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# Role of Ribosome Degradation in the Death of Starved *Escherichia coli* Cells

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**In *Escherichia coli* cultures limited for phosphate, the number of ribosomal particles was reduced to a small percentage of its earlier peak value by the time the viable cell count began to drop; the 30S subunits decreased more than the 50S subunits. Moreover, the ribosomal activity was reduced even more: these cells no longer synthesized protein, and their extracts could not translate phage RNA unless ribosomes were added. The translation initiation factors also disappeared, suggesting that they become less stable when released from their normal attachment to 30S subunits. In contrast, elongation factors, aminoacyl-tRNA synthetases, and tRNA persisted. During further incubation, until viability was reduced to $10^{-5}$, the ribosomal particles disappeared altogether, while tRNA continued to be preserved. These results suggest that an excessive loss of ribosomes (and of initiation factors) may be a major cause of cell death during prolonged phosphate starvation.**

Formidable methodological obstacles have discouraged investigation of the mechanism of the loss of viability of bacteria in the stationary phase (25), although the frequent exposure of bacteria to prolonged starvation in nature makes this an important problem. In approaching it we suggest that the theoretically predictable mechanisms of cell death fall into only three classes. The first mechanism is irreversible damage to a vital element in the genome. This seems unlikely to be a major response to starvation (unless a prophage is activated), since cells have elaborate devices for protecting the DNA. The second mechanism is irreversible damage to the cell membrane. Although this is clearly important in organisms that autolyze readily during starvation (such as *Streptococcus pneumoniae*), enterobacteria and many other organisms maintain their turbidity. Even stronger evidence against this mechanism is the observation of Postgate and Hunter (26) that starved *Aerobacter aerogenes* cells maintained an intact osmotic barrier after the viability count had dropped by 80%.

The third mechanism is the complete loss of certain species of macromolecules. These would not include most enzymes, the cofactors, any kind of RNA, or the components of various energy-transducing systems, since these products should all be restorable if their genes are intact and if the necessary building blocks and energy can be provided. However, the complete loss of any protein species required for protein synthesis should be lethal. Indeed, this mechanism has been demonstrated: temperature inactivation of a temperature-sensitive mutant elongation factor G rapidly destroyed viability (1). The same effect would be expected from the complete loss of ribosomes, of any soluble factors of translation or aminoacyl-tRNA synthetases, or of RNA polymerase.

The degradation of ribosomes seems a likely mechanism in this third class, since it is a well-known major response of bacteria to starvation (17). This adaptive process provides nucleotides and amino acids, and it might also provide energy in the form of nucleoside diphosphates if rRNA is degraded by polynucleotide phosphorylase—a mechanism for which there is fragmentary evidence (12–14, 18, 19). However, if the loss of functional ribosomes (or of other proteins required for protein synthesis) should be carried too far, causing them to fall below a critical level, the cell would no longer be viable.

Many early studies have examined the effects of various factors on the survival of starved bacteria (5, 16, 18, 26, 27), and others have examined the loss of total RNA or of ribosomes (2, 4–6, 12–14, 20, 22, 24, 28, 29). Montague and Dawes (21) connected the two problems by observing that the loss of viability in starved cells was associated with a decrease in both RNA and nucleotide content, but they concluded that the nucleotide loss might cause death by depriving the cell of energy. In this paper we examine the possibility that the loss of ribosomes is critical.

Most earlier studies of ribosome breakdown in starvation were carried out by abruptly transferring cells from growth conditions to a buffer. This procedure has the advantage of defining the composition of the starvation medium precisely. However, in nature cells tend to be deprived of a required food gradually, and their adaptive changes in composition may affect their survival. We have therefore studied starving cell cultures, rather than cells transferred to a buffer. To optimize measurements of the protein-synthesizing activity of cell extracts, we used a mutant of *Escherichia coli* lacking the periplasmic RNase I.

## MATERIALS AND METHODS

**Growth conditions.** *E. coli* D10, an RNase $I^-$, $Met^-$, lambda lysogenic K-12 strain (10), was used for most experiments. The media used were variations on a minimal medium composed of the following (per liter): 1 g of $(NH_4)_2SO_4$, 3 g of $KH_2PO_4$, 7 g of $K_2HPO_4$, 0.5 g of sodium citrate, 5 mg of $CaCl_2$, 100 mg of $FeSO_4$, 100 mg of L-methionine, and 4 g of glucose. To limit carbon or nitrogen we varied the concentration of glucose or of $(NH_4)_2SO_4$. For limiting phosphate the medium was as described above, except that 11 mg of $KH_2PO_4$ and 25 mg of $K_2HPO_4$ were used; 12.1 g of Tris per liter was added for buffering, and the medium was adjusted to pH 7.6 with HCl. The cultures (1.5 liters) were incubated with a 5% inoculum of an overnight culture in 6-liter flasks on a reciprocal shaker at 37°C. Growth was monitored with a Klett-Summerson colorimeter at 540 nm.

