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The relationship between intracellular pH, the pH gradient and potassium transport in *Escherichia coli*

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The capacity of *E. coli* cells to regulate intracellular pH (pH_i) during net potassium uptake has been investigated. The data show: (*a*) that cells sense their intracellular pH; (*b*) that the pH gradient (ΔpH) exerts a feedback regulation on pH_i; (*c*) that a mechanism of regulation of pH_i exists which may be independent of Na^+ [Zilberstein, Agmon, Schuldiner & Padan (1982) *J. Biol. Chem.* **257**, 3687–3691]; and (*d*) that cells have a limited capacity to raise their intracellular pH in the absence of net K^+ transport.

It is now established that a wide variety of bacteria maintain a relatively constant intracellular pH (pH_i) despite fluctuations in external pH (pH_o; for a review, see Padan *et al.*, 1981). To account for this phenomenon, two different activities are required, the first to raise pH_i when pH_o is acidic and the second to lower pH_i when pH_o is alkaline. One candidate for the former is that of active K^+ transport. It has been established in many organisms that the cytoplasmic pH is raised by the extrusion of protons during the electrogenic influx of K^+ (Bakker & Mangerich, 1981; Erechinska *et al.*, 1981; Kroll & Booth, 1981; Harold & Papineau, 1972; Tokuda *et al.*, 1981). Such a mechanism could account for the recovery of the cytoplasmic pH observed in *Escherichia coli* cells subjected to perturbation of pH_i by the rapid lowering of the external pH (Slonczewski *et al.*, 1982; R. G. Kroll & I. R. Booth, unpublished work). The second type of activity is the cation antiports (Fig. 1; Padan *et al.*, 1976; Krulwich *et al.*, 1979; Brey *et al.*, 1980), the activity of which could cause pH_i to be lowered below pH_o. Such activity could explain the maintenance of a constant intracellular pH at alkaline values of pH_o. This mechanism has been proposed to account for the observed acidification of the cytoplasm after perturbation of pH_i by rapid increases in pH_o (Zilberstein *et al.*, 1982). These two mechanisms of changing pH_i may be complementary aspects of pH_i regulation.

Abbreviations used: pH_o, external pH; pH_i, internal pH; ATPase, adenosine-5′-triphosphatase; ΔpH, pH gradient; $\Delta\Psi$, membrane potential; DMO, dimethyloxazolidinedione.

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The role of cation transport in pH_i regulation is clearly an important one. However, at present no detailed mechanisms exist for the transport systems and, as a consequence, the models of pH regulation lack definition. Investigators have to date preferred Na^+ and K^+ as regulatory cations. However, the proposal of either cation meets with difficulties, which have been discussed elsewhere (Booth & Kroll, 1983). Additional problems also exist for models of pH_i regulation. In particular there is a need to establish the load against which the regulatory mechanism must act. Thus it is important to know whether the prevailing tendency is for pH_i to fall or to rise and whether this is due to net metabolic H^+ consumption or generation, or to net transmembrane fluxes of H^+ as a result of leaks, transport or energy transduction (Raven & Smith, 1976; Sanders & Slayman, 1982; Kobayashi *et al.*, 1982; Booth & Kroll, 1983).

In order to study pH_i regulation it is necesary to find a way to perturb the cytoplasmic pH. Two successful methods have been developed. The first is the pH-shift experiment described above in which cells are subjected to a rapid change of pH_o in either the acid or the alkaline direction (Slonczewski *et al.*, 1982; Zilberstein *et al.*, 1982). The resulting perturbation of intracellular pH and the recovery to the normal value can then be studied. The second method relies upon the exchange of protons for K^+ during uptake of K^+ into K^+-depleted cells (Kroll & Booth, 1981). We have suggested that at pH_o 6.3 there was a relatively simple relationship between the extent of net K^+ uptake and the generation of a transmembrane pH gradient (ΔpH; Kroll & Booth, 1981). However, other groups observed that increasing the value of

