# Beryllium toxicity in *Neurospora crassa*

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**Abstract.** In *Neurospora crassa*, 0·44 mM $Be^{2+}$ caused half-maximal inhibition of growth and this inhibition could be fully counteracted by the addition of 2·5 mM $Ca^{2+}$ to the medium. $Mn^{2+}$ and $Mg^{2+}$ were less effective in reversing the growth inhibition caused by $Be^{2+}$ and the order of effectiveness was $Ca^{2+} > Mn^{2+} > Mg^{2+}$. $Fe^{3+}$ and $Zn^{2+}$ were ineffective in reversing $Be^{2+}$ toxicity.

Pyruvate, malate and succinate also reversed the growth inhibition caused by $Be^{2+}$ in *N. crassa*. Pyruvate restored growth by a mechanism not involving control of $Be^{2+}$ accumulation in the mould. The rate of utilisation of glucose by the mycelia grown in the presence of $Be^{2+}$ was reduced, while that of pyruvate was not affected. The results indicate that the primary metabolic lesion in $Be^{2+}$ toxicity in *N. crassa* is probably a block at some step(s) in the glycolytic sequence.

## Introduction

Beryllium salts are known to be highly toxic to mammals. Intravenous injection of $Be^{2+}$ leads to liver necrosis (Aldridge *et al*., 1949) and chronic toxicity has been encountered in the industry (Everest, 1964). As Witschi (1970) has remarked, beryllium causes 'bewilderingly diverse' effects, and there is as yet very little definite information concerning the key processes damaged by beryllium. Among a wide array of phosphotransferases and hydrolases studied, the concentration of $Be^{2+}$ for 50% inhibition of alkaline phosphatase was 10 $\mu$M and 5 $\mu$M for phosphoglucomutase; in contrast, most other enzymes are not inhibited even at 1 mM concentration (Thomas and Aldridge, 1966). Beryllium also inhibits DNA synthesis in regenerating rat liver at fairly low levels (Witschi, 1970).

Very little is known on the toxic effects of beryllium in micro-organisms. The high sensitivity of phosphoglucomutase to beryllium prompted us to examine whether a possible derangement of carbohydrate metabolism could be one consequence of beryllium toxicity. In this paper, we present a specific effect of beryllium on the glycolytic sequence in the mould *Neurospora crassa* and also some metal ion interactions in beryllium toxicity.

## Materials and methods

*Organism, media and growth*

The wild strain of *N. crassa*, Em 5297a was used in the experiments described below and the growth was in stationary cultures. The mould was grown on 10 ml

minimal salts medium containing 2% glucose in 50 ml pyrex conical flasks, at pH 4·8–5·0 and 30 ± 1° C. The composition of the medium and the growth conditions were the same as in earlier studies (Adiga *et al.*, 1962; Nirmala and Sivarama Sastry, 1973). In view of the possibility of the formation of insoluble beryllium phosphate, inorganic phosphate in the medium was replaced by 0·04% sodium $\beta$-glycerophosphate (Na$_2$C$_3$H$_5$ (OH)$_2$ PO$_4$ ·5$\frac{1}{2}$ H$_2$O) and 0·016% KCl. A sterile solution of $\beta$-glycerophosphate was separately added aseptically. In growth experiments, mycelia were harvested after 72 h thoroughly washed, dried overnight at 60° C and weighed. All experiments were run in duplicate and repeated at least four times. Mycelial weights were always reproducible to ± 1–2 mg in duplicate experiments.

All salts used were of the analytical reagent grade. Beryllium was always used as BeSO$_4$ · 4H$_2$O. The metal salts employed in growth reversal experiments were MgSO$_4$ · 7H$_2$O, CaCl$_2$ · 2H$_2$O, ZnSO$_4$ · 7H$_2$O and MnSO$_4$ · 4H$_2$O. In some studies, a 1 : 1 complex of ferric-EDTA was also used as a source of iron.

