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Interrelationships in Trace-Element Metabolism in *Aspergillus niger*

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The existence of an antagonism between heavy metals, e.g. cobalt, nickel, zinc, cadmium and copper, and essential ones, e.g. iron and magnesium, has been known for some time. The well-documented iron-deficiency 'chlorosis' in plants can be induced by high, extra-physiological concentrations of these heavy metals (Hewitt, 1951). Similar phenomena have not been studied as extensively in micro-organisms. Abelson & Aldous (1950) found that excess of cobalt, nickel, zinc, cadmium and manganese all interfered with magnesium metabolism in *Torulopsis utilis*, *Aerobacter aerogenes*, *Escherichia coli* and *Aspergillus niger*. The toxic effect on growth, with some of these organisms, could be counteracted by supplementing the culture media with magnesium. Healy, Cheng & McElroy (1955), working with *Neurospora crassa*, obtained evidence of deranged iron metabolism as

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well in metal toxicities, particularly that caused by cobalt; the decrease in the activity of iron-dependent enzymes, though not growth inhibition, could be corrected by small supplements of iron.

Recently, Sivarama Sastry, Adiga, Venkatasubramanyam & Sarma (1960*a*) have shown that, in *N. crassa*, the growth inhibition caused by cobalt and nickel toxicities can be competitively overcome by an adequate concentration of iron in the culture medium; in zinc toxicosis, iron reversed growth inhibition non-competitively. Although magnesium was also a powerful antagonist of these toxic metals, its mechanism of action was quite different from that of iron. In the light of these findings and the possible effect of metal toxicities on carbohydrate metabolism suggested by the work of Healy *et al.* (1955), interrelationships between cobalt, nickel and zinc toxicities and iron and magnesium have been investigated in the present work with the mould *A. niger*.

EXPERIMENTAL

Materials. All metal salts employed in these studies, $ZnSO_4,7H_2O$ (Merck), $CoCl_2,6H_2O$ (British Drug Houses Ltd.), $NiSO_4,6H_2O$ (May and Baker Ltd.), $MgSO_4,7H_2O$ (Merck) and ferric ammonium citrate (Fisher) were of analytical grade. The concentrations are expressed as μg. of metal/flask containing 10 ml. of medium.

Experimental organism. An acid-forming strain of *A. niger*, obtained from the National Collection of Type Cultures, Poona, India, was used. It was maintained by weekly subcultures on agar slants containing the basal medium described below with 0·1% each of malt and yeast extracts. A spore suspension in sterile, glass-distilled water, obtained from a 10-day-old culture and adjusted to 90% transmission in a photoelectric colorimeter, was used for inoculation.

Basal medium and culture conditions. A. niger was grown in 100 ml. Pyrex conical flasks, containing 10 ml. of culture medium, adjusted to pH 2·5–3·0, for 5 days at 30±1°.

The basal medium contained (g./100 ml.): glucose, 15; KH_2PO_4, 0·25; NH_4NO_3, 0·25; $MnSO_4,4H_2O$, 0·01; $MgSO_4,7H_2O$, 0·025; $ZnSO_4,7H_2O$, 0·00065.

It was essential to use solutions made up in glass-distilled water, and to clean all glassware with chromic acid followed by thorough washing with glass-distilled water, in order to obtain consistent and reproducible results.

All experiments were repeated at least four times, and the average values have been given in the results presented below.

Assay procedures. The mycelia were harvested after 5 days' growth, washed extensively with distilled water to remove salts and spores, dried at 60–80° overnight and weighed.

Total acid produced was estimated by titrating portions (2 ml.) of culture medium, at the end of the experiment, against 0·05 N-NaOH with bromothymol blue as indicator (Sivarama Sastry & Sarma, 1957). Citric acid was analysed in suitable portions of deproteinized culture fluid by the method of Saffran & Denstedt (1948)

Glucose utilization was determined by following its disappearance from the medium, by the method of Morris (1948) as modified by Trevelyan & Harrison (1952) with anthrone. Recovery experiments showed that interference from medium constituents other than glucose was negligible at the dilutions employed.

