# HEAVY METAL ACCUMULATION IN FILAMENTOUS FUNGI

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Colin Charles Townsley, B.Sc.

Department of Biological Sciences,
University of Keele.

## ABSTRACT

Heavy metal toxicity was monitored in filamentous fungi by observing the effects of incorporating metal salts in a solid glucose mineral salts (GMS) medium on fungal colony extension. Copper, cadmium and zinc toxicity to *Penicillium spinulosum*, *Trichoderma viride* and *Aspergillus niger* was investigated by monitoring the effects of added metal on biomass production when supplemented in a liquid GMS medium.

Metal accumulation was determined in actively growing mycelium in GMS medium initially buffered at pH 5.5 with 50.0 mM MES containing added metal at non-toxic concentrations. Accumulation of metals appeared to be maximal during the lag phase of growth. This was followed by an apparent reduction in mycelial metal during the linear growth phase. Exponential growth was not observed. The medium pH at inoculation was critical for effective metal uptake.

Metal uptake by non-growing fungal suspensions was investigated by harvesting mycelium in mid-linear growth phase and resuspending in 50.0 mM MES buffer (pH 5.5) containing added metal. Uptake was initially rapid indicating wall binding. No energy dependence was demonstrated in subsequent metal uptake. Similar uptake patterns were observed for mycelium in the presence and absence of 10.0 mM glucose, in the presence of 1.0 mM sodium azide, under anaerobic incubation and also in isolated cell walls. Only incubation at $4^{\circ}C$ reduced metal accumulation. Two possible metal binding sites were implicated in *Penicillium spinulosum* and these were shown to be non-specific for copper. Electron spin resonance measurements on copper loaded biomass indicated copper coordination to either 4 nitrogen or 2 nitrogen and 2 oxygen atoms. The evidence suggested that metal accumulation in filamentous fungi under the experimental conditions employed was a physical process, possibly by adsorption initially onto the cell wall, followed by internalisation by diffusion.

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CONTENTS

## 1. GENERAL INTRODUCTION

The essential elements of protoplasm circulate in the biosphere from environment to organism and back again in biogeochemical element cycles. Since organisms are constructed of elements it follows that they are indispensible in element cycling and it is becoming apparent that micro-organisms in particular are the major exponents of mineral turnover. According to Trudinger *et al* (1979), the influential role of microbes in chemical cycling can be attributed to an active involvement of elemental metabolism, and a passive role whereby element mobilisation in an ecosystem is an indirect result of microbial habitation in terms of redox changes, pH changes, organic material utilisation and production, weathering and other secondary physical and chemical means.

The primary involvement of microbes in biogeochemical cycling is as a result of metabolic processes. This involvement can be classified as one involving redox reactions, or one involving those reactions which result in phenomena such as biomethylation or alkylation. Many of these metabolic changes in the elemental state however, are dependent on the metal being accumulated and the main objective of this study was to examine the phenomenon of heavy metal accumulation specifically in filamentous fungi.

There has been a recent surge in interest in the area of metal accumulation in microbes, partly because of the improvement in the sensitivity of analytical instruments for metal determinations, but more importantly, because of the increased awareness of metal pollution and the possibilities of metal recovery from aqueous effluents containing soluble metallic residues at low concentrations.

The economic and political significance of metal accumulation by microorganisms has been realised relatively recently with the discovery that they can extract metals from very dilute aqueous solutions and can therefore act as biosorbents. According to Lundgren and Malouf (1983), in the last 40 years metal consumption in the world as a whole has escalated dramatically with the result that many metals have become scarce. With the rise in the industrial use of metals there has been a concomitant increase in the discharge of toxic metals into the environment, through metal-laden industrial effluents reaching sewage systems, agricultural run off, atmospheric contamination from fossil fuel burning, mine waste drainage, radioactive fuel processing and automobile exhaust systems. There are recommendations set out by the World Health Organisation for limits in metal release into the environment and this should result in increasingly stringent legislation. However, conventional techniques for metal recovery prior to discharge such as precipitation, ion exchange, electrolysis, evaporative-distillation, liquid-liquid extraction and solvent extraction (Flett and Pearson, 1975) are likely to become prohibitively expensive and inefficient at the new thresholds and it appears that the capacity of microbes for metal accumulation from very dilute solutions may be applicable to this problem. The attractive idea of treating metal bearing wastes with waste biomass from microbial processes has been proposed (Tsezos and Volesky, 1981; Tsezos, 1983).

On an academic level, there is a substantial body of literature devoted to the relationships between metals and bacteria, algae and yeasts with reference to toxicity, resistance and accumulation and transport, but comparative information for filamentous fungi is much more fragmented. The few reports which are available are often contradictory, particularly when reference is made to the involvement of metabolic energy in metal

uptake although these are usually in different experimental systems (Paton and Budd, 1972; Somers, 1963; Horikoshi *et al*, 1981). Also, most reports for metal uptake in fungi are for non-growing biomass (Budd, 1969, 1975, 1979; Paton and Budd, 1972 and Schneider and Lindberg, 1983). Furthermore, almost all reported work has been performed using a limited number of fungal species. For example there is a great deal of information available for *Neocosmospora vasinfecta* (Budd, 1969, 1975, 1979; Paton and Budd, 1972) and for *Neurospora crassa* (Somers, 1963; Slayman and Tatum, 1964; Slayman and Slayman, 1970; Venkateswerlu and Sivarama Sastry, 1970, 1973, 1979; Sivarama Sastry *et al*, 1962; Maruthi Mohan and Sivarama Sastry, 1983; Maruthi Mohan *et al*, 1984; Schneider and Lindberg, 1983). Most reports are also for a single metal. It seems also that a large proportion of the work on heavy metals and fungi has been carried out using spores in which the effects of metals on germination were studied (Miller and McCallan, 1957; Parry and Wood, 1958; Somers, 1961, 1963, 1966; Lowry *et al*, 1957; Okamoto *et al*, 1977). Although toxicity assessment in terms of spore germination is reasonably effective for fungicide screening, it is not necessarily applicable to evaluating toxicity in vegetative mycelium since spores are likely to be physiologically quite different. For this reason toxicity tests on vegetative mycelium need to be performed prior to accumulation studies in vegetative mycelium. It appears that most of the literature supports the view that there is a great deal of confusion about the association of heavy metals with filamentous fungal cells. This project attempted to clarify some of the ambiguity surrounding this area.

One of the initial aims of the project was to define and employ a simple, quick and effective technique for screening metal toxicity in filamentous fungi so that a working system for comparativity could be established using three fungi and three metals for the rest of the project.

The three fungi and the three metals used were selected from this primary study of the effects of five metals on the growth of seven filamentous fungi in solid medium. The three fungi, *Penicillium spinulosum*, *Trichoderma viride* and *Aspergillus niger* were chosen after consideration of their growth and sporulation characteristics in solid medium with regard to their responses to the presence of added metal and their relative ease of handling. The three metals, copper, cadmium and zinc were chosen for study at non-toxic concentrations. All three metals have ecological significance and differ in their toxic effects to fungi.

Copper is of interest since it is an essential element (Bowen, 1966) and is transported into fungal cells, but at elevated concentrations causes a toxic effect at the cell surface either by membrane damage or interference with membrane transport and normal membrane activities (Ross, 1975). Zinc is also of interest because it is an essential element (Bowen, 1966) yet it is relatively non-toxic, reputedly exerting its effect by creating a cellular magnesium deficiency (Adiga *et al*, 1961; Laborey and Lavollay, 1973). Cadmium on the other hand, has not been reported to be an essential element and appears to be very toxic.

The three metals are of interest ecologically in terms of their fungicidal activities and in terms of pollution. Tolerance to copper in *Poria spp.* has been reported by Levi (1969), in *physalospora obtusa* to copper in Bordeaux mixture (Taylor, 1953) and similarly in *Phytophthora infestans* (Horsfall, 1956). Massie *et al*, (1968) reported tolerance of *Sclerotinia homeocarpa* Bennett to a cadmium-containing fungicide.

There are numerous reports for anthropogenic deposition of copper into the environment (Babich and Stotzky, 1983). Similarly, cadmium emission has

recently been quantified in Europe (Nordberg, 1974; Hutton, 1983a, 1983b; Taylor, 1983). Zinc also, is emitted by industrial and domestic activity and has an adverse effect on the activity, ecology and population dynamics of microbiota (Babich and Stotzky, 1974). The study of copper, cadmium and zinc in terms of toxicity and uptake in fungi is justifiable because of their wide ranging effects on microorganisms and their ecological significance.

To facilitate metal accumulation experiments, there was the apparent need to separate biomass from metal containing medium and since this was impractical using solid medium, further experimentation was performed using liquid medium. The objective then was to assess the growth parameters in the liquid medium and this required the formulation of a standard inoculum and inoculation procedures.

In developing the experimental procedures for metal accumulation monitoring in growing fungi, a method for estimating the degree of metal complexing was necessary and this was established using electrochemical techniques. Fortunately, there did not appear to be a major complexing problem in this medium which had been specifically designed for metal uptake studies. Complexing has previously been reported to be a serious complicating factor in metal accumulation studies because it renders metal ions biologically unavailable for uptake, and this therefore influences metal toxicity as well as the quantitative amount of metal accumulated. The relevant literature is reviewed in chapter 2.

The rationale for using growing fungi initially for metal accumulation studies was based on a number of factors. Primarily, the aim was to observe uptake in growing fungal cultures since most of the published work has

been performed using non-growing suspensions of fungi. There is however, very little evidence to suggest that quantitative accumulation is the same in the two systems, or even that the accumulation is uniform with time in growing cultures throughout the growth cycle. It appears that the only reports available for growing uptake are those of Shatzman and Kosman (1978) using *Dactylium dendroides* and copper, Venkateswerlu and Sivarama Sastry (1979) using *Neurospora crassa* and cobalt, Failla and Weinberg (1977) using *Candida utilis* and zinc, and of Gadd and Griffiths (1978b) using copper and *Aureobasidium pullulans*. The report by Shatzman and Kosman (1978) was mainly concerned with the utilisation of copper and its role in the biosynthesis of copper-containing proteins, but the authors did investigate copper uptake by growing cells of *Dactylium dendroides*. Cobalt uptake by *Neurospora crassa* was limited to an investigation of accumulation over a 2 hour period in toxic levels of the metal. The other two citations refer to organisms which are either not filamentous (*Candida utilis*) or polymorphic (*Aureobasidium pullulans*). Using three metals and three fungi introduced a comparative angle to the work. It is worthy of note also that most of the reports in the literature tend to concentrate either on metal toxicity or on accumulation, when using non-growing suspensions of biomass for the uptake studies, and so it has been difficult in the past to relate metal accumulation to toxicity (i.e. when monitoring the effect on growth) even though the two parameters are likely to be related. By using growing cultures however, this allowed the author to observe toxicity and metal accumulation concurrently.

## 2. DETERMINATION OF HEAVY METAL TOXICITY IN SOLID MEDIUM.

### 2.1. Introduction.

It is well established that the physiological role of some heavy metals such as copper, manganese and zinc is essential for microbial growth (Bowen, 1966), yet at elevated concentrations the same metals often become toxic. As a consequence of this essentiality, the implication is that the heavy metals are in some way internalised. It is also well known that heavy metal resistance can be acquired by microorganisms, so it is clear that there could be an intimate relationship between metal toxicity, resistance and uptake of the metal. Simple and definitive techniques are required to monitor each of these parameters.

One method for illustrating the effect of zinc, lead and cadmium on leaf surface microflora composition has been demonstrated by monitoring the number of recognisably different colonies of bacteria, yeasts and fungi on control leaves artificially treated with metal salts (Gingell *et al*, 1976), Similar work has been reported by Bewley (1978, 1980) who used this technique as well as direct observations of the effects of heavy metals on fungal hyphae extension, on leaf surfaces. A great deal of work has appeared in the literature describing evaluations of metal toxicity to fungi on solid medium based on the effects of metals on mycelial proliferation. The effects of copper salts on the mycelial growth of wood-rotting fungi have been demonstrated using agar spot-plate cultures (Keino, 1950). Misra and Singh (1970) have used metal supplemented agar plates to determine the fungitoxicity of copper fungicides to *Alternaria tenuis* and *Helminthosporium oryzae*. Fungitoxicity of zinc in pablum extract agar was reported for a number of soil microfungi isolated from soil in the vicinity of a zinc smelter in Pennsylvania, based on the effects of zinc

on colony extenion (Jordan and Lechevalier, 1975). The value of the technique has been emphasised by Babich and Stotzky who have employed the plate method to demonstrate the toxicity of cadmium to a number of microorganisms (Babich and Stotzky, 1977a, 1977b, 1982), zinc toxicity (Babich and Stotzky, 1978), manganese toxicity (Babich and Stotzky, 1981) and the synergistic effects of copper and nickel to microbes (Babich and Stotzky 1983). Chromium toxicity has also been reported by Babich *et al* (1982b) as has the comparative toxicity of nickel to selected microbes (Babich *et al*, 1982a). The effects of combinations of simulated acid rain and cadmium or zinc on microbial activity in soil has been reported by Bewley and Stotzky (1983).

The first section of this study aims to demonstrate the degree of toxicity of cobalt, manganese, copper, cadmium and zinc to seven filamentous fungi. The metals were selected on the basis of their reputed toxic effects, to obtain a series of metals ranging in their effects from relatively inocuous to severely toxic. The fungi used were selected after consideration of parameters such as growth rates, sporulation ability and ease of handling.

By using different types of solid medium or by supplementing the medium with various substances, the plate method can be used to demonstrate the influence of medium design on metal toxicity by virtue of the complexing capacity of many medium constituents. A brewing yeast was found to be unaffected by 40 $\mu g\ ml^{-1}$ copper in a malt wort molasses fermentation but copper was toxic at 1-2 $\mu g\ ml^{-1}$ in a mineral salts medium (White and Munns, 1951). Mediation of copper toxicity to *Aerobacter aerogenes* was attributed to complexing by yeast extract and cysteine by Macloed *et al* (1967). Steemann Nielsen and Kamp-Nielsen (1970), suggested that pronounced copper

toxicity at a level of $5 \times 10^{-3}$ µg $ml^{-1}$ to *Chlorella pyrenoidosa* was due to an absence of complexing agents in the medium. Ramamoorthy and Kushner (1975), reported that 160.0 µg $ml^{-1}$ mercury were required in their complex medium before ionic mercury was detectable. At 2.0 µg $ml^{-1}$ mercury, only $2 \times 10^{-3}$ µg $ml^{-1}$ was actually present in the ionic form. Babich and Stotzky (1977b), also reported that the addition of certain clay minerals to soil provided protection against the fungistatic effects of cadmium.

The objective of the second part of this section on metal toxicity in solid media was to determine the influence of the medium constituents of two different types of medium on cadmium toxicity. One of the media used was a glucose mineral salts medium (GMS) which was relatively free of complexing agents, the other was that employed by Babich and Stotzky (1977a) containing mycological peptone. Complexing of added metal results in mitigation of toxicity because of the reduced biological availability of the metal and consequential reduced uptake. This is important in terms of medium design for subsequent experiments which investigate metal accumulation.

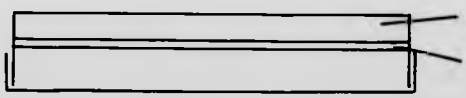
It is worthy of mention here that fungi were isolated from non-polluted environments since reports in the literature suggest that resistance to metals may result from exposure to metal and there are some suggestions that this may be due to reduced metal uptake (Basu *et al*, 1955; Starkey and Waksman, 1943). This would obviously have serious implications for metal uptake studies so the problem was avoided by using fungi isolated from non-polluted areas only.

## 2.2. Materials and Methods

### 2.2.1. Isolation of Fungal Cultures

A number of filamentous fungi were isolated from soil, rotting vegetation and the atmosphere in the vicinity of Keele University. Isolation from rotting vegetation and the atmosphere were carried out using isolation agar plates. Malt extract agar (MEA) (Oxoid), and Czapek's Dox (Oxoid) were used as isolation media. To prevent overgrowth by phycomycetes the media were supplemented with Rose Bengal (1:30 000 dilution), and bacterial growth was suppressed by the addition of streptomcycin (30.0 µg $ml^{-1}$).

For isolation of soil fungi 1.0 g of soil was suspended in 15.0 ml sterile carboxy methyl cellulose and shaken with glass beads to produce a fine soil suspension. 1.0 ml of this suspension was added to 100.0 ml molten tap water agar (TWA) (Oxoid). After thorough mixing 10.0 ml of the soil tap water agar mixture were poured onto isolation agar plates as a thin even layer.




All isolation plates were incubated at 25° C for 1 to 2 weeks. After incubation fungal colonies were selected for purification and further study. Purification of isolates was performed by repeated subculturing onto fresh MEA or Czapek's Dox. As a precaution all moulds were grown for a period of 10 days on MEA amended with penicillin at a concentration of 500 units $ml^{-1}$ to prevent bacterial contamination.

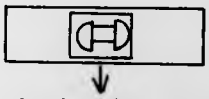
After purification the isolates were identified by microscopic examination of the characteristic reproductive structures. The drop-slide and thin-agar-film techniques were employed for the identification procedure using MEA and TWA.

a. Drop-slide technique.

MEA/TWA allowed to set.

Drop bissected.

Fungal plug placed between the 2 halves of the drop and covered with a coverslip.



Incubation in moist Petri dish at 25°C for 12-36 h.

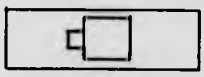
Microscopic examination.

b. Thin-agar-film technique.

MEA/TWA allowed to set.

Coverslip placed on drop prior to setting.

Fungal plug placed adjacent to thin agar film.



Incubation in moist Petri dish at 25°C for 12-36 h.

Microscopic examination.

Twelve fungi were successfully isolated, purified and identified. These included: *Penicillium spinulosum*, *Aspergillus niger*, *Trichoderma viride*, *Fusarium sp.*, *Botrytis sp.*, *Alternaria sp.*, *Dendryphion sp.*, *Trichothecium roseum*, *Thamnidium sp.*, *Mucor sp.*, *Rhizopus sp.* and *Mortierella sp.* Maintenance of the fungal isolates was carried out on potato dextrose agar slopes at 4°C with repeated subculturing every 3 months.

Chemicals.

All the chemicals employed in this study were of AnalaR or best available grade. Malt extract agar and Czapek's Dox agar were purchased from Oxoid Ltd., Wade Road, Baskingstoke, Hampshire, England., and all the other chemicals were obtained from either BDH Chemicals Ltd., Poole, England., or the Sigma Chemical Company Ltd., Poole, England.

Fresh stock metal solutions were prepared for each experiment in either distilled $H_2O$ or IM HCl. Metal chloride salts were used in all experiments unless otherwise stated.

Filters.

Filters used for harvesting mycelium were either Millipore HAWP (pore size 0.45 μM, 47 mm. diameter) or Whatman 541 fast ashless filters (7 cm. diameter).

Experimental pH.

The experimental pH for all work was 5.5 unless otherwise stated.

2.2.2. Toxicity tests on solid medium.

Metal toxicity was evaluated by monitoring the effects of added metal on radial colony extension on plates. Toxicity tests were carried out on a solidified glucose mineral salts medium. The medium composition per litre was as follows: Magnesium glycerophosphate ($C_3H_7MgO_6P.2H_2O$) 0.5g., $(NH_4)_2SO_4$ 2.0g., KCl 0.5g., $CaCl_2.6H_2O$ 0.25g., 2 (N-Morpholino) - ethane sulphonic acid (MES buffer) 9.76g., glucose 20.0g. 0.1ml of the trace element solution and 1.0ml of the vitamin solution were added per litre also (See appendix for composition). Agar (Oxoid No. 3) was added to a final concentration of 1.2% and the pH was adjusted to 5.5 using saturated KOH. pH 5.5 was used to ensure that the added metals were freely available in the ionic form and not hydroxylated as occurs at alkaline pHs.

IM solutions of copper, cadmium, cobalt, manganese and zinc were prepared in sterile distilled water from chloride salts of the metals. After filter sterilising dilution series were prepared in the range $1\text{-}10^{-4}$M. Appropriate additions of the metal salt were made to molten GMS medium to achieve final concentrations of 10, 100, 1000 and 10 000 µM. For ease of comparison with the literature it was decided to convert the metal concentrations to µg $ml^{-1}$ units and these represent the following concentrations respectively for copper, cadmium, zinc, manganese and cobalt: Copper 0.63, 6.3, 63.5, 635.0 µg $ml^{-1}$, cadmium 1.12, 11.2, 112.0, 1120.0 µg $ml^{-1}$, zinc 0.65, 6.5, 65.0, 650.0 µg $ml^{-1}$, manganese 0.55, 5.5, 55.0, 550.0 µg $ml^{-1}$ and cobalt 0.59,5.9, 59.0, 590.0 µg $ml^{-1}$.

Seven fungi including *P. spinulosum*, *A. niger*, *T. viride*, *Fusarium sp.*, *Botrytis sp.*, *Mucor sp.* and *Trichothecium roseum* were grown on GMS for several days at 25°C. Three replicate plates were inoculated with

5mm diameter discs of mycelium for each of the metal concentrations. The discs were placed centrally with fungal growth down to minimise sporulation and growth from the original GMS disc. Plates were incubated at 25°C until the colonies approached the edge of the plates. The experiment was performed in duplicate.

Growth was monitored for a minimum period of 3 days for fast growing fungi such as *Mucor sp.*, or for upto 20 days for the slower growing fungi such as *T. roseum*. The linear growth rate was established by measuring radial extension in 3 planes every 24h. and a mean growth rate was calculated in mm $day^{-1}$. Growth rates in the presence of added metal were compared to control plates with no added metal and the results are presented graphically as a plot of the percentage of the control growth rate for metal addition, against concentration of added metal.

Toxicity tests on 2 different types of solid medium.

Cadmium toxicity was determined using the mycelial extension technique on solidified GMS medium and on the medium used by Babich and Stotzky (1977a) in the concentration range 1-1000 µg $ml^{-1}$. The Babich and Stotzky medium composition per litre was as follows: $MgSO_4.7H_2O$ 0.5g., $KH_2PO_4$ 0.5g., NaCl 0.5g., Mycological peptone 10g., glucose 20g., agar 15g. The pH was adjusted to 5.9.

Four fungi including *P. spinulosum*, *T. viride*, *A. niger* and *Botrytis sp.* were grown on GMS medium for several days at 25°C. Three replicate plates of each medium were inoculated with 5mm discs of mycelium for each metal concentration and incubated for several days at 25°C. Growth was monitored in 3 directions and a comparison was made between the 2 media to determine if medium constituents such as mycological peptone

influenced metal availability and subsequent toxicity. By using percentage values of the control growth rates in the absence of added metal the complication caused by the inherent differences in growth rates on the 2 different media was avoided. The experiment was repeated for copper and with *T. viride*.

2.3. Results.

2.3.1. Toxicity tests on solid medium

Colony growth of all 7 fungi was typically linear in terms of radial extension. The variability in growth rates between the fungi is shown in table 2.3.1. For metal treated fungi, colony linear extension was expressed as a percentage of the control growth rate (no added metal) of the fungus in question on solid medium and plotted against added metal concentration. The effect of copper, cadmium, zinc, manganese and cobalt on growth is illustrated for *P. spinulosum*, *T. viride*, *A. niger*, *Botrytis sp.*, *Mucor sp.*, *Fusarium sp.*, and *Trichothecium roseum* in figures 2.3. 1-5. Radial extension of all metal treated fungal colonies was linear in terms of expansion across the surface of the plate expressed in units of mm $day^{-1}$ under all treatment conditions and in control plates.

Copper

The degree of copper toxicity to the fungi was variable. *T. viride* was notably resistant to copper in comparison with the other fungi showing slight inhibition at 63.5 µg $ml^{-1}$ and producing about 30% of the control growth rate even at 635.0 µg $ml^{-1}$. On the other hand *T. roseum* appeared to be particularly susceptible to copper since 6.35 µg $ml^{-1}$ was about 45% inhibitory, and 90% inhibitory at 63.5 µg $ml^{-1}$ $Cu^{++}$. Inhibition was complete at 635.0 µg $ml^{-1}$ for *A. niger*, *Botrytis sp.*, *Mucor sp.*, *Fusarium sp.*, and *T. roseum*. *Penicillium spinulosum* and *T. viride* were able to grow at about 28% and 30% of the controls at 635.0 µg $ml^{-1}$. The concentrations of copper which caused incipient (initial) and total inhibition of growth were variable between fungi. For example, *A. niger* did not grow at 635.0 µg $ml^{-1}$ but at 6.35 µg $ml^{-1}$ showed greater growth at about 80% of the control than did *P. spinulosum* (i.e. at about 64%

of the control) which exhibited 28% of the control fungus at 635 μg $ml^{-1}$. Many more examples of this phenomenon were apparent. Copper was generally very toxic.

Cadmium.

Again the toxicity of this metal to fungi was variable. The most resistant fungus, as observed with copper, appeared to be *Trichoderma viride* which exhibited about 55% growth as compared to controls at 112.0 μg $ml^{-1}$ $Cd^{++}$ and 10% at 1120.0 μg $ml^{-1}$. There was a degree of inhibitions ranging from 80% for *Fusarium sp.* to 10% for *T. viride* at concentrations as low as 1.12 μg $ml^{-1}$. *Fusarium sp.* and *T. roseum* were generally the two most susceptible fungi to cadmium. Slight growth was observed for *T. viride*, *P. spinulosum* and *A. niger* at 1120.0 μg $ml^{-1}$ but this concentration was completely inhibitory for *Botrytis sp.*, *Mucor sp.*, *Fusarium sp.* and *T. roseum*. As with copper, the concentration of cadmium which caused incipient inhibition was not correlated with the concentration which caused total inhibition of growth between different fungi. Cadmium was much more toxic than copper to most of the fungi tested under the conditions used experimentally, as shown by the fact that the range of inhibition was much narrower than that shown with the other metals.

Zinc.

Zinc apppeared to be generally less toxic than either copper or cadmium. *Penicillium spinulosum* was apparently the most resistant fungus tested at the higher concentration of 650.0 μg $ml^{-1}$ and showed about 77% of the control growth at this level of added zinc. *Trichoderma viride*

| Fungus | Growth rate (mm $day^{-1}$) |
|---|---|
| *Mucor sp.* | 11.5 |
| *Trichoderma viride.* | 10.5 |
| *Fusarium sp.* | 5.7 |
| *Botrytis sp.* | 5.5 |
| *Pencillium spinulosum.* | 3.5 |
| *Aspergillus niger.* | 2.2 |
| *Trichothecium roseum.* | 1.5 |

*Table* 2.3.1.

Linear growth rates of filamentous fungi on solidified Glucose Mineral Salts (GMS) medium.

also showed about 40% of the control growth at 650.0 μg $ml^{-1}$. *Fusarium sp.* appeared to be inhibited by 30% at 0.65 μg $ml^{-1}$ and 23% at 6.5 μg$ml^{-1}$, but was affected to a lesser degree at 65.0 μg $ml^{-1}$ with 40% inhibition than was *T. roseum* which only exhibited 21% of the control at the higher concentration. 650.0 μg $ml^{-1}$ was completely toxic to *Fusarium sp.*, *T. roseum*, *Botrytis sp.*, *Mucor sp.* and *A. niger*. The phycomycete *Mucor sp.* was apparently stimulated by low zinc concentrations showing 35% stimulation at 0.65 μg $ml^{-1}$ and 36% at 6.5 μg $ml^{-1}$ although 18% inhibition was recorded at 65.0 μg $ml^{-1}$.

Manganese.

Manganese appeared to be a relatively non-toxic metal showing a maximum of 10% inhibition at 0.55 μg $ml^{-1}Mn^{++}$. *T. roseum* exhibited a certain degree of susceptibility to manganese with 30% inhibition at 5.5 μg $ml^{-1}$, 30% at 55 μg $ml^{-1}$ and total inhibition at 550.0 μg $ml^{-1}$. It is noteworthy that at 550.0 μg $ml^{-1}$ manganese exhibited a wide range of effects from total inhibition for *Mucor sp.* and *T. roseum*, 80% inhibition for *Fusarium sp.*, 33%, 22% and 18% inhibition for *Botrytis sp.*, *P. spinulosum* and *T. viride* respectively, and an apparent stimulation of 21% for *A. niger*. This was the most wide ranging series of effects for any of the metals tried. Manganese was also the only metal which had no inhibitory effect on a fungus at the highest concentration tested. As with the other metals tested there appeared to be no clear correlation with the manganese concentration which was initially toxic and that which caused more serious inhibition of growth between different organisms. Manganese was the least effective metal tested overall in terms of growth inhibition.

*Figures* 2.3. 1-5 Heavy metal toxicity to filamentous fungi on solid medium.

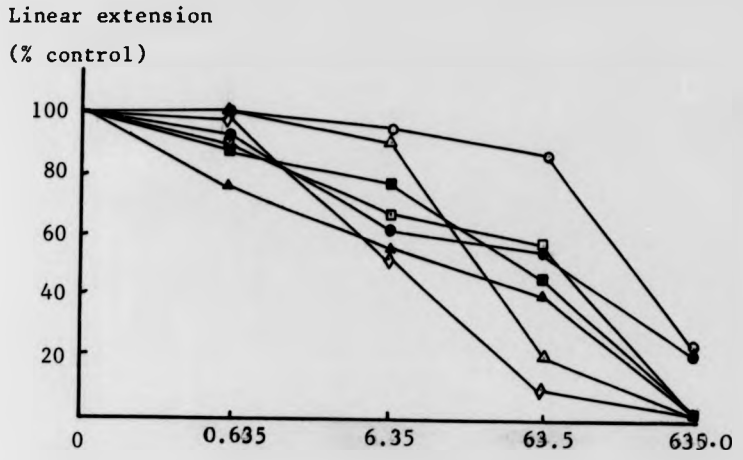



*Figure* 2.3.1.

- ● *P. spinulosum.*
- ○ *T. viride.*
- ■ *A. niger.*
- □ *Botrytis sp.*
- ▲ *Mucor sp.*
- △ *Fusarium sp.*
- ◊ *T. roseum.*

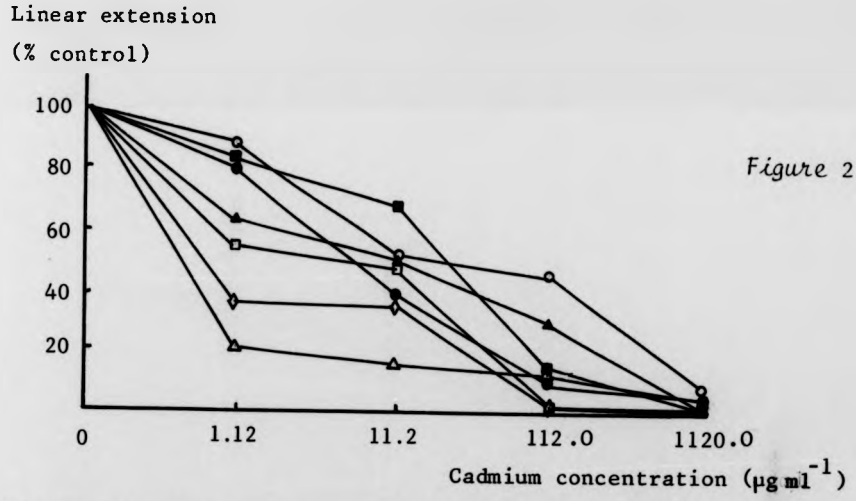



*Figure* 2.3.2.

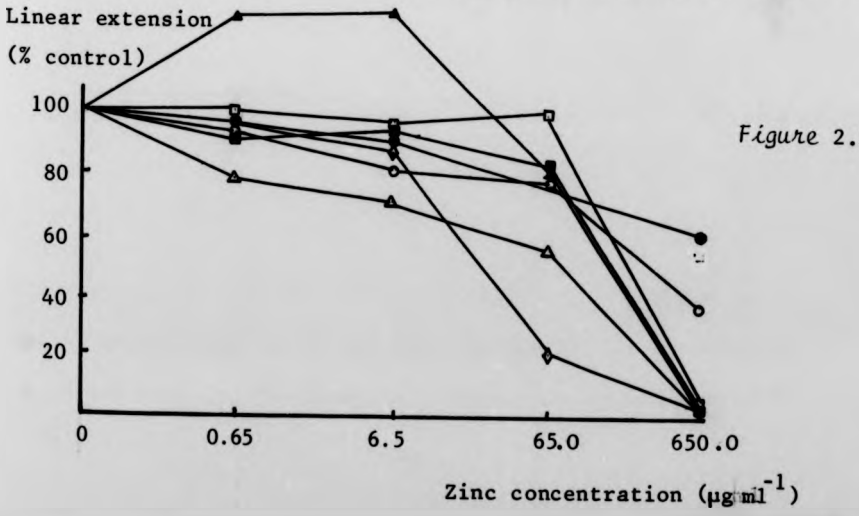



*Figure* 2.3.3.

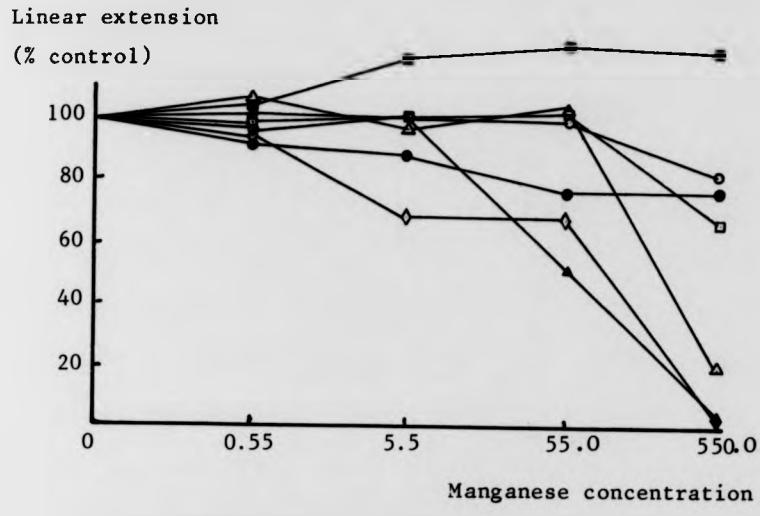



Figure 2.3.4.

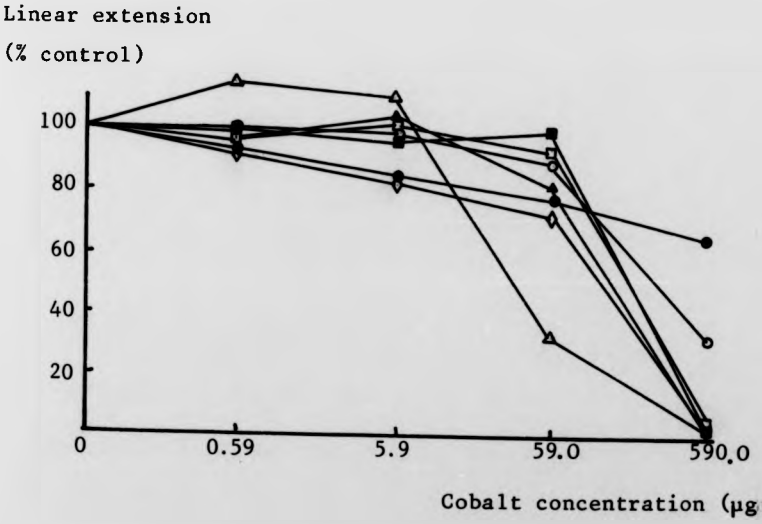



Figure 2.3.5.

Heavy metal toxicity to filamentous fungi on solid medium.

●, *P. spinulosum*; ○, *T. viride*; ■, *A. niger*; □, *Botrytis sp.*;

▲, *Mucor sp.*; △, *Fusarium sp.*; ◊, *Trichothecium roseum*.

Cobalt.

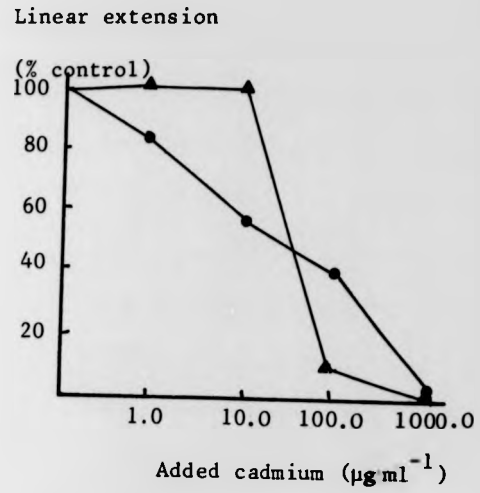
Cobalt, like manganese, appeared to be a relatively inocuous metal showing little toxicity upto 59.0 µg $ml^{-1}$ $Co^{++}$. With the exception of *Fusarium sp.* which showed 30% of control growth at 59.0 µg $ml^{-1}$, the other fungi tested were not inhibited by more than 25% at this concentration. At 590.0 µg $ml^{-1}$ however, all fungi were severely restricted in growth except for *T. viride* which exhibited about 38% control growth and *P. spinulosum*, which appeared to be the most resistant fungus, showing 70% of the control growth. *Fusarium sp.* was stimulated by 0.59 and 5.9 µg $ml^{-1}$ $Co^{++}$ by 12 and 10% respectively. At 59.0 µg $ml^{-1}$ however, *Fusarium sp.* was the most susceptible fungus tested and this confirms the lack of correlation between metal concentrations at which metal toxicity is first observed and those at which inhibition of growth is more serious for other fungi.

The general toxicity series for the metals tested was $Cd^{++} > Cu^{++} > Zn^{++} = Co^{++} > Mn^{++}$. The seven fungi were generally variable in response to the metals but *T. viride* often demonstrated a higher degree of resistance whilst *T. roseum* and *Fusarium sp.* were usually susceptible.

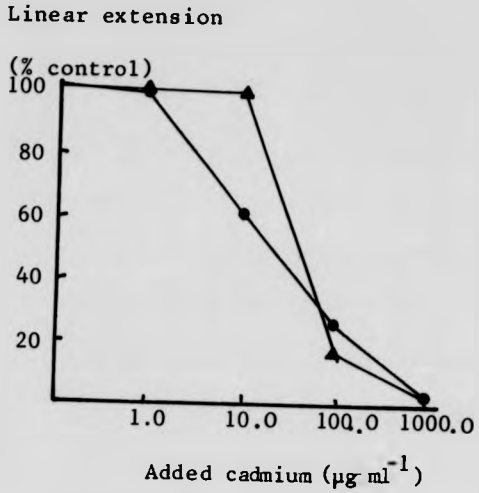
Toxicity tests on 2 different types of solid medium.

Figures 2.3. 6-9 illustrate the effect of cadmium on surface growth when added to GMS medium and the medium of Babich and Stotzky (1977a), of *P. spinulosum*, *T. viride*, *A. niger* and *Botrytis sp.* in an attempt to elucidate the influence of medium components on metal availability and toxicity. There appeared to be no difference in cadmium toxicity to *T. viride* in the range of cadmium concentrations from 1-1000 µg $ml^{-1}$ on the 2 media used, but toxicity to *P. spinulosum* at 1.0 and 10.0 µg $ml^{-1}$ and to *Botrytis sp.* at 10.0 µg $ml^{-1}$ $Cd^{++}$ appeared to be less severe on

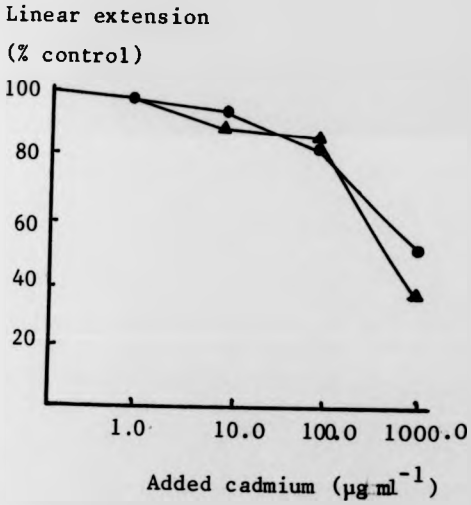
the Babich and Stotzky medium than on the GMS medium. Figure 2.3. 10 shows the effect of copper on the growth of *T. viride* on the same 2 media. Copper appeared to be more toxic at 63.5 µg $ml^{-1}$ $Cu^{++}$ on the GMS medium.



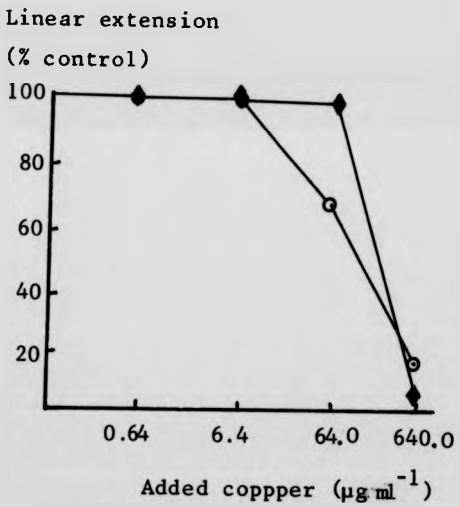

*P. spinulosum.*



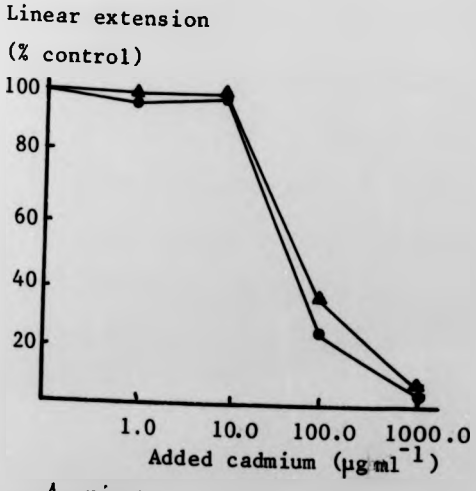

*Botrytis sp.*




*T. viride.*




*T. viride.*




*A. niger.*

*Figures* 2.3.6-10.
Cadmium and copper toxicity to filamentous fungi on 2 types of solid medium based on inhibition of colony linear extension.

- ●, Cadmium in GMS medium.
- ▲, Cadmium in Babich & Stotzky medium.
- ⊙, Copper in GMS medium.
- ♦, Coppper in Babich & Stotzky medium.

## 2.4. Discussion.

Although the growth rates of the 7 fungi tested varied substantially, the rates of colony extension were linear. The observed growth of the surface colonies on solid medium was typical of growth of filamentous fungi on solid substrates, since it is generally accepted that fungal hyphae grow at a constant rate. According to Trinci (1971), growth rate is linear and depends on the width of the peripheral portion of growth where hyphal growth is exponential and about equal to the specific growth rate.

In this section a rapid and effective technique was used for monitoring the effects of heavy metals on mycelial growth of filamentous fungi. The technique is of particular interest when screening tests are performed on a number of metals and several fungi. Unfortunately, the method was not universally applicable to all fungi since problems were encountered during the study with fungi that sporulated profusely, or produced irregularly shaped colonies. There is also the difficulty of metal complexing agents in the medium with special reference here to the agar complement. Furthermore, attempts to relate metal toxicity to accumulation on plates is very difficult because of the problems in separating mycelium from medium for subsequent metal analysis of the hyphae although Gadd (1983), has approached the problem with a degree of success. This author added metal salts to wells in the agar and monitored inhibition zones. However, differential toxicities of copper, cadmium, zinc, manganese and cobalt were clearly demonstrated in this study and some observations on fungal resistance were made, even though there are limitations of the method.

The pH of the medium was 5.5 to ensure that most of the added metal existed predominantly as the free divalent cation. Cadmium for example, forms $CdOH^+$ at pH 7.5 and $Cd(OH)_2$ at 9 (Babich and Stotzky, 1977a), so pH is clearly of great importance in metal availability.

Manganese appeared to be the least toxic metal tested overall and indeed was stimulatory with A. *niger*. Zinc and cobalt were also relatively inocuous and variable in their effects on fungal growth but, these were generally moderate. Copper was very toxic to most of the fungi tested but cadmium proved to be the most effective in inhibiting fungal growth.

Copper.

Although copper was demonstrated to be very toxic to most of the fungi tested in this study, typically showing deleterious effects at 6.35 to 63.5 µg $ml^{-1}$ *T. viride* was able to tolerate 63.5 and 635.0 µg $ml^{-1}$ $Cu^{++}$. It is interesting that this fungus grew rapidly at a rate of 10.5 mm $day^{-1}$ in controls.

There is little data available on plate toxicity in the literature for copper, but Babich and Stotzky (1983), reported that copper elicited toxicity to *Aspergillus flavipes* at 100.0 µg $ml^{-1}$ $Cu^{++}$. By comparison, the strain of A. *niger* used in this study showed slight susceptibility to copper at 0.635 µg $ml^{-1}$ and was seriously affected by copper at 63.5 µg $ml^{-1}$ shown by an inhibition of growth over controls amounting to about 45%. Misra and Singh (1971), showed that copper, in the form of copper containing fungicides such as Bordeaux Mixture, Blitox 50 and Fytolan was variable in its effects on surface growth of fungi. *Helminthosporium oryzae* was less susceptible to copper than *Alternaria tenuis*, as shown by

the fact that a higher copper concentration was required in *Helminthosporium oryzae* to achieve the $ED_{50}$ parameters shown by *Alternaria tenuis*. The authors attributed this to the fact that *H. oryzae* is a virulent pathogen whereas *A. tenuis* is saprophytic or weakly pathogenic. On the other hand, the data of Smith (1977) supported the suggestion that there is no correlation between saprophytic and parasitic activity and sensitivity to trace metals *in vitro*, in either plate testing using a solidified medium or in liquid culture. Unfortunately, Misra and Singh (1971) applied copper in dosages of $\mu g\ cm^{-2}$ so it is difficult to make direct comparisons with the data obtained in this study.

Cadmium.

Cadmium was the most toxic metal tested in this study, often becoming toxic at 1.12 $\mu g\ ml^{-1}\ Cd^{++}$. *Trichoderma viride* showed the greatest degree of tolerance at 112.0 $\mu g\ ml^{-1}\ Cd^{++}$.

According to Babich and Stotzky (1977a), 22 fungi could be categorised as sensitive, moderately resistant and resistant in their responses to cadmium. It is noteworthy that the authors classed *T. viride* as resistant and *A. niger* as moderately resistant and this is in agreement with the results obtained in this study. The strain of *T. viride* used by Babich and Stotzky showed 22% growth over the control at 10.0 $\mu g\ ml^{-1}\ Cd^{++}$ (Babich and Stotzky, 1977a, 1977b) and 26% growth as compared to controls at added cadmium concentrations of 25.0 $\mu g\ ml^{-1}$ (Babich and Stotzky, 1982). For the strain of *T. viride* used in this study, 60% of the control growth rate was achieved at 11.2 $\mu g\ ml^{-1}$ and 55% at 112.0 $\mu g\ ml^{-1}$ so the 'Keele' strain appeared to be rather more resistant. The strain of *A. niger* used by Babich and Stotzky showed reduced growth at 10.0 $\mu g\ ml^{-1}\ Cd^{++}$ and was completely inhibited at

100.0 μg $ml^{-1}$ (Babich and Stotzky, 1977a, 1977b), and was inhibited by 17% at 25.0 μg $ml^{-1}$ (Babich and Stotzky, 1982). In the strain used in this study growth was inhibited by 33% at 11.2 μg $ml^{-1}$ and 85% at 112.0 μg $ml^{-1}$ so this is in good agreement with published results. Other studies (Doyle *et al*, 1975), showed that growth of A. *niger* was initially reduced at 10.0 μg $ml^{-1}$ in heart-infusion broth and totally inhibited at 80.0 μg $ml^{-1}$.

The very toxic nature of cadmium was also indicated by Babich and Stotzky (1977a, 1977b) in that the exponential growth rates were reduced by as little as 0.5 μg $ml^{-1}$ $Cd^{++}$ in *Agrobacterium tumefaciens* and *Nocardia corallina*. Other studies have shown increases in generation time of *Escherichia coli* at 0.34 μg $ml^{-1}$ $Cd^{++}$ (Mitra *et al*, 1975). Although this data for organisms other than filamentous fungi is not directly comparable, it is interesting that in this study low cadmium levels of 1.12 μg $ml^{-1}$ inhibited *Fusarium sp.* and *T. roseum* by 80% and 62% respectively. Work by Babich and Stotzky (1977a, 1977b), suggested that only 2 fungi were inhibited by 1.0 μg $ml^{-1}$ $Cd^{++}$ and this was by 8% in the case of *Penicillium vermiculatum* and *Botrytis cinerea*. *Aureobasidium pullulans* was significantly inhibited by 2.2 μg $ml^{-1}$ $Cd^{++}$ as was *Pestalotiopsis sp.* and *Pleurophomella sp.* (Smith, 1977), and *Chaetomium sp.* by 1.5 μg $ml^{-1}$ $Cd^{++}$. Cadmium toxicity to *Aureobasidium pullulans* was also reported by Gadd (1983).

Zinc.

Zinc is a metal generally considered to be low in toxicity to fungi (Somers, 1961). Whilst zinc is an essential metal required for the activity of zinc containing enzymes such as glyceraldehyde 3-phosphatase, cysteine desulphydrase and aldolase it appears to be moderately toxic.

A degree of resistance was demonstrated in *P. spinulosum* and *T. viride* at 650.0 µg $ml^{-1}$, and *mucor sp.* appeared to be stimulated at 0.65 and 6.5 µg $ml^{-1}$. Zinc reputedly inhibits respiration (Nickerson, 1946) and growth of fungi (Smith, 1977). Work by Smith (1977), indicated that linear growth of *Chaetomium sp.* was suppressed by 130.0 µg $ml^{-1}$ as was *Cladosporium sp.*; and *Gnomonia platani*, *Epicoccum sp.* and *Pleurophomella sp.* were inhibited by 265.0 µg $ml^{-1}$ $Zn^{++}$.

Babich and Stotzky (1978) showed that zinc at concentrations at up to 65.0 µg $ml^{-1}$ did not affect fungi but 650.0 µg $ml^{-1}$ reduced mycelial growth of *Fusarium solani*, *Cunninghamella echinulata*, *A. niger*, *T. viride* and completely inhibited *Rhizopus solani*. Growth of *A. niger* was inhibited by 50% at 650.0 µg $ml^{-1}$ at pH 5.6, but the strain used in this study was completely inhibited at 650.0 µg $ml^{-1}$. Other work on zinc toxicity (Babich and Stotzky, 1983) based on $CO_2$ output showed that zinc had no effect on the lag period of *A. niger* when added to soil at pH 4.9 or acidified to pH 3.2. Addition of 10 000 µg $ml^{-1}$ $Zn^{++}$ to soil at both these pH values resulted in increasing the lag period by 1 day. Acidifying to pH 2.8 resulted in a 2 day extension of lag. The effect of soil on reducing the toxicity of zinc is apparent here since 1000.0 µg $ml^{-1}$ did not affect growth but in Sabouraud dextrose agar it was 50% toxic at 650.0 µg $ml^{-1}$. The role of pH is also of importance. Jordan and Lechevalier (1975) showed that most fungi isolated in the vicinity of a zinc smelter could tolerate fairly high doses of zinc. Although minimum toxic levels ranged from 6.5 to 130.0 µg $ml^{-1}$, most of the fungi tested were capable of tolerating at least 45.5 µg $ml^{-1}$ and exhibited 50% of the normal growth rate at this zinc level. This is in fair agreement with the zinc toxicity levels at 65.0 µg $ml^{-1}$ as shown in this study.

Manganese.

Manganese appeared to be a fairly non toxic metal except at elevated concentrations of 55.0 μg $ml^{-1}$ $Mn^{++}$ and above for most fungi, although there was a certain amount of variability. *Trichothecium roseum* was particularly susceptible to manganese showing toxic effects at 5.5 μg $ml^{-1}$. Manganese has been reported to be essential for the growth of A. *niger* by Kisser *et al* (1980), and its main role is as an activator phosphate transferases and decarboxylases.

*Aspergillus niger* was stimulated by manganese at concentrations greater than 0.55 μg $ml^{-1}$ up to 550.0 μg $ml^{-1}$. Babich and Stotzky (1981), reported that *Aspergillus conoides* was stimulated by 10, 50 and 100 μg $ml^{-1}$ which is in good agreement with the results of this study. Smith (1977) also observed that growth of *Aureobasidium pullulans* was promoted in the presence of 8.3 μg $ml^{-1}$ $Mn^{++}$. Stimulation of growth by low concentrations of metal ions has also been shown for copper and *Penicillium ochrochloron* by Stokes and Lindsay (1979), and for cadmium in *Scytalidium sp.*, *Pythium sp.* and *Dictyuchus sp.* (Duddridge and Wainwright, 1980). Low cadmium concentrations also stimulated *Lactobacillus acidophilus* and *Streptococcus faecalis* (Doyle *et al*, 1975). This phenomenon has been termed the Arndt-Schulz law of metal stimulation (Lamanna and Mallette, 1953) and has been interpreted as a requirement for more metal than is afforded by the normal micronutrient levels in the media, or alternatively, it may be that the metal causes an increase in cell membrane permeability which allows for a freer flow of nutrients into the cell, with a concomitant increase in metabolic activity. Smith (1977) interpreted growth stimulation in his linear extension experiments as a result of the anionic fraction of the metal nitrate supplement and not of the presence of the cation. In this study,

the author used chloride salts as the anionic fraction but according to Babich and Stotzky (1978) the influence of chlorides can be variable. These authors reported that the toxicity of zinc to fungi, bacteria and coliphages was unaffected, lessened or increased by the presence of high concentrations of NaCl. The increased toxicity of zinc in organisms where toxicity of zinc was elevated in the presence of high concentrations of NaCl, was not a result of a synergistic interaction between $Zn^{++}$ and elevated osmotic pressures, but of the formation of complex anionic Zn-Cl species that exerted greater toxicities than did cationic $Zn^{++}$. Conversely, in organisms where zinc toxicity was reduced, the decrease in zinc toxicity with increasing concentrations of NaCl probably reflected the decrease in the levels of $Zn^{++}$, due to the formation of Zn-Cl species, which was less inhibitory to these microbes than was $Zn^{++}$.

Smith (1977), designated manganese as one of the broadest spectrum growth suppressors for urban-tree phylloplane fungi. Babich and Stotzky (1981) reported that fungi exhibited a wide range of sensitivities to manganese. For example, *Scopulariopsis brevicaulis* and *Aspergillus giganteus* showed incipient inhibition at 100.0 μg $ml^{-1}$. *Aspergillus niger*, *T. viride* and *P. vermiculatum* were inhibited at 500.0 μg $ml^{-1}$ whilst a *Cephalosporium sp.* grew at up to 1000.0 μg $ml^{-1}$ and *Aspergillus clavatus* was unaffected even at 2000.0 μg $ml^{-1}$ $Mn^{++}$. The results of this study show some agreement with the data of Babich and Stotzky (1980). The strain of *T. viride* used in this study was inhibited by 10% at 549.0 μg $ml^{-1}$ and this is in agreement with the value of 500.0 μg $ml^{-1}$ as reported by Babich and Stotzky for incipient inhibition. However, the strain of *A. niger* used in this study showed no inhibition even at 549.0 μg $ml^{-1}$ and this probably reflects strain variation between the two isolates used in the different laboratories. Smith (1977)

demonstrated that manganese toxicity in phylloplane fungi such as *Gnomonia platani*, *Pleurophomella sp.* and *Aureobasidium pullulans* became apparent at 469.0 µg $ml^{-1}$ $Mn^{++}$ and inhibition was more severe or complete at 1311.0 µg $ml^{-1}$. Some of the fungi used in this study, for example *Fusarium sp.*, *Mucor sp.* and *T.roseum* appeared to be more susceptible to manganese than in other published work but this may have been due to factors such as design of the medium, that is, in terms of the presence of components which could potentially complex added metals or of the working pH. Alternatively, strain variation may have influenced results which could account for anomalies between laboratories.

Cobalt.

Cobalt appeared to be an inocuous metal in terms of fungal toxicity and only at concentrations of 590.0 µg $ml^{-1}$ was it appreciably effective in reducing surface mycelial growth with the exception of *Fusarium sp.* to which it was toxic at 59.0 µg $ml^{-1}$ $Co^{++}$.

There appears to be no relevant information in the literature on cobalt toxicity on solid medium. Toxicity data is available however, for cobalt in liquid medium with *Neurospora crassa* (Sivarama Sastry *et al*, 1962; Padmanabhan and Sarma, 1966 and Venkateswerlu and Sivarama Sastry, 1970). From these investigations and earlier work by Healy *et al* (1955), cobalt toxicity has been elucidated with regard to the influence of $Fe^{+++}$ and $Mg^{++}$. The toxicity of cobalt was considerably enhanced by decreasing the normally high magnesium concentration in the medium. Reversal of cobalt toxicity was possible with iron and magnesium, the reversal by magnesium being due to a suppression of the uptake of the toxic metal. Control of cobalt uptake was not apparent when iron was the counteracting metal. Cobalt toxicity itself in this fungus, may be

due to iron deficiency (Healy *et al*, 1955; Adiga *et al*, 1961) or may be a result of interference with mycelial haem synthesis (Padmanabhan and Sarma, 1966).

Toxicity tests on 2 types of solid medium.

Although there appeared to be no difference in cadmium toxicity to *T. viride* and *A. niger* on the 2 media used, toxicity to *P. spinulosum* at 1.0 and 10.0 µg $ml^{-1}$ $Cd^{++}$ and to *Botrytis sp.* at 10.0 µg $ml^{-1}$ appeared to be less severe on the medium used by Babich and Stotzky (1977a), than on the GMS medium. This could indicate that at low cadmium levels, some of the metal was unavailable due to complexing to components in the Babich and Stotzky medium and thus toxicity of the metal was reduced. Variation in the response of the different fungi may be related to their scavenging abilities or competitiveness for metal ions. A consideration of the medium constituents shows that the Babich and Stotzky medium contains potential complexing agents in the form of mycological peptone (1%) and the presence of inorganic phosphate may also lead to some precipitation of the added metal.

Ion specific electrodes have been used by Ramamoorthy and Kushner (1975) to study binding of $Hg^{++}$, $Pb^{++}$, $Cu^{++}$ and $Cd^{++}$ to bacterial growth media and media components such as yeast extract, peptone, tryptone, proteose peptone and casamino acids. They showed that all media and constituents bound large amounts of mercury, copper and lead but not cadmium. In this study copper toxicity was less severe at 63.5 µg $ml^{-1}$ $Cu^{++}$ in *T. viride* in the Babich and Stotzky medium than on the GMS medium and the results for cadmium were variable for different fungi. Ramamoorthy and Kushner (1975) showed that the affinity of the medium components for cations varied with the metal in question but in general the Irving-Williams

series for cation affinity to organic ligands was followed with $Hg^{++} >> Pb^{++} >> Cu^{++} >> Cd^{++}$ (Irving and Williams, 1948). The effects of complexing of cadmium did not appear to be very serious in this study, and this may be explained at least in part by the suggestion that cadmium does not seem to be complexed to such a great extent because of its low chelate stability constant. Fungi may have a high affinity for cadmium and this should also be considered in this respect.

## 3. GROWTH OF FUNGI IN LIQUID CULTURE

### 3.1. Introduction.

As a prerequisite to metal toxicity testing and accumulation experiments in liquid medium, it was considered necessary to assess fungal growth parameters in liquid agitated culture. Although much of the work on fungitoxicity of metal ions has been carried out on solid medium or in liquid medium but with the use of spores as inoculum, fewer workers have attempted to standardise a fragmented mycelium inoculum. The latter technique was considered to be preferable however, since interpretation of experiments which utilise spore inocula are often complicated by the effects of metal ions on spore germination. The aim of this section was to develop a standard inoculum preparation and inoculation technique using mycelial fragments, and to develop a method for estimating the mid-period of the rapid growth phase reproducibly.

### 3.2. Materials and Methods.

#### 3.2.1. Development of standard inoculum.

Three fungi were selected on the basis of their responses to heavy metals, reproducibility of growth patterns and ease of handling. The chosen fungi incorporated a range of tolerances to copper, cadmium and zinc, had high growth rates with reasonably reproducible growth curves, and were easy to handle in terms of mycelium fragmentation and spore production for subculturing purposes. The identification of the fungi employed was confirmed by the Commonwealth Mycological Institute as *Penicillium spinulosum Thom*, *Aspergillus niger van Tieghem* and *Trichoderma viride Pers.*

Initially, 100ml GMS medium contained in a 250ml Ehrlenmyer flask were inoculated with a 5mm diameter plug for each fungus from GMS plates. These were incubated for a period of 5 days with shaking at 200 rpm, at 25°C. After the 5 day incubation period, the single mass of culture was decanted into a sterile blender flask (MSE Atomix) and fragmented at full speed for up to 2 minutes, with water cooling every 30 seconds to prevent heating of the culture. During the fragmentation procedure, portions of the culture were periodically removed for microscopic examination to ensure adequate breakage and to monitor hyphae fracture, so that excessive debris production could be avoided. Two minutes appeared to be the most suitable blending time to achieve adequate breakage of the hyphae. The density of the fragment suspension was adjusted with sterile GMS medium so that a 1 in 20 dilution of the fragment suspension produced a reading of 0.50 absorbance units (1cm light path)at 650nm on a spectrophotometer (Pye Unicam SP6-400). The fragment suspension was then reinoculated into GMS medium, initially using 5.0 ml of the inoculum for 100 ml of the medium. Determination of the optimum inoculum size is dealt with in the next section. After 60 hours of incubation at 25°C with shaking at 200 rpm, subculturing was carried out into GMS medium using the aforementioned techniques for the inoculum preparation. Fungi were routinely subcultured 2 or 3 times before proceeding to experimental work.

3.2.2. Determination of optimum inoculum density for 100 ml GMS medium.

Thirty 250 ml Ehrlenmyer flasks containing 100 ml GMS medium were inoculated with 1.0 ml of a *P. spinulosum* fragment suspension prepared as previously described. Twenty seven of the inoculated flasks were incubated in an orbital shaker at 25°C. The contents of the 3 remaining flasks were collected by filtration using Millipore filters which had been dried for 18 hours at 105°C before weighing. The filtered fungal

material was then washed by rinsing twice with 100 ml distilled water. The filter plus filtered material was then redried at 105°C for 18 hours and the dry weight of the fungal material obtained. At 12 hour intervals, 3 replicate flasks were selected randomly from the orbital shaker and the contents collected by filtration. (At low culture density Millipore filters were used and at higher culture densities Whatman 541 filters were employed). Mycelial dry weights were obtained at the time of inoculation and at 12 hour intervals throughout the growth cycle. Growth was typically complete within 96 hours. Growth curves were constructed by plotting dry weight increase against time of incubation. The procedure was then repeated but using 2.0 and 5.0 ml inoculum to determine the optimum inoculum size for adequate and reproducible growth. The experiment was then repeated for *T. viride* and *A. niger*. The dry weight curves are shown in Figures 3.3.1a, 1b and 1c and 1.0 ml inoculum volume appeared to be appropriate and was used in all further experiments. It is worthy of note here that growth during the rapid period of proliferation appeared to be linear with time and exponential growth was not observed. Hereafter, all references to the rapid phase of growth will be termed linear growth.

3.2.3. Prediction of mid-linear growth phase of cultures.

To enable subculturing using mid-linear fungal cultures it was necessary to be able to predict when a particular culture was in this phase of growth. For each of three fungi growth curves were plotted using the 1.0 ml inoculum density and the period of mid-linear phase was estimated at 48-60 hours for all the fungi tested in the GMS medium under standard incubation conditions. (See Figures 3.3.1a, 1b and 1c).

3.2.4. Summary of standard techniques for inoculum preparation, inoculation and incubation.

Actively growing cultures in mid-linear phase were subcultured twice in GMS medium prior to experimentation. Mid-linear phase fragmented mycelium was prepared by homogenising cultures at full speed for 2 minutes, with periodic cooling and microscopic examination. The optical density of the fragment suspension was adjusted so that a 1 in 20 dilution with fresh GMS medium produced a reading of 0.50 absorbance units at a 1 cm path length. 1.0 ml of this suspension was routinely inoculated into 100 ml GMS medium and incubated for the required growth period at 25°C with shaking at 200 rpm on an orbital shaker.

## 3.3. Results

### 3.3.1. Determination of optimum inoculum density for 100 ml GMS medium.

The effects of varying inoculum size on dry weight curves for *P. spinulosum*, *T. viride* and *A. niger* are shown in Figures 3.3.1a, 1b and 1c. The most appropriate inoculum size was found to be 1.0 ml for the 100 ml GMS medium volume. Although 2.0 ml inoculum showed little difference in overall culture characteristics, the smallest feasible inoculum was chosen for subsequent use. The use of 5.0 ml inoculum resulted in slight increases in biomass production which appeard to be quite significant in the case of *P. spinulosum*. The growth rate during the period of rapid growth appeared to be linear and not exponential.

### 3.3.2. Description of growth morphologies of fungi in liquid agitated culture.

a. *Penicillium spinulosum*.

After 2 minutes of homogenisation in the blender, fragments of mycelium were produced which exhibited a small degree of branching. Blending for a period in excess of 2 minutes resulted in a loss of viability of the fragments.

A 1% inoculum level always resulted in pelleted growth. The pellets, which were white in appearance, seemed to be of a non-coagulating type, uniformly 2 to 2.5 mm in diameter by mid-linear phase and consisted of a tightly packed centre surrounded by a layer of dense filamentous hyphae. By mid-linear phase small fragments were observed to break from the pellets and these remained discrete. Loose filamentous growth was never observed in this fungus at pH 5.5 and no pigmentation was ever apparent in submerged culture.

It is important to note that the appearance of the cultures grown in flasks was similar to that of batch grown cultures in the stirred-tank fermenter (see chapter 7) at pH 5.5 except that there was more evidence of fragment breakage from the pellets. At pH 3.5, (fermenter cultures) pellets were not so compact and were smaller in diameter (about 1 mm), and pellet breakage seemed more significant. At pH 2.0 (fermenter cultures) growth was diffuse, often pulp-like and filamentous.

As shown in Figure 3.3.1a, the lag period in the flask batch culture lasted from 0-24 hours, linear growth from 24-72 hours, and stationary phase from 72-84 hours onwards. Fermenter cultures showed a similar growth pattern (see chapter 7). Dry weight increase appeared to be linear during the 24-60 hour period of growth. Maximal dry weights achieved were about 6-8 mg dry weight $ml^{-1}$ GMS medium in both flask cultures (pH 5.5) and fermenter cultures at pH 5.5, 3.5 and 2.0.

Some variation in apparent growth rate based on dry weight determinations was observed between different batches of cultures inoculated from different starter cultures. This was probably a reflection of the difficulty encountered in preparing a standard inoculum although all reasonable precautions were taken. Also it became apparent that fewer large pellets showed an overall slower culture growth than did a larger number of smaller pellets and the number of pellets depended on the state of the fragment inoculum, so great care was required in standardisation. Even so, some variation occurred. Fortunately, variation between cultures in the same batch was less of a problem but nevertheless was occasionally present.

The effect of inoculum size on subsequent dry weight increase during liquid culture of *P. spinulosum*, *T. viride* and *A. niger*.

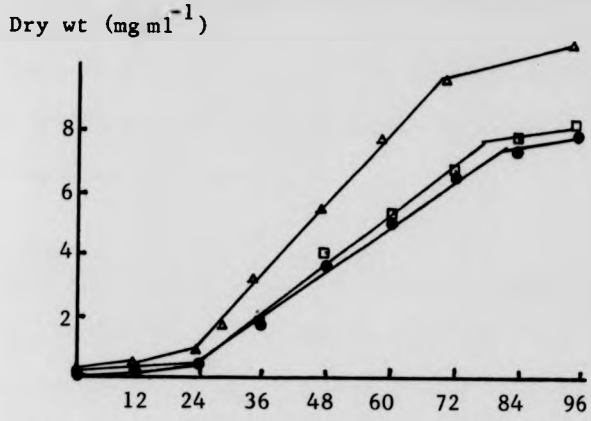



*Figure* 3.3.1a

●, 1.0ml inoculum
□, 2.0ml inoculum
△, 5.0ml inoculum
(for 100ml GMS).

*P. spinulosum*.

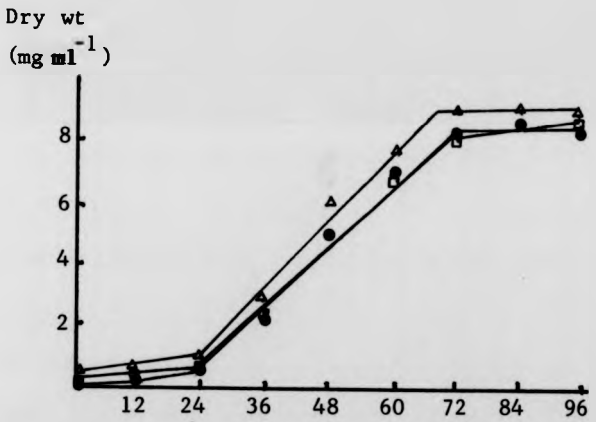



*Figure* 3.3.1b

*T. viride*.

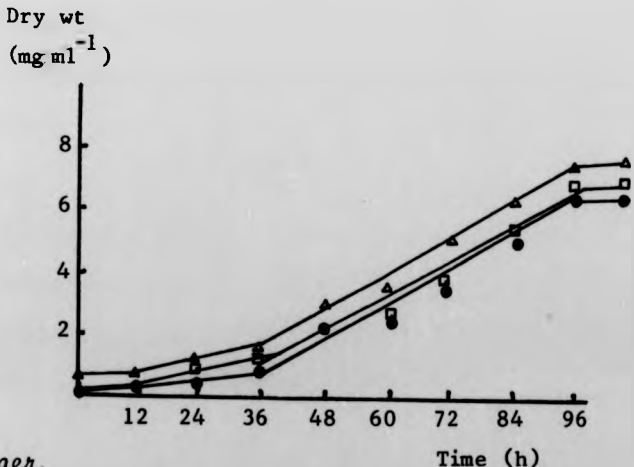



*Figure* 3.3.1c

*A. niger*.

b. *Trichoderma viride*.

The 2 minute blending procedure for *T. viride* produced fragments in which no branching was apparent. Reasonable growth could be achieved even if the blending was extended to 4 minutes.

In flask culture at pH 5.5 using a 1% inoculation density and standard incubation, the fungus always appeared to be morphologically diffuse, often pulp-like and filamentous. A small proportion of the culture (about 5%) appeared to consist of agglomerations of mycelium with hard packed centres so that the mycelium did not represent a homogeneous suspension with respect to the medium. Overall, the culture was filamentous and diffuse. The mycelium, which was white in appearance, seemed to be morphologically similar at pH 5.5, 3.5 and 2.0 when grown in the stirred-tank fermenter, and when grown in flasks (pH 5.5).

The lag period lasted for 12-24 hours, linear growth 36-60 hours and the onset of stationary phase occurred by about 72 hours. The growth curve is illustrated in Figure 3.3.1b. As with *P. spinulosum*, growth rates appeared to be somewhat variable between different batches of culture when inoculated from different starter cultures, but significant variation between cultures within a batch was not observed. Maximal dry weight production was approximately 8 mg dry weight $ml^{-1}$ GMS medium under all the conditions tested (i.e. flask and fermenter cultures at all tested pH values).

c. *Aspergillus niger*.

Fragments produced after 2 minutes of homogenisation were branched. The original starter culuture pellets from which the pellets were produced appeared to be harder and more compact than those of *P. spinulosum*. This

resulted in the production of larger fragments for *A. niger* than with *P. spinulosum* using the same blending duration. Blending for a period in excess of 2 minutes significantly reduced viability of the fragments.

A 1% inoculation density at pH 5.5 under standard incubation conditions produced cultures consisting of often variably sized pellets probably due to coagulation of fragments. The number of pellets per culture was usually visibly fewer than with *P. spinulosum* cultures. The number of pellets produced depended on the inoculum size, and an inoculum volume of less than 1.0 ml resulted in a culture consisting of a small number of very large agglomerated pellets, and this appeared to result in poor biomass productivity. The pellets produced were typically very dense and tightly packed, 1-4 mm in diameter with the majority spherical in shape, although often undefineable shapes were present. Loose filamentous growth was never observed at pH 5.5. Consistency of pellet sizes was more apparent in the fermenter cultures with pellets typically ranging from 0.5-2.0 mm in diameter, but they were very compact. Occasionally, rudimentary reproductive structures were observed in submerged cultures which were never apparent in *P. spinulosum* or *T. viride*. In all cultures the mycelium was initially white in appearance, but by the middle of the linear phase of growth the mycelium exhibited orange pigmentation, presumably due to the formation of carotenoid compounds. Incubation at pH 2.0 in the fermenter, produced mycelium which was diffuse but pigmentation was still observed.

As shown in Figure 3.3.1c, the growth rate was generally slower than for *P. spinulosum* and *T. viride* often with a longer lag period of 24-36 hours. This was probably due to coagulation of fragments at the time of inoculation so that fewer, larger pellets were produced, and hence the

rate of biomass production was reduced. The final culture density achieved was about 6.0 mg dry weight $ml^{-1}$ GMS medium but varied in the range 4 to 9.0 mg $ml^{-1}$ depending on the pellet morphology. Linear phase lasted from 36-84 hours after inoculation but again was rather more variable than with either of the other two fungi tested because of the heterogeneity in pelleting.

## 3.4. Discussion.

### Growth of fungi in liquid culture.

Quantitative growth data for a large range of fungi in submerged agitated culture is not available. The three basic phases of the idealised growth curve, lag, exponential and stationary phase as described by Pirt and Callow (1960) for homogeneous submerged mould cultures, were not recognisable in the fungi used in this study. In these studies using *P. spinulosum*, *T. viride* and *A. niger*, exponential phase was not observed, and a linear increase in biomass was apparent during the rapid phase of growth under the growth conditions used. This pattern of growth is similar to that reported for *Fusarium solani* in an aerated medium (Cochrane, 1958), and is most likely to be due to the fact that the mycelium was not homogeneously dispersed in the medium because of pellet formation in *P. spinulosum* and *A. niger*, or mycelial agglomeration in *T. viride*.

The phenomenon of fungal growth being limited to the terminal parts of hyphae in surface colony culture is probably applicable to pelleted growth also, except that the latter occurs in three dimensions. The spherical shape implies that growth of the composite hyphae making up a pellet is uniform and if the hyphal extension is linear, then the pellet radius should increase linearly with time. Trinci (1970), found that the radii of *Aspergillus midulans* pellets increased in accordance with the linear law. Emerson (1950) showed that the cube root of the volume or mass of *Neurospora sp.* pellets increased linearly with time and indeed, this was found to be true for *P. spinulosum* and *A. niger* (data not shown). This cube root law has also been shown by Trinci (1970). Obviously, the technique of volume measurement of biomass pellets was unsuitable for *T. viride* which produced filamentous growth. For this reason it was

preferred to use dry weight increase as a parameter for monitoring of growth in submerged culture. This was also suitable for following the progress of fermenter cultures. For all the fungi tested in this investigation, lag phase was followed by the usual period of rapid growth which will be referred to as linear phase hereafter.

Variation in the growth rates in liquid culture between batches was undoubtedly caused by the variable inoculum and hence the pelleting characteristics, but standardisation of a mechanical destruction technique is clearly difficult. Although the fragment suspension was prepared in a standard way and the optical density adjusted prior to inoculation, these precautions could not take account of differences in fragment size, fragment numbers and fragment viability between different inoculum preparations. One of the main problems with the technique was that inevitably mycelial debris was produced during hyphal breakage, and this was difficult to quantify in relation to the viable component of the inoculum. Plate counting techniques were avoided for a number of reasons; one important point is that plate counting would not provide meaningful data on the amount of mycelial debris because the method monitors living material and does not take account of the fact that part of a fragment might be viable with residual dead cell material attached as a result of wall fracturing. Thus, this would not help in assessing the degree of debris reliably. Another point is that a wholly viable fragment may not necessarily behave in the same way as a fragment which consists almost totally of dead material, so plate counting was considered to be inappropriate in this context. One further point is that any data obtained using plate counting would be retrospective, and therefore would not be particularly useful in monitoring viability of inocula. Furthermore, a methylene blue dye exclusion test used as a rapid colorimetric

viability test as reported by Bonora and Mares (1982) for single celled eucaryotic microorganisms, proved to be totally unsatisfactory for fragment viability testing because of the variable non-viable component of viable fragments which complicated results. The non-viable portion of fragments resulted from cell wall fracture thus producing fragments containing some viable cells together with some damaged wall material.

There is little information in the literature on the effects of blending cultures since conventially processes involving filamentous fungi have used spores as inocula. The use of mycelial fragments as typical vegetative material was thought to be preferable to the use of spores and this is supported by the work of Savage and Vander Brook (1946) which showed that the fermentation properties of viable fragments of a blended culture of *Penicillium chrysogenum* were normal. The authors were able to produce a blended culture with an increase in the number of viable growth centres with little or no injury to their growth or fermentative capacities. The objectives of this project were to investigate the effects of heavy metals in terms of toxicity and metal accumulation specifically on vegetative cells. Thus, a vegetative inoculum had to be employed since the influence of heavy metals on spore germination could give rise to ambiguous results.

As reported in the results section, inoculation of GMS medium with a 1% inoculum charge of fragmented mid-linear phase culture led to the production of different morphological growth characteristics for the three fungi. *Penicillium spinulosum* always produced pelleted growth at pH 5.5 which appeared to be non-coagulative and the pellets were uniform in their size characteristics. *Trichoderma viride* always produced loose filamentous growth and *A. niger* usually produced a mostly pelleted

culture with often variably sized units. According to Pirt (1975) little is known about the factors which determine whether or not mycelium will develop in the stromatic or pelleted form.

It is likely that inoculum size is important in determining patterns of pelleting, and it is generally recognised that high concentrations of propagules lead to filamentous growth and low concentrations lead to pelleted growth as reported for *P. chrysogenum* (Camici, *et al*, 1952). Galbraith and Smith (1969) reported that pellets of *A. niger* were formed from agglomerates of upto several hundred spores and that the number of spores per pellet depended on pH. For hyphal units, pelleting will depend on the interaction of hyphae at an early stage of growth and this prevents pellet formation in non-coagulating cultures. For coagulating forms such as *A. niger*, pellets are formed at all spore concentrations, and this was confirmed for fragments in the strain used in this study which showed no morphology changes at the different inoculum levels of 1, 2 and 5%.

In a useful review article by Metz and Kossen (1977) the effects of agitation, pH, $O_2$ tension and medium viscosity on pellet formation are discussed. The accepted view on agitation is that the greater the agitation the smaller and more compact are the pellets if they are formed.

In the experiments carried out in the fermenter with electronic pH control (chapter 7) it is worthy of note that, although at pH 5.5 both *P. spinulosum* and *A. niger* produced pelleted growth, this was not so distinct at pH 3.5, and at pH 2.0 both organisms produced filamentous growth. It is interesting that Galbraith and Smith (1969) working with *A. niger* reported that at pH 5.5 coagulation and pellet formation was very

pronounced whereas at pH 2.0 there was no evidence of coagulation. Intermediate pH produced very small pellets. They reported that pH had some effect on the spore surface properties and this may also be true for hyphal fragments. The surface charge of the spore is reportedly negative overall (Douglas *et al*, 1959), but Galbraith and Smith (1969) suggest that pH does not markedly affect this, and that it is more likely that cell wall protein and carbohydrate constituents are significantly influenced by pH. Metz and Kossen (1977) cited pH effects reported for *P. chrysogenum* by Pirt and Callow, (1959). In *P. chrysogenum* at pH 6, filamentous growth occurred and at higher pH values pellet formation took place. It is also noteworthy that Galbraith and Smith (1969) reported that changing the pH from 4.5 to 2.1 had no effect on dry weight increase in A. *niger* and on the rates of glucose and ammonia uptake. pH appeared to have no effect on biomass productivity in this study.

## 4. DETERMINATION OF HEAVY METAL TOXICITY IN LIQUID MEDIUM.

### 4.1. Introduction.

The primary objective was to determine the levels of metal which could be added to liquid medium without causing severe toxic effects. An effective rapid screening technique was employed in section 2 of this study for evaluating toxicity of heavy metals in solid medium to fungi. However, the method is unsuitable for studying metal uptake in solid medium because of the difficulty in separating the mycelium from the medium for metal analysis. For this reason toxicity tests in liquid medium were also performed, so that metal uptake in fungi growing in liquid medium could be monitored, at selected metal concentrations which were not seriously inhibitory to growth. Another aim was to determine if there was any relationship between quantitative metal uptake and growth inhibition in *P. spinulosum*, *T. viride* and *A. niger*.

