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The effect of copper on histidine biosynthesis in *Neurospora crassa*

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Abstract. The inhibition of growth of a wild strain of *Neurospora crassa* by Cu^{2+} is counteracted by histidine, histidine methyl ester, histidinol and Mn^{2+}. In the presence of Cu^{2+}, the total free amino acid content decreased by 30%. The decreased free amino acid pools of arginine, histidine and tyrosine were restored on the addition of Mn^{2+}. Histidinol phosphate phosphatase showed a decrease in activity in the presence of Cu^{2+}. This inhibition was reversed on the addition of excess Mn^{2+}. The data suggest that copper toxicity in the mould is due to suppression of histidine biosynthesis.

Keywords. *Neurospora crassa;* copper toxicity; histidine biosynthesis; histidinol phosphate phosphatase.

Introduction

Copper, toxic to several micro-organisms, affects glucose utilisation in *Escherichia coli* (Saklawska Szymonowa, 1953), in yeast (Antoine, 1963), and in *Lactobacillus* (Effinger, 1956). Copper also interferes with nitrogen metabolism in *Curvularia penniseti.* Under these conditions, free tyrosine was absent in the cultures in the presence of sublethal concentrations of copper (Tandon and Chandra, 1962). The effect of amino acids and other metabolic agents on the toxicity of copper to spores of *Stemphylium sarcinaeforme* and *Monilinia fructicola* was examined by Adam (1956). Very little is known on the interaction of copper with other metal ions like manganese and also copper with amino acids. Hence, it was proposed to elucidate the mechanism of copper toxicity in *Neurospora crassa.* This paper reports the role of Cu^{2+} on histidine biosynthesis.

Materials and methods

Chemicals

All chemicals and amino acids were of analytical grade obtained from British Drug House, India. Ninhydrin was a product of Fluka AG, Buchs, Switzerland. Histidinol dihydrochloride was prepared from L-histidine hydrochloride

Wosterfeld 1955). Histidinol phosphate was a kind gift of Dr. E. Hoffman of Kansas University, Michigan, USA. Histidine methyl ester dihydrochloride was obtained as a gift from Dr G. Tamu of College Station, Houston, Texas, USA.

Organism and growth conditions

A wild strain of *Neurospora crassa* Em 5297a employed in the present studies, was maintained on agar slants by weekly subcultures. The basal medium used for agar slants and far growth experiments was described earlier (Sivarama Sastry *et al.,* 1962a). The mould was grown in 50 ml conical flasks on 10 ml of basal medium, pH, 4·5–5·0. This medium contained ammonium tartrate and ammonium nitrate as the nitrogen sources. Tween–80 was added to the medium to suppress sporulation (Zalokar, 1954). The mycelia after 72h of growth were dried at 60–80° C overnight to obtain the dry wt for growth measurement. Various compounds and metal ions were added to the medium along with copper to ascertain their ability to counteract the toxicity. Restoration of 90% growth was used as criteria for reversal of toxicity.

Other estimations

The glucose content of the medium after the growth period was estimated by the method of Somogyi (1952). To measure histidine uptake, 24 h old mycelia were aseptically transferred, after proper washing with distilled water, to 10 ml medium containing histidine (0·12 mg/ml, 773·4μ mol). These mycelia were allowed to grow for a farther period of 24, 48 and 72 h. Aliquots at the end of the experiment were withdrawn and the histidine content was determined (Tabor, 1957). The 3-day old mycelia were subjected to wet digestion (Sivarama Sastry *et al.,* 1962b) and suitable aliquots were used to determine copper content using the oxaldihydrazide procedure (Gunnar, 1957).

The total nitrogen content of mycelial pads was measured by the micro-Kjeldahl method (Ballentine, 1957). To extract the free amino acids from 3-day old mycelial pads, the procedure of Fuerst and Wagner (1957) was employed and the total content of ninhydrin-positive material of these extracts was determined by the method described by Rosen (1957). The same extracts were used for the determination of individual amino acids by an automatic amino acid analyser. The procedure described by Ames *et al.* (1953) was used to extract the imidazoles from *N. crassa* mycelia and these were estimated as described above (Tabor, 1957).

