A Role for Zinc in the Structural Integrity of the Cytoplasmic Ribosomes of Euglena gracilis¹,²

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JUDITH A. PRASK³ AND DONALD J. PLOCKE
Department of Biology, Boston College, Chestnut Hill, Massachusetts 02167

ABSTRACT

Zinc deficiency in dark-grown Euglena gracilis Klebs, Z strain Pringsheim, results in the disappearance of cytoplasmic ribosomes. In contrast, ribosomes in zinc-sufficient Euglena are conserved, do not undergo turnover, and can be demonstrated at any stage of growth. The zinc content of ribosomes from zinc-deficient Euglena just prior to ribosomal disappearance is 300 to 380 micrograms of zinc per gram rRNA as compared to 650 to 1280 micrograms of zinc per gram rRNA in ribosomes from zinc-sufficient cells. Ribosomal disappearance is observed to involve a generalized disintegration process related to the lower content of zinc in the ribosomes. Reappearance of ribosomes requires the addition of zinc. It is proposed that adequate zinc may be essential for normal tertiary and quaternary structure of the cytoplasmic ribosomes of Euglena.

The association of divalent metal ions with ribosomes is a well established phenomenon. However, because of its prominent role both in subunit association and in in vitro protein synthesis, Mg²⁺ has dominated this area of study, almost to the exclusion of other metals. Studies in vitro with Mg²⁺ have been complemented by experiments in vivo, such as those of McCarthy (18) with E. coli and of Kennell and coworkers (14, 15, 19) with A. aerogenes, to determine the effects of varying intracellular Mn through starvation. The gradual loss of Mg²⁺ from the starved cells results in the disintegration, degradation, and final disappearance of ribosomes.

Other workers have suggested the involvement of metals in addition to Mg²⁺ in ribosomal structure. Working with the ribosomes of E. coli, Tal (35–37) found dialysis against Ni²⁺, Zn²⁺, Co²⁺, Fe²⁺, or Mn²⁺ to be more effective than that against Mg²⁺ in restoring near normal sedimentation values of ribosomal subunits which had previously been unfolded by treatment with EDTA. The deficiency of Zn²⁺ has been repeatedly associated with impaired protein metabolism in a variety of organisms including Neurospora (22, 23) and Mycobacterium (43).

Price and Vallee (26) studied zinc deficiency in Euglena gracilis and found that when zinc is limiting, growth is severely retarded. This impairment can be reversed, however, and full growth attained merely by the addition of adequate zinc. Wacker (41) observed that the levels of RNA and protein in zinc-deficient Euglena are markedly reduced whereas massive amounts of polynucleotide and amino acids accumulate. These findings, as well as those reported for zinc-deficient Neurospora and Mycobacterium, indicate that zinc deficiency results in a severe malfunction in the process of protein synthesis.

Wacker and Vallee (42) had demonstrated the presence of 12 different metals, including zinc, in the total RNA from a wide variety of biological species and suggested a functional role for these metals possibly related to protein synthesis. Since the ribosomes contain the major part of the RNA in the cells we decided to examine the effect of zinc deficiency on the ribosomes, specifically the cytoplasmic ribosomes of Euglena gracilis.

MATERIALS AND METHODS

Culturing of Cells. Euglena gracilis Klebs, Z strain Pringsheim, was grown in 2-liter Pyrex flasks containing 1 liter of chemically defined medium (26) prepared with Johnson-Matthey Speccure salts (Jarrell-Ash Co., Waltham, Mass.). Other components were purified on a column of Dowex A-1 chelating resin (Dow Chemical Co.) with the exception of thiamine and vitamin B₆ which had no special treatment. All cultures were grown on a rotary shaker in the dark at 30 C. Glassware was treated as described by Price and Vallee (39). Deionized water (Continental Water Conditioning Co.) was used in all experiments.

Zinc-sufficient medium contained 1 mg of zinc per liter; the limiting amounts used varied from 10 μg to 30 μg of zinc added per liter. Inoculations were made from a transfer medium containing 10 μg of zinc per liter. Growth was monitored by turbidity measurements with a Klett colorimeter and a No. 54 filter.