The metals chosen for study were selected because of their differences in fungitoxicity based on observations from section 2 and also because of their reputed ecological importance as discussed in the general introduction.

The literature on metal toxicity in solid medium has already been discussed but it is relevant here also. There has however, been a great deal of work carried out on metal toxicity in liquid medium both in relation to spores and mycelium, mainly because of the importance of heavy metals in fungicides. Uptake of metals, toxicity, inhibition of germination and antidoting has been extensively investigated with regard to conidia as discussed in the general introduction. Toxicity of heavy metals to fungal mycelia has also been studied in the last decade but to a lesser degree.

Parry and Wood (1958) reported toxicity tests using *Botrytis cinerea* with copper sulphate and phenyl mercuric acetate in liquid media. The toxicity of high concentrations of zinc has been reported by a number of workers. Adiga *et al* (1961) demonstrated zinc toxicity in *A. niger*. The effects of zinc on *Neurospora crassa* were demonstrated by Sivarama Sastry *et al* (1962) and on *A. niger* by Laborey and Lavollay (1973). Paton and Budd (1972) indicated that zinc inhibited growth of *Neocosmospora vasinfecta* by 90% at 6500 µg $ml^{-1}$ $Zn^{++}$. Chandra and Banerjee (1973) reported that *Trichophyton rubrum* was very sensitive to the action of lead, copper and cadmium and toxic effects of zinc were also noted. The effects of iron, zinc, manganese and copper on *Cephalothecium roseum* causing pink rot of apple was reported by Thind and Madan (1975). Okamoto and Fuwa (1974) reported copper toxicity for *P. ochrochloron*, *P. chrysogenum* and *A. niger*. Dwivedi and Pandey (1977) reported toxic effects of iron, zinc, manganese and copper for six soil microfungi in liquid culture. Smith (1977) reported that there was considerable variation in growth inhibition in urban-tree phylloplane fungi in the presence of cadmium, copper, manganese, aluminium, chromium, nickel, iron, lead, sodium and zinc. The addition of lead and cadmium to media supporting aquatic fungi resulted in inhibitory effects, while zinc at relatively higher concentrations showed no effect on growth (Duddridge and Wainwright, 1979). Inhibition of growth of *P. ochrochloron* at elevated copper concentrations was reported by Stokes and Lindsay (1979). Toxicity of copper in *Neurospora crassa*, and the alleviation of toxicity by iron and manganese has been reported by Venkateswerlu and Sivarama Sastry (1979).

Although toxicity assessment in terms of spore germination is reasonably effective for fungicide screening it is not necessarily applicable

to evaluating toxicity to vegetative mycelia, since spores are likely to be different physiologically to mycelia. Clearly, care has to be taken in relating the published effects of metals in fungi, particularly if comparisons between spore and vegetative material are to be made because of different media used, fungal strains *etc.*, and tests really need to be carried out prior to an uptake study under one's own experimental conditions, until a single standardised toxicity test is devised and accepted for general use.

4.2. Materials and Methods.

Copper was added to the GMS medium as copper chloride to produce final concentrations of 0.635, 6.35, 63.5 and 635.0 µg $ml^{-1}$ $Cu^{++}$. Cadmium chloride was added to produce final concentrations of 1.22, 11.2, 112.0 and 1120.0 µg $ml^{-1}$ $Cd^{++}$, and zinc, also added as the chloride salt, to form 0.65, 6.5, 65.0, and 650.0 µg $ml^{-1}$ $Zn^{++}$. The concentrations are not identical because they represent conversions from uM units to facilitate comparison with published work. Control flasks contained no added metal.

Three replicate flasks were prepared for each metal, metal concentration and for each of the 3 fungi tested. The fungal inocula were prepared in the standard manner after 2 subcultures of the stock organisms and were actively growing in mid-linear phase at the time of homogenisation and inoculation. Standard inoculation and incubation procedures were employed.

At the time of inoculation, and at 12 hour intervals for 84 hours, 3 replicate flasks were selected for controls, each metal, metal concentration and each fungus. Collection of biomass was either by filtration onto Millipore or Whatman 541 filters or by centrifugation at 4000 rpm, depending on the mycelial density. The retained biomass was washed with 200 ml distilled water and used as an estimate of dry weight.

Dry weight curves were constructed by plotting biomass increase against time throughout the incubation period. Figures 4.3.1a, 1b and 1c illustrate the effects of copper addition on the growth of *P. spinulosum*, *T. viride* and *A. niger* as compared to controls with no added metal. Figures 4.3.2. and 3. show similar responses for cadmium and zinc.

4.3. Results.

Copper.

The results for copper toxicity are shown in Figures 4.3.1a, 1b and 1c. Copper became toxic at 6.35 μg $ml^{-1}$ $Cu^{++}$ and was totally inhibitory at 63.5 and 635.0 μg $ml^{-1}$ to *P. spinulosum*. The response of *T. viride* was similar to that of *P. spinulosum* except that there appeared to be slight growth at 63.5 μg $ml^{-1}$ and the inhibition at 6.35 μg $ml^{-1}$ was not so significant. *Aspergillus niger* was unaffected by copper upto 6.35 μg $ml^{-1}$ and slight growth was observed at 63.5 μg $ml^{-1}$. *Aspergillus niger* appeared to be the most tolerant fungus tested with respect to copper in liquid medium. This is in contrast to the situation on solid medium where *T. viride* demonstrated the highest degree of resistance. Copper toxicity in liquid GMS medium was similar to that on solid medium in the case of *P. spinulosum*. However, copper was more toxic by a factor of ten to *T. viride* in liquid GMS medium and also it was more toxic to *A. niger* in liquid medium.

Cadmium.

The results for cadmium toxicity are illustrated in Figures 2a, 2b and 2c. Cadmium was severely toxic to *P. spinulosum* at 1.12 μg $ml^{-1}$ $Cd^{++}$ with only 15% of the control dry weight being achieved after 80 hours at incubation. *Trichoderma viride* on the other hand, appeared to be much more tolerant to cadmium than *P. spinulosum* with toxicity not becoming apparent until the cadmium concentration in the medium was 112.0 μg $ml^{-1}$ $Cd^{++}$. Furthermore, there was still some slight growth at 1120.0 μg $ml^{-1}$. *Aspergillus niger* showed an intermediate response with cadmium showing toxic effects at 11.2 μg $ml^{-1}$ $Cd^{++}$. The reduction in growth observed at 11.2 μg $ml^{-1}$ was very significant however, and no increase in biomass was seen at cadmium concentrations of 112.0 and 1120.0 μg $ml^{-1}$. Toxicity of

cadmium to *T. viride* was similar on solid and in liquid GMS medium. Cadmium however was more toxic to *P. spinulosum* by a factor of ten in liquid GMS and a similar effect was seen with *A. niger*.

Zinc.

The results for zinc toxicity are illustrated in Figures 4.3.3a, 3b and 3c. Zinc only became severely toxic to *P. spinulosum* at 650.0 µg $ml^{-1}$ $Zn^{++}$, although there was a slight reduction in growth rate at 65.0 µg $ml^{-1}$. Zinc appeared to be slightly more toxic to *T.viride* than to *P. spinulosum* with 100% inhibition occurring at 650.0 µg $ml^{-1}$. Zinc was most toxic to *A. niger* at 65.0 µg $ml^{-1}$ in comparison to *P. spinulosum* and *T. viride*. At 650.0 µg $ml^{-1}$, zinc was 100% inhibitory to *A. niger*. There appeared to be little difference between the levels of toxicity to the three fungi tested when the zinc was incorporated into the liquid or solid GMS medium.

When the metals were added to the liquid GMS medium prior to inoculation, *A. niger* appeared to be the most resistant fungus to copper, whilst *T. viride* showed most resistance to the presence of cadmium. *Penicillium spinulosum* however, was notably susceptible to cadmium. With the exception of *T. viride* cadmium appeared to be the most toxic metal tested with all 3 fungi used. The exception for *T. viride* was that copper appeared to be more toxic than cadmium since a relatively high degree of tolerance was demonstrated with *T. viride* for cadmium. Copper showed an intermediate level of toxicity whilst zinc was relatively non-toxic overall. The three fungi showed different sensitivity series for the metals tested. The toxicity series for *P. spinulosum* was Cd > Cu > Zn; for *T.viride* Cu > Cd > Zn and for *A. niger* Cd > Cu > Zn.

Effect of copper concentration on fungal biomass production in liquid culture.

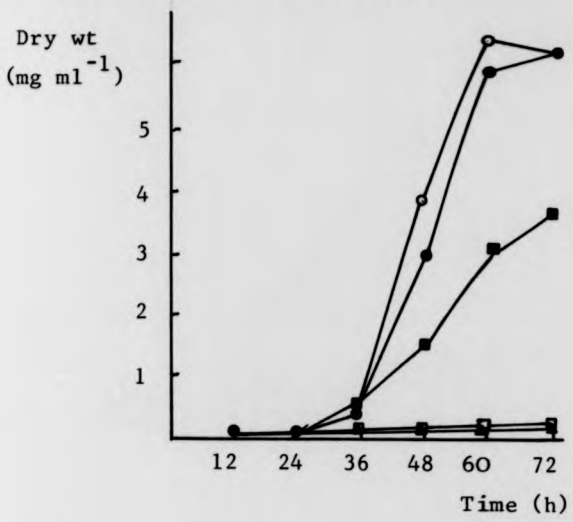



*Figure* 4.3.1a

- ● Control
- ○ 0.635 µg ml$^{-1}$
- ■ 6.35 µg ml$^{-1}$
- □ 63.5 µg ml$^{-1}$
- ▲ 635.0 µg ml$^{-1}$

*P. spinulosum*

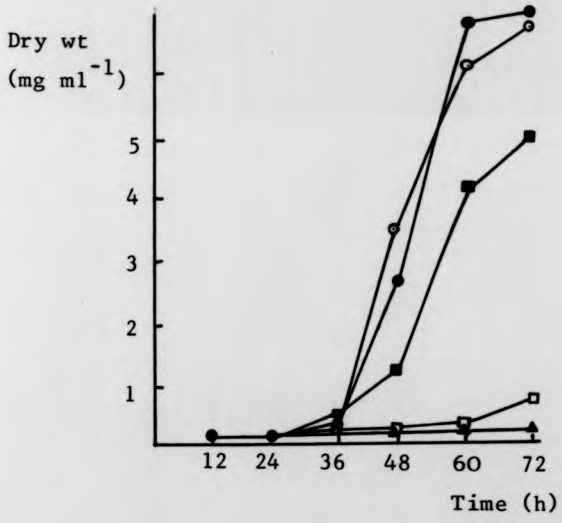



*Figure* 4.3.1b

*T. viride*

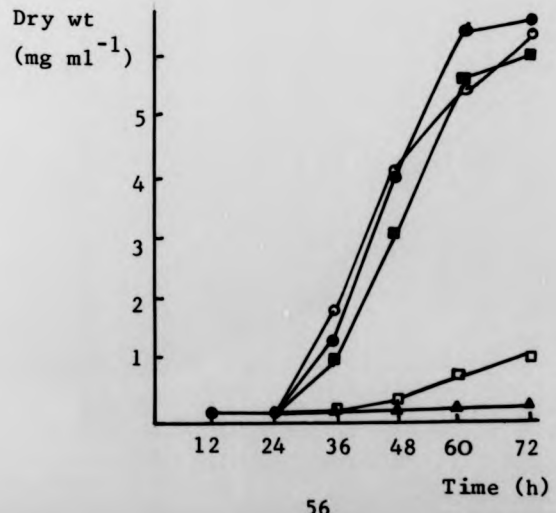



*Figure* 4.3.1c

*A. niger*

Effect of cadmium concentration on fungal biomass production in liquid culture.

Figures 4.3.2a,2b,2c.

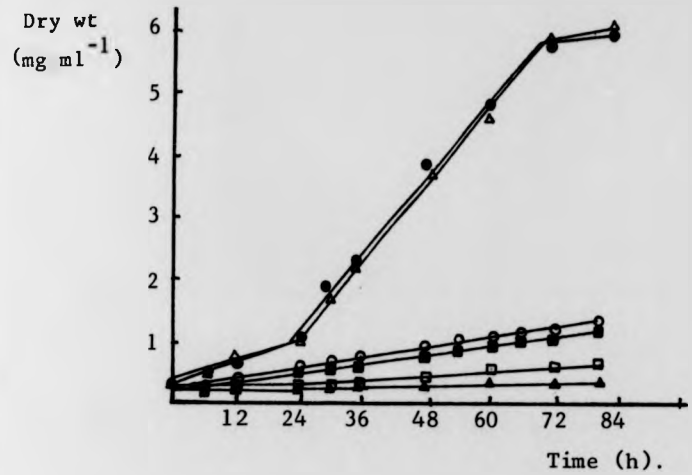



Figure 4.3.2a

- Control
- 0.11 µg ml$^{-1}$ Cd++
- 1.12 µg ml$^{-1}$ Cd++
- 11.2 µg ml$^{-1}$ Cd++
- 112.0 µg ml$^{-1}$ Cd$^{++}$
- 1120 µg ml$^{-1}$ Cd$^{++}$

*P. spinulosum.*

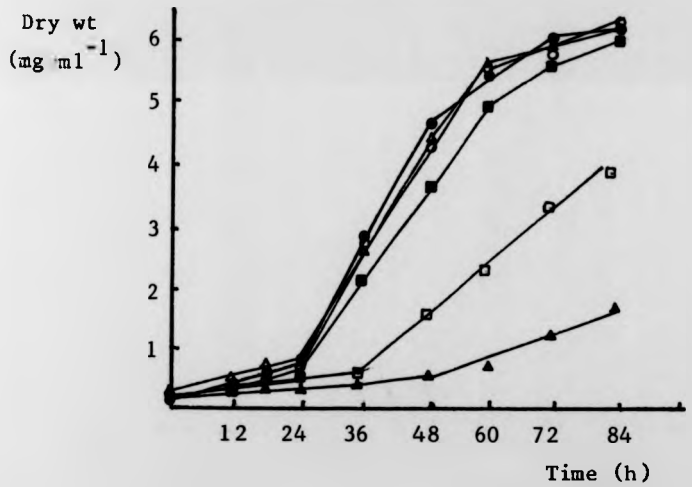



Figure 4.3.2b

*T. viride.*

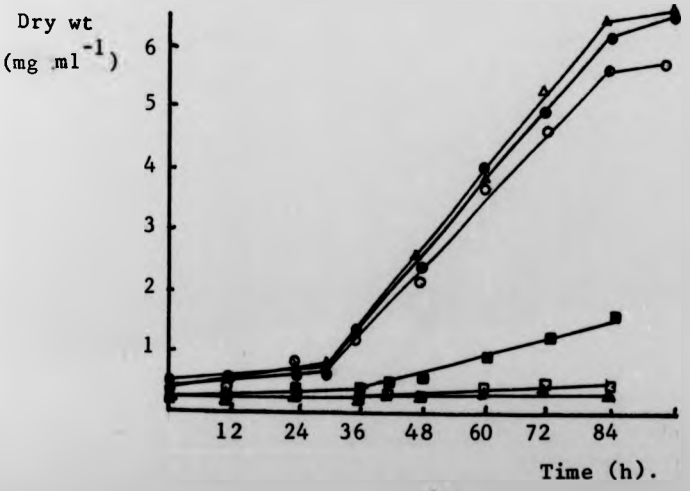



Figure 4.3.2c

*A. niger.*

Effect of cadmium concentration on fungal biomass production in liquid culture.

Figures 4.3.2a,2b,2c.

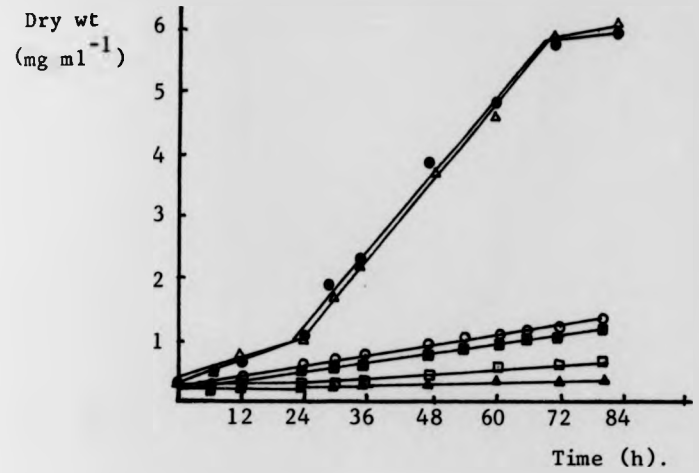



*Figure* 4.3.2a

- Control
- 0.11 μg ml$^{-1}$ Cd++
- 1.12 μg ml$^{-1}$ Cd++
- 11.2 μg ml$^{-1}$ Cd++
- 112.0 μg ml$^{-1}$ Cd$^{++}$
- 1120 μg ml$^{-1}$ Cd$^{++}$

*P. spinulosum.*

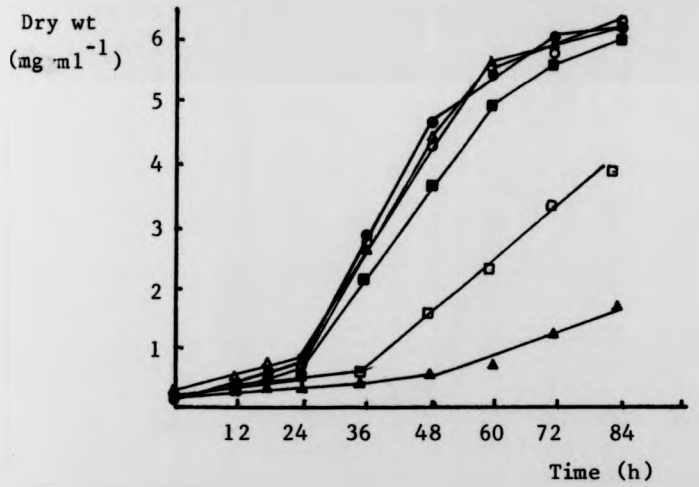



*Figure* 4.3.2b

*T. viride.*

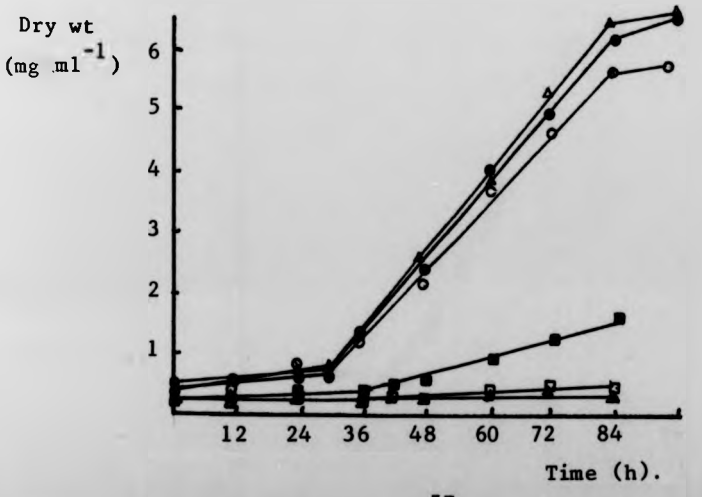



*Figure* 4.3.2c

*A. niger.*

Effect of zinc concentration on fungal biomass production in liquid culture.

Figures 4.3.3a,3b,3c.

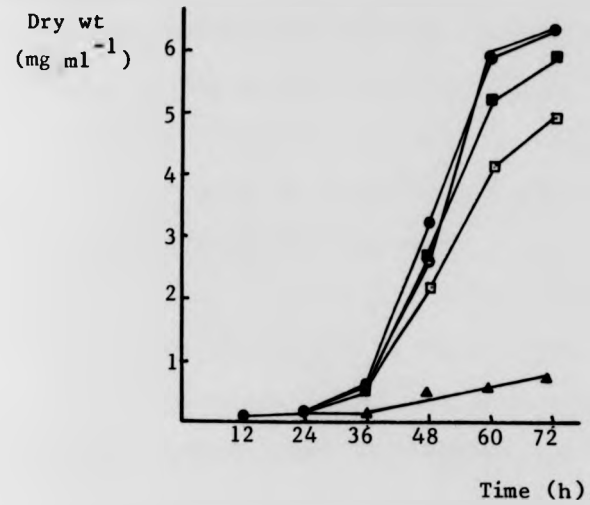



Figure 4.3.3a

- Control
- 0.65 µg ml$^{-1}$
- 6.5 µg ml$^{-1}$
- 65.0 µg ml$^{-1}$
- 650.0 µg ml$^{-1}$

*P. spinulosum.*

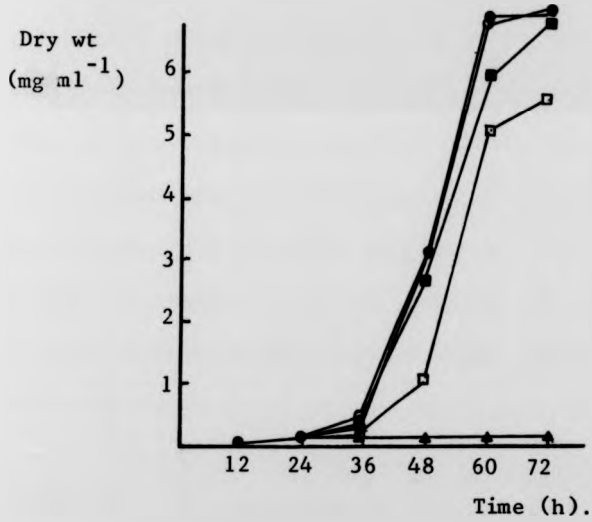



Figure 4.3.3b

*T. viride.*

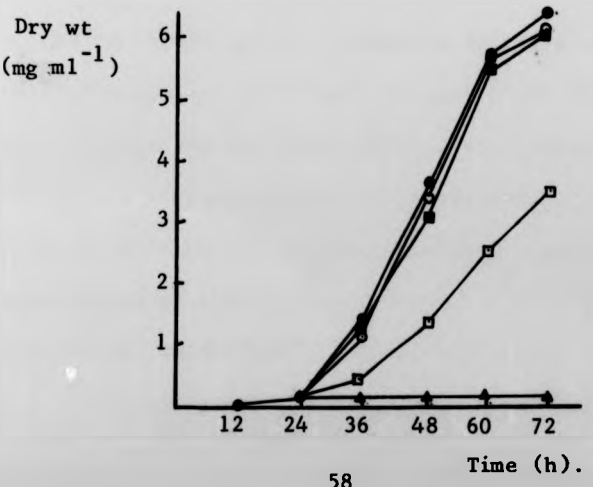



Figure 4.3.3c

*A. niger.*

4.4. Discussion.

In liquid medium copper appeared to be very toxic to *P. spinulosum* with inhibitory effects initially occurring at 6.35 μg $ml^{-1}$ $Cu^{++}$ and inhibition being complete at 63.5 μg $ml^{-1}$. *Trichoderma viride* was similar in its response to copper. *Aspergillus niger* appeared to be very slightly more resistant than the other 2 fungi tested showing no appreciable effect at 6.35 μg $ml^{-1}$. It is interesting that copper appeared to be more toxic to *T. viride* and *A. niger* in liquid medium than on solid medium although there was no appreciable difference in relative toxicities for *P. spinulosum*. This peculiarity may be related to copper complexing effects by the agar complement in the solid medium which may reduce the availability of metal to *T. viride* and *A. niger*. It may also be possible that *P. spinulosum* is a more efficient scavenger for metal than either of the other 2 fungi in such a way that the organism can compete with the agar for added metal. One other possible explanation could be that it is just not feasible to relate toxicity in liquid medium where the toxicant is ambient, to toxicity in aerial mycelium (surface growth on plates) where there is less contact with the metal ion and translocation effects may be operative.

There are some conflicting reports in the literature on this matter. Parry and Wood (1958) reported that toxicity of copper was lower in agar media than in liquid media. Growth in 300.0 μg $ml^{-1}$ $Cu^{++}$ only occurred at the air-medium interface so a proportion of the mycelium was in fact aerial. Chandra and Banerjee (1973) also reported that *Trichophyton rubrum* only grew as a surface culture in liquid medium amended with 8.0 μg $ml^{-1}$ $Cu^{++}$ which appeared to be the minimum inhibitory concentration. This is about the same degree of toxicity as observed in this study. For liquid culture toxicity tests, Smith (1977), showed that copper caused no significant

growth suppression at 8.7 μg $ml^{-1}$ in *A. pullulans*, *Chaetomium sp.*, *Epicoccum sp.*, *Gnomonia platani* or *Pestaliotiopsis sp.*. On solid medium however, although the degree of toxicity for *Chaetomium sp.* was similar at 8.7 μg $ml^{-1}$ $Cu^{++}$, in the case of *Epicoccum sp.* copper was less inhibitory on solid medium only causing growth reduction at 18.0 μg $ml^{-1}$ $Cu^{++}$. This may be expected since the agar present in the medium could complex the added copper, reduce its availability and hence reduce its toxicity to the fungus. Alternatively, it may be a characteristic of aerial mycelial growth in that there is less contact with the medium and therefore the fungus may seem more resistant as a consequence of the metal not being translocated along the hyphae. It is interesting to note that Stokes and Lindsay (1979) showed that dry weight production in 0, 50.0, 100.0 and 1000.0 μg $ml^{-1}$ $Cu^{++}$ was improved for *P. ochrochloron* under conditions of agitated incubation and submerged culture, and uptake of copper was lower than in the static cultures which exhibited surface growth. This lower uptake of copper in shaken culture would explain the increased dry weight production, although one might have expected shaking to increase copper uptake due to improved contact with the copper containing medium. It would be difficult to explain however, how increased contact with the ambient medium containing copper in liquid agitated cultures, resulted in a lower copper accumulation, than in surface growing colonies which have less contact with the medium. One possible explanation is that the physiology and metabolism of the fungus is different under the two incubation conditions such that increased medium contact results in reduced copper transport. It may be that as a consequence of better medium contact (and therefore of better contact with the copper), copper transport is more finely controlled. In this study using *P. spinulosum*, *T. viride* and *A. niger*, the majority of the metal and fungus combinations showed that metal toxicity was more severe

in submerged culture in liquid medium, than in surface colonies on solid medium. This suggests that more metal is accumulated under shaking conditions, but it must be remembered that there was the probability of reduced copper availability in solid medium owing to complexing by agar. Surface growth of the *P. ochrochloron* as observed by Stokes and Lindsay (1979) occurred on liquid medium in the form of a pellicle, and not on an agar-solidified version of their medium, so the experimental conditions were not directly comparable to this study.

It is worthy of note here that Smith (1977) observed no stimulatory effects of metals in liquid culture although it was a common feature on solid medium. A parallel situation was observed in this study with the three fungi used, and this also may bear some relation to possible physiological differences in submerged and aerial mycelium.

Further work has been reported on mycelial copper toxicity to a number of organisms, but care has to be taken in making direct comparisons with the results observed in this study because of the different experimental conditions under which the trials were performed, in terms of medium, strains of organisms, the origin of the fungi used *etc.*. In particular, care has to be taken when making comparisons with data obtained on resistant organisms such as *P. ochrochloron*.

Okamoto and Fuwa (1974) reported that *P. ochrochloron* grew well in saturated copper sulphate whereas *A. niger* was inhibited by 200.0 $\mu g\ ml^{-1}$ $Cu^{++}$ and *P. chrysogenum* by 100.0 $\mu g\ ml^{-1}$. These latter two organisms appeared to be less susceptible to the presence of copper than the three fungi studied under the conditions of this investigation, but careful consideration of the experimental conditions of these workers, reveals that the pH

at which the tests were performed was 4.0. This may explain the lower toxicity of the copper than was observed with the Keele strains of *P. spinulosum*, *A. niger* and *T. viride* at pH 5.5, which would result in greater copper uptake since there would be less competition for binding sites because of the lower concentration of $H^+$ ions at this pH. The extreme resistance of *P. ochrochloron* was demonstrated by growing at concentrations of copper up to $10^5$ µg $ml^{-1}$ $Cu^{++}$ in the medium (Okamoto, *et al* 1977).

Copper toxicity levels for various fungi, which were in reasonable agreement with the levels observed in this study, were reported by Thind and Mira Madan (1975). The authors reported that *Cephalothecium roseum* grew at upto 10.0 µg $ml^{-1}$ $Cu^{++}$ and total inhibition occurred at 100.0 µg $ml^{-1}$ at pH 6.0. Stimulation was apparent at 1.0 µg $ml^{-1}$. Dwivedi and Pandey (1977) also showed that concentrations of copper in excess of 5.0 µg $ml^{-1}$ inhibited most of the fungi he tested. Venkateswerlu and Sivarama Sastry (1979) showed that copper was 50% inhibitory at 5.0 µg $ml^{-1}$ to *Neurospora crassa* on a nitrate medium. Earlier work however, had shown that 80.0 µg $ml^{-1}$ $Cu^{++}$ was 50% inhibitory on a normal nitrogen medium which contained ammonium tartrate and 0.52% ammonium nitrate (Sivarama Sastry *et al*, 1962, Venkateswerlu and Sivarama Sastry, 1973) which represents a 16 fold decrease in toxicity over the nitrate medium (0.52% sodium nitrate as the sole nitrogen source). The enhanced toxicity in the sodium nitrate medium was apparently related to a decrease in nitrite reductase activity. The N source in the GMS medium used in this study was ammonium sulphate, but the levels of copper toxicity were similar to those with sodium nitrate as the N source as reported by Venkateswerlu and Sivarama Sastry (1979). Gadd and Griffiths (1978b) reported that a sensitive strain of *Aureobasidium pullulans* was completely inhibited by

130.0-540.0 μg $ml^{-1}$ $Cu^{++}$, although a tolerant strain grew at these levels although 50% cell death occurred.

The results of this study showed that cadmium was severely toxic at concentrations as low as 1.12 μg $ml^{-1}$ $Cd^{++}$ to *P. spinulosum* whereas the fungus *T. viride* demonstrated a degree of resistance, growing well at 112.0 μg $ml^{-1}$ and some growth was apparent even at 1120.0 μg $ml^{-1}$. However, a doubling of the lag period was apparent at the higher concentration. *Aspergillus niger* by comparison was intermediate in terms of susceptibility to cadmium. Cadmium was more toxic in liquid medium to *P. spinulosum* and *A. niger* than in solid medium but the level of inhibition was similar under both conditions for *T. viride*. According to the Irving-Williams series for stability of chelates (Irving and Williams, 1948) complexing of cadmium should not have been a serious problem, but care should be taken when attempting to extrapolate data on stability constants for metal complexes with oxine, dithizone and various other substances as reported by the authors, to conditions in microbial growth media which can be continually modified by the presence of metabolising microorganisms.

Cadmium has been found to be a severely toxic metal by many other workers. Abelson and Aldous (1950) showed that toxicity to *Escherichia coli* occurred in the range 0-20.0 μg $ml^{-1}$ $Cd^{++}$ but the inhibition was reduced by the presence of magnesium at all concentrations upto 200.0 μg $ml^{-1}$ $Mg^{++}$. Chandra and Banerjee (1973) showed that the minimum inhibitory concentration for cadmium to *Trychophyton rubrum* was 15.0 μg $ml^{-1}$, but growth occurred only at the medium air interface with no submerged mycelium. This degree of susceptibility appeared to be similar to that exhibited by *A. niger* as observed in this study. Smith (1977) showed

that dry weights of fungi were reduced in comparison to controls in the presence of 1.5 µg $ml^{-1}$ $Cd^{++}$ for *Chaetomium sp.*, and *Pestalotiopsis sp.* Although cadmium was toxic in plate culture at 1.5 µg $ml^{-1}$ to *Chaetomium sp.*, *A. pullulans* was susceptible only at 2.2 µg $ml^{-1}$, and most of the other fungi were unaffected by this level of cadmium in the solidified medium. Cadmium then, was less toxic in solid medium according to the data of Smith. Levels of toxicity at 1.5 µg $ml^{-1}$ are similar to the response of *P. spinulosum* at 1.12 µg $ml^{-1}$ in this study. Duddridge and Wainwright (1980) showed that cadmium was toxic to three aquatic fungi, *Scytalidium sp.*, *Pythium sp.* and *Dictyuchus sp.* at 2.5 and 5.0 µg $ml^{-1}$ $Cd^{++}$. Cadmium is clearly a very toxic metal to most fungi. At the opposite end of the spectrum is *P. ochrochloron* which was capable of tolerating 1000.0 µg $ml^{-1}$ although it was totally inhibited at 20 000.0 µg $ml^{-1}$ (Okamoto, *et al* 1977). It is interesting though that the authors reported that spore germination was only inhibited at 20 000.0 µg $ml^{-1}$ in this fungus. Miller and McCallan, (1957) reported that exposure of the spores of *Neurospora sitophila*, *Monilinia fructicola* or *A. niger* van Tieghem to cadmium at a concentration of 5-40 000.0 µg of $Cd^{++}$ per g of spores, for 0.02 hours and 2.08 hours had little effect on spore germination. Only after 20-25 hours of exposure to the metal was there significant inhibition of germination.

Zinc appeared to be the least toxic metal in this study. Toxicity to *P. spinulosum* and *T. viride* was elicited at 650.0 µg $ml^{-1}$ $Zn^{++}$ although *A. niger* appeared to be quite susceptible showing about 40% inhibition at 65.0 µg $ml^{-1}$ and total inhibition at 650.0 µg $ml^{-1}$. Zinc toxicity was marginally greater in liquid medium than in the solidified version. The variability in metal toxicity to the three fungi tested in the two types of medium suggests that these differences are more a function of the

different metal properties such as charge characteristics and ionic radii, and not so much due to the submerged versus aerial mycelial growth forms. For example, copper toxicity was more severe on solid medium to *T. viride*, but cadmium and zinc toxicity were essentially similar on the two media for this fungus. On the other hand, toxicity of copper to *P. spinulosum* was similar in both media, but cadmium was more toxic in the liquid GMS medium to this fungus. Thus there appears to be no clear relationship between the toxicity of the three metals studied and the type of fungal growth resulting from surface or submerged culture.

The relative toxicity of zinc to different fungi reported in the literature is difficult to compare with the results in this study mainly because there is no standard toxicity test medium. Problems are encountered in comparing zinc toxicity in medium containing variable amounts of magnesium, since this cation is intimately associated with the effects of zinc. Adiga *et al* (1961) and Laborey and Lavollay (1973) have reported that zinc toxicity in *A. niger* manifests itself as a magnesium deficiency. Laborey and Lavollay (1977) showed that $Cd^{++}$ toxicity was related to magnesium presence but they also implicated $Ca^{++}$ in this phenomenon. In the presence of high concentrations of magnesium, the toxicity of cadmium was strongly reduced by calcium in *A. niger*. Similar effects were noted for *A. niger* with interaction of cadmium, zinc and magnesium. Zinc toxicity was antagonised by the presence of magnesium.

Sivarama Sastry *et al* (1962), investigated the effects of increasing the concentrations of zinc in a normal and minimal magnesium medium and found that in medium containing 49.3 μg $ml^{-1}$ $Mg^{++}$ (normal) 200.0 μg $ml^{-1}$ $Zn^{++}$ could be tolerated by *Neurospora crassa* (although there was 40.8% inhibition of growth). In a medium containing only 5.0 μg $ml^{-1}$ $Mg^{++}$

(minimal) a mere 25.0 µg $ml^{-1}$ $Zn^{++}$ caused a 41.95% inhibition of growth. Thus reducing the magnesium content of the medium by a factor of 10, caused an 8 fold increase in zinc toxicity. Although $Fe^{++}$ also reduced zinc toxicity, it did not reduce uptake of the metal as occurred with magnesium. For the purpose of comparison, the magnesium content of the GMS medium used in this study was 52.75 µg $ml^{-1}$. The degree of zinc toxicity on the GMS medium was not high, certainly not apparent at 25.0 µg $ml^{-1}$ $Zn^{++}$, and this may be due to the normal magnesium content as shown by Sivarama Sastry *et al*, (1962). Abelson and Aldous (1950) also reported enhanced zinc toxicity at 2.0 µg $ml^{-1}$ to *Escherichia coli* in medium deficient in magnesium.

*Neocosmospora vasinfecta* appeared to tolerate higher zinc concentrations than any of the fungi in this investigation since it grew as well in 650.0 µg $ml^{-1}$ $Zn^{++}$ as in control flasks with 1.625 µg $ml^{-1}$ and toxicity was only apparent at 6500.0 µg $ml^{-1}$ as shown by a 90% reduction in growth by Paton and Budd,(1972). Thind and Mira Madan (1975) also reported that *Cephalothecium roseum* mycelium and sporulation was stimulated at upto 200.0 µg $ml^{-1}$ with only slight inhibition at 400.0 µg $ml^{-1}$. Smith (1977) reported that zinc was toxic to *Epicoccum sp.*, *Gnomonia platani* and *Pestalotiopsis sp.* at 130.0 µg $ml^{-1}$ in liquid media but not to *Aureobasidium pullulans*. On plates however, this level of zinc was toxic to *A. pullulans* and 265.0 µg $ml^{-1}$ $Zn^{++}$ was toxic to *Epicoccum sp.* Duddridge and Wainwright (1980) showed that zinc had no effect at 0-10.0 µg $ml^{-1}$ on 3 aquatic fungi and that there was no relationship to the amount of carbon or nitrogen in the medium. Zinc uptake was reduced however, in the medium containing low C and N. According to Okamoto *et al* (1977), zinc was very inocuous to *P. ochrochloron* and spore germination was not inhibited at 10 000.0 µg $ml^{-1}$ $Zn^{++}$.

The authors were not able to establish the toxicity limit. Mycelial growth however, was slightly reduced at 1000.0 µg $ml^{-1}$ but at 100 000.0 µg $ml^{-1}$ inhibition still amounted to only 50%. Miller and McCallan (1957) showed also that zinc on short exposure to spores, including those of *A. niger* had no effect on germination. Even at a zinc loading of 100 000.0 µg $g^{-1}$ spores, 25.4 hours of exposure only caused 27% inhibition of germination of *Neurospora sitophila* spores.

The general toxicity series for the metals used in this study for *P. spinulosum* and *A. niger* with Cd > Cu > Zn, and for *T. viride* with Cu > Cd > Zn, agrees well with the general pattern observed by Horsfall (1956), who reported the following series in order of decreasing toxicity: Ag > Hg > Cu > Cd > Cr > Ni > Pb > Co > Zn > Fe > Ca.
The series published by Bedford (1936), for *Alternaria tenuis* and *Botrytis fabae* is similar except for lead and nickel. Treen-Sears *et al* (1984) reported the following series for *Rhizopus javanicus*:
Cu > Co > Ni > Mn > Mb. Zinc had no effect on growth.
For *P. ochrochloron*, Okamoto *et al* (1977) reported the following toxicity series:
Hg > Ni > Tl > Co > Cr > Cd, > Pb, > Fe > Mn, > Zn & Cu. It is apparent that there are some major differences when highly resistant atypical organisms are studied. Obviously, there does appear to be a trend for normally susceptible fungi to metals, but the usefulness of these toxicity series is limited since they are dependent on many factors related to the metal, the fungus and the environment. Toxicity tests do however serve a useful purpose as a prerequisite to metal accumulation studies, and can sometimes be used to relate metal resistance to metal uptake characteristics of particular fungi. The next section of this investigation was a study of metal accumulation.

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Hg > Ni > Tl > Co > Cr > Cd, > Pb, > Fe > Mn, > Zn & Cu. It is apparent that there are some major differences when highly resistant atypical organisms are studied. Obviously, there does appear to be a trend for normally susceptible fungi to metals, but the usefulness of these toxicity series is limited since they are dependent on many factors related to the metal, the fungus and the environment. Toxicity tests do however serve a useful purpose as a prerequisite to metal accumulation studies, and can sometimes be used to relate metal resistance to metal uptake characteristics of particular fungi. The next section of this investigation was a study of metal accumulation.

## 5. DEVELOPMENT OF EXPERIMENTAL PROCEDURES FOR METAL ACCUMULATION EXPERIMENTS USING ACTIVELY GROWING FUNGI.

### 5.1. Preliminary experiments.

A number of preliminary procedural checks were performed prior to metal accumulation experiments and these will be described initially. These include the monitoring of metal retention by experimental apparatus and filters, an evaluation of metal complexing capability by medium constituents and metabolic products of the fungi secreted during growth which would affect metal availability, and an evaluation of the influence of the MES buffer on metal accumulation.

#### 5.1.1. Glassware preparation.

All analytical and sampling glassware was soaked for 24 hours in DECON detergent (2%). After rinsing in deionised water the glassware was soaked in 2% reagent grade HCl for a further 24 hours and then rinsed with either deionised or distilled water.

#### 5.1.2. Metal retention of experimental sampling apparatus.

Apparatus such as plastic centrifuge tubes and glassware was checked for metal retaining qualities. The apparatus under investigation was first filled with a metal chloride solution (copper, cadmium or zinc) at concentrations of 1.0, 10.0 and 100.0 $\mu g\ ml^{-1}$ metal prepared in either 4% $H_2SO_4$, distilled $H_2O$ or GMS medium. The centrifuge tubes and glassware were sealed with parafilm to prevent evaporation and left to stand at room temperature for the duration of the experiment. Total metal content of the solutions was assayed after 0, 24 and 96 hours by atomic absorption spectrophotometry (AAS) to monitor for losses due to absorption to plastic or glass. No detectable loss was observed in centrifuge tubes, sample collecting flasks or in volumetric flasks

after the 96 hours duration of the experiment, as a further check the apparatus was washed in 10% $H_2SO_4$ and no metal was detectable in these rinses as established by AAS.

5.1.3. An evaluation of metal content of filters and their metal retaining capacities.

The digestion procedure used for filters was as described in procedure B of the analytical methods committee (1967). Millipore filters were dried overnight at 105°C, cooled in a desiccator and weighed. Each filter was then placed in a separate flask containing 1.0 ml conc $H_2SO_4$ (AnalaR grade) and heated to 400°C on a heating block until charred. At this point 2.0 ml of 50% $H_2O_2$ were added dropwise to the flask after removal from the heating block. The samples were then reheated until all the $H_2O_2$ had boiled off and the conc $H_2SO_4$ fumed. Further additions of $H_2O_2$ were made if there was any evidence of residual yellow colouration due to organics presence. The process was repeated until the acid was colourless. After cooling, the sample vessel was rinsed down with distilled $H_2O$ and the contents transferred to appropriate volumetric flasks and made up to volume with distilled $H_2O$. The use of this digestion technique enabled total metal content of the samples to be established. The samples were analysed for copper, cadmium and zinc using AAS to determine the basal metal content of the filters. The procedure was repeated for the Whatman 541 filters. The Millipore filters contained no detectable copper or cadmium, but contained 0.1 µg $Zn^{++}$ $g^{-1}$ filter. Paper filters contained 0.48, 0.03 and 0.65 µg $g^{-1}$ dry weight copper, cadmium and zinc respectively.

As well as investigating the basal metal contents of the filters, the ability of the filters to retain metal from filtered solutions was

monitored. 100 ml volumes of 1.0, 10.0 and 100.0 µg $ml^{-1}$ solutions of copper, cadmium and zinc chloride were prepared and filtered through individual Millipore and Whatman 541 filters. The filters were then washed vigourously by filtering a further 500 ml distilled $H_2O$. They were then dried at 105°C overnight and weighed and subsequently digested and assayed for metal as previously described. No copper or cadmium was retained by Millipore filters but they did absorb zinc from the 100.0 µg $ml^{-1}$ solution to attain a final total content of 0.86 µg $Zn^{++}$ $g^{-1}$ filter. Whatman 541 filters retained no metal from the solution containing 1.0 µg $ml^{-1}$ copper, cadmium or zinc, but absorbed zinc to attain a total content of 1.09 and 1.33 µg $Zn^{++}$ $g^{-1}$ filter from solutions of 10.0 and 100.0 µg $ml^{-1}$ $Zn^{++}$ respectively. They also absorbed copper to attain a final copper loading of 0.59 and 0.62 µg $Cu^{++}$ $g^{-1}$ filter from 10.0 and 100.0 µg $ml^{-1}$ $Cu^{++}$ solutions respectively. Cadmium was retained from a 100.0 µg $ml^{-1}$ $Cd^{++}$ solution resulting in a concentration of 0.05 µg $Cd^{++}$ $g^{-1}$ dry weight of the filter. As a consequence of the high metal retaining characteristics of the paper filters, the use of Whatman 541 filters was restricted, and only Millipore filters were employed with metal concentrations upto 15.0 µg $ml^{-1}$ metal. Paper filters where used, were not digested. Instead, the mycelium was scraped from them and used directly in the metal analyses. The filters were then discarded.

## 5.2. Preliminary experiments.

### Electrochemical and voltammetric analysis for the determination of complexation capacities of medium constituents and spent medium.

### 5.2.1. Introduction.

Electrode measurements involve electron transfer which is the measured parameter. Each oxidation state of a metal has a characteristic electron transfer reaction which occurs at specific electrode potentials. If there is more than one oxidation state of the metal ion, these can therefore be distinguished by electrochemical techniques. Since electrochemical measurements are restricted to soluble species of a known oxidation state, they provide information on the distribution of species within a particular solution. (Bond, 1980).

Two direct methods for determining the degree of metal complexation to medium constituents were employed, direct current polarography (DCP), and anodic stripping voltammetry (ASV). These techniques provide a quantitative measurement of the free electroactive metal ion in terms of the polarographic wave height or peak height, relative to standard wave heights produced by standard additions so that a standard curve can be constructed by plotting wave height against added metal concentration. The techniques also provide the possibility for a qualitative evaluation of complex formation, reversibility and irreversibility, complex stability and ion speciation by virtue of half-wave potential shifts and the degree and direction of the shift.

Non-reversible complexes are characterised by a reduction in wave height and a shift in half-wave potential, the bigger the shift in the half-wave potential, the more stable is the complex, since more energy is required to bring about the reduction or oxidation of the electoactive

species. Reversibility of complexes can be demonstrated by utilising the cylic scanning capability of most modern machines.

5.2.2. Materials and Methods.

5.2.2.1. Medium constituent complexing capacity and spent medium analysis.

Samples of MES buffer, magnesium glycerophosphate, $(NH_4)_2SO_4$, at the same concentrations as in the normal medium were prepared and supplemented with copper chloride to obtain a final concentration of 2.5 µg $ml^{-1}$ $Cu^{++}$ in 50 ml volumetric flasks made up with $10^{-3}$ M NaCl (AnalaR grade) solution as the supporting electrolyte, and adjusted to pH 5.5 with saturated KOH. A control 2.5 µg $ml^{-1}$ $Cu^{++}$ sample was prepared consisting of copper chloride and $10^{-3}$ M NaCl only, again adjusted to pH 5.5 with saturated KOH or strong HCl.

Similarly, samples containing the metabolic inhibitors sodium azide, 2,4 dinitrophenol (2,4 DNP) and sodium monoiodoacetate were prepared in the supporting electrolyte, to a final concentration of 1.0 mM. The sodium azide and 2,4 DNP were adjusted to pH 5.5, the monoiodoacetate was adjusted to pH 4.0. Again, the final copper concentration was 2,5 µg $ml^{-1}$.

Samples of spent medium obtained from mid linear growth phase cultures of *P. spinulosum*, *T. viride* and *A. niger* were readjusted to pH 5.5, and copper chloride and AnalaR NaCl was added to obtain a final concentration of 2.5 µg $ml^{-1}$ and $10^{-3}$ M respectively. Similar samples were prepared for $Zn^{++}$ and $Cd^{++}$ at 2.5 µg $ml^{-1}$ concentrations.

Sampled DCP using a dropping mercury electrode (DME) was performed on a 174A EG and G PARC Polarographic Analyser connected to a static

mercury dropping electrode (SMDE). The initial potential was set at -1.0 V to include the redox potentials for all 3 metals during a scan at a rate of 50 mV $sec^{-1}$ in the positive direction. The current range of 0.5 μA was found to be the most appropriate in this operation mode.

Acknowledgement.
Polarographic equipment used in this subsection 5.2.2.1. was that belonging to the Oceanography Department, Liverpool University.
I gratefully acknowledge Dr. C.M.G. Van den Berg for his permission to use the equipment and his assistance where needed.

5.2.2.2. Complexing capacity of spent medium during the growth of fungi.

Since mid-linear phase spent medium appeared to cause some reduction in the availability of added metals, it was decided to monitor the complexing of metals throughout the growth of the fungus to determine if complexing could be related to the presence of secreted metabolites in the medium.

This section of the work was performed on a Metrohm 663 VA Stand with a static mercury drop electrode (SMDE) coupled to a Metrohm 626 Polarecord analyser. Deoxygenation was carried out with oxygen-free-nitrogen.

A series of replicate flasks containing 100 ml GMS medium were inoculated in the standard manner using *P. spinulosum* initially. At the time of inoculation and at every subsequent 24 hours during the growth cycle, 2 replicate flasks were selected and the mycelium collected by filtration and then discarded. 20 ml of the filtrate was used in the polarographic analysis. The sample was deaerated and then supplemented

with either copper, cadmium or zinc chloride solutions to obtain final concentrations of 2.5 µg $ml^{-1}$ metal. Anodic stripping voltammetry (ASV) was performed by employing a hanging mercury drop with linear sweep on differential pulse mode 50. The scan rate used was 10 mV $sec^{-1}$ and the current range was 5.0 µA. For copper estimations, the initial potential was set at -0.40 V for cadmium at -0.80 V and for zinc it was set at -1.20 V. Scanning was carried out in the positive direction, with redox potentials occurring for copper at 0 V, for cadmium at -0.56 V and for zinc at -0.98V. The procedure was repeated for *T. viride* and A. *niger* and the results are illustrated in figures 5.2.3.4. to 11 and Table 5.2.3.1a and b.

5.2.2.3. The effect of pH on half wave potentials and peak height in voltammetric traces.

As a control for the experiment on complexing capacity of spent medium during the growth cycle it was considered necessary to check that pH modifications did not influence the polarographic waves obtained in the experiment. ASV runs in the positive direction were performed on fresh GMS medium and $10^{-3}$ M NaCl with added metal at a concentration of 2.5 µg $ml^{-1}$ at pH 5.5 and 2.0. Any alteration in the wave parameters at the two pHs would indicate that chemical modifications in the electrolyte had occurred and this would render estimations of complexing very difficult. If on the other hand, no changes were apparent in the waves at the two pHs, then any alteration in the peak characteristics must have been due to an actual change in the medium brought about by fungal metabolism.

5.2.3. Results.

5.2.3.1. Medium constituent complexing capacity and spent medium analysis.

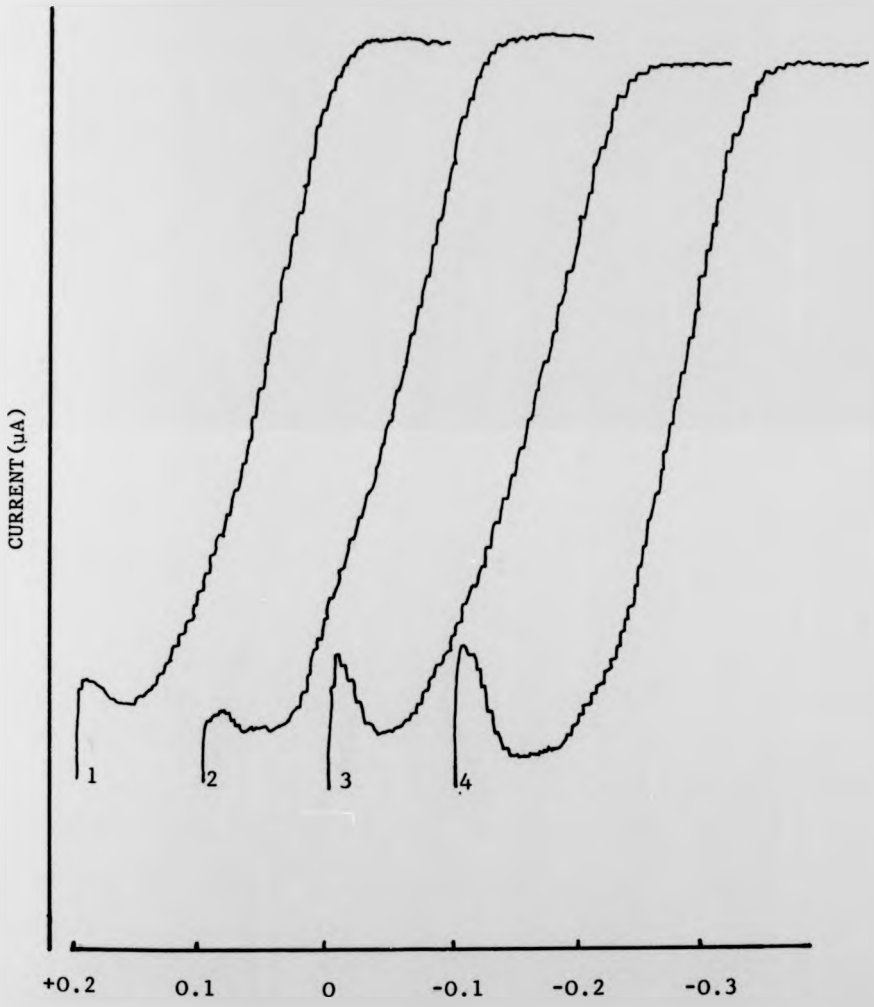
Figure 5.2.3.1. shows the DC polarographic waves for copper added at 2.5 µg $ml^{-1}$ to control flasks (electrolyte only as $10^{-3}$ NaCl), 50.0 mM MES buffer, magnesium glycerophosphate and $(NH_4)_2SO_4$ all prepared in $10^{-3}$ M NaCl as the supporting electrolyte. The identical traces showing the same half wave potential and wave height suggest that there is no complexing of copper by these medium constituents.

Figure 5.2.3.2. shows the DC polarographic wave for copper added at 2.5 µg $ml^{-1}$ to control flasks (electrolyte only), and also the waves produced when metabolic inhibitors were added to the supporting electrolyte containing copper. The traces demonstrate that 1.0 mM monoiodoacetate had no effect on the half wave potential or peak height although 2,4 DNP slightly reduced the half wave potential (slight shift in the positive direction), and also increased the wave height slightly. The most dramatic effect was seen with the addition of sodium azide to a final concentration of 1.0 mM. The azide caused a reduction in wave height by about 50% and also the half wave potential was shifted in the negative direction indicating a more negative redox potential for the copper which is attributable to the formation of a copper complex with the azide.

Figure 5.2.3.3. illustrates the DC polarographic waves for copper added at 2.5 µg $ml^{-1}$ to control flasks and to spent medium of mid linear cultures of *P. spinulosum*, *T. viride* and *A. niger*. Complexing of the copper is clearly demonstrated in all cases as shown by the significant modifications in the polarogram parameters.

*Figure* 5.2.3.1.

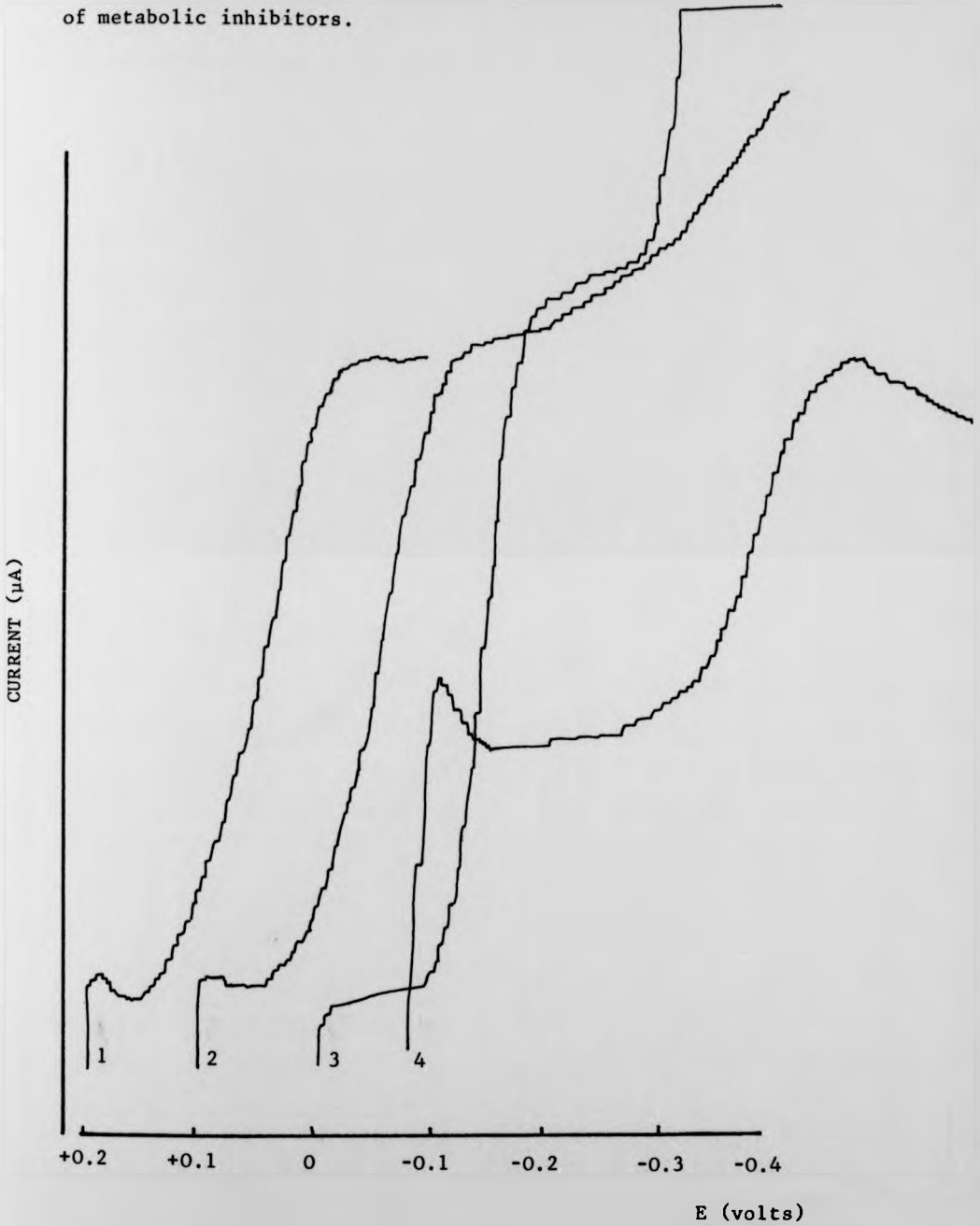
Medium constituent polarographic traces for copper.




1. Control
2. MES
3. Mg glycerophosphate
4. $(NH_4)_2\ SO_4$

*Figure* 5.2.3.2.

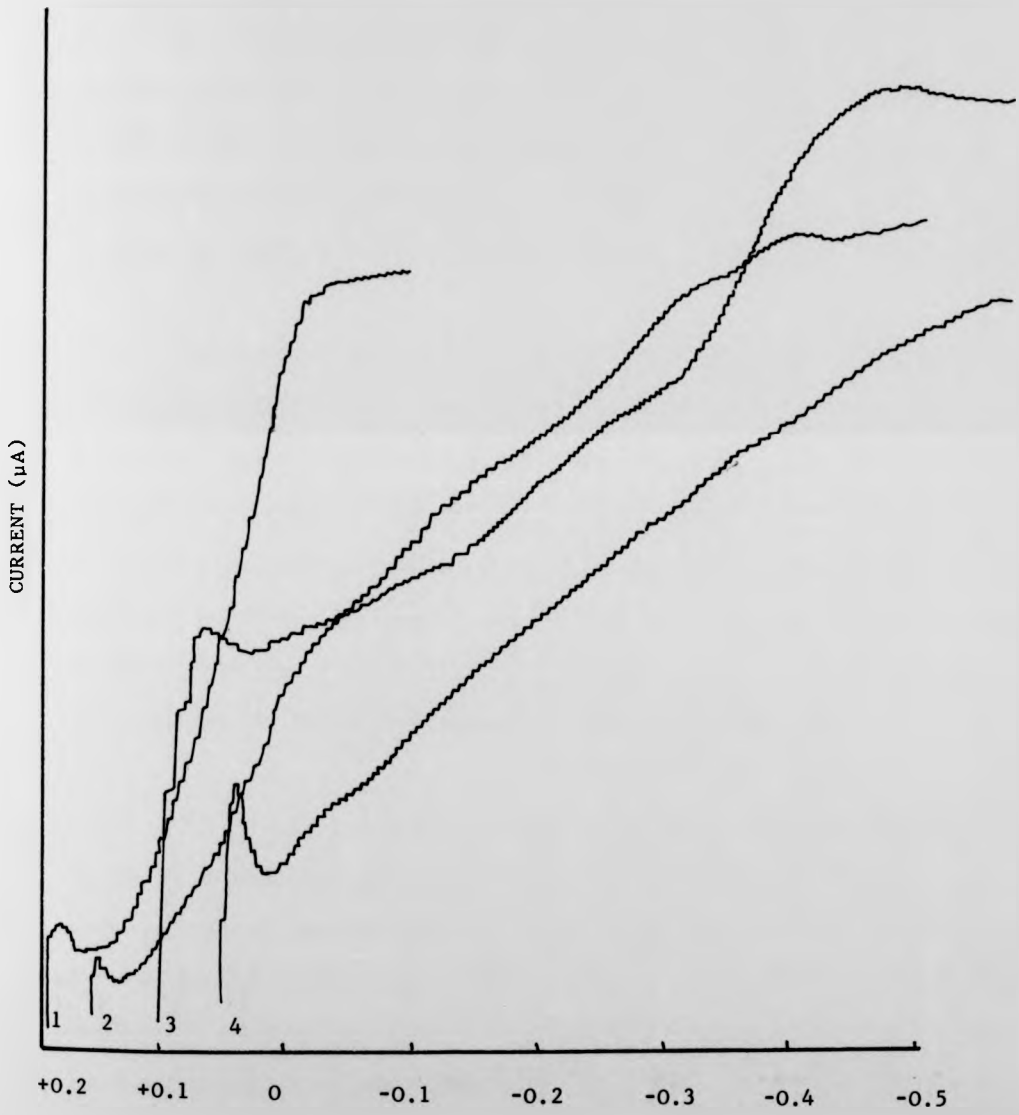
Polarographic traces for 2.5 µg $ml^{-1}$ copper in the presence of metabolic inhibitors.




1. Control (no metabolic inhibitor)
2. monoiodoacetate
3. 2,4DNP
4. Sodium Azide

*Figure* 5.2.3.3.

Polarographic traces for 2.5 µg $ml^{-1}$ copper prepared in spent medium collected at mid-linear phase of growth.




1. Control
2. Spent medium *P.spinulosum*
3. Spent medium A. *niger*
4. Spent medium *T. viride*

5.2.3.2. Complexing capacity of spent medium during the growth of fungi.

Figure 5.2.3.4. shows the polarogram for GMS medium containing 2.5 µg $ml^{-1}$ $Cu^{++}$. Figures 5.2.3.5-7 show the effect of increasing the growth time in GMS medium (24, 48 and 72 hours) on the ASV waves produced when copper was added at a concentration of 2.5 µg $ml^{-1}$. Figures 5.2.3.8-11 show results for cadmium and figures 5.2.3.12-15 for zinc. The figures illustrated are for growth medium which had supported the growth of *P. spinulosum*, and although not shown diagrammatically, the effects were identical for *T. viride* and *A. niger*, and the effects after 72 hours of growth are shown in Tables 5.2.3.1a and 1b for all three fungi.

In Figures 5.2.3.4.-7 it can be seen that with increasing time of support of growth for *P. spinulosum*, the ASV peak for copper at 2.5 µg $ml^{-1}$ appeared to be modified especially at pH 5.5. At this pH, the half wave potential was shifted in the negative direction and there was a reduction in peak height as a consequence of microbial growth. At pH 2.0 however, there was no shift in the half wave potential and only a slight reduction in peak height was recorded. It seems that copper was complexed at the higher pH of 5.5 but not at 2.0.

In Figures 5.2.3.8-11 it can be seen that with increasing time of growth in the GMS medium with *P. spinulosum*, this resulted in modifications of the ASV peak for cadmium but more so at pH 2.0 than at 5.5. At pH 5.5, there was no effect on the half wave potential and peak height. At pH 2.0, there was a shift in the half wave potential in the positive direction with a concomitant reduction in peak height. In the case of cadmium, it seems that complexing effects were more important at the lower pH of 2.0 and this may be of some importance in the availability of cadmium at mid-linear phase of growth when the pH is reduced to 2.0.

Figures 5.2.3.12-15 illustrate that increasing the period of growth time in the GMS medium using *P. spinulosum*, resulted in modifications of the ASV peak for zinc at 2.5 μg $ml^{-1}$, but as with cadmium, the effect was more marked at pH 2.0 than at pH 5.5. This is interesting because of the chemical similarity of zinc and cadmium. At pH 5.5, there was a slight shift to the right (positive direction) in the half wave potential and a reduction in peak height. At pH 2.0 there was a more dramatic shift to the right (positive direction) in the half wave potential and a bigger reduction in peak height, particularly after 72 hours of growth. Zinc availability may therefore be affected by pH reductions to 2.0 during growth.

In Tables 5.2.3.1a and 1b it can be seen that the modifying capacity in respect of the medium of all three fungi tested appeared to be similar, in that spent medium from the 3 fungi produced similar changes in the half wave potential shifts and peak height reductions for the ASV peaks produced for 2.5 μg $ml^{-1}$ copper, cadmium and zinc prepared in spent medium which had supported growth for 72 hours.

5.2.3.3. <u>The effect of pH on wave characteristics in voltammetric traces</u>.

In Figures 5.2.3.16-18 it is shown that for copper, cadmium and zinc reductions in pH from 5.5 to 2.0 in $10^{-3}$ M NaCl and fresh GMS medium, resulted in no modifications in the half wave potentials and peak heights for ASV traces produced at 2.5 μg $ml^{-1}$ metal concentration.

Complexing capacity of spent medium during the growth of fungi.

ASV waves for 2.5 $\mu g\ ml^{-1}$ copper prepared in spent medium after supporting growth of *P. spinulosum*.

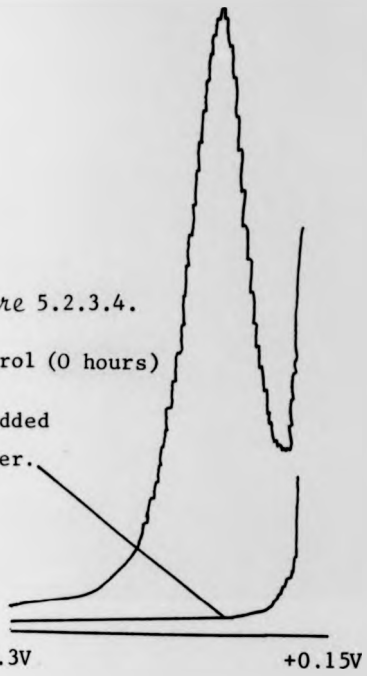



*Figure* 5.2.3.4.

GMS controls showing same wave parameters at pH 5.5 and 2.0.

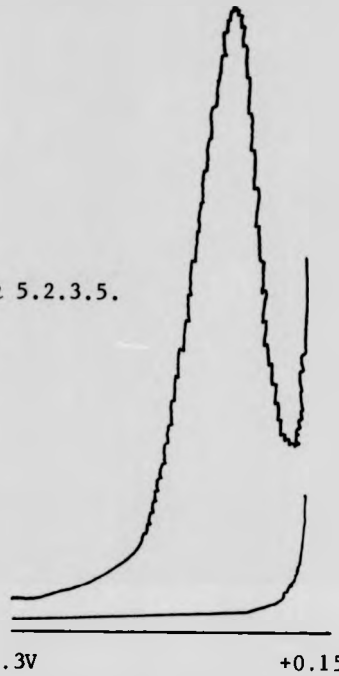



*Figure* 5.2.3.5.

24 h.

No change in waves after supporting growth for 24 h, pH 5.5 and 2.0.

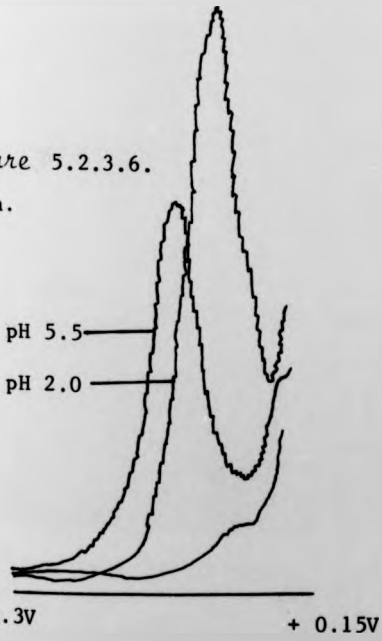



*Figure* 5.2.3.6.

48 h.

Half wave potential shift to left(-ve) at pH 5.5 with reduction in peak ht.

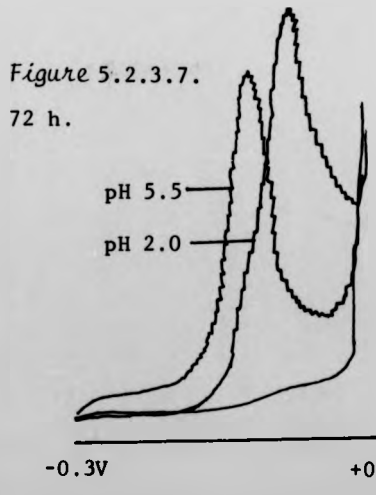



*Figure* 5.2.3.7.

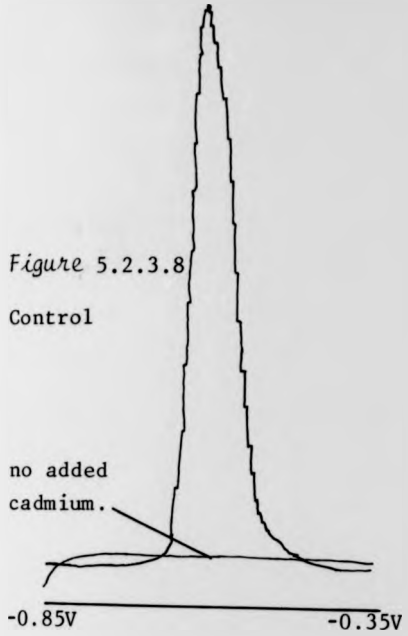
72 h.

As 48 h, but also slight reduction in peak ht. at pH 2.0.

Complexing capacity of spent medium during the growth of fungi- ASV waves for 2.5 µg $ml^{-1}$ cadmium prepared in spent medium after supporting growth.

*Figure* 5.2.3.8

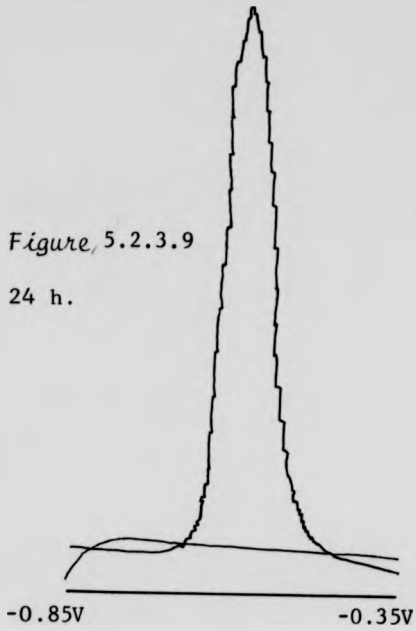
Control




GMS controls showing same wave parameters at pH 5.5 and 2.0.

*Figure* 5.2.3.9

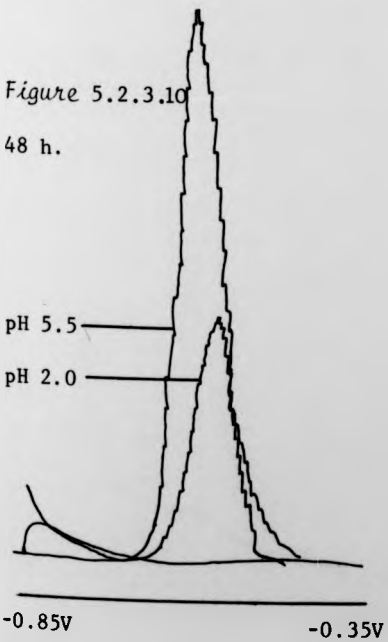
24 h.




No change in waves after supporting growth for 24 h. at pH 5.5 or 2.0

*Figure* 5.2.3.10

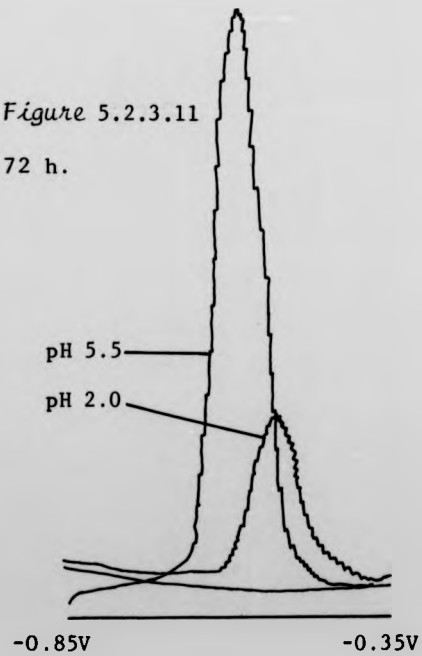
48 h.




No change at pH 5.5 but slight right(+) shift in half wave potential and reduced peak ht. at pH 2.0.

*Figure* 5.2.3.11

72 h.

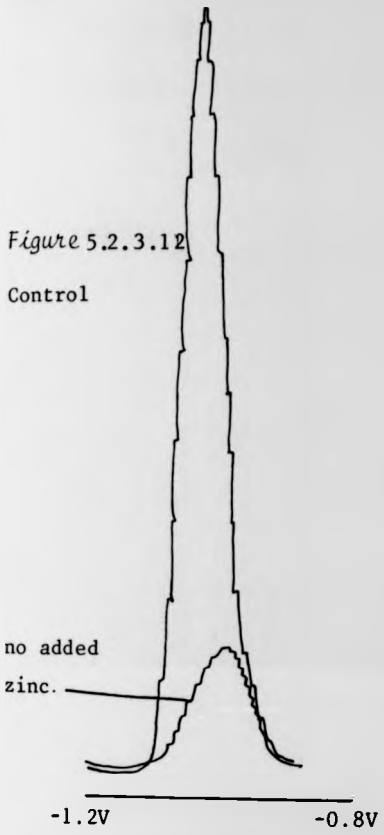



As 48 h. but further reduction in peak ht. at pH 2.0.

ASV waves for 2.5 $\mu g\ ml^{-1}$ zinc prepared in spent medium after supporting fungal growth.

*Figure* 5.2.3.12

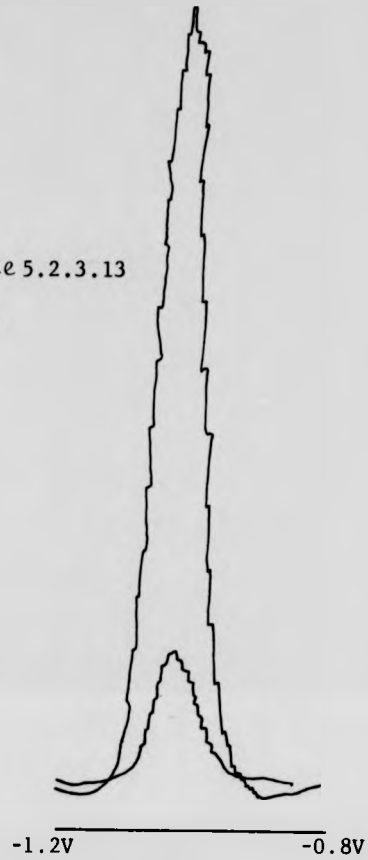
Control




GMS controls showing same wave parameters at pH 5.5 and 2.0.

*Figure* 5.2.3.13

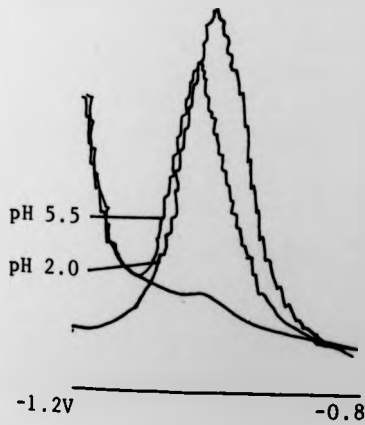
24 h.




No change in waves after supporting growth for 24 h. at pH 5.5 and 2.0.

*Figure* 5.2.3.14

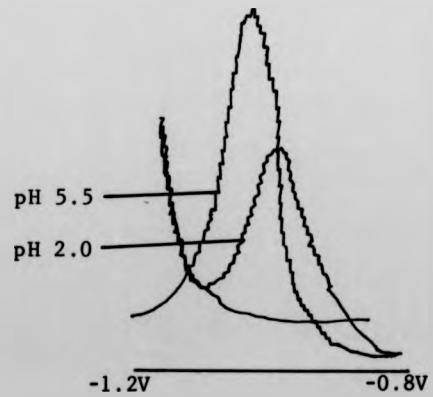
48 h.




Half wave potential shifts to right (+) at pH 5.5 and 2.0 and reductions in peak ht. Shift most marked at pH 2.0.

*Figure* 5.2.3.15

72 h.




Half wave potential shifts to right (+ve) at pH 5.5 and 2.0 with peak ht. reductions. Most dramatic at pH 2.0.

*Table* 5.2.3.1a and 1b.

Wave pattern modifications produced from 2.5 μg $ml^{-1}$ metal solutions prepared in spent medium utilised for fungal growth for 72 hours.

| Fungus | Metal | Half wave shift. pH 5.5 | Peak ht. |
|---|---|---|---|
| *P. spinulosum* | $Cu^{++}$ | - shift | Reduced |
| | $Cd^{++}$ | No change | No change |
| | $Zn^{++}$ | Sl. + shift | Reduced |
| *T. viride* | $Cu^{++}$ | Sl. - shift | Reduced |
| | $Cd^{++}$ | No change | Sl. red. |
| | $Zn^{++}$ | + shift | Reduced |
| *A. niger* | $Cu^{++}$ | - shift | Sl. red. |
| | $Cd^{++}$ | No change | Sl. red. |
| | $Zn^{++}$ | + shift | Reduced |

| Fungus | Metal | Half wave shift. pH 2.0 | Peak ht. |
|---|---|---|---|
| *P. spinulosum* | $Cu^{++}$ | No change | Sl. reduced |
| | $Cd^{++}$ | + shift | Reduced |
| | $Zn^{++}$ | + shift | Reduced |
| *T. viride* | $Cu^{++}$ | No change | Sl. reduced |
| | $Cd^{++}$ | + shift | Reduced |
| | $Zn^{++}$ | + shift | Reduced |
| *A. niger* | $Cu^{++}$ | No change | Sl. reduced |
| | $Cd^{++}$ | + shift | Reduced |
| | $Zn^{++}$ | + shift | Reduced |

5.2.4. Discussion

At pH 5.5 there appeared to be some complexing of copper by substances accumulating as a result of fungal growth. At this pH there appeared to be little complexing of cadmium, although the zinc peak suffered a shift in the half wave potential in the positive direction and a reduction in wave height, suggesting that fungal growth had some influence on the chemistry of zinc in the GMS medium, perhaps producing complexes with different groups from those complexing copper.

At pH 2.0 copper did not appear to be complexed, although the half wave potential for cadmium and zinc were shifted in the positive direction. The results for copper were as expected with the spent medium causing some complexation at pH 5.5 but not at pH 2.0. It was however surprising, to find that cadmium and zinc were affected in some way at pH 2.0 and this is very difficult to explain. The fact that cadmium and zinc were influenced by low pH in this way may affect the availability of these metals in the GMS medium at the mid-linear stage of growth when the pH of the medium is reduced to about 2.0.

As a check that pH itself did not cause alterations in the characteristics of the waves, voltammetric runs were performed at pH 5.5 and 2.0 on $10^{-3}$ M NaCl and fresh GMS medium which had not supported the growth of fungi. The rationale was that if the waves produced at the different pH values on these media were the same, the alterations in the waves in spent medium must have been due to a modification of the medium as a result of microbial growth, and not as a consequence of some chemical peculiarity of the electrochemical technique. In fact lowering the pH in these two systems (fresh GMS and NaCl), had no effect on the ASV traces so this appears to confirm that cadmium and zinc are in some way

*Table* 5.2.3.1a and 1b.

