Magnesium Starvation of *Aerobacter aerogenes*

III. Protein Metabolism

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ABSTRACT

The metabolism of the ribosomal and soluble protein components of *Aerobacter aerogenes* was examined during its incubation in a Mg^{++} -deficient medium. Bacteria were exposed to leucine- H^3 during the exponential growth period preceding Mg^{++} starvation, and extracts were prepared after intervals of starvation and were centrifuged through gradients of sucrose to separate ribosomal from soluble proteins. Ribosomal proteins synthesized during the preceding exponential growth were slowly lost from the ribosomes; after 8 hr of starvation, few, if any, sedimented with ribosomes. Losses of total protein, together with the known rate of ribosome decay during Mg^{++} starvation, suggested that these ribosomal proteins are ultimately degraded to acid-soluble products and account for all protein lost by the starving cells. These conclusions were supported by studies of Mg^{++} starvation in a uracil-requiring strain of *A. aerogenes*: during uracil starvation a smaller fraction of the proteins synthesized were ribosomal, and the fraction of protein which subsequently decayed during Mg^{++} starvation was correspondingly less. During recovery from Mg^{++} starvation, proteins, lost from disintegrated ribosomes, were not detectably reutilized into new particles even before their degradation to acid-soluble products was complete. Synthesis of soluble proteins continued for more than 24 hr of starvation at a rate per milliliter close to 45% of the instantaneous rate per milliliter of the exponentially growing bacteria at the time Mg^{++} was removed. This value agreed with that found previously for synthetic rates of deoxyribonucleic acid, transfer ribonucleic acid, and ribosomal ribonucleic acid during starvation relative to rates during exponential growth.

A deficiency of any of the major components required for normal growth generally results in a marked decrease in protein synthesis by the organism. For example, protein synthesis per hour is only 3 to 5% of the total protein in *Escherichia coli* during nitrogen starvation (18, 22), 3% in *Aerobacter aerogenes* during carbon starvation (10), and not detectable during K^+ starvation of *E. coli* (14). When *E. coli* is starved of phosphate, the level of protein doubles during the first 4 hr (2), but there is less than a doubling during the subsequent 20 hr (*unpublished data*). Similarly, in sulfur starvation of *E. coli*, protein synthesis continues for a short time before depletion of endogenous reserves, and then becomes extremely slow (20).

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Mg^{++} is a required cofactor for many enzyme activities and is a key structural component of ribosomes (see Discussion). However, in contrast to the above starvations, *A. aerogenes* continues to synthesize soluble proteins for more than 24 hr of Mg^{++} starvation at approximately 45% of the instantaneous rate per milliliter of the cells growing exponentially at the start of the starvation. This value (about 50% per hr per total initial protein) agrees with the rates of synthesis of nucleic acids in Mg^{++} -starved compared with exponentially growing cells (7), and is more than 10 times the rate of protein synthesis observed during starvation for any of the other major components of a simple inorganic salts-carbon medium (carbon, nitrogen, phosphorus, potassium, or sulfur).

The significance of this continuing balanced synthesis of macromolecules at these rates will be considered in this and the following paper (8).

MATERIALS AND METHODS

General procedures. The organisms *A. aerogenes* strains 1033 and U3, the conditions of culture for exponential growth and for starvation, preparation of extracts, and centrifugation through gradients of sucrose were described in the preceding papers (6, 7). The methods used to normalize the total radioactivities recovered from sucrose gradients for quantitative comparisons are the same as those used for the study of nucleic acid metabolism (7), except that radioactive amino acids were used as exogenous precursors, and radioactivity in protein was estimated as described below.

Estimation of radioactivity in protein. H^3 and C^{14} contents of protein were estimated by bringing a cell sample (<100 μ g, dry weight) to 5% trichloroacetic acid at 0 C; after 30 min or longer, the sample was heated at 90 C for 30 min and the remaining acid-insoluble material was chilled and collected on a membrane filter (0.8- μ ; Millipore Filter Corp., Bedford, Mass.), and was prepared for counting as previously described (6).

