sliding velocity does not depend solely upon 'local' variables such as ATP concentration, but is also controlled by a mechanism closely associated with the mechanism for the initiation of rhythmic bending waves. The nature of such control mechanisms is still unknown. It would be interesting in this connection to study the effect of imposed vibration on the beating of demembranated flagella in various concentrations of ATP.

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