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Role of Protein Synthesis in the Survival of Carbon-Starved *Escherichia coli* K-12

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In a typical *Escherichia coli* K-12 culture starved for glucose, 50% of the cells lose viability in ca. 6 days (Reeve et al., J. Bacteriol. 157:758–763, 1984). Inhibition of protein synthesis by chloramphenicol resulted in a more rapid loss of viability in glucose-starved *E. coli* K-12 cultures. The more chloramphenicol added (i.e., the more protein synthesis was inhibited) and the earlier during starvation it was added, the greater was its effect on culture viability. Chloramphenicol was found to have the same effect on a *relA* strain as on an isogenic *relA*$^+$ strain of *E. coli*. Addition of the amino acid analogs *S*-2-aminoethylcysteine, 7-azatryptophan, and *p*-fluorophenylalanine to carbon-starved cultures to induce synthesis of abnormal proteins had an effect on viability similar to that observed when 50 μg of chloramphenicol per ml was added at zero time for starvation. Both chloramphenicol and the amino acid analogs had delayed effects on viability, compared with their effects on synthesis of normal proteins. The need for protein synthesis did not arise from cryptic growth, since no cryptic growth of the starving cells was observed under the conditions used. From these and previous results obtained from work with peptidase-deficient mutants of *E. coli* K-12 and *Salmonella typhimurium* LT2 (Reeve et al., J. Bacteriol. 157:758–763, 1984), we concluded that a number of survival-related proteins are synthesized by *E. coli* K-12 cells as a response to carbon starvation. These proteins are largely synthesized during the early hours of starvation, but their continued activity is required for long-term survival.

Starving bacteria are of interest in both an ecological and an applied context, and we have been interested in characterizing the physiological changes that occur during starvation (12, 16, 22). We have previously presented evidence (16) that protein degradation plays a role in the survival of carbon-starved bacteria. Mutants of *Escherichia coli* and *Salmonella typhimurium* that were deficient in protein degradation were found to possess a greatly decreased stability under conditions of carbon starvation. Although these mutants had no innate deficiency in their protein-synthetic machinery, they were unable to synthesize protein at the same rate as their corresponding wild-type strains during carbon starvation. This suggested that amino acids derived from protein degradation were utilized by these cells for new protein synthesis and that their increased susceptibility to carbon starvation originated from their inability to synthesize these proteins.

In *E. coli* cells subjected to carbon starvation, the rate of protein synthesis drops to about 20% of the initial rate during the first hour of starvation (16) and then remains roughly constant for at least the next 47 h (unpublished data). We present evidence here that this protein synthesis is important for the survival of carbon-starved *E. coli* K-12; inhibition of normal protein synthesis during starvation greatly compromised survival.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The *E. coli* K-12 wild type used in this study was a Stanford strain. Strains NF161 (*metB argA spoT1 relA*$^+$), NF162 (*metB argA spoT1 relA1*), and NF859 (*metB argA spoT*$^+$ *relA*$^+$) (3) were provided by J. Gallant. M9 medium (16) was used for the growth and starvation of all strains. For growth, the medium was supplemented with 0.4% glucose; in experiments with the NF strains, the growth medium was also supplemented with 0.3 mM methionine and 0.3 mM arginine (Sigma Chemical Co.). All chemicals were reagent grade. Cultures were grown at 37°C as described previously (16). The *relA* phenotypes of the NF strains were monitored periodically by using growth inhibition by serine hydroxamate as described by Pao and Gallant (13).

Starvation for carbon. Mid-log-phase cells were harvested and subjected to carbon starvation as described previously (16). For the NF strains, the starvation medium was supplemented with 0.3 mM methionine and 0.3 mM arginine. Chloramphenicol and the amino acid analogs DL-7-azatryptophan, *p*-fluoro-DL-phenylalanine, and *S*-2-aminoethyl-L-cysteine were from Sigma.

