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Interrelationships in Trace-Element Metabolism in Metal Toxicities in a Cobalt-Resistant Strain of *Neurospora crassa*

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A strain of *Neurospora crassa* was isolated by training the mould to grow on media containing high concentrations of Co^{2+}. This strain, the Co^R strain, exhibited approximately tenfold the resistance of the parent strain to Co^{2+} and Ni^{2+} but not to Zn^{2+} or Cu^{2+}. Co^{2+} toxicity in the Co^R strain was reversed by Mg^{2+} but not by Fe^{3+}. Also, Co^{2+} did not affect iron metabolism in this strain. It is suggested that the mechanism of resistance in the Co^R strain involves an alteration in the pattern of iron metabolism such that the latter is no longer adversely affected by toxic concentrations of Co^{2+}. The Co^R strain is genetically stable and is most probably a result of a resistance mutation in *N. crassa* induced by Co^{2+}.

In *Neurospora crassa*, Co^{2+} toxicity results in a derangement of iron metabolism that is reflected in a decrease in the activities of iron-dependent enzymes such as catalase (Healy *et al.*, 1955), a growth inhibition that can be reversed by iron (Sivarama Sastry *et al.*, 1962*a*) and the formation of an iron-binding compound (a siderochrome) owing to iron deficiency induced by excess of Co^{2+} (Padmanabhan & Sarma, 1964, 1966). This antagonism between Co^{2+} and iron is predominantly at an intracellular level. About 60% of Co^{2+} taken up by *N. crassa* mycelia is present intracellularly (Venkateswerlu & Sivarama Sastry, 1970). Excess of Fe^{3+} does not, however, control overall Co^{2+} uptake (Sivarama Sastry *et al.*, 1962*a*), and the converse is also true (Padmanabhan & Sarma, 1966). One possible way by which Fe^{3+} reverses Co^{2+} toxicity is by bringing about a translocation of intracellular Co^{2+} from sites where Co^{2+} is toxic to sites where it is non-toxic (Sivarama Sastry *et al.*, 1962*a*).

The present paper reports the isolation of, and some features of trace-element metabolism in, a strain of *N. crassa* which has ten times the resistance of the parent wild strain to Co^{2+} and Ni^{2+} but not to Zn^{2+} or Cu^{2+}. Studies with this strain suggest that its resistance to Co^{2+} is a consequence of a release of the control exerted by Co^{2+} on iron metabolism. The results obtained are discussed from the standpoint of interrelationships in metal-ion metabolism in *N. crassa*.

Experimental

Chemicals

Metal salts used were $NiSO_4,6H_2O$, $CuSO_4,5H_2O$, $ZnSO_4,7H_2O$, $FeCl_3,6H_2O$, and ferric ammonium citrate, $MgSO_4,7H_2O$, $CoSO_4,7H_2O$ (containing 0.01% Ni^{2+} and 0.005% Zn^{2+}) and sodium β-glycerophosphate [$Na_2C_3H_3(OH)_2PO_4,5\frac{1}{2}H_2O$]. Fe^{3+}–EDTA was prepared by mixing ferric ammonium sulphate [$FeNH_4(SO_4)_2,12H_2O$] and Na_2EDTA in 1:1 molar ratio and adjusting the mixture to pH 4.8–5.0. All chemicals were products of British Drug Houses [BDH Laboratories Chemicals Division, Glaxo Laboratories (India) Ltd., Bombay-1, India] and were of analytical grade. Yeast extract (Bacteriological) was purchased from A. Costantino and C. Favria, Italy. Solutions used for media were made up in glass-distilled water.

Metal concentrations are expressed in terms of the metal contributed by the respective salt employed.

Organisms, media and growth

The wild-type strain *Neurospora crassa Em*5297*a* was used in some experiments. It was grown and maintained on the 'normal magnesium' basal medium as described by Sivarama Sastry *et al.* (1962*a*). A Co^{2+}-resistant strain was derived from this parent strain by adapting it to grow on toxic concentrations of Co^{2+}, and isolating a pure, resistant culture as described in detail below. For this resistant strain, the basal medium employed differed from the 'normal magnesium' medium in that KH_2PO_4 was replaced by 0.015% KCl and 0.04% sodium β-glycerophosphate, the latter being autoclaved separately and added aseptically; the resistant strain was maintained by weekly subcultures on agar slopes (3% agar) containing, in addition, 2mg of Co^{2+}/ml and 0.4% yeast extract.

In growth experiments, the moulds were grown in Pyrex conical flasks (50ml) containing 10ml of culture medium, for 3 days at 30±1°C. The medium was

adjusted, when necessary, to pH 4.8–5.0 with NaOH, and the final pH was unchanged at the end of the growth period in all experiments. The mycelia were harvested, washed thoroughly with water, dried at 60–80°C overnight and weighed to measure growth. Growth experiments were repeated a minimum of four times.

