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Escherichia coli Intracellular pH, Membrane Potential, and Cell Growth

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We studied the changes in various cell functions during the shift to alkaline extracellular pH in wild-type *Escherichia coli* and in strain DZ3, a mutant defective in pH homeostasis. A rapid increase in membrane potential ($\Delta\psi$) was detected in both the wild type and the mutant immediately upon the shift, when both cell types failed to control intracellular pH. Upon reestablishment of intracellular pH − extracellular pH and growth in the wild type, $\Delta\psi$ decreased to a new steady-state value. The electrochemical proton gradient ($\Delta\mu_{H+}$) was similar in magnitude to that observed before the pH shift. In the mutant DZ3, $\Delta\psi$ remained elevated, and even though $\Delta\mu_{H+}$ was higher than in the wild type, growth was impaired. Cessation of growth in the mutant is not a result of cell death. Hence, the mutant affords an interesting system to explore the intracellular-pH-sensitive steps that arrest growth without affecting viability. In addition to $\Delta\mu_{H+}$, we measured respiration rates, protein synthesis, cell viability, induction of β-galactosidase, DNA synthesis, and cell elongation upon failure of pH homeostasis. Cell division was the only function arrested after the shift in extracellular pH. The cells formed long chains with no increase in colony-forming capacity.

The primary proton pumps linked to either electron transport or ATP hydrolysis have a dual physiological role in bacterial cells. They maintain a proton gradient across the cytoplasmic membrane which serves as an energy transmitter for processes such as ATP synthesis, transport of solutes, and locomotion (6, 14). In addition, the procaryotic primary proton pumps participate in the control of the cytoplasmic pH (16, 17). In *Escherichia coli* and other bacteria which grow optimally at neutral pH, the intracellular pH (pH_i) − extracellular pH (pH_o) (ΔpH) maintained by the proton pumps changes over the range of growth-supporting (pH_o) so that the pH_i remains constant at pH 7.6 to 7.8 (16, 17). A ΔpH of 0 is observed at pH_o 7.8, but above or below this pH_o, the ΔpH becomes either more acidic or more basic inside. In both alkalophiles and acidophiles, pH homeostasis is also dependent on the primary proton pumps and pH is maintained at 9.5 or 6.5, respectively (17; T. A. Krulwich and A. A. Guffanti, Adv. Microb. Physiol., in press).

A recently developed, rapid method of pH_i determination has made it possible to follow the time course and sequence of changes in pH occurring upon transfer of logarithmically growing *E. coli* cells to media of different external pH values (22). After a shift in pH_o of wild-type bacteria from pH_o 7.2 to 8.3, 8.6, 8.8, or 6.4, the ΔpH across the membrane collapsed to zero within 30 s. Subsequently, ΔpH was built up and pH homeostasis was reestablished over 3 to 6 min.

Analysis of the properties of mutants defective in the Na^+/H^+ antiporter in *E. coli* (22, 23) and alkalophilic bacteria (11; Krulwich and Guffanti, in press) has led to the conclusion that the collaborative functioning of the antiporter and the primary proton pumps allows pH homeostasis at alkaline pH. The *E. coli* mutant DZ3 behaves like the wild type after transfer from pH 7.2 to 6.7 but cannot control the pH_i and is unable to grow above pH 8.3. In contrast, in the wild type, both pH homeostasis and optimal growth are maintained up to pH_o 8.8 (22, 23).

The membrane potential ($\Delta\psi$) and the ΔpH make up the proton electrochemical gradient ($\Delta\mu_{H+}$). Change in one of the components is often accompanied by a compensatory change in the other, so that the $\Delta\mu_{H+}$ supply remains constant. It was therefore interesting to study the $\Delta\psi$ during the drastic changes in ΔpH that occur when the pH_o is increased.

With both the wild type and the mutant, failure of the cells to readjust the pH_i to 7.8 stopped growth, and the recovery of the pH homeostasis always preceded resumption of growth. These findings raised the possibility of a tight coupling between the two processes (23). This coupling may be explained by a reaction(s) needed for growth that is very sensitive to the pH_i. Indeed, mutant DZ3 stopped growing and controlling its pH_i above pH_o 8.3 but did not lose viability for at least 12 h at the nonpermissive pH_o, up to pH_o 9. Both the mutant and the wild-type cells lost viability at pHs above 9. Hence, the mutant affords a system for exploring the pH_i-sensitive step(s) that can stop growth without affecting viability.

We have measured respiration rates, $\Delta\mu_{H+}$, protein synthesis, cell viability, induction of β-galactosidase, DNA synthesis, and cell elongation in relation to failure of pH homeostasis. Cell division was the only function studied that was arrested after the shift in pH_o.