In all the figures presented, growth, acid production and glucose-utilization values have been plotted as percentage of control values (i.e. values reached in each case with unsupplemented basal medium given above) against metal concentration (μg. of metal/flask).

RESULTS

In Figs. 1–3 are presented the effect of increasing toxic concentrations of Zn, Co and Ni respectively on growth, acid production and glucose utilization by *A. niger*. These results show that Ni is the most toxic, followed by Zn and Co, in that order. Nickel (400 μg.), Zn (1000 μg.) or Co (1200 μg.) give a growth inhibition of about 60%. Cobalt and Ni decrease growth throughout the concentration range, whereas, with up to 600 μg. of Zn, the decrease is somewhat gradual and thereafter becomes much more pronounced. Since, of the three metals, Zn is the only one that is normally needed by *A. niger* for growth as well as acid production (Steinberg, 1919; Shu & Johnson, 1948; Tomlinson, Campbell & Trussel, 1950; Nicholas, 1952), a greater tolerance of *A. niger* to excess of Zn, as shown by Fig. 1, is not unexpected.

Acid production, calculated on the basis of acid accumulated per 100 mg. of mycelial weight produced, exhibits a rapid and continuous fall with increase of Co and Ni. Zinc in amounts up to 250 μg. does not have an adverse influence in this

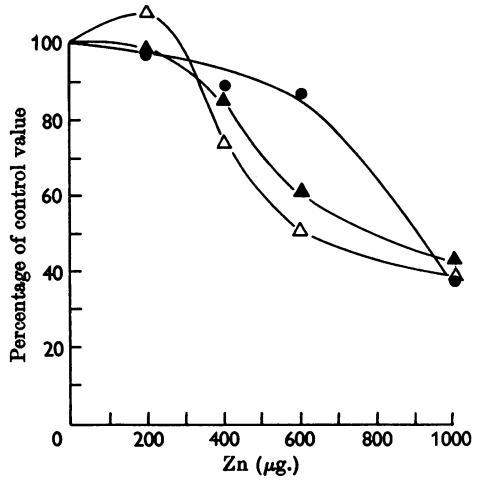

Fig. 1. Effect of zinc toxicity on *Aspergillus niger*. For experimental details see text. ●, Growth; ▲, glucose utilized; △, acid produced.

respect; on the other hand, a consistent, though small, increase in acid has always been obtained with 200 μg. of Zn. Further, estimations of citric acid have shown that the citric acid values run in parallel with total acid. When the relative effects of Co, Ni and Zn are compared, however, it becomes evident that Ni is most inhibitory towards acid production, since with 100 μg. of Ni (Fig. 3) the inhibition in acid formation is about 62 % even when the corresponding decrease in growth is only 12 %. Neither Co nor Zn has such a pronounced influence under comparable conditions (Figs. 1, 2).

Glucose utilization, on the basis of mg. of glucose used/100 mg. of mycelial weight produced, has also been plotted as a percentage of the control values in Figs. 1–3. These Figures reveal that the extent of utilization of the carbon source is also impaired concomitantly with growth in all metal toxicities. At concentrations of the metals producing over 50 % decrease in growth, the glucose consumption does not exceed the decrease in growth. These values, which are about 50 % of control, i.e. 250–300 mg. of glucose/100 mg. of dry mycelium under the present experimental conditions, may represent the minimal rates of utilization of glucose for synthesis of cell material.

The effect of supplementation of the culture medium with Mg at various concentrations was studied in detail as regards its ability to counteract inhibition in growth, acid production and glucose utilization. In all subsequent experiments, *A. niger* was grown with 400 μg. of Ni, 1000 μg. of Zn or 1200 μg. of Co, which produced severe toxicity. These concentrations have been chosen, since, under the experimental conditions employed, they produce comparable extents of inhibition of growth. The effect of Mg supplementation was studied in the range 0–2000 μg. All values given in Figs. 4–6 have been depicted on the basis of response to supplemented Mg. The values corresponding to 'zero Mg' in these Figures are obtained in the toxic state on the basal medium alone, with the normal Mg content (246·8 μg. of Mg/10 ml.).

