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DIRECT MEASUREMENTS OF THE STIFFNESS OF ECHINODERM SPERM FLAGELLA

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SUMMARY

1. The stiffness (flexural rigidity) of some echinoderm sperm flagella was measured, using a flexible glass microneedle.

2. Values of $0{\cdot}3$–$1{\cdot}5 \times 10^{-21}$ N m^2 were obtained for the stiffness of live flagella which were immobilized with CO_2-saturated sea water.

3. The immobilized live flagellum was uniform in stiffness along its entire length, except in a particular plane of imposed bending in which flexible regions were observed.

4. Demembranated flagella (*Hemicentrotus pulcherrimus*) in an ATP-free solution were about ten times stiffer ($1{\cdot}1 \times 10^{-20}$ N m^2) than immobilized live ones ($0{\cdot}5$–$0{\cdot}9 \times 10^{-21}$ N m^2). The stiffness was decreased by addition of ATP to the solution and became equivalent to that of live ones when the solution contained 10 mM ATP.

5. In the demembranated flagella, the effects of ADP and ATP on the stiffness were similar. Other nucleotide phosphates and inorganic phosphate did not reduce the stiffness.

6. Young's modulus of microtubules is estimated to be 2–5×10^9 Nm^2 on the basis that the microtubules have no tight connexion with one another in immobilized live flagella.

INTRODUCTION

Detailed knowledge of the mechanical properties of the flagellum is important in understanding the mechanism of flagellar movement. The bending of the flagellum must be an active process generated in it or a passive one due to external forces applied to it (cf. Sleigh, 1974). Baba (1972) measured the stiffness (flexural rigidity) of compound cilia of *Mytilus* gill in the course of their beating stroke with a flexible glass microneedle. Lindemann, Rudd & Rikmenspoel (1973) determined the stiffness of impaled bull sperm flagella from the rate of recoil of the flagellum from passive bending with a microprobe. In bull sperm flagella, the axoneme of $9+2$ microtubules is surrounded by coarse fibres and it is possible that the stiffness of the coarse fibres contributes greatly to the stiffness of the flagellum.

In the present study, stiffness of a flagellum which has a simple basic structure consisting of $9+2$ microtubules, the echinoderm sperm flagellum, was measured by bending it with a flexible glass microneedle.

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MATERIALS AND METHODS

The spermatozoa used were from the sea urchins *Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus*, the starfish *Asterina pectinifera* and the sand dollar *Astriclypeus manni*. Semen was collected by injecting 0·5 M-KCl into the body cavity in the case of sea urchins and sand dollar and by dissecting testes out of the bodies in the case of starfish. The semen was diluted to 1000 times with artificial sea water (ASW, containing 0·47 M-NaCl, 55 mM-$MgSO_4$, 10 mM-$CaCl_2$, 5 mM-KCl, and 1 mM-$NaHCO_3$, pH 8·2). At that time, most spermatozoa of sea urchins and sand dollars swam actively, but those of starfishes were inactive with straight flagella. The sperm suspensions of sea urchins and sand dollars were diluted 100 times with CO_2-saturated artificial sea water (CO_2-ASW), pH 5, which immobilized the sperm flagella. Spermatozoa recovered their motility when they were transferred to normal ASW again. In the case of starfish, immobilized sperm suspension was obtained by diluting the semen with normal ASW. Immobilized starfish spermatozoa were also prepared by activating them with ASW containing 1 mM histidine (Fujii *et al.* 1955), followed by dilution into CO_2-ASW.

Demembranated spermatozoa of the sea urchin (*H. pulcherrimus*) were prepared according to the method of Gibbons & Gibbons (1972) with a little modification: one volume of semen which had been diluted 1:9 with CO_2-ASW was added to 20 volumes of extraction solution (0·15 M-KCl, 2 mM-$MgSO_4$, 0·5 mM-EDTA, 0·5 mM-β-mercaptoethanol, 2 mM-Tris-HCl buffer, and 0·04% (v/v) Triton X-100, pH 8·0). The mixture was gently agitated for 40 s at room temperature and kept at 0 °C for up to 2 h until use. These demembranated spermatozoa were diluted 1:100 into working solution. Three kinds of working solutions were used. WS-1 contained 0·15 M-KCl, 2 mM-$MgSO_4$, 0·5 mM-EDTA, 0·5 mM-EGTA, 5 mM-DTT (dithiothreitol) and 20 mM-Tris-HCl buffer, pH 8·0; WS-2 had the same composition as WS-1 except that $MgSO_4$ was omitted; WS-3 had the same composition as WS-1 except that Tris-HCl buffer was exchanged for 20 mM-Na_2HPO_4-NaH_2PO_4 buffer. The demembranated spermatozoa could be reactivated in WS-1 and WS-3, if an appropriate amount of ATP was added (0·05 mM ATP was enough to induce the reactivation while 0·005 mM was not). Demembranated spermatozoa in WS-2, lacking $MgSO_4$, could not be reactivated by addition of ATP.