When extracting nucleic acids from proteins by hot trichloroacetic acid (23), it is important that the temperature does not rise above 90 C because of significant hydrolysis of bacterial protein. As much as 20% of the protein may become acid-soluble after heating for 30 min in 5% trichloroacetic acid in a boiling-water bath (Fig. 1). This has been verified by colorimetric determinations of the release of peptides (13).

Estimation of contents of ribosomal protein (R-protein) and soluble protein (S-protein). We have defined

R-proteins and S-proteins on the basis of sedimentation characteristics of proteins from extracts prepared and centrifuged in 10^{-4} M Mg^{++} for 3 hr through a 5 to 20% gradient of sucrose in a SW39 rotor (Spinco). The protein sedimenting in the lower two-thirds of the gradient contains the structural R-proteins, as well as unfinished polypeptides which are being synthesized on the particles. However, the time required for synthesis plus release of most or all polypeptides is very short (1 or 2 sec; 17), so that an extremely small fraction of the soluble protein is associated with the particles. Second, there is a significant difference in the kinetics of formation of ribosomal RNA (rRNA) and R-protein. Whereas the former molecules appear to mature relatively slowly to a final state in a completed ribosome (16), during which time they may be relatively unstable (5), the R-proteins sediment at a rate characteristic of completed particles very soon after incorporation of exogenous amino acids (1). As a result of these two features of ribosome and protein synthesis, bacteria exposed to labeled amino acids for a very short time have the same approximate distribution of radioactivity between their R-protein and S-protein fractions as do bacteria exposed for a very long time. For example, we have exposed exponentially growing *A. aerogenes* to leucine- H^3 for 10 sec. One portion was chilled (no chase), a second was chased with unlabeled leucine for 5 min, and a third portion was chased for 20 min. Upon subsequent sucrose gradient centrifugation of the extracts, the percentage of H^3 in the R-protein region (fractions 1 through 21 in Fig. 2) was 35, 28, and 28, respectively,

FIG. 1. Radioactivity remaining in precipitated form in 5% trichloroacetic acid at the temperatures shown. Bacteria were grown on uniformly labeled L-leucine- C^{14} and uracil-5,6- H^3 to label protein and nucleic acids, respectively. Equal portions of culture were chilled and brought to 5% trichloroacetic acid at 0 C. After 30 min, each sample was incubated at a specified temperature and for a specified time, terminated by chilling. The remaining insoluble material was collected on filters for counting.

whereas cells labeled for four generations had 29% in the same region. Therefore, the radioactivity associated with the ribosomes even after a very short pulse is a measure of newly-synthesized R-protein.

Relative rates of synthesis. Unless specified otherwise, rates of synthesis of components refer to rates per milliliter of culture. Compared with rates during Mg^{++} starvation, the instantaneous rate of synthesis of a component by exponentially growing bacteria is that rate derived from the slope of the tangent to the curve of accumulation in 1 ml of the same cells, growing exponentially at the start of Mg^{++} starvation (7). By expressing rates on this basis, we are comparing the biosynthetic activities which persist during the starvation to those present in the exponentially growing cells at the onset of the starvation.

Radioactive amino acids. Uniformly labeled L-arginine- C^{14} (240 mc/mmmole), L-leucine- C^{14} (240 mc/mmmole), and L-leucine-4,5- H^3 (4 c/mmmole) were products of New England Nuclear Corp., Boston, Mass.

