A STUDY OF PROTOPLASMIC STREAMING IN *NITELLA* BY LASER DOPPLER SPECTROSCOPY

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ABSTRACT Laser light scattered from particles in the streaming protoplasm of a living cell is shifted in frequency by the Doppler effect. The spectrum of the scattered light can be measured and interpreted to infer details of the velocity distribution in the protoplasm. We have developed this approach to study the protoplasmic streaming in the fresh-water alga *Nitella*. Our results indicate a characteristic flow pattern to which diffusion makes a negligible contribution. No difference in the velocity of particles of different size is indicated. The streaming velocity varies linearly with temperature with a supraoptimal temperature of 34°C, and the velocity distribution becomes narrower at high temperatures. The protoplasmic streaming can be inhibited by laser light, and this effect has been used to study the photoresponse of the algae. Using beam diameters of about 50 μm, we have shown that the inhibition is very local, becoming minimal at a displacement of about 200 μm in the upstream direction and 400 μm in the downstream direction. Prolonged exposure produces a bleached area free of chloroplasts, which is three orders of magnitude less sensitive to photoinhibition.

INTRODUCTION

Protoplasmic streaming, the organized flow of protoplasm within a living cell, has been an active area of scientific investigation for at least two hundred years. A comprehensive review of all work up to 1959 has been given by Kamiya (1). The central objective of the modern work in this area is to elucidate the molecular mechanism of the application of the motive force. Inseparable from this objective is a need for careful and detailed physical characterization of the flow distribution within the cell and its response to external stimuli. Past studies have been conducted almost exclusively by optical microscopy and cinematography. We report here the application of laser light scattering spectroscopy to the study of protoplasmic streaming. An account of our first experiments of this kind has been published elsewhere (2).

The organism which we have chosen for study is the common fresh-water alga *Nitella flexilis*. We have concentrated our studies on the long internodal cells. The protoplasm flows forth and back the length of the internodal cell in two channels which are wound in a slight helical pattern. The two channels are separated by narrow regions, usually termed indifferent zones, in which there is no streaming. The cell protoplasm is contained in a narrow cylindrical annulus surrounding the large vacuole. A cross section of the *Nitella* internodal cell is shown in Fig. 1. The streaming endo-

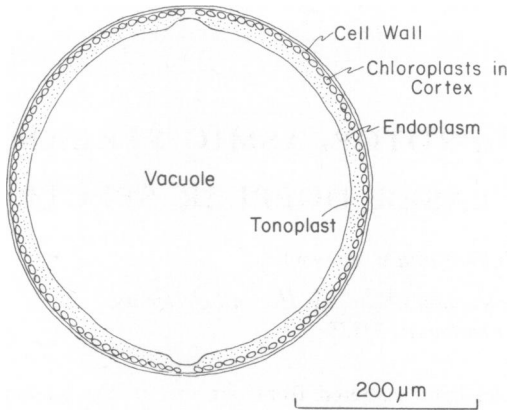

FIGURE 1 A cross section of an internodal cell of *Nitella* showing the endoplasm confined to the annulus between the cortex and the tonoplast, a membrane separating the endoplasm from the vacuole of the cell. The tonoplast separates the two channels of the endoplasm by contact with the ectoplasm at the indifferent zone.

plasm lies between the stationary cortex and the tonoplast, the membrane between the endoplasm and the vacuole. In the indifferent zones the tonoplast approaches the cortex and excludes the endoplasm. The chloroplasts are imbedded in the cortex, except that no chloroplasts are found in the indifferent zones.

The protoplasmic streaming in *Nitella* is readily observable by optical microscopy. Large particles which are carried with the flow can be seen to move approximately parallel to the walls of the channel at velocities of up to 100 μm/s. Observation by optical microscopy has been extended by the introduction of microcinematography (3, 4). From these studies a great deal has been learned about the details of the flow in the cell. However, there are certain limitations inherent in this technique, the most obvious being that observation is limited to particles large enough to be visible with a light microscope and must rely on human detection and interpretation. A further difficulty is that the velocity of a particle must be determined by measuring the time elapsed to traverse a known distance, so that it is not possible to measure velocities at a localized point in the cell. These difficulties are overcome to a considerable degree by employing the technique of laser Doppler spectroscopy, which has the additional advantages of being fast, convenient, and very precise.

Laser light scattering has become a common and useful technique for the study of the hydrodynamics of biological macromolecules (5–7). Yeh and Cummins first demonstrated that the Doppler shift of scattered laser light could be measured to determine the velocities of particles in a flowing medium (8). Recently this technique has been applied to the determination of electrophoretic velocities (9) and velocities of blood flow in living organisms (10, 11). In 1974 we reported the first application of laser Doppler spectroscopy to the measurement of protoplasmic streaming velocities in a living cell (2). The Doppler-shifted spectra from internodal and leaf cells of

Nitella flexilis were obtained in just a few seconds, and it was demonstrated that the spectra could reliably be interpreted as the velocity profile of all particles in the small region illuminated by the laser. In this paper we report further characterization of the streaming by laser light scattering, including the response of the cells to temperature and intense light.