Isolation of the Co^{2+}-resistant strain

Spores from a 6-day-old slant of *N. crassa Em5297a* were inoculated on to agar slants containing the basal medium for the resistant strain (see the previous section) and Co^{2+} (100 μg/ml). Growth was poor, but after 10 days there was some sporulation. The spores were transferred successively three times to similar slants at intervals of 7–10 days. The fourth slant showed good growth, and these spores were inoculated on to a slant containing 200 μg of Co^{2+}/ml. Again the growth was poor but, with a second subculturing, satisfactory adaptation to this concentration of Co^{2+} was attained. This process was repeated by using slants containing successively increasing concentrations of Co^{2+}, i.e. 500 μg, 1 mg and finally 2 mg of Co^{2+}/ml. On the second slant containing 2 mg of Co^{2+}/ml the strain exhibited excellent growth. (In contrast, when spores from the parent wild strain were directly inoculated on to slants containing 2 mg of Co^{2+}/ml, there was no detectable growth even after several weeks.) Further adaptation to higher concentrations of Co^{2+} was not attempted since no greater increase in metal resistance was discernible in preliminary experiments.

The possibility remained that this resistant strain was not homogeneous. To obtain a pure strain, the following isolation procedure, based on that of Webber & DeSerres (1965), was adopted. A suspension of conidia of the Co^{2+}-resistant isolate was filtered aseptically through glass wool to remove contaminating hyphal fragments and then diluted suitably with sterile water. Samples (0.5 ml) of this suspension were plated out in Petri dishes on to 25 ml of solid agar medium made up from the basal medium used for the Co^{2+}-resistant strain, also containing 3% agar, 0.4% yeast extract, 2 mg of Co^{2+}/ml, 0.001% nicotinamide and 1.5% sorbose (to obtain colonial growth of the mould). The plates were incubated at 30±1°C for 5 days. Under these conditions 100–150 discrete colonies (2–5 mm diameter) were observed. The largest single colonies were isolated, and tested for resistance to Co^{2+} by determining growth attained in 72 h in liquid medium at increasing concentrations of Co^{2+}. The isolate showing maximal tolerance to Co^{2+} was once again plated out as described above. The isolate from this second plating showed reproducible and maximal resistance to Co^{2+} and is the one used in the present study, designated hereafter as the Co^R strain.

To test for the homogeneity of the resistance phenotype, the Co^R strain was again plated out on sorbose medium and six colonies were picked out at random. These isolates were tested for resistance to Co^{2+} and behaved similarly to the Co^R strain (see the Results section).

The stability of the Co^{2+} resistance of the Co^R strain was examined by transferring spores from a stock slant successively to agar slants free of Co^{2+} at weekly intervals. Resistance to Co^{2+} was determined after every two or three such passages up to a maximum of 20 transfers. In view of the results of Webb (1970*a*), the stability of resistance was also checked by subculturing the Co^R strain twice, sequentially, on slants made up with medium containing no Co^{2+} and minimal magnesium, i.e. 50 μg of Mg^{2+}/10 ml of medium (Sivarama Sastry *et al.*, 1962*a*).

Assay of iron-binding compound

The extraction procedure for determining the iron-binding compound excreted by *Neurospora* mycelia was based on that described by Padmanabhan & Sarma (1964). In view of the small amounts excreted by the Co^R strain, amounts were scaled up. Culture fluid from three flasks was pooled, filtered, and portions (25 ml) were taken; 2.5 ml of $FeCl_3$ (1 mg of Fe^{3+}/ml) was added. After 15 min the precipitate that formed was removed by centrifugation (2500 *g*; 15 min). The supernatant was saturated with $(NH_4)_2SO_4$ and extracted twice with benzyl alcohol (5 ml) to remove the iron-binding compound. The benzyl alcohol layers were pooled and a portion (4 ml) was shaken with 44 ml of water–ether (1:10, v/v) in a separating funnel. The lower aqueous layer, which separated on standing, was drawn off (4 ml). A second extraction was performed by adding 4 ml of water.

The aqueous layers were pooled, evaporated to 2 ml on a sand bath at 100°C and the Folin-positive material therein was determined by the method of Subramanian *et al.* (1965), which assays siderochromes under these conditions. Values are expressed in terms of E_{660}, as measured in a Coleman Junior spectrophotometer. A blank was run in an identical manner with uninoculated medium containing suitable concentrations of Co^{2+} and the resulting E_{660} was deducted.

Metal-ion uptake by mycelia

When the Co^{2+} or Ni^{2+} content of mycelia was to be determined, mycelia were thoroughly washed, dried and wet-digested (Sivarama Sastry *et al.*, 1962*b*). The Co^{2+} content of digests was determined with nitroso-R salt as follows. To a suitable volume of the digest (containing 2–16 μg of Co^{2+}) 0.1 ml of conc. HCl was added, followed by 0.1 ml of 30%

H_2O_2. After 5min, 2ml of 33% sodium acetate (trihydrate), 0.1 ml of 33% KF, 1 ml of 0.2% nitroso-R salt and 1 ml of conc. HNO_3 were added, in that order, and mixed well. The total volume was made to 6ml with water. After 5min the colour intensity was read at 525nm in a Coleman Junior spectrophotometer. The Ni^{2+} content of digests was assayed with dimethylglyoxime (Sandell, 1959).