MATERIALS AND METHODS

Bacteria and growth media. The *E. coli* K-12 strains used in this study were CS71 (*gltC metB lacYI*) and DZ3 (*gltC metB lacYI phs*) (23). The cells were grown on minimal medium A (5) lacking citrate, supplemented with L-methionine (50 μg/ml), and containing 0.5% glycerol as the carbon source. Solid media were prepared by the addition of 1.5% agar (Difco). The L broth used for viable counts contained KCl instead of NaCl.

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Growth under controlled pH. Cells were grown in batch culture under controlled pH conditions as previously described (22). KOH was added at a rate of either 2.25 to 3.6 or 13.5 to 21.6 meq/min, and the titrations lasted 3 min and 30 s, respectively.

Determination of $\Delta\psi$ and ΔpH under growth conditions. pH_i and $\Delta\psi$ were estimated from the distribution across the cell membrane of [^{14}C]5,5-dimethyloxazolidine-2,4-dione, [^{14}C]methylamine, or [^{3}H]tetraphenylphosphonium, as previously described (22). Ten milliliters of a cell suspension (0.1 to 0.2 mg of cell protein per ml) was quickly transferred from the growth vessel into a prewarmed (37°C), 100-ml flask containing 9 μM [^{14}C]methylamine (10 Ci/mol) or 0.32 mM [^{14}C]5,5-dimethyloxazolidine-2,4-dione (120 Ci/mol) for pH_i measurements or 9 μM [^{3}H]tetraphenylphosphonium (275 Ci/mol) and 1 mM potassium EDTA (pH 7) for $\Delta\psi$ measurements. The suspension was incubated with continuous shaking at 37°C for 1 min for pH_i measurements and for 2 min for $\Delta\psi$ measurements. At given times the suspension was filtered through a glass fiber filter (GF/C Whatman, 25-mm diameter). The filters were transferred into toluene-Triton scintillation liquid and assayed for radioactivity in a Tricarb scintillation counter.

Determination of respiration rates and synthesis of protein and DNA. Cell suspensions (0.1 to 0.2 mg of cell protein per ml) were rapidly transferred into a prewarmed (37°C) flask, and incubation continued with shaking. The pH_o of these samples remained the same as that of the suspensions in the pH stat for at least 3 min. The oxygen content was measured in 2.0-ml samples with a Yellow Springs Ohio oxygen electrode and a model RE511 recorder (Goerz, Vienna, Austria).

For determination of protein or DNA synthesis, 3 ml of the growing cell suspension was used. [^{14}C]leucine (2.5 Ci/mol) or [^{3}H]thymidine (30 Ci/mmol) was added to reach 40 μM and 33 nM, respectively. After 2 min of incubation, the samples were mixed with 3 ml of 5% trichloroacetic acid, kept for 15 min at 4°C, filtered on GF/C glass fiber filters (Whatman), washed with 5% trichloroacetic acid, and assayed for radioactivity. The rates of incorporation were linear for at least 5 min.

Determination of β-galactosidase. Samples (5 ml) were withdrawn and incubated with isopropyl-1-thio-β-D-galactoside (10^{-3} M) at 37°C. The reaction was stopped by the addition of 1 drop of chloroform and 1 drop of 0.1% sodium dodecyl sulfate to 0.5 ml of the suspension. After vigorous mixing, the permeabilized cells were kept on ice until assay of β-galactosidase activity by the method of Miller (13).

Protein determination. Protein was determined by the method of Lowry et al. (12).

Materials. [^{14}C]methylamine was obtained from The Radiochemical Centre, Amersham, England; [^{14}C]5,5-dimethyl-2,4-oxazolidinedione, [^{14}C]leucine, and [^{3}H]thymidine were obtained from New England Nuclear Corp., Boston, Mass.; and [^{3}H]tetraphenylphosphonium was obtained from the Nuclear Center, Negev, Israel.

RESULTS

Changes in pH_i and $\Delta\psi$ on slow and rapid pH_o transitions in wild-type cells. As previously shown (22), respiring *E. coli* wild-type cells growing logarithmically at pH 7.2 maintain a ΔpH of 0.6 (basic inside) which yields a pH_i of 7.8 (Fig. 1A). Under identical conditions, a $\Delta\psi$ of 86 mV was determined (Fig. 1A), which is lower by 40 mV than the value previously observed in resting cells (24) and about 15 mV lower than those reported by others in growing cells (1, 9). This difference in $\Delta\psi$ cannot be explained by the different techniques used, since the $\Delta\psi$ of resting cells as determined by the present technique is 125 mV (data not shown), which is identical to that previously measured (24). We therefore believe that growing cells have a low $\Delta\psi$ and thus a low $\Delta\psi_{H+}$ as compared with nongrowing cells.

