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Photoresponses of the Purple Nonsulfur Bacteria *Rhodospirillum centenum* and *Rhodobacter sphaeroides*

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We have measured the photoresponse of two purple nonsulfur bacteria, *Rhodobacter sphaeroides* and *Rhodospirillum centenum*, under defined conditions in a light beam propagating at 90° to the optical axis of the microscope. This beam presented cells with a steep gradient of intensity perpendicular to the direction of propagation and a shallow gradient in the direction of light propagation. *R. centenum*, a species that reverses to change direction, accumulated in the light beam, as expected for a "scotophobic" response, while *R. sphaeroides*, which stops rather than reverses, accumulated outside the light beam. We also compared the behavior of liquid-grown *R. centenum*, which swims by using a single polar flagellum, to that of surface-grown *R. centenum*, which swarms over agar by using many lateral flagella and has been shown to move as colonies toward specific wavelengths of light. When suspended in liquid medium, both liquid- and surface-grown *R. centenum* showed similar responses to the light gradient. In all cases, free-swimming cells responded to the steep gradient of intensity but not to the shallow gradient, indicating they cannot sense the direction of light propagation but only its intensity. In a control experiment, the known phototactic alga *Chlamydamonas reinhardtii* was shown to swim in the direction of light propagation.

There has been interest and controversy for many years about whether free-swimming phototrophic bacteria can sense the direction of light as well as its intensity, i.e., whether they show true phototaxis (3, 4, 12, 14). It is well-known that phototrophic bacteria, including *Chromatium*, *Rhodospirillum*, and *Thiospirillum* spp., reverse direction when swimming through a light/dark boundary, causing them to be trapped on the light side of the boundary (reviewed in reference 6). In 1953, Clayton demonstrated that a step down in light intensity of approximately 1% could cause a response in *Rhodospirillum rubrum* (5). These organisms accumulated in specific regions of a light spectrum corresponding to the absorption spectrum of their light-harvesting pigments. Examination of the behavior of these cells showed that they reversed direction when they swam out of an actinic wavelength, indicating that they can respond to changes in the intensity of particular wavelengths of light.

We were interested in whether free-swimming phototrophic bacteria can also sense the direction of propagation and move toward a light source. Large gliding cyanobacteria can certainly sense the direction of light and orient themselves in a favorable direction (12). Filamentous, gliding cyanobacteria show true phototactic orientation, i.e., movement oriented with respect to the direction of a light source, gliding either toward the light source (positive phototaxis) or away from it (negative phototaxis). In addition, these cells can sense the intensity of the light. The mechanisms used for directional sensing are still unresolved, but they involve calcium-dependent changes in electrical potential (11).

Rhodobacter sphaeroides and *Rhodospirillum centenum* were chosen for these experiments since they show different free-swimming patterns. *R. sphaeroides* swims by rotating its flagellum unidirectionally, stopping and starting rotation, and reorienting during stops (2). In response to a step down in light intensity, cells stop transiently, adapt, and then swim off in a new direction (15). Liquid-grown *R. centenum* swims by using a polar flagellum which reverses rotation, causing the cells to reverse swimming direction when presented with a step down in light intensity. On agar surfaces, *R. centenum* induces lateral flagella, which enable the cells to move over the agar. The colony moves as a unit and appears to be able to sense a change not only in the light intensity but also in the direction of the light (17, 18). Is this effect true phototaxis, or is it a colonial manifestation of the scotophobic response? We have compared the photoresponses of these two species to light presented as a beam which provided both a shallow and steep light gradient and a direction of propagation.

