FACTORS INFLUENCING THE FUNGISTATIC ACTION OF 8-HYDROXYQUINOLINE (OXINE) AND ITS METAL COMPLEXES

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[*Manuscript received January* 2, 1951]

Summary

The fungistatic effect of oxine against *Aspergillus niger* is greatly reduced if the medium is first freed of cupric Cu, and toxicity increases with increasing amounts of Cu. The toxicity is overcome by lowering the pH of the medium even in the presence of quite large amounts of Cu.

Removal of ferrous Fe slightly reduces the fungistatic activity of oxine but deficiencies of Zn, Mn, or Mo have no effect. Oxine inhibition is not reversed by the addition of high concentrations of Co, Zn, Mn, Fe, or Cu and only partially by Mo.

The toxicity of seven chelators was tested in the presence and absence of Cu and, of these, four showed an increase in fungistatic action in the presence of Cu.

These findings are discussed in relation to the possible mode of action of oxine and its metal complexes on fungal cells.

I. Introduction

Several workers have postulated that the toxicity of oxine to microorganisms may be due to its ability to form chelate complexes with essential trace metals, which are then rendered unavailable for metabolic processes (Zentmyer 1943; Albert 1944). This view was supported by further work when Zentmyer (1944) demonstrated that oxine inhibition could be reversed by Zn, and Albert *et al.* (1947) found strong positive correlation between bacteriostatic activity and chelating power in a series of oxines, and demonstrated reversal of oxine inhibition by Co for Gram-positive bacteria, and by Zn and Fe for Gram-negative bacteria. Gale (1949) found that oxine inhibited glutamic acid assimilation in *Staphylococcus aureus,* but the addition of Mn, Co, and Fe annulled this.

That the toxicity of oxine was not entirely due to its ability to render essential metals unavailable was suggested by Mason (1948) who found that Cu oxinate was more toxic against some phytopathogenic fungi than was oxine itself. The present paper supports this view, as it has been found that in the absence of Cu, oxine is not fungistatic even at relatively high concentrations. In addition to Cu, ferrous Fe also increases the fungistatic activity of oxine. Similar findings for bacteria have recently been made by Rubbo, Albert, and Gibson (1950). No reversal of inhibition by high concentrations of Co, Zn, Fe, Cu, or Mn was obtained, but slight reversal occurred with large amounts of Mo.

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II. Materials and Methods

(i) *Test Organisms.*—Most of the work was carried out using *Aspergillus niger,* strain 543R, but several others, viz. 540 and Mulder, gave similar results. Stock cultures were grown in tubes of liquid medium freed from trace elements as described below. Inoculum was prepared by suspending one loopful of spores in 10 ml. of twice glass-distilled water, and seeding each flask with one drop from a sterile pipette previously cleaned with aqua regia.

(ii) *Medium.*—A nutrient solution of the following composition was used: sucrose, 50 g.; KNO_3, 5 g.; K_2HPO_4, 2.5 g.; $MgSO_4.7H_2O$, 0.25 g.; distilled H_2O, 1000 ml. The following optimum amounts of trace elements were added to one litre of medium: Fe (ferrous), 200 μg.; Zn, 180 μg.; Cu (cupric), 40 μg.; Mn (manganous), 20 μg.; Mo (molybdate), 10 μg.; and B (borate), 10 μg.

(iii) *Removal of Trace Metals.*—As the amount of trace metals present is a critical factor in oxine inhibition, all glassware was cleaned by immersion in aqua regia overnight, followed by three rinses with water from a tinned copper still and three with double glass-distilled water.

The medium was freed from Fe, Zn, Cu, and Mn by repeated extraction with an 0.5 per cent. solution of oxine in redistilled carbon tetrachloride at pH 9, and with a solution of purified diphenylthiocarbazone (dithizone) in carbon tetrachloride at pH 6. Excess chelating agents were removed by several shakings with redistilled carbon tetrachloride at pH 9, 8, 6, and 4. In all cases the pH was adjusted either with glass-distilled HCl or NH_4OH. The degree of deficiency of various trace metals obtainable in this medium at pH 7.0 was determined by growing *A. niger* (543R) in flasks containing 25 ml. of purified medium with optimum amounts of all essential trace elements added except the one under test.

