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Accumulation of Glutamate by *Salmonella typhimurium* in Response to Osmotic Stress

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Salmonella typhimurium **accumulates glutamate in response to osmotic stress. Cells in aerobic exponential growth have an intracellular pool of approximately 125 nmol of glutamate mg of protein^{-1}. When cells were grown in minimal medium with 500 mM NaCl, KCl, or sucrose, 290 to 430 nmol of glutamate was found to accumulate. Values were lower when cells were harvested in stationary phase. Cells were grown in conventional medium, harvested, washed, resuspended in the control medium or in medium with osmolytes, and aerated for 1 h. With aeration, glutamate was found to accumulate at levels comparable to those observed in exponential cultures. Antibiotics inhibiting protein synthesis did not affect glutamate accumulation when cells were aerated. Strains with mutations in glutamate synthase (*glt*) or in glutamate dehydrogenase (*gdh*) accumulated nearly normal levels of glutamate under these conditions. A double (*gdh glt*) mutant accumulated much less glutamate (63.9 nmol mg of protein^{-1}), but a 1.9-fold excess accumulated when cells were aerated with osmotic stress. Methionine sulfone, an inhibitor of glutamate synthase, did not prevent accumulation of glutamate in cells aerated with osmotic stress. Glutamate dehydrogenase is thought to have minimum activity when ammonium is limiting. Resuspending cells with limiting ammonium reduced glutamate production but did not eliminate accumulation of excess glutamate when cells were osmotically stressed. Amino oxyacetic acid, an inhibitor of transamination reactions, did not prevent accumulation of excess glutamate. The results indicate that neither glutamate dehydrogenase nor glutamate synthase is solely responsible for the excess glutamate that accumulates. It can be calculated that this excess glutamate accounts for only 1.0% of the cell's capacity to make glutamate. Very small changes in some of the reactions involved in glutamate metabolism could account for this.**

The response of bacteria to osmotic stress is a global response involving not only derepression of functions to facilitate the cell's adaptation to the situation (3, 7, 19, 20, 27) but also the activity of cellular enzymes and regulatory proteins. The amount of unbound water decreases and the concentration of metabolic solutes increase in response to osmotic stress (12, 13). The absolute amount of K^+ increases in nearly all cells (12, 13, 19, 20, 24). The activity of the intracellular water is altered by these changes, and this affects the activity of many enzymes and regulatory proteins (12, 13, 38, 52).

Many bacteria accumulate organic compounds (19, 20, 57), including glutamate, in response to osmotic stress (7, 12, 44, 45). Glutamate accumulates to act as an osmolyte to increase the intracellular concentration of solutes to reduce loss of water by osmosis (32, 41). Glutamate also acts as a counter ion for the K^+ that accumulates (24). It has been shown that K^+ glutamate stimulates protein-DNA interactions, suggesting that the accumulation of glutamate could be involved in regulation of expression of functions made in response to osmotic stress (38, 52).

It is not always certain whether the response of a cell to osmotic stress is a response to the stress per se. For example, it has been shown that the accumulation of trehalose in response to osmotic stress is mediated by the two-component mechanism that regulates entry into stationary growth (25, 29, 30). The differential production of *ompF* and *ompC*, responsible for production of major porins in response to osmotic stress, has been studied in detail (16, 19, 20). However, the porin composition also responds not only to the osmolarity of the growth medium but also to many other stimuli (16). There is often cross talk between two-component regulatory mechanisms (60). The rationale for the response of porin composition to osmotic stress has never been clear. This response to osmotic stress could be fortuitous.

Glutamate is central to ammonium assimilation. Every amino group passes through the glutamate-glutamine couple (51). Assimilation is rigorously regulated by the nitrogen regulation (*ntr*) system, a complex two-component regulatory system (1, 51) with a unique sigma factor and an enhancer for some of the functions (34, 35, 50). Appended to this, at least in *Klebsiella aerogenes* and perhaps in other enteric coliforms, is the *nac* (nitrogen assimilation control) regulatory system, which is superimposed on some, but not all, nitrogen-regulated functions (4–6, 43). In addition, in *Escherichia coli* there is a function in the *glt* operon, *gltF*, that regulates some functions involved in ammonium assimilation (14, 15).

