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Cytoplasmic pH Mediates pH Taxis and Weak-Acid Repellent Taxis of Bacteria

MAY KIHARA AND ROBERT M. MACNAB*

Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06511

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Bacteria migrate away from an acid pH and from a number of chemicals, including organic acids such as acetate; the basis for detection of these environmental cues has not been demonstrated. Membrane-permeant weak acids caused prolonged tumbling when added to *Salmonella* sp. or *Escherichia coli* cells at pH 5.5. Tethered *Salmonella* cells went from a prestimulus behavior of 14% clockwise rotation to 80% clockwise rotation when 40 mM acetate was added and remained this way for more than 30 min. A low external pH in the absence of weak acid did not markedly affect steady-state tumbling frequency. Among the weak acids tested, the rank for acidity (salicylate > benzoate > acetate > 5,5-dimethyl-2,4-oxazolidinedione) was the same as the rank for the ability to collapse the transmembrane pH gradient and to cause tumbling. At pH 7.0, the tumbling responses caused by the weak acids were much briefer. Indole, a non-weak-acid repellent, did not cause prolonged tumbling at low pH. Two chemotaxis mutants (a *Salmonella* mutant defective in the chemotaxis methylesterase and an *E. coli* mutant defective in the methyl-accepting protein MCP I) showed inverse responses of enhanced counterclockwise rotation in the first 1 min after acetate addition. The latter mutant had been found previously to be defective in the sensing of gradients of extracellular pH and (at neutral pH) of acetate. We conclude (i) that taxes away from acid pH and membrane-permeant weak acids are both mediated by a pH-sensitive component located either in the cytoplasm or on the cytoplasmic side of the membrane, rather than by an external receptor (as in the case of the attractants), and (ii) that both of these taxes involve components of the chemotaxis methylation system, at least in the early phase of the response.

Bacterial chemotaxis is undoubtedly the best understood biological behavioral system at the present time (14, 17, 42).

Beginning with the demonstration by Adler (1) that neither transport nor metabolism of sugars and amino acids is necessary for tactic responses to occur, and continuing with detailed genetic, biochemical, and physiological studies in a number of laboratories, a dogma has emerged that the first events in the sensory transduction process are the binding of a chemoeffector to an external receptor site and the transmission of information regarding this event to the cytoplasmic side of the membrane. Results obtained in the course of studies of motor function in bacteria have led us to conclude that this dogma is not valid in the case of taxis away from weak-acid repellents or an acidic pH.

The bacterial flagellar motor is the final effector in the chain of events initiated by chemical and other sensory information, modulation of motor function being the basis of the migration to more favorable environments. The motor is modulated in the simplest possible way. It is placed in either of two discrete states, namely, counterclockwise (CCW) or clockwise (CW) rotation (5, 16, 40). Spontaneous alternation between these states occurs with a characteristic constant probability, and this results in the cell alternating between translational motion (swimming) and chaotic angular motion (tumbling). Favorable tactic stimuli, in the form of temporal gradient information, result in enhanced probabilities of remaining in the CCW state and hence a prolongation of swimming intervals in the favorable direction (6, 18).

Much progress has been made toward an understanding of this behavioral system, which is proving to be a useful model system of sensory transduction and motor response, but many fundamental points are still obscure. For example, it is not known what determines the unstimulated switching probabilities of the motor, nor is how these probabilities are perturbed in stimulated cells known. A number of genes are known to be involved in chemotaxis, and characterization of their gene products continues to be a

profitable approach to understanding the phenomenon. Another approach is to explore nonmacromolecular properties of the cell that might affect motor function. There are strong a priori reasons for supposing that such properties, and especially those properties intimately involved with the energetic state of the cell, are likely to be important, since cells give tactic responses to such energy-related parameters as oxygen, light, pH, and proton ionophores.

Proton electrochemical potential, or proton motive force (PMF), plays a complex role in motor function. It is the energy source for rotation (9, 12, 15, 19–22, 38, 39) and is probably also the common parameter by which a variety of energy perturbations are sensed by the switching mechanism of the motor, a sudden decrease in PMF transiently enhancing CW rotation and a sudden increase in PMF transiently suppressing it (16a, 22, 26, 45). In a previous study from this laboratory, it was shown that the steady-state PMF level also has a regulatory effect on switching probabilities. A long-term decrease in PMF, provided it is sufficient to take the motor below the state of saturation (where speed is PMF independent [12, 39]), causes a long-term suppression of CW rotation (11).

The observation (11) that *Bacillus subtilis* at pH 5.5 became slow and smooth swimming when the transmembrane pH difference (ΔpH) was partially collapsed by nigericin or weak acids, such as acetate, was consistent with PMF regulation of motor switching and with the equivalence of electrical potential ($\Delta\psi$) and ΔpH as energy sources for the motor (12, 20, 39). However, when *Salmonella* or *Escherichia coli* at pH 5.5 was subjected to ΔpH collapse by acetate, the behavior was dramatically different; not only did the cells remain motile, but the motility consisted of vigorous tumbling.

How could these very different behavioral responses between gram-positive and gram-negative bacteria be reconciled? The reduction or, in the limit, the abolition of motility in *B. subtilis* by acetate was understandable in view of the dominance of the ΔpH component of PMF in that species at pH 5.5 (12, 39). However, *E. coli* maintains a substantial $\Delta\psi$ component (80 to 100 mV [8, 12, 27]) even at low pH. The fact that motility in *E. coli* was still fairly vigorous despite ΔpH collapse by acetate indicated that the remaining component of PMF, $\Delta\psi$, was sufficiently large that the motors were still close to saturation; since PMF affects switching probabilities only below the saturation region, reduction of PMF by ΔpH collapse would not be expected to cause suppression of tumbling. However, this still left the marked enhancement of tumbling unexplained.

Regulation of motor switching by cytoplasmic pH. When the method of ΔpH collapse is to lower the cytoplasmic pH to the external value, the cell has been perturbed in at least two ways: its PMF has been reduced, and its cytoplasmic pH has been altered from the value to which it is normally regulated at the prevailing external pH. The latter aspect led us to hypothesize that it is low cytoplasmic pH that causes enhancement of tumbling. This hypothesis suggested two further hypotheses, one concerning tactic responses to pH gradients and one concerning tactic responses to gradients of weak acids.

pH taxis. It has been known for many years that bacteria, including *E. coli*, migrate in pH gradients to an optimum position in the vicinity of neutral pH (32, 37, 48). When external pH is increased from acid values toward neutrality, the PMF in gram-negative species like *E. coli* gets smaller, because the progressive loss of an inwardly directed ΔpH component is not fully compensated for by the increase in the $\Delta\psi$ component (8, 12, 27). Therefore, it is evident that migration from an acid pH to a neutral pH cannot simply be an example of "energy taxis," analogous to phototaxis or aerotaxis. Rather, the cells must in some way be capable of sensing pH per se. If changes in external pH are reflected, permanently or transiently, by changes in cytoplasmic pH, a component of motor regulation sensitive to cytoplasmic pH could provide the basis for pH taxis.