The live or demembranated sperm suspensions were poured into a chamber of 1 mm depth. One of the spermatozoa was fixed by sucking on its head with a braking micropipette (cf. Hiramoto, 1974) inserted through one of the side openings into the chamber. Then the position of the flagellum (100–200 μm below the coverslip) and its inclination were adjusted by tilting and rotating the micropipette so that the flagellum could be clearly observed along its entire length. The stiffness of the flagellum was determined as follows (Fig. 1*a*). A force was applied to the middle region of the flagellum (P_1 in Fig. 1*a*) by pushing it with a flexible microneedle (Nm) inserted into the chamber while the flagellum was supported with a stiff microneedle (Ns) at its distal region (D, about 30 μm from the base). The force applied to the flagellum at P_1 was determined from the amount of displacement of the microneedle Nm ($\overline{P_1P_2}$) when the flagellum was removed from Nm. The strength of the microneedles was

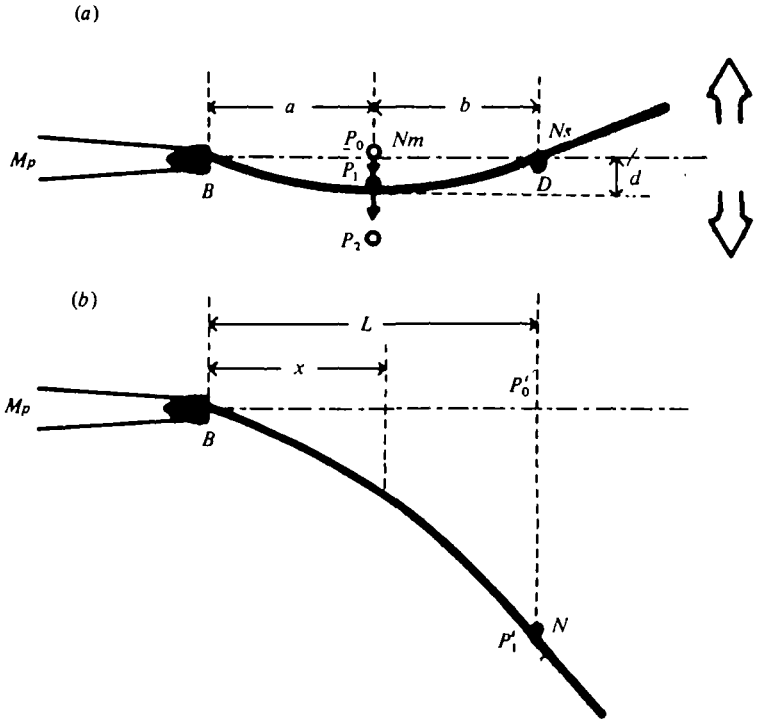

Fig. 1. Technique for measuring the stiffness of immobilized sperm flagella. (*a*) The head of the sperm is fixed at the tip of the micropipette (Mp), and the distal portion of the flagellum is supported with a microneedle (Ns) at point D. Another microneedle (Nm) pushes the flagellum at point P_1, having displaced this point of the flagellum from point P_0. The needle moves to P_2 after the removal of the flagellum. The stiffness is determined from Eqn (1) (see Materials and Methods). (*b*) The fixed flagellum is pushed with a microneedle (N) at point P_1 and is laterally displaced. The relative stiffness is calculated at points every 2 μm along the flagellum.

determined before experimentation (cf. Yoneda, 1960), and was $0{\cdot}8–5 \times 10^{-12}$ N (1 μm displacement at the tip). The calibration errors of the microneedles were within 20%. The change of position of the microneedle and deformation of flagella were photographically recorded on 35 mm film after they attained the equilibrium positions (about 5 s or more after application of a force or removal of flagellum).

