Effects of Reduced Energy Production on Protein Degradation, Guanosine Tetraphosphate, and RNA Synthesis in *Escherichia coli*\(^*\)

(Received for publication, March 1, 1977, and in revised form, November 2, 1977)

**ANN C. ST. JOHN**\(^*\) and **ALFRED L. GOLDBERG**

*From the Department of Physiology, Harvard Medical School, Boston, Massachusetts 02115*

In *Escherichia coli* (strain A33, ATCC No. 27873) protein degradation increases and RNA synthesis falls under conditions that reduce the cell's capacity to generate ATP (e.g. glucose or phosphate starvation). To test whether reduced energy production might signal these responses, cellular ATP content was lowered to different extents with various concentrations of inhibitors of electron transport. Concentrations of cyanide or azide which caused a moderate reduction (30 to 50%) in ATP content led to the cessation of growth, a decrease in RNA synthesis, and a doubling in the rate of breakdown of normal cell proteins. Under these conditions, the degradation of abnormal cell proteins containing canavanine was not altered. In contrast, concentrations of cyanide or azide that lowered ATP by 80% or more reduced the degradation of both normal and abnormal proteins below rates found in growing cells. Thus, two distinct effects of reduced ATP production were demonstrated: 1) a moderate inhibition of energy metabolism accelerated the breakdown of normal cell proteins in similar fashion to starvation; 2) a drastic reduction of ATP levels inhibited the degradation of all cell proteins.

Both rel \(^*\) and rel \(-\) cells increase protein degradation when treated with these inhibitors of respiration. Under these conditions both strains show a marked accumulation of guanosine-3'-diphosphate-5'-diphosphate (ppGpp). Thus in addition to the well established changes in ppGpp synthesis determined by levels of charged tRNA, ppGpp content also depends on the intracellular energy levels. The accumulation of ppGpp when ATP production was inhibited resulted primarily from a 6- to 9-fold decrease in the degradaion of this nucleotide, in accord with earlier reports on cells deprived of glucose.

Addition of tetracycline, an inhibitor of ppGpp synthesis, to cells treated with cyanide or azide caused a rapid decrease in ppGpp levels and lowered protein catabolism toward the levels found in growing cells. Tetracycline also reduced protein breakdown in cells deprived of a carbon source or shifted from aerobic to anaerobic condition, but this inhibitor did not affect proteolysis in growing cells. These findings suggest that increased levels of ppGpp are essential for the stimulation of protein breakdown. Addition of tetracycline to cells treated with cyanide or undergoing an energy step-down caused a small increase in uracil incorporation into RNA, but RNA synthesis was still much slower than in growing cells. Thus ppGpp may contribute to, but is not primarily responsible for, the decreased RNA synthesis in cells with reduced energy production.

When *Escherichia coli* are deprived of amino acids, a carbon source, or essential inorganic ions, the overall rate of protein breakdown increases 2- to 3-fold (1-3). Recent studies have implicated guanosine-5'-diphosphate-3'-diphosphate (ppGpp) in the regulation of protein degradation in *E. coli* (3, 4). Thus the lack of an amino acid (4, 5) or aminoacyl-tRNA (6) leads to a stimulation of proteolysis in rel \(^*\) cells, but not in rel \(-\) cells, which fail to accumulate ppGpp\(^2\) under such conditions. This enhancement of protein breakdown occurs simultaneously with a dramatic inhibition of a number of other growth-related processes, including the synthesis of ribosomal and transfer RNA, ribosomal proteins, and phospholipids, all of which appear to be regulated by levels of ppGpp (3, 7).

Upon starvation for a source of carbon (5), potassium (8), or phosphate (8, 9), or upon "energy step-down" (10), rel \(^*\) cells as well as rel \(^*\) increase protein catabolism and decrease RNA synthesis (7). Under these conditions both rel \(^*\) and rel \(-\) strains contain high levels of ppGpp (3, 7, 11, 12). An attractive hypothesis would be that this accumulation of ppGpp triggers the enhancement of proteolysis (3) and the concomitant fall in RNA synthesis (7). However, the involvement of ppGpp in the inhibition of RNA synthesis (13, 14) and enhancement of protein breakdown (3, 9) is still a matter of controversy.

Since the enhancement of protein breakdown and the accumulation of ppGpp during carbon or ion starvation occur in both rel \(^*\) and rel \(-\) strains, these changes are probably not signaled by a decrease in the supply of aminoacyl-tRNA (3).


