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Effects of Molybdenum, Copper and Iron on Some Enzymes in *Neurospora crassa*

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SUMMARY: Diverse enzyme changes were obtained in *Neurospora crassa* grown at deficient, optimal and toxic levels of Mo, Cu and Fe. These included mutual antagonisms between the metals, single effects of one element or additive ones of more than one element. Cytochrome *c* oxidase content, enhanced by Cu treatment, was decreased by increasing Mo; the reverse relationship was obtained with nitrate reductase. Cytochrome *c* and nitrite reductases, however, were depressed by amounts both of Cu and Mo which were toxic to growth; the effects were additive. Single effects were as follows: TPNH diaphorase and acid phosphatase were depressed by Cu and Mo excess respectively; the iron enzymes and the two diaphorases were more active when Fe was increased from deficiency to sufficiency level. High iron concentrations increased nitrate reductase but depressed the nitrite system. Molybdenum is shown to inhibit acid phosphatase competitively *in vitro*, presumably by forming a molybdo-phosphate addition compound with the substrate. Cu dispersed this complex and restored the enzyme activity to normal. Vanadate or tungstate had a similar effect to molybdate in depressing the enzyme but Cu was found to reverse the inhibition.

Thus it appears that antagonisms between metals in nutrition experiments may be related to their effects on certain enzymes as illustrated here for Cu, Mo and Fe in *Neurospora*.

Antagonisms between nutrients are well established in plants and animals but little is known how these affect cell metabolism. Healy, Cheng & McElroy (1955) showed that the effect of Co excess in depressing iron-containing enzymes in *Neurospora crassa* was partially reversed by increasing Fe in the culture solution. van Reen (1953) demonstrated that Zn toxicity in the rat decreased the amount of depressed liver catalase and cytochrome *c* oxidase and that this effect was remedied by adding copper sulphate to the diet.

This paper amplifies a preliminary report in which it was shown that Cu and Mo were reciprocally antagonistic towards cytochrome *c* oxidase and nitrate reductase in *Neurospora crassa* (Commissiong & Nicholas, 1957); the effects of iron concentration on some enzymes are also considered.

METHODS

Culture medium. *Neurospora crassa*, macroconidial wild type 5297a, was grown in the following medium: sucrose, 20 g.; Na tartrate, 1 g.; KH_2PO_4 , 3 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; NaCl, 0.1 g.; CaCl_2 , 0.1 g. After removing Fe, Cu and Mo from the macronutrients (Nicholas, 1952), the following micronutrients were added: biotin, 5 $\mu\text{g.}$; Zn as $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 200 $\mu\text{g.}$; Mn as $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 20 $\mu\text{g.}$; Cu as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ at 0, 0.08 mg. or 10 mg.; Mo as

$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ at 0, 0.02 mg. or 200 mg.; Fe as $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ at 0, 0.2 mg. or 35 mg.; distilled water, 1 l.; pH 4.8. The three concentrations of Fe, Cu and Mo represented deficiency (0), normal (1), and toxicity concentrations (2), based on the dry weight yields of the felts.

Purification of culture media. Fe, Cu and Mo were removed from the macro-nutrients by the copper co-precipitation and quinoline methods (Nicholas, 1952). The micronutrient elements Fe, Cu, Mn, Zn and Mo were used as 'spectroscopically pure' compounds supplied by Johnson and Mathey (Hatton Garden, London, E.C.1). Biotin (25 $\mu\text{g.}$) dispensed in phosphate buffer (pH 7) was supplied in ampoules by the Ashe Laboratories (Leatherhead, Surrey). The culture medium (200 ml.) was dispensed in 1 l. Erlenmeyer flasks, and after sterilizing at 10 lb./sq.in. for 10 min. and cooling, various amounts of Fe, Cu and Mo were added to their respective containers. The media were then inoculated with a spore suspension of *Neurospora crassa* 5297a in glass-distilled water and incubated for 4 days at 30° in the dark. The cultures were shaken periodically to prevent sporulation.

