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Cobalt Toxicity and Iron Metabolism in *Neurospora crassa*

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1. Increasing concentrations of cobalt in the medium result in increased production of an iron-binding compound and a corresponding fall in catalase activity of *Neurospora crassa*. 2. Cobalt rapidly depletes the medium of iron by enhancing the rate of iron uptake by the mycelium. 3. With toxic amounts of cobalt there is a fall in bound ^{59}Fe and haem ^{59}Fe as well as a decreased incorporation of [2-^{14}C]glycine into the mycelial haem fraction. The production of the iron-binding compound precedes the fall in the iron-dependent systems mentioned. 4. The ^{59}Fe bound to the iron-binding compound acts as a better iron source for haem synthesis in cell-free extracts as compared with $^{59}FeSO_4$. 5. Cobalt inhibits iron incorporation into protoporphyrin in cell-free extracts but is not itself incorporated to an appreciable extent.

Cobalt toxicity has been related to iron deficiency in *Aspergillus niger* (Adiga, Sivarama Sastry, Venkatasubramanyam & Sarma, 1961). A direct cobalt–iron antagonism is also demonstrable in *Neurospora crassa* (Sivarama Sastry, Adiga, Venkatasubramanyam & Sarma, 1962). Healy, Cheng & McElroy (1955), in studies at the enzymic level, concluded that cobalt toxicity is comparable with straight iron deficiency in *N. crassa*. A further correlation in this organism has been the isolation of an iron-binding compound (X) from the culture fluid when the organism is grown under conditions of cobalt toxicity or straight iron deficiency (Padmanaban & Sarma, 1964). The iron complex of the iron-binding compound (XFe) serves as a good source of iron for the parent organism to maintain normal growth, catalase and a few non-haem iron enzyme activities. Further, this organic iron is incorporated into the mycelium much faster than inorganic iron or a simple chelate like ferric citrate (Padmanaban & Sarma, 1965). Preliminary studies show that this iron-binding compound belongs to the siderochrome (Bickel *et al.* 1960) class of compounds.

A study of the interference of cobalt with the iron metabolism of the mould *N. crassa* has been carried out and the results are presented below.

EXPERIMENTAL

Culture and growth conditions. N. crassa Em 5297a (wild) was used. The composition of the medium was as described by Sivarama Sastry *et al.* (1962). The organism was grown in 50ml. Pyrex conical flasks in 10ml. of basal medium at 30° without shaking.

Effect of cobalt on ^{59}Fe *uptake.* The organism was grown with 800μg. of cobalt/10ml. of basal medium and optimum concentrations of iron (1μg./10ml. of basal medium) as [^{59}Fe]ferric citrate. After growth for the required period, the mycelia were removed, washed free of adhering radioactivity, weighed and made into a fine suspension with 5ml. of water. A portion was used to measure the total radioactivity incorporated, by using a DSS–5 scintillation detector attached to a decade scaler (Nuclear–Chicago Corp., Des Plaines, Ill., U.S.A.). The rest of the sample was precipitated with cold trichloroacetic acid (final concn. 10%, w/v). The precipitate was washed four times with cold trichloroacetic acid and the radioactivity on the precipitate was measured after digestion with acid. The trichloroacetic acid-precipitable ^{59}Fe is referred to below as bound ^{59}Fe. The results are expressed in terms of mycelial dry weight. The dry weight/fresh weight ratio was 1:5.

Effect of cobalt on ^{59}Fe *and* [2-^{14}C]*glycine incorporation into the mycelial haem fraction.* The mycelia grown in the presence of cobalt and the appropriate tracer were washed free of adhering radioactivity and the haemin was isolated from the mycelial acetone-dried powder according to the following method of Labbe & Nishida (1957). A sample (1g.) of the acetone-dried powder was suspended in 5ml. of water, and 100ml. of a solvent mixture of acetic acid saturated with $SrCl_2$ and acetone (1:3, v/v) was added. Since insufficient haemin could be isolated to permit subsequent purification, 50mg. of carrier haemin was added. The mixture was kept overnight and then briefly heated to the boiling point of the solvent and filtered. The residue was washed thrice with acetic acid–acetone mixture and haemin was isolated from the filtrate. The radioactivity of a known amount of isolated and recrystallized haemin was taken as a measure of the radioactivity incorporated into the mycelial haem fraction.

