

## *Mini review*

# **Roles of trace metals in transcriptional control of microbial secondary metabolism**

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Received September 25, 1989

**Summary.** Secondary metabolism in bacteria and fungi requires a much narrower range of environmental concentrations of key trace metals than that permitted for primary metabolism. The cells acquire appropriate quantities of the key metals at the initiation of the shift from primary to secondary metabolism. Evidence suggests that these essential micronutrients participate in regulating the expression of genes responsible for synthesis of secondary metabolites and/or morphological alterations associated with cellular differentiation.

**Key words:** Secondary metabolism – Iron – Manganese – Zinc

## **Introduction**

The study of secondary metabolism, which has long been of interest to natural product chemists and to pharmacognosists, has become an important field of endeavor for microbial and plant physiologists during the past half century. Advances in this area of study have contributed to discovery of novel compounds and to markedly improved yields of useful pharmacological agents. More recently, molecular geneticists have begun to examine the mechanisms responsible for the shift from the replicating vegetative phase into the mature, functionally differentiated cell. Elucidation of biological principles in regard to the control of microbial and plant cell secondary metabolism and differentiation ultimately should be useful for increasing our understanding of animal cell differentiation and dedifferentiation.

Although secondary metabolism and differentiation ostensibly can be dissociated (e.g., cells that form an excessive amount of a particular secondary product may be unable to complete normal morphogenesis), the two processes of cell maturation are considered to be induced by a common regulatory mechanism (Weinberg 1971; Katz and Demain 1977). During the phase of rapid cell multiplication, in either batch (Bu<sup>+</sup>Lock

1961) or continuous (Hostalek 1979) culture, the maturational shift is suppressed. As the rate of growth begins to decline, gene products required for morphological and/or metabolic differentiation are synthesized in those cells that recently ceased dividing (Hostalek 1979; Zahner 1979; Grootwassink and Gaucher 1980).

Though still incomplete, the evidence acquired during the past 15 years indicates that regulation of such maturational processes primarily occurs at the level of transcription. Accordingly, genes that code proteinaceous secondary metabolites (e.g., bacterial exotoxins) and enzymes required for the synthesis of low-molecular-mass secondary substances (e.g., peptide antibiotics) are derepressed, whereas some genes that had been activated during the growth phase are repressed.

The synthesis of bacterial and fungal secondary metabolites, as well as of structures required for cytodifferentiation, has a much narrower tolerance for a range of environmental concentrations of specific trace metal(s) than does vegetative growth of the producer cells (Weinberg 1970, 1977). Regulatory processes dependent on the quantitative adjustment of nutrients that are continuously required for other normal cellular activities may seem hazardous (Rutter et al. 1973). Yet, increasingly, reports are becoming available that bacteria and fungi indeed utilize inorganic micronutrients to regulate transcription of processes of cell maturation. These reports are discussed below.

## **Identification of the key trace metals**

The systematic study of the control of secondary metabolism began in the first decade of the 20th century with attempts to improve the yield of exotoxin from cultures of *Corynebacterium diphtheriae* for vaccine production. During the first third of the century, the quantities of this protein that were synthesized remained unpredictable. By 1935, it was recognized that the variability was associated with the amount of iron that contaminated the cultured cells either by leaching

from the walls of the fermentation vessels or from ingredients of the medium. One of the discoverers of this fact commented: "So narrow is the zone (of iron concentration) in which (diphtheria) toxin is obtained and so sharp the peak of maximal production that this single uncontrolled factor must have played a greater role in any previous experiments than the specific conditions supposedly under investigation." (Mueller 1941).

During the 1930s and 1940s, numerous secondary metabolites produced by fungi and actinomycetes began to be identified. Unlike the bacterial exotoxins which often are proteins and therefore synthesized via ribosomal systems, the fungal and actinomycetal metabolites generally are low-molecular-mass products that are assembled by synthetase complexes. Here, too, micronutrients soon were recognized as key factors affecting yield. A pioneer researcher noted: "Without a doubt, it is possible to alter pigment excretion of any fungus seriously by trace element nutrition" (Foster 1949).