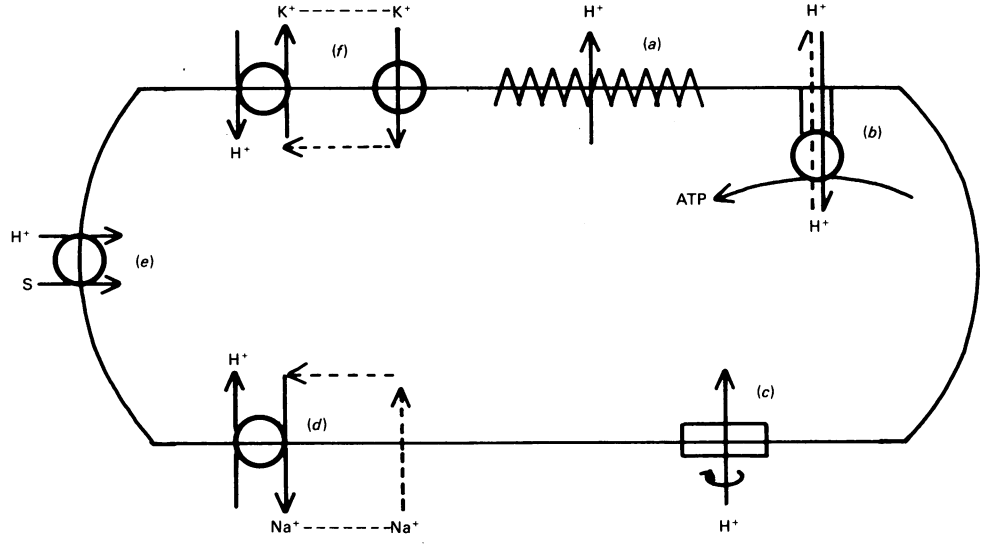

Fig. 1. *Ionic circuits in aerobic E. coli*
Respiration (*a*) drives the extrusion of protons from the cell, which may re-enter via a number of routes; (*b*) the adenosine-5′-triphosphatase; (*c*) the flagellar rotor; (*d*) the Na^+/H^+ antiport; (*e*) solute symport; and (*f*) K^+/H^+ antiport. In (*d*) and (*f*) the presence of a Na^+ leak and active uptake systems for K^+ respectively, together with their respective antiports, constitute the Na^+ and K^+ cycles.

pH_o led to the generation of progressively smaller pH gradients as a consequence of K^+ uptake (Bakker & Mangerich, 1981; Tokuda *et al.*, 1981). This apparent contradiction could be resolved if the final pH gradient were the result of different systems each acting either to raise or to lower pH_i. Thus, at slightly alkaline external pH (pH_o 7.1–7.6), K^+ uptake might provoke the increase of pH_i to an unacceptably high value with subsequent reduction to an optimum value. The present study was undertaken to ascertain if this was the case.

We have investigated pH regulation in *E. coli* by using K^+ transport to provoke large changes in pH_i. The data suggest: (*a*) that cells sense their intracellular pH; (*b*) that ΔpH exerts a feedback regulation on pH_i; (*c*) that the mechanism of this regulation does not involve Na^+ ions; and (*d*) that K^+ transport may be the natural mechanism to raise pH_i.

Materials and methods

All chemicals were purchased from BDH (AnalaR grade), except thiamin, Mes (4-morpholine-ethanesulphonic acid), Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid), Bistris propane {1,3-bis[tris(hydroxymethyl)methylamino]propane} and chloramphenicol, which were from Sigma. Radiochemicals were purchased from Amersham International, Amersham, Bucks., U.K., except for [7-^{14}C]benzoic acid, which was from New England Nuclear Corp.

Bacterial strains

E. coli K12 Frag 1 (*thi-1, rha-4, lac-Z82, gal-33*) was kindly supplied by Dr. W. Epstein, University of Chicago, Chicago, IL, U.S.A.

All strains were maintained as freeze-dried cell suspensions and on slopes of Yudkin's complex medium (Booth *et al.*, 1979).

Preparation of K^+-depleted cell suspensions

E. coli Frag 1 was grown overnight on glucose minimal medium (Epstein & Kim, 1971) with 100 μM-K^+ present as the limiting nutrient.

In the morning the cells were diluted in fresh growth medium containing only 20 μM-K^+ and incubated until growth had ceased due to K^+ limitation, as previously described (Kroll & Booth, 1981). Cell suspensions were harvested and washed at 4°C by centrifugation at 16000*g* for 5 min. The cells were washed three times in cold alkali-metal-free buffer (10 mM-Mes, 10 mM-Hepes or 10 mM-Bistris propane, 150 mM-choline chloride and 50 μg of chloramphenicol/ml) previously adjusted to either pH 5.3 or 7.1 with choline hydroxide or HCl. The cell suspensions were stored on ice for up to 4 h.

Measurement of internal pH and of membrane potential

The pH gradient and membrane potential across the membrane were measured as previously described (Kroll & Booth, 1981). The external pH was

monitored continuously and was maintained constant by addition of small aliquots of choline hydroxide. From a knowledge of ΔpH and pH_o, pH_i was calculated.