Viability determinations. The viability of starving cultures was determined by spreading serial dilutions of cultures on M9 agar plus 0.02% glucose as described previously (16). For the NF strains, the agar medium was supplemented with 0.3 mM methionine and 0.3 mM arginine. Culture half-lives were measured by drawing the straight line of best fit through the viability data obtained after 1 day of starvation.

Determination of protein synthesis. Two methods were used for measurement of protein synthesis. In the first, 1 ml of culture was added to 10 μl of 0.1-mg/ml leucine and 4 μCi of L-[4,5-^{3}H]leucine (59.2 Ci/mmol; New England Nuclear Corp.) in a 5-ml shake tube. The leucine concentration was thus 7.7 μM, and the specific radioactivity of the label was 520 Ci/mol. The culture was shaken 10 min at 37°C, at which time 0.75 ml was withdrawn and pipetted into 75 μl of 50% trichloroacetic acid. The resulting precipitates were washed and counted for radioactivity as described previously (16). In the second method, leucine (13 μg per ml) and [^{3}H]leucine (2.5 μCi per ml) were added to the cultures at zero time for starvation, for a final leucine concentration of 0.1 mM and a

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specific radioactivity of 25 Ci/mol. Trichloroacetic acid precipitates were formed and counted at desired intervals thereafter. These two methods yielded the same results.

Monitoring the fate of DL-*p*-fluorophenyl[^{14}C]alanine. To 60 ml of carbon-starved cells, 6 μCi of DL-*p*-fluorophenyl [^{14}C]alanine (20 mCi/mmol; Research Products International) were added at zero time for starvation. Duplicate 0.5-ml aliquots of this culture were counted to obtain total culture disintegrations per minute. The rest of the culture was divided into one 6-ml aliquot and 10 5-ml aliquots in serum vials fitted with cap and bucket assemblies as described by Harrison et al. (7). At desired intervals, duplicate 0.5-ml aliquots were withdrawn from the 6-ml culture and filtered through Gelman GN-6 filters (25 mm), which were counted to determine intracellular analog. A 0.2-ml volume of 5 N H_2SO_4 was added to duplicate, sealed 5-ml cultures, by using a syringe inserted through the serum cap, and 0.15 ml of β-phenylethylamine (Sigma) was added to the filter paper in the buckets with a syringe. After shaking 1 h at 37°C, the acidified cultures were filtered through GN-6 filters, which were counted to determine radioactivity in protein. The filter papers were removed from the plastic buckets and counted to determine disintegrations per minute in CO_2. Counts per minute were converted to disintegrations per minute by using an internal standard.

Starvation culture cross-feeding experiments. A logarithmic *E. coli* K-12 culture at a density of about 7×10^7 cells per ml was divided into two. To one (culture A), 0.4 μCi of [^{14}C]glucose ([*U*-^{14}C]D-glucose, 295 mCi/mmol; ICN Radiochemical Division) per ml was added. To the other (culture B), no additions were made. After about four generations of growth, both cultures were harvested, washed twice in M9 minus glucose, and suspended to the same density in M9 minus glucose.

To determine total initial culture disintegrations per minute, duplicate 0.5-ml aliquots of culture A were counted at zero time for starvation. At desired intervals during starvation, 16 ml of culture A and 10 ml of culture B were centrifuged. Duplicate 1-ml aliquots of the culture A supernatant were counted to determine supernatant counts in culture A.

At each time point, the centrifuged culture B (unlabeled) cells were suspended in 10 ml of the culture A (labeled) supernatant. Of this suspension, 4 ml was placed in a serum vial fitted with a cap and bucket assembly and was shaken at 37°C for 100 min, at which time the disintegrations per minute in CO_2 and in macromolecules were determined as described above. The remainder of the suspension was incubated separately in a serum vial at 37°C for 100 min, at which time cellular disintegrations per minute were determined, also as described above.