RESULTS

Dissociation of R-protein from ribosomes during Mg^{++} starvation. The loss of R-protein during Mg^{++} starvation is shown in Fig. 2. Bacteria were exposed to H^3 -leucine during five generations of exponential growth and subsequently were starved of Mg^{++} for the times indicated. Extracts were prepared and centrifuged through gradients of sucrose, and the resulting distributions of radioactivity were normalized for quantitative comparison (6). R-protein was lost from particles very soon after removal of Mg^{++} from the medium, and by 8 hr very little, if any, prelabeled protein sedimented with the ribosomes. The sedimentation pattern of protein from bacteria starved for 20 hr was almost identical to the 8-hr pattern in Fig. 2. In contrast, the content of S-protein, labeled in the preceding period of normal growth, had not changed significantly even after 20 hr of Mg^{++} starvation.

It was shown previously that during Mg^{++} starvation a given rRNA molecule remains stable for at least 90 min after its synthesis (6). In contrast, the sedimentation rates of the 30 and 50S ribosomal subunits began to change within minutes after removal of Mg^{++} from the medium (Fig. 1 in the first paper of this series). Therefore, the results in Fig. 2 indicate that one of the first events leading to changed sedimentation rates of the particles is loss of protein while the rRNA remains protected against enzymatic attack until later.

Fate of R-proteins dissociated from ribosomes. An R-protein molecule released from a particle could (i) be degraded by proteases, (ii) be reutilized into new particles, or (iii) remain stable but not be reutilized. Reutilization is considered because of the suggestion that R-proteins actually

FIG. 2. Sedimentation characteristics of protein synthesized in the preceding exponential growth as a function of time of Mg^{++} starvation. *Aerobacter aerogenes* was grown for five generations of exponential growth in the presence of leucine- H^3 (20 μ g/ml, 14 μ c/mg), and then was washed and resuspended in MMG - Mg^{++} , containing unlabeled leucine (100 μ g/ml) and was incubated for 20 hr. At the times indicated, extracts were prepared for sucrose gradient centrifugation. Hot acid-precipitable counts per minute were normalized so that counts per minute recovered were proportional to counts per minute per milliliter of culture (Materials and Methods). Symbols: $\circ$, exponentially growing bacteria; $\triangle$, 25-min Mg^{++} starvation; $\bullet$, 8-hr Mg^{++} starvation.

may be stored in the bacteria before use (11), and also because of the claim that R-protein released during a long starvation of *E. coli* can be reutilized during the subsequent period of recovery (19).

Our results indicated that little, if any, of the R-protein made during the preceding exponential growth phase was reutilized to make new ribosomes when *A. aerogenes* cells were recovering from 20 hr of Mg^{++} starvation. In fact, the following observations suggest that by 8 hr virtually all of the R-protein released from decayed particles has become acid-soluble. First, we compared the loss during Mg^{++} starvation of the whole cell RNA made in the preceding period of exponential growth with the loss of the whole cell protein which had been made in the same period. After 20 hr of starvation, 55% of this RNA became acid-soluble, and this was entirely due to a loss of 75% of the rRNA; i.e., transfer RNA (tRNA) is stable (6). Since 28% of the leucine in *A. aerogenes* protein is present in R-

proteins, then, if only R-proteins became acid-soluble, and if all the R-proteins associated with the decayed rRNA did so, 21% ($0.75 \times 28\%$) of the total protein would become acid-soluble. The observed losses are shown in Table 1. At all times, the loss of rRNA was paralleled by a loss of protein equivalent to that expected if the R-protein from these degraded particles, and only this protein, was destroyed.

It is still possible that degradation of S-proteins contributes to protein losses and that the relative constancy of the S-protein content in Fig. 2 reflects the presence of R-proteins which have replaced degraded S-proteins in the S-protein fraction. Without criteria for identifying R-protein accurately, this is difficult to discount rigorously. However, the following experiment suggests that this alternative possibility is not valid.

