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FLEXURAL RIGIDITY OF SINGLET MICROTUBULES ESTIMATED FROM STATISTICAL ANALYSIS OF THEIR CONTOUR LENGTHS AND END-TO-END DISTANCES

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Microtubules in solutions, observed under a dark-field microscope, show incessant Brownian movement such as translational, rotational and flexing motion. A large number of microtubules, spontaneously stuck to the under surface of a coverslip, were photographed and the contour lengths and end-to-end distances of their images were measured. From the statistical analysis of the contour lengths and end-to-end distances, a value for the parameter λ representing the flexibility of singlet microtubules was estimated to be $\lambda = (6.8 \pm 0.8) \cdot 10^{-3} \mu\text{m}^{-1}$. From the value of λ , the elastic modulus for bending, ϵ , and Young's modulus, Y , of singlet microtubules were computed to be $\epsilon = \sim 10^{-16} \text{ dyne}\cdot\text{cm}^2$ and $Y = \sim 10^9 \text{ dyne}\cdot\text{cm}^{-2}$, respectively. The microscopic elastic constant, k , of bonding between two tubulin monomers neighboring along the singlet microtubule was computed to be $k = \sim 10^2 \text{ dyne}\cdot\text{cm}^{-1}$. A singlet microtubule is an order of magnitude as strong against bending and as weak against stretching as an F-actin filament.

Introduction

Indirect immunofluorescence staining of intact cells revealed that the framework of living cells, called the 'cytoskeleton', is made up of F-actin filaments, intermediate filaments and microtubules [1–5]. Recently it has been thought that these filaments structurally support the overall shape of cells. It thus became important to study the structural strength and rigidity of individual filaments, but only the mechanical properties of F-actin filaments have been reported [6–8].

Our purpose in this research has been to measure the flexural rigidity of singlet microtubules.

The principles of the measurement are as follows: (1) Singlet microtubules in solution can be observed under a dark-field microscope equipped with a high-brightness light source [9]; (2) When observed, these microtubules show incessant Brownian movement such as translational, rotational and flexing motion. If their images are recorded on photographs, the contour length, L , and end-to-end distance, R , of each microtubule can be measured. If an image of a microtubule is continuously recorded on videotapes or cinefilms, its L and R become measurable from frame to frame of the recorded images; (3) From the statistical analysis of L and R of filamentous polymers, we can estimate their flexural rigidity [10,11].

In the present study, a large number of microtubules, spontaneously stuck to the under surface of a coverslip, were photographed under a dark-field microscope, L and R of their images were statistically analyzed on the basis of Kratky-

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Abbreviations: Mes, 4-morpholineethanesulfonic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid.

Porod's equation [10], and values of the inverse of the statistical length, λ , the elastic modulus for bending, ϵ , and Young's modulus, Y , of singlet microtubules were estimated. A value of the microscopic elastic constant, k , of bonding between two tubulin monomers neighboring along a singlet microtubule was also estimated. Mechanical properties of singlet microtubules were compared with those of F-actin filaments and bacterial flagella.

In the following study [12], an image of a microtubule will be continuously recorded on a videotape with a dark-field microscope and a high-sensitivity TV camera. From the value of L and the time-averaged value of R , the λ value will be statistically estimated for each microtubule, and the average value of the parameter λ will be compared with the λ value estimated in the present study.

