*Proc. Natl. Acad. Sci. USA*
Vol. 73, No. 12, pp. 4387–4391, December 1976
Biochemistry

# Change in membrane potential during bacterial chemotaxis*

(*Escherichia coli*/triphenylmethylphosphonium uptake/attractants/repellents)

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*Communicated by Henry Lardy, September 13, 1976*

ABSTRACT **To find out if there are changes in membrane potential during bacterial chemotaxis, we measured the membrane potential of *Escherichia coli* indirectly by use of the permeating, lipid-soluble cation triphenylmethylphosphonium. Addition of attractants or repellents to the bacteria brought about a hyperpolarizing peak (as well as additional, later changes in membrane potential). This peak was shown to be a part of the chemotactic mechanism based on the following evidence: (*i*) All attractants and repellents tested gave this peak while chemotactically inert chemicals did not. (*ii*) Mutants lacking galactose taxis failed to give the peak with galactose but did with another attractant and with repellents. (*iii*) Methionine, required for chemotaxis, is also required for production of this peak. (*iv*) A mutant in a control gene (*flaI*), unable to synthesize flagella and cytoplasmic membrane proteins related to motility and chemotaxis, failed to give the peak. (*v*) Paralyzed (*mot*) mutants gave little or none of the peak. Generally nonchemotactic (*che*) mutants, on the other hand, did give this peak.**

**Very likely there are ion fluxes that bring about this change in membrane potential. We discuss the possible role of the *mot* gene product as an ion gate controlled by a methylation-demethylation process in response to attractants and repellents acting through their chemoreceptors.**

Motile bacteria are attracted to certain chemicals and repelled by others (for reviews of chemotaxis, see refs. 1 and 2). The motility is caused by rotation of the flagella (3–5), and the chemicals are detected by chemoreceptors (6). When bacteria encounter an increasing concentration of attractant, they swim smoothly without tumbling (7–9), due to counterclockwise rotation of the flagella (10); when they encounter an increasing concentration of repellent, they tumble (11), due to clockwise rotation of the flagella (10). How do the chemoreceptors signal the flagella which way to rotate?

One proposal is that a change in membrane potential is involved in the signalling process (see references cited in refs. 1 and 2). This could be related to what is already established for eukaryotic excitable cells: in response to sensory stimuli or neurotransmitters potential changes occur in protozoa, sensory cells, and nerve and muscle cells (12–14). Faust and Doetsch (15) and Caraway and Krieg (16) have shown that a variety of drugs that alter excitable membranes influence the motility of a number of bacterial species. Ordal and Goldman have shown that uncouplers of oxidative phosphorylation, which depolarize cells, cause tumbling of the bacterium *Bacillus subtilis* (17). However, a measure of membrane potential in bacteria undergoing chemotaxis has not yet been reported.

Since bacteria are too small for the direct measurement of membrane potential by insertion of microelectrodes, we measured membrane potential indirectly by the use of a permeating, lipid-soluble cation, a method pioneered by V. P. Skulachev, E. A. Liberman, and their associates: such an ion that freely diffuses across the cell membrane distributes itself between the cells and the medium in accordance with the membrane potential (for reviews and references see refs. 18–20). We used the cation triphenylmethylphosphonium ($TPMP^+$) (18–20).

In this article we present evidence that attractants and repellents cause changes in $TPMP^+$ level of *Escherichia coli* bacteria and that these changes are brought about by the chemotactic mechanism. Based on the work of others (18–20), we interpret changes in $TPMP^+$ level to reflect changes in membrane potential. It is not yet known if these changes represent a signal from the chemoreceptors to the flagella or if they are the consequence of flagellar function.

## MATERIALS AND METHODS

**Growth and Preparation of Bacteria.** All strains used are derivatives of *E. coli* K-12 and have been described (6, 21–25). Bacteria previously adapted in Vogel-Bonner medium (25) containing 50 mM glycerol and, when needed, 1 mM D-galactose were grown fresh in the same medium (100 ml in a 1-liter flask with rotary shaking) from an initial $OD_{590}$ of 0.1 to a final $OD_{590}$ of 0.6–0.8. (An $OD_{590}$ of 1.0 corresponds to about $7 \times 10^8$ bacteria per ml and about 140 $\mu$g of cell protein per ml; results are reported in terms of mg of cell protein, but in fact only $OD_{590}$ was ever measured.) The bacteria were washed at room temperature by centrifugation twice in 0.1 M Tris-HCl (pH 8.1). The pellets were resuspended gently to preserve motility. Finally the bacteria, still motile, were suspended in 1 ml of the same buffer.