THEORY

The theory of laser light scattering spectroscopy, including velocity measurements from Doppler shifts, is well understood and has been summarized in numerous reviews, the most recent by Chu (7). We present here only the minimal theoretical results necessary for the interpretation of our data in simple terms.

The important parameters are defined in Fig. 2, which is a schematic representation of the experimental apparatus. The incident laser light is monochromatic with incident wave vector $\mathbf{k}_I$, which has the direction of the propagation of the light and has magnitude $2\pi/\lambda$, where λ is the wavelength of the light in the scattering medium, i.e. in the protoplasm. Light is scattered by the optical inhomogeneities in the cell, including the particles in the protoplasm. The scattered light is collected at a well-defined angle by a pair of small apertures which define, after a correction for refraction, the scattered wave vector $\mathbf{k}_S$. The angle between the incident and scattered wave vectors is called the scattering angle θ. The vector difference between the scattered and incident wave vectors is called the scattering vector $\mathbf{K}$, whose magnitude can be obtained from geometry to be $(4\pi/\lambda)\sin(\theta/2)$, assuming that λ and λ_S are approximately equal. For the purposes of this discussion, all of these variable are defined in the scattering medium.

Whereas the incident light is monochromatic, the scattered light has a frequency distribution imposed upon it because some of the scattering particles are in motion. If these particles are in motion because of translational diffusion, the theory predicts

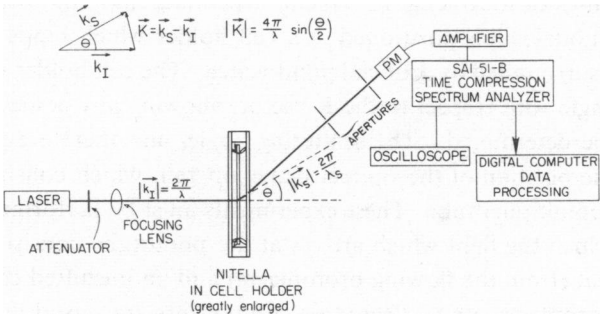

FIGURE 2 A diagram of the experimental apparatus. Laser light, attenuated and focused, is incident on a *Nitella* cell surrounded by artificial pond water and gently bound in a cell holder. Apertures determine the scattering angle θ after a correction for refraction. All parameters in the diagram are defined in the scattering medium. The AC component of the photocurrent is amplified and spectrum analyzed in real-time. The spectrum is continuously monitored and displayed on an oscilloscope. An averaged spectrum can be obtained in a few seconds.

that the spectrum will be a Lorentzian frequency distribution centered at the incident frequency with a half-width in hertz of $DK^2/2\pi$, where D is the translational diffusion coefficient of the particles and K is the magnitude of the scattering vector defined before. If the particles are in directed motion with a velocity $\mathbf{v}$, the theory predicts that the scattered light will be shifted in frequency by the Doppler effect and that the magnitude of the frequency shift in hertz will be $|\mathbf{K}\cdot\mathbf{v}|/2\pi$, which can be written $K\,v\cos\alpha/2\pi$, where α is the angle between $\mathbf{K}$ and $\mathbf{v}$.

The analysis of light scattering spectra from flowing particles can be complicated by a number of factors (12). For the benefit of readers who are not familiar with the theory, we describe here the general principles which will be used in this report. If an assembly of particles is undergoing directed flow in which diffusion and other broadening effects are negligible, the Doppler-shifted spectrum has a one-to-one correspondence between frequency and velocity so that the velocity distribution is directly manifest in the frequency distribution. If the particles are not all of the same size and composition, however, the relative contribution to the light scattering spectrum from each particle will be roughly proportional to the square of the mass of the particle, so that large particles make by far the greatest contribution. The relative contribution of each particle to the scattered intensity spectrum will also be a function of scattering angle, because large particles have a reduced intensity at high angles due to the interference of the scattered light from different regions of the particle.

EXPERIMENTAL METHOD

The experimental apparatus is presented schematically in Fig. 2. The incident laser is either a Spectra-Physics Model 125A helium-neon laser or a Spectra-Physics Model 165 argon ion laser (Spectra-Physics, Inc., Mountain View, Calif.). The laser light is greatly attenuated to avoid inhibiting the streaming (discussed later), and the power is measured with a Spectra-Physics Model 404 power meter. The light is then focused with biconvex lenses of focal length 3–20 cm, depending upon the beam size desired. The *Nitella* internodal cell is positioned in a cell holder which binds it gently at the ends. The cell is surrounded by artificial pond water. The cell holder may be oriented at any desired angle with respect to the $\mathbf{K}$ vector, allowing any desired component of the velocity to be determined. The scattering angle, and therefore the $\mathbf{K}$ vector, is determined by the position of the optical detection rail, which consists of two apertures and a photomultiplier tube. These experiments must be performed in the heterodyne mode, in which the light which arrives at the phototube consists of a Doppler-shifted component (from the flowing protoplasm) and an unshifted component (from the cell wall, chloroplasts, etc.). These two components are mixed at the phototube, and the output of the tube, which acts as a nonlinear detector for electric fields, includes the difference "beats" which are the Doppler shift frequencies. The output of the phototube is amplified and then frequency analyzed by a SAICOR Model 51-B time-compression real-time spectrum analyzer. In a typical experiment, the spectrum is measured with a 1 Hz resolution, which takes only 1 s to obtain. Normally a number