Taxis away from weak acids. A number of repellents for *Salmonella* sp. and *E. coli* are weak acids (47, 48). Among these is acetate, which was the agent used to collapse ΔpH when we made the preliminary observation of enhanced tumbling described above. This led us to consider whether the long-term tumbling associated with a substantial reduction of cytoplasmic pH by acetate at a low external pH and the brief tumbling when acetate is added in the vicinity of neutral pH might be related, i.e., whether weak-acid taxis, as well as pH taxis, might be mediated by changes in cytoplasmic pH.

In this paper, we report in detail the effects of lowered cytoplasmic pH on the motility of *Salmonella* and *E. coli*, in support of the hypotheses outlined above; we found that at concentrations that completely collapse ΔpH, acetate and several other membrane-permeant weak acids cause prolonged tumbling at a low pH, but only brief tumbling at a neutral pH. We conclude that weak-acid repellents are recognized not by receptors, as has been suggested previously (47, 48), but by the effect which they have on cytoplasmic pH.

MATERIALS AND METHODS

Bacterial strains. The analysis of the effects of weak acids on motility and ΔpH was carried out with *Salmonella* sp. strain ST1, a wild-type strain selected for good motility (3), and with *Salmonella* sp. strain SL3625, a leaky *flaAIII* mutant that is wild type otherwise (50) and can be tethered without special treatment. Confirmation of the basic results was made in *E. coli* by using strains RP437 (29) and AN180 (7), which are wild type with respect to motility and taxis. A variety of *Salmonella* and *E. coli* strains carrying mutations in chemotaxis genes were tested for response to acetate at low pH (see Table 3). The ATPase-defective *E. coli* strain AN120 (7) was used for experiments regarding ATP levels.

Media and culture conditions. Cells were grown with shaking at 25°C in nutrient broth (Difco Laboratories) containing 0.5% NaCl and any necessary nutritional supplements to a final density of 1.5×10^9 cells per ml (optical density at 650 nm, 0.8).

They were then centrifuged at $3,000 \times g$ for 5 min at 5°C, washed, and suspended in tethering medium (10 mM potassium phosphate, 0.1 mM EDTA, 67 mM NaCl) at the desired pH.

Chemicals. Carbonyl cyanide-*m*-chlorophenyl hydrazone, chloramphenicol, luciferin-luciferase extract FLE-50, nalidixic acid, phosphoenolpyruvate, and rifampin were obtained from Sigma Chemical Co. 5,5-Dimethyl-2,4-oxazolidinedione (DMO) was from Pfaltz and Bauer, sodium acetate and benzoic acid were from Baker, sodium salicylate was from Mallinckrodt, indole was from Matheson, Coleman and Bell, and [7-^{14}C]benzoic acid (26 mCi/mmol) was from New England Nuclear Corp.

Salmonella antiflagellar antibody was obtained from Lee Laboratories. *E. coli* antiflagellar antibody was prepared in this laboratory by using a conventional protocol.

Tethering procedures. The leaky Fla$^-$ mutant was tethered directly; other strains were first sheared in a Waring blender and then tethered in the presence of chloramphenicol, as described previously (13).

Microscopic observations. Observations and video recording of free-swimming and tethered cells were made in dark field as described previously (11). For measurements of short responses (<1 min) a rapid-mixing temporal gradient apparatus (18) was used. Long-term behavior of free cells was studied in a bridged cover slip arrangement.

Analysis of tethered cell data. For analyses of rotational behavior we employed a novel snapshot method that permits a relatively rapid estimate to be made of the relative extents of CW rotation and CCW rotation, without the need for timing individual intervals. For each datum point, brief video records (a few seconds each) were made of a number of fields of tethered cells. Then, at an arbitrary time point (indicated by a video time-date generator reading to 0.01 s; model VTG302ad; Impossible Electronic Techniques, Inc.) in the middle of each record, each rotating cell was scored as being either in CW rotation or in CCW rotation. The scores of the records were then combined (yielding data for at least 50 cells) to give the mean fraction of the population that was in CW rotation at any arbitrary time during the interval spanned by the records. The data obtained in this way were in good agreement with the data obtained on the mean fraction of time in CW rotation by the more tedious process of timing individual intervals of CW rotation and CCW rotation (see below).

(The two approaches yield ensemble and time averages of the system, respectively. The equivalence of the two averages for events with time-independent probability is the subject of the ergodic hypothesis of statistical thermodynamics.)

Measurement of ΔpH. Measurement of ΔpH was carried out by the technique of flow dialysis (33), using the protocol described previously (11), except that in place of [^{14}C]DMO (pK 6.3) we used [^{14}C]benzoate (pK 4.2) because it provides greater sensitivity at low pH values. The chemical concentration of the [^{14}C]benzoate in these experiments was 12 μM.

Data were plotted and analyzed with a Hewlett-Packard 41C programmable calculator/printer. Differential uptake was calculated from the vertical displacement between linear regressions of the fractions before and after addition of weak acid or carbonyl cyanide-*m*-chlorophenyl hydrazone.

Measurement of ATP levels. ATP was measured by the luciferin-luciferase assay, as described previously (11).

RESULTS

Effects of acetate on motility. Wild-type *Salmonella* sp. strain ST1 suspended in tethering medium at pH 5.5 displayed random motility, i.e., an alternation between swimming and tumbling. However, when 40 mM acetate was added, the cells tumbled vigorously and continuously; 20 min later, they showed some translational motion, but still tumbled far more frequently than untreated cells. This behavior was particularly striking because, although a variety of circumstances (notably repellent addition) are known to cause transient tumbling, only one other circumstance (addition of methyl ester analogs of the methyl-accepting chemotaxis proteins) resulting in long-term tumbling has been described (25) for wild-type *Salmonella* or *E. coli*. Similar results were obtained when *E. coli* strain RP437 or AN180 was subjected to 40 mM acetate, although motility was impaired somewhat.