Wave pattern modifications produced from 2.5 μg $ml^{-1}$ metal solutions prepared in spent medium utilised for fungal growth for 72 hours.

| Fungus | Metal | Half wave shift. pH 5.5 | Peak ht. |
|---|---|---|---|
| *P. spinulosum* | $Cu^{++}$ | - shift | Reduced |
| | $Cd^{++}$ | No change | No change |
| | $Zn^{++}$ | Sl. + shift | Reduced |
| *T. viride* | $Cu^{++}$ | Sl. - shift | Reduced |
| | $Cd^{++}$ | No change | Sl. red. |
| | $Zn^{++}$ | + shift | Reduced |
| *A. niger* | $Cu^{++}$ | - shift | Sl. red. |
| | $Cd^{++}$ | No change | Sl. red. |
| | $Zn^{++}$ | + shift | Reduced |

| Fungus | Metal | Half wave shift. pH 2.0 | Peak ht. |
|---|---|---|---|
| *P. spinulosum* | $Cu^{++}$ | No change | Sl. reduced |
| | $Cd^{++}$ | + shift | Reduced |
| | $Zn^{++}$ | + shift | Reduced |
| *T. viride* | $Cu^{++}$ | No change | Sl. reduced |
| | $Cd^{++}$ | + shift | Reduced |
| | $Zn^{++}$ | + shift | Reduced |
| *A. niger* | $Cu^{++}$ | No change | Sl. reduced |
| | $Cd^{++}$ | + shift | Reduced |
| | $Zn^{++}$ | + shift | Reduced |

# PAGINATION ERROR

5.2.4. Discussion

At pH 5.5 there appeared to be some complexing of copper by substances accumulating as a result of fungal growth. At this pH there appeared to be little complexing of cadmium, although the zinc peak suffered a shift in the half wave potential in the positive direction and a reduction in wave height, suggesting that fungal growth had some influence on the chemistry of zinc in the GMS medium, perhaps producing complexes with different groups from those complexing copper.

At pH 2.0 copper did not appear to be complexed, although the half wave potential for cadmium and zinc were shifted in the positive direction. The results for copper were as expected with the spent medium causing some complexation at pH 5.5 but not at pH 2.0. It was however surprising, to find that cadmium and zinc were affected in some way at pH 2.0 and this is very difficult to explain. The fact that cadmium and zinc were influenced by low pH in this way may affect the availability of these metals in the GMS medium at the mid-linear stage of growth when the pH of the medium is reduced to about 2.0.

As a check that pH itself did not cause alterations in the characteristics of the waves, voltammetric runs were performed at pH 5.5 and 2.0 on $10^{-3}$ M NaCl and fresh GMS medium which had not supported the growth of fungi. The rationale was that if the waves produced at the different pH values on these media were the same, the alterations in the waves in spent medium must have been due to a modification of the medium as a result of microbial growth, and not as a consequence of some chemical peculiarity of the electrochemical technique. In fact lowering the pH in these two systems (fresh GMS and NaCl), had no effect on the ASV traces so this appears to confirm that cadmium and zinc are in some way

complexed at pH 2.0 as a consequence of microbial modification of the medium. Copper on the other hand, was complexed at pH 5.5 and not at pH 2.0. Therefore, it seems that in the experiments which investigate metal accumulation (next section) in actively growing fungi where the pH during growth falls to 2.0 in mid-linear phase of growth, copper availability ought to be increased at the lower pH, but in the case of cadmium and zinc there may be some detrimental change in availability possibly due to complexing to fungal metabolites. This does not of course necessarily mean that the fungi cannot compete with other complexing agents for metal ions and actually still accumulate metal.

## 5.3. Preliminary experiments

### An evaluation of the efficiency of MES buffer and determination of its possible utilisation.

The objective of this investigation was to monitor the effectiveness of the buffering capacity of the MES buffer at various concentrations and to determine if the buffer is metabolised.

### 5.3.1. Materials and Methods.

#### 5.3.1.1. Effect of MES concentration on pH profiles during active growth of *Penicillium spinulosum*.

One litre Erhlenmyer flasks containing 400 ml GMS medium were prepared and inoculated with 4.0 ml of a fragment suspension of *P. spinulosum* to assess the concentration of MES buffer required to maintain the pH at 5.5 during the growth of the fungus. Each experimental flask was prepared in triplicate and contained either 50.0, 100.0, 250.0 or 500.0 mM MES buffer.

Incubation was at 25°C with shaking. Every 12 hours for 60 hours of incubation, samples were removed in triplicate from the flasks. At the early stages of growth 50 ml samples were necessary because of the low density of culture, but as the biomass density increased, progressively smaller samples were taken. The samples were collected by filtration and the filtrate retained for pH measurements. Figure 5.3.2.1. shows the effect of MES concentration on the maintenance of initial pH with time in culture.

#### 5.3.1.2. Determination of MES buffer utilisation by actively growing *Penicillium spinulosum*.

Two batches of 3, 1 litre flasks containing 400 ml GMS medium were prepared, one batch containing 5.0 mg $ml^{-1}$ glucose and the other

batch containing 10.0 mg $ml^{-1}$ glucose. The objective of this experiment was to grow the *P. spinulosum* until the added glucose became limiting, and then to determine by means of a dry weight plot if the MES was utilised as a substrate which ought to be depicted by a biphasic growth curve.

Each set of 3 flasks was inoculated and incubated as described previously, and sampled every 12 hours for dry weight determinations. Figures 5.3.2.2a and 2b illustrate dry weight increase with time for the two glucose concentrations, superimposed on plots for glucose utilisation. A glucose assay kit (Sigma reagent kit No. 15 UV) was used for glucose concentration determinations in the spent medium.

5.3.1.3. Glucose assay.

The glucose contents of the spent medium were assayed by employing a spectrophotometric technique based on the conversion of glucose to glucose 6-P by ATP in the presence of hexokinase, coupled with the subsequent reduction of NADP to NADPH. NADPH absorbs strongly at 340 nM whilst NADP does not absorb at this wavelength. Thus, the reaction can be followed by monitoring the change in absorbance at 340 nM since the formation of NADPH is directly proportional to the glucose concentration.

The assay solution was used at 25°C for glucose estimations. A 1:10 dilution of the spent medium using distilled $H_2O$ was carried out to enable measurements to be made in the appropriate glucose concentration range. After thorough mixing, 3.0 ml of the assay solution were pipetted into a cuvette and the background absorbance measured at 340 nM using distilled $H_2O$ as the blanking solution, in a

Pye Unicam Spectrophotometer (Model SP6-400). 0.02 ml of the diluted spent medium were added to the assay solution in the cuvette and carefully mixed. The absorbance was read at completion of the conversion of NADP to NADPH. The glucose concentration was calculated by subtraction of initial from the final absorbance reading and multiplying by a conversion factor.

( (Final-initial absorbance) x 4400).

5.3.2. Results.

5.3.2.1. Effect of MES concentration on pH profiles during active growth of *Penicillium spinulosum*.

Figure 5.3.2.1. shows that MES at all concentrations tested maintained the pH at 5.5 throughout the inoculation procedure (this included the addition of 100.0 µL of the metal salt prepared in 1.0 M HCl), and for the first 24 hours of growth. By 36 hours however, the pH had fallen to around 5.0 in the presence of 50.0 and 100.0 mM MES and by 48 hours the pH was between 2.0 and 2.5. The MES concentrations of 250.0 and 500.0 mM appeared to be capable of satisfactorily maintaining the pH at 5.5 for 36 hours, after which the pH dropped to 5.0 in both cases by 48 hours of growth. 500.0 mM MES was the only concentration which was able to maintain the pH above 5.0 for the duration of the growth cycle. 250.0 mM MES was able to maintain the pH at 5.0 until late linear phase, after which the pH dropped to 3.8 after 60 hours of growth.

250.0 and 500.0 mM MES concentrations were considered to be impractical for extended usage because of the prohibitive costs involved. It was subsequently demonstrated that the pH at inoculation is crucial for metal uptake as shown by experiments with electronic pH control in a fermenter and this will be discussed in Chapter 7. It was decided to use 50.0 mM MES to ensure that the pH was maintained at 5.5 during

the inoculation procedure, since pH control during later stages of growth did not appear to be very important in metal uptake.

5.3.2.2. Determination of MES buffer utilisation by actively growing *Penicillium spinulosum*.

Figures 5.3.2.2a and 2b show the relationship between glucose utilisation from an initial concentration of 5.0 mg $ml^{-1}$ and 10.0 mg $ml^{-1}$ respectively, and biomass production in the presence of 50.0 mM MES. Biomass production ceased in both cases after glucose depletion and there appeared to be no evidence of a biphasic growth curve. It appears therefore, that the MES was not utilised for biomass production after the glucose had been metabolised.

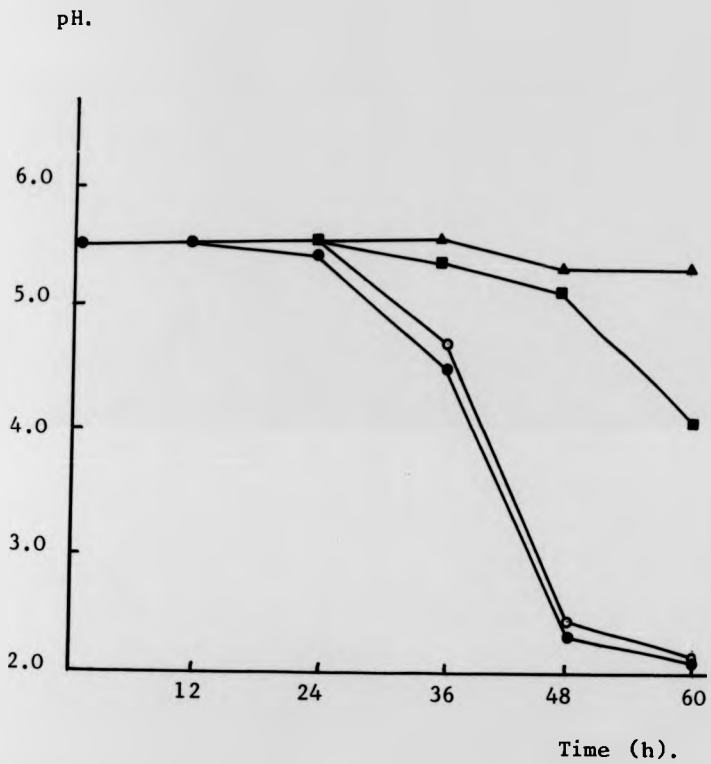



*Figure* 5.3.2.1.

Influence of MES concentration on the buffering of medium pH during growth of *P. spinulosum* in GMS medium.

●, 50.0 mM MES

o, 100.0 mM MES

■, 250.0 mM MES

▲, 500.0 mM MES

Glucose utilisation and biomass production by *P. spinulosum* from initial glucose levels of 5 and 10 mg $ml^{-1}$ in the presence of 50.0 mM MES

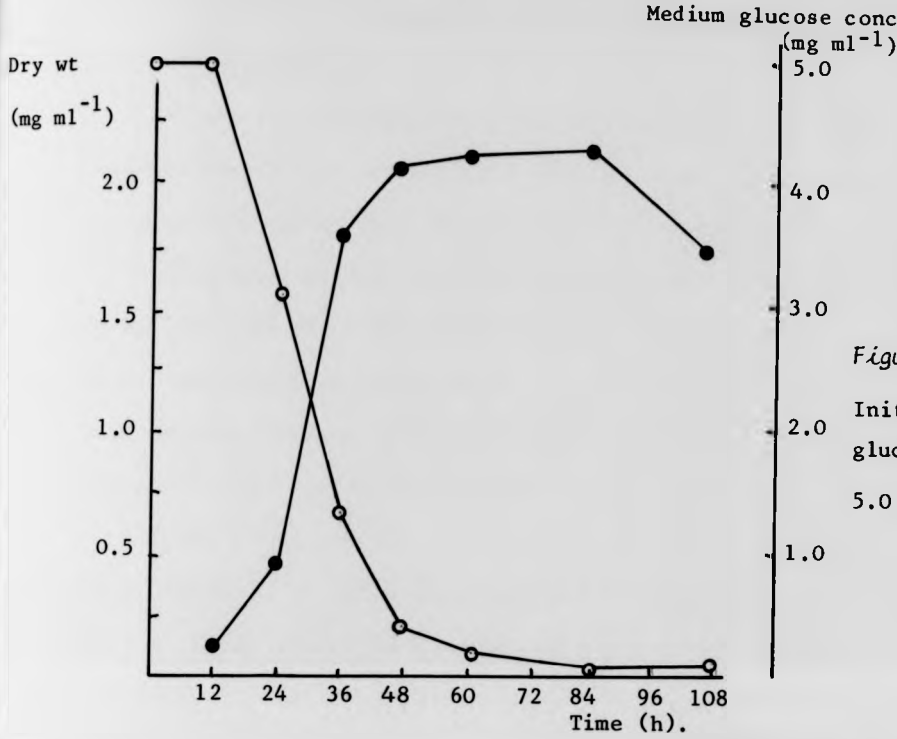



*Figure* 5.3.2.2a Initial glucose 5.0 mg. $ml^{-1}$

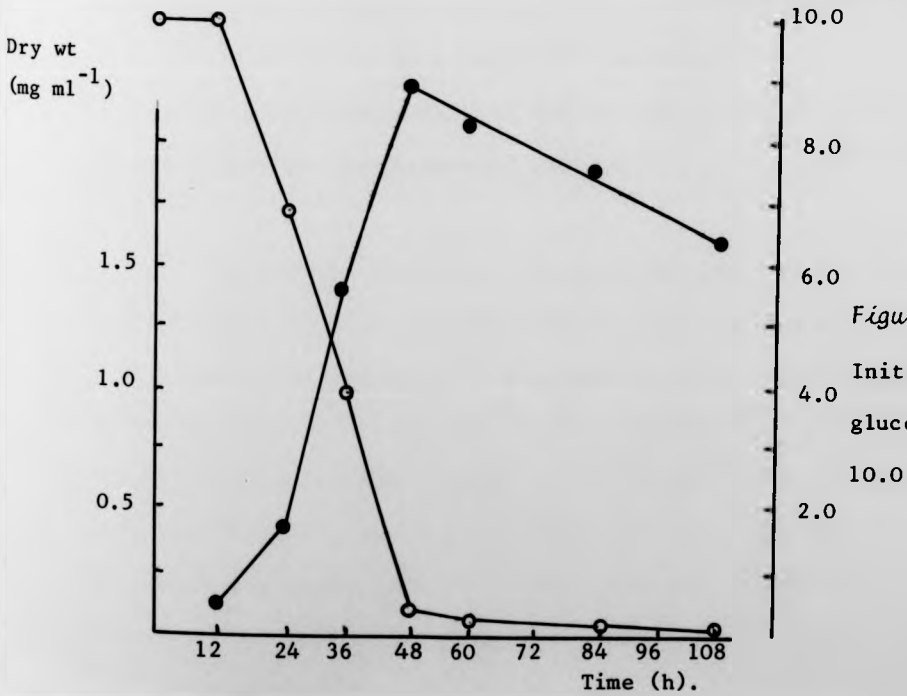



*Figure* 5.3.2.2b Initial glucose 10.0 mg $ml^{-1}$

●, Biomass production (mg $ml^{-1}$); ○, Medium glucose content (mg $ml^{-1}$).

## 6. METAL ACCUMULATION IN ACTIVELY GROWING FUNGI.

### 6.1. Introduction.

The relationship between metal transport and fungal growth has received very little attention. Jennings (1976) considered the relationship between the rates of uptake or efflux which are measured in resting mycelium and those which are present in growing mycelium, to be initially the most important question. The problem is that flux measurements have to be performed on non-growing mycelium since the measurements of metal concentrations are difficult to relate to a dynamic system. In fact most metal uptake studies are carried out on resting mycelium, usually on resting exponential phase material, but it is difficult to say for certain that comparisons of metal uptake at this phase, between different organisms, is necessarily an accurate representation of the true situation for metal uptake for the whole of the growth cycle. Also there appears to have been no comparative study of uptake of metals in a large number of filamentous fungi or of a variety of metals at a number of concentrations. Thus, a coherent body of information has not been obtained under standardised conditions to enable genuine comparisons to be made.

The toxicity experiments have so far indicated a range of differential toxicities of metals and a range of tolerances of fungi to the metals. It may well be that there is an intimate relationship between metal toxicity and the quantitative amount of metal accumulated, and this can best be investigated by performing a comparative study to determine whether or not a relationship is consistent for all fungi. The use of growing cells is more applicable than non-growing cells in this respect, since it is easier to estimate the effects of toxic metals on growing cells than on non-growing cells by observing the

effects of metals on fungal growth. It is unlikely that resistance would be conferred on all fungi by virtue of a reduction in metal uptake, but it is certainly a possible mechanism for tolerance of high concentrations of metal. This type of resistance has been reported by Chopra (1971, 1975) for cadmium resistance of *Staphylococcus aureus* and for *Penicillium* and *Aspergillus sp.* which can survive in saturated copper sulphate (Basu *et al*, 1955; Starkey and Waksman, 1943).

The importance of studying metal uptake in growing organisms has been reported by a limited number of workers. Copper uptake has been studied in *Escherichia coli* by Baldry and Dean (1980b) and the authors showed that the stage of growth had a profound effect on metal uptake. One report has appeared in the literature for metal uptake in a growing yeast culture. Zinc accumulation in *Candida utilis* has been studied by Failla and Weinberg (1977) and the authors reported that uptake was cyclic in terms of the growth cycle. Metal uptake in *Euglena sp.* occurred specifically in lag-phase (Kempner and Miller, 1972). Gadd and Griffiths (1978b) reported that copper uptake in the polymorphic fungus *Aureobasidium pullulans* peaked in lag-phase and declined in exponential phase.

There is a limited number of reports for metal uptake in growing filamentous fungi. Copper uptake has been studied in *P. ochrochloron* (Stokes and Lindsay, 1979), and in *Dactylium dendroides* (Shatzman and Kosman, 1978). Magnesium uptake has been studied in *Neurospora crassa* (Viotti *et al*, 1971). All the authors reported that uptake was influenced in some way by the stage of growth of the fungus under investigation. Venkateswerlu and Sivarama Sastry (1970) have shown energy dependent cobalt uptake in growing suspensions of

*Neurospora crassa* over a 2 hour incubation period. The authors however chose to perform the experiments at toxic concentrations of the metal.

The primary objective of this section of the study was to carry out a comparative study of copper, cadmium and zinc accumulation in *P. spinulosum*, *T. viride* and *A. niger* at a range of metal concentrations, throughout the growth cycle. Another aim was to investigate quantitative uptake in relation to toxicity, to determine if there was a possible trend towards metal exclusion in fungi which appeared to show tolerance. It is important to note however, that organisms were not isolated specifically for their resistance to metals.

## 6.2. Materials and Methods.

Copper, cadmium and zinc accumulation in actively growing cultures of *P. spinulosum*, *T. viride* and *A. niger* was monitored by incorporating metal chloride salts into the GMS growth medium prior to inoculation. Metal uptake was measured during the growth of the organisms tested.

### 6.2.1. Experimental procedure for metal accumulation study.

For each experimental run of one metal at a single concentration with one fungus, batches of 72 replicate Ehrlenmyer 250 ml flasks containing 100 ml GMS medium at pH 5.5 were used for the growth of the organisms. Sixty of these experimental flasks were supplemented with appropriate volumes of filter sterilised metal chloride salt prepared in 1 M HCl to obtain final concentrations of 1.0, 2.5, 5.0, 7.5, 10.0 and 15.0 µg $ml^{-1}$ copper and cadmium, and 1.0, 5.0, 10.0, 20.0, 35.0 and 50.0 µg $ml^{-1}$ zinc. The volume of metal chloride added was 0.1 ml to prevent excessive volume changes in the medium. MES buffer was employed throughout these experiments at a concentration of 50.0 mM. The metal concentrations chosen for investigation were either non-toxic (zinc) or toxic but non-lethal (copper and cadmium) as previously demonstrated in toxicity tests in liquid and solid GMS medium. Zinc was used at the higher concentrations because it was relatively non-toxic.

The remaining 12 flasks served as control flasks and had no metal addition. All flasks were equilibriated to 25°C before they were inoculated. The flasks were inoculated simultaneously using 1.0 ml of a fungal fragment suspension prepared from an actively growing culture which had been subcultured twice. Incubation was at 25°C with shaking at 200 rpm in an orbital shaker.

By sampling periodically throughout the growth of the cultures for all the fungi, metals and metal concentrations tested, and monitoring dry weight increases in controls and in metal supplemented cultures, an estimation of the effect of metal type and concentration on growth could be established whilst simultaneously quantitatively determining the amount of metal bound to the fungus or accumulated at specific phases of growth. The experiment was performed for all three metals at the specified concentrations with the three fungi *P. spinulosum*, *T. viride* and *A. niger*.

6.2.2. Sampling procedure.

At 12 hour intervals throughout the growth cycle of the fungi, a minimum of 5 replicate cultures were randomly selected from the 60 experimental flasks and 2 from the control flask series. It is worthy of note here, that one sample constituted a whole culture and 5 replicate samples were therefore 5 individual but identically prepared cultures. The biomass was separated from the growth medium by filtration onto preweighed Millipore filters when the fungal density was low, or by centrifugation at 4000 rpm when the density was high. Two, 100 ml washings with distilled $H_2O$ were carried out for each sample. Centrifuged fungal material was placed in preweighed acid washed 100 ml conical flasks. Each biomass sample was then dried at 105°C for 18 hours and reweighed for dry weight determinations.

Initial samples at time 0 hours actually represented samples taken after 10 minutes after inoculation. Each sample for 0 hour sampling was staggered by 4 minutes at the inoculation procedure to allow time for filter sampling. The spent medium from the samples was also retained for analysis for metal content and for pH monitoring.

This procedure was partly precautionary in that spent medium metal and the metal content of the complementary fungal sample was used to ensure that metal removal from the system was due to accumulation by the fungus only, and not lost in other ways such adsorption to glassware. These measurements also confirmed that the effective concentration of the added metal did not increase due to evaporation of water from the medium during incubation. In addition to this, spent medium metal analysis provided a direct method of measuring metal removal from the growth medium and was therefore a useful indicator of metal removal and possible consequential metal limitation. This was particularly important at the low metal concentrations with effective metal accumulating fungi (e.g. 1.0 μg $ml^{-1}$ $Cu^{++}$ and *P. spinulosum*). By taking pH measurements information was provided about buffer effectiveness and possible reductions in metal availability during experiments due to pH alterations during growth.

6.2.3. Sample digestion and metal analysis.

The sample digestion method used was the $H_2SO_4/H_2O_2$ technique as described in procedure B of the Analytical Methods Committee (1967). Sample digestion was as described in section 5.1.3. (Page 69).

After digesting, samples were assayed immediately for total metal content by atomic absorption spectrophotometry (AAS) on a Perkin Elmer Atomic Absorption Spectrophotometer model 280 using 4% $H_2SO_4$ as a blank solution. Standard metal solutions were prepared in 4% $H_2SO_4$ in all cases. Copper and cadmium weredetermined in the range 0-2.0 μg $ml^{-1}$, and zinc in the range 0-1.0 μg $ml^{-1}$ since in these ranges the relationship between metal concentration and absorbance is linear.

The spent medium samples which had been stored at $2^{o}C$ were assayed directly by AAS using GMS medium as a blank. This blank GMS medium was normal GMS medium but without the added complement of the trace element solution. It is perhaps worthy of note here that the contribution of the medium trace metal solution to the total copper and zinc contents in flasks with added metal was very small. The contribution of copper from the added trace metal solution was not measureable on the AAS but a concentration of 0.17 $\mu g\ ml^{-1}\ Zn^{++}$ was detectable in control flasks due to the zinc content of the medium. These were regarded as negligible in comparison to the concentrations of added metal. There was no cadmium present in the trace element solution.

6.2.4. Influence of MES buffer on metal accumulation in actively growing fungi.

The influence of 50.0 mM MES on metal accumulation in growing fungi was determined by comparing uptake in buffered and unbuffered systems. Thirty replicate flasks each containing 100 ml GMS medium were prepared. A further batch of 30 flasks containing 100 ml GMS medium minus the MES buffer were prepared. Each set of flasks was adjusted to pH 5.5 before the addition of 0.1 ml of $10^{5}\ \mu g\ ml^{-1}\ Cd^{++}$ dissolved in 1 M HCl to produce a final concentration of 100.0 $\mu g\ ml^{-1}\ Cd^{++}$. The buffered flasks remained at pH 5.5 whilst in the unbuffered flasks the pH fell to 3.2 on addition of the metal. All the flasks were then inoculated with *T. viride* in the standard way and incubated under standard conditions. Three replicate flasks were periodically removed from the shaker upto 144 hours of incubation and the biomass collected by filtration or centrifugation and then washed in distilled $H_2O$. Dry weights of the samples were obtained. Metal analysis was performed to determine the cadmium contents of the mycelium and the pH was monitored of the spent medium immediately after sampling.

## 6.3. Results.

### 6.3.1. Metal accumulation, dry weight and pH profiles during growth of fungi.

Figures 6.3.1.-45 show similar patterns for copper, cadmium and zinc accumulation for all the concentrations tested with *P. spinulosum*, *T. viride* and *A. niger*. The reduction in pH of the GMS medium during growth of the fungi was directly proportional to the density of biomass produced. The drop in pH to 2.0 was reproducible for all three fungi by the mid-late linear phase of growth.

Accumulation of copper, cadmium and zinc at all the concentrations tested with the three fungi appeared to be maximal during the lag phase of growth. The pH at this time was maintained at 5.5. Following the high initial metal content there was an apparent dilution of metal on a per cell basis during the linear phase of growth which was initially very rapid as linear growth proceeded suggesting that immediately after lag phase metal uptake was much slower or had ceased. Although there was a reduction in pH to about 2.0 during mid-late linear phase, the rapid fall in metal content occurred before this, during the end of the lag phase and early linear phase. Therefore, the reduction in pH was unlikely to be a significant factor in this metal reduction phenomenon since the pH was normally above 5.0 at the time it occurred.

At very low metal concentrations it is possible that there was total removal of metal from the system and that this could account for the rapid reduction in metal content of the mycelium as growth proceeded. This is possible in the case of *P. spinulosum* and copper addition at the low concentrations due to the effective copper-removing

capability of the fungus (about 85% copper removal from 1.0 µg $ml^{-1}$ added copper). Cadmium and zinc removal on the other hand by the three fungi was negligible in most cases, but the reduction phenomenon was demonstrated in all cases suggesting that the reduction was not a result of metal limitation by depletion from the medium as a consequence of fungal accumulation.

Key for figures 6.3.1 to 6.3.45.

● , mycelial metal content (µg $g^{-1}$ dry weight).

▲ , pH.

■ , dry weight (mg $ml^{-1}$).

Copper uptake from 1.0 µg $ml^{-1}$ $Cu^{++}$ amended in GMS medium by *P. spinulosum*, *T. viride* & *A. niger*.

*Figures 6.3.1.-3*

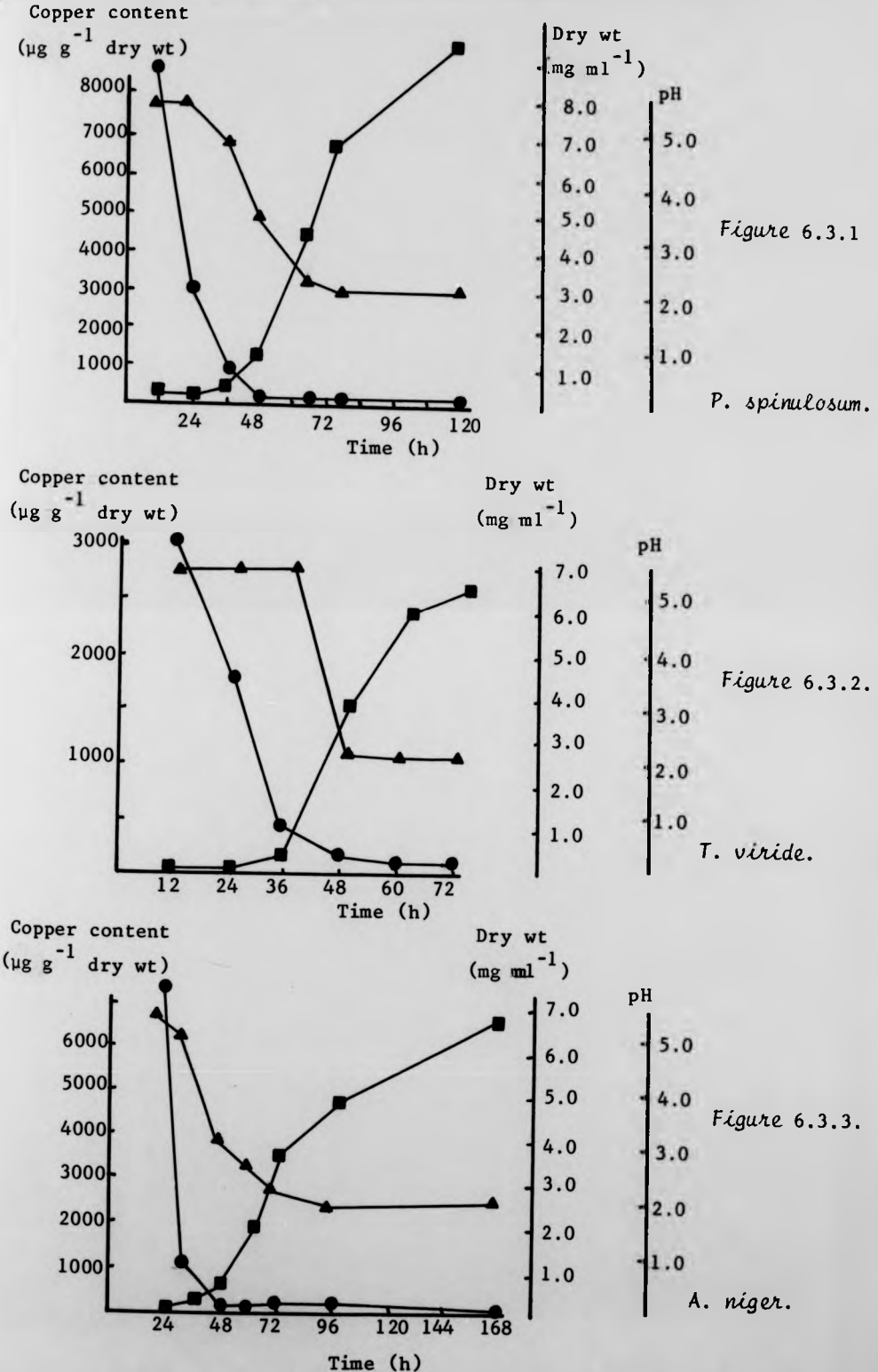



*Figure 6.3.1* *P. spinulosum.*

*Figure 6.3.2.* *T. viride.*

*Figure 6.3.3.* *A. niger.*

●, mycelial metal content(µg $g^{-1}$); ▲, pH; ■, dry weight (mg $ml^{-1}$).

Figures 6.3.4-6

Copper uptake from 2.5 µg $ml^{-1}$ $Cu^{++}$ amended in GMS medium by *P. spinulosum*, *T. viride* & *A. niger*.

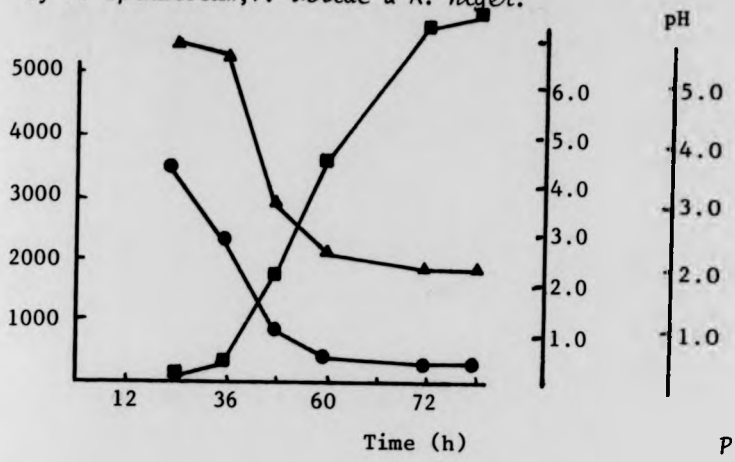



*Figure 6.3.4.*

*P. spinulosum*

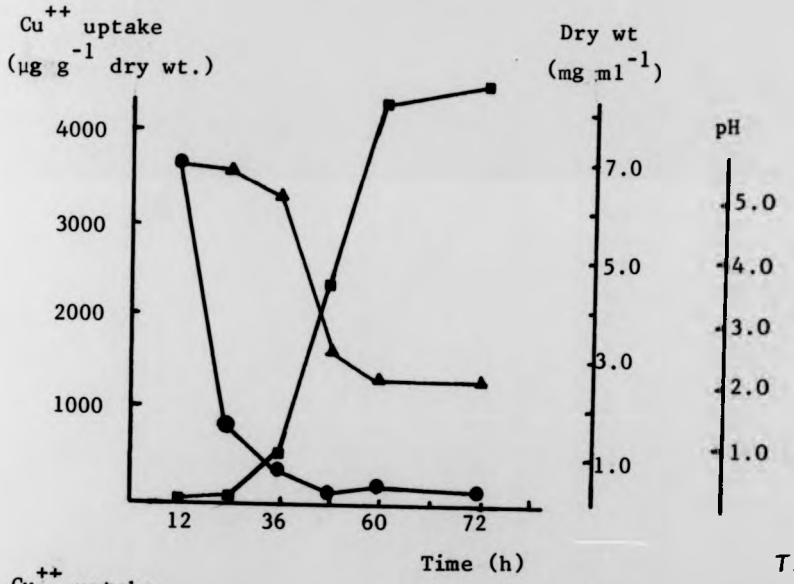



*Figure 6.3.5.*

*T. viride*

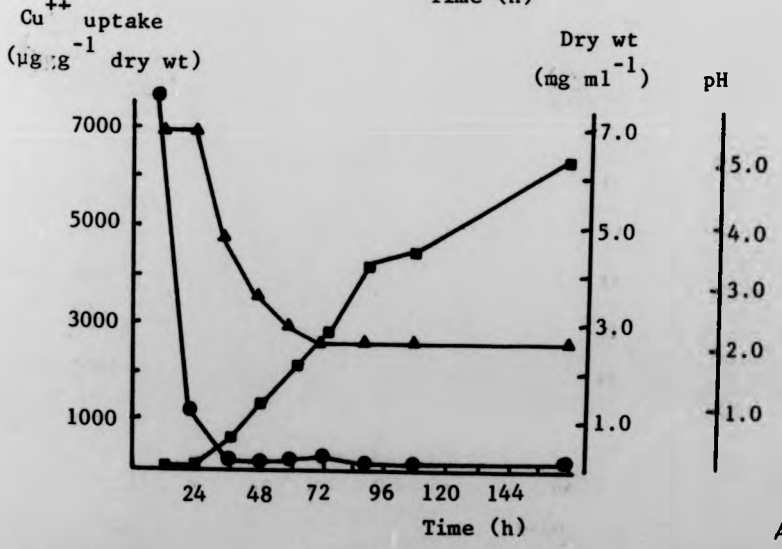



*Figure 6.3.6.*

*A. niger*

Copper uptake from 5.0 µg $ml^{-1}$ $Cu^{++}$ amended in GMS medium by *P. spinulosum*, *T. viride* & *A. niger*.

Figures 6.3.7-9

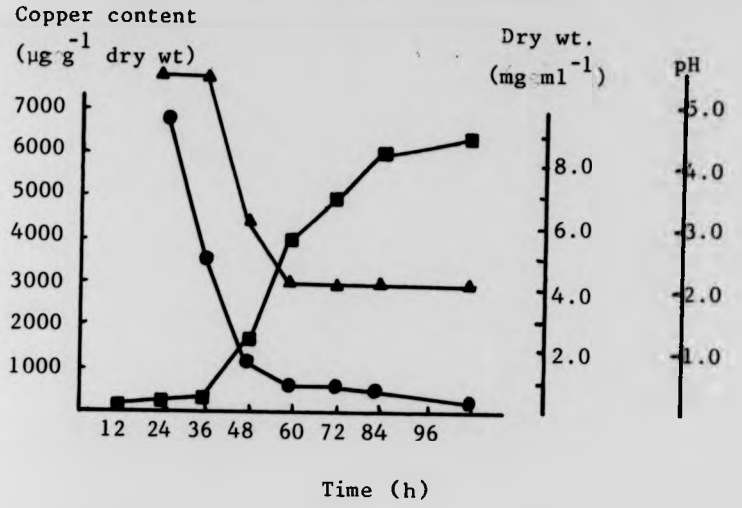



*Figure* 6.3.7.

*P. spinulosum.*

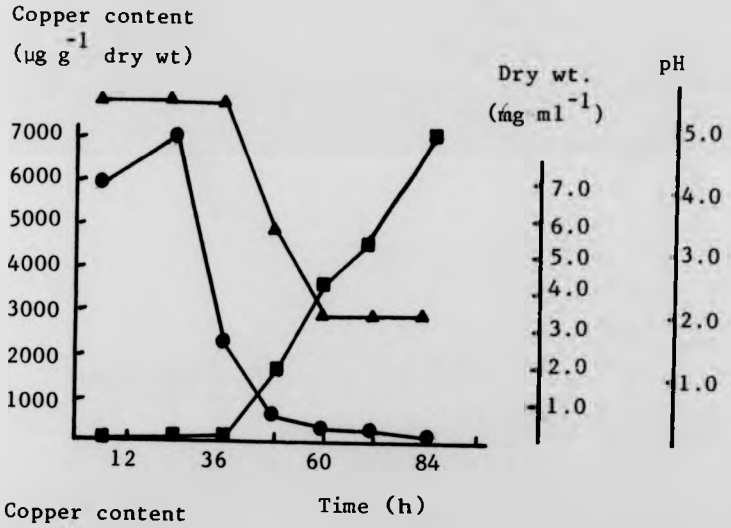



*Figure* 6.3.8.

*T. viride.*

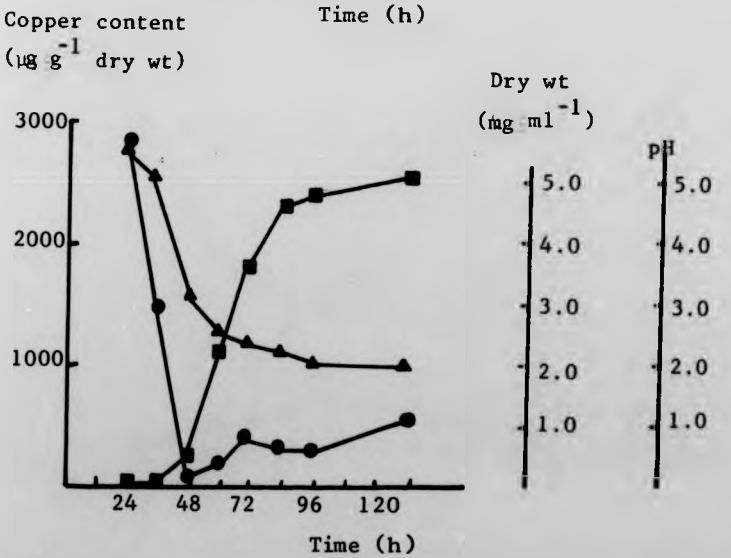



*Figure* 6.3.9.

*A. niger.*

Copper uptake from 7.5 µg $ml^{-1}$ $Cu^{++}$ amended in GMS medium by *P. spinulosum*, *T. viride* & *A. niger*.

Figures 6.3.10-12

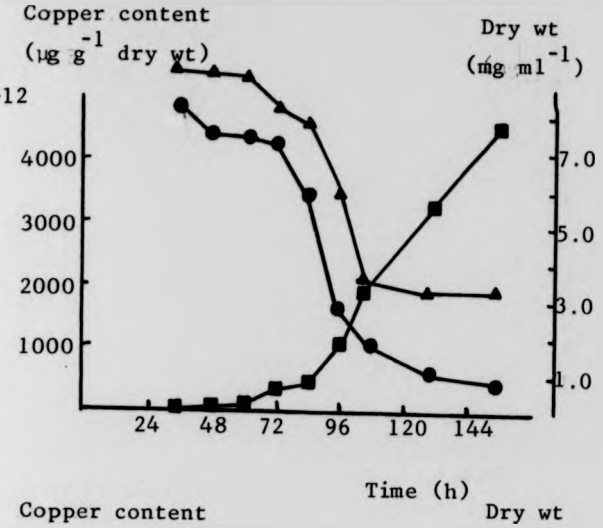



Figure 6.3.10

*P. spinulosum.*

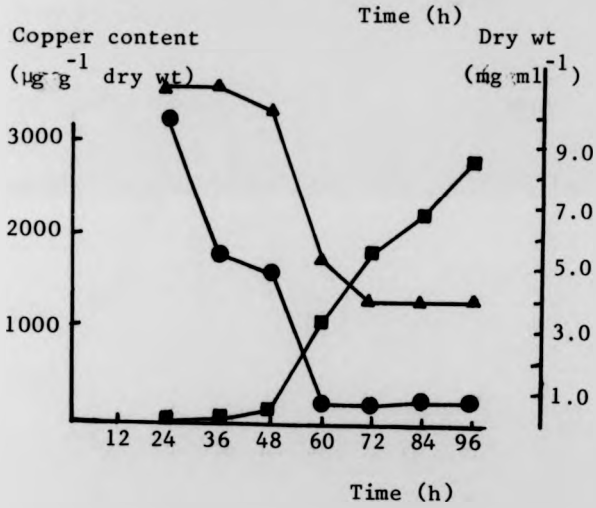



Figure 6.3.11

*T. viride.*

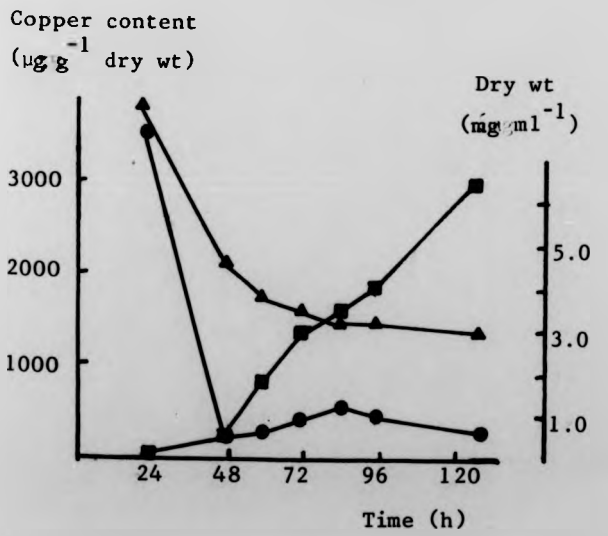



Figure 6.3.12

*A. niger.*

Copper uptake from 10.0 µg $ml^{-1}$ $Cu^{++}$ amended in GMS medium by *P. spinulosum*, *T. viride* & *A. niger*.

*Figures* 6.3.13-15

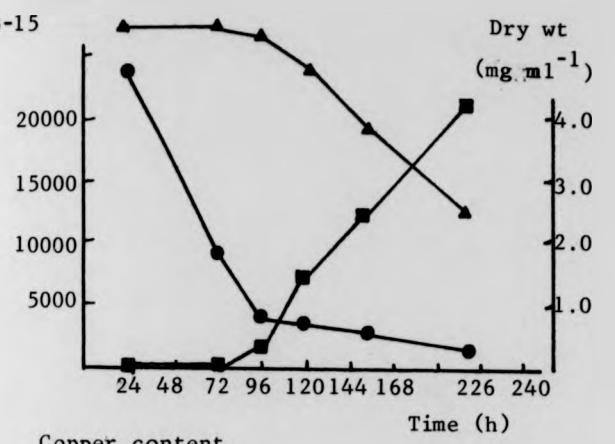



*Figure* 6.3.13

*P. spinulosum*.

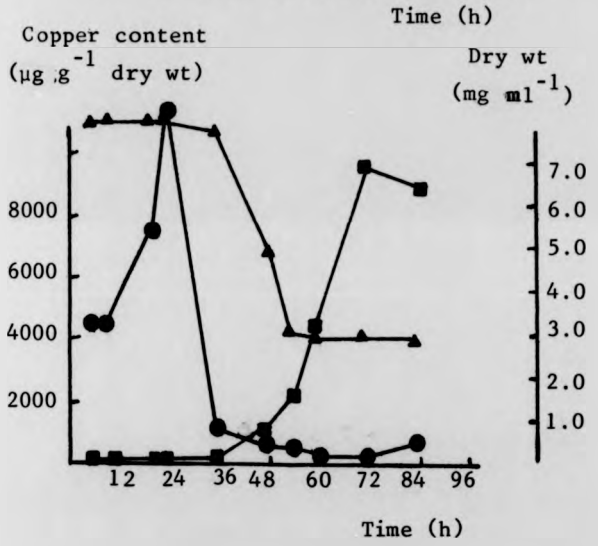



*Figure* 6.3.14

*T. viride*.

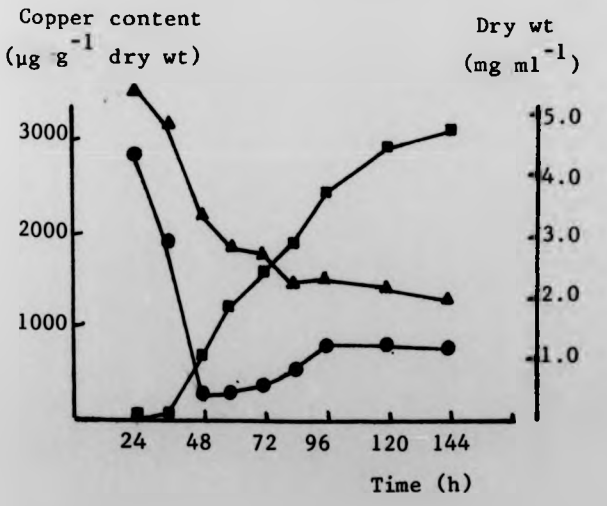



*Figure* 6.3.15

*A. niger*.

Zinc uptake from 1.0 µg $ml^{-1}$ $Zn^{++}$ amended in GMS medium by *P. spinulosum*, *T. viride* & *A. niger*.

Figure 6.3.16

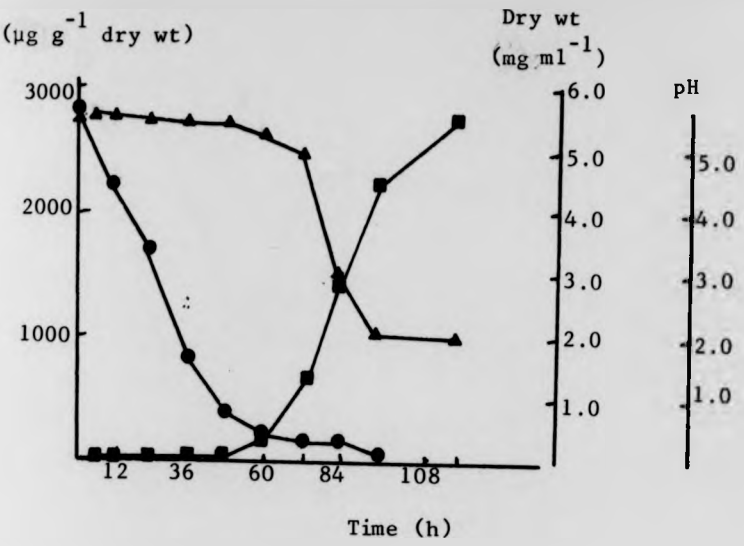



Figure 6.3.16

*P. spinulosum*.

Zinc uptake from 5.0 µg $ml^{-1}$ $Zn^{++}$ amended in GMS medium by *P. spinulosum, T. viride* & *A. niger.*

*Figures* 6.3.17-19

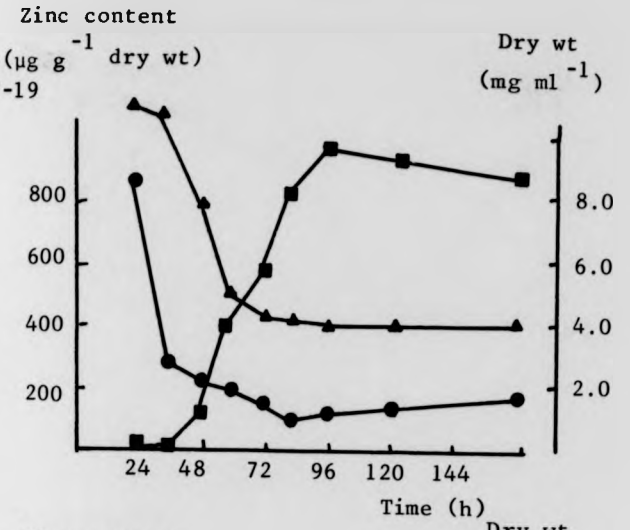



*Figure* 6.3.17

*P. spinulosum.*

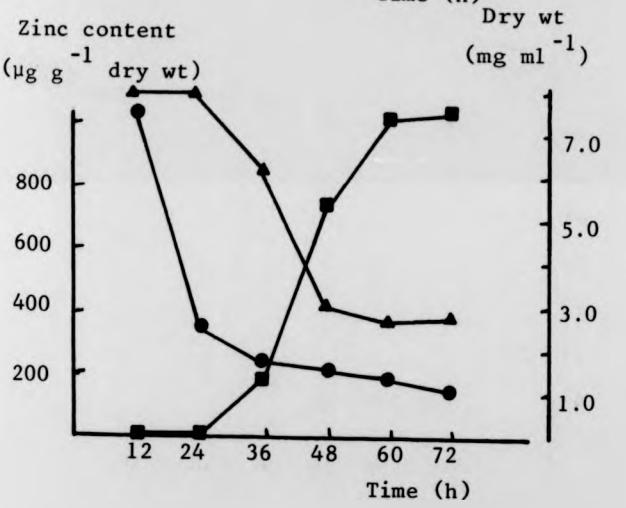



*Figure* 6.3.18

*T. viride.*

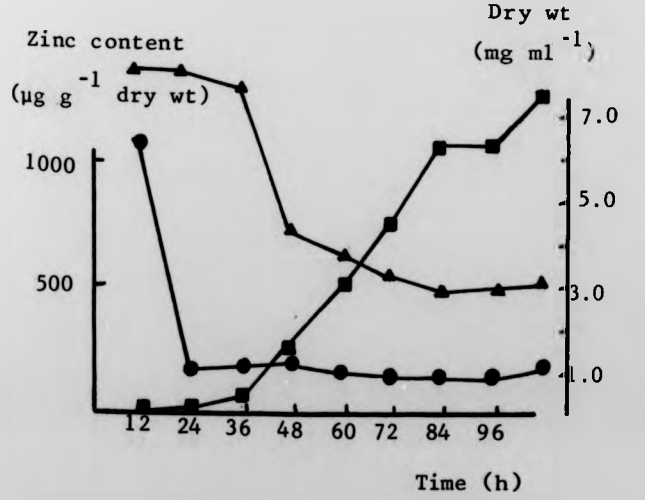



*Figure* 6.3.19

*A. niger.*

Zinc uptake from 10.0 µg $ml^{-1}$ $Zn^{++}$ amended in GMS medium by *P. spinulosum, T. viride* & *A. niger.*

*Figures* 6.3.20-22

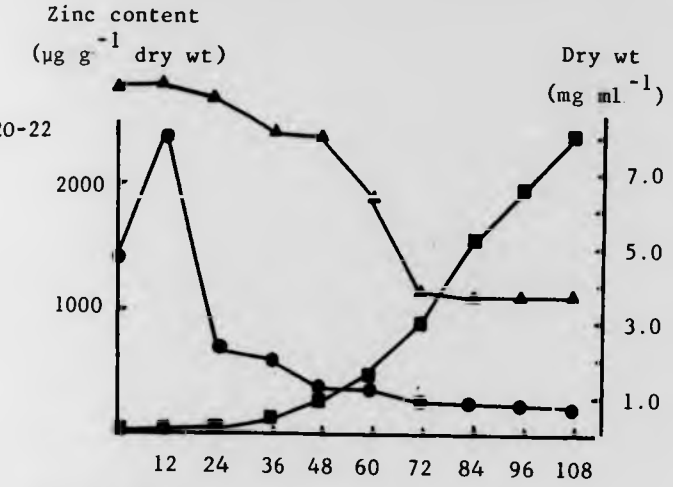



*Figure* 6.3.20

*P. spinulosum.*

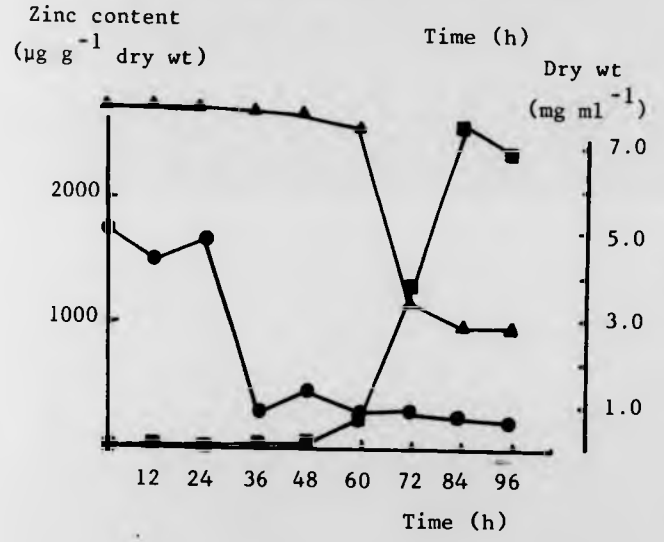



*Figure* 6.3.21

*T. viride.*

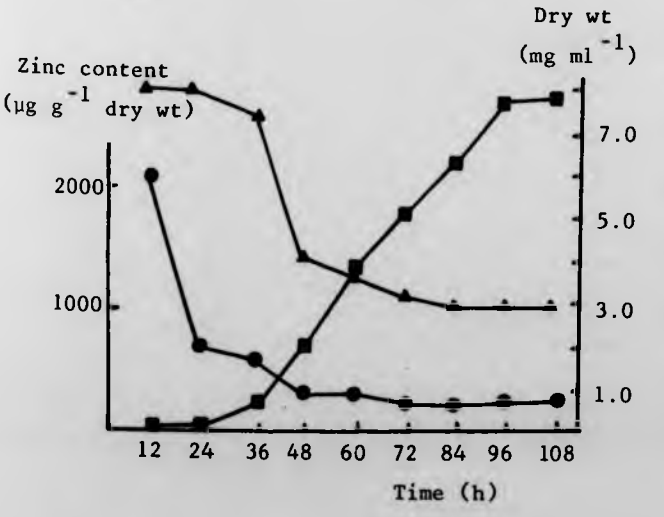



*Figure* 6.3.22

*A. niger.*

The effect of pH on the half wave potential and peak height in ASV traces. (Scans in positive direction).

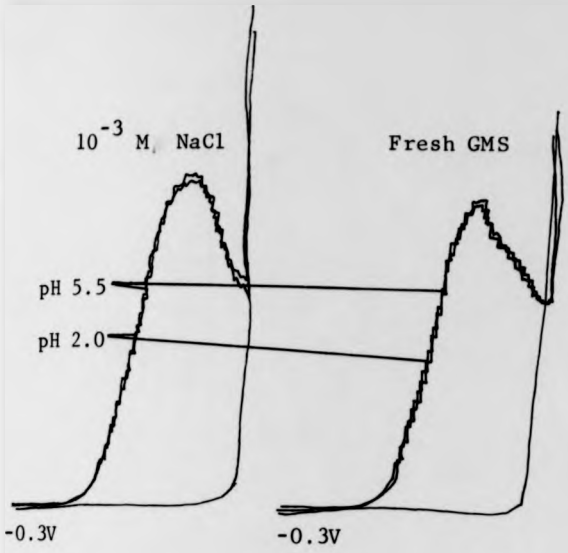



*Figure* 5.2.3.16.
ASV waves for 2.5µg $ml^{-1}$ $Cu^{++}$ in $10^{-3}$ M. NaCl and fresh GMS at pH 5.5 and 2.0. No change in parameters with pH.

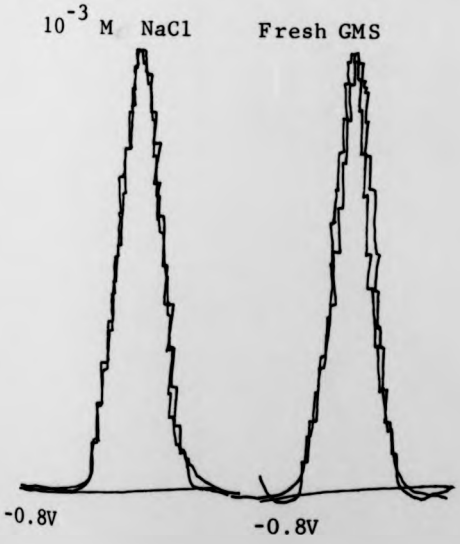



*Figure* 5.2.3.17.
ASV waves for 2.5 µg $ml^{-1}$ $Cd^{++}$ in $10^{-3}$ M NaCl and fresh GMS medium at pH 5.5 and 2.0. No change in the wave characteristics were observed with pH.

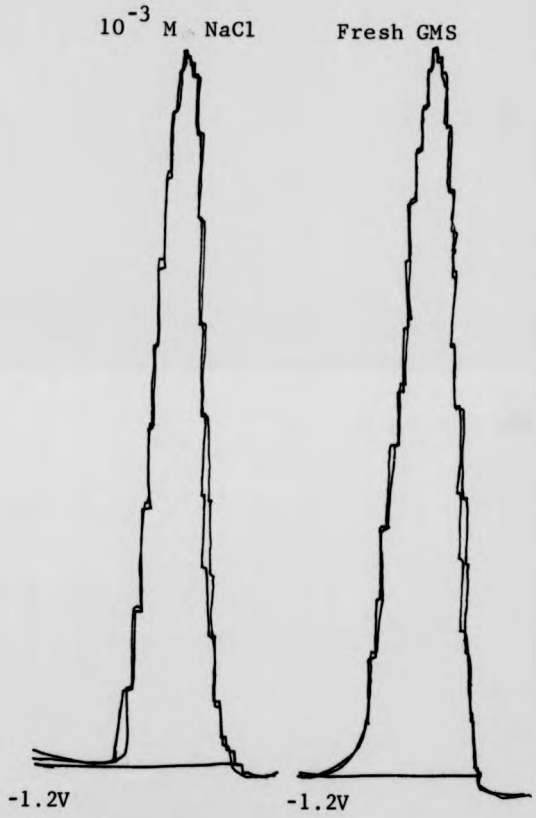



*Figure* 5.2.3.18.
ASV waves for 2.5µg $ml^{-1}$ $Zn^{++}$ in $10^{-3}$ M NaCl and fresh GMS at pH 5.5 and 2.0. There were no wave modifications with changes in pH.

Zinc uptake from 20.0 µg $ml^{-1}$ $Zn^{++}$ amended in GMS medium by *P. spinulosum*, *T. viride* & *A. niger*.

*Figures* 5.3.23-25

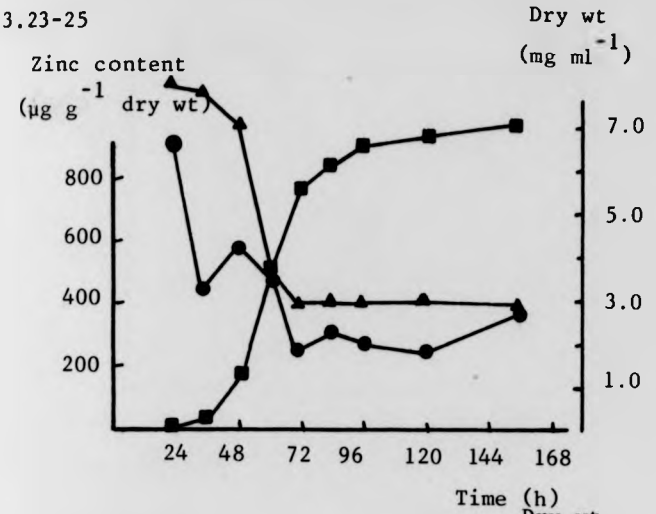



*Figure* 6.3.23

*P. spinulosum.*

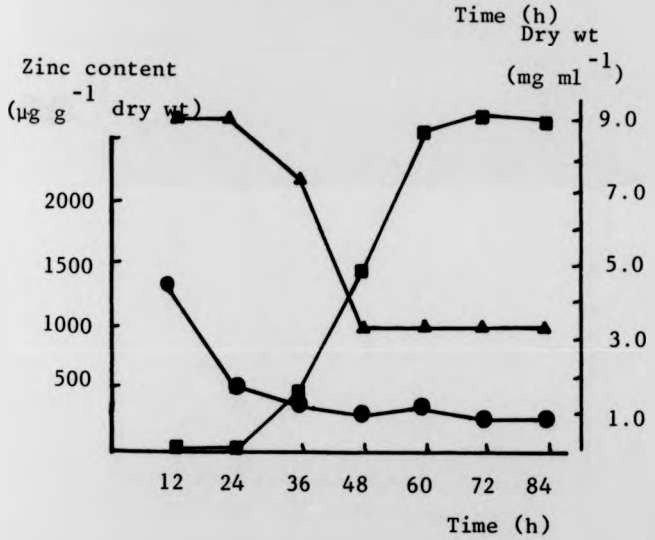



*Figure* 6.3.24

*T. viride.*

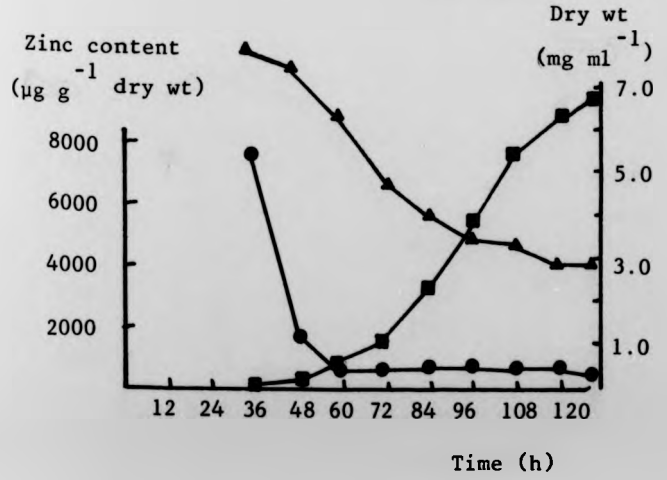



*Figure* 6.3.25

*A. niger*

Zinc uptake from 35.0 µg $ml^{-1}$ $Zn^{++}$ amended in GMS medium by *P. spinulosum*, *T. viride* & *A. niger*.

*Figures* 6.3.26-28

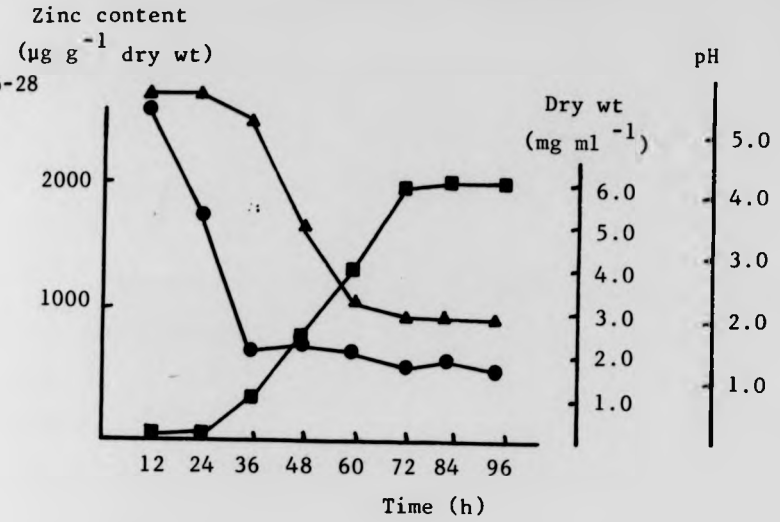



*Figure* 6.3.26

*P. spinulosum.*

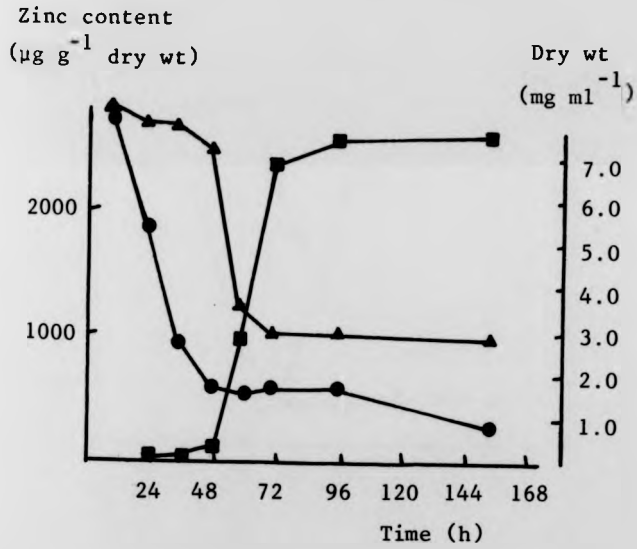



*Figure* 6.3.27

*T. viride.*

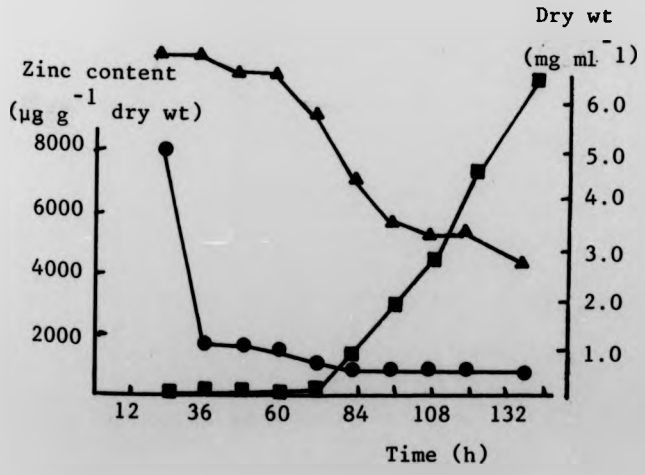
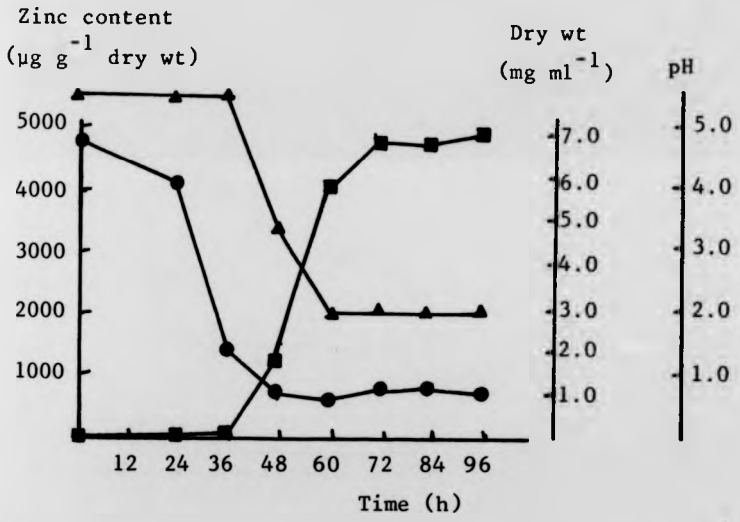



*Figure* 6.3.28

*A. niger.*

Zinc uptake from 50.0 µg $ml^{-1}$ $Zn^{++}$ amended in GMS medium by *T.viride*.

*Figure* 6.3.29




*T. viride.*

Cadmium uptake from 1.0 µg $ml^{-1}$ $Cd^{++}$ amended in GMS medium by *P. spinulosum, T. viride* & *A. niger*.

*Figures* 6.3.30-32

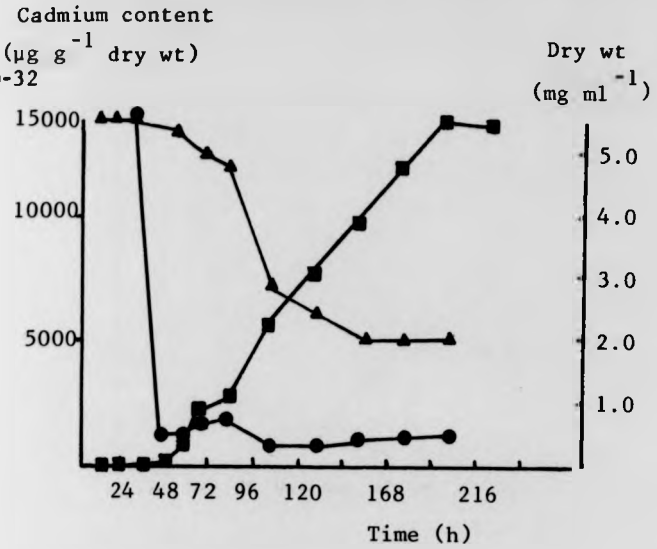



*Figure* 6.3.30

*P. spinulosum.*

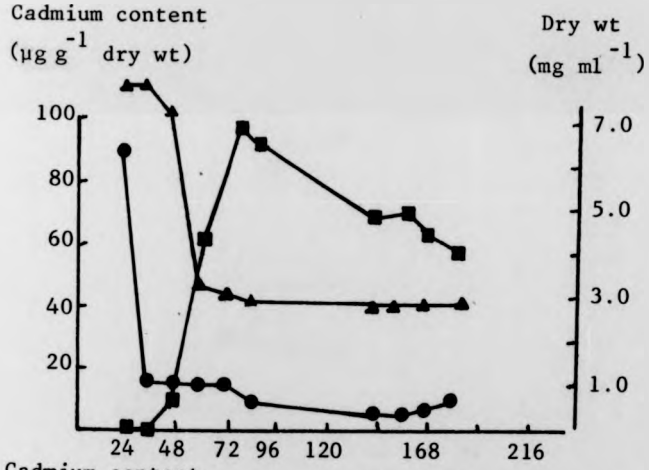



*Figure* 6.3.31

*T. viride.*

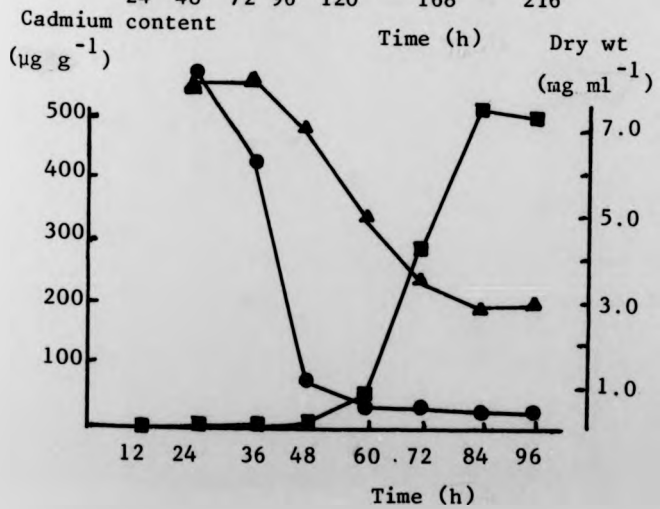



*Figure* 6.3.32

*A. niger.*

Cadmium uptake from 2.5 μg $ml^{-1}$ $Cd^{++}$ amended in GMS medium by *P. spinulosum*, *T. viride* & *A. niger*.

*Figures* 6.3.33-35

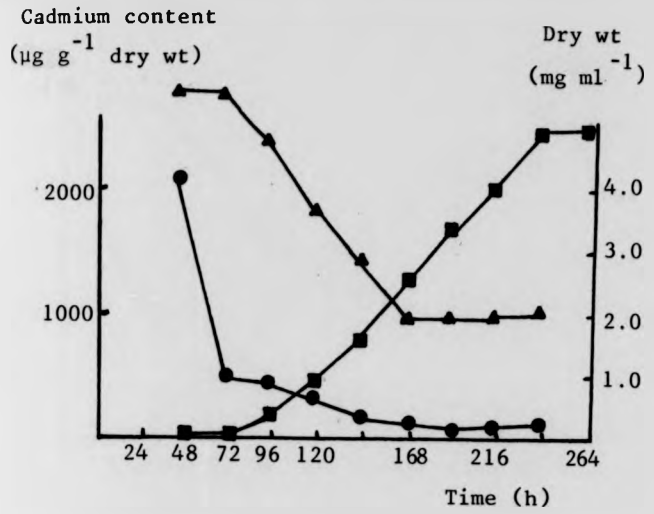



*Figure* 6.3.33

*P. spinulosum.*

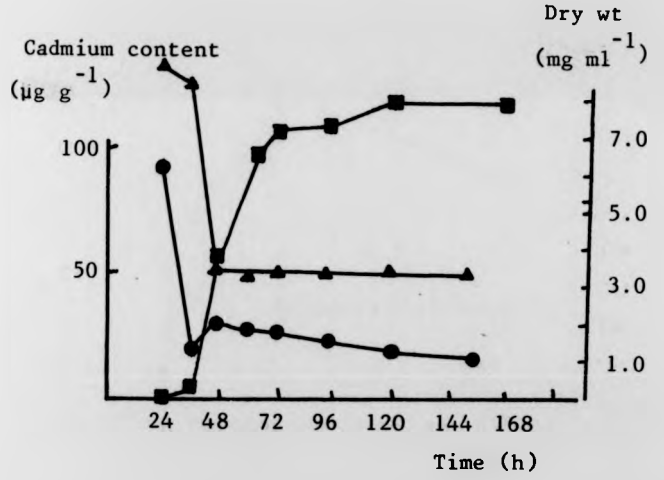



*Figure* 6.3.34

*T. viride.*

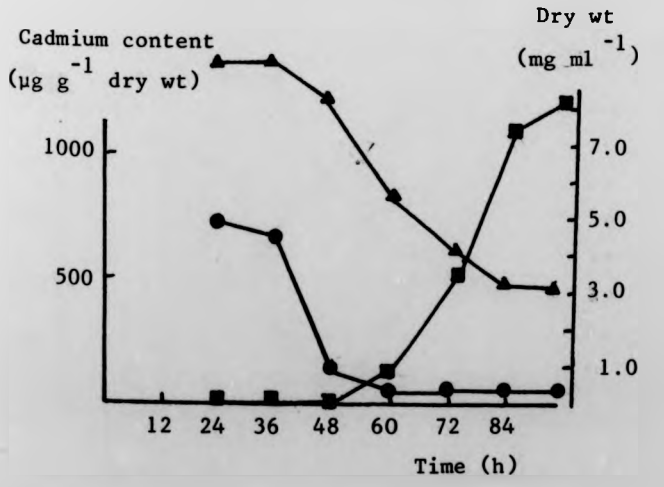



*Figure* 6.3.35

*A. niger.*

Cadmium uptake from 5.0 µg $ml^{-1}$ $Cd^{++}$ amended in GMS medium by *P. spinulosum, T. viride* & *A. niger*.