Of the ten biologically important transition series

metals (i.e., atomic numbers 23–30, 42, and 48), three have been identified as having a key role in the biochemical and morphological maturation of diverse groups of microorganisms. To facilitate secondary metabolism and differentiation in species of *Bacillus* and in some species of fungi, the concentration of manganese must be carefully adjusted. In contrast, for many other groups of procaryotes, including actinomycetes, the quantity of iron is critical for the shift to cell maturation. In the case of some actinomycetes, as well as many groups of fungi, the concentration of zinc is paramount (Table 1). It is not yet clear why secondary metabolic processes and differentiation by actinomycetes often require the adjustment of both iron and zinc, whereas other procaryotes generally respond to the manipulation of only iron or manganese.

In the majority of cases examined, the metabolic shift occurs over a relatively small range of concentrations of the critical metal. For example, >75% of maximal growth of actinomycetes is observed over a range of concentrations of iron that is nearly three orders of

**Table 1.** Secondary metabolic products and structures that are synthesized through a range of concentrations of a key metal that is much narrower than the range that permits growth of the producer cells

(A) Iron				
Procaryotes			Fungi	
Small molecular products	Protein products	Differentiated structures	Products	
Actinomycin D <sup>1</sup> , actinorubin <sup>2</sup> , alkyl quinolones <sup>2</sup> , antimycin <sup>3</sup> , candidicin <sup>1</sup> , chloramphenicol <sup>2</sup> , grisein <sup>1</sup> , hydrogen cyanide <sup>3</sup> , mitomycin <sup>2</sup> , monensin <sup>1</sup> , neomycin <sup>1</sup> , neopurpuratum <sup>1</sup> , prodigiosin <sup>3</sup> , pyocyanine <sup>3</sup> , streptomycin <sup>1</sup> , tetracycline <sup>1</sup>	Exotoxins of <i>Aeromonas hydrophila</i> <sup>3</sup> , <i>Clostridium tetani</i> <sup>2</sup> , <i>Corynebacterium diphtheriae</i> <sup>4</sup> , <i>Pseudomonas aeruginosa</i> <sup>5</sup> , <i>Shigella dysenteriae</i> <sup>3</sup> , <i>Staphylococcus aureus</i> <sup>2</sup> and <i>Vibrio cholerae</i> <sup>3</sup> ; elastase, alkaline protease and lipase of <i>Pseudomonas aeruginosa</i> <sup>6</sup>	Cysts of <i>Azotobacter vinelandii</i> <sup>7</sup> ; spores of <i>Clostridium bifermentans</i> <sup>8</sup>	Patulin <sup>2</sup> , penicillin <sup>2</sup> , stemphyloxin <sup>9</sup>	
(B) Manganese				
<i>Bacillus</i> species		<i>Actinomycetes</i>	<i>Fungi</i>	
Products	Differentiated structure	Product	Products	Differentiated structures
Anthrax protective antigen <sup>10</sup> , bacillin <sup>2</sup> , bacitracin <sup>2</sup> , D-glutamyl polypeptide <sup>2</sup> , mycobactin <sup>2</sup> , subtilin <sup>2</sup> , surfactin <sup>11</sup>	Endospores <sup>2</sup> , developmental entities, bacteriophage <sup>2</sup> , transfectants <sup>2</sup> , transformants <sup>2</sup>	Neomycin <sup>2</sup>	Alternariol <sup>12</sup> , fusarin C <sup>13</sup> , malformin <sup>2</sup> , patulin <sup>14</sup> , rubratoxin <sup>3</sup>	Cleistothecia <sup>15</sup> , oogonia <sup>2</sup>
(C) Zinc				
Actinomycete products		Fungal products	Fungal structures	
Actinomycin D <sup>1</sup> , candidicin <sup>1</sup> , candihexin <sup>1</sup> , chloramphenicol <sup>1</sup> , kanamycin <sup>3</sup> , neomycin <sup>1</sup> , streptomycin <sup>1</sup>		Aflatoxin <sup>3</sup> , alternariol <sup>12</sup> , cynodontin <sup>3</sup> , ergotamine <sup>3</sup> , fusaric acid <sup>2</sup> , furarin C <sup>13</sup> , gentisyl alcohol <sup>3</sup> , griseofulvin <sup>3</sup> , lysergic acid <sup>3</sup> , malformin <sup>3</sup> , naphtharazine <sup>3</sup> , ochratoxin <sup>3</sup> , patulin <sup>3</sup> , penicillin <sup>3</sup> , rubratoxin <sup>3</sup> , versicolorin <sup>16</sup> , zearalenone <sup>3</sup>	Oogonia <sup>3</sup> , sclerotia <sup>3</sup> , vesicles <sup>3</sup>	

