THE STIFFNESS OF THE FLAGELLA OF IMPALED BULL SPERM

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ABSTRACT The elastic rigidity (stiffness) of impaled motionless bull sperm flagella has been determined by a manipulatory technique which permitted direct analytical treatment of the experimental system. The effects of external ATP and ADP were measured. It was found that ATP acts as a plasticizing agent, while ADP does not. The stiffness measured for flagella in a medium without ATP was 15 times greater than the value measured with 10 mM ATP present. The rigor-like stiffness measured with no ATP present is reversible with ATP and seems to be correlated to a transition in the state of the contractile system.

INTRODUCTION

Any comprehensive model of the behavior of ciliary and flagellar systems is dependent on a reliable estimate of the mechanical properties of the system. The presently available estimates of the stiffness of flagellar and ciliary systems have been obtained by analysis of the motion of the active systems (Rikmenspoel, 1965 *b;* Rikmenspoel, 1966; Rikmenspoel and Sleigh, 1970). In such an analysis the value of the stiffness obtained is meaningful only if the theory used in the analysis applies. The method used by Rikmenspoel to obtain a stiffness estimate for bull sperm flagella is based on the assumption that the wave form reflects the mechanical properties of the system (Machin, 1958; Rikmenspoel, 1965 *b*). This assumption has been questioned by other investigators (Browkaw, 1971; Lubliner and Blum, 1971). Because of this problem we have determined the stiffness of the flagella of bull sperm in a manner which should be independent of the model for the contractile event.

The stiffness of flagella has also been assumed heretofore to be expressible as the elastic rigidity, a well-defined physical property of solids, and theoretical work on cilia and flagella has proceeded on this assumption. In the present study the behavior of inactivated flagella is compared with that of a purely elastic model.

We have also tried to determine whether the passive stiffness reflects the condition of the contractile system. We have previously reported that external ATP and ADP

will enter impaled bull sperm and are able to sustain flagellar activity (Lindemann and Rikmenspoel, 1971, 1972 *a* and *c*). The effect of ATP and ADP on stiffness is presently reported.

METHODS

Bull semen diluted to five times its volume with citrate-egg yolk diluent (Rikmenspoel, 1965 *a*) was generously supplied by the Eastern Artificial Insemination Cooperative (Ithaca, New York). Sperm were centrifuged twice and resuspended each time in 2 ml of the working medium which contained: 0.072 M potassium methylsulfonate, 0.163 M sucrose, 0.005 M magnesium sulfate, 2–5 μM calcium chloride, 0.007 M sodium lactate, and 2% dibasic sodium phosphate buffer solution (0.1 M). pH of the medium was 7.5. This working medium was developed for use in microdissection studies on bull sperm (Lindemann and Rikmenspoel, 1972 *a*).

Sodium ATP and ADP were added to a 10 ml sample of working medium to obtain the concentrations desired for each experiment. The pH of this solution was adjusted to 7.5 before the addition of 3–4 drops of the prepared sperm suspension. 1 ml of the final preparation was then placed in a special microscope slide chamber (Lindemann and Rikmenspoel, 1971) for observation.

The chamber temperature was maintained at 20°C during experiments. The experimental medium has a viscosity at this temperature which is not measurably different from that of citrate-egg yolk diluent at 37°C (using the viscometer described in Lindemann and Rikmenspoel, 1972 *b*). The drag coefficient (k_0) applied in earlier stiffness determinations (Rikmenspoel, 1965 *b*) was therefore used in the present work.

Observations and film records were made using a Zeiss Universal microscope (Carl Zeiss, Inc., New York) and a Bolex H-16 motion picture camera (Paillard S. A., Sainte-Croix, Switzerland). The microscope was equipped with a Zeiss × 40 water immersion objective which has a working distance of 2 mm between the objective and the slide. A drawn glass capillary microprobe was used to impale and manipulate individual sperm. The probe was held in a piezoelectric driver (Rikmenspoel and Lindemann, 1971) which made possible the rapid advance of the probe to impale a sperm head. The piezoelectric driver was in turn mounted on a Brinkmann CP VI manipulator (Brinkmann Instruments, Inc., Westburg, N. Y.). A detailed description of the apparatus appeared in an earlier paper (Lindemann and Rikmenspoel, 1971).

