Proc. Natl. Acad. Sci. USA
Vol. 75, No. 9, pp. 4175–4179, September 1978
Biochemistry

RNA metabolism, manganese, and RNA polymerases of zinc-sufficient and zinc-deficient *Euglena gracilis*

(RNA content and base composition/zinc deficiency)

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ABSTRACT **The three major RNA classes from zinc-sufficient [(+Zn)] and zinc-deficient [(−Zn)] *Euglena gracilis* have been separated by affinity chromatography on oligo(dT)- and *N*-{*N'*-[*m*-(dihydroxyboryl)phenyl]succinamoyl}aminoethyl (DBAE)-celluloses. The total RNA content and the ribosomal and transfer RNA fractions are the same in (+Zn) and (−Zn) cells. In (−Zn) cells, the messenger RNA fraction increases, and its altered base composition reveals additional bases and a 2-fold increase in the (G+C)/(A+U) ratio. Since the intracellular content of manganese increases in (−Zn) cells, we have examined its role in determining these changes in RNA composition. An increase in the Mn^{2+} content from 1 to 10 mM in assays with RNA polymerases I and II from (+Zn) cells and those with the single RNA polymerase from (−Zn) cells decreases the ratio of UMP to CMP incorporated from 1.7 to 1.0, 2.1 to 0.8 and 3.5 to 0.4, respectively. Thus, Mn^{2+} concentration can significantly alter the products of the enzymatic action of RNA polymerases from both (+Zn) and (−Zn) *E. gracilis* cells.**

Zinc is central to normal nucleic acid synthesis and function (1). Its deficiency retards and, ultimately, arrests growth in species of all phyla. The eukaryote *Euglena gracilis* has long served both to delineate the zinc-dependent cell constituents that are essential to cell division and growth (2) and to identify the consequences of zinc deficiency on the synthesis, composition, and function of DNA and RNA and on cell division. Zinc deficiency alters the DNA and protein content and the incorporation of [^{3}H]uridine into the RNA of these cells (3–5). Moreover, zinc-deficient [(−Zn)] *E. gracilis* accumulate other metals—e.g., Mn, Fe, Cr, and Ni—some of which also affect DNA and RNA function (3, 4). Zinc-sufficient [(+Zn)] cells contain three RNA polymerases (6, 7) which seem to be replaced by a single, unusual RNA polymerase in (−Zn) cells (unpublished results). These observations have led to an investigation of the effects of zinc deficiency on the characteristics of the classes of RNA of these cells. (−Zn) cells contain nearly the same amount of transfer and ribosomal RNA but also twice as much messenger RNA as (+Zn) cells. Moreover, there is a significant difference in the base composition of mRNA fractions of (−Zn) and (+Zn) cells. By varying the concentration of Mn^{2+} in a cell-free system employing the single RNA polymerase from (−Zn) cells as well as RNA polymerases I and II from (+Zn) cells, we have obtained RNAs whose base compositions emulate those of mRNA from (−Zn) *E. gracilis*.

MATERIALS AND METHODS

Isolation of RNA Classes. The growth of *E. gracilis*, strain Z, in (+Zn) or (−Zn) media, containing 10^{-5} M or 10^{-7} M Zn^{2+}, respectively, has been described (2–4). All cultures were harvested in early stationary phase; unless stated otherwise procedures were carried out at 0–4°. (+Zn) or (−Zn) cells, 20 gm, were washed and suspended in 60 ml of 0.1 M Tris·HCl, pH 7.6. Cells were counted in triplicate by using a hemocytometer. The cells were ruptured as described (6, 7) and particulate matter was removed from the resultant cellular homogenate by centrifugation at 17,000 × *g* for 15 min. An equal volume of phenol, cooled prior to use, was added to the cellular homogenate and the resultant mixture was stirred for one hr in a hood. The phenol mixture was spun at 17,000 × *g* for 10 min at 23°, and the clear aqueous layer was collected and maintained at 4°. The phenol and interphase layers were homogenized, one volume of 0.1 M Tris·HCl, pH 7.6 was added, and the procedures repeated. The resultant aqueous layers were mixed with 3 volumes of absolute ethanol and 0.1 volume of 10% NaCl and the RNA was then precipitated after it was stirred for 90 min at 4°. It was collected by centrifugation at 48,000 × *g*, and then dissolved in, and dialyzed against, buffer for 24 hr. The dialyzed sample was lyophilized, weighed, and stored at −70°. The total RNA concentration was determined by an orcinol method and calculated per cell for each sample.