^{14}C measurements were made in a Geiger–Müller end-window counter attached to a decade scaler (Nuclear–Chicago Corp.). Appropriate corrections due to self-absorption, background and radioactive decay were applied.

Effect of cobalt on ^{59}Fe *incorporation into protoporphyrin in cell-free extracts.* The procedure employed was essentially the same as described by Nishida & Labbe (1959) involving the estimation of radioactivity associated with the enzymically formed haemin from ^{59}Fe and protoporphyrin. The 72hr.-old normal mycelia (10g. fresh wt.) were ground in 50ml. of 0·1M-phosphate buffer, pH7·5, in the presence of 100mg. of Tween 20 and glass powder by using a pestle and mortar and centrifuged at 15000*g* for 20min. after keeping the homogenate stirred for 1hr. This enzyme preparation was incubated with the other constituents in amounts as used by Labbe & Hubbard (1961) for 2hr. under N_2 at 37° in a Dubnoff metabolic shaker. The reaction was stopped with the acetic acid–acetone mixture, and haemin was isolated after adding 10ml. of carrier blood, by the procedure described above. The recrystallized haemin was dissolved in alkaline pyridine and portions were used to measure radioactivity and haemin content.

Catalase assay. Catalase activity was determined in phosphate buffer extracts of the mycelia by the procedure described by Ramachandran & Sarma (1954).

Protein content. The method of Lowry, Rosebrough, Farr & Randall (1951) was used to estimate protein in the enzyme samples.

Estimation and isolation of the iron-binding compound For estimation the method employed by Neilands (1957) was used. To 3ml. of the culture fluid, 1ml. of $FeCl_3,6H_2O$ (1mg. of iron/ml.) solution was added and centrifuged. The extinction of the supernatant was measured at 440mμ, since XFe has an absorption maximum at this wavelength. The iron-binding compound (X) was isolated as the iron complex (XFe) from the cobalt-toxic culture fluid by the addition of $FeCl_3,6H_2O$ followed by $(NH_4)_2SO_4$ saturation, benzyl alcohol extraction and subsequent purification as described by Padmanaban & Sarma (1964). $X^{59}Fe$ was isolated after adding $^{59}FeCl_3$ directly to the cobalt-toxic culture fluid. The iron content in XFe was estimated, after wet digestion of the sample, by the *o*-phenanthroline method (Sandell, 1950).

RESULTS

A reciprocal relationship exists in *N. crassa* between the production of the iron-binding compound and catalase activity and this is governed by the iron status of the growth medium (Padmanaban & Sarma, 1965). Increasing concentrations of cobalt cause an increase in the production of the iron-binding compound and a corresponding fall in catalase activity (Table 1). The fall in the production of the iron-binding compound at the highest concentration of cobalt employed could be due to the severe inhibition of the growth of the organism.

Thus the results presented in Table 1 simulate conditions of iron deficiency and it was decided to examine whether cobalt causes an iron deficiency by inhibiting the uptake of this essential metal by the organism. Cobalt does not inhibit iron uptake but enhances instead the rate of incorporation (Table 2). The ^{59}Fe incorporated by the mycelium grown with cobalt is double the corresponding value for the normal mycelium at the end of 24hr. growth. This indicates that cobalt enhances the rate of ^{59}Fe incorporation into the mycelium in addition to its growth-inhibitory effects, and this contributes to the considerably higher iron concentration, when

Table 1. *Effect of increasing concentrations of cobalt on growth, catalase activity and iron-binding compound production in* N. crassa *at the end of 72hr. growth*

Experimental details are given in the text.

