Magnesium and the Growth of Escherichia coli*

JOAN E. LUSK,† R. J. P. WILLIAMS,§ AND EUGENE P. KENNEDY

From the Department of Biological Chemistry and the Laboratory of Biophysics of the Peter Bent Brigham Hospital, Harvard Medical School, Boston, Massachusetts 02115

SUMMARY

The growth of cells of Escherichia coli has been studied in media with varying amounts of magnesium. Experiments in which the content of magnesium in the medium and in the cell have been directly measured reveal that the growth rate of magnesium-limited wild type cells is half-maximal at extracellular concentrations of 1 to 3 \times 10^{-6} \text{ M}. Mutants have been isolated which require 100 times this concentration for half-maximal rate of growth. Supplementation of the medium with calcium or strontium enables the mutants to grow on the same low levels of magnesium as the wild type even though calcium or strontium cannot replace magnesium as an essential growth factor.

Cells harvested in the exponential phase of growth from medium not limited in magnesium lose one-third to two-thirds of their total magnesium upon treatment with ethylenediaminetetraacetate. In contrast, magnesium-starved cells retain nearly all of their magnesium when treated with EDTA. In a sonic extract of magnesium-starved cells, 15% of the total magnesium is not sedimentable at 100,000 \times g. When such an extract is passed over Sephadex G-25 or G-50, 75 to 80% of the magnesium is free to diffuse into the gel.

Magnesium is unquestionably essential to living cells. It is required for the activity of many enzymes and is needed for the preservation of the structure of ribosomes (1). In addition, studies of magnesium starvation (2), and the effects of ethylenediaminetetraacetate (3) and of osmotic shock (4) suggest that the ion is important for the maintenance of the permeability barrier of the bacterial cell. The stabilization of spheroplasts by magnesium ions (5, 6) also suggests their involvement in the integrity of the cell membrane.

Ribosomal stability in vitro is known to depend on the presence of magnesium (1). Similarly, when living bacteria are deprived of magnesium, extensive turnover (degradation and synthesis) of their ribosomes occurs (7-11). Ribosomal protein is degraded. The ribosomal RNA remains acid-precipitable, but it is not reused for the concurrent formation of ribosomes, which are synthesized de novo. In these studies of the fate of ribosomes during starvation for magnesium, the actual magnesium content of the starved cells was not determined. Therefore, it was not possible to correlate the observed effects on ribosomal structure with changes in magnesium content of the cells, as the similar effects in vitro can be correlated with magnesium concentration. One of the aims of the present work was to observe the actual variation in magnesium content during starvation for the metal.

The level of cellular magnesium has not been measured in previous studies of the changes in permeability affected by treating the cells with EDTA. We have investigated the time course and extent of removal of cellular magnesium by EDTA in an attempt to correlate the movements of this ion with the observed effects on permeability.

We have also isolated mutants of Escherichia coli strain A324 which have an altered dependence of growth rate on the concentration of magnesium in the medium. These mutants require an extracellular magnesium concentration 100-fold higher than that which supports maximal growth rate of the wild type.

MATERIALS AND METHODS

Bacteria—E. coli strain A324, derived from K-12, was a gift from Dr. S. Luria. It is F-, i-tyr+a*, requires thiamine and proline, and is streptomycin-resistant.

Medium—Medium I contained 1% Casamino acids (Difco), KCl (5 mM), K$_2$SO$_4$ (0.5 mM), KH$_2$PO$_4$ (1 mM), Te$_3$SO$_4$ (1.8 \mu M), and Tris-HCl (0.1 M), pH 7.4. Thiamine-HCl (2 \mu g per ml) and the desired concentration of MgSO$_4$ were added after autoclaving. Medium I without added magnesium contained 5 to 8 \mu M magnesium from contaminants in the KH$_2$PO$_4$ and Casa-
minos, and also derived from the metal caps of the shake flasks during autoclaving.