To make the cells permeable (ref. 26 and reference to L. Leive cited there) to $TPMP^+$, they were incubated for 6 min at 36° with occasional agitation; then $K^+$-EDTA was added to 10 mM and the incubation was continued for 3 more min at 36°. Now the motility has been lost. The EDTA was then removed quickly by a 10-fold dilution with ice-cold 0.1 M potassium phosphate (pH 6.6) followed by immediate centrifugation in the cold at 13,000 rpm for 7 min. The bacteria were washed twice with the same ice-cold phosphate buffer, and the final pellet was resuspended in 10 mM cold potassium phosphate (pH 6.6) to an $OD_{590}$ of 20. These cells, now motile again, were kept on ice until use.

**Uptake of $TPMP^+$.** A slight modification of the procedure of Schuldiner and Kaback (20) was used. From the ice-cold stock of cells, samples were withdrawn into a 50-ml test tube and diluted 2-fold with a room-temperature solution of 10 mM potassium phosphate (pH 6.6) containing 20 mM $MgSO_4$. When used, L-methionine (0.1 mM) was added at this point. The bacteria were kept for 5 min at room temperature, and D-lactate (20 mM), $Na^+$ tetraphenylboron (2 $\mu$M) (K&K Laboratories), and finally [$^3$H]$TPMP^+$ $Br^-$ (10 $\mu$M) (114 Ci/mol, a gift of Dr. H. R. Kaback) were added. At various times a 50-$\mu$l sample was transferred to a 5-ml tube, quickly diluted with 2 ml of 0.1 M LiCl at room temperature, rapidly filtered on a Uni-pore polycarbonate membrane (0.6 $\mu$m pore size; Bio-Rad Labs), and washed with 2 ml of 0.1 M LiCl; the entire procedure

Abbreviation: $TPMP^+$, triphenylmethylphosphonium.

* Presented at the Tenth International Congress of Biochemistry, Hamburg, Germany, July 25–31, 1976.

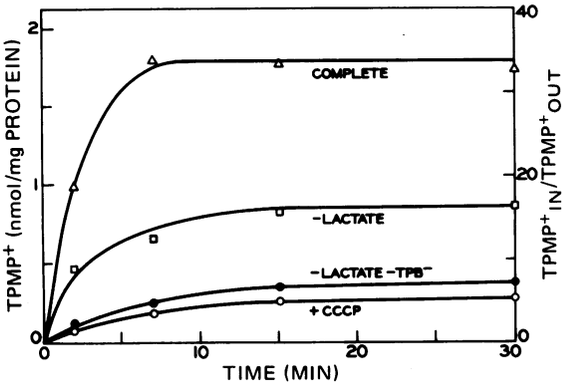


FIG. 1. Uptake of TPMP+ by chemotactically wild-type *E. coli* strain AW574. See *Materials and Methods* for details. (△) The complete system included 20 mM D-lactate, 2 μM $Na^+$ tetraphenylboron$^-$ and 10 μM [$^3$H]TPMP+ $Br^-$. (□) As in complete system except that D-lactate was missing. (●) As in complete system except D-lactate and tetraphenylboron$^-$ (TPB) were missing. (○) As in complete system, but $10^{-5}$ M carbonylcyanide *m*-chlorophenylhydrazone (CCCP) was present from the time of addition of TPMP+. The concentration of TPMP+ inside the cells was calculated using a value of 5.4 μl of intracellular water per mg of cell protein, according to Winkler and Wilson (see ref. 20).

