Nucleic Acids and Metals. III. Changes in Nucleic Acid, Protein, and Metal Content as a Consequence of Zinc Deficiency in *Euglena gracilis**

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As a consequence of zinc deficiency the RNA content of *Euglena gracilis* is decreased, that of amino acids is increased, and the DNA content is doubled. In addition, there is a massive accumulation of acid-insoluble polyphosphate. Concomitantly the metal content of the deficienct organisms is markedly altered. As zinc content decreases that of other metals rises; manganese, particularly, increases 60-fold in photosynthesizing zinc-deficient *Euglena*. The results suggest that the lesion characteristic of zinc deficiency reflects the participation of the metal in the synthetic process leading from RNA to protein. The data afford an opportunity to localize the site of action of this element, which has been found in RNA's and many enzymes from virtually all phyla.

The involvement of metal ions in virtually all phases of metabolism has generated increasingly successful attempts to define their mechanism of action. The compositional association of metals with proteins has long been recognized and their functional essentiality in the biological action of many enzymes has been repeatedly demonstrated (Vallee, 1961).

The occurrence of metals in nucleic acids has not been appreciated until quite recently, and the discernment of their role in the structure and function of nucleic acids has been the subject of conjecture (Wacker and Vallee, 1959; Fuwa *et al.*, 1960).

The introduction of metal deficiencies in appropriate organisms has been a rewarding approach to the understanding of their mode and locus of action. For this reason the problem has been approached by measuring the effect of metal deprivation on the nucleic acid and protein metabolism of a microorganism. A suitable organism for such studies should have the following characteristics: a plant-like nutrition, which allows the metal content of the growth medium and the organism to be controlled rigidly; ease of cell breakage to permit ready isolation and characterization of the subcellular material; vigorous growth and metabolism to amplify the alterations induced by metal deficiency; and finally homogeneity of cell populations to permit unambiguous interpretation of the analytical and functional changes brought about by the deficiency.

The flagellate *Euglena gracilis* fulfills these criteria. It was further selected for study because it can be grown on a simple well-defined medium from which metals can be easily removed (Price and Vallee, 1962). Since zinc is particularly ubiquitous in nature, has been found in the RNA from all phyla examined thus far, and is required as a functional component of a number of different enzymes, this metal was chosen for study. Previous investigations, moreover, have already demonstrated that growth of *Euglena gracilis* is severely reduced in a zinc-deficient medium (Price and Vallee, 1962).

As a consequence of zinc deprivation of these organisms their protein and RNA are markedly reduced while there is a concomitant increase in amino acids and polyphosphate. In addition, zinc deficiency leads to a mitotic arrest as indicated by a doubling of the DNA content of the deficient cells.

METHODS

Euglena gracilis[1] was grown in the presence and absence of light in a synthetic medium containing glutamic and malic acids, sucrose, vitamin B_{12}, thiamine, and a mineral mixture. The mixture of glutamic and malic acids was rendered metal-free by repeated extractions with dithizone in carbon tetrachloride (Price and Vallee, 1962). Metals were removed from sucrose solution by passage over a chelating resin (Bio-Rad. AG 50 x 8). Spectrographically pure salts (Johnson and Matthey, Ltd., London) were used to constitute the mineral mixture except for KH_2PO_4, $MgSO_4$, and $Ca(NO_3)_2$, which were reagent grade salts purified by extraction with dithizone in carbon tetrachloride. The purified, zinc-deficient medium contained less than 10^{-9} moles of zinc per liter. The complete zinc-sufficient medium differed from the zinc-deficient one only by the addition of 1.5×10^{-5} M $ZnCl_2$. The organisms

