Interrelationships in Trace-Element Metabolism in Metal Toxicities in *Neurospora crassa*

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The influence of iron on the toxicity of the heavy metals cobalt, nickel and zinc to *Neurospora crassa* has been studied from an enzymic point of view by Healy, Cheng & McElroy (1955). These authors noted that the activity of several iron-dependent enzymes in this organism was reduced in these toxicities and could be partially corrected by iron supplements to growing cultures; however, growth was not restored by the concentrations of iron employed. Previously Abelson & Aldous (1950) had shown that heavy-metal toxicities in several other organisms affected magnesium metabolism and were also directly related to the concentration of magnesium in growth media. Somewhat similar results with respect to toxic metal-magnesium interactions have also been obtained by Lavollay (1959). Such relationships have been studied extensively from the standpoint of growth as well as metabolic patterns in *Aspergillus niger* by Adiga, Sivarama Sastry, Venkatasubramanyam & Sarma (1961). These studies showed, for the first time, that iron also can completely suppress the growth-inhibitory effect of metal toxicities in *A. niger*, thus resembling magnesium in this respect. However, the similarity between the effects of iron and magnesium appears to be manifested only at the level of growth, and to be dependent on the organism studied, as shown by the work of Adiga, Sivarama Sastry, Venkatasubramanyam & Sarma (1962b) with the larvae of the insect *Corcyra cephalonica* St. In view of these results interactions between iron and magnesium on the one hand and of cobalt, nickel and zinc on the other have been studied in *N. crassa*. A preliminary report of part of this study has been presented (Sivarama Sastry, Adiga, Venkatasubramanyam & Sarma, 1960a).

EXPERIMENTAL

Organism. The wild strain of *Neurospora crassa* Em 5297a used in this study was obtained through the courtesy of Dr H. K. Mitchell, California Institute of Technology,

Pasadena, Calif., U.S.A. It was maintained by weekly subcultures on agar slants containing the basal medium described below. A spore suspension (from a 7-day-old culture) of nearly 90% transmission was used for inoculation.

Basal medium and culture technique. *N. crassa* was grown in 50 ml. Pyrex conical flasks containing 10 ml. of culture medium, adjusted to pH 4.8–5.0, for 3 days at $30 \pm 1^\circ$. The mycelia were then harvested, washed thoroughly, dried at $60\text{--}80^\circ$ overnight and weighed.

The basal medium used was the same as that used by Sivarama Sastry, Ramaiah & Sarma (1958b) and contained (g./100 ml.): glucose, 2; KH_2PO_4 , 0.3; NH_4NO_3 , 0.2; ammonium tartrate, 0.1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05; NaCl, 0.01; CaCl_2 , 0.01. Trace elements included were ($\mu\text{g.}/100\text{ ml.}$): zinc, 20; manganese, 20; copper, 8; iron, 2; molybdenum, 2. Biotin was added to give a final concentration of $0.5\text{ }\mu\text{g.}/100\text{ ml.}$

The chemicals used were of analytical grade and glass-distilled water was employed in the making of all solutions. Cobalt was added to the basal medium where required as $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, nickel as $\text{NiSO}_4 \cdot 7\text{H}_2\text{O}$ and zinc as $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$. Iron was supplied as ferric ammonium citrate, and was added aseptically after being separately autoclaved. All metal concentrations have been given in this study as $\mu\text{g.}$ of metal/10 ml. of medium. All experiments have been repeated a minimum of four times.

Procedures for the radioactivity experiments. In experiments where the effects of iron and magnesium on the uptake and accumulation of cobalt and zinc were studied, ^{60}Co (carrier-free) and ^{65}Zn (100 mc/g.), both purchased from The Radiochemical Centre, Amersham, Bucks., were included together with the required amounts of non-radioactive metal salts in the growth media at concentrations indicated in the Tables. After 3 days, the mycelia were washed till the washings were free of radioactivity, dried, weighed as before and finely powdered. Samples of the powdered mycelia (10–20 mg.) were put in suitable Pyrex test tubes of uniform thickness and the radioactivities determined with a DS5-5 well-type scintillation detector attached to a 151A type decade scaler (Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.). Counting voltages used were 1250 v for ^{60}Co and 1200 v for ^{65}Zn . The counting procedure for mycelial samples was checked for any errors consequent on small variations in the thickness of the Pyrex tubes employed, and for possible self-absorption due to sample size. Under the conditions of the present study these errors were negligible. All samples were counted for adequate time-intervals, depending on the count rate, so that the counting error was within $\pm 2\%$.