\(\text{2} \quad \text{The abbreviation used is: ppGpp, guanosine-3'-diphosphate-5'-diphosphate}\)

\(\text{3} \quad \text{A. C. St. John and A. L. Goldberg, manuscript in preparation.}\)
Furthermore, the mechanism of ppGpp accumulation on amino acid starvation appears to differ from that on glucose deprivation. While lack of aminocyclo-1-RNA leads to an accelerated synthesis of ppGpp only in rel A+ strains (15), several groups (12, 16, 17) have shown that step-down conditions or glucose starvation reduces the rate of degradation of ppGpp in rel A+ and rel A- cells. Therefore, some alteration other than deprivation. While lack of aminoacyl-tRNA leads to an accelerated synthesis of ppGpp only in rel A+ strains (15), several groups (18, 19) have noted that treatment of E. coli with energy inhibitors can stimulate protein degradation. These findings appear surprising since a severe inhibition of energy metabolism is known to block intracellular protein catabolism (3, 18). We therefore investigated systematically the effects of decreased ATP generation on protein degradation and RNA synthesis in rel A+ and rel A- cells by providing various concentrations of inhibitors of energy metabolism. In addition, we have studied the effects of reduced energy production on ppGpp levels in an attempt to determine whether ppGpp may play a role in regulating protein breakdown and RNA synthesis under these conditions.

**Materials and Methods**

E. coli A-33 rel A+ (arg-, trp-) and A-33 rel A- (arg-, trp-) were kindly provided by Dr. B. Davis (Harvard Medical School). Cells were grown with aeration at 37°C in a basal salts medium (6) containing sodium succinate (2 g/liter) as the sole carbon source and arginine and tryptophan (30 mg/ml). Growth was estimated by measuring the turbidity of the cell suspension with a Klett-Sumner colorimeter (green filter) or a Gilford spectrophotometer (550 nm). An OD660 of 1.0 was equivalent to 95 Klett units and to 4 x 10^8 cells/ml.

Protein breakdown was measured after growth for two generations in medium supplemented with 1-(4,5-H)leucine (0.1 μCi/ml) (6). The cells were collected on Millipore filters and washed with medium lacking [3H]leucine and containing 120 μg/ml of nonradioactive leucine. Degradation of protein was then estimated from the release of [3H]leucine from protein into trichloroacetic acid-soluble form, as described previously (22). Each value is the average of two determinations which generally agreed within 5%. To measure the degradation of analog-containing proteins, growing cells were washed twice with arginine-free medium and resuspended at a density of 10^9 cells/ml in the same medium supplemented with [14C]canavanine (0.1 μCi/ml). After 10 min, the cells were washed and resuspended at a density of 3 x 10^8 cells/ml in medium containing 120 μg/ml of arginine. Release of [14C]canavanine into acid-soluble form was then measured (22).

RNA synthesis was measured by the incorporation of [2,4,14C]uracil into material precipitable with 5% trichloroacetic acid and collected on glass-fiber filter discs. The growth medium was supplemented with 0.05 μCi of [14C]uracil/ml (0.56 μCi/μl). The acid-precipitable radioactivity which was incorporated into DNA (resistant to hydrolysis by 1.0 N KOH for 16 h at 37°C) was subtracted from that in the total trichloroacetic acid precipitate to determine the incorporation into RNA. The specific activity of the [14C]uracil was measured by counting an aliquot of the culture medium, and the nanomoles of uracil incorporated into RNA was expressed relative to the turbidity of the culture (OD660).

The levels of ATP were estimated after extracting the cells with 4% trichloroacetic acid. The acid was extracted with 4 volumes of ether, and ATP was measured in duplicate by the firefly luciferase assay described previously by Stanley and Williams (25). Lyophilized firefly lanterns were obtained from Sigma Chemical Co.


To measure ppGpp levels, cells were grown in a low phosphate medium (pH 7.4) that contained per liter: 7.26 g of Tris, 2.4 g of (NH₄)₂SO₄, 0.295 g of MgSO₄·7 H₂O, 0.298 g of KCl, 0.054 g of Na₂HPO₄·7 H₂O, and 0.174 g of NaCl. Sucinate and the required amino acids were sterilized and added separately. The medium was supplemented with 100 μCi/ml of 32P, and after 60 min the levels of ppGpp were estimated by the method of Cashel (15). The breakdown of ppGpp was measured by a modification of the procedure of Gallant et al. (16). Cells were incubated for 30 min in Tris medium containing succinate and 0.2 μM 32P (100 μCi/ml) in the absence of arginine and tryptophan or in the presence of energy inhibitors. After 30 min, tetraacycline (50 μg/ml) was added to the cultures to prevent further synthesis of ppGpp, and samples were taken at regular intervals for assay of ppGpp content.