In the enteric coliform bacteria, ammonium can be assimilated by the production of glutamate from two reactions: glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH). It is thought that GOGAT functions when ammonium is limiting and that GDH functions when ammonium is present in concentrations greater than 1 mM (10, 11, 22, 46, 51). Glutamate can also be produced by the degradation of proline or arginine in *Salmonella typhimurium* (28, 42). Glutamate can be produced by the transamination of 2-ketoglutarate by using whatever sources of assimilated ammonium that are available to the cell (17). Finally, glutamate can be produced by the deamination of glutamine (28). These pathways can be consid-

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TABLE 1. Strains used in this study

Strain	Genotype(s)	Source or reference
S. typhimurium		
TA831	del *hisF645*	S. Kustu, University of California, Berkeley
SK705	del *hisF645 zcd-2*::Tn*10gdh51*	S. Kustu
SK2207	del *hisF645 gltB629*	S. Kustu
SK2214	del *hisF645 gltB629 zcdS-2 gdh51*	S. Kustu
JB2112	*dhuA hisJ5601 gal2395* del *gltB833-cod* del *gdh71 recA1*	46
LT-2	Wild type	J. Ingraham, University of California, Davis
E. coli MC4100	*LacU169 araD139 rpsL thiA flbB relA* F^-	R. Valentine, University of California, Davis

ered salvage pathways, routes that are not normally used by the cell. They do not, of course, result in the assimilation of ammonium, only in the synthesis of glutamate.

It has been proposed that the excess glutamate that accumulates in response to osmotic stress is synthesized by GDH (20, 44, 45). GDH is stimulated by K^+, and nearly all cells accumulate this ion when they are osmotically stressed. This could provide for the increase in glutamate pools observed (44). However, when cells are resuspended with carbonyl cyanide-*meta*-chlorophenylhydrazone, K^+ transport is inhibited, yet cells continue to synthesize excess glutamate when they are osmotically stressed (49). GDH is also stimulated by an increase in the pH. Osmotic stress causes an increase in the intracellular pH, and it has been proposed that this increase in pH and the concomitant stimulation of GDH account for the accumulation of glutamate (45). However, it has been shown that the pH remains elevated for only a short time and that the production of excess glutamate continues (23). These observations prompt questions about the role of GDH in the production of the excess glutamate.

This work was undertaken to determine the role of GDH in the accumulation of excess glutamate in response to osmotic stress. Using mutants blocked in GDH and GOGAT activity and conditions that minimize these reactions, we have shown that neither reaction is responsible for the excess glutamate.

MATERIALS AND METHODS

Cultures. The strains used in this study are described in Table 1. Strain SK705 has no GDH when tested by Western blots (immunoblots). In strain SK2207, neither subunit of GOGAT was detectable by Western blots (33b). To the best of our knowledge, work with these strains has not been published previously.

Growth. Cells were grown in M63 (47) with 0.1% glucose as the carbon source supplemented with 10 μM histidine for *his* strains. The M63 was supplemented with 0.02 μM ferric sulfate · $7H_2O$ and 20 μM $CaCl_2$. M63 medium has an osmolarity of 260 mosM. M63 with 500 mM NaCl measured with a Wescor osmometer has an osmolarity of 1250 mosM. Cells were grown at 30°C in an incubator shaker in Erlenmeyer flasks filled to no more than 20% capacity. The flasks were fitted with side arms permitting optical measurements.