Measurement of K^+ transport

K^+ uptake was determined by measurement of extracellular ion concentration in cell-free filtrates by using a flame photometer (Booth *et al.*, 1979). In some experiments, radioactive $^{42}K^+$ was used to measure net K^+ uptake by collecting cell samples on Millipore filters. The filters were immediately washed with two aliquots (2ml) of K^+-free buffer before immersion in Triton X-100-based scintillant {Triton X-100, 333ml; toluene, 667ml; PPO (2,5-diphenyloxazole), 4g; POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene], 0.1g} and counted in a Packard Tri-Carb 300C scintillation counter.

Respiration rates

The rate of respiration was monitored as previously described (Kroll & Booth, 1981).

Measurement of glucose consumption rate

K^+-depleted cells were incubated with 300μM-glucose at pH_o 7.1 in the incubation system described above. At intervals, two 1ml aliquots were transferred to Eppendorf centrifuge tubes and spun for 20s at 12000*g* in a Mechanika Precyzyjna microcentrifuge type 320a (Burkard Scientific Sales Ltd., Uxbridge, Middx., U.K.). A portion (200μl) of the supernatant was removed and incubated with 1ml of Sigma glucose oxidase reagent for 45min at room temperature. The A_{450} of the samples and of known standards was measured and the rate of glucose consumption of the cells calculated.

Sigma glucose oxidase reagent consists of one PGO enzyme capsule (Sigma) dissolved in 100ml of distilled water and *o*-dianisidine dihydrochloride added to a final concentration of 40μg/ml.

Measurement of acid production

Acid production was measured with a pH-microelectrode (Probion, model 103/E7) and a Beckman pH-meter (model 101901) coupled to a Rikadenki multichannel pen recorder (model B341) as previously described (Collins & Hamilton, 1976). The buffering capacity of the medium was reduced 5-fold to allow accurate quantification of the acid production and the overall pH drift was restricted to 0.2 pH unit.

Results

Investigation of the relationship between K^+ uptake and ΔpH generation required manipulation of the intracellular pH of the cells. This was achieved by washing K^+-depleted cells with pH 5.3 buffer and subsequent incubation at the desired pH. Prepared thus, cells washed at pH 5.3 exhibited an intracellular pH of 6.4. Subsequent incubation with glucose in buffer of any desired pH between 6.4 and 7.6 led to the development of a small pH gradient (Fig. 2). Alternatively, cell suspensions which were washed with pH 7.1 buffer instead of pH 5.3 maintained pH_i close to that value,

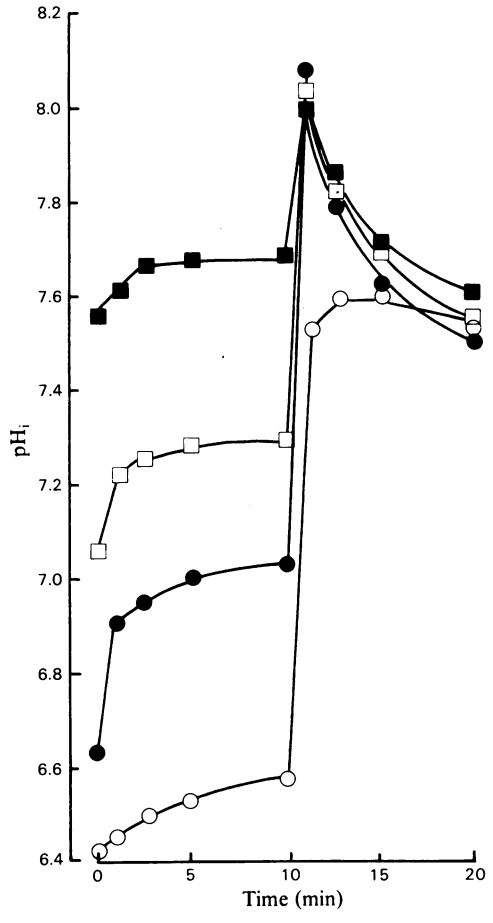

Fig. 2. *Time course of pH_i change during K^+ uptake*
Potassium-depleted cells of *E. coli* Frag 1 were washed three times in a buffer system comprising 10mM-Mes, 10mM-Bistris propane, and 150mM-choline chloride, pH 5.3. The cell suspensions were incubated at 2mg/ml at pH_o 5.3 (○) 6.8 (●), 7.15 (□) or 7.6 (■). The intracellular pH was determined by the transmembrane distribution of a weak acid (benzoate, 2μM; dimethyloxazolidine, 3μM) using a centrifugation-based assay. After the first time-point, glucose (1mM) was added and the incubation continued. After 10min, KCl (750μM) was added and pH_i monitored for a further 10min.