**Results**

**Effect of Reduced Energy Production on Protein Degradation**—To examine the effects of reduced ATP production, potassium cyanide, an inhibitor of electron transport, was added to A-33 rel A+ during growth on succinate. Under such conditions, anaerobic glycolysis cannot occur, and ATP production is dependent on oxidative phosphorylation. Preliminary experiments indicated that a gradual reduction in cellular ATP levels could be achieved by addition of different concentrations of cyanide (ranging between 10 and 2000 μM KCN). The cellular ATP content was similar after 30 and 60 min of incubation with KCN.

The effects of various levels of KCN on rates of protein degradation are shown in Fig. 1. Control cells had a 90-min doubling time and degraded intracellular proteins at a rate of 2.5%/h. Treatment with 10 μM KCN reduced ATP content by 24%, slowed the doubling time to 120 min, and increased protein degradation slightly (by 20%). At a KCN concentra-
tation of 100 \textmu M KCN, growth ceased, ATP fell by 36%, and the rate of protein breakdown doubled (5.4%/h) (Fig. 1). The stimulation of proteolysis was thus similar in magnitude to that seen upon starvation of cells for various essential nutrients.

By contrast, treatment with 2000 \textmu M KCN, which reduced ATP content by 90%, caused protein breakdown to fall to half the rate found in control cells. This result is in accord with earlier findings (3, 18-21) that a minimal amount of ATP (approximately 20% of the amount found in growing cells) is required for all types of protein catabolism.

The effects of graded decreases in ATP production on rates of protein breakdown were examined in a series of experiments with KCN or azide. Treatment of E. coli A-33 rel A+ with increasing concentrations of NaN, (0.5 to 25 mM) affected protein degradation in a similar fashion to KCN (Fig. 1); that is, low concentrations of NaN, promoted proteolysis, while very high concentrations blocked this process. Although the concentrations of azide that influenced protein breakdown were much higher than those of KCN, the effects of these inhibitors on proteolysis appeared very similar when the data were related to their effects on cellular ATP content (Fig. 2). At concentrations that lowered energy levels more than 25% (50 to 250 \textmu M KCN or 0.5 to 5.0 mM NaN,), growth stopped and protein catabolism was stimulated 2-fold (Fig. 2). When ATP levels decreased by 80% or more, the rate of protein degradation was less than the control rate (Fig. 2). Thus, in a large series of experiments, reducing energy production had two opposite effects on protein catabolism: 1) when ATP levels were reduced moderately (30 to 50%), a 2-fold stimulation in protein breakdown occurred; 2) a drastic reduction in ATP levels led to an inhibition of protein catabolism.

Additional experiments examined whether inhibition of energy production promotes protein degradation in rel A- cells as it does in rel A+. As shown in Fig. 3, a 2- to 3-fold stimulation of protein catabolism occurred in A-33 rel A- when ATP levels were reduced by 40 to 50% with KCN. Under these conditions, growth did not occur. Further lowering of ATP levels led to an inhibition of proteolysis. Thus a direct inhibition of energy metabolism, like starvation for a carbon source or inorganic ions (3, 8), enhances protein catabolism in a similar manner in rel A+ and rel A- strains.

**Effect of Reduced Energy Production on Degradation of Abnormal Proteins**—Although various types of starvation promote the degradation of normal proteins, they do not increase the hydrolysis of abnormal proteins, which occurs rapidly even in growing cells (3, 22, 24). In treatment with energy inhibitors induces the same cellular response as occurs during starvation, a moderate inhibition of ATP production should not stimulate the degradation of abnormal proteins (e.g., those containing amino acid analogs). Therefore the rate of breakdown of proteins containing the arginine analog, [14C]canavanine, was measured in the presence of various levels of potassium cyanide (0 to 2 mM). Such proteins were degraded 6 times more rapidly than those containing arginine.

The breakdown of analog-containing proteins did not increase significantly when ATP was reduced by 25 to 50% (Table I) in contrast to results with normal cell proteins and actually appeared to decrease when ATP fell by 50%. When ATP levels decreased by 80% or more, the rate of hydrolysis of [14C]canavanine-proteins fell significantly. These findings are in accord with previous reports (3, 19-21, 24) that a low level of ATP is essential for the breakdown of both normal and abnormal polypeptides.

