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Motility Response of *Rhodobacter sphaeroides* to Chemotactic Stimulation

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Tethered rotating cells of *Rhodobacter sphaeroides* varied widely in their stopping frequency; 45% of cells showed no stops of longer than 1 s, whereas others showed stops of up to several seconds. Individual cells alternated between stops and rotation at a fairly constant rate, without continuous variation. Addition of the chemoattractant propionate to free-swimming cells of *R. sphaeroides* increased the mean population swimming speed from 15 to 23 μm s^{-1}. After correction for nonmotile cells, the percentage swimming at less than 5 μm s^{-1} dropped from approximately 22 to 8, whereas the percentage swimming at greater than 50 μm s^{-1} increased from 6 to 15. However, cells already swimming did not swim faster after propionate addition; the increase in the mean population speed after propionate addition was caused by an increase in the mean run length between stops from 25 to 101 μm. The increased run length was the result of a drop in both the stopping frequency and the length of a stop. Addition of propionate over the range of 10 μM to 1 mM decreased the stopping frequency; this decrease was almost entirely blocked by benzoate, a competitive inhibitor of propionate transport. The chemoattractants acetate and potassium had the same effect as propionate on the distribution of stopping frequency, which demonstrated that this is a general behavioral response to chemotactic stimulation. Adaptation to propionate stimulation was slow and very variable, cultures frequently showing little adaptation over 30 min. This characteristic may be the result of the lack of a highly specific chemosensory system in *R. sphaeroides*.

Bacteria swim by the rotation of one or more flagella driven by the electrochemical proton gradient (22). Enteric bacteria such as *Salmonella typhimurium* and *Escherichia coli* have several flagella coupled by mechanical forces into a bundle, the counter-clockwise rotation of which results in smooth swimming (21). Periodically brief periods of clockwise rotation cause the bundle to fly apart and the cell to tumble. Chemoattractants cause a suppression of tumbling as the bacterium moves up the concentration gradient, with consequent longer periods of smooth swimming (5, 6, 19).

The usual mechanism for detection of a chemical gradient by bacteria is via cell membrane receptors (methyl-accepting chemotaxis proteins [MCPs]) that span the cytoplasmic membrane (13). Adaptation to the continued presence of a chemoattractant is mediated by methylation of the MCPs at specific glutamate residues. Not all chemotactic responses are mediated by this system, however; sugars of the phosphotransferase system require group translocation with an involvement of enzyme II (1, 7, 9, 26), whereas positive aerotaxis in *S. typhimurium* requires electron transport and is sensed by cytochrome *o* (20, 27). The electrical membrane potential has also been shown to be involved in chemotaxis to sugars in *Spirochaeta aurantia* (12).

Motility and chemosensing in the purple photosynthetic bacterium *Rhodobacter sphaeroides* are very different from the activities described in enteric bacteria. *R. sphaeroides* has a single subpolar flagellum that rotates only clockwise; direction changing of the cell is not caused by counter-rotation of the flagellum but by Brownian motion when the flagellum stops rotating (4). The principal chemoattractants for *R. sphaeroides* include the weak organic acids, which are strong repellents in enteric bacteria (28). MCPs have not been identified in this species, and there appears to be an obligate requirement for transport in chemotactic signaling (16, 28). Chemotaxis in *R. sphaeroides* is sensitive to the addition of proton ionophores, which suggests a possible involvement of the proton motive force (PMF). Indeed, since the phototactic and aerotactic responses of *R. sphaeroides* require electron transport coupled to a change in the PMF, cells may integrate signals from several sources via the PMF (3). Little is known of how chemotaxis affects the swimming behavior of *R. sphaeroides*. The highly variable unstimulated swimming behavior and the lack of a tumbling response means there is not a clear transition to smooth swimming upon addition of a chemoattractant. Populations of cells show a wide range in speed and swimming pattern that requires the use of computer tracking to analyze (P. S. Poole, D. R. Sinclair, and J. P. Armitage, Analo Biochem., in press). Because of the lack of a sophisticated MCP signaling system in *R. sphaeroides*, we felt it important to determine how motor function and hence swimming patterns are changed by chemoattractants.