**Analytical Procedures—**Suspensions of cells were chilled in ice and centrifuged at 10,000 × g for 10 min. The pellets were washed with cold Tris-HCl (0.05 M, pH 7.4) and resuspended in distilled deionized water. Protein was measured by the method of Lowry et al. (12). Magnesium was measured by atomic absorption spectroscopy. The procedure of Ida, Fuku, and Wacker (13) was modified so that the saturated oxime solution of 1 N in HCIO₄ was as well as 1 N in HCl. The magnesium content of bacteria determined by this method agreed with that found in control samples which had been ashed, and with values obtained by emission spectroscopy. The wash with Tris buffer did not alter the magnesium content of the cells.

Potassium was determined in the Technicon Autoanalyzer of the Clinical Center Laboratory of the Peter Bent Brigham Hospital.

For measurement of total RNA, samples of the culture were chilled in ice and brought to 5% in trichloracetic acid. After at least 30 min at 0°, the samples were centrifuged and the precipitates washed successively with cold 5% trichloracetic acid and ethanol. The precipitate was hydrolyzed in 0.5 N KOH at 100° for 20 min. After acidification with an equal volume of 1 N HClO₄ in the cold, a portion of the supernatant solution was analyzed for ribose by the orcinol reaction. E. coli ribosomal RNA (a gift from Dr. D. G. Comb) or AMP was used as a standard.

**Mutagenesis—**Ethyl methane sulfonate (0.07 ml) was added to 5 ml of a culture of exponentially growing cells of strain A24 at a density of 5 × 10⁸ per ml in Medium I containing 10 mM magnesium. After incubation for 2 hours at 37°, the culture was diluted 10-fold with the same medium. The cell density was then allowed to increase 100-fold to allow expression of the mutation.

Mutagenesis with N-methyl-N′-nitro-N-nitrosoguanidine was carried out according to the method of Adelberg, Mandel, and Chen (14).

**Selection—**The mutagenized cells were treated with penicillin in several cycles according to the method of Lubin (15). Penicillin was added to cultures growing in Medium I supplemented with 0.01 mM magnesium. After 2 hours, portions were filtered and washed, and the cells were allowed to grow to stationary phase in Medium I containing 10 mM magnesium. Portions of these cultures, now enriched in mutants, were suitably diluted in a sterile sodium chloride solution (0.9%) and plated. Plates for growth of mutants requiring high concentrations of magnesium were prepared from Medium I containing 10 mM MgSO₄, 25 mg per liter of streptomycin sulfate, and 2% agar. Colonies were replicated (16) on similar plates containing no added magnesium. Agar for these low magnesium plates was previously purified by dialysis against 0.05 M Tris-HCl, pH 7.4, containing 1 mM EDTA and 5 mM NaCl until free of magnesium. Followed by dialysis against 0.05 M Tris-HCl, pH 7.4, containing 5 mM NaCl. Colonies that failed to replicate appeared as 1% of the total population after two cycles of treatment with penicillin. These were picked and their magnesium requirement further tested in liquid medium; approximately 5% failed to grow at low concentrations of magnesium. Clones of the mutants made with nitrosoguanidine with low reversion rate were screened by growth in 10 mM magnesium and plating

**RESULTS**

**Relation between Total Growth and Available Magnesium—**

Flasks containing different concentrations of magnesium were inoculated with small inos of cells of strain A24 and shaken for 24 hours at 37°. The cells were collected and washed by centrifugation as described under "Materials and Methods," and the total protein of the culture was determined as a measure of cell growth. The results are shown in Fig. 1.

The final value for total protein in the culture was directly proportional to the available magnesium up to initial concentrations of about 3 × 10⁻⁹ M magnesium. Such magnesium-limited cells contained 0.06 μmole of magnesium per mg of protein, as determined by direct measurement.

The amount of growth achieved with limiting amounts of magnesium was not affected by the addition of KCl (0.05 M), CaCl₂ (10⁻³ M), or higher amounts of phosphate (0.01 M).

**Variations in Magnesium and Potassium Content of Cells—**

Cells of strain A24 were grown in Medium I under conditions of magnesium limitation (10⁻³ M MgSO₄ added initially) or magnesium excess (10⁻² M initial concentration). Samples of cells were taken during the exponential phase of growth or during stationary phase.

