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pH homeostasis in *Escherichia coli*: Measurement by ^{31}P nuclear magnetic resonance of methylphosphonate and phosphate

(pH regulation/intracellular pH/protonmotive force/Fourier-transform NMR)

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ABSTRACT **The intracellular pH of *Escherichia coli* cells, respiring on endogenous energy sources, was monitored continuously by ^{31}P NMR over an extracellular pH range between 5.5 and 9. pH homeostasis was found to be good over the entire range, with the data conforming to the simple relationship**

$$\text{intracellular pH} = 7.6 + 0.1(\text{external pH} - 7.6)$$

so that the extreme values observed for intracellular pH were 7.4 and 7.8 at external pH 5.5 and 9, respectively. As well as inorganic phosphate, we employed the pH-sensitive NMR probe methylphosphonate, which was taken up by glycerol-grown cells and was nontoxic; its pK_a of 7.65 made it an ideal probe for measurement of cytoplasmic pH and alkaline external pH.

Given the pH sensitivity of most enzymatic reactions, the ability to regulate cytoplasmic pH is likely to be a valuable attribute for any cell, and especially for a free-living unicellular organism such as a bacterium that encounters substantial variation in the pH of its environment.

A number of studies have been made of the dependence of the internal pH (pH_{int}) of bacteria upon the value of the external pH (pH_{ext}) (1–8). These have generally indicated that, at pH_{ext} 7.5 or lower, homeostasis of pH is fairly good. However, among these studies there is no clear agreement as to whether bacteria can maintain the inverted (inside more acid) pH gradient necessary for homeostasis at pH_{ext} above 7.5. The variable results reported may stem partly from the difficulty of keeping cells adequately energized at alkaline pH, but there are also questions regarding the assumption that membrane-permeant basic probes such as methylamine equilibrate passively (9). ^{31}P NMR has been employed successfully for measurement of pH_{int} in bacteria (5, 10), but the chemical shift of inorganic phosphate (P_i), the species usually monitored, is pH insensitive above pH 8, because the pK_a is 6.8 at physiological ionic strengths. Any failure of regulation at alkaline pH would therefore not be readily detected by phosphate chemical shift experiments.

For our studies both of molecular mechanisms of pH regulation (11, 12) and of pH taxis of bacteria (13, 14) we felt it was important to establish the degree of homeostasis of a population of cells as pH_{ext} was progressively adjusted over a wide range. We have used ^{31}P NMR to monitor both pH_{int} and pH_{ext} rapidly and continuously, employing for the alkaline region a synthetic probe, methylphosphonate [Me*P*; $CH_3PO(OH)_2$], which is taken up by the cells, has a pH-sensitive chemical shift that is well resolved from the shifts of phosphorus metabolites, and has a pK_a of 7.6 that enables measurement of both pH_{int} and pH_{ext}; for acid pH_{ext} we employed the resonance of P_i. Our results indicate that homeostasis of pH in respiring *Escherichia coli* cells is extremely good (7.6 ± 0.2) over a pH_{ext} range of about 5.5 to 9.

MATERIALS AND METHODS

Measurement of ^{31}P NMR Spectra. ^{31}P NMR spectra were obtained at 145.8 MHz on a Bruker WH360 wide-bore spectrometer. Free induction decays were accumulated in 2-min blocks, using 35° pulses at a repetition rate of 10 s^{-1}. The low magnetic field drift (<0.01 ppm hr^{-1}) obviated the use of a field frequency lock.

The titration curves of Me*P* and P_i in buffer were constructed by addition of successive portions of 1 M NaOH, measurement of pH by electrode, and measurement of the ^{31}P chemical shift with respect to the pH-insensitive reference resonance (at 0.49 ppm) of glycerophosphocholine, present at 0.3 mg ml^{-1}. [All chemical shifts are reported on a scale with the resonance of 85% phosphoric acid placed at the origin, and with higher frequency (downfield) resonances given positive values in accordance with IUPAC convention.]