Preparation of cell-free extracts. The mycelial felts from each of the twenty-seven treatments, collected separately in sintered Buchner funnels, were washed thoroughly with glass-distilled water and frozen overnight at -17°. They were then homogenized, in groups of nine, in four times their weight of cold 0.1 M-phosphate buffer (pH 7.5) in a mortar and then in a Ten Broeck glass macerator at 0°. The homogenates were centrifuged at 3000 g for 10 min. at 4° and the supernatant solutions used for enzyme assays.

Cofactors and other compounds. Diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN) (both of 95 % purity) and cytochrome *c* were obtained from the Sigma Chemical Company, U.S.A. Reduced DPN was prepared enzymically by the alcohol dehydrogenase method of Pullman, Colowick & Kaplan (1952) and reduced TPN by using the isocitric dehydrogenase enzyme from acetone powder of pig heart. Boiled pig heart extract, centrifuged at 18,000 g for 20 min. at 4° was used as a source of flavine adenine dinucleotide (FAD).

Catalase activity was measured at 37° by the perborate method of Feinstein (1949). The reaction mixture contained 8 ml. 1.5 % (w/v) $\text{NaBO}_3 \cdot 4\text{H}_2\text{O}$ (pH 6.8); 1.9 ml. 0.1 M-phosphate buffer (pH 6.8); 0.1 ml. enzyme extract; this was incubated at 37° for 5 min. Then 10 ml. 2 N- H_2SO_4 was added and the solution titrated with 0.05 N- KMnO_4 . The values are expressed as m-mole perborate degraded/5 min./mg. protein.

Peroxidase was determined by the spectrophotometric method of Smith, Robinson & Stotz (1949). The reaction mixture contained 1.45 ml. McIlvaine's buffer (pH 6.0); 1.0 ml. 10^{-3} reduced m-2:6-dichloroindophenol; 0.5 ml. 0.1 M- H_2O_2 ; 0.05 ml. enzyme extract. The oxidation of the dye was followed at 625 $m\mu$. at 15 sec. intervals. The unit of activity was the change in $\log I_0/I$ of 0.001/min. calculated between 15 and 75 sec./mg. protein.

DPNH and TPNH *oxidases* ('diaphorase' systems) were measured by following dye reduction at 625 $m\mu$. The reaction mixture used was: 2.85 ml. 0.1 M-phosphate buffer (pH 7.5); 0.15 ml. 1×10^{-3} M-2:6-dichloroindophenol;

0.1 ml. 0.1 M-KCN; 0.05 ml. DPNH (10^{-3} M) or TPNH ($2 \mu\text{mole/ml.}$); 0.05 ml. enzyme extract.

Cytochrome c oxidase was estimated by the method of Smith & Stotz (1949). The reaction mixture contained 1.95 ml. 0.1 M-phosphate (pH 6.8); 1.0 ml. 10^{-3} reduced m-2:6-dichloroindophenol; 0.2 ml. 1×10^{-4} M-cytochrome *c*; 0.05 ml. enzyme extract. The unit of activity was the change in $\log I_0/I$ of 0.001/min. calculated between 15 and 75 sec./mg. protein.

DPN-*cytochrome c reductase* was measured at 551 m μ . The reaction solution contained 2.65 ml. 0.1 M-phosphate buffer (pH 7.5); 0.03 ml. 0.1 M-KCN; 0.2 ml. 0.1 M-MgCl₂.6H₂O; 0.1 ml. cytochrome *c*, 3×10^{-3} M; 0.1 ml. DPNH (10^{-3} M); 0.1 ml. enzyme extract.