Assay of catalase

Freshly harvested, 3-day-old *N. crassa* mycelia (50–150mg fresh wt.) were washed extensively with ice-cold water, blotted to remove adherent moisture and extracted, by grinding, with twice their weight of glass powder and 7ml of phosphate buffer (Na_2HPO_4–NaH_2PO_4, 0.05M, pH7.0) at 0°C. The extract was centrifuged at 4°C (10000*g*; 15min) and the supernatant was collected. The residue was further extracted with 3ml of the phosphate buffer and centrifuged as described above. The supernatants were pooled. After suitable dilution, a portion (1ml) of the extract was incubated with 1ml of 0.1M-H_2O_2 (in phosphate buffer) at 30°C and the catalase activity was determined by a permanganate titration procedure (Ramachandran & Sarma, 1954). Catalase activity was expressed as mmol of H_2O_2 decomposed/5min per mg of protein. Protein concentrations in mycelial extracts were determined by the method of Lowry *et al.* (1951) with bovine serum albumin (Cohn fraction V; Mann Research Laboratories, New York, N.Y., U.S.A.) as standard.

Results

Characteristics of the Co^R strain

The isolated Co^R strain grew very much like the parent strain, except that the spores formed were pale-yellowish brown in colour, in contrast with the rose-pink spores of the parent strain *N. crassa Em5297a*. This characteristic feature was retained by the Co^R strain when it was maintained on agar slants containing Co^{2+} (2mg/ml). On transference to slants free of Co^{2+}, the spore colour began to intensify after the tenth transfer and by 18–20 transfers was indistinguishable from that of the parent strain; however, metal resistance was unaffected (see below). This is probably indicative of a suppression of spore-pigment production in the presence of Co^{2+}. The Co^R strain was otherwise indistinguishable from the parent strain and was fully prototrophic.

The homogeneity of the Co^R phenotype was established as described in the Experimental section. The growth of six isolates, derived from the Co^R strain, as a function of Co^{2+} concentration is summarized in Table 1; all isolates are inhibited alike by Co^{2+}.

The Co^R strain was subcultured on Co^{2+}-free medium 20 times at weekly intervals, and also twice on a Co^{2+}-free medium also low in Mg^{2+} (50μg of Mg^{2+}/10ml, in contrast to the normal concentration of 493.7μg of Mg^{2+}/10ml always provided in the medium; Sivarama Sastry *et al.*, 1962*a*). The metal-resistance pattern remained unchanged by both treatments. Thus the resistance of the Co^R strain to Co^{2+} and Ni^{2+} (see below) is stable.

Metal-ion toxicity in the Co^R strain

The effect of increasing concentrations of Co^{2+}, Ni^{2+} and Cu^{2+} on the growth of the Co^R and wild-type strains is depicted in Figs. 1, 2 and 3 respectively; 50% growth inhibition is obtained with 600μg of Co^{2+}/10ml in the wild-type strain and with 5500μg of Co^{2+}/10ml in the Co^R strain. The corresponding values for Ni^{2+} are 300μg/10ml and 3000μg/10ml respectively. From previous work (Sivarama Sastry *et al.*, 1962*a*) it is known that 1000μg of Zn^{2+}/10ml results in 50% inhibition of growth in the wild-type strain; in separate experiments this was found to be so in the Co^R strain as well. The results in Fig. 3 are noteworthy in that a different pattern of response obtains for Cu^{2+}. Half-maximal growth results at 800μg of Cu^{2+}/10ml in the wild-type strain, and at only 400μg of Cu^{2+}/10ml in the Co^R strain, indicating that the latter is twice as sensitive to Cu^{2+} as the parent strain.

Table 1. *Homogeneity of Co^R strain with respect to Co^{2+} resistance*

Growth of isolates I–VI in the absence of Co^{2+} was 36.0, 36.6, 40.4, 42.4, 39.2 and 36.3mg respectively. Period of growth was 72h. Experimental details are given in the text.

Concn. of Co^{2+} (mg/10ml)	Growth of isolate (% of control)						
	I	II	III	IV	V	VI	Mean
2.0	86.2	83.0	83.7	84.9	100.0	83.0	86.8
4.0	75.0	73.7	68.1	66.7	78.8	74.5	74.5
6.0	50.0	48.3	50.0	51.9	45.5	47.9	48.9
8.0	34.7	33.2	38.1	32.6	25.6	33.8	33.0

Metal-ion uptake in toxic concentrations of metals in the Co^R strain

The uptake of Co^{2+} and Ni^{2+} by the Co^R strain during growth in the presence of inhibitory concentrations of these two metals is also shown in Figs. 1 and 2. At half-maximal growth, the Co^R strain accumulates 275μg of Co^{2+}/100mg dry wt. and 79μg of Ni^{2+}/100mg dry wt., in toxic concentrations of Co^{2+} and Ni^{2+} respectively; in contrast, the corresponding values for the parent strain are 47.6μg of Co^{2+} and 10.3μg of Ni^{2+} (Sivarama Sastry *et al.*, 1962*a*). Extrapolation from Figs. 1 and 2 shows that at the latter concentration of accumulated metal, the Co^R strain is inhibited by only about 5–7%. Thus the Co^R strain can tolerate very much higher inhibitory metal concentrations than the parent strain.