A Nikon phase contrast microscope with BM 40× objective and HKW 10× ocular was used throughout the experiments. All measurements were carried out at room temperature (20 ± 1 °C).

If the flagellum is regarded as a uniform elastic rod and if it is assumed that the flagellum is supported freely both at the joint of the flagellum to the sperm head (B in Fig. 1*a*) and at the point where the supporting microneedle attaches (D in Fig. 1*a*), and that the deformation of flagellum is small enough, the average stiffness (*EI*) of the flagellum is expressed by

$$EI = \frac{Fa^2b^2}{3(a+b)\,d}, \qquad (1)$$

where F is the force applied to the flagellum at the point P_1; a and b the distances of P_0 from B and from D, respectively; and d the displacement of point P_1 ($\overline{P_0P_1}$) by the application of the force.

The relative stiffness of the elements of the flagellum at various points along the flagellar shaft was determined by the method shown in Fig. 1 (*b*). A flagellum fixed by the micropipette at the head of the spermatozoon (as mentioned above) was bent by a microneedle in the direction normal to the flagellar axis. If the curvature of the flagellum at distance x from the base (B in Fig. 1 *b*) is κ, the stiffness at this point, $EI_{(x)}$, is represented by

$$EI_{(x)} = (L-x)\,F/\kappa, \tag{2}$$

where L is the length between B and the point P_0', where the force, F, is applied. Relative values of stiffness at various points along the flagellum could be expressed by the function of $(L-x)/\kappa$, because F is constant in this case. In practice, the curvature at a point of the flagellum was obtained from the angle formed by two tangents at the points on the flagellum 2 μm apart from the point in question in opposite directions, one distal and the other proximal.

RESULTS

The stiffness of flagella of live spermatozoa

The stiffness of flagella of live spermatozoa was determined after they were immobilized as described in Materials and Methods. The mean stiffness of the entire length of the flagellum was measured by the method shown in Fig. 1 (*a*). The flagellum bent by application of external force regained its original straight shape when the force was removed unless the deformation was too large (greater than about 15 μm for $\overline{P_0P_1}$). Deformation of flagella was usually kept within 5 μm ($\overline{P_0P_1}$). Every flagellum was bent in two opposite directions, as shown by arrow heads in Fig. 1 (*a*). Usually, the values for both directions were equal within an experimental error so that the mean value was taken as the value for the flagellum in a particular plane of imposed bending. When the external force was applied, the tangent to the flagellum at its base usually appeared to deviate from the initial flagellar axis as illustrated in Fig. 1, especially in the case of the demembranated flagellum, suggesting that equation (1) was appropriate for analysis of the stiffness.

When the measurements were repeated in the same flagellum, the range of the stiffness values was within $\pm 30\%$ of the mean value, provided that the plane of imposed bending was unchanged. On the other hand, the values varied considerably even in the same flagellum when the plane of imposed bending was changed by rotating the flagellum about its axis. For example, the range of measured values of stiffness was determined to be $0{\cdot}3$–$1{\cdot}1 \times 10^{-21}$ N m^2 for an individual flagellum of *P. depressus* when the measurement was carried out at 30° increments about the flagellar axis, and $0{\cdot}6$–$1{\cdot}4 \times 10^{-21}$ N m^2 in the case of *A. manni*. Ranges for the species studied are given in Table 1.

The difference in stiffness of a single flagellum at various regions along the flagellar shaft was determined by the method shown in Fig. 1 (*b*). Fig. 2 shows typical results obtained with a *P. depressus* spermatozoon, where the relative stiffness is plotted against the distance from the base. The value would be constant if the flagellum were a uniform elastic rod. In this case, the results shown by open circles suggest that the flagellum had a more or less uniform stiffness along its axis. When the measurement