Nitrate reductase was measured by the method of Nicholas, Nason & McElroy (1954). The assay method consisted of adding 0.1 ml. enzyme extract to a solution containing 0.1 ml. 0.1 M-KNO₃; 0.1 ml. boiled pig heart extract; 0.1 ml. 10^{-4} M-KCN; 0.1 ml. 1×10^{-3} M-TPNH; 0.1 ml. 0.1 M-phosphate buffer (pH 7.5). The mixture was incubated for 10 min. at 25° and the reaction stopped by adding 1.0 ml. 0.001 % (w/v) *N*-(1-naphthyl)-ethylenediamine dihydrochloride reagent followed by 0.5 ml. 1 % (w/v) sulphanilamide reagent. The volume was made up to 5 ml. with glass-distilled water and read on the Spekker absorptiometer, after 10 min., at 540 m μ . Control tests without TPNH were used to correct for the turbidity of the enzyme preparation. Units of activity are expressed as m $\mu\text{mole NO}_2^-$ formed in 10 min./mg. protein.

Nitrite reductase was determined by the method described by Medina & Nicholas (1957). The test consisted of adding 0.1 ml. enzyme to a solution containing 0.2 ml. 10^{-3} M-NaNO₂; 0.1 ml. boiled pig heart extract; 0.1 ml. DPNH (10^{-3} M) and 0.25 ml. 0.1 M-pyrophosphate buffer (pH 7.5). After 10 min. incubation at 25°, 0.1 ml. 1.0 M-Ba acetate and 3.15 ml. cold absolute ethanol were added. The mixture was stirred well, left at 4° for 30 min. and the precipitate centrifuged down; 0.5 ml. of the supernatant fluid was then taken and the colour developed as in the nitrate reductase assay.

Alkaline and acid phosphatases were estimated by the method of Bessey, Lowry & Brock (1946) in which the breakdown of *p*-nitrophenylphosphate to *p*-nitrophenol formed in 30 min./mg. protein was determined at alkaline and acid pH.

Protein was determined by the Folin method as described by Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Enzyme assays were made on the extracts from the mycelial felts of each of the twenty-seven treatment combinations; the results were analysed statistically.

Iron-containing enzymes

Effect of iron concentration. Catalase, peroxidase, cytochrome *c* oxidase and its reductase were all affected by an increase in iron concentration as illustrated in Fig. 1. Their means showed in each case a significant increase from the Fe₀

to the Fe_1 level. Cytochrome *c* oxidase was depressed at Fe concentrations that depressed growth.

Effect of copper. Of the four iron-containing enzymes examined, only cytochrome *c* oxidase and cytochrome *c* reductase were significantly affected by the copper treatment. From deficiency to the toxicity level of the metal, based on growth measurements, catalase activity decreased steadily.

Fig. 1. Ordinates: means of units enzyme activity/mg. protein (units see Methods). Abscissae: deficiency (0), sufficiency (1) and toxicity (2) levels for Fe, Cu and Mo in the culture solution, based on dry weight yield of the felts.

The response of cytochrome *c* oxidase and cytochrome *c* reductase to an increase in copper varied markedly. Cytochrome *c* oxidase was significantly increased from the deficiency to the high levels that depressed yields of the felts by 50%. Cytochrome *c* reductase, however, was significantly depressed at the highest concentration of the metal.

Effect of molybdenum. The enzymes differed in their response to molybdenum. Although the mean values for catalase or peroxidase activity were not significantly different for the various Mo treatments there was a tendency

for an increase in activity of these two enzymes from deficiency to the sufficiency level, followed by a depression at concentrations that depressed felt weight.

Cytochrome *c* reductase was significantly depressed by increasing Mo from deficiency to values that were associated with a depression in the yields of felts. The oxidase showed a similar trend and the difference between the Mo₁ and Mo₂ treatments was significant.

In vitro experiments. The only effect of adding Cu or Mo *in vitro* at 1 µg. to 0.1 ml. extract of normal felts was to increase the endogenous rates of peroxidase and cytochrome *c* oxidase.

Fig. 2. Ordinates: means of units enzyme activity/mg. protein (units see Methods). Abscissae: deficiency (0), sufficiency (1) and toxicity (2) levels for Fe, Cu and Mo in the culture solution, based on dry weight yield of the felts.

