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Glutamate is required to maintain the steady-state potassium pool in *Salmonella typhimurium*

(glutamate synthase/glutamine synthetase/glutamine synthetase adenylyltransferase/potassium channel/osmoregulation)

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ABSTRACT **In many bacteria, accumulation of K^+ at high external osmolalities is accompanied by accumulation of glutamate. To determine whether there is an obligatory relationship between glutamate and K^+ pools, we studied mutant strains of *Salmonella typhimurium* with defects in glutamate synthesis. Enteric bacteria synthesize glutamate by the combined action of glutamine synthetase and glutamate synthase (GS/GOGAT cycle) or the action of biosynthetic glutamate dehydrogenase (GDH). Activity of the GS/GOGAT cycle is required under nitrogen-limiting conditions and is decreased at high external ammonium/ammonia (NH_4^+) concentrations by lowered synthesis of GS and a decrease in its catalytic activity due to covalent modification (adenylylation by GS adenylyltransferase). By contrast, GDH functions efficiently only at high external NH_4^+ concentrations, because it has a low affinity for NH_4^+. When grown at low concentrations of NH_4^+ (≤2 mM), mutant strains of *S. typhimurium* that lack GOGAT and therefore are dependent on GDH have a low glutamate pool and grow slowly; we now demonstrate that they have a low K^+ pool. When subjected to a sudden NH_4^+ upshift, strains lacking GS adenylyltransferase drain their glutamate pool into glutamine and grow very slowly; we now find that they also drain their K^+ pool. Restoration of the glutamate pool in these strains at late times after shift was accompanied by restoration of the K^+ pool and a normal growth rate. Taken together, the results indicate that glutamate is required to maintain the steady-state K^+ pool —apparently no other anion can substitute as a counter-ion for free K^+— and that K^+ glutamate is required for optimal growth.**

K^+ is an essential nutrient in all environments (reviewed in ref. 1), and a large proportion of it is bound to macromolecules (2). At high external osmolalities, bacteria increase their internal K^+ concentration and accumulate solutes such as glutamate, trehalose, proline, and glycinebetaine to maintain turgor pressure for optimal growth (reviewed in ref. 3). Accumulation of K^+ is an early response to hyperosmotic shock (4) and is accompanied by glutamate accumulation (2, 5, 6). In bacteria and a number of archaea, K^+ and glutamate pools are the largest cation and anion pools, respectively, and glutamate is the predominant free amino acid (7–11). Even in media of low osmolality (≈105 mosmolal), enteric bacteria maintain a glutamate pool in excess of 22 nmol/mg dry weight (12, 13), which corresponds to a concentration of ≈11 mM. This is probably above the concentration needed for the maximal rate of synthesis of other nitrogen-containing compounds derived from glutamate, because the growth rates of enteric bacteria remain near optimum at 105 mosmolal.

There are several K^+ transport systems in *Escherichia coli*, and presumably other enteric bacteria: the Kdp, Trk, and Kup systems function in uptake and the KefB and KefC systems in efflux (reviewed in ref. 14). K^+ efflux might also occur through channels such as the mechanosensitive channels MscL and MscS (15) and the Kch channel, a homologue of eukaryotic K^+ channels that was discovered by genome analysis (16). The different K^+ transport systems/channels presumably function in a coordinated fashion to maintain an optimal intracellular K^+ concentration. Glutamate is regarded as the major counterion for free K^+ during osmoadaptation (6, 12), but it is not known whether this relationship is obligatory.

In *Salmonella typhimurium*, as in a number of other bacteria, there are two pathways for synthesis of glutamate (see Fig. 1*A*): the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle (17) and biosynthetic glutamate dehydrogenase (GDH). For each turn of the GS/GOGAT cycle, one molecule of ammonium/ammonia (NH_4^+) and one molecule of 2-oxoglutarate are assimilated into glutamate. Because GS has a high affinity for NH_4^+ ($K_m < 0.2$ mM; ref. 18) and the synthesis of glutamate is coupled to ATP hydrolysis, the GS/GOGAT pathway functions efficiently even at low NH_4^+ concentrations. By contrast, the GDH pathway functions efficiently only at high NH_4^+ concentrations, because GDH has a relatively low affinity for NH_4^+ ($K_m > 1$ mM; refs. 17 and 18). In *gltB/D* mutant strains, which lack a functional GOGAT, glutamate synthesis depends upon GDH and can be limited at low external NH_4^+ concentrations (13).