Materials and Methods

Partially purified tubulin was prepared from porcine brain by the method of Shelanski et al. [13] with some modifications. (1) Blood vessels were removed from porcine brain tissue. The brain tissue was washed three times with 10 mM sodium phosphate buffer (pH 6.8) containing 0.24 M sucrose and 10 mM MgCl₂. The tissue was then minced in a reassembly buffer solution (10 mM Mes-NaOH/1 mM ATP/50 mM KCl/0.5 mM MgSO₄/1 mM EGTA, pH 6.8) with a Waring blender for 3 min and further homogenized with a Teflon homogenizer for 3 min. The brain homogenate was centrifuged at 48 000 $\times g$ for 40 min. All procedures described above were carried out at 0°C. (2) The supernatant was mixed with an equal volume of the reassembly buffer containing 8 M glycerol, incubated at 35°C for 40 min, and centrifuged at 100 000 $\times g$ for 40 min at 35°C. (3) The pellet was suspended in the cold reassembly buffer, kept at 0°C for 10 min, and centrifuged at 100 000 $\times g$ for 60 min at 0°C. Step 2 (tubulin polymerization at 35°C) and step 3 (tubulin depolymerization at 0°C) were twice repeated for further purification of tubulin. The supernatant finally obtained in step 3 was used as a tubulin fraction in our experiments. SDS-gel electrophoresis showed that about 85% of the protein in this fraction was tubulin.

Protein concentration was determined by the biuret reaction [14], calibrated with solutions of bovine serum albumin of known concentration.

Temperature of the 2 mg/ml solution of tubulin was raised from 0°C to 25°C. This initiated tubulin polymerization to microtubules in the reassembly buffer solution. The viscosity of the tubulin solution, while in the process of polymerization, was repeatedly measured with Ostwald-type capillary viscometers having 0.6–0.8 ml of capacity and about 30 s of flow time for the reassembly buffer solution at 25°C. The solution viscosity increased to its maximal level about 0.5 h after initiation of the tubulin polymerization. According to electron-microscopic observation, singlet microtubules having cylindrical structure existed in the solution for at least 1.5 h after completion of the increase in the solution viscosity.

A drop of the reassembled microtubule solution was placed on a glass-slide and covered with a coverslip. The glass-slide was placed on a stage of a dark-field microscope (type SUR-K, a product of Nikon Co., Japan) equipped with a high-brightness light source [9]. Total magnification of the microscope was 600 \times , and observation was carried out within 1.5 h after completion of the increase in the solution viscosity in a room maintained at 25 \pm 1°C. While microtubules in the solution showed incessant Brownian movement, some of them came near and spontaneously stuck to the under surface of the coverslip. The stuck microtubules were photographed on a Kodak Tri-X Panfilm with an exposure time of 3–7 s.

Photographs of microtubules were projected onto a graphic-digitizer (type AM-02, a product of Kontron Messgerade Co. F.R.G.). The total magnification of the microtubule images was 3000 \times on the digitizer. It was possible to measure image length within an accuracy of 0.1 mm on the digitizer.

The only microtubules considered in this analysis were longer than 2 μm and shorter than 70 μm in length. Microtubules shorter than 2 μm could not be clearly distinguished from dusts and other particles, and those longer than 70 μm were too long to be projected onto the digitizer. We only utilized images which were in focus from one end to the other of the microtubule and those which were not folded or broken along their contours.

Some microtubules stuck to the under surface of the coverslip only at a part of their contours, and the off part was sometimes out of focus of the microscope. Images of such microtubules were not utilized.

We assume that a microtubule is a semiflexible chain which is straight in shape at mechanical equilibrium in the absence of Brownian movement. The mean-squared end-to-end distance, $\langle R^2 \rangle$, of a semiflexible chain is related to its contour length, L : i.e.,

$$\langle R^2 \rangle = f_3(\lambda, L) = (\exp(-2\lambda L) - 1 + 2\lambda L)/(2\lambda^2) \quad (1)$$

for a three dimensional chain [10], and

$$\langle R^2 \rangle = f_2(\lambda, L) = 2(\exp(-\lambda L) - 1 + \lambda L)/\lambda^2 \quad (2)$$

for the same chain adsorbed to a two dimensional substrate [15], where λ is the inverse of the statistical length of the chain. Qualitatively speaking, the parameter λ is a measure of chain stiffness or flexibility; that is, the more flexible the chain, the larger the parameter λ .

We assumed that the length measurement in our experiment was precise, since the relative error of the length measurement on the digitizer was less than 0.6% for a 10-μm-length filament. Let L_j and R_j be the contour length and end-to-end distance of the j th filament image divided by the total magnification, respectively. Let σ_j be the dispersion of R_j .