After seven days' incubation at 30°C., the mycelium from each flask was harvested, dried, and weighed. In medium with all trace elements provided, the average weight of mycelium was 0.31 g., while with deficiencies of Fe, Zn, Cu, and Mn, average weights of 0.01, 0.00(5), 0.16, and 0.19 g. respectively were obtained.

With Fe deficiency, the mycelial mat was thin, white, with a few small black spore heads; with Zn deficiency the mat was fragile and produced a few large black sporangia; in Cu-deficient medium the growth was fairly poor, white, and devoid of spores; while with Mn deficiency no mat was formed, only isolated floating papery colonies resembling puffed wheat.

(iv) *Tests for Toxicity.*—The ability of oxine to inhibit fungal growth was estimated by the dry weights of mycelium obtained after seven days' incubation at 30°C. in 25 ml. of purified medium to which known amounts of trace metals and oxine had been added. Conical Pyrex flasks of 100 ml. capacity covered with inverted beakers were used in duplicate or triplicate throughout the work. While work was in progress, it was found that the pH of the medium influenced the toxicity of oxine, and that below pH 5.0, there was no toxic effect up to a

concentration of oxine of 12 mg./l. In cases of extreme inhibition, there was either no growth at all or insufficient to reduce pH appreciably. Since the medium was poorly buffered and as *A. niger* produces large amounts of acid, daily examinations of the cultures sometimes revealed slight recovery if enough mycelium developed to lower the pH sufficiently.

III. Experimental Results

(*a*) *Effect of Cu on Oxine Toxicity*

While purifying media from heavy metals, it was observed that residual traces of oxine were fungistatic except in the absence of Cu. Therefore an experiment was designed to determine the interactions of Cu and oxine by growing *A. niger* (543R) in purified medium at pH 6 and 7 containing various combinations ranging from Cu, 0 to 60 μg., and oxine, 0 to 12 mg./l., together with optimum amounts of other essential trace elements. The results are set out in Table 1 and the type of growth obtained for certain treatments is shown in Plate 1.

Table 1

Effect of copper on toxicity of oxine to *Aspergillus niger*, 543R

Conc. of Cu		Conc. of Oxine*		Mean Wt. of Mycelium from 25 ml. Medium (g.)	
(μg./l.)	Molarity	(mg./l.)	Molarity	pH 6	pH 7
0	0	0	0	0.17	0.16
0	0	2	1.38×10^{-5}	0.17	0.19
0	0	4	2.76×10^{-5}	0.17	0.19
0	0	8	5.52×10^{-5}	0.10	0.10
0	0	12	8.27×10^{-5}	0.00	0.00
20	3.14×10^{-7}	0	0	0.25	0.30
20	3.14×10^{-7}	0.5	3.45×10^{-6}	0.24	0.23
20	3.14×10^{-7}	0.75	4.6×10^{-6}	0.19	0.16
20	3.14×10^{-7}	1	6.90×10^{-6}	0.03	0.01
20	3.14×10^{-7}	1.25	8.62×10^{-6}	0.00 (3)	0.00
20	3.14×10^{-7}	1.5	10.3×10^{-6}	0.00	0.00
40	6.29×10^{-7}	0	0	0.29	0.31
40	6.29×10^{-7}	0.25	1.72×10^{-6}	0.24	0.00
40	6.29×10^{-7}	0.5	3.45×10^{-6}	0.24	0.00
40	6.29×10^{-7}	0.75	4.6×10^{-6}	0.00	0.00
60	9.43×10^{-7}	0	0	0.27	0.32
60	9.43×10^{-7}	0.25	1.72×10^{-6}	0.01	0.00
60	9.43×10^{-7}	0.5	3.45×10^{-6}	0.00	0.00

* One molecule of oxine combines with two atoms of divalent metal.