The various classes in this mixture of RNAs were separated by a series of affinity columns. Cytoplasmic mRNA contains a poly A segment that is absent from other RNAs. Oligo(dT)-cellulose will bind only the poly A-containing mRNA and, thus, affords a rapid purification method (8). Oligo(dT)-cellulose (Collaborative Research, Waltham, MA) was swollen for 24 hr in 0.01 M Tris·HCl, pH 7.4, packed into a column (1 × 24 cm), and equilibrated for 12 hr at room temperature with binding buffer B (0.01 M Tris·HCl, pH 7.4/0.5 M NaCl/10% glycerol). Lyophilized RNA, 10–15 mg, was suspended in 10 ml of binding buffer and applied to the column at a flow rate of 10 ml/min. Under these conditions, only the mRNA binds to the oligo(dT)-cellulose. The mRNA was eluted with 0.01 M Tris·HCl, pH 7.4/10% glycerol and elution was monitored by absorbance at 254 nm. The RNAs that do not bind to the oligo(dT)-cellulose column were chromatographed on a *N*-{*N'*-[*m*-(dihydroxyboryl)phenyl]succinamoyl} aminoethyl (DBAE)-cellulose affinity column which binds transfer RNA and separates it from the bulk of ribosomal RNA and the minor acylated fractions of transfer RNA (9). DBAE-cellulose was hydrated at 4° in binding buffer C (0.2 M NaCl, 0.01 M $MgCl_2$/0.05 M 4-methyl morpholine, pH 7.7/20% ethanol). Prior to use, new cellulose was conditioned for a few cycles by absorption and desorption with yeast tRNA obtained commercially. RNA that did not bind to the oligo(dT)-cellulose was first dialyzed against the binding buffer C and then applied to DBAE-cellulose at a rate of 3 column volumes per hr. The column was washed with an additional 3–4 column volumes of binding buffer to elute any amino acylated tRNA or ribosomal RNA bound nonspecifically to the cellulose; the tRNA was then eluted with 0.02 M NaCl/0.05 M Na acetate, pH 5.

Abbreviations: (−Zn), zinc-deficient; (+Zn), zinc-sufficient; DBAE-cellulose, *N*-{*N'*-[*m*-(dihydroxyboryl)phenyl]succinamoyl}aminoethyl-cellulose.

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Table 1. RNA content* of zinc sufficient, (+Zn), and deficient, (−Zn), *E. gracilis*

	Total RNA, $\mu g/10^6$ cells	Ribosomal, %†	Transfer, %†	Messenger, %†
(+Zn)	20 ± 5	79 ± 5	15 ± 3	6 ± 2
(−Zn)	19 ± 5	74 ± 4	15 ± 3	11 ± 3

* Values are the mean ± SD of six preparations.
† Percent of total RNA.

Sequential use of these columns allowed separation of the three major classes of RNA.

Base Composition of RNA Classes. The individual messenger, transfer, and ribosomal RNA from both (+Zn) and (−Zn) cells were dialyzed against metal-free distilled water for 24 hr at 4°. The samples were lyophilized and then hydrolyzed using 0.1 ml of 72% (wt/vol) $HClO_4$ per mg of RNA.

A 3-μl sample of the hydrolyzed bases was subjected to high-pressure liquid chromatography (Micromiretics Model 7115-24, Micromiretics, Norcross, GA) as previously reported (10). Each base in the sample was identified both by its retention time and by analysis of its characteristic UV spectrum (10).

Effect of Mn^{2+} on RNA Polymerase Function. RNA polymerase was isolated from (−Zn) cells and characterized by methods employed previously to purify RNA polymerase I, II, and III from (+Zn) cells (6, 7) (unpublished results). Solutions for the assay of RNA polymerase contained 10 μg of purified enzyme, 10 μmol of dithiothreitol, and 20 μg of heat-denatured calf thymus DNA in a total volume of 0.1 ml. We examined the effect of different concentrations of Mn^{2+} on the incorporation of [^{3}H]UMP, [^{3}H]AMP, or [^{3}H]CMP by assaying with 5 μCi of [^{3}H]UTP (25 Ci/mmol), 5×10^{-4} μmol of UTP, plus 5×10^{-3} μmol each of GTP, ATP, and CTP; with 5 μCi of [^{3}H]ATP (20 Ci/mmol), 5×10^{-4} μmol of ATP plus 5×10^{-3} μmol each of UTP, GTP, and CTP; or with 5 μCi of [^{3}H]CTP (21 Ci/mmol), 5×10^{-4} μmol of CTP, plus 5×10^{-3} μmol each of GTP, ATP, and UTP.