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TABLE I

FRACTIONATION OF *Euglena gracilis* BY THE SCHNEIDER PROCEDURE AND MEASUREMENTS MADE ON EACH FRACTION

Fraction	Measurements
Whole *Euglena*	cell count, dry weight, packed cell volume, nitrogen, phosphorus
10% TCA, 6 hr, 4°	
Centrifugation	
Precipitate	Supernatant = acid-extractable fraction: A 268.5, phosphorus, nitrogen
95% Ethanol, 90°	
Centrifugation	
Precipitate	Supernatant = phospholipid fraction: phosphorus, nitrogen
2 × 3:1 ethanol-ether, 70°	
Centrifugation	
Precipitate	Supernatant = total nucleic acid fraction: A 268.5, phosphorus, nitrogen, diphenylamine,
2 × 5% TCA, 95°, 10 min	
Centrifugation	
Precipitate = protein fraction	nitrogen, phosphorus
	+ Charcoal
	Centrifugation
Charcoal + RNA and DNA	Supernatnant = polyphosphate: A 268.5, nitrogen, phosphorus

were grown at 23° on a rotary shaker in 1 liter of medium contained in 2-liter Erlenmeyer flasks stoppered with sterile cloth and cotton plugs. Some organisms were grown in complete darkness; light-grown cultures were illuminated by 4 General Electric fluorescent bulbs (F48T12) 4 feet above the cultures. Growth was determined by measuring the absorbance of the culture in a Klett colorimeter with a No. 54 filter.

Cultures were harvested and transferred to a 1-liter polyethylene centrifuge cone and sedimented at 2000 rpm for 15 minutes in an International refrigerated centrifuge. The medium was decanted and the cells were washed with metal-free water and centrifuged again. The sedimented cells were then suspended in metal-free water to yield a thick slurry containing about 10 to 20 g of organisms per 100 ml. Duplicate 2-ml aliquots of this concentrate were then fractionated by a modification of the Schneider procedure (Schneider, 1945, 1946). Duplicate aliquots were also obtained for measurement of dry weight, total nitrogen, total phosphate, packed cell volume, and metal content. After dilution in 1% formaldehyde cells were counted in a hemocytometer. Phosphorus was measured by the method of Ames (Ames and Dubin, 1960), nitrogen by the Kjeldahl procedure (Swift, 1938), DNA by the diphenylamine method (Seibert, 1940), and total nucleic acids by their absorption at 268.5 mμ. RNA was estimated spectrophotometrically as the difference in absorption at 268.5 mμ after the subtraction of DNA measured by the diphenylamine procedure. Acid-insoluble polyphosphate was measured by determining the residual phosphate of the total nucleic acid fraction after absorption of the nucleic acids on Norite (Harold, 1960). An outline of these procedures is given in Table I. Amino acid nitrogen is defined as the total acid-extractable nitrogen minus nucleotide nitrogen determined by the absorbance at 268.5 mμ. Metal analyses were performed by emission spectroscopy using the porous cup method described previously (Vallee and Neurath, 1955). Reagent grade chemicals were used throughout. Glassware and reagents were treated to remove contaminating metals as previously described (Thiers, 1957). Metal-free water was prepared by passage over a mixed bed ion exchange resin (Wacker and Vallee, 1959).

RESULTS

When *Euglena* are grown in the dark in a medium containing only 1.5×10^{-7} M of added

TABLE II

PHOSPHORUS AND NITROGEN CONTENT OF ZINC SUFFICIENT AND DEFICIENT *Euglena* AND OF THE FRACTIONS OBTAINED BY THE SCHNEIDER PROCEDURE; DARK-GROWN CELLS HARVESTED AT THE PLATEAU OF THE GROWTH CURVE

	Zinc-Sufficient				Zinc-Deficient			
	Phosphorus (μM/2 ml)	(mean)	Nitrogen (μg/2 ml)	(mean)	Phosphorus (μM/2 ml)	(mean)	Nitrogen (μg/2 ml)	(mean)
Whole *Euglena*	26.4		10.2		54		6.0	
	30.4	(30.7)	10.5	(10.5)	40	(49.0)	6.6	(6.3)
	35.2		10.8		53		6.1	
Acid-extractable	10.0		2.5		16.0		3.0	
	16.3	(14.2)	2.5	(2.5)	19.3	(16.7)	3.0	(3.0)
	14.5		2.6		15.8		3.0	
	16.0		2.5		15.8		3.0	
Phospholipid	3.2		3.2		1.8		0.3	
	3.2	(3.5)	3.1	(3.0)	2.5	(2.1)	0.4	(0.4)
	3.8		3.0		[a]		0.4	
	3.8		2.8		[a]		0.3	
Protein	1.5		5.0		0.6		1.8	
	1.6	(1.6)	5.0	(5.0)	0.6	(0.7)	1.9	(1.8)
	1.7		[a]		0.7		1.8	
	1.7		[a]		0.7		1.7	
Total nucleic acid	12.0		1.2		30.0		1.1	
	13.8	(12.3)	1.2	(1.2)	26.4	(27.6)	1.2	(1.1)
	11.6		1.2		27.0		1.1	
	11.8		1.2		27.0		1.1	
Σ of fractions		31.6		11.7		47.1		6.3
% of recovery		102		111		96		100