DL-malic acid (product of Eastman Kodak, Rochester, NY, USA) and succinic acid (BDH) were adjusted to pH 4·8–5·0 with NaOH and included in media, where needed. Sodium pyruvate (product of E. Merck, Darmstadt, W. Germany) was separately sterilised and added aseptically as required. All compounds tested for their ability to counteract Be$^{2+}$ toxicity were always added along with Be$^{2+}$ in growth media prior to inoculation with the organism.

In experiments where utilisation of glucose or pyruvate by preformed mycelia was studied, the following procedures were adopted. *N. crassa* was grown for 48 h with or without 0·33 mM Be$^{2+}$ on the minimal medium described above but with 0·8% glucose, and one drop of Tween-80 per 100 ml medium to suppress sporulation (Zalokar, 1954). In preliminary experiments, this level of glucose was found to be the minimal concentration for adequate growth. This inhibitory level of Be$^{2+}$ was chosen, on the basis of preliminary experiments, to obtain convenient rates of glucose utilisation. After 48 h of growth, mycelia were thoroughly washed with sterile water, transferred aseptically to carbon-free minimal medium and incubated for 18–20 h to deplete the endogenous substrates. Starved mycelia were once again washed thoroughly and used in flotation experiments as described below.

For determining rates of glucose utilisation, about 200 mg fresh wt of mycelia from normal or beryllium cultures, were suspended in 40 ml of minimal medium, containing 1 mg glucose per ml. For studying pyruvate utilisation, 100 mg (fresh wt) mycelia were suspended in 20 ml medium containing 0·25 mg sodium pyruvate per ml. Mycelia were shaken in the above media, for 4 h at 30° C, in a reciprocal shaker, at a rate of about 10–15 strokes per min. Aliquots were removed at different time intervals for assay of glucose or pyruvate.

In some experiments, after flotation, mycelia were thoroughly washed to free them of glucose or pyruvate, ground with 10% ice-cold trichloroacetic acid, and centrifuged at 10,000 g in the cold for 20 min. The supernatant was collected and assayed for glucose, according to the method of Nelson (1944) and for pyruvate as described by Friedemann and Haugen (1943) as described below.

In experiments where the amount of glucose utilised during growth was to be determined, the medium was decanted, mycelia washed thoroughly with water and

medium and washings were made up to 25 ml. Suitable aliquots were removed and assayed for glucose.

*Analytical methods*

The uptake of $Be^{2+}$ by *N. crassa* was examined in 72 h-old mycelia from cultures grown with 0·44 mM $Be^{2+}$ with or without supplements that counteracted beryllium toxicity. Mycelia were extensively washed with glass-distilled water and subjected to wet digestion with conc. $HNO_3$, $HClO_4$ and HCl according to the procedure of Sivarama Sastry *et al.* (1962a). The digested residues were dissolved in water, adjusted to pH 7, and aliquots removed for beryllium assay with Fast Sulphon Black F, as described by Cabrera and West (1963), except that absorbance of all solutions including the dye blank were measured against water blank at 630 nm.

## Results

*Reversal of $Be^{2+}$ toxicity by metal ions*

The effect of increasing concentrations of beryllium on the growth of *N. crassa* is shown in figure 1. Growth inhibition (50%) occurred at 0·44 mM concentration and this concentration was routinely used in the subsequent experiments. In these experiments, the medium contained 2 mM $Mg^{2+}$, a level normally employed for the growth of *N. crassa* (Sivarama Sastry *et al.,* 1962b).
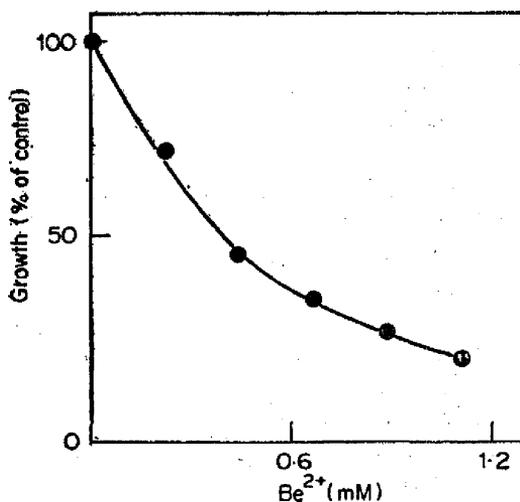


**Figure 1.** Effect of beryllium concentration on the growth of *Neurospora crassa*, Em 5297a.
Growth period 72 h on 10 ml medium.