The tumbling was not a consequence of damage to the motor, since addition of the attractant serine (1 mM) to *Salmonella* sp. strain ST1 caused 1.5 min of smooth swimming, followed by relaxation to the tumbling state.

When *Salmonella* cells (strain ST1 or, more conveniently, the leaky Fla$^-$ mutant SL3625) were tethered, the basis of the long-term tumbling was found to be a long-term bias toward CW rotation, as expected from other studies (16). As Fig. 1 shows, motor function shifted from a mean prestimulus value of 14% CW ro-

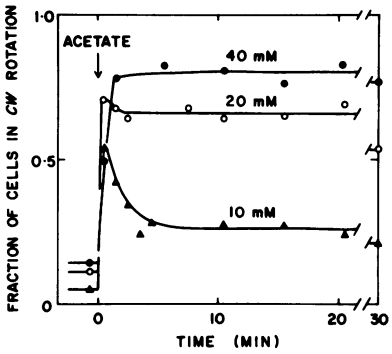

FIG. 1. *Responses at pH 5.5 of tethered Salmonella sp. strain SL3625 cells to addition of acetate at different concentrations. Response was measured as the fraction of cells in a population that were in CW rotation at a given instant; therefore, it is an ensemble average, which is numerically equivalent to a time average for a population of cells with a constant switching probability on the time scale of the measurement (see text). Each datum point is based on the behavior of ca. 150 cells.*

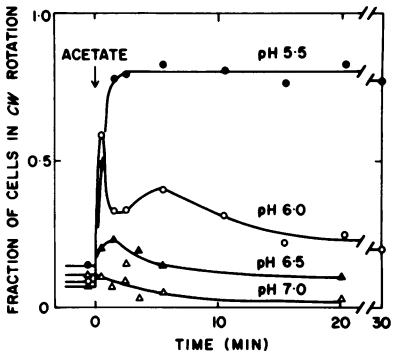

FIG. 2. *Responses at different pH values of tethered Salmonella sp. strain SL3625 cells to addition of 40 mM acetate.*

tation to 80% CW rotation when 40 mM acetate was added to cells tethered at pH 5.5. The shift was complete within 1 min, and 30 min later the strong bias toward CW rotation remained. After 1 h, slight relaxation (to 61% CW rotation) was observed. At lower acetate concentrations, relaxation was more evident; 10 mM acetate caused a rapid increase to 54% CW rotation, followed by relaxation to 25% CW rotation (still much higher than the prestimulus level) by 5 min.

Data obtained at pH 5.0 were similar to those obtained at pH 5.5. When higher external pH values were used (Fig. 2), the maximum CW bias attained upon addition of 40 mM acetate was less extreme, and the cells relaxed considerably. At pH 6.0, a rapid increase to 58% CW rotation was followed by a reproducible undershoot and then a gradual relaxation from 40% CW rotation to a final value of 22% CW rotation by 15 min. At pH 6.5, the maximum extent of CW rotation was 23%, and relaxation was complete in less than 10 min.

In free-swimming cells, the tumbling response to addition of 40 mM acetate was much briefer (30 s) at pH 7.0 than at pH 5.5, but even at pH 8.0 a distinct tumbling response (ca. 5 s) could still be detected in the temporal gradient apparatus.

We wished to know whether both CW → CCW and CCW → CW switching probabilities were perturbed by acetate addition at low pH. The data for Fig. 1 and 2 were obtained by a snapshot analysis of video records of tethered cells (see above), a technique that yields information on the relative probabilities of the two states but not on the mean residence times in them. Therefore, video records of *Salmonella* sp. strain SL3625 were analyzed in detail to obtain this information. In a 1-min interval before acetate addition at pH 5.5, the mean CCW and CW intervals of 10 reversing cells were 2.48 ± 0.38 and 0.38 ± 0.12 s, respectively. In similar measurements made 5 min after addition of 40 mM acetate, the mean CCW and CW intervals were 0.68 ± 0.18 and 3.90 ± 1.04 s, respectively. Thus, both switching probabilities were perturbed in a reciprocal manner, as has been noted in other contexts previously (11). These data correspond to 13 and 85% CW rotation before and after acetate addition, respectively; when we included nonreversing cells in the calculation (14% rotated exclusively CCW before acetate addition; 6% rotated exclusively CCW and 17% rotated exclusively CW after acetate addition; data based on inspection of 73 and 48 cells, respectively), we obtained values of 11 and 83% CW rotation, respectively. These time averages are in good agreement with the ensemble averages of 14 and 80% before and after addition of acetate (Fig. 1), respectively, indicating that data obtained by the snapshot method give a valid estimate of the time-average behavior of cells.

Effects of other weak acids on motility at pH 5.5 and 7.0. The enhancement of CW rotation might be specific to acetate (which is known to be a repellent for both *E. coli* [48] and *Salmonella* [47]), or it might be a consequence of ΔpH collapse. If the latter explanation were correct, any membrane-permeant weak acid ought to have a similar effect. The naturally occurring compounds acetate, benzoate, and salicylate and the synthetic compound DMO have been used in radiolabeled forms (at low concentrations) for the measurement of ΔpH across cell membranes (33), precisely because they are membrane-permeant weak acids. Therefore, we examined these

compounds for possible effects on motility. Like acetate, benzoate caused prolonged tumbling in *Salmonella* sp. strain ST1 at pH 5.5 (Table 1). Salicylate and DMO caused tumbling, but also caused a progressive deterioration in motility, which prevented us from determining the cessation of the tumbling response. Therefore, the values in Table 1 for these two acids are lower boundaries.

We also subjected strain ST1 to these four weak acids at pH 7.0 in a temporal gradient apparatus. Responses (Table 1) were much briefer and required much higher concentrations than at pH 5.5.

In contrast to the prolonged tumbling obtained with weak-acid repellents at pH 5.5, 10^{-4} M indole, which at neutral pH is a stronger repellent than acetate (47, 48), caused only a 7-s tumbling response at pH 5.5.

Effects of weak acids on ΔpH. It was next necessary to establish whether the weak acids at the concentrations causing prolonged tumbling did in fact appreciably alter the cytoplasmic pH. Using the technique of flow dialysis and [^{14}C]benzoate at a low concentration (12 μM) as the probe, we made measurements of ΔpH on *Salmonella* sp. strain ST1 at pH 5.5 and different concentrations of each weak acid. Table 2 shows the minimum concentrations of the weak acids needed to cause complete collapse (for at least 10 min) of the cytoplasmic pH to the external value of pH 5.5, along with the respective pK's and the concentrations giving the maximum behavioral effect. This table shows that rankings of the four compounds with respect to acidity, effectiveness in collapsing ΔpH, and effectiveness in causing tumbling were the same, namely, salicylate > benzoate > acetate > DMO.