Cobalt added (μg./10ml. of medium)	Growth (mg. dry wt.)	Catalase activity (ml. of 10mM-$KMnO_4$ consumed/mg. of protein/5min.)	Iron-binding compound ($E_{440m\mu}$)
0	42·8	50·2	0·03
50	46·0	35·4	0·10
200	37·2	20·2	0·16
600	28·1	13·1	0·20
800	22·0	9·0	0·22
1000	13·2	5·7	0·07

Table 2. *Effect of cobalt on total and bound* ^{59}Fe *in the mycelia as a function of growth period in* N. crassa

The basal medium contained in 10ml. 24·6mμc (1·0μg. of iron) as [^{59}Fe]ferric citrate. Experimental details are given in the text.

Period of growth (hr.)	Normal		Cobalt-toxic	
	Total ^{59}Fe (mμg.atoms/100mg. dry wt.)	Bound ^{59}Fe (mμg.atoms/100mg. dry wt.)	Total ^{59}Fe (mμg.atoms/100mg. dry wt.)	Bound ^{59}Fe (mμg.atoms/100mg. dry wt.)
24	64·2	10·6	558·1	45·4
30	42·7	10·5	218·7	15·2
48	38·5	9·8	85·5	7·2
54	32·3	9·3	74·6	6·3
72	30·6	9·2	53·7	5·7

Table 3. *Effect of cobalt on catalase, iron-binding compound and haem* ^{59}Fe *in the mycelia as a function of growth period in* N. crassa

The basal medium contained in 10 ml. 120·9 mμc (1 μg. of iron) as [^{59}Fe]ferric citrate. Experimental details are given in the text.

	Normal treatment				Cobalt-toxic treatment			
Period of growth (hr.)	Growth (mg. dry wt.)	Catalase activity (ml. of 10 mM-$KMnO_4$/mg. of protein/5 min.)	Iron-binding compound ($E_{440m\mu}$)	Haem ^{59}Fe (μμg.atoms/10 mg. of haemin/100 mg. dry wt.)	Growth (mg. dry wt.)	Catalase activity (ml. of 10 mM-$KMnO_4$/mg. of protein/5 min.)	Iron-binding compound ($E_{440m\mu}$)	Haem ^{59}Fe (μμg.atoms/10 mg. of haemin/100 mg. dry wt.)
24	8·2	7·2	–	74·0	3·2	8·5	0·04	81·0
48	37·0	39·2	—	91·0	19·0	10·4	0·12	73·0
72	46·2	52·3	0·03	109·9	25·2	11·0	0·22	66·0

Table 4. *Effect of cobalt on* [2-^{14}C]*glycine incorporation into the mycelial haem fraction of* N. crassa

The basal medium contained in 10 ml. 250·0 mμc (48·0 μg. of glycine) of [2-^{14}C]glycine. Experimental details are given in the text.

Treatment	Total incorporation (μmoles/g. dry wt.)	Incorporation into haem (mμmoles/10 mg. of haemin/g. dry wt.)
Normal	12·0	6·9
Cobalt-toxic	17·1	4·7

Table 5. *Effect of cobalt on* ^{59}Fe *incorporation into protoporphyrin in cell-free extracts of* N. crassa

The incubation mixture in 2 ml. contained: iron ($^{59}FeSO_4$), 24 mμmoles; cobalt ($^{60}CoCl_2$), 30 mμmoles; protoporphyrin, 30 mμmoles; tris (pH 7·8), 180 μmoles; ascorbic acid, 40 μmoles; enzyme, 0·5 ml. containing 6 mg. of protein/ml. The iron-binding fraction prepared from XFe was added in 0·1 ml. and in slight excess equivalents of the iron present in the incubation mixture. The amount of radioactivity included as ^{59}Fe or ^{60}Co was 250·0 mμc. Experimental details are given in the text.