MATERIALS AND METHODS

Chemicals. *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), sodium propionate, and sodium acetate were obtained from Sigma Chemical Co. All other reagents were of analytical grade.

Bacteria and growth media. *R. sphaeroides* WS8 (wild type) was obtained from W. R. Sistrom. Cultures were grown on medium A of W. R. Sistrom under tungsten filament illumination and anaerobic conditions at 25°C as previously described (3). Cells were harvested in late log phase at a density of approximately 10^9 cells ml^{-1} and suspended in 10 mM HEPES (pH 7.2) sparged with N_2 for at least 45 min.

Computer tracking. Cells were sealed in optically flat

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microslides (Camlab, Cambridge, United Kingdom) and observed with a Nikon Optiphot microscope; this procedure maintained cells in an illuminated anaerobic environment during the experiment. For rotation analysis, cells were tethered with antibody raised against flagellar filament and sealed in microslides, and their rotation was tracked in real time by using an image analysis system as described elsewhere (Poole et al., in press). Free-swimming cells were tracked for up to 3 min, with typically 200 cells tracked. Individual cells were tracked for 0.4 s, with a high level of discrimination against noncellular material to allow accurate detection of stopped cells. Data were output as discrete track speeds for each cell, and the speeds of all cells were averaged to give a measure of population behavior. Since the tracking system can detect stopped cells, the effect of chemoattractants on stopping frequency can be accurately determined. All assays were conducted in pairs by first measuring the free-swimming speed distribution of untreated cells, followed by direct measurement of the speed distribution of cells plus attractant. At least three replicates were performed before the significance of a speed change was determined by the use of a paired *t* test.

Motility traces. Bacteria were viewed with a Nikon Optiphot phase-contrast microscope, using a 100× E plan apo oil immersion objective. The image was transferred to a Panasonic Vidicon video camera via an extension tube and 2.5× imaging lens. This image was videotaped on a Sony Umatic video recorder, and individual cell tracks were traced on acetate sheets.

Measurement of membrane potential. At alkaline pHs, the electrical component or membrane potential makes up almost all of the PMF (23). The membrane potential of cells was measured by monitoring the absorbance changes of carotenoid pigments in the cytoplasmic membrane by using a DW2000 dual-wavelength spectrophotometer (SLM-Aminco) (2, 8, 17). Samples were incubated under a stream of nitrogen in the spectrophotometer for 20 min to allow equilibration. The carotenoid absorbance was measured by using the wavelength pair 523-510 nm and the bandshift in response to actinic illumination at 90° with saturating light at 871 nm. The total membrane potential was measured at the end of the experiment by the addition of 20 μM CCCP to completely collapse the potential.

RESULTS

Tethered cells. Figure 1 shows the rotation for 5 min of a typical cell of *R. sphaeroides* tethered by its flagellum. Rotation stopped for 1 s or longer six times over this period; stops of shorter duration resulted in a transient decrease in the rotation rate. Although there were spikes where the apparent rotation rate increased above the mean of 2.4 Hz, they were very short and caused in part by quantization of the rotation rate by the image analysis system. Analysis of 18 cells for approximately 10 min each gave a mean rotation rate of 3.4 Hz (standard deviation, 0.7 Hz). The variability between cells is shown by the absence of stops of longer than 1 s in 8 of the 18 cells. Absolute stop times varied from less than 0.5 s, which is the current limit of detection of the computer system, to several seconds. With the exception of stops, cells rarely showed large changes in the mean rotation rate for prolonged periods. This observation suggests that individual flagellar motors do not continuously vary their rotation rates but instead alternate between a stopped state and a full rotation rate. Antibody-tethered cells of *R. sphaeroides* appeared to rotate more slowly and showed fewer stops than did cells which spontaneously tethered themselves to the slides, which suggested that the antibody may have had an effect on rotation. Because of this observation, the effects of chemoattractants on behavior of free-swimming *R. sphaeroides* were investigated.