Reversal of metal-ion toxicity by Mg^{2+}

The work of Sivarama Sastry *et al.* (1962*a*) has shown that Mg^{2+} as well as Fe^{3+} can reverse the growth inhibition caused by Co^{2+} and Ni^{2+} in the parent strain. Hence, this aspect has been studied in the Co^R strain. Figs. 4 and 5 show the results obtained with added Mg^{2+} (in addition to 493.7μg of Mg^{2+}/10ml contained in the medium). As in the parent strain, Mg^{2+} can evidently bring about complete reversal of Co^{2+} and Ni^{2+} toxicities by suppressing the uptake of the toxic metal ions.

Effect of Fe^{3+} on metal toxicities in N. crassa strains

In studies with the parent strain (Sivarama Sastry *et al.*, 1962*a*), Fe^{3+} was added as ferric ammonium citrate, and at the concentrations involved citrate itself had little effect. However, trials with ferric ammonium citrate in the Co^R strain showed that Co^{2+} and Ni^{2+} toxicities could be reversed by very high concentrations of ferric ammonium citrate, but that this effect was solely due to the extremely high concentrations of citrate added along with Fe^{3+}. Consequently, several alternative means of supplementing with Fe^{3+} were sought. Eventually it was

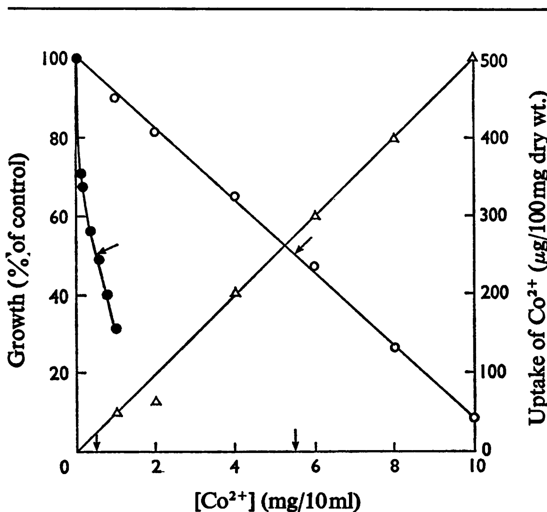

Fig. 1. *Co^{2+} toxicity in N. crassa*

For experimental details see the text. ●, Growth of parent strain (*Em5297a*); ○, growth of Co^R strain; △, uptake of Co^{2+} in the Co^R strain. Weights of control mycelia (no Co^{2+}) were 37.5mg in the Co^R strain and 41.3mg in the parent strain. Arrows indicate points corresponding to half-maximal growth and the concentration of toxic metal for the two strains involved.

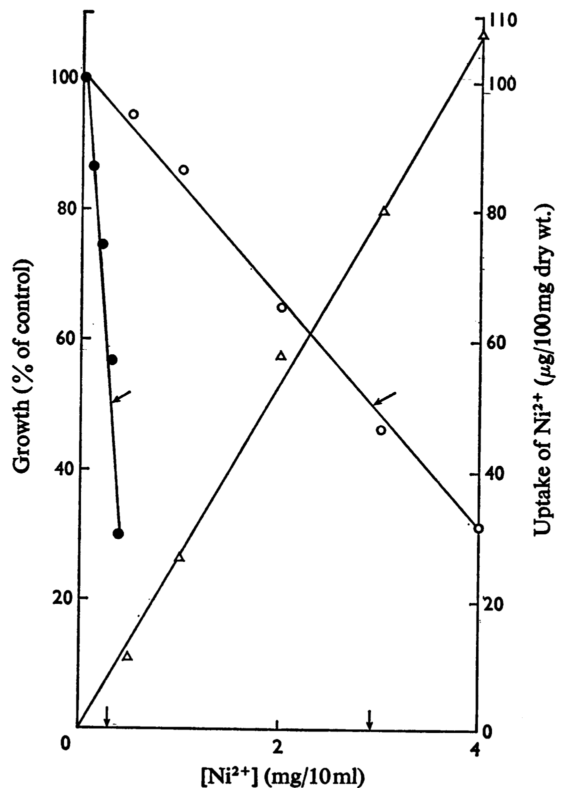

Fig. 2. *Ni^{2+} toxicity in N. crassa*

For experimental details see the text. ●, Growth of parent strain; ○, growth of Co^R strain; △, uptake of Ni^{2+} in the Co^R strain. Weights of control mycelia (no Ni^{2+}) were 36.5mg in the Co^R strain and 40mg in the parent strain. Arrows indicate points corresponding to half-maximal growth and the concentration of toxic metal for the two strains involved.

