Dependence of the Putrescine Content of *Escherichia coli* on the Osmotic Strength of the Medium*

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**SUMMARY**

Putrescine is the polyamine present in highest concentration in *Escherichia coli*; however, few specific functions for it are known. We found that cells growing in nutrient broth supplemented with high concentrations of NaCl, KCl, MgCl₂, or sucrose had greatly reduced levels of cellular putrescine. On the basis of osmotic strength, all four solutes produced similar decreases in putrescine content. In contrast, glycerol had little effect on the amount of cellular putrescine. Cellular spermidine content was not affected by any of the additives. When cells were grown in high NaCl, pools for most amino acids increased; a few remained the same or decreased slightly.

A sudden increase in the osmolarity of the medium led to a rapid excretion of cellular putrescine while there was no decrease in spermidine or free amino acids. This loss of putrescine could be blocked by sodium azide or sodium arsenate. The mechanism of putrescine excretion has two components; one is dependent on high concentrations of potassium ion in the medium, and the other is not. Cells grown in the presence of 11 mM potassium and then put into potassium-free medium containing 0.1 mM potassium and resuspended in low salt medium did increase 6-fold within 2 min at reexposure to low salt medium.

Putrescine (1,4-diaminobutane) and spermidine (1-aminopropyl-1,4-diaminobutane) are the polyamines found in highest concentrations in *Escherichia coli*. The putrescine content is usually several-fold greater than that of spermidine (1); in vivo both polyamines turn over slowly if at all (2, 3). In *E. coli* both polyamines have been implicated in various aspects of *in vitro* RNA and protein synthesis (4). Spermidine is more effective than putrescine in all such systems in which both polyamines have been examined.

Few specific functions for putrescine are known. It is a precursor of spermidine (2, 5). Hirschfield et al. (6) described a mutant of *E. coli* in which putrescine synthesis could be blocked by growth on arginine-containing medium. The slow growth of these cells was greatly stimulated by adding putrescine, and much less by spermidine. Morris and Jorstad (7) found that reducing the intracellular putrescine of another mutant to 1% of normal decreased the doubling rate by only 10%. Inouye and Pardee (8) found that if an arginine auxotroph of *E. coli* was starved for arginine for 1 hour, addition of arginine caused the cells to divide synchronously. Putrescine was specific in abolishing this synchrony. The authors suggested that an increase in the molar ratio of putrescine to spermidine might be a critical factor for cell division; however, this ratio was at least twice as much affected by a decrease in the relative spermidine content as by an increase in the relative putrescine content. All other proposed specific functions for putrescine require relatively low concentrations of putrescine and do not explain the high levels found in autotrophic *E. coli*.

We postulated that high concentrations of putrescine might play some role in maintaining the cellular ionic or osmotic balance. Evidence presented here demonstrates that mono- and divalent...
ions and several sugars, present in the medium, can reduce the cellular putrescine content without affecting the spermidine content. Glycerol, which freely penetrates the cell (9, 10), does not reduce the cellular putrescine content.

A sudden increase in the osmolarity of the medium leads to a prompt excretion of cellular putrescine, but not spermidine. Because the cellular K⁺ content rises under the same circumstances (11), we suspected that K⁺ uptake and putrescine excretion might be coupled. Evidence presented here demonstrates that the rate of putrescine excretion is dependent on the K⁺ concentration in the medium. A preliminary report of this work has appeared (12).

MATERIALS AND METHODS

Bacteria and Media—E. coli B and K12-112 (λ h) (referred to as K12(λ) in the text) were from our laboratory stock. Low salt nutrient broth contained 8.00 g of Difco nutrient broth per liter. M9 medium contained 18.2 mM KH₂PO₄, 38.5 mM Na₂HPO₄, 15.5 mM NH₄Cl, 7.23 mM glucose, and 0.63 mM MgSO₄. K⁺ medium (11 mM) contained 11.0 mM KCl, 23.2 mM Na₂HPO₄, 9.35 mM NH₄Cl, 11.0 mM Na₂HPO₄, 8.82 mM glucose, and 1.00 mM MgSO₄ (pH 6.92). Media with low concentrations of Na⁺ were constructed by replacing the sodium phosphate buffer with potassium or ammonium phosphate buffers of the same ionic strength. Media with different concentrations of K⁺, NH₄⁺, or Mg²⁺ were made by replacing some or all of the KCl, NH₄Cl, or MgSO₄ with NaCl or Na₂SO₄, so that constant Cl⁻ and SO₄²⁻ concentrations were maintained. Medium at pH 8.0 was made by adjusting the phosphate buffer stock solution to pH 8.0 with NaOH. The final phosphate concentration in medium at pH 8.0 was the same as in the standard 11 mM K⁺ medium. Viable cell concentrations were determined by diluting culture aliquots by adjusting the phosphate buffer stock solution to pH 8.0 with NaOH (unless otherwise noted). Viable cell concentrations were maintained. Medium at pH 8.0 was made by adjusting the phosphate buffer stock solution to pH 8.0 with NaOH. The final phosphate concentration in medium at pH 8.0 was the same as in the standard 11 mM K⁺ medium. Viable cell concentrations were determined by diluting culture aliquots by adjusting the phosphate buffer stock solution to pH 8.0 with NaOH (unless otherwise noted).