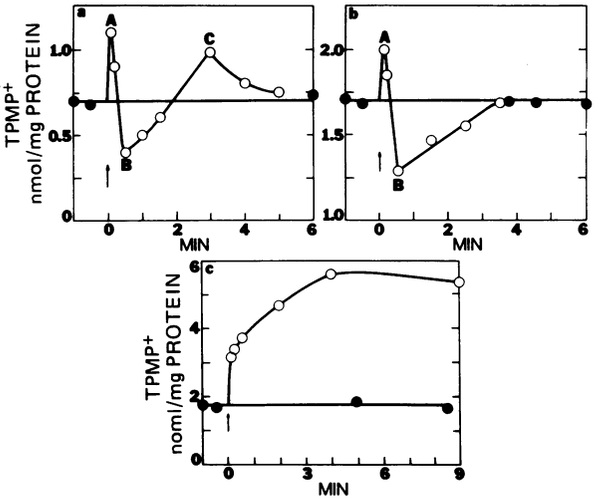


FIG. 2. Change in cellular level of TPMP+ upon addition of attractants or repellents. The uptake of TPMP+ was first allowed to reach a plateau value as in Fig. 1. Then at about 15 min ("zero time") attractant or repellent was added (○); (●) no attractant or repellent was added. (a) D-Galactose (10 mM) was added at zero time (arrow) to chemotactically wild-type, $Gal^+$, $Met^-$ AW546. L-Methionine (0.1 mM) was present. Peak A represents a hyperpolarization of 21 mV. In eight experiments done with this strain the average ratio of cellular TPMP+ after and before the addition of galactose was 1.48, with a standard deviation of 0.19. (b) DL-α-Methylaspartate (2 mM) was added at zero time (arrow) to chemotactically wild-type AW574. Peak A represents a hyperpolarization of 6 mV, whereas in AW546, with its lower plateau value (see Fig. 2a), α-methylaspartate gave a hyperpolarization of 19 mV. (c) A mixture of acetate (15 mM), indole (0.2 mM), and L-leucine (30 mM) was added at zero time (arrow) to strain AW574. The hyperpolarization represents 73 mV. These data and data shown in panel b came from the same experiment.

should take no more than 20 sec. (Some batches of filters have a slow filtration rate; in that case the concentration of bacteria must be slightly reduced.) Filters were finally dried and put into scintillation fluid (4 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis[2(5′-phenyloxazolyl)]benzene per liter of reagent grade toluene); radioactivity was then determined.

**Effect of Attractants and Repellents.** After the uptake of TPMP+ has reached its plateau (15–20 min), 50-μl samples were transferred into 5-ml tubes. After 5 min, attractant or repellent (or nothing) was added ("zero time"); at various times later, each sample was assayed for TPMP+ content of the cells.

A study was made of the effect of omitting the attractant or repellent from the LiCl solution used for dilution and washing of the sample; this would result in dilution of the chemical, and it is known that dilution of an attractant causes tumbling (8) and dilution of a repellent causes smooth swimming (11). For attractants it did not matter if the chemical was present or not in the LiCl solution, so it was not routinely added. For repellents, the results reported were obtained with repellent in the LiCl solution at the same concentration as present before dilution of the sample.

## RESULTS

**Measurement of Membrane Potential.** In this work changes of membrane potential were followed by measuring the variations of the TPMP+ concentration inside the bacterial cells (see *Introduction*).

In agreement with the work of others (19, 20), Fig. 1 shows that the uptake of TPMP+ is stimulated by an energy source (D-lactate) and by the addition of the lipophylic anion tetraphenylboron. The mechanism of this latter stimulation is not fully understood (see references cited in ref. 19). Uptake of TPMP+ is inhibited by agents that collapse the membrane potential such as carbonylcyanide *m*-chlorophenylhydrazone (Fig. 1) (19, 20). Addition of carbonylcyanide *m*-chlorophenylhydrazone (10 μM) or of the potassium ionophore valinomycin (20 μM) to cells that have reached a maximal accumulation of TPMP+ results in a discharge of 70% of the cation within 4 min (data not shown).

Using the Nernst equation, one can calculate from the ratio of TPMP+ inside to that outside the cells (Fig. 1, top curve) that the membrane potential is −85 mV. This value varies from strain to strain and is, for example, −65 mV in the strain shown in Fig. 2a. These values are in good agreement with those reported by others (19, 20).