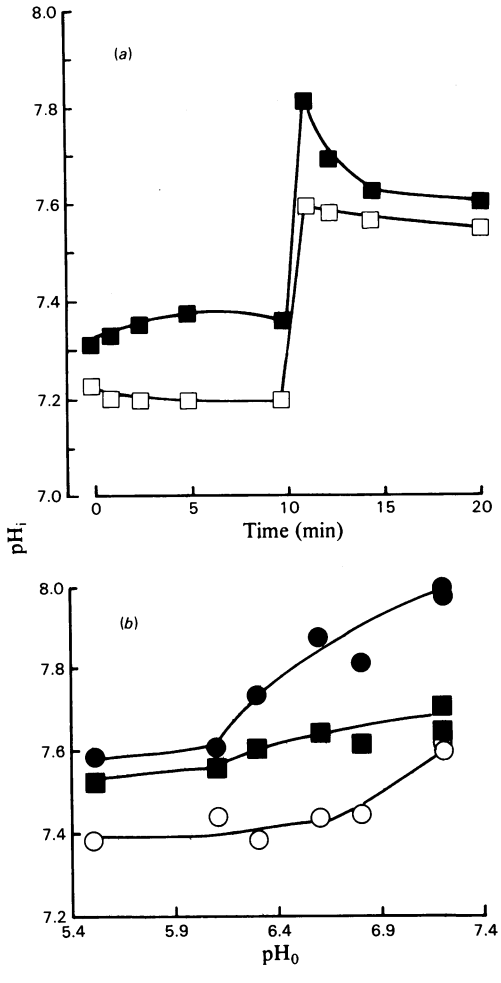

Fig. 3. *Effect of pH_o on overshoot of pH_i*
Potassium-depleted cells of *E. coli* Frag 1 were washed at pH 7.1 with the buffer system used at pH 5.3. (*a*) The cells were incubated at pH_o 5.3 (□) and pH_o 7.1 (■) and pH_i was determined in the presence of glucose as in Fig. 2. (*b*) The cells were incubated as in (*a*), but at a range of pH_o values; the initial (○), peak (●) and final (■) values of pH_i are taken from time-course plots similar to those in (*a*).

even when incubated at pH 5.3 (Fig. 3). Addition of glucose to such cell suspensions did not markedly affect the value of the cytoplasmic pH. These two washing procedures allowed the manipulation of pH_i before the onset of K^+ uptake.

Effect of pH_o and pH_i on K^+ uptake

The extent of K^+ uptake was not affected by the initial values of either pH_o or pH_i. Thus K^+-depleted cells incubated with glucose and 750 μM-KCl took up 190 ± 11 ng-ion of K^+/mg dry wt. of cells, independent of both pH_o and pH_i. Net potass-

Table 1. *Potassium uptake by K^+-depleted cells of E. coli Frag 1*
Potassium-depleted cell suspensions were prepared and incubated as in Fig. 2. K^+ uptake was measured as described in the Materials and methods section using $^{42}K^+$ as a tracer (specific radioactivity 10–60 mCi/μmol). Net K^+ uptake was measured by flame photometry as previously described (Kroll & Booth, 1981) at an external concentration of 750 μM.

pH_o	Initial rate of K^+ uptake (nmol of K^+/min per mg of cells)	Total K^+ uptake (nmol of K^+/mg of cells)
5.3	68 ± 19	195 ± 7
5.8	60 ± 9	191 ± 4
6.3	79 ± 8	191 ± 11
6.8	81 ± 12	192 ± 2
7.3	81 ± 9	194 ± 4
8.0	72 ± 18	185 ± 10

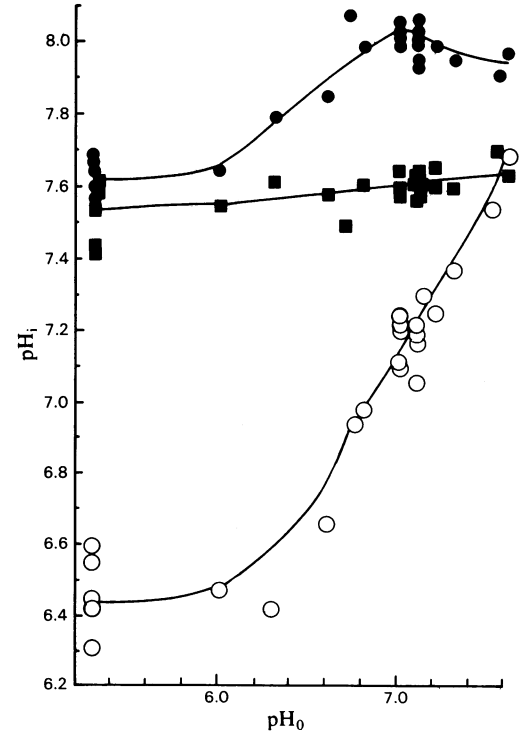

Fig. 4. *Influence of pH_o on the initial, peak and final values of pH_i of K^+-depleted E. coli incubated with K^+*
Potassium-depleted cells of *E. coli* Frag 1 were washed at pH 5.3 and incubated at a range of pH_o values in the presence of 1 mM-glucose. The initial (○), peak (●) and final (■) values of pH_i were determined from plots similar to those in Fig. 2, during K^+ uptake.

ium uptake was biphasic, with an initial rapid phase (20s) and a subsequent slower uptake lasting 4–5min (results not shown). This pattern was not affected by pH_o, although the initial rate was 20% slower at pH_o5.3 than at pH_o7.1 (Table 1).