Decay of protein synthesized during uracil starvation. If only R-proteins decay during Mg^{++} starvation, then the percentage of the total H^3 -protein which is lost should be less when at the start a smaller fraction of the total labeled protein is R-protein. When labeled during uracil starvation, only 17% of labeled protein of *A. aerogenes* strain U3 (uracil-less) is ribosomal, in contrast to 28% in exponentially grown bacteria (Fig. 3). Such labeled cells were washed and then resuspended in a medium containing uracil but deficient in Mg^{++} , and after 20 hr of Mg^{++} starvation, total hot acid-insoluble radioactivity was de-

termined. If 75% of the R-proteins made during the preceding uracil starvation were lost during 20 hr of Mg^{++} starvation, then about 13% ($0.75 \times 17\%$) of the total labeled cell protein would be lost; we have observed losses of 7 to 10% in such an experiment. The differences between such small losses (13% compared with 7 to 10%) may reflect experimental errors, but the results clearly show that, when a *larger* fraction of the labeled protein is S-protein, a *smaller* percentage of the total protein decays during the subsequent 20 hr of Mg^{++} starvation; i.e., when 72% of the total protein is S-protein, 22% decays. During uracil starvation, 83% of the total protein synthesized is S-protein and 7 to 10% decays.

These data, along with the results shown in Fig. 2 and Table 1 suggest that all R-proteins released from ribosomes are *ultimately* destroyed; by 8 hr, virtually all of the dissociated R-protein made in the preceding period of normal growth has been degraded. This breakdown of R-proteins accounts for most, if not all, of the protein degradation during Mg^{++} starvation, although, as indicated above, it is not possible to rule out a very slow decay of S-proteins.

Possible reutilization of R-protein during recovery. We have also looked for evidence of reutilization of released intact R-proteins in the assembly of ribosomes during "recovery" from Mg^{++} starvation. Bacteria with H^3 -protein were starved for 20 hr without additional labeled amino acid and then incubated in MMG to recover; extracts were prepared before and after recovery and were centrifuged through gradients of sucrose. There was no transfer of H^3 from the S-protein to the R-protein fraction even during significant ribosome synthesis. This result is consistent with the previous conclusion that none of the dissociated R-proteins remain to be reutilized.

From Table 1 and the foregoing conclusions it appears that at 3 hr of Mg^{++} starvation about one-third of the R-protein made in the preceding exponential growth, which ultimately decays, is still acid-insoluble. It is possible that during the early phase of starvation, before these proteins are completely destroyed, their reutilization could be detected. Since some R-proteins are released very soon after removal of Mg^{++} from the medium (Fig. 2), we have also looked for reutilization of recently released R-proteins. Total protein was labeled with H^3 -leucine during five generations of exponential growth as well as during the subsequent Mg^{++} starvation of only 90 min. During the last 20 min of starvation, newly synthesized protein was labeled by the presence of arginine- C^{14} . After washing, the cells were resuspended in MMG to allow recovery. Gradient

TABLE 1. Losses of RNA and protein, made in the preceding period of exponential growth, from *Aerobacter aerogenes* during Mg^{++} starvation^a

Starvation time hr	Per cent acid-insoluble counts/min		
	(1) RNA (observed)	(2) Protein (expected)	(3) Protein (observed)
0	100	100	100
3	80	92	87
8	52	81	83
20	45	79	80

^a Exponentially growing bacteria were exposed to labeled uracil or to labeled leucine for five generations before incubation in MMG — Mg for 20 hr with excess unlabeled precursor. Column 1 gives the total acid-insoluble counts per minute from uracil, showing losses of rRNA (6). Column 2 gives the expected acid-insoluble counts per minute from leucine if the only protein which became acid-soluble during the starvation were R-protein and if every ribosome which lost its rRNA also lost all its R-protein. Column 3 gives the observed losses of acid-insoluble leucine (see text).