**RNA Synthesis upon Inhibition of Energy Generation**—The stimulation of protein breakdown in various types of starvation and step-down conditions is associated with a marked reduction in RNA synthesis (3, 7, 10). We therefore studied the effects of graded inhibition of energy metabolism on RNA synthesis in A-33 rel A+. Addition of 10 \textmu M KCN slowed growth and reduced the incorporation of uracil into RNA by approximately 40%. Treatment with 50 or 100 \textmu M KCN, which led to a 2-fold stimulation in protein catabolism (Figs. 1 and 2), almost completely blocked growth and RNA synthesis.

**Fig. 2.** The relationship between the rate of protein breakdown and the ATP content of A-33 rel A+ cells treated with cyanide or azide. A series of experiments (similar to that in Fig. 1) was conducted in which cells were treated with various concentrations of cyanide or azide. In each experiment the rates of protein breakdown and the ATP levels were compared in the inhibitor-treated cultures and controls. Since the rate of protein breakdown in the control cultures varied from day to day for unknown reasons, the changes in proteolysis upon treatment with cyanide or azide are plotted as the rate of protein breakdown in the experimental culture relative to that in a control culture. Each point represents the data from a single culture in which both the ATP level and rate of protein breakdown were determined. In the cultures (from right to left) were treated with 0, 10, 25, 50, 100, 500, and 2000 \textmu M KCN; control ATP level = 1.54 nmol/OD. \(*\), cultures were treated with 0, 50, 100, 250, and 1000 \textmu M KCN; control ATP level = 1.53 nmol/OD. \(*\), cultures were treated with 0, 10, 25, 50, 100, 500, 1600, and 9000 \textmu M KCN; control ATP level = 1.46 nmol/OD. \(*\), cultures were treated with 0, 0.5, 1.0, 2.5, 5.0, 10.0, and 25.0 mM NaN; control ATP level = 1.26 nmol/OD.

**Fig. 3.** The relationship between the rate of protein breakdown and the ATP content of A-33 rel A+ treated with cyanide. The rates of protein breakdown and the levels of ATP in cultures treated with 0, 50, 100, 500, and 1000 \textmu M KCN. These values are expressed relative to a control culture growing on succinate. Data are plotted as in Fig. 2. Additional data consistent with this relationship have been obtained in several experiments.
Influence of ATP Production on ppGpp and Protein Degradation

Table I

Effects of treatment with KCN on ATP content and breakdown of proteins containing [14C]carnosine

Cells growing on succinate-minimal medium containing arginine were washed twice with arginine-free medium and resuspended in the latter medium supplemented with [14C]carnosine (0.1 µCi/ml). After 10 min, the cells were washed and resuspended in 5 ml of medium containing 120 µg/ml of arginine. One milliliter was added to each flask containing 10 ml of the same medium and the indicated concentration of KCN. The appearance of radioactivity in acid-soluble form was then measured in the usual fashion (22). Similar results were obtained in two additional experiments.

<table>
<thead>
<tr>
<th>KCN concentration (µM)</th>
<th>ATP level</th>
<th>Protein breakdown</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% control</td>
<td>% ATP min</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>16.3</td>
</tr>
<tr>
<td>10</td>
<td>90</td>
<td>17.9</td>
</tr>
<tr>
<td>50</td>
<td>60</td>
<td>14.5</td>
</tr>
<tr>
<td>100</td>
<td>45</td>
<td>13.5</td>
</tr>
<tr>
<td>500</td>
<td>20</td>
<td>8.6</td>
</tr>
<tr>
<td>1000</td>
<td>13</td>
<td>9.6</td>
</tr>
<tr>
<td>2000</td>
<td>10</td>
<td>4.7</td>
</tr>
</tbody>
</table>

The effects of KCN on RNA synthesis in succinate medium are similar to those observed by Nazar and Wong (25, 26) with rel A+ and rel A- strains growing on glucose. These workers demonstrated a preferential inhibition of RNA synthesis that could not be explained simply by a fall in the supply of nucleotide triphosphate precursors (26).

Involvement of ppGpp in These Alterations in Protein Breakdown and RNA Synthesis - To examine whether ppGpp might be responsible for the enhancement of protein catabolism (Figs. 2 and 3) and the decrease in RNA synthesis (Table I), we measured the levels of ppGpp in cells treated with cyanide or azide. When ATP levels fell by 40 to 50% with 100 µM KCN and by about 30% with 2.5 mM NaN₃, ppGpp content increased 5- to 10-fold. This large increase in ppGpp correlates with a doubling of protein breakdown and a drastic fall in RNA synthesis. Surprisingly when ATP levels were severely depleted (by 90%) with 500 µM KCN or 25 mM NaN₃, ppGpp content was necessary to study cells with an initially high level of this nucleotide. Therefore, the control culture was starved for arginine and tryptophan prior to the addition of tetracycline. (Previously Fieil et al. (28) showed that depletion of aminoacyl-tRNA does not affect the degradation of ppGpp although it increases synthesis of this compound.)