Isolation, Separation, and Quantitation of Polyamines—Aliquots of 50 ml were removed from the bacterial culture and chilled on ice. Optical density (at 600 nm; Beckman DU spectrophotometer), pH, and viable cell concentration were measured. Culture aliquots were then centrifuged at 4° and 2000 × g and 4° for 10 min. Pellets were taken up in ice-cold 0.25 M HClO₄ to a total volume of 5 ml (17), allowed to stand in an ice bucket for 10 min, and centrifuged at 2000 × g for 10 min. Pellets were saved for protein estimation (16). Supernatants were then adjusted to pH 7.1 with 1 N KOH and the KClO₄ precipitates collected by centrifugation at 2000 × g and 4° for 10 min. Supernatants were then adjusted to pH 2.0 with concentrated HCl, and 1.0 to 2.0 ml aliquots were analyzed for amino acid composition with a Beckman model MS instrument.

Radioactive Chemicals and Assay—[1-¹⁴C]Putrescine-2 HCl (109 μCi per mg) and spermidine ([aminoguanidino]-[1-¹⁴C]ethylmethyleneamine-3 HCl) (41.7 μCi per mg) were obtained from New England Nuclear. Radioactivity was determined with a Packard liquid scintillation spectrometer (model 3310) in 5 or 10 ml of counting solution which consisted of 4.0 g of 2,5-diphenyloxazole (Packard), 0.05 g of 1,4-bis[2-(4-methyl-5-phenyl- o xoazoly)]-benzene (Packard), and 120 g of naphthalene (Eastman Kodak) in 1 liter of p-dioxane (Eastman Kodak).

Measurement of Potassium Uptake by E. coli B—Measurement of cellular potassium content was made by the method of Zarleno and Schultz (18) with slight modifications. Aliquots of 1 ml were rapidly filtered onto type B-6 Schleicher and Schuell filters. Cells from cultures in 11 mM K⁺ medium were washed twice with 2 ml of 0.2 M sucrose (special enzyme grade, Mann Research Laboratories); cells in 11 mM K⁺, 0.4 M sucrose medium were washed with 0.6 M sucrose. The filters were placed in α-toluou counting vials (Nuclear-Chicago), and 1 drop of concentrated, redistilled nitric acid (G. Frederick Smith Chemical Company, Columbus, Ohio) was added to each filter. After 30 min, 2 ml of 15 mM lithium standard solution (Instrumentation Laboratories) were added to each vial, and potassium values were determined with a flame photometer (Instrumentation Laboratories, model 143). Potassium contents were corrected for blank filters which contained an average of 0.088 μequ of potassium.

RESULTS

Polyamine Content of Bacteria Grown at Different Osmotic Strengths—Cells were initially grown in low salt nutrient broth which contains low concentrations of several cations including Na⁺, 10 mM; K⁺, 25 mM; Ca²⁺, 0.69 mM; Mg²⁺, 0.31 mM (determined on the single bottle of Difco nutrient broth used for all experiments). Growth at increased concentrations of NaCl, KCl, MgCl₂, or sucrose, up to 0.6 osmol per liter, reduced the putrescine content of E. coli B by more than 5-fold (Fig. 1). Growth at high concentrations of glycerol did not bring about a comparable reduction in putrescine content. The spermidine content (Fig. 1) varied only slightly with growth in media of different osmotic strengths. E. coli K12(λ) showed similar decreases in putrescine content with increasing concentrations of NaCl and MgCl₂; again, spermidine content was unaffected (data not shown).

The following experiments show that the low putrescine content observed with cells grown in high osmolarity media is not due to artificial loss of cell constituents. (a) Centrifugation at...
either 4° or 37° and rinsing of cells in 0.15 M or 0.6 M NaCl caused only slight variations in putrescine content and no loss of cell viability. Washing of cells in sucrose solutions or adding sucrose to washed cells had little effect on the polyamine content (described in the legend to Fig. 1). (b) The pH of our bacterial cultures varied only between pH 5.8 and 6.9 and did not affect the putrescine content. Larger variations of pH affect the putrescine content (13). (c) Cellular putrescine content did not correlate with changes in growth rates. (d) E. coli B, grown in 11 mM K⁺ medium in the presence of 0.4 M NaCl, showed increases in the pools of most amino acids (Table I, columns B and C), whereas the putrescine concentration was reduced.