In the absence of Cu at both pH 6 and 7 there was no inhibition of growth at concentrations of oxine up to 4 mg., marked reduction of growth at 8 mg./l., and no mycelium at 12 mg./l. At a concentration of 20 μg. Cu/l., growth ceased at pH 6 in the presence of 1.5 mg. oxine/l., while at pH 7, it ceased at

1.25 mg. oxine/l. With 40 μg. Cu/l., no growth appeared at pH 6 and 7 in the presence of 0.75 and 0.25 mg. oxine/l. respectively. Finally at 60 μg. Cu/l., growth failed at pH 6 and 7 at 0.5 and 0.25 mg. oxine/l. respectively. In the absence of oxine, Cu showed no toxicity even at the highest concentrations.

These results indicate that the higher the concentration of added Cu, the greater the toxicity of oxine. This is more marked at pH 7 than at pH 6. These facts suggest that Cu oxinate is the toxic substance.

(*b*) *Effect of pH on Oxine Toxicity*

The difference in behaviour of oxine in the presence of Cu at pH 6 and 7 suggested an experiment to test the effect of varying the pH from 2 to 10 in the presence and absence of Cu. The results are summarized in Table 2.

TABLE 2
INFLUENCE OF pH ON TOXICITY OF OXINE TO *ASPERGILLUS NIGER*, 543R IN THE PRESENCE AND ABSENCE OF COPPER

Conc. of Cu		Conc. of Oxine*		Mean Wt. of Mycelium from 25 ml. Medium (g.)								
(μg./l.)	Molarity	(mg./l.)	Molarity	pH 2	pH 3.5	pH 4	pH 5	pH 6	pH 7	pH 7.5	pH 8	pH 8.5 and 10
0	0	0	0	0.15	0.16	0.18	0.18	0.17	0.16	0.16	0.13	0.00
0	0	3	2.7×10^{-5}	0.20	0.17	0.20	0.18	0.17	0.19	0.12	0.07	0.00
40	6.29×10^{-7}	0	0	0.25	0.31	0.31	0.31	0.29	0.31	0.30	0.24	0.00
40	6.29×10^{-7}	3	2.7×10^{-5}	0.23	0.31	0.27	0.20	0.00	0.00	0.00	0.00	0.00

* One molecule of oxine combines with two atoms of divalent metal.

When both Cu and oxine were omitted, typical Cu-deficient mycelium was obtained and the weight did not vary significantly over the pH range from 2 to 7.5; there was a slight decrease at pH 8 and no growth at higher pH values. In the absence of Cu and in the presence of oxine growth remained unchanged from pH 2 to 7; slight reduction occurred at pH 7.5, and no growth above pH 8.

At the optimum concentration of Cu of 40 μg./l. with no oxine added, normal weights of mycelium were obtained from pH 3.5 to 7.5; a slight reduction occurred at either end of this range, viz. pH 2 and 8, while no mycelium developed above pH 8. At the optimum level of Cu when oxine was added there was normal growth from pH 2 to 5, slight toxicity at pH 5, and complete inhibition at higher pH values. This indicates that the toxicity of oxine is greatly reduced at low pH even in the presence of Cu. It suggests that toxic Cu oxinate is decomposed or is rendered less harmful by acidity.

(*c*) *Effect of Other Metals on Oxine Toxicity*

It was thought that other trace metals might also increase the toxicity of oxine to *A. niger* (543R), so an experiment was set up in the presence and absence of oxine, using media at pH 7 deficient in Fe, in Zn, in Mn, or partially deficient in Mo.