Growth was estimated from the A_{550}. An A of 1.00 was equivalent to 2.2×10^9 cells per ml in strain TA831. An A of 1.00 was found to be equivalent to 0.401 mg of protein ml^{-1} when cells were grown in M63 and to 0.519 mg ml^{-1} when cells grown in M63 with NaCl. When cells were grown, harvested, resuspended, and aerated for 60 min, an A of 1.00 was found to be equivalent to 0.442 mg of protein (40) in cells aerated in M63 and to 0.497 when cells were aerated in M63 with NaCl. When cells were grown with NaCl, concentrated by centrifugation, and resuspended in an equal volume of water, the protein content was much lower, 20 to 50% lower, depending on growth conditions. The supernatant contained the protein that was lost from the cells. Apparently, osmotic stress causes cells to lose a substantial fraction of their protein. Protein determinations were made for the cultures, that is, the cells suspended in the growth medium.

In the control medium, at 30°C, wild-type cells grew with a generation time of approximately 1 h. With 500 mM NaCl to cause osmotic stress, cells grew with a generation time of approximately 4.5 h. The mutants, particularly the double mutant, grew more slowly under all conditions.

When cells were grown in conventional M63 medium and shifted to M63 with osmolytes, cells were collected by filtration or centrifugation in a clinical centrifuge for 5 min at room temperature. Usually 5 ml of cells were collected and resuspended in 10 ml of medium. The Glt^- double mutant strains were grown with 1 mM glutamate. Glt^- cells were washed after collection on the filter with 4 × 2.5 ml of M63 without amino acids or a carbon source. When cells were aerated with limiting ammonium, they were washed with 4 × 2.5 ml of M63 without ammonium. The washed cells were resuspended in M63 without ammonium or in M63 with 1 mM ammonium and glucose. Histidine was always present. Cells were incubated for 60 min after being shifted to medium with a high osmolarity. The rate of glutamate accumulation was found to be linear for at least 60 min.

Enzyme assays. For assays of GDH and GOGAT, cells were harvested by centrifugation, washed once in 100 mM phosphate buffer (pH 7), and broken in a French pressure cell. Cell debris was removed by centrifugation at 10,000 × *g* for 30 min in a refrigerated preparative centrifuge. Cell extracts were dialyzed against 10 mM phosphate buffer (pH 7) overnight with three changes of the buffer. Enzymes were assayed by conventional spectrophotometric techniques to measure the oxidation of NADH or NADPH (11).

Glutamate determinations. Glutamate was determined spectrophotometrically by the method of Witt (61), with bovine GDH and acetyl pyridine dinucleotide. This method is linear between 5 and 150 nmol of glutamate ml^{-1}. We found the reproducibility of the assays to be improved by using a twofold-greater concentration of acetyl pyridine dinucleotide and by incubating the reaction mixtures for 3 to 4 h. The results were plotted, and a linear regression was fitted. The y intercept, determined from the regression line, was subtracted from all values as a 0 value. The slope of the regression line was used to calculate the concentration of glutamate. Assays were routinely done in triplicate. All the data presented represent the means of at least three independent experiments.

For glutamate determinations, cells were collected on cellulose filters (0.45-μm pore size). The filter was placed in a test tube, and the test tube was chilled in an ice bath. Glutamate

TABLE 2. Glutamate production in response to osmotic stress

Osmolyte[a]	Amt of glutamate (nmol mg of protein^{-1})[b]		
	Exponential phase	Stationary phase	Shift
None (control)	126.2	50.6	155.8
NaCl	315.6	258.7	418.5
KCl	287.2	258.9	324.8
Sucrose	309.7	297.9	445.7
Glycerol	202.9	53.0	167.3

[a] NaCl, KCl, and sucrose were present at a final concentration of 500 mM. Glycerol was present at a concentration of 720 mM.

[b] Results for strain TA831 are presented. Protein values for cells growing with KCl and sucrose were assumed to be comparable to the values for cells growing with NaCl. Cells were harvested in stationary growth phase or mid-exponential growth phase (A_{550} of 0.4 to 0.5) or were grown to mid-exponential growth phase, harvested, and resuspended in medium with the osmolytes (shift) and aerated for 60 min.

was extracted from the cells by the addition of 5.0 ml of boiling glass-distilled water. The tube filled with the hot water was vortexed vigorously. After the tube had cooled, the filter was removed, and the cells were pelleted by centrifugation. The supernatant was then filtered through a second cellulose filter (pore size, 0.2 μm). This second filtration contributed to greater reproducibility. Results were comparable with both cellulose acetate and cellulose nitrate filters.

