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Excretion of pyruvate in nickel toxicity in wild type and Ni^{2+} resistant mutants of *Neurospora crassa*

P. MARUTHI MOHAN* and K. SIVARAMA SASTRY†
Department of Biochemistry, Osmania University, Hyderabad 500 007, India
* Present address: Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India

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Abstract. The parent wild strain *Neurospora crassa* Em 5297a and three Ni^{2+} resistant *Neurospora crassa* mutants have been shown to excrete pyruvate into the culture medium in Ni^{2+} and Co^{2+} toxicities. Ni^{2+} has a more pronounced effect in this regard. The excretion is progressive with growth inhibition and is abolished by Mg^{2+} in all strains and by Fe^{3+} partially in the Em strain but not in *Neurospora crassa* Ni^{R1}. Pyruvate, citrate and malate supplementation reverse growth inhibition caused by excess Ni^{2+}, but with concomitant suppression of Ni^{2+} accumulation. It is suggested that one of the features of Ni^{2+} toxicity in *Neurospora crassa* is a derangement in carbohydrate metabolism at step(s) beyond pyruvate and that this is possibly due to decreased in *vivo* activity of Mg^{2+} dependent processes.

Keywords. *Neurospora crassa;* nickel-resistant *N. crassa*; nickel toxicity; pyruvate excretion.

Introduction

Toxicities of heavy metal ions such as Co^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+} and Be^{2+} have been extensively studied in *Neurospora crassa* and *Aspergillus niger* (Maruthi Mohan and Sivarama Sastry, 1983; Naidu *et al.,* 1979; Venkateswerlu and Sivarama Sastry, 1972, 1979; Sivarama Sastry *et al.,* 1962a,b; Adiga *et al.,* 1961, 1962) and it is evident that disturbed Fe^{3+} or Mg^{2+} metabolism is one of the major underlying mechanisms. Such an interaction between essential metal ions such as Fe^{3+} or Mg^{2+} could affect carbohydrate utilization. Decreased acid production in *A. niger* in metal toxicities is partially reversed by supplemented Mg^{2+} (Adiga *et al.,* 1961). In yeast, Ni^{2+}, under certain conditions, inhibits anaerobic glucose utilization and this has been suggested to be due to Ni^{2+} inhibition of alcohol dehydrogenase (Fuhrmann and Rothstein, 1968). Pyruvate oxidation by cell suspensions of *Aerobacter aerogenes* is inhibited by Co^{2+} (Webb, 1970). On the other hand, in *N. crassa,* in Be^{2+} toxicity, utilization of glucose is inhibited but not that of pyruvate and it has been postulated that Be^{2+} inhibits glycolysis at some step(s) prior to pyruvate (Naidu *et al.,* 1979).

Growth inhibition of *A. niger* due to Co^{2+}, Ni^{2+} and Zn^{2+} toxicities can be reversed by added intermediates of carbohydrate metabolism, especially malate and pyruvate

† To whom correspondence should be addressed.

(Sivarama Sastry *et al.,* 1962a). Pyruvate reverses Be $^{2+}$ toxicity without suppression of Be^{2+} uptake (Naidu *et al.,* 1979). Enhancement of uptake of metal ions could also be involved; for instance, Mg $^{2+}$ uptake is enhanced by glucose, pyruvate, succinate and α-ketoglutarate in *A. aerogenes* cell suspensions (Webb, 1970). Thus, toxic metal ion interactions with carbohydrate metabolism are complex.

Recently we have isolated three Ni^{2+} resistant mutants of *N. crassa* wherein toxic metal ion interaction with essential ions such as Fe^{3+} and Mg^{2+} is dependent on the nature of the mutant involved (Maruthi Mohan and Sivarama Sastry, 1983). These mutants, *N. crassa* Ni^{R1}, Ni^{R2} and Ni^{R3} respectively, while being four-fold resistant to N i^{2+}, differ in cross-resistance to other metal ions such as Co^{2+}, Zn^{2+}, and Cu^{2+}. In all these mutants, Mg^{2+} reverses Ni^{2+} toxicity by suppressing the uptake of Ni^{2+}. In the case of *N. crassa* Ni^{R3}, Mg^{2+} counteracts Co^{2+} toxicity by a similar mechanism. However, in *N. crassa* Ni^{RI} and Ni^{R2}, Mg^{2+} reversal of Co^{2+} toxicity does not cause a decrease in Co^{2+} uptake. In view of such strain specific differences in toxic ion-Mg^{2+} interaction and the observation that the pyruvate was excreted into the culture medium as a result of Co^{2+} and Ni^{2+} toxicities in these mutants, we have examined some features of pyruvate excretion. The results presented here show that pyruvate excretion due to Ni^{2+} toxicity is suppressed by Fe^{3+} and Mg^{2+}.