When magnesium was not limiting (10⁻³ M), the magnesium to protein ratio fell only slightly as the cells passed from ex-
MAGNESIUM AND POTASSIUM CONTENT OF E. COLI

Strain A324 was grown at 37° in Medium I to which the indicated magnesium concentration had been added. When the culture reached exponential or stationary phase, the cells were chilled, harvested, washed with Tris-Cl (0.05 M, pH 7.4), and resuspended in distilled deionized water. Protein and magnesium were determined as stated under "Materials and Methods." Potassium was determined on unwashed cells, because washing with Tris was found to remove most of the cellular potassium.

<table>
<thead>
<tr>
<th></th>
<th>Magnesium</th>
<th>Potassium</th>
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<tbody>
<tr>
<td>Cells grown on 10^-2 M magnesium</td>
<td>0.195 ± 0.03 (6)</td>
<td>0.72</td>
</tr>
<tr>
<td>Exponential phase</td>
<td>0.145 ± 0.01 (2)</td>
<td>0.175, 0.35</td>
</tr>
<tr>
<td>Stationary phase</td>
<td>0.155 ± 0.02 (16)</td>
<td>0.79</td>
</tr>
<tr>
<td>Cells grown on 10^-4 M magnesium</td>
<td>0.079 ± 0.014 (23)</td>
<td>0.62, 0.62</td>
</tr>
<tr>
<td>Exponential phase</td>
<td>0.079 ± 0.014 (23)</td>
<td>0.62, 0.62</td>
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Values given are mean ± standard deviation. The number given in parentheses is the number of determinations.

The initial, exponential rate of growth of cells in 10^-1 M magnesium is fully as great as in 10^-2 M magnesium. Apparently the slightly lower content of magnesium of the cells at 10^-2 M (Table I) does not affect the growth rate.

The magnesium-starved cells grown into stationary phase have a distinctly higher content of potassium than stationary cells grown on excess (10^-4 M) magnesium.

"Bound" and "Soluble" Intracellular Magnesium—Strain A324 was grown in Medium I containing 10^-2 M magnesium until early stationary phase. The medium at this point contained 10^-4 M or less magnesium, which eliminated the need for washing the cells. The cells were harvested by centrifugation, resuspended in water, sonically disrupted, and centrifuged at 3000 × g for 10 min to remove whole cells, which yielded a cell-free dispersion with a protein concentration of 12 mg per ml and 1.3 pmol of potassium per mg.

The extract was centrifuged for 30 min at 30,000 × g, after which one-fourth of the total magnesium was in the pellet and the remainder (1 pmol per ml) in the supernatant. The 30,000 × g supernatant was next centrifuged at 100,000 × g for 2 hours. Half of the magnesium then appeared in the pellet. The remaining magnesium in the supernatant was not evenly distributed, with 2 to 4 times as much magnesium in the bottom half of the soluble fraction as in the top. The top half of this high speed supernatant contained a total magnesium concentration of 2 × 10^-4 M, or 15% of the initial magnesium concentration of the sonic extract. Ten-fold dilution of the 30,000 × g supernatant with distilled water made no difference in the relative distribution of soluble and pellet-bound magnesium upon subsequent centrifugation at 100,000 × g.

If the fraction of the total cellular magnesium not sedimented at 100,000 × g in the experiment just described is accepted as an operational description of "soluble magnesium," then the approximate concentration of intracellular soluble magnesium can be calculated in the following way. From data of Epstein and Schultz (17) the content of intracellular water in E. coli K-12 is 1.8 ml per g, dry weight, or (assuming 50% of the dry weight is protein) 3.6 μl per mg of protein. If the same ratio holds in magnesium-starved cells, the value of 0.09 μm of total magnesium per mg of protein in the experiment just described represents 25 μm of mg of cell water. If 15% of this total magnesium is in the soluble fraction, then this corresponds to an intracellular concentration of about 4 mM soluble magnesium. The validity of this calculation depends principally upon the assumption that dilution of the contents of the cell during sonic disruption does not appreciably influence the affinity of the sedimentable macromolecules for magnesium.

The bulk of the intracellular magnesium is not tightly bound to macromolecular constituents of E. coli. In two experiments, in which sonic extracts of magnesium-starved cells of A324 were passed over Sephadex G-25 or G-50, only 18% and 29%, respectively, of the total magnesium was recovered in the macromolecular fractions.