ΔpH generation during K^+ uptake

The value of pH_o has a profound effect on the magnitude of the pH gradient generated during K^+ uptake. At pH_o5.3, K^+ uptake resulted in an increase in pH_i from 6.5 to 7.6 within 45s (Fig. 2). Thereafter the internal pH declined slightly to pH_i7.55 and was then constant. As the value of pH_o was increased, the pattern of ΔpH generation changed (Fig. 4). Above pH_o6.3 there was a rapid alkalinization of the cytoplasm, followed by acidification to pH_i7.6 (Figs. 2 and 4). It was noteworthy that, although the initial and peak values of pH_i were all different, there was a consistent end point of pH_i7.6 ± 0.05 (Fig. 4). These experiments suggest that the cells can sense and regulate pH_i. Thus, during K^+ uptake, pH_i overshoots the regulated value and then undergoes a controlled reduction to a value close to pH_i7.6.

The previous experiments suggested that overshoot occurred whenever the initial value of pH_i was close to the regulated value (pH_i7.6). However, pH_o was used to vary pH_i, and thus two variables (pH_i and pH_o) were present in each experiment. K^+-depleted cells washed at pH7.1, which retain high internal pH even when incubated at acid external pH were used to separate the effects of pH_o and pH_i. In such cells incubated at pH_o5.3 the onset of K^+ uptake did not provoke overshoot of pH_i (Fig. 3*a*). Rather the intracellular pH rose from pH_i7.1 to pH_i7.6 and was thereafter constant. Similar cell suspensions incubated at pH_o7.1 did exhibit overshoot of pH_i during K^+ uptake (Fig. 3*a*). As pH_o was varied between these extremes, extensive overshoot occurred only above pH_o6.4 (Fig. 3*b*). This is similar to the observations made on cells in which pH_i was a pH_o-dependent variable (Fig. 4). However, when the initial value of pH_i was relatively constant the occurrence of overshoot was dependent on the value of pH_o. This suggests that the mechanism by which excessive alkalinization of the cytoplasm is prevented is activated either by pH_o directly or indirectly through the magnitude of ΔpH, since this increases as pH_o decreases.

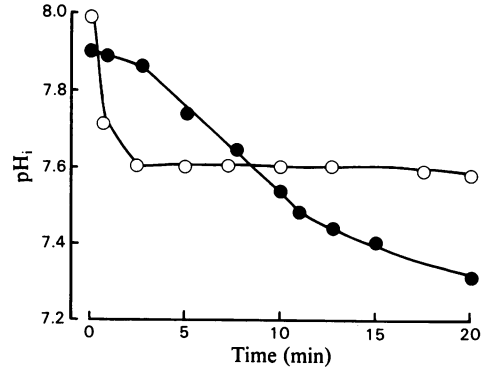

Fig. 6. *Decline of intracellular pH in the presence and absence of glucose*
Potassium-depleted cells were washed at pH_o7.5 in the buffer system described in Fig. 1. The cells were subsequently incubated at pH_o7.1 in the presence (○) or absence (●) of glucose (1 mM) and pH_i determined as in Fig. 1.

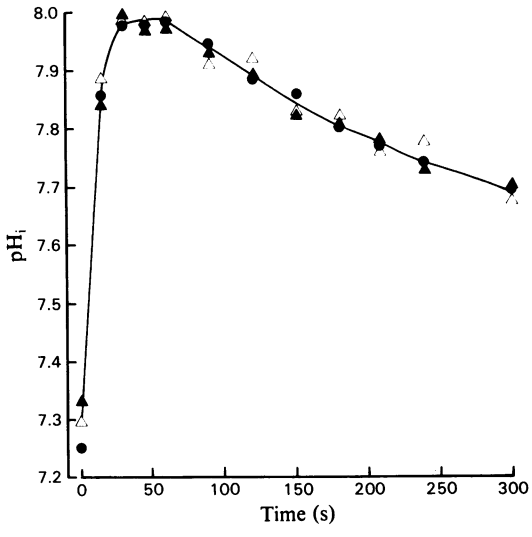

Fig. 5. *Effect of Na^+ on the kinetics of pH_i decline*
Cells were prepared as described in Fig. 1 and incubated with 1mM-glucose at pH_o7.1. At $t = 0$, 750μM-KCl was added and pH_i monitored as previously described (Fig. 1). At 1min before addition of K^+, NaCl was added as follows: (●) no Na^+ addition; (△), 1mM-NaCl; (▲), 10mM-NaCl. In the absence of added Na^+, the contaminating level in the buffer was found to be 38μM.