**Polyamine Content after Sudden Increase in Osmolarity of Medium—**

E. coli B, previously labeled with [¹⁴C]putrescine in low salt nutrient broth, was transferred to nutrient broth containing (a) 0.3 M NaCl, or (b) 0.6 M sucrose, or (c) 0.6 M glycerol. Within 5 min the ¹⁴C content of the cells in NaCl or sucrose decreased more than 10-fold; the ¹⁴C content of the cells in glycerol decreased about 20% (Fig. 2). (p-Xylose, d-arabinose, and maltose were similar to sucrose in their ability to bring about a loss of ¹⁴C from the cells (data not shown).) In a similar experiment the ¹⁴C released from cells after a shift to high salt nutrient broth (0.6 M NaCl) was identified as ¹⁴C]putrescine by extraction into butanol-1 (13) and chromatography.

To confirm these results, cellular putrescine and spermidine contents were measured chemically after a shift to high salt nutrient broth (0.6 M NaCl). Spermidine values remained at the low salt level for 2 hours after adding NaCl, whereas the putrescine content fell 6-fold in 9 min and remained depressed for at least 2 hours (Fig. 3, circles).

As a control for the experiment in Fig. 2, cells were shifted to 0.3 M NaCl, 0.6 M sucrose, or 0.6 M glycerol on ice. After 15 min intracellular ¹⁴C was measured as for the other samples. Little or no ¹⁴C was lost from the cells during this time. The fact that cold could inhibit the rapid putrescine loss suggested that the process might be energy dependent.

Therefore, the effect of a sudden increase in salt concentration was repeated in the presence of 6 mM sodium azide or 10 mM NaCl.

**Table 1**

Amino acid pools of E. coli B in high osmolarity medium

| Amino acid          | Δ Low salt control | B. NaCl added | C. Grown in NaCl | C. Grown in NaCl |
|---------------------|-------------------|---------------|-----------------|-----------------|
| Alanine             | 17                | 32            | 50              |
| Arginine            | <0.10             | 0.13          | 0.23            |
| Asparagine and glutamine | 3.2           | 16            | 16              |
| Aspartic acid       | 1.9               | 2.5           | 2.5             |
| Half cystine        | <0.10             | <0.10         | 1.0             |
| Glutamic acid       | 78                | 280           | 330             |
| Glutathione         | 120               | 340           | 390             |
| Glycine             | 1.5               | 6.2           | 3.2             |
| Histidine           | <0.10             | 1.8           | 0.56            |
| Isoleucine          | 1.1               | 2.5           | 0.68            |
| Leucine             | 1.0               | 6.1           | 3.1             |
| Lysine              | 1.6               | 14            | 2.8             |
| Methionine          | 3.8               | 5.3           | 14              |
| Ornithine           | <0.10             | 0.19          | 1.1             |
| Phenylalanine       | 2.0               | 3.2           | 0.78            |
| Serine              | 0.63              | 2.3           | 1.3             |
| Threonine           | 3.0               | 2.7           | 1.9             |
| Tyrosine            | 2.1               | 3.6           | 0.67            |
| Valine              | 16                | 8.8           | 9.2             |

Nanumoles of putrescine-2 HCl per mg of cell protein

| Milligrams of total cell protein | Total viable cell number |
|---------------------------------|--------------------------|
| 15.8                            | 7.4 x 10⁹                |
| 24.6                            | 5.0 x 10⁹                |
| 11 x 10⁹                        | 11 x 10⁹                |

Fig. 1. Effect of osmolarity of the medium on polyamine content of E. coli B. Results are calculated as μg of total cellular polyamine hydrochloride per mg of protein. A fresh overnight culture of E. coli B grown in low salt nutrient broth was diluted 1:100 with nutrient broth containing NaCl (○—○), MgCl₂ (●—●), KCl (■—■), sucrose (□—□) or glycerol (▲—▲), and the cultures were grown to an optical density of 0.300 to 0.400. The cultures were then chilled and analyzed for polyamines; sucrose cultures were washed in 0.2 M sucrose instead of 0.15 M NaCl. Washing cultures grown in low salt nutrient broth with 0.2 M sucrose or adding 0.4 ml of 0.6 M sucrose to the pellet of a low salt culture washed with 0.15 M NaCl produced putrescine-2 HCl contents of 27 and 24 μg per mg of protein, respectively (compare to points at 0 osmols per liter). Spermidine-3 HCl values for these controls were 11 and 8.2 μg per mg of protein, respectively.
change in salt concentration per se. However, when cells were
tion for cultures grown in high salt and not a response to the
a shift to high salt reflects the normal rate of putrescine excre-
the process may directly or indirectly require energy.

fact that two inhibitors could block putrescine loss implies that
experiments with inhibitors affected the spermidine level. The
blocked the putrescine loss (data not shown). None of the ex-
used. Sodium arsenate (neutralized to pH 6.8 before use) also
inhibited the loss of putrescine, even at the low concentration
for NaCl alone and sodium azide plus NaCl indicates that azide
affected by the particular solute used to increase the osmolarity
with additions of 0.35 M NaCl or 0.7 M sucrose. The same rates
of the medium, the experiment shown in Fig. 4B was repeated
above. At the end of the experiment these were filtered and
counted in the same manner as the other samples. The values for
these samples are plotted as the time = 0 points in the figure.