of these spectra are summed in the averager included in the spectrum analyzer in order to improve the signal-to-noise ratio. The time for collection of most of the spectra was around 1 min. Both the individual spectra from the analyzer and the averaged spectrum from the averager are viewed on oscilloscopes during the course of an experiment, allowing a nearly instantaneous monitoring of the velocity distribution in the illuminated region. After a complete averaged spectrum has been taken, the output is plotted by an X-Y recorder and/or recorded digitally on punched paper tape for subsequent computer analysis.

Nitella flexilis samples were purchased from Carolina Biological Supply Co., Burlington, N.C. The samples were grown in soil-pond water medium under normal room fluorescent light at room temperature. To perform an experiment we chose a healthy internodal cell, third or fourth from the newest cell on the growth end. These cells are normally about 3 or 4 cm in length. The cell is excised by cutting outside the nodes on either end. It is then positioned in the cell holder and allowed to rest until it achieves a steady streaming rate as determined by the real-time output of the spectrum analyzer. Cells treated in this way were observed to maintain a normal rate of streaming and response to external stimuli for many days. Before and after performing experiments, the cells were observed with a Nikon LK-E research microscope with phase contrast optics (Nikon, Inc., Garden City, N.Y.). During the course of the experiment the cells are continually observed with a Nikon EPOI Shopscope, which was fitted with a reticle to measure beam diameters and positions.

RESULTS

A sample spectrum obtained from a *Nitella* internodal cell at room temperature is presented in Fig. 3. The scattering angle θ was 28.8°. The center of the Doppler-shifted peak is 93 Hz, which corresponds to a velocity of 72 μm/s (T = 25.3°C). The distribution of spectral intensity about 93 Hz is quite narrow and uniform, indicating that a large portion of the protoplasm is flowing with about the same velocity. There is little intensity at higher frequencies, and the level at frequencies greater than 150 Hz is presumed to be the background due to "shot noise" in the phototube. We have found no evidence of any velocities above this region. There is considerable intensity at lower frequencies, indicating that at least some of the particles are impeded or are in a region of the protoplasm which flows more slowly. In the course of the experiments described in this report, we have taken more than a thousand spectra of this form.

A distinct advantage of studying protoplasmic streaming by light scattering is the ability to use the light scattering angular factors discussed briefly in the theory section. We have therefore extensively studied the angular dependence of the light scattering spectra. The first feature to investigate was the dependence of the Doppler shift of the center of the peak on the scattering angle, and hence on the scattering vector $\mathbf{K}$. For a monodisperse set of scatterers flowing at constant velocity the Doppler shift should be proportional to $\mathbf{K} \cdot \mathbf{v} = Kv\cos\alpha$. The results of the determination of the frequency

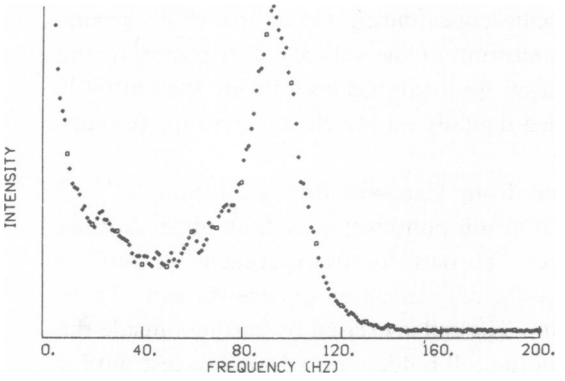

FIGURE 3

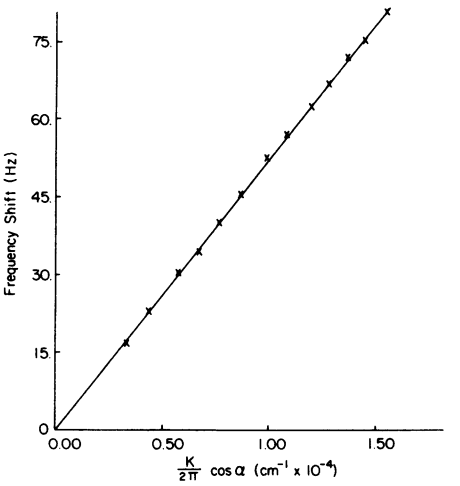

FIGURE 4

FIGURE 3 A typical spectrum of the scattered light from an illuminated region of flowing protoplasm showing a narrow, Doppler-shifted peak at 93 Hz. The frequencies observed are directly proportional to velocities in the scattering volume. This frequency peak corresponds to a streaming velocity of 72 μm/s.