Ion antagonisms are well known in micro-organisms and metabolically important metal ions such as $Mg^{2+}$ or $Fe^{3+}$ can reverse heavy metal toxicities (Sivarama Sastry *et al.*, 1962b; Sivarama Sastry, 1963). Hence the ability of several physiologically important metal ions in reversing beryllium toxicity shown in figure 2.
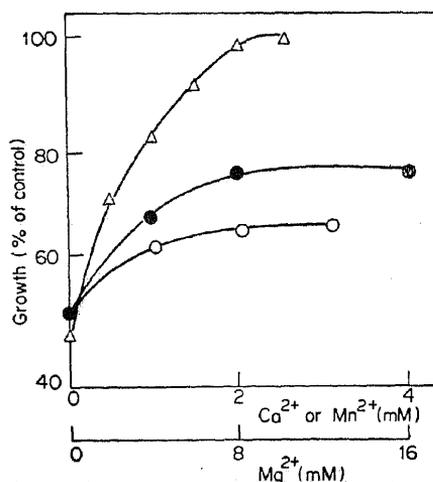
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**Figure 2.** Reversal of $Be^{2+}$ toxicity in *N. crassa* by metal ions.
Inhibitory concentration of $Be^{2+}$ was 0·44 mM medium, in all cases. The medium (10 ml) was supplemented with $Be^{2+}$ (0·44 mM) which caused 50% inhibition of growth. This medium was supplemented with metal ions at concentrations indicated. Growth in the absence of any added metal ion was normalised to 100. Growth in control, $Ca^{2+}$, $Mn^{2+}$ or $Mg^{2+}$ was 40·3, 39·5, 39·7 and 43·5 mg respectively.

$\triangle$—$\triangle$,  $Ca^{2+}$
●—●,  $Mn^{2+}$
O—O,  $Mg^{2+}$

The effects of only those metal ions which were either partially or fully capable of counteracting beryllium toxicity are represented. It might be seen that only $Ca^{2+}$ at a concentration of 2 mM completely reversed growth inhibition due to beryllium toxicity. (The minimal medium used contained 0·9 mM $Ca^{2+}$). The next most effective metal ion was $Mn^{2+}$, the maximal growth being about 78% of the control at 2·7mM. The least effective was $Mg^{2+}$, maximal growth obtained being 67% of the control at a supplemented level of 8·2 mM (the minimal medium used contains 2 mM $Mg^{2+}$). Other metal ions tested including $Zn^{2+}$ and $Fe^{3+}$ (as ferric-EDTA complex) did not counteract beryllium toxicity. A strong anatgonism between $Be^{2+}$ and $Ca^{2+}$ was apparent.

*Reversal of $Be^{2+}$ toxicity by intermediates of carbohydrate metabolism*

The next aspect studied was the possible influence of beryllium on carbohydrate metabolism. The ability of various intermediates of carbohydrate metabolism to counteract beryllium toxicity is depicted in figure 3. Full restoration of growth was obtained with 12 mM DL-malic acid, 40mM sodium pyruvate or 25·5 mM succinate (as indicated by the arrows in fig. 3). Malate appeared to be the most effective metabolite.
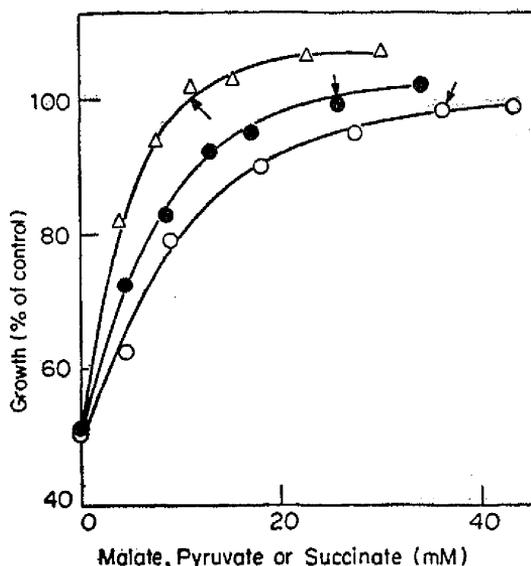
**Figure 3.** Effect of malate, succinate and pyruvate on $Be^{2+}$ toxicity in *N. crassa*. Concentration of $Be^{2+}$ used was 0·44mM. Growth of control mycelia (No $Be^{2+}$) was 40·0 mg in all cases and was normalised to 100. Experimental details, see text.