(The result that stronger acids had a greater ability to collapse ΔpH is not obvious a priori. Several factors are likely to be involved, including the permeabilities of the protonated and unprotonated forms and the relative amounts present of each. A weaker acid would have a higher fraction in the more permeable protonated form, but would release fewer protons upon entry. At any rate, from the point of view of the present paper the most important correlation is between ability to cause ΔpH collapse and ability to cause tumbling.)

At lower concentrations of the weak acids, partial collapse of ΔpH was followed by a progressive recovery. Figure 3 illustrates this phenomenon after addition of 1.5 mM benzoate (which is not metabolizable); similar results were obtained with 3 mM acetate, even in the presence of 140 mM glycerol. These results suggest that the cells have some capacity to increase the rate of proton pumping when a proton leak is introduced.

For all four compounds, the concentrations for maximal behavioral effect were sufficient to cause complete ΔpH collapse; in fact, they were about a factor or two higher than the threshold concentrations in this regard. Since a small residual ΔpH (ca. 0.2 unit or less) is very difficult to detect by flow dialysis, the threshold concentration for total collapse may be underestimated, or the discrepancy may be an artifact of the different conditions (e.g., cell density) under which the two types of measurement have to be made. (Measurements of ΔpH in Table 2 were made in the presence of 1% [vol/vol] glycerol to maximize ΔpH before weak acid addition. However, whether glycerol was present or not, 15 mM acetate appeared to cause complete collapse of ΔpH.)

Indole (10^{-4} M), as expected from its nonacidic

TABLE 1. *Tumbling response of Salmonella sp. strain ST1 to membrane-permeant weak acids*

Concn (mM)	Response time (min)[a]							
	Salicylate		Benzoate		Acetate		DMO	
	pH 5.5	pH 7.0	pH 5.5	pH 7.0	pH 5.5	pH 7.0	pH 5.5	pH 7.0
1.25	0.2 (M)[b]	ND[c]	ND	ND	ND	ND	ND	ND
2.5	0.5 (M)	ND	0.2 (V)	ND	0.2 (V)	ND	ND	ND
5	(>1.5) (S)	<0.1 (M)	1.0 (V)	0 (V)	0.3 (V)	<0.1 (V)	ND	ND
10	— (P)[d]	0.2 (M)	7 (M)	<0.1 (V)	0.5 (V)	0.1 (V)	0 (V)	ND
20	ND	0.5 (M)	(>10) (S)	0.2 (V)	3 (V)	0.3 (V)	0.5 (V)	0 (V)
40	ND	(>1) (S)	— (P)	0.3 (V)	>10 (V)	0.3 (V)	1.0 (M)	<0.1 (V)
80	ND	ND	ND	0.8 (M)	ND	0.5 (V)	(>3) (S)	0.2 (M)

[a] Time interval during which the majority of the cells were tumbling continuously after weak acid addition. When motility was severely impaired, responses were difficult to evaluate, and such responses are given in parentheses. Measurements at pH 5.5 were made under a bridged cover slip and at pH 7.0 in the temporal gradient apparatus.

[b] Letters in parentheses are estimates of vigor: V, vigorous; M, moderate; S, slow; P, paralyzed.

[c] ND, Not determined.

[d] —, Not applicable.

TABLE 2. *Comparison of the properties of four membrane-permeant weak acids*

Weak acid	pK	Concn for ΔpH collapse at pH 5.5 (mM)[a]	Concn for maximum tumbling response at pH 5.5 (mM)[b]
Salicylate	3.0	2	5
Benzoate	4.2	10	20
Acetate	4.8	15	40
DMO	6.3	50	80

[a] Measured by flow dialysis. The concentration shown is the minimum concentration causing complete collapse of ΔpH in *Salmonella* sp. strain ST1 for at least 10 min.

[b] Data are for *Salmonella* sp. strain ST1 and are taken from Table 1. The concentration shown is the concentration causing a tumbling response of more than 10 min or, in the case of salicylate and DMO, the subparalyzing concentration causing the maximum tumbling response.

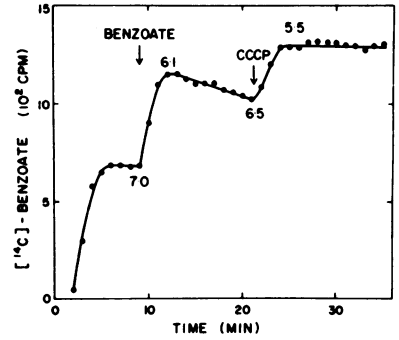

FIG. 3. *Partial collapse and recovery of ΔpH in Salmonella ST1 after addition of 1.5 mM benzoate at pH 5.5. The values of the cytoplasmic pH at different points in time are indicated directly on the graph. ΔpH was measured by uptake of [^{14}C]benzoate in a flow dialysis apparatus. The ordinate shows the radioactivity in the effluent and is proportional to the concentration of benzoate in the extracellular medium. The proton ionophore carbonyl cyanide-m-chlorophenyl hydrazone (CCCP) (100 μM) was added at the end of the run to collapse the PMF. Complete and permanent collapse of ΔpH was observed when 10 mM benzoate was used.*

character and in agreement with its failure to produce prolonged tumbling at pH 5.5, had no effect on ΔpH at pH 5.5.

To assess behavioral effects at different pH values, the ability of acetate to collapse ΔpH was examined as a function of external pH. At external pH values of 5.5, 6.0, and 6.5, the same concentration (15 mM) was necessary for complete collapse of ΔpH. This may reflect a balance between two opposing factors as external pH was increased, namely, decreasing permeability (because of decreasing concentration of the permeable protonated form) and decreasing ΔpH to be collapsed.