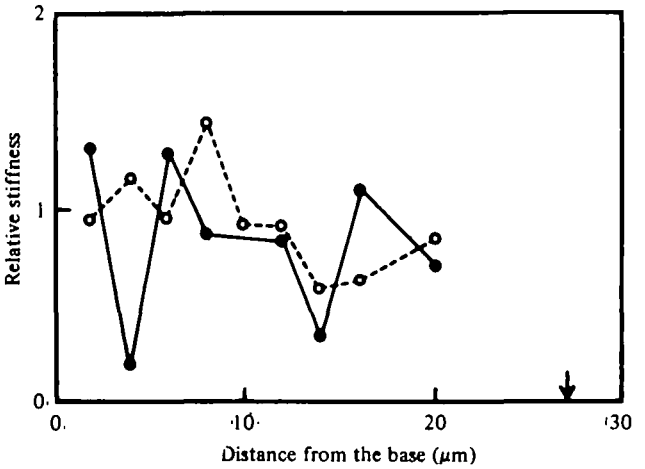

Fig. 2. Variation of relative stiffness along the flagellum. The flagellum is pushed with a microneedle at a point 27 μm (arrow) from the base. ○, Stiffness in a particular plane of imposed bending. ●, Stiffness measured after 60° rotation about the flagellar axis.

was carried out after 60° rotation about the flagellar axis, however, two flexible regions were recognized, as shown by solid circles in Fig. 2. These flexible regions were observed at 4 and 14 μm from the base and the stiffness values of these regions were $\frac{1}{8}$ and $\frac{1}{3}$ of the mean value of the other regions (adopted to be unity in this figure). Such flexible regions were often observed in one orientation of the plane of imposed bending, within an angular range of about 30°. Two flexible regions were sometimes observed, as in the case shown in Fig. 2; with other spermatozoa only one flexible region was found. The position of the flexible regions varied along the flagella, although if there were two, the distance between them was more or less constant (10–15 μm). The length of each flexible region was usually smaller than 4 μm.

The stiffness of demembranated sperm flagella

The spermatozoa of the sea urchin *H. pulcherrimus* were demembranated with Triton X-100, and suspended in the working solutions. The stiffness was measured by the method shown in Fig. 1(*a*). The stiffness was $1{\cdot}1 \times 10^{-20}$ N m^2 in all the working solutions without ATP, which is about ten times that of immobilized live flagella (Fig. 3). When ATP was applied at concentrations greater than 0·05 mM, in WS-1 and WS-3, the demembranated spermatozoa were reactivated as reported by Gibbons & Gibbons (1972), whereas they could not be reactivated in WS-2 (without magnesium) even in the presence of ATP. As shown in Fig. 3, the higher the ATP concentration was, the more flexible the flagella became. Also, the higher the ATP concentration, the less time was taken to attain the equilibrium position when the external force was applied to or removed from the flagella. The stiffness measured for the demembranated flagella in WS-2 containing 10 mM ATP was almost equal to that of immobilized live spermatozoa. The flexible regions as observed in live spermatozoa were not recognized in the demembranated spermatozoa, and the deviation of their stiffness values was smaller.

Phosphate did not alter the stiffness of flagella: the flagella in WS-3, which contained 20 mM phosphate, were as stiff as those in WS-1 and WS-2 (Fig. 3).