FIG. 3. Sedimentation characteristics of proteins synthesized in *Aerobacter aerogenes* U3 during exponential growth compared with those synthesized during a subsequent period of uracil starvation. Bacteria, labeled for five generations of exponential growth in the presence of leucine- H^3 ($20 \mu\text{g/ml}$, $14 \mu\text{C/mg}$) plus uracil ($40 \mu\text{g/ml}$), were washed and resuspended in MMG lacking uracil and incubated for 60 min. During the last 20 min of uracil starvation, the bacteria were exposed to C^{14} -leucine ($0.2 \mu\text{g/ml}$, $125 \mu\text{C/mg}$) and then to an excess of leucine- C^{13} ($100 \mu\text{g/ml}$) for 5 min. Hot acid-precipitable counts per minute as well as optical density at $260 \text{ m}\mu$ from fractions after gradient centrifugation of extracts are shown. Counts per minute were not normalized; instead, the S-protein peaks were plotted to be almost coincident to emphasize the decreased ratio of synthesis in the same cells of R-protein/S-protein during uracil starvation compared with exponential growth. Incorporation of amino acids during 20 min of uracil starvation is about 5% of that incorporated during 20 min of exponential growth. Symbols: $\square$, optical density at $260 \text{ m}\mu$; $\circ$, H^3 counts per minute; $\bullet$, C^{14} counts per minute.

centrifugation of extracts (Fig. 4) gave no evidence for measurable reincorporation of any R-proteins into new ribosomes during a period of recovery in which significant amounts of new ribosomal material were being made.

Proteins synthesized during Mg^{++} starvation. Protein synthesis was estimated by arginine incorporation into hot trichloroacetic acid-insoluble material. Because of the higher content of basic proteins in the particles, the percentage of arginine present in the ribosomes was 40% of the total protein arginine in the bacteria. The relative contents of arginine and leucine in R-proteins compared with S-proteins was in approximate agreement with the amino acid analyses of these proteins in *E. coli* (24).

Bacteria were exposed to arginine- C^{14} for 30 sec and then to excess unlabeled arginine for 5 min. The chase was terminated by chilling, and extracts were prepared and centrifuged through gradients of sucrose. Two such gradients are shown in Fig. 5. Instead of normalizing total

counts per minute to correct for losses (6), we have adjusted the S-protein peaks to be approximately coincident in order to emphasize the changed character of the proteins being formed from exogenous amino acids. These experiments indicate that, as starvation progresses, a progressively decreasing proportion of the incorporated arginine is in R-proteins. Upon recovery, however, the percentage of total exogenous amino acids entering R-protein returns to normal (Table 2).

Absolute rates of protein synthesis. Rates of total protein synthesis during Mg^{++} starvation were estimated by the incorporation of labeled amino acid into protein during either a very short (pulse) or a long exposure. Both procedures gave essentially similar results with regard to relative rates of protein synthesis. A typical pulse-chase experiment is shown in Fig. 6. It should be noted that estimations of protein synthesis by amino acid incorporation are less complicated than are estimations of nucleic acid synthesis by purine or

pyrimidine incorporation (7), because the total amino acid pool fraction in bacteria is "chased" very quickly by exogenous amino acid. This observation has often been misinterpreted to mean that amino acids, in acid-soluble form in the cell, exchange freely with amino acids in the medium. It is clear from Fig. 6 that a fraction, at least, do

FIG. 4. Changing sedimentation characteristics of total (top) and newly synthesized (bottom) protein in *Aerobacter aerogenes* after 25-min recovery in MMG subsequent to 90 min of Mg^{++} starvation. Bacteria were labeled with leucine- H^3 (20 $\mu g/ml$, 14 $\mu g/mg$) during five generations of exponential growth as well as during 90 min of Mg^{++} starvation. During the last 20 min of starvation, the cells were exposed to arginine- C^{14} (6.4 $\mu g/ml$, 2.5 $\mu g/mg$). Half of the culture was chilled and to the second half was added Mg^{++} plus excess (100 $\mu g/ml$) unlabeled arginine and leucine, and it was incubated for 25 min of recovery. Hot acid-precipitable H^3 and C^{14} counts per minute from fractions collected after gradient centrifugation of extracts were normalized to correct for all losses. Symbols: $\circ$, extracts from bacteria starved for 90 min; $\bullet$, extracts from bacteria starved for 90 min and then allowed to recover in MMG for 25 min.