Enzyme assay

Histidinol phosphate phosphatase (EC 3·1·3·15) was extracted from 3-day old mycelia using 0·1 M triethanolamine-HCl buffer, pH 7·1 and assayed according to the method described by Ames (1957). The method of Lowry *et al.* (1951) was employed for the determination of the protein content of enzyme extracts. The specific activity is expressed as μmol phosphate liberated per mg protein in 30 min at 30° C.

Results

Effects of copper on glucose uptake

Glucose uptake (550 mg/100 mg mycelial dry wt) in presence of Cu^{2+} (1·5 mM $CuSO_4$) was not different from the uptake in control culture (543 mg/100 mg dry wt).

Effect of amino acids and other compounds on copper toxicity

The reversal of copper toxicity by several amino acids (arginine, cysteine, glutamic acid, glycine, histidine, leucine, methionine, phenylalanine, proline and tryptophan); metal ions (cobalt, magnesium, manganese, molybdenum and zinc) and certain other compounds (aminoimidazole carboxamide, glucose, glutathione, histidine methyl ester dihydrochloride, histidinol, histidonol phosphate and imidazole) was studied. Of all the metal ions examined only Mn^{2+} (3·6 mM $MnSO_4$) was found to be effective in counteracting the toxicity. Amongst amino acids, only histidine (0·8 mM) or its methyl ester (0·3 mM) were found to counteract the inhibitory effect of copper (figure 1). The inhibition could be reversed by a Cu^{2+}/histidine molar ratio of 2 and a Cu^{2+}/histidinemethyl ester ratio of 3·3.

Histidinol phosphate, an intermediate in the later stages of histidine biosynthesis in *N. crassa* (Ames and Mitchell, 1955) could not reverse copper toxicity. Histidinol (0·56 mM) could, however, bring about a complete reversal of the growth inhibitory effects of Cu^{2+}. Mn^{2+}, histidine, histidine methyl ester and histidinol could not reverse copper toxicity when ammonium nitrate and ammonium tartrate

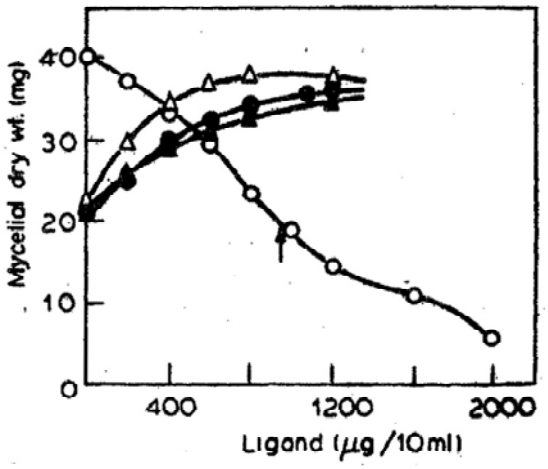

Figure 1. Copper toxicity in *N. crassa* and its reversal by histidine or histidinol or histidine methyl ester. The organism was grown on 10 ml of basal medium containing copper. Arrow. mark indicates 50% growth inhibition concentration. In the reversal experiments, histidine or histidinol or histidine methyl ester was aseptically added before inoculation along with 1·5 mM Cu^{2+}. The *x* axis indicates the amount of Cu^{2+} or histidine or histidinol phosphate or histidine methyl ester. O–O copper; Δ–Δ histidine; ●–● histidinol; ▲–▲ histidine methyl ester.

were replaced by sodium nitrate as the sole nitrogen source in the medium (Subramanian and Sarma, 1968).