**Change in Membrane Potential Upon Addition of Attractants and Repellents.** Attractants that can be transported and metabolized gave three waves of change in TPMP+ level. The example of the attractant D-galactose (27) in wild-type *E. coli* is shown in Fig. 2a. Evidence will be presented that the first wave ("phase A"), a hyperpolarization, results from chemotaxis; that the second wave ("phase B"), a depolarization, appears to result from transport; and that the third wave ("phase C"), a hyperpolarization, appears to result from metabolism of the chemical.

L-Serine, a transported metabolizable amino acid attractant (28), also gave rise to these three phases (data not shown). The attractant, DL-α-methylaspartate, an aspartate analog that is transported but is not oxidized (28), produced phases A and B but did not appear to produce phase C (Fg. 2b).

The repellents, acetate, indole, and L-leucine (4), when combined, were a potent mixture: Fig. 2c shows that its addition resulted in a large increase in uptake of TPMP+, which declined slowly after the maximum shown. Diluted 10-fold, the mixture gave about ⅕ the increase in TPMP+, and now the hyperpolarization ("phase I") was followed by a second phase of depolarization ("phase II"), as for attractants (data not shown). Apparently at high concentrations the mixture gave such a large phase I that phase II was obscured.

Acetate by itself, at a concentration present in the full-strength mixture, produced about ¼ as large an increase in TPMP+ level as did the mixture (data not shown).

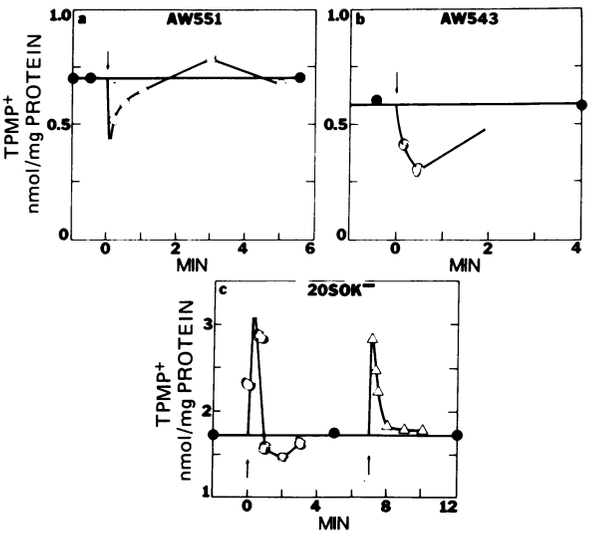


FIG. 3. Change in cellular level of $TPMP^+$ upon addition of galactose to mutants defective in galactose taxis, transport, or metabolism. The uptake of $TPMP^+$ was first allowed to reach a plateau value as in Fig. 1. Then at about 15 min ("zero time") galactose was added (○, △); (●) no galactose added. (a) (○) D-Galactose (10 mM) was added at zero time (arrow) to strain AW551, which is $Gal^+$, $Met^-$ and has a defective galactose binding protein that prevents galactose taxis but serves for galactose transport (see *text*). L-Methionine (0.1 mM) was present. (b) (○) D-Galactose (10 mM) was added at zero time (arrow) to strain AW543, a $Gal^+$, $Met^-$ galactose binding protein mutant that allows neither galactose taxis nor galactose transport via the β-methylgalactoside permease; the galactose permease is intact (see *text*). L-Methionine (0.1 mM) was present. (c) (○) 10 mM D-galactose was added at zero time (arrow); (△) 1 mM D-galactose added at 7 min (arrow). The strain was $20SOK^-$, normal for galactose taxis but defective in the several transport systems for galactose and also $Gal^-$ (see *text*).