Figures 6.3.36-38

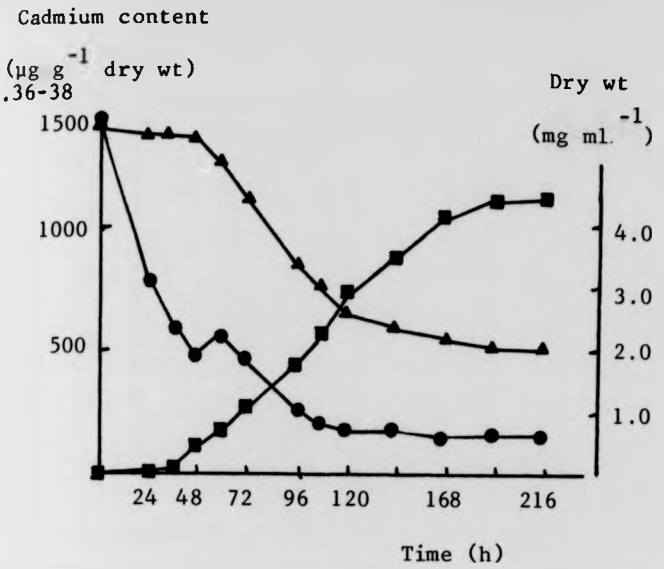



*Figure* 6.3.36

*P. spinulosum.*

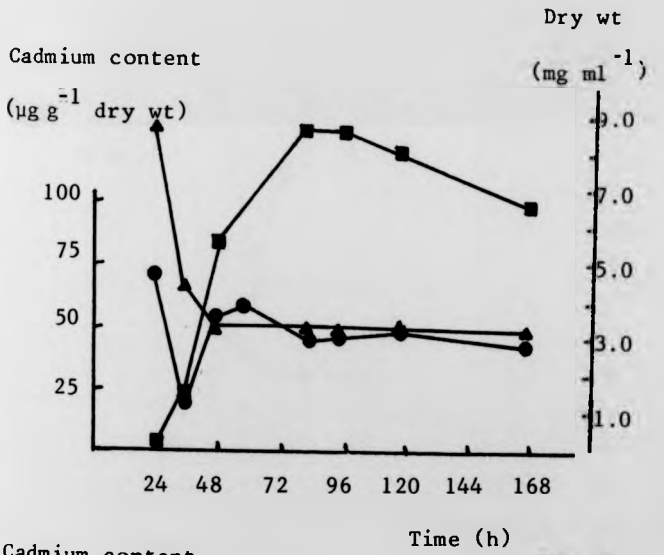



*Figure* 6.3.37

*T. viride.*

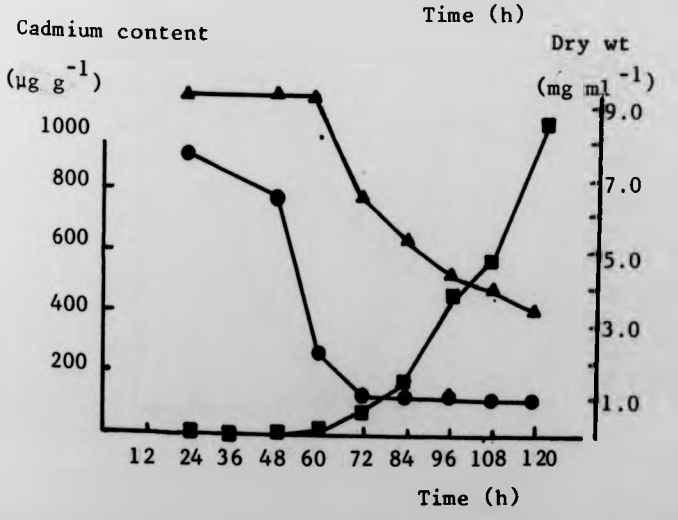



*Figure* 6.3.38

*A. niger.*

Cadmium uptake from 7.5 µg ml$^{-1}$ $Cd^{++}$ amended in GMS medium by *P. spinulosum, T. viride* & *A. niger.*

*Figures* 6.3.39-41

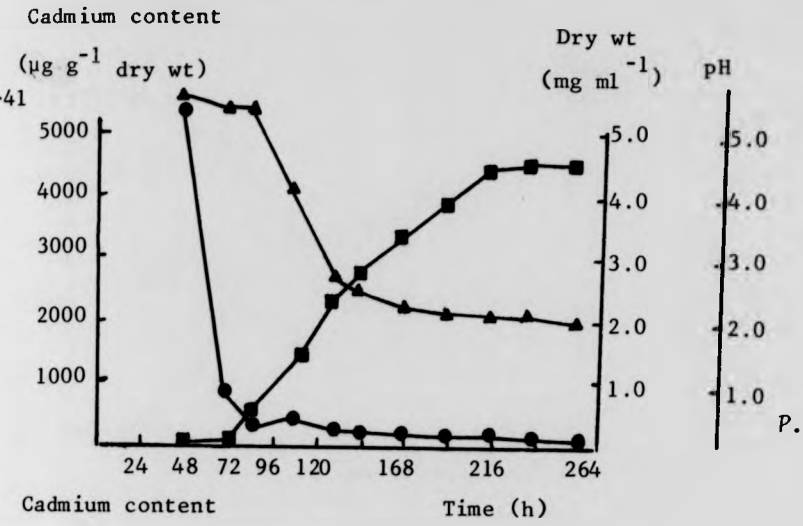



*Figure* 6.3.39

*P. spinulosum.*

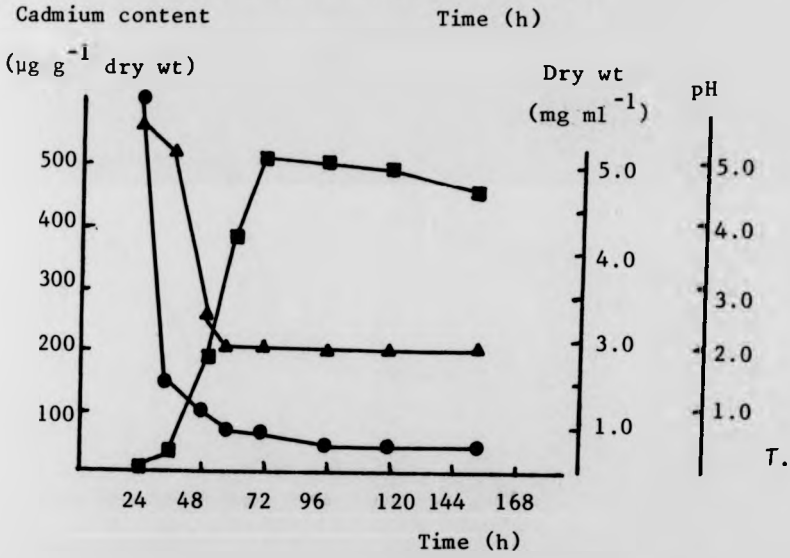



*Figure* 6.3.40

*T. viride.*

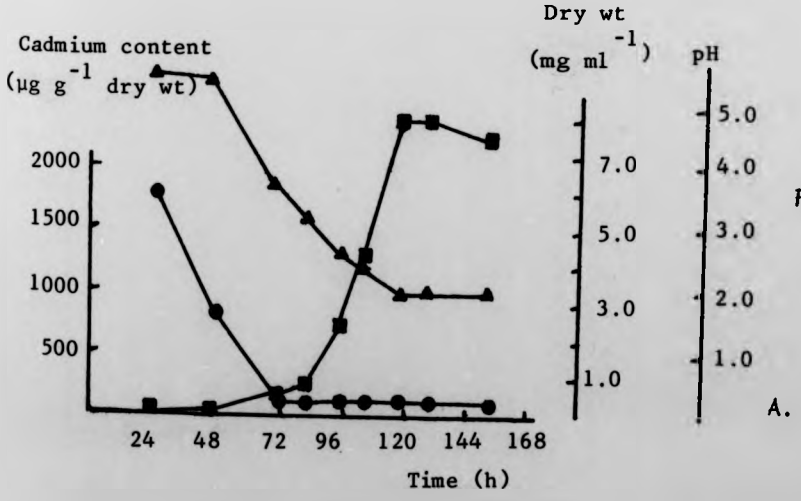



*Figure* 6.3.41

*A. niger.*

Cadmium uptake from 10.0 μg $ml^{-1}$ $Cd^{++}$ amended in GMS medium by *P. spinulosum, T. viride* & *A. niger.*

*Figures* 6.3.42-44

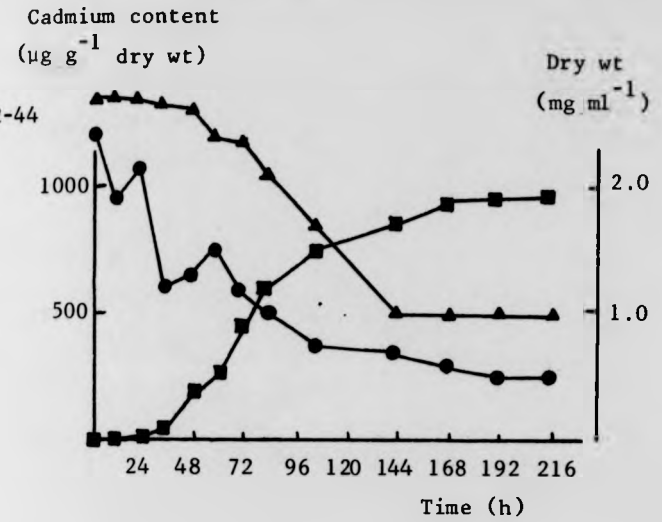



*Figure* 6.3.42

*P. spinulosum.*

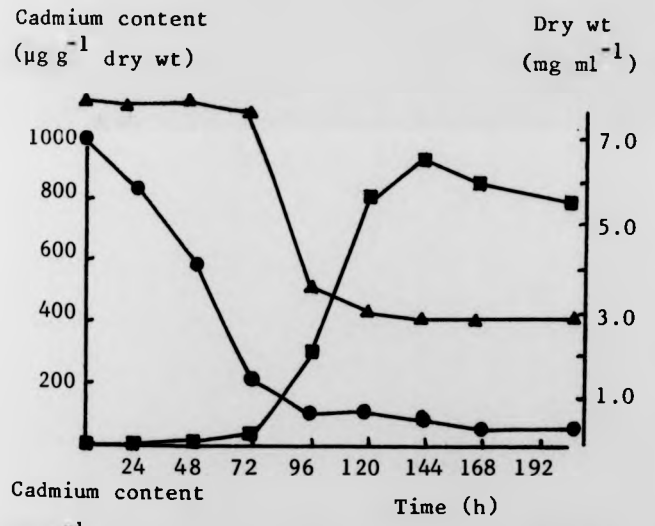



*Figure* 6.3.43

*T. viride.*

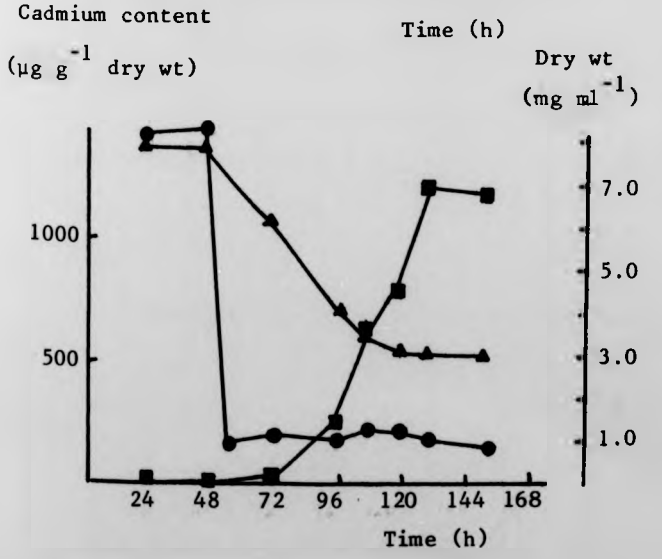
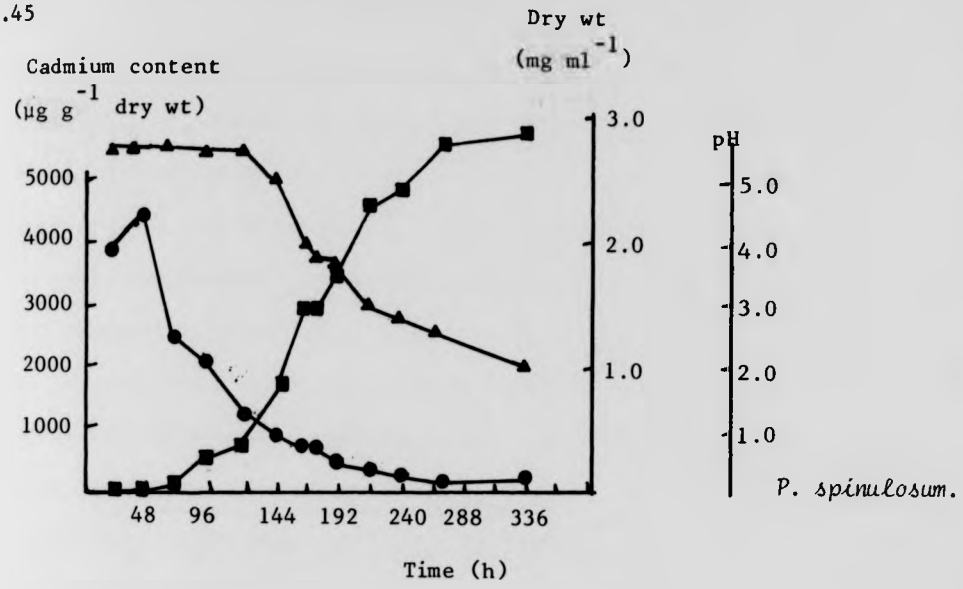



*Figure* 6.3.44

*A. niger.*

Cadmium uptake from 15.0 µg $ml^{-1}$ $Cd^{++}$ amended in GMS medium by *P. spinulosum*,

*Figure* 6.3.45



6.3.2. Mid linear phase metal contents of actively growing fungi.

Table 6.3.1. illustrates that copper accumulation appeared to be greatest in *P. spinulosum* with a mid-linear phase copper content of 2758.8 (± 177.0) µg $Cu^{++}$ $g^{-1}$ dry weight of the fungus accumulated from a GMS medium with copper added to a final concentration of 10.0 µg $ml^{-1}$. The copper contents of *P. spinulosum* at mid-linear phase were proportional to the concentration of added copper in the medium, but not directly, as shown by an appreciable rise in copper content at 7.5 and 10.0 µg $ml^{-1}$ $Cu^{++}$ and this may be related to the fact that copper started to exert toxic effects at these concentrations. Thus, copper levels may have been elevated due to cell membrane damage and subsequent binding of copper to cellular constituents.

Uptake of copper in *T. viride* was similar to that for *P. spinulosum* at 1.0 µg $ml^{-1}$ with copper contents at 151.6 (± 10.2) and 172.9 (± 10.2) µg $g^{-1}$ dry weight respectively. At copper concentrations of 2.5 µg $ml^{-1}$ and above the metal content of *T. viride* was significantly lower than in *P. spinulosum*. There appeared to be no proportionality in the amount of copper accumulated with concentration, for example, at 1.0 µg $ml^{-1}$ $Cu^{++}$ more copper was accumulated than at 2.5 µg $ml^{-1}$ (151.6 to 97.9 µg $g^{-1}$ dry weight) and at 5.0 µg $ml^{-1}$ $Cu^{++}$ more copper was accumulated than at 7.5 and 10.0 µg $ml^{-1}$ $Cu^{++}$. The amount of copper accumulated from 1.0 and 10.0 µg $ml^{-1}$ (151.6 and 201.3 µg $g^{-1}$ dry weight respectively) was similar, in contrast to the situation with *P. spinulosum*.

*Aspergillus niger* accumulated less copper from equivalent concentrations than did *P. spinulosum* at all the copper levels tested but the comparison with *T. viride* was not so simple. At 1.0 µg $ml^{-1}$ $Cu^{++}$, *A. niger* accumulated less copper than did *T. viride* but at 2.5, 7.5 and

*Table* 6.3.1.

Copper, cadmium and zinc contents of *P. spinulosum*, *T. viride* and *A. niger* when grown in GMS medium with added metal, at mid-linear phase of growth.

| Metal | Metal concentration ($\mu g\ ml^{-1}$) | Mycelial metal content in mid-linear phase ($\mu g\ g^{-1}$ dry wt). | | |
|---|---|---|---|---|
| Copper | 1.0 | 172.9 (±10.2) | 151.6 (±10.2) | 82.2 (±2.1) |
| | 2.5 | 470.8 (±22.1) | 97.9 (±3.3) | 151.0 (±9.1) |
| | 5.0 | 527.6 (±19.1) | 329.6 (±9.2) | 181.0 (±18.1) |
| | 7.5 | 1123.0 (±88.5) | 219.6 (±19.6) | 421.8 (±44.3) |
| | 10.0 | 2758.8 (±177.0) | 201.3 (±14.1) | 360.6 (±28.8) |
| Zinc | 1.0 | 143.2 (±4.5) | ——— | ——— |
| | 5.0 | 199.7 (±12.5) | 216.5 (±8.7) | 142.4 (±2.7) |
| | 10.0 | 266.0 (±12.3) | 310.9 (±11.4) | 207.6 (±3.6) |
| | 20.0 | 521.8 (±19.3) | 275.1 (±10.4) | 452.6 (±13.8) |
| | 35.0 | 756.8 (±19.7) | 552.0 (±22.0) | 808.9 (±13.3) |
| | 50.0 | ——— | 713.3 (±59.8) | ——— |
| Cadmium | 1.0 | 74.1 (±1.8) | 12.8 (±0.7) | 34.1 (±0.8) |
| | 2.5 | 91.9 (±2.3) | 31.4 (±2.2) | 69.3 (±1.4) |
| | 5.0 | 212.4 (±12.6) | 54.1 (±3.3) | 126.6 (±2.7) |
| | 7.5 | 230.4 (±17.7) | 100.7 (±3.7) | 156.4 (±3.0) |
| | 10.0 | 514.4 (±93.0) | 112.6 (±6.0) | 224.4 (±6.8) |
| | 15.0 | 743.1 (±133.6) | ——— | ——— |
| | | *P. spinulosum* | *T. viride* | *A. niger* |

10.0 µg $ml^{-1}$ the situation was reversed. This may have been in part due to the fact that *A. niger* showed an increasing metal content with increasing metal concentration (except at 10.0 µg $ml^{-1}$ $Cu^{++}$), whereas *T. viride* showed no clear relationship.

Zinc accumulation in *P. spinulosum* was concentration dependent with a zinc content of 143.2 (± 4.5) µg $g^{-1}$ dry weight at 1.0 µg $ml^{-1}$ $Zn^{++}$ reaching 756.8 (± 19.7) µg $g^{-1}$ dry weight at 35.0 µg $ml^{-1}$ added zinc. Even when considering the differences in atomic weights, zinc uptake appears to be lower than copper at most concentrations on a molar basis. Zinc uptake was lower in *T. viride* than in *P. spinulosum* at 20.0 and 35.0 µg $ml^{-1}$ $Zn^{++}$ but slightly greater at 5.0 and 10.0 µg $ml^{-1}$. It is interesting that at the higher concentrations, *T. viride* accumulated less zinc than *P. spinulosum*, but zinc was more toxic to *T. viride*. *Aspergillus niger* accumulated least zinc from 5.0 and 10.0 µg $ml^{-1}$ $Zn^{++}$ of the three fungi tested although the differences were small. At 20.0 and 35.0 µg $ml^{-1}$ added zinc, metal accumulation was similar in *P. spinulosum* and *A. niger*.

*Penicillium spinulosum* accumulated more cadmium than did *A. niger* and *T. viride* with *A. niger* acquiring intermediate mycelial cadmium contents. For the three fungi tested cadmium uptake was generally lower than copper and zinc even when the differences in atomic weights have been accounted for. For all three fungi, cadmium uptake was concentration dependent. Interestingly, cadmium was severly toxic to *P. spinulosum* and this fungus accumulated more cadmium than either of the other two fungi tested. *Trichoderma viride* accumulated the least cadmium and was notably tolerant of added cadmium upto 112.0 µg $ml^{-1}$ $Cd^{++}$.

6.3.3. Influence of MES buffer on metal accumulation in actively growing *Trichoderma viride*.

On metal addition (0.1 ml in 1.0 M HCl into 100 ml GMS medium) the buffered system containing 50.0 mM MES in the GMS medium remained at pH 5.5 whereas in the system with no MES buffer the pH immediately fell to 3.5. (Figures 6.3.46 and 6.3.47). Concomitant with this reduction in pH the cadmium accumulation in lag phase was reduced from 14 083.0 $\mu g\ g^{-1}$ dry weight to 4761.0 $\mu g\ g^{-1}$ dry weight. The growth in the unbuffered system was slightly quicker than in the buffered system but this was probably due to the smaller metal loading, and consequential smaller toxic effects. In both cases, pH reductions with growth were directly proportional to biomass production, dropping to 2.0 by the end of linear growth. The typical metal reductions at lag and early linear phase were observed in both systems.

The mid-linear phase cadmium contents for the buffered and unbuffered systems were 921.4 and 132.1 $\mu g\ g^{-1}$ dry weight respectively, so reducing the pH initially did seem to have an effect on subsequent metal content in mid-linear phase. However, by the end of the growth cycle these values were 502.9 and 549.9 $\mu g\ g^{-1}$ dry weight respectively. Initial pH clearly influences cadmium accumulation in shake flasks.

Cadmium uptake from 100.0 µg $ml^{-1}$ by *T. viride* in a) buffered and b) unbuffered GMS.

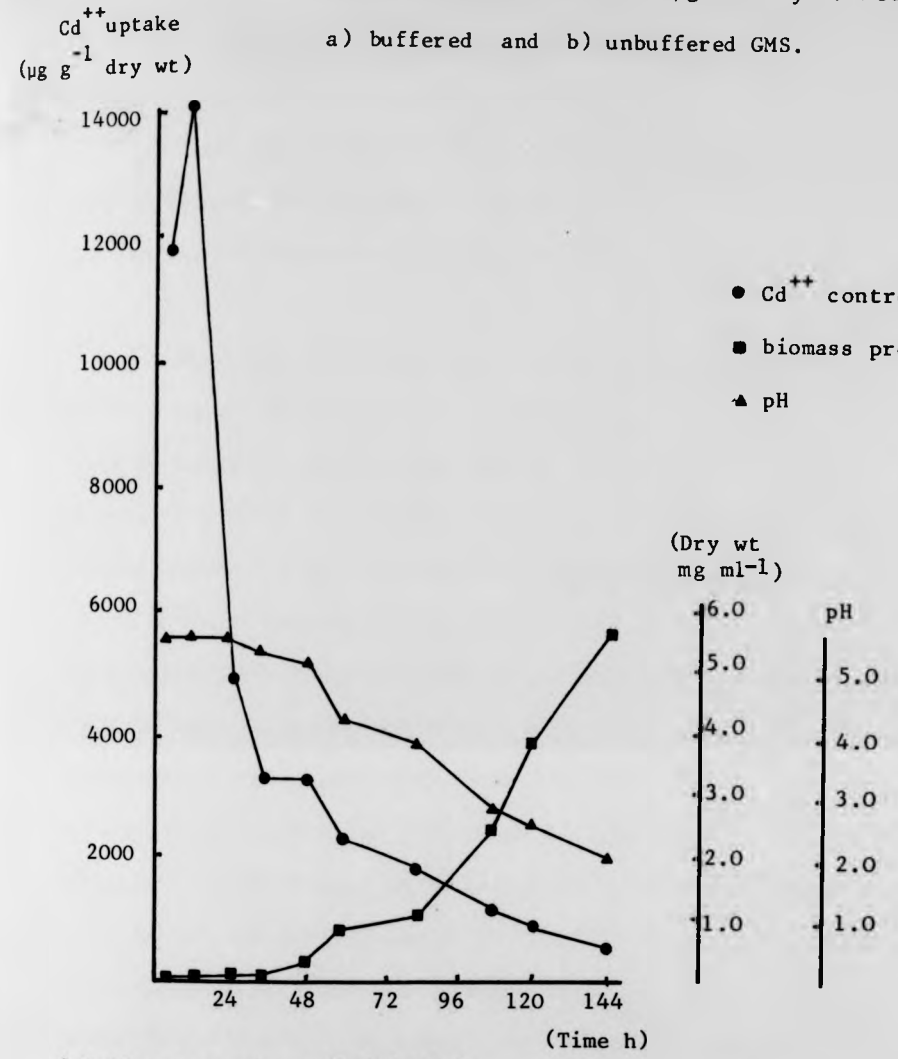



*Figure* 6.3.46

a) buffered at pH 5.5 (initially)

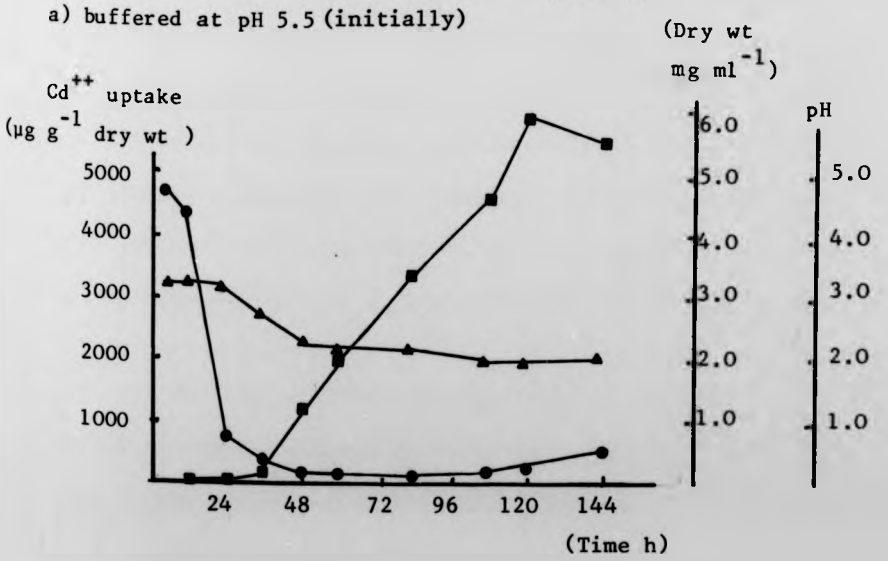



*Figure* 6.3.47

b) unbuffered

6.4. Discussion.

The metal uptake profiles throughout the growth of the three fungi indicated that accumulation was dependent on the growth stage of the fungus, and the metal type and also the exogenous metal concentration.

The range of uptake capacity of the fungi was diverse ranging from 23 212.1 μg $g^{-1}$ dry weight in *P. spinulosum* incubated with 10.0 μg $ml^{-1}$ $Cu^{++}$ after 24 hours of growth which was the highest accumulation recorded representing 2.32% dry weight of the fungus, to 5.5 μg $g^{-1}$ dry weight for cadmium accumulation in *T. viride* growing in the presence of 1.0 μg $ml^{-1}$ $Cd^{++}$ which was the lowest recorded representing 0.0055% dry weight of the fungus. These metal loading values are in agreement with the data of Baldry and Dean, (1980a) where uptake of copper as % dry weight accounted for 0.05 to 0.2% of the fungal dry weight for the moulds tested. Uptake of copper was as high as 0.5% of dry weight in bacteria. The variation in metal uptake of the three fungi in this study could be related to differences in cell wall architecture and/or components, metal uptake mechanisms and intracellular binding proteins, and the variation is likely to be genetic and not adaptive since the strains used were isolated from unpolluted environments and all apparent differences would be inherent. Maximum metal uptake by baker's yeast was 0.16% of the dry weight of the organisms (Lange de la Camp and Steinmann, 1953), by *Fusarium oxysporum*, 1.85% (Hartman, 1976); by a *Scenedesmus sp.*, 1.1% (Stokes, 1975); by *Saccharomyces cerevisiae*, 1% (Kikuchi, 1964) and by *Bacillus megaterium*, 0.0152% (Krueger and Kolodziej, 1976).

The levels of metal accumulated in the three fungi of this study was often concentration dependent although this was variable. Baldry and Dean (1980c) have reported similar dependences on the concentration of metal

present. The uptake of copper by a heavy metal resistant strain of *Penicillium ochrochloron* usually increased as the copper concentration in the medium was increased up to 1000.0 μg $ml^{-1}$. At the higher concentrations however, it remained constant at 0.1% of the dry weight (Okamoto *et al*, 1977). Bedford (1936) also found that the copper content of a resistant *Penicillium sp.* did not vary from 6% when the copper content of the medium was above 2000.0 μg $ml^{-1}$.

The most interesting phenomenon of the heavy metal accumulation studies in growing cultures was the reduction in mycelial metal content observed in early linear phase of growth. There are a number of possible explanations for this result but it is unlikely that any one of them could be wholly responsible for the apparent reduction of biomass metal content.

The nature of the inoculum preparation was brutal and almost certainly resulted in a loss of viability of a proportion of the mycelium, although this was difficult to quantify. The debris produced in the homogenising procedure may account for the rapid and extensive initial accumulation.

It is possible that binding of metals by dead cells contributed to initial high metal content since at the higher concentrations tested the dilution effect seemed often to be more prominent. This could possibly be explained by an initial elevated cell death rate, leading to the higher initial metal content. At the lower metal concentrations, cell death would not be so significant and indeed the reduction in biomass loading was not so apparent.

Another possible explanation for the reduction of metal content in early linear phase was limitation of metal availability during the growth of the fungi either by removal as a result of fungal accumulation, complexing by medium constituents or released metabolites, or perhaps by pH effects.

It is unlikely that metal removal from the medium could account for reduction in bound metal since only a very small proportion of the metal is removed by lag phase material because of the low density of biomass present in the culture at this stage. However, when the fungal density increased to the linear phase levels, metal removal was quite significant, particularly at the low metal concentrations when effective metal accumulating fungi were tested. For example, *P. spinulosum* removed about 70% of the copper from the medium containing 1.0 $\mu g\ ml^{-1}$ $Cu^{++}$. Typical amounts of metal removed however amounted to 10-25% only, so in most cases substantial amounts of residual metal were present in the medium even at the end of linear phase. However, the pattern of metal accumulation was similar to that reported by Shatzman and Kosman (1978) for the fungal *Dactylium dendroides*, in which copper accumulation experiments were performed in copper deficient medium.

Complexing to medium constituents was not a problem as shown by the polarographic studies so this could not have led to a reduction in metal availability. Particular care was taken over the choice of buffer. Tris buffer binds copper, as does citrate. Inorganic phosphate buffers often result in precipitation of metals (Baldry and Dean, 1980b). MES buffer was chosen since according to Good *et al*, (1966), it buffers well at pH 5.5 and binds very little copper. Other medium constituents were shown to be of little or no significance in metal complexing as

demonstrated using polarography. Medium and complexing problems have been discussed elsewhere in the literature (Ramamoorthy and Kushner, 1975; Duddridge and Wainwright, 1980; Gadd and Griffiths, 1978a). Complexing by metabolites assumed to be present in spent medium did occur with respect to certain metals at various pHs as shown by the polarographic studies. Copper was complexed at pH 5.5 by the spent medium but not at pH 2.0. Therefore, at mid linear phase when the pH dropped to 2.0 copper availability in terms of complexing should not have been a problem. Cadmium and zinc on the other hand, did show signs of complexing at pH 2.0 which is both surprising and difficult to explain. Control experiments in which the pH was modified in GMS and $10^{-3}$ M NaCl suggested that pH did not in fact influence the parameters of the polarographic and voltammetric waves so suggesting that this was a real effect and that the medium had in some way been affected such that the cadmium and zinc was complexed at pH 2.0 and not at 5.5. The important point to remember is that complexing was not apparently a problem at the time of the dilution so this does not explain the phenomenon. The fact that the dilution effect was bigger at the higher metal concentrations where one might expect complexing to be less significant anyway, tends to refute the idea that complexing could account for the dilution effects in mycelial metal contents.

One possible explanation for the reduction in mycelial copper during growth could have been the effect of pH since it is well known that pH reductions cause a reduction in metal uptake and this is discussed in the next section. By the time of mid-linear phase, the medium pH had fallen from 5.5 to 2.0, and this lower pH would certainly constrain further metal uptake in linear phase although it is unlikely that the reduction in metal loading could have been caused singly by the pH fall because they occurred at different times of the growth cycle. The reduction in metal content was apparent before there was a significant fall in pH.

The role of pH in metal accumulation was studied further using automated pH control and this is reported in the next section. The problem was investigated in this section of the work though with limited success, by eliminating MES buffer from the medium so that buffering capacity of the normal medium could be evaluated and correlated with metal uptake. By omitting the MES it was clear that pH did have a role to play in cadmium uptake from 100.0 μg $ml^{-1}$ cadmium by *T. viride*. In the unbuffered system on addition of the metal (in 1.0 M HCl) the pH immediately fell to 3.5 whereas in the buffered system it fell only to 5.4. The drop in pH of the unbuffered system resulted in a 66% reduction in cadmium uptake in lag phase. Mid-linear phase cadmium levels were also reduced in the unbuffered system by 85% but by stationary phase the metal content of the mycelium in both systems was similar (502.9 and 549.9 μg $g^{-1}$ for the buffered and unbuffered systems respectively). Clearly, the initial cadmium uptake was influenced by pH which in turn had an effect on the mid-linear phase metal content. The similar levels at stationary phase probably represent the complement of metal which is either most tightly bound on the cell wall or intracellular, and did not appear to be affected by an initial reduction in metal uptake. The reduction in metal binding effect was observed irrespective of the pH, although reducing the pH caused a smaller reduction. It seems therefore that there must have been some overriding factor in the metal reduction effect, although the influence of pH may be of some significance.

The metal reduction effect reported by Gadd and Griffiths (1978b) in *Aureobasidium pullulans* in copper uptake studies was attributed to reductions in pH with growth of a copper-tolerant strain. At 127-508 μg$ml^{-1}$ $Cu^{++}$ uptake by the tolerant strain was maximal in lag phase and declined in exponential phase. Since only 4% of the added metal was removed there

was no limitation in copper availability. Gadd and Griffiths (1978b) concluded that reduced uptake in the tolerant strain conferred resistance upon it when compared to the higher levels of uptake in the sensitive strain. According to Gadd and Griffiths (1980), the tolerance of the same resistant strain was apparently due to a pH reduction from 4.5 to 3.5. This pH reduction caused a smaller metal uptake so that exponential growth resumed, but this would have the same effect in the sensitive strain, if it had grown and released $H^+$ ions into the medium. The most likely reason for tolerance was an initial lower capacity for copper uptake so that growth could proceed, $H^+$ ions would be produced and released into the medium, hence detoxifying the copper by reducing the possibility for uptake. On the other hand, the sensitive strain accumulated more copper initially and was therefore inhibited. It is interesting that *T. viride* used in this study accumulated low metal levels in general, and was particularly resistant to cadmium.

Another possible explanation for the decreased metal binding phenomenon is that there may have been a physiological change in the fungus during lag phase in response to the high levels of mycelial metal, which could have resulted in the much slower subsequent uptake during linear phase. This change could be structural although this is unlikely, or perhaps electrical as a result of a change in surface potential. There is very little information on the surface charge of fungal mycelial surfaces although there have been electrophoretic studies on bacteria, yeasts and fungal spores (work cited by Douglas, *et al*, 1959).

Failla and Weinberg (1977) reported that zinc accumulation by the yeast *Candida utilis* was high during lag and late exponential phase, but negligible in early exponential and stationary phase. These authors

suggested the possibility of a regulatory process for zinc uptake which could account for these results. The reduction in intracellular zinc during early exponential phase could not be explained by an efflux system, and neither was there a limitation in metal availability. Also the phenomenon did not appear to be related to medium changes during growth. The authors concluded that zinc accumulation was dependent on the zinc status of the yeast and commented that it was not obvious why there should be an energy and temperature dependent active transport system for zinc. Although the authors suggested that the stringent control of zinc uptake was possibly related to post-exponential phase events such as secondary metabolism and differentiation, neither the mechanism nor the function was elucidated.

Further reports for metal accumulation have appeared for actively growing microorganisms which show the metal reduction phenomenon, but there still appears to be no adequate explanation. It is interesting that a metal reduction effect was reported by Baldry and Dean (1980b), for *Escherichia coli* growing in the presence of copper at 5.0 $\mu g\ ml^{-1}$ $Cu^{++}$, but quite different characteristics in the pattern of growth and accumulation were observed for the bacterium when compared to the results for the filamentous fungi studied in this investigation. Copper uptake increased at the end of exponential phase and passed through a sharp maximum in the succeeding deceleration phase and settled in stationary phase at a level of one-quarter of the maximum value. The same pattern was observed at different copper concentrations and for five organisms. The authors did report that at the metal reduction stage, the pH was reduced from 7.0 to 5.0, but by maintaining the pH at 5.0 using an autotitrator this did not keep the copper at its maximum value of 0.20% dry weight with copper added at 5.0 $\mu g\ ml^{-1}$. No explanation was offered for the observation.

Failla and Weinberg (1977) showed that there was no zinc uptake by the yeast *Candida utilis* in early exponential phase. Stokes and Lindsay (1979) reported two different uptake patterns for copper in *Penicillium ochrochloron* which depended on the stage of growth when the copper was added in the range 10-5000.0 $\mu g\ ml^{-1}\ Cu^{++}$. When the fungus was grown for 7 days, then the copper added, a maximum uptake was achieved at day 1 with $4 \times 10^5\ \mu g\ g^{-1}$ from 5000.0 $\mu g\ ml^{-1}\ Cu^{++}$. The metal content of the fungus then decreased with time upto day 14. Uptake was concentration dependent. When the fungus was inoculated directly into the copper containing medium, the uptake appeared to increase with time and the highest concentration factor was at day 7. However, the uptake in the first system was more efficient with a concentration factor of 4000 at day 7 from 100.0 $\mu g\ ml^{-1}\ Cu^{++}$, whereas in the second situation it was only 28.5. These patterns of accumulation are dissimilar to the uptake profiles demonstrated for the fungi used in this study, but *P. ochrochloron* is atypical anyway in its degree of metal tolerance.

One other explanation for the reduction in metal content during linear phase in the fungi used in this study, is the possibility that an efflux system was operative. This theory was dismissed by Failla and Weinberg (1977) in *C. utilis* for zinc accumulation, and there appeared to be no significant increases in the residual metal levels in the medium with the growth of *P. spinulosum*, *T. viride* and *A. niger*. Although in most cases any increase in residual metal would be small, it ought to have been measureable in the examples where dramatic reductions were apparent.

Although the function of these patterns of accumulation are unknown it is possible that high initial uptake is a scavenging mechanism

resulting in high metal concentrations localised outside the cell, perhaps on the cell wall. It may then be possible for the fungus to release the ions from their bound locality and this may result in internalisation either by diffusion or an active mechanism. Released $H^+$ ions may well be involved in releasing cations from the binding sites so that diffusion could occur along a concentration gradient, from a high concentration in the vicinity of the cell membrane to a lower intracellular concentration. In *Achlya sp.* Le John *et al* (1974) reported that $Ca^{++}$ stored in the cell wall complex using a glycopeptide as a vehicle for this function was released by $N_6$ (substituted)-adenine (cytokinins). Release of Calcium was cytokinin dependent. Not only cytokinins were able to release the $Ca^{++}$ when the physiological state of the cell demanded this, but also benzimidazole and adenine derivatives, nucleotides, nucleosides and methyl zanthines were among more than 40 agents tested. It is possible that similar mechanisms operate for heavy metal release from the cell wall, and $H^+$ ions would seem to be a reasonable candidate. Uptake of metals by non-growing suspensions would help to elucidate whether or not there is an active system involved for heavy metal uptake in the filamentous fungi tested in this study and this will be discussed in a later section.

The mid-linear phase metal contents of the three fungi tested with the three metals at various concentrations indicated that accumulation was concentration dependent. In *T. viride* and *A. niger* though, maximum uptake seemed to occur at moderate copper concentrations. There appeared to be great variability in the capacity for metal accumulation between different fungi and different metals.

Copper uptake by *P. spinulosum* was markedly greater than in either of the other fungi tested, particularly at 7.5 and 10.0 μg $ml^{-1}$ added copper. It may be that copper caused an increased cell permeability at these elevated concentrations, especially since *P. spinulosum* was very susceptible to copper toxicity, so the high levels of binding could have been due to binding to intracellular proteins for example. On the other hand, the cell wall binding sites in *P. spinulosum* may have had a higher affinity for copper than the sites in the other fungi, or perhaps were of different composition or structural architecture. It could also be that the fungi were different in terms of their apparent ratio of cell wall binding to intracellular uptake, and it may be that fungi with a high metal uptake capacity have a greater capacity for cell wall adsorptive uptake. There is also the possibility that localisation onto organelles or specific proteins once the metals have been internalised could account for differences in uptake. The influence of charge on uptake is also significant and this may be influenced by medium pH. Obviously, the metals themselves are important in determining uptake since charge characteristics, ionic size parameters *etc.* are almost certainly involved. Differences in metal accumulation are apparent and could depend on one or many of the above factors but the fact that variability occurs supports the view that comparative studies are necessary.

There does not appear to be any clear relationship between quantitative metal uptake and the degree of toxicity. In the case of copper, toxicity was similar to all fungi, yet uptake was dramatically greater in *P. spinulosum*. In the case of cadmium however, *P. spinulosum* was very susceptible and accumulated more cadmium than *T. viride* or *A. niger*. *Trichoderma viride* accumulated least cadmium and demonstrated a fair degree of tolerance to the metal. *Aspergillus niger* accumulated

an intermediate amount of cadmium and showed intermediate toxicity responses. Toxic responses to cadmium then, do appear to depend on the amount of the metal accumulated. Zinc was relatively non-toxic to all fungi and the differences in accumulation were not very dramatic. It is possible that relationships between uptake and toxicity hold for non-essential elements but more studies would be required before any firm conclusions could be drawn.

The quantities of metal accumulated compare favourably with levels of uptake reported in the literature for actively growing fungi. Duddridge and Wainwright (1980) reported slightly higher levels of cadmium and zinc accumulated in *Pythium sp.*, *Dictyuchus sp.* and a *Scytalidium sp.* than observed in this study but they were of the same order of magnitude. Interestingly, copper accumulation from 10.0 μg $ml^{-1}$ in *P. spinulosum* at a level of 2758.8 μg $g^{-1}$ dry weight is in fairly close agreement with the figure reported for *P. ochrochloron* of 3450.0 μg $g^{-1}$ dry weight (Stokes and Lindsay, 1979). The importance of strain variation here is emphasised, by the fact that Okamoto *et al*, (1977) reported an accumulation of a modest 250.0 μg $g^{-1}$ dry weight for the same organism in a medium containing 10.0 μg $ml^{-1}$ $Cu^{++}$. Cadmium and zinc accumulation in *P. ochrochloron* was lower than in *P. spinulosum* as reported by Okamoto *et al*, (1977). Baldry and Dean (1980a)reported data showing variation in terms of metal uptake between *Penicillium* species and two strains of *P. spinulosum*.

It is clear that metal uptake is dependent on the precise experimental conditions in terms of medium, metal and fungal physiology. The next section of the study is concerned with the influence of pH on metal uptake in growing fungi comparing buffered systems with experiments in fermenters with electronic pH control.

## 7. THE EFFECT OF pH ON HEAVY METAL ACCUMULATION AND TOXICITY.

### 7.1. Introduction.

The influence of pH on heavy metal availability and therefore on uptake and toxicity in microorganisms is critical. At acidic pH, metals are generally regarded as free cations but as the pH becomes alkaline, hydroxide and oxide precipitates are formed which render the metal unavailable to microbes. There are many reports in the literature on the effects of pH on metal uptake and toxicity in bacteria, algae, protozoa, yeasts and filamentous fungi and it is apparent that the role of pH in metal accumulation is quite variable. Most of the published work however, supports the view that there is usually a pH optimum for metal accumulation with reduced uptake if the pH is adjusted in the alkaline or acid direction.

A number of acidophilic bacteria of the *Ferrobacillus-Thiobacillus* group were isolated from acid drainage from a copper mine by Ehrlich (1963) and the author proposed that the resistance was due to decreased uptake of copper at low pH. Further information in bacterial studies has been reported by Babich and Stotzky (1977) who showed that cadmium toxicity was pH dependent for a number of bacteria, whilst Brock (1969) suggested that metal resistance in acidophilic bacteria was due to competition for metal binding sites by $H^+$ ions and this would consequently cause a reduction in metal ion uptake.

Information on algal metal accumulation has been supplied by Hart and Scaife (1977) who reported that growth inhibition of the alga *Chlorella pyrenoidosa* was more pronounced at pH 7 than 8 suggesting that cadmium removal from the medium and its deposition in the algal cells was pH dependent. Bennett (1969) reported that there was prolific

growth of algae in acid mine streams whilst Ehrlich (1963) isolated protozoa from an acid mine stream containing copper. Norberg and Persson (1984) also showed reduced copper and cadmium uptake by the bacterium *Zoogloea ramigera* with low pH.

The role of pH in metal accumulation in yeasts has also received some attention. The relationship between pH and copper availability has been investigated by Avakyan (1971) using *Candida utilis*. The influence of pH was demonstrated by the fact that complexes with amino acids were less toxic at pH values of 7 than at lower pH values. The direct relationship between pH and metal uptake in yeasts has been illustrated by a number of workers including Fuhrmann and Rothstein (1968) investigating nickel uptake in *Saccharomyces cerevisiae*, Okorokov *et al* (1979) working on manganese uptake by *S. cerevisiae* and Fuhrmann (1974) studying uptake of manganese in *S. cerevisiae* where the workers showed that the pH value for optimum uptake was between 5 and 7.0. The rate of zinc uptake by *C. utilis* decreased with increasing pH between 4.8 and 8.2 (Failla *et al*,1976). Calcium uptake by *Saccharomyces pombe* and calcium and strontium uptake by *S. cerevisiae* increased markedly with increasing pH upto 8.0 (Boutry *et al* 1977, Roomans *et al* 1979) and decreased again at higher pH values (Boutry *et al*, 1977).

There are also a large number of publications on the effects of pH on metal uptake in filamentous fungi. Decreased uptake of copper by fungi at low pH has been noted by Horsfall (1956) who suggested that this could be important in determining tolerant behaviour to copper containing fungicides. Many other workers have also reported that low pH apparently reduces uptake and/or toxicity of a number of metals in various fungi (Bedford, 1936; Sato, 1939; Starkey and Waksman, 1943;

Lowry *et al*, 1957; Young, 1961; Levi, 1969; Venkateswerlu and Sivarama Sastry, 1970; Le John *et al*, 1974; Singh, 1977; Shumate *et al*, 1978 and Gadd and Griffiths, 1980). A more detailed study was carried out by Starkey (1973) using the fungus *Scytalidium sp.* and 11 other fungi. The fungus grew at pH 0.3 to 2.0 in the presence of 63550.0 $\mu g\ ml^{-1}$ added copper but was sensitive to 2.54 $\mu g\ ml^{-1}$ near neutrality. *Aspergillus niger*, *Penicillium notatum* and *Trichoderma koningi* tolerated 25.42 to 2542.0 $\mu g\ ml^{-1}$ at pH 2.0 to 7.0. The critical pH range for copper was reported as being between 4.2 and 5.0 and the author suggested that above this pH copper penetrated the cells, below it uptake was reduced or ceases. Ashida (1965) attributed this resistance to reduction of cell permeability and the production of copper binding compounds such as cysteine and a change in enzymatic activities. An alternative explanation for this phenomenon has been tendered by Okamoto *et al*, (1977) with the author postulating that reduction of copper uptake was due to competition with $H^+$ ions and this was the mechanism conferring resistance on the fungus *Penicillium ochrochloron*.

The biology of a copper swamp has been studied and soil fungi were isolated by Kendrick (1962). Also a brown fungus was isolated from acid soil and from acid solutions in an industrial plant and a uranium mine (Sigler and Carmichael, 1974). The fungus was characterised as a new *Scytalidium sp.*, and the resistance to the metal was undoubtedly due to the low pH. Similarly, *Scytalidium sp.* isolated from soil and extremely acid and high salt concentration tolerant was reported by Gould, *et al*, (1974).

According to Babich and Stotzky (1977a and 1977b) cadmium toxicity was potentiated at pH 8 or 9 to *Aspergillus niger*, *Trichoderma viride*

and *Rhizopus stolonifer* using plate-testing techniques. It is interesting to note that Stokes and Lindsay (1979) reported that copper uptake in *Penicillium ochrochloron* and subsequent concentration in the fungus showed no correlation with pH over the range 4.5 to 6.5. Babich and Stotzky (1981) showed that there was no consistent trend in the effect of pH on manganese toxicity to fungi.

The importance of the role of pH in metal uptake in fungal material employed as biosorbents for removing metals from solutions, has been emphasised by a number of workers. In two U.S. Patents (Drobot (1981) 4,293,333 and Drobot and Lechavelier (1981), 4,293,334), a process was described for recovering metal in insoluble form from an aqueous medium wherein the pH was maintained in the range 1 - 3 (claim 14), for recovery of base and precious metals. This seems rather low in the light of other relevant available information but it is likely to be a representative value of many typical industrial effluents. An operational pH of 6.5 was employed by Muzzarelli *et al* (1980) for the recovery of copper and mercury using columns of chitosan-glucan prepared from *Aspergillus niger*, although this pH is approaching the value where copper may precipitate. Biosorption of uranium and thorium by *Rhizopus arrhizus* was markedly affected by solution pH (Tsezos and Volesky, 1981, 1982a, 1982b and Tsezos, 1983). In general, lower biosorptive uptake was exhibited by the fungus at pH 2.0 than at pH 4.0, and no discernible difference in uptake was observed between pH 4 and 5.0 which was optimal for metal recovery. Tobin *et al* (1984), reported that the biosorbent capacity of *R. arrhizus* biomass was markedly pH dependent. This was explained in terms of the effect of pH on metal absorption sites in the biomass containing phosphate, carboxylate and other functional groups.

Although there is a battery of information in general terms on the effects of pH on metal uptake and toxicity in fungi, there appears to be no comparable metal uptake data between different experimental situations with respect to pH and growth. During the growth of the fungus, the pH was reduced (as was observed in the flask cultures), but in stirred-tank fermenter cultures the pH was maintained at the initial pH electronically. Although Gadd and Griffiths (1980) reported that pH fell during the growth of a copper-tolerant strain of *Aureobasidium pullulans*, and that this appeared to be involved with copper tolerance due to reduced copper uptake, the authors did not attempt to maintain the pH at the initial level of 4.5 to 4.7 for comparison in parallel studies.

There is no indication in the literature on whether or not optimum pH is critical for metal uptake throughout the growth cycle or merely at specific periods such as lag phase, exponential or stationary phase in growing cultures. By comparing mid-linear phase material of growing fungi under flask conditions (i.e. with pH fall from 5.5 to 2.0 during growth), and of fungi grown with electronically maintained pH at 5.5 throughout the growth cycle in a stirred-tank fermenter, it ought to be possible to determine if subsequent metal loading in linear phase depends on the initial pH only, or a constant pH during growth. Further information could be obtained by investigating lag phase biomass.

The aim of this section of the project was to obtain data on the influence of pH on metal contents at various stages of fungal growth in the stirred-tank fermenter, so that comparisons could be made with the results obtained using flask cultures (see preceding section) where the pH was reduced during growth. In this way, the period of crucial pH control for optimum metal uptake in terms of the fungal growth cycle could be established.

7.2. Materials and Methods.

7.2.1. Operation of stirred-tank fermenter.

The stirred-tank fermenter was an LH Engineering fermenter, type CC 1500 with a working volume of 1800 ml. Temperature was maintained at 25°C, pH at 5.5 ± 0.1 unit, aeration rate at 2 litres per minute and agitation at 1000 rev $min^{-1}$. Inoculum was prepared as previously described for flask cultures and 20.0 ml of inoculum was injected into the vessel. Dissolved oxygen levels did not appear to fall below 75% by late linear phase, when measurement became impossible due to fungal accretion onto the oxygen electrode. Growth medium used was GMS but without the complement of MES buffer.

7.2.2. Sampling of fungal mycelium.

Samples, usually 25-100.0 ml depending on the mycelial density were collected in triplicate in sterile universal bottles at appropriate time intervals. The cells were separated by filtration onto Whatman 541 or Millipore filters. Filtered cells were washed with 200 ml distilled $H_2O$.

Relatively large samples in triplicate were required to obtain significant amounts of material for weighing and metal analysis, particularly at the early stages of growth, and this resulted in considerable volume changes as growth proceeded. For this reason experimental runs were often repeated several times under identical conditions to obtain a sufficient number of samples at each time interval so that growth curves or metal uptake profiles could be constructed from the composite data. It was considered unsatisfactory to reduce the working volume from 1800 ml to below 1500 ml. For this reason much of the comparative data obtained from the fermenter initially was at mid-linear phase only.

7.2.3. Mid-linear phase determination.

Samples were collected at various time intervals and the mycelium separated from the GMS medium (minus MES buffer) by filtration. Cells were dried and weighed and the filtrates were retained for glucose assay. The glucose assay was described previously in section 5.2.1.3. The relationship between dry weight and glucose utilisation is presented graphically for experiments using either vortex or baffled mixing of the fungal culture.

7.2.4. Metal uptake by growing cultures in the fermenter.

Mid-linear phase metal content determinations.

Copper uptake was determined in *P. spinulosum* by incubating cells in GMS medium containing 1.0, 2.5 or 5.0 μg $ml^{-1}$ $Cu^{++}$ in the fermenter vessel. The temperature was maintained at 25°C and the pH was held at either 5.5, 3.5 or 2.0 ± 0.1 units by automatic addition of 2 M $HNO_3$ or 2 M KOH. Control experiments had no added copper. Three, 100 ml samples were collected at mid linear phase and copper determinations performed as described previously on the mycelium. Similar experiments were performed for zinc and cadmium at 2.5 μg $ml^{-1}$ concentration with *P. spinulosum*. The experiments at 2.5 μg $ml^{-1}$ were replicated at least 3 times and in some cases 5 times.

7.2.5. Lag phase metal content determinations.

To determine accurately the influence of pH on lag phase accumulation, separate studies had to be performed because of the large sample volumes required at this stage of growth since the mycelial density was very low. The inoculation and incubation procedure was as described earlier but three 250 ml samples were collected after 24 hours and analysed for metal content.

7.2.6. Uptake profile throughout the growth cycle.

The quantitative metal uptake during the whole of the fungal growth cycle was obtained from composite data of 4 runs because of the difficulty encountered in collecting large samples. Experimental runs were performed in duplicate and samples collected at 12, 24, and 36 hours of incubation, and then duplicated reruns were carried out and the cells sampled at 36, 48, 60, 72 and 84 hours. There appeared to be no difficulty in expressing the data as a single run and the results are graphically presented using the composite data. All three fungi were tested with each of the three metals.

7.2.7. Evaluation of copper toxicity in fermenter experiments.

In a separate study copper toxicity was determined at pH 5.5, 3.5 and 2.0 using 1.0, 2.5 and 5.0 $\mu g\ ml^{-1}\ Cu^{++}$. Composite data prepared as described in the previous subsection was used to illustrate the effect of copper on fungal growth at the various pH values.

7.3. Results.

7.3.1. Mid-linear phase determination.

Vortex mixing was found to be the most suitable agitating mechanism in terms of reproducibility of results because of the problems of accretion in the baffled system which caused difficulty in obtaining representative samples of the culture. Mid-linear phase was estimated to occur at a glucose concentration of 10 g litre$^{-1}$ in the vortex mixing system. The results are presented graphically in Figure 7.3.1a and 1b.

7.3.2. Metal uptake by growing cultures in the fermenter.

Mid-linear phase metal content determinations.

The uptake of copper from the trace solution (i.e. controls) ranged from 8.6 to 12.4 µg g$^{-1}$ dry weight of the fungus in the fermenter cultures and was 15.3 µg g$^{-1}$ dry weight in the flask cultures. This variation is very likely to be a reflection of the difficulty in analysing the very low metal contents in the control cultures which were assayed at the limits of detection of the atomic absorption spectrophotometer.

The effect of pH on mid-linear phase copper contents of *P. spinulosum* is shown in Table 7.3.1a. There appeared to be a marked effect on copper content when the pH was reduced from 5.5 to 3.5 and 2.0 with copper additions at 1.0, 2.5 and 5.0 µg ml$^{-1}$ $Cu^{++}$. This data was necessarily produced from single runs in the fermenter because of the typical long down-time associated with batch runs in fermenters. For this reason the standard errors quoted are for samples of a single run and not for separate experiments. The reduction in pH from 5.5 to 3.5 resulted in a fall in copper content of 10%, 26% and 38% over the contents at the higher pH of 5.5 at added copper concentrations of 1.0, 2.5 and 5.0 µg ml$^{-1}$ $Cu^{++}$

respectively. A reduction in pH from 5.5 to 2.0 at the same respective copper concentrations resulted in a bigger decrease in copper content as compared to the mycelial contents at pH 5.5 of 56%, 82% and 65%.

Also illustrated in Table 7.3.1a is the comparison of mycelial copper contents of fungi grown in shake flasks where the initial pH of 5.5 fell during growth to 2.0, with stirred-tank fermenter cultures where the pH was maintained at 5.5 throughout growth. It is noteworthy that at 1.0 and 2.5 $\mu g\ ml^{-1}$ added copper, uptake appeared to be higher in the shake flask cultures. At 5.0 $\mu g\ ml^{-1}$ $Cu^{++}$ copper levels at mid-linear phase were higher in the fermenter system but not greatly so. A fall in pH during growth seemed to have little effect on mid-linear phase mycelial copper contents, but the initial pH appeared to be more critical.

It is interesting that zinc uptake from the trace metal solution which was determined in samples from the fermenter to demonstrate the significance of pH, if any, on trace uptake, appeared to be unaffected by the pH. The results for zinc uptake from the trace solution are illustrated in Table 7.3.1b and it appears that reducing the pH from 5.5 to 2.0 had no appreciable effect on the mid-linear phase metal content, even though mycelial contents at the higher levels of added metal were influenced by pH, as shown in Table 7.3.2b.

Since it was considered desirable to have statistical proof that pH at the initial time of inoculation was important for mid-linear phase metal loading, the experiments were replicated at metal concentrations of 2.5 $\mu g\ ml^{-1}$ regardless of the lengthy down-times. Tables 7.3.2a, 2b and 2c illustrate the effect of pH on copper, cadmium and zinc uptake

from added levels of the metal of 2.5 μg $ml^{-1}$ by *P. spinulosum*. On reducing the pH from 5.5 to 3.5 copper contents were reduced by 26% and zinc was reduced by 16%. The mid-linear phase cadmium content appeared to be reduced much more significantly by this pH change with a decrease of 59%. On reducing the pH from 5.5 to 2.0 mycelial metal contents were reduced by 82%, 45% and 64% for copper, zinc and cadmium respectively. Interestingly, the uptake of metal from the trace solution did not appear to be affected by these pH changes. Although the reduction in pH from 5.5 to 3.5 is indicated as producing a statistically significant ($p = 0.025$) change in the amount of copper uptake from the trace solution after cadmium addition (Table 7.3.2.c), this is likely to be a peculiarity of the statistical process due to the small sample (3 replicate runs) and the coincidental small standard error obtained in this case. The statement that the copper content is significantly different is probably invalid in this instance and the copper uptake from the trace solution could be said to be constant irrespective of pH change.

7.3.3. Lag phase metal content metal determintions.

The mid-linear phase metal contents of fungi were not affected by a reduction in pH late in the growth cycle but were greatly influenced by the initial pH value. Table 7.3.3. illustrates the influence of pH on lag phase accumulation in flask cultures and stirred-tank fermenter cultures. Although there was no significant difference in copper contents at pH 5.5 in either system in lag or mid-linear phase, by reducing the initial pH to 2.0 there appeared to be a dramatic reduction of copper content in lag phase mycelium in the fermenter from 2983.5 (± 311.3) μg $g^{-1}$ dry weight to 82.4 (± 5.7) μg $g^{-1}$ dry weight of the fungus. This was accompanied by the fall in mid-linear copper

from added levels of the metal of 2.5 μg $ml^{-1}$ by *P. spinulosum*. On reducing the pH from 5.5 to 3.5 copper contents were reduced by 26% and zinc was reduced by 16%. The mid-linear phase cadmium content appeared to be reduced much more significantly by this pH change with a decrease of 59%. On reducing the pH from 5.5 to 2.0 mycelial metal contents were reduced by 82%, 45% and 64% for copper, zinc and cadmium respectively. Interestingly, the uptake of metal from the trace solution did not appear to be affected by these pH changes. Although the reduction in pH from 5.5 to 3.5 is indicated as producing a statistically significant ($p = 0.025$) change in the amount of copper uptake from the trace solution after cadmium addition (Table 7.3.2.c), this is likely to be a peculiarity of the statistical process due to the small sample (3 replicate runs) and the coincidental small standard error obtained in this case. The statement that the copper content is significantly different is probably invalid in this instance and the copper uptake from the trace solution could be said to be constant irrespective of pH change.

7.3.3. Lag phase metal content metal determintions.

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Figs 7.3.1a & 1b. Glucose utilisation by *P. spinulosum* and biomass production during batch culture in a fermenter with vortex and baffled mixing.

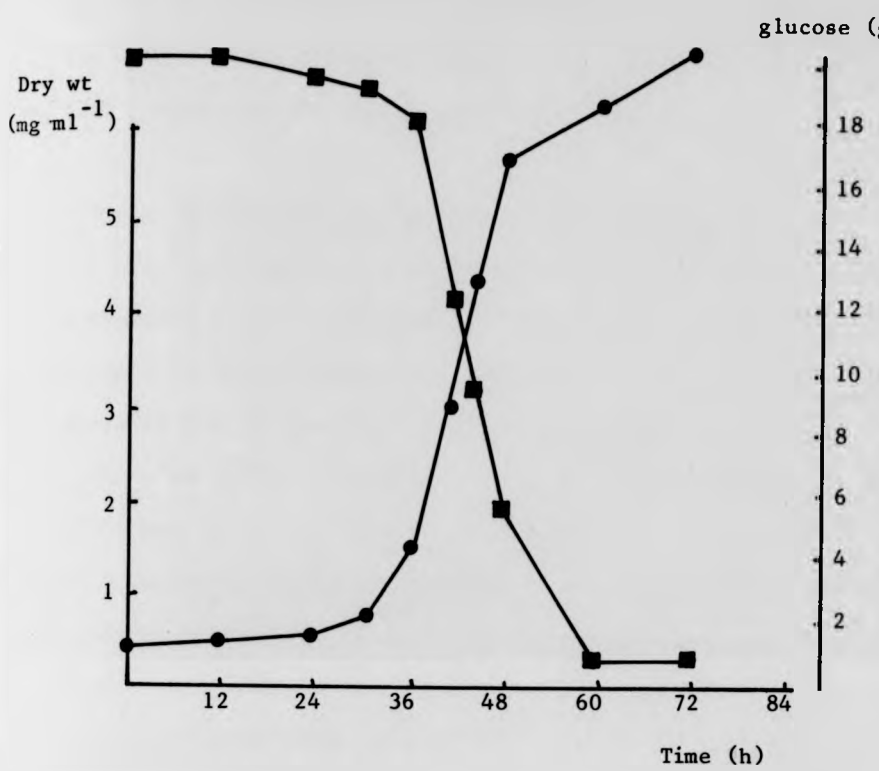



*Figure* 7.3.1a

Vortex mixing

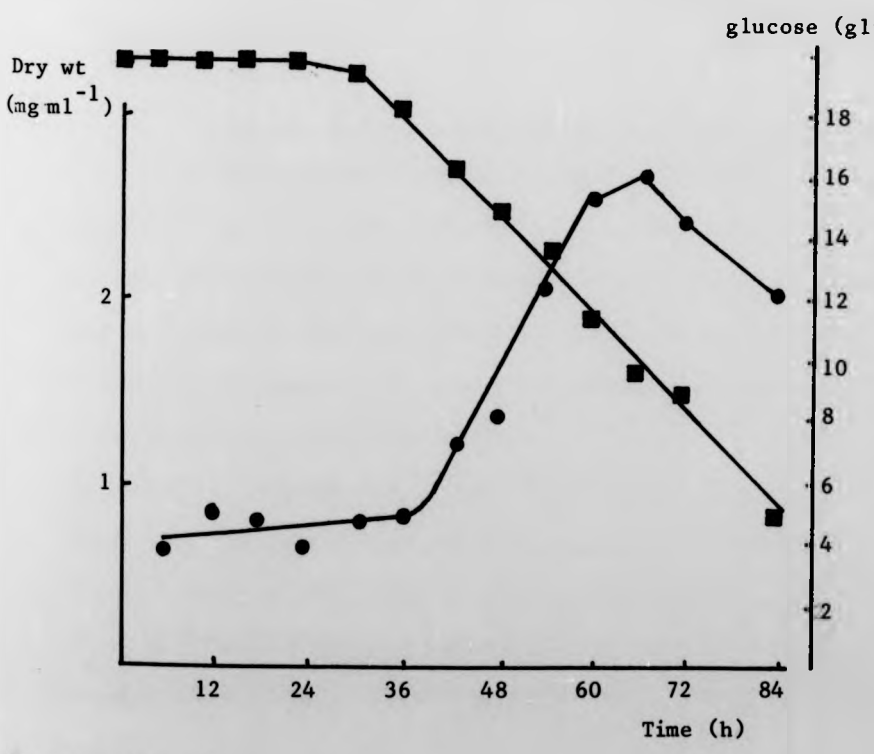



*Figure* 7.3.1b

Baffled mixing

● biomass production

■ glucose utilisation

content as already described. Clearly, initial culture pH was critical for uptake of metal in lag phase and this appears to influence the metal content of mid-linear phase mycelium.

7.3.4. Uptake profile throughout the growth cycle.

The metal uptake profiles for copper, cadmium and zinc accumulation in *P. spinulosum*, *T. viride* and *A. niger* are shown in Figures 7.3.2-10. When these profiles for maintained pH during the whole of the growth cycle at pH 5.5 are compared to the plots for the shake flask cultures (Figures 6.3.1-45) there is apparently little difference in the pattern of accumulation or the quantitative amount of metal taken up. There appeared to be a high initial accumulation, followed by a period of rapid reduction in metal content in lag or early linear phase as was observed in the shake flask cultures. Fungal mid-linear phase metal contents were similar in both systems for all the metals and fungi tested.

7.3.5. Evaluation of copper toxicity in fermenter experiments.

By observing the effects of added copper at 1.0, 2.5 and 5.0 $\mu g\ ml^{-1}$ on dry weight production of *P. spinulosum* at pH 5.5, 3.5 and 2.0, the influence of pH on copper toxicity could be evaluated. Figures 7.3.11-13 show the effects of copper addition at these various pH levels when compared to control growth curves grown in GMS with no added copper. At pH 5.5, 1.0 and 2.5 $\mu g\ ml^{-1}\ Cu^{++}$ increased the lag period by 12 hours and 5.0 $\mu g\ ml^{-1}\ Cu^{++}$ by 24 hours and in the latter case the growth rate appeared to be slowed down also. At pH 3.5, 1.0 $\mu g\ ml^{-1}\ Cu^{++}$ had no effect on growth but 2.5 and 5.0 $\mu g\ ml^{-1}\ Cu^{++}$ slightly increased the lag period. At pH 2.0, 1.0 $\mu g\ ml^{-1}\ Cu^{++}$ had no effect on growth but 2.5 and 5.0 $\mu g\ ml^{-1}\ Cu^{++}$

increased the lag period. The growth rate was also reduced at pH 2.0 in the presence of 5.0 μg $ml^{-1}$ $Cu^{++}$.

Clearly, copper was more toxic at pH 5.5 than at pH 3.5 or 2.0 and this was almost certainly due to reduced uptake as demonstrated in the earlier subsections of this chapter. It should be remembered however, that the morphology of the fungus was modified by altering the pH as described and discussed in section 3 of the study.

*Table* 7.3.1a.

Comparison of copper uptake by growing cultures of *P. spinulosum* in batch flask culture and in batch fermenter culture from 1.0, 2.5 and 5.0 $\mu g\ ml^{-1}$ copper and the effect of pH on uptake.

| Added $Cu^{++}$ ($\mu g\ ml^{-1}$) | 1.0 | 2.5 | 5.0 |
|---|---|---|---|
| Experimental conditions | Copper uptake ($\mu g\ g^{-1}$ dry wt) | Copper uptake ($\mu g\ g^{-1}$ dry wt) | Copper uptake ($\mu g\ g^{-1}$ dry wt) |
| Flask culture pH 5.5 | 173.0 ±10.2 | 470.8 ±22.1 | 527.6 ±19.1 |
| Fermenter pH 5.5 | 131.2 ±4.2 | 413.9 ±13.4 | 596.5 ±7.4 |
| Fermenter pH 3.5 | 117.5 ±1.5 | 304.5 ±8.3 | 368.4 ±12.9 |
| Fermenter pH 2.0 | 57.7 ±1.3 | 76.4 ±4.1 | 207.8 ±11.1 |

Flask results are mean values of at least 10 replicates (± S.E. of mean). Fermenter results are values for single experiments (± S.E of mean of samples from the single run.)

*Table* 7.3.1b.

Effect of pH on zinc uptake from trace solution in GMS medium.

| Added $Cu^{++}$ ($\mu g\ ml^{-1}$) | 1.0 | 2.5 | 5.0 |
|---|---|---|---|
| Experimental conditions | Zinc uptake ($\mu g\ g^{-1}$) | Zinc uptake ($\mu g\ g^{-1}$) | Zinc uptake ($\mu g\ g^{-1}$) |
| Fermenter pH 5.5 | 108.3 ±5.2 | 109.0 ±3.1 | 102.4 ±2.0 |
| Fermenter pH 3.5 | 101.4 ±4.7 | 122.5 ±3.8 | 100.5 ±4.0 |
| Fermenter pH 2.0 | 100.2 ±2.0 | 118.3 ±8.6 | 109.3 ±1.4 |

*Tables* 7.3.2a, 2b and 2c.

Effect of pH on mid linear mycelium copper, zinc and cadmium contents of growing cultures of *P. spinulosum* in GMS medium amended with 2.5 $\mu g\,ml^{-1}$ metal, and the effect on the uptake of copper and zinc from the trace metal solution during fermenter batch culture.

GMS medium amended with 2.5 $\mu g\,ml^{-1}$ copper.

| pH | $Cu^{++}$ uptake from added $Cu^{++}$ ($\mu g\ g^{-1}$ dry wt). | $Zn^{++}$ uptake from trace solution($\mu g\ g^{-1}$ dry wt) |
|---|---|---|
| 5.5 | 413.9 ($\pm$13.4) | 109.9 ($\pm$3.1) |
| 3.5 | 304.5 ($\pm$8.3) | 122.5 ($\pm$3.8) |
| 2.0 | *76.4 ($\pm$4.1) | 118.3 ($\pm$8.6) |

*Table* 7.3.2a

Results based on the mean of 5 replicate runs ($\pm$ S.E. of the mean).

* Significantly different $p_{0.025.5.5}$

GMS medium amended with 2.5 $\mu g\,ml^{-1}$ zinc.

| pH | $Zn^{++}$ uptake from added $Zn^{++}$ ($\mu g\ g^{-1}$ dry wt). | $Cu^{++}$ uptake from trace solution($\mu g\ g^{-1}$ dry wt) |
|---|---|---|
| 5.5 | 190.3 ($\pm$13.1) | 16.5 ($\pm$1.8) |
| 3.5 | *160.4 ($\pm$2.3) | 13.9 ($\pm$1.6) |
| 2.0 | *105.6 ($\pm$7.7) | 18.0 ($\pm$1.0) |

*Table* 7.3.2b

Results based on the mean of 3 replicate runs ($\pm$ S.E. of the mean).

*Significantly different $p_{0.025.3.3}$

GMS medium amended with 2.5 $\mu g\,ml^{-1}$ cadmium.

| pH | $Cd^{++}$ uptake from added $Cd^{++}$ ($\mu g\ g^{-1}$ dry wt) | $Cu^{++}$ uptake from trace solution($\mu g\ g^{-1}$ dry wt) | $Zn^{++}$ uptake from trace solution($\mu g\ g^{-1}$ dry wt) |
|---|---|---|---|
| 5.5 | 401.7 ($\pm$19.2) | 21.4 ($\pm$2.0) | 76.6 ($\pm$4.3) |
| 3.5 | *166.0 ($\pm$4.3) | *20.3 ($\pm$0.1) | 79.5 ($\pm$5.6) |
| 2.0 | *144.4 ($\pm$5.5) | 19.4 ($\pm$0.3) | 102.8 ($\pm$0.9) |

Results based on the mean of 3 replicate runs ($\pm$ S.E. of the mean).

*Significantly different $p_{0.025.3.3}$

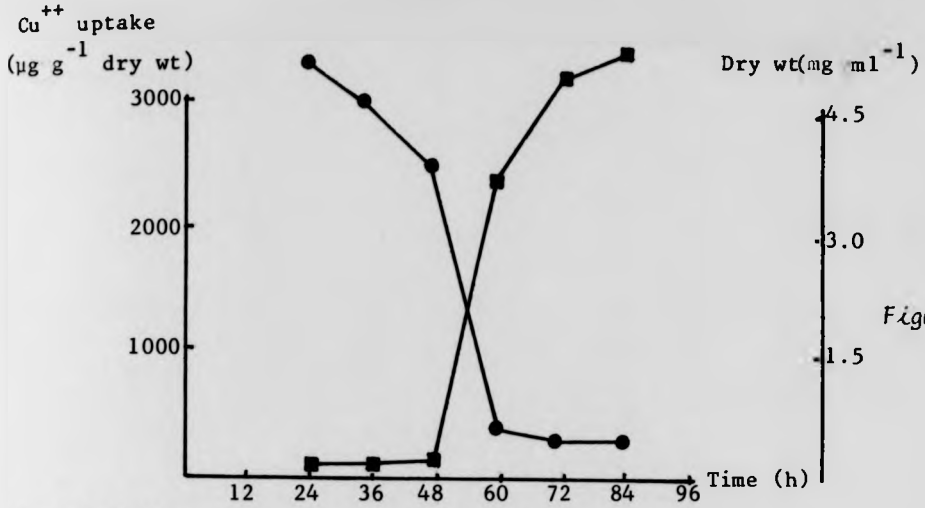
*Table* 7.3.2c.

*Table* 7.3.3.

Influence of initial culture pH on copper uptake by *P. spinulosum* from 2.5 $\mu g\ ml^{-1}\ Cu^{++}$ in batch flask cultures and in fermenter batch cultures.

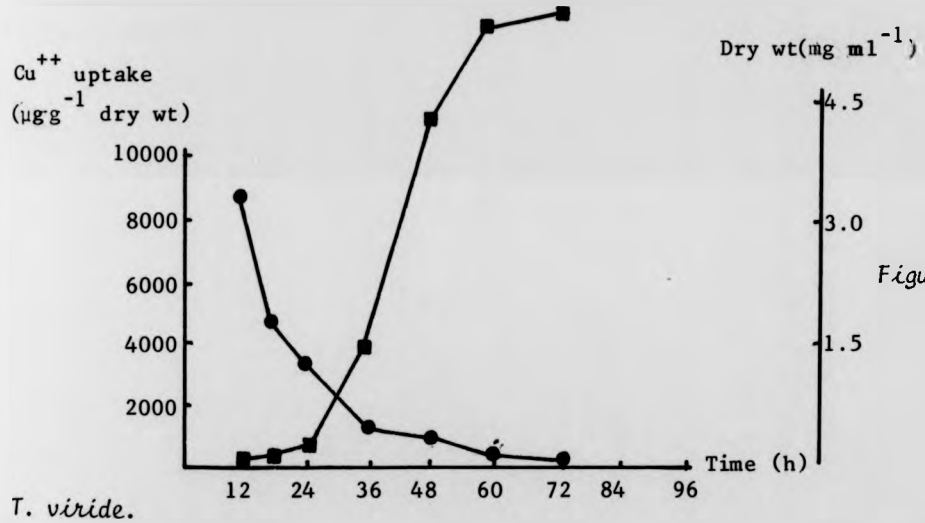
| | $Cu^{++}$ uptake by lag-phase culture. ($\mu g\ g^{-1}$ dry wt.) | $Cu^{++}$ uptake by mid-linear phase culture. ($\mu g\ g^{-1}$ dry wt.) |
|---|---|---|
| Flask culture Initial pH 5.5 | 3625.7 (±72.7) | 470.8 (±22.1) |
| Fermenter culture Initial pH 5.5 | 2983.5 (±311.3) | 413.9 (±13.4) |
| Fermenter culture Initial pH 2.0 | 82.4 (±5.7) | 76.4 (±4.1) |

Values indicated for flask cultures at lag phase are means based on at least 5 replicate flasks (± S.E. of the mean).
Values indicated for flask cultures at mid linear phase are mean values based on at least 10 replicate flasks (± S.E. of the mean).
Results for batch cultures grown in the fermenter are based on the mean of 2 replicate runs for the lag values, and 5 replicate runs for the mid-linear values.



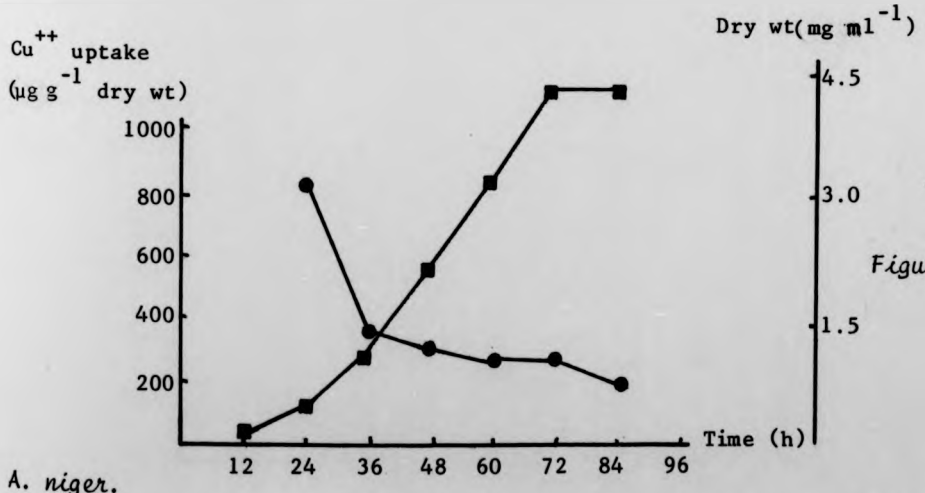

*P. spinulosum.*

Figure 7.3.2.




*T. viride.*

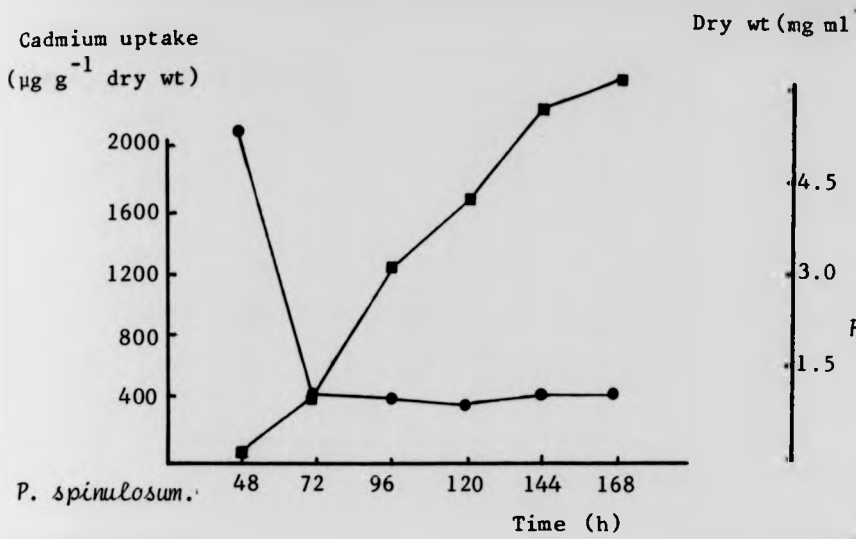
Figure 7.3.3.




*A. niger.*

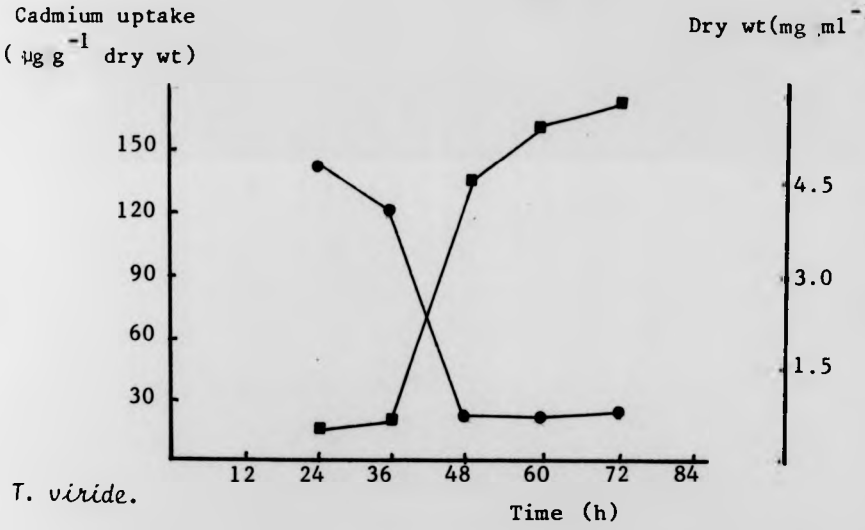
Figure 7.3.4.

*Figures* 7.3.2-4 Copper uptake in stirred-tank fermenter from 2.5 µg ml$^{-1}$ at pH 5.5. ●, copper content; ■, dry wt.



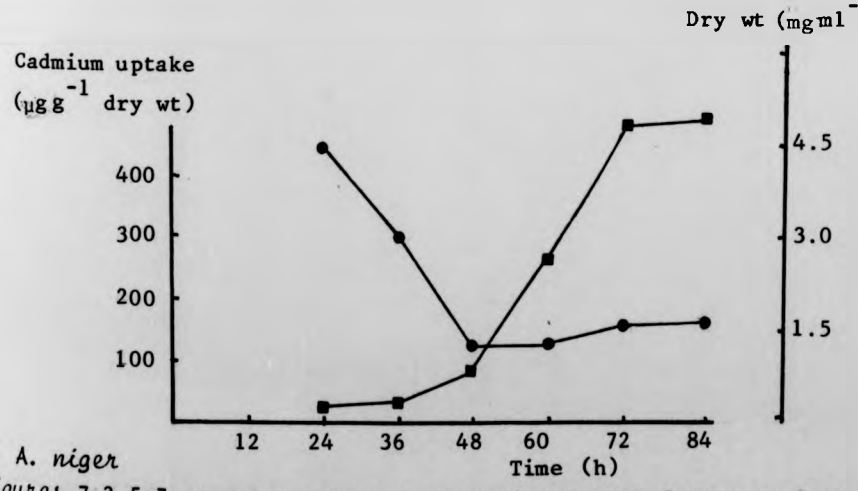

*P. spinulosum.*

*Figure* 7.3.5.




*T. viride.*

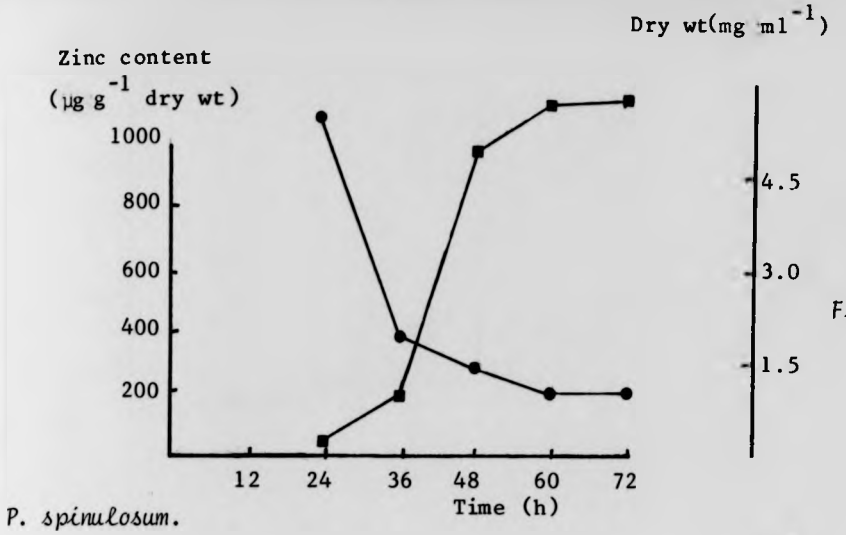
*Figure* 7.3.6.




*A. niger*

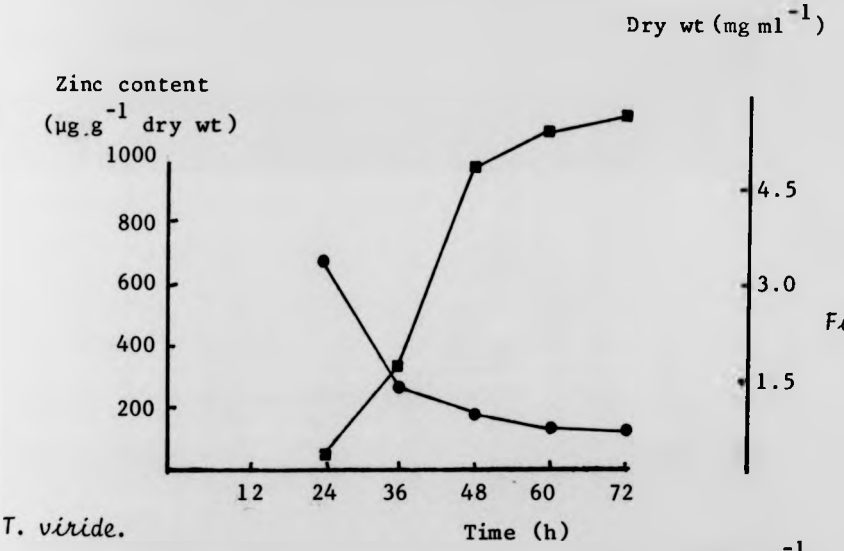
*Figure* 7.3.7.