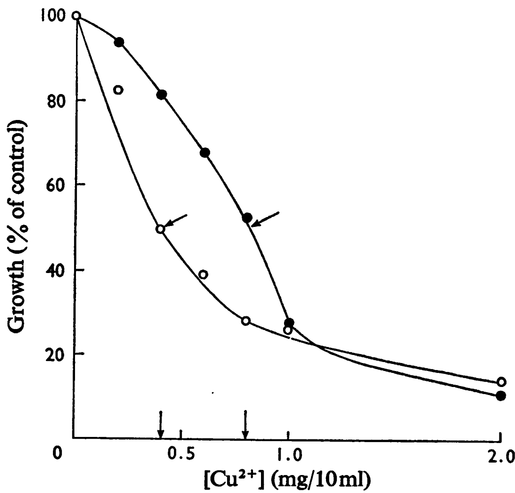

Fig. 3. *Cu^{2+} toxicity in N. crassa*

For experimental details see the text. ●, Growth of parent strain; ○, growth of Co^R strain; weights of control mycelia (no Cu^{2+}) were 38.6mg and 42.0mg in the Co^R strain and the 'normal' strain respectively. Arrows indicate points corresponding to half-maximal growth and the concentration of toxic metal for the two strains involved.

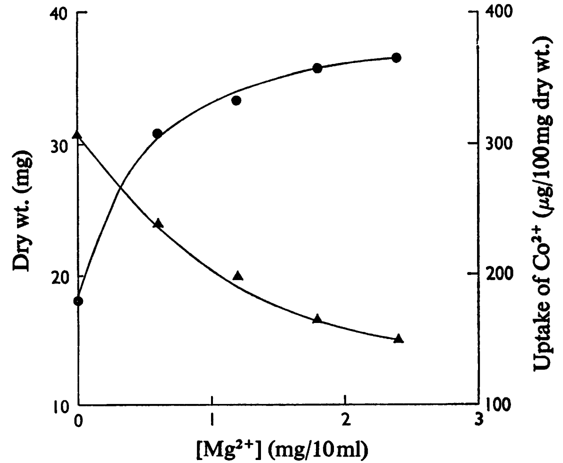

Fig. 4. *Effect of Mg^{2+} on Co^{2+} toxicity in N. crassa Co^R strain*

Toxic concentration of Co^{2+} used was 6mg of Co^{2+}/10ml of medium. For experimental details see the text. ●, Growth; ▲, uptake of Co^{2+}.

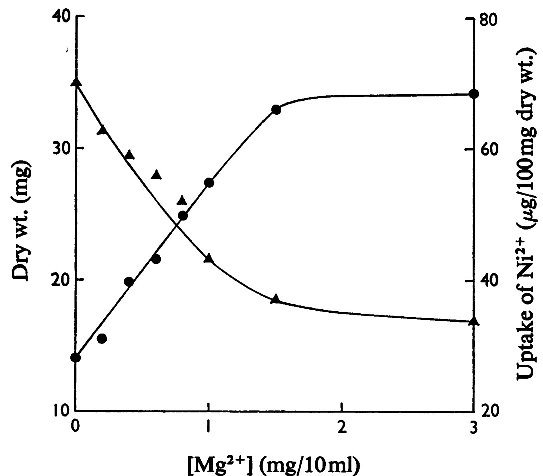

Fig. 5. *Effect of Mg^{2+} on Ni^{2+} toxicity in N. crassa Co^R strain*

Toxic concentration of Ni^{2+} used was 3mg of Ni^{2+}/10ml of medium. For experimental details see the text. ●, Growth; ▲, uptake of Ni^{2+}.

found that the best source of Fe^{3+} was a Fe^{3+}–EDTA (1:1) complex. Since Fe^{3+}–EDTA was not used in the earlier work (Sivarama Sastry *et al.*, 1962*a*) in initial experiments, the effect of added Fe^{3+}–EDTA on Co^{2+} and Ni^{2+} toxicities was studied in the parent strain. The results obtained are shown in Fig. 6; toxicity due to 600µg of Co^{2+}/10ml is fully reversed by 200µg of Fe^{3+}/10ml, and that due to 300µg of Ni^{2+}/10ml is completely counteracted by 100µg of Fe^{3+}/10ml, when the source of Fe^{3+} is Fe^{3+}–EDTA. It is noteworthy that Fe^{3+}–EDTA is much more effective than ferric ammonium citrate as a source of Fe^{3+} since full growth of the parent strain is obtained with a Fe^{3+}: toxic metal ratio of 1:3; in contrast, when ferric ammonium citrate is the source of Fe^{3+}, the corresponding ratio is 1:2 (Sivarama Sastry *et al.*, 1962*a*).