Magnesium is most effective with Zn, 200 μg. of additional Mg restoring growth to 94 % of the control; thereafter the growth curves reach a plateau (Fig. 4). In Ni and Co toxicities, the effect of Mg is similar except that much higher amounts (600 μg. for Ni and 2000 μg. for Co) are needed for comparable reversal (Fig. 4). If the ratio of total

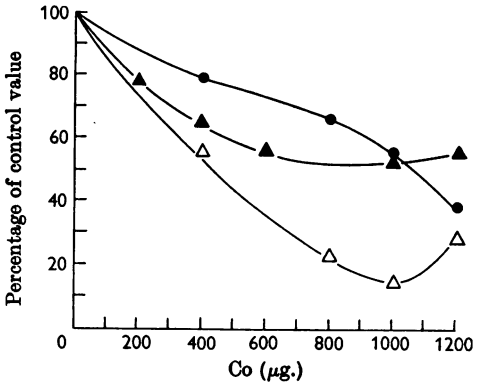

Fig. 2. Effect of cobalt toxicity on *Aspergillus niger*. For experimental details see text. ●, Growth; ▲, glucose utilized; △, acid produced.

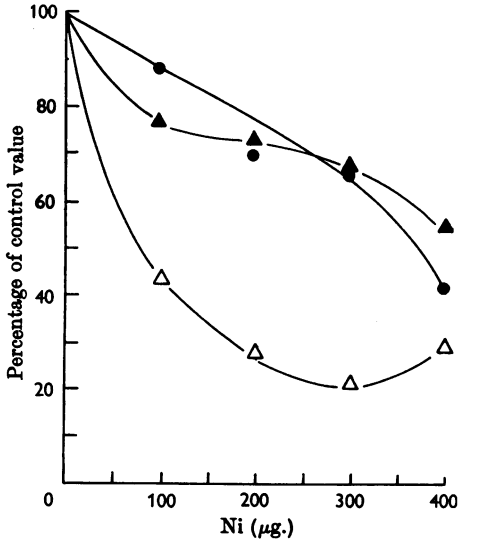

Fig. 3. Effect of nickel toxicity on *Aspergillus niger*. For experimental details see text. ●, Growth; ▲, glucose utilized; △, acid produced.

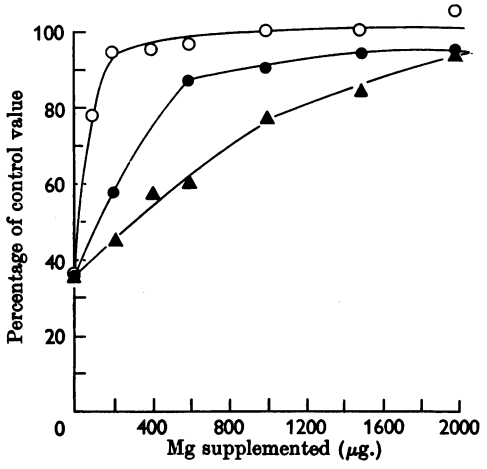

Fig. 4. Influence of magnesium on growth of *Aspergillus niger* in metal toxicities. For experimental details see text. ○, Zinc toxicity; ●, nickel toxicity; ▲, cobalt toxicity.

Mg in the medium to metal required to give an ultimate growth of 90% of control is calculated for the three different toxicities, the values are 0·45 for Zn, 1·7 for Co and 2·1 for Ni.

The influence of Mg on acid production in the metal toxicities is shown in Fig. 5. Restoration of acid formation to normal occurs only in Zn and Ni toxicities; the small adverse effect of Mg in Co toxicity, even at a concentration of 2000 μg., where growth is normal, is not significant.

The data presented in Fig. 6 showing the influence of Mg on glucose utilization in metal toxicities, and those of Figs. 4 and 5, again indicate that complete counteraction by Mg occurs only with the metabolic derangements caused by Zn and Ni and that Mg is only partially effective in Co toxicity. Even with Zn and Ni, much higher concentrations of Mg than those required to restore growth to normal are needed to bring acid and glucose values to control levels, indicating that the effect on the latter two parameters is not necessarily only associated with increase in growth.