$\triangle$—$\triangle$, DL-maleate
●—●, succinate
O—O, pyruvate

*Effects of metal ions and other compounds on $Be^{2+}$ uptake in N. crassa*

The effects of metal ions as well as of pyruvate, succinate and malate could, *a priori*, be related to the possible suppression of $Be^{2+}$ uptake by *N. crassa*. Although the reversal of beryllium toxicity by malate and succinate is accompanied by the suppression of the uptake of $Be^{2+}$ by the mycelium, this is not so when toxicity is reversed by $Ca^{2+}$ or by pyruvate (table 1). The reversal by $Ca^{2+}$ suggested a competitive interaction between the two ions in the intracellular space.

*Effect of $Be^{2+}$ on carbohydrate metabolism*

In view of the data of figure 3 and table 1 it appeared likely that the observed counteraction of beryllium toxicity by pyruvate is a reflection of an adverse effect of $Be^{2+}$ on carbohydrate metabolism at some step(s) prior to pyruvate.

It is evident from figures 4 and 5 that mycelia grown in a medium containing $Be^{2+}$ utilise glucose at a diminished rate as compared to mycelia grown in the absence of $Be^{2+}$, but utilise pyruvate at an almost identical rate. These mycelia were also examined for their intracellular concentration of free glucose (reducing sugar) as well as pyruvate at the end of the flotation period and were found to contain no detectable amounts of either compound. Thus the values shown in figures 4 and 5 truly represent glucose or pyruvate metabolised by the respective mycelia. In flotation experiments, the increase in mass was usually small ($\approx$ 5%) as compared to the fresh weight of the mycelia.

*A. Naidu et al.*

**Table 1.** Uptake of $Be^{2+}$ by *N. crassa mycelia*.

| Supplements to the basal medium containing $Be^{2+}$ (0·44 mM) | Concentration[a] (mM) | $Be^{2+}$ content of mycelia (µg/100 mg. dry wt.) |
|---|---|---|
| None | .. | $21·64 \pm 2·13$[b] |
| Pyruvate | 36·4 | $23·56 \pm 1·74$ |
| Malate | 11·2 | $4·15 \pm 2·21$ |
| Succinate | 25·5 | $3·66 \pm 1·70$ |
| Calcium | 2·0 | $21·02 \pm 1·89$ |

[a] Concentrations were the lowest at which inhibition was reversed.
[b] S.D. of mean.

In view of the above results, the total amount of glucose utilised by *N. crassa* during growth in a medium containing beryllium as well as pyruvate, succinate and malate, was also determined.

It was interesting to observe that in the presence of beryllium as well as under conditions where toxicity was reversed, the total amount of glucose utilised in 72 h per unit wt of mycelium was not appreciably altered (table 2). Further, in these experiments 94·2% of added pyruvate was utilised.

