OBSERVATION OF FLAGELLATION OF SPERMATOZOA BY DEPOLARIZED LASER LIGHT SCATTERING

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ABSTRACT Depolarized laser light-scattering theory was applied to derive the autocorrelation function of laser light scattered by motile spermatozoa, assuming that each spermatozoon is a chain of rotatable rigid ellipsoids of revolution and also that the rotational velocity about an axis perpendicular to the symmetry axis of the ellipsoid is constant for times of the order of the characteristic decay time of the autocorrelation function. The rotations are produced by flagellar movements of the spermatozoa. The correlation function thus obtained was related to the second-order coefficient of a Legendre polynomial expanison of the rotation of the direction angle of the ellipsoidal axis. The experimental fact that the correlation function for dead spermatozoa of sea urchin resembled that for flagella mechanically separated from spermatozoa indicated to us that the depolarized light was scattered mainly by flagella. The rotational velocity distribution of the flagella was determined by comparing the theoretical analysis with the experimentally obtained correlation functions for the motile and dead spermatozoa. The value of the average velocity caused by the flagellation, 230 rad/s, was in good agreement with that measured under an optical microscope.

INTRODUCTION

Measurement of the effects of chemical reagents and environmental factors such as pH, temperature, and electromagnetic waves on the motility of microorganisms is important in biology and medicine. These effects relate directly to the physical origin of taxis and hazards. More specifically, the motility of spermatozoa is an important factor in fertility (1) and thus information concerning spermatozoon motility is essential to the field of artificial insemination.

In this report we apply depolarized light-scattering techniques to the study of motile microorganisms (in this case, spermatozoa), for the first time. Laser light-scattering spectroscopy has been well recognized as an appropriate tool for determining the average swimming velocity distribution of motile microorganisms such as bacteria (2–5) and spermatozoa (6–9), but it has been usual to observe the polarized component of the scattered laser light, the spectrum of which reflects mainly translational motions of the scattering particles. We observed instead the depolarized component of the laser light scattered by spermatozoa in semen and have obtained important information about flagellar movements, which are responsible for spermatozoon motility.

The intensity of the depolarized scattered light depends on the molecular anisotropy of the polarizability tensor of the macromolecules that scatter the laser light (10–13). Generally, it can be said that the intensity of the depolarized component of scattered light is much weaker

than the polarized. Therefore, it is technically much more difficult to extract the depolarized component of scattered light from the strong background light of the polarized component except at very low scattering angles, when the time constant for decay of the polarized spectrum becomes very long (14).

Solutions of Tobacco Mosaic Virus (11, 12) and DNA (13) have been studied with depolarized forward light scattering. These reports clarified the mechanism of rotational diffusion of thermally activated macromolecules. Few other reports in which this technique was used have appeared, however. A part of the experimental results described in this report was published elsewhere for rapid circulation (15).

DEPOLARIZED SCATTERING THEORY OF MOTILE SPERMATOZOA

In this section we will apply depolarized scattering theory to a system consisting of motile spermatozoa and will deduce a formula by which the parameters of rotary motions reflecting the flagellation of the spermatozoa are related to the observables of depolarized laser light scattered by spermatozoa.

The depolarized laser light scattered by particles originates from the optical anisotropy of the particle (10–13). As a first approximation, a spermatozoon can be regarded as a chain of rigid ellipsoidal (optically uniaxial) particles. As a result, depolarized scattering theory is applicable to the spermatozoon. To begin with, we will develop the depolarized scattering theory of the rigid and ellipsoidal particles that rotate by their internal force, so as to obtain the normalized field correlation function $G_n^{(1)}(\tau)$, which is defined as $\langle \mathbf{E}(0)^* \mathbf{E}(\tau) \rangle / \langle |\mathbf{E}(0)| \rangle^2$, where $\mathbf{E}(t)$ is the depolarized component of the light scattered by the particles at time t. Note that the depolarized component is proportional to $(\alpha_\parallel - \alpha_\perp)$, where $\alpha_\parallel$ and $\alpha_\perp$ are particle-fixed polarizability components parallel to the symmetry axis of the particle and perpendicular to the axis, respectively (14).