Nickel estimation. The uptake of nickel in various conditions in nickel toxicity was measured by the estimation of nickel in the dry mycelia, after wet-ashing with nitric acid

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and perchloric acid, by the colorimetric method with dimethylglyoxime, with citrate added to avoid interference due to iron (Sandell, 1950). Where the mycelia were from cultures grown with large amounts of iron, the values were checked for recoveries of nickel and found to be accurate to within $\pm 5\%$. All experiments were repeated a minimum of four times.

RESULTS

The dependence of heavy-metal toxicity on the magnesium concentration in the growth media was investigated in several organisms by Abelson & Aldous (1950), but corresponding results were lacking for *N. crassa*. The medium employed in the present work had a magnesium concentration of $493.7 \mu\text{g./10 ml.}$, which is commonly used in studies with *N. crassa* (Nicholas & Commissiong, 1957). This concentration was thought to be much above the actual minimal requirement for this metal under the present experimental conditions. The simple omission of magnesium from the basal medium, achieved by replacing magnesium sulphate by sodium sulphate to supply an equivalent amount of sulphur, resulted in a total suppression of growth. To such a magnesium-deficient medium graded amounts of magnesium chloride hexahydrate were added to provide $0\text{--}50 \mu\text{g.}$ of magnesium/10 ml. and the growth response was measured (Fig. 1). Mycelial growth nearly equal to that reached on the normal medium ($493.7 \mu\text{g.}$ of magnesium/10 ml.) was obtained with $50 \mu\text{g.}$ of magnesium/10 ml. In subsequent experiments the toxicities of

metals were studied under both these conditions, which are referred to as 'normal magnesium' and 'minimal magnesium' respectively.

To fix the toxic concentrations of cobalt, nickel and zinc and to study variations in these with the magnesium content of the medium, growth of the mould was followed as a function of increasing concentrations of toxic metals on 'minimal magnesium' and 'normal magnesium' media (Figs. 2, 3 and 4).

The concentrations of cobalt giving approximately half-maximal growth are $100 \mu\text{g./10 ml.}$ and $1000 \mu\text{g./10 ml.}$ on 'minimal magnesium' and 'normal magnesium' media respectively; the corresponding values are 40 and $400 \mu\text{g.}$ of nickel/10 ml. and 200 and $2000 \mu\text{g.}$ of zinc/10 ml. Further, the tenfold increase in toxicity with a similar order of decrease in the magnesium concentration in the medium is very significant and indicates a direct correlation as in other organisms.

In experiments where the reversal of inhibition was studied, the following concentrations ($\mu\text{g./10 ml.}$) of metals were used in 'normal magnesium' and 'minimal magnesium' media respectively to obtain severe inhibition of growth: cobalt, 1000 and 150; zinc, 2000 and 250; nickel, 400 and 60. Preliminary experiments at these concentrations, with high concentrations of iron, showed that complete counteraction of the inhibition of growth could be obtained with iron, and, since there was an apparent dependence of growth on the ratio between toxic metals and iron on the 'normal magnesium' medium, several ratios were tested. The results are shown in Tables 1 and 2.

Fig. 1. Magnesium requirement of *Neurospora crassa*. The experimental details are given in the text.

Fig. 2. Influence of the magnesium concentration on the toxicity of cobalt to *Neurospora crassa*. The experimental details are given in the text. $\blacktriangle$, Growth on 'minimal magnesium' medium; $\bullet$, growth on 'normal magnesium' medium.

These results demonstrate that complete reversal of growth can be obtained with iron in *N. crassa* as with *A. niger* (Adiga *et al.* 1961) and *Corcyra cephalonica* St. (Adiga *et al.* 1962*b*). Within the limits of experimental error, these results indicate a competitive antagonism between cobalt and iron and between nickel and iron, and a non-competitive relationship between zinc and iron, as judged by the criterion of defined growth

response to fixed ratios of inhibitor to counter-acting factor (Adiga, Sivarama Sastry & Sarma, 1962*a*).