Recovery from Magnesium Starvation—Cells were starved for magnesium by growth in Medium I containing 10^-2 M magnesium until early stationary phase. One volume of this culture was diluted into 10 volumes of the same prewarmed medium. There was a short lag before exponential growth was resumed (Fig. 2). The RNA content of the culture, however, began immediately to increase at the normal exponential rate for steady state growth in this medium. Protein synthesis attained this rate only when the RNA to protein ratio had reached the normal value for exponential cells (0.1 mg per mg of protein). This behavior is in agreement with results of McCarthy (8) and Suzuki and Hayashi (18) for E. coli and of Kennell and Magasanik (7) for Aerobacter aerogenes. Magnesium regain was rapid, magnesium to protein ratios characteristic of exponential cells being reached somewhat before maximal growth rate.

Growth on Limiting Concentrations of Magnesium—As the growth of the culture shown in Fig. 2 continued, the medium became depleted of magnesium. The growth rate began to decrease before the cellular magnesium content was appreciably lowered, half maximal rate of growth occurring when 1 to 3 μM magnesium remained in the medium. Once the supply of magnesium was exhausted, the total RNA content of the culture remained constant for several hours and then slowly decreased. Protein concentration and turbidity of the culture continued to increase slowly, increasing 10% during the next 12 hours.

Dependence of Growth Rate on Extracellular Magnesium—The concentration of magnesium at which the rate of growth of the wild type is half-maximal was defined by experiments such as shown in Fig. 2. Rate constants k were obtained from the equation A = Aoe^kt, where A is the optical density of the culture at time t. A plot of log (k/kmax−k) against log (Mg)extracellular gave a straight line with intercept of the logarithm of the apparent K_m for magnesium (Fig. 3). The
slope of the line, in this case near unity, indicates the apparent order of the process rate-limited in magnesium. For growth rate, the apparent $K_m$ for magnesium of strain A324 is 1 to 3 $\mu$M.

Loss of Magnesium from Cells Treated with EDTA—Cells of A324 in exponential or stationary phase were treated with 1 mM EDTA at pH 8.0 and at 28°. The resulting release of magnesium from the cells is shown in Fig. 4.

Stationary phase cells starved for magnesium always released less magnesium to the medium than did stationary phase cells grown in excess magnesium. These results with stationary phase cells were quite reproducible, but results with exponential phase cells tended to be more variable, with losses ranging from one-third to two-thirds of total cellular magnesium, whether grown on limiting or excess magnesium.

The loss of cellular magnesium was essentially complete within the first 5 min of incubation with EDTA. Loss of magnesium from the cells during the next hour paralleled a slow decrease in cellular protein and could have been a result of lysis of the cells.

![Fig. 4. Loss of magnesium from cells treated with EDTA.](http://www.jbc.org/)

When the incubation with EDTA was carried out at 3–4°, the efflux of magnesium was more extensive than at 28°. Only 10% of the magnesium of exponential cells and 30% of that of magnesium-starved cells remained bound to the cell pellet after treatment at this low temperature. The loss in the cold was at least as rapid as at 28°, being nearly complete with the first 5 min.

Mutants Which Require High Levels of Magnesium for Growth—Mutagenesis and selection were carried out as described under “Materials and Methods.” The mutant A324-1, obtained by treatment with ethyl methane sulfonate, has a reversion rate so high that within 24 hours any magnesium-limited culture...
growth of the wild type strain of A324 whether grown at high or low levels of magnesium. Strontium appears to be about as effective as calcium for A324-1, but less so for A324-2. 

The magnesium content of A324-1 and A324-2 growing on 10 mM magnesium or on 0.01 mM magnesium plus 1 mM calcium is similar to that of the parental strain growing on high or low magnesium, respectively. All three strains contain the same low levels of magnesium during magnesium starvation, although this state occurs at extracellular magnesium concentrations of 1 µM for A324 and at 100 µM for A324-1 or A324-2. Magnesium starvation of the mutants is reversible by the addition of magnesium or calcium, rapid exponential growth being resumed after a lag of 1 to 2 hours.