All reagents were obtained from commercial sources and were used as received.

RESULTS

Comparable values for glutamate accumulation were obtained when cells were harvested by filtration, by centrifugation for 5 min in a clinical centrifuge or by rapid collection in a microcentrifuge. We found no difference in glutamate levels when glutamate was extracted by the addition of boiling water and when cells were boiled for 5 min. In some experiments, cells were collected by filtration and boiling water was passed through the cells. Again, this did not affect glutamate levels. Extracting the glutamate with 95% ethanol, drying the sample, and resuspending the sample in water gave comparable results. The extraction and stability of glutamate does not appear to be critical. We found that boiling a solution of glutamine for 5 min and then cooling the mixture on ice did not cause glutamate to be formed. Glutamine pools in cells do not contribute to the glutamate measured.

Accumulation of glutamate in response to osmotic stress. The results of measurements of accumulation of glutamate in cells collected in stationary phase and exponential phase and in cells grown to mid-exponential phase, collected, and resuspended in medium with a high osmolarity are shown in Table 2. These data show that regardless of growth conditions, cells accumulated excess glutamate when they were osmotically stressed. When cells in stationary growth phase (overnight cultures) were harvested and the glutamate accumulation was determined, cells that had been grown with osmotic stress had higher levels of glutamate than did cells grown in the control medium (Table 2). Compared with cells in stationary growth, cells in mid-exponential growth accumulated somewhat greater amounts of glutamate when they were grown with osmotic stress (Table 2). Cells grown in M63, harvested, and resuspended in medium causing osmotic stress (a shift) were found to accumulate glutamate at a linear rate for 60 min (31). The levels were a little higher than those found in exponential-phase cells (Table 2).

Potassium had an effect comparable to that of sodium when present in excess. Sucrose at 500 nM, with a lower osmolarity than that of NaCl or KCl at 500 mM, caused comparable amounts of glutamate to accumulate (Table 2). It was found that cells growing with 720 mM sucrose (providing an osmolarity comparable to 500 mM NaCl) did not accumulate appreciably more glutamate (data not presented).

Glycerol enters cells by facilitated diffusion and does not affect the turgor of cells (36). Values for glutamate were highly variable when glycerol was present. When cells were harvested in log phase, glycerol appeared to stimulate glutamate accumulation. However, when cells were harvested after overnight growth or after they had been shifted to medium with glycerol, glutamate did not accumulate in excess (Table 2).

Glutamate accumulation and stationary growth phase. Recent evidence indicates that at least some osmotically regulated functions (e.g., trehalose synthesis) are derepressed when cells enter stationary growth phase (29, 30). Accumulation of glutamate in response to osmotic stress does not appear to be a function associated with stationary phase. Cells were grown in the control medium with 0.1% glucose to stationary phase and incubated overnight. Presumably, these cells were thoroughly in stationary phase. These cells had no detectable glutamate pools. We found when these stationary cells were collected and suspended in fresh medium with NaCl, more than 500 nmol of glutamate mg of protein^{-1} accumulated after incubation for 60 min. Cells resuspended in the control medium accumulated 80 nmol mg of protein^{-1}. These cells grew very little when they were resuspended in the fresh medium during this interval. Glutamate production is not a consequence of stationary phase metabolism.