Slow (3-min) alkalinization of the medium to pH 8.3 elicited transient changes in pH_i in wild-type cells (22; Fig. 1A). After the shift in pH_o and the 1 min needed for the measurement, the ΔpH was already reduced to 0. Subsequently, a reversed ΔpH (acid inside) was built up, reaching 0.5 U after 6 to 10 min, which results in a pH_i of 7.8, i.e., the pH homeostatic state. An increase in $\Delta\psi$ of 50 mV was observed after the shift in pH_o and the 2 min needed for measurement (Fig. 1A). The $\Delta\psi$ increased further, reaching the maximal value of 150 mV after 5 min. During the following 5 to 10 min, however, the $\Delta\psi$ decreased by about 15 mV, reaching a steady-state value of about 135 mV. It should be emphasized that this decrease in $\Delta\psi$ occurred while the pH_i became more acidic than the pH_o (Fig. 1A). Thus, as a result of the oscillations of its parameters after pH shift, the $\Delta\mu_{H+}$ first rapidly increased to 145 mV and then decreased more slowly to 105 mV by the time pH homeostasis had been achieved.

A very similar pattern of events occurred when the medium of wild-type cells was shifted from pH_o 7.2 to 8.6 (Fig. 2A). The $\Delta\psi$ and $\Delta\mu_{H+}$ increased when the ΔpH became zero. The $\Delta\psi$ then decreased, and reversion of ΔpH occurred. The optimal pH homeostasis was achived only after about 20 min (22). After this transition, the $\Delta\mu_{H+}$ was 110 mV, as is seen after the smaller shift from 7.2 to 8.3.

After rapid (30-s) alkalinization of the medium from pH 7.2 to 8.3, an initial phase of the ΔpH transition was unraveled (Fig. 3). Because of the shift in pH_o, the ΔpH changed from 0.6 (basic inside) to 0.45 (acid inside), thus still yielding a pH_i of 7.85. This residue of the artificially imposed ΔpH decreased rapidly, however, and disappeared after 4 min. The initial increase in $\Delta\psi$ was too rapid to follow even after fast titration, but clearly the maximum was attained when the ΔpH became 0. After 30 min, the $\Delta\psi$ decreased, the ΔpH was rebuilt, and a constant steady-state pH_i was reestablished at 7.85.

Although similarity in pattern and final steady-state values were obtained for $\Delta\mu_{H+}$, the absolute values of both the $\Delta\psi$ and ΔpH in the rapid titration system were lower by 20 mV and 0.05 U, respectively, as compared with the values obtained in the 3-min titration system (cf. Fig. 1 and 3). Furthermore, after rapid titration and oscillations of the $\Delta\mu_{H+}$ components (Fig. 3), the growth resumed and the doubling time was 2 h (data not shown). This was slower by 30 min than that of the slow-titrated system (22). The difference between the slow and rapid titration systems was more pronounced when the shift from pH 7.2 to 8.6 was carried out (Fig. 4). Although the final $\Delta\mu_{H+}$ was similar to that observed after the smaller shift in pH, the $\Delta\psi$ after the initial increase showed a smaller decline, and the ΔpH attained a value of only 0.55 (acid inside), yielding a new steady-state pH_i of 8.1. The resumed growth rate was drastically reduced to a doubling time of 3 h. Hence, we concluded that the rapid pH shift was deleterious to the cells, but it nevertheless allowed the detection of the initial kinetics of the pH changes.

At alkaline pH, DZ3 exhibits only the initial oscillations of the $\Delta\mu_{H+}$ parameters. The mutant DZ3, which was shown to be impaired in the Na^+/H^+ antiporter activity and in pH