Function of the GS/GOGAT cycle is controlled by modulation of the synthesis and catalytic activity of GS (reviewed in ref. 19; see Fig. 1*B*). Under nitrogen-limiting conditions, synthesis of GS is elevated and the enzyme is in its unmodified, catalytically active form. When excess NH_4^+ is added to nitrogen-limited cultures, GS is rapidly adenylylated by GS adenylyltransferase (20–22) and thereby inactivated. In *glnE* mutant strains, which lack GS adenylyltransferase and are therefore unable to modify GS, the glutamate pool is rapidly depleted when nitrogen-limited cultures are subjected to a sudden NH_4^+ upshift; the drop in glutamate is due to uncontrolled synthesis of glutamine, not to excretion into the medium (22).

In this study we used *gltB/D* and *glnE* mutant strains of *S. typhimurium* to decrease the intracellular glutamate pool by different means—limiting the external NH_4^+ concentration in the first instance or suddenly increasing its concentration in the second. Depletion of the glutamate pool, which was known to be accompanied by a growth defect in each case (13, 22), resulted in a decrease in the steady-state K^+ pool. We conclude that glutamate plays an important role in osmoregulation by retaining an optimal intracellular level of K^+. A preliminary report of this work has been presented.†

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. Strains were derived from *S. typhimurium* LT2. Congenic strains SK2633

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Abbreviations: GS, glutamine synthetase; GOGAT, glutamate synthase; GDH, glutamate dehydrogenase.

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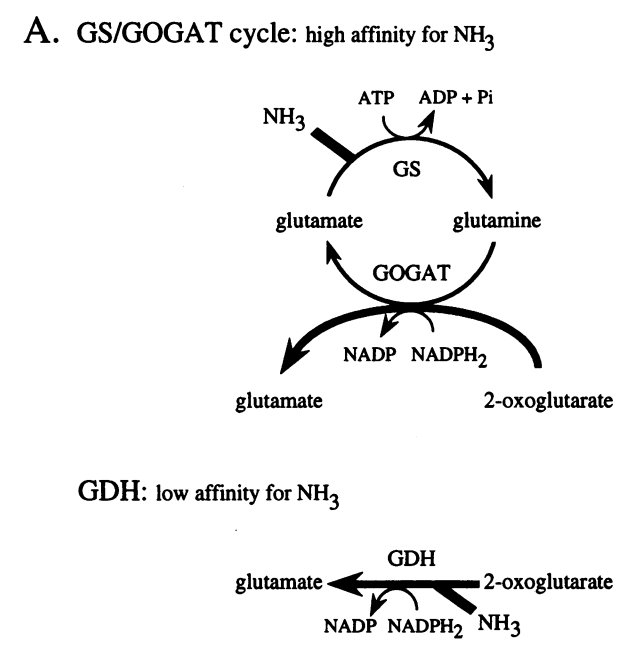

FIG. 1. The two biosynthetic pathways for glutamate (*A*) and covalent modification of glutamine synthetase in enteric bacteria (*B*). Bold arrows in *A* indicate net synthesis of glutamate.

(wild-type), SK2630 (*gltB629*), and SK2626 (*gltB/D627*) (13) were used for hyperosmotic shock experiments; the latter strains lack GOGAT activity. Congenic strains SK2994 (wild-type), SK2996 (*glnE122*), and SK 2995 (*glnE121*) (22) were used for NH_4^+ upshift experiments; the latter strains lack GS adenylyltransferase activity.