It was found that, in the absence of Fe, the weights of mycelium with 0, 0.75, and 2 mg. oxine/l. were 0.05, 0.04, and nil respectively. In the presence of 200 μg. Fe/l. and the absence of oxine the mycelial weight was 0.31 g., but when oxine was present in either concentration, no growth was obtained. This indicates that Fe aggravates the fungistatic action of oxine, but to a lesser degree than Cu. This suggests that Fe oxinate is less fungistatic than Cu oxinate. The effect is more noticeable in the absence of Cu than of Fe because the weight of mycelium in Cu-deficient medium is still quite appreciable. Unlike Cu and Fe, deficiencies of Zn, Mn, and Mo did not lessen the toxicity of oxine.

Further work was done at pH 6 on the effect of different concentrations of ferrous Fe on the toxicity of oxine at various levels (Table 3).

TABLE 3

EFFECT OF FERROUS IRON ON TOXICITY OF OXINE TO *ASPERGILLUS NIGER*, 543R

Conc. of Oxine*		Mean Wt. of Mycelium from 25 ml. Medium at Different Conc. of Fe (g.)		
(mg./l.)	Molarity	200 μg./l.	700 μg./l.	1200 μg./l.
0	0	0.29	0.27	0.27
0.25	1.72×10^{-6}	0.24	0.21	0.18
0.5	3.45×10^{-6}	0.24	0.00	0.00
0.75	4.6×10^{-6}	0.00	0.00	0.00

* One molecule of oxine combines with two atoms of divalent metal.

It is apparent that, in the absence of oxine, normal growth occurred even up to the highest concentration of Fe used. At 0.25 mg. oxine/l. growth was almost normal at 200 μg. Fe/l. but declined slightly at higher levels of Fe. At 0.5 and 0.75 mg. oxine/l., growth ceased at 700 and 200 μg. Fe/l. respectively. This shows that the higher the concentration of added Fe the greater the toxicity of oxine.

Until now the work had been concerned with the effect of trace metals in increasing the toxicity of oxine. Since other authors had shown that certain trace metals could reverse oxine inhibition this was now tested by adding Zn, Mn, Fe, Cu, Co, and Mo singly to a purified medium containing optimum amounts of essential elements. The concentration of additional trace metals ranged from the optimum amount up to 1000 μg./l., i.e. amounts greater than were necessary to combine with the oxine that was added at the level of 0.7 mg./l.

In the absence of oxine, in no instance were these amounts of added trace elements inhibitory. In the presence of oxine the addition of Zn, Mn, Fe, or Cu did not lessen toxicity since no growth occurred, but in the presence of 1000 μg. Mo slight growth appeared after three days' incubation. This suggests that Mo is the only trace element capable of reversing the fungistatic activity of oxine.

(*d*) *Fungistatic Activity of Other Chelators*

It was of interest to know whether chelators other than oxine possessed fungistatic activity, and whether or not this was influenced by the Cu content of the medium. Consequently *A. niger* (543R) was grown in purified medium at pH 7 containing 0 or 40 μg. Cu/l., together with optimum amounts of other essential elements, after the addition of various chelators separately in the following concentrations: sodium diethyldithiocarbamate, cupferron, or *o*-phenanthroline from 0 to 10 mg./l.; 4-methyl-1 : 2-dimercaptobenzene (dithiol) or diphenylthiocarbazone (dithizone) from 0 to 20 mg./l.; 1-hydroxyphenazine or 6-hydroxy-*m*-phenanthroline from 0 to 3 mg./l.

It was found that sodium diethyldithiocarbamate or cupferron was not toxic at the highest levels, either in the presence or absence of Cu. At the highest concentration of *o*-phenanthroline, growth was only slightly inhibited in the presence of Cu, but totally supressed in its absence. In this case Cu reversed the toxic nature of this chelator. With dithiol or dithizone (20 mg./l.), the fungus was not inhibited in Cu-deficient medium, but growth was retarded somewhat when Cu was supplied. 1-Hydroxyphenazine and 6-hydroxy-*m*-phenanthroline, which closely resemble 8-hydroxyquinoline in possessing chelating groups in the same positions, behaved somewhat like oxine by partially inhibiting the growth of the fungus in the presence of Cu, but not in its absence.