Metal source	Metal incorporated (μμg. atoms/10 mg. of haemin)
$^{59}FeSO_4$	87·2
$^{59}FeSO_4$*	8·4
$^{59}FeSO_4$ + 30 mμmoles of $CoCl_2$	76·9
$^{59}FeSO_4$ + 60 mμmoles of $CoCl_2$	73·0
$^{59}FeSO_4$ + 150 mμmoles of $CoCl_2$	52·9
$^{59}FeSO_4$ + iron-binding fraction	257·1
$^{59}FeSO_4$ + iron-binding fraction*	98·0
$^{59}FeSO_4$ + iron-binding fraction + 30 mμmoles of $CoCl_2$	221·4
$^{59}FeSO_4$ + iron-binding fraction + 60 mμmoles of $CoCl_2$	211·3
$^{59}FeSO_4$ + iron-binding fraction + 150 mμmoles of $CoCl_2$	130·6
$^{60}CoCl_2$	9·5

* Non-enzymic incorporation (enzyme denatured or omitted).

the results are expressed on a unit mycelial weight basis. However, bound ^{59}Fe shows a significant decrease at the end of the growth period (72 hr.) in cobalt-toxic mycelia, indicating that the cobalt–iron antagonism is intracellular in this organism.

To detect the primary site of induced iron deficiency, the effects of cobalt on growth, catalase activity, iron-binding compound production, bound ^{59}Fe and haem ^{59}Fe in the mycelia were determined as a function of the growth period. From the results presented in Tables 2 and 3 it is evident that, at 24 hr. of growth in the presence of cobalt, catalase activity, bound ^{59}Fe and haem ^{59}Fe values are higher than or comparable with those of the normal mycelia. The iron-binding compound can be detected in the culture fluid even at this period of growth under cobalt-toxic conditions. The subsequent decrease in bound ^{59}Fe and haem ^{59}Fe thus indicates an effect of cobalt at the level of iron utilization for the synthesis of key metabolites.

The fall in haem ^{59}Fe values can be due to two factors, namely an effect of cobalt on the synthesis of the porphyrin moiety and a possible competition between cobalt and iron at the level of incorporation into the porphyrin nucleus. The former aspect was examined by growing the organism under cobalt-toxic conditions in the presence of [2-^{14}C]-glycine. At the end of 72 hr. growth cobalt does not inhibit total [2-^{14}C]glycine incorporation into the mycelia but inhibits incorporation into the haem fraction (Table 4).

Previously it was found that XFe can act as a good source of iron for *N. crassa* and is incorporated much faster into the mycelium than is inorganic iron of a simple chelate like ferric citrate. Also, the incorporation of inorganic iron or ferric citrate is

Table 6. *Iron incorporation from* $X^{59}Fe$ *and* $^{59}FeSO_4$ *into protoporphyrin in cell-free extracts of* N. crassa

The low specific activity of the isolated $X^{59}Fe$ necessitated the use of high concentrations of substrates. The incubation mixture in 5ml. contained: iron ($^{59}FeSO_4$ or $X^{59}Fe$), 0·5 μmole; protoporphyrin, 0·6 μmole; tris (pH 7·8), 360 μmoles; ascorbic acid, 80 μmoles; enzyme, 2ml. containing 6mg. of protein/ml. The amount of radioactivity included was 65·1 mμc. Experimental details are given in the text.

Iron source	Iron incorporated (mμg. atoms/10mg. of haemin)
$^{59}FeSO_4$	12·1
$^{59}FeSO_4$*	1·3
$X^{59}Fe$	23·7
$X^{59}Fe$*	10·7

* Non-enzymic incorporation (enzyme denatured or omitted).