Cell recycling apparatus. The cell recycling apparatus used in the cryptic growth studies consisted of a 1-liter culture flask, from which cells were constantly withdrawn by a Sarns model S10K-II pump and cycled through a hollow-fiber filter unit and then back to the original culture flask. The filter unit was an Accurel filtration module (Ghia Membrana, Pleasanton, Calif.) consisting of three extruded polypropylene fibers (18-mm inside diameter) encased in a single shell. The fiber pore size was 0.2 μm, and the length of the fibers was 30 cm. Fresh starvation medium from a reservoir was drawn into the system at the same rate as used starvation medium was extruded through the filter.

The culture (volume, 240 ml) was pumped through the system at a rate of 300 ml per min, and filtrate was drawn off at a rate of 150 ml per h. Thus, the medium was turned over at a rate of about 1 culture volume every 100 min. Experiments were carried out in a warm room maintained at 37°C. Samples for viability determination were withdrawn periodically via a sample port in the side of the culture flask.

TABLE 1. Effect of varying concentrations of chloramphenicol on carbon-starved *E. coli* K-12[a]

Chloramphenicol concn (μg/ml)	Protein synthesis rate at 30 min (% control[b])	Culture half-life (% control[c])
0	100	100
10	28	55
50	9	35
100	6	20

[a] An *E. coli* K-12 culture was grown to a density of ca 2.5×10^8 cells per ml, harvested, and suspended in growth medium minus glucose, as described in the text. The culture was then divided into four 50-ml aliquots. One aliquot was used as a control. To the others, 10, 50, and 100 μg of chloramphenicol per ml, respectively, were added. The half-lives of the cultures and the protein synthesis rate in each culture after 30 min of starvation were measured as described in the text.

[b] 100% = 160 pmol of leucine incorporated per ml of culture in 10 min.

[c] 100% = 80 h.

Miscellaneous methods. β-Galactosidase activity was induced with isopropyl-β-D-thiogalactopyranoside (Sigma) and assayed with *o*-nitrophenyl-β-galactopyranoside (Sigma) as described by Clark and Switzer (2). Epifluorescence microscopy to determine whole-cell counts and electron microscopy to determine cell morphology and size were carried out as described by Amy and Morita (1).

RESULTS

Effect of chloramphenicol on protein synthesis and viability of carbon-starved *E. coli*. If protein synthesis is important for the survival of carbon-starved bacteria, then inhibition of protein synthesis by antibiotics should result in decreased stability of the cells. We found that chloramphenicol added at zero time of starvation inhibited protein synthesis in carbon-starved *E. coli* K-12, the extent of inhibition depending upon the concentration of chloramphenicol used (Table 1). In turn, increasing concentrations of chloramphenicol caused a progressive decrease in the culture half-life during starvation.

In another set of experiments, 100 μg of chloramphenicol per ml of culture was added to identical starving cultures at different times during starvation, and the effect on culture half-life was determined (Fig. 1). Regardless of when chloramphenicol was added, it lowered the culture half-life by at least 60%. However, up to 9 h of starvation, the earlier the inhibitor was added, the more pronounced was the reduction in culture half-life. After 9 h, the effect of chloramphenicol on culture half-life remained roughly the same regardless of the time of addition.

Even when chloramphenicol was added at zero time of starvation, loss of viability did not occur until after at least 5 h of starvation. When it was added after ≥3 h of starvation, loss of viability did not commence until after 24 h, as was the case with the control culture (data not shown).

Effect of *relA* mutation on survival during carbon starvation. Chloramphenicol and other inhibitors of protein synthesis depress the stringent response in cells starved for amino acids (8). This is presumably because cessation of protein synthesis allows a buildup of charged tRNA in the cell, abolishing the signal for increased synthesis of guanosine tetraphosphate (6, 19). Whether chloramphenicol de-

presses the stringent response in cells subjected to prolonged carbon starvation is not known, but it is conceivable that it does. Prolonged starvation for carbon is likely to create amino acid scarcity in the cell, and the resulting buildup of uncharged tRNA would then elicit the stringent response in the same manner as in amino acid-starved cells.