**DISCUSSION**

The mechanisms of the uptake and accumulation of magnesium by cells of *E. coli* are not known. The cations could be bound to the abundant anions of the cell or concentrated by an active transport system. *E. coli* ribosomes are known to bind magnesium in vitro. The maximum magnesium to phosphorus ratio is 0.55 at 10 mM magnesium (20, 21). If this ratio were applicable in vivo, the RNA of the cell [0.94 ± 0.1 µmol of RNA-P per mg of protein in exponential cells and 0.44 ± 0.1 µmol of RNA-P per mg of protein in magnesium-limited cells] could bind more than 3 times the observed magnesium content. The actual capacity of ribosomes in vivo to bind magnesium will be altered by the concentrations of other cations present in the cell, for example by potassium and polyamines. Nevertheless, a large proportion of the cellular magnesium is probably bound to ribosomes.

The proportionality of cellular magnesium to RNA content is in agreement with this concept. Experiments with *A. aerogenes* in a chemostat (22) have shown that cellular magnesium to RNA ratios vary only slightly under several different conditions of growth.

Other evidence suggests that magnesium is accumulated in the cell by active transport as well as by binding to intracellular anions. According to McCarthy (8), cells starved of magnesium for 20 hours contain only 5% of the amount of ribosomes found in exponential cells. If binding to ribosomes were the only means of holding magnesium within the cell, one would expect that upon the disappearance of the ribosomes magnesium would leak into the medium. Yet the extracellular magnesium remains on the order of 1 µM during starvation of more than 24 hours; the cellular magnesium to protein ratio decreases only because of protein synthesis and not because of excretion of magnesium. Such maintenance of a concentration gradient supports the argument either that there are nonribosomal binding substances within the cell, or that the internal magnesium is retained by an active transport system. Such a transport system may well be necessary to supply the levels of free magnesium required by many intracellular enzymes. For example, *E. coli* DNA polymerase (23) and amino acid-activating enzymes (24, 25) require more than 1 mM magnesium for full activity in vitro.

In their study of the intracellular content of magnesium in *E. coli* strain ML 35, Hurwitz and Rosano (26) presented data suggesting that the growth of this organism may take place at maximal initial rates at concentrations as low as 10⁻³ M magnesium in the medium. However, their figures apparently refer
to the concentration of magnesium added to the medium, and they do not report the levels of magnesium present as contaminants of other constituents. Since the amount of contaminating magnesium derived from ordinary analytical grade reagents may be significant (5 to 8 μM in the medium we have used) and since the concentration of magnesium falls rapidly when this constituent is growth-limiting, it is important to measure the actual amounts of magnesium in the medium at various rates of growth.

Hurwitz and Rosano (26) also reported that the concentration of freely soluble magnesium within the cell was almost exactly the same as the concentration in the growth medium, from which they concluded that no accumulation of soluble magnesium occurs against a concentration gradient in these cells. Since their figures for magnesium in the medium apparently refer to initial concentrations of added magnesium it is not easy to interpret this finding. Hurwitz and Rosano (26) concluded that the intracellular level of soluble magnesium may be as low as 5.8 μM in cells growing in magnesium-depleted medium, which leads them to speculate that spermidine (the level of which rises in these cells) may be more important than magnesium in protein synthesis in vivo. However, as mentioned above, magnesium is required for the activity of a very wide range of enzymes which carry out vital functions other than protein synthesis, and it is highly unlikely that spermidine can replace magnesium as a cofactor for each such essential enzyme. For this reason, we have reinvestigated the problem. Our results, with a direct measurement of the magnesium content of the supernatant fraction of sonically disrupted cells, indicate that 15% of the total cellular magnesium is soluble magnesium as defined operationally by this test. This would correspond to a concentration of soluble magnesium ion within the cell of about 4 mM, a value in fair agreement with estimates (27) of the magnesium content of mammalian cells, based on the activity of magnesium-dependent enzymes. Measurements of the amount of magnesium which is free to diffuse from broken cells into Sephadex G-50 gel indicate that about 80% of the total magnesium is in equilibrium with a species of low molecular weight.