Reduced protein synthesis and sparing of glutamate. It has been proposed that since osmotic stress slows growth dramatically, protein synthesis and the need for amino acids are reduced. This would spare the glutamate produced by the cell, and the amino acid would accumulate (37). If glutamate accumulates because protein synthesis is inhibited when cells are osmotically stressed, the addition of antibiotics that inhibit protein synthesis should have the same effect. Chloramphenicol (25 μg ml^{-1}) inhibited growth of the cultures completely. This antibiotic caused glutamate levels to increase in unstressed cells (243.3 and 159.3 nmol of glutamate mg of protein^{-1} for cultures with and without chloramphenicol, respectively), indicating that some sparing does occur. When cells were resuspended in medium with the antibiotic and 500 mM NaCl to induce osmotic stress, still more glutamate accumulated (492.6 and 453.5 nmol of glutamate mg of protein^{-1} for cultures with and without chloramphenicol, respectively). Comparable results were obtained with streptomycin and kanamycin. These results suggest that the excess glutamate is not accumulated simply by sparing. This experiment also shows that de novo protein synthesis is not required for increased production of glutamate.

Although this experiment showed that sparing cannot account for the glutamate made when cells were shifted to a medium with high osmolarity, it does not preclude sparing when cells are grown in steady state with osmotic stress. Decreased protein synthesis should result in reduced synthesis of amino acids, and this would spare glutamate.

Glutamate production in mutant strains. A role for GDH or GOGAT was tested with mutants defective in these activities. The results of this experiment are shown in Table 3. Note that in these experiments, the single mutants were grown to mid-exponential phase, harvested, washed, and resuspended in the control medium or medium with 500 mM NaCl without glutamate. These data show that neither mutation had much

TABLE 3. Glutamate production in mutant strains

Strain	Genotype	Glutamate (nmol mg of protein^{-1})[a]	
		Without NaCl (control)	With NaCl
TA831	Wild type	150.8	422.9
SK705	*gdh*	134.4	383.2
SK2207	*glt*	131.1	381.2
SK2214	*glt gdh*	61.3	139.5
LT2	Wild type	149.0	455.7

[a] Cells were grown to mid-exponential phase in M63 with 1 mM glutamate, harvested by filtration, washed with M63 without glutamate, and resuspended in M63 alone and M63 with NaCl (500 mM) without glutamate. Cells were harvested after aeration for 1 h.

TABLE 4. Effect of inhibitors of enzymes involved in glutamate synthesis

Inhibitor	Enzyme activity[a]			Glutamate (nmol mg of protein^{-1})[b] in medium	
	GDH	GOGAT	TA	Without NaCl	With NaCl
None (control)	+	+	+	155.8	418.5
NH_4 (1 mM)	−	+	+	134.2	403.5
MTSF	+	−	+	70.9	282.7
$-NH_4$, MTSF	−	−	+	50.5	141.5
AOA	+	+	−	189.5	488.9

[a] Enzymes include GDH, inactive when NH_4^+ is limiting; GOGAT, inactive with the addition of 1 mM MTSF; and transaminase (TA), inactive with the addition of 5 mM AOA. +, enzyme is active; −, the enzyme is inactive in this situation.

[b] Cells were grown in M63 with histidine, collected by filtration, washed with 4 × 2.5 ml of M63 without NH_4^+, and resuspended in the medium as indicated. Cells were incubated for 1 h and harvested by filtration. Unless noted otherwise, cells were incubated in conventional M63 medium with 15 mM ammonium.

effect on glutamate levels when cells were grown in medium with high levels of ammonium (M63 is 15 mM with respect to ammonium). Cells still accumulated excess glutamate when they were osmotically stressed.

The double mutant *gdh glt* was grown with 1 mM glutamate. When cells were shifted to medium without glutamate, glutamate levels were much lower. Osmotic stress still caused a 2.6-fold excess of glutamate to accumulate (Table 3).

The *gdh* mutant did not have detectable levels of enzyme by Western blot. Neither subunit of GOGAT was detectable in the *glt* mutant measured by Western blot (33b). The glutamate produced in response to osmotic stress could not be due to leakiness of the *gdh* and *glt* mutations. The K_m value of ammonium for GDH is 1 mM (10, 11, 51). It has been proposed that the affinity of GDH for ammonium is enhanced by osmotic stress (the K_m value is lowered). We found that the *glt* mutant (which uses GDH for glutamate production) grew more slowly than did wild-type cells on medium with 1 mM ammonium. However, relative to that of wild-type cells, cell growth was reduced a comparable amount with the addition of 500 mM NaCl, indicating that the affinity of the enzyme for ammonium is not affected by the intracellular response to osmotic stress.