It should be noted that, even when length measurement is accurate, the dispersion of the end-to-end distance R need to be considered, since R is a stochastic variable, but L is not. Let us imagine an ensemble of semiflexible filaments of contour-length L . If we measure L and R of the filaments in the ensemble, the obtained L -value will be always the same (within the accuracy of length measurement), but the R -value will distribute about its mean. The width of this distribution is the dispersion of the end-to-end distance R .

Since the microtubules statistically analyzed in our experiment, were the ones stuck to the under surface of the coverslip, the expectation value of the parameter λ was estimated so that it might minimize the squared sum of residuals between R_j

and its expectation $f_2(\lambda, L_j)^{1/2}$ weighted with $1/\sigma_j^2$,

$$S(\lambda) = \sum_j \left(R_j - f_2(\lambda, L_j)^{1/2} \right)^2 / \sigma_j^2 = \min \quad (3)$$

We assume that σ_j is given by

$$\sigma_j = c \quad (4)$$

where c is an unknown constant. Then, we have

$$S(\lambda) = \sum_j \left(R_j - f_2(\lambda, L_j)^{1/2} \right)^2 / c^2 \quad (5)$$

from Eqns. 3 and 4.

The dispersion ξ_λ of the parameter λ was estimated in the following way [16]. Let λ_0 be the value of the parameter λ that minimizes the right-hand side of Eqn. 5. Let N be the number of microtubules measured. We computed an expectation value of c by substituting λ_0 and the expectation value of $S(\lambda)$ ($= N - 1$) into Eqn. 5. Then, we computed the expectation value of σ_λ by substituting the expectation value of c into

$$1/\sigma_\lambda^2 = \sum_j \left\{ \frac{\partial [f_2(\lambda, L_j)^{1/2}]}{\partial \lambda} \Big|_{\lambda_0} \right\}^2 / \sigma_j^2 \quad (6)$$

The elastic modulus for bending, ϵ , of a semi-flexible filament has been given in terms of the inverse of the statistical length, λ , as

$$\epsilon = k_B T / (2\lambda) \quad (7)$$

where k_B and T are the Boltzmann constant and absolute temperature, respectively [11]. Note that Eqn. 7 is different from the relation between ϵ and λ adopted in Refs. 7 and 8,

$$\epsilon = 3k_B T / (4\lambda) \quad (F1)$$

Equation F1 was first given by Harris and Hearst [17], but their theory has some inconsistencies in the stiff limit of filament flexibility. Since a singlet microtubule of several tens of μm in length is a rather stiff filament, Eqn. 7 is better than Eqn. F1. Anyway, the ϵ value calculated by using Eqn. F1 is only 1.5-times as large as the value presented in the text.

The elastic modulus for bending, ϵ , of a uniform elastic rod is equal to the product of Young's modulus, Y , and the moment of inertia of the cross section of the rod, I :

$$\epsilon = Y \cdot I \quad (8)$$

We assume that a singlet microtubule is a cylinder of radius r assembled from n protofilaments of radius a and that, even when a singlet microtubule is bent, there does not occur sliding between protofilaments in the microtubule. Then, the moment of inertia of the cross section, I , of a singlet microtubule is given by

$$I = n(\pi a^4/4 + \pi r^2 a^2/2) \quad (9)$$

When the values of λ , a , r , and n are known, the elastic modulus for bending, ϵ , and Young's modulus, Y , of a singlet microtubule can be calculated using Eqns. 7, 8 and 9.

Young's modulus, Y , is related to the microscopic elastic constant, k , of bonding between two neighboring monomers along the polymer by the equation

$$Y = kX_0/(\pi a^2) \quad (10)$$

where X_0 is the center-to-center distance of monomers in the polymer [18].