Flavoprotein enzymes

Effect of iron. The contents of DPNH diaphorase, nitrate and nitrite reductases were significantly changed by the iron treatments as shown in Fig. 2. The TPNH diaphorase showed a gradual increase from the deficiency to the

toxicity level, based on yield of felts. The DPNH diaphorase was significantly increased in activity from the Fe₀ to the Fe₁ level.

Nitrate and nitrite reductases showed opposite effects in their response to iron at the highest concentration; the former enzyme activity was stimulated whereas that for the nitrite reductase was depressed.

Effect of copper. Increasing copper from sufficiency to toxicity concentrations based on the weight of mycelial felts, depressed both DPNH and TPNH diaphorases, these effects, however, just failed to reach significance for the latter enzyme. Nitrate and nitrite reductases were significantly depressed at the highest concentrations of copper.

Effect of molybdenum. Molybdenum status did not significantly affect the diaphorase enzymes although a high level of the micronutrient tended to depress them. Nitrate reductase increased from Mo₀→Mo₁→Mo₂ whereas nitrite reductase showed the opposite effect.

In vitro studies. The addition of Cu or Mo at 1 µg. to 0.1 ml. extract of normal felts had no effect either on DPNH or TPNH diaphorases. Nitrate and nitrite reductases were, however, depressed at this Cu concentration.

Phosphatases

Alkaline phosphatase was not changed by the treatments given whereas the acid phosphatase was significantly depressed when molybdenum was in excess as shown in Fig. 3.

Fig. 3. Ordinates: means of units enzyme activity/mg. protein (units see Methods). Abscissae: deficiency (0), sufficiency (1) and toxicity (2) levels for Fe, Cu and Mo in the culture solution, based on dry weight yield of the felts.

In vitro experiments. Alkaline phosphatase was unchanged by the addition either of Cu or Mo to the extracts. The results of *in vitro* experiments with acid phosphatase are shown in Table 1. Molybdate markedly depressed acid phosphatase whereas Cu stimulated its activity. The addition of Cu partially

reversed the inhibition by molybdate whereas Fe, Mn and Zn were without effect. Vanadate or tungstate also markedly depressed the enzyme.

Table 1. *The effect of metal addition in vitro on the activity of acid phosphatase*

Values as percentage of normal

Control	FeCl ₃	MnCl ₂	ZnSO ₄	CuSO ₄	(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	NH ₄ VO ₃	Na ₂ WO ₄ ·2H ₂ O	Cu + Mo
100	94	100	107	118	30	19	20	46

1 μ g. of each metal added to the reaction mixture which consisted of: 1 ml. acid phosphatase reagent and 0.05 ml. enzyme extract incubated at 38° for 30 min. and made to a final volume of 5 ml. with 0.06N-NaOH.

Fig. 4

Fig. 5

Fig. 4. Lineweaver-Burk (1934) plot of the competitive inhibition of acid phosphatase activity by sodium molybdate. Assay mixtures consisted of 1 ml. acid phosphatase reagent, 0.05 ml. enzyme extract and 0.05 ml. of either distilled water or a solution of Na₂MoO₄ of 8 μ g. Mo/ml. The mixtures were incubated at 38° for 30 min. and then 3.9 ml. of 0.06N-NaOH were added to make a final volume of 5 ml. The intensity of the *p*-nitrophenol colour developed was read on the Spekker Absorptiometer at 540 m μ . 1/V, reciprocal of the intensity of the *p*-nitrophenol at 540 m μ . $\times 10^4$; 1/S, reciprocal of substrate concentration where the normal substrate concentration is considered as unity. x—x, no inhibitor; O—O, with molybdate.