Bull sperm prepared as described above stick to the glass slide by their heads; their flagella usually remain free from the slide and retain motility. Under these circumstances the cell was localized well enough to permit dissection or manipulation with the microprobe.

We have reported earlier (Lindemann and Rikmenspoel, 1971; Lindemann and Rikmenspoel, 1972 *a*) that bull sperm which have been impaled with a microprobe will lose all activity shortly after impalement if ATP and ADP are not present in the external medium. It was also shown that the range of ATP concentrations which will activate impaled bull sperm is 0.05–1 mM. Outside of this range cells are inactive after impalement. Furthermore, while concentrations of 5–15 mM ADP have been shown to be effective in producing flagellar activity in impaled cells this action can be completely blocked by concentrations of ATP greater than 1 mM (Lindemann and Rikmenspoel, 1972 *c*). It is therefore possible to obtain a wide range of ATP and ADP concentrations in the external medium of the bull sperm which will allow deactivation of the flagellum by impalement.

Cells were deactivated by impalement at external ATP and ADP concentrations which do not activate the flagellum. Each cell was first impaled through the head with the micro-

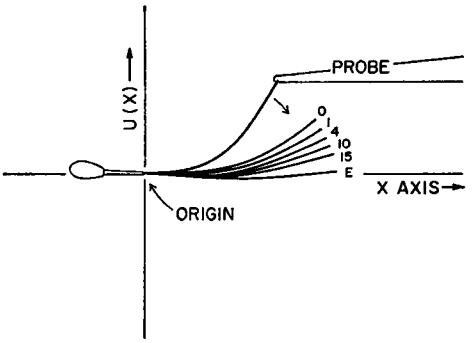


FIGURE 1 A tracing of relaxation of sperm in 10 mM ATP. Several frames are omitted after release from the electrode. Frames used in analysis are traced and numbered. The coordinates used in model analysis are shown. Position E is the equilibrium position of the flagellum after relaxation.

probe and the head was pressed to the slide to insure that it was securely stuck. The probe was passed under the flagellum to determine that it was free from the slide and then positioned near the distal end of the flagellum. Once in position the probe was used to displace the flagellum laterally (typically 5–30 μm). The flagellum usually sprang away from the probe as the lateral displacement was increased. The relaxation of the flagellum to its initial position was filmed (Fig. 1). To avoid error due to interference by the water boundary layer near the slide, cells with the flagellum clearly above the plane of the slide were preferentially selected. An effort was made to measure several cells at each ATP or ADP concentration and take several measurements on each cell. Data reported for each set of conditions are based on the recording of at least two cells each measured at least twice.

The film records were screened and those flagella of which the relaxations did not encounter interference from debris in the medium were selected for analysis. Frame-by-frame analysis of films was carried out on a Vanguard motion analyzer (Vanguard Instrument Corp., Melville, N. Y.). The change in the deviation of the flagellum from the equilibrium position was measured as the flagellum returned to its equilibrium position. An accuracy of ± 0.1 μm was obtainable using the Vanguard motion analyzer and selecting a well-focused portion of the flagellum. Camera speed was determined from timing marks on the edge of the film emulsion.

THEORY

The behavior of a passive flagellum under the conditions described above (refer to Fig. 1) is such that a simple mathematical description can be used to determine the stiffness. The flagellum constitutes a rod of known length l. The rod is secured at one end, and during relaxation it is free at the other end. Therefore, the boundary conditions which can be assigned to this system are:

$$0 = U(x = 0) = \frac{dU(x = 0)}{dx} = \frac{d^2U(x = l)}{dx^2} = \frac{d^3U(x = l)}{dx^3}, \qquad (1)$$

where x is the coordinate along the length of the flagellum, $U(x)$ is the displacement

of the flagellum away from the equilibrium position, and l is the x coordinate of the tip of the flagellum. A small amplitude approximation of the equation of motion of an elastic rod in a viscous medium is:

$$\frac{\partial^4 U(x, t)}{\partial x^4} = -c \frac{\partial U(x, t)}{\partial t}, \tag{2}$$

where

$$c = \frac{k_0}{IE}, \tag{3}$$

where IE is the elastic rigidity or stiffness, t is time, and k_0 is the effective drag coefficient. Eq. 2 is a fourth order partial differential equation which can be solved for the boundary conditions specified in Eq. 1 by substituting

$$U(x, t) = \exp(-t/\tau)\, Z(x). \tag{4}$$

We separate the variables and obtain

$$\frac{d^4}{dx^4} Z(x) = \lambda^4 Z(x), \tag{5}$$

where

$$\lambda^4 = c/\tau. \tag{6}$$

For the boundary conditions:

$$Z(0) = Z'(0) = Z''(l) = Z'''(l) = 0, \tag{7}$$

Eq. 5 yields a series of solutions which can be found by standard techniques (see Sommerfeld, 1949). The secular equation which gives the eigenvalues for λ is

$$\cosh \lambda l \cdot \cos \lambda l = -1. \tag{8}$$

The solutions of Eq. 8 can be found numerically:

$$\lambda_j = \mu_j / l, \ (j = 1, 2, 3 \ldots), \tag{9}$$

with

$$\mu_1 = 1.87 \ldots$$

$$\mu_2 = 4.69 \ldots$$

$$\mu_3 = 7.85 \ldots \text{ etc.}$$

Since the experimental conditions impart a monotonic bend on the flagellum the shape of the flagellum largely conforms to the first mode represented by λ_1. Each

of the modes represented by μ_1, μ_2 . . . of Eq. 9 has a decay time $\tau_{1,2}$ . . . . By combining Eqs. 3, 6, and 9 it can be seen that

$$1/\tau_j = \left(\frac{\mu_j}{l}\right)^4 \cdot \frac{IE}{k_0}. \tag{10}$$

For j larger than 1 the rate of decay $1/\tau_j$ is much faster than that of the first mode ($j = 1$). In the experimental case we may therefore write for the measured decay time τ_e

$$1/\tau_e = \left(\frac{\mu_1}{l}\right)^4 \cdot \frac{IE}{k_0}. \tag{11}$$

The values of k_0, and l are known (Rikmenspoel, 1965 *b*) and are $k_0 = 2.1 \times 10^{-2}$ dyn cm^{-2}-s and $l = 0.005$ cm. Solving for IE in terms of τ_e we can write:

$$IE = 1.06 \times 10^{-12}/\tau_e \text{ dyn cm}^2. \tag{12}$$

Using this formula stiffness values were found for experimentally measured decay times.

Bull sperm flagella are known to taper significantly over their length. Since Eq. 12 applies to an untapered rod, stiffness values obtained using this equation must be corrected to take the taper of the flagella into account. A suitable approximation of the taper is given by (Rikmenspoel, 1965 *b*):

$$R = R_0\left(1 - \frac{x}{L}\right), \tag{13}$$

which is the form of a trunkated cone, where L is the taper length (at which the radius is 0) and R_0 is the radius at the head end of the flagellum and R is the radius at x. IE is dependent on the fourth power of the radius and can be expressed as:

$$IE = I_0E\left(1 - \frac{x}{L}\right)^4. \tag{14}$$

A computer program was available for a large amplitude form of Eq. 2 and was used to simulate a relaxing flagellum. This equation has been put forth by Rikmenspoel and Rudd (1972) and will be discussed in full elsewhere. The initial conditions for the computer simulation were modeled after the experimental conditions where:

$$IE\frac{d^2U}{dx^2} = F(l - x), \tag{15}$$

F is the force applied at the tip of the flagellum to displace it.

Computations were done to simulate the relaxations of a rod at different tapers (l/L) to determine the shape of the relaxing rod as taper is varied. This allowed us

to find a taper value for the flagella by correlating the experimental tail shape during relaxation with the computer simulated tail shapes for various tapers, and the correction factor due to the taper which applies to the value of *IE* derived with Eq. 12.