*Figures* 7.3.5-7 Cadmium uptake in the stirred-tank fermenter from 2.5 µg ml$^{-1}$ at pH 5.5. ●, cadmium content; ■, dry weight.



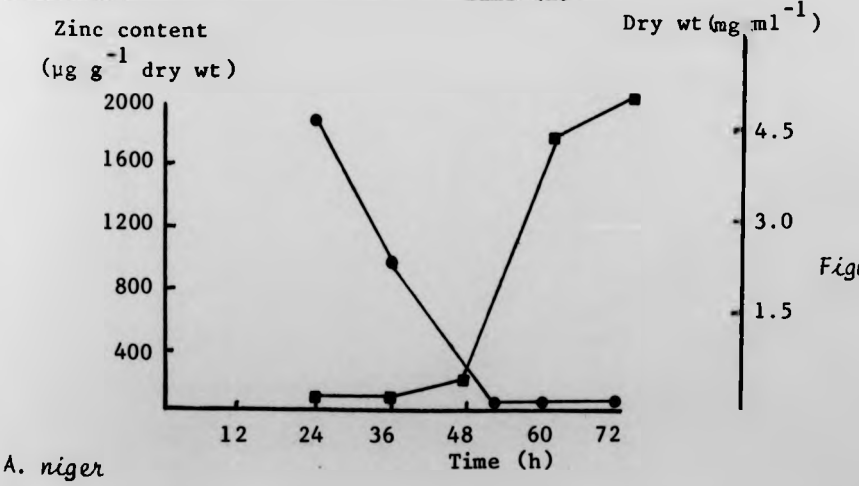

*P. spinulosum.*

*Figure* 7.3.8.




*T. viride.*

*Figure* 7.3.9.




*A. niger*

*Figure* 7.3.10

*Figures* 7.3.8-10 Zinc uptake in the stirred-tank fermenter from 2.5 µg ml$^{-1}$ at pH 5.5. ●, zinc content; ■, dry weight.

Figures 7.3-11-13. Effect of pH on copper toxicity in *P. spinulosum* in batch culture in the stirred-tank fermenter.

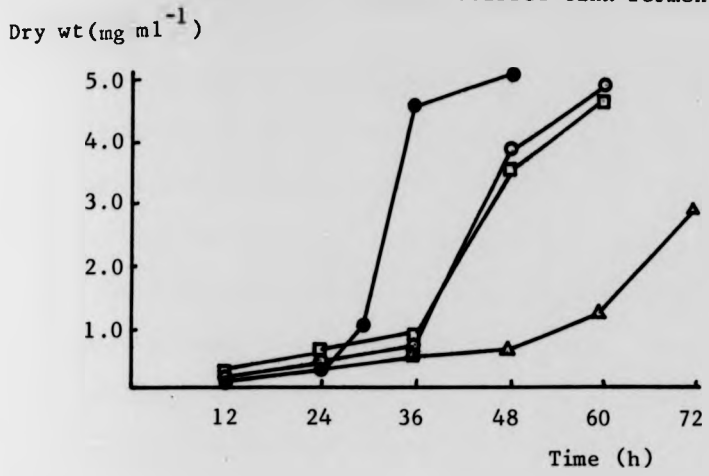



*Figure* 7.3.11

pH 5.5

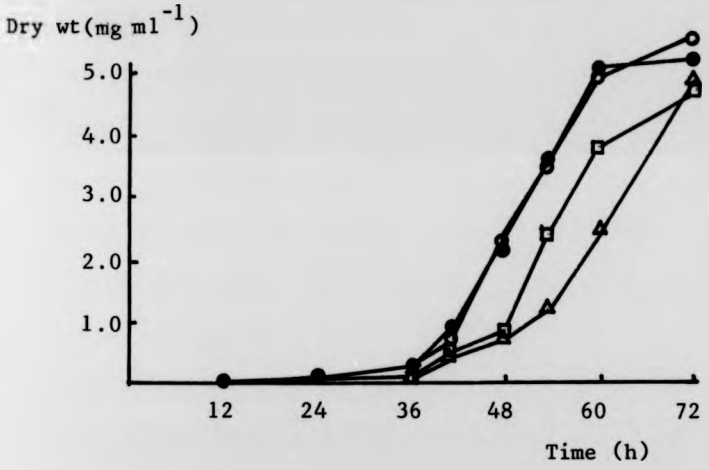



*Figure* 7.3.12

pH 3.5

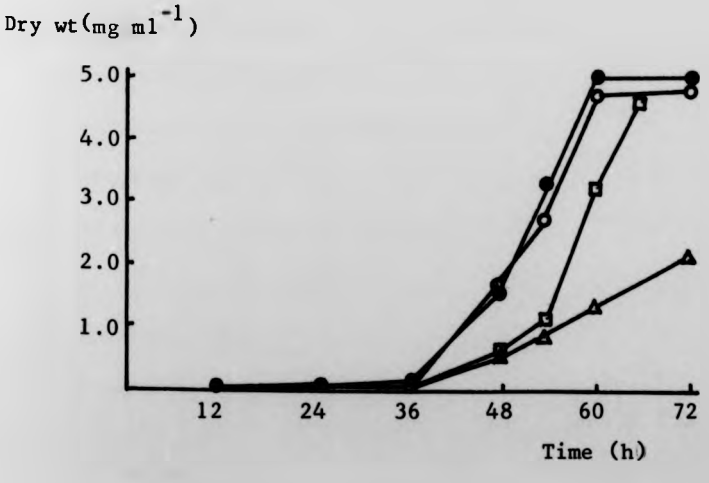



*Figure* 7.3.13

pH 2.0

●, Control; ○, 1.0 μg ml$^{-1}$ $Cu^{++}$; □, 2.5 μg ml$^{-1}$ $Cu^{++}$; △, 5.0 μg ml$^{-1}$ $Cu^{++}$.

7.4. Discussion.

As shown by the mid-linear phase copper uptake levels (Table 7.3.1) in the fermenter with maintained pH, uptake of the metal was pH dependent since copper loading was reduced as the pH was lowered at 1.0, 2.5 and 5.0 μg $ml^{-1}$ added copper. It is interesting though that the uptake of zinc in the same system from the trace element solution was not pH dependent and this is difficult to explain. The pH dependence appeared to be based on the metal status of the medium, that is, when the metal was present in trace amounts there appeared to be no pH dependence whilst in the relatively higher concentrations uptake was dependent on pH. It is possible that the scavenging mechanism at trace concentrations is very efficient and because of the essentiality of copper, the uptake was so effective that it was independent of pH. This may be related to possible differing efficiencies of a number of binding sites; at low concentrations a high affinity binding site may be operational which is independent of pH, perhaps because it is specific for metallic ions. At higher metal concentrations, a low affinity binding site may be operational which could be pH dependent due to a lack of specificity for metal ions in that it could also bind $H^+$ ions when present at high concentrations at low pH. It is interesting to speculate on the mechanism of essential metal uptake from trace amounts in acidophilic organisms. In these cases also, the mechanism for metal scavenging must be very effective and may resemble the proposed high affinity binding system. As shown in Tables 7.3.2a, 2b and 2c. the situation was similar for zinc and cadmium at 2.5 μg $ml^{-1}$ as for copper in that uptake was pH dependent. In virtually all the cases though, the complementary metals present in the trace metal solution were unaffected by pH changes.

Also shown in Table 7.3.1. is the effect of falling pH during growth on mid-linear phase metal levels compared to those at pH 5.5 when maintained throughout the growth cycle in the fermenter studies. The fall in pH during growth produced similar mid-linear phase copper loadings as in the fermenter cultures. This implies that there is very little uptake during linear phase and that the metal taken up in lag phase is not apparently removed by the presence of high ambient concentrations of $H^+$ ions. It is possible that mid-linear phase is more important for consistent regulation of internalisation of metal from its location possibly on the cell wall, rather than *de novo* uptake onto the fungus at this stage.

The uptake of metals in lag phase (Table 7.8.3.), was very pH dependent so it is clear from this data that it is the initial pH which is responsible for influencing lag phase metal loading and this in turn affects mid-linear phase metal contents, implying that there was not much uptake in linear phase. The fall in pH during growth though, did not affect metal loading in mid-linear phase as demonstrated by the similarity of mid-linear phase metal contents at pH 5.5 reducing to 2.0 in linear phase (flask cultures) and maintained pH of 5.5 (fermenter cultures). The metal loading during the whole of the growth cycle (Figures 7.3.2-10) produced from composite data in the fermenter was very similar to that obtained in the flask cultures so this confirms the view that the initial pH is crucial and that pH during the later stages of growth is not so important.

The effect of initial pH on uptake was reflected in the effect of pH on copper toxicity (Figures 7.3.11-13). A lower initial pH, producing a smaller metal accumulation, resulted in decreased metal

toxicity to the fungus. Gadd and Griffiths (1980) reported that decreasing the pH from 4.5 to 3.2 reduced copper uptake in a tolerant and a sensitive strain of *Aureobasidium pullulans*, but the apparent toxicity in percentage terms was less in the tolerant strain due to a lower inherent uptake capacity of the fungus. For the tolerant strain in copper free medium, the pH fell during growth from 4.7 to 2.2. In copper containing medium supporting the tolerant strain the pH fell from 4.5 to 2.2 but at a slower rate. This is not particularly surprising since the growth of the fungus was retarded. For the sensitive strain, in copper free medium, the pH fell from 4.7 to 2.1 as with the tolerant strain, but in copper containing medium it was reduced by a mere 0.5 units to 4.0, but obviously this is a direct result of the inhibition of growth and therefore the fungus could not probably produce and release protons. When the initial pH was 3.2, there was the typical fall in pH with the sensitive strain indicating reduced metal uptake at the lower pH. If the authors had attempted to maintain the pH at the initial level, the resistance in the tolerant strain would have been demonstrated without the added complication of the continuously falling pH value with its concomitant reduction in metal uptake.

There are a number of explanations in the literature on how pH affects metal accumulation in fungi, but it should be remembered that the morphology of the experimental fungi can be quite different at various pH values, often with pelleting occurring at the higher pH values. There may be changes in cell wall architecture or of wall components at the various pH values and there is almost certainly an electrical charge change in the cell wall, which may in fact account for the changes in pelleting characteristics. For the yeast *Candida utilis* Khovrychev

*et al* (1976) reported that at a growth inhibiting pH value of 2.35 leading to a decrease in the population density, the total amino acid content was practically unchanged compared with pH 4.5 but the amounts of glutamate and asparate in the cell decreased. Since glutamate was reduced this also led to a reduction in the amount of arginine since glutamate is an arginine precursor. However, according to Khovrychev *et al* (1978), specific inhibition of protein or RNA synthesis did not occur when the pH of the medium was low. Pirt and Callow (1959) demonstrated an increase in length of hyphae of *Penicillium chrysogenum* with decrease in pH from 7.4 to 6.0 which they interpreted as an increase in the resistance of the cells to shear effects with decrease in pH value. This hypothesis implies that the cell wall structure or composition depends on the pH of the environment during growth. Douglas *et al* (1959) reported the electrophoretic mobilities for spores of *Fusarium solani*, *Mucor ramannianus*, *Bacillus subtilis*, *Penicillium cyclopium* and *P. spinulosum*, over the pH range 1.3 to 12.7 in media of ionic strength 0.05 and at 25°C. Electrophoretically the spores of the 4 fungi were clearly differentiated from one another and this may be important in the diverse quantitative uptake capacities of different organisms. Concerning the chemical nature of the fungal spore surfaces, it was possible to make suggestions by comparison with the equivalent mobilities against pH curves for model particles such as polysaccharide, peptide and lipid. On this basis, the two *Penicillium species*, appeared to have wax or lipid surfaces, possibly with a polysaccharide component in the case of *P. spinulosum*. The curves for *Fusarium solani* and *Mucor ramannianus* suggested quite different surfaces from the two *Penicillium species*. Mobility steps were apparent at pH 2-4 suggesting COOH groups. Norris (1976) reported that electrophoretic studies showed an electronegative charge at low pH

resulting from the dissociation of all surface acidic groups such as carboxyl and phosphoryl. Norris reported that although the functional groups were protonated, the conditions favoured metal-functional group complex formation. It is unfortunate that similar data is not available for whole mycelia instead of spores buttechnical difficulties may militate against this.

Some information on the chemical effects of pH alterations on cell walls is also available. According to Tsezos and Volesky (1982a) the decrease in total uranium uptake by *Rhizopus arrhizus* was attributable to a number of factors dependent on pH since the solution pH affected three parameters. The first parameter was hydrolysis of the chitin amine. Low pH increases $H_3O^+$ concentration and therefore intensifies the competition between $H_3O^+$ and uranium ions for the chitin complexation sites. The second parameter was uranium adsorption, which is a process which depends significantly on the physical and chemical characteristics of the adsorbate. At pH below 2.5, uranium exists as $UO_2^+$, whereas at pH values greater than 2.5 it hydrolyses extensively. Hydrolysis is accompanied by significant reduction in solubility and lower solubility promotes adsorption. Therefore high pH increases adsorption. The third parameter was reported as being hydrolysis of the uranium-chitin complex.

According to Gale and Wixson (1979), carboxyl groups typically found in pectin, and other related polysaccharides which are normal constituents of plant cell walls, carboxyl groups of dicarboxylic amino acids present in proteins and polypeptide complexes, carboxyl groups of fatty acids, the phosphate groups of nucleic acids phospholipids, lipoproteins, and some polysaccharides, sulphydryl and organo sulphate

groups all provide ample anionic sites at physiological pH. Chelate stability, and the ability to displace other cations, is partly a function of ionic radius. Effective ionic radius in turn, is determined partly by the natural tendency of strongly charged substances to become hydrated by building up concentric layers of polarised water molecules. The extent of hydration appears to be related to charge density and this is affected by pH and ionic strengh of the medium. These physical factors and the presence of organic ligands, manifest themselves in the ultimate process of cation exchange among the available anionic sites so obviously the effect of pH on metal uptake is a very complicated phenomenon.

The influence of pH on metal binding sites in *R. arrhizus* has been discussed by Tobin *et al* (1984). At pH 4.0 the primary amines would be positively charged and therefore not be expected to interact with cations. Many of the carboxylate groups would be neutral; however, the $H^+$ ions could be displaced relatively easily by the metal ions. The uptake of the uranyl ion was lower at pH 2.5 than at 3.5, which is consistent with competition for binding sites with protons. Most of the phosphate groups present as mono and diesters would have a negative charge above pH 3.0 and therefore would be strongly involved in metal binding. Hydroxyl and amide functional groups are very weak bases and could only form weak bonds with the metals. It is likely the authors suggested, that most of the metal binding sites contain carboxylate or phosphate ligands or both. These primary bonds may be augmented by association with other groups including hydroxyl groups. If the uptake was due to electrostatic attraction of the solvated ions, then the most important groups at pH 4 would be the phosphates since they would be negatively charged at this pH.

It is not clear how the effect of pH could influence the mycelial metal reduction at early linear phase observed in this study since there appeared to be no effect of pH on total mid-linear phase loading. It is possible that $H^+$ ions could be intimately and significantly involved in internalisation processes such as displacing metal ions from the cell wall in ion exchange processes where the $H^+$ ion is in a relatively high concentration, and the metal ions were localised on the cell wall during lag phase. As the fungus grows and metabolises it could produce $H^+$ ions and this could conceivably displace bound metal ions and therefore influence total metal loading. The fact that there appeared to be no difference in mid-linear phase loading with maintained or falling pH, implies however, that the $H^+$ ions did not reduce the total loading in mid-linear phase although they may still have been involved in the displacement of metal ions which could then be internalised. Although the total loading was not affected in this study by the pH drop, there is evidence in the literature that $H^+$ ions can indeed displace metal ions from microbial cells. Blocking of metal binding sites by $H^+$ ions would explain the observation of Douglas *et al* (1959) in that reducing the pH from 5.5 to 2.0 caused a reduction in the electrophoretic mobility to the anode for all the organisms he tested, that is, the surface charge became less negative and this would result in reduced metal binding. It is likely that high metal ion binding in lag phase would also affect the surface charge in a similar way and reduce further binding in linear phase as a consequence. Both $H^+$ and metal ions are probably involved in this reduced metal binding during linear phase. Other workers including Marquis *et al* (1973) have reported that in *Streptococcus species* cell walls have a tendency to bind $H^+$ ions over metal ions. Treen-Sears *et al* (1984) suggested that mycelium of *R. arrhizus* sequestered essential metals, thereby making them unavailable

for growth. However, as the pH dropped during the period of acid production, metal displacement occurred and continued slow growth was observed. This would be expected since biosorption in *Rhizopus sp.* is an ion-exchange-complexation phenomenon and is pH dependent (Treen-Sears *et al*, in press). This agrees with the suggestions for $H^+$ ions in metal uptake in this study. Useful information on the effects of $H^+$ efflux on metal displacement and internalisation could be obtained by monitoring metal loading on the internal and external portions of the fungus whilst measuring $H^+$ efflux in growing cultures. This would however be technically very difficult due to the problems of making flux measurements on dynamic systems and the difficulties of separating external and internal uptake since most chelating substances used to quantify external removable metal do not necessarily remove tightly bound metal and may also disturb internal ionic metal status. There is also the problem of heterogeneity of portions of the hyphae in growing mycelium, for instance, the actively growing tip is very likely to be quite distinct from the older parts of the mycelium in terms of components, architecture and metal uptake systems. Useful information could be obtained on internal and external metal uptake by looking at the effects of pH on metal uptake by isolated protoplasts and cell walls. The merits of these techniques are discussed in a later section.

Although not strictly comparable to divalent cation uptake, it is interesting that monovalent cation influx such as that of potassium in *Neurospora sp.* is accompanied either by the efflux of sodium and hydrogen ions during net transport, or by efflux of potassium ions during steady state exchange. According to Slayman and Slayman (1970) at low extracellular pHs such as 4.6, net uptake of potassium was a simple exponential process obeying Michaelis kinetics. At high pH however,

(8-9), potassium uptake became considerably more complex, and could be resolved into two distinct components. The so called fast component (time constant 1.2 minutes) was matched quantitatively by a rapid loss of sodium attributable to ion exchange within the cell wall. The slower component (10.9 minute time constant) was thought to be a carrier mediated mechanism. The fast component including the exchange of ions may well be a similar process to that occurring in lag phase in this study in growing fungi.

It is clear that further information on various aspects of metal uptake in *P. spinulosum*, *T. viride* and *A. niger* is required and one important factor is localisation on the cell wall for example, particularly in lag phase. It would also be very useful to know how many binding sites in the cell wall are present, since the possibility of pH affecting a low affinity binding site only, would imply that there is more than one site available. Kinetic analysis of the initial binding would also be of use here. Also the mechanism of internalisation requires elucidation to determine if there is any metabolic involvement. Protons could of course be involved in delocalising metal ions from the cell wall independent of internalisation. These factors will be investigated in the subsequent sections of this study.

## 8. HEAVY METAL ACCUMULATION BY NON-GROWING SUSPENSIONS OF FILAMENTOUS FUNGI.

### 8.1.1. General Introduction

In the preceding sections of this study some interesting aspects of quantitative metal accumulation have been demonstrated in growing filamentous fungi. Unfortunately, metal transport processes in growing cells are masked by the growth kinetics of the organism, and hence it is impossible to perform flux measurements on dynamic systems. Also experiments designed to monitor transport in filamentous fungi which involve temperature effects, a variety of substrates and metabolic inhibitors, militate against the measurement of metal transport in growing mycelium, since these treatments in themselves would affect the metabolic activity of the cell, and hence modify the metal transport system if energy was involved. Thus, the use of non-growing suspensions of fungi simplifies the study of transport processes.

Most workers investigating metal uptake in non-growing suspensions have shown by kinetic analysis that uptake is a multiphasic system, involving wall binding components, usually as a prerequisite to intracellular accumulation, which in some cases has been shown to be energy dependent. Wakatsuki *et al* (1979) have demonstrated the uptake parameters for copper in the yeast *Debaryomyces hansenii* over a 120 minute incubation period. According to these authors a biphasic uptake system involving rapid binding onto the cell wall was followed by intracellular transport in this yeast. However, the data of Ross (1977) shows that exposure of a non-growing suspension of cells of *Saccharomyces cerevisiae* to 1.02 μg $ml^{-1}$ copper resulted in 90% loss of viability within 10 minutes. It is likely therefore that the high concentration of copper 6.35 μg $ml^{-1}$ used by Wakatsuki *et al* (1979) would have resulted in substantial cell death. Some of the

experiments of Wakatsuki *et al* (1979) were carried out in 0.9% NaCl. It is conceivable that the toxicity of copper was reduced by the formation of Cu-Cl complexes. Reduced toxicity of zinc (Babich and Stotzky, 1978) and cadmium (Babich and Stotzky, 1982) as a result of Zn-Cl or Cd-Cl complexes has been reported but data is not available for copper. As Wakatsuki *et al* (1979) do not give data on cell viability, their data must be treated with caution.

Schneider and Lindberg (1983) showed that zinc uptake in *Neurospora* was a biphasic uptake process with an initial rapid phase which lasted for 1 minute and was followed by a slower mechanism which was linear for at least 10 minutes. Budd (1969) showed that $K^+$ uptake in *Neocosmospora vasinfecta* was dependent on metabolic energy and kinetic analysis showed that there were two kinetically distinct uptake mechanisms for $K^+$ (Budd,1975). Budd (1979) reported similar mechanisms for $Mg^{++}$ uptake in the same fungus. Paton and Budd (1972) showed that zinc uptake was also a biphasic process in *Neocosmospora vasinfecta*, with the internalisation phase being energy dependent. Le John *et al* (1974) investigated non-growing intact cell suspensions for calcium transport studies and the influence of cytokinins and sulphydryl group reacting agents in *Achlya sp.* Again uptake was dependent on two components, with a low molecular weight calcium binding glycopeptide in the cell wall being involved in the initial uptake phase mechanism, and a calcium uptake component associated with the cell membrane as the second uptake phase.

The study of metal uptake in non-growing cells is justifiable also with regard to the recent resurgence in interest in the possibility of using

non-growing cells for metal recovery from industrial effluents containing low levels of metallic residues. Many industrial effluents contain toxic substances other than metals which would preclude the use of growing cells for the treatment of many metalliferous effluents. The use of suspensions of fungi has received a lot of study for metal recovery purposes perhaps due to their relative ease of handling when compared to bacteria.

Tsezos and Volesky (1981, 1982a, 1982b) and Tsezos (1983) have used suspensions of *Rhizopus arrhizus* to demonstrate uranium and thorium uptake by the fungal material. Using various techniques such as X-ray energy dispersion analysis, electron microscopy and infra red spectroscopy and by producing adsorption isotherm plots, the authors reported that uranium uptake was a three phase uptake system whilst that for thorium was a two phase system.

Tobin *et al* (1984) using *R. arrhizus* produced a biosorbent material from 70h old cells by washing and grinding them and then resuspending the product in metal solutions including $La^{+++}$, $Mn^{++}$, $Cu^{++}$, $Zn^{++}$, $Cd^{++}$, $Pb^{++}$, $UO_2^{++}$ and $Ag^{+}$. This material, although not intact, appeared to behave in some respects as a non-growing suspension of biomass. Muzzarelli *et al* (1980) have produced biosorbents from *Aspergillus niger* by manufacturing metal sequestering columns of chitosan-glucan polymer extracts. Treen-Sears *et al* (1984) have also used formulations of broken portions of mycelium of *Rhizopus javanicus* and they showed that the status of the growing mycelium prior to these treatments was important. For example, the eventual metal uptake capacity of the suspended mycelial material was improved by adding mineral salts to the medium once growth had commenced. Galun *et al* (1983) studied the uptake of uranium by suspension of *Penicillium digitatum*, and demonstrated its inhibition by iron (Galun *et al* 1984). They further

emphasised the potential for metal recovery by fungal suspensions by recycling the material after removing uranium from preloaded biomass with alkali carbonates, EDTA, ammonia and organic acids. Suspensions of various fungi were used by Drobot (1981) (U.S. Patent 4,293,333) to recover a range of metals from aqueous solvents. The author suggested that growth could be promoted in the effluent by the addition of nutrients to the contacting solution including oils, alcohols, citric acid, ammonium salts, amino acids and a co-precipitate of calcium carbonate and calcium citrate to provide inorganic carbonate and organic nutrients. Alternatively the author suggested that non-growing biomass could be added directly to the effluent to effect metal recovery. In either case the growth status of the fungi used in these trials was not easy to determine from the information provided by the author. Drobot and Lechavelier (1981) (U.S. Patent 4,293,334) suggested that metals could be recovered from aqueous solutions with killed fungi.

The primary objective of studying metal uptake in non-growing cell suspensions, was to investigate the metal transport process with particular emphasis on elucidating the role of the metabolism, if any, by observing the effects of the presence of metabolic inhibitors, the presence and absence of glucose and the influence of temperature on metal uptake. Further, these studies were designed to investigate the role of the cell wall in metal uptake in non-growing cells by studying competitive uptake with other divalent cations, by observing uptake by cell wall and chemically modified cell wall preparations, and by studying initial binding kinetics. Information on the molecular relationships of metals with non-growing fungal cells was obtained by performing electron spin resonance (ESR) measurements on metal loaded fungal cells. This work is included in the appendix.

## METAL ACCUMULATION BY NON-GROWING SUSPENSIONS OF FILAMENTOUS FUNGI - METABOLIC DEPENDENCE AND CELL WALL INVOLVEMENT.

### 8.1.2. Introduction.

There appears to be little information in the literature on metal localisation in filamentous fungi and that which is available has typically been obtained by one of two reported techniques. The first technique relies on the fact that heavy metals are electron dense and can therefore be detected by electron microscopic techniques. Lindegren *et al* (1972) reported that copper used as a stain in electron microscopy studies appeared to attach to the nuclei and chromosomes in yeasts. Precipitation of mercury in electron dense bodies has been noted in the hyphae of *Chrysosporum pannorum* (Williams and Pugh, 1974). Similarly, Paton and Budd (1972) have demonstrated electron dense bodies in *Neocosmospora vasinfecta* after growth in zinc containing medium.

The other technique has been used to determine the amounts of metal which is desorbable from the fungus and therefore assumed to be located on the outside of the cell. Non-desorbable metal is that which is either tightly bound or internalised.

Paton and Budd (1972) have determined the amount of zinc which is desorbable from *Neocosmospora vasinfecta* after zinc accumulation from zinc containing medium. This removable zinc was attributed to surface binding of zinc which the authors termed phase 1 uptake. Horikoshi *et al* (1981) have reported that most of the absorbed uranium on actinomycetes was released by washing the organisms with an EDTA solution, suggesting that the accumulation of uranium depended on physico-chemical adsorption at the cell surface, rather than on biological activity, and that uranium

is coupled to the cells by ligands which were easily substituted with EDTA. Venkateswerlu and Sivarama Sastry (1970) reported that the maximal amount of $Co^{++}$ leached out by EDTA from mycelia that had taken up cobalt was about 30%, was rapid and largely completed within 30 minutes. The authors suggested that loss of intracellular $Co^{++}$ due to the flotation of mycelium in EDTA was quite small and that it was probable that the $Co^{++}$ leached out by EDTA was predominantly surface bound metal ion. The elution of toxic cations was studied by Lowry *et al* (1957) from ascospores of *Neurospora sp.* and the authors showed that ATP, dihydroxyphenyl alanine and EDTA were the most successful. They suggested that since cations are restricted to the exterior of dormant ascospores, the adsorption sites on the surface of the cell can serve as a reservoir of ions which can penetrate the cell as soon as germination of the cell begins. Tests of the relative efficiency of various washing treatments (water, 0.1N HCl, non radioactive $CuSO_4$, 0.1% cetyltrimethyl-ammonium bromide and $1X\ 10^{-2}$ M EDTA) in removing the copper from *Neurospora crassa* and *Alternaria tenuis* spores showed that the two fungal species differed (Somers, 1963). The copper binding sites on *A. tenuis* were much more accessible to exchange and chelation than those on *N. crassa*. The exchangeable copper has fungistatic properties since Müller and Biedermann (1952) have shown that the germination of copper treated spores of *Alternaria tenuis* can be restored by EDTA treatment. Galun *et al* (1983) have shown that uranium adsorbed in a precultured mycelial biomass was 94 to 99% extractable by alkali carbonates, whilst the amount removable by EDTA was only 61%. Norberg and Persson (1984) also reported similar release of metals from flocculated particles of *Zoogloea ramigera* using acid treatments and investigated the possibility of recycling desorbed biomass. It is interesting that according to Galun *et al* (1984), the effectiveness of fungi as extractants was concentration dependent but after the initial EDTA treatment, the capacity

for adsorbing uranium from $UO_2Cl_2$ increased markedly. On the other hand, Schneider and Lindberg (1983) reported that uptake of zinc by *Neurospora crassa* was inhibited by a previous exposure to 0.1 mM EDTA and the authors suggested that the EDTA caused a loss of a required protein. Similarly, Somers (1966) reported that *N. crassa* conidia which were briefly washed in a non-toxic concentration of hydrochloric acid and then reincubated with solutions of copper sulphate accumulated less metal and were also less susceptible to copper. This was attributed to copper exclusion due to the binding of hydrogen ions to the superficial cation receptor sites.

In this study, it was decided to investigate the role of the cell wall in metal accumulation by apportioning uptake into desorbable and non-desorbable uptake, using EDTA as the desorbent. The mechanism of internalisation was investigated by attempting to demonstrate metabolic dependence by observing the effects of glucose, metabolic inhibitors and temperature reduction on metal accumulation.

The metabolic dependence of metal uptake has been reviewed for the yeasts by Borst Pauwels (1981). Glucose appears to stimulate copper uptake in *Saccharomyces cerevisiae* (Ross, 1977), zinc uptake in *Candida utilis* (Failla and Weinberg, 1977), zinc uptake by *Saccharomyces cerevisiae* (Ponta and Broda, 1970) and cadmium and cobalt uptake in *S. cerevisiae* (Norris and Kelly, 1977). According to Paton and Budd (1972), in the filamentous fungus *Neocosmospora vasinfecta*, glucose inhibited phase 2 uptake from 65 $\mu g\ ml^{-1}$ at all concentrations tested from 0.1 mM to 90.0 mM during 20 minutes. Phase 2 uptake was that which was energy dependent. Inhibition was curvilinear with respect to glucose concentration and amounted to 46% at90.0mM glucose. Treating the mycelium for 15 minutes

in 1.0 mM glucose inhibited subsequent phase 2 uptake by 18%, but interestingly, if 5.0 mM phosphate at pH 6.5 was also present during the incubation, this inhibition fell to 10% (Paton and Budd, 1972). It is also of interest that Rothstein *et al* (1985) reported that 25% of the added manganese to fresh baker's yeast was surface bound in less than 3 minutes, regardless of glucose additions to the medium. However, in the presence of phosphate and glucose, there was, in addition to the surface binding, a continuous absorbtion of manganese which was markedly stimulated by $K^+$.

Metabolic inhibitors appear to affect the two phases of uptake in different ways. Somers (1963) used two techniques to determine the effect of respiratory inhibitors and thiol reagents on copper uptake by *Neurospora crassa* conidia. Both methods (see discussion for details) showed that whilst these agents reduced copper uptake, the mechanism was unaffected by the uncoupler of oxidative phosphorylation, 2-4 dinitrophenol (DNP). Paton and Budd (1972) demonstrated that in phase 1 of zinc uptake in *Neocosmspora vasinfecta* (i.e. surface binding) there was no effect on adding $NaN_3$, but phase 2 (energy dependent uptake) was stongly inhibited though not completely at 0.1 and 1.0 mM $NaN_3$ and this was not fully reversible. Norris and Kelly (1977) demonstrated the effect of CCCP on metal cation accumulation in *S. cerevisiae*. Surface binding was independent of the uncoupler but the intracellular accumulation was depressed by CCCP. It is worthy of note that Schneider and Lindberg (1983) showed that both the rapid and slow phases of zinc accumulation in *N. crassa* were inhibited with azide but this may have been as a result of complexing of zinc by the azide. Le John *et al* (1977) reported that $Ca^{++}$ transport was insensitive to metabolic inhibitors. Horikoshi *et al* (1981) showed that the uptake of uranium by *Actinomyces levoris* and *Streptomyces viridochromogenes* was

not affected by a 30 minute treatment with DNP or $NaN_3$ and uptake was attributed to a non-metabolic process.

The effects of temperature on metal uptake have been shown to be similar to the effects of metabolic inhibitors. The uptake of heavy metals in relation to temperature has been studied in *Saccharomyces cerevisiae* and *Pseudomonas aeruginosa* by Shumate *et al* (1978). These authors showed that metal uptake was temperature dependent and that there was an optimum temperature for metal uptake. Zinc accumulation and the influence of temperature has been demonstrated in *C. utilis* (Failla and Weinberg, 1977). Also effects of temperature have been demonstrated in *S. cerevisiae* for copper uptake (Ross, 1977) and in *S. cerevisiae* for zinc uptake (Ponta and Broda, 1970). Similar dependencies for cadmium uptake have been shown in the alga *Chlorella pyrenoidosa* by Hart and Scaife (1977) and in lichens by Beckett and Brown (1984).

The effect of temperature has been studied for $Cd^{++}$ uptake in *Achlya sp.*(Le John *et al*, 1974) and for $Cd^{++}$ uptake in *Aureobasidium pullulans* (Gadd and Mowll, 1983). Also the influence of temperature has been demonstrated for $Zn^{++}$ uptake in *Neocosmospora vasinfecta* (Paton and Budd, 1972), for $Cu^{++}$ uptake in *A. niger*, *Penicillium glaucum* and *Dematium pullulans* (Sato, 1939), and for thorium and uranium uptake in *Rhizopus arrhizus* (Tsezos and Volesky, 1981, 1982a, 1982b and Tsezos, 1983). The importance of temperature in commercial recovery of platinum, iridium, ruthenium, rhodium, gold, iron, zinc, copper, aluminium and nickel in *Cladosporium sp.*, *Penicillium sp.* and 2 black fungi has been emphasised in two U.S. patents (Drobot 1981, Drobot and Lechavelier, 1981).
The effect of temperature on zinc uptake in *A. niger* has been studied

(Babich and Stotzky, 1978) and on copper and mercury uptake by a chitin-chitosan complex of A. *niger* (Muzzarelli *et al*, 1980). The influence of temperature on metal uptake in *P. spinulosum*, *T. viride* and A. *niger* was investigated to elucidate the effect of temperature on metal uptake with particular emphasis on whether metabolic energy was involved in uptake by non-growing cell suspensions.

Similarly, the effects of anaerobic incubation on metal uptake have been investigated with regard to distinguishing metabolic uptake from passive uptake, but there appear to be some conflicting reports in the literature. Ponta and Broda (1970) showed that zinc uptake into the interior of the cell in *S. cerevisiae* did not strongly depend on the presence of air, but this is not surprising since under conditions of low oxygen tension, energy would be derived from glucose fermentation. Energy dependent zinc uptake would be more dependent on the glucose concentration. Baldry and Dean (1980b) reported that copper uptake during batch culture of *Escherichia coli* was reduced under conditions of oxygen shortage. Other reports however, contradict this and Somers (1963) showed that the uptake of copper by conidia of *N. crassa* was 25% greater in a $N_2$ atmosphere than under standard aerobic incubation. In *Neocosmospora vasinfecta* Paton and Budd (1972) showed that although anaerobiosis had no effect on surface binding of zinc initially, after 3 hours of anaerobic incubation subsequent surface binding was reduced by 25 to 30%. Normal aeration caused the amount of surface bound uptake to increase slightly. This implies that the binding sites are metabolically turned over. Anaerobiosis produced the strongest inhibition of phase 2 (energy dependent) zinc uptake, amounting to 88% after 2 hours. However, in this case, uptake was fully restored on returning to aerobic conditions, even after 230 minutes of anaerobiosis. According to McBrien (1980), the toxicity of copper is

substantially greater when the metal ion is applied to cells under anoxic conditions. The increase in toxicity occurs because Cu(11) is reduced to Cu(1) which is stable under anoxia and is more toxic than the oxidised species, and not because of increased uptake under anaerobic conditions. According to McBrien, anoxic potentiation has been observed in mammalian cells in tissue culture, in bacterial, algal, fungal and in yeast cells.

The main objective of this section of the work was to determine if there was any metabolic dependence in metal uptake in non-growing suspensions of filamentous fungi. Another aim was to determine if the cell wall was involved in localisation of metals in fungi, and to attempt to quantify metal internalisation using desorption techniques. The rationale was that over a 2 hour incubation period, if the metal was intracellularly accumulated, the proportion of non-desorbable metal ought to increase and the proportion of desorbable metal would decrease.

## 8.2. Materials and Methods

### 8.2.1. DEVELOPMENT OF EXPERIMENTAL PROCEDURES

#### 8.2.1.1. Preparation of non-growing fungal suspension.

The aim was to produce a standard slurry of uniform consistency of mid-linear phase fungus of known water content, in which the density of mycelium was such that typically less than 10-15% metal removal occurred from experimental metal solutions in uptake trials.

Following 2 subcultures in 100 ml GMS medium, a series of 1 litre flasks containing 400 ml GMS medium was inoculated with 10 ml of mid-linear phase fragmented culture. The inoculum density was higher than for normal culturing to achieve a denser culture consisting of smaller mycelial units. Cultures grown in this way reached mid-linear phase after 48 hours of standard incubation.

After 48 hours of growth, the cultures were filtered and washed with 2 X 100 ml distilled $H_2O$. The mycelium obtained from 2 cultures was bulked by resuspending the contents of the 2 flasks in 400 ml 50.0 mM MES (pH 5.5) at 25$^{o}$C and reincubated for a period of time; this incubation period was normally 30 minutes to allow equilibriation, but when starved cultures were required this period was extended to 12 hours. After these periods, the filtering and washing procedure was repeated to remove any metabolites which may have been released during the incubation. Resuspending in fresh MES produced a mycelial slurry which was ready for use.

#### 8.2.1.2. Liquid content of slurry.

The liquid content of the mycelial slurry had to be determined prior to addition to metal solutions so that corrections in the metal concentrations could be made.

25 ml samples of the suspension were collected and filtered to apparent dryness and the filtrate was retained. The volume of the filtrate was measured to determine the liquid content of the 25 ml volumes of slurry. The resulting volume was used to calculate the additional aqueous content of the experimental uptake flasks when 5.0 ml of the slurry was added to 100 ml volume of MES plus metal solution. Before addition of the fungal suspension, appropriate adjustments of the volume of metal solutions added were made to correct for the extra liquid content. 5.0 ml of the fungal suspension typically was suspended in 3.5 ml water for all fungi tested (i.e. 70% water). It should be remembered that this water content does not relate to the actual water content of the fungal protoplasm, it refers only to the water involved in suspending the fungal pellets.

8.2.1.3. Effect of culture age on copper uptake by *Penicillium spinulosum*.

Separate unstarved mycelial suspensions were prepared from actively growing *P. spinulosum* cultures at 36, 48 and 60 hours of growth after inoculation so that mycelium in early, mid and late linear phase was obtained. In this way the effect of culture age on copper uptake could be investigated. To twelve, 250 ml conical flasks containing 100 ml 50.0 mM MES (pH 5.5) plus 10.0 mM glucose and supplemented with copper chloride to a final concentration of 2.5 $\mu g\ ml^{-1}$, 5 ml aliquots of the 36 hour fungal suspension were added and incubated at 25°C with shaking at 200 rpm. After 5, 30, 60 and 120 minutes of incubation, 3 flasks were selected and the mycelium collected for copper analysis. Similarly, copper uptake profiles were obtained for the 48 and 60 hour old mycelium.

8.2.1.4. Determination of the optimum desorbing medium for recovering accumulated metal from metal loaded biomass.

The aim of these experiments was to determine the most efficient desorption medium for removing metal from metal-loaded biomass. By

investigating desorption after 5 minutes and 2 hours incubation in metal, it should be possible to determine whether or not metal is internalised over 2 hours and to what extent. That is, if metal is internalised in 2 hours, then a smaller percentage of total metal should be recoverable than would be the case for the 5 minute incubation.

An unstarved mycelial slurry was prepared as described previously and 5 ml were added to each of thirty 250 ml conical flasks containing 50.0 mM MES with added copper to a final concentration of 2.5 $\mu g\ ml^{-1}$ (corrected for the mycelial slurry water content) at pH 5.5. All flasks were incubated at 25°C with shaking at 200 rpm. Batches of 3 replicate flasks were used for each treatment with a 4 minute stagger between batches to facilitate management of sampling.

After 5 minute and 2 hour incubations in copper, the batches of copper loaded mycelium were separated from the copper solution by filtration. The samples were washed with 200 ml of distilled $H_2O$ and split into 2 portions. One portion of each of the 3 replicate samples for both incubation times were placed in preweighed collection flasks for dry weight and copper determinations. The remaining portion of each of the 3 replicate samples was placed in one of 5 desorption media. The desorption media used were distilled $H_2O$, basal GMS medium at pH 5.5, 50.0 mM MES plus 1.0 mM EDTA at pH 5.5, phthalate buffer at pH 4.0 and basal medium plus 1.0 mM EDTA at pH 5.5. These were incubated in the standard way for 15 minutes. The biomass was then filtered and the dry weights and copper contents determined. Potassium contents of each sample were monitored to check for membrane damage caused by EDTA. The dilutions necessary for $K^+$ measurements were made in a 1000.0 $\mu g\ ml^{-1}$ lanthanum chloride solution to prevent interference in A.A.S. determinations. The experiment was repeated for cadmium and zinc.

8.2.1.5. Determination of optimum EDTA concentration and treatment time for copper desorption from *P. spinulosum*.

The aim of these experiments was to determine the most appropriate concentration of EDTA for copper desorption from copper loaded biomass and to determine the optimum desorbing time. 24 portions of unstarved copper loaded *P. spinulosum* were prepared using the aforementioned method. Copper loading was carried out using a 30 minute incubation in 2.5 μg $ml^{-1}$ $Cu^{++}$ solution. Three biomass samples were used directly for dry weight determinations and copper content analyses. Three replicate portions from the 21 remaining, were used for each EDTA treatment. 15 minute incubation treatments were carried out in 50.0 mM MES at pH 5.5 containing 0.1, 0.25, 0.5, 1.0, 2.5 and 5.0 mM EDTA and also in 1.0 mM EDTA alone, after which the mycelium was collected for dry weight determination and copper analysis. The experiment was then repeated using 50.0 mM MES containing 1.0 mM EDTA but with 5, 10, 15, 20, 30 and 40 minute desorption times to determine the optimum desorption period.

8.2.1.6. Effect of starvation on copper uptake from 5.0 μg $ml^{-1}$ Copper by *Penicillium spinulosum*.

The aim was to determine the effect of varying lengths of starvation on subsequent metal uptake capacity and also to determine the effect of starvation on desorption of accumulated copper. Three replicate 1 litre Ehrlenmyer flasks containing 400 ml of a dense suspension of *P. spinulosum* in 50.0 mM MES were prepared and incubated in the normal way. After 1, 2, 4, 6, 12, 24, 48 and 96 hours of incubation, 10 ml of the starving culture were withdrawn from each of the 3 replicate flasks and suspended in 3 separate 250 ml Ehrlenmyer flasks containing 100 ml 50.0 mM MES (pH 5.5) to which had been added copper chloride to a final concentration of 5.0 μg $ml^{-1}$ (corrected). The 3 replicates were then incubated for 2 hours. The copper

loaded biomass was then filtered and washed with 200 ml distilled $H_2O$. Half the samples were used directly for dry weight determinations and copper analyses, whilst the other portions of the samples were resuspended in desorption medium and then analysed. All subsequent experiments utilised unstarved mycelium unless otherwise stated.

8.2.1.7. Effect of glucose concentration on copper uptake from 5.0 μg $ml^{-1}$ copper by a non-growing suspension of *Penicillium spinulosum*.

The aim of the experiment was to determine if glucose at a range of concentrations from 0.55 to 111.11 mM influenced copper uptake by a non-growing 12 hour starved culture of *P. spinulosum*. To each of eighteen 250 ml Ehrlenmyer flasks containing 100 ml 50.0 mM MES (pH 5.5) plus varying amounts of glucose and containing copper at 5.0 μg $ml^{-1}$, was added 5.0 ml of the fungal slurry. MES containing copper but no glucose was used in control flasks. Batches of 3 replicate flasks at each glucose concentration were employed. The suspensions were incubated for 2 hours in the copper solutions and then analysed for copper. All subsequent experiments, including ones which investigated the effects of glucose on metal uptake, utilised unstarved mycelium since the presence of glucose did not appear to enhance uptake in starved mycelium.

8.2.1.8. Effect of anion component on zinc and copper uptake by non-growing *Penicillium spinulosum* from 5.0 μg $ml^{-1}$ metal in 50.0 mM MES and GMS medium.

The objective of this experiment was to determine whether the anionic component of added metal salts (chloride or sulphate) had any influence on the quantitative uptake of the metal cation both in MES and in GMS medium. An unstarved mycelial suspension was prepared. Twelve 250 ml Ehrlenmyer flasks containing 100 ml 50.0 mM MES (pH 5.5) plus 10.0 mM glucose and twelve containing 100 ml GMS medium were supplemented with metal

solutions to produce final concentrations of 5.0 μg $ml^{-1}$ $Cu^{++}$ or $Zn^{++}$. Batches of 3 replicate flasks of MES and 3 of GMS medium were supplemented with zinc sulphate. Further batches of 3 replicate flasks for each medium were prepared using zinc chloride, copper sulphate and copper chloride. To each batch of triplicate flasks, 5.0 ml of the mycelial suspension was added and these were incubated under standard conditions for 2 hours. The biomass was filtered and analysed for metal using AAS.

8.2.1.9. Effect of metabolic inhibitors on copper uptake from 2.5 μg $ml^{-1}$ by *Penicillium spinulosum* with preincubation with inhibitor and simultaneous incubation with inhibitor and copper.

The aim of the experiment was to determine the effect of metabolic inhibitors on copper uptake, when fungi were preincubated in the presence of the metabolic inhibitor and when the metabolic inhibitor was included in the experimental flask. 600 ml of a *P. spinulosum* slurry were prepared. 200 ml of this suspension were used for the preincubation experiments, 200 ml for controls with no inhibitor treatment and 200 ml for the simultaneous copper and metabolic inhibitor experiments.

To each of twelve 250 ml Ehrlenmyer flasks containing 99.0 ml 50.0 mM MES at pH 5.5 plus 10.0 mM glucose, was added 1.0 ml of a 100.0 mM solution of sodium monoiodoacetate to produce a final concentration of 1.0 mM inhibitor. The pH was adjusted to 3.5 with concentrated HCl. Twelve flasks containing 1.0 mM 2,4 DNP and 1.0 mM azide were also prepared but adjusted to pH 5.5. 5.0 ml of the fungal slurry were added and incubated for 30 minutes under standard conditions. After the 30 minute incubation in the presence of inhibitor the mycelium was filtered and washed with distilled $H_2O$ and resuspended in further flasks containing 100 ml MES

(50.0 mM, pH 5.5) at pH 5.5 plus 10.0 mM glucose containing 2.5 μg $ml^{-1}$ copper. After 5, 30, 60 and 120 minutes of incubation, 3 replicate flasks for each inhibitor were selected and the biomass analysed for copper. To 12 control flasks with no inhibitor added, and 12 flasks with MES plus glucose plus each inhibitor (pH 5.5) for simultaneous metal and inhibitor treatment all containing 2.5 μg $ml^{-1}$ copper, were added 5.0 ml aliquots of the fungal suspension and these were incubated under standard conditions. Sampling was carried out after 5, 30, 60 and 120 minutes for controls and flasks containing inhibitor. The copper and $K^+$ contents for all samples were determined.

8.2.1.10 Demonstration of the effectiveness of the metabolic inhibitors used by measurement of oxygen uptake using an oxygen electrode.

The effectiveness of the metabolic inhibitors used in this study was checked using a Rank oxygen electrode connected to an M.S.E. Spectroplus oxygen meter with a recorder attachment. The Spectroplus was set to zero by adding sodium dithionite after electrode preparation and connection and this represented 0% saturation. Water was passed through the electrode jacket at 25°C.

3.0 ml distilled water were added to the reaction chamber. The Spectroplus was then adjusted to 100%. Operation of the electrode was checked by adding sodium dithionite after which a reading of 0-5% saturation was obtained within 30 seconds. After these operational checks, the contents of the reaction chamber were aspirated and the chamber rinsed with distilled $H_2O$.

3.0 ml of a medium containing 1.0 mM glucose and 0.05 M $KH_2PO_4$ at pH 5.5 were placed in the reaction chamber. This was mixed to achieve 100%

saturation. Ten pellets of mid-linear phase *P. spinulosum* were carefully suspended in the medium in the chamber and readings were taken immediately for $O_2$ uptake. The amount of biomass added was approximately 0.05% $^{w}/v$. When the trace showed 50% $O_2$ utilisation, 3.0 µL of a 1.0 M sodium monoiodoacetate solution were added to produce a final concentration of 1.0 mM. The effect on oxygen uptake was monitored. The experiment was repeated at pH 5.5 with monoiodoacetate added at 5.0, 10.0 and 50.0 mM concentrations. The experiment was also repeated for *T. viride* and *A. niger* using 1.0 mM monoiodoacetate at pH 3.5. The effectiveness of 2,4 DNP and sodium azide in inhibiting oxygen uptake was also investigated. Since the azide was so effective at 1.0 mM concentration this was used in the majority of subsequent inhibitor experiments.

8.2.1.11 Resuspension of experimental mycelium in GMS medium and $K^+$ loss measurements as biomass viability checks.

The objective of this work was to ensure that fungi were not killed by incubation in 2.5 µg $ml^{-1}$ metal by resuspending in GMS medium and monitoring growth and also by monitoring $K^+$ loss during the experiment. A slurry of mid-linear phase *P. spinulosum* was prepared. 5.0 ml aliquots were added to fifteen 250 ml Ehrlenmyer flasks containing 100 ml 50.0 mM MES at pH 5.5 supplemented with copper chloride to a final concentration of 2.5 µg $ml^{-1}$. After 5, 30, 60, 120 and 360 minutes of incubation in the copper solution, 3 replicate flasks were selected and the mycelium harvested by filtration. To maintain sterility, all filtration was carried out in a laminar flow cabinet using sterilised Buchner equipment and sterilised Whatman 541 filters. Half the mycelium of each of the 3 samples was retained for metal analysis whilst the remaining portions were resuspended in 400 ml GMS medium and reincubated. Metal loaded mycelium was added to the GMS medium to form a suspension of approximately 0.1% $^{w}/v$. The

resuspended mycelium was sampled at 0, 6, 12 and 24 hours after inoculation to monitor dry weight increase.

$K^+$ contents were measured for all samples simultaneously to the copper analyses. The procedure was repeated for *T. viride* and *A. niger* and also for all three experimental fungi with 2.5 μg $ml^{-1}$ cadmium.

## 8.3. Results.

### 8.3.1. Development of experimental procedures.

#### 8.3.1.1. Effect of culture age on copper uptake by *Penicillium spinulosum*.

Figure 8.3.1.1. shows the effect of culture age on copper uptake by *P. spinulosum* from a 50.0 mM MES solution containing 2.5 μg $ml^{-1}$ $Cu^{++}$ (pH 5.5). It is clear that the younger the original culture is, the higher is the subsequent accumulation under non-growing conditions. 48 hours was chosen as the ideal culture age for all further experiments since at this time the yield of biomass was reasonably adequate.

Norberg and Persson (1983) showed that cadmium and copper removal from solution by *Zoogloea ramigera* reached a maximum when the cultures were 6 to 8 days old. On the other hand, Horikoshi *et al* (1981) showed that uranium uptake in *Streptomyces viridochromogenes* did not change significantly with the age of the culture.

#### 8.3.1.2. Determination of the optimum desorption medium for removing accumulated metal from *Penicillium spinulosum*.

The results are shown in Figure 8.3.1.2. For both 5 minute and 2 hour incubations in copper the subsequent desorption of the loaded copper appeared to be similar indicating that very little of the accumulated metal was internalised over the 5 minute to 2 hour incubation period. There was no apparent difference for copper, cadmium or zinc.

Distilled $H_2O$ alone proved to be unsatisfactory as a desorbent whilst basal medium pH 5.5, 50.0 mM MES plus 1.0 mM EDTA pH 5.5, phthalate buffer pH 4.0 and basal medium plus 1.0 mM EDTA pH 5.5, gave similar results

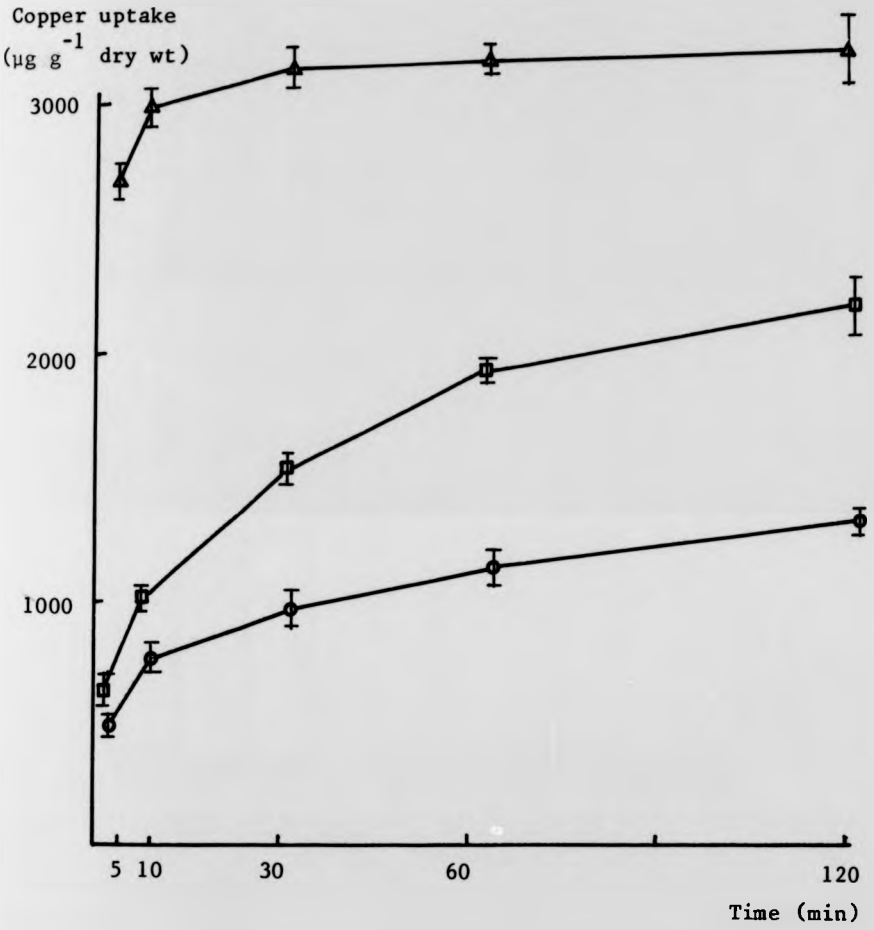
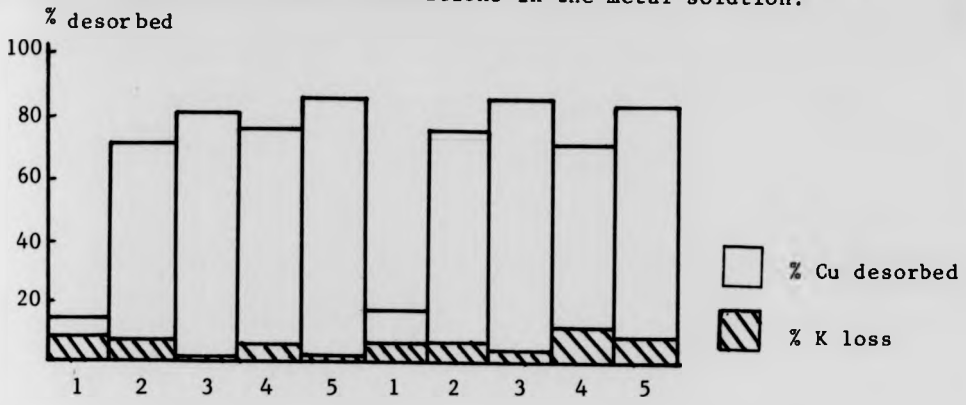



*Figure* 8.3.1.1.

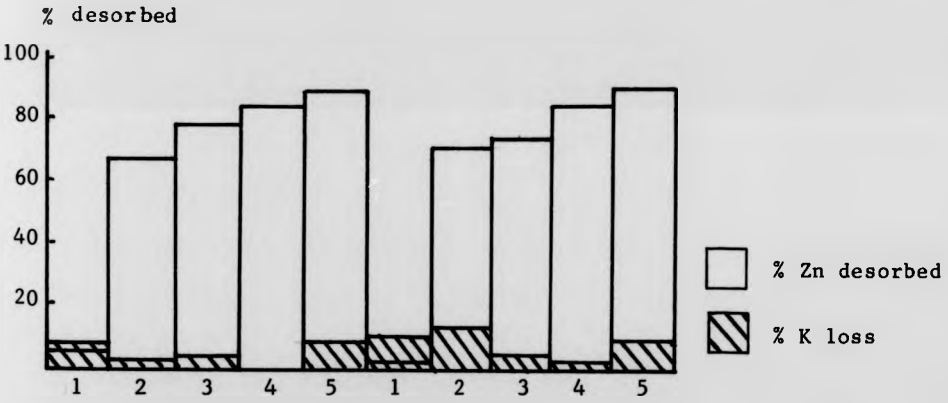
Effect of culture age on copper uptake by non growing suspensions of *P. spinulosum* suspended in 50mM MES containing 2.5 µg ml$^{-1}$ $Cu^{++}$.
△, 36h culture; □, 48h culture; ○, 60h culture.

*Figure* 8.3.1.2.

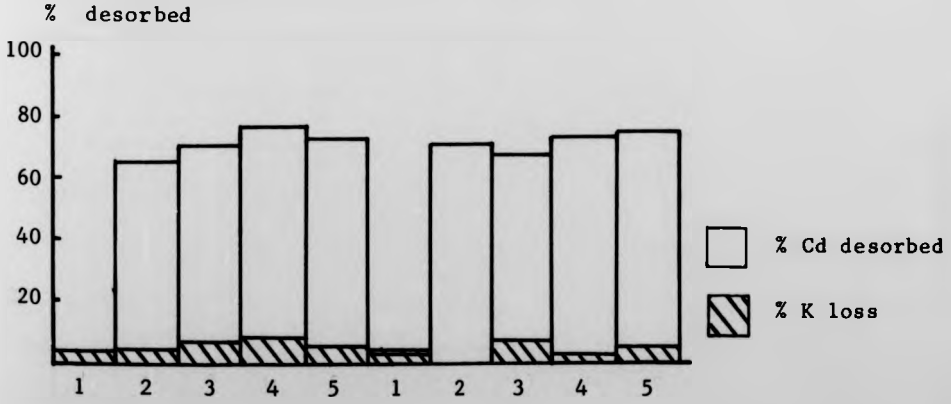
Determination of the optimum desorbing medium for copper, cadmium and zinc after uptake by *P. spinulosum* from 50mM MES containing 2.5μg $ml^{-1}$ metal with 5 minute and 2 hour incubations in the metal solution.




Copper. 5 min incubation 2 hour incubation




Zinc. 5 min incubation 2 hour incubation




Cadmium.

1, distilled water; 2, Basal GMS medium; 3, 50 mM MES plus 1 mM EDTA; 4, Phthalate buffer pH 4.0; 5, Basal GMS medium plus 1 mM EDTA.

with copper desorption values between 60 and 80%. 50.0 mM MES plus 1.0 mM EDTA adjusted to pH 5.5 was chosen for all subsequent work since it achieved good levels of desorption whilst $K^+$ losses of less than 7% were routinely recorded. (Typically $K^+$ loss was substantially less than this value). The indication was that cell membrane damage was minimal.

8.3.1.3. Determination of optimum EDTA concentration for copper desorption from *Penicillium spinulosum*.

The results are illustrated in Figure 8.3.1.3. 0.1 and 0.25 mM EDTA desorbed only 27 and 45% of the total copper respectively and were considered unsatisfactory. 0.5, 1.0, 2.5 and 5.0 mM removed between 60 and 70% of the preloaded copper. 1.0 mM was chosen as the optimum concentration for subsequent experiments since it achieved a high level of desorption with minimal $K^+$ loss. 1.0 mM EDTA alone achieved almost the same degree of copper removal as 50.0 mM MES plus 1 mM EDTA but the $K^+$ loss associated with the 1.0 mM EDTA treatment was slightly larger.

8.3.1.4. Determination of optimum time in 1 mM EDTA for desorption of accumulated copper in *Penicillium spinulosum*.

The results are shown in Table 8.3.1.1. After the loading of *P. spinulosum* pellets with copper by incubation for 30 minutes in 2.5 μg $ml^{-1}$ the minimum time of desorption treatment in 50.0 mM MES plus 1.0 mM EDTA to achieve over 70% copper removal was 15 minutes. Although 20 and 30 minute treatments produced slightly improved levels of desorption, 15 minutes was the time used in all experiments because of the increased risk of greater $K^+$ losses with extended incubations in EDTA.

*Figure* 8.3.1.3.

Determination of optimum EDTA concentration for desorption of accumulated copper from *P. spinulosum* after 30 minute incubation in 2.5µg $ml^{-1}$ $Cu^{++}$. (15 minute desorption treatment in EDTA).

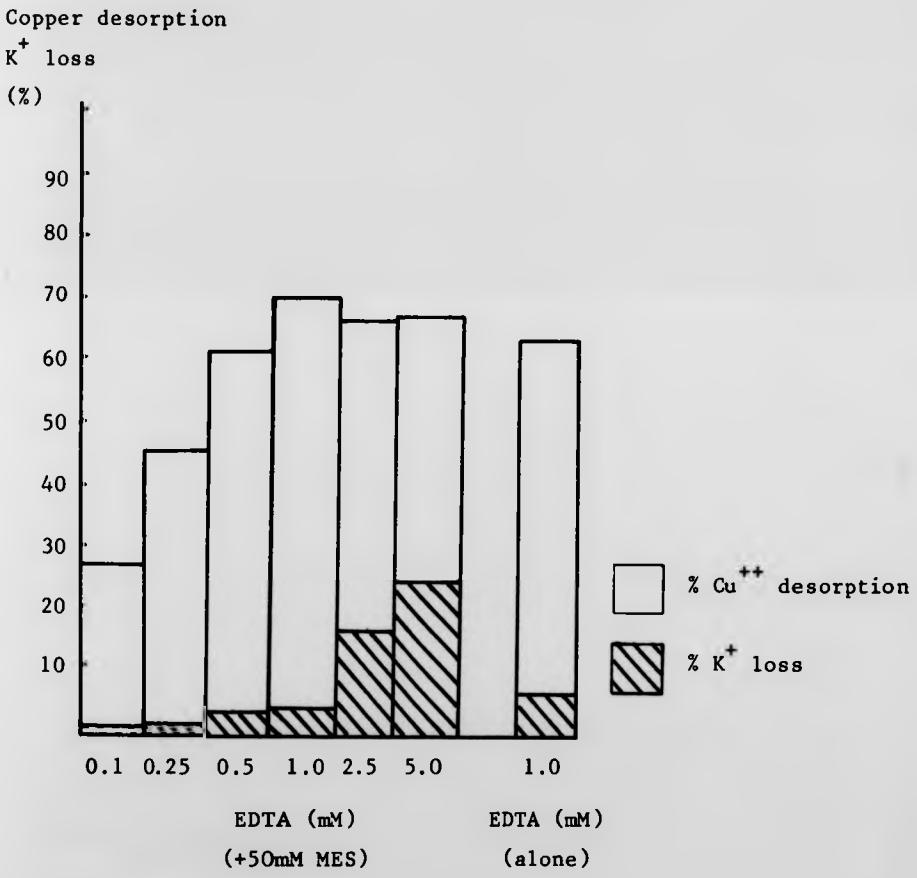



*Table* 8.3.1.1.

Determination of optimum time in 1mM EDTA for desorption of accumulated copper from *P. spinulosum* after 30 minute incubation in 2.5µg $ml^{-1}$ $Cu^{++}$.

| Time in EDTA (m) | 5 | 10 | 15 | 20 | 30 | 40 |
|---|---|---|---|---|---|---|
| % $Cu^{++}$ desorbed | 44.1 | 60.3 | 73.9 | 76.2 | 75.0 | 71.2 |

8.3.1.5. Effect of starvation on subsequent copper uptake from 5.0 μg $ml^{-1}$ by a non-growing suspension of *Penicillium spinulosum*.

Figure 8.3.1.4. shows the effect of prestarvation on subsequent copper uptake from 5.0 μg $ml^{-1}$ $Cu^{++}$ over a 2 hour incubation period. There appeared to be a slight increase in the subsequent copper uptakes after 1, 2, 4, 6 and 12 hour starves in 50.0 mM MES (pH 5.5). On increasing the starvation time from 12 to 24 hours there was a dramatic reduction in the capability of the fungus to accumulate copper and this trend was also evident after 48 and 96 hours of starvation. 12 hours was the maximum starvation period used in experiments since discolouration of mycelium occurred after 24 hours. Also after 24 hours the ease with which mycelium could be handled became progressively worse, particularly with regard to filtering.

Table 8.3.1.2. shows that the proportion of non-desorbable copper altered with the degree of starvation over the 96 hour period of starvation. The percentage of desorbable copper increased when the starvation was extended from 1 to 2 hours, perhaps indicating that the fungus was increasingly unable to internalise copper after 1 hour of starvation.

8.3.1.6. Effect of glucose concentration on copper uptake from 5.0 μg $ml^{-1}$ copper by a non-growing suspension of *Penicillium spinulosum*.

Figure 8.3.1.5. shows the effect of glucose concentration on copper uptake by a 12 hour starved non-growing suspension of *P. spinulosum*. Glucose did not appear to increase the amount of copper accumulated in starved cultures. In fact there appeared to be a slight reduction in uptake for the addition of 0.55 mM glucose, but

*Figure* 8.3.1.4.

Effect of starvation time in 50mM MES on subsequent $Cu^{++}$ uptake from 5 $\mu g\,ml^{-1}$ $Cu^{++}$ by *P. spinulosum* over a 2 hour incubation period.

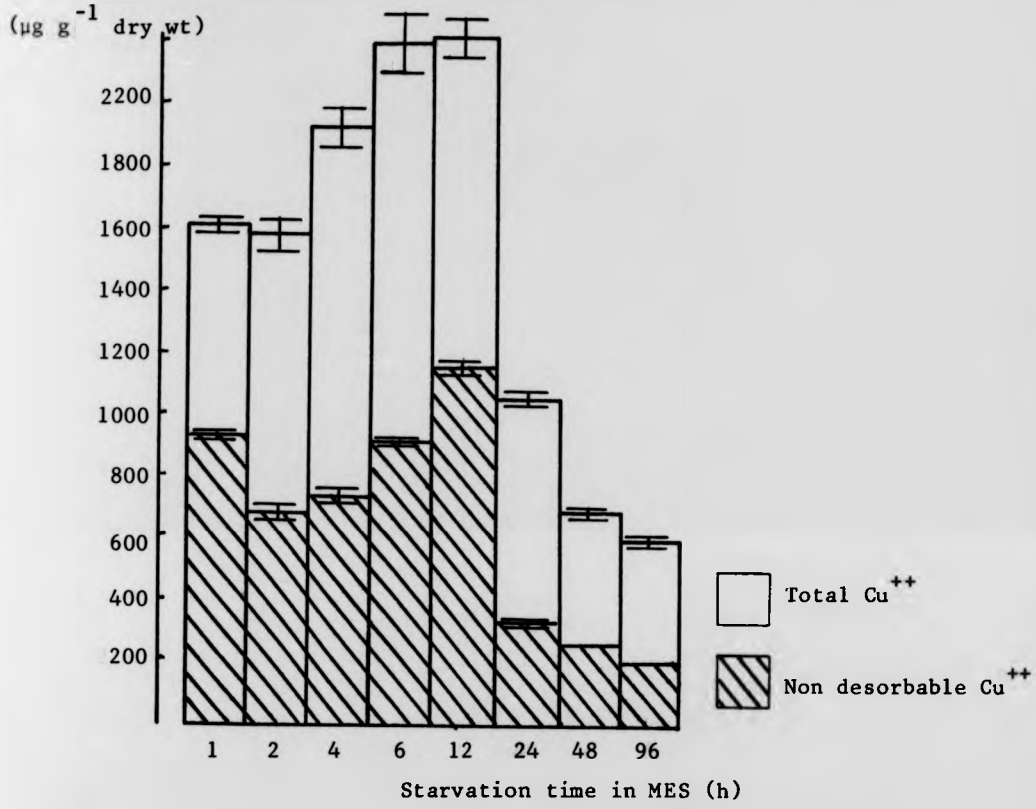



*Table* 8.3.1.2.

Effect of starvation time in 50mM MES on subsequent $Cu^{++}$ desorption after $Cu^{++}$ accumulation by *P. spinulosum* over a 2 hour incubation period.

| Starvation time (h) | 1 | 2 | 4 | 6 | 12 | 24 | 48 | 96 |
|---|---|---|---|---|---|---|---|---|
| % $Cu^{++}$ desorption | 42.7 | 59.4 | 63.1 | 59.3 | 47.7 | 67.3 | 65.9 | 67.9 |

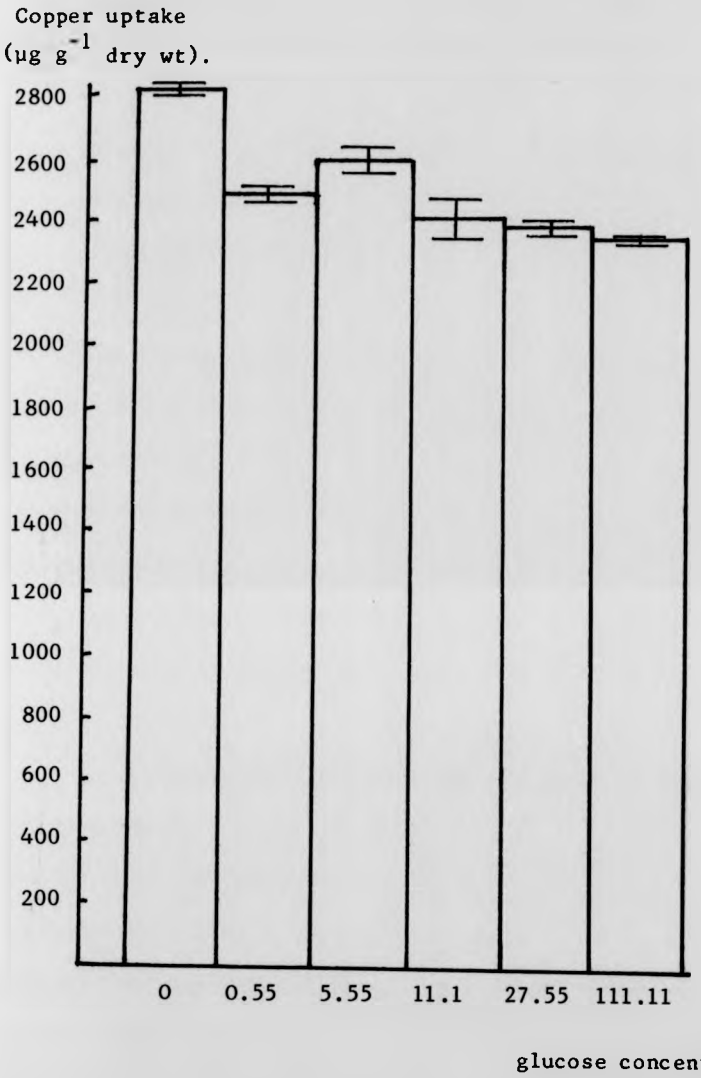



*Figure* 8.3.1.5.

Effect of glucose concentration on copper uptake from 50mM MES containing 5.0 µg ml$^{-1}$ $Cu^{++}$ by a non growing suspension of *P. spinulosum*.

there was no further reduction upto the highest glucose concentration tested at 111.11 mM. Glucose was normally present in uptake flasks at a concentration of 10.0 mM for all subsequent experiments.

8.3.1.7. Influence of anionic component of $Zn^{++}$ and $Cu^{++}$ salts used in zinc and copper uptake experiments from 5.0 µg $ml^{-1}$ using *Penicillium spinulosum*.

The histogram shown in Figure 8.3.1.6 shows that there was no clear difference in cation accumulation from the metal salt whether the salt was of the chloride or the sulphate form. This was the case in GMS medium and 50.0 mM MES although it is interesting to note that lower copper and zinc accumulation occurred in the GMS medium than in the MES and this is almost certainly due to competition by other cations such as $Mg^{++}$ and $Ca^{++}$. ($Mg^{++}$ was present in the medium at 52.75 µg $ml^{-1}$ and $Ca^{++}$ at 45.74 µg $ml^{-1}$.)

The fact that the anionic component of the copper salt had no effect on copper accumulation was in agreement with the results reported by Baldry and Dean (1980b) for copper and the bacterium *Escherichia coli*. The bacterium grew without a lag in the presence of 10.0 µg $ml^{-1}$ $Cu^{++}$ irrespective of whether the copper was added as cupric acetate, cupric chloride, cupric nitrate or cupric sulphate. Since the copper salts are fully ionised in solution it would be expected that there would be little difference in copper uptake from alternative copper salts. This evidence however, appears to be in direct conflict with the data of Bedford (1936), which suggested that for a *Penicillium sp.* which was capable of growth in Czapek's Dox liquid medium saturated with cupric sulphate, was inhibited at concentrations of 10000.0, 6000.0 and 1500.0 µg $ml^{-1}$ $Cu^{++}$ when the copper was added as cupric nitrate, cupric chloride or cupric acetate respectively. It should

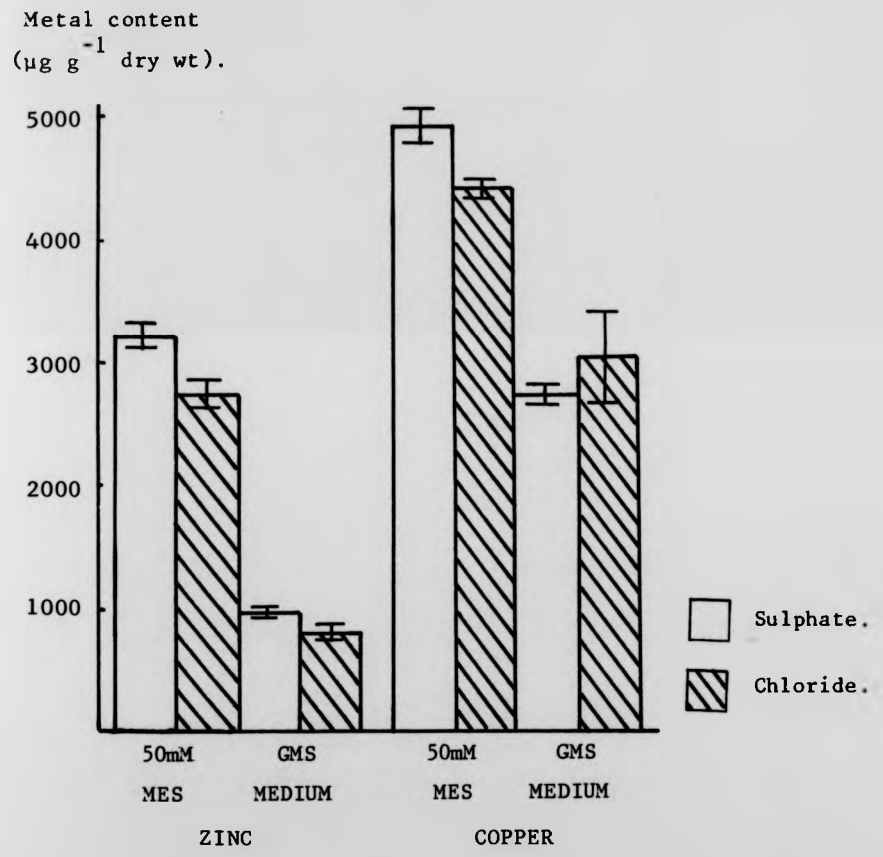



*Figure* 8.3.1.6.

Effect of the anionic component of the metal salt used in zinc and copper uptake experiments with non-growing suspensions of *P. spinulosum* suspended in 50mM MES or GMS medium supplemented with metal sulphate or chloride to obtain a final metal conentration of 5.0µg ml$^{-1}$.

be noted that these were far from physiological concentrations and also the fungus used by Bedford was atypical in terms of its apparent copper resistance, so these results may be anomalous. It is interesting that although the presence of nitrate in the medium had no effect on copper uptake in *E. coli*, the presence of $Cl^-$ in the suspending medium depressed the copper uptake (Baldry and Dean, 1980c). The toxicity of zinc to fungi, bacteria and coliphages was unaffected, lessened or increased by the addition of high concentrations of NaCl (Babich and Stotzky, 1978). The formation of a complex anionic Zn-Cl species exerted greater toxicity in some organisms, although in other organisms this may have resulted in reduced toxicity due to an apparent decrease in $Zn^{++}$ concentration. Babich and Stotzky (1982) also reported that the toxicity of cadmium to fungi decreased as the $Cl^-$ concentration of seawater was increased, indicating lower toxicity of Cd-Cl complexes than of $Cd^{++}$. It is interesting that Nickerson (1946) reported that a 65.4 μg $ml^{-1}$ concentration of zinc as $ZnCl_2$ or $Zn(NO_3)_2$ reduced the respiration of mycelial mats of *Trichophyton rubrum* to an equivalent extent. However, a 1124.0 μg $ml^{-1}$ concentration of cadmium as $CdCl_2$ reduced, but as $CdSO_4$ increased, respiration of the fungus. It seems that the response of organisms to the anionic component of a metal salt is variable.

8.3.1.8. The effect of metabolic inhibitors on copper uptake from 2.5 μg $ml^{-1}$ copper by a non-growing suspension of *Penicillium spinulosum*.

Figures 8.3.1.7a, 7b, 7c, show that preincubation for 30 minutes in 1.0 mM monoiodoacetate, 2,4 DNP and sodium azide had little effect on subsequent copper uptake from 2.5 μg $ml^{-1}$ $Cu^{++}$ by *P. spinulosum*. This suggests that the proportion of uptake of copper which is due to metabolic

*Figures* 8.3.1.7a,b,c.

The effect of metabolic inhibitors on copper uptake from 2.5 µg $ml^{-1}$ $Cu^{++}$ by non-growing *P. spinulosum* under preincubation with inhibitor and simultaneous incubation of *P. spinulosum* with inhibitor and copper.

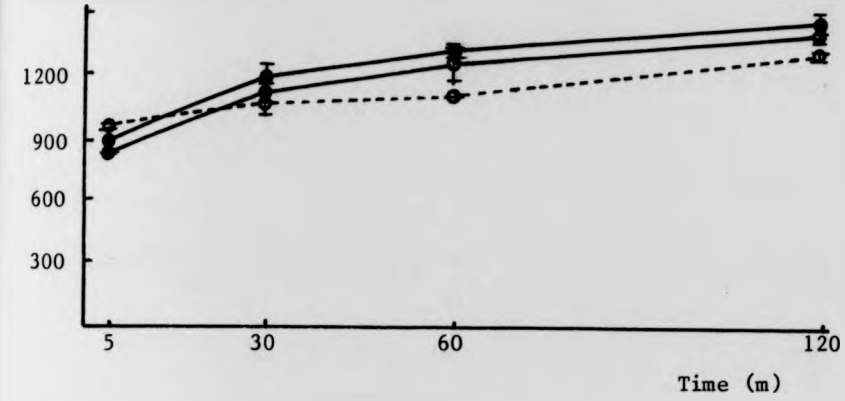



*Figure* 8.3.1.7a

Monoiodoacetate.

