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 ion-free low salt medium, the putrescine content approached the same or decreased slightly.

When cells growing in high salt medium were transferred to low salt medium, the putrescine content approached the low salt value only after 140 min. However, the ability of cells to take up [¹⁴C]putrescine from the medium did increase 6-fold within 2 min after resuspension in low salt medium.

[¹⁴C]Spermidine was not metabolized to a measurable extent over 70 min in either low salt or high salt nutrient broth cultures. Because the cellular spermidine contents were similar in the two cultures and because [¹⁴C]spermidine was not catalyzed in either culture, the rates of spermidine synthesis must also be similar, even though the precursor (putrescine) pool sizes are quite different.

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. (9) 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 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. Glyceral, 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 obtained 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, 35.5 mM Na₂HPO₄, 15.5 mM NH₄Cl, 7.28 mM glucose, and 0.58 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 NaSO₄ 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 in phosphate buffer (3.16 g of Na₂HPO₄, 3.0 g of KH₂PO₄, and 4.0 g of NaCl per liter, pH 7.2) and plating on tryptone agar.

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°C and 2000 x g for 10 min. Pellets were washed with 0.6 M sucrose (special enzyme grade, Mann Laboratories) ; cells in 11 mM K⁺, 0.4 M sucrose medium were washed with 0.6 M sucrose. The filters were placed in a cold room (1°C) or incubator (37°C). After decanting supernatants, cell pellets were frozen.

The method of Dubin and Rosenthal (13) was used to quantitate polyamines. Polyamines were extracted from bacteria into 0.5 N trichloroacetic acid, hydrolyzed in 6 N HCl to convert acetylated polyamines to the parent compounds, and separated by descending paper chromatography on Whatman No. 1 paper in butanol-1-acetic acid-pyridine-water (4 : 1 : 1: 2). (Sonication of the trichloroacetic acid extracts (Branson sonifier, model 575, setting 4, two 30-sec bursts on ice) did not increase the amount of polyamine recovered.) Polyamine bands were stained, extracted, and estimated by the method of Raina and Cohen (14), as modified by Raina et al. (15). Putrescine and spermidine standards (Calbiochem) were chromatographed in every experiment. As an aid to identification of bands, 0.1 μCi of [³⁵S]putrescine or [³⁵S]spermidine was added to some trichloroacetic acid extracts, and the areas of radioactivity were located with a radiochromatogram scanner (Packard, model 7200). This method also established that very little if any of the radioactive polyamines were degraded during the analysis. Recovery of radioactive spermidine and putrescine ranged from 88 to 95%.

The method of Lowry et al. (16) was used to determine total cellular protein in the trichloroacetic acid precipitates, using bovine plasma albumin (Armour Pharmaceutical Company) as standard.

Analysis of Amino Acid Pools—Cultures for analysis of amino acid pools were rapidly chilled on ice and centrifuged at 3000 x g and 4°C for 3 min. Pellets were then taken up in ice-cold 0.25 N HClO₄ to a total volume of 5 ml (17), allowed to stand in an ice bucket for 10 min, and centrifuged at 2000 x g and 4°C for 10 min. Pellets were saved for protein estimation (18). Supernatants were adjusted to pH 7.1 with 1 N KOH and the KClO₄ precipitates collected by centrifugation at 2000 x g and 4°C for 10 min. Supernatants were then adjusted to pH 8.0 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, ⁴-¹⁴C]Putrescine-2 HCl (109 μCi per mg) and spermidine ([aminopropyl]-[¹, ⁴-¹⁴C]tetramethylenediamine-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-oxazolyl)]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 Zarlingo and Schultz (18) with slight modifications. Aliquots of 1 ml were rapidly filtered on 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 a cold room (1°C) or incubator (37°C). 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 μeq 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⁺, 0.63 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 osmole 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 generation times; thus, changes in putrescine content are not caused by 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 NaCl (0.6 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). (d) 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

![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 (□—□), glucose (■—■) 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 0.1 M 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 osmoles per liter). Spermine-3 HCl values for these controls were 11 and 8.2 μg per mg of protein, respectively.

Table I

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Control</th>
<th>NaCl added</th>
<th>Grown in NaCl</th>
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<tr>
<td>Alanine</td>
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<td>0.25</td>
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<tr>
<td>Asparagine</td>
<td>&lt;3.2</td>
<td>16</td>
<td>16</td>
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<tr>
<td>Glutamine</td>
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<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.0</td>
<td>6.1</td>
<td>3.1</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.6</td>
<td>14</td>
<td>2.8</td>
</tr>
<tr>
<td>Methionine</td>
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<td>5.3</td>
<td>11</td>
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<td>Ornithine</td>
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<tr>
<td>Tyrosine</td>
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<td>0.67</td>
</tr>
<tr>
<td>Valine</td>
<td>16</td>
<td>8.8</td>
<td>9.2</td>
</tr>
</tbody>
</table>

| Nanomoles of putrescine-2 HCl per mg of cell protein | 150 | 12 | 12 |
| Milligrams of total cell protein | 17.9 | 15.8 | 24.6 |
| Total viable cell number | 7.4 x 10^10 | 5.0 x 10^10 | 11 x 10^9 |
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 × 10^9 cells per ml and incubated at 37°C for 15 min with 0.08 μCi per ml of [14C]putrescine. The cells were then sedimented at about 2000 × g (Sorvall bunsen centrifuge) for 10 min at 37°C 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 (○—○), 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 μm, 25 mm in diameter). (Aliquots which were filtered immediately 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, 0.15 M NaCl solutions 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.