RESULTS

The time-course of the displacement of the flagella while relaxing towards the equilibrium position was plotted on a semilogarithmic scale. The first point used in plotting $[U(x, 0)]$ was always taken one or more frames after the release of the flagellum from the probe. This insured that the flagellum was already in the process of relaxing at time zero. Two plots of the data appear in Fig. 2. The same cell was often measured at two positions along the flagellum (also shown in Fig. 2). The decay times varied very little with position, serving to verify that a first mode solution of the motion equation is adequate.

The stiffness measured for the flagella of impaled sperm after correction for taper (see below) in a medium containing no ATP or ADP was 53×10^{-12} dyn cm^2 (see Table I). Therefore, without ADP or ATP the flagella were 30 times stiffer than the

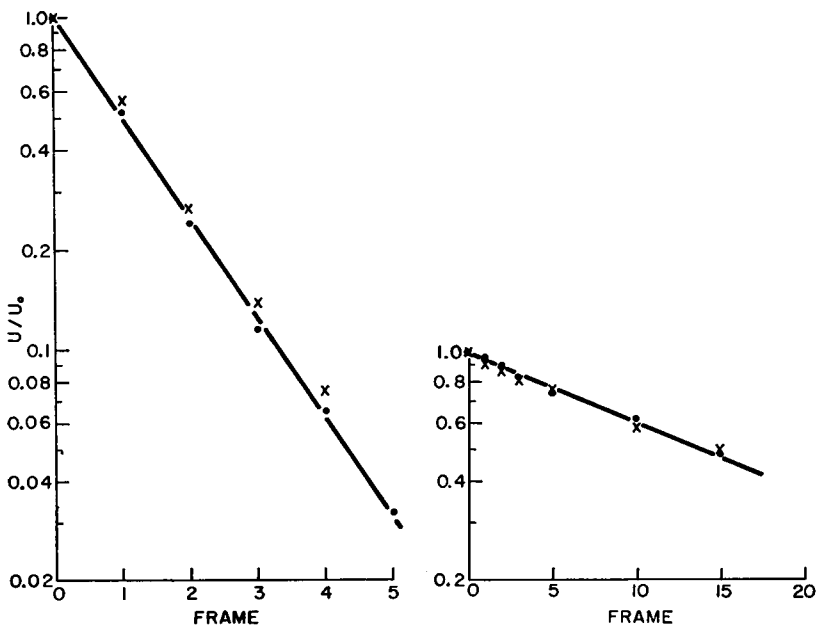


FIGURE 2 The relaxation of flagella. Vertical axis is displacement (U) divided by displacement at first frame analyzed (U_0). Left: relaxation of bull sperm flagellum without ATP present. ×, measured halfway down the length of the flagellum; ●, measured 15 μm proximal to the tip. Note that the two graphs have different horizontal scales. Right: relaxation of bull sperm flagellum in 10 mM ATP. ×, measured near the tip of the flagellum; ●, measured 10 μm proximal to the tip.

TABLE I

AVERAGE DECAY TIMES ($\bar{\tau}_e$) AND STIFFNESS VALUES (IE) FOR BULL SPERM WITH EXTERNAL ATP AND ADP VARIED

Ejaculates	Condition	No. of measurements	$\bar{\tau}_e$	IE	I_0E, corrected for taper
			ms	10^{-12} *dyn cm²*	
1, 2, and 5	Impaled, no ATP or ADP	9	36 ±10	29	53
5	Impaled 0.01 mM ATP	4	43 ±7	24	44
5	Impaled 0.02 mM ATP	5	36 ±4	29	53
5	Impaled 0.02 mM ADP	5	39 ±7	27	49
4	Impaled 2 mM ATP	4	172 ±22	6.1	11
4	Impaled 2 mM ATP 10 mM ADP	4	166 ±39	6.4	12
1 and 2	Impaled 5 mM ATP 5 mM ADP	5	213 ±84	5.0	9.0
5	Impaled 10 mM ATP	5	462 ±30	2.3	4.1
4	Impaled 10 mM ATP 2 mM ADP	5	501 ±114	2.1	3.8
3	Impaled 10 mM ATP 10 mM ADP	5	477 ±87	2.2	4.0
1	Stopped with KCN, not impaled	4	40 ±7	26	47
1	Stopped with KCN, impaled 5 mM ATP 5 mM ADP	5	162 ±24	6.5	12