<sup>1</sup> Weinberg (1989), <sup>2</sup> Weinberg (1977), <sup>3</sup> Weinberg (1986), <sup>4</sup> Tai and Holmes (1988), <sup>5</sup> Lory (1986), <sup>6</sup> McKellar et al. (1987), <sup>7</sup> Page (1983), <sup>8</sup> Ahmed et al. (1988), <sup>9</sup> Manulis et al. (1984), <sup>10</sup> Ristroph and Ivins (1983), <sup>11</sup> Cooper et al. (1981), <sup>12</sup> Coupland and Niehaus (1987), <sup>13</sup> Jackson et al. (1989), <sup>14</sup> Scott et al. (1986b), <sup>15</sup> Zonneveld (1975), <sup>16</sup> Failla and Niehaus (1986a)

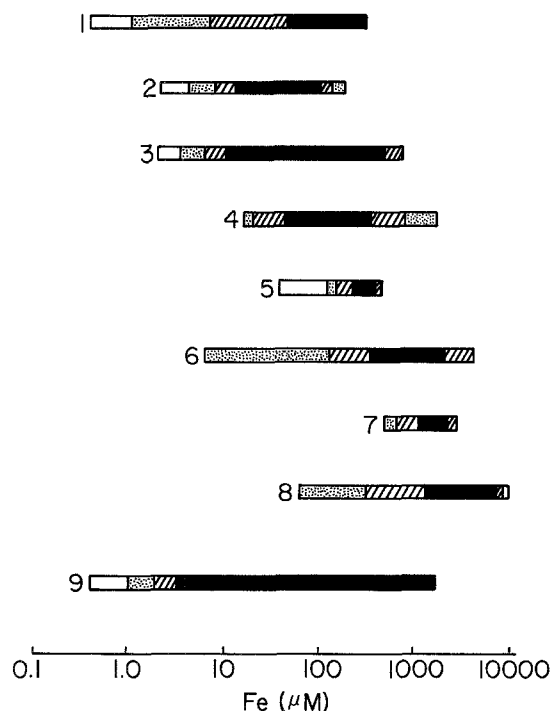


Fig. 1. Comparison of effect of iron on vegetative growth with that on yield of actinomycetal secondary metabolites. Reproduced with permission from Fig. 1 of Weinberg (1989). Shaded area = 75%–100% of maximal yield; cross-hatched area = 50%–75% of maximal yield; stippled area = 25%–50% of maximal yield; blank area = 0%–25% of maximal yield. Key: (1) grisein; (2) neomycin; (3) streptomycin; (4) actinomycin; (5) tobramycin; (6) candicidin; (7) monensin; (8) neopurpuratin; (9) vegetative growth

magnitude. In contrast, the mean of the range that permits >75% of maximal yield of eight typical secondary metabolites is <80% of one order of magnitude (Fig. 1). Similar contrasts in the permissible concentrations of iron required occur with other procaryotes, irrespective of whether the secondary metabolite is a low-molecular-mass molecule or a protein exotoxin (Weinberg 1986).

In some systems, iron is required for the production of protein secondary metabolites or of the synthetases of small molecules. In other systems, iron inhibits production. If the key metal present in a culture is less than the amount that represses or derepresses the secondary process, more of the element will be needed for the regulatory activity. In contrast, if the quantity already present is excessive, reduction of the amount by chelation or precipitation may permit the metal to exert its function. In a few cases, however, a small amount of the metal may inhibit the system whereas a higher amount may stimulate the process. In such cases, the functional protein is assumed to have two metal binding sites: at low concentration, the metal would combine with a high-affinity site. At the higher concentration, the metal would bind to the second, weaker, site thus resulting in alteration of the configuration of the protein.

Generally, a linear relationship is observed between the quantity of metabolite and the amount of iron when the metal concentration is plotted on a logarithmic

scale. This relationship is observed also with manganese for *Bacillus* species and with zinc for fungi and actinomycetes.

In some reports, additional trace metals appear to be active at supra-physiological concentrations. Often such observations simply indicate contamination of one reagent-grade metal salt with a small amount of another micronutrient. For instance, synthesis of a secondary metabolic lipopeptide, surfactin, by *Bacillus subtilis* required 1  $\mu\text{M}$  manganese or 1300  $\mu\text{M}$  iron (Cooper et al. 1981). In a different study, analysis revealed that 1300  $\mu\text{M}$  ferric chloride was contaminated with 3  $\mu\text{M}$  manganese (Scott et al. 1986b).