RESULTS

E. gracilis grown in the presence of zinc (+Zn) contained 20 μg of RNA/10^6 cells, a value virtually unaltered by zinc deficiency (−Zn) (Table 1). Sequential chromatography on oligo(dT)- and DBAE-celluloses resolved the total RNA into three fractions. Chromatography on oligo(dT)-cellulose separated the mRNA from approximately 90% or more of the total remainder. The unbound fraction was then applied to a DBAE-cellulose column that separates transfer RNA from ribosomal RNA. The amounts of ribosomal, transfer, and messenger RNAs of (+Zn) cells were equivalent to the values reported previously for *E. gracilis* grown to early stationary phase (11). The amount of total RNA in (−Zn) cells was virtually the same, the fraction of rRNA was slightly less, and that of tRNA was essentially the same as those in (+Zn) cells (Table 1). In marked contrast, the mRNA content of (−Zn) cells, 11% of the total RNA, was almost twice that of (+Zn) cells.

The values for the base composition of ribosomal, transfer, and messenger RNAs from (+Zn) cells were also virtually the same as those reported for *E. gracilis* when other methods were utilized (11). The purine and pyrimidine contents of rRNA were identical for (+Zn) and (−Zn) cells (Table 2). However, the analysis of the four major bases found in tRNA demonstrate that the guanine content decreased from 34% in (+Zn) to 24% in (−Zn) cells, whereas the cytosine content increased from 27% to 38%, respectively. The contents of adenine and uracil were identical.

The base composition of mRNA from (+Zn) and (−Zn) cells differed strikingly (Table 2). Fig. 1 compares the chromatogram of a hydrolysate of mRNA from (+Zn) cells with that of one from (−Zn) cells. Based on the elution volumes from the high-pressure liquid chromatography system and the UV spectra of each fraction, the former revealed only four peaks identified as uracil (U), guanine (G), cytosine (C), and adenine (A). In contrast, the mRNA hydrolysate from (−Zn) cells contained *seven major* peaks and several minor ones. The uracil, guanine, cytosine, and adenine fractions in this chromatogram also have been identified by their characteristic UV spectra. The elution volumes and UV spectra of the remaining fractions differed from that of the four major bases in mRNA from (+Zn) cells, and from that of a number of other known bases—e.g., thymine, hypoxanthine, xanthine, 5-methylcytosine—and are not as yet identified but not found in mRNA from (+Zn) cells. In multiple analyses, the (G+C)/(A+U) ratios of different mRNA samples from (+Zn) cells have all ranged from 1.4 to 1.7, whereas these ratios of corresponding samples from (−Zn) cells have ranged from 2.0 to 4.3. Thus, in mRNA from (−Zn) cells, the ratio of the known major purine and pyrimidine bases was consistently greater than in that from (+Zn) cells and, furthermore, mRNA from (−Zn) cells contained additional bases.

Mechanisms by which zinc deficiency might affect RNA metabolism have emphasized the essentiality of zinc for the function of DNA and RNA polymerases (4–6), and the importance of Mn^{2+} or Mg^{2+} for the activity of these enzymes has also been noted. However, generally, the possible synergism or antagonism of these and other metals in nucleotide polymerase action and, specifically, their effect(s) on the base composition of the resultant RNA product, have not been examined critically. These (−Zn) cells contain 35- and 5-fold greater amounts of Mn^{2+} and Mg^{2+}, respectively (3, 4) and, hence, the effects of these ions on the metabolism of these en-

Table 2. Base composition of *E. gracilis* RNA

	+Zn			−Zn		
Base	Ribosomal	Transfer	Messenger	Ribosomal	Transfer	Messenger
Guanine (G)	36	34	35	36	24	25
Cytosine (C)	26	27	26	26	38	49
Adenine (A)	21	19	21	22	20	10
Uracil (U)	17	20	18	16	18	16

The data are expressed as $\frac{\mu g \text{ of each base}}{\mu g \text{ G} + \text{C} + \text{A} + \text{U}} \times 100$. The mRNA from (−Zn) cells contains a number of additional bases (Fig. 1) that are not included in this calculation. Each value is the mean of three analysis.