A similar reduction in the degradation of ppGpp was found when rel A+ cells were treated with azide. In cells treated with a concentration of NaN₃ that promotes proteolysis (2.5 mM), the half-life of ppGpp was 82 s. In addition, the ratio of ATP production on ppGpp and protein degradation

Table II

Effects of KCN on RNA synthesis in E. coli

Cells were grown in succinate-minimal medium to a density of 5 x 10⁹/ml, collected on a Millipore filter, and resuspended in 7 ml of sucinate medium containing 10 µg of uracil/ml. One milliliter was added to each flask containing 10 ml of the same medium and the indicated concentration of KCN. At zero time, [14C]uracil (0.05 µCi/ml) was added to the flasks, and the incorporation of [14C]uracil into RNA was measured. These data are typical of results obtained in two separate experiments.

<table>
<thead>
<tr>
<th>KCN concentration (µM)</th>
<th>RNA synthesis (pmol uracil/OD h)</th>
<th>% control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.3</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>1.9</td>
<td>63</td>
</tr>
<tr>
<td>50</td>
<td>0.46</td>
<td>14</td>
</tr>
<tr>
<td>100</td>
<td>0.23</td>
<td>7</td>
</tr>
<tr>
<td>500</td>
<td>0.07</td>
<td>2</td>
</tr>
<tr>
<td>1000</td>
<td>0.03</td>
<td>1</td>
</tr>
</tbody>
</table>

Table III

Levels of ATP and ppGpp in E. coli during inhibition of energy metabolism

A-33 rel A+ were grown initially in Tris medium containing 1.0 mM Na₂HPO₄. Cells were collected, washed twice, and resuspended in Tris medium containing 0.2 mM Na₂HPO₄. Cyanide or azide were then added at the indicated concentrations. For measurement of ppGpp and ATP levels, a portion of each culture was supplemented with 100 µCi/ml of ^32P. After 60 min, ppGpp and ATP levels were determined. Each value for ppGpp represents the mean ± the standard error when the number of experiments, indicated in parentheses, exceeded two. The relative ATP content was determined both by the chromatographic technique described by Cashel (15) and by the firefly luciferase assay (23), and the results of several determinations were averaged. The values for ATP content are the means from two separate experiments, which agreed within ±10%. These values are also consistent with the changes in ATP levels observed in many other experiments (see Figs. 2 and 3, Table I).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Condition</th>
<th>ATP level (µM)</th>
<th>ppGpp level pmol/KD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-33 rel A+</td>
<td>Control</td>
<td>100</td>
<td>54 ± 14 (4)</td>
</tr>
<tr>
<td>10 µM KCN</td>
<td>94</td>
<td>69 ± 20 (3)</td>
<td></td>
</tr>
<tr>
<td>2.5 mM NaN₃</td>
<td>68</td>
<td>361 ± 34 (3)</td>
<td></td>
</tr>
<tr>
<td>100 µM KCN</td>
<td>53</td>
<td>516 ± 55 (4)</td>
<td></td>
</tr>
<tr>
<td>500 µM KCN</td>
<td>14</td>
<td>602 ± 195 (9)</td>
<td></td>
</tr>
<tr>
<td>25 mM NaN₃</td>
<td>9</td>
<td>185 ± 39 (2)</td>
<td></td>
</tr>
<tr>
<td>A-33 rel A-</td>
<td>Control</td>
<td>100</td>
<td>41 ± 6 (2)</td>
</tr>
<tr>
<td>100 µM KCN</td>
<td>48</td>
<td>175 ± 25 (2)</td>
<td></td>
</tr>
<tr>
<td>No succinate</td>
<td>50</td>
<td>227</td>
<td></td>
</tr>
</tbody>
</table>
Influence of ATP Production on ppGpp and Protein Degradation

Levels of ppGpp were present in both cultures. Tetracycline (50 μg/ml) was added to both cultures at zero time to block ppGpp synthesis. The decrease in ppGpp levels with time was measured as described in Table III. Similar results were obtained in two separate experiments.