**Table 2.** Utilisation of glucose by *N. crassa* during growth in a medium containing beryllium (0·44 mM).

| Supplements to the basal medium[a] | Concentration (mM) | Mycelial dry wt (mg) | Glucose utilised | |
|---|---|---|---|---|
| | | | Total (mg) | mg/mg dry weight |
| Control (No $Be^{2+}$) | .. | 40·3 | $196·9 \pm 1·84$[c] | 4·99 |
| None | Nil | 20·5 | $108·0 \pm 2·20$ | 5·15 |
| Pyruvate[b] | 36·4 | 38·7 | $198·3 \pm 2·20$ | 5·12 |
| Succinate | 25·5 | 41·0 | $198·5 \pm 1·50$ | 4·82 |
| Malate | 11·2 | 41·2 | $197·8 \pm 2·20$ | 4·80 |

[a] In all cases, the growth medium contained 200 mg glucose per 10 ml.
[b] In this case, pyruvate (37·7 mg) was also found to be utilised by the mycelia during the growth period of 72 h.
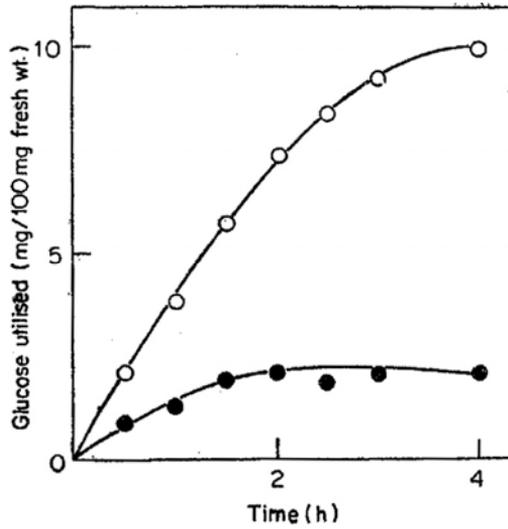[cc] S. D. of mean.

**Figure 4.** Utilisation of glucose by *N. crassa* grown in a medium containing $Be^{2+}$ (0·33 mM).
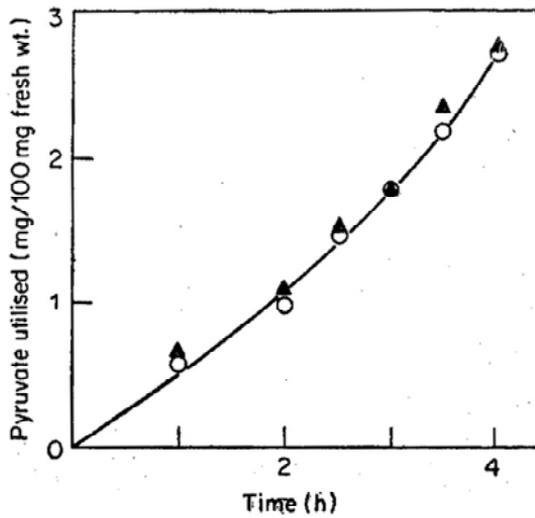
O—O, Normal mycelia
●—●, $Be^{2+}$-grown mycelia



**Figure 5.** Utilisation of pyruvate by *N. crassa* mycelia grown in a medium containing $Be^{2+}$ (0·33 mM)

O—O, Normal mycelia
▲—▲, $Be^{2+}$-grown mycelia.

## Discussion

Beryllium toxicity has not so far been studied in any detail in micro-organisms. The results presented in this paper indicate, for the first time, an unusual effect of beryllium toxicity on carbohydrate metabolism in *N. crassa* as well as some interactions between the trace elements.

Abelson and Aldous (1950) were among the first to show that the toxicity of several bivalent metal ions can be suppressed by $Mg^{2+}$, at fairly high levels, in several bacteria. Subsequently, Adiga *et al.* (1962) found that toxicities of cobalt, nickel and zinc to *Aspergillus niger* can be counteracted by either $Mg^{2+}$ or $Fe^{3+}$. Sivarama Sastry *et al.* (1962b) showed that the toxicity of the same metal ions to *N. crassa* is similarly annulled by $Mg^{2+}$, predominantly by a process involving control of uptake of the toxic metal ion.