Mechanism of overshoot

Current models of pH regulation in prokaryotes and eukaryotes would predict that the fall in pH_i during overshoot was due to one of the following: (*a*) Na^+/H^+ or K^+/H^+ exchange (Padan *et al.*, 1981); (*b*) Cl^-/HCO_3^- exchange (Roos & Boron, 1982); or (*c*) production of acid in the cytoplasm (Sanders & Slayman, 1982; Kobayashi *et al.*, 1982). These possibilities were examined in our experimental system.

Overshoot exhibited no Na^+-dependence in the range 38μM–10mM. Identical rates of decay of pH_i

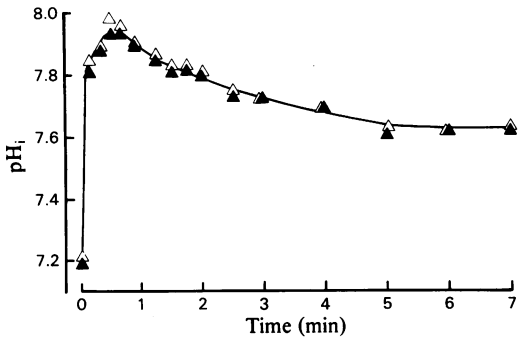

Fig. 7. *Rate of decline of pH_i in cells metabolizing glucose or glycerol*

Cells were grown to K^+ depletion on minimal medium containing either glucose or glycerol as sole carbon and energy source. Cells were subsequently treated as indicated in Fig. 1, with incubation at pH_o 7.1 in the presence of the relevant carbon source: glycerol (△); glucose (▲). At $t = 0$, K^+ (750 μM) was added and pH_i and pH_o were recorded as described in Fig. 1.

were observed over this concentration range (Fig. 5). The possible involvement of Cl^- was investigated by replacing choline chloride with sorbitol as osmoticum and adding 0.5 mM-K_2SO_4 to initiate K^+ uptake in the presence or absence of 10 mM-choline chloride. The absence of chloride had no effect on either the extent of overshoot (peak values of pH_i 8.0 and 8.05 in the presence and absence of Cl^- respectively) or the rate of decay of pH_i ($t_{\frac{1}{2}}$ approx. 90 s in both the presence and absence of Cl^-).

The decline of pH_i showed no significant dependence on external K^+. K^+-depleted cells washed at pH 7.5 exhibited an internal pH close to pH_i 8. Incubation of these cell cells at pH_o 7.1 in the absence of K^+ led to an uncontrolled decline of pH_i (Fig. 6). The addition of glucose accelerated the decline of pH_i, but the fall was controlled and the intracellular pH stabilized at pH_i 7.6 (Fig. 6).

The acidification of the cytoplasm could arise from glucose-derived acidic metabolites. One may calculate that the proportion of glucose which is not oxidized to CO_2 is significant and may be kinetically competent to explain some of the fall in pH_i. The consumption rate of glucose was 13.2 ± 2.3 nmol/min per mg of cells. Complete oxidation of the glucose according to the equation:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \qquad (1)$$

would result in a respiratory rate of 79.2 nmol of O_2/min per mg of cells. Addition of K^+ to glucose-respiring cells at pH_o 7.1 resulted in a respiratory rate of 35.9 ± 5.4 nmol of O_2 consumed/min per mg. Thus only 45% of the glucose consumed can be accounted for by respiration. However, the true stoichiometry for glucose metabolism in *E. coli* has been reported to be:

$$C_6H_{12}O_6 + 4O_2 \rightarrow 4CO_2 + 4H_2O + CH_3COOH \qquad (2)$$

(Stouthamer & Bettenhausen, 1977). In this case the expected respiratory rate is 52.8 nmol/min per mg of cells and the observed rate accounts for 68% of the glucose. Clearly there is a discrepancy in the rates, which indicates that not all the glucose is oxidized. This is supported by measurements of the rate of acid production. K^+-depleted cells produce acid at 43 ± 5 ng-ion of H^+/min per mg of cells. As H_2CO_3 (eqn. 1), this acid would be equivalent to 54%, and as H_2CO_3 plus acetate (eqn. 2) 65%, of the sugar utilized. On the basis of known values of the cytoplasmic buffering power (40–50 mmol of H^+/pH unit per litre of cell water; Collins & Hamilton, 1976; Sanders & Slayman, 1982; Slonczewski *et al.*, 1982), acidic metabolites derived from glucose could contribute to the acidification of the cytoplasm. However, this mechanism is not competent to account for the failure of cells washed at pH 7.1 to exhibit overshoot.