Effect of acetate on ATP. There is a direct link between PMF and ATP production via the membrane-bound ATPase. Also, several of the central catabolic enzymes (notably hexokinase [41] and phosphofructokinase [46]) have been found to be pH sensitive in the species where this has been examined. Therefore, since ATP is a substrate for the chemotactic methylation system (2, 4), we wished to know how ATP levels were affected by acetate addition at low pH. The results of one of several experiments directed at this question are shown in Fig. 4. The *E. coli uncA* mutant AN120 (which is incapable of interconverting PMF and ATP) was subjected to 50 mM acetate at pH 6.0; within 2 min, the cytoplasmic ATP concentration had fallen from a prestimulus level of 2.2 mM to ca. 1.7 mM, and it declined gradually thereafter. Simultaneous behavioral observations indicated immediate and almost continuous tumbling for 5 min and then a gradual increase in the extent of translational motion. However, even at 40 min cells were much more tumbly than the control. Similar decreases in ATP levels were noted when wild-type *Salmonella* was exposed to 50 mM acetate at pH 6.0, even in the presence of 50 μg of rifampin per ml and 20 μg of nalidixic acid per ml (to block ATP use for macromolecular synthesis), or when phosphoenolpyruvate (for which *Salmonella* has a transport system [36]) was used as a carbon source in an attempt to bypass pH-sensitive glycolytic enzymes (data not shown).

Effects of pH jumps on motility. Although it has been demonstrated that bacteria migrate in pH gradients (32, 37, 48), responses to temporal pH gradients have not been described for either *Salmonella* or *E. coli*. By analogy with

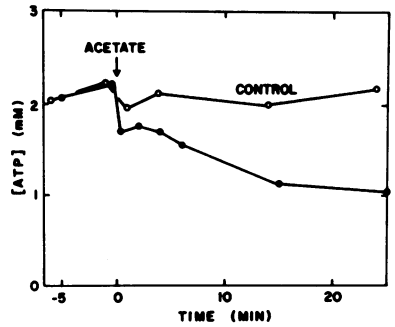

FIG. 4. *Effect of 50 mM acetate on the cytoplasmic ATP concentration of E. coli AN120 (uncA) in a solution containing 10 mM phosphate, 0.1 mM EDTA, and 1% (vol/vol) glycerol (pH 6.0). No acetate was added to the control sample.*

responses to organic attractants and repellents, one would expect migration from an acid pH to a neutral pH in a spatial assay to be reflected in a temporal assay by suppression of tumbling upon shifting from the acid pH to the neutral pH or by enhancement of tumbling upon shifting from neutral to acid conditions or both. These expectations were fulfilled, with one additional feature.

When *Salmonella* sp. strain ST1 was subjected to a pH jump from 5.5 to 7.0 in the temporal gradient apparatus, a 45-s smooth response was observed. The reverse jump (pH 7.0 to 5.5) caused a brief (10-s) smooth response, which was then replaced by 2 min of tumbling before relaxation to the prestimulus behavior. *E. coli* RP437 showed similar, but somewhat briefer, responses. The brief period of smooth swimming upon shifting to the low pH was observed in both species and may have been a response to energization of the cells by imposition of a large ΔpH component of PMF.

Responses of chemotaxis mutants to collapse of ΔpH. Mutations in a number of genes affect chemotactic responses. We wished to establish whether any of these mutants would show anomalous behavior upon ΔpH collapse. Of particular interest was the *tsr* gene of *E. coli*, because this had been found by Adler and colleagues (24, 48) to be necessary for normal repellent responses to low pH and to fatty acids.

Mutants in medium at pH 5.5 were subjected to acetate at a concentration of 40 mM or to acetate at a lower concentration if impairment of motility at 40 mM was pronounced. In general, *E. coli* mutants were less able to withstand the high acetate concentration than were mutants of *Salmonella*. Table 3 shows the steady-state behaviors (at least 2 min after acetate addition) of the various mutants. The majority of smooth mutants were susceptible to acetate, but some (*E. coli cheC*, *Salmonella cheC*, *E. coli cheD*, *Salmonella cheY*, *E. coli tar tsr*) failed to show any tumbling. In the case of mutants with a considerable prestimulus level of tumbling, it was difficult to determine whether there was any enhancement. However, in at least two mutants, *Salmonella* sp. strain SL4041 (*cheB*) and *E. coli* RP4368 (*tsr*), there was a distinct initial smooth response, which lasted about 45 and 30 s, respectively.

We tethered these two strains in order to quantitate their motor responses to ΔpH collapse. The result for *Salmonella* sp. strain SL4041 (*cheB*) is shown in Fig. 5. Whereas the wild type showed a monotonic rise from 14 to 80% CW rotation when 40 mM acetate was added, as described above (Fig. 1 and 2), the *cheB* mutant dropped transiently from its high prestimulus level of 83% to a minimum of 13% CW rotation after 15 s (in agreement with the smooth response observed in free cells), before approaching a steady-state level (essentially the same as the prestimulus value) by about 7 min.

When tethered (Fig. 6), the *E. coli tsr* mutant RP4368 showed almost complete suppression of CW rotation in the first 30 s after addition of 10 mM acetate, but then CW rotation steadily increased to 57% after 7 min. This is much higher than the prestimulus value (11%) and indicates that the *tsr* mutation does not prevent long-term CW enhancement. The wild-type parent strain RP437 showed CW enhancement immediately after addition of 17.5 mM acetate, reaching a maximum of 70% 15 min later. (The increase in CW bias was much slower for *E. coli* than for *Salmonella* [Fig. 5 and 6]. We have not further investigated this difference between the two species.) The acetate concentrations were the highest that could be used for these strains without seriously impairing motility. When subjected to 10 mM acetate, strain RP437 again showed only long-term enhancement (data not shown).

DISCUSSION

From the results described above, we conclude that some component of the regulation system of the flagellar motor is a pH-sensitive device. Whereas the state of the motor does not appear to be sensitive to the steady-state value of the external pH (prestimulus data of Fig. 2), lower cytoplasmic pH values favor CW rotation.

The evidence for this conclusion may be summarized as follows. (i) Weak acids caused tumbling in free cells and enhanced CW rotation in tethered cells. (ii) The behavioral effects of these weak acids were much more pronounced at a low pH. (iii) The four weak acids tested ranked the same with respect to acidity, ability to collapse ΔpH, and ability to perturb behavior. And (iv) the concentration of each weak acid necessary for maximum effect on behavior was sufficient to cause complete and permanent collapse of ΔpH.

