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Sensory electrophysiology of bacteria: Relationship of the membrane potential to motility and chemotaxis in *Bacillus subtilis*

(cyanine dye/triphenylmethylphosphonium/tumble regulator)

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ABSTRACT The relationship of membrane potential to motility and chemotaxis of *Bacillus subtilis* has been tested by using the fluorescence of a cyanine dye as a probe of the potential. The dye fluorescence was found to be an indicator of membrane potential by correlation with triphenylmethylphosphonium ion distribution and with changes due to anaerobicity and ionophore addition. When the potential was sufficient for motility and constant over time, it was found that the absolute level of the potential did not affect the swimming behavior of the bacteria. Transient alteration of the membrane potential did, however, lead to changes in swimming behavior. Attractants were found to alter the swimming behavior of the bacteria without altering the membrane potential. Thus, change of the overall membrane potential of a normal *B. subtilis* is not required for chemotaxis, but such a change is sensed by the bacteria just as changing levels of attractants and repellents are sensed.

Many types of cells identified with sensory systems respond with transient or permanent changes in membrane potential when confronted with specific stimuli. Behavior of this sort is seen in cells as diverse as sensory neurons in complex mammalian systems (1) and the unicellular protozoa (2). The alteration in membrane potential is a signal that can then be transmitted to another site and lead to an appropriate behavioral response.

Among the simplest organisms to show sensory behavior are the chemotactic bacteria, such as *Salmonella typhimurium*, *Escherichia coli*, and *Bacillus subtilis* (for review see refs. 3–5). The ability of bacteria to migrate in response to gradients of attractants or repellents results from their ability to control their tumbling frequency (6, 7). In a homogeneous environment the bacteria show a random pattern of swimming in which periods of swimming in approximately straight lines (smooth swimming or running) alternate with periods of abrupt changes in direction (tumbling). The bacteria migrate by suppressing tumbling when moving in a favorable direction and increasing tumbling when moving in an unfavorable direction. The sensing system involves a bacterial memory which can be rationalized in terms of a parameter called the response regulator (6, 8).

That membrane potential can affect the swimming pattern and might thus be involved in chemotaxis was indicated by findings that a number of membrane-active drugs (9, 10), as well as inhibitors and uncouplers of oxidative phosphorylation (11, 12), can influence motility. Ordal and Goldman (11, 12), and later de Jong *et al.* (13), concluded that transient changes in potential cause swimming behavior changes. Szmelcman and Adler (14) have recently found an increase in triphenylmethylphosphonium uptake when attractants or repellents are added to *E. coli*. In studies in our laboratory (15, 16), perturbations of the electron transport chain, and hence of the ability of the bacteria to generate a membrane potential, were found to affect the swimming behavior of the bacteria and to be additive with other sensory stimuli.

These results strongly implicated membrane potential in some way with chemotactic behavior, but what was needed was more direct information on membrane potential during the rapid chemotactic response. Bacteria are too small to allow direct measurement with microelectrodes, but in recent years cyanine dyes have been used as probes of membrane potential in bacteria (17), erythrocytes (18), and neurons (19). Cyanine dyes, however, provide only an empirical, qualitative correlation with membrane potential. On the other hand, the equilibrium distribution of permeant cations, such as triphenylmethylphosphonium ion, has been quantitatively correlated with membrane potential in bacteria (20, 21) and membrane vesicles (22, 23), but the chemotactic response is probably shorter than the time required for equilibration of these ions. In this study, therefore, we have correlated cyanine dye fluorescence with membrane potential as established with permeant cations and then correlated bacterial behavior with the cyanine dye fluorescence. The results provide a consistent picture of the role of membrane potential in chemotactic behavior.

MATERIALS AND METHODS

Bacteria. *B. subtilis* strain W168 is wild type for chemotaxis and has no specific growth requirements. More than 90% of the bacteria were motile and showed the normal nongradient pattern of alternating smooth swimming and tumbling that we shall call "random" behavior.