The synthesis of exotoxin A by *Pseudomonas aeruginosa* was suppressed by either 2  $\mu\text{M}$  iron sulfate, or 1000  $\mu\text{M}$  manganese sulfate, or 1000  $\mu\text{M}$  copper sulfate (Blumentals et al. 1987). No information was provided on the amount of iron contamination of the latter two salts. In another report, the production of diphtheria exotoxin was suppressed by 3  $\mu\text{M}$  iron or by 2350  $\mu\text{M}$  manganese, 120  $\mu\text{M}$  copper, 500  $\mu\text{M}$  cobalt, or 2350  $\mu\text{M}$  nickel (Groman and Judge 1979). The amounts of iron contamination of the four other metal salts were stated by the commercial supplier to be less than that needed for suppression of toxigenesis. Unfortunately, the actual quantities of iron were not verified experimentally.

Cadmium, generally categorized as a non-essential trace metal, apparently can substitute for zinc in the regulation of secondary metabolism in some laboratory situations. For example, in a mutant strain of *Aspergillus parasiticus* grown in a zinc-deficient culture, 1  $\mu\text{M}$  cadmium caused a twofold increase in vegetative growth and a >60-fold increase in synthesis of versicolorin (Failla and Niehaus 1986b). Cadmium has a well-documented affinity for metallothionein; this binding of small amounts of the metal may release sufficient endogenous zinc to overcome the zinc limitation of growth and maturation. Alternatively, cadmium may substitute for zinc as an activator of regulatory proteins.

In procaryotes and mitochondria, superoxide dismutases require iron or manganese, whereas the cytoplasm of eucaryotes contains the enzyme with copper and zinc. Williams (1982) has suggested that perhaps eucaryotes do not use iron or manganese in the cytoplasmic enzyme because the reduced forms of these ions can be mutagenic. The mutagenic potential might present a greater danger to long-lived multicellular organisms than to relatively short-lived procaryotes. Perhaps in those eucaryotic systems in which manganese or iron regulate secondary metabolism, mitochondrial rather than nuclear genes are involved. Other factors that could influence selection of specific metals include the relative levels of their availability as well as the redox potential of the environmental niches in which transport would occur. Cellular uptake of these inorganic nutrients is considered next.

#### Regulation of acquisition of the key metals

The well-defined quantity of the key metal(s) essential

for initiation of secondary metabolism and cell maturation must be present at the temporal phase of the culture in which the cells are competent to acquire and utilize the element. If the metal is present too early in the culture cycle, the cells often refrain from activating their transport system until such time as the element is needed. Otherwise, they would be forced to store the metal in a safe compartment. If the metal becomes available after the time at which it normally would be utilized, the secondary process will be less efficient or may fail to occur.

Accordingly, the narrow quantitative requirement for key metal(s) for the control of maturation should be reflected in a well-regulated control of uptake of the element(s) during the latter portion of the growth phase. For example, cells of *Candida utilis* have a high capacity for uptake of zinc during the initial lag phase and again in late exponential phase (Failla and Weinberg 1977). During the early and mid-exponential phase, the  $V_{\max}$  of the transport system was  $0.22 \text{ nmol Zn}^{2+} \text{ min}^{-1} \text{ mg}^{-1} \text{ dry mass}$ ; in late exponential phase, it was increased 17-fold to  $3.65 \text{ nmol Zn}^{2+} \text{ min}^{-1} \text{ mg}^{-1} \text{ dry mass}$ . Predictably, iron, whose concentration is not critical for control of fungal secondary metabolism, should be accumulated throughout the batch growth cycle at a steady rate; this was, indeed, the case. Likewise, in fungal systems such as *C. utilis* in which manganese does not control the secondary process, cellular uptake and content of this metal remains relatively constant throughout the batch culture cycle (Parkin and Ross 1986).

In *Aspergillus parasiticus*, zinc overload suppressed the formation of the polyketide, versicolorin A (Failla and Niehaus 1986a). In this system, the rate of accumulation of the metal was increased threefold as the culture progressed from 20 h to 60 h post-inoculation. In *Alternaria alternata*, 10  $\mu\text{M}$  zinc was required for alternariol synthesis; inclusion of 10  $\mu\text{M}$  manganese resulted in a threefold increase in yield (Coupland and Niehaus 1987). Manganese was inactive in the absence of zinc. As in *A. parasiticus*, the time of addition of zinc was critical for production of alternariol. For maximal synthesis, the metal was required when the mycelium had attained 15% of its potential biomass. If addition was delayed until full biomass had been reached, zinc failed to stimulate alternariol production.