In experiments with cells, the absolute chemical shift scale was chosen such that extracellular Me*P* and P_i gave the same value for pH_{ext} at the beginning of the experiment; this procedure was facilitated by the fact that the pH dependences of the chemical shifts of the two probes are in the opposite direction (see Table 1). The chemical shifts of cytoplasmic Me*P* and P_i were assumed to follow the same titration behavior as in buffer. This assumption is supported by the excellent agreement between the cytoplasmic pH values reported by the two probes (see Fig. 2 and 3), especially in view of the opposite direction of the pH dependences; also, in non-cell experiments, we found no significant dependence of chemical shift of either probe upon ionic strength, provided the latter was moderately high.

Cell Growth and Treatment. *E. coli* FRAG1, a wild-type strain (15), was grown at 37°C on M9 minimal medium (16) with 0.5% glycerol as a carbon source. About 3 liters of cells in logarithmic phase (OD_{650} *ca.* 0.6) was cooled to below 10°C with continuous oxygenation, and then centrifuged (4000 × *g*, 10 min), washed, and resuspended to 33% wt/vol wet pellet in ice-cold buffer [20 mM citric acid/20 mM 1,4-piperazinediethanesulfonic acid (Pipes)/20 mM 2-{[tris(hydroxymethyl)methyl]-amino}ethanesulfonic acid (Tes)/20 mM *N,N*-bis(2-hydroxyethyl)glycine (Bicine)/80 mM NaCl/5 mM KH_2PO_4, adjusted to pH 7 with NaOH] plus 5 mM Me*P* and Antifoam B (Dow) at 10 μl ml^{-1}. This buffer was devised to provide adequate and approximately constant buffering capacity over the entire pH range of the experiments. No exogenous carbon source was

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Abbreviations: Δ*p*, protonmotive force; ΔpH, transmembrane pH difference; Δψ, membrane potential; $\Delta\psi_{crossover}$, membrane potential at zero ΔpH; Me*P*, methylphosphonate; $pH_{crossover}$, pH at zero ΔpH; pH_{ext}, external pH; pH_{int}, intracellular pH.

Table 1. Chemical shift characteristics of Me*P* and P_i

Probe	Chemical shift, ppm: Monobasic form	Dibasic form	Difference	pK_a*
Me*P*	24.90	21.05	−3.85	7.65
P_i	0.65	3.15	2.50	6.83

* Estimated by fitting the data to the Henderson–Hasselbalch equation.

used. ^{31}P NMR spectra were measured on 15 ml of the cell suspension in a 20-mm-diameter tube, with vigorous oxygenation maintained throughout. The oxygenation method employed a stream of 1000 ml min^{-1} above the position of the NMR observation coil and twin streams, of 100 ml min^{-1} each, at the bottom of the sample tube. The temperature was regulated at 25 ± 1°C. When the internal Me*P* had risen to a detectable level (typically after 25 min), pH_{ext} was progressively altered by adding either 1 M HCl or 1 M NaOH at 1 ml hr^{-1} via a syringe pump (model 341A, Sage Instruments, Cambridge, MA).

RESULTS

Me*P* as a Probe of Alkaline pH. The pH-dependent chemical shift characteristics of Me*P* and P_i in buffer are compared in Table 1. The Me*P* resonances lay about 22 ppm downfield of those of P_i. As the monobasic form of Me*P* was titrated through to the dibasic form, there was an upfield shift of 3.85 ppm; the corresponding titration of P_i caused a shift that was smaller (2.50 ppm) and occurred in the opposite direction—i.e., downfield. The chemical shifts observed for both molecules in buffer showed excellent conformity to the Henderson–Hasselbalch equation. The pK_a of 7.65 obtained for Me*P* was virtually identical to the pH of the cytoplasm (see below), whereas the pK_a of P_i was approximately one pH unit lower. These values, measured in buffer of relatively high ionic strength, are somewhat lower than literature values [7.9 for Me*P* (17) and 7.21 for P_i] measured under standard conditions. By restoration of the original pH with acid after a base titration and vice versa we established that further increase in ionic strength had a negligible effect on the chemical shifts or pK_a of either molecule.