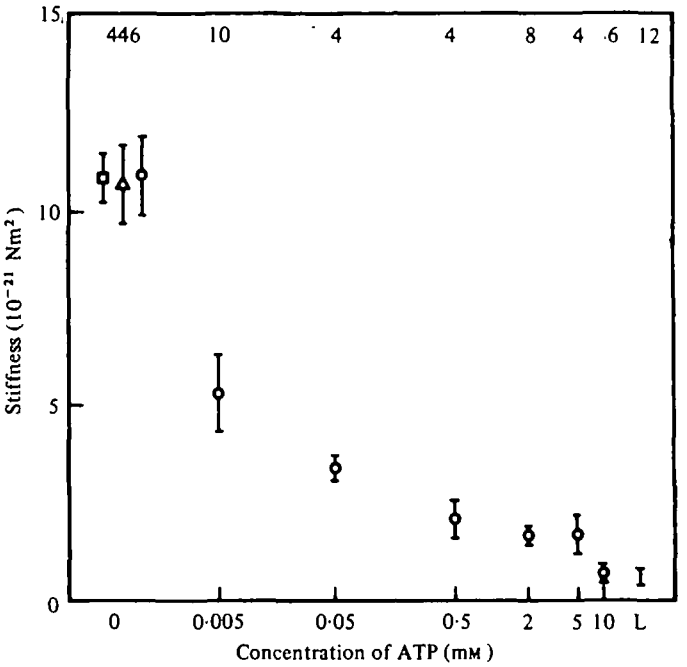

Fig. 3. Change in the stiffness of flagella with ATP concentration. The stiffness was measured in WS-2 (without magnesium) containing various concentrations of ATP (○). Numbers of spermatozoa measured are shown at the top of the figure. The stiffness of flagella in WS-1 and those in WS-3 (both of them with magnesium but without ATP) are shown by □ and △, respectively. The stiffness of immobilized live flagella is shown at the right end of the figure (indicated by L). Vertical bars at each point represent the standard deviation.

The effects on the stiffness of demembranated flagella of nucleotide phosphates other than ATP were examined at a concentration of 2 mM in WS-2. Among these phosphates, only ADP had an effect similar to that of ATP (Table 2).

DISCUSSION

It is possible that the stiffness values of sea urchin and sand dollar sperm flagella determined in the present study are different from those of spermatozoa in normal sea water, because the measurements were carried out with spermatozoa immobilized by immersing in CO_2-ASW with low pH. However, it would seem that neither CO_2 nor low pH has large effects on flagellar stiffness because the stiffness observed in starfish spermatozoa immobilized by CO_2-ASW after activation by histidine-ASW was similar to the stiffness observed in immobilized spermatozoa dissected into normal ASW.

The stiffness of flagella was calculated using equation (1) assuming that the flagella were uniform straight elastic rods. This is supported by the observation that echinoderm sperm flagella have a uniform structure, consisting of 9+2 microtubules, along the length of the flagella (Afzelius, 1959). It was also assumed that the flagella were supported freely at both ends, and this is supported by the observation that the microneedle could be moved smoothly along the flagellum, keeping a contact with it,

Table 1. *The stiffness of flagella in live spermatozoa, immobilized by CO_2 except where noted*

	Stiffness (10^{-21} N m^2)	No. of spermatozoa measured
Pseudocentrotus depressus	0·3–1·2	7
Hemicentrotus pulcherrimus	0·5–0·9	12
Astriclypeus manni	0·6–1·5*	7
Asterina pectinifera		
ASW†	0·6–1·2	4
CO_2-ASW‡	0·7–1·0	5

* One spermatozoon measured gave a value $3{\cdot}5 \times 10^{-21}$ N m^2, but others were in this range.
† Spermatozoa were suspended in ASW after dissection from the testes.
‡ Spermatozoa were suspended in CO_2-ASW after activation by histidine-ASW.

Table 2. *The effect of various nucleotide phosphates, at a concentration of 2 mM in WS-2, upon demembranated* H. pulcherrimus *sperm flagella*

Nucleotide phosphate	Stiffness (mean ± S.D. in 10^{-21} N m^2)	No. of spermatozoa measured
ATP	1·9 ± 0·2	7
ADP	2·0 ± 0·5	5
AMP	8·9 ± 1·9	6
c-AMP	9·0 ± 0·5	4
GTP	9·7 ± 0·7	6
None (rigour state)	10·9 ± 1·0	6

and that the angle between the head axis and the flagellar one at the base was fairly changeable by application of external forces. If the flagellar axis were fixed to the head and/or midpiece at its base, stiffness, EI^*, would be given by

$$EI^* = \frac{Fa^3}{12d}\left\{4 - \frac{a(2a+3b)^2}{(a+b)^3}\right\}, \tag{3}$$

where a, b, d, and F are the same measure used in equation (1). Because the microneedle was applied at the middle point of the flagellum between the two supported points in almost all of the present experiments (where $a = b$), $EI^* = 7Fa^3/96d = 0{\cdot}44EI$. Therefore the stiffness values would be calculated to be 0·44 times the values shown in Tables 1 and 2 if the flagellum were rigidly supported at its base.

The difference in stiffness depending on the plane of imposed bending and the appearance of flexible regions in a particular plane of imposed bending, observed in this study, may have a role in determining the bending wave plane of the flagella because there seems to be no difference in the efficiency of the active bending force on various planes of bending (Brokaw, 1977). However, this can only be resolved by further study.