IV. Discussion

It has already been mentioned that a number of authors consider that the toxicity of oxine might be due to its ability to chelate with trace metals, either in the medium or more probably on or inside the cells, and thus deprive organisms of metals essential for metabolism. This argument gained support from the observation that high concentrations of certain metal cations can reverse oxine inhibition.

While this may be partly true for *A. niger*, it is not the whole explanation, since the addition of optimal amounts of Cu and Fe greatly increases the fungistatic action while their removal lessens it. This suggests that Cu and Fe oxinates are more toxic than free oxine, which at pH 7 occurs almost entirely as the neutral molecule (Albert and Magrath 1947). It is difficult to see why they should be so toxic except that at this pH, Cu and Fe oxinates are much more soluble in organic solvents than in water, whereas with oxine this partition is not so marked, and tests showed that coloured metal complexes were readily adsorbed from water by *A. niger* mycelium, which possesses a lipoid-protein cell membrane. It seems likely that neutral, relatively inert molecules like Cu and Fe oxinates are adsorbed onto vital cell interfaces, not by ionic or covalent linkages, but rather by van der Waal forces. Since the degree of adsorption and bacteriostatic activity of heterocyclic bases are partly determined by the area and flatness of the molecule (Albert, Rubbo, and Burvill 1949), it is conceivable that metal oxinates, which are flat molecules with over twice the area of the oxine molecule, owe part of their high toxicity to their large surface

area. If this were true, then it is difficult to understand why other metal oxinates with similar flat molecular areas and with stability constants between those of Cu and Fe oxinates, e.g. those of Co and Zn (Maley and Mellor 1949) are not equally effective fungistats at pH 7.

Assuming that adsorption has occurred, and that the toxic action takes place on some cell surface, it is still more difficult to speculate how a relatively stable molecule like Cu oxinate acts fungistatically. It seems unlikely that such small amounts of Cu oxinate could adversely poise the redox potential of the fungus. There is a possibility that Cu oxinate might act as a reversible H carrier and promote complete oxidation of some essential metabolite (Albert and Falk 1949). If Cu oxinate functions by immobilizing other metallic cations essential for the mould, then probably this could be done only after displacing the Cu. A cell component, with chelating properties and having a stronger avidity for Cu than oxine, might do this by donating H, e.g. substances with thiol groups (Bernheim and Bernheim 1939). The oxine cations thereby produced might then become the intra-cellular poison. The Cu in Cu oxinate might also be displaced by other metal cations capable of forming more stable complexes, e.g. Pd, or by the mass action effect of abnormally high concentrations of cations with lower stability constants, e.g. Co, Zn, and Mn (Maley and Mellor 1949). This does not seem to happen except with Mo. Therefore the mode of fungistatic action of Cu oxinate (or of Fe oxinate) remains unknown.

The observation that the toxicity of Cu oxinate is nullified by lowering the pH to 4 suggests that at this acidity most of the metal complex has been decomposed, giving a mixture of Cu^{++} ions, oxine cation, and the neutral molecule. This seems reasonable as the pH for 50 per cent. dissociation of Cu oxinate is near this value. Since the pK_{NH} of oxine is 5.03 (Albert and Magrath 1947) then at pH 4 a higher concentration of oxine cation would be present than of the undissociated oxine, while at still lower pH values the amount of oxine cation would rise still further and the amounts of neutral oxine and Cu oxinate would become almost negligible. It has been shown that the bacteriostatic activity of heterocyclic bases is directly proportional to their degree of cationic ionization (Albert, Rubbo, and Burvill 1949). It is therefore surprising that the toxicity of oxine does not again increase as the pH approaches 2.