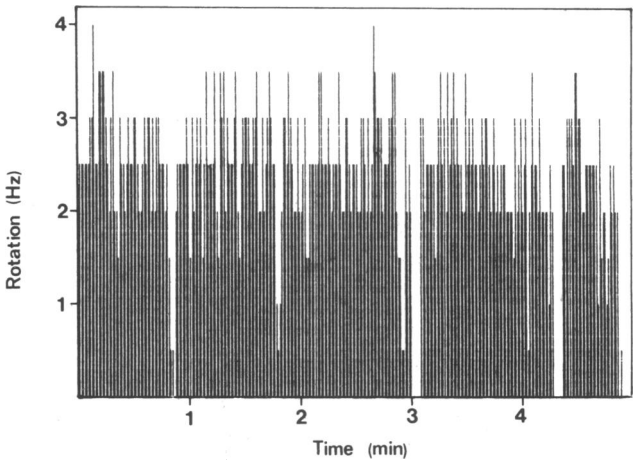

FIG. 1. Rotation of cells of *R. sphaeroides* that were antibody tethered by the flagellum. Rotation rates were analyzed and averaged for each second.

Free-swimming behavior. The distribution of speed for free-swimming cells of *R. sphaeroides* WS8 varied widely, from 0 to more than 120 μm s^{-1} (Fig. 2A). The high percentage of cells at low speed was the result of both motile cells that had stopped and genuinely nonmotile cells.

One of the strongest chemoattractants for *R. sphaeroides* is propionate, and addition of propionate to cells caused a significant increase in the mean swimming speed, from 15 to 23 μm s^{-1} (Fig. 2). Although these data might suggest that propionate increased the speed at which individual cells swam, data presented later show that the change in the mean population swimming speed was due to a decrease in the number of stopped cells. The percentage of stopped cells (speed of <5 μm s^{-1}) decreased from 37 to 23, whereas the percentage of cells showing fast runs (speed of >50 μm s^{-1}) increased from 6 to 15 after propionate addition. Since 10 to 15% of cells were nonmotile even in highly motile cultures, there was always an overestimation of the number of cells temporarily stopped. This fact implies that the real percentage of stopped cells during the course of the experiment changed from approximately 22 to 8 after propionate addition. Such data strongly suggest that the attractant suppressed stopping and enhanced fast runs. Starvation of freshly harvested cells often increased the degree of stimulation by chemoattractants, although the effect varied from culture to culture. Addition of chloramphenicol (50 μg ml^{-1}) did not inhibit the change in stopping frequency, which indicated that there was no requirement for protein synthesis. Propionate addition at concentrations of 10 μM to 1 mM caused a reduction in stopping frequency, as indicated by an increase in the mean population swimming speed (Table 1). The precise response at any concentration varied considerably, with 1 mM causing apparent speed increases of between 30 and 70% in different batches of cells and at different times after suspension in HEPES buffer.

Addition of acetate, which is also a strong attractant for strain WS8, shifted the mean population swimming speed from 15 to 24 μm s^{-1} (Fig. 3). The percentage of cells