Growth Conditions. A spore stock of W168 was inoculated into a medium consisting of 1% tryptone, 0.5% NaCl, 0.14 mM $CaCl_2$, 0.2 mM $MgCl_2$, and 0.01 mM $MnCl_2$ (medium one) (24). The bacteria were grown to an OD_{590} of 0.5–0.8 and then harvested by centrifugation. Unless otherwise stated, the bacteria were then resuspended and washed three times in the chemotaxis buffer of Ordal and Goldman (11), which contains glycerol, lactate, phosphate, $CaCl_2$, $MgCl_2$, and $MnCl_2$.

Chemicals. Valinomycin and carbonylcyanide *m*-chlorophenylhydrazone (CCCP) were obtained from Sigma and kept as 1 mM stock solutions in 95% ethanol. The cyanine dye, 3,3′-dipropylthiodicarbocyanine iodide [diS-C_3-(5)] (18), was a gift of A. S. Waggoner of Amherst College and was kept as a 1 mM stock solution in 95% ethanol. [^{3}H]Triphenylmethylphosphonium bromide ([^{3}H]TPMP) (specific activity 114 mCi/mmol) was a gift of H. R. Kaback of the Roche Institute. Labeled TPMP was mixed with unlabeled TPMP to give a stock solution of 10 mM TPMP (measured activity = 4 mCi/mmol) for use in distribution experiments.

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Abbreviations: TPMP, triphenylmethylphosphonium bromide; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; diS-C_3-(5), 3,3′-dipropylthiodicarbocyanine iodide.

Microscopy. Bacteria were observed with a Leitz microscope and tungsten light source as described before (16). Presence of the cyanine dye at dye/bacteria ratios used in these experiments did not affect the motility of the bacteria, although photodynamic effects were observed at higher ratios.

Fluorescence Measurements. Fluorescence measurements were made on either a Perkin-Elmer MPF-2A or Spex Fluorolog fluorimeter. Measurements were made at a bacterial concentration of about 2×10^8 cells per ml ($OD_{590} = 0.3$) and a diS-C_3-(5) concentration of 167 nM. Excitation and emission wavelengths of 622 nm and 670 nm with band widths of 10 nm were used (18). The bacteria were kept well aerated by a device that stirs the bacteria within the cuvette as measurements are made. Mixing time with this stirring device is under 1 sec. Agents to be tested were added to the stirring bacteria from ethanolic stock solutions. The ethanol alone did not affect the swimming behavior of the bacteria. Control experiments were performed with phospholipid vesicles or in the absence of bacteria.

[^{3}H]TPMP Distribution Measurements. The method used was based on similar methods reported by Schuldiner and Kaback (22) and used by Szmelcman and Adler (14). The bacteria were grown to an OD_{590} of 0.5–0.8 in medium one and then harvested and washed three times in chemotaxis medium. After the last washing, the bacteria were resuspended to an OD_{590} of 20 in chemotaxis buffer and kept on ice. The bacteria were then diluted with an equal volume of chemotaxis buffer at room temperature and were stirred at room temperature for 5 min. Then sodium tetraphenylboron was added to a final concentration of 2 μM, followed immediately by addition of [^{3}H]-TPMP from the stock solution to a final concentration of 10 μM. The bacteria remained motile throughout these procedures. At appropriate times, 50-μl portions of the bacterial suspension were then transferred to 5-ml test tubes, where they were quickly diluted with 2 ml of 0.1 M LiCl, filtered through Bio-Rad uni-pore polycarbonate filters (0.45-μm pore size), and washed with an additional 2 ml of 0.1 M LiCl. The filters were then air dried, placed in liquid scintillation vials with 10 ml of Handifluor (Mallinckrodt), and assayed for radioactivity in a Packard model 3375 spectrometer. Background radioactivity was determined in the absence of cells and was negligible.

Effects of Chemicals on [^{3}H]TPMP Distribution. To test for the effects of attractants and repellents on TPMP distribution, the bacteria prepared as above were incubated with the [^{3}H]TPMP for 15 min with continuous vigorous stirring. The bacterial suspension (50 μl) was then quickly added to a shaking 5-ml test tube already containing 5 μl of 10 times concentrated repellent or attractant to be tested. At various times after this addition, the bacteria were filtered and the amount of [^{3}H]-TPMP retained was determined as above. Addition of chemotaxis buffer served as a control.