sodium arsenate. (These relatively low concentrations blocked
any increase in cell titer without killing cells in either high or
low salt nutrient broth.) Sodium azide by itself caused a small
loss of cellular putrescine (Fig. 3), but comparison of the curves
above. When cells were suspended in 0.4 M NaCl medium con-
Fig. 2. Effect of NaCl, sucrose, and glycerol on release of
[14C]putrescine from E. coli B. A 13-ml culture was grown in low
salt nutrient broth to about 1.3 x 10^8 cells per ml and incubated
at 37° for 15 min with 0.08 μCi per ml of [14C]putrescine. The cells
were then sedimented at about 2000 × g (Sorvall dasc centrifuge)
for 10 min at 37° and twice washed in 13 ml of fresh, warm, low salt
nutrient broth and centrifuged. Cells were then taken up in 13 ml
of warm, low salt nutrient broth and 2.5 ml were added to 2.5 ml
of low salt nutrient broth (----), nutrient broth plus 0.6 M
NaCl (O---O), nutrient broth plus 1.2 M sucrose (□□□□) or
nutrient broth plus 1.2 M glycerol (△△△△). At the indicated
times 1-ml samples were withdrawn, chilled quickly on ice and
filtered onto Schleicher and Schuell filters (type B-6, pore size
0.45 μ, 25 mm in diameter). (Aliquots which were filtered im-
mEDIATELY but not chilled produced the same results.) Filters
were washed with about 12 ml of cold 0.15 M NaCl and counted in
5 ml of scintillation fluid. As a control, at time = 0 part of the
prelabeled culture was chilled and then 0.5-ml aliquots were
diluted into cold, 0.5-ml aliquots of the four solutions mentioned
above. At the end of the experiment these were filtered and
counted in the same manner as the other samples. The values for
these samples are plotted as the time = 0 points in the figure.

It was possible that the excretion of [14C]putrescine following
a shift to high salt reflects the normal rate of putrescine excre-
tion for cultures grown in high salt and not a response to the
change in salt concentration per se. However, when cells were
grown and previously labeled with [14C]putrescine in high salt
nutrient broth (0.3 M NaCl), they excreted 14C in high salt at a
very slow rate similar to cells which were grown, labeled, and
incubated in low salt medium. A similar experiment using 11
mm K+ medium gave identical results.

Effect of External Potassium Concentration on [14C]Putrescine
Excretion. E. coli B, grown in 11 mM K+ medium (chemically
defined medium with calculated osmolarity of 0.14) and labeled
with [14C]putrescine, excreted 64% of the cellular 14C within 3
min after being transferred to 11 mM K+, 0.4 M NaCl medium
(Fig. 4A). Cells maintained in low osmotic strength medium
lost putrescine very slowly (about 4% in 3 min). In contrast
to loss of putrescine, amino acid pools were not depleted by
transfer of cells to high osmolarity medium (Table I, column B).
As shown in Fig. 4B, reducing the external K+ concentration
significantly reduced the rate of excretion of cellular [14C]putres-
cine; cells lost only 28% of the 14C after 3 min in no K+, 0.4 M
NaCl medium. To establish that this rather slow excretion rate was not af-
fected by the particular solute used to increase the osmolarity of
the medium, the experiment shown in Fig. 4B was repeated
with additions of 0.35 M NaCl or 0.7 M sucrose. The same rates
of [14C]putrescine excretion were observed, indicating that the
high concentration of Na+ present in the experiment shown in
Fig. 4D could not partially substitute for K+.

The reduction in the rate of [14C]putrescine excretion is reversi-
ble. When cells were suspended in 0.4 M NaCl medium con-
taining no K+ and made 11 mM in KCl 2 min later, they promptly
excreted 61% of the cellular 14C in the next 3 min (Fig. 4C).

The rate of [14C]putrescine excretion in 0.4 M NaCl medium
was measured with several different concentrations of K+, rang-
ing from 0.2 mM to 11 mM. Below 0.4 mM K+, the rate of excre-
tion was slow and varied little with the external K+ concen-
tration. At higher K+ concentrations, the rate of putrescine excretion increased dramatically. The concentration of K+ which produced a half-maximum rate of [14C]putrescine excre-
tion was 0.69 mM.

Nutrient broth contains 2.5 mM K+. Therefore, the previ-
ously mentioned studies of putrescine excretion in nutrient broth
Osmolarity of Medium and E. coli Putrescine Content

FIG. 4. Ability of E. coli B grown in 11 mM K+ medium to excrete [14C]putrescine after a sudden increase in the osmolarity of the medium. For each of the experiments shown, a 5-ml culture of E. coli B was grown to optical density 0.400 in 11 mM K+ medium, incubated with 1 μCi of [14C]putrescine at 37° for 15 min, centrifuged, and washed once in 5 ml of 11 mM K+ medium. Cells were then taken up in 8 ml of 11 mM K+ medium, distributed to two tubes, and centrifuged. In Part A, one pellet was resuspended in 7 ml of 11 mM K+ medium (○—○); the other pellet was resuspended in 7 ml of 11 mM K+, 0.4 mM NaCl medium (Δ—Δ). At the times shown 1 ml aliquots were filtered onto Schleicher and Schuell filters, and the cellular 14C was determined. In Part B, one pellet was resuspended in 7 ml of medium lacking NaCl and KCl (○—○), the other was resuspended in 7 ml of 0.4 mM NaCl medium lacking KC1 (△—△). In Part C the cells were put in the same two media as in Part B. At 2 min both solutions were made 11 mM in KC1 by the addition of 0.495 ml of 0.1 M KC1 (arrow). The cpm measured after KCl addition were corrected for dilution.

cultures (Figs. 2 and 3) were not affected by low K+ concentration in the medium.