Our photobehavioral assay was designed to examine the response of a population of individual cells in a microscope field to light emitted from an optical fiber. The fiber was oriented at 90° to the optical axis and positioned such that the light beam was projected horizontally across the field of the microscope (Fig. 1). In this way, net movement of cells toward or away from the light source, as well as accumulation of cells in the light source, could be determined. Cells in the light beam scattered light into the microscope, in a manner similar to that used in dark-field microscopy. Light scattering was proportional to the number of cells in the light beam; thus, the flux of cells could be observed by measuring changes in light intensity. If cells simply responded to the step down in light, i.e., were scotophobic, they should accumulate uniformly in all parts of the beam. If the cells were also phototactic, they should move toward the optical fiber. This system allowed us to assay all these possibilities under the controlled conditions of a microscope slide. As a control, we also examined the behavior of the

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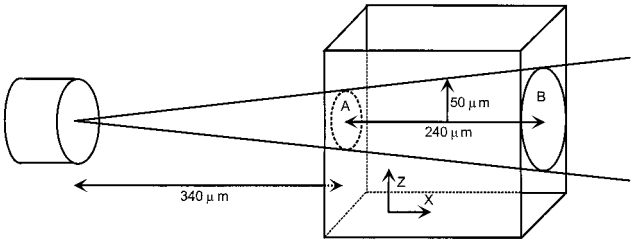

FIG. 1. Diagram of the light beam propagating from the tip of the fiber through the volume of the slide. The cube represents the volume of the slide in the field of the microscope. The distance from one edge of the field, point A, to the opposite side of the field, point B, is 240 μm. The radius of the beam at the center of the field is 50 μm. Measurements were taken from a single image (as in Fig. 2). The microscope views the experiment from above.

alga *Chlamydomonas reinhardtii* (which is known to exhibit true phototaxis) (14, 19).

MATERIALS AND METHODS

Bacterial strains and growth conditions. Wild-type *R. centenum* ATCC 51521 was a gift from Carl Bauer. Liquid cultures were grown photoheterotrophically at 42°C in PYVS medium (0.3% peptone, 0.3% yeast extract, 0.4% soytone, 20 ng of vitamin B_{12} per ml, 1.5 μg of biotin per ml) in a sealed tube 6 to 8 in. from a 75-W incandescent light bulb (8). These cells were examined in the photoresponse assay in the medium in which they were grown. Swarm cells were grown as described by Ragatz et al. (18). Briefly, 1 ml of early-stationary-phase liquid culture was concentrated by centrifugation to 50 μl, and 8 μl of this suspension was applied to a PYVS swarm plate (0.8% agar), allowed to dry for a short time, and then incubated in the dark for 4 h or until dense growth was apparent at the site of inoculation. This incubation allowed the induction of lateral flagella. Swarming was initiated by placing the plate in front of a tungsten spot light bulb (150 W). The cells were incubated in this fashion until the colony had moved a distance equal to at least one colony diameter and the number of flagella (see below) indicated that the cells were fully differentiated into swarmer cells. Cells from the leading edge of the swarming colony were resuspended in a small volume of distilled water. This apparently removed extracellular polysaccaride, which allowed the swarmer cells to swim in liquid medium (4a). The motile cells were then examined in the photoresponse assay.

R. sphaeroides WS8NΔop was grown photoheterotrophically at 25°C in minimal succinate medium 12 to 15 in. from a 52-W incandescent light bulb as previously described (1). Cells were harvested in early log phase, when the complement of light-harvesting and reaction center complexes was low, conditions under which the step-down photoresponses are maximal (10). The cells were assayed in succinate medium.

C. reinhardtii was grown photoautotrophically at 22°C in basic culture medium (Ward's Biology, Rochester, N.Y.) 12 to 15 in. from a 52-W incandescent light bulb on an 18-h light, 6-h dark cycle. The cells were assayed in their growth medium.

Visualization of flagella. Surface-grown cells were resuspended in sterile deionized water, as described above (4a). Liquid-grown cells were tested in the culture medium in which they were grown. A 3-μl volume of cell suspension was placed on a microscope slide and covered with a 22- by 22-mm cover glass. A 3-μl volume of Flagella Stain (Carr-Scarborough Corp., Decatur, Ga.) was placed at the edge of the cover glass and allowed to move under it by capillary action. The cells were stained for approximately 5 min and then examined by phase-contrast microscopy.