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Effects of Tetracycline on Protein Breakdown and RNA Synthesis upon Inhibition of Energy Production—To test whether this accumulation of ppGpp in cells treated with energy inhibitors is necessary for the increased proteolysis and decreased RNA synthesis, we examined the effects of tetracycline on these processes. Cells were treated with 50 μM KCN to enhance protein breakdown. When tetracycline was added to a portion of the culture, protein degradation decreased toward the rates found in growing cells. The decrease in ppGpp levels with time was measured as described in Table III. Similar results were obtained in two separate experiments.

Addition of tetracycline did not restore ATP to normal levels.5 In addition, the effect of tetracycline on RNA synthesis was measured with [3H]uracil incorporation into RNA (data not shown). These findings thus indicate that the stimulation of proteolysis found in cells growing on succinate or glucose does not depend upon ppGpp or protein synthesis. Therefore the basal rate of protein degradation found in growing cells does not depend upon ppGpp or protein synthesis.

In addition, the effect of tetracycline on RNA synthesis was measured in cells treated with 50 μM KCN (Table IV). This concentration of KCN reduced uracil incorporation into RNA by 90% (Table IV). Addition of tetracycline to such cultures caused a small but reproducible increase in uracil incorporation, although RNA synthesis was still far below levels seen in growing cultures. Similarly, in cells deprived of carbon source or cells shifted to anaerobic conditions, RNA synthesis fell dramatically, but addition of tetracycline increased incorporation into RNA slightly (Table IV). It is possible that this small rise in uracil incorporation induced by tetracycline reflects an enhancement of uracil transport. However in additional experiments, similar effects of tetracycline were observed when RNA synthesis was measured with [3H]uracil incorporation into RNA. Furthermore, RNA synthesis also fell dramatically when succinate-grown cells were administered KCN 15 min after they had been pretreated with tetracycline to prevent ppGpp synthesis (data not shown). These findings thus indicate that the accumulation of ppGpp may contribute in a minor way to the reduced uracil incorporation; however, a marked inhibition of RNA synthesis can occur upon energy restriction without continued production of this nucleotide.

Effects of Tetracycline on Protein Breakdown and RNA Synthesis upon Inhibition of Energy Production—To test whether this accumulation of ppGpp in cells treated with energy inhibitors is necessary for the increased proteolysis and decreased RNA synthesis, we examined the effects of tetracycline on these processes. Cells were treated with 50 μM KCN to enhance protein breakdown. When tetracycline was added to a portion of the culture, protein degradation decreased toward the rates found in growing cells (Fig. 5). Addition of tetracycline did not restore ATP to normal levels.5

These findings thus suggest that the stimulation of proteolysis induced by cyanide requires the continued production of ppGpp. Tetracycline caused a similar reduction in the rate of protein degradation in cells deprived of a carbon source or cells shifted from aerobic to anaerobic conditions (Table IV). However, this antibiotic did not reduce the low levels of proteolysis found in cells growing on succinate or glucose. Therefore the basal rate of protein degradation found in growing cells does not depend upon ppGpp or protein synthesis.

In addition, the effect of tetracycline on RNA synthesis was measured in cells treated with 50 μM KCN (Table IV). This concentration of KCN reduced uracil incorporation into RNA by 90% (Table IV). Addition of tetracycline to such cultures caused a small but reproducible increase in uracil incorporation, although RNA synthesis was still far below levels seen in growing cultures. Similarly, in cells deprived of a carbon source or cells shifted to anaerobic conditions, RNA synthesis fell dramatically, but addition of tetracycline increased incorporation into RNA slightly (Table IV). It is possible that this small rise in uracil incorporation induced by tetracycline reflects an enhancement of uracil transport. However in additional experiments, similar effects of tetracycline were observed when RNA synthesis was measured with [3H]uracil incorporation into RNA. Furthermore, RNA synthesis also fell dramatically when succinate-grown cells were administered KCN 15 min after they had been pretreated with tetracycline to prevent ppGpp synthesis (data not shown). These findings thus indicate that the accumulation of ppGpp may contribute in a minor way to the reduced uracil incorporation; however, a marked inhibition of RNA synthesis can occur upon energy restriction without continued production of this nucleotide.