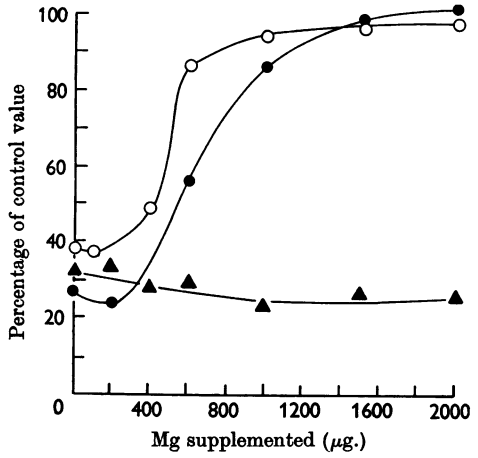

Fig. 5. Influence of magnesium on acid production by *Aspergillus niger* in metal toxicities. For experimental details see text. ○, Zinc toxicity; ●, nickel toxicity; ▲, cobalt toxicity.

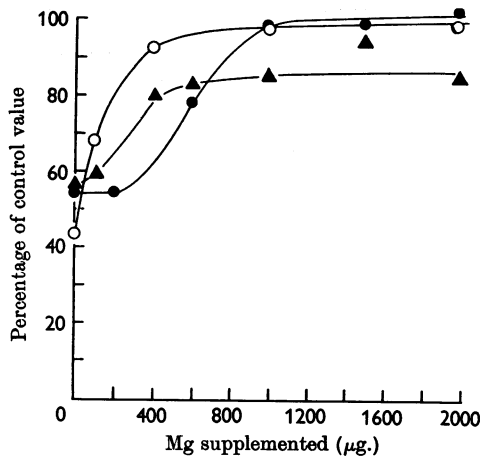

Fig. 6. Influence of magnesium on glucose utilization by *Aspergillus niger* in metal toxicities. For experimental details see text. ○, Zinc toxicity; ●, nickel toxicity; ▲, cobalt toxicity.

In view of our earlier findings that Fe can also suppress metal toxicities in *N. crassa* (Sivarama Sastry *et al.* 1960*a*), competitively in Co and Ni toxicities and non-competitively in Zn toxicity, this aspect was investigated in *A. niger*. Iron, like Mg, counteracts completely the growth inhibition by Zn, Co and Ni (Fig. 7). However, if the growth curves are compared with the corresponding ones with Mg (Fig. 4), the quantitative difference between Fe and Mg becomes obvious. Whereas the concentrations of Mg required to reverse growth retardation differ markedly with the metal toxicity concerned, in striking contrast, a uniformly high concentration of Fe in the range 1600–2000 μg. is always needed.

Fig. 8 shows the influence of Fe on glucose utilization by *A. niger* in metal toxicities. A significant improvement due to Fe takes place only in Co toxicity. Moreover, in no instance does Fe alter the amount of acid accumulated.

The effect of Mg and Fe on citric acid formation by *A. niger* in metal toxicities has not been investigated throughout the concentration range employed here. However, a few experiments were

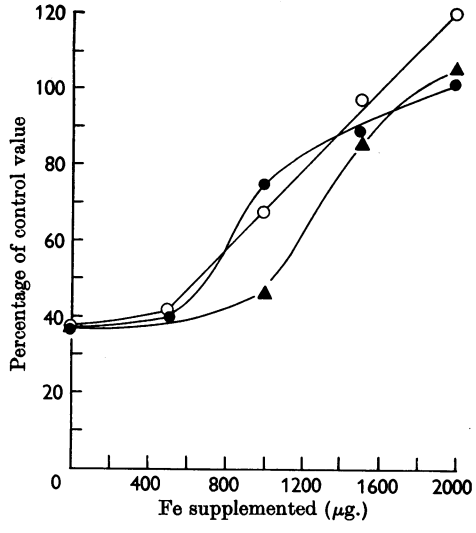

Fig. 7. Influence of iron on growth of *Aspergillus niger* in metal toxicities. For experimental details see text. ○, Zinc toxicity; ●, nickel toxicity; ▲, cobalt toxicity.

carried out to examine whether both total acid and citric acid values were restored to normal under conditions of reversal of toxicities. Results of one such typical experiment are presented in Table 1.