The KCN inhibited samples were in working medium which contained 4 mM deoxyglucose. 40 μl of 0.1 M KCN was administered to these preparations during microscopic observation. Measurements were restricted to cells which were active before impalement or KCN inhibition.

stiffness estimated by Rikmenspoel for motile sperm (1.8×10^{-12} dyn cm²). When ATP was included in the medium at a concentration of 2 mM, the flagella were markedly less stiff. The stiffness could be decreased still further by concentrations of ATP greater than 2 mM. At 10 mM ATP the average measured stiffness was 4.0×10^{-12} dyn cm². ADP is not capable of mediating a similar effect, since the stiffness does not show any correlation to the concentration of that compound. The results of the stiffness determination for passive flagella under conditions of varied ATP and ADP concentration are compiled in Table I.

The results of KCN inhibition with cells that are not impaled is similar to the effect of impalement when ATP is not present. The KCN induced stiffening can be reversed if the cells are opened after inhibition and ATP is present externally. This indicates that the chemical event which raises the stiffness in the absence of ATP is not an irreversible change accompanying cell death.

Correction for Taper

The shape of the curves computed for rods of varied taper when compared with the measured profiles of flagella allowed the interpolation of an approximate taper value for bull sperm flagella. An example comparison of experimental and computed curves appears in Fig. 3. The curve presented for the real flagellum has been corrected for natural shape irregularities by subtracting the real equilibrium position from the measured displacement. A taper value (l/L) of 0.6–0.7 was obtained in this way. This analysis was carried out for three cells spanning the stiffness range and the results for each were consistent within the limit of accuracy of the technique (± 0.1).

From the computer simulation it was also possible to find the change in the decay time of a rod of constant I_0E as the taper is varied (Fig. 4). From this relation a correction factor may be interpolated for a flagellum with a taper of 0.65. This value is 1.8 ± 0.2 and has been used to correct the stiffness values obtained using the analytical solution for an untapered rod. This correction appears in the last column of Table I.

The final corrected stiffness values have been plotted as a function of ATP concentration in Fig. 5. The graph includes values from experiments where ADP was present, but is plotted only as a function of ATP concentration. The ATP activation range for impaled sperm (from Lindemann and Rikmenspoel, 1972 *a*) has been indicated on the graph. Measurements within that range of ATP concentration were not possible because of the motion of the flagella after impalement. It can be seen that ATP in concentrations below the value necessary for activation elicited no

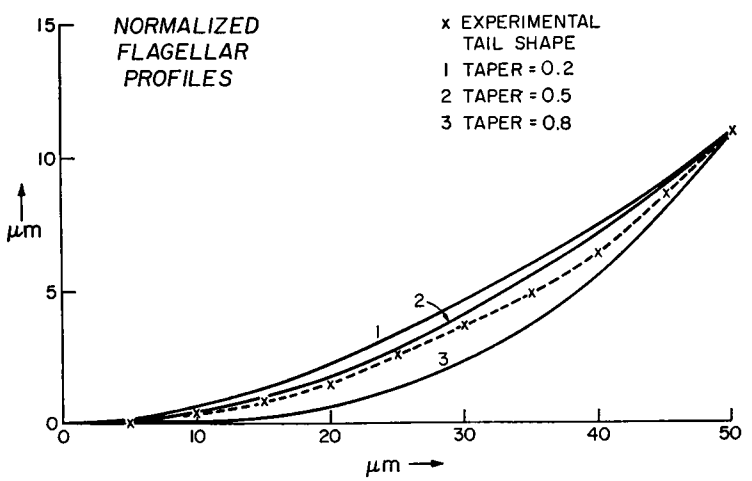
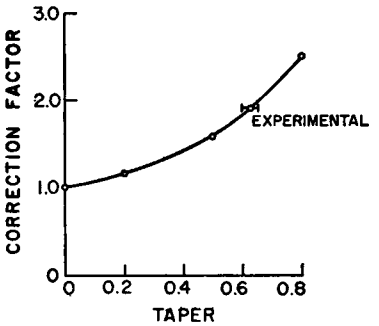


FIGURE 3 Taper determination. Solid curves are plotted from computed relaxation of 50-μm elastic rods at tapers (l/L) of 0.2, 0.5, and 0.8. The dashed line corresponds to the measured shape of a relaxing flagellum with the same displacement at its tip as the computed models. Interpolation between curves at five points along the x axis was used to determine the taper of the experimental curve, the average value is 0.65.



