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Oxine, Ferric Oxine and Copper Oxine as Inhibitors of Growth and Differentiation of *Allomyces macrogynus*

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Abstract

Oxine is an inhibitor of growth and differentiation in *A. macrogynus*. Growth was inhibited by as little as 4 μg/ml and the first effect observed was inhibition of nucleic acid synthesis, followed by inhibition of protein synthesis. Consistent inhibition by oxine of the development of zoosporangia in starving plants required a concentration of 60 μg/ml. The degradation of RNA, always observed in starving plants, continued in the presence of oxine, showing that this is an independent phenomenon. When glucose was available, oxine increased the synthesis of trehalose without a comparable effect on glycogen synthesis. Cycloheximide increased trehalose synthesis to an even greater extent. The inhibition of glucose metabolism by oxine was concentration- and time-dependent. Oxine inhibition was reversible on transfer to nutrient solutions.

Both iron oxine and copper oxine bind rapidly to the fungal walls. It is suggested that the site of binding is wall galactopeptide and that cell transport systems are disorganized. In the presence of glucose plants recovered from inhibition by ferric oxine without transfer. Observations are not in agreement with a theory that oxine inhibits transcription by chelating Mg and Mn.

Introduction

Oxine (8-hydroxyquinoline) has been used as an inhibitor of bacteria and fungi for many years. The lethal effect of oxine on bacteria is due to iron oxine and is reversed by cobalt salts, but the inhibition of fungi by metal oxines is not reversible by cobalt ions (Albert 1973).

Fraser and Creanor (1974) found that oxine inhibited the synthesis of mRNA and rRNA in growing cultures of the fission yeast, *Schizosaccharomyces pombe*. Creanor *et al.* (1975) assayed enzymes from cultures which had been exposed to oxine at a concentration of 50 μg/ml. Some enzyme activities declined within 30 min of exposure while others increased. This increased production of enzymes was prevented by the addition of cycloheximide which inhibits protein synthesis. Fraser and Creanor (1974, 1975) suggested that the inhibition of RNA synthesis was due to binding of Mn^{2+} and Mg^{2+} ions, although they were aware that oxine is a poor chelator of these ions. Their *in vitro* inhibition of RNA synthesis did not commence below 1 mM oxine and was not complete until 5 mM yet the concentrations of 50 μg/ml which they found effective *in vivo* approximates 0·3 mM.

The differentiation of *Allomyces macrogynus* Emerson to produce zoosporangia in conditions of starvation or near-starvation provides a system in which oxine

inhibition can be studied *in vivo* without the addition of magnesium or manganese salts. The time of transcription of the stable mRNA for the whole process may be determined by adding actinomycin D at 10-min intervals until there is no longer inhibition of development (Youatt 1976 and references therein). Oxine has proved to be a more stable and less expensive alternative inhibitor for this purpose (Youatt 1980*a*).

In previous studies the differentiating plants of *A. macrogynus* were analysed during the process and found to undergo a degradation of RNA and redistribution of phosphate (Youatt 1980*b*). Analyses of changes in carbohydrates were also made, to follow accumulation of glycogen and trehalose in the presence of glucose and sodium glutamate as well as to follow the synthesis of wall glycan and wall chitin (Youatt 1980*c*). In this paper plants were exposed to oxine, iron oxine and copper oxine and the suspending media and the plants were analysed as in previous studies (Youatt 1980*a*, 1980*b*, 1980*c*). This has allowed determination of which processes are directly related to the differentiation process.

Methods

The methods used in this work have already been described: culture methods using the defined medium which contains four amino acids and glucose are given in Youatt (1980*a*); fractionation of plants and analytical methods for protein, RNA and phosphate are described by Youatt (1980*b*); and assays of glucose, glycogen, trehalose, wall glycan and wall chitin by Youatt (1980*c*).

Samples required dilution of at least 1 : 20 for the assay of glucose; the problems of interference with the assay by oxine encountered by Creanor *et al.* (1975) did not then arise.