Measurement of photoresponses. The optical fiber (8-μm core, 125-μm diameter, 0.11 numerical aperture (NA) in air, 0.14 NA in water [Newport Corp., Irvine, Calif.]) was coupled to a Zeiss (Thornwood, N.Y.) 100-W tungsten-halogen light source via a Zeiss infinity-corrected 20× 0.75-NA objective mounted on a Newport precision fiber coupler. The parallel beam from the light source was projected directly into the back of the objective. The heat filter was removed from the collector lens housing to allow transmittance of infrared light, the optimal light for inducing photoresponses in purple nonsulfur bacteria. A simple shutter was inserted between the light source and the objective. The amount of light transmitted by the fiber was measured by placing the tip of the fiber in contact with the face of a quantum radiometer (Li-Cor, Lincoln, Nebr.). A direct reading of 0.1 μmol of photons m^{-2} s^{-1} was obtained; dividing by the area of the sensor (4.7×10^{-5} m^2), approximately 2×10^3 μmol of photons s^{-1} through the fiber was obtained. This is a rough estimate, since the sensor is calibrated with the full area of the face illuminated (12a). The absolute intensity, although sufficient to do the experiment, was not as important as the gradients presented to the cells. As the beam diverged in the X direction, its intensity decreased as a function of cross-sectional area. The beam at point A was threefold brighter than at point B, whereas in the Z direction the intensity decreased in a Gaussian profile from a maximum at the center to essentially zero at the edge of the beam (Fig. 1). Thus, we have a shallow gradient in the X direction and a sharp gradient in the Z direction. This is a method similar to that described by Moss et al. for the study of *Chlamydomonas* phototaxis (13).

Experiments with *C. reinhardtii* used a fiber with a 50-μm core and a 125-μm diameter. This was necessary due to the large size (10 to 15 μm in diameter) and great swimming speed (≈60 μm/s [swimming speed measured as described in reference 16]) of this organism. The cells swam through the relatively narrow beam used for the bacteria in less than 2 s (if they swam across the center of the beam), which is approximately the length of time required for a response in *C. reinhardtii* (for a review, see reference 7).

The Zeiss Axioplan 2 microscope was fitted with a 40×, 0.75-NA phase-contrast objective, a model C2400-80 image intensifier (Hamamatsu Corp, Tokyo, Japan), and a Dage-MTI (Michigan City, Ind.) charge-coupled device (CCD) camera. The voltage setting on the intensifier and the gain and contrast settings on the CCD camera were determined empirically and were identical for all experiments. The final image represented a 240- by 220-μm area of the slide. The experiments were recorded with a Sony EVC-100 Hi-8 video recorder.

Cells were examined in slide chambers approximately 0.2 mm deep, 1 cm wide, and 2.2 cm long. These were constructed by attaching two cover glasses to a slide (parallel to the slide surface, leaving a 1-cm gap between them) with vacuum grease. A third cover glass was attached to the first two (across the gap) with vacuum grease, creating a tunnel. Both ends of the chamber were left open. The cell suspension (approximately 5×10^8 cells ml^{-1}) was then added, and the chamber was mounted on the microscope and incubated for several minutes to allow the center of the chamber to become less aerobic. Although both species showed photoresponses under aerobic conditions, the responses were stronger under anaerobic conditions (10). The tip of the optical fiber was then inserted under the bridging cover glass and held in place by attaching it to the microscope stage. The tip of the fiber was imaged by phase-contrast microscopy and positioned by adjusting the position and rotation of the stage so that the light beam produced was horizontal with respect to the camera. The microscope was focused on the core of the fiber. The tip of the fiber was then moved one microscope field (approximately 200 μm) out of view. The microscope light source was turned off, and the cells were kept in darkness for several minutes to allow them to distribute randomly through the chamber. At the start of the experiment, the shutter was opened. Light scattered by the cells was amplified by the image intensifier and imaged on the CCD camera. Data were recorded on video tape.

Data from the video tape were digitized on a personal computer at 5-, 10-, or 20-s intervals with a Metamorph (Universal Imaging Corp.) image analysis package with a Matrox MVP-AT (Dorval, Quebec, Canada) video capture board. At each time interval, 30 video frames (1 s) were added by using the sum 16-bit function of Metamorph and stored to disk. The intensity profile was measured across the beam on both the right and left sides of each captured image (Fig. 2). The baseline was subtracted, and the area under the curve was calculated. The data are presented as this area as a function of time.