FIGURE 4 A linear least-squares plot of frequency shift of the peak frequency in the spectrum versus $(K/2\pi)\cos\alpha$. K is the magnitude of the scattering vector and α is the angle between the streaming direction and the scattering vector. Since large particles contribute proportionately less intensity at higher scattering angles, we interpret the linearity of this plot to constitute convincing evidence that particles in the region of maximum streaming velocity are carried with the same velocity regardless of size.

shift as a function of scattering angle are presented in Fig. 4. These experiments were performed by illuminating the same volume on one cell for all measurements. The predicted linear dependence is observed to an extremely high degree of precision. The slope of the line is the velocity of the protoplasm parallel to the channel, which by a linear least-squares fit is determined to be 56.5 μm/s (T = 20.0°C). This sample of scatterers, however, is by no means monodisperse, and in fact, the distribution of size of scatterers undoubtedly ranges over several orders of magnitude. As was pointed out earlier, the large particles contribute proportionately less of the scattering intensity at higher scattering angles. We therefore interpret the linearity of this plot to constitute convincing evidence that particles in the region of maximum streaming velocity are carried with the same velocity regardless of size.

It is also significant to observe the width of the Doppler-shifted spectrum as a function of scattering angle. The theory predicts that the width of the shifted peak will be proportional to K^2 if it is broadened by diffusion and to K if it represents a distribution of directed velocities. A plot of spectral half-widths as a function of K is presented in Fig. 5. These half-widths are determined by measuring the half-width at half-height of the high frequency half of the Doppler-shifted peak. Error bars are drawn at each point to indicate the maximum variation which could be attributed to the interpreta-

tion procedure. Each point in Fig. 5 represents the average of several determinations on several different cells. The line in Fig. 5 is a fit of all except the highest angle data with the constraint that the line must pass through the origin. To within the precision of the data, it is clear that a linear dependence on K is obtained, indicating no substantial contribution to the width from diffusion. It is interesting, however, that at higher scattering angles, where the experiment is biased somewhat in favor of smaller particles, there is a slight upward deviation of the data. This deviation may be indicative either of the increased contribution of diffusion for small particles or of a greater dispersion in the streaming velocities of small particles. Based on the linearity of spectral half-widths with the first power of K which we have consistently observed, we have interpreted the spectra obtained as resulting from the Doppler shifts of a medium with a velocity distribution which is directly reflected in the scattering spectrum. Most of our experiments have been performed at low scattering angle where this approximation has the greatest validity, and in no case do we feel that the limits of this approximation exceed the uncertainty in the data.

The interpretation of the Doppler profile as purely a reflection of the velocity distribution requires two further assumptions. The first is that the distribution of particles in the flowing endoplasm is random, so that all parts of the endoplasm are represented equally. The endoplasm is about 20 μm wide, and the particles for the most part are of the order of μm, so this assumption is reasonable but certainly not rigorous. The second essential assumption is that secondary scattering (the scattering of a photon by two scatterers before it exits the wall) is insignificant. Secondary scattering leads to significant depolarized intensities, so we have measured the depolarization

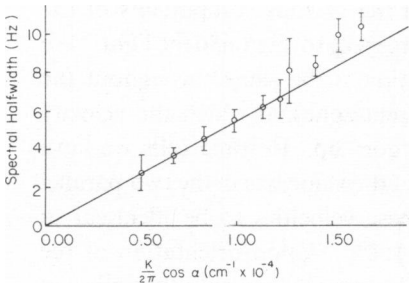

FIGURE 5

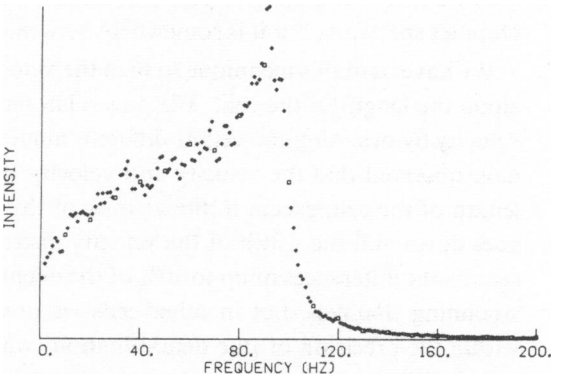

FIGURE 6

FIGURE 5 Spectral half-width of the Doppler-shifted peak plotted against $(K/2\pi)\cos\alpha$. The linearity of this dependence implies that diffusion makes only a small contribution to the spectrum. The deviation upward at high angles likely indicates that for the motion of smaller particles, diffusion may be more important.