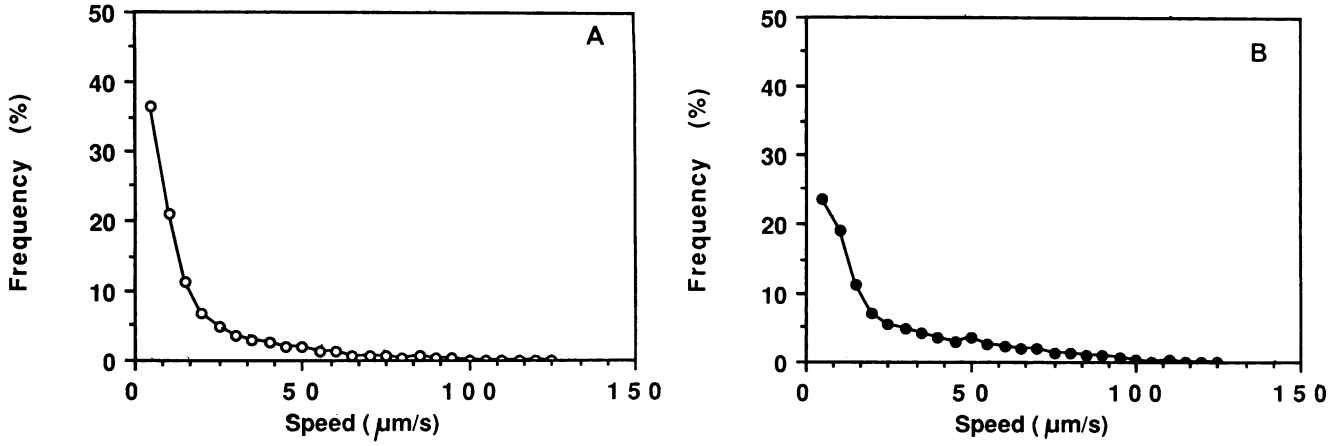

FIG. 2. Frequency polygon of the effect of propionate addition on the speed distribution of free-swimming cells of *R. sphaeroides*. (A) Control cells in HEPES buffer. (B) Cells after addition of 1 mM propionate. Data are from 25 experiments and approximately 4,600 tracked cells for each sample. The means and standard errors for control and propionate-treated cells were 15 ± 0.26 and 23 ± 0.35 μm s^{-1}, respectively, which are significantly different at a probability level of 99%.

swimming at less than 5 μm s^{-1} decreased from 42 to 31, whereas the percentage of cells swimming at more than 50 μm s^{-1} increased from 6 to 17.

Acetate and propionate belong to the same class of chemoattractant for strain WS8 and inhibit each other's transport (16). To test a different class of attractant, potassium was added to unstimulated cells; this addition caused a decrease in stopping frequency, as reflected in the apparent increase in speed from 14 to 22 μm s^{-1} (Fig. 4). Once again there were qualitative shifts in the distribution of cell speed, with the percentage of cells swimming at less than 5 μm s^{-1} dropping from 48 to 30 and the percentage swimming at more than 50 μm s^{-1} increasing from 7 to 12.

Inhibition of the chemotactic response. Benzoate is a known competitive inhibitor of propionate transport and chemotaxis, while itself moving only passively across the membrane of *R. sphaeroides* and causing no chemotactic response (16). Addition of 2 mM benzoate did not cause any significant effect on population behavior but did block the response caused by the addition of 50 μM propionate (Table 1).

Mechanism of population speed change. Although the mean speed of a population of *R. sphaeroides* increased significantly upon addition of the chemoattractant propionate, acetate, or potassium, this fact does not by itself indicate that individual cells were tending to swim faster. Addition of a chemoattractant could actually cause the flagellar motor to rotate faster, resulting in an increase in the swimming speed of individual cells; alternatively, a chemoattractant may change the frequency or duration of stops. Either change in behavior would increase the mean swimming speed of a population of cells, which is what is measured by the tracking computer. When all cell speeds of less than 50 μm s^{-1} were removed from the data, the cell tracts left were those of bacteria which continued swimming smoothly throughout the experiment and exhibited no stops during the 0.4-s measured time. A real change in speed would be seen as an increase in the average speed of cells swimming at more than 50 μm s^{-1}. It is assumed that since these cells are on average swimming below the possible maximum, they would show at least a partial increase in speed in response to chemoattractants. When cell speeds of less than 50 μm s^{-1} were subtracted from the speed distributions before and after propionate addition, the average speed changed from 68 to 69 μm s^{-1}, respectively, which is not a significant change (Fig. 2). This finding suggested that the motor itself was not rotating faster after addition of propionate but that changes in motor stopping frequency were more important.

TABLE 1. Effect of propionate and benzoate on the free-swimming speed of *R. sphaeroides*[a]

Addition	Speed (%)
None	100
Propionate	
0.01 mM	121
0.05 mM	143
1 mM	137
Benzoate (2 mM)	105
Benzoate (2 mM) + propionate (0.05 mM)	111

[a] All treatments are the average of at least four replicates. Values for the treatments with 2 mM benzoate and 2 mM benzoate plus 0.05 mM propionate are not significantly different from the control value at the 95% probability level. Values for the treatments with 0.05 and 1 mM propionate are significantly different from the control value at the 95% probability level, whereas the value for the 0.01 mM propionate treatment is significantly different at the 94% probability level.