The influence of iron on metal toxicities has also been investigated under 'minimal magnesium' conditions. From the results of Table 3, wherein the minimal concentrations of iron needed to give maximal reversal of growth inhibition are shown, the antagonistic concentrations are markedly different from those obtaining in 'normal magnesium' media. Further, the amount of iron required is uniformly higher in all cases when the magnesium concentration is minimal.

The possibility that the restoration of growth was associated with a control of the uptake of toxic metal by iron and magnesium was then

Fig. 3. Influence of the magnesium concentration on the toxicity of nickel to *Neurospora crassa*. The experimental details are given in the text. ▲, Growth on 'minimal magnesium' medium; ●, growth on 'normal magnesium' medium.

Fig. 4. Influence of the magnesium concentration on the toxicity of zinc to *Neurospora crassa*. The experimental details are given in the text. ▲, Growth on 'minimal magnesium' medium; ●, growth on 'normal magnesium' medium.

Table 1. *Competitive reversal by iron of cobalt and nickel toxicities to Neurospora crassa in 'normal magnesium' medium*

The experimental details are given in the text. The weight of the control mycelium was 41.2 mg.

Ratio toxic metal:Fe	...	Mycelial dry wt. (mg.)					
		No Fe	2:0.3	2:0.5	2:0.75	2:1.0	2:1.5
Concn. of toxic metal (μg./10 ml.)							
Cobalt							
1000		23.5	32.5	33.5	38.0	40.0	—
1200		12.5	29.6	33.0	41.0	40.0	—
1500		8.4	31.5	33.0	34.5	40.0	—
	Mean		31.2	33.1	37.8	40.0	—
Nickel							
400		24.2	—	36.3	—	37.5	41.8
500		17.7	—	33.4	—	40.1	41.2
700		8.4	—	27.7	—	35.0	40.6
	Mean		—	32.5	—	37.5	41.2

explored under all the conditions of toxicity studied (Tables 4, 5 and 6). In these studies, the concentrations of iron were those found previously to be optimum for growth. At the lower concentrations of toxic metals used in 'minimal magnesium' conditions, the uptake of toxic metal was also studied by including 500 $\mu\text{g.}$ of magnesium ('normal magnesium') as well, for purposes of comparison as well as to note the effect of magnesium. In toxicity due to higher concentrations of metals in 'normal magnesium' medium, the

influence of magnesium was examined by further supplementing with 2000 $\mu\text{g.}$ of magnesium/10 ml., a concentration needed for maximal reversal of the inhibition of growth. The suppression of the uptake of metal by magnesium is clearly evident. With iron, however, in none of the three metal toxicities in either 'minimal magnesium' or 'normal magnesium' media is there any decrease in the amount of metal accumulated. This points to distinctly different modes of action of iron and magnesium.

Table 2. *Reversal by iron of zinc toxicity in Neurospora crassa on 'normal magnesium' medium*

The experimental details are given in the text.

Concn. of metals ($\mu\text{g./10 ml.}$)	Mycelial dry wt. (mg.)
Control (none)	39.0
Zn (2000)	23.1
Zn (2000) + Fe (1000)	35.5
Zn (2000) + Fe (2000)	38.5
Zn (2500)	14.2
Zn (2500) + Fe (1250)	31.0
Zn (2500) + Fe (2500)	35.0
Zn (3000)	8.5
Zn (3000) + Fe (1500)	17.0
Zn (3000) + Fe (3000)	23.0

Table 3. *Reversal by iron of metal toxicities in Neurospora crassa on 'minimal magnesium' medium*

The experimental details are given in the text.

Concn. of metals ($\mu\text{g./10 ml.}$)	Mycelial dry wt. (mg.)
Control (none)	41.0
Co (150)	17.8
Co (150) + Fe (200)	40.0
Ni (60)	10.4
Ni (60) + Fe (100)	39.3
Zn (250)	23.8
Zn (250) + Fe (1000)	41.4

DISCUSSION

The complexity of metal interaction under conditions of metal toxicities is illustrated by the present results. The only other investigation of this nature with *N. crassa* so far is that of Healy *et al.* (1955), but the present experiments differ from this previous work in several respects, e.g. the ratio of surface to volume of medium used, the extent of aeration and the duration of the experimental period (4 days in the previous work). Consequently the pattern of results is also somewhat different, the order of increasing toxicity of metals being zinc, cobalt, nickel (zinc, nickel, cobalt in the previous study). Also, with the higher iron concentrations tested in the present work, complete reversal of metal toxicities by iron has been demonstrated.