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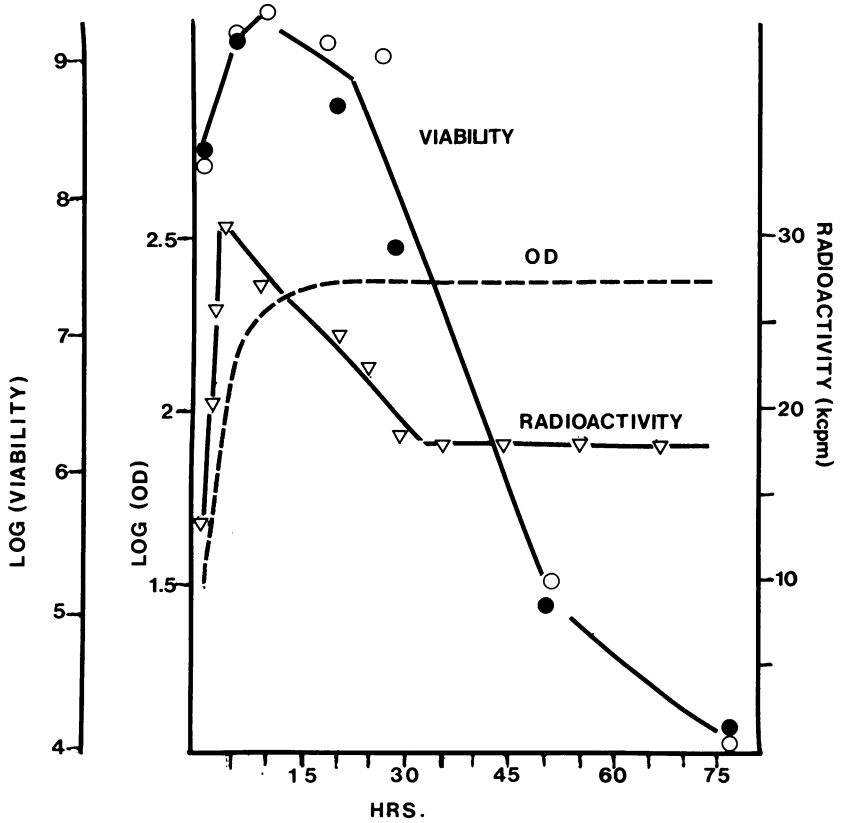


FIG. 1. Nucleic acid content, viability, and optical density (OD) during phosphate limitation. *E. coli* cultures were incubated in phosphate-limited medium containing 5 μg of [$^3$H]uridine per ml. Optical density (– – –), TCA-precipitable radioactivity (▽), and viability (○) were determined as described in Materials and Methods. Viability in a culture incubated without uridine (●) was also measured.

**Buffers.** TKM buffer contained 10 mM Tris hydrochloride (pH 7.6), 50 mM KCl, and 10 mM magnesium acetate. TKMD buffer contained, in addition, 1 mM dithiothreitol.

**Viable cell counts.** Viable cell counts were determined by making 10-fold serial dilutions in the minimal medium without a carbon source, plating in duplicate on LB medium or minimal medium plates, and incubating at 37°C overnight. LB medium plates contained, per liter, 10 g of tryptone (Difco Laboratories), 7.5 g of NaCl, 2 g of yeast extract, and 18 g of Bacto-Agar.

**Measurement of labeled nucleic acid.** Cells were grown with 1 μCi (5 μg) of [$^3$H]uridine per ml. At various times 100-μl samples were transferred to 2 ml of 5% trichloroacetic acid (TCA) on ice. After 30 min the precipitates were collected on glass fiber filters (Whatman GA/C), washed three times with 5% TCA and once with 1% acetic acid, dried under a heat lamp, and counted in scintillation fluid.

**Measurement of protein synthesis in cells.** At various times a 1-ml sample of culture was incubated with [$^{14}$C]leucine (0.1 μCi; ca. 300 mCi/mmol) for 30 min at 37°C. Incorporation was measured by one of the following procedures. (i) In experiments involving many samples, 25-μl portions were spotted onto a previously numbered Whatman 3MM chromatography paper strip. The strips were immersed in 5% TCA at 0°C for 10 min and then at 100°C for 15 min, cooled, washed three times with 95% ethanol, dried under a heat lamp, and counted as described above. (ii) When only a few samples were tested, 100-μl portions were transferred to 2 ml of 5% TCA and held at 100°C for 15 min. The precipitates were then collected, washed, dried, and counted.

**S30 extracts.** Extracts were made from cultures of at least 1.5 liters as described previously (32). After completion of nascent polypeptides (to convert all polysomes to free ribosomes) by incubation at 15°C for 15 min, the cells were harvested by centrifugation and washed once with cold TKM buffer. The pellet was frozen with solid $CO_2$-ethanol and stored at −70°C. The cells were lysed by being ground in the cold with 1.5 times their weight of alumina, and DNase I (RNase-free; Worthington Diagnostics) was then added (2 μg/g of cells), unless otherwise indicated. The lysate was diluted with an equal weight of cold TKMD buffer and centrifuged at 12,000 × *g* for 10 min to remove cell debris and alumina. The supernatant solution was centrifuged at 30,000 × *g* for 30 min, and the supernatant was dialyzed against cold TKMD buffer for 3 h. The resulting S30 preparation was frozen in small portions in solid $CO_2$-ethanol and stored at −70°C.

**In vitro protein synthesis.** The activity of the S30 preparations was determined by incubating 1.0 $A_{260}$ unit for 30 min at 37°C in a reaction mixture containing the following (per 0.1 ml): 50 μg of phage R17 RNA, 50 mM Tris hydrochloride (pH 7.6), 40 mM $NH_4Cl$, 9 mM magnesium acetate, 2 mM dithiothreitol, a source of energy (0.1 mM Tris-ATP, 2 mM Tris-GTP, 5 mM phosphoenolpyruvate, or 1.5 μg of pyruvate kinase), 2 μM [$^{14}$C]valine (210 Ci/mol), and 19 other amino acids at 50 μM each. S100 extracts, crude initiation factors, and ribosomes (washed with 1 M $NH_4Cl$) were prepared from *E. coli* D10 as described previously (32). Purified initiation factors (IFs) were generous gifts of H. F. Kung (Roche Institute of Molecular Biology, Nutley, N.J.).