Copper uptake
(µg $g^{-1}$ dry wt).

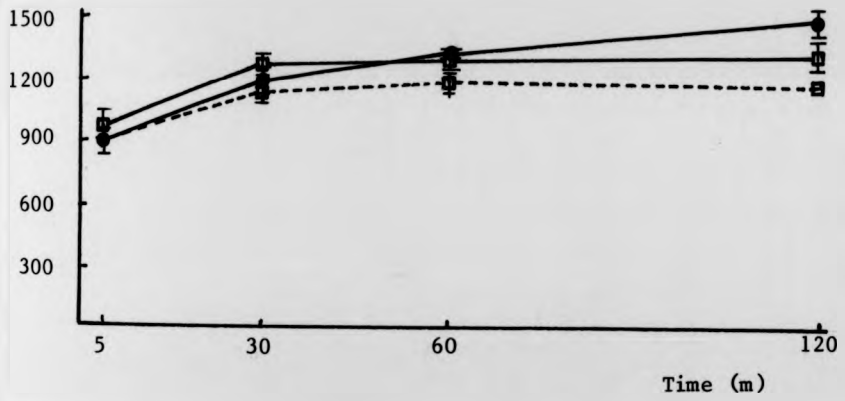



*Figure* 8.3.1.7b

2,4 DNP

Copper uptake
(µg $g^{-1}$ dry wt).

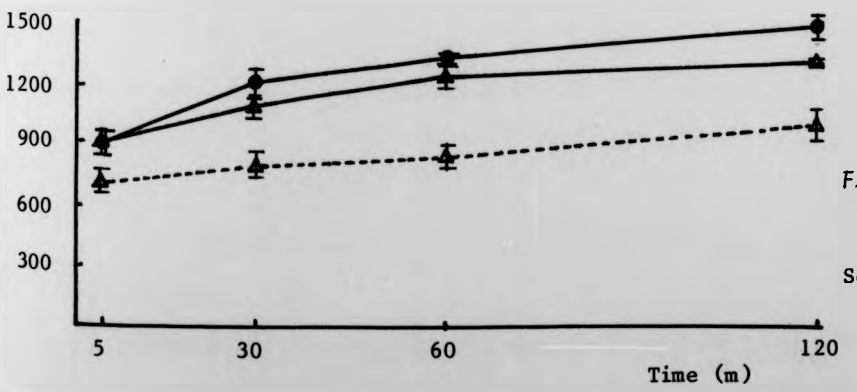



*Figure* 8.3.1.7c

Sodium azide.

●,Controls with no inhibitor treatment; ○, solid line represents preincubation with monoiodoacetate; broken line represents simultaneous incubation with monoiodoacetate and copper; □,2,4 DNP; △, sodium azide.

energy is negligible. There appeared to be a slight reduction in copper uptake when the fungus was incubated in copper and monoiodoacetate simultaneously. For 2,4 DNP the reduction was more clear. In the case of simultaneous azide and copper incubation, there was a marked reduction in apparent copper accumulation and this could be explained by the complexing ability of azide as observed in the polarographic studies. All subsequent experiments designed to study the effects of metabolic inhibitors on metal uptake were carried out by preincubating the fungus in inhibitor followed by a thorough washing prior to incubation in metal solution.

8.3.1.9. Demonstration of the effectiveness of the metabolic inhibitors used.

Table 8.3.1.3. shows the results for the demonstration of the effectiveness of the metabolic inhibitors used in this study. Oxygen uptake by *P. spinulosum* was inhibited by 36.8% with 1.0 mM monoiodoacetate at pH 5.5. At pH 3.5, inhibition was improved at 55.6% and subsequent preincubations with monoiodoacetate were performed at this reduced pH. Increasing the monoiodoacetate concentration at pH 5.5 to 5.0 mM produced 71.6% inhibition of oxygen uptake but further increases in inhibitor concentration upto 50.0 mM had little effect on oxygen uptake. In the presence of 1.0 mM azide, oxygen uptake was reduced by 96.1% in *P. spinulosum* and this inhibitor was chosen for most subsequent studies. 2,4 DNP also appeared to cause inhibition of oxygen uptake although this is difficult to explain since uncouplers characteristically stimulate oxygen uptake.

*Trichoderma viride* appeared to be more sensitive to 1.0 mM monoiodoacetate at pH 5.5 and 3.5 than was *P. spinulosum* with oxygen uptake being inhibited by 43.8 and 100% at the two respective pH values. The azide proved to be less effective with *T. viride* than with *P. spinulosum*

*Table* 8.3.1.3.

Effect of metabolic inhibitors on oxygen uptake in filamentous fungi.

| Experimental condition | Oxygen uptake ($\mu$Mol $O_2$ $ml^{-1}$ $H_2O$) | % inhibition $O_2$ uptake |
|---|---|---|
| *P. spinulosum* control pH 5.5 | 0.0272 | ---- |
| *P. spinulosum* + 1mM iodoacetate pH 5.5 | 0.0172 | 36.8 |
| *P. spinulosum* + 5mM iodoacetate pH 5.5 | 0.0077 | 71.6 |
| *P. spinulosum* + 10mM iodoacetate pH 5.5 | 0.0079 | 70.9 |
| *P. spinulosum* + 50mM iodoacetate pH 5.5 | 0.0058 | 78.7 |
| *P. spinulosum* + 1mM iodoacetate pH 3.5 | 0.0121 | 55.6 |
| *P. spinulosum* control pH 5.5 | 0.0330 | ---- |
| *P. spinulosum* + 1mM azide pH 5.5 | 0.0013 | 96.1 |
| *P. spinulosum* control pH 5.5 | 0.0309 | ---- |
| *P. spinulosum* + 1mM 2,4 DNP pH 5.5 | 0.0129 | 58.3 |
| *P. spinulosum* + 5mM 2,4 DNP pH 5.5 | 0.0071 | 77.1 |
| *T. viride* control pH 5.5 | 0.0430 | ---- |
| *T. viride* + 1mM iodoacetate pH 3.5 | 0.0242 | 43.8 |
| *T. viride* + 5mM iodoacetate pH 3.5 | 0.0000 | 100.0 |
| *T. viride* control pH 5.5 | 0.0455 | ---- |
| *T. viride* + 1mM azide pH 5.5 | 0.0125 | 72.6 |
| *T. viride* + 2mM azide pH 5.5 | 0.0085 | 81.3 |
| *T. viride* + 5mM azide pH 5.5 | 0.0061 | 86.5 |
| *T. viride* + 10mM azide pH 5.5 | 0.0000 | 100.0 |
| *A. niger* control pH 3.5 | 0.0294 | ---- |
| *A. niger* + 1mM iodoacetate pH 3.5 | 0.0199 | 32.3 |
| *A. niger* control pH 5.5 | 0.0302 | ---- |
| *A. niger* + 1mM azide pH 5.5 | 0.0038 | 87.4 |

$O_2$ content of air saturated $H_2O$ at 760 mm Hg pressure is 0.258 $\mu$Mol $O_2$ $ml^{-1}$ $H_2O$ at 25°C.

however, with oxygen uptake inhibitions of 72.6 and 96.1% respectively at 1.0 mM concentrations of inhibitor. 10.0 mM azide was completely inhibitory to *T. viride*. 1.0 mM azide was found to be more effective in inhibiting oxygen uptake than was 1.0 mM monoiodoacetate with *A. niger*. Both inhibitors were slightly less effective with *A. niger* than with *P. spinulosum*. 1.0 mM azide was used in all comparative studies of the effect of metabolic inhibitors on metal uptake, and was used in the manner described for preincubation.

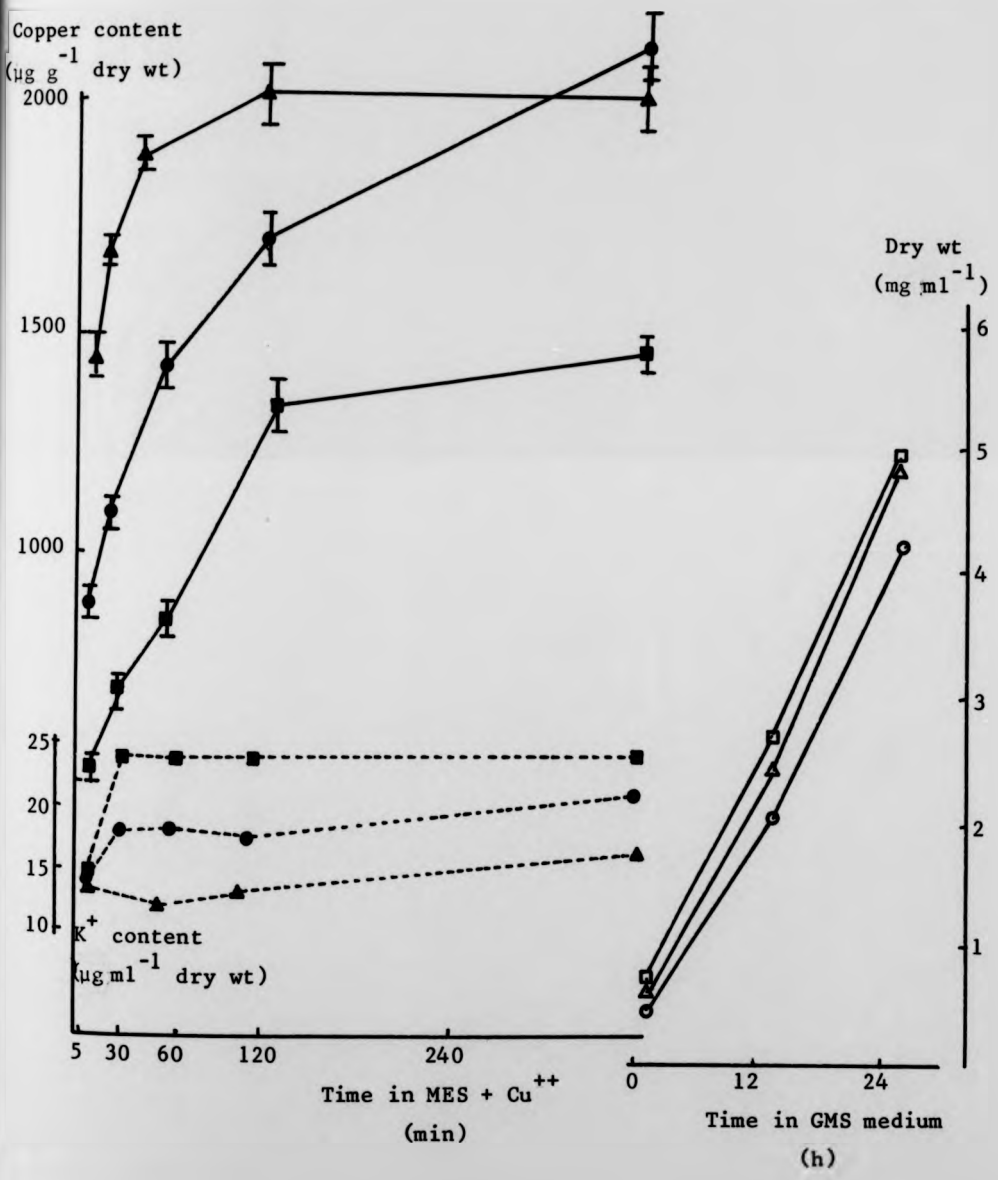
8.3.1.10. Monitoring of fungal viability and $K^+$ loss checks.

Figure 8.3.1.8a shows that copper loaded biomass was capable of immediate growth when resuspended in fresh GMS medium after 6 hour incubations in 2.5 μg $ml^{-1}$ $Cu^{++}$. $K^+$ contents appeared to be variable for the 3 fungi. There appeared to be no viability loss.

Figure 8.3.1.8b shows the effect of cadmium loading on the subsequent growth of the 3 fungi after 6 hour incubations in 2.5 μg $ml^{-1}$ $Cd^{++}$. On resuspension in the GMS medium *A. niger* and *T. viride* showed a slight lag before linear growth was apparent. *Penicillium spinulosum*, which was susceptible to the presence of cadmium, demonstrated a 12 hour lag before growth proceeded. $K^+$ contents were less variable than with the copper loading experiment. There appeared to be no appreciable $K^+$ loss from *P. spinulosum* in the presence of cadmium over the 6 hour incubation period.

*Figure* 8.3.1.8a

Relative mycelial $K^+$ contents during copper uptake from 2.5 $\mu g\ ml^{-1}$ $Cu^{++}$ by fungi and growth of copper loaded biomass when resuspended in GMS medium after copper treatment.




Closed symbol solid line represents copper uptake.

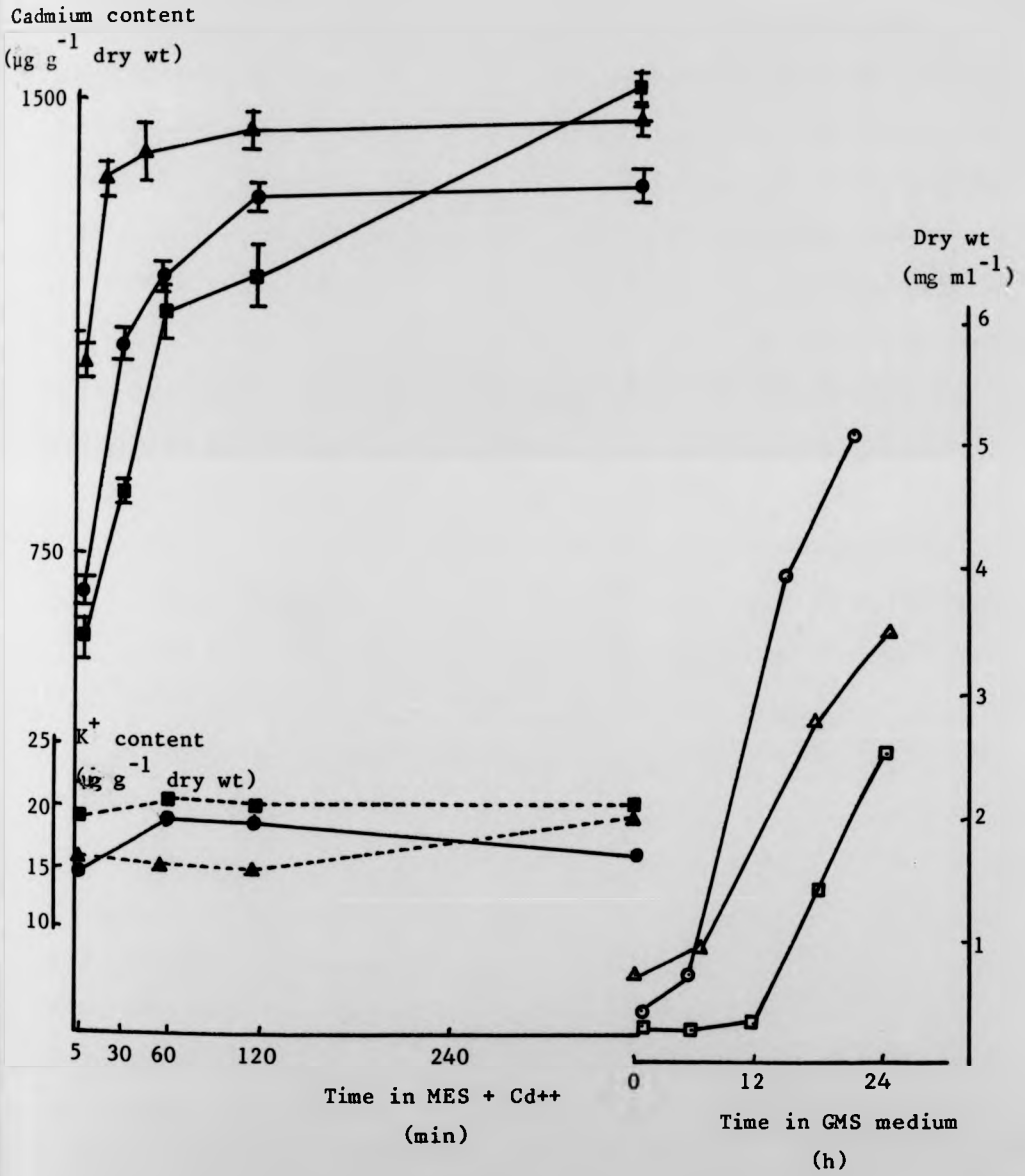
Closed symbol broken line represents $K^+$ content.

Open symbol solid line represents dry wt. increase in GMS.

■,*P. spinulosum*; ●*T. viride*; ▲A. *niger*.

*Figure* 8.3.1.8b

Relative mycelial $K^+$ contents during cadmium uptake from 2.5μg $ml^{-1}$ $Cd^{++}$ by fungi and growth of cadmium loaded biomass when resuspended in GMS medium after cadmium treatment.




Closed symbol solid line represents cadmium uptake.

Closed symbol broken line represents $K^+$ content.

Open symbol solid line represents dry wt. increase in GMS.

■ □ , *P.spinulosum*; ● ○ , *T. viride*; ▲ △ , *A. niger*.

8.2.2. Materials and Methods.

8.2.2.1. EFFECT OF GLUCOSE, METABOLIC INHIBITORS AND TEMPERATURE ON METAL UPTAKE IN NON-GROWING FUNGAL SUSPENSIONS.

To elucidate the mechanism of mycelial uptake, the influence of glucose, metabolic inhibitors and temperature on total and non-desorbable metal uptake was investigated. A fungal slurry of mid-linear phase, unstarved mycelium was prepared and used to determine the uptake of copper, cadmium and zinc from 2.5 μg $ml^{-1}$ solutions. Unstarved cultures were used in glucose experiments since the starving procedure appeared to increase metal uptake slightly (Section 8.3.1.5). Starving would also introduce an age difference between mycelium used in studies on the effect of glucose, and those experiments investigating the influence of temperature and metabolic inhibitors on metal uptake. Uptake over 2 hour incubation periods was compared in the following situations:

i. Metal contained in 50.0 mM MES only at 25°C at pH 5.5.

ii. Metal contained in 50.0 mM plus 10.0 mM glucose at pH 5.5.

iii. Metal contained in 50.0 mM MES plus 10.0 mM glucose using fungal material which had been preincubated in metabolic inhibitor (pH 5.5).

iv. Metal contained in 50.0 mM MES at 4°C at pH 5.5.

Fungal density was adjusted to prevent metal removal from experimental solutions in excess of 10%.

i. The uptake of copper from a 50.0 mM MES solution at pH 5.5 containing 2.5 μg $ml^{-1}$ $Cu^{++}$ was determined over a 2 hour period. Batches of 15 suspensions of *P. spinulosum* were incubated at 25°C with shaking at 200 rpm for 1, 5, 30, 60 and 120 minutes. Three replicate batches of fungal suspensions were employed per time period. At the end of the incubation period the fungal samples were collected by filtration and washed. Half of each sample was analysed directly for copper and $K^{+}$ content whilst copper was desorbed from the remaining portion of each sample under the standard conditions. Uptake was expressed in μg $g^{-1}$ dry weight for each incubation period. Plots of uptake against time for total metal and non-desorbable metal contents were

constructed to demonstrate metal uptake profiles. The experiment was repeated for cadmium and zinc using *T. viride* and *A. niger*.

ii) The effect of glucose on copper uptake by non-growing suspensions was determined by repeating the procedure as in (i) above but using 50.0 MM MES plus 10.0 mM glucose.

iii) The effect of metabolic inhibitors on metal uptake was determined by preincubating fungal material in 50.0 mM MES containing 1.0 mM sodium azide at pH 5.5 for 30 minutes. After the preincubation period, the mycelium was filtered and washed with distilled $H_2O$ and resuspended in 50.0 mM MES plus 10.0 mM glucose containing the metal supplement and the procedure for determining metal uptake was repeated as in (i). All 3 fungi were tested with copper, cadmium and zinc using azide, but the effects of monoiodoacetate and 2,4 DNP were also investigated using various fungus and metal combinations. Monoiodoacetate preincubation was carried out at pH 3.5.

iv) The influence of temperature on metal uptake was investigated using the procedure as outlined in (i) except the experimental temperature was $4^{o}C$ instead of $25^{o}C$. The experiment was performed for all 3 fungi with copper, cadmium and zinc at 2.5 $\mu g\ ml^{-1}$ metal concentrations. A detailed investigation of the effect of temperature on copper uptake in *P. spinulosum* was carried out as described in the next section.

8.2.2.2. Effect of anaerobiosis on metal uptake by non-growing suspensions of fungi.

By incubating non-growing fungi under aerobic and anaerobic conditions, the influence of aerobic metabolism on metal uptake could be

established. By replacing metal loaded fungi which had been incubated anaerobically, into an aerobic environment, the effect of aerobic metabolic processes on prior loading could be monitored.

20 ml of a suspension of *P. spinulosum* were added to eight, 1 litre flasks containing 400 ml of 50.0 mM MES. These were incubated at 25°C with shaking, but 4 of the flasks were aerated using a filtered air stream whilst 4 were incubated anaerobically by bubbling $O_2$-free $N_2$ through the suspension. After a 30 minute equilibriation period, copper chloride was added to produce a final concentration of 2.5 µg $ml^{-1}$ $Cu^{++}$. After 5, 30, 60 and 120 minute periods of incubation three, 125 ml samples of suspension were taken from the anaerobic and aerobic flasks. The mycelium was collected by filtration. The samples were split for direct analysis of total copper and for non-desorbable copper content measurements.

In a separate 1 litre flask, the effect of 1 hour incubation under anaerobic conditions in the presence of metal, followed by 1 hour aerobic incubation on copper uptake after 2 hours was investigated. The suspension was pre-equilibriated for 30 minutes prior to metal addition. The flask was then incubated anaerobically for 1 hour followed by aerobic incubation for 1 hour, and the mycelium was then harvested for copper and $K^+$ analysis. As a viability check, small amounts of mycelium (about 20 pellets) were harvested from the anaerobic flasks after the 2 hour incubations and resuspended in 3 separate flasks of fresh GMS medium. These were incubated under standard conditions for 36 hours and the subsequent growth was monitored periodically by determining dry weight production. All experiments were performed in duplicate and repeated using cadmium and zinc. Zinc was also tested with *T. viride* and cadmium with *A. niger*.

8.3.2. Results.

8.3.2.1. EFFECT OF GLUCOSE, METABOLIC INHIBITORS AND TEMPERATURE ON METAL UPTAKE IN NON-GROWING FUNGAL SUSPENSIONS. INVESTIGATION OF METABOLIC INVOLVEMENT.

Metal accumulation in 50.0 mM MES at 25°C.

The metal uptake profile for unstarved mid-linear phase fungi suspended in 50.0 mM MES containing 2.5 μg $ml^{-1}$ metal at pH 5.5 was similar for copper, cadmium and zinc and for *P. spinulosum*, *T. viride* and *A. niger* as shown in Figures 8.3.2.1-9.

Uptake appeared to be initially rapid over the first 5 minutes of incubation, typically accounting for about 50% of the total uptake after 2 hours of incubation in the metal solution. The rate of metal accumulation slowed over the remainder of the experimental period and in most cases uptake appeared to be complete within 1 hour. There was little or no further accumulation between 1 and 2 hours of incubation.

The profile for non-desorbable metal followed a similar pattern as shown in Figures 8.3.2.1-9 and the data presented in Table 8.3.2.1a shows that the non-desorbable metal accounted for a proportion of the total metal accumulated over the incubation period ranging from 11.1% for *P. spinulosum* and cadmium, to 90.6% for *A. niger* and zinc. There was no clear pattern of differing proportions of non-desorbable metal between 5 minute and 2 hour incubations. Therefore, no firm conclusions can be drawn about the progress of metal internalisation.

Metal accumulation in 50.0 mM MES plus 10 mM glucose at 25°C.

Also shown in Figures 8.3.3.1-9 are the uptake profiles for unstarved fungi in 50.0 mM MES plus 10.0 mM glucose at 25°C. It appeared that

the uptake profiles obtained were identical to those for controls in MES only for all the metal and fungus combinations tested. Glucose appeared to have little effect on the quantitative uptake of metal over the 2 hour incubation period. Similarly, non-desorbable metal uptake (Figures 8.3.2.1-9) was not very different from control non-desorbable values and was variable, accounting for 16.8 to 79.6% of the total metal content as shown in Table 8.3.2-16.

The effect of glucose on modifying the proportion of non-desorbable metal between 5 minute and 2 hour incubations in the metal solution was inconsistent, so it is difficult to draw conclusions about the effect of glucose on metal internalisation.

Metal accumulation in 50.0 mM MES after preincubation in 1.0 mM azide (25°C).

The results are illustrated in Figures 8.3.2.1-9. Copper uptake and cadmium uptake was reduced by preincubation in 1.0 mM azide in *P. spinulosum* and *T. viride* respectively. With these exceptions there was little difference between uptake in control flasks and accumulation of total and non-desorbable metal following preincubation in inhibitor for any of the other metal fungus combinations. Non-desorbable metal contents accounted for about the same proportions of total metal accumulated as in controls (data presented in Table 8.3.2.1c), but the inherent variability made comparisons impossible. The reduction in the uptake of cadmium after preincubation in the inhibitor by *T. viride* was accompanied by a reduction in the amount of non-desorbable metal accumulated. Other metabolic inhibitors, including 2,4 DNP and monoiodoacetate had no effect on subsequent metal uptake as shown in Figures 8.3.2.10a-h.

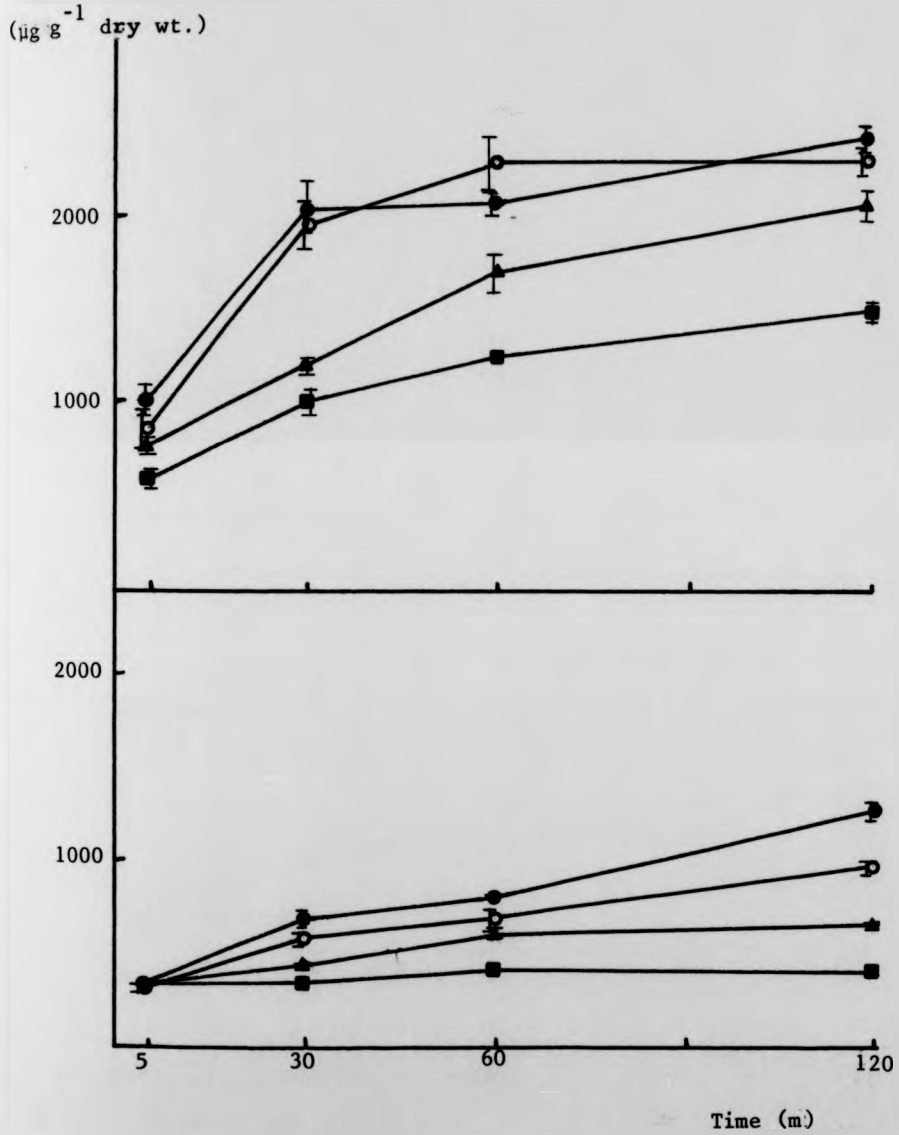
Metal accumulation in 50.0 mM MES at 4°C.

Also shown in Figures 8.3.2.1-9 are the effects of lowering the incubation temperature from 25°C to 4°C. This resulted in a reduction in metal accumulation in the systems tested without exception. Although the pattern of uptake was similar to that obtained for controls at 25°C, the quantitative reduction in total metal accumulation was typically around 50%. In all cases, initial binding was reduced also.

There was no obvious increase in the proportion of non-desorbable metal content as compared to controls (Figures 8.3.2.1-9) after 5 minute and 2 hour incubations. This is also illustrated in Table 8.3.2.1d. The indication is that temperature has no defineable influence on the amount of metal internalised under the conditions of these experiments.

*Figure* 8.3.2.1

Copper uptake from MES containing 2.5 µg $ml^{-1}$ $Cu^{++}$ by a non-growing suspension of *P. spinulosum* under various incubation conditions.

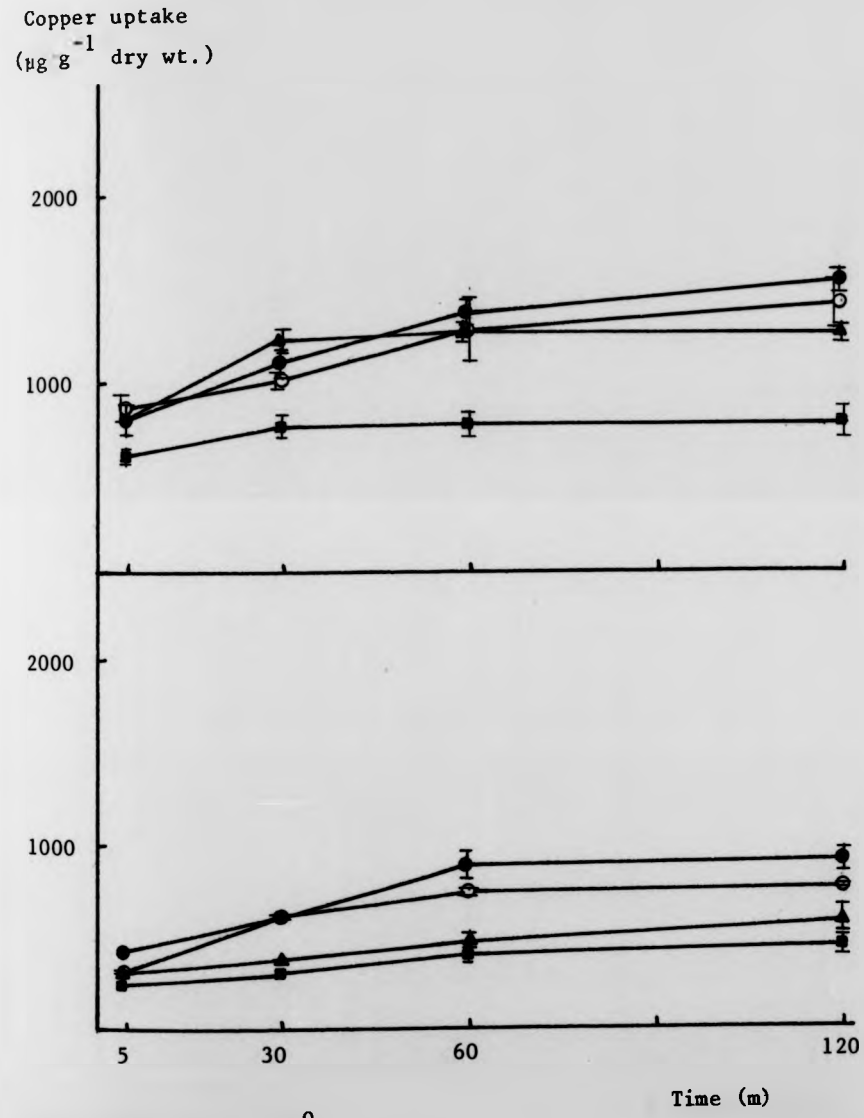



- O MES only at 25°C.
- ▲ MES plus 1mM sodium azide.
- ● MES plus 10mM glucose.
- ■ MES only at 4°C.

Upper figure represents total copper accumulated, lower non-desorbable.

*Figure* 8.3.2.2.

Copper uptake from 50 mM MES containing 2.5 μg $ml^{-1}$ $Cu^{++}$ by a non-growing suspension of *T. viride* under various conditions.




○ 50 mM MES only at 25°C

▲ 50 mM MES plus 1 mM sodium azide

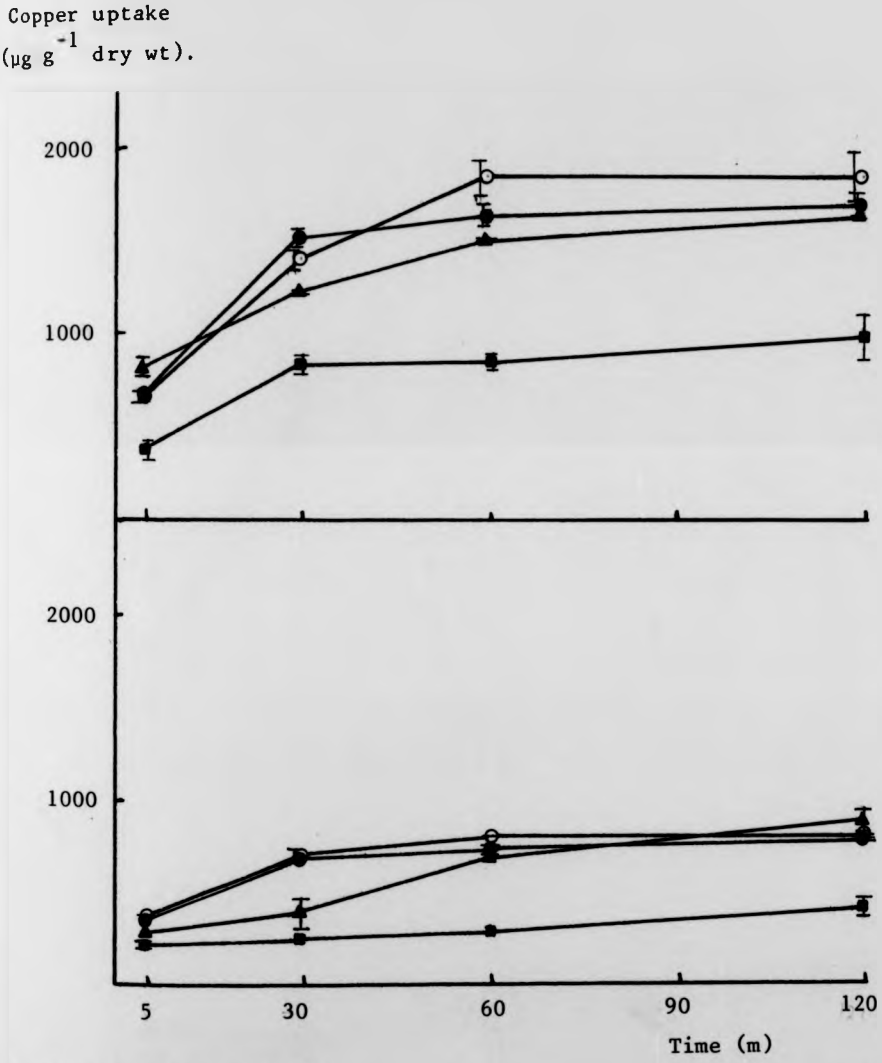
● 50 mM MES plus 10 mM glucose

■ 50 mM MES only at 4°C

Upper figure represents total copper accumulated.

Lower figure represents non-desorbable copper.

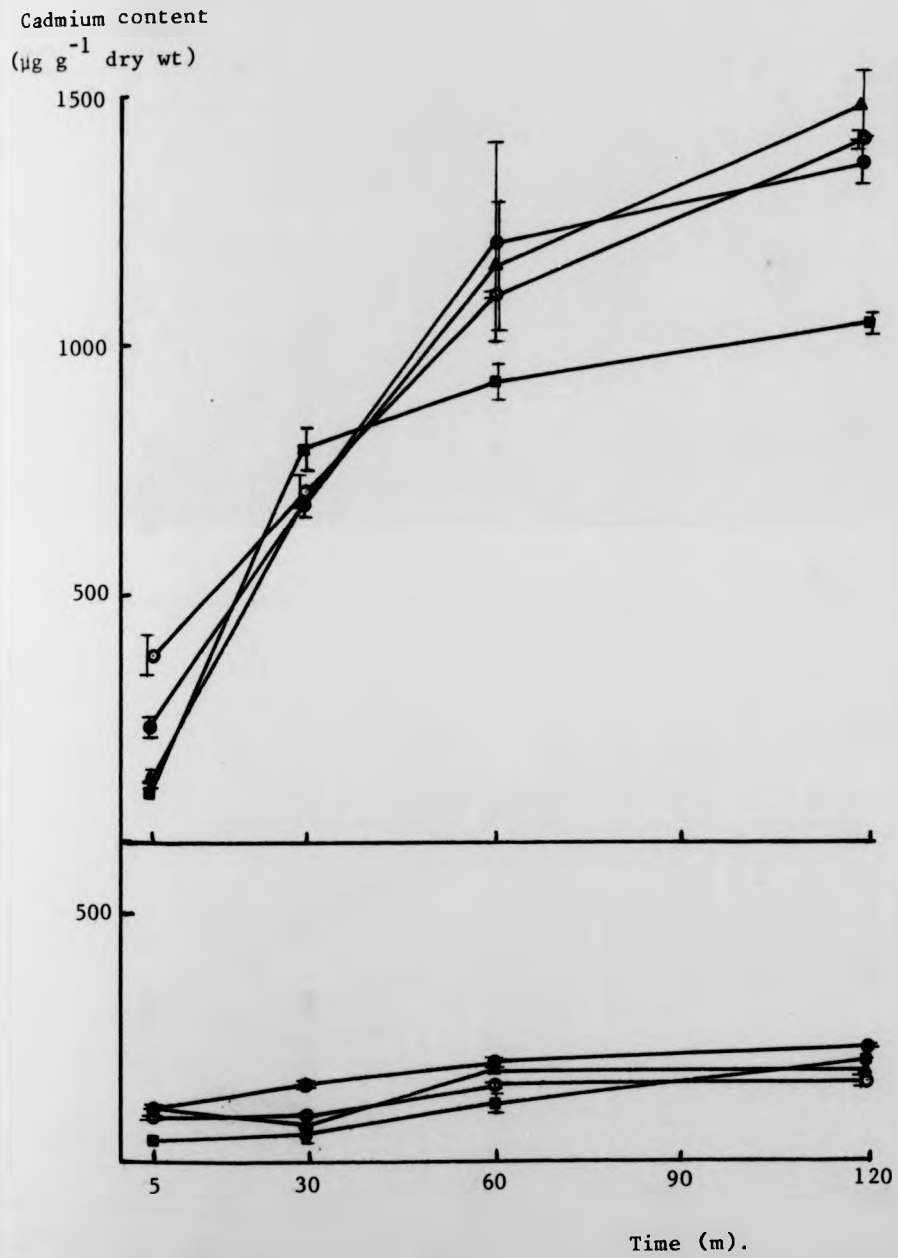
*Figure* 8.3.2.3.
Copper uptake from 50 mM MES containing 2.5 µg $ml^{-1}$ $Cu^{++}$ by a non-growing suspension of A. *niger* under various conditions.




○ 50 mM MES only at 25°C.
▲ 50 mM MES plus 1 mM sodium azide.
● 50 mM MES plus 10 mM glucose.
■ 50 mM MES only at 4°C.
Upper figures total copper
Lower figures non-desorbable copper.

*Figure* 8.3.2.4.

Cadmium uptake from 50 mM MES containing 2.5 $\mu g\ ml^{-1}$ $Cd^{++}$ by a non-growing suspension of *P. spinulosum* under various conditions.




○ 50 mM MES only at 25°C.

▲ 50 mM MES plus 1 mM sodium azide.

● 50 mM MES plus 10 mM glucose.

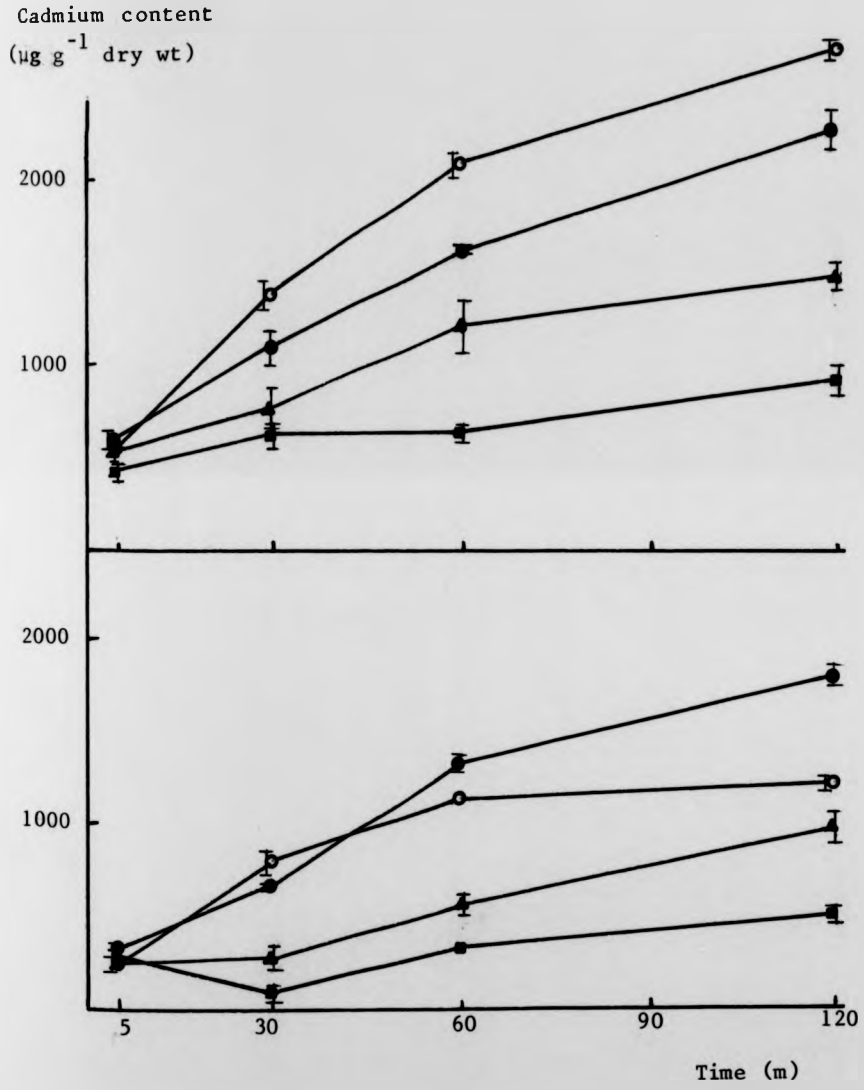
■ 50 mM MES only at 4°C.

Upper figures total cadmium.

Lower figures non-desorbable cadmium.

*Figure* 8.3.2.5.

Cadmium uptake from 50 mM MES containing 2.5µg $ml^{-1}$ $Cd^{++}$ by a non-growing suspension of *T. viride* under various conditions.




○ 50 mM MES only at 25°C.

▲ 50 mM MES plus 1mM sodium azide

● 50 mM MES plus 10 mM glucose.

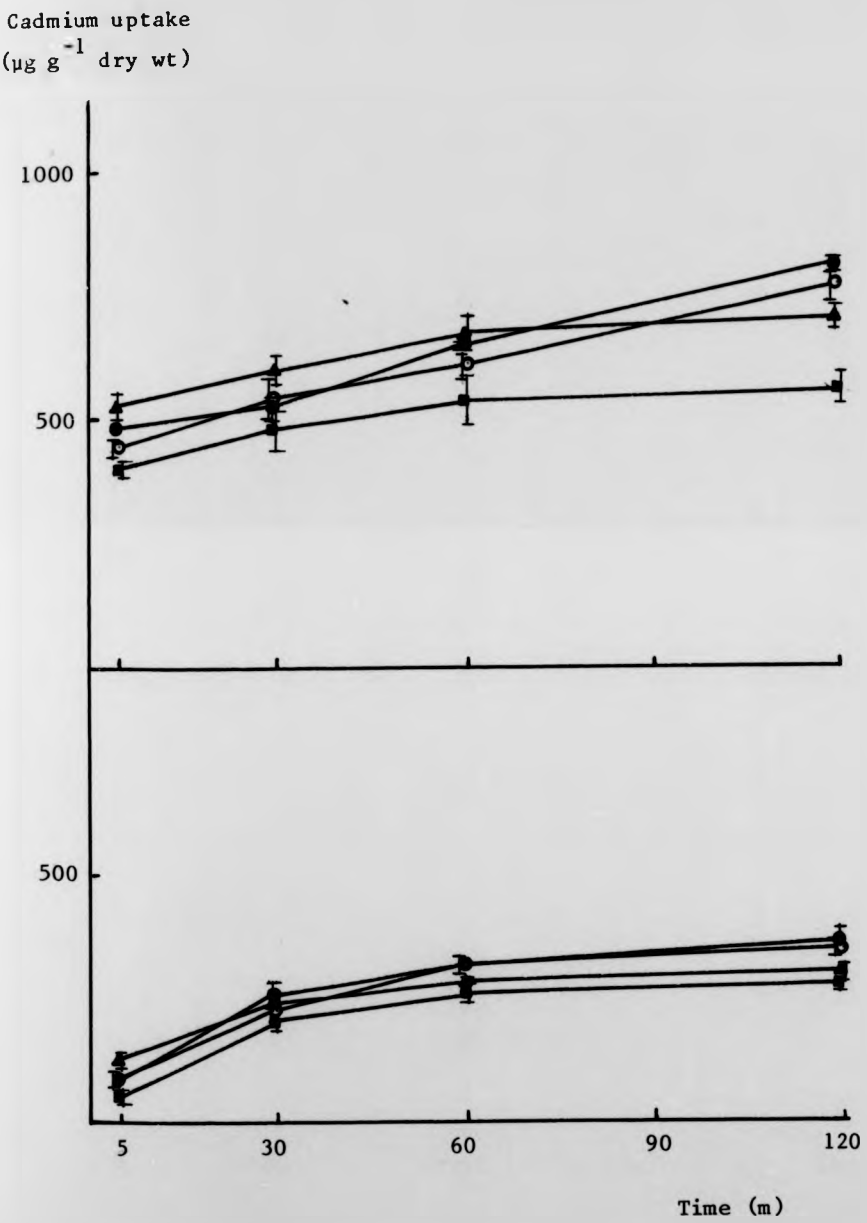
■ 50 mM MES only at 4°C.

Upper figures total cadmium.

Lower figures non-desorbable cadmium.

*Figure* 8.3.2.6.

Cadmium uptake from 50 mM MES containing 2.5 µg $ml^{-1}$ $Cd^{++}$ by a non-growing suspension of *Aspergillus niger* under various conditions.




○ 50 mM MES only at 25°C.

▲ 50 mM MES plus 1 mM sodium azide.

● 50 mM MES plus 10 mM glucose.

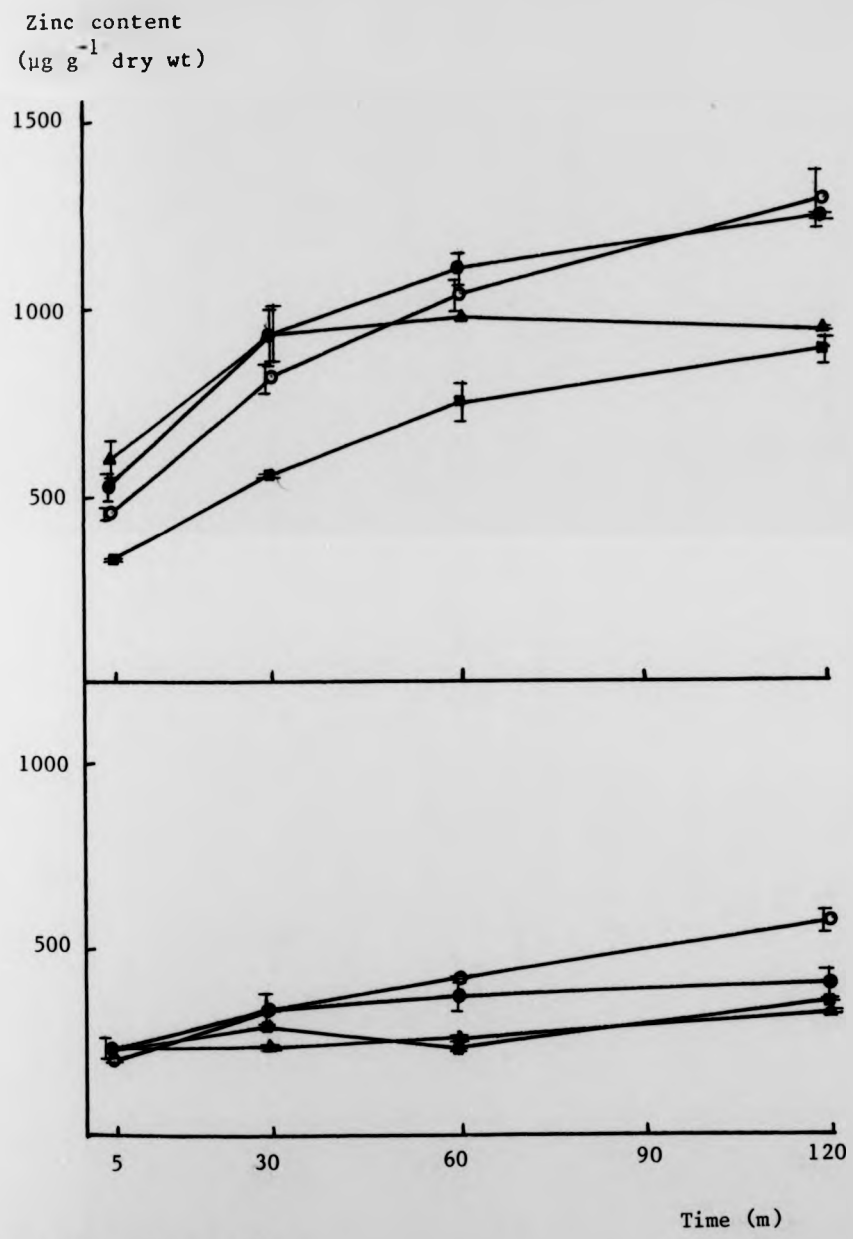
■ 50 mM MES only at 4°C.

Upper figures total cadmium.

Lower figures non-desorbable cadmium.

*Figure* 8.3.2.7.

Zinc uptake from 50 mM MES containing 2.5 μg $ml^{-1}$ $Zn^{++}$ by a non-growing suspension of *P. spinulosum* under various conditions.




○ 50 mM MES only at 25°C

▲ 50 mM MES plus 1 mM sodium azide

● 50 mM MES plus 10 mM glucose

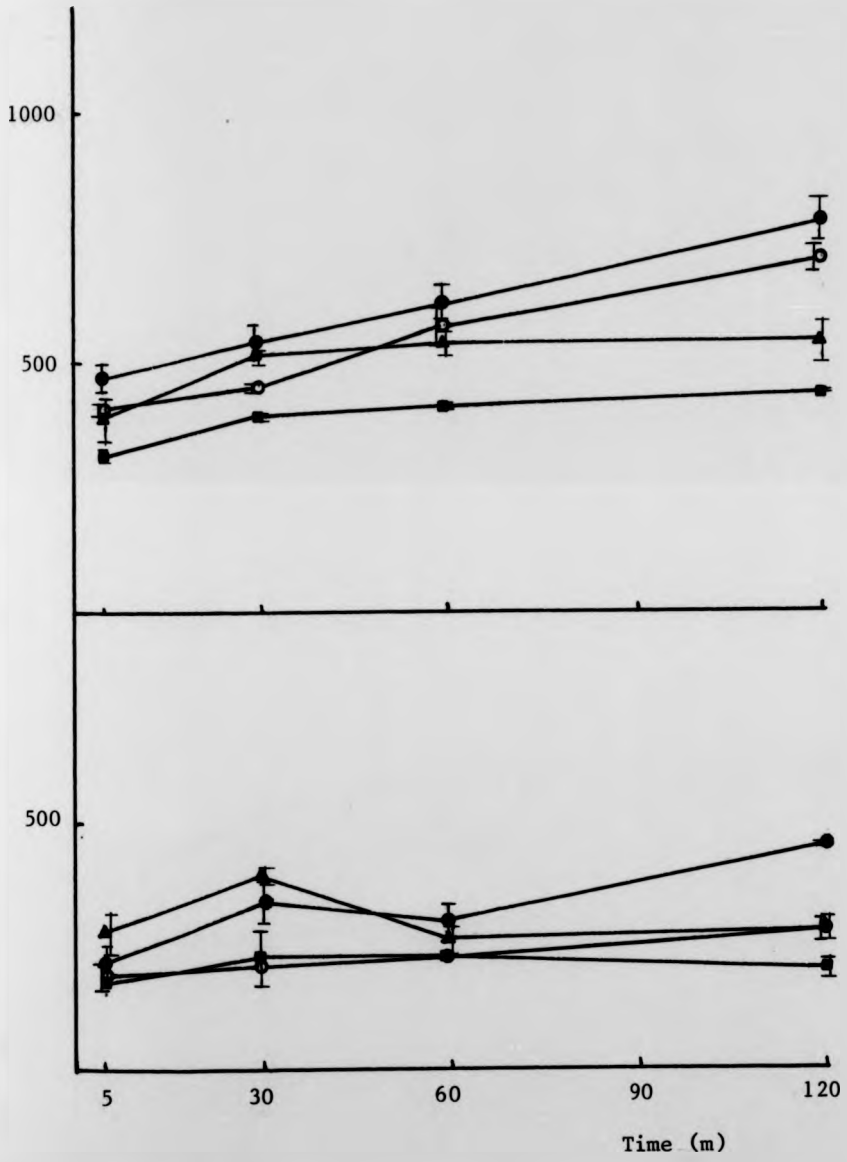
■ 50 mM MES only at 4°C.

Upper curves total zinc

Lower figures non-desorbable zinc.

*Figure* A.3.2.8.

Zinc uptake from 50 mM MES containing 2.5 $\mu g\,ml^{-1}$ $Zn^{++}$ by a non-growing suspension of *T. viride* under various conditions.




○ 50 mM MES only at 25°C

▲ 50 mM MES plus 1 mM sodium azide

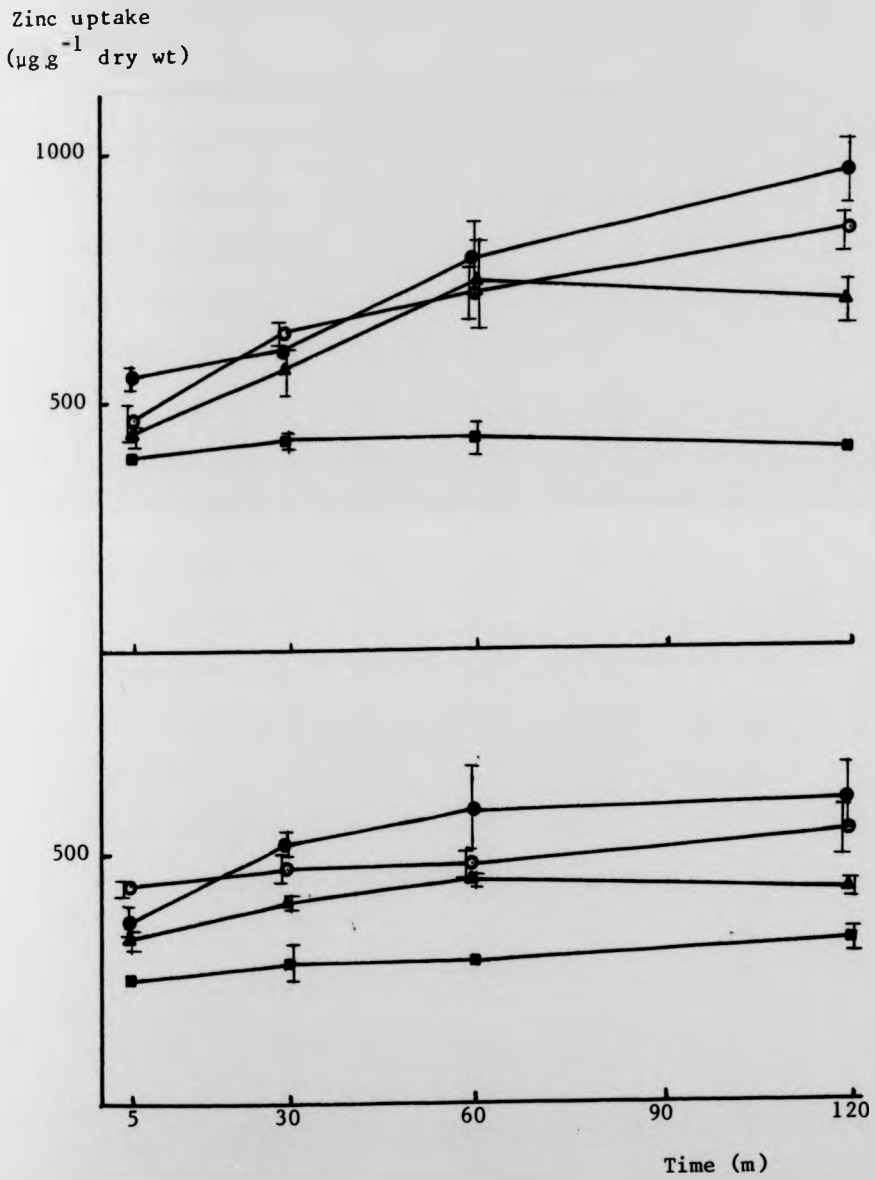
● 50 mM MES plus 10 mM glucose

■ 50 mM MES only at 4°C.

Upper figures total zinc uptake

Lower figures non-desorbable zinc uptake.

*Figure* 8.3.2.9.
Zinc uptake from 50 mM MES containing 2.5 µg $ml^{-1}$ $Zn^{++}$ by a non-growing suspension of *Aspergillus niger* under various conditions.




○ 50 mM MES only at 25°C
▲ 50 mM MES plus 1 mM sodium azide
● 50 mM MES plus 10 mM glucose
■ 50 mM MES only at 4°C
Upper curves represent total zinc uptake
Lower curves represent non-desorbable zinc uptake.

*Figure* 8.3.2.10a-h.

Effect of metabolic inhibitor pretreatment on subsequent heavy metal uptake by suspensions of non-growing filamentous fungi.

a. Copper uptake by *P. spinulosum* from 50.0mM MES containing 2.5 μg $ml^{-1}$ $Cu^{++}$.
o , control; □ , preincubation in 1.0mM monoiddoacetate.

b. Copper uptake by *P. spinulosum* from 50.0mM MES containing 1.0 μg $ml^{-1}$ $Cu^{++}$.
o , control; ■ , preincubation in 1.0mM 2,4DNP

c. Cadmium uptake by *P. spinulosum* from 50.0mM MES containing 2.5 μg $ml^{-1}$ $Cd^{++}$.
o , control; □ , preincubation on 1.0mM monoiodoacetate.

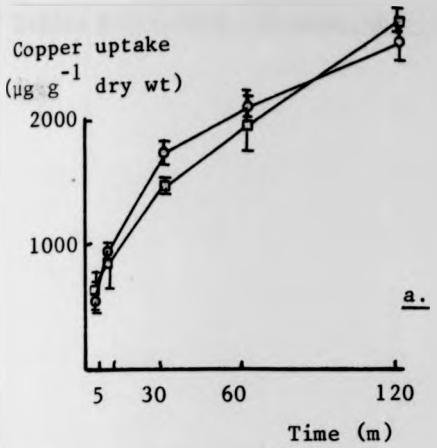
d. Cadmium uptake by *P. spinulosum* from 10.0 μg $ml^{-1}$ $Cd^{++}$ in 50.0mM MES
o , control; □ , preincubation in 1.0mM monoiodacetate.

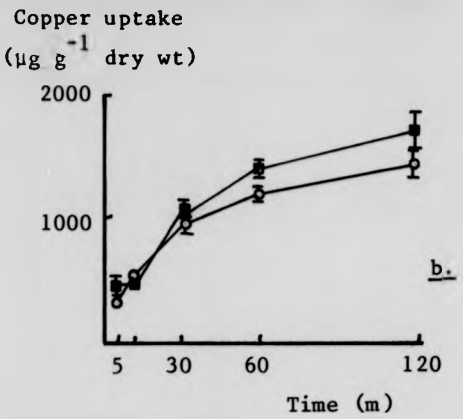
e. Zinc uptake by *P. spinulosum* from 50.0mM MES containing 5.0 μg $ml^{-1}$ $Zn^{++}$.
o , control; ■ , preincubation in 1.0mM 2,4 DNP.

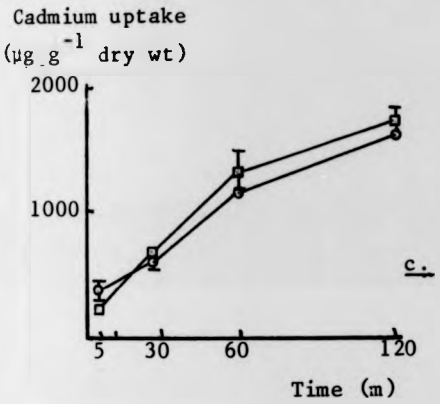
f. Zinc uptake by *P. spinulosum* from 50.0mM MES containing 10.0 μg $ml^{-1}$ $Zn^{++}$.
o , control; □ , preincubation in monoiodoacetate. (1.0mM).

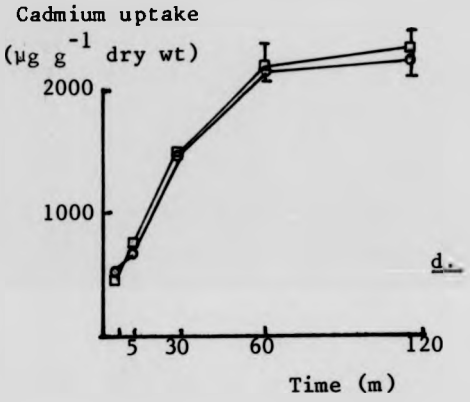
g. Copper uptake by *T. viride* from 50.0mM MES containing 2.5 μg $ml^{-1}$ $Cu^{++}$.
o , control; □ , preincubation in 1.0mM monoiodoacetate; ,
2,4 DNP.

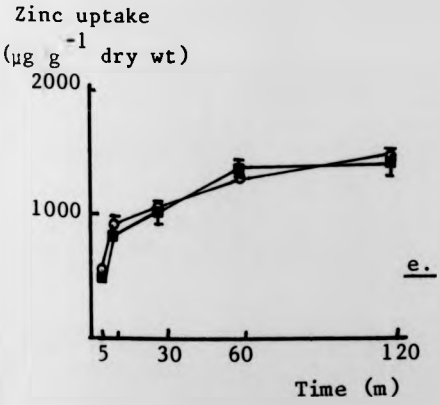
h. Copper uptake by A. *niger* from 50.0mM MES containing 5.0 μg $ml^{-1}$ $Cu^{++}$.
o , control; □ , 1.0mM monoiodoacetate preincubation treatment.

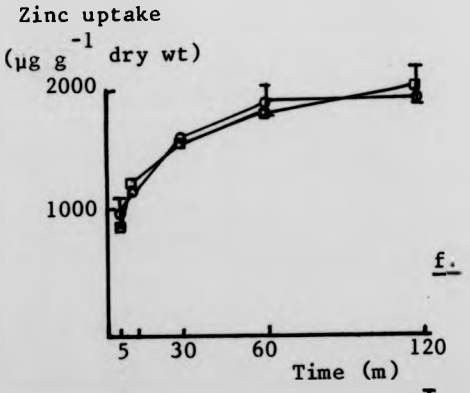

Copper uptake
(µg g -1 dry wt)
2000
1000
5
30
60
120
Time (m)
a.

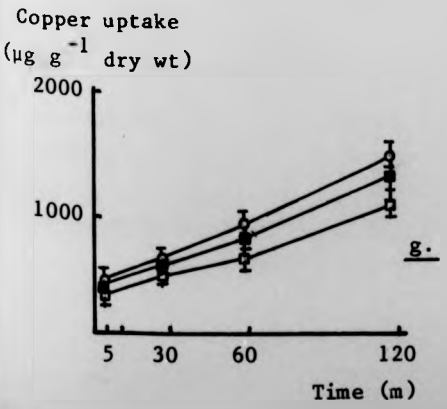

Copper uptake
(µg g -1 dry wt)
2000
1000
5
30
60
120
Time (m)
b.

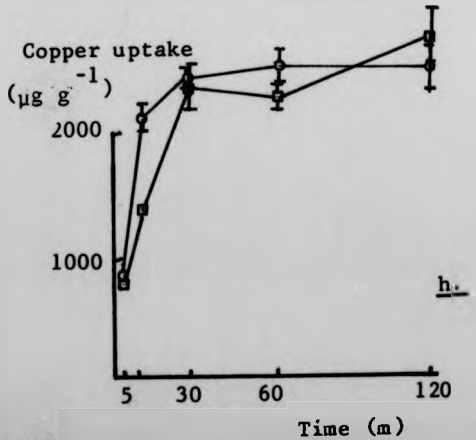

Cadmium uptake
(µg g -1 dry wt)
2000
1000
5
30
60
120
Time (m)
c.


Cadmium uptake
(µg g -1 dry wt)
2000
1000
5
30
60
120
Time (m)
d.


Zinc uptake
(µg g -1 dry wt)
2000
1000
5
30
60
120
Time (m)
e.


Zinc uptake
(µg g -1 dry wt)
2000
1000
5
30
60
120
Time (m)
f.


Copper uptake
(µg g -1 dry wt)
2000
1000
5
30
60
120
Time (m)
g.


Copper uptake
(µg g -1)
2000
1000
5
30
60
120
Time (m)
h.

*Tables* 8.3.2.1a-d Proportions of non-desorbable metal after 5 minute and 2 hour incubations in metal solutions under various incubation conditions.

*Table* 8.3.2.1a. Controls (50.0 mM MES only at 25°C).

| Incubation Time | 5 min $Cu^{++}$ | 2 hr $Cu^{++}$ | 5 min $Cd^{++}$ | 2 hour $Cd^{++}$ | 5 min $Zn^{++}$ | 2 hr $Zn^{++}$ |
|---|---|---|---|---|---|---|
| | Proportion | | metal | desorbed | (%) | |
| *P. spinulosum* | 35.0 | 41.0 | 21.4 | 11.1 | 45.5 | 45.1 |
| *T. viride* | 35.8 | 54.2 | 46.9 | 44.3 | 44.6 | 44.4 |
| *A. niger* | 58.1 | 44.21 | 19.7 | 44.7 | 90.6 | 66.3 |

*Table* 8.3.2.1b. 50.0 mM MES plus 10.0 mM glucose.

| Incubation Time | 5 min $Cu^{++}$ | 2 hr $Cu^{++}$ | 5 min $Cd^{++}$ | 2 hr $Cd^{++}$ | 5 min $Zn^{++}$ | 2 hr $Zn^{++}$ |
|---|---|---|---|---|---|---|
| | Proportion | | metal | desorbed | (%) | |
| *P. spinulosum* | 32.6 | 52.9 | 39.6 | 17.0 | 50.8 | 31.3 |
| *T. viride* | 53.9 | 58.4 | 59.5 | 79.6 | 56.4 | 66.92 |
| *A. niger* | 51.0 | 44.9 | 16.8 | 43.1 | 65.9 | 62.5 |

*Table* 8.3.2.1c. 50.0 mM MES plus 1.0 mM sodium azide.

| Incubation Time | 5 min $Cu^{++}$ | 2 hr $Cu^{++}$ | 5 min $Cd^{++}$ | 2 hr $Cd^{++}$ | 5 min $Zn^{++}$ | 2 hr $Zn^{++}$ |
|---|---|---|---|---|---|---|
| | Proportion | | metal | desorbed | (%) | |
| *P. spinulosum* | 40.9 | 30.5 | 73.0 | 12.7 | 32.0 | 34.9 |
| *T. viride* | 40.6 | 42.1 | 43.4 | 65.10 | 77.7 | 53.3 |
| *A. niger* | 34.6 | 53.7 | 27.2 | 42.4 | 79.0 | 60.1 |

*Table* 8.3.2.1d. 50.0 mM MES only at 4°C

| Incubation Time | 5 min $Cu^{++}$ | 2 hr $Cu^{++}$ | 5 min $Cd^{++}$ | 2 hr $Cd^{++}$ | 5 min $Zn^{++}$ | 2 hr $Zn^{++}$ |
|---|---|---|---|---|---|---|
| | Proportion | | metal | Desorbed | (%) | |
| *P. spinulosum* | 51.2 | 27.5 | 33.5 | 19.4 | 83.9 | 40.10 |
| *T. viride* | 34.6 | 52.9 | 63.3 | 52.4 | 60.7 | 54.5 |
| *A. niger* | 57.7 | 40.4 | 14.1 | 50.2 | 63.2 | 78.2 |

8.3.2.2. Quantitative uptake of metal in growing and non-growing cells.

A comparison of non-growing biomass and actively growing biomass metal accumulation capacities reveals a marked difference between the two systems. The data presented in Table 8.3.2.2. shows that non-growing suspensions of mid-linear phase fungal material were able to accumulate significantly greater quantities of metal ion than were actively growing cultures in mid-linear phase.

The values in Table 8.3.2.2. show the apparent differences in accumulation from 2.5 μg $ml^{-1}$ for copper and cadmium. There is no data available for zinc at 2.5 μg $ml^{-1}$ for actively growing mycelium, but comparison with data for uptake from 5.0 μg $ml^{-1}$ zinc with growing material and 2.5 μg $ml^{-1}$ zinc for non-growing suspensions, demonstrates that there is a greater affinity for zinc in the non-growing biomass.

The data for *T. viride* and cadmium is particularly noteworthy with growing material capable of accumulating 31.4 (± 2.2) μg $g^{-1}$ dry weight whilst similar, but non-growing suspensions of the biomass achieved a final cadmium content of 2727.4 (± 57.1) μg $g^{-1}$ dry weight after 2 hour incubations in the metal solution. pH reductions in mid-linear phases of growing suspensions may be responsible at least in part for these differences in metal uptake, although it is likely also that physiological differences in the fungi are involved.

*Table* 8.3.2.2.

Comparison of metal accumulation from 2.5 $\mu g\ ml^{-1}$ by actively growing fungi in mid-linear phase and non-growing suspensions of mid-linear phase fungi.

| Fungus. | $Cu^{++}$ uptake ($\mu g\ g^{-1}$) actively growing | $Cu^{++}$ uptake ($\mu g\ g^{-1}$) non growing |
|---|---|---|
| *P. spinulosum.* | 470.8 ($\pm$22.1) | 2388.6 ($\pm$42.2) |
| *T. viride.* | 97.9 ($\pm$3.3) | 1439.1 ($\pm$149.1) |
| *A. niger.* | 151.0 ($\pm$9.1) | 1844.2 ($\pm$104.7) |
| Fungus. | $Zn^{++}$ uptake ($\mu g\ g^{-1}$) actively growing | $Zn^{++}$ uptake ($\mu g\ g^{-1}$) non growing |
| *P. spinulosum.* | *199.7 ($\pm$12.5) | 1303.8 ($\pm$82.0) |
| *T. viride.* | *216.5 ($\pm$8.7) | 647.2 ($\pm$31.7) |
| *A. niger.* | *142.4 ($\pm$2.7) | 835.9 ($\pm$35.0) |
| Fungus. | $Cd^{++}$ uptake ($\mu g\ g^{-1}$) actively growing | $Cd^{++}$ uptake ($\mu g\ g^{-1}$) non growing |
| *P. spinulosum.* | 91.9 ($\pm$2.3) | 1434.8 ($\pm$27.7) |
| *T. viride.* | 31.4 ($\pm$2.2) | 2727.4 ($\pm$57.1) |
| *A. niger.* | 69.3 ($\pm$1.4) | 766.5 ($\pm$28.4) |

Data for growing mycelium is based on a minimum of 5 replicate cultures.

Data for non-growing mycelium is based on 3 replicate samples and duplicated.

* Uptake from 5.0 $\mu g\ ml^{-1}$ $Zn^{++}$ in GMS medium.

8.3.2.3. Effect of anaerobiosis on metal uptake by non-growing suspensions of biomass.

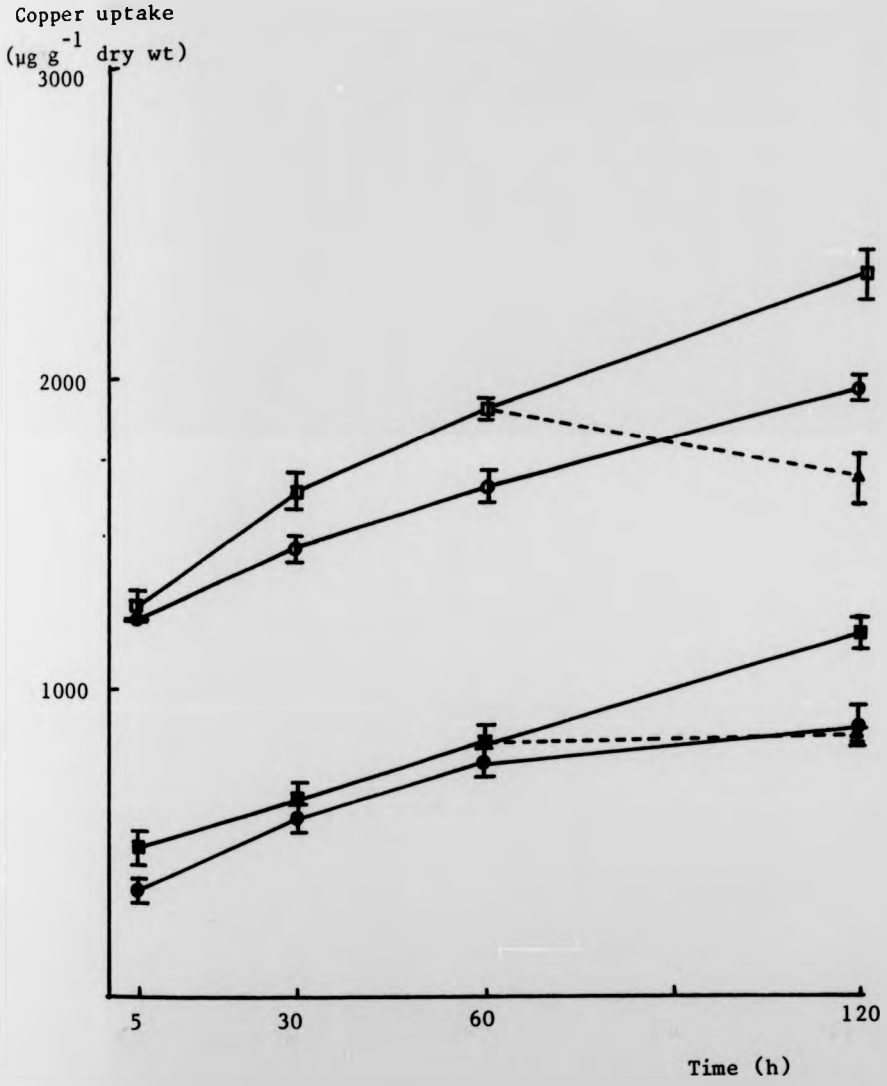
The effect of anaerobic incubation in the presence of metal is illustrated in Figures 8.3.2.11-15. Copper, cadmium and zinc accumulation are presented for *P. spinulosum*, cadmium for *A. niger* and zinc for *T. viride*.

The uptake pattern was typical with rapid accumulation over the first 5 minutes followed a period of decelerating uptake upto 2 hours. Total and non-desorbable metal content of all fungi was elevated by anaerobic incubation. 1 hour incubation under anaerobic conditions, followed by 1 hour incubation under normal aeration resulted in an increase in metal content over controls incubated aerobically throughout the incubation period after 1 hour, and this was reduced to a value approximating to control values after the second period of aerobic incubation. Non-desorbable metal showed a similar pattern.

Figures 8.3.2.16-20 show the growth of metal loaded biomass after incubation under anaerobic conditions when washed and resuspended in fresh GMS medium. There appeared to be no adverse effect on the growth characteristics of the fungi tested after metal-loading in anaerobic conditions.

*Figure* 8.3.2.11.

Copper uptake from 50mM MES containing 2.5µg $ml^{-1}$ $Cu^{++}$ by a non-growing suspension of *P. spinulosum* under aerobic and anaerobic ($O_2$ free $N_2$ bubbling) incubation at 25°C.




○ Total $Cu^{++}$ uptake under aerobic incubation.

□ Total $Cu^{++}$ uptake under anaerobic incubation.

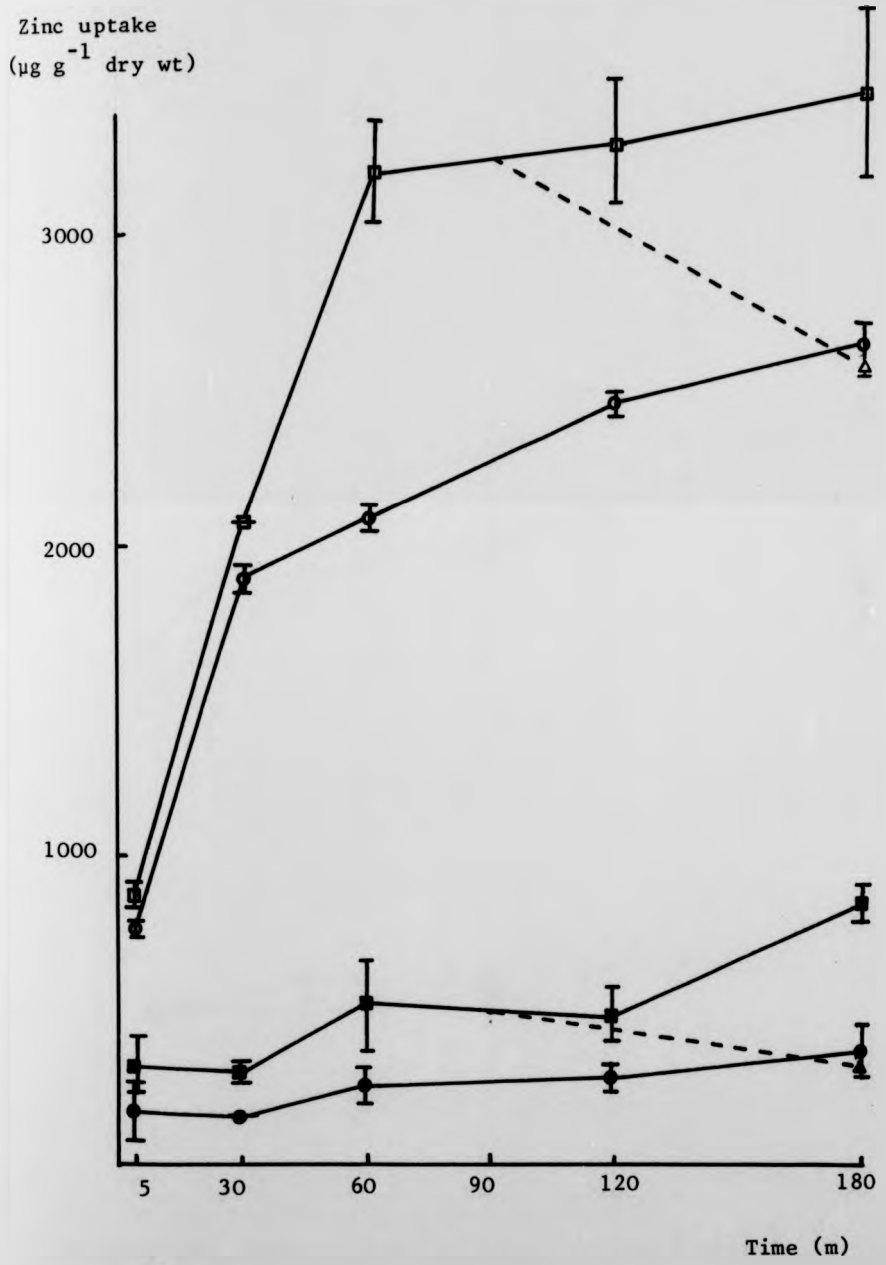
● Non-desorbable $Cu^{++}$ content after aerobic uptake and 1mM EDTA treatment.