Obviously, we need not take account of the rotation of the particle around its symmetry axis (14). Therefore, the rotation of the particle is denoted by the rotation vector θ which makes $\mathbf{n}(0)$ into $\mathbf{n}(\tau)$, where $\mathbf{n}(0)$ and $\mathbf{n}(\tau)$ are unit vectors representing the orientation angles of the symmetry axis of the particle at times 0 and τ, respectively. Unit vectors $\mathbf{n}(0)$ and $\mathbf{n}(\tau)$ in the particle frame (x, y, z) at time 0 are shown in Fig. 1. Assuming that the initial directions of axes of the ellipsoids and rotation vectors θ are random, we calculate the normalized field correlation function as follows (14):

$$G_n^{(1)}(\tau) = \langle P_2(\cos \theta) \rangle, \tag{1}$$

where $\theta = |\boldsymbol{\theta}|$, $P_n(x)$ stands for the nth order Legendre's polynomial for a variable x, and $\langle\ \rangle$ denotes an ensemble average. As θ is the rotation angle, the angular distribution $f(\theta)$ on the spherical surface is expressed as follows,

$$f(\theta) = \sum_{n=0}^{\infty} a_n(\tau) P_n(\cos \theta). \tag{2}$$

Putting Eq. 2 into Eq. 1, we get the expression of $G_n^{(1)}(\tau)$ as follows:

$$G_n^{(1)}(\tau) = \frac{4}{5} \pi\, a(\tau). \tag{3}$$

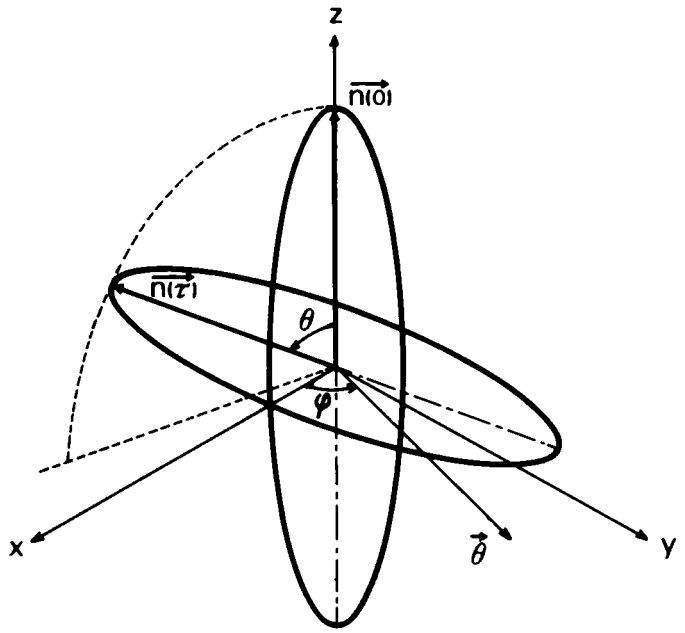

FIGURE 1 Unit vectors $\mathbf{n}(0)$ and $\mathbf{n}(\tau)$ in the particle frame (x, y, z) at time 0. θ is the rotation vector, and ϕ denotes the direction of θ.

This equation means that the correlation function of the depolarized laser light gives us the second-order coefficient of the angular distribution function expanded by Legendre's polynomials.

Although the rotating force generated by the microorganisms is represented by constant torque, the rotation angles will be distributed around an average rotation angle as a result of thermal agitation. Since a particle is assigned to be a part of the flagellum we may neglect rotation around the symmetry axis of the particle and obtain the angular distribution easily by solving the rotational diffusion equation under constant torque (see Appendix):

$$f(\theta) = \sum_{n=0}^{\infty} \frac{2n + 1}{4\pi} U_n(\tau) P_n(\cos \omega_0 \tau) P_n(\cos \theta), \tag{4}$$

where $\omega_0 \tau$ stands for the rotation angle of the particle due to the torque during time τ:

$$U_n(\tau) = \exp [-Dn(n + 1)\tau], \tag{5}$$

and D stands for the rotational diffusion constant. Putting Eq. 4 into Eq. 1, we obtain the correlation function:

$$G_n^{(1)}(\tau) = U_2(\tau) P_2(\cos \omega_0 \tau). \tag{6}$$

If one assumes that rotation velocities of individual particles remain unchanged over the measurement time, then Eq. 6 can be rewritten with the rotation velocity distribution $p(\omega_0)$ as follows:

$$G_n^{(1)}(\tau) = U_2(\tau) \int_0^{\infty} d\omega_0 \, p(\omega_0) P_2(\cos \omega_0 \tau). \tag{7}$$