Me*P* was taken up by glycerol-grown *E. coli* cells to an extent that readily permitted detection by NMR, but when glucose or succinate was used as the carbon source no resonance corresponding to cytoplasmic Me*P* was seen. α-Glycerophosphate (5 mM) caused a transient efflux of Me*P* (data not shown). Uptake may be occurring via the α-glycerophosphate uptake system, which has a rather broad specificity that includes phosphonates (18). For glycerol-grown cells, the initial uptake of Me*P* (at an external concentration of 6 mM) was 0.5 mM min^{-1}. After an hour, the cytoplasmic concentration began to level off

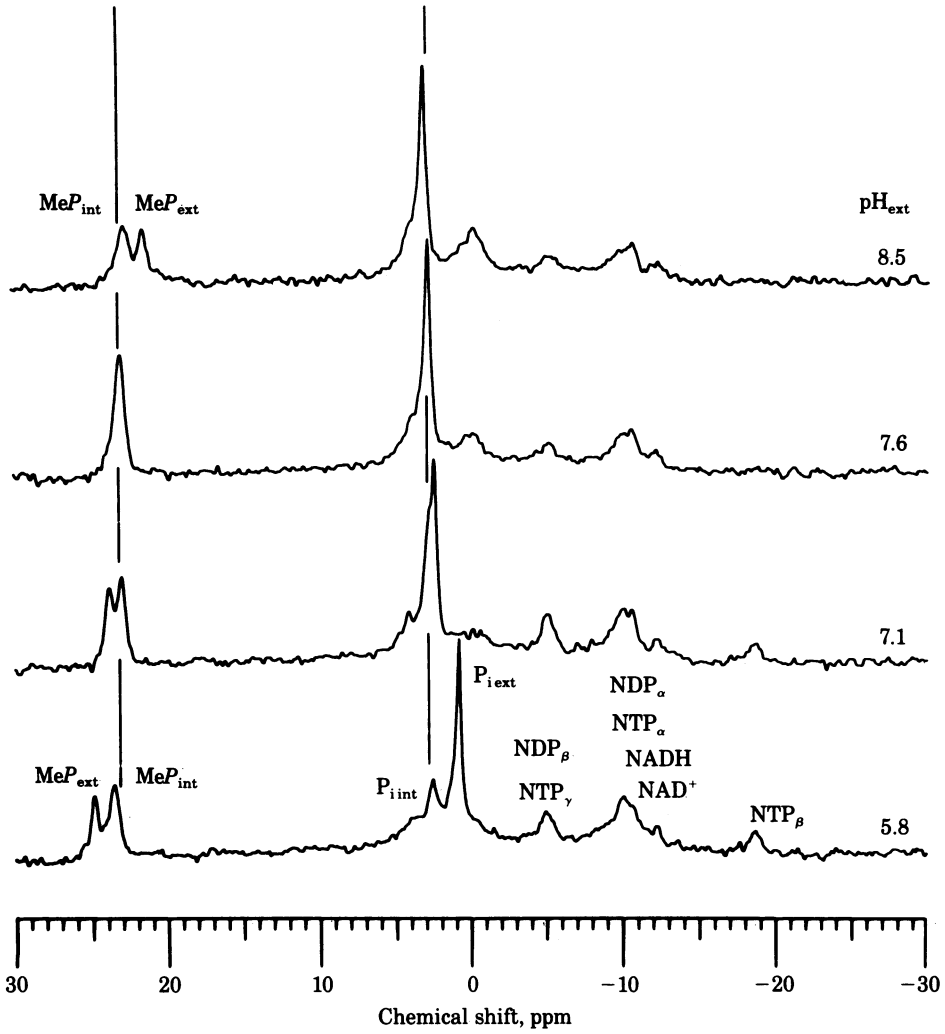

FIG. 1. ^{31}P NMR spectra of respiring *E. coli* cells in buffer at various values of pH_{ext}. The buffer contained Me*P* as well as P_i. Vertical lines indicate the positions of the Me*P* and P_i resonances at the crossover pH of 7.6, where ΔpH is zero. Above that pH, the external Me*P* resonance crosses over the internal resonance, whereas the external and internal resonances of P_i are not resolved.

at about 12 mM,[‡] at which point the external concentration had fallen to 4 mM. We found Me*P* to be nontoxic to *E. coli* at the concentration (5 mM) at which we used it as a probe, as judged by normal growth rate (doubling time of 68 min at 37°C in M9 medium plus 0.5% glycerol) and ATP levels measured by NMR.