Growth experiments were performed aerobically at 37°C. The full-strength minimal medium was sodium-based N^-C^- medium, which contains (per liter) Na_2SO_4 (0.8 g), Na_2HPO_4 (11.0 g), $NaH_2PO_4{\cdot}H_2O$ (4.8 g), $MgSO_4{\cdot}7H_2O$ (0.1 g), and NaCl (2.5 g). The diluted minimal medium was 0.2 × N^-C^- double-buffered (DB), which contains (per liter) Na_2SO_4 (0.16 g), Na_2HPO_4 (4.4 g), $NaH_2PO_4{\cdot}H_2O$ (1.9 g), $MgSO_4{\cdot}7H_2O$ (0.1 g), and NaCl (0.5 g). Both media were supplemented with glycerol as a carbon source (0.2 or 0.4%, as indicated) and NH_4Cl (2 or 10 mM, as indicated) or proline (10 mM) as a nitrogen source and 1 mM KCl. For hyperosmotic shock, additional NaCl was added to diluted minimal medium to a final concentration of 0.5 M. For NH_4^+ upshift, NH_4Cl was added to full-strength minimal medium to a final concentration of 10 mM. The measured osmolalities of 0.2 × N^-C^- DB, 0.2 × N^-C^- DB plus 0.5 M NaCl, and N^-C^-, containing the carbon and nitrogen sources, were 156, 1047, and 393 mosmol, respectively (measured with an Advanced Instruments wide-range osmometer, model 3WII).

Strains were grown overnight in nutrient broth medium and then subcultured into the medium used for a particular experiment, except that NH_4Cl was provided at 5 mM instead of 2 mM. After the cultures had reached saturation, cells were harvested by centrifugation, washed once with the medium to be used subsequently, and inoculated into warmed fresh medium to a low cell density (OD_{650} of 0.05 for cells grown on NH_4^+ and 0.15 for cells grown on proline). Cultures were subjected to hyperosmotic shock or NH_4^+ upshift after the cells had undergone one to two doublings.

Measurement of Cytoplasmic K^+. Cells from 1- or 2-ml samples were rapidly harvested by filtration through Millipore HAWP filters (mixture of cellulose acetate and nitrate; 0.45-μm pore size; 25 mm) (12) that had been pretreated in 1% nitric acid overnight. The cells were then immediately washed twice with 1 ml warmed growth medium of the same composition (and therefore osmolality) but lacking K^+. K^+ was extracted from the cells for 1 hr at room temperature by suspending the filters in 5 ml of 1% HNO_3 containing 50 μM NaCl in 16 × 150 mm Fisher glass tubes; cell debris was then removed by filtration. K^+ levels were determined by atomic absorption spectrometry (Perkin–Elmer spectrophotometer, model 2380) by comparison with KCl standards in 1% HNO_3 containing 50 μM NaCl. To minimize the error caused by K^+ contamination from filters and glass tubes, K^+ levels of blank controls (filters) were determined in duplicate, and the average value was subtracted from the experimental samples.

Analysis of Amino Acid Pools. A "no-harvest" protocol was used to extract glutamate and glutamine from cells, and their amounts were measured as described using α-aminoadipate as internal standard (13, 22, 23). Control experiments indicated that glutamate pools in samples harvested by filtration for K^+ measurements were the same as in samples treated by the "no-harvest" protocol.