To establish further that the responses described above are due to added Fe^{3+} and not due to possible effects of the EDTA moiety, the effect of Fe^{3+}–EDTA on the decrease in catalase activity and the enhanced excretion of an iron-binding compound (two metabolic alterations in Co^{2+} toxicity in the normal strain) was studied in the normal strain. The results obtained (Table 2) clearly show that Fe^{3+}–EDTA reverses all affected parameters in Co^{2+} toxicity without suppressing Co^{2+} accumulation, and are in agreement with the hypothesis that Co^{2+} toxicity in *N. crassa Em5297a* is equivalent to an iron deficiency that can be counteracted by added Fe^{3+} (Sivarama Sastry *et al.*, 1962*a*; Padmanabhan & Sarma, 1966).

Possible reversal of Co^{2+} and Ni^{2+} toxicities by Fe^{3+}–EDTA was next studied in the Co^R strain. Two types of control experiments were performed. First, along with toxic concentrations of Co^{2+} and Ni^{2+}, EDTA alone was supplemented at various concentrations. In the presence of 6mg of Co^{2+}/10ml or 3mg of Ni^{2+}/10ml, with up to 6.66mg of EDTA/10ml (equivalent to that introduced along with 1mg of Fe^{3+}/10ml) the growth of the Co^R strain was unaffected by EDTA; greater concentrations resulted in a further decrease in growth. Thus the EDTA anion *per se* cannot reverse metal toxicity. Secondly, as shown in Table 3, Fe^{3+}–EDTA alone was added to control cultures of the Co^R strain at several concentrations to see whether this complex itself was inhibitory at high dosages. Again, up to a concentration of 6mg of Fe^{3+}/10ml (as Fe^{3+}–EDTA), the iron complex was neither stimulatory nor inhibitory.

In reversal experiments, Fe^{3+}–EDTA was included along with Co^{2+} or Ni^{2+}, as shown in Table 3. Since in the parent strain a 1:3 ratio of Fe^{3+} (as Fe^{3+}–EDTA):toxic metal resulted in normal growth, if Fe^{3+} could reverse Ni^{2+} toxicity in the Co^R strain growth should have been normal with 3mg of Ni^{2+}/10ml plus 1ml of Fe^{3+}/10ml; however, this does not occur (Table 3). On the same basis, it is evident that Fe^{3+} cannot counteract Co^{2+} toxicity as well. This

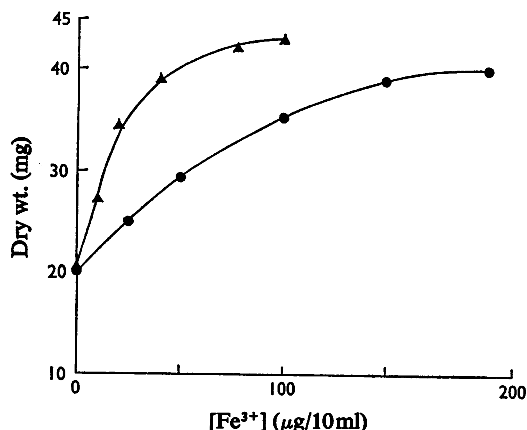

Fig. 6. *Effect of Fe^{3+}–EDTA complex on Co^{2+} and Ni^{2+} toxicities in N. crassa Em5297a*

Toxic concentrations used were 600μg of Co^{2+}/10ml and 300μg of Ni^{2+}/10ml. For experimental details see the text. ●, Reversal of Co^{2+} toxicity; weight of control mycelium (no Co^{2+}) was 40.3mg. ▲, Reversal of Ni^{2+} toxicity; weight of control mycelium (no Ni^{2+}) was 42.2mg.

Table 3. *Effect of Fe^{3+}–EDTA complex on metal toxicities in the Co^R strain*

For experimental details see the text. Fe^{3+} was added as Fe^{3+}–EDTA complex. In series (C), Fe^{3+}–EDTA alone was tested. No other metals were added.

Concn. of metal (mg/10ml)	$[Fe^{3+}]$ (mg/10ml)	Mycelial dry wt. (mg)
(A) Control (None)	Nil	37.9
Co^{2+} (6.0)	Nil	17.8
	0.25	16.9
	1.00	17.1
	2.00	17.6
	4.00	17.6
	6.00	11.0
(B) Control (None)	Nil	38.8
Ni^{2+} (3.0)	Nil	18.7
	0.5	19.1
	1.0	18.6
	2.0	18.6
	4.0	18.1
	6.0	17.4
(C) Control (None)	Nil	39.1
	0.5	39.1
	1.0	40.1
	2.0	40.3
	4.0	40.5
	6.0	40.2

Table 2. *Effect of iron as Fe^{3+}–EDTA complex on the catalase activity and concentrations of Co^{2+} and iron-binding compound (XFe) in Co^{2+} toxicity in N. crassa Em5297a*

For experimental details see the text. Amounts of metal supplemented are per 10ml of culture medium. Catalase specific activity is expressed as mmol of H_2O_2 decomposed/5min per mg of protein.