Fractionation of Cells. Cells were disrupted in a French Press in Buffer I of Rawson and Stutz (27) at 10,000 pounds/square inch, the suspension from which was examined with a light microscope to be sure that cell breakage was essentially complete. The suspension was fractionated by centrifugation (34) at 1000g for 10 min and at 20,000g for 30 min in a Sorvall RC-2 centrifuge and SS-34 rotor. The 20,000g supernatant was centrifuged for 1.5 to 2 hr at 50,000 rpm in an IEC B-50 ultracentrifuge and A321 rotor to yield the 100,000g pellet. This pellet was washed with deionized water and was stored at −70 C until used.

Sucrose Gradients. To demonstrate the presence of ribosomes, the 100,000g pellet was resuspended in Buffer II of Rawson and Stutz (27) with the aid of a 1-ml glass tissue homogenizer and layered on 12-m1 linear gradients of 0.3 to 1.1 M sucrose prepared in Buffer II. The gradients were cen-

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³A predoctoral Fellow of the National Aeronautics and Space Agency.
trifuged for 2½ hr at 40,000 rpm in a IEC SB 283 rotor, B-50 centrifuge. The tubes were pierced, and fractions were collected and diluted with 3 ml of deionized water for reading in a Shimadzu QV-50 spectrophotometer at 232, 260, and 280 nm. Under these conditions the predominant peak on the gradient is the 87S (27, 28) or 89S (20) monosome. We shall hereafter refer to this as the 87S peak.

To detect free ribosomal RNA, cell extracts (20,000g supernatants) were layered onto gradients prepared for this purpose according to Rawson and Stutz (27) in Buffer III and run for 18 hr in a IEC SB 112 rotor at 25,000 rpm, BD-2 centrifuge. Control Euglena RNA was prepared from the 100,000g pellets of normal cells by the SDS2 method of Rawson and Stutz (27).

For the detection of ribosomal subunits, the 100,000g pellets were layered onto sucrose gradients prepared as for ribosomes except that the magnesium concentration was lowered to 0.1 mM. These gradients were centrifuged for 5 hr in a IEC SB 112 rotor at 25,000 rpm in a BD-2 centrifuge.

When phosphate, zinc, or 32P measurements were to be made on the ribosomes, the peak fractions from sucrose gradients were pooled, resedimented at 100,000g, and resuspended in deionized water with the aid of a 1-ml glass tissue homogenizer.

Analyses. Phosphate was measured by the method of Ames and Dubin (1). Atomic absorption was used for zinc measurements (7, 8); standards were prepared from a Specpure zinc rod (Jarrell-Ash Co.) dissolved in dilute metal-free HCl and diluted with deionized water.

Chemicals. Chloramphenicol and cycloheximide were obtained from Sigma Chemical Co., St. Louis, Missouri. Sucrose used in the growth media and for gradients was the analytical grade obtained from Mallinckrodt Chemicals, St. Louis, Missouri, and contained insignificant amounts of zinc as determined by atomic absorption. Metal-free HCl (Aristar) was obtained from Gallard Schlesinger Chemical Co., Carle Place, New York.

Radioisotopic Experiments. For 32P experiments, a uniformly labeled inoculum was obtained by growing the cells in adequate zinc (1 mg per liter) with 0.5 mc 32P per liter (Na32PO4, carrier free, Iso/Serve Division, Cambridge Nuclear Co., Cambridge, Mass.) for 4 days. These cells were then harvested under sterile conditions, washed in sterile medium without isotope, and used as the inoculum for fresh medium without isotope. The individual fractions or the pooled peaks from sucrose gradients were diluted with deionized water to a total of 15 ml per scintillation vial (plus 1 drop of chloroform to retard microbial growth at room temperature) and were counted in a Nuclear Chicago Unilux liquid scintillation counter, Model 6850, detecting in this case the Cerenkov radiation (16).