RESULTS

C. reinhardtii. *C. reinhardtii* cells exhibited the classic phototactic response in our system. They entered the beam at random, and then the majority of the cells turned and swam toward the fiber. When the cells reached the face of the fiber (50 μm in diameter), they either ran into the fiber and then bounced off into the dark or simply swam past the fiber into the dark. Thus, there was no accumulation of cells in the beam. We quantified the response by counting the number of cells swimming toward (546 cells) and away from (150 cells) the fiber during a 57-min period (Fig. 3).

***R. centenum* liquid-grown cells.** *R. centenum* liquid-grown cells exhibited the standard scotophobic response; that is, they swam into the light beam and became trapped. Accumulation became apparent after only a few seconds, increased rapidly over about 50 s, and was maximal after 150 s. Swimming cells collected in the light beam at the same initial rate at both sides of the image (Fig. 4), indicating that they were being trapped in the higher intensity of the light beam. However, over the period of the experiments (which was up to 20 min), there was no sign of cells moving toward or away from the source of the light as judged by the scattered intensity of the population as well as observation of individual swimming cells. If there was a net movement of cells toward the fiber, we would expect a corresponding drop in the cell concentration on the side of the image away from the tip of the fiber; however, this was not the

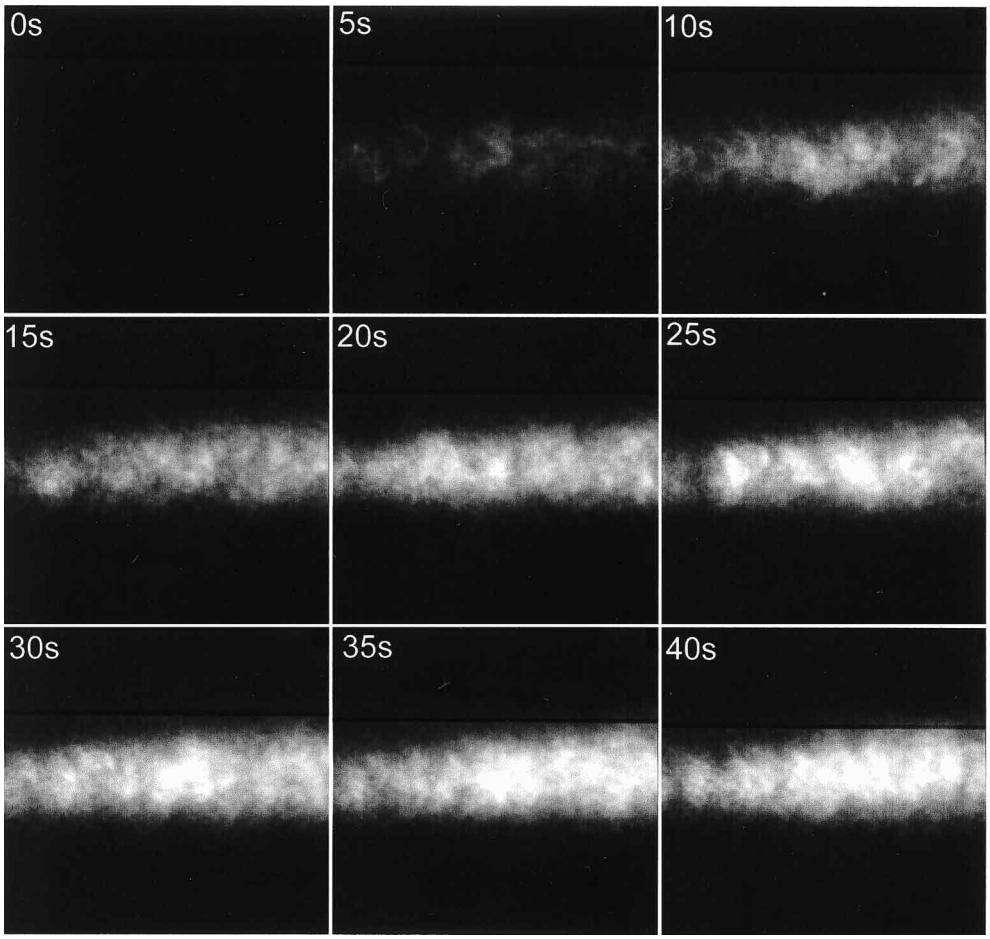

FIG. 2. Time series of *R. centenum* liquid-grown cells accumulating in the light beam. The results obtained in 1 s (30 frames) were averaged every 5 s. The left-hand side of each image is closest to the light source, with the beam propagating to the right. Intensity measurements are shown in Fig. 4.