Motility track analysis. To confirm that the behavioral change observed after chemotactic stimulation was a change in stopping frequency and stopping time, the patterns of motility tracks for up to 10 s before and after propionate stimulation were traced and compared. The mean and standard deviation for swimming lengths of 53 cells were 25 ± 14 μm before propionate addition, compared with 101 ± 86 μm for 26 cells after propionate addition. This finding clearly demonstrates that the run time had increased almost certainly as a result of a decrease in stopping frequency after propionate addition. Visual examination of swimming also revealed that the duration of stops decreased significantly. Therefore, addition of propionate caused a decrease both in the number and in the duration of stops of *R. sphaeroides*.

Adaptation to chemoattractants. An important part of the response to chemoattractants is the ability to adapt to a stimulus by returning to the prestimulus behavior. Figure 5 shows a typical adaptation response in *R. sphaeroides* WS8. The apparent speed of propionate-stimulated cells dropped to the control level within approximately 15 min. However, adaptation times were extremely variable, with some cul-

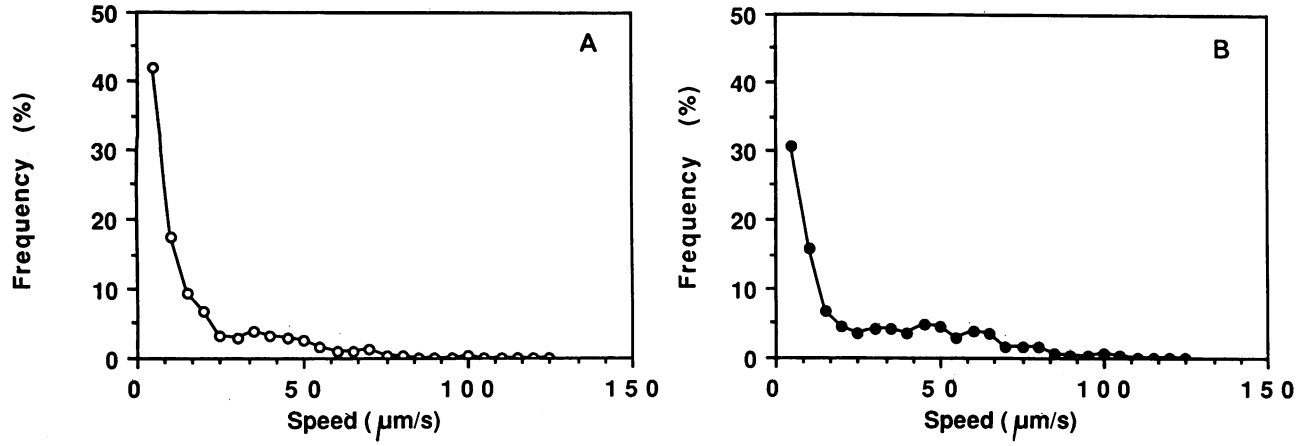

FIG. 3. Frequency polygon of the effect of acetate addition on the speed distribution of free-swimming cells of *R. sphaeroides*. (A) Control cells in HEPES buffer. (B) Cells after addition of 1 mM acetate. Data are from seven experiments and approximately 1,250 tracked cells for each sample. The means and standard errors for control and acetate-treated cells were 15 ± 0.51 and 24 ± 0.68 μm s^{-1}, respectively, which are significantly different at a probability level of 99%.

tures showing no adaptation after 30 min and others adapting within 10 min.

To test the ability of cells stimulated with one attractant to respond to an attractant in a different chemoeffector group, strain WS8 was suspended in HEPES buffer with 1 mM KCl, and the ability of the cells to respond to propionate addition was measured. These cells still showed a 68% increase in the mean population speed upon propionate addition, which reflected a decrease in stopping frequency. This observation demonstrates the ability of the cells to respond to an additional attractant.