FIGURE 6 Frequency spectrum from the parabolic laminar flow of a solution of 0.481 μm polystyrene latex spheres flowing between parallel plates of 20 μm separation. The scattering angle is 14.9°. The most probable velocity from this spectrum is 138 μm/s. The distribution of velocities typical of parabolic flow is accurately reflected in the Doppler spectrum.

ratio of the scattered light. The ratio of depolarized to polarized intensity is 0.02 ± 0.01. Moreover, the depolarized spectra are of the same form as the polarized spectra, indicating that the depolarized scattering comes largely from birefringent material which is flowing with the same velocity profile as the remainder of the protoplasm. The effects of secondary scattering are therefore presumed to be negligible.

It is important to note that in these experiments the illuminated region which is viewed by the photodetector includes an entire cross section of the flowing endoplasm, so the velocity distribution reflected in the spectrum is the velocity distribution from one side of the endoplasm to the other. The most striking feature of the velocity distribution measured by these experiments is that most of the particles have a velocity within 10% of the mode velocity of the distribution. This is a significantly more uniform distribution than is obtained from parabolic laminar flow between parallel plates, which is the model system most closely related to the narrow channel of flow in *Nitella*. For comparison we have constructed a model which consists of two parallel plates separated by 20 μm. The plates are several centimeters long, so that if fluid is allowed to flow between the plates at velocities comparable to protoplasmic streaming velocities, a parabolic laminar profile will be established. The spectrum from a solution of 0.481 μm diameter polystyrene spheres flowing in the model is presented in Fig. 6. The peak velocity of flow was 138 μm/s, which corresponds to the maximum flow velocity in the parabolic profile. The shape of the spectrum is consistent with theoretical predictions for parabolic flow in this system. The spectrum in Fig. 6 is to be contrasted with the protoplasmic streaming spectrum in Fig. 3. Clearly the protoplasmic streaming flow profile is a more uniform distribution. The streaming profile is by no means a plug-flow pattern, which would produce a single sharp peak in the Doppler spectrum, but it is somewhere between plug-flow and parabolic.

We have used this technique to map the velocities of the streaming at various points along the length of the cell. We have also measured the various components of the velocity by orienting the cell at different angles with respect to the incident light. We have observed that the velocity and velocity distribution is the same throughout the length of the cell, except in the vicinity of the indifferent zones, at which the velocity goes down and the width of the velocity distribution goes up. In some cells we have seen slight differences of up to 10% of the magnitude of the velocities in the two parallel streaming channels, but in other cells we observe these velocities to be identical to within the precision of our determination, which is 1–2%. Upon orientation of the cell at different angles with respect to the incident light, we observe only the expected angular decrease of the Doppler shift magnitude, with no apparent change in velocity distribution. If the cell is oriented so that the velocity of flow in the streaming channel is normal to the scattering vector, then the only component of the spectrum which remains is the low-frequency curve which seems to be centered at zero frequency and which may be attributable to diffusion, vibration, intracellular vibrational motion, amplitude modulation, or to the slight component of the velocity of particles which are not traveling exactly normal to the **K** vector.

Temperature Dependence of the Streaming

The most obvious environmental factor which may affect the streaming rate is the temperature of the cell. This has been recognized for many years, and a number of studies of the effects of temperature on the streaming velocity in *Nitella* have been published (13–17). Because light scattering measures the velocity distribution as well as the most probable velocity normally reported by microscopic observation, we have deemed it worthwhile to study the effects of temperature on the protoplasmic streaming velocity distribution as reflected in the light scattering spectrum. The temperature was controlled by placing the cells in a bath of medium which was maintained at the desired temperature by thermal contact with a much larger bath. In these experiments, it was very important to allow the cell an adequate amount of time to adjust after changing to a new temperature. This often took as much as a half hour even when the temperature was changed in small steps of only a few degrees centigrade. Stability was determined by monitoring the instantaneous output of the spectrum analyzer and waiting until the spectral peak was in a stable position for at least 5 min. This is probably a more rigorous test than has been applied in such studies before. Lambers (13) reported temperature shock only when the temperature was changed by 20° or so, and Tazawa (16) reports that the change of the velocity with changing temperature was "instantaneous." We observed all of the cells to be quite sensitive to temperature changes. The reason for this is not known, although a large number of intentional temperature jump experiments have been done (ref. 1, p. 88).

We have measured the temperature dependence of the most probable streaming velocity, determined by the peaks of the Doppler-shifted spectra over the temperature range 4–35°C. The results of this study are presented in Fig. 7, which is a plot of streaming velocity as a function of temperature. The data points on this plot include studies on 11 different internodal cells. No particular dependence on the direction of temperature change was observed. The spread in the data represents primarily variations among different cells; experiments on a single cell often gave a much smoother function. The data have been fit to a straight line by least-squares computer analysis, and the slope is 2.6 μm/s · °C. These results are in quite good quantitative and qualitative agreement with other measurements of this phenomenon using optical microscopy (13–17). The two striking aspects of this dependence of streaming velocity on temperature are the reproducible linearity and the large magnitude of the change in velocity with temperature. A review of attempts to explain these observations has been given by Kamiya (ref. 1, p. 83). The factors discussed include oxygen solubility, diffusion rates of ATP, and the temperature dependence of viscosity in the cell. Another factor could be the increase in metabolic rate with temperature. At present the temperature dependence of the streaming is well characterized but poorly understood.