Note that $G_n^{(1)}(\tau)$ is expressed as a product of two contributions: one from the rotational Brownian motion and the other from the rotatory motion of the particles caused by the constant torque. We thus obtain a theoretical expression for the correlation function of the depolarized light scattered by rigid and optically ellipsoidal particles. When ω_0 is zero, $G_n^{(1)}(\tau) = \exp(-6D\tau)$. This expression has been used in analyzing the data obtained in the depolarized scattering experiments, e.g., on Tobacco Mosaic Virus (11,12) and DNA (13).

Since we are assuming a spermatozoon to be a chain of rigid particles, ω_0 reflects the rotatory motions of the particles caused by the flagellation of the spermatozoa. When interactions between the particles exist, $U_n(\tau)$ depends on the model of the structure of the spermatozoon, and becomes much more complicated than the expression in Eq. 5. However, considering that $U_n(\tau)$ is the nth order coefficient of a Legendre polynomial expansion of the thermaly activated part of the motions, these findings do not affect the procedure for obtaining the expressions for the correlation function such as Eq. 7.

By transforming Eq. 7, we obtain the rotation velocity distribution caused by flagellation as follows:

$$p(\omega_0) = \frac{16}{3\pi} \int_0^\infty d\tau (G_n^{(1)}(\tau)/U_2(\tau) - 1/4) \cos 2\omega_0 \tau. \tag{8}$$

As the specimen system is expected to be stationary, the ensemble average operation in Eq. 1 can be replaced by the time average operation. Therefore, we determine $G_n^{(1)}(\tau)$ and $U_2(\tau)$ by measuring the depolarized light scattered by motile spermatozoa and by dead but diffusing spermatozoa, respectively. Thus we obtain the rotation velocity distribution by using the data obtained experimentally and by the relation given by Eq. 8. Note that the formulae shown in Eqs. 7 and 8 do not depend on the differences between planar (16, 17) or helical flagellation.

EXPERIMENTAL METHOD AND APPARATUS

A photoelectron-counting technique was used to obtain the spectrum of the depolarized component of the light scattered by a solution of spermatozoa. Fig. 2 shows a schematic diagram of the apparatus used for observing the depolarized component of the scattered light. The optical arrangement is similar to that previously developed by Wada et al. (11). It consists of a He-Ne laser, a polarizer, a rectangular specimen cell, an analyzer whose optical axis lies perpendicular to that of the polarizer, and a photoelectron-counting system (18).

The scattering angle was chosen to be almost 0° to avoid the strong background light due to the incident light and the polarized component of the forward scattering light. The deviation of the angle from 0° was ~0.2°, so that the intensity of the polarized component could be suppressed to a level ~30 times weaker than the depolarized component at the surface of the photocathode of the photomultiplier. The time correlation of the light intensity of the depolarized component was thus measured in a homodyne mode.

The photoelectron-counting system used in these experiments consists of a photomultiplier (9558 QB;

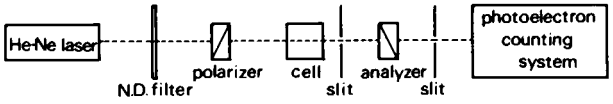

FIGURE 2 Scheme of the optical arrangement for observing the depolarized component of the laser light scattered by spermatozoa and flagella.

EMI Technology, Sunnyvale, Calif.) whose photocathode is cooled to ~200°K, a discriminator, a pulse-interval digitizer, a data channel interface, and a minicomputer system. The pulse-interval digitizer digitizes the time intervals between neighboring pulses in a photoelectron pulse train. The data channel interface transfers the data from the digitizer directly to the magnetic core memory of the computer system. The computer system is used to process the stored data of pulse intervals into the autocorrelation function of time. The details of this photoelectron-counting system have been previously described (18).