Effect of Medium Osmolarity and Potassium Concentration on Polyamine Content of E. coli B—Putrescine content (measured chemically) is a function of medium osmolarity but not of K+ concentration. Low cellular concentrations of putrescine (23 to 24 μg of total putrescine-2 HCl per mg of protein) resulted in a low cellular putrescine content (2.2 to 3.3 μg per mg of protein), regardless of the K+ concentration. The spermidine contents were similar in all cultures (approximately 7.9 μg of total spermidine-3 HCl per mg of protein).

Table II

| Medium | K+ | 0.7 M Sucrose | Mg2+ | NH4+ | Na+ | Fraction of 14C lost in 3 min
|--------|----|---------------|------|------|-----|-----------------|
| A      | 0.1 mM | — | + | + | + | 0.003 |
| B      | 11.0 mM | — | + | + | + | 0.49 |
| C      | 11.0 mM | — | — | + | + | 0.54 |
| D      | 11.0 mM | — | + | — | + | 0.47 |
| F      | — | + | — | + | + | 0.06 |
| G      | — | — | — | — | — | 0.13 |

a A (+) indicates the concentration found in 11 mM K+ medium; a (−) indicates absence of the particular ion.

* The rates of [14C]putrescine excretion in media B and E were slightly less than the excretion rates shown in Fig. 4 because the sucrose concentration in media B and E was 0.7 M, whereas the NaCl concentration in Fig. 4 was 0.4 M (equivalent to an osmotic contribution of about 0.8 M).

Effect of External Ammonium, Magnesium, Sodium, and Rubidium Concentrations and pH on [14C]Putrescine Excretion—A culture of E. coli B grown in 11 mM K+ medium, labeled with [14C]putrescine, washed, and divided into seven portions which were centrifuged separately. The pellets were then resuspended in various media (Table II). The cells in suspension A (low osmolarity control) lost 14C at the slow rate characteristic of cells in media of low osmotic strength. Cultures B, C, and D, which all contained 11 mM K+ and 0.7 M sucrose, lost [14C]putrescine rapidly. Cultures E, F, and G, which all contained 0.7 M sucrose but lacked K+, had intermediate rates of [14C]putrescine excretion. Thus, the absence of Mg2+ and NH4+ or the absence of Na+ did not significantly alter the excretion patterns. In similar experiments 30 mM Mg2+ or 11 mM Rb+ could not substitute for K+ (data not shown).

The effect of pH on the rate of putrescine excretion was measured over the range of pH 6.4 to 7.3. E. coli B was grown in 11 mM K+ medium (pH 0.9), labeled with [14C]putrescine, washed in growth medium, and split into four portions before the final centrifugation. The cells were put in the same two media as in Part B. At 2 min both solutions were made 11 mM in KCl by the addition of 0.495 ml of 0.1 M KCl (arrow). The cpm measured after KCl addition were corrected for dilution.

Effect of Medium Osmolarity and Potassium Concentration on Putrescine Content of E. coli B—Putrescine content (measured chemically) is a function of medium osmolarity but not of K+ concentration. Low cellular concentrations of putrescine in high osmolarity medium are not a consequence of a K+-dependent, rapid excretion of putrescine. E. coli B grown in 11 mM K+ or 0.1 mM K+ medium contained large and similar amounts of putrescine (23 to 24 μg of total putrescine-2 HCl per mg of protein). Growth in high osmolarity medium (0.6 M sucrose) resulted in a low cellular putrescine content (2.2 to 3.3 μg per mg of protein), regardless of the K+ concentration. The spermidine contents were similar in all cultures (approximately 7.9 μg of total spermidine-3 HCl per mg of protein).

Effect of Potassium Concentration on Growth of E. coli B—In order to determine the ability of cells grown in low concentrations of K+ to excrete putrescine, we measured the generation times of E. coli B at 37° in media containing different amounts of K+. In media containing greater than 0.04 mM K+, the optical
density (at 600 nm) of the culture doubled every 44 min. At K⁺ concentrations of 0.002 mM or less, the optical density doubled every 220 min. At intermediate K⁺ concentrations, the optical density increased at first increased logarithmically with a doubling time of 44 min and then changed to a doubling time of 220 min. The point at which this change occurred varied directly with the K⁺ concentration; for example, in 0.01 mM K⁺ medium, the optical density increased with a doubling time of 44 min to optical density 0.132 and then decreased over the following 30 min to a doubling time of 220 min. To avoid these changes in growth rate, cells were grown in 0.1 mM K⁺, a concentration which is low and yet maintains cells in rapid growth to an optical density greater than 0.700.