[a] Lost in processing.

zinc, growth is markedly reduced compared to the normal medium containing 1.5×10^{-5} M of zinc (Fig. 1). On the addition of zinc at any point on the growth curve normal growth resumes after a short lag period. Addition of other metals, amino acids, nitrogenous bases, or nucleotides does not restore normal growth.[2] The growth curves of the light-grown organisms are essentially the same as those of organisms grown in the dark (Fig. 1).

Zinc sufficient and deficient organisms, grown in the dark, were harvested at various times during growth to determine their nucleic acid and protein content; during the lag phase on day 7; in the log phase on day 9; and at the plateau of the growth curve on day 13. In each instance, dry weight, packed cell volume, numbers of cells, and metal content were measured. The cells then were fractionated by a modification of the Schneider procedure as shown in Table I. The measurements performed on each of the fractions obtained are indicated. Total nitrogen and phosphorus content of normal and zinc-deficient cells harvested at the plateau, as well as the nitrogen and phosphorus contents of each of the fractions of these cells obtained by the Schneider procedure, are shown in Table II. Triplicate determinations were made on whole *Euglena*, and duplicate analyses of two separate Schneider procedures were carried out on the individual fractions. Recovery of phosphorus in the fractions varies from 96 to 102% of the total phosphorus, and recovery of the

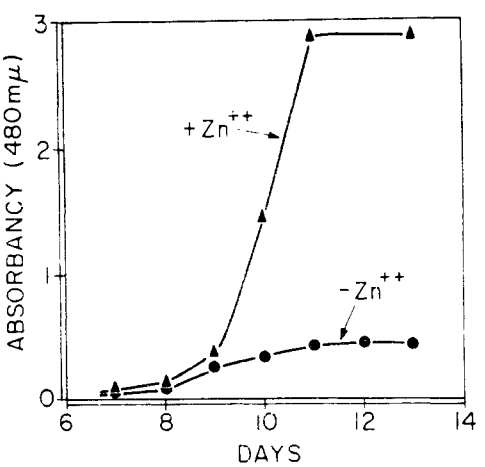

FIG. 1.—Growth of zinc-sufficient and zinc-deficient *Euglena gracilis*. Sufficient medium contains 1.5×10^{-5} M Zn^{++}; deficient medium contains a limiting amount of zinc (1.5×10^{-7} M). Cultures were grown in the dark.

[2] All metals with known biological function are included in the zinc deficient medium. Substitution of 1.5×10^{-5} M $CdCl_2$ for zinc did not promote growth. The addition of an amino acid mixture of 20 amino acids or the addition of a mixture of adenine, guanine, cytosine and uracil or their respective mononucleotides failed to restore normal growth in the zinc deficient cultures.

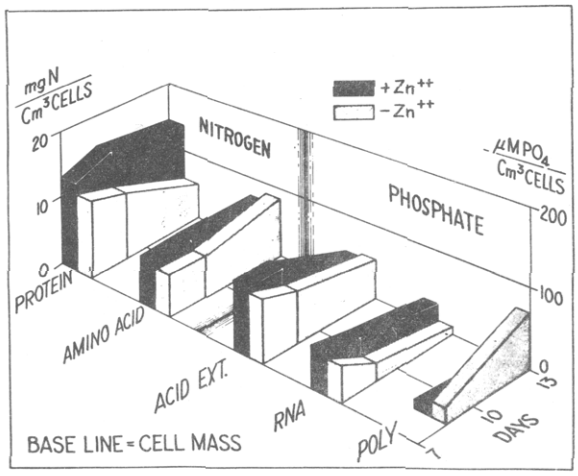

FIG. 2.—Changes in the major nitrogen and phosphate containing fractions of zinc sufficient and deficient *Euglena* as a function of the growth phase when cell mass is employed as a baseline. Cultures were harvested and fractionated at 7, 9, and 13 days after inoculation, when the cells are in lag phase, in logarithmic growth, and at the plateau of the growth curve, respectively, as indicated. Cultures were grown in the dark.