FIG. 5. Sedimentation characteristics of *Aerobacter aerogenes* proteins synthesized during exponential growth ($\circ$) or during Mg^{++} starvation after 8 hr of incubation in Mg^{++} -free medium ($\bullet$). Bacteria were exposed to arginine- C^{14} (3 $\mu g/ml$, 70 $\mu g/mg$) for 30 sec and then to excess arginine- C^{14} (100 $\mu g/ml$) for 5 min before chilling. Hot acid-precipitable counts per minute recovered after gradient centrifugation of extracts were not normalized for quantitative comparisons. The S-protein peaks were plotted to be almost coincident to emphasize the decreased ratio of synthesis of R-protein/S-protein during Mg^{++} starvation.

not; they are "chased" into protein rather than out of the cell (17).

A comparison of the total incorporation after either 30 sec of labeling or 5 min of chase indicates that the rate of protein synthesis per milliliter of culture declined during the first few hours of Mg^{++} starvation. It then remained approximately constant for more than 20 hr, at a rate close to 30% of the instantaneous rate in the exponentially growing population at the onset of starvation.

Since, in comparison with incorporation by exponentially growing cells, more of the total incorporated exogenous arginine entered S-proteins during Mg^{++} starvation (Table 2), the actual rate of S-protein synthesis was somewhat higher than 30% of normal, whereas that of R-proteins was lower (Table 3). S-protein synthesis was fairly constant after the first few hours at a rate close to one-half the instantaneous rate of exponentially growing cells. This relative value for S-protein synthesis is the same as that found for deoxyribonucleic acid (DNA), rRNA, and tRNA syntheses during Mg^{++} starvation (7).

DISCUSSION

Reutilization of R-protein. There is considerable interest at this time in the possible reassembly

TABLE 2. Percentage of exogenous arginine- C^{14} incorporated into ribosomal proteins of *Aerobacter aerogenes* growing exponentially (0 time), or after incubation in a Mg^{++} -free medium for the times indicated^a

Period of growth	Time	R-protein (per cent of total)
	hr	
Exponential	0	41
Starvation	0.5	37
	3.5	27
	8	21
	20	16
Recovery	0.25	42
	1.5	41.5

^a Also shown are the percentages in bacteria after periods of recovery (plus Mg^{++}) subsequent to 20 hr of Mg^{++} starvation (last two lines). Bacteria were exposed to arginine- C^{14} for 30 sec and then to excess arginine- C^{12} for 5 min before chilling. Extracts were prepared, and the distribution of newly synthesized proteins was estimated from the fractions obtained after sucrose gradient centrifugation (see Materials and Methods).

of dissociated ribosomes, and studies of the process may further our understanding of ribosome structure and synthesis. Recent reports indicate that it is possible to reconstitute a ribosome in vitro after part of its protein has been dissociated (3, 25, 25a). It is possible that the same process could occur in vivo. A study of the formation of protein-deficient ribosomes during treatment of *E. coli* with chloramphenicol led Kurland and Maaløe (11) to conclude that there is a supply of free ribosomal protein in the normal cell, and that, in the absence of in vivo polypeptide synthesis, this protein is "consumed" for the assembly of the incomplete "CM-particles."

Nakada and Marquisee (19) concluded that R-protein made during exponential growth of *E. coli* was released during Mg^{++} deficiency and after 24 hr of starvation this same protein could then be reutilized intact into new particles being formed during recovery. We did not observe these results with *A. aerogenes*. In fact, our results suggest that after several hours there are few, if any, of these R-proteins left to be reutilized in this organism.