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 for NaCl alone and sodium azide plus NaCl indicates that azide inhibited the loss of putrescine, even at the low concentration used. Sodium arsenate (neutralized to pH 6.8 before use) also blocked the putrescine loss (data not shown). None of the experiments with inhibitors affected the spermidine level. The fact that two inhibitors could block putrescine loss implies that the process may directly or indirectly require energy.

It was possible that the excretion of [14C]putrescine following a shift to high salt reflects the normal rate of putrescine excretion 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 containing 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. 4A).

As shown in Fig. 4B, reducing the external K+ concentration significantly reduced the rate of excretion of cellular [14C]putrescine; 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 affected 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 reversible. When cells were suspended in 0.4 M NaCl medium containing 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+, ranging from 0.2 mM to 11 mM. Below 0.4 mM K+, the rate of excretion was slow and varied little with the external K+ concentration. At higher K+ concentrations, the rate of putrescine excretion increased dramatically. The concentration of K+ which produced a half-maximum rate of [14C]putrescine excretion was 0.09 mM.

Nutrient broth contains 2.5 mM K+. Therefore, the previously mentioned studies of putrescine excretion in nutrient broth...
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 contents of putrescine in the range of 6.4 to 7.3. E. coli B grown in 11 mm K⁺ medium (pH 6.9), 2 mm K⁺ medium (pH 6.0), and 11 mm K⁺ medium (pH 5.9). All media except D and G contained sodium phosphate buffer, media D and G contained ammonium phosphate buffer (see "Materials and Methods").

**Table II**

| Medium | K⁺ | 0.7 M Sucrose | Mg⁺² | NH₄⁺ | Na⁺ | Fraction of [¹⁴C] lost in 3 min
|--------|----|----------------|------|------|-----|------------------------
| A      | 0.1 mm  | -   | +   | +   | +   | 0.003
| B      | 11.0 mm | +   | +   | +   | +   | 0.47
| C      | 11.0 mm | +   | +   | -   | +   | 0.06
| D      | 11.0 mm | -   | +   | -   | +   | 0.13
| E      | -      | +   | +   | +   | -   | 0.08

- 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 was 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 Mg⁺² and NH₄⁺ or the absence of Na⁺ did not significantly alter the excretion patterns. In similar experiments 30 mm Mg⁺² 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 6.9), labeled with [¹⁴C]putrescine, washed in growth medium, and split into four portions before the final centrifugation. Pellets were resuspended in 10 ml of (a) 11 mm K⁺ medium (pH 6.9), (b) 11 mm K⁺ medium (pH 6.9), (c) 11 mm K⁺ medium (pH 6.9), and (d) 11 mm K⁺ medium (pH 6.9). Aliquots for determination of cellular [¹⁴C]putrescine were removed at times up to 10 min. After 15 min the pH values were (a) 6.5; (b) 6.4; and (c) 7.3; and (d) 7.1. This difference in pH is known to affect the levels of putrescine in stationary cultures of E. coli (2, 13); however, both of our cultures in 0.7 M sucrose (b) and (d) lost cellular [¹⁴C]putrescine at the same rapid rate. The two cultures in medium without sucrose (a) and (c) lost [¹⁴C] at slow and similar rates. Thus, pH did not affect the rate of [¹⁴C]putrescine excretion over the range of 6.4 to 7.3.

**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°C 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 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 K1 medium, labeled with [14C]putrescine, washed twice, and then resuspended in 11 mM K2 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 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, and then incubated in 0.1 mM K+ medium. At various times aliquots were made 0.4 x in NaCl 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+, Mg2+, 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 0.050 mM Mg2+ could grow logarithmically to optical density 0.600. Concentrations of Na+ from 0.1 mM to 6.0 x 10-1 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 growing in high salt nutrient broth (0.6 mM NaCl) was centrifuged at 37° and resuspended in low salt nutrient broth. Cells began to grow log rithmically 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 osmolality, 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 in 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
would be rate limiting in the proposed coupling of K+ uptake and putrescine excretion. Kf concentrations were used to assure that putrescine excretion have only long term effects on the spermidine level. Maintenance of a high spermidine content in the presence of a reduced nutrient broth-Spermidine in E. coli turns over very slowly, if at all. Exposure to 0.4 M sucrose (Fig. 6). The K+ uptake was similar 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.

with low putrescine content were measured after a sudden exposure to 0.4 M 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 putrescine (precursor) pool might require an alteration in the enzyme system which forms spermidine (2, 5) or an alteration in the catabolism of spermidine.