Effects of Magnesium Starvation on Cell Physiology—The effects of magnesium starvation on the metabolism of protein and RNA vary quantitatively with the species of bacterium and with possible depletion of the source of carbon in the medium. Kennell and Kotoulas (9) found that the content of 30 S and 50 S ribosomes of a culture of A. aerogenes remained constant for 22 hours of magnesium starvation. They emphasized the importance of supplemental additions of a source of carbon (glucose) for maintenance of the ribosomes. In earlier work with the same organism, Kennell and Magasanik (7) had found the concentration of ribosomal RNA per volume of culture to decrease by 30% during 20 hours of deprivation of magnesium. They observed that combined deprivation of magnesium and glucose resulted in a much more rapid degradation of the ribosomes.

The ribosomes of E. coli appear to be even more sensitive to magnesium starvation than those of A. aerogenes. McCarthy (8) and Suzuki and Hayashi (18) found only a small percentage of the exponential content of ribosomes in E. coli after 20 hours of magnesium starvation. Similarly, Natori, Nozawa, and Muzuno (11) found that the total ribosomal RNA decreased to about one-third of its initial volume after 15 hours of magnesium starvation. Starvation for carbon may have occurred in both cases, although only to an extent comparable to that of A. aerogenes in the work of Kennell and Magasanik (7). Degradation of the E. coli ribosomes, however, seemed to be much more extensive. In both species, ribosomal RNA was synthesized during magnesium starvation (9, 11), and its subsequent degradation followed a similar path to acid-soluble fragments via a tRNA-like polymer. The difference seemed to be in the relative rates of ribosomal synthesis and degradation.

The continuation of protein synthesis during magnesium starvation must depend on the presence of functional ribosomes. In accordance with the proposed greater sensitivity of its ribosomes, growth of E. coli as measured by optical density was found to cease within a few hours after the onset of magnesium starvation (8, 11). In contrast, A. aerogenes continued net protein synthesis during 20 hours of magnesium starvation even under conditions of limited source of carbon (7). When additional carbon was supplied (10), net protein synthesis by magnesium-limited A. aerogenes continued at 45% of the exponential rate for at least 48 hours. The present work with E. coli indicates that protein synthesis during magnesium starvation continues at only a small percentage of the maximal rate, but definitely does continue for at least 24 hours. The difference between this and previous results with E. coli could perhaps be the result of the different media and strains employed. It is probable that the level of total protein, like that of total RNA, reflects a delicate balance between synthesis and degradation.

Effects of EDTA—The effects of EDTA on the cell have been ascribed to its chelation of divalent cations and their consequent removal from the cell. The rapid release of magnesium into solutions of EDTA is similar to the time course of susceptibility to osmotic shock (4) and to actinomycin (3). To attribute these effects solely to the loss of magnesium is not possible, however, because many other metals (e.g. barium, iron, and calcium) are also removed to a large extent by EDTA. Theoretically, magnesium could be held inside the cell, bound externally to its surface, or both. It was expected that surface magnesium would be readily removed by EDTA even in the cold. Internal magnesium would have to cross a lipid membrane to be removed from the cell. Its efflux therefore should be strongly dependent upon temperature. The observed increase in rate and extent of removal of magnesium in the cold is anomalous, if all effects are attributed to EDTA alone. Cold Tris itself, however, has effects on permeability, particularly on the retention of nucleotides (28). It seems probable that cold Tris has damaged the cells in a way that allows EDTA to remove more of their magnesium. Cold Tris alone has never been observed to remove magnesium from the cells.

Mutants Which Require High Concentrations of Magnesium—In selecting mutants unable to grow on low concentrations of magnesium, we had hoped to obtain strains with an alteration in the assumed transport system for magnesium. The primary lesion in the mutants actually isolated is not known. When supplemented with relatively high levels of calcium (or strontium), the mutants utilize exogenous magnesium as well as wild type. The action of calcium is obscure, since its presence does not reduce the cellular content of magnesium. Furthermore, mutant cells in a medium with high levels of calcium and limiting magnesium cease growth when the magnesium is exhausted. These facts show that calcium cannot be substituted for magnesium. A more detailed biochemical analysis will be needed to determine the locus of altered function in these mutants.

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