RESULTS

Typical growth patterns observed under the conditions of this study for Euglena in adequate zinc (1 mg/liter) and in various limiting amounts of zinc are shown in Figure 1.

To determine the effects of zinc deficiency on the cytoplasmic ribosomes, zinc-deficient Euglena (grown in 10 µg Zn/liter) were harvested in stationary phase (days 7–10 after inoculation), and the cell contents (both the 20,000g supernatant and the 100,000g pellet) were examined by sucrose gradient; no ribosome peak could be demonstrated. To elucidate the

* Abbreviation: SDS: sodium dodecyl sulfate

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Fig. 1. Effect of zinc concentration on the growth of Euglena gracilis. Typical growth curves are shown for Euglena grown in the dark in adequate zinc (1 mg/liter), in limiting amounts (10–30 µg/liter) and with no added zinc in the medium.

events leading to the loss of demonstrable ribosomes, cells grown in 10 µg Zn/liter were harvested daily beginning at day 3 after inoculation, and the 100,000g pellets were examined by sucrose gradient as above. Figure 2 shows the typical sequence of events in such an experiment: the presence of a definite ribosomal peak up to a certain stage of growth, after which it disappears. The ribosomes disappear from the cells within a 24-hr period at approximately the onset of stationary phase, which begins between days 4 and 5 in zinc deficiency (Fig. 1), and during which period the cell density may increase 10 to 15%. Even in the absence of a distinct peak on day 5, evidence of ribosomes is indicated by gradient fractions with a A 300/ A 260 ratio greater than 1.5 (24). In later stages (day 6), there is neither a ribosomal peak nor any region in which this ratio reaches 1.5 (Fig. 2d). However, when 1 mg zinc is added to a culture in the condition shown on day 6 (Fig. 2d), full growth is attained by day 9 and is accompanied by the reappearance of ribosomes (Fig. 2e).

An attempt was made to determine whether other metals could substitute for zinc in this recovery process. Addition of Ni2+, Co2+, or Mg2+ in final concentrations of 1.0 to 0.01 mM failed to take the place of Zn2+ in this process.

A more defined picture of the nature and timing of ribosomal disappearance is obtained by sampling a single culture during the 24-hr period between days 4 and 5 (hours 96 to 120) (Fig. 3). In contrast with the single, homogeneous 87S peak of normal Euglena ribosomes, the ribosomal profile of zinc-deficient cells shows signs of heterogeneity very early in this 24-hr period (Fig. 3). During the first half of the period the ribosomal profile changes only gradually (Fig. 3, a, b, c); however, very soon thereafter a much more rapid change occurs, and the 87S peak disappears completely. However, there is no proportional and concomitant increase in material in
In these studies 1-liter batches of cells were harvested over the period of ribosomal breakdown and the cell extracts (20,000g supernatants) were divided into equal aliquots. One aliquot served as the control and was placed immediately in ice with no further treatment. To the other aliquots was added a solution of a Sepacpure salt of one of the following metals to make the final concentration: 0.01 mM Zn\(^{2+}\), 0.1 mM Co\(^{2+}\), or 0.1 mM Ni\(^{2+}\). This mixture was then incubated at 30 C for 1 hr, after which the controls and test samples were centrifuged at 100,000g, and the resulting pellets were examined by means of sucrose gradients. No ribosomes could be demonstrated in any of these samples nor in further experiments in which incubation time, temperature, and buffer ionic strength were varied.

Having established the disappearance of ribosomes from zinc-deficient Euglena, it was necessary to evaluate this disappearance in terms of the normal course of events for ribosomes in zinc-sufficient cells. To determine whether the ribosomes are conserved or whether they rapidly undergo turnover during the exponential phase, cultures (1 mg zinc/liter) inoculated with cells uniformly labeled with \(^{32}\)P were harvested daily throughout exponential growth. The ribosomes were recovered from sucrose gradients and assayed for \(^{32}\)P and total phosphorus content. It is evident from the data in Figure 4 that the total counts present in the ribosomes of the inoculum (day 0) remain constant throughout 4 days of exponential growth, despite a 20-fold increase in cell mass (grams of zinc), attempts were made to reconstitute ribosomes \textit{in vitro} through the addition of zinc and other metals to cell extracts.