Fig. 1 illustrates the properties of Me*P* and P_i as probes of external and cytoplasmic pH in *E. coli*. At pH_{ext} 5.8, the internal and external resonances of both probes are clearly resolved, with a "mirror-image" relationship that derives from the opposite direction of the pH dependences. At pH_{ext} 7.1, the Me*P* resonances are still well resolved, whereas the resonance from internal P_i now appears only as a shoulder on the external P_i resonance. In this pH range, resolution of P_i resonances is especially difficult because in highly energized cells internal P_i pools tend to be low; this is not the case with Me*P*. At the crossover pH of 7.6 (where ΔpH is zero) both probes show a single resonance, as expected. The P_i resonance remains unresolved above the crossover pH because deprotonation is essentially complete, whereas the resonance from external Me*P* emerges as a well-resolved peak upfield from the internal resonance. Because of its greater pH sensitivity in this range, Me*P* also shows much more clearly than P_i the small but real dependence of pH_{int} upon pH_{ext} (compare positions of the internal resonance of Me*P* at pH_{ext} 5.8, 7.6, and 8.5 in Fig. 1). We have found that for both probes the cytoplasmic resonance is broader, and has a shorter relaxation time, than that from the external buffer. The reason for these differences is not understood.

Effect of External pH upon Cytoplasmic pH. Fig. 2 shows the results of an experiment in which glycerol-grown *E. coli* cells, suspended in buffer plus Me*P*, were subjected to a decrease in pH_{ext} from 7.55 to 5.6 over a 45-min period followed by an increase to 8.7 over a 70-min period. Wherever the pH of a given compartment fell within the usable titration range of both probes, the agreement between the reported pH values was excellent. Me*P* gave less scatter than P_i in estimates of pH_{int}, because these were close to its apparent pK_a (7.65). The homeostasis displayed in Fig. 2 is very good, but not perfect, with pH_{int} ranging between 7.4 at pH_{ext} 5.6 and 7.8 at pH_{ext} 8.7, with a crossover value of 7.6. The deviation is systematic rather than random, as is evident in Fig. 3, where the data from the same experiment are presented as variation in ΔpH as a function of pH_{ext}. The close fit (correlation coefficient of 0.997) of the data to a linear function indicates that the deviation of pH_{int} from the crossover pH is proportional to the deviation of pH_{ext} from that value: i.e.,

$$(pH_{int} - pH_{crossover}) = k(pH_{ext} - pH_{crossover}).$$

In this particular experiment k was 0.11 and $pH_{crossover}$ was 7.63. The degree of homeostasis varied slightly from experiment to experiment. In another experiment (carried out on two successive batches of cells, one used for measurement from around neutral pH_{ext} down to 5.5 and the other from around neutral pH_{ext} up to 9.0) we found $k = 0.07$ (correlation coefficient 0.996) with a crossover pH of 7.63 (data not shown).

Cells maintained for prolonged periods at extreme acid or alkaline pH_{ext} had a tendency to lose homeostasis—i.e., ΔpH eventually began to collapse. Removal of the oxygen supply had the same effect (data not shown).

Referring back to Fig. 1, it can be seen that nucleotide triphosphate levels (judged by the NTP_β resonance) were much lower at alkaline pH_{ext}. We have consistently observed this pattern, although the drop was not always as marked as in Fig. 1.

‡ These calculations are based on an assumed cytoplasmic volume of 0.5 ml per g wet weight.