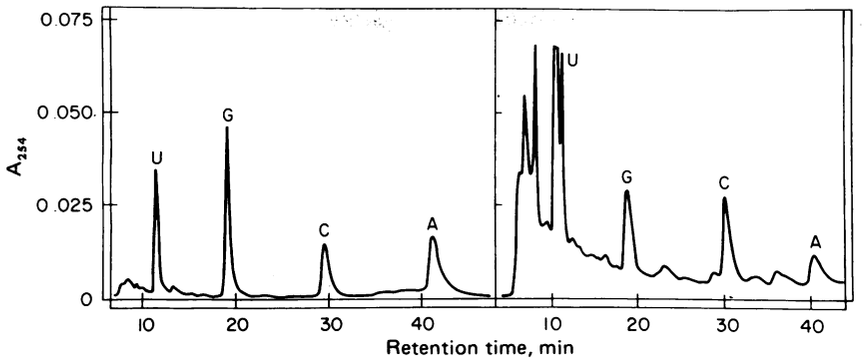

FIG. 1. The bases of *E. gracilis* mRNA from (+Zn) cells (*Left*) and (−Zn) cells (*Right*) were analyzed by high-pressure liquid chromatography. A 3-μl sample of an acid hydrolysate was used. The retention time and UV spectra differ for each base, allowing for base separation and identification. The mRNA from (+Zn) cells contains only the four major bases, uracil, guanine, cytosine, and adenine. In contrast, the mRNA from (−Zn) cells contains additional, unknown bases. Moreover, the ratios of (G+C)/(A+U), 1.6 and 2.9 for (+Zn) cell and (−Zn) cell mRNA, respectively, differ strikingly.

zymes could be significant. Hence, we have determined the effects of various concentrations of Mn^{2+} on the incorporation of bases into RNA generated by RNA polymerases I and II from (+Zn) cells and the single RNA polymerase from (−Zn) *E. gracilis*, as shown in Table 3. With RNA polymerase I or II from (+Zn) cells, increasing the Mn^{2+} concentration from 1 to 10 mM decreased the ratio of UMP to CMP incorporated from 1.7 to 1.0 and 2.1 to 0.8, respectively. In assays of the single RNA polymerase from (−Zn) cells, this ratio also decreased from 3.5 to 0.4. Thus, the base composition of RNA synthesized by polymerases from either cell type varies as a function of the Mn^{2+} concentration.

DISCUSSION

Zinc was first shown to be essential for cell division over 100 years ago (12), and during the last 30 years its universal importance as well as the biologic and pathologic consequences of its deficiency on prokaryotes, single cell eukaryotes, and non-mammalian and mammalian systems has been documented (13). Characteristically, zinc deficiency arrests cell proliferation and results in developmental and teratologic abnormalities of mammals (14). Evidence for the essentiality of zinc to DNA and RNA synthesis and cell division has emerged only in the course of the past decade (3–7, 15–26), providing a biochemical basis for an understanding of the roles of this element in normal and abnormal growth and development. Our studies with *E. gracilis* have aimed to identify those processes that arrest cell division, growth, and development when zinc is limiting. It became apparent early that altered synthesis of DNA alone does not account for the metabolic and cytologic properties of (−Zn) cells whose chemical properties included concomitant derangements of RNA and protein metabolism (3, 4). Indeed, the isolation of three zinc RNA polymerases from (+Zn) *E. gracilis* (6, 7; unpublished results) reinforced the conclusion that RNA metabolism of (−Zn) cells could be affected and prompted the study both of function of the RNA polymerases and the consequences to these organisms. (−Zn) *E. gracilis* contains a single, unusual zinc RNA polymerase but its sensitivity to α-amanitin differs from that of the three RNA polymerases found in normal (+Zn) cells (unpublished results). This difference in the number of RNA polymerases of (−Zn) cells emphasized the profound effect of the deficiency of this metal on these systems and made the investigation of its products imperative.

Table 3. Effect of $[Mn^{2+}]$ on relative UMP/CMP incorporation by *E. gracilis* RNA polymerases

Mn^{2+}, mM	RNA polymerase I	RNA polymerase II	Single enzyme from (−Zn) cells
1	1.7	2.1	3.5
5	1.4	1.7	1.5
10	1.0	0.8	0.4

* Analogous results are obtained for relative UMP/AMP incorporation.