Results and Discussion

An example of photographs of microtubules observed under a dark-field microscope is shown

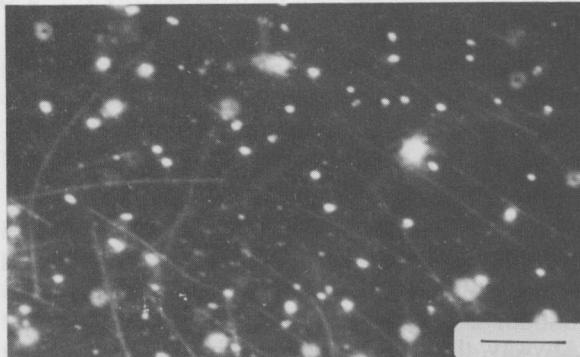


Fig. 1. Singlet microtubules photographed under a dark-field optical microscope. Bar represents 10 μm .

in Fig. 1. The contour lengths and the end-to-end distances of about 250 microtubules were measured. The observed values of data (L_j , R_j) were substituted into Eqns. 5 and 6, and the expectation values of the parameter λ and its dispersion σ_λ were estimated to be $\lambda \pm \sigma_\lambda = (6.8 \pm 0.8) \cdot 10^{-3} \mu\text{m}^{-1}$. The expectation values of λ and σ_λ are dependent on the form of σ_j . If we assume that σ_j is given by

$$\sigma_j = c \cdot L_j \quad (\text{F2})$$

instead of Eqn. 4, for example, we then obtain the expectation values of $\lambda \pm \sigma_\lambda = (7.3 \pm 0.8) \cdot 10^{-3} \mu\text{m}^{-1}$. Through the values of λ and σ_λ are dependent on the form of σ_j , the order of magnitude of their values remains the same for reasonable hypothesis on σ_j .

Fig. 2 shows the relation between the contour length (L) and the ratio of the end-to-end distance to the contour length (R/L). Dots in Fig. 2 are the observed data (L_j , R_j/L_j). A solid line in Fig. 2 shows the $L - R/L$ relation,

$$\bar{R}/L = [2(\exp(-\lambda L) - 1 + \lambda L)]^{1/2}/(\lambda L)^2 \quad (11)$$

with the λ value of $6.8 \cdot 10^{-3} \mu\text{m}^{-1}$. A dotted line in Fig. 2 is $R/L = 1$. Fig. 2 clearly shows that the observed pairs of data (L_j , R_j/L_j) distribute defi-

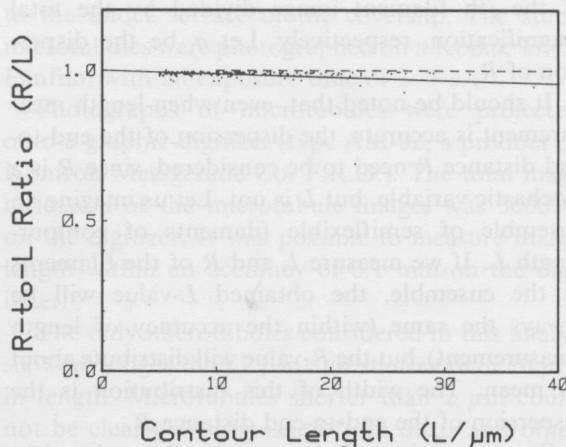


Fig. 2. The relation between L and R/L where L and R are the contour length and end-to-end distance, respectively. A solid line shows the $L - \bar{R}/L$ relation (Eqn. 11) with the λ value of $6.8 \cdot 10^{-3} \mu\text{m}^{-1}$, and a dotted line shows $R/L = 1$. Dots are the observed data (L_j , R_j/L_j).

TABLE I

MECHANICAL PROPERTIES OF F-ACTIN FILAMENTS, BACTERIAL FLAGELLA AND SINGLET MICROTUBULES

An F-actin filament was assumed to consist of two elastic rods of radius 1 nm. A bacterial flagellum was assumed to be a cylinder whose outer and inner radii are 6 nm and 5.5 nm, respectively. A singlet microtubule was assumed to be a cylinder of radius 11 nm assembled from 13 protofilaments of radius 1 nm.