Figs. 2, 3 and 4 show that in *N. crassa*, as in other organisms (Abelson & Aldous, 1950), the three metals tested become more toxic as the magnesium supply is reduced. Also there is a linear decrease of growth with a progressive increase of zinc and nickel, whereas the parabolic curve found in cobalt toxicity (Fig. 2) is probably indicative of major toxic effects of cobalt even at the concentration inducing half-maximal growth. Possibly the binding of cobalt by this mould, unlike that of other metals, is not linearly dependent on the concentration of cobalt in the medium throughout the concentration range. In *A. niger*

Table 4. *Influence of iron and magnesium on the uptake of ^{60}Co by Neurospora crassa in cobalt toxicity*

The experimental details are given in the text. The ^{60}Co added gave 33.6×10^4 counts/min./10 ml.

Concn. of metals ($\mu\text{g./10 ml.}$)	Mycelial dry wt. (mg.)	$10^{-4} \times ^{60}\text{Co}$ uptake (counts/min./ 100 mg. dry wt.)	Co uptake ($\mu\text{g./100 mg.}$ dry wt.)
A. 'Normal magnesium' medium			
Control (none)	29.0	26.2	—
Co (1000)	13.0	1.6	47.6
Co (1000) + Fe (500)	33.4	1.7	50.6
Co (1000) + Mg (2000)	22.0	0.6	17.9
B. 'Minimal magnesium' medium			
Control (none)	28.0	17.0	—
Co (150)	15.0	8.2	36.6
Co (150) + Fe (200)	33.5	8.6	38.4
Co (150) + Mg (500)	28.9	1.7	7.6

Table 5. *Influence of iron and magnesium on the uptake of ^{65}Zn by Neurospora crassa in zinc toxicity*

The experimental details are given in the text. The ^{65}Zn added gave 65.6×10^3 counts/min./10 ml.

Concn. of metals ($\mu\text{g./10 ml.}$)	Mycelial dry wt. (mg.)	$10^{-3} \times ^{65}\text{Zn}$ uptake (counts/min./ 100 mg. dry wt.)	Zn uptake ($\mu\text{g./100 mg.}$ dry wt.)
A. 'Normal magnesium' medium			
Control (none)	32.0	62.3	—
Zn (2000)	15.0	4.9	149.4
Zn (2000) + Fe (1000)	40.0	7.4	225.6
Zn (2000) + Mg (2000)	32.0	3.8	115.8
B. 'Minimal magnesium' medium			
Control (none)	28.4	51.1	—
Zn (250)	13.5	37.5	142.9
Zn (250) + Fe (1000)	31.0	40.6	154.7
Zn (250) + Mg (500)	25.0	12.8	46.8

Table 6. *Influence of iron and magnesium on the uptake of nickel by Neurospora crassa in nickel toxicity*

The experimental details are given in the text.

Concn. of metals ($\mu\text{g./10 ml.}$)	Mycelial dry wt. (mg.)	Ni uptake ($\mu\text{g./100 mg.}$ dry wt.)
A. 'Normal magnesium' medium		
Control (none)	42.5	—
Ni (400)	25.4	10.3
Ni (400) + Fe (300)	45.9	10.0
Ni (400) + Mg (2000)	39.1	5.8
B. 'Minimal magnesium' medium		
Control (none)	39.0	—
Ni (60)	24.4	9.4
Ni (60) + Fe (100)	38.1	8.6
Ni (60) + Mg (500)	35.3	3.7

also cobalt toxicity does not resemble zinc and nickel toxicities in several respects (Adiga *et al.* 1961).

The results on the counteracting ability of iron (Tables 1, 2 and 3) are in line with the findings with *A. niger* (Adiga *et al.* 1961) and *Corcyra* larvae (Adiga *et al.* 1962b), and suggest that such interactions known hitherto mainly in plants (Hewitt, 1951) are of more general occurrence. Further, the competitive relationships found (Table 1) are restricted to 'normal magnesium' conditions; higher concentrations of iron are required when the magnesium concentration is smaller, namely 'minimal magnesium' (Table 3), suggesting that the effectiveness of iron is related to the nutritional status of the mould with respect to magnesium.