TABLE IV
Effects of Tetracycline on protein degradation and RNA synthesis in E. coli during step-down conditions

For measurements of protein breakdown, A-33 rel A+ were grown in succinate-minimal medium containing [3H]leucine (0.1 μCi/ml). After 2 generations, the cells were harvested, washed in medium without succinate containing 120 μg/ml of nonradioactive leucine, and resuspended in 3 ml of the same medium. One milliliter was added to 10 ml of the same medium supplemented with succinate or succinate plus 50 μM KCN. Cultures growing on minimal medium with glucose (5 g/liter) as the sole carbon source were labeled, harvested, washed, and resuspended on a similar medium or one lacking glucose. To induce anaerobiosis, N2 was bubbled vigorously through the culture throughout the period of study. For each condition, similar results were obtained in two additional experiments. A portion of each culture was treated with 50 μg/ml of tetracycline. Protein breakdown was measured in the usual fashion and uracil incorporation into RNA was measured as described in Table II. The enhancement of proteolysis upon anaerobiosis was smaller repeatedly than that occurring in the other conditions.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Protein breakdown</th>
<th>RNA synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of control</td>
<td>mmol uracil/OD680 min</td>
</tr>
<tr>
<td>Succinate</td>
<td>1.1</td>
<td>0.360</td>
</tr>
<tr>
<td>Succinate + KCN</td>
<td>3.1</td>
<td>0.034</td>
</tr>
<tr>
<td>+ Glucose</td>
<td>1.6</td>
<td>0.278</td>
</tr>
<tr>
<td>- Glucose</td>
<td>3.9</td>
<td>0.017</td>
</tr>
<tr>
<td>Glucose aerobic</td>
<td>1.7</td>
<td>0.243</td>
</tr>
<tr>
<td>Glucose anaerobic</td>
<td>2.9</td>
<td>0.077</td>
</tr>
</tbody>
</table>

5 Unpublished observations.
These studies demonstrate that a moderate inhibition (30 to 50%) of the cell's ability to generate ATP leads to a stimulation of protein catabolism (Fig. 2), as well as a dramatic reduction in growth and RNA synthesis (Table II). Such regulatory effects on the degradation of normal cell proteins should not be confused with the well-established requirement for a low amount of ATP for all types of protein breakdown (19-21).

Recent in vivo (20) and in vitro studies (20, 29) have indicated that high energy phosphates in some way are essential in the degradative process. In accord with this conclusion, we found that concentrations of cyanide or azide that lowered ATP levels by 80% or more reduced the catabolism of both normal and abnormal proteins below rates found in growing cells (Fig. 2, Table I).

Presumably these effects of partial energy restriction are related to the adaptations that occur during starvation for a carbon source or essential ions. These findings support the simple hypothesis that a decrease in cellular high energy phosphates may signal the increase in protein catabolism (3) as well as the fall in RNA synthesis (25, 26) and other anabolic processes in starving cells. In fact, deprivation of E. coli for a source of carbon (7, 19), potassium or phosphate, or step-down conditions (14, 25) leads to a decrease in ATP content at least as large as that induced by 50 to 100 μM KCN. Furthermore, a number of similarities exist between the effects of energy inhibitors and those of starvation for carbon or inorganic ions: both promote the hydrolysis of normal proteins 2- to 3-fold, both are effective in rel A+ and rel A- cells and neither increases the degradation of abnormal proteins (3).

Although very different concentrations of azide or cyanide were necessary to cause comparable stimulation of protein breakdown, these agents had indistinguishable effects when protein breakdown was related to changes in cellular ATP content (Fig. 2). In related studies, addition of KCN was also found to stimulate protein breakdown in E. coli growing in the presence of a complete supply of amino acids. Thus the increase in proteolysis probably is signaled by the decreased energy production rather than the lack of amino acids. Possibly the ATP levels themselves or some closely related parameter (e.g. the concentration of another nucleotide) regulates the rates of protein degradation and ppGpp production. At present it is still unresolved whether the changes in RNA synthesis during energy step-down are directly signaled by reduced ATP concentrations. Gallant et al. (14) have argued that the changes in ATP levels during energy step-down are too small to account for the fall in RNA synthesis in such cells. However, small decreases in ATP content may be amplified in some fashion (e.g. by increases in ppGpp levels). On the other hand, some parameter indirectly related to ATP levels, such as adenylate charge (30), the levels of other nucleotides, the concentrations of NAD+ or NADP+, etc., may vary more dramatically upon treatment with KCN or NaN3 and may be a more sensitive measure of energy production than cellular ATP content.