Determination of Cell Dry Weight. Dry weights were determined for *gltB*$^+$ and *gltB*$^-$ strains in the medium used for the hyperosmotic shock experiment of Fig. 2 *A* and *C*. NaCl was added to the experimental culture at an OD_{650} of ≈0.1, and both experimental and control cultures (100 ml each) were harvested at an OD_{650} of ≈0.4. After cooling to 0°C for 5 min, cells were collected by centrifugation at 16,000 × *g* for 10 min, washed twice by suspending in 100 ml of double-distilled water (0°C) and centrifugation, and suspended to a total volume of 10 ml with water. Two samples (4.5 ml each) were placed in preweighed aluminum boats, dried at 60°C until all visible water had evaporated, and then dried under vacuum to constant weight. For cultures subjected to hyperosmotic shock, losses of optical density in medium and washes were negligible. However, for control cultures of the *gltB*$^+$ and *gltB*$^-$ strains, losses were 39% and 33%, respectively, and dry weights per ml·OD_{650} were corrected for these losses. Dry weights were determined for *glnE*$^+$ and *glnE*$^-$ strains in the medium used for the NH_4^+ upshift experiment of Fig. 3. They were determined 10 min and one generation after shift, as described. Losses of OD in medium and washes were negligible.

RESULTS

Pools of Glutamate and K^+ in *gltB/D* Strains upon Hyperosmotic Shock. When a control culture of the wild-type (*gltB*$^+$) strain SK2633 was grown in dilute minimal medium (see *Materials and Methods*) with 2 mM NH_4^+ as a nitrogen source, both its glutamate and K^+ pools were constant during the exponential phase (23 and 287 nmol/ml·OD_{650}, respectively; Fig. 2 *A* and *B*). [At the end of exponential growth when NH_4^+ was being exhausted from the medium, both pools decreased (to 8 and 248 nmol/ml·OD_{650}, respectively)]. When an exponentially growing culture was exposed to hyperosmotic shock by addition of NaCl to 0.5 M final concentration, both the glutamate and K^+ pools showed a large transient increase [similar to that seen by Dinnbier *et al.* (5) for *E. coli*]. At 30 min post-shock, these pools reached 168 and 574 nmol/ml·OD_{650},