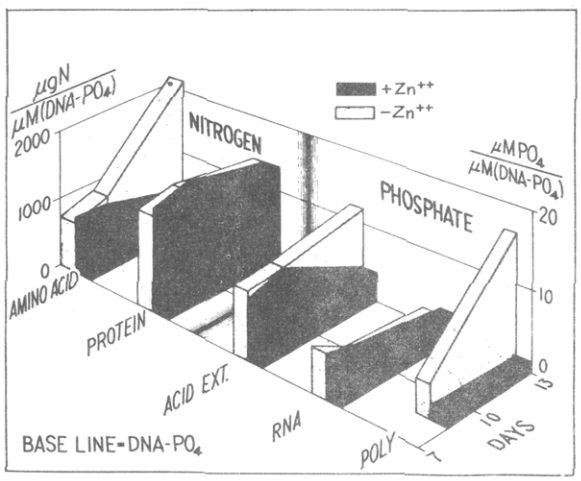

FIG. 3.—Changes in the major nitrogen and phosphate containing fractions of zinc sufficient and deficient *Euglena* as a function of the growth phase when DNA phosphate is employed as a baseline.

nitrogen in the fractions varies from 100 to 111% of the total nitrogen. These data are representative of the over-all precision and accuracy of the fractionation scheme as well as the precision of the phosphorus and nitrogen analyses.

Figure 2 shows the changes in the major nitrogen and phosphate fractions of zinc sufficient and deficient *Euglena* as a function of the growth phase when cell mass is employed as the baseline. During lag phase these parameters are virtually identical in both. However, as growth continues the effect of zinc deficiency on these cellular constituents becomes apparent. There is a progressive

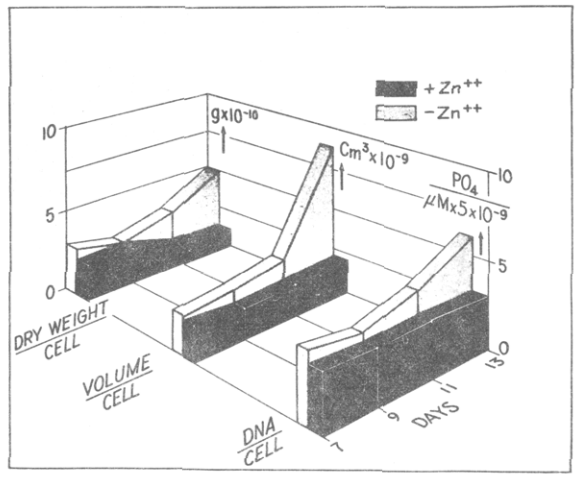

FIG. 4.—Changes in the dry weight, volume, and DNA content per cell of zinc sufficient and deficient *Euglena* as a function of the growth phase. The dark-grown cultures were harvested at 7, 9, 11, and 13 days after inoculation, as indicated.

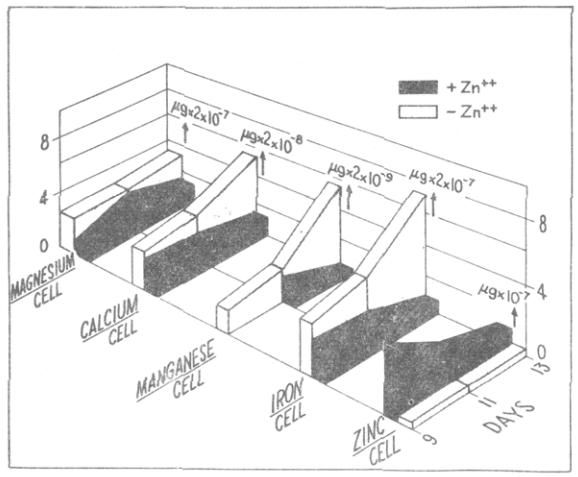

FIG. 5.—Changes in the metal content per cell of zinc sufficient and deficient *Euglena* as a function of the growth phase. The dark-grown cultures were harvested at 9, 11, and 13 days after inoculation, as indicated.