Ribosomes were stored at −70°C in TKMD buffer plus 10% glycerol.

**Analysis of ribosomal particles and rRNA in sucrose gradients.** To determine the distribution of ribosomal particles in S30 preparations, we centrifuged 1.0-$A_{260}$-unit samples through 10 to 30% linear sucrose gradients in TKM buffer (Beckman SW50.1 rotor, 2 h, 45,000 rpm, 3°C). The gradients were examined in an Isco analyzer by measuring the $A_{254}$.

To determine the size distribution of the rRNA, we centrifuged the S30 preparations through a Sephadex G-25 column equilibrated with Tris hydrochloride (to remove $K^+$ introduced with the TKM buffer). After being mixed with 1% sodium dodecyl sulfate to free the rRNA from proteins, samples were analyzed as previously described (31), except that 5 to 20% sucrose gradients of 12 ml were used for centrifugation (Beckman SW41 rotor, 14 h, 31,000 rpm, 14°C).

**Reagents.** Tris-ATP, Tris-GTP, Tris, pyruvate kinase, phosphoenolpyruvate, dithiothreitol, and amino acids were from Calbiochem-Behring or Sigma Chemical Co. All radioactive materials were from Du Pont Co. or New England Nuclear Corp.

## RESULTS

**Choice of conditions of starvation.** Cells of RNase $I^-$ *E. coli* D10 were grown in media containing excess [$^3$H]uridine and limited for nitrogen [0.01 g of $(NH_4)_2SO_4$ per liter], carbon (0.2 g of glucose per liter), or phosphate (11 mg of $KH_2PO_4$ and 25 mg of $K_2HPO_4$ per liter). After reaching its peak, the radioactivity of the cells decreased at a similar rate under the three kinds of starvation. Loss of viability and cessation of protein synthesis appeared somewhat earlier with phosphate limitation, and so in further studies we used these conditions.

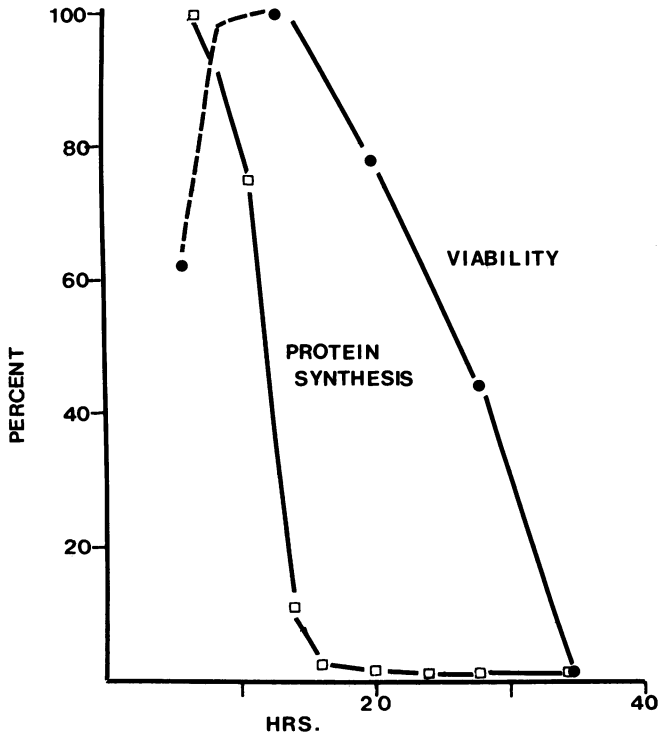


FIG. 2. In vivo protein synthesis and viable counts. Cultures were incubated as described in the legend to Fig. 1 but without added uridine. The peak (100%) viable count was $9.6 \times 10^8$ cells per ml. Samples were incubated with [$^{14}$C]leucine for 30 min, and protein synthesis was expressed as the percentage of maximal incorporation (100%: 61,000 cpm). The values are expressed on a linear scale; the broken line represents uncertainty between the datum points but reflects results obtained in other experiments.

TABLE 1. Protein synthesis by extracts from phosphate-limited cells[a]

| Addition | [$^{14}$C]valine incorporation (cpm) by indicated cells | | |
|---|---|---|---|
| | Exponential phase | Early stationary phase | Intermediate phase |
| None | 38,300 | 36,900 | 776 |
| Ribosomes | 39,800 | 45,000 | 844 |
| IFs | 52,300 | 50,100 | 2,151 |
| IFs + ribosomes | | | 36,100 |
| IFs + 30S subunits | 44,800 | 51,600 | 9,300 |
| IFs + 50S subunits | 51,200 | 50,300 | 1,300 |

[a] The cultures were harvested successively at 4, 10, and 29 h; the viable cell counts were $3.4 \times 10^9$/ml and $1.3 \times 10^9$/ml in the early stationary and intermediate cultures, respectively. One $A_{260}$ unit of each S30 extract was assayed for [$^{14}$C]valine incorporation in 30 min with phage R17 RNA as the message. The components added were 60 μg of $NH_4Cl$-washed ribosomes, 35 μg of crude IFs, 20 μg of 30S subunits, and 40 μg of 50S subunits.

Although strain D10 is lysogenic for phage lambda, cell death did not appear to be due to the induction of phage lysis, for the optical density remained constant while the viability decreased extensively (Fig. 1), and no lambda phage particles could be detected in the culture supernatant in a phage spot test. Moreover, the kinetics of cell death were similar, although somewhat slower, in *E. coli* MRE600, which is not lysogenic. The kinetics were also slower in *E. coli* ATCC 27257, which is not RNase $I^-$. Further experiments were done with strain D10.