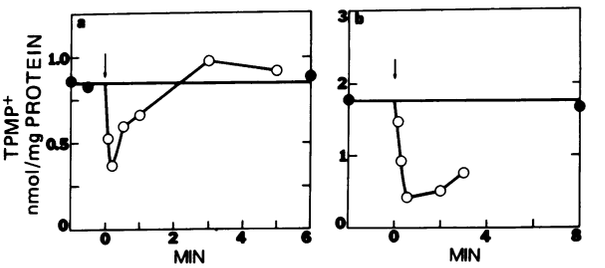


FIG. 4. Effect of methionine on change in cellular level of $TPMP^+$ caused by attractants. The uptake of $TPMP^+$ was first allowed to reach a plateau as in Fig. 1. Then at about 15 min ("zero time") attractant was added (○); (●) no attractant added. (a) D-Galactose (10 mM) was added at zero time (arrow) to the $Met^-$ strain AW546. Methionine was omitted. These data were obtained in the same experiment as, and are to be compared with, those of Fig. 2a, where methionine was present. (b) DL-α-Methylaspartate (2 mM) was added at zero time (arrow) to the $Met^-$ strain 2-46, and methionine was omitted. In the same experiment, data were collected for DL-α-methylaspartate with methionine present; this result looks exactly like that pictured in Fig. 2b and is therefore not shown.

The chemotactically inert chemicals glycerol, L-arginine, or L-lysine (27–29) at 10 mM failed to give the first phase, but did produce the second one. L-Galactose at 10 mM, which is not sensed, transported, or metabolized by *E. coli* (27), caused little or no change in $TPMP^+$ content of the cells.

**Change in Membrane Potential Upon Addition of Galactose to Mutants Defective in Chemotaxis, Transport, or Metabolism of Galactose.** A powerful tool for dissecting the multiphasic response to an attractant is the use of mutants blocked at various stages. The galactose binding protein is the recognition component both for galactose transport via the β-methylgalactoside permease (30), and also for galactose taxis (22). The second major transport system for galactose, the galactose permease (31), does not use the galactose binding protein (30) and does not serve for galactose taxis (6). Mutants in these various functions were tested.

AW551, mutated in *mglB*, the structural gene for the galactose binding protein, produces a protein that retains the ability to bind galactose and to transport it via the β-methylgalactoside permease, but it has lost galactose taxis (22, 25). Galactose metabolism is normal (25). As shown in Fig. 3a, phase A is missing, while phases B and C are present. The responses to another attractant, α-methylaspartate, and to the repellent mixture were normal.

AW543 is a mutant in the *mglB* gene that has lost both its capacity to transport galactose via the β-methylgalactoside permease and its chemotactic response towards this sugar. This mutant appears to have a normal galactose permease since it grows on galactose (22, 25). Fig. 3b shows that phase A is absent while phase B occurs. (The experiment was not carried out long enough to detect phase C.) The responses to α-methylaspartate and to the repellent mixture were normal.

$20SOK^-$ has normal galactose binding protein (30) and is normal for galactose taxis (6). However, the strain is a mutant in the *mglA* and *mglC* genes of the β-methylgalactoside permease (25) and also in the gene for galactose permease (31); consequently, it is highly defective in the transport of galactose (6, 30, 31). In addition, the strain has a mutation in the galactokinase gene and therefore fails to metabolize galactose (6). Fig. 3c shows that phase A is present but phase B and very likely phase C are absent at 1 mM galactose. Phase B is weak at 10 mM galactose (Fig. 3c); this may represent residual transport of galactose at this high concentration or contamination by glucose, known to be present in the galactose.

**Requirement of Methionine for Change in Membrane Potential.** L-Methionine is known to be required for chemotaxis and for tumbling in *E. coli* and *Salmonella* (see ref. 1 for review). As shown in Fig. 4, methionine is required for phase A of the galactose response (Fig. 4a) and for phase A of the response to α-methylaspartate (Fig. 4b). In contrast, phase B does not require methionine (Fig. 4a and b). Neither does phase C require methionine (Fig. 4a).

When methionine was omitted, phase I of the response to the repellent acetate was reduced by about ⅔ (data not shown).

**Change in Membrane Potential in Generally Nonchemotactic Mutants.** Mutations in several *E. coli* genes, *cheA*, *B*, *C*, and *D*, can lead to total loss of chemotaxis; motility is retained, but usually it is either smooth (no tumbling) or tumbly (nearly constant tumbling) (23, 32). Surprisingly, the effect of an attractant, α-methylaspartate, and of the repellent mixture on $TPMP^+$ level was found to be close to wild-type in *cheA*593, *cheB*590, and *cheC*497 (23).