The data in Figures 2 and 3 indicate that the extent of ribosomal disappearance is fairly complete. However, in view of the rapid reappearance of ribosomes when Zn is added to cultures with no intact ribosomes (see below), the possibility exists that RNA and protein components of the ribosomes remain intact and merely recombine when zinc is added.

To investigate this possibility, the 100,000g supernatants and pellets from cells harvested at 12-hr intervals during the period of ribosomal disappearance (days 3½ to 5) were examined by means of appropriate sucrose gradients for intact rRNA and subunits. The inoculum for these cultures was uniformly labeled with \(^{32}\)P to allow detection of intact ribosomal components in concentrations too low to be demonstrated by \(A_{200,000}\). No intact ribosomal components could be demonstrated on the gradients either by means of \(A_{200,000}\) or by \(^{32}\)P counts.

As a further test of the hypothesis that intact components remain and merely recombine when zinc is added to the culture, attempts were made to reconstitute ribosomes \textit{in vitro} through the addition of zinc and other metals to cell extracts. In these studies 1-liter batches of cells were harvested over the period of ribosomal breakdown and the cell extracts (20,000g supernatants) were divided into equal aliquots. One aliquot served as the control and was placed immediately in ice with no further treatment. To the other aliquots was added a solution of a Sepacpure salt of one of the following metals to make the final concentration: 0.01 mM Zn\(^{2+}\), 0.1 mM Co\(^{2+}\), or 0.1 mM Ni\(^{2+}\). This mixture was then incubated at 30 C for 1 hr, after which the controls and test samples were centrifuged at 100,000g, and the resulting pellets were examined by means of sucrose gradients. No ribosomes could be demonstrated in any of these samples nor in further experiments in which incubation time, temperature, and buffer ionic strength were varied.

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Fig. 4. Conservation of ribosomes during exponential growth in normal Euglena. Cultures inoculated with uniformly labeled cells were harvested daily during exponential growth and the ribosomes were isolated from sucrose gradients. Total ribosomes recovered were measured for cpm \( ^{32}P \) (■), and μg P (▲). Also shown are the grams of cells harvested (○) and the final growth in Klett units for that culture (▲).

harvested per liter, in turbidity measured by Klett units, and in the total phosphorus content of the ribosome population.

To examine the state of ribosomes in nongrowing cells of stationary phase, the ratio of cpm of \( ^{32}P \) to \( A_{600} \) was used as a measure of the existing ribosomes. This ratio was determined for ribosome peak fractions from cultures which had been inoculated with uniformly labeled cells and harvested daily for 4 days into stationary phase, days 7 through 10 (Fig. 5). The cpm \( ^{32}P/A_{600} \) in the ribosome peak remains constant at 30,000 in cells which have clearly stopped growing.

One explanation for the instability of ribosomes in zinc deficiency is that zinc participates directly in the maintenance of ribosomal structure. This was examined by the analysis of ribosomes for zinc content using atomic absorption. The number of atoms of zinc per ribosome was calculated from the zinc content per gram of ribosomal RNA based on the combined molecular weight of Euglena cytoplasmic ribosomal RNAs of 2.15 × 10^6 daltons (17). The results of these experiments are shown in Table I. In all cases the zinc content of the ribosomes from zinc-sufficient cells is at least double that of ribosomes from zinc-deficient cells just prior to ribosomal disappearance.

As described above (Fig. 2), the reappearance of ribosomes and recovery of exponential growth can be effected in deficient cultures merely by the addition of sufficient zinc. A second requirement for the recovery process was found to be protein synthesis. When 1 mg of zinc was added to 1 liter of zinc-deficient cultures on day 6 along with 20 μg of cycloheximide, a potent inhibitor of protein synthesis by the 80S class of ribosomes (26), there was neither any increase in cell mass nor was there any reappearance of ribosomes in these cells. However, when 1 mg of zinc was added to cultures containing 2 g per liter of chloramphenicol, recovery of growth and ribosomal reappearance occurred, but usually to the extent of less than 70% of the control value without inhibitor.