Supplements (μg/10ml)	Dry wt. (mg)	Mycelial Co^{2+} (μg/100mg dry wt.)	Catalase specific activity	XFe excreted (E_{660} units/5ml of medium)
Nil (control)	39.9	Nil	1.4	0.18
Co^{2+} (600)	20.1	47.5	0.4	1.97
Co^{2+} (600)+Fe^{3+} (200)	39.0	46.6	1.3	0.20

ineffectiveness of Fe^{3+} in reversing Co^{2+} and Ni^{2+} toxicities in the Co^R strain is in sharp contrast with the full counteraction that obtains in the parent strain.

Catalase activity and excretion of iron-binding compound in the Co^R strain

The results obtained are consistent with the conclusion that Co^{2+} toxicity in the Co^R strain, unlike that in the parent strain (Sivarama Sastry *et al.*, 1962*a*), is not associated with deranged iron metabolism. It should therefore be expected that in the Co^R strain, in Co^{2+} toxicity, there should be neither a decrease in catalase activity nor an excretion of the iron-binding compound (XFe). These aspects were studied and the results obtained are summarized in Tables 4 and 5. The results are fully in accordance with expectation, and it is clear that, in Co^{2+} toxicity in the Co^R strain, parameters indicative of deranged iron metabolism are unaffected.

Discussion

The Co^R strain of *N. crassa* studied has about tenfold the resistance of the 'parent' strain to both Co^{2+} and Ni^{2+}, but is just as sensitive to Zn^{2+}. One peculiar feature is its greater sensitivity to Cu^{2+}, which remains unexplained. As mentioned above, in the parent strain Co^{2+} toxicity is equivalent to iron deficiency, brought about by a derangement of iron utilization by excess of Co^{2+}; excess of iron can reverse all affected parameters, including growth, without suppressing the uptake of Co^{2+}. In contrast, in the Co^R strain, growth inhibition caused by excess of Co^{2+} or Ni^{2+} cannot be reversed by Fe^{3+}. Further, in Co^{2+} toxicity in the Co^R strain, catalase activities remain normal and no iron-binding compound is excreted. Hence it is evident that the mechanism of resistance is associated with an altered pattern of iron metabolism in this strain, such that excess of Co^{2+} no longer affects iron utilization. To our knowledge, this is the first instance of a phenomenon of this nature.

Also, the Co^R strain accumulates very much higher concentrations of Co^{2+} in Co^{2+} toxicity than does the parent strain. It has been found (G. Venkateswerlu & K. Sivarama Sastry, unpublished work) that around 50% of the Co^{2+} taken up by this strain is present intracellularly, indicating that the mechanism of resistance does not involve the development of a permeability barrier for the inhibitory metal. This ability to tolerate much higher mycelial concentrations of Co^{2+} without concomitant inhibition of iron metabolism suggests that the systems involved in the utilization of iron in the Co^R strain differ from those in the parent strain, in regard to their sensitivity to toxic ions such as Co^{2+} and Ni^{2+}.

However, as in the parent strain, toxicity of Co^{2+} as well as Ni^{2+} is reversed by Mg^{2+} in the Co^R strain, but at a much lower ratio of Mg^{2+} to toxic metal. In many bacteria there is good evidence (Abelson & Aldous, 1950; Webb, 1970*a*,*b*) that Mg^{2+} antagonizes heavy-metal toxicities. This is also true in *N. crassa* (Sivarama Sastry *et al.*, 1962*a*) and in *Aspergillus niger* (Adiga *et al.*, 1961). Such antagonism is manifested predominantly at the uptake level and is related to the fact that the same transport mechanism handles Mg^{2+} and a variety of bivalent metals. Consequently, the much higher efficiency with which Mg^{2+} reverses Co^{2+} and Ni^{2+} toxicities in the Co^R strain (as compared with the parent strain) is indicative of a probable change in the affinity of the transport system operating in the Co^R strain; it is possible that, in the Co^R strain, this system has a much higher affinity for Mg^{2+} than for toxic ions such as Co^{2+} and Ni^{2+}.

Table 4. *Effect of Co^{2+} toxicity on catalase activity in the Co^R strain*

For experimental details see the text. Catalase specific activity is expressed as mmol of H_2O_2 decomposed/5min per mg of protein.

Supplements (mg/10ml)	Growth (mg dry wt.)	Catalase specific activity
Nil (control)	37.5	2.42
Co^{2+} (2.0)	28.5	2.48
Co^{2+} (4.0)	24.6	2.09
Co^{2+} (6.0)	17.6	2.51

Table 5. *Formation of iron-binding compound (XFe) in Co^{2+} toxicity in N. crassa*

For experimental details see the text. Values for XFe represent total calculated E_{660} units/25ml of culture medium.