Membrane potential. Measurement of the steady-state membrane potential of *R. sphaeroides* showed that there was no increase in membrane potential upon addition of propionate (Fig. 6). Response to chemoattractants therefore did not appear to occur as a result of a simple increase in the steady-state membrane potential causing an increase in swimming speed. This finding also shows that the increase in chemotactic response after starvation of cells was not caused by the fact that propionate acted simply as an energy source.

DISCUSSION

Previous studies have shown that the chemotactic responses of *R. sphaeroides* are linked to the transport of the chemoattractant and that this activity may involve the PMF (16). Lowering the membrane potential by addition of proton ionophores caused a concomitant loss of propionate taxis and transport even though motility was still at the control level (J. P. Armitage, P. S. Poole, C. J. Ingham, and A. Crallagher, submitted for publication). Clearly, such a system is very different from an MCP-dependent system, which uses a specific set of receptors to detect and adapt to chemoattractants. Since the mechanism of detection is apparently so different, it was of great interest to determine whether the mechanisms of response and possible adaptation are also different.

Analysis of tethered rotating *R. sphaeroides* revealed that there were large differences among cells in the frequency of stops of longer than 1 s. Almost 45% of the cells showed no stops of 1 s or longer when examined for 10 min, whereas others showed stops of up to several seconds. Most individ-

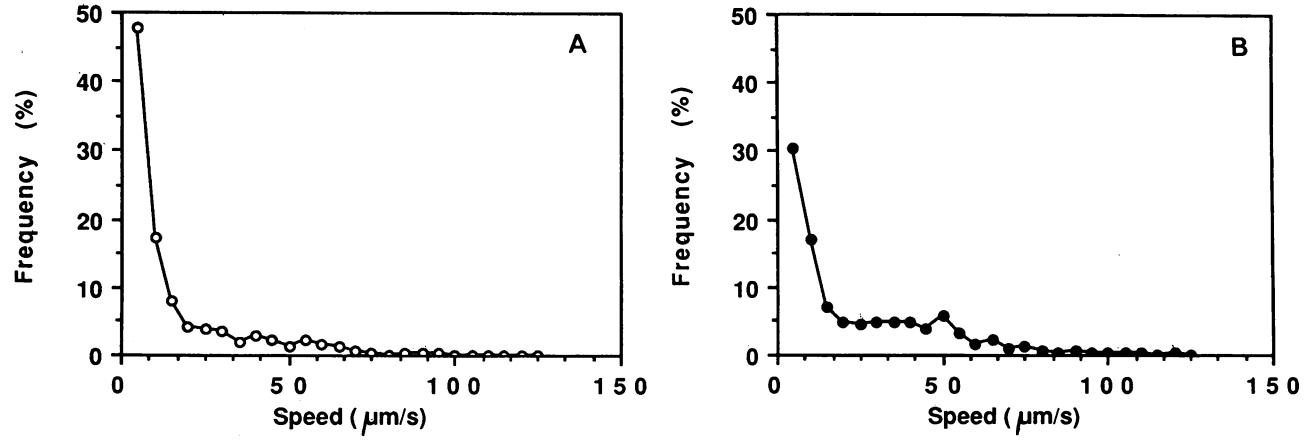

FIG. 4. Frequency polygon of the effect of potassium addition on the speed distribution of free-swimming cells of *R. sphaeroides*. (A) Control cells in HEPES buffer. (B) Cells after addition of 1 mM KCl. Data are from three experiments and approximately 500 tracked cells for each sample. The means and standard errors for control and KCl-treated cells were 14 ± 0.74 and 22 ± 1.00 μm s^{-1}, respectively, which are significantly different at a probability level of 95%.