Glycerol-metabolizing cells produce acid more slowly than those with glucose (17.9 ± 3.9 ng-ion of H^+/min per mg of cells), which indicates a lower metabolic rate. These cells exhibit an overshoot identical with that shown by cells incubated with glucose (Fig. 7). This result further suggests that acidification of the cytoplasm is not closely coupled to the metabolism of the energy source.

Discussion

In the present study we have attempted to study pH_i regulation in *E. coli* through direct perturbation of the intracellular pH as a consequence of K^+ uptake. K^+-depletion of cells allowed us to set both pH_i and pH_o and to impose major perturbations of intracellular pH. This arises due to the effective exchange of intracellular H^+ for external K^+ (Kroll & Booth, 1981). Under these experimental conditions, two K^+-transport systems are operative, namely **Kdp** and **TrkA**; however, all the effects observed are independent of the mechanism of K^+ transport. Identical results were obtained with the wild-type and two mutants each lacking either the **Kdp** or the **TrkA** systems (strains Frag 5 and TK2240 respectively; Rhoads *et al.*, 1976). Thus the overshoot phenomenon observed here is not a property of the K^+-uptake system, but reflects the pH_i-regulation mechanism.

The capacity to regulate pH_i invokes the ability to sense and to effect controlled changes in intracellular pH. These abilities are demonstrated in *E.*

coli in the present paper. Whenever the intracellular pH rose above pH_i 7.6 it subsequently reverted to close to this value. One can predict the peak value of pH_i from a knowledge of the cytoplasmic buffering power (Collins & Hamilton, 1976) and the net K^+/H^+ exchange (Kroll & Booth, 1981). Cells incubated at pH_o 7.6 (initial pH_i 7.7) with K^+ should exhibit a peak value of the intracellular pH greater than pH_i 8.7. The maximum value observed in our studies was pH_i 8.1, which rapidly declined to pH_i 7.6 (Fig. 4). This suggests that the intracellular pH is sensed and activates a mechanism allowing net entry of protons, causing pH_i to fall.

Intracellular pH does not seem to be the sole determinant of the occurrence of overshoot. Cells with a pre-established pH gradient did not exhibit overshoot during K^+ uptake (Figs. 3*a* and 3*b*). An explanation for this effect could be feedback activation of the proton entry route by ΔpH. This is supported by the data presented here. Firstly, cells with an initial pH_i 7.1–7.2 do exhibit overshoot if ΔpH is small (pH_o 7.1; Fig. 3*a*), but not if ΔpH is large (pH_o 5.3–6.4; Fig. 3*b*). Secondly, the rate of decline of pH_i was dependent on the peak value of ΔpH, increasing as ΔpH increased (Fig. 2). At higher values of pH_o, when the peak value of ΔpH is small (Figs. 2 and 4), the collapse of pH_i is also rapid, and consequently the proton entry pathway cannot be simply dependent on ΔpH. Rather one envisages either a net electrogenic H^+ entry system or an ATP-dependent mechanism that is controlled by both pH_i and ΔpH.

In animal cells pH_i regulation is effected through $Na^+/H^+/HCO_3^-/Cl^-$ fluxes, which may be more or less tightly coupled, depending on the organism (Roos & Boron, 1982). In *E. coli* the Na^+/H^+ antiport has been reported to effect the controlled collapse of pH_i in growing cells subjected to a rapid alkalinization of the growth medium (Zilberstein *et al.*, 1982). Genetic evidence was presented in support of this hypothesis. Such a system would be an ideal candidate for collapsing pH_i during overshoot by allowing internal Na^+ to exchange for external protons. The evidence does not favour this explanation of our data. Increasing the external $[Na^+]$ from 38 μM to 10 mM did not stimulate the rate of collapse of pH_i. It has been suggested that only low concentrations of Na^+ are necessary to effect pH regulation (Krulwich *et al.*, 1982; Padan *et al.*, 1981). At 38 μM external Na^+ the combined activity of an electrogenic antiport ($2H^+/Na^+$; Schuldiner & Fishkes, 1978) and an inward Na^+ leak (Booth & Kroll, 1983) would set the intracellular Na^+ concentration in the range 0.8–38 μM, depending on the relative activities of the two systems. The total Na^+ content of the cells would be in the range 1–48 pmol/mg of cells on the basis of a cytoplasmic volume of 1.2 μl/mg of cells (Kroll & Booth, 1981). A fall of pH_i of 0.1 unit/min would require a net H^+ flux of 7 nmol/min per mg of cells, and consequently the internal Na^+ itself would not be sufficient to cause this. Increasing the external Na^+ should increase that inside the cell and thus make more Na^+ available for exchange. However, this was found not to occur.