Fig. 5. Lineweaver-Burk (1934) plot showing competitive inhibition of copper by molybdate on acid phosphatase (*in vitro*). Assay mixtures consisted of 1 ml. acid phosphatase reagent, 0.05 ml. enzyme extract, 0.05 ml. Na₂MoO₄·2H₂O at 0, 1.0, 2.1 or 5.2 $\times 10^{-6}$ M. Na₂MoO₄·2H₂O and 0.05 ml. CuSO₄·5H₂O at 0.6, 0.9, 1.2 and 1.5 $\times 10^{-6}$ M. The mixtures were incubated at 38° for 30 min. and then 3.85 ml. 0.06N-NaOH were added to make a final volume of 5 ml. The intensity of the *p*-nitrophenol colour developed was read on the Spekker absorptiometer at 540 m μ . 1/V, reciprocal of the intensity of the *p*-nitrophenol at 540 m μ . $\times 10^4$; 1/S, reciprocal of the amount of copper added per assay. □—□, no molybdenum; x—x, 1.0 $\times 10^{-6}$ M-Na₂MoO₄·2H₂O; △—△, 2.1 $\times 10^{-6}$ M-Na₂MoO₄·2H₂O; O—O, 5.2 $\times 10^{-6}$ M-Na₂MoO₄·2H₂O.

Fig. 4 shows that molybdate inhibits the enzyme competitively. The K_m for the enzyme-substrate complex was calculated to be 1.6×10^{-3} M and the dissociation constant for the enzyme-inhibitor complex was 0.68×10^{-5} M.

The results in Fig. 5 demonstrate the competitive nature of the inhibition of Cu by molybdate (*in vitro*) on acid phosphatase.

DISCUSSION

Although it is well established that Mo and Cu are antagonistic in animal nutrition experiments, little work has been done on enzyme changes which occur in the tissues under these conditions. Changes in enzyme pattern in *Neurospora crassa* given different amounts of Cu, Mo and Fe, reported in this paper, illustrate that some key enzymes, drastically decreased by one of these metals, may be partially restored by increasing one of the other two in the culture solution. Thus the activity of cytochrome *c* oxidase was enhanced by adding Cu, whereas amounts of Mo that decreased felt weight, decreased it. A similar result was obtained by van Reen (1953) in the rat where adding Cu alleviated the effect of Zn in depressing cytochrome *c* oxidase in the liver. The effect of Cu in increasing this enzyme is in keeping with the suggestion that Cu is a component of the DPNH oxidase system (Green, Basford & Mackler, 1956).

The opposite effect was obtained with nitrate reductase, a molybdo-flavo-protein, where an increase in Mo enhanced its activity and Cu depressed it. The addition of Cu also depressed the enzyme *in vitro* but the addition of Mo failed to restore the low activity of nitrate reductase prepared from Mo-deficient felts (Nicholas, Nason & McElroy, 1954).

Cytochrome *c* reductase was decreased when Cu was in excess, in contrast to a substantial increase in its oxidase, whereas iron treatments increased both enzymes. The effects of Cu excess on cytochrome *c* reductase is unlikely to be caused by interference with haem-iron because the oxidase activity is enhanced under similar conditions. Cu excess may, therefore, affect the non-haem-iron which is only present in the reductase system.

A number of enzymes decreased by toxic concentrations of one of the metals were either not increased by varying the concentrations of the other two in the culture solution or were depressed further. Thus, it appears that nutrient antagonisms are reflected by some enzymes only and in this study these were metallo-proteins.

Acid phosphatase was depressed by increasing Mo from sufficiency to toxicity levels (based on dry weight of the felts), thus confirming a similar effect observed in higher plants (Spencer, 1954). Molybdate is shown to be a competitive inhibitor of copper in acid phosphatase *in vitro*. Vanadate and tungstate had a similar inhibitory effect to molybdate, presumably because they too form complexes in acid solution with *p*-nitrophenyl phosphate, the substrate for the phosphatase. It is noteworthy that alkaline phosphatase was unaffected because the substrate-molybdate complex is unstable at pH 10.3; the pH at which the assay is made.

In these experiments, substantial differences were required between mean values before significant effects were obtained. This was in the main due to the limited number of replicates that could be conveniently handled in enzyme work of this nature. For this reason some effects known to occur in previous experiments just failed to reach significance in these studies, e.g. a greater catalase and peroxidase activity in felts given optimal Mo (Nicholas, 1956).

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