Calculation of Membrane Potential from TPMP Distribution. The external [TPMP] was determined from the specific activity of the [^{3}H]TPMP and the [TPMP] in the external medium after correcting for uptake by the bacteria. The internal [TPMP] was calculated from the amount of TPMP retained by the bacteria on the filter and the internal volume of the bacteria, which was found to be about 2 ml/g dry weight of cells (OD_{590} of 0.1 equals 50 μg dry weight of cells per ml) by determination of sucrose-impermeable space. The membrane potential was then calculated from the Nernst equation (21).

RESULTS

Effects of Oxygen on Fluorescence and Swimming Behavior. The oxygen level available to the bacteria affects the fluorescence intensity of the cyanine dye diS-C_3-(5) and also the bacterial swimming behavior, as shown in Fig. 1. If cyanine dye is added to a well-aerated suspension of bacteria, a steady baseline of fluorescence develops. If the well-aerated cells are stirred under nitrogen instead of air, the fluorescence initially remains constant but then increases rapidly to a new plateau value two to three times above baseline. This increase in fluorescence parallels the utilization of available oxygen by the bacteria as measured with an oxygen electrode. If the anaerobic bacteria are provided with air, the fluorescence rapidly decreases to the baseline level. Because these bacteria would be expected to be unable to maintain a high membrane potential in the absence of oxygen, these results indicate that depolarization of the membrane leads to an increase in diS-C_3-(5) fluorescence. This correlation of increased dye fluorescence with decreased potential appears to hold in all the experiments reported here.

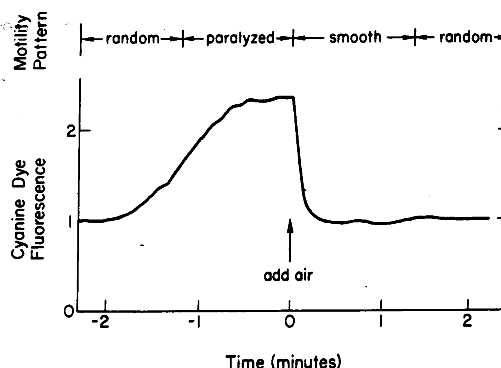

FIG. 1. Effects of oxygen on the fluorescence of diS-C_3-(5) and on the motility of *B. subtilis*. The bacteria were prepared in chemotaxis medium and the fluorescence and motility patterns were determined. The bacteria were allowed to stir under nitrogen in the fluorescence cuvette for 3 min until the point indicated by the arrow, at which time the nitrogen was replaced by air. Motility measurements were made in the presence of the cyanine dye. Short periods of constant tumbling were sometimes observed before the period of paralysis. Fluorescence intensity in this and subsequent figures is given in arbitrary units, where the baseline fluorescence in chemotaxis buffer with bacteria is given the value 1.

The swimming behavior of the bacteria is also dependent on the oxygen level. Well-aerated bacteria remain motile for long periods in chemotaxis medium, but under anaerobic conditions *B. subtilis* rapidly becomes immotile. If the immotile anaerobic bacteria are suddenly exposed to oxygen, they will regain motility in a few seconds. For the first 30–90 sec after the sudden exposure to oxygen (a temporal gradient) the bacteria show smooth swimming only, after which they regain a random swimming pattern.

Effect of Altering Membrane Potential with Valinomycin on Dye Fluorescence and Swimming Behavior. The effect of added valinomycin, which is an electrogenic potassium-specific ionophore and thus affects the membrane potential, on the fluorescence of diS-C_3-(5) in the presence of bacteria is dependent on the external concentration of potassium. As shown in Fig. 2, if the bacteria are suspended in a medium free of potassium (100 mM sodium phosphate buffer, pH 7), added valinomycin will cause a slight decrease in the fluorescence of the dye, which is consistent with the expected hyperpolarization of the membrane under those conditions (cf. ref. 25). In the presence of increasing amounts of external potassium, however, the fluorescence change seen on the addition of valinomycin is from the small decrease to a very large increase, which indicates a depolarization.