The present studies compare the amounts of RNAs synthesized by both (+Zn) and (−Zn) cells. They show that while a single zinc RNA polymerase appears to be characteristic of the latter, zinc deficiency does not alter the total RNA content per (−Zn) cell, and remarkably each of the RNA classes—i.e., ribosomal, transfer, and messenger—are formed (Table 1). Thus, changes in the *amount* of each of these classes would not appear to account for the biologic effects of zinc deficiency in *E. gracilis*. However, the base composition of mRNA from (−Zn) and (+Zn) cells differs remarkably as demonstrated by the 2-fold increase of its (G+C)/(A+U) base ratio and the presence of significant amounts of bases other than uracil, guanine, cytosine, and adenine (Fig. 1). Obviously, though the *sequence* of bases in mRNA coding for different proteins would vary, for nearly all mRNAs the ratio of (G+C)/(A+U) has been found to be uniform with the exception of the mRNA from the posterior silk gland of *Bombyx mori* which has an unusually high G + C content. This specialized tissue synthesizes large quantities of a single protein, of course (27). Changes in mRNA composition to the extent observed between (+Zn) and (−Zn) cells are unusual indeed, and have not been reported previously in cells deprived of essential nutrients or as a function of growth or cell cycle stage. The demonstration of an altered composition of mRNA from (−Zn) cells suggests that, in *E. gracilis*, translation may be altered and lead either to the formation of products with unusual amino acid composition or to changes in the rate of synthesis of specific proteins that may be either essential for or inhibitory to cellular function. Abnormalities of protein metabolism in (−Zn) *E. gracilis* (3) and plants (28) have been shown, albeit only in terms of amino acid and total protein content. The effects of such alterations in protein metabolism could be decisive and result in the arrest of cell division in (−Zn) organisms because ongoing protein synthesis is required for this process (29–31). Similarly, if extended to other systems, derangements in the amino acid composition and in the quantity of proteins formed or in the synthesis of proteins involved in

the formation of tissues and organs could lead to the developmental abnormalities characteristic of zinc-deficient mammals (14).

Present understanding of mRNA synthesis and metabolism provides some possible clues for the basis of the unusual composition of this RNA species in (−Zn) cells. Thus, the mRNA products found in (−Zn) cells could result from the accumulation of mRNA molecules either more stable than those of other mRNA species and persisting in these cells or coding for large amounts of specific protein(s). They could also be the consequence of alterations in processes that determine or regulate the incorporation of bases into mRNA normally, including altered base sequences or misreading of the DNA template, abnormal function of RNA polymerases, and post transcriptional modifications of the mRNA molecules. The latter usually occur through endonuclear cleavage of 3′ and 5′ ends of the RNA formed and either addition of poly(A) or methylation (33). Moreover, mRNA molecules may be cleaved and various segments could combine to form novel species (34). Thus, derangements of any of these and of yet other processes involved in the regulation of the base composition and sequence of normal mRNAs could result in those of (−Zn) cells.

To initiate the process of examining these, we have elected to study first the effect of Mn^{2+} on the product of RNA polymerases in view of the accumulation of metals in (−Zn) cells (3, 4). Both Mn^{2+} and Mg^{2+} activate RNA polymerases and are thought to be involved in binding of the nucleotide substrate to the enzyme. Moreover, *in vitro* they are known to affect the composition of the nucleic acid products formed by micrococcal DNA polymerase (35), *Escherichia coli* RNA polymerase (36), and viral reverse transcriptases (37). When incubated *in vitro* in the presence of either Mn^{2+} or Mg^{2+}, these enzymes synthesize nucleic acid products with a different composition. Our studies of both RNA polymerases I and II from (+Zn) cells of the single RNA polymerase from (−Zn) cells demonstrate that the base composition of the resultant RNA also depends markedly on the *concentration* of the activating metal in the ambient environment, in this case Mn^{2+} (Table 3). It would seem that Mn^{2+} affects the interaction between the polymerases and the bases of the template or the nucleotide substrates. The mechanism for this interaction and the dependence of the composition of the RNA product on both the activating metal and its concentration are unknown. However, in this regard, it is pertinent that the stability constants for nucleotide–metal, nucleoside–metal, and base–metal complexes differ not only for each metal relative to the four major bases but also for each base relative to different metals (38, 39). Thus, the differences in the interaction between metals—e.g., Mn^{2+}—and the bases, present in *both* the template and substrate for RNA polymerase, could be determinants of the base composition of RNA synthesized *in vitro*. This suggests that the increased Mn^{2+} concentration of (−Zn) cells might result in altered RNA metabolism *in vivo*.