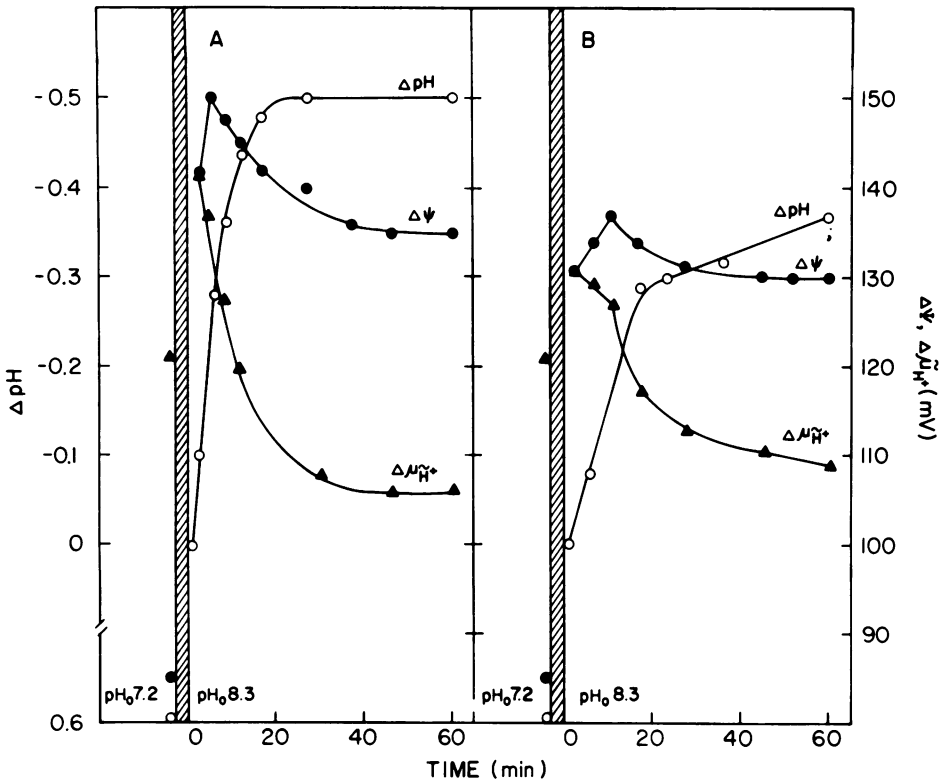

FIG. 1. $\Delta\psi$, ΔpH, and $\Delta\mu_{H^+}$ in growing *E. coli* cells after a slow shift in pH_o from 7.2 to 8.3. *E. coli* CS71 (A) and DZ3 (B) cells were grown in a pH stat at pH 7.2 to mid-logarithmic phase (0.1 mg of cell protein per ml). Samples of 0.5 M KOH were then automatically added at a rate of 2.4 meq/min to shift the pH to 8.3. The titration was finished within 3 min, as marked by the hatched bar. At given times, 10-ml samples were withdrawn for determination of $\Delta\psi$ (●) and ΔpH (○) with tetraphenylphosphonium or methylamine as described in the text. $\Delta\mu_{H^+}$ (▲) was calculated in millivolts with the equation $\Delta\mu_{H^+} = (\Delta\psi - 58\ \Delta pH)$.