The absolute values for mycelial weights, total acid production and glucose utilization are also recorded. These data serve to indicate that Mg does, in fact, restore citric acid values concomitantly with those for total acid.

Lastly, a fundamental difference between Fe and Mg reversal of metal toxicities deserves mention. In all cases, only in Mg-supplemented cultures, normal sporulation occurred when growth was enhanced, the extent of sporulation running almost parallel with the improvement in growth. However, even when Fe restored growth completely to normal, there was little, if any, spore formation.

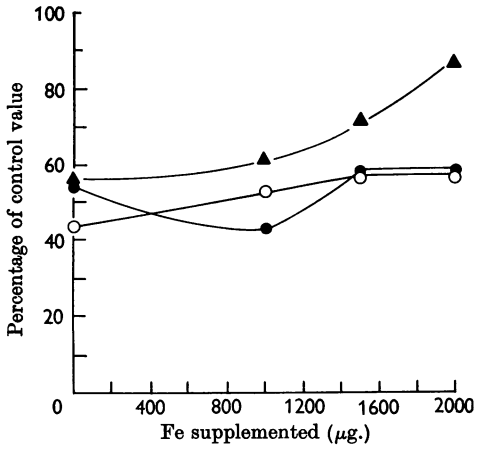

Fig. 8. Influence of iron on glucose utilization by *Aspergillus niger* in metal toxicities. For experimental details see text. ○, Zinc toxicity; ●, nickel toxicity; ▲, cobalt toxicity.

DISCUSSION

The principal features of the present investigation are the marked differences not only in the quantitative aspects of cobalt, nickel and zinc toxicities in *A. niger*, but also the variations in the patterns of counteraction of these metal toxicities by iron and magnesium. Iron and magnesium show a similarity only in reversal of growth inhibition. Whereas iron restores growth values to normal, it has no effect on inhibition of acid production and is only partially beneficial in glucose utilization by *A. niger* in cobalt toxicity, being practically ineffective in this respect in the other toxicities studied. The fact that iron by itself depresses acid production by *A. niger* has been recognized by earlier workers (Tomlinson *et al.* 1950), and hence the absence of an increase in acid yields concomitantly with enhancement of growth by iron is not unexpected.

Since therefore both iron and magnesium can counteract growth inhibition, without necessarily influencing the rate of carbohydrate utilization, a reversal of metal toxicities with respect to growth, even when complete, need not inevitably involve a concomitant reversal of other metabolic derangements. This is strikingly illustrated by the response in acid-production values in metal toxicities to magnesium (Fig. 5). In cobalt toxicity, in contrast with nickel and zinc toxicities, wherein magnesium restores both growth and acid production to normal values, magnesium is completely without effect on the latter process.

In *N. crassa*, Healy *et al.* (1955) have observed that iron, at concentrations at which it has little influence on the retardation of growth due to cobalt toxicity, partially restores the activity of some of the iron-dependent enzymes. Much

Table 1. *Effects of iron and magnesium on metal toxicities in* Aspergillus niger

Acid produced was expressed as ml. of 0·05 N-NaOH required/2 ml. of culture medium. For all experimental details see text. Supplements when added were (mg.): Zn, 1·0; Co, 1·2; Ni, 0·4; Mg, 2; Fe, 2.

Supplements to 10 ml. of basal medium	Mycelial dry wt.		Acid produced		Citric acid formed		Glucose consumed	
	mg.	Percentage of control	Total	Percentage of control	mg./100 mg. of mycelium	Percentage of control	mg./100 mg. of mycelium	Percentage of control
None (control)	195	100·0	22·1	100·0	100·0	100·0	468·0	100·0
Zn	79	40·5	2·0	9·0	2·8	2·8	252·0	54·0
Zn + Mg	197	101·0	22·2	102·0	180·0	180·0	459·0	98·0
Zn + Fe	231	118·5	1·4	6·3	2·8	2·8	271·0	58·0
Co	72	37·0	1·9	8·6	3·2	3·2	264·0	56·0
Co + Mg	196	100·0	3·9	17·7	18·0	18·0	355·0	76·0
Co + Fe	198	102·0	3·6	16·3	2·7	2·7	419·0	89·0
Ni	72	37·0	2·1	9·5	4·4	4·4	284·3	61·0
Ni + Mg	190	98·0	18·6	84·5	154·0	154·0	494·0	105·5
Ni + Fe	224	115·0	2·7	12·2	10·3	10·3	299·5	64·0