The effect of added valinomycin on the swimming behavior of the bacteria is also dependent on the external potassium

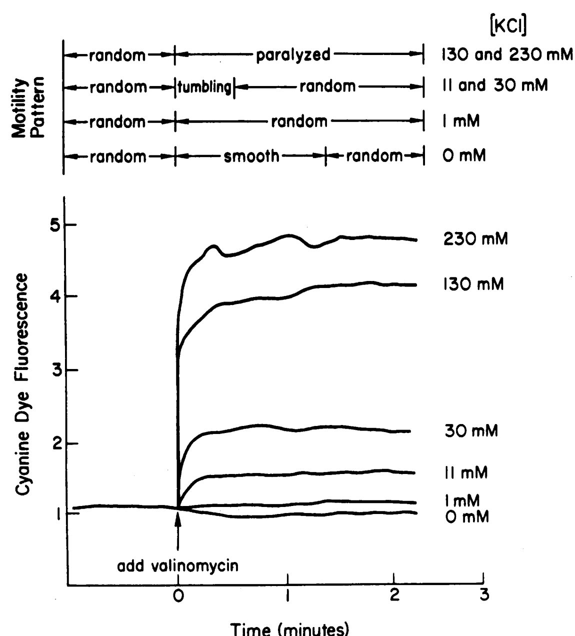

FIG. 2. Effects of added valinomycin on the fluorescence of diS-C_3-(5) and on the motility of *B. subtilis*. The bacteria were suspended in 100 mM sodium phosphate buffer, pH 7, with the indicated external concentrations of KCl. After equilibration with the dye for several minutes in the particular medium, 5 μM valinomycin was added at the time indicated. Fluorescence and motility patterns were then determined. Short periods of constant tumbling were sometimes observed before paralysis occurred. Fluorescence units are arbitrary as in Fig. 1.

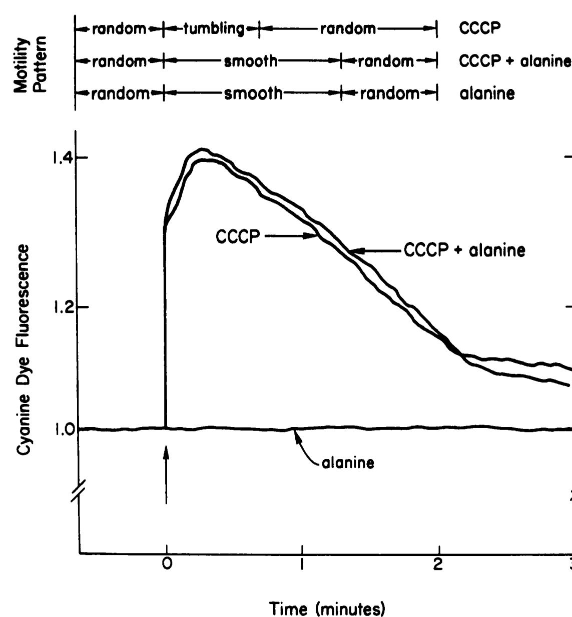

FIG. 3. Effects of CCCP and L-alanine addition on the fluorescence of diS-C_3-(5) and on the motility patterns of *B. subtilis*. The bacteria were prepared in chemotaxis medium in the presence of the cyanine dye and the fluorescence and motility patterns were determined. After 1 min of equilibration with the cyanine dye, 10 mM L-alanine, 0.3 μM CCCP, or 10 mM L-alanine and 0.3 μM CCCP were added at the time indicated by the arrow. Fluorescence units are arbitrary as in Fig. 1. The difference between the two curves is not significant.

concentration. In potassium-free medium (100 mM sodium phosphate buffer, pH 7), addition of valinomycin leads to a transient period of completely smooth swimming for about 30–60 sec. The swimming pattern then returns to random. If the external potassium concentration is greater than 100 mM, addition of valinomycin is followed by a rapid and complete paralysis of the bacteria. At external potassium concentrations of about 1–100 mM, addition of valinomycin is followed by brief periods of constant tumbling, with the swimming behavior of most of the bacteria returning to random after 10–30 sec, although some bacteria are paralyzed.