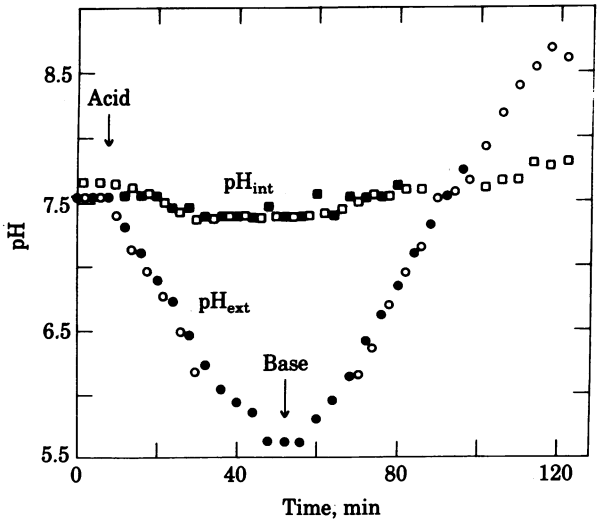

FIG. 2. Dependence of pH_{int} of *E. coli* upon pH_{ext}. pH values were measured by the chemical shift of both Me*P* and P_i. At the time indicated by the first arrow, pumping of 1 M HCl was begun; at the second arrow, the pump was switched to 1 M NaOH. Data were accumulated continuously and stored in 2-min blocks; in the figure, data points represent two-block (4-min) averages, staggered for the two probes for clarity of presentation. Data points based on Me*P* below pH 6.1 and P_i above pH 7.8 have been omitted because they lie outside the useful titration range of the respective probes. ■, Internal P_i; □, internal Me*P*; ●, external P_i; ○, external Me*P*.

DISCUSSION

The manner in which cytoplasmic pH of bacteria changes with external conditions is important from several points of view. It prescribes the conditions under which intracellular reactions take place, it is the empirical basis for an exploration of the molecular mechanisms of pH regulation, it is implicated in tactic responses, and it prescribes the magnitude of ΔpH, which is one of the two components of protonmotive force, a central energetic parameter for many functions of the bacterial cell. In the

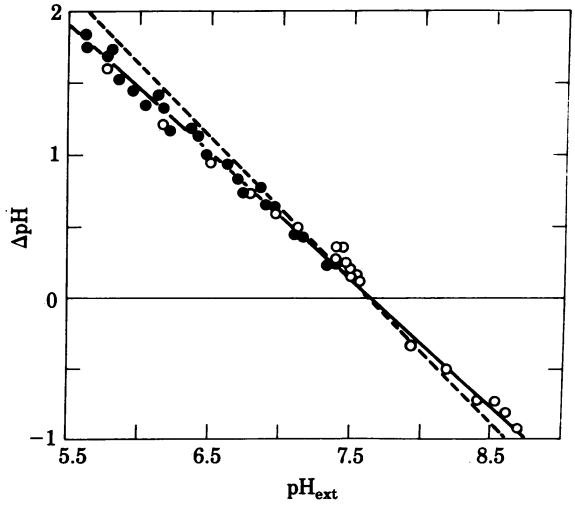

FIG. 3. Transmembrane pH difference (ΔpH) in *E. coli* as a function of pH_{ext}. Data are the same as used for Fig. 2, and measurements made as pH_{ext} was being raised and lowered are combined. The linear regression (continuous line) was made on the data from both Me*P* and P_i; it has a slope of −0.89, a zero-crossing of 7.63, and a correlation coefficient of 0.997. The broken line, with a slope of −1, represents perfect homeostasis with pH_{int} constant at 7.63 at all pH_{ext}. ●, P_i; ○, Me*P*.

latter regard, the issue of homeostasis at alkaline pH becomes particularly important, because such homeostasis implies a *negative* contribution to protonmotive force.