By using this system, the light intensity correlation function $G_n(\tau) = \langle I(t) \cdot I(t+\tau) \rangle / \langle I(t) \rangle^2$ is obtained, where $I(t)$ stands for the instantaneous light intensity at the photocathode at time t and the bracket refers to a time average operation with respect to t. $G_n(\tau)$ is related to the normalized field correlation function $G_n^{(1)}(\tau)$. When the fluctuation of the permeability of the scattering source obeys Gaussian statistics and the incident laser light irradiates the specimen in a single mode, the following relation holds (19):

$$G_n(\tau) - 1 = c\,|G_n^{(1)}(\tau)|^2, \tag{9}$$

where c is a function of the number of coherence areas (20) in the observed volume, and ≤ 1.

From the experimentally obtained quantity $G_n(\tau)$, the time independent part can be eliminated to yield the time-dependent part, $\gamma(\tau)$. $\gamma(\tau)$ is normalized, so that $\gamma(0)$ is equal to 1. In this case, therefore, $\gamma(\tau) = |G_n^{(1)}(\tau)|^2$.

With the use of the relation described above, Eq. 8 can be written as:

$$p(\omega_0) = \frac{16}{3\pi} \int_0^\infty d\tau \left(\sqrt{\gamma(\tau)} / \sqrt{\gamma'(\tau)} - 1/4 \right) \cos 2\omega_0 \tau, \tag{10}$$

where $\gamma(\tau)$ and $\gamma'(\tau)$ are the time-dependent part of the intensity correlation function for motile and dead spermatozoa, respectively.

SPECIMEN AND EXPERIMENTAL RESULTS

Spermatozoa of sea chestnut *(Anthocidaris crassispina)*, which belong to the family of *Echinometridae* (sea urchin) and can be obtained only in Japan in summer, were used in these experiments. The spermatozoa were obtained by injecting a 500-mM KCl solution into a mature sea urchin of ~6 cm diameter. The spermatozoa obtained in this way were at rest, but when diluted with sea water they began to swim. The spermatozoa were used right after being diluted into sea water to a final concentration of 10^7 sperm/ml. All of the measurements were carried out within a few minutes of obtaining the spermatozoa.

In the optical arrangement shown in Fig. 2, the scattering volume is $\sim 10^{-2}$ ml. Therefore, the sample contained $\sim 10^5$ spermatozoa in the scattering volume. The field scattered by such a number of microorganisms is expected to obey Gaussian statistics (21). Moreover, the field has already been found experimentally to obey the statistics (22).

Fig. 3 shows a set of experimentally obtained (15) correlation functions $G_n(\tau)$ and $\log \gamma(\tau)$ of the depolarized light scattered from the specimen prepared as described above in natural sea water. In this experiment, the measurement was performed 5 min after the spermatozoa were diluted into sea water. As seen in Fig. 3, the overall decay characteristic of $G_n(\tau)$ and $\gamma(\tau)$ resembles the one for the polarized light scattering from the specimen of motile microorganisms (2, 3). We obtained 552 μs as the characteristic decay time γ_0, defined as $\gamma(\tau_0)/\gamma(0) = 1/e$.

The characteristic decay times τ_0 changed with time, as shown in Fig. 4; 150 min after the

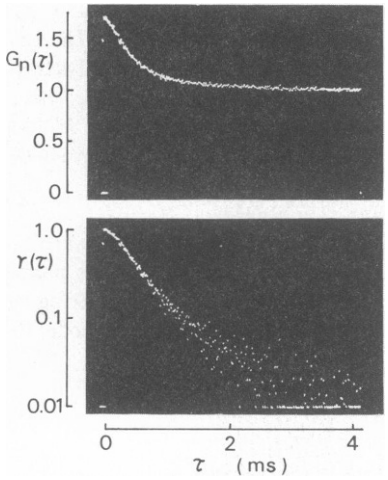

FIGURE 3

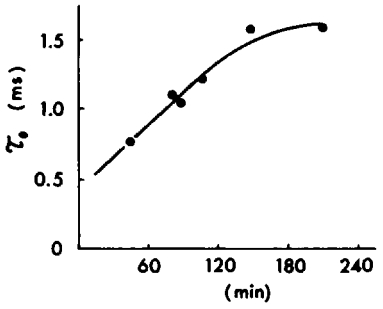

FIGURE 4

FIGURE 3 Representative set of the intensity correlation function of the depolarized laser light scattered by motile spermatozoa, where $G_n(\tau)$ stands for the normalized light intensity correlation function and $\gamma(\tau)$ is the normalized time-dependent part of $G_n(\tau)$. The data were obtained 5 min after the spermatozoa were diluted into the sea water. They were still moving actively. The measurement was done at room temperature with a scattering angle of 0.2°.