As we have previously suggested, the capacity of Na^+ to act in pH_i regulation depends not only on the properties of the antiport but also on those of the leak pathway by which Na^+ enters the cell. To effect pH_i collapse in our system the available Na^+ would need to cycle through the cell at a rate approximately equivalent to the H^+ influx required to effect a fall of 0.1 pH unit/min. This is necessary to counteract the K^+ uptake, which continues during overshoot and which predisposes pH_i to rise. At 38 μM-Na^+ the cytoplasmic pool would need to turn over between 150 and 7000 times/min. The latter value is more reasonable, since antiport activity would be expected to outstrip that of the leak, thus reducing the intracellular Na^+ pool and maintaining the Na^+ gradient. The leak pathway must then possess a high affinity for Na^+, a high velocity, and exhibit appropriate control to explain overshoot satisfactorily.

In those experiments in which the increase in pH_i is controlled during K^+ uptake (Figs. 3*a* and 3*b*, pH_o 5.3–6.4), the problem faced by an explanation based on Na^+ is even greater. Here the rate of Na^+ cycling must considerably exceed that in the situation where overshoot occurs, since the predisposition of pH_i to rise is much greater.

We found no evidence of a role for Cl^- in either the development or the collapse of ΔpH. In animal cells HCO_3^- is usually coupled to the flux of Cl^- (Roos & Boron, 1982). We have not rigorously examined HCO_3^- as a possible effector of pH_i control; it clearly has a potential role, since it is a universal product of aerobic metabolism. Raven (1972) has reported that most decarboxylases of the cell have unhydrated CO_2 as their product. To play a role in pH regulation, the CO_2 would need to dissolve in cytoplasmic water; subsequent pumping of HCO_3^- out of the cell would leave one proton behind, causing pH_i to fall. Although there is no strong evidence, it seems likely that CO_2 exit occurs as the gas rather than as either carbonic acid or HCO_3^- (J. A. Raven, personal communication). Our observed acidification of the medium in the presence of glucose is consistent with this suggestion.

Finally, K^+/H^+ antiport has been suggested to effect pH_i homoeostasis (Plack & Rosen, 1980; Kroll & Booth, 1981). Intracellular K^+ exchanging for external H^+ would cause a reduction in pH_i. The attraction of this model lies in the ubiquitous

occurrence of high cytoplasmic K^+ concentrations in bacterial cells, and the disadvantage in the central role this cation plays in osmoregulation (Epstein & Laimins, 1980). Thus if K^+ were to be excreted, it would then be taken up again to maintain osmotic balance. In the present context, since K^+ uptake was used to provoke the increase in pH_i, there seems to be little to be gained from such a model.

In conclusion, these experiments show that cells of *E. coli* truly regulate their cytoplasmic pH. The mechanism by which this is achieved is not known, but the evidence presented sets the character of the system. Thus pH_i regulation involves the sensing of both pH_i and pH_o and the controlled acidification of the cytoplasm. We favour an ionic mechanism of acidification rather than the production of metabolic acids, as it is the one which fits most readily with the sensitivity of overshoot to pH_o. The evidence also suggests that the Na^+/H^+ antiport is unlikely to be involved, although only the isolation of mutants with the antiport gene deleted will confirm this. Finally, it is noteworthy that regulation at pH_i 7.6 requires the elevation of the intracellular pH to above this value. Thus, in the presence of glucose, a series of steady-state values of pH_i were achieved which were below pH_i 7.6 (Figs. 1, 2 and 3). These were maintained despite high rates of respiration and hence proton cycling. This implies that alkalinization of the cytoplasm is not readily achieved by the cells unless K^+ transport is occurring. At first sight this contradicts the observations of other groups, where alkalinization of the growth medium provoked pH_i to increase (Zilberstein *et al.*, 1982). However, this may be explained by the instantaneous reduction of the protonmotive force in response to the pH shift (ΔpH changes from -36mV to $+36$mV), causing a reduction of H^+ influx through the ATPase. Consequently pH_i would rise due to a change in the balance of H^+ extrusion and re-entry. Under less extreme conditions it seems likely that net K^+ uptake may play a direct role in pH_i regulation acting to raise the cytoplasmic pH (Booth & Kroll, 1983).

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