As in the above earlier studies glucose–glutamate solutions contained glucose and sodium glutamate in a molar ratio of 3 : 1. Most commonly, 6 mM glucose sufficed but with experiments of more than 6 h duration 8 or 9 mM glucose was supplied.

Oxine was recrystallized from ethanol. The stock solution was prepared fresh for each experiment and contained 10 mg oxine dissolved in aqueous ethanol (2 ml of ethanol followed by 8 ml of water). Iron oxine chelate (1 : 1) was obtained by adding 0·018 ml of 60% (w/v) $FeCl_3$ to an oxine of the same concentration and the pH was adjusted to 6. Crystalline copper oxine separated from the solution when copper sulfate was added and could be dissolved only at a concentration of oxine equivalent to 0·2 mg/ml.

Ferric ion was assayed colorimetrically in media and extracts of plants. Standard solutions were freshly prepared dilutions of $FeCl_3$ containing 5–50 μg Fe^{3+} per millilitre. The biological samples were acidified where necessary with 2 M HCl. Equal volumes of sample and 2 M KCNS were combined and the colour was measured at 470 nm.

Results

Effective Concentration of Oxine in Different Conditions of Exposure to It

Cultures after 16 h incubation in the defined medium (Youatt 1980*a*) were still developing vegetatively. Oxine at a concentration of as little as 4 μg/ml caused all development to cease in 2 h. Plants from the same culture transferred to glucose–glutamate solution required 10 μg/ml to prevent development of sporangia. The plants, when suspended in water, were inhibited by 60 μg/ml from making sporangia while at 40 μg/ml the process was sometimes entirely blocked and at others delayed for about 3 h. With suboptimal concentrations of actinomycin D the same delay was observed and the sporangia produced in both of these conditions were unable to release spores.

After exposure to oxine for 24 h the cultures and suspensions all showed reversal of inhibition if transferred either to culture media or to glucose–glutamate solution.

Analyses of Plants Exposed to Oxine in Different Media

Cultures growing in defined medium

Vegetative cultures in defined medium were incubated with oxine at a concentration of 40 μg/ml and without oxine from 16 to 21 h after inoculation. Microscopically, the control cultures extended and branched their hyphae during this time and the inhibited cultures showed only some distortion of the hyphal tip as evidence that wall synthesis had not ceased immediately. The changes in wall glycan and wall chitin were at the limit of detection and, from 2 h, no synthesis of walls could be detected in the presence of oxine.

Compared with the control culture, the net synthesis of RNA in the first hour was inhibited 70% in the presence of oxine and no further change was detected from 2–3 h. Protein synthesis continued at the same rate as the control culture for 1 h but ceased completely in 3 h. In this respect the culture resembled the fission yeast (Fraser and Creanor 1974) and observations were consistent with the initial inhibition of the synthesis of RNA.

The growing culture metabolized glucose at an increasing rate as the plants grew during the 5 h experiment. With oxine present, the initial rate of metabolism of glucose was maintained for 1 h and thereafter declined but glucose metabolism had not ceased at 5 h. The growing culture maintained constant levels of 20–30 μg/mg dry weight of glycogen and 10–15 μg/mg of trehalose until vegetative growth ceased. Then both commenced to rise to new peak concentrations like those found in the earlier work [approximately 100 μg/mg for glycogen and 35 μg/mg for trehalose (Youatt 1980*c*)]. In the presence of oxine the concentration of trehalose immediately began to rise and increased at a faster rate to a peak of about 70 μg/mg. Glycogen synthesis also commenced on addition of oxine but the rate of synthesis and the peak concentrations reached were not increased.

Cultures transferred to water

Plants suspended in water were more severely starved and underwent chemical changes only during the development of one zoosporangium. The changes, recorded previously (Youatt 1980*b*, 1980*c*), included the degradation of RNA and release of phosphate. Glycogen was maintained at approximately constant low levels and trehalose was released. Oxine did not produce changes in these patterns, unless possibly to have caused an earlier release of the trehalose.