To measure the catabolism of spermidine by E. coli B, a 30-ml logarithmically growing culture in low salt nutrient broth and a 30-ml logarithmically growing culture in high salt nutrient broth (0.6 M NaCl) were incubated at 37° for 20 min with 1 μCi of [14C]spermidine (41.7 μCi per mg) and centrifuged at 2160 X g for 10 min. Then, the pellets from low salt and high salt nutrient broth were washed twice in 20 ml of low salt nutrient broth or high salt nutrient broth, respectively. They were next resuspended and incubated at 37° in 11 mM K+, 0.4 M sucrose medium, was about 27% lower than the same ratio in 11 mM K+ medium. No correction was made for this change in optical density.

![Graph](image-url)  
Fig. 5. Uptake of [14C]putrescine by E. coli B in high and low salt nutrient broth. Cells in high salt nutrient broth (0.6 mM NaCl) were grown to an optical density of 0.200, centrifuged at 37° and 2000 X g for 10 min, and re-suspended in warm, high salt nutrient broth. At the times shown (−40 to −20 min), aliquots were removed for optical density determination and incubation with [14C]putrescine as described below. At −15 min cells were centrifuged at 37° and then re-suspended at 0 min in low salt nutrient broth. At +50 min the culture was again centrifuged at 37° and at +70 min was re-suspended in warm, low salt nutrient broth. Uptake was measured by incubating 1 ml of culture with 1 ml of high or low salt nutrient broth containing 2 μCi of [14C]putrescine for 3 min at 37°. The incubation mixture was filtered onto Schleicher and Schuell filters and washed with 10 ml of 0.15 M NaCl; 30 sec after adding cells to filters, the filters were placed in 5 ml of counting solution. Washing the filters with cold 0.5 M NaCl did not alter the radioactivity recovered. If the filters were allowed to dry on the suction apparatus, they lost radioactivity. Results were corrected for sham incubations (no cells present) and normalized by dividing by the optical density of the solution at each time point. Radioactivity in the high salt sham incubations was less than 30% of that in the experimental samples; radioactivity in low salt sham incubations was generally less than 0.5% of experimental.

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.
cursor (putrescine) pool changes greatly. This could come about by close regulation of enzyme activity or by rate-limiting amounts of enzyme being present in both cultures. Also, the \( K_m \) for putrescine of the spermidine biosynthetic enzyme could be so low that the observed changes in putrescine concentration might not alter enzymatic activity.

**DISCUSSION**

We have shown that the putrescine content of *E. coli* varies inversely with the osmotic strength of the growth medium (Fig. 1). Medium containing glycerol was an exception. We have also shown that the sudden addition of any of a variety of charged or uncharged solutes (but not glycerol) produces a rapid loss of intracellular putrescine (Figs. 2 and 3).

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 osmotic 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 streptomyacin (14), were effective in *E. coli* 15 TAU, which normally excretes much more putrescine into the medium than do strains B and K12 (22). The loss of substantial amounts of putrescine in the presence of levorphanol, streptomycin, or chilling took more than an hour, whereas our cells exposed to high osmotic strengths lost putrescine 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. Hurwitz et al. (22) 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.

Smith and Richards (24) reported another example of salt concentrations affecting polyamines; K\(^{+}\)-deficient barley and cabbage 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 osmolality. A change in turgor pressure is a likely trigger for putrescine excretion. Bolten 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\(^{+}\). Glycerol, which does not cause putrescine excretion, penetrates the cell freely (9, 10) and would not be expected to lower the turgor pressure. Thus, a change in turgor pressure appears to be a likely signal for the excretion of putrescine.

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 putrescine excretion. Resupplementing K\(^{+}\) restored the high rate of putrescine excretion within a few minutes. Na\(^{+}\), NH\(_4\)^\(^{+}\), Rb\(^{+}\), and Mg\(^{2+}\) did not substitute for K\(^{+}\) (Table II).

In fact, putrescine excretion may be coupled with the uptake of K\(^{+}\). The \( K_m \) for potassium uptake after a sudden increase in the medium osmolality is approximately 1 mM (26); the \( K_m \) 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 putrescine excretion (Fig. 3) appear to require metabolic energy. However, it seems that K\(^{+}\) uptake is not dependent upon putrescine 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 uptake, 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 osmolality medium. Our observations 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 increased under these conditions (Table I).

The data on putrescine excretion, K\(^{+}\) uptake (11), and glutamate increase can be integrated in the following hypothesis: shifts from low osmolality media to high osmolality media force gram-negative bacteria to suddenly increase their interior osmolality 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).

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