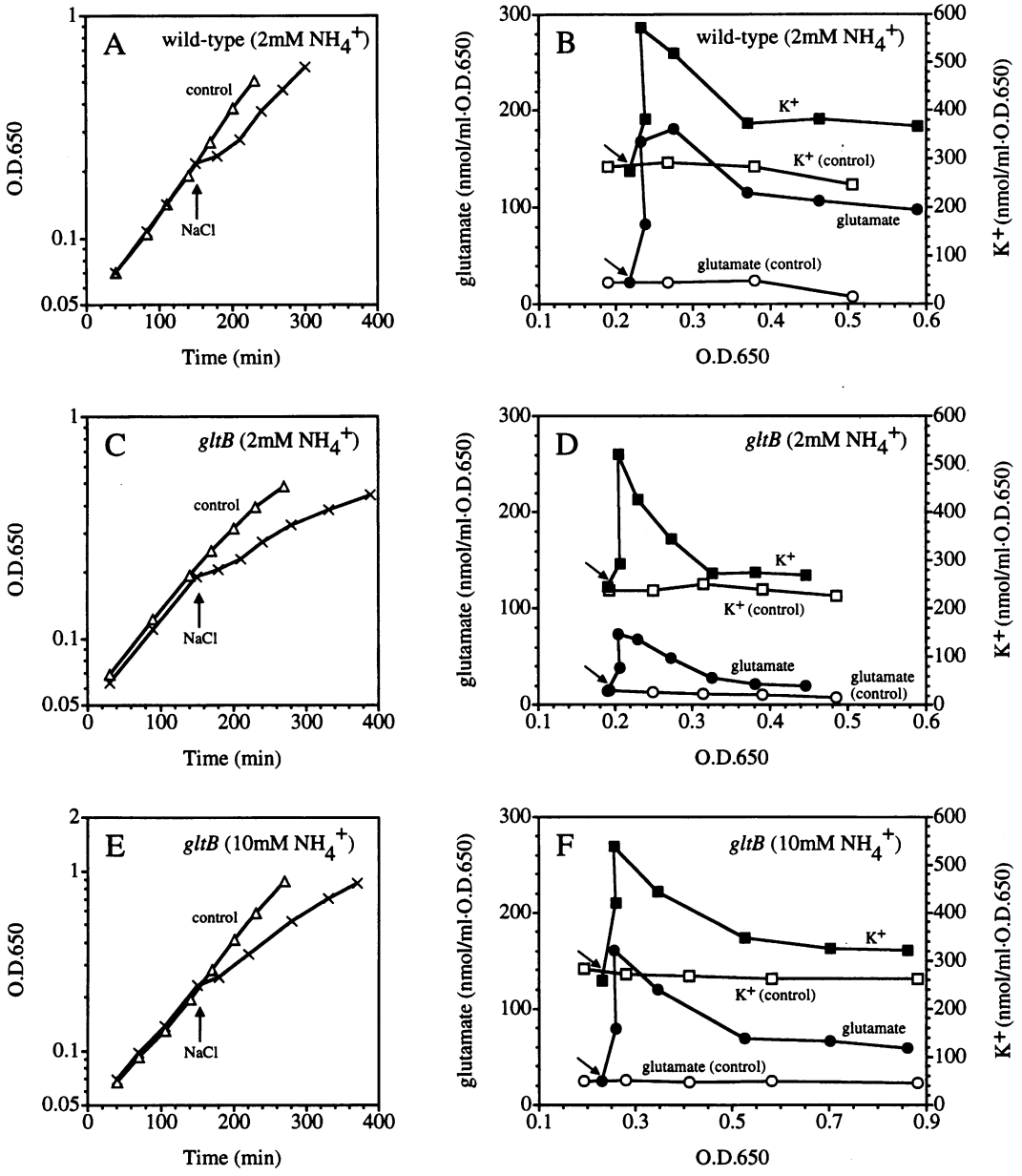

FIG. 2. Growth responses and pools of glutamate and K^+ in a *gltB*$^+$ and a *gltB* mutant strain upon hyperosmotic shock. The dilute minimal medium contained 0.2% glycerol as a carbon source and 2 or 10 mM NH_4Cl as a nitrogen source, as indicated. At an OD_{650} of ≈0.2 (time, 150 min), experimental cultures (crosses in *A*, *C*, and *E*) were subjected to hyperosmotic shock by addition of NaCl to a final concentration of 0.5 M (indicated by arrows). Cultures without added NaCl (open triangles) served as controls. Pools of glutamate (circles) and K^+ (squares) were measured as described (panels *B*, *D*, and *F*). Closed symbols are used for experimental cultures and open symbols for control cultures. The first three points for experimental cultures were taken just preshock and at 10 and 30 min postshock, respectively. Dry weight values (mg per ml·OD_{650}) for SK2633 (*gltB*$^+$) and SK2630 (*gltB*) were 0.46 and 0.45, respectively, in dilute medium and 0.28 and 0.26, respectively, after "adaptation" to 0.5 M NaCl. (*A* and *B*) SK2633 (*gltB*$^+$) with 2 mM NH_4Cl as a nitrogen source. The doubling time of the control culture was 63 min and that of the culture "adapted" to NaCl was 89 min. (*C* and *D*) SK2630 (*gltB*) with 2 mM NH_4Cl as a nitrogen source. The doubling time of the control culture was 78 min and that of the culture "adapted" to 0.5 M NaCl increased from 121 to 260 min as NH_4^+ was depleted from the medium. (*E* and *F*) SK2630 (*gltB*) with 10 mM NH_4Cl as a nitrogen source. The doubling time of the control culture was 62 min and that of the culture "adapted" to 0.5 M NaCl increased from 92 to 136 min.

respectively. As the culture adapted to the high external osmolality and re-established exponential growth at a slightly slower rate (Fig. 2*A*), the pools of both K^+ and glutamate gradually decreased to constant levels that were higher than those before shock (107 and 375 nmol/ml·OD_{650} for glutamate and K^+, respectively; Fig. 2*B*). At this point the glutamate pool had increased by 4.6-fold and the K^+ pool by 31%. Conversion to nmol per mg of dry weight gave preshock values for glutamate and K^+ of 50 and 624, respectively, and values after adaptation to hyperosmotic shock of 382 and 1339, respectively.