**Changes in nucleic acid content and in viable cell number.** As a first test for the possible correlation of cell death with extensive degradation of ribosomes, cultures were grown in phosphate-limited medium containing excess [$^3$H]uridine, and samples taken at various times were assayed for acid-precipitable counts and viable cell number.

The content of [$^3$H] from uridine in the cells reached a peak during the exponential phase and then began to decrease well before the viable cell number leveled off (Fig. 1). When the culture reached its maximal viable cell count the nucleic acid content had decreased to about 70% of its maximal value. Loss of mRNA could not be large enough to account for all of this loss (ca. 30%), which was observed consistently. Evidently, in response to the slowing of growth the "stable" RNA (15) not only ceased to accumulate ("shift-down") but also began to be degraded before growth ceased.

During further incubation the $^3$H content in the cells continued to decrease, leveling off in various experiments at 30 to 50% of the maximum. This level was reached at about a 99% loss in the viable cell count, and it showed little further decrease during further loss of viability, down to $10^{-5}$. The time of onset of the loss of viability varied from 12 to 20 h.

Although rRNA is known to constitute the bulk of the total incorporated nucleotide in cells in steady-state growth, it became a small fraction of the counts in cells by the stage of moderate viability loss (50 to 80%). An analysis of an extract of such cells prepared without DNase showed that 40% of the acid-precipitable counts were sensitive to RNase (as

TABLE 2. Effect of IFs on protein synthesis by an extract from phosphate-starved cells[a]

| Addition | [$^{14}$C]valine incorporation (cpm) |
|---|---|
| None | 696 |
| IF1 | 602 |
| IF2 | 1,200 |
| IF3 | 1,100 |
| IF1 + IF3 | 570 |
| IF2 + IF3 | 1,610 |
| IF1 + 2 + 3 | 3,370 |

[a] The intermediate extract from Table 1 was assayed for R17 RNA-directed [$^{14}$C]valine incorporation with 0.5 $A_{260}$ unit of extract and 30 μg of $NH_4Cl$-washed ribosomes per 0.05 ml of reaction mixture. The supplements were 0.18 μg of IF1, 1 μg of IF2, and 0.25 μg of IF3.

expected of tRNA, but not of ribosomes) and that 40% were sensitive to DNase.

**Protein synthesis in cells.** Samples of phosphate-limited cultures were taken at intervals and incubated for 30 min with added [$^{14}$C]leucine. Although one would expect net protein synthesis to be severely impaired in cells in the stationary phase, labeling with [$^{14}$C]leucine might have detected low-level incorporation, perhaps associated with turnover. However, as Fig. 2 shows, when the culture had begun to lose viability (by 20 to 50%), it had lost all detectable ability to incorporate a labeled amino acid (<2% of the value at late exponential growth).

When such a culture was supplemented with phosphate, it resumed substantial protein synthesis within less than 1 h (data not shown). Although the changes during this restoration proved too complex to interpret, this result indicates that the culture still retained a source of energy.

To test whether the loss of protein-synthesizing capacity was due to the limitation of ribosomes or of some other component, we examined extracts for their content of ribosomal particles and for their capacity to synthesize protein when supplemented with various components. Cells were harvested at several stages.

**Protein synthesis in extracts.** Large-scale cultures were harvested during exponential growth (at an optical density of 120 to 140 Klett units) and also after reaching a steady reading (at 180 to 200 Klett units). Samples were also taken at intervals from a third culture that was incubated further; as a rapid, indirect test for the intermediate stage, they were monitored for their ability to incorporate a labeled amino acid as described above. When this incorporation ceased the cells were harvested, and a late culture was harvested 10 to 12 h later. (The inferred viability of 20 to 50% and $10^{-5}$, respectively, were verified.) We refer to the stage of moderate loss as "intermediate."

S30 extracts (treated with DNase) were prepared, and they yielded only about one-third as much $A_{260}$ material from the intermediate and late cultures as from the exponential cultures extracted at the same turbidity. Cell breakage by a French press rather than by grinding with alumina yielded similar results. The extracts were diluted to a uniform $A_{260}$ value in assays for their ability to incorporate [$^{14}$C]valine. (We did not attempt to calculate the S30 concentration in terms of cells directly, because the efficiency of recovery of ribosomes would not be expected to be uniform.)

With excess phage RNA as the messenger, extracts from cells in the early stationary phase had approximately the same protein-synthesizing activity per $A_{260}$ unit (and hence somewhat less per cell) as extracts from cells in the exponential phase (Table 1). The loss in the intermediate culture (or in the late culture), however, was dramatic: the extracts had no detectable activity. Since mRNA and ATP were provided, the cells at this stage evidently lacked some other required component.

**Components required for restoration of activity.** To identify the defect, we supplemented extracts from an intermediate culture with various components of the protein-synthesizing system, including salt-washed ribosomes (which lack IFs). With polyuracil, which bypasses physiological initiation, as the messenger, translation required the addition of ribosomes (data not shown). However, with an initiating system, translating phage R17 RNA, translation required IFs as well (Table 1).

When separate purified IFs were used along with ribosomes, IF3 and IF2 each had a slight effect, but all three IFs were required for a maximal response (Table 2). In contrast, the elongation factors of protein synthesis and the aminoacyl-tRNA synthetases were not significantly lost in intermediate cultures, for in the presence of added ribosomes and IFs the further addition of S100 extract (at several different concentrations) was not stimulatory (data not shown).