To determine the time course of ribosomal reappearance, cells were sampled at 2- to 3-hr intervals over the 24-hr period after addition of zinc. A total of four cultures in two separate experiments were measured for the 0- to 12-hr period, and one culture was assayed for the 12- to 24-hr period. Growth patterns of these cultures are shown in the upper part of Figure 6. Sucrose gradient separations of ribosome samples from each of the 0- to 12-hr cultures are shown; as early as 2 hours after zinc is added ribosome peaks can be observed.

The maximum growth rate observed during 0 to 12 hr after zinc is added is 4% per hr in contrast to the minimum rate of 17% per hr which occurs from 12 to 15 hr (Fig. 7, upper). The growth pattern appears to be a reflection of the ribosomal population (amount of 87S material on the basis of total \( A_{600} \) units/gram cells in the 87S peak in Fig. 6) which remains constant over 2 to 9 hr after zinc is added (range of 0.152 ± 0.015 unit \( A_{600} \)), in contrast to the marked increase in 87S material observed at 12 hr (0.298 unit \( A_{600} \); Fig. 7, lower).

DISCUSSION

The cytoplasmic ribosomes of Euglena gracilis are profoundly affected under conditions of zinc deficiency. The

![Figure 5](https://example.com/figure5.png)

FIG. 5. Stability of ribosomes during stationary phase in normal Euglena. Cultures inoculated with uniformly labeled cells were harvested daily for 4 days after entering stationary phase. The ribosome profiles were determined on sucrose gradients and the individual fractions from the ribosome peaks were measured for cpm \( ^{32}P \) and \( A_{600} \). The ratio of cpm \( ^{32}P/A_{600} \) in these peaks is given (○) as are the grams of cells harvested (○) and the final growth in Klett units for that culture (▲).

Table I. Zinc Content of Cytoplasmic Ribosomes from Zinc-sufficient and Zinc-deficient Euglena

<table>
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<th>Euglena</th>
<th>Experiment No.</th>
<th>μg Zn Added per Liter</th>
<th>Day of Harvest</th>
<th>μg Zn per g RNA ( ^{32}P )</th>
<th>Atoms Zn per Ribosome</th>
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<td>4</td>
<td>310, 385</td>
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1 Measured as μg Pi/sample × 10 = μg RNA/sample.

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abrupt disappearance of 87S ribosomes from zinc-deficient cells is in sharp contrast to the well defined 87S peak which can be demonstrated well into stationary phase in normal zinc-sufficient cells. It seems unlikely that the disappearance is simply the result of an impairment of ribosomal synthesis during zinc deficiency. In the first place, there is little or no turnover of ribosomes in zinc-sufficient cells during either exponential growth or in stationary phase. Secondly, the increase in cell mass during the time interval in which the ribosomes disappear from the cells is 20% or less, which would eliminate the possibility that the ribosomes are simply being diluted out. It would appear, then, that ribosomes synthesized by zinc-deficient cells may be fundamentally unstable and consequently much more susceptible to breakdown than those of cells with a much higher zinc content. Zinc measurements suggest that this fundamental difference in stability may be directly related to the zinc content of the ribosomes, and that a certain minimal zinc content may be required for ribosomal integrity. Comparison of the ribosomal zinc content with the total cellular zinc in stationary cells (42) reveals only a 2-fold difference in zinc content in the ribosomes from normal and zinc-deficient cells, while the intracellular zinc varies by a factor of 14 (from 192 μg/g dry weight for normal as compared to 14 μg/g dry weight for deficient cells). These data argue against a merely adventitious binding of zinc to the ribosomes and support the possibility of a structural role for zinc in the ribosomes.