The overall shape of the velocity distribution varies little with temperature, but the magnitude of the width of the Doppler-shifted peak changes substantially with respect to the magnitude of the shift. Although the half-width data are quite imprecise for these experiments, the general trend is that the distribution of velocities becomes

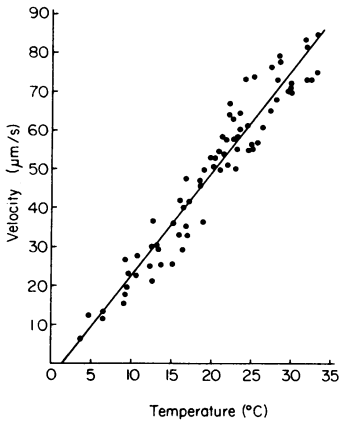

FIGURE 7

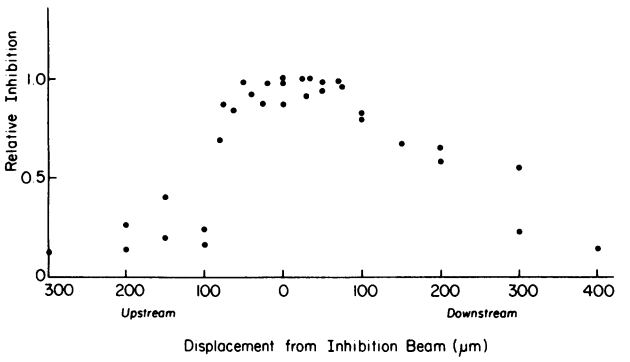

FIGURE 8

FIGURE 7 The variation in protoplasmic streaming velocity with temperature. Streaming velocities from the Doppler-shifted peaks, measured from 11 different internodal cells after the streaming rate had equilibrated to its new value for at least 5 min, show a linear dependence on temperature. Stable streaming values are not obtained above 34°C, the supraoptimal temperature for streaming, above which the cell irreversibly ceases streaming. The scatter of values on the graph indicates the biological variability.

FIGURE 8 The variation of "relative inhibition" of the streaming protoplasm with distance upstream and downstream from the inhibited region. A low intensity laser beam of λ = 632.8 nm is viewed by the photodetector as it probes either side of a laser beam of λ = 514.5 nm, which has intensity greater than the threshold for inhibition. The scattering from the inhibiting beam is attenuated by optical filters so that only the scattering from the observation beam is detected. "Relative inhibition" is a rough index based on the width of a spectrum or the position of a Doppler-shifted peak, if one is discernable. It is zero for normal uninhibited values of the Doppler shift and is one for an unshifted peak of minimum width. The inhibition is very local and is more extensive on the downstream side of the inhibiting beam.

narrower at higher temperatures, varying from a half-width which is about 12% of the shift at low temperature to a half-width which is only about 8% of the shift at around 30°C. This was not previously known, and it is not clear what the best explanation for it would be. The general principle involved is probably that the width is due to the dissipative forces which resist the flow of protoplasm, and these forces are presumably less effective at higher temperatures, as evidenced also by the higher rate of flow.

When the temperature is raised to just above 34°C, the velocity can be observed to decrease in time over a period of about an hour, after which the cell dies. If the temperature is lowered before the streaming ceases, it will continue to stream at low temperatures for an indefinite period of time, but at a lower rate than a healthy cell, indicating that some irreversible damage has been done. The temperature of 34°C is therefore called the supraoptimal temperature. Our value of 34°C for the supraoptimal temperature in *Nitella* internodal cells is in exact agreement with the first report of this phenomenon, which was given by Lambers (13). This sharp transition in cell behavior at a distinct and reproducible temperature is reminiscent of a phase transition. We therefore have performed differential thermal analysis experiments on

these cells in collaboration with Professor Edward Barrall at the University of Connecticut Institute for Materials Sciences. The experiments were performed on a DuPont Thermal Analyzer No. 900 (DuPont Instruments, Wilmington, Dela.), with a sensitivity of 5 mcal/s. On this instrument we were able to detect no first-order phase transitions over the temperature range 20–40°C. However, it was observed that upon heating above the supraoptimal temperature of 34°C, the heat capacity of the sample above 25°C was irreversibly decreased. This presumably is indicative of some phase transition or denaturation of small magnitude. However, the magnitude of the thermal effect was just slightly greater than the sensitivity of the instrument, so further characterization of this transition was not attempted.