Inhibitors of enzymes involved in glutamate synthesis. Methionine sulfone (MTSF) is a very effective inhibitor of GOGAT in vitro in *K. aerogenes* (9). Although this question has not been addressed for *S. typhimurium*, 1 mM MTSF was found to inhibit growth of *S. typhimurium* in M63. The addition of 1 mM glutamate relieved this inhibition. A comparable situation was observed in *K. aerogenes*. Presumably, MTSF has a comparable inhibitory effect in *S. typhimurium*. It is possible that MTSF inhibits some other key reaction in cells and that the addition of glutamate reduces transport of MTSF. However, the data for *K. aerogenes* do not support this interpretation.

When GDH was limited by resuspending cells with 1 mM ammonium (Table 4, strain SK705), glutamate production was comparable to that observed in the *gdh* mutant (Table 3). Excess glutamate accumulated. When GOGAT was inhibited by the addition of MTSF (Table 4), glutamate levels were reduced. However, excess glutamate was still accumulated in response to osmotic stress. When GDH activity was reduced by limiting NH_4^+ and GOGAT was inhibited by the addition of MTSF (Table 4), the levels of glutamate were reduced to values similar to those observed for the double mutant (Table 3). A series of experiments in which cells were washed in medium without ammonium and aerated for 60 min without ammonium were done. Presumably, under these conditions, the intracellular concentration of ammonium would be negligible. Values were comparable to those obtained with the double mutant (Table 3).

Transamination of 2-ketoglutarate by using assimilated ammonium sources is inhibited by amino oxyacetic acid (AOA) (33a, 50a, 56). Glutamate levels were about 50 nmol mg of protein^{-1} higher in both the controls and cells under osmotic stress with AOA, indicating that glutamate can be spared. Excess glutamate still accumulated in osmotically stressed cells inhibited with AOA (Table 4).

Effect of K^+ and glutamate. In *E. coli* (2), we found that GDH is stimulated by K^+ in vitro with crude cell extracts (2, 44). We found (by analysis of a linear plot of specific activity versus the K^+ concentration) that 60 mM K^+ stimulated GDH by 50%. In contrast, 297.5 mM K^+ inhibited GOGAT by 50%. By using the values for K^+ and cytoplasmic water from the study by Caley et al. (12), the intracellular concentration of K^+ varied from 215 mM in unstressed cells to 805 mM in osmotically stressed cells. The stimulation of GDH and inhibition of GOGAT by K^+ could be physiologically significant. In *E. coli*, we found both GDH and GOGAT to be inhibited by excess glutamate. Glutamate (219 mM) inhibited GDH by 50%, while 210.5 mM glutamate inhibited GOGAT by 50% (2). By using the values for cytoplasmic water determined by Cayley et al. (12), cells growing in 250 mosM medium (the controls were grown in M63 medium) have 2.08 μl of cytoplasmic water mg (dry weight)$^{-1}$. This is equivalent to 3.79 μl mg of protein^{-1} (33). A concentration of 150 nmol of glutamate mg of protein^{-1} (the values observed in unstressed cells) would be equivalent to 39.58 nmol μl^{-1}. Cells growing in 1,260 mosM medium (M63 with 500 mM NaCl) have 1.04 μl of cytoplasmic water mg (dry weight)$^{-1}$ (12, 13). The concentrations of glutamate that accumulate in stressed cells would be equivalent to 317 mM glutamate. Inhibition of these two activities by the accumulated glutamate could occur in osmotically stressed cells. We did not encounter levels of glutamate greater than approximately 600 nmol mg of protein^{-1} in any experiments. Accumulation of glutamate could limit its production.