V. Acknowledgments

The authors wish to acknowledge financial assistance from the Commonwealth Research Grant. They are also indebted to Dr. E. G. Mulder for providing his strain of *Aspergillus niger*, to Dr. F. P. Dwyer for his advice and gifts of certain chelators, to Miss A. E. Healy for typing the manuscript, and to Mr. S. Woodward-Smith of the Department of Medical Artistry, University of Sydney, for the photograph.

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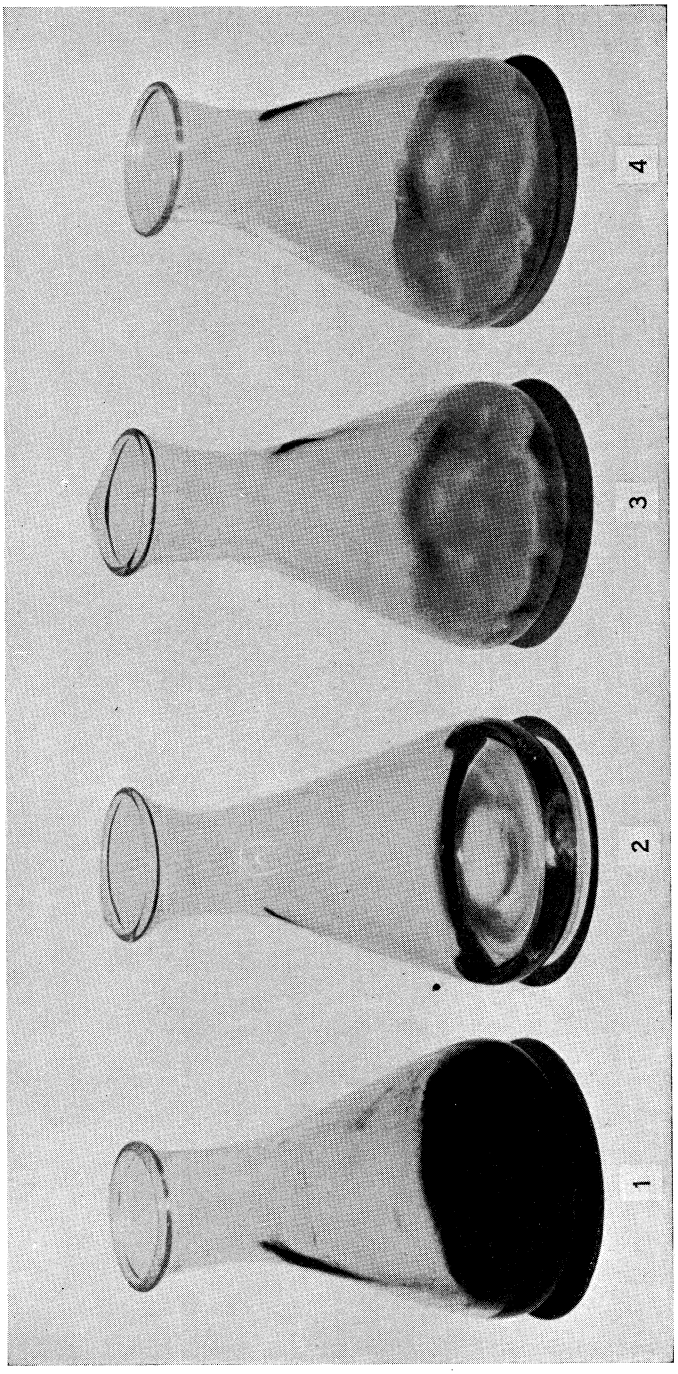

Effect of two concentrations of Cu on growth of *Aspergillus niger*, 543R, in the presence and absence of oxine. L. to R.: 1. Cu, 40 µg./l.; oxine, nil; 2. Cu, 40 µg./l.; oxine, 0.75 mg./l. 3. Cu, 20 µg./l.; oxine, nil. 4. Cu, 20 µg./l.; oxine, 0.75 mg./l.