A comparison of the amount of metal accumulated in mycelia on 'normal magnesium' and 'minimal magnesium' (Tables 5 and 6) indicates that there is a correlation between the order of toxicity of metals and the intracellular concentrations that the mould can tolerate: e.g. nickel is the most toxic, and the nickel values are quantitatively the smallest found. Again, since the mycelial concentrations of the toxic metals, in all cases of inhibited growth, are nearly identical both in

'normal magnesium' and 'minimal magnesium' conditions, these values apparently determine the final mycelial weight attained in absence of a counteracting metal. Also the greater fraction of the extracellular metal is taken up from the 'minimal magnesium' medium. A similar dependence of iron uptake on the magnesium concentration would explain the observed differences in the iron requirement for growth reversal under the two conditions of magnesium supply.

Further, from the results of Tables 4, 5 and 6, which show that only with the reversal by magnesium of metal toxicities is a suppression of the uptake of toxic metal encountered, the difference in the modes of action of iron and magnesium is apparent. These results imply that, when adequate concentrations of iron are present, *N. crassa* can tolerate a comparatively higher total intracellular concentration of the toxic metals and still grow normally. It is likely that iron primarily effects a redistribution of these metals from sites where they are toxic to others where they are comparatively non-toxic. Even when magnesium reverses growth inhibition there are still appreciable amounts of the toxic metals present in the mycelia. Hence a partial redistribution of metals is probably adequate to permit normal rates of many vital metabolic reactions; the amount of any toxic metal retained in the presence of magnesium would then be assumed to represent the non-toxic fraction from the standpoint of overall growth.

Comparison of the present results with those for *A. niger* (Adiga *et al.* 1961) and *Corcyra* larvae (Adiga *et al.* 1962b) emphasizes the complexity and species variations encountered in metal-toxicity phenomena. The counteracting ability of iron would seem to be more general than that of magnesium. Further, these metals act quite differently in metabolism. Thus, in *A. niger*, magnesium reverses inhibitions in growth as well as in several metabolic activities; iron affects only the growth picture. One peculiar feature of metal toxicities both in higher organisms, e.g. the rat

(Smith & Larson, 1946; van Reen & Pearson, 1953), and in lower ones, e.g. *Corcyra* larvae (Sivarama Sastry, Radhakrishna Murthy & Sarma, 1958*a*; Sivarama Sastry & Sarma, 1958), is that growth inhibition can be reversed independently of other parameters. This also would seem to be of general occurrence.

Though metal antagonisms can ultimately be explained on the basis of a control of one metal uptake by another, the present results emphasize that this control could be either extracellular or intracellular. Such control processes also differ with the nature of the cells in question. For instance, in contrast with the results with *N. crassa*, iron does suppress total uptake of ^{65}Zn in erythrocytes (Sivarama Sastry, Viswanathan, Ramaiah & Sarma, 1960*b*). Thus a metal may control the uptake of another in one type of cell but not in another.

SUMMARY

1. The toxicity of cobalt, nickel and zinc to the growth of *Neurospora crassa* was studied as a function of magnesium supply. The extent of toxicity of all the metals was considerably enhanced by decreasing the normally high magnesium concentration in the medium to a minimal value for optimum growth.

2. Reversal of metal toxicities was possible with iron as well as magnesium. The antagonism between iron and the toxic metal was competitive, at high magnesium concentrations, for cobalt and nickel toxicities only. With a minimal magnesium supply a much higher ratio of iron to toxic metal was necessary in all cases to achieve normal growth rates.

3. The correlation between mycelial accumulation of the toxic metal and the growth of the mould was investigated under various conditions. In the

absence of a counteracting metal there was a direct relationship between these two parameters.

4. The reversal of metal toxicities by magnesium is due to a suppression of the uptake of toxic metal. Further, such a control of metal uptake does not occur when iron is the counteracting metal.

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Particulate Ribonucleoprotein Components of *Hevea brasiliensis* Latex

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During the isolation of a soluble nucleoprotein component of natural-rubber latex from freeze-dried latex 'serum' it was noted that a very small amount of additional polynucleotide material was present in the sediment obtained from this recon-

stituted serum by centrifuging at high *g* (McMullen, 1959).

The present paper shows that most of this particulate nucleic acid is located in the 'bottom fraction' freeze-dried material as obtained from