Table 1 further shows a difference in the survival of active 30S and 50S subunits. When purified subunits were added separately to the intermediate extract along with crude IFs, the 30S particles had a moderate effect, but the 50S particles had virtually none. The addition of 70S ribosomes, however, restored a high level of activity, comparable to that in the exponential-phase S30 extract (although lower if compared in terms of the equivalent number of cells). It thus appears that at this stage essentially all the 30S subunits and also the IFs (which normally are bound to these subunits in the cell) were inactivated and that the number of active 50S subunits was severely reduced.

**Analysis of ribosomal components.** As Fig. 3C shows, the S30 extract of the intermediate culture contained some 30S and 70S (as well as 50S) particles. Moreover, the 30S subunits were degraded faster than the 50S subunits, paralleling the difference in their inactivation shown in the preceding section. Thus, the $A_{254}$ ratio in the 30S, 50S, and 70S regions was 1:2:5 in the exponential and early stationary phases of growth, whereas in the intermediate phase it was 1:11:2 (i.e., a total 30S/50S subunit molar ratio of about 1:3).

An analysis of the rRNA (Fig. 4) extracted from ribosomes by sodium dodecyl sulfate confirmed these results. The $A_{260}$ ratio of 16S to 23S rRNA was 1:2 in exponential cells (Fig. 4A) but 1:4 in intermediate cells (Fig. 4C), indicating a shift in the molar ratio from 1:1 to about 1:2.

Figure 4 further demonstrates a striking decrease in the ratio of rRNA to tRNA during starvation. In extracts of exponential-phase cells (treated with DNase and sodium dodecyl sulfate), the 16S and 23S regions together represented 85 to 90% of the total $A_{260}$, but this value was reduced to 30 to 40% in extracts of early-stationary-phase cells and to only 10 to 15% in extracts of intermediate-phase cells. RNA close to the top of the gradient (ca. 4S) accounted for the rest.

Clearly, the incorporated labeled uridine that persists after extensive degradation of the ribosomes is all located in the 4S region and is maintained at a steady level (Fig. 1). Moreover, because the degraded rRNA yielded no detectable intermediates, it is unlikely to accumulate a significant amount of 4S fragments. Accordingly, the tRNA appears to be entirely stable and to constitute virtually all of the 4S peaks in these experiments, and so the 4S RNA provides a

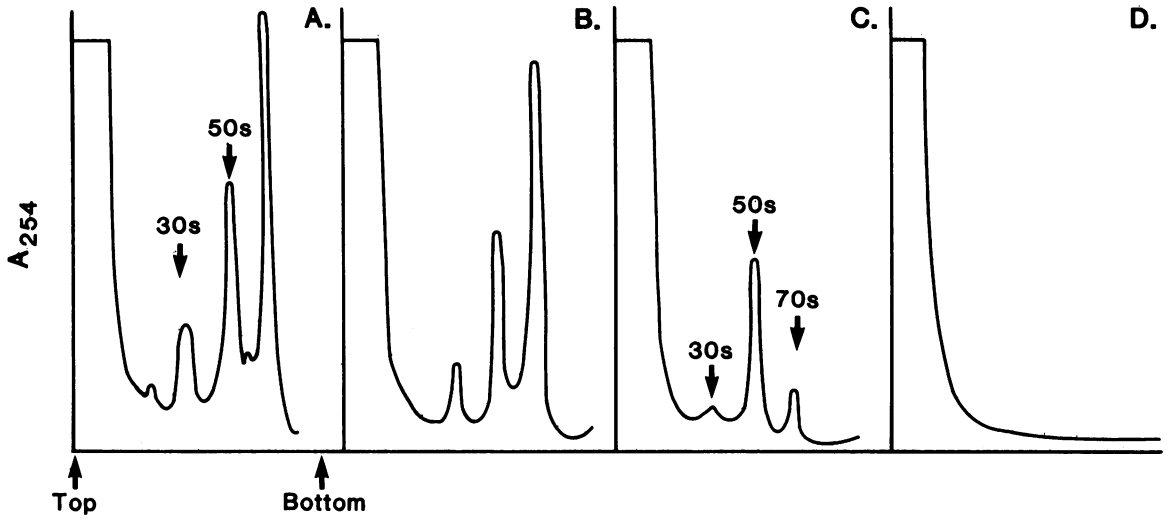


FIG. 3. Ribosomal profiles of extracts. Extracts were prepared from the cells described in Table 1 at different phases: A, exponential; B, early stationary; C, intermediate; and D, late (12 h after intermediate; viability reduced by $10^5$). Each was adjusted to the same $A_{260}$ and analyzed in a sucrose gradient as described in Materials and Methods.

tentative basis for converting the ribosome concentrations in various extracts, analyzed at equal $A_{260}$ values, into terms of equivalent numbers of cells. We can calculate that in intermediate cells the standardized extract was concentrated around fivefold (since the tRNA was shifted from 15 to 85% of the total RNA). Hence, in this culture the ribosomes, reduced to 15% of the $A_{260}$, were in reality reduced to an average of ca. 3% of what their concentration was in exponential cells. Since the ratio of 50S to 30S subunits doubled at this stage, the 30S subunits were evidently reduced to ca. 2%, and the 50S subunits were evidently reduced to ca. 4%.

An additional interesting feature in Fig. 3 is that the peaks of the surviving subunits in an inactive extract were sharp, without any significant broadening. Evidently, the disintegration of a subunit, once past an early stage, proceeded rapidly to completion (on the time scale of these experiments). Similarly, the rRNA molecules were rapidly degraded after their release from disintegrating ribosomes, as shown by the sharpness of the peaks of 16S and 23S RNAs in Fig. 4C. The same appears to be true of the released ribosomal proteins: two-dimensional gel electrophoresis (11) did not detect any in an S100 fraction (data not shown).