The effect of histidine on copper toxicity was examined at various growth stages of the mould. The results are depicted in figure 2. When histidine was added after 24 h of growth (in the presence of 1·5 mM, Cu^{2+}) the mycelia could recover

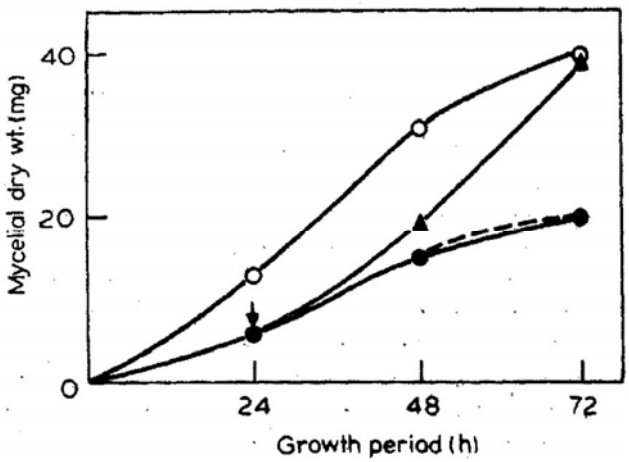

Figure 2. Effect of histidine on coper toxicity in *N. crassa* at various growth stages. O—O control (no additions); ●—● Cu^{2+} (1·5 mM) Δ—Δ histidine added at 24 h growth. Arrow indicates time of histidine addition. When histidine was added at 48 h, it did not reverse the inhibition of growth caused by Cu^{2+}.

from the toxicity. Addition of histidine after 48 h of growth in the presence of Cu^{2+} could not reverse the growth inhibition. The ability of toxic cultures to accumulate histidine was also studied. The results indicated that 24 h old mycelium grown on Cu^{2+} (1·5 mM) accumulated twice as much histidine (178 μmol/100 mg dry wt) as the control mycelium of the same age (90 μmo1/100 mg dry wt). Such an accumulation was not observed in the case, of mycelia where the toxicity could be reversed by Mn^{2+} (114 μmol/100 mg dry wt).

The effect of histidine on copper uptake during toxicity was also examined. The intracellular copper concentration values in control and toxic cultures were 966 and 1290 μg /100 mg dry wt of mycelia, respectively. These data reveal that histidine can counteract copper toxicity without lowering significantly its uptake by the organism.

Effect of copper on free amino acid pool

In contrast to the above observation, the data of table 1 reveal a 32% decrease of the total ninhydrin-positive material, in. mycelia grown in the presence of Cu^{2+}. However, not much difference can be noticed in the total nitrogen content between the control and toxic groups. The results also indicate a 43 % decrease in histidine content and a 52% decrease in aiginine content. Under these conditions, a marked decrease was noticed in tyrosine content. These amino acid levels can be restored to normal values on administration of excess Mn^{2+}. An increase in histidine content was observed in manganese-treated toxic cultures. It is also seen that

Table 1. The effect of copper on nitrogen metabolism in *N. crassa* and its reversal of its toxicity by manganese.

	Control (No additions)	Toxic (contains 1000 μg copper/10 ml medium)	Reversal (contains 1000 μg copper + 2000 μg manganese/10 ml medium)
(a) Total nitrogen content[b] (mg/100 mg dry wt.)	7·2 (100)[c]	8·4 (116)	8·8 (122)
(b) Total ninhydrin-positive material (μ mol/100 mg dry wt)	44·1 (100)	29·5 (68·5)	39·3 (89)
(c) Histidine content[a] (μ mol/100 mg dry wt)	0·7 (100)	0·4 (57)	1·1 (157)
(d) Arginine content[a] (μ mol/100 mg dry wt)	1·99 (100)	0·95 (48)	1·87 (94)
(e) Tyrosine content[a] (μ mol/100 mg dry wt)	0·22 (100)	Not detectable	0·16 (79)
(f) Total imidazole content (μ mol/100 mg dry wt)	104 (100)	153 (147)	102 (100)

Myeelia grown under different condition for 3 days were collected, washed, and used in the above experiments.

Numbers indicated in parentheses are percentage values.

[a] As determind by amino acid analyser.