The importance of this conclusion extends beyond the elucidation of the biochemical consequences of zinc deficiency, however, because they point to a mechanism by which environmental factors—e.g., the concentration of metals such as Mn^{2+}—could regulate, modify, and influence transcription of the DNA template. There is abundant evidence for such roles of Mn^{2+} and Mg^{2+} in regulating other important aspects of cellular metabolism [e.g., phosphotransfer reactions (40)] exemplified by the control of the enzyme-mediated covalent modification reactions, such as adenylylations and phosphorylation (41), on the activities of glutamine synthetase of *E. coli* or muscle phosphorylase. Thus, the presence of either Mg^{2+} or Mn^{2+} alters the specificity of glutamine synthetase (42).

Conjointly, these observations suggest the existence of hitherto unrecognized variables in the regulation of transcription and translation in eukaryotic cells such as *E. gracilis*. The decrease in zinc content of cells and consequent accumulation of other metals suggests that attention to metal–metal antagonism or synergism should be considered in understanding cell division and development (43–46).

Our previous study of the effects of cadmium on the morphology and cell division of *E. gracilis* is a pertinent example. Although (−Zn) cells take up cadmium added to the culture media, (+Zn) cells do not. The presence of cadmium *then* induces multinucleation, blocks in cytokinesis, etc., in these (−Zn) cells, which are *not* observed when grown in the absence of cadmium (43). Thus, metal–metal interactions previously recognized in numerous organisms and tissues and often described as "imbalance," "antagonism," "synergism," or "conditioned deficiencies" (44–46) may be phenomenological examples of derangements in this metal-dependent regulation of cellular metabolism, much as the mechanisms underlying their symptomatic recognition have not been carried out. Overall, the present studies emphasize the essential role of zinc in cell division and development. Further, they direct attention to and call for reexamination of modes of regulation of the metabolism of nucleic acid and of the synthesis of protein by metals.

This work was supported, in part, by Grants-in-Aid GM 15003 from the National Institutes of Health, of the Department of Health, Education and Welfare. K.H.F. is an Investigator of the Howard Hughes Medical Institute.

1. Vallee, B. L. (1977) in *Biological Aspects of Inorganic Chemistry*, ed. Dolphin, D. (Wiley, New York), pp. 37–70.
2. Price, C. A. & Vallee, B. L. (1962) *Plant Physiol.* **37**, 428–433.
3. Wacker, W. E. C. (1962) *Biochemistry* **1**, 859–865.
4. Falchuk, K. H., Fawcett, D. W. & Vallee, B. L. (1975) *J. Cell Sci.* **17**, 57–78.
5. Falchuk, K. H., Krishan, A. & Vallee, B. L. (1975) *Biochemistry* **14**, 3439–3444.
6. Falchuk, K. H., Mazus, B., Ulpino, L. & Vallee, B. L. (1976) *Biochemistry* **15**, 4468–4475.
7. Falchuk, K. H., Ulpino, L., Mazus, B. & Vallee, B. L. (1977) *Biochem. Biophys. Res. Commun.* **74**, 1206–1212.
8. Hirsch, M. & Penman, S. (1974) *J. Mol. Biol.* **83**, 131–142.
9. McCutchan, T. F., Gilham, P. T. & Soll, D. (1975) *Nucleic Acids Res.* **2**, 853–864.
10. Falchuk, K. H. & Hardy, C. (1978) *Anal. Biochem.*, in press.
11. Brawerman, G. (1968) in *The Biology of Euglena*, ed. Buetow, D. E. (Academic, New York), Vol. 2, pp. 110–119.
12. Raulin, J. (1869) *Ann. Sci. Natl. Botan. Biol. Vegetale* **11**, 93.
13. Vallee, B. L. (1959) *Physiol. Rev.* **39**, 443–490.
14. Hurley, L. S. (1969) *Am. J. Nutr.* **22**, 1332–1339.
15. Wacker, W. E. C. & Vallee, B. L. (1959) *J. Biol. Chem.* **234**, 3257–3262.
16. Fuwa, K., Wacker, W. E. C., Druyan, R., Bartholomay, A. F. & Vallee, B. L. (1960) *Proc. Natl. Acad. Sci. USA* **46**, 1298–1307.
17. Shin, Y. A. & Eichhorn, G. L. (1968) *Biochemistry* **7**, 1026–1032.
18. Prask, J. A. & Plocke, D. J. (1971) *Plant Physiol.* **48**, 150–156.
19. Slater, J. P., Mildvan, A. S. & Loeb, L. A. (1971) *Biochem. Biophys. Res. Commun.* **44**, 37–43.
20. Scrutton, M. C., Wu, C. W. & Goldwait, D. A. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 2497–2501.
21. Auld, D. S., Livingston, D., Kawaguchi, H. & Vallee, B. L. (1974) *Biochem. Biophys. Res. Commun.* **57**, 967–972.
22. Auld, D. S., Livingston, D., Kawaguchi, H. & Vallee, B. L. (1975) *Biochem. Biophys. Res. Commun.* **62**, 296–302.
23. Falchuk, K. H., Hardy, C., Ulpino, L. & Vallee, B. L. (1977) *Biochem. Biophys. Res. Commun.* **77**, 314–319.