In both rel A+ and rel A- cells, the increase in protein breakdown during treatment with inhibitors of respiration (Figs. 2 and 3) was associated with a marked accumulation of ppGpp (Table III) (31). Increased levels of ppGpp have previously been observed upon starvation for a carbon source or energy step-down in both rel A+ and rel A- cells (11-16). This nucleotide appears to mediate the changes in RNA synthesis (7, 32) and protein breakdown (3, 4) during starvation for amino acids or aminoacyl-tRNA. A number of other growth-related processes also are inhibited upon amino acid starvation apparently as a consequence of the high ppGpp concentrations (7). The present studies and others (7) linking ppGpp levels to energy metabolism raise the possibility that the rise in this nucleotide plays a similar role in other types of starvation as in amino acid deprivation. It would clearly be advantageous for a cell to shut down energy-consuming processes (e.g. synthesis of ribosomal RNA and proteins) when its ability to generate ATP is reduced. In addition increased breakdown of proteins in energy-poor cells should provide amino acids that can be used for the synthesis of catabolic enzymes (1, 3) or possibly even as substrates for energy production.

The experiments with tetracycline (Figs. 4 and 5) suggest that the accumulation of ppGpp is essential for the stimulation of protein breakdown upon inhibition of energy production. A critical assumption of this argument is that tetracycline serves as a selective inhibitor of ppGpp synthesis by blocking the stringent factor (27). In accord with this view, tetracycline causes a rapid fall in ppGpp (Fig. 4) that precedes the reduction in proteolysis toward basal levels (4). Although tetracycline also inhibits protein synthesis, its ability to reduce proteolysis in starved cells cannot be explained simply by this effect. It remains possible that tetracycline has some unknown additional effect causing the reduction in protein breakdown. However, at the concentration used here, tetracycline does not affect the degradation of abnormal proteins or normal proteins in growing cells (Table IV) nor the rates of RNA synthesis or levels of ATP. While these results are all consistent with a necessary role for ppGpp in the stimulation of proteolysis, proof of this conclusion will require demonstration of an effect of ppGpp on proteolysis in cell-free extracts.

In contrast, the accumulation of ppGpp in energy-restricted cells does not by itself seem to account for the dramatic fall in RNA synthesis. Addition of tetracycline to KCN-treated cells or glucose-starved cells only partially reversed the inhibition of uracil incorporation into RNA (Table IV). Thus the rise in ppGpp may contribute in part to the fall in RNA synthesis, although another mechanism must be primarily responsible for this adaptation. Two groups (13, 14) have also questioned whether the fall in RNA synthesis on carbon starvation and step-down is signaled by ppGpp. Gallant et al. (14) demonstrated under such conditions a new guanosine nucleotide that may be involved in the inhibition of RNA synthesis. It is also possible that a moderate fall in ATP and other RNA precursors may limit RNA synthesis upon treatment with KCN or azide. However, Nazar and Wong (26) have presented evidence refuting this idea.

The buildup of ppGpp under conditions in which energy production is inhibited, such as starvation for a carbon source (16), step-down (17), or treatment with cyanide or azide (Fig. 4), involves a marked decrease in the degradation of this nucleotide. In fact, most, if not all, of the 5- to 10-fold accumulation of ppGpp during treatment with KCN or NaN3, can be accounted for by the 6-fold reduction in ppGpp degradation. It is possible that some enhancement of ppGpp synthesis (as occurs on amino acid deprivation) may also occur in rel A- cells under energy restriction. The limited data in Table III, in fact, suggest a greater accumulation of ppGpp in rel A+ cells than in rel A-, which would be consistent with some enhancement of ppGpp synthesis. It has been suggested (3, 10, 22) that bacteria contain two...
systems for protein degradation with distinct physiological roles. One is responsible for the basal rate of protein breakdown in growing cells. This constitutive system protects against the accumulation of abnormal proteins and functions independently of ppGpp (i.e., in the presence of tetracycline) (Table IV). A second degradative response is evident under poor nutritional conditions when cellular levels of ppGpp are high (Table III, Fig. 5). These experiments and related studies (3, 4, 6, 11) indicate two mechanisms through which the acceleration of proteolysis may occur: (a) a lack of aminoacyl-tRNA in relA+ cells leads to accelerated synthesis of ppGpp and thus increased protein catabolism; (b) a moderate fall in ATP production leads to an accumulation of ppGpp (through a decrease in its degradation) and to increased proteolysis. Possibly ppGpp causes an activation of the cell's proteolytic machinery, or it may somehow make certain cell proteins more sensitive to pre-existent degradative enzymes. This issue is presently being investigated.

Acknowledgments—We are deeply grateful to Miss Kathy Conklin and Mr. Eric Rezenthal for their expert assistance in these studies, and Miss Claire Kollin and Mrs. Elsa Fox for their assistance in the preparation of this manuscript.

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Effects of reduced energy production on protein degradation, guanosine tetraphosphate, and RNA synthesis in Escherichia coli.

A C St John and A L Goldberg


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