The collapse of ΔpH by weak acids has the following secondary consequences for the cell. (i) A partial compensation by hyperpolarization occurs (34; M. A. Snyder, J. B. Stock, and D. E. Koshland, Jr., personal communication). However, this cannot be the cause of enhanced tumbling, since variation of membrane potential by other means (namely, by varying external pH) does not affect tumble frequency (prestimulus data of Fig. 2) (11). (ii) ATP pools decrease (Fig. 4), but again, this cannot be responsible for the behavioral change, since the onset of tumbling is virtually instantaneous; also, as described pre-

TABLE 3. *Effect of acetate on the steady-state behavior at pH 5.5 of various chemotaxis mutants of E. coli and Salmonella*

Species	Strain	Reference(s)	Genotype[a]	Steady-state behavior[b]	
				Before acetate addition	After acetate addition[c]
E. coli	RP437	30	Wild type	Random	Tumbly
Salmonella sp.	ST1	3	Wild type	Random	Tumbly
E. coli	RP4604	29	*cheA116*	Smooth	Tumbly
Salmonella sp.	ST1002	43	*cheA*	Smooth	Tumbly
E. coli	RP4845	28	*cheB274*	Tumbly	Tumbly
Salmonella sp.	SL4041	44, 49, 50	*cheB111*	Tumbly	Tumbly[d]
E. coli	RP4156	28	*cheC181*	Smooth	Smooth
E. coli	RP4493	Parkinson[e]	*cheC* (*scyA2*)	Tumbly	Tumbly
Salmonella sp.	ST203	51	*cheC303*	Smooth	Smooth
Salmonella sp.	ST134	13, 35	*cheC84*	Inverse random	Inverse random[f]
E. coli	RP4792	30	*cheD192*	Smooth	Smooth
E. coli	RP4602	Parkinson[e]	*cheW113*	Smooth	Tumbly
Salmonella sp.	ST202	51	*cheW302*	Smooth	Tumbly
E. coli	RP4332	31	*cheR202*	Smooth	Random
Salmonella sp.	ST1038	43	*cheR*	Smooth	Tumbly
E. coli	RP4315	Parkinson[e]	*cheY201*	Smooth	Tumbly
Salmonella sp.	ST112	43	*cheY262*	Smooth	Smooth
E. coli	RP4767	Parkinson[e]	*cheZ281*	Tumbly	Tumbly
Salmonella sp.	ST171	51	*cheZ221*	Tumbly	Tumbly
E. coli	RP4324	Parkinson[e]	*tar-52Δ1*	Smooth	Tumbly
E. coli	RP4368	10; Parkinson[e]	*tsr-518* (*cheD*)[g]	Random	Tumbly[d]
E. coli	RP4372	Parkinson[e]	*tar-52Δ1 tsr-518*	Smooth	Smooth

[a] Genotype with respect to motility and chemotaxis. Other (e.g., auxotrophic) mutations are present in some strains (see original references). Gene symbols follow the unified convention proposed by Koshland, Parkinson, and others. In *E. coli*, the original symbol for *cheR* was *cheX*; in *Salmonella*, *cheA* was *cheP*, *cheB* was *cheX*, *cheC* was *cheU*, *cheY* was *cheQ*, and *cheZ* was *cheT*.

[b] Smooth, Swimming only; random, wild-type alternation between swimming and tumbling; inverse random, alternation between inverse swimming and tumbling (13).

[c] Behavior at least 2 min after addition. The acetate concentration was generally 40 mM for *Salmonella* and 20 mM for *E. coli*; for strains whose motility was severely impaired by these concentrations, lower values were used.

[d] Smooth initial response. See text and Fig. 5 and 6 for more detailed examinations of these strains.

[e] J. S. Parkinson, unpublished data.

[f] The proportion of time in inverse smooth swimming was greater than before acetate addition.

[g] This strain is now believed (30) to be a double mutant in the closely linked *tsr* and *cheD* loci (which may in fact be the same gene). However, the phenotype of this strain is Tsr$^-$, not Che$^-$.

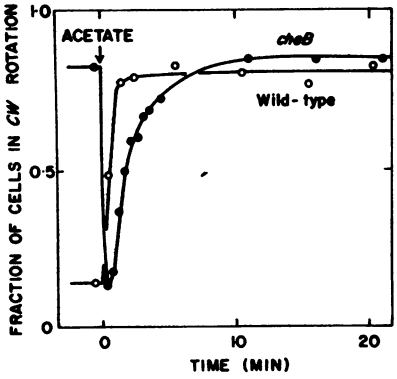

FIG. 5. *Response at pH 5.5 of a methylesterase-defective Salmonella mutant (strain SL4041, cheB)* (●) *to addition of 40 mM acetate. The response of strain SL3625* (○) *(wild type with respect to motor function) is shown for comparison.*

viously (11), the ATP pool can be reduced to much lower values (0.2 mM) by arsenate treatment, without altering the level of tumbling. Therefore, it seems likely that cytoplasmic pH affects the chemotaxis system directly.

Is the regulation of the motor by cytoplasmic pH utilized in any aspect of cell behavior? Two plausible contexts come to mind, namely, the tactic responses that are exhibited to gradients of pH and weak acids. The results obtained in this study strongly suggest that motor regulation by cytoplasmic pH is involved in both contexts.

pH taxis. Although pH taxis has been demonstrated to be part of the behavioral repertoire of bacteria (32, 37, 48), the nature of the signal initiating the response has not been determined.

One is first tempted to inquire whether pH taxis is another example of energy (PMF) taxis, as exemplified by aerotaxis and phototaxis. Al-

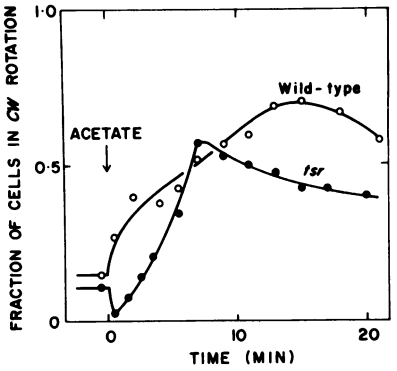

FIG. 6. *Response at pH 5.5 of an E. coli tsr mutant (strain RP4368) (●), defective in methyl-accepting protein MCP I, to addition of acetate. The response of the parent strain (RP437) (○) is shown for comparison. The concentrations used were 10 and 17.5 mM, respectively, the highest concentrations that could be used without impairing motility in these strains.*

though this may explain migration of cells from an alkaline pH to a neutral pH, it cannot explain migration from an acidic pH to a neutral pH, because in the process of such a migration the PMF of cells is diminishing, not increasing. ΔpH is, of course, diminishing even more rapidly than PMF as a whole in this situation so that, unless one invokes hypothetical ΔpH and $\Delta\psi$ sensors of opposite polarity, ΔpH cannot be the signal; since ΔpH and $\Delta\psi$ are equivalent as energy sources for the motor (12, 20, 39) and exert similar steady-state (11) and transient (22, 23) regulatory effects on it, such an inverted polarity seems implausible. This suggests that pH per se is involved, rather than some energy parameter such as ΔpH or PMF.