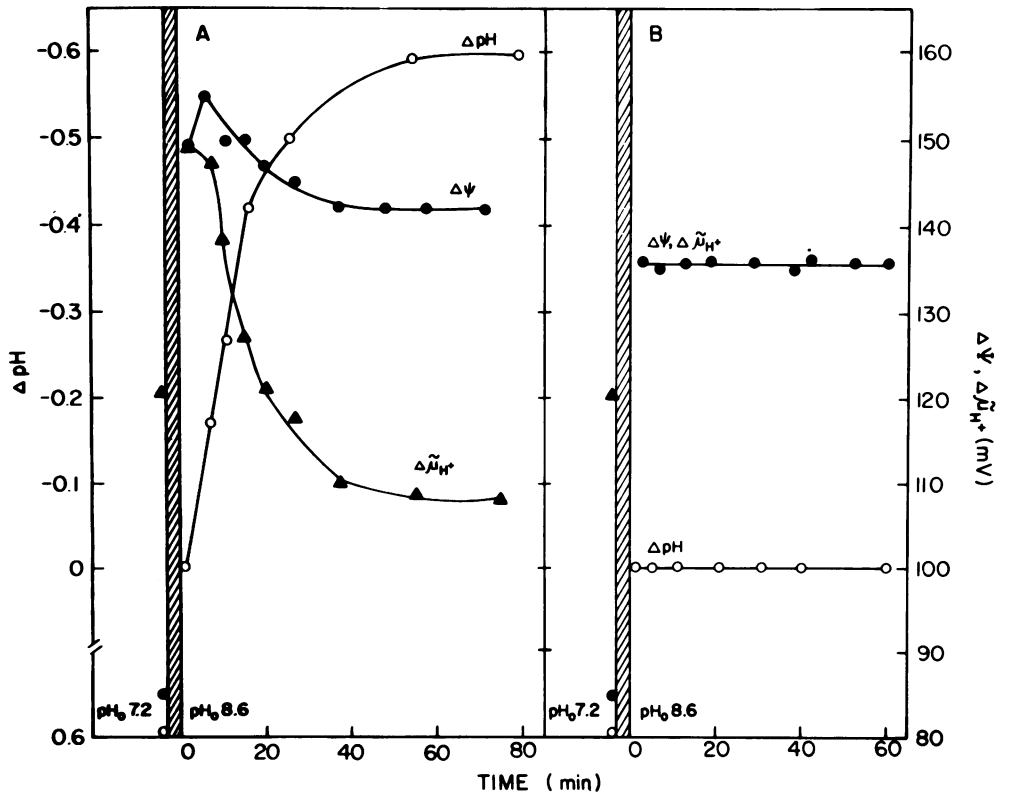

FIG. 2. $\Delta\psi$, ΔpH, and $\Delta\mu_{H^+}$ in growing *E. coli* cells after a slow shift of pH_o from 7.2 to 8.6. *E. coli* CS71 (A) and DZ3 (B) cells were grown at pH 7.2 to mid-logarithmic phase. Samples of 0.5 M KOH were then added at a rate of 2.6 meq/min to shift the pH to 8.6. At given times, samples were withdrawn for determination of ΔpH and $\Delta\psi$ as described in the text.

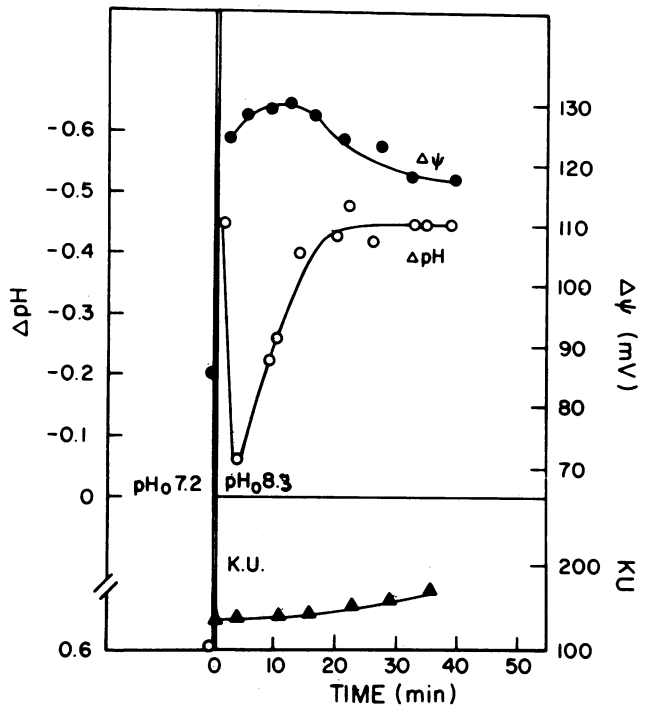

FIG. 3. Δψ, ΔpH, and growth rate of *E. coli* cells after a rapid shift of pH_o from 7.2 to 8.3. *E. coli* CS71 cells were grown at pH 7.2 and transferred to pH 8.3 as described in the legend to Fig. 1, except the rate of KOH addition was such that the final pH was reached within 30 s. At given times, Δψ (●) and ΔpH (○) were monitored. Growth (▲) was followed by determining the Klett units (KU) of the culture with filter 42.

homeostasis above pH 8.3 (22, 23), was tested with respect to $\Delta\mu_{H+}$ after both slow (3-min) and fast (30-s) alkalinization. After the slow increase in pH_o from 7.2 to 8.3, the ΔpH of 0.6 (basic inside) was dissipated as in the wild-type cell but then increased to only 0.35 U (acid inside) and only after 20 to 30 min (Fig. 1B). Under these conditions, resumption of cell growth showed slow kinetics similar to those of cytoplasm acidification (22; Fig. 1B). The growth rate of the mutant was about half that of the wild type after such a shift in pH. The Δψ changed initially in a manner similar to that observed with the wild type (Fig. 1B), i.e., by rapidly increasing after the pH shift while the ΔpH was collapsing. However, the maximum Δψ obtained was 15 mV lower than that of the wild type at the same stage. The new steady-state $\Delta\mu_{H+}$ was about 110 mV, however, similar to that of the wild type. Both a slow (Fig. 2B) and a rapid (Fig. 4B) increase in pH_o from 7.2 to 8.6 caused an immediate, rapid rise of Δψ of the mutant cells, from 80 mV to 125 to 136 mV, and this remained constant for at least 40 min. The initial collapse of the ΔpH was seen only after rapid alkalinization, but no restoration of the ΔpH occurred and no growth of cells followed (Fig. 4B and 3B). It should be emphasized that the final $\Delta\mu_{H+}$ of the mutant cells (137 mV) was higher than that of the wild-type cells (110 mV) (Fig. 2 and 4).