[b] As determined by the micro-Kjeldahl method.

[c] Normalising the value in the control to 100.

the total imidazole content in mycelium from Cu^{2+}-treated cultures is 47% higher than in either the control mycelia or mycelia grown in presence of Mn^{2+}.

Effect of copper on histidinol phosphate phosphatase

Since only histidinol and histidine were able to counteract copper toxicity but not histidinol phosphate, the enzyme involved in the conversion of histidinol phosphate to histidinol was assayed. The specific activity of this enzyme is 0·162 in Cu^{2+} inhibited cultures as compared to the value of 0·404 observed in control mycelia. The results clearly indicate that the activity of this enzyme is inhibited to a significant extent in presence of Cu^{2+} , the inhibition being reversed by administration of Mn^{2+} (specific activity being 0·436).

Discussion

Earlier it had been shown that copper toxicity as manifested by growth inhibition in *N. crassa,* cultured on a medium containing ammonium nitrate and ammonium

tartrate as sole nitrogen sources, could be reversed by Mn^{2+} (Venkateswerlu and Sivarama Sastry,1979). This could not be achieved on a medium where the nitrogen sources, ammonium nitrate and ammonium tartrate, were replaced by sodium nitrate. Manganese was able to bring about this effect without suppressing the intracellular concentration of Cu^{2+}. This observation stimulated us to probe further into the role of Mn^{2+} on the growth of this mould. A similar effect in mitigating copper toxicity is shown by histidine in the present studies. This reversal could not be due to the formation of a copper-histidine complex since such complexes are known to be unstable under the pH conditions of the present growth studies (Yoshimo and Maki, 1966; Kaden and Zuberbuhler, 1966). It has also been observed by us that histidine methyl ester, whose copper complex has a weaker stability constant than that of histidine (Puplikova *et al.*, 1966), is more efficient in counteracting copper toxicity. Similarly, histidinol could alleviate copper toxicity, while its phosphate ester or imidazole were ineffective. Even histidine could reverse copper toxicity only when the mould was grown in a medium containing ammonium salts as the nitrogen source, but not in a medium where sodium nitrate was the sole nitrogen source. This effect is reminescent of the effect of Mn^{2+} on copper toxicity.

Histidine is also effective in reversing the toxicity if it is added during the first 48 h of growth but not later. It is also observed that histidine does not lower the intracellular copper concentration, a behaviour similar to the counter action by manganese (Venkateswerlu and Sivarama Sastry, 1979). Further, it is also evident that the toxic cultures have a greater ability for histidine uptake than the control cultures. However, the uptake may be related to the intracellular concentration of histidine, which is governed by its biosynthesis, utilisation and degradation. Under these conditions there was a decrease of not only histidine but also arginine, and only traces of tyrosine were present (table 1). These amino acid levels were restored to a marked extent on addition of Mn^{2+}. Though the content of arginine and tyrosine is decreased under toxic conditions they could not reverse the growth inhibition by copper (data not given), in a manner similar to histidine. This may be due to a histidine mediated control of arginine biosynthesis (Carsiotis *et al.*, 1974). A similar regulation of aromatic amino acid biosynthesis has also been reported in *Bacillus subtilis* (Nester, 1968).

Our results indicate that under conditions of copper toxicity, the activity of the enzyme histidinol phosphate phosphatase decreases. It is restored to normal levels, on addition of Mn^{2+} to the cultures. Brenner and Ames (1971) as well as Houston and Margaret (1974) have reported that the EDTA inhibition of this enzyme can be released by Mn^{2+}. Earlier, it was also pointed out that this enzyme has a divalent metal ion requirement in *N. crassa* since the enzyme was found to be sensitive to 8-hydroxy-quinolme (Ames, 1957). The accumulation of increased imidazole compounds in toxic cultures which disappear on supplementation of excess Mn^{2+} lends further support to the suggestion that Mn^{2+} is essential for the biosynthesis of histidine and that this process is interfered with, in the presence of Cu^{2+}.

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