**Change in Membrane Potential in Paralyzed Mutants.** A mutation in the *mot* (for motility) gene leads to paralysis of the bacteria: the flagella are synthesized and appear morphologically and chemically normal, but they do not function (24, 33, and earlier references cited there). Two *mot* mutants, M524 and M526 (24), were tested for response of $TPMP^+$ levels to addition of galactose, α-methylaspartate, and repellent mixture. Phase A of the response to the two attractants was missing, while phase B was present. (The response was not followed long enough to detect phase C for galactose.) The response to the repellent mixture was markedly reduced (⅕–⅓ of the wild-type level).

**Change in Membrane Potential in a Nonflagellated Mutant.** *FlaI* is a control gene whose mutation causes loss of synthesis of the entire flagellum (34) as well as cytoplasmic membrane components required for motility and chemotaxis: the methyl-accepting chemotaxis protein (21) and the *mot* gene product (33). A *flaI* mutant, 1016 (34), was tested and found to be missing phase A of the responses to galactose and α-methylaspartate. Phase B was present for the two attractants. Phase C was present for the metabolizable attractant, galactose. Response of the membrane potential to the repellent mixture was 95% blocked.

## DISCUSSION

Using $TPMP^+$ as an indirect probe, we have now found that the membrane potential changes when attractants or repellents are added to bacteria. (It is conceivable that earlier changes in membrane potential have been overlooked here because they would be too fast to be measured by the present technique.)

**Change in Membrane Potential Upon Addition of Attractants.** In the case of attractants, there is a triphasic response when transport and metabolism of the chemical can occur (see, for example, Fig. 2a).

*Phase A.* The evidence that this wave of hyperpolarization is a part of the mechanism for chemotaxis is here summarized. (*i*) All attractants tested give phase A, while the chemotactically inert chemicals tested do not. (*ii*) Mutants lacking galactose taxis fail to give phase A when galactose is added (Fig. 3a and b), though phases B (Fig. 3a and b) and C (Fig. 3a) are present. (*iii*) Methionine is required for chemotaxis, and it is required for phase A but it is not needed for the later phases (Fig. 4a and b). (*iv*) A mutant in a control gene (*flaI*), unable to synthesize flagella as well as cytoplasmic membrane proteins related to motility and chemotaxis, fails to give phase A, but does give the remaining phases. Thus, production of phase A is under this same control, which indicates that phase A is part of the mechanism for motility or chemotaxis. (*v*) Paralyzed (*mot*) mutants fail to give phase A.

*Phase B.* This wave of depolarization appears to result from transport, since all transportable chemicals tested give phase B while nontransportable ones do not (Fig. 3c, 1 mM galactose). Phase B does not require methionine (Fig. 4) and is not affected by mutations that eliminate motility and chemotaxis. Depolarization indicates a decreased level of energy; since transport uses energy, it is understandable that depolarization might accompany transport.

*Phase C.* This hyperpolarizing wave is obtained with those chemicals that can be metabolized, such as galactose in wild-type bacteria (Fig. 2a) or serine, while it does not appear to be obtained with the nonmetabolizable ones, such as galactose in a galactokinaseless mutant (Fig. 3c) or α-methylasparate (Fig. 2b). Phase C is not affected by omission of methionine (Fig. 4a) or by mutations that eliminate motility and chemotaxis. We suggest that phase C hyperpolarization reflects the additional energy made available by use of metabolizable chemicals.

**Change in Membrane Potential Upon Addition of Repellents.** Repellents bring about a wave of hyperpolarization, phase I (Fig. 2c), followed by depolarization, phase II. Arguments similar to those presented for phase A of the attractant response suggest that phase I results from chemotaxis. Phase II may well be due to transport of the repellents, comparable to phase B for attractants.