**Excretion of [¹⁴C]Putrescine by E. coli B Grown in 0.1 mM K⁺ Medium—** Cultures grown in 0.1 mM K⁺ medium were able to excrete only 22% of the [¹⁴C]putrescine after 3 min in 0.1 mM K⁺, 0.4 mM NaCl medium. Addition of 11 mM K⁺ after 7 min initially produced a slow rate of putrescine excretion. This rate of excretion appeared to increase at later times. Thus, cultures grown in 0.1 mM K⁺ medium are not immediately able to excrete putrescine rapidly in 11 mM K⁺, 0.4 mM NaCl medium.

To quantitate the time required for regeneration of the K⁺-dependent excretion mechanism, a culture of E. coli B was grown in 0.1 mM K⁺ medium, labeled with [¹⁴C]putrescine, washed twice, and then resuspended in 11 mM K⁺ medium. Periodically, aliquots were made 0.4 x in NaCl and incubated for 3 min to measure the rate of [¹⁴C]putrescine excretion. The ability of cells to excrete [¹⁴C]putrescine began to increase immediately after resuspension in 11 mM K⁺ medium; the fraction of [¹⁴C]excreted in 3 min doubled 3 min after resuspension and tripled by 10 min after resuspension. The maximal rate of excretion (70% of [¹⁴C]excreted in 3 min) was reached after 15 min.

To determine the time required for cells to lose the ability to excrete [¹⁴C]putrescine rapidly, cells were grown in 11 mM K⁺ medium, labeled with [¹⁴C]putrescine, washed, and then incubated in 0.1 mM K⁺ medium. At various times aliquots were made 0.4 x in NaCl and 11 mM in K⁺ and incubated for 3 min. The cells retained the ability to excrete [¹⁴C]putrescine rapidly for more than 50 min of growth in low K⁺ medium (data not shown).

**Excretion of [¹⁴C]Putrescine by E. coli B Grown in Low Concentrations of Ammonium, Magnesium, and Sodium Loss—** In contrast to low K⁺ medium, E. coli B grown in low concentrations of NH₄⁺, Mg⁺², or Na⁺ produced the usual rapid excretion of [¹⁴C]putrescine in 11 mM K⁺, 0.7 mM sucrose medium lacking the specific cation under study. The lowest concentration of NH₄⁺ which would support logarithmic growth past optical density 0.400 was 7 mM. Cells in 0.005 mM Mg⁺² could grow logarithmically to optical density 0.600. Concentrations of Na⁺ from 0.1 mM to 6.0 x 10⁻² mM allowed the optical density to increase logarithmically to 0.175; the growth rate then gradually slowed over several generations. Therefore, low Na⁺ cultures were grown only to optical density 0.150 before labeling.

**Polyamine Content after Decreasing Salt Concentration of Nutrient Broth and M9 Medium—** E. coli B grown in high salt nutrient broth (0.6 mM NaCl) was centrifuged at 37° and resuspended in low salt nutrient broth. Cells began to grow logarithmically within 2 min. The putrescine content rose from a high salt value of 0.69 μg ((total putrescine-2 HCl)/milliliters of culture x optical density of culture)) to 1.33 μg of putrescine at 9 min after resuspension in low salt nutrient broth. This was followed by a gradual increase to 3.0 μg of putrescine at 80 min after resuspension, two generations later. This putrescine value was only half of the final low salt value. There was little change in the spermidine content.

The putrescine content of E. coli B in low salt (regular M9 medium ((3.9 μg of putrescine-2 HCl)/milliliters of culture x optical density of culture)) was only about half the value for low salt nutrient broth; however, high concentrations of NaCl resulted in reduction of the putrescine content.

Cells growing in high salt M9 medium (0.3 x NaCl added; calculated osmolality, 0.49; K⁺ concentration, 18.2 mM) resumed growth within 5 min after resuspension in low salt M9 medium (calculated osmolarity, 0.19). As expected, the spermidine content changed very little. The putrescine content increased slowly ((from 0.8 to 1.0 μg of putrescine-2 HCl)/milliliters of culture x optical density of culture)) and approached the value for cells grown in low salt M9 medium (3.2 μg of putrescine) 2 hours after resuspension (two generations of growth in low salt medium).

The changes in polyamine levels after transfer from high to low salt medium were similar in both nutrient broth and the chemically defined medium, M9. The putrescine content returned to the low salt level very slowly in both media; therefore, high putrescine content is not a prerequisite for resumption of rapid growth. If putrescine has specific cellular functions other than an adjustment to the osmolality of the medium, these functions must require very low levels of putrescine.