**Acidification during incubation.** In phosphate-limited cultures buffered with 100 mM Tris, the pH decreased from 7.6 to 5.0 during 20 h of incubation. The acidity accelerated cell

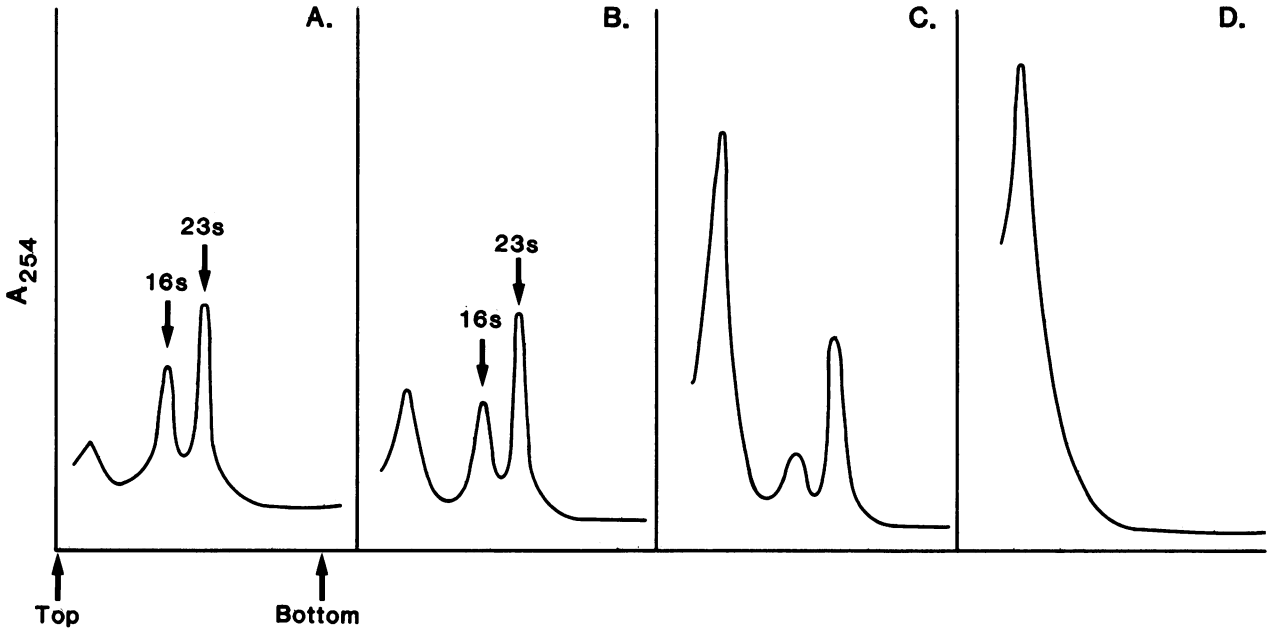


FIG. 4. Analysis of RNA size distribution. The extracts from Fig. 3A to D were treated with sodium dodecyl sulfate and analyzed for RNA profiles by centrifugation in a sucrose gradient as described in Materials and Methods.

death, for when the pH was adjusted back to 7.0 with NaOH the viability was sustained for at least an extra 24 h. However, the mechanism of loss of viability did not appear to depend on acidity: when viability did drop in the culture at pH 7.0, the correlated loss of capacity for protein synthesis and loss of ribosomal content were essentially the same as those described above (data not shown).

Since helices of ribosomes form in growing *E. coli* cells when the pH is allowed to decrease from 7.0 to 5.0 (23) and since in extracts these sediment with the discarded fragments of the cell envelope, it seemed possible that the acidification developing in our culture might be introducing artifacts into our analyses. However, when we separated membrane-associated from non-membrane-associated ribosomes by fractionating lysates (after a clearing spin at 3,000 × *g*) in a triphasic sucrose density gradient as previously described (3), the proportion of ribosomes recovered with the membrane fraction did not change between the early-stationary- and intermediate-phase cultures, although the total number of ribosomes fell substantially.

## DISCUSSION

As previously observed with various bacteria under conditions of decelerating growth (12, 18, 19, 24), *E. coli* cells in a phosphate-limited minimal medium began to degrade ribosomes before growth had ceased (Fig. 1). During further incubation in the stationary phase, by the time the viability loss reached 50 to 80% (intermediate cultures), 50% or more of the total nucleic acid (Fig. 1), including most of the ribosomes, had been lost, and protein synthesis was no longer detectable. (At this stage the starved cultures had not depleted their source of carbon and energy, for they resumed protein synthesis when supplemented with phosphate.) During a further decrease in viability to less than $10^{-5}$ (late cultures), the turbidity and the nucleic acid content (predominantly tRNA and DNA) remained essentially constant. These findings on nucleic acid loss are in harmony with those of Dawes et al. for *Peptococcus* (4, 21) or *E. coli* (6) cells incubated in buffer. We obtained similar results with cultures limited for carbon or for nitrogen.

As the culture runs out of exogenous phosphate and the ribosomes are degraded, the limited phosphate is presumably redistributed into the DNA, tRNA, phospholipid, and lipopolysaccharide of the increasing number of smaller cells. Some might also be accumulated in nonutilizable compounds (e.g., shed lipopolysaccharide) in the medium, as has been observed for nitrogenous compounds from cells incubated in buffer (30); however, since our focus was on the fate of the machinery of protein synthesis we did not examine this possibility.