	λ (μm^{-1})	ϵ (dyne \cdot cm 2)	Y (dyne \cdot cm $^{-2}$)	k (dyne \cdot cm $^{-1}$)
F-actin	$1.7 \cdot 10^{-1}$	$1.5 \cdot 10^{-17}$	$\sim 10^{11}$	$\sim 10^4$
Bacterial Flagella	—	$2.2 \cdot 10^{-15}$	$\sim 10^{11}$	—
Singlet Mictotubules	$6.8 \cdot 10^{-3}$	$4.5 \cdot 10^{-16}$	$\sim 10^9$	$\sim 10^2$

nitely below the line $R/L = 1$. This means that microtubules are not rigid but semiflexible.

The elastic modulus for bending, ϵ , was computed from the above value of λ and Eqn. 7 to be $\epsilon = 4.5 \cdot 10^{-16}$ dyne \cdot cm 2 . Let $n = 13$, $a = 1$ nm and $r = 11$ nm in Eqn. 9 [19], then the moment of inertia of the cross section, I , is $I = 2.5 \cdot 10^{-25}$ cm 4 . The values of ϵ and I were substituted into Eqn. 8, and Young's modulus, Y , was calculated to be $Y = 1.8 \cdot 10^9$ dyne \cdot cm $^{-2}$. Let $X_0 = 4$ nm in Eqn. 10 [18], then the microscopic elastic constant, k , is estimated to be $k = 1.4 \cdot 10^2$ dyne \cdot cm $^{-1}$. Though the values of Y and k strongly depend on such structural parameters as n , a , r and X_0 in Eqn. 9 and 10, the order of magnitude of their values remains the same for reasonable values of the structural parameters.

The value of a adopted here is smaller than the reported value of the radius 2.5 nm of a protofilament [19]. The reason is because the contacting area of two tubulin monomers neighboring along the protofilament will be smaller than $\pi(2.5 \text{ nm})^2$. A protofilament is here assumed to be mechanically equivalent to a uniform elastic rod of radius 1 nm.

Table I shows the values of the inverse of the statistical length, λ , the elastic modulus for bending, ϵ , Young's modulus Y , and the microscopic elastic constant, k , of F-actin [6–8, 18], of bacterial flagella [20], and of singlet microtubules. If we compare the values of Y in Table I, we can easily notice that the singlet microtubules consist of the most elastic material of all. It should be noted that the material elasticity of the singlet microtubules has no relation with the fact that polymerization and depolymerization of tubulin are very sensitive

to the environmental conditions, such as temperature and pH. The former is related to the second derivative of the intermonomer interaction free energy at the minimum, and the latter has a relation with the sensitiveness of the trough depth and of the barrier height of the interaction free energy to environmental conditions [18]. Comparing the values of ϵ in Table I, we conclude that a singlet microtubule is an order of magnitude as strong against bending as an F-actin filament. The force constant for stretching a filament is given by $n \cdot k$, where n is the number of protofilaments in the filament. The value of n is 13 for a microtubule, and is 2 for an F-actin filament. From these values of n and the values of k in Table I, we conclude that a singlet microtubule is an order of magnitude as weak against stretching as an F-actin filament. The microtubule could then serve well as a main cross beam of cytoskeleton in a living cell, and not as an elastic component.

Okuno and Hiramoto measured the flexural rigidity of echinoderm sperm flagella in the relaxed state to be $\epsilon = (3-7) \cdot 10^{-13}$ dyne \cdot cm 2 [21]. They assumed that the elastic component of sperm flagella mainly consisted of microtubules and that the nine outer doublets and the two central singlets in the sperm flagella independently worked without any connection among them. They then estimated Young's modulus, Y , of the doublet microtubule to be $Y = (2-5) \cdot 10^{10}$ dyne \cdot cm $^{-2}$.

Young's modulus of the doublet microtubules estimated by Okuno and Hiramoto is an order of magnitude as large as that of the singlet microtubules reported by the present authors. At the present stage of study, however, we can not conclusively say why these two groups have reported

such different values of the Young's modulus. Further studies on the mechanical properties of the singlet microtubules are necessary.

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