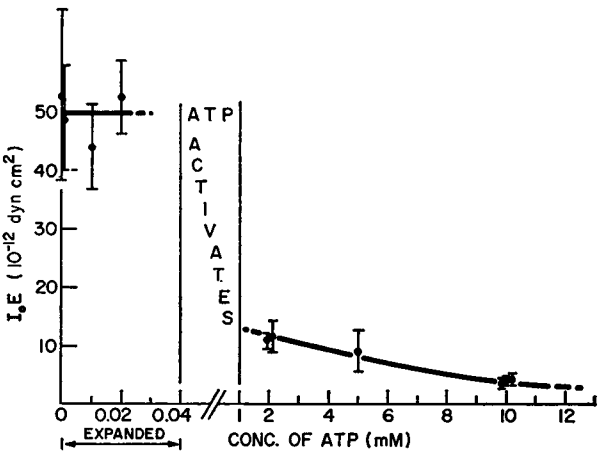


FIGURE 4

FIGURE 5

FIGURE 4 Correction factor due to taper which must be applied to stiffness values obtained with Eq. 12 (see text). The best estimate for the taper of bull sperm flagella (see Fig. 3) gives a correction factor of 1.8 ±0.2.

FIGURE 5 The stiffness of bull sperm flagella plotted as a function of ATP concentration. Measurement was not possible in the portion of the graph labeled "ATP activates" due to the active motion of sperm in this ATP range. Some of the points plotted are from experiments where ADP was also present (multiple points at some ATP concentrations). Note that this does not affect the grouping of points.

marked change in stiffness. Above the activation range the stiffness values are consistantly lower and do not exceed 12×10^{-12} dyn cm^2, which is less than one-fourth of the average value of 49×10^{-12} dyn cm^2 for cells at ATP concentrations below 0.05 mM. Therefore, a large discontinuity in the stiffness occurs across the activation range which represents an ATP concentration change of only 1.95 mM. A much smaller change in stiffness is associated with increasing ATP concentration above the activation range.

The value of 4.0×10^{-12} dyn cm^2 obtained for the stiffness of bull sperm flagella at 10 mM ATP concentration differs by approximately a factor of two from the estimate of 1.8×10^{-12} dyn cm^2 made by Rikmenspoel (1965 *b*) for the active system. Extrapolating Fig. 5 to greater ATP concentrations could possibly lower the minimum stiffness value. Direct measurement, however, becomes difficult for values of τ_e greater than 500 ms. At 10 mM ATP flagella often would not return all the way to the original equilibrium position. This may be due to the sensitivity of a flaccid flagellum to interference from debris and water boundary layers. It could also represent a hysteresis due to a viscoelastic component at high ATP concentrations. The observed exponential decay of the flagellum when related to the original equilibrium position, however, indicates that the second explanation is rather unlikely.

DISCUSSION

ATP has long been known to plasticize glycerinated muscle (Szent-Gyorgyi, 1951; Bozler, 1951, 1954). The conditions necessary for rigor, activation, and relaxation of muscle are well defined. We have now been able to demonstrate a similar set of conditions for rigor, activation, and relaxation in the flagella of impaled bull sperm. One difference, however, separates the behavior of the two systems. There is an apparent lack of an activating substance which could serve a role similar to that of Ca^{++} in muscle (Bishop and Hoffmann-Berling, 1955; Lindemann and Rikmenspoel, 1972 *a*).