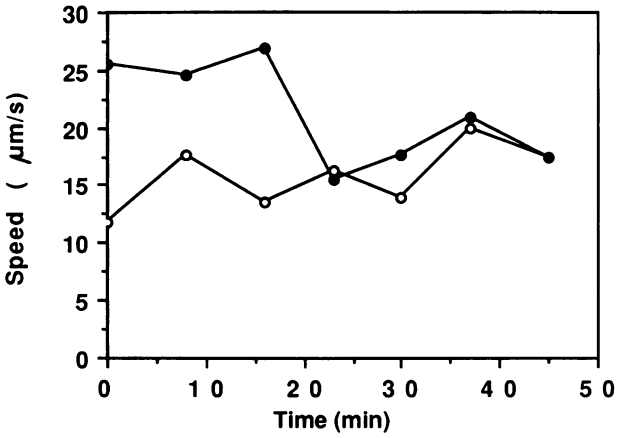

FIG. 5. Adaptation of *R. sphaeroides* to propionate addition. Symbols: ○, control cells; ●, cells plus 1 mM propionate.

ual cells did not vary the rate of rotation in a continuous fashion; instead, they switched between rotating at a set level and stops. However, antibody-tethered cells did not appear to rotate as fast or stop as frequently as did cells that spontaneously tethered themselves to cover slips. The lower rate of rotation in antibody-tethered than in naturally tethered cells suggested that the antibody or other conditions of tethering might have impeded cell body rotation. Furthermore, the large differences in stopping frequency among tethered cells implied that the analysis of a chemotactic response in one cell may have little relevance to a population of cells. To examine true population responses, without the possibility of antibody interference, we therefore examined free-swimming cells.

Addition of propionate, acetate, or potassium to *R. sphaeroides* increased the mean population speed by 53, 60, and 57%, respectively (Fig. 2 through 4). The measured increase in mean population speed was not, however, caused by an increase in the rate of flagellar rotation, since comparison of the speed of cells already swimming smoothly before and after propionate addition showed no significant change. This finding suggests that the apparent change in speed was due to qualitative changes in stopping behavior. Such changes were reflected in the decrease in the number of cells swimming at less than 5 μm s^{-1} after addition of a chemoattractant and in the approximate doubling in the number of cells showing runs of more than 50 μm s^{-1}. As a direct measure of the swimming pattern, motility traces were examined; the mean run length between stops was found to increase from 25 to 101 μm after propionate addition. This increase in run length is a measure of the decrease in stopping frequency. The duration of stops also appeared to decrease. Therefore, the general response of *R. sphaeroides* to addition of a chemoattractant was a decrease in the frequency and duration of stops; this response resulted in cells moving up a concentration gradient, reorientation during the stops being brought about mainly by Brownian motion. There is evidence that *Rhizobium meliloti* also decreases the frequency of stops of its peritrichous flagellar bundle in response to a chemoattractant (11). However, the stop times are less than 0.1 s, unlike the highly variable stop times of *R. sphaeroides*. The highly complex swimming pattern of *Spirochaeta aurantia* is also changed in response to a chemoattractant by an increase in smooth swimming (10). While all of these systems lack the tumbles of *E. coli* and show widely differing responses to attractants, they all move up a concentration gradient by increasing smooth swimming. This fact reinforces the essential unity of temporal sensing in bacteria.

It has been shown that blocking the uptake of propionate with benzoate inhibits chemotaxis as measured by using plug plates and chemotaxis wells (16). The use of the temporal assay described here demonstrates that benzoate also inhibits the behavioral response to propionate. This finding supports the suggestion that the minimum requirement for chemotaxis is transport of the attractant.

There was no change in the steady-state membrane potential after propionate addition, which demonstrates that the response was not the result of alteration of the stopping frequency by a metabolic increase in the steady-state membrane potential (Fig. 6). A similar conclusion has been reached by titrating the membrane potential of *R. sphaeroides* with CCCP and concomitantly measuring propionate taxis as well as several other parameters (Armitage et al., submitted). It was shown that the membrane potential could be reduced to less than 20% of the maximum before propionate taxis was severely inhibited.