It is thus possible that the deleterious effect of chloramphenicol on starvation survival arises from an effect of the antibiotic on the stringent response rather than from its inhibition of protein synthesis. This effect of chloramphenicol would be analogous to that of a *relA* mutation, since mutants in the *relA* locus lack the ability to respond to uncharged tRNA with an increased synthesis of guanosine tetraphosphate (5). If this explanation holds, then *relA* mutants should be less stable under carbon starvation than the wild type. Furthermore, chloramphenicol should have little effect on the carbon starvation survival of a *relA* mutant.

To test this, we subjected isogenic *relA*$^+$ and *relA* K-12 strains (NF161 and NF162, respectively) to carbon starvation as described above, and we monitored the viability behavior of each strain in the presence or absence of chloramphenicol (Fig. 2). The response of both strains to starvation was identical. Furthermore, chloramphenicol caused the same degree of reduction in culture half-life during carbon starvation in both strains. Thus, the effect of chloramphenicol on protein synthesis per se appeared to be important in causing its deleterious effect on survival during starvation.

The two strains used in the above experiments also possessed a *spoT* mutation. However, the starvation behavior of the closely related strain NF859, which is *spoT*$^+$, established that this mutation did not influence the results; NF859 showed a survival pattern identical to that of the other strains in the presence as well as in the absence of chloramphenicol (Fig. 2).

Effects of amino acid analogs on viability of carbon-starved *E. coli*. Continued protein synthesis played a role in survival of carbon-starved bacteria, as shown by the above data, but it is unclear from this data whether the proteins resulting from this synthesis are important factors in survival or whether the act of synthesizing protein itself is beneficial to the cells. Since starvation results in elevated rates of ribosomal RNA degradation (9), factors that stabilize ribosomes may be beneficial during starvation, and continued protein synthesis might have this effect. To choose between these alternatives, we added amino acid analogs to

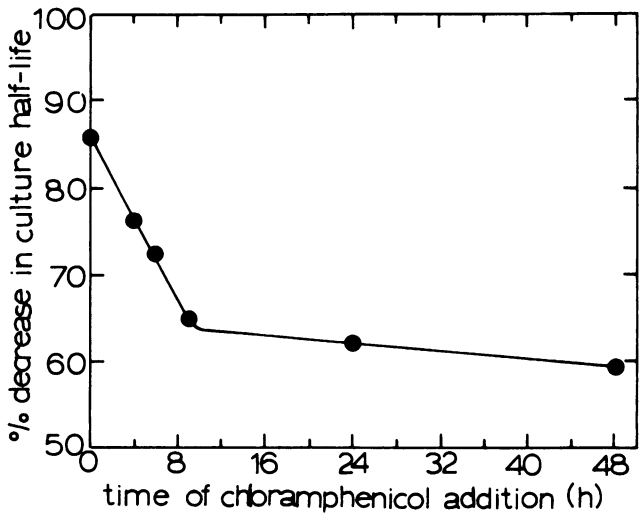

FIG. 1. Differential effect of chloramphenicol added at different times during carbon starvation of *E. coli* K-12. A 300-ml culture of *E. coli* K-12 was grown to a density of ca. 2.5 × 10^8 cells per ml, harvested, and suspended in growth medium minus glucose, as described in the text. At zero time for starvation, the culture was divided into 50-ml aliquots, and 100 μg of solid chloramphenicol per ml was added to different cultures at the times shown. The survival of the cultures was monitored over a period of 7 days, as described in the text. Half-lives for the control culture and for the cultures to which chloramphenicol was added at 0 or 6 h of starvation are averages calculated from the results of two separate experiments. All other half-lives are calculated from a single experiment. Results are expressed as the percent decrease in culture half-life compared with that of a control culture (half-life, 142 h) to which chloramphenicol was never added.