higher concentrations of iron are necessary to correct the growth inhibition in *N. crassa* (Sivarama Sastry *et al.* 1960*a*). The effects of iron and magnesium in *A. niger* suggest that similar marked differences prevail as regards reversal of the metabolic derangements. In conditions when growth is restored to normal without concomitant reversal of enzymic or metabolic changes, the organism may possibly achieve normal rates of synthesis or degradation of vital metabolites with the limited amounts of the enzymes available.

The nature of the effects of magnesium and iron in suppression of metal toxicities in *A. niger* appears to be different. If, as suggested by Abelson & Aldous (1950), the primary influence of magnesium is a control of toxic-metal uptake, which presumably results in the depression of the intracellular concentrations of the toxic ions to concentrations at which they become non-toxic, it would be natural to expect all adverse influences of metal toxicities to be simultaneously annulled. Since this does not happen in cobalt toxicity (Figs. 4, 5), the control of ion uptake alone is not perhaps the only mechanism involved in the reversal of metal toxicities by magnesium.

The quantitative aspects of magnesium reversal (Fig. 4) show that, at a fairly low ratio of magnesium: zinc (0·45:1), the growth of the mould is normal. The corresponding ratios are considerably higher for cobalt and nickel. Further, in cobalt toxicity alone (Fig. 5) magnesium has no influence on acid production. From these results it is probable that zinc toxicity is largely equivalent to a conditioned magnesium deficiency, whereas the picture in cobalt toxicity indicates an iron deficiency. Such a conclusion is further justified by the fact that iron enhances the rate of glucose utilization only in cobalt toxicity (Fig. 8).

The effects of iron on nickel and zinc toxicities (Figs. 7, 8) are probably attributable to a suppression of toxic-metal accumulation at intracellular sites controlling growth, rather than to antagonism at a metabolic level. In equine erythrocytes, for example, Fe^{3+} ions have been shown to be a powerful inhibitor of ^{65}Zn uptake (Sivarama Sastry, Viswanathan, Ramaiah & Sarma, 1960*b*).

In any event, the multiplicity of metabolic derangements observed and the fact that iron and magnesium show marked differences both qualitatively and quantitatively suggests that a large number of cellular functions are impaired and that intracellular concentrations affecting each process are probably quite different. The presence of full sporulation in cultures whose growth is restored to normal by supplemented magnesium and the absence of such an effect with iron are also in line with such a possibility. The observed responses to iron and magnesium at the various concentrations studied may indicate controlling effects at different intracellular loci.

SUMMARY

1. Cobalt, nickel and zinc toxicities have been studied in detail in the mould *Aspergillus niger* from the point of view of their adverse influence on growth, acid formation and overall glucose utilization. At toxic concentrations, all these parameters were impaired to different degrees at various metal concentrations.

2. The counteracting effect of iron and magnesium when supplemented with the toxic metals has been examined. Either of these metals could overcome growth inhibition completely, iron at concentrations uniformly in the range 160–200 μg./ml. of culture medium, and magnesium at concentrations varying markedly with the metal toxicity involved. Only magnesium supplementation permitted normal sporulation concomitantly with growth.

3. Magnesium restored growth, acid formation and glucose utilization to normal levels in all cases except in cobalt toxicity, where it had no influence on acid formation and promoted glucose consumption only to the extent of about 87 % of the control. Iron reversed the impaired glucose utilization partially in cobalt toxicity alone. In all toxicities it was ineffective with regard to acid production.

4. The results have been discussed, and it has been suggested that zinc toxicity corresponded to a conditioned magnesium deficiency and that due to cobalt to an iron deficiency.

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