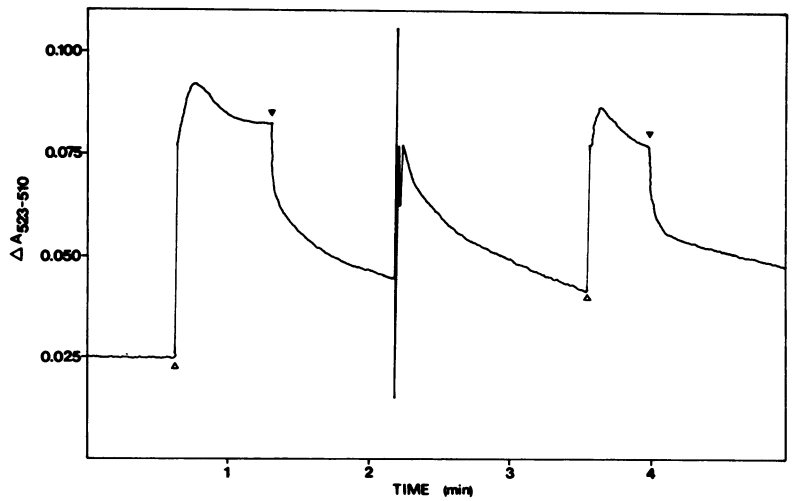

FIG. 6. Membrane potential of *R. sphaeroides* measured from the carotenoid bandshift. Symbols: △, actinic light source turned on; ▲, actinic light source turned off. Propionate (1 mM) was added just after 2 min. The transient spike is caused by opening the spectrophotometer.

Although it has been shown that *R. sphaeroides* lacks MCPs, the free-swimming behavior of *R. sphaeroides* does return to the prestimulus state with time after addition of propionate, which indicates an ability to adapt to stimulation (Fig. 5). However, there was no consistent time period for adaptation to the addition of a set concentration of attractant; some cultures partially adapted within 10 min, whereas others showed little adaptation within 30 min. These very long periods for adaptation to stimulation by weak acids in *R. sphaeroides* can be compared with the equally long periods for adaptation of *E. coli* to repulsion by weak acids (18). In *E. coli*, the repulsion response appeared to be caused by a drop in the intracellular pH, sensed by the MCP Tsr. The degree of increased tumbling in *E. coli* was directly related to the acidity of the weak acid. Internal pH sensing does not appear to be involved in sensing in *R. sphaeroides*; indeed, benzoate did not cause any change in behavior compared with that seen with acetate or propionate, whereas potassium did (16). Despite the lack of MCPs, cells of *R. sphaeroides* suspended in the presence of the attractant 1 mM potassium were able to respond normally to propionate addition, demonstrating the ability to adapt to one attractant and allow response to a second attractant. The large and variable adaptation time may be a result of chemotactic sensing requiring the transport or metabolism (or both) of an attractant rather than a specific receptor system. Numerous factors, including growth, membrane synthesis, transport, and general cellular metabolism, will influence such a fundamental sensing system.

Double mutants of *E. coli* lacking both the methyltransferase and methylesterase for MCP modification can still respond to chemoattractants although they lack the ability to adapt to the stimulus via methylation of MCPs (29–31). These double mutants do eventually partially adapt to a stimulus but over a long time period. Weis and Koshland (31) have shown that the chemotactic response to aspartate in these mutants requires a very steep concentration gradient. This finding indicates that methylation in *E. coli* is required for a normal sensing response in addition to adaptation to chemotactic stimulation. The methylation of the MCP system of enteric bacteria appears to be involved in fine tuning the chemotactic response to a given magnitude of stimulation, whereas phosphorylation of Che proteins is involved in signal transduction (14, 15, 24, 25, 32). *R. sphaeroides*, which lacks the MCP system, resembles these double mutants of *E. coli* in its poor adaptation after the addition of a chemoattractant. In terms of the enteric model, *R. sphaeroides* does not have an MCP system with which to reset its sensitivity to continued stimulation by a chemoattractant. These results suggest that MCP-independent sensing may be a basic response of cells, whereas MCP sensing adds a very sensitive and sophisticated control system. It is now apparent that the mechanism of signal transduction in *R. sphaeroides* may be very different from that in enteric bacteria. This is currently an area of active investigation.

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