increase in amino acid nitrogen and a concomitant decrease in protein and RNA. The acid-extractable phosphate does not change, but there is a great increase in acid-insoluble polyphosphate. All of the changes are most marked at the plateau of the growth curve.

The lesion is similar when DNA phosphate is employed as a baseline (Fig. 3). Again, amino acids accumulate and both protein and RNA are decreased. According to this baseline acid-extractable phosphate and acid-insoluble polyphosphate are both increased.

These data demonstrate a marked impairment in both RNA and protein synthesis in zinc-deficient *Euglena* when measured on the basis of either cell mass or DNA content. Microscopic examina-

tion of the zinc-deficient cells demonstrates another aspect of the lesion: the individual cells are very large. The weight, volume, and DNA content per cell of sufficient and deficient *Euglena* are shown as a function of the growth phase, documenting the increase in cell size (Fig. 4). The size of the zinc-deficient cells increases progressively until, at the plateau, their weight and volume are about 5-fold greater than those of the sufficient cells. In addition, the DNA content of the deficient cells increases progressively, until finally it is 1.95 times that of the normal zinc-sufficient organisms. The DNA content of the normal cells remains constant at 1.7×10^{-8} μM DNA·PO_4 per cell at all phases of growth.

The metal content of normal and deficient organisms was measured spectrographically at three phases of growth (Fig. 5). Most striking is the profound decrease in the zinc content of the deficient cells. The reduction in zinc content is already evident during logarithmic growth and persists in the cells harvested at the plateau of the growth curve. Moreover, the zinc content of the deficient cells, harvested when growth has ceased, is even less when expressed with dry weight used as a baseline. Thus, the zinc content of normal cells is 192 μg/g dry weight, whereas the deficient cells contain only 14 μg/g dry weight.

In contrast to the decrease in zinc there is a striking increase in the content of all other metals in the deficient cells. The increase per cell varies from 3- to 18-fold for magnesium and manganese respectively.

The studies reported so far were all performed on dark-grown organisms. Similar measurements were, however, performed on illuminated cells, which exhibit all changes found in the dark-grown organisms (Table III). A 60-fold increase in the manganese content of the light-grown deficient cells constitutes the only major difference observed between organisms grown under the two conditions (Fig. 6).

Discussion

In previous communications we reported the presence of metals in RNA obtained from various species (Wacker and Vallee, 1959) and the stabilization of RNA structure by metals of the first transition series (Fuwa *et al.*, 1960). The biological significance of these observations has, however, been in doubt. In view of the multiple functions now assigned to specific RNA's either in the activation of amino acids (Hoagland, 1960) as templates for protein synthesis (Gros *et al.*, 1961; Nirenberg and Matthaei, 1961) or as the carriers of virus infectivity (Fraenkel-Conrat *et al.*, 1957), the investigation of the functional role of metals in RNA could be approached by several different avenues. In each of these systems, however, experimental obstacles are encountered which make it impossible to control the presence of a metal as a single variable (Holley and Lazar, 1961). These experimental difficulties are due to the nature of the assay systems used for the measurement of activity, since contaminating metals may be introduced by the assay systems.

It is, however, possible to overcome these obstacles through yet another approach: the induction of a deficiency of a metal known to be present in RNA. If the metal is a functionally significant component of RNA its lack should be reflected in alterations of processes mediated by RNA, *e.g.*, protein synthesis. Although RNA contains a number of different metals (Wacker and Vallee, 1959), zinc was chosen for these studies, since in addition to its presence in RNA it is particularly ubiquitous in nature and is known to be necessary for growth of many species, and its function as an essential component of a number of enzymes has been well documented (Vallee, 1961).