Effect of Altering Membrane Potential with CCCP on Dye Fluorescence and Swimming Behavior. Addition of CCCP, which is an uncoupler of oxidative phosphorylation and would be expected to lead to depolarization, to a bacterial suspension containing diS-C_3-(5) is followed by an increase in fluorescence, but the extent and permanence of the increase depends on the concentration of CCCP added. The addition of 1 μM CCCP is followed by an abrupt 2- to 3-fold increase in fluorescence, but lower CCCP concentrations cause a smaller increase. When CCCP is added to only 0.3 μM, there is an initial rapid 40% increase in the fluorescence followed by a slower decrease in the fluorescence over the course of 1–2 min, as is shown in Fig. 3.

The bacterial swimming behavior is also changed by the addition of CCCP, and the changes noted are dependent on the concentration of CCCP added. At low concentrations of CCCP (0.1 μM) we find, in agreement with Ordal and Goldman (11), that addition of CCCP leads to a transient period of constantly tumbling behavior followed by a return to normal swimming behavior. Addition of CCCP to 1.0 μM or higher causes a brief period of constant tumbling followed by complete paralysis of the bacteria.

Effects of Chemoeffector on Fluorescence Intensity and Swimming Behavior. When alanine, which is a strong attractant for *B. subtilis* (26, 27), is added to the bacteria, a change in the swimming behavior of the bacteria results, but there is little or no change in the fluorescence of diS-C_3-(5) as shown in Fig. 3. Furthermore, addition of alanine to the bacteria simultaneously with addition of CCCP is followed by a fluorescence change similar to that observed upon CCCP addition alone, although the behavioral change is completely different. When 0.3 μM CCCP, which causes transient tumbling of bacteria as noted above, is added to the bacteria simultaneously with 10 mM alanine, the attractant effect of the alanine is strong enough to overcome the repellent effect of the CCCP (cf. ref. 28), and the bacteria respond to the addition of the two chemicals with a period of smooth swimming followed by a return to random swimming behavior. This is similar to additive effects of attractants and repellents that have been reported before (29). Although simultaneous addition of alanine with the CCCP overcomes the behavioral effect of CCCP, the change in fluorescence of diS-C_3-(5) that follows addition of CCCP alone is not significantly affected by the simultaneous addition of alanine.

Correlation of TPMP and Fluorescence Intensity with Membrane Potential. To have more than one indication of membrane potential, the fluorescence of diS-C_3-(5) was correlated with TPMP distribution. Measurement of the transmembrane TPMP distribution by a method similar to that of Schuldiner and Kaback (22) gave, in different experiments, values of −60 to −75 mV (internal negative) for the membrane electrical potential in this strain of *B. subtilis*. This potential can be altered by addition of valinomycin and external potassium or the uncoupler CCCP.

With this knowledge of the value of the membrane potential under different conditions, we correlated fluorescence changes of the cyanine dye with the numerical value of the membrane potential, and the results are shown in Fig. 4. There is a good

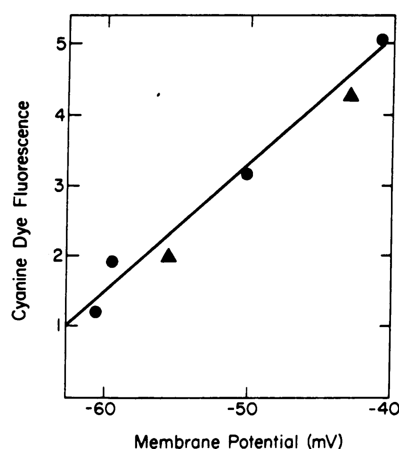

FIG. 4. Correlation of the membrane potential as determined by TPMP distribution with changes in the fluorescence of diS-C_3-(5). The membrane potential was measured by [^{3}H]TPMP distribution and then the fluorescence of diS-C_3-(5) in the presence of *B. subtilis* was measured under similar conditions, as described in *Materials and Methods*. Measurements were made either in the presence of valinomycin and various external concentrations of potassium (●) or in the presence of low concentrations of CCCP (▲). Points determined in the presence of CCCP have been corrected for quenching of the fluorescence by the CCCP as determined from control experiments with phospholipid vesicles. The absolute value of the membrane potential varied somewhat among different experiments, but the same correlation with fluorescence intensity was always observed. Fluorescence units are arbitrary as in Fig. 1.