Cell physiology and viability upon failure of pH homeostasis. Despite the defective pH homeostasis and cessation of growth above a pH_o of 8.3, the mutant DZ3 did not die for at least 12 h when maintained at pH 8.8 (22; Fig. 5). The existence of a range of pH_o at which the pH_i equals ph_o, while growth ceases without loss of viability, allowed us to explore the sensitivity of different metabolic reactions to the pH_i (Fig. 5). Cell growth, as measured by the increase in total protein, colony-forming capacity, or increase in light

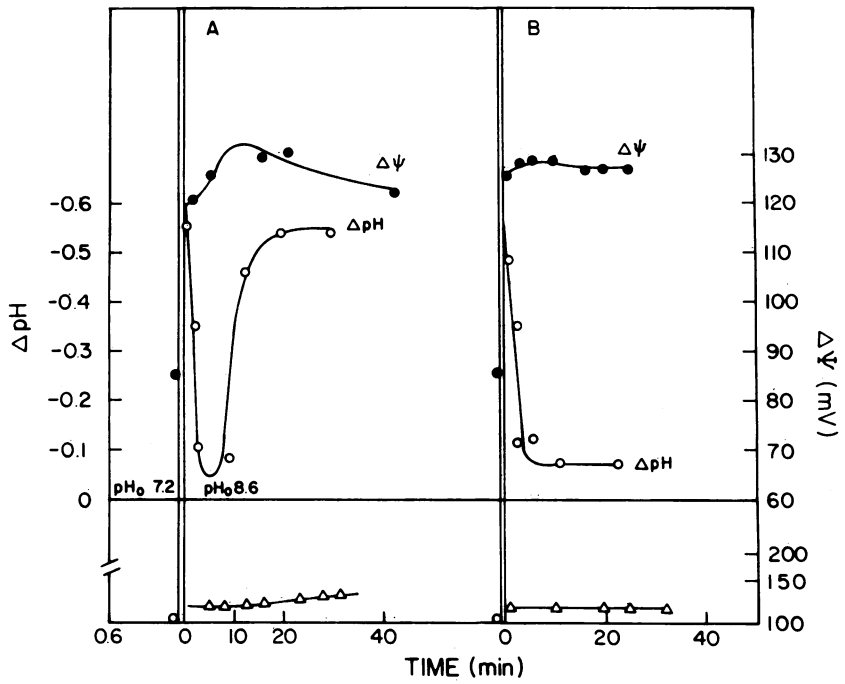

FIG. 4. Δψ, ΔpH, and growth rate of *E. coli* cells after a rapid shift of pH_o from 7.2 to 8.6. *E. coli* CS71 (A) and DZ3 (B) cells were grown at pH 7.2 and transferred to pH 8.6 as described in the legend to Fig. 3. Δψ (●), ΔpH (○), and growth (▲) were monitored as described in the legend to Fig. 3.