Photoresponse of Nitella

The protoplasmic streaming in the internodel cells of *Nitella flexilis* is inhibited by intense light. For this reason the laser beam is greatly attenuated before it reaches the alga. The inhibition of the streaming is proportional to the intensity of the light in the illuminated region of the alga, with a threshold intensity of about 2 W/cm^2 below which no effects of the light are seen. This threshold intensity may vary from cell to cell and will generally be a function of the wavelength of the light, although our experiments to determine this dependence have not been reproducible. Our experiments were normally performed with a beam diameter of about 50 μm, so the laser power was attenuated to about 40 μW. If the threshold power is exceeded, the streaming can be observed to decrease, normally quite suddenly after an exposure time of 1–5 min. Reducing the power will completely reverse this effect. If a cell is inhibited by light at one point and then the beam is moved to another point, the cell is observed to be streaming normally (2). The room lights during these experiments were dimmed, but a control experiment in which the cell was illuminated by an incandescent lamp showed no response of the streaming velocity or pattern to this light, which had an intensity of less than 1 $\mu W/cm^2$.

The photoinhibition of the streaming by laser light was utilized in an experiment to determine the lower limit of the spatial domain over which the streaming could be inhibited. This experiment was performed by using two lasers of different wavelengths. The helium-neon laser (λ = 632.8 nm) was used as the observation laser, and its intensity was kept below the threshold intensity. An argon ion laser (λ = 514.5 nm) was used to inhibit the streaming with an intensity above the threshold intensity by at least a factor of 10. The inhibiting beam was held stationary while the observation beam was moved about in the vicinity to study the distribution of velocity. An optical filter blocked the inhibiting beam from the detection optics. Within the radius of the inhibiting beam, the observation beam produced scattered light with a low-frequency spectrum centered at zero frequency. As the observation beam was moved away from the inhibiting beam, the spectrum became broader and then became a clearly Doppler-shifted peak, whose shift magnitude increased with displacement until a normal spectrum with the normal Doppler shift was obtained. The data for these experiments are presented in Fig. 8. We have defined the term "relative inhibition," which is a rather

rough index based on the width of the spectrum or the center of the Doppler-shifted peak if one was clearly discernible. A relative inhibition of one means that the spectrum was an unshifted peak of minimum width, and a relative inhibition of zero corresponds to the normal uninhibited value for the Doppler shift. From Fig. 8 it is clear that the inhibition is complete for about one beam diameter and then falls off quite sharply. It is interesting that the distance over which the inhibition is effective is different on the two sides of the region by about a factor of two. The direction of displacement in these experiments was parallel to the flow in directions we have labeled "upstream" and "downstream." The inhibition is effective over a significantly longer distance on the downstream side. This can be interpreted to imply either that the mechanism of streaming is such that photoinhibition has greater effect downstream or that the effect of light is to put an impediment in the flow much like a stone in a stream which will have a greater effect downstream. At present these two possibilities are not distinguished.

If the inhibiting beam is maintained in one position on the cell for a half hour or longer, the spot becomes a "window" in which all of the chloroplasts have been detached. This process has been used to great advantage by workers doing optical microscopy experiments (18, 19). We have observed the velocity distribution in the window region after the inhibiting beam has been removed, and we obtain results consistent with these earlier reports. The window "recovers" over a period of about a day and obtains a normal velocity distribution even though it is free of chloroplasts. A very interesting observation on the bleached windows was their extremely high tolerance to high intensities of laser light. The threshold intensity for photoinhibition of streaming in a window region was 2,000 W/cm^2, fully a thousand times greater than the threshold for a normal cell. We interpret this result to mean that the photoresponse is mediated to a large degree by the chloroplasts.

DISCUSSION

The ultimate goal of research on protoplasmic streaming is a molecular explanation for the generation of the motive force. A number of investigators have concluded that the location of the generation of the motive force is at the interface between the endoplasm and ectoplasm or cortical gel (1, 20–24). Bundles of microfilaments, similar to actin-containing microfilaments in other motile systems, are situated at this interface and are thought to play a role in the mechanism (1, 19, 21, 22, 24–26). Fibrils lying along the ectoplasm can be observed to run parallel to the direction of streaming. There are many reports of organelle movement along these fibrils in cells of *Nitella* and *Chara* (19, 21, 27). One recent report suggests that these fibrils are a framework for filaments extending into the endoplasm and that streaming occurs via propagating bending waves of these filaments (19). The details of the mechanism remain obscure.

One direct manifestation of any proposed mechanism would be the velocity distribution which it would produce (28–30). The single most significant advantage of the laser light scattering experiment is that in each experiment a complete velocity dis-

tribution is determined in a matter of seconds. This velocity distribution includes the contribution of particles which may be too small to see in a microscope, but which are large enough to scatter significant intensities of light. We have shown that diffusion can be neglected at all but very high scattering angles, allowing the interpretation of frequency and velocity in a one-to-one correspondence. Fig. 3 therefore represents the velocity distribution in the flowing portion of the cell, with the peak of the Doppler-shifted curve corresponding to the velocity normally reported by microscopic observation. The only substantial reservation we have about this interpretation pertains to the possibility that particles of different sizes may be constrained to certain regions of the streaming channel. In that event, our distribution would unduly weight that region of the channel in which the larger particles are most prevalent. Most of the particles present in the protoplasm are significantly smaller than the streaming channel, and we therefore do not expect this source of error to be large, but we cannot say that it is negligible. The broadening of the spectrum by the transit time of particles through the beam can be shown to be a minor source of the spectral width in our experiments.