DISCUSSION

Tempest et al. (59) first reported accumulation of glutamate in cells grown with osmotic stress. Measures (44) reported accumulation of glutamate but no other amino acids in a

variety of bacteria grown in complex undefined medium with osmotic stress. With *E. coli*, grown in a complex undefined medium, Measures reported a 150% increase in glutamate pools with the addition of 650 mM NaCl. Since cells were grown in a complex undefined medium, the values are not comparable. These investigators offered evidence suggesting that GDH could be responsible for the excess glutamate.

Csonka and Hanson (20), citing a personal communication, have proposed that the excess glutamate made in response to osmotic stress is made by GDH. However, the techniques used to determine this were not described and no data were provided.

Csonka (18) reported 136 nmol of glutamate mg of protein^{-1} when *S. typhimurium* was grown in M63 medium and 290 nmol mg of protein^{-1} when cells were grown in medium with 650 mM NaCl. He also found 10 and 142 nmol of glutamine in the two cultures. Glutamate and glutamine were measured by amino acid analysis. This was only a twofold increase in glutamate levels (18). McLaggan et al. (43a) also reported that glutamine as well as glutamate levels increased in *E. coli* grown with osmotic stress. These investigators used high-performance liquid chromatography to separate cytoplasmic compounds and quantified them by optical *A*.

Dinnbier et al. (23) reported 150 nmol of glutamate mg of protein^{-1} in *E. coli* grown in minimal medium, with 680 nmol mg of protein^{-1} accumulating after the cells were shifted to medium causing osmotic stress. This represented a 4.5-fold increase in glutamate levels. Glutamate was measured by nuclear magnetic resonance.

Caley et al. (12, 13), also working with *E. coli* K-12, reported 0.06 umol of glutamate mg (dry weight)$^{-1}$, equivalent to 86 nmol of glutamate mg of protein^{-1} in cells grown in minimal defined medium with osmolarity lower than that of M63 and the equivalent of 329 nmol in cells grown in medium with 500 mM NaCl. Again, this represented a fourfold increase. These investigators also used nuclear magnetic resonance to measure glutamate levels.

Alternative mechanisms. It has been suggested that bacteria could utilize histidine to supply the glutamate. *S. typhimurium* cannot use histidine as a sole source of ammonium (28). Many of the experiments were repeated with strain LT2, wild type with respect to histidine, so that no exogenous histidine was present, and the results were comparable. Therefore, histidine degradation to glutamate is not likely to be involved.

There could be a new pathway for de novo synthesis of glutamate expressed when cells are osmotically stressed. This would explain why the double mutant, lacking both GDH and GOGAT, accumulates glutamate under these conditions. However, we found that the two double Glt$^-$ mutants remained auxotrophic for glutamate when cells were plated on M63 with 500 mM NaCl.

It has also been suggested that cells excrete much of the glutamate produced under normal conditions and that with osmotic stress they retain a greater portion. We found less than 5 nmol of glutamate ml^{-1} in the growth medium (31). Cells grown without osmotic stress do not appear to excrete glutamate. By using published values for intracellular water in stressed cells (12, 13), it can be determined that 5 nmol ml^{-1} is comparable to an intracellular concentration of 2 mM.

The glutamate that accumulates in response to osmotic stress could be derived from the degradation of cellular protein. A gram (dry weight) of bacterial cells includes 500 μmol of glutamate and glutamine (33). If one cell weighs 2.8×10^{-13} g, it can be calculated that one cell contains 1.4×10^{-10} μmol of glutamate and glutamine. From Materials and Methods, 1 mg of protein = 5.4×10^9 cells; therefore, 5.4×10^9 cells would contain 760 nmol of glutamate and glutamine. We found that 400 to 600 nmol of glutamate mg of protein^{-1} (2.2×10^9 cells) accumulated. Virtually all the protein in the cell would have to be degraded to account for the excess glutamate that accumulates.