Materials and methods

Chemicals

Sodium pyruvate, α-ketoglutaric acid, oxaloacetic acid, L-malic acid, citric acid and Fe^{3+}-EDTA were analytical grade products, purchased from British Drug House Chemicals Division, Glaxo laboratories, Bombay. All other chemicals and reagents used were of analytical grade.

Organisms and growth

Neurospora crassa Em 5297a (wild type) and the Ni^{2+} resistant strains (Ni^{RI}, Ni^{R2} and Ni^{R3}) were grown for 72 h at pH 4·8-5·0, for 72 h in experiments involving preformed mycelia in media as described earlier (Maruthi Mohan and Sivarama Sastry, 1983). In experiments wherein the effects of pyruvate, malate and citrate were to be examined, these solutions, adjusted to pH 4·8-5·0, were separately sterilized and added aseptically to growth media along with Ni^{2+}.

Identification and estimation of α-ketoacids

At the end of the growth period (72 h), the medium was collected quantitatively, and pooled with mycelial washings. When required, this was concentrated to a known volume. Ketoacids herein were identified by paper chromatography after conversion to their 2,4-dinitrophenylhydrazones (Block *et al.,* 1958). Aliquots of the extracts were adsorbed on Dowex-1 (formate), and ketoacids eluted with 6N formic acid, concentrated and chromatographed (Busch *et al.,* 1962). Pyruvate was assayed in the culture medium (Friedmann, 1943).

Estimation of Ni^{2+}

Mycelia were subjected to wet digestion after thorough washing to free them of adhering Ni^{2+} (Sivarama Sastry *et al.,* 1962c) and Ni^{2+} content in digests estimated with dimethylglyoxime (Sandell, 1959).

Results

Initial experiments with the parent strain (*N. crassa* Em 5297a) and the three Ni^{2+} resistant mutants showed significant ketoacid excretion only in the presence of Ni^{2+} and Co^{2+} at a concentration which caused 50% growth inhibition (I_{50}). Chromatographic analyses established that the ketoacid was mostly pyruvate; the only others found were traces of α-ketoglutarate and oxaloacetate.

Features of pyruvate excretion at I_{50} levels of Co^{2+} and Ni^{2+} are shown in tables 1 and 2. Despite the I_{50} values being different from three mutants, they all excreted

Table 1. Pyruvate excretion in Ni^{2+} toxicity in *N. crassa.*

Strain	Conc. of Ni^{2+} (mM) I_{50}	Dry wt. of** mycelia (mg/flask)	Ni^{2+} uptake (pg/100 mg dry/wt.)	Pyruvate* (mg/100 mg dry wt.)
Em 5297a	1·0	21	14	2·97
Ni^{R1}	1·0	29	39	0·50
	4·0	15	189	4·25
Ni^{R2}	4·0	14	329	4·1
Ni^{R3}	4·0	14	10	4·8

N. crassa strains grown under Ni $^{2+}$ toxic conditions (except for the lower Ni $^{2+}$ level in the case of Ni ^{R1}, which was not inhibitory) for 72 h at 30 ± 1°C in 10 ml medium.
* Values represent pyruvate excreted into the culture medium.
** Weight of control mycelia (No Ni^{2+}): Em, 45; Ni^{R1}, 30; Ni^{R2}, 29; Ni^{R3}, 28.

Table 2. Pyruvate excretion in Co^{2+} toxicity in *N. crassa.*

Strain	I_{50} Conc. of Co^{2+} (mM)	Dry wt. of mycelia* (mg/flask)	Co^{2+} uptake (μg/100 mg dry wt.)	Pyruvate** (mg/100 mg dry wt.)
Em 5297a	1·0	23	22	0·8
Ni^{R1}	6·0	14	375	1·4
Ni^{R2}	6·0	15	481	2·1
Ni^{R3}	2·0	13	57	1·8

N. crassa strains were grown under Ni^{2+} toxic conditions for 72 h at 30 ± 1°C in 10 ml medium.
* Weight of control mycelia (No Ni^{2+}): EM, 45; $Ni^{R!}$, 30; Ni^{R2}, 29; Ni^{R3}, 28. (controls)
** Values represent pyruvate excreted into the culture medium.

approximately the same amount of pyruvate. However, quantitatively, more pyruvate was excreted in Ni^{2+} toxicity. It may be noted that at 1 mM Ni^{2+} concentration, which was not toxic to *N. crassa* Ni^{R1}, the parent strain Em excreted more pyruvate than the mutant. Consequently, further studies were conducted on Ni^{2+} toxicity in *N. crassa* Ni^{R1} (as a typical Ni^{2+} resistant strain). Results presented in figures 1 and 2 show that the pyruvate excretion was a function of growth inhibition with increasing Ni^{2+} concentration in both the strains.