FIGURE 4 Observation time dependence of the characteristic decay time τ_0. τ_0 is defined to satisfy the relation $\gamma(\tau_0)/\gamma(0) = 1/e$. Observation time is from the onset of the dilution of spermatozoa through the time when the measurement is done.

spermatozoa were diluted, τ_0 was found to be 1.6 ms and remained constant. At the same time, the decay characteristic of the light intensity correlation function $G_n(\tau)$ changed as the time between the spermatozoa dilution and the measurement increased, and approached a single exponential type, as shown in Fig. 5. The spermatozoa in the specimen, whose correlation function is shown in Fig. 5, were observed by the optical microscope to be immobile. The exponential decay is expected for the light intensity correlation function of the depolarized component of light scattered from a thermally fluctuating solution of anisotropic macromolecules (10). The characteristic decay time at the time immediately after the dilution of spermatozoa is expected to be 400 μs, a value obtained by extrapolating the experimental data from Fig. 4.

The depolarized component of the light scattered by the spermatozoa is composed of two elements: one from the light scattered by the heads of the spermatozoa and the other scattered by the flagella. Since the heads of the spermatozoa occupy a larger volume than the tails, you might expect the heads to scatter more light than the tails. However, the heads are almost spherical and optically isotropic, so that they do not contribute much to the depolarized component of the scattered light. When the head is taken to be an ellipsoid of revolution with long and short axes of 1 μm and 0.5 μm (1, 16, 17), respectively, then the calculated value τ_0 from the rotational diffusion constant at thermal equilibrium, 1.25 s, does not agree with the observed value of 1.6 ms. If the contribution from the head were a major portion in the depolarized component of the light scattered by immobile spermatozoa, we would find the observed value of the characteristic time to be similar to the calculated value.

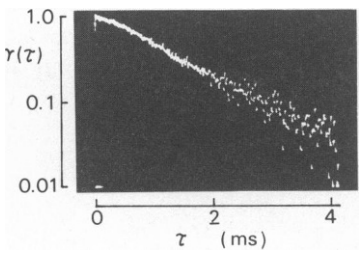

FIGURE 5

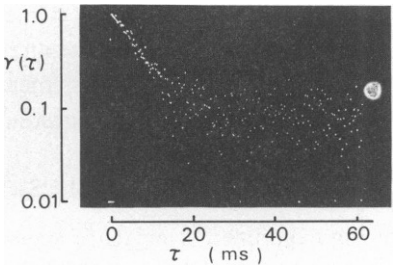

FIGURE 6

FIGURE 5 Representative correlation function for the spermatozoa that are almost dead. The data were obtained 325 min after the spermatozoa were diluted into the sea water.

FIGURE 6 Intensity correlation function obtained for dead spermatozoa suspended in Ca^{2+}- and Mg^{2+}-free ASW at room temperature.

To demonstrate that the depolarized component of scattered light derives mainly from the light scattered by the flagella, we show the representative correlation functions for dead spermatozoa (Fig. 6) and flagella separated from spermatozoa (Fig. 7) in Ca^{2+}- and Mg^{2+}-free artificial sea water (ASW). The flagella were obtained by the following method: the spermatozoa in ASW were broken off into heads and flagella in a few minutes with a homogenizer. The flagella alone were collected with a separative centrifuge. ASW was used because the flagella could be easily separated from the spermatozoa in that medium. We found τ_0 for the dead spermatozoa and for the flagella in ASW to be 7.3 ms and 4.0 ms; respectively. Those values are in good agreement with each other, considering the mechanical damage to flagella in the separating procedure. We conclude, therefore, that the depolarized component must be due to the light scattered mainly by the flagella.