Previous studies of pH regulation in *E. coli* (2–7) have shown that ΔpH is substantial at acidic pH_{ext} (1.5–2 pH units at a pH_{ext} of 6), and decreases as pH_{ext} is increased, indicating some degree of regulation. In several such studies the decrease in ΔpH with increasing pH_{ext} was reported to be more or less quantitative, so that pH_{int} remained fairly constant. For example, Padan *et al.* (2), measuring ΔpH by uptake of the weak acid 5,5-dimethyloxazolidine-2,4-dione, obtained values of 8.0 and 7.7 for pH_{int} at pH_{ext} of 6.0 and 8.0, respectively. Felle *et al.* (7) obtained a constant value of 7.5 for pH_{int} of cells between pH_{ext} 5.5 and 7.5, as did Ramos *et al.* (3) for vesicles. Booth *et al.* (6) reported only partial regulation, with an increase of 0.4 pH units in pH_{int} over the pH_{ext} range 6.3 to 8. Navon *et al.* (5), using ^{31}P NMR to measure pH directly (rather than ΔpH), found that the data from a number of separate experiments at pH_{ext} between 6 and 8 had a statistical scatter of about 0.5 pH unit, but they reported no systematic variation with pH_{ext}.

Most of these studies, with one notable exception (4), have indicated that at some moderately alkaline value (in the range 7.5 to 8) the ΔpH reaches zero, at which point the only contribution to protonmotive force is $\Delta\psi$. Relatively few measurements have been reported for more extreme alkaline pH_{ext}, where the important question arises of whether the cells can maintain their cytoplasm at a pH more acid than the environment. Detection of such an inversion by weak acid exclusion is technically difficult. Padan *et al.* (2) employed instead the uptake of the weak base methylamine and reported an inverted ΔpH, with pH_{int} rising more slowly than pH_{ext}, reaching a value of 8.4 at pH_{ext} 8.9. However, it has been reported (9) that methylamine can be actively accumulated by *E. coli* via an NH_4^+ transport system, and indeed Booth *et al.* (6) observed a 4- to 5-fold uptake of the base by *E. coli*, even at a pH_{ext} of 5.9; this raises questions concerning the meaning of uptake of methylamine at higher pH_{ext}. Guffanti *et al.* (8), also using methylamine uptake, have examined pH regulation in an alkalophilic *Bacillus* species, and reported an inverted ΔpH that reached a maximum value of 2.5 units, acid inside, at pH_{ext} 11.5. They concluded that methylamine uptake could be either active or passive, depending on the ionic composition of the buffer. Evidence for an inverted ΔpH has been provided by NMR experiments of Navon *et al.* (5), in which the pH_{int} was estimated to be 7.6 at pH_{ext} about 8; the inversion was undoubtedly real, but the pH values have considerable uncertainty because they are at the upper limit of the useful range of P_i as a probe.

The present study went beyond those mentioned above in that it was an attempt to continuously monitor pH_{int} in *E. coli* cells while pH_{ext} was being altered over a wide range. It demonstrates that these cells are capable of a high degree of pH homeostasis, maintaining pH_{int} close to 7.6 even when the pH_{ext} is as low as 5.5 or as high as 9. Any deviation of pH_{ext} from 7.6 is reflected only to the extent of about 10% as a deviation in pH_{int} (Figs. 2 and 3).

The protocol followed had the following features that strengthen our conclusions regarding homeostasis: (*i*) Both pH_{int} and pH_{ext} rather than their difference, ΔpH, were measured. (*ii*) Measurements were made continuously on a single cell sample as pH_{ext} was slowly varied. (*iii*) Cells were fully aerobic at all times while the measurements were being made. (*iv*) Two pH probes were employed, with useful ranges that overlapped and permitted measurements between pH 5.5 and 9. (*v*) In contrast to the use of membrane-permeant weak acids or bases, the extent of uptake of the probe, and the uptake mechanism (active vs. passive), were immaterial provided uptake was sufficient for detection of the resonance signal. (*vi*) No disruption of the normal state of the cell (by treatments such as permeabilization with EDTA or addition of valinomycin) was employed; the synthetic NMR probe, Me*P*, was shown to be nontoxic. [In experiments in which it is important to monitor pH_{int} immediately upon sample introduction into the NMR spectrometer, Me*P* could therefore be added prior to cell harvesting.]