case. The response was saturated after about 150 s (Fig. 4), almost certainly because the cells within a few hundred micrometers have a high probability of swimming across the light/dark boundary and, once there, become trapped by the scotophobic response. However, cells farther from the illuminated region are many times less likely to move into the light beam. After cells within about 1 mm from the illuminated region become trapped, the rate of movement into the beam becomes a property of an unbiased random walk. The higher cell density on the side away from the light source is probably due to the larger beam width in that area (Fig. 4). As the circumference of the beam gets larger, there are more cells close to the boundary which can enter the beam.

***R. centenum* surface-grown cells.** Swarmer cells were tested in the photoassay within 5 min of suspension in deionized water. They remained hyperflagellated for approximately 40 min after suspension, as determined by flagellar staining and direct observation by phase-contrast microscopy (data not shown). Unlike liquid-grown cells, swarmer cells swam much faster in the light than they did in the dark, suggesting that they were light limited. Dark-incubated cells required 10 to 15 s to reach full speed upon illumination. As expected, individual cells were clearly motile in the beam of the photoresponse assay but did not swim preferentially in the direction of the fiber as did *C. reinhardtii* (data not shown). The slow swimming of the cells in the dark resulted in a slower entry of cells into the beam, as demonstrated by the slower rise in Fig. 5 than in Fig. 4. There was no increase in the number of cells closest to the light source compared with those away from the light source, suggesting that there was no net movement of cells (Fig. 5).

R. sphaeroides. Unexpectedly, *R. sphaeroides* showed a steady linear decline in the number of cells in the light path (Fig. 6). The cells both close to and away from the light source behaved identically, again indicating no ability to sense the direction of the light (Fig. 6). The steady exodus from the light beam continued until the cells cleared an area of the sample chamber corresponding to the light path (Fig. 7).

DISCUSSION

The above data clearly show that free-swimming purple nonsulfur bacteria in liquid medium do not sense the direction of light but simply respond to a change in light intensity. We have shown that a truly phototactic organism (*Chlamydomonas*) swims toward the light source in our experimental setup (Fig. 3). Neither bacterium under discussion exhibited this behavior under any condition tested (Fig. 4 to 6).

The photoresponses observed were all due to a decrease in the light intensity, a step-down response. The two species investigated have very different swimming patterns. Liquid-grown *R. centenum* uses its polar flagellum to swim forward

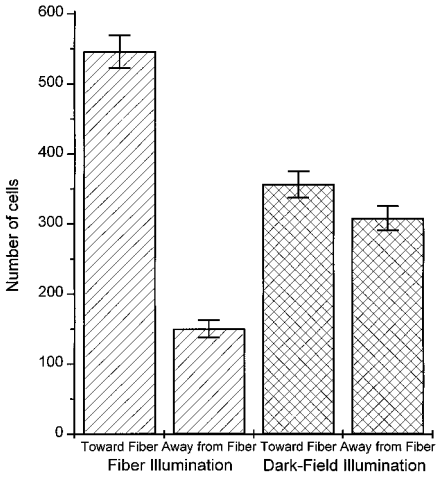

FIG. 3. Bar graph of *C. reinhardtii* swimming direction. The cells were examined with a 4× objective. Illumination was provided either by the optical fiber (diagonal lines) or by standard dark-field illumination (crossed lines). The cells were recorded on video tape. The direction of cell swimming was scored by counting the number of cells which crossed a vertical line (drawn across the beam projected by the fiber) on the video monitor. Error bars are ± the square root of the number of cells.