The *gltB* mutant strain SK2630 grew more slowly than the *gltB*$^+$ strain in 2 mM NH_4^+, both at low external osmolality and, more obviously, after adaptation to high osmolality (Fig. 2*C*). The direct cause of the growth defect was a limitation in glutamate synthesis by GDH at low concentrations of NH_4^+, particularly at high external osmolality (ref. 13; Fig. 2*D*). The glutamate pool of the *gltB* mutant strain was 13 nmol/ml·OD_{650} at low external osmolality, which was ≈56% that of the *gltB*$^+$ strain. After adaptation to high osmolality, the glutamate pool of the mutant strain was 24 nmol/ml·OD_{650}, which was only 22% that of the *gltB*$^+$ strain and represented an

increase of only ≈1.8-fold. The values for the K^+ pool of the *gltB* mutant strain were 241 and 273 nmol/ml·OD_{650} before and after adaptation to high osmolality, respectively; the latter value was only 13% higher than the former and was considerably lower than the corresponding value for the *gltB*$^+$ strain. Conversion to nmol per mg of dry weight gave preshock values for glutamate and K^+ of 29 and 536, respectively, and values after adaptation to hyperosmotic shock of 92 and 1050, respectively. Similar results were obtained with a second *gltB/D* strain, SK2626 (data not shown).

Although the *gltB* mutant strain clearly failed to maintain a normal steady-state K^+ pool after adaptation to high osmolality, it showed a remarkably normal transient increase in this pool immediately following hyperosmotic shock. At 30 min after addition of NaCl, its K^+ pool had jumped to 522 nmol/ml·OD_{650} (Fig. 2*D*), nearly the same as that of the *gltB*$^+$ strain (Fig. 2*B*). This was the case even though the glutamate pool of the *gltB* mutant strain (73 nmol/ml·OD_{650}) was less than half that of the *gltB*$^+$ strain at this time.

The growth defect of the *gltB* mutant strain could be partially alleviated by increasing the external NH_4^+ concentration to 10 mM (Fig. 2*E*) and thereby allowing more rapid synthesis of glutamate by GDH. Under these conditions, the glutamate and K^+ pools were 24 and 269 nmol/ml·OD_{650}, respectively, at low external osmolality. They increased to 65 and 332 nmol/ml·OD_{650}, respectively, after adaptation to high osmolality (Fig. 2*F*), which represented increases of 2.7-fold and 23%. At high external osmolality, both pools were intermediate between those of the *gltB*$^+$ and *gltB* mutant strains grown with low NH_4^+, as was the growth rate of the culture. The same was true for a second *gltB/D* strain, SK2626 (data not shown). Taken together, the results of these experiments indicate that steady-state levels of K^+ are correlated with glutamate levels, but that transient accumulation of K^+ can occur upon hyperosmotic shock even though glutamate levels are low.

Pools of Glutamate and K^+ in *glnE* Strains upon NH_4^+ upshift. When a culture of the wild-type (*glnE*$^+$) strain SK2994 was subjected to NH_4^+ upshift after growth on proline as the sole nitrogen source, it quickly adenylylated GS (22) and adopted a more rapid growth rate (Fig. 3*A*, open triangles). As shown previously, the glutamate pool increased somewhat after shift (2.5-fold in Fig. 3*B*, from 30 to 73 nmol/ml·OD_{650} at 60 min after shift). The K^+ pool, which was 273 nmol/ml·OD_{650} before shift, also increased. In contrast, the *glnE* mutant strain SK2996, which fails to adenylylate GS (22), had a large growth defect after shift (Fig. 3*A*, crosses), which persisted for approximately two generations. As a consequence of excess catalytic activity of GS, the glutamate pool in this strain dropped to ≈25% of its preshift value at 10 min after shift (from 28 to 7 nmol/ml·OD_{650}) and was similarly low at 60 min after shift (9 nmol/ml·OD_{650}). This value was ≈10% that of the *glnE*$^+$ strain. As observed previously (22), the drop in glutamate was accompanied by a massive increase in glutamine, much of which was excreted into the medium at late times after shift (data not shown). The drop in glutamate was accompanied by a drop in K^+, which reached its lowest value of 156 nmol/ml·OD_{650} at 60 min after shift. This value was only ≈50% that of the *glnE*$^+$ strain, whereas preshift values were identical. The absolute loss of K^+ (117 nmol/ml·OD_{650} = 273–156) was much greater than the loss of glutamate (19 nmol/ml·OD_{650} = 28–9). Similar results were obtained upon NH_4^+ upshift of a second *glnE* strain, SK2995 (data not shown).