The velocity distribution implied by the data in Fig. 3 is more uniform than parabolic flow, but certainly not a plug flow. We observe no velocities in the cell which are above the distribution about the most probable velocity. The peak at low frequency and the increased intensity on the low-frequency side of the Doppler-shifted peak may be the result of shearing near the boundaries of the endoplasmic flow. The velocity gradient in the endoplasm near the endoplasm-ectoplasm interface should contribute low frequencies to the spectrum. Donaldson has shown from streaming velocities fit to theoretical models and stress/rate-of-strain relations that the shearing zone at the endoplasm-ectoplasm interface is of the order of 0.05 μm to 0.2 μm (31). Such a narrow region could only be the source of our low frequency intensity if it exerts a retarding force on particles elsewhere in the cytoplasm. Shearing of vacuolar sap could also contribute low frequencies to the spectrum from the lower shearing velocities. However, this source of scattering intensity appears to be small since the vacuoles, in the internodal cells studied, lack any obvious suspended inclusions or optical inhomogeneities. Also, the low frequencies in the light scattering spectrum change very little when the beam passes through the center of the vacuole rather than through the edge of the cell. One possibility which has not been eliminated is that the low frequency part of the spectrum comes from the gross motion of parts of the cell which are not related to protoplasmic streaming. A final possibility is that the low-frequency part of the spectrum is the result of the wavelike motions of filaments which have been suggested by Allen (19). These filaments are reported to be waving with a broad distribution of frequencies between 0.5 Hz and 5 Hz, which exactly corresponds to the low-frequency part of our distribution. Judging by the relative intensity of this part of our spectrum, however, we conclude that it is unlikely that the waving filaments account for a large portion of our low-frequency intensity. The presence of these filaments would probably be masked by the other sources of low-frequency intensity, so that we can make no definitive statement about their presence based on our data.

The facility with which this technique can measure velocity distributions has made it possible for us to perform the first complete study of the dependence of the distribution on temperature. The linear dependence of the most probable velocity on temperature is verified, and the biological variability of the cell response to temperature can be estimated from our data on a number of different cells. The distribution of velocities becomes relatively narrower at higher temperature throughout the range of temperatures studied. This could be due to weakened dissipative forces that resist the flow such as decreased viscous forces, or to the increased activity of the motive apparatus because of increased ATP production and rate of diffusion. The supraoptimal temperature for protoplasmic streaming in *Nitella* is verified to be 34°C. Efforts to locate thermal transitions or denaturation near the supraoptimal temperature by differential thermal analysis showed a gross shift in heat capacity when the cell is heated above the supraoptimal temperature.

A further advantage of the laser light scattering technique is the facility with which the photoresponse of the cell can be studied. Plant cells possess a complicated bioelectric response to light stimulation that includes not only the thylakoid membranes of chloroplasts, but also the cytoplasmic membrane of the cell (32–38). Protoplasmic streaming in plants has been shown to be stimulated by low intensities of light and inhibited by high intensities of light. The threshold above which no significant stimulation of streaming occurs has been shown to be 7×10^{-2} mW/cm^2 in *Nitella clavata* (33). Our experiments have shown that the threshold below which no significant inhibition of the streaming occurs is 2 W/cm^2 when the illumination is confined to a very small region of the cell. We find therefore a large plateau range of intensities in which the incident beam may be without affecting the streaming rate in the cell. Using a second, more intense beam, we have been able to perform the first experiments which measure the spatial extent of the inhibition (Fig. 8), and we have shown that the inhibition is greater on the downstream side. We have made efforts to determine the wavelength dependence of the inhibition of streaming, and we have found variability of about a factor of two from different experiments on the same cell, and about the same variation from one cell to another. Within this level of uncertainty, we have seen no difference in the ability of blue, green, or red light to inhibit the streaming. This is in sharp contrast to the results of Bottelier quoted in Kamiya, in which a difference of over two orders of magnitude was seen for different wavelengths inhibiting the streaming in an *Avena* coleoptile cell (ref. 1, p. 91).

A significant result in our study of the photoresponse of *Nitella* cells is the greatly increased threshold for light-induced streaming inhibition in "window" regions in which the chloroplasts have been exfoliated by prolonged exposure to an intense laser beam. A recent study on *Nitella* by Palevitz and Hepler has shown the microfilaments to be closely associated with the chloroplast envelope (26). The microfilaments are observed to lie adjacent to the chloroplast and may be attached to the chloroplast envelope. Our experiments show that the photoresponse of streaming is three orders of magnitude less sensitive in the region free of chloroplasts, supporting the link between chloroplasts and the streaming mechanism.

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