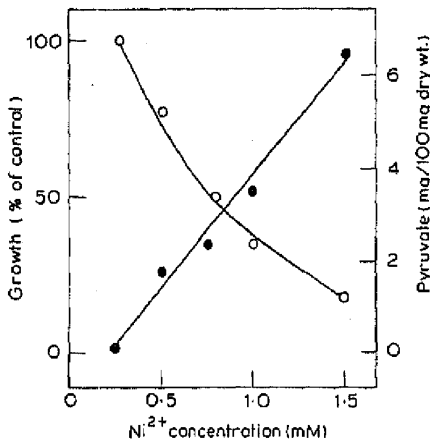

Figure 1. Influence of Ni^{2+} toxicity on growth and pyruvate excretion in *N. crassa* Em 5297a. *N. crassa* Em was grown with different levels of Ni^{2+} for 72 h at 30 ± 1°C. Media and mycelial washings were pooled and the pyruvate content estimated.
For experimental details see text (O), Growth; (●) pyruvate.

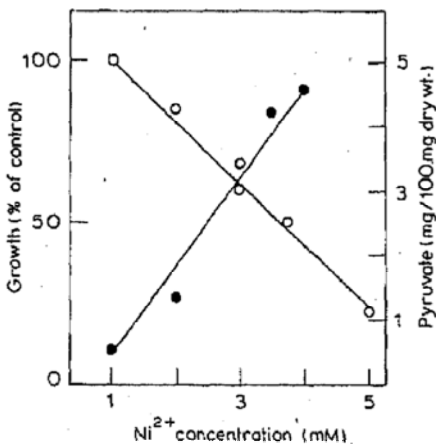

Figure 2. Influence of Ni^{2+} toxicity on growth and pyruvate excretion in *N. crassa* Ni^{R1}. *N. crassa* Ni^{R1} was grown with different levels of Ni^{2+} for 72h at 30 ± 1°C.
Experimental details as in figure 1. (O) Growth; (●)pyruvate.

Earlier studies had shown that Ni^{2+} toxicity in the Em strain was fully counteracted by Fe^{3+} and Mg^{2+} ions and the reversal of inhibition by Mg^{2+}, but not by Fe^{3+}, was associated with suppression of Ni^{2+} uptake (Sivarama Sastry *et al.*, 1962b). The effects

of these two ions on pyruvate excretion were therefore studied under conditions of reversal of Ni^{2+} toxicity. Pyruvate excretion was fully inhibited by 3 mM Mg^{2+}. On the other hand, 2mM Fe^{3+} -EDTA complex (Fe^{3+}-EDTA complex was found to be a better source of iron for *N. crassa,* Venkateswerlu and Sivarama Sastry, 1973) reversed growth inhibition fully, but reduced pyruvate excretion only by about 50%. In a typical experiment, on a cell dry weight basis, pyruvate accumulated in the presence of Ni^{2+} (mg/100 mg dry wt.) was 2·97 as compared to 1·53 in presence of Ni^{2+} and 2 mM Fe^{3+} -EDTA. Likewise in *N. crassa* Ni^{R1}, Mg^{2+} at 8 mM concentration prevented pyruvate excretion due to 4mM Ni^{2+}; Fe^{3+} was not tested herein, since it does not counteract Ni^{2+} toxicity in *N. crassa* Ni^{R1} (loc. cit).

The influence of added pyruvate, citrate and malate on Ni^{2+} toxicity in Em and Ni^{R1} strains was also examined. The results (table 3) show that all of them, at concentrations which reversed growth inhibition, suppressed Ni^{2+} uptake.

Table 3. Effects of pyruvate, citrate and malate on Ni^{2+} toxicity in *N. crassa.*

Strain	Organic* acid	Dry wt. (mg)	Ni^{2+} uptake (μg/100 mg dry wt.)
Em 5297a	—	12	14
	Pyruvate	41	4·9
	Citrate	40	5·0
	Malate	42	7·3
	—	14	189
Ni^{R1}	Pyruvate	32·5	73·7
	Citrate	28.5	15·9
	Malate	30	23·2

* Organic acids were supplemented at 100 mg/10 ml medium. Toxic concentration of Ni^{2+} used: Em, 1·0mM; Ni^{R1}, 4·0mM. Growth for 72h at 30 ±1°C, pH 5·0. Control weights of mycelia (with no Ni^{2+} in medium) were; Em, 50 mg; Ni^{R1}, 31 mg.