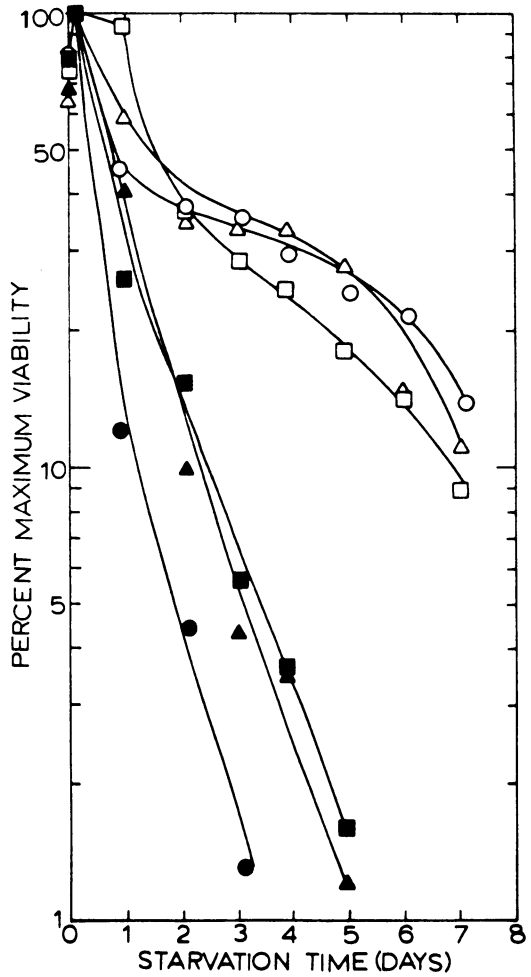

FIG. 2. Carbon starvation survival of *relA1* and *relA*$^+$ *E. coli* K-12 strains in the presence and absence of chloramphenicol. Cultures (100 ml each) of *E. coli* NF161, NF162, and NF859 were grown to densities of ca. 4.5 × 10^8 cells per ml, harvested, and suspended in growth medium minus glucose as described in the text. At zero time for starvation, each culture was divided into two 30-ml cultures, and 100 μg of solid chloramphenicol per ml was added to one culture of each strain. Viability was monitored as described in the text. Open symbols represent controls; closed symbols represent cultures to which chloramphenicol was added. Symbols: ○ and ●, NF161 (*relA*$^+$ *spoT1*); △ and ▲, NF162 (*relA1 spoT1*); □ and ■, NF859 (*relA*$^+$ *spoT*$^+$).

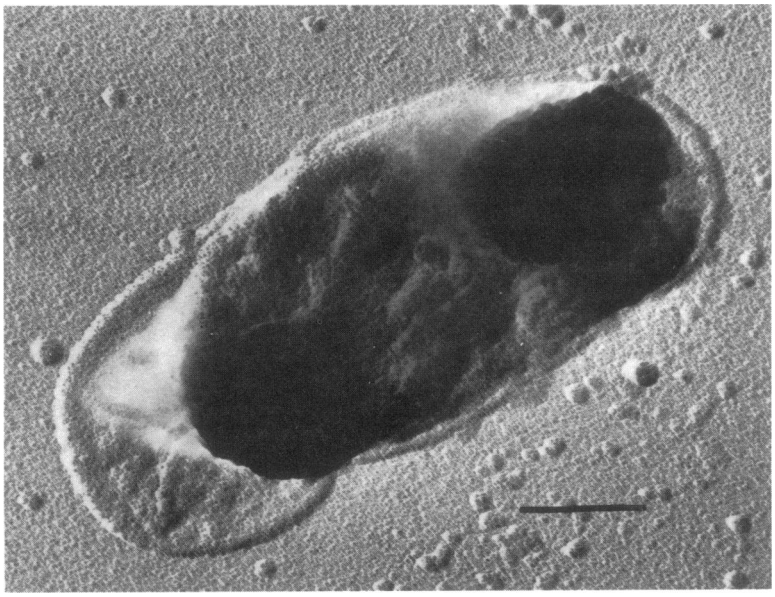

FIG. 3. Electron micrograph of *E. coli* K-12 cell after 170 h of starvation. Note that the cytoplasmic contents have shrunken away from the cell envelope, which maintains its original size and shape throughout starvation. Bar, 0.5 μm.