correlation between the cyanine dye fluorescence and the membrane potential as determined by TPMP, which supports the use of the cyanine dye as an indicator of membrane potential in the chemotaxis experiments.

Effects of Chemoeffectors and CCCP on Membrane Potential as Measured by TPMP. When 1.0 μM CCCP was added to bacteria equilibrated with TPMP, there was a rapid 70% loss of TPMP from the bacteria. This result is to be expected if CCCP lowers the membrane potential, because the bacteria normally accumulate TPMP at a higher concentration than the outside medium due to their interior negative potential. The addition of alanine is followed by a small transient drop in the total TPMP retained, but the level of TPMP returns to the original level in a few seconds. When chemotaxis buffer without attractant was added to the TPMP-equilibrated bacteria, a small transient rise in the retained TPMP occurred.

DISCUSSION

Cyanine Dye Fluorescence as a Probe of Membrane Potential. Results reported here provide strong support for the correlation of the cyanine dye fluorescence with the overall membrane potential in *B. subtilis*. Previous studies with the cyanine dye in other systems also showed this correlation (ref. 25; reviewed in ref. 30) but the empirical nature of the probe requires its calibration in the system to be investigated. There is an excellent correlation of cyanine dye fluorescence with distribution of the TPMP, and previous studies by Kaback *et al.* (22, 23), Skulachev (31), and others have provided strong evidence that TPMP indeed measures membrane potential. Furthermore, fluorescence intensity changes also follow the alterations of membrane potential expected due to anaerobicity, valinomycin, and CCCP. These results,and others to be reported elsewhere (J. B. Miller and D. E. Koshland, unpublished data), support the conclusion that the fluorescence intensity of the cyanine dye diS-C_3-(5) is a rapid indicator of overall membrane potential in *B. subtilis*.

Membrane Potential Changes and Swimming Behavior. When the membrane potential of *B. subtilis* is altered, the swimming behavior of the bacteria is also altered. For instance, when the bacteria are highly depolarized they are paralyzed. Thus, in the absence of oxygen or in the presence of CCCP or valinomycin with high concentrations of external potassium, there is a large increase in cyanine dye fluorescence, indicating a large depolarization, and the bacteria are immotile. Because motility has been shown to be dependent on the energized membrane state (32), this result is not surprising.

When the membrane potential is above the level needed for motility, changes in this level have the same effect on swimming behavior as chemoeffector gradients. When the membrane potential as measured by cyanine dye fluorescence increases, the bacteria show a period of constant smooth swimming (tumbling suppressed) and then return to the random swimming pattern. Changes in attractant levels lead to this same swimming pattern. When the bacteria are subjected to agents such as CCCP or valinomycin in the presence of intermediate concentrations of external potassium, which decreases the potential, a period of transient tumbling followed by a return to the random pattern is observed.

The fact that the bacteria return to the normal random pattern in the absence of a gradient, despite the fact that the membrane potential is maintained at different constant levels, indicates that the absolute level of membrane potential does not control the swimming behavior. Thus, as for concentrations of attractants and repellents, it is a change in and not the absolute level of the overall membrane potential that leads to changes in the swimming behavior.

Chemoeffectors and Membrane Potential. When chemoeffectors such as alanine are added to the bacteria, a change in the swimming pattern results but no change in the membrane potential is observed. Alanine has been shown to be a strong attractant for some strains of *B. subtilis* (refs. 26 and 27; but cf. ref. 33) and its sudden additon to the bacteria causes a period of smooth swimming with tumbling suppressed before a return to the random swimming behavior. Despite this alteration in swimming behavior there is no change in the fluorescence of the cyanine dye, indicating no substantial change in membrane potential. The same conclusion is reached with the simultaneous addition of CCCP and alanine because the membrane potential change measured by the cyanine dye is the same as found upon addition of CCCP alone, although the behavioral change is completely different. This is a case where depolarization of the membrane is observed, yet smooth swimming results, which shows a lack of correlation of the absolute value of the membrane potential with behavioral response.