It is possible that a certain fraction of the R-proteins is reused very soon after release from a disintegrating particle; at any time, the free reserve of R-protein would be very small so that re-

utilization could not be measured by the type of experiment shown in Fig. 4. The reduced rates of incorporation of exogenous arginine into R-proteins compared with S-proteins during Mg^{++} starvation (Fig. 5) could reflect a process whereby part of the requirement for new ribosomal proteins is being met by R-proteins released from decaying particles. However, different types of experiments would probably be necessary to prove

FIG. 6. (top) Incorporation of arginine- C^{14} into *Aerobacter aerogenes* protein (hot acid-precipitable counts per minute) during Mg^{++} starvation or exponential growth. Bacteria labeled for five generations of exponential growth with H^3 -leucine (20 $\mu g/ml$, 14 $\mu c/mg$) were washed and resuspended in MMG - Mg^{++} containing unlabeled leucine (100 $\mu g/ml$). After an indicated period, a small volume (10 ml) was exposed to arginine- C^{14} (3 $\mu g/ml$, 70 $\mu c/mg$) for 30 sec and then to arginine- C^{12} (100 $\mu g/ml$). C^{14} counts per minute of the exponentially growing cells were normalized by the H^3 content to time zero on the curve of H^3 per milliliter during Mg^{++} starvation to compare rates of protein synthesis in 1 ml of bacteria growing exponentially just before initiation of Mg^{++} starvation to that during the subsequent 20 hr of starvation (7). (bottom) Relative rates of protein synthesis estimated from the total incorporation after 30-sec pulse as shown above. Zero-time is defined as 100%. Abscissa gives hours of starvation before the pulse of arginine- C^{14} .

TABLE 3. *Relative rates of synthesis (%) of soluble and ribosomal proteins during Mg⁺⁺ starvation^a*

Time	S-proteins	R-proteins
hr		
0	100	100
3	45	22
8	43	15
20	50	12

^a The relative rate of total protein synthesis, from Fig. 6 (bottom), at a given time of Mg⁺⁺ starvation is used with the data of Table 2 to derive relative rates of S-protein and R-protein synthesis at that time; e.g., (34%) (27/41) gives 22% for relative rate of R-protein synthesis at 3 hr of Mg⁺⁺ starvation.

that reutilization does occur in this system, e.g., it could be determined if N¹⁵-labeled R-proteins made at an earlier time became associated with newly synthesized rRNA.

Ribosome-limited nucleic acid and protein synthesis. How does Mg⁺⁺ limit biosynthesis in these bacteria? This metal plays an important role in at least two cellular functions which are both directly concerned with biosynthesis: (i) the activation of many enzymes and (ii) the function of the ribosomes (see below). Nonetheless, the relative amounts of the S-protein and nucleic acid components (7) being synthesized in the absence of exogenous Mg⁺⁺ is the same as when Mg⁺⁺ is present. Furthermore, and perhaps more remarkable, these rates of synthesis are close to half the instantaneous rates extant in the exponentially growing bacteria at the initiation of Mg⁺⁺ starvation.

Recent measurements of the Mg⁺⁺ content in *A. aerogenes* fractions indicate that the bulk of intracellular Mg⁺⁺ is associated with the ribosomes (D. W. Tempest, *personal communication*). When prepared in extracts, these structures readily and reversibly associate into larger particles if the Mg⁺⁺ concentration is raised, or they dissociate to smaller units if it is lowered (27). The optimal Mg⁺⁺ concentration for the functioning of ribosomes in polypeptide synthesis *in vitro* is very close to 0.01 M, suggesting that it is the larger particles which are active. Furthermore, this activity is quite sensitive to changes in Mg⁺⁺ concentration either above or below the optimum; e.g., at 0.001 M Mg⁺⁺ the activity is only 7% of the maximum (26).

As is well-known, this same ion is probably the most common metal-cofactor for those enzymes for which metal requirements have been determined (28). Thus, the reduced biosynthetic ac-

tivities of Mg⁺⁺-starved bacteria could reflect either (i) the role of this ion in maintaining competent ribosomes or (ii) its importance as a co-factor for one or more enzymes. We favor the former alternative for the following reasons.