As synthesis of GS was repressed in the *glnE* strains and its amount was decreased by dilution (22), the growth rates, glutamate pools, and K^+ pools of the strains increased (Fig. 3 *A* and *B* and data not shown). When cultures were transferred to fresh medium to allow additional growth, the doubling times and pools were very similar to those for the *glnE*$^+$ strain (legend to Fig. 3). These results indicated that glutamate was required to maintain the K^+ pool and that K^+ glutamate was needed for optimal growth.

DISCUSSION

By using mutant strains of *S. typhimurium* with a primary defect in glutamate metabolism, we showed that glutamate is required to maintain the steady-state K^+ pool. Apparently no other anion can substitute for glutamate as the counter-ion for free K^+. Moreover, normal K^+ glutamate pools are required for optimal growth. Whether slow growth at low K^+ glutamate is due to abnormal intracellular turgor (refs. 12 and 14; see below), an inappropriate internal salt concentration/ionic strength, both, or neither is not clear.

The mutant strains we used were of two sorts. The *gltB/D* strains have a low glutamate pool when limited for NH_4^+ as the nitrogen source. They are unable to maintain a normal K^+ pool under these conditions, particularly at high external osmolalities, and they grow less rapidly than wild-type (congenic *glt*$^+$)

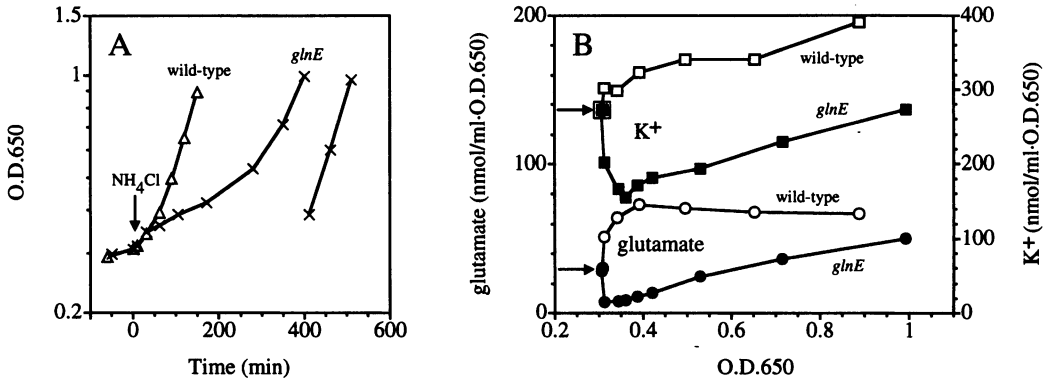

FIG. 3. Growth response and pools of glutamate and K^+ in a *glnE* mutant strain upon NH_4^+ upshift. Full-strength minimal medium contained 0.4% glycerol as a carbon source and 10 mM proline as a nitrogen source. NH_4Cl was added to a final concentration of 10 mM at time 0, as indicated by the arrow. (*A*) Growth curves for SK2994 (*glnE*$^+$; open triangles) and SK2996 (*glnE*; crosses). At an OD_{650} of 0.96 (395 min postshift), SK2996 was diluted 3-fold into fresh, warmed medium. Preshift doubling times for SK2994 and SK2996 were 782 and 869 min, respectively. Apparent "doubling times" between 30 and 60 min postshift were 150 and 432 min, respectively. At an OD_{650} of ≈0.8, doubling times were 68 and 106 min, respectively, and after dilution, the doubling time of SK2996 was 72 min. Dry weight values (mg per ml·OD_{650}) for SK2994 (*glnE*$^+$) 10 min and one generation after shift were 0.39 and 0.40, respectively. Those for SK2996 (*glnE*) were 0.37 and 0.43, respectively. (*B*) Pools of glutamate (circles) and K^+ (squares) for SK2994 (*glnE*$^+$; open symbols) and SK2996 (*glnE*; closed symbols). The first four points in each case were from samples taken just preshift and at 10, 30, and 60 min postshift, respectively. The values of glutamate and K^+ for SK2996 after dilution were 55 and 290, 65 and 314, and 80 and 355 nmol/ml·OD_{650}, respectively, at times 410, 460, and 510 min postshift.

strains. Interestingly, when challenged by hyperosmotic shock, they can increase K^+ levels transiently by almost as much as a *glt*$^+$ strain, which indicates that glutamate is not required for this very large initial response. McLaggan *et al.* (6) reached the same conclusion regarding the initial response based on the fact that an *E. coli* strain placed in a medium lacking NH_4^+ and therefore unable to accumulate glutamate nevertheless showed a normal transient increase in K^+ upon hyperosmotic shock.