The stiffness of the demembranated flagella in working solutions without ATP was about ten times that of immobilized live flagella. This may be because they were in rigour state (Gibbons & Gibbons, 1974). It seems that ATP 'plasticizes' the demembranated flagella and reduces their stiffness. Even a concentration of 0·005 mM rendered flagella more flexible than those in ATP-free working solutions, although

they were unable to generate bending waves. This state may correspond to that observed by Gibbons & Gibbons (1974) where the rigour waves of flagella gradually relaxed to a straight shape by their own elasticity. The relationship between ATP concentration and the strength of its effect may be interpreted if ATP is capable of inducing the dynein arms to detach from adjacent microtubules (Warner, 1978) so that the shear resistance between the microtubules decreases. Magnesium appeared indispensable for inducing flagellar movement (Hoffmann-Berling, 1955; Gibbons & Gibbons, 1972).

The ability of ADP to produce effects upon flagellar stiffness, similar to those of ATP, may be explained by its being converted to ATP via a myokinase system in the axoneme (Brokaw, 1961; Brokaw & Gibbons, 1973) or the contamination of ADP used by traces of ATP. ADP has already been observed to plasticize the stiffness in the demembranated sea urchin sperm flagella (Gibbons & Gibbons, 1974), amputated bull sperm flagella (Lindemann *et al.* 1973) and amputated starfish sperm flagella (Okuno & Hiramoto, 1976). ADP has also been observed to induce spontaneous beating (Lindemann & Rikmenspoel, 1972; Okuno & Hiramoto, 1976).

Brokaw (1966) estimated that more than 4 mM ATP should be necessary for the concentration at the base of the sea urchin sperm flagellum if ATP is supplied by diffusion from the base through the flagellar shaft. Nevo & Rikmenspoel (1970) estimated the necessary concentration as 16 mM by similar assumptions using different coefficients in the sea urchin sperm flagellum. The amount of ATP in sea urchin spermatozoa was measured by Rothschild & Mann (1950) and by Hultin (1958). Taking into account the volume of spermatozoa, their concentration in semen and the distribution of ATP, the concentration of ATP was estimated to be in the range of the values mentioned above. It is therefore reasonable that the stiffness of demembranated flagella in the WS-2 solution with 10 mM ATP is equivalent to that of live immobilized ones.

The stiffness observed in the present study are less than those obtained by Lindemann *et al.* (1973) in bull sperm flagella, which were 5×10^{-20} N m^2 in the absence of ATP and 4×10^{-21} N m^2 with 10 mM ATP. This is possibly because bull sperm flagella contain coarse fibres around the 9+2 microtubules, but Baba (1972) has recorded even higher values in cilia which have a similar structure to those of the present study; values of $2\text{–}3 \times 10^{-19}$ N m^2 in the component cilium of the compound cilia of *Mytilus* gill. In this case, the higher values may be because they were derived from experiments with beating flagella. If the elastic component of flagella mainly consists of microtubules of the axoneme, the stiffness of flagella is given by EI, where E is Young's modulus of microtubules and I is the second moment of area of the cross section of the axoneme. If it is assumed that nine outer doublets and two central singlets operate without any connexions with one another, the second moment of the axoneme, I_f, is given by Holwill (1965) as $1{\cdot}3 \times 10^{-31}$ m^4. This state is considered to correspond to the 'relaxed state' which is assumed to occur in the immobilized live flagella and the demembranated flagella in WS-2 with high concentration of ATP in the present experiments. If the microtubules act with tight connexion, the second moment of the axoneme is given by 3×10^{-29} m^4 (Baba, 1972). If the cross bridges of dynein arms are made among the outer doublet microtubules in beating cilia as Baba (1972) assumed, the difference between his results and the present study could be

explained to a certain extent. Furthermore, there is a possibility that he overestimated the stiffness of component cilia because he did not take into consideration the possible effects of cementing substance connecting component cilia. However, his values still seem higher than those of rigour flagella in the present study.

From the lowest values of stiffness of immobilized live flagella in the present study. $0{\cdot}3$–$0{\cdot}7 \times 10^{-21}$ N m^2 (Table 1), Young's modulus of microtubules can be estimated to be 2–5×10^9 N m^{-2} assuming the second moment of the cross section of the axoneme is $1{\cdot}3 \times 10^{-31}$ m^4, as mentioned above. This value for Young's modulus is reasonable for a protein fibre.

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