In accord with the experimental hypothesis the data demonstrate that, in addition to the previously documented (Price and Vallee, 1962) arrest of growth of *Euglena gracilis*, zinc deficiency is

Table III

Distribution of the Major Nitrogen and Phosphate Fractions of Light-Grown Zinc Sufficient and Deficient *Euglena gracilis* Harvested at the Plateau of the Growth Curve

	Zinc-Sufficient	Zinc-Deficient
Amino acid nitrogen	2.0 μg/cc[a]	8.0 μg/cc
Protein nitrogen	10.1 μg/cc	6.1 μg/cc
RNA phosphate	18.3 μM/cc	8.8 μM/cc
Acid-insoluble polyphosphate	8.7 μM/cc	107 μM/cc
DNA phosphate	1.65×10^{-8} μM/cell	2.31×10^{-8} μM/cell

[a] cc = cubic centimeter of packed cells.

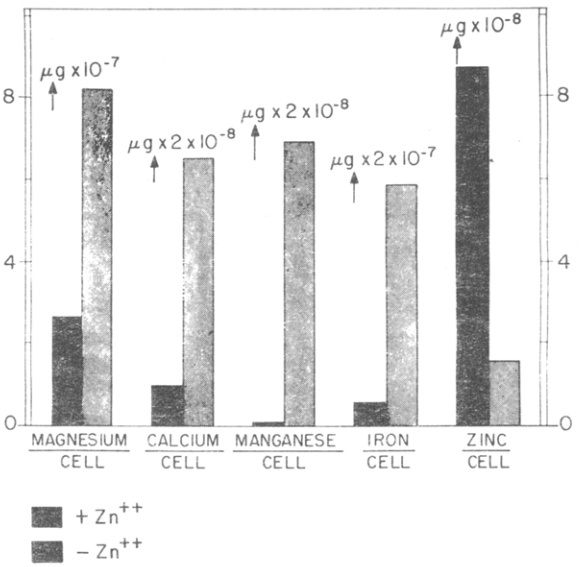

Fig. 6.—Metal content per cell of light-grown zinc sufficient and deficient *Euglena* harvested at the plateau of growth.

also accompanied by major alterations in protein and nucleic acid content of the organisms. The lesion resulting from zinc deficiency consists of an impairment of protein synthesis, indicated by an increase in protein precursors, *e.g.*, amino acids, and a decrease in RNA and protein. In addition, the expansion of the size of individual cells and the doubling of their DNA content demonstrates a mitotic arrest as a consequence of zinc deficiency.

The earliest attempts to define the growth arrest of zinc deficiency in chemical terms demonstrated a marked impairment of protein metabolism of *Neurospora crassa* (Nason 1950) though the locus of the action of the metal could not be determined. Although alterations in the activity of specific enzymes including tryptophane-desmolase, required for the synthesis of tryptophane, were found, these did not appear to account for the over-all decrease in protein content of the deficient organisms, and a direct effect of the zinc deficiency on protein synthesis was in fact postulated (Nason 1950; Nason *et al.*, 1951). Subsequent studies of the effects of zinc deficiency in tomato plants confirmed the decrease in protein synthesis (Possingham, 1956).

In addition to the previously documented increase in amino acids (Possingham, 1956) and decrease in protein in zinc-deficient *Euglena gracilis*, RNA is found to be decreased and the DNA content is increased. Thus changes in the major molecular species involved in the protein synthetic process have been identified. Analogous results have been obtained in *Mycobacterium smegmatis* made deficient in zinc and iron (Winder and O'Hara, 1962).

On the basis of the present data it cannot be stated unequivocally that the observed effects on protein and nucleic acid metabolism derive directly from a deficiency of zinc in the RNA of these organisms. However, since zinc is known to be a functional component of a number of enzymes it is entirely possible that its lack could bring about an as yet unidentified enzyme defect in the synthesis of some component of the protein synthetic process. It is clear that zinc deficiency is not overcome by the addition of amino acids, purines, pyrimidines, or their nucleotides, and this fact suggests that the effects of zinc deficiency are not brought about solely by a failure to synthesize these precursors. Further attempts to localize the specific sites of action of zinc in this system have not been made.