In analyzing cells at various stages of starvation we standardized the extracts in terms of $A_{260}$ rather than in terms of cell number or weight, because extraction was not quantitatively reproducible. However, the results could be converted into terms of cells on the basis of the content of tRNA, which appeared, as a first approximation, to be quantitatively retained in the starved cells (see below). This calculation indicated that in the intermediate cultures only ca. 4% of the 50S subunits and 2% of the 30S subunits, measured either as particles (Fig. 3) or as extracted RNA (Fig. 4), had survived. If the assumption of quantitative retention of tRNA is incorrect, the concentration of these residual ribosomal particles would be even lower.

These figures also confirmed the observation of Kaplan and Apirion (13, 14) that the disintegration of a subunit, once begun, proceeds rapidly to completion: after extensive degradation of the ribosomes the residual particles (and their extracted RNA) still yielded sharp peaks in a sucrose gradient. (However, particles of diminished S value have been observed under some conditions of starvation [20].) Similarly, as might be expected from the rapid degradation of ribosomal proteins formed in the presence of excess rRNA (7, 8), we could not detect in the cytosol any of the proteins released from the disintegrating ribosomes.

In extracts from intermediate cultures the loss of ribosomal activity was even more complete than the loss of particles: no translation of phage RNA was seen unless ribosomes and IFs were added (Tbles 1 and 2). If the particles tested had fully retained their activity it would have been detected, and so it appears that there is a significant lag between inactivation and the initiation of a rapid breakdown. Whether these changes are initiated by a nuclease or by a protease is not known.

In marked contrast to the IFs, the elongation factors and the amino acid-activating enzymes had not undergone any apparent loss in intermediate cultures, since the addition of S100 to their extracts did not stimulate translation. The tRNA also was stable in starving cells, in contrast to rRNA. Thus, even in late cultures, with a complete loss of ribosomal particles and rRNA, the cells maintained a relatively high, steady level of incorporated [$^3$H]uridine (Fig. 1), which was identified as 4S RNA (Fig. 4D) and DNA. Since protein synthesis by extracts of intermediate cells (supplemented with ribosomes and IFs) was not stimulated by S100 extracts, these cells evidently retained a high level of functional tRNA. It thus appears that even though the synthesis of tRNA is coordinately regulated with that of rRNA in steady-state growth at various rates (15), during starvation tRNA is maintained at a level that permits the dwindling supply of ribosomes to function efficiently.

Interestingly, starvation appears to decrease the stability of only those components of the protein-synthesizing system that it causes to be more exposed, whereas those whose state is unchanged remain stable. Thus, the IFs are almost entirely bound to 30S subunits and to initiation complexes in growing cells, whereas during starvation these disappear, causing the IFs to accumulate as free proteins. In contrast, elongation factor and the amino acid-activating enzymes are already largely free in growing cells, whereas elongation factor Tu, which is stabilized by attachment to elongation factor or to aminoacyl-tRNA in growing cells (9), retains these attachments in starved cells. Finally, the release of RNA from the degraded ribosomes in starving cells would expose sequences that are normally covered; the tRNAs, in contrast, are partly free during growth as well as starvation.

The breakdown of ribosomes may also be promoted by a lack of adaptedness to the free state. Thus, starvation causes the polysomes to be converted to free ribosomes, whereas the latter are infrequent in growing cells. The more rapid elimination of 30S subunits than of 50S subunits (and their greater lability at an elevated temperature [33]) may have a similar explanation: in steady-state growth the "native" 30S subunits are mostly complexed with IFs, whereas the 50S subunits are free; also, the accumulation of free ribosomes in starvation would, by equilibration, increase the concentration of both free subunits.

Since one cannot hope to demonstrate that starved cells on the threshold of loss of viability have only one active ribosome or very few active ribosomes, it seems impossible to obtain direct proof for our proposal that ribosome degradation (and the associated loss of IFs) may be a major cause of death in starving cells. However, this hypothesis leads to

a prediction that can be tested: mutations that affect the kinetics of ribosome breakdown should also affect the kinetics of cell death. Meanwhile, the absence of detectable active ribosomes in the whole population by the time of only 50% loss of viability and the continued loss of ribosomal particles during more extensive killing support this idea, and we know of no evidence for any alternative. If this hypothesis is correct, ribosome degradation would be a double-edged sword: it initially has adaptive value for starved cells, but if carried too far it becomes suicidal.