cells subjected to carbon starvation. These conditions should allow protein synthesis to continue, but the proteins formed are likely to be defective (4, 17). Three analogs, DL-7-azatryptophan (a tryptophan analog), *S*-2-aminoethyl-L-cysteine (a lysine analog), and *p*-fluoro-DL-phenylalanine (a phenylalanine analog) were tested. When added individually at 50 μg per ml, these analogs exerted nearly identical effects on the viability of the carbon-starved cells, reducing the culture survival rate to about the same degree as did 50 μg of chloramphenical per ml. As was the case with chloramphenicol, the analogs had a delayed effect on culture viability; no effect was observed until after 24 h of starvation, even when the analogs were added at zero time for starvation (data not shown).

The interpretation of these results was confounded by the finding that the analogs did decrease the rate of protein synthesis. In a separate experiment (data not shown), we found that azatryptophan, aminoethylcysteine, and fluorophenylalanine decreased the rate of protein synthesis in starving cells during the first hour of starvation by 46, 35, and 27%, respectively. However, only 7-azatryptophan had a prolonged effect on protein synthesis; cells treated with the other two analogs showed normal rates of protein synthesis after 90 min.

Overall, it is most likely that the deleterious effect of amino acid analogs on the starving cells was due to the synthesis of faulty proteins rather than to their effect in decreasing the rate of protein synthesis. Several results supported this conclusion. (i) Whereas the effects of the analogs on the rate of protein synthesis varied widely, their effects on the culture half-life of the starving cells were the same. (ii) Even though 7-azatryptophan only partially inhibited bulk protein synthesis during the first hour of starvation, we found that no active β-galactosidase was synthesized in response to the exposure of starving azatryptophan-treated cells to isopropyl-β-D-thiogalactopyranoside, whereas control starved cells synthesized appreciable levels of active enzyme (data not shown). These results indicate that azatryptophan incorporation resulted in synthesis of inactive β-galactosidase. (iii) By feeding DL-*p*-fluorophenyl [^{14}C]alanine to cells at zero time for starvation, we were able to directly determine the fate of this analog. By 4 h of starvation, all of the labeled analog transported into the cell had been incorporated into protein, and no label was respired.

Lack of cryptic growth in carbon-starved *E. coli* cultures. The findings presented above strongly suggest that continued protein synthesis enhances the survival capability of carbon-starved *E. coli* K-12 cultures. One possibility is that this protein synthesis is part of an overall cryptic growth of the culture, i.e., multiplication of part of the starving culture on nutrients released by dying or lysing cells or both (14, 15, 18). We conducted an investigation of this possibility.

When we measured electron micrographs of carbon-starved *E. coli* cells at various points during starvation, we found no appreciable change in average cell length, even after 6 days of starvation. The number of whole cells, measured by epifluorescent counting of culture samples taken out to 6 days of starvation, remained constant throughout starvation, even while 50% of the cells in the culture lost viability. Thus, there was little change in outer cell dimensions during starvation, and no cell lysis was detectable.

Although cell lysis evidently did not occur during starvation, nonviable cells might have released nutrients into the medium, making them available for use by viable cells. In electron micrographs, a marked decrease in protoplast size was evident as starvation progressed (Fig. 3). Using ^{14}C-labeled starving cells as described above, we found that this decrease could be correlated with the loss of low-molecular-weight cellular material into the starvation milieu. By 117 h of starvation, 25% of the ^{14}C-labeled material inside the labeled cells had been extruded into the medium. However, less than 1% of this material was taken up or utilized by other cells in the culture. We concluded that the material released into the surrounding medium by starving cells was virtually useless to these cells and did not promote growth in other starving cells. In agreement with this conclusion, the survival of starving *E. coli* cells in a recycled culture, whose medium was removed and replaced with fresh medium at a constant rate, was identical to that of cells in a batch culture.