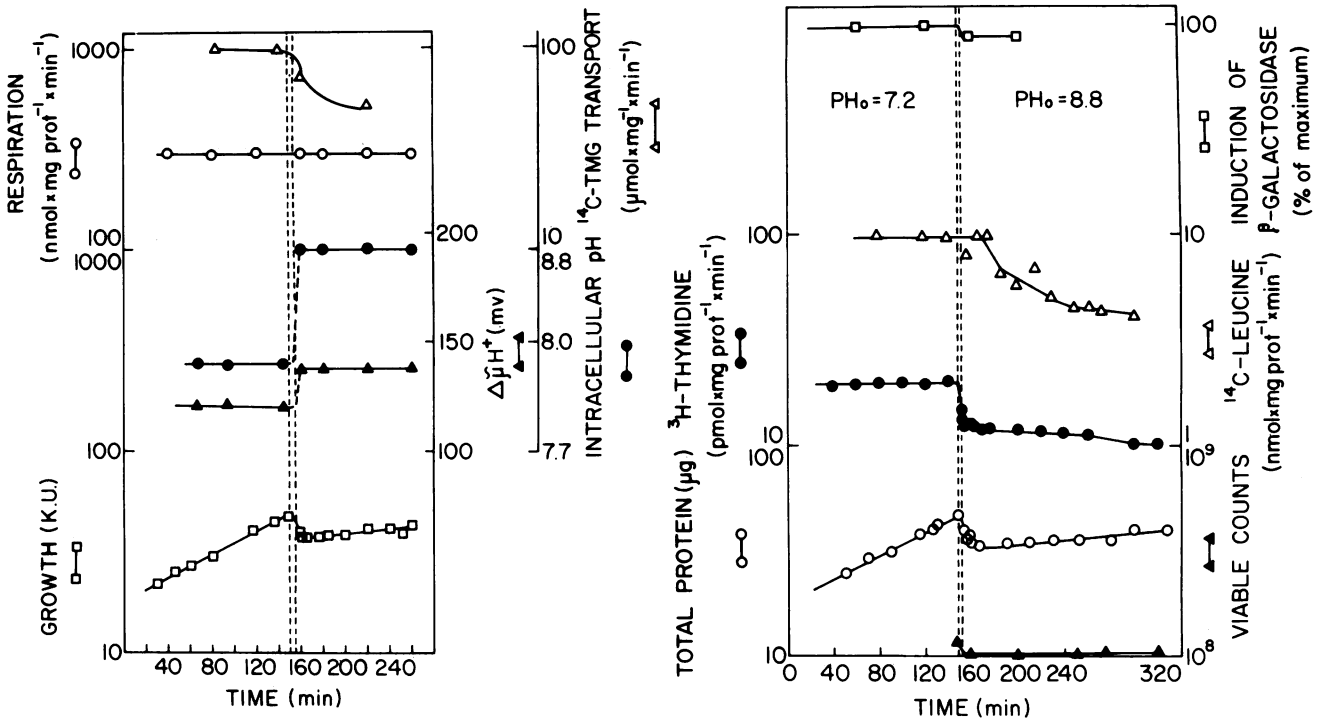

FIG. 5. Growth rate, cell viability, energy transduction, and synthesis of protein and DNA in *E. coli* DZ3 upon transfer to a nonpermissive pH. *E. coli* DZ3 cells were grown in a pH stat at pH 7.2. At given times, samples were withdrawn and various parameters were monitored. The pH_o was then changed to 8.8 as described in the legend to Fig. 2, and the measurements were continued by methods described in the text.

scattering of the cell suspensions, stopped immediately after the shift in pH_o, after a small degree (10%) of cell lysis. The respiration rate remained unchanged after the transition to pH 8.8, and the magnitude of $\Delta\mu_{H^+}$ increased somewhat. The initial rate of active transport of TMG after the shift was 70% of the control and decreased only slowly during the ensuing 50 min. The rate of protein synthesis, as measured by the initial rate of leucine incorporation, was unimpaired for 15 min and then decreased, reaching 50% of the control rate at 100 min. In addition, the induction ability of β-galactosidase, which requires both transcription and translation, was constant for at least 40 min after the shift to pH 8.8. The rate of DNA synthesis, as measured by the initial rate of thymidine incorporation, decreased rapidly to 60% of its original value within 3 to 5 min and thereafter continued to decrease slowly. Before the pH shift, most of the cells were single, and only about 13% were in pairs (Table 1). At the end of 3 h at pH 8.8, most cells were in pairs, and chains of three and four bacterial cells were also observed. After longer incubation at the nonpermissive pH, long chains of up to eight cells were observed (Table 1). When the cultures were shifted back to pH 7.2 after 2 h at pH 8.8, the rate of protein synthesis returned (within 30 min) to the initial rate at pH 7.2 (data not shown), and the cell number doubled. Thereafter, the cells continued to grow at the rate characteristic at pH 7.2 (1.1-h doubling time).

DISCUSSION

Using a rapid filtration technique for cell separation, we monitored the $\Delta\psi$ in *E. coli* cells under conditions identical to those previously used to determine the effects of change in external pH on ΔpH (22). Furthermore, the introduction of rapid alkalinization allowed us to discern the events within 1 to 2 min after the shift in pH_o. We found a ΔpH (acid inside) and an increase in $\Delta\psi$ after alkalinization of cells growing at 7.2 to 8.3 or 8.6. Then, within 2 to 3 min, failure of pH homeostasis occurred, during which time ΔpH collapsed but $\Delta\psi$ increased further.

A net flux of protons down the ΔpH imposed by the shift is directed outwards, in the same orientation as that of the respiratory-driven proton pumps. The outwardly directed ΔpH should allow the $\Delta\psi$ formed by the proton pumps to increase. Indeed, a drastic rise in the latter was observed after the pH shift; the $\Delta\psi$ reached a maximum when the imposed ΔpH was abolished (Fig. 1 through 4). Participation of other transport activities which would allow outwardly directed electrogenic proton movements cannot be ruled out.

TABLE 1. Shift in pH_o to 8.8 induces filament formation in DZ3 cells[a]

Incubation conditions	% Chains with indicated no. of cells per chain:			
	1	1.1–2	2.1–4	4.1
pH 7.2	86	14		
pH 8.8, 3 h	8	85	7	
pH 8.8, 6 h	4	30	48	18

[a] *E. coli* DZ3 cells were grown in a pH stat at pH 7.2 to mid-logarithmic phase. Samples of 0.5 M KOH were then automatically added at a rate of 2.7 meq/min to shift the pH_o to 8.8. The titration was finished within 3 min. At given times, samples were removed and observed under a phase-contrast microscope. The numbers of cells per chain were calculated from measurements of chain length divided by the average length of the individual cell as determined under these growth conditions (2.08 ± 0.15). In each group, at least 60 cells were measured.