Biosynthesis of the polyketide patulin by *Penicillium urticae* in a medium that contained 140  $\mu\text{M}$  zinc required 20  $\mu\text{M}$  manganese (Scott and Gaucher 1986). During the 3-h transition from vegetative growth to the stationary phase (22–25 h post-inoculation), cellular manganese acquisition increased by 40–50%. The first pathway enzyme for patulin production was detected at about 20 h and the remaining pathway enzymes appeared coordinately at about 24 h. In contrast, manganese-deficient cultures did not synthesize patulin, although the first enzyme in the biosynthetic pathway continued to produce 6-methylsalicylic acid (Scott et al. 1986b). Vegetative growth was not modulated by alteration in manganese availability.

In the procaryote *Bacillus*, manganese likewise is

the key metal in the shift to secondary metabolism and maturation. Organisms in this genus rapidly accumulate the metal early in exponential growth and then maintain a relatively constant quantity per cell during the remainder of the vegetative period. At 3 h into the sporulation process, manganese accumulation begins again (Silver and Jasper 1977). In other genera of procaryotes, iron would be expected to be assimilated early in the growth cycle and again at the time that vegetative growth is slowing. Such kinetics have been observed in cultures of *Agmenellum quadruplicatum*, a cyanobacterium (Hardie et al. 1983).

### The molecular site of action of the key metals

The exotoxin of *C. diphtheriae* was discovered in 1895, the control of its synthesis by iron in 1935, and the approximate site of action of iron in 1975. In the latter year, Murphy et al. (1976) isolated a  $\beta$ -phage mutant lysogen of *C. diphtheriae* that partially was insensitive to iron suppression of toxigenesis. Presented data were consistent with the authors' model that association of iron with an aporepressor results in the binding of the holoprotein to the operator of the phage *tox* operon, thereby preventing transcription. Moreover, inhibition of toxigenesis was identical after the addition either of 16  $\mu\text{M}$  iron or 1.2  $\mu\text{M}$  rifampin, an inhibitor of RNA polymerase reinitiation (Murphy et al. 1978). Additional evidence was provided by Kaczorek et al. (1985) who showed that the level of toxin mRNA was considerably higher in cells cultured in low-iron medium than in medium with high iron.

Further insight regarding the suppressor influence of iron on transcription of *tox* has been obtained by construction of a *tox-galK* transcriptional fusion (Tai and Holmes 1988). A fragment of the *tox* operon containing the regulatory region was cloned into a *galK* transcription vector such that expression of *galK* activity was controlled by the *tox* promoter; *tox* promoter activity was then monitored in the wild type and in an iron-uptake-defective mutant of *Escherichia coli* under both high- and low-iron growth conditions. Induction of galactokinase was prevented by iron. Measurement of *tox*-specific mRNA showed that this regulation occurred at the level of transcription.

In *E. coli*, the genes encoding the biosynthesis of siderophores and their corresponding outer-membrane-protein receptors are organized as operons whose transcription is negatively regulated via a protein repressor Fur that uses iron as a co-repressor (Bagg and Neilands 1987). The Fur protein was found to be essential for iron regulation of the  $\beta$ *tox* promoter (Tai and Holmes 1988). The nucleotide sequence of the latter shared 10 of the 19 nucleotides with the consensus sequence of several *fur*-regulated genes of *E. coli* (Tai and Holmes 1988). The degree of similarity suggested that the Fur protein and iron regulate the *tox* promoter in *E. coli* by the same mechanism by which they regulate the iron-uptake operons.

Iron-mediated regulation of the synthesis of exo-

toxin A by *Ps. aeruginosa* likewise has been shown to occur at the level of transcription. Lory (1986) observed that the sharp increase in exotoxin A mRNA in late exponential and early stationary phases of the batch culture cycle is blocked by extracellular iron at a half-maximal concentration of 10  $\mu$ M. The characteristic linear relationship between the logarithm of the iron concentration and the quantity of exotoxin A, as well as of exotoxin A mRNA, was demonstrated. The author suggested that iron could (a) bind to and activate a repressor, (b) bind to and inactivate a transcriptional activator, or (c) act at a post-transcriptional level by altering the rate of mRNA turnover.