Measurement of the TPMP distribution in the presence of alanine gradients shows a small and reproducible transient decrease in the amount of TPMP retained within the bacteria, which indicates a decrease in membrane potential. The TPMP distribution returns to baseline after 1 min. An opposite result is seen to occur with the buffer control. These phenomena are not yet fully understood.

These results can now be examined in the light of previous studies on the role of membrane potential in chemotaxis. Our results support the conclusion of Ordal and Goldman that alteration of the membrane potential of *B. subtilis* does alter the swimming pattern of the bacteria, but that the absolute level of potential is not correlated with tumbling frequency (11, 12); de Jong *et al.* also concluded that transient changes in potential lead to changes in swimming behavior (13). The results do not agree, however, with the conclusion of Szmelcman and Adler from studies with TPMP distribution that there is a change in membrane potential in *E. coli* correlated with addition of at-

tractants or repellents (14). One possibility to explain this discrepancy is that there is a fundamental difference between the sensory systems of Gram-positive *B. subtilis* and Gram-negative *E. coli*, as was suggested to explain differences of repellent sensing (12). Several studies, however, support the conclusion that they have fundamentally similar chemotaxis mechanisms (e.g., compare ref. 34 with ref. 35). Other possibilities are the difference in surface structures of the two types of bacteria, the question of what TPMP measures when used as a kinetic probe, the availability of oxygen, and differences in conditions, but the reason is not clear at this time.

Relationship of the Membrane Potential to the Response Regulator. The results reported here lead to the conclusion that the overall membrane potential is not the tumble frequency regulator in *B. subtilis*, because it is possible to alter tumbling frequency without altering membrane potential. It is clear, however, that a change in the overall membrane potential can induce periods of swimming or tumbling in the same way as a change in the level of a chemoeffector. Thus, it would appear that a change, but not the absolute value, of the membrane potential can be detected by the sensory system of the bacterium and this alters the pattern of swimming behavior. The results do not exclude the possibility that some specialized potential or ion transport system—for example, a specifically designed sodium/potassium antiport mechanism—is involved in tumble regulation. In such a case, a change in the overall membrane potential would be affecting a localized potential or ion distribution, perhaps in the vicinity of the flagella. The two potentials would, however, need to be coupled in such a way that the localized potential system was sensitive only to temporal changes in the overall membrane potential.

Because it has recently been shown (23, 36, 37) that the membrane electrical potential is interconvertible with the membrane proton gradient, the role of the proton gradient in chemotaxis has been investigated. From results to be reported elsewhere (J. B. Miller and D. E. Koshland, unpublished data), it is shown that alteration of the proton gradient has effects on the bacterial swimming behavior similar to the effects seen on alteration of the electrical potential, but neither the proton gradient nor total protonmotive force could be identified as the tumble regulator.

It is perhaps not surprising that the bacterium does not use the gross membrane potential as the tumble frequency regulator. This membrane potential, according to chemiosmotic theory (38), is used as an energy source for oxidative phosphorylation, active transport, and also for motility itself. To require a change in the overall potential of the energy system in order to achieve chemotaxis would be a cumbersome method for controlling one facet of the bacterial economy. On the other hand, it is logical that the chemotaxis system should respond to temporal changes in membrane potential. Because the membrane potential is vital to the economy of the cell, an alteration in its level is a signal of significant changes in the environment that should lead to an appropriate behavioral response. So far, the bacterial chemotaxis system has been found to respond to such widely diverse phenomena as changes in nutrient level, light (15, 16), oxygen and other electron acceptors, temperature (39), metal ion concentrations (40), and now membrane potential. These phenomena must all provide signals to the tumble frequency regulator, which then leads to the appropriate behavioral response.

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