Experimentally, $\gamma(\tau)$ in Eq. 10 can be easily determined through Eq. 9 from $G_n(\tau)$ of the result for the depolarized light scattering on the solution of almost dead spermatozoa in Fig. 5. Therefore, we can get the distribution function $p(\omega_0)$ for the beating flagella through Eq. 10.

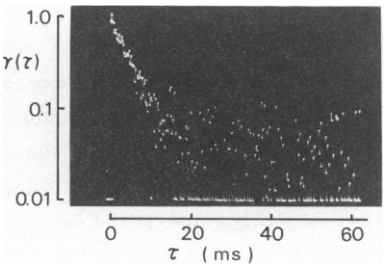

FIGURE 7

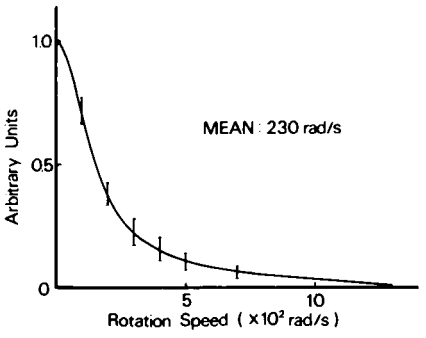

FIGURE 8

FIGURE 7 Intensity correlation function obtained for flagella separated from sea urchin spermatozoa. The flagella were suspended in Ca^{2+}- and Mg^{2+}-free ASW at room temperature.

FIGURE 8 Rotation velocity distribution of motile spermatozoa, which originates from the flagellation. The distribution is calculated from Eq. 10 for the data shown in Figs. 3 and 5.

The result is shown in Fig. 8 for the specimen of spermatozoa corresponding to the correlation function $G_n^{(1)}(\tau)$ in Fig. 3. The average angular frequency $\bar{\omega}_0$ obtained is 230 rad/s from this figure. This value of $\bar{\omega}_0$ is in good agreement with the beat number of flagella, 30–40 beats/s observed for the spermatozoa of sea chestnut under an optical microscope (16, 17).

DISCUSSION AND CONCLUSION

The following experimental evidence suggests that the function $G_n(\tau)$ or $\gamma(\tau)$ is due to the depolarized component of the scattered light. First, the intensity of the depolarized component was found to be 30 times stronger than the intensity of the polarized light at the surface of the photocathode; second, the characteristic decay time of 400 μs was far shorter than expected for the polarized component of light scattered from a similar specimen. We found the τ_0 to be 426 μs for the polarized component at the scattering angle $\psi = 43°$ (22), suggesting that τ_0 for the polarized component at the scattering angle 0.2° should be 90 ms, since the angular dependence of τ_0 for the polarized component is known to be inversely proportional to $\sin^2(\psi/2)$ (2, 3, 9). These two experimental findings suggest that the light measured was the depolarized component of the light scattered by spermatozoa.

The depolarized component of the scattered light is mainly due to the light scattered by the flagella. The following two bits of experimental evidence suggest the inference described above. First, the calculated value corresponding to the rotational diffusion constant at thermal equilibrium for the heads of spermatozoa is too large compared with that observed experimentally and shown in Fig. 5. Second, we found that the values of τ_0 for the dead spermatozoa and for the flagella shown in Figs. 6 and 7 are in good agreement. The values of τ_0 shown in Figs. 5 and 6 are at variance. We think the difference originates from the difference of the experimental solvents: one is natural sea water from the difference of the experimental solvents: one is natural sea water and the other is Ca^{2+}- and Mg^{2+}-free ASW.

The observed correlation function for dead spermatozoa shown in Fig. 5 is expected to reflect Brownian motion of the flagella. A more detailed analysis of the obtained data will give us more information on the length and the stiffness of the flagella (23). But when $U_2(\tau)$ is determined experimentally, as shown in Fig. 5, information concerning the length and stiffness of the flagella does not affect the procedure of obtaining the distribution of the rotation velocity of the flagella.