ADP does not affect the stiffness measured for impaled bull sperm flagella. It does, however, induce motility at 5–15 mM concentration if ATP is not present. Sperm activated by ADP do not appear to be extremely stiff. ADP probably acts on the contractile system only after localized conversion to ATP via a myokinase system in bull sperm. The ATP produced could then act to plasticize the flagellum. A determination of the stiffness of ADP activated sperm is in progress and should decide this point.

The flagellar stiffness at high ATP concentrations is a factor of two greater than the value found earlier for motile sperm from a theoretical analysis of the flagellar motion (the "dynamic" stiffness). The very rigid condition of the sperm flagella at low ATP concentrations is probably caused by the contractile elements. As relaxation of the contractile elements is induced by ATP the minimum stiffness obtainable would be that of the passive flagellar sheath plus the remaining contribution of the relaxed contractile fibers (see Bahr and Zeitler, 1964; Fawcett, 1958; Rothschild, 1962 for details of the structure).

The stiffness change we measure could be caused by a change in the Young's modulus of one or more of the components of the flagellum. It could, however, also represent a variation in the degree of cross bridging of the internal force producing elements with each other. It has been demonstrated that in sea urchin flagella the outer tubules of the axoneme do indeed form ATP sensitive cross bridges (Summers and Gibbons, 1971). Therefore, it is not unlikely that changes in cross bridging between the internal active elements of a bull sperm flagellum could account for the stiffness change we observe with ATP.

The measurement presented here for bull sperm and the recent work of Baba on Mytilus gill cilia (Baba, 1972) both yield stiffness values greater than those predicted from motion analysis (Rikmenspoel, 1965 *b;* Rikmenspoel and Sleigh, 1970). Our study, however, shows clearly that a certain component of the stiffness can be widely modified depending on the amount of ATP present, and that this change is probably due to a transition in the state of the active force producing elements.

The measurements described in this paper give a value for the stiffness IE of the flagellum, proportional to the drag coefficient k_0 of the flagellum, according to Eq. 11. The value for k_0 used in the above was identical with that used in the dynamic

wave analysis. Gray and Hancock (1955) have derived for a waving flagellum

$$k_0 = -\frac{4\pi\eta}{0.5 + \ln\left(\frac{a}{2\lambda}\right)}, \qquad (16)$$

where γ is the radius of the cross-section of the flagellum, λ is the wavelength, and η the external viscosity.

The value for k_0 obtained from Eq. 16 has been generally accepted for waving flagella because the correct values for the propulsive effect of flagella are obtained with it (Holwill, 1966). In our present experimental condition a wavelength for the flagellum cannot be meaningfully defined, however. During the return of our impaled flagella to the equilibrium position the motion is more like that of a rod moving parallel to its axis. The drag coefficient in that case is (Lamb, 1952)

$$k_0 = \frac{4\pi\eta}{0.5 - \gamma - \ln\left(\frac{Va}{4\nu}\right)}, \qquad (17)$$

where $\gamma = 0.577$ is the constant of Euler, V is the transverse velocity of the rod, and $\nu = 0.01$ stokes is the kinematic viscosity. The drag coefficient of Eq. 17 is different for different V, which in our case corresponds to different ATP concentrations.

As characteristic values for V and a to be inserted in Eq. 17 can be taken those halfway down the flagellum. The taper of the flagellum will to some extent compensate the increase of V towards the tip. When the ATP concentration is 10 mM, the characteristic values for $V \approx 20$ μm/s and $a \approx 0.3$ μm, give with Eq. 17 a value of $k_0 = 0.0095$ dyn cm^{-2} s, less than half the value used by us to arrive at the stiffness shown in Table I. The lower value of k_0 would give for sperm in 10 mM ATP a stiffness $I_0E = 1.8 \times 10^{-12}$ dyn cm^2, which is identical with the one found from the dynamic analysis.

Since the flagellum in our experiments was always curved (see Fig. 1), it cannot be considered strictly as a straight rod moving parallel to its axis and the value of $k_0 = 0.0095$ dyn cm^{-2} s should be considered a lower limit for the value of k_0 which is really applicable.

It is clear, however, that the passive value for the stiffness at high ATP concentration converges to the dynamic value. The latter value should, therefore, be considered as the meaningful one for theoretical analysis of flagellar motility.

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