Cultures transferred to glucose–glutamate solution

Plants in glucose–glutamate solution differed from the vegetative cultures in that oxine did not so rapidly inhibit wall synthesis and the hyphal length approximately doubled in the first 2 h of exposure. However, branching of the hyphae did not occur. Corresponding increases in wall glycan and chitin were measured in this 2 h period and were unchanged thereafter.

Plants in water or glucose–glutamate solution showed no increase in the total content of RNA. Characteristically RNA was degraded and the phosphate redistributed in the plants and medium (Youatt 1980*b*). Oxine at concentrations which prevented the development of sporangia had no effect on the redistribution of phosphate.

Youatt (1980*c*) showed that plants transferred to glucose–glutamate solution immediately began to accumulate trehalose and glycogen. In the presence of oxine there was no change in glycogen synthesis but increased accumulation of trehalose was observed. Concentrations of 70–100 μg/mg dry weight were found where control suspensions accumulated only 30–35 μg/mg dry weight. Because increases in glycogen and trehalose were observable within 10 min of transfer it seemed likely that the enzymes required were already present. The use of cycloheximide at a concentration of 100 μg/ml confirmed this (Fig. 1). The initial rate of synthesis of glycogen was the same in the presence of cycloheximide but this declined and the peak content reached at 3 h was 85 μg/mg compared with 120 μg/mg in the control or with oxine. The presence of cycloheximide increased trehalose production to an even greater extent than was achieved with oxine and a peak of 150 μg/mg dry weight was reached.

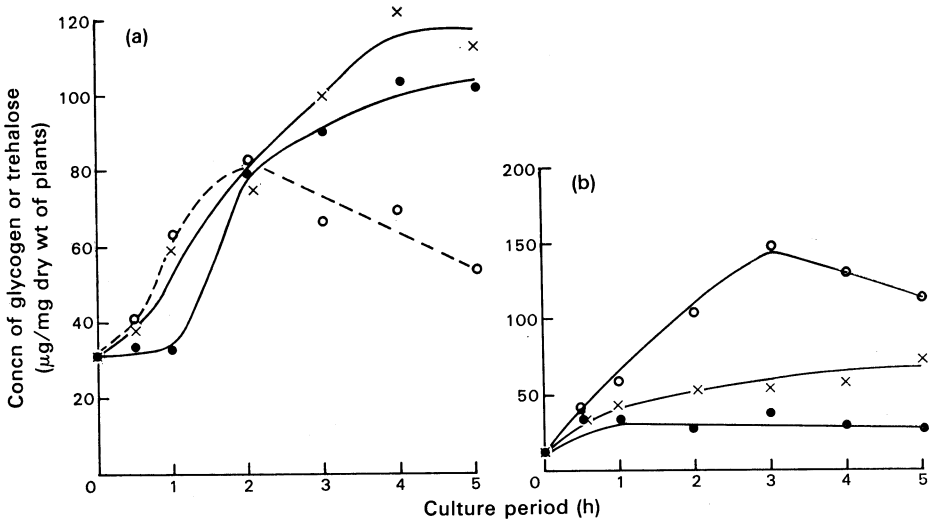

Fig. 1. Synthesis of glycogen (*a*) and trehalose (*b*) in the presence of oxine (60 μg/ml, ×) and cycloheximide (100 μg/ml, ○). Cultures grown for 16 h on defined medium and suspended in a solution containing 9 mM glucose and 3 mM sodium glutamate with a plant density of 0·6 mg dry weight/ml. ● Control.

The rate of metabolism of glucose by suspensions of 2 mg/ml dry weight was measured with an initial 6 mM glucose and 2 mM glutamate containing oxine at concentrations of 0, 4, 10, 40 μg/ml. The initial metabolic rate per hour for 2 mM glucose was maintained for 3 h with oxine concentrations of 0, 4, and 10 μg/ml and for almost 2 h with 40 μg/ml. After inhibition was established the concentration of glucose was readjusted to 5–6 mM in each suspension. The rates then measured were 1·2, 0·7, 0·5 and 0·2 mM/h with oxine at concentrations of 0, 4, 10 and 40 μg/ml. The final rate was measured between 4·5–6·5 h after the addition of oxine.