As shown by the initial behaviors at different pH values in Fig. 2, steady-state external pH cannot itself be the signal. An external pH receptor linked to a subsequent component capable of adaptation to a changing external pH is possible in principle; such an arrangement is responsible for transduction of gradient information in the case of sugars such as glucose, galactose, and ribose. However, the fact that a dramatic change in behavior occurs upon shifting cytoplasmic pH at a constant external pH strongly suggests that cytoplasmic pH is the parameter responsible.

Bacteria regulate their cytoplasmic pH values rather closely (8, 12, 27), and it is appropriate to ask whether a change in external pH could generate a large enough change in cytoplasmic pH to perturb motor switching appreciably. This is a question which will require further study, but it should be noted that the conclusion that cytoplasmic pH is closely regulated is based on steady-state measurements. It seems likely that cells subjected to a decrease in external pH experience a transient decrease in cytoplasmic pH to a value more extreme than the eventual reregulated value; preliminary measurements by ^{31}P nuclear magnetic resonance (J. L. Slonczewski, J. R. Alger, and R. M. Macnab, unpublished data) indicate that such transient overshoots occur. Thus, steady-state measurements may underestimate the magnitude of the cytoplasmic pH signal in response to a change in external pH.

It should also be remembered that the bacterial sensing system is known to be a remarkably sensitive one. *Salmonella* cells can detect a temporal change in serine concentration of 0.1%/s (18) and modulate their switching probabilities sufficiently to ensure effective migration in spatial gradients. If a cytoplasmic pH sensor of comparable sensitivity were responsible for pH taxis, it could detect temporal cytoplasmic pH gradients of 4×10^{-4} pH unit per s; a spatial gradient of 0.1 pH unit per mm in the external medium (corresponding to about 3×10^{-3} pH unit per s for a swimming bacterium) could be attenuated 10-fold as a cytoplasmic signal and still be detected by such a sensor.

Thus far we have discussed only the excitation mechanism of pH taxis and not the adaptation mechanism by which a cell returns to its prestimulus behavior in pH jump experiments. In the case of attractants, which are recognized by external receptors, adaptation is mediated by the chemotaxis methylation system (42). In the case of pH taxis, if the change in cytoplasmic pH (either upon pH shift or upon addition of weak acids) were transient, adaptation would follow automatically. As mentioned above, preliminary measurements of pH by nuclear magnetic resonance indicate that such transient changes in pH do occur. However, if this is the sole adaptation mechanism, a permanent alteration of the cytoplasmic pH ought to cause a permanent alteration of motor behavior. This was observed when the cytoplasmic pH was collapsed to an external value of 5.5 or lower; cells retained a permanent CW bias (Fig. 2). At pH 6.0 and above, however, cells showed a progressively increasing capacity to recover CCW rotational ability (Fig. 2) under conditions where flow dialysis indicated that ΔpH remained collapsed. This suggests that adaptation is possible even at a constant cytoplasmic pH; the absence of adaptation at pH 5.5 could be the result of an excitation too extreme to be counteracted by the adaptation mechanism or of a disabling of the adaptation mechanism.

The behaviors of the *E. coli tsr* and *Salmonella cheB* mutants also argue against a simple

model in which there is a one-to-one correspondence between the value of the cytoplasmic pH and the state of the motor, because in these strains the short-term (ca. 1-min) effect of lowering the cytoplasmic pH could be distinguished from the long-term effect. Unlike the wild types, both of these mutants suppressed CW rotation initially. The *tsr* mutant then showed a long-term enhancement of CW rotation, which was comparable to the wild-type response; the *Salmonella cheB* mutant resumed its usual high CW bias with no further enhancement. These results suggest that there is more than one component of motor regulation affected by lowered cytoplasmic pH and that both mutants have inverse responses with respect to the earlier component only. (The fact that acetate is functionally an attractant for *E. coli tsr* mutants at pH 7.0 [24] indicates a dominance of the early inverted phase under these conditions.)

Interpretation of the adaptation of cells subjected to ΔpH collapse by weak acids was complicated by another feature of motor regulation, namely, the CCW bias that results whenever PMF and motor speed are reduced by metabolic intervention (11). To collapse ΔpH by weak acids, these compounds had to be used at high concentrations (on the order of 10 mM), which were close to or above the threshold for impairment of motility. This was most noticeable in the cases of salicylate and DMO (for which we were unable to find conditions for indefinite vigorous tumbling), but even with acetate and benzoate there was a slight decrease in speed; also, if for any reason a culture was less vigorous than usual before addition of the weak acid, the further deterioration was marked, and the tumbling response was less distinct and less long-lived. Several of the *che* mutants tested were particularly susceptible to impairment of motility, even by acetate, and once again it was not possible to obtain vigorous long-lasting tumbling in these cases. Therefore, the behavioral adaptation to lowered cytoplasmic pH upon addition of weak acids could be a result of progressive onset of a CCW bias (derived from lowered cytoplasmic pH). A prediction from this is that if conditions can be obtained whereby ΔpH can be collapsed without any impairment of motility, the behavioral change should be permanent. The long-term tumbling observed in permeabilized *E. coli* after nigericin is added (34) could be an example of "benign" ΔpH collapse.

It is evident from the foregoing discussion that the mechanism of adaptation in pH taxis is still uncertain.

Does the system that mediates pH taxis utilize any components of the system that mediates chemotaxis? The failure of some smooth *che* mutants to tumble in response to a lowered cytoplasmic pH is not necessarily indicative of a specific failure to process pH information and may simply reflect an unstimulated phenotype too extreme to be perturbed. However, two mutants (*tsr* and *cheB*) processed pH information incorrectly, giving smooth responses in the period immediately after the lowering of cytoplasmic pH (Fig. 5 and 6). This indicates that the system for processing pH information employs either the *tsr* and *cheB* gene products or components strongly interacting with them. Both of these proteins are components of the chemotaxis methylation system, the former as a substrate (MCP I) and the latter as a methylesterase, but it remains to be seen whether the methylation status of MCP I is perturbed during pH taxis. In the case of chemotaxis, a change in methylation status has been found to correlate with the process of adaptation rather than excitation (42). Thus, for example, methyltransferase (*cheR*) mutants can still be excited by chemotactic stimuli. We find that this also applies to lowered cytoplasmic pH, since *cheR* mutants, like the wild type, showed enhanced tumbling (Table 3).