markedly increased when the iron-binding fraction prepared from XFe is added (Padmanaban & Sarma, 1965). The effect was therefore examined of the iron-binding fraction on iron incorporation into protoporphyrin, with and without cobalt, in cell-free extracts (Table 5). In the presence of the iron-binding fraction, ^{59}Fe incorporation into protoporphyrin is significantly enhanced as compared with when $^{59}FeSO_4$ alone is used. The superiority of this type of organic iron is further emphasized since intact $X^{59}Fe$ has been found to act as a better iron source than $^{59}FeSO_4$ (Table 6). When the organic iron is used as the iron source the non-enzymic incorporation into protoporphyrin is high and is of the same order as that of enzymic incorporation of inorganic iron. Cobalt inhibits ^{59}Fe incorporation even in the presence of the iron-binding fraction, significantly when present in definite excess of iron. However, when cobalt is used in amounts equivalent to and in place of iron, its incorporation into protoporphyrin is less than 10% of that of iron.

DISCUSSION

Increasing concentrations of cobalt result in increased production of the iron-binding compound and a corresponding fall in catalase activity (Table 1). An identical picture has been obtained when there is a decrease in the iron concentration of the medium from the optimum level (Padmanaban & Sarma, 1965). This indicates a conditioned iron deficiency brought about by cobalt.

Cobalt does not produce an iron deficiency by inhibiting the iron uptake of the organism from the medium. Instead, maximal iron incorporation into the mycelium takes place within 24hr. under cobalt-toxic conditions. However, in the mycelium, bound ^{59}Fe, haem ^{59}Fe and catalase activity show a significant decrease in cobalt toxicity at 72hr. of growth, although these parameters are either higher or not affected at 24hr. of growth as compared with the normal mycelia. Thus an intracellular manifestation of cobalt–iron antagonism is evident that probably requires the build-up of a certain concentration of cobalt in the mycelium at the competition sites. Cobalt enhances iron uptake in *Candida guilliermondii* but there is a decrease in the trichloroacetic acid-precipitable iron and the iron incorporated into the supernatant and particulate fractions (Enari, 1958).

The iron-binding compound is secreted into the medium even at 24hr. of growth in cobalt toxicity. An iron chelate similar to XFe was detected in the *N. crassa* mycelium grown under normal conditions with optimum amounts of iron (Padmanaban & Sarma, 1965). This indicates that the formation of the iron chelate in the mycelium is a very early phase in iron transport and is interfered with in cobalt toxicity, owing to the depletion of iron from the medium, leading to the secretion of the iron-binding compound into the medium before the other iron-dependent systems are affected. The mechanism of the secretion of the iron-binding compound in large amounts is not clear and some of the possibilities for this type of a phenomenon have been discussed by Neilands (1961). The formation of the iron chelate under favourable conditions can take place at the cell surface itself, since cation-binding sites are known to be located at the periphery of the cell, as for example in yeast (Rothstein & Hayes, 1956).

The intracellular cobalt–iron antagonism is evident at the level of haem synthesis. Here, cobalt inhibits the synthesis of the porphyrin moiety and in cell-free extracts inhibits iron incorporation into protoporphyrin significantly when present in definite excess of iron. Cobalt has been found to inhibit incorporation of radioactive glycine into haem by preparations of rabbit bone marrow (Laforet & Thomas, 1956). However, in *N. crassa* cobalt itself is incorporated into protoporphyrin to a poor extent even though it inhibits iron. Labbe & Hubbard (1961) have shown that the rat-liver iron–protoporphyrin chelating enzyme can utilize both iron and cobalt for the respective haem formation with equal facility. Species specificity appears to be an important factor in determining the metal specificity of these enzymes. For example, a specific cobalt–porphyrin synthase is present in *Clostridium tetanomorphum*, which exhibits very little haem-synthase activity (Porra & Ross, 1965).

The metabolic potency of $X^{59}Fe$ as a better iron source than $^{59}FeSO_4$ for haem synthesis in cell-free

extracts emphasizes the possibility that this type of organic iron formed in the cell can act as an iron donor for haem synthesis *in vivo*. Such a possibility has already been envisaged (Prelog, 1964), and it is held that, at least in micro-organisms, the siderамines, the growth-promoting compounds of the siderochromes, may play an important part in the enzymic incorporation of iron into porphyrins.

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