and periodically reverse. Therefore, when faced with a step down in light intensity, the cells reverse direction and become trapped in the light beam (Fig. 4). *R. sphaeroides*, on the other hand, changes direction by transiently stopping flagellar rotation, and Brownian motion reorients the cell during this stop. Thus, when swimming over a light/dark boundary, the *R. sphaeroides* cell stops, adapts, and then swims off in a new direction. If the reorientation is truly random, the chance of that cell swimming back into the cone of light is less than 50%. The photoresponse in *R. sphaeroides* therefore resulted in a phototrophic bacterium being trapped not in the light but in the dark (Fig. 6), a curious result but one that is consistent with the idea that *R. sphaeroides* is not sensing light but, rather, integrating multiple stimuli sensed through changes in the common electron transport chain to move toward areas of maximum electron transport rate (9, 10). Previous data have shown that *R. sphaeroides* responds to changes in the rate of electron transfer by a transient stop followed by adaptation, whether the stimulus was a reduction in light, oxygen, or dimethyl sulfoxide (DMSO). Data have also shown that electron transfer components are shared between respiratory and photosynthetic pathways and that the pathways interact, with oxygen, for example, reducing any light-induced responses. *R. sphaeroides* is therefore responding to changes in electron transfer by stopping, reorienting, and swimming in a new direction. If the new direction continues to cause a reduction in electron transfer, the cell stops again, but if it is a direction where the electron transfer rate increases, it continues to swim normally. In a gradient of oxygen or an anaerobic acceptor such as DMSO, the cells create their own steep gradient as a result of metabolism of the acceptor. This is not the case with light,

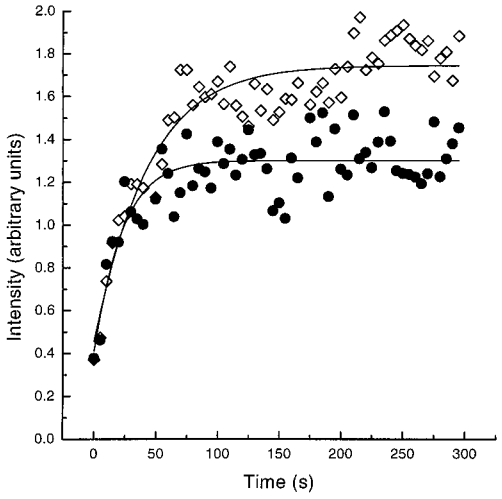

FIG. 4. Plot of *R. centenum* liquid-grown cells accumulating in the light beam versus time. Open diamonds represent the right-hand side of the image, and solid circles represent the left-hand side of the image, as depicted in Fig. 2. The intensity profile was measured on the left and right sides of each captured image. The baseline was subtracted, and the area under the curve was calculated. The data are presented as the area under the curve versus time.

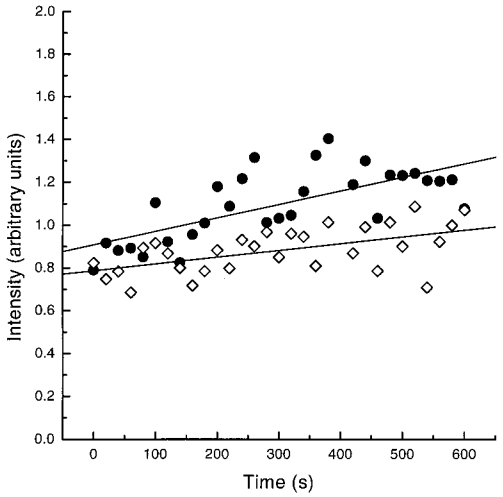

FIG. 5. Plot of *R. centenum* surface-grown cells accumulating in the light beam versus time. The results obtained in 1 s (30 frames) were averaged every 20 s. See the legend to Fig. 4 for details.