**Ionic Basis for Phase A of Attractant Response and for Phase I of Repellent Response.** These changes in membrane potential very likely result from changes in ion fluxes. Attractants and repellents both produce a hyperpolarizing wave, but the ion responsible for the hyperpolarization would seem to be different in the two cases, since addition of attractants suppresses tumbling (7, 8) while addition of repellents increases tumbling (11). Since hyperpolarization can produce *either* smooth swimming or tumbling, it appears that the membrane potential *per se* does not determine the frequency of tumbling, but rather specific ions must.

The duration of the first phase is considerably shorter than the duration of change in tumbling frequency in response to attractants. Possibly the latter duration represents the time it takes for the cell to re-establish its initial ion concentration. For the repellents the time courses of the potential and behavioral responses were parallel during the time observed.

Whatever the ions in the first phase, *E. coli* must have ion gates through which the ions flow, and these gates must open and close in response to sensory stimuli.

**Role of Methylation.** Methionine is required for chemotaxis and for tumbling, and it functions, apparently via *S*-adenosylmethionine, to methylate a methyl-accepting chemotaxis protein (21) (for a review see ref. 1).

A striking result reported here is the requirement of methionine for the change in membrane potential caused by chemotaxis. We suggest that a methylation-demethylation process controls the quantity or nature of ions that flow through the ion gate(s) involved in chemotaxis.

Certain generally nonchemotactic (*che*) mutants fail to methylate the methyl-accepting chemotaxis protein (21), but one of these (*cheB590*) tested here did not block the change in membrane potential that is a part of chemotaxis. Therefore we suggest that there must be two sites for methylation—the methyl-accepting chemotaxis protein and the ion gates invoked here. The *che* gene products must act after (or in parallel with) the change in membrane potential, since these products are not needed for the change in membrane potential to occur.

**Role of the *mot* Gene Product.** Flagella work by rotating, either clockwise leading to tumbling or counterclockwise leading to smooth swimming (3–5, 10). The energy for this rotation is not ATP, but rather the intermediate of oxidative phosphorylation, presumably the proton motive force (35). Some workers (4, 18) have suggested that an ion flux drives a rotary motor that turns the flagella. Recently the *mot* gene product has been located in the cytoplasmic membrane (33), but the function of this protein is unknown. Possibly an altered *mot* protein could paralyze the bacteria by preventing this ion flux.

A very interesting result of the present study is the finding that *mot* mutants are blocked in the change in membrane potential involved in chemotaxis. Our result could be explained if the *mot* protein is an ion gate. This gate interacts with the chemoreceptors to produce changes in ion fluxes (hence in membrane potential) and it is also required for rotation of the flagella. The changes in ion fluxes, brought about by methylation of the gate, are analyzed by the *che* gene products, which tell the flagellar motor which way to rotate†. It is possible that the changes in membrane potential that we observe are not the signal from the chemoreceptors to the flagella but rather are the consequence of a change in ion fluxes through the *mot* gate at the base of the flagella.

The following problems remain to be solved: identifiction of the ions involved, isolation and characterization of the ion gates, understanding how sensory stimuli (acting through

† More detailed models depend on whether the chemoreceptors and the *mot* product are located at the base of the flagellum or are distributed all around the cell membrane.

sensory receptors) turn these gates on and off, learning how ion fluxes might rotate flagella, and determining how *che* gene products control the direction of rotation.

It will be interesting to see how much these mechanisms resemble any counterparts in the eukaryotic cell's response to sensory stimuli or to neurotransmitters‡.

This work was supported by a Public Health Service Grant (AIO8746) from the National Institutes of Health (National Institute of Allergy and Infectious Diseases), the National Science Foundation (PCM75-21007), and the Graduate School of the University of Wisconsin-Madison. S.S. is indebted to the North Atlantic Treaty Organization for a contribution towards his postdoctoral stipend. We are grateful to Dr. Melvin Simon for strains provided. We are particularly very thankful to Dr. Ronald Kaback for [$^3$H]TPMP$^+$ and for advice on its use. We thank Margaret M. Dahl for technical help.

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‡ Receptor potentials, synaptic potentials, and action potentials ordinarily are waves of depolarization (14), but hyperpolarizing waves are known for the receptor potential of the vertebrate rod (14) and for the posterior receptor potential of *Paramecium* (12).

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