Data presented here show that $Mg^{2+}$ cannot effectively overcome $Be^{2+}$ toxicity in *N. crassa*. This is interesting in view of the fact that $Be^{2+}$ can apparently partly replace $Mg^{2+}$ in *A. niger* (Steinberg, 1946) and in tomato plants (Hoagland, 1952) suggesting that in some systems $Mg^{2+}$ and $Be^{2+}$ can act at the same metabolic site(s). Also, in the case of phosphoglucomutase, though $Mg^{2+}$ cannot reverse inhibition once established by $Be^{2+}$, it can diminish its inhibitory effect (Aldridge and Thomas, 1966). Similarly in rats, $Be^{2+}$ toxicity is known to inhibit breakdown of $\alpha$-ketoglutarate, malate and succinate, and $Mg^{2+}$ can counteract this toxicity (Mukhina, 1967).

Although the exact metabolic importance of $Ca^{2+}$ to *N. crassa* is not understood, it is an essential nutrient for this mould (Castel and Bertrand, 1954; Castel, 1956). The data of figure 2 and table 1 show that $Ca^{2+}$ completely reverses $Be^{2+}$ toxicity in *N. crassa* without suppressing overall uptake of $Be^{2+}$ by this mould. This would suggest that there is a metabolic antagonism between these two ions in *N. crassa*. Alternatively, $Ca^{2+}$ could bring about redistribution of $Be^{2+}$ in the intracellular space of *N. crassa* by displacing $Be^{2+}$ from intracellular sites at which $Be^{2+}$ exerts its toxicity. A similar possibility has been earlier suggested by Sivarama Sastry *et al.* (1962b) for an analogous pattern of $Fe^{3+}$ counteraction of $Co^{2+}$ toxicity in *N. crassa*. The relative efficiencies of $Ca^{2+}$, $Mn^{2+}$ and $Mg^{2+}$ in reversing $Be^{2+}$ toxicity indicate the quantitative aspects of metal ion interactions.

Another significant feature of $Be^{2+}$ toxicity in *N. crassa* is the impairment of carbohydrate metabolism. The decreased rate of utilisation of glucose, in the presence of excess $Be^{2+}$ is evidently growth limiting in this mould. Since, under these conditions, pyruvate utilisation is unaffected, it would appear that one major effect of $Be^{2+}$ toxicity is an inhibition of some step(s) involved in the conversion of glucose to pyruvate. The counteraction by pyruvate cannot be ascribed to diauxic growth on pyruvate, since, concomitantly with the restoration of the growth rate, glucose utilisation is also brought back to normal in short term experiments with preformed *N. crassa* mycelia; possible suppression of $Be^{2+}$ uptake by pyruvate is ruled out (table 1).

The data of figure 4 and tables 1 and 2 indicate that in the presence of added pyruvate, overall utilisation of glucose is enhanced *in vivo* and this probably accounts for the observed normal rates of growth. It is likely that in $Be^{2+}$ toxicity, due to the decreased rate of conversion of glucose to pyruvate, adequate concentrations of the intermediates of the tricarboxylic acid cycle are not attained *in vivo*. As a

consequence, the rate of carbohydrate metabolism would be less than optimal, and growth would be restricted.

It is evident that the locus of attack is between glucose and pyruvate but the step(s) that are actually blocked by $Be^{2+}$ in *N. crassa* are not evident. From the studies of Thomas and Aldridge (1966) it is known that phosphoglucomutase is most potently inhibited by $Be^{2+}$. However, this enzyme is not required for growth of this mould on glucose. Enolase (Malmstrom, 1955) and hexokinase (Thomas and Aldridge, 1966) are also inhibited by $Be^{2+}$, but the concentrations required for significant inhibition are higher than 5 mM $Be^{2+}$. Since the growth of *N. crassa* is markedly inhibited even at 0·44 mM $Be^{2+}$, it is unlikely that the inhibition of these enzymes is responsible.

It is interesting to note that $Be^{2+}$ inhibits glycolysis in rat liver slices (Klemperer, 1950). Mukhina (1967) has found that, in rats, $Be^{2+}$ toxicity results in diminished rates of oxidative breakdown of $\alpha$-ketoglutarate, malate and succinate. In view of the present results, it would appear that considerable species differences exist in regard to the toxic effects of $Be^{2+}$.

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