The average rotation velocity $\bar{\omega}_0$ shown in Fig. 8 is in good agreement with the angular frequency of flagella observed for the spermatozoa of sea chestnut under an optical microscope (16, 17). Note that the result shown in Fig. 8 was obtained under the assumptions that the flagellum could be approximated as a chain of rotatable rigid ellipsoids, and also that the rotation velocity of the ellipsoid could be considered unchanged for the characteristic decay time of dead spermatozoa at thermal equilibrium. The former assumption seems reasonable considering the structure of the flagella; considering the average beat number of flagella in spermatozoa, the assumption that the velocity is unchanged in a few milliseconds also seems to be a fairly good approximation.

In this article, we have reported a formulation of depolarized scattering theory of motile spermatozoa, and have applied depolarized light-scattering techniques to a study of the flagellation of motile spermatozoa. Analysis of experimental results gave us the rotation

velocity distribution that reflects the flagellation of the spermatozoa. The average velocity of 230 rad/s obtained by the procedure derived from the depolarized scattering theory is consistent with the beat number of flagellation, 30–40 beats/s (16, 17), observed under an optical microscope.

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APPENDIX

In this section we derive the distribution of the rotation angle of the rigid and ellipsoidal particle under constant torque and thermal agitation, neglecting the rotation around the symmetry axis. Then we can obtain the Langevin equation of the particle under constant torque **T** as follows,

$$\frac{d\omega(t)}{dt} = -\zeta\omega(t) + \frac{\mathbf{T} - \mathbf{R}(t)}{I}, \tag{A-1}$$

where $\omega(t)$ is the velocity of the particle around the axis perpendicular to the symmetry axis, ζ, the friction coefficient; I, the moment of inertia around the axis perpendicular to the symmetry axis; and $\mathbf{R}(t)$, the collision force. In other words, Eq. A-1 represents the motion of unit vector **n** pointing along the symmetry axis of the particle.

Defining ω_0 as $\mathbf{T}/I\zeta$, we can replace $\omega(t)$ in Eq. A-1 by $\omega_0 + \omega'(t)$ as follows:

$$\frac{d\omega'(t)}{dt} = -\zeta\omega'(t) + \frac{\mathbf{T} - \mathbf{R}(t)}{I}, \tag{A-2}$$

where ω_0 and ω' are the average velocity and the fluctuation around the average velocity, respectively. Eq. A-2 is similar to the Langevin equation describing the motion of a particle activated thermally without other external force. Therefore Eq. A-2 gives us the well-known type of a diffusion function as follows:

$$\frac{\partial f}{\partial t} = D\nabla^2 f, \tag{A-3}$$

where f denotes the angular distribution and D is the rotational diffusion constant. Note that the distribution function obtained by solving Eq. A-3 should be the function expressed in the frame (θ', ϕ'), which rotate with the velocity ω_0.

By solving Eq. A-3 under the condition that the initial **n** lies on the point $(\theta' = 0, \phi' = 0)$, we can get the distribution function, as follows,

$$f(t, \theta', \phi') = \sum_{n=0}^{\infty} \frac{2n+1}{4\pi} U_n(t) P_n(\cos\theta'), \tag{A-4}$$

where $U_n(t)$ is defined as $\exp[-Dn(n+1)t]$ and P_n is the nth order Legendre's function. Therefore, the angular distribution function under constant torque is obtained by rewriting Eq. A-4 using the

coordinates (θ, ϕ) of the frame which does not rotate, as follows:

$$f(t, \theta, \phi) = \sum_{n=0}^{\infty} \frac{2n+1}{2\pi} U_n(t) \left\{ \frac{1}{2} P_n(\cos \omega_0 t) P_n(\cos \theta) + \sum_{m=0}^{n} \left[\frac{(n-m)!}{(n+1)!} P_n^m(\cos \omega_0 t) P_n^m(\cos \theta) \times (\cos m\phi_0 \cos m\phi + \sin m\phi_0 \sin m\phi) \right] \right\}, \quad \text{(A-5)}$$

where ω_0 is $|\omega_0|$, ϕ_0 gives the direction of ω_0, and P_n^m is the associated Legendre's function. When the direction of ω_0 is at random, the angular distribution function is obtained as follows:

$$f(t, \theta) = \sum_{n=0}^{\infty} \frac{2n+1}{4\pi} U_n(t) P_n(\cos \omega_0 t) P_n(\cos \theta).$$

We have hereby derived the expression described in Eq. 4.

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