First, a brief review of the Mg⁺⁺ requirements of 25 Mg⁺⁺-dependent enzymes, chosen at random from *Methods in Enzymology* (S. P. Colowick and N. O. Kaplan [ed.], Academic Press, Inc., New York), showed that most of them exhibit maximal activity with concentrations of Mg⁺⁺ between 10⁻⁴ to 10⁻³ M. This is 10 to 100 times lower than that concentration required for maximal polypeptide synthesis, *in vitro* (see above). A few required only trace amounts, but for two of the enzymes the concentrations specified were close to 0.01 M Mg⁺⁺. However, in contrast to the Mg⁺⁺ dependency of ribosomes for polypeptide synthesis, the Mg⁺⁺ dependency data indicate a rather broad concentration range for high activity; e.g., the optimal Mg⁺⁺ concentration for an RNA-inhibitable DNA endonuclease is 7 × 10⁻³ M, but this enzyme is 66% as active with a Mg⁺⁺ concentration of only 10⁻³ M (12).

Of course, extrapolations from *in vitro* observations to the *in vivo* situation demand reservation. However, if, as is apparently true, Mg⁺⁺-starved bacteria can maintain an intracellular Mg⁺⁺ concentration and content high enough to maintain a large fraction of the ribosomes (with their weak affinity for the metal) in a fully active state, then it seems likely that during this time enzyme activities never become the limiting factor for metabolism.

Second, during Mg⁺⁺ starvation, the content of ribosomes per milliliter of culture which are actually functioning in polypeptide synthesis is lower than that in the bacteria at the start of the starvation. The extent of this decrease is difficult to estimate, but, while the total content of normally sedimenting 16 and 23S rRNA remains approximately constant at prestarvation levels for more than 22 hr, a large fraction is in free 30, 50, and 70S particles, which accumulate during various stages in the sequential steps of breakdown (6), rather than in the larger structures which are responsible for protein synthesis (21). Thus, it seems quite likely that at least half of the normally sedimenting rRNA in these bacteria could be contained in nonfunctioning particles, accounting for the reduction in protein synthesis to about one-half of the instantaneous rate per milliliter in the same cells growing exponentially. From the known function of ribosomes in peptide bond formation, it is to be expected that the cellular content of functional ribosomes could limit protein synthesis. It had been shown previously that rates of ribosome formation during recovery

of Mg^{++} -starved bacteria were proportional to ribosome contents (9, 15). It has been suggested that this latter limitation might simply reflect the limitation on R-protein synthesis which parallels the limitation on overall protein synthesis.

However, why should ribosome content limit the synthesis of rRNA, let alone that of DNA and tRNA? It is conceivable that the synthesis of each of these nucleic acids is dependent upon an unstable enzyme which is continuously replenished; thus, its content, limited by the rate of polypeptide synthesis, would be fixed at a level determined by the constant content of functional ribosomes. In fact, a specific "initiator" protein has been postulated by Jacob, Brenner, and Cuzin (4) to play a role in the regulation of DNA synthesis. Otherwise, one must invoke a role for ribosomes over and above their known role in protein synthesis; e.g., expression of all gene activities could be dependent upon direct contact of the ribosomes with individual DNA regions or their complementary RNA.

However, an important alternative to this hypothesis is considered in the last of these papers (8). Although the primary effect of Mg^{++} deficiency on biosynthetic activities is probably traceable to the importance of the metal for ribosomes, it does not necessarily follow from this that ribosomes are *directly* limiting the synthesis of all nucleic acids and proteins. It will be shown that, after several hours of Mg^{++} starvation, most cells in the population are inert, and only a small fraction of the total DNA of the culture is present in those cells which are responsible for biosynthetic activities. Therefore, although the restricted content of ribosomes may somehow limit the number of active chromosomes in the viable cells, the rates of synthesis of all nucleic acids could then be *directly* limited by the latter.

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