Overall, our findings showed that, although *E. coli* cells starved for carbon underwent marked changes in internal morphology, little, if any, cryptic growth occurred in our starved cultures.

DISCUSSION

To our knowledge, the data presented in this paper represent the first demonstration of a requirement for synthesis of active proteins for the survival of carbon-starved bacteria. The addition of chloramphenicol to *E. coli* cells starved for carbon inhibited protein synthesis and markedly increased the death rate during starvation. Studies with *relA* mutants indicated that the deleterious effect of chloramphenicol on starvation survival was not mediated through interference with the stringent response but rather was due to protein synthesis inhibition. Cells starved in the presence of amino acid analogs also exhibited decreased survival. The enhanced survival of starved bacteria able to synthesize protein was not a result of cryptic growth of these bacteria; we found that little, if any, cryptic growth occurred in starving cultures under the conditions used.

A few points about our studies with chloramphenicol are particularly noteworthy. First, the extent of reduction in survival during starvation was proportional to the extent of inhibition of protein synthesis by chloramphenicol (Table 1). This result is consistent with our previous observations with peptidase-deficient mutants of *E. coli* and *S. typhimurium*. In these mutants, the survival rate during starvation correlated directly with the rate of protein breakdown which, by making free amino acids available, in turn determined the rate of protein synthesis (16, 21). Also, it is striking that chloramphenicol exerted the greatest effect on survival when added in the early phase of starvation. After the first hour of starvation, carbon-starved *E. coli* cells maintained a constant rate of protein synthesis for many hours, and the addition of chloramphenicol at any time caused a virtually complete inhibition of this protein synthesis (data not shown). Yet, the effect of chloramphenicol on survival remained dependent on the time of its addition during starvation for the next 8 h. Thus, the temporal aspect of the chloramphenicol effect did not stem from the fact that its addition at different times caused different net changes in the rate of protein synthesis. Rather, it appears that proteins synthesized maximally during the first 9 h of starvation are the most crucial for survival. The hypothesis that these proteins, synthesized at the onset of starvation, play a role in long-term survival is supported by the observation that the addition of chloramphenicol or amino acid analogs at zero time for starvation had a delayed effect on culture viability.

Willetts (20) demonstrated the presence of a pool of labile proteins in logarithmically growing *E. coli*, and it is possible that some of the protein synthesis occurring at the onset of starvation serves to replenish these rapidly degraded proteins. However, this resynthesis of previously existing proteins would not explain the relatively greater importance to survival of protein synthesis during the initial hours of starvation; resynthesis of labile proteins would be expected to occur at the same rate throughout starvation. Studies with mutants unable to initiate the degradation of proteins during starvation would be helpful in discerning to what extent the resynthesis of preexisting proteins plays a role in starvation survival.

Starving cells very likely synthesize proteins whose purpose is to increase the probability of escaping starvation. It is well-established that organisms subjected to growth under carbon limitation derepress the synthesis of catabolic enzymes, thus amplifying their potential to make use of diverse carbon substrates and thereby escaping carbon limitation (10, 11). The same phenomenon probably occurs at the onset of starvation. What substrate may present itself in the milieu of a starving bacterium is, of course, unpredictable, and a general derepression of catabolic enzymes would serve the function of priming the organism to use new substrates without a lag if they become available.

However, the essential biosynthetic activity at the onset of starvation that is indicated by the data presented in this paper appears to be concerned with the synthesis of proteins which help the bacterium to withstand the starvation stress per se, rather than to escape it. A cell entering starvation faces a fundamentally altered physiological situation. The cell metabolism, formerly geared to growth, must be reoriented to maintenance. Endogenous energy reserves must be mobilized, and the cell must survive in the absence of multiplication. These changes probably require marked physiological and structural changes in the cells, and the proteins synthesized at the onset of starvation might help to achieve this change. Studies now under way are aimed at a direct investigation of these starvation proteins.

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