Inhibition of Plant Growth by Iron Oxine

Iron oxine [as the Fe(III) 1 : 1 complex] at the equivalent concentration of 40 μg/ml was added to suspensions of plants in either water or glucose–glutamate solution. The plants were seen to clump instantaneously and microscopic observation showed

the rhizoids to be stained and entangled. The plant masses on filter papers appeared black. Assay of the supernatant medium by absorbance and colorimetric assay of iron showed that the chelate was bound within seconds of its addition and the plants then began to leak contents to the medium. Plants were fractionated as in the earlier work (Youatt 1980*b*) and fractions were analysed for bound iron. Also, to avoid changes of pH, plants were fractionated by homogenization in water and walls were cleaned by protease action and further washing. Low molecular weight fractions were separated by membrane filtration. No iron of low molecular weight was found in the extracts, indicating that iron was bound to polymers. After exposure to iron oxine at concentrations of 10 and 40 μg/ml, as the chelate, 60–70% and 40% of the iron was recovered from the walls, respectively.

Plants exposed to iron oxine in water did not develop sporangia but soon became vacuolated and the treatment seemed to be lethal. Ferric chloride solutions were not inhibitory at the same molarity. Plants exposed in glucose–glutamate solution began to undergo hyphal extension after 3–4 h and at approximately 9 h made sporangia, either zoosporangia or resistant sporangia, depending on the availability of glucose. The walls of plants which recovered in the presence of the inhibitor remained coloured though the considerable increase in wall content diluted the colour.

Analyses of these plants showed that they did not utilize glutamate until hyphal elongation commenced. Glucose metabolism was reduced to 50% of the control rate but continued while glucose was available. No changes were observable in the distribution of phosphate initially but after a 2 h lag loss of RNA and redistribution of phosphate occurred. The rates of synthesis of both glycogen and trehalose were reduced initially to 25% of the control rates.

Additional Observations of the Effect of Oxine Chelates on Plant Growth

Heat-killed plants bound Fe(III) oxine as readily as living plants. By the decline in absorbance at 255 nm it was seen that Fe(II) oxine was similarly bound and the plants were stained as before. Copper(II) oxine was also bound rapidly but copper itself was as toxic as the chelate.

Using absorbance to detect free oxine and the chelate it was found necessary to provide an excess of Fe(II) to obtain a single peak at 255 nm. With a large excess of Mg^{2+} ions it was still not possible to obtain a single peak and with this effect at such an excess it did not appear useful to pursue the use of magnesium oxine as a growth inhibitor.

Discussion

The major purpose of this study was to further an understanding of the control of differentiation in *A. macrogynus*. The observation that oxine prevented the development of zoosporangia but did not modify the degradation of RNA allowed this to be recognized as a phenomenon related to the starvation conditions and of no special relevance to sporangium development.

Youatt (1980*c*) suggested that the synthesis of trehalose was of some regulatory significance, mainly because trehalose was synthesized but not utilized subsequently by the plants. The results with oxine and with cycloheximide show that the enzymes involved are already present but are regulated in the growing plants. When balanced vegetative growth ceased and glucose was available the synthesis of trehalose was

immediately increased. The observation, that cycloheximide increased trehalose synthesis to a greater extent than oxine did, suggests that regulation is achieved either by an unstable protein or by the product of an unstable enzyme. This will be the subject of further study. Crystalline trehalose was obtained from plants in the presence of cycloheximide and shown to be the α,α-isomer by X-ray crystallography and optical rotation (Youatt, unpublished data).

The observation that plants developed zoosporangia which were unable to release spores in the presence of suboptimal concentrations of oxine and actinomycin was also of interest. In earlier studies with haploid plants it was possible to add actinomycin D too late to block development of the male gametangium and too early to allow development of the female gametangium (Youatt 1976). It now seems possible to distinguish, as a separate transcription, the preliminary development of the zoosporangium and, as a subsequent development, the completion of spores.