**Uptake of [¹⁴C]Putrescine after Decreasing Salt Concentration—** The ability of E. coli B to take up [¹⁴C]putrescine from the medium was studied before and after transferring cells from high salt (0.6 mM NaCl) to low salt nutrient broth (Fig. 5). Cells were centrifuged out of the original high salt medium and resuspended in fresh high salt medium at the start of the experiment to remove any putrescine which the cells might have excreted during growth. After resuspension the cells were able to take up very little radioactive putrescine. After a second centrifugation and resuspension in low salt nutrient broth, the cells took up 9-fold more [¹⁴C]putrescine after only 2 min and 15-fold more after 20 min.

Occasional low values in the plateau region (40 to 120 min, Fig. 5) are probably due to lysis of cells during filtration. The plateau itself might have been caused by dilution of isotope with unlabeled putrescine excreted from the cells. To eliminate this possibility cells were again centrifuged and resuspended in low salt medium at the time shown by the arrow (Fig. 5). No change in uptake occurred; therefore, isotope dilution was not significant. Putrescine uptake was not affected by centrifugation and resuspension in any part of the experiment.

**Potassium Uptake in Bacteria with High or Low Putrescine Content—** Because a sudden increase in the osmolality of the medium causes both a rapid uptake of K⁺ (11) and rapid excretion of putrescine, K⁺ uptake might be influenced by the level of putrescine in the cell. It is possible to obtain cells growing logarithmically in 11 mM K⁺ medium which contain small amounts of putrescine. If a culture in 11 mM K⁺ medium is made 0.4 mM NaCl, the cells rapidly excrete putrescine and begin to grow logarithmically after a lag period of about 40 min. If these growing cells are centrifuged at 37° and resuspended in 11 mM K⁺ medium (no NaCl supplement), they continue logarithmic growth, but the putrescine content remains at most 25% of normal for more than 40 min.

Increases in cellular K⁺ of a control culture and a culture
with low putrescine content were measured after a sudden exposure to 0.4 mM sucrose (Fig. 6). The K⁺ uptake was similar in both, therefore, the cellular putrescine content did not influence K⁺ uptake.

Turnover of [14C]Spermidine in Low Salt and High Salt Nutrient Broth—Spermidine in E. coli turns over very slowly, if at all (2, 3), so that rapid fluctuations in the putrescine pool should have only long-term effects on the spermidine level. Maintenance of a high spermidine content in the presence of a reduced external potassium concentration was used to assure that putrescine excretion would be rate limiting in the proposed coupling of K⁺ uptake and putrescine excretion.

1 This concentration of sucrose was used to make the results comparable to those of Epstein and Schultz (11). High external K⁺ concentrations were used to assure that putrescine excretion would be rate limiting in the proposed coupling of K⁺ uptake and putrescine excretion.
was made 0.6 M in NaCl; the ratio increased 697, in the presence
density to milligrams of protein increased 47y0 after the culture
occur without loss of putrescine. The ratio of culture optical
volume can be observed as an increase in turbidity of the culture
studies in a paper given at the Los Angeles meeting of the Ameri-
might carry polyamines out of the cell. This decrease in cell
bound Mg+2, and ribosome-bound putrescine was unchanged.

It is unlikely that the rapid loss of intracellular putrescine
which occurs following a sudden increase in the osmotic strength
of the medium is due to passive leakage through a nonspecifically
damaged membrane. First, treatment of cells with toluene or
butanol-1, which are known to damage cell membranes, releases
both putrescine and spermidine from the cell (19). If high os-
monic strength damaged the membrane similarly, it should bring
about a release of spermidine as well as putrescine. Second, if
the membrane were damaged, the cell might lose its amino acid
pools. However, addition of NaCl to cultures of E. coli and
other gram-negative organisms does not cause loss of amino acids
from the cellular pool (Ref. 17 and our Table I). Third, if the
loss of putrescine were passive, this loss should not be blocked
by metabolic inhibitors. However, metabolic inhibitors and
cold did block loss of putrescine (Figs. 2 and 3).

Even with an intact membrane, increasing the osmotic strength
of the medium could produce a rapid loss of cell water which
might carry polyamines out of the cell. This decrease in cell
volume can be observed as an increase in turbidity of the culture
(20). In our experiments (Fig. 3) an increase in turbidity did
occur without loss of putrescine. The ratio of culture optical
density to milligrams of protein increased 47% after the culture
was made 0.6 M in NaCl, the ratio increased 69% in the presence
of NaCl and sodium azide. Thus, azide-treated cells lost water
without losing putrescine.

Only two agents, other than high pH (13) and chilling (3),
have been reported to reduce the cellular putrescine content of
E. coli without decreasing spermidine. These nonphysiologic
agents, levorphanol (21) and streptomycin (14), were effective
in E. coli 15 TAU, which normally excretes much more putres-
cine than the medium than do strains B and K12 (22). The loss
of substantial amounts of putrescine in the presence of levor-
phanol, streptomycin, or chilling took more than an hour,
whereas our cells exposed to high osmotic strengths lost putres-
cine within 3 min (Figs. 2, 3, and 4).