Glutamate could be spared when cells are grown with osmotic stress because the rate of protein synthesis and the need for amino acids are reduced (37). Since glutamate provides nearly all the amino moieties in cells, this is an appealing model. When cells were shifted from the control medium to medium with chloramphenicol, some excess glutamate accumulated. However, even greater quantities of glutamate accumulated with osmotic stress. Chloramphenicol inhibits protein synthesis completely, so the glutamate that accumulates under these conditions should represent the maximum levels that can accumulate because of sparing. When AOA was present, glutamate utilization as a substrate for transamination reactions should have been inhibited, allowing an excess of glutamate to be spared. Excess glutamate accumulated in cultures with AOA. Yet, more glutamate accumulated when cells were osmotically stressed (Table 4). Both of these experiments involved cells shifted from the control medium to medium with osmotic stress. In cells growing in steady state, excess glutamate could accumulated because protein synthesis is slower with osmotic stress and amino acid biosynthesis is decreased.

Studies of mutants. All three mutants, Gdh$^-$, Gogat$^-$, and the mutant lacking both activities, accumulated glutamate in response to osmotic stress (Table 3). The single *gdh* or *glt* mutations caused glutamate levels to decrease only a little. Apparently, the activities compensate for one another.

The *gdh* mutation did not prevent accumulation of excess glutamate, indicating that this reaction is not responsible for the excess glutamate as has been suggested (20, 44, 59). In the double *gdh glt* mutant, glutamate levels were lower than those in wild-type cells. Nevertheless, excess glutamate accumulated in the double mutant with osmotic stress. This suggests that minor salvage pathways can provide significant amounts of glutamate. Since a nearly threefold excess of glutamate accumulated when the double mutant was shifted and aerated in medium without glutamate, these salvage pathways appear to be stimulated by osmotic stress and the accompanying changes in the intracellular situation.

Inhibitors. Inhibiting GOGAT with MTSF did not prevent accumulation of excess glutamate in response to osmotic stress. Reducing GDH activity by limiting ammonium did not prevent osmotic stress. The addition of AOA to inhibit transamination of 2-ketoglutarate by using assimilated sources of nitrogen did not prevent accumulation in response to osmotic stress. All of these techniques affected glutamate levels. None prevented accumulation of glutamate. This indicates that no one reaction can account for the production of excess glutamate in osmotically stressed cells.

The model. A typical bacterial cell contains nearly 10,000 μmol of assimilated ammonium g (dry weight)$^{-1}$ (33). If bacterial cells are 55% protein, then this is equivalent to 18.1 μmol of NH_4^+ mg of protein^{-1}. Bacteria growing with a generation time of 1 h would need to assimilate ammonium, that is, to synthesize glutamate, at a rate of 303 nmol min^{-1} mg of protein^{-1}. The rate of synthesis of excess glutamate can be calculated as 315.6 − 126.2 = 189.4; 189.4/60 = 3.2 nmol mg of protein^{-1} min^{-1} (Table 2). This represents 1.0% of a cell's capacity to make glutamate [(3.2 nmol/303 nmol) × 100]. The activities of enzymes involved in glutamate metabolism would not have to change dramatically to account for the accumulation of excess glutamate in response to osmotic stress. Since

every amino group in cells is ultimately derived from glutamate (glutamine is synthesized from glutamate) and these amination reactions are reversible, there are literally hundreds of reactions that could provide the glutamate.

Rhizobium meliloti accumulates K^+ and glutamate when the organism is osmotically stressed. This has been studied in some detail (8, 26, 39). Ammonium assimilation in *R. meliloti* is unlike that in enteric coliform bacteria (21, 53, 54, 58). In this bacterium neither GDH nor GOGAT appears to be responsible for the production of the excess glutamate. However, unlike the situation in *S. typhimurium*, production of excess glutamate is completely blocked by the addition of AOA, indicating that transamination of 2-ketoglutarate provides the glutamate.

In summary, our data show that *S. typhimurium* accumulates glutamate in response to osmotic stress. This accumulation of excess glutamate is an active response of the cell to osmotic stress and not a metabolic accident. Osmotic stress alters the activity of enzymes involved in glutamate metabolism, and this results in increased pools. No one reaction is responsible for this excess glutamate.

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