In differentiating plants oxine at a concentration of 100 μg/ml had no detectable effect on sporangium development after transcription was complete. This means that the inhibitor has a high degree of specificity for the transcription of mRNA because a very wide range of enzymes is involved in the development, release and germination of spores. Inhibition by oxine was time- and concentration-dependent and therefore the higher concentration of oxine would be valuable for studying the stages of transcription referred to above.

It is confirmed that oxine inhibits the transcription of mRNA but the results are not compatible with the view that the mode of inhibition is by chelation of Mg^{2+} and Mn^{2+}. Were this the case then oxine should be a more effective inhibitor of plants suspended in water than of plants in a culture medium with high concentrations of Mg^{2+}. The reverse was true.

Inhibition by oxine was shown to depend on the availability of nutrients as well as on the concentration of oxine. It appeared that oxine reached its site of action rapidly enough because differentiation was delayed even when it was not completely blocked. It appears more reasonable to postulate the requirement of some metabolic step to complete the inhibition by oxine. An excessive emphasis on chelation may be obscuring other possible modes of action for oxine.

It is not proposed to investigate further the action of oxine as an inhibitor of mRNA transcription but attention is drawn to the fact that the transcription of stable mRNA in differentiating plants offers what is, in many ways, a simpler system for study than the growing culture where the inhibition is followed rapidly by a cessation of all measurable change.

It is possible to suggest a site of binding in the walls for the metal oxines. When estimating the protein content of the walls of *A. macrogynus* (Youatt 1977) it was found that the copper complexes precipitated the galactopeptide fraction (unpublished observation). Fultz and Sussman (1966) used an immunofluorescent technique to demonstrate that galactose residues were exposed in the walls of rhizoids and masked in the hyphal walls. This would account for the selective staining of the rhizoids which was observed with iron and copper oxines. It would be of interest to know whether other fungi with wall galactans bind metal oxines in this fashion. The observations suggest that the sudden binding of the chelates to the walls produced a severe disorganization of cell transport systems and a loss of contents from the plants. In the presence of nutrients these were subsequently replaced and develop-

ment proceeded after a lag of about 2 h. A similar lag occurred in the recovery from pressure-induced vacuoles (Youatt 1980*a*).

References

Albert, A. (1973). 'Selective Toxicity.' Ch. 10. pp. 370–7. (Chapman and Hall: London.)

Creanor, J., May, J. W., and Mitchison, J. M. (1975). The effect of 8-hydroxyquinoline on enzyme synthesis in the fission yeast *Schizosaccharomyces pombe*. *Eur. J. Biochem.* **60**, 487–93.

Fraser, R. S. S., and Creanor, J. (1974). Rapid and selective inhibition of RNA synthesis in yeast by 8-hydroxyquinoline. *Eur. J. Biochem.* **46**, 67–73.

Fraser, R. S. S., and Creanor, J. (1975). The mechanism of inhibition of ribonucleic acid synthesis by 8-hydroxyquinoline and the antibiotic lomofungin. *Biochem. J.* **147**, 401–10.

Fultz, S. A., and Sussman, A. S. (1966). Antigenic differences in the surfaces of hyphae and rhizoids in *Allomyces*. *Science* (*Wash., D.C.*) **152**, 785–7.

Youatt, J. (1976). The effect of actinomycin D on the formation of gametangia and mitosporangia in *Allomyces*. *Arch. Microbiol.* **109**, 163–6.

Youatt, J. (1977). Chemical nature of *Allomyces* walls. *Trans. Br. Mycol. Soc.* **69**, 187–90.

Youatt, J. (1980*a*). Selective production of resistant sporangia in suspensions of *Allomyces*. *Trans. Br. Mycol. Soc.* **75**, 334–6.

Youatt, J. (1980*b*). Degradation of nucleic acid by *Allomyces macrogynus* during the production of zoosporangia and resistant sporangia. *Aust. J. Biol. Sci.* **33**, 393–401.

Youatt, J. (1980*c*). Changes in the carbohydrates of *Allomyces macrogynus* during the selective development of either zoosporangia or resistant sporangia. *Aust. J. Biol. Sci.* **33**, 505–11.

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