■ Non-desorbable $Cu^{++}$ content after anaerobic uptake and 1mM EDTA treatment.

△ Total $Cu^{++}$ content after uptake for 1h under anaerobic incubation followed by 1h incubation under normal aeration, Non-desorbable $Cu^{++}$, ▲.

*Figure* 8.3.2.12.

Zinc uptake from 50mM MES containing 5.0 µg $ml^{-1}$ $Zn^{++}$ by a non-growing suspension of *P. spinulosum* under aerobic and anaerobic ($O_2$ free $N_2$ bubbling) incubation at 25°C.




○ Total $Zn^{++}$ uptake under aerobic incubation.

□ Total $Zn^{++}$ uptake under anaerobic incubation.

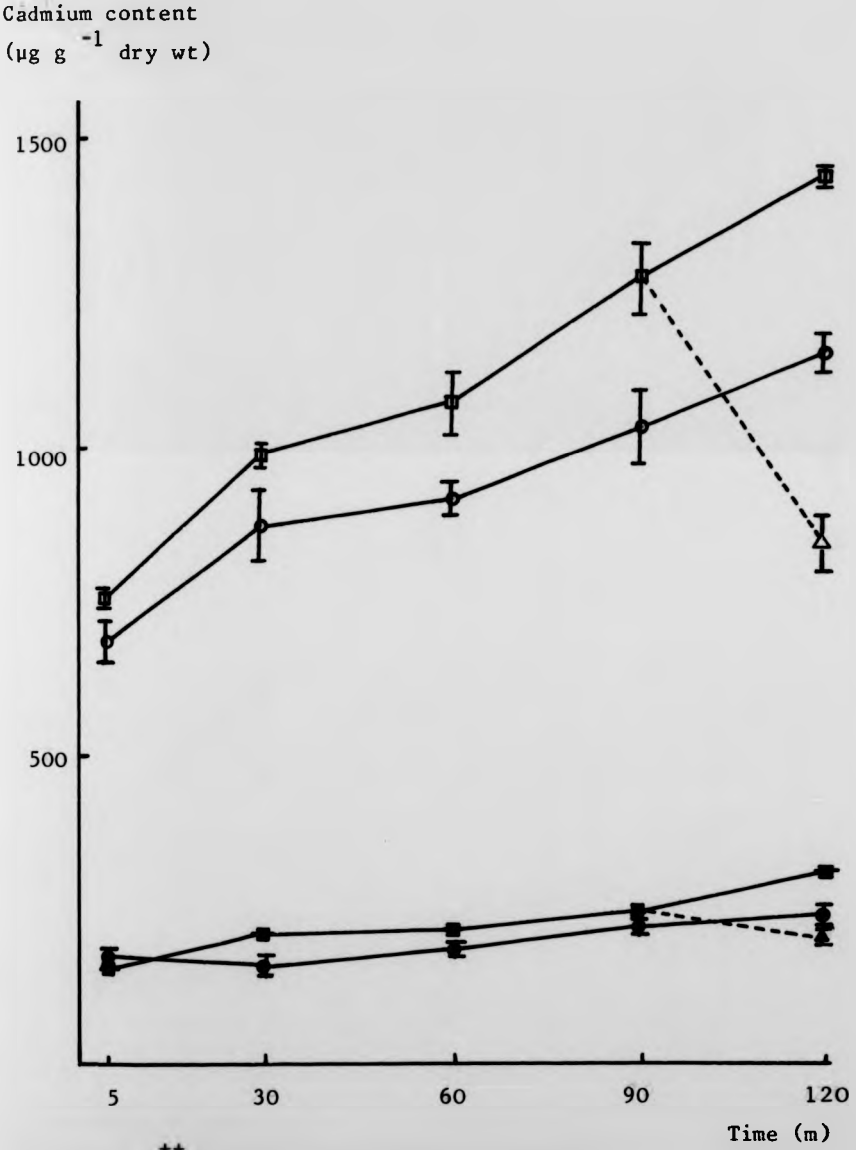
● Non-desorbable $Zn^{++}$ content after aerobic uptake and 1mM EDTA treatment.

■ Non-desorbable $Zn^{++}$ content afer anaerobic uptake and 1mM EDTA treatment.

△ Total $Zn^{++}$ content after uptake for 1 h under anaerobic incubation followed by 1h incubation under normal aeration. Non-desorbable $Zn^{++}$, ▲.

*Figure* 8.3.2.13.

Cadmium uptake from 50mM MES containing 2.5 µg $ml^{-1}$ $Cd^{++}$ by a non-growing suspension of A. *niger* under aerobic and anaerobic ($O_2$ free $N_2$ bubbling) incubation at 25°C.




○ Total $Cd^{++}$ uptake under aerobic incubation.

□ Total $Cd^{++}$ uptake under anaerobic incubation.

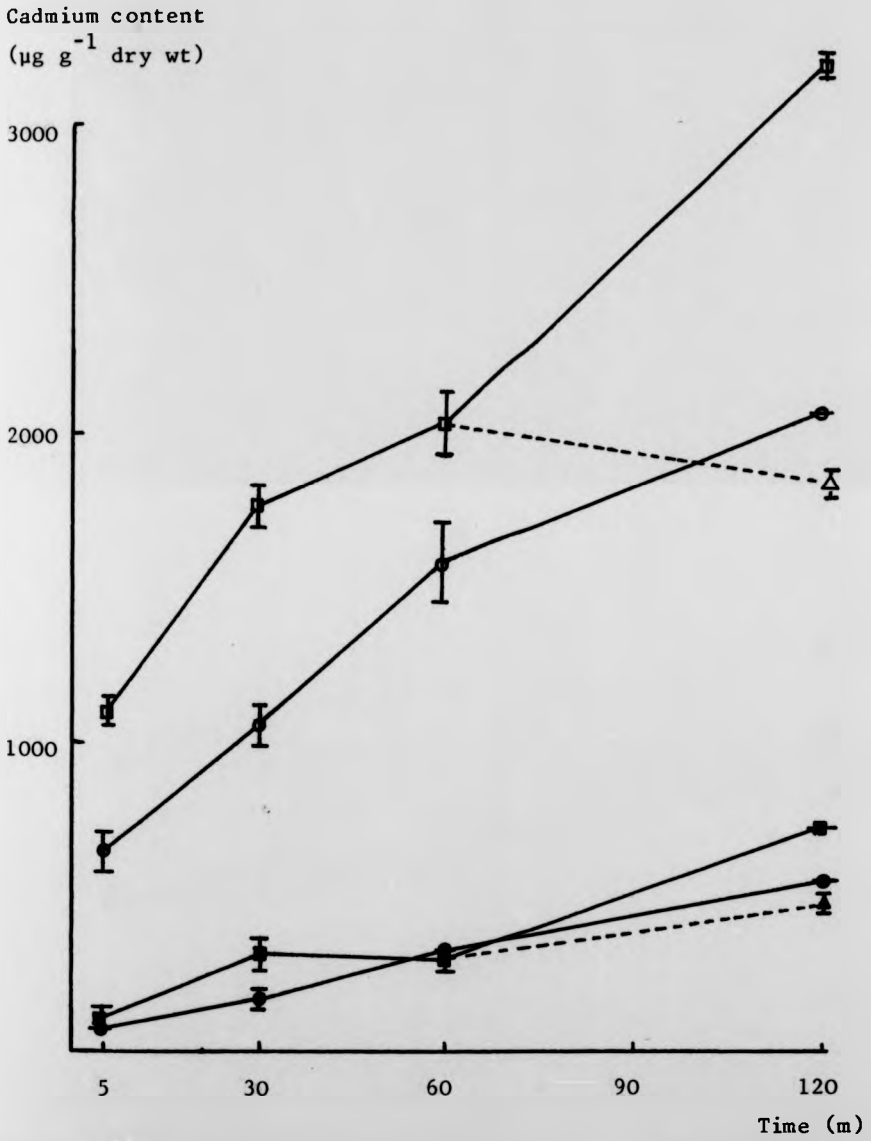
● Non-desorbable $Cd^{++}$ content after aerobic uptake and 1mM EDTA treatment.

■ Non-desorbable $Cd^{++}$ content after anaerobic uptake and 1mM EDTA treatment.

△ Total $Cd^{++}$ content after uptake for 1h under anaerobic incubation followed by 1h incubation under normal aeration. Non-desorbable $Cd^{++}$, ▲.

*Figure* 8.3.2.14.

Cadmium uptake from 50mM MES containing 2.5 µg $ml^{-1}$ $Cd^{++}$ by a non-growing suspension of *P. spinulosum* under aerobic and anaerobic ($O_2$ free $N_2$ bubbling) incubation at 25° C.




○ Total $Cd^{++}$ uptake under aerobic incubation.

□ Total $Cd^{++}$ uptake under anaerobic incubation.

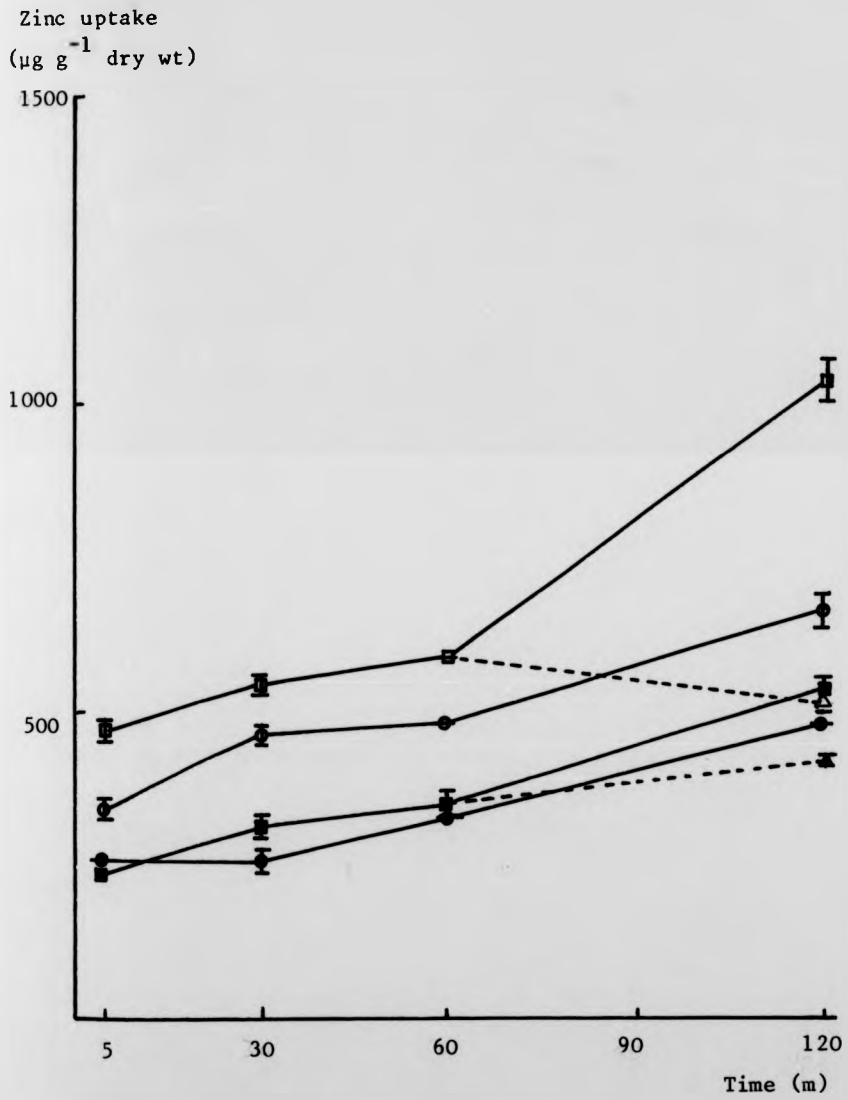
● Non-desorbable $Cd^{++}$ content after aerobic uptake and 1mM EDTA treatment.

■ Non-desorbable $Cd^{++}$ content after anaerobic uptake and 1mM EDTA treatment.

△ Total $Cd^{++}$ content after uptake for 1h under anaerobic incubation followed by 1h incubation under normal aeration. Non-desorbable $Cd^{++}$, ▲ .

*Figure* 8.3.2.15.

Zinc uptake from 50mM MES containing 2.5 µg $ml^{-1}$ $Zn^{++}$ by a non-growing suspension of *T. viride* under aerobic and anaerobic ($O_2$ free $N_2$ bubbling) incubation at 25°C.




○ Total $Zn^{++}$ uptake under aerobic incubation.

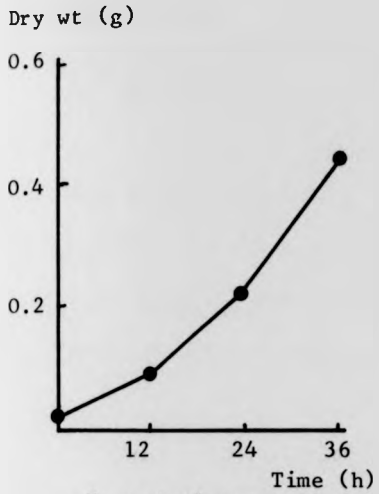
□ Total $Zn^{++}$ uptake under anaerobic incubation.

● Non-desorbable $Zn^{++}$ content after aerobic uptake and 1mM EDTA treatment.

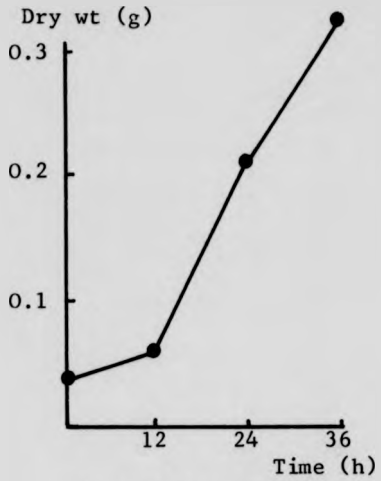
■ Non-desorbable $Zn^{++}$ content after anaerobic uptake and 1mM EDTA treatment.

△ Total $Zn^{++}$ content after uptake for 1h under anaerobic incubation followed by 1h incubation under normal aeration. Non-desorbable $Zn^{++}$, ▲.

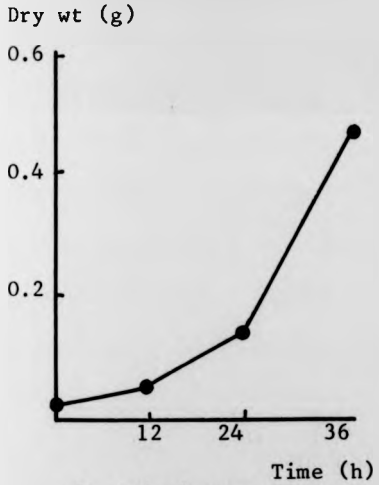
*Figures* 8.3.2.16-20



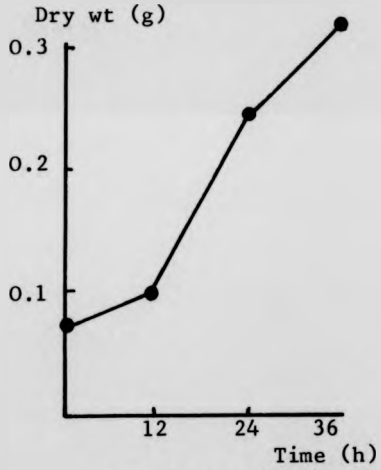

*P. spinulosum* $Zn^{++}$



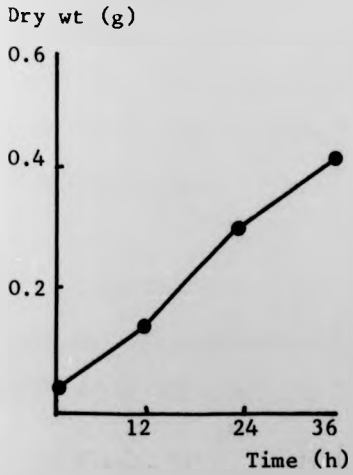

*P. spinulosum* $Cu^{++}$




*P. spinulosum* $Cd^{++}$




*T. viride* $Zn^{++}$




*A. niger* $Cd^{++}$

Graphs to show biomass growth after 2 hours in anaerobic conditions with 2.5 µg $ml^{-1}$ metal salt when resuspended in GMS medium.

8.4. Discussion.

The pattern of metal uptake in unstarved non-growing cells (Figures 8.3.2.1-9) was similar to those described by other workers using non-growing mycelium over short incubation periods (Paton and Budd, 1972). The described pattern was also similar for other organisms (Ponta and Broda, 1970; Horikoshi *et al*, 1981; Norris and Kelly, 1977 and Wakatsuki *et al*, 1979) and in fungal conidia (Somers, 1963).

Over the initial 5 minutes, uptake appeared to be very rapid for all the metals tested in all 3 fungi and it is likely that this period represented surface binding by adsorption to binding sites. The rate of uptake decreased over the subsequent 50 minutes upto 1 hour with little or no further uptake upto 2 hours of incubation. There appeared to be some degree of batch variation in terms of quantitative metal uptake between experiments, although this was not observed within experiments. This was almost certainly due to slight physiological differences in fungi between different suspension preparations. This pattern of accumulation was identical in controls, in fungi treated with metabolic inhibitors, in cells with additional glucose and in cells in which incubation was carried out at reduced temperature. The decrease in rate of uptake after 5 minutes may be indicative of an internalisation process which could be active or passive. The various treatments set out to determine which of the two possible mechanisms was operative under the conditions of these experiments.

Most workers have reported that the initial binding in fungi is a process of adsorption (Lowry *et al*, 1957; Budd, 1969, 1975, 1979; Paton and Budd, 1972). This has also been reported to be the case

specifically in yeasts (Ponta and Broda, 1970; Failla and Weinberg, 1977; Norris and Kelly, 1977 and Wakatsuki *et al*,1979). Somers (1963) reported that the uptake of copper by conidia of *Neurospora crassa* was initially an ion-exchange reaction which was followed by permeation throughout the cell but there was no evidence of an adsorption process. The slower phase of uptake has been reported to have metabolic dependence in fungi (Budd, 1969, 1975, 1979; Paton and Budd, 1972) and in yeasts (Ponta and Broda, 1970; Failla and Weinberg, 1977; Norris and Kelly, 1977). It should be mentioned that the profile for non-desorbable metal was similar to the profiles for total metal uptake in all cases. Desorption data results have to be interpreted cautiously because it is difficult to determine the exact location from where the metal was originally desorbed. The metal is likely to be easily desorbed from low affinity sites on the cell wall, such as the additional adsorption sites reported for uranium and thorium in *R. arrhizus* by Tsezos and Volesky (1982a, 1982b). Desorption of tightly bound metal ions on the other hand, is likely to be much more difficult, such as for example, those ions involved in the formation of coordination and nucleation sites (Tsezos and Volesky, 1982b). Also the amount of metal which is removable may very well depend on the initial ambient metal concentration. For example, at low metal concentrations high affinity binding sites may be involved whereas at higher concentrations these and other lower affinity sites may be involved as reported by Budd (1975, 1979) for filamentous fungi and by Rothstein and Hayes (1956) for yeasts. It is likely that metal desorption from high affinity sites is much less effective than from low affinity binding sites. It is also possible that highly effective chelating agents such as EDTA may remove intracellular metal, although an attempt was made to monitor this by checking for $K^+$ losses from the cell. $K^+$ losses ranged from 0 to 15% over the 15 minute incubation in EDTA and these losses were probably due to the EDTA causing membrane leakiness. Also during incubation

in the presence of metal ion exchange mechanisms can occur whereby $K^+$ ions are exchanged for metal ions. $K^+$ exchange and efflux in response to metal uptake for the purpose of maintaining electroneutrality, has been reported by a number of workers. According to Okamoto *et al* (1977) $K^+$ increased in mycelium of *Penicillium ochrochloron* at toxic nickel levels, whereas at high concentrations of copper and zinc, there was a marked reduction in cellular $K^+$ content. Most reports however, have been for yeasts, and $K^+$ exchange for metal ions has been observed for cobalt uptake (Fuhrmann and Rothstein, 1968; Fuhrmann, 1973; Norris and Kelly, 1977) and manganese uptake (Okorokov *et al*, 1979). Lichko *et al* (1980) have shown that manganese accumulation into vauoles was accompanied by a decrease in vacuolar $K^+$ concentration. In *S. cerevisiae*, but not in *Sporobolomyces roseus*, Mowll and Gadd (1983) showed that $K^+$ was lost due to membrane damage in reponse to zinc uptake. Passow *et al* (1961) also discussed $K^+$ loss from erythrocytes after lead accumulation.

It was initially proposed by the author that metal desorption as a proportion of total metal uptake ought to become less significant as metal was internalised because the ever increasing amount of intracellular metal should not be desorbable, so the proportion of cell wall associated metal should become less significant. However, the results in Tables 8.3.2.1a, 1b, 1c and 1d suggest that there was no consistent trend in the amounts of metal desorbable after 5 minute and 2 hour incubations for control cultures, those treated with glucose, metabolic inhibitors and a reduced temperature. This may be due to a lack of sensitivity of the technique since the amount of internal metal may be quite small after 2 hours of incubation although if one takes account of the desorption data, the amount of non-desorbable metal (and therefore possibly internal metal) did range from 11.1 to 90.6%. Although as already mentioned, this may be due in part to tightly bound

metal, but it is not possible to speculate confidently on the variability of the tightness of binding and the degree of internalisation in different fungi.

The overall results from the investigation of metabolic dependence suggested that there was little or no active uptake of metallic cations under the conditions of these experiments.

The design of the experiments should have produced results similar to those reported by Paton and Budd (1972) since the experimental conditions were very similar. Paton and Budd (1972) monitored zinc uptake from 6.5 μg $ml^{-1}$ zinc by non-growing suspensions of *Neocosmospora vasinfecta*. These authors were able to show that zinc uptake was a two-stage process. Phase 1 was believed to represent adsorption to negatively charged groups in the hyphal surface-membrane whilst phase 2 appeared to be a more slowly established process of transport into the cytoplasm. Phase 1 showed no metabolic dependence and was not influenced by low temperature, $NaN_3$ or anaerobiosis but was reduced in the presence of various other divalent cations. Phase 2 on the other hand was strongly inhibited by low temperature, $NaN_3$ and anaerobiosisand exhibited carrier-type kinetics. Paton and Budd used pH 6.5 as their experimental pH as opposed to 5.5, but this is unlikely to havecaused the dramatic differences observed.

It is worthy of note that Venkateswerlu and Sivarama Sastry (1970) showed energy dependent uptake of cobalt in preformed, washed and resuspended growing mycelium of *Neurospora crassa*. These authors stated that they chose to carry out uptake experiments at 200.0 μg $ml^{-1}$ cobalt because the metal was toxic to preformed mycelium at this concentration. The mycelia though were still viable after 2 hours of incubation. These authors showed that both surface binding and overall uptake were temperature dependent.

Transport of cobalt was depressed by azide, dinitrophenol and fluoride. No account was taken of possible interference of growth kinetics.

It is interesting that the work of Somers (1963) showed that in contrast to these results, in ascospores of *Neurospora crassa* uptake was almost totally by an energy independent system. The uptake of copper from 19.0 μg $ml^{-1}$ copper was dependent on temperature and an activation energy suggestive of a physical uptake mechanism was calculated. Other cations were able to compete for the metal binding sites at the fungal surface. Respiratory inhibitors and thiol reagents were able to reduce the uptake of copper whilst the mechanism was unaffected by 2,4 DNP. The reaction of iodoacetate with cellular sulphydryl groups blocked these sites to copper binding. Anaerobic incubation increased copper uptake by 25%.

It is clear that although the experimental conditions and the physiological states of the fungi were similar to those employed by Paton and Budd (1972, the energy dependent phase 2 zinc uptake shown in *Neocosmospora vasinfecta* was not reproducible in *P. spinulosum*, *T. viride* or *A. niger*. Similarly, copper and cadmium uptake was not by an energy dependent mechanism in these three fungi. On the other hand, the characteristics of metal uptake in this study appeared to show some similarity to the patterns of uptake demonstrated for ascospores of *N. crassa* by Somers (1963) when these cells were subjected to copper solutions.

The presence of glucose did not appear to have any marked effect on metal uptake in any of the fungi tested over the 2 hour incubation period. The rationale of the experiments was that if there was an energy dependent uptake system, then total uptake ought to be enhanced in the presence of glucose. If on the other hand, there had been a reduction of metal uptake

in the presence of glucose, this could have been indicative of a metabolic efflux system or of competition for uptake of the metal by glucose. It is likely that the binding of metal to the cell surface accounted for a very large proportion of the total uptake which could have masked any metabolic uptake.

It is interesting though that in *Neocosmospora vasinfecta*, glucose inhibited energy dependent phase 2 uptake of zinc from a solution containing 65.4 µg $ml^{-1}$ at all the glucose concentrations tested from 0.1 mM to 90.0 mM over a 20 minute period. Inhibiton was curvilinear with respect to glucose and amounted to 46% at 90.0 mM glucose (Paton and Budd, 1972). In *P. spinulosum*, it is interesting that the presence of glucose caused slight reductions in copper uptake from 10.0 µg $ml^{-1}$ ranging from 7.6% to 13.9% at 5.6 mM and 27.7 mM glucose respectively (section 8.2.1.7). It appears that glucose either competes for uptake under these conditions, or is involved in promoting a metabolic efflux system. In fact Paton and Budd (1972) reported that preincubating in glucose at 1.0 mM concentration reduced subsequent phase 2 binding by 18%, which is of the same order as the reduction observed in this study.

The expected result of glucose stimulation of metal uptake has been observed in yeast studies.

In *Saccharomyces cerevisiae* Ross (1977) reported a dramatic enhancement of copper uptake in the presence of 10.0 mM glucose so that 70% of the available copper was removed by the experimental cells in 10 minutes from 1.02 µg $ml^{-1}$ copper. Omission of the glucose reduced copper binding to less than 20% in 60 minutes. With the enhancement of uptake there was a concomitant increase in toxicity. Ross (1977) concluded that copper uptake was energy dependent since the conditions that favoured fermentation caused an increase in copper uptake.

Failla and Weinberg (1977) suggested that removal of glucose from the medium caused an overall reduction of total zinc uptake in *Candida utilis*. For stationary phase cells, the authors showed that non-growing cells from a low glucose culture were far less able to accumulate zinc than normally grown cells. Addition of glucose to cells that had been in stationary phase for 24 hours grown on 16.5 or 5.5 mM glucose, caused increases in accumulation of 180 and 11% respectively. This indicated that although glucose limitation caused a decrease in the quantitative amount of zinc accumulated and that glucose was able to stimulate uptake, energy status was not the only factor involved. The authors hypothesised that the zinc status of the cells controlled the quantity of metal that could be accumulated subsequently and that glucose was a mediator.

The metabolic inhibitor studies on metal uptake demonstrated slight reductions in metal uptake in some cases after preincubation in 1.0 mM azide. This tends to support the argument that most of the metal is located on the cell wall. The fact that preincubation in monoiodoacetate or 2,4 DNP had no significant effect in reducing total uptake or that azide reduced copper and zinc uptake in *P. spinulosum* and cadmium in *T. viride* by a small amount only, suggests that there was either no metabolic uptake or that the metabolic uptake was so small that it was masked by the major proportion of uptake onto the cell wall which is not an energy dependent process. It is interesting that simultaneous incubation of the metal with azide caused a marked reduction in metal uptake and the results from the polarographic studies suggest that this was as a result of metal complexation by the azide.

In contrast to these findings, Paton and Budd (1972) reported that although phase 1 binding was unaffected by sodium azide treatment, phase 2

uptake was severely inhibited in the presence of metabolic inhibitors. Also the effect of 0.1 mM $NaN_3$ did not appear to be fully reversible.

Somers (1963) studied the effects of seven metabolic inhibitors on copper uptake by conidia of *Neurospora crassa*. He first preincubated cells in inhibitor, then added metal, whilst in another set of experiments he added inhibitor and metal concurrently. In the first type of experiment the complication of reaction between copper and the inhibitor was avoided. Both sets of experiments showed that respiratory inhibitors and thiol reagents reduced the copper uptake by 2 to 36% whilst 2,4 DNP did not affect the uptake mechanism. Preincubation in inhibitor was the most effective method of inhibiting uptake using monoiodoacetate, resulting in 31% inhibition as opposed to 11% inhibition when copper and monoiodoacetate were added simultaneously. Monoiodoacetate and p-chloromercuribenzoate were assumed to block copper binding by reaction with cellular sulphydryl groups. The results of Somers suggest that in *Neurospora crassa* conidia, a small proportion of copper uptake is linked to respiration. According to Somers (1963), sodium azide had no effect on copper uptake on preincubation of the conidia in the inhibitor. Azide only became effective in reducing uptake when the metal and inhibitor were applied simultaneously, but this was probably due to complexing. In this study though preincubation in sodium azide reduced metal uptake slightly. In general the small levels of inhibition shown by Somers are in agreement with the results observed in this study, indicating that most of the uptake in *N. crassa* conidia and the mycelium of the fungi used in this study, was due to surface binding.

In a study of cobalt uptake in growing cells of *N. crassa* over a 2 hour incubation period, Venkateswerlu and Sivarama Sastry (1970) were

able to demonstrate that about 40% of the total uptake was rapid surface binding. The authors showed that azide, DNP and fluoride inhibited uptake by about 50%. According to Tissieres *et al* (1953), azide inhibited respiratory metabolism by 50%. The results obtained using the oxygen electrode (section 8.2.1.10) suggested that 1.0 mM azide was more effective than this, with 72.6, 87.4 and 96.1% inhibition of oxygen uptake in *T. viride*, *A. niger* and *P. spinulosum* respectively. In the study of Venkateswerlu and Sivarama Sastry (1970) short term uptake (20 minutes) was inhibited by 19, 5 and 6% by azide, 2,4 DNP and fluoride respectively. This could have been caused by complexation of the metal or by competition for binding sites between the metal and inhibitor. The small degrees of inhibition observed could suggest that uptake in the fungi in this study was mostly due to surface binding. Thus, the inhibitors affected primarily the transport of cobalt into the mycelial cells and not surface binding. A value of 40% for surface binding of cobalt uptake was obtained by extrapolating from a time course experiment and occurred in 20 minutes (EDTA desorption studies showed 30% surface binding). The authors must have assumed that there was no further binding after this period. It may be of some significance that the authors used 200.0 µg $ml^{-1}$ $Co^{++}$ which was chosen for its toxicity, and therefore the results obtained are not likely to be typical for normal uptake processes.

In growing cells of *Dactylium dendroides*, Shatzman and Kosman (1978) reported that 2,4 DNP and $CN^-$ did not inhibit copper uptake and thus ascribed uptake to an energy-independent process. Growing material however, is likely to produce complicated results because the model is a dynamic one.

The results of anaerobic incubation under deoxygenated $N_2$ was rather surprising in that there was a 20 to 30% increase in total metal uptake over the 2 to 3 hour incubation period, even though there appeared

to be no loss of viability. This increase in total uptake was paralleled by an increase in non-desorbable uptake presumed to represent intracellular uptake over the control cultures incubated under standard aeration. These findings may support the idea that a metabolic efflux system is operational under aerobic conditions, since under anaerobic incubation, when the efflux system would be inhibited, the total and intracellular metal levels increased. Another possible explanation is that under anaerobic incubation the fungus could produce a metabolite which could bind metal in excess of the normal amounts, and this might account for the higher apparent uptake. Also the proportion of wall bound component (desorbable metal) was usually lower under anaerobic incubation than under aerobic incubation possibly suggesting that under anaerobic conditions, the internal metal proportion increased, perhaps again implying that there was an inoperable efflux system under anaerobic incubation.

When the fungi were incubated anaerobically for 60 minutes in the presence of the metals and then reaerated for a further 60 minutes, it is interesting that the total and non-desorbable metal content of the fungi started to decrease. This again may be indicative of a metabolic efflux system which operates under aerobic conditions. If metabolites were produced under anaerobic conditions (which could possibly bind metal), the metal would be likely to remain at the same level on reaeration until it was eventually delocalised and pumped out of the cell. It is interesting though that the decline in metal on reaeration was so rapid, suggesting that the effect may have been a chemical one, perhaps involving oxidation state changes. There appears to be no data available on different binding potentials of the various oxidation states of copper.

Paton and Budd (1972) demonstrated the opposite response to an anaerobic atmosphere for phase 2 uptake in the fungus *Neocosmospora vasinfecta*. Although there was no effect on phase 1 uptake, zinc

accumulation in phase 2 was inhibited by 88% after 2 hours of anaerobiosis but uptake was fully restored after returning to aerobic conditions even after 230 minutes of anaerobic incubation. Clearly, zinc uptake in this organism is dependent on metabolism, and yet in *P. spinulosum* and *T. viride* zinc uptake was stimulated by anoxic conditions. It is interesting that in *N. crassa* conidia, copper uptake under anaerobic incubation was increased, (Somers, 1963) as in this study, whilst in *Neocosmospora vasinfecta* phase 1 binding was unaffected by anoxia.

The results obtained in this work are in agreement with the data of Somers (1963), which showed that for copper uptake in conidia of *N.crassa* there was a 25% increase in total uptake after 30 minutes in an atmosphere of $O_2$ free $N_2$ from a copper solution containing 17.0 µg $ml^{-1}$ copper. This was similar to the situation reported by Hassal (1962) who found that copper was taken up by *Chlorella vulgaris* to a greater extent under anaerobic conditions. Somers (1963) postulated that sites were available under anaerobic conditions for copper binding which were more easily oxidised. According to McBrien (1980), of the two copper species $Cu^{+}$ and $Cu^{++}$, $Cu^{+}$ is unstable in aqueous solutions in the presence of oxygen or oxidising metal ions such as $Fe^{+++}$ and is rapidly oxidised to $Cu^{++}$. In a variety of different types of cell, for example in algae (McBrien and Hassall, 1965, 1967) and bacteria (Beswick *et al*, 1976), the toxic effects of copper were greater when the ions were applied in anoxic conditions than under aerobic conditions. The increased toxicity was due to the fact that $Cu^{+}$ was substantially more toxic than $Cu^{++}$.

Reducing the temperature to 4°C in all cases reduced total and non-desorbable metal uptake. The rapid binding phase over 5 minutes appeared to be slightly reduced as well as the slower uptake phase upto 2 hours.

Paton and Budd (1972) reported that reducing the incubation temperature from 25°C to 3°C reduced phase 1 zinc uptake in *Neocosmospora vasinfecta* by 3.6%. This finding is in contrast to the result demonstrated in this study where surface binding appeared to be reduced at decreased temperature. Paton and Budd (1972) also showed that phase 2 energy dependent zinc uptake was significantly reduced by 69.1% and 73.5% after 60 minutes and 2 hours incubation at 3°C.

In *N. crassa* conidia, there appeared to be a definite temperature dependence for copper binding (Somers 1963). Venkateswerlu and Sivarama Sastry (1970) also showed that surface binding of cobalt to growing cells of *N. crassa* was directly proportional to temperature increase. Also Tsezos and Volesky (1981) showed that the uptake capacity of *Rhizopus arrhizus* was influenced by temperature, in that a temperature increase favoured uptake of Uranium and Thorium.

Muzzarelli *et al* (1980) reported that the efficiency of copper and mercuric ion adsorption by a chitosan-glucan complex from *Aspergillus niger* was more efficient at 60 than at 20°C.

Beckett and Brown (1984) reported that in experiments lasting 30 minutes, extracellular cadmium uptake in the lichen *Peltigera horizontalis*, increased with increasing temperature in the range 0 to 30°C. Clearly, temperature effects are very important in physical metal uptake processes.

It is worth noting here that active phases of metal uptake in fungi showed temperature dependence also (Paton and Budd, 1972; Venkateswerlu and Sivarama Sastry, 1970), in lichens (Beckett and Brown, 1984) and in yeasts (Ponta and Broda, 1970; Norris and Kelly, 1977; Failla and Weinberg, 1977; and Ross, 1977).

The results of this section of the study do seem to indicate that uptake in non-growing suspensions of mycelium is not primarily an energy dependent system, although as already mentioned, it may be that a small proportion of the total uptake was active, but was masked by the wall binding component. The likelihood is though, that uptake occurs by an initial process of adsorption followed by an internalisation process which is either active (not demonstrated in this study) or passive, for example, a process of diffusion. It is possible that uptake is more easily shown to be metabolically dependent in growing cultures. It would seem feasible to the author, that lag phase growing cultures and non-growing mid-linear phase mycelium take up metal predominantly by adsorption, thus resulting in the similar quantitative metal contents. It is possible that there may be a metabolic uptake process in growing mycelium during linear phase, which is inducible under conditions of low metal status. It would seem extravagant to expend energy on metal accumulation during linear phase, when an adequate supply of metal ions could be afforded by a slow diffusive process. This could occur from a location where metal ions are in high concentration in the vicinity of the cell membrane, perhaps having been released from the cell wall by protons. This could be a credible mechanism for metal uptake when there is a high content of metal localised on the cell wall, and since the ions are required in very small amounts anyway. Somers (1963) commented that the copper taken up by spores is trapped at cell sites so there would be no need to invoke an active transport mechanism for metal ion accumulation. Metabolic dependence could be important however, when there is no ion store on the cell wall perhaps in very young mycelium for example, or under conditions of metal limitation or in acidic environments. The recent use of protoplasts of fungal cells for metal uptake studies (Gadd *et al*, 1984) could provide additional evidence for this hypothesis by determining whether or not protoplasts can accumulate metals actively in the absence of a cell wall, and therefore in the absence of an ion reservoir. Great care is

required when interpreting results for protoplast metal uptake because of the possibility of exposure of previously hidden binding sites, and also the fact that cell wall regeneration begins almost immediately (Dooijewaard-kloosterziel *et al*, 1973). One area of further investigation of great interest and potential usefulness may be to study the cell wall-less mutant of *Neurospora crassa* (Emmerson, 1963) to investigate further the role of the cell wall *in situ*. Section 13 in this study deals with the role of the cell wall in metal uptake by fungi.

The applicability of metabolic dependence studies depends on the proportion of metal uptake that is actually energy dependent. It is difficult to demonstrate the role of metabolism in systems where most of the uptake seems to be a physical process which masks all other possible processes as has been observed in *P. spinulosum*, *T. viride* and *A. niger*.

Paton and Budd (1972) were fortunate to have chosen a system to study with an easily recognisable metabolic phase of transport into the cell and have been able to develop an idea of the proportion of metal on the cell wall. Somers (1963) on the other hand, was not able to demonstrate a very high degree of metabolic dependence but regarded a small proportion of the uptake to the internal part of the cell as energy dependent. The majority of the copper in the conidia of *N. crassa* was surface bound, Somers (1963) whilst in the growing mycelium of the same fungus, only 40% of cell cobalt was bound on the cell wall in 2 hours (Venkateswerlu and Sivarama Sastry 1970). This may reflect structural differences between spores and mycelium and also whether the mycelium is growing or not, as well as strain variation. In fact Somers (1963) reported that cell walls of *Penicillium italicum* were particularly effective in accumulating copper whilst the walls of *N. crassa* showed a relatively low affinity. Morphological

differences in terms of wall structure are also implicated by the fact that in *Alternaria tenuis*, 96% of the adsorbed copper was removed by EDTA whilst in *N. crassa* this figure was only 33%. It is interesting that Gadd and Griffiths (1978a) stated that in most organisms studied, the amount of metal bound by surfaces was insignificant when compared to the amounts that can be taken up by energy requiring processes. The authors cannot have given adequate consideration to the report of Somers (1963). It is noteworthy that more recently, Horikoshi *et al* (1981) dismissed any form of energy dependent uptake and suggested that uranium uptake in actinomycetes was almost wholly attributable to surface adsorption.

It appeared that on comparing mid linear phase metal uptake of growing suspensions with non-growing (mid linear phase) suspensions of mycelium, that there was a marked difference between the two systems. (Table 8.3.2.2.). In the non-growing suspensions of fungi significantly greater quantities of metal were accumulated than would have been anticipated from the results using growing mycelium. This was particularly notable in the case of *T. viride* which accumulated 31.4 $\mu g\ g^{-1}$ dry weight cadmium in the growing state and 2727.4 $\mu g\ g^{-1}$ dry weight in the non-growing state from 2.5 $\mu g\ ml^{-1}$ cadmium. It would be interesting to speculate on the possibility that the non-growing mycelium behaved like the lag-phase material of a growing suspension, where the accumulation was very high. One possible explanation for the higher accumulation in non-growing suspensions is that there is no competition for binding sites in these experiments whilst in the growing mycelium there is likely to be competition from other metallic cations present in the growth medium as trace elements. If this were the case though, one might not expect to observe high lag phase metal levels, and it is doubtful whether the amount of competing cation could account for the discrepancy of this magnitude. More data on competition is presented in chapter 11.

## 9. EFFECT OF TEMPERATURE ON COPPER UPTAKE FROM 2.5 µg $ml^{-1}$ $Cu^{++}$ BY NON-GROWING *Penicillium spinulosum*.

### 9.1. Introduction.

Biological phenomena are exceedingly complex and involve temperature dependent physical and chemical phenomena. Whether these are chemical or physical, they depend on molecular activities which in turn are influenced by temperature (Hoar, 1975). For descriptive purposes, the $Q_{10}$ value can be useful as the critical thermal increment. In general, $Q_{10}$ values associated with physical processes such as diffusion are less than 1.5, whilst thermochemical (enzymatic) reactions range from 2.0 to 3.0.

The effect of temperature on biological reactions is also described by the Arrhenius equation:

$$\text{Ln}\ \frac{k_2}{k_1} = \frac{A}{R}\left[\frac{1}{T_1} - \frac{1}{T_2}\right]$$

Where k is the reaction velocity constant, T is the absolute temperature, R is the gas constant and A is the Arrhenius constant.

According to Hoar (1975), the value of the Arrhenius constant A for many biological processes falls between 1 and 25 Kcals $mol^{-1}$ and can be referred to as the apparent activation energy. Arrhenius introduced the hypothesis of an activated state. According to this theory all elementary rate processes (diffusion, solubility, adsorption *etc.*) can be considered unstable equilibria between reactants, in the normal state and in an activated state. The A constant then, represents the energy which molecules in their initial state must acquire before they can participate in a

reaction. It is the energy of activation for the particular process and remains constant over a limited temperature range. Generally, low A values of 1 to 5 Kcals $mol^{-1}$ define physical processes, and higher values upto 25 Kcals $mol^{-1}$ are active processes.

Borst Pauwels (1981) has calculated Arrhenius constants for the data of Ponta and Broda (1970) who worked on zinc uptake in *Saccharomyces cerevisiae* and reported a figure of 15 Kcals $mol^{-1}$. Similarly for the data of Norris and Kelly (1977) he reported a value of 15 Kcals $mol^{-1}$ for cobalt uptake. For manganese uptake an activation energy of 18 Kcals $mol^{-1}$ was found (Fuhrmann, 1974). These authors reported that uptake of these metals was an active process and the calculated Arrhenius constants confirm this. An Arrhenius constant of 10.5 Kcals $mol^{-1}$ was calculated for copper uptake by spores of *Neurospora crassa* by Somers (1963), but the author suggested that this was rather high for a simple exchange process and he cited the work of Danielli (1958) which indicated that even higher temperature coefficients for passive diffusion processes were possible.

In the preceding section, it was reported that lowering incubation temperature resulted in a reduction in total metal uptake throughout the uptake profile. The aim of this investigation was to determine if any metabolic dependence could be established by appraising $Q_{10}$ values and Arrhenius constants for metal uptake after short incubations (presumed mostly adsorption) and longer incubations (presumed adsorption and some internalisation).

## 9.2. Materials and Methods.

The uptake of copper from 2.5 µg $ml^{-1}$ $Cu^{++}$ in 50.0 mM MES buffer, pH 5.5 plus 10.0 mM glucose by *P. spinulosum* was investigated at 5, 15, 25,

35 and 45°C over an incubation period of 60 minutes. Samples were harvested in triplicate at 1, 5, 10, 20, 30, 40, 50 and 60 minutes of standard incubation to monitor the effect of temperature on uptake against time. $Q_{10}$ values were calculated from rate plots for each incubation time, the rationale being that short incubation uptakes would provide $Q_{10}$ values indicative of passive processes and the longer incubation time uptake $Q_{10}$ values would be suggestive of metabolic processes, if metabolic energy was involved in the uptake of copper. A similar experimental rationale was proposed for calculated values of activation energies obtained from the same data.

9.3. Results.

The influence of temperature on copper uptake by non-growing suspensions of *P. spinulosum* is depicted in Figures 9.3.1a, 1b and 1c. Uptake profiles at 5, 15, 25, 35 and 45°C are shown in Figures 9.3.1a and the typical pattern of rapid initial accumulation followed by slower uptake is illustrated for all the temperatures tested.

Figure 9.3.1b shows that the rate of copper uptake after each time period was directly proportional to the incubation temperature. $Q_{10}$ values of 1.1 to 1.3 indicate that the uptake was a physical process and that there was apparently no metabolic involvement.

Figure 9.3.1c is an Arrhenius plot of the data and activation energies calculated for the various periods of uptake suggest that uptake occurred by a physical process. Calculated activation energies were 2.94, 3.36, 4.07, 4.62 and 5.33 Kcals $mol^{-1}$ after 1, 5, 20, 30 and 60 minutes of incubation respectively. An Arrhenius constant of 6.51 Kcals $mol^{-1}$ was calculated for the 60 minute incubation period which had been corrected for the initial period of rapid accumulation (i.e. 5 minutes).

*Figure* 9.3.1a Effect of temperature on quantitative copper uptake by non-growing suspensions of *P .spinulosum* from 50mM MES containing 2.5 µg $ml^{-1}$ $Cu^{++}$ in the temperature range 5-45°C.

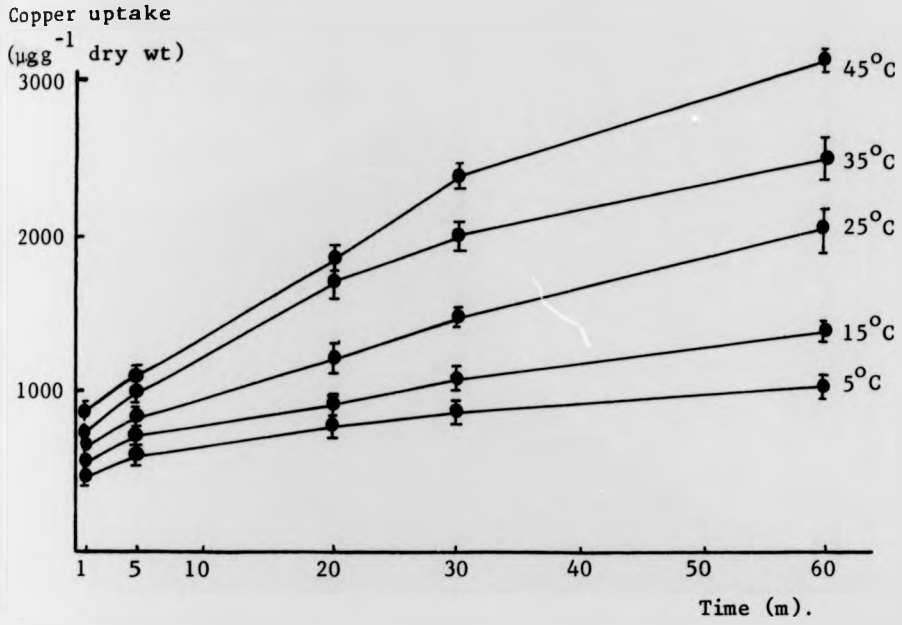
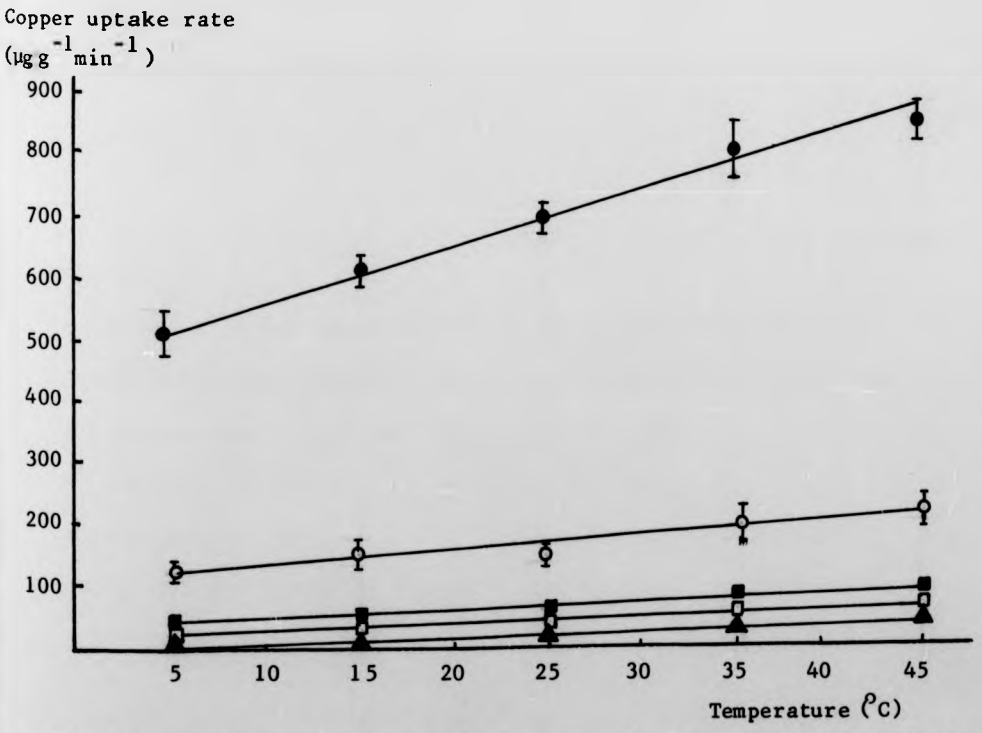



*Figure* 9.3.1b Effect of temperature on copper uptake rate by *P .spinulosum*. (Experimental conditions as above).




●, Uptake rate after 1 min.; ○, 5 min.; ■, 20 min.; □, 30 min.; ▲60 min.

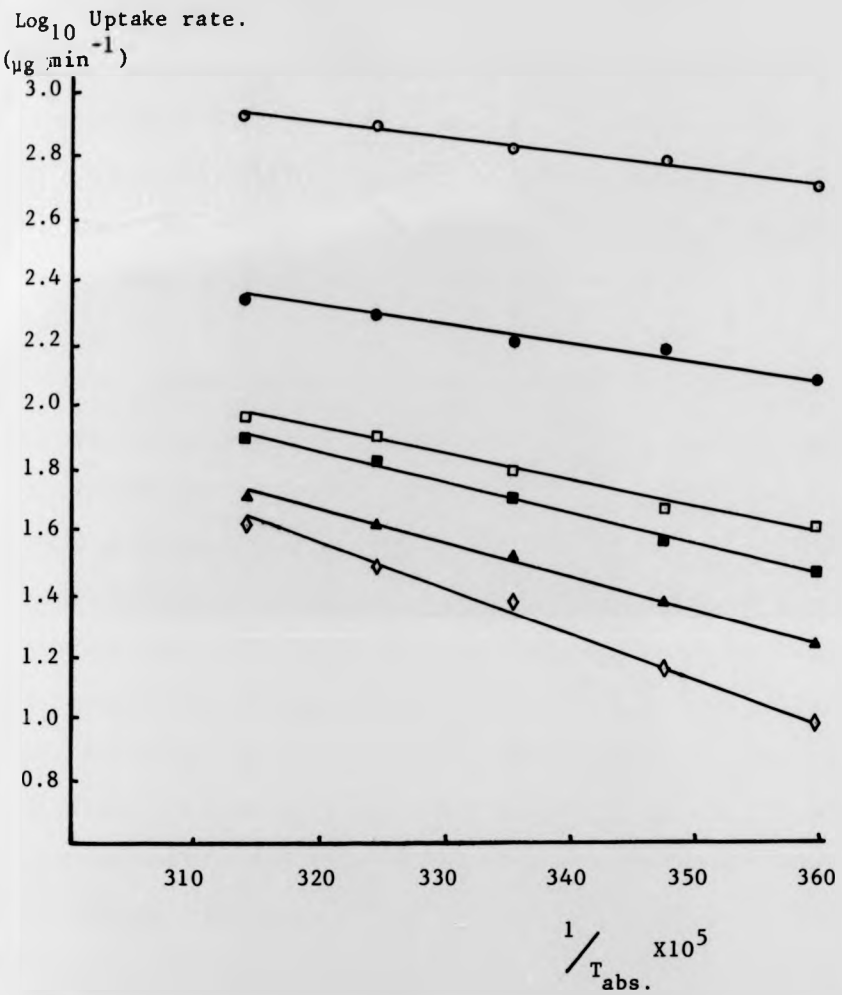


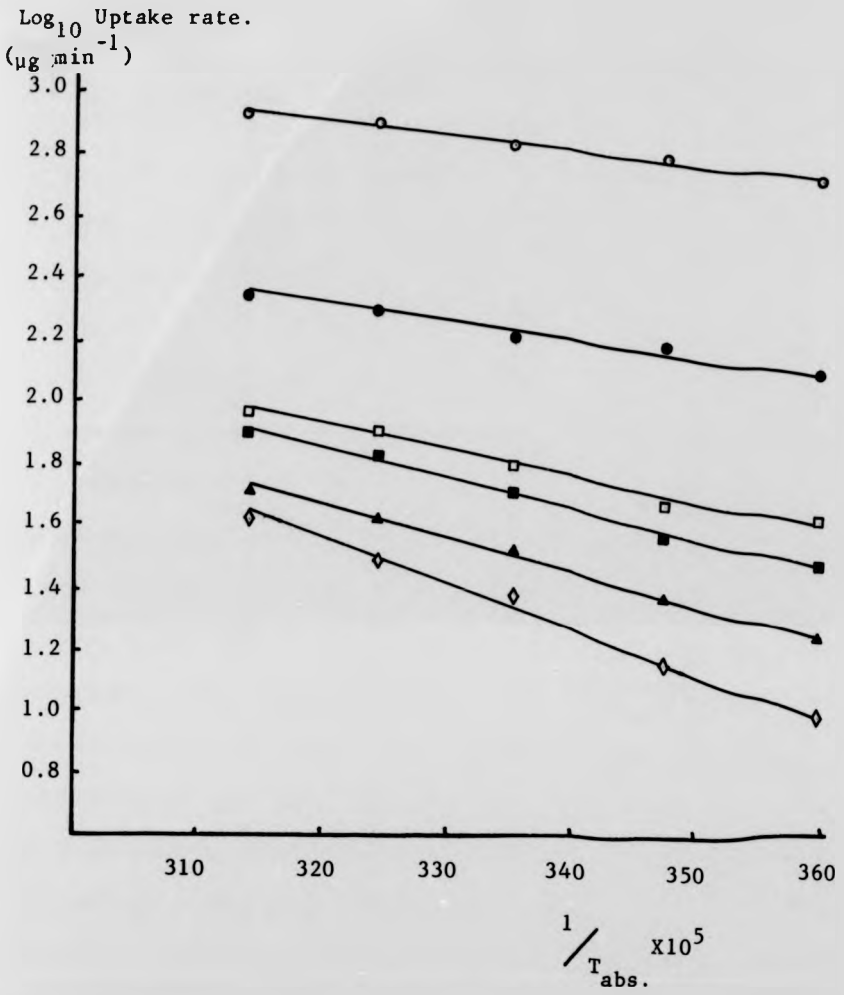

*Figure* 9.3.1c

Arrhenius plots for copper uptake by non growing suspensions of *P. spinulosum* from 50mM MES containing 2.5 µg ml$^{-1}$ $Cu^{++}$ at pH 5.5.

o , uptake over 1 min.; ● , uptake over 5 min.;

□ , uptake over 20 min.; ■ , uptake over 30 min.;

▲ , uptake over 60 min.; ◊ , uptake over 60 min corrected for initial adsorptive uptake during the first 5 min. of incubation.

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o , uptake over 1 min.; ● , uptake over 5 min.;

□ , uptake over 20 min.; ■ , uptake over 30 min.;

▲ , uptake over 60 min.; ◊ , uptake over 60 min corrected for initial adsorptive uptake during the first 5 min. of incubation.

9.4. Discussion.

In *P. spinulosum* $Q_{10}$ values were calculated for all incubation times ranging from 1 to 60 minutes and ranged from 1.1 to 1.3. In general $Q_{10}$ values for diffusion and other physical processes are less than 1.5 so it seems that there was little demonstrable metabolic dependence in copper uptake under the conditions of these experiments.

Venkateswerlu and Sivarama Sastry (1970) showed that cobalt uptake was temperature dependent in *N. crassa* and they claimed that the $Q_{10}$ values were very different for the 2 hour and 20 minute incubations. These were calculated as 180 µg $Co^{++}$ $g^{-1}$ and 30 µg $Co^{++}$ $g^{-1}$ dry weight respectively. According to the authors then, it seemed that the initial binding phase although dependent on temperature, was not so markedly affected as the longer incubation. The authors suggested that the evidence supports the theory of a 2 phase uptake process, with the second phase being metabolically mediated. The actual $Q_{10}$ values, calculated from the data of Venkateswerlu and Sivarama and Sastry (1970) (by the author) appear to be 1.15 and 1.10 µg $min^{-1}$ after 2 hours and 20 minutes in the cobalt solution. There does not appear to be any evidence for metabolic uptake in respect of the temperature data. Ponta and Broda (1970) reported $Q_{10}$ values of 2 to 2.7 for zinc uptake in *S. cerevisiae* which is more suggestive of an active system.

In the range 5 to 45°C, *P. spinulosum* showed temperature dependence and by plotting $\log_{10}$ uptake rate (µg $min^{-1}$) against $^{1}/T_{abs} \times 10^{5}$ a straight line relationship was produced. From this Arrhenius plot, activation energies for the various incubation times were calculated. For the total copper uptake these ranged from 2.94 after 1 minute to 5.33 Kcals $mol^{-1}$

after 60 minutes of incubation. The total uptake after 60 minutes was corrected for the adsorption by subtracting the 5 minute uptake value and an activation energy of 6.51 Kcals $mol^{-1}$ was determined. These values are all indicative of a physical process of copper uptake but the fact that the figure increased with incubation time suggests that there is an energy barrier following the initial adsorption process and this could be represented by the physical barrier of the cell membrane. All the values appear to be rather low in comparison to the value of 10.5 Kcals $mol^{-1}$ for copper uptake in *N. crassa* conidia as reported by Somers (1963), but he suggests that this value is very high. The range of activation energies for biological processes and enzymatic reactions is 1 to 25 Kcals $mol^{-1}$ and physical processes such as diffusion and adsorption are typically at the lower end (Hoar, 1975). Higher temperature coefficients for passive diffusion processes than the value reported by Somers (1963) have also been reported by Danielli (1958). The values obtained for *P. spinulosum* all fall below the calculated values for active uptake as reported by Borst Pauwels (1981) for yeast divalent cation uptake. For zinc uptake the activation energy was 15 Kcals $mol^{-1}$ in *S. cerevisiae* (Ponta and Broda, 1970), for cobalt 15 Kcals $mol^{-1}$ in *S. cerevisiae* (Norris and Kelly, 1977), and for manganese 18 Kcals $mol^{-1}$ (Fuhrmann, 1974).

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## 10. COPPER UPTAKE BY HEAT-KILLED CELLS OF *Penicillium spinulosum*.

### 10.1 Introduction.

In the light of the results of the preceding sections which have demonstrated that metal uptake does not occur by a metabolically mediated process, it was decided to investigate the metal uptake properties of heat-killed mycelium.

### 10.2 Method.

A mycelial slurry was prepared in the usual way and split into 2 portions. One portion was incubated at 25°C whilst the remaining portion was heated at 85°C for 2 hours. The mycelium from both suspensions was harvested and washed with distilled $H_2O$. To each of twelve, 250 ml Ehrlenmyer flasks containing 100 ml 50.0 mM MES plus 10.0 mM glucose plus copper at a final concentration of 2.5 µg $ml^{-1}$ $Cu^{++}$, was added a 5.0 ml aliquot of the heat-killed mycelium. To a further batch of identical flasks, living non-growing mycelium was added. Flasks were then incubated and the mycelium harvested from triplicate flasks after 1, 30, 60 and 120 minutes of incubation. The copper loaded heat-killed and living mycelium was then analysed for copper content by AAS.

### 10.3 Results.

Mycelium of *P. spinulosum* which had been heat-killed by a 2 hour incubation at 85°C showed the typical uptake profile with 2.5 µg $ml^{-1}$ $Cu^{++}$. When compared to the uptake in control mycelium (Figure 10.3.1.), it is apparent that the heat-killed mycelium had a much higher capacity for copper, particularly during the period of rapid binding. Initial binding

of the heat-killed mycelium exhibited a 9 fold increase in affinity of the mycelium for copper and this was probably as a result of the destruction of cell membranes allowing for a free flow of cations into the cell and consequential binding to intracellular debris. After 2 hours of incubation, the heat-killed mycelium had accumulated 2.5 times the amount of copper accumulated in control biomass.

## 10.4 Discussion.

It was apparent that heat-killed mycelium of *P. spinulosum* had a higher affinity for copper than did living mycelium. It should be noted however, that the killed mycelium aggregated and was difficult to handle and this caused problems with rinsing. Although extra care was taken with the rinsing procedure it is nevertheless conceiveable that some copper was not thoroughly washed free and this could have manifested itself in the higher apparent copper binding.

With the help of an enrichment culture, Chiu (1972) isolated a fungal culture from sewage which could accumulate uranium from solution. Uranium was taken up by living as well as dead mycelia. Horikoshi *et al* (1981) showed that heat-killed actinomycete cells had the same uranium uptake capacity as living cells. Similarly, Lowry *et al* (1957) reported that 15 minute boiling of *Neurospora tetrasperma* ascospores had no effect on silver, uranium, copper, thorium and aluminium uptake. The uptake of copper, uranium, manganese and cadmium was increased in microalgae by boiling cells in water for upto 20 minutes (Horikoshi *et al*, 1977). In *Chlorella regularis* (Horikoshi *et al*, 1981) the amount of uranium taken up by heat-killed cells was 4 times that taken up by living cells. Other workers have shown increased metal uptake by killed cells. Ponta and Broda (1970) demonstrated increased uptake of zinc in *Saccharomyces cerevisiae*,

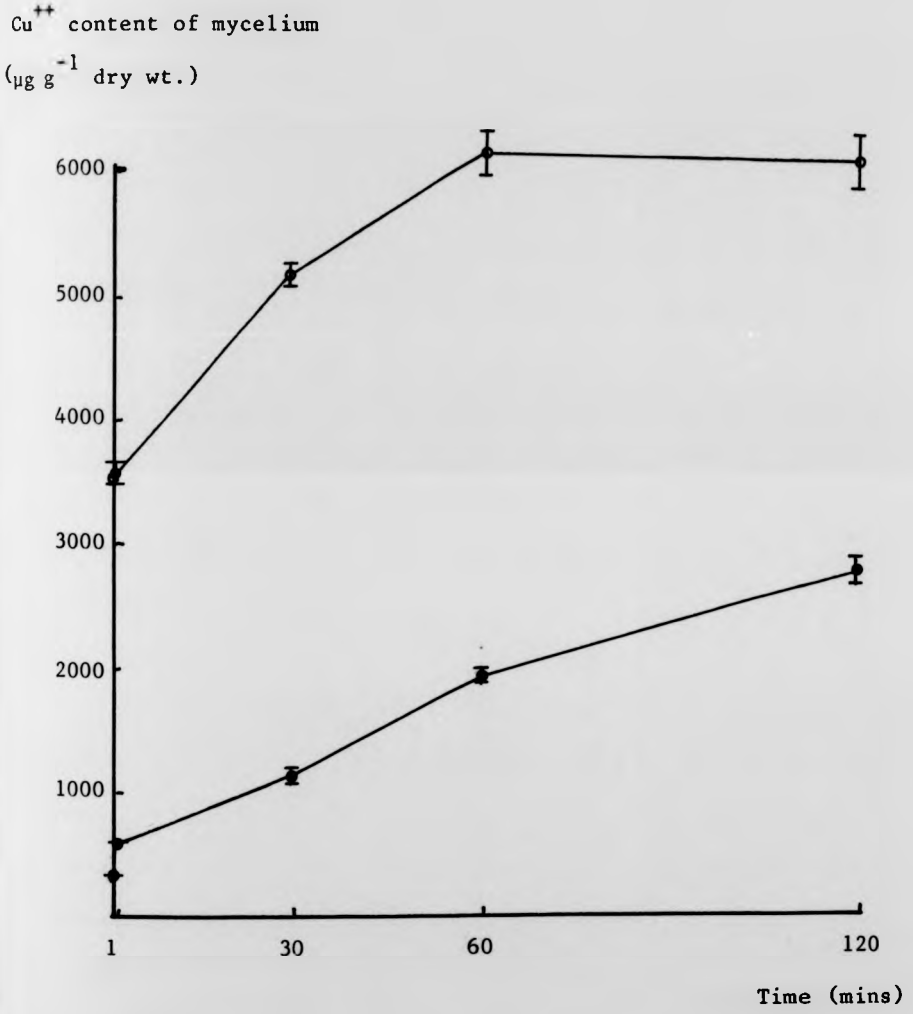



*Figure* 10.3.1.

Effect of heat killing on copper uptake from MES at pH 5.5 supplemented with 2.5 µg $ml^{-1}$ $Cu^{++}$ by *P. spinulosum*. ●,control mycelium at 25°C; o,mycelium heated at 85°C prior to incubation with copper.

whilst Galun *et al* (1983) reported increased uptake of uranium in *Penicillium digitatum*. Tsezos and Volesky (1981) reported that disrupted cells of *Rhizopus arrhizus* accumulated 10% more uranium than whole cells. On the other hand, Duddridge and Wainwright (1980) reported that heat-killing actually reduced the uptake capacity of a number of metals in *Pythium sp.*, *Scytalidium sp.* and a *Dictyuchus sp.* and the authors ascribed this to an alteration or destruction by heat, of some metal binding ligands present on the fungus cell wall. Somers (1963) however, commented that the denatured protein of dead boiled cells was a better copper complexing agent than was native protein.

The importance of improved biosorptive capacity of killed cells was emphasised in a U.S. patent (Drobot and Lechavelier, 1981) for a metal recovery process. The inventors suggested that the fungi were to be killed in the process of the invention, by heating in an aqueous medium in the range 85 to 150$^{o}$C.

The fact that dead cells can accumulate metals to a high degree implies that the phenomenon may be associated with the cell wall, and for this reason, and the fact that the cell wall seems to be closely involved in metal uptake in whole cells (growing and non-growing), chapter 13 of this investigation was devoted to a study of copper uptake by a cell wall preparation of *P. spinulosum* and chemically modified cell walls of the same fungus.

## 11. COMPETITION EXPERIMENTS FOR COPPER UPTAKE BY *Penicillium spinulosum*.

### 11.1. Introduction.

The effects of competing cations on metal uptake in microorganisms has received a great deal of attention and much of the recent work in relation to algae, protozoa and bacteria has been reviewed by Gadd and Griffiths (1978a) and by Babich and Stotzky (1980). The effects of competition by metals for yeast cation uptakehave been reviewed by Borst Pauwels (1981). However, all three reviews require updating and the inclusion of some notable omissions.

Studies on the effects of competing cations on metal uptake can be organised into a number of categories. One area of study has been directed towards an understanding of binding to the surface of cells, and particularly towards understanding the specificity of binding sites. These effects of competing cations can normally be subdivided into a) those which reduce uptake or b) those which enhance uptake by additive or synergistic mechanisms. Another area of study has concentrated on the effects of competing cations on metal internalisation and again there are reports of stimulatory and inhibitory effects. Much of the interest has been related to cation relationships in toxicity studies.

Lowry *et al* (1957) showed that copper could be displaced from the surface of *Neurospora tetrasperma* ascospores by $Na^{+}$, $K^{+}$, $Mg^{++}$ and $Ca^{++}$. The consequence was that the spores which had previously been prevented from germinating because of the presence of a copper coating, germinated successfully. Rothstein *et al* (1958) showed that the uptake mechanism for $Mg^{++}$ and $Mn^{++}$ in *Saccharomyces cerevisiae*, was highly selective as compared to $Ca^{++}$, $Sr^{++}$ and $UO_2^{++}$, whereas the binding affinity was greatest for

$UO_2^{++}$ with little discrimination between $Mg^{++}$, $Cu^{++}$, $Mn^{++}$ and $Sr^{++}$. In contrast to surface bound cations which were completely exchangeable, the absorbed cations were not exchangeable. Somers (1963) reported that $K^{+}$, $Mg^{++}$ and $Ca^{++}$ were able to compete for binding sites on conidia of *Neurospora crassa*, and that the degree of competition depended on the concentration and valency of the ions. Paton and Budd (1972) showed that phase 1 zinc uptake (surface binding) in *Neocosmospora vasinfecta* decreased in the presence of equimolar concentrations of $Cu^{++}$ and $UO_2^{++}$ by 70%. Cobalt, $Mn^{++}$, $Ni^{++}$ and $Mg^{++}$ decreased zinc uptake by upto 39% but iron actually increased uptake by 12%. Budd (1979) demonstrated that a 10 fold excess of radioactive $Mg^{++}$ reduced the adsorption of non-radioactive $Mg^{++}$ and that $Ca^{++}$, $Zn^{++}$ and $Mn^{++}$ occupied $Mg^{++}$ binding sites. Doyle *et al* (1980) observed the displacement of $Mn^{++}$ from the cell wall of *Bacillus subtilis* by $Ni^{++}$, $Ca^{++}$, $Mg^{++}$, $La^{++}$ and $Li^{++}$. The $Mn^{++}$ was present on the cell wall at near saturation levels. Tsezos and Volesky (1982a) showed that $Zn^{++}$ and $Fe^{++}$ depressed $UO_2^{++}$ uptake by *Rhizopus arrhizus* but there was no effect on $Th^{+++}$ uptake (Tsezos and Volesky, 1982b). Tsezos (1983) indicated that increasing the concentration of the co-ion ($Zn^{++}$, $Cu^{++}$ or $Fe^{++}$) from 100.0 to 1000.0 μg $ml^{-1}$, decreased $UO_2^{++}$ uptake by chitin by upto 39%, and that iron was the most effective ion in this role. Galun *et al* (1984) reported that ferric chloride interferes with $UO_2^{++}$ uptake from uranyl chloride by *Penicillium digitatum*. Nickel, $Cu^{++}$, $Zn^{++}$, $Cd^{++}$ and $Pb^{++}$ failed to approach the activity of the $Fe^{+++}$ ion. The authors attributed this to the fact that the iron was trivalent and the other ions were divalent. The difference in effects could not be explained in terms of variation in ionic radii since the U(Vl) oxo-ion is very large. Beckett and Brown (1984) showed that cadmium uptake in the lichen *Peltigera praetextata* was inhibited by monovalent cations. Cadmium uptake appeared to be stimulated by $Cu^{++}$ and there seemed to be no distinction between extra and intracellular uptake in this respect.

Abelson and Aldous (1950) indicated that the toxicity of $Ni^{++}$, $Co^{++}$, $Cd^{++}$, $Zn^{++}$ and $Mn^{++}$ to *Escherichia coli*, *Aerobacter aerogenes*, *Torulopsis utilis* and *A. niger* was influenced by the $Mg^{++}$ level in the medium. Low $Mg^{++}$ levels led to increased toxicity. Budd (1979) reported that total $K^{+}$ transport in *Neocosmospora vasinfecta* was decreased by half in 5 fold excess of $Ru^{++}$, and was unaffected by 100 fold excess of $Na^{++}$. Copper and $Mg^{++}$ had no intermediate effect. Paton and Budd (1972) showed that energy dependent uptake of zinc was decreased by 80% by $UO_2^{++}$. Copper, $Ni^{++}$ and $Co^{++}$ were less inhibitory and $Fe^{++}$ and $Fe^{+++}$ stimulated uptake by 40%. Budd (1979) showed that at 10 fold molar excess of $K^{+}$ and $Ca^{++}$ there was no effect on $Mg^{++}$ transport in the same fungus, but $Mn^{++}$ and $Zn^{++}$ were inhibitory. Venkateswerlu and Sivarama Sastry (1970) showed that cobalt uptake by *Neurospora crassa* was influenced by $Mg^{++}$. A $Co^{++}/Mg^{++}$ weight ratio of 1:2 reversed cobalt toxicity and $Mg^{++}$ severely depressed $Co^{++}$ uptake. It was suggested that the system that transports $Mg^{++}$ was also involved with $Co^{++}$ uptake. According to Laborey & Lavollay (1973) the toxicity of cadmium to *A. niger* resulted from antagonism with zinc and magnesium. According to Failla *et al* (1976), $Zn^{++}$ uptake in *Candida utilis* was not related to the general cation uptake system reported by Rothstein *et al* (1958), and zinc uptake occurred by a specific energy-dependent pathway, and was unaffected by several other cations but inhibited by cadmium. Failla and Weinberg (1977) showed that the presence of 0.2 mM $Cu^{++}$, $Mn^{++}$, $Fe^{++}$ or $Co^{++}$ had no effect on zinc uptake, but in 0.2 mM $Cd^{++}$, $Zn^{++}$ uptake was only 17% of the control uptake. The zinc status had no effect on iron uptake however. Laborey and Lavollay (1977), showed that $Ca^{++}$ had an antitoxic effect towards $Cd^{++}$. In the presence of high concentrations of $Mg^{++}$, the toxicity of $Cd^{++}$ was nullified by $Ca^{++}$, and this was related to the control of ion penetration. Norris and Kelly (1977) showed that $Co^{++}$ and $Cd^{++}$ appeared to be accumulated via a general cation uptake system

in *Saccharomyces cerevisiae*, with limited specificity related to ionic radii of the cations. Okamoto *et al* (1977) reported that in *Penicillium ochrochloron* there appeared to be a marked reduction in the cellular contents of $K^{+}$ and $Mg^{++}$ when $Cu^{++}$ and $Zn^{++}$ were present in higher concentrations in the medium. Baldry and Dean (1980c) showed that the addition of $Fe^{+++}$ and $Al^{+++}$ to a medium supporting *E. coli* prevented $Cu^{++}$ accumulation, and although less active than $Fe^{+++}$ or $Al^{+++}$ in this respect, other trivalent cations were effective in reducing $Cu^{++}$ uptake. Divalent cations were much less effective and $Na^{+}$ and $K^{+}$ had a negligible effect.

Enhancement of metal uptake in response to the presence of interacting cations has been reported by a number of workers. Gesswagner and Altmann (1967) showed that the $Zn^{++}$ content of *S. cerevisiae* cells was increased specifically by $Cu^{++}$ whilst $Mn^{++}$ content was unaffected. This was attributed to an increase in -SH groups and amino groups in the yeast cell. According to Fuhrmann and Rothstein (1968), $Co^{++}$ enhanced $Zn^{++}$ uptake, and $Zn^{++}$ enhanced $Co^{++}$ uptake, whereas $Ca^{++}$ inhibited both $Zn^{++}$ and $Co^{++}$ uptake. According to Norris and Kelly (1979) $Cu^{++}$ caused a seven fold increase in the initial uptake of $Ma^{++}$ and doubled the uptake of $Ni^{++}$ in *S. cerevisiae*. The total final metal content of the cells was the same however. Babich and Stotzky (1983) showed a synergistic interaction between $Ni^{++}$ and $Cu^{++}$ although the mechanism was not elucidated.

The aim of this section of the study was to demonstrate the influence of the cationic constituents of the GMS medium on metal uptake in non-growing suspensions of fungi. Also the uptake of copper by non-growing *P. spinulosum* was to be investigated in the presence of competing cations over a 2 hour incubation period. In this way the effects of the presence of co-ions on adsorptive and total copper uptake could be determined, in terms of possible stimulatory or inhibitory effects.

11.2. Materials and Methods.

11.2.1. Comparison of copper uptake from GMS medium and 50.0 mM MES.

5.0 ml aliquots of a mycelial suspension of *P. spinulosum* were added to twelve 250 ml Ehrlenmyer flasks containing 100 ml GMS medium or 50.0 mM MES adjusted to pH 5.5 containing 2.5 µg $ml^{-1}$ $Cu^{++}$. The flasks were incubated under standard conditions and the mycelium harvested from 3 replicate flasks after 5, 30, 60, 120 and 360 minutes of incubation. Copper analyses were then carried out. The experiment was then repeated for *T. viride* and *A. niger*

11.2.2. Effect of medium constituents on copper uptake.

Since the apparent uptake in medium was lower than in MES buffer, the effect of individual medium components on copper uptake by *P. spinulosum* from a 5.0 µg $ml^{-1}$ $Cu^{++}$ was investigated. Copper content of mycelium was assayed after 5, 60, 120 and 360 minutes of incubation in various solutions containing $Cu^{++}$. Three replicate samples at each time interval were employed. The solutions used were $(NH_4)_2SO_4$, 50.0 mM MES pH 5.5, and 50.0 mM MES plus magnesium glycerophosphate at the normal GMS medium concentrations. A further experiment was performed which compared the influence of 50.0 mM MES pH 5.5, 50.0 mM MES plus magnesium glycerophosphate and 50.0 mM MES pH 5.5 plus double the normal complement of magnesium glycerophosphate.

11.2.3. Copper uptake in the presence of competing cations at 1.0 mM concentrations.

The aim was to investigate the influence of competing cations at 1.0 mM concentrations on copper uptake from 2.5 µg $ml^{-1}$ $Cu^{++}$ (pH 5.5) by

*P. spinulosum* to determine the degree of specificity, if any, of binding. The cations employed were manganese, magnesium, cobalt and zinc. Copper uptake experiments were performed as described above in 50.0 mM MES pH 5.5 containing 2.5 µg $ml^{-1}$ $Cu^{++}$ and also in the same but containing 1.0 mM magnesium, manganese, cobalt or zinc. Standard incubation techniques were employed throughout. The uptake of copper and the competing ion was monitored as was $K^{+}$ loss. The percentage of control copper uptake was calculated for each competing cation at 1 minute and 2 hour incubation periods.

## 11.3. Results.

### 11.3.1. Comparison of copper uptake from GMS medium and 50.0 mM MES

Figures 11.3.1a, 1b, 1c illustrate the uptake of copper when added to 50.0 mM MES or GMS medium at pH 5.5 to a final concentration of 2.5 µg $ml^{-1}$ by *P. spinulosum*, *T. viride* and *A. niger*. Although all three fungi demonstrated the typical uptake pattern in both the MES and the GMS it was apparent that accumulation was much more efficient in MES. GMS medium appeared to cause a reduction in uptake of about 50% over the 6 hour incubation period.

### 11.3.2. Effect of medium constituents on copper uptake.

The effect of $(NH_4)_2SO_4$ and magnesium glycerophosphate on copper uptake in *P. spinulosum* is illustrated in Figure 11.3.2. The uptake of copper in control mycelium suspended in 50.0 mM MES pH 5.5 showed the typical accumulation pattern. Mycelium suspended in $(NH_4)_2SO_4$ plus 50.0 mM MES resulted in levels of copper accumulation which were not greatly different to the uptake in control mycelium. Copper accumulation in magnesium glycerophosphate plus 50.0 mM MES pH 5.5 and double-strength

magnesium glycerophosphate plus 50.0 mM MES pH 5.5 was reduced however. Double strength magnesium glycerophosphate resulted in a greater reduction of copper uptake than did the normal strength magnesium glycerophosphate.

Since magnesium glycerophosphate was not responsible for complexing metals as demonstrated in the polarography section (5), the depressed copper contents of mycelia suspened in this solution must have been due to competition for binding sites with the magnesium ions.

11.3.3. Copper uptake in the presence of competing cations at 1.0 mM concentrations.

The competitive effect of manganese, magnesium, cobalt and zinc at 1.0 mM concentration on the uptake of copper from 2.5 µg $ml^{-1}$ $Cu^{++}$ by *P. spinulosum* is illustrated in Figure 11.3.3. and Table 11.3.1.