The inverted response of the *tsr* mutant and the normal response of the *tar* mutant to a lowered cytoplasmic pH provide additional support for the conclusion that this is the signal for pH taxis, since it had been shown previously that *tsr* mutants were unable to respond to pH gradients (and gave inverted responses to weak acids), whereas *tar* mutants responded normally (24, 48).

Cytoplasmic pH and PMF are intimately related, and both parameters have now been found to play a role in the regulation of the flagellar motor. The mechanism in the two cases must be considerably different, because in contrast to regulation by cytoplasmic pH, regulation by PMF was not found to require any component of the chemotactic transducing system (11).

Weak-acid taxis. The initial observations of enhanced tumbling were made with acetate, a known repellent. If the repellent action of acetate is a consequence of its effect on cytoplasmic pH rather than of its specific recognition by a receptor, any agent causing ΔpH collapse should act as a repellent and should have a greater effect at a low pH. The results obtained with four weak acids, including a synthetic one (DMO) for which there is no reason to postulate the existence of a receptor, clearly support this prediction.

In earlier studies of negative chemotaxis (47, 48), it was found that certain repellents competitively inhibit the response to others; these results were interpreted in terms of receptor classes. Whereas the abstract concept has be-

come a reality in the case of various sugar receptors (by virtue of their isolation and characterization), no repellent receptor has yet been identified. The results presented above suggest that the original interpretation of repellent competition classes requires revision, at least in the case of weak acids. The fatty acids acetate, propionate, and butyrate were assigned to a single class in *Salmonella* (47) and *E. coli* (48). (Additional evidence [34] now argues against the original assignment [48] of benzoate and salicylate to a separate class from the fatty acids in *E. coli*.) We now reinterpret this, not in terms of a common "fatty acid receptor," but in terms of a common mode of action, namely, collapse of ΔpH. For example, if 20 mM acetate has already completely collapsed the cytoplasmic pH value to the external value, the addition of 2 mM propionate should have little effect.

In a recent study (34), Repaske and Adler have independently concluded that weak-acid repellents (and, additionally, weak-base attractants) are detected via their effects on cytoplasmic pH.

Thus, the mechanisms of weak-acid taxis and pH taxis seem to be the same. The simplest mechanism (Fig. 7) would involve a protein (either cytoplasmic or membrane bound and exposed to the cytoplasm) which, when protonated, is altered in such a way as to initiate a signal to the motor; if this simple mechanism is correct, the term *proton receptor* seems appropriate. In this modified sense fatty acids would act through a common receptor, but one whose specificity was for protons rather than for fatty acids. Since the methylesterase and one of the methyl-accepting proteins are needed for a normal response to a lowered cytoplasmic pH (Fig. 5 and 6), pH-sensitive hydrolysis of the methylated protein may be involved. It is interesting in this regard that the methylesterase of *Salmonella* is more active at pH 5.5 than at neutral pH (J. B. Stock and D. E. Koshland, Jr., personal communication).

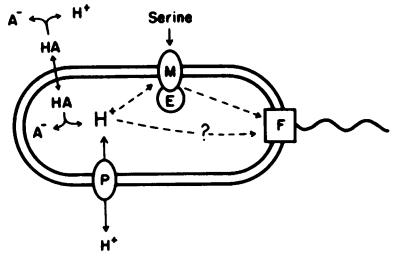

FIG. 7. *Model for the sensing of pH and weak acids by bacteria. A decrease in the external pH perturbs the regulation of cytoplasmic pH, which is under the control of system(s) P, causing a transient decrease in cytoplasmic pH. Likewise, the addition of a weak-acid repellent (e.g., acetate), which permeates the membrane in the protonated form (HA), results in proton transfer into the cytoplasm. A lowered cytoplasmic pH alters the state of the methyl-accepting chemotaxis protein MCP I (M), possibly with respect to demethylation by the methylesterase (E). This results in a signal to the flagellar motor (F) to develop a clockwise bias, and the cell tumbles. Cytoplasmic pH may also affect the motor directly. As well as sensing cytoplasmic pH, M is a sensor for extracellular serine and for temperature.*

We wish to emphasize that the reinterpretation of the mechanism of repellent action is thus far confined to those compounds whose membrane permeabilities and acidities are such that they can collapse ΔpH. How other repellents, such as indole, act remains to be elucidated.

The magnitude of the drop in the cytoplasmic pH upon addition of weak acids should determine the extent (defined as percent CW rotation) of the response and also its duration before the cell adapts, either as a result of restoring its cytoplasmic pH or as the result of an adaptation process in the sensory mechanism, if such a process exists. This was found to be the case, responses at a low external pH being more extreme and much more prolonged. Therefore, one might suppose that a weak acid can only act as a repellent if the external pH is below the prestimulus cytoplasmic value, i.e., if there is an inwardly directed proton gradient to be collapsed. This supposition is not necessarily correct; the addition of a weak acid would result in acidification of the cytoplasm even in the absence of a preexisting ΔpH if the diffusion potential of the weak acid transiently drove the uptake of protons faster than they could be pumped out. This may explain the brief responses to weak acids that were observed even at pH 8.0.

What does the avoidance of a low pH and weak acids mean in terms of survival advantage to a cell? Are both responses advantageous, or is one a fortuitous consequence of the mechanism that has evolved for expressing the other? It is obvious that extremes of pH are harmful; exposed surface structure begins to denature, the capacity to regulate cytoplasmic pH is strained beyond its limits, and numerous cellular functions (including motility itself) fail. The migration from environments of moderately acid pH can then be viewed as escape before conditions become excessively harmful. In light of the results of the present study, the advantage of avoiding weak acids can now be understood in similar terms; at a constant pH (say, pH 6.0), which may be well within normal tolerance limits of a cell, a gradient of weak acid warns of a nearby environment where that same pH value may become intolerable, because the proton po-

tential across the membrane can be short-circuited by the weak acid.

Because many enzymes show a marked pH dependence, the regulation of cytoplasmic pH is extremely important for the proper functioning of any cell. In higher animals, the bulk of this task falls to specialized organs, such as the lungs and the kidneys; elevated acid levels in the extracellular fluids result in increased acid excretion. An individual bacterial cell has no such environmental protection and must perform the task individually; in ways that are still poorly understood, cytoplasmic pH acts as a signal for its own regulation via transport systems. However, it is now apparent that cytoplasmic pH acts also as a signal for the behavioral response of bacteria, enabling them to avoid environments that would overtax their ability to regulate cytoplasmic pH by physiological means alone.

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