Only a few studies have been published on the relationship
of polyamine levels and salt concentrations in the medium. Hur-
witz2 reported that the spermidine content of E. coli grown in
0.01 mM Mg+2 was 20 times greater than that of cells grown in
1 mM Mg+2. Hurwitz and Rosano (23) found that over the
range of 0.001 to 10 mM Mg+2, the amount of spermidine bound
to ribosomes varied inversely with the amount of ribosome-bound Mg+2; and ribosome-bound putrescine was unchanged.

2 According to Cohen and Raina (22) C. Hurwitz reported these
studies in a paper given at the Los Angeles meeting of the Ameri-
can Society for Microbiology, May 1966.

Smith and Richards (24) reported another example of salt con-
centrations affecting polyamines; K+-deficient barley and cab-
bage leaves and red clover plants contained more putrescine
than the normal plants.

The experiments in our paper help to define the mechanism
by which E. coli excretes putrescine after an increase in osmo-
larity. A change in turgor pressure is a likely trigger for pu-
trescine excretion. Bolton et al. (25) found that increasing the
osmotic strength of the medium produced a temporary decrease
in turgor pressure of E. coli and cessation of nucleic acid and
protein synthesis. Later, macromolecular syntheses resumed
as the turgor pressure increased. Of the various compounds used
in our experiments, sucrose and the salts are known to penetrate
the cell very slowly (20) and would be expected to reduce the
turgor pressure of the cell. All of these agents produce rapid
putrescine excretion in the presence of adequate K+.

Epstein and Schultz (11) proposed that the cell achieves an
increase in turgor pressure through an osmoregulatory pump
which takes up K+ in exchange for H+, the H+ being provided
by an increase in metabolic acids. We have demonstrated that,
in addition to uptake of K+, the cell also excretes putrescine
after an increase in the osmolality of the medium. Furthermore,
the rapid excretion of putrescine is K+ dependent (Fig. 4). Lack
of K+ in the medium caused a severe decrease in the rate of pu-
trescine excretion. Resupplementing K+ restored the high rate
of putrescine excretion within a few minutes. Na+, NH4+,
Rb+, and Mg+2 did not substitute for K+ (Table II).

In fact, putrescine excretion may be coupled with the uptake
of K+. The Km for potassium uptake after a sudden increase
in the medium osmolarity is approximately 1 mM (26); the Km
for potassium stimulation of putrescine excretion is 0.69 mM.
Also, the rates of putrescine excretion (Fig. 4) and K+ uptake
(Ref. 26 and Fig. 6) are similar. Both K+ uptake (11) and pu-
trescine excretion (Fig. 3) appear to require metabolic energy.
However, it seems that K+ uptake is not dependent upon pu-
trescine excretion. Fig. 6 shows that cells with reduced content
of putrescine took up almost normal amounts of K+ following
an increase in medium osmolality. Therefore, if cation excretion
is necessary for K+ uptake and if putrescine facilitates this up-
take, substitutes must exist when the intracellular putrescine
concentration is low.

Tempest et al. (17) have demonstrated that the concentrations
of free amino acids, and particularly glutamate, increased when
E. coli was transferred to high osmolarity medium. Our ob-
servations confirm these data (Table I). Since glutamate is a
precursor of putrescine, a reduction in putrescine synthesis
could account for part of the increase in glutamate. Pools of
ornithine and arginine, direct precursors of putrescine, also
increase under these conditions (Table I).

The data on putrescine excretion, K+ uptake (11), and gluta-
mate increase can be integrated in the following hypothesis;
shifts from low osmolarity media to high osmolarity media force
gram-negative bacteria to suddenly increase their interior osmo-
larity in order to maintain a positive turgor pressure. The
mechanisms for increasing the interior osmolality include K+
uptake (11) and synthesis of amino acids, primarily glutamate
(Ref. 17 and our Table I).
After an increase in medium osmolarity, putrescine\textsuperscript{2+} excretion could be used by the cell to help balance the increase in internal positive charge from K\textsuperscript{+} uptake. Putrescine\textsuperscript{2+} excretion would play an even more important role in reducing the large increases in internal ionic strength resulting from K\textsuperscript{+} uptake, with a minimum expense of osmotically active solute. Putrescine\textsuperscript{2+} excretion would reduce the net increase in internal positive charge by 28\% and internal ionic strength by 56\%, while reducing the net increase in internal osmolarity by only 14\% (calculated from Fig. 1 and Ref. 11 for an increase of 0.8 osmole per liter). For an increase of 0.4 osmole per liter, the loss of internal ionic strength by putrescine\textsuperscript{2+} excretion would almost exactly balance the increase in ionic strength from K\textsuperscript{+} uptake.

Synthesis of negatively charged molecules, such as glutamate, would serve to maintain charge balance and would increase internal osmolarity at the same time. By coordinated K\textsuperscript{+} uptake, putrescine\textsuperscript{2+} excretion, and glutamate synthesis, the cell could achieve substantial increases in internal osmolarity while minimizing changes in internal positive charge and ionic strength.

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Dependence of the Putrescine Content of *Escherichia coli* on the Osmotic Strength of the Medium

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