It must be emphasized that we have demonstrated homeostasis for cells under only one set of conditions, namely, fully aerobic metabolism utilizing endogenous energy sources. Anaerobiosis, even when a glycolytic substrate is available, impairs the ability to regulate pH_{int} (2, 5). Because our measurements were made under no-growth conditions, it is clear that the pH regulatory mechanism(s) must utilize preexisting components of the cell without the requirement for synthesis of new components.

In the experiments reported here, pH_{ext} was being changed at about 0.05 unit min^{-1} (Fig. 2), and homeostasis was close to perfect under those conditions. We have found, however, that if pH_{ext} is changed abruptly, there is an appreciable change in pH_{int} initially before recovery of homeostasis (unpublished observations). Such short-term perturbations are likely to be involved in pH taxis (13, 14).

Homeostasis of pH_{int} in alkaline environments places a heavy energy demand on the cell. At pH_{ext} 9, there is an outwardly directed proton chemical potential, equivalent to about 70 mV; this has to be subtracted from the membrane potential, to give the protonmotive force that is available for performing work such as ATP synthesis, transport, and motility. Recent studies suggest, however, that $\Delta\psi$ compensates appreciably for changes in ΔpH, at a rate of about 0.4 mV/mV (a ΔpH of 1 being energetically equivalent at 25°C to a potential of −59 mV) between pH_{ext} 6 and 8 (7, 19, 20). A combination of this result

$$\Delta\psi = \Delta\psi_{crossover} + 0.4 \times 59\Delta pH$$

with our finding regarding pH homeostasis

$$\Delta pH = 0.9(pH_{crossover} - pH_{ext})$$

suggests the following relationship for protonmotive force (Δp) in respiring *E. coli*

$$\Delta p = \Delta\psi - 59\Delta pH$$

$$= \Delta\psi_{crossover} - 0.54 \times 59(pH_{crossover} - pH_{ext})$$

which with $pH_{crossover} = 7.6$ and $\Delta\psi = -140$ mV at pH_{ext} 7.6 (20) yields

$$\Delta p = -140 + 32(pH_{ext} - 7.6).$$

If compensation continues at this rate up to pH_{ext} 9, the protonmotive force will have been reduced to less than −100 mV.

The requirement of vigorous respiration for pH regulation and maintenance of protonmotive force is likely to be especially critical at high pH_{ext}. Also, processes that are contingent on the magnitude of the protonmotive force are likely to progressively fail as pH_{ext} becomes more alkaline. It is interesting in this regard that motility (which is driven by protonmotive force) is observed to decrease dramatically above pH_{ext} 8.5 (21), and that in the present study nucleotide triphosphate pools were found to be depleted at alkaline pH_{ext} (Fig. 1).

The primary pumps of the bacterial cell are proton pumps driven either by electron transport (NADH oxidation) or ATP hydrolysis. Because of the limited electrical capacitance of the cell membrane, the development of an appreciable inwardly

directed (i.e., alkaline inside) ΔpH demands that some other positively charged species be accumulated in the cell (or, equivalently, that a negative charge be extruded); it is generally assumed that K^+ fulfills this role. The development of an appreciable outwardly directed ΔpH, in contrast, demands that more protons enter the cell than were pumped out and that some other positively charged species be extruded to compensate; it has variously been suggested that Na^+/H^+ (2, 22, 23) or K^+/H^+ (11, 24) antiporters are responsible. However, we found that pH homeostasis appeared to be normal in cells grown and assayed in the absence of Na^+, and also in cells grown in the presence of K^+, but assayed in K^+-free buffer. Further, an alkaline-sensitive mutant KHA1, which is defective with respect to K^+/H^+ antiport (12) regulated pH_{int} as well as wild-type even at alkaline pH_{ext} (unpublished observations). The mechanism of pH homeostasis therefore remains unclear.

Finally, we may note that Me*P* should be a useful NMR probe in other studies in which measurement of alkaline pH_{ext} is desired. If it is taken up into the cytoplasm, as was the case with glycerol-grown *E. coli*, it can also be used for measurement of pH_{int}. Its pK_a of 7.6 makes it an ideal probe in this regard.

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