24. Kotsiopoulos, P. S. & Mohr, S. C. (1975) *Biochem. Biophys. Res. Commun.* **67,** 979–987.
25. Auld, D. S., Atsuya, I., Campino, C. & Valenzuela, P. (1976) *Biochem. Biophys. Res. Commun.* **69,** 548–554.
26. Wardzilak, T. M. & Benson, R. W. (1977) *Biochem. Biophys. Res. Commun.* **76,** 247–252.
27. Suziki, Y. & Brown, O. D. (1972) *J. Mol. Biol.* **63,** 409–429.
28. Possingham, T. V. (1956) *Aust. J. Biol. Sci.* **9,** 539.
29. Gelfant, S. (1966) *Methods Cell Physiol.* **2,** 359–395.
30. Brunori, A., Avanzi, S. & D'Amato, F. (1966) *Mutat. Res.* **3,** 305–313.
31. Epifanova, O. I. & Terskikh, V. J. (1969) *Cell Tissue Kinet.* **2,** 75–93.
32. Van't Hof, J. (1974) in *Cell Cycle Controls,* eds. Padilla, G. M., Cameron, I. L. & Zimmerman, A. (Academic, New York), pp. 77–85.
33. Brawerman, G. (1974) *Annu. Rev. Biochem.* **43,** 621–642.
34. Gelinas, R. E. & Roberts, R. J. (1977) *Cell* **11,** 533–544.
35. Littman, R. M. (1971) *J. Mol. Biol.* **61,** 1–23.
36. Pogo, A. D., Littau, V. C., Allfrey, V. G. & Mirsky, A. E. (1967) *Proc. Natl. Acad. Sci. USA* **57,** 743–750.
37. Sirover, M. A. & Loeb, L. (1976) *Biochem. Biophys. Res. Commun.* **70,** 812–817.
38. Weser, U. (1968) *Struct. Bonding (Berlin)* **5,** 41–67.
39. Eichhorn, G. L. (1974) in *Inorganic Biochemistry,* ed. Eichhorn, G. L. (Elsevier, Amsterdam, Netherlands), Vol. 2, pp. 1191–1243.
40. Lardy, H. (1951) in *Phosphorus Metabolism,* eds. McElroy, W. D. & Glass, B. (John Hopkins, New York), Vol. 1, pp. 477–499.
41. Holzer, H. (1969) *Adv. Enzymol.* **32,** 297–326.
42. Stadtman, E. R., Shapiro, B. M., Ginsburg, A., Kingdon, H. S. & Denton, M. D. (1968) *Brookhaven Symp. Biol.* **21,** 378–396.
43. Falchuk, K. H., Fawcett, D. W. & Vallee, B. L. (1975) *J. Submicrosc. Cytol.* **7,** 139–152.
44. Woolley, D. W. (1947) *Physiol. Revs.* **27,** 308–333.
45. Ershoff, B. H. (1948) *Physiol. Revs.* **28,** 107–137.
46. Martin, G. J. (1951) *Biological Antagonism* (Blakiston, New York), pp. 412–434.