Several items of experimental data seem to suggest that protein synthesis itself is directly impaired. The effects of zinc deprivation do not simply constitute a nonspecific failure of oxidative metabolism to provide sufficient energy for these processes, since a striking accumulation of acid-insoluble polyphosphate constitutes one of the major characteristics of the deficient cells. Studies in several microbial species have demonstrated that polyphosphate is synthesized from ATP (Kornberg *et al.*, 1956; Muhammed, 1961; Zaitseva *et al.*, 1960). The accumulation of this high-energy inorganic polyphosphate in the deficient cells is evidence that the energy-generating systems are competent. Moreover, the presence of increased amounts of polyphosphate in these organisms is in keeping with similar increases in polyphosphate in other species (Winder and O'Hara, 1962) as an accompaniment of reduced protein synthesis. The accumulation of polyphosphate has, in fact, been considered to be the specific consequence of its failure to be utilized in the synthesis of RNA and protein (Mudd *et al.*, 1958). The nature of the molecular defect responsible for the lesion of zinc-deficient organisms cannot be localized precisely by the present data nor, in fact, can a decision be made with certainty whether the over-all lesion is the result of a single or multiple molecular defects. Recent studies, however, support the idea that a failure of protein synthesis may be considered the primary event. It has been shown that DNA replication eventually ceases in the absence of protein synthesis in bacteria. When protein synthesis is blocked under conditions which permit replication of DNA, synthesis of DNA continues only until the organism has doubled the DNA content of each genome (Maaløe and Hanawalt, 1961; Hanawalt *et al.*, 1961). Thus, it could be predicted that the mitotic arrest in the present system is the result of a failure of protein synthesis which precludes cell division and continued replication of DNA. In regard to the effect of zinc deficiency in producing a mitotic arrest it should be pointed out that deficiencies of other metals, *e.g.*, iron (Winder and O'Hara, 1962), manganese (Richter, 1961), and magnesium (Webb, 1951a,b), all of which are known to be present in RNA (Wacker and Vallee, 1959), bring about a similar metabolic result.

The measurements of metal content of these organisms serve to substantiate the validity of the experimental approach. The marked reduction in zinc is telling documentation of the severity of zinc deprivation. The demonstration that zinc-deficient cells accumulate other metals is in keeping with previous observations on the biological effects of metal deficiency. In many species, the deprivation of one metal is accompanied by the accumulation of others, or conversely the intoxication with a single metal may produce a reduction in the content of others. This observed ion antagonism is so general as to constitute virtually a biological generality, but the underlying chemical and physical basis has not as yet been ascertained (Smith, 1962).

Since *Euglena gracilis* is capable of carrying out photosynthesis, a unique opportunity is offered to measure the consequences of zinc deficiency and its manifestations when such a major change in cell metabolism is suddenly induced. This consideration is the more intriguing when it is recalled that zinc has in the past been thought to be essential in chlorophyll synthesis (Schwartz, 1956).

The lesion in zinc deficiency of organisms further stressed by exposure to light is virtually identical to that of the dark-grown organisms. The marked increase in manganese content of the zinc-deficient photosynthesizing cells represents the single exception. The rather specific alteration in the concentration of manganese occurring in conjunction with active photosynthesis in the zinc-deficient organisms may represent an unsought for yet significant result of this study. Manganese is known to be required in the photosynthetic process (Richter, 1961; Pirson, 1955; Tanner *et al.*, 1960). The accumulation of this ion in association with the depletion of zinc, another metal said to be involved in this process, suggests that this system may be exploited experimentally to define the precise role of metals in the photosynthetic process.

Since these experiments were undertaken in an effort to determine the potential functional significance of the presence of metals in RNA, it is of considerable interest that these major alterations in nucleic acid and protein metabolism are the result of a deficiency of one of the metals known to be present in RNA. The identification and localization of the molecular defect(s) induced by zinc deprivation seems feasible, and this system offers promise of a conclusion concerning the functional role of metals in RNA.

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