Subsequently, Chen et al. (1987) reported that the exotoxin A gene inserted into either non-toxigenic strains of *Ps. aeruginosa* or into *E. coli* was prevented from transcribing exotoxin A mRNA by 100  $\mu$ M iron. However, these authors cautioned that the metal might be functioning to enhance degradation of mRNA transcripts. In a different study on exotoxin A synthesis by *Ps. aeruginosa*, 20  $\mu$ M iron inhibited formation of the toxin-specific mRNA (Blumentals et al. 1987).

Transcription of the genes that code for Shiga-like toxin (SLT-1) in *E. coli* increased markedly when excess iron was chelated by 2,2'-dipyridyl (De Grandis et al. 1987) and the SLT-1 operon was shown to be negatively regulated by the *fur* gene product (Calderwood and Mekalanos 1987). The latter authors noted that the negative transcriptional regulation of both siderophore-based iron-uptake systems and SLT production by *fur* is an example of coordinate expression of virulence factors that is triggered by the exceedingly low availability of free iron in mammalian tissues. A 21-base-pair dyad repeat in the promoter of the SLT-1 operon is identical to similar dyads in the promoter regions of three other *fur*-regulated genes and might represent the operator binding site for the iron-containing Fur protein.

That iron participates in transcriptional regulation of acquisition of the metal is not unique to procaryotes. In human erythroleukemic K562 cells, for example, intracellular iron suppresses production of transferrin receptor mRNA (Rao et al. 1986).

Manganese, like iron, probably functions at the level of transcription. In *E. coli*, the metal can substitute for ferrous ion in the Fur protein and can generate an active repressor in vitro (Bagg and Nielands 1987). In the patulin-forming system of *P. urticae*, manganese neither modulated synthetase activities nor influenced translational processes (Scott et al. 1986a). Production of patulin-specific mRNA was prevented when actinomycin D, an inhibitor of RNA synthesis, was added simultaneously with manganese at 26 h. In contrast, actinomycin D was inactive if added at 28 h (2 h after manganese). On the other hand, if cycloheximide, an inhibitor of translation, were added either at 26 or 28 h, the patulin synthetases were absent. In view of the present information on the site of action of iron in maturational processes, it might be rewarding to search for accessory transcriptional factors that require manganese as a cofactor.

Zinc has multiple roles in gene transcription (Wu

and Wu 1987). Recent studies have focused on the eucaryotic and viral transcriptional regulatory proteins that contain DNA-binding residues characterized as finger-loops (Sunderman and Barber 1988). These repeat moieties of about 30 amino acids are stabilized by a zinc atom complexed in tetrahedral coordination to two cysteines and two histidines. Potential metal-binding finger-loops are often found in the nucleic-acid-binding proteins (Berg 1986). Might the regulatory role of zinc in fungal and actinomycetal secondary metabolism and maturation involve stabilization of such finger-loops in specific activators or repressors?

## Perspectives

The remarkable sensitivity of secondary metabolism to small fluctuations in trace mineral conditions raises numerous questions for further research. For instance, why do the key metals differ among actinomycetes, most other bacteria, and *Bacillus*? Although it is tempting to conclude the existence of a unitary role of the key metals that control secondary metabolism and cell maturation, much more work is needed especially with those systems that are regulated by manganese and/or zinc. In addition to participation in regulation of transcription, the key metals might also have important roles in translation and in synthetase activity. Moreover, the mechanism whereby the process of adjustment of the quantity of key metal(s) is linked to the stage of the culture cycle at which the element(s) function remains unknown.

To what extent are secondary metabolism and differentiation of cells of green plants and of animals controlled by trace metals? Inasmuch as secondary processes can now be studied in cell (rather than tissue or organ) cultures, the latter question can be investigated with the precision formerly available only for microorganisms. Many aspects of secondary metabolism and differentiation are shared by cells of plants, animals, and microorganisms (Weinberg 1983). In each of the three organismic groups, the secondary process requires completion of several cycles of cell replication followed by derepression of a programmed set of genes. In each taxonomic group, the quantity and quality of specialized products formed by the maturing cells are under genetic, nutritional, and environmental influences. Thus, it would not be surprising if trace metal(s) were to be identified whose permissible range of concentrations would be much narrower for secondary metabolism than for vegetative growth of plant and animal cells.

*Acknowledgement.* My sincere thanks to Dr. Mark L. Failla for his review of this and previous manuscripts and for numerous provocative discussions on the manifold ramifications of trace element physiology.

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