The other mutant strains we employed, *glnE* strains, drain their glutamate pool into glutamine when they are shifted from nitrogen-limiting to NH_4^+-excess conditions—conditions opposite to those that cause glutamate limitation in *gltB/D* strains. Again, loss of glutamate is accompanied by loss of K^+ and a profound growth defect. The loss of K^+ occurs despite the fact that there is no decrease in the ATP pool (22) and no change in external osmolality. Upon NH_4^+ upshift, *glnE* strains deplete their glutamate pool to ≈10% the level in a congenic *glnE*$^+$ strain. [Because glutamate is not found in the medium (see *Materials and Methods*), it must be metabolized rather than excreted.] The *glnE* strains excrete K^+ and have a K^+ pool only ≈50% that of the *glnE*$^+$ strain. The gradual recovery of an optimal growth rate by these strains is correlated with replenishment of the glutamate and K^+ pools. Although the glutamate pool initially drops to 20 nmol per mg of dry weight = ≈10 mM, we do not think that slowed growth is due to defects in biosynthesis, because this pool is comparable to that in a wild-type strain growing at low external osmolality (≈11 mM). Moreover, proline, the product whose synthesis requires the highest concentration of glutamate (24), was provided in the medium as the preshift nitrogen source. Preliminary calculations indicate that decreases in turgor in *glnE* strains upon NH_4^+ upshift should be minimal because decreases in the glutamate and K^+ pools are accompanied by large compensatory increases in the glutamine pool (ref. 22; unpublished data).

It will be of interest to determine how *glnE* strains excrete K^+ upon NH_4^+ upshift, specifically whether they excrete it through a known efflux system(s) or channel(s). It will also be interesting to know whether K^+ excretion is controlled ("gated") directly by glutamate or occurs indirectly as a consequence of glutamate depletion, for example, in response to the charge imbalance that is created.

In addition to possible changes in turgor and salt concentration that may accompany a decrease in the levels of the two major ionic solutes K^+ and glutamate, there is a more fundamental problem of electrical balance. As K^+ and glutamate pools decrease in *glnE* strains after NH_4^+ upshift (Fig. 3*B* and legend), changes in the absolute value of the K^+ pool are always several times larger than those in the glutamate pool. By analogy to the case when K^+ and glutamate pools are increased in wild-type strains in response to increases in external osmolality (e.g., Fig. 2*B* and legend), two K^+ ions may be exchanged for one putrescine (doubly charged) to maintain electroneutrality (25, 26). Otherwise, the cytoplasm would presumably become highly acidic as K^+ was exchanged for H^+.

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