Evidence for the participation of transition elements in the preservation of the structure of ribosomal components has been advanced by a number of investigators. Transition elements have striking effects on the tertiary structure of RNA molecules (4, 12) which are over and above the effects of hydrogen bonding (9). Hydration (29), unfolding (35, 36, 37), and swelling (38) are manifestations of the conformational changes caused by removal of metals from ribosomal subunits by EDTA. Since the stability constants for complexes of EDTA with Zn⁡\(^{2+}\), Fe⁡\(^{2+}\), Ni⁡\(^{2+}\), Co⁡\(^{2+}\), and Mn⁡\(^{2+}\) are three to ten orders of magnitude greater than for the complexes of EDTA with Mg⁡\(^{2+}\) (30), it is not unlikely that EDTA would be effective in removing any transition elements bound to the ribosomes. It is not surprising, therefore, that the addition of Mg⁡\(^{2+}\) alone, or of any other single metal ion, fails to reverse entirely the effects of prolonged dialysis of ribosomes against EDTA (10, 35, 36, 37).

In addition to their possible structural role, divalent cations such as zinc may also protect ribosomal RNA from attack by ribonuclease. While it is true that relatively high concentrations of metal ions (10⁻⁴ M or higher) are necessary to inhibit many types of ribonuclease examined in vitro (5, 6, 21, 33), an effective metal ion concentration of this order of magnitude might be attained locally within the ribosome with the consequent inhibition of ribonuclease activity. Lowering the concentration of metal ions within the ribosome might then result in increased nuclease activity. However, in similar sys-

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**Fig. 6.** Reappearance of ribosomes during recovery of *Euglena* from zinc deficiency. Sucrose gradients are shown for the 100,000g pellets from zinc-deficient cells sampled before zinc is added (a), and at 2 hr (b), 4 hr (c), 6 hr (d), 9 hr (e), and 12 hr (f) after zinc is added to the culture. The 100,000g pellets from the samples removed from culture (Fig. 7, upper) were resuspended in 2 ml of Buffer II and 1.5 ml of the clarified suspensions was layered onto 12-ml sucrose gradients (0.3-1.1 M in Buffer II). The gradients were centrifuged for 2 hr at 40,000 rpm and 0°C. The 87S peak is marked; left on the abscissa corresponds to the bottom of the gradient.

**Fig. 7.** Growth pattern of *Euglena gracilis* during recovery from zinc deficiency. Upper: The growth pattern is shown for four different cultures from two separate experiments in which cells were grown in 30 μg Zn/liter for 5½ days to the deficient state, 1 mg zinc was added, and each culture was sampled for 0-12 hrs after the addition of zinc. Samples of about 150 ml were removed at the designated times from an original volume of 1 liter. One of these cultures (●) provided the data for Fig. 6. Another culture (▼) was sampled just before zinc was added and was left undisturbed until sampling occurred from 12 to 24 hr after zinc was added. Slopes of the curves are shown. Lower: The amount of total ribosomal material (total A₂₆₀ units/gram of cells from Fig. 6) in the 87S peak is presented for each sample (2, 4, 6, 9, and 12 hr after zinc was added).
tems intact ribosomes and ribosomal subunits subjected to digestion by ribonuclease retained their original sedimentation values (3, 11, 31, 40), and the fragmented RNA was not released from the ribosomes (3, 31). It seems unlikely, therefore, that increased nuclease activity alone would result in ribosomal disappearance, which appears rather to be due to an intrinsic defect in the ribosomes of deficient cells as a consequence of the lowered ribosomal zinc concentration.

It is proposed, then, that in the absence of adequate zinc, defective ribosomes are produced, and that these ribosomes may undergo unfolding when the intracellular zinc reaches a certain critical low value, allowing attack by ribonucleases and possibly proteases in areas otherwise inaccessible in normal, tightly-packed ribosomes. The resulting RNA fragments and proteins would remain in the supernatant under the conditions of digestion employed in this study, accounting for the inability to detect intact ribosomal RNA in the fragmented cells at any point during or after ribosomal breakdown.

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LITERATURE CITED