All four competing cations reduced copper uptake and this was most significant at the early stages of uptake. Zinc inhibited uptake by 51.7% after 1 minute, magnesium by 41.8%, manganese by 40.9% and cobalt by 38.4%. After 2 hours of incubation however, the competitive effects of the manganese, magnesium and cobalt were less significant in reducing uptake with inhibition amounting to around 10% of the uptake shown by controls. Magnesium however, inhibited copper uptake by 23.5% even after 2 hours of incubation.

11.4. Discussion.

The reduction in copper binding and uptake over the 6 hour incubation period in *P. spinulosum*, *T. viride* and *A. niger* (Figures 11.3.1a,1b,1c in GMS medium as opposed to MES could be explained in terms of complexing

*Figure* 11.3.1a,1b,1c.

Copper uptake by non growing fungi suspended in GMS medium and 50.0 mM MES containing 2.5 µg $ml^{-1}$ $Cu^{++}$.

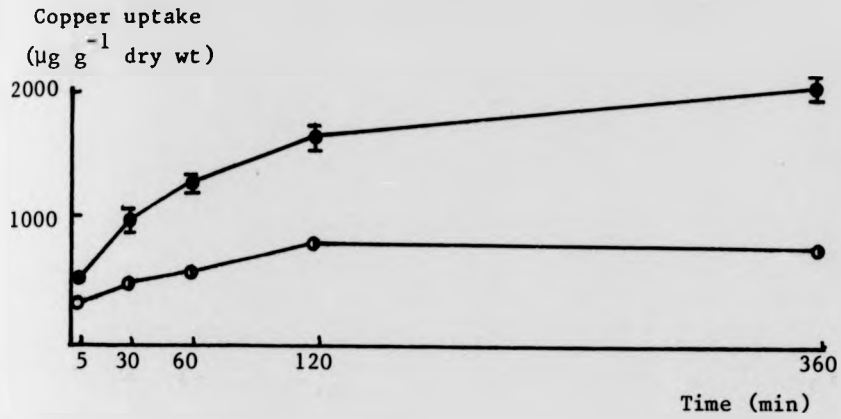



*Figure* 11.3.1a

*P. spinulosum*

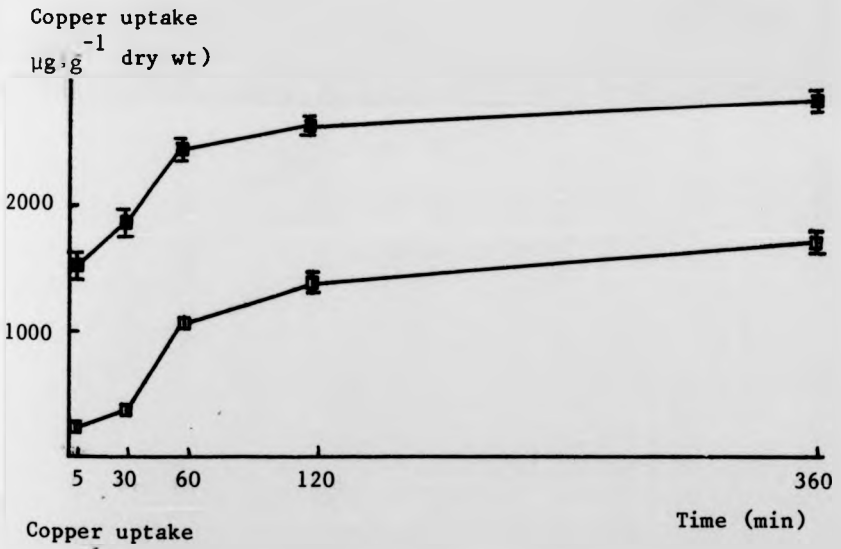



*Figure* 11.3.1b

*T. viride*

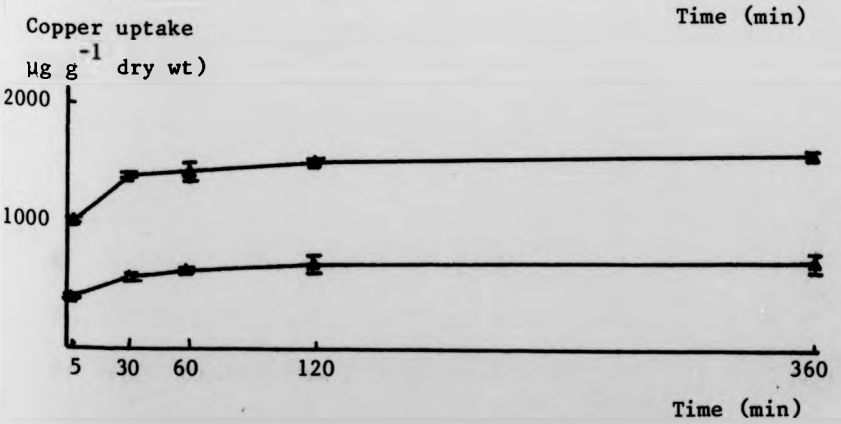



*Figure* 11.3.1c.

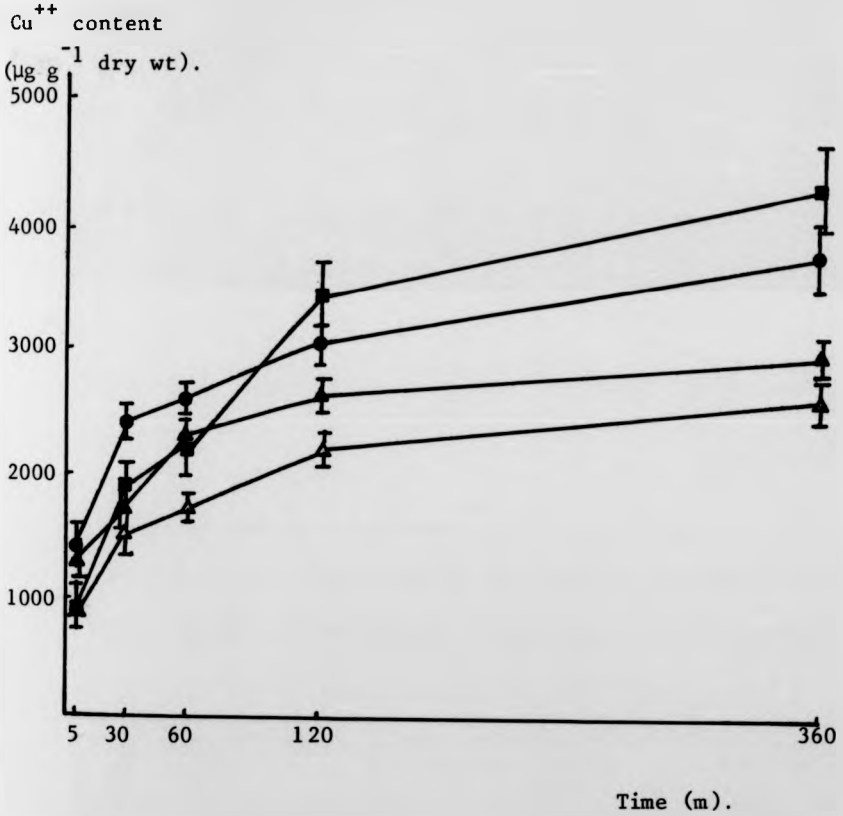
*A. niger*

Fungal suspension in 50 mM MES pH 5.5 ● ■ ▲

Fungal suspension in GMS medium pH 5.5 ○ □ ▲

*Figure* 11.3.2.

The influence of medium constituents on copper accumulation by non-growing suspensions of *P. spinulosum* from 5.0$\mu$g $ml^{-1}$ $Cu^{++}$.

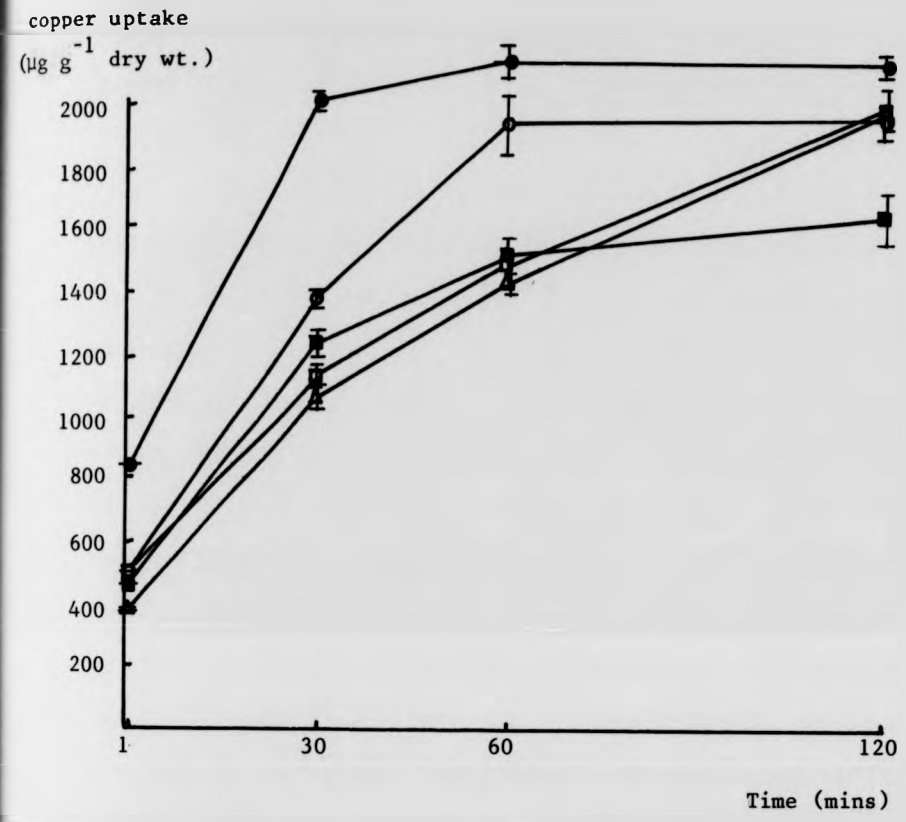



●, control uptake in 50mM MES only.

■, 50mM MES plus ammonium sulphate.

▲, 50mM MES plus magnesium glycerophosphate.

△, 50mM MES plus double-strength magnesium glycerophosphate.




ure 11.3.3. Copper uptake from 2.5µg ml$^{-1}$ in the presence of competing cations at 1mM concentration. ● ,control incubated in copper only; ⊙ plus 1mM manganese; ■ magnesium; □ cobalt; △ zinc.

| Competing cation (1 mM) | Copper uptake after 1 min. incubation. (% control) | Copper uptake after 2 hour incubation. (% control) |
|---|---|---|
| Control | 100.0 | 100.0 |
| manganese | 59.1 | 90.9 |
| magnesium | 58.2 | 76.5 |
| cobalt | 61.6 | 91.6 |
| zinc | 48.3 | 91.4 |

11.3.1. Copper uptake by *P. spinulosum* in the presence of competing cations expressed as a percentage of control uptake in $Cu^{++}$ only.

of copper by medium constituents. This hypothesis can be disregarded however, since the polarographic studies indicated that the medium constituents were not involved in metal complexing (see section 5).

A more likely explanation could be that competition for binding sites with other cations present in the GMS medium may have been responsible for the reduced copper binding. The reduction was quite marked though, but this is not surprising since the magnesium content of the medium was 52.75 $\mu g\ ml^{-1}$ medium and the calcium content was 45.75 $\mu g\ ml^{-1}$, so these concentrations were significantly higher than the added copper concentration of 2.5 $\mu g\ ml^{-1}$. There may well have been another factor involved in the reduced binding in growth medium, and this could be related to growth itself, particularly after 6 hours of incubation since proliferation in the GMS medium could have resumed. The results shown in Figure 11.3.2. however, support the view that the medium cationic components do compete with copper for binding and uptake. Ammonium sulphate incubation did not reduce copper uptake, but the presence of the magnesium glycerophosphate effectively reduced the amount of copper accumulated.

The reduction of copper uptake after 1 minute in the presence of competing cations (Figure 11.3.3) was almost certainly due to the competitive effects of the added ions for the copper binding sites, suggesting that they are not specific for copper. In the light of this, it is difficult to explain the high affinity for copper of growing cells of *P. spinulosum*, if the binding sites show no specificity for copper. The fact that the apparent inhibition of copper uptake was lower after a longer period of incubation may have been a result of internalisation of metal. Loss of viability and subsequent binding to intracellular components does not explain the reduction in inhibition with extended incubation since there

appeared to be no reduction in viability and no appreciable $K^+$ loss. It is interesting that $Mg^{++}$ was the most inhibitory metal after 2 hours of incubation, so the relative affinities of the ligands for $Cu^{++}$ and $Mg^{++}$ must have been similar. Reduction in copper accumulation in the presence of other divalent cations is in agreement with the findings of other workers in a number of types of microorganisms (Lowry *et al*, 1957; Somers, 1963; Okamoto *et al*, 1977; Wakatsuki *et al*, 1979; Baldry and Dean, 1980a).

According to Babich and Stotzky (1980), there is little information on the mechanisms whereby one heavy metal increases or decreases the toxicity of a second heavy metal. The authors suggest that the antagonistic interactions may result from competition between the metals for common sites at the cell surface. Another mechanism of antagonism between heavy metals may involve the sorption of one heavy metal to the amorphous complex of the other heavy metal. In amphibian and mammalian cells, different heavy metals bind to specific ionogenic sites on the surface of the cells. Synergistic interactions between heavy metals on microbial cells may result from the adsorption of both metals to the surface of the cell, with the adsorption of one metal increasing the permeability of the cell membrane to the second metal.

Competition of copper uptake with other divalent cations has highlighted the significance of the cell wall in initial non-specific binding of copper in *P. spinulosum*. The next chapter investigates the kinetics of the initial binding.

## 12. KINETIC ANALYSIS OF INITIAL BINDING OF COPPER TO *Penicillium spinulosum*.

### 12.1. Introduction.

There have been numerous reports on kinetic analysis of metal uptake in most types of microorganisms but general consideration has been almost exclusively directed towards the energy-dependent uptake system (Venkateswerlu and Sivarama Sastry, 1970; Ponta and Broda, 1970; Budd, 1969, 1975, 1979; Paton and Budd, 1972; Norris and Kelly, 1977; Failla and Weinberg, 1977 and Shumate *et al*, 1978, 1980). There has been relatively little work carried out on the kinetics of surface binding, although some workers have indicated that uptake of metals usually occurs with a primary initial binding phase. Paton and Budd (1972) reported that zinc uptake in *Neocosmospora vasinfecta* was a two phase process with the first phase believed to represent adsorption to negatively charged groups in the hyphal surface membrane. The kinetics of $K^+$ adsorption have been studied in the same fungus by Budd (1975) over the initial contact time of 0 to 12 minutes, and the authors reported that adsorption of $K^+$ could be attributed to two distinct $K^+$ binding entities at the mycelial surface.

Adsorption is commonly observed during cation uptake in fungi (Rothstein and Hayes, 1956; Budd and Harley, 1962; Ponta and Broda, 1970; Paton and Budd, 1972) as in other organisms, but according to Budd (1975), it is less conspicuous with the monovalent cations than with divalent or polyvalent cations. Adsorption of $K^+$ was observed in yeast (Rothstein and Hayes, 1956), where it displaced $Mn^{++}$ from one of the two binding sites for this ion. The association constant calculated for their data was very close to that for site 2 in *Neocosmospora vasinfecta* (Budd, 1975).

In *Neurospora sp.* $K^+$ adsorption at pH 5.8 involved a single binding site (Slayman and Slayman, 1970), so it is clear that there is a lot of diversity in the surface binding properties of filamentous fungi.

Wakatsuki *et al* (1979) reported that double reciprocal plots of the uptake velocity of copper against copper concentration produced straight lines between 0.64 μg $ml^{-1}$ $Cu^{++}$ and 6.40 μg $ml^{-1}$, but two lines were observed between 0.64 and 315.0 μg $ml^{-1}$ $Cu^{++}$. Although these results are for total uptake over a 60 minute incubation period, the results showed (according to the authors) that copper bound to defined sites having a high affinity for copper at low concentrations, whilst at high concentrations copper bound to not only the sites having a high copper affinity but also to the various sites having lower affinities. The authors suggested that the uptake velocity of copper may be dependent on the efficiency of the formation of stable complexes of copper at the cell surface and that it may depend on the number of copper binding sites and the molecular forms of the copper compounds. In fact though, at 315.0 μg $ml^{-1}$ $Cu^{++}$ it is likely that cell membrane damage would have occurred and that binding would be to intracellular components as well as surface groups.

Since a large amount of metal has been shown to bind to the cell wall of fungi in this study, the objective of this section of the work was to investigate surface binding and to determine the number of operational binding sites for copper in the wall of *P. spinulosum* at various copper concentrations, so that the binding properties reported by other workers in various fungi could be evaluated.

12.2. Materials and Methods.

12.2.1. Determination of linear uptake during initial binding.

A mycelial slurry was prepared as previously described using the fungus *P. spinulosum*. Flasks containing 1.0, 2.5, 5.0, 7.5 and 10.0 µg $ml^{-1}$ $Cu^{++}$ in 100 ml, 50.0 mM MES pH 5.5 were prepared and equilibriated to 25°C. The copper concentrations had been corrected for the water content of the slurry.

Five ml of the slurry were drawn into a wide-tipped syringe and rapidly discharged into the copper solutions. The procedure was performed in triplicate. Incubation was carried out (with shaking) for 10, 20, 30, 45, 60, 120, 180, 240 and 300 seconds after which the fungal material was separated from the copper solution by rapid vacuum filtration (3 seconds for 105 ml of filtrate) onto Whatman 541 filters. The mycelium was then collected for dry weight and copper content determinations after immediate washing with 200 ml distilled $H_2O$.

12.2.2. Determination of initial binding kinetics.

The uptake of copper over the first minute of incubation appeared to be a linear process in the copper concentration range 1.0-10.0 µg $ml^{-1}$. The above experiments were repeated in the range 1.0 - 250.0 µg $ml^{-1}$ $Cu^{++}$ over a 10 second incubation period to enable kinetic analysis over a wide concentration range. Michaelis Menten (B vs F) Lineweaver Burke (I/B vs I/F) and Scatchard ($B/F$ vs B) plots of the data were constructed.

## 12.3. Results.

### 12.3.1. Determination of the linear uptake phase of initial binding.

Figure 12.3.1. illustrates the time course of copper binding to *P. spinulosum* over a 5 minute incubation period in the concentration range 1.0 to 10.0 μg $ml^{-1}$ $Cu^{++}$. Copper uptake over the whole of the incubation period was concentration dependent with the amount of metal bound increasing with the higher metal concentrations. The maximum rate of uptake was clearly in the first 10 seconds of incubation. It was not possible to quantify the rate of uptake in contacting periods of less than 10 seconds for practical reasons, but it is likely that the copper binding was instantaneous.

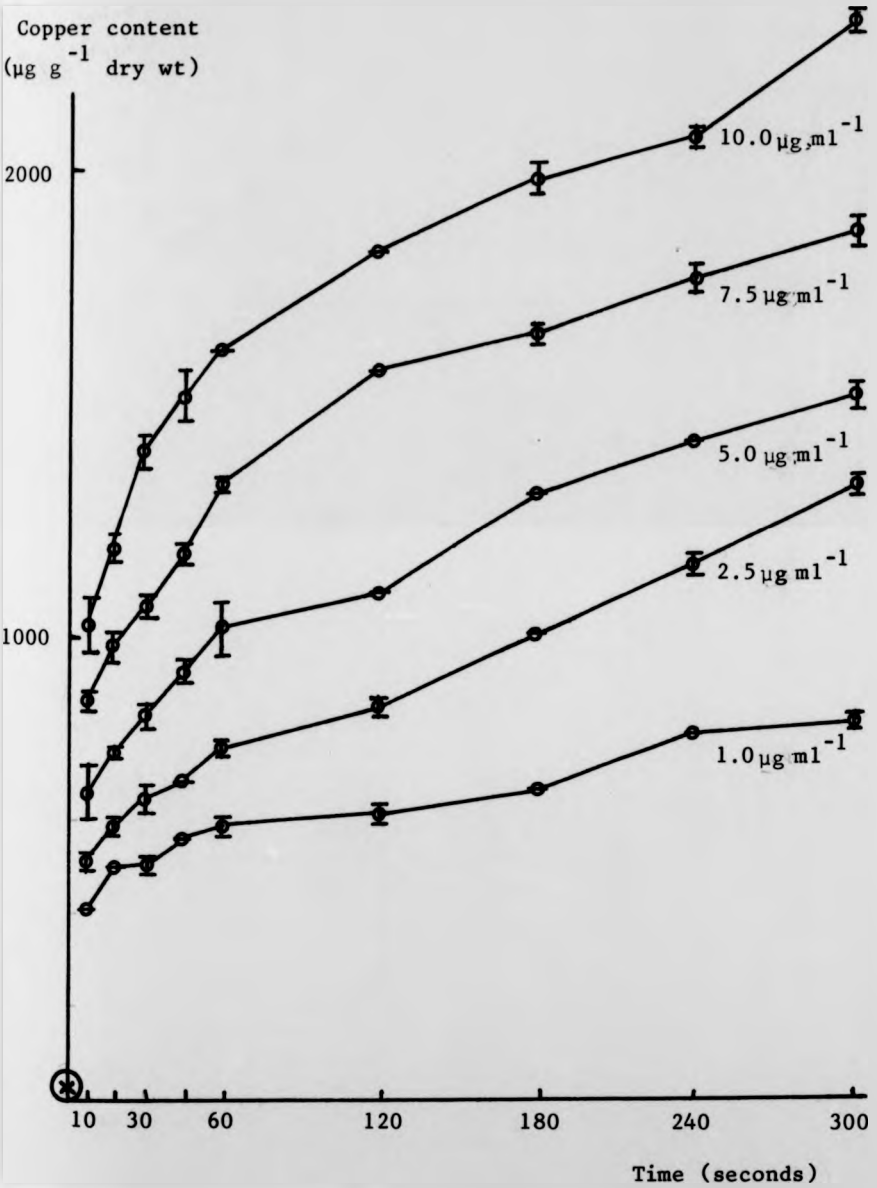
The amount of copper bound for the first 60 seconds of incubation at all the copper concentrations tested was virtually linear. The uptake over the subsequent 2 to 5 minutes of incubation was fairly linear, but the rates of uptake over this period of incubation were slower than during the first minute. The difference in rates between the incubation times 0-1 minute and 2-5 minutes increased as the metal concentration increased, and this appeared to be due to faster binding rates during the first minute, as the metal concentration increased. The rates of uptake appeared to be similar at all the metal concentrations tested over the latter 2 to 5 minutes of incubation.

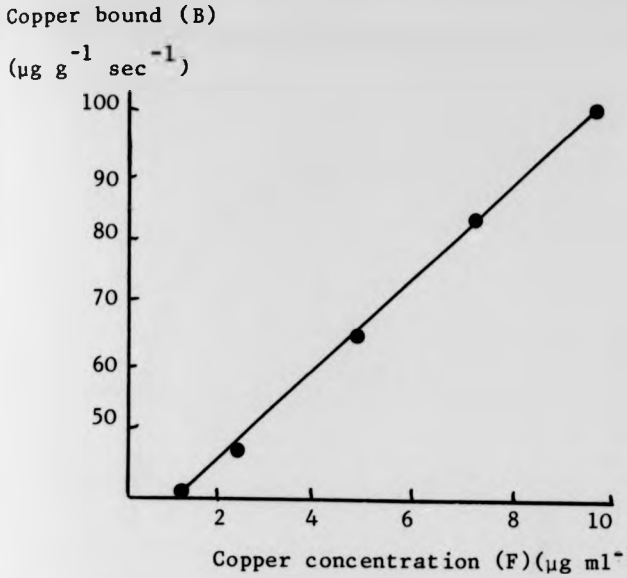
### 12.3.2. Determination of initial binding kinetics.

Figures 12.3.2a, b, c, d, e, f, illustrate the binding kinetics of copper after a 10 second incubation period. Figure 12.3.2a shows a Michaelis Menton plot for copper uptake in the concentration range

*Figure* 12.3.1.

Effect of copper concentration on initial copper binding by *P. spinulosum* in the range 1-10.0 $\mu g\, ml^{-1}$ $Cu^{++}$ over 5 minutes.






*Figures* 12.3.2a, 2b, 2c.

Copper uptake kinetics in the concentration range 1.0-10.0 µg ml$^{-1}$ in *Penicillium spinulosum*

*Figure* 2a

Michaelis-Menten plot.

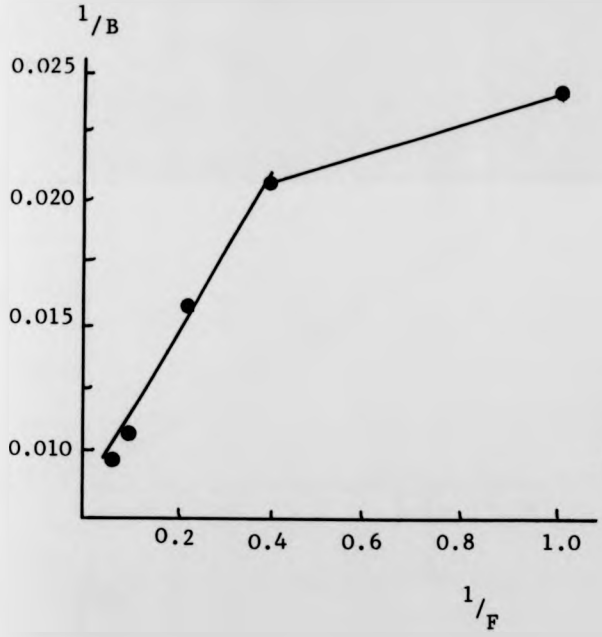



*Figure* 2b

Lineweaver-Burke plot.

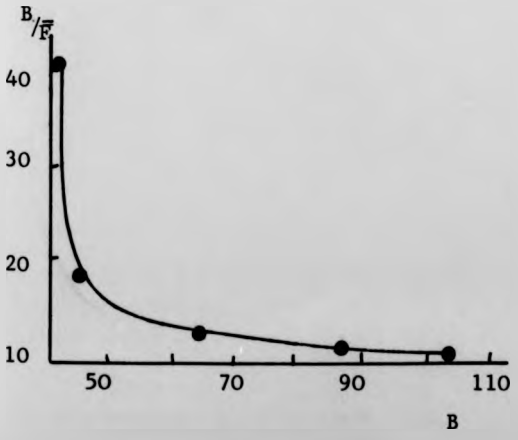


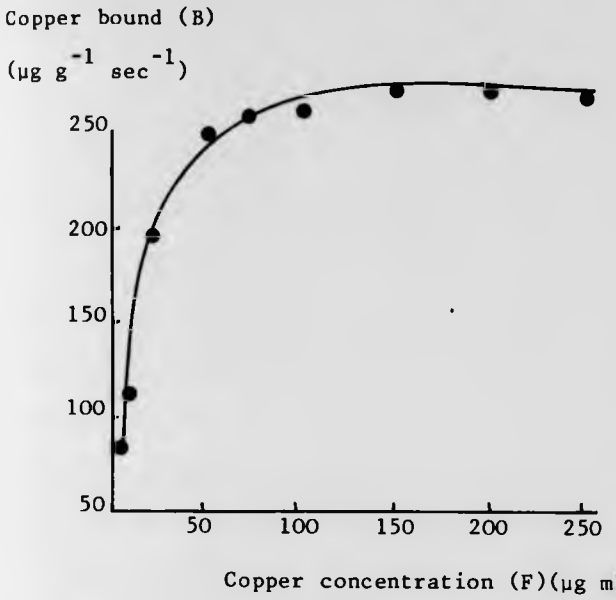

*Figure* 2c

Scatchard plot.

F = copper concentration (µg ml$^{-1}$)

B = copper bound (µg g$^{-1}$ sec$^{-1}$)

$\bar{F}$ = free copper concentration (µg ml$^{-1}$)

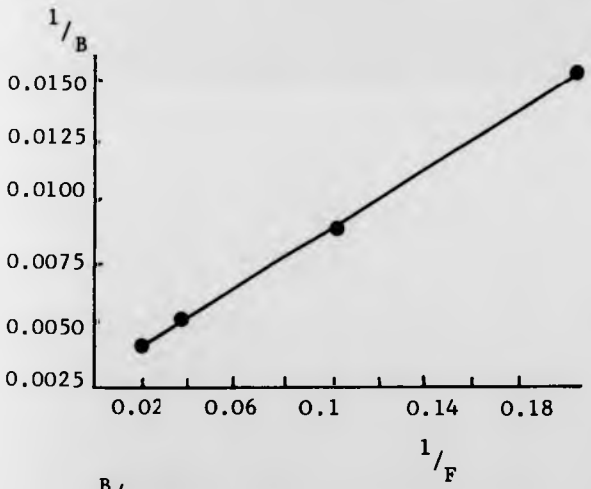



*Figures* 12.3.2d, 2e, 2f.

Copper uptake kinetics in the concentration range 5.0-250.0 µg ml$^{-1}$ in *P. spinulosum*.

*Figure* 2d

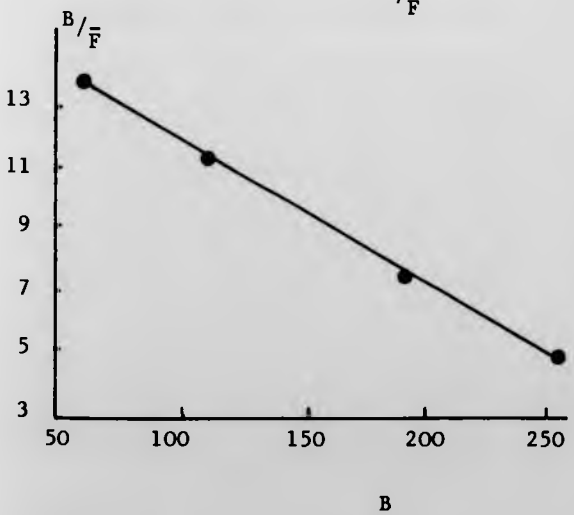
Michaelis-Menton plot




Copper uptake kinetics in the concentration range 5.0-50.0 µg ml$^{-1}$.

*Figure* 2e

Lineweaver-Burke plot.




Copper uptake kinetics in the concentration range 5.0-50.0 µg ml$^{-1}$

*Figure* 2f

Scatchard plot

1.0-10.0 μg $ml^{-1}$ $Cu^{++}$, and it is clear that the fungus did not saturate even at the highest concentration and that the uptake rate varied in a directly proportional manner with concentration. Transformation of the data into the form of a Lineweaver-Burke plot is shown in Figure 12.3.2b, and the lack of a single straight line is suggestive of a binding process involving more than one binding site in this concentration range. This was confirmed by further transformation of the data into the form of a Scatchard plot (Figure 12.3.2c) and again two binding sites are indicated. The $K_d$ (dissociation constant) of the binding site operational between 1.0 and 5.0 μg $ml^{-1}$ $Cu^{++}$ was 0.30 μg $ml^{-1}$ $Cu^{++}$ and the $B_{max}$ (maximum binding capacity) was 55.0 μg $g^{-1}$ dry wt $sec^{-1}$, and this represents a relatively high affinity binding site when compared to the same parameters calculated for the uptake data obtained in the copper concentration range 5.0 to 50.0 μg $ml^{-1}$ $Cu^{++}$.

Figure 12.3.2d illustrates the Michaelis Menten plot for 5.0 to 250.0 μg $ml^{-1}$ $Cu^{++}$ and saturation occurred at about 50.0 μg $ml^{-1}$ $Cu^{++}$. The Lineweaver-Burke and Scatchard plots (Figures 12.3.2e and f respectively) are indicative of a single binding species in the concentration range 5.0 to 50.0 μg $ml^{-1}$ $Cu^{++}$ and a $K_d$ of 122.0 μg $ml^{-1}$ and a $B_{max}$ of 300.0 μg $g^{-1}sec^{-1}$ was calculated from the plots. Clearly, this binding site has a much lower affinity than the site which was operational at 1.0 to 5.0 μg $ml^{-1}$ $Cu^{++}$, although both binding entities may be functional at higher metal concentrations.

12.4. Discussion.

Most of the increase in copper binding to *P. spinulosum* with increasing copper concentration appeared to occur in the first minute of incubation so clearly the initial adsorptive process was concentration dependent (Figure 12.3.1).

The binding kinetic data in the copper concentration range 1.0 to 5.0 μg $ml^{-1}$ $Cu^{++}$ was indicative of a two binding site system present in the cell wall of *P. spinulosum*. These findings bear some resemblance to the results reported for $Mg^{++}$ adsorption in *Neocosmospora vasinfecta* (Budd, 1979), in that the presence of two mycelial surface species binding $Mg^{++}$ with different affinities were indicated in this fungus. These sites were also non-specific for $Mg^{++}$ and probably represented general cation exchange sites. This is supported by the observation that $Mg^{++}$ adsorption involved exchange for both $K^{+}$ and $H^{+}$ ions. Interestingly, in this study it appeared that binding sites for copper also were not specific in *P. spinulosum* as shown by the competition studies. Two $K^{+}$ adsorption sites also exist in *N. vasinfecta* (Budd, 1975). The relative and absolute abundances for $K^{+}$, $Zn^{++}$ and $Mg^{++}$ binding sites did not agree well in *N. vasinfecta*, and the authors suggested that it is likely that the supply of a given binding species at the mycelial surface varies with the growth conditions. This type of variation in the supply of binding species under different growth conditions might explain however the reduced metal uptake in linear phase of actively growing fungi. The physiological role of cation adsorption has received little attention.

Budd (1975) reported interesting anomalous results for $K^{+}$ binding to the two binding sites. Adsorption of $K^{+}$ to site 2 (predominantly at

high KCl concentrations) was easily and completely reversible, whereas adsorption to site 1 (predominantly at low KCl concentrations) was not. Similarly, the contrasting effects of dinitrophenol on adsorption indicated that site 1 only was sensitive to the inhibitor. It would be interesting to speculate on the possibility of a metabolic dependence of binding to high affinity sites in *P. spinulosum*, but this is unlikely.

The importance of the cell wall in metal uptake has been further emphasised in this section of the study with particular reference to the role of initial binding. The following chapter attempts to elucidate the role of the cell wall in binding with regard to cell wall components, especially chitin, by comparing uptake of metal by commercial chitin with isolated purified cell walls of fungi, and chemically modified cell walls.

## 13. INVESTIGATION OF THE BINDING CONSTITUENTS OF THE CELL WALL OF *Penicillium spinulosum*.

### 13.1. Introduction.

The significance of the cell wall in metal binding has been demonstrated and this final section was designed to investigate the cell wall binding constituents with reference to copper and *P. spinulosum*.

The major sites of copper binding and the chemical basis for selectivity by the *P. spinulosum* cell wall was studied by comparing the uptake of copper by pure cell wall, chitin and various chemically modified cell wall preparations. There appears to be little information in the literature on chemical modification of isolated fungal cell walls although there have been a number of studies of metal uptake in fragments of mycelium (Tobin *et al*, 1984; Treen-Sears *et al*, 1984) which had been dried and ground prior to metal uptake studies, and also in chitin and cell walls of *Rhizopus arrhizus* (Tsezos and Volesky, 1981, 1982a, 1982b; Tsezos, 1983), and in a chitin-chitosan complex of *Aspergillus niger* (Muzzarelli *et al*, 1980). There has been a much more intensive investigation of metal binding to cell walls of bacteria, and of techniques of chemical modification of bacterial cell walls with particular emphasis on the genus *Bacillus* (Beveridge and Murray, 1976, 1980; Doyle *et al*, 1980; Beveridge *et al*, 1982) and in *Staphylococcus aureus* (Cutinelli and Galdiero, 1967).

13.2. Materials and Methods.

13.2.1. Comparison of copper uptake by a cell wall preparation of *Penicillium spinulosum* and commercial chitin.

The technique used for this part of the study was a modification of techniques after Bartnicki-Garcia and Nickerson (1961), Rizza and Kornfeld (1969) and Novaes-Ledieu and Garcia-Mendoza (1981). A cell wall preparation of mid linear phase mycelium of *P. spinulosum* was employed in this study. The cell walls were prepared in the following way: Ten, 400 ml volume mid-linear phase cultures at 4°C were decanted separately into an Atomix blender flask (MSE) and homogenised for 2 minutes at full speed. Care was taken at all times to maintain the preparation below 10°C to prevent enzymic degradation of the cell walls. The hyphal suspension was then submitted to the action of a Braun cell disintegrator (B. Braun Apparatebau, Melsungen, Germany) with glass beads (0.45-0.50 mm in diameter) for 3 minutes at full speed with frequent cooling with liquid carbon dioxide. The procedure was continued until complete disruption was apparent by phase contrast microscopic examination.

The homogenates were decanted to separate the glass beads and the disrupted cell suspension centrifuged at 400 g for 5 minutes. The supernatant was discarded. The cell walls were then purified by washing at least 5 times using mechanical wrist-action shaking for 30 minutes in each of the following solutions: 10% sucrose, 1.0 M NaCl, 2% sodium lauryl sulphate. The cell walls were further treated with 15 washings in cold distilled $H_2O$ and recovered by centrifugation. Hyphal cell walls appeared to be free from cytoplasmic material when stained with methylene blue and observed with phase contrast microscopy. No extinction was detectable in the distilled $H_2O$ supernatants at 260 and 280 nm indicating that there

was no cytoplasmic contamination. The cell walls were then lyophilised and stored at room temperature.

To compare metal adsorption by the cell wall preparation and chitin, 0.01% suspensions of the cell wall, commercial crude chitin, and commercial purified chitin (Sigma chemical corporation), were prepared in 50.0 mM MES buffer adjusted to pH 5.5. Small volumes of copper chloride were added to produce final concentrations of 2.5 $\mu g\ ml^{-1}\ Cu^{++}$.

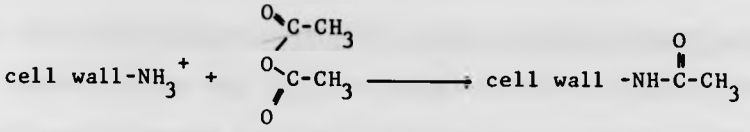
For each preparation, batches of 12 suspensions were incubated at 25°C with shaking for 1, 5, 30 and 60 minutes. Three replicate batches of suspensions were employed for each time period. At the end of the incubation period, the samples were collected by Millipore filtration and washed with distilled $H_2O$. Dry weights were obtained and copper analysis was carried out by AAS. Copper contents of untreated suspensions were determined. The purified chitin contained no copper.

13.2.2. Copper uptake by a preparation of *Penicillium spinulosum* cell wall and by chemically modified cell walls from 50.0 mM MES containing 2.5 $\mu g\ ml^{-1}\ Cu^{++}$.

The same cell wall preparation was used as in the above procedure. Four portions of 1g of the cell walls were modified using the following methods after Doyle *et al* , (1980).

13.2.2a. Amino acetylation was performed by the addition of 100.0 µL quantities of acetic anhydride to 40 ml of a well stirred wall suspension (7.5 mg cell wall per ml distilled $H_2O$) in a 50% saturated sodium acetate

solution. The anhydride (total volume of 1200 ml) was added at 12 minute intervals with continuous shaking of the suspension. The acetylated wall was then centrifuged, washed with cold distilled $H_2O$ and resuspended in 1.0 M hydroxylamine at pH 8.0, to effect the removal of O-acetyl groups. The amino-acetylated wall was then rewashed after filtration and stored at $4^oC$ until used.



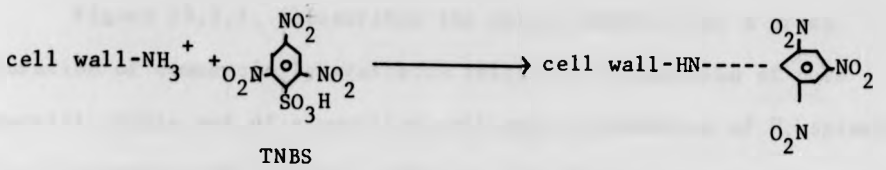
acetic anhydride

13.2.2b. Succinylation of cell walls was carried out by adding 300 mg solid succinic anhydride to 40 ml of a cell wall suspension in 1.0 M sodium carbonate at pH 8.0. The anhydride was added at 10 minute intervals in 30 mg quantities. The modified walls were then treated with 1.0 M hydroxylamine at pH 8.0 as for the acetylated cell walls, washed and stored at $4^oC$. Succinic anhydride treatment introduces a further negative charge into the cell wall by reducing the numbers of positive amino groups by binding to them.

succinic anhydride

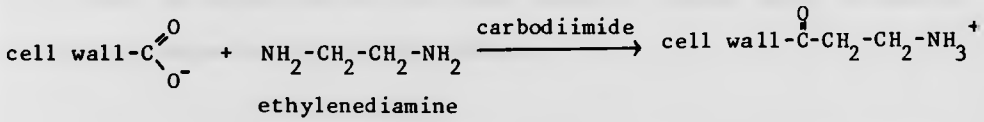
13.2.2c. The bulky trinitrobenzenesulphonic acid (TNBS) was introduced into the amino groups of the cell wall using the following procedure. To 40 ml of a wall suspension in 4% (wt/vol) sodium bicarbonate solution at pH 7.9, was added a 4.0 ml sample of TNBS (2 mg $ml^{-1}$ in distilled $H_2O$). The addition of TNBS was performed 3 times at intervals of 30 minutes with continuous shaking at 200 rpm. The modified walls were then washed

with distilled $H_2O$ and stored at $4^oC$.




TNBS

13.2.2d. The carbodiimide-nucleophile reaction with carboxyl groups was used to alter the negative groups in the cell wall. 300 mg of cell wall were suspended in 20 ml of 1.0 M ethylenediamine. The water soluble carbodiimide, 1-ethyl-3,3-dimethyl amino propyl-carbodiimide was added to the suspensions to a final concentration of 1.0 M. All suspensions were prepared in 0.2 M $CaCl_2$ at pH 4.75 which acted as a weak buffer. Maintenance of pH at 4.75 was carried out by the addition of dilute HCl. After the pH was stabilised, incubation was carried out for a further 2 hours with shaking. The walls were then centrifuged with 0.1 M sodium acetate at pH 5.0 and then with distilled $H_2O$. The modified walls were stored at $4^oC$ until used.




ethylenediamine

Copper uptake by 0.01% suspenisons of untreated cell walls (controls) and modified cell walls was compared over a 2 hour incubation period. The effect of ligand modification on subsequent copper uptake is shown in the results section. Modification of whole cells was attempted butmycelial agglomeration led to major difficulties in copper treatments so the procedure was abandoned.

13.3. Results.

Figure 13.3.1. illustrates the uptake profile for a crude preparation of commercially available chitin, a preparation of pure commercial chitin and of a purified cell wall preparation of *P. spinulosum*, and for comparison the uptake profile for the intact non-growing mycelium is also indicated. The uptake by the crude chitin was clearly much greater than the pure chitin or purified cell wall preparation and this was probably due to constituent differences particularly of possible protein inclusions and other substances which could bind metal in the crude chitin. The pure chitin accumulated relatively similar amounts of copper to the purified cell wall preparation, but the initial uptake was more pronounced in the pure chitin. It is possible that this was a result of the different physical nature of the two preparations. It is interesting that when compared to the intact mycelium uptake, the cell wall uptake constituted about 25-30% of the intact mycelium copper levels. Although the crude chitin accumulated copper more rapidly than the intact cells, the final levels of copper after 60 minutes of incubation appeared to be very similar.

Figure 13.3.2. shows the uptake profiles over a 2 hour incubation period in 2.5 $\mu g\ ml^{-1}$ $Cu^{++}$ of a cell wall preparation of *P. spinulosum* and of chemically modified cell walls.

Ethylenediamine treatment and TNBS treatment increased the uptake of copper over the first hour of incubation, but there was little discernible difference in copper loadings between these treatments and the control untreated cell wall after 2 hours of incubation. Acetic anhydride treatment dramatically increased copper binding by four fold after 1 minute of contact with copper, but the final increase after the

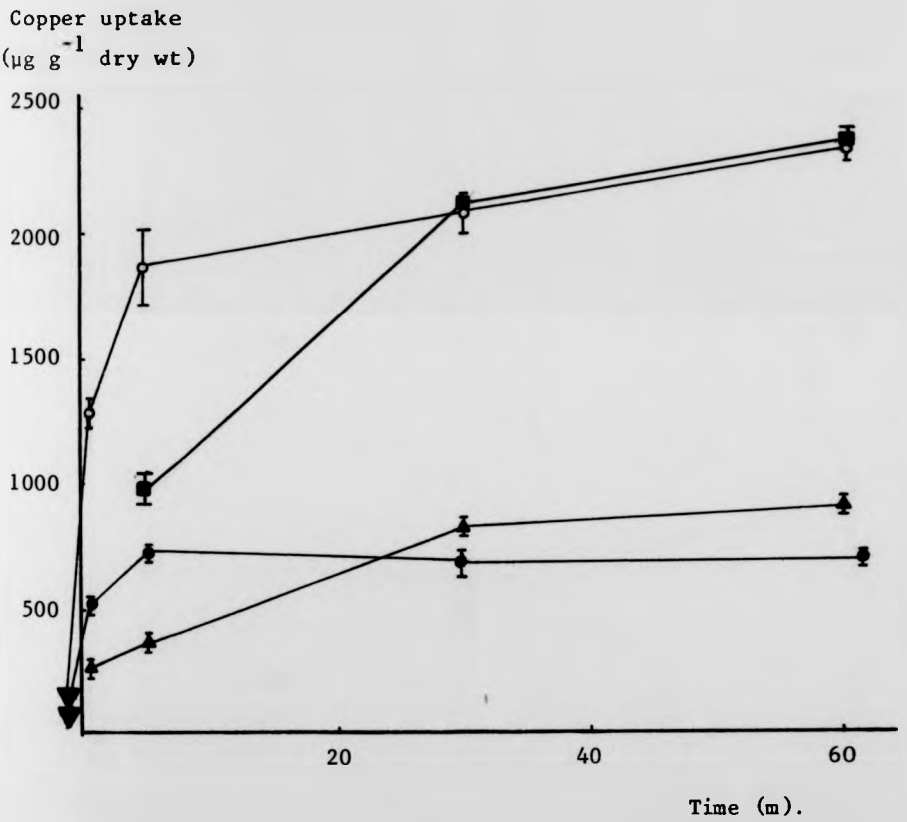



*Figure* 13.3.1.

Copper uptake from 2.5 µg ml$^{-1}$ $Cu^{++}$ by a crude preparation of chitin , ● ; a preparation of pure chitin, ▲ ; and by a cell-wall preparation of *P. spinulosum*. ●. Initial copper contents ▼ .

Copper uptake by intact cells for comparison, ■ .

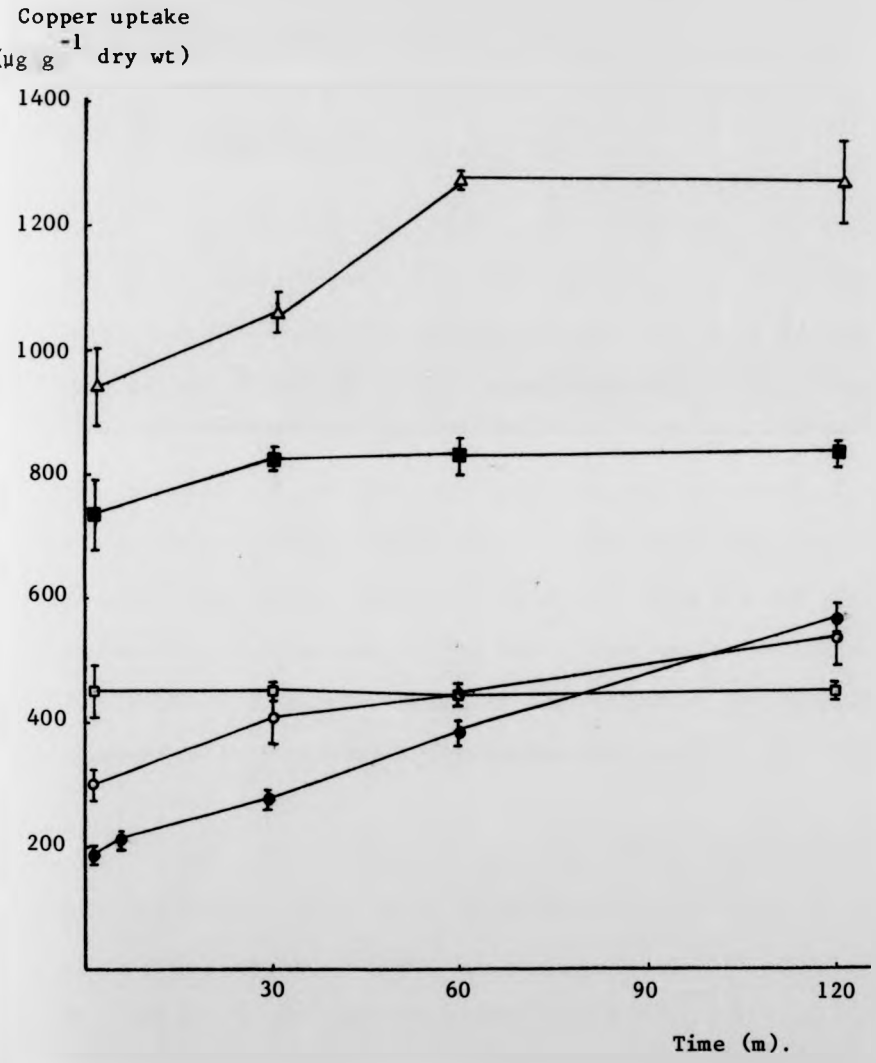



*Figure* 13.3.2.

Copper uptake from 50mM MES containing 2.5µg ml$^{-1}$ $Cu^{++}$ by a preparation of cell-walls of *P. spinulosum* and by chemically modified cell-walls. ●, control cell-walls; □, cell-walls treated with trinitrobenzene-sulfonic acid (TNBS); ○, ethylenediamine treatment; ■, treatment with acetic anhydride; △, modification with succinic anhydride.

whole of the 2 hour incubation was about 1.75 fold over the control cell walls. The most marked increase in cell wall binding was brought about by treating the cell wall preparation with succinic anhydride and this resulted in a five fold increase in copper uptake over controls after 1 minute of contact with the copper solution, and an eventual three fold increase after 2 hours of incubation.

## 13.4 Discussion

The fact that the crude chitin accumulated more copper than any of the other prepared biosorbents was probably due to contaminating protein moieties and other binding agents. The purified cell wall preparation accumulated similar amounts of metal as did the pure chitin. It is interesting that when compared to the intact mycelium copper uptake, the uptake by the purified cell wall represented a 25-30% proportion of the uptake shown by intact cells. This would imply that the amount of metal which would normally be desorbable from the mycelium would be around 25% assuming that all the bound metal could be released. The desorption data however, suggested that 59-65% of the total copper accumulated was desorbable from non-growing mycelium of *P. spinulosum*.

Any interpretation of purified cell wall data has to be approached with caution since there is the possibility that extra, previously unexposed binding sites could be revealed. It would also be difficult to estimate the possible consequences of the purification treatments, particularly with reference here to the detergent washing which would almost certainly result in a protein loss. Examination of uptake characteristics of KOH extracted cells would provide useful information on the role of protein in total uptake. It is difficult to explain the fact that the initial binding figure in the cell walls, was

only about 25% of the total copper uptake in whole cells, since one might expect that the initial binding would be similar in both the cell wall preparation and in the intact mycelium (after 1 minute, for example). Also one might expect that the major difference in metal contents of cell walls and intact cells would only occur after extended periods of incubation (for example, 1 hour), after which time the metal could have been internalised into the intact mycelium, so accounting for the ultimately higher uptake in the whole cells. The results could perhaps be explained in terms of the intact cells having a higher affinity for metal then purified cell walls and this could be due to the loss of binding substances in the cell wall matrix which resulted in the lower binding.

The effects of chemical modification of cell walls for *P. spinulosum* is in general agreement with the information reported by Doyle *et al* (1980) for cell walls of the bacterium *Bacillus subtilis*. Succinic anhydride treatment introduces an extra negative charge into the cell wall and the authors reported that this resulted in an increase in the number of binding sites for $Mn^{++}$ although there was no change in the affinity between the metal and the ligand. In the case of copper and the fungus *P. spinulosum*, succinic anhydride treatment also resulted in a dramatic increase in binding of the metal. Acetic anhydride treatment introduces acetyl groups and reduces the number of ammonium groups in *B. subtilis* walls and Doyle *et al* (1980) reported that this resulted in an increase in the number of binding sites for $Mn^{++}$, $Ca^{++}$ and $Na^{+}$ and increased the affinity for $Mn^{++}$ and $Na^{+}$. Similarly, copper binding was enhanced in *P. spinulosum* after modification using acetic anhydride. TNBS modification introduces a bulky group into the cell wall thereby reducing the number of ammonium groups. According to

Doyle *et al* (1980), this increased the number of binding sites for $Ca^{++}$ and $Na^{+}$. The affinity for $Ca^{++}$ was decreased whilst for $Na^{+}$ there was an increase in affinity. There was no effect on the number of binding sites for $Mn^{++}$ although there was an increase in affinity. The authors attributed this to the possibility that ammonium ions were acting as competitive counter ions. In the case of *P. spinulosum*, this treatment had little effect on overall $Cu^{++}$ binding although initial binding was elevated to twice the control binding level. Ethylenediamine treatment results in the replacement of carboxyl groups with positive charges. This resulted in decreasing the number of binding sites and the affinity for metals in *B. subtilis*. In the *P. spinulosum* cell wall, this treatment appeared to have little effect on copper loading.

Doyle *et al* (1980) summarised their findings by suggesting that carboxyl and amino groups were important in regulating interaction between cations and cell walls. The major disagreement with the ethylenediamine treatment may suggest that carboxyl groups are not so significant in fungal cell walls, although this is rather unlikely. It may well be that in this case, the chemical modification was unsuccessful, particularly when one considers the dissimilarity of fungal and bacterial cell walls. Unfortunately, in the case of copper uptake and *P. spinulosum*, there is no available evidence to confirm the effectiveness of the chemical treatments, and this would constitute a basis for further investigation of these phenomena. The agreement with some of the data of Doyle *et al* (1980), who did experimentally confirm the modifications, may suggest by analogy that the fungal cell walls were similarly modified.

It is interesting that Doyle *et al* (1980) suggested that the metals were binding to the same sites on the cell wall, and this would

agree with the results obtained in the competition experiments by this author in the fungus *P. spinulosum*. Doyle *et al* (1980) reported that an explanation for the striking differences in affinities between the metals and the cell walls is lacking, although they suggest that the spatial arrangement of amino acids may be a factor in controlling the strength of the metal ion-cell wall-ligand interactions. Clearly, more research is required into the structural components of cell walls so that the role of the cell wall in metal uptake at the ionic level can be understood. Initial studies using electron spin resonance techniques may be of particular importance in this respect and more attention should be directed towards this. Preliminary studies of this nature have been carried out in a collaborative study using electron spin resonance (ESR) and these are reported in the Appendix.

## 14. GENERAL DISCUSSION.

The toxicity tests on solid and liquid medium have indicated that there was no correlation between metal essentiality and the degree of toxicity which the metal elicited in this study. Whilst zinc and manganese for example were relatively non-toxic, copper was extremely effective in inhibiting fungal growth, and yet all three metals were essential elements, (Bowen, 1966). Cadmium on the other hand, was the most toxic metal tested on both solid and liquid medium and is not reported to be an essential element.

The essentiality of copper appears to be due to the requirement of certain enzymes for this ion since enhanced protein levels and acid phosphatase activity have been observed at optimum copper levels. Jones and Greenfield (1984) reviewed the toxic effects of copper to yeasts and suggested that the fungitoxic effects are directed towards two primary cellular sites; the first effect was reported as being directed at soluble enzymes whereby specific metabolic lesions are produced at inhibitory copper concentrations. The second site of action was shown to be at the membrane. At very high copper concentrations, irreversible membrane damage occurs which results in accelerated viability loss. Toxicity appears to be due to effects on soluble enzymes and interference with essential element transport, for example, magnesium transport. Zinc is an essential element (Bowen, 1966) and its role is in the maintenance of activity of a host of zinc containing enzymes. It enhances riboflavin synthesis, activates acid and alkaline phosphatases and generally increases the protein content of cells. Toxic concentrations influence a whole range of membrane functions by subtle interactions leading to deoptimisation of membrane performance.

The toxicity tests on solid medium were of limited application because of the difficulties in medium separation for uptake studies and also because of the possible complexation problems caused by agar. The technique did at least serve however, to provide the author with rapid preliminary information on toxicity for a fairly large number of metals and fungi. Whilst cadmium was the most toxic metal tested, copper was very toxic, zinc and cobalt were moderate (but variable), and manganese was relatively non-toxic. The following toxicity series was proposed: For *Penicillium spinulosum* and *Aspergillus niger* $Cd^{++} >> Cu^{++} >> Zn^{++}$ and for *Trichoderma viride* $Cu^{++} >> Cd^{++} >> Zn^{++}$. The results obtained in the solid and liquid media agreed well considering the presence of the agar complement in the solid medium. Also though, the results were complicated by the difference in hyphal morphology observed on the two media. In liquid medium the hyphae were fully submerged whilst in the solid medium part of the mycelium was aerial and this may have led to differences in hyphal morphology and as a consequence the behaviour of the fungus may have been different in the two systems. As discussed in chapter 4, the toxicity series obtained was in agreement with the data of Bedford (1936), Horsfall (1956), Okamoto *et al* (1977) and Treen-Sears *et al* (1984). Whilst there appears to be a general trend for fungitoxicity of metal ions, the usefulness of these toxicity series is limited because they are dependent on many factors which can influence metal binding. These factors could include the environment with respect to pH and temperature but also the organism itself can influence the metal binding. For example, metals could be complexed by secreted metabolites in the medium as well as being affected by pH changes. Thus in metal binding and uptake, and hence in toxicity, the organic ligand-metal relationship is very dependent on the environmental conditions and can therefore be very variable.

Passow *et al* (1961), suggested that the relative affinities of heavy metals for ligands such as $-OH_5COOH$, $-SO_3H_2$, -SH, $-NH_2$-imidazole should permit predictions concerning the sites of heavy metal action on living cells. This may be true under standardised uniform conditions and indeed, heavy metals are bound by amines or simple amino acids in the following order of decreasing affinity under certain conditions: $Hg^{++} > Cu^{++} > Ni^{++} > Pb^{++} > Zn^{++} > Co^{++} > Cd^{++} > Mn^{++} > Mg^{++} > Ca^{++} > Ba^{++}$. Similarly, the affinity towards $COO^-$ groups can be represented by $Cu^{++} > Ni^{++} > Zn^{++} > Co^{++} > Mn^{++} > Mg^{++} > Ca^{++} > Ba^{++}$ Passow *et al* (1961).

It would be an elegant procedure if one could reliably apply these methods in order to relate a toxicity series for metals to relative affinities for ligands in fungi, to enable prediction of binding sites and sites of toxic action, but given the number of problems, this would be very difficult to achieve, particularly since no one has attempted to define standard working conditions. The first problem would be in defining the participation of hydroxyl groups of water in complex formation, which would obviously influence the electrochemical properties of the metal. The second difficulty would be that all biological ligands contain dissociable protons so there is an apparent competition for binding sites, and this is further complicated by surface charges and a general requirement for electroneutrality in the ambient medium. The contribution of the cell wall has been demonstrated by monitoring metal uptake in isolated cell walls but it is not clear if the composition or the architecture of the cell wall remains constant throughout the growth cycle. Temperature, pH, interference from other cations, and the physiological state of the organism can influence metal binding affinities as demonstrated in this study, so an attempt to relate these factors to produce a general series of toxicity to microorganisms would seem to be rather inappropriate.

Many workers have attempted to explain the differential toxicities of metals to microorganisms. Somers (1966) discussed the role of metals in terms of toxicity at various regions of the cell; at the outside of the cell membrane, at the cell membrane and within the protoplast. Somers (1961) showed that for 24 metal cations directed against *Alternaria tenuis* and *Botrytis fabae* their $ED_{50}$ values conformed to an exponential relationship with electronegativity. Bowen (1966) has confirmed that there is some correlation between electronegativity, insolubility of sulphides and stability of chelates. This author also reported that although a toxicity series for metals can be demonstrated in fungi and plants, there is no single fungitoxicity series.

There are indications in the literature that there is an interaction of metals in terms of uptake and subsequent toxicity of metals with similar ionic radii. Norris and Kelly (1977) suggested that the stronger interaction between cadmium and calcium than with cadmium and manganese was due to the similarity of the ionic radii of cadmium and calcium (0.097 and 0.099 nM respectively). (The crystal ionic radius of manganese is 0.080 nM for comparison). Further work by Norris and Kelly (1979) showed that zinc uptake by the yeast *Candida utilis* was inhibited slightly more by cadmium than by calcium and this may be due to chemical similarity of cadmium and zinc. Tobin *et al* (1984), have reported that *Rhizopus arrhizus* adsorbed a variety of metal cations and the amount of metal taken up was directly related to the ionic radii of $La^{+++}$, $Mn^{++}$, $Cu^{++}$, $Zn^{++}$, $Cd^{++}$, $Hg^{++}$, $Pb^{++}$, $Ag^{+}$ and $UO_2^{++}$.

Thus the ionic characteristics of metals seem to influence where they bind and the degree of binding and perhaps this could be related to uptake and toxicity of heavy metals. Abelson and Aldous (1950) however, suggested that if one attempted to enlarge upon the qualitative agreement

between the physical-chemical binding of cations and toxicity, a number of difficulties would appear. Firstly, organisms do not constitute reversible chemical systems, secondly, in a non-equilibrium system, mobility and reaction rates are the governing factors and thirdly, binding of cations can occur at a number of sites.

It has been recognised that microorganisms can develop adaptive resistance in response to gradually increasing metal concentrations, but in addition, organisms can also be isolated that exhibit high levels of genetic resistance against some metal ions in their naturally occurring state. Many mechanisms of resistance are however demonstrated by microorganisms.

Microbial hydrogen sulphide production often has significant effects on metal toxicity since most heavy metals form insoluble sulphides with $H_2S$. For example, copper precipitation has been observed in *Poria vaillantii* (Levi, 1969), and it is possible that this may have been copper sulphide. Binding of metals by organic substances present in the microbial environment can influence metal toxicity. In *A. niger* a lead chelate complex with DL-cysteine has been shown to produce pronounced swellings in the mycelium. As fungal growth proceeded, the lead chelate decomposed forming free $Pb^{++}$ ions which in turn formed chelates with citric acid produced by the fungus (Zlochevskaya, 1968). Oxalic acid production has been linked with copper tolerance of several species of *Poria* (Levi, 1969). Crystals produced by *Poria monticola* growing on a copper containing medium were shown to be copper oxalate. Reduced metal toxicity has also been observed in the fungus *Endothia parasitica* which can produce oxalic acid (Englander and Corden, 1971). Intracellular organic substances such as sulphydryl compounds have been implicated in

mercury complexing in a mercury tolerant strain of A. *niger* (Ashworth and Amin, 1964). In some cases the resistance against metal ions is manifested by a reduced uptake of the ion. Bacterial resistance to some heavy metals can be controlled by genes as extrachromosomal resistance (R) factors or plasmids and in *Staphylococcus aureus*, the mechanism of cadmium resistance results from a permeability change in the cells, so that resistant cells containing the plasmid do not accumulate cadmium (Chopra, 1971, 1975). There is no evidence for plasmid mediated metal resistance in fungi however. At high metal concentrations, intracellular precipitation of metals can occur which results in a detoxification of the metal due to compartmentalisation. For example, electron dense bodies, presumed to contain zinc have been observed in *Neocosmospora vasinfecta* after growth in zinc containing medium (Paton and Budd, 1972).

The toxicity tests provided the author with an idea of acceptable working levels of metals to enable accumulation studies at non-toxic concentrations. The procedures highlighted fungi which had reliable growth parameters and also demonstrated degrees of metal tolerance. The toxicity study was a useful and important prerequisite to uptake studies.

The results of the accumulation experiments in growing fungi with relation to toxicity, did not however provide the author with clear evidence to support a defined relationship between quantitative metal uptake and the degree of toxicity shown by that metal. For example, copper toxicity was similar in *P. spinulosum*, *T. viride* and *A. niger*, yet uptake of copper was dramatically greater in *P. spinulosum* than in either of the other fungi. On the other hand, in the presence of cadmium, *P. spinulosum* was the most susceptible fungus and accumulated more of this

metal than did *T. viride* or *A. niger*. *Trichoderma viride* which accumulated least cadmium, was the most resistant fungus tested. Zinc was relatively non toxic to all fungi and differences in accumulation were relatively small. More comparative data of this sort is required before firm conclusions can be drawn on this phenomenon.

The pattern of metal accumulation in growing filamentous fungi was interesting, but the function of a process whereby uptake is initially high in lag phase followed by a period of reduced metal uptake on a per cell basis in linear phase is difficult to speculate on. It might be expected that metal requirements would be high during the periods of rapid growth, but in fact metal loadings appeared to be reduced at this time. The most likely explanation is that in fungi a scavenging mechanism may operate initially, which could result in high metal concentrations localised in the vicinity of the cell membrane. The evidence from the isolated cell wall metal uptake studies, adsorption kinetics studies and the electron spin resonance work has indicated that this localisation is most likely to be on the cell wall. From consideration of the results of the studies designed to elucidate metabolic involvement in metal uptake, it appeared that at least under the conditions in which the experiments were performed, the localised metals were not internalised by an energy dependent mechanism. This is in agreement with the data of some workers, whilst it is in dispute with some evidence of other workers, and this is discussed in the relevant chapters. The variability in the requirement for energy for metal uptake observed by researchers, is very likely to be as a result of differing conditions used in various laboratories and of the choice of species of fungus studied. The active zinc uptake in *Neocosmospora vasinfecta* as reported by Paton and Budd (1972) could not be demonstrated in this study even though the experimental conditions, except for a difference in pH, were as similar as practically possible. This supports the idea that the variation is a characteristic of the fungi themselves.

The fact that in growing cultures the pH was difficult to maintain in buffered systems, led the author to believe that pH may have been influential in the metal reduction phenomenon in linear phase of growing cultures. In fact the fermenter studies which utilised electronically maintained pH, showed that it was only the initial pH which was critical for metal-loading in lag phase and that this loading in turn influenced linear phase metal contents. The pH effect in lag phase was probably by virtue of competition for binding sites by divalent cations and protons and this is suggestive of a physical, probably adsorptive process for initial metal binding.

The mechanism by which the localised metals are transposed from the cell wall to the internal portion of the cell can only be speculated on from the data available from this study, but there is circumstantial evidence for an involvement of pH. It appears that energy is not involved, so it is possible that metal ions are displaced from their location on the cell wall by a gradually increasing concentration of $H^+$ ions due to metabolic activities, resulting in the formation of a concentration gradient of metallic cations outside the cell membrane, with a high concentration outside the cell and a lower one in the cytoplasm. This could possibly allow internalisation of metal by a process of diffusion. The results from the experiments designed to observe the effects of temperature on uptake, tend to suggest that this could be the case because of the low activation energy of uptake which was observed. The intracellular requirement for metal is very small anyway, so under conditions in which a concentration gradient can be set up, it is possible that a mechanism of this type could fulfill the cation requirement. Under conditions of metal limitation where a gradient may not be possible, an energy dependent process may then be involved. Further studies should consider the uptake of metals from much lower metal concentrations in an attempt to promote a possible metabolic uptake system.

It is unfortunate that the data from the two experimental situations (i.e. growing and non-growing cells) have to be combined in order to produce a working hypothesis for metal uptake. As a result of the difficulties of making uptake measurements in a dynamic growing system, and of performing metabolic inhibitor and temperature studies on growing cells, some of the information has to be gleened from experiments involving non-growing cell suspensions. On the other hand, toxicity tests are more appropriate on growing mycelium, although one might argue that toxicity tests might be better applied at the biochemical level since the effect of metals on growth is a rather indirect way of observing metal toxicity. One problematic feature of combining the results obtained from two experimental systems is the assumption that the metal uptake process is the same in both. This assumption is confounded however, by the fact that there was a large discrepancy in the quantitative amount of metal accumulated in the two systems, so although there appeared to be no metabolic involvement in metal uptake in non-growing cells, it would be dangerous to suggest that this would necessarily also be the case for growing fungi. The implication in the past has been that one could extrapolate results from non-growing cells and apply them to the situation where cells are growing. The evidence presented by the author however, indicates that this extrapolation is not necessarily appropriate under all circumstances.

The role of the cell wall in uptake has been indicated in this study but there is very little information on metal uptake by isolated protoplasts, except for a recent report by Gadd *et al* (1984). There are no reports showing that an active transport system is present in isolated fungal protoplasts. Active uptake in protoplasts should not be regarded as the mechanism which is necessarily operational under all conditions anyway. Obviously, an isolated protoplast, just as an isolated cell wall,

is likely to produce atypical results because of the exposure of previously unexposed binding sites, but even so, information from an isolated cell wall and its complementary protoplast would be of enormous value. This would be particularly interesting if one could demonstrate active uptake in protoplasts from a fungus, which when intact did not show any metabolic involvement in metal uptake because of the presence of a reservoir of metal ions on the cell wall. Furthermore, the ultimate aim ought to be to study metal uptake in a regenerating protoplast in an attempt to relate metal uptake with the formation and incorporation of quantifiable cell wall components.

The influence of pH in metal displacement from the cell wall and subsequent internalisation, could further be investigated by monitoring metal loading on the internal and external portions of the fungus whilst measuring $H^+$ efflux in growing fungal cultures. This could be approached by separating the two systems (i.e. the cell wall component and the protoplast), although again the same complications of non-physiological conditions manifest themselves. Progress in studies of metal accumulation by growing cells could be achieved by observing the phenomenon in continuous culture, where at least growth is relatively uniform and balanced, in contrast to the batch culture system.

The results of this study indicate some of the characteristics of metal accumulation in filamentous fungi in growing cultures, but there are still many problems to be solved, with special reference here to the mechanisms of uptake. Once the quantitative metal accumulation has been investigated, researchers would then be able to approach the problem of measuring influx or transport into the cell in growing cells. Although Jennings (1976) has outlined some preliminary ideas for studying glucose transport in growing fungi, much more information is required for other

nutrients including metals. Jennings (1969) provided equations for plant cells relating growth to transport and the same author (1976) suggested that adaptation of these equations for the growth of fungal cells might form the basis of a theoretical approach to transport in growing filamentous fungi.

Apart from the academic requirement for further information in the area of metal accumulation in fungi, it is likely there will be increased pressure from water authorities, conservation groups and eventually the hydrometallurgical industries, for the development of better metal recovery processes possibly exploiting microorganisms. Filamentous fungi in particular may be of some interest because of their ease of handling.

By comparing quantitative metal uptake in non-growing biomass and growing mycelium it became clear that it would not be feasible to use growing cells in metal recovery systems because of the reduction in binding of metal in linear phase of growth. This problem, and the fact that difficutlies would be encountered in treating highly toxic effluents would constrain the use of a self regenerating system in many applications. It would seem sensible to make use of the finding that non-growing cells achieved superior metal contents to growing mycelium, and it is conceivable that waste biomass from other commercial activities exploiting fungi could be used directly for effluent treatments.

There is enormous scope for further research in this area in terms of optimisation of metal sequestering with regard to temperature, pH, and the physiological state of the fungus *etc*. Also work needs directing towards improving the specificity of biosorbents for certain metals,

including precious or radioactive metals, in mixed effluents. Furthermore, the role of other effluent components such as other metals, acids, solvents, chelating agents, solids, detergents *etc.* needs to be thoroughly investigated in relation to the efficiency of metal recovery processes. Basic academic research into the phenomenon of heavy metal accumulation and transport in microorganisms could facilitate the development and application of efficient microbial metal recovery processes in industry.

## 15. Appendix.

### Electron spin resonance measurements on copper loaded *Penicillium spinulosum*

### 15.1. Introduction.

Some information on the functional groups involved in copper binding has been obtained in this study, and by using electron spin resonance (ESR) techniques, information on copper coordination to specific functional groups can be obtained.

The structural implications derived from the analysis of electron paramagnetic resonance (EPR) spectra of natural and artificial copper proteins have been investigated by Peisach and Blumberg (1974) and these authors presented a method of relating chemical structure of the protein to the EPR parameters A and g. The chemical state of copper in *Chlorella regularis* has been investigated using EPR by Nakajima *et al* (1977, 1981), and the authors reported that the A and g parameters of the cupric ion suggested that the ion was tetragonally coordinated by two nitrogen and two oxygen atoms. Tsezos (1983) reported that an ionic copper (11) chitin complex was detectable in *Rhizopus arrhizus*. The A and g parameters of the EPR spectrum suggested that the ligands were coordinated via at least two oxygen atoms and one nitrogen was involved with the chitin-metal coordination, and an axially symmetrical copper (11) complex with chitin, through an amine nitrogen has been suggested by Mattar and Tsezos (unpublished). In an electron spin resonance survey of fungal phenolic polymers and melanins, Saiz-Jimenez and Shafizadeh (1984) found that the phenolic polymers from *Epicoccum purpuascens* and *Penicillium funiculosum* displayed hyperfine splitting, indicating the presence of

transition metal bonding. The copper complex ESR parameters were similar to those reported for mixed complexes of copper, bipyridyl and dicarboxylic acids, suggesting that nitrogen containing groups from the proteinaceous moiety were involved in the process.

## 15.2. Materials and Methods.

Dr. J.B. Raynor of the Chemistry Department, University of Leicester, is gratefully acknowledged for his kind assistance in performing and analysing ESR measurements of copper loaded biomass.

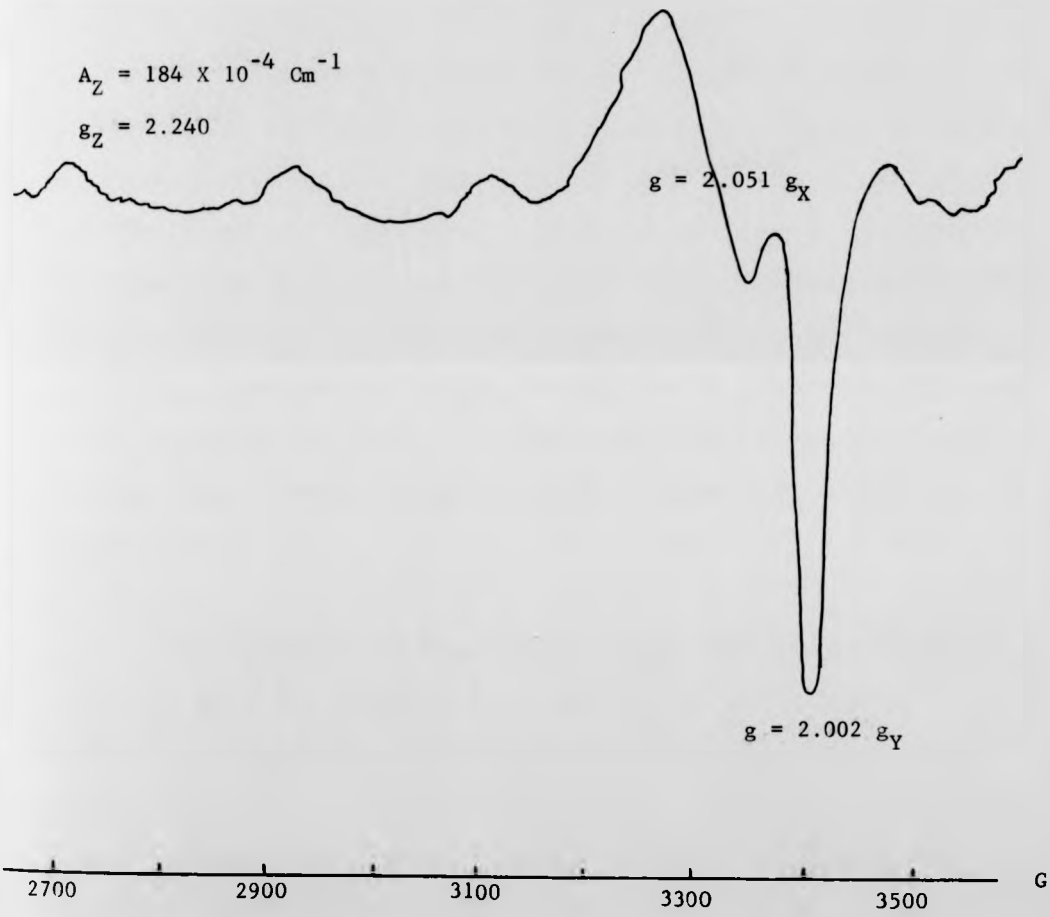
*Penicillium spinulosum* was loaded with copper from a 2.5 µg $ml^{-1}$ $Cu^{++}$ solution in 50.0 mM MES pH 5.5 and lyophilised. Operation temperature was 140°C, microwave power 15 d B 61 mW, field modulation intensity 10 $G_{pp}$, gain 5 x $10^5$ with a time constant of 1 second. The field intensity had a mid range of 3100 G, a scan range of 1000 G and a scan time of 200 seconds.

## 15.3. Results.

The ESR spectrum of copper loaded *P. spinulosum* is shown in Figure 15.3.1. The spectrum shows the typical $Cu^{++}$ signal. From the g and A values ($g_x$ = 2.051, $g_y$ = 2.002, $g_z$ = 2.240 and $A_z$ = 184 x $10^{-4}$ $cm^{-1}$) the copper can be ascribed to type 2 (non-blue). Also it is not apparently bonded to sulphur, and it is likely that it is bonded to 4 nitrogen or 2 nitrogen and 2 oxygen atoms in the equatorial plane. There was no evidence of dimer formation. The sharp and symmetric line in the higher field (3400G) is probably that of free radicals such as quinones.

*Figure* 15.3.1.

Electron Spin Resonance spectrum for copper loaded P. *spinulosum* biomass.




Temperature 140 K., Microwave power 15 dB 61 mW., Field modulation intensity 10 $G_{pp}$., Gain 5 X $10^5$, Time constant 1 second, Field intensity mid range 3100 G, field intensity scan range 1000 G, Scan time 200 seconds.

Discussion.

The results obtained from the ESR studies are in good agreement with those for copper binding in *Chlorella regularis* (Nakajima *et al*, 1977, 1981), in *Rhizopus arrhizus* (Tsezos, 1983) and in phenolic polymers produced by *Penicillium funiculosum* (Saiz-Jimenez and Shafizadeh, 1984).

The ESR spectrum of copper ions in *P. spinulosum* has the g anisotrophy and the hyperfine structure of the copper nucleus, coordinated to either 4 nitrogens or 2 nitrogens and 2 oxygens in the equatorial plane as suggested by the relationship of the $g_z$ and $A_z$ values by extrapolation from results for naturally occurring copper proteins (Peisach and Blumberg, 1974). On the basis of chemical and physical evidence other than ESR, it has been suggested that a ligand to the metal in *Pseudomonas fluorescens* and in parsley plastocyanin is a sulphur atom (Finazzi-Agro *et al*, 1970). There does not appear to be any sulphur involvement in *P. spinulosum* however.

The parameters for *P. spinulosum* copper binding are remarkably similar to those for *Polyporus versicolor* laccase and for human ceruloplasmin, suggesting that the coordination geometry may be similar. Electron spin resonance spectra of two phenolic polymers studied by Saiz-Jimenez and Shafizadeh (1984), showed resonance with parameters g 2.256 to 2.267 and 2.067 to 2.069 and A of 168 to 170 and this could have arisen from a copper protein of the laccase type. Laccases oxidise and polymerise phenols to produce phenolic polymers and melanins.

According to Peisach and Blumberg (1974), it is usually found that the ligands binding copper are arranged in a distorted octahedral

environment about the metal ion with 4 ligands, disposed in an approximate plane including the copper ion, lying close to the copper and thus strongly bonded. The other two ligands are arranged on a straight line including the copper and perpendicular to the plane.

When interpreting ESR data, it must be noted that the ESR of the copper is governed by the chemical nature and charge state of the close lying ligand atoms to the metal atom. Further information on the coordination of copper in fungal cell walls ought to be obtainable from ESR measurements. Information for binding of Cu (1) could be gained from experiments designed to measure Cu (11) by ESR and total copper bound by AAS. There is clearly scope for further use of ESR studies in attempting to relate copper toxicity to its valence state and likely different quantitative accumulation patterns.

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