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## LITERATURE CITED

1. **Aharonowitz, Y., and E. Z. Ron.** 1976. Translocation in *Bacillus subtilis*: irreversible inactivation of elongation factor G and viability of a temperature-sensitive mutant. J. Bacteriol. **125**:1229–1231.
2. **Ben-Hamida, F., and D. Schlessinger.** 1966. Synthesis and breakdown of ribonucleic acid in *Escherichia coli* starving for nitrogen. Biochim. Biophys. Acta **119**:183–191.
3. **Caulfield, M. P., S. Horiuchi, P. C. Tai, and B. D. Davis.** 1984. The 64 kilodalton membrane protein of *Bacillus subtilis* is also present as a multiprotein complex on membrane-free ribosomes. Proc. Natl. Acad. Sci. USA **81**:7772–7776.
4. **Dawes, E. A.** 1976. Endogenous metabolism and survival, p. 19–53. *In* T. R. G. Gray and J. R. Postgate (ed.), The survival of vegetative microbes. Cambridge University Press, Cambridge.
5. **Dawes, E. A., and D. W. Ribbons.** 1964. Some aspects of the endogenous metabolism of bacteria. Bacteriol. Rev. **28**:126–149.
6. **Dawes, E. A., and D. W. Ribbons.** 1965. Studies on the endogenous metabolism of *Escherichia coli*. Biochem. J. **95**:332–343.
7. **Dennis, P. P.** 1974. Synthesis and stability of individual ribosomal proteins in the presence of rifampicin. Mol. Gen. Genet. **134**:39–47.
8. **Fallon, A. M., C. S. Jinks, M. Yamamoto, and M. Nomura.** 1979. Expression of ribosomal protein genes cloned in a hybrid plasmid in *Escherichia coli*: gene dosage effects on synthesis of ribosomal proteins and ribosomal protein messenger ribonucleic acid. J. Bacteriol. **138**:383–396.
9. **Furano, A. V.** 1975. Content of elongation factor Tu in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **72**:4780–4784.
10. **Gesteland, R. F.** 1966. Isolation and characterization of ribonuclease I mutants of *Escherichia coli*. J. Mol. Biol. **16**:67–84.
11. **Geyl, D., A. Bock, and K. Isono.** 1981. An improved method for two-dimensional gel electrophoresis: analysis of mutationally altered ribosomal proteins of *Escherichia coli*. Mol. Gen. Genet. **181**:309–312.
12. **Horiuchi, T.** 1959. RNA degradation and DNA and protein synthesis of *E. coli* B in a phosphate deficient medium. J. Biochem. (Tokyo) **46**:1467–1480.
13. **Kaplan, R., and D. Apirion.** 1975. The fate of ribosomes in *Escherichia coli* cells starved for a carbon source. J. Biol. Chem. **250**:1854–1863.
14. **Kaplan, R., and D. Apirion.** 1975. Decay of ribosomal ribonucleic acid in *Escherichia coli* cells starved for various nutrients. J. Biol. Chem. **250**:3174–3178.
15. **Maaloe, O., and N. O. Kjeldgaard.** 1966. Control of macromolecular synthesis. W. A. Benjamin, New York.
16. **Mallette, M. F., C. I. Cowan, and J. J. R. Campbell.** 1964. Growth and survival of *Escherichia coli* in medium limited in phosphate. J. Bacteriol. **87**:779–785.
17. **Mandelstam, J., and H. Halvorson.** 1960. Turnover of protein and nucleic acid in soluble and ribosome fractions of nongrowing *Escherichia coli*. Biochim. Biophys. Acta **40**:43–49.
18. **Maruyama, H., and D. Mizuno.** 1965. The participation of ribonuclease in the degradation of *Escherichia coli* ribosomal ribonucleic acid as revealed by oligonucleotide accumulation in the phosphorous-deficient stage. Biochim. Biophys. Acta **108**:593–604.
19. **Maruyama, H., and D. Mizuno.** 1970. Ribosome degradation and the degradation products in starved *Escherichia coli*. I. Comparison of the degradation rate and of the nucleotide pool between *Escherichia coli* B and Q-13 strains in phosphate deficiency. Biochim. Biophys. Acta **199**:159–165.
20. **Maruyama, H., and S. Okamura.** 1972. Ribosome degradation and the degradation products in starved *Escherichia coli*. V. Ribonucleoprotein particles from glucose-starved cells. J. Bacteriol. **110**:442–446.
21. **Montague, M. D., and E. A. Dawes.** 1974. The survival of *Peptococcus prevotii* in relation to the adenylate energy charge. J. Gen. Microbiol. **80**:291–299.
22. **Nath, K. K., and A. L. Koch.** 1971. Protein degradation in *Escherichia coli*. III. Strain differences in the degradation of protein and nucleic acid resulting from starvation. J. Biol. Chem. **246**:6956–6957.
23. **Nauman, R. K., D. Silverman, and H. Voelz.** 1971. Ribosomal helices: formation in *Escherichia coli* during acidic growth. J. Bacteriol. **107**:358–360.
24. **Okamura, S., H. B. Maruyama, and T. Yanagita.** 1973. Ribosome degradation and the degradation products in starved *Escherichia coli*. VI. Prolonged culture during glucose starvation. J. Biochem. (Tokyo) **73**:915–922.
25. **Postgate, J. R.** 1976. Death in macrobes and microbes, p. 1–18. *In* T. R. G. Gray and J. R. Postgate (ed.), The survival of vegetative microbes. Cambridge University Press, Cambridge.
26. **Postgate, J. R., and J. R. Hunter.** 1962. The survival of starved bacteria. J. Gen. Microbiol. **29**:233–263.
27. **Postgate, J. R., and J. R. Hunter.** 1963. The survival of starved bacteria. J. Appl. Bacteriol. **26**:295–306.
28. **St. John, A. C., and A. L. Goldberg.** 1980. Effects of starvation for potassium and other inorganic ions on protein degradation and ribonucleic acid synthesis in *Escherichia coli*. J. Bacteriol. **143**:1223–1233.
29. **Strange, R. D., F. A. Dark, and A. G. Ness.** 1961. The survival of stationary phase *Aerobacter aerogenes* stored in aqueous suspension. J. Gen. Microbiol. **25**:61–76.
30. **Strange, R. E., H. E. Wade, and A. G. Ness.** 1963. The catabolism of proteins and nucleic acids in starved *Aerobacter aerogenes*. Biochem. J. **86**:197–203.
31. **Tai, P. C., and J. L. Ingraham.** 1971. A ribosome-like particle accumulated at low temperature by a cold-sensitive mutant of *Salmonella typhimurium*. Biochim. Biophys. Acta **232**:151–166.
32. **Tai, P. C., B. J. Wallace, E. L. Herzog, and B. D. Davis.** 1973. Properties of initiation-free polysomes of *Escherichia coli*. Biochemistry **12**:609–615.
33. **Tal, M., A. Silberstein, and K. Moyer.** 1977. *In vitro* reassembly of 30S ribosomal subunits following their specific destruction by thermal shock. Biochim. Biophys. Acta **479**:479–496.