Discussion

In *N. crassa* (Sivarama Sastry *et al.,* 1962b), *A. niger* (Adiga *et al.,* 1961) and in several bacteria (Abelson and Aldous, 1950) there is good evidence that some of the effects of heavy metal toxicities are due to an induced deficiency of Mg^{2+} caused by excessive intracellular toxic ion concentrations. The present study suggests that the excretion of pyruvate in Ni^{2+} toxicity in *N. crassa* is probably due to deranged Mg^{2+} metabolism. In the case of added Fe^{3+}, growth inhibition due to Ni^{2+} is fully counteracted but there is only a partial suppression of pyruvate excretion.

In *N. crassa,* Fe^{3+} counteracts Ni^{2+} toxicity by apparently translocating intracellular Ni^{2+} from sites wherein Ni^{2+} is toxic to others at which Ni^{2+} binding is not inhibitory (Sivarama Sastry *et al.,* 1962b). Consequently, the limited ability of Fe^{3+} to suppress pyruvate excretion even when it restores the growth rate to normal suggests that the intracellular sites at which Ni^{2+} is inhibitory to carbohydrate metabolism are

those which depend on Mg^{2+}. The reversal of Ni^{2+} toxicity by added intermediates such as pyruvate, citrate and malate is not a metabolic effect but apparently related to limiting Ni^{2+} uptake.

It has been suggested earlier that in *A. niger*, the primary metabolic lesions in carbohydrate metabolism due to metal toxicities are at steps involved in the formation of pyruvate and malate (Sivarama Sastry *et al.,* 1962a). Be $^{2+}$ toxicity inhibits glycolysis at some step(s) prior to pyruvate (Naidu *et al*., 1979). The present data indicate that Ni^{2+} (and Co^{2+}) toxicity in *N. crassa* affect not the biosynthesis of pyruvate but rather its subsequent metabolism. Thus, the site(s) of action of different metal ions on carbohydrate metabolism depend, apparently, both on the metal ion in question as well on the organism.

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References

Abelson, P. H. and Aldous, E (1950) *J. Bacteriol,* **60,** 401.

Adiga, P. R., Sivarama Sastry, K., Venkatasubramanyam, V. and Sarma, P. S. (1961) *Biochem. J.,* **81,** 545.

Adiga, P. R., Sivarama Sastry, K. and Sarma, P. S. (1962) *Biochim. Biophys. Acta,* **64,** 546.

Block, R. J., Durrum, E. L. and Zweig, G. (1958) *Paper Chromatography and Paper Electrophoresis* 2nd edition, (New York: Academic Press).

Busch, H., Hurlbert, R. B. and Van R. Potter (1962) *J. Biol. Chem.,* **196,** 717.

Friedmann, T. E. and Haugen, G. E. (1943) *J. Biol. Chem.,* **147,** 415.

Fuhrmann, G. F. and Rothstein, A. (1968) *Biochim. Biophys. Acta,* **163,** 331.

Maruthi Mohan, P. and Sivarama Sastry, K. (1983) *Biochem. J.,* **212,** 205.

Naidu, A., Nirmala, J. and Sivarama Sastry, K. (1979) *J. Biosci.,* **1,** 279.

Sandell, E. B. (1959) *Colorimetric Determination of Traces of Metals,* (New York: Interscience Publishers), p. 672.

Sivarama Sastry, K., Adiga, P. R., Padmanabhan, G. and Sarma, P. S. (1962a) *Arch. Biochem. Biophys.,* **99,** 352.

Sivarama Sastry, K., Adiga, P. R., Venkatasubramanyam, V. and Sarma, P. S. (1962b) *Biochem, J.,* **85,** 486.

Sivarama Sastry, K., Raman, N. and Sarma, P. S. (1962c) *Anal Chem.,* **34,** 1302.

Venkateswerlu, G. and Sivarama Sastry, K. (1973) *Biochem. J.,* **132,** 673.

Venkateswerlu, G. and Sivarama Sastry, K. (1972). *Indian J. Biochem. Biophys.,* **16,** 84.

Webb, M. (1970) *Biochim. Biophys. Acta,* **222,** 428.