After the initial failure to control pH_i, the pH homeostatic mechanism began to function and a net influx of protons occurred, until the pH_i reached 7.8 and was maintained at that value thereafter. Since ΔpH is 0 at the beginning of this influx and the $\Delta\psi$ decrease is parallel to the acidification, it is likely that this proton movement is an electrogenic process driven by the $\Delta\psi$. We have proposed that the mechanism responsible for the acidification of the cytoplasm at alkaline pH_o is the Na^+/H^+ antiporter (17). Others have suggested a role for the K^+/H^+ antiporter in pH homeostasis (3, 4). The Na^+/H^+ antiporter was shown to be electrogenic at high pH and electroneutral at low pH (18). Furthermore, in the present work, monitoring $\Delta\psi$ and ΔpH after the shift in pH_o showed that the initial transitions, which precede the acidification of the cytoplasm in the wild type, are also present in the Na^+/H^+ antiporter-defective mutant DZ3; although the $\Delta\psi$ of the mutant reached a somewhat lower peak, it increased drastically after the pH_o shift, and the ΔpH imposed by the shift decreased to 0. Strikingly, neither the reestablishment of the ΔpH (acid inside) nor the accompanying decrease in $\Delta\psi$ could be seen in the mutant beyond 8.3, implying that the pH adaptation is linked to an electrogenic Na^+/H^+ antiporter.

Two physiological phases could be distinguished after the shift to alkaline pH: an initial failure to control pH_i, followed by pH homeostasis. It may be suggested that events of the first phase signal and affect the latter phase. The initial rapid increase in $\Delta\psi$ may activate the Na^+/H^+ antiporter electrogenic activity that acidifies the cytoplasm and permits pH homeostasis. In this case, the antiporter may be voltage dependent, as has been suggested for halophiles (11). Also, changes in pH_i or pH_o or both could control the antiporter activity.

Transient failure of pH homeostasis has also been detected when pH_o is made acid (22) or when, at constant pH_o, a high concentration of a weak acid is added (21). The recovery of pH_i depends in each case on the load imposed on the system. The capacity of the pH homeostatic mechanism appears to be a function of both the span of the shift in pH_o and the rate at which the change occurs. In the present paper, we show that even the shift from 7.2 to 8.3 may be deleterious if made within 30 s; i.e., $\Delta\psi$ did not rise to the maximum, pH_i failed to reach the optimal steady-state value, and subsequent growth was slowed down (Fig. 3). It is possible that the larger and faster the shift, the greater the damage caused to intracellular components or the larger the leakage of intracellular material. This may then impede the rate and capacity of ΔpH restoration.

The steady-state values of $\Delta\mu_{H+}$ at pH 7.2 before the shift in pH and at pH 8.3 and 8.6 after the shift in pH are very similar, amounting to 130 to 135 mV. Even in the mutant DZ3 at the nonpermissive pH, the $\Delta\mu_{H+}$ is high and constant. In all cases, the high $\Delta\mu_{H+}$ is maintained by a very efficient compensation of the $\Delta\psi$ for the pH. This appears to characterize the bacterial membrane and is understandable in view of the need in bacteria of pH homeostasis and, simultaneously, a constant and high $\Delta\mu_{H+}$.

In the present work, we postulate that the need for pH homeostasis during growth is not due to a general pH sensitivity of cytoplasmic proteins, but that there is a specific pH-sensitive function. For example, the mutant DZ3 that cannot grow and control its pH_i beyond pH_o 8.3 remains fully viable, up to pH 9. At the pH nonpermissive for growth, the respiration rate was not affected; the $\Delta\psi$ of the mutant was above normal and drove the transport of the lactose analog TMG. Protein synthesis decreased slowly and reached 50% at 100 min. Since total protein changed very little, we suggest that breakdown of protein was enhanced. The decrease in the rate of DNA synthesis and induction of β-galactosidase decreased at most to 50% 2 h after the pH shift and, like protein synthesis, cannot account for the growth arrest, which occurred immediately after the shift in pH_o. Since at the alkaline pH the cells do not separate and form long chains, we suggest that a process of the cell division is the pH-sensitive step. The possibility that pH_i may serve a central role in regulation of metabolism and cell growth has gained support in eucaryotes and procaryotes (2, 7, 8, 15, 19, 20, 21).

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