Parent strain			Co^R strain		
Concn. of Co^{2+} (mg/10ml)	Dry wt. (mg)	XFe formed	Concn. of Co^{2+} (mg/10ml)	Dry wt. (mg)	XFe formed
Nil (control)	39.0	0.825	Nil (control)	37.5	0.275
0.30	33.6	8.45	2.0	28.1	0.300
0.60	20.6	10.325	6.0	18.2	0.275

The resistance of the Co^R strain to Co^{2+} and Ni^{2+} is quite stable and is not lost on repeated transfers to a Co^{2+}-free medium. Metal resistance is also stable on a low-Mg^{2+} medium; in contrast, metal resistance in several bacteria is quickly lost when the organisms are subcultured on media low in Mg^{2+} (Webb, 1970*a*). Hence it seems likely that the Co^R strain is a result of a mutation induced by Co^{2+} in *N. crassa*. Although this is the first instance of this type in *N. crassa*, there are other examples in the literature of metals inducing mutation. Garassini *et al.* (1966) have reported mutation induction by Co^{2+} in *Candida utilis*. In *Aspergillus flavus*, barium treatment results in a mutant that loses the ability to produce aflatoxin (Lee & Townsley, 1968). Exposure to Cu^{2+} results in strains resistant to Cu^{2+} in *Saccharomyces cerevisiae* (Antoine, 1964, 1965; Brenes-Pomales *et al.*, 1955). Mn^{2+}, Cu^{2+}, Co^{2+} and Ni^{2+} all induce respiratory deficiency in yeast with high frequency, though it has been suggested that this is due to action at the cytoplasmic level (Lindegren *et al.*, 1958). In many such cases the resistance is under genetic control, and is often stable (Ashida, 1965). In the Enterobacteriaceae, the R-factors that mediate resistance to metals are episomal in nature (Smith, 1967). It is difficult to say from the present results whether the mutation to Co^{2+} resistance in *N. crassa* is chromosomal or extrachromosomal until a regular genetic analysis is performed. However, it is clear that the Co^R strain is not the result of a selection by Co^{2+} of a tolerant population from the parent conidia of the 'normal' strain, since in repeated trials (G. Venkateswerlu & K. Sivarama Sastry, unpublished work) no traces of growth were seen even after many weeks, when conidia of the parent strain were directly put on to agar media containing high concentrations of Co^{2+}.

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References

Abelson, P. H. & Aldous, E. (1950) *J. Bacteriol.* **60**, 401–413
Adiga, P. R., Sivarama Sastry, K., Venkatasubramanyam, V. & Sarma, P. S. (1961) *Biochem. J.* **81**, 545–550
Antoine, A. (1964) *Exp. Cell. Res.* **36**, 73–85
Antoine, A. (1965) *Exp. Cell Res.* **37**, 278–291
Ashida, J. (1965) *Annu. Rev. Phytopathol.* **3**, 153–174
Brenes-Pomales, A., Lindegren, G. & Lindegren, C. C. (1955) *Nature (London)* **176**, 841–842
Garassini, L. A., Florenzano, G. & Balloni, W. (1966) *Ric. Sci.* **36**, 65–67
Healy, W. B., Cheng, S. & McElroy, W. D. (1955) *Arch. Biochem. Biophys.* **54**, 206–214
Lee, E. G. H. & Townsley, P. M. (1968) *J. Food Sci.* **33**, 420–423
Lindegren, C. C,, Nagai, S. & Nagai, H. (1958) *Nature (London)* **182**, 446–448
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Padmanabhan, G. & Sarma, P. S. (1964) *Arch. Biochem. Biophys.* **108**, 362–363
Padmanabhan, G. & Sarma, P. S. (1966) *Biochem. J.* **98**, 330–334
Ramachandran, L. K. & Sarma, P. S. (1954) *J. Sci. Ind. Res. Sect. B.* **13**, 115–117
Sandell, E. B. (1959) *Colorimetric Determination of Traces of Metals*, pp. 672–673, Interscience Publishers, New York
Sivarama Sastry, K., Adiga, P. R., Venkatasubramanyam, V. & Sarma, P. S. (1962*a*) *Biochem. J.* **85**, 486–491
Sivarama Sastry, K., Raman, N. & Sarma, P. S. (1962*b*) *Anal. Chem.* **34**, 1302–1303
Smith, D. H. (1967) *Science* **156**, 1114–1115
Subramanian, K. N., Padmanabhan, G. & Sarma, P. S. (1965) *Anal. Biochem.* **12**, 106–112
Venkateswerlu, G. & Sivarama Sastry, K. (1970) *Biochem. J.* **118**, 497–503
Webb, M. (1970*a*) *Biochim. Biophys. Acta* **222**, 428–439
Webb, M. (1970*b*) *Biochim. Biophys. Acta* **222**, 440–446
Webber, B. B. & DeSerres, F. J. (1965) *Proc. Nat. Acad. Sci. U.S.* **53**, 430–437