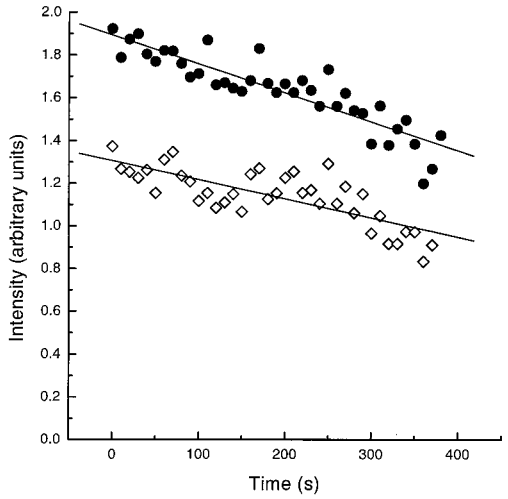

FIG. 6. Plot of *R. sphaeroides* liquid-grown cells accumulating in the light beam versus time. The results obtained in 1 s (30 frames) were averaged every 10 s. See the legend to Fig. 4 for details.

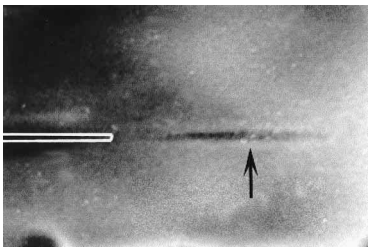

FIG. 7. Image of the microscope slide preparation of *R. sphaeroides* directly after the conclusion of the experiment in Fig. 6. The slide was removed from the microscope and photographed. A diagram of the fiber has been drawn over the image to indicate the previous position of the fiber (white lines). The light is propagating from left to right as in Fig. 1. The black arrow shows the zone of clearing where the cells have left the light beam. The elapsed time from the start of the experiment to removal of the fiber was approximately 20 min.

which is a permanent gradient. Free-swimming cells in a clear environment, such as liquid medium, would be unlikely to encounter a 1% reduction in intensity in the few seconds required for temporal sensing, although this may be possible for cells in dense microbial mats. The data suggest that for free-swimming cells, a reversal response to a sharp light/dark step down will bring the cells back into the light but that the motility response of *R. sphaeroides* may have evolved to cope with different gradients. This implies that in the natural environment, oxygen and other terminal electron acceptors may play a more important role than light in controlling the movement of *R. sphaeroides.*

Colonies of surface-grown *R. centenum* have been shown to move toward or away from light in a way that suggests that they can sense the direction of the light (17, 18). When cells were moved from these active swarms into liquid medium, the individual cells proved unable to sense the direction of the light, although the light/dark boundary was sensed (Fig. 5). There are no data on whether cells resuspended from swarm plate-grown colonies swim by using lateral or polar flagella. However, *R. centenum* has a single sensory pathway controlling responses to light and chemical gradients (4a). If this is the case, it should make no difference whether the lateral or polar flagella are being used, since they should both be controlled by the single sensory pathway. The observation that individual cells from a colony showing a phototactic response do not show phototaxis indicates that phototaxis is a colonial response. In the experiment of Ragatz et al., colonies of *R. centenum* moved down the intensity gradient toward the light source (18). This effect could be explained by scotophobia if one considers that the leading edge of the colony shades the rest of the colony. Any cell which moves from the leading edge into the colony will experience a drop in intensity and will reverse direction. Thus, the net movement is toward the light even though the total intensity is lower. It would be instructive to measure the intensity of light inside the colony by using microsensors.

Given these results, is there a role for photoresponses of the purple nonsulfur bacteria in the natural environment, and, if not, why are photoresponses seen? It seems unlikely that photoresponses are a major environmental response in free-swimming bacteria under conditions where there are shallow light gradients. These would not produce the change in intensity during the periods of smooth swimming required to cause a response, and, as shown above, free-swimming bacteria are unable to sense the direction of light. Why, then, do bacteria show photoresponses? Recent data have shown that the responses to light, oxygen, and alternative oxidants such as DMSO are integrated (9, 10). Cells probably respond to a change in the rate of electron transport through common components of the electron transfer chain. Any change in the rate of electron transfer, probably measured as a change in the redox state of one of the components, will cause a change in behavior. It seems likely that the steep change in light intensity provided in our experiment is one effector of this redox sensor. The primary role of this redox sensor is to keep the bacteria in an environment where the electron transport rate is maximal.

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